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The impact on food safety

Doctoral thesis

NTNU
Norwegian University of Science and Technology
Thesis for the Degree of
Philosophiae Doctor
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Trondheim, May 2022

Gunn Merethe Bjørge Thomassen

Summary (English)

Food safety is strongly dependent on good hygiene in the processing environment. The bacterial contamination load in a salmon processing facility is affected by the salmon that enters the facility, the sea water that is pumped in together with the fish, carry-over from staff members and transportable equipment, and to a small extent, the air. Additionally, cleaning and disinfection procedures aims to counteract the mentioned factors and to keep the bacterial numbers in the facility as low as possible. There is a growing concern that the daily use of disinfectants in the food processing environment acts as a selective pressure on the bacteria present, favouring those with high tolerance, and that this can lead to increased tolerance and resistance towards the disinfectant in certain bacteria over time. Co-selection of bacteria with antibiotic resistance properties has also been indicated in connection with disinfection tolerance.

This thesis aimed at studying the development and dynamics in the resilient bacterial communities in a newly opened salmon processing facility during the first year of production and, to characterize bacterial isolates from these communities in order to gain more knowledge about the resilient bacteria, what properties they hold and the direct and indirect impact they might have on the food safety. In this work, a newly developed sequencing-based method, ON-rep-seq, was explored in a small-scale strain differentiation experiment of *Listeria monocytogenes* strains and in a large-scale species classification experiment on a diverse set of isolates originating from the salmon processing environment.

In the general contamination level, an overall increase in bacterial numbers were observed for contact surfaces in the slaughter department during the one-year study. On contact surfaces in the filleting department, on non-contact surfaces and on the fish no such trend over time was observed but rather a high variation between different time points for sampling. Overall, the bacterial flora detected was dominated by *Pseudomonas* spp. (46%) and other psychrotrophic, gram-negative bacteria as *Acinetobacter* spp. (14%), *Serratia* spp. (6%), *Chryseobacterium* spp. (5%) and *Aliivibrio* spp. (3%). But when classifying the bacterial isolates to species- and strain-level by ON-rep-seq method, highly point-specific bacterial communities were revealed.

The genus *Pseudomonas* is known to be the dominant genus in many food processing environments. Members of this genus are also known to be important spoilage bacteria in many different food products, they are non-fastidious, and some have a high tolerance

towards several antimicrobial agents. In addition, many of them are psychrotrophic and notorious biofilm producers, resulting in excellent survival in humid, low-temperature food processing environments. Characterization of a set of presumptive *Pseudomonas* isolates from the salmon processing facility was performed by classification based on sequencing of the *rpoD* housekeeping gene (or 16S rRNA gene for non-*Pseudomonas*) biofilm forming capacity at 12 °C and, susceptibility towards a panel of antibiotics of clinical and veterinary relevance. This revealed that *P. fluorescens* was most likely the dominant species among these isolates together with other species in the *P. fluorescence* group. The results from this work also elucidated the inconclusive and confusing taxonomy of the large and numerous *Pseudomonas* genus, which in addition to the 297 validly named and described species, also holds several hundred unclassified and undescribed strains. Several of the isolates in this work have highest similarity to unclassified strain of *Pseudomonas* or low similarity to any registered species at all and should be further studied as potential new species. In this set of isolates a high rate of resistance towards the clinically relevant antibiotics ampicillin and amoxicillin and, the veterinary relevant antibiotics florfenicol and oxolinic acid was observed. The resistance rate was much higher among *Pseudomonas* isolates than among non-*Pseudomonas* isolates. Despite this, no genetical antimicrobial resistance determinants was detected among the 30 *Pseudomonas* isolates that were subjected to whole genome sequencing, indicating that the resistance is caused by other mechanisms or genetical determinants not present in the ResFinder database.

A smaller, randomly selected set of isolates from the same pool was analysed for tolerance towards common disinfectants used in the salmon industry. This revealed that several isolates had tolerance towards the user concentration of the disinfectant (1%). One isolate survived 4X user concentration and must be considered as resistant to the disinfectant. Most of the isolates had a higher tolerance towards the disinfectant in biofilm state than in planktonic state. Five selected isolates were grown in multispecies biofilm together with the pathogen *L. monocytogenes* and the survival after disinfectant challenge was observed. The results indicate that the *Pseudomonas* multi-species biofilm served as a shelter for *L. monocytogenes* resulting in high survival of the pathogen after disinfection, despite that it was eradicated by the same disinfection treatment when grown in single-species biofilm.

This work shows that bacteria commonly found in food processing environments can inhabit traits that i) makes them more persistent in the environment, ii) can aid in the protection of

potential pathogens in the environment, and by that have an indirect impact on the food safety.

ON-rep-seq method was used in strain separation om *L. monocytogenes* isolates detected in the salmon processing facility in relation with an incident of reoccurring *L. monocytogenes* positive samples in a gutting machine in routine sampling. In this case the method could separate between three *Listeria* strains, one *L. innocua* and two *L. monocytogenes*. WGS analysis of the same strains did not separate the strains any further. The work in this thesis shows that ON-rep-seq has a high potential in strain separation of some bacteria as *L. monocytogenes*. In addition, it has a potential in species-level identification for most bacteria in complex communities in food processing environments. However, novel bacteria (not present in databases), not complete draft genomes, or misclassified genomes, will reduce the resolution of taxonomic classification of the method and additionally, some complex genera, as *Pseudomonas*, is just as difficult to classify by this method as it is with other methods.

Sammendrag (norsk)

God mattrygghet er sterkt avhengig av god hygiene i prosessmiljøet hvor maten blir produsert. Den bakterielle forurensningen i et lakseforedlingsanlegg påvirkes av laksen som kommer inn i anlegget, sjøvannet som pumpes inn sammen med fisken, kontaminering overført fra ansatte og transportabelt utstyr, og, til en viss grad, luften. I tillegg har prosedyrene for vask- og desinfeksjon som mål å motvirke de nevnte faktorene og å holde bakterietallet i anlegget så lavt som mulig. Det er en økende bekymring for at daglig bruk av desinfeksjonsmidler i matproduksjonsmiljøet utøver et selektivt press på bakteriene som er til stede, og dermed favoriserer de med høy toleranse, og at dette kan føre til økt toleranse og resistens mot desinfeksjonsmiddelet i noen bakterier over tid. Det finnes også indikasjoner på at seleksjon for bakterier med økt toleranse for desinfeksjonsmidler også selekterer for bakterier med antibiotikaresistensegenskaper.

Målet med dette prosjektet var å studere utviklingen og dynamikken i gjenstridige bakteriesamfunn i et nyåpnet lakseforedlingsanlegg gjennom det første operasjonelle året og å karakterisere bakterieisolater fra disse samfunnene for å få mer kunnskap om de tilstedeværende bakteriene, hvilke egenskaper de har, samt den direkte og indirekte innvirkningen de kan ha på mattryggheten. I dette arbeidet ble en nylig utviklet sekvenserings-basert metode, ON-rep-seq, utforsket i et småskala eksperiment for å differensiere *Listeria monocytogenes*-stammer fra hverandre, samt i et større skala for å klassifisere på et stort og variert sett med bakterieisolater fra lakseforedlingsmiljø.

For det generelle kontamineringsnivået ble det observert en økning i bakterietall for kontaktflatene i slakteriavdelingen i løpet av denne studien. På kontaktflater i filetavdelingen, ikke-kontaktflater og på fisken ble det ikke observert en slik trend over tid, men heller en høy variasjon mellom ulike tidspunkt for prøvetaking. Totalt sett var den påviste bakteriefloraen dominert av *Pseudomonas* spp. (46%) og andre psykrotrofe, gramnegative bakterier som *Acinetobacter* spp. (14%), *Serratia* spp. (6%), *Chryseobacterium* spp. (5%) og *Aliivibrio* spp. (3 %). Men når bakterieisolatene ble klassifisert til arts- og stammenivå ved hjelp av ON-rep-seq-metoden, ble punktspesifikke bakteriesamfunn avdekket.

Pseudomonas- slekten er kjent for å være den dominerende bakterieslekten i mange matforedlingsmiljøer. Mange arter i denne slekten er også kjent for å være viktige forringelsesbakterier i mange ulike matprodukter, de har beskjedne næringskrav, og noen har høy toleranse mot flere antimikrobielle midler. I tillegg er mange av dem psykrotrofe og

velvillige biofilmprodusenter, noe som resulterer i utmerket overlevelse i fuktige og kjølige matforedlingsmiljøer. Karakterisering av et utvalg presumptive *Pseudomonas*-isolat fra lakseforedlingsanlegget ble utført ved klassifisering basert på sekvensering av husholdningsgenet *rpoD* (eller 16S rRNA-genet for ikke-*Pseudomonas*), evne til biofilmdannelse ved 12 °C og sensitivitet for et panel bestående av 16 antibiotika med klinisk eller veterinær relevans. Disse analysene viste at *P. fluorescens* mest sannsynlig var den dominerende arten blant disse isolatene, i tillegg til andre arter i *P. fluorescens*-gruppen. Resultatene fra dette arbeidet belyste også den komplekse og forvirrende taksonomien til den tallrike *Pseudomonas*-slekten, som i tillegg til de 297 gyldig navngitte og beskrevne artene, også har flere hundre uklassifiserte og ubeskrevne stammer. Flere av isolatene i dette arbeidet hadde høyest likhet med uklassifiserte stammer av *Pseudomonas* eller lav likhet med noen registrerte arter i det hele tatt og bør studeres videre som muligens nye arter.

I dette settet med bakterieisolater ble det observert en høy grad av resistens mot de klinisk relevante antibiotikaene ampicillin og amoxicillin og de veterinærmedisinrelevante antibiotikaene florfenikol og oksolinsyre. Resistensraten var mye høyere blant *Pseudomonas*-isolater enn blant ikke-*Pseudomonas*-isolater. Til tross for dette ble ingen genetiske antimikrobielle resistens-determinanter påvist blant de 30 *Pseudomonas*-isolatene som ble helgenomsekvensert, noe som indikerer at den fenotypiske resistensen er forårsaket av andre mekanismer, eller at de aktuelle genetiske determinantene ikke er til stede i ResFinder-databasen.

Et mindre, tilfeldig utvalgt sett med isolater fra samme samling ble analysert for toleranse mot et desinfeksjonsmiddel som er mye brukt i lakseindustrien. Dette forsøket viste at flere isolater hadde toleranse for brukerkonsentrasjonen av desinfeksjonsmidlet (1%). Ett isolat overlevde 4X brukerkonsentrasjon og må anses som resistent mot desinfeksjonsmidlet. De fleste isolatene hadde en høyere toleranse overfor desinfeksjonsmidlet i biofilmtilstand enn i planktonisk tilstand. Fem utvalgte isolater ble dyrket i multispecies-biofilm sammen med den patogene *L. monocytogenes* og overlevelsen etter at biofilmen ble utsatt for desinfeksjons ble observert. Resultatene indikerer at *Pseudomonas* multispecies-biofilm fungerte som en beskyttelse for *L. monocytogenes*, noe som resulterte i høy overlevelse av *L. monocytogenes* etter desinfeksjon til tross for at *L. monocytegenes* i singelspecies biofilm ble fullstendig utslettet.

Dette arbeidet viser at bakterier som ofte finnes i matprosesseringsmiljøer kan inneha egenskaper som i) gjør dem mer persistente i miljøet, ii) kan hjelpe til med å beskytte potensielle patogener i miljøet, og dermed kan de ha en indirekte innvirkning på mattryggheten.

ON-rep-seq metoden ble brukt til å skille ulike stammer av *L. monocytogenes* påvist i lakseprosesseringsanlegget i forbindelse med gjentatte påvisninger av *L. monocytogenes* i en sløyemaskin ved rutinemessig prøvetaking. I dette tilfellet kunne metoden skille isolatene i tre *Listeria*-stammer, en *L. innocua* og to *L. monocytogenes*. WGS-analyse av de samme stammene kunne ikke skille stammene ytterligere fra hverandre. Arbeidet i denne oppgaven viser at ON-rep-seq har et stort potensial innen stammeseparasjon av bakterier som *L. monocytogenes*. I tillegg har den et potensial for identifikasjon av bakterie på artsnivå for de fleste bakterier i komplekse samfunn som matprosesseringsmiljøer er. Imidlertid vil nye bakterier (ikke til stede i databaser), ikke ufullstendige genom eller feilklassifiserte genom redusere oppløsningen av den taksonomiske klassifiseringen for metoden. I tillegg er noen komplekse bakterieslekter, som *Pseudomonas*, like vanskelige å klassifisere med denne metoden som de er med andre metoder.

Preface

This PhD was financed by the project OPTiMAT (Optimal Utilization of Marine Food Resources), a multidisciplinary project funded by the Norwegian University of Science and Technology (NTNU). The PhD position was funded for four years with 25 % duty work for the Department of Biotechnology and Food Science.

Most of the practical work was carried out in the laboratories of the Food Science group at campus Kalvskinnet. Sampling was done at a salmon slaughterhouse, that wish to remain anonymous. All sequencing services was outsourced.

Originally, a research stay at the University of Copenhagen (KU), Department of Food Science, Section for Food Microbiology and Fermentation was planned. Due to the ongoing pandemic and travel restrictions at that time, this was postponed several times and eventually cancelled. This caused some complications and changes late in the project and in addition, the analysis that were planned to perform during the stay was reduced in extent and outsourced to the research group at KU.

During the period as a PhD-candidate I have also been closely involved in the supervision of three Bachelor groups and six Master students. Some of these student's work has contributed directly or indirectly to parts of this thesis. The most direct contributions are valued by co-authorship in paper III and IV. Additionally, I have participated with poster contributions at three international conferences with poster contributions, EFFoST international conference in 2017 and 2021 and the 1st ReFood conference in 2018.

Thesis outline and list of papers

This thesis is submitted for the degree of Philosophiae Doctor (PhD) and consists of two parts. The first part is an introduction to the research topics with a short review and discussion up against my own findings, which are indicated by reference to Papers I-IV. This part is divided into chapters where chapter 1 gives an introduction to the background for this project, Chapter 2 states the goals and specific research objectives, Chapter 3 explains the sampling strategy used to collect all the biological material used in this work, Chapter 4-6 address the different topics relevant to the project and, Chapter 7 addresses the relevance of this research for the food industry. Conclusions and future perspectives are given in Chapter 8. The second part of the thesis consists of the four scientific papers produced from this work, whereof Paper I is published in MicrobiologyOpen (Wiley & Sons), Paper II is submitted to Food Microbiology (Elsevier) and currently under review. Paper III and IV are presented here as manuscripts, but they will be polished and submitted to relevant journals shortly after submitting this thesis.

I. Thomassen, G. M. B., Krych, L., Knøchel, S., Mehli, L., 2021. ON-rep-seq as a rapid and cost-effective alternative to WGS for species level identification and strain level discrimination of *Listeria monocytogenes* contamination in a salmon processing plant. MicrobiologyOpen 10, e1246.

II. Thomassen, G. M. B., Krych, L., Knøchel, S., Mehli, L., 2022. Bacterial community development and diversity during the first year in a new salmon processing plant. Submitted to Food Microbiology March 31st, currently in review.

III. Thomassen, G. M. B., Tennfjord, C. E., Reiche, T., Bringsli, R., Mehli, L. 2022. Antibiotic resistance properties among *Pseudomonas* spp. associated with salmon processing plant. Manuscript. (Accepted for publication in *Microorganisms* 13.07.2022)

IV. Thomassen, G. M. B., Reiche, T., Hjørungnes, M., Bringsli, R., Mehli, L. 2022. Antimicrobial resistance in *Pseudomonas* spp. biofilm – a possible shelter for *Listeria monocytogenes*. Manuscript.

List of abbreviations

AC	-	Aerobic Count
AMR	-	Anti-Microbial Resistance
AOAC	-	Association of Official Analytical Collaboration International
APC	-	Aerobic Psychrotrophic Count
ARG	-	Antibiotic Resistance Gene
ATP	-	Adenosin Triphosphate
BLASTn	-	Basic Logical Alignment Search Tool - nucleotide
CFU	-	Colony Forming Units
CLSI	-	Clinical and Laboratory Standards Institute
CLSM	-	Confocal Laser Scanning Microcopy
C&D	-	Cleaning and Disinfection
DDAC	-	Dodecyl dimethyl ammonium chloride
DDBJ	-	DNA Data Bank of Japan
DNA	-	Deoxyribonucleic Acid
EC	-	European Commission
ECDC	-	European Centre for Disease Prevention and Control
eDNA	-	Extracellular DNA
EDTA	-	Ethylene diamine tetra acetate
EFSA	-	European Food Safety Authority
ENA	-	European Nucleotide Archive
EPS	-	Extracellular Polymeric Substances
EUCAST	-	European Committee on Antimicrobial Susceptibility Testing
FCS	-	Food contact surface
FPE	-	Food Processing Environment
HGT	-	Horizontal Gene Transfer
HTS	-	High Throughput Sequencing
L&H	-	Long and Hammer agar
LPSN	-	List of Procaryotic names with Standing in Nomenclature
MAP	-	Modified Atmosphere Packaged
MBC	-	Minimum Biocidal Concentration
MBEC	-	Minimum Biofilm Eradication Concentration
MIC	-	Minimum Inhibitory Concentration

MLSA	-	Multilocus Sequence Analysis
MLST	-	Multilocus Sequence Typing
NCBI	-	National Center for Biotechnology Information
NFCS	-	Non-food contact surface
NGS	-	Next Generation Sequencing
ON-rep-seq	-	Oxford Nanopore Technology-based rep-PCR amplicon sequencing
ONT	-	Oxford Nanopore Technology
QAC	-	Quaternary ammonium compounds
QC	-	Quality Control
QS	-	Quorum Sensing
RAPD	-	Random Amplification of Polymorphic DNA
RDP	-	Ribosomal Database Project
RefSeq	-	NCBI Reference Sequence Database
rRNA	-	Ribosomal Ribonucleic Acid
PAA	-	Peracetic acid
PacBio	-	Pacific Bioscience
PCR	-	Polymerase Chain Reaction
PFGE	-	Pulsed Field Gel Electrophoresis
PsC	-	Pseudomonas Count
PVC	-	Polyvinyl chloride
Rep-PCR	-	Repetitive element palindromic PCR
TGS	-	Third Generation Sequencing
VBNC	-	Viable but non-culturable
WGS	-	Whole Genome Sequencing

Table of contents

1 Introduction

High quality, minimally processed, fresh fish products that are safe to eat are in high demand. The Norwegian salmon industry accounts for about 50 % of the world total salmon products, producing nearly 1,4 million tons per year and exporting about 1,1 million tons (NSC, 2022). For this a reasonable shelf life is required. Additionally, the large-scale use of salmon in unheated dishes like sushi and traditional products like cold smoked and cured salmon, underlines the importance of microbial safety and quality of the raw material.

All food processing facilities are under constant microbial pressure caused by both raw materials entering the facility, staff members and more. Main factors contributing to the total microbial load in a modern salmon processing facility are shown in Figure 1. It is well known that the hygiene in food processing facilities is of essential significance for the microbial quality of the finished product. As all other food processing facilities, the salmon slaughterhouses are subject to strict requirements for cleaning and disinfection routines to ensure optimal quality of their products. Nevertheless, it seems that microorganisms residing in the environment and production equipment lead to products being constantly contaminated by the same or similar bacteria. A general perception is that this is caused by bacteria forming biofilm in the production equipment (Carrascosa et al., 2021).

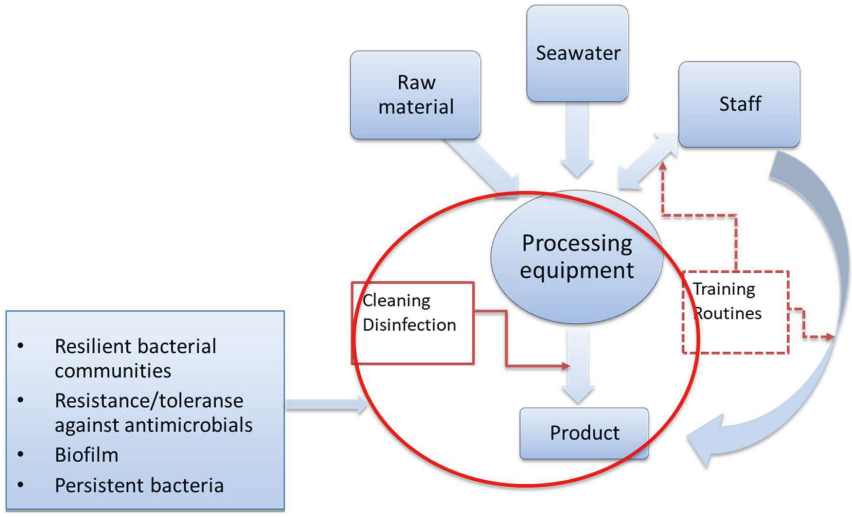

Figure 1: Main factors affecting the microbial load in a salmon processing facility and subsequently the finished product. In read squares factors meant for protecting the product against bacterial contamination. The read circle indicates the area of focus in this thesis. Challenges related bacteria within this area are mentioned.

Biofilms are communities consisting of different species and strains of microorganisms, which are established on different surfaces and entangled in a self-produced matrix. Biofilms can be formed everywhere in environments where microorganisms are present, the humidity high enough and some organic matter is available (Flemming et al., 2016). In food producing facilities areas of special concern are those difficult to clean, pipes and tubes, inside advanced equipment etc. (Alvarez-Ordóñez et al., 2019, Mizan et al., 2015, Van Houdt and Michiels, 2010).

Most bacteria can form biofilm and, it has been suggested that life in biofilm is the preferred way of living for many bacteria as it increases the microorganisms' resistance to external influences, like desiccation, chemicals, and mechanical interruptions (Flemming et al., 2016). Also a number of pathogenic bacteria such as *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, are prone to form biofilm (Blackman and Frank, 1996, Jeong and Frank, 1994, Langsrud et al., 2016, Møretrø and Langsrud, 2004, Rode et al., 2007, Silagyi et al., 2009, Stepanović et al., 2004). Detection of such pathogens in food processing environments (FPEs), and specially of *L. monocytogenes* is relatively common and causes large concerns for the facilities. This bacterium can cause mild to moderate food poisoning and flu-like symptoms, or no symptoms at all, in otherwise healthy people. But, in young children, elderly, pregnant women or others with compromised immune systems, an infection can become serious or even fatal (FHI, 2017). The detection of such pathogenic bacteria in the product, or in the processing equipment may lead to withdrawal of entire batches of product, food loss and subsequently large financial losses and a weakened reputation for the company. A worst-case scenario is that contaminated products go undetected out on the marked and infects consumers.

Recent research indicates that the background microbiota in the food processing environment may be of great influence on the survival and establishment of specific pathogenic bacteria (Fagerlund et al., 2021, Lianou et al., 2020). It is therefore of interest to investigate such background microbiota to get more knowledge about the resident bacteria, the dynamics and the interspecies interactions in the bacterial communities.

1.1 The concepts of biofilm, persistent bacteria, and resilient bacterial communities

Biofilms are formed by microorganisms attaching to a surface by different attachment mechanisms like pili, fimbriae, or other (Flemming et al., 2016). Irregularities on the surfaces like small cracks, crevasses, or niches, where the bacterial cell are protected against mechanical disruption and shear forces, are more prone to be colonized by biofilm (Srey et

al., 2013). Remnants of organic matter will also support the growth and survival of bacterial cells (Coughlan et al., 2016), making food processing environments the ideal environment for biofilm establishment. Subsequently of the cell's attachment to the surface, biofilm will start developing as more microorganisms arrive and microcolonies are formed. In these microcolonies, the cells will begin to form an extracellular matrix consisting mainly of polysaccharides, proteins, DNA, and lipids (Flemming et al., 2016). This matrix serves as a protective layer on top of the microcolonies. It helps new cells to attach, as well as protecting the cells from external influences such as mechanical impact, desiccation, or chemicals (Burmølle et al., 2014, Truelstrup Hansen and Vogel, 2011). Environmental biofilms usually consist of many different microorganisms with different properties. Because the bacteria live in such communities, the properties of one single strain present can benefit the whole community. For example, if a species or strain is resistant to a chemical used for cleaning and/or disinfection, the resistant cells will be unaffected by the chemical and the biofilm matrix will be at least partly intact. It has also been shown that in biofilms there can be some kind of communication and collaboration between the organisms (Quorum Sensing, QS) (Flemming et al., 2016) as well as the exchange of DNA (horizontal gene transfer, HGT) between the various organisms in the biofilm (Coughlan et al., 2016). This is of high interest to monitor when it comes to antibiotic resistance genes (ARG). ARGs are often situated on plasmids or other mobile genetic elements and might easily be transmitted to other bacterial cell by HGT (Van Meervenne et al., 2014).

In the food industry biofilm formation is of high concern as it can develop in piping systems, tanks, and other kinds of processing equipment, more or less regardless of the type of surface (Mizan et al., 2015). In the meat and the salmon industry old, worn-out parts as e.g., conveyor belts have been shown to be hotspots for the buildup of biofilm (Fagerlund et al., 2017, Langsrud et al., 2016). In a laboratory study where small coupons of old conveyor belts was used as attachment surface for biofilm they showed that the most profound biofilm buildup was in the small microgrooves on the backside of the conveyor belts (Fagerlund et al., 2017). In the industry, such non-food contact surfaces are likely to not be the main focus during cleaning and disinfection and hence the risk for biofilm build up is substantial. Wagner et al. (2020) identified biofilm hotspots in a meat processing environment by quantifying bacteria and analyzing for biofilm matrix components. They found that non-food contact surfaces (NFCS) like drains and water hoses were hotspots for biofilm development. On the food contact surfaces (FCS) the buildup of a biofilm will be constantly disrupted by

both the food product passing and by the regular cleaning and disinfection (C&D) routines. In such cases it can be more accurate to speak about persistent bacteria or persistent communities. However, the study by (Wagner et al., 2020) also reported that 10 % of their food contact surfaces were identified as biofilm hotspots in addition to 8 % of their non-food contact surfaces.

Resilience is the capacity of systems to recover their initial state or function after a disturbance (Carvalho et al., 2019). In ecology, the concepts of resilience and resistance is complementary. Resistance refers to the insensitivity to a disturbance as e.g., antibiotic or disinfection treatment and resilience focus on the recovery of the system after the disruption.

The expression "persistent" has mostly been used on specific bacterial strains (often pathogens) that are repeatedly detected in the same facility over a prolonged time. Many studies have addressed the issue of which properties are associated with persistent strains versus non-persistent strains, but no unambiguous answer in regard to adhesion properties, tolerance towards disinfectants etc. has been established. There are also studies investigating the interactions between these persistent strains and other bacteria commonly found in food processing environments. But also in these studies, an unambiguous answer is lacking (Fagerlund et al., 2021, Lianou et al., 2020).

1.2 Limitations in current methods for microbiological analysis in food industry

Classical methods for microbiological analysis in the food industry is enrichment and plate count methods. Such methods are commonly used for both hygiene evaluation (total aerobic plate count) and to detect specific pathogens (*L. monocytogenes*, *S. aureus*, etc.) or contamination indicators (*E. coli* or coliforms). But these methods are very limited when it comes to characterising or identifying the bacteria. The last two decades sequencing technology has dramatically developed and become much more available and affordable then earlier. Today, whole genome sequencing (WGS) is commonly used in both surveillance and source tracking in outbreaks of pathogenic bacteria (Nadon et al., 2017, Van Walle et al., 2018, Zhang et al., 2020) and the method has a huge potential. But the method requires comprehensive data analysis and data interpretation and, the bioinformatic infrastructure and expertise necessary to do this can be difficult to find in food producing companies (Jagadeesan et al., 2019a, Jagadeesan et al., 2019b, Oakeson, 2017, Quainoo et al., 2017). Therefore, there is a great need for solutions and methods that can aid these companies in making use of the possibilities that lies in the sequencing technology and sequencing

methods. It is of importance that such methods are reliable, efficient, easy to use and adjustable to suite different company's needs.

2 Aims and specific research objectives

The main goal of this project was to gain more knowledge and understanding of the resilient and/or persistent bacterial communities in production equipment in the salmon industry, as well as the effect these communities have on food safety and product quality. The focus was placed on bacteria that remain in the production equipment and environment, in a newly started salmon processing facility, after cleaning and disinfection. Emphasis was also placed on the identification of isolates to species/strain-level to obtain a more detailed knowledge about the bacterial diversity in a food processing environment and, to study their properties regarding biofilm formation and antimicrobial tolerance.

Specific research objectives:

1. Explore new methods for species identification and strain differentiation. (**Paper I and II**)

2. Elucidate the ability of specific bacteria to survive and persist in production equipment after cleaning and disinfection. Bacteria isolated from production equipment and environment should be identified to species and possibly strain level and characterized. (**Paper II-IV**)

3. Increase the knowledge about the development and formation of persistent bacterial communities and their resilience in a new production line in a salmon processing facility from start-up and one year onwards. (**Paper II**)

4. Increase the knowledge about antimicrobial tolerance in bacteria commonly found in food processing environments and investigate how these properties affect the survival and persistence of other bacteria (e.g. pathogens) in the food processing environment. (**Paper III and IV**)

3 Sampling strategy

The starting point for all work performed in **Papers II-IV** in this thesis was the longitudinal sampling in a newly started salmon processing plant. The sampling was done on 23 fixed sampling points in the processing environment (Figure 2) on thirteen different occasions during the first year of production. The inlet water was sampled every time and additionally, skin and gills of whole gutted fish and fish fillet was also sampled at four occations. To focus on the resilient bacteria all sampling was done on cleaned, disinfected surfaces in the morning before production start. A detailed description of the sampling procedure is given in **Paper II**.

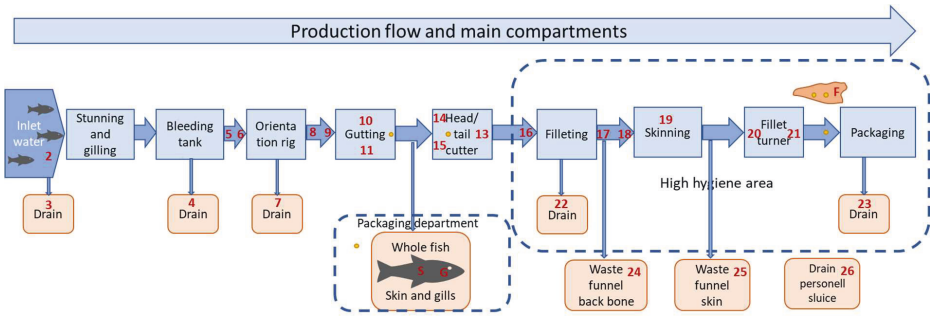

Figure 2 (adapted from Paper II): Schematic diagram of the processing facility with marks for the sampling points. Main equipment and machinery are drawn in light blue squares, conveyors in dark blue arrows, while sampled drains and waste funnels (non-contact surfaces) are drawn in orange. Sampling points are marked with red numbers (2-26). Product samples were taken of filets (F) just before packaging in addition to swab samples of skin (S) and gills (G) of whole fish ready for packaging. Yellow dots indicate where the *L. monocytogenes* analysed in **Paper I** were detected.

The different sampling points varied in category (water, food contact surface (FCS), non-food contact surface (NFCS), fish etc.) and in sampling area. A description of the sampling points is given in Table 1. For quantification of the different bacterial parameters tested, a calculation to CFU/cm^2 was done for all surface samples, CFU/g for fish fillet samples and CFU/mL for water samples.

Table 1: Overview of the different sampling points, category (FCS=food contact surface in slaughter department or filleting department, NFCS=non-food contact surface, F=fish), sampling type, water, cloth, swab or fish fillet, and approximate sampling area.

Sampling point	Sampling point category	Sampling type	Sampling area/volume	Isolates studied in Paper
2. Inlet water		Water	100 ml	II, III, IV
3. Drain under inlet	NFCS	Cloth	30x30 cm	
4. Drain under bleeding tank	NFCS	Cloth	30x30 cm	
5. Conveyor	FCS, slaughter	Cloth	30x30 cm	
6. Conveyor	FCS, slaughter	Cloth	30x30 cm	
7. Drain under orientation rig	NFCS	Cloth	30x30 cm	III, IV
8. Slide above conveyor	FCS, slaughter	Cloth	30x30 cm	II, III, IV
9. Slide above conveyor	FCS, slaughter	Cloth	30x30 cm	
10. Gutting machine, suction	FCS, slaughter	Swab	10x10 cm	II, III, IV
11. Gutting machine, holder	FCS, slaughter	Swab	10x10 cm	II
13. Tail cutter	FCS, slaughter	Cloth	90x10 cm	II
14. Head cutter knife	FCS, slaughter	Cloth	2 x Ø25 cm	II, III, IV
15. Head cutter, holder	FCS, slaughter	Swab	10x10 cm	II
16. Peg band before fileting	FCS, fillet	Swab	5x20 cm	
17. Conveyor after fileting	FCS, fillet	Cloth	30x30 cm	II
18. Conveyor before skinning	FCS, fillet	Cloth	30x30 cm	II, III, IV
19. Skinning machine	FCS, fillet	Cloth	30x30 cm	II
20. Filet turner, slide	FCS, fillet	Cloth	30x30 cm	II
21. Filet turner, arm	FCS, fillet	Cloth	30x30 cm	II
22. Drain under filet turner	NFCS	Cloth	30x30 cm	III, IV
23. Drain under packaging	NFCS	Cloth	30x30 cm	
24. Waste funnel, backbone	NFCS	Cloth	30x30 cm	
25. Waste funnel, skin	NFCS	Cloth	30x30 cm	
26. Drain, personnel sluice	NFCS	Cloth	30x30 cm	
F. Fish fillet before packaging	Fish	Fish fillet	25 g	II, III, IV
S. Skin, gutted whole fish	Fish	Swab	10x10 cm	III, IV
G. Gills, gutted whole fish	Fish	Swab	Gills on both side of fish	III, IV

The *Listeria monocytogenes* isolates studied in **Paper I** was detected during the facility's own quality control sampling. The location of where the isolates were detected is indicated with yellow dots in Figure 2. Some of these isolates were detected during production and some before production start. The isolates were detected from two different gutting machines and two different Head and tail cutters. The exact sampling spot and area of these samples differs from the sampling done in this work as the QC sampling in this case was done with the aim of detecting *Listeria*, and not for quantification.

4 Analysing and studying food-related microbial ecology

When analyzing bacterial communities there are many considerations that must be taken, and choice of method must be done for each step in the process according to which questions that are to be answered. In research other methods are available then those commonly used in routine analysis in food industry. For food producing facilities the choice of method for analysis is also important, but food businesses are obliged to use strictly validated methods. More information and extensive reviews on different microbiological methods for identification of bacteria and detection of pathogens in general can be found elsewhere. In this work the focus is on methods relevant for the salmon industry and the most problematic pathogen for the salmon industry, *Listeria monocytogenes*.

4.1 Hygiene control in food processing facilities

Sampling in FPE for microbiological parameters as Total Aerobic Count or detection of specific pathogens or indicator bacteria should be done at regular basis in all food producing facilities and is usually a part of their Quality Control system. All food producing facilities are obliged to develop and comply to a sampling routine based on HACCP principles and good hygiene practice appropriate to the relevant production. General guidelines on how to perform the sampling for reliable results are stated in the European Commission Regulation (EC) No 2073/2005 (EuropeanCommision, 2005). These guidelines are supplemented with specific guidelines regarding testing for *L. monocytogenes* for producers of food where this bacteria is an issue (Carpentier, 2012), and a practical guide for design and implementation of an environmental monitoring system for food producers are presented by Spanu and Jordan (2020). In addition to culture based microbial testing methods, ATP measurements are often used in hygiene control as it is a quick and easy method to detect residues of organic matter (Nivens et al., 2009).

The recommended sampling procedures are suitable for downstream microbiological analysis by conventional culturing methods and involves swabbing surfaces with dry or pre-moistened swabs, sponges or cloths before dilutions and spread plating or, in case of pathogen detection, an enrichment step before plating. The sampling done in **Paper II** and **III** was performed according to the mentioned guidelines. When sampling FPEs for biofilm or persistent bacteria the focus is mostly on detecting the various bacteria present but, if the focus is on detecting biofilm formation it can also be necessary to test for other, biofilm associated components like proteins, carbohydrates, uronic acid and extracellular DNA (Maes et al., 2017, Wagner et al., 2020). In this work the focus was on which bacteria could be detected, and sampling for biofilm components was not performed.

4.2 Pathogen detection and subtyping

Microbiological analysis of food and the FPE has traditionally been limited to Total aerobic plate count for hygiene control of surfaces and bacterial load in food product and, detection of specific pathogens or indicator organisms by performing validated culture dependent methods which often include several enumeration steps in selective culture media. Food industry or external laboratories are obliged to use validated methods for microbiological analyses of food and FPE but, as many of these validated conventional methods are rather time consuming the interest for more rapid methods is high. Methods for pathogen detection like plating on chromogenic media can shorten the analysis time by a day or two by simplifying confirmation steps (Greenwood et al., 2005), and methods based on selective enrichment and detection by specific polymerase chain reaction (PCR) and plating only of samples positive in PCR are gaining popularity in industry (e.g. iQCheck BioRad, SureTect™ ThermoFisher, FoodProof® BIOTECON Diagnostics GmbH). Several of these methods are now validated and approved by AOAC International, AFNOR and/or NordVal but as these methods/kits are specific for each pathogen, each kit must be validated separately. In some cases, as for *E. coli* O157:H7, the kit is made for specific testing of this strain, but for other bacteria as e.g., *L. monocytogenes* strain differentiation is not possible with the kits and downstream isolation of the bacteria is necessary for further characterization. In research purposes many different methods has been presented for rapid detection and/or identification of *L. monocytogenes* and other pathogens (Välimaa et al., 2015) but not many has been extensively used in routine analysis for the industry.

In surveillance of specific pathogens or outbreak investigations it is not only necessary to detect the pathogen, but also to differentiate on strain level. In research, several methods for strain separation have been used, all with different strengths and weaknesses (Wiedmann, 2002a, Wiedmann, 2002b). Phenotype based subtyping like serotyping and biochemical assays (e.g API, Vitek, BIOLOG) have been extensively used in clinical and public health laboratories, also for subtyping of foodborne pathogens in case of illness and outbreak situations, but for source tracking and surveillance in industrial settings the usage of such methods is scarce.

The genotypic fingerprint method Pulsed-field gel electrophoresis (PFGE) has been much used for subtyping of many different bacteria and has been considered as the gold standard for bacterial typing (Neoh et al., 2019) as it provides high discriminatory power, high reproducibility and has shown good epidemiological concordance for subtyping of *L.*

monocytogenes (Luque-Sastre, 2015). PFGE has been used in several studies for source tracking of *L. monocytogenes* (Fugett et al., 2007, Pažin et al., 2018, Swaminathan et al., 2001) and EFSA (European Food Safety Authority) has developed a standard operating procedure for the method (Roussel et al., 2014). Other genotyping methods used for *Listeria* subtyping is RAPD (random amplification of polymorphic DNA), ribotyping, and MLST (multilocus sequence typing) (Wiedmann, 2002a). Additionally, Harvey et al. (2004) showed that rep-PCR (Repetitive Extragenic Palindromic PCR) had a discriminatory power almost similar to PFGE for *L. monocytogenes*.

As mentioned, all these methods used for research purposes has a good potential and can provide useful information in industry, but they have not been extensively used in routine analyses in, or for, the industry. This is most likely because they require specific equipment, instrumentation, and trained personnel and therefore are not easily implemented in the existing routine analyses.

4.3 Sequencing technology and strategy

The sequencing technology has developed enormously during the last decades. The Sanger sequencing technology was first introduced in 1977 by Sanger (1977). The technology has been significantly refined since then and is known to provided high accuracy (99,99 %) and read lengths of approximately 1000bp. It has been, and still is, very important when it comes to sequencing of single genes, as done for 16S rRNA gene and *rpo*D gene in **Paper II** and **III** of this work.

The next generation sequencing (NGS) technology, or High Throughput Sequencing (HTS) arouse during the 2000 decade and key features were high throughput and high accuracy but, with capacity of only short reads (50-300 bp depending on technology). The features and different technologies under the term NGS has been excellently reviewed by Goodwin et al. (2016) and van Dijk et al. (2018) among others. The leading technology within NGS has for many years been Illumina technology, mainly because of relatively low cost per sequenced base enabled by their very high throughput. In this work, Illumina NovaSeq technology was used for WGS in **Paper I** and **III**. But even though the Illumina NovaSeq instruments has an impressively high throughput (22 000-67 000 Mb/h) it still has the drawback of only short reads (2x150 bp) resulting in complex data analysis and the need for strong computational power.

With third generation sequencing (TGS) provided by PacBio (Pacific Bioscience) and Oxford Nanopore Technology (ONT) (Loman and Watson, 2015), came the long read lengths (190 000 bp on ONT) and single cell sequencing. These technologies are still up and coming and has in their early days experienced some challenges when it comes to accuracy. Instruments from these two companies are based on very different technologies and the Oxford Nanopore Technology stands out with the technology that seem to have the biggest potential in food industrial settings due to the small and practical size of the instrument(s). The long reads gained from these sequencing platforms has proven useful as supplement to short-read sequencing for e.g., closing gaps in genomes. Hybrid sequencing for achieving closed procaryotic genomes is getting more common (Chen et al., 2020, Derakhshani et al., 2020). In this strategy the long reads are used as a backbone structure while the short reads increase the coverage and provide the depth.

4.4 Sequencing based analyses for bacterial classification

With the emergence of the sequencing technology many new methods for detection, identification and classification have been introduced. Sequencing of housekeeping genes for identification and classification has been proven valuable and, sequencing of 16S rRNA gene has for some time been the gold standard for bacterial classification (Janda and Abbott, 2007).

Despite its extensive use and usefulness in bacterial classification, sequencing of 16S rRNA gene is not without limitations. It has a rather low phylogenetic power at species level and a low discriminatory power for some genera (**Paper II**)(Gomila et al., 2015, Janda and Abbott, 2007). E.g. within the genus *Pseudomonas* species separation and identification has been found to be very difficult based on 16S rRNA gene (Gomila et al., 2015, Özen and Ussery, 2012). According to List of Prokaryotic names with Standing in Nomenclature, the *Pseudomonas* genus includes 297 validly registered species and additionally a couple of hundred species not validly published (Parte et al., 2020)(accessed 09.05.2022), making it a large, diverse and phylogenetic complex group which is especially difficult to classify (**Paper II** and **III**). For this reason, also other housekeeping genes has been explored for usage in classification. In case of *Pseudomonas, rpo*B, *rpo*D, and *gyr*B has been suggested (Ait Tayeb et al., 2005, Mulet et al., 2009, Yamamoto et al., 2000) and, Mulet et al. (2010) suggested to use a multi-locus sequence analysis (MLSA) approach including all four of these genes (16S, *rpo*B, *rpo*D, *gyr*B). Girard et al. (2020) demonstrated that sequencing of the *rpoD* gene alone could accurately assign environmental isolates to specific phylogenetic groups and in 95% of

the cases provided a taxonomic affiliation concordant with WGS. This was the background for choosing the *rpoD* gene for classification of the *Pseudomonas* isolates in **Paper II** and **III**.

With the rise of sequencing technology, the use of Whole genome sequencing (WGS) as a typing tool for bacterial strains has been suggested (Salipante et al., 2015). WGS analysis has a higher resolution and better reproducibility then other methods as it theoretically can distinguish between strains with one single nucleotide difference. However, the WGS method generates huge amounts of data, and it requires specific bioinformatic skills to analyze these data. Several online tools are available for identification or classification of bacterial isolates based on WGS data. Larsen et al. (2014) compared several of these and found that among the tested identification methods KmerFinder had the highest accuracy. The method examines the number of co-occurring k-mers between query and DNA-sequences in a database. In the mentioned study it correctly identified 93-97 % of the isolates in the evaluation set. This tool was used in **Paper I** and **III** to verify the identification of the whole genome sequenced isolates.

The development of sequencing technology and the decreasing prizes has resulted in a continuous increasing number of WG-sequenced isolates. Using the information gained from this in classification and phylogenetic studies has resulted in the proposition of many new species in addition to reorganization of previously established species (Allard and Kraft, 2016, Gomila et al., 2015, Lalucat et al., 2020, Thompson et al., 2015).

4.5 Surveillance and source tracking of pathogens in food industry

So far the use of WGS has proven to be a valuable method in source tracking in cases of disease outbreaks. And when the outbreak is caused by foodborne pathogens this becomes relevant also for the food industry (Sekse et al., 2017). It has been predicted that WGS will be the new gold standard for strain differentiation, characterization, and epidemiological analysis, as it can replace both traditional typing methods, detection of resistance genes and many other sequence-based investigation (Kwong et al., 2015, Quainoo et al., 2017). It has been well demonstrated that it has the highest discriminatory power and resolution if compared to other molecular typing methods (Moura et al., 2017, Stasiewicz et al., 2015). WGS has been successfully used in several disease outbreak situations, both in retrospect and in real-time (Jackson et al., 2016, Joensen et al., 2014, Kvistholm Jensen et al., 2016, Nouws et al., 2020, Schjørring et al., 2017) and, its technical superiority is indisputable. However, WGS analysis requires high competence in microbiology, molecular genetics, bioinformatics

and more. The studies referred to here are coordinated and performed by large research groups, public health institutions, or food authorities who are in possession of such competence. In recent years also some of the larger food producing companies has become increasingly interested in applying WGS for surveillance of pathogens in FPE and products (Jagadeesan et al., 2019b, Klijn et al., 2020). But, for implementing this technology as a routine tool the necessary investments are too high, and the necessary competence can be difficult to establish (Klijn et al., 2020). Additionally, the big variety of analysis approaches that are available is pointed out as an obstacle for food businesses (Jagadeesan et al., 2019a) and there is a need for time effective, cost effective, semi-automated pipelines, designed to suite the company's needs (Nastasijevic et al., 2017). Several commercial software are available that can provide standardized or custom set of data analyses, but using these requires some initial knowledge on tested organisms in order to customize the analysis. There is also an increasing number of online webtools freely available and several commercial software packages under paid license are also available, and they all have their pros and cons (Jagadeesan et al., 2019a, Quainoo et al., 2017). In this work the commercial software package, Geneious Prime (Dotmatics) was used in **Paper III**, and additionally, several freely available webtools was used in **Papers I-III**.

ON-rep-seq, introduced by (Krych et al., 2019), is a newly developed method that combines the Rep-PCR fingerprint method (Versalovic et al., 1991) with sequencing of the generated DNA fragments by Oxford Nanopore Technology. This results in highly discriminating sequences which allows for accurate taxonomic identification for most bacterial isolates and in many cases, it also provide excellent strain differentiation (**Paper I** and **II**). In **Paper I** this method was used to analyse 20 presumptive *L. monocytogenes* isolates. From this set of isolates the method differentiated three strains. The taxonomic classification revealed that one group of three isolates was not *L. monocytogenes* but rather *L. innocua* that had been misinterpreted when grown on selective and differential agar plates (Rapid'L.mono (BioRad) and Brilliance *Listeria* Differential agar (Oxoid)). The results from ON-rep-seq analysis were evaluated by WGS of the isolates and no further differentiation of the strains was obtained. By this we demonstrated how ON-rep-seq may serve as a rapid and cost-effective method for species level identification and strain level discrimination of *Listeria* species.

The material in **Paper I** was however very limited and, a larger set of isolates will be necessary to evaluate the discriminatory power of ON-rep-seq more thorough. A large set of *L. monocytogenes* isolates is currently being analysed in another project in our research group

and, in **Paper II** the method was used to analyse bacteria detected from the food contact surfaces in the salmon processing facility, which will be discussed in the next chapter.

4.6 Analysis of bacterial communities

When analysing bacterial communities there is always a question on whether to use culture dependent or culture independent methods. Culture independent methods like metagenomic sequencing by shot-gun approach or microbial profiling approach with amplification of 16S is gaining popularity as the sequencing technology becomes more available and affordable. A huge potential lies within these methods (McHugh et al., 2021, Solden et al., 2016) and it is even possible to assemble whole, closed genomes from the genomic fragments produced by shotgun metagenomic sequencing (Ercolini, 2017, Sharon and Banfield, 2013). These methods have the advantage that they can process and analyse many samples at the same time and that they for large sets of samples are less laborious and gives more information than conventional methods. They can also detect the non-culturable cells/bacteria that are present which, in the case of sampling in food producing environment, can include cell that are damaged or stressed because of the chemical and mechanical stress induced by C&D actions. Through the last few years metagenomics and other sequencing-based methods have been used in several studies to analyse the background microbiota in food processing environments (Alexa et al., 2020, Zwirzitz et al., 2020). The main disadvantages with these methods are, for shot-gun sequencing, that the bioinformatical analysis is quite complicated, and for amplicon sequencing that the resolution in classification analysis mostly will be on genus level. Additionally, the sampling procedures for such studies are of high importance as these samples are not only sensitive to contamination of live bacteria, but also dead bacteria and other DNA contamination. Additionally, DNA extraction and bias in downstream analysis is a huge challenge, especially when sampling from cleaned surfaces as the biomass from such samples are expected to be very low (McHugh et al., 2021).

When using culture dependent methods, the obvious limitations are in the choice of media and growth conditions that will cause a bias in the samples, favouring the strains with high growth rate under the selected conditions. Additionally, stressed, and viable but non-culturable (VBNC) cells can escape detection. One advantage with the culture dependent methods is the possibility to isolate certain bacterial strains of interest, and this was the reason for choosing culture dependent methods in **Paper II** and **III** of this work. The isolation of colonies was followed by using ON-rep-seq method to classify the isolates to species level and to differentiate closely related strains. In **Paper II** we demonstrated that

ON-rep-seq provided good species-level identification for most of the isolates and additionally could differentiate between different strains. However, we revealed some shortcomings when dealing with little studied species. This is probably because ON-rep-seq is dependent on a database of whole genome sequences. If there are no WGS data for å species similar enough, the species will be reported as Unclassified or the different consensus reads will match different, relatively close related species and, the interpretation can be difficult or even wrong.

4.7 Database issues

Next- and third-generation sequencing technology has become a powerful tool in microbial community studies. Such studies rely on search against public databases for taxonomic assignment of the species. Sequencing and analysis of the 16S rRNA gene has served as a cornerstone in the analysis of both single isolates and bacterial communities and, specialized 16S rRNA databases has been developed for this, e.g., Greengenes, RDP (ribosomal database project) and SILVA (Edgar, 2018). Common for most of these databases is that they rely on the users to provide metadata for each submission and most of the taxonomic annotations that are given are based on predictions from a sequence rather than authoritative assignments based on thorough studies of isolates or type strains (Bagheri et al., 2020, Edgar, 2018). Additionally, most of these databases do not have any method for detecting errors in the metadata provided by the user, and this can potentially lead to propagation of errors (Bagheri et al., 2020).

Database issues are always relevant regardless of which sequence-based approach that are being used. In **Paper II** and **Paper III,** we experienced several of these issues. The ON-rep-seq method used for analysing 20 *Listeria* isolates in **Paper I** provided an excellent classification and strain differentiation on that set of isolates and, the WGS-based analysis done did not give any further differentiation. In **Paper II** the same method was used on a much larger and diverse set of isolates. For most genera we obtained a good species classification and strain differentiation while other species remained unclassified. Additionally, even though the method gave good species classification within most genera, within the genus *Pseudomonas* it gave ambiguous results and classification was difficult. ON-rep-seq amplifies several DNA fragments from the bacterial genomes and is therefore dependent on whole genome sequences or a custom built database of sequences from isolates previously analysed by ON-rep-seq. ON-rep-seq uses the Kraken2 database (Wood et al., 2019) which is built on bacterial, archaeal and viral genomes that are available in The

Reference Sequence (RefSeq) procaryotic collection. RefSeq is a curated, non-redundant collection of well-annotated sequences. The collection is continuously growing as new data becomes publicly available and, in August 2021 the RefSeq prokaryotic collection contained genomes from 14606 species (Sayers et al., 2021). Previously undescribed species and all species not whole genome sequences are not present in this database. Many of the isolates collected in this work are environmental isolates with no immediate clinical or industrial interest and have not been fully sequenced. Therefore, they will not find a match in the database, resulting in no, poor, or wrong classification.

On the other side of the scale, we find the large, comprehensive nucleotide databases Genbank, European Nucletide Archive (ENA) and DNA Data Bank of Japan (DDBJ). These databases have daily exchange of data and for that reason they are relatively similar. Genbank contains a total of 9.9 trillion base pairs and a fair 1% of these comes from bacteria (Sayers et al., 2020). When performing a BLASTn search it is possible to search against all sequences in these databases, but it is reasonable to use some of the filtering possibilities in the search to narrow it down. For the isolates unclassified by ON-rep-seq in **Paper II** a partial sequencing (1100 bp) of the 16S rRNA gene and subsequent BLASTn search performed. This resulted in a species or genus classification for most of the isolates and most of them were found to belong to the genera *Pseudomonas, Photobacterium, Chryseobacterium, Shewanella*. One problem with the 16S rRNA gene is the redundancy of the databases and the previously mentioned issue with user provided metadata where the taxonomic annotation is based on a prediction of the sequence. A wrong annotation can lead to more wrong annotations which can provide confusing or ambiguous results for later BLASTn users. Additionally, in some genera the differences in the 16S rRNA gene between the species is relatively small and it is not possible to differentiate the species.

As mentioned in chapter 4.4 species within the genus *Pseudomonas* are difficult to classify by sequencing approach. One of the main reasons for this is the databases. The genus *Pseudomonas* comprises of 297 validly described and named species according to LPSN, and additional 201 not validly described (Parte et al., 2020), and in NCBI Taxonomy the list of unclassified *Pseudomonas* sp. is endless. It has been documented that as much as 30% of the sequenced genomes were annotated to the wrong species (Gomila et al., 2015). As the *rpoD* gene has been used in MLSA analysis of *Pseudomonas,* the database for this gene is comprehensive but not as redundant as 16S rRNA gene database. For this reason, the *Pseudomonas* isolates not classified to species by ON-rep-seq in **Paper II** and the

presumptive *Pseudomonas* isolates detected on *Pseudomonas* CFC Selective agar in **Paper III** were attempted to classify by sequencing of the *rpoD* gene.

5 Bacterial communities in food processing environments

5.1 Biofilm and persistent bacteria in the salmon industry

In the Norwegian salmon industry, the challenges with biofilm and persistent bacteria are well known. Many of the facilities report about similar challenges and the same problematic areas (Løvdal, 2017). Areas that have been pointed out as high-risk areas for biofilm are non-food contact surfaces like drains, inside ceiling of de-slimers, and water hoses for cleaning (Løvdal, 2017). Additionally, advanced mechanical equipment with both contact and non-contact surfaces like gutting machines, head-cutters, skin-off machines are areas where biofilm build up may occur and the risk for contamination of the food product is high (**Paper I**, **Paper II**, (Møretrø et al., 2016)). On the food contact surfaces the buildup of a mature biofilm is less likely for several reasons. These surfaces are more likely to be designed with hygiene in focus and they are object to thorough cleaning and disinfection which regularly will disrupt attached cells/starting biofilm. This, however, does not mean that there are no remaining cells on these surfaces. It has been reported in several studies aerobic cell counts of >3,0 log CFU/cm^2 on food contact surfaces after cleaning and disinfection (Møretrø et al., 2016)(**Paper II**). At present, there are no general acceptance limits for hygiene samples. Querido et al. (2019) uses a limit of >10 CFU/cm^2 for assessing the hygienic condition and refers this to an old (2001) European Commission guideline.

Which microorganisms that are present in the biofilm in a production facility will depend on a several factors. What is in/on the raw materials, temperature in the production room, methods for washing and disinfection, etc. In a salmon processing facility, most microorganisms enter the production line via the raw material and the water pumped in together with it. Therefore, the bacterial flora is likely to be dominated by Gram negative, psychrotrophic and psychrophilic bacteria like *Pseudomonas* spp., *Shewanella* spp., *Photobacterium* spp. (Bagge-Ravn et al., 2003, Gram and Dalgaard, 2002, Gram and Huss, 2000, Møretrø and Langsrud, 2017, Møretrø et al., 2016)(**Paper II**). Bacterial cells remaining on the surfaces after cleaning and disinfection may proliferate in humid environment, especially if organic matters are present, and subsequently contaminate the product. Which bacteria that will proliferate and dominate on the cleaned surfaces will depend on the surrounding conditions and the growth demands of the bacteria. Additionally, there will be a constant and inevitable re-introduction of new bacteria entering the facility together with the salmon and water that are pumped into the facility during production.

When analyzing isolates to species/strain level, as we did in **Paper II**, the recontamination from equipment to fillet is not apparent. Only five of eighteen strains of the species/strains identified on the salmon fillets were detected also in the equipment on the same day, but three of these strains were detected at several sampling points the same day and can be considered as dominant in the facility on that day. The principle of transfer of bacteria from surface to product has been demonstrated in several experimental settings (Midelet and Carpentier, 2002, Midelet and Carpentier, 2004, Sheen, 2008, Truelstrup Hansen and Vogel, 2011) and this contamination route is, and must be, acknowledged. Møretrø et al. (2016) concluded in their study that a recontamination with *Pseudomonas* spp. and *Shewanella* spp. from the equipment to the finished salmon fillet had happened.

5.2 Spoilage bacteria associated with biofilm

Bacteria in the genera *Pseudomonas*, *Shewanella, Serratia* and *Photobacterium* are known spoilage bacteria in cold-stored fish (Alfaro et al., 2013, Dalgaard et al., 1997, Gram and Dalgaard, 2002, Lerfall et al., 2018, Parlapani and Boziaris, 2016, Parlapani et al., 2015).

The significance of *Photobacterium* spp. and specially *Ph. phosphoreum* in spoilage of fish and seafood has been well documented (Dalgaard, 1995, Dalgaard et al., 1997) and, it has been reported as the main spoilage bacteria in vacuum and modified atmosphere packaged salmon (Jääskeläinen et al., 2019, Macé et al., 2012) and saithe (Lerfall et al., 2018). However, this important spoilage bacterium is not associated with the FPE and is reported to be absent from processing equipment in salmon processing plants, even though it was highly present in seawater and fish entering the plant, on the fish throughout the process line and, on the finished product (Møretrø et al., 2016)(**Paper II**). This indicate that bacteria of this genus are not an important part of the persistent bacterial communities in FPE.

Shewanella spp. are gram negative, facultatively anaerobic rods found in both freshwater and seawater. They are commonly found on the surface of live fish and are therefore commonly found in the fish processing environments (Møretrø et al., 2016). In this work *Shewanella* was not detected in the processing equipment (**Paper II)**, but a few isolates were detected from the inlet water and from the skin of the fish when samples were inoculated onto *Pseudomonas* CFC agar (**Paper III**). There might be a negative bias in this because we identified isolates grown on L&H agar in **Paper II** and isolates from *Pseudomonas* CFC agar in **Paper III**. *Shewanella* spp. are more inclined to be detected from Iron agar where they will grow as black colonies due to production of H_2S. Colonies from Iron agar was not isolated in this work and the abundance of *Shewanella* spp. might therefore be underestimated.

However, the quantification of H_2S producing bacteria revealed very low numbers of these in the processing equipment (**Paper II**). H_2S producing bacteria were mostly detected on the non-food contact surfaces (drains, waste funnels, inlet water, gutting machine suction) and, on a few occasions on the head cutter knife. But these bacteria were also detected on the fish skin, gills and on the fillet. This is in concordance with the findings of (Møretrø et al., 2016) who detected *Shewanella* on the fish, conveyors and other equipment in the slaughter department and on the ice stored fillets. Bacteria in the genus *Shewanella* are normally not pathogenic, but they are associated with spoilage of fish and fish products (Dalgaard, 1995, Gram and Dalgaard, 2002, Hozbor et al., 2006). Particularly the species *S. putrefaciens* is known to cause spoilage of fish by producing trimethylamine and a bad odor and thus cause spoilage of the fish product (Dalgaard, 1995, Jørgensen and Huss, 1989), but also *S. baltica* has shown a significant spoilage potential in fish (Fu et al., 2018, Vogel et al., 2005). *Shewanella*'s importance in the persisting bacterial communities is difficult to assess based on the findings in this work. However they are present in the processing environment and Møretrø et al. (2016) detected *Shewanella* isolates with identical sequence profiles (394 bp of 16S rRNAgene) both in the processing equipment and on fillets. But most likely this contamination comes from the raw material and not because of persistence in the equipment.

Serratia spp. are Gram negative bacteria belonging to the family *Enterobacteriaceae*. At the time of writing there are 23 species validly published under LPSN (Parte et al., 2020). Some of these (*S. liquefaciens*, *S. grimesii*, *S. proteamaculans*, *S. quinivorans*, *S. ficaria*, *S. fonticola* and *S. plymuthica*) are known to grow at low temperatures and CO_2-enriched anoxic atmospheres (Schuerger, 2016) and, they can be prevalent in vacuum packaged and MAP food products (Säde et al., 2013). Both *S. proteamaculans* and *S. liquefaciens* is present in various spoiled seafood products, also in unprocessed salmon (Begrem et al., 2021) and has been reported to have a high spoilage potential (Begrem et al., 2021, Joffraud et al., 2006, Macé et al., 2012). I this work *S. proteamaculans* was detected on a few occasions on food contact surfaces and once in inlet water (**Paper II** and **III**). *S. liquefaciens* was detected only in the last two samplings. But at these two samplings it was detected at three and six sampling points respectively, in addition to the salmon fillet from the last sampling (**Paper II**). Additionally, *S. liquefaciens* and a few *S. fonticola* were detected from fish skin samples grown on *Pseudomonas* CFC agar (**Paper III**). This indicate that *S. liquefaciens* can become a considerable part of the persisting bacterial communities in a FPE when first introduces but, according to this study, they are probably not among the first colonizers. Considering the

spoilage potential they possess, and that they seemingly can contaminate from the equipment to the fish fillet, they are likely to have a high impact on the shelf-life of the finished product.

Pseudomonas spp. are ubiquitous in the environment and they inherent many different properties that makes them of special interest to control in FPEs (**Paper II, III, IV**). i) Most species of this genus are psychrotrophic or psychrophilic and therefore grow well in cold-stored food (Molin and Ternström, 1982), ii) they are known to be able to form biofilm in a production environment and on a number of different surfaces (Tolker-Nielsen, 2004, Wang et al., 2018, Weiss Nielsen et al., 2011)(**Paper IV**), iii) they have modest nutritional requirements and, they often have a high tolerance or resistance to various antimicrobial agents (**Paper III** and **IV**). Some of these properties will be discussed more in detail later.

Pseudomonas spp. has been shown to have a pronounced spoilage potential in various food products (Maes et al., 2019, Raposo et al., 2016) as they can produce various volatile compounds and shows a high enzyme activity of both proteases, lipases, pectinases and lecithinases (Kumar et al., 2019, Rajmohan et al., 2002). *Pseudomonas* spp. is often pointed out as one of the main spoilage bacteria in both milk and dairy products (Arslan et al., 2011), meat and poultry (Hinton et al., 2004) and in seafoods (Jääskeläinen et al., 2019, Macé et al., 2012, Mikš-Krajnik et al., 2016, Xie et al., 2018). Due to their ubiquitous nature and high biofilm forming capabilities (**Paper IV**) they are commonly found in the FPE for various food groups, both meat and dairy (Stellato et al., 2017), poultry (Hinton et al., 2004) and, especially in seafood facilities they are often found to be the dominating bacteria (Bagge-Ravn et al., 2003, Møretrø et al., 2016)(**Paper II**).

Even though the *Pseudomonas* genera are highly associated with all different food groups there are indications that different species are associated with different food types and/or processing types. Stellato et al. (2017) found that *P. fragi* and *P. fluorescens* was dominating in both meat and dairy environment, and that the two species co-occurred in the same micro-environments. Møretrø et al. (2016) found that *P. gessardii* and *P. libanensis* were the most prevalent in salmon and the salmon processing environment. In our study *P. fluorescens*, or species closely related to *P. fluorescens*, seemed to be the most prevalent, but with several different strains present (**Paper II** and **III**). Stellato et al. (2017) suggests that different strains of the same species have different adaptation efficiency, which lead to different resilient bacterial communities at different places. Also Møretrø et al. (2016) detected isolates with different sequence profile at the different sampling spots. This kind of "point-specific

communities" was also indicated in the results from **Paper II** and **III**. In **Paper II** we saw that different strains were detected from different sampling points, but often at high proportions.

The study of biofilm forming capability in **Paper IV** showed that the different strains had variating ability to form biofilm under the given conditions. The surrounding conditions will also affect which strains that will thrive best. Liu et al. (2015) studied the biofilm forming characteristics of *P. lundensis* and found that the biofilm production was much higher at 4 °C than at 30 °C, and Xie et al. (2018) showed that *P. fluorescens* have a higher spoilage potential at 4 °C than at 30 °C. Adding up all the discussed issues, it seems that members of the genus *Pseudomonas* is one of the most important groups of bacteria to control in the salmon processing environment.

5.3 Pathogens in biofilm

As mentioned in chapter 1, many potential pathogenic bacteria such as *Escherichia coli*, *Listeria monocytogenes*, *Aeromonas* spp., *Vibrio* spp. have been associated with production of biofilm (Blackman and Frank, 1996, Bonsaglia et al., 2014, Jeong and Frank, 1994, Mizan et al., 2015, Møretrø and Langsrud, 2004, Rode et al., 2007, Silagyi et al., 2009, Stepanović et al., 2004).

Despite this, it seems that *L. monocytogenes* is the one that are most associated with prolonged persistence in FPEs (Brandt, 2014, Fagerlund et al., 2021, Ferreira et al., 2014, Vázquez-Sánchez et al., 2017). Which bacteria that causes most challenges for the food producing companies will vary due to which product is being produced, raw material, temperature at different steps and numerous other factors, not to forget domestic processing conditions and how the product is meant to be treated before eating. In the Norwegian salmon industry *L. monocytogenes* is the one pathogenic bacterium that causes most concern. There are several reasons for this: i) *L. monocytogenes* is the causative agent for listeriosis. In healthy adults, the infection dose is relatively high (> 10,000 cells) and the disease is mild or asymptomatic (FHI, 2017). In children, elderly and other immune compromised, the infective dose is much lower, and the disease can become very serious or even deadly. Pregnant women who are infected can also be asymptomatic, but the infection can cause harm to the unborn child, premature birth or stillbirth. ii) *Listeria* spp. has the ability to grow and multiply, even at low temperatures. Although cold storage (4 °C) will slow down the growth, it will not prevent the bacteria from multiplying in the product. This means that especially lightly processed products that are stored for a prolonged time, such as cold smoked or cured

fish are particularly high-risk products in regard to this bacterium. iii) It has been shown that *L. monocytogenes* and other *Listeria* species have the ability to form biofilm on surfaces of stainless steel, glass, PVC (Hingston et al., 2013, Szlavik et al., 2012, Truelstrup Hansen and Vogel, 2011) and, iv) *Listeria* spp. is very tolerant to desiccation, high salt concentration and variation in pH (Hingston et al., 2017, Truelstrup Hansen and Vogel, 2011, Vogel et al., 2010). These points together make the salmon industry's concern about *L. monocytogenes* in its product very real as it makes the lightly processed salmon product the ideal home for the bacterium.

Listeria monocytogenes and other *Listeria* spp. are commonly found in soil, fresh water and seawater, drains and sewage (Linke et al., 2014, Sauders et al., 2012) and, as it is present in seawater it is more or less inevitable that the bacteria enters the salmon processing facilities when the fish and seawater is pumped in. A Norwegian study that was conducted to register the prevalence of *Listeria* in seawater and equipment installations associated with the salmon in sea pens pointed towards dead fish and the well boats used to transport the fish from sea pens to slaughter facilities (Haldorsen, 2019). Additionally, they detected *L. monocytogenes* in the fish feed. This can be some of the reason why the gutting machine suctions are reported as a high-risk spot and problematic area for *L. monocytogenes* contamination (Løvdal, 2017) (**Paper I** and **II**). During our sampling for **Paper II** all the samples were analysed for the presence of *Listeria* spp. We detected *L. innocua* at two occasions, but no *L. monocytogenes*. However, *L. monocytogenes* was detected in the facility during production by the facilities own QC-personnel. This indicates that there were transient strains of *L. monocytogenes* entering the facility during this time period. As mentioned in **Paper II**, the facility had a problem with reoccurring *L. monocytogenes* in the gutting machines which led to an extraordinal disassembly and cleaning of the equipment. The same problem reoccurred later in several of the gutting machines at the facility and in **Paper I** we compared 20 *Listeria* isolates that had been isolated from the gutting machines and downstream in the processing lines. Our results showed that 15 of the isolates were most probably the same strain, indicating that this strain might have been established in the equipment at that time or, the same strain was reintroduced from an external source several times (**Paper I**).

A lot of research has been conducted in order to understand why *L. monocytogenes* can persist in various FPEs for a prolonged time. It is of interest to understand the mechanisms involved, as well as to find ways to prevent it. Recent research has indicated that the composition of the background microbiota in the environment is just as important for the

establishment of *L. monocytogenes* as the specific traits of each *L. monocytogenes* strains, and that a good match between the background microbiota and the specific *L. monocytogenes* strain is of high importance (Fagerlund et al., 2021, Lianou et al., 2020). This highlights the need for more knowledge about background microbiota in FPEs at species and/or strain level.

5.4 Interspecies interactions in biofilm and bacterial communities

Interactions between the inhabitants of a biofilm is of high interest and not very well understood (Burmølle et al., 2014, Giaouris et al., 2015). It is difficult to study such interactions because they may depend on several different factors that can be difficult to mimic in laboratory experiments. However, several studies have been done but each study can only cover a small part of the picture and the results are difficult to compare and sometimes pointing in different directions.

Multispecies biofilms has been described as the bacteria's "protective clothing" in extreme environments (Yin et al., 2019), and several mechanisms for this has been described. A review paper by Sanchez-Vizuete et al. (2015) describes four main mechanisms that can protect pathogens in multispecies biofilms against disinfectants and points out a few important ones. One is that the extracellular matrix makes the cells in the biofilm protected due to reduced diffusion of the antimicrobial agent, leaving the inner layers of the biofilm well protected. The protective effect of the matrix can be enhanced by some specific compound produced by one species (Flemming, 2011). Secondly, the spatial organization of the inhabitants in the biofilm will affect the bacterial survival when exposed to antimicrobial agents. The occurrence of persister cells is an example of this mechanism. Persister cells are subpopulations of dormant of slow-growing variants of regular cells that are highly tolerant to antimicrobial agents (Lewis, 2010). For these cells chemicals attacking cell division will have reduced effect because the cells are not dividing, and chemicals attacking the cell membrane will not reach the cells at high enough concentration to cause damage. Thirdly, interspecies communication like Quorum Sensing (QS) is when specific compounds produced by some bacterial cells induce a coordinated response for development of genetic competence, regulation of virulence or biofilm formation (Jayaraman and Wood, 2008). Such cell-to-cell communication has been suggested to induce the expression of genes that can increase the resistance to the antimicrobial agent in the biofilm (Hassett et al., 1999). Finally, genetic plasticity is pointed out as a mechanism that aids the survival of pathogens in multispecies biofilms. This is based on the physical proximity of cells of different species and the presence of extracellular DNA (eDNA) in the matrix will facilitate HGT between species.

HGT has been pointed out as an important driving force of the spreading of antimicrobial resistance genes (Barlow, 2009). Chiang et al. (2013) demonstrated that the extracellular DNA produced by *P. aeruginosa* worked as a shield against aminoglycosides because the negatively charged extracellular DNA binds the positively charged aminoglycoside.

A special concern when it comes to multispecies biofilms in FPE is that the biofilms protect potential pathogens against the regular cleaning and disinfection procedures. This principle was demonstrated in **Paper IV** where a multispecies biofilm of five different *Pseudomonas* spp. with variable tolerance to a PAA disinfectant were grown in biofilm together with *L. monocytogenes*. The survival of *L. monocytogenes* after disinfection treatment increased drastically when grown in the multispecies biofilm then when grown in biofilm alone. In the described experiment we suspected the high tolerance towards the PAA disinfectant in some of the *Pseudomonas* strains that were used to be the cause of the biofilm survival. I addition, *L. monocytogenes* has been shown to migrate to the inner layers of a biofilm (Fagerlund et al., 2017, Puga et al., 2016), which we also suspected was the case in our study.

The survival and growth of *L. monocytogenes* in dual or multispecies biofilm with regard to FPEs has been studied a lot and the results indicate that the faith of *L. monocytogenes* strongly depends on the background microbiota. Carpentier and Chassaing (2004) grew *L. monocytogenes* in duo-culture biofilms with 29 different bacteria isolated from FPE after cleaning and disinfection (C&D). Of these strains 16 decreased *L. monocytogenes* CFU counts in biofilm, 11 strains had no obvious effect and four strains seemed to increase *L. monocytogenes* biofilm CFU counts. Hassan et al. (2004) demonstrated that *L. monocytogenes* survived at higher numbers on a condensate forming surface when attached in a pre-existing *Pseudomonas putida* biofilm than alone. Similarly, Guðbjörnsdóttir et al. (2005) showed that colonisation of *L. monocytogenes* on smooth stainless steel surfaces was enhanced in the presence of mixed *Pseudomonas* spp. biofilm. Daneshvar Alavi and Truelstrup Hansen (2013) studied the dynamics of biofilm formation and survival after desiccation of *L. monocytogenes* in dual species biofilm with three common spoilage bacteria (*P. fluorescens, S. proteamaculans* and *S. baltica*) and concluded that the fate of *L. monocytogenes* during formation of biofilm and desiccation depended on the characteristics of the co-cultured bacterium. Puga et al. (2016) demonstrated that an old biofilm of *P. fluorescens* changed its structure to become more compact when *L. monocytogenes* was introduced into the biofilm, rendering the biofilm more resistant to antimicrobials, while (Giaouris et al., 2013) shows that the presence of *L. monocytogenes* in dual biofilm strongly

increases the resistance of *P. putida* towards the disinfectant agent benzalkonium chloride. It has also been shown that different strains of *L. monocytogenes* shows large variation of competitive growth when grown together with various combinations of background microbiota (Heir et al., 2018). All these findings underpin the relevance and importance of analysing the bacterial communities in FPE more detailed than before and preferably down to species or stain level. It also highlights the need for evaluating cleaning and disinfection agents not only on single strains, but on multispecies biofilms with bacterial strains relevant for FPEs.

5.5 Spatial and temporal variations in microbiota

It is now an established truth that most FPEs are not sterile but rather host a small amount of persistent (according to a loose definition) or transient bacteria/microorganisms. It is also general accepted that old surfaces are more prone to biofilm growth/residing bacteria than new and smooth surfaces. Before starting the sampling in the salmon processing facility**,** we hypothesized that the number of resilient bacteria would increase over time and that a more or less stabile microbiota would establish over time. We did see an increase in the general bacterial load over time (**Paper II**). When analysing FCSs in slaughter department, FCSs in filleting department and, NFCSs separately the increase was most obvious in FCSs in slaughter department. In the filleting department the increase was not as distinct. A reasonable explanation for this is that in the salmon processing facility the processing line is split into six lines in the filleting department. We sampled only one line, but the same line each time. During this first year of production in the facility, the facility was not always run at full capacity, which led to the filleting line of our focus was not used every day. This means that the surfaces in the filleting department were not exposed to the same wear and tear as the surfaces in the slaughter department. During the sampling we also registered a high variability in CFU counts between each sampling at the same sampling points (**Paper II**). This we believe are explained by rotating, intense cleaning routines at NFCSs and by minor variations in manual cleaning procedures on FCSs.

For the microbial colonization of a new environment, it has previously been described that the initial period will be characterized by a fluctuating microbiota with high diversity before a more stable microbiota is established (Cobo-Díaz et al., 2021, Lax et al., 2014). In **Paper II** we documented that *Pseudomonas* was the dominating genera overall. It was present in 92 % of the samples. Over time the relative abundance of *Pseudomonas* decreased from 69 % form the sampling before start-up to 31 % one week after start-up. At the sampling done 5 months

after start-up the relative abundance of *Pseudomonas* was 50 %, then it decreased to 48 % and 41 % for sampling 10 and 12 months after start-up. This is quite similar to what Cobo-Díaz et al. (2021) described for a newly started meat processing facility and it indicates that the genus *Pseudomonas* is an important part of the initial colonizing bacteriota in different FPEs even though it varies a bit in the relative abundance from one sampling to the other. The spatial distribution of bacteria showed that *Pseudomonas* was detected on all the different surfaces, and it was found in 93 % of the analysed surface samples. This again shows that this genus is very important in the colonization of FPEs.

Most studies of the microbiota in FPEs only report the present bacteria on genus level (Cobo-Díaz et al., 2021, Johnson et al., 2021, Zwirzitz et al., 2020). In this work we attempted to analyse isolates down to species or even strain level. This approach revealed highly point-specific bacterial communities (**Paper II**). We documented a low diversity within each sample but a high diversity between samples. Only a few of the species/strains detected were detected at several sampling points and time points, and the few strains that reoccurred over time did not reoccur at the same sampling point. A probable explanation for this is that in this case we still had the fluctuating microbiota and did not have any stable bacterial communities at the sampling points tested. It is however reasonable to believe that a more stable microbiota could have been detected if the sampling had continued for a longer period or if we had tested at other sampling points as some more unreachable, non-food contact surfaces (NFCS) and worn-out parts.

6 Bacterial tolerances towards antimicrobial agents

Good cleaning and disinfection routines in food processing facilities are of highest importance and the disinfectants used should be highly efficient at inhibiting the bacteria present. Some widely used disinfectants in the food industry contains glutaraldehyde, sodium hypochlorite, quaternary ammonium compounds (QAC) or peracetic acid (PAA) as main active ingredient. In Norwegian salmon industry QAC-based or PAA-based disinfectants is most used. PAA is a strong oxidizing agent that disrupts several important functions in the bacterial cells like the membrane and intracellular proteins (Kitis, 2004), while QACs primarily disrupts the cell membrane (Gerba, 2015). In addition to the main active ingredient, commercial disinfectants also contain other chemicals such as surfactants that can help increase the efficiency of the disinfectant, or stabilizers to prevent deterioration of the active ingredient (Briñez et al., 2006). Some of these additives, e.g hydrogen peroxide and acetic acid used to stabilize PAA, or EDTA added in QAC disinfectants, may provide additional antimicrobial effect and/or increase the effectiveness of the main active ingredient (Cruz and Fletcher, 2012, Langsrud and Sundheim, 1997). In the assessment of disinfectants, it is therefore important to consider both the effect of the commercial product as well as the specific effect of the main active ingredient.

Over the years, concerns have been raised on whether the extensive use of disinfectants and antimicrobial agents can cause the exposed bacteria to adapt and increase their tolerance towards the agent, and even become resistant to the agent over time (Langsrud and Sundheim, 1997, Aase et al., 2000). There are also studies on antimicrobial resistance (AMR) that indicates cross-resistance between disinfectants and antibiotics (Langsrud and Sundheim, 1997, Lemaître et al., 1998, Soumet et al., 2012), highlighting the urgence to study and monitor the level of tolerance and resistance in food associated bacteria.

Antibiotic resistance and tolerance towards disinfectants are increasingly often discussed in connection with each other, and there are some differences and concept definitions that need to be established. A recent review from Bland et al. (2022) highlights the need for clarification of the terminology used and suggests the following: Antimicrobial is a general term for substances that effectively prevents growth of or that kills microorganisms. Antibiotics are antimicrobial agents used to combat infections in animals or humans, while pesticides are antimicrobial agents applied to the environment. Sanitizers and disinfectants are subcategories of pesticides. US regulations specify sanitizers for use on food contact surfaces and disinfectants mainly for use on non-food contact surfaces while European

regulations use the term disinfectants for both (Bland et al., 2022), as will I in the following text.

Regarding the concepts of resistance, tolerance, and susceptibility, CLSI (CLSI, 2022) and EUCAST (EUCAST, 2022) have established robust and defined systems for the assessment of antibiotics (used in **Paper III**). But for disinfectants there are no such system and a need for clarification of the terms is evident. Bland et al. (2022) argues in favour of reserving the term "resistant" for bacterial isolates that are not inhibited or inactivated at the recommended user concentration. The term "sensitive" should be used for strains that are inhibited or inactivated by the disinfectant at several orders of magnitude below the recommended user concentration, while "tolerant" could be used for isolates that are inhibited or inactivated by recommended user concentrations but shows better survival at higher concentrations than the sensitive isolates.

Common methods for investigating the resistance properties of bacterial strains towards antibiotics are disc diffusion susceptibility test or MIC (minimum inhibitory concentration) according to CLSI or EUCAST guidelines. When evaluating the efficiency of a disinfectant for use on FCSs the gold standard is that it must result in a 5 $\log_{10}$ reduction in cell number of specific test organisms (Bland et al., 2022, EPA, 2012). Harrison et al. (2010) presented a microtiter method for testing MIC, minimum bactericidal concentration (MBC), minimum biofilm eradication concentration (MBEC) and $\log_{10}$-reduction, that is suitable for testing both antibiotics and disinfectants. This procedure was used in **Paper IV** for analysing the efficiency of a PAA based disinfectant and for the antibiotic florfenicol. For disinfectants, MIC values are of less interest in food industry because the disinfected surfaces are usually rinsed with water to prevent residues of disinfectant in the food products, and resident bacteria on the surfaces will therefore not be exposed to the disinfectant for a prolonged time. On the contrary, MBC and MBEC values should be of high interest as these better reflect the scenario in a food processing facility. In contrast to disinfectants, antibiotics are meant to have a longer exposure time. Hence for florfenicol the MIC values are more relevant than MBC and MBEC values.

6.1 Tolerance and resistance towards disinfectants in FPE associated bacterial isolates

Cleaning and disinfection routines in food producing facilities do not aim to sterilize the surfaces but rather to reduce the number of bacteria to a minimum and to eradicate potential pathogenic bacteria. Antimicrobial efficiency tests are usually performed on monocultures of laboratory bacterial strains in planktonic state. In recent years a greater awareness around the

problems of biofilm in food processing facilities has risen and the need for strategies to eradicate biofilms is evident.

Bacterial communities in food processing environments are repeatedly subjected to a selective pressure that favours those communities and/or individuals that are able to adapt to the chemical stress imposed by cleaning agents and disinfectants. The mechanisms involved in increased tolerance to disinfectants are many, and not all of them are completely understood. The tolerance and adaptation to QACs has been frequently studied and several mechanisms has been documented. On an individual cell level, mechanisms that can facilitate such adaptation includes efflux pump systems (Jiang et al., 2016, Aase et al., 2000) and modification of fatty acid composition in the cell membrane, while on community level biofilm formation and production of extracellular polymeric substances (EPS) can aid all the different bacteria present (Flemming, 2011, Puga et al., 2016, Yin et al., 2019). Several genetic determinants for increased QAC tolerance have been described in different bacteria species (Jiang et al., 2017, Kücken et al., 2000, Møretrø et al., 2017, Sundheim et al., 1998). E.g. in *L. monocytogenes* the *qacH* and *brcABC* genes/gene complexes have been shown to provide increased tolerance to QACs (Møretrø et al., 2017). However, the biocidal concentration to kill bacterial strains harbouring these determinants is still much lower than user concentration and the significance of these genes are therefore debatable (Kastbjerg and Gram, 2012).

For PAA disinfectants there are also some studies demonstrating tolerances in a few bacterial strains (**Paper IV**)(Chang et al., 2005b, Zook et al., 2001). *E. coli* has been reported to adapt to the chemical and *P. aeruginosa* showed changes in the transcription of membrane proteins after exposure to sublethal concentrations (Chang et al., 2005b, Zook et al., 2001). The mechanisms behind this in not evident but multidrug efflux pumps are suspected to be involved. But, there are to date no reports of specific PAA resistance genes in any microorganisms (Bland et al., 2022).

There is a high variation in tolerance towards PAA disinfectants between different bacterial strains **(Paper IV**)(Fagerlund et al., 2017). In **Paper IV** we investigated 11 *Pseudomonas* isolates and three other isolates (*L. monocytogenes*, *S. liquefaciens*, *A. hydrophila*) for their tolerance against a PAA-based commercial disinfectant (Aqua DES Foam PAA) in both planktonic state and in biofilm (MIC, MBC and MBEC values). In this study we found that all isolates except one *Pseudomonas* isolate was inhibited in planktonic state by

concentrations 4- to 8-fold lower than the lowest concentration used in the salmon processing facility (1%). But, in biofilm state, four of the *Pseudomonas* isolates had continued growth after exposure to the lowest user concentration (1%) and, only one *Pseudomonas* isolate reached a 5 $\log_{10}$ reduction in viable cell count after exposure. According to the definitions stated by Bland et al. (2022) these isolates can be considered as tolerant to the disinfectant. One *Pseudomonas* isolate showed still considerable growth and did not reach a 5 $\log_{10}$ reduction after exposure to 4X the user concentration (4%) and must be considered resistant, according to Bland et al. (2022). Our findings here demonstrate a high variation in strain sensitivity to the disinfectant and there is a risk that some strains will not be inhibited by the regular C&D procedure. The reason why this one isolate inhabits this high tolerance towards the disinfectant is not known. The same isolate was subject to whole genome sequencing in **Paper III** and screened for acquired antimicrobial resistance determinants by ResFinder 4.1 (Bortolaia et al., 2020, Clausen et al., 2018, Zankari et al., 2017). No resistance determinants were found. However, in the disc diffusion assay it was characterized as resistant to antibiotics of three different antibiotic groups and hence classified as multidrug resistant (**Paper III**).

Resistance to oxidative disinfectants is mainly associated with biofilm growth (Langsrud et al., 2003a). Bridier et al. (2011) demonstrated by fluorescent staining and confocal laser scanning microscopy (CLSM) a uniform and linear loss of cell viability in *P. aeruginosa* biofilm exposed to PAA, indicating that the greater resistance of biofilm cells is not due to limitations of penetration of the chemical. Multidrug efflux systems as the MexXY-OprM in *P. aeruginosa* are believed to take part in the bacterial cells act of escaping the action of chemicals (Poole, 2007). It has been demonstrated that the oxidative stress imposed on the bacteria when exposed to PAA or hydrogen peroxide stimulates the expression of a gene called PA5471 that is required for MexXY expression (Chang et al., 2005a, Chang et al., 2005b). This means that the bacteria upregulate the MexXY-OprM efflux system when exposed to PAA and thereby pumps the chemical agent out of the cell and escapes the damaging effect.

If this mechanism, or a similar mechanism, is the case in the high-tolerant *Pseudomonas* isolate in **Paper IV**, or if the high resistance is only due to biofilm formation and extracellular polymeric substances is not known. Langsrud et al. (2003b) discovered that a *P. fluorescens* isolate from food industry developed higher resistance to dodecyl dimethylammonium chloride (DDAC) when exposed to gradually higher concentrations. The

increased resistance was followed by loss of flagella and increased slime production and additionally cross resistance to several antibiotic agents but, not to PAA. It is however likely that increased slime (EPS) production as a stress response can result to increased tolerance also to PAA.

6.2 Multispecies biofilms show increased tolerance to disinfectants

There are numerous studies on how various pathogens survive in multispecies biofilm and on how the multispecies communities/biofilms shelter the pathogens from external stress. Several mechanisms for interspecies interaction in biofilms have been described (Burmølle et al., 2014, Sanchez-Vizuete et al., 2015). Many of these studies use laboratory strains in their experiment, but in the recent years it has become more common to imitate the food production environment by constructing the multispecies biofilms based on isolates from the food processing environment of interest, and to adjust experimental conditions to be more similar to the FPE. It is however difficult to mimic all the factors from the FPE in a laboratory experiment. One will need to choose which parameters to mimic and which to overlook based on the questions that seeks to be answered. To be able to understand the interactions in bacterial communities under various stress exposure, it is of huge help to know the reactions in each individual strain involved.

In **Paper IV** we demonstrated how a *L. monocytogenes* strain in single species biofilm was totally eradicated by exposure to a 1% PAA disinfectant, while the same strain grown in a multispecies biofilm of five *Pseudomonas* isolates, some with high tolerance to the disinfectant, survived the same treatment. In a study of multispecies biofilms based on isolates from the meat industry, Fagerlund et al. (2017) showed that *L. monocytogenes* together with *P. putida* and *P. fluorescens* survived C&D routines better than other representative isolates from the same environment. There are many studies on *L. monocytogenes* tolerance towards disinfection agent but, as summarized in the review by (Bland et al., 2022) there are to date no proof of resistance in *L. monocytogenes* to any disinfectant. The results stated in **Paper IV** supports this conclusion but in addition, we demonstrate how the tolerance or resistance properties of other bacteria, in this case *Pseudomonas* spp., shelters *L. monocytogenes* against the disinfectant.

Most studies on multispecies biofilm resistance or tolerance towards various disinfecting agents shows that multispecies biofilm are more resistant than single species biofilms (Li et al., 2021). The interactions within the biofilm can be both cooperative, competitive or neutral. The growth rates of the five *Pseudomonas* isolates used in multispecies biofilm in **Paper IV**

were tested under the same conditions as used in the biofilm experiment. They all had a very similar growth curve when grown alone and when grown together the mixed culture had a slightly steeper curve and reached a slightly higher max (data not published). This indicates a neutral relationship between these strains. But when plating the culture just before it reached stationary phase showed that it varied a lot between the parallels which of the strains that was dominating. This points towards a basic internal competition of nutrients. *L. monocytogenes* grown together with the *Pseudomonas* mix in the biofilm experiment reached significantly lower CFU in both suspension and biofilm than when grown alone. However, the relative number of *L. monocytogenes* to *Pseudomonas* in biofilm increased over time, while decreasing in suspension (**Paper IV**). This can be explained by *L. monocytogenes* moving to the inner layers of the biofilm, as has been documented by Fagerlund et al. (2017) and (Puga et al., 2016).

6.3 Antibiotic resistance in bacteria associated with salmon processing environment

The bacterial flora isolated from the salmon processing facility is likely to be affected by the bacterial flora surrounding the live salmon in the sea pens, also when it comes to antibiotic resistance properties. In Norwegian aquaculture the most used antibiotics are florfenicol and oxolinic acid (NORM/NORM-VET2020). Also in Chilean aquaculture (second largest salmon producer in the world) florfenicol is the most used, followed by oxotetracycline (Miranda et al., 2018). The difference between the two counties' use of antibiotics is in the amount. In Norway an annual usage of 223 kg is registered (NORM/NORM-VET2020), while in Chile the annual usage between 2010 and 2019 was 143200-563200 kg (Soto, 2020). It is a general perception that the level of antibiotic resistance will be lower in areas with less use of antibiotics than in areas with high use as the selective pressure will be lower. Both Miranda and Rojas (2007) and Fernández-Alarcón et al. (2010) found high levels of resistance against florfenicol in bacteria associated with salmon aquaculture in Chile. In both studies the resistance was suspected to be caused partly by the specific resistance gene *floR* and partly by intrinsic resistance mechanisms. Also in both studies, the main proportion of resistant bacteria belonged to *Pseudomonas*.

In **Paper III** we investigated the antibiotic resistance properties of isolates from a salmon slaughterhouse isolated from *Pseudomonas* CFC selective agar by disc diffusion assay. Of these isolates 68 % belonged to *Pseudomonas* and the rest to *Aeromonas*, *Acinetobacter*, *Morganella*, *Serratia*, *Shewanella*, *Stenotrophomonas*, and *Pseudoalteromonas* or the family *Enterobacteriaceae*. This analysis demonstrated a high level of resistance among both the

Pseudomonas isolates and the non-*Pseudomonas* isolates towards the β-lactams ampicillin (AMP) and amoxycillin (AML). Additionally, a high level of resistance was observed among the *Pseudomonas* isolates towards oxolinic acid (OA) and florfenicol (FFC) with 92% and 84% respectively. Among the non-*Pseudomonas* isolates the level of resistance to OA and FFC was 22% and 6% respectively. In **Paper IV** the MIC, MBC and MBEC values for some of the same isolates were investigated and two isolates showed MIC values of 2400 µg/mL or higher and additionally seven isolates had MIC values between 150-300 µg/ml. This high level of resistance was rather surprising. Even though most of these bacterial species are not the cause of any infection of where florfenicol or oxolinic acid is used as treatment, it is important to monitor the resistance among bacteria associated with aquaculture. I also want to point out here the similarity of several of our florfenicol resistant isolates to the newly described fish pathogen *Pseudomonas* sp. J380 strain commented on in **Paper III**.

6.4 Detection of antibiotic resistance genes (ARGs)

Many antibiotic resistance properties are facilitated by the presence of specific antimicrobial resistance genes (ARGs). These genes are often located on plasmids or other transposable genetic elements in the bacteria's genome. This means that these genes are "easily" transferred between bacteria, both intra- and inter-species, by different mechanisms recapitulated as horizontal gene transfer (HGT) (Burmeister, 2015).

To detect ARGs one can run one or several specific PCRs with primers designed to target the gene of interest. This has been the regular way to detect ARGs for several years and it is still very useful if simply the detection of the gene or genes is the objective and if the genes you are looking for are known and few in number. But often one is interested in if a bacterial strain carries any AMR at all and in that case running a PCR for every possible ARG is extremely laborious. With the emergence of sequencing technologies, it has become more common to screen for ARGs by performing whole genome sequencing and mine the data for known antibiotic resistant determinants by comparing to a database. There are several of these databases available e.g., ResFinder, NCBI AMRFinder Plus, ARGminer, MEGAres, NDARO, SARG and CARD (Papp and Solymosi, 2022). Some of these databases contains only acquired resistance genes and some also include AMR associated mutations. Additionally, MEGAres includes biocide- and metal resistance genes (Papp and Solymosi, 2022) retrieved from the BacMet database (Pal et al., 2014). All of these databases require some level of bioinformatics skills to use. ResFinder is developed to be an easy-to-use webtool, with a curated database, where filtered sequencing read files or assembled genome

or contigs files can be uploaded on the webpage, and the results are received in few minutes. For this reason, it was the selected tool in search for antibiotic resistance gene in both **Paper I** and **Paper III**.

As mentioned in chapter 6.3, the *Pseudomonas* isolates analysed in **Paper III** showed phenotypic resistance to several different antibiotics in the disc diffusion assay. However, a search in ResFinder 4.1 database with WGS data from 30 of these isolates resulted in none detected antimicrobial resistance determinants. This is in contrast to the findings of Heir et al. (2021) in *Pseudomonas* isolates originating from Norwegian retail chicken meat, where they found a total of more than 70 different antibiotic resistance associated genes in 31 *Pseudomonas* isolates. The two most likely explanation is that (Heir et al., 2021) searched several databases in contrast to only one database as used in **Paper III** and, that the bacterial isolate from chicken had more of the acquired antibiotic resistance genes than isolates from the salmon processing facility.

7 Relevance for the food industry

This thesis has its focus on the food processing environment in Norwegian salmon industry, and all sampling has been done in one facility. But the challenges that are highlighted are relevant for all salmon processing facilities in arctic and subarctic areas, and many of the challenges is also recognizable for, and transferrable to, food processing businesses in other sectors or geographical areas.

7.1 The implication of biofilm problems in food industry

The most obvious implication of biofilm in the FPE for the food industry is the risk for transfer of biofilm bacteria to the food product, as commented in Chapter 5.1. The transfer of spoilage bacteria will have a negative effect on the shelf life of the product. Reduced shelf life means shorter window of when the product is available for customers, hence a risk for lower income. It also reduces the possibilities for export and transfer of the product over longer distances. Additionally, a shorter shelf life will increase the risk of the food products ending up as food waste. In case of a product being spoiled before the stated end of shelf-life date, the spoiled product might reach the customer and, by that, have negative implications in the company's reputation and subsequently reduced sale.

If pathogenic bacteria are present in the biofilm and transferred to the product this can of course threaten the health of the customers and potentially lead to serious food borne infections (Control), 2018, Ferreira et al., 2014, Ricci et al., 2018). Such incidents, if the source of infection is traced back to a specific product, the consequences for the company can be considerable with withdrawal of huge amounts of food products (Olsen et al., 2005).

From the research discussed in this thesis and the results presented in **Paper I-IV** it is clear that the cleaning and disinfection routines in food industry is essential to ensure safe food products of good quality. It is important that the disinfection agents used are highly efficient, also towards bacteria not necessarily seen as a threat and, towards multispecies biofilms.

7.2 The use of sequencing-based methods in the food industry

Conventional methods for analysing hygiene control samples and for pathogen detection in food industry are laborious and slow. As the demand for fresh food, and especially seafood, is increasing it is of high importance to get the quick answers on the microbiological samples so the fresh products can be released on the marked. Therefore, the interest for rapid and reliable methods are of high interest and sequencing -based methods has caught the interest of the food industry. In an international scale, some large companies have implemented WGS in

their surveillance of pathogens (discussed in Chapter 4.5). But in a Norwegian scale, only the largest food processing companies have access to their own labs for microbiological analysis. Other food companies rely on outsourcing this to external labs for such analysis. To my knowledge sequencing-based analysis is not done as routine analysis, but some labs are looking into it and the interest is rising. WGS is a powerful tool in the comparison of bacterial strains in surveillance and source tracking of pathogens and, the concept may seem as the ultimate method, it can in many cases be a bit over the top.

Some food processing facilities that have their own lab for microbial analysis, as the facility sampled in this work, store isolates when they detect pathogens like *L. monocytogenes*. The number of isolates can be high and, in situation where they suspect a persistent strain in the facility it can be of interest to analyse many isolates. In such situations the ON-rep-seq method (**Paper I** and **II**) can serve as a screening method of a high number of isolates before doing WGS, or it can even provide enough information for the facility without having to do WGS.

7.3 The food value chain as a carrier of antimicrobial resistance

Antimicrobial resistance (AMR) has been pointed out as one of the major public health challenges of the 21st century (ECDC, 2020, Murray et al., 2022) and, the food value chain has been pointed out as a source of transmission, development and spreading of AMR (Cobo-Díaz et al., 2021, FAO/WHO, 2019).

One of the key driving forces in the emergence of AMR is the extensive use and misuse of antimicrobial agents in many countries (O´Neill, 2014; WHO, 2019), both in human and livestock medicine. AMR bacteria may be introduced into the food chain as a result of disease treatment in livestock animals or growing produce/plants, the food chain may act as a carrier of these and subsequently expose the consumers to AMR bacteria (Cobo-Díaz et al., 2021, Hudson et al., 2017). Aquaculture systems has been described as “genetic hotspots” for gene transfer and spreading of ARGs (Watts et al., 2017). Antibiotics given to farmed fish are usually administered through the fish feed (Ibrahim et al., 2020) hence, unconsumed feed is sedimented on the sea bed close to the fish farms and this provides a selection for bacteria with high tolerance to antibiotics. This way, bacteria with AMR properties are moved down the seafood value chain and contribute to the dissemination of ARGs (Sørum, 2005).

Among the isolates analysed for antibiotic resistance in **Paper III** a high level of phenotypic resistance was detected, but when a selection of 30 isolates that showed resistance towards

four or more classes of antibiotics were whole genome sequenced and screened for acquired resistance genes, none such genes were detected. In this set of isolates the resistance is probably caused by intrinsic mechanisms as e.g., multidrug efflux pumps, and this type of resistance is not believed to be the cause of the spreading of antimicrobial resistance.

8 Concluding remarks and future perspectives

A newly opened salmon processing facility has an excellent starting point for keeping the processing equipment and environment clean and at a high hygienic standard. The background microbiota in a FPE is often overlooked because it mainly consists of innocuous bacteria "unknown" for the industry. The detailed knowledge of the bacterial communities in the food processing environment on species level can be significant for improving cleaning and disinfection routines, it will be of help to evaluate the shelf life of the product and it will be of help securing the food safety.

In **Paper II** we documented that most of the tested surfaces had low levels of bacterial contamination with a few exceptions and problematic spots. An overall increase in bacterial numbers were observed for contact surfaces in the slaughter department only. On contact surfaces in the filleting department, non-contact surfaces and on the fish no, such trend was observed but rather a high variation between different time points for sampling.

The bacterial flora detected was highly dominated by *Pseudomonas* spp. (46% of isolates from L&H medium) When classifying the bacterial isolates to species/strain-level by ON-rep-seq method we revealed that the bacterial communities were highly point specific with only a few species/strains that were detected several times.

One of the hygienic problematic spots we detected during the one-year sampling period was the gutting machine suction unit where a clear increase in detected bacterial contamination was observed over time. During the time of sampling the facility experienced reoccurring detection of *L. monocytogenes* in the same gutting machine as tested in this study and one other of the same design. The 20 *Listeria* isolates we analyzed in **Paper I** originated from the two gutting machines and their downstream processing lines. In our study we confirmed that 15 of the 20 isolates were indeed of the same strain and this probably spread from the gutting machines and further into the processing line. **Paper I** was also a proof-of-concept study to investigate if the newly developed ON-rep-seq method had potential in strain differentiation of *L. monocytogenes* strains. ON-rep-seq gave the same strain differentiation as WGS did, and further analyses on the WGS data with easy-to-use bioinformatic tools gave no further differentiation of the strains. By this we demonstrated that the recently developed ON-rep-seq method is a promising, rapid, cost-effective method for strain differentiation of *L. monocytogenes*, that can serve as a pre-screening of isolates before WGS or in some situations replace the need for WGS.

After applying the ON-rep-seq method to a larger and more diverse set of isolates in **Paper II** we suggest that ON-rep-seq has a potential in species-level identification for most bacteria in complex communities as food processing environments but, it may fall short on less studied species due to lack of available similar whole genome sequences and for some highly diverse and complex genera as *Pseudomonas*.

The most prevalent genus detected in the salmon processing environment was *Pseudomonas* (**Paper II**). Isolates of this genus were further characterized by their biofilm forming capacity (**Paper IV**), tolerance and resistance towards antimicrobial agents (**Paper III and IV**) and dynamic in multispecies biofilm (**Paper IV**). In **Paper III** we demonstrated that within the genus *Pseudomonas* the level of phenotypic antimicrobial resistance is high, with 92% an 84% of the Pseudomonas isolates being resistant to oxolinic acid and florfenicol respectively, two important antibiotics in aquaculture. Additionally, we demonstrated that the high level of resistance in this set of *Pseudomonas* isolates is most likely due to intrinsic properties like efflux pumps as none acquired resistance genes were detected by ResFinder in the 30 *Pseudomonas* isolates that were whole genome sequenced. Based on these and earlier described finding, these highly resistant *Pseudomonas* strains might pose an indirect risk to food safety even though they are not pathogenic themselves.

In **Paper IV** we demonstrate a high variation in the biofilm forming capabilities among the same set of *Pseudomonas* isolates under the current conditions. A randomly selected set of these isolates subjected to susceptibility testing towards a common disinfectant in salmon industry and the antibiotic florfenicol used in the salmon farming industry. The isolates demonstrated a high tolerance towards the PAA-based industry disinfectant and florfenicol. Additionally, in a multispecies biofilm experiment it was demonstrated that their strong and disinfection tolerant biofilm can serve as a shelter for other bacteria like the pathogen *Listeria monocytogenes*.

This work shows that bacteria in the background microbiota of food processing facilities can inhibit traits that i) makes them more persistent in the environment, ii) can aid in the protection of potential pathogens. This work also demonstrates an accurate and cost-effective method for strain-differentiation of the persistent pathogen *L. monocytogenes* that cause a lot of concern in the food industry. The method also has a great potential in strain-differentiation of many other bacteria however, shortcomings has been detected when it comes to unknown

or little described species that are not whole genome sequenced and, some highly complex genera like e.g., *Pseudomonas* where the taxonomy is diverse and unconfirmed.

Future perspectives:

The number of studies of microbial diversity, microbial communities and biofilm in food processing environments has increased during the last years and much knowledge has been gained. However, various food processing environments are not uniform. Each spot or area has its own complex microbial community with the internal interactions and external excitations that might cause. But there will usually be some common factors between all the differences in the details and it is important to both see the main lines and to find the possible important information hidden in the details. It is not realistic to believe that every food processing business should know exactly which bacteria that are present in the food processing environment at all times. But, having a fairly good knowledge about the dominating microbiota in the environment can be of significance for improving cleaning and disinfection routines by being able to target the present bacteria with the best suited cleaning and disinfection agents. This can subsequently help to improve both the shelf life and the food safety of the product.

The drastic development in sequencing technology has been crucial for all kinds of microbial diversity studies, also food microbiology related studies. It has been demonstrated that metagenomic shotgun sequencing can provide full genomes of bacteria present in the sample and provide strain-level resolution of complex communities (Ercolini, 2017, Sharon and Banfield, 2013). By applying such methods, we gain more insight, but we also find more questions for researchers to search for an answer. An important thing to keep in mind is how these methods and possibilities can be implemented in the food industry and how the possibilities are presented and communicated to the industry.

Some of the applications that sequencing technology provides are already in use by authorities at large scale in surveillance of pathogens and in investigation of disease outbreak. Some of the large international corporations with large research departments do also use such technologies. Smaller, national companies, like the one object for sampling in this thesis, do not use any sequencing service as a routine. They are however interested, but there is a gap between how these methods are used in research today and how they can be of use in an industrial setting. The obvious obstacles are the necessary investment in sequencing equipment, computer power and data storage and most of all, personnel with the

bioinformatic skills needed for analyzing the data. Additionally, much of the development on sequencing equipment has been on reaching a high throughput, while for the food industry a more flexible pipeline would be more preferrable.

A method like ON-rep-seq can be more manageable for a typical large Norwegian food producer, if the method is further streamlined and the personnel are well trained. It can relatively easy be implemented in their existing surveillance program for pathogens like *L. monocytogenes* as showed in **Paper I**. In addition, it can be used for other pathogens e.g. *E. coli*, *Salmonella* spp., and probably also *Campylobacter*, *Yersinia* and more. For the commercial analytical laboratories this could be a suitable method to offer to customers and use it as a pre-screening before doing WGS on selected isolates. There is however still a way to go before this technology and this method can be commonly applied in the food industry. For industrial application further effectivization and streamlining of the procedure will be necessary in addition to training of QC-personnel and establishing the infrastructure.

Another approach for rapid pathogen detection that has potential for implementation in already existing procedures is quasimetagenomics (Commichaux et al., 2021, Wagner et al., 2021). In this approach extraction of total DNA from short-time incubated enrichment medium is performed with subsequent metagenomic analysis. This is demonstrated to detect *L. monocytogenes* in samples from enrichment medium after only four hours of incubation in contrast to the regular 24-48 hours. This approach can also be relatively easy implemented in the analysis procedure commonly used today as it is based on sequencing of DNA from the enrichment step in the procedure already in use. But also, for this method, the obstacle is the data analysis and, development of easy-to use tools or programs for this will be necessary.

In case of analyzing the bacterial communities within food processing facilities, this can be very relevant for companies producing fermented or cured products, where the microbiota or the "domestic flora" might have a more direct impact, and a desirable impact on the food product(s), e.g., dairies (cheese, yogurt), breweries (wine, beer) or smoke houses (smoked and cured salmon). In the type of salmon processing facility where the sampling for this thesis was done the perception of the importance in knowing the background microbiota might be lacking today. Their focus is on the pathogens and how to combat them. But with the recent research pointing out the importance of the background bacterial communities and how the properties of these can affect the survival and persistence of pathogens, this can change. And maybe, further up the road, sequencing analysis of hygiene samples will be

everyday routine in most food processing companies. As sequencing technology emerges it is reasonable to believe that various new sequencing-based methods for early detection and species/strain differentiation will be suggested.

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ORIGINAL ARTICLE

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ON-rep-seq as a rapid and cost-effective alternative to whole-genome sequencing for species-level identification and strain-level discrimination of *Listeria monocytogenes* contamination in a salmon processing plant

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Abstract

Identification, source tracking, and surveillance of food pathogens are crucial factors for the food-producing industry. Over the last decade, the techniques used for this have moved from conventional enrichment methods, through species-specific detection by PCR to sequencing-based methods, whole-genome sequencing (WGS) being the ultimate method. However, using WGS requires the right infrastructure, high computational power, and bioinformatics expertise. Therefore, there is a need for faster, more cost-effective, and more user-friendly methods. A newly developed method, ON-rep-seq, combines the classical rep-PCR method with nanopore sequencing, resulting in a highly discriminating set of sequences that can be used for species identification and also strain discrimination. This study is essentially a real industry case from a salmon processing plant. Twenty *Listeria monocytogenes* isolates were analyzed both by ON-rep-seq and WGS to identify and differentiate putative *L. monocytogenes* from a routine sampling of processing equipment and products, and finally, compare the strain-level discriminatory power of ON-rep-seq to different analyzing levels delivered from the WGS data. The analyses revealed that among the isolates tested there were three different strains. The isolates of the most frequently detected strain (n = 15) were all detected in the problematic area in the processing plant. The strain level discrimination done by ON-rep-seq was in full accordance with the interpretation of WGS data. Our findings also demonstrate that ON-rep-seq may serve as a primary screening method alternative to WGS for identification and strain-level differentiation for surveillance of potential pathogens in a food-producing environment.

KEYWORDS
foodborne pathogen, *Listeria monocytogenes*, ON-rep-seq, Oxford Nanopore technology, whole-genome sequencing

1 | INTRODUCTION

Intra-species variability exists in the bacterial genome (Abee et al., 2016; Sela et al., 2018) and therefore strain-level discrimination of pathogens is a key factor for the identification and subsequent elimination of a contamination source at a food processing plant. The significance of *Listeria monocytogenes* as a foodborne pathogen is well documented (Buchanan et al., 2017; Farber & Peterkin, 1991; Gandhi & Chikindas, 2007), and through the years different microbial typing methods, more or less labor-intensive, have been used to identify and differentiate this pathogen at the strain level (Jadhav et al., 2012; Wiedmann, 2002). During the last decades, development in sequencing technologies and whole-genome sequencing (WGS) has rapidly been changing bacterial strain identification analysis in the food industry. WGS is now becoming a more available and affordable molecular tool and is proposed to be the new primary typing tool for strain identification of *L. monocytogenes* (Moura et al., 2017). It has already been successfully used to investigate and characterize outbreaks of listeriosis (Jackson et al., 2016; Kvistholm Jensen et al., 2016; Schjørring et al., 2017). *L. monocytogenes* is a highly heterogeneous, omnipresent, psychrotolerant pathogen (Moura et al., 2016), able to survive and persist in food processing plants for years (Fagerlund et al., 2016). The possibility of *L. monocytogenes* contamination in food products from residual cells in the equipment represents a serious concern, especially in the ready-to-eat (RTE) food industry (EFSA, 2018; Fonnesbech Vogel et al., 2001). Many food processing plants have therefore implemented a comprehensive testing regime to detect this pathogen in raw materials, processing environment, equipment, and food products (Carpentier & Léna, 2012; EuropeanCommision, 2005). Whenever a food processing plant experience frequent detection of *L. monocytogenes* it raises the question of whether the contamination is due to a persistent strain or transient strains. Identification at the strain level and source tracking are therefore crucial to recognize possible "hot spots" for accommodating the pathogen.

Sequence-based typing, and in particular whole genome sequencing (WGS), are proposed to replace pulse-field gel electrophoresis (PFGE) as the primary typing method for *L. monocytogenes* (Moura et al., 2017) as well as for other foodborne pathogens (Oakeson et al., 2017). Nevertheless, PFGE, MLST (multilocus sequence type), and other typing methods will remain relevant techniques for smaller laboratories also in years to come (Neoh et al., 2019) because of the significant investments necessary to implement WGS in strain typing (Nouws et al., 2020).

In theory, WGS can differentiate strains on a single nucleotide level and it has a resolution superior to PFGE and MLST (Salipante et al., 2015; Stasiewicz et al., 2015), and is gaining support in both outbreak investigation, surveillance, and source tracking of pathogenic bacteria (Nadon et al., 2017; Van Walle et al., 2018; Zhang et al., 2020). So, WGS analysis generated with short-read technology offered by Illumina sequencing platforms is cost-effective, accurate, and offers a low sequencing cost per base however with the limitations of short reads and challenging genome assembly (Kwong et al., 2015; Xu et al., 2020). Additional important drawbacks of the WGS as a molecular tool for institutions lacking bioinformatics infrastructure and expertise is the comprehensive data analysis and data interpretation (Oakeson et al., 2017). There is, however, a variety of WGS data analysis pipelines available (Jagadeesan, Baert, et al., 2019; Quainoo et al., 2017), ranging from methods that require extensive bioinformatics expertise to commercial software packages which can be challenging to use (Amézquita et al., 2020; Jagadeesan, Gerner-Smidt, et al., 2019). Nevertheless, studies have shown that source tracking with WGS data from *L. monocytogenes* was possible from these platforms with default settings (Jagadeesan, Gerner-Smidt, et al., 2019; Oakeson et al., 2017).

The third-generation sequencing technologies allow for long sequencing reads of single molecules which simplifies the reconstruction of the molecules and de novo assembly of genomes. One of the cheapest (~$1000) and most commonly used is a MinION sequencer commercialized in 2014 by Oxford Nanopore Technologies (ONT) (Jain et al., 2015; Loman & Watson, 2015). In its early days, this technology had limitations due to the high error rate and relatively low throughput (Kilianski et al., 2015; van Dijk et al., 2018). Since then the technology has matured significantly with a reduced error rate and higher throughput (Karst et al., 2021). Considering ONT's latest release, Flongle, which is a $90 adapter for the MinION transportable sequencing platform, the sequencing cost is now considerably decreased.

The classical fingerprinting method, repetitive sequence-based PCR (rep-PCR) was introduced in 1991 by Versalovic et al. (1991) and has been shown to have equal discriminatory power as PFGE for subtyping *Listeria monocytogenes* (Chou & Wang, 2006). By combining rep-PCR with the sequencing of the amplicons with the ONT sequencing platform Krych et al. (2019) presented a new method called ON-rep-seq. This method combines the discriminative power of rep-PCR fingerprinting with access to the sequence information for each DNA fragment which we earlier only knew as bands on a gel. This gives a set of highly discriminating sequences which allows for accurate taxonomic identification and in many cases strain-level differentiation (Krych et al., 2019).

This study aimed to explore the use of ON-rep-seq as (1) a screening method in a real industry case for identification and differentiation of putative *L. monocytogenes* isolated during routine sampling of processing equipment and products and (2) to evaluate the strain level discrimination results with WGS.

2 | MATERIALS AND METHODS

2.1 | Sampling in processing plant and preparation of isolates

Routine sampling in the salmon processing plant was performed according to the company's guidelines. Environmental testing was performed both at fixed and rotational sampling points every day, before, during, and after the processing of the salmon. Analysis

of the samples was performed at the in-house laboratory of the processing plant following the iQ-Check *Listeria* spp. kit (Bio-Rad) procedure. All PCR-positive samples were plated on Rapid'L.mono agar plates (Bio-Rad). From all plates that contained colonies with typical characteristics of *L. monocytogenes* colony, the material was frozen at −20°C and stored in the Microbank™ system (Pro-Lab Diagnostics) before being transported to NTNU and further stored at −80°C. Two gutting machine lines repeatedly tested positive for *L. monocytogenes* and therefore, 20 isolates deriving from different time points and places on these lines were selected for further investigations (Table 1).

Upon analysis, the isolates were propagated on Brain Heart Infusion agar (BHIA; CM1136) and repropagated at a minimum twice. Their growth and appearance on Brilliance Listeria Differential agar (BLA; CM1080) was investigated after incubation at 37°C for 24 ± 2 h.

Note, DNA extraction was performed by using the Genomic Micro AX Bacteria+ Gravity-kit (102–100 M, A&A BIOTECHNOLOGY) according to the manufacturer's procedure. The RNAse treatment was included in the procedure. The DNA was eluted in the neutralized elution buffer. Also, DNA quality was checked on agarose gel and DNA concentrations were estimated by spectrophotometric measurement using BioTek PowerWave XS, Take3 plate, and Gen5 2.0 software. DNA (30 μl, ~40 ng/μl) was sent on ice with overnight shipment to Novogene UK Sequencing laboratory and another 30 μl (~40 ng/μl) of DNA was subjected to ON-rep-seq sequencing at the University of Copenhagen, Denmark.

2.2 | Whole-genome sequencing

2.2.1 | Library construction and sequencing details

At the sequencing laboratory, DNA purity and integrity were again controlled and accurate DNA concentration was measured by Qubit® 3.0 fluorometer quantification. The genomic DNA was randomly sheared into fragments of about 350 bp and library construction was done by using the NEBNext® DNA Library Prep Kit. End repairing, dA-tailing, and ligation of NEBNext® adapter were done before the fragments were PCR enriched by P5 and indexed P7 oligos. Purification and quality check of the products was performed before sequencing. The sequencing strategy was paired-end sequencing with a read length of 150 bp at each end, performed on an Illumina® NovaSeq™ 6000 sequencing platform.

Base-calling was done with CASAVA v1.8 software and the raw read dataset was subject to quality filtering. Paired reads containing either adapter contamination, more than 10% uncertain nucleotides or reads with low-quality nucleotides (base quality $Q \leq 5$) constituting more than 50% of either read, was removed to obtain high-quality reads.

TABLE 1 Sampling points and sampling dates of the 20 *Listeria* isolates from the two gutting machines with frequently positive *L. monocytogenes* samples and downstream in the processing lines. The presumptive identifications from the processing plant in-house laboratory are listed

Isolate ID	Sampling point	Sampling date	ID Rapid'L.mono
SL3.179	Gutting machine 3	28.06.2019	*L. monocytogenes*
SL3.189	Gutting machine 3	08.07.2019	*L. monocytogenes*
SL3.212	Gutting machine 3	31.07.2019	*L. monocytogenes*
SL3.296	Gutting machine 3	23.10.2019	*L. monocytogenes*
SL6.141	Gutting machine 6	21.05.2019	*L. monocytogenes*
SL6.206	Gutting machine 6	25.07.2019	*L. monocytogenes*
SL6.212	Gutting machine 6	31.07.2019	*L. monocytogenes*
SL6.218	Gutting machine 6	06.08.2019	*L. monocytogenes*
HK1.329h	Head and tail cutter 1	25.11.2019	*L. monocytogenes*
HK1.329v	Head and tail cutter 1	25.11.2019	*L. monocytogenes*
HK1.337	Head and tail cutter 1	03.12.2019	*L. monocytogenes*
HK3.297	Head and tail cutter 3	24.10.2019	*L. monocytogenes*
HK3.331	Head and tail cutter 3	27.11.2019	*L. monocytogenes*
HK3.357	Head and tail cutter 3	23.12.2019	*L. monocytogenes*
PK.141	Packaging department	21.05.2019	*L. monocytogenes*
F1K1.353	Filleting machine 1 quality scanner1	19.12.2019	*L. monocytogenes*[a]
F1K2.353	Filleting machine 1 quality scanner 2	19.12.2019	*L. monocytogenes*
FS.171	Fillet salmon	20.06.2019	*L. monocytogenes*
SwF1.296	Swab fillet	23.10.2019	*L. monocytogenes*[a]
SwF1.357	Swab fillet	23.12.2019	*L. monocytogenes*[a]

[a]Inconclusive, suspected to be *L. monocytogenes*.

2.2.2 | Genomic characterization based on WGS data

The whole-genome sequences were analyzed by using the online web-based tools developed by the Center for Genomic Epidemiology (CGE, 2020). The high-quality reads from Illumina PE150 sequencing were used as templates and uploaded to the CGE server. The typing tool KmerFinder (Clausen et al., 2018; Hasman et al., 2014; Larsen et al., 2014) was used to identify the species based on Kmers (length = 16 bases), while MLST 2.0 (Larsen et al., 2012), was used to determine the sequence type based on the seven conventional MLST loci. For the 17 isolates identified as *L. monocytogenes* the MLST configuration *Listeria monocytogenes* was chosen, and for the three isolates identified as *L. innocua*, the MLST configuration, *Listera* was chosen.

Average Nucleotide Identity (ANI) is a measure used to compare the genome sequences of two prokaryotic organisms and calculate the ANI value. Here, the online ANI Calculator from ChunLab (Yoon et al., 2017), based on the OrthoANI algorithm, was used to do pairwise comparisons of all the isolates in the dataset.

To show the relationship between the *L. monocytogenes* isolates a phylogenetic tree based on SNPs was constructed using the CGE webtool CSI Phylogeny 1.4 (Kaas et al., 2014). Three reference genomes were included in the tree (Table 2). To give a better visualization the result file in Newick format was uploaded to another web tool, iTol (Letunic & Bork, 2019). The phylogenetic tree was rooted at the reference strain *L. monocytogenes* EGD-e.

Further on, genotypic characterization and phenotypic predictions were made on acquired antimicrobial resistance genes using ResFinder 3.2 (Zankari et al., 2012), virulence-associated genes using VirulenceFinder 2.0 (Joensen et al., 2014) with default settings (the threshold for ID = 90%, minimum length = 60%) and pathogenic genes using PathogeneFinder 1.1 (Cosentino et al., 2013) for bacteria in the phylum Firmicutes. Detection of plasmids was performed using the online web tool PlasmidFinder 1.2 (Carattoli et al., 2014) for Gram-positive bacteria with the following settings: threshold for minimum identity = 80% and minimum coverage = 60%. To investigate if any of the isolates carried a truncated *inlA* gene, the sequences of each isolate's *inlA* gene were submitted to the NCBI webtool ORFfinder and analyzed for premature stop codons (PMSC).

2.2.3 | Comparison to other published isolates by NCBI Pathogen Detection

The WGS data from each isolate was submitted to NCBI SRA. Sequence data for pathogens submitted to SRA are regularly picked up by the NCBI Pathogen Detection project, assembled, and compared to all other assemblies in the same taxonomic group (NCBI, 2016). Isolates in the same SNP cluster differ with <50 SNPs and within each cluster, a phylogenetic tree is constructed based on a maximum compatibility algorithm (Cherry, 2017). The "Search and Highlight" function was used to find other isolates associated with salmon, fish, seafood, and food processing environment.

2.3 | Oxford Nanopore Technology based rep-PCR amplicon sequencing

2.3.1 | ON-rep-seq library preparation

The Rep-PCR reaction mix contained 5 µl PCRBIO HiFi buffer (5×), 0.25 µl of PCRBIO HiFi Polymerase (PCR Biosystems Ltd), 4 µl of (GTG)5 primers (5 µM), 1 µl of DNA (~20 ng/µl) and nuclease-free water to a total volume of 25 µl. The Rep-PCR thermal conditions were as follows: Denaturation at 95°C for 5 min; 30 cycles of 95°C for 30 s, 45°C for 1 min and 62°C for 4 min; followed by final elongation at 72°C for 5 min using SureCycler 8800 (Agilent).

The barcoding Rep-PCR reaction mix contained 12 µl of PCRBIO UltraMix (PCR Biosystems Ltd, London, United Kingdom) 2 µl of corresponding repBC primer (10 µM), 1 µl of PCR product from Rep-PCR-1 and nuclease-free water to a total volume of 25 µl. Incorporation of ONT compatible adapters was performed using dual-stage PCR where first 3 cycles provide optimal annealing of (GTG)5 regions, following 10 cycles of denaturation 5 min; 3 cycles of 95°C for 30 s, 45°C for 1 min and 62°C for 4 min; followed by 10 cycles of 95°C for 30 s, 65°C for 1 min and 72°C for 4 min and final elongation at 72°C for 5 min. After Rep-PCR-2 samples were pooled using 10 µl of each sample. The pooled library was cleaned with AMPure XP beads (Beckman Coulter Genomics) in volumes 100:50 µl respectively. The bead pellet was washed with 80% ethanol and re-suspended in 100 µl of nuclease-free water.

The pooled and bead-purified library was measured with Qubit® dsDNA HS Assay Kit (Life Technologies) and 66 ng of the library was used as an input to the End-prep step in 1D amplicon by ligation protocol (ADE_9003_v108_ revT_18Oct2016) with one adjustment: 80% ethanol instead of 70% was used for all washing steps.

The sequencing was performed using the R9.4.1 flow cell.

2.3.2 | Data collection, base calling, demultiplexing, and trimming

Data were collected using Oxford Nanopore software: GridION 19.12.2 (https://nanoporetech.com). Guppy 4.4.0 toolkit was used to base call raw fast5 to fastq (https://nanoporetech.com) and demultiplex based on custom adapters.

2.3.3 | Correction and base location of peaks

Peaks are identified in LCp expressed as sequencing length (*x*-axis) by the number of reads (*y*-axis) by fitting local third order polynomials in a sliding window of size 1/50 of the *x*-span across the *x*-axis, followed by calculation of the first- and second-order

TABLE 2 Overview of the seven reference genomes that are used in the different analyses in this study

		Which analyses included in			
Reference strain	**GenBank accession number**	**ON-rep-seq**	**WGS**	**SNP phylogeny**	**Ortho ANI**
Listeria monocytogenes EGD-e	GCA_000196035.1_ASM19603v1	X	X	X	X
Listeria innocua Clip11262	GCF_000195795.1_ASM19579v1	X	X		X
Listeria monocytogenes LO28	GCA_000168675.1_ASM16867v1	X			
Listeria monocytogenes N53-1	GCA_000382945.1_ASM38294v1	X			
Listeria monocytogenes 12067	NA	X			
Listeria monocytogenes R479a	GCF_000613085.1			X	X
Listeria monocytogenes T1-037	GCF_003002675.1			X	X

TABLE 3 Each isolates accession BioSample number, of where both WGS and ON-rep-seq data are available, are listed together with sequencing quality data from WGS and ON-rep-seq respectively

		WGS			
Isolate ID	**Accession number/BioSample**	**No. raw reads pairs**	**Effective reads (%)**	**Average depth (X)[a]**	**Coverage at least 4X (%)[b]**
SL3.179	SAMN21435073	4,968,125	99.95	400.18	95.57
SL3.189	SAMN21435074	4,932,455	99.97	380.95	95.57
SL3.212	SAMN21435075	4,676,698	99.96	379.05	95.57
SL3.296	SAMN21435076	5,085,190	99.94	404.65	95.56
SL6.141	SAMN21435077	4,545,360	99.95	366.83	95.57
SL6.206	SAMN21435078	4,587,296	99.97	373.45	95.57
SL6.212	SAMN21435079	4,146,831	99.95	335.28	95.57
SL6.218	SAMN21435080	5,273,298	99.96	416.48	95.57
HK1.329h	SAMN21435081	3,975,803	99.95	327.09	95.55
HK1.329v	SAMN21435082	4,254,40	99.94	343.39	95.55
HK1.337	SAMN21435083	5,292,498	99.95	421.29	95.56
HK3.297	SAMN21435084	4,247,379	99.95	344.86	95.56
HK3.331	SAMN21435085	4,101,054	99.94	334.49	95.56
HK3.357	SAMN21435086	4,085,114	99.91	322.14	95.56
PK.141	SAMN21435087	3,656,738	99.95	294.98	95.56
F1K1.353	SAMN21435090	3,450,091	99.93	284.19	92.98
F1K2.353	SAMN21435089	4,400,199	99.98	334.92	95.13
FS.171	SAMN21435088	4,495,916	99.94	348.41	95.13
SwF1.296	SAMN21435091	3,866,277	99.92	315.47	92.98
SwF1.357	SAMN21435092	4,628,147	99.89	368.21	92.98

[a]Average depth of mapped (against reference strain) reads at each site, calculated by the number of bases in the mapped reads dividing by size of the assembled genome.

[b]The percentage of the assembled genome with ≥4X coverage at each site.

derivatives. Only peaks with intensity higher than baseline, defined as a moving boxcar (zero-order polynomial) in a broad window (4 times the size of the window used for calculation of the derivative) are used for further analysis. The identified peaks are ordered based on the height, and a representative fragment is used for database matching.

Sequences containing quality scores (fastq files) resolved within each peak were retrieved using Cutadapt v1.15 (Martin, 2011) and corrected with Canu v1.6 (Koren et al., 2017) using the following parameters: genomeSize = 5k, minimumReadLength = 200, correctedErrorRate = 0.05, corOutCoverage = 5000, corMinCoverage = 2 and minOverlapLength = 50. The corrected reads were sorted by length and clustered by cluster_fast from VSEARCH (Rognes et al., 2016), using the following options: -id of 0.9, -minsl of 0.8, -sizeout, and min_cons_pct of 20. The purpose of this step is to detect structural sequence variants of similar length. Subsequently, consensus

sequences were sorted by size (coverage), and those with a minimum coverage size of 50× were kept for downstream analyses. A detailed description of the LCp comparison algorithm is given in the original work (Krych et al., 2019). Kraken2 (Wood & Salzberg, 2014) metagenomic classifier was used for the classification of corrected reads.

3 | RESULTS

3.1 | Whole-genome sequencing of the 20 *Listeria* isolates

The total amount of raw data generated by WGS was 23.0 GB, with the amount of raw data for each isolate varying between 1.1 GB to 1.6 GB. After filtering out low quality and adapter contaminations the amount of clean data for each isolate were between 99.91 to 99.98% of the raw data. Detailed quality metrics for each isolate are shown in Table 3. The SRA BioSample accession numbers for each isolate are also listed in the table.

3.1.1 | Taxonomic identification reveals two different *Listeria* species

The online classifier KmerFinder predicted 17 of the isolates to be *L. monocytogenes*, while the prediction of the three remaining isolates was *L. innocua* (Table 4).

3.1.2 | MLST profiling indicates two strains within 17 isolates of *L. monocytogenes*

Further differentiation of the isolates with the online typing tool MLST revealed 15 of the 17 isolates to be of sequence type (ST) 37 while the last two were of ST8 (Table 4).

The phylogenetic tree based on SNPs supported the similarity of the *L. monocytogenes* isolates clustering in two different groups in perfect correlation with the MLST sequence type (Figure 1). The two isolates F1K2.353 and FS.171 belong to the ST8 cluster together with ST8 reference strain *L. monocytogenes* R479a, while the other 15 isolates belonging to ST37 cluster together with ST37 reference strain *L. monocytogenes* T1-037. Both groups differentiated from *L. monocytogenes* EGD-e reference strain.

3.1.3 | ANI analysis did not identify any strain-level differences

Pairwise Average Nucleotide Identity (ANI) between the isolates and four reference genomes, revealed a 100.00% similarity between the 15 *L. monocytogenes* of ST37 (Figure 2). This indicated that they are all the same strain. Compared to the *L. monocytogenes* T1-037 reference genome, also ST37, these isolates all had ANI values of 99.95%–99.96%. The ST8 isolates, F1K2.353 and FS.171 had an ANI value of 99.97% to each other and 99.92% and 99.98% respectively to the *L. monocytogenes* R479a (ST8) reference genome. ANI values between ST8 and ST37 isolates varied between 99.29%–99.35%, but between ST8 isolates and the reference strain *L. monocytogenes* T1-037 it was down to 98.97%–98.98%. The three *L. innocua* isolates had ANI values of 100.00% to each other and 99.97% to *L. innocua* Clip11262 reference genome (Figure 2) and they cannot be differentiated from each other or *L. innocua* Clip11262 by this method.

3.1.4 | Antibiotic resistance genes, virulence genes, and pathogen genes showed no additional strain-level differences

All the *L. monocytogenes* isolates in this selection carried the *fosX* gene coding for fosfomycin resistance with a sequence identity of 98.76% for ST8 isolates and 99.25% for ST37 isolates. No resistance genes included in the ResFinder 3.2 database were detected in the *L. innocua* isolates.

The *L. monocytogenes* isolates carried a large number of virulence genes. In the ST8 isolates, 21 known virulence genes with 100% ID to sequence in the database could be identified, and additionally 62 virulence genes with 98.0%–99.9% ID (Table 3). The ST37 isolates carried 24 known virulence genes with 100% ID to sequence in the database and 57 virulence genes with 98.0%–99.9% ID (Table 3).

When analyzing the *L. monocytogenes* isolates for a possible truncated *inlA* gene, it was confirmed that all isolates constituted a full length (2403 bp) *inlA* gene with a 98.54% and 98.21% identity for ST8 and ST37 isolates, respectively. The NCBI webtool ORFfinder reported no in-frame premature stop codons for any of the isolates.

The selected isolates in this study, including the *L. innocua* isolates, were predicted to be human pathogens by the web tool PathogenFinder 1.1 with probability 0.812 for the ST37 isolates, 0.808 for ST8 isolates, and 0.818 for *L. innocua* isolates. However, the *prfA* gene coding for positive regulatory factor A (PrfA) in *L. monocytogenes*, was absent from the *L. innocua* isolates.

3.1.5 | No strain-specific plasmids were found

When applying the default settings (95% identity, 60% coverage) in the webtool PlasmidFinder 1.2 no plasmids could be detected in the 17 *L. monocytogenes* isolates (Table 4). Lowering the identity cutoff to 80% enabled the detection of the rep26 sequence of pLM5578 (84% ID) (Gilmour et al., 2010) and the rep26 sequence of PLGUG1 originally isolated from *L. grayi* (Kuenne et al., 2010) in the *L. monocytogenes* ST8 isolate, F1K2.353. Interestingly the three *L. innocua* isolates were found to carry a plasmid with 100% similarity to plasmid pLM33 which is commonly found in food-related lineage II *L. monocytogenes* strains (Canchaya et al., 2010).

TABLE 4 Overview over species identification and strain discrimination obtained from ON-rep-seq data and identification, strain discrimination, and genotypic traits identified from WGS data. For comparison, the corresponding information for reference genomes *L. monocytogenes* EGD-e and *L. innocua* Clip11262 are also included

	ON-rep-seq	WGS				
Isolate ID	Taxonomy assignment	ID KmerFinder	MLST	No. virulence genes (ID >98%)	Predicted human pathogen (prob.)	Plasmids
Reference		*L. monocytogenes* EGD-e	35	87	Yes (0.812)	–
Reference		*L. innocua* Clip11262	–	0	Yes (0.812)	pLI100 (rep26) pLI100 (repUS65) pLI100 (rep32)
F1K1.353	*L. innocua*	*L. innocua*	–	2	Yes (0.818)	pLM33 (rep25)
F1K2.353	*L. monocytogenes*	*L. monocytogenes*	8	83	Yes (0.808)	–
FS.171	*L. monocytogenes*	*L. monocytogenes*	8	83	Yes (0.808)	–
HK1.329h	*L. monocytogenes*	*L. monocytogenes*	37	81	Yes (0.812)	–
HK1.329v	*L. monocytogenes*	*L. monocytogenes*	37	81	Yes (0.812)	–
HK1.337	*L. monocytogenes*	*L. monocytogenes*	37	81	Yes (0.812)	–
HK3.297	*L. monocytogenes*	*L. monocytogenes*	37	81	Yes (0.812)	–
HK3.331	*L. monocytogenes*	*L. monocytogenes*	37	81	Yes (0.812)	–
HK3.357	*L. monocytogenes*	*L. monocytogenes*	37	81	Yes (0.812)	–
PK.141	*L. monocytogenes*	*L. monocytogenes*	37	81	Yes (0.812)	–
SL3.179	*L. monocytogenes*	*L. monocytogenes*	37	81	Yes (0.812)	–
SL3.189	*L. monocytogenes*	*L. monocytogenes*	37	81	Yes (0.812)	–
SL3.212	*L. monocytogenes*	*L. monocytogenes*	37	81	Yes (0.812)	–
SL3.296	*L. monocytogenes*	*L. monocytogenes*	37	81	Yes (0.812)	–
SL6.141	*L. monocytogenes*	*L. monocytogenes*	37	81	Yes (0.812)	–
SL6.206	*L. monocytogenes*	*L. monocytogenes*	37	81	Yes (0.812)	–
SL6.212	*L. monocytogenes*	*L. monocytogenes*	37	81	Yes (0.812)	–
SL6.218	*L. monocytogenes*	*L. monocytogenes*	37	81	Yes (0.812)	–
SwF1.296	*L. innocua*	*L. innocua*	–	2	Yes (0.818)	pLM33 (rep25)
SwF1.357	*L. innocua*	*L. innocua*	–	2	Yes (0.818)	pLM33 (rep25)

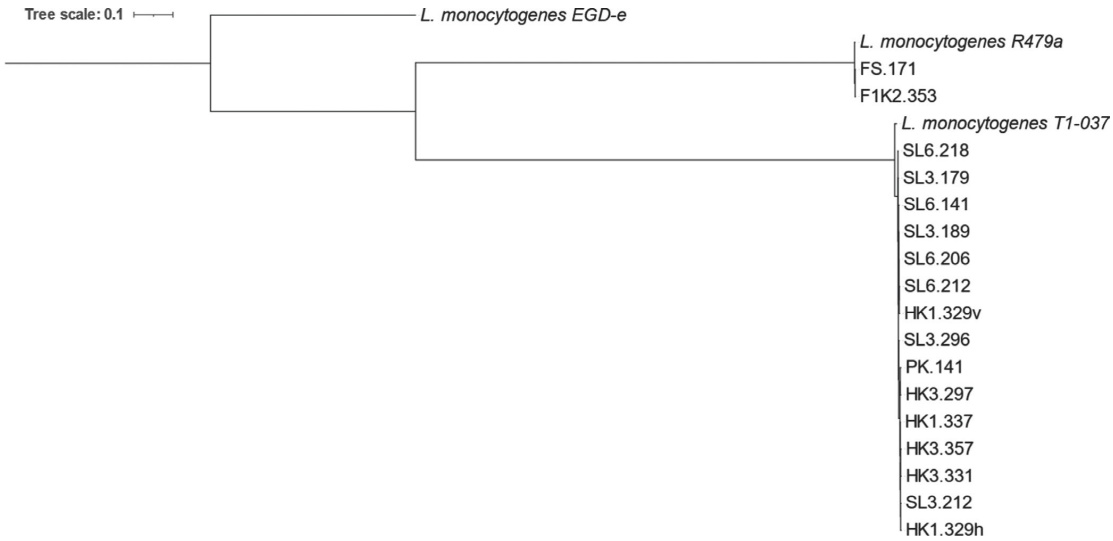

FIGURE 1 A phylogenetic tree of the 17 *L. monocytogenes* isolates based on SNPs. The *L. monocytogenes* clusters in two groups corresponding to their MLST sequence type. The two isolates F1K2.353 and FS.171 belong to ST8 and cluster together with the ST8 reference strain *L. monocytogenes* R479a while the other 15 isolates belong to ST37 and cluster together with the ST37 reference strain *L. monocytogenes* T1-037. The tree was rooted at the reference strain *L. monocytogenes* EGD-e

	F1K1.353	F1K2.353	FS.171	HK1.329h	HK1.329v	HK1.337	HK3.297	HK3.331	HK3.357	PK.141	SL3.179	SL3.189	SL3.212	SL3.296	SL6.141	SL6.206	SL6.212	SL6.218	SwF1.296	SwF1.357	Li Clip11262	Lm EGD-e	Lm R479a	Lm TI037
F1K1.353	100.00																							
F1K2.353	88.40	100.00																						
FS.171	88.54	99.97	100.00																					
HK1.329h	88.74	99.30	99.35	100.00																				
HK1.329v	88.70	99.30	99.34	100.00	100.00																			
HK1.337	88.74	99.30	99.34	100.00	100.00	100.00																		
HK3.297	88.74	99.30	99.34	100.00	100.00	100.00	100.00																	
HK3.331	88.74	99.30	99.35	100.00	100.00	100.00	100.00	100.00																
HK3.357	88.74	99.30	99.35	100.00	100.00	100.00	100.00	100.00	100.00															
PK.141	88.74	99.30	99.35	100.00	100.00	100.00	100.00	100.00	100.00	100.00														
SL3.179	88.71	99.29	99.34	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00													
SL3.189	88.69	99.29	99.33	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00												
SL3.212	88.74	99.30	99.35	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00											
SL3.296	88.69	99.29	99.33	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00										
SL6.141	88.71	99.30	99.34	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00									
SL6.206	88.70	99.30	99.33	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00								
SL6.212	88.70	99.30	99.34	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00							
SL6.218	88.70	99.29	99.33	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00						
SwF1.296	100.00	88.40	88.55	88.75	88.71	88.75	88.75	88.74	88.75	88.75	88.72	88.70	88.75	88.71	88.72	88.71	88.71	88.72	100.00	SwF1.357				
SwF1.357	100.00	88.40	88.54	88.74	88.70	88.74	88.74	88.74	88.74	88.74	88.71	88.69	88.75	88.70	88.71	88.70	88.70	88.71	100.00	100.00	Li Clip11262			
Li Clip11262	99.97	88.42	88.54	88.73	88.70	88.73	88.73	88.73	88.73	88.73	88.71	88.69	88.74	88.69	88.71	88.69	88.69	88.69	99.97	99.97	100.00	Lm EGD-e		
Lm EGD-e	88.41	99.02	99.07	99.24	99.22	99.24	99.24	99.24	99.24	99.24	99.23	99.23	99.24	99.23	99.23	99.23	99.23	99.23	88.42	88.42	88.47	100.00	Lm R479a	
Lm R479a	88.55	99.92	99.98	99.35	99.34	99.35	99.35	99.35	99.35	99.35	99.34	99.34	99.35	99.33	99.34	99.34	99.34	99.34	88.55	88.54	88.52	99.07	100.00	Lm TI037
Lm TI037	88.42	98.97	98.98	99.96	99.95	99.95	99.96	99.96	99.95	99.96	99.95	99.95	99.96	99.95	99.95	99.95	99.95	99.95	88.42	88.42	88.42	99.01	98.98	100.00

FIGURE 2 OrthoANI matrix showing the average nucleotide identity between the isolates. The ANI values between the isolates and some closely related strains are also included. Based on a cut-off for ANI value of <99% to separate strains, this means that by the OrthoANI method the 17 *L. monocytogenes* isolates in this study are indistinguishable from each other. The cut-off for species-level discrimination is <95%. ANI values between *L. innocua* and *L. monocytogenes* strains in this study are 88–89% and give clear interspecies discrimination

3.1.6 | NCBI pathogen detection pipeline assigned the *L. monocytogenes* isolates in two different SNP clusters

When picked up by the NCBI Pathogen Detection project the *L. monocytogenes* isolates in this study was assigned to two different SNP clusters, the group of 15 isolates was assigned to SNP Cluster PDS000032941.106 (393 isolates), while the group of two isolates was assigned to SNP Cluster PDS000025311.185 (1093 isolates). Figure 3 displays subtrees of the phylogenetic trees of these two SNP clusters with the isolates from this study together with the closest related isolates within the respective NCBI Pathogen Detection SNP cluster.

According to this analysis, the group of 15 isolates differs by a maximum of 4 SNPs from each other, while the two other isolates differ by only one SNP.

3.2 | Analysis of 20 *Listeria* isolates with ON-rep-seq is in accordance with the WGS data regarding species level classification and strain level discrimination

3.2.1 | Species-level classification

Classification of corrected reads from LCPs in 20 isolates identified 17 isolates as *L. monocytogenes* and three as *L. innocua*.

FIGURE 3 Maximum compatibility phylogenetic tree of *L. monocytogenes* isolates generated by NCBI Pathogen Detection pipeline. (A) shows a subtree of SNP cluster PDS000032941.106 where 15 of the *L. monocytogenes* isolates from this study was assigned, together with the isolates most closely related. (B) shows a subtree of SNP cluster PDS000025311.185 where two of the *L. monocytogenes* isolates from this study was assigned, together with the isolates most closely related according to this analysis

3.2.2 | Strain-level discrimination

The read length count profiles (LCps) from the sequenced Rep-PCR products identified three unique profiles among the selected isolates (Figures 4 and 5). Among 17 *L. monocytogenes* isolates two unique clusters of LCps were distinguished with two and 15 isolates (Figure 5). No differentiation in LCp profiles could be observed among three *L. innocua* isolates (Figure 4).

4 | DISCUSSION

Species-level and strain-level discrimination of microorganisms is essential for a food processing plant to track microbial contamination sources in the value chain. Intra-species variability exists in important characteristics such as virulence, pathogenicity, and drug resistance. During seven months a bacterial isolate can change due to environmental conditions, isolation, and culturing can generate new SNPs (Allard et al., 2012), and sequences from the same contamination source are most likely not identical even though they are of the same origin (Pightling et al., 2018).

In this study, a set of 20 putative *Listeria monocytogenes* isolates from a salmon processing plant were identified to species and differentiated down to strain level with ON-rep-seq and the results were evaluated by WGS. The isolates, originally detected through routine sampling in the processing plant, were selected from different time points and sampling points in the processing facility, with a focus on two gutting machines where *L. monocytogenes* had repeatedly been detected. The ON-rep-seq method separated the isolates into three distinct groups with unique LCps (read length count profiles). The taxonomic classification performed on the consensus reads from each peak revealed that these groups were two different *L. monocytogenes* strains and one *L. innocua* strain. This differentiation is in agreement with our former work where we described the relationship between unique LCps and associated strains (Krych et al., 2019). Testing novel methods on real industry case isolates is significant, and in this study, ON-rep-seq was able to unravel differences and similarities between the isolates. Results as unique LCps differentiating between strains, as presented here, will inform the quality control personnel at the processing plant that with high probability it is the same strain that caused the repeatedly positive tests in the gutting machines and head cutters. All the 15 *L. monocytogenes* isolates from the same area in the factory have the same LCp, while the two isolates from the filleting area have a different LCp and the *L. innocua* strains a third profile, and they all differ from the *L. monocytogenes* reference strains. The visualization of the strain differentiation in a heat map allows for an easy and intuitive interpretation of strain similarity. However, the classification in the ON-rep-seq method cannot identify exactly which strains they are unless they can be compared with identical LCps from a larger set of strains in a database.

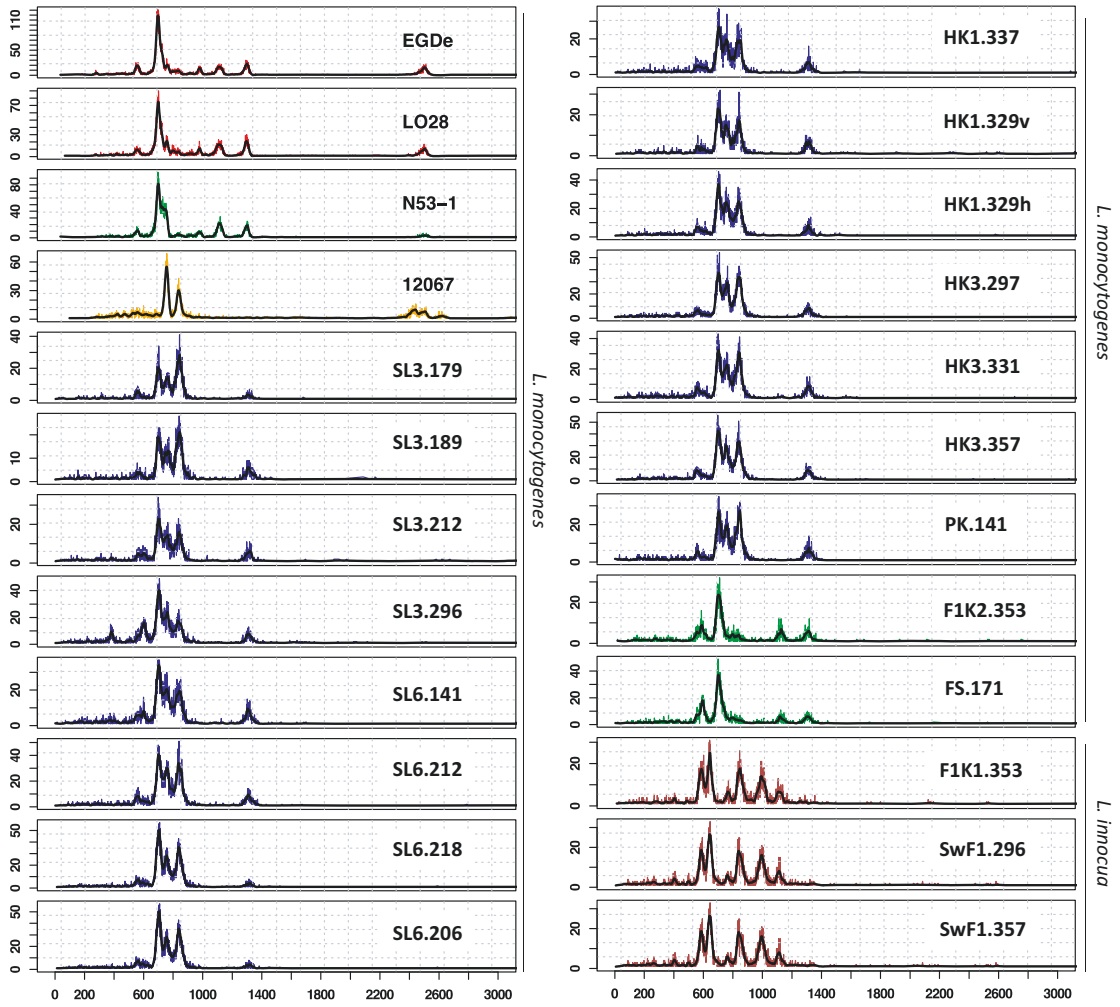

FIGURE 4 LCps (read length count profiles) generated from 20 putative *L. monocytogenes* isolates sampled from a salmon processing plant. The curves are a function of read length and abundance, where the position of the peak on the x-axis corresponds to the length of the sequence and the height of the peaks corresponds to abundance. The four LCps at the top left are from reference strains analyzed in an earlier project (Krych et al., 2019). The two closely related strains EGD-e and LO28 have previously been shown to be indistinguishable from each other than by SNP analysis. As is the case here as they have the same LCp. The two other strains N53-1 and 12067 clearly show different profiles. Fifteen of the isolates analyzed in this study show the same LCp (blue) and are expected to be the same strain. Two of the isolates show an LCp (green) different from (c) but similar to each other, while three isolates show a third LCp (brown). This indicates that among the 20 isolates there are three different strains

So, WGS has currently the highest discrimination power as to resolution compared to other molecular typing techniques. However, making use of the power in this technology requires a high level of bioinformatic competence and computer infrastructure. Several commercial units provide a standardized or custom set of data analyses, yet this approach requires initial knowledge on tested organisms to customize the analysis. An increasing number of online web tools, free or paid, and several commercial softwares are also available, which all have their pros and cons (Jagadeesan, Baert, et al., 2019; Quainoo et al., 2017). In 2017 PulseNet International published their vision that WGS should be used by all public health laboratories to identify, characterize and subtype food pathogens for better and more accurate source tracking (Nadon et al., 2017). In the aftermath of this, the use of WGS among food companies was discussed in an industry workshop in 2019 (Amézquita et al., 2020). One of the barriers discussed was the development of expertise in sequencing and bioinformatics that is necessary, as well as the concern for the requirement of computer infrastructure and data storage needed (Amézquita et al., 2020).

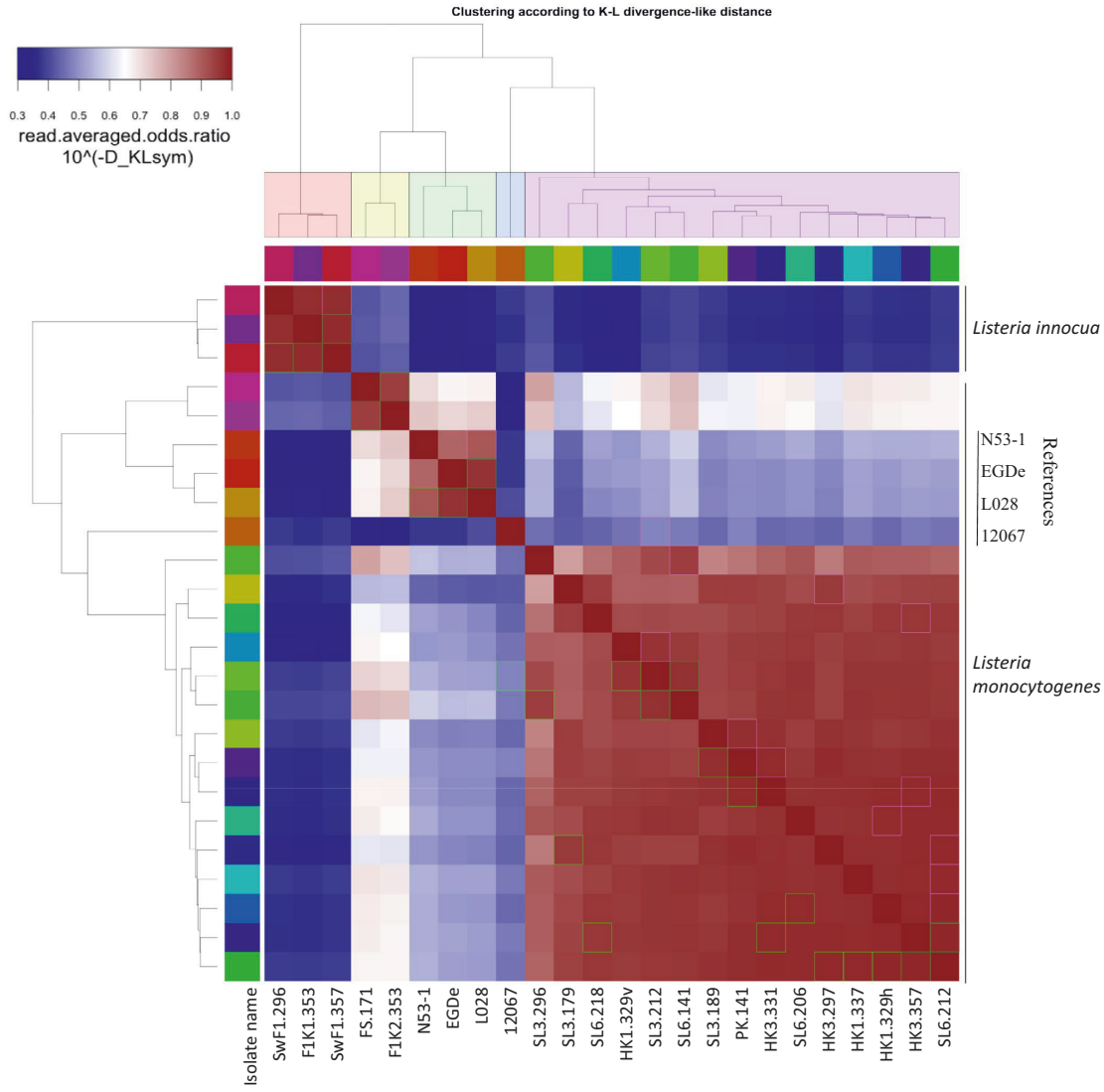

FIGURE 5 Heatmap showing the similarity (10^(-D_KLsym)) between 20 isolates and 4 references, with clusters according to cut-off=0.09. The three isolates (SwF1.296, SwF1.357, and F1K1.353) found to be *L. innocua* are clearly different from all the *L. monocytogenes* strains. The large group of 15 isolates with the same LCp cluster together, as do the two last isolates. This corresponds perfectly with the MLST classification based on WGS data

Based on the WGS data, the isolates in this study were further characterized into sequence types (MLST). In correspondence with the identical LCps from the ON-rep-seq analysis, the group of two identical *L. monocytogenes* strains was identified as ST8 and the group of 15 *L. monocytogenes* strains as ST37.

The two isolates of *L. monocytogenes* ST8 were originally detected in the filleting area, the first isolate, FS.171, from salmon fillet and the second isolate, F1K2.353, in a filleting machine six months later. Strain ST8 has earlier been linked to a multi-country outbreak of listeriosis in Denmark, Germany, and France in 2015–2018 which was due to the consumption of salmon products (EFSA, 2018). In addition, ST8 has been identified repeatedly over three years in a salmon processing plant in Denmark (Schmitz-Esser et al., 2015). In Norway, *L. monocytogenes* ST8 has been frequently detected in one salmon slaughterhouse for 13 years (Fagerlund et al., 2016). All this demonstrates that *L. monocytogenes* of this ST can be persistent, and it can cause listeriosis. *L. monocytogenes* ST37 has been detected in both food products and food processing environments associated with meat, dairy, and vegetables, respectively (Cabal et al., 2019; Stessl et al., 2020;

Tomáštíková et al., 2019). It is however suspected to be a less persistent strain than ST8 (Muhterem-Uyar et al., 2018).

The phylogenetic analyses done, both by CSI Phylogeny and NCBI Pathogen Detection confirms the grouping of the isolates demonstrated by ON-rep-seq. CSI Phylogeny SNP tree in Figure 1 indicates that the *L. monocytogenes* isolates cluster in two different groups in exact accordance with the ON-rep-seq LCps, and the MLST sequence type, additionally, both groups are somewhat different from the reference strain *L. monocytogenes* EGD-e. The three *L. innocua* strains are not included in the SNP phylogenetic tree as their relationship to the *L. monocytogenes* are too distinct.

In NCBI Pathogen Detection phylogenetic tree the two groups of isolates are assigned to two different SNP clusters which means that the groups differ by >50 SNPs. Within the group of 15 isolates, the isolates differed by a maximum of 4 SNPs while there was only one SNP difference between the two isolates in the small group. This supports the conclusion that all the isolates within each group are the same strain and that we are dealing with two strains of *L. monocytogenes* in this material. The minimum SNP difference to a clinical isolate is 25–29 SNPs and the closest environmental isolate is an isolate from a dairy barn in Finland from 2015 with a 27–31 SNP difference. Within this SNP cluster, there are no other isolates with a registered association to salmon, only four isolates associated with fish (herring) and one isolate associated with seafood, namely a seafood factory in Ireland. There is a group of five isolates associated with food processing environment collected in the UK in 2011 with a minimum SNP difference of 34.

The two isolates (FS.171 and F1K2.353) assigned to SNP Cluster PDS000025311.185 belong to ST8. In this analysis the minimum SNP difference from our isolates to a clinical isolate, namely from a case of human listeriosis in Germany in 2018, is 25 and 26 SNPs. Within this SNP cluster there are 15 other isolates associated with salmon, mostly smoked salmon. Three of these come from salmon processing facilities in Norway, the closest being 32–33 SNPs different from the isolates in this study. Five additional isolates in this SNP cluster were associated with fish or seafood and 11 isolates were reported to come from the processing environment.

The average nucleotide identity (OrthoANI values) indicated a high degree of conservation among the different isolates. In this analysis, all the 15 isolates of ST37 had an ANI value to each other of 100.00% which indicates that they are most likely the same strain. Considering an ANI cutoff value of <99% to differentiate between strains, these strains cannot be differentiated with the ANI index. The two ST8 isolates share an ANI value of 99.97% and are by this method considered to be the same strain. The ANI values between strains of different ST were all >99.00%, which means that none of the *L. monocytogenes* isolates in this study can be differentiated from each other by this method.

None of the *L. monocytogenes* isolates in this study carried a truncated *inlA* gene. The virulence factor internalin A in *L. monocytogenes*, encoded by *inlA*, plays a critical role in crossing the intestinal barrier to give a systemic infection in humans (Olier et al., 2005). Clinical isolates of *L. monocytogenes* usually carry a fully functional *inl*A gene (Gorski et al., 2016). Different mutations in this gene can lead to premature stop codons (PMSC) (Van Stelten et al., 2010) and have been identified in 45%–50% of food isolates analyzed (Upham et al., 2019; Van Stelten et al., 2010). This can indicate a lower potential of pathogenesis (Olier et al., 2005) and this gene has been suggested as a genetic marker for risk assessment (Upham et al., 2019). In this study, all the *L. monocytogenes* isolates carried a full length and predictably fully functional *inl*A gene meaning that they must be considered as a severe risk for human infection if they contaminate the food product.

In the analysis of pathogenicity done with PathogenFinder, all the isolates, including *L. innocua*, were predicted to be human pathogens. However, the *prf*A gene, coding for positive regulatory factor (PrfA) of *L. monocytogenes*, was not present in the *L. innocua* isolates. This factor regulates and activates most of the known virulence genes by binding to a palindromic *prf*A recognition sequence located in the promoter region (Glaser et al., 2001; Greene & Freitag, 2003). This means that many of the genes involved in pathogenesis will not be expressed in these isolates even though they are present and therefore these isolates are probably not pathogenic. The *prf*A gene was present in all *L. monocytogenes* isolates in full length and with 100% identity to the reference gene.

In this study, the isolates used for analysis were selected based on when and where they were detected in the processing plant, and in connection to the area with frequent *Listeria* detection. The analyses done revealed a low diversity in the tested isolates and thereby give a limited base for a thorough evaluation of the ON-rep-seq method. However, it shows that in this specific industry case it was indeed the same strain (group of 15 similar isolates) causing the repeatedly positive tests in the two gutting machines and downstream equipment. All the tested isolates were detected throughout seven months. This is a relatively short time to evaluate if the strains are persistent strains or transient strains. However, the result from this study supports that there is a strain that persists in equipment and environment in the processing plant for these seven months.

Daily, many industries cannot afford long-term studies on strain persistence, and the main information regarding putative contaminations is limited to the species level typing, namely the presence/absence of *L. monocytogenes*. In cases where strain tracing is necessary e.g. in presence of frequent positive tests for *Listeria* in certain areas or equipment, swift preventative action is needed followed by the validation of the action. In such cases, a fast, reliable, and cost-effective approach is desirable.

For routine analysis of *Listeria*, many companies use a two-step method, first iQ-Check™ *Listeria* spp. PCR Detection Kit, secondly, positive samples plated on Rapid'L.mono agar plates. On these plates, *L. monocytogenes* usually appear as blue/green colonies with no colored halo whereas e.g *L. innocua* appear as white colonies. There are exceptions, however, where white colonies were identified as *L. monocytogenes*, and blue colonies were confirmed as *L. innocua*. (Greenwood et al., 2005), as was also the case for some of the isolates in this study. Those three isolates (F1K1.353, SwF1.296, and SwF1.357) did not show lecithinase activity when grown on

TABLE 5 Comparison of commercial prices for traditional typing methods, 16S sequencing, WGS, and ON-rep-seq

Typing method	Unit price (1–10 units/ next 10)	Additional preparation costs (DNA-extraction, QC)	Additional one-time cost	Total cost for 20 isolates
PFGE	170 €/89 €			2590 €[a]
Serotyping	180 €/117 €			2970 €[a]
WGS	85 €	2 €	27 €	1767 €[a]
16S (Sanger seq)	4 €	4 €		160 €[a]
ON-rep-seq	10 €–2 €[c]	4 €		280 €[b]

[a]Commercial prices.
[b]Estimated price based on the price for one Flongle, library preparation, and necessary working hours.
[c]The price for each sample if 96 samples are analyzed simultaneously on the Flongle.

BLA, which indicates that they are not *L. monocytogenes* but another *Listeria* species. However, the isolates were not excluded from the study based on this considering they had been identified as *L. monocytogenes* by the company's analysis on Rapid'L.mono agar and when grown on Rapid'L.mono in our laboratory the inconclusive morphology (areas with blue halo) was confirmed. It was therefore of interest to get a thorough analysis of these isolates as well. The identification of *L. innocua* in this study highlights the difficulty for the processing plant to correctly differentiate *Listeria* even at the species level with the methods available.

Many companies have established a comprehensive test regime to detect and eliminate *L. monocytogenes* from their value chain and this system can include storage of presumptive *L. monocytogenes* isolates in case of tracking and tracing of source contamination. It must be acknowledged that; the more they test – the more they find, and for some processing plants, this can lead to several hundred isolates a year. Performing WGS on hundreds of isolates is not applicable due to the costs, workload, data processing, and data storage needed (Amézquita et al., 2020; Jagadeesan, Gerner-Smidt, et al., 2019). Sequencing a small number of isolates in a tracing situation will be the most likely scenario but selecting the most representative isolates for this might be a challenge. As demonstrated here the ON-rep-seq method gives sufficient information for preliminary source tracking of pathogens in the food industry to serve as a screening method before doing WGS and can in some cases even serve as an alternative method to WGS.

ON-rep-seq as a fast-screening method offers much more accurate taxonomic identification than 16S rRNA gene sequencing with simultaneous access to a strain level discrimination comparable to that obtained from the WGS. Table 5 lists some commercial prices for different traditional typing methods and compares them to sequencing-based methods as Sanger sequencing of 16S rRNA gene, WGS, and the novel method ON-rep-seq. This overview shows that the cost of ON-rep-seq is within the same range as that of Sanger sequencing, making it 8 to 10 times more cost-effective than the alternative typing methods delivering similar information regarding identification and differentiation. The method can be introduced to facilities at a very low cost since the MinION sequencing platform is available at about $1000. Furthermore, the possibility for analysis of up to 96 isolates on a Flongle, which is the cheapest flow cell available so far ($90), ensures low running costs with the highest resolution level that offers comparable resolution to WGS in terms of classification.

5 | CONCLUSION

With this study, we demonstrate that the recently developed fingerprinting method combined with nanopore sequencing called ON-rep-seq is a promising, rapid, cost-effective, and less laborious alternative to the whole genome sequencing for species-level identification and strain level discrimination of *Listeria* species.

From a set of 20 isolates, 17 *L. monocytogenes* and 3 *L. innocua* were identified and the *L. monocytogenes* isolates were further differentiated into two strains. The analysis done on WGS data showed the same, and no further differentiation of the isolates was obtained.

The material in this study is however very limited. To evaluate the discriminatory power of ON-rep-seq more thoroughly a more diverse set of isolates will be necessary.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

Gunn Merethe Bjørge Thomassen: Conceptualization (equal); Formal analysis (equal); Writing-original draft (lead); Writing-review & editing (equal). **Lukasz Krych:** Conceptualization (equal); Formal analysis (equal); Writing-review & editing (equal). **Susanne Knøchel:** Writing-review & editing (equal). **Lisbeth Mehli:** Conceptualization (equal); Writing-original draft (supporting); Writing-review & editing (equal).

ETHICS STATEMENT

None required.

DATA AVAILABILITY STATEMENT

Sequence reads from whole-genome sequencing and ON-rep-seq are available at the NCBI repository under the BioProject number PRJNA763206: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA763206. The ON-rep-seq data analysis toolbox is available on GitHub: https://github.com/lauramilena3/On-rep-seq and Zenodo: https://doi.org/10.5281/zenodo.3384841

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Paper II

Bacterial community development and diversity during the first year of production in a new salmon processing plant

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Abstract

The bacterial diversity and load on equipment in food processing facilities is constantly influenced by raw material, water, air, and staff. Despite regular cleaning and disinfection, some bacteria may persist and thereby potentially compromise food quality and safety. Little is known about how bacterial communities in a new food processing facility gradually establish themselves.

Here, the development of bacterial communities in a newly opened salmon processing plant was studied from the first day and during the first year of operation. In order to focus on the persisting bacterial communities, surface sampling was done on strategical sampling points after cleaning and disinfection. To study the diversity dynamics, isolates from selected sampling and time points were identified by ON-rep-seq method supplemented by 16S rRNA gene or *rpoD* gene sequencing (for *Pseudomonas*). An overall increase in bacterial numbers was only observed for contact surfaces in the slaughter department. In the filleting department, on non-contact surfaces, and on the fish no such trend was observed but rather a

high variation between different time points. Changes in temporal and spatial diversity and community composition were observed. Our study also suggests that ON-rep-seq has a potential for species-level identification for complex communities in food processing environments.

Keywords: Bacterial community, species-level identification, ON-rep-seq, food processing environment, food safety, food spoilage

Abbreviations[1]

[1] AC Aerobic count

APC Aerobic psychrotrophic count

C&D Cleaning and disinfection

FPE Food processing environment

PsC Pseudomonas count

1 Introduction

Microorganisms are constantly introduced into food processing facilities via raw material, water, equipment and staff, compromising both the shelf life of the product and the food safety. In a salmon processing facility, the cold marine water environment and the following chilled processing facilities will favor Gram negative, psychrotrophic bacteria (Guðbjörnsdóttir et al., 2005, Langsrud et al., 2016, Møretrø et al., 2016). *Pseudomonas* spp, *Enterobacteriaceae* and *Acinetobacter* spp. are generally dominant on food processing surfaces in seafood, meat and dairy industry (Møretrø and Langsrud, 2017) whereas *Pseudomonas* spp. and *Shewanella* spp. was the most common bacteria detected in the salmon processing environment (Møretrø et al., 2016). Many bacterial species can attach to surfaces and form biofilm. This ability is a species-specific feature causing challenges in the food industry (Mizan et al., 2015, Møretrø et al., 2016). The possibility for transmission of bacteria from food contact surfaces to the food product is also well documented (Hinton et al., 2004, Midelet and Carpentier, 2002, Møretrø et al., 2016, Sheen, 2008, Truelstrup Hansen and Vogel, 2011), which highlights the importance of preventing the formation of bacterial biofilm in food processing environment.

The food processing facilities routines on cleaning and disinfection (C&D) aim to keep the bacterial load as low as possible, however, environmental bacteria can survive the C&D treatment when exposed to the concentrations used in industrial environments (Fagerlund et al., 2017). Additionally, some types of equipment and surfaces used in the food industry might be difficult to clean. Together, these issues may result in residual bacteria forming a persisting background microbiota (Nivens et al., 2009).

Persisting bacteria can form biofilm structures that can host and protect potentially pathogenic bacteria such as *Escherichia coli* and *Listeria monocytogenes* (Giaouris et al., 2013, Gomes et al., 2017, Langsrud et al., 2016, Schwering et al., 2013). *L. monocytogenes* is of profound

concern for the food industry, also for the salmon industry in Norway, and there are numerous studies on how *L. monocytogenes* survive and persist in biofilms in food producing facilities, as reviewed by Fagerlund et al. (2021) and Lianou et al. (2020). These reviews summarize that both strain variability in *L. monocytogenes* and interactions with the background microbiota affect the survival and persistence of pathogens. The persistence of some *L. monocytogenes* in food producing environments is suggested to be caused by the match between the specific *L. monocytogenes* strain and the surrounding microbiome, and not solely because of specific intrinsic traits (Fagerlund et al., 2021). This highlights the significance in identifying the background bacterial microflora in food processing premises down to species level.

Microbial analysis in food processing environments has traditionally been limited to conventional spread plating and culture dependent methods. But as sequencing technology has developed, sequencing based methods has become the standard in research of microbial communities and other microbial analysis. In recent years also the food industry has taken interest in sequencing-based methods. So far it has mostly been used in the detections and identification of pathogens and for source tracking of disease outbreaks (Jagadeesan et al., 2019, Klijn et al., 2020, Nouws et al., 2020, Painset et al., 2019, Sekse et al., 2017), but as the industry gets more familiar with the possibilities of the technology, the number of applications is likely to increase. We believe that making use of state-of-the-art sequencing technology can help to provide additional knowledge about the background microbiota in food processing facilities and by that push forward towards high quality and long shelf life of the food product.

The aim of this study was to analyze the development and dynamics, both quantitatively and qualitatively, of the residing bacterial communities in the food processing environment of a newly opened salmon processing facility. And, for the identification of bacterial isolates to

85 species-level, explore the potential of a third generation DNA sequencing based method, ON-
86 rep-seq (Krych et al., 2019).

2 Materials and methods

2.1 Sampling in cleaned and disinfected processing environment

The sampling location was a newly opened salmon processing facility at the coast of Mid-Norway. The facility receives salmon from several marine farming locations in the region, and the fish is pumped into the facility directly from the well boat without the use of waiting pens. The facility produces gutted whole fish packaged in Styrofoam boxes with ice, whole fillets packaged in Styrofoam boxes and frozen, and vacuum-packed portioned fillets with or without skin. Bacterial sampling in the facility was performed thirteen times throughout the first year of production (at day 0, 6, 13, 20, 40, 75, 110, 131, 159, 229, 271, 320, 362) at the same 24 fixed sampling points each time (Figure 1). A short description of the sampling points is given in Table 1. The sampling points were chosen in collaboration with the Quality Manager and the Cleaning Manager at the facility. Samples of fish fillet and swab samples of skin and gills of gutted whole fish were collected at four different occasions. All sampling was performed after cleaning, disinfection and air drying in the mornings before startup of normal production. Sampling was performed by swabbing 100 cm^2 with a sterile swab (Promedia ST-25 PBS, r-biopharm, Germany) in 10 ml phosphate buffered saline (PBS) or by swabbing 900 cm^2 (30 x 30 cm) with sterile clothes pre-moistened with 25 ml PBS (Sodibox, France). The choice of swabbing method depended on the type and area of the surface. Clothes and swabs were kept cold during the transportation (3 h) from the facility to the lab. Spread plating were performed the same day.

2.2 Quantification of general and specific bacteria

Additional PBS was added to the bag with the sampling cloth to a total weight of 50 g more than a clean, unused cloth in its bag, before it was mashed in a Stomacher for 30 seconds. The cloth was aseptically removed from the bag and ten-fold serial dilutions of the liquid was

made before plating on several growth media. Swab samples were also serially diluted 10-fold before plating on growth media.

Total aerobic count (AC) and H_2S-producing bacteria were analyzed on Iron Agar (IA) (Oxoid, CM0964) with L-cyctein added to 0.04% final concentration, incubated at 22 ℃ for 72 h. Aerobic psychrotrophic counts (APC) were analyzed on Long & Hammer agar (LH) (van Spreekens, 1974) with Fe(III)NH_4Citrat added to a final concentration of 0.025%, incubated at 15 ℃ for 5 days, according to NMKL Method No 184. Analysis for *Pseudomonas* spp. (PsC) was performed on Oxoid™ *Pseudomonas* CFC selective agar (CFC) (CM0559/SR0103, Thermo Fisher Scientific) incubated at 25 ℃ for 48 h, while *E. coli* and other coliform bacteria was analysed on Oxoid™ Chromogenic Coliform Agar (Oxoid, CM1205) incubated at 37 ℃ for 24 h. Detection of *Listeria* spp. was performed according to the Oxoid Listeria Precise™ Method with minor adjustments. Ten mL of the stomacher-liquid or 3 mL of the swab liquid was added to 90 mL or 27 mL respectively of ONE Listeria Enrichment Broth (Oxoid, CM1066B) giving a 10-fold dilution and incubated at 30 ℃ for 24 h. Positive samples for presumptive *Listeria* spp, seen by a color change in the broth from brown to black, were streaked (10 µL) on to *Brilliance*™ *Listeria* Differential Agar plates (Oxoid, CM1080B with added *Brilliance*™ Listeria Selective Supplement SR0227E and *Brilliance*™ Listeria Differential Supplement SR0228E,). Negative samples were left at 30 ℃ for up to 7 days to also detect damaged, stressed, and slow growing *Listeria* strains.

2.3 Preparation of isolates and DNA extraction

Colonies were picked from LH agar plates for isolation and further analysis. For randomized picking of colonies, plates with 10-100 colonies were preferred and an aim of 20 colonies per sampling point was used. Plates containing more than 40 colonies were divided in two and colonies picked from one half. Plates with more colonies were divided in equal sectors and

137 picking was done in one of the sectors (1/2, ¼, 1/8). The isolates were repropagated minimum
138 twice before they were frozen in TSB w/20% glycerol at -80 ℃.
139 Isolates from sampling time 0 (before startup), 1 (one week after startup), 8 (5 months after
140 startup) and 12 (12 months after startup) and selected sampling points (2, 8, 10, 11, 13, 14, 15,
141 17, 18, 20, 22) (Table 1) were selected for further analysis and thereby thawed and plated on
142 LH agar again, incubated at 15 ℃ for 5 days and repropagated twice. DNA extraction from
143 the isolates was done with Micro AX Bacteria Gravity-kit (A&A Biotechnology, Poland)
144 following the producer's procedure. DNA quality and integrity was checked by running 10 µL
145 DNA on 1% agarose gel containing GelRed (Biotium, USA) and visualization under UV-light
146 in a G:box (Syngene, USA). DNA concentration was measured spectrophotometrically by a
147 PowerWaveXS (Biotek®,USA) and Take3 plate with software Gen5 2.0. For isolates
148 resulting in low DNA concentration by this procedure, DNA extraction was performed again
149 with Micro AX Bacteria+ Gravity-kit (A&A Biotechnology, Poland) which include
150 mutanolysin treatment for lysing Gram-positive bacteria.
151
152 **2.4 Classification of isolates by ON-rep-seq method**
153 DNA was normalized (1 ng/µL) and subject to ON-rep-seq analysis at University of
154 Copenhagen. Library preparation and amplifications was performed as described by Krych et
155 al. (2019). In brief: A Rep-PCR with REP primers (GTG)5 was performed to amplify
156 fragments of the DNA before the dual step barcoding Rep-PCR was done to incorporate the
157 Oxford Nanopore Technology (ONT) compatible adapters. The samples were pooled, and the
158 library purified before final DNA quantification measurement and end preparation according
159 to 1D amplicon by ligation protocol (ADE_9003_v108_ revT_18Oct2016), and finally
160 loading of the library on a R9.4.1 flow cell.

Data were collected using Oxford Nanopore software: GridION 19.12.2 (https://nanoporetech.com). Guppy 4.4.0 toolkit was used to base call raw fast5 to fastq and demultiplex based on custom adapters. Further, the ON-rep-seq data analysis toolbox which is available from github repository (https://github.com/lauramilena3/On-rep-seq), was used to classify the isolates (Krych et al., 2019). From the sequenced amplicons a read length count profiles (LCp) were generated for each sample and a corrected consensus read for all the reads in each peak were generated. Kraken2 (Wood and Salzberg, 2014) metagenomic classifier was used for classification of corrected reads based on NCBI database. For visualization of D_KLsym distance on bacterial LCp, heatmaps was generated based on Ward.D clustering method and modified heatmap3 from R library.

2.5 Sequencing of 16S rRNA gene or *rpoD* gene

Isolates that were not sufficiently classified by ON-rep-seq method were subjected to 16S rDNA sequencing. The universal 16S primers 338f (Huse et al., 2008) and 1492r (Turner et al., 1999) resulting in an amplicon of 1154 bp and covering V3-V9 variable regions were used. PCR reactions were performed with 25 µL reactions containing 1x PCR buffer, 200 µM of each nucleotide, total concentration of $MgCl_2$ at 650 µM, 0.4 µM each primer, 2.5 U Taq polymerase (Qiagen), and 50–100 ng template DNA. The PCR amplification cycles were as follows: Initial denaturation at 95 °C for 15 min, 25 cycles of denaturation at 95 °C for 60 s, annealing for 30 s at 58 °C, and extension at 72 °C for 60 s, followed by a final extension at 72 °C for 5 min.

Isolates identified as *Pseudomonas* genus by ON-rep-seq, without a clear species classification were subjected to sequencing of the *rpoD* housekeeping gene with primers PsEG30F/PsEG790R, resulting in a 760 bp product (Mulet et al.,2009). The PCR reactions

were performed with 25 µL reactions containing 1x PCR buffer, 200 µM of each nucleotide, total concentration of $MgCl_2$ at 650 µM, 0.5 µM each primer, 2.5 U Taq polymerase (Qiagen), and 50–100 ng template DNA. The PCR amplification cycles were as follows: Denaturation at 95 °C for 15 min, 30 cycles of denaturation at 95 °C for 60 s, annealing at 55 °C for 60 s, and extension at 72 °C for 60 s, followed by a final extension at 72 °C for 5 min. PCR products were enzymatically purified by ExoSAP-IT™ (Thermo Fischer Scientific, USA) procedure which entail incubation at 37 °C for 15 min to degrade remaining primers and nucleotides followed by inactivation at 80 °C for 15 min. A quality control of the purified PCR products was performed, and the PCR products were prepared for sequencing according to Eurofins LightRun sequencing acquirements.

Classification of the isolates was done by comparison to sequences currently available in the NCBI database (www.ncbi.nlm.nih.gov/BLAST) using BLASTN search.

2.6 Statistical data analyses

Statistical analyses were performed using IBM SPSS Statistics 27. To analyze the difference in bacterial load for sampling points of different categories (non-food contact surfaces (NFCS), food contact surface (FCS) slaughter dep., FCS filet dep., and fish), a One-way ANOVA with post hoc Tukey test was done. Correlation between the bacterial parameters Aerobic count, aerobe psychrotrophic count, *Pseudomonas* spp. count, H_2S-producing bacteria and coliform count was calculated by a bivariate correlation analysis for Pearson's coefficient.

208 **3 Results**

209 **3.1 High variability in bacterial cell counts between the sampling points**

210 A total of 312 different samples were collected after cleaning and disinfection, from 24
211 different sampling points throughout a newly opened salmon processing facility (Figure 1).
212 Of these samples, 26 (8%) were negative for all parameters checked.
213 The bacterial counts ranged from zero to 5.9 log CFU/cm^2, 5.8 log CFU/cm^2 and 5.3 log
214 CFU/cm^2 for AC, APC and PsC respectively (Figure 2). Not surprisingly, the NFCSs had the
215 highest bacterial count among the environmental samples, with an average of 1.9 log
216 CFU/cm^2, 2.2 log CFU/cm^2 and 1.9 log CFU/cm^2 for AC, APC and PsC respectively.
217 Average bacterial count for these three parameters on FCS in the slaughter department were
218 0.4 log CFU/cm^2, 1.0 log CFU/cm^2 and 0.8 log CFU/cm^2, and on FCSs in the filet department
219 0.2 log CFU/cm^2, 0.3 log CFU/cm^2 and 0.2 log CFU/cm^2 for AC, APC and PsC respectively.
220 The gutting machine suction stands out with the highest variability in bacterial cell counts for
221 both AC, APC and PsC ranging from zero to 5.8 log CFU/cm^2 (Figure 2). The inlet water had
222 an average AC of 2.3 log CFU/mL, APC of 3.4 log CFU/mL and PsC of 2.1 CFU/mL.
223 Moreover, fish filet, skin and gills of round fish were sampled at four occasions during the
224 time period. The bacterial counts varied between the samplings and no clear trend was
225 observed (Figure 2).
226 A significant difference (ANOVA, posthoc Tukey $p<0.001$) in bacterial load could be noticed
227 between the three groups of surface sampling points: NFCS, FCS in slaughter department and
228 FCS in filet department for three of the parameters, AC, APC and PsC.
229
230 There was a high correlation between AC, APC and PsC for the different sampling points,
231 with Pearson correlation coefficient at 0.863, 0.844 and 0.859 for AC vs. APC, APC vs. PsC
232 and PsC vs. AC respectively ($p<0.01$).

Coliforms and H_2S-producing bacteria were only sporadically detected and mostly at low numbers. The head cutter knife was the only FCS where coliforms was detected more than once. H_2S-producing bacteria was overall more frequently detected, mostly on the NFCSs. Yet, the correlation between H_2S-producing bacteria and coliforms towards all the above-mentioned bacterial parameters was significant at 0.01 level.

During the time frame of the sampling, no *L. monocytogenes* was detected on the cleaned and disinfected surfaces. *L. innocua* was detected twice; one time on the head cutter knife and one time in the drain below filleting machine.

3.2 The total bacterial load in the facility increased during the first year of processing

To assess the development of the general bacterial load on the FCSs over time, the average bacteria count for all contact sampling points was calculated (Figure 3). On FCS in slaughter department (Figure 3A) an increase in the bacterial load was seen for both AC, APC and PsC, but the increase was most obvious for AC. For FCSs in the filleting department (Figure 3B) a similar trend was observed but the increase was not as distinct as in the slaughter department. For the NFCS, it was observed a slight increase in bacterial load over time. The bacterial load on the fish filets did not increase over time.

3.3 Microbial profiling by ON-rep-seq and 16S rRNA gene amplicon sequencing

A total of 520 isolates were identified by ON-rep-seq resulting in the detection of 75 unique taxa belonging to 27 different genera. Of all these isolates 78% were identified to species level, additional 8% was identified to genus level and 14% remained unclassified. All isolates that were not classified by ON-rep-seq and the isolates that only reached a genus-level classification were subjected to sequencing of the 16S rRNA gene. When combining the

identification from ON-rep-seq analysis and 16S rDNA sequencing 84 unique taxa were detected, and these belonged to 34 different genera. From this combined identification 85% of the isolates were identified to species level, 12% were identified to genus level and 4% still remained unclassified.

Members of the genus *Pseudomonas* were the most abundant in this combined classification and accounted for 26% (n = 22) of the taxa and 46% of all isolates (Figure S1). Other frequently detected species were *Acinetobacter* spp. (14%), *Serratia* spp. (6%), *Chryseobacterium* spp. (5%) and *Aliivibrio* spp. (3%). The most prevalent genera among these isolates that were subjected to 16S rDNA sequencing were *Chryseobacterium* (n = 15), *Photobacterium* (n = 11), *Shewanella* (n = 8) and *Glutamicibacter/Arthrobacter* (n = 6). Isolates classified as for example *Chryseobacterium* spp. or *Shewanella* spp. by 16S sequencing had a few consensus reads from ON-rep-seq that was annotated to different species of *Chryseobacterium* or *Shewanella* respectively. For isolates classified as *Photobacterium* spp. many consensus reads were assigned as unclassified, several reads were assigned to various *Vibrio* spp. and additionally one or two consensus reads were assigned to genera like *Photobacterium*, *Aliivibrio*, *Providencia*, *Pantoea* and *Actinobacillus*. *Photobacterium*, *Vibrio* and *Aliivibrio* are closely related and belong to the family *Vibrionaceae* while the closest commons classification level for *Providencia*, *Pantoea* and *Actinobacillus* is on class level (*Gammaproteobacteria*). Of the eleven isolates that belonged to the genus *Photobacterium*, five were most similar to *Photobacterium phosphoreum*, according to 16S rRNA gene sequencing. All the isolates classified as *Shewanella* by 16S rRNA gene sequencing had highest similarity to *S. algidipiscicola*.

Only 8% of *Pseudomonas* isolates obtained a clear species classification by ON-rep-seq. Additional 42% were assigned to different unclassified strains of *Pseudomonas*. The remaining 50% of *Pseudomonas* isolates could not be classified by this method. For some

283 *Pseudomonas* isolates the classification was ambiguous as the different consensus reads were
284 assigned to different species. Isolates of *Pseudomonas* genus that did not obtain a clear
285 species classification by ON-rep-seq were subjected to sequencing of the *rpoD* gene (n =
286 101). Of these 101 *Pseudomonas*, 62 isolates (61%) obtained a clear species classification, *P.*
287 *fluorescens* being the most abundant (43%) (Figure S2). Several of the isolates that were most
288 similar to the unclassified *Pseudomonas* strains Myb193, FDAARGOS_380, LG1D9 or
289 NC02, had the highest similarity to *P. fluorescens* by *rpoD* analysis with similarities between
290 98.58-99.72%, 98.15-100%, 98.72-99.57% and 99.72-99.86% respectively. If counting these
291 in addition to the strains already classified as *P. fluorescens*, makes *P. fluorescens* by far the
292 most abundant species in this material accounting for 23% of all the isolates. Additionally,
293 eight isolates (8%) were assigned to different strains of unclassified *Pseudomons* spp. while
294 the remaining 31 isolates had a high similarity to several different species and could not be
295 classified with certainty or could not be assigned to any known species. Fifteen of these 31
296 isolates had a similarity to other *rpoD* sequences of less than 98% and could be considered as
297 previously undescribed species (Girard et al., 2020) while the remaining isolates had high
298 similarity to several *Pseudomonas* spp. belonging to different groups and subgroups.
299
300 **3.4 Spatial and temporal dynamics of the bacterial communities**
301 Species of *Pseudomonas* were more abundant on the equipment and environment surfaces
302 than in inlet water and on the salmon fillet (Figure 4). When comparing all the analyzed
303 sampling points, *Pseudomonas* was present in 94% of them. *Photobacterium* was only
304 detected in the inlet water and on the salmon fillet, while *Vibrio* was only detected in the inlet
305 water. *Aliivibrio* and *Psychrobacter* was only detected in inlet water and on conveyor belt in
306 the slaughter department and do not seem to be carried further into the facility. The genera
307 *Janthinobacterium*, *Leucobacter*, *Comamonas*, *Morganella*, *Pseudochrobactrum*,

Arthrobacter, *Pedobacter*, *Sphingobacterium* and *Galactobacter* were only detected as singletons.

3.5 Species/strain-level resolution reveals time and point-specific communities

ON-rep-seq analysis assigned most of the isolates to species level, but some isolates only to genus level (Figure 4). Using dynamic classification gives a high resolution and reveals that only a few species are detected at several sampling points or time (Supplemental Table S1). Of all the detected taxa, 61% (n = 51) were detected in only one sample. Only 10% (n = 8) of the taxa were detected in five samples or more. Five of these taxa belonged to different groups, species, or strains of *Pseudomonas* genus while the rest belonged to *Microbacterium* sp., *Serratia liquefaciens* and the remaining group of unclassified isolates. In addition, only 12% (n = 10) of the taxa were detected both in the equipment and on the salmon fillet. Of these taxa, seven belonged to *Pseudomonas* genus while the rest belonged to *Microbacterium* sp., *Serratia liquefaciens* and the remaining group of unclassified isolates. The overall strain diversity is visualized in heatmaps generated by ON-rep-seq method depicting the strains diversity (Supplemental figures S3-S8).

4 Discussion

In this study we aimed to classify members of the bacterial communities in the equipment and the food processing environment of a newly opened salmon processing plant by sampling the same sampling points 13 times through a period of one year after start-up. We identified most of the isolates to species-level by using a 3rd generation sequencing based method, ON-rep-seq, and by this documented the development and dynamics of the bacterial communities during this period.

Initially quantification of different bacterial groups from cleaned and disinfected surfaces in the salmon processing environment was performed with standard cultivation methods and as expected, the bacterial counts were generally low. However, the microbial diversity was relatively high. Presently, there are no general acceptance limits for hygiene samples, however, NSW Food Authority states in its guidelines for environmental swabbing that a total viable count of >10 CFU/cm^2 (average over time) is unacceptable on cleaned and disinfected FCSs in meat and poultry abattoirs (NSW, 2012). It has also been indicated that a contamination level below 2,5 CFU/cm^2 after cleaning and disinfection should be achievable (Griffith, 2016).

In this study we observed that the limit of 2,5 CFU/cm^2 (0,4 log CFU/cm^2) was achievable but not always achieved, neither was the limit of less than 10 CFU/cm^2 (2 log CFU/cm^2). This is in concordance with the findings from Møretrø et al. (2016) where they reported AC on Iron agar to be > 3 log CFU /cm^2 for the most contaminated FCSs.

As the processing plant was entirely new and the equipment had only been used for test runs prior to our first sampling, a general increase of bacterial load on the surfaces over time was expected. This was observed on the FCSs in the slaughter department and slightly on the FCSs in the filleting department. Through this first year of production, the facility was not always run at maximum capacity, hence, all the production lines in the filleting department

were not used every day. This affected the cleaning and disinfection (C&D) routines in the facility. C&D of the production lines in the filleting department was performed if the line had been used or were to be used the next day. Based on information from the facility about when the production line of our focus had been used, there was no correlation between recent C&D and bacterial cell counts.

No general increase over time was observed on the NFCSs but rather a considerable variability in cell counts from one sampling to the next. The high variability (0-5,9 log CFU/cm^2) in the NFCS can probably be explained by the cleaning and disinfection routines in the facility where they practiced an extraordinary disinfection of the drains and waste funnels rotational in different parts of the facility.

One NFCS of special interest is the gutting machine suction. We observed a considerable increase in bacterial count for both total AC, APC and PsC in the suction of the gutting machine during the first 9 months (Supplemental Figure S9). The sampled unit is a special steel pipe that sucks out the viscera of the fish by using vacuum. The end of the pipe can be in contact with the abdominal cavity of the fish and, with prolonged use parts may be worn out and the possibility of flush back is present (personal communication, QC manager at facility). This exact equipment is known to be a high risk area for bacterial contamination and also for *Listeria monocytogenes* colonization due to low accessibility for cleaning (Løvdal, 2017). During our study the facility experienced an increasing problem with frequent detection of *L. monocytogenes* in this specific equipment. Because of this, an extraordinary disassembly and cleaning measures were effectuated by the facility personnel between our samplings 10 and 11. This resulted in bacterial counts at this point dropping to below the detection limit for all bacterial parameters except AC in Iron agar (IA). In our study, this sampling point is characterized by the highest variability in bacterial cell counts.

375 The bacterial communities associated with salmon, production equipment and salmon fillet is
376 dominated by Gram-negative, psychrotrophic bacteria and, according to Broekaert et al.
377 (2011), LH agar is the best suited medium to analyze for these communities. The bacteria
378 detected on IA and on LH agar will in many cases be partly overlapping depending on the
379 environmental conditions, therefore the high correlation between the total AC on IA and APC
380 on LH was expected. Broekaert et al. (2011) also showed that most species of psychrotrophic
381 *Pseudomonas* grew well on LH agar, and due to the high prevalence of *Pseudomonas* spp. in
382 food processing environments (Cobo-Díaz et al., 2021, Maes et al., 2019, Møretrø et al.,
383 2016), a correlation between *Pseudomonas*, APC and AC was expected.
384 It is increasingly common to analyze bacterial communities by culture independent methods
385 as metagenomic sequencing or sequence based microbial profiling (Alexa et al., 2020,
386 McHugh et al., 2021, Solden et al., 2016, Zwirzitz et al., 2020). In this study however, it was
387 of our interest to get bacterial isolates from the samples. For this reason, a culture-based
388 approach was chosen and subsequently the high throughput sequencing-based method, ON-
389 rep-seq was used to analyze and differentiate the isolates down to species or strain level. We
390 have previously used this method for differentiating between *L. monocytogenes* isolates from
391 a specific industry case (Thomassen et al., 2021), and here we explore it at a much larger and
392 more diverse set of isolates.
393 The ON-rep-seq method classified 78% of the isolates to species or strain level, 8% to genus
394 level and 14% remained unclassified. Many of the isolates that were only identified to genus
395 level belonged either to the genus *Pseudomonas* or to a less described species in the
396 respective genus. ON-rep-seq classification resolution relays on the database completeness
397 and quality. Hence, its performance is largely affected if the reference genomes in the
398 database are not complete or misclassified. For this reason, our results were supplemented

with 16S rRNA gene amplicon sequencing for isolates unclassified by ON-rep-seq, as curated databases for 16S rRNA genes could provide additional information despite lower resolution. Isolates classified as *Chryseobacterium* spp., *Shewanella* spp., or *Photobacterium* spp. by 16S rDNA sequencing were not sufficiently classified by ON-rep-seq. The ambiguous classification of isolates from these genera most likely occurs because of the lack of matching sequences in the database. While *Chryseobacterium* spp. and *Shewanella* spp. could be assigned to the correct genus, isolates of *Photobacterium* spp. were wrongly assigned to *Vibrio* spp. in some cases.

Of all the isolates identified, almost half (46%, n = 237) belonged to the genus *Pseudomonas*. This was not unexpected as *Pseudomonas* spp. has been found to be the most common bacteria in several different food premises regardless of sampling method or choice of analyzing method (Cobo-Díaz et al., 2021, Gram and Huss, 2000, Maes et al., 2019, Møretrø et al., 2016, Parlapani and Boziaris, 2016). The species classification obtained by ON-rep-seq for isolates belonging to *Pseudomonas* genus was relatively low as 50% of the isolates could not be classified. *Pseudomonas* isolates where a species classification could not be called with confidence were subject to sequencing of the *rpoD* gene (n = 101). Sequencing of this housekeeping gene has been suggested as an effective and accurate tool for identification and classification of *Pseudomonas* isolates by Girard et al. (2020). In our case it resolved a species classification for 61% (n = 62) of the *Pseudomonas* isolates subjected to this analysis. The most abundant species according to the *rpoD* sequencing was *P. fluorescens* or uncertain species of *P. fluorescens* group.

It was clear that the species level identification resolution of ON-rep-seq for *Pseudomonas* was significantly reduced compared to other genera. By using two different methods in the attempt to classify the *Pseudomonas* isolates we obtained a higher number of reliable species classifications but also some partly contradictive taxonomy assignment between ON-rep-seq

and *rpoD* sequencing were registered. ON-rep-seq assign many *Pseudomonas* isolates to *P. koreensis* species because this were the species where most of the consensus reads had the best match. But with many of the other consensus reads matching several different *P. fluorescens* strains, this species assignment is questionable. However, Gomila et al. (2015) revealed that several *Pseudomonas* strains previously assigned as *P. fluorescens* clustered intertwined with *P. koreensis* subgroup and close to *P. koreensis* type strains in phylogenetic analysis based on four concatenated housekeeping genes (16S rRNA, *rpoB*, *rpoD* and *gyrB*). The same study also reported that about 30% of sequenced genomes of non-type strains were not correctly assigned at the species level and 20% were not identified at all. The genus *Pseudomonas* is one of the largest bacterial genera with almost 200 recognized species and over 500 full genomes available in Genbank (Koehorst et al., 2016, Nikolaidis et al., 2020). This makes species classification of *Pseudomonas* very complicated (Gomila et al., 2015, Lalucat et al., 2020, Özen and Ussery, 2012), particularly for methods that relay on shotgun sequencing or extragenic regions sequencing. It is therefore clear that poor performance of ON-rep-seq on classification of *Pseudomonas* spp. is related to the meagre quality of the databases.

However, the LCp profile comparison with heatmaps generated by the ON-rep-seq method indicates rather high strain diversity in the analyzed samples (Supplemental figures S3-S8). Figure S5 contains all the *Pseudomonas* isolates from this study and shows the complexity of this genus. The intertwined clustering of *P. koreensis* and *P. fluorescens*, as reported by (Gomila et al., 2015), can also be seen in the Figure S5, but here also other species are intertwined. Additionally, it is apparent that isolates classified as the same species/strain do not consistently cluster together. In Figure S6 all the *Acinetobacter* isolates are compared, and the clustering for this genus is much more consistent with the species classification than it is for *Pseudomonas*. It has earlier been reported that rep-PCR, is a well-suited method to

differentiate between strains within the genus of *Acinetobacter* (Pasanen et al., 2014, Snelling et al., 1996). And, as rep-PCR is the first step in ON-rep-seq, a good differentiation of *Acinetobacter* spp. was achieved. In our study there is a difference in the diversity between the *Pseudomonas* set of isolates and the *Acinetobacter* set. But the picture we see here (Supplemental figure S5) also indicates that the genetic diversity within *Pseudomonas* genus, in addition to previous mentioned database issues, makes the species classification by this method difficult.

In this study the bacterial communities remaining after C&D seem to vary both through time and space, and many of the detected species were only detected once and at one point, though at a high number. The diversity within the samples was generally low but the diversity between the samples was high. This phenomena with a high day-to-day diversity have also been observed in other studies (Cobo-Díaz et al., 2021, Johnson et al., 2021). In our case we suspect that the reason for this was that the sampled surfaces were daily object to a thorough C&D procedure but, with minor variations from day to day due to manual labor, resulting in variable number of remaining bacteria every day. As very few of the detected bacterial strains were detected at several sampling points and time points, it is not apposite to speak about persistent bacteria based on these results. However, isolates with highest similarity to species/strains *Pseudomonas* sp. MYb193, *Pseudomonas fluorescens*, *Pseudomonas koreensis*, *Pseudomonas* FDAARGOS_380 and to *Serratia liquefaciens*, reoccurred several times, but rarely at the same sampling point.

Of all the bacterial species detected in this study several of them are known spoilage organisms. *Pseudomonas* spp. has been reported to be the main spoilage organism in iced freshwater fish (Gram and Dalgaard, 2002), in tropical brackish water shrimp stored at 0 °C (Dabadé et al., 2015), and in gutted sea bream (Parlapani et al., 2015) among others.

473 Both *Photobacterium phosphoreum*, which was detected on several occasions in this study,
474 and various *Shewanella* spp. are well-known spoilage bacteria in fish (Dalgaard et al., 1997,
475 Gram and Dalgaard, 2002, Gram and Huss, 2000). All the isolates classified as *Shewanella* by
476 16S rRNA gene sequencing had highest similarity to *S. algidipiscicola*. This species is
477 reported to both reduce TMAO and to produce H_2S (Satomi et al., 2007), thus it must be
478 considered as a spoilage bacterium. In addition, *Microbacterium* sp., *Acinetobacter* sp.,
479 *Stenotrophomonas* sp. and several other of those detected in low numbers, have been shown
480 to have spoilage potential (Maes et al., 2019). Based on this we must assume that the detected
481 bacterial flora poses a significant risk for spoilage of the salmon filets produced.
482

5 Conclusion

In this study of bacterial communities in a salmon processing plant we have documented a generally low contamination level on food contact surfaces but with a few questionable spots. We saw a general increase in contamination level on food contact surfaces, especially in the slaughter department through the first year of production. Bacterial load on salmon filet at the end of the production line does not increase.

A diverse psychrotrophic bacterial community, highly dominated by *Pseudomonas* spp. was detected, and most of the detected species have been reported to have a spoilage potential in seafood.

By classification of bacterial isolates to species-level and differentiating between strains we revealed point-specific bacterial communities, which indicates limited number of persistent bacteria. The detailed knowledge of the bacterial communities on species level can be significant for improving cleaning and disinfection routines and, it can be helpful in evaluating the shelf life and the food safety of the product.

The ON-rep-seq method has a potential in species-level identification for most bacteria in these complex bacterial communities but as also reported for other methods, it has difficulties in clear species classification within the highly divergent *Pseudomonas* genus. Additionally, novel bacteria (not present in databases), non-complete draft genomes, or misclassified genomes, will reduce the resolution of taxonomic classification by this method.

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Author contribution:

Gunn Merethe Bjørge Thomassen: Conceptualization (equal); writing – original draft (lead); formal analysis (equal); writing – review and editing (equal); **Lukasz Krych**: Conceptualization (equal); formal analysis (equal); writing – review and editing (equal); **Susanne Knøchel**: Conceptualization (equal); Writing – review and editing (equal); **Lisbeth Mehli**: Conceptualization (equal); writing – original draft (supporting); writing – review and editing (equal).

Data Availability Statement: The ON-rep-seq data analysis toolbox is available from github repository https://github.com/lauramilena3/On-rep-seq

Ethics statement: None required

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700 **Table 1:** Overview of the different sampling points, category (FCS=food contact surface in slaughter
701 department or filleting department, NFCS=non-food contact surface, F=fish), sampling type, water,
702 cloth, swab or fish fillet, and approximate sampling area.

Sampling point	Sampling point category	Sampling type	Sampling area/volume
2. Inlet water		Water	100 ml
3. Drain under inlet	NFCS	Cloth	30x30 cm
4. Drain under bleeding tank	NFCS	Cloth	30x30 cm
5. Conveyor	FCS, slaughter	Cloth	30x30 cm
6. Conveyor	FCS, slaughter	Cloth	30x30 cm
7. Drain under orientation rig	NFCS	Cloth	30x30 cm
8. Slide above conveyor	FCS, slaughter	Cloth	30x30 cm
9. Slide above conveyor	FCS, slaughter	Cloth	30x30 cm
10. Gutting machine, suction	FCS, slaughter	Swab	10x10 cm
11. Gutting machine, holder	FCS, slaughter	Swab	10x10 cm
13. Tail cutter	FCS, slaughter	Cloth	90x10 cm
14. Head cutter knife	FCS, slaughter	Cloth	2 x Ø25 cm
15. Head cutter, holder	FCS, slaughter	Swab	10x10 cm
16. Peg band before fileting	FCS, fillet	Swab	5x20 cm
17. Conveyor after fileting	FCS, fillet	Cloth	30x30 cm
18. Conveyor before skinning	FCS, fillet	Cloth	30x30 cm
19. Skinning machine	FCS, fillet	Cloth	30x30 cm
20. Filet turner, slide	FCS, fillet	Cloth	30x30 cm
21. Filet turner, arm	FCS, fillet	Cloth	30x30 cm
22. Drain under filet turner	NFCS	Cloth	30x30 cm
23. Drain under packaging	NFCS	Cloth	30x30 cm
24. Waste funnel, backbone	NFCS	Cloth	30x30 cm
25. Waste funnel, skin	NFCS	Cloth	30x30 cm
26. Drain, personnel sluice	NFCS	Cloth	30x30 cm
F. Fish fillet before packaging	Fish	Fish fillet	25 g
S. Skin, gutted whole fish	Fish	Swab	10x10 cm
G. Gills, gutted whole fish	Fish	Swab	Gills on both side of fish

703

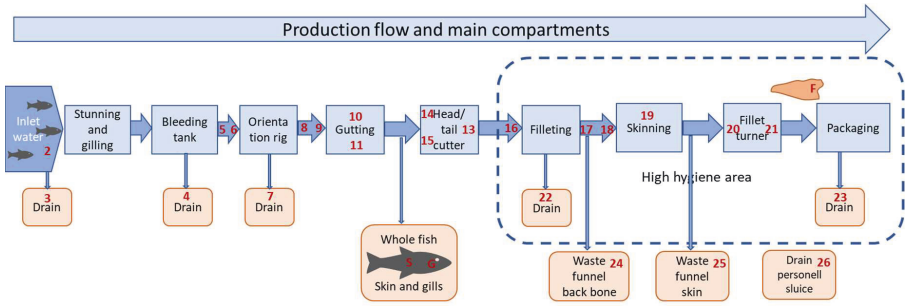


Figure 1: Schematic diagram of the processing facility. Main equipment and machinery are drawn in light blue squares, conveyors in dark blue arrows, while sampled drains and waste funnels (non-contact surfaces) are drawn in orange. Sampling points are marked with red numbers (2-26). Product samples were taken of filets (F) just before packaging in addition to swab samples of skin (S) and gills (G) of whole fish ready for packaging.

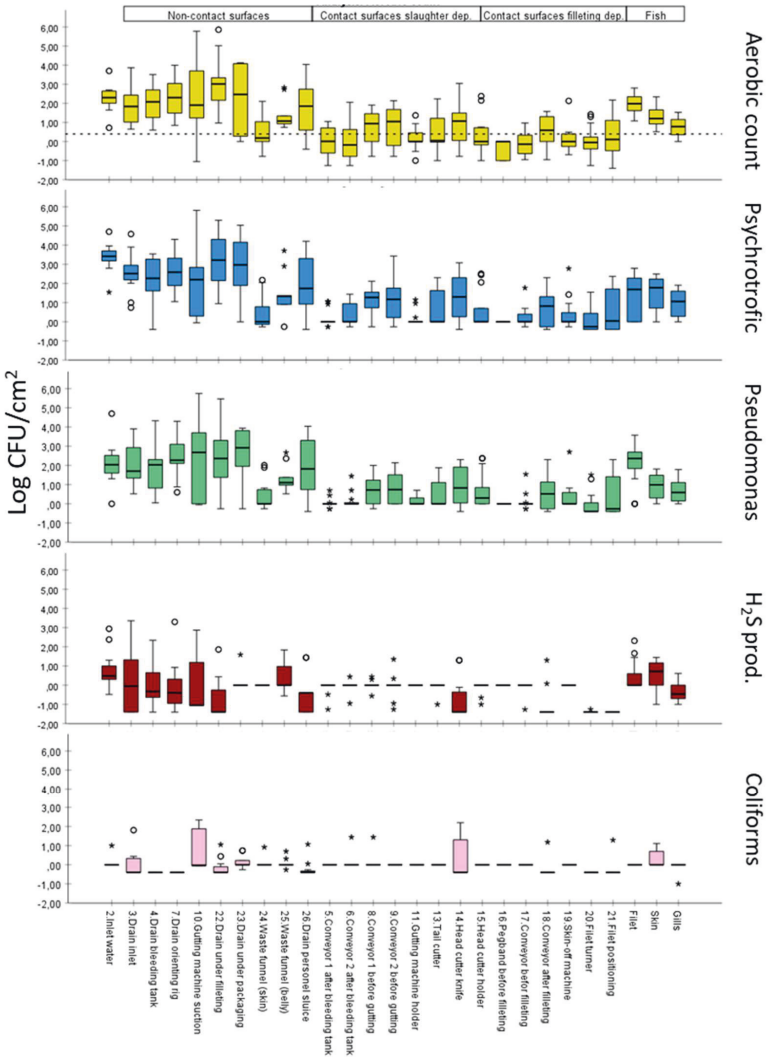


Figure 2: Spatial variation in bacterial load. Aerobic cell count, Psychrotrofic cell count, *Pseudomonas* spp., H_2S-producing bacteria and coliforms from each sampling point.The analysis for aerobic count and H_2S-producing bacteria has a lower detection limit then the analysis for Psychrotrofic count, *Pseudomonas* and coliforms and negative log values means that the cfu/cm^2 was between 0 and 10. The boxes indicate the interquartile range of the data, the black line inside each box is the median and the whiskers extend to the most extreme values within 1,5 x interquartile range. Outliers are market with * or °. The sampling points on the x-axis is divided into four different sample categories, non-contac surfaces, contact surfaces slaghter department, contact surfaces fillet department and fish, as indcated on top of the figure. The dotted line at log 2,5 cfu/cm^2 for aerobic count indicate the acceptance level for aerobic cell count on cleaned and disinfected surfaces s stated by Griffith (2016). Note that sampling point 2: inlet water is included in this figure to show the bacterial level in the water (in log CFU/ml), but since it is not included in the surface category, it was omited from further statistical analyses. The log CFU value for "Filet" is in cfu/g, while the rest are in log cfu/cm^2 due to the nature of the samples.

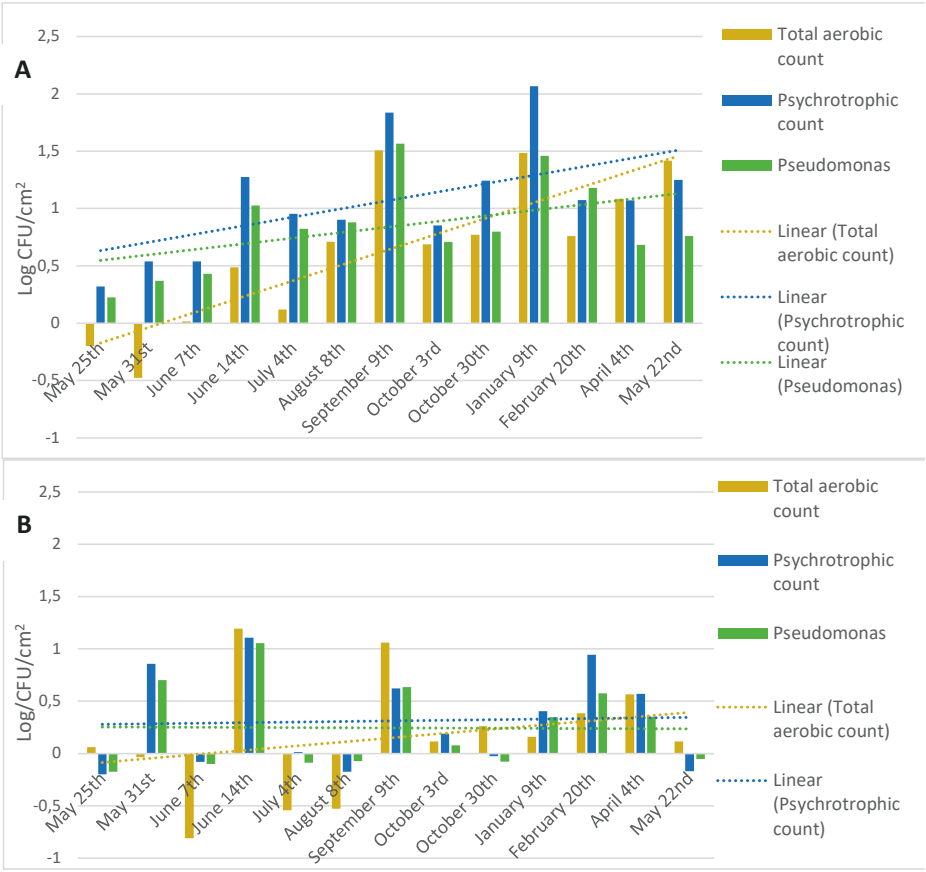
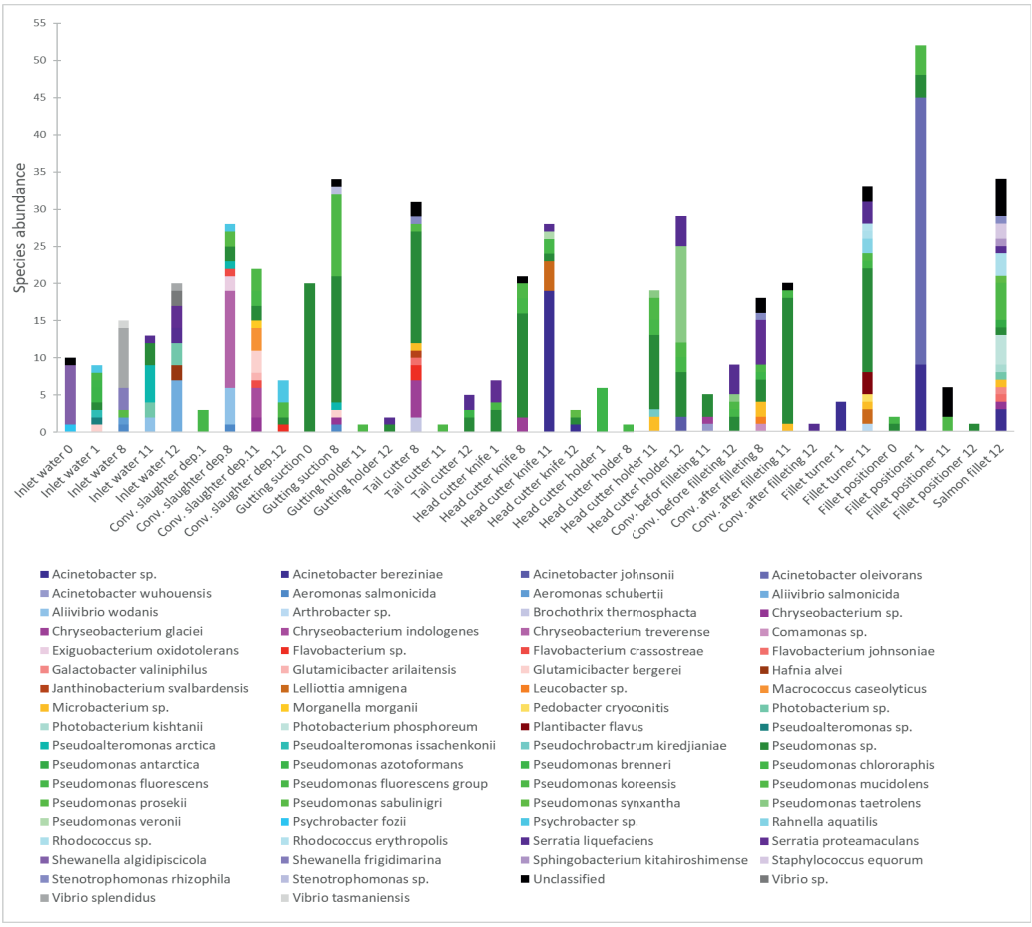


Figure 3: Trend in bacterial load over time. The diagrams show the average log CFU/cm^2 for, **A**: the food-contact surfaces in the slaughter departmend and **B**: the food-contact surfaces in the filleting department, and how it developed over time from the first sampling in May (before start-up of regular production in the facility) to the last sampling in May one year after. All samples were taken in the morning after cleaning and disinfection, before production startup. The trendlines indicates the development for the bacterial counts during the sampling period. For contact surfaces in the slaughter department (A), an increase in bacterial count for both aerobic, aerobic psychrotrophs and for *Pseudomonas* was observed. For the sampling points in the filleting department (B) no general increase was observed, but rather a high variation in bacterial counts between different sampling points.


Species abundance
55
50
45
40
35
30
25
20
15
10
5
0
Inlet water 0
Inlet water 1
Inlet water 8
Inlet water 11
Inlet water 12
Conv. slaughter dep.1
Conv. slaughter dep.8
Conv. slaughter dep.11
Conv. slaughter dep.12
Gutting suction 0
Gutting suction 8
Gutting holder 11
Gutting holder 12
Tail cutter 8
Tail cutter 11
Tail cutter 12
Head cutter knife 1
Head cutter knife 8
Head cutter knife 11
Head cutter knife 12
Head cutter holder 1
Head cutter holder 8
Head cutter holder 11
Head cutter holder 12
Conv. befor filleting 11
Conv. before filleting 12
Conv. after filleting 8
Conv. after filleting 11
Conv. after filleting 12
Fillet turner 1
Fillet turner 11
Fillet positioner 0
Fillet positioner 1
Fillet positioner 11
Fillet positioner 12
Salmon fillet 12
Acinetobacter sp.
Acinetobacter bereziniae
Acinetobacter johnsonii
Acinetobacter oleivorans
Acinetobacter wuhouensis
Aeromonas salmonicida
Aeromonas schubertii
Aliivibrio salmonicida
Aliivibrio wodanis
Arthrobacter sp.
Brochothrix thermosphacta
Chryseobacterium sp.
Chryseobacterium glaciei
Chryseobacterium indologenes
Chryseobacterium treverense
Comamonas sp.
Exiguobacterium oxidotolerans
Flavobacterium sp.
Flavobacterium cassostreae
Flavobacterium johnsoniae
Galactobacter valiniphilus
Glutamicibacter arilaitensis
Glutamicibacter bergerei
Hafnia alvei
Janthinobacterium svalbardensis
Lelliottia amnigena
Leucobacter sp.
Macrococcus caseolyticus
Microbacterium sp.
Morganella morganii
Pedobacter cryoconitis
Photobacterium sp.
Photobacterium kishtanii
Photobacterium phosphoreum
Plantibacter flavus
Pseudoalteromonas sp.
Pseudoalteromonas arctica
Pseudoalteromonas issachenkonii
Pseudochrobactrum kiredjianiae
Pseudomonas sp.
Pseudomonas antarctica
Pseudomonas azotoformans
Pseudomonas brenneri
Pseudomonas chlororaphis
Pseudomonas fluorescens
Pseudomonas fluorescens group
Pseudomonas koreensis
Pseudomonas mucidolens
Pseudomonas prosekii
Pseudomonas sabulinigri
Pseudomonas synxantha
Pseudomonas taetrolens
Pseudomonas veronii
Psychrobacter fozii
Psychrobacter sp
Rahnella aquatilis
Rhodococcus sp.
Rhodococcus erythropolis
Serratia liquefaciens
Serratia proteamaculans
Shewanella algidipiscicola
Shewanella frigidimarina
Sphingobacterium kitahiroshimense
Staphylococcus equorum
Stenotrophomonas rhizophila
Stenotrophomonas sp.
Unclassified
Vibrio sp.
Vibrio splendidus
Vibrio tasmaniensis

Figur 4: Species abundance. Barchart demonstrating bacterial abundance for each sampling point. The x-axis is sorted by sampling point and sampling time (0, 1, 8, 11, 12). Missing points means that there are no isolates collected from that point either because of overgrown plates (Conveyor slaughter dep. time 0) or there were no growth (the rest). The identification of bacterial community has been conducted using ON-rep-seq and 16S rRNA gene amplicon sequencing.

Captions for Supplemental material

Supplemental Table S1: Detailed overview of when and where the isolates were detected. Sampling points are denoted with e.g 0-2 where the first number (0 in this case) denotes the sampling time and the second number (2 in this case) is the sampling spot. Samplingspot number i according to table 1. For better visualization green color is added in the cells of where the bacterial strains are detectes. The darker color the higher number of isolates form that spot. Blue color in the tax ID cell indicates that isolats of that TaxID occures several times. The darker color the more frequent. TaxIDs only detected in the salmon fillet is indicated with a pale apricot color.

Supplemental Figure S1: Overview of the relative abundance of aerobic psychrotrophic isolates (n = 520) identified from the salmon processing facility by ON-rep-seq and 16S rRNA gene amplicon sequencing.

Supplemental Figure S2: Classification of *Pseudomonas* isolates by sequencing *rpo*D. *Pseudomonas* isolates that could not be unambiguously classified down to species level with ON-rep-seq were subjected to sequencing of *rpoD* gene. This provided a species identification for 61% of them. Isolates mentioned as unclassified *Pseudomonas* sp. in the figure had the highest similarity to different unclassified strains of Pseudomonas. Isolates mentioned as Pseudomonas sp. were either impossible to distinguish to one species by the two methods used or they had a sequence similarity < 98% to any other registered *rpoD* sequence in the NCBI sequence database.

Supplemental Figures S3-S8: Overall strain diversity visualized in heatmaps generated by ON-rep-seq method (D_KLsym distance of bacterial LCp, heatmaps was generated based on Ward.D clustering method and modified heatmap3 from R library). The set of isolates is divided into six groups for better visualization.

Supplemental Figure S9: Development of bacterial count over time in sampling point10: gutting machine suction.

Supplemental Table S1: Detailed overview of when and where the isolates were detected. Sampling points are denoted with e.g 0-2 where the first number (0 in this case) denotes the sampling time and the second number (2 in this case) is the sampling spot. Samplingspot number i according to table 1. For better visualization green color is added in the cells of where the bacterial strains are detectes. The darker color the higher number of isolates form that spot. Blue color in the tax ID cell indicates that isolats of that TaxID occures several times. The darker color the more frequent. Taxids only detected in the salmon fillet is indicated with a pale apricot color.

Tax ID	Frequency in total	0-2	0-10	0-21	1-2	1-8	1-14	1-15	1-20	1-21	8-2	8-8	8-10	8-11	8-13	8-14	8-15	8-18	11-2	11-8	11-13	11-14	11-15	11-17	11-18	11-20	11-21	12-2	12-8	12-11	12-13	12-14	12-15	12-17	12-18	12-21	12-F
Pseudomonas	36	0	1	1	1	0	3	0	0	3	0	0	3	0	5	1	0	0	2	1	0	1	2	0	0	1	0	0	1	1	2	0	3	2	0	1	1
Pseudomonas sp. Mfb193	58	0	19	0	0	0	0	0	0	0	0	0	7	0	7	10	0	2	0	0	0	0	5	0	4	4	0	0	0	0	0	0	0	0	0	0	0
Shewanella algidipiscicola	8	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Psychrobacter fozii	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Unclassified	19	1	0	0	0	0	0	0	0	0	0	0	1	0	2	1	0	2	0	0	0	0	0	0	1	2	4	0	0	0	0	0	0	0	0	0	5
Pseudomonas fluorescens	12	0	0	1	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	2	1	0	0	0	0	0	0	0	0	0	2	0	0	0	0
Glutamicibacter bergerei	5	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudomonas brenneri	9	0	0	0	1	0	0	6	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Pseudoalteromonas	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudomonas azotoformans	2	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Psychrobacter	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudoalteromonas issachenkonii	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Acinetobacter bereziniae	35	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	19	0	0	0	0	0	0	0	0	0	0	0	0	0	3
Acinetobacter olevorans DR1	36	0	0	0	0	0	0	0	0	0	36	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudomonas koreensis	18	0	0	0	0	0	0	0	0	4	0	0	11	1	0	2	0	0	0	3	1	0	1	0	0	1	2	0	2	0	0	0	2	1	0	0	5
Pseudomonas fluorescens group	2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Serratia proteamaculans 568	12	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0
Vibrio splendidus	9	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Aeromonas schubertii	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Vibrio tasmaniensis LGP32	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Shewanella frigidimarina NCIMB 400	3	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aeromonas salmonicida subsp. pectinolytica 34mel	2	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudomonas salvaliregil	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aliivibrio wodanis	7	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Flavobacterium crassostreae	2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudoalteromonas arctica A 37-1-2	7	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Exiguobacterium oxidotolerans	2	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chryseobacterium treverense	13	0	0	0	0	0	0	0	0	0	13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudomonas proteli	2	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudomonas sp. FDAARGOS_380	37	0	0	0	0	0	0	0	0	0	2	7	0	2	2	0	0	0	1	0	0	0	3	13	5	0	0	0	0	0	0	0	2	0	0	0	0
Psychrobacter sp. P11F6	4	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0
Chryseobacterium	3	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Stenotrophomonas sp. LM091	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aeromonas salmonicida	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Microbacterium sp. TPU 3398	7	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	2	0	0	0	0	2	0	1	1	0	0	0	0	0	0	0	0	0	0	0
Janthinobacterium svalbardensis	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chryseobacterium glaciei	8	0	0	0	0	0	0	0	0	0	0	0	0	0	5	2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Flavobacterium johnsoniae UW101	2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Brochothrix thermosphacta	2	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Stenotrophomonas rhizophila	3	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Flavobacterium	3	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Pseudomonas sp. NS1(2017)	3	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0
Pseudomonas mucoidens	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudomonas sp. NC02	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	3	0	0	2	0	0	0	0	0	1	0	0	0	0
Pseudomonas fluorescens R124	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Leucobacter	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Comamonas	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudomonas sp. LG109	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Photobacterium	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	1
Pseudomonas sp. M30-35	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Serratia liquefaciens ATCC 27592	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	3	0	2	0	1	2	0	4	4	1	0	1
Chryseobacterium indologenes	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Macrococcus caseolyticus JCSC5402	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chryseobacterium sp. StRB126	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Glutamicibacter arilaitensis Re117	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Morganella morganii	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudomonas veronii	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lelliottia amnigena	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0
Pseudomonas taetrolens	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	11	1	0	0
Pseudochrobactrum kiredjianiae	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Acinetobacter wuhouensis	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudomonas fluorescens A506	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Arthrobacter sp. An36	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Plantibacter flavus	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0
Rhodococcus erythropolis R138	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Rhodococcus sp. BH4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Rahnella aquatilis CIP 78.65 = ATCC 33071	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0
Pseudomonas chlororaphis	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Pedobacter cryoconitis	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Aliivibrio salmonicida LFI1238	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	0	0	0	0	0	0	0	0	0
Acinetobacter	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Vibrio	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
Hafnia alvei	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0
Acinetobacter johnsonii XBB1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0
Pseudomonas syrxantha BG33R	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
Staphylococcus equorum	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Rhodococcus	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
Photobacterium phosphoreum	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
Sphingobacterium kitahiroshimense	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Galactobacter valinilyticus	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Pseudomonas antarctica	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Microbacterium	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Photobacterium kishitanii	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Sum	520	10	20	2	9	3	7	6	4	52	15	28	34	1	31	21	1	18	13	22	1	28	19	5	20	33	6	20	7	2	5	3	29	9	1	1	34
Number of different taxa	83																																				

520

Supplement material Figure S1 and Figure S2

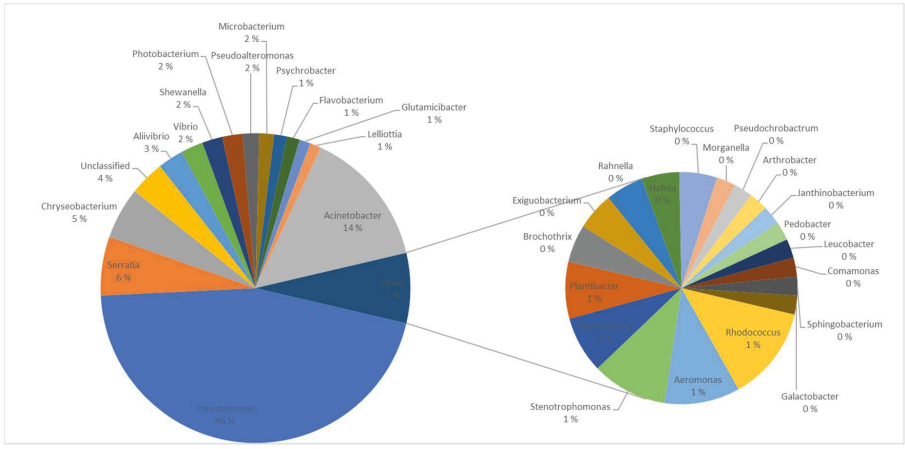


Figure S1: Overview of the relative abundance of aerobic psychrotrophic isolates (n = 520) identified from the salmon processing facility by ON-rep-seq and 16S rRNA gene amplicon sequencing.

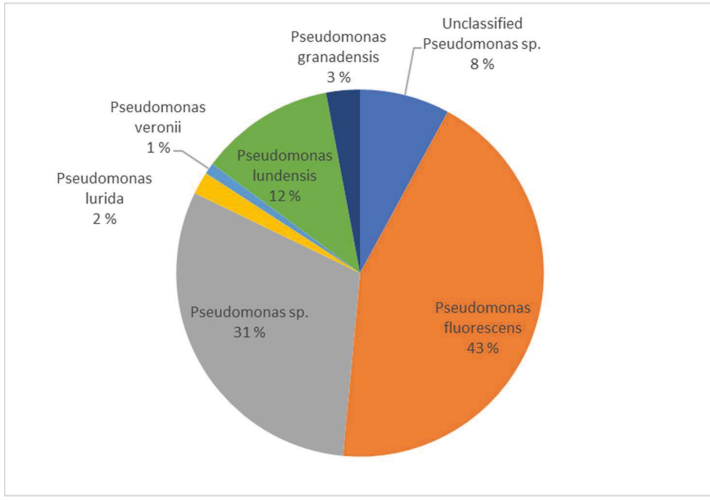
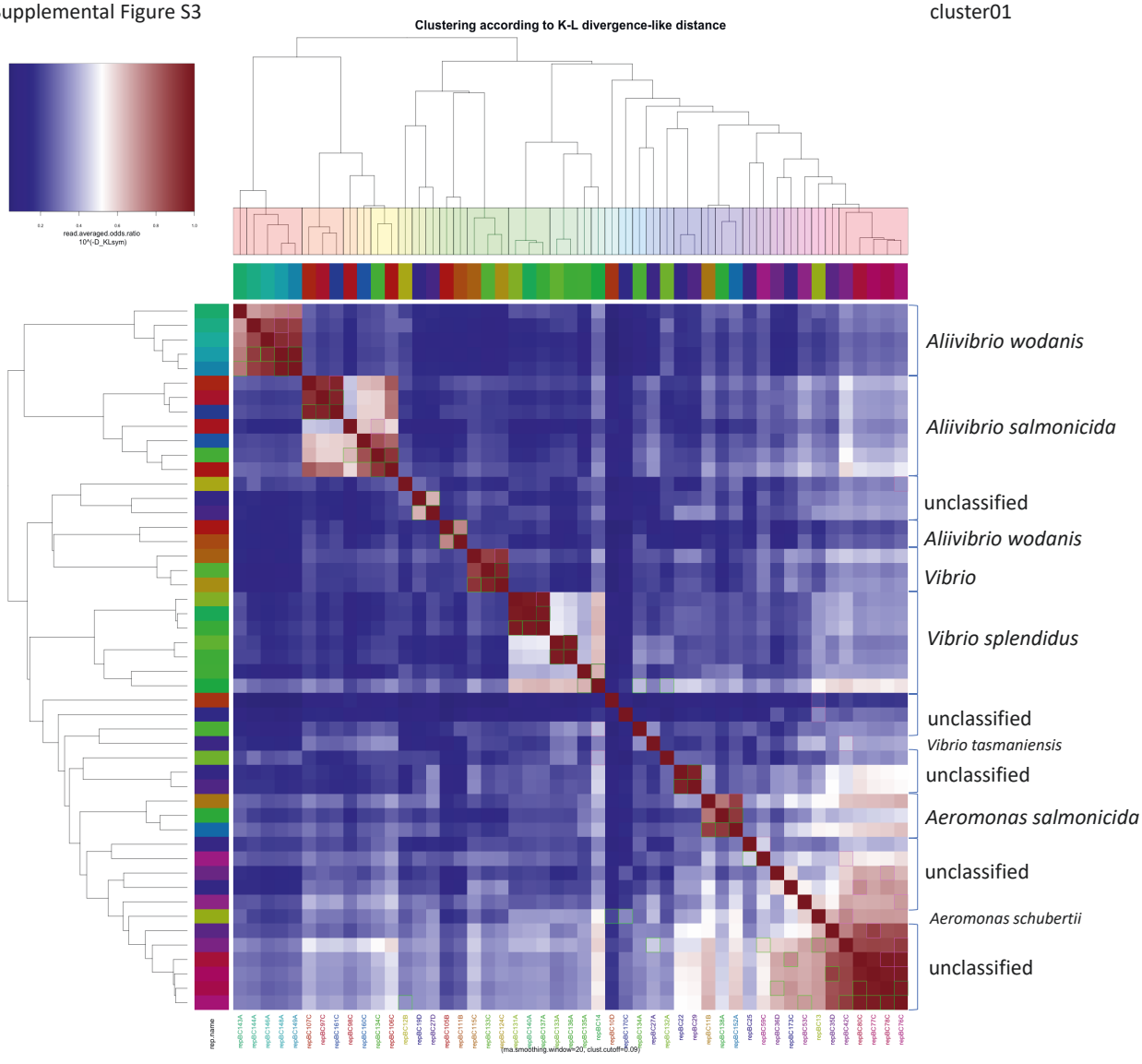


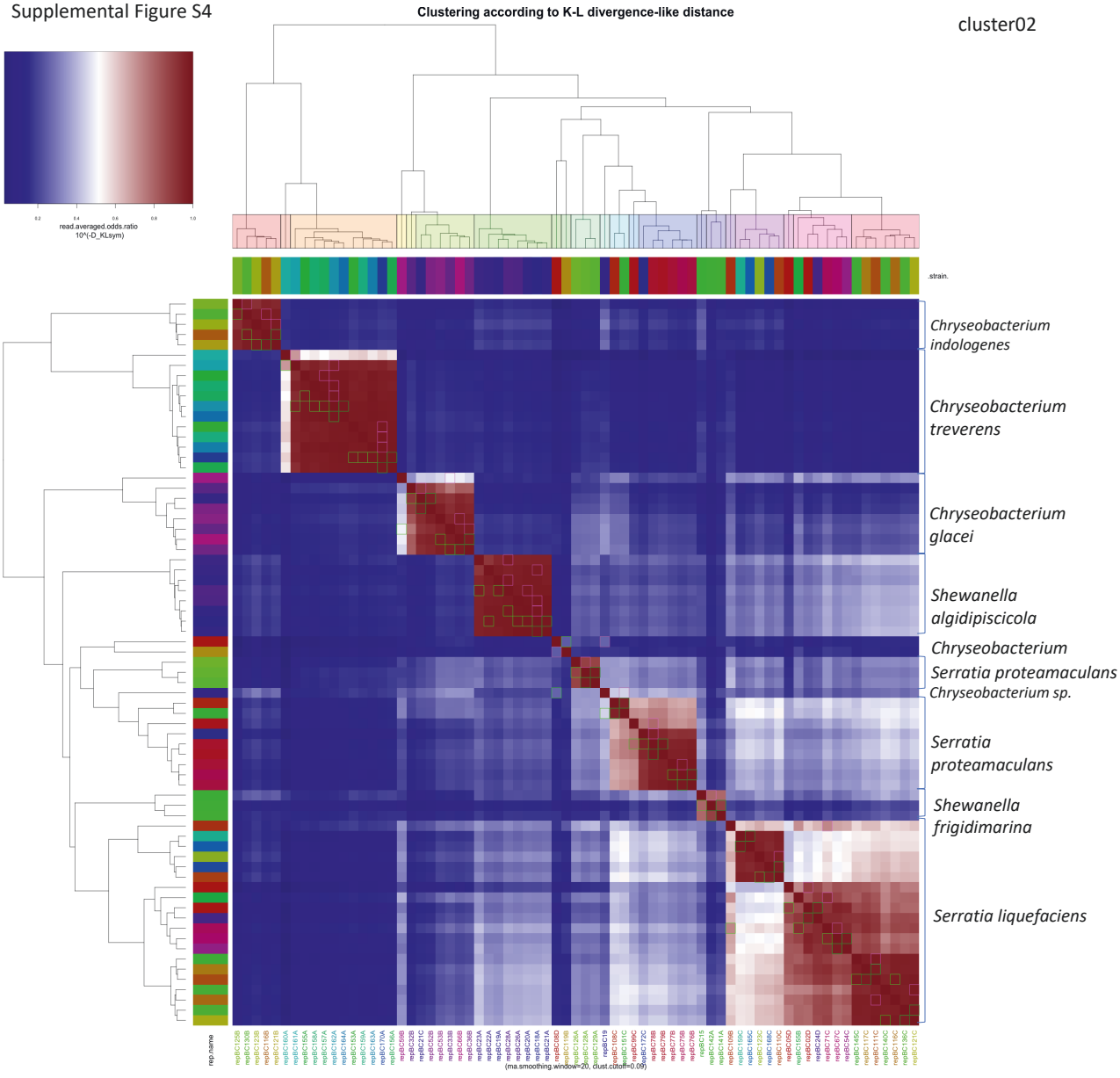
Figure S2: Classification of *Pseudomonas* isolates by sequencing *rpo*D. *Pseudomonas* isolates that could not be unambiguously classified down to species level with ON-rep-seq were subjected to sequencing of *rpoD* gene. This provided a species identification for 61% of them. Isolates mentioned as unclassified *Pseudomonas* sp. in the figure had the highest similarity to different unclassified strains of Pseudomonas. Isolates mentioned as Pseudomonas sp. were either impossible to distinguish to one species by the two methods used or they had a sequence similarity < 98% to any other registered *rpoD* sequence in the NCBI sequence database.

Supplemental Figure S3

cluster01

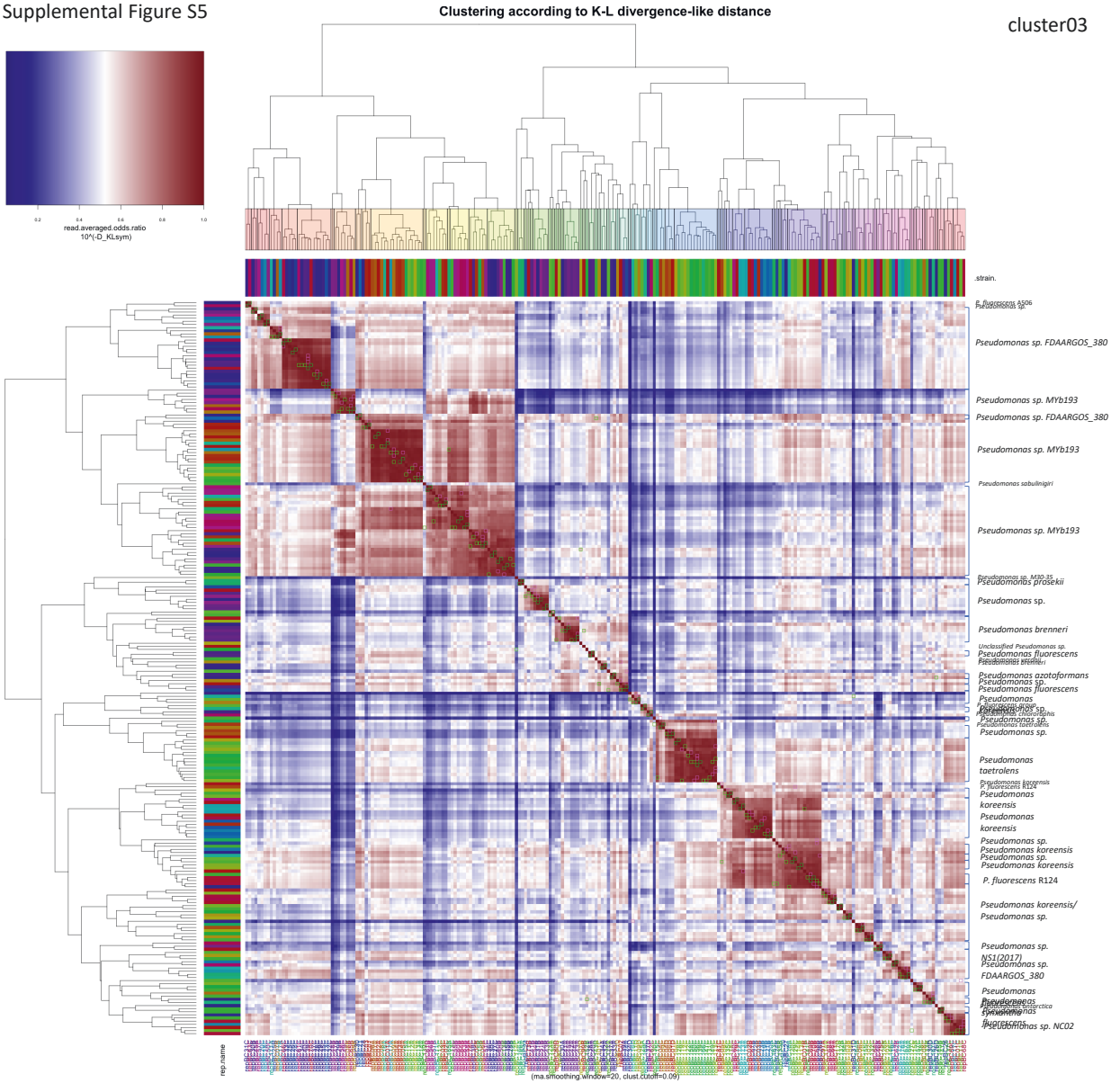


Supplemental Figure S4

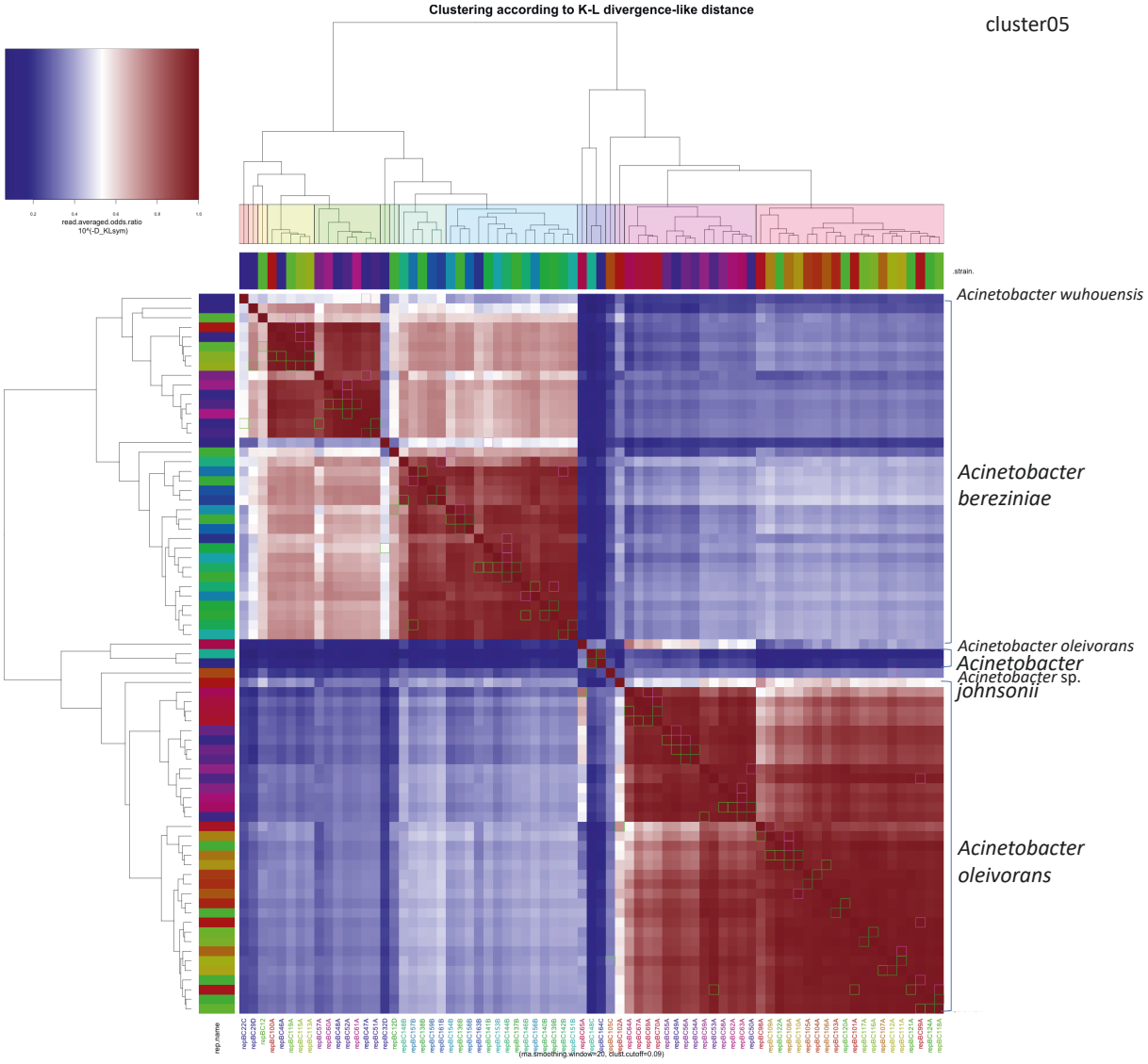


cluster02

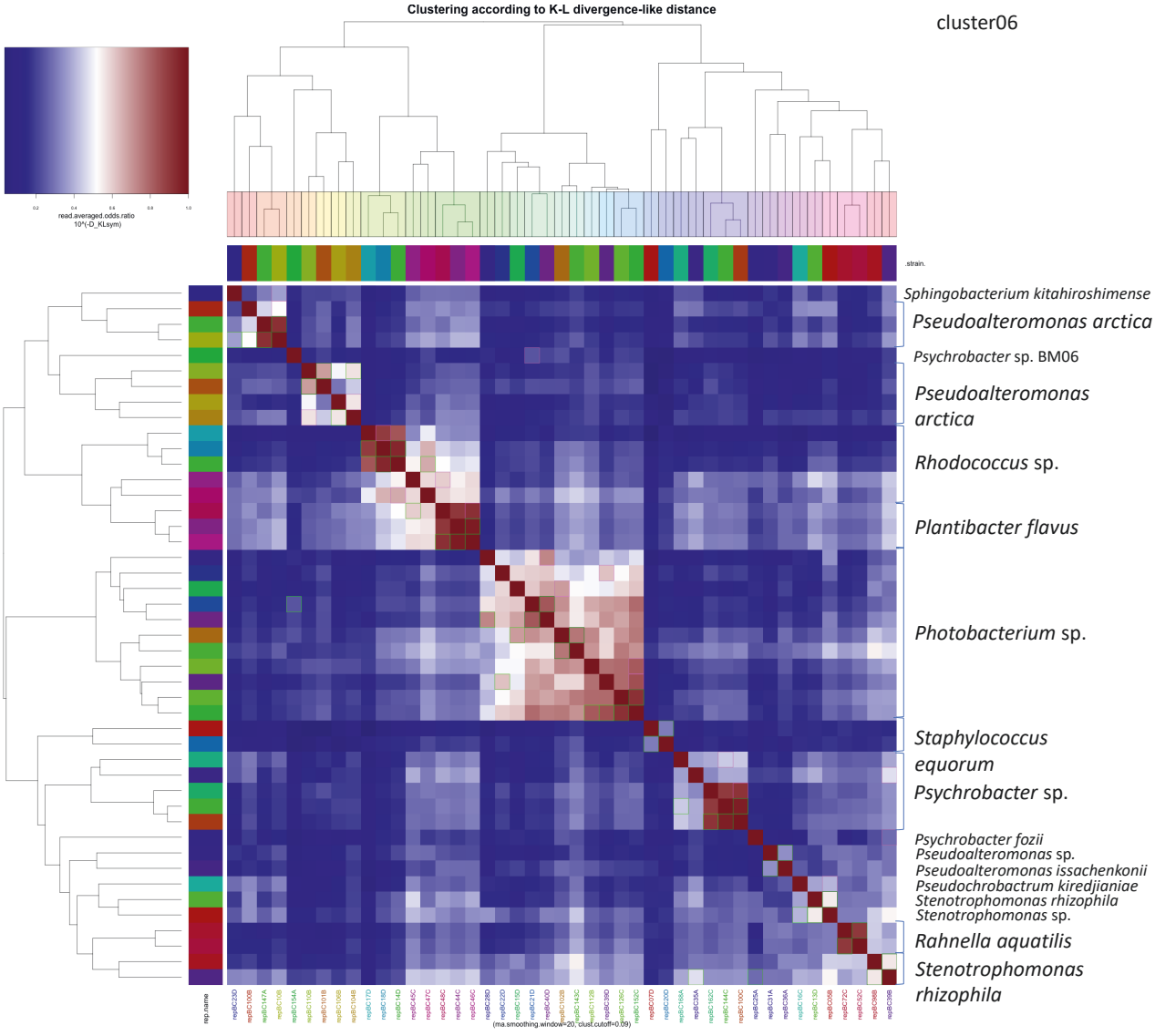
Supplemental Figure S5



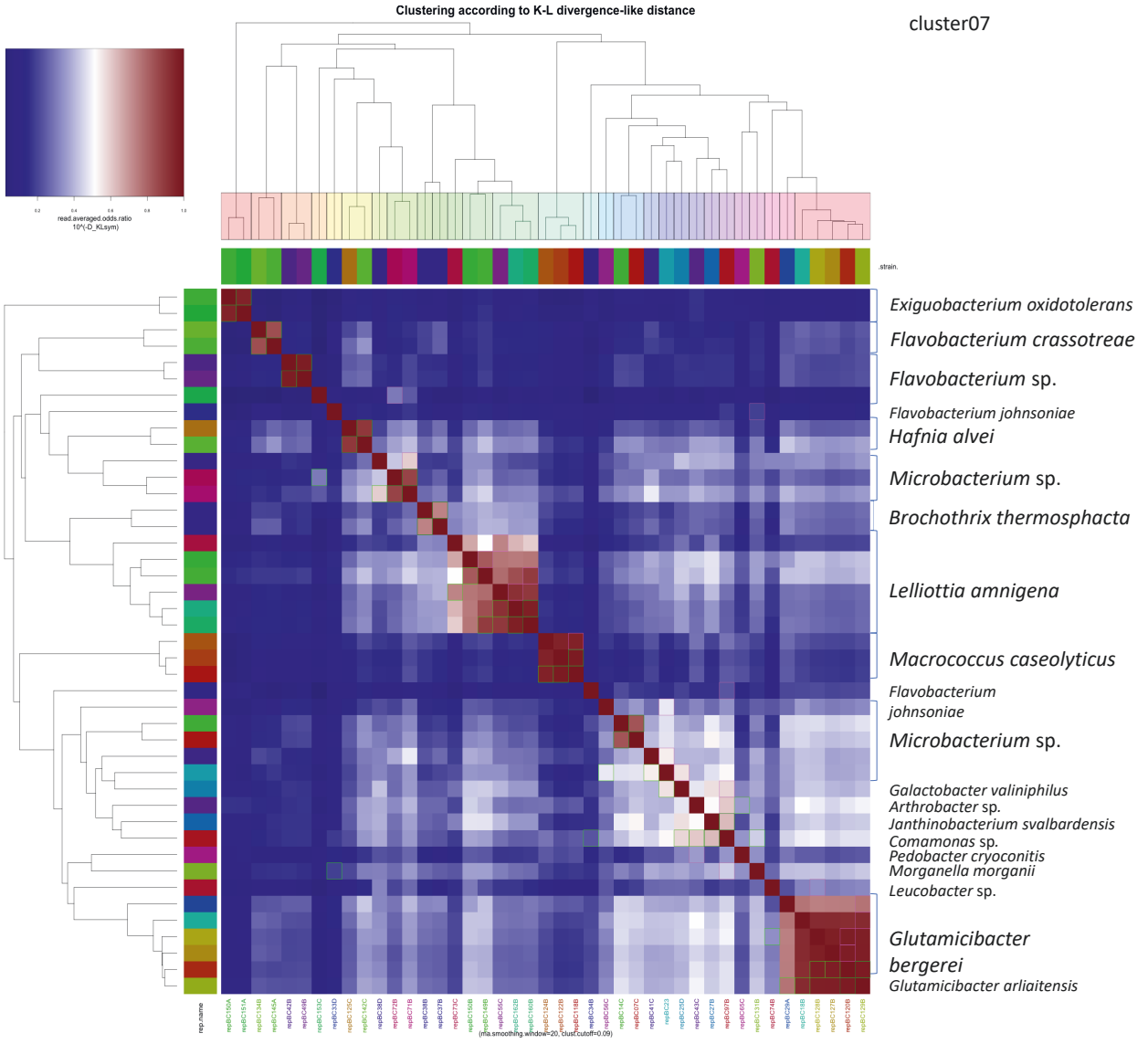
Supplemental Figure S6

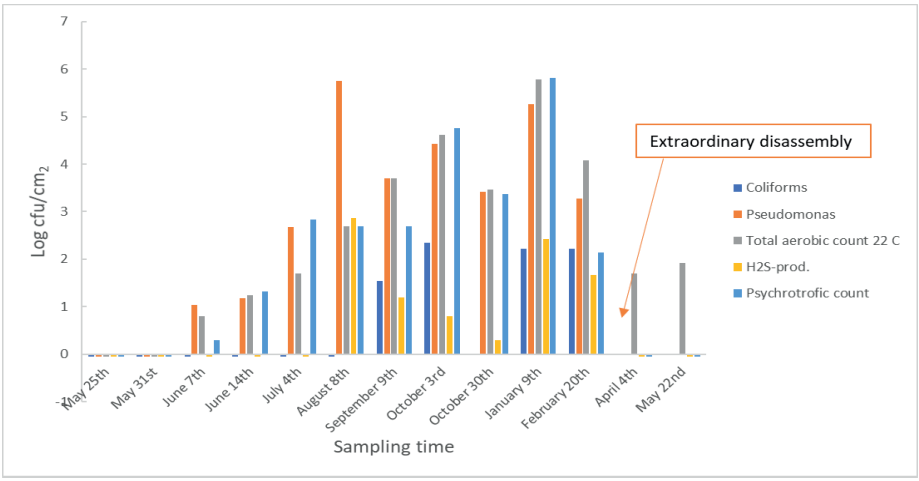


Supplemental Figure S7



Supplemental Figure S8




Log cfu/cm2
7
6
5
4
3
2
1
0
May 25th
May 31st
June 7th
June 14th
July 4th
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September 9th
October 3rd
October 30th
January 9th
February 20th
April 4th
May 22nd
Sampling time
Extraordinary disassembly
Coliforms
Pseudomonas
Total aerobic count 22 C
H2S-prod.
Psychrotrofic count

Paper III

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Antibiotic resistance properties among *Pseudomonas* spp. associated with salmon processing environments

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Abstract

Continuous monitoring of antimicrobial resistance in bacteria along the food value chain is crucial for the assessment of human health risks. It is suspected that uncritical use of antibiotics in farming and livestock animals for years is one of the main reasons for increased antibiotic resistance in many bacteria. In this study we aimed to identify a set of 222 presumptive *Pseudomonas* isolates originating for a salmon processing environment, and to examine the phenotypic and genotypic antibiotic resistance profile in these isolates. Of all the analyzed isolated 68% belonged to *Pseudomonas*, and the most abundant species were *P. fluorescens*, *P. azotoformans, P. gessardii, P. libanesis, P. lundensis, P. cedrina and P. extremaustralis*, but as many as 27 % of *Pseudomonas* isolates could not be classified to species level. Phenotypic susceptibility analysis by disc diffusion method revealed a high level of resistance towards antibiotics ampicillin, amoxicillin, cefotaxime, ceftriaxone, imipenem and the fish farming relevant florfenicol and oxolinic acid among *Pseudomonas* isolates. Whole genome sequencing and subsequent analysis of AMR determinants by ResFinder webtool revealed that none of the isolates contained any acquired resistance determinants, hence the phenotypic resistance is most likely caused by intrinsic efflux pump systems.

Keywords: Pseudomonas spp., food processing environment, antibiotic resistance,

Introduction

Antimicrobial resistance (AMR) is one of the major public health challenges of the 21st century (ECDC, 2020, Murray et al., 2022, O'Neill, 2014, WHO, 2014). The emergence of AMR leads to the ineffectiveness of common antibiotics and increasing failure rate in treatment of infections, resulting in rising mortality rates for common infectious diseases (Capita and Alonso-Calleja, 2013, ECDC, 2020). One of the key driving forces in this evolving problem is the extensive use and misuse of antimicrobial agents (FAO/WHO, 2019, O'Neill, 2014). Antimicrobials are used for a variety of different reasons and across many different sectors in the anthroposphere (FAO/WHO, 2019) and the primary purpose of antimicrobials is to kill or inhibit the growth of microorganisms (Schwarz et al., 2004). Bacteria present in the food value chain is affected by antibiotics used to treat livestock and farmed animals as well as disinfection and sanitation agents used in the environment of the animals, in slaughterhouses and in downstream processing facilities. The strong selective pressure this puts on microorganisms promotes the development of tolerance and resistance properties (ECDC, 2020, Parmley, 2012). The food chain contributes to the transmission of AMR through contamination of food products by resistant bacteria at different stages in the value chain and thereby function as a vehicle for AMR dissemination (FAO/WHO, 2019, Sanseverino et al., 2018). Consequently, the food value chain may expose humans towards bacteria with antimicrobial resistance (Hudson et al., 2017). Estimates for the use of antibiotics in livestock production predicts that it in 2030 will have increased by 67% globally when compared to the consumption data in 2010 (Van Boeckel et al., 2015). This development is directly related to the continued intensification of industrial livestock production systems (Van Boeckel et al., 2015).

Aquaculture systems are “genetic hotspots” for gene transfer and spreading of antibiotic resistance genes (ARGs), as they naturally contain high numbers of diverse bacteria (Watts et

al., 2017). In the aquaculture industry antibiotics are normally administered orally through the fish feed (Ibrahim et al., 2020). However, unconsumed feed is released into the sediments underneath fish farms and may contribute to AMR development in the environmental and the fish microflora (Marshall and Levy, 2011). Hence, bacteria with AMR properties are moved down the seafood value chain and can promote dissemination of AMR genes (Sørum, 2005). The most widely used antibiotics in the aquaculture sector globally belong to three different classes: quinolones (oxolinic acid, flumequine, and enrofloxacin), tetracyclines (oxytetracycline) and phenicols (florfenicol) (Miranda et al., 2013). In Norwegian aquacultures florfenicol and oxolinic acid are the most used antibiotics (NORM/NORM-VET2020). Also in Chile, the second largest salmon producer worldwide, florfenicol is the most commonly used antibiotic, followed by oxytetracycline (Miranda et al., 2018). The use of antibiotics in Norwegian aquaculture is extremely low due to successful introduction of systematic vaccination programs (NORM/NORM-VET2020). While Chilean aquaculture had an annual usage of 143200 – 563200 kg during the period from 2010 – 2019 (Soto, 2020), the use of antibiotics in the Norwegian aquaculture has for the last two years (2019-2020) been 222-223 kg pr year (NORM/NORM-VET2020).

The main focus of AMR is on clinical isolates and a few indicator bacteria, and it seems there are very few studies investigating the occurrence of AMR among other bacteria in food and in the food value chain in Norway. A recent study concerning antibiotic resistance in *Aeromonas* spp. from seafood products did however reveal that 98% of tested isolates were highly resistant to several antibiotics (Lee et al., 2021). In *Pseudomonas* spp. isolated from Norwegian chicken meat over three decades, 18% of the isolates showed resistance to more than three antibiotics and a high number of resistance determinants were detected (Heir et al., 2021). Hence, more studies are needed to give better insight into the occurrence of antibiotic resistance in bacteria associated with the food value chain.

Pseudomonas is the dominant bacterial genus in food processing facilities and is a common part of the microflora of many different food products (Heir et al., 2021, Møretrø et al., 2016, Stellato et al., 2017, Thomassen et al., Submitted). The only species of *Pseudomonas* that is considered a human pathogen is *P. aeruginosa,* but this species is not a common part of the microflora in food products (Heir et al., 2021). Additionally, *P. putida* has been reported as an opportunistic human pathogen (Kim et al., 2012, Peter et al., 2017). *Pseudomonas* spp. have been reported as major spoilage bacteria in aerobically stored, chilled fish and in processing equipment, as *P. fluorescens, P. lundensis, P. libanensis, P. gessardii* and *P. veronii*, have been detected in salmon filet and processing equipment (Møretrø et al., 2016). The aim of this study was to examine the occurrence of antibiotic resistance in *Pseudomonas* isolates, and other common bacteria from a salmon processing environment, phenotypically by the disk diffusion method and genotypically by whole genome sequencing. The isolates were collected from a Norwegian salmon processing plant over the first year the processing plant was operative.

2 Materials and methods

2.1 Sampling and preparation of isolates

The sampling was done in a newly opened salmon processing facility at the coast of Mid-Norway which receives salmon from several marine farming locations in the region. The fish is pumped into the facility directly from the well boat without the use of waiting pens and the products produced at the facility are gutted, whole fish, whole fillets (with or without skin) and vacuum-packed portioned fillets with or without skin. Samples for this study were collected at four different time points throughout the first year of production and from seven sampling points, both food contact surfaces and non-food contact surfaces (Table 1). Additionally, samples of fish fillet and swab samples of skin and gills of gutted whole fish were collected. All sampling of surfaces were done after cleaning and disinfection. Sampling was performed by swabbing 100 cm^2 with a sterile swab (Promedia ST-25 PBS, r-biopharm, Germany) in 10 ml phosphate buffered saline (PBS) or by swabbing 900 cm^2 (30 x 30 cm) with sterile clothes pre-moistened with 25 ml PBS (Sodibox, France). The choice of swabbing method depended on the type and area of the surface. Cloths and swabs were kept cold during the transportation (3h) from the facility to the lab. Dilutions and plating were performed the same day. Bacteria from the swab samples were subjected to various analytical methods, including isolation on the selective growth media *Pseudomonas* CFC Selective agar (CM0559 and SR0103, Oxoid Ltd.). After quantification single colonies were picked and re-propagated minimum twice before they were transferred to TSB w/20% glycerol and stored at -80 °C.

[Table 1]

2.2. Classification of presumptive *Pseudomonas* isolates by sequencing of *rpoD* gene or 16S rDNA gene

A total of 222 presumptive *Pseudomonas* spp. isolates from selected sampling points were subjected to *rpoD* or 16S rDNA sequencing (Table S1). DNA extractions were done by DNeasy Blood & Tissue Kit (Qiagen) and performed according to the producer's procedure (DNeasy Blood & Tissue Handbook, July 2006).

As all these isolates were isolated from *Pseudomonas* CFC Selective agar they were considered presumptive *Pseudomonas* spp. and they were subjected to PCR for the *rpoD* housekeeping gene with primers PsEG30F (5'-ATYGAAATCGCCAARCG-3') and PsEG790R (5'-CGGTTGATKTCCTTGA-3'), resulting in a 760 bp product (Mulet et al., 2009). The PCR reactions were performed with 25 µl reactions containing 1x PCR buffer, 200 µM of each nucleotide, total concentration of $MgCl_2$ at 650 µM, 0.5 µM each primer, 2.5 U Taq polymerase (Qiagen), and 50–100 ng template DNA. The PCR amplification cycles were as follows: Initial denaturation at 95 °C for 15 min, 30 cycles of denaturation at 95 °C for 60 s, annealing for 60 s at 55 °C, and extension at 72 °C for 60 s, followed by a final extension at 72 °C for 5 min. Visualization of the PCR products was done on a 1 % agarose gel. As the primers should be specific for the genus *Pseudomonas*, missing band of the correct size was considered an indication for the isolate being non-*Pseudomonas*. These isolates were subjected to PCR with the universal 16S primers 338f (5'-ACTCCTACGGGAGGCAGCAG-3') (Huse et al., 2008) and 1492r (5'-GGTTACCTTGTTACGACTT-3') (Turner et al., 1999), resulting in an amplicon of 1154 bp and covering V3-V9 variable regions. PCR reactions were performed with 25 µL reactions containing 1x PCR buffer, 200 µM of each nucleotide, total concentration of $MgCl_2$ at 650 µM, 0.4 µM each primer, 2.5 U Taq polymerase (Qiagen), and 50–100 ng template DNA. The PCR amplification cycles were as follows: Initial denaturation at 95 °C for 15 min, 25 cycles of denaturation at 95 °C for 60 s, annealing for 30 s at 58 °C, and extension at 72 °C for 60 s, followed by a final extension at 72 °C for 5 min.

PCR products was enzymatically purified by ExoSAP-IT™ (Thermo Fischer Scientific, USA) procedure which entail incubation at 37 ℃ for 15 min to degrade remaining primers and nucleotides followed by inactivation at 80 ℃ for 15 min. Purified PCR products was quality controlled and prepared for sequencing according to Eurofins LightRun sequencing acquirements. Classification of sequences was done by using BLASTN and comparison to sequences currently available in the NCBI database (www.ncbi.nlm.nih.gov/BLAST).

A phylogenetic three was constructed of selected *rpoD* sequences by using Geneious Prime v2022.1.1. The trimmed sequences were aligned and cut to equal length (~700 bp) and used to construct a phylogenetic tree (neighbor joining tree with Jukes–Cantor distance measure and bootstrap (100 replicates)). The constructed tree was exported to iTol and processed for better visualization (Letunic and Bork, 2021).

2.3 Antibiotic susceptibility by disc diffusion assay

The antibacterial susceptibility profiles of isolates from *Pseudomonas* CFC agar were performed using the conventional disk diffusion assay on 16 different antimicrobials from seven different classes. The selection of antibiotics for this screening was mainly based on the most used antibiotics in Norway in both human and veterinary medicine and, the two antibiotics florfenicol and oxolinic acid that is most used in aquaculture according to (NORM/NORM-VET2020). The test was conducted in accordance with the guidelines from European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2021) with modification in regard to incubation temperature and time. A 0.5 McFarland standard suspension was used for inoculum standardization of all isolates and the reference cultures *Pseudomonas aeruginosa* CCUG 17619 and *E. coli* CCUG 17620. A few isolates that did not grow on conventional Mueller Hinton plates were grown on equivalent plates containing

sheep blood. Disks (Oxoid) containing the following antibacterial agents were used: ampicillin (AMP, 10 µg), amoxicillin (AML, 30 µg), piperacillin /tazobactam (TZP, 36 µg), piperacillin (PRL, 30 µg), cefotaxime (CTX, 30 µg), ceftriaxone (CRO, 30 µg), ceftazidime (CAZ, 30 µg), meropenem (MEM, 10 µg), imipenem (IPM, 10 µg), ciprofloxacin (CIP, 1 µg), amikacin (AK, 30 µg), tobramycin (TOB, 30 µg), doxycycline (DO, 30 µg), tetracycline (TET, 30 µg), oxolinic acid (OA, 2 µg) and florfenicol (FFC, 30 µg). Plates were incubated at 25 ± 2 °C for 20 hours. Zones of inhibition were interpreted in accordance with EUCAST breakpoint table (EUCAST, 2022). For organisms where no guidelines exist, interpretative criteria for similar antimicrobial or organism combinations were used (Baron et al., 2021, Miranda et al., 2016). Multidrug resistant (MDR) strains in this study were defined as being resistant to antibiotics in three or more of the antimicrobial classes analyzed (Magiorakos et al., 2012).

2.4 Whole genome sequencing

Thirty Pseudomonas isolates were selected based on phenotypic resistance to antibiotics of four or more classes, for further characterization by whole genome sequencing. High quality DNA was extracted by using the Genomic Micro AX Bacteria+ Gravity-kit (102-100M, A&A BIOTECHNOLOGY, Poland) according to the manufacturer's procedure. RNAse treatment was included in the procedure. The quality of the DNA was checked on agarose gel and DNA concentrations were estimated by spectrophotometric measurement using BioTek PowerWave XS, Take3 plate and Gen5 2.0 software. DNA samples were sent on ice with overnight shipment to Novogene UK Sequencing laboratory. DNA purity and integrity was again controlled, and accurate DNA concentration was measured by Qubit® 3.0 fluorometer quantification at the sequencing laboratory. The genomic DNA was randomly sheared into short fragments, then end-repaired and A-tailed before Illumina adapters were ligated. A

PCR amplification of the fragments with adapters were performed before size selection and purification. The sequencing strategy was paired-end sequencing with read length of 150 bp at each end, performed on Illumina® NovaSeq™ 6000 sequencing platform.

Base calling was done with CASAVA v1.8 software and the raw read dataset was subject to quality filtering. Paired reads containing either adapter contamination, more than 10 % uncertain nucleotides or reads with low quality nucleotides (base quality $Q \geq 5$) constituting more than 50 % of either read, was removed to obtain high quality reads.

2.5 Data analysis of sequences

The whole genome sequences were analyzed by using the online web-based tools developed by Center for Genomic Epidemiology (CGE). The high-quality read files were used as templates and uploaded to the typing tool KmerFinder 3.2 (Hasman et al., 2014, Larsen et al., 2014, Clausen et al., 2018) to identify the species based on Kmers (length=16 bases). The high-quality clean reads (fastq) were then assembled in Geneious Prime 2022.1.1 (https://www.geneious.com) by mapping to the reference genomes of which they were most similar to according to KmerFinder. The read sets were paired during import to Geneious by using BBmerge, Paird end (inward pointing) with insert size: 350 bp. No trimming was done on the sequences at this point as this was performed by Novogene at an earlier step. Normalization on the sequence reads was performed by BBNorm v.38.84 with default settings; Target coverage level=40, Min depth=6 and no error correction. Assemblies for each isolate was generated by mapping to suitable references according to previous analysis. Geneious mapper was used with settings; Medium-Low sensitivity and iteration up to five times. The consensus sequences were extracted to fasta files with the lowest stringency to get fewest ambiguous bases.

To analyze the isolates for acquires antimicrobial resistance genes the high quality read files were uploaded to the ResFinder 4.1 webtool (Bortolaia et al., 2020, Clausen et al., 2018, Zankari et al., 2017) with default settings (threshold for ID = 90%, Min. length = 60%), all antimicrobial configurations and species = Other.

A phylogenetic tree of the 30 isolates and relevant reference genomes downloaded from GenBank was generated with Fast mode in the webtool NDtree 1.2 (Joensen et al., 2014, Kaas et al., 2014, Leekitcharoenphon et al., 2014). The newick file based on UPGMA algorithm from NDtree was uploaded to iTol (Letunic and Bork, 2021) for better visualization, and the tree was rooted at the *P. aeruginosa* outgroup.

Pairwise Average Nucleotide Identity (ANI) values between the 30 isolates and ten reference genomes was calculated using CJ bioscience's online ANI Calculator from ChunLab (Yoon et al., 2017), which is based on the OrthoANIu algorithm.

3 Results

3.1 Species diversity on *Pseudomonas* CFC agar and their relation

Presumptive *Pseudomonas* isolates (n = 222) from fish (skin, gills and filet) and seven sampling points in a salmon processing plant were collected during the first production year in a salmon processing plant. Partial sequencing of the *rpoD* gene (~700 bp) and the 16S rRNA gene (~1100 base pairs) were used for classification of the isolates. The majority of the isolates (68%) were classified within the *Pseudomonas* genus, other identified genera were *Aeromonas*, *Acinetobacter*, *Morganella*, *Serratia*, *Shewanella*, *Stenotrophomonas*, and *Pseudoalteromonas* (Figure S1). Four isolates were only classified as members of the family *Enterobacteriaceae* and for nine isolates no sequence was obtained not. In the *Pseudomonas* genus 23 different species were detected, with *P. fluorescens* being the most abundant (42%; Figure S1). In total 28% of the *Pseudomonas* isolates could not be classified to species. Most abundant of the other *Pseudomonas* spp. was *P. azotoformans, P. gessardii, P. libanensis, P. lundensis, P. cedrina and P. extremaustralis,* which all belonged to the *P. fluorescens* group (Lalucat et al., 2020).

A phylogenetic three was constructed of selected *rpoD* sequences. A large group of the isolates clustered close to *P. fluorescens* (Table S1) and clustered to species within the *P. fluorescens* group. Isolates from different sampling points and different sampling times were broadly distributed across the whole phylogenetic tree (Figure 1) e.g., LJP374 from sampling 2 eight months after the startup of the facility and LJP883 from the fish, skin and gills sampled 12 months after startup seem to be closely related (Figure 1). One isolate sampled after eight months from inlet water, LJP343, had a high similarity to an isolate from the slaughter department, LJP760, sampled after 12 months.

Strains with highest similarity to *P. fluorescens* could be identified at all sampling times and all sampling points in this study while *P. lundensis, P. gessardi, P. cedrina* and *P. azotoformans* could be identified at multiple sampling times and sampling points (Table S1). *P. extremaustralis* could only be identified on the salmon's skin and *P. aguilliseptica* only on the gills.

[Figure 1]

3.2 Phenotypic antimicrobial susceptibility

Susceptibility to 16 different antibiotics grouped in seven different classes was used in these analyses. All the 222 isolates from colonies grown on *Pseudomonas* CFC agar were selected for antibiotic susceptibility testing where the *Pseudomonas* isolates constituted 68%. Seven of the isolates (three *Pseudoalteromonas*, one *Stenotrophomonas*, and three *Pseudomonas*) did not meet the criteria of growth on Mueller-Hinton agar plates required in the guidelines, and susceptibility could not be determined. The non-*Pseudomonas* isolates were mainly resistant to ampicillin and amoxycillin, however, resistance to in total 12 of the 16 antibiotics were detected among these isolates. *Serratia* spp. and *Stenotrophomonas* spp. were most resistant to the antibiotics tested (Table S2).

The largest contributor to antibiotic resistance in this material is *Pseudomonas* spp. (Figure 2). As expected, a large proportion of these isolates were resistant to ampicillin and amoxycillin, 92 and 87%, respectively (Figure 2). Resistance to oxolinic acid and the fluorinated derivative of chloramphenicol, florfenicol, with 92 and 84% respectively, was also detected. Further, resistance to the cephalosporins, cefotaxime and ceftriaxone were observed in 56 and 40 % of the *Pseudomonas* isolates while resistance to ciprofloxacin could be observed in 9.5% (Figure 2; Table S2). Among these *Pseudomonas* isolates, no resistance to amikacin or tobramycin could be observed.

[Figure 2]

Most of the *Pseudomonas* isolates from the first sampling time multidrug resistant (MDR). Isolates were detected from only three sampling points at this sampling time, conveyor belt slaughter (CSL) in the slaughter department, gutting machine suction (G) and inlet water (IW). These includes the only isolate resistant to six different antibiotic classes (LJP028) (Table 2, Table S2). This isolate, classified as *P. fluorescens* was additionally the only isolate resistant to doxycycline in this material. At sampling time two, isolates were retrieved from five sampling points (Table 2). The isolates from inlet water (IW) were mainly resistant to one or more of ampicillin, amoxicillin, oxolinic acid and florfenicol. One of these isolates *P. fluorescens* (LJP316) was additionally resistant to ciprofloxacin. From the third sampling time the most resistant isolates were *P. gessardii* (LJP706, LJP707) isolated from the drain (DS) in the slaughter department. These isolates were the only isolates resistant to TZP in this material (Table 2, Table S2). The resistance properties within the isolates from fish fillet, skin and gills were highly diverse. Isolates resistant to less than 3 classes (0-2) constituted 25% of these isolates. The most resistant isolates from this group LJP844 (*P. azotoformans*), LJP889 (*P. fluorescens*) and LJP888 (*P. libanensis*) were isolated from the fish skin. Two of the three *Pseudomonas* isolates susceptible to all tested antibiotics in this study were detected in this group.

[Table 2]

3.4 Genomic characterization based on WGS data

The typing tool KmerFinder 3.2 provided a classification for the strain most similar to each isolate (Table S4) together with a score that gives the total number of matching Kmers between the query and the template and, Query Coverage (%) and Template Coverage (%) which gives the percentage of input query Kmers that match the template and the template

coverage respectively. Depth gives an estimation of the sequencing depth. For seven of the isolates the best match from KmerFinder obtained low values for Kmer match between query sequence and template sequence, which indicates low similarity to any other genome in the database.

The phylogeny of the 30 *Pseudomonas* isolates subjected to WGS is visualized in Figure 3. The tree shows two main groups, one small cluster with the tree isolates LJP316, LJP321 and LJP379 together with the reference strain *Pseudomonas* sp. NIBR-H.19 and one large group where all the rest of the isolates and references in smaller subgroups, including species *P. sivasensis*, *P. gessardi*, *P. fluorescens*, *P. synxantha*, *P. libanensis* and the unclassified *Pseudomans* sp. FDAARGOS_380 and *Pseudomonas* sp. J380. An overview of all reference genes and genomes used in this study can be found in Table S3.

Eight of the isolates (LJP026, LJP028, LJP031, LJP039, LJP040, LJP043, LJP044, LJP045) clustering together with *Pseudomonas* sp. J380 originated from the first sampling but from two different sampling points, conveyor and gutting machine in the slaughter department of the facility. Two other isolates (LJP418 and LJP426) highly similar to the eight, originated from the second sampling and the head cutter which is downstream of the previously mentioned sampling point. Five additional isolates from samplings two and three are closely related to this group. These were detected in the gutting machine and the head cutter during sampling two, and on a conveyor in the slaughter department and on fish skin during sampling three.

[Figure 3]

The pairwise calculated ANI values (Figure S2) supports the clusters in the phylogenetic tree By using the ANI value cut of at ≥ 99.00% for strains and ≥ 96.50% for species (Girard et al., 2021, Girard et al., 2020), these pairwise ANI calculations between the isolates and ten

reference genomes, revealed 11 different species among our isolates as indicated by the coloured boxes in Figure 3.

The 30 isolates that were subjected to WGS were selected based on phenotypic resistance to four or more classes of antibiotics. None of these isolates carried any acquired antibiotic resistance genes according to the ResFinder 4.1 database.

Discussion

Pseudomonas spp. are recognized as major food spoilers in the food industry, in salmon processing plants (Møretrø et al., 2016), in poultry (Heir et al., 2021) and in the dairy and meat industry (Stellato et al., 2017).

The origin of the material in this study was colonies grown on *Pseudomonas* CFC Selective agar after sampling in a salmon processing facility in a period of one year. Among the *Pseudomonas* isolates analysed in this study 23 different species were detected, with *P. fluorescens* being the most abundant. Other abundant species were *P. azotoformans*, *P. gessardii*, *P. libanesis*, *P. lundensis*, *P. cedrina* and *P. extremaustralis*, which all belong to the *P. fluorescens* lineage according to Girard et al. (2021), though different groups and subgroups. However, as much as 32% of the isolates detected from *Pseudomonas* CFC Selective agar belonged to other genera than *Pseudomonas*. These were classified as species of *Aeromonas*, *Acinetobacter*, *Morganella*, *Serratia*, *Shewanella*, *Stenotrophomonas*, and *Pseudoalteromonas*. It is commonly known that bacteria from other genera are able to grow on *Pseudomonas* CFC Selective agar (Heir et al., 2021, Tryfinopoulou et al., 2001). As many of the non-*Pseudomonas* detected on *Pseudomonas* CFC Selective agar in this study were species known as commonly occurring bacteria in food processing environments and, also as potential spoilage bacteria, they were included in the further analyses and served as a basis of comparison in the phenotypic antibiotic susceptibility experiment.

Even though the reports of *Pseudomonas* spp. in various environments are frequent, the reported species varies a lot. E.g. in the salmon industry, reported *Pseudomonas* species are *P. fluorescens, P. lundensis, P. libanensis, P. gessardii* and *P. veronii* (Møretrø et al., 2016). In meat and dairy environments *P. fragi* and *P. fluorescens* were found to be most prevalent (Stellato et al., 2017), while Heir et al. (2021) reports species of the *P. fluorescens* group to

be the most prevalent in chicken meat. As seen in our results, many of the isolates (27%) had highest similarity to various unclassified *Pseudomonas* spp., hence no species classification was achieved. Additionally, some isolates could not be differentiated between two or more known species due to equally high similarity to the different species. The genus *Pseudomonas* is large and complex with, until now, more than 300 validly described species (Girard et al., 2021) and additionally several hundred unclassified strains. For many years the most common way to identify bacteria has been sequencing of the 16S rRNA gene, but this method has limitations in resolution on species level, especially for the large and heterogenous *Pseudomonas* genus. For this group, sequencing of 16S rRNA gene can delineate the three main lineages (*P. aeruginosa*, *P. pertucinogena* and *P. fluorescens*) but in most cases it cannot differentiate environmental isolates at the species level (Lalucat et al., 2020). A MLST approach including the genes 16S rRNA, *rpoB*, *rpoD* and *gyrB* has been shown to provide a better resolution for *Pseudomonas* species identification (Mulet et al., 2010). But sequencing of four genes is laborious and expensive, hence sequencing of the *rpoD* has been suggested and proven to be an accurate and inexpensive alternative for identification of large sets of environmental *Pseudomonas* isolates (Girard et al., 2020, Mulet et al., 2009).

Among the 30 *Pseudomonas* isolates that were subjected to WGS, 21 had highest similarity to different unclassified *Pseudomonas* sp. according to KmerFinder. Four isolates had the highest similarity, though not very high, to *P. fluorescens* (LJP030, LJP707) or *P. synxantha* (LJP374, LJP883), but according to *rpoD* all three of these (LJP030, LJP374, LJP883) were most similar to *P. fluorescens* with a nucleotide identity above the cutoff limit of ≥98.0% as recommended by Girard et al. (2020), while LJP707 was classified as *P. gessardi*. This discrepancy can be explained by database issues as the KmerFinder database contains only the high-quality complete and annotated genomes, while the classification by *rpoD* was done

by performing a BLASTn search in Genbank which contains more than 40 000 registered *rpoD* sequences for *Pseudomonas*.

Average Nucleotide Identity (ANI) calculations is a widely used method to compare two bacterial genomes for classification. It is common to consider ANI values of ≥95.0% to indicate the species boundaries (Goris et al., 2007, Jain et al., 2018) but in the work with *Pseudomonas* species delineation (Girard et al., 2021) set the cutoff at ≥96.5% to classify isolates to the same species and considered ANI values between 95.0% and 96.5% to be ambiguous. Isolates LJP844 and LJP728, most likely the same strain, has ANI values between 95.23% and 95.57% when compared to the large group highly similar to reference genome *Pseudomonas* sp. J380.

Several of the isolates in this study were closely related to *Pseudomonas* sp. J380. Ten of the isolates must be considered the same strain and additionally five is most likely of the same species, according to the calculated ANI values. *Pseudomonas* sp. J380 is described as an opportunistic pathogen in cunners (*Tautogolabrus adspersus*) and lumpfish (*Cyclopterus lumpus*) which are used as cleaner fish in salmon farming (Umasuthan et al., 2021). Most of the farmed fish and also the different cleaner fish species are susceptible to bacterial infections, and for this florfenicol is often prescribed (Grave and Helgesen, 2018, Kverme et al., 2019). In this study, all the 15 isolates highly similar to *Pseudomonas* sp. J380 were resistant to florfenicol in the disc diffusion assay. This information should be of interest for the veterinary medicine society and taken into consideration when prescribing antibiotic to cleaner fish populations.

Isolate LJP030, classified as *P. fluorescens* by *rpoD* sequencing (Id: 99.35%), has the highest similarity, though not so high, to *P. fluorescens* PF08 according to KmerFinder (Table S4) and do not cluster with any other isolates of references in the phylogenetic tree. The isolate

has the highest ANI value when compared to *P. fluorescens* PF08 at 93.32% and next to *P. gessardi* (92.93%) and LJP707 (92.85%). Several of our analysis points towards this isolate belonging to *P. fluorescens* species, but the ANI values are below the cutoff for species delineation. Also, the ANI value of the two *P. fluorescens* reference genomes ATCC13525 and PF08 (84.82%) is below the cutoff. It is not clear if this is caused by high heterogeneity within the *P. fluorescens* species or if it is caused by mis annotations in the database (Gomila et al., 2015). This issue, the fact that 21 of our 30 sequenced genomes is most similar to unclassified strains of *Pseudomonas* and the high rate of unclassified species by *rpoD* sequencing demonstrates the difficulties arising in *Pseudomonas* classification and shows that in many cases even WGS cannot call the species identity with confidence.

In Norway, the use of antibiotics in aquaculture is very low (223 kg (norm vet) due to an efficient vaccination program in fingerlings (Midtlyng et al., 2011), however oxolinic acid and florfenicol are the main antibiotics used (222 kg) in aquaculture in Norway. Florfenicol is the fluorinated derivative of chloramphenicol and is solely used in veterinary medicine (Li et al., 2020, Schwarz et al., 2004). A small number of antibacterial agents are registered for use in aquaculture in the U.S., Canada, and Europe. Among these are antibiotic classes such as: macrolides, β-lactams (amoxicillin), fenicols (florfenicol), tetracyclines (oxytetracycline) and quinolones (oxolinic acid) (Lunestad and Samuelsen, 2008). In Chile, florfenicol is also the first choice of antibiotics in salmon production (Miranda et al., 2018, Ramírez et al., 2022). As a consequence of the use of antibiotics, resistance in different bacterial species is building up and might contribute to a global health problem.

The lowest susceptibility detected, was against/to the abovementioned veterinary antibiotics florfenicol and oxolinic acid. Additionally, a few isolates were susceptible to ampicillin and amoxycillin. It was expected to see high levels of resistance towards these antibiotics as it is well documented that the human pathogen *Pseudomonas aeruginosa* display resistance to

several antibiotics from different classes, e.g. aminoglycosides, quinolones and from the majority of related β-lactam antibiotics e.g. ampicillin (Hancock and Speert, 2000, Ryan, 2000). Low susceptibility of *Pseudomonas* spp. to ampicillin has also been documented in other parts of the food industry (Decimo et al., 2016, Meng et al., 2020). Our results show that among these environmental isolates, not all *Pseudomonas* demonstrates this resistance. Therefore, we find it relevant to also report these results.

A large proportion of our *Pseudomonas* isolates were resistant to florfenicol (82%). Similar results were achieved in *Pseudomonas* sampled close to a mussel farm and in shellfish in Chile (Miranda and Rojas, 2007, Ramírez et al., 2022). Buschmann et al. (2012) showed that also in the sediments beneath the fish cages florfenicol- and oxolinic acid-resistant bacteria could be detected and thereby increase the proportion of antimicrobial-resistant bacteria in the environment.

Resistance to other β-lactams, in this case carbapenems as imipenem and meropenems, were observed in this study, 27 and 5 % respectively. This is in line with results from *Pseudomonas* spp. from Norwegian poultry where 26 and 13 % were resistant to imipenem and meropenem (Heir et al., 2021). In the dairy industry a variation in resistance pattern in *Pseudomonas* spp. has been observed. *Pseudomonas* isolates from raw milk were highly resistant to imipenem (95%), and to a lower extent resistant to meropenem (28%) (Meng et al., 2020) while the opposite occurred in *Pseudomonas* isolates from bulk tank milk, highly resistant to meropenem (56%) and to a lesser extent to imipenem (Decimo et al., 2016).

The quinolones are a class of highly effective antibiotics extensively used in human medicine and consequently their use in animal production has been severely restricted by the WHO, however, their use in animal production is not prohibited in many countries (Collignon et al., 2016).

447 The search in ResFinder database with sequence reads from WGS did not reveal any acquired
448 resistance genes in these isolates. This means that none of the ARGs registered in the
449 comprehensive ResFinder database were present in our isolates, hence all resistance observed
450 in the isolates most likely originates from intrinsic mechanisms. E.g., is the resistance against
451 carbapenems in *Pseudomonas* spp. shown to be mostly mediated via efflux pumps (intrinsic
452 resistance), especially in water environment (Tacão et al., 2015). Intrinsic resistance among
453 resistant bacteria, including pseudomonads is observed earlier (Kerry et al., 1994, Sengeløv et
454 al., 2003).

455 *P. aeruginosa* is a well-studied human pathogen within the genus *Pseudomonas* and is known
456 to inhabit high intrinsic resistance to several different antibiotics. The mechanisms behind
457 such resistance can include a low outer membrane permeability, multidrug efflux pump
458 systems as e.g. MexAB-OprM or Mex XY-OprM (Goli et al., 2018) and, the production of
459 inactivating enzymes like β-lactamases (Breidenstein et al., 2011). Some of these intrinsic
460 resistance mechanisms can confer resistance to multiple antibiotics at once (Kakoullis et al.,
461 2021). It can be reasonable to anticipate that the mechanisms causing resistance in other
462 *Pseudomonas* spp. can be the same or similar to those described in *P. aeruginosa*. In the
463 study by Heir et al. (2021) genes encoding the MexAB-OprM efflux system were detected in
464 29 of 31 strains. It is not unlikely that some of these genes are present also in the isolates of
465 our study, but as such genes are not in the ResFinder database, they were not detected by this
466 approach.

467 One of the mechanisms associated with florfenicol resistance is the presence of the *floR* gene
468 encoding florfenicol/chloramphenicol specific efflux pumps (Schwarz et al., 2004). However,
469 a study by Fernández-Alarcón et al. (2010) found that florfenicol resistance not necessarily
470 correlates with the presence of *floR* gene. In that study florfenicol MIC values among Gram
471 negative bacteria were determined in the presence and absence of specific efflux pump

inhibitors. High MIC values were detected among bacteria both positive and negative for the *floR* gene. Further, Fernández-Alarcón et al. (2010) pointed out that non-specific multi-drug efflux pump systems may be involved in resistance mechanisms. Likewise, Adesoji and Call (2020) reported a high occurrence of florfenicol resistance among *Pseudomonas* spp. in combination with a low prevalence of the *floR* gene. Moreover, lower resistance levels towards erythromycin, kanamycin, and fosfomycin were observed in *Pseudomonas* strains of food origin than those reported in literature for clinical isolates.

Antibiotic resistance could be detected in a various of species in this study however *P. gessardii* was the only species resistant to TZP. The findings of Heir et al. (2021) indicated taxa specific differences in resistance properties. In this material only four isolates were susceptible to all antibiotics tested. Of these, one was classified as *P. brenneri*, one as *P. anguilliseptica,* one as *P. fluorescens* and one unclassified. The *P. brenneri* and *P. anguilliseptica* were the only isolates of the respective species, and the *P. fluorescens* was one susceptible isolate among many resistant. Among the other species detected, variable resistance profiles were seen and there is no clear indication of taxa specific resistance profiles.

A prerequisite for using the inhibition zone interpretation criteria in the disc diffusion assay is an incubation of MHA (Müller-Hinton agar) plates at 35 ±2 ℃ for 16-18 hours. However, as most of the tested isolates were psychrophiles and could not grow at high temperatures, the assay for these was conducted at 25 ℃ for 20 hours instead. Smith and Kronvall (2015) demonstrated that the precision in sets of disc diffusion zones decreases with lower incubation temperature and increased time. The lower incubation temperature is probably the reason why we registered a slight deviation in the zone diameter for our reference strains for a few of the antibiotics (Table S2). Similar issues when testing psychrophilic bacteria has been reported earlier (Baron et al., 2021, Miranda et al., 2016). Thus, it is clearly necessary to

497 develop interpretive criteria allowing lower incubation temperatures to meet the need for
498 resistance testing of aquatic isolates. The Clinical Laboratory Standard Institute (CLSI) have
499 published standard test protocols for both disc diffusion assay and MIC methods for
500 incubation on 28 ± 2°C of 22 ± 2°C, but this method and break-point tables were not
501 accessible at the time of the experiment.
502

Conclusion

Pseudomonas isolates origination from a salmon processing environment are diverse with many species represented. But the complex and confusion taxonomy of the genus *Pseudomonas* makes it difficult to provide confident taxonomic assignments for many of the isolates. However, in this study isolates belonging to the *P. fluorescens* group is highly dominating. The isolates show a high level of phenotypic resistance towards a panel of antibiotics with 84% of them being resistant towards three of more classes of antibiotics and hence must be considered as multidrug resistant. This resistance is most likely not caused by any acquired antimicrobial resistance genes, as none such genetic resistance determinants was detected in the set of 30 isolates subjected to whole genome sequencing, but more likely by intrinsic stress response and/or efflux pump systems which is known to be frequent among *Pseudomonas* spp. As the level of acquired resistance genes is low, the probability of spreading of the resistance within this food processing environment and further into the food value chain is small. However, the high level of phenotypic resistance is concerning and should be monitored. And we would like to point out the finding of resistance to florfenicol in isolates with very high genomic similarity to *Pseudomonas* sp. J380, which was recently described as the cause of bacterial infections in different cleaner fish species.

Additionally, the level of resistance among isolates in this study did not increase with the time of sampling. This indicate that the selective pressure in the food processing environment did not induce increased resistance.

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Author contributions:

Gunn Merethe Bjørge Thomassen: Conceptualization (equal); writing – original draft (lead); formal analysis (equal); writing – review and editing (equal); Illustrations (equal); **Thorben Reiche**: Formal analysis (equal); writing – review and editing (equal); **Christine Eikås Tennfjord**: Formal analysis (equal); writing – review and editing (equal); **Lisbeth Mehli**: Conceptualization (equal); writing – original draft (supporting); writing – review and editing (equal); Illustrations (equal).

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Tables and figures for Pseudomonas AMR

Table 1: Sampling points

Table 1: Overview of the different sampling points, sampling point category; CSS=contact surface slaughter department, NCS=Non-contact surface, CSF=contact surface filleting department, F=fish, sampling type; water, cloth, swab or fish fillet, and approximate sampling area.

Sampling point	Sampling point category	Sampling type	Sampling area
Inlet water	Contact surface, slaughter	Water	100 ml
Drain slaughter dep.	Non-contact	Cloth	30x30 cm
Conveyor slaughter dep.	Contact, slaughter	Cloth	30x30 cm
Gutting machine, suction	Contact, slaughter	Swab	10x10 cm
Head cutter knife	Contact, slaughter	Cloth	2 x Ø25 cm
Conveyor fillet dep.	Contact, fillet	Cloth	30x30 cm
Drain fillet dep.	Non-contact	Cloth	30x30 cm
Fish fillet before packaging	Fish	Fish fillet	25 g
Skin, gutted whole fish	Fish	Swab	10x10 cm
Gills, gutted whole fish	Fish	Swab	Gills on both side of fish

Table 2: Resistance profiles of the classified *Pseudomonas* population based on disk diffusion with corresponding breakpoint values (EUCAST 2022). All isolates are LJP, only isolate numbers displayed in the columns. CSL; conveyor slaugther, G; gutting machine suction, IW; inlet water, HCK; head cutting knife, CSK; conveyor skinning, DS; drain slaughter, DF; drain filleting department, S1-5; skin five different fishes, F 1-5; Fillet five different fish.

Sampl. time	Sampl. point	Isolate NO (LJP)	Taxonomic classification	Resistance profiles	Res. to # antib. classes
1	CSL	035, 040	*Psedudomonas fluorescens* (2)	AMP, AML, CTX, CRO, IPM, OA, FFC	5
1	CSL	044	*Pseudomonas fluorescens*	AMP, AML, CTX, CRO, CAZ, IPM, OA, FFC	5
1	CSL	045	*Pseudomonas fluorescens*	AMP, AML, CTX, CRO, CIP, OA, FFC	4
1	CSL	042	*Pseudomonas fluorescens*	AMP, AML, CTX, CRO, OA, FFC	4
1	CSL	033, 038	*Pseudomonas tolaasii* (2)	AMP, AML, CTX, CRO, IPM, OA, FFC	5
1	CSL	037	*Pseudomonas umsongensis*	AMP, AML, CTX, CRO, IPM, OA, FFC	5
1	CSL	043	*Pseudomonas synxantha*	AMP, AML, CTX, CRO, IPM, CIP, OA, FFC	5
1	CSL	046	*Pseudomonas sp*	AMP, AML, CTX, IPM, OA, FFC	5
1	CSL	032, 034, 041	Unclassified *Pseudomonas* (3)	AMP, AML, CTX, CRO, OA, FFC	4
1	CSL	039	*Pseudomonas cedrina*	AMP, AML, CTX, CRO, IPM, OA, FFC	5
1	G	028	*Pseudomonas fluorescens*	AMP, AML, CTX, CRO, IPM, CIP, DO, OA, FFC	6
1	G	030	*Pseudomonas fluorescens*	AMP, AML, CTX, CRO, MEM, IPM, OA, FFC	5
1	G	027	*Pseudomonas reactans*	AMP, AML, CTX, CRO, IPM, OA, FFC	5
1	G	029	Unclassified *Pseudomonas*	AMP, AML, IPM, OA, FFC	4
1	G	026	*Pseudomonas azotoformans*	AMP, AML, CTX, CRO, IPM, CIP, OA FFC	5
1	G	031	*Pseudomonas cedrina*	AMP, AML, CTX, IPM, OA, FFC	5
1	IW	009	*Pseudomonas brenneri*	susceptible	0
2	IW	310, 326	*Pseudomonas fluorescens* (2)	AMP, AML, OA, FFC	3
2	IW	316	*Pseudomonas fluorescens*	AMP, AML, CIP, OA, FFC	3
2	IW	321	*Pseudomonas fluorescens*	AMP, AML, CTX, OA, FFC	4
2	IW	314, 315	*Pseudomonas guineae* (2)	OA	1
2	IW	309	*Pseudomonas marincola*	OA, FFC	2
2	IW	312	*Pseudomonas pseudoalcaligenes*	AMP	1
2	IW	313, 320	Unclassified *Pseudomonas* (2)	OA, FFC	2
2	IW	311	Unclassified *Pseudomonas*	AMP	1
2	IW	327, 329	Unclassified *Pseudomonas* (2)	OA	1
2	CSL	339	*Pseudomonas fluorescens*	AMP, AML, OA, FFC	3
2	G	366, 371	*Pseudomonas azotoformans* (2)	AMP, AML, IPM, OA, FFC	4
2	G	375	*Pseudomonas azotoformans*	AMP, AML, CTX, CRO, OA, FFC	4
2	G	360	*Pseudomonas cedrina*	AMP, AML, CTX, IPM, OA, FFC	5
2	G	362	*Pseudomonas fluorescens*	AMP, AML, CTX, CRO, OA, FFC	5
2	G	383	*Pseudomonas fluorescens*	AMP, AML, CTX, OA, FFC	4
2	G	374	*Pseudomonas fluorescens*	AMP, AML, CTX, CRO,CAZ, CIP, OA, FFC	4
2	G	364	*Pseudomonas fluorescens*	AMP, AML, CTX, CRO, IPM, OA, FFC	5

2	G	370	*Pseudomonas fluorescens*	AMP, AML, CTX, CRO, OA, FFC	4
2	G	373a	*Pseudomonas fluorescens*	AMP, AML, IPM, OA, FFC	4
2	G	369	*Pseudomonas fluorescens*	AMP, AML, OA, FFC	3
2	G	363, 381, 382, 384, 385	*Pseudomonas fluorescens* (5)	AMP, AML, OA, FFC	3
2	G	379	*Pseudomonas fluorescens*	AMP, AML, TET, OA, FFC	4
2	G	365, 372	Unclassified *Pseudomonas*	AMP, AML, OA, FFC	3
2	G	367	Unclassified *Pseudomonas*	AMP, AML, CTX, IPM, OA, FFC	5
2	G	368, 376	Unclassified *Pseudomonas* (2)	AMP, AML, CRO, IPM, OA, FFC	5
2	G	380	Unclassified *Pseudomonas*	AMP, AML, CRO, IPM, CIP OA, FFC	5
2	HCK	421	*Pseudomoans lurida*	AMP, AML, CTX, CRO, OA, FFC	4
2	HCK	422	*Pseudomonas fluorescens*	AMP, AML, CTX, CRO, OA, FFC	4
2	HCK	419, 423, 425	*Pseudomonas fluorescens* (3)	AMP, AML, OA, FFC	3
2	HCK	417, 426	*Pseudomonas fluorescens* (2)	AMP, AML, CTX, CRO, IPM, CIP, OA, FFC	5
2	HCK	418	*Pseudomonas marginalis*	AMP, AML, CTX, CRO, IPM, CIP, OA, FFC	5
2	CSK	344	*Pseudomonas azotoformans*	AMP, AML, CTX, CRO, IPM, OA, FFC	5
2	CSK	341	*Pseudomonas fluorescens*	AMP, AML, CTX, CRO, IPM, OA, FFC	5
2	CSK	343	Unclassified *Pseudomonas*	AMP, AML, CRO, OA, FFC	4
3	DS	710	*Pseudomonas azotoformans*	AMP, AML, CTX, OA, FFC	4
3	DS	713	*Pseudomonas fluorescens*	AMP, AML, OA, FFC	3
3	DS	705	*Pseudomonas fluorescens*	AMP, AML, OA, FFC	3
3	DS	706	*Pseudomonas gessardii*	AMP, AML, TZP, CTX, CRO, MEM, OA, FFC	5
3	DS	707	*Pseudomonas gessardii*	AMP, AML, TZP, PRL, CTX, CRO, MEM, OA, FFC	5
3	DS	714	*Pseudomonas gessardii*	AMP, AML, CTX, CRO, OA, FFC	4
3	DS	716	*Pseudomonas gessardii*	AMP, AML, TZP, CTX, CRO, MEM, OA, FFC	5
3	DS	718	*Pseudomonas fluorescens*	AMP, AML, OA, FFC	3
3	DS	708, 711, 715	Unclassified *Pseudomonas* (3)	AMP, AML, CTX, OA, FFC	4
3	DS	712	Unclassified *Pseudomonas*	AMP, AML, CTX, OA, FFC	4
3	DS	709	Unclassified *Pseudomonas*	AMP, AML, OA, FFC	3
3	CSL	722	*Pseudomonas fluorescens*	AMP, AML, CTX, , IPM, OA, FFC	5
3	CSL	721, 719	*Pseudomonas fluorescens* (2)	AMP, AML, CTX, CRO, IPM, OA, FFC	5
3	CSL	720, 727	*Pseudomonas fluorescens* (2)	AMP, AML, OA, FFC	3
3	CSL	725	*Pseudomonas fluorescens*	AMP, AML, OA, FFC	3
3	CSL	726	*Pseudomonas fluorescens*	AMP, AML, CTX, CRO, MEM, IPM, OA, FFC	5
3	CSL	728	*Pseudomonas paralactis*	AMP, AML, CTX, IPM, CIP, OA, FFC	5
3	CSL	724	*Pseudomonas poae*	AMP, AML, CTX, CRO, OA, FFC	4
3	CSL	723	Unclassified *Pseudomonas*	AMP, AML, CTX, CRO, MEM, IPM, OA, FFC	5
3	HCK	760	*Pseudomonas putida*	AMP, AML, CRO, OA, FFC	4
3	CSK	788	*Pseudomonas lundensis*	AMP	1
3	DF	796	*Pseudomonas fluorescens*	AMP, AML, CTX, CRO, OA, FFC	4
3	DF	799, 800, 801	Unclassified *Pseudomonas* (3)	AMP, AML, CTX, CRO, OA, FFC	4
3	DF	802	Unclassified *Pseudomonas*	AMP, AML, CTX, OA, FFC	4
3	DF	795	*Pseudomonas fluorescens*	AMP, AML, CTX, CRO, OA, FFC	4

3	DF	798	*Pseudomonas fluorescens*	AMP, AML, OA, FFC	3
3	DF	797	*Pseudomonas azotoformans*	AMP, AML, CTX, CRO, IPM,OA, FFC	5
3	G2	823	*Pseudomonas anguilliseptica*	susceptible	0
3	S1	844	*Pseudomonas azotoformans*	AMP, AML, CTX, CRO, IPM, CIP, OA, FFC	5
3	S1	840	*Pseudomonas extremaustralis*	AMP, AML, CTX, OA, FFC	4
3	S5	910	*Pseudomonas extremaustralis*	AMP, AML, CTX, CRO, OA, FFC	4
3	S1	843	*Pseudomonas extremaustralis*	AMP, AML, CTX, OA	3
3	S5	899	*Pseudomonas fluorescens*	AMP, AML, OA, FFC	3
3	S1	836	*Pseudomonas fluorescens*	AMP, AML, OA, FFC	3
3	S3	867	*Pseudomonas fluorescens*	AMP, AML, CIP, OA, FFC	3
3	S4	880	*Pseudomonas fluorescens*	AMP, AML, CTX, OA, FFC	4
3	S4	881	*Pseudomonas fluorescens*	AMP, AML, OA, FFC	3
3	S4	883	*Pseudomonas fluorescens*	AMP, AML, CTX, CRO, IPM, CIP, OA, FFC	5
3	S4	889	*Pseudomonas fluorescens*	AMP, AML, CTX, CRO, MEM, IPM, OA, FFC	5
3	S5	906	*Pseudomonas fluorescens*	AMP, AML, CTX, CRO, OA, FFC	4
3	S5	907, 908	*Pseudomonas fluorescens* (2)	AMP, AML, OA	2
3	S1	845	*Pseudomonas fluorescens*	AMP, AML, OA, FFC	3
3	S2	850	*Pseudomonas fluorescens*	AMP	1
3	S2	859	*Pseudomonas fluorescens*	susceptible	0
3	S3	864	*Pseudomonas fluorescens*	AMP, AML , OA	2
3	S3	865	*Pseudomonas fluorescens*	AMP, AML	1
3	S3	866	*Pseudomonas fluorescens*	AML	1
3	S4	887	*Pseudomonas fluorescens*	AMP	1
3	S5	912	*Pseudomonas fragi*	AMP, AML, PRL, CTX, OA, FFC	4
3	S1	835	*Pseudomonas gessardii*	AMP, AML, CTX, OA, FFC	4
3	S4	893	*Pseudomonas libanensis/fluorescens*	AMP, AML, OA	2
3	S4	882	*Pseudomonas libanesis*	AMP, AML, OA, FFC	3
3	S4	888	*Pseudomonas libanesis*	AMP, AML, CTX, CRO, IPM, OA, FFC	5
3	F2	833	*Pseudomonas lundensis*	AMP, OA, FFC	3
3	S5	905	*Pseudomonas lundensis*	AMP, OA, FFC	3
3	S5	904	*Pseudomonas lundensis*	AMP	1
3	S5	895	*Pseudomonas veronii*	AMP, AML, CTX, CRO, OA, FFC	4
3	S4	884	*Pseudomonas libanensis*	AMP, AML, CTX, OA, FFC	4
3	S5	896	Unclassified *Pseudomonas*	AMP, AML, CTX, CRO, OA, FFC	4
3	S1	846	Unclassified *Pseudomonas*	AMP, AML, CTX, CRO, OA, FFC	4
3	S3	863, 870	Unclassified *Pseudomonas* (2)	AMP, AML, CTX, CRO, OA, FFC	4
3	S1	838, 839, 842, 837	Unclassified *Pseudomonas* (4)	AMP, AML, CTX, OA, FFC	4
3	S1	841	Unclassified *Pseudomonas*	AMP, AML, OA, FFC	3
3	S2	848	Unclassified *Pseudomonas*	AMP, AML, CTX, OA, FFC	4
3	S4	879	Unclassified *Pseudomonas*	AMP, AML, OA, FFC	3
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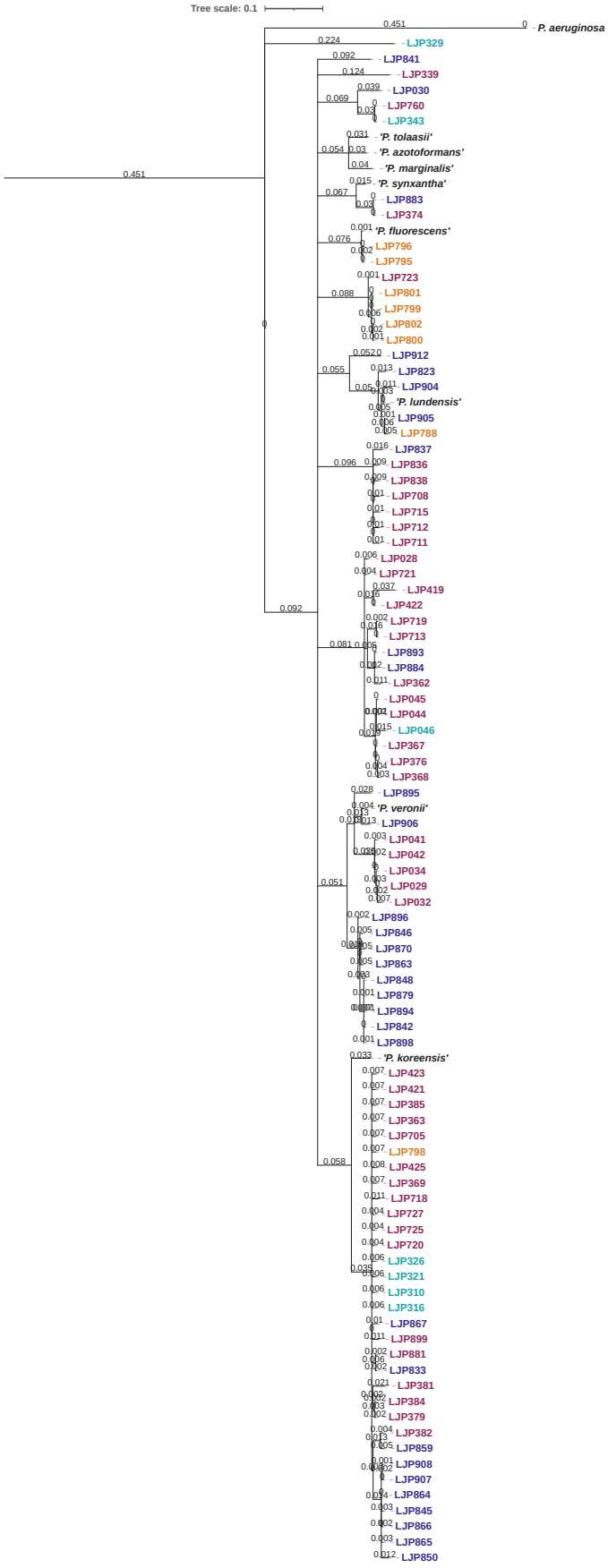
3	S5	898, 903	Unclassified *Pseudomonas* (2)	AMP, AML, CTX, OA	3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LJP368
LJP895
'P. veronii'
LJP906
LJP041
LJP042
LJP034
LJP029
LJP032
LJP896
LJP846
LJP870
LJP863
LJP848
LJP879
LJP894
LJP842
LJP898
'P. koreensis'
LJP423
LJP421
LJP385
LJP363
LJP705
LJP798
LJP425
LJP369
LJP718
LJP727
LJP725
LJP720
LJP326
LJP321
LJP310
LJP316
LJP867
LJP899
LJP881
LJP833
LJP381
LJP384
LJP379
LJP382
LJP859
LJP908
LJP907
LJP864
LJP845
LJP866
LJP865
LJP850

Figure 1: Phylogenetic distribution of Pseudomonas isolates (n = 89) based on partial sequencing of the rpoD gene. The trimmed sequences were aligned and cut to equal length (700 bp) and used to construct a phylogenetic tree (neighbor joining tree with Jukes–Cantor distance measure and bootstrap (100 replicates)). Sampling point of isolation is indicated by colour; inlet water (light blue), salmon slaughter department (dark pink), fillet department (orange), skin, gills and fish fillet (dark blue). The rpoD sequence of nine relevant reference strains are included and *P. aeruginosa* were used as an outgroup.

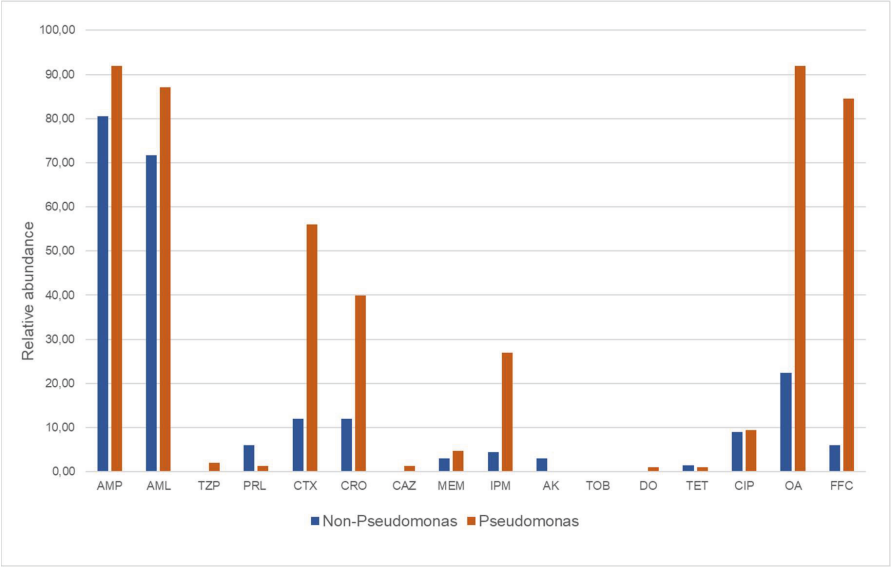


Figure 2: Relative abundance of *Pseudomonas* and non-*Pseudomonas* isolates sampled from different sampling points in a salmon processing facility harbouring phenotypical resistance towards sixteen different antibiotics. AMP-ampicillin, AML-amoxycillin, TZP-piperacillin/tazobactam, PRL-piperacillin, CTX-cefotaxime, CRO-ceftriaxone, CAZ-ceftazidime, MEM-meropenem, IPM-imipenem, AK-amicacin, TOB-tobramycin, DO-doxycycline, TET-tetracycline, CIP-ciprofloxacin, OA-oxacilinic acid, FFC-florfenicol.

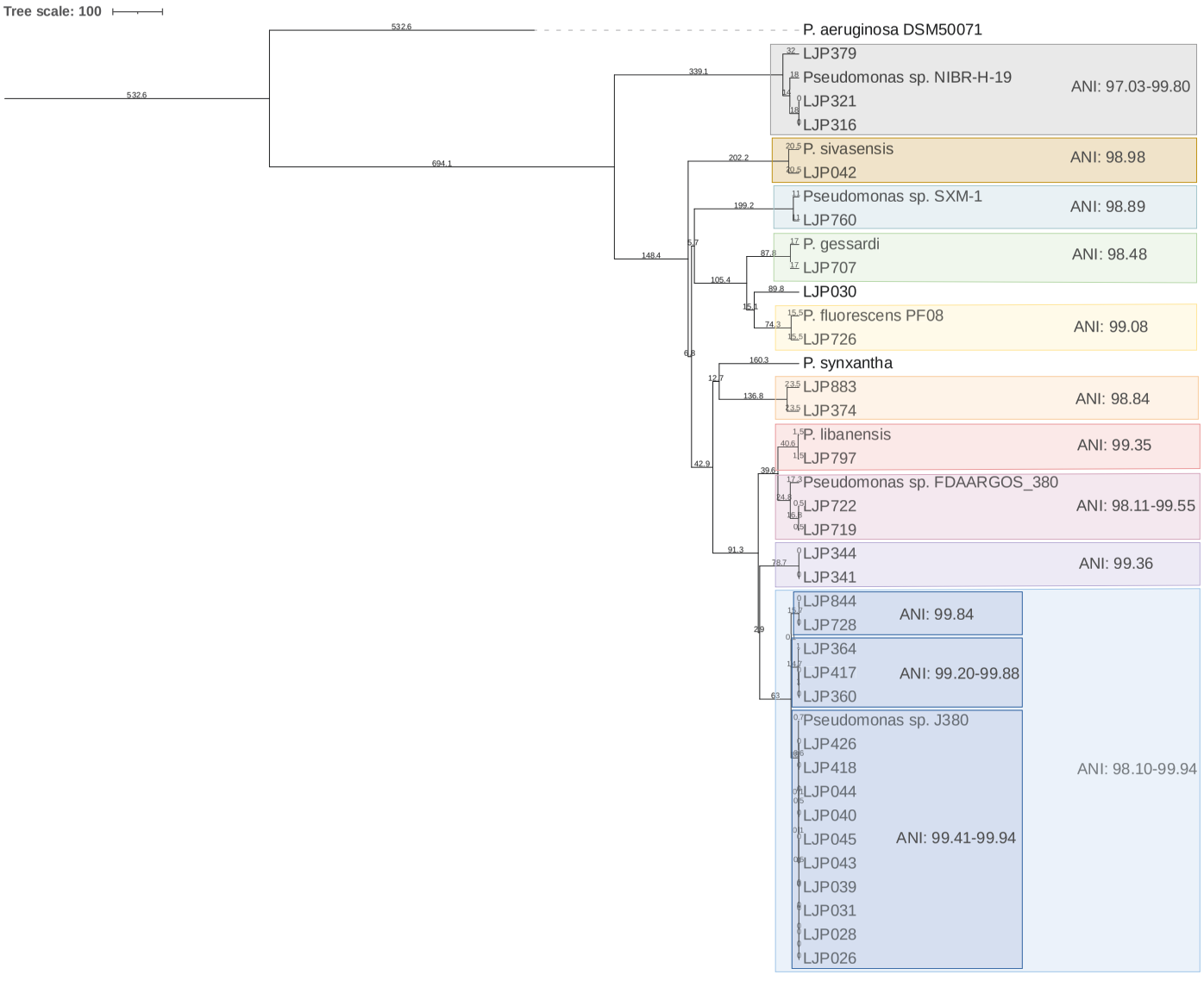

Tree scale: 100
P. aeruginosa DSM50071
LJP379
Pseudomonas sp. NIBR-H-19
LJP321
LJP316
ANI: 97.03-99.80
P. sivasensis
LJP042
ANI: 98.98
Pseudomonas sp. SXM-1
LJP760
ANI: 98.89
P. gessardi
LJP707
ANI: 98.48
LJP030
P. fluorescens PF08
LJP726
ANI: 99.08
P. synxantha
LJP883
LJP374
ANI: 98.84
P. libanensis
LJP797
ANI: 99.35
Pseudomonas sp. FDAARGOS_380
LJP722
LJP719
ANI: 98.11-99.55
LJP344
LJP341
ANI: 99.36
LJP844
LJP728
ANI: 99.84
LJP364
LJP417
LJP360
ANI: 99.20-99.88
Pseudomonas sp. J380
LJP426
LJP418
LJP044
LJP040
LJP045
LJP043
LJP039
LJP031
LJP028
LJP026
ANI: 99.41-99.94
ANI: 98.10-99.94

Figure 3: Phylogenetic tree based on draft genome assemblies of 30 environmental isolates of *Pseudomonas* spp. and ten reference genomes with *P. aeruginosa* as an outgroup. The UPGMA phylogenetic tre was generated by the online webtool NDTree and exported to iTOL for post processing. The different clusters are marked in different colours and the intra-group ANI values are included. The main groups here all have intra group ANI values ≥ 96.5% and are considered to belong to the same species. The large group highly similar to reference *Pseudomonas* sp. J380 is divided in three smaller clusters and the intra subgroup ANI values are noted. These intra subgroup ANI values are >99.0% and the isolates in each subgroup are considered to be the same strain.

Supplement material, Table S1

Table S1:

Isolates used in this study. Time and sample point for the origin of each isolate is listed, together with classification based on sequencing of either 16S rRNA gene or *rpo* D gene.

Isolat ID	Sample time	Sample point	Highest similarity to	Target gene for ID
LJP007	May2018	Inlet water	*Pseudoalteromonas* sp.	16S
LJP008	May2018	Inlet water	*Pseudoalteromonas distincta*	16S
LJP009	May2018	Inlet water	*Pseudomonas brenneri*	rpoD
LJP010	May2018	Inlet water	*Pseudoalteromonas* sp.	16S
LJP011	May2018	Inlet water	*Pseudoalteromonas* sp.	16S
LJP012	May2018	Inlet water	*Stenotrophomonas rhizophila*	16S
LJP013	May2018	Inlet water	*Pseudoalteromonas* sp.	16S
LJP014	May2018	Inlet water	*Pseudomonas fluorescens*	rpoD
LJP015	May2018	Inlet water	*Pseudoalteromonas issachenkonii*	16S
LJP026	May2018	Gutting, suction	*Pseudomonas azotoformans*	16S
LJP027	May2018	Gutting, suction	*Pseudomonas reactans*	16S
LJP028	May2018	Gutting, suction	*Pseudomonas fluorescens*	rpoD
LJP029	May2018	Gutting, suction	*Pseudomonas* sp.	rpoD
LJP030	May2018	Gutting, suction	*Pseudomonas fluorescens*	rpoD
LJP031	May2018	Gutting, suction	*Pseudomonas cedrina*	16S
LJP032	May2018	Conveyor S	*Pseudomonas* sp.	rpoD
LJP033	May2018	Conveyor S	*Pseudomonas tolaasii*	16S
LJP034	May2018	Conveyor S	*Pseudomonas* sp.	rpoD
LJP035	May2018	Conveyor S	*Pseudomonas fluorescens*	rpoD
LJP037	May2018	Conveyor S	*Pseudomonas umsongensis*	16S
LJP038	May2018	Conveyor S	*Pseudomonas tolaasii*	16S
LJP039	May2018	Conveyor S	*Pseudomonas cedrina*	16S
LJP040	May2018	Conveyor S	*Pseudomonas fluorescens*	rpoD
LJP041	May2018	Conveyor S	*Pseudomonas* sp.	rpoD
LJP042	May2018	Conveyor S	Pseudomonas fluorescens	rpoD
LJP043	May2018	Conveyor S	*Pseudomonas synxantha*	16S
LJP044	May2018	Conveyor S	*Pseudomonas fluorescens*	rpoD
LJP045	May2018	Conveyor S	*Pseudomonas fluorescens*	rpoD
LJP046	May2018	Drain F	*Pseudomonas* sp.	rpoD
LJP308	Oct2018	Inlet water	*Aeromonas piscicola*	rpoD
LJP309	Oct2018	Inlet water	*Pseudomonas marincola*	16S
LJP310	Oct2018	Inlet water	*Pseudomonas fluorescens* strain	rpoD
LJP311	Oct2018	Inlet water	*Pseudomonas* sp.	rpoD
LJP312	Oct2018	Inlet water	*Pseudomonas pseudoalcaligenes*	16S
LJP313	Oct2018	Inlet water	*Pseudomonas* sp.	rpoD
LJP314	Oct2018	Inlet water	*Pseudomonas guineae*	16S
LJP315	Oct2018	Inlet water	*Pseudomonas guineae*	16S
LJP316	Oct2018	Inlet water	*Pseudomonas fluorescens*	rpoD
LJP317	Oct2018	Inlet water	*Morganella psychrotolerans*	16S
LJP318	Oct2018	Inlet water	*Aeromonas salmonicida*	16S

LJP319	Oct2018	Inlet water	*Morganella psychrotolerans*	16S
LJP320	Oct2018	Inlet water	*Pseudomonas* sp.	rpoD
LJP321	Oct2018	Inlet water	*Pseudomonas fluorescens*	rpoD
LJP323	Oct2018	Inlet water	*Morganella psychrotolerans*	16S
LJP324	Oct2018	Inlet water	*Morganella psychrotolerans*	16S
LJP325	Oct2018	Inlet water	*Aeromonas bestiarum*	16S
LJP326	Oct2018	Inlet water	*Pseudomonas fluorescens* strain	rpoD
LJP327	Oct2018	Inlet water	*Pseudomonas* sp.	rpoD
LJP328	Oct2018	Inlet water	*Aeromonas hydrophila*	16S
LJP329	Oct2018	Inlet water	*Pseudomonas* sp.	rpoD
LJP332	Oct2018	Inlet water	*Morganella psychrotolerans*	16S
LJP333	Oct2018	Inlet water	*Morganella psychrotolerans*	16S
LJP334	Oct2018	Inlet water	*Morganella psychrotolerans*	16S
LJP335	Oct2018	Inlet water	*Morganella psychrotolerans*	16S
LJP339	Oct2018	Conveyor S	*Pseudomonas fluorescens*	rpoD
LJP341	Oct2018	Conveyor F	*Pseudomonas fluorescens*	rpoD
LJP342	Oct2018	Conveyor F	*Serratia* sp.	16S
LJP343	Oct2018	Conveyor F	*Pseudomonas* sp.	rpoD
LJP344	Oct2018	Conveyor F	*Pseudomonas azotoformans*	16S
LJP345	Oct2018	Conveyor F	*Enterobacteriaceae* bacterium ENUB8	16S
LJP346	Oct2018	Conveyor F	*Enterobacteriaceae* bacterium ENUB8	16S
LJP347	Oct2018	Conveyor F	*Serratia proteamaculans*	16S
LJP360	Oct2018	Gutting, suction	*Pseudomonas cedrina*	16S
LJP362	Oct2018	Gutting, suction	*Pseudomonas fluorescens*	rpoD
LJP363	Oct2018	Gutting, suction	*Pseudomonas fluorescens*	rpoD
LJP364	Oct2018	Gutting, suction	*Pseudomonas fluorescens*	rpoD
LJP365	Oct2018	Gutting, suction	*Pseudomonas* sp.	16S
LJP366	Oct2018	Gutting, suction	*Pseudomonas azotoformans*	16S
LJP367	Oct2018	Gutting, suction	*Pseudomonas* sp.	rpoD
LJP368	Oct2018	Gutting, suction	*Pseudomonas* sp.	rpoD
LJP369	Oct2018	Gutting, suction	*Pseudomonas fluorescens*	rpoD
LJP370	Oct2018	Gutting, suction	*Pseudomonas fluorescens*	rpoD
LJP371	Oct2018	Gutting, suction	*Pseudomonas azotoformans*	16S
LJP372	Oct2018	Gutting, suction	*Pseudomonas* sp.	16S
LJP373	Oct2018	Gutting, suction	*Pseudomonas fluorescens*	rpoD
LJP374	Oct2018	Gutting, suction	*Pseudomonas fluorescens*	rpoD
LJP375	Oct2018	Gutting, suction	*Pseudomonas azotoformans*	16S
LJP376	Oct2018	Gutting, suction	*Pseudomonas* sp.	rpoD
LJP378	Oct2018	Gutting, suction	*Stenotrophomonas rhizophila*	16S
LJP379	Oct2018	Gutting, suction	*Pseudomonas fluorescens*	rpoD
LJP380	Oct2018	Gutting, suction	*Pseudomonas* sp.	rpoD
LJP381	Oct2018	Gutting, suction	*Pseudomonas fluorescens*	rpoD
LJP382	Oct2018	Gutting, suction	*Pseudomonas fluorescens*	rpoD
LJP383	Oct2018	Gutting, suction	*Pseudomonas fluorescens*	16S
LJP384	Oct2018	Gutting, suction	*Pseudomonas fluorescens*	rpoD
LJP385	Oct2018	Gutting, suction	*Pseudomonas fluorescens*	rpoD
LJP417	Oct2018	Head cutter	*Pseudomonas fluorescens*	rpoD

LJP418	Oct2018	Head cutter	*Pseudomonas marginalis*	16S
LJP419	Oct2018	Head cutter	*Pseudomonas fluorescens*	rpoD
LJP420	Oct2018	Head cutter	*Stenotrophomonas rhizophila*	16S
LJP421	Oct2018	Head cutter	*Pseudomonas lurida*	rpoD
LJP422	Oct2018	Head cutter	*Pseudomonas fluorescens*	rpoD
LJP423	Oct2018	Head cutter	*Pseudomonas fluorescens*	rpoD
LJP424	Oct2018	Head cutter	*Stenotrophomonas* sp.	16S
LJP425	Oct2018	Head cutter	*Pseudomonas fluorescens*	rpoD
LJP426	Oct2018	Head cutter	*Pseudomonas fluorescens*	rpoD
LJP658	May2019	Inlet water	*Pseudoalteromonas distincta*	16S
LJP659	May2019	Inlet water	*Pseudomonas fluorescens*	rpoD
LJP660	May2019	Inlet water	*Pseudoalteromonas distincta*	16S
LJP661	May2019	Inlet water	*Pseudomonas fluorescens*	rpoD
LJP705	May2019	Drain S	*Pseudomonas fluorescens*	rpoD
LJP706	May2019	Drain S	*Pseudomonas gessardii*	rpoD
LJP707	May2019	Drain S	*Pseudomonas gessardii*	rpoD
LJP708	May2019	Drain S	*Pseudomonas* sp.	rpoD
LJP709	May2019	Drain S	*Pseudomonas* sp.	rpoD
LJP710	May2019	Drain S	*Pseudomonas azotoformans*	16S
LJP711	May2019	Drain S	*Pseudomonas* sp.	rpoD
LJP712	May2019	Drain S	*Pseudomonas* sp.	rpoD
LJP713	May2019	Drain S	*Pseudomonas fluorescens*	rpoD
LJP714	May2019	Drain S	*Pseudomonas gessardii*	rpoD
LJP715	May2019	Drain S	*Pseudomonas* sp.	rpoD
LJP716	May2019	Drain S	*Pseudomonas gessardii*	rpoD
LJP717	May2019	Drain S	*Stenotrophomonas* sp.	16S
LJP718	May2019	Drain S	*Pseudomonas fluorescens*	rpoD
LJP719	May2019	Conveyor S	*Pseudomonas fluorescens*	rpoD
LJP720	May2019	Conveyor S	*Pseudomonas fluorescens*	rpoD
LJP721	May2019	Conveyor S	*Pseudomonas fluorescens*	rpoD
LJP722	May2019	Conveyor S	*Pseudomonas libanensis*	16S
LJP723	May2019	Conveyor S	*Pseudomonas* sp.	rpoD
LJP724	May2019	Conveyor S	*Pseudomonas poae*	16S
LJP725	May2019	Conveyor S	*Pseudomonas fluorescens*	rpoD
LJP726	May2019	Conveyor S	*Pseudomonas fluorescens*	rpoD
LJP727	May2019	Conveyor S	*Pseudomonas fluorescens*	rpoD
LJP728	May2019	Conveyor S	*Pseudomonas paralactis*	16S
LJP760	May2019	Head cutter	*Pseudomonas putida*	rpoD
LJP761	May2019	Head cutter	*Serratia liquefaciens*	16S
LJP762	May2019	Head cutter	*Enterobacteriaceae* bacterium	16S
LJP763	May2019	Head cutter	*Serratia liquefaciens*	16S
LJP764	May2019	Head cutter	*Serratia liquefaciens*	16S
LJP765	May2019	Head cutter	*Enterobacteriaceae* bacterium	16S
LJP766	May2019	Head cutter	*Serratia liquefaciens*	16S
LJP788	May2019	Conveyor F	*Pseudomonas lundensis*	rpoD
LJP794	May2019	Conveyor F	*Serratia liquefaciens*	16S
LJP795	May2019	Drain F	*Pseudomonas fluorescens*	rpoD

LJP796	May2019	Drain F	*Pseudomonas fluorescens*	rpoD
LJP797	May2019	Drain F	*Pseudomonas azotoformans*	16S
LJP798	May2019	Drain F	*Pseudomonas fluorescens*	rpoD
LJP799	May2019	Drain F	*Pseudomonas* sp.	rpoD
LJP800	May2019	Drain F	*Pseudomonas* sp.	rpoD
LJP801	May2019	Drain F	*Pseudomonas* sp.	rpoD
LJP802	May2019	Drain F	*Pseudomonas* sp.	rpoD
LJP823	May2019	Gills	*Pseudomonas anguilliseptica*	rpoD
LJP825	May2019	Fillet2	*Serratia liquefaciens*	16S
LJP826	May2019	Fillet2	*Serratia liquefaciens*	16S
LJP827	May2019	Fillet2	*Serratia liquefaciens*	16S
LJP828	May2019	Fillet2	*Serratia liquefaciens*	16S
LJP829	May2019	Fillet2	*Serratia liquefaciens*	16S
LJP830	May2019	Fillet2	*Serratia liquefaciens*	rpoD
LJP831	May2019	Fillet2	*Serratia liquefaciens*	rpoD
LJP832	May2019	Fillet2	*Serratia liquefaciens*	16S
LJP833	May2019	Fillet2	*Pseudomonas lundensis*	rpoD
LJP835	May2019	Skin1	*Pseudomonas gessardii*	rpoD
LJP836	May2019	Skin1	*Pseudomonas fluorescens*	rpoD
LJP837	May2019	Skin1	*Pseudomonas* sp.	rpoD
LJP838	May2019	Skin1	*Pseudomonas* sp.	rpoD
LJP839	May2019	Skin1	*Pseudomonas* sp.	rpoD
LJP840	May2019	Skin1	*Pseudomonas extremaustralis*	16S
LJP841	May2019	Skin1	*Pseudomonas* sp.	rpoD
LJP842	May2019	Skin1	*Pseudomonas* sp.	rpoD
LJP843	May2019	Skin1	*Pseudomonas extremaustralis*	16S
LJP844	May2019	Skin1	*Pseudomonas azotoformans*	rpoD
LJP845	May2019	Skin1	*Pseudomonas fluorescens*	rpoD
LJP846	May2019	Skin1	*Pseudomonas* sp.	rpoD
LJP847	May2019	Skin1	*Serratia liquefaciens*	16S
LJP848	May2019	Skin2	*Pseudomonas* sp.	rpoD
LJP849	May2019	Skin2	*Serratia liquefaciens*	16S
LJP850	May2019	Skin2	*Pseudomonas fluorescens*	rpoD
LJP851	May2019	Skin2	*Serratia liquefaciens*	16S
LJP854	May2019	Skin2	*Serratia proteamaculans*	rpoD
LJP856	May2019	Skin2	*Serratia liquefaciens*	16S
LJP857	May2019	Skin2	*Serratia liquefaciens*	16S
LJP858	May2019	Skin2	*Serratia liquefaciens*	16S
LJP859	May2019	Skin2	*Pseudomonas fluorescens*	rpoD
LJP860	May2019	Skin2	*Stenotrophomonas rhizophila*	16S
LJP861	May2019	Skin2	*Serratia liquefaciens*	16S
LJP862	May2019	Skin2	*Serratia liquefaciens*	16S
LJP863	May2019	Skin3	*Pseudomonas* sp.	rpoD
LJP864	May2019	Skin3	*Pseudomonas fluorescens*	rpoD
LJP865	May2019	Skin3	*Pseudomonas fluorescens*	rpoD
LJP866	May2019	Skin3	*Pseudomonas fluorescens*	rpoD
LJP867	May2019	Skin3	*Pseudomonas fluorescens*	rpoD

LJP868	May2019	Skin3	*Aeromonas salmoncida*	16S
LJP869	May2019	Skin3	*Serratia liquefaciens*	16S
LJP870	May2019	Skin3	*Pseudomonas* sp.	rpoD
LJP871	May2019	Skin3	*Acinetobacter pittii*	16S
LJP873	May2019	Skin3	*Serratia liquefaciens*	16S
LJP874	May2019	Skin3	*Serratia fonticola*	16S
LJP875	May2019	Skin3	*Shewanella baltica/putrefaciens*	16S
LJP876	May2019	Skin4	*Serratia liquefaciens*	16S
LJP877	May2019	Skin4	*Shewanella putrefaciens*	16S
LJP878	May2019	Skin4	*Serratia liquefaciens*	16S
LJP879	May2019	Skin4	*Pseudomonas* sp.	rpoD
LJP880	May2019	Skin4	*Pseudomonas fluorescens*	rpoD
LJP881	May2019	Skin4	*Pseudomonas fluorescens*	rpoD
LJP882	May2019	Skin4	*Pseudomonas libanensis*	rpoD
LJP883	May2019	Skin4	*Pseudomonas fluorescens*	rpoD
LJP884	May2019	Skin4	*Pseudomonas libanensis*	rpoD
LJP885	May2019	Skin4	*Serratia fonticola*	16S
LJP886	May2019	Skin4	*Aeromonas salmoncida*	16S
LJP887	May2019	Skin4	*Pseudomonas fluorescens*	rpoD
LJP888	May2019	Skin4	*Pseudomonas libanensis*	rpoD
LJP889	May2019	Skin4	*Pseudomonas fluorescens*	rpoD
LJP891	May2019	Skin4	*Serratia liquefaciens*	16S
LJP892	May2019	Skin4	*Serratia fonticola*	16S
LJP893	May2019	Skin4	*Pseudomonas libanensis*	rpoD
LJP894	May2019	Skin5	*Pseudomonas* sp.	rpoD
LJP895	May2019	Skin5	*Pseudomonas veronii*	rpoD
LJP896	May2019	Skin5	*Pseudomonas extremaustralis*	rpoD
LJP897	May2019	Skin5	*Aeromonas salmoncida*	16S
LJP898	May2019	Skin5	*Pseudomonas* sp.	rpoD
LJP899	May2019	Skin5	*Pseudomonas fluorescens*	rpoD
LJP900	May2019	Skin5	*Aeromonas hydrophila*	16S
LJP901	May2019	Skin5	*Aeromonas sp.*	16S
LJP903	May2019	Skin5	*Pseudomonas* sp.	rpoD
LJP904	May2019	Skin5	*Pseudomonas lundensis*	rpoD
LJP905	May2019	Skin5	*Pseudomonas lundensis*	rpoD
LJP906	May2019	Skin5	*Pseudomonas fluorescens*	rpoD
LJP907	May2019	Skin5	*Pseudomonas fluorescens*	rpoD
LJP908	May2019	Skin5	*Pseudomonas fluorescens*	rpoD
LJP909	May2019	Skin5	*Shewanella baltica/putrefaciens*	16S
LJP910	May2019	Skin5	*Pseudomonas extremaustralis*	rpoD
LJP912	May2019	Skin5	*Pseudomonas fragi*	rpoD

Supplement material table S2

Table S2: Disc diffusion test for 222 isolates and 16 antibiotics belonging to seven different classes. The numbers indicates the zone diameter. Resistance according to EUCAST breakpoint table are highlighted in red.

				beta-lactams				Cephalosporins			Carbapenemems		Aminoglycosides		Tetracylines		Quinolones		Amphenicol
Sample point		Isolate	Identified spiecies	Ampicillin AMP10	Amoxycillin AML30	Piperacillin/ Tazobactam TZP36	Piperacillin PRL30	Cefotaxime CTX30	Ceftriaxone CRO30	Ceftazidime CAZ30	Meropenem MEM10	Imipenem IPM10	Amikacin AK30	Tobramycin TOB30	Doxycycline DO30	Tetracycline Te30	Ciprofloxacin CIP1	Oxolinic acid OA2	Florfenicol FFC30
S3	12	LIP871	*Acinetobacter pittii*	12	14	23	20	21	19	24	31	30	20	24	25	21	22	18	0
2	8	LIP325	*Aeromonas bestiarum*	0	0	25	25	42	38	36	32	27	26	23	32	34	38	39	36
S5	12	LIP900	*Aeromonas hydophila*	0	0	24	22	41	39	35	8	11	31	25	28	31	37	39	33
S5	12	LIP901	*Aeromonas* sp.	0	0	19	12	36	37	30	27	27	22	24	28	31	35	37	31
2	8	LIP328	*Aeromonas hydrophila*	0	0	23	22	39	32	33	33	28	23	25	19	15	40	39	31
2	8	LIP308	*Aeromonas pisciola*	0	0	27	26	40	39	37	31	25	24	24	29	32	38	35	34
S3	12	LIP868	*Aeromonas salmonicida*	0	0	27	22	42	37	35	35	32	22	23	28	32	36	36	37
S4	12	LIP886	*Aeromonas salmonicida*	0	0	23	23	42	39	37	28	26	24	26	28	34	37	32	35
S5	12	LIP897	*Aeromonas salmonicida*	0	0	20	20	40	31	35	31	27	24	24	26	30	36	38	34
2	8	LIP318	*Aeromonas salmonicida*	0	0	27	24	40	37	36	30	25	24	24	29	24	34	37	35
14	12	LIP762	*Enterobacteriaceae* bacterium	0	0	21	22	29	29	26	33	29	21	23	22	25	29	29	20
14	12	LIP765	*Enterobacteriaceae* bacterium	0	7	22	20	28	28	26	33	28	21	23	22	23	28	28	18
18	8	LIP345	*Enterobacteriaceae* bacterium	16	19	25	27	29	31	30	31	30	23	25	23	24	29	31	19
18	8	LIP346	*Enterobacteriaceae* bacterium	21	19	23	28	31	31	32	34	30	23	26	24	24	29	32	19
Reference			**Escherichia CCUG 17620**	16	14	25	26	34	33	30	34	30	21	24	23	24	36	29	21
2	8	LIP317	*Morganella psychrotolerans*	0	0	30	30	37	36	33	35	25	25	26	23	26	33	31	31
2	8	LIP335	*Morganella psychrotolerans*	22	0	29	27	36	32	33	35	30	24	26	32	27	29	34	33
2	8	LIP324	*Morganella psychrotolerans*	0	0	26	26	35	26	29	35	27	24	27	24	30	35	32	29
2	8	LIP333	*Morganella psychrotolerans*	0	0	30	31	37	40	34	36	25	26	28	24	30	35	33	29
2	8	LIP334	*Morganella psychrotolerans*	0	0	27	26	33	36	32	35	24	24	27	25	30	34	30	29
2	8	LIP319	*Morganella psychrotolerans*	0	0	26	27	34	38	29	29	27	23	26	24	26	38	40	26
2	8	LIP323	*Morganella psychrotolerans*	0	0	27	28	34	37	31	35	25	25	30	24	28	39	39	25
2	8	LIP332	*Morganella psychrotolerans*	0	0	27	26	34	37	32	35	27	25	29	25	31	38	39	27
2	0	LJP008	Pseudoalteromonas distincta	38	35	40	40	38	48	45	42	34	31	33	31	28	39	31	32
2	12	LJP 658	Pseudoalteromonas distincta	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
2	12	LJP 660	Pseudoalteromonas distincta	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
2	0	LJP 013	Pseudoalteromonas sp.	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
2	0	LJP 015	Pseudoalteromonas issachenkonii	30	31	29	30	43	42	41	37	32	24	28	26	27	35	34	29
2	0	LJP 007	Pseudoalteromonas sp.	35	39	40	43	45	46	40	45	37	31	37	34	34	43	34	39
2	0	LJP 011	Pseudoalteromonas sp.	35	34	39	40	39	44	44	39	34	28	32	33	32	37	33	33
2	0	LJP 010	Pseudoalteromonas sp.	34	33	38	38	43	45	43	41	34	28	32	32	31	33	34	34
10	0	LIP029	*Psedudomans* sp.	0	0	26	26	15	15	27	27	16	25	28	24	23	26	15	8
8	0	LIP035	Pseudomonas fluorescens	0	0	25	28	12	12	26	26	16	25	26	22	24	26	16	8
14	8	LIP421	Pseudomoans lurida	0	0	24	24	0	0	19	24	20	26	26	22	22	28	17	0
Reference			***Pseudomonas aeruginosa***	7	7	27	27	21	19	30	31	25	25	29	17	20	23	0	0
G2	12	LIP823	Pseudomonas anguilliseptica	17	17	31	31	33	38	38	46	36	32	36	32	38	37	33	20
10	8	LIP366	Pseudomonas azotoformans	0	0	27	27	18	19	26	25	19	27	26	24	23	31	19	9
S1	12	LIP844	Pseudomonas azotoformans	0	0	27	28	12	12	27	18	14	27	28	27	26	24	12	0
18	8	LIP344	Pseudomonas azotoformans	0	0	25	24	0	0	26	27	16	25	26	22	20	27	16	0
10	8	LIP371	Pseudomonas azotoformans	0	0	29	27	17	16	28	24	14	26	27	28	24	27	17	8
10	0	LIP026	Pseudomonas azotoformans	0	0	27	28	12	13	25	27	16	26	28	26	24	24	14	11
10	8	LIP375	Pseudomonas azotoformans	0	0	23	24	0	0	26	27	23	25	27	21	22	28	14	0
22	12	LIP797	Pseudomonas azotoformans	0	0	23	24	0	0	26	25	17	22	25	22	23	30	15	0
7	12	LIP710	Pseudomonas azotoformans	0	0	26	28	13	15	27	25	24	26	29	24	23	29	16	8
2	0	LIP009	Pseudomonas brenneri	28	29	37	36	41	46	43	41	38	32	34	30	29	42	36	35
10	8	LIP360	Pseudomonas cedrina	0	0	27	27	8	14	26	23	15	26	26	27	26	27	17	0
8	0	LIP039	Pseudomonas cedrina	0	0	24	26	12	10	26	25	15	26	27	23	24	26	14	11
10	0	LIP031	Pseudomonas cedrina	0	0	26	26	14	14	26	27	18	27	28	26	26	27	17	12
8	0	LIP043	Pseudomonas synxantha	0	0	25	26	14	0	24	26	15	24	26	24	23	24	12	9
S1	12	LIP846	Unclassified Pseudomonas	0	0	24	25	0	12	23	25	23	28	28	20	22	26	12	0
S3	12	LIP863	Unclassified Pseudomonas	0	0	23	24	11	10	26	25	28	27	27	23	22	26	16	0
S3	12	LPJ 870	Unclassified Pseudomonas	0	0	23	24	0	13	24	26	26	26	29	24	24	28	15	0
S5	12	LPJ 896	Unclassified Pseudomonas	0	0	26	24	8	11	25	30	27	29	30	26	25	27	15	0
S5	12	LPJ 910	Pseudomonas extremaustralis	0	0	22	23	0	12	26	22	25	28	29	24	23	29	15	0
S1	12	LIP 840	*Pseudomonas extremaustralis*	0	0	31	31	11	14	34	34	36	25	26	33	31	29	19	14
S1	12	LIP 843	*Pseudomonas extremaustralis*	0	0	24	25	11	16	29	24	27	21	23	21	22	31	18	19
S5	12	LPJ 899	Pseudomonas fluorescens	0	0	23	24	18	22	25	32	27	25	26	19	20	30	17	0
S1	12	LPJ 836	Pseudomonas fluorescens	0	0	22	23	17	18	23	30	32	26	27	24	24	33	22	0

S1	12	LPJ 838	Unclassified Pseudomonas	0	0	27	27	0	14	25	33	27	26	27	19	22	26	15	10
S1	12	LPJ 839	Unclassified Pseudomonas	0	0	26	26	0	16	26	33	32	27	27	20	23	26	16	9
S3	12	LPJ 867	Pseudomonas fluorescens	0	0	21	22	15	18	22	25	31	26	25	21	21	25	16	0
S4	12	LPJ 880	Pseudomonas fluorescens	0	0	28	27	14	16	28	24	24	26	27	23	25	31	20	0
S4	12	LPJ 881	Pseudomonas fluorescens	0	0	21	21	17	19	24	32	32	25	26	22	22	30	17	0
S4	12	LPJ 883	Pseudomonas fluorescens	0	0	26	26	0	0	27	28	19	26	27	24	24	24	10	0
S5	12	LPJ 906	Pseudomonas fluorescens	0	0	25	27	8	0	26	23	24	28	29	25	25	33	16	9
S5	12	UP 907	*Pseudomonas fluorescens*	0	0	23	24	17	20	24	30	32	25	26	21	21	29	18	21
S5	12	UP 908	*Pseudomonas fluorescens*	0	0	22	22	17	30	24	29	29	25	24	20	21	28	17	21
22	12	UP 796	Pseudomonas fluorescens	0	0	26	28	7	0	27	31	27	30	31	30	28	29	19	8
10	8	UP 383	Pseudomonas fluorescens	0	0	24	25	12	14	26	28	26	28	28	22	24	30	15	0
8	12	UP 722	Pseudomonas fluorescens	0	0	26	25	10	14	26	25	18	28	29	25	25	29	15	0
22	12	UP 800	Pseudomonas fluorescens	0	0	24	25	0	10	24	26	27	26	29	22	21	29	17	9
22	12	UP 801	Pseudomonas fluorescens	0	0	25	25	0	13	26	24	26	26	28	25	23	30	17	8
22	12	UP 795	Pseudomonas fluorescens	0	0	27	27	0	0	29	31	25	30	30	29	26	29	19	8
10	8	UP 362	Pseudomonas fluorescens	0	0	23	24	0	0	25	26	21	24	26	20	20	28	14	0
14	8	UP 422	Pseudomonas fluorescens	0	0	23	23	0	0	24	26	22	25	26	21	22	27	13	0
10	8	UP 374	Pseudomonas fluorescens	0	0	27	27	0	0	16	29	24	26	28	27	22	25	12	8
14	8	UP 419	Pseudomonas fluorescens	0	0	25	25	15	17	26	34	24	26	26	25	25	27	17	9
7	12	UP 713	Pseudomonas fluorescens	0	0	27	29	16	18	29	29	25	28	28	22	24	32	21	11
8	12	UP 721	Pseudomonas fluorescens	0	0	25	25	0	14	27	24	18	26	28	24	25	30	15	0
8	12	UP 719	Pseudomonas fluorescens	0	0	27	29	10	11	26	26	19	29	29	27	25	31	18	0
10	0	UP 028	*Pseudomonas fluorescens*	0	0	25	28	12	0	26	25	16	25	26	12	23	25	20	10
8	0	UP 040	Pseudomonas fluorescens	0	0	26	27	13	11	27	26	16	25	27	22	22	28	15	9
8	0	UP 044	Pseudomonas fluorescens	0	0	25	26	11	0	0	26	15	25	27	24	24	27	15	10
8	0	UP 045	Pseudomonas fluorescens	0	0	38	27	13	13	27	28	26	26	28	24	26	25	13	11
18	8	UP 341	Pseudomonas fluorescens	0	0	24	23	0	0	26	31	15	25	26	19	19	27	15	0
10	8	UP 364	Pseudomonas fluorescens	0	0	26	27	14	13	26	23	14	25	25	24	25	26	21	0
14	8	UP 426	Pseudomonas fluorescens	0	0	24	25	0	0	25	27	13	23	25	25	25	25	11	0
10	8	UP 370	Pseudomonas fluorescens	0	0	24	25	10	11	25	27	21	25	26	22	20	30	15	0
10	0	UP 030	Pseudomonas fluorescens	0	0	24	24	12	8	24	15	19	25	26	22	22	27	14	0
10	8	UP 373a	Pseudomonas fluorescens	0	0	27	28	16	16	28	24	14	26	28	27	25	26	15	9
14	8	UP 417	Pseudomonas fluorescens	0	0	26	25	13	13	26	23	12	24	27	26	25	24	13	0
S1	12	UP 845	*Pseudomonas fluorescens*	0	0	26	26	17	22	26	32	36	25	26	25	23	29	20	12
S2	12	UP 850	*Pseudomonas fluorescens*	0	14	24	23	31	29	30	34	34	21	25	24	24	27	30	24
S2	12	UP 859	*Pseudomonas fluorescens*	13	14	23	26	32	31	29	34	31	22	25	24	22	31	31	19
S3	12	UP 864	Pseudomonas fluorescens	0	0	23	19	31	32	30	30	25	20	23	21	21	31	22	20
S3	12	UP 865	*Pseudomonas fluorescens*	0	0	23	23	32	29	29	28	27	22	23	21	20	31	31	19
S3	12	UP 866	*Pseudomonas fluorescens*	15	0	25	26	34	30	26	36	31	22	24	20	22	34	30	23
10	8	UP 363	Pseudomonas fluorescens	0	0	22	31	18	21	24	35	33	26	25	20	19	30	20	9
10	8	UP 369	Pseudomonas fluorescens	0	0	24	23	17	22	25	36	33	25	26	20	20	30	20	10
10	8	UP 379	Pseudomonas fluorescens	0	0	22	22	19	19	24	33	27	26	26	21	10	29	18	0
10	8	UP 381	Pseudomonas fluorescens	0	0	22	22	17	18	24	23	25	24	24	20	18	29	19	0
10	8	UP 382	Pseudomonas fluorescens	0	0	21	21	16	18	23	30	32	24	26	19	19	28	17	0
10	8	UP 384	Pseudomonas fluorescens	0	0	23	24	18	21	25	33	29	25	26	20	20	30	18	0
10	8	UP 385	Pseudomonas fluorescens	0	0	22	23	18	21	25	34	29	25	26	20	19	29	17	0
14	8	UP 423	Pseudomonas fluorescens	0	0	21	21	17	19	23	31	30	25	26	19	18	30	17	0
14	8	UP 425	Pseudomonas fluorescens	0	0	19	21	16	18	22	29	31	23	25	19	18	28	16	0
2	0	UP 014	Pseudomonas fluorescens	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
2	12	UP 661	Pseudomonas fluorescens	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
2	12	UP 659	Pseudomonas fluorescens	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
7	12	UP 705	Pseudomonas fluorescens	0	0	25	25	20	22	26	32	26	25	27	23	22	29	17	0
7	12	UP 718	Pseudomonas fluorescens	0	0	21	22	15	15	20	29	30	25	26	23	21	30	20	0
22	12	UP 798	Pseudomonas fluorescens	0	0	24	24	18	20	27	34	32	26	29	20	20	33	17	10
8	8	UP 339	*Pseudomonas fluorescens*	0	0	26	26	15	18	27	42	25	25	26	21	22	29	17	0
S4	12	UP 887	*Pseudomonas fluorescens*	10	10	24	24	31	31	31	31	27	21	23	22	22	32	29	17
S4	12	UP 889	*Pseudomonas fluorescens*	0	0	24	23	0	0	25	17	18	25	26	25	26	31	20	8
8	0	UP 042	Pseudomonas fluorescens	0	0	24	25	0	0	22	36	24	25	26	23	23	29	18	10
2	8	UP 310	Pseudomonas fluorescens	0	0	22	22	18	20	25	36	31	26	28	25	23	32	19	13
2	8	UP 316	Pseudomonas fluorescens	0	0	22	22	18	31	25	25	31	25	27	25	23	23	13	0
2	8	UP 321	Pseudomonas fluorescens	0	0	21	20	9	19	24	35	29	26	27	23	22	30	18	13
2	8	UP 326	Pseudomonas fluorescens	0	0	21	21	18	20	24	36	32	27	27	24	22	30	20	12
8	12	UP 720	Pseudomonas fluorescens	0	0	23	26	19	20	26	33	35	26	27	24	24	34	21	10
8	12	UP 725	Pseudomonas fluorescens	0	0	23	24	18	21	25	30	32	27	26	25	26	33	22	9

8	12	LJP 727	Pseudomonas fluorescens	0	0	23	24	19	20	25	32	34	28	26	26	27	33	22	12
8	12	LJP 726	Pseudomonas fluorescens	0	0	23	24	0	0	23	10	12	25	25	21	24	32	19	0
S5	12	LJP 912	*Pseudomonas fragi*	0	0	26	16	0	15	28	30	27	29	29	24	22	30	16	0
S1	12	LJP 835	Pseudomonas gessardii	0	0	26	34	0	14	27	34	23	26	28	28	26	26	16	0
7	12	LJP 706	Pseudomonas gessardii	0	0	17	18	0	0	23	12	25	28	28	21	23	26	11	9
7	12	LJP 707	Pseudomonas gessardii	0	0	16	17	0	0	22	13	29	28	28	23	24	27	11	0
7	12	LJP 714	Pseudomonas gessardii	0	0	20	19	0	0	25	19	26	28	29	23	25	30	14	9
7	12	LJP 716	Pseudomonas gessardii	0	0	17	18	0	0	24	15	32	28	29	21	25	28	14	9
2	8	LJP 314	Pseudomonas guineae	23	29	30	31	33	34	34	48	39	31	31	27	29	35	28	24
2	8	LJP 315	Pseudomonas guineae	25	28	29	31	33	30	31	46	38	30	31	28	28	35	27	26
S4	12	LJP 893	*Pseudomonas libanensis*	0	0	27	27	16	15	27	28	22	25	26	23	23	31	22	23
S4	12	LJP 884	*Pseudomonas libanensis*	0	0	26	27	13	17	28	28	23	25	26	22	23	31	21	0
S4	12	LJP 882	Pseudomonas libanesis	0	0	27	29	15	16	28	26	23	25	27	24	24	33	22	0
S4	12	LJP 888	Pseudomonas libanesis	0	0	24	24	0	0	25	18	17	26	28	26	27	30	20	0
18	12	LJP 788	Pseudomonas lundensis	13	9	22	25	31	30	29	31	26	22	23	22	23	32	32	17
F2	12	LJP 833	Pseudomonas lundensis	0	10	27	29	23	24	26	32	25	26	27	24	22	26	19	11
S5	12	LJP 905	Pseudomonas lundensis	0	8	27	27	23	25	28	34	29	27	27	23	24	27	21	11
S5	12	LJP 904	*Pseudomonas lundensis*	0	12	29	29	25	26	27	31	27	27	26	25	28	26	30	28
S1	12	LJP 841	Unclassified Pseudomonas	0	0	28	28	17	16	29	33	26	29	31	25	25	31	17	0
14	8	LJP 418	Pseudomonas marginalis	0	0	24	24	0	0	24	26	13	23	25	24	25	23	12	0
2	8	LJP 309	Pseudomonas marincola	20	24	24	25	30	29	31	38	34	28	29	28	28	32	24	11
8	12	LJP 728	Pseudomonas paralactis	0	0	28	27	0	14	27	19	13	27	28	28	28	22	10	0
8	12	LJP 724	Pseudomonas poae	0	0	25	25	0	12	28	25	20	27	28	23	27	29	15	0
2	8	LJP 312	Pseudomonas psudoalcaligenes	11	22	29	31	30	29	36	46	44	24	28	25	29	39	32	26
14	12	LJP 760	Pseudomonas putida	0	0	31	33	16	0	32	27	30	30	29	26	26	38	24	11
10	0	LJP 027	Pseudomonas reactans	0	0	26	27	14	0	23	25	16	26	27	23	23	28	15	8
22	0	LJP 046	Pseudomonas sp.	0	0	27	25	13	16	27	30	19	27	27	22	20	29	17	0
8	0	LJP 032	Pseudomonas sp.	0	0	24	24	0	0	20	36	25	26	26	24	24	30	19	9
8	0	LJP 034	Pseudomonas sp.	0	0	24	24	0	0	21	39	23	25	26	23	22	28	19	9
8	0	LJP 041	Pseudomonas sp.	0	0	25	25	0	0	23	29	23	26	27	22	21	29	18	10
10	8	LJP 367	Pseudomonas sp.	0	0	26	25	8	17	27	25	15	26	27	25	23	27	16	0
10	8	LJP 368	Pseudomonas sp.	0	0	28	27	16	13	28	36	14	25	27	28	26	26	16	0
10	8	LJP 376	Pseudomonas sp.	0	0	27	26	15	13	26	23	13	25	26	26	25	26	15	0
10	8	LJP 380	Pseudomonas sp.	0	0	25	26	12	12	25	33	13	24	25	27	25	24	15	0
S1	12	LJP 842	*Pseudomonas* sp.	0	0	25	25	12	16	27	32	29	27	28	24	23	38	16	8
S2	12	LJP 848	*Pseudomonas* sp.	0	0	27	28	13	16	28	30	30	28	30	22	21	30	17	10
S4	12	LJP 879	*Pseudomonas* sp.	0	0	26	27	16	27	27	27	22	25	25	21	22	32	22	0
S5	12	LJP 894	*Pseudomonas* sp.	21	10	24	27	34	30	26	35	32	21	24	20	22	34	31	22
S5	12	LJP 898	*Pseudomonas* sp.	0	0	26	27	12	16	28	30	28	26	29	24	20	29	16	20
S5	12	LJP 903	*Pseudomonas* sp.	0	0	27	28	0	20	29	35	33	19	30	25	25	29	19	25
S1	12	LJP 837	*Pseudomonas* sp.	0	0	26	27	12	18	27	33	34	27	30	20	19	28	16	11
7	12	LJP 708	Pseudomonas sp.	0	0	26	27	9	18	27	34	26	27	29	23	20	29	16	0
7	12	LJP 711	Pseudomonas sp.	0	0	25	25	0	18	24	34	29	28	31	21	22	33	16	12
7	12	LJP 712	Pseudomonas sp.	0	0	28	29	13	22	30	37	28	29	31	21	21	33	19	9
7	12	LJP 715	Pseudomonas sp.	0	0	27	26	0	21	27	35	25	30	30	23	25	34	18	0
7	12	LJP 709	Pseudomonas sp.	0	0	23	24	17	20	26	32	32	27	28	23	25	31	18	10
2	8	LJP 313	Pseudomonas sp.	21	22	26	25	29	31	32	44	39	29	29	27	28	35	28	12
2	8	LJP 320	Pseudomonas sp.	23	24	30	29	31	30	33	43	39	27	29	31	30	35	28	13
2	8	LJP 311	Pseudomonas sp.	12	16	25	26	28	31	31	46	39	29	30	31	30	37	29	17
22	12	LJP 799	Pseudomonas sp.	0	0	22	24	0	12	25	23	25	27	30	22	20	31	16	0
22	12	LJP 802	Pseudomonas sp.	0	0	24	25	0	14	25	24	27	26	29	22	23	32	17	0
8	12	LJP 723	Pseudomonas sp.	0	0	23	24	8	0	24	9	12	26	27	24	27	32	20	0
10	8	LJP 365	Pseudomonas sp.	0	0	26	27	16	17	27	35	25	25	26	23	22	30	21	9
10	8	LJP 372	Pseudomonas sp.	0	0	23	23	18	29	24	35	33	26	28	22	21	29	17	10
18	8	LJP 343	Pseudomonas sp.	0	0	31	31	15	0	30	27	26	28	27	24	25	37	28	10
2	8	LJP 327	Pseudomonas sp.	22	26	28	28	29	32	29	34	36	30	30	24	26	30	23	18
2	8	LJP 329	Pseudomonas sp.	22	27	28	29	29	30	30	47	40	29	29	27	29	36	26	16
8	0	LJP 033	Pseudomonas tolaasii	0	0	27	27	13	13	25	29	17	25	27	23	25	28	16	9
8	0	LJP 038	Pseudomonas tolaasii	0	0	26	28	11	11	27	25	16	26	27	22	24	28	15	10
8	0	LJP 037	Pseudomonas umsongensis	0	0	27	27	13	12	27	27	16	26	27	23	23	28	14	10
S5	12	LJP 895	Pseudomonas veronii	0	0	30	30	12	18	28	30	26	28	28	25	26	33	19	12
S4	12	LJP 885	Serratia fonticola	0	0	30	21	23	20	32	33	31	24	25	23	26	34	32	25
S2	12	LJP 858	Serratia liquefaciens	0	13	26	22	32	29	29	33	31	23	25	23	22	27	29	19
F2	12	LJP 831	Serratia liquefaciens	11	10	25	24	32	31	32	31	27	22	23	21	22	31	31	20

F2	12	UP 830	Serratia liquefaciens	11	11	24	25	33	31	30	31	31	21	23	22	20	32	31	18
18	8	UP 347	Serratia proteamaculans	19	18	24	26	31	32	29	33	31	23	25	23	23	28	29	20
S2	12	UP 854	Serratia proteamaculans	0	13	23	19	32	30	29	34	30	21	23	23	22	27	30	19
18	8	UP 342	Serratia sp.	11	12	21	23	27	26	26	32	29	21	24	22	24	30	30	20
S3	12	UP 874	Serratia fonticola	0	0	25	30	29	26	31	30	27	22	24	23	24	30	30	22
S4	12	UP 892	Serratia fonticola	0	0	28	9	9	9	31	33	26	22	24	21	22	32	31	24
14	12	UP 764	Serratia liquefaciens	0	12	22	23	29	30	29	34	29	21	23	22	24	28	28	20
20	12	UP 794	Serratia liquefaciens	0	0	22	22	16	19	27	12	29	23	24	23	23	31	19	21
F2	12	UP 825	Serratia liquefaciens	11	11	24	25	32	29	29	30	27	20	23	23	24	33	31	16
F2	12	UP 826	Serratia liquefaciens	12	11	24	25	31	32	30	31	26	20	23	22	24	31	30	17
F2	12	UP 827	Serratia liquefaciens	12	10	25	27	32	33	31	32	28	22	24	23	25	33	32	20
F2	12	UP 828	Serratia liquefaciens	10	10	24	25	30	31	29	32	28	22	24	22	24	33	31	18
F2	12	UP 829	Serratia liquefaciens	10	10	23	25	32	31	29	32	25	20	23	21	24	32	30	17
F2	12	UP 832	Serratia liquefaciens	9	11	23	24	30	30	31	32	27	21	23	22	24	32	30	18
S1	12	LPJ 847	Serratia liquefaciens	0	0	23	24	0	8	24	27	24	27	26	21	24	27	14	0
S2	12	UP 849	Serratia liquefaciens	12	12	23	23	30	31	30	32	30	20	23	23	23	28	28	18
S2	12	UP 851	Serratia liquefaciens	12	12	22	24	31	32	30	32	30	20	24	24	24	27	30	18
S2	12	UP 856	Serratia liquefaciens	9	15	23	23	30	31	31	31	31	21	24	23	23	27	29	19
S2	12	UP 857	Serratia liquefaciens	9	15	24	24	30	32	31	33	30	21	24	24	24	29	31	18
S2	12	UP 861	Serratia liquefaciens	0	12	26	26	24	27	28	33	26	27	27	24	27	26	19	11
S2	12	UP 862	Serratia liquefaciens	0	0	27	28	11	16	28	30	26	27	27	24	27	33	16	12
S3	12	UP 869	Serratia liquefaciens	9	9	22	23	30	29	28	28	26	20	23	22	24	30	28	20
S3	12	UP 873	Serratia liquefaciens	10	0	23	25	30	31	30	29	27	22	24	21	23	33	30	21
S4	12	UP 876	Serratia liquefaciens	0	0	31	30	15	28	30	29	29	27	31	26	27	33	19	14
S4	12	UP 878	Serratia liquefaciens	9	11	23	24	30	30	29	31	27	22	23	21	23	32	30	18
S4	12	UP 891	Serratia liquefaciens	9	10	24	24	31	30	29	29	27	22	23	22	24	31	29	20
14	12	UP 761	Serratia liquefaciens	0	11	21	20	28	28	25	33	27	20	22	21	24	27	27	17
14	12	UP 763	Serratia liquefaciens	0	0	21	23	29	27	26	34	30	23	25	22	24	32	30	21
14	12	UP 766	Serratia liquefaciens	0	11	21	22	27	28	26	34	28	21	23	21	21	30	30	19
S3	12	UP 875	Shewanella baltica/putrefaciens	22	15	32	31	35	33	34	36	35	27	26	31	28	29	31	38
S5	12	UP 909	Shewanella baltica/putrefaciens	24	20	32	31	34	32	32	35	36	26	27	32	31	29	31	40
S4	12	UP 877	Shewanella putrefaciens	21	15	31	31	36	32	31	35	36	27	27	31	27	27	32	36
S2	12	UP 860	*Stenotrophomonas rhizophila*	0	0	20	14	15	14	24	32	8	10	21	31	23	19	26	26
10	8	UP 378	Stenotrophomonas rhizophila	0	0	24	22	13	10	24	32	26	21	23	28	23	20	26	25
14	8	UP 420	Stenotrophomonas rhizophila	0	0	24	25	13	11	27	34	26	21	24	27	24	20	27	26
14	8	UP 424	Stenotrophomonas sp.	0	0	24	24	14	11	25	32	24	17	20	28	26	14	24	24
7	12	UP 717	Stenotrophomonas sp.	21	16	26	26	29	29	28	42	10	10	17	33	24	19	25	26
2	0	UP 012	Stenotrophomonas rhizophila	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d

Supplemet material, Table S3

Table S3: Reference strains used in this study. Accession number for each sequence is given and RefSeq assembly accession number is given if available. An X indicates in which analysis the reference strain has been used.

Reference strain	Accession number	Genome assembly	Phylogenetic tree rpoD	Mapping WGS	Phylogenetic tree WGS
Pseudomonas sp. J380	NZ_CP043060.1	GCF_009827115.1		X	X
Pseudomonas fluorescens strain PF08	NZ_CP032618.1	GCF_003626995.1		X	X
Pseudomonas synxantha strain R6-28-08	NZ_CP027756.1	GCF_003851555.1	X	X	X
Pseudomonas gessardi LMG 21604	NZ_FNKR01000003.1	GCF_900101185.1		X	X
Pseudomonas sivasensis BsEB-1	NZ_CP090029.1	GCF_021391435.1		X	X
Pseudomonas libanensis DMSP-1	NZ_CP034425.1	GCF_003952245.1		X	X
Pseudomonas sp. FDAARGOS_380	NZ_CP023969.1	GCF_002591235.1		X	X
Pseudomonas sp. SXM-1	NZ_CP038001.1	GCF_004379315.1		X	X
Pseudomonas sp. NIBR-H-19	NZ_CP089304.1	GCF_021228675.1		X	X
Pseudomonas aeruginosa DSM 50071	NZ_CP012001.1	GCF_001045685.1		X	X
Pseudomonas lundensis	NZ_CP062158.2	GCA_001020725.3	X		
Pseudomonas koreensis LMG 21318	NZ_LT629687.1	GCF_001605965.1	X		
Pseudomonas marginalis H21	NZ_LACF01000008.1	GCF_000967935.1	X		
Pseudomonas tolaasii NCPPB 2192	NZ_PHHD01000001.1		X		
Pseudomonas azotoformans LMG 21611	NZ_LT629702.1	GCF_900103345.1	X		
Pseudomonas veronii R02	NZ_CP018420.1		X		
Pseudomonas aeruginosa PAO1	NC_002516.2	GCF_000006765.1	X		
Pseudomonas fluorescens ATCC 13525	NZ_LT907842.1		X		

Supplement material

Table S4: **Overview of the 30 Pseudomonas isolates that were whole genome sequenced and the results from KmerFinder. The Score is the total number of matching Kmers between the query and the template. Query Coverage (%) is the percentage of input Kmers that match the temlate, while Template Coverage (%) is the template coverage. Depth gives an estimation of the sequencing depth. Isolates with low similarity to any genome in RefSeq is highlighted in pale red.**

	KmerFinder				
Sample ID	(Reference used for mapping in Geneious assembly)	**Score**	**QC**	**TC**	**Depth**
LJP028	Pseudomonas sp. J380	55199978	89,89	90,67	313,01
LJP030	Pseudomonas fluorescens strain PF08	14002675	31,79	36,28	83,96
LJP040	Pseudomonas sp. J380	43982064	91,07	90,66	249,40
LJP042	Pseudomonas fluorescens strain W-6	35155798	77,53	84,88	209,32
LJP044	Pseudomonas sp. J380	36951100	90,90	90,67	209,53
LJP045	Pseudomonas sp. J380	45522160	89,70	90,68	258,13
LJP341	Pseudomonas fluorescens strain Pt14	36639634	78,09	87,32	224,53
LJP364	Pseudomonas sp. J380	31257867	72,20	78,84	177,25
LJP374	Pseudomonas synxantha strain R6-28-08	8998674	26,02	24,88	47,41
LJP417	Pseudomonas sp. J380	29039469	72,85	78,79	164,67
LJP426	Pseudomonas sp. J380	52040578	81,99	91,06	295,09
LJP316	Pseudomonas sp. Seg1	7935265	18,07	19,11	43,42
LJP321	Pseudomonas sp. Seg1	7509073	18,30	18,82	41,08
LJP379	Pseudomonas sp. Seg1	7420084	17,49	18,59	40,60
LJP719	Pseudomonas sp. FDAARGOS_380	24073438	65,02	72,53	132,71
LJP726	Pseudomonas fluorescens PF08	27529487	71,36	85,01	165,06
LJP883	Pseudomonas synxantha strain R6-28-08	15013660	24,46	23,83	79,09
LJP760	Pseudomonas sp. SXM-1	31293894	66,85	79,88	159,86
LJP026	Pseudomonas sp. J380	42561232	91,39	90,69	241,34
LJP344	Pseudomonas fluorescens strain Pt14	40834458	78,44	87,38	250,24
LJP797	Pseudomonas libanensis	37593720	84,04	93,00	215,49
LJP844	Pseudomonas sp. J380	31832144	68,24	77,86	180,50
LJP031	Pseudomonas sp. J380	46030063	89,93	90,67	261,01
LJP039	Pseudomonas sp. J380	45909919	89,30	90,68	260,33
LJP360	Pseudomonas sp. J380	33156322	71,70	79,03	188,01
LJP043	Pseudomonas sp. J380	38075047	89,80	90,67	215,90
LJP418	Pseudomonas sp. J380	38673555	82,26	91,05	219,30
LJP707	Pseudomonas fluorescens strain PF08	12405343	24,67	31,74	24,67
LJP722	Pseudomonas sp. FDAARGOS_380	27593533	65,09	72,56	152,11
LJP728	Pseudomonas sp. J380	33123129	69,10	77,93	187,82

Supplement material, Figure S1

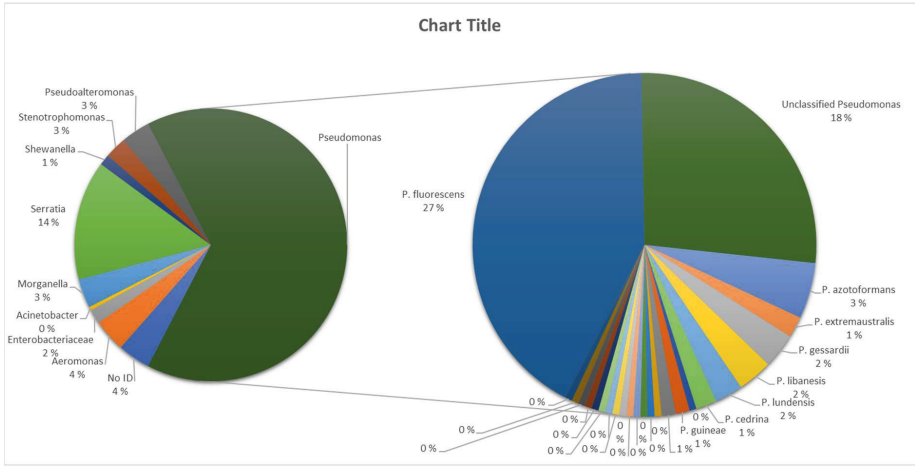
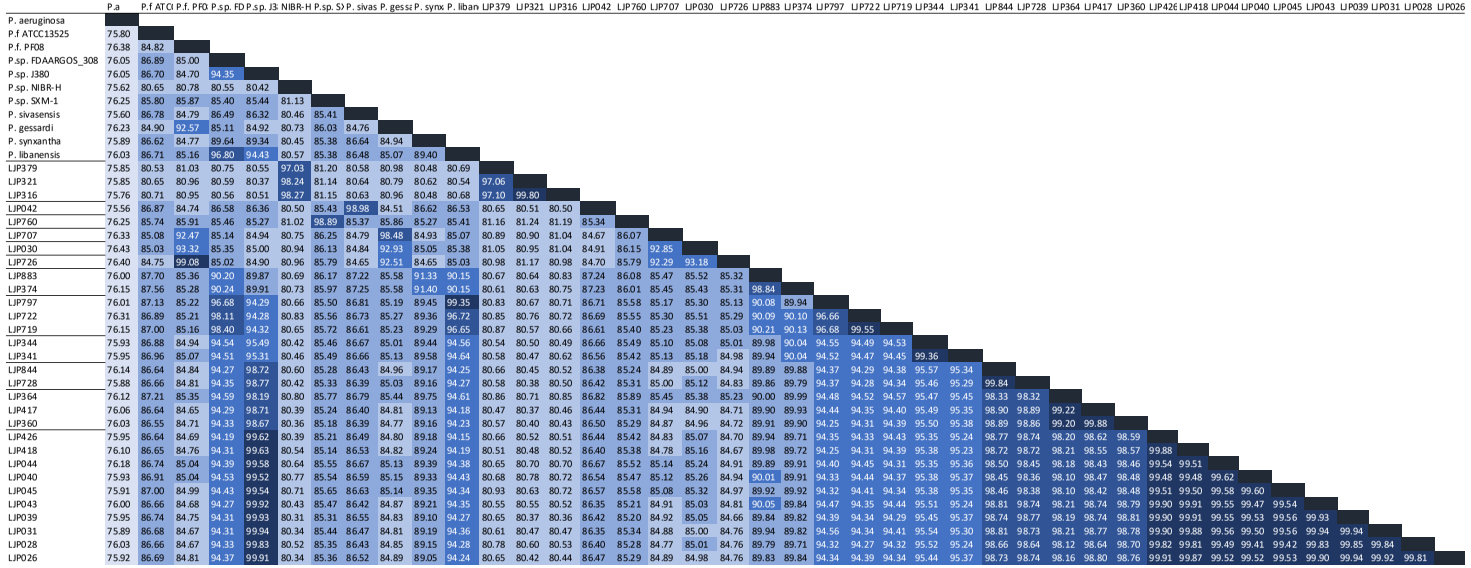


Figure S1: Overall classification of isolates from *Pseudomonas* CFC Selective agar identified in this study. The left pie shows genus level classification. 65% of the isolates belonged to genus Pseudomonas. Species level classification of these are shown in the right pie.

Supplement material, Figure S2



	P.a	P.f ATCC	P.f. PFO	P.sp. FD	P.sp. J3	NIBR-H	P.sp. S	P. sivas	P. gess	P. synx	P. liban	LJP379	LJP321	LJP316	LJP042	LJP760	LJP707	LJP030	LJP726	LJP883	LJP374	LJP797	LJP722	LJP719	LJP344	LJP341	LJP844	LJP728	LJP364	LJP417	LJP360	LJP426	LJP418	LJP044	LJP040	LJP045	LJP043	LJP039	LJP031	LJP028	LJP026
P. aeruginosa																																										
P.f ATCC13525	75.80																																									
P.f. PF08	76.38	84.82																																								
P.sp. FDAARGOS_308	76.05	86.89	85.00																																							
P.sp. J380	76.05	86.70	84.70	94.35																																						
P.sp. NIBR-H	75.62	80.65	80.78	80.55	80.42																																				
P.sp. SXM-1	76.25	85.80	85.87	85.40	85.44	81.13																																			
P. sivasensis	75.60	86.78	84.79	86.49	86.32	80.46	85.41																																		
P. gessardi	76.23	84.90	92.57	85.11	84.92	80.73	86.03	84.76																																	
P. synxantha	75.89	86.62	84.77	89.64	89.34	80.45	85.38	86.64	84.94																																
P. libanensis	76.03	86.71	85.16	96.80	94.43	80.57	85.38	86.48	85.07	89.40																															
LJP379	75.85	80.53	81.03	80.75	80.55	97.03	81.20	80.58	80.98	80.48	80.69																														
LJP321	75.85	80.65	80.96	80.59	80.37	98.24	81.14	80.64	80.79	80.62	80.54	97.06																													
LJP316	75.76	80.71	80.95	80.56	80.51	98.27	81.15	80.63	80.96	80.48	80.68	97.10	99.80																												
LJP042	75.56	86.87	84.74	86.58	86.36	80.50	85.43	98.98	84.51	86.62	86.53	80.65	80.51	80.50																											
LJP760	76.25	85.74	85.91	85.46	85.27	81.02	98.89	85.37	85.86	85.27	85.41	81.16	81.24	81.19	85.34																										
LJP707	76.33	85.08	92.47	85.14	84.94	80.75	86.25	84.79	98.48	84.93	85.07	80.89	80.90	81.04	84.67	86.07																									
LJP030	76.43	85.03	93.32	85.35	85.00	80.94	86.13	84.84	92.93	85.05	85.38	81.05	80.95	81.04	84.91	86.15	92.85																								
LJP726	76.40	84.75	99.08	85.02	84.90	80.96	85.79	84.65	92.51	84.65	85.03	80.98	81.17	80.98	84.70	85.79	92.29	93.18																							
LJP883	76.00	87.70	85.36	90.20	89.87	80.69	86.17	87.22	85.58	91.33	90.15	80.67	80.64	80.83	87.24	86.08	85.47	85.52	85.37																						
LJP374	76.15	87.56	85.28	90.24	89.91	80.73	85.97	87.25	85.58	91.40	90.15	80.61	80.63	80.75	87.23	86.01	85.45	85.43	85.31	98.84																					
LJP797	76.01	87.13	85.22	96.68	94.29	80.66	85.50	86.81	85.19	89.45	99.35	80.83	80.67	80.71	86.71	85.58	85.17	85.30	85.13	90.08	89.94																				
LJP722	76.31	86.89	85.21	98.11	94.28	80.83	85.56	86.73	85.27	89.36	96.72	80.85	80.76	80.72	86.69	85.55	85.30	85.51	85.29	90.09	90.10	96.66																			
LJP719	76.15	87.00	85.16	98.40	94.32	80.65	85.72	86.61	85.23	89.29	96.65	80.87	80.57	80.66	86.61	85.40	85.23	85.38	85.03	90.21	90.13	96.68	99.55																		
LJP344	75.93	86.88	84.94	94.54	95.49	80.42	85.46	86.67	85.01	89.44	94.56	80.54	80.50	80.49	86.66	85.49	85.10	85.08	85.01	89.98	90.04	94.55	94.49	94.53																	
LJP341	75.95	86.96	85.07	94.51	95.31	80.46	85.49	86.66	85.13	89.58	94.64	80.58	80.47	80.62	86.56	85.42	85.13	85.18	84.98	89.94	90.04	94.52	94.47	94.45	99.36																
LJP844	76.14	86.64	84.84	94.27	98.72	80.60	85.28	86.43	84.96	89.17	94.25	80.66	80.45	80.52	86.38	85.24	84.89	85.00	84.94	89.89	89.88	94.37	94.29	94.38	95.57	95.34															
LJP728	75.88	86.66	84.81	94.35	98.77	80.42	85.33	86.39	85.03	89.16	94.27	80.58	80.38	80.50	86.42	85.31	85.00	85.12	84.83	89.86	89.79	94.37	94.28	94.34	95.46	95.29	99.84													
LJP364	76.12	87.21	85.35	94.59	98.19	80.80	85.77	86.79	85.44	89.75	94.61	80.86	80.71	80.85	86.82	85.89	85.45	85.38	85.23	90.00	89.99	94.48	94.52	94.57	95.47	95.45	98.33	98.32												
LJP417	76.06	86.64	84.65	94.29	98.71	80.39	85.24	86.40	84.81	89.13	94.18	80.47	80.37	80.46	86.44	85.31	84.94	84.90	84.71	89.90	89.93	94.44	94.35	94.40	95.49	95.35	98.90	98.89	99.22											
LJP360	76.03	86.55	84.71	94.33	98.67	80.36	85.18	86.39	84.77	89.16	94.23	80.57	80.40	80.43	86.50	85.29	84.87	84.96	84.72	89.91	89.90	94.25	94.31	94.39	95.50	95.38	98.89	98.86	99.20	99.88										
LJP426	75.95	86.64	84.69	94.19	99.62	80.39	85.21	86.49	84.80	89.18	94.15	80.66	80.52	80.51	86.44	85.42	84.83	85.07	84.70	89.94	89.71	94.35	94.33	94.43	95.35	95.24	98.77	98.74	98.20	98.62	98.59									
LJP418	76.10	86.65	84.76	94.31	99.63	80.54	85.14	86.53	84.82	89.24	94.19	80.51	80.48	80.52	86.40	85.38	84.78	85.16	84.67	89.98	89.72	94.25	94.31	94.39	95.38	95.23	98.72	98.72	98.21	98.55	98.57	99.88								
LJP044	76.18	86.74	85.04	94.39	99.58	80.64	85.55	86.67	85.13	89.39	94.38	80.65	80.70	80.70	86.67	85.52	85.14	85.24	84.91	89.89	89.91	94.40	94.45	94.31	95.35	95.36	98.50	98.45	98.18	98.43	98.46	99.54	99.51							
LJP040	75.93	86.91	85.04	94.53	99.52	80.77	85.54	86.59	85.15	89.33	94.43	80.68	80.78	80.72	86.54	85.47	85.12	85.26	84.94	90.01	89.91	94.33	94.44	94.37	95.38	95.37	98.45	98.36	98.10	98.47	98.48	99.48	99.48	99.62						
LJP045	75.91	87.00	84.99	94.43	99.54	80.71	85.65	86.63	85.14	89.35	94.34	80.93	80.63	80.72	86.57	85.58	85.08	85.32	84.97	89.92	89.92	94.32	94.41	94.34	95.38	95.35	98.46	98.38	98.10	98.42	98.48	99.51	99.50	99.58	99.60					
LJP043	76.00	86.66	84.68	94.27	99.92	80.43	85.47	86.42	84.87	89.21	94.35	80.55	80.55	80.52	86.35	85.21	84.91	85.03	84.81	90.05	89.84	94.47	94.35	94.44	95.51	95.24	98.81	98.74	98.21	98.74	98.79	99.90	99.91	99.55	99.47	99.54				
LJP039	75.95	86.74	84.75	94.31	99.93	80.31	85.31	86.55	84.83	89.10	94.27	80.65	80.37	80.36	86.42	85.20	84.92	85.05	84.66	89.84	89.82	94.39	94.34	94.29	95.45	95.37	98.74	98.77	98.19	98.74	98.81	99.90	99.91	99.55	99.53	99.56	99.93			
LJP031	75.89	86.68	84.67	94.31	99.94	80.34	85.44	86.47	84.81	89.19	94.36	80.61	80.47	80.47	86.35	85.34	84.88	85.00	84.76	89.94	89.82	94.56	94.34	94.41	95.54	95.30	98.81	98.73	98.21	98.77	98.78	99.90	99.88	99.56	99.50	99.56	99.94	99.94		
LJP028	76.03	86.66	84.67	94.33	99.83	80.52	85.35	86.43	84.85	89.15	94.28	80.78	80.60	80.53	86.40	85.28	84.77	85.01	84.76	89.79	89.71	94.32	94.27	94.32	95.52	95.24	98.66	98.64	98.12	98.64	98.70	99.82	99.81	99.49	99.41	99.42	99.83	99.85	99.84	
LJP026	75.92	86.69	84.81	94.37	99.91	80.34	85.36	86.52	84.89	89.05	94.24	80.65	80.42	80.44	86.47	85.29	84.89	84.98	84.76	89.83	89.84	94.34	94.39	94.34	95.44	95.37	98.73	98.74	98.16	98.80	98.76	99.91	99.87	99.52	99.52	99.53	99.90	99.94	99.92	99.81

Figure S2: Average Nucleotide Identity (ANI) matrix with pairwise comparisons av the genomes. The ANI values are calculated using CJ Bioscience's online ANI Calculator. Values ≥ 99.00 (darkest blue) indicate that the two isolates are the same strain. Values ≥96.5 indicate that the two belong to the same species.

Paper IV

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