Master's thesis

NTNU
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Phenotypic and Genotypic Characterization of Antimicrobial Resistance in *Pseudomonas* spp. isolated from the Salmon Industry

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Supervisor: Lisbeth Mehli
Co-supervisor: Gunn Merethe B. Thomassen
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Abstract

Controlling antimicrobial resistance (AMR) and achieving improved public health requires multisectoral approaches like the One Health initiative. The food industry is also involved in the complex challenges imposed by AMR. Continuous AMR monitoring in the food value chain is therefore essential for the assessment of risks to human health.

This study aimed to examine the phenotypic and genotypic antimicrobial resistance of *Pseudomonas* spp. isolated from a Norwegian salmon processing plant. This included generating baseline data on the prevalence of both disinfectant and antibiotic resistance among these *Pseudomonas* spp..

We identified 33 isolates by 16S rRNA sequencing and found the majority of the isolates belonging to *Pseudomonas* spp. (70%). These 33 isolates in addition to 129 previously identified isolates (mostly *Pseudomonas* spp.) were characterized by polymerase chain reaction (PCR) genotyping for the detection of seven antimicrobial resistance genes (*qacH, qacEΔ1, bcrABC, sulI, tetG, ampC* and *floR*). Faint amplicons were detected for several genes, but these could not be confirmed by Sanger sequencing. The antibiotic susceptibility profiles were determined of 62 isolates using the disk diffusion method. A high prevalence of ampicillin resistance (79%) and florfenicol resistance (66%) was found, while none of the isolates were resistant towards tetracycline. In vitro biofilm screening in peg lid reactors, revealed the biofilm formation capabilities among 38 bacterial isolates. A total of 88% of the isolates with high biofilm formation capabilities belonged to *Pseudomonas* species.

The MBEC^{TM} assay was used for high-throughput antimicrobial susceptibility testing of biofilms. Two common disinfectants and one antibiotic (florfenicol) were included in the test. The six *Pseudomonas* isolates and one *L. monocytogenes* isolate selected for the test were generally less susceptible towards antimicrobial agents in biofilm state, contra planktonic state. The recommended user concentration and contact time of the disinfectants were effective against isolates in planktonic state. However, the results suggested that the recommended user concentration and contact time was insufficient to eradicate biofilms. The observed minimum inhibitory concentration (MIC) of florfenicol against the selected isolates ranged between 300.00 and >2400.00 µg/mL florfenicol.

In this study we elucidated the high biofilm formation capabilities among *Pseudomonas* spp. and the subsequent effect of biofilm formation on antimicrobial susceptibility. The high occurrence of the phenotypic ampicillin and florfenicol resistance could not be explained by the genotypic resistance mechanisms that we investigated. Further studies are needed to investigate the prevalence of these resistances in the salmon industry and to assess the risk of dissemination through horizontal gene transfer.

Sammendrag

Antimikrobiell resistens (AMR) utgjør en alvorlig trussel mot verdens folkehelse. For å motarbeide denne trusselen kreves det multisektorale tilnærminger. Én helse-prinsippet er et godt eksempel på en slik tilnærming og kan være avgjørende i kampen mot AMR. Verdikjeden for mat er en viktig sektor som også er involvert i de komplekse utfordringene som AMR utgjør. For å kunne vurdere risikoen for utviklingen og spredningen av AMR i matkjeden kreves det en systematisk resistensovervåkning.

Hovedmålet med denne studien var å karakterisere fenotypisk og genotypisk antimikrobiell resistens hos *Pseudomonas* spp. isolert fra et norsk prosesseringsanlegg for laks. Dette involverte analyser knyttet til bakteriell resistens mot både antibiotika og desinfeksjonsmidler.

I denne studien ble 33 isolater identifisert ved sekvensering av 16S rRNA, flertallet av isolatene tilhørte *Pseudomonas* spp. (70%). Polymerasekjedereaksjonen (PCR) ble gjennomført for å påvise syv antimikrobielle resistensgener (*qacH, qacEΔ1, bcrABC, sulI, tetG, ampC* og *floR*) blant 162 isolater. Derav 33 isolater som ble identifisert i denne studien og 129 tidligere identifiserte isolater (hovedsakelig *Pseudomonas* spp.). Svake DNA bånd ble påvist for flere resistensgener, men disse kunne ikke bekreftes ved Sanger-sekvensering. Den bakterielle følsomheten overfor antibiotika ble analysert hos 62 isolater ved bruk av diskdiffusjonsmetoden. Det ble påvist en høy forekomst av ampicillinresistens (79%) og florfenikolresistens (66%), mens ingen av isolatene var resistente mot tetracyklin.

Evnen til biofilmdanelse ble undersøkt hos 38 bakterieisolater ved bruk av in vitro biofilmreaktorer. Totalt 88% av isolatene med høy evne til biofilmdannelse tilhørte *Pseudomonas*-arter. Videre ble det gjennomført en MBEC^{TM} analyse for å kartlegge og sammenligne den antimikrobielle følsomheten av utvalgte isolater i planktonisk tilstand og i biofilmtilstand. I denne analysen ble seks *Pseudomonas*-isolater og ett *L. monocytogenes*-isolat testet overfor to desinfeksjonsmidler og ett antibiotikum (florfenicol). Isolatene viste generelt lavere følsomhet overfor de antimikrobielle midlene i biofilmtilstand, sammenlignet med når isolatene var i en planktonisk tilstand. Den anbefalte brukskonsentrasjonen og kontakttiden for desinfeksjonsmidlene viste seg å være effektiv mot isolatene i planktonisk tilstand. Brukskonsentrasjonen og kontakttiden var imidlertid ikke tilstrekkelig for å utrydde isolatene i biofilmtilstand. Den antimikrobielle følsomhetstesten viste også at den minimale hemmende konsentrasjonen (MIC) av florfenikol overfor de utvalgte isolatene varierte mellom 300,00 og > 2400,00 µg/mL florfenikol.

I denne studien belyste vi den høye evnen til biofilmdannelse blant *Pseudomonas*-arter og effekten av biofilmdannelse på antimikrobiell følsomhet. Den høye forekomsten av fenotypisk ampicillin- og florfenikolresistens kunne ikke forklares med de genotypiske resistensmekanismene som ble undersøkt. Det konkluderes med at det er behov for ytterligere studier for å undersøke forekomsten av bakterier med resistensegenskaper i laksenæringen og for å vurdere risikoen knyttet til AMR spredning gjennom horisontal genoverføring.

Preface

This master thesis was carried out at the Norwegian University of Science and Technology (NTNU) at the department of Biotechnology and Food Science. The thesis accounted for 45 ECTS-credits and was a part of the M.Sc. program Food and Technology.

All experiments related to the project were carried out at the chemistry and microbiology laboratories of NTNU in Kalvskinnet (Trondheim, Norway) during the autumn and spring period of 2020/2021. The thesis was carried out in support of a PhD project executed by the doctoral research fellow Gunn Merethe B. Thomassen and financed by OPTiMAT (Optimal Utilization of Marine Food Resources). OPTiMAT is an interdisciplinary research project that aimed to promote optimal utilization of marine food resources with an emphasis on technology and product quality in the seafood chain.

I would like to express my gratitude to my main supervisor associate professor Lisbeth Mehli and co-supervisor doctoral research fellow Gunn Merethe B. Thomassen. I appreciate their continuous support and motivation. I would also like to thank the department of Biotechnology and Food Science and OPTiMAT for the financial support.

Furthermore, I would like to thank Dr. Trond Møretrø for airmailing two reference strains for antimicrobial resistance genes, *Listeria monocytogenes* MF 4624 and MF 5634.

Last but not least, I would like to express my appreciation to Dr. Stefan Bruns for proofreading.

My personal interest for the field of microbiology gave rise to high expectations for the project. These expectations have truly been met, the work has been highly interesting and the learning outcome was significant.

Trondheim, 25. May 2021

Thorben Reiche

Signature

Table of contents

1.0 Introduction 1

1.1 Antimicrobial resistance 1

1.2 AMR in the seafood value chain 3
1.2.1 Use of antibiotics in the aquaculture sector 3
1.2.2 Use of disinfectants in the food industry 4

1.3 The genus of Pseudomonas 5

1.4 Mechanisms of antimicrobial resistance 6
1.4.1 Natural resistance 6
1.4.2 Acquired resistance 6
1.4.3 Molecular mechanisms of drug resistance 7
1.4.4 Molecular mechanisms of disinfectant resistance 9
1.4.5 Effect of biofilm formation on resistance properties 10
1.5 Aim of this study 11

2.0 Materials and methods 12

2.1 Description of bacterial isolates from salmon processing plant 12
2.1.1 Inclusion of various reference strains and positive controls 12

2.2 Overview of the applied experimental methodology 13

2.3 Identification of 36 bacterial isolates by sequencing of 16S rRNA 13
2.3.1 Amplification of 16S rRNA 13
2.3.2 Visualization of PCR products 14
2.3.3 Purification and quantification of PCR products for sequencing 14

2.4 Genotypic detection of antimicrobial resistance 15
2.4.1 Cultivation and DNA isolation of reference strains 16
2.4.2 Establishment of PCR assays and detection of ARGs among reference strains 16
2.4.3 Detection of 7 ARGs among 162 bacterial isolates 17
2.4.4 Designing new *floR* and *floR2* primer pairs 18
2.4.5 Primer functionality testing 18

2.5 Phenotypic testing of antibiotic resistance 19
2.5.1 McFarland standards 19
2.5.2 Disk diffusion antibiotic susceptibility test 19

2.6 In vitro biofilm screening 20
2.6.1 Screening of in vitro biofilm formation capabilities among bacterial isolates 20
2.6.2 In vitro susceptibility test of bacterial isolates in peg lid biofilm reactors 22
2.6.3 Calculations and statistical analysis 24

3.0 Results 25

3.1 Identification of 36 bacterial isolates by sequencing of 16S rRNA 25

3.2 Genotypic detection of antimicrobial resistance 26
3.2.1 Establishment of PCR assays and detection of ARGs among reference strains 26
3.2.2 Detection of 7 ARGs among 162 bacterial isolates 26
3.2.3 Functionality of the new *floR* and *floR2* primer pairs 27
3.2.4 Detection of *floR* among 162 bacterial isolates using primers from this study 29

3.3 Phenotypic testing of antibiotic resistance 30
3.3.1 Disk diffusion test results of 6 reference strains 30
3.3.2 Disk diffusion test results of 62 bacterial isolates 30

3.4 Screening of in vitro biofilm formation capabilities among bacterial isolates 31

3.5 In vitro susceptibility test of bacterial isolates in peg lid biofilm reactors *32*
3.5.1 Aqua Des Foam PAA - effect of cumulative concentrations on isolates in planktonic and biofilm state 33
3.5.2 Suma Bac D10 - effect of cumulative concentrations on isolates in planktonic and biofilm state 36
3.5.3 Florfenicol - effect of cumulative concentrations on isolates in planktonic and biofilm state 38
3.5.4 Variation and reproducibility of the analysis 42

4.0 Discussion 43

4.1 Identification of bacterial isolates *43*

4.2 Genotypic detection of antimicrobial resistance *43*

4.3 Phenotypic testing of antibiotic resistance *44*

4.4 Biofilm formation capabilities *46*

4.5 Susceptibility test of bacterial isolates in peg lid biofilm reactors *46*

5.0 Conclusion 49

6.0 Future perspectives 50

7.0 References 51

Appendix A – Overview of 165 bacterial isolates included in this study

Appendix B – PCR cycling conditions for PCR assays

Appendix C – Identification results – overview of BLAST-hits

Appendix D – Gel image of PCR assay for *bcrABC* – detection of faint amplicons

Appendix E – Overview of results for ARG screening and disk diffusion

1.0 Introduction

1.1 Antimicrobial resistance

Antimicrobial resistance (AMR) is one of the major public health challenges of the 21st century (ECDC, 2018; O´Neill, 2014; WHO, 2014). The emergence of AMR is a threat to global public health that can lead towards the ineffectiveness of common antibiotics. Consequently, the treatment of infections may become more difficult resulting in rising mortality rates for common infectious diseases (Capita et al., 2013; ECDC, 2018).

One of the key driving forces in this evolving problem is the extensive use and misuse of antimicrobial agents (O´Neill, 2014; WHO, 2019). Antimicrobials are used for a variety of different reasons and across many different sectors in the anthroposphere (WHO, 2019). The primary purpose of antimicrobials is to kill or inhibit the growth of microorganisms (Schwarz et al., 2001). However, the use of antimicrobials applies a strong selective pressure on microorganisms and consequentially promotes the development of tolerance and resistance properties (ECDC, 2021; Parmley et al., 2012). Moreover, resistance towards one antimicrobial agent may facilitate the development of other resistances (Parmley et al., 2012) while human and animal activity contributes to transferring AMR into different sectors and ecosystems (WHO, 2019).

In the recent years a consensus has been reached that the challenges imposed by AMR are far too complex to be addressed within isolated sectors (Capita et al., 2013; Graham et al., 2019; O´Neill, 2014; Parmley et al., 2012). Multisectoral approaches like the One Health perspective will be essential in overcoming the looming AMR crisis (WHO, 2017). Additionally, the One Health approach enables highlighting links between different sectors in context of potential AMR promotion (Parmley et al., 2012).

The food value chain is one of the promoters of AMR (Hudson et al., 2017). There is evidence that the food chain contributes to the transmission and development of AMR (WHO, 2019). Food products can be contaminated by resistant bacteria at different stages in the value chain and function as a vehicle for AMR dissemination (WHO, 2019). Consequently, the food value chain may expose humans towards bacteria with antimicrobial resistance (Hudson et al., 2017).

The development of AMR in the food chain can be linked to the use of antimicrobial agents (Hudson et al., 2017), including antimicrobials used for animal and plant disease treatment as well as antimicrobials used for prevention and control (Marshall et al., 2011). Estimates for the use of antimicrobials in livestock production predict that in 2030 antimicrobial consumption 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).

Sørum (2005) suggested that resistances among aquatic bacteria develop faster than bacteria originating from terrestrial animals. In the aquaculture industry antimicrobials 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 towards AMR development in the environment and microflora of fish (Marshall et al., 2011). Hence, bacteria with AMR properties are moved down the seafood value chain and promote dissemination (Sørum, 2005).

Additional concerns regarding AMR development in the food value chain have been raised by the extensive use of disinfectants (Cadena et al., 2019; Møretrø et al., 2017b; WHO, 2019). In today's food industry disinfectants are extensively used as a part of the daily washing routine for removing and preventing the spread of unwanted microorganisms (WHO, 2019). Unfortunately, such extensive use may promote resistance and tolerance towards disinfectants and cross-resistance to other biocides such as antibiotics (Fernandez Marquez et al., 2017).

Bacteria within the genus *Pseudomonas* were shown to possess resistance and tolerance properties towards both antibiotics (Heir et al., 2021) and disinfectants (Langsrud et al., 1997). *Pseudomonas* spp. are ubiquitous spoilage organisms in the seafood industry (Hatje et al., 2014) and are known for their biofilm formation capabilities (Mann et al., 2012). Biofilms increase protection against harsh conditions and dilute antimicrobials (Drenkard, 2003). Bacteria within biofilms can therefore be exposed to sub-lethal concentrations of antimicrobials for prolonged periods of time, which provides perfect conditions for promoting AMR development (Drenkard, 2003). Biofilms do not only offer increased protection but also offer additional favorable effects for bacteria, e.g., bacteria can interchange specific resistance genes through horizontal gene transfer and consequently accelerate the bacterial adaption to environmental variations (Pace et al., 2005). A potential outcome from this is increased dissemination of resistance associated genes within biofilms, which can decrease bacterial susceptibility towards antimicrobials. (Pace et al., 2005)

In summary, AMR among foodborne bacteria is an evolving problem. Bacteria with resistance properties can conceivably pose a big challenge in the whole food value chain that affects both, the quality and the safety of food products. Ultimately, AMR development in the food industry sector may affect the health care sector as well by proliferating cross-resistance to medically important antibiotics.

1.2 AMR in the seafood value chain

The aquaculture sector is one of the fastest growing food productions sectors in the world and in 2014 half of the globally consumed fish originated from aquacultures (FAO, 2014). The intensification of aquafarming has led to higher fish densities, stressful conditions for the fish and production sites at new geographical locations (Sanseverino et al., 2018). The risk of disease and the need for treatment is amplified by these factors. In light of climate change and accompanying increasing surface water temperatures it becomes more likely that new fish pathogens will emerge (Håkonsholm et al., 2020). Surges in diseased fish may be met by an intensified use of antimicrobials that further stimulates the emergence of AMR in the aquaculture (Sanseverino et al., 2018). Subsequently, disseminating bacteria with AMR properties in the food value chain. These concerns where already raised by several studies (Lee et al., 2021; Marshall et al., 2011; Sørum, 2005).

At the same time, recent consumer trends show the demand of less processed foods with less preservatives (Hoel et al., 2019). In the seafood category the demand for ready-to-eat sushi products grew significantly in the last years. However, the consumption of these products without further heat treatment also increases the risk of exposure towards foodborne bacteria (Hoel et al., 2019) and potentially also bacteria with AMR properties.

1.2.1 Use of antibiotics in the aquaculture sector

In the aquaculture sector antibiotics are utilized for controlling bacterial fish diseases. (Miranda et al., 2013). The globally most widely used antibiotics in the aquaculture sector belong to three different categories: 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 but fortunately the successful introduction of systematic vaccination programs has drastically decreased the use of antibiotics in the Norwegian aquaculture since the 1980s (NORM/NORM-VET, 2019).

Residues of antibiotics may also enter aquaculture systems through agricultural runoffs. A recent report by the European Commission concluded that levels of antibiotic residues in European aquaculture systems are currently of no concern (Sanseverino et al., 2018). However, it was also stated that more empirical data are needed for robust conclusions and that further investigations should be carried out (Sanseverino et al., 2018).

In general, the prevalence of antimicrobial resistance in bacteria from Norwegian livestock animals is low according to the annual surveillance program NORM/NORM-VET (2019). Only a few studies have investigated the prevalence of bacteria with antimicrobial resistance properties in Norwegian seafood. Lee et al. (2021) reported a high prevalence of antibiotic resistance among *Aeromonas* species isolated from Norwegian retail sushi. Most of the isolates were multidrug resistant. It was also stated that there is a need for further studies on the prevalence of genetic determinants associated with resistance in bacteria from Norwegian farmed fish (Lee et al., 2021). A study by Håkonsholm et al. (2020) investigated *Vibrio* spp. isolated from the Norwegian marine environment. The prevalence of resistance was low but clinically important resistance genes were found in some isolates. The importance of continuous research and AMR surveillance was also pointed out by Håkonsholm et al. (2020).

Antibiotics have different modes of action on bacteria (Figure 1) including the inhibition of cell wall synthesis, the inhibition of metabolic pathways and the inhibition of protein synthesis (Sanseverino et al., 2018). The common denominator of those mechanisms is that they interfere with bacterial growth and proliferation with the purpose of killing or inhibiting the bacteria. Antibiotics that cause bacterial inhibition are described as bacteriostatic, while those killing bacteria are bactericidal (Sanseverino et al., 2018). Florfenicol is an example of a bacteriostatic antibiotic which impairs the protein synthesis by reversive binding to the 50S ribosome subunit (Davis et al., 2014).

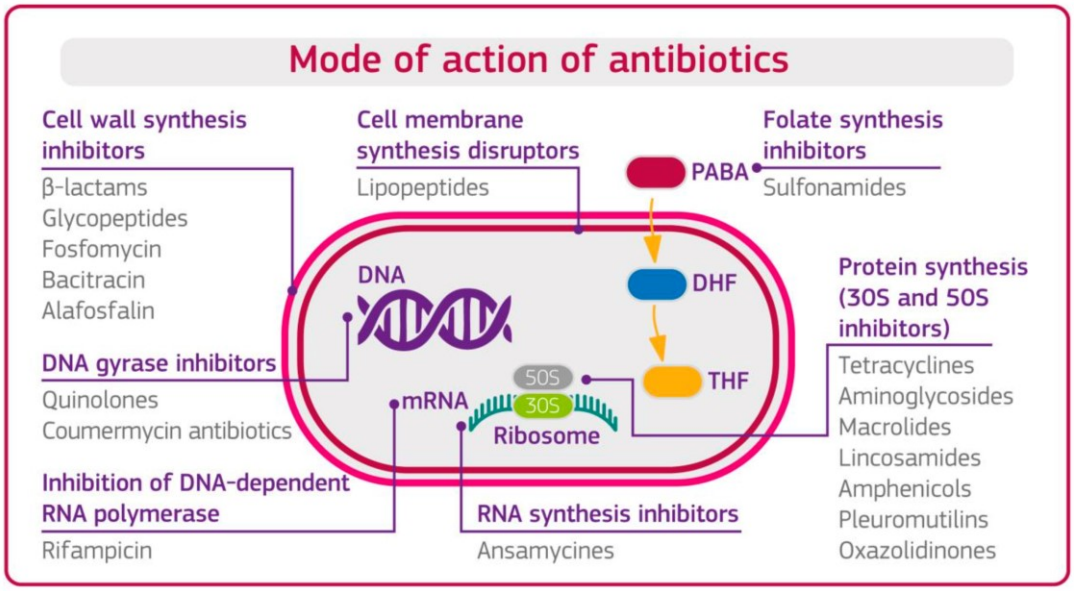


Figure 1: Illustration of the mode of action associated with different categories of antibiotics (Sanseverino et al., 2018).

1.2.2 Use of disinfectants in the food industry

Within the food industry disinfectants are commonly used in sanitation routines (Mc Carlie et al., 2020). Quaternary ammonium compounds (QAC) and peroxygens are among the most widely used disinfectants in the food industry (Fagerlund et al., 2017). The modes of action associated with these disinfectants are fundamentally different from the one for antibiotics. Peroxygen compounds like peracetic acid are strong oxidants that produce free radicals which can cause severe cell damage (McDonnell et al., 1999). This includes the denaturation of proteins and enzymes as well as an increase in cell wall permeability and an impairment of DNA replication (McDonnell et al., 1999). QAC-based disinfectants, such as benzalkonium chloride, are cationic detergents. Their primary mode of action is the disruption of cell membranes but other damaging effects have also been reported including DNA degradation (McDonnell et al., 1999).

Several studies have reported that continuous exposure to QAC can cause bacteria to develop a tolerance and resistance properties (Langsrud et al., 1997; Mc Carlie et al., 2020). Bacteria belonging to *Pseudomonas* and *Staphylococcus* species with resistance properties towards QAC were already identified in the Norwegian food industry previously (Heir et al., 2021; Heir et al., 1995). However, a study from 2002 demonstrated that the prevalence of bacteria with QAC-resistance isolated from the Norwegian food industry was low (Sidhu et al., 2002).

1.3 The genus of *Pseudomonas*

Bacterial species within the genus of *Pseudomonas* are Gammaproteobacteria that belong to the order of Pseudomonadales (Peix et al., 2018). *Pseudomonas* spp. are obligate aerobe, Gram-negative, rod shaped and non-spore-forming bacteria (Zago et al., 2009). They are one of the most ubiquitous bacterial species in the world and by the time of writing around 200 different species have already been identified (Peix et al., 2018). The prevalence of *Pseudomonas* spp. has been widely detected in the natural and human environment, including extreme environments such as the Antarctica and the Atacama desert in South America. In the human environment some species like *P. aeruginosa* are opportunistic pathogens (Zago et al., 2009). *P. aeruginosa* is common in health care settings due to the high prevalence of virulence factors and antibiotic resistance properties that are associated with this species (Wu et al., 2015). They are also highly adaptive to different environmental conditions. Infections caused by *P. aeruginosa* are mostly only seen in patients with underlying health conditions, such as cancer and cystic fibrosis, but are unfortunately often serious and difficult to treat (Wu et al., 2015).

Pseudomonas spp. are also widespread in the food chain and are a common part of the microflora of many different food products (Heir et al., 2021). However, the prevalence of *Pseudomonas* spp. in food products is mainly associated with food spoilage and not with food safety. Species belonging to *P. aeruginosa* are not a common a part of the microflora in food products (Heir et al., 2021).

Pseudomonas spp. have also been described as one of the most important spoilage organisms in aerobically stored chilled fish products (Møretrø et al., 2016). This is especially true for species belonging to *P. fluorescens* and *P. ludensis* which can be explained by their metabolic versatility and their psychotropic nature that enables growth at temperatures below 10 C° (Liu et al., 2015). The spoilage mechanisms of these species can be linked to their production of proteolytic and lipolytic enzymes (Heir et al., 2021) that cause off-odors and denature muscle proteins resulting in a reduction of the muscle water holding capacity (Xie et al., 2018).

In a previous study *Pseudomonas* spp. were found to be the predominant species in salmon processing plants after sanitation routines (Møretrø et al., 2016). The main source of *Pseudomonas* spp. in processing plants is likely the gut microbiota of the salmon that was found to have *Pseudomonas* spp. in high abundance previously (Cantas et al., 2011). Furthermore, the high prevalence and persistence of *Pseudomonas* spp. in food production environments is related to their high biofilm formation capabilities (Heir et al., 2021). These capabilities involve huge advantages in terms of growth and survival of harsh environmental condition.

On the basis of the ubiquity of *Pseudomonas* spp. in the seafood industry these microorganisms have become an important area of research; with regards to their spoilage potential, the development of antimicrobial resistances and the underlying resistance mechanisms. Miranda et al. (2013) pointed out that there is a need for studies on the prevalence of antimicrobial resistance in the aquaculture sector, specifically *P. fluorescens* species.

1.4 Mechanisms of antimicrobial resistance

1.4.1 Natural resistance

Antimicrobial resistance is regulated on the genetic level and conferred by a spectrum of complex molecular mechanisms commonly categorized into naturally and acquired resistances (excellently reviewed in Reygaert, 2018). Natural resistances are conferred by inherent genes that have evolved over the course of time (Ray et al., 2017). The genetic determinants associated with a natural resistance are mainly encoded in the chromosomal DNA and passed on to the next generation during cell replication (Ray et al., 2017). These characteristics are therefore universal within a bacterial species. Natural resistances can be further classified into intrinsic and induced resistances (Reygaert, 2018). Both classifications refer to naturally occurring genes. However, genes conferring intrinsic resistance are permanently expressed whereas genes that confer induced resistance are only expressed after exposure to antimicrobials (Reygaert, 2018).

1.4.2 Acquired resistance

Apart from naturally occurring resistances bacteria can also acquire resistances through mutations in existing genes or by a horizontal transfer of genes (Parmley et al., 2012). Mutations are small erroneous changes in the DNA sequences of the genetic material and can occur during DNA replication. The consequences of mutations are often harmful to the bacterial cell but occasionally favorable and causative to the acquisition of resistance (Reygaert, 2018). Horizontal gene transfer is the exchange of mobile genetic elements between bacteria which can occur across different bacterial species and genera (Van Hoek et al., 2011). The ability to exchange genes gives rise to a rapid development of resistance among bacteria (Ray et al., 2017). Horizontal gene transfer is initiated by the processes of conjugation, transformation and transduction (Figure 2) (Ray et al., 2017).

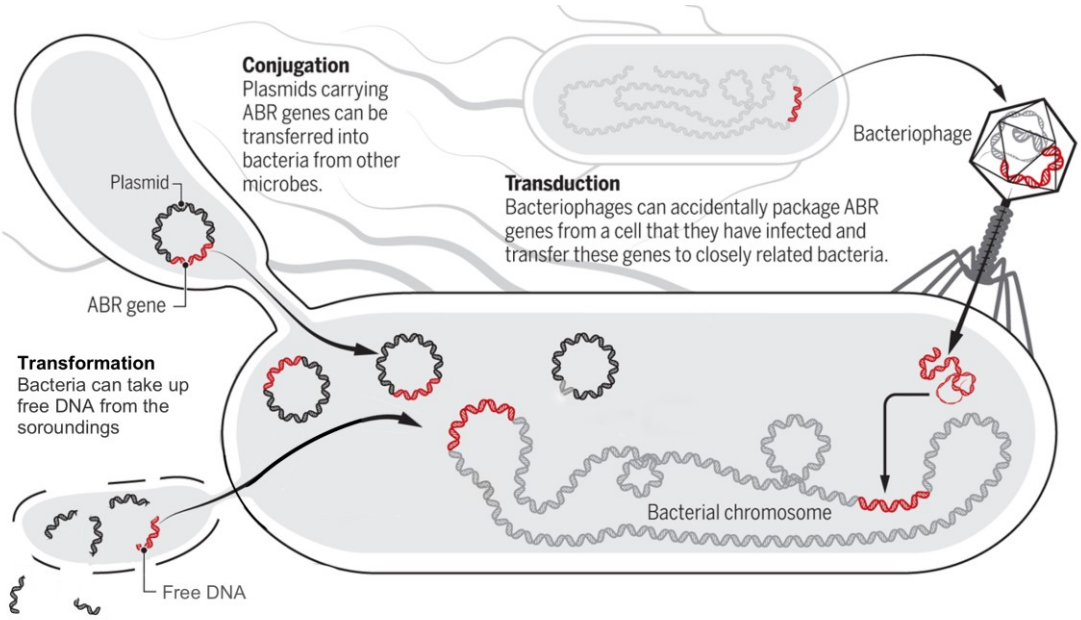


Figure 2: Overview of the processes involved in the horizontal gene transfer (adapted from MacLean et al., 2019).

Conjugation is the horizontal gene transfer from one cell to another through direct contact by the pilus (Ray et al., 2017). The genetic material that can be subjected to transfer are generally plasmids and transposons (Van Hoek et al., 2011). Plasmids are small ring-structured DNA-molecules located in the cytoplasm of some bacterial cells and typically harbor non-essential genes (Partridge et al., 2018). Thus, plasmids are associated with genes that provide beneficial attributes for the bacteria and frequently contain resistance genes. In fact, it has been reported that a single plasmid can even harbor multiple resistance genes (Cox et al., 2017). Plasmids are not always allocated equally during cell division because they replicate independently (Martinsen et al., 2021).

In contrast to plasmids, transposons are dependent on the incorporation in either plasmids or chromosomes for replication (Van Hoek et al., 2011). Transposons are small mobile pieces of DNA and are, like plasmids, able to transfer genes between bacteria which includes resistance genes (Partridge et al., 2018). However, transposon have a larger host range than plasmids (Roberts, 2008). Additional genetic elements associated with the dissemination of resistance genes are the integrons that are generally found as a part of plasmids or transposon (Partridge et al., 2018). The integron itself is not considered to be mobile but contains mobile gene cassettes (Domingues et al., 2012).

The horizontal gene transfer process of transformation differs from conjugation because exogenous DNA from the surroundings can be directly taken up by the cell (Ray et al., 2017). In this process no direct cell-to-cell contact is needed. After the uptake of DNA it can further be subjected to chromosomal incorporation. The DNA may originate from the genetic material of disrupted bacteria after cell lysis (Ray et al., 2017). Nevertheless, plasmids and transposons may also be subjected to transformation (Lorenz et al., 1994).

The horizontal gene transfer process of transduction involves bacteriophages transferring bacterial DNA (Willi et al., 1997). Bacteriophages are viruses targeting bacterial cells for self-replication. In some cases the bacteriophages can carry and transfer mobile genetic elements from one cell to another, including resistance genes (Willi et al., 1997).

1.4.3 Molecular mechanisms of drug resistance

The resistance mechanisms in prokaryotes are conferred by a range of complex biochemical processes (Cox et al., 2013; Kumar, 2017). The most common natural mechanism of resistance is low membrane permeability that provides resistance against specific types of antimicrobials (Fajardo et al., 2008). Low membrane permeability is typically associated with Gram negative bacteria due to their distinctive multilayer cytoplasmic membrane. The permeability is regulated by small pores, i.e., integral proteins in the membrane (Cox et al., 2013). The diffusion of molecules across the membrane is facilitated by channels in the pores that allow the uptake of essential nutrients but restrict the penetration of antimicrobials (Cox et al., 2013). This mechanism of resistance has been observed in members of the *P. aeruginosa* species (Ray et al., 2017).

Further common resistance mechanisms are efflux pump systems (Cox et al., 2013). These systems can be chromosomally encoded as a part of the natural resistance mechanisms or as a part of the acquired resistances through transference by mobile genetic elements (Cox et al., 2013; Ray et al., 2017). Efflux pumps are capable of actively transporting antimicrobials out of the cell to maintain non-lethal antimicrobial concentrations within the cell. The mechanism of these pumps is dependent on energy which is either provided by adenosine triphosphate

(ATP) hydrolysis or by the proton motive force (Cox et al., 2013). Some of these pumps are only capable of transporting specific drugs while others are more advanced and can transport a wide variety of different compounds (Ray et al., 2017). The efflux pumps are commonly categorized into five families (Figure 3): the ATP-binding cassette (ABC) family, the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, the resistance-nodulation-cell division (RND) family and the multidrug and toxic compound extrusion (MATE) family (Reygaert, 2018).

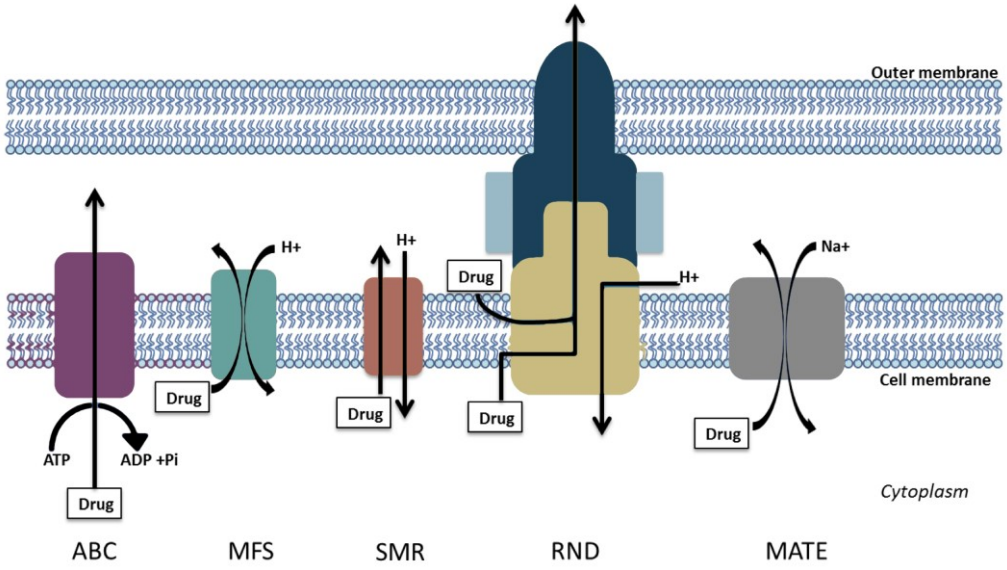


Figure 3: Illustration of the five families of efflux pump systems (adapted from Reygaert, 2018).

Efflux pumps that belong to the ABC transporter family can transport a wide variety of different compounds. This includes nutrients, like proteins and polysaccharides, but also toxins and drugs (Reygaert, 2018). For instance, the novel gene *optrA* is encoding an ABC transporter associated with drug efflux of linezolid, chloramphenicol and florfenicol (Wang et al., 2015). *optrA* has previously been identified located on plasmids in *Enterococcus* species (Wang et al., 2015). The common denominator for all of the ABC transporters is that they are fueled by ATP molecules (Reygaert, 2018). in contrast to efflux pumps in the MFS family, which are driven by the proton motive force by symport or antiport systems (Reygaert, 2018). Examples of resistance genes encoding MFS pumps are *floR* (Kadlec et al., 2007), *fexA* (Kehrenberg et al., 2004) and *pexA* (Lang et al., 2010) all conferring florfenicol resistance. Both *floR* and *fexA* have previously been identified on mobile genetic elements and may be subjected to horizontal gene transfer (Zhao et al., 2016).

Resistance genes belonging to the *tet* family (*tetA, tetB, tetC* and *tetG*) are also encoding MFS efflux pumps and are associated with tetracycline resistance (Olowe et al., 2013). These genes are often found on conjugative elements such as plasmids and transposons according to Roberts (2005). Several of the *tet* genes have been previously found in *Pseudomonas* spp. (Adesoji et al., 2015; Meng et al., 2020).

The SMR efflux pumps are likewise driven by the proton motive force. It has been reported that these pumps only have a narrow range of substrates and drug efflux is not common (Reygaert, 2018).

Efflux pumps belonging to the RND family operate via an antiport mechanism and are frequently associated with drug efflux (Reygaert, 2018). One of the most characterized RND

efflux pump associated with natural resistance is the chromosomally encoded MexA-MexB-OprM system in *P. aeruginosa* (Aeschlimann, 2003; Ray et al., 2017). The substrates of this multidrug efflux pump include a broad spectrum of different antibiotics, such as chloramphenicol, tetracycline and trimethoprim. Aeschlimann (2003) reported that the minimum inhibitory concentration of specific antibiotics decreased by more than a hundredfold in the absence of MexA-MexB-OprM. This pump system has also been detected in *P. fluorescens* isolated from the food value chain (Heir et al., 2021; Quintieri et al., 2019).

The last of the five efflux pumps families are the MATE transporters (Reygaert, 2018). MATE transporters use Na^+ ions as an energy source and are associated with quinolone resistance. However, MATE transporter are not well characterized in bacteria (Reygaert, 2018).

Other essential mechanisms of resistance involve the use of enzymes for drug degradation by hydrolytic cleavage or drug modification by the addition of functional groups (Kumar, 2017; Ray et al., 2017). Examples of these enzymes are the β-lactamase enzymes with the ability to inactivate β-lactam drugs by hydrolysis (Kumar, 2017). Genes encoding the production of β-lactamases are located in the chromosome of some bacterial species but can also be found on plasmids (Song et al., 2006). For instance, the gene bla_{TEM-1} that encodes extended spectrum β-lactamases has been identified on plasmids (Haghighatpanah et al., 2016). Another example is *ampC* conferring resistance against penicillins and some first generation cephalosporins. *ampC* has also been identified in *Pseudomonas* spp. previously (Heir et al., 2021).

Some bacteria have also evolved mechanisms of resistance that modify the drug target, either by altering it or by replacing the drug target entirely (Kumar, 2017). The process of drug target alteration involves enzymes, such as the erythromycin 23S ribosomal RNA methyltransferase. The production of this enzyme has been connected to the presence of the resistance genes *ermA* and *ermB* (Min et al., 2008). The resistance gene *cfr* is likewise associated with enzymes that alter the drug target (Kumar, 2017). It has been reported that *cfr* is frequently plasmid-borne and confers resistance against chloramphenicol and florfenicol among other antibiotics (Kumar, 2017). This gene is closely associated with resistance mechanisms in *Pseudomonas* spp. (EMA, 2014).

The replacement of drug targets can also result in drug resistance. This mechanism has been associated with the resistance gene *sulI* conferring resistance towards the clinically important sulfonamides (Radu et al., 2021). This gene has likewise been identified in *Pseudomonas* spp. (Meng et al., 2020).

1.4.4 Molecular mechanisms of disinfectant resistance

The mechanisms of disinfectant resistance can also be subjected to horizontal gene transfer. In fact, it has been revealed that horizontal gene transfer is the main route of acquisition for this type of resistance (Mc Carlie et al., 2020). Disinfectant resistance genes were identified on mobile genetic elements previously and in some cases these genes co-existed with antibiotic resistance genes (Kim et al., 2018).

The molecular mechanisms of resistance towards drugs and disinfectants are related although the primary mechanisms conferring resistance towards disinfectants are efflux pumps (Mc Carlie et al., 2020). Examples are the frequently characterized and plasmid-borne efflux pumps encoded by genes belonging to the *qac* family (Chapman, 2003). These are associated

with a resistance towards QAC. For instance, the gene *qacEΔ1* encodes an efflux pump that belongs to the major facilitator superfamily (MFS) that was identified on integrons in Gram negative bacteria (Chapman, 2003).

The gene *qacH* has also indicated to play an important role in resistance towards QAC. This gene is coding for a small multi drug exporter (SMR) located in the outer membrane of the bacteria cells (Colinon et al., 2010). Additionally, *bcrABC* is associated with QAC resistance, more specifically benzalkonium chloride. It was detected in resistant strains of *L. monocytogenes*. (Martínez-Suárez et al., 2016). *bcrABC* is encoding an efflux system in in the ATP-binding cassette (ABC) family.

Studies have also reported induced natural resistance among *P. aeruginosa* species upon exposure towards benzalkonium chloride that involved the downregulation of porins in the cellular membrane (Mc Carlie et al., 2020).

Mechanisms of defense towards oxidizing disinfectants, such as peracetic acid, are different from those associated with QAC resistance (Chapman, 2003). Multigene systems as *soxRS* and *oxyR* have been reported to prevent and repair damage caused by radicals (Chapman, 2003). Systems like these are a part of the natural stress response towards environmental factors (Ray et al., 2017). Additional defense mechanisms towards oxidants are biofilm formation capabilities (Chapman, 2003).

1.4.5 Effect of biofilm formation on resistance properties

Biofilms are microbial communities enfolded in a matrix of self-produced polymeric substances (Abe et al., 2020). The formation of biofilms plays an imperative role in the resistance properties among bacteria (Hall et al., 2017). In fact, it has been reported that biofilms increase the tolerance towards antibiotics by more than a hundred-fold compared to cells in planktonic state (Ceri et al., 1999). This can be explained by the synergistic effects of the multifactorial defense mechanisms in biofilms (Figure 4) including the slow penetration of antimicrobials, a manifold of intercellular interactions and persister cells (Hall et al., 2017).

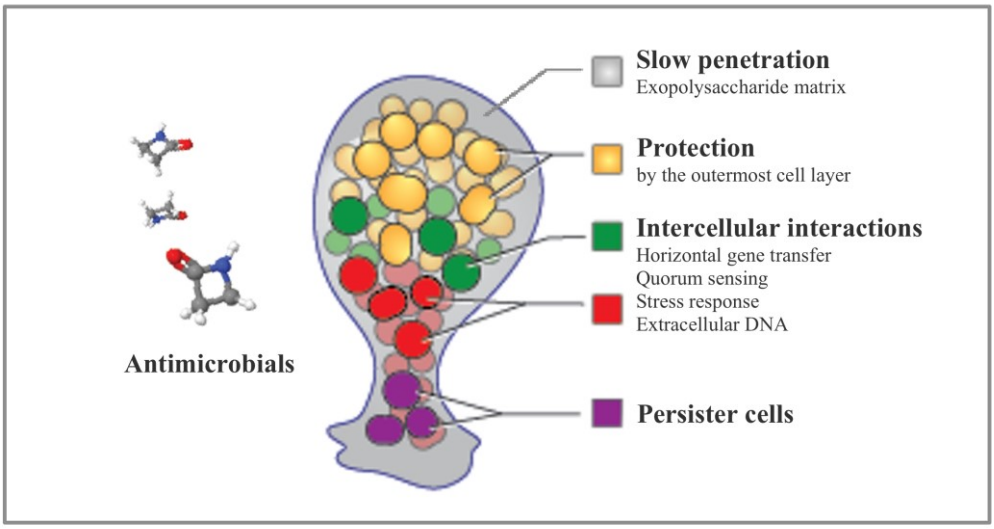


Figure 4: Resistance and tolerance mechanisms of biofilms (adapted from Hall et al. (2017) and UW (2020)).

Biofilms have also been described as hot spots for horizontal gene transfer where genes are frequently being exchanged (Abe et al., 2020). The intercellular proximity in biofilms is expected to promote gene transfer (Reygaert, 2018). Both conjugation and transformation are major routes of gene transfer whereas gene transduction is less common. However, in some cases bacteriophages in biofilms have been observed to even support the life cycle and strengthen the biofilm structure (Abe et al., 2020). An additional process of horizontal gene transfer is also taking place in biofilms, namely the membrane vesicle mediated transfer of genes. This kind of transfer is especially abundant in marine ecosystems (Biller et al., 2014). Membrane vesicles are small circular lipid particles that are released from the membrane carrying nutrients and potentially also resistance genes. Further, the vesicles can be absorbed by surrounding bacterial cells (Abe et al., 2020). This process was observed in biofilms of *P. aeruginosa* (Murphy et al., 2014).

Bacteria in biofilm state also show a decreased susceptibility towards disinfectants. Houari et al. (2007) reported a fourfold reduction in the susceptibility towards benzalkonium chloride of *P. aeruginosa* in planktonic state when compared to the biofilm state. Biofilms also protect bacteria from oxidizing disinfectants since these react with components of the biofilm (Chapman, 2003). The reactivity of the oxidant is decreased as it diffuses further into the biofilm, thus bacteria living deep inside of the biofilm are protected. Studies have shown that *P. aeruginosa* biofilms display a reduced susceptibility towards oxidants (Cochran et al., 2000).

The efficacy of disinfectants against bacteria is highly dependent on two factors: concentration and contact time (West et al., 2018). However, these factors are generally defined on the basis of susceptibility tests undertaken with bacteria in planktonic state (Fagerlund et al., 2017). Increased focus on the efficacy of disinfectant against biofilms is needed considering the omnipresence of biofilms.

1.5 Aim of this study

The aim of this study was to examine and characterize phenotypic and genotypic antimicrobial resistance among biofilm-forming *Pseudomonas* spp. that were isolated from a Norwegian salmon processing plant. This included generating baseline data on the prevalence of specific genetic determinants associated with disinfectant and antibiotic resistance among these *Pseudomonas* spp.. The phenotypic characterization included the determination of the antibiotic susceptibility profiles among these *Pseudomonas* spp., including florfenicol, which is commonly used in the Norwegian aquaculture sector.

Furthermore, this study assesses the biofilm formation capabilities among a selection of these *Pseudomonas* spp. and thereafter evaluates the effectiveness of three antimicrobial agents towards *Pseudomonas* spp. in planktonic state contra biofilm state.

The main objectives of the experimental work were (i) the identification of bacterial species, (ii) the detection of antimicrobial resistance genes (ARGs), (iii) the testing of antibiotic susceptibility (iv) the in vitro screening of biofilms.

2.0 Materials and methods

2.1 Description of bacterial isolates from salmon processing plant

The bacterial isolates included in this study originated from a salmon slaughterhouse facility in Norway. Swab sampling procedures were performed at several sampling locations over a period of one year (mid 2018 – mid 2019). The sampling was performed ahead of production start after the facility was routinely cleaned. Bacteria from the swab samples were subjected to various analytical methods, including isolation on the selective growth media *Pseudomonas* CFC (CM0559 and SR0103, Oxoid Ltd.). Presumptive *Pseudomonas* species were transferred to a growth broth containing a cryoprotectant (20% glycerol) and stored at -80 °C. This experimental work was undertaken by doctoral research fellow Gunn Merethe B. Thomassen.

A total of 142 isolates from eight different sampling locations were included in this study (Table 1). These were previously identified in Boyko (2020). However, 36 isolates were not successfully sequenced and could not be identified on species level. Additional 20 *Listeria* isolates by Thomassen et al. (2021) and 3 *S. aureus* isolates (Mehli et al., 2017) were included in this study, resulting in a combined quantity of 165 bacterial isolates (cf. Appendix A).

Table 1: Sample collection spots of 142 bacterial isolates.

Spot No.	Sampling location	Quantity of isolates
2	Water from well boat	**38**
7	Drains at the orientation rig	**14**
8	Steel board behind the orientation rig	**25**
10	Suction unit at the gutting machine	**30**
14	Head cutting machine	**17**
18	Conveyor in front of skinning machine	**8**
20	Filet turner	**1**
22	Drains at the filet turner	**9**
	Total	**142**

2.1.1 Inclusion of various reference strains and positive controls

A selection of 9 reference strains was also included in this study (Table 2). These strains were identified in literature and either purchased from international culture collections or obtained from personal contacts. Several of these reference strains were classified at biosafety level 2 and therefore all laboratorial work with these strains was carried out at the laboratory for pathogens with enhanced safety measures.

Table 2: Characteristics and involvement of reference strains in different experiments.

Reference strain/positive control	Characteristics	Reference	Detection of ARGs	Disk diffusion	Biofilm screening
Escherichi coli CCUG 17620	Positive for *ampC* and used for suscpetibility testing	Black et al. (2005), CLSI (2012b)	✓	✓	✓
Pseudomonas aeruginosa CCUG 17619	Used for suscpetibility testing	CLSI (2012b)	✓	✓	✓
Pseudomonas aeruginosa CCUG 59347	Positive for *floR2*, *sulI, qacEΔ1 and tetG*	NCBI (2016)*	✓	✓	✓
Pseudomonas aeruginosa NCTC 13717	Positive for *floR2*, *sulI, qacEΔ1 and tetG*	NCBI (2016)*	✓		
Acinetobacter baumannii NCTC 13305	Positive for *floR*	NCBI (2016)*	✓	✓	✓
Listeria innocua CCUG 15531	Common quality control strain	-	✓		
Listeria innocua CCUG 44813	Common quality control strain	-	✓		
Listeria monocytogenes MF 4624 (ST8)	Positive for *bcrABC*	Møretrø et al. (2017b)	✓	✓	✓
Listeria monocytogenes MF 5634 (ST8)	Positive for *qacH*	Møretrø et al. (2017b)	✓	✓	✓

**Pathogen detection browser by NCBI (2016)*

2.2 Overview of the applied experimental methodology

The main tasks of the experimental work (Figure 5) required implementing various laboratory based methods of analysis. Only validated and academically recognized methods were selected (Altschul et al., 1997; CLSI, 2012a; Harrison et al., 2010; Innovotech, 2015; Madigan, 2015, p. 343; McFarland, 1907). The exact execution of these methods will be presented in the following chapters.

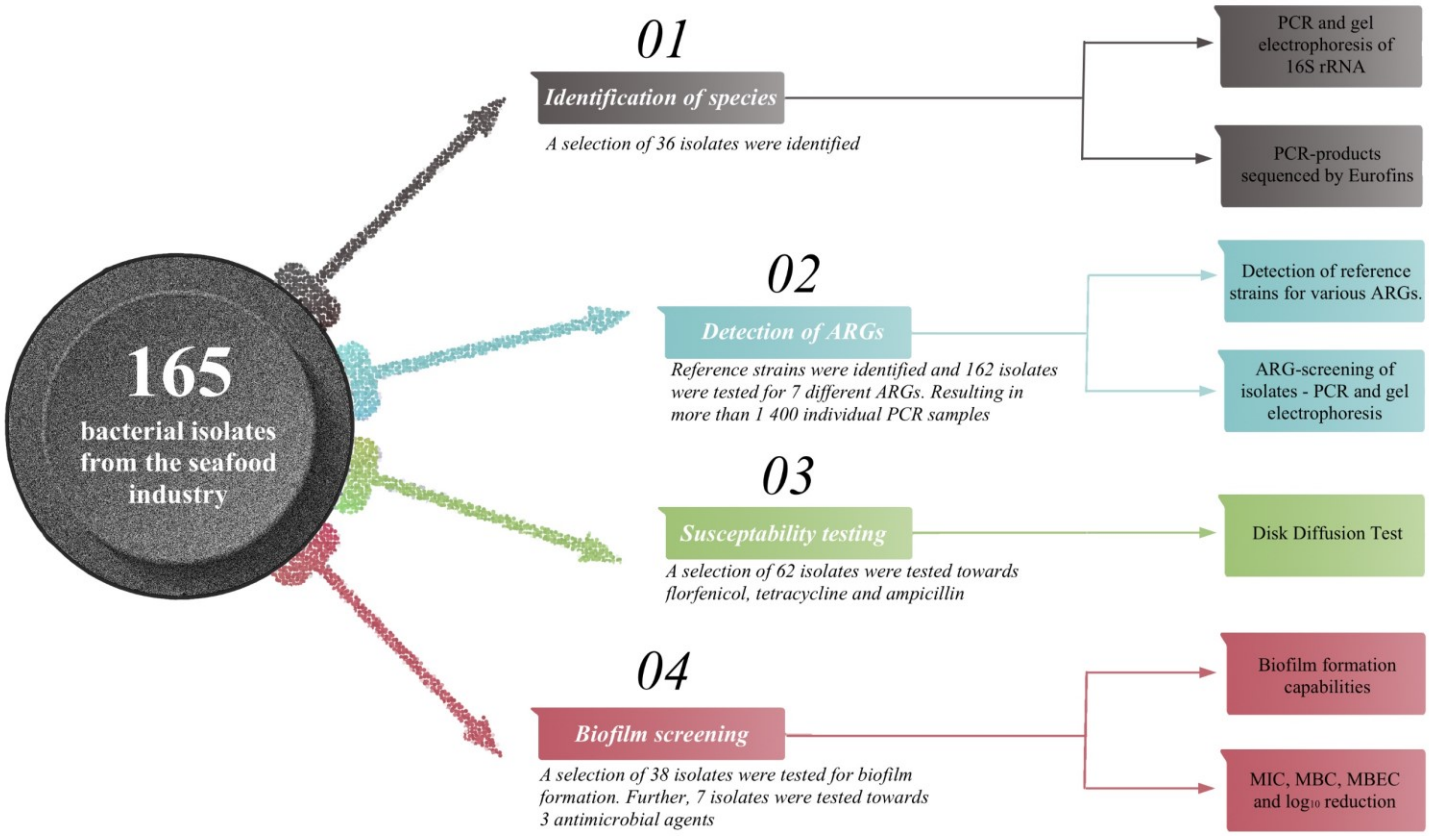


Figure 5: A selection of 165 bacterial isolates from the salmon industry were analyzed.

2.3 Identification of 36 bacterial isolates by sequencing of 16S rRNA

2.3.1 Amplification of 16S rRNA

The polymerase chain reaction (PCR) reaction was performed using 1.5 µL template DNA (Boyko, 2020) and 23.5 µL master mix (Table 3). Primer pairs and cycling conditions are shown in Table 4 and Appendix B, respectively. Control samples of the master mix were included, using 1.5 µL of nuclease free water instead of template DNA. The PCR-reaction was carried out in a C1000™ thermal cycler (Bio-Rad Inc).

Table 3: Composition of master mix used for PCR.

Components	Supplier	Stock Concentration	Volume per reaction (µL)	Final Concentration
PCR buffer	203205, © Qiagen	10X	2.5	1X
$MgCl_2$	203205, © Qiagen	25 mM	(0.5)*	0.5 mM
dNTP mix	201901, © Qiagen	10 nM	0.5	200 µM
Primer f	Merck KGaA Sigma-Aldrich	10 µM	0.5	0.2 µM
Primer r	Merck KGaA Sigma-Aldrich	10 µM	0.5	0.2 µM
HotStar Taq DNA Polymerase	203205, © Qiagen	5.0 units/µL	0.125	0.625 units/reaction
Distilled nuclease free water	AM9937 Invitrogen™	-	19.375 (18.875)*	-
Volume	-	-	23.5	-
Template DNA	-	-	1.5	-
Total Volume	-	-	25	-

**MgCl₂ only used for specific ARGs*

Table 4: Primer pair used for PCR of 16S rRNA.

Target gene	Name	f/r	Sequence	Amplicon length (bp)	Reference
16S rRNA	*338F*	f	5-ACTCCTACGGGAGGCAGCAG-3	1150	Huse et al. (2008)
	1492R	r	5-TACGGYTACCTTGTTACGACT-3		Turner et al. (1999)

2.3.2 Visualization of PCR products

A stock solution for the electrophoresis buffer was prepared (50X TAE; Tris base (71003-490, VWR; 121.00 g), glacial acetic acid (1.00063, EMSURE®; 28.55 mL), EDTA (0.5 M, pH 8.0, 50.00 mL) and deionized water (to volume 500.00 mL)). The working solution was 1X TAE (40.0 mM Tris, 20.0 mM acetic acid and 1.0 mM EDTA).

PCR products were analyzed on a 150 mL, 1.5% gel containing agarose (11500727, SeaKem® LE Agarose), 1X TAE buffer and 10.0 µL GelRed DNA stain (#41003, Biotium). The PCR products were mixed with 2.0 µL of 6X DNA loading dye (R0611, ThermoFischer Scientific) and 10.0 µL were added to each well. GeneRuler 1 kb Plus DNA ladder (SM1331, Thermo Fischer Scientific) was used as a size marker. Electrophoresis was performed with a Sub-Cell® GT System (Bio-Rad Inc.) at 90 volts for 90 minutes. A Syngene™ G:Box with the accompanying software GeneSys G:Box Chemi-XRQ was used for gel imaging.

2.3.3 Purification and quantification of PCR products for sequencing

The PCR Product Cleanup ExoSAP-IT™ was used and the procedure was carried out according to default specifications (78201.1.ML, Thermo Fischer Scientific, Applied Biosystems™).

The yield and purity of the PCR products were analyzed spectrophotometrically. Samples were measured with 2 parallels by a microplate spectrophotometer (PowerWaveXS Microplate, BioTek®) in connection with Gen5 2.0 data analysis software. The absorbance readings were carried out at 260 nm and 280 nm. It was ensured that the A_{260}/A_{280} ratio was approximately between 1.7 and 2.0.

Samples were prepared for external sequencing at Eurofins Genomics (Cologne, Germany) in accordance with their specifications. DNA concentrations were adjusted to 25-30 ng/µl for PCR products >1000 bp and 15-20 ng/µl for products <1000 bp. Samples were then sent to Eurofins Genomics for Sanger sequencing (Lightrun Tube service). Results were obtained as FASTA files and analyzed using nucleotide BLAST (Basic Local Alignment Search Tool) (available at https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1997).

2.4 Genotypic detection of antimicrobial resistance

The detection of ARGs consisted of two main tasks. First, identifying reference strains, i.e. positive controls for the different ARGs and establishing PCR assays and thereafter, detecting ARGs among 162 bacterial isolates. A selection of 17 different ARGs (Table 5) were included in the project, associated with both antibiotic resistance and disinfectant tolerance.

Table 5: Overview of ARGs and primer pairs.

Antimicrobial resistance	Target gene	Name	f/r	Sequence	Amplicon length (bp)	Reference
Ampicillin	*ampC*	ampCF	f	5-CCTCTTGCTCCACATTTGCT-3	189	Shi et al. (2013)
		ampCR	r	5- ACAACGTTTGCTGTGTGACG - 3		
β-Lactam	bla_{TEM-1}	$bla_{TEM-1}f$	f	5-CATTTTCGTGTCGCCCTTAT-3	167	
		$bla_{TEM-1}r$	r	5-GGGCGAAAACTCTCAAGGAT-3		
Tetracycline	*tetA*	*tetAf*	f	5-GCTACATCCTGCTTGCCTTC-3	210	
		tetAr	r	5-CATAGATCGCCGTGAAGAGG-3		
	tetC	*tetCf*	f	5-CTTGAGAGCCTTCAACCCAG-3	418	
		tetCr	r	5-ATGGTCGTCATCTACCTGCC-3		
	tetG	*tetGf*	f	5-GCTCGGTGGTATCTCTGCTC-3	468	
		tetGr	r	5-AGCAACAGAATCGGGAACAC-3		
Sulphonamide	*sulI*	*sulIf*	f	5-CGGCGTGGGCTACCTGAACG-3	433	
		sulIr	r	5-GCCGATCGCGTGAAGTTCCG-3		
Trimetoprim	*dfrA17*	*dfrA17f*	f	5-TTGAAAATTTCATTGATTG-3	474	
		dfrA17r	r	5-TTAGCCTTTTTTCCAAATCT-3		
Quaternary ammonium compounds	*qacH*	qacHf	f	5-ATGTCATATCTATATTTAGC-3	366	Müller et al. (2013)
		qacHr	r	5-TCACTCTTCATTAATTGTAATAG-3		
	qacEΔ1	qacEΔ1F	f	5-TAGCGAGGGCTTTACTAAGC-3	300	Xiao-Min et al. (2014)
		qacEΔ1R	r	5-ATTCAGAATGCCGAACACCG-3		
Benzalkonium chloride	*bcrABC*	bCf5f	f	5-GGAGGGTAATCATGTCAG-3	1312	Elhanafi et al. (2010)
		bCf5r	r	5-GTATAATCCGGATGCTGCCC-3		
Multidrug resistance	*cfr*	cfrF	f	5-GCAGGTTGGGAGTCATTTTG-3	198	Zhao et al. (2016)
		cfrR	r	5-ACGGTTGGCTAGAGCTTCAC-3		
Phenicols and oxazolidinones	optrA	optrAF	f	5-AAACACTTATGGGTGGTGTGG-3	188	
		optrAR	r	5-CTGAAATGAGCCAAGAGCAG-3		
Florfenicol (phenicol specific exporter genes)	*fexA*	fexAF	f	5-TCGCTGTTCTTGTGTTCGTC-3	186	
		fexAR	r	5-ACAGCCCCATCAGAGTCATC-3		
	fexB	fexBF	f	5-TTGGGTCGTAAGTGGTGTTG-3	185	
		fexBR	r	5-CAGCTCCTTGAAACATTCTACC-3		
	pexA	pexAF	f	5-ACAGTGCAGGTCGAAGAACC-3	215	
		pexAR	r	5-TGCATTACCAATCGACATCC-3		
	floR	floRF	f	5-GCTTTAGCGCCGGTATGG-3	120	
		floRR	r	5-GACAGTGGCGAAGGCAAAG-3		
	floR	floliF	f	5-GCGATATTCATTACTTTGGC-3	426	Faldynova et al. (2003)
		floliR	r	5-TAGGATGAAGGTGAGGAATG-3		
	floR	floZF	f	5-GGCTTTCGTCATTGCGTCTC-3	678	Zhang et al. (2009)
		floZR	r	5-ATCGGTAGGATGAAGGTGAGGA-3		
	floR	floTHF	f	5-TCGCCCGGTATTCCTTAATCG-3	963	This study
		floTHR	r	5-TGAAGGTGAGGAATGACGGC-3		
	floR2	flo2THF	f	5-GCCTTTGTTGCGTTTCGTCT-3	456	
		flo2THR	r	5-CGCGAAGGCCAAGCTAAATC-3		

2.4.1 Cultivation and DNA isolation of reference strains

Cultivation of bacterial sub colonies

A total of 9 reference strains (Table 2) were cultivated on tryptic soy agar (TSA) (84602.0500, VWR). The growth media used in entire experimental work of this study was prepared according to the default procedure provided by the manufacturer. Plates were incubated at 37 °C for 24 hours. Sub colonies were further streaked out and incubated at the same conditions. For each strain a uniform colony appearance was confirmed.

Extraction of metagenomic DNA

The DNA isolation kit *Genomic Micro AX Bacteria Gravity Flow* (A&A Biotechnology, Poland) was used for DNA isolation in accordance to the included protocols (cat. #102-100M and cat. #102-100). Step 1 of both protocols was slightly altered, i.e. well-defined colonies were transferred directly to the suspension buffer instead of using bacteria suspensions and discarding the supernatant after centrifugation. Isolated DNA was stored at -20 °C.

The yield and purity of the isolated DNA was analyzed spectrophotometrically, using the same procedure as described for the PCR products in Section 2.3.3. DNA concentrations were adjusted to 50-100 ng/µl.

In order to verify successful DNA isolation, PCR and gel electrophoresis of 16S rRNA were conducted as described in Section 2.3.

2.4.2 Establishment of PCR assays and detection of ARGs among reference strains

PCR and gel electrophoresis were used to detect the presence of 17 ARGs (Table 6). The analysis was carried out as described in Section 2.3. Template DNA for reference strains from this study was used in addition to DNA from Boyko (2020) and Mehli et al. (2017) for bacterial isolates. Master mixes for *qacH, qacEΔ1* and *bcrABC* were prepared with $MgCl_2$ (Table 3).

The PCR for *bcrABC* and *qacH* was conducted several times with different cycling conditions (cf. Appendix B) for temperature optimalization. Furthermore, a gradient PCR was carried out for *optrA* and bla_{TEM-1} in order to empirically determinate the best annealing temperature from eight annealing temperatures between 55-65 °C (cf. Appendix B).

Table 6: Overview of reference strains and bacterial isolates included in analysis for the detection of positive controls for ARGs (103 PCR reactions).

Reference strains	**ARGs**															
	ampC	*bla* $_{TEM-1}$	*tetA*	*tetC*	*tetG*	*sulI*	*dfr-A17*	*qacH*	*qac-EΔ1*	*bcr-ABC*	*cfr*	optrA	*fexA*	*fexB*	pexA	*floR/ floR2*
Escherichi coli CCUG 17620	✓	✓	✓	✓	✓		✓	✓		✓	✓	✓	✓	✓	✓	✓
Pseudomonas aeruginosa CCUG 17619	✓	✓	✓	✓	✓		✓	✓		✓	✓	✓				
Pseudomonas aeruginosa CCUG 59347		✓				✓		✓	✓	✓	✓		✓	✓	✓	✓
Pseudomonas aeruginosa NCTC 13717		✓				✓		✓	✓	✓	✓		✓	✓	✓	✓
Acinetobacter baumannii NCTC 13305											✓		✓	✓	✓	✓
Listeria innocua CCUG 15531								✓		✓						
Listeria innocua CCUG 44813								✓		✓						
Listeria monocytogenes MF4624						✓		✓	✓	✓	✓		✓	✓	✓	
Listeria monocytogenes MF5634						✓		✓	✓	✓	✓		✓	✓	✓	
Staphylococcus aureus S160		✓														
Staphylococcus aureus S227		✓														
Staphylococcus aureus S229		✓														
Pseudomonas brenneri LJP 009											✓	✓				
Stenotrophomonas rhizophila LJP 012											✓	✓				
Pseudomonas spp. LJP 029	✓	✓	✓	✓	✓		✓			✓						
Pseudomonas fluorescens LJP 423	✓	✓	✓	✓	✓		✓			✓						
Pseudomonas gessardii LJP 716	✓	✓			✓		✓	✓		✓						
Pseudomonas putida LJP 760	✓	✓			✓		✓	✓	✓	✓						

2.4.3 Detection of 7 ARGs among 162 bacterial isolates

The presence of 7 different ARGs (*qacH, qacEΔ1, bcrABC, sulI, tetG, ampC* and *floR)* was analyzed among 162 bacterial isolates, resulting in almost 1200 individual PCR reactions. Reference strains were included in all set-ups. The PCR assay and gel electrophoresis were carried out as described in Section 2.3 with template DNA by Boyko (2020) and (Thomassen et al., 2021).

To confirm the presence of resistance genes, a selection of PCR products was sent to Eurofins Genomics for Sanger sequencing. This included a total of 9 positive *bcrABC* products, 5 *sulI* products and 8 *floR* products. The products were purified and quantified as described in Section 2.3.3. Sequencing results were analyzed with BLAST.

2.4.4 Designing new *floR* and *floR2* primer pairs

In hindsight of unsatisfactory PCR and sequencing results with *floR* primer pairs developed by Zhao et al. (2016) an attempt was made to design new *floR/floR2* based primer pairs.

The specific DNA region associated with the *floR* gene is located on the *pMBSF1* plasmid of *Escherichia coli* (GenBank accession no.: AJ518835.1). *Pseudomonas aeruginosa* CCUG 59347 (GenBank accession no.: VTBD01000032.1) was used as a source for the DNA region of the related *floR2* gene. The sequences were compared to other *floR/florR2* sequences using the multiple sequence alignment tool Clustal Omega (EMBL-EBI, 2021) (available at https://www.ebi.ac.uk/Tools/msa/clustalo/) to confirm the uniformity. The NCBI primer designing tool was used (Ye et al., 2012) (available at https://www.ncbi.nlm.nih.gov/tools/primer-blast/) with the preliminary identified *floR* sequence (Figure 6) and *floR2* sequence as templates.

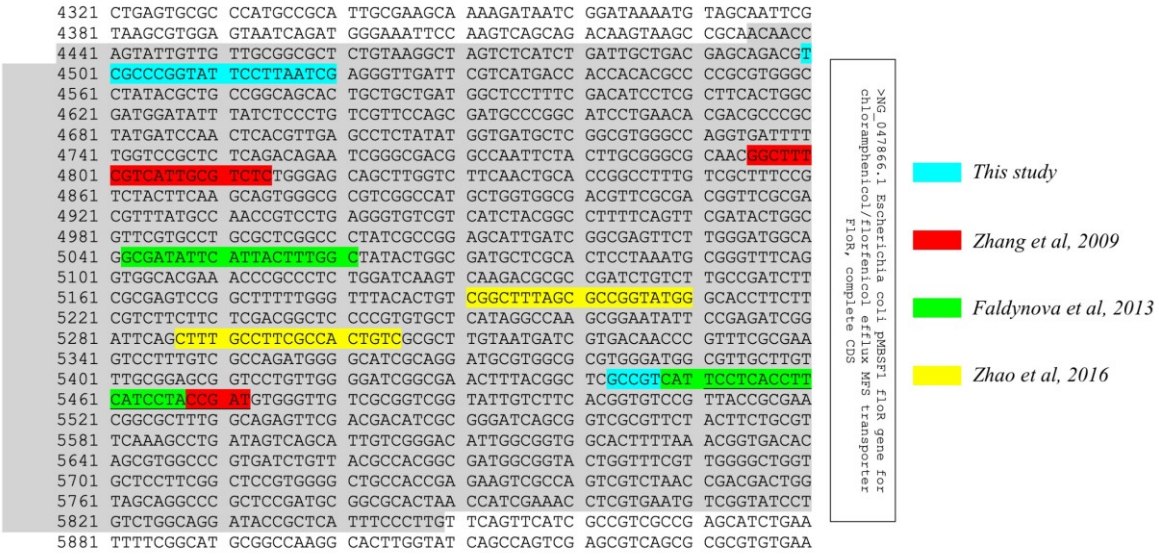


Figure 6: DNA region of floR, located on the plasmid pMBSF1. Primer pairs of different origin are highlighted with colors. Some of the reverse primers are overlapping.

2.4.5 Primer functionality testing

A PCR and gel electrophoresis was performed on the newly designed primer pair floTHF/floTHR (*floR*) and flo2THF/flo2THR (*floR2*), in addition to the *floR* primers described by Faldynova et al. (2003) and Zhang et al. (2009), in order to assess the primer functionality. The assay was conducted as described in Section 2.3 with template DNA from reference strains harboring *floR* and *floR2*.

From this PCR a total of 7 PCR products were purified and quantified as described in Section 2.3.3. Products were prepared for sequencing with the reverse *and* the forward primer. A total of 14 samples were sent for sequencing analysis to Eurofins Genomics.

Additionally, the designed primer pair for *floR* was used in a PCR assay to repeat the *floR*-screening among the 162 previously tested bacterial isolates. The PCR and gel electrophoresis were conducted analogue to the procedure described in Section 2.3.

2.5 Phenotypic testing of antibiotic resistance

2.5.1 McFarland standards

McFarland standardization was used as a reference for adjusting the turbidity of bacteria suspensions and henceforth ensuring a consistent cell density of each inoculum (McFarland, 1907). McFarland standard number 0.5 and 1.0 were prepared (Table 7). The turbidity was verified by measuring the optical density (OD) at wavelength 625 nm using a spectrophotometer (Shimadzu UV1800). According to CLSI (2012a) and Hardy Diagnostics (2020) the OD_{625} values are between 0.08 – 0.13 for standard number 0.5 and 0.14 – 0.17 and for standard number 1.0, respectively.

Table 7: Required reagents to prepare McFarland standards (Harrison et al., 2010).

McFarland standard number	Volume of 1.0% $BaCl_2$ (mL)	Volume of 1.0% H_2SO_4 (mL)	Approximate cell number in matching bacterial suspension (CFU/mL)	Approximate cell number in inoculum used for biofilm cultivation (CFU/mL)
0.5	0.050	9.950	1.5×10^8	5.0×10^6
1.0	0.100	9.900	3.0×10^8	1.0×10^7

2.5.2 Disk diffusion antibiotic susceptibility test

A total of 62 isolates and 6 reference strains were tested towards 3 different types of antibiotics. Isolates were selected in order to get a representative selection from every sampling location and date from the salmon slaughterhouse facility. The test was conducted in accordance to the guidelines from CLSI (2012a). The procedural manual by the Philippine Aquaculture Department (Tendencia, 2004) was used as assisting material.

A few colonies of repropagated bacteria were transferred to glass tubes containing 10.0 mL of 0,9% saline (NaCl from VWR: 27810.295). The concentration of bacterial suspensions was calibrated by matching their transmittance to McFarland standard 0.5 using a turbidimeter (BioLog 21907). The standardized inoculum was thereafter transferred by a sterile cotton swab to Müller-Hinton agar plates (CM0337, Oxoid Ltd.) and spread out evenly. Antibiotic disks were placed on each agar plate (florfenicol; CT1754B, 30,0 µg, ampicillin; CT0003B, 10,0 µg and tetracycline; CT0053B, 30,0 µg supplied by Oxoid Ltd.). Plates were incubated at 35 ± 2 °C for 16-18 hours.

After incubation, the diameter of the inhibition zone around each antibiotic disk was registered and measured. The zone diameter was interpreted in accordance to Table 8.

*Table 8: Bacteria species sorted after their corresponding interpretative inhibition zone diameter. Categorized as resistant **(R)** , intermediate **(I)** and susceptible **(S)**.*

Bacteria Order/Family/Genus	Interpretative data for Florfenicol (mm)			Reference	Interpretative data for Ampicillin (mm)			Reference	Interpretative data for Tetracycline (mm)			Reference
	(R)	(I)	(S)		(R)	(I)	(S)		(R)	(I)	(S)	
Pseudomonas spp.	≤ 14	-	>14	Miranda et al. (2007)	≤ 13	-	>13	Miranda et al. (2007)	≤14	-	>14	Miranda et al. (2007)
Enterobacterales	≤14	15-18	≥19	This study	≤13	14-16	≥17	CLSI (2012b)	≤11	12-14	≥15	CLSI (2012b)
Aeromonas spp.	≤14	15-18	≥19	This study	≤ 13	-	>13	This study	≤11	12-14	≥15	CLSI (2012b)
Acinetobacter spp.	≤14	15-18	≥19	This study	≤ 13	-	>13	This study	≤11	12-14	≥15	CLSI (2012b)
Listeria monocytogenes	≤14	15-18	≥19	This study	<16	-	≥16	EUCAST (2021)	≤14	15-18	≥19	This study
Veterinary pathogens	≤14	15-18	≥19	Tendencia (2004)	-	-	-	-	≤14	15-18	≥19	Tendencia (2004)

2.6 In vitro biofilm screening

The in vitro biofilm screening consisted of two experiments. The first experiment was a analysis to investigate biofilm formation capabilities among a selection of isolates. The experiment was set to form the basis for evaluating and selecting isolates for the next experiment. Thereafter, in the next experiment, the selected isolates were tested towards antimicrobial agents in order to determine the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), minimum biofilm eradication concentration (MBEC) and $\log_{10}$ reduction.

The methodology for both experiments was based on the procedural manual for the MBEC™ assay from Innovotech (2015) and a protocol from the Nature Publishing Group (Harrison et al., 2010). However, with some modifications described below.

The first step for both experiments was to grow subcultures. A total of 44 isolates were recultivated and stored refrigerated (4 °C) for a maximum of 2 weeks. Fresh colonies were prepared at the start of each experiment. In order to simulate realistic conditions for the production environment in the seafood industry the temperature for biofilm cultivation was set to 12 °C. Biofilms were cultivated with ½ concentration of tryptic soy broth (TSB) (84675.0500 VWR). Tween 20 and PCA was supplied by VWR (0777-1L) (84608.0500).

Furthermore, in the initial phase of the experiments, an analysis of optimization was performed to investigated whether the well plate lid affected the optical density measurements.

2.6.1 Screening of in vitro biofilm formation capabilities among bacterial isolates

A total of 38 bacterial isolates and 6 reference strains were tested for biofilm formation capabilities. Figure 7 illustrates the execution of the experiment step by step in a flow diagram. In brief, a standardized inoculum of each isolate is prepared and verified. Afterwards, each inoculum was transferred to a 96 well plate with peg lid (Nunc™ MicroWell™ and Immuno™ TSP Lids, Thermo Scientific) and incubated. During incubation, planktonic cells are growing in the wells, while biofilm is formed on the pegs.

Optical density (OD_{650}) of the planktonic growth is measured right before incubation, and after incubation. The peg lid with biofilm formation is transferred to a recovery plate and the biofilm is dislodged by sonication (Branson 5800 Ultrasonic Cleaner). Thereafter, OD_{650} is measured of the well plate with the recovered cells from the biofilm.

The obtained data from the optical density measurements are used to calculate the biofilm formation capability of each isolate. Mean OD_{650} values from the recovered cells of the biofilm are subtracted mean OD_{650} values from the planktonic cell growth. The OD values of the planktonic growth are always higher than OD values of biofilm growth. Small negative differences between these values are therefore indicating good biofilm formation capabilities. Isolates were categorized into high capabilities ($\Delta OD_{650} \gtrapprox$ -0,100), medium (-0,200 $\lessapprox \Delta OD_{650} \gtrapprox$ -0,100) and low ($\Delta OD_{650} \lessapprox$ -0,200).

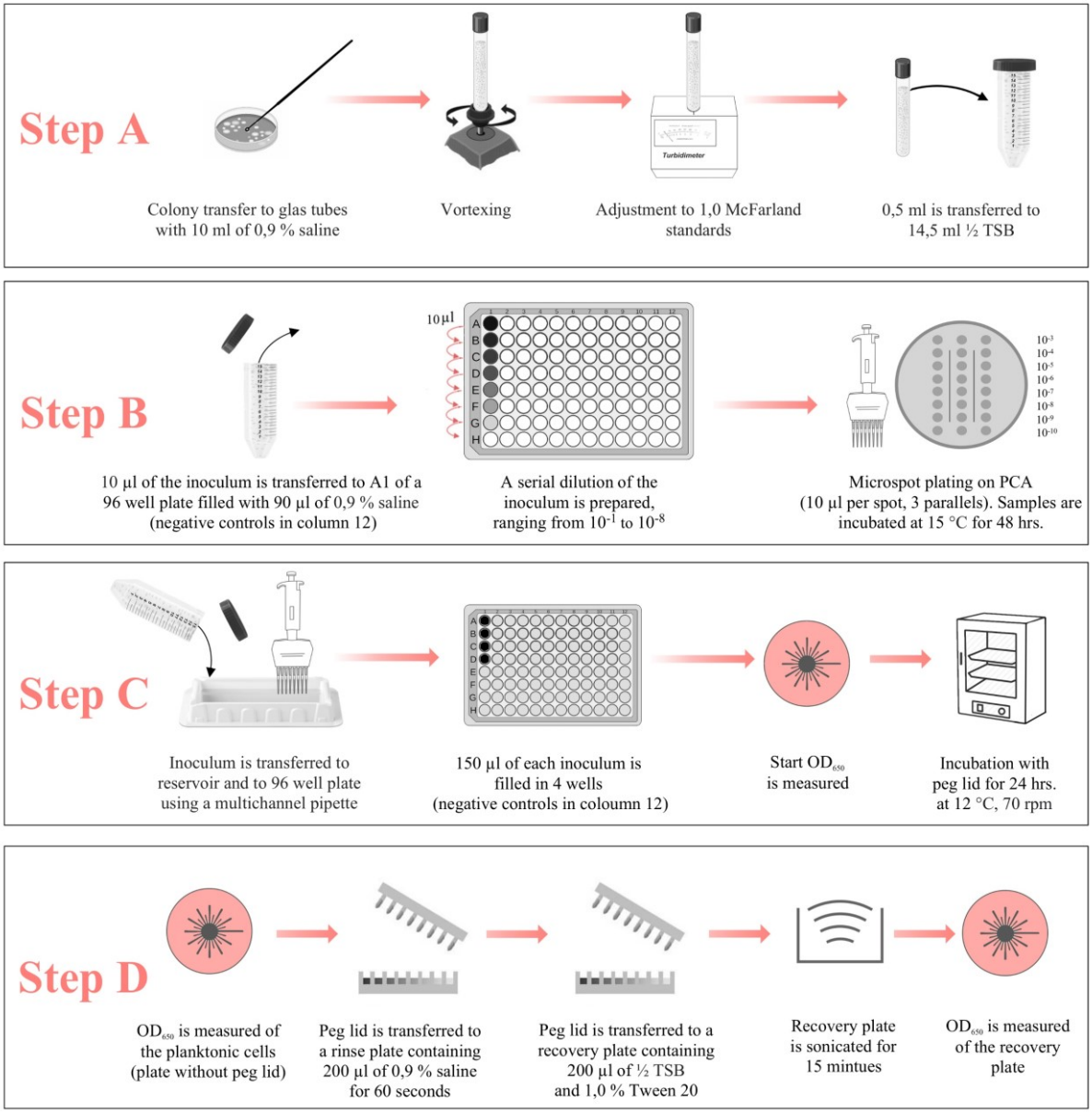


Figure 7: Step by step flow diagram for the analysis of biofilm formation capabilities. ***A:*** *Inoculum preparation by McFarland standardization using a turbidimeter.* ***B:*** *Inoculum verification by serial dilution in a 96 well plate and spot plating for viable cell count (VCC).* ***C:*** *Preparation of a 96 well plate with a total of 22 bacterial isolates, each with 4 parallels. Start OD_{650} is measured by a microplate spectrophotometer.* ***D:*** *Optical density (OD_{650}) is measured of the planktonic cells in the well plate, and the recovered cells from the peg lid after sonication. 2-3 parallels were checked by VCC (as shown in step B).*

2.6.2 In vitro susceptibility test of bacterial isolates in peg lid biofilm reactors

A total of 6 bacterial isolates and 1 reference strain were selected for biofilm susceptibility testing and tested towards 3 different antimicrobial agents, 2 disinfectants and 1 antibiotic.

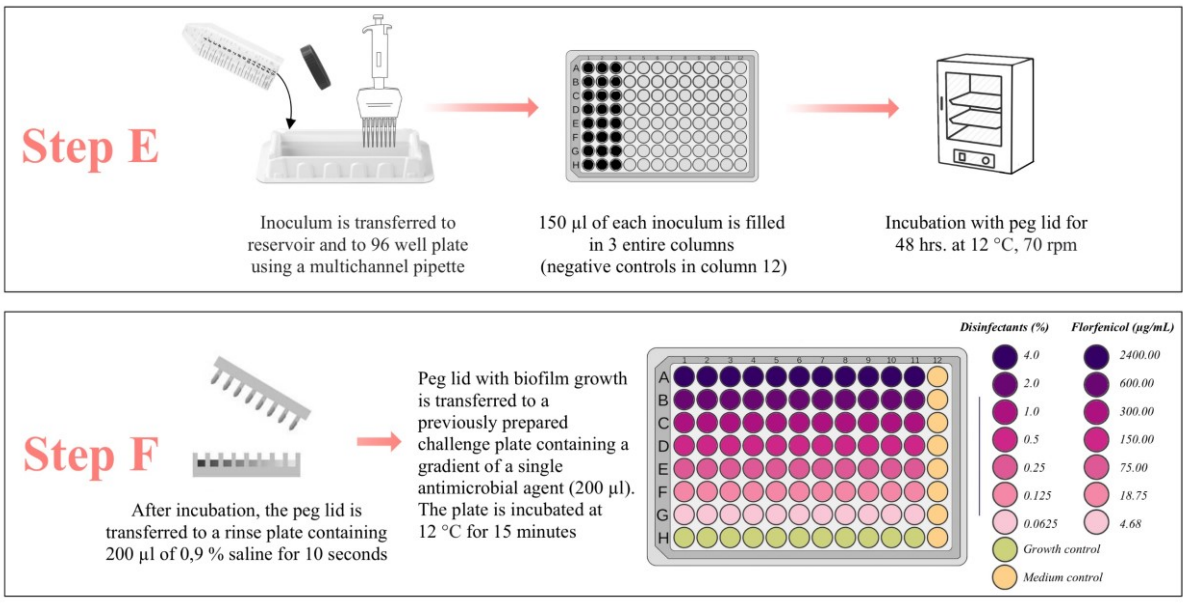
Table 9: Overview of the antimicrobials included in the susceptibility test.

Antimicrobial	Supplier	Active substance	Recommended user concentration	Recommended contact time
Suma Bac D10	L-100849171, Lilleborg	Benzalkonium chloride	1.0%	5-15 minutes
Aqua DES Foam PAA	H608, Aquatic Chemistry	Peracetic acid	1.0% is used at the salmon processing plant 1.5-3.0% is recommended by the producer	5-15 minutes
Florfenicol	F1427, Sigma Aldrich	Florfenicol	-	24 hours

Cumulative concentrations of the disinfectants (Table 9) were used in the analysis, ranging from 0.0625% - 4.0% (diluted with TSB, ½ conc.). A stock solution of analytical standard florfenicol for drug analysis (Table 9) was prepared by solving florfenicol in a small volume of dimethyl sulfoxide (DMSO) (1.16743.1000, EMPLURA®). Thereafter, a working solution (2400.00 µg/mL florfenicol) was prepared with ½ TSB. Cumulative concentrations of florfenicol were used in the analysis, ranging from 4.68 - 2400.00 µg/mL, based on Miranda et al. (2007).

Step A and B (Figure 7) *"Inoculum preparation and verification"* was carried out as described in the previous experiment. The remaining steps of the experiment are described in the flow diagram (step E-K, Figure 8).

In brief, parallels of each inoculum were transferred to 96 well plates and set for incubation. The peg lid with biofilm formation was placed into a challenge plate containing a gradient of a single antimicrobial agent. During the contact time the biofilm on the peg lid is shedding planktonic cells into the challenge plate. Afterwards, the challenge plate was separated from the peg lid, and used for determining MIC and MBC. Concurrently, the biofilm on the peg lid was recovered in a new 96 well plate and dislodged by sonication. The plate with the recovered cells from the biofilm was used for determining MBEC and $\log_{10}$ reduction subsequently.



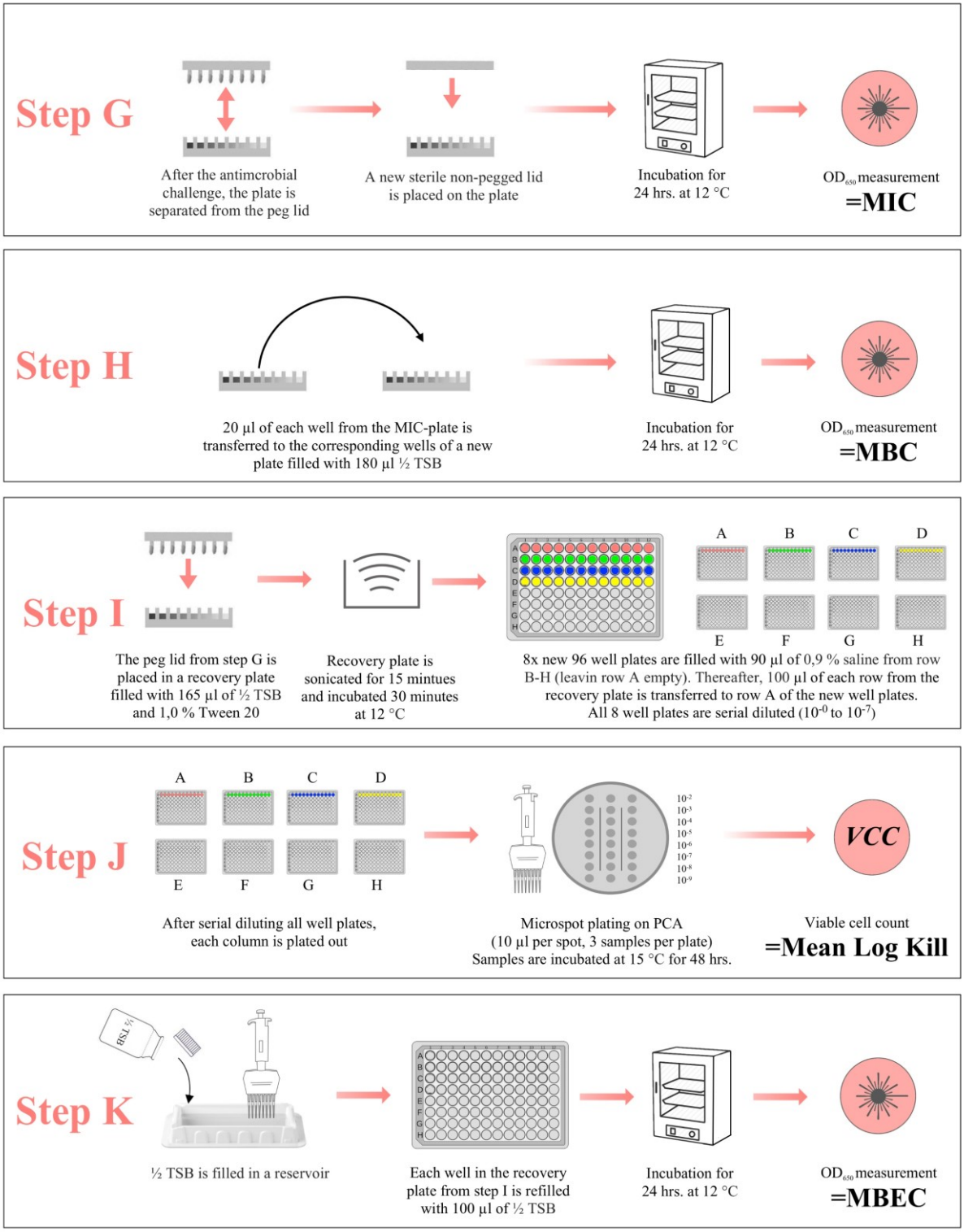


Figure 8: Step by step flow diagram for the susceptibility test. ***E:*** *Preparation of a 96 well plate with a total of 4 bacterial isolates, each with 3 parallels.* ***F:*** *Antimicrobial challenge. The florfenicol challenge plate was incubated with peg lid for 24 hours at 12 °C, and thereafter not incubated before measuring MIC.* ***G:*** *Determination of MIC. One isolate of each MIC plate was checked by VCC (as shown in step B).* ***H:*** *Determination of MBC.* ***I:*** *Biofilm recovery.* ***J:*** *Determination of mean log kill.* ***K:*** *Determination of MBEC.*

2.6.3 Calculations and statistical analysis

The biofilm experiments were conducted in quadruplicates or triplicates. The collected data were processed in accordance with Innovotech (2015) and Harrison et al. (2010). Data are provided as mean ± standard deviation of the mean (SD). The limit of detection (LoD) for OD measurements was defined as: $LoD = mean_{control} + 3\ SD_{control}$ (Armbruster et al., 2008).

Inter- and intra-assay coefficients of variation (% CV) were calculated for evaluating precision and reproducibility. The CVs were calculated on non-log transformed data (Canchola et al., 2017).

One-way analysis of variance (ANOVA) was performed for detecting significant differences ($p<0.05$) in the mean log kill. All data were log-transformed in order to achieve equal variance and normal distribution. Duncan's multiple range test was performed for data comparison ($p<0.05$) in accordance with a MBEC study by Hossain et al. (2020). The data were analyzed in the software SPSS statistics (Version 25, IBM).

3.0 Results

3.1 Identification of 36 bacterial isolates by sequencing of 16S rRNA

Positive DNA bands for the 16S rRNA gene of all 36 isolates were detected right above the 1000 bp mark of the DNA ladder. A total of 33 out of 36 bacterial isolates were successfully identified from the sequencing results.

The majority of these presumptive *Pseudomonas* isolates were confirmed as *Pseudomonas* and accounted for 70% of the isolates. The remaining 30% belonged to six different genera (Figure 9). A complete overview of the sequencing results, including the identification accuracy is presented in Appendix C.

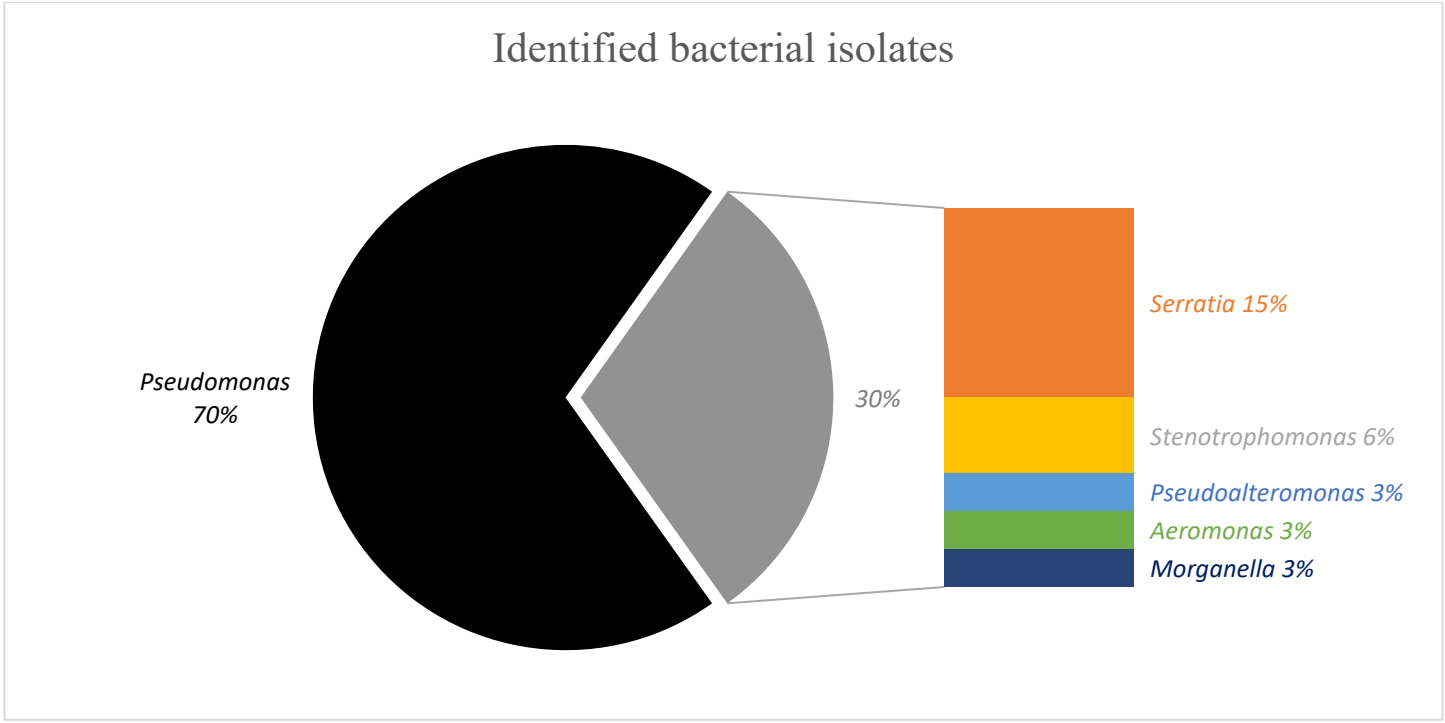


Figure 9: Overview of the successfully identified bacterial isolates.

3.2 Genotypic detection of antimicrobial resistance

3.2.1 Establishment of PCR assays and detection of ARGs among reference strains

Different reference strains were tested for the presence of resistance genes in order to use them as positive controls in the subsequent PCR assays with the bacterial isolates from the salmon processing plant. The PCR and gel electrophoresis, confirmed the presence of amplicons in 12 out of 103 PCR reactions. The amplicons accounted for 8 different ARGs among 6 reference strains (Table 10). The detected ARG-profiles in the reference strains were matching with those described in Table 2, Section 2.1.1.

Table 10: Reference strains for 8 different ARGs were confirmed by PCR and gel electrophoresis.

Target Gene	Amplicon size	Reference strain
qacH	366 bp	*Listeria monocytogenes* **MF 5634**
bcrABC	1312 bp	*Listeria monocytogenes* **MF 4624**
ampC	189 bp	*Escherichia coli* **CCUG 17620**
tetG	468 bp	*Pseudomonas aeruginosa* **NCTC 13717** *Pseudomonas aeruginosa* **CCUG 59347**
floR	120 bp	*Acinetobacter baumannii* **NCTC 13305**
floR2	404 bp	*Pseudomonas aeruginosa* **NCTC 13717** *Pseudomonas aeruginosa* **CCUG 59347**
sulI	433 bp	*Pseudomonas aeruginosa* **NCTC 13717** *Pseudomonas aeruginosa* **CCUG 59347**
qacEΔ1	115 bp	*Pseudomonas aeruginosa* **NCTC 13717** *Pseudomonas aeruginosa* **CCUG 59347**

Reference strains for *tetA, tetC, bla*$_{TEM-1}$*, optrA, dfrA17, cfr, fexA, fexB* and *pexA* were not detected and PCR assays could not be established. These ARGs were therefore not included in any further analysis. Faint amplicons were detected for *bla*$_{TEM-1}$ and *optrA*, and a gradient PCR was conducted. However, none of the eight different annealing temperatures resulted in clear visible amplicons for either *bla*$_{TEM-1}$ or *optrA*.

3.2.2 Detection of 7 ARGs among 162 bacterial isolates

Amplicons of correct sizes were detected for all reference strains included in the different PCR assays (*qacH, qacEΔ1, bcrABC, sulI, tetG, ampC* and *floR*), and all negative controls were blank.

Faint amplicons were detected for 8 bacterial isolates in the PCR assay for *bcrABC* (Appendix D). The size of the amplicons was consistently around 1600 bp. The remaining 154 isolates tested negative towards *bcrABC.* The PCR assay for *qacEΔ1*, resulted likewise in the detection of faint amplicons, for a total of 23 bacterial isolates. However, the amplicons were of different sizes, mostly around the 1000 bp mark.

Furthermore, faint amplicons of different sizes around 700 bp were detected for a number of 17 bacterial isolates in the PCR for *sulI*. The PCR for *floR* resulted in clear visible amplicons for the isolate *P. pseudoalcaligene* (LJP 312) and *A. hydrophila* (LJP 328) with sizes of approximately 400 bp and 1000 bp, respectively. Additionally, faint amplicons were detected for 27 bacterial isolates, with varying sizes around 1000 bp.

The PCR assays for *qacH, tetG* and *ampC* did not result in the detection of amplicons for any of the 162 bacterial isolates. A complete overview of the results for the detected ARGs among the bacterial isolates, is presented in cf. Appendix E.

Verification of the detected amplicons by sequencing

Sequences were obtained from 4 *floR* amplicons. Only one sequence resulted in a BLAST-hit for the *floR* gene, henceforth in the reference strain *Acinetobacter baumannii* NCTC 13305 (Table 11). The remaining 3 sequences were not matching with *floR*, however the sequences were matching with the identity of the analyzed bacterial species.

A total of 18 amplicons were not successfully sequenced (9 amplicons for *bcrABC*, 5 for *sulI* and 4 for *floR*).

Table 11: One amplicon resulted in a BLAST-hit for floR.

Target gene	Size (bp)	Primer pair	Primer ref.	Bacterial isolate/strain	BLAST-hit	Seq. length	Max Score	Query Cov.	E value	Per. Id.
floR	*120*	f-5-GCTT TAGC GCCGG TATGG-3 r-5-GAC AGTGG CGAAG GCAAAG -3	*Zhao et al. (2016)*	*Acinetobacter baumannii* **NCTC 13305**	Acinetobacter baumannii strain RCH52 chloramphenicol and florfenicol resistance protein (floR)	71bp	116	90.00%	1e-22	100.00%
				Escherichia coli **CCUG 17620**	Escherichia coli strain ATCC 25922 chromosome, complete genome	293bp	503	96.00%	5e-138	99.65%
				Pseudomonas aeruginosa **CCUG 59347**	Pseudomonas aeruginosa strain PAC1 chromosome, complete genome	353 bp	627	99.00%	2e-175	99.71%
				Aeromonas hydrophila **LJP 328**	Aeromonas hydrophila strain 3206 chromosome, complete genome	638bp	966	98.00%	0.0	94.26%

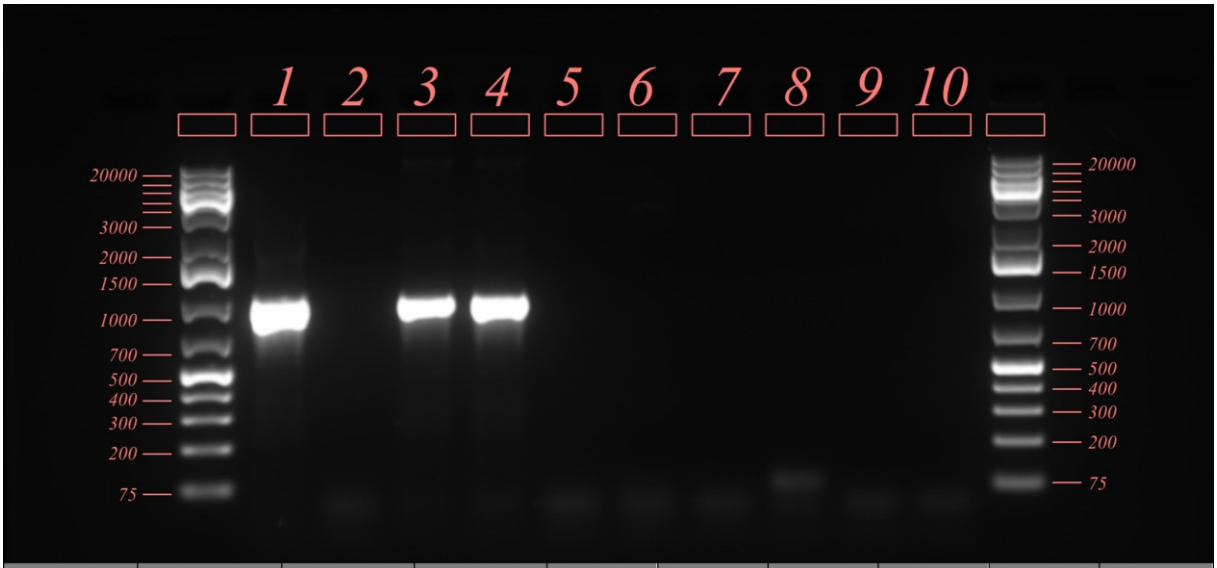
3.2.3 Functionality of the new *floR* and *floR2* primer pairs

On the basis of unsatisfactory PCR and sequencing results with *floR* primer pairs developed by Zhao et al. (2016) new *floR/floR2* primers were designed.

The functionality of the designed primer pair floTHF/floTHR (*floR*) and flo2THF/flo2THR (*floR2*) was successfully verified by PCR and gel electrophoresis with the corresponding positive control strains. A total of 5 preliminary *floR* positive bacterial isolates (faint amplicons) were included in the reaction. All of the 5 isolates tested negative with the new primer pairs (Figure 10 and 11).

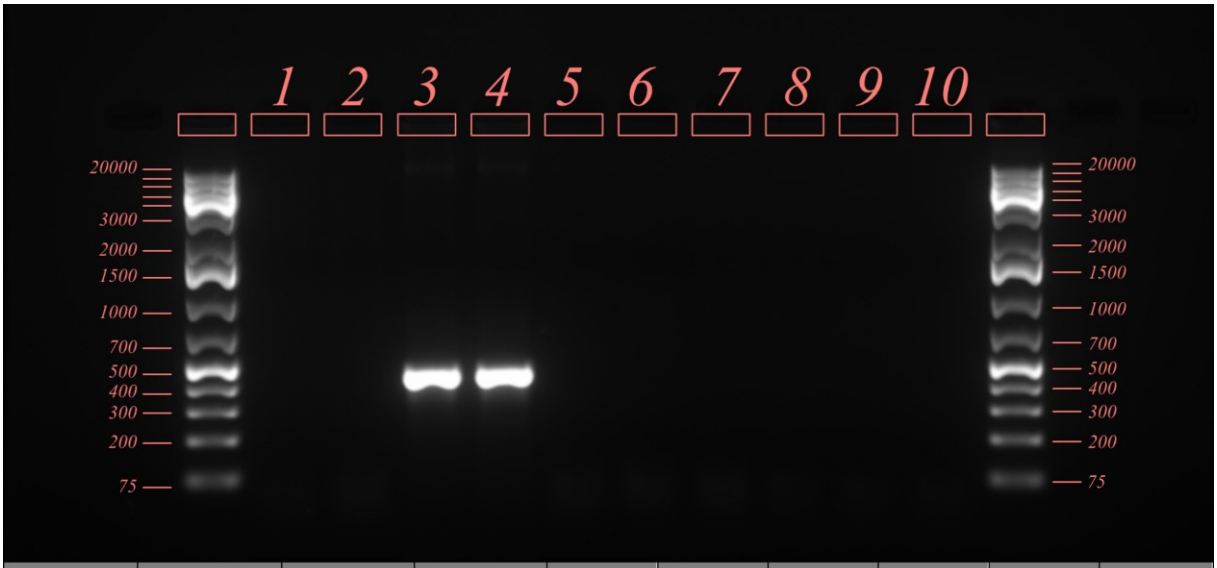
In the PCR assay with the new *floR* primer pair, clear amplicons were detected for both *A. baumannii* (NCTC 13305) and both of the *P. aeruginosa* strains (CCUG 59347, NCTC 13717). The amplicons were of consistent sizes around 1000 bp. The results for the *floR2* primer pair showed amplicons (~500 bp) for both of the *P. aeruginosa* strains, however *A. baumannii* tested negative.

The PCR with the *floR* primers by Faldynova et al. (2003) and Zhang et al. (2009) resulted in the detection of clear amplicons for *A. baumannii*. Both of the *P. aeruginosa* strains tested negative.



1	2	3	4	5	6	7	8	9	10
Acinetobacter Baumannii NCTC 13305	*Escherichia coli CCUG 17620*	*Pseudomonas aeruginosa CCUG 59347*	*Pseudomonas aeruginosa NCTC 13717*	*LJP 312*	*LJP 328*	*LJP 343*	*LJP 344*	*LJP 347*	*Control sample*

Figure 10: Gel electrophoresis image from PCR-reactions with the new floTHF/floTHR (floR) primer pair. The detected DNA-bands were approximately 1000 bp long.



1	2	3	4	5	6	7	8	9	10
Acinetobacter Baumannii NCTC 13305	*Escherichia coli CCUG 17620*	*Pseudomonas aeruginosa CCUG 59347*	*Pseudomonas aeruginosa NCTC 13717*	*LJP 312*	*LJP 328*	*LJP 343*	*LJP 344*	*LJP 347*	*Control sample*

Figure 11: Gel electrophoresis image from PCR-reactions with the new flo2THF/flo2THR (floR2) primer pair. The detected DNA-bands were approximately 500 bp long.

Verification of floR/floR2 amplicons by sequencing

All of the 7 PCR amplicons from the PCR reactions analyzed with different *floR/floR2* primers were successfully sequenced with the forward and reverse primer (Table 12). Moreover, all of the obtained sequences were matching with the corresponding *floR* and *floR2* gene.

Table 12: Sequencing results for reference strains analyzed with different floR primer pairs.

Target gene	Primer ref.	Size (bp)	Reference strain	Primer	BLAST-hit	Seq. length	Max Score	Query Cov.	E value	Per. Id.
floR	*Zhang et al. (2009)*	678	*Acinetobacter baumannii* **NCTC 13305**	f: 5-GCGATATT CATTACTTTGGC-3	Acinetobacter baumannii strain RCH52 chloramphenicol and florfenicol resistance protein (floR) gene	646bp	1171	98%	0.0	99.84%
				r: 5-ATCGGTAGG ATGAAGGTGAGG A-3		639bp	1158	99%	0.0	99.53%
	Faldynova et al. (2003)	426	*Acinetobacter baumannii* **NCTC 13305**	f: 5-GCGATATT CATTACTTTGGC-3	Acinetobacter baumannii strain RCH52 chloramphenicol and florfenicol resistance protein (floR) gene	394bp	710	98%	0.0	99.49%
				r: 5-TAGGATGA AGGTGAGGAATG-3		167bp	309	100%	3e-80	100.00%
	This study	963	*Acinetobacter baumannii* **NCTC 13305**	f: 5-TCGCCCGG TATTCCTTAATCG-3	Acinetobacter baumannii strain RCH52 chloramphenicol and florfenicol resistance protein (floR) gene	916bp	1668	99%	0.0	100.00%
				r: 5-TGAAGGTG AGGAATGACGGC-3		918bp	1661	99%	0.0	99.45%
			Pseudomonas aeruginosa **CCUG 59347**	f: 5-TCGCCCGGT ATTCCTTAATCG-3	Salmonella enterica subsp. enterica SRC19 floR2 gene for chloramphenicol/florfenicol efflux MFS transporter FloR2	927bp	1676	97%	0.0	100.00%
				r: 5-TGAAGGTGA GGAATGACGGC-3		926bp	1677	98%	0.0	99.78%
			Pseudomonas aeruginosa **NCTC 13717**	f: 5-TCGCCCGG TATTCCTTAATCG-3	Salmonella enterica subsp. enterica SRC19 floR2 gene for chloramphenicol/florfenicol efflux MFS transporter FloR2	923bp	1679	98%	0.0	100.00%
				r: 5-TGAAGGTG AGGAATGACGGC-3		927bp	1674	98%	0.0	99.56%
floR2	*This study*	456	*Pseudomonas aeruginosa* **CCUG 59347**	f: 5-GCCTTTGTT GCGTTTCGTCT-3	Salmonella enterica subsp. enterica SRC19 floR2 gene for chloramphenicol/florfenicol efflux MFS transporter FloR2	416bp	763	100%	0.0	99.76%
				r: 5-CGCGAAGGC CAAGCTAAATC-3		412bp	752	100%	0.0	99.52%
			Pseudomonas aeruginosa **NCTC 13717**	f: 5-GCCTTTGTT GCGTTTCGTCT-3	Salmonella enterica subsp. enterica SRC19 floR2 gene for chloramphenicol/florfenicol efflux MFS transporter FloR2	416bp	763	100%	0.0	99.76%
				r: 5-CGCGAAGGCC AAGCTAAATC-3		433bp	763	97%	0.0	99.29%

3.2.4 Detection of *floR* among 162 bacterial isolates using primers from this study

Faint amplicons were detected for 2 bacterial isolates (LJP 040 and 027) right above the 1000 bp mark of the DNA ladder. Additional 12 faint amplicons of consistent size (500 bp) were detected (cf. Appendix E). Isolates LJP 040 and 027 were subjected to a PCR assay with *floR* primers by Zhang et al. (2009) and *floR2* primers (from this study), however both tested negative.

3.3 Phenotypic testing of antibiotic resistance

3.3.1 Disk diffusion test results of 6 reference strains

The disk diffusion test was performed on 6 reference stains and 62 selected bacterial isolates. The quality control strain (*E.coli* CCUG 17620) performed successfully, the zone diameter of the quality control strain was within the recommended limits (Table 13). Reference strains belonging to *L. monocytogenes* species (MF 4624 and MF 5634) were susceptible towards all antibiotics (florfenicol, ampicillin and tetracycline). The test results for the remaining reference strains are presented in cf. Appendix E.

Table 13: Measured diameter and reference diameter of quality control strain.

Reference strain	Zone Diameter (mm)					
	Florfenicol 30.0 µg		Ampicillin 10.0 µg		Tetracycline 30.0 µg	
	Measured	Reference	Measured	Reference	Measured	Reference
Escherichia coli CCUG 17620	24 mm	22-28 mm (Oxoid Ltd., 2020)	18 mm	16-22 mm (CLSI, 2012b)	24 mm	18-25 mm (CLSI, 2012b)

3.3.2 Disk diffusion test results of 62 bacterial isolates

A high prevalence of ampicillin resistance (79%) was found among the selected bacterial isolates (Figure 12). Florfenicol resistance was observed in 66% of the isolates. None of the isolates were resistant against tetracycline, however one isolate was categorized as intermediate. The specific zone diameters of the isolates are presented in cf. Appendix E.

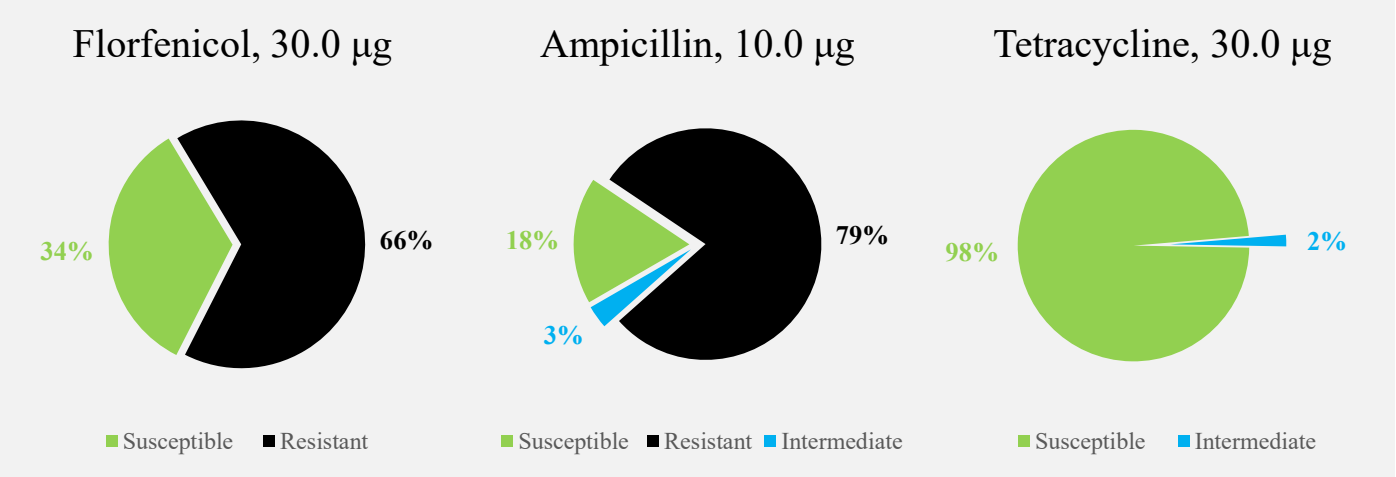


Figure 12: Antimicrobial susceptibility patterns of 62 bacterial isolates.

The percentual prevalence of resistance was considerably higher among *Pseudomonas* isolates (n=50) compared to bacterial isolates (n=12) belonging to other species. A total of 88% and 82% of these *Pseudomonas* isolates were resistant against ampicillin and florfenicol, respectively. The remaining bacterial isolates showed 42% and 0% resistance against ampicillin and florfenicol, respectively.

3.4 Screening of in vitro biofilm formation capabilities among bacterial isolates

At the outset of the biofilm screening it was investigated if the optical density measurements of the 96 well plates were affected by the well plate lids. The intra-assay coefficient of variation (n=22) of well plate measurements with lid was higher (11.0%) compared to measurements without lid (3.2%), as shown in Figure 13. Consequently, all well plates were measured without lids in the whole experiment.

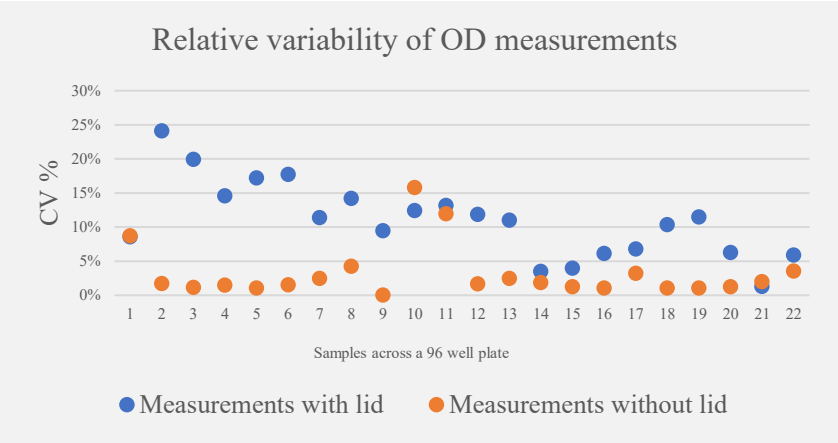


Figure 13: The CVs of measurements with well plate lid were generally >10%, contra <4% without lid.

The mean inoculum concentration of the selected 38 bacterial isolates and 6 reference strains for the experiment was 6.4 ± 0.56 log CFU/mL. A total of 16 out of 38 bacterial isolates showed high capabilities for biofilm formation, 2 showed medium capabilities and 8 low capabilities (Figure 14). A total of 88% of isolates with high biofilm formation capabilities belonged to *Pseudomonas* species. Isolates with low capabilities were predominantly other species, and *Pseudomonas* spp. accounted only for 25%. The results of the remaining 12 bacterial isolates and 6 reference strains were found to be below the limit of detection (LoD).

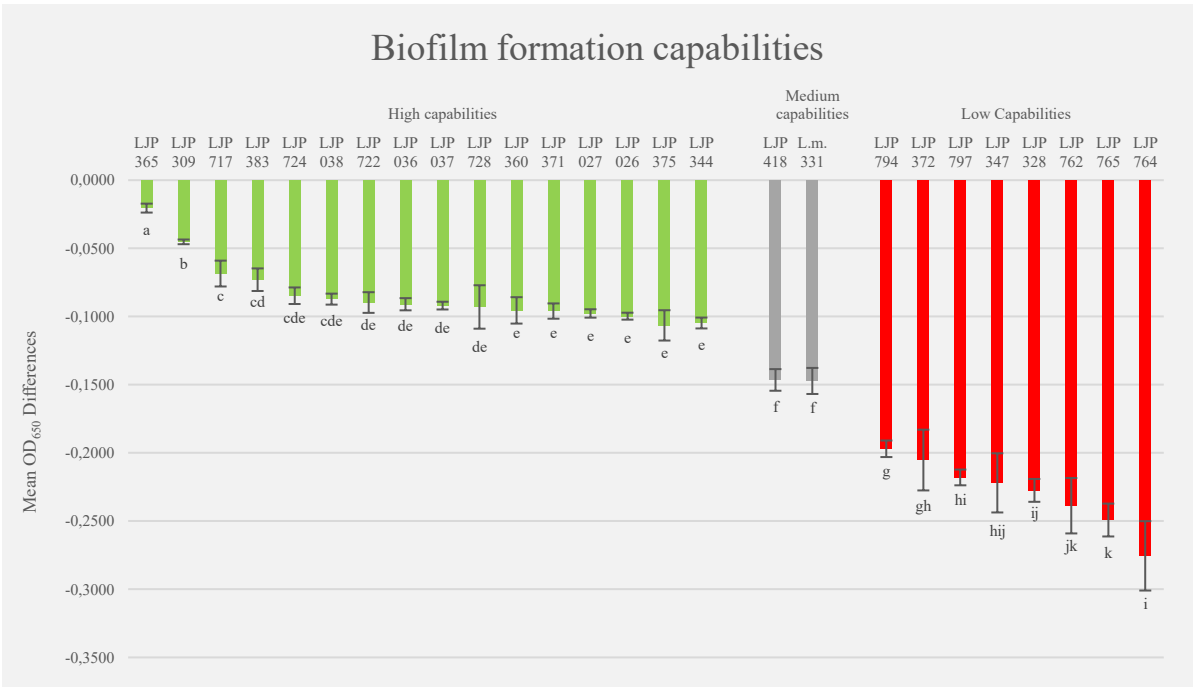


Figure 14: Biofilm formation capabilities (high, medium and low). Sample bars marked with different letters (a, b, c etc.) are significantly different based on Duncan's multiple range test (p<0.05).

The viable cell count (VCC) was determined for 5 bacterial isolates and compared to OD values from the experiment (Figure 15). The statistical analysis showed no significant difference ($p<0.05$) in the mean log (CFU/mL) difference between isolates LJP 722, LJP 371, LJP 347 and LJP 764. However, isolate *L. monocytogenes* (L.m. 331) was significantly different ($p<0.05$) from the other isolates.

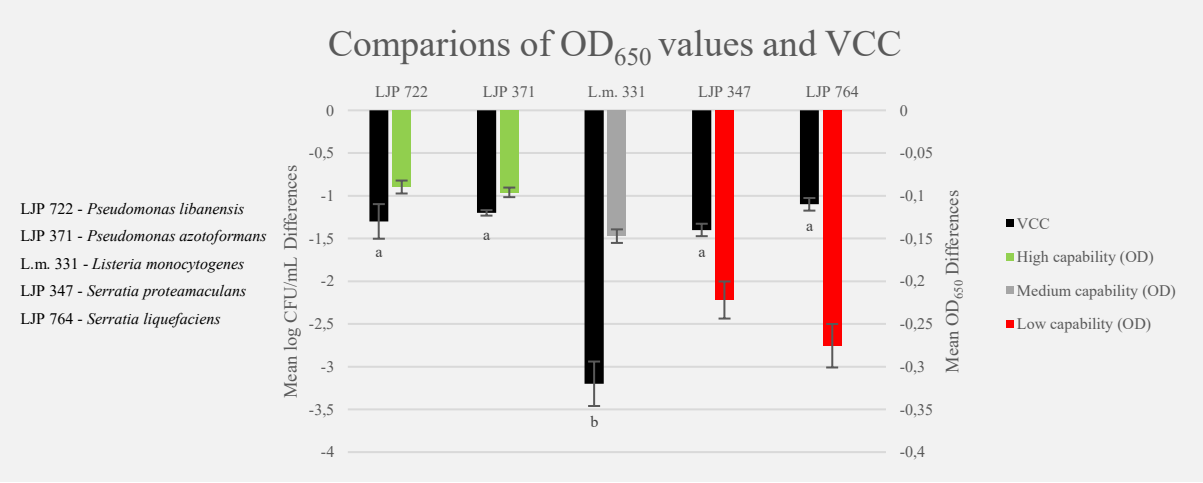


Figure 15: VCC and OD values. Sample bars marked with different letters are significantly different ($p<0.05$).

3.5 In vitro susceptibility test of bacterial isolates in peg lid biofilm reactors

Bacterial isolates and reference strains were selected for the experiment on the basis of previous test results (Table 14). Most of the selected isolates were resistant against florfenicol and ampicillin, showed high or medium biofilm formation capabilities and harbored possible ARGs (faint amplicons). *L. monocytogenes* MF 4624 was included since the strain was clearly positive for *bcrABC* (Møretrø et al., 2017b), associated with tolerance and resistance towards benzalkonium chloride.

Table 14: A total of 7 isolates were selected for the susceptibility test. Results for the ARG screening marked with green represent positive strains, i.e. detection of clear DNA bands. Yellow cells represent the detection of faint amplicons. Red cells represent negative test results.

Isolate ID	Bacterial species	Biofilm formation	Disk Diffusion			ARG screening							
		OD_{650} difference	FFC 30.0 µg	AMP 10.0 µg	TET 30.0 µg	*floR/ floR2	**floR/ floR2	sulI	tetG	ampC	qacH	qacE-Δ1	bcr-ABC
LJP 042	*Pseudomonas fluorescens strain W-6*	-0,080	R	R	S								
LJP 316	*Pseudomonas fluorescens*, strain: ATCC 17573	-0,100	R	R	S								
LJP 321	*Pseudomonas fluorescens* strain: ATCC 17573	-0,101	R	R	S								
LJP 760	*Pseudomonas putida* strain CFBP 5933	-0,081	S	R	S								
Ref.	*Listeria monocytogenes* MF 4624	Below LoD	S	S	S	n.d.	n.d.		n.d.	n.d.			
LJP 040	*Pseudomonas fluorescens* strain PF59	-0,071	R	R	S								
LJP 788	*Pseudomonas lundensis*	-0,185	S	S	S								

** PCR assay with floR primers by Zhao et al. (2016)*
*** PCR assay with floR primers from this study*

Complications with the ultrasonic water bath used for sonication at the laboratory compromised several experiments, worth 2-3 weeks of laboratorial work. The quality of the analysis was deemed insufficient for these experiments and results are thus not shown in this study. However, the experiments were successfully repeated.

The concentrations of the inoculum used in the different susceptibly tests were approximately around ~7 log CFU/mL. However, the inoculum concentration for the test with Aqua Des Foam PAA was 6.73 ± 0.47 log CFU/mL and significantly lower ($p<0.05$) than the inoculum concentration for Suma Bac D10 (7.03 ± 0.33 log CFU/mL) and florfenicol (6.98 ± 0.32 log CFU/mL). There was no significant difference ($p<0.05$) between the inoculum concentration for the test with Suma Bac D10 and florfenicol.

3.5.1 Aqua Des Foam PAA - effect of cumulative concentrations on isolates in planktonic and biofilm state

The Aqua Des Foam PAA MIC values (Figure 16) of the isolates varied between 0.25% and 2.0% with an exposure time of 15 minutes at 12 °C. Isolate LJP 042 exhibited a high MIC value of 2.0%, however with a high standard deviation. The remaining 5 isolates showed MIC values of 0.25%. Isolate MF 4624 did not have sufficient growth.

The VCC (Table 15) of isolate LJP 042 did not correspond with the spectrophotometrically measured MIC and showed a MIC value of 1.0%. Additionally, the VCC of LJP 040 showed an identical MIC value of 1.0%.

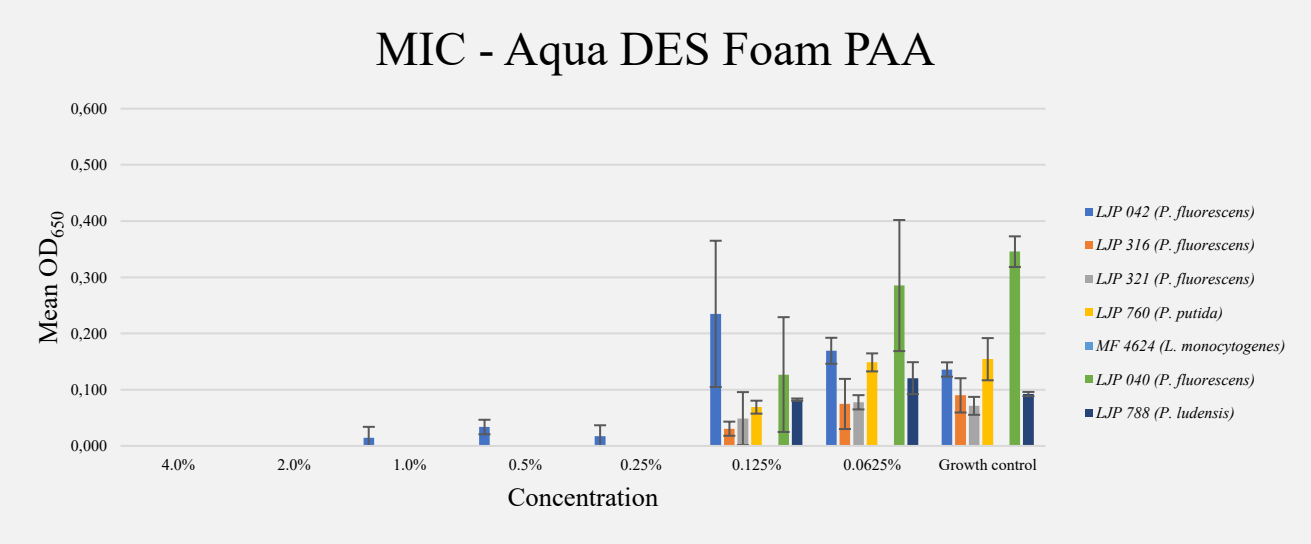


Figure 16: Optical density measurements of MIC values for Aqua DES Foam PAA.

Table 15: Heatmap for the MIC-check by viable cell count (log CFU/mL). Color intensity increases with cell concentration

Isolate	Concentrations of Aqua Des Foam PAA							
	4.0%	2.0%	1.0%	0.5%	0.25%	0.125%	0.0625%	Growth control
LJP 042	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	6.0 ± 0.09	7.3 ± 0.09	8.1 ± 0.09	9.5 ± 0.11	9.5 ± 0.04
LJP 040	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.8 ± 1.73	6.3 ± 0.12	8.0 ± 0.13	8.3 ± 0.03	9.0 ± 0.15

The MBC values of all bacterial isolates were lower than the recommended user concentration of 1.0%, and most isolates exhibited MBC values lower than 0.5% (Figure 17). The MBEC values (Figure 18) were notably higher compared to MIC and MBC values. A total of 4 isolates showed MBEC values of 2.0%, while 2 isolates showed values of 0.5%.

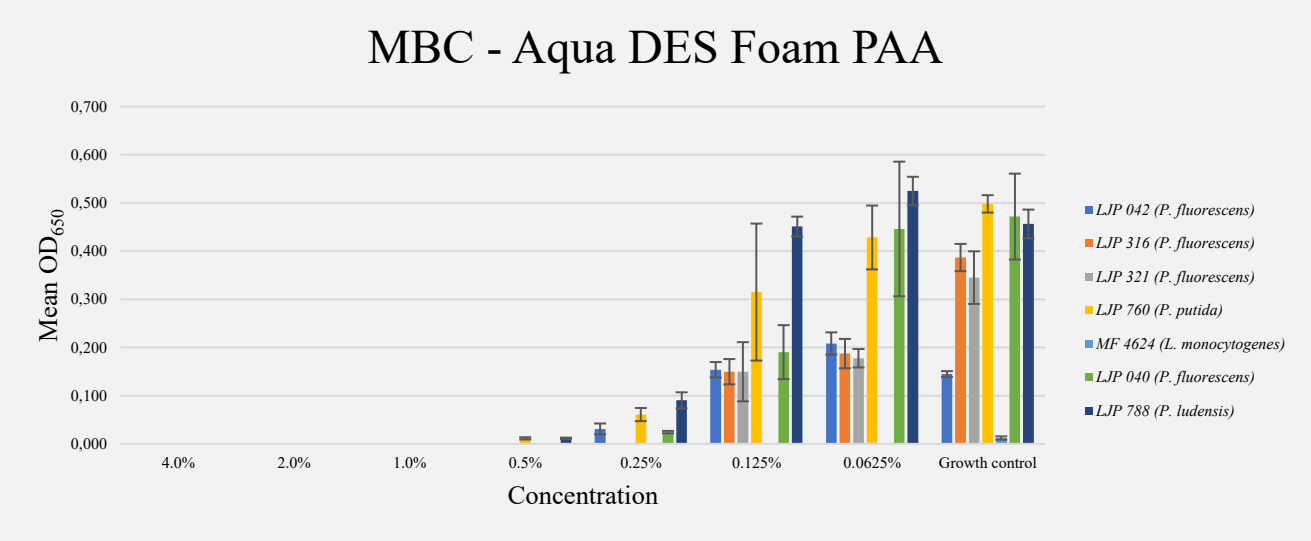


Figure 17: Optical density measurements of MBC values for Aqua DES Foam PAA.

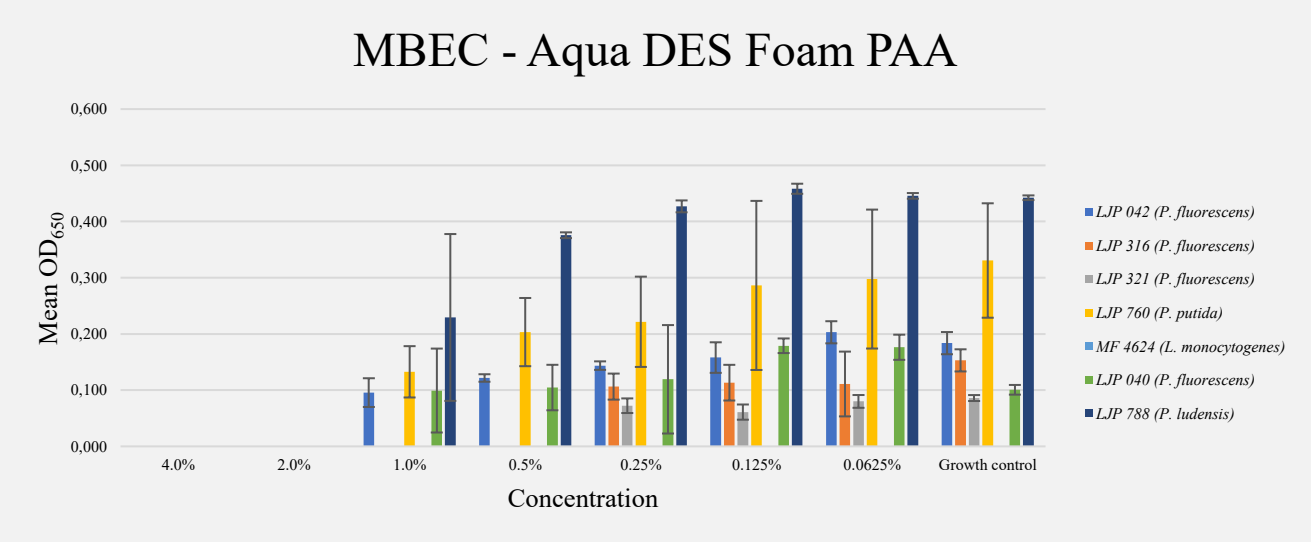


Figure 18: Optical density measurements of MBEC values for Aqua DES Foam PAA.

The mean log kill results of Aqua DES Foam PAA (Figure 19) showed less than 1.0 log CFU/mL reduction for 5 isolates exposed to the user concentration (1.0%) of the disinfectant. The mean log kill of 6 isolates was significantly higher ($p<0.05$) at 2.0% compared to the user concentration of 1.0%. At the concentration of 2.0%, 4 isolates showed a log reduction between 2.5 – 4.0 log CFU/mL, while 3 isolates were completely inactivated (6.0 – 7.0 log CFU/mL reduction). There was no significant difference ($p<0.05$) in the mean log kill between 1.0% and 2.0% for isolate LJP 316. At the concentration of 4.0% only one isolate survived, however, with a log reduction of approximately 7.0 CFU/mL and a high standard deviation. The remaining 6 isolates were completely inactivated at 4.0%.

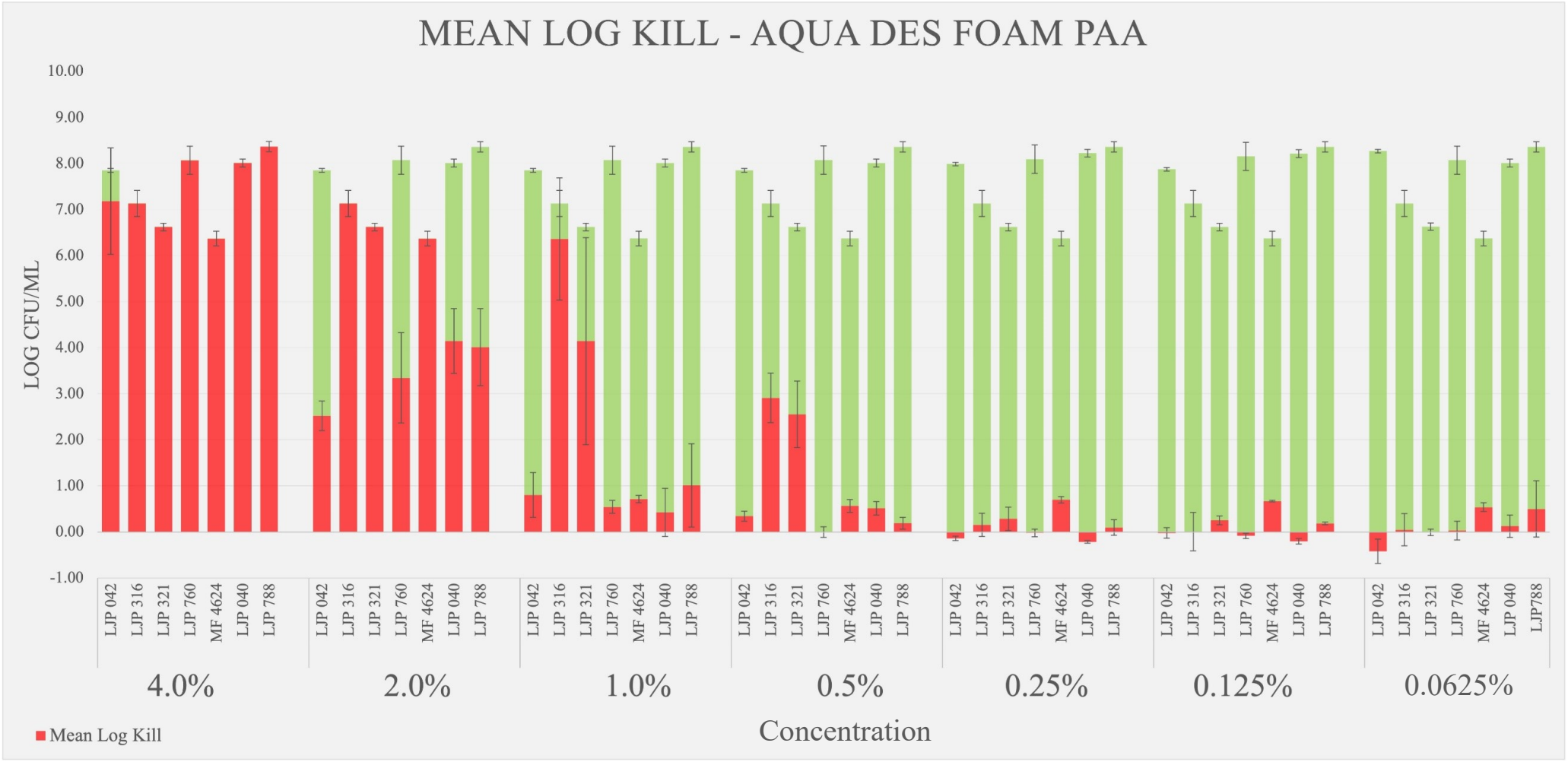


Figure 19: Mean log kill of Aqua DES Foam PAA (VCC). Green pillars are showing the growth of each isolate exposed to different concentrations of the disinfectant. The log reduction of each isolates is shown in red.

3.5.2 Suma Bac D10 - effect of cumulative concentrations on isolates in planktonic and biofilm state

The OD measurements for determining MIC and MBC values were affected by the strong visible color of Suma Bac D10. The collected data was incoherent and is therefore not shown. The VCC of isolates LJP 042 and LJP 040 showed respective MIC values of 0.125% and 0.25% (Figure 20) with an exposure time towards Suma Bac D10 of 15 minutes at 12 °C. The results for MBEC (Figure 21) showed that most of the isolates were inactivated at the lowest concentration (0.0625%) of the disinfectant. However, isolates LJP 042 and LJP 040 exhibited MBEC values of 2.0% and 0.25%, respectively.

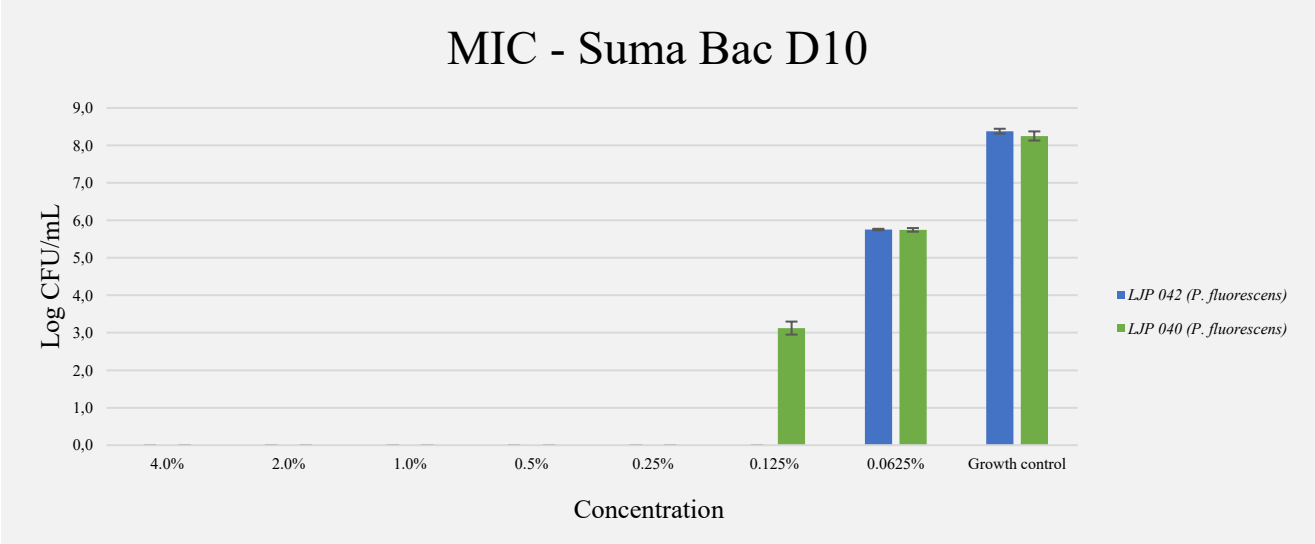


Figure 20: MIC-check by viable cell count (log CFU/mL).

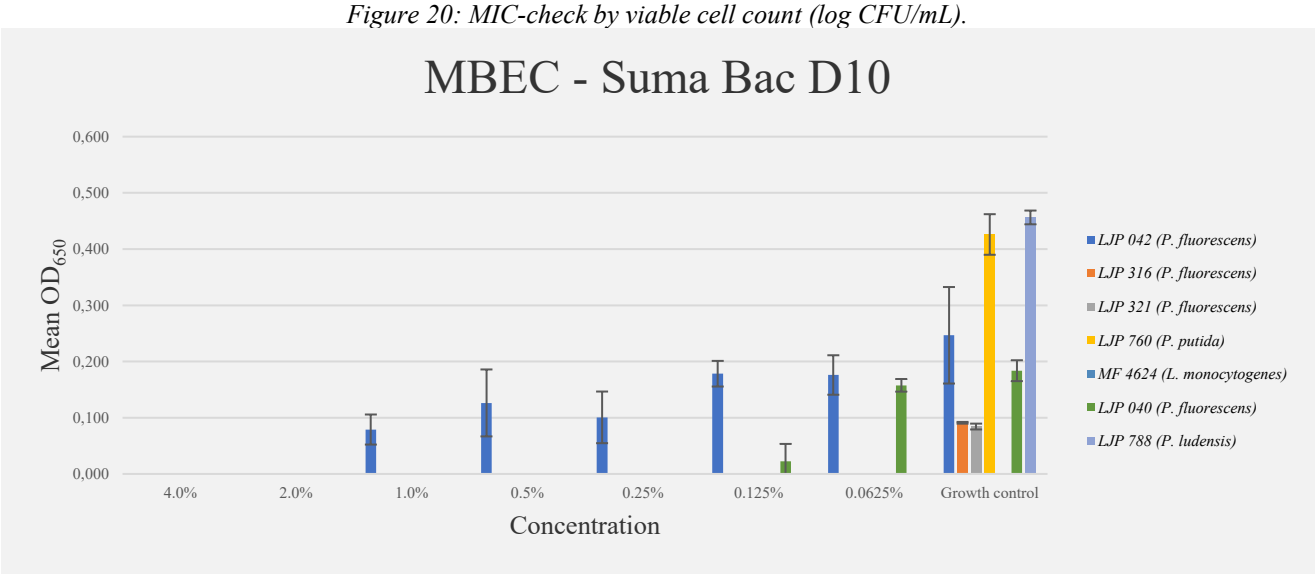


Figure 21: Optical density measurements of MBEC values for Suma Bac D10.

The mean log kill results of Suma Bac D10 (Figure 22) showed complete inactivation for 5 isolates at a concentration of 0.25% (6-8 log CFU/mL reduction). Isolates LJP 042 and LJP 040 survived the user concentration (1.0%), however with a log reduction of approximately 4.5 CFU/mL. All of the isolates were completely inactivated at 2.0%. At user concentration (1.0%), the mean log kill of 5 isolates was significantly higher ($p<0.05$) by Suma Bac D10 compared to the mean log kill by Aqua DES Foam PAA of the same isolates. There was no significant difference ($p<0.05$) in the mean log kill between Suma Bac D10 and Aqua DES Foam PAA for isolates LJP 316 and LJP 321 at user concentration.

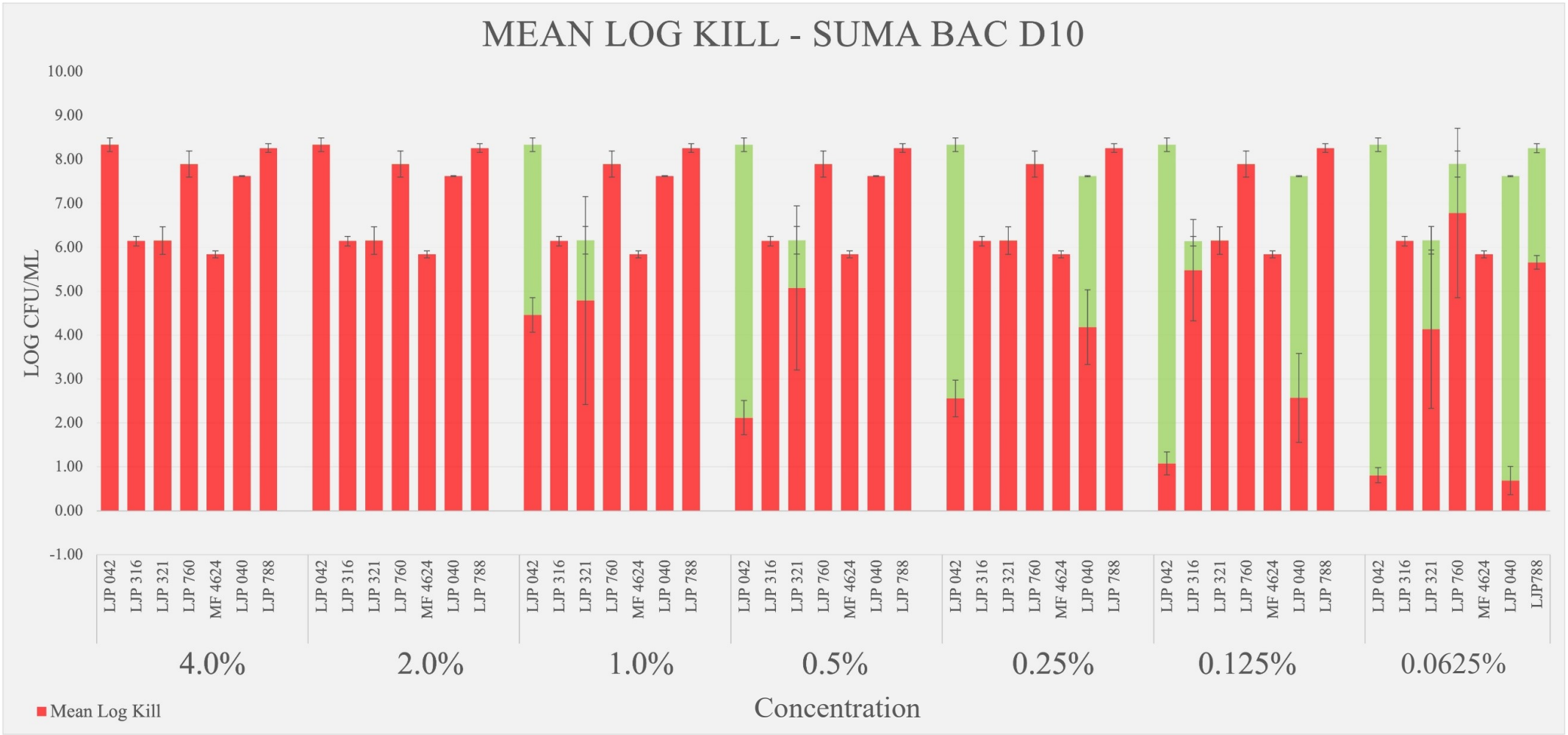


Figure 22: Mean log kill of Suma Bac D10 (VCC). Green pillars are showing the growth of each isolate exposed to different concentrations of the disinfectant. The log reduction of each isolates is shown in red.

3.5.3 Florfenicol - effect of cumulative concentrations on isolates in planktonic and biofilm state

The florfenicol MIC values of the isolates varied between approximately 300.00 and >2400.00 µg/mL (Figure 23) with an exposure time of 24 hours at 12 °C. Isolate LJP 042 exhibited the highest MIC value of >2400.00 µg/mL, while LJP 040 showed a MIC value of 2400.00 µg/mL. A total of 3 isolates showed MIC values of 300.00 µg/mL, isolate MF 4624 did not have sufficient growth.

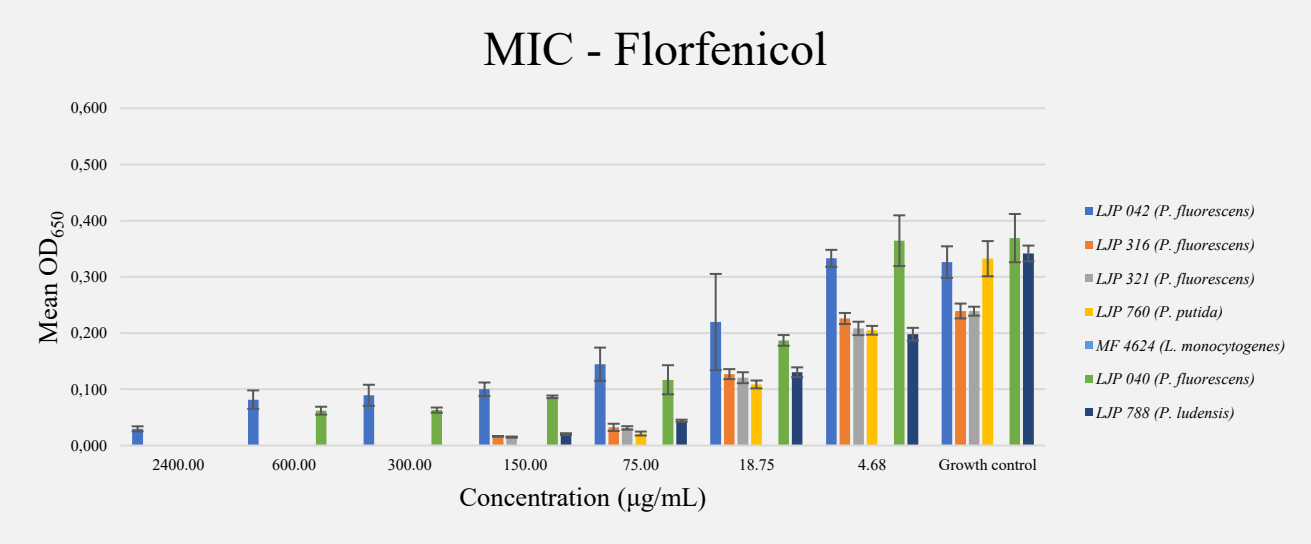


Figure 23: Optical density measurements of MIC values for florfenicol.

The results for the MIC-check by VCC of isolates LJP 042 and LJP 040 (Table 16) showed that both isolates survived the highest florfenicol concentration of 2400.00 µg/mL.

Table 16: Heatmap for the MIC-check by viable cell count (log CFU/mL). Color intensity increases with cell concentration.

Isolate	Concentrations of florfenicol							
	2400.00 µg/mL	**600.00 µg/mL**	**300.00 µg/mL**	**150.00 µg/mL**	**75.00 µg/mL**	**18.75 µg/mL**	**4.68 µg/mL**	**Growth control**
LJP 042	7.3 ± 0.07	8.4 ± 0.09	8.3 ± 0.05	8.4 ± 0.06	8.5 ± 0.11	9.0 ± 0.23	9.0 ± 0.02	9.1 ± 0.05
LJP 040	4.8 ± 0.06	5.3 ± 0.06	6.6 ± 0.05	8.2 ± 0.09	8.6 ± 0.03	9.1 ± 0.13	9.4 ± 0.02	9.3 ± 0.3

The florfenicol MBC values (Figure 24) for the isolates where slightly lower than the MIC values. Isolate LJP 042 exhibited a MBC of 600.00 µg/mL florfenicol. A total of 4 isolates showed MBC values of 300.00 µg/mL, while 1 isolate hade a MBC of 150.00 µg/mL florfenicol.

A total of 3 isolates showed florfenicol MBEC values >2400.00 µg/mL florfenicol (Figure 25). Further, 2 and 1 isolate showed MBEC values of 2400.00 µg/mL and 600.00 µg/mL, respectively.

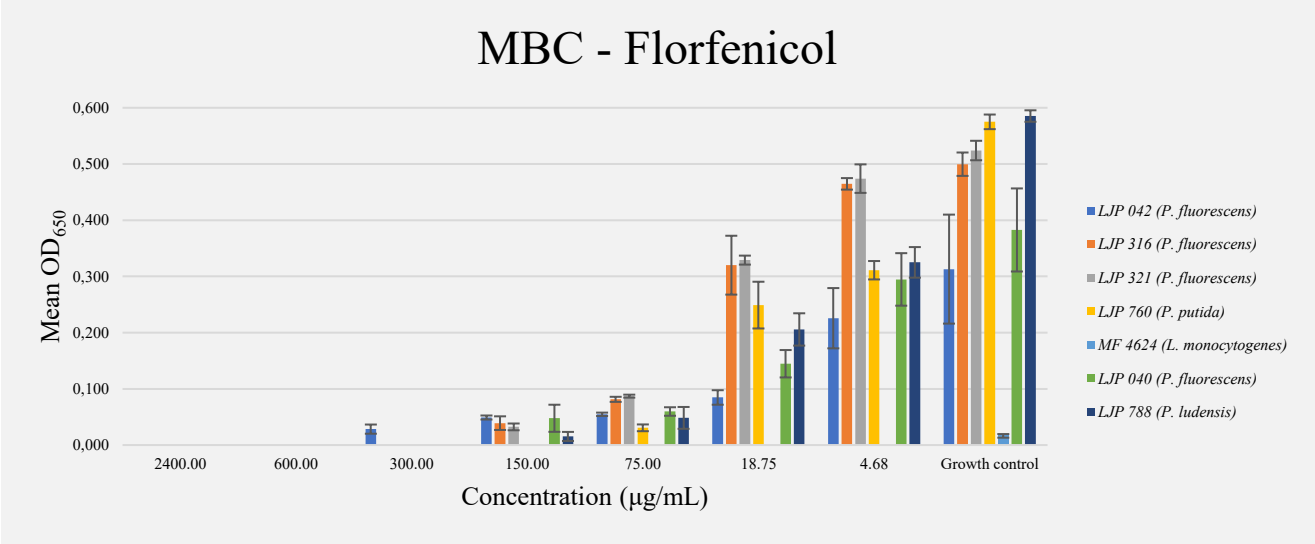


Figure 24: Optical density measurements of MBC values for florfenicol.

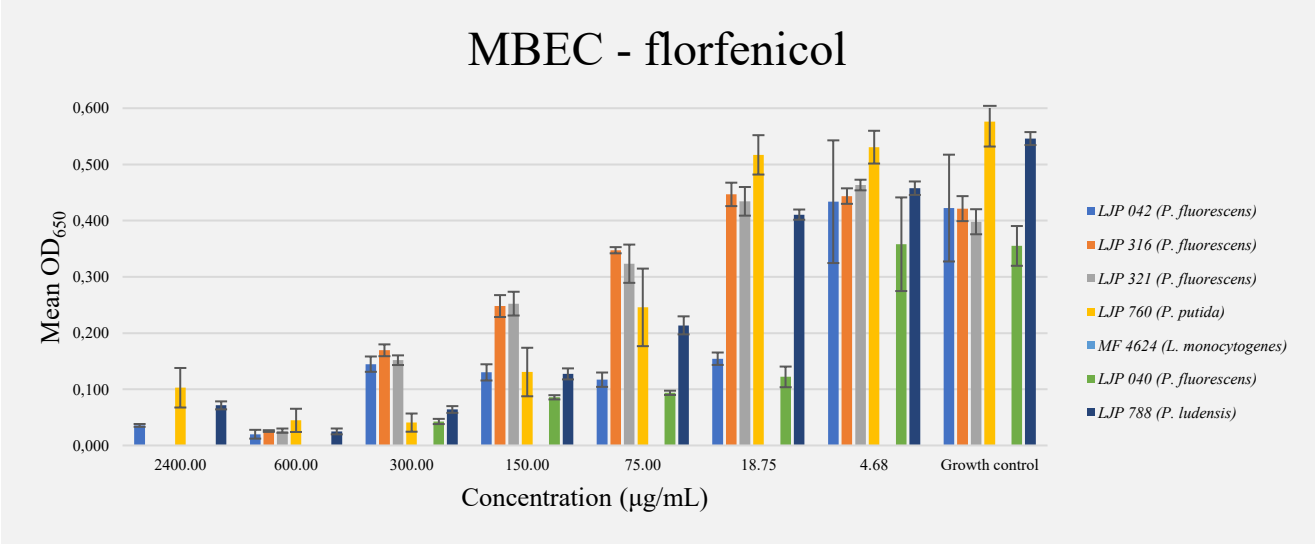


Figure 25: Optical density measurements of MBEC values for florfenicol.

The mean log kill results of florfenicol (Figure 26) showed less than 2.0 log (CFU/mL) reduction for all isolates at the highest concentration of florfenicol (2400.00 µg/mL). Overall, florfenicol appeared to have a low impact on survival of the isolates tested. However, it was observed that florfenicol affected colony sizes. For example, *P. ludensis* (LJP 788) showed approximately the same VCC after being exposed to 2400.00 µg/mL and 75.00 µg/mL florfenicol. However the parallels exposed to high concentrations of florfenicol had distinctively smaller colonies (Figure 27). The differences in colony size were observed for all isolates.

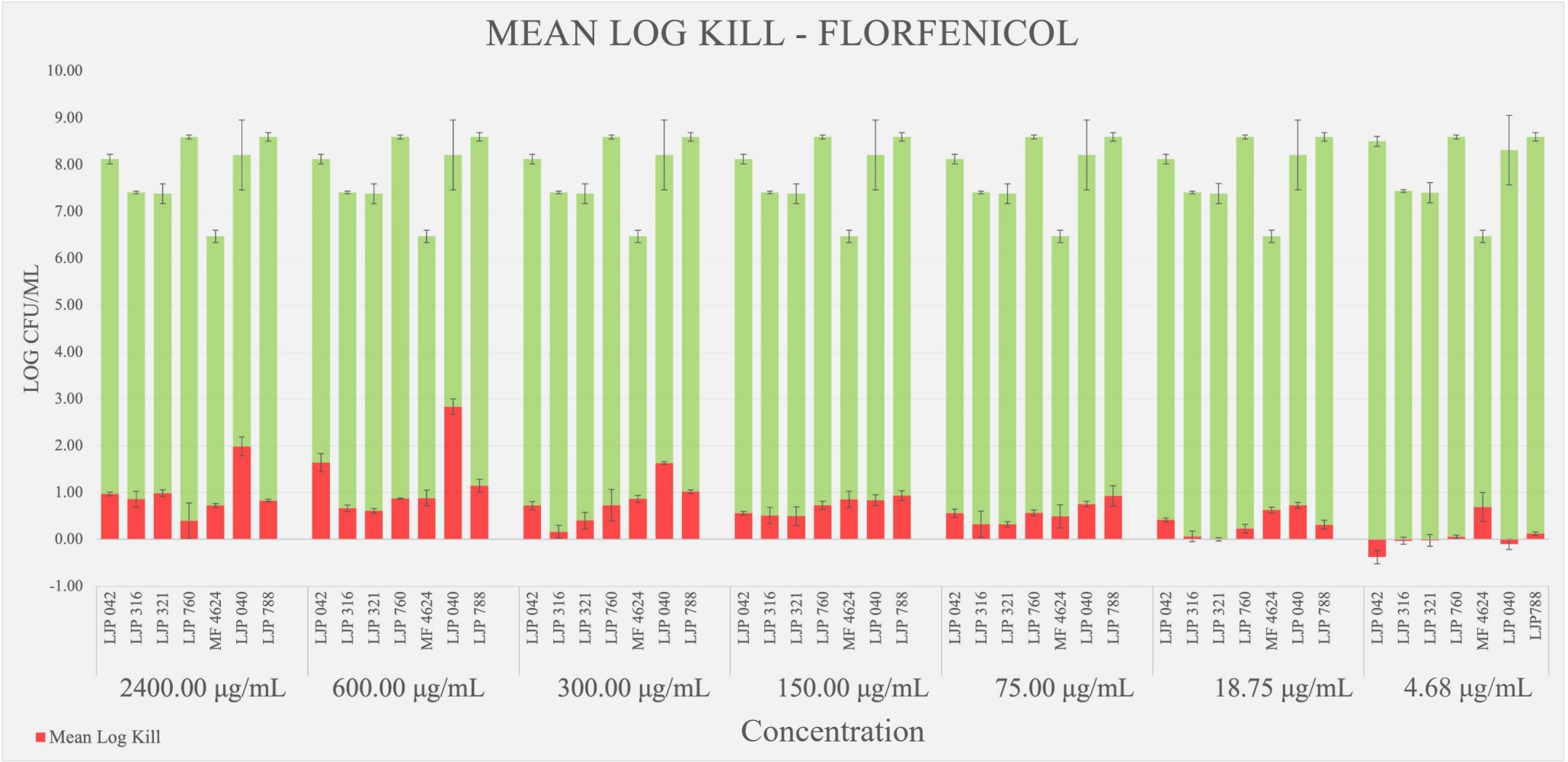


Figure 26: Mean log kill of florfenicol (VCC). Green pillars are showing the growth of each isolate exposed to different concentrations of the disinfectant. The log reduction of each isolates is shown in red.

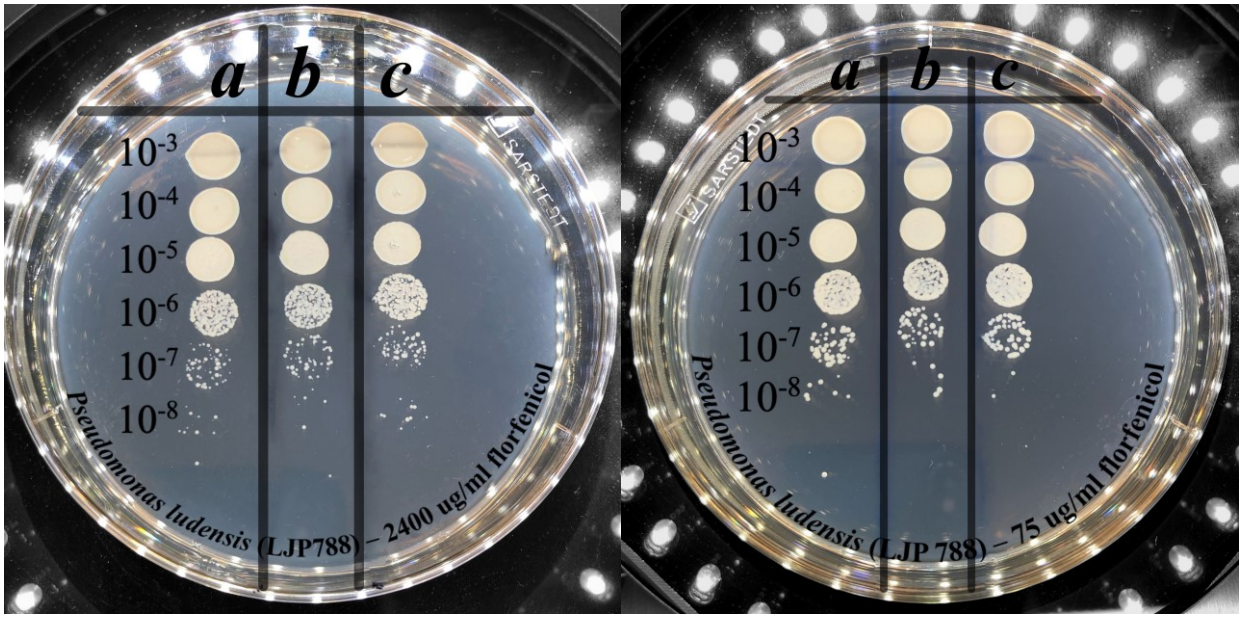


Figure 27: Two petri dishes with 3 parallels of Pseudomonas ludensis (LJP 788). The isolate on the left side was exposed towards 2400.00 µg/mL of florfenicol, and showed smaller colonies compared to the isolate on the right side (75.00 µg/mL).

3.5.4 Variation and reproducibility of the analysis

The inter-assay (n=6) CV was calculated from OD_{650} and VCC values of growth control means from well plates used in the test with Aqua DES Foam PAA, Suma Bac D10 and florfenicol. The intra-assay (n=21) CV was calculated from OD_{650} and VCC values of growth control triplicates on each well plate. The coefficients calculated from VCC were generally higher than the ones calculated from optical density measurements (Table 17).

Table 17: Coefficients of variation (CV)

Assay	Intra-assay coefficient of variation	Inter-assay coefficient of variation
Viable cell count (VCC) - Mean log kill	27.0%	62.5%
Optical density (OD_{650}) - MIC	8.0%	36.2%
Optical density (OD_{650}) - MBEC	7.6%	36.8%

4.0 Discussion

4.1 Identification of bacterial isolates

The identification by 16S rRNA sequencing is a simple and accurate method for identifying bacterial isolates on the genus level (Janda et al., 2007). In this study we identified most of the isolates not only on the genus level but also on the species level. The identification on species level was estimated as rather uncertain given the reduced accuracy of 16S rRNA sequencing on the species level (Janda et al., 2007). A higher taxonomic resolution could have been obtained by *gyrB* and *rpoD* sequencing (Yamamoto et al., 2000). Furthermore, genome-based approaches could also be used for phylogenetic classification in taxonomy and are likely superior to conventional methods with respect to accuracy and reproducibility (Meier-Kolthoff et al., 2019; Yoon et al., 2017). These methods were not applied in our analysis due to complexity and time limitations.

In this study the majority of the 36 presumptive *Pseudomonas* species were successfully confirmed as *Pseudomonas* species (70%). The taxonomic profiles of the identified isolates were generally resembling the results by Boyko (2020), where isolates from the same sampling locations were identified. Boyko (2020) identified comparable proportions of *Pseudmonas* spp. in addition to several other species. Ten of the isolates identified in this study belonged to different genera mostly within the order of Enterobacterales which indicates a limited performance of the selective growth media *Pseudomonas* CFC. Nevertheless, similar findings were published by Tryfinopoulou et al. (2001).

Overall, the most predominant species within the selection of identified *Pseudomonas* isolates was *P. azotoformans.* This species is traditionally known as a plant pathogen (Fang et al., 2016) but was also detected in the microbiota of aquacultured fish previously (Ruzauskas et al., 2018). *P.azotoformans* is also closely related to *P. fluorescens* that is commonly associated with fish microbiota (Gram et al., 2001).

4.2 Genotypic detection of antimicrobial resistance

In the initial phase of the genotypic detection procedure for the analysis of antimicrobial resistances, we successfully confirmed positive controls for eight ARGs for establishing reliable PCR assays. Positive controls in the form of reference strains are used as markers for the validation and assessment of the accuracy in detection methods (Wilder, 2019). They are commonly used in studies investigating the prevalence of ARGs (Bergeron et al., 2017; Müller et al., 2013).

In the subsequent ARG screening among the bacterial isolates the large majority tested negative towards all eight ARGs. We detected faint amplicons for several isolates that were mostly of incorrect size. Interspecies disparities in the sequences of identical ARGs provide an explanation for this observation. According to Alcock et al. (2019) there are different variants of the same resistance genes and genes may be subjected to mutations. In this study, *L. monocytogenes* isolates were used as positive controls in PCR assays for *qacH* and *bcrABC*. We suspect that amplicon size and primer specificity may differ between *L. monocytogenes* and *Pseudomonas* spp. in PCR assays targeting these genes. The consequence of this could be the detection of faint amplicons of incorrect sizes. No faint amplicons could be confirmed by sequencing in this study. The ambiguous PCR results could

therefore also be a consequence of insufficient primer specificity or suboptimal PCR cycling conditions, resulting in non-specific binding products (Abd-Elsalam, 2003). The sequencing was neither successful for most of the clear DNA bands representing amplicons from the positive control strains. Whether the primer design was optimal for sequencing analysis is therefore questionable (Eurofins, 2021).

The PCR assay of *floR* was one of the assays where we detected large numbers of faint amplicons of different sizes. The faint amplicons may represent the prevalence of the *floR* related gene *floR2*. Yet, *floR2* specific primers could not be found in published literature. Consequentially, we designed new *floR2* and *floR* primers that aimed to improve the specificity of the PCR assay.

We verified both primer pairs (floTHF/floTHR and flo2THF/flo2THR) by PCR and the amplicons were successfully sequenced. The *floR2* primers were found to be *floR2* specific. On the contrary, the new *floR* primers showed binding towards both *floR* and *floR2* and were therefore not specific towards *floR* only. However, primers by Faldynova et al. (2003) and Zhang et al. (2009) showed clear *floR* specificity in this study. These results could be valuable for future PCR assays that target these genes and try distinguishing between them.

Ultimately, the PCR based approach for the detection of ARGs has shown a number of disadvantages. The results were partially inconclusive and the method is highly dependent on specific primer pairs. Additionally, the approach is quite time consuming when large quantities of different ARGs are analyzed. These drawbacks might be alleviated by metagenomic-analysis which can detect a broad spectrum of different ARGs simultaneous (Yang et al., 2014).

4.3 Phenotypic testing of antibiotic resistance

The disk diffusion method is a standardized method for susceptibility testing with widespread application (Andrews et al., 2001; CLSI, 2012a; Matuschek et al., 2014). We used the disk diffusion method for the determination of the antibiotic resistance patterns of 62 bacterial isolates and 7 reference strains. In general, a high prevalence of ampicillin and florfenicol resistance was detected. However, no isolate was resistant towards tetracycline. The resistance profiles were comparable to the profiles published by Miranda et al. (2007) that investigated bacterial isolates from Chilean fish farms. These isolates belong predominantly also to *Pseudomonas* spp.. A high occurrence of florfenicol and ampicillin resistance was found, while the prevalence of resistance towards tetracycline was low. A recent study by Lee et al. (2021) found also similar resistance profiles among bacteria from aquatic origin. It was reported that *Aeromonas* species isolated from Norwegian seafood showed a high prevalence of ampicillin and florfenicol resistance.

Low effectiveness of florfenicol towards *Pseudomonas* species has been reported (EMA, 2014). The primary mechanism that confers florfenicol resistance is associated with the *floR* gene encoding florfenicol specific efflux pumps (EMA, 2014; Schwarz et al., 2004). However, our results do not allow correlating the high occurrence of phenotypic florfenicol resistance with genotypic resistance mechanisms that are conferred by the *floR* gene.

A study by Fernández-Alarcón et al. (2010) found that florfenicol resistance not necessarily correlates with the presence of *floR*. Florfenicol MIC values among gram 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 et al. (2020) reported a high occurrence of florfenicol resistance among *Pseudomonas* spp. in combination with a low prevalence of the *floR* gene. Additional ARGs associated with florfenicol resistance have been identified, including *cfr, fexA, fexB* (Long et al., 2006) and *optrA* (Wang et al., 2015). Nevertheless, the prevalence of these genes could not be analyzed in our study, since reference strains were not identified.

According to the annual NORM/NORM-VET (2019) report, florfenicol is the most utilized antibiotic in the Norwegian aquaculture and has been used for more than ten years. The high occurrence of florfenicol resistance might be a consequence of the long-term use of florfenicol in the Norwegian aquacultures. Despite a far lower usage of antibiotics in the Norwegian aquaculture than in other countries (Miranda et al., 2018), while Norway is the largest salmon producer in the world (Iversen et al., 2020). In 2016 383 tons of antibiotics were used in the Chilean salmon industry (Miranda et al., 2018) whereas only 201 kilograms were used in Norwegian fish farms (NORM/NORM-VET, 2019). In fact, the antibiotic use is more than 1.500 times higher per ton of produced salmon in Chile compared to Norway (Miranda et al., 2018).

The result of our disk diffusion tests showed also that most of the bacterial isolates exhibited resistance towards ampicillin (79%). The resistance among *Pseudomonas* species could be explained by their ability to produce beta-lactamase enzymes, providing resistance towards beta-lactam drugs (Falodun et al., 2020). The production of these enzymes can be linked to the presence of genes like *ampC* and bla_{TEM-1} (Shi et al., 2013). However, in this study the phenotypic ampicillin resistance could not be explained by the presence of *ampC*.

Phenotypic resistance towards tetracycline was not detected for any of the tested bacterial isolates. Tetracycline was by far the most utilized antibiotic in the Norwegian fish farms in the 1980s (Svanevik et al., 2021) but is almost completely phased out in recent years. (NORM/NORM-VET, 2019).

The disk diffusion method has shown to be relatively easy to perform while being highly reproducible (Jorgensen et al., 2015). The major limitation of the method is the necessity for standardized interpretative data for different organisms (Jorgensen et al., 2015). Finding adequate species and drug dependent interpretative data was challenging and assumptions had to be made. The lack of organism specific florfenicol interpretative data has already been addressed by several studies (Bowker et al., 2010; Hariharan et al., 2004). We used florfenicol interpretative data for common veterinary pathogens by Tendencia (2004) for species belonging to Enterobacterales, *Listeria*, *Aeromonas* and *Acinetobacter.* Official interpretative data for ampicillin and tetracycline were used as far as available (CLSI, 2012b; EUCAST, 2021). For *Pseudomonas* species, we used interpretative data by Miranda et al. (2007).

4.4 Biofilm formation capabilities

The initial screening of the biofilm formation capabilities in this study demonstrated that isolates with high capabilities were predominantly belonging to *Pseudomonas* spp.. These results correspond with several other studies where *Pseudomonas* spp. was found to have high biofilm formation capabilities (Mann et al., 2012; Møretrø et al., 2017a; Quintieri et al., 2019; Ude et al., 2006). The results for the biofilm formation capabilities were also resembling the findings by Boyko (2020), where isolates from the same sampling locations were analyzed.

Liu et al. (2015) reported that specific *Pseudomonas* strains have the capability of forming biofilms faster at low temperatures (4 – 10 °C) than at elevated temperatures of ~30 °C. We used an incubation temperature of 12 °C in order to simulate realistic conditions for the production environment in salmon processing plants. This could explain the high biofilm formation capabilities among *Pseudomonas* spp. that we observed. However, the low temperature was also negatively affecting the growth of some isolates and most of the reference strains. A possible explanation would be the mesophilic nature of the reference strains *P. aeruginosa* (Barbier et al., 2014), *E. coli* (Kumar et al., 2013) and *A. baumanii* (Dekic et al., 2018). As a consequence growth was insufficient and below the limit of detection.

The viable cell count did not correspond directly to the measured OD values that we measured. Interspecies morphological differences provide a possible explanation for this observation. Identical OD values for growth measurements in different species do not necessarily represent identical VCCs because cell sizes may be dissimilar (Harrison et al., 2010). For instance, the cell size of *Pseudomonas* spp. are 0.5 to 1.0 × 1.5 to 5.0 µm (Diggle et al., 2020) whereas *L. monocytogenes* cells are clearly smaller with 0.4 to 0.5 × 0.5 to 2.0 µm (PHE, 2020). This could explain why the mean differential VCC of *L. monocytogenes* did not agree with the OD measurements in this study. Furthermore, differences in intra- and inter-species aggregation have been reported, including waxy aggregations, floccular masses and slimes (Ude et al., 2006). These differences would likely also affect OD measurements and could conceivably explain why the significant difference ($p<0.05$) in biofilm formation between *Serratia* spp. and *Pseudomonas* spp. measured by OD was not reflected by the VCC.

The VCC from the experiment did also reveal that bacteria concentrations below 1.0×10^7 CFU/mL were not detected by the OD measurements. This is clearly a considerable disadvantage and has also been previously reported by Biesta-Peters et al. (2010) and Clais et al. (2015).

4.5 Susceptibility test of bacterial isolates in peg lid biofilm reactors

The susceptibility test with Aqua DES Foam PAA and Suma Bac D10 demonstrated that the disinfectants were effective against most of the tested isolates in planktonic state at user concentration (1.0%). In other words, MIC and MBC values were equivalent to user concentration or lower for most isolates. However, the effectiveness of both disinfectants was lower towards isolates in sessile state, i.e., biofilms. The MBEC values were distinctively higher than MIC and MBC values. These findings underline that bacteria in biofilm exhibit enhanced tolerance to disinfectants that were previously reported by several studies (Azizoglu et al., 2015; Steenackers et al., 2012).

Our susceptibility test showed clear differences in the effectiveness between Aqua DES Foam PAA and Suma Bac D10. Generally, the MIC and MBEC values were higher for Aqua DES Foam PAA than for Suma Bac D10. Hence, the effectiveness of the prior was lower. This was confirmed by a significantly higher ($p<0.05$) mean log kill of most isolates by Suma Bac D10 at user concentration (1.0%).

The user concentration of Aqua DES Foam PAA is 1.0% in the salmon processing plant from this study (Undisclosed, 2021). Our results demonstrated that the mean log kill by the user concentration of Aqua DES Foam PAA was generally lower than 1.0 log CFU/mL for most isolates. However, the producer of the disinfectant is recommending dosages of 1.5 - 3.0% (Aquatic, 2018) and with these concentrations the test results showed significantly higher ($p<0.05$) mean log kill values. Complete inactivation of most isolates was achieved at 4.0% of Aqua DES Foam PAA which demonstrates that the recommendations by the producer of the disinfectant are appropriate. The off-label use by the industry must be evaluated as deficient and not effective against biofilm-forming bacteria.

In the MBEC experiment with Suma Bac D10, isolate *P. fluorescens* (LJP 042) clearly surpassed the other isolates with a MBEC value eight times higher compared to the other isolates. This could be explained by the presence of the benzalkonium chloride associated resistance gene *bcrABC* because LJP 042 exhibits a faint amplicon for this gene which indicates a correlation between genotypic and phenotypic resistance. The presence of *bcrABC* could not be confirmed by sequencing, i.e., the high MBEC value may as well be a consequence of the high biofilm formation capability of the isolate.

The reference strain *L. monocytogenes* MF 4624 harbors the gene *bcrABC* (Møretrø et al., 2017b) and was therefore included in the susceptibility test. Unfortunately, the growth of the strain was insufficient and OD measurements were below the limit of detection. The limited growth was likely caused by the low incubation temperature chosen. The VCC of the mean log kill revealed growth of *L. monocytogenes* MF 4624 around 6.0 log CFU/mL but the presence of *bcrABC* did not seem to provide increased tolerance towards Suma Bac D10 compared to the other isolates.

Susceptibility tests for florfenicol showed MIC values between 300.00 and >2400.00 µg/mL florfenicol. Miranda et al. (2007) and Adesoji et al. (2020) reported comparable MIC values among *Pseudomonas* spp., mostly ranging from >512 to >1024 µg/mL florfenicol. While Ho et al. (2000) reported MICs between 0.78 and >100.00 µg/mL florfenicol for a selection of *P. fluorescens* strains with an aquatic origin. Florfenicol MIC values of more than 2400.00 µg/mL have not been reported previously to our knowledge. In accordance with this, the florfenicol MIC values were evaluated as relatively high in comparison with previous studies. The high MIC values corresponded also well with the results from the disk diffusion tests where most isolates were categorized as resistant.

The florfenicol MBEC values were generally higher than MIC values and demonstrated increased tolerance for most isolates in biofilm contra planktonic state. These findings are supported by previous studies where bacteria in biofilm drastically increased their tolerance towards antibiotics compared to planktonic cells (Ceri et al., 1999; Olson et al., 2002).

The isolate of *P. putida* (LJP 760) showed a relatively low florfenicol MIC of 150.00 µg/mL. This agrees with the results derived from the disk diffusion test where LJP 760 tested as susceptible for florfenicol. However, the MBEC value of the isolate was more than 15 times higher (>2400.00 µg/mL). This result may indicate that high biofilm capabilities could outperform resistance capabilities. Additionally, the mean log kill of florfenicol was less than 2.0 log CFU/mL for all isolates at the highest concentration. Nevertheless, the results indicated that florfenicol is causing a growth delay of the recovered cells from the biofilm since differences in colony sizes were observed. This could be explained by the bacteriostatic nature of action associated with florfenicol (Davis et al., 2014).

Lastly, we calculated the coefficients of variation, commonly used for accessing variation and reproducibility within laboratorial assays (Bastarache et al., 2011; Clais et al., 2015).

The intra-assay coefficient of variation was calculated to assess the variation between triplicates and quadruplicates. In this study, we found that the well plate lid affected the variability. The intra-assay CV for OD measurements with lid was measured at 11.0% whereas the CV reduced to 3.2% without a lid. According to Clais et al. (2015) and Salimetrics (2021) intra-assay CVs below 10% are within acceptable levels and we consequently chose to perform measurements without lid for minimizing variability. The difference observed may be explained by condensation on the inside of the lid from bacterial activity or dust particles on the top of the lid after incubation that contribute to aberrant OD measurements.

The intra-assay CV for OD measurements of MIC and MBEC plates were 8.0% and 7.6%, respectively and reflected acceptable variability between triplicates in the experiment. However, the intra-assay CV for viable cell counts of the mean log kill was considerable higher (27.0%). This could partially be a consequence of preparing serial dilutions to perform VCCs, as several cell counts were performed at high dilution factors in this study. Every dilution step induces variation and it can be assumed that the CV increases in correlation to the dilution factor. Clais et al. (2015) reported likewise high CV percentages for viable cell counts at high dilution factors.

The inter-assay coefficient of variation was calculated to assess the plate-to-plate repeatability of the assays and acceptable levels are usually below 15% (Clais et al., 2015; Salimetrics, 2021). Surprisingly, the inter-assay CVs were remarkably higher in this study. They ranged from 36% to 62%. The high variability could be explained by the significant difference ($p<0.05$) between the inoculum concentration for Aqua DES Foam PAA and the other inoculum concentrations. This could further be linked to the McFarland standardization, possibly giving rise to variation and inadequate repeatability. However, all inoculum concentrations were within 1.0 log CFU/mL of the desired starting cell number and were therefore within the acceptable levels recommended by Harrison et al. (2010).

5.0 Conclusion

This study aimed to examine the phenotypic and genotypic antimicrobial resistance among *Pseudomonas* spp. isolated from a Norwegian salmon processing plant. The majority of the isolates were successfully identified as *Pseudomonas* species. The prevalence of resistance genes conferring antibiotic and disinfectant resistance was generally low among the isolates. Our disk diffusion test demonstrated that most of the *Pseudomonas* species were resistant towards ampicillin and florfenicol. Prevalence of tetracycline resistance was not detected. The high occurrence of the phenotypic resistance could not be explained by the genotypic resistance mechanisms that we investigated. The resistant isolates did not carry any resistance gene related to that particular antibiotic. Discrepancies between phenotypic and genotyping resistance is also present in other studies.

The results in this study substantiated the high biofilm formation capabilities among *Pseudomonas* spp. at temperatures equivalent to those observed in salmon processing plants. Further, we elucidated the strong effect of biofilm formation on the antimicrobial susceptibility patterns of *Pseudomonas* spp. Isolates in biofilm states were generally less susceptible towards antimicrobial agents compared to isolates in planktonic state.

The disinfectants Suma Bac D10 and Aqua Des Foam PAA were effective against the selected *Pseudomonas* spp. in planktonic state at user concentrations of 1.0%. Although, substantially less effective against the selected *Pseudomonas* spp. in biofilm state. The effectiveness of Suma Bac D10 was significantly higher at user concentrations of 1.0%. compared to Aqua Des Foam PAA.

The effect of cumulative florfenicol concentrations on *Pseudomonas* isolates demonstrated the low-level susceptibility among these isolates. Corresponding to the high prevalence of florfenicol resistance detected in the disk diffusion test. The overall high occurrence of florfenicol resistance might be a consequence of the long-term use of florfenicol in the Norwegian aquaculture sector. Although, we cannot conclude whether the observed resistance is acquired or a part of the natural resistance mechanisms found in *Pseudomonas* spp. Further studies are needed to investigate florfenicol resistance in the Norwegian salmon industry and to assess the risk of the acquisition and dissemination of resistance by horizontal gene transfer.

6.0 Future perspectives

Tackling the AMR crisis and henceforth achieving better public health requires multisectoral approaches like the One Health initiative. The food industry is also involved in the complex challenges imposed by AMR. Continuous AMR monitoring in the food industry and the aquaculture sector is therefore essential for the assessment of risks to human health.

Our study provides a basis for further investigations on the prevalence of AMR among *Pseudomonas* species from Norwegian salmon processing plants. Increased focus should be directed on *Pseudomonas* spp. based on their predominance in salmon processing plants and other food industry sectors. The relevance of this species increases also in connection with the increased seafood consumption and especially raw seafood consumption.

More knowledge is also needed in regards to the development of disinfectant resistance and possible cross-resistance towards medically important antibiotics. It is also important to consider the involvement of biofilm formation in the development of AMR.

Metagenomic approaches for detecting AMR have previously shown great potential and could be performed in future studies. Transcriptomics could also be used to monitor changes in gene expression. Lastly, there is also a need for species specific interpretative data for the disk diffusion test for a more accurate determination of susceptibility patterns.

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Appendix A

Overview of 165 bacterial isolates included in this study

Table 1: Overview of 142 bacterial isolates with results for identification and biofilm formation capabilities by Boyko (2020). Green indicates good capabilities, white indicates medium and red low. Grey values are invalid.

Isolate ID	Date	Sampling location	Identified species	Biofilm formation
LJP 007	25.05.2018	2	*Pseudoalteromonas* sp. strain Arc7-671 16S ribosomal RNA gene, partial sequence	-0.008
LJP 008	↓	2	*Pseudoalteromonas distincta* strain 20KNS10Z3 16S ribosomal RNA gene, partial sequence	-0.004
LJP 009		2	*Pseudomonas brenneri* strain PF37 RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.098
LJP 010		2	*Pseudoalteromonas* sp. strain BOMB.9.10.22 16S ribosomal RNA gene, partial sequence	0.006
LJP 011		2	*Pseudoalteromonas* sp. strain Arc7-671 16S ribosomal RNA gene, partial sequence	0.005
LJP 012		2	*Stenotrophomonas rhizophila* strain KR2-13 16S ribosomal RNA gene, partial sequence	-0.126
LJP 013		2	Missing sequence	-
LJP 014		2	*Pseudomonas fluorescens* strain PF67 RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.098
LJP 015		2	*Pseudoalteromonas issachenkonii* strain 3B 16S ribosomal RNA gene, partial sequence	-0.007
LJP 026		10	Missing sequence	-
LJP 027		10	Missing sequence	-
LJP 028		10	Missing sequence	-
LJP 029		10	*Pseudomonas* sp. CCUG 62357 partial *rpoD* gene for DNA-directed RNA polymerase subunit D, strain CCUG 62357	-0.082
LJP 030		10	*Pseudomonas fluorescens* strain PF67 RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.103
LJP 031		10	Missing sequence	-
LJP 032		8	*Pseudomonas* sp. CCUG 62357 partial *rpoD* gene for DNA-directed RNA polymerase subunit D, strain CCUG 62357	-0.143
LJP 033		8	Missing sequence	-
LJP 034		8	*Pseudomonas* sp. CCUG 62357 partial *rpoD* gene for DNA-directed RNA polymerase subunit D, strain CCUG 62357	-0.107
LJP 035		8	*Pseudomonas fluorescens* strain PF59 RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.060
LJP 036		8	Missing sequence	-
LJP 037		8	Missing sequence	-
LJP 038		8	Missing sequence	-
LJP 039		8	Missing sequence	-
LJP 040		8	*Pseudomonas fluorescens* strain PF59 RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.071
LJP 041		8	*Pseudomonas* sp. CCUG 62357 partial *rpoD* gene for DNA-directed RNA polymerase subunit D, strain CCUG 62357	-0.072
LJP 042		8	*Pseudomonas fluorescens strain* W-6 chromosome, complete genome	-0.080
LJP 043		8	Missing sequence	-
LJP 044		8	*Pseudomonas fluorescens* strain PF59 RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.071
LJP 045		8	*Pseudomonas fluorescens* strain PF59 RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.075
LJP 046		22	*Pseudomonas* sp. CFSAN084952 chromosome, complete genome	-0.062
LJP 308	31.10.2018	2	*Aeromonas piscicola* strain CECT 7443 RNA polymerase sigma-70 factor (*rpoD*) gene, partial cds	-0.210
LJP 309	↓	2	Missing sequence	-
LJP 310		2	*Pseudomonas fluorescens rpoD* gene for DNA-directed RNA polymerase subunit D, partial cds, strain: ATCC 17573	-0.095
LJP 311		2	*Pseudomonas* sp. P6169 RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.055
LJP 312		2	Missing sequence	-
LJP 313		2	*Pseudomonas* sp. M30-35, complete genome	-0.052
LJP 314		2	*Pseudomonas guineae* strain OX1110 16S ribosomal RNA gene, partial sequence	-0.164
LJP 315		2	*Pseudomonas guineae* strain OX1110 16S ribosomal RNA gene, partial sequence	-0.113
LJP 316		2	*Pseudomonas fluorescens rpoD* gene for DNA-directed RNA polymerase subunit D, partial cds, strain: ATCC 17573	-0.100
LJP 317		2	*Morganella psychrotolerans* strain C11 16S ribosomal RNA gene, partial sequence	-0.112
LJP 318		2	*Aeromonas salmonicida* strain JF11 16S ribosomal RNA gene, partial sequence	-0.216
LJP 319		2	*Morganella psychrotolerans* strain U2/5 16S ribosomal RNA gene, partial sequence	-0.104
LJP 320		2	*Pseudomonas* sp. M30-35, complete genome	-0.028
LJP 321		2	*Pseudomonas fluorescens rpoD* gene for DNA-directed RNA polymerase subunit D, partial cds, strain: ATCC 17573	-0.101
LJP 323		2	*Morganella psychrotolerans* strain U2/5 16S ribosomal RNA gene, partial sequence	-0.091
LJP 324		2	*Morganella psychrotolerans* strain C11 16S ribosomal RNA gene, partial sequence	-0.125
LJP 325		2	*Aeromonas bestiarum* strain ESV-384 RNA polymerase sigma-70 factor (*rpoD*) gene, partial cds	-0.187
LJP 326		2	*Pseudomonas fluorescens rpoD* gene for DNA-directed RNA polymerase subunit D, partial cds, strain: ATCC 17573	-0.213
LJP 327		2	*Pseudomonas* sp. strain P12Ogen RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.155
LJP 328		2	Missing sequence	-
LJP 329		2	*Pseudomonas* sp. strain P12Ogen RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.192
LJP 332		2	*Morganella psychrotolerans* strain U2/5 16S ribosomal RNA gene, partial sequence	-0.094
LJP 333		2	*Morganella psychrotolerans* strain C11 16S ribosomal RNA gene, partial sequence	-0.088
LJP 334		2	*Morganella psychrotolerans* strain C11 16S ribosomal RNA gene, partial sequence	-0.093
LJP 335		2	Missing sequence	-
LJP 339		8	Missing sequence	-
LJP 341		18	*Pseudomonas fluorescens* strain PF59 RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.089
LJP 342		18	*Serratia* sp. QZS4_4 16S ribosomal RNA gene, partial sequence	-0.244
LJP 343		18	*Pseudomonas* sp. SXM-1 chromosome, complete genome	-0.086
LJP 344		18	Missing sequence	-
LJP 345		18	*Enterobacteriaceae bacterium* ENUB8 16S ribosomal RNA genes, partial sequence	-0.252
LJP 346		18	*Enterobacteriaceae bacterium* ENUB8 16S ribosomal RNA genes, partial sequence	-0.228
LJP 347		18	Missing sequence	-

LJP 360		10	Missing sequence	-
LJP 362		10	*Pseudomonas fluorescens* strain PF100 RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.144
LJP 363		10	*Pseudomonas fluorescens* strain PF85 RNA polymerase sigma factor (*rpoD*) gene, complete cds	-0.150
LJP 364		10	*Pseudomonas fluorescens* strain PF59 RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.101
LJP 365		10	Missing sequence	-
LJP 366		10	Missing sequence	-
LJP 367		10	*Pseudomonas* sp. CCUG 66625 partial *rpoD* gene for DNA-directed RNA polymerase subunit D, strain CCUG 66625	-0.089
LJP 368		10	*Pseudomonas* sp. CCUG 66625 partial *rpoD* gene for DNA-directed RNA polymerase subunit D, strain CCUG 66625	-0.126
LJP 369		10	*Pseudomonas fluorescens* strain PF85 RNA polymerase sigma factor (*rpoD*) gene, complete cds	-0.087
LJP 370		10	*Pseudomonas fluorescens* strain PF64 RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.141
LJP 371		10	Missing sequence	-
LJP 372		10	Missing sequence	-
LJP 373a		10	*Pseudomonas fluorescens* strain PF80 RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.102
LJP 374		10	*Pseudomonas fluorescens* strain PF 53 RNA polymerase sigma factor *RpoD* (*rpoD*) gene, complete cds	-0.325
LJP 375		10	Missing sequence	-
LJP 376		10	*Pseudomonas* sp. CCUG 66625 partial *rpoD* gene for DNA-directed RNA polymerase subunit D, strain CCUG 66625	-0.108
LJP 378		10	*Stenotrophomonas rhizophila* strain KR2-13 16S ribosomal RNA gene, partial sequence	-0.081
LJP 379		10	*Pseudomonas fluorescens* strain PF85 RNA polymerase sigma factor (*rpoD*) gene, complete cds	-0.297
LJP 380		10	*Pseudomonas* sp. CCUG 66625 partial *rpoD* gene for DNA-directed RNA polymerase subunit D, strain CCUG 66625	-0.108
LJP 381		10	*Pseudomonas fluorescens* strain PF85 RNA polymerase sigma factor (*rpoD*) gene, complete cds	-0.286
LJP 382		10	*Pseudomonas fluorescens* strain PF85 RNA polymerase sigma factor (*rpoD*) gene, complete cds	-0.114
LJP 383		10	Missing sequence	-
LJP 384		10	*Pseudomonas fluorescens* strain PF85 RNA polymerase sigma factor (*rpoD*) gene, complete cds	-0.260
LJP 385		10	*Pseudomonas fluorescens* strain PF85 RNA polymerase sigma factor (*rpoD*) gene, complete cds	-0.105
LJP 417		14	*Pseudomonas fluorescens* strain PF80 RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.109
LJP 418		14	Missing sequence	-
LJP 419		14	*Pseudomonas fluorescens* strain PF56 RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.066
LJP 420		14	*Stenotrophomonas rhizophila* strain VITDW0102 16S ribosomal RNA gene, partial sequence	-0.094
LJP 421		14	*Pseudomonas lurida* strain MYb11 chromosome, complete genome	-0.251
LJP 422		14	*Pseudomonas fluorescens* strain PF100 RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.165
LJP 423		14	*Pseudomonas fluorescens* strain PF85 RNA polymerase sigma factor (*rpoD*) gene, complete cds	-0.080
LJP 424		14	Missing sequence	-
LJP 425		14	*Pseudomonas fluorescens* strain PF85 RNA polymerase sigma factor (*rpoD*) gene, complete cds	-0.099
LJP 426		14	*Pseudomonas fluorescens* strain PF59 RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.169
LJP 658	22.05.2019	2	*Pseudoalteromonas distincta* strain 20KNS10Z3 16S ribosomal RNA gene, partial sequence	0.004
LJP 659	↓	2	*Pseudomonas fluorescens* strain PF85 RNA polymerase sigma factor (*rpoD*) gene, complete cds	0.010
LJP 660		2	*Pseudoalteromonas distincta* strain 20KNS10Z3 16S ribosomal RNA gene, partial sequence	0.011
LJP 661		2	*Pseudomonas fluorescens rpoD* gene for DNA-directed RNA polymerase subunit D, partial cds, strain: ATCC 17573	-0.109
LJP 705		7	*Pseudomonas fluorescens* strain PF85 RNA polymerase sigma factor (*rpoD*) gene, complete cds	-0.220
LJP 706		7	*Pseudomonas gessardii* partial *rpoD* gene for DNA-directed RNA polymerase subunit D, type strain CIP 105469T	-0.119
LJP 707		7	*Pseudomonas gessardii* partial *rpoD* gene for DNA-directed RNA polymerase subunit D, type strain CIP 105469T	-0.106
LJP 708		7	*Pseudomonas* sp. Irchel 3E20 partial *rpoD* gene for *RpoD*, strain Irchel 3E20	-0.170
LJP 709		7	*Pseudomonas* sp. Irchel s2f8 partial *rpoD* gene for *RpoD*, isolate s2f8_A10	-0.194
LJP 710		7	*Pseudomonas azotoformans* partial 16S rRNA gene, isolate SW_HL_6_52	-0.023
LJP 711		7	*Pseudomonas* sp. Irchel 3E20 partial *rpoD* gene for *RpoD*, strain Irchel 3E20	-0.133
LJP 712		7	*Pseudomonas* sp. Irchel 3E20 partial *rpoD* gene for *RpoD*, strain Irchel 3E20	-0.133
LJP 713		7	*Pseudomonas fluorescens* strain PF56 RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.070
LJP 714		7	*Pseudomonas gessardii* partial *rpoD* gene for DNA-directed RNA polymerase subunit D, type strain CIP 105469T	-0.097
LJP 715		7	*Pseudomonas* sp. Irchel 3E20 partial *rpoD* gene for *RpoD*, strain Irchel 3E20	-0.121
LJP 716		7	*Pseudomonas gessardii* partial *rpoD* gene for DNA-directed RNA polymerase subunit D, type strain CIP 105469T	-0.092
LJP 717		7	Missing sequence	-
LJP 718		7	*Pseudomonas fluorescens* strain PF85 RNA polymerase sigma factor (*rpoD*) gene, complete cds	-0.088
LJP 719		8	*Pseudomonas fluorescens* strain PF56 RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.074
LJP 720		8	*Pseudomonas fluorescens rpoD* gene for DNA-directed RNA polymerase subunit D, partial cds, strain: ATCC 17573	-0.085
LJP 721		8	*Pseudomonas fluorescens* strain PF56 RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.066
LJP 722		8	Missing sequence	-
LJP 723		8	*Pseudomonas* sp. rDWA119 partial *rpoD* gene for DNA-directed RNA polymerase subunit D, isolate rDWA119	-0.298
LJP 724		8	Missing sequence	-
LJP 725		8	*Pseudomonas fluorescens rpoD* gene for DNA-directed RNA polymerase subunit D, partial cds, strain: ATCC 17573	-0.073
LJP 726		8	*Pseudomonas fluorescens* strain PF89 RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.232
LJP 727		8	*Pseudomonas fluorescens rpoD* gene for DNA-directed RNA polymerase subunit D, partial cds, strain: ATCC 17573	-0.072
LJP 728		8	Missing sequence	-
LJP 760		14	*Pseudomonas putida* partial *rpoD* gene for DNA-directed RNA polimerase subunit D, strain CFBP 5933	-0.081
LJP 761		14	*Serratia liquefaciens* strain FG3 chromosome, complete genome	-0.217
LJP 762		14	Missing sequence	-
LJP 763		14	*Serratia liquefaciens* strain FG3 chromosome, complete genome	-0.208
LJP 764		14	Missing sequence	-
LJP 765		14	Missing sequence	-
LJP 766		14	*Serratia liquefaciens* strain FG3 chromosome, complete genome	-0.209
LJP 788		18	*Pseudomonas lundensis rpoD* gene for RNA polymerase sigma-70 factor, partial cds	-0.185
LJP 794		20	Missing sequence	-
LJP 795		22	*Pseudomonas fluorescens* strain I5B RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.115
LJP 796		22	*Pseudomonas fluorescens* strain I5B RNA polymerase sigma factor (*rpoD*) gene, partial cds	-0.111
LJP 797		22	Missing sequence	-

LJP 798		22	*Pseudomonas fluorescens* strain PF85 RNA polymerase sigma factor (*rpoD*) gene, complete cds	-0.199
LJP 799		22	*Pseudomonas* sp. rDWA116 partial *rpoD* gene for DNA-directed RNA polymerase subunit D, isolate rDWA116	-0.179
LJP 800		22	*Pseudomonas fluorescens* strain FW300N2E3, complete genome	-0.422
LJP 801		22	*Pseudomonas fluorescens* strain FW300N2E3, complete genome	-0.331
LJP 802		22	*Pseudomonas* sp. rDWA116 partial *rpoD* gene for DNA-directed RNA polymerase subunit D, isolate rDWA116	-0.374

Table 2: Overview of 20 Listeria-isolates from salmon processing plant (Thomassen et al., 2021).

Isolate ID	Date	Sampling location	Identified species
F1K2.353 (ST8)	19.12.19	Filleting machine no. 1, quality scanner no. 2	*Listeria monocytogenes*
SL3.189 (ST37)	08.07.19	Gutting machine no. 3	*Listeria monocytogenes*
HK3.331 (ST37)	27.11.19	Head and tail cutter no. 3	*Listeria monocytogenes*
SL6.212 (ST37)	31.07.19	Gutting machine no. 3	*Listeria monocytogenes*
SL6.141 (ST37)	21.05.19	Gutting machine no. 6	*Listeria monocytogenes*
PK.141 (ST37)	21.05.19	Packaging department	*Listeria monocytogenes*
FS.171 (ST8)	20.06.19	Filet salmon	*Listeria monocytogenes*
SL3.179 (ST37)	28.06.19	Gutting machine no. 3	*Listeria monocytogenes*
SL6.206 (ST37)	25.07.19	Gutting machine no. 6	*Listeria monocytogenes*
SL3.212 (ST37)	31.07.19	Gutting machine no. 3	*Listeria monocytogenes*
SL6.218-59 (ST37)	05.08.19	Gutting machine no. 6	*Listeria monocytogenes*
SL6.218-60 (ST37)	05.08.19	Gutting machine no. 6	*Listeria monocytogenes*
SwF1.296 (ST37)	23.10.19	Swab filet	*Listeria innocua*
SL3.296 (ST37)	23.10.19	Gutting machine no. 3	*Listeria monocytogenes*
HK3.297 (ST37)	24.10.19	Head and tail cutter no. 3	*Listeria monocytogenes*
HK1.329h (ST37)	25.11.19	Head and tail cutter no. 1	*Listeria monocytogenes*
HK1.329v (ST37)	25.11.19	Head and tail cutter no. 1	*Listeria monocytogenes*
HK.3.357 (ST37)	23.12.19	Head and tail cutter no. 3	*Listeria monocytogenes*
SwF1.357 (ST37)	23.12.19	Swab filet	*Listeria innocua*
F1K1.353 (ST37)	19.12.19	Filleting machine no. 1, quality scanner no. 1	*Listeria innocua*

Table 3: Overview of 3 bacterial isolates from Mehli et al. (2017).

Isolate ID	Identified species
S160	*Staphylococcus aureus*
S227	*Staphylococcus aureus*
S229	*Staphylococcus aureus*

Appendix B

Cycling conditions for PCR assays

Table 1: Cycling conditions for different ARGs used for PCR assays.

Target Gene	Steps in the PCR reaction							Reference
	Initial denaturation	Denaturation	Hybridization	Elongation	Reaction cycles	Final elongation	Cool down	
16S rRNA	95 °C, 15 min	95 °C, 60 sec	58 °C, 30 sec	72 °C, 60 sec	30	72 °C, 5 min	12 °C, ∞	This study
ampC	94 °C, 15 min	94 °C, 30 sec	58 °C, 30 sec	72 °C, 60 sec	35	72 °C, 7 min	12 °C, ∞	Shi et al. (2013)
bla_{TEM-1}								
bla_{TEM-1}	94 °C, 15 min	94 °C, 30 sec	55-65 °C, 30 sec	72 °C, 60 sec	35	72 °C, 7 min	12 °C, ∞	Gradient PCR
tetA	94 °C, 15 min	94 °C, 30 sec	55 °C, 30 sec	72 °C, 60 sec	35	72 °C, 7 min	12 °C, ∞	Shi et al. (2013)
tetC								
tetG								
sulI								
dfrA17	94 °C, 15 min	94 °C, 60 sec	55 °C, 60 sec	72 °C, 90 sec	35	72 °C, 10 min	12 °C, ∞	Shi et al. (2013)
qacEΔ1	94 °C, 15 min	93 °C, 30 sec	55 °C, 30 sec	72 °C, 60 sec	35	72 °C, 5 min	12 °C, ∞	Xiao-Min et al. (2014)
qacH	95 °C, 15 min	94 °C, 40 sec	56 °C, 40 sec	72 °C, 25 sec	30	72 °C, 5 min	12 °C, ∞	Müller et al. (2013)
*qacH**	95 °C, 15 min	94 °C, 30 sec	56 °C, 90 sec	72 °C, 30 sec	30	72 °C, 10 min	12 °C, ∞	Møretrø et al. (2017b)
bcrABC	95 °C, 15 min	94 °C, 40 sec	62 °C, 45 sec	72 °C, 25 sec	30	72 °C, 5 min	12 °C, ∞	Müller et al. (2013)
bcrABC	95 °C, 15 min	94 °C, 30 sec	60 °C, 90 sec	72 °C, 90 sec	30	72 °C, 10 min	12 °C, ∞	Møretrø et al. (2017b)
bcrABC	95 °C, 15 min	94 °C, 40 sec	62 °C, 45 sec	72 °C, 90 sec	30	72 °C, 5 min	12 °C, ∞	adjusted
fexA	95 °C, 15 min	95 °C, 30 sec	60 °C, 30 sec	72 °C, 30 sec	40	72 °C, 5 min	12 °C, ∞	Zhao et al. (2016)
fexB								
cfr								
pexA								
optrA								
floR								
optrA	94 °C, 15 min	94 °C, 30 sec	55-65 °C, 30 sec	72 °C, 60 sec	35	72 °C, 7 min	12 °C, ∞	Gradient PCR
floR	94 °C, 15 min	94 °C, 50 sec	54 °C, 50 sec	72 °C, 60 sec	45	72 °C, 10 min	12 °C, ∞	Zhang et al. (2009)
floR	94 °C, 15 min	94 °C, 30 sec	55 °C, 30 sec	72 °C, 30 sec	35	72 °C, 10 min	12 °C, ∞	Yoo (2013)
floR	94 °C, 15 min	94 °C, 40 sec	57 °C, 45 sec	72 °C, 60 sec	35	72 °C, 10 min	12 °C, ∞	This study
floR2	94 °C, 15 min	94 °C, 30 sec	57 °C, 30 sec	72 °C, 30 sec	35	72 °C, 5 min	12 °C, ∞	This study

Appendix C

Identification results – overview of BLAST-hits

Table 1: A total of 33 bacterial isolates were identified in this study, 3 sequences were missing.

Isolate ID	BLAST-hit	Sequence length (bp)	Percentage identity	Query coverage
LJP 013	*Pseudoalteromonas* sp. S-1 16S ribosomal RNA gene, partial sequence	1140	99.47%	99%
LJP 026	*Pseudomonas azotoformans* strain C 16S ribosomal RNA gene, partial sequence	326	97.55%	100%
LJP 027	*Pseudomonas reactans* gene for 16S ribosomal RNA, partial sequence, isolate: B0813	119	94.12%	100%
LJP 028	Missing sequence	-	-	-
LJP 031	*Pseudomonas cedrina subsp. fulgida* gene for 16S rRNA, partial sequence, strain: C247	126	99.21%	99%
LJP 033	*Pseudomonas tolaasii* strain Pt102 16S ribosomal RNA gene, partial sequence	291	99.65%	98%
LJP 036	Missing sequence	-	-	-
LJP 037	*Pseudomonas umsongensis* strain SeaQual_P_81/6 16S ribosomal RNA gene, partial sequence	400	98.75%	100%
LJP 038	*Pseudomonas tolaasii* strain Pt102 16S ribosomal RNA gene, partial sequence	292	99.65%	96%
LJP 039	*Pseudomonas cedrina* strain JM10 16S ribosomal RNA gene, partial sequence	527	98.86%	100%
LJP 043	*Pseudomonas cf. synxantha* V4.BP.03 partial 16S rRNA gene	1088	99.72%	99%
LJP 309	*Pseudomonas marincola* strain SeaQual_P_B24 16S ribosomal RNA gene, partial seq	825	99.39%	100%
LJP 312	*Pseudomonas pseudoalcaligenes* strain MA3 16S ribosomal RNA gene, partial sequence	957	96.37%	97%
LJP 328	*Aeromonas hydrophila* strain 23-C-23 chromosome, complete genome	1127	99.55%	99%
LJP 335	*Morganella psychrotolerans* strain 769 16S ribosomal RNA gene, partial sequence	1119	99.64%	99%
LJP 339	Missing sequence	-	-	-
LJP 344	*Pseudomonas azotoformans* strain 7RMB(B) 16S ribosomal RNA gene, partial sequence	524	94.27%	96%
LJP 347	*Serratia proteamaculans* strain KB49 16S ribosomal RNA gene, partial sequence	1132	99.82%	99%
LJP 360	*Pseudomonas cedrina* strain Cl-10 16S ribosomal RNA gene, partial sequence	1042	99.81%	100%
LJP 365	*Pseudomonas* sp. strain CM-CNRG 623 16S ribosomal RNA gene, partial sequence	906	98.56%	99%
LJP 366	*Pseudomonas azotoformans* partial 16S rRNA gene, isolate SW_HL_6_52	993	99.60%	100%
LJP 371	*Pseudomonas azotoformans* strain B26 16S ribosomal RNA gene, partial sequence	1122	99.55%	100%
LJP 372	*Pseudomonas* sp. strain SeaQual_P_B791/7 16S ribosomal RNA gene, partial sequence	938	98.06%	98%
LJP 375	*Pseudomonas azotoformans* strain D116S ribosomal RNA gene, partial sequence	1050	99.62%	99%
LJP 383	*Pseudomonas fluorescens* strain c50 16S ribosomal RNA gene, partial sequence	335	96.40%	99%
LJP 418	*Pseudomonas marginalis* strain PZG_A16 16S ribosomal RNA gene, partial sequence	1102	99.46%	100%
LJP 424	*Stenotrophomonas* sp. strain B10 16S ribosomal RNA gene, partial sequence	549	99.45%	100%
LJP 717	*Stenotrophomonas* sp. strain CDB16 16S ribosomal RNA gene, partial sequence	815	98.90%	100%
LJP 722	*Pseudomonas libanensis* strain EB367 16S ribosomal RNA gene, partial sequence	828	99.88%	99%
LJP 724	*Pseudomonas poae* strain XJX-5 16S ribosomal RNA gene, partial sequence	427	99.30%	100%
LJP 728	*Pseudomonas paralactis* strain RTFHPD 264 16S ribosomal RNA gene, partial sequence	887	98.42%	99%
LJP 762	*Serratia grimesii* strain 5DXQ9 16S ribosomal RNA gene, partial sequence	870	98.96%	99%
LJP 764	*Serratia liquefaciens* strain S1 chromosome, complete genome	1068	99.81%	99%
LJP 765	*Serratia liquefaciens* strain S1 chromosome, complete genome	1119	99.64%	99%
LJP 794	*Serratia liquefaciens* strain S1 chromosome, complete genome	1050	99.90%	99%
LJP 797	*Pseudomonas azotoformans* strain D1 16S ribosomal RNA gene, partial sequence	901	98.78%	99%

Appendix D

Gel image of PCR assay for *bcrABC* – Detection of faint amplicons

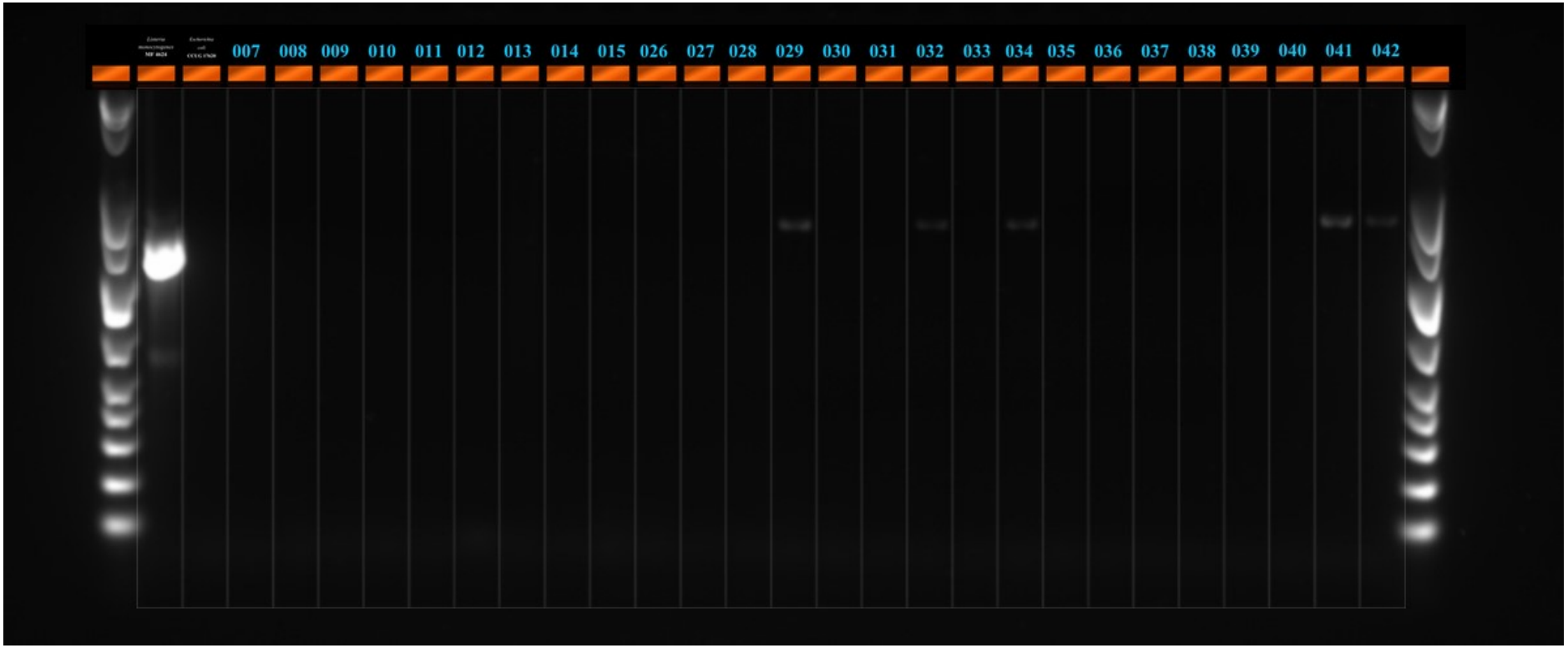

Figure 1: Gel image from the PCR assay for bcrABC. L. monocytogenes MF 4624 and E. coli CCUG 17620 were used as positive and negative controls, respectively. Faint amplicons were detected for isolates LJP 029, LJP 032, LJP 034, LJP 041 and LJP 042.

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Appendix E

Overview of results for ARG screening and disk diffusion

Table 1: Overview of bacterial isolates and reference strains with respective results for the disk diffusion test and ARG screening. Green cells represent positive strains, i.e. detection of clear DNA bands. Yellow cells represent the detection of faint amplicons. Red cells represent negative test results, i.e. no detection of DNA bands.

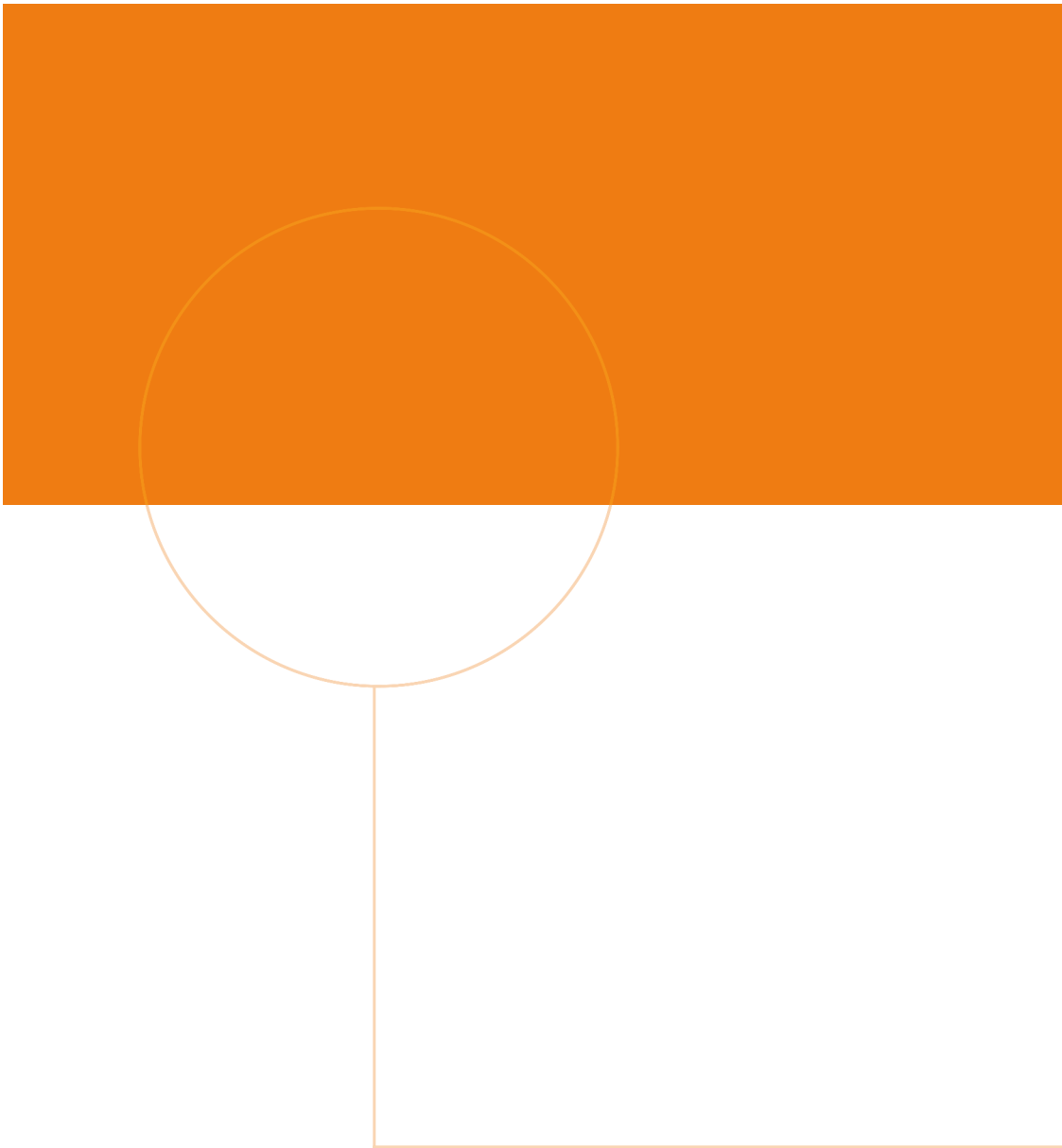
Isolate ID	Identified species	Disk Diffusion - Zone Diameter (mm)			Antimicrobial Resistance Genes (ARGs)							
		Florfenicol, 30.0 µg	Ampicillin, 10.0 µg	Tetracycline, 30.0 µg	*floR/ floR2	**floR/ floR2	sulI	tetG	ampC	qacH	qacE-Δ1	bcr-ABC
Ref.	*Pseudomonas aeruginosa* CCUG 17619	< 7 mm (R)	< 7 mm (R)	18 mm (S)	n.d.	n.d.	n.d.				n.d.	
Ref.	*Pseudomonas aeruginosa* CCUG 59347	< 7 mm (R)	< 7 mm (R)	< 7 mm (R)					n.d.			
Ref.	*Pseudomonas aeruginosa* NCTC 13717	n.d.	n.d.	n.d.					n.d.			
Ref.	*Escherichia coli* CCUG 17620	24 mm (S)	18 mm (S)	24 mm (S)			n.d.				n.d.	
Ref.	*Acinetobacter baumannii* NTCT 13305	< 7 mm (R)	< 7 mm (R)	20 mm (S)			n.d.	n.d.	n.d.	n.d.		n.d.
Ref.	*Listeria monocytogenes* ST8 MF 4624	30 mm (S)	29 mm (S)	29 mm (S)	n.d.	n.d.		n.d.	n.d.			
Ref.	*Listeria monocytogenes* ST8 MF 5634	30 mm (S)	28 mm (S)	30 mm (S)	n.d.	n.d.		n.d.	n.d.			
Ref.	*Listeria innocua* CCUG 15531	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	
Ref.	*Listeria innocua* CCUG 44813	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		n.d.	
LJP 007	*Pseudoalteromonas* sp. strain Arc7-671	n.d.	n.d.	n.d.								
LJP 008	*Pseudoalteromonas distincta* strain 20KNS10Z3	n.d.	n.d.	n.d.								
LJP 009	*Pseudomonas brenneri* strain PF37	n.d.	n.d.	n.d.								
LJP 010	*Pseudoalteromonas* sp. strain BOMB.9.10.22	n.d.	n.d.	n.d.								
LJP 011	*Pseudoalteromonas* sp. strain Arc7-671	n.d.	n.d.	n.d.								
LJP 012	*Stenotrophomonas rhizophila* strain KR2-13	n.d.	n.d.	n.d.								
LJP 013	*Pseudoalteromonas* sp. S-1 16S	n.d.	n.d.	n.d.								
LJP 014	*Pseudomonas fluorescens* strain PF67	n.d.	n.d.	n.d.								
LJP 015	*Pseudoalteromonas issachenkonii* strain 3B	n.d.	n.d.	n.d.								
LJP 026	*Pseudomonas azotoformans* strain C	13 mm (R)	< 7 mm (R)	25 mm (S)								
LJP 027	*Pseudomonas reactans* gene	9 mm (R)	< 7 mm (R)	24 mm (S)								
LJP 028	n.d.	n.d.	n.d.	n.d.								
LJP 029	*Pseudomonas* sp. CCUG 62357	11 mm (R)	< 7 mm (R)	25 mm (S)								
LJP 030	*Pseudomonas fluorescens* strain PF67	8 mm (R)	< 7 mm (R)	25 mm (S)								
LJP 031	*Pseudomonas cedrina subsp. fulgida*	8 mm (R)	< 7 mm (R)	25 mm (S)								
LJP 032	*Pseudomonas* sp. CCUG 62357	n.d.	n.d.	n.d.								
LJP 033	*Pseudomonas tolaasii* strain Pt102	11 mm (R)	< 7 mm (R)	24 mm (S)								
LJP 034	*Pseudomonas* sp. CCUG 62357	n.d.	n.d.	n.d.								
LJP 035	*Pseudomonas fluorescens* strain PF59	n.d.	n.d.	n.d.								
LJP 036	n.d.	n.d.	n.d.	n.d.								
LJP 037	*Pseudomonas umsongensis* strain SeaQual_P_81/6	12 mm (R)	< 7 mm (R)	25 mm (S)								
LJP 038	*Pseudomonas tolaasii* strain Pt102	8 mm (R)	< 7 mm (R)	24 mm (S)								
LJP 039	*Pseudomonas cedrina* strain JM10	9 mm (R)	< 7 mm (R)	23 mm (S)								
LJP 040	*Pseudomonas fluorescens* strain PF59	9 mm (R)	< 7 mm (R)	25 mm (S)								
LJP 041	*Pseudomonas* sp. CCUG 62357	n.d.	n.d.	n.d.								
LJP 042	*Pseudomonas fluorescens strain W-6 chromosome*	12 mm (R)	< 7 mm (R)	22 mm (S)								
LJP 043	*Pseudomonas cf. synxantha* V4.BP.03	n.d.	n.d.	n.d.								
LJP 044	*Pseudomonas fluorescens* strain PF59	11 mm (R)	< 7 mm (R)	24 mm (S)								

LJP 045	*Pseudomonas fluorescens* strain PF59	n.d.	n.d.	n.d.								
LJP 046	*Pseudomonas* sp.	**9 mm (R)**	**< 7 mm (R)**	**24 mm (S)**								
LJP 308	*Aeromonas piscicola* strain CECT 7443	n.d.	n.d.	n.d.								
LJP 309	*Pseudomonas marincola* strain SeaQual_P_B24	n.d.	n.d.	n.d.								
LJP 310	*Pseudomonas fluorescens* strain: ATCC 17573	n.d.	n.d.	n.d.								
LJP 311	*Pseudomonas* sp. P6169	n.d.	n.d.	n.d.								
LJP 312	*Pseudomonas pseudoalcaligenes* strain MA3	**14 mm (R)**	**9 mm (R)**	**23 mm (S)**								
LJP 313	*Pseudomonas* sp. M30-35	n.d.	n.d.	n.d.								
LJP 314	*Pseudomonas guineae* strain OX1110	**22 mm (S)**	**23 mm (S)**	**27 mm (S)**								
LJP 315	*Pseudomonas guineae* strain OX1110	n.d.	n.d.	n.d.								
LJP 316	*Pseudomonas fluorescens*, strain: ATCC 17573	**10 mm (R)**	**< 7 mm (R)**	**24 mm (S)**								
LJP 317	*Morganella psychrotolerans* strain	n.d.	n.d.	n.d.								
LJP 318	*Aeromonas salmonicida* strain JF11	n.d.	n.d.	n.d.								
LJP 319	*Morganella psychrotolerans* strain U2/5	n.d.	n.d.	n.d.								
LJP 320	*Pseudomonas* sp. M30-35	n.d.	n.d.	n.d.								
LJP 321	*Pseudomonas fluorescens* strain: ATCC 17573	**11 mm (R)**	**< 7 mm (R)**	**22 mm (S)**								
LJP 323	*Morganella psychrotolerans* strain U2/5	**30 mm (S)**	**< 7 mm (R)**	**31 mm (S)**								
LJP 324	*Morganella psychrotolerans* strain C11	n.d.	n.d.	n.d.								
LJP 325	*Aeromonas bestiarum* strain ESV-384	n.d.	n.d.	n.d.								
LJP 326	*Pseudomonas fluorescens* strain: ATCC 17573	n.d.	n.d.	n.d.								
LJP 327	*Pseudomonas* sp. strain P12Ogen	**23 mm (S)**	**28 mm (S)**	**28 mm (S)**								
LJP 328	*Aeromonas hydrophila* strain 23-C-23	**29 mm (S)**	**< 7 mm (R)**	**12 mm (I)**								
LJP 329	*Pseudomonas* sp. strain P12O	n.d.	n.d.	n.d.								
LJP 332	*Morganella psychrotolerans* strain U2/5	n.d.	n.d.	n.d.								
LJP 333	*Morganella psychrotolerans* strain C11	n.d.	n.d.	n.d.								
LJP 334	*Morganella psychrotolerans* strain C11	n.d.	n.d.	n.d.								
LJP 335	*Morganella psychrotolerans* strain 769	n.d.	n.d.	n.d.								
LJP 339	n.d.	n.d.	n.d.	n.d.								
LJP 341	*Pseudomonas fluorescens* strain PF59	**10 mm (R)**	**< 7 mm (R)**	**24 mm (S)**								
LJP 342	*Serratia* sp. QZS4_4	n.d.	n.d.	n.d.								
LJP 343	*Pseudomonas* sp. SXM-1	**13 mm (R)**	**< 7 mm (R)**	**27 mm (S)**								
LJP 344	*Pseudomonas azotoformans* strain 7RMB	**10 mm (R)**	**< 7 mm (R)**	**25 mm (S)**								
LJP 345	*Enterobacteriaceae bacterium* ENUB8	**24 mm (S)**	**21 mm (S)**	**24 mm (S)**								
LJP 346	*Enterobacteriaceae bacterium* ENUB8	**23 mm (S)**	**20 mm (S)**	**24 mm (S)**								
LJP 347	*Serratia proteamaculans* strain KB49	**25 mm (S)**	**21 mm (S)**	**24 mm (S)**								
LJP 360	*Pseudomonas cedrina* strain Cl-10	**11 mm (R)**	**< 7 mm (R)**	**29 mm (S)**								
LJP 362	*Pseudomonas fluorescens* strain PF100	n.d.	n.d.	n.d.								
LJP 363	*Pseudomonas fluorescens* strain PF85	n.d.	n.d.	n.d.								
LJP 364	*Pseudomonas fluorescens* strain PF59	n.d.	n.d.	n.d.								
LJP 365	*Pseudomonas* sp. strain CM-CNRG 623	**12 mm (R)**	**< 7 mm (R)**	**25 mm (S)**								
LJP 366	*Pseudomonas azotoformans*	**11 mm (R)**	**< 7 mm (R)**	**25 mm (S)**								
LJP 367	*Pseudomonas* sp. CCUG 66625	n.d.	n.d.	n.d.								
LJP 368	*Pseudomonas* sp. CCUG 66625	n.d.	n.d.	n.d.								
LJP 369	*Pseudomonas fluorescens* strain PF85	**9 mm (R)**	**< 7 mm (R)**	**21 mm (S)**								
LJP 370	*Pseudomonas fluorescens* strain PF64	**9 mm (R)**	**< 7 mm (R)**	**26 mm (S)**								
LJP 371	*Pseudomonas azotoformans* strain B26	**8 mm (R)**	**< 7 mm (R)**	**27 mm (S)**								
LJP 372	*Pseudomonas* sp. strain SeaQual_P_B791/7	n.d.	n.d.	n.d.								
LJP 373a	*Pseudomonas fluorescens* strain PF80	n.d.	n.d.	n.d.								
LJP 374	*Pseudomonas fluorescens* strain PF 53	**13 mm (R)**	**< 7 mm (R)**	**26 mm (S)**								
LJP 375	*Pseudomonas azotoformans* strain D1	**8 mm (R)**	**< 7 mm (R)**	**24 mm (S)**								
LJP 376	*Pseudomonas* sp. CCUG 66625	n.d.	n.d.	n.d.								
LJP 378	*Stenotrophomonas rhizophila* strain KR2-13	n.d.	n.d.	n.d.								
LJP 379	*Pseudomonas fluorescens* strain PF85	**10 mm (R)**	**< 7 mm (R)**	**22 mm (S)**								
LJP 380	*Pseudomonas* sp. CCUG 66625	**13 mm (R)**	**< 7 mm (R)**	**26 mm (S)**								

LJP 381	*Pseudomonas fluorescens* strain PF85	n.d.	n.d.	n.d.								
LJP 382	*Pseudomonas fluorescens* strain PF85	n.d.	n.d.	n.d.								
LJP 383	*Pseudomonas fluorescens* strain c50	**12 mm (R)**	**< 7 mm (R)**	**25 mm (S)**								
LJP 384	*Pseudomonas fluorescens* strain PF85	n.d.	n.d.	n.d.								
LJP 385	*Pseudomonas fluorescens* strain PF85	n.d.	n.d.	n.d.								
LJP 417	*Pseudomonas fluorescens* strain PF80	n.d.	n.d.	n.d.								
LJP 418	*Pseudomonas marginalis* strain PZG_A16	**9 mm (R)**	**< 7 mm (R)**	**26 mm (S)**								
LJP 419	*Pseudomonas fluorescens* strain PF56	**12 mm (R)**	**< 7 mm (R)**	**23 mm (S)**								
LJP 420	*Stenotrophomonas rhizophila* strain VITDW0102	n.d.	n.d.	n.d.								
LJP 421	*Pseudomonas lurida* strain MYb11	**10 mm (R)**	**< 7 mm (R)**	**23 mm (S)**								
LJP 422	*Pseudomonas fluorescens* strain PF100	n.d.	n.d.	n.d.								
LJP 423	*Pseudomonas fluorescens* strain PF85	**9 mm (R)**	**< 7 mm (R)**	**18 mm (S)**								
LJP 424	*Stenotrophomonas* sp. strain B10	**30 mm (S)**	**< 7 mm (R)**	**27 mm (S)**								
LJP 425	*Pseudomonas fluorescens* strain PF85	n.d.	n.d.	n.d.								
LJP 426	*Pseudomonas fluorescens* strain PF59	n.d.	n.d.	n.d.								
LJP 658	*Pseudoalteromonas distincta* strain 20KNS10Z3	n.d.	n.d.	n.d.								
LJP 659	*Pseudomonas fluorescens* strain PF85	n.d.	n.d.	n.d.								
LJP 660	*Pseudoalteromonas distincta* strain 20KNS10Z3	n.d.	n.d.	n.d.								
LJP 661	*Pseudomonas fluorescens* strain: ATCC 17573	n.d.	n.d.	n.d.								
LJP 705	*Pseudomonas fluorescens* strain PF85	n.d.	n.d.	n.d.								
LJP 706	*Pseudomonas gessardii* strain CIP 105469T	**12 mm (R)**	**< 7 mm (R)**	**25 mm (S)**								
LJP 707	*Pseudomonas gessardii* strain CIP 105469T	n.d.	n.d.	n.d.								
LJP 708	*Pseudomonas* sp. Irchel 3E20	**12 mm (R)**	**< 7 mm (R)**	**22 mm (S)**								
LJP 709	*Pseudomonas* sp. Irchel s2f8	n.d.	n.d.	n.d.								
LJP 710	*Pseudomonas azotoformans* SW_HL_6_52	**10 mm (R)**	**< 7 mm (R)**	**25 mm (S)**								
LJP 711	*Pseudomonas* sp. Irchel 3E20 strain Irchel 3E20	n.d.	n.d.	n.d.								
LJP 712	*Pseudomonas* sp. Irchel 3E20 strain Irchel 3E20	n.d.	n.d.	n.d.								
LJP 713	*Pseudomonas fluorescens* strain PF56	n.d.	n.d.	n.d.								
LJP 714	*Pseudomonas gessardii* strain CIP 105469T	n.d.	n.d.	n.d.								
LJP 715	*Pseudomonas* sp. Irchel 3E20 strain Irchel 3E20	n.d.	n.d.	n.d.								
LJP 716	*Pseudomonas gessardii* strain CIP 105469T	n.d.	n.d.	n.d.								
LJP 717	*Stenotrophomonas* sp. strain CDB16	n.d.	n.d.	n.d.								
LJP 718	*Pseudomonas fluorescens* strain PF85	**9 mm (R)**	**< 7 mm (R)**	**21 mm (S)**								
LJP 719	*Pseudomonas fluorescens* strain PF56 RNA	n.d.	n.d.	n.d.								
LJP 720	*Pseudomonas fluorescens* strain: ATCC 17573	n.d.	n.d.	n.d.								
LJP 721	*Pseudomonas fluorescens* strain PF56	n.d.	n.d.	n.d.								
LJP 722	*Pseudomonas libanensis* strain EB367	**15 mm (S)**	**< 7 mm (R)**	**28 mm (S)**								
LJP 723	*Pseudomonas* sp. rDWA119	**13 mm (R)**	**< 7 mm (R)**	**23 mm (S)**								
LJP 724	*Pseudomonas poae* strain XJX-5	**8 mm (R)**	**< 7 mm (R)**	**26 mm (S)**								
LJP 725	*Pseudomonas fluorescens* strain: ATCC 17573	n.d.	n.d.	n.d.								
LJP 726	*Pseudomonas fluorescens* strain PF89	n.d.	n.d.	n.d.								
LJP 727	*Pseudomonas fluorescens* strain: ATCC 17573	n.d.	n.d.	n.d.								
LJP 728	*Pseudomonas paralactis* strain RTFHPD 264	**12 mm (R)**	**< 7 mm (R)**	**29 mm (S)**								
LJP 760	*Pseudomonas putida* strain CFBP 5933	**19 mm (S)**	**11 mm (R)**	**21 mm (S)**								
LJP 761	*Serratia liquefaciens* strain FG3	**21 mm (S)**	**12 mm (R)**	**20 mm (S)**								
LJP 762	*Enterobacteriaceae bacterium* Poky4i	**24 mm (S)**	**14 mm (I)**	**22 mm (S)**								
LJP 763	*Serratia liquefaciens* strain FG3	n.d.	n.d.	n.d.								
LJP 764	*Serratia liquefaciens* strain	**24 mm (S)**	**14 mm (I)**	**21 mm (S)**								
LJP 765	*Enterobacteriaceae bacterium* Poky4i	**23 mm (S)**	**12 mm (R)**	**21 mm (S)**								
LJP 766	*Serratia liquefaciens* strain FG3	n.d.	n.d.	n.d.								
LJP 788	*Pseudomonas lundensis*	**24 mm (S)**	**18 mm (S)**	**23 mm (S)**								
LJP 794	*Serratia liquefaciens* strain	n.d.	n.d.	n.d.								
LJP 795	*Pseudomonas fluorescens* strain I5B	**23 mm (S)**	**13 mm (R)**	**21 mm (S)**								
LJP 796	*Pseudomonas fluorescens* strain	**24 mm (S)**	**18 mm (S)**	**23 mm (S)**								

LJP 797	*Pseudomonas azotoformans* strain D1	**22 mm (S)**	**16 mm (S)**	**20 mm (S)**								
LJP 798	*Pseudomonas fluorescens* strain PF85	n.d.	n.d.	n.d.								
LJP 799	*Pseudomonas* sp. rDWA116	**22 mm (S)**	**15 mm (S)**	**22 mm (S)**								
LJP 800	*Pseudomonas fluorescens* strain FW300N2E3	n.d.	n.d.	n.d.								
LJP 801	*Pseudomonas fluorescens* strain FW300N2E3	n.d.	n.d.	n.d.								
LJP 802	*Pseudomonas* sp. rDWA116	n.d.	n.d.	n.d.								
F1K2.353	*Listeria monocytogenes*	**28 mm (S)**	**27 mm (S)**	**27 mm (S)**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
SL3.189	*Listeria monocytogenes*	n.d.	n.d.	n.d.								
HK3.331	*Listeria monocytogenes*	**28 mm (S)**	**26 mm (S)**	**27 mm (S)**								
SL6.212	*Listeria monocytogenes*	n.d.	n.d.	n.d.								
SL6.141	*Listeria monocytogenes*	n.d.	n.d.	n.d.								
PK.141	*Listeria monocytogenes*	n.d.	n.d.	n.d.								
FS.171	*Listeria monocytogenes*	n.d.	n.d.	n.d.								
SL3.179	*Listeria monocytogenes*	n.d.	n.d.	n.d.								
SL6.206	*Listeria monocytogenes*	n.d.	n.d.	n.d.								
SL3.212	*Listeria monocytogenes*	n.d.	n.d.	n.d.								
SL6.218-59	*Listeria monocytogenes*	n.d.	n.d.	n.d.								
SL6.218-60	*Listeria monocytogenes*	n.d.	n.d.	n.d.								
SwF1.296	*Listeria innocua*	n.d.	n.d.	n.d.								
SL3.296	*Listeria monocytogenes*	n.d.	n.d.	n.d.								
HK3.297	*Listeria monocytogenes*	n.d.	n.d.	n.d.								
HK1.329h	*Listeria monocytogenes*	n.d.	n.d.	n.d.								
HK1.329v	*Listeria monocytogenes*	n.d.	n.d.	n.d.								
HK.3.357	*Listeria monocytogenes*	n.d.	n.d.	n.d.								
SwF1.357	*Listeria innocua*	n.d.	n.d.	n.d.								
F1K1.353	*Listeria innocua*	n.d.	n.d.	n.d.								

** PCR assay with floR primers by Zhao et al. (2016)*
*** PCR assay with floR primers from this study*



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