Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

Ingrid M. Duister

Identification and characterization of *Listeria monocytogenes* originating from the salmon processing value chain

Master's thesis in MSBIOTECH
Supervisor: Lisbeth Mehli
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Preface

This thesis marks the end of my time as a master student at the Institute for Biotechnology and Food Science at the Norwegian University of Science and Technology (NTNU) Trondheim. This thesis was part of the TraceListeria research projected, which was an interdisciplinary project between a private seafood company in Norway, the University of Copenhagen (KU) and NTNU. The project was funded in most part by the Norwegian Seafood Research Fund, as well as NTNU, KU and the seafood company which I have chosen to keep anonymous throughout the study. TraceListeria aimed to better understand the occurrence, contamination points, and paths of infection for *Listeria monocytogenes* by systematically screening the entire value chain of the seafood production industry.

I wish to take this opportunity to express my gratitude towards my main thesis supervisor, dr. Lisbeth Mehli. Lisbeth has shown immense kindness, compassion, and support during the entire process of completing my thesis work. At times the stress seemed overwhelming, and that is when her support came through the most as she motivated me to do the very best and be the very best that I can be. For this, I will be eternally grateful. In addition, I must thank my co-supervisor Heidi Camilla Sagen-Ohren, from Aquatiq, for her industry relevant expertise in analyzing the results obtained during the study. Her insights were invaluable to the project.

It would be amiss if I did not take this opportunity to thank everybody working in the lab for their emotional and technical support. I would like to focus especially on John-Kristian Jameson. Without his good wit and technical skills in the lab, this thesis would not be what it is. He made the long days in the lab easier with his good company, and I wish him all the best in all his future endeavors. Ingrid Margrethe Boneng is the student I worked most closely with during my time in the lab, as she also worked on the TraceListeria project. She provided me with a discussion partner, and we have had many good conversations that have helped me grow into a more reflective person.

Finally, I must thank my family. My mother introduced me to the wonders of science and nature at an early age, and without her I would obviously not be where I am today. Alongside my father they instilled a solid work ethic in me that has allowed me to blindly follow my ambitions knowing I have the support of my family behind me, always. I love you and thank you for everything.

Abstract

The identification, source tracking and surveillance of foodborne pathogens such as *Listeria monocytogenes* (*L. monocytogenes*) are crucial for the food industry to limit major outbreaks with severe consequence of human life. Several techniques within strain-level differentiation of pathogens have been developed, with whole-genome sequencing (WGS) being the most discriminatory. However, WGS has not been implemented in large by the food industry as it requires the right infrastructure, high bioinformatical skill and is a very expensive method for large scale studies. The need for a faster, cheaper, and more user-friendly method has been developed. A method combining classic Rep-PCR with Oxford Nanopore sequencing (ON-rep-seq) has been developed recently. In this study, we demonstrate the ability of the ON-rep-seq assay to differentiate isolates of *L. monocytogenes* down to the strain level with a sensitivity comparable with that of WGS. In total 160 isolates from the filleting department of a salmon processing plant were analyzed using ON-rep-seq which showed a low level of genetic diversity among filleting department isolates. Based on ON-rep-seq results, 10 isolates were sent off for further characterization with WGS. The genomic data from the WGS assay shows a relationship between length count profile (LCp) plot morphology, obtained from ON-rep-seq, with sequence types (STs) obtained through Multi Locus Sequence Typing (MLST). Three different STs were observed in the filleting department, ST37 (n = 7), ST8 (n = 2) and ST637 (n = 1). To expand the collection of isolates, six isolates from the processing plant's own quality control team were included as well as all six ST8 isolates obtained earlier in the value chain examined by the TraceListeria project. Five of the quality control isolates were characterized as ST37 and the last as ST8. A phylogenetic analysis of high-quality single nucleotide polymorphisms (hqSNPs) was also performed which clustered isolates into groups corresponding to their STs. The isolates were also exposed to a variety of *in vitro* characterization assays such as antimicrobial susceptibility, cold tolerance, salt tolerance and acid tolerance. The phenotypic results were compared to WGS data. The antimicrobial susceptibility assay displayed almost no resistance to seven of the antibiotics tested, with the exception of cefoxitin to which *L. monocytogenes* is innately resistant. The WGS data supports these observations. The isolates' growth was not inhibited when exposed to cold, salt or acid, but somewhat limited. No relationship between ST and increased or decreased tolerance to these stress factors could be discerned.

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1. Introduction

Norway is known for its long coastline which alongside the arctic climate has created an environment for easy access to good quality seafood, and the subsequent development of seafood farming as demand has increased over time. Seafood is such a large industry that it makes up the third largest export after crude oil and gas, exporting a record high 3,1 million tons worth NOK 120,8 billion (~USD 13,4 billion) in 2021. This was a 14 percent increase from the previous year (*Record high Norwegian seafood exports in 2021*, 2022; sentralbyrå, 2022). This industry sector is hence vital to the economy of Norway. The increased demand for fresh, chilled fish over the past decades has posed a problem for the producer, namely the management of pathogenic, foodborne bacteria. Major foodborne outbreaks can have a substantial damaging impact on public health and the seafood industry. Therefore, microbial control is a key factor for increasing food safety as well as shelf life.

1.1. Listeria monocytogenes

Listeria monocytogenes (*L. monocytogenes*) is a Gram-positive bacterium first discovered in 1926 as a pathogen in rabbits and was later in 1929 discovered to also cause disease in humans (Cossart, 2007; Murray et al., 1926). Despite the scientific community's understanding of *L. monocytogenes*' pathogenicity, it was not linked to foodborne illness until a larger outbreak of listeriosis in 1983 in Canada (Schlech et al., 1983). Now it is widely understood that the main route of infection is through contaminated food sources. *L. monocytogenes* is the causative agent of listeriosis, a dangerous foodborne disease, especially for immunocompromised people and pregnant woman with a mortality rate between 20-30% (Hamon et al., 2006). Over 2500 cases of human listeriosis are reported in the EU every year, which collects data from 28 member states and 4 non-member states, making listeriosis the fifth most common foodborne disease. The highest proportions of infection was found in ready-to-eat (RTE) foods and especially seafood products such as smoked fish (European Food Safety et al., 2021). Therefore, controlling and preventing the contamination of *L. monocytogenes* in food processing plants has become a top priority for both producers and consumers to preserve public health.

L. monocytogenes is a member of the *Listeria* genus, which contains 19 species of non-spore-forming, facultative anaerobe, Gram-positive bacteria (Camejo et al., 2011; Ryser et al., 2019). *L. monocytogenes* is one of six closely related species that form a group referred to as *Listeria sensu stricto*, while the remaining 13 species are more phylogenetically distant from this group (Ryser et al., 2019). Within the genus only *L. monocytogenes* and *L. ivanovii* are considered pathogenic, although *L. ivanovii* is mostly found in ruminants and human infection

is extremely rare (Guillet et al., 2010; Orsi et al., 2011; Ryser et al., 2019). As a result, *L. monocytogenes* is the main pathogen of public health concern.

1.1.1. Listeriosis

The susceptibility of a host to *L. monocytogenes* predominantly depends on cell-mediated immunity, and most cases occur in individuals with diminished cell-mediated immunity. Consequently, the infectious dose is not known (Hamon et al., 2006; Schuchat et al., 1991). Listeriosis can materialize in two main ways, either invasive or non-invasive infection (Allerberger & Wagner, 2010; Schuchat et al., 1991; Swaminathan & Gerner-Smidt, 2007).

With non-invasive infection the symptoms are usually mild compared to the invasive kind and usually manifest as gastroenteritis. Individuals become ill within 24-48 hours of exposure to food contaminated with *L. monocytogenes*, and experience symptoms such as nausea, vomiting and diarrhea (Doganay, 2003; Swaminathan & Gerner-Smidt, 2007). The illness produces non-specific symptoms in this instance, so many cases go undiagnosed and the illness usually resolves itself without medical intervention (Swaminathan & Gerner-Smidt, 2007). In a few cases, however, gastroenteritis can lead to invasive listeriosis (Schlech & Acheson, 2000; Swaminathan & Gerner-Smidt, 2007).

Invasive listeriosis most commonly manifests as septicaemia or meningitis in immunocompromised individuals. In pregnant woman the disease either manifests as perinatal listeriosis or neonatal listeriosis. During pregnancy cell-mediated immunity is impaired, and a listerial infection can occur, most often during the third trimester (Allerberger & Wagner, 2010; Doganay, 2003; Schlech & Acheson, 2000; Swaminathan & Gerner-Smidt, 2007). Perinatal listeriosis may result in stillbirths, spontaneous abortion, or premature labor whilst neonatal listeriosis may result in neonatal distress of the infant such as meningitis. The latter occurs because *L. monocytogenes* can cross the fetoplacental barrier and propagate in the placenta, which is how it is able to infect a fetus when the mother has been exposed to contaminated food (Schlech & Acheson, 2000; Swaminathan & Gerner-Smidt, 2007). This ability to cross several blood barriers is also what leads to symptoms such as septicaemia and meningitis in adults (Hamon et al., 2006; Swaminathan & Gerner-Smidt, 2007). Sepsis may occur in compromised hosts, and will result in severe illness with fever, nausea, vomiting and possible acute respiratory distress and multi-organ system failure (Doganay, 2003). Meningitis is the most commonly recognized listerial infection and occurs when *L. monocytogenes* crosses the blood-brain barrier (Doganay, 2003; Hamon et al., 2006). The illness can either be acute or subacute

and carry symptoms such as high fever, stiffness in neck muscles, and involuntary movement such as tremors or seizures (Doganay, 2003).

If left untreated, invasive listeriosis is fatal, but *L. monocytogenes* is susceptible to wide range of antibiotics (Allerberger & Wagner, 2010; Doganay, 2003). Acquired antimicrobial resistance is rare in *L. monocytogenes*, with the exception of fosfomycin (Allerberger & Wagner, 2010; Swaminathan & Gerner-Smidt, 2007). Interestingly, the susceptibility of *L. monocytogenes* to antibiotics has not changed significantly since the latter half of the 20th century (Allerberger & Wagner, 2010; Andriyanov et al., 2021). The microbe is naturally susceptible to most β-lactam antibiotics such as penicillins, meropenem and ampicillin with the exception of cephalosporins, such as cefoxitin, to which it shows an intrinsic resistance (Allerberger & Wagner, 2010; Swaminathan & Gerner-Smidt, 2007; Troxler et al., 2000). This is likely due to two specific penicillin-binding proteins (PBP), namely PBP3 and PBP5 (Hakenbeck & Hof, 1991). For patients with β-lactam allergies trimethoprim-sulphamethoxazole or erythromycin could be used as an alternative (Allerberger & Wagner, 2010). Furthermore, *L. monocytogenes* is generally susceptible to fluoroquinolones and tetracycline (Swaminathan & Gerner-Smidt, 2007; Troxler et al., 2000).

1.1.2. Virulence

The pathophysiology of *L. monocytogenes* infection is thought to start in the gastrointestinal tract, as contaminated foods are the main sources for both epidemic and sporadic cases of listeriosis (Vázquez-Boland José et al., 2001). It is important to note that not all strains of *L. monocytogenes* cause disease in humans, in fact more than 90% of human infection is caused by three of the bacterium's 13 serotypes, mainly 4b, 1/2a and 1/2b (Chen & Knabel, 2007; Orsi et al., 2011; Pontello et al., 2012; Swaminathan & Gerner-Smidt, 2007). In the gastrointestinal tract, about 20h after ingestion, *L. monocytogenes* infects intestinal epithelial cells through the interaction of its surface protein internalin A (InlA), encoded by *inlA*, and the epithelial cadherin protein (E-cadherin) (Allerberger & Wagner, 2010; Hamon et al., 2006; Pamer, 2004). E-cadherin is a transmembrane protein where its intracellular domain forms a complex with actin proteins of the cytoskeleton through catenins, which are cadherin binding proteins. InlA binds to the extracellular domain of E-cadherin which triggers a cytoskeletal rearrangement of actin to allow for entry of *L. monocytogenes* (Cossart & Lecuit, 1998; Hamon et al., 2006; Shen et al., 2013). Another surface protein expressed by *L. monocytogenes* allows for bacterial entry into a range of other cells such as phagocytes, this is internalin B (InlB), encoded by *inlB*, which triggers the endocytic pathway by binding to the Met receptor (Hamon et al., 2006; Pamer,

2004). Met is a protein tyrosine kinase, and binding of InlB activates the activity of Met which induces the cytoskeletal rearrangement of actin required for uptake (Hamon et al., 2006). *L. monocytogenes* enters the host cell through the invagination of its cell wall around the bacterium which creates a vacuole. After entry the bacterium releases virulence factor listeriolysin O (LLO), encoded within the *hly* gene, which is a pore-forming cytotoxin that lyses the membrane of the vacuole. This facilitates bacterial release into the cell's cytoplasm (Cossart & Lecuit, 1998; Hamon et al., 2006; Ryser et al., 2019). LLO also has β-hemalytic acitivity that creates a distinctive phenotype on blood agar plates, a key identification criterion (Lecuit, 2020). Two phospholipases are also expressed and secreted to aid in the escape from the vacuole; phosphoinositide-phospholipase C (plcA) and phosphatidylinositol-specific phospholipase C (plcB), encoded by *plcA* and *plcB*, respectively. However, they are not expressed to the same extent as LLO. Once in the cytoplasm, *L. monocytogenes* will begin to multiply and with that a number of genes are upregulated to facilitate; growth, phagosomal lysis, actin-based motility and cell-to-cell spreading (Ryser et al., 2019). Intracytoplasmic cell-to-cell spreading is the result of actin-based mobility. The ability to spread from cell to cell without coming into contact with the extracellular matrix allows *L. monocytogenes* to circumvent circulating antibodies or other bactericidal compounds. Thereby the bacterium can propagate through tissue and cause serious infection. It mobilizes itself within the cytoplasm by polymerizing actin asymmetrically along its surface, generating an actin tail that propels it. This reaction is mediated by the surface protein ActA (Figure 1). *L. monocytogenes* is also able to disseminate into the bloodstream after uptake into epithelial or phagocytic cells and can spread to other parts of the host organism and cross tight barriers such as the blood-brain barrier and the fetoplacental barrier (Hamon et al., 2006; Lecuit, 2020).

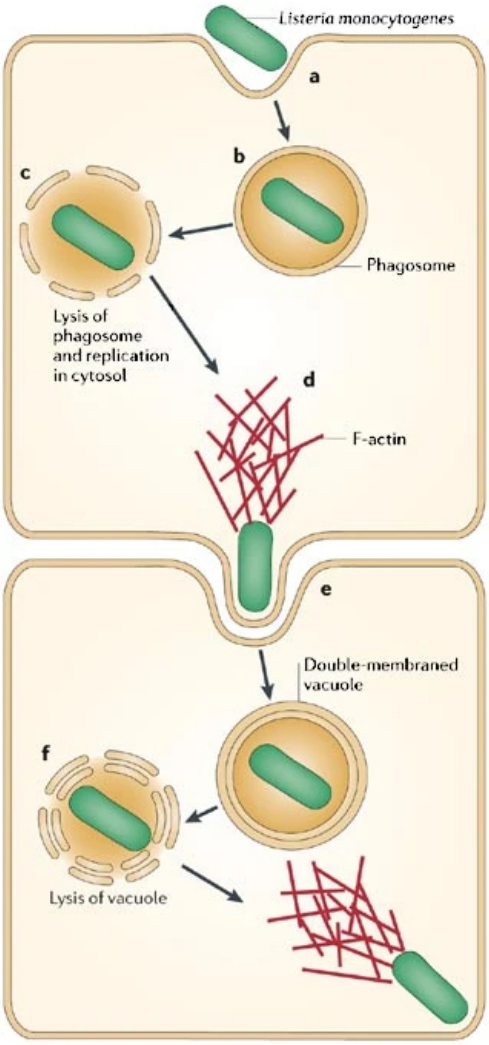

Figure 1 *– Entry of* L. monocytogenes *into a host cell and propagation through tissue. (a) Internalin interaction between either E-cadherin or MET receptor and subsequent cytoskeletal rearrangement that is required for the invagination of the cell wall around the bacterium, (b) uptake of bacterium in vacuole, (c)* L. monocytogenes *secretes the pore-forming toxin LLO and phospholipases plcA and plcB which lyses the membrane of the vacuole and promotes the escape of the bacterium into the cytoplasm where it multiplies, (d) The surface protein ActA polymerizes actin to create a tail that propels it through the cytoplasm, (e) the actin based mobility facilitates intracytoplasmic cell-to-cell spreading, (f) the bacterium again secretes LLO, plcA and plcB to escape from the double-membraned vacuole, continue the cycle and propagate through tissue. Copyrights obtained from (Hamon et al., 2006).*

1.1.3. Listeria monocytogenes in food processing plants

L. monocytogenes is ubiquitous in environments such as soil and water and is resistant to a range of physical and chemical agents. The bacterium is facultative anaerobe and can therefore survive in limited oxygen atmospheres, as well as refrigerated environments (T < 4°C), high salt concentrations (20%) and moderate to low acidity (pH 4.4-9.6) (Farber & Peterkin, 1991). *L. monocytogenes* achieves optimal growth at a temperature of 37°C, which is common for mammalian pathogens (Ryser et al., 2019). It is also able to form biofilms which protects the bacteria further from outside stressors. Biofilms are structured ecosystems for microorganisms that pose a threat in the food production industry, as they can persist over long periods and give way for persistent strains of pathogenic bacteria to continually contaminate the production line (Tompkin, 2004; van der Veen & Abee, 2011). These biofilms often form in environments with high moisture and access to organic material for nutrition where bacteria produce extracellular polymeric substances (EPS) to cling to a surface. From there it recruits other microorganisms which organize into a highly synergistic society (Donlan & Costerton, 2002). Biofilms are of

huge concern in the food processing industry as even after cleaning the processing environment might appear visually clean and pass inspection. When sampling the equipment before production, the results will also not detect the presence of bacteria. However, as the production line begins to move, the biofilms hidden in niches between close fitting materials such as metal-to-metal or metal-to-plastic will start to leak out into the production line. This occurs because of the vibrations of the equipment and water dislodging the microorganisms, leading to them contaminating the food (Tompkin, 2004).

Eliminating contamination of *L. monocytogenes* remains a challenge due to these traits, and the focus has shifted to limiting the contamination by implementing hazard analysis and critical control point (HACCP) programs. HACCP is a management tool with the objective to surveil critical control points (CCPs) in the food production chain. Hazard analysis at these CCPs establishes critical limits, monitoring procedures to determine whether limits have been exceeded, and corrective actions if control is lost. In addition to solid record-keeping procedures and effective documentation, these measurements help ensure food safety (Ropkins & Beck, 2000). A strong HACCP program is a prerequisite for any food business to comply with the microbial criteria and rules dictated by the Commission of the European Communities Reguluation (EC) No 2073/2005 (Community), 2005). A HACCP program with frequent testing for pathogenic bacteria and corrective actions to limit contamination is hence vital for hindering large foodborne outbreaks (Tompkin, 2004).

Foods that undergo several handling steps, processing environments and equipment are more likely to be contaminated by foodborne pathogens. This is probably because the complex equipment in larger processing plants is difficult to clean and sanitize, and the constant movement along a production line increases the likelihood of the food encountering a contaminated surface (Kovačević et al., 2012). In a salmon processing plant for instance, there are a lot of steps involved in the processing of fish to a RTE product. Within these steps large equipment such as gutting lines, conveyor belts and rollers, head cutters and slicers, to name a few, are involved. All this equipment has hollow areas and close-fitting material, and there is also large amounts of water involved in the processing of salmon. All these factors create sites where moisture accumulates and *L. monocytogenes* can grow and persist (Løvdal et al., 2017). In a study done by Nofima AS samples taken from equipment in Norwegian salmon processing plants directly after cleaning showed only a marginal decrease in the number of samples positive for *L. monocytogenes* (Heir & Langsrud, 2013). This indicates that some strains are very persistent in the processing environment. It is important to establish whether a strain is

persistent or transient, to differentiate between a one-time breach in protocol or whether the issue of contamination is endemic.

A brief overview of foodborne listeriosis outbreaks in Europe over the last decade, the contaminated RTE food source, Multi Locus Sequence Type (MLST) and consequence of human life is given in Table 1. The high mortality rate underlines the importance of surveillance of *L. monocytogenes* in any food production facility.

Table 1 – Overview of foodborne listeriosis outbreaks in Europe since 2012, number of cases linked to the outbreak, number of deaths resulting from illness, the identified contaminated food and MLST of *L. monocytogenes* strain isolated.

Year(s)	No. cases	No. deaths (%)	Food product	MLST	Reference
2014-2019	22	5 (23)	Cold-smoked salmon	8	(EFSA, 2019b)
2015-2018	41	6 (15)	Frozen vegetables	6	(European Food Safety et al., 2021)
2015-2018	12	4 (33)	Cold-smoked salmon	8	(EFSA, 2018)
2017-2019	21	3 (14)	RTE meat	6	(EFSA, 2019a)
2018-2019	112	7 (6)	Blood sausage	6	(Halbedel et al., 2020)
2014	41	17 (41)	RTE meat	224	(Kvistholm Jensen et al., 2016)
2012-2016	26	3 (12)	Turkey meat	8	(Gelbíčová et al., 2018)
2015-2018	34	10 (29)	Soft cheese	6	(Nüesch-Inderbinen et al., 2021)
2013-2015	10	4 (40)	Cold-smoked salmon	391	(Gillesberg Lassen et al., 2016)
2013-2015	10	3 (30)	Cold-smoked halibut	6	(Gillesberg Lassen et al., 2016)

1.1.4. Detection and subtyping L. monocytogenes in food processing plants

There are several methods to detect and isolate *L. monocytogenes* from the food processing environment. More conventional methods utilize enrichment media and phenotypic characteristics to identify the presence of *L. monocytogenes* and these methods are still widely in use today as they are fairly cheap and easy to perform on-site. The most common selective agents employed to suppress competing microflora for *Listeria species* (*Listeria spp.*) are nalidixic acid to suppress Gram-negative bacteria, acriflavine to suppress other Gram-positive bacteria and cycloheximide to inhibit the growth of yeasts or fungi. Other antimicrobials such as lithium chloride are also often used (Jantzen et al., 2006). The enrichment is then plated onto enrichment plates where the colonies can be examined for phenotypical traits. The Thermo Scientific™ Brilliance™ *Listeria* Agar Base (BLA) used in this study to isolate and differentiate between *Listeria spp.* and *L. monocytogenes* utilizes nalidixic acid and lithium chloride, polymyxin B, X-glucoside and lastly amphotericin to suppress yeast and fungi. X-glucoside is a chromogen that is cleaved by β-glucosidase which is common to all *Listeria spp*. To further characterize pathogenic *L. monocytogenes* or *L. ivanovii* the medium contains

lecithin, which is hydrolyzed by the enzyme lecithinase specific to these species. This creates a distinctive opaque precipitate around the colonies (Figure 2) (ThermoFisherScientific). As it requires very little training to perform this procedure, it is readily used even with the advancements in DNA technology over the last 20 years. However, one must note that although this method can differentiate between *Listeria spp.* and other microorganisms, as well as between *Listeria spp.* and *L. monocytogenes* and *L. ivanovii*, it is not species or strain specific. The method is also time consuming as the enrichment and plating of the bacteria takes days to gain results. Therefore, it is recommended that this method be used in addition to other methods that can differentiate based on genotype and not only phenotype.

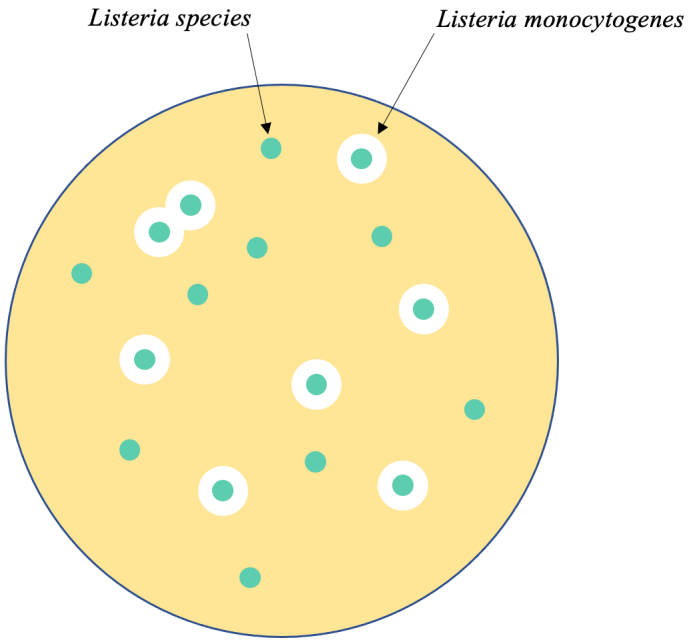

***Figure 2** - BLA plate schematic for how* L. monocytogenes *can be phenotypically distinguished from* Listeria spp. L. monocytogenes *will hydrolyze lecithin which creates an opaque zone around the colonies. All* Listeria spp. *will show as green colonies on BLA. Modified from (ThermoFisherScientific).*

A method for quick detection of *Listeria spp.* and *L. monocytogenes* also builds on the enrichment of the microorganisms using a selective broth. After initial cultivation the DNA in the solution can be isolated and used in a real-time quantitative polymerase chain reaction (qPCR) (Jantzen et al., 2006). The qPCR assay is a modification to the original PCR method that includes fluorescent quantification of the nucleic product (Jantzen et al., 2006; Mackay, 2004). PCR utilizes deoxyribose nucleoside triphosphates (dNTPs), oligonucleotide primers and a thermostable DNA polymerase to amplify a DNA sequence in a set of cycles. First, the DNA needs to be denatured and this is done by increasing the temperature to ~95°C. The reason for using a thermostable polymerase is evident in this step as it will not denature at high temperatures. The temperature is then decreased to ~60°C to allow the primers to anneal to the target sequence before it is increased again to ~70°C for the elongation of the target sequence.

These steps are repeated in several cycles, amplifying the DNA sequence exponentially. The cycles are preceded by an initial denaturation of the DNA and succeeded by a final extension of the sequence. In ordinary PCR analyses, the product of the chain reactions is loaded onto a agarose gel for identification (Rahman et al., 2013). The amplicon detection process is what differs qPCR from conventional PCR assays, as the oligonucleotides used are labeled with molecules capable of fluorescing. The fluorescence is then detected and measured by a sensor in the thermocycler in real time, giving results instantly and excluding the need for post amplified procedures such as gel electrophoresis (Mackay, 2004; Rahman et al., 2013). The Suretect™ kits used for the qPCR assay in this study has primers specific to either *Listeria spp.* or *L. monocytogenes* which allows for identification on the species level. Although, this assay will not discriminate down to the strain level of the microorganisms.

As previously mentioned, more than 90% of foodborne outbreaks of listeriosis are caused by only 3 serotypes of *L. monocytogenes* (Chen & Knabel, 2007; Orsi et al., 2011; Pontello et al., 2012; Swaminathan & Gerner-Smidt, 2007). Therefore, strain-level discrimination has become increasingly more important in the prevention, tracing, and surveillance of foodborne listeriosis outbreaks. A PCR assay was introduced in 1994 by Versalovic. et al (1994) that utilizes the interspersed repetitive sequences as a binding site for primers (Versalovic et al., 1994). Repetitive extragenic palindromic (Rep) sequences are highly conserved repetitive DNA sequences first discovered in *Escherichia coli* and *Salmonella typhirium* (Stern et al., 1984). By using them as binding sites for primers and amplifying the regions between the repetitive sequences, amplicons of various sizes can be separated with electrophoresis thereby creating a distinctive pattern on a gel, like a genomic fingerprint. This Rep based PCR (Rep-PCR) assay only requires a single primer set to be used for DNA fingerprinting of several organisms even down to the strain level (Versalovic et al., 1994). This assay has been used for discriminating between serotypes of *L. monocytogenes* in several studies, which is a helpful technique when examining and source tracking isolates from food processing plants (Chou & Wang, 2006; Jeršek et al., 1999; Jersek et al., 1996).

Rep-PCR is, however, unable to taxonomically identify the isolate species. By combining this PCR assay with the sequencing of the amplicons using Oxford Nanopore technologies, Krych et. al (2019) presented a modified PCR assay, called ON-rep-seq (Krych et al., 2019). Sequences that were earlier only known as fingerprint patterns on a gel can now be used for accurate taxonomic identification, often down to the strain level (Chou & Wang, 2006; Thomassen et al., 2021). In 2021 Thomassen et. al (2021) presented a study where ON-rep-seq was used to prove that isolates from the same strain of *L. monocytogenes* persistently

contaminated the head and tail cutter in the same Norwegian salmon processing plant examined in this study. They also found that the strain level discrimination provided by ON-rep-seq was in accordance with whole genome sequencing (WGS) data of the isolates. Thereby, presenting ON-rep-seq as a primary, cheaper screening method alternative to WGS for strain level differentiation and identification (Krych et al., 2019; Thomassen et al., 2021).

Serotyping is often used in tracing of foodborne outbreaks of listeriosis, as only a few serotypes are responsible for majority of outbreaks (Chen & Knabel, 2007; Orsi et al., 2011; Pontello et al., 2012; Swaminathan & Gerner-Smidt, 2007). If a major foodborne outbreak is caused by a 4b serotype strain, foods testing positive for other serotypes can then likely be ruled out as having been the source of contamination. *L. monocytogenes* have different surface antigens, either somatic (O) or flagellar (H). There are 15 different subtypes of O antigens (I-XV), and 4 H antigen subtypes (A-D). Each serotype can be determined by their unique combinations of O and H antigens using antisera (Table 2) (Liu, 2006; Seeliger & Höhne, 1979). The *Listeria* species *Listeria seeligeri* also contain serotypes 1/2a, 1/2b 3b, 4a, 4b, 4c and 6b. This creates a problem in the serological assay's ability to differentiate isolates unless a species differentiation method is implemented beforehand. In addition the high costs of the antisera required for the assay has limited this method for widespread clinical use (Liu, 2006). Serotyping can also be performed by multiplex-PCR, but this method although more cost- and time effective cannot distinguish isolates to specific serotypes. Instead it clusters isolates into broader serogroups (Kérouanton et al., 2010).

Table 2 – Composition of somatic (O) and flagellar (H) antigens in *Listeria monocytogenes* serotypes. Modified from Seeliger & Höhne (1979) and Liu et al. (2006).

Serotype	O antigens	H antigens
1/2a	I, II	A, B
1/2b	I, II	A, B, C
1/2c	I, II	B, D
3a	II, IV	A, B
3b	II, IV	A, B, C
3c	II, IV	B, D
4a	(V), VII, IX	A, B, C
4b	V, VI	A, B, C
4c	V, VII	A, B, C
4d	(V), VI, VIII	A, B, C
4e	V, VI, (VIII), (IX)	A, B, C
7	XII, XIII	A, B, C
5	(V), VI, (VIII), X	A, B, C
6a	V, (VI), (VII), (IX), XV	A, B, C
6b	(V), (VI), (VII), IX, X, XI	A, B, C

Pulsed-field gel electrophoresis (PFGE) is a highly reproducible genetic subtyping method that uses restriction enzymes to digest DNA and yield hundreds of fragments. These fragments are thereafter separated with conventional agarose gel electrophoresis and form very distinctive patterns, much like Rep-PCR, and compared to a standard (Liu, 2006). The method is equally discriminatory in strain-level differentiation as Rep-PCR, however, more laborious as the complex banding patterns are technically demanding and tedious to interpret. So, this method takes longer and is in addition more expensive than Rep-PCR (Chou & Wang, 2006; Liu, 2006). Software has been developed, for example by Bionumerics, where gel images can be uploaded and analyzed electronically. The major disadvantage with this is that the PFGE standard protocols for an organism needs followed and run correctly to obtain distinctive banding patterns and a good quality gel image. Otherwise the software will not work optimally and give erroneous results (Jensen et al., 2017). The method also offers no taxonomical identification of isolates without including a standard, just as conventional Rep-PCR.

Whole genome sequencing (WGS) is regarded as the most discriminatory method in detection, identification, and differentiation of pathogenic foodborne bacteria as it has many advantages. Isolates can be differentiated by a single nucleotide. They can be taxonomically identified with basic local alignment search tools (BLAST) which compare query sequences to sequences in databases of established species. The large amount of genomic data allows for accurate tracing of strains with familiar similarity and thereby determine persistence of bacteria in processing plants (Allard et al., 2016; Hurley et al., 2019). Also, phenotypic characteristics such as tolerance to cold, salt, acid and antibiotics can be largely determined *in silico* by looking for specific protein sequences in the genome (Cotter et al., 2005; Hurley et al., 2019; Ryan et al., 2010). There are many different commercialized WGS techniques available, which will be discussed further in section 1.3. However, WGS to this day has not made sense to implement locally in microbial surveillance of food processing plants as its largest disadvantage is the expertise required to run the assay. High technical knowledge for the bioinformatical interpretation of the data, and laborious work creating DNA libraries is required for current available assays (Pallen et al., 2010; Pop & Salzberg, 2008; Rossen et al., 2018). The cost of running WGS, although steadily declining, is still a large enough disadvantage to not implement it in smaller laboratories (Köser et al., 2012; Rossen et al., 2018). A compromise to lower the cost to differentiate microbes in a food processing plant would be to sequence only parts of the genome, such as the 16S region. However, in the case of *L. monocytogenes* it does not provide adequate strain-level discrimination (Czajka et al., 1993).

An overview of the prices for the different subtyping and differentiation techniques mentioned in this chapter is given in Table 3.

Table 3 – Comparison of commercial prices for traditional typing methods, 16S sequencing, WGS and ON-rep-seq for 20 isolates. Currency = Euro (€). Modified from Thomassen et al. (2021).

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

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

1.2. Whole genome sequencing

As mentioned in section *1.1.4* there is a variety of commercially available WGS techniques. The technology has developed leaps and bounds since Frederick Sanger first published the genomic sequence of bacteriophage ΦX174 in 1977 (Sanger et al., 1977).

1.2.1. First generation sequencing (Sanger)

Sanger sequencing is often also referred to as the chain termination method as it involves DNA synthesis of a complementary template strand using normal 2'-deoxynucleotides (dNTPs) followed by a termination of synthesis using 2',3'-dideoxynucleotides (ddNTPs). This generates a set of nested fragments where the ratio of dNTP/ddNTP determines the frequency of chain termination. These fragments can be separated using gel electrophoresis and analyzed to ascertain the DNA sequence (Metzker, 2005; Sanger et al., 1977). However, research conducted in the 1980s into fluorescent dyes has rendered the electrophoresis method largely obsolete. In the case of this automated method the ddNTPs are tagged with fluorescent dyes specific for each nucleotide. As the fragments pass by a laser, the light emitted is measured for wavelengths specific for a colour correlating to a nucleotide. As each fragment passes by the laser, based on the length of the fragment, the sequence is given (Metzker, 2005; Prober et al., 1987; Smith et al., 1987; Smith et al., 1986). Sanger sequencing was long regarded as the most reliable form of sequencing, however, it is a very laborious and time-consuming procedure. For instance, The Human Genome Project took 10 years to complete (Venter et al., 2001). To that point Sanger sequencing is not used in large scale genomic projects, but the mark it has left on the world of genomic research is undeniable. It has paved the way for the rapid development of cheaper,

faster sequencing technologies since the time of the Human Genome Project. These new procedures were aptly named, next generation sequencing (NGS) technologies. The development of these technologies has led to a dramatic drop in the price of sequencing of whole-genomes, thereby making them cheaper than Sanger sequencing (Wetterstrand, 2021).

1.2.2. Next generation sequencing (Illumina)

The most predominant NGS technology to emerge, and by many considered the "new gold standard" in sequencing, is Illumina sequencing (Slatko et al., 2018). What it has in common with most NGS platforms is that it can rapidly sequence a genetic element through massive parallel sequencing. Meaning the platform sequences millions of smaller, overlapping DNA fragments simultaneously (Figure 3), limiting the bias of the sequencing. For example, if one read has a base error, there are millions of overlapping fragments correctly sequenced that can be used for base error correction. The collection of fragments corresponding to a given sequence is called a DNA library. This gives NGS an advantage in capturing mutations than Sanger sequencing (Behjati & Tarpey, 2013). The length of the fragments depends on the NGS platform, for instance Illumina requires fragments to be 36-300bp long (Bruijns et al., 2018).

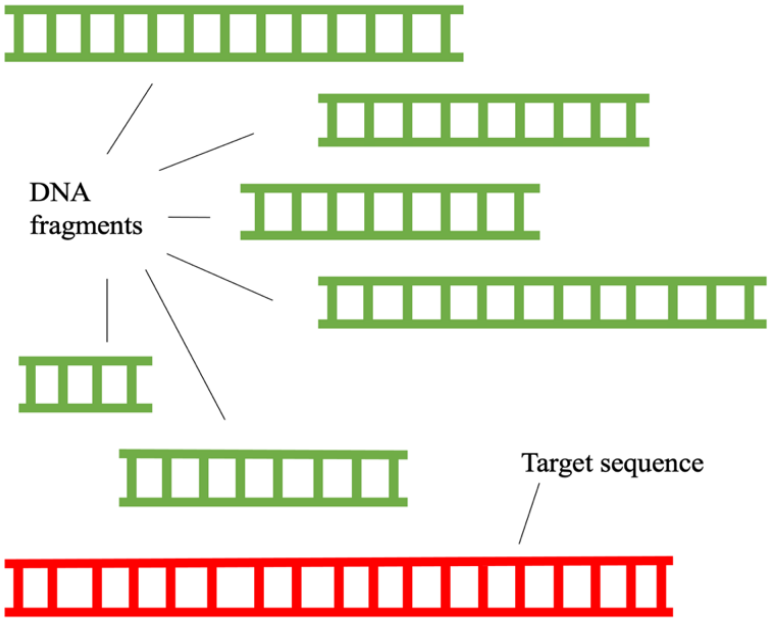

Figure 3 *– Concept of massive parallel sequencing used by most NGS platforms. By fragmenting DNA and sequencing the overlapping fragments, the genetic sequence is provided.*

The fragments are ligated with a barcode sequences, known oligonucleotide sequences, specific to each sample used for identification in the raw data. The barcode sequences have complementary oligonucleotide sequences on the Illumina flow cell that act as anchor points for the fragments. The DNA fragments attach to these anchor points on one end, and then bend

over to attach to another anchor point that is complementary to the barcode sequence on the other end, creating a bridge. (Clark et al., 2019). The DNA is then subjected to repeated rounds of amplification, creating clonal clusters on the flow cell to increase the output signal during sequencing. Illumina sequences the DNA through synthesis, incorporating modified nucleotides to the template strand. Similar to Sanger sequencing, these nucleotides are tagged with a fluorescent label with colours corresponding to each specific nucleotide. As these modified nucleotides are incorporated into the complementary strand, they emit the fluorescence which is detected by a sensor (Clark et al., 2019; Metzker, 2005; Prober et al., 1987; Slatko et al., 2018; Smith et al., 1987; Smith et al., 1986). The fluorescent detection of the sequence has cut the time it takes to sequence the entire genomes down to a few days from sampling, and with a reported accuracy of 99,9% it is no wonder that Illumina has largely replaced Sanger sequencing when it comes to WGS. Some disadvantages the platform has is the short fragments required, as long fragments produce too much background noise during fluorescence detection. The sample preparation for the construction of these DNA libraries required are therefore laborious and the data assembly to obtain the sequence requires high skilled training. The equipment needed for Illumina sequencing also consists of large benchtop machines (Ari & Arikan, 2016). There was a growing want among the scientific community for a smaller sequencing platform, preferably one that can be taken out into the field, and which required minimal preparation of samples. This desire led to the development of what is often called third generation or large fragment single molecule sequencing technologies (Eisenstein, 2012; van Dijk et al., 2018).

1.2.3. Third generation sequencing (Oxford Nanopore)

Oxford Nanopore technologies (ON) has become one of the major players to produce a platform that utilizes large fragments of single DNA molecules for sequencing instead of fragments. They also offer one unit, the MinION, which is the size of a USB stick (Eisenstein, 2012). The DNA molecule, whether fragmented or not, is ligated with a barcode adapter sequence. As well as providing a reference sequence to identify the sample, the adapters are DNA-protein complexes with a tightly bound enzyme which ensures the movement of the DNA through the nanopore (Slatko et al., 2018; van Dijk et al., 2018). Therefore, there is some degree of library preparation with ON, but since the DNA is not fragmented as with Illumina, the sample workflow is greatly simplified and the turnaround time reduced remarkably (Kafetzopoulou et al., 2018; Petersen et al., 2019). The nanopore consists of a protein complex with two chambers which are filled with an electrolyte solution. On top of the complex is a motor protein which

unwinds the double helix and drives the DNA molecule through the nanopore via a ratcheting motion. The two chambers are separated from one another by a membrane that can be exposed to an electrical current (Slatko et al., 2018; van Dijk et al., 2018). The chamber that is located on the same side of the motor protein and DNA infeed is referred to as *cis* whilst the chamber on the other side of the membrane is referred to as *trans* (van Dijk et al., 2018). When the membrane is subjected to a constant voltage, the fluctuation in the current when a DNA strand moves through the pore can be observed and characterized as each nucleotide provides a characteristic electronic signal (Petersen et al., 2019; Slatko et al., 2018; van Dijk et al., 2018). The change in current is given in so-called "squiggle plots" (Figure 4) (van Dijk et al., 2018). The plot is assembled in real-time, making ON the only platform to offer real-time sequencing results (Petersen et al., 2019).

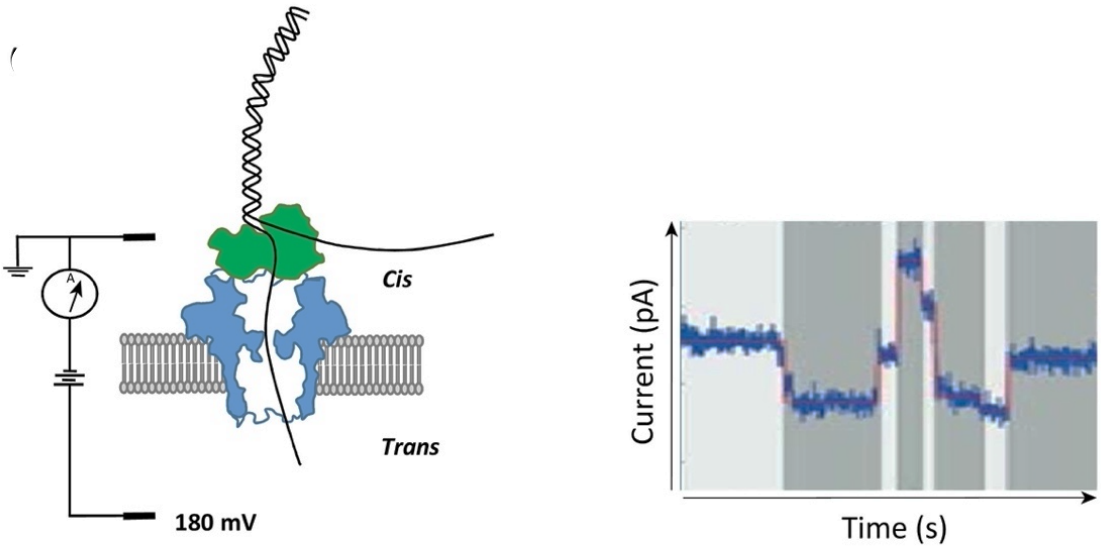

Figure 4 *- Schematic representation of ON technology flow cell. The nanopore consists of two chambers (cis and trans) that are filled with ionic solutions and separated by an electrically charged membrane. Withing the membrane is a nano-sized pore that the nucleic acid can pass through. The nucleic acid is driven through the pore electrophoretically which is controlled by a motor protein (green). The electrical charge over the membrane is constant, but as a nucleic acid passes through the membrane the charge changes. This shift in electrical charge is recorded in real time and graphically represented in "squiggle plots". These plots can then be converted to FASTQ files. Copyrights obtained from (van Dijk et al., 2018).*

ON seems likely to be the future in sequencing, but even though they are one of major players on the sequencing market its implementation is limited when compared to Illumina. ON has many advantages over Illumina such as simple workflow, reduced turnaround time, ability for high-throughput sequencing and long read lengths (Kafetzopoulou et al., 2018; Petersen et al., 2019). The technology, conversely, has some disadvantages the largest being the high error rate (10-15%) (Kafetzopoulou et al., 2018; Laver et al., 2015; Petersen et al., 2019; Stefan et al., 2022).

1.3. Source tracking pathogens

Source tracking aims to identify the origins of microbial contamination in the food value chain so that remedial action can be implemented. The methods for source tracking dangerous microbes in the food value chain may vary widely, but all have a common purpose which is to differentiate them beyond the species and subspecies level (Wiedmann, 2019). Many of the molecular subtyping methods used in source tracking pathogens is mentioned in section *1.1.4*. Multi-country foodborne outbreaks may occur over prolonged time periods, the spatial and temporal differences might make accurate surveillance and tracking of pathogens to its source difficult. However, through molecular subtyping of bacterial isolates clusters of the genetic type causing foodborne outbreaks can rapidly be detected (Wiedmann, 2019). The genetic sequence is inherited vertically, from one cell to its daughter cells, and by harvesting subtype data it can be used to reconstruct a microbe's evolutionary history. This study of phylogenetics is a powerful tool used in foodborne outbreak detection and source tracking (Allard et al., 2018). Molecular subtyping is also used for in-plant source tracking, as the persistence of bacterial subtypes in different equipment and processing environments can be determined (Wiedmann, 2019).

As mentioned, WGS is considered the most discriminatory subtyping method as it can differentiate isolates down to a single nucleotide. Since the introduction of WGS, open databases like the National Center for Biotechnology Information (NCBI) and the European Nucleotide Archive (ENA) have been established. Here entire genome assemblies, raw sequencing data and, if known, functional annotations are uploaded. These databases have proved vital food production surveillance (Allard et al., 2016; Harrison et al., 2020; Sayers et al., 2021). By uploading sequences obtained from regular testing or in concurrence with a research project to such a database allows for links between, food, environmental and clinical isolates to be phylogenetically revealed. The databases match the query sequences against their previously collected data through alignment and calculate the statistical significance of matches. The input data does not need to be whole genome sequences but can be amino acid sequences or snippets of sequences, for instance from specific genes or loci (Allard et al., 2018; Harrison et al., 2020; Sayers et al., 2021).

The analysis of WGS data usually requires high bioinformatic skills which is a major limiting factor for its widespread adoption by the industry (Barretto et al., 2021). Pipelines for more user-friendly analysis of WGS data have been developed. For example, the Center for Genomic Epidemiology (CGE) at the Technical University of Denmark (DTU) provides a pipeline that analyzes high quality single nucleotide polymorphisms (hqSNPs) and infers a

phylogeny based on the alignment of hqSNPs between isolates (Kaas et al., 2014). The CGE also has a MLST service which characterizes isolates using sequences of internal fragments of seven housekeeping loci (Bartual et al., 2005; Camacho et al., 2009; Griffiths et al., 2010; Jaureguy et al., 2008; Larsen et al., 2012; Lemee et al., 2004; Wirth et al., 2006). For each loci, the different sequences are assigned as distinct alleles, and the alleles at each of the seven loci define the isolate's sequence type (ST). Closely related isolates will have identical STs, or STs that differ at a few loci whilst unrelated isolates will have totally different STs (Urwin & Maiden, 2003). As MLST is based in nucleotide sequences, it is very discriminatory and by far its greatest attribute is the portability of that data as it can be uploaded to the internet (Maiden et al., 1998). This allows for exchange of epidemiological results and development between laboratories on a global spatial scale. In general, analysis of SNPs even with simpler pipelines developed require more bioinformatical skill than MLST. MLST for example does not require the use of external reference genomes, and provides appropriate differentiation of *L. monocytogenes* subtypes for source tracking studies (Jagadeesan et al., 2019).

1.4. Aims of this study

The work conducted for this thesis was extensive and warranted several aims:

1. Detection of *Listeria spp.* or *L. monocytogenes* in selected sampling point, within the filleting department of a Norwegian salmon processing plant, using classical media enrichment techniques and real-time PCR.
2. Propagation of colonies to pure cultures and isolation of DNA for ON-rep-seq analysis.
3. Selection of isolates for WGS based off ON-rep-seq results, and subsequent DNA isolation.
4. *In silico* characterization of whole-genome sequences through hqSNPs and MLST analysis, in addition to detecting the number of virulence genes and acquired antibiotic resistance genes using online pipelines.
5. *In vitro* characterization of isolates through antimicrobial susceptibility testing and stress response to cold, salt and acid.

2. Materials and Methods

The work conducted for this thesis was part of a larger research project between a private seafood production company, the Norwegian University of Science and Technology (NTNU) Department of Food Science, and the University of Copenhagen (KU) Department of Food Science. The research project named, TraceListeria, aimed to better understand the occurrence, contamination points, and paths of infection for *L. monocytogenes* by systematically screening the entire value chain of the seafood production industry. So far, the source tracking of *L. monocytogenes* has largely been limited to facilities and equipment in the abattoirs and processing plants. The risk for contamination in the sea phase has not been as scrupulously investigated. TraceListeria included samples from the feed production, feed transport and distribution systems, the production of salmon in production enclosures, the well boats that transport the salmon to the abattoir for slaughter and finished products to encompass the entire value chain of production. Originally the finished product to be studied was only whole fish in boxes, however, the project group decided to include isolates from the company's own quality control team and the processing plant's filleting department to expand the data base. The salmon arriving at the filleting department is where this study begins (Figure 5).

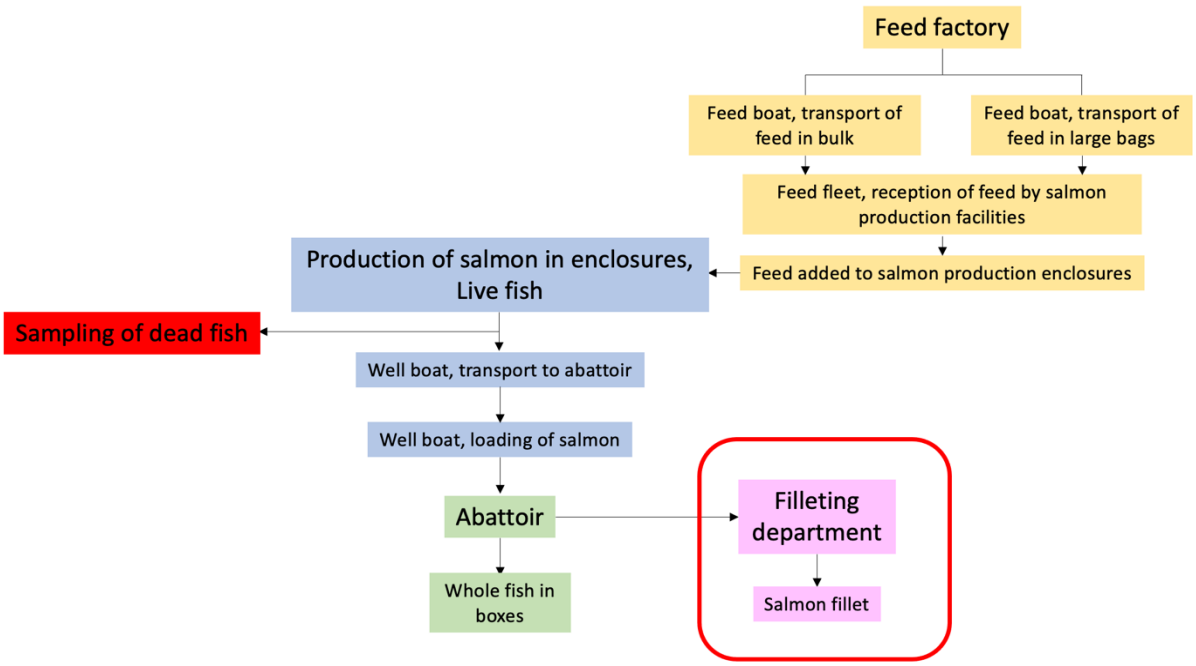

Figure 5 *- Entire value chain in the production of salmon screened by the TraceListeria project. Samples were taken at every point and screened for* L. monocytogenes *starting at the production of feed and the transport of the feed to the production enclosures, the feed phase is highlighted in yellow boxes. The next phase marked in blue was the sea phase, which is the production of salmon in net pens, samples of dead fish were collected along with live fish in this phase, seen in red. The fish were then transported out of the sea phase into the processing phase at the abattoir, the finished products were either whole fish in box or salmon fillet from the filleting department. The filleting department, highlighted in purple and outlined with red is the focus of this study.*

2.1. Sampling at the salmon processing plant

Due to the COVID-19 pandemic and restrictions that followed, it was not allowed to sample on-site at the salmon processing plant. A sampling scheme was constructed that instructed employees at the processing plant how, when, and where to take the samples required by this study. After the samples were obtained, they were sealed and sent to Trondheim by boat. Samples were acquired when the salmon produced in the value chain examined by the TraceListeria project arrived the filleting department.

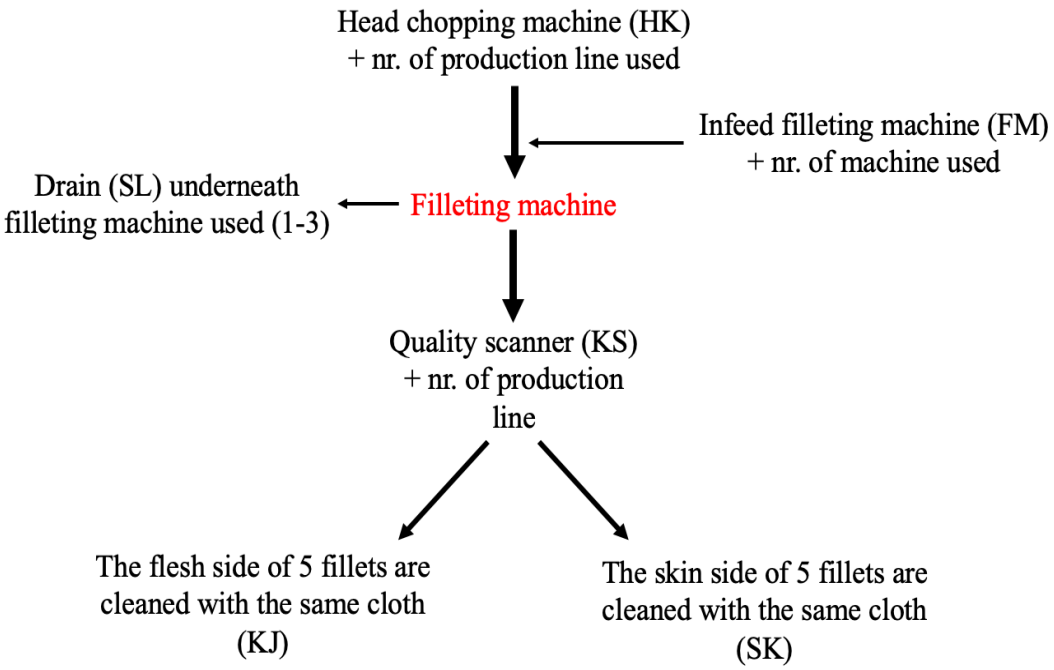

Figure 6 *– Processing line and sampling points in the filleting department of a salmon processing plant. The environments in black writing were the sampling points for this study.*

The employees collected the samples from predetermined control points in the processing plant shown in black writing in Figure 6. Control points were selected for this study based on the processing plant's experience with previous "hot spots" for *L. monocytogenes*, results obtained in the study by Thomassen et al. (2021) and technical guidelines published by the European Union Reference Laboratory for *Listeria monocytogenes* (EURL *Lm*) on sampling for the detection of *L. monocytogenes* (Carpentier & Barre, 2012). This technical guideline was published to compensate for the gaps in the International Standard ISO 18593 specific to *L. monocytogenes* (Standardization, 2018). The international standard does not cover when sampling should be done, or which areas should be included. Both ISO 18593 and the EURL *Lm* technical guide should be used when implementing the rules set by (EC) Regulation No 2073/2005, article 5.2 (Community), 2005). The technical guide emphasizes sampling areas where the bacterium is able to grow and persist, which are often hard to reach places in the

equipment that might be difficult to clean without disassembly. As there is a lot of water involved during the processing of salmon many wet and soiled areas where *L. monocytogenes* can grow exists in either non-food surfaces such as the drains and food contact surfaces such as gutting lines, conveyor belts, head cutters and slicers. These are all recommended by EURL *Lm* to sample. The technical guide also stresses the importance of sampling during production and not directly after cleaning. Preferably at the end of production or at a minimum 2h into it. That is because cells remain in these harbourage sites mentioned above, despite cleaning and remain undetected. During processing these cells become dislodged due to the vibration of the equipment and liquid like water coming into contact with these sites (Carpentier & Barre, 2012).

The samples were collected using a sterile cloth that was wiped over the equipment and finished product before being placed in a sterile bag, closed, and prepared for sending (Figure 7). Using either a cloth, sponge or swap to wipe the equipment is the only appropriate method for *L. monocytogenes* according to the guidelines published in ISO 18593:2018 (Standardization, 2018).

The fish from the TraceListeria production enclosures arrived at the abattoir for processing 5 separate weeks during the summer of 2021, see Table 4.

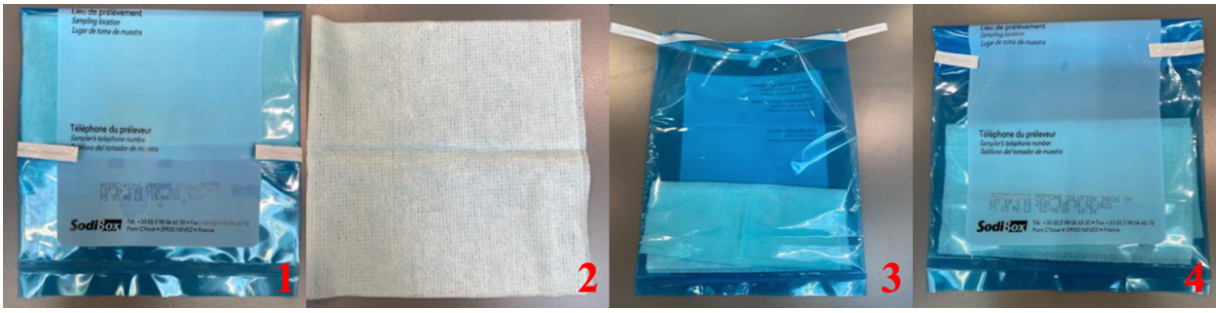

Figure 7 *– Sample cloth used in study. 1, unused cloth in bag. 2, the sterile sample cloth. 3, sample cloth put back in bag after sampling. 4, bag with used sample cloth is sealed by folding the bag three times and fastening the white strips.*

Table 4 – Overview of samples from the filleting department at salmon processing facility O-A in the TraceListeria value chain, the week and date they were sampled, and how many samples were taken at each control point. (KJ) = Flesh side of 5 fillets, (SK) = Skin side of 5 fillets, (SL) = Drain underneath filleting machine, (HK) = Head chopping machine, (FM) = Infeed filleting machine, (KS) = Quality scanner.

Week	Sample date	Fish from production site	Sample points (Fil)					
			KJ	SK	SL	HK	FM	KS
22	01.06.2021	O-B	3	3	3	3	3	3
23	10.06.2021	O-A	4	4	3	4	4	4
26	20.06.2021	O-A	4	4	3	4	4	4
29	20.07.2021	O-A	3	3	2	2	2	2
34	24.08.2021	O-B	3	3	2	2	2	2
Total no. samples			**17**	**17**	**13**	**15**	**15**	**15**

2.2. Selective enrichment and identification of samples

Selective enrichment was performed by adding 90 g of liquid Thermo Scientific™ Oxoid™ 24 *Listeria* Enrichment Broth (LEB) and 4,4 mL supplement buffer to the sample bags and incubated at 37°C for 24h. LEB allows for optimal growth of *Listeria* spp. and is designed for use in PCR systems. After cultivating overnight, 1,5 mL of the cloth extract was transferred to 1,5 mL Eppendorf tubes. The Suretect™ *Listeria* species PCR assay kit included its own lysis step and primers specific for *Listeria* spp. and was used for the initial screening of all samples following the manufacturer's procedure. The VWR Doppio thermocycler was used for the lysis step, and the Thermo Scientific™ QuantStudio 5 qPCR was used for the amplification process. The following thermal conditions were used for the lysis and qPCR assays:

Lysis		**qPCR**		
37°C	10min	95°C	7min	
95°C	2min	95°C	5sec	50 cycles
		60°C	45sec	

All samples that tested positive for *Listeria* spp. were reanalyzed with the SureTect™ *Listeria monocytogenes* PCR Assay kit with primers for specific detection of *L. monocytogenes*. The *Listeria* spp. positive samples were simultaneously plated onto Thermo Scientific™ Brilliance™ Listeria Agar (BLA) and incubated at 37°C for 24h. *Listeria* spp. will grow as green colonies on BLA, but due to the lecithin which is hydrolyzed by lecithinase specific to *L. monocytogenes*, samples containing *L. monocytogenes* will have a distinct opaque precipitate around their colonies (Figure 2). After 24h the BLA plates were compared to the *Listeria* spp. and *L. monocytogenes* qPCR assays to determine whether a precipitate could be established around colonies from samples that tested positive for *L. monocytogenes*. After incubation, 5 distinct colonies from each sample were picked and streaked into their own sector of new BLA plates (A-E) and incubated at 37°C for 24h. After another inspection for green colonies and precipitate, each of the 5 isolates from every sample was streaked onto their own Thermo Scientific™ Oxoid™ Brain Heart Infusion Agar (BHIA) plate and incubated at 37°C for 24h. These overnight plate cultures were used to make freeze stocks for storage at -80°C, and downstream DNA isolation.

2.3. DNA isolation

The isolates' genomic DNA was isolated and purified using Genomic Micro AX Bacteria+ Gravity-kit (102-100M A&A Biotechnology) according to the manufacturer's procedure. The

exception being that no liquid overnight cultures were made, instead colonies of overnight agar cultures were added to the suspension buffer. This kit was chosen because it isolates the DNA without fractioning it, as long intact DNA fragments are preferable for ON sequencing assays. Roughly 7-8 colonies of similar sizes and morphologies were needed to extract at least 40 ng/µL DNA. The concentrations were measured with the BioTek PowerWave XS, Take 3 plate, and Gen5 2.0 software. From the isolated DNA that measured above the 40 ng/µl threshold, 50 µL was sent on ice overnight to KU for the ON-rep-seq assay or for WGS.

When the DNA was isolated for WGS the proteinase K step was extended to 30 min instead of 10 min to ensure purity of DNA extract without contaminants such as proteins and amino acids.

2.4. Oxford Nanopore Technologies based Rep-PCR amplicon sequencing

The methods used during the ON-rep-seq assay have previously been described by Krych et al. (2019) and Thomassen et al. (2021). It is important to note that this part of the study was not completed at NTNU, but by dr. Krych's team at KU, and therefore the details of the methods will not be described here. Instead, an outline of the procedure and the intention of each step is given in summary.

ON-rep-seq is a method that combines classic Rep-PCR with NGS technology and allows for taxonomic identification down to species and strain level. The first step is a normal Rep-PCR.

The second PCR step attaches barcodes to the Rep sequences. The thermal conditions of the Rep-PCR-2 are slightly more complicated than the first, because ON compatible adapters need to be incorporated through a dual-stage PCR. The first stage provides optimal annealing conditions, while the next stage allows for the best hybridization of full adapters.

After the second Rep-PCR every sample was pooled together. Samples were not pooled in equimolar concentrations, as one expects differences in the length of the amplified regions between Rep sequences of different species/strains. The sequencing was performed using the R9.4.1 flow cell.

The data was collected using Oxford Nanopore software. Peaks were identified in Length Count profiles (LCp) expressed as the sequencing length along the x-axis by the number of reads along the y-axis. Sequences containing quality scores resolved within each peak were retrieved and corrected. The corrected reads were then sorted by length and clustered. This step detects structural sequence variants of similar length, thereby, consensus sequences were sorted by size. Finally, a metagenomics program was used to classify the corrected reads (Figure 8).

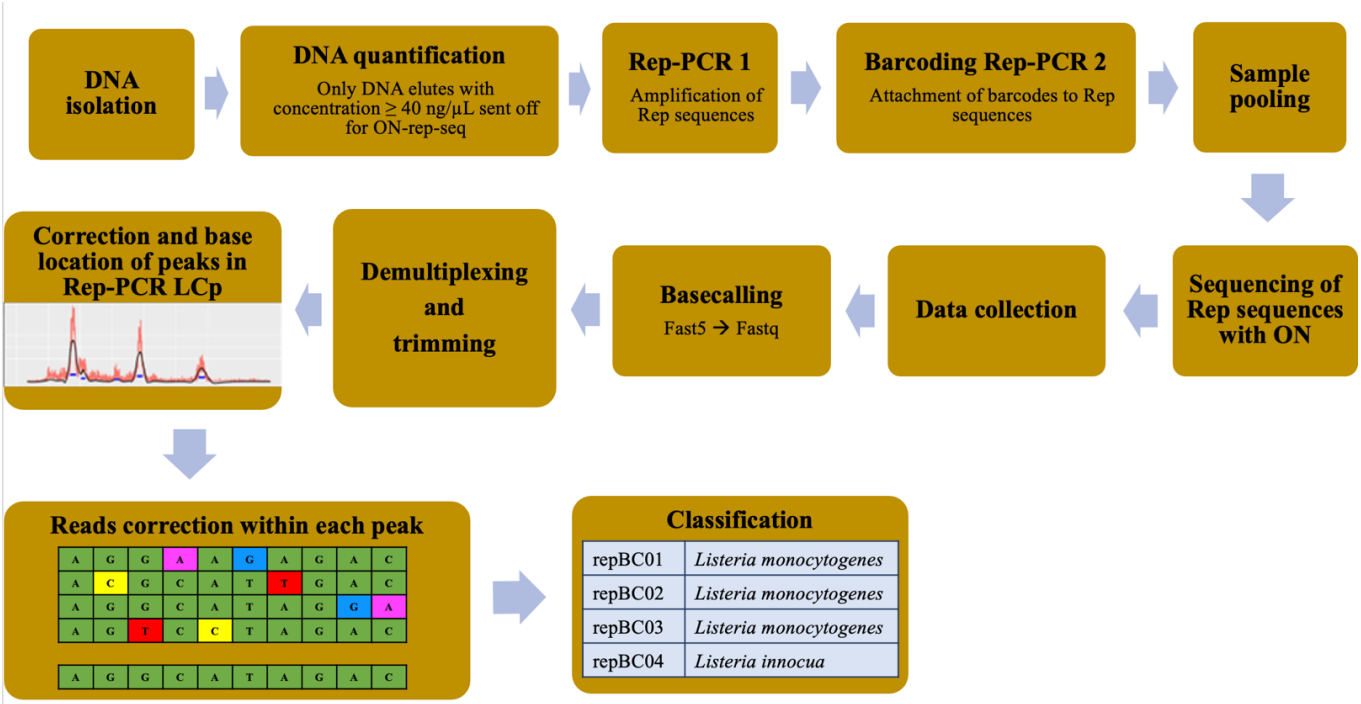

Figure 8 *– Schematic overview of ON-rep-seq pipeline. First the DNA needs to be isolated and quantified. Only eluates with concentrations ≥ 40 ng/µL were used downstream for ON-rep-seq analysis. The first PCR step amplifies the Rep sequences in the DNA. The second PCR step attaches barcodes with adapters to the ends of the Rep sequences. The samples are then pooled together before being sequenced with ON. The data is collected with ON software and after initial basecalling and demultiplexing of the raw data, the Fastq files are used to generate LCps based on sequences length distribution. Reads within each peak are clustered, corrected, and taxonomically classified using the improved quality reads. Modified from Krych et al. (2019).*

2.5. Whole genome sequencing

In excess of 300 isolates were analyzed with the ON-rep-seq assay for the TraceListeria project, whereof a total of 57 isolates were selected for WGS. Once more, it is important to note that this assay was not conducted at NTNU, but by dr. Krych's team at KU. The platform chosen to execute this assay was again Oxford Nanopore.

2.5.1. Genomic characterization based on WGS data

The methods described in this section have previously been published by Thomassen et al. (2021). Of the 57 isolates sent off for WGS, 10 isolates were from the filleting department. The filleting department isolates sent off for WGS consisted mostly of isolates from the infeed filleting machine, the drain underneath the filleting machine, and the quality scanner. The reason for this selection was that the quality scanner and infeed machine especially were identified as "hot spots" for *L. monocytogenes* contamination during selective enrichment and identification. However, to expand the data base of this study and to observe possible similarities between isolates across the value chain, it was decided to include six isolates obtained earlier in the TraceListeria project. These were chosen after an initial screening for

sequence types (STs) with the CGE MLST (v2.0.9), based on seven conventional MLST loci (Bartual et al., 2005; Camacho et al., 2009; Griffiths et al., 2010; Jaureguy et al., 2008; Larsen et al., 2012; Lemee et al., 2004; Wirth et al., 2006). As all WGS isolates were taxonomically identified as *L. monocytogenes* the corresponding MLST configuration was chosen. Isolates characterized as ST8 were added to the data base of this study. Two of these isolates stemmed from feed samples and three from feed distribution samples. For the same reasons as well as to give a temporal overview of contamination, six isolates from the processing plant's own quality control team taken in 2019 and 2020 were included. Thereby making 22 of the 57 isolates selected for WGS applicable to this particular study (Table A2).

The isolates were chosen based on inclusion of the entire value chain, all isolates had to be taxonomically identified as *L. monocytogenes* by the ON-rep-seq assay, and some duplicate isolates stemming from the same sample. The reason for duplicates is to observe genetic similarity or diversity in one given sample. WGS was used in this study to compare it to the sensitivity and accuracy of the ON-rep-seq assay to estimate whether it can be used for strain-level discriminatory source tracking in food production value chains. In addition, the WGS data was screened for antibiotic resistance genes and virulence genes to assess the results obtained by phenotypical characterization of isolates (chapter 2.6). Isolates were screened for; acquired antibiotic resistance genes using CGE ResFinder (v4.1) (Bortolaia et al., 2020; Camacho et al., 2009; Zankari et al., 2017), and virulence genes using CGE VirulenceFinder (v2.0.3) (Camacho et al., 2009; Joensen et al., 2014; Malberg Tetzschner et al., 2020). The default settings with threshold for ID = 90% and minimum length = 60% was used.

Finally, the relatedness of the 22 WGS isolates was analyzed by creating a phylogenetic tree with CGE CSIPhylogeny (v1.4). CSIPhylogeny identifies and filters SNPs, performs site validation and infers a phylogeny based on alignment of the high quality SNPs (Kaas et al., 2014). The genome assembly of *L. monocytogenes* EGD-e (accession no. NC_003210) was used as the reference genome, and the FASTA files for the 22 WGS isolates were uploaded. The default settings were not changed for this run:

Select min. depth at SNP positions = 10x, Select min. relative depth at SNP positions = 10%, Select min. distance between SNPs (prune) = 10bp, Select min. SNP quality = 30, Select min. read mapping quality = 25, Select min. Z-score = 1.96, Use altered FastTree = Yes.

The results were imported as a Newick file that could be uploaded into the web-based program Interactive Tree of Life (iTol, v6.5.4) to visualize the phylogenetic tree (Letunic & Bork, 2021).

2.6. Characterization

2.6.1. Antimicrobial susceptibility test

Table 5 – Antibiotics used in susceptibility testing, their corresponding concentration, and the zone diameter breakpoints for each. Susceptibility to an antibiotic is indicated by S ≥ when zones are equal to or greater than the breakpoint. Resistance to an antibiotic is indicated by R < when zones less than the breakpoint. The intermediate category is not listed but it falls between the values of the susceptible and resistant breakpoints. If those values are the same, the intermediate category does not exist.

		Zone diameter breakpoints (mm)	
Antimicrobial agent	**Concentration**	**S ≥**	**R <**
Tetracycline (TE)*	30µg/disc	22	19
Erythromycin (E)	10µg/disc	25	25
Ciprofloxacin (CN)*	10µg/disc	50	21
Sulfamethoxazole-trimethoprim (SXT)	25µg/disc	29	29
Meropenem (MEM)	10µg/disc	26	26
Ampicillin (AMP)	10µg/disc	16	16
Penicillin G/Benzylpenicillin (P)	1µg/disc	13	13
Cefoxitin (FOX)	30µg/disc	N/A	N/A

*Zone diameter breakpoints from *Staphylococcus aureus*

The Mueller-Hinton fastidious organism (MHF) agar plates used were ordered ready-made from Montebello Diagnostics AS (product 10132). They differ from regular MH plates by supplementing the agar with 5% mechanically defibrinated horse blood and 20mg/l β-NAD, and thereby facilitate growth for fastidious organisms such as *L. monocytogenes*. The procedure used and described here is derived from the manual of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2022).

The MHF plates must all be inoculated with an equal density of cells. Therefore, a 0.5 McFarland turbidity standard was first made by adding 0,5 mL 0,048 mol/L $BaCl_2$ to 99,5 mL 0,18 mol/L H_2SO_4 and mixed thoroughly. A spectrophotometer was used to measure the density of the suspension at a wavelength of 625nm. The absorbance of the standard should measure between 0,08 and 0,13, the one made in this study had an absorbance of 0,12. The suspension was distributed in a turbidimeter tube and sealed before further use. The 0.5 McFarland standard is equivalent to ~1-2 x 10^8 colony forming units per mL (CFU/mL).

A saline solution was made for the cell suspensions by mixing 9g NaCl with 1 L distilled water and autoclaved at 121°C for 15min. The turbidimeter was turned on at the start of every assay as it needed 15min to warm up before calibration. The instrument was calibrated for this procedure by inserting a glass tube containing distilled water and dialing up the needle to 100%. Then the McFarland standard was inserted into the turbidimeter which measured 66% turbidity. Fresh overnight BHIA plate cultures were prepared for direct colony suspension. A sterile loop

was used to pick morphologically similar colonies and suspend them in 10 mL saline, approximately 7-8 colonies were needed in the beginning to come close to ~66% turbidity. It was very important for both the colony suspensions and McFarland standard to be vortexed thoroughly before turbidity could be measured. If the turbidity measured above 66% a sterile loop was again used to pick 1-2 more colonies and suspended, if the turbidity measured below 66% a sterile pipette was used to add some more saline to the suspension. Once the turbidity matched the standards the inoculum was plated on MHF agar plates with a sterile cotton swab by swabbing in three directions to ensure an even lawn on every plate. Since eight antibiotics were used, two MHF plates for each isolate were prepared, as only 4 discs effectively fit on one plate to avoid crossing of inhibition zones (Figure 9). The antibiotics chosen for this study were based on antibiotics commonly used for the treatment of listeriosis, as well as one reference antibiotic which *L. monocytogenes* is known to have an innate immunity against (Table 5). A Bunsen burner was lit to disinfect the plyers used to apply the antibiotic discs to the plates, between plates, as to avoid cross-contamination between isolates. Once the disks were applied to the agar the plates were placed into a 2,5 L anaerobe jar with which in turn was closed with a lid. Before closing a Thermo Scientific™ Oxoid™ CO_2 Gen™ sachet was placed into the jar which will generate a 4-6% CO_2 atmosphere within the jar. Finally, the plates were placed into the incubator at 37°C for 16h. The method states that the plates may be in the incubator for 16-20h before zone measurement, but all plates were taken out of the incubator after 16h to ensure homogeneity of the readings. Only nine isolates were processed at a time to stay within the 15-15-15-minute rule: use the inoculum suspension within 15 minutes of preparation, apply disks within 15 minutes of inoculation and incubate plates within 15 minutes of disk application. After incubation the plates were inspected for a confluent lawn of growth without any single colonies. The diameter of the inhibition zones, where it was clear, for each antimicrobial agent was measured with a ruler from the front of the plate with the lid removed, see the black dotted line in Figure x. The diameter was then compared to EUCAST breakpoint tables for susceptibility and resistance zones. The EUCAST breakpoint tables for *L. monocytogenes* do not include all antibiotics used in this study (Table 5), but as previously described by Khen et al. (2015) the breakpoint tables of *Staphylococci* can be utilized to estimate susceptibility or resistance (Khen et al., 2015).

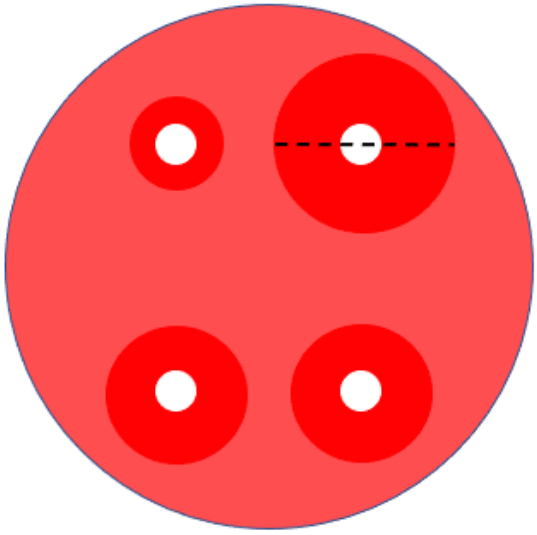

Figure 9 *- MHF plate configuration with antibiotic discs and possible inhibition zones.*

2.6.2. Cold tolerance assay

The methods described here are modified from methods described previously (Arguedas-Villa et al., 2010; Hingston et al., 2017). Overnight cultures grown on BHIA were standardized to 10^8 CFU/mL using the McFarland standard prepared for the antimicrobial susceptibility test. Approximately 7-8 colonies were transferred to 10mL peptone saline solution or as many as it took to reach the same turbidity as the McFarland standard. The 10^8 CFU/mL standardized isolates were then diluted in Thermo Scientific™ Oxoid™ Brain Heart Infusion Broth (BHIB) to a final volume and density of 50mL and 10^3 CFU/mL. This was then divided into 10 15mL tubes, where 5 were placed in an incubator set to 12°C and 5 were placed in an incubator set to 37°C. These temperatures were chosen as a standard and the storage facility of the salmon processing plant keeps its temperature at 12°C. The isolates were measured spectrophotometrically 5 times at λ = 625 nm over the course of 10 days, and for each measurement one of the 5 tubes was taken out and discarded after use. The measurements were taken on day 1, 2, 4, 6 and 10. The isolates subjected to this assay originated from the isolates of the TraceListeria project selected for WGS. A standard curve was created by measuring both absorbance and calculating CFU/mL of selected isolates from this group. This section of the study was completed alongside another master student who studied the rest of the isolates selected for WGS selected in the TraceListeria project. The growth-curve-isolates were diluted at each measurement and plated onto Tryptic Soy Agar (TSA) with 6% yeast extract to enumerate CFU/mL. The standard curve was used to calculate CFU/mL for all other isolates which were, as mentioned, only measured spectrophotometrically.

The log_{10} values of CFU/mL was calculated and these values were plotted into the ComBase Online DMFit (v3.5), a web-based application to fit bacterial curves where a linear phase is preceded and followed by a stationary phase (Baranyi & Roberts, 1994). The application calculates the maximum growth rate of each isolate (μ_{max}) which is then compared to the average μ_{max} of all 22 WGS isolates. If the deviation was > or < than 1 from the median μ_{max} the isolates were considered tolerant or sensitive, respectively, according to Hingston et al. (2017). The log_{10} data was also subjected to a univariate analysis of variance (ANOVA) with Tukey HSD tests, utilizing IMB SSPS Statistics (v28.0.1) to assess statistical significance of differences in growth between STs when exposed to a temperature of 12°C. In addition, a growth plot of Log_{10} CFU/mL (y-axis) against time (x-axis) was created based on estimated marginal means to visualize the variation in growth between STs. The analysis was performed using the following parameters:

Univariate analysis parameters:

Dependent Variable = log_{10} CFU/mL ***Fixed Factors*** = Sequence type and Time

Plot parameters:

Horizontal Axis = Time ***Separate Lines*** = Sequence type ***Chart type*** = Line chart

Post Hoc Test parameters:

Factors = Sequence type and Time ***Equal Variances Assumed*** = Tukey

2.6.3. Salt and acid tolerance assay

The methods for this tolerance assay have been previously described (Bergholz et al., 2010; Hingston et al., 2017). The 22 isolates sent for WGS relevant to this study were grown overnight on BHIA at 37°C, colonies were picked and added to a peptone saline solution, compared to a McFarland standard as mentioned previously, to a density of 10^8 CFU/mL. They were then diluted in either BHIB supplemented with 6% (w/w) NaCl or BHIB with pH 5 (adjusted with 1M HCl) to a final concentration of 10^7 CFU/mL. In 96-well-plates, 200 µL from the NaCl or pH cultures were added in triplicates (technical replicates). One plate with isolates in just BHIB without stress and one plate containing blank BHIB were prepared in addition. These plates were then incubated at room temperature (~22°C), and absorbance measured at λ = 600nm every 60min for 24h on the BioTek Powerwave XS with software Gen (v2.0). The blank BHIB plate was included as the environment of the BioTek Powerwave XS is not sterile, to see the level of possible outside contamination affecting the results. The data was imported into Microsoft Excel for processing. Room temperature was chosen since the incubation temperature

as the BioTek Powerwave XS operates at that temperature and cannot be changed. The data from this assay was also subjected to a univariate ANOVA and Tukey HSD test as described in section *2.6.2*, with the exception being that the dependent variable was absorbance in this assay instead of $\log_{10}$ CFU/mL. The maximum growth rate could not be calculated for this assay as it was not possible to find a constant for the media used (BHIB), therefore only the growth rate during exponential phase was calculated and used as μ_{max} to calculate fold decrease as described by (Muchaamba et al., 2019). Fold decrease = μ_{max} isolate exposed to stress / μ_{max} isolate not exposed to stress. To calculate the growth rate, all absorbance data was converted to $\log_{10}$ values and plotted into a graph. The exponential phase was identified within the graph as being between two points that created a straight exponential line. The graph was then fitted with an exponential trend line. From this trend line a formula $y = Ae^{Bx}$ can be extracted where B = growth rate. It was decided for this study that the trend line had to fit the curve to an R^2-value $\geq 0{,}99$ (Figure 10).

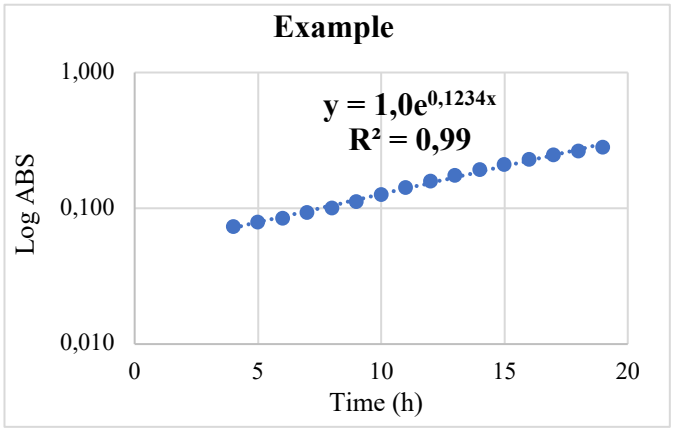

Figure 10 - Example of exponential growth plot from which the growth rate can be extrapolated as growth rate = B in the formula $y = Ae^{Bx}$, outlined in red

3. Results

3.1. Identification of Listeria spp. *and* Listeria monocytogenes

The results of the qPCR show that the infeed filleting machine is the point of most frequent contamination for *Listeria spp.* and *L. monocytogenes*, with 10 and 5 positive samples respectively. This represents 67% and 33% of FM samples. The drain underneath the filleting machine and the quality scanner had the second highest number of positive samples for *Listeria spp.* with 8 which corresponds to 62% and 53%, respectively. However, the drains had less samples positive for *L. monocytogenes*, only 1 compared to the quality scanners' 4. So, only 8% of drain samples were positive for *L. monocytogenes* compared to 27% for the quality scanners. The flesh and skin sides of the finished salmon filet product had 6 and 5 positive *Listeria spp.* samples which constitutes 35% and 29% of total samples, respectively. The skin side of the fish did not test positive for *L. monocytogenes* at any time. The flesh side on the other hand had 1 positive *L. monocytogenes* sample, 6% of total samples, during this study. Finally, the head chopping machine tested positive for *Listeria spp.* only once during this study and not for *L. monocytogenes*. That means only 7% of total HK samples were positive for *Listeria spp.* (Figure 11).

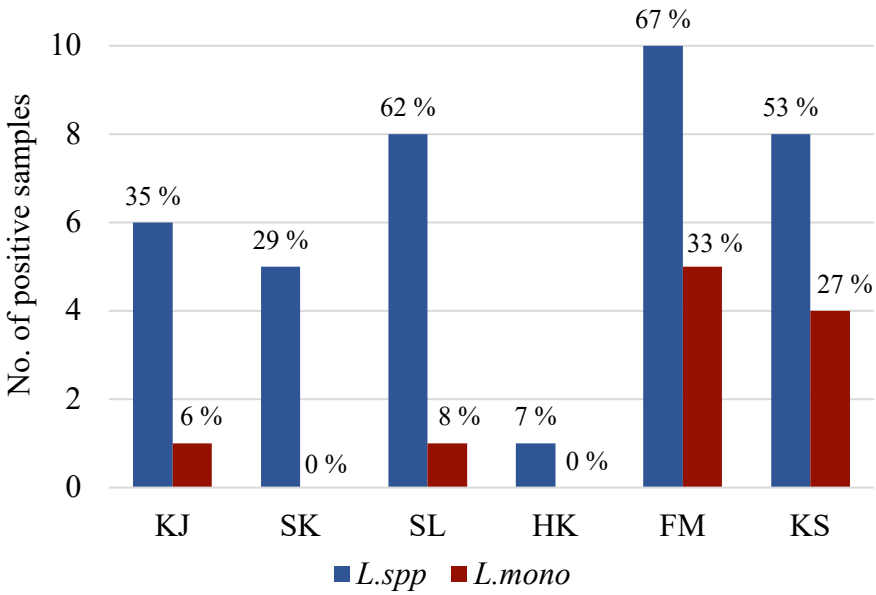

Figure 11 *– Total number of samples from each predetermined sample point in the filleting department of a salmon processing facility that tested positive for* Listeria spp. *(Dark blue) and* L. monocytogenes *(Red). The percentage of total samples that tested positive for either* Listeria spp. *or* L. monocytogenes *is indicated above each column. The sample points chosen were the flesh side of 5 salmon fillets (KJ), the skin side of 5 salmon fillets (SK), the drains underneath the filleting machines (SL), the headchopping machines (HK), the infeed filleting machines (FM) and finally the quality scanners (KS).*

Out of the 38 samples that tested positive for *Listeria spp.* and were plated on BLA, only 32 showed growth and could be isolated for downstream treatment (Table A1). One from the

infeed filleting machine, quality scanner, flesh side and skin side of the filets samples each did not grow on plates. Two of the drain samples did not show growth after transfer from enrichment broth to BLA plates. All isolates that were identified as *L. monocytogenes* by the qPCR analysis, and showed growth on agar plates, contained the opaque zone around colonies as predicted (Figure 12).

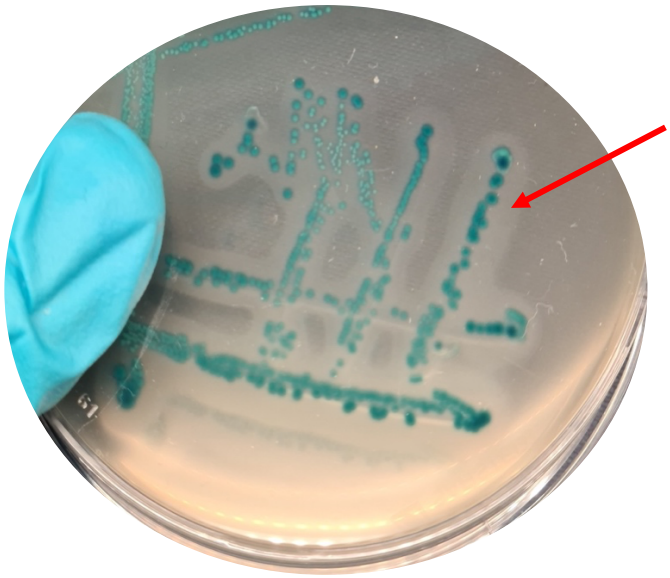

Figure 12 *– BLA plate for U23 FilFM-1, which was identified as* L. monocytogenes *through the qPCR assay and also showed the distinct opaque zone around the colonies, specific for* L. monocytogenes (indicated with red arrow).

3.2. ON-rep-seq species- and strain-level discrimination

Three heatmaps were generated by dr. Krych's team at KU for the TraceListeria project, one for each ON-rep-seq run, wherein each pixel is the comparison between two isolates. In total 115 isolates from the filleting department went through DNA isolation and subsequent ON-rep-seq analysis alongside isolates from the whole TraceListeria project.

The colour of the pixel determines the genetic similarity between isolates, a darker blue colour indicates low homogeneity between isolates whilst a darker red colour indicates high homogeneity between isolates. In Figure 13 on the next page the heat map for TraceListeria isolates sent in for ON-rep-seq analysis in September 2021 is portrayed. It shows low genetic diversity between most isolates, however, there seem to be three distinct families where different isolates cluster. A zoomed in look of the first cluster shows that the fillet department isolates, marked in red, tend to cluster with feed and environmental isolates. In all three heatmaps this pattern of filleting isolates clustering with other fillet isolates and feed and environmental isolates emerges (Figure A1 and A2).

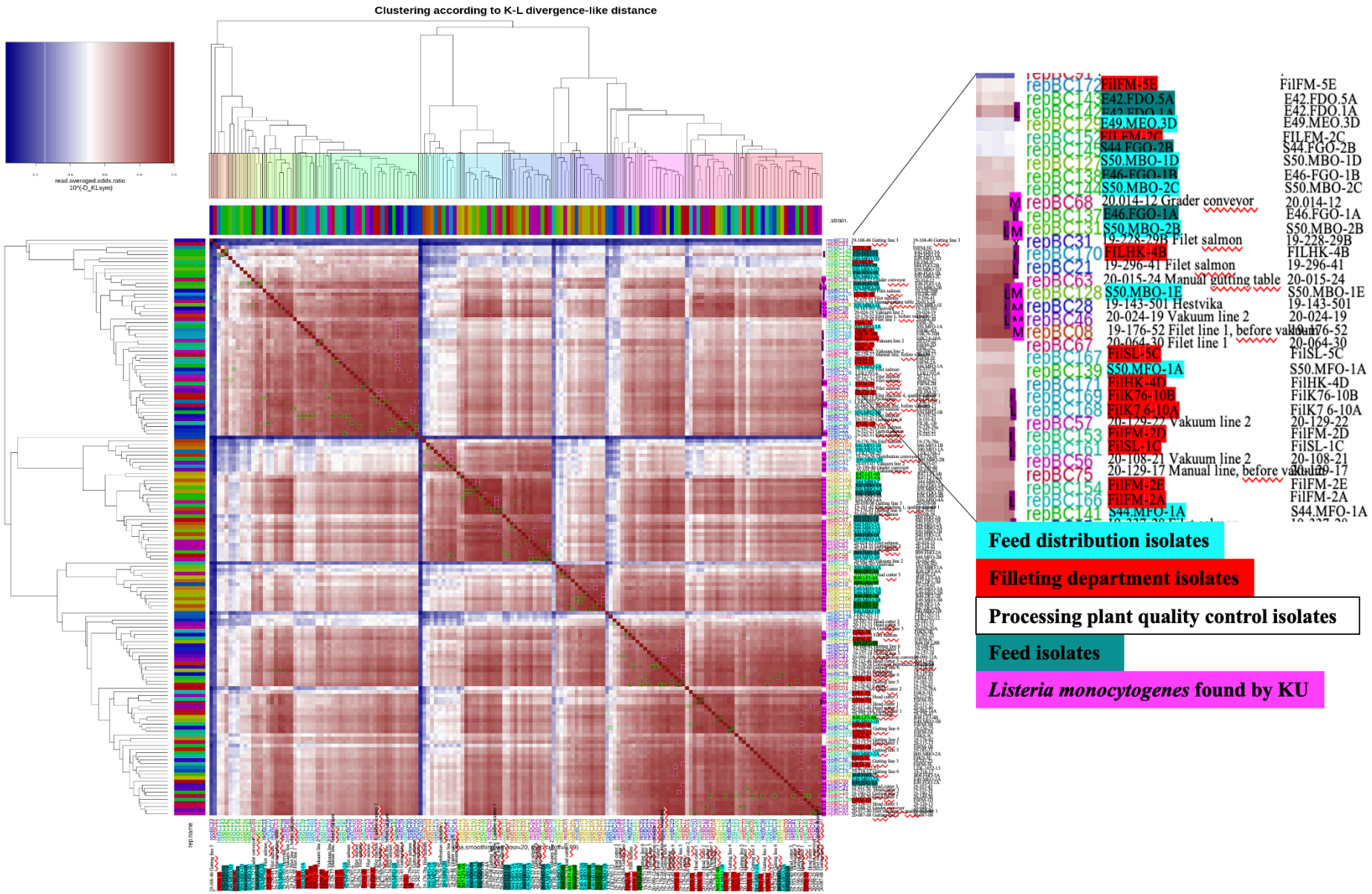

Figure 13 *– Heat map generated by LCp clustering, September 2021. The strains are ordered in a phylogenetic tree based on LCp differentiation in both the top x-axis and left y-axis, whilst their ON-rep-seq barcode ID and TraceListeria ID is given along the bottom bottom x-axis and right y-axis. Each pixel is the comparison between two isolates, and the darker the red colour of the pixel is the more similar the LCp plots between the isolates are. The more genetically similar the isolates are the more they tend to cluster. ON-rep-seq bioinformatical analysis was conducted by dr. Krych's team at KU.*

Based on ON-rep-seq results 57 *L. monocytogenes* isolates spanning the entire TraceListeria project were chosen for WGS. The criteria for WGS selection were that the entire value chain of the salmon production industry was included, and that the isolates had to be characterized as *L. monocytogenes* through the qPCR assay, classical media cultivation and ON-rep-seq assay. Ten of the 57 isolates selected for WGS stemmed from the filleting department. Twelve isolates obtained throughout the TraceListeria project were included to broaden the picture of contamination throughout the value chain over time. Six of these twelve isolates stemmed from feed and environmental samples taken in 2021, and the other six isolates were obtained by the processing plant's own quality control team in 2019 and 2020 (Table A2). Out of the ten filleting department isolates chosen for WGS, the ON-rep-seq analysis provided two distinct LCp plots. One distinct plot was seen for two of the ten isolates, which were duplicates from a sample collected of quality scanner 4 in week 26. The other distinct plot was observed in four of the isolates (Figure 14). Discernable LCp plots were not available for all of the ten isolates. For instance, the LCp plot for U22 FilFM-2B had too much background noise to discern a distinct morphology for comparison (Figure A3). Therefore, the similarity between isolates from the same sample point could not be manually determined.

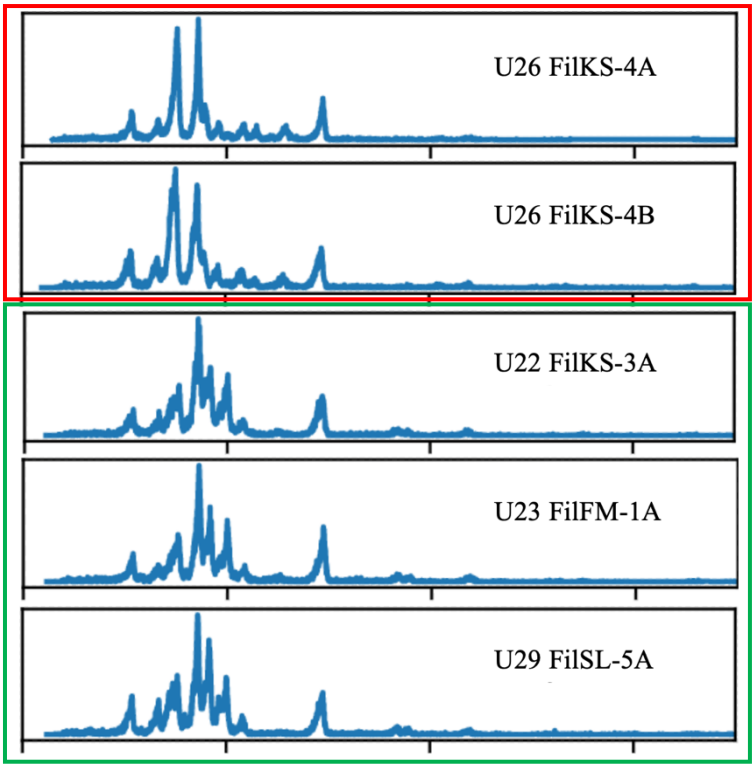

Figure 14 *– LCp plots for 5 of the 10 filleting department isolates selected for WGS, displaying two distinct morphologies observed after ON-rep-seq analysis. The first, outlined in red, belongs to two of the isolates chosen for WGS. The isolates are duplicates from a sample obtained from quality scanner 4 during the processing of salmon in week 26. The second morphology observed, outlined in green, was seen in all other isolates sent off for WGS, with the exception of U22 FilFM-2B which had too much background noise to distinguish any distinct morphology.*

3.3. Whole genome sequencing

The differentiation of the 22 isolates selected in this study with the Center of Genomic Epidemiology (CGE) at DTU, online sequence typing tool MLST revealed three sequence types. Only one isolate, U22 FilFM-2B, belonged to ST637, whilst 9 isolates were subtyped as ST8 and the remaining 12 isolates as ST37. The duplicates for U26 FilKS-4 were both ST8, in addition to one quality control sample from the gutting line in 2020. All other isolates from the filleting department and quality control team were subtyped as ST37 (Table 6).

Within each subtype, all isolates that were characterized as the same ST, had the same amount of virulence genes. So, all ST8 isolates had 83 virulence genes, all ST37 isolates had 82 virulence genes and U22 FilFM-2B had only six virulence genes. All isolates carried the *fosX* gene which encodes fosfomycin resistance, but no other acquired antibiotic resistance genes were found in any of the genome assemblies.

Table 6 – Genomic characterization of 22 WGS isolates, and one reference genome, based on WGS data. Each isolates' corresponding WGS barcode ID is given along with the identified sequence type based on seven conventional MLST loci, the number of virulence genes with a sequence identity higher than 98%, and the number of acquired antibiotic resistance genes.

Isolate ID	WGS barcode ID	MLST	No. virulence genes (ID>98%)	No. antibiotic resistance genes
S40.FHO.1D	TL01-02	8	83	1
S50.FGO.3C	TL01-05	8	83	1
S09.MBO.2A	TL02-02	8	83	1
S40.MBO.2B	TL02-10	8	83	1
S44.MFO.2C	TL03-01	8	83	1
S44.MFO.2E	TL03-02	8	83	1
U26 FilKS-4B	TL04-06	8	83	1
U22 FilKS-3A	TL04-07	37	82	1
U23 FilFM-1A	TL04-08	37	82	1
U26 FilKS-4A	TL04-09	8	83	1
U29 FilSL-5A	TL04-10	37	82	1
U22 FilFM-1E	TL04-11	37	82	1
U22 FilFM-2B	TL04-12	637	6	1
U22 FilFM-3A	TL05-01	37	82	1
U22 FilKS-3E	TL05-02	37	82	1
U23 FilFM-1B	TL05-03	37	82	1
20-038-08	TL05-04	8	83	1
19-170-54	TL05-05	37	82	1
19-218-60	TL05-06	37	82	1
LEK-1652-13	TL05-07	37	82	1
19-357-43	TL05-08	37	82	1
20-048-25	TL05-09	37	82	1
L. monocytogenes EGD-e	Reference	35	88	1

The amount of raw data varied between the three subtypes. All ST37 isolates' FASTA file sizes were 2,96 MB, ST8 isolates' file sizes were 3,07 ± 0,013 MB and ST637 had a file size of 2,95 MB.

The phylogenetic tree seen in Figure 15 based on hqSNPs supported the similarities between isolates, clustering them into two distinct group, with one outlier U22 FilFM-2B. The two groups corresponded to the two identified ST, ST8 and ST37. The reference genome of *L. monocytogenes* EGD-e was included to see whether both groups differentiated from it.

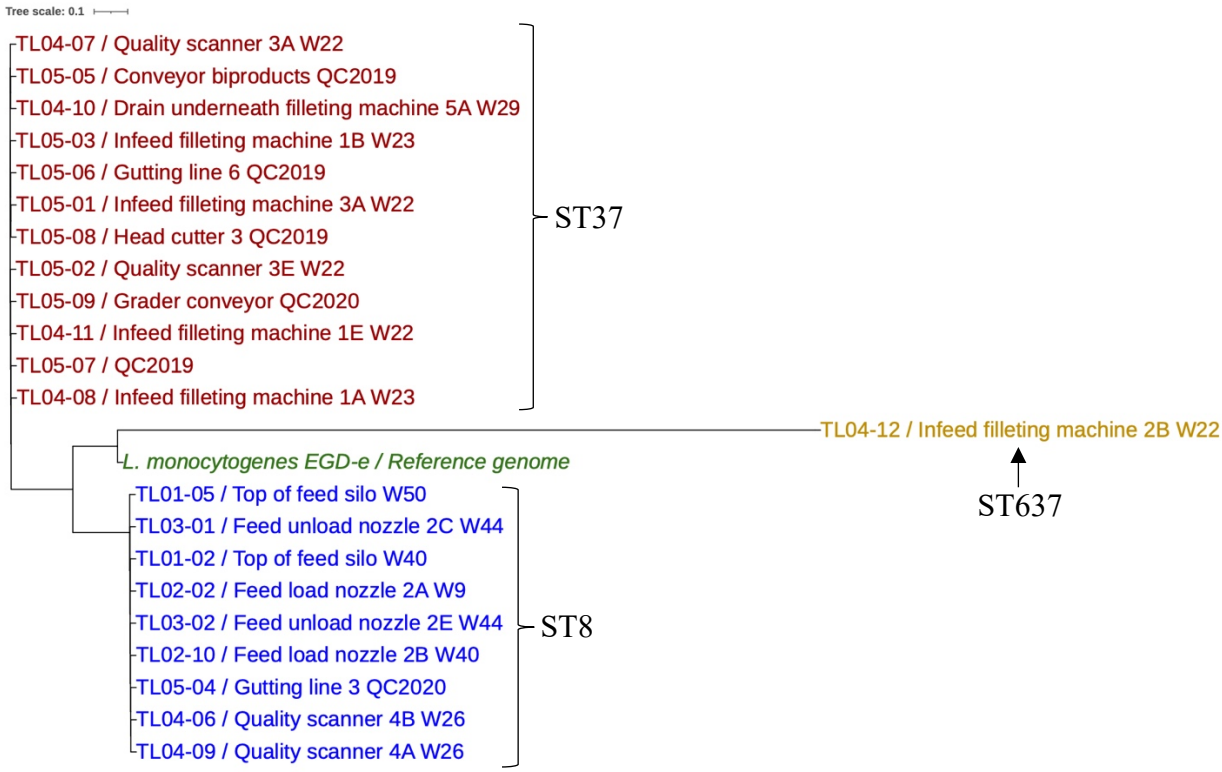

Figure 15 *– Phylogenetic tree of the 22* L. monocytogenes *isolates in addition to reference sequence L. monocytogenes EGD-e based on SNPs. The isolates cluster into two groups which correspond with their annotated ST. The top cluster consists of isolates that make up ST37, while the bottom cluster consists of isolates from ST8. Since U22 FilFM-2B belongs to ST637 and is the only isolate to do so, it does not form a cluster.*

All isolates, with the exception of U22 FilFM-2B, contained five specific virulence genes needed by *L. monocytogenes* for invasive listeriosis to occur. U22 FilFM-2B did not contain any of these virulence genes (Table 7).

Table 7 – Specific virulence genes observed in WGS data for all isolates applicable to this study. These 5 genes; *inlA*, *actA*, *plcA*, *plcB* and *hly* are needed for invasive listeriosis. X = isolate contains virulence gene with ID > 98%, - = isolate does not contain virulence gene

Isolate ID	Barcode	*inlA*	*actA*	*plcA*	*plcB*	*hly*
S40.FHO.1D	TL01-02	x	x	x	x	x
S50.FGO.3C	TL01-05	x	x	x	x	x
S09.MBO.2A	TL02-02	x	x	x	x	x
S40.MBO.2B	TL02-10	x	x	x	x	x
S44.MFO.2C	TL03-01	x	x	x	x	x
S44.MFO.2E	TL03-02	x	x	x	x	x
U26 FilKS-4B	TL04-06	x	x	x	x	x
U22 FilKS-3A	TL04-07	x	x	x	x	x
U23 FilFM-1A	TL04-08	x	x	x	x	x
U26 FilKS-4A	TL04-09	x	x	x	x	x
U29 FilSL-5A	TL04-10	x	x	x	x	x
U22 FilFM-1E	TL04-11	x	x	x	x	x
U22 FilFM-2B	TL04-12	-	-	-	-	-
U22 FilFM-3A	TL05-01	x	x	x	x	x
U22 FilKS-3E	TL05-02	x	x	x	x	x
U23 FilFM-1B	TL05-03	x	x	x	x	x
20-038-08	TL05-04	x	x	x	x	x
19-170-54	TL05-05	x	x	x	x	x
19-218-60	TL05-06	x	x	x	x	x
LEK-1652-13	TL05-07	x	x	x	x	x
19-357-43	TL05-08	x	x	x	x	x
20-048-25	TL05-09	x	x	x	x	x
L. monocytogenes EGD-e	Reference	x	x	x	x	x

3.4. Characterization

3.4.1. Antimicrobial susceptibility test

In total 81 isolates, three replicates from 29 samples, were exposed to an antimicrobial susceptibility test including eight antibiotics (Table 5, Table A3). The antibiotic tetracycline creates a zone larger than the breakpoint of 19 mm, required to be deemed resistant, for all isolates. In addition, none of the isolates fall within the intermediate category 19-22 mm. All isolates tested susceptible to the antibiotic as the zones where ≥ 25mm (Figure 16).

E does not have an intermediate category, and two isolates were measured below the resistance breakpoint of 25mm. One isolate, U26 FilKJ 11-15 B, did not produce a zone at all. The other isolate that falls within the resistant category, U26 FilSL-4D, had a zone diameter of 24 mm. From all the isolates subjected to this study, 14 were observed to have zone diameters of 25mm, the breakpoint limit between resistant and susceptible (Figure 16).

CN has a very large intermediate category (21-50 mm). All isolates fall within the intermediate category as the zone diameters measured between 22 mm and 27 mm.

SXT does not have an intermediate category, isolates are either susceptible at zone diameters ≥ 29 mm or resistant at values below that. The lowest diameter measured in this study was 30 mm, placing all isolates in the susceptible category (Figure 16).

Two isolates, U22 FilFM-2A and U26 FilKJ 11-15B, subjected to MEM fall within the resistant category having zone diameters of 25 mm which is below the breakpoint of 26 mm. One isolate, U29 FilFM-2A, had a zone diameter of 26 mm, exactly the breakpoint limit. The resistant isolate U26 FilKJ 11-15B also tested as resistant for E (Figure 16).

One isolate, U34 FilFM-2A, did not have a zone around the AMP disc and is thereby the only isolate which falls within the resistant category. All other isolates fall within the susceptible category with the second smallest zone diameter measured at 19 mm, and the majority of isolates having zone diameters between 23 mm and 25 mm, above the 16 mm breakpoint. AMP does not have an intermediate susceptible category (Figure 16).

There were 10 isolates observed to have zone diameters at the breakpoint limit between resistant and susceptible, as there is no intermediate category, around the P discs. This limit is at 13 mm. However, as all isolates were ≥ 13 mm, they were susceptible (Figure 16).

The vast majority of isolates (n = 62) had no visible zone around the FOX antimicrobial discs. Eight isolates had a zone diameter of 11 mm and another three had diameters of 14 mm (Figure 16).

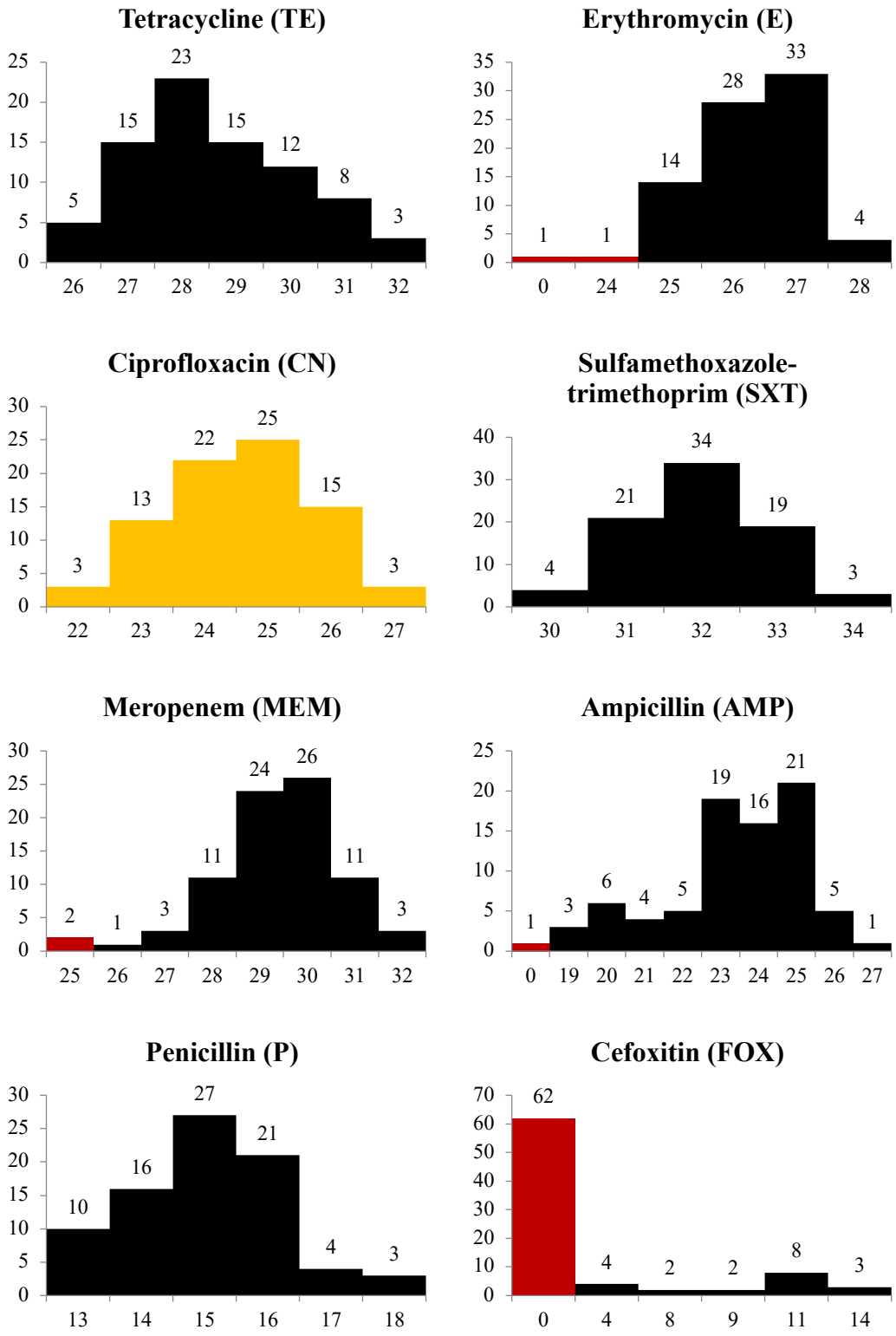

Figure 16 *– Histograms for each antibiotic used in this study and their respective zones of inhibition measured on all isolates. The y-axis displays the frequency of isolates at specific zones of inhibition measured in mm along the x-axis.* ***Red*** *columns signify the resistant breakpoints,* ***black*** *columns signify the susceptible breakpoints and* ***yellow*** *columns signify the intermediate category.*

A summary of the antimicrobial susceptibility test results is given below (Table 8), in addition to displays of selected MHF agar plates after completed testing (Figure 17, 18).

Table 8 – Antimicrobial profiles of 81 *Listeria spp.* selectively isolated from the filleting department.

	Number of isolates (%)		
Antimicrobial agent	**Susceptible**	**Intermediate**	**Resistant**
Tetracycline (TE)	81 (100)	0	0
Erythromycin (E)	79 (98)	0	2 (2)
Ciprofloxacin (CN)	0	81 (100)	0
Sulfamethoxazole-trimethoprim (SXT)	81 (100)	0	0
Meropenem (MEM)	79 (98)	0	2 (2)
Ampicillin (AMP)	80 (99)	0	1 (1)
Penicillin G/Benzylpenicillin (P)	81 (100)	0	0
Cefoxitin (FOX)	N/A	N/A	62 (77)

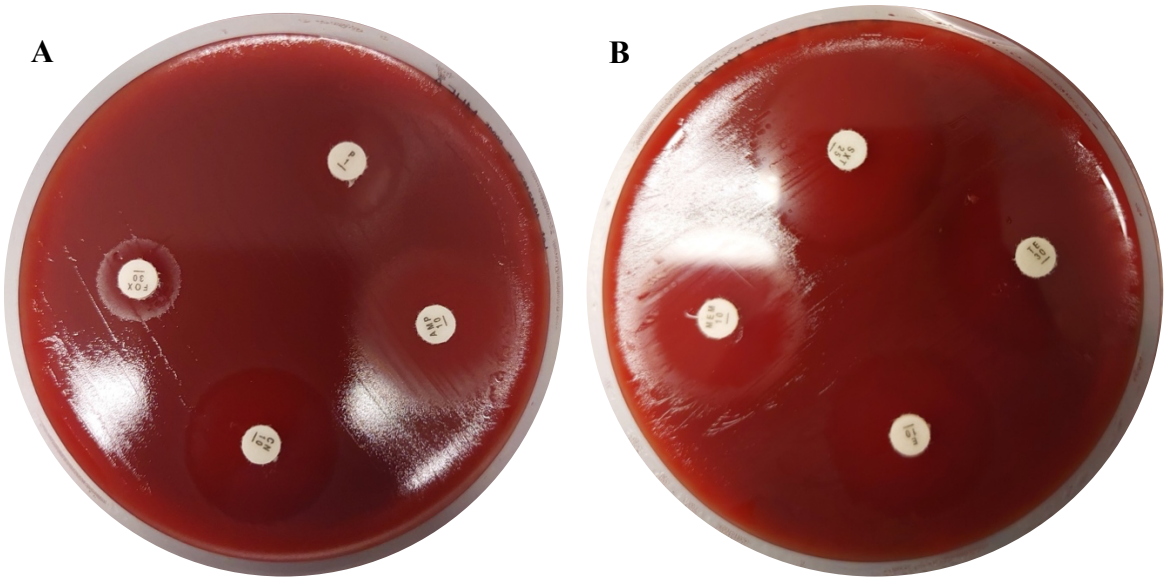

Figure 17 *– MHF agar plates for U34 FilSL-1C (A) Plate with benzylpenicillin, cefoxitin, ampicillin and ciprofloxacin discs (B) Plate with meropenem, sulfamethoxazole-trimethoprim, tetracycline, and erythromycin discs.*

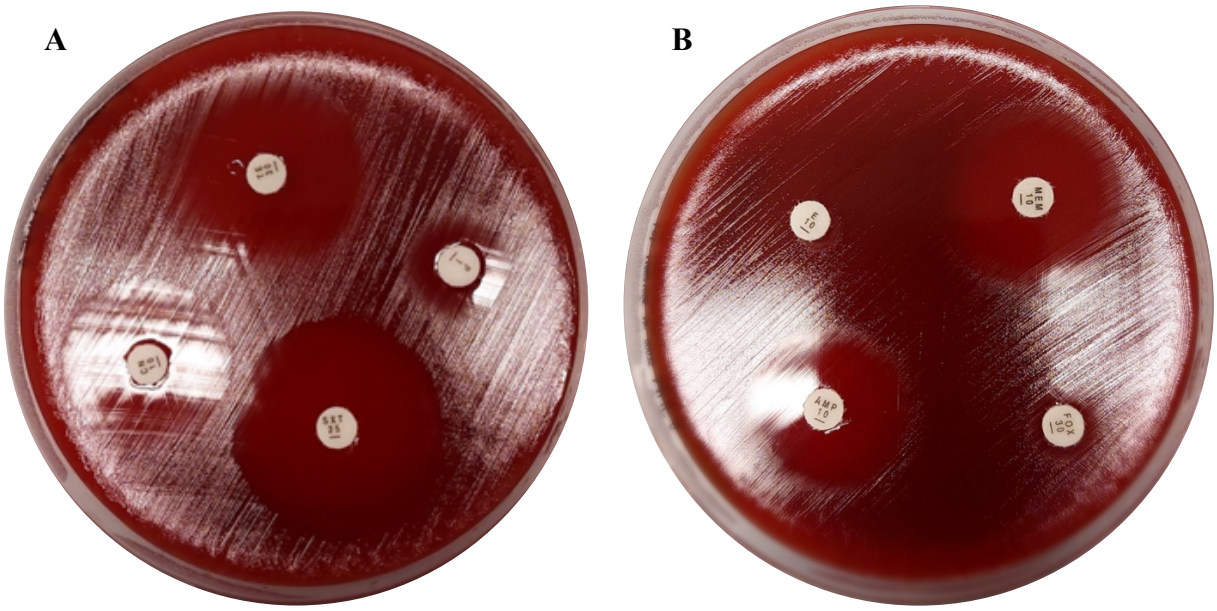

Figure 18 *– MHF agar plates for U26 FilKJ 11-15B* ***(A)*** *Plate with sulfamethoxazole-trimethoprim, tetracycline, benzylpenicillin, and ciprofloxacin discs,* ***(B)*** *Plate with ampicillin, erythromycin, meropenem and cefoxitin discs.*

3.4.2. Cold tolerance assay

This test was performed only on the isolates selected for WGS. A selection of isolates were used to make a standard curve to enumerate CFU/ml based off absorbance measurements (Figure 19, Table9).

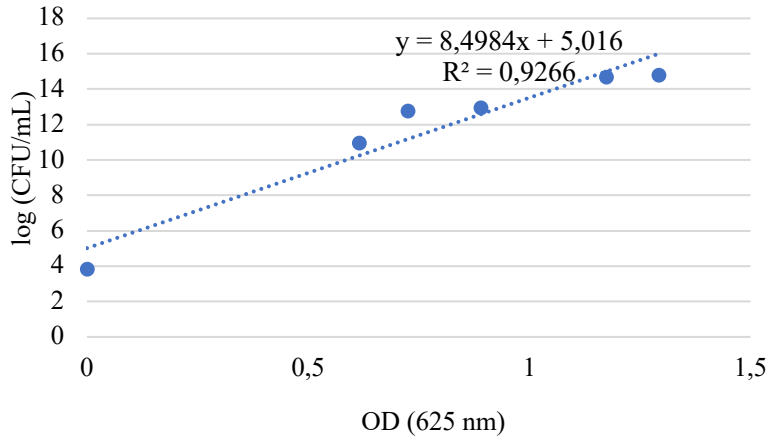

Figure 19 *– Standard curve for enumerating CFU/ml based off absorbance measurements at OD 625nm given by a selection of isolates.*

Table 9 – Isolates selected for absorbance vs. CFU/mL standard curve, to gain formula for enumerating all isolates spectrophotometrically.

Isolate ID	Sample point
S44.MBO.2C	Feed distribution, feed load nozzle, week 44 2020, feed factory F-C
S40.MBO.2B	Feed distribution, feed load nozzle, week 40 2020, feed factory F-C
H18.HFO.7C	Whole fish in box product, week 18 2021, processing facility O-A
R47.LF3.1C	Live fish, production enclosure 8, week 47 2020, production facility O-C
R47.DF3.6A	Dead fish, production enclosure 8, week 47 2020, production facility O-C
U22 FilFM-3A	Infeed filleting machine 3, week 22 2021, processing facility O-A
20-048-25	Quality control, grader conveyor, 2020, processing facility O-A

Isolates were exposed to two different temperatures, 37°C and 12°C to evaluate ability to grow at lower temperatures. No isolates displayed any tolerance to cold exposure. One isolate, U22 FilKS-3A, had a differential μ_{max} of -1,16 and was classified as sensitive (Figure 20A).

The STs show no statistically significance in growth variation when exposed to a temperature of 12°C between them (P = 0,328) (Figure 20B).

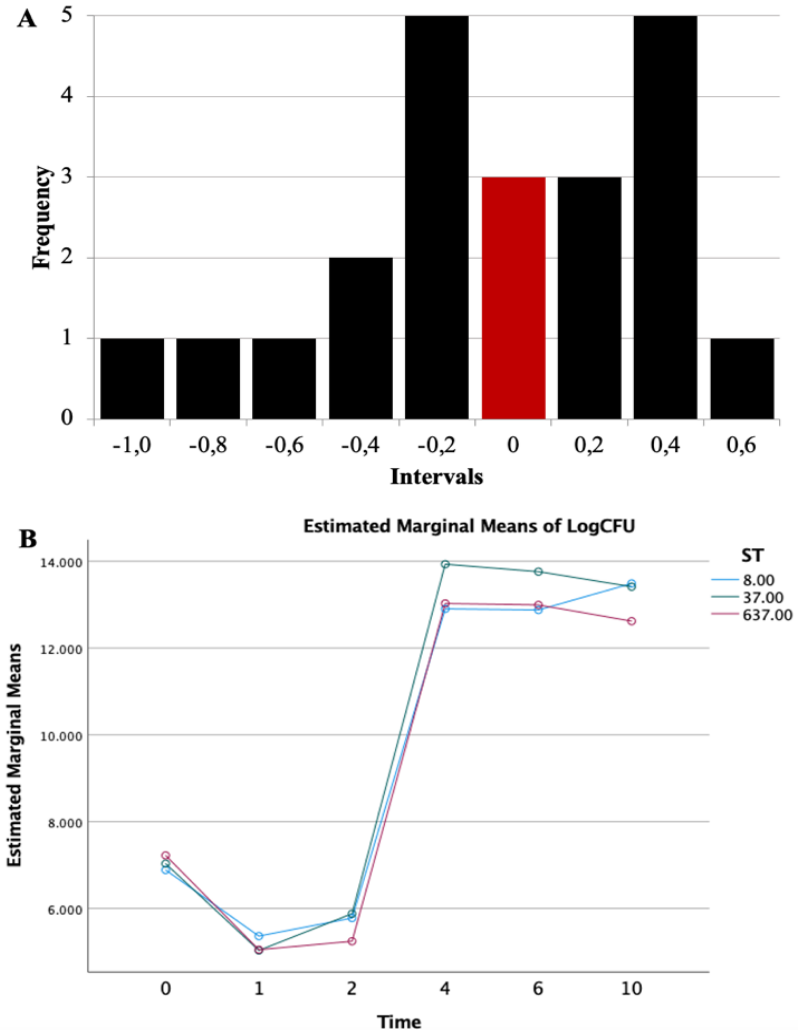

Figure 20 *– **(A)** Distribution of isolates at intervals of 0,2 µmax from the median µmax (red column). **(B)** Estimated marginal means of log_{10} CFU/mL growth curve for the three STs included in this study from the filleting department, as well as five ST8 isolates from the TraceListeria value chain and six isolates from the processing plant's own quality control team. The Figure presents stress tolerance variation among the STs when exposed to a temperature of 12°C over a period of 10 days. Measurements were taken day 0, 1, 2, 4, 6, and 10.*

Incubated at 37°C, the isolates reached exponential phase within the first day and by the 2nd day most had entered the death phase as CFU/mL concentrations started decreasing (Figure 21). Incubated at 12°C, the isolates' lag phase lasted until at least the second day of measuring CFU/mL. By day 4 all were in exponential phase, by day 6 all isolates were in stationary phase. The isolates remained in stationary phase for the rest of the experiment at 12°C. The isolates exposed to 12°C did not reach the same concentration as many of the isolates incubated at 37°C before entering stationary/death phase (Figure 21).

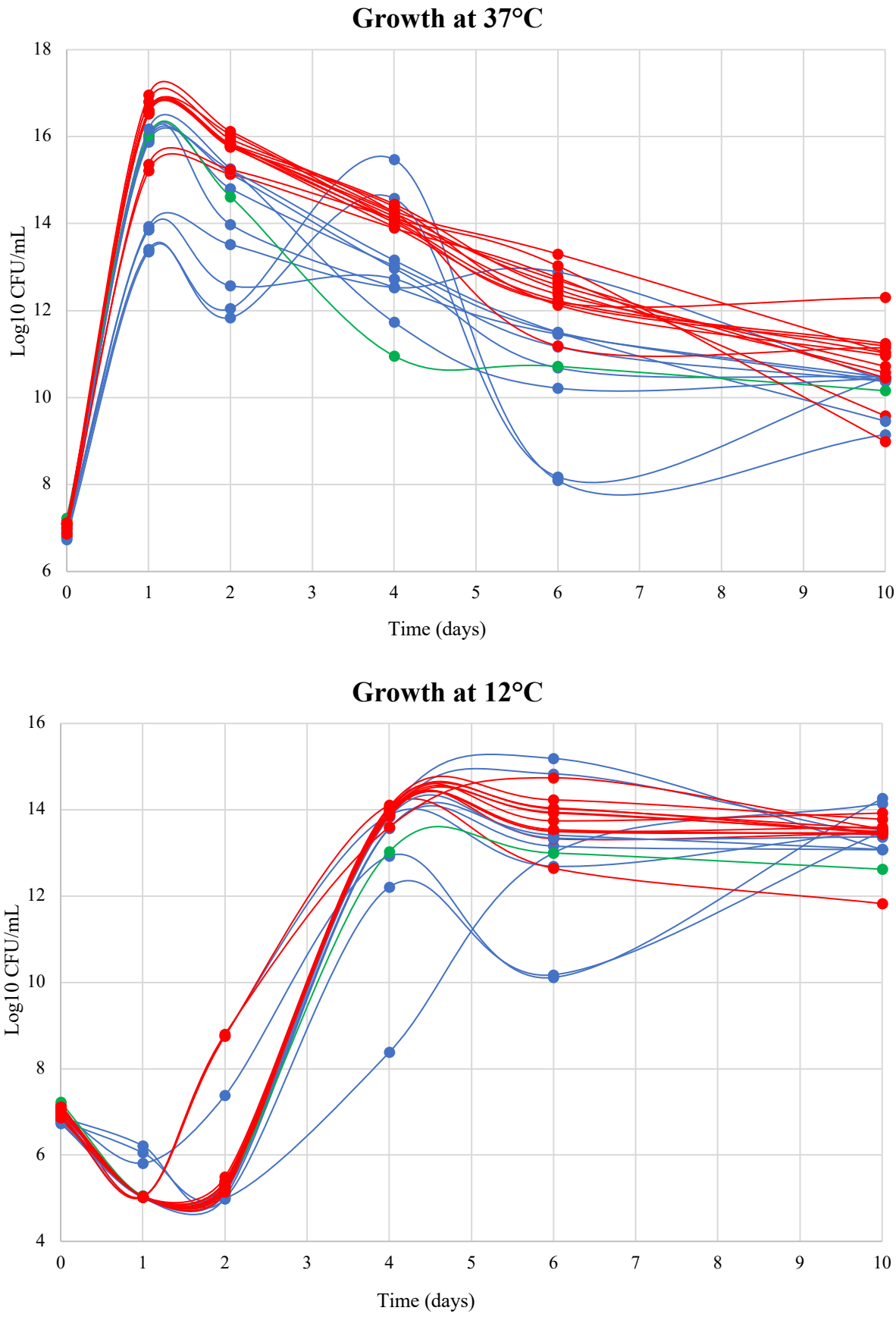

Figure 21 *– Growth curve for isolates incubated at two different temperatures (TOP) isolates incubated at 37°C, (BOTTOM) isolates incubated at 12°C.* ***Red*** *lines indicate isolates classified as ST37,* ***blue*** *lines ST8 and* ***green*** *ST637.*

3.4.3. Salt and acid tolerance assay

When adding stress components such as salt or acid the growth of *L. monocytogenes* was restricted compared to when no stress was added to the media (Figure 22). When not exposed to stress the isolates displayed a normal, sigmoidal growth curve. A blank BHIB plate was added to check for potential contaminations, which occurred within the last 5 hours of the analysis. However, the addition of stress factors did not stop growth, nor delay the lag phase of the isolates. The growth curve for pH seems to flatten out towards the end, whilst the salt curve seems to still be increasing (Figure 22).

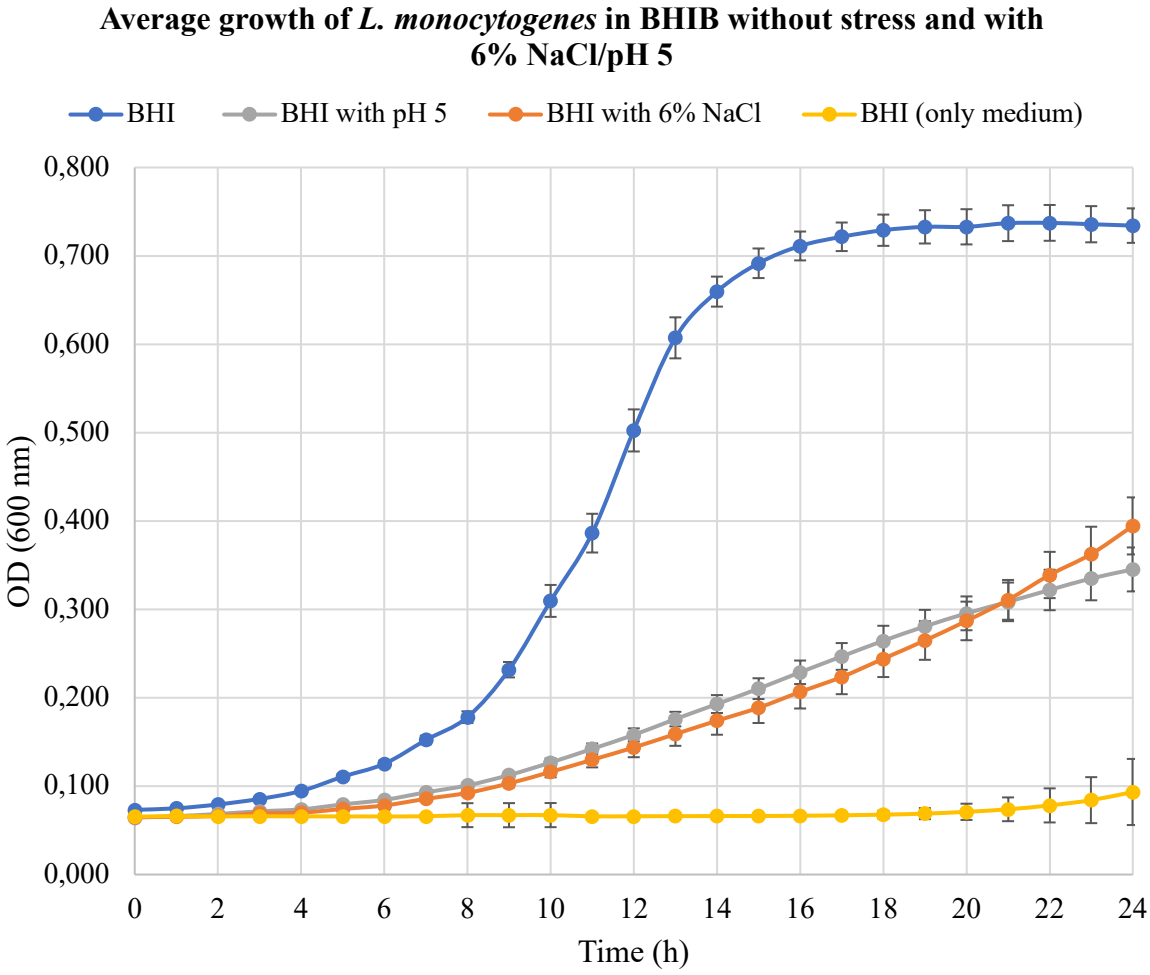

Figure 22 *– Growth curve of median ABS values for* L. monocytogenes *isolates exposed to three different environments; without any stressor, with lowered pH (pH = 5), and increased salt concentration (6% NaCl). A plate of blank BHIB was also analyzed to determine amount of outside contamination during ABS measurements.*

There was some variation observed in the growth between isolates when exposed to stress factors such as salt or acid, but the variation is low (Figure 23).

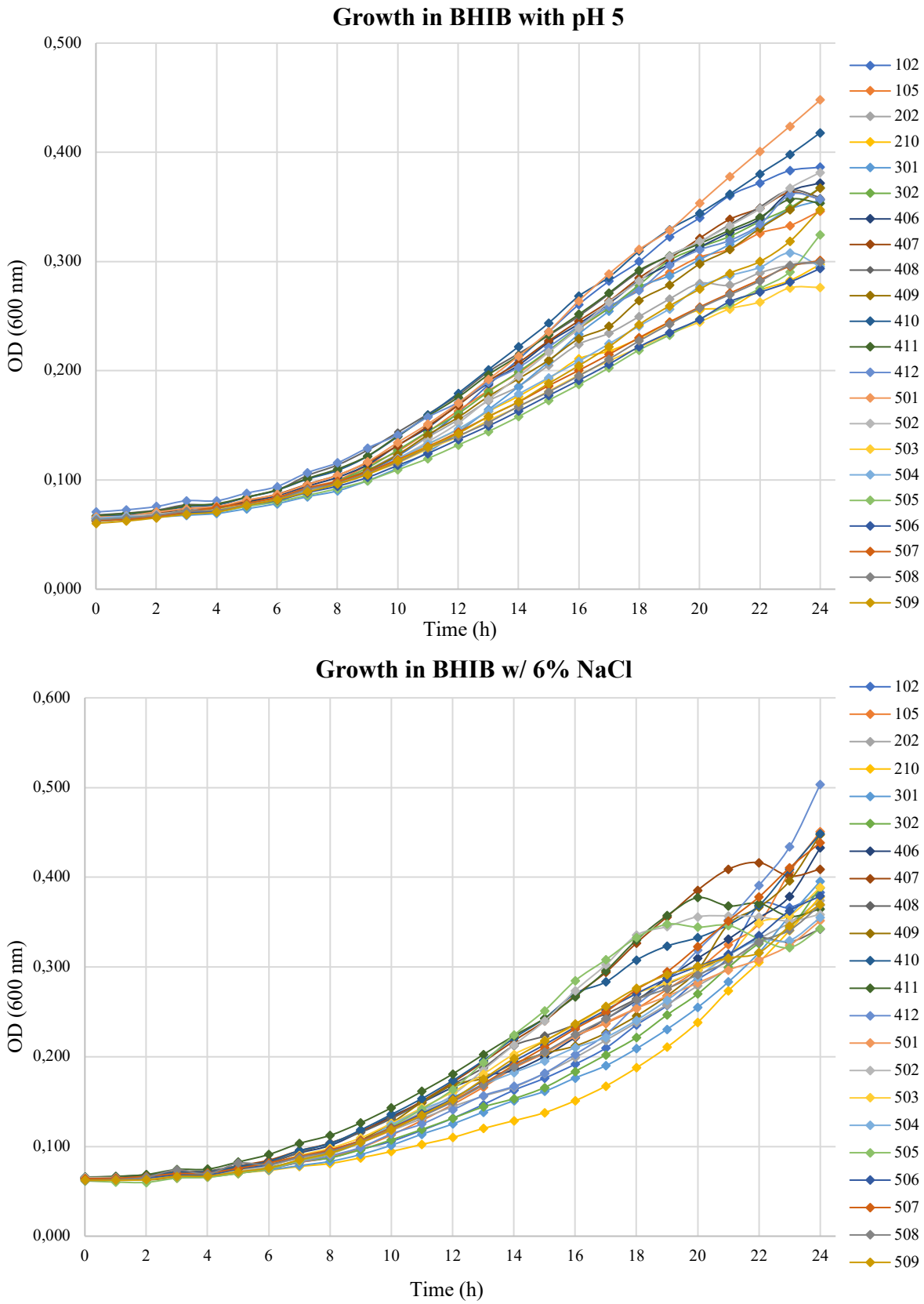

Figure 23 *– Growth curve for all isolates when exposed to BHIB with pH lowered to 5 with HCl (TOP) or increased salt concentration (6% NaCl) (BOTTOM). The numbers referring to an isolate is derived from their WGS Barcode ID, minus TL0 in front (i.e 102 = TL01-02).*

The fold decrease when *L. monocytogenes* isolates were exposed to lowered pH = 5, varied between 1,9-2,9. When exposed to salt the fold decrease varied between 1,8-2,9. They show a low level of differentiation in growth (Figure 24).

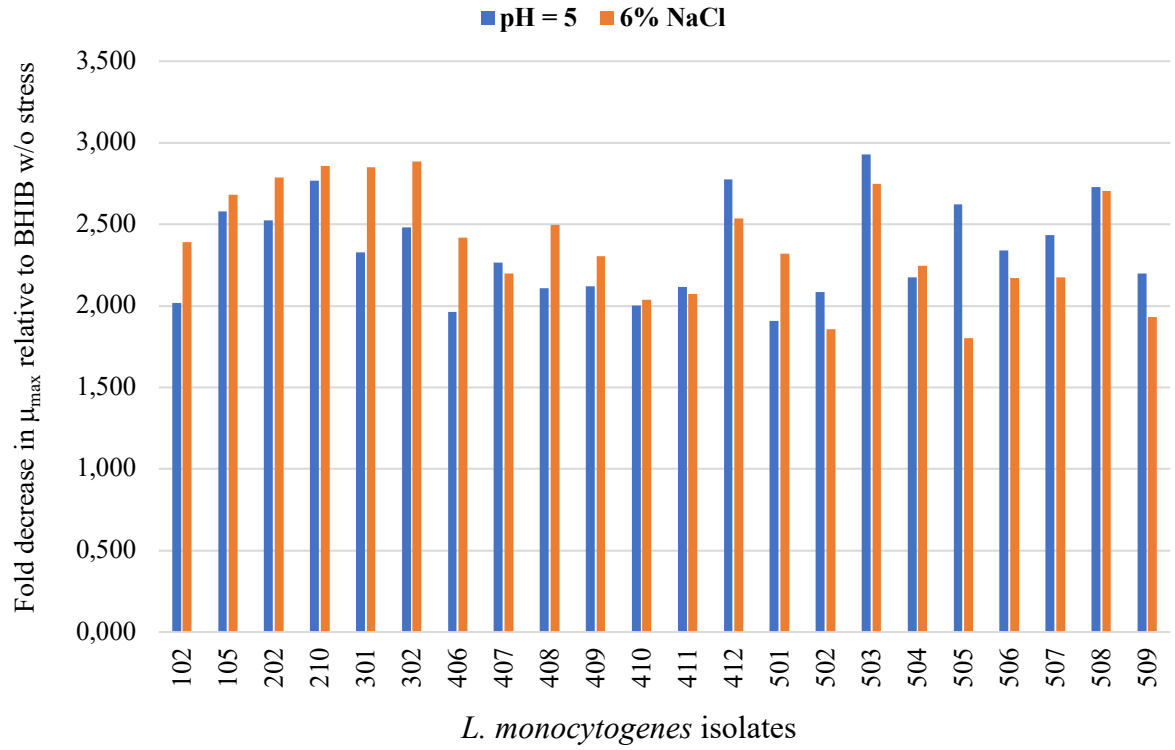

Figure 24 *– Fold decrease in growth rate among* L. monocytogenes *exposed to BHIB with pH lowered to 5 with HCl indicated in blue, and BHIB with increased salt concentration (6% NaCl) indicated in orange.*

There was a larger degree of variation among STs when exposed to an increased salt concentration (6% NaCl) when compared to lowered pH (pH = 5). Interestingly, ST8 and ST37 showed a statistically significant similarity in growth when exposed to an acidic environment (P = 1.000), but when exposed to elevated salt concentration ST37 is statistically significantly different from ST8. ST637 was included in the ANOVA, despite only consisting of one single isolate. ST637 shows a variance from ST37 and ST8 during growth in both conditions, however, more notably in low pH (Figure 25).

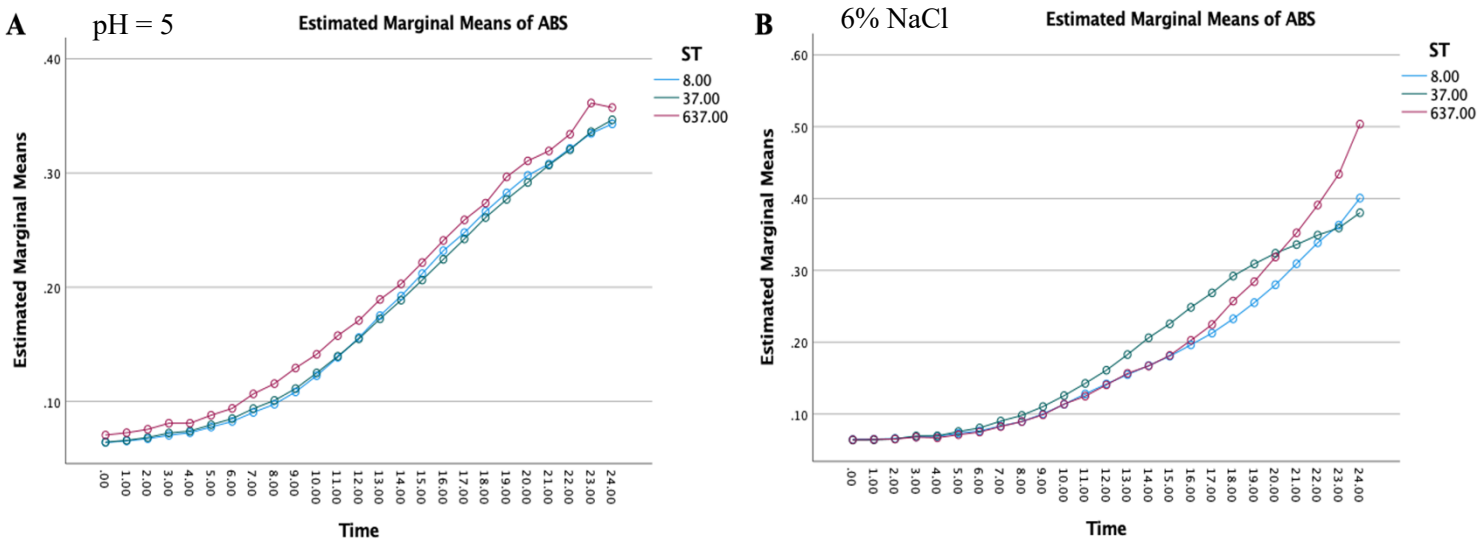

Figure 25 *– Estimated marginal means of absorbance growth curve for the three STs included in this study from the filleting department, as well as five ST8 isolates from the greater TraceListeria project and six isolates from the processing plant's own quality control team. The Figures present stress tolerance variation among the STs over 24h (y-axis).*

(A) *Growth curve for ST8 (blue), ST37 (green) and ST637 (red) subjected to lowered pH = 5.*

(B) *Growth curve for ST8 (blue), ST37 (green) and ST637 (red) subjected to increased salt concentration (6% NaCl).*

4. Discussion

Species- and strain-level differentiation of microorganisms is essential for source tracking contamination sources in the value chain of any food processing facility (Wiedmann, 2019). The seafood industry is consistently affected by *L. monocytogenes* contamination, which can have severe consequences on human life during foodborne outbreaks (European Food Safety et al., 2021). Highlighting the need for strong internal surveillance and implementation of HACCP strategies to ensure food safety for consumers.

In this study isolates were obtained through 5 processing cycles by sampling from a Norwegian salmon processing plant. Samples were taken during processing to increase the likelihood of obtaining *Listeria spp*. When the samples arrived at NTNU they were subjected to a selective enrichment process and real-time PCR assay, and results were compared with results from conventional plating techniques. The isolates were then identified and differentiated down to the strain-level with ON-rep-seq and evaluated by WGS. Finally, isolates were exposed to characterization assays to determine strain variability in stress tolerance.

The results of the qPCR assay were obtained within 30 hours, proving it is a quick, initial screening method for pathogenic bacteria in a food processing plant. It identified the presence of *Listeria spp.* in all sample points of the production line. The assay identified the presence of *L. monocytogenes* in most sample points, with the exception of the head cutter and the skin side of the finished fillet product. This shows that *L. monocytogenes* is present in both the raw material and the processing environment. The infeed filleting machine immediately jumps out as a major contamination point with the highest number of positive samples for both *Listeria spp.* and *L. monocytogenes*. The infeed machine had been pointed out as a "hot spot" for *L. monocytogenes* by the processing plant's own quality control team. Due to the machine's high complexity of many moving parts, the constant water being pumped through the processing system, and difficulty cleaning, it creates an environment favorable for growth of *L. monocytogenes* (Kovačević et al., 2012). *L. monocytogenes* is known to form biofilms which aid in its persistent survival within the processing environment. Cells within these biofilms get dislodged when the machinery begins moving and water gets flushed through the system, allowing for the recontamination of pathogenic bacteria such as *L. monocytogenes* (Tompkin, 2004; van der Veen & Abee, 2011). This poses the question whether the contamination from the FM machine is caused by transient strains, or whether there is a persistent strain affecting the processing line. From these initial results one can begin to assume that the head chopping machine is not ground zero for contamination as it only tested positive for *Listeria spp*. once

and never for *L. monocytogenes*. This is in stark contrast to the study done by Thomassen et al. (2021) where samples taken in 2017 showed that the head chopping machine was one of the major contamination points in the processing plant. It suggests that whatever corrective strategies were implemented by the processing plant might have worked. However, a test positive for *Listeria spp.* is an indication that the surrounding environment has the correct conditions to allow growth of *L. monocytogenes* as well (Orsi & Wiedmann, 2016). Interestingly, both the drains underneath the filleting machines and quality scanners had similar number of positive *Listeria spp.* samples (n = 8), but the drains only had one positive *L. monocytogenes* sample during the entire study. Compared to the quality scanner, which was the second worst offender after the infeed machine in number of positive for *L. monocytogenes* samples (n = 4) (Figure 11). One would assume that the drain would be a favorable environment to promote growth of *L. monocytogenes* strains that are able to form biofilms (Donlan & Costerton, 2002; van der Veen & Abee, 2011). If *L. monocytogenes* was present its growth might have been suppressed by other microorganisms such as *Pseudomonas fragi* or *Staphylococcus xylosus* (Carpentier & Cerf, 2011; Carpentier & Chassaing, 2004; Norwood & Gilmour, 2001). However, the enrichment procedure done after sampling should ensure for selective growth of *Listeria spp.* and if they are present, they should have been detected through the qPCR assay. The qPCR assay is good for initial, rapid, and cost-effective screening of equipment, surfaces, and product for pathogenic bacteria with little training to conduct the assay (Berrada et al., 2006; O'Grady et al., 2009). It could at least be used to detect areas where conditions are favorable for growth. Further genotypic analysis such as ON-rep-seq or WGS is required for more discriminatory characterization and strain-level differentiation of isolates (Chen & Knabel, 2007; Orsi et al., 2011; Pontello et al., 2012; Swaminathan & Gerner-Smidt, 2007). These downstream assays will provide more genetic information needed for accurate source tracking of this dangerous microbe through the salmon processing plant. It should be noted that out of the 38 samples that tested positive for *Listeria spp.* and were subsequently inoculated onto BLA plates, only 32 isolates grew and could be used for downstream analysis. The reason for these false-positive PCR results might be best explained by the presence of *Listeria spp.* cells, however, not viable ones. The qPCR method is highly sensitive and not many cells are needed for a positive result (Postollec et al., 2011).

The ON-rep-seq method revealed very low genetic diversity among isolates obtained from the filleting. During LCp plot clustering, the filleting department isolates would cluster with the quality control isolates from 2019 and 2020, as well as isolates from feed and the distribution of feed to production enclosures. This indicates that there is not much genetic diversity among

isolates across the entire salmon production value chain when studying these samples. Two distinct groups with unique LCP plots could be discerned from the assay, indicating that at a minimum two different strains are present in the processing environment (Figure 14). During the first ON-rep-seq run, several of the LCp plots contained too much background noise to manually identify differences in peaks (Figure A3). However, the ON-rep-seq assay was still able to taxonomically identify all isolates, as previously shown by Krych et al. (2019) and Thomassen et al. (2021). Any future studies done on the isolates obtained in this study should therefore consider sending those isolates with LCp plots containing background noise for a new round of ON-rep-seq to positively determine genetic relationships between all isolates in this study, and the TraceListeria project as a whole.

Unique LCp plots will be able to inform the quality control team with high probability that the same strains are causing recontamination of the processing environment. The heatmap generated by LCp plot clustering allows for easy interpretation of strain similarity. Yet, the classification through the ON-rep-seq method cannot identify precisely which strains isolates are without comparison of identical LCp plots in a database, which currently does not exist. The ability of the ON-rep-seq assay to differentiate bacterial isolates down to the strain-level has been proven in previous studies (Krych et al., 2019; Thomassen et al., 2021). If a LCp plot database is developed, ON-rep-seq could present as a cheaper, strain-level differentiation alternative to WGS.

WGS currently has the most discriminatory power of resolution compared to other subtyping techniques, but requires high levels of bioinformatical skills and computer infrastructure (Barretto et al., 2021). To aid in the analysis of genomic data, several online pipelines and commercial softwares have been developed. All these pipelines have pros and cons which should be considered before use (Jagadeesan et al., 2019). In this study pipelines developed by the Center for Genomic Epidemiology at DTU were used for hqSNPs and MLST analysis (Bartual et al., 2005; Camacho et al., 2009; Griffiths et al., 2010; Jaureguy et al., 2008; Kaas et al., 2014; Larsen et al., 2012; Lemee et al., 2004; Wirth et al., 2006).

Based on WGS data, isolates were characterized into ST8, ST37 and ST637 (Table 6). The ST8 isolates were duplicates obtained from the same quality scanner 4 sample. Isolates of ST8 and ST37 were also observed in the feed factory and feed transport and distribution systems, however, not during the sea phase of the value chain examined by the TraceListeria project (Figure A4). ST37 was the most prevalent ST throughout the value chain examined here, and is also true for the study done by Thomassen et al. (2021) at the same processing plant (Lerfall et al., 2022). Two ST8 isolates were also observed by Thomassen et al. (2021) in the filleting

department, one from a salmon fillet and the other from quality scanner 2 (Thomassen et al., 2021). In this study the two ST8 isolates were duplicates, also from a quality scanner in the filleting department, however not from the same processing line (U26 FilKJ-4A and 4B). *L. monocytogenes* ST8 has been linked to two foodborne listeriosis outbreaks before due to the consumption of cold-smoked salmon products between 2014-2019 (EFSA, 2018, 2019b) (Table 1). In Norway, ST8 has continuously been detected in a salmon abattoir for over a decade (Fagerlund et al., 2016). All these factors demonstrate that this ST can persist in processing facilities and are pathogenic, which is of major concern in regard to food safety. *L. monocytogenes* ST37 has been detected in products and facilities processing meat, dairy, and vegetables, but no major foodborne outbreaks have been linked to this ST in Europe (Cabal et al., 2019; Stessl et al., 2020; Tomastikova et al., 2019).

To expand the collection of isolates for this study, all ST8 isolates from the TraceListeria value chain (n = 6), were characterized along with the filleting department isolates. Also, six isolates from the processing plant's quality control team from 2019 and 2020 were included. The quality control isolates were characterized as ST37 with the exception of 20-038-08, which was characterized as ST8. It has been suggested that ST8 is more persistent than ST37 (Muhterem-Uyar et al., 2018), but the results of this study does not necessarily support this claim as ST37 was the most prevalent even in isolates from 2019 and 2020. Therefore, it seems that it is the same strains of *L. monocytogenes* causing repeated positive tests from the processing environment and product, especially the infeed filleting machines.

The phylogenetic analysis done by CSI Phylogeny confirms the grouping of isolates characterized by MLST, as the tree in Figure 15 indicates that isolates cluster into two groups in accordance with their ST. The U22 FilFM-2B isolate did not cluster as it was the only isolate to be characterized as ST637. The two groups differ slightly from the reference genome, *L. monocytogenes* EGD-e, which is required for hqSNPs analysis. This pipeline, although simplified for more user-friendly bioinformatic analysis required more steps, parameters, and a reference genome to be completed. On the other hand, the MLST pipeline did not require a reference genome and only required the uploading of sequences before analysis. As pointed out by Jagadeesan et al. (2019), MLST provided adequate differentiation for source tracking. It was discussed whether reference genomes for each ST should have been implemented in the phylogeny analysis to see if isolates would differentiate further within each cluster. However, the use of only *L. monocytogenes* EGD-e was deemed discriminatory enough for this study. A future study could reanalyze the results obtained here with the inclusion of more reference genomes.

When comparing the LCp plots from ON-rep-seq analysis to the phylogenetic and MLST analysis, one can observe the high similarity between plots from isolates with the same ST and a distinct difference from isolates with different STs. In Figure 14, the isolates outlined in green were characterized as ST37 and the isolates outlined in red as ST8. This relationship between LCp plot morphology and MLST was also observed by Thomassen et al. (2021). Again, it must be pointed out that not all isolates had discernable plots. Any future studies should therefore consider reanalyzing isolates with disruptive plots to ascertain the relationship between LCp plots and ST. However, it seems that ON-rep-seq has the ability to differentiate *L. monocytogenes* down to the strain-level with a resolution comparable with WGS, for the fraction of the price (Table 3).

During *in silico* characterization of the WGS isolates' genomic data with the virulence finder pipeline, a homology was observed between isolates of the same ST. All ST8 isolates had 83 virulence genes (ID > 98%), the same as observed by Thomassen et al. (2021) in ST8 isolates from the same processing plant. All ST37 isolates contained 82 virulence genes, which is one more than observed by Thomassen et al. (2021), but the study does not specify which virulence genes were observed. Therefore, it cannot be determined which virulence gene has been acquired. The isolate U22 FilFM-2B only contained 6 virulence genes. The sequences were further analyzed for five specific virulence genes required for the microbe to manifest as invasive listeriosis, the five genes were *inlA*, *actA*, *plcA*, *plcB* and *hly* (Hamon et al., 2006) (Figure 1). Isolate U22 FilFM-2B did not contain any of the five virulence genes required for invasive listeriosis, bringing into question its pathogenic ability. The five genes were observed in all ST8 and ST37 isolates, severely increasing the interests in the surveillance of these strains due to the foodborne danger they might pose. These five specific virulence genes have been previously observed in ST8 strains isolated from fish processing environments in Denmark, Norway and Poland (Fagerlund et al., 2016; Wieczorek et al., 2020). Strains of ST37 have been reported to have the same five virulence genes before as well (Alvarez-Molina et al., 2021; Šteingolde et al., 2021). As mentioned, ST8 has been linked to foodborne listeriosis outbreaks before (EFSA, 2018, 2019b).

The WGS isolates' genomic data was also screened for acquired antibiotic resistance genes, of which only one was found in all isolates, which was resistance towards fosfomycin encoded by the *fosX* gene. So, the strains which are suspected to be persistent within the processing environment have not acquired any additional antibiotic resistance genes over time when comparing the quality control isolates from 2019 and 2020 with the isolates obtained in this study. This is in line with previous studies that acquired antimicrobial resistance is rare in *L.*

monocytogenes, and that the susceptibility towards antibiotics does not change much over time for this bacteria (Allerberger & Wagner, 2010; Andriyanov et al., 2021). Acquired resistance towards only fosfomycin in *L. monocytogenes* is known (Allerberger & Wagner, 2010; Swaminathan & Gerner-Smidt, 2007), but due to lack of space on MHF agar plates it was not tested during the *in vitro* antimicrobial resistance characterization. Further studies on the isolates from this study and future isolates obtained through regular sampling should consider adding fosfomycin to the antibiotics tested to unconditionally confirm this, although it is well established. Here one must note that the antimicrobial susceptibility testing was performed on isolates before the selection of isolates to be sent off for WGS. Therefore, many more isolates were exposed to this assay than the other characterization assays; cold, lowered pH and increased salt concentration which only included the 22 isolates chosen for WGS. In total 81 isolates were exposed to the antimicrobial susceptibility test, which consisted of three replicates from 27 different samples obtained from the product and processing environment in the filleting department. Cefoxitin was used as a control antibiotic for resistance, as *L. monocytogenes* has an intrinsic resistance towards this antimicrobial agent likely due to specific PBPs (Allerberger & Wagner, 2010; Hakenbeck & Hof, 1991; Swaminathan & Gerner-Smidt, 2007; Troxler et al., 2000). Out of the 81 isolates subjected to the antimicrobial susceptibility test, 62 (77%) had no zone of inhibition which is as expected (Table 8). Since *L. monocytogenes* has an innate immunity to this antibiotic, none of the isolates should have a zone of inhibition. The most likely explanation for this discrepancy is that some form of contamination of other microorganisms has occurred, either during the inoculation of colonies in the saline solution before turbidity measurement, or from blockage of sterile airflow in the LAF-bench. Future studies could perhaps look at the genomic data for the isolates which were susceptible to see whether there is some disruption in the genes encoding the PBPs. All other antibiotics used in this study (TE, E, CN, SXT, MEM, AMP and P) were antibiotics used in the treatment of listeriosis (Allerberger & Wagner, 2010; Swaminathan & Gerner-Smidt, 2007; Troxler et al., 2000). Two isolates were observed to be resistant to E and also MEM, whilst one isolate was resistant to AMP (Table A3). Isolate U26 FilKJ 11-15B was resistant to both E and MEM, but neither this isolate nor the other resistant isolates were sent off for WGS. Hence, this phenotypical observation cannot be confirmed with genomic data. Future studies should consider exposing these isolates to this assay again and simultaneously send them off for WGS for genomic characterization. Due to the very low level of genetic diversity among the filleting department isolates observed in the ON-rep-seq assay and WGS it seems unlikely that these

isolates contain acquired antibiotic resistance genes. Most likely some contamination occurred, or the discs applied to the MHF plates of these isolates were defect.

Finally, the 22 isolates selected for WGS were subjected to a variety of stress factors to establish tolerance, and whether there was any relation between ST and increased or lowered tolerance to; lowered temperature (12°C), lowered pH (pH = 5) and increased salt concentration (6% NaCl). The growth rate of the isolates was restricted when exposed to any of the three stress factors, but growth was not inhibited by cold, salt or acid exposure. During the cold tolerance assay, the isolates had a longer lag phase when incubated at 12°C compared to incubation at the optimum growth temperate of 37°C. However, when exponential phase was reached within 96h at 12°C $\log_{10}$ CFU/mL concentrations were not far below the maximum measured on average at 37°C before entering death phase. Death phase could not be observed at 12°C, only stationary phase which the isolates stayed within for the duration of the assay (Figure 21). Strains of *L. monocytogenes* are able to grow at temperatures as low as, and perhaps lower than 4°C (Farber & Peterkin, 1991; Hingston et al., 2017). The temperature chosen for this study was due to the temperature in the processing facility examined. No statistically significant relationship could be observed between ST and tolerance to low temperature. The variation of growth between isolates might not become apparent when exposed to 12°C, and future studies could attempt exposing isolates to lower temperatures to see a possible increase in variation. A temperature of 12°C might aide in the persistence of *L. monocytogenes* at the processing plant as they are still able to grow and perhaps stay within stationary phase long enough for more nutrients to become accessible during new processing cycles.

No statistically significant relationship between ST and tolerance could be observed in isolates when exposed to a heightened salt concentration. When exposed to a lowered pH, ST37 and ST8 isolates were statistically significant similar in tolerance, whilst ST637 was statistically significant different. As only a single isolate was characterized as ST637, the results of the ANOVA analyses does not have a solid statistical foundation in regard to this ST (Figure 25). Future studies could attempt to obtain more isolates of ST637 and expose them to the same stress factors as this study to conclusively determine any relationship between ST and tolerance. On average, the isolates had a similar tolerance to pH and salt (Figure 22). The lag phase was also not affected when compared to the microtiter plate where isolates grew in BHIB without any stress factors, but the growth rate in the exponential phase is lower for the isolates when exposed to either lowered pH or increased salt concentration. The fold decrease between isolates in each stress factor also did not vary greatly (Figure 24). For the most part isolates grew twice as slow in either stress factor when compared to the control plate without stress.

It should be noted that the methods, especially for the cold tolerance assay, had to be heavily modified to accommodate availability of resources and time for the study. This led to several observed sources of error in this study. Firstly, the OD of 625nm for the cold-tolerance assay was not given in the method by Hingston et al. (2017) and was chosen as that was the OD used during preparation of the McFarland standard. The methods of both Hingston et al. (2017) and Muchaamba et al. (2019) specify the use of a OD of 600nm for the salt and acid tolerance assays, which was then also done in this study. This disallowed the use of those ABS measurements for conversion to CFU/mL values as in the cold tolerance assay. The DMFit model could therefore not be used, and a maximum growth rate was not obtained for the WGS isolates when exposed to lowered pH or increased salt concentration. Secondly, the number of measurements taken in the cold tolerance assay led to maximum growth rates at 37°C that made no sense, such as negative values. This was due to the large variations in CFU/mL concentrations between each measurement and the lack of a stationary phase observed. Future studies should therefore include more measurements to expand the number of datapoints for this type of analysis.

It is highly unlikely for a food product undergoing processing to only be exposed to one factor limiting growth of microorganisms. Any future studies should therefore consider exposing the isolates to several limiting factors simultaneously to see whether certain STs are able to withstand that stress better compared to other STs. It has been reported that isolates containing a full length *inlA* genes show an increased tolerance to cold, salt and acid (Hingston et al., 2017; Kovacevic et al., 2013). This statement does not hold true in this study, however, as the ST637 isolate does not contain any of the five invasive listeriosis genes, including *inlA*, but it showed no decreased tolerance to any of the stress factors in this study (Table 8). Future studies into the stress tolerance of *L. monocytogenes* should consider screening genomic data for other genetic markers that could explain increased or decreased tolerance, such as plasmids, which was not done in this particular study. Strains of *L. monocytogenes* have been shown to be able to grow in conditions with 9-14% NaCl, even up to 20%, and in acidic conditions with pH = 4 (Farber & Peterkin, 1991; Shabala et al., 2008). So similar to the cold tolerance assay, perhaps the conditions set in this study were not strict enough to extrapolate any differences in tolerance in correspondence to ST. Future studies should therefore also consider, in addition to combining stressors, to examine tolerance to a variety of pH conditions and salt concentrations.

The results of this study show that ON-rep-seq has the ability to differentiate isolates of *L. monocytogenes* down to the strain level with a resolution comparable with WGS, which has also been reported in previous studies (Krych et al., 2019; Thomassen et al., 2021). The ON-

rep-seq method could potentially allow for a far cheaper and more food industry applicable way of classifying and differentiating bacteria. It was observed that strains from ST37 and ST8 are persistent within the processing environment as they were repeatedly observed in isolates from 2019 up until 2021, but the same cannot be concluded for ST637. The antimicrobial susceptibility test results concur with former findings that acquired antibiotic resistance in *L. monocytogenes* is rare (Allerberger & Wagner, 2010; Swaminathan & Gerner-Smidt, 2007). Finally, no growth could be inhibited for any isolate when exposed to any of the stress factors discussed previous, and no relationship between increased or decreased tolerance to stress and ST could be ascertained.

5. Conclusion

This study demonstrates the ability of the qPCR assay to quickly identify the presence of *L. monocytogenes* in the processing environment and products in a Norwegian salmon processing plant. The qPCR results were confirmed through conventional plating methods, using Thermo Scientific™ Brilliance™ *Listeria* Agar (BLA). These initial results revealed that the infeed filleting machines, and quality scanners were "hot spots" for *L. monocytogenes* contamination. The BLA plates were used downstream to propagate colonies into pure cultures so the DNA of the isolates could be isolated for ON-rep-seq analysis. The ON-rep-seq assay shows that there is a low level of genetic diversity among the filleting department. Based on ON-rep-seq results, isolates taxonomically classified as *L. monocytogenes* and spanning the entire TraceListeria value chain were selected for WGS. By characterizing whole-genome sequences, three STs were observed in the filleting department, ST8, ST37 and ST637. A phylogenetic tree created with a hqSNPs analysis clustered isolates into groups corresponding to their STs, confirming MLST is an adequate subtyping technique for source tracking *L. monocytogenes*. ST37 was the most prevalent ST observed in this study. Isolates belonging to ST37 and ST8 have been repeatedly isolated within the processing facility since 2017. Further characterization of isolates within these two STs detected five genes (*inlA*, *actA*, *plcA*, *plcB* and *hly*) required for invasive listeriosis, and they therefore have the potential to cause foodborne outbreaks. The isolates obtained in this study have no additional acquired antibiotic resistance genes with the exception of resistance to fosfomycin, and this pattern of susceptibility was observed during *in vitro* antimicrobial susceptibility testing. Finally, isolates selected for WGS were exposed to a cold-, salt- and acid tolerance assay. Growth could not be inhibited in this study but was limited. Only one isolate, U22 FilKS-3A, was considered sensitive to a temperature of 12°C. The sensitivity of isolates to the three stress factors is not dependent ST.

Future studies should further analyze the genome sequences of the isolates for genetic markers that could explain the ability of ST8 and ST37 to persist within the processing facility. The stress response assays should be redone, including more measurements for the cold tolerance assay, and mixing stress factors to observe the combined effect of them on restricting growth of *L. monocytogenes*. For the isolates which has non-discernable LCp plots one could consider reisolating their DNA for a new ON-rep-seq analysis to confirm that LCp plots correspond to specific STs. Lastly, continuous sampling and subtyping of isolates from the processing plant will aide in source tracking *L. monocytogenes* to specific contamination points within the value chain.

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Appendix

Table A1 – Samples tested positive for *Listeria spp.* which managed to grow on BLA after detection, and number of samples tested positive for *L. monocytogenes* along with their sample point, week sampled, study's sample ID, date sampled and date qPCR performed.

Sample point + production line/machine used	Week	Sample ID IMD	Date sampled	Date qPCR	*L.*spp	*L.mono*
Infeed filleting machine 1	22	U22 FilFM-1	01.06.2021	03.06.2021	1	1
Infeed filleting machine 2	22	U22 FilFM-2	01.06.2021	03.06.2021	1	1
Infeed filleting machine 3	22	U22 FilFM-3	01.06.2021	03.06.2021	1	1
Drain underneath filleting machine 1	22	U22 FilSL-1	01.06.2021	03.06.2021	1	0
Quality scanner 3	22	U22 FilKS-3	01.06.2021	03.06.2021	1	1
Head chopping machine 4	23	U23 FilHK-4	10.06.2021	12.06.2021	1	0
Infeed filleting machine 1	23	U23 FilFM-1	10.06.2021	12.06.2021	1	1
Infeed filleting machine 2	23	U23 FilFM-2	10.06.2021	12.06.2021	1	0
Infeed filleting machine 5	23	U23 FilFM-5	10.06.2021	12.06.2021	1	0
Drain underneath filleting machine 5	23	U23 FilSL-5	10.06.2021	12.06.2021	1	0
Quality scanner 4	23	U23 FilKS-4	10.06.2021	12.06.2021	1	0
Flesh side of fish filet nr 6-10	23	U23 FilKJ 6-10	10.06.2021	12.06.2021	1	0
Drain underneath filleting machine 4	26	U26 FilSL-4	20.06.2021	01.07.2021	1	0
Drain underneath filleting machine 5	26	U26 FilSL-5	20.06.2021	01.07.2021	1	0
Quality scanner 4	26	U26 FilKS-4	20.06.2021	01.07.2021	1	1
Quality scanner 5	26	U26 FilKS-5	20.06.2021	01.07.2021	1	1
Flesh side of fish filet nr 11-15	26	U26 FilKJ 11-15	20.06.2021	01.07.2021	1	1
Skin side of fish filet nr 1-5	26	U26 FilSK 1-5	20.06.2021	01.07.2021	1	0
Skin side of fish filet nr 11-15	26	U26 FilSK 11-15	20.06.2021	01.07.2021	1	0
Infeed filleting machine 2	29	U29 FilFM-2	20.07.2021	22.07.2021	1	1
Drain underneath filleting machine 5	29	U29 FilSL-5	20.07.2021	22.07.2021	1	1
Quality scanner 2	29	U29 FilKS-2	20.07.2021	22.07.2021	1	0
Quality scanner 5	29	U29 FilKS-5	20.07.2021	22.07.2021	1	1
Flesh side of fish filet nr 11-15	29	U29 FilKJ 11-15	20.07.2021	22.07.2021	1	0
Infeed filleting machine 1	34	U34 FilFM-1	24.08.2021	26.08.2021	1	0
Infeed filleting machine 2	34	U34 FilFM-2	24.08.2021	26.08.2021	1	0
Drain underneath filleting machine 1	34	U34 FilSL-1	24.08.2021	26.08.2021	1	0
Quality scanner 2	34	U34 FilKS-2	24.08.2021	26.08.2021	1	0
Flesh side of fish filet nr 1-5	34	U34 FilKJ 1-5	24.08.2021	26.08.2021	1	0
Flesh side of fish filet nr 11-15	34	U34 FilKJ 11-15	24.08.2021	26.08.2021	1	0
Skin side of fish filet nr 1-5	34	U34 FilSK 1-5	24.08.2021	26.08.2021	1	0
Skin side of fish filet nr 6-10	34	U34 FilSK 6-10	24.08.2021	26.08.2021	1	0
TOTAL					**32**	**11**

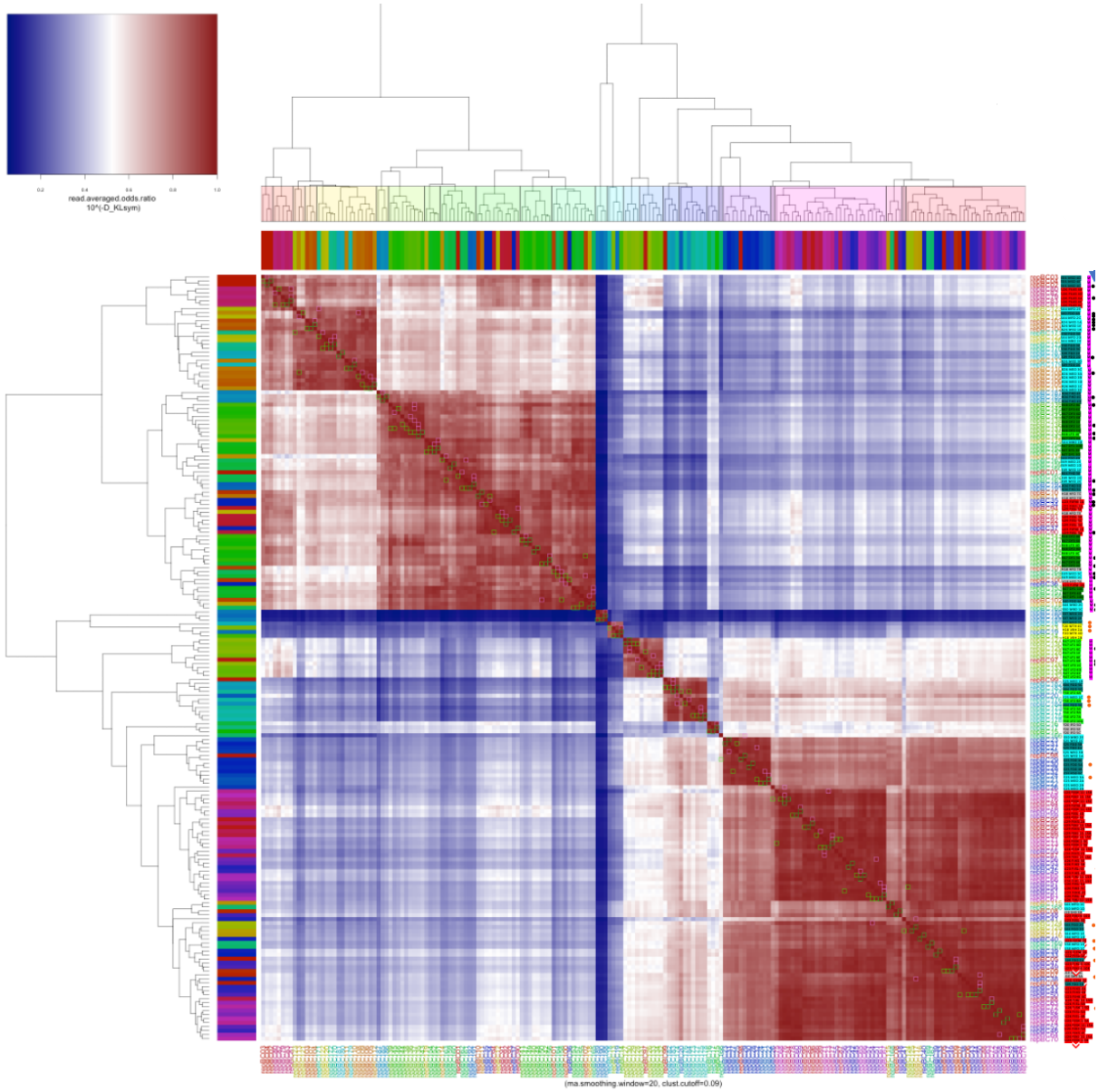


Figure A1 *– Heatmap generated from ON-rep-seq run 1, November 2021. Isolates in red are from the filleting department, turquoise are environmental isolates, blue-green are feed isolates, grey are slaughterhouse isolates, light-green are live fish isolates from netpens, dark-green are dead fish and yellow are well boat isolates.*

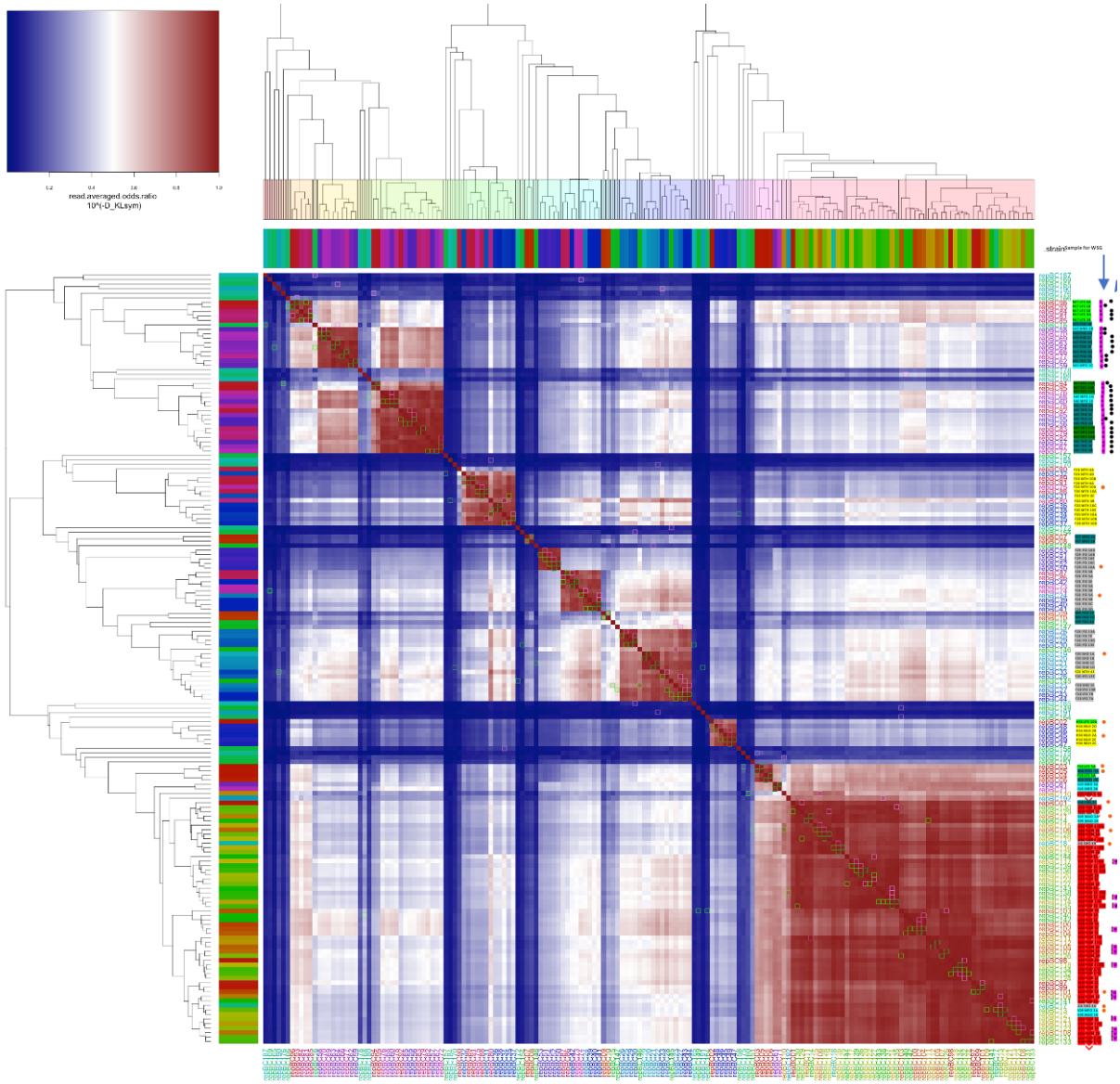


Figure A2 *– Heatmap generated from ON-rep-seq run 2, November 2021. Isolates in red are from the filleting department, turquoise are environmental isolates, blue-green are feed isolates, grey are slaughterhouse isolates, light-green are live fish isolates from netpens, dark-green are dead fish and yellow are well boat isolates.*

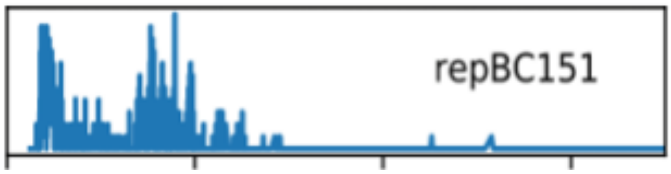


Figure A3 *– LCp plot for U22 FilFM-2B*

Table A2 – Isolates applicable to this study chosen for WGS based on ON-rep-seq assay results.

Isolate	Sample point and time
S40.FHO.1D	Feed sample, bottom of feed silo, week 40 2020, feed fleet O-A
S50.FGO.3C	Feed sample, top of feed silo, week 50 2020, feed fleet O-A
S09.MBO.2A	Environmental sample, feed load nozzle, week 9 2021, feed factory F-C
S40.MBO.2B	Environmental sample, feed load nozzle, week 40 2020, feed factory F-C
S44.MFO.2C	Environmental sample, feed unload nozzle, week 44 2020, feed fleet O-A
S44.MFO.2E	Environmental sample, feed unload nozzle, week 44 2020, feed fleet O-A
U26 FilKS-4B	Filleting sample, quality scanner 4, week 26 2021, production facility O-A
U22 FilKS-3A	Filleting sample, quality scanner 3, week 22 2021, production facility O-A
U23 FilFM-1A	Filleting sample, infeed filleting machine 1, week 26 2021, production facility O-A
U26 FilKS-4A	Filleting sample, quality scanner 4, week 26 2021, production facility O-A
U29 FilSL-5A	Filleting sample, drain under filleting machine 5, week 29 2021, production facility O-A
U22 FilFM-1E	Filleting sample, infeed filleting machine 1, week 22 2021, production facility O-A
U22 FilFM-2B	Filleting sample, infeed filleting machine 2, week 22 2021, production facility O-A
U22 FilFM-3A	Filleting sample, infeed filleting machine 3, week 22 2021, production facility O-A
U22 FilKS-3E	Filleting sample, quality scanner 3, week 22 2021, production facility O-A
U23 FilFM-1B	Filleting sample, infeed filleting machine 1, week 23 2021, production facility O-A
20-038-08	Quality control sample, 2020, gutting line 3, production facility O-A
19-170-54	Quality control sample, 2019, conveyor for biproducts heads tails, production facility O-A
19-218-60	Quality control sample, 2019, gutting line 6, production facility O-A
LEK-1652-13	Quality control sample from processing facility O-A, 2019.
19-357-43	Quality control sample, 2019, head cutter 3, production facility O-A
20-048-25	Quality control sample, 2020, grader conveyor, production facility O-A

Table A3 – Zone diameters in cm of inhibition for all 81 isolates subjected to antimicrobial susceptibility testing. TE = Tetracycline, E = Erythromycin, CN = Ciprofloxacin, SXT = Sulfamethoxazole-trimethoprim, MEM = Meropenem, AMP = Ampicillin, P = Penicillin, FOX = Cefoxitin.

	TE	E	CN	SXT	MEM	AMP	P	FOX
U22 FilSK 11-15 A	2,7	2,6	2,4	3,1	2,9	2,2	1,5	0
U22 FilSK 11-15 C	2,7	2,5	2,3	3,2	3	2,3	1,6	0
U22 FilSK 11-15 E	2,8	2,5	2,5	3,1	3	2,4	1,45	0
U22 FilFM-1 B	2,8	2,55	2,4	3,15	2,9	1,9	1,45	0,4
U22 FilFM-1 D	2,85	2,55	2,35	3,2	2,9	2,2	1,5	0,4
U22 FilFM-1 E	2,7	2,5	2,4	3,2	2,8	2,3	1,4	0
U22 FilFM-2 A	2,95	2,65	2,45	3,1	2,5	2,4	1,5	0
U22 FilFM-2 C	2,95	2,55	2,45	3,1	2,8	2,3	1,45	0
U22 FilFM-2 D	2,8	2,55	2,4	3,05	2,8	2,1	1,6	0
U22 FilFM-3 C	2,8	2,55	2,4	3,2	3	2,1	1,3	0
U22 FilFM-3 D	2,8	2,5	2,4	3,1	2,95	2,4	1,4	0
U22 FilFM-3 E	2,7	2,55	2,5	3,1	3,1	2,45	1,5	0
U22 FilKS-3 A	2,8	2,7	2,4	3,2	3,05	2,45	1,6	0
U22 FilKS-3 B	2,75	2,6	2,4	3,2	3,05	2,5	1,55	0,9
U22 FilKS-3 C	2,9	2,6	2,5	3,1	3,05	2,5	1,55	0,8
U23 FilKJ 6-10 B	2,8	2,65	2,25	3,1	2,8	2,3	1,6	0
U23 FilKJ 6-10 C	2,8	2,6	2,2	3,3	2,9	2,4	1,5	0
U23 FilKJ 6-10 E	2,8	2,7	2,25	3,2	2,9	2,4	1,7	0
U23 FilSL-5 A	2,8	2,6	2,2	3,3	2,9	2,3	1,55	0
U23 FilSL-5 B	2,7	2,6	2,3	3,2	2,9	2,4	1,65	0
U23 FilSL-5 E	2,8	2,5	2,3	3,3	3	2,3	1,6	0
U23 FilHK-4 A	2,7	2,7	2,4	3,3	2,9	2,55	1,5	0,4
U23 FilHK-4 B	2,8	2,7	2,35	3,4	2,9	2,5	1,3	0,4
U23 FilHK-4 D	2,6	2,75	2,4	3,2	3	2,55	1,65	0
U23 FilFM-1 B	2,9	2,75	2,4	3,2	3,05	2,3	1,45	0
U23 FilFM-1 C	2,8	2,7	2,45	3,25	2,95	2,4	1,45	0
U23 FilFM-1 D	2,6	2,65	2,5	3,1	3	2,3	1,4	0
U23 FilFM-2 A	2,8	2,7	2,3	3,2	2,9	2,45	1,5	0
U23 FilFM-2 C	2,8	2,7	2,4	3,2	2,95	2,5	1,3	0
U23 FilFM-2 E	2,7	2,6	2,3	3,25	2,9	2,45	1,5	0
U26 FilKJ 11-15 B	2,9	0	2,7	3,3	2,5	2,2	1,45	0
U26 FilKJ 11-15 D	3,15	2,7	2,5	3,2	2,9	2,45	1,65	0
U26 FilKJ 11-15 E	2,85	2,6	2,45	3,35	2,9	2,3	1,5	0
U26 FilSK 11-15 A	2,9	2,7	2,6	3,05	2,9	2,5	1,6	0
U26 FilSK 11-15 C	3	2,55	2,3	3,15	2,9	1,9	1,6	0
U26 FilSK 11-15 D	2,7	2,6	2,4	3,1	2,8	1,85	1,55	0
U26 FilSL-4 C	2,8	2,45	2,5	3,15	2,7	2,35	1,35	1,1
U26 FilSL-4 D	2,75	2,4	2,4	3,1	2,65	2,25	1,55	0,8
U26 FilSL-4 E	2,9	2,45	2,3	3,05	2,85	2,3	1,55	0
U26-FilSL-5 A	3	2,7	2,4	3,25	3	1,95	1,3	0
U26-FilSL-5 B	3	2,7	2,4	3,25	3,1	2	1,2	0
U26-FilSL-5 C	3,1	2,7	2,6	3,3	3,1	2	1,4	0
U26 FilKS-4 B	3,2	2,7	2,55	3,4	3,2	2,2	1,5	0
U26 FilKS-4 C	3,05	2,7	2,6	3,25	3,1	2,05	1,35	0
U26 FilKS-4 E	2,9	2,7	2,65	3,3	3,2	2,3	1,6	0
U26 FilKS-5 A	2,55	2,7	2,3	3,25	2,9	2,55	1,5	0
U26 FilKS-5 B	2,7	2,7	2,4	3,2	2,9	2,5	1,5	0
U26 FilKS-5 E	2,7	2,8	2,3	3,2	2,9	2,35	1,4	0
U29 FilKJ 11-15 A	2,6	2,6	2,4	3,2	2,8	2,5	1,3	0
U29 FilKJ 11-15 D	2,7	2,65	2,2	3,25	2,8	2,5	1,3	0
U29 FilKJ 11-15 E	2,7	2,6	2,3	3,3	3	2,45	1,3	0
U29 FilSL-5 B	3	2,6	2,55	3,1	3,1	2	1,8	1,4
U29 FilSL-5 C	3	2,55	2,5	3	2,95	2,4	1,6	1,4
U29 FilSL-5 D	2,8	2,7	2,5	3,05	3,1	2,4	1,3	1,4
U29 FilFM-2 A	2,9	2,45	2,5	3	2,6	2,2	1,5	0
U29 FilFM-2 C	3,05	2,45	2,5	3,2	2,8	2,4	1,55	0
U29 FilFM-2 E	2,9	2,65	2,45	3,05	2,7	2,3	1,4	1
U29 FilKS-2 B	2,8	2,6	2,6	3,3	3	2,6	1,5	1
U29 FilKS-2 D	3,05	2,7	2,6	3,2	2,95	2,5	1,65	1

U29 FilKS-2 E	3	2,65	2,5	3,3	2,8	2,5	1,55	1
U29 FilKS-5 A	3	2,6	2,5	3,25	2,95	2,4	1,5	1,1
U29 FilKS-5 C	2,9	2,7	2,6	3,2	3	2,5	1,5	1
U29 FilKS-5 D	3	2,7	2,6	3,2	3	2,7	1,55	1
U34 FilKJ 1-5 C	2,8	2,7	2,65	3,3	2,9	2,5	1,5	0,9
U34 FilKJ 1-5 D	2,75	2,6	2,6	3,15	2,9	2,6	1,5	0
U34 FilKJ 1-5 E	2,7	2,6	2,6	3,1	3	2,3	1,4	0
U34 FilSK 1-5 A	2,9	2,5	2,55	3,1	2,95	2,3	1,55	0
U34 FilSK 1-5 B	2,9	2,65	2,5	3,1	3	2,3	1,55	0
U34 FilSK 1-5 C	2,9	2,65	2,5	3,15	3	2,3	1,4	0
U34 FilSL-1 B	2,7	2,65	2,5	3,2	2,9	2,5	1,4	0
U34 FilSL-1 C	2,75	2,5	2,5	3	2,9	2,4	1,3	0
U34 FilSL-1 E	2,9	2,7	2,6	3,1	3	2,45	1,5	0
U34 FilFM-1 A	2,6	2,6	2,5	3	2,8	2,3	1,5	0
U34 FilFM-1 B	3	2,5	2,3	3,2	3	2,4	1,4	0
U34 FilFM-1 E	2,7	2,6	2,6	3,15	2,8	2,45	1,4	0
U34 FilFM-2 A	3,05	2,6	2,5	3,2	2,95	0	1,7	0
U34 FilFM-2 B	3,1	2,5	2,4	3,2	3	2,3	1,55	0
U34 FilFM-2 D	3,05	2,5	2,45	3,2	2,9	2,4	1,45	0
U34 FilKS-2 C	3,15	2,7	2,5	3,2	3,1	2	1,4	0
U34 FilKS-2 D	3	2,6	2,4	3,2	3	2	1,4	0
U34 FilKS-2 E	3,05	2,75	2,6	3,2	3,15	2,1	1,35	0

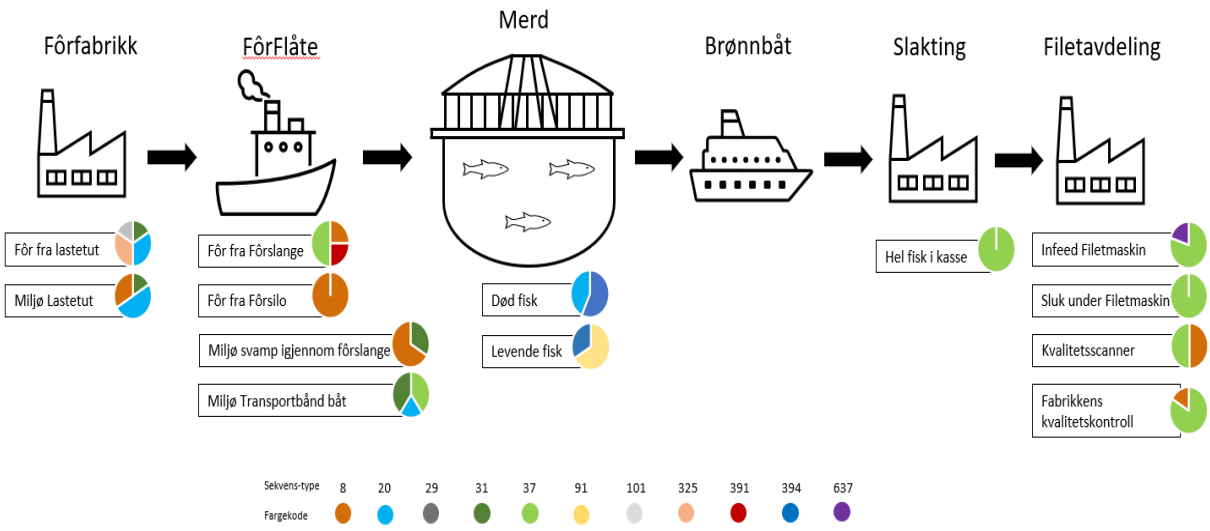
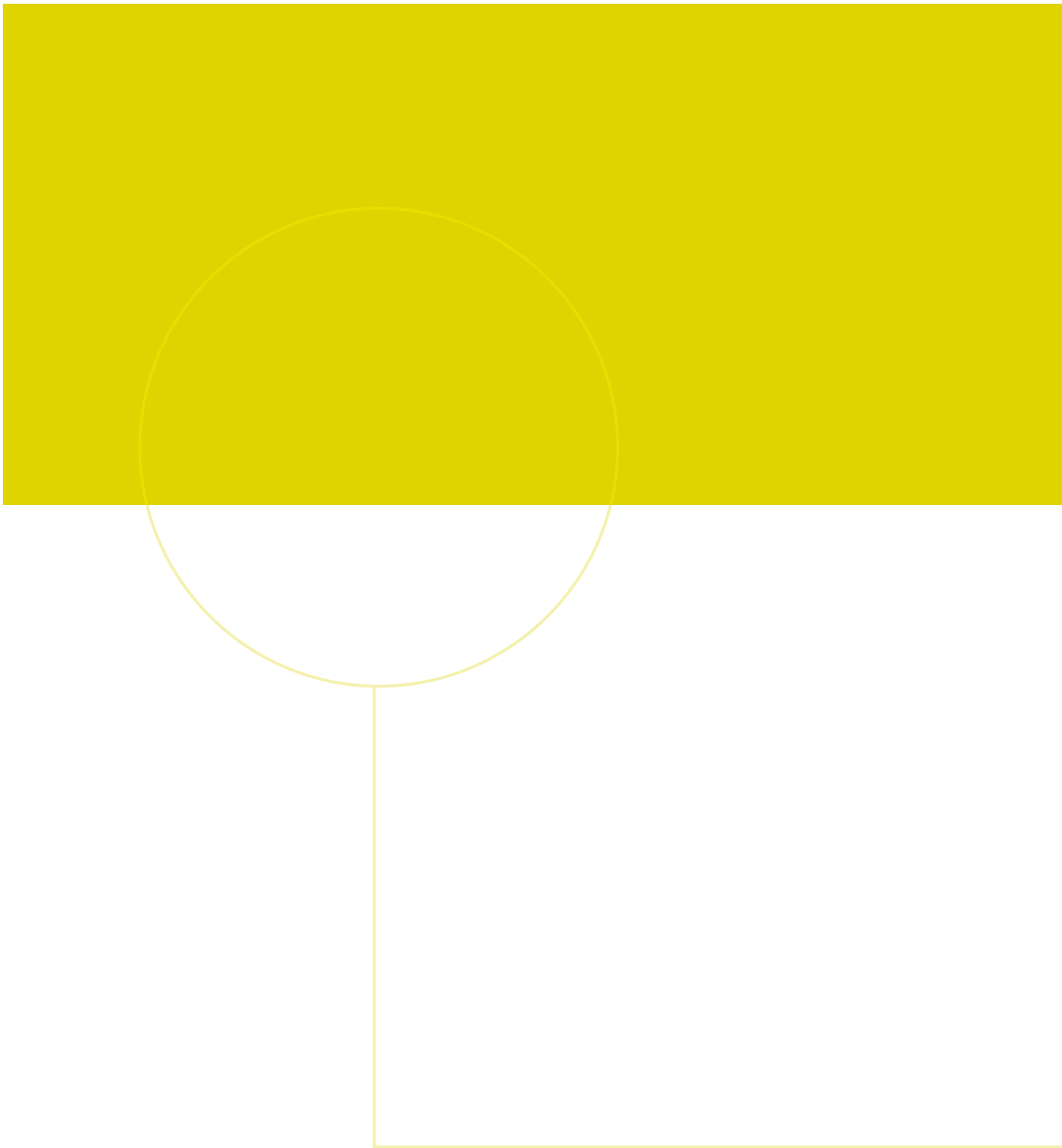


Figure A4 *– MLST observed throughout the entire value chain examined by the TraceListeria project. Starting with the feed factory and on to the feed fleet, salmon production enclosures, well boats, abattoir and finally the filleting department. ST colour codes: ORANGE=ST8, LIGHT BLUE = ST20, DARK GREY = ST29, DARK GREEN = ST31, LIGHT GREEN = ST37 YELLOW = ST91, LIGHT GREY = ST101, PINK = ST325, RED = ST391, DARK BLUE = ST394, PURPLE = ST637. Figure obtained from (Lerfall et al., 2022).*



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