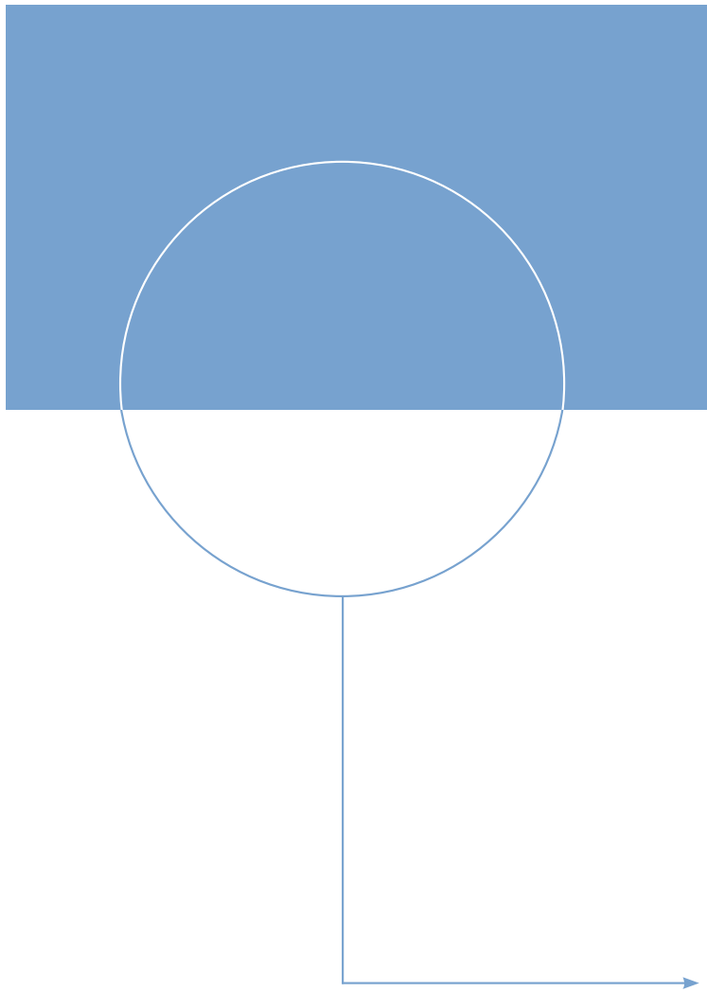

Master's thesis

2022

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# Genetic Characterization and Stress Tolerance Profiling of *Listeria monocytogenes* from the Salmon Processing Value Chain

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# Genetic Characterization and Stress Tolerance Profiling of *Listeria monocytogenes* from the Salmon Processing Value Chain

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## ABSTRACT

The food-borne pathogen *Listeria monocytogenes* can cause invasive illness in humans through entrance of food value chains. The pathogen is especially a concern for safety of ready-to-eat foods, and tracking of food-borne outbreaks is important to identify contamination sources in value chains.

The aim of this study was to genetic characterize, and examine stress tolerance of 35 *L. monocytogenes* isolates from the salmon processing value chain in Norway, originated from 2020 to 2021. This study aimed to examine possible dominant *L. monocytogenes* strains in the value chain, and identify possible contamination sources.

Characterization of genetic features was based on whole-genome sequencing with Oxford Nanopore Technology, and included MLST profiling, phylogenetic analysis based on SNPs, identification of antibiotic resistance genes, and identification of virulence genes. A total of nine sequence types were identified (ST20, ST29, ST31, ST37, ST91, ST101, ST325, ST391, ST394), and ST20, ST37, and ST394 were dominant strains.

The *fosX* gene conferring fosfomycin resistance was identified for all *L. monocytogenes* isolates, and the heat resistance gene, *ClpL* was identified for all ST31 isolates. Important virulence genes (*actA*, *hly*, *inlA*, *inlB*, *plcA*, *plcB*) were detected for ST20, ST29, ST37, ST91, ST101, and ST394.

Stress tolerance profiling included cold (12 °C), salt (5.4% NaCl), and acid (pH 5.05) tolerance examination. Cold tolerance was observed for six *L. monocytogenes* isolates (ST20, ST31, ST37). For growth in salt or acid, maximum growth rate decreased for all bacterial isolates relative to stress-free conditions, and significant differences in maximum growth rate between the strains were also detected.

A possible contamination source in the salmon value chain includes the feed section, but further samplings should be performed to draw a more solid conclusion. Limits in this study were lack of data points in the value chain, and incomplete growth curves for stress tolerance examination. Further work should include examination of different levels of stress, stress factors in combination, and heat resistance of *L. monocytogenes*.

# SAMMENDRAG

Den matbårne patogene bakterien *Listeria monocytogenes* kan gi alvorlig sykdom i mennesker ved å entre verdikjeder i matindustrien. Denne patogene bakterien er spesielt en bekymring for tryggheten til spiseklare produkter, og sporing av matbårne utbrudd er viktig for å identifisere kontamineringspunkter i verdikjeder.

Målet med dette studiet var å genetisk karakterisere og undersøke stresstoleranse for 35 *L. monocytogenes*-isolater fra verdikjeden til lakseprosessering i Norge, isolert fra 2020 til 2021. Målet var å undersøke mulige dominante stammer av *L. monocytogenes*, og mulige kontamineringskilder i verdikjeden.

Karakterisering av genetiske egenskaper var basert på helgenomsekvensering med Oxford Nanopore-teknologi, og inkluderte MLST-analyse, fylogenetisk analyse basert på SNPs, identifisering av antibiotikaresistensgener og identifisering av virulensgener. Totalt ble ni sekvenstyper identifisert (ST20, ST29, ST31, ST37, ST91, ST101, ST325, ST391, ST394). ST20, ST37, og ST394 var dominante stammer. Genet *fosX* som koder for fosfomycin-resistens ble detektert for alle *L. monocytogenes*-isolater, og *ClpL* som koder for varmeresistens ble detektert for alle ST31-isolater. Viktige virulensgener (*actA*, *hly*, *inlA*, *inlB*, *plcA*, *plcB*) ble detektert hos ST20, ST29, ST37, ST91, ST101, og ST394.

Stresstoleranse-profilering av *L. monocytogenes* inkluderte undersøkelse av kulde- (12 °C), salt- (5.4% NaCl), og syretoleranse (pH 5.05). Kuldetoleranse var observert hos seks bakterieisolater (ST20, ST31, ST37). For vekst i salt eller syre avtok maksimal vekstrate for alle bakterieisolater i forhold til i stress-frie forhold, og signifikante forskjeller i maksimum vekstrate mellom stammene ble detektert.

En mulig kontamineringskilde i verdikjeden til lakseprosessering er fôr-avdelingen, men videre prøvetaking bør gjennomføres for å trekke en mer solid konklusjon. Begrensninger i dette studiet var mangel på antall datapunkter i verdikjeden og ufullstendige vekstkurver for undersøkelse av stresstoleranse. Fremtidig arbeid bør inkludere undersøkelse av ulike nivåer av stress, stressfaktorer i kombinasjon og varmeresistens av *L. monocytogenes*.

# PREFACE

This thesis was written for my Master's degree in Chemical Engineering and Biotechnology with specialization in Biotechnology at Norwegian University of Science and Technology (NTNU), Trondheim. It was carried out from January to June 2022 at Department of Biotechnology and Food Science at NTNU, and accounted for 30 ECTS-credits.

The subject of this thesis is related to tracing of pathogenic bacteria in food value chains, and it is written in association to the TRACEListeria project at NTNU. All experimental work have been carried out at the chemistry, microbial, and pathogen laboratories at NTNU Trondheim, campus Kalvskinnet.

I would like to express my gratitude to my supervisor, Associate Professor Lisbeth Mehli for encouraging and guiding me in this thesis. Thank you for giving me advice, feedback, and motivating me though-out this time. I would also like to thank everyone working on the TRACEListeria project, making it possible for me to write my thesis on a fascinating and important research topic. I am grateful to Fiskeri- og Havbruksnæringens Forskningsfinansiering (FHF), NTNU, University of Copenhagen, and Lerøy Midt AS for financially funding the project. I would also acknowledge the laboratory staff at NTNU Kalvskinnet, Associate Professor Kirill Mukhatov, and fellow Master's student Ingrid Duister who were always present to help me with my research whenever I needed them. Finally, I would thank my family and friends for their endless support, and for always believing in me.

Ingrid Margrethe Boneng
Trondheim, 16 June 2022

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# ACRONYMS

ActA actin assembly-inducing protein
ADI arginine deiminase
ANOVA analysis of variance
ATR acid tolerance response
BHI brain heart infusion broth
BHIA brain heart infusion agar
BLA brilliance Listeria agar
Cap cold acclimatization proteins
CGE Center of Genomic and Epidemiology
Csp cold shock proteins
FHF Fiskeri- og Havbruksnæringens Forskningsfinansiering
GAD glutamate decarboxylase
InlA internalin A
InlB internalin B
LLO listeriolysin O
MLST multi-locus sequence typing
NCBI National Center of Biotechnology Information
NTNU Norwegian University of Science and Technology
OD optical density
ON-rep-seq Oxford Nanopore-based rep-PCR-based sequencing
ONT Oxford Nanopore technology
PCR polymerase chain reation
pHi internal pH
PLCs phospholipases C
RTE ready-to-eat
Sig. significance level
SNPs single nucleotide polymorphisms
ST sequence type
TSA tryptic soy agar
WGS whole-genome sequencing

# 1 INTRODUCTION

## 1.1 FEATURES OF THE FOOD-BORNE PATHOGEN *Listeria monocytogenes*

*Listeria monocytogenes* is a Gram-positive, facultative anaerobe, food-borne pathogen with the capability of causing the serious invasive illness listeriosis (Lungu et al., 2009). The pathogen belongs to the bacterial Genus *Listeria*, which composes 20 known species by 2020 (Quereda et al., 2020). *L. monocytogenes* is one of two species within the Genus that is pathogenic, and the only one of these two that normally infects humans (Olaimat et al., 2018). The most usual way humans get infected are by eating food contaminated with *L. monocytogenes*.

*L. monocytogenes* species comprises 14 serotypes, which are subtype discrimination based on somatic (O) and flagellar (H) antigens (Borucki & Call, 2003). Serotypes 1/2a, 1/2b, 1/2c, and 4b are most frequently presented in food and clinical isolates (Muchaamba et al., 2022), and these serotypes (except 1/2c) cause listeriosis (Borucki & Call, 2003).

*L. monocytogenes* is intracellular, and it can penetrate, survive and grow within cells in the gastrointestinal tract or macrophages (Lungu et al., 2009). Listeriosis can lead to sepsis (blood poisoning) (NHI, 2021), meningitis (inflammation of the tissue covering the brain) or meningoencephalitis (inflammation of the brain and covering tissue) (Johns Hopkins Medicine, 2022). Additionally, it can lead to stillbirth of pregnant women. Even though listeriosis is rare, the disease is serious due to the fatality rate of 20-30% (Schuchat et al., 1991). People with weakened immune systems, pregnant women, newborns, and elderly are most postponed for the disease (Olaimat et al., 2018). Table 1.1 presents listeriosis occurrence per 100,000 individuals for the general population and some of the risk groups.

Table 1.1: Listeriosis occurrence per 100,000 individuals for the general population, and groups of people postponed for the disease. The table is adapted from Boneng (2021). The number of occurrence for the groups are collected from (Olaimat et al., 2018).

| General population | Pregnant women | Newborns | Elderly |
|---|---|---|---|
| 0.7 | 12 | 3.4 | 10 |

*L. monocytogenes* is ubiquitous (Colagiorgi et al., 2017), facultative anaerobe, and can withstand various environmental conditions (Roberts et al., 2020). The pathogen can grow in low temperature, low pH, high salt concentration, and high water activity ($a_w$) (Farber, 2000). Growth conditions are presented in Table 1.2.

The pathogen also has the ability of biofilm formation (Colagiorgi et al., 2017). Biofilms are structures of bacterial cells embedded in a self produced mixture of polysaccharides, proteins, and DNA Reygaert, 2018), protecting the organism from stresses in the environment (Colagiorgi et al., 2017). Formation of biofilms contribute to increased resistance towards attack of the host immune system (Reygaert, 2018), and food cleaning processes (Olaimat et al., 2018).

Table 1.2: Growth conditions of *L. monocytogenes*, including temperature (T), pH, salt concentration ($C_{NaCl}$), and water activity ($a_w$). Optimal (opt.) conditions are given for T and pH. The table is adapted from Boneng (2021), and modified. Values for T, pH and $a_w$ are collected from Lungu et al. (2009), and $C_{NaCl}$ from Olaimat et al. (2018).

| T [°C] | pH | $C_{NaCl}$ (w/v) | $a_w$ |
|---|---|---|---|
| 0-45 | 4.1-9.6 | 0-20% | >0.90 |
| (opt. 30-37) | (opt. 7) | | |

## 1.2 *Listeria monocytogenes* IN FOOD INDUSTRY

The ubiquitous nature of *L. monocytogenes* results in several ways of entrance into food chains, and it has been detected in a variety of food products (NicAogáin and O'Byrne, 2016, Skowron et al., 2018). The pathogen can enter food industry through soil (agriculture), seawater (aquaculture), and within food processing environments. It has been found that *L. monocytogenes* incidence is higher in farms than in natural environments (minimal human interference), suggesting that occurrence of the pathogen is associated with human and animal presence (NicAogáin & O'Byrne, 2016). *L. monocytogenes* has been detected in food products such as fish, meat, fruit, vegetables, and unpasteurized dairy products (Skowron et al., 2018, Olaimat et al., 2018).

If the food processing environment is growth favourable for *L. monocytogenes*, it can lead to persister strains - i.e. long term survival strains (Fagerlund A. et al., 2016). Persistence for several years in food processing environments has been found, and it can cause recurrent cross-contamination of food products (NicAogáin & O'Byrne, 2016). The persistent strains might be a result of established strains that have adapted to the environment, or frequent introduction of same subtype strains (Fagerlund A. et al., 2016).

*L. monocytogenes* is a well-known challenge in the fish farming industry. The bacteria can be found in the water environment of the farm industry, and on the outer surface, in the gastrointestinal tract or in the gills of the fish present. The main ways of *L. monocytogenes* contamination in fish processing plants are by spread from bowel to other tissues within the fish, or by cross-contamination due to insufficient processing hygiene (use of contaminated equipment, lack of good cleaning processes). In the salmon processing value chain, the pathogen is most commonly detected in feed and filleting area (Skowron et al., 2018).

The ability of growing in refrigerator temperatures makes *L. monocytogenes* a particular concern for safety of food consumed without reheating or cooking, referred to as ready-to-eat (RTE) foods (Kathariou, 2002). The current trend of eating fresh and natural food has increased the demand for RTE foods. Minimal processing technology have been developed to meet the demand of the costumers, but consumption of RTE foods could potentially increase risk towards foodborne pathogens (Lee et al., 2021). Post-processing levels of *L. monocytogenes* contamination in food is usually low, but the potential of regrowth in RTE foods presents the highest risk to consumers (Hingston et al., 2017). Figure 1.1 illustrates transmission routes of *L. monocytogenes* into food value chains.

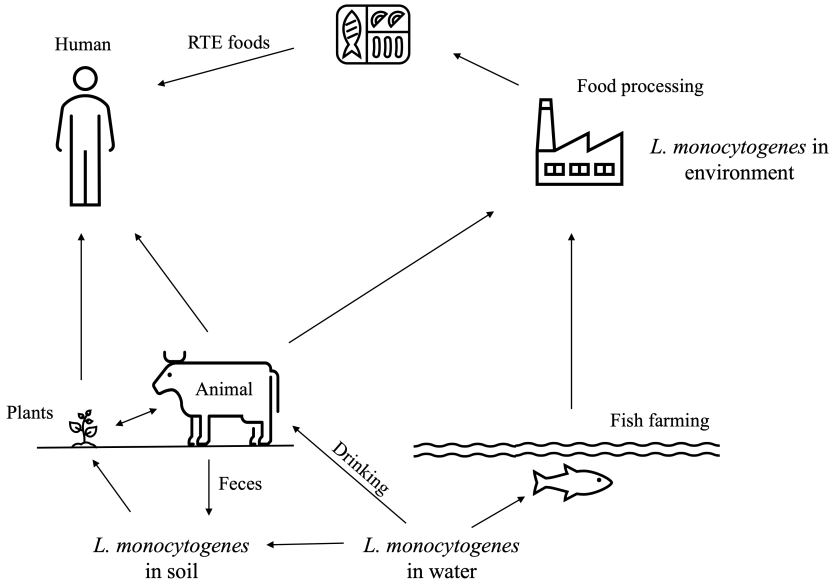


Figure 1.1: Possible transmission routes of *L. monocytogenes* in the food industry. The bacteria can enter food value chains through soil, water or food processing environment. *L. monocytogenes* from the soil can transfer to plants (crops, fruits, vegetables etc.), and further to humans or animals eating the plants. From water it can transfer to fish or to animals through the drinking water. The bacteria can enter a food processing value chain via infected animals or fish, and eventually transmit to humans via the food product (especially via ready-to-eat foods because of minimal processing). The figure is adapted from Quereda et al. (2021), and modified based on the literature in this section.

## 1.3 STRESS TOLERANCE OF *Listeria monocytogenes*

Stress factors are used in the food industry to control unwanted microbial growth and to ensure food safety (Wiktorczyk-Kapischke et al., 2021). Microorganisms have adapted to function optimally in their normal environment, and if they are exposed to extreme environmental changes, the cell is exposed to stress. As a response, microorganisms have evolved physiological and genetic mechanisms to survive these changes. The extent of the environmental changes and the ability of the microbe to adapt determine whether the cell is killed, growth retarded, or growth rate decreased. The adaption to changes in the environment take place in the lag phase (also called acclimation phase), and an extension of lag phase is associated with stress response (Beales, 2004).

*L. monocytogenes* encounters many stress factors, such as low temperatures, low pH, and osmotic (salt) stress (Bucur et al., 2018). Stress tolerance of the pathogen is described in the following sections.

### 1.3.1 *Cold tolerance*

Low temperatures are used to limit growth of microorganisms (Wiktorczyk-Kapischke et al., 2021). Cold makes bacterial membranes more rigid, reduce enzymatic reaction rates, and decrease uptake level and transport of molecules (NicAogáin & O'Byrne, 2016). Adaption to temperature changes is very common among microbes because temperature has an impact on all reactions of the cell (Berry & Foegeding, 1997).

The mechanisms allowing growth at low temperatures for *L. monocytogenes* involve stabilization of macro-molecule structures, compatible solutes uptake, and maintenance of cellular membrane fluidity (Wemekamp-Kamphuis et al., 2004). When bacteria are exposed to a fast decrease below optimal temperature, the cell experience cold shock, and cold shock proteins (Csp) are synthesized in response (Wiktorczyk-Kapischke et al., 2021). These proteins enable replication of DNA, transcription to RNA, and translation to proteins at low temperatures. Genetic expression of up to 50 different cold shock proteins is activated, depending on the species and extent of temperature drop (Beales, 2004). Four Csp have been identified for *L. monocytogenes*, and these are synthesized in increased amounts from 30 °C to 10 °C, according to Wemekamp-Kamphuis et al. (2004). After cold shock, the bacterial cell will acclimatize by synthesizing cold acclimatization proteins (Cap). These proteins support the action of Csp (Wiktorczyk-Kapischke et al., 2021).

The $\sigma^B$ factor is also important for cold stress response in *L. monocytogenes* (Becker et al., 2000). This factor ensures appropriate response to environmental changes by up-regulation of approximately 300 genes in the organism (Guerreiro et al., 2020). The $\sigma^B$ factor is activated in

response to stress (such as temperature decrease), and it modulates accumulation of solutes called compatible solutes (Becker et al., 2000). These support the Csp and Cap action in *L. monocytogenes* (Wiktorczyk-Kapischke et al., 2021). Compatible solutes and the associated mechanism will be further described in the next subsection.

Many cold tolerant microorganisms have the ability of increasing membrane fluidity by adjusting fatty acid composition of the membrane phospholipids when they are exposed to cold. Decrease in temperature leads to changes in fluidity of the membrane bi-layer due to order-disorder transition of the fatty acids in the membrane lipids. The state of the fatty acids will go from fluid (disorder) to a more ordered form due to the temperature decrease. Cold tolerant microorganisms adapt to these changes by incorporating fatty acids with lower melting points into the membrane lipids, resulting in maintenance of membrane fluidity (Berry & Foegeding, 1997). *L. monocytogenes* can adapt to cold by adjusting membrane fluidity through fatty acid alteration (Becker et al., 2000). The alteration includes branching in the methyl end and shortening of the fatty acids (Wemekamp-Kamphuis et al., 2004).

### 1.3.2 *Salt tolerance*

Salt is an effective preservative and antibacterial agent because it reduces water activity ($a_w$) of foods (Henney et al., 2010), contributes to osmotic stress, and affects surface tension (Wiktorczyk-Kapischke et al., 2021). Osmotic stress leads to loss of water from the microbial cell and decrease in cytoplasmic volume, causing cellular death or retard growth (Henney et al., 2010, Beales, 2004). Additionally, osmotic stress has the ability of causing protein or DNA destruction, and decrease of membrane fluidity. It has also been suggested to limit oxygen solubility and force cells to expend energy to exclude sodium ions from the cell (Wiktorczyk-Kapischke et al., 2021, Henney et al., 2010). It is therefore fundamental for the bacterial cells to adapt to the environmental osmolarity for the ability to grow and survive (Sleator & Hill, 2002).

*L. monocytogenes* can withstand up to 20% NaCl in the surroundings (Olaimat et al., 2018), and the main osmotic stress response involves compatible solutes (Wiktorczyk-Kapischke et al., 2021). These solutes are small organic molecules that help the cell maintain turgor during high osmolarity (Sleator & Hill, 2002), such as betaine found in spinach and carnitine found in meat (Becker et al., 2000). Compatible solutes can in general be synthesized by the bacterial cell or be transported from the environment. If they are produced internal, the solutes are usually high soluble, pH neutral end product metabolites. Exogenous solutes are typically amino acid derivatives synthesized by plants, or peptides in foods (Beales, 2004). *L. monocytogenes* cannot

synthesize compatible solutes *de novo*, and rely on transport from the surroundings (Wiktorczyk-Kapischke et al., 2021).

Three compatible solute transporters have been identified for *L. monocytogenes*. All transporters are $\sigma^B$-dependent (2021), and activated by increased osmolarity (Becker et al., 2000). The BetL and Gbu transporters provide long-term protection against osmolarity during low and high salt stress, respectively. Both transporters are responsible for glycine betaine transport. The transporter OpuC is also important for salt stress response, and is responsible for transport of carnitine (Wiktorczyk-Kapischke et al., 2021).

Reduction of $a_w$ leads to loss of turgor pressure (Beales, 2004) (the intracellular force pushing the plasma membrane against the cell wall (Beauzamy et al., 2014)) due to loss of water from the cell, or an increase in external solutes that cannot cross the cell membrane. To restore the turgor pressure, the cell can raise the level of compatible solutes (Beales, 2004). The response to increased osmolarity involving compatible solutes includes two steps. Firstly, the levels of $K^+$ and glutamate increase as a response to the water flux that is triggered along the osmolaric gradient (Sleator & Hill, 2002). Secondly, compatible solutes are accumulated intracellular (Wiktorczyk-Kapischke et al., 2021). The solutes then function to maintain cell turgor, stabilize enzyme function, protect the cell from salinity and desiccation, in addition to high and low temperature (Sleator & Hill, 2002). Figure 1.2 illustrates response to osmotic stress by compatible solutes.

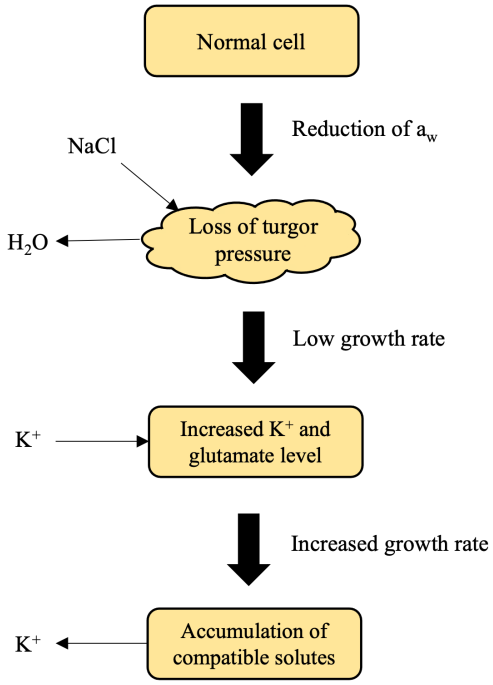


Figure 1.2: Adaption to osmotic stress of bacterial cells by compatible solutes. Reduction of water activity ($a_w$) leads to loss of water from the cell or an increase in external solutes that cannot cross the membrane, and thus loss of turgor pressure. The growth rate then decreases as the cell experience stress. Further, the levels of $K^+$ and glutamate are increased, and compatible solutes accumulated intracellular to restore turgor pressure and increase growth rate. The figure is adapted from Beales (2004), and modified.

Likewise for cold stress, the membrane function of bacterial cells is also disrupted by salt stress (**MuhammadJamil2012SalinityContents**). When $a_w$ is decreased, the membrane lipids can adopt a non-bilayer phase (2004). Salt tolerant microorganisms can adapt by changing composition of the membrane (Gandhi & Shah, 2016), which mainly occur in the head group of the lipids. An increase in anionic phospholipids and/or glycolipids is the most common alteration of lipid composition. This stress response has been observed among several microorganisms, including food-borne pathogens (Beales, 2004).

### 1.3.3 *Acid tolerance*

Use of acid in foods are common to inhibit microbial growth. Acid increases proton ($H^+$) concentration, resulting in protonation of biological molecules. This can affect charge, structure and function of important cellular molecules, leading to interruption of homeostasis of the cell (Arcari et al., 2020)(the process of maintaining cell stability while adjusting to environmental changes)(Billman, 2020). It is essential for the bacterial cell to maintain pH homeostasis, and both internal pH (pHi) and pH from the surroundings must therefore be

regulated by the cell (Guan & Liu, 2020). To do that, bacteria use a passive or active mechanisms, including increase of cellular buffer capacity or use of a proton pump, respectively (Wiktorczyk-Kapischke et al., 2021).

Four mechanism have been suggested to confer maintenance of pH homeostasis in *L. monocytogenes*. The glutamate decarboxylase (GAD) system confers survival in low pH (<4.5) (2021). GAD elevates pHi of the cell by converting extracellular glutamate to $\gamma$-aminobutyrate acid, and subsequently exchange the latter for glutamate at the cell membrane by an antiporter. An intracellular proton is consumed as a result, contributing to maintaining homeostasis (Cotter et al., 2001). The GAD system helps bacteria survive in low pH food products such as juice and yoghurt, and can even confer survival in gastric juice. Resistance to mild pH stress is conferred by $F_0F_1$-ATPase, and the arginine deiminase (ADI) system is activated by extremely low pH. The fourth system is the acid tolerance response (ATR), which works at the same time as the $F_0F_1$-ATPase and GAD system. Interaction of these three contribute to survival and adaption to acid stress of *L. monocytogenes* (Wiktorczyk-Kapischke et al., 2021).

The ability of *Listeria monocytogenes* to survive and adapt to acidic conditions is predicted to play an important role in the infection cycle, and several mechanisms have been suggested for acid stress treatment of this pathogen (Ryan et al., 2009, Bucur et al., 2018). *L. monocytogenes* has to withstand low pH for the capability to grow in acidic foods, manage to pass through the gastric barrier, and to survive within infected cells (Ryan et al., 2009).

## 1.4 VIRULENCE FACTORS AND INFECTION CYCLE OF *Listeria monocytogenes*

As described, *L. monocytogenes* can cause the invasive illness listeriosis (Lungu et al., 2009). The reason for the high death rate of the disease is partly attributed to the intracellular lifestyle of *L. monocytogenes* and the location of lesions. The brain is included in these locations, making it hard to treat the disease due to the inaccessibility of the pathogen to drugs. Thus, the therapeutic choices is limited (Scortti et al., 2018).

The pathogenesis of *L. monocytogenes* relies on virulence genes that are coordinately regulated by the PrfA transcriptional activator. The virulence genes are selectively induced by the activator inside the host, thus preventing unnecessary production of virulence factors when it is living in other environments (and not infecting a living host) (2018). Several important virulence factors have been identified for *L. monocytogenes*.

Bacterial uptake of the host cell are mediated by the surface proteins internalin A (InlA) and/or internalin B (InlB) encoded by *inlA*

and *inlB*, respectively. The differences between these proteins lay in the receptors they bind on the host cell, and the permissiveness of the host. Humans are permissive to both InlA and InlB (Phelps et al., 2018). A premature stop codon has been revealed for the *InlA* gene, functioning as a determinant for *L. monocytogenes* virulence. The premature stop codon results in truncated InlA, meaning the whole protein is not translated. The result is lower virulence properties of the bacteria (Fravalo et al., 2017).

Upon host entrance, the *L. monocytogenes* is confined with a vacoule (Phelps et al., 2018). Listeriolysin O (LLO) and two phospholipases C (PLCs) mediate escape from the vacoule into cytosol of the host cell, where the pathogen is capable of dividing (Portnoy et al., 2002). LLO is encoded by *hly* (Price et al., 2018), PLCA by *plcA*, and PLCB by *plcB*. Once entered into the host cytosol, actin assembly-inducing protein (ActA) induce polymerization of host actin filaments and mediate motility. The bacteria uses the resulting force that is generated by the actin polymerization to move intracellularly, and from cell to cell. Upon cell-to-cell spread, the bacteria is surrounded by a double-membraned vacoule. The escape is mediated the same way as for the primary vacoule, and new infection cycles are repeated (Portnoy et al., 2002). Figure 1.3 shows a schematic overview of *L. monocytogenes* infection cycle.

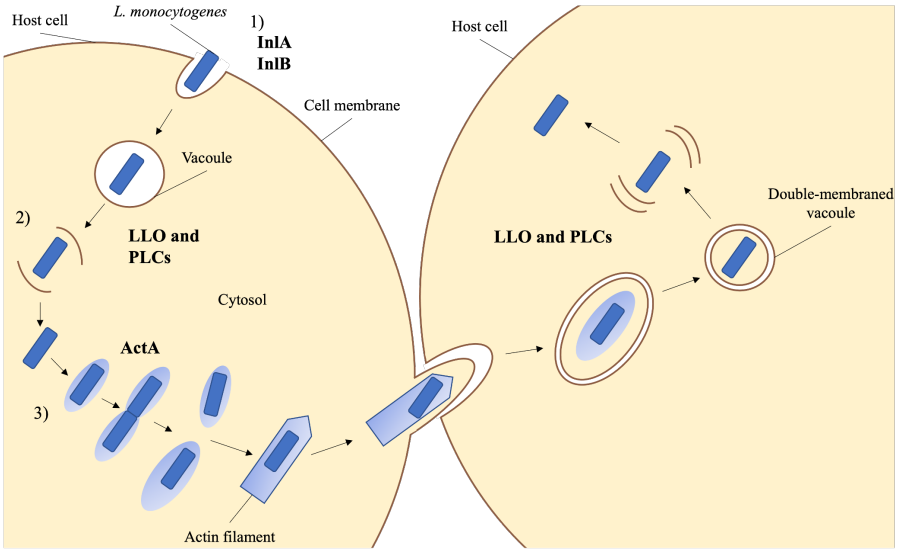


Figure 1.3: Infection cycle of *L. monocytogenes*. 1) Host entrance is mediated by InlA and/or InlB, and the pathogen is confined with a vacoule upon infection. 2) Vacoular escape into cytosol are mediated by LLO and PLCs. 3) ActA mediates actin nucleation in addition to intracellular and cell-to-cell motility. Upon spread to a new cell, *L. monocytogenes* is surrounded by a double-membraned vacoule, and a new infection cycle is repeated by the same mechanisms as described. The figure is adapted from Portnoy et al. (2002), and modified.

## 1.5 NANOPORE SEQUENCING BY OXFORD NANOPORE TECHNOLOGY

Whole-genome sequencing (WGS) provides full information of the entire bacterial genome with high resolution (Quainoo et al., 2017), and several WGS techniques are available. The first generation of sequencing technology came about forty years ago with the Sanger sequencing. The next-generation sequencing followed, and made sequencing cheaper and faster. A third-generation has appeared, increasing quality of the reads (van Dijk et al., 2018).

Oxford Nanopore technology (ONT) introduced the third-generation, and includes long-read sequencing (Wick et al., 2019). Nanopore sequencing by ONT requires little sample preparation, is based on a PCR-free protocol (Oxford Nanopore Technologies, 2021), and provides low-cost long read data (Baseggio et al., 2021). ONT is based on applying a voltage difference on a membrane in a flow cell containing two chambers (*cis* and *trans*) (van Dijk et al., 2018), and passing single-stranded DNA through a nanopore on the membrane. The resistance of the membrane will be affected by the nucleotides in the DNA-sequence when they are passing the pore (Wick et al., 2019). The nucleotide-sequence can be indicated by measuring the current of the membrane several thousand times per second, presenting it graphically in a "squiggle plot", and processing the data by a data analysis software. Unwinding of double-stranded DNA and movement of one of the single-strands through the nanopore is promoted by an enzyme (helicase or polymerase) bound to a DNA-protein complex, referred to as adapters (van Dijk et al., 2018). Figure 1.4 presents the Oxford Nanopore technology schematic.

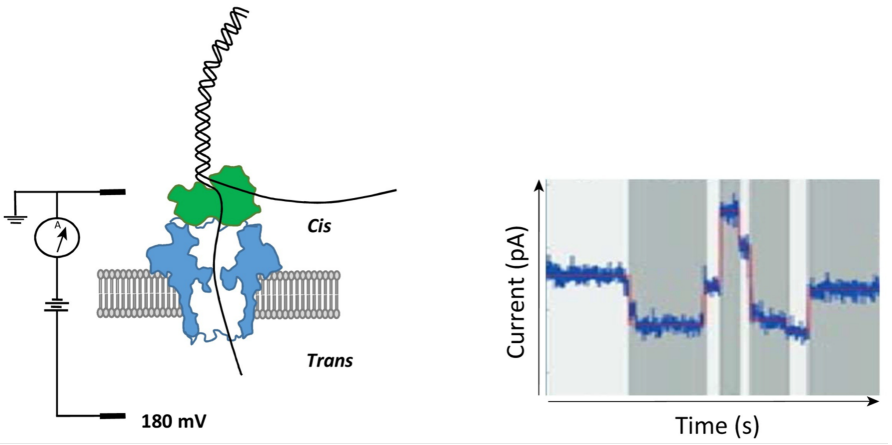


Figure 1.4: Representation of Oxford Nanopore Technologies. A membrane containing nanopores are placed in a flow cell, separating the *cis* and *trans* chambers of the cell. DNA is unwound and one strand passed through the pore on the membrane by an adapter (DNA-protein complex bound to an enzyme). Membrane current is measured as the single-stranded DNA passes through, and graphically presented in a "squiggle plot". The figure is adapted from van Dijk et al. (2018).

## 1.6 WHOLE-GENOME SEQUENCING FOR TRACKING OF FOOD-BORNE OUTBREAKS

It has earlier been a challenge to distinguish closely related bacterial strains in food-borne outbreaks, and to identify virulence or resistance features of these strains. During earlier outbreaks, concurrent genetic analyses have not been performed, resulting in limitations of phylogenic information provided. Additionally, the conventional methods used for characterization of pathogens have limited genomic resolution, and the approaches of the analyses have often been target-specific (Quainoo et al., 2017).

The safety in food industry can be revolutionized by WGS analyses, and it is now increasingly used for routine analysis (Pightling et al., 2018). Contamination sources of illness outbreaks can be identified accurately due to WGS (Quainoo et al., 2017). The variety of WGS tools allow real-time or retro-perspective molecular epidemiological analyses of pathogens, and different genomic variations can be assessed due to the precision of WGS - e.g. single nucleotide polymorphisms (SNPs) or allellic profiles (Chen et al., 2017).

Analysts have been hesitant to implementation of WGS due to high costs, but output and analysis speed have been increased, and cost been reduced the latter years as described in Section 1.5. WGS can therefore provide a faster and more affordable analysis in tracking of food-boorne outbreaks than earlier (Quainoo et al., 2017). However, it is also important to mention that bioinformatics and computer skills

are required (Thomassen et al., 2021), and guidance for interpreting the analyses lack to some extent. Pightling et al. (2018) also points out that different bacteria populations contain different levels of genetic diversity due to evolutionary forces, which must be considered when interpreting the analyses. SNPs are the results of these forces (2018), and they are genomic variants at single base positions in the DNA (Gunter, 2022). Because of these variations, identical genome sequences of isolates from same source of contamination cannot be expected (Pightling et al., 2018).

WGS is used to epidemiologically investigate listeriosis outbreaks (Zhang et al., 2020). A worldwide network for sharing genome sequencing data has been launched by the United States, and National Center of Biotechnology Information (NCBI) have generated a SNP-based WGS tree based on daily updates of the data. The tree contains data from more than 14 000 *L. monocytogenes* genomes (per 2017) (Chen et al., 2017).

When bacterial isolates sampled from the environment are to be whole-genome sequenced for target-specific analysis, some preparations are required. Environmental samples are grown on selective media to verify the species of target, the colonies are purified, and DNA can then be extracted for purification prior to sequencing. Isolation of DNA should lead to extraction with good quality and quantity, and without contamination of RNA or proteins. Examples of DNA quality is presented in Figure 1.5, and sample A) presents a qualified DNA sample (Novogene, 2019). Specific segments of the DNA can be amplified by polymerase chain reation (PCR), and primers can be used to target the segment of interest (Gupta, 2019). However, nanopore sequencing with ONT does not require amplification of the DNA with PCR, as described in Section 1.5 (Oxford Nanopore Technologies, 2021).

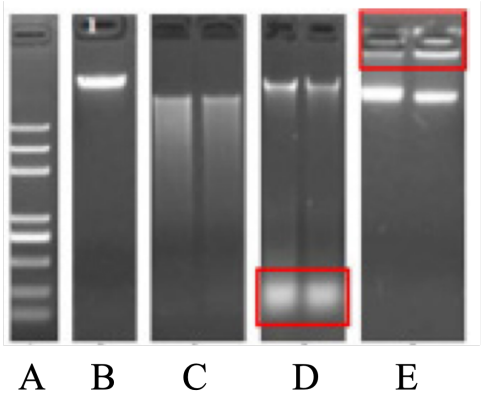


Figure 1.5: Examples of DNA quality for isolated DNA: 1) DNA marker 2), DNA sample with good quality, 3) degraded DNA, 4) DNA contaminated with RNA (red square) 5) DNA contaminated with protein (red square). The figure is adapted from Novogene (2019).

## 1.7 MLST PROFILING FOR STRAIN-LEVEL DISCRIMINATION

There are differences between *L. monocytogenes* strains, and a variety of methods have been applied for strain-level differentiation. Some strains are highly pathogenic, whereas others are less or even not virulent to the host. As shown earlier with serotyping, Multi-locus sequence typing (MLST) method is one tool commonly used for the differentiation of strains (Wu et al., 2016). This method characterize bacterial isolates within a species by using sequences of internal fragments of selected house-keeping genes (loci). The isolates are characterized based on what allele they possess from each house-keeping gene, and accordingly they are assigned to a sequence type (ST) (PubMLST, 2018). Figure 1.6 illustrates MLST analysis for strain-discrimination.

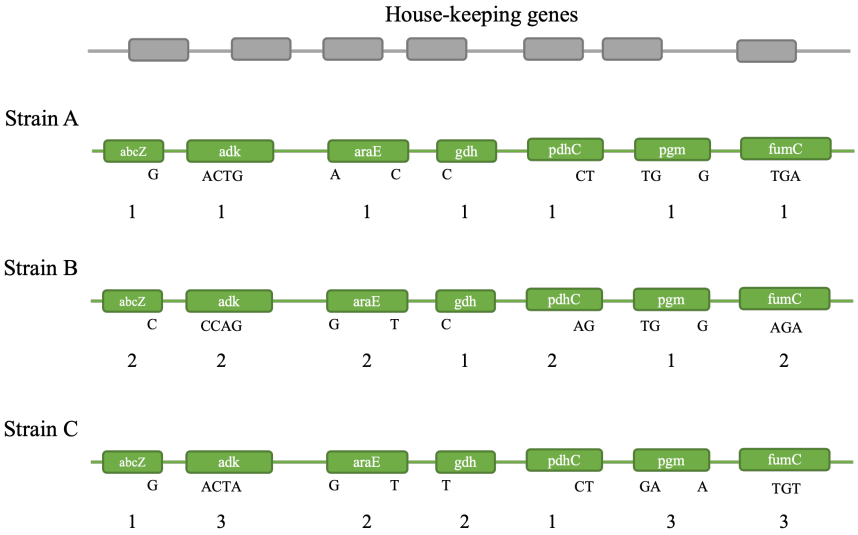


Figure 1.6: Schematic representation of Multi-locus sequence typing analysis. The figure illustrates an example for discrimination of three bacterial strains (A, B, C). Sequences in given loci of the bacterial isolates examined are screened for identity with a known sequence for each locus of a selection of house-keeping genes. For each locus, if there are differences in the sequence, it is considered a new allele. For instance, strain B has a difference in nucleotide sequence compared to strain A in the first house keeping gene (sequence abcZ). Thus, it is considered a new allele, assigned by a unique allele number (number 2 in this case). Based on what allele each isolate posses for each house-keeping gene, they are assigned a sequence type. The figure is adapted from bioMérieux (2022).

## 1.8 AIM OF STUDY

The aim of this study was to characterize genetic features and phenotypic stress tolerance profiles of *Listeria monocytogenes* originating from the salmon processing value chain in Norway, and possibly identify contamination sources in the value chain.

All bacterial isolates were whole-genome sequenced, and the sequencing data was analysed for genetic characterization. The analyses included MLST profiling, phylogenetic analysis based on SNPs, identification of antibiotic resistance genes, and selected virulence genes important for infection.

Stress tolerance profiling included examination of bacterial growth in cold, salt, and acid stress. The cold tolerance analysis included growth at the temperature in salmon slaughter house in the value chain. Salt and acid conditions were selected based on growth conditions for *L. monoocytogenes*.

# MATERIALS AND METHODS

# 2

## 2.1 ORIGIN OF BACTERIAL ISOLATES AND PROJECT BACKGROUND

This study was performed in association to the TRACEListeria project (FHF, 901591) at NTNU. The project aims to trace *Listeria monocytogenes* in the salmon processing value chain. The value chain has been limited to distribution of feed, production of salmon in the sea, transport of fish to slaughter house, filet department, and processing and packing of fish. Figure 2.1 presents an overview of the salmon value chain. Samplings have been performed at three salmon processing plants in the central Norway from mid 2020 to mid 2021.

Analysis of the samples was performed following the iQ-Check spp. kit (Bio-Rad) procedure. All bacterial isolates with positive PCR products were grown on the selective media Rapid' L.mono for verification of *L. monocytogenes*. The isolates were stored at NTNU at −80 °C. Screening of the presumptive *L. monocytogenes* isolates was performed by the sequencing method, Oxford Nanopore-based rep-PCR-based sequencing (ON-rep-seq). A total of 56 *L. monocytogenes* isolates from different points of origin in the value chain were selected for WGS based on the screening.

The 56 *L. monocytogenes* isolates were examined in two master's theses. All bacterial isolates from the filet department, and a selection of the isolates from the feed fabric were examined in another master's study. That study included 21 of the 56 *L. monocytogenes* isolates. The remaining 35 isolates from the feed fabric and other points of the value chain were examined in this study. This is illustrated in Figure 2.2.

It is important to state that sampling, screening and selection of the bacterial isolates were performed in association to the TRACEListeria project at NTNU, prior to this master's thesis. WGS of the bacterial isolates was conducted in this study, and the main focus is the 35 *L. monocytogenes* isolates selected for this thesis.

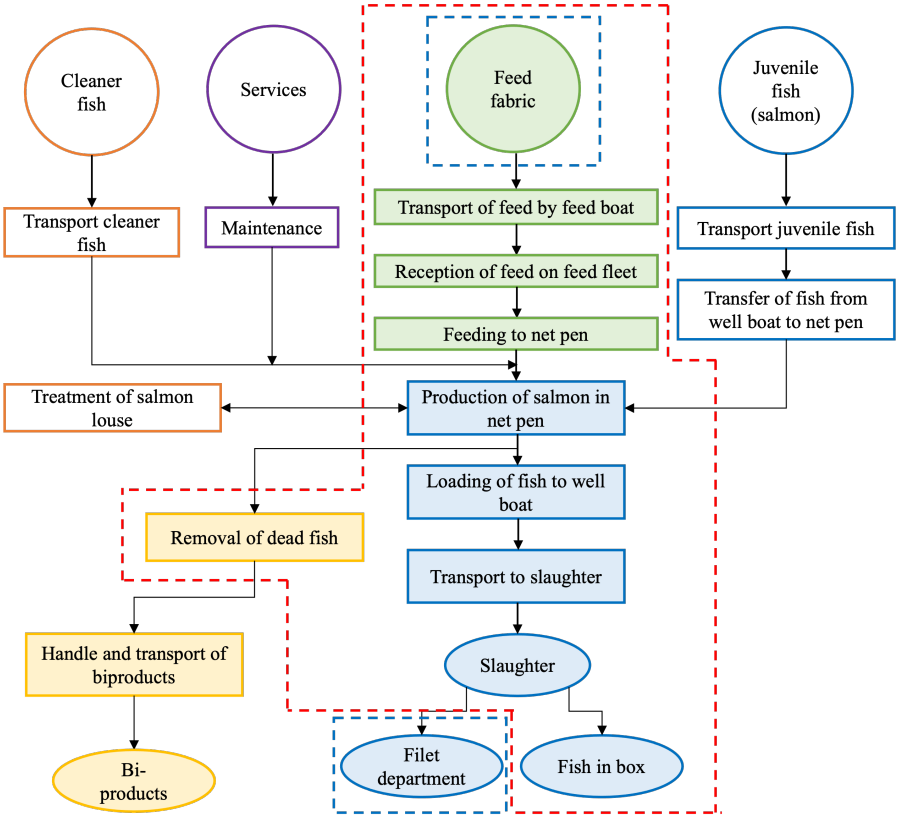


Figure 2.1: Overview of the salmon value chain examined in the TRACEListeria project. Out of a total of 56 bacterial isolates selected for WGS, were 35 selected for further examination in this study, and 21 for another master's study. The origin of the bacterial isolates is marked (red dashed line: the 35 isolates selected for this study; blue dashed line: the 21 isolates selected for the other master's study.

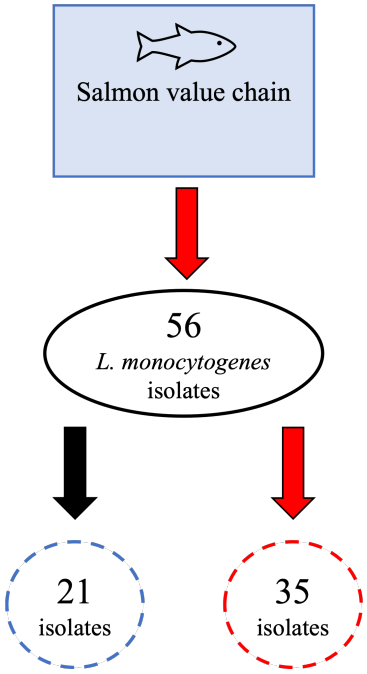


Figure 2.2: Overview of bacterial isolate selection. A total of 56 *L. monocytogenes* isolates from the salmon processing value chain were selected for WGS in association to the TRACEListeria project. These isolates were further examined in this study (35 isolates) and another master's study (21 isolates).

## 2.2 DESCRIPTION OF THE 35 BACTERIAL ISOLATES EXAMINED IN THIS STUDY

In this study, 35 *L. monocytogenes* isolates from the salmon processing value chain in Norway were examined. The bacterial isolates were sampled from October 2020 to May 2021, and originated from the feed or sea area of the value chain. Sampling points and number of isolates sampled from each point is presented in Table 2.1. A detailed overview of isolate ID, point and date of origin for all individual isolates is given in Appendix A.

Table 2.1: Location and sampling points in the salmon processing value chain for the 35 *L. monocytogenes* isolates examined in this study. Number (No.) of isolates from each sampling point and location are given.

| Location | Sampling point | No. of isolates |
|---|---|---|
| Feed | Feed | 9 |
| Feed | Environment and swamp samples | 11 |
| Sea | Living fish | 6 |
| Sea | Dead fish | 7 |
| Sea | Fish in box | 2 |

## 2.3 OVERVIEW OF APPLIED EXPERIMENTAL METHODOLOGY

For the experimental work, several laboratory based methods of analysis were implemented. All methods are based on other studies and/or established tools ((Bortolaia et al., 2020), (Doran, 2013), (Harrison et al., 2010), (Hingston et al., 2017), (Joensen et al., 2014), (Kaas et al., 2014), (Larsen et al., 2012), (Letunic & Bork, 2021)). The experimental work can be divided into phenotypic and genotypic analyses of the 35 *L. monocytogenes* isolates. Figure 2.3 illustrates the experimental work, and all methods are described in the following sections.

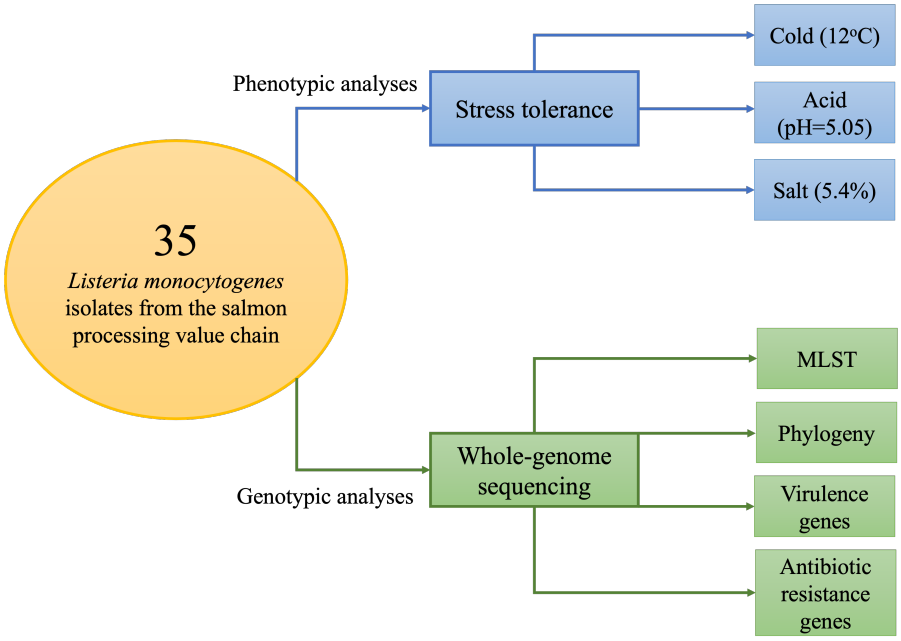


Figure 2.3: Overview of applied methodology. A selection of 35 *L. monocytogenes* isolates from the salmon processing value chain were analysed. Phenotypic analyses included examination of stress tolerance (cold, acid, salt). All bacterial isolates were whole-genome sequenced, and several genetic analyses performed on the resulting sequencing data (multi-locus sequence typing (MLST), phylogenetic tree construction, examination of virulence genes and antibiotic resistance genes).

## 2.4 WHOLE-GENOME SEQUENCING OF *Listeria monocytogenes* ISOLATES

All 35 *Listeria monocytogenes* isolates examined in this study were whole-genome sequenced by Oxford Nanopore technology. The work prior to WGS was performed in cooperation with the other master's student who examined the remaining 21 out of the 56 bacterial isolates selected for WGS.

### 2.4.1 *DNA isolation and preparation for sequencing*

DNA was isolated from all bacterial isolates by using Genomic Micro AX Bacteria+ Gravity-kit (102-100 M, A&A BIOTECHNOLOGY) according to manufacturer's instructions, with the exception of the proteinase K step. This step was extended from 10 min to 30 min to ensure high quality DNA, and to avoid contamination (proteins, RNA etc.) of the DNA.

Gel electrophoresis was run to verify successful DNA isolation. A 150 mL gel with 1.2% (w/v) agarose was prepared. A stock solution of buffer for electrophoresis was prepared (500 mL 50x TAE buffer: 121 g Tris Base, 28.55 mL acetic acid, 50.0 mL 0.5 M EDTA, deionized waster as remaining volume) prior to gel preparation, and the working solution was 1x TAE buffer (40.0 mM Tris base, 20.0 mM acetic acid, 1.0 mM EDTA). To prepare the gel, 150 mL 1x TAE buffer was added to 1.8 g agarose in a flask, and heated and mixed every 30 sec until the agarose was dissolved in the buffer (approximately 2.5 min). The agrose/buffer solution was cooled to approximately 65 °C, and 8 µL gel red was added. The solution was poured into a casting apparatus containing a gel tray with casting gates suitable to make an appropriate number of wells. The solution was left in the apparatus until the gel was solidified (approximately 30 min). The casting gates was then removed, and the gel was removed from the apparatus and ready for use. Gel preparation is summarized in Table 2.2.

Table 2.2: Preparation of a 150 mL gel with 1.2% (w/v) agarose for gel electrophoresis. All agents are listed along with suppliers and mass (m) or volume (V).

| Agent | Supplier | m/V |
|---|---|---|
| Agarose | 50005, SeaKem LE Agarose | 1.8 g |
| 1x TAE buffer | Tris base: 71003490, VWR; Acetic acid: 1.00063 Emsure | 150.0 mL |
| Gel red | 41003, Biotium | 8.0 µL |

To run the gel electrophoresis, the gel was placed in an electrophoresis apparatus, and 1x TAE buffer was added until the gel was covered.

For each sample, 5 µL 6x DNA loading buffer (R0611, Thermo Fisher Scientific) was added to 10 µL DNA. GelPilot 100 bp Plus Ladder (239045, Qiagen) was used as DNA marker. Gel electrophoresis was run for 40 min on 75 V by Sub-Cell ® GT system (Bio-Rad Inc). A Syngene$^{TM}$G: Box and the image acquisition software, GeneSys G: Box Chemi-XRQ, were used for gel imaging.

DNA concentration was measured for successfully isolated DNA verified by gel electrophoresis. A minimum DNA concentration of 40 ng/µL was required for WGS. The data analysis software Gen5 2.0 and a micro-plate spectrophotometer (PowerWaveXS Microplate, BioTek) were used, and 2 µL DNA from each isolate was added as duplicates to a micro-plate for measurement. TE buffer (12090015, Thermo Fisher Scientific) was used for calibration of the spectrophotometer prior to use.

The DNA samples for WGS were packed and sent on ice to Technical University of Denmark for sequencing. WGS was performed according to Oxford Nanophore method.

### 2.4.2 *Analysis of sequencing data*

Different genetic analyses were performed on the sequences of the 35 *L. monocytogenes* isolates. A variety of tools provided by Center of Genomic and Epidemiology (CGE) were used, and the files from WGS uploaded on FASTA format for analysis. MLST 2.0 (available at https://cge.food.dtu.dk/services/MLST/) was used to determine sequence types of the bacterial isolates by multi-locus sequence typing based on seven conventional loci. *L. monocytogenes* was selected as MLST configuration, and the remaining settings by default. A phylogeny tree based on SNPs was constructed using the tool CSI Phylogeny (available at https://cge.food.dtu.dk/services/CSIPhylogeny/) by default to show relationship between the bacterial isolates. A reference genome was included (*Listeria monocytogenes* EGD-e)[1]. The result file in Newick format was uploaded to the tool iTOL v6 from Interactive Tree of Life (available at https://itol.embl.de/upload.cgi) to manage and display the phylogeny tree. The different groupings of the tree were colour coded based on revealed sequence types from MLST 2.0. The tree was rooted at the reference.

Identification of acquired antibiotic resistance genes using ResFinder 4.1 (available at https://cge.food.dtu.dk/services/ResFinder/), and identification of acquired virulence genes using VirulenceFinder 2.0 (available at https://cge.food.dtu.dk/services/VirulenceFinder/) were performed for genotypic characterization and predictions of phenotypic features. For ResFinder, "other" was selected species due to no option for selecting *Listeria*. Default settings were used for VirulenceFinder (Threshold ID = 90%, minimum length = 60%), and "*Lis-*

1 GenBank accession number: GCA000196035.1ASM19603v1

*teria"* as selected species. All services used for genetic analyses are presented in Table 2.3.

Table 2.3: Services used for analyses of sequencing data from WGS of 35 *L. monocytogenes* isolates. The tools are provided by Center of Genomic Epidemiology or Interactive Tree of Life.

| Service | Description | Citation |
|---|---|---|
| ResFinder 4.1 | Identification of acquired antibiotic resistance genes | Bortolaia et al. (2020) |
| VirulenceFinder 2.0 | Identification of acquired virulence genes | Joensen et al. (2014) |
| MLST 2.0 | MLST | Larsen et al. (2012) |
| CSI Phylogeny | Infer phylogeny based on SNPs | Kaas et al. (2014) |
| iTOL v6 | Display and management of phylogeny tree | Letunic and Bork (2021) |

## 2.5 STRESS TOLERANCE PROFILING OF *Listeria monocytogenes* ISOLATES

Examination of stress tolerance for 35 *L. monocytogenes* isolates was performed according to Hingston et al. (2017), with some modifications due to availability of equipments, safety measures when working with pathogenic bacteria, and time restrictions.

### 2.5.1 *Cultivation and standardization of bacterial isolates prior to examination of stress tolerance*

All growth media used for the experimental work was prepared and stored according to the manufacturer´s instructions. Each *L. monocytogenes* isolate was cultivated on brilliance Listeria agar (BLA) (CM1080, SR0227, SR0228, Oxoid Ltd), and incubated for 22-24 h at 37 °C. One colony was transferred from the overnight culture to brain heart infusion agar (BHIA) (CM1136, Oxoid Ltd), and incubated at the same conditions.

To standardize cell concentration of the *L. monocytogenes* isolates for the stress tolerance examinations, a McFarland 0.5 turbidity standard was prepared according to Harrison et al. (2010). Preparation of 15 mL standard, and cell concentration corresponding to the standard is presented in Table 2.4. It was controlled that optical density (OD) of the standard measured at 625 nm was within the range of 0.08-0.13 by a spectrophotometer (Shimadzu UV1800) to verify the turbidity of the standard. It was stored sealed in the dark at room temperature, and used within the shelf life of six months.

Table 2.4: Preparation of McFarland 0.5 turbidity standard, according to Harrison et al. (2010). A total volume (V) of 15 mL was prepared by mixing 1.0% (w/v) $BaCl_2$ and 1.0% (v/v) $H_2SO_4$. Volume of each agent, and the number of cells in bacterial suspension corresponding to turbidity of the standard ($C_{cell}$) is given.

| V 1.0% (w/v) $BaCl_2$ [mL] | V 1.0% (v/v) $H_2SO_4$ [mL] | $C_{cell}$ [CFU/mL] |
|---|---|---|
| 0.075 | 14.925 | $1.5 \cdot 10^8$ |

Cell concentration in suspension for each *L. monocytogenes* isolate was standardized to $1.5 \cdot 10^8$ CFU/mL by using the McFarland 0.5 standard as reference. Inoculum was prepared by adding 10 mL of peptone water (LP0034, Oxoid Ltd) to sterile glass tubes, and adding colonies from the overnight culture on BHIA for each isolate. The turbidity was measured at 590 nm by a turbidimeter (21907, BioLog). Cell colonies were added to the inoculum until the turbidity was equal to the McFarland 0.5 standard (65±2% light transmittance). If turbidity was higher than the standard (light transmittance<65±2%),

peptone water was added and measurement repeated. All tubes with inoculum or standard were mixed thoroughly prior to turbidity measurements. Figure 2.4 shows a flow chart illustration the standardization of cell concentration prior to stress tolerance examination.

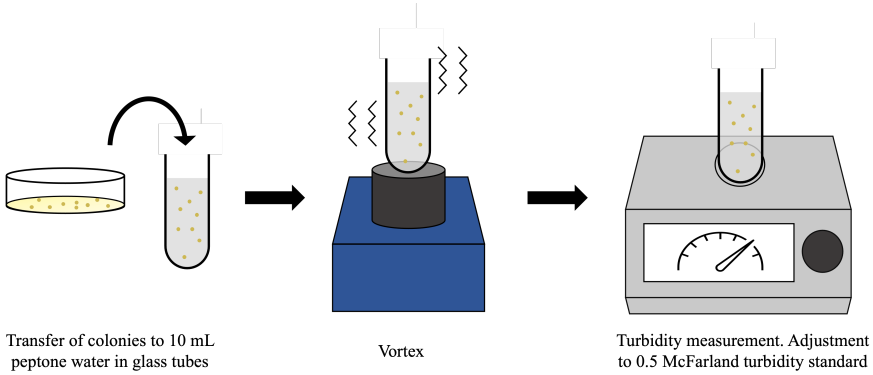


Figure 2.4: Flow chart for standardization of bacterial cell concentration for 35 *L. monocytogenes* isolates. For each isolate, bacterial cells were transferred to peptone water, vortexed, and the turbidity of the suspension measured by a turbidimeter. The turbidity of the bacterial suspension was adjusted to the 0.5 McFarland turbidity standard.

### 2.5.2 *Cold tolerance examination of the bacterial isolates*

To examine cold tolerance, the *L. monocytogenes* isolates were incubated in cold, and growth was measured spectroscopically over a time period. The aim was to examine the temperature in the salmon slaughter, packing and processing area, which is 12 °C. Optimal growth temperature for *L. monocytogenes* was used as 37 °C control for optimal growth (Table 1.2).

Prior to cold tolerance examination, standardized cell suspension of each isolate ($1.5 \cdot 10^8$ CFU/mL) was diluted to approximately $1.5 \cdot 10^3$ CFU/mL in brain heart infusion broth (BHI) (CM1135, Oxoid Ltd) by a five-fold dilution series. Five parallels of 5 mL diluted cell suspension were incubated in 15 mL tubes at 12 °C, and at optimal temperature (37 °C). $OD_{625}$ was measured prior to incubation (0 hours), and after 24, 48, 96, 140 and 244 hours of incubation (one parallell per measurement) by a spectrophotometer (Shimadzu UV1700). OD was not measurable prior to incubation (0 h), and growth measurements were therefore performed by colony counting. Prior to counting, 100 µL of isolate suspension was plated on tryptic soy agar (TSA) (84602.0500, VWR) containing 6% yeast extract (LP0021B, Oxoid Ltd.), and incubated for 22-24 h at 37 °C. The colonies were counted by using a manual colony counter (435000, Interscience).

Standard curves (at 12 °C and 37 °C) were constructed to assume concentration (CFU/mL) from $OD_{625}$ by interpolation. Colony count-

ing and $OD_{625}$ measurements were performed on selected isolates from different points of origin in the salmon value chain (seven in total) each day to construct the curves. $Log_{10}(CFU/mL)$ vs. $OD_{625}$ was plotted, and the average values for the selected isolates used. A line was fitted using linear regression.

The resulting growth curves ($log_{10}(CFU/mL)$ vs. time) for the bacterial isolates were fitted to the Baranyi and Roberts model using DMFit on the ComBase browser (available at https://browser.combase.cc/DMFit.aspx). Maximum growth rate ($\mu_{max}$) was determined for each isolate. Classification of tolerance was based on method described by Hingston et al. (2017), but modified due to only one replication per isolate, and to restrictions in number of measurements. Isolates were considered tolerant or sensitive to cold stress if the deviation of $\mu_{max}$ from the median was > or < than 1, respectively. All remaining isolates were considered intermediate tolerant. The median was selected as standard rather than the average value to avoid influence of very stress sensitive isolates, according to Hingston et al. (2017).

Figure 2.5 shows a flow chart over the experimental procedure applied for cold tolerance examination, as described in this section.

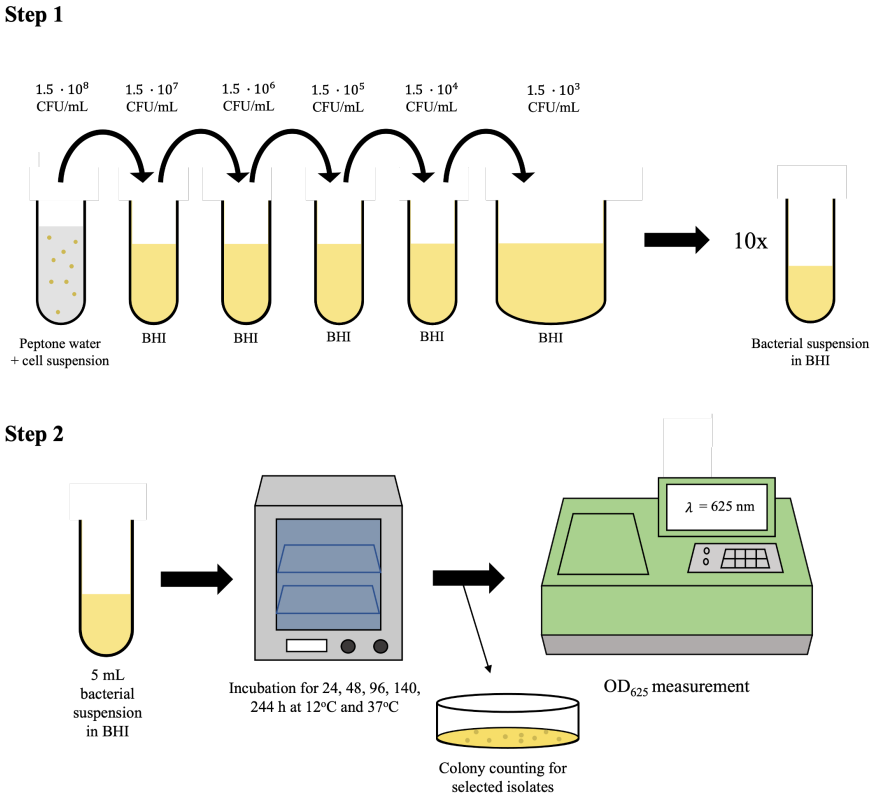


Figure 2.5: Flow chart for cold tolerance profiling of 35 *L. monocytogenes* isolates. **Step 1:** dilution of standardized ($1.5 \cdot 10^8$ CFU/mL) cell suspension to $1.5 \cdot 10^3$ CFU/mL. Five parallels for 12 °C, and five for 37 °C were prepared for each bacterial isolate. **Step 2:** Diluted cell suspension incubated at 12 °C and 37 °C. $OD_{625}$ was measured after 24, 48, 140, 244 hours. Colony counting was performed prior to incubation (0 hours) because $OD_{625}$ was not measurable, and for each time interval for selected isolates to construct standard curves.

### 2.5.3 *Acid and salt tolerance examination of the bacterial isolates*

For examination of salt tolerance of 35 *L. monocytogenes*, growth in 5.4% NaCl was examined. Growth in pH 5.05 was examined for acid tolerance. These growth conditions were selected based on growth conditions for *L. monocytogene* (Table 1.2). BHI with NaCl to obtain 5.4% NaCl in diluted bacterial suspension was prepared. BHI with pH adjusted by 1 M HCl were also prepared to obtain diluted bacterial suspensions with pH 5.05. A pH meter (PHM210, MeterLab$^{TM}$) was used to adjust pH. BHI without stress factors (one parallel with bacteria, and one parallel without) was used as reference.

For each bacterial isolate, standardized bacterial cell suspension ($1.5 \cdot 10^8$ CFU/mL) was diluted to approximately $1.5 \cdot 10^7$ CFU/mL in BHI with NaCl. Final volume was 10 mL diluted bacterial suspension in 5.4% NaCl in a 15 mL tube. The same was repeated for BHI

with acid. The bacterial suspensions were mixed thoroughly, and 200 µL from each suspension were added as triplicates to sterile 96-well micro-plates with lid (351172, Falcon®). The plates were incubated in room temperature, covered with the lid and in a sealed box. $OD_{600}$ was recorded each hour for a total of 24 hours to measure growth. The data analysis software Gen5 2.0 and a micro-plate spectrophotometer (PowerWaveXS Microplate, BioTek) were used for the measurements. The resulting growth curves were fitted in Excel (version 16.61.1) by plotting $OD_{600}$ vs. time, and a line fitted exponentially. $\mu_{max}$ was determined for all bacterial isolates according to Doran (2013).

A flow chart of the main steps for the salt and acid stress tolerance experiments is presented in Figure 2.6 to summarize the procedure described in this section.

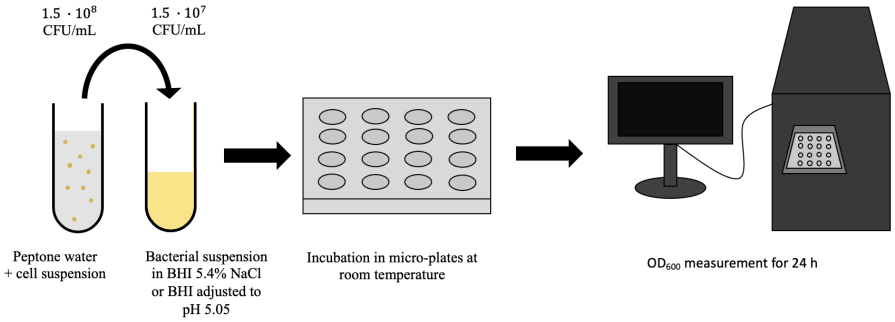


Figure 2.6: Flow chart for salt- and acid tolerance profiling for 35 *L. monocytogenes* isolates. Standardized bacterial cell suspensions ($1.5 \cdot 10^8$ CFU/mL) were diluted to $1.5 \cdot 10^7$ CFU/mL for each isolate, and incubated at room temperature. $OD_{600}$ was measured each hour for 24 hours for each isolate in salt stress (5.4% NaCl), acid stress (pH 5.05), and no stress conditions, in addition to growth media (BHI) without bacteria.

### 2.5.4 *Statistical analysis*

To detect if there were differences in stress tolerance between the *L. monocytogenes* strains, one-way analysis of variance (ANOVA) were used. Tukey post hoc test were used to determine which isolates that were different. The maximum growth rate ($\mu_{max}$) was used for basis of the statistical analysis, and to determine if the bacterial isolates could be distinguished. The software SPSS Statistics (version 28.0.1.0, IBM) was used to perform the statistical analysis with significance level (Sig.) 0.05. All p-values $\leqslant$ 0.05 were considered significant. The statistical analysis required n>1 for all variables, and *L. monocytogenes* isolates were excluded if they were the only bacterial isolate within a identified strain.

# 3 RESULTS

## 3.1 WHOLE-GENOME SEQUENCING OF *Listeria monocytogenes* ISOLATES

DNA was successfully isolated from all 35 *Listeria monocytogenes* isolates for whole-genome sequencing, and DNA concentration for isolated DNA from the bacterial isolates are presented in Table 3.1.

Table 3.1: DNA concentration of isolated DNA for the 35 *L. monocytogenes* isolates examined in this study.

| Isolate ID | DNA concentration [ng/µL] |
|---|---|
| 101 | 72.5 |
| 103 | 199.1 |
| 104 | 55.5 |
| 106 | 50.4 |
| 107 | 65.8 |
| 108 | 58.2 |
| 109 | 68.2 |
| 110 | 70.9 |
| 111 | 76.7 |
| 112 | 60.2 |
| 201 | 70.0 |
| 203 | 60.0 |
| 204 | 50.4 |
| 205 | 59.1 |
| 206 | 98.9 |
| 207 | 79.8 |
| 208 | 72.7 |
| 209 | 74.2 |
| 211 | 118.9 |
| 212 | 59.2 |
| 303 | 73.6 |
| 304 | 70.7 |

Gel documentation of qualified DNA for 56 *L. monocytogenes* isolates selected for WGS is presented in Figure 3.1, and Figure 3.2, and DNA of the 35 *L. monocytogenes* examined in this study are indicated on the gels (yellow).

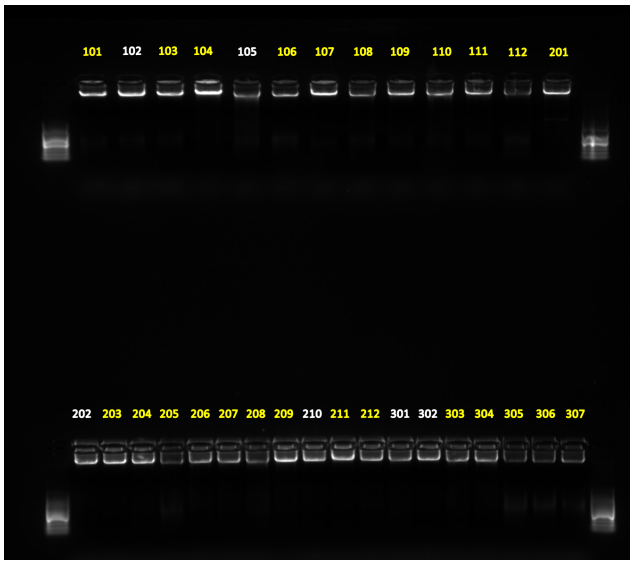


Figure 3.1: Gel image from gel electrophoresis on DNA from 56 *Listeria monocytogenes* isolates selected for WGS. Isolate ID are indicated, and the 35 *L. monocytogenes* isolates examined in this study are marked in yellow.

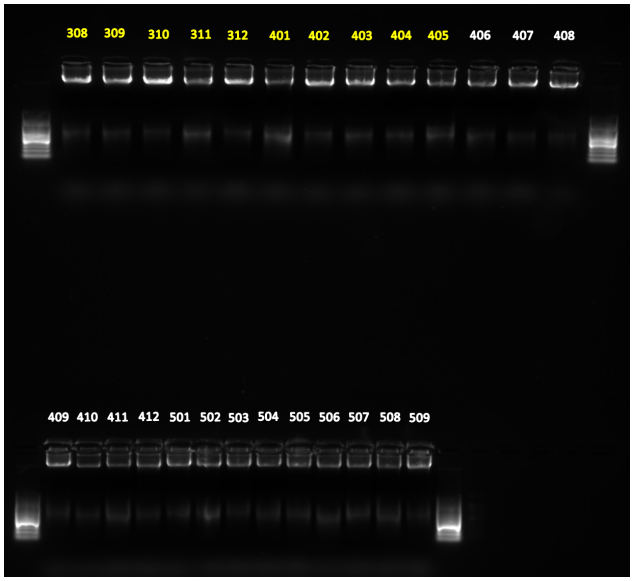


Figure 3.2: Gel image from gel electrophoresis on DNA from 56 *Listeria monocytogenes* isolates selected for WGS. Isolate ID are indicated, and the 35 *L. monocytogenes* isolates examined in this study are marked in yellow.

The sequencing data generated by WGS of the 35 *Listeria monocytogenes* isolates were generated on FASTA format with an amount of data ranging from 2.9 to 3.1 MB per isolate file.

### 3.1.1 *MLST profiling revealed nine sequence types*

MLST profiling of the 35 *L. monocytogenes* isolates with the online service MLST 2.0 revealed nine sequence types in total: ST20 (n=9), ST29 (n=1), ST31 (n=5), ST37 (n=6), ST91 (n=4), ST101 (n=1), ST325 (n=2), ST391 (n=1), and ST394 (n=6). Figure 3.3 presents sequence type distribution in percentage for all *L. monocytogenes* isolates examined in this study. An overview of sequence types for the individual isolates is attached to Appendix A.

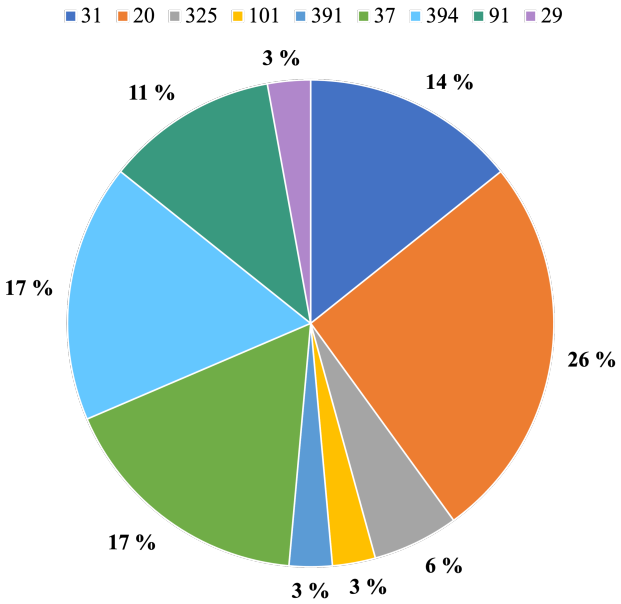


Figure 3.3: Distribution of sequence types (ST) for 35 *L. monocytogenes* isolates revealed by MLST 2.0 (available at https://cge.food.dtu.dk/services/MLST/). Nine ST were identified in total (ST20, ST29, ST31, ST37, ST91, ST101, ST325, ST391, ST394), and ST 20 accounted for the majority (26%).

The phylogenetic tree constructed based on SNPs by the online service CSI Phylogeny, and displayed by iTOL v6, clustered the 35 *L. monocytogenes* isolates in nine groups. The same ST was identified from different sampling points in the salmon value chain. MLST 2.0 revealed the reference strain (*L. monocytogenes* EGD-e) to be ST35, and the reference was differentiated from all nine groups in the pylogenetic tree. Figure 3.4 presents the phylogenetic tree.

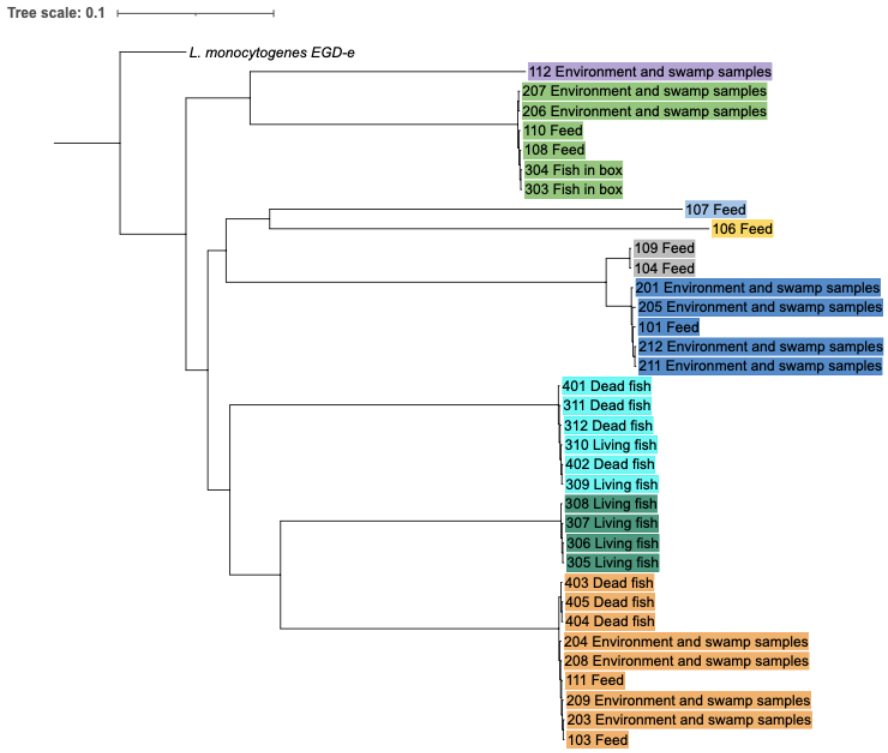


Figure 3.4: Phylogenetic tree for 35 *L. monocytogenes* isolates based on SNPs, constructed and displayed by CSI Phylogeny and iTOL v6 (available at https://cge.food.dtu.dk/services/CSIPhylogeny/ and https://itol.embl.de/upload.cgi). The *L. monocytogenes* isolates clustered in nine groups. The point of origin, and sequence types of the bacterial isolates are indicated on the phylogenetic tree (ST20: orange, ST29: purple, ST31: dark blue, ST37: green, ST91: turquoise, ST101: yellow, ST325: grey, ST391: media blue, ST394: light blue). The reference strain (*L. monocytogenes* EGD-e) differentiated from all nine groups. The phylogenetic tree was rooted at the reference strain.

The distribution of sequence types of the 35 *L. monocytogenes* isolates in association to sampling points in the salmon value chain are presented in Figure 3.5. ST20 and ST37 were present in both feed and sea area. ST29, ST31, ST101, ST325, and ST391 were detected in the feed area, and ST91 and ST394 only in the sea area.

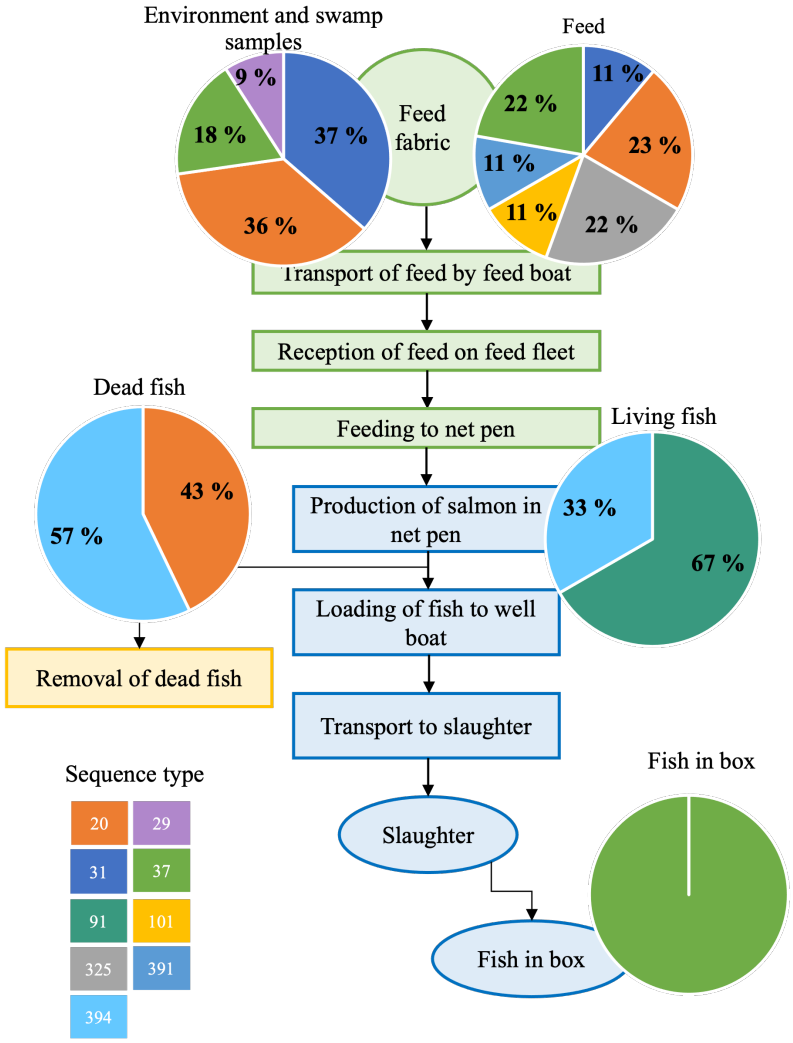


Figure 3.5: Distribution of sequence types for 35 *L. monocytogenes* isolates in association to sampling points in the salmon value chain. The bacterial isolates originated from the feed area (environment and swamp sample, feed) and/or from the sea area (dead fish, living fish, fish in box). The sequence types of the bacterial isolates origins are indicated on the figure (ST20: orange, ST29: purple, ST31: dark blue, ST37: green, ST91: turquoise, ST101: yellow, ST325: grey, ST391: media blue, ST394: light blue).

### 3.1.2 *Identification of acquired antibiotic resistance genes showed heat resistance for only ST31*

Examination of acquired antibiotic resistance genes (with ID>90%) with the service ResFinder 4.1, revealed that the antibiotic resistance profile was identical for all *L. monocytogenes* isolates within a sequence type. All 35 *L. monocytogenes* isolates carried the gene *fosX*. This gene encodes fosfomycin resistance. All bacterial isolates being ST31 carried the *ClpL* gene encoding heat resistance, with a sequence identity of 99.95%. Table 3.2 presents the antibiotic resistance genes and sequence identity (ID) for all ST.

Table 3.2: Acquired antibiotic resistance genes (with identification sequence>90%) identified for 35 *L. monocytogenes* isolates. The antibiotic resistance profile was identical for all *L. monocytogenes* isolates within a sequence type (ST). *fosX* encoding fosfomycin resistance was identified for all ST, and *ClpL* encoding heat resistance was identified for ST31. Sequence identity (ID) for the identified genes are presented. The resistance genes were identified by ResFinder 4.1 (available at https://cge.food.dtu.dk/services/ResFinder/).

| ST | Antibiotic resistance genes (ID>90%) | ID (%) |
|---|---|---|
| 20 | *fosX* | 97.26 |
| 29 | *fosX* | 97.26 |
| 31 | *fosX*, *ClpL* | 98.01, 99.95 |
| 37 | *fosX* | 99.25 |
| 91 | *fosX* | 98.76 |
| 101 | *fosX* | 98.01 |
| 325 | *fosX* | 98.01 |
| 391 | *fosX* | 97.51 |
| 394 | *fosX* | 97.76 |

### 3.1.3 *Identification of virulence genes showed that the actA gene was not carried by all sequence types*

Identification of virulence genes with the online service VirulenceFinder 2.0, revealed that the virulence profile was identical for all isolates within a sequence type. A high number of virulence genes with ID>98% were identified for the different sequence types.

Selected virulence genes important for *L. monocytogenes* infection (*hly*, *inlA*, *inlB*, *plcA*, and *plcB*) were carried by all sequence types with a sequence identity (ID) ranging from 97.5% to 99.87%. When analyzing for a possible truncated *inlA* gene, a full length gene (2403/2403 base-pairs) was confirmed for all isolates with identity from 97.75% to 99.25% (2405/2403 bp was identified for ST29).

The *actA* gene was carried by ST20, ST29, ST37, ST91, ST101, and ST394 with identity from 97.45% to 99.01%. The gene was not identified for ST31, ST325 and ST391. Number of virulence genes, and sequence identity (ID) for the selected genes important for infection for all sequence types are presented in Table 3.3.

Table 3.3: Number (No.) of virulence genes with sequence identity (ID)>98% for the different sequence types (ST) revealed for 35 *L. monocytogenes* isolates. ID for selected virulence genes important for infection (*actA*, *hly*, *inlA*, *inlB*, *plcA*, and *plcB*) is given with a threshold of 90%. The virulence genes were identified by VirulenceFinder 2.0 (available at https://cge.food.dtu.dk/services/VirulenceFinder/).

| | No. virulence | ID (%) | | | | | |
|---|---|---|---|---|---|---|---|
| ST | genes (ID>98%) | *actA* | *hly* | *inlA* | *inlB* | *plcA* | *plcB* |
| 20 | 81 | 98.02 | 99.87 | 98.59 | 99.47 | 98.74 | 99.77 |
| 29 | 73 | 97.5 | 99.62 | 97.75 | 99.47 | 99.27 | 99.77 |
| 31 | 72 | - | 99.94 | 99.21 | 99.37 | 98.32 | 99.77 |
| 37 | 78 | 99.01 | 99.94 | 98.21 | 99.68 | 98.22 | 99.77 |
| 91 | 79 | 97.45 | 99.75 | 98.96 | 99.42 | 99.16 | 99.77 |
| 101 | 72 | 98.28 | 99.94 | 97.79 | 99.0 | 98.95 | 99.66 |
| 325 | 71 | - | 99.94 | 99.25 | 99.37 | 98.22 | 99.77 |
| 391 | 71 | - | 99.94 | 98.0 | 99.26 | 98.95 | 99.08 |
| 394 | 76 | 98.54 | 99.87 | 97.84 | 99.47 | 98.64 | 99.77 |

## 3.2 COLD TOLERANCE OF *Listeria monocytogenes* ISOLATES

Growth at 12 °C was examined for 35 *L. monocytogenes* isolates, and individual growth curves ($\log_{10}$(CFU/mL) vs. time) for all bacterial isolates at 12 °C, and at optimal growth temperature (37 °C) are presented in Figure 3.6 and Figure 3.7, respectively. Average growth curve for all 35 bacterial isolates, and the 21 remaining *L. monocytogenes* isolates from the selection of 56 isolates for WGS, are also presented on the figures (black dashed line).

The standard curves (at 12 °C and at 37 °C) constructed for assuming bacterial cell concentration from $OD_{625}$ for the cold tolerance examination for *L. monocytogenes* isolates are attached to Appendix B.

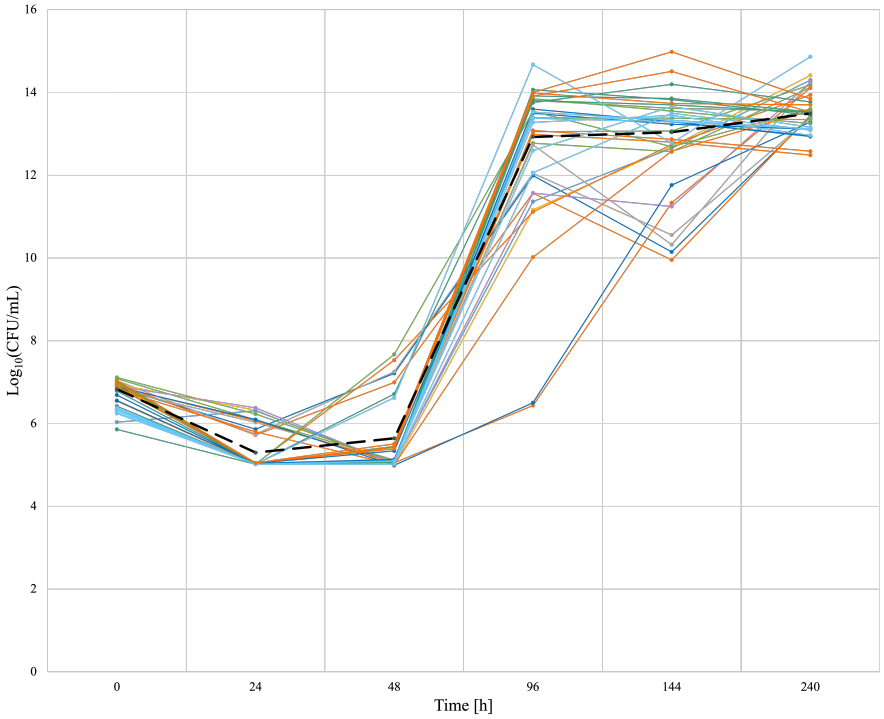


Figure 3.6: Individual growth curves ($\log_{10}$(CFU/mL) vs. time) for 35 *L. monocytogenes* isolates at 12 °C. sequence type of the bacterial isolates are indicated on the growth curves (ST20: orange (103, 111, 203, 204, 208, 209, 403, 404, 405), ST29: purple (112), ST31: dark blue (101, 201, 205, 211, 212), ST37: green (108, 110, 206, 207, 303, 304), ST91: turquoise (305, 306, 307, 308), ST101: yellow (106), ST325: grey (104, 109), ST391: media blue (107), ST394: light blue (309, 310, 311, 312, 401, 402)). Average growth curve based on growth for all bacterial isolates in this study, and the 21 remaining *L. monocytogenes* isolates from the selection of 56 isolates for WGS, is presented (black, dashed line).

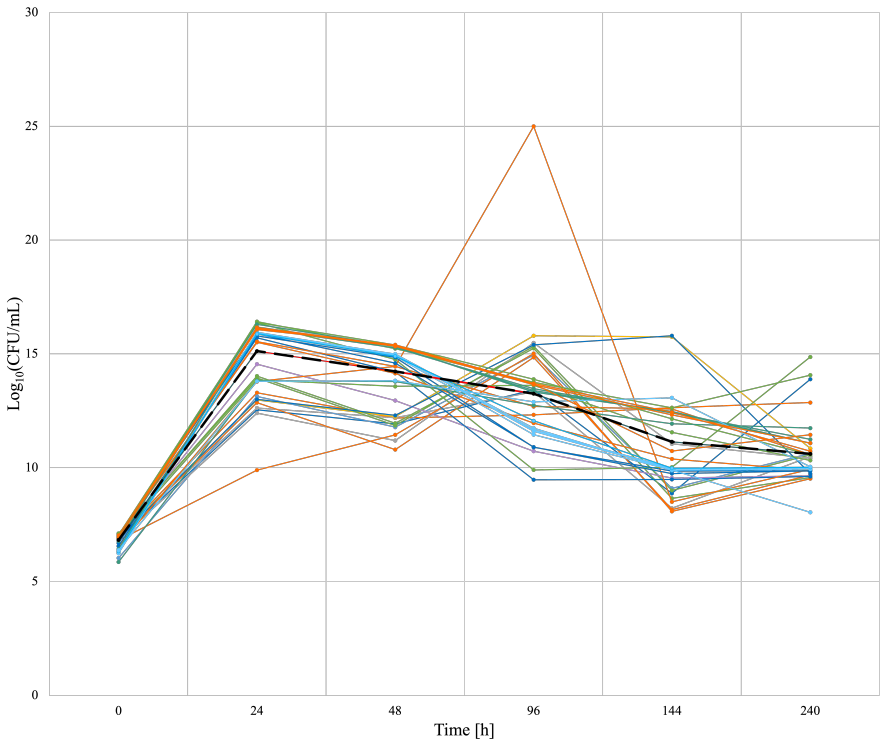


Figure 3.7: Individual growth curves ($\log_{10}$(CFU/mL) vs. time) for 35 *L. monocytogenes* isolates at optimal growth temperature (37 °C). sequence type of the bacterial isolates are indicated on the growth curves (ST20: orange (103, 111, 203, 204, 208, 209, 403, 404, 405), ST29: purple (112), ST31: dark blue (101, 201, 205, 211, 212), ST37: green (108, 110, 206, 207, 303, 304), ST91: turquoise (305, 306, 307, 308), ST101: yellow (106), ST325: grey (104, 109), ST391: media blue (107), ST394: light blue (309, 310, 311, 312, 401, 402)). Average growth curve based on growth for all bacterial isolates in this study, and the 21 remaining *L. monocytogenes* isolates from the selection of 56 isolates for WGS, is presented (black, dashed line).

The average growth curve at 12 °C for the *L. monocytogenes* isolates showed lag phase for approximately 48 hours, growth from approximately 48-96 hours, and stationary phase from approximately 96 hours. The average growth curve for the optimal temperature for the *L. monocytogenes* isolates did not show lag phase, but growth was observed for approximately 24 hours, and bacterial cell density decreases from approximately 24 hours.

Cold tolerance distribution based on maximum growth rates ($\mu_{max}$) of the 35 *L. monocytogenes* isolates is presented in Figure 3.8. Tolerance was observed for 6 bacterial isolates being ST20 (203, 208, 209), ST31 (211, 212), and ST37 (303). The deviation from median was >12.5 $h^{-1}$ for all tolerant isolates. One-way ANOVA revealed that the differences in $\mu_{max}$ between the sequence types are not significant (p=0.384), and the Tukey post hoc test also revealed that the ST cannot be distin-

guished based on $\mu_{max}$ for growth at 12 °C. Results of the statistical analysis are attached to Appendix D.

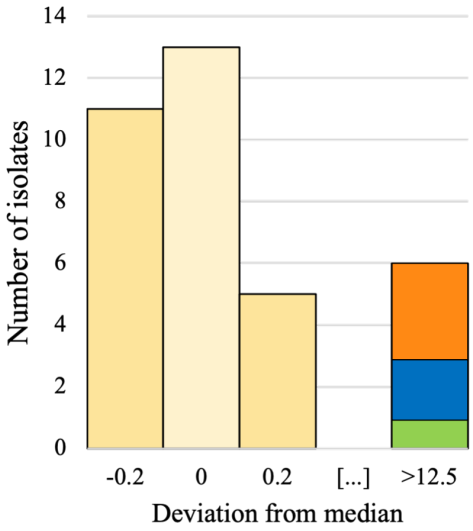


Figure 3.8: Cold tolerance distribution of 35 *L. monocytogenes* isolates. The isolates were considered tolerant or sensitive if the deviation from the median maximum growth rate was > or < than 1, respectively. Tolerance was observed for 6 bacterial isolates being ST20 (orange: 203, 208, 209), ST31 (dark blue: 211, 212), and ST37 (green: 303).

## 3.3 SALT TOLERANCE AND ACID TOLERANCE OF *Listeria monocytogenes* ISOLATES

Bacterial growth during salt stress (5.4% NaCl), and acid stress (pH 5.05) were examined for the *L. monocytogenes* isolates. Average growth curves ($OD_{600}$ vs. time) for salt stress (orange) and acid stress (grey) for all bacterial isolates examined in this study, and the 21 remaining *L. monocytogenes* isolates selected for WGS, are presented in Figure 3.9. Bacterial growth in growth media without addition of salt or acid (blue), and growth media without bacteria or addition of salt or acid (yellow) were used as references, and average growth curves are also presented for the references.

The average growth curves for the L. monocytogenes isolates show a decrease in growth for the bacterial isolates in salt stress and acid stress compared to stress-free conditions. For both stress conditions, lag-phase is observed for approximately 8 or 9 hours. Growth is observed for the rest of the time period, and stationary phase was not reached after 24 hours. In stress-free conditions, lag phase is observed for approximately 8 hours, exponential phase from approximately 8-13 hours, and stationary phase from approximately 13-24 hours.

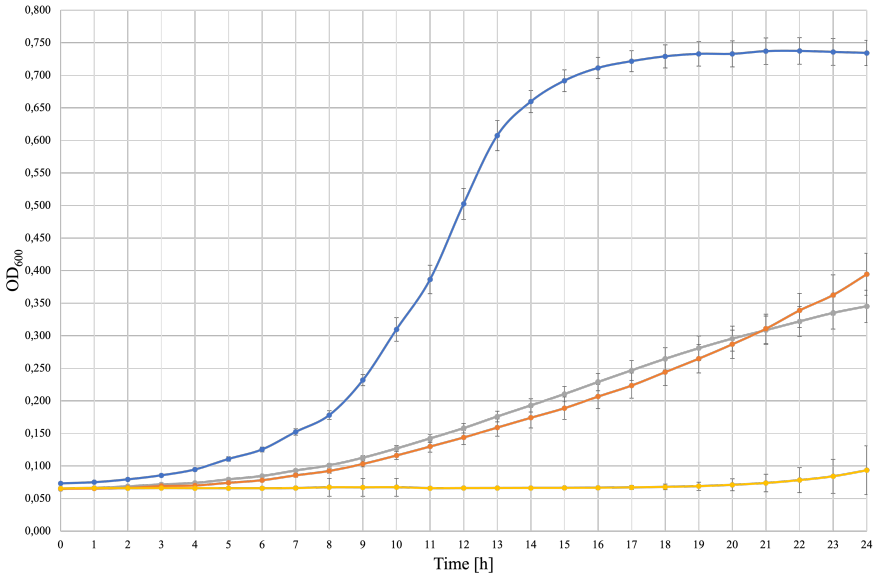


Figure 3.9: Average growth curves ($OD_{600}$ vs. time) for growth in salt stress (orange; 5.4% NaCl) and acid stress (grey; pH 5.05) for 35 *L. monocytogenes* isolates examined in this study, and 21 additionally *L. monocytogenes* isolates from the selection of 56 isolates for WGS. Bacterial growth in stress-free conditions (blue), and media without bacteria (yellow) were used as reference.

### 3.3.1 *Salt tolerance examination showed decrease in maximum growth rate and sequence types discrimination*

Figure 3.10 presents individual growth curves ($OD_{600}$ vs. time) for 35 *L. monocytogenes* isolates in salt stress (5.4% NaCl). Growth was detected for all bacterial isolates.

Fold decrease was calculated for 35 *L. monocytogenes* isolates to compare growth in salt stress to stress-free conditions. It was found that the maximum growth rate ($\mu_{max}$) for the *L. monocytogenes* isolates in average decreased with a factor of 2.615 in salt stress relative to no stress conditions. Fold decrease was positive for all bacterial isolates. Figure 3.11 presents fold decrease for $\mu_{max}$ in salt stress relative to $\mu_{max}$ in BHI without stress conditions for all *L. monocytogenes* isolates examined in this study.

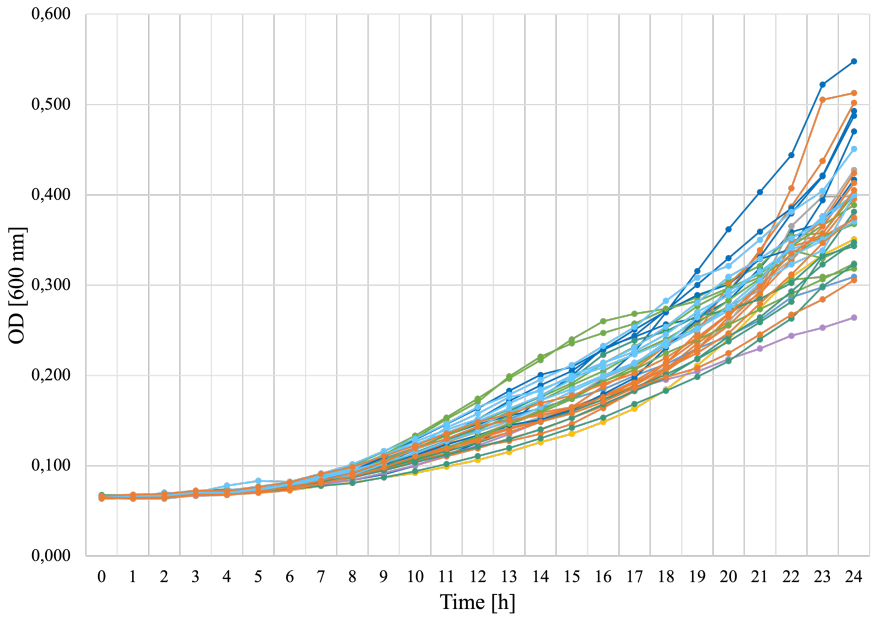


Figure 3.10: Individual growth curves ($OD_{600}$ vs. time) for growth in salt stress (5.4% NaCl) for 35 *L. monocytogenes* isolates. sequence type of the bacterial isolates are indicated on the growth curves (ST20: orange (103, 111, 203, 204, 208, 209, 403, 404, 405), ST29: purple (112), ST31: dark blue (101, 201, 205, 211, 212), ST37: green (108, 110, 206, 207, 303, 304), ST91: turquoise (305, 306, 307, 308), ST101: yellow (106), ST325: grey (104, 109), ST391: media blue (107), ST394: light blue (309, 310, 311, 312, 401, 402)).

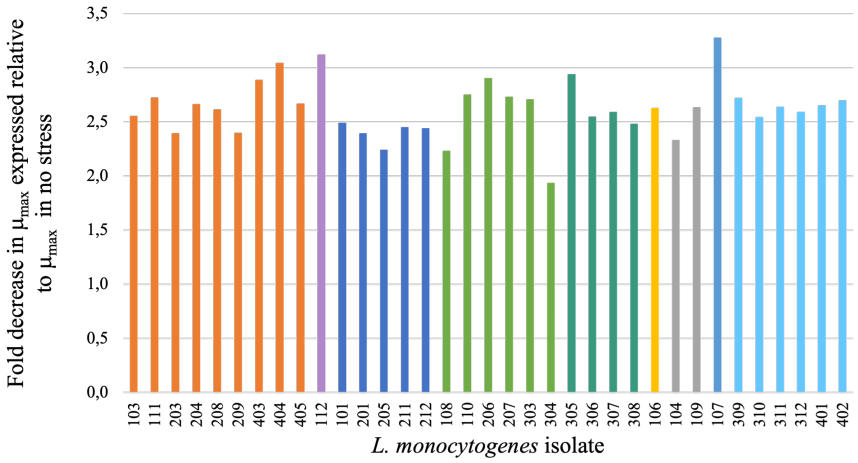


Figure 3.11: Fold decrease of maximum growth rate ($\mu_{max}$) in salt stress (5.4% NaCl) relative to $\mu_{max}$ in stress-free conditions (only BHI) for 35 *L. monocytogenes* isolates. Sequence type of the bacterial isolates are indicated (ST20: orange, ST29: purple, ST31: dark blue, ST37: green, ST91: turquoise, ST101: yellow, ST325: grey, ST391: media blue, ST394: light blue).

One-way ANOVA revealed that the differences in $\mu_{max}$ between the sequence types are significant (p=0.05) for growth in 5.4% NaCl. Tukey post hoc test revealed that $\mu_{max}$ for ST31 is significantly higher than $\mu_{max}$ for ST91. No other significant differences in $\mu_{max}$ were found. Results of the statistical analysis are attached to Appendix D.

### 3.3.2 *Acid tolerance examination showed decrease in maximum growth rate and sequence types discrimination*

Figure 3.12 presents individual growth curves ($OD_{600}$ vs. time) for 35 *L. monocytogenes* isolates in acid stress (pH 5.05). Growth was detected for all bacterial isolates.

Fold decrease was calculated for 35 *L. monocytogenes* isolates to compare growth in acid stress to stress-free conditions. It was found that $\mu_{max}$ for the *L. monocytogenes* isolates in average decreased with a factor of 2.428 in acid stress compared to stress-free conditions. Fold decrease was positive for all bacterial isolates. Figure 3.13 presents fold decrease of $\mu_{max}$ in acid stress relative to $\mu_{max}$ in BHI without stress conditions for all *L. monocytogenes* isolates.

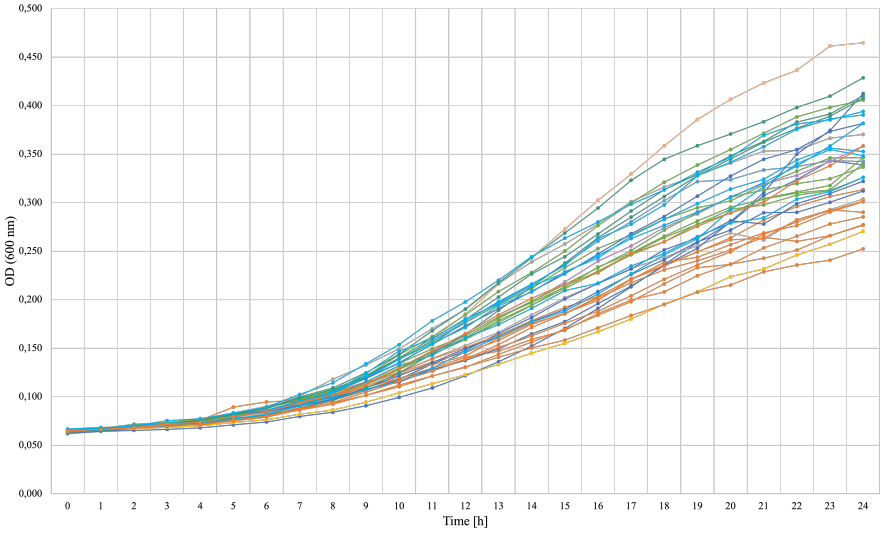


Figure 3.12: Individual growth curves ($OD_{600}$ vs. time) for 35 *L. monocytogenes* isolates in acid stress (pH 5.05). sequence type of the bacterial isolates are indicated on the growth curves (ST20: orange (103, 111, 203, 204, 208, 209, 403, 404, 405), ST29: purple (112), ST31: dark blue (101, 201, 205, 211, 212), ST37: green (108, 110, 206, 207, 303, 304), ST91: turquoise (305, 306, 307, 308), ST101: yellow (106), ST325: grey (104, 109), ST391: media blue (107), ST394: light blue (309, 310, 311, 312, 401, 402)).

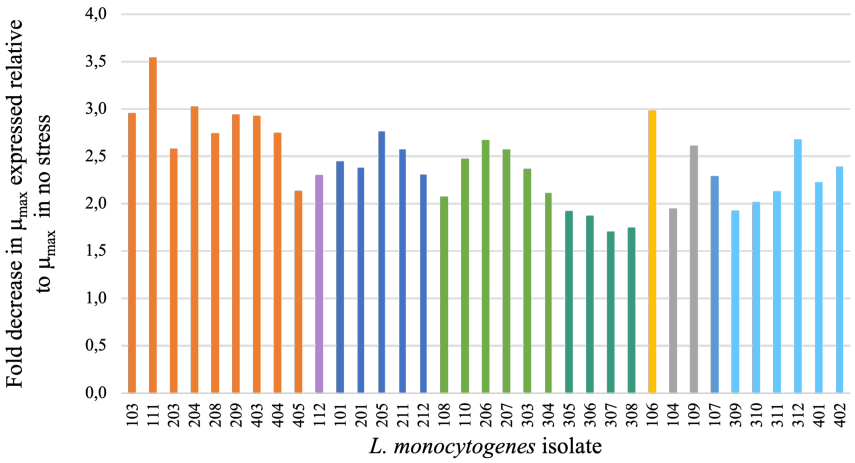


Figure 3.13: Fold decrease of maximum growth rate ($\mu_{max}$) in acid stress (pH 5) relative to $\mu_{max}$ in stress-free conditions (only BHI) for 35 *L. monocytogenes* isolates. sequence type of the bacterial isolates are indicated (ST20: orange, ST29: purple, ST31: dark blue, ST37: green, ST91: turquoise, ST101: yellow, ST325: grey, ST391: media blue, ST394: light blue).

One-way ANOVA revealed that the differences in $\mu_{max}$ between the sequence types are significant ($p<0.001$) for growth in acid stress. Tukey post hoc test revealed that $\mu_{max}$ for ST91 are significantly higher than $\mu_{max}$ for ST20, 31, 37 and 325. $\mu_{max}$ for ST394 are significantly higher than $\mu_{max}$ for ST20. Results of the statistical analysis are attached to Appendix D.

# 4 DISCUSSION

## 4.1 GENETIC ANALYSIS OF *Listeria monocytogenes*

In this study, a selection of 35 *Listeria monocytogenes* isolates from the salmon processing value chain in Norway was examined for potential properties important for entering a processing plant. The bacterial isolates were selected from 56 *L. monocytogenes* isolates sampled in association to the TRACEListeria project at NTNU. The remaining 21 isolates were examined in another master's study conducted by another master's student. The 35 bacterial isolates examined in this study were genetic characterized based on sequencing data generated by WGS with Oxford Nanopore technology, and examination of stress tolerance properties was performed. The 35 *L. monocytogenes* isolates originated from the feed area (feed, environment and swamp samples), or from the sea area (living fish, dead fish, fish in box) of the salmon value chain (Table 2.1).

WGS has become an important and widespread analysis tool in the food industry for tracking of food-borne outbreaks (Pightling et al., 2018). Identification of contamination sources for pathogens in food value chains (Quainoo et al., 2017), and genetic characterization of pathogens can be performed based on WGS (Chen et al., 2017). It is also important to discriminate microbes in the food industry on strain-level for tracking food-borne outbreaks because different strains within a species can have different properties (Wu et al., 2016), making for instance some bacterial strains more resistant to food-cleaning processes, and some more virulent than other strains. Strain-discrimination of the *L. monocytogenes* isolates examined in this study was performed by the MLST method.

MLST profiling of the 35 *L. monocytogenes* isolates revealed nine sequence types (ST20, 29, 31, 37, 91, 101, 325, 391, 394) (Figure 3.3). The majority of the bacterial isolates were ST20 (26%), and other large groups were ST37 (17%) and ST394 (17%). ST20 and ST37 were found in both the feed and sea area (Figure 3.5). It is therefore a possibility that these sequence types can be transmitted through the value chain, and that the feed section may be a possible source of contamination for *L. monocytogenes* in living fish, dead fish, and fish in box. However, it is important to examine the whole value chain (include slaughter house, feed boat, well boat etc.) for a more solid conclusion on the sequence type distribution and possible contamination sources.

By considering the remaining 21 bacterial isolates sampled in association to the TRACElisteria project, a larger part of the salmon

value chain would have been included in this study for tracking of *L. monocytogenes*. This was performed by Lerfall et al. (2022). The sequence types 20, 37, and 394 were still revealed to be dominant in the value chain, but ST37 was the most dominant ST (31% of the 56 *L. monocytogenes* isolates). This ST was tracked in the filet department in addition to feed and fish in box. ST37 is to some extent persistent, and *L. monocytogenes* of this type has been detected in several other food products and processing plants of meat, dairy and vegetables (Stessl et al., 2020). Fagerlund et al. (2022) examined 769 *L. monocytogenes* isolates from nine meat and six salmon processing facilities in Norway collected from 1990 to 2020. They sampled 306 of the bacterial isolates examined from salmon processing factories, and identified ST37 in one salmon processing plant.

Lerfall et al. (2022) detected ST20 among 16% of the 56 *L. monocytogenes* isolates, and this ST was not detected in any additional points than revealed by MLST profiling of the 35 bacterial isolates in this master's study. Jennison et al. (2017) performed MLST profiling of 244 *L. monocytogenes* from meat, dairy, seafood, fruit, vegetables, animals, environment, and humans from 1931 to 2015 in Australia. They found that ST20 only comprised isolates of animal origin (sheep and goats), and suggested that this may be a niche for ST20. These findings are not in compliance with the findings in this master's study, whereas ST20 was one of the most dominant types in the salmon value chain. Fagerlund et al. (2022) which examined *L. monocytogenes* from meat and salmon processing facilities over three decades also identified ST20 in two salmon processing plants in Norway.

Longitudinal studies for tracking of food-borne pathogens is important for examination of persistent strains. The 35 *L. monocytogenes* isolates included in this master's study was sampled over a time period of eight months. Sampling for a longer time period could have provided information on possible established strains that have adapted to the food processing environment. There is also a possibility that other sequence types would have been detected if sampling was performed at other time periods.

As described in the introduction, *L. monocytogenes* can be found in the outer surface, intestines or gills of fish in fish farming industry (Skowron et al., 2018). Sampling of the bacterial isolates has not been performed on salmon intestines, and should therefore be included in further examinations. Lerfall et al. (2022) also point out that further sampling is necessary to draw a more solid conclusion on contamination sources in the salmon value chain, and that the fish intestines should be included in a future sampling plan.

The sequence types found in individual cases (ST29, 101, 391) in this master's study was not found in any other points of the value chain by Lerfall et al. (2022). There is a possibility that these strains are less tolerant or less persistent than other strains found in higher

numbers. Further samplings may reveal if the salmon value chain is not an appropriate environment for the sequence types 29, 101, and 391.

Phylogenetic analysis based on SNPs was performed for the 35 *L. monocytogenes* isolates, and a phylogenetic tree was constructed (Figure 3.4). The bacterial isolates were clustered in nine groups in perfect accordance with the MLST analysis. The reference genome was found to be ST35, and differentiated from all nine groups, also in accordance with the sequence types identified for the 35 bacterial isolates by MLST.

### 4.1.1 *Identification of acquired antibiotic resistance genes*

Genetic analysis of bacteria is important to characterize variability, trends, and new emerging pathogens with new properties. Bacteria adapt to environmental changes, and can change rapidly and generate new SNPs (Thomassen et al., 2021). In the analysis of acquired antibiotic resistance genes, *fosX* encoding fosfomycin resistance was carried by all 35 *L. monocytogenes* isolates (Table 3.2). According to Scortti et al. (2018) is *fosX* universally conserved in *L. monocytogenes* and part of the core genome. These findings are based on analysis of genomic sequences from 1 696 *L. monocytogenes* isolates from different countries, and over a thousand core-genome MLST sub-types performed by Scortti et al. (2018). Thus, the *fosX* gene does not confer acquired resistance, but intrinsic resistance. This means that the gene is not a result of mutations or gene transfer in individual bacteria cells (Munita J. M. & Arias C. A., 2016), but the gene is a natural part of the species genome conferred by inherited genes (Ray et al., 2017). Scortti et al. (2018) also found that the *fosX* gene is suppressed by the PrfA transcriptional regulator for virulence genes. When PrfA is activated by host signals upon infection of pathogenic *L. monocytogenes*, the influx of fosfomycin in the bacterial cell was increased (2018). Therefore it could be interesting to examine the connection between pathogenicity and antimicrobial resistance in a further study, especially for fosfomycin because this antimicrobial has in fact been reported to be a useful drug for listeriosis treatment (2018).

The gene *ClpL* conferring heat resistance was carried by all bacterial isolates being ST31 (Table 3.2). Pöntinen et al. (2017) found pLM58 plasmid-borne ClpL in *L. monocytogenes* isolates. They found that the *ClpL* gene conferred increased survival and growth at 55 °C. Detection of plasmids should therefore be performed on the bacterial isolates to examine if the pLM58 plasmid might be present and a possible carrier for the *ClpL* gene. Growth examinations in heat should also be performed to obtain a heat tolerance profile of the bacterial isolates, and to reveal if isolates carrying *ClpL* grow better at elevated

temperatures compared to *L. monocytogenes* isolates not carrying the heat resistance gene.

### 4.1.2 *Identification of acquired virulence genes*

A high number of virulence genes was detected for the 35 *L. monocytogenes* isolates (Table 3.3). The important virulence genes for infection that was examined (*actA*, *hly*, *inlA*, *inlB*, *plcA*, *plcB*), were identified for all bacterial isolates being ST20, ST29, ST37, ST91, ST101, and ST394. These strains may therefore be virulent and a severe risk to humans. However, because expression of the virulence genes are regulated by the PrfA transcriptional activator (Scortti et al., 2018), presence of the *prfA* gene (encoding PrfA (Selling et al., 2010)) should be examined in a further study. If *prfA* is not present, the isolates are most likely not virulent.

The *actA*-gene was not identified for for the sequence types 31, 325 and 391. The *actA* gene encodes the protein ActA, which constitutes one of the major virulence factors of *L. monocytogenes* (Darji et al., 1998). It is required for intracellular motility and cell-to-cell spread (Portnoy et al., 2002). Thus, these strains may not be pathogenic.

The *inlA* gene encodes a surface protein (InlA) mediating bacterial entrance in host cells (Phelps et al., 2018). Mutations in *inlA* can lead to premature stop codons, resulting in a truncated gene and possible lower pathogenesis. Premature stop codons have been identified for a high number of *L. monocytogenes* isolates from food (Van Stelten et al., 2010). A full length *inlA* profile were identified among all bacterial isolates in this study. Therefore, it is a possibility of pathogenesis (specially for ST20, 29, 37, 91, 101, 394), and the isolates constitutes a severe risk for humans if food products are contaminated.

## 4.2 COLD TOLERANCE PROFILING

*Listeria monocytogenes* can survive and grow at temperatures from 0 °C-45 °C (Table 1.2) (Lungu et al., 2009). When temperature decreases, the bacterial cell is exposed to cold stress, and stress response (stabilizing of macro-molecules, uptake of compatible solutes, and membrane fluidity maintenance) (Wemekamp-Kamphuis et al., 2004)) is activated (Wiktorczyk-Kapischke et al., 2021).

Bacterial growth at 12 °C was examined for the cold tolerance analysis of 35 *L. monocytogenes* isolates. This temperature was selected for the analysis because it is the temperature found in the slaughter house of the salmon value chain. The optimal growth temperature for *L. monocytogenes* is 30 °C-37 °C (Olaimat et al., 2018). Bacterial growth at 37 °C was also examined as a control temperature on optimal growth. It was expected to observe bacterial growth at both cold (12 °C) and optimal (37 °C) growth temperature, but with a decrease

in growth rate at 12 °C compared to the optimal temperature. Maximum growth rate ($\mu_{max}$) was found for each *L. monocytogenes* isolate by fitting growth curves using the Baranyi and Robert model.

For bacterial growth at 12 °C, the average growth curve for the *L. monocytogenes* isolates showed lag phase from approximately 0-48 hours, growth phase from 48-96 hours, and stationary phase from 96-240 hours (Figure 3.6). The individual growth curves for the 35 *L. monocytogenes* isolates showed similar growth trends in general. Growth was observed for all bacterial isolates at 12 °C, as expected. The food processing environment is therefore favourable for *L. monocytogenes* growth, which can lead to established bacterial strains in the value chain (Fagerlund A. et al., 2016).

For optimal growth temperature (37 °C), the average growth curve for the *L. monocytogenes* isolates showed no lag period, but growth phase from approximately 0-24 hours, and a decrease in the bacterial cell concentration from 24-240 hours was shown (Figure 3.7). The decreased cell concentration indicates that the bacterial cells finally entered death phase, where the microbes can no longer replicate due to lack of nutrients and/or occurrence of cellular damage (Brown, 2022). Similar growth trends between the individual bacterial growth curves were in general observed at 37 °C. The bacterial concentration of one *L. monocytogenes* isolate (209) was extreme (high) at 96 hours compared to the measured concentrations of the remaining *L. monocytogenes* isolates, but there is a possibility that it is due to contamination of the bacterial suspension tube.

By comparing the growth curves for *L. monocytogenes* growth at 12 °C and at 37 °C, an extended lag phase was observed for bacterial growth at cold temperature compared to optimal temperature. Adaption to physical stress in the surroundings is associated with an extended lag phase because the bacteria needs to adapt to the changes before growth can be promoted (Beales, 2004).

The bacterial cell concentrations were in general high for both cold and optimal temperature. The maximum $\log_{10}$CFU/mL-value for growth at 37 °C was approximately 15, compared to approximately 13.5 at 12 °C. Prior to the cold tolerance experiment, a bacterial suspension for each *L. monocytogenes* isolate was prepared and the cell concentration of the suspension was standardized to $1.5 \cdot 10^8$ CFU/mL by turbidity measurements with a McFarland 0.5 standard as reference. The cell suspension was then diluted to $1.5 \cdot 10^3$ CFU/mL, and this was the start concentration for the cold tolerance experiment. This bacterial suspension ($1.5 \cdot 10^3$ CFU/mL) was plated, incubated at 37 °C for 22-24 hours, and the colonies were counted. At 0 hours, the $\log_{10}$CFU/mL-value of the growth curves (found by colony counting) was approximately 7, which corresponds to a bacterial concentration of $10^7$ CFU/mL. Thus, the bacterial concentration in suspension found by colony counting did not correspond to $1.5 \cdot 10^3$ CFU/mL.

Possible explanations may be that the shelf life of the McFarland 0.5 standard was shorter than six months, and therefore the standard did not correspond to $1.5 \cdot 10^8$ CFU/mL. Another possible error source is that the dilution series were performed manually, increasing the chance of errors. Inaccurate pipetting (volume errors, bacterial contamination from the pipette tip) or contamination from the surroundings are some possible explanations.

Stress tolerance of the *L. monocytogenes* isolates were determined based on $\mu_{max}$. Cold tolerance was observed for six bacterial isolates, but the majority of *L. monocytogenes* isolates were intermediate tolerant (n=29). Non was cold sensitive (Figure 3.8). The tolerant isolates originated from feed (n=5) and fish in box (n=1), and included the sequence types 20 (n=3), 31 (n=2), and 37 (n=1). The spread of cold tolerance among the bacterial isolates were small, and there is a possibility that a lower temperature should have been examined to obtain more information of the differences in cold tolerance among the bacterial isolates.

More growth data should have been obtained for a more solid conclusion on the cold tolerance distribution among the *L. monocytogenes* isolates. A higher number of OD-measurements during growth would have resulted in more accurate growth curves for the *L. monocytogenes* isolates. The $\mu_{max}$-values were in general lower for bacterial growth at 37 °C compared to 12 °C (Appendix C). $\mu_{max}$ was even negative for some *L. monocytogenes* isolates for growth at 37 °C. This is not expected because 37 °C is the optimal growth temperature for *L. monocytogenes*, and the bacterial growth curves indicate that the slope of the average growth curve during growth phase is higher at 37 °C than at 12 °C. A possible explanation for why $\mu_{max}$-values for the *L. monocytogenes* isolates are higher at 12 °C may be due to few OD-measurements during growth phase. The decrease in bacterial cell density due to entrance of death phase at 37 °C also influence the fit of the growth curves by the Baranyi and Robert model. It is therefore possible that another model than Baranyi and Robert could have been a better fit to estimate $\mu_{max}$. As a result, a solid conclusion on the tolerance distribution cannot be drawn, and the cold tolerance experiment should be repeated in future studies.

## 4.3 SALT TOLERANCE AND ACID TOLERANCE PROFILING

*L. monocytogenes* has been reported to grow and/or survive in salt concentration from 0-20% (Olaimat et al., 2018), and at pH 4.1-9.6 (Lungu et al., 2009) (Table 1.2). Salt stress response in *L. monocytogenes* involve uptake of compatible solutes by compatible solute transporters (Wiktorczyk-Kapischke et al., 2021), and survival in acid stress are conferred by several mechanisms elevating internal pH of the cell (Cotter et al., 2001).

Salt tolerance and acid tolerance of 35 *L. monocytogenes* isolates were examined in this study by growing the bacterial isolates in elevated salt concentration (5.4% NaCl), and at lower pH (5.05) than optimum. Bacterial growth was monitored by measuring $OD_{600}$ hourly for 24 hours. Medium without addition of salt or HCl was used as a reference.

The average growth curve for *L. monocytogenes* isolates in salt stress (5.4% NaCl) showed that growth rate for the bacterial isolates decreased compared to bacterial growth in stress-free conditions (Figure 3.9). The average growth curve for the *L. monocytogenes* isolates in stress-free conditions showed growth phase from approximately 8-13 hours, and stationary phase from approximately 13-24 hours. In salt stress, the growth phase was observed from approximately 8 hours, and the bacterial isolates were still growing at 24 hours.

Growth was observed for all individual *L. monocytogenes* isolates in salt stress (5.4% NaCl) (Figure 3.10). Fold decrease was calculated for all bacterial isolates, comparing the maximum growth rate in salt stress to $\mu_{max}$ in stress-free conditions (Figure 3.11). For each *L. monocytogenes* isolate, it was demonstrated that $\mu_{max}$ decreased in salt stress relative to $\mu_{max}$ in stress-free conditions. The average fold decrease factor for all bacterial isolates grown in salt stress was 2.615, meaning that $\mu_{max}$ in salt stress in general was more than halved relative to $\mu_{max}$ in stress-free conditions. However, no ST showed higher fold decrease trend relative to the other sequence types. By examining bacterial growth at a higher salt concentration than 5.4%, a larger extent of salt tolerance variations could have been identified for the *L. monocytogenes* isolates.

*L. monocytogenes* has to encounter acid stress in the gastrointestinal tract and phagocytic vacoule during the infection cycle, and therefore is survival and adaption to acid cruical for pathogenicity. Observed differences in acid stress tolerance between the bacterial isolates could therefore indicate differences in host pathogenicity, and thus link acid tolerance to virulence property (Muchaamba et al., 2019). The average growth curve for *L. monocytogenes* isolates in acid stress (pH 5.05) showed that growth rate for the bacterial isolates decreased compared to bacterial growth in stress-free conditions. At pH 5.05, growth phase was observed from approximately 8 hours, and growth was still detected at 24 hours. However, the slope of the average bacterial growth curve for acid stress showed a decreasing trend at 24 hours. This suggests that stationary phase for the bacterial isolates could have been entered within the next hour(s) in acid stress.

Growth was observed for all individual *L. monocytogenes* isolates in acid stress (Figure 3.12). Fold decrease of $\mu_{max}$ for each bacterial isolate grown in pH 5.05 relative to $\mu_{max}$ for bacterial growth in stress-free conditions was also calculated for acid tolerance profiling. It was demonstrated that $\mu_{max}$ decreased for the bacterial isolates

growing in acid stress compared to growing in stress-free conditions. The average fold decrease factor for all bacterial isolates was 2.428. This means that the maximum growth rate for the *L. monocytogenes* isolates was more than halved in acid stress compared to stress-free conditions. ST20 showed a trend of high fold decrease. However, if stress tolerance was examined at a lower pH value, more variations in acid tolerance of the bacterial isolates could have been obtained.

Hingston et al. (2017) examined stress tolerance of 166 *L. monocytogenes* from food processing environments and humans in Canada and Switzerland, and measured bacterial growth in 6% NaCl, and at pH 5 every 30 min until all isolates reached stationary phase (approximately 26 hours). They detected both tolerance and sensitivity in both salt and acid, and detected variations in tolerance profiles among the 166 *L. monocytogenes* isolates. After 24 hours, the 35 *L. monocytogenes* isolates examined in this master's study were still growing in both salt and acid. Thus, the growth should have been monitored for a longer time period according to Hingston et al. (2017). It was not performed in this study due to limits in availability of instruments. Measurements were obtained hourly instead of every 30 min due to time limits (measurements were done manually).

A more solid conclusion on salt tolerance and acid tolerance of the *L. monocytogenes* isolates could have been drawn if broader sets of growth curves were obtained for the isolates. The $\mu_{max}$ values might have been higher for the bacterial isolates grown in salt stress and in acid stress if more growth phase data were obtained, especially for bacterial growth in salt stress. The average curve for *L. monocytogenes* growth in salt stress indicates that bacterial cell concentration was still increasing at 24 hours, as already described (Figure 3.9).

## 4.4 CONSIDERATIONS AROUND STATISTICAL ANALYSIS FOR STRESS TOLERANCE PROFILING

One-way ANOVA and Tukey post hoc test with Sig. 0.05 were used to determine if there were significant differences between $\mu_{max}$ of the *Listeria monocytogenes* strains for growth in stress conditions (cold, salt, acid). The sequence types represented by only one bacterial isolate (n<2; ST29, 101, 391) were excluded because basing statistics on one group in a variable will not give any variance, and a post hoc test cannot be performed for these variables. Another important note is that the variables did not have the same amount of groups. For the statistical analysis, a higher amount of groups in each variable, and balanced measurements (same amount of groups) would have resulted in a more steady conclusion.

It was found that the differences between $\mu_{max}$ of the sequence types (with n>1) were not significant for the cold tolerance experiment, but it was significant for the salt and acid tolerance experiment

(Appendix D). This means that the sequence types cannot be distinguished based on $\mu_{max}$ for growth at 12 °C, but $\mu_{max}$ of the ST can be distinguished for growth in 5.4% NaCl and at pH 5.05.

The Tukey post hoc test were performed to reveal which ST that could be distinguished based on their $\mu_{max}$. The post hoc test did not reveal anything for the cold tolerance experiment because significant differences between the sequence types were not found.

The Tukey post hoc tests for the salt tolerance examination of the 35 *L. monocytogenes* isolates revealed that $\mu_{max}$ for ST31 was significantly higher than $\mu_{max}$ for ST91. The $\mu_{max}$ for the bacterial isolates excluded from the statistical analysis (ST101: 106, ST391: 107, ST29: 112) were in general low relative to $\mu_{max}$ of the ST included in the statistical analysis, for the salt tolerance experiment. $\mu_{max}$ of ST29 (*L. monocytogenes* isolate 112) and $\mu_{max}$ of ST391 (*L. monocytogenes* isolate 107) were lower than $\mu_{max}$ of ST91 (which had the lowest growth rate of the ST included in the statistical analysis). It could therefore be interesting to examine a higher number of isolates of ST29 and ST391 to reveal if the growth rates of these sequence types could possible be significantly different from some of the other ST, and possible salt sensitive.

For bacterial growth in acid stress, the Tukey post hoc test revealed that $\mu_{max}$ for ST91 were significantly higher than $\mu_{max}$ for ST20, ST31, ST37 and ST325 at pH 5.05. $\mu_{max}$ for ST394 was significantly higher than $\mu_{max}$ for ST20. The lowest average growth rate was observed for ST20 ($\mu_{max}$=0.0835 $h^{-1}$), and the highest $\mu_{max}$ for ST91 ($\mu_{max}$=0.1214 $h^{-1}$). ST101, which was excluded from the statistical analysis, showed a low growth rate at pH 5.05, and it could also be interesting to examine a higher number of bacterial isolates from this strain.

## 4.5 COMBINATION OF STRESS FACTORS

Stress tolerance profiling of 35 *Listeria monocytogenes* was performed, and included growth examination of cold stress (12 °C), salt stress (5.4% NaCl), and acid stress (pH 5.05).

In food preservation of almost all foods, several preservative methods are used in combination. This is called hurdle technology, and has become more prevalent now because the principles and interactions of major preservative factors is better known (Singh & Shalini, 2016). Smart hurdle technology are topical for minimizing processing of foods (2016), and among the most important hurdles used in food preservation are temperature (cold), water activity (salt), and acidity found. Koutsoumanis and Sofos (2005) modelled growth response of *L. monocytogenes* as function of temperature, pH, and $a_w$, and showed that combined use of hurdles have an effect on growth of the pathogen. It is indicated that combination of stress factors are necessary for food safety, and stress tolerance profiling of *L. monocy-*

*togenes* should therefore include the stress factors cold, salt and acid in combination in a further study. Additionally, different levels of stress should be included in a further study to obtain a more detailed stress tolerance profiles, and variations in stress tolerance among the *L. monocytogenes* isolates. The different levels of stress should include different temperatures, different salt concentrations and different levels of acidity.

# 5 CONCLUSION

The aim of this study was to genetically and phenotypically characterize *Listeria monocytogenes* isolates originating from the salmon processing value chain in order to differentiate between the isolates and possibly identify the source of a contamination in the processing plant.

MLST-profiling revealed nine sequence types among the bacterial isolates (ST20,ST29, ST31, ST37, ST91, ST101, ST325, ST391, ST394), and a phylogenetic tree clustered the isolates in groups in perfect accordance with the sequence types. Dominant sequence types were ST20, ST37, and ST394. Based on these results one might suggest that the feed part of this chain can be a possible contamination source of *L. monocytogenes* in fish (living and dead), and fish in box.

The *fosX* gene conferring fosfomycin resistance was carried by all bacterial isolates. Additionally, all isolates being ST31 carried the heat resistance gene, *ClpL*.

A high number of virulence genes was identified. The important virulence genes for listeriosis development, *hly*, *inlB*, *plcA*, *plcB*, and full length-profile of *inlA* were identified for all bacterial strains. The *actA* gene was identified for ST20,ST29, ST37, ST91, ST101, ST394. These bacterial strains constitutes a severe risk for humans if food products are contaminated.

At 12 °C, cold tolerance was detected for six *L. monocytogenes* isolates from the feed and slaughter section (ST20, ST31, and ST37). Salt tolerance or acid tolerance was not detected for any bacterial isolates, but the maximum growth rate decreased in stress-conditions compared to stress-free conditions. Maximum growth rate was significantly higher for ST31 compared to ST91 in 5.4% NaCl, and it was significantly higher for ST91 compared to ST20, ST31, ST37, and ST325 in pH 5.05. Additionally, $\mu_{max}$ was significantly higher for ST394 than for ST20. Further studies are necessary to draw a more solid conclusion on the stress tolerance profiles of the *L. monocytogenes* isolates.

# 6 LIMITS AND FURTHER WORK

*Listeria monocytogenes* is a challenge in the food industry, especially for RTE foods. Genetic characterization of pathogens is important to track possible contamination sources in food value chains. The pathogen can grow in a wide range of environmental conditions, and it is crucial to monitor stress tolerance to ensure food safety.

This study provides an overview of *L. monocytogenes* strains present at several points of the salmon value chain in Norway during a time period of eight months. Further samplings over a longer time period should be performed to reveal persistent strains, and to draw a solid conclusion on contamination sources. Further samplings should also include salmon intestines.

Identification of selected important virulence genes for pathogenesis was provided in this study. Further studies should examine presence of the PrfA transcriptional activator to characterize pathogenesis of the bacterial isolates. This regulator activates important virulence genes upon host infection.

The stress tolerance examination performed in this study provides a basis for further investigations on cold, salt, and acid tolerance. Growth should be measured within shorter time intervals for cold tolerance examination, and for a longer time periods (to provide a full growth curve) for the salt and acid tolerance experiment. Additionally, different temperatures, salt concentrations, and acidity levels should be examined to reveal tolerance and sensitivity limits of the bacterial isolates. Different stress conditions should also be examined in combination. The *ClpL* gene conferring heat resistance was revealed for ST31 isolates, and heat tolerance should also be included in further stress tolerance examinations.

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# OVERVIEW OF BACTERIAL ISOLATES ORIGIN AND RESULTS OF WGS

A

Table A.1 presents point and date of origin, and sequence type (ST) for all 35 *Listeria monocytogenes* examined in this study. Date of origin could not be found for the isolates 104, 211, and 212, but the isolates were sampled at some point during 2020-2021 according to the TRACEListeria project.

Table A.1: Overview of point and date of origin, and sequence types (ST) for the 35 *L. monocytogenes* isolates examined in this study. Date of origin could not be found for the isolates 104, 211, and 212. [1]Environment and swamp samples.

| Isolate ID | Point of origin | Date of origin [mm.dd.yy] | ST |
|---|---|---|---|
| 101 | Feed | 10.08.20 | 31 |
| 103 | Feed | 10.08.20 | 20 |
| 104 | Feed | - | 325 |
| 106 | Feed | 03.05.21 | 101 |
| 107 | Feed | 02.02.21 | 391 |
| 108 | Feed | 02.02.21 | 37 |
| 109 | Feed | 10.21.20 | 325 |
| 110 | Feed | 03.04.21 | 37 |
| 111 | Feed | 10.08.20 | 20 |
| 112 | E&s [1] | 02.22.21 | 29 |
| 201 | E&s[1] | 12.18.20 | 31 |
| 203 | E&s[1] | 10.08.20 | 20 |
| 204 | E&s[1] | 11.06.20 | 20 |
| 205 | E&s[1] | 10.08.20 | 31 |
| 206 | E&s[1] | 12.14.20 | 37 |
| 207 | E&s[1] | 12.14.20 | 37 |
| 208 | E&s[1] | 12.14.20 | 20 |
| 209 | E&s[1] | 12.18.20 | 20 |
| 211 | E&s[1] | - | 31 |
| 212 | E&s[1] | - | 31 |

| Isolate ID | Point of origin | Date of origin [mm.dd.yy] | ST | |
|---|---|---|---|---|
| 303 | Fish in box | 05.18.21 | 37 | |
| 304 | Fish in box | 05.18.21 | 37 | |
| 305 | Living fish | 11.30.20 | 91 | 52.6 |
| 306 | Living fish | 11.30.20 | 91 | 49.9 |
| 307 | Living fish | 11.30.20 | 91 | 84.5 |
| 308 | Living fish | 11.30.20 | 91 | 49.4 |
| 309 | Living fish | 12.01.20 | 394 | 54.3 |
| 310 | Living fish | 12.01.20 | 394 | 54.3 |
| 311 | Dead fish | 11.30.20 | 394 | 48.4 |
| 312 | Dead fish | 12.01.20 | 394 | 132.4 |
| 401 | Dead fish | 12.01.20 | 394 | 83.7 |
| 402 | Dead fish | 11.30.20 | 394 | 51.7 |
| 403 | Dead fish | 11.30.20 | 20 | 87.4 |
| 404 | Dead fish | 11.30.20 | 20 | 127 |
| 405 | Dead fish | 11.30.20 | 20 | 50.8 |

# STANDARD CURVES FOR COLD TOLERANCE PROFILING

# B

Figure B.1 presents the standard curves (12 °C and 37 °C) constructed for cold tolerance profiling of 35 *L. monocytogenes* isolates in this study.

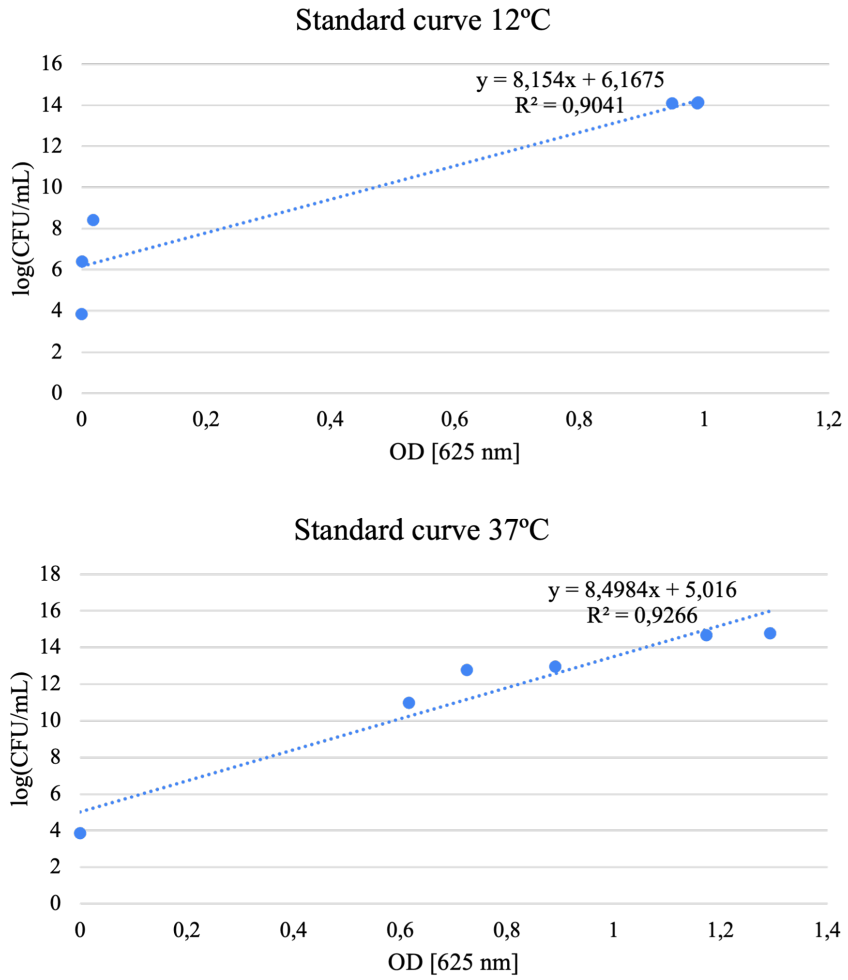


Figure B.1: Standard curves (12 °C and 37 °C) for cold tolerance profiling of 35 *L. monocytogenes* isolates. The curves were constructed to assume concentration (CFU/mL) from $OD_{625}$ by interpolation.

# MAXIMUM GROWTH RATES FOR STRESS TOLERANCE PROFILING

C

Table C.1 presents the maximum growth rates ($\mu_{max}$) for 35 *L. monocytogenes* isolates for stress tolerance profiling of the bacterial isolates. $\mu_{max}$ is presented for bacterial growth in cold stress (12 °C), optimal growth temperature (37 °C), salt stress (5.4% NaCl), acid stress (pH 5.05), and in media without cold, salt, or acid (stress-free).

Table C.1: Overview of maximum growth rate ($\mu_{max}$) of 35 *L. monocytogenes for stress tolerance profiling*. isolates $\mu_{max}$ is presented for bacterial growth in cold stress (12 °C), optimal growth temperature (opt. T; 37 °C), salt stress (5.4% NaCl), acid stress (pH 5.05), and stress-free conditions.

| Isolate | $\mu_{max}$ [$h^{-1}$] | | | | |
|---|---|---|---|---|---|
| ID | 12 °C | 37 °C | 5.4% NaCl | pH=5.05 | Stress-free |
| 101 | 0.0362 | 0.0141 | 0.1019 | 0.1038 | 0.2535 |
| 103 | 0.0338 | 0.0133 | 0.0956 | 0.0826 | 0.2438 |
| 104 | 0.0326 | -0.0047 | 0.0954 | 0.1143 | 0.2222 |
| 106 | 0.0499 | 0.0.2640 | 0.0866 | 0.0763 | 0.2272 |
| 107 | 0.0551 | 0.0035 | 0.0749 | 0.1072 | 0.2451 |
| 108 | 0.0551 | -0.006 | 0.0954 | 0.1028 | 0.2127 |
| 109 | 0.0410 | 0.0054 | 0.0889 | 0.0898 | 0.2340 |
| 110 | 0.0611 | -0.0255 | 0.0869 | 0.0967 | 0.2388 |
| 111 | 0.1250 | -0.0198 | 0.0925 | 0.0711 | 0.2516 |
| 112 | 0.0421 | -0.0167 | 0.0739 | 0.1002 | 0.2304 |
| 201 | 0.1280 | 0.2810 | 0.0953 | 0.0957 | 0.2278 |
| 203 | 13.651 | 0.1300 | 0.0923 | 0.0857 | 0.2206 |
| 204 | 0.0908 | 0.0024 | 0.0879 | 0.0774 | 0.2339 |
| 205 | 0.0758 | -0.0097 | 0.1045 | 0.0846 | 0.2339 |
| 206 | 0.0759 | 0.0088 | 0.0908 | 0.0987 | 0.2633 |
| 207 | 0.0768 | 0.0094 | 0.0899 | 0.0944 | 0.2425 |
| 208 | 12.843 | -0.0205 | 0.0883 | 0.0842 | 0.2305 |
| 209 | 12.759 | 9.8210 | 0.0968 | 0.0789 | 0.2317 |
| 211 | 14.234 | -0.0203 | 0.0940 | 0.0894 | 0.2301 |
| 212 | 13.311 | -0.0079 | 0.0985 | 0.1041 | 0.2400 |

| Isolate | $\mu_{max}$ [$h^{-1}$] | | | | |
|---|---|---|---|---|---|
| ID | 12 °C | 37 °C | 5.4% NaCl | pH=5.05 | Stress-free |
| 303 | 13.302 | -0.0129 | 0.0832 | 0.0952 | 0.2250 |
| 304 | 0.0723 | -0.0044 | 0.1203 | 0.1099 | 0.2324 |
| 305 | 0.0787 | -0.0393 | 0.0801 | 0.1229 | 0.2354 |
| 306 | 0.0790 | -0.0384 | 0.0822 | 0.1120 | 0.2093 |
| 307 | 0.1860 | 0.0082 | 0.0818 | 0.1246 | 0.2117 |
| 308 | 0.0805 | 0.0001 | 0.0886 | 0.1259 | 0.2195 |
| 309 | 0.0770 | -0.0674 | 0.0846 | 0.1198 | 0.2300 |
| 310 | 0.0785 | -0.0072 | 0.0890 | 0.1125 | 0.2263 |
| 311 | 0.0768 | -0.0230 | 0.0894 | 0.1107 | 0.2355 |
| 312 | 0.0734 | -0.0227 | 0.0931 | 0.0900 | 0.2408 |
| 401 | 0.0669 | -0.0236 | 0.0854 | 0.1018 | 0.2262 |
| 402 | 0.0900 | 0.0021 | 0.0884 | 0.0996 | 0.2382 |
| 403 | 0.0813 | -0.0401 | 0.0831 | 0.0821 | 0.2398 |
| 404 | 0.0793 | -0.0215 | 0.0763 | 0.0846 | 0.2321 |
| 405 | 0.0775 | -0.0354 | 0.084 | 0.1050 | 0.2238 |

# STATISTICAL ANALYSIS: RESULTS FROM SPSS

D

Figure D.1, Figure D.2, and Figure D.3 present results of statistical analysis to examine differences in $\mu_{max}$ between sequence types for stress tolerance profiling of 35 *L. monocytogenes*. The analysis was performed in SPSS Statistics, and the figures are adapted from this software.

**Tests of Between-Subjects Effects**

Dependent Variable: μ_max

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 150.200[a] | 5 | 30.040 | 1.100 | .384 |
| Intercept | 111.451 | 1 | 111.451 | 4.081 | .054 |
| ST | 150.200 | 5 | 30.040 | 1.100 | .384 |
| Error | 710.013 | 26 | 27.308 | | |
| Total | 1071.000 | 32 | | | |
| Corrected Total | 860.212 | 31 | | | |

a. R Squared = .175 (Adjusted R Squared = .016)

**Homogeneous Subsets**

μ_max

Tukey HSD[a,b,c]

| ST | N | Subset 1 |
|---|---|---|
| 325.00 | 2 | .0368 |
| 394.00 | 6 | .0771 |
| 91.00 | 4 | .1061 |
| 37.00 | 6 | 2.2739 |
| 20.00 | 9 | 4.4156 |
| 31.00 | 5 | 5.5570 |
| Sig. | | .637 |

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 27.308.

a. Uses Harmonic Mean Sample Size = 4.303.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = 0.05.

Figure D.1: Results of one-way ANOVA (left table) and Tukey post hoc test (right table) for the cold tolerance examination of 35 *L. monocytogenes* isolates. Maximum growth rate ($\mu_{max}$) was set as dependent variable, and sequence types (ST) as independent variable. ST with n<2 (ST29, ST101, ST391) were excluded from the statistical analysis. P-value is 0.384, and there are not significant differences between the sequence types.

**Tests of Between-Subjects Effects**

Dependent Variable: μ_max

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | .001[a] | 5 | .000 | 2.592 | .050 |
| Intercept | .213 | 1 | .213 | 3870.843 | <.001 |
| ST | .001 | 5 | .000 | 2.592 | .050 |
| Error | .001 | 26 | 5.508E-5 | | |
| Total | .266 | 32 | | | |
| Corrected Total | .002 | 31 | | | |

a. R Squared = .333 (Adjusted R Squared = .204)

**μ_max**

Tukey HSD[a,b,c]

| ST | N | Subset 1 | Subset 2 |
|---|---|---|---|
| 91.00 | 4 | .0832 | |
| 394.00 | 6 | .0883 | .0883 |
| 20.00 | 9 | .0885 | .0885 |
| 325.00 | 2 | .0922 | .0922 |
| 37.00 | 6 | .0943 | .0943 |
| 31.00 | 5 | | .0988 |
| Sig. | | .277 | .328 |

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 5.508E-5.

a. Uses Harmonic Mean Sample Size = 4.303.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = 0.05.

Figure D.2: Results of one-way ANOVA (left table) and Tukey post hoc test (right table) for salt tolerance examination 35 *L. monocytogenes* isolates. Maximum growth rate ($\mu_{max}$) was set as dependent variable, and sequence types (ST) as independent variable. ST with n<2 (ST29, ST101, ST391) were excluded from the statistical analysis. P-value is 0.05, and there are significant differences between the sequence types. The post hoc test shows that $\mu_{max}$ for ST31 is significantly higher than $\mu_{max}$ for ST91.

**Tests of Between-Subjects Effects**

Dependent Variable: μ_max

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | .005[a] | 5 | .001 | 10.914 | <.001 |
| Intercept | .265 | 1 | .265 | 3211.421 | <.001 |
| ST | .005 | 5 | .001 | 10.914 | <.001 |
| Error | .002 | 26 | 8.249E-5 | | |
| Total | .317 | 32 | | | |
| Corrected Total | .007 | 31 | | | |

a. R Squared = .677 (Adjusted R Squared = .615)

**μ_max**

Tukey HSD[a,b,c]

| ST | N | Subset 1 | Subset 2 | Subset 3 |
|---|---|---|---|---|
| 20.00 | 9 | .0835 | | |
| 31.00 | 5 | .0955 | .0955 | |
| 37.00 | 6 | .0996 | .0996 | |
| 325.00 | 2 | .1021 | .1021 | |
| 394.00 | 6 | | .1057 | .1057 |
| 91.00 | 4 | | | .1214 |
| Sig. | | .059 | .575 | .154 |

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 8.249E-5.

a. Uses Harmonic Mean Sample Size = 4.303.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = 0.05.

Figure D.3: Results of one-way ANOVA (left table) and Tukey post hoc test (right table) for acid tolerance examination 35 *L. monocytogenes* isolates. Maximum growth rate ($\mu_{max}$) was set as dependent variable, and sequence types (ST) as independent variable. ST with n<2 (ST29, ST101, ST391) were excluded from the statistical analysis. P-value is <0.001, and there are significant differences between the sequence types. The post hoc test shows that $\mu_{max}$ for ST91 is significantly higher than $\mu_{max}$ for ST20, ST31, ST37, ST325. $\mu_{max}$ for ST394 is significantly higher than for ST20.