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Developing methods for detecting virulence and chemical resistance-related genes of *Listeria monocytogenes*

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ABSTRACT

Developing methods for detecting virulence and chemical resistance-related genes of Listeria *monocytogenes*

Listeria *monocytogenes* is a pathogen capable of surviving several commonly used chemical purifying agents, like quaternary ammonium compound-based ones. It provides a challenge for food processing facilities dealing with the *L.monocytogenes* infestation. This thesis provides the theoretical approach to detecting *L.monocytogenes* pathogenicity and chemical tolerance by detecting the genes most often implicated in virulence and chemical resistance through the use of PCR primers. It offers a literature overview of previously discovered information about Listeria and its pathogenicity and chemical tolerance factors and suggests an experiment plan for PCR primer design.

KEY WORDS: Listeria *monocytogenes*; PCR primer design; *L.monocytogenes* virulence; *L.monocytogenes* chemical tolerance

CERCS: B110 Bioinformatics, medical informatics, biomathematics, biometrics

Meetodid *Listeria monocytogenes* puhastuskemikaalide vastast resistentsust ja virulentsust põhjustavate geenide tuvastamiseks

Listeria monocytogenes on patogeen, mis suudab ellu jääda erinevate levinud puhastusreagentide juuresolekul nagu Kvaternaarsed ammooniumkatioonidel põhinevad kemikaalid. See tekitab probleeme toiduainetööstuses tegutsevatele firmadele, kes peavad rinda pistma *Listeria monocytogenes* saastusega. Käesolev bakalaureusetöö annab ülevaate *L.monocytogenes* virulentsuse ja kemikaalide resistentsusega seotud geenidest ning meetoditest nende analüüsiks. Töö eksperimentaalses osas pakutakse välja bioinformaatiliste meetodite tegevuskava, mille tulemusel oleks võimalik PCR abil tuvastada geene, mis võivad olla seotud *L.monocytogenes* resistentsusega puhastuskemikaalide suhtes.

MÄRKSÕNAD: *Listeria monocytogenes*; PCR praimerite disain; *L.monocytogenes* virulentsus; *L.monocytogenes* resistentsus kemikaalidele

CERCS: B110 Bioinformaatika, meditsiiniinformaatika, biomatemaatika, biomeetrika

CONTENTS

LIST OF ABBREVIATIONS

ADBAC - alkyldimethylbenzylammonium chloride

BAC - benzalkonium chloride

DDAC - diallyldimethylammonium chloride

LGR - leucine-rich repeat

MucBP - Mucin-Binding Protein

NCBI - National Center for Biotechnology Information

NGS - Next-generation sequencing

QAC - quaternary ammonium compound

SMR - small multidrug resistance

WGS - Whole-genome sequencing

INTRODUCTION

Listeria monocytogenes is a pathogenic Gram-positive, facultative bacteria belonging to the genus Listeria, that is capable of infecting humans and animals with listeriosis, a disease with particularly high fatality rate. It is mainly consumed through food, like dairy products and fish, through the contamination of food production facilities. *L.monocytogenes* especially is notable for its ability to thrive in extreme circumstances, setting it apart as an extraordinary pathogen through utilizing a set of different regulatory systems. Over the years since *Listeria monocytogenes* discovery in 1927 there have been a lot of breakthroughs regarding the mechanisms with which *L.monocytogenes* infects organisms. WGS further advanced our knowledge about the differences between strains of *L.monocytogenes* that were pathogenic and the ones that were present in foods but didn't cause an infection.

While listeriosis presents itself in a variety of severity of its symptoms, it is especially dangerous for pregnant women, infants, adults aged 65 or older, and people with weakened immune systems. Some *Listeria monocytogenes* have innate resistance to several sanitary measures, causing contamination to persist for possibly years, making detection of the bacteria to prevent future outbreaks and health risks more urgent.

This thesis focuses on developing methods for detecting the strains of *L.monocytogenes* that are virulent and have natural resistance to cleaning chemicals as well as giving an overview of our current knowledge about *Listeria monocytogenes*. It aims to provide a theoretical approach for *L.monocytogenes* strains recognition based on genes most commonly implicated in pathogenicity and QAC tolerance.

First chapter of the literature overview consists of a chapter describing symptoms and characteristics of listeriosis, an illness caused by *L.monocytogenes*, in both animals and humans. Next chapter follows it up with main qualities differentiating the Listeria genus species from each other. Third chapter is a description of *Listeria monocytogenes*, its lineage variety and genetic composition. It also includes information about WGS, as WGS studies have played a major role in discovering the exact genetic composition of *L.monocytogenes*. The chapter closes off with the comparison of *L.monocytogenes* with its closest related species. The fourth and fifth chapters focus on *L.monocytogenes* virulence and chemical tolerance factors, respectively, and genes

implicated in encoding for them. The experimental part of this paper includes an experimental plan with detailed steps to detect pathogenicity and QAC tolerance using PCR analysis.

1. LITERATURE OVERVIEW

1.1 LISTERIOSIS - AN OVERVIEW

Listeriosis is a bacterial infection caused by several strains of Listeria. The main source of the disease and the focus of this thesis is *Listeria monocytogenes*, although *Listeria ivanovii* has been reported to be the cause of the infection in some cases. (Troxler, R et. al, 2000)

Listeriosis can be gravely dangerous, with symptoms ranging from meningitis to sepsis. The bacteria mainly spreads through the consumption of foods among animals and humans alike and the treatments mainly involve antibiotics. However, *L.monocytogenes* is naturally resistant to multiple types of antibiotics and has been documented developing antibiotic resistance, making the prevention of the spread of the bacteria that much more urgent. (Olaimat, A.N. et al., 2018)

1.1.1 Listeriosis in animals:

Listeriosis in animals is characterized by being a disease that is primarily dealt with during winter. Outbreaks are typically caused by spoiled silage, which causes the multiplication of Listeria through its less acidic pH. The change of the feeding successfully prevents the further spread.

Most commonly listeriosis expresses itself in ruminants as inflammation of the brain, rapidly affecting the population and causing death in the first 24-48 hours after first symptoms, unless met with aggressive therapy. The cattle are typically affected less, and are more prone to recovery. The typical number of animals affected is not high, being around 2%, but in some circumstances can reach up to 30% in sheep.

Other forms of listeriosis among animals include abortion and diarrhea, with recent increase among cases with several forms occurring alongside each other in the same flock.

Listeriosis is fairly uncommon in pigs and follows the rapid course of the disease, causing death in three to four days.

As Listeria has been identified in a significant number of feces and milk of apparently healthy animals, it is important to handle materials that are suspected to be infected with the bacteria with

caution. Infected milk can be especially hazardous as the bacteria is capable of surviving some forms of pasteurization. (Scott, P. R., 2014)

1.1.2 Listeriosis in humans:

Listeriosis in humans is a relatively rare illness with high mortality rates of up to 30%. (Mead et al., 1999) It is caused by an opportunistic pathogen *Listeria monocytogenes*. The disease is of especial danger to groups of population with pre-existing vulnerabilities such as hospital patients, pregnant women and the elderly (WHO, 2004), however it can also affect people without risk factors (Hernandez-Milian, 2014). Despite low rates of infection, the involvement of RTE and other industrially produced foods cause listeriosis to be among the most impactful foodborne illnesses. (Van Stelten, A. et al., 2010)

It is caused by an opportunistic pathogen *Listeria monocytogenes*. Listeriosis is mainly diagnosed in industrialized countries and it is not yet clear whether the difference between the rates of the spread of listeriosis represent the geographical, dietetic and food storage differences or if the cause of the differences lies in the rates of diagnosis. (WHO, 2004)

WHO differentiates between invasive and non-invasive listeriosis. Invasive type is characterized by its aggressiveness, having a high mortality rate of up to 30% (Mead et al., 1999), while a non-invasive type causes symptoms of gastroenteritis after a show period of incubation (Aureli et al., 2000). Non-invasive outbreaks are typically caused by consumption of high doses of *L.*m*onocytogenes* by otherwise healthy individuals. (WHO, 2004)

1.2 LISTERIA GENUS

1.2.1 Listeria genus: Characteristics

Listeria is a genus mainly known for its close association with listeriosis and pathogenicity to mammals. Currently 17 species of Listeria are known, with 9 of them being described in 2009. (Orsi & Wiedmann, 2016)

Common characteristics of Listeria include: rod-like shape, gram-positivity, ability to grow at temperatures as low as 4 °C, motility, positive catalase reaction, inability to reduce nitrate to nitrite, positive reaction in the Voges-Proskauer test, ability to ferment D-arabitol, α-methyl D-glucoside, cellobiose, D-fructose, D-mannose, N-acetylglucosamine, maltose, and lactose, and inability to ferment inositol, L-arabinose, and D-mannitol. (Orsi & Wiedmann, 2016)

Pathogenicity is actually not that frequent among Listeria genus, being only found in 2 out of 17 species, as well as a few strains of *L.innocua*. (Troxler, R et. al, 2000)

1.2.2 Listeria genus: Species

Listeria genus includes 18 species (Figure 1), taking into account the *Listeria goaensis* that was discovered in 2018. (Doijad, S. P., et al. , 2018)

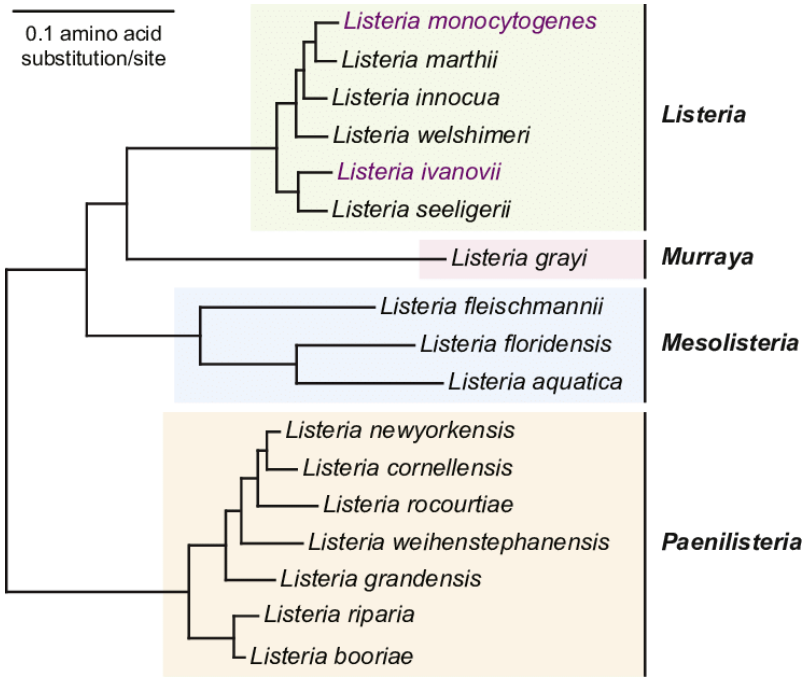

***Figure 1**: "Phylogenomic tree of Listeria species. Listeria monocytogenes and Listeria ivanovii are highlighted in purple letters (by pathogenicity)." (Lebreton, A. et al., 2016)*

Main subgroups that can be defined are *Listeria sensu strictu* and *Listeria sensu lato*, that, in turn, can be divided into three separate monophyletic groups. *Listeria sensu strictu* includes species such as *L.*m*onocytogenes*, *L.seeligeri*, *L.ivanovii*, *L.welshimeri*, and *L.innocua* and is characterized by ability to grow at temperatures as low as 4 °C, motility, positive catalase reaction, inability to reduce nitrate to nitrite, positive reaction in the Voges-Proskauer test, ability to ferment D-arabitol, α-methyl D-glucoside, cellobiose, D-fructose, D-mannose, N-acetylglucosamine, maltose, and lactose, and inability to ferment inositol, L-arabinose, and D-mannitol. (Orsi & Wiedmann, 2016) *Listeria sensu lato*, on the other hand, includes *L.marthii*, *L.rocourtiae*, *L.weihenstephanensis*, *L.grandensis*, *L.riparia*, *L.booriae*, *L.fleischmannii*, *L.floridensis*, *L.aquatica*, *L.newyorkensis*, and *L.cornellensis* and is characterized by non-virulence, non-motility (except for *Listeria grayi*), ability to reduce nitrate (except for *Listeria floridensis*), and the negative result for the Voges-Proskauer test (except for *L.grayi*). Unlike all other Listeria species, species in *Listeria sensu lato* are not able to grow below 7 °C. (Orsi & Wiedmann, 2016)

As the focus of this thesis is directed towards *Listeria monocytogenes* species, it's virulence is the one we will explore in this paper.

1.3 LISTERIA *MONOCYTOGENES*

Listeria monocytogenes (Figure 2) is an opportunistic pathogen found in RTE and animal foods with the third highest mortality rate among all bacterial foodborne pathogens in the US. (CDC, 2013) While relatively uncommon in the EU, the number of cases has been rapidly growing, causing a reason for more vigilant control of the food production process (Pontello, M. et al., 2012). *L.*m*onocytogenes* can be found in the environment, like soils and feces, and the bacteria has the natural ability to persist in food processing facilities for months and possibly years, despite the sanitation measures. (Stoller, A. et al., 2019)

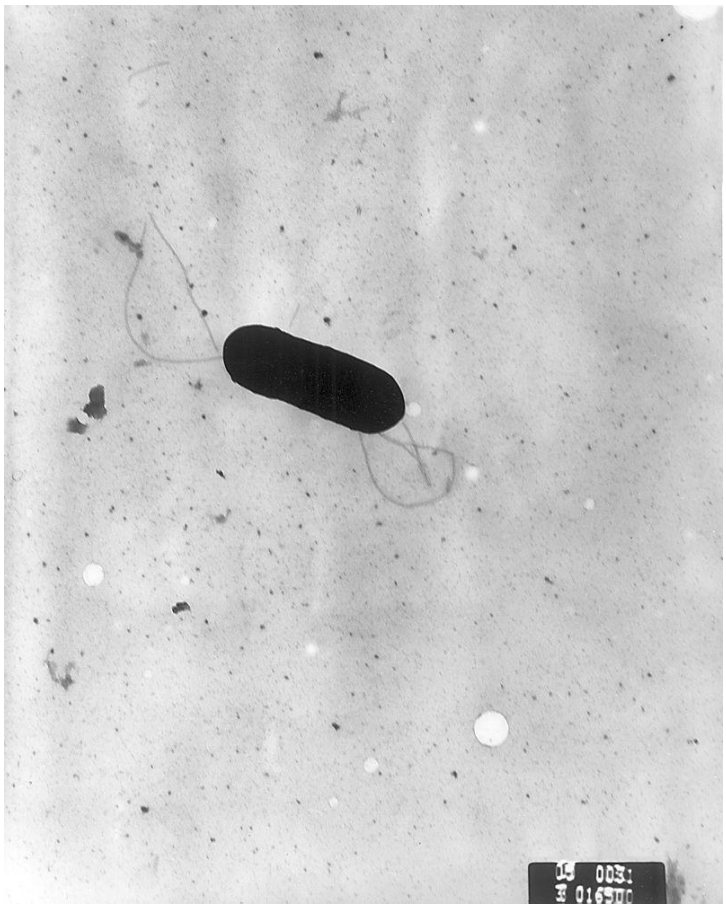

Figure 2*: Electron micrograph of a flagellated Listeria monocytogenes bacterium, Magnified 41,250X. This media comes from the Centers for Disease Control and Prevention's Public Health Image Library (PHIL), with identification number #2287.*

Typically, *L.monocytogenes* is divided into 4 main phylogenetic divisions, called **lineages**. (Orsi, R. H., et al., 2011) Lineage I and II are the most commonly observed ones in studying listeria, as serotypes 4b and 1/2b make up the division I and 1/2c with 1/2a make up the division II, commonly found in human clinical cases and animal listeriosis cases, respectively. (Borucki, M. K. et al., 2003) The third and fourth lineages are comparatively rarer and are mainly isolated from animal sources. (Orsi, R. H., et al., 2011)

1.3.1 Lineage variety

As mentioned before, *L.monocytogenes* is divided into 4 highly distinct lineages. (Orsi, R. H., et al., 2011) Each of these lineages is composed of the serotypes, with lineage I being composed of serotypes 1/2b, 3b and 4b, lineage II being composed of serotypes 1/2a, 1/2c and 3a, and lineage

III containing serotypes 4a and 4c, with serotype 7 being undefined due to limited availability of its strains. In this chapter we will go into more detail into what is a distinction between those groups in relation to *L.monocytogenes* characteristics.

Lineage I has been most commonly recognized as being linked to the majority of human listeriosis outbreaks through serotypes 4b and 1/2b. (Jeffers, G., et al., 2001) (Orsi, R. H., et al., 2011) Overall, lineage I strains seem to be much more common among human listeriosis cases in the USA when they are in Northern Europe. (Jeffers, G., et al., 2001) (Lukinmaa, S., et al., 2003) This phenomenon does not seem to be caused by the differences in exposure as lineage I strains seem to be overrepresented in human cases in comparison to their frequency in foods. (Gray et al., 2004)

There also seems to be a connection between the condition of a patient and a serotype of *L.monocytogenes*, with 1/2b isolates being associated with patients with underlying illnesses, while 4b with pregnant patients, as well as 4b being the most common serotype with association to human listeriosis cases regardless of their condition otherwise. (McLauchlin, J., 1990)

Some outbreaks have been associated with lineage II serotype 1/2a, which is the serotype that also appears to be more common in listeriosis cases in Finland and in Sweden. (Lukinmaa, S., et al., 2003) (Parihar, et al., 2008)

Lineage I appears to have a higher pathogenicity overall in comparison to lineage II (Pinner, R., et al., 1992) It seems that a big part of the difference in virulence lies in *inlA* containing premature stop codons in lineage II isolates despite being fully intact in 1/2b and 4b serotypes. (Gaillard, J. L., et al., 1991) All of those factors together lead to a hypothesis that lineage I's increased representation is connected to lineage II showing reduced virulence.

Lineage III and IV are relatively rare, being mostly isolates from soil and other rural habitats rather than foods and food-associated environments (Wiedmann, M., et al., 1997) One of the main differences of lineages I and II from lineages III and IV is a defect or complete absence of *inlJ* gene, which leads to a conclusion that some isolates of III and IV lineages may be lacking pathogenicity in mice, (Liu, D., et al., 2006) however further research is required into virulence of those two lineages.

In terms of formation of biofilms, lineage I is better at their formation in comparison to lineage II, but overall relationship between biofilms and serotypes is hard to pinpoint and leads to contradicting results from different studies. (Djordjevic, D., et al., 2002) (Borucki, M. K., et al., 2003)

1.3.2 Genotypic characteristics of *Listeria monocytogenes*

As previously mentioned, typically the population structure of *L.monocytogenes* is divided into four lineages, and the species is largely clonal in nature. The lineages are well-defined, differing from each other by 56–168 fixed nucleotide differences. Majorities of allelic sequences are lineage-specific, with few exceptions found being possibly related to experimental errors.

Phylogenetic tree of the species shows that the split between lineage 1 and 2 is the most recent one, with concatenated sequences in lineage 2 diversifying before those in lineage 1.

The clonal complexes are more often identified in the lineage 2, and overall lineages 1 and 2 account for 94% of all isolates. Genetic diversity of the species is largely associated with mutation. (Haase, J. K., et al., 2014)

Typically, the *L.monocytogenes* virulence is associated with the regulatory gene cluster *prfA*, and it is believed that *Listeria spp*. have at some point majorly lost the virulence factors rather than *L.monocytogenes* becoming virulent. (Den Bakker, H. C., et al., 2010) Genes that are highly conserved among the *L.monocytogenes* include genes such as *vip*, *plcA*, *plcB*, *bsh* and *ami*. (Tan, M. F., et al., 2015) All of those genes seem to play a role in the species virulence and so will be talked about in more detail in a later chapter.

Internalin proteins InlA, InlB InlC and InlJ are known to be preserved in the majority of lineage 1 and 2 strains and have also been identified as virulence markers (*inlA*, *inlC* and *inlJ*). (Liu, D., et al., 2007) InlA is a listerial surface protein associated with internalization of the intestinal epithelial cells. (Gaillard, J. L., et al., 1991) InlB is required for invasion in the endothelial cells and is not associated with the lineage 4. (Parida, S. K., et al., 1998) (Liu, D., et al., 2007)

While InlA and InlB are well-studied proteins, IntlJ's association with virulence has been discovered recently and it's exact purpose is not yet well known but it's been discovered to act as an adhesin during in vivo infection. (Sabet, C., et al., 2008)

1.3.3 Genome sequencing

WGS is a widely-used technique in the fields of scientific research and medicine. It allows for a quality, high-resolution characterization of pathogens, furthering research in areas of antibiotic resistance and virulence. NGS instrumentalization has allowed WGS to be much more attainable, lowering the costs. (Gautam, S. S., et al., 2019)

Main steps of the technique, also shown on Figure 3, are as follows:

1. DNA extraction.

DNA is extracted from the cells. It is then eluted, treated with RNase and purified.

2. DNA shearing

Purified DNA is cut into short strands of known length by using "molecular scissors" enzymes.

3. DNA library preparation

The DNA library is then prepared for sequencing. A DNA library is the collection of cut DNA fragments, multiplied using the PCR method.

4. DNA library sequencing

The library is put through a sequencer. The nucleotide sequences making up fragments are determined (DNA reads).

5. DNA sequence analysis

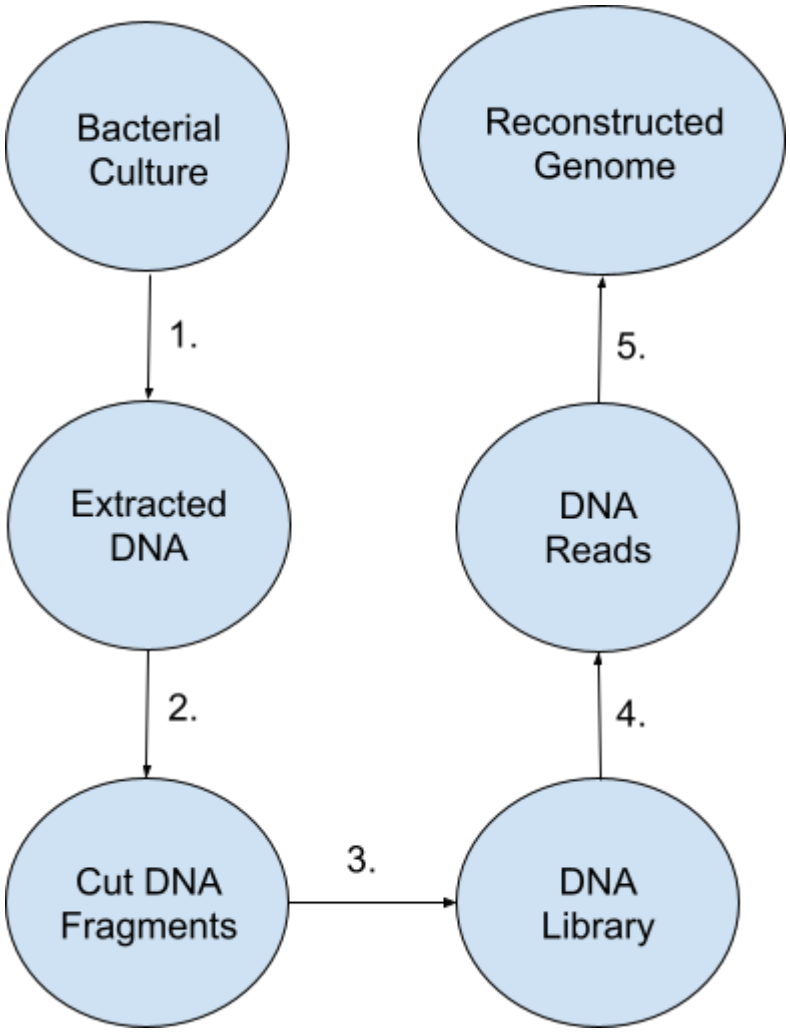

Figure 3*: Step-by step process of WGS process.* 1. DNA is extracted from the culture; 2. DNA is cut into fragments; 3. Fragments are quantified into a DNA library; 4. DNA library is sequenced into DNA reads; 5. DNA reads are put together through computer programs into a genome.

DNA reads are put together through various scripts into DNA sequences. That genome sequence is the final result of the WGS and is available for further analysis.

Studies involving WGS of *L.monocytogenes* have provided a lot of insight into the way this pathogen operates. As of April 2021, NCBI's RefSeq database contains 237 *L.monocytogenes* genomes, furthering our understanding of the genetic composition of the bacteria as well as differences in its natural diversity between strains (retrieved from https://www.ncbi.nlm.nih.gov/genome/browse#!/prokaryotes/159/ (09.04.2021)).

1.3.4 Comparison between *L.monocytogenes* and other Listeria species

L.monocytogenes as a species most notably differs from the majority of other listeria species with its pathogenicity. To analyze the source of its virulence and evolution of the species, it is most commonly compared against *L.innocua*, *L.marthii* and *L.ivanovii*. (Den Bakker, H. C., Bundrant B. N., et al., 2010) (Schardt, J., et al., 2017) (Den Bakker, H. C., Cummings, C. A., et al., 2010)

The main reasons for choosing those specific species is as follows: *L.innocua* is the species most closely related to *L.monocytogenes* (Tan, M. F., et al., 2015), *L.marthii* is closely related to *L.innocua* and can be mischaracterized as such, sharing a lot of biochemical similarities (Orsi, R. H., et al.l, 2016) and *L.ivanovii* is the only other listeria strain that is pathogenic (Bergey, D. H., et al., 1984).

1.3.3.1 *L.marthii* and *L.innocua*

When trying to determine the differences between genomes of *L.innocua* and *L.monocytogenes*, which are closely related but differ in pathogenicity, it is important to understand what mechanism causes the change. The variance between those two species seem to be related to mechanisms of horizontal gene transfer: transmission of plasmids, transposons and phages as well as uptake of DNA. An example of such mechanism occurring would be the plasmid found in *L.innocua* but not in *L.monocytogenes* that is predicted to encode resistance to heavy metals. (Buchrieser, C., et al., 2003)

1.3.3.2 *L.ivanovii*

L.ivanovii is a livestock pathogen that is similar in physiology and habitat to *L.monocytogenes*. The comparison between the two species becomes of higher importance to figure out the difference that causes *L.ivanovii* to be so rare in clinical specimens. (Schmid, M. W., et al., 2005) It is also one of the least commonly isolated Listeria sensu strictu species, with some studies failing to isolate it at all. (Orsi & Wiedmann, 2016)

While *L.ivanovii* mainly infects sheep, it has occasionally been found in bovines and humans. However, in human cases the isolate is usually found in the patient's blood with no clear

transmission route. (Chand, P. & Sadana, J.R., 1999) (Gill, P. A., et al., 1997) (Guillet, C., et al., 2010)

The role of *prfA* in *L.ivanovii* is taken up by the transcription regulator that is highly homologous to it and also belongs to the Crp-Fnr family. (Lampidis, R., et al., 1994)

1.4 VIRULENCE

1.4.1 Difference between *L.monocytogenes* strains in virulence, the most prominently virulent strains

L.monocytogenes virulence factors can be divided in two groups. The first group consists of the six genes that are named together as *prfA* virulence cluster, also known as Listeria pathogenicity island, or LiPI. Those genes are *prfA*, *plcA*, *hly*, *mpl*, *actA*, and *plcB*. The second group of virulence factors consists of the members of the internalin family. (Dramsi, S., et al., 1993) (Dramsi, S., et al., 1996)

L.monocytogenes's virulence is a criteria that greatly varies from strain to strain. The main three strains that are used to identify and analyze the pathogenicity of the bacteria are EGD-e, 10403S and EGD, that had their genomes sequenced in 2001, 2008 and 2014, respectively. (Bécavin, C. et al., 2014)

EGD-e varies greatly from the other two, being more related to the 1/2c than 1/2a strains (Ragon, M., et al. 2008), while EGD and 10403S are genetically very close, despite 10403S strain lacking the point mutation in the transcriptional regulator PrfA, which contributes to the expression of several virulence genes. (Bécavin, C. et al., 2014)

1.4.2 LiPI (*prfA*, *plcA*, *hly*, *mpl*, *actA*, *plcB*)

Main function of the *prfA* involves encoding for the motility and the survival of the bacteria in the host cell. (Den Bakker, H. C., Bundrant B. N., et al., 2010) *PrfA* cluster genes require careful regulation by a variety of mechanisms and are tightly controlled. (Johansson, J., & Freitag, N.E., 2019) Temperature appears to play an important role in activation of the mRNA translation of the cluster. (Aguilar-Bultet, L., et al., 2018)

LiPI is a subset of genes that are directly regulated by *prfA*. LiPI-1 includes *hly*, *plcA*, *prfA*, *mpl*, *actA* and *plcB*. (Milohanic, E., et al., 2003) Two types of LiPIs are especially important in analysing *L.monocytogenes* virulence and they are also extensively researched. While there are 4 known LiPIs overall, the main focus of the research and of this thesis, subsequently, goes to the LiPI-1 and LiPI-4 for such quality as commonness and hypervirulence, respectively. LiPI-3, although identified in outbreaks and present in half of lineage I strains, mainly contributes to virulence of the bacteria in vivo. (Orsi, R. H., Bakker, H. C. de., et al., 2011)

LiPI-1 is the most important virulence factor in *L.monocytogenes*, being found in the overwhelming majority of the strains. (Hurley, D., et al., 2019) Those genes produce several virulence factors: listeriolysin O (encoded by the ***hly*** gene), phospholipases A (encoded by ***plcA***), phospholipases B (encoded by ***plcB***), zinc metalloproteinase (encoded by ***mpl***), actin assembly-inducing protein (encoded by ***actA***) and the transcriptional activator PrfA (encoded by ***prfA***). (Aguilar-Bultet, L., et al., 2018)

LiPI-4 is known to be able to enhance invasion of the bacteria through the activation of cellobiose-type phosphotransferase systems. LiPI-4 is typically found with the infection-associated isolates that are considered hypervirulent. Reduced pathogenicity, on the other hand, has been found to display some premature stop codons in virulence factors (*prfA*, *actA*, etc.), leading to nonfunctional proteins. (Maury, M. M., et al., 2016)

Overall, the most important gene of the LiPI subset appears to be *prfA*, as strains lacking *prfA* are avirulent. (Rolhion, N., & Cossart, P., 2017)

1.4.3 Internalins

Second group of virulence factors contributing to *L.monocytogenes* outbreak potential are internalins, proteins that help in adherence to and invasion of host cells. They aren't required for strain to be virulent but appear to contribute to its pathogenicity. (Orsi, R. H., Bakker, H. C. de., et al., 2011)

There are 6 main internalin genes (*inlA*, *inlB*, *inlC*, *inlK*, *inlF*, *inlJ*) that are involved in invasion (*inlA*, *inlB*), adherence (*inlF*, *inlJ*), cell-to-cell spread (*inlC*), and autophagy evasion (*inlK*). (Lim,

S. Y., et al., 2016) The *inlA* and *inlB* genes are most commonly known for encoding surface proteins required for successful invasion of cells (*inlA* is required for invasion of epithelial placental cells and epithelial intestinal cells while *inlB* is required for invasion of hepatocytes and fibroblasts (Orsi, R. H., Bakker, H. C. de., et al., 2011)). (Bierne, H., et al, 2007) The *inlC* encoded protein appears to play a role in the eventual spread of the pathogen from cell-to-cell. The exact role of less studied internalins is not fully clear yet, despite some apparent associations. (Orsi, R. H., Bakker, H. C. de., et al., 2011) There are typically multiple versions of each gene encoding for internalins. (Schmid, M. W., et al., 2005)

Overall, there are few main characteristics of the internalin protein group. Those characteristics include LGR domains that are responsible for host-to-cell interactions, domans adjacent to LRR, recognition motifs and MucBP repeats. (Aguilar-Bultet, L., et al., 2018) *L.monocytogenes* internalins are also larger than those of other Listeria species, bound to the cell wall by their C-terminus. (Navarre, W. W., & Schneewind, O., 1999)

When it comes to lineage gene variance of internalins, there do appear to be some patterns. The *inlG*, *inlF* and *inlH* have only been discovered in the lineage II strains (Jia, Y., et al., 2007), *inlF* and *inlH* are not found at all in lineage III (Jia, Y., et al., 2007) and *inlJ* is absent from lineage IV. (Orsi, R. H., Bakker, H. C. de., et al., 2011)

1.4.4 Other genes (*vip*, *bsh* and *ami*)

While the internalins and the LiPI are two of the most well-studied groups of virulence factors of *L.monocytogenes*, there are also other genes that have been found to have a connection to the pathogenicity of this bacteria. Those genes are *vip*, *bsh* and *ami*. The *vip* gene encodes for the recognition (LPXTG) surface protein that binds to the cell wall of the Listeria and interacts with the gp96 protein of the host during the invasion. There has also been some evidence to suggest that Vip protein is interfering with the immune response of the host through signalling. (Cabanes, D., et al., 2005) The *vip* gene is present in lineage I, most strains of lineage iI but is absent in lineage III, while all strains of *L.innocua* and *L.marthii* that are non-virulent appear to lack this gene. (Tan, M. F., et al., 2015). Bile salt hydrolase is encoded by the *bsh* gene. BSH plays an important role in making sure *L.monocytogenes* is surviving in the intestines due to the resistance

to bile salts. (Begley, M.,et al., 2005) The *ami* gene is known for encoding Ami, autolysis amidase, a protein playing an important role in the adhesion to the host process. (Asano, K., et al., 2012)

1.5 *LISTERIA MONOCYTOGENES* GROWING RESISTANCE

While *Listeria monocytogenes* is most commonly recognized for its pathogenicity, it is far from it's only remarkable trait. There has been growing concern regarding *L.monocytogenes* growing resistance to antibiotics and its natural resistance, to heat and to the cleaning chemicals. Some evidence has been discovered to support the claim of some resistance of *L.monocytogenes* to heat and cold. (Jiang, X., et al., 2012; Garedew, L., et al., 2015; Haubert, L., et al., 2016; Ballom, K. F., et al., 2020) There is also substantial research done in terms of discovering genes contributing to the antibiotic resistance in *L.monocytogenes*, (Noll, M., et al., 2018) but this thesis focuses on the cleaning chemical resistance specifically.

In this subchapter of the thesis we will summarize everything we know about the *L.monocytogenes* growing resistance to the cleaning chemicals and the genes involved in encoding for processes creating that resistance.

1.5.1 Quaternary ammonium sanitizers tolerance

The most popular and common disinfectant products are those that include QACs in their formulation. The two types of QAC that are most prevalent in sanitizing products are ADBAC and DDAC. QACs are considered to be low-level disinfectants and are effective against bacteria, viruses and some fungi. They function by interfering with the function of the cell membrane, resulting in lysis. (Fu, E., et al., 2007)

BAC is a commonly used disinfectant in the food industry and a common QAC.

In a study conducted by Mereghetti et al. (2000) an association has been observed between the isolates of 1/2 serotype and increased QAC tolerance, possibly explaining lack of strains from lineage II in samples found in food relative to lineage I.

There are multiple genes, linked to commonly recognized tolerance factors: ***emrE***, which encodes for an efflux pump, Tn6188 transposon, ***bcrABC*** cassette, ***tmr*** and ***emrC***, a gene encoding for an efflux pump EmrC. (Kovacevic, J., et al., 2016) (Müller, A., et al., 2013) (Elhanafi, D., et al., 2010) (Dutta, V., et al., 2014) (Kropac, A. C., et al., 2019) They vary in how common they are in *L.monocytogenes* strains. Based on the WGS performed by Hurley, D., et al., 2019, genes encoding tolerance for BAC has been found in 62% of the isolates studied. The most common gene happened to be *emrC* followed by *bcrABC* and *qacH-Tn6188* (transposon Tn6188 harbours the transporter QacH (Müller, A., et al., 2014)).

1.5.1.1 Tn6188

Tn6188 is a transposon discovered in 2013 by Müller, A., et al.. Found to be closely related to other transposons, such as Tn554, Tn558, Tn559 and Tn5406, found in *Staphylococcus aureus* and various *Firmicutes*. It encodes three consecutive transposase genes: *tnpA*, *tnpB*, and *tnpC*. It also encodes for a protein with similarity to EmrE and Smr, QacH, which have been found to be responsible for exporting the disinfectant from the bacteria. (Bay, D. et al., 2008) This transposon's function appears to be encoding for bacteria's tolerance to QAC as well as being recognized to perform a similar role in other bacterial genera. (Müller, A., et al., 2013)

In the study by Hurley D., et al., (2019) Tn6188 transposon was found in 14% of the isolates of *L.monocytogenes*.

1.5.1.2 *emrC*

The plasmid pLMST6 was first identified by Kremer P. H., et al. (2017). It is associated with ST6 isolates that are more frequent in severe cases. The *emrC* gene is present on this plasmid. The gene's main function in the *L.monocytogenes* is connected to encoding a QAC efflux pump. There also appeared to be an association between strains carrying the plasmid and resistance to some antibiotics (amoxicillin and gentamicin) (Kremer P. H., et al. 2017)

Kropac, A. C., et al. (2019) found a 1.6% prevalence of this plasmid in their study while in the study by Hurley D., et al., (2019) it was found in 25% of the isolates of *L.monocytogenes*.

Kropac, A. C., et al. (2019) also confirmed the hypothesis of the effect of the plasmid on the virulence potential of *L.monocytogenes*.

1.5.1.3 *emrE*

LGI1 is a 50-kb genomic island that appears to play an important role in *L.monocytogenes* tolerance of QAC. The *emrE* gene is found on locus 1862 of the LGI1 element, encoding for the efflux pump. Deletion of the *emrE* gene leads to reduced survival in the presence of QAC cleaning chemicals. (Kovacevic, J., et al., 2016)

1.5.1.4 bcrABC

The *bcrABC* cassette was characterized in 2010 by Elhanafi, D., et al., 2010 based on the 1998-1999 listeria outbreak strains. The cassette is located in the 80-kb plasmid, plM80, which was found to induce resistance to BAC and to cadmium. There are three open reading frames responsible for the BAC tolerance in the plM80 plasmid: *bcrA*, *bcrB* and *bcrC*. Removing the plasmid left the strain susceptible to BAC. (Nelson, K. E., et al., 2004) It has also been found that *bcrABC* significantly lowers transcription at lower temperatures. The transcriptional regulator *bcrA* appears to have transcriptional control of the cassette, while *bcrB* and *bcrC* are SMR genes. (Elhanafi, D., et al., 2010)

In the study by Hurley D., et al., (2019) *bcrABC* cassette was found in 19% of the isolates of *L.monocytogenes*.

2. THE AIMS OF THE THESIS

The aims of this thesis are to:

- Create an overview of the *Listeria monocytogenes* virulence and chemical resistance studies
- Formulate an experimental plan based on published works on how to detect virulence and chemical resistance genes using PCR analysis.

3. EXPERIMENTAL PLAN

3.1 Materials and Methods

3.1.1 Data Sources

Main data source for this thesis would be NCBI. The NGS sequencing data would be downloaded from NCBI SRA database (https://www.ncbi.nlm.nih.gov/sra). Currently (14.05.2021) there are over 38 thousand samples containing raw reads of *Listeria monocytogenes* WGS available through the database. Data format used by the sources is fastq. Genes that potentially could be targeted by designed primers are summarized in Table 1 and Table 2.

Table 1: Summarizing table of genes associated with chemical resistance

Gene	**Location**	**Description**	**Reference**
qacH	Tn6188	encoding for quaternary ammonium compound-resistance protein QacH	Müller, A., et al. (2013)
emrC	pLMST6	encoding for efflux pumps EmrC and EmrE, respectively	Kropac, A. C., et al. (2019)
emrE	LGI1		Kovacevic, J., et al. (2016)
bcrABC	pLM80	resistance cassette consisting of a transcriptional regulator *bcrA*, and SMR protein family transporters *bcrBC*	Elhanafi, D., et al. (2010)

Table 2: Summarizing table of genes associated with virulence

Gene	Location	Description	Reference
hly	LiPI	encoding for listeriolysin O virulence factor	Milohanic, E., et al. (2003)
plcA		encoding for phospholipases A	
plcB		encoding for phospholipases B	
mpl		encoding for zinc metalloproteinase	
actA		encoding for actin assembly-inducing protein	
prfA		encoding for the transcriptional activator PrfA, the main virulence factor of L.*monocytogenes*	
inlA	locus tag lmo0433	encoding for InlA surface protein required for invasion of epithelial placental cells	Bierne, H., et al. (2007)
inlB	locus tag lmo0434	encoding for InlB surface protein required for invasion of hepatocytes and fibroblasts	
inlC	locus tag lmo1786	encoding for InlC protein that plays a role in the cell-to-cell spread	Lim, S. Y., et al. (2016)
inlK	locus tag HCB09_RS11760	encoding for InlK protein that plays a role in the autophagy evasion	
inlJ	locus tag HCB09_RS08430	encoding for InlJ protein that plays a role in the bacterial adhesion to host cells	
inlH	locus tag lmo0263	exact role of InlH is not fully clear yet	Orsi, R. H., Bakker, H. C. de., et al. (2011)
vip	locus tag lmo0320	encoding for Vip protein that interferes with host's immune response	Cabanes, D., et al. (2005)
bsh	locus tag lmo2067	encoding for BSH, ensuring survival in the intestines of the host	Begley, M.,et al. (2005)
ami	locus tag lmo2558	encoding for Ami protein that plays a role in bacterial adhesion to host cells	Asano, K., et al. (2012)

For the future wet lab experiment testing primers we would request the bacterial samples of *L.monocytogenes* from UT collaborators.

3.1.2 Tools for analysing data

3.1.2.1 Cleaning reads (fastp)

The fastp program is a powerful tool used for preprocessing the genomic data. Its main strength lies in its fast speed: fastp is 2 to 5 times faster than other tools for preprocessing the genomic data, like Cutadapt. It is also capable of performing more operations, as it unites functions of quality control, adapter trimming and read pruning and filtering, making it a more efficient alternative. (Chen, S., et al., 2018)

3.1.2.2 Genome assembly (SPAdes)

SPAdes is a de novo sequence assembly algorithm that takes reads as input in the FASTQ format. Its functionality includes assembly of bacterial genomes from short reads, bacterial metagenomes, eukaryotic transcriptomes and small eukaryotic genomes. It can't be used for long-read assembly alone and it is not recommended to run SPAdes for large genomes. (Prjibelski, A., et al., 2020)

3.1.2.3 BLAST program

Basic Local Alignment Search Tool (BLAST) is a commonly used program for comparison and detection of the genetic sequences. BLAST requires a query sequence (genetic information of the genes chosen) and target sequences (our database sequencing data taken from NCBI). This tool's main strengths lay in the trade-off between the quality and speed of the search, which it achieves by modifying and refining blastp program's search criteria and abilities. (Altschul, S. F., et al., 1997)

3.1.2.4 Design of PCR primers (Primer3)

Primer3 is a set of programs that are used for PCR primer design. There are two main ways to use Primer3 for primer design: through the command-line interface and through the web interface.

Main advantages of using Primer3 tools are the open-source code, easily available web service and its capability to be incorporated and used in tandem with other software. (Untergasser, A., et al., 2012) Program primer3_masker is implemented into the Primer3 program, making it possible to find and mask the error-prone regions of the DNA template before conducting the PCR primer design. The primer3_masker program, in particular, is advantageous, as it is based on frequency of k-mers, so therefore can be applied to any genome sequence. (Kõressaar, T., et al., 2018)

3.2 Discussion

The experimental plan is presented on the diagram on Figure 4. More details regarding the steps are given here.

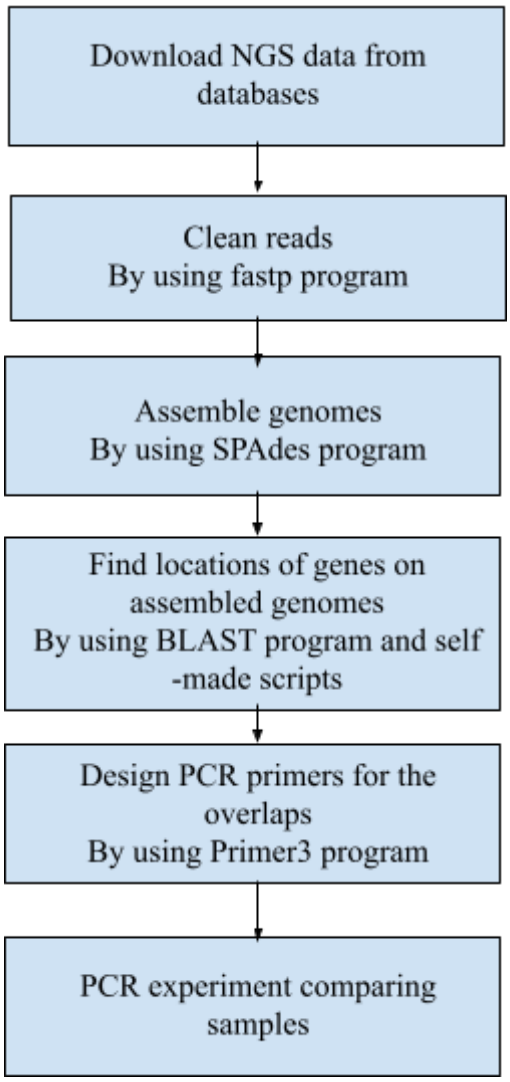

Figure 4: Block diagram representing the experimental plan.

The first step is to obtain sequencing data from the NCBI SRA database. The result of that would be a fastq file with raw reads of *L.monocytogenes* WGS data. The next step is to clean those reads

by using the fastp program, performing the quality check and pruning the reads. The result is a preprocessed fasta file. That file is then assembled into genomes using the SPAdes program. Then we locate the specific genomic elements we are looking for on newly assembled genomes by using the BLAST program. Our target genes are genes from LiPI (*prfA*, *actA*) and internalin *inlA* due to how common they are to detect virulence and *emrC*, *bcrABC* and *tnpA* to detect the tolerance to QAC. The resulting overlaps are the genome sequences that we will then target when designing primers. The experimental primers are designed by using the Primer3 program.

The next stage of the experiment would be to conclude a real-life PCR experiment, testing the made primers and comparing the samples that were found to be virulent and resistant to the samples that were found non-pathogenic and lacking in tolerance to cleaning chemicals. That comparison would prove whether the primers correctly detect the strains. For concluding that experiment, we would request the bacterial samples of *L.monocytogenes* from UT collaborators, as there are samples from the fish processing facility that demonstrate resistance to cleaning chemicals. The result of such experiment would be the PCR gel pictures depicting the presence of resistance and virulence related genes through the PCR product band.

After concluding the test we would need to run a control experiment to confirm the correlation between presence and absence of those genes in positive and negative control. It is also important to note, however, that the presence of target genes do not necessarily always mean presence of virulence factors, even if correlation is present. It is possible we would need to conclude some additional tests on variation in those target genes. There are few ways to do that, one of which would be to use k-mer-based strategy for identification of our genetic markers, as that way it is possible for us to accurately predict the pathogen's phenotype. (Aun, E., et al., 2018)

4 CONCLUSION

Listeria monocytogenes is Gram-positive pathogen that causes listeriosis through the consumption of contaminated food. It is capable of surviving several commonly used chemical purifying agents, which causes severe issues for food processing plants dealing with infestation. This paper provides the theoretical approach to detecting *L.monocytogenes* pathogenicity and chemical tolerance by detecting the genes most often implicated in virulence and chemical resistance through the use of PCR primers as well as a literature overview for *Listeria monocytogenes* virulence and QAC resistance studies.

As this thesis is theoretical and offers methodology for detecting virulence and chemical resistance genes, there is a need for an experimental study to confirm the effectiveness of the method. If the proposed above method is functional, it would be possible to expand it to genes beyond the ones chosen in this thesis.

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