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# Molecular Mechanisms behind the Interactions between *Listeria monocytogenes* LL195 and a German Deli Meat "Lyoner"

von Philipp-Michael Beindorf

aus Pasewalk

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**Aus dem Veterinärwissenschaftlichen Department der Tierärztlichen Fakultät der**

**der Ludwig-Maximilians-Universität München**

**Lehrstuhl für Lebensmittelsicherheit und -analytik**

**Arbeit angefertigt unter der Leitung von:** Univ.-Prof. Dr. Claudia Guldimann, Ph.D.

**Mitbetreuung durch:** Dr. Irmak Şah, Ph.D., Ph.D.

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| **Dekan:** | Univ.-Prof. Dr. Reinhard K. Straubinger, Ph.D. |
| **Berichterstatter:** | Univ.-Prof. Dr. Claudia Guldimann, Ph.D. |
| **Korreferent:** | Prof. Dr. Frank Ebel |

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Meiner Großmutter

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# LIST OF ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| BHI | Brain heart infusion |
| bp | Base pairs |
| CFU | Colony forming units |
| Csp | Cold-schock proteins |
| DNA | Deoxyribonucleic acid |
| EC | European Commission |
| EFSA | European Food Safty Agency |
| EU | European Union |
| FDA | Food and Drug Administration |
| g | Grams |
| GRAS | Generally recognized as safe |
| Gr (+) | Gram positive |
| HACCP | Hazard Analysis and Critical Control Point |
| HMW | High-molecular-weight |
| HTH | Helix-turn-helix |
| Kb | Kilobase |
| LB | Luria Bertani |
| LIPI-1 | Listeria pathogenicity island-1 |
| min | Minute |
| PCR | Polymerase chain reaction |
| PG | Peptidoglycan |
| PrfA | Regulatory factor A |
| OD | Optical dense |
| R-M | Restriction-modification |
| RTE | Ready-to-eat |
| sec | Second |
| SEM | Scanning electron microscope |
| Tn | Transposon |
| TnSeq | Transposon insertion sequencing |
| WT | Wildtype |
| YOPI | Young, Old, Pregnant, Immunocompromised |
| % | Percent |
| µg | Microgram |
| µl | Microliter |
| °C | Centigrade Degree |

# I. INTRODUCTION

Listeriosis is a potentially fatal food-borne disease that is caused by *Listeria monocytogenes* (*L. monocytogenes*), a zoonotic pathogen capable of infecting both humans and animals. It was first isolated from a patient with meningitis in France in 1921 (DUMONT und COTONI, 1921) and then characterized and named by Murray in 1926 (MURRAY et al., 1926). The understanding that consuming certain foods could cause human cases of listeriosis became clear in the 1980s.

In the following years, there were repeated major outbreaks (LINNAN et al., 1988; BILLE, 1989; MCLAUCHLIN et al., 1991; GOULET et al., 1993) worldwide (SCHLECH III et al., 1983). As a result, considerable efforts have been made to mitigate the risk of listeriosis. For instance, the Hazard Analysis and Critical Control Point System (HACCP), originally developed by NASA in the 1960s to ensure food safety for astronauts, has proven to be highly effective. When applied to the food industry, it has helped to identify and control critical points in food production, storage, and distribution, thereby significantly reducing the risk of listeriosis outbreaks.

As a result, since the 1990s, occurrences of large listeriosis outbreaks affecting over 50 individuals have become increasingly uncommon (BAYERISCHES LANDESAMT FÜR GESUNDHEIT UND LEBENSMITTELSICHERHEIT, 09.08.2022). However, it remains a persistent issue that continues to be relevant. According to the latest zoonoses report by the European Food Safety Agency (EFSA), listeriosis was the fifth most common food-borne illness in the European Union in 2022 (EU). With a case number of 2,738, cases of listeriosis in 2022 were far rarer than Campylobacter diseases at the top of the list, which were reported with 137,107 cases. However, when comparing the hospitalization rate (96% for listeriosis, 23.5% for Campylobacter) and a mortality rate (18.1% vs. 0.04%), listeriosis poses by far the highest risk of severe consequences among the food borne diseases that are common in the EU (THE EUROPEAN UNION ONE HEALTH 2022 ZOONOSES REPORT, 2023).

In particular, foods from fish, fishery products, and deli meats are frequently involved in outbreaks (THE EUROPEAN UNION ONE HEALTH 2022

ZOONOSES REPORT, 2023). Although some of these products undergo a heating step that should ensure their safety, there is a risk of re-contamination after the heat step in the production process, particularly during steps like slicing and packaging (SHEEN und HWANG, 2008). The ability of *L. monocytogenes* to form biofilms and its ubiquitous occurrence contribute in the environment to its potential to colonize food production facilities and contaminate food products (PIYUSH KUMAR et al., 2022).

The phenotypic characteristics of *L. monocytogenes* vary on different food matrices (SPANU et al., 2014) and *L. monocytogenes* exhibits specific regulatory responses to environmental stress factors relevant to the food production environment such as pH, temperature, osmotic, oxidative, or abiotic stresses, resulting in altered gene expression profiles. These impressive ability to adapt to different environmental stresses allow *L. monocytogenes* to survive and proliferate in food or food processing facilities, making it a challenging food-borne pathogen to control in the food industry (BUCUR et al., 2018; GUERREIRO et al., 2020). To understand the mechanisms behind these adaptations, numerous studies have been conducted using various research techniques, including genomic and transcriptomic analyses to investigate the gene expression profiles, proteomic and metabolomic analyses to understand metabolic processes, molecular biology techniques to examine the function of specific genes. As a result, the findings from these studies provided valuable insights into the mechanisms that mediate *L. monocytogenes* resilience under stress. However, it is important to note that many of these findings were obtained using laboratory culture media, which may not accurately represent the complex environments encountered in real world food settings. Studies that were performed in food matrices show that the expression levels of stress response (FALEIRO et al., 2003; MELO et al., 2015), virulence (RANTSIOU et al., 2012; WIŚNIEWSKI et al., 2022) and adhesion genes (VAZQUEZ-ARMENTA et al., 2020; SCHIAVANO et al., 2021) can vary between various food matrices and also between laboratory media and food matrices (RANTSIOU et al., 2012). Most of the available data on the resilience of *L. monocytogenes* under different stress conditions have been established using laboratory reference strains like 10403S, EGD or EGDe. While these are well characterized, they do not represent the clinically important clonal complexes CC1, CC4, and CC6 (BÉCAVIN et al., 2014; MAURY et al., 2019). In addition,

these reference strains may have lost crucial characteristics through laboratory passaging, and the obtained data should be evaluated by taking the genetic background of strain into consideration (BÉCAVIN et al., 2014; HSU et al., 2020). Therefore, to expand and refine the existing data, there is a need for research using clinically relevant strains interacting with relevant food matrices.

The main objective of this study was to screen a classic transposon mutagenesis library in an outbreak strain of *L. monocytogenes* on a ready-to-eat (RTE) meat product as a relevant food matrix, to analyze the molecular mechanisms underlying *L. monocytogenes* resilience under these conditions. By confirming candidate genes, we aimed to create a ‘metabolic map’ that elucidates the interactions between individual pathogen-matrix pairs.

# II. AIM AND SCOPE OF THE THESIS

The aim of this thesis was to gain a better understanding of the fitness contribution of individual genes within the genome of a *L. monocytogenes* outbreak strain through screening a transposon mutagenesis library and to investigate growth dynamics of individual mutants on a RTE food matrix.

As stated in the introduction chapter, our current understanding of how *L. monocytogenes* behaves under different environmental conditions primarily relies on well-characterized laboratory reference strains, leading to a potential knowledge gap in our understanding of the interactions between outbreak strains and various environmental conditions.

RTE foods are one of the primary sources of human listeriosis, with deli meats figuring prominently: According to a meta-analysis study that reviewed 100 studies from all over the world, *L. monocytogenes* has a prevalence of 2.9 % in sliced, RTE deli meats (CHURCHILL et al., 2019). We therefore chose a German deli meat (Lyoner-type sausage) as a model food matrix to study the fitness effect of individual genes by using a transposon mutagenesis library in an outbreak strain of *L. monocytogenes*, LL195. Data on the molecular mechanisms underlying the interaction of *Listeria* with Lyoner could help to implement specific and data-based control measures to improve food safety related to *L. monocytogenes*.

# III. LITERATURE REVIEW

## 1. Food-borne diseases and pathogens

Food-borne diseases are a significant public health concern, affecting millions of people worldwide each year, with an estimated 600 million cases and 420,000 deaths occurring annually due to the consumption of unsafe food (WORLD HEALTH ORGANIZATION, 2015; LEE und YOON, 2021). These numbers reveal that approximately 7.69 % of the global population is affected by food-borne diseases each year. Furthermore, about 7.5 % of all annual deaths can be attributed to food-borne diseases (LEE und YOON, 2021). These diseases can be caused by microbial pathogens such as bacteria, viruses, and parasites, as well as chemical contaminants, natural toxins, allergens, physical hazards (TAUXE, 2002; WORLD HEALTH ORGANIZATION, 2024). Among them, microbial pathogens are one of the most common causes of food-borne illnesses, highlighting the importance of effective food safety measures to prevent the contamination of foods with bacteria as well as their growth and spread. Food-borne pathogens can contaminate food at any stage of the food supply chain, from farm to fork, including primary production, manufacturing, processing, and consumption. Symptoms of food-borne diseases can range from mild gastrointestinal distress to severe, life-threatening conditions, and may include nausea, vomiting, diarrhea, abdominal cramps, and fever. People with a weakened immune system, such as elderly people, pregnant women, newborns, and immunocompromised individuals are at increased risk for severe food-borne illnesses. This vulnerable group is often referred to as YOPI (Young, Old, Pregnant, Immunocompromised) and they are more likely to experience severe symptoms (JACKSON und MEAH, 2018). The symptoms and the incubation periods of the most important food-borne pathogens have been well studied and are summarized in Table 1, adapted from (GRUMEZESCU und HOLBAN, 2018).

**Table 1:** Symptoms, onset of symptoms, and responsible microorganisms or toxins for the major food-borne illnesses (GRUMEZESCU und HOLBAN, 2018)

| Approximate time to symptoms | Signs and Symptoms | Organism | Illness | Food source |
|---|---|---|---|---|
| **2-6 h** | Severe nausea and vomiting, abdominal cramps, diarrhea and fever, prostration, dehydration, headache, muscle cramping, and transient changes in blood pressure and pulse rate | *Staphylococcus aureus* | *Staphylococcus aureus* intoxication | Unrefrigerated or improperly refrigerated meats and meat products, poultry and egg products; salads; milk and dairy products |
| **4-90 h** | Watery or bloody diarrhea, abdominal cramps, nausea, vomiting, fever | *Vibrio parahaemolyticus* | *Vibrio parahaemolyticus* gastroenteritis | Undercooked or raw seafood or fish, oysters and fishery products |
| **6-48 h** | Diarrhea, fever, abdominal cramps, vomiting, nausea, headache | *Salmonella* spp. | Salmonellosis | Raw eggs, poultry, meat, unpasteurized milk or juice, cheese, chocolate, contaminated raw fruits and vegetables, spices, salads |

**Table 1:** Continued

| | | | | |
|---|---|---|---|---|
| **8-24 h** | Abdominal cramps, watery diarrhea, rarely vomiting and fever | *Clostridium perfringens* | *Clostridium perfringens* enteritis | Meats, poultry, vegetables (spice and herbs), raw and processed foods |
| **10-16 h** | Abdominal cramps, watery diarrhea, nausea, vomiting, pain | *Bacillus cereus* | *B. cereus* gastroenteritis | Meats, stews, gravies, boiled or fried rice, spices, dried foods, milk, dairy products, vegetable dishes, fish, pasta, salads |
| **12-24 h** | Acute gastroenteritis may include vomiting, nausea, fever, chills, abdominal pain, watery diarrhea | Miscellaneous Enterobacteriaceae | Miscellaneous Enterobacteriaceae enteric | Dairy products, raw shellfish, raw vegetables |
| **12-36 h** | Vomiting, abdominal pain, diarrhea, fatigue, blurred vision, double vision, muscle weakness, slurred speech, difficulty in swallowing, dry mouth, headache, dizziness, constipation | *Clostridium botulinum* | Botulism | Home-canned vegetables, fish and fish products, condiments, meat and meat products, soups, mushrooms |
| **12h – 21 days** | Vomiting, diarrhea, abdominal pain, fever, bleeding within the skin, nausea, chills, pain in the extremities | *Vibrio vulnificus* | *Vibrio vulnificus* infection | Undercooked or raw seafood |

**Table 1:** Continued

| | | | | |
|---|---|---|---|---|
| **20-50 h** | Fever, chills, abdominal pain, nausea, diarrhea, vomiting | *Plesiomonas shigelloides* | *Plesiomonas shigelloides* enteric infection | Contaminated water, raw shellfish, improperly cooked or raw foods and seafoods |
| **24-48 h** | Dysentery-like symptoms, blood and mucus in the stool, abdominal cramps, mild fever, vomiting | *Aeromonas hydrophila* | *Aeromonas* enteritis | Seafood, snails, drinking water, vegetables |
| **24-36 h** | Abdominal pain, diarrhea, mild fever, vomiting | *Yersinia enterocolitica* | Yersiniosis | Raw milk and milk products, meats, fish, seafood |
| **1-3 days** | Pain on swallowing, high fever, headache, nausea, vomiting, malaise, rhinorrhea | *Streptococcus* spp. | *Streptococcus* spp. intoxication | Pasteurized and raw milk, eggs, cooked seafood, salads |
| **1-3 days** | Profuse watery diarrhea, severe dehydration, abdominal pain and vomiting, with rice-water stools | *Vibrio cholera* | Cholera | Seafood, vegetables |
| **1-6 days** | Watery diarrhea, abdominal cramps, vomiting, high fever, nausea, malaise | *E. coli* | *E. coli* infection | Water or food contaminated with feces, raw milk from infected animals, vegetables |
| **1-7 days** | Abdominal cramps, fever, diarrhea, vomiting, pus or mucus in stools, tenesmus | *Shigella* spp. | Shigellosis | Raw or uncooked foods, contaminated drinking water, salads and vegetables, raw milk and dairy products |

**Table 1:** Continued

| | | | | |
|---|---|---|---|---|
| **2-5 days** | Bloody diarrhea, abdominal cramps, fever, vomiting, nausea, headache, muscle pain | *Campylobacter spp.* | Campylobacteriosis | Raw and undercooked poultry, meat, unpasteurized milk, contaminated drinking water, vegetables, seafood |
| **3-6 days** | Varying from mild diarrhea to severe bowel damage, chills, fever, headache | *Francisella tularensis* | Tularemia | Milk and undercooked meats from infected animals (often rabbits and hares) |
| **Days to several weeks** | Influenza-like symptoms such as fever, headache, muscle aches, stiff neck, confusion, loss of balance, convulsions, nausea, vomiting, diarrhea, abortions | *Listeria monocytogenes* | Listeriosis | Unpasteurized and raw milk and milk products, meat, deli meats, raw and smoked fish and seafood, raw vegetables |
| **10-20 days** | Nausea, high fever, abdominal pain, headache, rashes, loss of appetite | *Salmonella typhi* and *Salmonella paratyphi* | Typhoid fever, paratyphoid fever | Dairy products, meat products, eggs, seafood, fruits, vegetables |
| **2 weeks** | Very high fever, severe headache, muscle aches, chills, profuse sweating, nausea, vomiting, diarrhea, dry cough, abdominal cramps, chest pain | *Coxiella burnetii* | Q fever | Contaminated unpasteurized milk or dairy products |

**Table 1:** Continued

| | | | | |
|---|---|---|---|---|
| **3 weeks** | Intermittent fever, lassitude, sweat, headache, chills, | *Brucella* spp. | Brucellosis | Unpasteurized goat´s or |

| | | | | |
|---|---|---|---|---|
| | constipation, arthralgias, generalized aching, weight loss, anorexia, malaise, joint and muscle pain, arrhythmia, edema, chest pain, meningoencephalitis, stiff neck, confusion or seizures, spondylitis | | | shepp´s milk and products made from the milk of infected animals |
| **Variable** | Poor feeding response, irritability, jaundice, grunting respirations, instability of body temperature, seizures, brain abscess, hydrocephalus, developmental delay | *Enterobacter sakazakii* | *Cronobacter* infection | Contaminated powdered infant formula, milk powders, cheese products, other dried foods |
| **Months to years** | Fever, night sweats, fatigue, loss of appetite, weight loss, chronic cough, bloodstained sputum, chest pain, diarrhea, abdominal pain | *Mycobacterium bovis* and *mycobacterium caprae* | Tuberculosis | Raw and unpasteurized milk and milk products, raw or undercooked meats of infected animals |

While there are many known pathogens that can cause food-borne illnesses, some of the most significant ones include *Campylobacter*, *Salmonella*, *L. monocytogenes*, Shiga Toxin producing *Escherichia coli*, and *Yersinia*. In the 27 EU Member States and the United Kingdom, 5763 food-borne outbreaks were registered in 2022, with 48605 people confirmed to be infected and 64 fatalities. This number of deaths was one of the highest in the EU in the last 10 years. Norovirus caused the highest number of infections, while *Salmonella* Enteritidis was responsible for the highest number of outbreaks. However, listeriosis stands out as one of the most severe food-borne diseases, with the highest case fatality (18.1%) and hospitalization (96 %) rates among reported cases with 28 deaths in 2022. (THE EUROPEAN UNION ONE HEALTH 2022 ZOONOSES REPORT, 2023).

## 2. The Genus Listeria and *Listeria monocytogenes*

### 2.1. Characterization

As of February 2024, the genus Listeria consists of 21 recognized species and six subspecies, which can be divided into two distinct clades: sensu stricto and sensu lato (ORSI und WIEDMANN, 2016). The sensu stricto group comprises all Listeria spp. that likely interacts with mammals, whereas the sensu lato group consists of species classified as environmental bacteria. This classification is based on the observation that Listeria species from the sensu stricto group have been detected in the feces or gastrointestinal tract of asymptomatic animals and in foods of animal origin. In contrast, species from sensu lato group have only been isolated from the environment or food contact surfaces, suggesting a less direct association with mammalian hosts (SCHARDT et al., 2017; ORSI et al., 2024). The Listeria sensu stricto group comprises to a group of 10 species: *L. monocytogenes, L. seeligeri*, *L. welshimeri*, *L. innocua*, *L. ivanovii*, *L. marthii*, *L. farberi*, *L. immobilis*, *L. cossartiae*, and *L. swaminathanii*. These species are all closely related to, *L. monocytogenes,* which was the first species identified in 1924 (ORSI et al., 2024). Although the Listeria sensu lato originally referred to all species within the genus Listeria, recent publications have used the term “sensu lato” to refer specifically to those Listeria species that are less phylogenetically related to *L. monocytogenes* (i.e., those species not in the Listeria sensu stricto group). The Listeria sensu lato currently includes 18 species (*L. grayi, L.*

*fleischmannii, L. floridensis, L. aquatica, L. valentina, L. thailandensis, L. goaensis, L. ilorinensis, L. costaricensis, L. rustica, L. portnoyi, L. cornellensis, L. newyorkensis, L. rocourtiae, L. weihenstephanensis, L. grandensis, L. booriae, and L. riparia*) (ORSI et al., 2024).

**Table 2:** The sensu stricto group of the genus Listeria

| species | subspecies | isolated from | Reference |
|---|---|---|---|
| ***L. monocytogenes*** | | a variety of diseases in widely different host species including man | (SEASTONE, 1935) |
| ***L. innocua*** | | | (SEELIGER, 1981) |
| ***L. seeligeri*** | | Adult mice | (ROCOURT und GRIMONT, 1983) |
| ***L. welshimeri*** | | Adult mice | (ROCOURT und GRIMONT, 1983) |
| ***L. ivanovii*** | *ivanovii* | Listeriosis patient | (SEELIGER et al., 1984) |
| | *londoniensis* | Listeriosis patient | (BOERLIN et al., 1992) |
| ***L. marthii*** | | the natural environment, Finger Lakes National Forest | (GRAVES et al., 2010) |

**Table 3:** the sensu lato group of the genus Listeria

| species | subspecies | isolated from | Reference |
|---|---|---|---|
| ***L. grayi*** | *grayi* | different living beings | (ROCOURT et al., 1992) |
| | *murrayi* | vegetation | (ROCOURT et al., 1992) |
| ***L. rocourtiae*** | | pre-cut lettuce | (LECLERCQ et al., 2010) |
| ***L. fleischmannii*** | *fleischmannii* | from cheese | (BERTSCH et al., 2013) |
| | *coloradonensis* | on a cattle ranch | (DEN BAKKER et al., 2013) |
| ***L. weihenstephanensis*** | | water plant | (LANG HALTER et al., 2013) |
| ***L. aquatica*** | | agricultural and natural environments | (DEN BAKKER et al., 2014) |
| ***L. cornellensis*** | | agricultural and natural environments | (DEN BAKKER et al., 2014) |
| ***L. grandensis*** | | agricultural and natural environments | (DEN BAKKER et al., 2014) |
| ***L. riparia*** | | agricultural and natural environments | (DEN BAKKER et al., 2014) |
| ***L. newyorkensis*** | | food processing environments | (WELLER et al., 2015) |

**Table 3:** Continued

| | | |
|---|---|---|
| ***L. booriae*** | food processing environments | (WELLER et al., 2015) |
| ***L. costaricensis*** | food processing drainage system | (NÚÑEZ-MONTERO et al., 2018) |
| ***L. goaensis*** | mangrove swamps | (DOIJAD et al., 2018) |
| ***L. thailandensis*** | fried chicken | (LECLERCQ et al., 2019) |
| ***L. valentina*** | water trough and faeces of healthy sheep | (QUEREDA et al., 2020) |

Recent research suggests that additional species may be identified in the future, as new Listeria isolates obtained from soil have not yet been classified (CARLIN et al., 2021). Among all Listeria species, only two are considered pathogenic: *L. monocytogenes* and *L. ivanovii*. While *L. ivanovii* is predominantly an animal pathogen affecting ruminants and sheep (VAZQUEZ-ARMENTA et al., 2020), *L. monocytogenes* is responsible for the vast majority of human cases (THE EUROPEAN UNION ONE HEALTH 2022 ZOONOSES REPORT, 2023).

*L. monocytogenes* is widely distributed in nature and can be commonly found in soil, where it lives as a saprophyte on decomposing plant matter (VÁZQUEZ-BOLAND et al., 2001; LINKE et al., 2014). While it primarily exists in this saprophytic state, it is capable of making the transition into a pathogen following its ingestion by a susceptible host, highlighting the importance of understanding its transmission routes (FREITAG et al., 2009). *L. monocytogenes* is a highly adaptable pathogen that can tolerate a wide range of environmental conditions. One of the key characteristics of *L. monocytogenes* is its ability to adapt to, resist, survive, and even grow under challenging conditions including food related stress factors. This adaptability allows the pathogen to persist in various food environments. One of its most notable features is its ability to grow at low

temperatures, with a minimum growth temperature of around 4 °C. This makes it a particular concern in refrigerated foods, as it can continue to multiply even under refrigeration.

In addition to its psychotropic nature, *L. monocytogenes* is also capable of tolerating a wide range of pH levels, from acidic to alkaline conditions. It can survive in environments with a pH as low as 4.7 and as high as 9.2, making it highly versatile in terms of the types of food it can contaminate.

Furthermore, *L. monocytogenes* is also able to withstand large differences in osmolarity, meaning it can survive in environments with varying salt concentrations. This makes it a potential concern in a variety of foods, from deli meats to fresh produce.

Overall, the adaptability of *L. monocytogenes* to diverse environmental conditions makes it a challenging pathogen to control and a significant concern for food safety (PHAN-THANH und MONTAGNE, 1998; BUCUR et al., 2018).

*L. monocytogenes* is a rod-shaped, gram positive, facultative anaerobic, bacterium with a size of 0.5×2–3 µm. As a facultative anaerobe, it is capable of growing in both aerobic and anaerobic conditions (VÁZQUEZ-BOLAND et al., 2001; PIZARRO-CERDÁ und COSSART, 2019). It is a catalase positive, non spore-forming and non-capsule producing bacterium. As a low G+C bacterium, its genomic DNA contains a lower proportion of guanine and cytosine bases (VÁZQUEZ-BOLAND et al., 2001). It is an intracellular pathogen capable of growing and replicating within host cells, including macrophages and non-phagocytic cells (COSSART, 2011). *L. monocytogenes* is motile at the temperatures between 10-25 °C, during which it forms 4-6 flagella per cell. These whip-like structures that help the bacterium to swim and move in its environment (PEEL et al., 1988). The motility of *L. monocytogenes* is important since it contributes to its ability to colonize various ecological niches and invade host tissues during infection (QUEREDA TORRES et al., 2021; SIBANDA und BUYS, 2022).

Understanding the genetic diversity and population structure of *L. monocytogenes* is crucial for epidemiological investigations, as it allows for the identification of strains associated with specific outbreaks and sources of contamination. *L. monocytogenes* exhibits a high degree of genetic diversity. The serotyping system, which is based on somatic (O) and flagellar (H) antigens, identifies 13 serovars. These serovars represent diverse genetic groups of strains, highlighting the extensive genetic variability within the *L. monocytogenes* population. However, only four serotypes - 1/2a, 1/2b, 1/2c, and 4b - are responsible for the majority of human listeriosis cases. The population structure of *L. monocytogenes* can be further divided into three lineages. Lineage I includes serotypes 4b, 1/2b, 3b, 4d, 4e, and 7, while Lineage II encompasses serotypes 1/2a, 1/2c, 3a, and 3c and lineage III contains serotypes 4a and 4c (COSSART, 2011). There are several methods to classify and divide listeria into subgroups, including PCR (DOUMITH et al., 2004), pulsed-field gel electrophoresis (PFGE) (NEVES et al., 2008), multilocus variable-number tandem-repeat analysis (MLVA) (MARTÍN et al., 2018), multilocus sequence typing (MLST) (RAGON et al., 2008), and core genome MLST (cgMLST) (MOURA et al., 2016). MLST, which involves sequence analysis of specific housekeeping genes (DOUMITH et al., 2004), has been used to identify clonal complexes of *L. monocytogenes* that are distributed geographically or temporally (CHENAL-FRANCISQUE et al., 2011). *L. monocytogenes* clonal complexes are groups of closely related strains that share similar genetic characteristics. CC1, CC2, CC4, and CC6 are hypervirulent clones that are highly associated with human listeriosis. On the other hand, CC9 and CC121 are associated with food processing environments and various food such as dairy products, and meat products and have shown to be hypovirulent due to mutations in virulence genes (MAURY et al., 2019).

## 2.2. Listeriosis in humans

*L. monocytogenes* is a facultative pathogen that can lead to serious health consequences. In healthy and immunocompetent individuals, oral intake of *L. monocytogenes* may result in no to mild symptoms, which typically manifest as self-limiting gastroenteritis or flu-like symptoms (RADOSHEVICH und COSSART, 2018).

Nevertheless, *L. monocytogenes* can cause severe health problems, particularly in susceptible individuals. Clinical presentations include febrile gastroenteritis, septicemia, neonatal infection, meningoencephalitis and miscarriage (SIEGMAN-IGRA et al., 2002; DREVETS und BRONZE, 2008). While pasteurization and cooking can kill *L. monocytogenes*, RTE foods are often the cause of illness because RTE products are often consumed immediately by the consumer without reheating. Recontamination often occurs prior to packaging, even if these foods undergo a heating step during production. After ingestion, *L. monocytogenes* can spread via the blood stream and establish itself in the central nervous system causing meningoencephalitis or the placenta, causing abortion (BUCHANAN et al., 2017). In Germany, listeriosis cases have been subject to mandatory reporting since 2001. As a result, around 500 cases are registered each year, with the majority affecting pregnant women (HOF et al., 2007). This underscores the importance of implementing effective prevention strategies and control measures to reduce the public health burden of *L. monocytogenes* infections, particularly among high-risk populations.

Food safety strategies and measures vary between countries, reflecting differences in regulatory approaches. For instance, the United States maintain a strict zero-tolerance policy for *L. monocytogenes* in food, while European regulations reflect a risk-based approach taking into account the relatively high minimal infectious dose of *L. monocytogenes* and allow the presence of minimal amounts under specific conditions.

### 2.2.1. The intracellular infection cycle

The intracellular infection cycle takes place in the following steps (DE LAS HERAS et al., 2011):

(1) *L. monocytogenes* utilizes two surface proteins, internalin A and internalin B, for cell entry. These proteins interact with the surface ligands E-cadherin and c-Met of the host cell via their leucin-rich repeat domains.

(2) This interaction initiates a process similar to phagocytosis, in which the bacterium is taken up into the host cell.

(3) Once inside the cell, *L. monocytogenes* employs three membrane damaging factors: the pore-forming toxin listeriolysin O and the phospholipases PlcA and PlcB, which lyse the vacuole membrane.

(4) Upon release into the host cell cytosol, *L. monocytogenes* can multiply using hexophosphates from the host cell, which are be taken up via the Hpt permease.

(5) A major advantage of this intracellular infection strategy is that *L. monocytogenes* can spread directly from cell to cell, thereby evading the host's extracellular defense mechanisms. This cell-to-cell spread is facilitated by the surface protein ActA.

(6) During the invasion of the neighboring cells, the bacteria protrude as structures called listeriopods. This process is assisted by internalin C, which reduces the tension between the cell walls.

(7) After invasion, the double membrane vacuole in the newly infected cell is dissolved, allowing a new round of bacterial proliferation to begin

One of the most important proteins for this cycle is the central regulatory factor A (PrfA). It can be described as a key regulatory protein that plays a significant role in coordinating the expression of essential virulence factors and it play a crucial in the infection cycle because it regulated all nine of the genes mentioned in the intracellular infection cycle above. PrfA ensures the timely and effective expression of virulence genes that enable bacteria to infect and replicate within host cells (SCORTTI et al., 2007). To ensure that the PrfA regulon is only active during the infection of a host cell and not while *L. monocytogenes* is residing in the environment, its activation is selectively regulated. This regulation is thermoregulated and responsive to the 37°C and low pH levels. The PrfA regulon

remains inactive at ambient temperatures, but when the bacterium encounters the elevated temperatures and acidic conditions within a hosts stomach, the regulon is activated, allowing for the coordinated expression of virulence factors necessary for infection (FREITAG et al., 2009; NEUHAUS et al., 2013). PrfA consists of two identical subunits as a homodimer and the symmetrical PrfA dimer activates transcription by binding through its Helix-turn-helix (HTH) pair to a palindromic 'PrfA box' (SCORTTI et al., 2007; DESHAYES et al., 2012). *PrfA* is a part of the Listeria pathogenicity island-1 I (LIPI-1), which also includes the *inlAB* operon, the *inlC* and *hpt* monocistrons (WIKTORCZYK-KAPISCHKE et al., 2021). LIPI-1 includes key virulence factors, such as LLO (hly gene), InlA, InlB, ActA, PlcA, PlcB, Mpl, InlC, Hpt and PrfA itself. In addition to these nine key virulence determinants that are tightly regulated by PrfA, Transcriptomic profiling and proteomic analyses have revealed that PrfA also has a regulatory role on the expression of as many as 145 other *L. monocytogenes* genes (MILOHANIC et al., 2003).

## 2.3. Stress response mechanisms of *L. monocytogenes*

### 2.3.1. Alternative sigma factors

*L. monocytogenes* employs several mechanisms to adapt to challenging environmental conditions; for instance, when the bacterium is ingested orally, it must rapidly adapt to the harsh conditions of the host's stomach. *L. monocytogenes* uses different sigma factors to regulate gene expression in response to changing environmental conditions. Sigma factors are multi-domain subunits of the RNA polymerase, play a critical role in transcription initiation by altering the promoter recognition specificity of the enzyme (PAGET, 2015). *L. monocytogenes* has one housekeeping sigma factor ($\sigma^A$) and four different alternative sigma factors ($\sigma^B$, $\sigma^C$, $\sigma^H$ and $\sigma^L$) (GLASER et al., 2001). Among them, σB plays a crucial role in regulating the transcription of several virulence and stress-response genes (CHATURONGAKUL et al., 2008). For example, σB is very important for survival in food, food-processing environments or in the stomach of the host, as it regulates the expression of genes that mediate survival under acid stress, osmotic stress, oxidative stress, cold stress and nutrient deficiency or energy stress (KAZMIERCZAK et al., 2003; SUE et al., 2004).

In addition to σB, *L. monocytogenes* encodes other alternative sigma factors, such as σH and σL. σH participates in the transition from exponential growth phase to stationary phase, sporulation, nutrient transport and the regulation of several other transcription factors and cell wall proteins. Moreover, σH appears to be essential for survival in phagocytes and epithelial cells, as it facilitates the escape of *L. monocytogenes* from phagosomes (MEDRANO, 2018). On the other hand, σL contributes to osmotolerance and also provides some resistance against the antibacterial peptide mesentericin (ROBICHON et al., 1997).

σB also acts synergistically with the positive regulatory factor A (PrfA), the master regulator of virulence gene expression in *L. monocytogenes*. These two proteins coregulate genes involved in bile resistance and internalization. Most importantly, σB modulates *prfA* transcription via through direct transcriptional activation and indirect post transcriptional repression under some environmental conditions (GABALLA et al., 2019).

### 2.3.2. Temperature stress

*L. monocytogenes* produces various proteins that help protect the bacterium from damage caused by environmental stressors. These proteins include heat-shock proteins, which help the bacterium cope with high temperatures by stabilizing the proteins and prevent them from improper folding and aggregation; cold-shock proteins, which aid in adaptation to low temperatures, by various strategies such as enabling replication, transcription, and translation at low temperatures (SCHÄRER et al., 2013; MUCHAAMBA et al., 2021) and oxidative stress response proteins, which counteract the harmful effects of reactive oxygen species (WIKTORCZYK-KAPISCHKE et al., 2021).

OppA, Ctc, GroEL, and DnaK are considered particularly important for cold stress adaptation. The OppA protein is important for the accumulation of short peptide substrates, facilitating bacterial growth under cold stress (BOREZEE et al., 2000). CtC, is a general stress protein, which supports the adaptation to high osmolarity conditions (GARDAN et al., 2003). GroEL and DnaK, both molecular chaperones, assist in the degradation of damaged proteins or initiate refolding, which is also essential for coping with heat stress-induced protein damage (GAHAN et al., 2001). Under extreme heat stress, above 45 °C, *L. monocytogenes* synthesizes heat shock proteins to prevent unproductive protein aggregation.

Furthermore, ferritin-like protein has been found to be present at both temperature extremes, indicating its significance in adapting to various environmental stressors (AGOSTON et al., 2009).

### 2.3.3. Agr System

Biofilms can form on food processing equipment or packaging materials, increasing the risk of contamination. Biofilms provide a protective environment for bacteria, including *L. monocytogenes*, allowing them to survive and persist in harsh conditions, which may contribute to the challenges in eliminating this pathogen from food processing environments (COLAGIORGI et al., 2017). The accessory gene regulator (Agr) system in *L. monocytogenes* is associated with quorum-sensing, the sensing of environmental stresses and the coordination of appropriate responses within the bacterial population (GUARIGLIA-OROPEZA et al., 2014). These functions contribute to the establishment of biofilms and virulence regulation under severe conditions (AUTRET et al., 2003; RIEU et al., 2007; RIEDEL et al., 2009; BANERJI et al., 2022). In *L. monocytogenes*, the agr system comprises four genes (*agrA, agrB, agrC and agrD*) organized as an operon. Specifically, *agrA* encodes a response regulator protein that modulates gene expression in response to environmental signals and cell density, *agrB* is responsible for processing of agrD into a mature auto-inducing peptide (ZETZMANN et al., 2016), *agrC* encodes a two-component histidine kinase protein that senses AIP (autoinducing peptides) concentration in the environment and initiates signal transduction pathways, and agrD serves as a precursor peptide, which is considered to positively regulates the expression of agr system.

# 3. *L. monocytogenes* in foods and food processing environments

## 3.1. Environmental origins and transmission routes along the food chain

*L. monocytogenes* is ubiquitous in the environment, and there are many potential sources through which this bacterium can enter the feed and food chain (HAASE et al., 2014). *L. monocytogenes* can establish itself in various niches in agricultural production operations or food processing plants, mainly via contamination with soil or fecal excretion from a wide range of hosts, including human or wild and domestic animals (BELIAS et al., 2022). This problem is exacerbated by the fact that some individuals in the host pool may be asymptomatic carriers, thus unknowingly spreading the pathogen (SCHODER und WAGNER, 2012).

*L. monocytogenes* can persist in food processing plants or equipment for extended periods of time. Especially in humid environments, it can spread rapidly from the food processing environment to food products (JEMMI und STEPHAN, 2006; GANDHI und CHIKINDAS, 2007).

Several studies have reported the presence of *L. monocytogenes* in different farm, pet and game animals. In Germany *L. monocytogenes* was detected in the feces of 1-10% of healthy pigs, hens, sheep, horses, dogs, and cats and >30 % of cows (WEBER et al., 1995). A study in healthy red deer found *L. monocytogenes* in tonsils, rumen and stomach contents, liver, intestinal lymph nodes, cecum content and feces (42 % animals were positive) and wild boars (25 % animals were positive) (WEINDL et al., 2016). These findings suggest that a significant number of animals could be asymptomatic carriers of *L. monocytogenes*. Additionally, it is estimated that up to 10% of the German human population may harbor *L. monocytogenes* in their intestines (BAYERISCHES LANDESAMT FÜR GESUNDHEIT UND LEBENSMITTELSICHERHEIT, 09.08.2022).

Potential transmission routes of *L. monocytogenes* along the food chain include the farm environment where *L. monocytogenes* can be introduced through animal feed, contaminated water, poor-quality silage or feces of animals. Once established, it can spread within a farm, contaminating various areas including soil, vegetation, and animal housing (RODRIGUEZ et al., 2021).

1. Primary production: During primary production, such as plant cultivation or animal rearing, *L. monocytogenes* can contaminate products through direct or indirect contact with contaminated soil, water, or surfaces in the production environment or infected animals (GONZALES-BARRON et al., 2023).

2. Harvest and post-harvest handling: Contamination can occur during harvesting, handling, and transportation of products, either directly from the environment or through cross-contamination from other products or equipment (MARIK et al., 2020).

3. Food processing: *L. monocytogenes* can be introduced to or spread within food processing facilities through contaminated raw materials or inadequate sanitation practices. The bacterium can form biofilms on surfaces such as equipment, floors and drains making it difficult to eliminate (CAMARGO et al., 2017).

4. Distribution and retail: Improper storage conditions or cross-contamination during transportation, distribution, or at retail outlets can lead to further spread of the bacterium (LIANOU und SOFOS, 2007).

5. Food preparation and consumption: Inadequate cooking or handling practices in households, restaurants, or other food service establishments can result in contamination or growth of *L. monocytogenes* in foods.

These potential transmission routes are crucial for identifying critical control points and implementing effective preventive measures to reduce the risk of *L. monocytogenes* contamination along the entire food chain.

### 3.2. Outbreaks related to *L. monocytogenes*

Although most cases of listeriosis are sporadic, outbreaks occur frequently and involve various food matrices. The table provided below summarizes notable outbreaks where more than 100 cases were involved and the first recognized outbreak related to *L. monocytogenes* in different food matrices (Table 5).

**Table 5:** Some Major Listeriosis Outbreaks

| Outbreak year | Location | Food vehicle | Cases | Reference |
|---|---|---|---|---|
| 1981 | Canada | Coleslaw | 41 | (SCHLECH III et al., 1983) |
| 1983-1987 | Switzerland | Vacherin Mont dór cheese | 122 | (BILLE, 1989) |
| 1985 | California (USA) | Mexican-style cheese | 142 | (LINNAN et al., 1988) |
| 1987-1989 | England | Paté | 300 | (MCLAUCHLIN et al., 1991) |
| 1992 | France | Jellied pork tongue | 279 | (GOULET et al., 1993) |
| 1997 | Italy | Corn | 2930 | (AURELI et al., 2000) |
| 2011 | USA | Fresh whole cantaloupe | 147 | (MCCOLLUM et al., 2013) |
| 2017/18 | South Africa | RTE processed meat products | 1060 | (SMITH et al., 2019) |

### 3.3. *L. monocytogenes* in ready-to-eat (RTE) food, specifically RTE meat

RTE food refers to any food product that is intended for direct consumption, without the need for further preparation, such as cooking, reheating, or processing in any other way (EUROPEAN FOOD SAFETY AUTHORITY, 21 December 2023). These foods are typically prepared and packaged by the manufacturers, making them convenient and easily accessible for consumers. RTE foods include a variety of products, such as fresh or pre-cut fruits and vegetables, deli meats, salads, sandwiches or cheese (HILLIER-BROWN et al., 2017).

While raw products like fruit and vegetables inherently bear the risk of *L. monocytogenes* contamination, thermal processing like cooking or

pasteurization is generally effective in eliminating *L. monocytogenes*, provided that the appropriate temperatures are maintained. However, post-processing recontamination is possible due to inadequate hygiene in the food processing environment. Factors such as unclean machinery and/or insufficient personnel hygiene practices can contribute to the reintroduction of *L. monocytogenes* or other pathogens into the food products (BAYERISCHES LANDESAMT FÜR GESUNDHEIT UND LEBENSMITTELSICHERHEIT, 09.08.2022). For deli meats, the slicing and packaging stages of food production present a particularly high risk for contamination by *L. monocytogenes* from equipment surfaces, packaging materials, and handling by personnel. The increased potential for cross-contamination at these stages makes it crucial to implement and adhere to strict food safety protocols, including proper sanitation, hygienic handling practices, and regular microbial testing. By addressing these high-risk points in the production process, the likelihood of contamination can be significantly reduced, thereby ensuring the safety and quality of ready-to-eat food products (SHEEN und HWANG, 2008).

Foods of animal origin that are frequently contaminated with *L. monocytogenes* comprise cooked sausage (in particular if sliced to a RTE format), sausage and meat pâtés, short- or quick- matured raw sausage varieties (such as onion sausage and tea sausage), raw meat products (like tartare, minced meat), raw unpasteurized milk and dairy products made from it (e.g. raw milk cheese), soft cheeses (especially those with red smear formation like Romadur and Roquefort), and seafood products like cold smoked salmon (BAYERISCHES LANDESAMT FÜR GESUNDHEIT UND LEBENSMITTELSICHERHEIT, 09.08.2022). Because their high water activity ($a_w$-value) and nutrient content makes many RTE foods a good growth substrate for bacteria, their safety relies on refrigeration to prevent bacterial growth. However, *L. monocytogenes* is psychrotrophic, meaning it can still grow at refrigerator temperatures, albeit more slowly. This ability allows *L. monocytogenes* to multiply and potentially reach dangerous levels in these products over time, in particular where high initial contamination levels are combined with abusive refrigeration temperatures above 4 °C (ZIEGLER et al., 2018; ZIEGLER et al., 2019). Therefore, contaminated RTE foods are the primary transmission vehicles for human *L. monocytogenes* infections (SELF et al., 2019; HALBEDEL et al., 2020; THOMAS et al., 2020).

It is assumed that a value of 100 CFU/g or more poses a direct risk to human health. While in the USA a zero-tolerance approach with regard to *L. monocytogenes* in RTE foods is taken, the European laws take this relatively high infectious dose into account and aim to keep the contamination levels in RTE foods below 100 CFU/g throughout the shelf life. (European Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs).

**Table 6:** Food safety criteria (EUROPEAN UNION, 2005)

| **Food category** | **Sampling-plan** | | **Limits** | | **Analytical reference method** | **Stage where the criterion applies** |
|---|---|---|---|---|---|---|
| | **n** | **C** | **m** | **M** | | |
| RTE foods intended for infants and RTE foods for special medical purposes | 10 | 0 | Absence in 25 g | | EN/ISO 11290-1 | Products placed on the market during their shelf-life |
| RTE foods able to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes | 5 | 0 | 100 cfu/g | | EN/ISO 11290-2 | Products placed on the market during their shelf-life |
| | 5 | 0 | Absence in 25 g | | EN/ISO 11290-1 | Before the food has left the immediate control of the food business operator, who has produced it |
| RTE foods unable to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes | 5 | 0 | 100 cfu/g | | EN/ISO 11290-2 | Products placed on the market during their shelf-life |

In the list above, n describes the number of random samples to be taken and c the permitted number of samples taken, which may be above m or between m and M. If the manufacturer can prove to the competent authorities that 100 CFU/g are not exceeded during the entire shelf life, these apply to him. If he is unable to do so, samples from the food company must not contain any *L. monocytogenes* in 25 g.

# 4. Lyoner

## 4.1. Classification according to the German Food Code

According to the guidelines for meat and meat products of the German Food Code, the chicken Lyoner used in this study is classified in the sub-category “finely chopped emulsified sausage” (BUNDESMINISTERIUM FÜR ERNÄHRUNG UND LANDWIRTSCHAFT, 20.10.2022).

## 4.2. Composition

The name of the product (Hähnchen-Lyoner) already provides some information on the composition of the sausage. The first part of the name "Hähnchen" (chicken) indicates that all of the material of animal origin used for this product is derived from chicken, i.e. meat, connective and fat tissue. As an ingredient of meat products "meat" only refers to the skeletal muscles with attached or embedded fat and connective tissue as well as embedded lymph nodes, nerves and blood vessels. The second part of the name “Lyoner” is a type of emulsified sausage, which is specified in the German Food Code as a cured sausage, which means that nitrite curing salt is used, resulting in the characteristic color of Lyoner. The Food Code further states that "Lyoner" is filled in medium or large caliber casings during production. In addition, certain quality criteria must be met, such as a minimum amount of at least 8% of meat protein free of connective tissue protein. This so-called "BEFFE" is defined as total protein minus the sum of foreign protein, foreign non-protein nitrogen compounds and connective tissue protein (BUNDESMINISTERIUM FÜR ERNÄHRUNG UND LANDWIRTSCHAFT, 20.10.2022). The raw materials raw chicken meat, chicken fat, drinking water in the form of ice and nitrite curing salt a sausage meat

batter, which is then filled into appropriate casings and scalded using either steam or submersion in hot water at a temperature of at least 75 °C.

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*Listeria monocytogenes* uses *de novo* purine synthesis to enhance fitness in


Lyoner-type sausage

Philipp-Michael Beindorf [a,*], Jule Anna Horlbog [b], Irene Esteban Cuesta [a], Claudia Guldimann [a], Irmak Şah [a]

[a] *Food Safety and Analytics, Faculty of Veterinary Medicine, Ludwig-Maximilians-University Munich (LMU), Scho¨nleutnerstrasse 8, 85764, Oberschleissheim, Germany*

[b] *Institute for Food Safety and Hygiene, Vetsuisse Faculty, University of Zürich, Zürich, Switzerland*

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## A B S T R A C T

*Listeria monocytogenes* is an important food-borne pathogen with high hospitalization and case fatality rates. To cause disease, *L. monocytogenes* must gain access to a specific food matrix and in many cases be able to grow before the food is consumed. The fitness of this pathogen differs between individual foods and depends on its ability to adapt to various environmental stressors that are highly specific in each food matrix. Deli meats are an important cause of infections with *L. monocytogenes*. Here, we screened a transposon mutagenesis library in

*L. monocytogenes* LL195 with 2640 individual mutants on a Lyoner-type deli meat to understand the fitness effect of individual genes. After the determination of 10 candidate mutants with confirmed phenotypes on Lyoner, in- frame deletion mutants of these genes were created by allelic replacement. The fitness effects of these in-frame mutants were then confirmed by growth experiments under cold stress and on Lyoner separately, and in a combined condition of both. Mutants with deletions in the cytosine-specific DNA modification methyltransferase *sau3AIM* and the penicillin-binding protein *pbp-B* were impaired in their growth at cold temperatures in rich medium as well as on Lyoner, suggesting a temperature-dependent phenotype. In contrast, the *purB* deletion mutant exhibited reduced fitness that was specific to growth on Lyoner. Our results indicate an important role for the *sau3AIM* and *pbp-B* genes in cold stress adaptation, while *purB*, which is a central component of purine biosynthesis, may play a more specialized role on the fitness of *L. monocytogenes* during growth on Lyoner.

## 1. Introduction

*Listeria monocytogenes* is the causative agent of listeriosis, which re- mains one of the most severe food-borne illnesses in the European Union (The European Union One Health 2021 Zoonoses Report, 2022) and worldwide. The latest available report by the European Food Safety Agency (EFSA) documents 23 foodborne outbreaks and 2183 cases with a case-fatality rate of 9% in the EU in 2021 (The European Union One Health 2021 Zoonoses Report, 2022). Ready-to-eat (RTE) foods like smoked fish, soft cheeses, and deli meats, are overrepresented among foods implicated in listeriosis cases and outbreaks (Lopez-Valladares et al., 2018). The primary reason these products represent a high risk is that there is either no killing step during their production or, in case of deli meats, events of re-contamination during processes like slicing. Sliced RTE deli meats have frequently been implicated in *L. monocytogenes* outbreaks and show a global prevalence of listeriosis of

2.9 % (with considerable heterogeneity) as determined by a systematic review and meta-analysis (Churchill et al., 2019). Several quantitative microbial risk analysis models estimate that most cases in the EU and the USA originate from RTE deli meats (Sampedro et al., 2022). The heating step in the production of most deli meats makes intact cooked sausages safe for consumption. The high number of listeriosis cases associated with deli meats results from post-processing contamination of the finished product during slicing and packaging (Sheen & Hwang, 2008). Once *L. monocytogenes* is present on a given food, the intrinsic factors such as nutrient content, pH, water activity ($a_w$), presence of antimicrobial compounds and extrinsic factors such as oxygen availability, storage and transportation conditions determine whether and at what rate the pathogen is able to grow. As a saprophyte, *L. monocytogenes* is ubiquitously present in the natural environment and has a unique ability to adapt and survive under challenging conditions, including food- related stress conditions (Bucur et al., 2018). They can grow in a wide pH range (4.7–9.2), at temperatures from —0.5 to 45 °C, in high salt concentrations, and in the presence of preserving additives like bacteriocins.

As facultative anaerobes, they may also grow under anaerobic conditions (Chaturongakul et al., 2008).

Challenge tests on different food matrices show that phenotypic characteristics of *L. monocytogenes* vary on different food matrices (Spanu et al., 2014). Several studies have been conducted to establish genes and molecular mechanisms that underlie its adaptation to different niches to date: the alternative sigma factor $\sigma^B$ acts as a global transcriptional regulator under many different stress conditions such as acidic, osmotic, or oxidative conditions, cold stress and nutrient limitations (Guerreiro et al., 2020). The response of *L. monocytogenes* to specific environmental stressors typically results in global changes in gene and protein expression profiles, and specific regulatory responses to pH, temperature, osmotic, oxidative, or abiotic stresses are well documented (reviewed in Bucur et al., 2018; Guerreiro et al., 2020). However, these studies were typically carried out in laboratory culture media. According to the limited studies done in food matrices, gene expression profiles of different *L. monocytogenes* strains varied significantly when they were grown in various foods (Olesen et al., 2010; Rantsiou et al., 2012). There are also variations in the expression level of the stress response, virulence, and adhesion genes between laboratory media and food matrix (Rantsiou, Mataragas, et al., 2012). One draw- back of transcriptome studies is that a significant change in gene expression does not always significantly affect fitness (Feder & Walser, 2005). Therefore, screening random mutants for fitness effects of individual genes on the food matrix complements and expands these data. Most of the available data on the resilience of *L. monocytogenes* in the food environment has been established using well-characterized laboratory reference strains such as EGD-e and 10403S. However, these strains do not represent clinically important clonal complexes, nor do they cover the clinically relevant lineage II or serotype 4b, and passaging may have led to the loss of crucial pathophysiological characteristics (B´ecavin et al., 2014). Therefore, results should be interpreted not only considering the experimental conditions but also in the light of the genetic background of the bacterial strains used (Fux et al., 2005). In summary, there is a significant knowledge gap in our understanding of the interactions between clinically relevant strains of *L. monocytogenes* and the food

matrices they are most associated with.

However, a better understanding of the molecular mechanisms behind the resilience of *L. monocytogenes* on different food matrices is crucial for the development of tailored and effective control methods. Therefore, the aim of this study was to elucidate the fitness contribution of individual genes of *L. monocytogenes* and to investigate their growth dynamics on a high-risk food matrix. We chose sliced Lyoner sausage as a model matrix because (i) Lyoner-type sausages are very popular in Europe, (ii) as deli-meats they are often associated with listeriosis cases, and (iii) their similarities with South-African "polony" that was responsible for the world's largest listeriosis outbreak in 2017–2018 (Smith et al., 2019). In this study, to overcome the disadvantages of using reference strains, we chose to work with an outbreak, serotype 4b strain of *L. monocytogenes* (Weinmaier et al., 2013).

## 2. Materials and methods

### 2.1. *Bacterial strains, and growth conditions*

The *L. monocytogenes* strain LL195 was used for the following experiments. This strain was isolated during an outbreak in Switzerland between 1983 and 1987, which was associated with Vacherin Mont-d'Or cheese (Weinmaier et al., 2013). *L. monocytogenes* LL195 was grown in Brain Heart Infusion (BHI, Merck KGaA, Germany) broth or on BHI agar (BHI + 1,5 % Agar Technical LP0012B, Oxoid Deutschland GmbH, Germany) unless otherwise stated. *Escherichia coli* Top10 strain was used as a host for the cloning experiments and grown in Luria Bertani (LB, Carl Roth GmbH, Germany) Broth or on LB agar (LB, Carl Roth GmbH + 1,5 % Agar Technical, Oxoid Deutschland GmbH, Germany) plates.

### 2.2. *Cloning of the transposon library*

To clone the Tn-library in LL195, we used pJZ037 and the protocol described by Zemansky et al. (Zemansky et al., 2009). pJZ037 contains a 1423 bp transposon that integrates into "TA" recognition sites via a mariner C9 transposase. In short, the plasmid pJZ037 was prepared using the Qiagen QIAprep Spin Miniprep kit (Qiagen, Cat. 27,104). To obtain electrocompetent *L. monocytogenes* LL195, cells were grown in 50 ml BHI (Biorad, Hercules, USA) with 0.5 M sucrose (Fluka Honeywell, Charlotte, USA) at 37 °C with shaking at 200 rpm until they reached an $OD_{600}$ of 0.2. After addition of 10 µg/ml penicillin (Sigma-Aldrich, St. Louis, USA) and further incubation for another 2 h at 37 °C with shaking, the culture was cooled on ice, centrifuged at 5000×*g* at 4 °C and washed twice with 1 M HEPES (Sigma-Aldrich, St. Louis, USA) with 0.5 M sucrose. The resulting pellet was resuspended in 0.4 ml 1 M HEPES with 0.5 M sucrose. Aliquots of 100 µl of these electrocompetent cells were used to electroporate 1 µg pJZ037 in 0.1 mm cuvettes and 1.8 kV for 5 ms, followed by the addition of 1 ml BHI and incubation for 1 h at 30 °C without shaking. Dilutions of the resulting cultures were plated on BHI plates containing 7.5 µg/ml chloramphenicol (Sigma-Aldrich, St. Louis, USA) and incubated at 30 °C (permissive for plasmid replication) for 72 h. Colonies were then plated onto BHI agar plates containing 1 µg/ml

erythromycin (Sigma-Aldrich, St. Louis, USA) and incubated at 43 °C (the non-permissive temperature for plasmid replication) for 48 h to cure the plasmid. From these plates, colonies that were resistant to erythromycin but sensitive to chloramphenicol were determined by replica-plating, picked and subcultured into 96-well plates. An addi- tional passage in BHI with 1 µg/ml erythromycin at 43 °C, 24 h and control to confirm no growth in BHI with 7.5 µg/ml chloramphenicol at 30 °C, 24 h were performed in this 96-well format. The *L. monocytogenes*

LL195 genome size is 2,904,662 bp (Weinmaier et al., 2013), and we aimed to produce a transposon insertion at least every 1200 bp on average. Therefore, mutant collection was continued until a collection of at least 2420 mutans was achieved. The final transposon library contains a collection of 2640 individual

mutants, corresponding to a Tn insertion roughly every 1100 bp and was stored at —80 °C in 96-well plates in BHI containing 1 µg/ml erythromycin and 15 % glycerol (Sigma-Aldrich, St. Louis, USA).

### 2.3. *Screening of the transposon library on a German deli meat*

For the pre-screening, the frozen library was inoculated into 96-well plates (U bottom, Greiner BioOne, Germany), each containing 200 µl of BHI broth, using a 96 pin-replicator made of stainless steel. The plates were then incubated overnight at 37 °C with continuous shaking at 250 rpm in a microplate shaker (88-861-024 Fisherbrand™, Thermo Fisher, USA). Each plate contained three replicate wells with the wild type (WT) strain as a control. These pre-cultures were subcultured 1:100 into fresh BHI in 96 well plates and incubated until the WT strain reached early log phase corresponding to $OD_{600}$ of 0,3–0,4 and $10^8$ CFU/ml (Bio-photometer 6131, Eppendorf SE, Germany) at 37 °C with 250 rpm shaking. This culture was used to inoculate miniaturized matrix pieces in 96-well plates. For the matrix, we chose slices of boiled chicken sausage called Lyoner (REWE BIO Hähnchen, REWE Markt GmbH, Germany). Using a custom-made stainless-steel punch (square base with 96 bars with diameter of 0.5 cm), pieces weighing an average of 45.3 mg were extracted and placed into individual wells of a 96-well plate. To ensure uniform positioning at the bottom of the wells, the plates were centrifuged (Eppendorf SE, Germany, Centrifuge 5804 R) at 500×*g* for 1 min. A total of $10^5$ cells were inoculated on these slices of Lyoner and incubated at 8 °C for 48 h. Growth at the $t_0$ time point of the inoculum was compared with the growth on the food matrices after incubation at 8 °C for 48 h by using the running drop method on oxford agar (OX, Merck KGaA, Germany) (Küchbacher, Cossart, & Pizarro-Cerda, 2021). For this, 10 µl of the liquid cultures were carefully dispensed at the top of Oxford agar plates and tilted to allow the drops to run the length of the plate in parallel tracks. In this manner, six samples can be plated on one plate. The plates were then incubated at 37 °C. Following the incubation period, the colonies were counted to determine the colony-forming units (CFUs).

The propagation of the mutant library was compared with the propagation of the WT strain, *L. monocytogenes* LL195. This method was used to identify candidate mutants whose growth on Lyoner differed from the WT. The phenotype of these candidates was confirmed in a larger volume and compared with that of the WT. For this, candidate mutants were grown on BHI agar plates overnight at 37 °C. A few colonies were transferred into 5 ml BHI broth in tubes (Test Tube Soda glass, 100 × 16,00 × 0,8 - 1 mm, round bottom, VWR International GmbH, Germany) and were incubated overnight (MaxQ 6000, Thermo Fisher, USA) at 37 °C with continuous shaking at 200 rpm. They were then subcultured 1:100 into 5 ml fresh BHI broth and incubated until the WT strain reached early exponential phase corresponding to $OD_{600}$ of 0, 3–0,4 corresponding to roughly $10^8$ CFU/ml at 37 °C with 250 rpm shaking. Two hundred microliters of the main culture were inoculated onto a slice of Lyoner (10 g, diameter of each slice was 9,3 cm), placed in sterile petri dishes (633,180, Greiner BioOne, Germany), and incubated at 8 °C for 48 h. Quantification was performed on oxford agar (Merck KGaA) plates that were incubated at 37 °C for 24 h. These larger volume screenings were repeated three times.

### 2.4. *Identification of transposon insertion sites in candidate mutants*

In candidate mutants with a confirmed phenotype on Lyoner, candidate genes were identified by determining the exact location of the Tn in the genome using a slightly modified, nested PCR protocol as described in (Zemansky et al., 2009). Primers used to identify the exact location of transposon are listed in Supplementary Table 1. A colony touchdown PCR was performed using the Tn1 and Arb1 primers. The PCR mixture contained 0.5 U of Taq DNA Polymerase (GoTaq® DNA Polymerase, Promega Corporation, USA), 1*X* GoTaq DNA Polymerase Buffer, 200 µM of each dNTP (Meridian Bioscience Inc., USA) and 0.2 µM of each primer in a final volume of 50 µl. The Touch-down PCR program was designed as follows: pre-denaturation at 95 °C for 5 min, followed by 16 cycles of denaturation at 98 °C for 30 s, primer annealing starting at 48 °C with a 1

°C decrease per cycle for 30 s, and elongation at 72 °C for 2 min. This was followed by an additional 16 cycles of denaturation at 98 °C for 10 s, primer annealing at 61 °C for 30 s, and elongation at 72 °C for 2 min, and a final extension at 72 °C for 5 min. The products were cleaned up with the QIAquick® PCR Purification Kit (cat. No. 28104, QIAGEN GmbH, Germany) following the manufacturer's protocol. 1 µl of these PCR products was employed as templates for a second PCR which is performed to increase specificity. The second PCR mixture was prepared in the same manner as the first PCR but this time with the primers Tn3 and Arb2. The second Touch-down PCR program was designed as follows: pre-denaturation at 98 °C for 3 min, followed by 20 cycles of denaturation at 98 °C for 10 s, primer annealing starting at 60 °C with a 0,5 °C decrease per cycle for 30 s, and elongation at 72 °C for 2 min. This was followed by an additional 20 cycles of denaturation at 98 °C for 10 s, primer annealing at 50 °C for 30 s, and elongation at 72 °C for 2 min, and a final extension at 72 °C for 5 min.

Subsequently, all samples were sequenced (Eurofins Genomics Europe Shared Services GmbH, Germany). The resulting sequences were aligned to the LL195 genome to determine the transposon insertion sites (Geneious Prime, Biomatters Inc., USA). Among those confirmed candidate genes, we selected candidates for the creation of in-frame, non-polar deletion mutants according to the following criteria: genes that were identified with several independent Tn insertions, genes that belong to a pathway that was identified as relevant several times, and, if applicable, genes in processes that might offer the potential for food safety interventions.

### 2.5. *Creation of in-frame deletion mutants*

To exclude polar effects and multiple transposon insertions, in- frame, non-polar mutants of the candidate genes were generated in a LL195 background. To generate in-frame deletion mutants by allelic replacement, pKSV7 (Smith & Youngman, 1992) and pMAD (Arnaud et al., 2004) suicidal plasmids were used. The genomic DNA of *L. monocytogenes* LL195 was isolated by using the ISOLATE II Genomic DNA Kit (Bioline GmbH, Germany). The quantity and the quality of the genomic DNA was measured by nanophotometer (NP80-Mobile, SN M81074, IMPLEN GmbH, Germany). Mutants were created using splicing by overlap extension (SOE). Two separate PCRs were performed to amplify the upstream and downstream regions of each candidate gene using primers shown in Supplementary Table 2. The PCR mixture contained 1× of Q5 High-Fidelity 2× Master Mix (New Englands Biolabs GmbH, Germany), and 0.5 µM of each primer in a final volume of 50 µl. The PCR program was designed as follows: pre-denaturation at 98 °C for 30 s, followed by 35 cycles of denaturation at 98 °C for 10 s, primer annealing at 55 °C for 30 s, and elongation at 72 °C for 20 s with a final extension at 72 °C for 2 min.

PCR products were purified with the QIAquick PCR Purification Kit according to manufacturer's instructions. Subsequently, overlap extension PCR was performed using the purified fragments as templates, resulting in the fusion of these fragments. The PCR mixture contained 1× of Q5 High-Fidelity 2× Master Mix (New Englands Biolabs GmbH, Germany), and 0.5 µM of each primer (Primer A and Primer D) in a final volume of 50 µl. The PCR program was designed as follows: pre- denaturation at 98 °C for 30 s, followed by 35 cycles of denaturation at 98 °C for 10 s, primer annealing at 59 °C for 30 s, and elongation at 72 °C for 40 s and a final extension at 72 °C for 2 min.

The final PCR products were ligated into pMAD (candidates: hypothetical protein 2, *Sau3AIM*), at the *Bam*HI-HF and *Nco*I-HF restriction sites (New England Biolabs GmbH, Germany) or into pKSV7 (candidates: *pbp-B*, *purB*, *DNA binding protein*, hypothetical protein 1, *Yham*), at the *Sac*I-HF and *Sal*I-HF restriction

sites (New England Biolabs GmbH, Germany). The ligated products were introduced into *E. coli* Top10 electrocompetent cells by electroporation. Transformed cells were plated on LB with ampicillin (100 µg/ml) agar and positive colonies were screened by PCR for the presence of the insert and sequenced. Plasmids with the correct insert were isolated from *E. coli* using the Qiagen QIAprep® MiniPrep Kit. These plasmids were electroporated into *L. monocytogenes* LL195 and transformants were selected on BHI + chloramphenicol (10 µg/ml, Carl Roth GmbH, Germany) for pKSV7 and casein-peptone soymeal-peptone broth (TSB, Merck KGaA, Germany) + erythromycin (5 µg/ml, Merck KGaA, Germany) + X-gal (50 µg/L) for pMAD.

For pKSV7: A few colonies from the transformant plates were selected and propagated in BHI + chloramphenicol (10 µg/ml) medium for 5–6 generations at 42 °C with shaking at 70 rpm for plasmid integration. Colonies in which the plasmid had chromosomally integrated in

*L. monocytogenes* were chosen from those that grew on BHI + chloramphenicol (10 µg/ml) agar plates. These selected colonies were further propagated in BHI without antibiotics at 30 °C with shaking at 70 rpm for at least 14 generations to eliminate the plasmid. Several colonies were screened to detect the loss of chloramphenicol resistance through replica plating on both BHI + chloramphenicol (10 µg/ml) and BHI plates. Among those displaying chloramphenicol sensitivity, identification of individuals harboring a mutant allele within the chromosome was achieved using PCR with A and D primers and subsequently confirmed by sequencing.

For pMAD: A few colonies from the transformant plates were chosen, pooled, and cultured in TSB + erythromycin (5 µg/ml) at 37 °C with shaking at 120 rpm for 5 h. Subsequently, the temperature was shifted to 42 °C, and incubation continued overnight to force the integration of the plasmid into the LL195 chromosome. Blue colonies, in which the plasmid had chromosomally integrated in *L. monocytogenes*, were chosen from those that grew on TSB + erythromycin (5 µg/ml) and X-gal (50 µg/L) agar plates. These selected colonies were further propagated in TSB without antibiotics at 37 °C with shaking at 120 rpm for at

least 14 generations to eliminate the plasmid. Several white colonies were screened to detect the loss of erythromycin resistance through replica plating on both TSB + erythromycin (5 μg/ml) and TSB plates. Among those displaying erythromycin sensitivity, identification of individuals harboring a mutant allele within the chromosome was achieved using PCR with A and D primers and subsequently confirmed by sequencing. All in-frame deletion mutants were stored at —80 °C in 35% glycerol.

### 2.6. *Confirmation of the phenotype using in frame deletion mutants*

Seven out of ten in-frame deletion mutants were successfully created: *DNA binding protein*, hypothetical protein 1, *Yham*, hypothetical protein 2, *Sau3AIM*, *pbp-B*, and *purB*. These were then used to confirm the phenotype on 10 g slices of Lyoner, using the same protocol as described for large volume confirmation screening of the transposon mutants above. However, this time, the main cultures were prepared in culture tubes containing 5 ml of BHI medium. To determine the effect of temperature, these experiments were carried out at 37, 25 and 8 °C in BHI and on Lyoner, separately and repeated three times.

### 2.7. *Scanning electron microscopic (SEM) imaging*

The in-frame deletion mutants *Sau3AIM*, *pbp-B*, and *purB* strains exhibited significant differences compared to the WT, and we followed up on the hypothesis that the mutations may cause morphological changes with imaging. The strains were cultured in 5 ml BHI overnight at 37 °C with shaking. These pre-cultures were diluted 1:100 into fresh BHI and incubated at 37 °C with shaking until the $OD_{600}$ reached 0,3–0,4 which is the exponential phase of the culture. These cultures were inoculated into fresh BHI and incubated at 12 °C for 48 h. One milliliter of each 37 °C and 12 °C cultures were pelleted and washed with PBS

twice and then fixed by resuspending the cells in equal amounts of 2% paraformaldehyde (Alfa Aesar GmbH & Co. KG, Germany) and incu- bating at room temperature for 2 h. The samples were stored at 4 °C until imaging. One drop of liquid samples was coated with a gold/palladium layer (approx. 2 nm) in a sputter coater and examined under high vacuum conditions in a field emission SEM (Philipps XL 30SFEG, Netherlands) at 5 KV using the in-lens secondary electron detector. These samples were also visualized by Gram staining under the light microscope.

### 2.8. *Data analysis*

All analyses were performed in RStudio version 2023.09.1 + 494 (RStudio Team, 2015) using the packages lme4 version 1.1–34 (Bates et al., 2015), sjPlot version 2.8.15 (Lüdecke, 2023), dplyr version 1.1.2 (Wickham, 2011) and ggplot2 version 3.4.2 (Wickham, 2016). To assess the effect of individual in-frame deletion mutants on the fitness of the strain a linear mixed effect model was calculated with lme4. It models log10 fold changes (defined as the log10 CFU/g or ml difference before and after the relevant stress condition) as a function of the mutant strain, temperature (8 °C vs 25 °C) and medium (Lyoner vs. BHI). An artificial variable created from the combination of the biological replicate, mutant and temperature was used as a random effect in the model to control for differences between experimental replicates. To verify that all in-frame deletion mutants had the same growth rate in BHI, an ANOVA was performed on the time it took the individual strains to reach an $OD_{600}$ of 0.4.

## 3. Results

### 3.1. *Phenotype of the L. monocytogenes LL195 transposon insertion library on Lyoner*

In this study, we screened almost 2640 transposon insertion mutants on a miniature Lyoner model. This initial small volume screening revealed that while the WT strain was able to grow on Lyoner at 8 °C (1,88 log10 CFU/g ±0,23), the growth of 106 mutants was reduced by at least 0,5 log10 CFU/g or they showed no growth on Lyoner compared to the WT strain, suggesting that the genes affected by the transposon insertion might be important for growth on Lyoner. Additionally, 16 mutants exhibited better growth on Lyoner compared to the WT strain, suggesting that the genes affected in these mutants might hinder growth on Lyoner. To efficiently handle the large number of mutants in the library, we initially conducted a small volume screening. This small volume screening allowed us to quickly obtain an initial overview of the mutants' growth patterns and identify potential candidates that exhibited notable differences in growth on Lyoner compared to the WT strain. This pre-screening process was essential in prioritizing the most promising mutants for subsequent analysis in a larger volume. As a next step, we performed a second, larger volume screening on Lyoner with a total of 122 candidate mutants in triplicate.

In the subsequent larger volume screening, no significant difference in growth was found among the 16 mutants that had initially demonstrated enhanced growth compared to the WT strain on Lyoner during the small volume screening. However, out of 106 candidate mutant strains that initially exhibited reduced, or no growth compared to the WT strain, 13 mutants were confirmed to have a negative effect on the growth of the respective strains on Lyoner (Table 1). The fact that genes, *sau3AIM* and *pbp-B*, and the promoter region of the *DNA binding protein* were hit by several transposon insertions confirmed the feasibility of the method.

### 3.2. *Identification of the candidate genes*

Transposon insertion sites of the candidate genes were identified using a slightly modified nested PCR protocol as described by (Zemansky et al., 2009). The localization of the transposon insertion sites, and their genetic context is shown in Fig. 1.

### 3.3. *Growth profiles of the in-frame deletion mutants*

To confirm the observed phenotypic changes in the mutants, we created in-frame deletion mutants by SOE PCR and screened them on Lyoner. Among the initial thirteen confirmed candidates, there were several that were located within the same genes, resulting in ten candidate genes (Fig. 1). Out of these, a total of 7 in-frame deletion mutants (*DNA binding protein*, hypothetical protein 1, *Yham*, hypothetical protein 2, *Sau3AIM*, *pbp-B*, and *purB),* were successfully created. Despite several attempts to create in-frame deletion mutants for *manY*, *manZ* and *prsa2* using pKSV-7 and pMAD, these efforts remained unsuccessful.

All seven in-frame mutants had the same growth rate as the WT in BHI at 37 °C and took an average of 3.32 h (sd = 0.2 h) to grow to an $OD_{600}$ of 0.4 (*p*-value for the comparison of growth time to $OD_{600}$ of 0.4 by mutant: 0.20).

While none of the seven in-frame mutants showed a growth defect at 37 °C in BHI, the *sau3AIM ($p < 0.01$), pbp-B ($p < 0.01$)* and *purB* ($p = 0.04$) mutants were impaired when grown under the tested stress conditions (Fig. 2).

The growth defect of the *sau3AIM* and *pbp-B* mutants was temperature dependent, since the *sau3AIM* and *pbp-B* mutants showed reduced fitness in BHI at 8 °C compared to the WT. This growth defect was also matrix dependent, since these mutants showed reduced fitness on the

**Table 1**

Genes that affected *L. monocytogenes* LL195 fitness on Lyoner.

| Mutant Name | Locus Tag | Interval | Disrupted Gene | Protein Name | Growth Difference Compared to WT (log reduction) | Function |
|---|---|---|---|---|---|---|
| P28C2<br>P28C3<br>P28C10 | BN389_03290 | 346,524 … 347,923 | *sau3AIM* | Cytosine Specific DNA Modification Methlytransferase | No Survival | Methylates C on GATC sequence and protect the host DNA from cleavage |
| P21G11<br>P30C4 | BN389_04850 | 493,081 … 495,132 | *pbp-B* | Penicillin Binding Protein | No Survival | Has a role in the cell wall assembly, cell division and cell wall reshaping |
| P29H8 | BN389_01150 | 115,272 … | *manY-manZ* | Phosphoenol- | 0,82 ± 0,2 | A major carbohydrate active |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | pyruvate- | | transport system, |
| | BN389_01160 | 116,078, 116,100 … 117,011 | | dependent sugar phosphotransferase system | | involves in mannose transport |
| P1B3 | BN389_7980 | 796,753 … 797,505 | Hypothetical Protein 1 | – | 0,76 ± 0,07 | Similar to transcriptional regulators of the GnTr family in *L. monocytogenes* |
| P24A5 | BN389_01040 | 99,035 … 104,914 | Hypothetical Protein 2 | – | 0,72 ± 0,16 | Similar to HisZ, a regulatory subunit of the ATP phosphoribosyltransferase |
| P18F2 | BN389_27,880 | 2,850,688 … | DNA Binding | – | 1,29 ± 0,40 | Similar to XRE family transcriptional |
| P16F10 | | 2,851,515 | protein | – | | regulator |
| | BN389_22,520 | 2,301,560 | *prsA2* | Post translocation | 2,18 ± 0,61 | Promotes the activity and stability |

| | | | | | | |
|---|---|---|---|---|---|---|
| P16E1 | | … | | chaperone | | of 2 |
| | | 2,302,441 | | | | virulence factors in *L. monocytogenes* |
| P27B9 | BN389_18,000 | 1,826,027 | *purB* | Adenylosuccinate lyase | 1,11 ± 0,19 | Takes part in the purine biosynthesis pathway, |
| | | … | | | | affect the colonization ability of |
| | | 1,824,717 | | | | *L. monocytogenes* in gastrointestinal tract |
| P16E1 | BN389_22,530 | 2,303,471 | *yham* | – | 2,18 ± 0,61 | 3′ to 5′ exonuclease activity |
| | | … | | | | |
| | | 2,302,482 | | | | |

Lyoner matrix at both temperatures compared to the WT. (Fig. 3).

On the other hand, the *purB* mutant had a fitness effect that was only apparent while growing on Lyoner matrix as shown by its significantly lower mean log fold change on Lyoner compared to the WT at both 8 °C and 25 °C. This mutation did not affect fitness in a temperature-dependent way, since its growth was the same as the WT in BHI at 37 °C and 8 °C.

### 3.4. *Morphology of the mutant strains compared to the wild type*

Given the growth differences of the *pbp-B, sau3AIM* and *purB* mutant under some or all tested stress conditions, we wondered whether these mutations might cause morphological changes in the cells. This hypothesis was based on the fact that PBP-B is involved in cell wall synthesis, and PurB is part of the purine synthesis pathway that has a broad involvement in cellular processes including the expression of cell wall components.

A Gram-stain revealed that while *pbp-B* mutant and WT cells grown at 12 °C and 37 °C looked normal, *purB* mutants had increased frequency of elongated cells when they were grown at 37 °C, and in *sau3AIM* mutants elongated cells were observed at both temperatures.

We therefore proceeded to analyze the cells grown at 37 °C by SEM (Fig. 4). These images show a high frequency of elongated cells of *purB* mutants that seem to have formed rudimentary division septa without fully separating the two daughter cells. While some incomplete divisions were also visible in the *sau3AIM* mutant cells, there was a high frequency of very long cells absent of any visible signs of attempts to divide. In contrast, the *pbp-B* mutant did not show any morphological differences compared to the WT.

## 4. Discussion

### 4.1. *To ensure reliable results, independent confirmation of small volume screening of a transposon library is necessary*

Out of 122 candidate mutants from the initial screen in the miniature "Lyoner model", 13 mutants were confirmed to have a phenotype when repeated on a larger scale (corresponding to a confirmation rate of 0.15). In comparable studies using high-throughput screening of individual transposon mutant libraries in *L. monocytogenes* followed by confirmation in larger volumes, comparable confirmation rates of 0.12 (range: 0.03–0.19) (Alonso et al., 2014; Hingston et al., 2015; Mains et al., 2021; Narayanan et al., 2022) were observed. Potential reasons for the relatively low confirmation rates possibly lay in the infeasibility of replicates in large screens and small volumes causing more variation, or a combination of the two. This highlights the need for confirmation of results from transposon insertion libraries in larger volumes and with in-frame deletion mutants. Potential reasons for the fact that we were unable to create three (*manY, ManZ and prsa2*) out of the 10 target mutants (Fig. 1) include the possibility that the transposon inserted in a way that left some residual function intact, whereas a in-frame deletion mutant would abolish all activity. It is also possible that the transposon insertion left some *cis*- or *trans*-acting regulatory sequences intact that we may have attempted to delete in the in-frame mutant. Strain-specific effects may be possible, since a *prsa2* mutant has been successfully created by others in *L. monocytogenes* 10403S (Alonzo & Freitag, 2010). In other organisms, a *manXYZ* mutant was viable in *Klebsiella pneumoniae* (Bieler et al., 2006).

### 4.2. *The sau3AIM and pbp-B in-frame mutant showed a temperature- dependent phenotype*

While the *sau3AIM* and *pbp-B* deletion mutants grew at the same rate to $OD_{600}$ as the WT at 37 °C, the mutations affected fitness under all tested stress conditions compared to the WT. Taking these results into

consideration, we concluded that these mutations cause a general growth defect under stress conditions. *L. monocytogenes* encodes five high-molecular-weight penicillin binding proteins (HMW PBPs): PBP- A1, PBP-A2, PBP-B1, PBP-B2, PBP-B3, that are all present in the LL195 genome (where they are annotated as *pbpF, pbpB, ponA, pbpC* and *pbpC*) (Weinmaier et al., 2013). The one identified here was annotated as penicillin-binding-protein-B (*pbp-B*) in LL195 and corresponds to *pbc-3/lmo0441* in EGDe and LMRG00133 in 10403S. In agreement with other authors (Rismondo et al., 2015), the in-frame deletion mutant for *pbp-B* did not have a discernible phenotype when grown in BHI at 37 °C. A deletion of the *Bacillus subtilis* homologue of *pbp-B*, *pbpC,* did not produce a phenotype either (Murray et al., 1996). However, to our knowledge none of these authors tested their mutants under cold stress. In this study, the *pbp-B* mutant showed at least a 2.5 $\log_{10}$ CFU/g reduction when incubated under any condition we tested under cold temperatures. This effect seems to be only present at cold temperatures, since Rismondo et al. (Rismondo et al., 2015) did not observe reduced

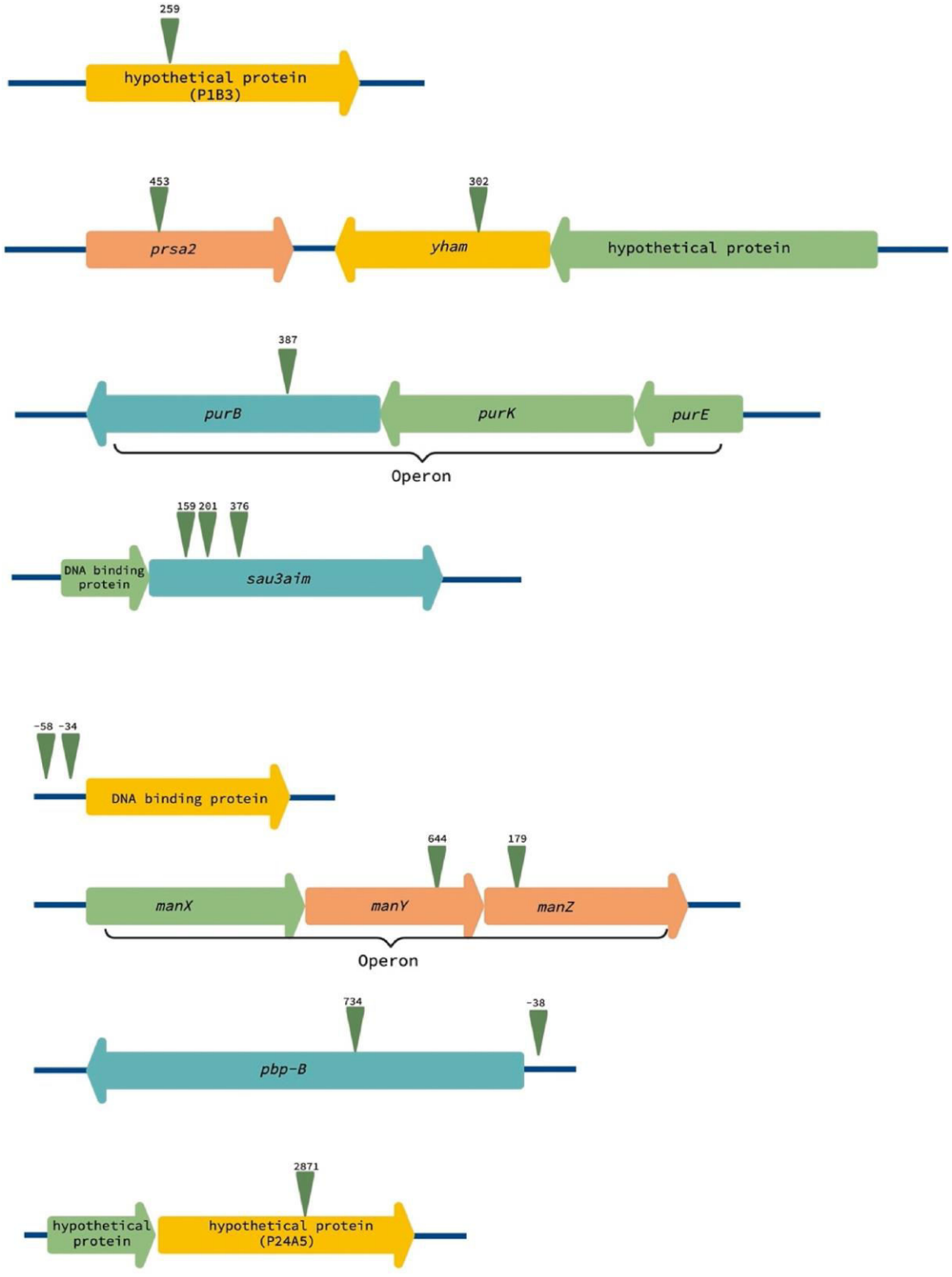


**Fig. 1.** Localization of transposon insertion sites. Upper green arrows represent sites of transposon insertions, and numbers above these arrows correspond to mapped transposon locations.

growth of a *pbp-3* mutant in EGD-e at 42 ˚C compared to 37 ˚C, and in accordance with this study the *pbp-B* mutant grown at 37 ˚C also showed normal morphology in SEM images (Fig. 4). A possible reason for this is that PBP-B might play a role in the reorganization of the cell wall during cold stress, and it might be interesting to see whether the observed cold phenotype aggravates in a cold-shock protein mutant background. Consistent with this theory suggesting a role in cell wall organization but not in cell division, PPB-3 localized to the cell wall, but not to the di- vision septum (Guinane et al., 2006; Rismondo et al., 2015). Since PPB-3 has no transglycosylase domain (Rismondo et al., 2015), its role is solely

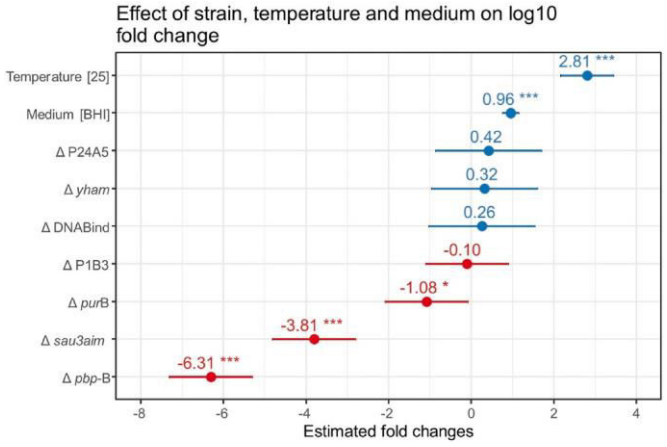


**Fig. 2.** Linear mixed effect model estimates for $\log_{10}$ fold changes of the in- frame mutants after growth for 48 h on Lyoner at 8 ˚C and 25 ˚C, as well as in BHI at 8 ˚C. Data points for mutants denoted by an asterisk were significantly different from the WT grown under the same condition. Data points for medium and temperature denoted with an asterisk were significantly different from the respective other condition, e.g. growth on Lyoner or growth at 8 ˚C.

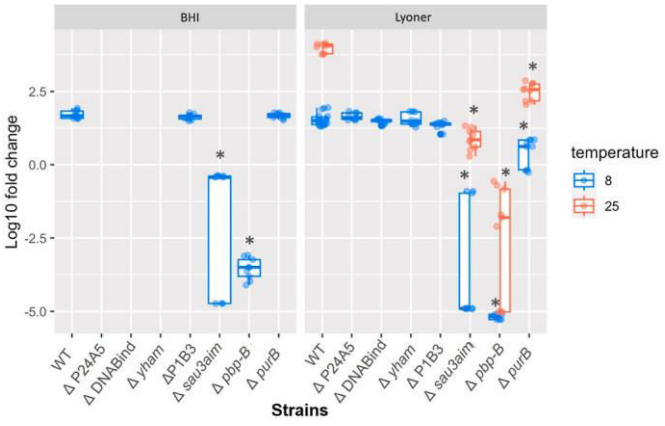


**Fig. 3.** Log fold change of the in-frame mutants after growth for 48 h on Lyoner at 8 °C and 25 °C, as well as in BHI at 8 °C. Data points denoted by an asterisk were significantly different from the WT grown under the same condition.

in the transpeptidation of peptidoglycan. Possibly, under standard lab- oratory conditions there might be functional redundancies between the different HMW PBPs, as suggested and demonstrated by other authors (Guinane et al., 2006; Rismondo et al., 2015) and supported by the normal morphology of the cells in SEM images. In this context, only PBP B2 has been demonstrated to be essential (Guinane et al., 2006; Rismondo et al., 2015). This redundancy may not be effective enough to balance the effect of a *pbp-B* mutation under the stress conditions tested here. Future studies on the expression of the different pbp's under cold conditions may clarify this question.

The *sau3AIM* gene encodes the methylase of a restriction- modification system that was first described in *Staphylococcus carnosu*s (Seeber et al., 1990). Sau3AIM methylates the cysteine in the recognition site GATC which in turn prevents cleavage by the enzyme *Sau*3AI. In particular, *L. monocytogenes* strains with serotype 4b showed a high prevalence of the *sau3AIM* restriction-modification system in their genome (Yildirim et al., 2004), fitting with its presence in LL195 that is also a serotype 4b strain (Weinmaier et al., 2013).

One possible explanation for the reduced fitness of the *sau3AIM* mutant at low temperatures may be that the activity of the cognate restriction enzyme *Sau*3AI is temperature dependent. We might speculate that the *L. monocytogenes Sau*3AI is not active at higher temperatures and therefore cells might be able to tolerate the missing methylation by Sau3AIM due to the lack of restriction activity. This is, however, contradicted by the fact that commercially available *Sau*3AI restriction enzymes have a temperature optimum at 37 °C. Whether the *L. monocytogenes* Sau3AIM enzyme is an exception would have to be investigated.

Temperature-dependent R-M systems have been described in *L. monocytogenes* (Jae-Won & Sophia, 2009) as well as in other organ- isms, e.g., *Lactococcus lactis* (O'Driscoll et al., 2004) and *Streptococcus lactis* (Sanders & Klaenhammer, 1984). In *L. monocytogenes*, a temperature-dependent phage resistance that is mediated by a temperature-dependent R-M system (LmoH7) with the recognition site GTATCC (Kim et al., 2012) has been shown in ECII strains (Jae-Won & Sophia, 2009). In these strains, the optimal expression of the R-M system was at 19 °C and decreased with increasing temperatures.

Whether there is a link between the temperature-dependent phenotype of the *sau3AIM* and *pbp-B* mutant with established temperature- dependent regulatory systems in *L. monocytogenes* remains to be investigated. For instance, virulence gene expression in *L. monocytogenes* is regulated via a temperature-sensitive riboswitch in the transcriptional regulator PrfA that mediates virulence gene translation at host body

temperatures of 37 °C, while this is suppressed at temperatures below 30 °C (Loh et al., 2009). It would be interesting to see whether the temperature-dependent effect of the *sau3AIM* and *pbp-B* mutants is affected in a PrfA* background where PrfA is constitutively active (Vega et al., 2004) or in a *prfA* null mutant. However, in the latest screen of the PrfA regulon at 37 °C, neither *sau3AIM* nor *pbp-B* were identified as PrfA dependent (Henderson et al., 2020).

### 4.3. *PurB is important for growth on Lyoner*

In contrast, the negative fitness effect of a *purB* deletion was only evident when the strains were grown on Lyoner, but not in BHI at either 8 or 37 °C. We therefore concluded that the product of *purB* is positively affecting growth on Lyoner, while it is not needed for efficient growth in BHI at optimal as well as cold temperatures. In *L. monocytogenes*, *purB* expression is transcriptionally co-regulated by the alternative sigma factors SigB and SigL at 3 °C in BHI but not at 37 °C (Mattila et al., 2020).

PurB is part of the purine biosynthesis pathway in *L. monocytogenes* (Ogata et al., 1999). Purines are essential for DNA replication, RNA transcription, and protein translation. Either of these mechanisms might be responsible for the elongated phenotype of *purB* mutant cells (Fig. 4) that we observed when they were grown at 37 °C. Bacteria can either synthesize purines *de novo* by a metabolically costly pathway, or through salvaging pre-formed nucleobases from the environment. PurB catalyzes two reactions within the *de novo* purine synthesis pathway (KEGG RN: R01083, RN:R04559 (Kanehisa & Sato, 2020), both of which yield fumarate as a side product. The *de novo* purine synthesis pathway is relevant for bacterial fitness in environments that do not offer abundant nucleobases, such as the intracellular environment during infection. Accordingly, attenuated virulence of purine biosynthesis mutants has been demonstrated in *L. monocytogenes* (Faith et al., 2012) as well as in *Salmonella* Typhimurium (McFarland & Stocker, 1987), *Brucella abortus* (Alcantara et al., 2004), *Brucella melitensis* (Crawford et al., 1996), *E. coli* (Shaffer et al., 2017) and *Staphylococcus aureus* (Connolly et al., 2017).

*L. monocytogenes* auxotrophs for purine biosynthesis have also shown severe fitness effects when grown in minimal medium supplemented with single carbon sources (Narayanan et al., 2022) and in porcine bile (Dowd et al., 2011). Since both Lyoner and BHI as growth substrates are based on meat that should offer an

abundance of exogenous nucleobases, it is unlikely that *L. monocytogenes* would meet a shortage of nucleobases that results in the need for *de novo* purine biosynthesis under the observed growth conditions. Also, a polar effect of the *purB* mutation on other genes within the same transcriptional unit can be excluded since the phenotype was confirmed in a in-frame, non-polar *purB* mutant. It is possible

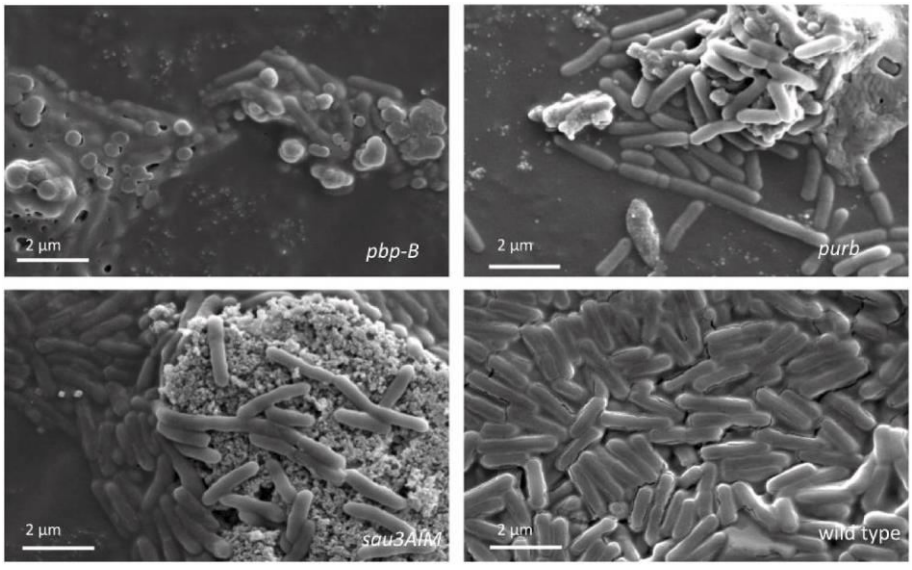


**Fig. 4.** SEM images of the *pbp-B, purB, sau3AIM* mutants and the WT, grown at 37°

that the observed fitness effect of the *purB* mutant during growth on Lyoner is due to an inability to efficiently use the exogenous purines present in meat, the lack of fumarate that is produced by the reactions catalyzed by PurB or due to other, yet unknown pleiotropic effects of PurB. We therefore hypothesize that some difference between the growth condition in Lyoner and BHI triggers a fitness effect due to the absence of *purB* in the mutant.

There are obvious differences in the composition and nutrient supply provided by a complex food matrix like Lyoner sausage and BHI as a laboratory medium.

Lyoner sausage is specified as a cured (1.6–1.9 % nitrite curing salts) cooked sausage containing tendon-free meat, a fat content of around 20%, and a pH of 6–6.2, according to the German specifications for meat products (Landwirtschaft, 2022). Carbon sources are limited in meat, which is addressed by supplementing BHI medium with glucose as a carbon source.

Therefore, Lyoner in comparison to BHI has a lower pH (pH Lyoner: around 6, BHI: 7.4), higher fat and salt content (Lyoner: NaCl approximately 0.3 M NaCl, BHI: 0.08 M) and offers a lower abundance of carbon sources. In addition, various spices added to Lyoner sausage may exert stress on the microbial populations in Lyoner sausage (Liu et al., 2017). It is possible that either of these differences may cause the observed growth defect of the *purB* mutant.

These differences may also affect the pathways responsible for purine import into the cell. In the absence of *de novo* purine synthesis in a *purB* mutant, *L. monocytogenes* would have to rely solely on the import of nucleobases via the purine salvage pathway. Several genes have previ- ously been shown to participate in this pathway: *lmo 1845* (Krawc- zyk-Balska et al., 2021), *lmo 1884* (Krajewski et al., 2017), *lmo 1885* (Knudsen et al., 2016), *lmo0573* (Ellinger et al., 2023), *pyrR* (Knudsen et al., 2016), *lmo2254* (Ledala et al., 2010), *lmo0456* (Fischer et al., 2022) and *lmo1839/pyrP* (Fischer et al., 2022). One might speculate that some of the specific conditions during growth in Lyoner may render this pathway ineffective and therefore be responsible for the observed phenotype. Some limited information is available on the regulatory mechanisms of the above genes. *lmo 1845* (Wurtzel et al., 2012), *lmo1885* and *lmo0573* (Ellinger et al., 2023) depend on riboswitches for their expression. *Lmo1885* was differentially expressed after exposure to sublethal concentrations of tetracycline and co-trimoxazole (Knudsen et al., 2016). *Lmo0573* was downregulated under anaerobic conditions (Ferrari et al., 2017) and upregulated in BHI compared to during host infection (Eimerman, 2011). Lmo2254 was downregulated in biofilms (Assisi et al., 2021), in response to iron limitation (Ledala et al., 2010), and during acid exposure (Li, 2020). This last regulatory mechanism might hint towards the purine import pathway being less effective at pH 6.4 than pH 7.4.

In summary, our study shows a role of the methylase Sau3AIM and the penicillin binding protein Pbp-3 for the growth of *L. monocytogenes* under food-associated stress conditions and of the adenylsuccinate lyase *purB* specifically for efficient growth of *L. monocytogenes* LL195 in Lyoner, a food matrix that is highly relevant as a vehicle for human infections with *L. monocytogenes*. This effect may in the future be exploited for highly targeted interventions to increase food safety. For instance, small molecule libraries of substances that are generally recognized as safe (GRAS) by the FDA may be screened for their effect on *purB* expression and provide an inhibitor that could serve as a food additive to reduce the fitness of *L. monocytogenes* in Lyoner and similar products like Polony. Currently, the use of designer temperate phage is being discussed as a potential strategy to mitigate the antibiotic resistance crisis in human therapy (Jaroszewicz et al., 2022). Once integrated into the host genome, these designer prophages are engineered to be unable to excise from the host genome to repress phage-mediated spread of antibiotic resistance, while they express repressors of virulence gene expression and therefore decrease virulence of the host bacteria. These same mechanisms may also be put to future use to reduce the fitness of pathogens in food and to complement the use of strictly lytic phage, thus broadening the available tools. However, these novel strategies are currently only applicable in experimental setups due to a lack of data on the long-term interaction of designer prophages with their hosts as well as regulatory hurdles.

## V. CRediT authorship contribution statement

**Philipp-Michael Beindorf:** Writing – original draft, Methodology, Investigation, Data curation. **Jule Anna Horlbog:** Writing – review & editing, Methodology. **Irene Esteban Cuesta:** Writing – review & edit- ing, Formal analysis, Conceptualization. **Claudia Guldimann:** Writing – review & editing, Writing – original draft, Resources, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Irmak S¸ ah:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

## VI. Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## VII. Data availability

Data will be made available on request.

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## VIII. Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2024.110393.

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# V. DISCUSSION

Given the severe health implications of listeriosis and the widespread presence of *L. monocytogenes* in the environment, it is crucial to understand the factors that influence bacterial growth and survival under various environmental conditions, enabling the development of effective strategies to prevent and control *L. monocytogenes* contamination in food production and processing environments.

In the context of this thesis, the genetic factors contributing to the growth of *L. monocytogenes* on a German deli meat, called Lyoner, were investigated using a transposon mutagenesis library in an outbreak strain of *L. monocytogenes*. By employing an outbreak strain and a relevant food matrix, we aimed to overcome the limitations associated with laboratory reference strains and laboratory-based food models, to obtain results more representative of real-world situations.

Our high-throughput screening approach of this transposon mutagenesis library of the outbreak strain LL195, consisting of 2640 individual mutants, identified 122 candidate mutants with confirmed phenotypes on Lyoner. To optimize the efficiency and cost-effectiveness of this process, initially a small-volume screening was performed. This approach enabled the rapid and cost-effective identification of potential candidates with observable phenotypes. While the small-volume screening was instrumental in simplifying the process, it is possible that some potential candidates may have been overlooked due to the limitations of this approach.

This limitation could in the future be mitigated by the use of TnSeq (transposon sequencing). TnSeq is a high-throughput method that combines transposon mutagenesis with next-generation sequencing to systematically investigate the genotype-phenotype relationships (VAN OPIJNEN et al., 2009). It is a powerful technique that offers several advantages over traditional transposon (Tn) mutagenesis libraries for studying gene function and bacterial fitness. Tn-seq also enables high-throughput analysis, allowing researchers to simultaneously examine thousands of mutants within a single experiment (CAIN et al., 2020). This is a significant improvement over classic Tn mutagenesis libraries, where individual mutants typically need to be screened one at a time. At the beginning of this study, a Tn-seq library was not available for *L. monocytogenes* and we chose to

use the traditional transposon (Tn) mutagenesis library approach that yielded valuable results from screening this library on an RTE meat product in terms of food-pathogen interactions. A Tn-seq library in *L. monocytogenes* EGDe has since been established through collaboration with the Robert Koch Institute (FISCHER et al., 2022) under a DFG project. Future research involving screening the Tn-Seq library on Lyoner may reveal novel genes and mechanisms associated with *L. monocytogenes* stress response and growth, in addition to confirming the result of this thesis study.

As discussed in the paper, among the 106 candidate mutant strains initially displaying reduced or no growth compared to the wild-type (WT) strain, 13 mutants (listed in the publication as Table 1) were confirmed to have a negative impact on the growth on Lyoner, corresponding to a confirmation rate of 0.15 after larger volume screening. This aligns with findings from similar studies (ALONSO et al., 2014; HINGSTON et al., 2015; MAINS et al., 2021; NARAYANAN et al., 2022). Analysis of transposon insertion sites in these confirmed candidates revealed that several candidates had mutations within the same genes, e.g., *sau3AIM* and *pbp-B*, along with the promoter region of a DNA binding protein. This confirmed the feasibility of the method and lead to the identification of a total of 10 candidate genes.

Because these mutants were created using transposons, genes were rendered inactive by insertion of the whole transposon into the genome. While this reliably disrupts gene function, the method has downsides: it can not be excluded that these large insertion act *in trans*, e.g. via polar effects. And while statistically, only one insertion per cell should be present, mutants with multiple insertions cannot be excluded. To confirm that the observed phenotype was due to the mutation that we identified, clean deletion mutants of the candidate genes were cloned. We attempted in-frame deletion mutants of the 10 candidate genes using either pMAD (ARNAUD et al., 2004), and in parallel if this failed pKSV-7 (SMITH und YOUNGMAN, 1992) shuttle vectors. However, it was only possible to successfully create in-frame deletion mutants for 7 of the 10 genes. There may be several potential reasons why three of the 10 target mutants (*manY*, *ManZ* and *prsa2*) could not be created, as was detailed in the discussion section of the paper. Beyond this, larger gene sizes can represent technical challenges and reduce deletion efficiency. However, we excluded gene size as a limiting factor, as the

genes were of similar size as successfully created mutants. Both pMAD and pKSV-7 plasmids were used in the cloning: where one failed the other was attempted. For the successful mutants, we suspect that the success of one plasmid over the other was largely due to chance – they are both derived from a common origin and share structural similarities (SMITH und YOUNGMAN, 1992; ARNAUD et al., 2004). Therefore, the failures are probably not attributable to differences between the plasmids themselves.

A fitness effect was confirmed in clean mutants for *pbp-B, sau3AIM* and *purB*. Since all experiments were performed at refrigeration temperature to simulate the food environment, we aimed to exclude that these mutations caused a general growth defect at low temperatures. Thererfore, growth experiments were repeated on Lyoner and in rich medium at different temperatures.

Penicillin-binding proteins (PBPs) are membrane-associated proteins that play a critical role in the biosynthesis of peptidoglycan (PG), the primary component of bacterial cell walls. The discovery and naming of these proteins are rooted in their unique ability to bind the β-lactam antibiotic penicillin, highlighting their essential function in maintaining bacterial cell integrity and the potential for exploiting their role in developing antimicrobial strategies (SHARIFZADEH et al., 2020). Although the *pbp-B* deletion mutant showed growth rates similar to those of the WT strain at 37 °C, the fitness of the mutant was noticeably impaired under all tested stress conditions compared to the WT. Considering the observed growth impairment of the *pbp-B* in-frame deletion mutant under various stress conditions, it was concluded that a general growth defect is caused by this mutation when the bacteria are exposed to various stress conditions.

While previous experiments did not reveal significant phenotypic changes in *pbp-B* mutants under normal growth conditions (MURRAY et al., 1996; RISMONDO et al., 2015), our study found a substantial reduction in colony-forming units (CFU) at cold temperatures. As indicated before, this cold stress-specific effect suggests a potential role for PBP-B in cell wall reorganization during cold stress, supported by its localization to the cell wall. The study highlights the need for further investigations into the expression patterns of different high-molecular-weight penicillin binding proteins (HMW PBPs) under varying stress conditions to better understand functional redundancies and specific stress responses in *L. monocytogenes*. Further studies on the expression of the various PBPs under

cold conditions could provide more insights into this matter.

Restriction-modification (R-M) systems are bacterial defense mechanisms that protect the cell from foreign DNA invasion, such as bacteriophages or plasmids. These systems consist of two essential components: a restriction enzyme and a methylase. Upon entry of foreign DNA into the cell, the restriction enzyme cleaves the unmethylated foreign DNA at specific recognition sites, rendering it non-functional and protecting the host cell from invasion. The host cell's own DNA is protected by the methylase that methylates the recognition sites to protect them from cleavage by the restriction enzyme. R-M systems thus plays a crucial role in maintaining the integrity of the bacterial genome and provide precise cellular protection against genetic intruders (RODIC et al., 2017; SHAW et al., 2023).

The *sau3AIM* gene encodes a methylase involved in a restriction-modification system, methylating the cysteine in the GATC recognition site to inhibit cleavage by Sau3AI. This system is not present in all *L. monocytogenes* strains with a high association to *L. monocytogenes* serotype 4b strains (YILDIRIM et al., 2004), including LL195. This underscores its association with specific serotypes and potential roles in genetic regulation within these strains.

The *sau3AIM* deletion mutant grew at 37 °C at the same rate as the WT, reaching a similar OD of 600 for comparison. However, under cold stress conditions, the mutant exhibited a significant reduction in fitness, with no viable colonies detected. One potential explanation for the reduced fitness of the *sau3AIM* mutant at lower temperatures is the temperature-dependent activity of the Sau3AI restriction enzyme. Although commercially available Sau3AI enzymes typically function optimally at 37 °C, the possibility of *L. monocytogenes* Sau3AI having different temperature requirements warrants further investigation. This idea is supported by documented temperature-dependent restriction-modification systems in *L. monocytogenes* and other organisms, highlighting the need for detailed studies to understand the temperature-dependent behaviors of these systems, including potential exceptions to standard enzyme activity profiles.

The potential correlation between the temperature-dependent phenotype of the *sau3AIM* and *pbp-B* mutants and established temperature-dependent regulatory systems in *L. monocytogenes* requires further investigation. For instance,

virulence gene expression in *L. monocytogenes* is controlled by a temperature-sensitive riboswitch in the transcriptional regulator PrfA, which mediates virulence gene translation at host body temperatures of 37°C but is suppressed at temperatures below 30 °C (LOH et al., 2009). It would be worthwhile to investigate whether the temperature-dependent effect of the *sau3AIM* and *pbp-B* mutants is altered in a PrfA* background where PrfA is constitutively active (VEGA et al., 2004) or in a prfA null mutant. However, neither *sau3AIM* nor *pbp-B* were identified as PrfA-dependent in a recent screen of the PrfA regulon at 37 °C (HENDERSON et al., 2020). Cold-shock proteins (Csps) aid in adaptation to low temperatures through mechanisms that aid replication, transcription, and translation at low temperatures (SCHÄRER et al., 2013; MUCHAAMBA et al., 2021). In addition to their well-established function in cold stress adaptation, Csps have been implicated in various other stress responses, highlighting their versatility and importance in bacterial survival. Csps can also regulate genes indirectly, e.g., by effects on transcriptional regulators. The Csp-dependent regulation of PrfA expression supports this idea, as the LIPI-1 genes in *L. monocytogenes*, which are controlled by PrfA, showed differential expression in the absence of Csps (SCHÄRER et al., 2013; MUCHAAMBA et al., 2021). In terms of the temperature dependent fitness effect of the tested genes, it may be interesting to consider the potential interactions between Csp and other regulatory proteins in *L. monocytogenes*.

Purines are essential components of nucleic acids and play a vital role in various cellular processes, including DNA replication, RNA transcription, and protein translation (KUMARI, 2018). The proper functioning of the purine biosynthesis pathway, which includes *purB*, is crucial for maintaining the growth and metabolic activities of *L. monocytogenes* under different growth conditions (OGATA et al., 1999). The negative fitness effect of a *purB* deletion was noticeable only when the strains were cultivated on Lyoner, but not in BHI at either 8 or 37 °C. As a result, it was concluded that growth on Lyoner is positively influenced by the product of *purB*, while it is dispensable for efficient growth in BHI at optimal as well as cold temperatures. In *L. monocytogenes*, *purB* expression is transcriptionally co-regulated by the alternative sigma factors σB and σL at 3 °C in BHI but not at 37 °C (MATTILA et al., 2020). While σB regulates the expression of genes that mediate survival under acid stress, osmotic

stress, oxidative stress, cold stress and nutrient deficiency or energy stress (KAZMIERCZAK et al., 2003; SUE et al., 2004), σL contributes to osmotolerance and also provides some resistance against the antibacterial peptides (ROBICHON et al., 1997). One interesting avenue for future research would be to generate mutants in the *purB* gene within regulatory pathway null mutants, such as the *sigB, sigL* or *prfA* mutant. This approach would allow to investigate the intricate interplay between *purB* and the regulatory network in *L. monocytogenes*. By studying the impact of *purB* mutations in the absence of a functional regulator, we can gain deeper insights into how these two components collectively contribute to the bacterium's ability to adapt to various environmental conditions and regulate virulence factors.

Future research may identify the exact mechanisms by which differences in growth conditions between Lyoner and BHI trigger a fitness effect in the *purB*-mutant. Research into *purB* and other genes that are associated with this pathway, may delve deeper into elucidating the precise regulatory mechanisms governing these genes under various environmental conditions. Investigating the interplay between *purB* and other regulatory factors, such as alternative sigma factors or global regulators, could provide valuable insights into the broader regulatory network controlling purine biosynthesis. Since *purB* was found to be specifically crucial for the efficient growth of *L. monocytogenes* LL195 in Lyoner, one potential approach could involve screening small molecule libraries of substances that are generally recognized as safe (GRAS) by the FDA for their effect on *purB* expression. This could lead to the identification of inhibitors that could serve as food additives to reduce the fitness of *L. monocytogenes* in Lyoner and similar products like Polony.

# VI. CONCLUSION AND OUTLOOK

In conclusion, our study demonstrated the significant roles of the methylase *sSau3AIM*, the penicillin binding protein *pbp-3*, and the adenylsuccinate lyase *purB* in the growth of *L. monocytogenes* under food-associated stress conditions. Notably, *purB* was found to be specifically crucial for the efficient growth of *L. monocytogenes* LL195 in Lyoner, a food matrix that is highly relevant as a vehicle for human infections with *L. monocytogenes*. Future research should focus on the expression of the various PBPs under cold conditions and potential interactions between Csps and other regulatory proteins in *L. monocytogenes* in terms of the temperature dependent fitness effect of the tested genes. Generating mutants in the *purB* gene within regulatory pathway null mutants offers a promising approach to understanding how *purB* and regulatory networks collectively influence the bacterium's adaptation to diverse environments. The findings of this study along with the future research may pave the way for the development of targeted interventions to enhance food safety in the future.

# VII. ZUSAMMENFASSUNG

*L. monocytogenes* ist ein wichtiger lebensmittelassozierter Krankheitserreger, der weltweit zu Erkrankungen mit hohen Hospitalisierung- und Todesfallraten führt. Insbesondere die Fähigkeit zum Wachstum unter Kühltemperaturen und eine hohe Resilienz gegenüber typischen lebensmittelassoziierten Stressbedingungen machen dieses Pathogen zu einer Priorität für die Lebensmittelsicherheit. Der Erreger kann jahrelang in Betrieben persistieren und aus dem Betriebsumfeld in fertige Produkte gelangen. Vor allem ready- to- eat (RTE) Produkte bergen ein besonderes Risiko, da diese meist ohne weiteren Erhitzungsschritt roh verspeist werden. Von Challenge-tests ist bekannt, dass unterschiedliche RTE Lebensmittel sehr unterschiedliche Wachstumsbedingungen bieten und entsprechend vielfältige Anforderungen an die Fitness des Erregers stellen. Allerdings sind sehr viele der verfügbaren Daten zur Fitness unter Stress von *L. monocytogenes* anhand von Laborstämmen und in Labormedien ermittelt worden, die nur bedingt die reale Situation wiederspiegeln. Die vorliegende Arbeit untersucht die Wechselwirkungen eines klinisch relevanten Ausbruchsstamms (LL195) mit der Wurstsorte Lyoner. Unter Zuhilfenahme einer Transposon-Mutagenese-Bibliothek mit 2640 individuellen Mutanten wurde der Fitness-Effekt einzelner Gene untersucht. In einem ersten high-throughput screening gelang es vielversprechende Kandidatengene zu identifizieren, welche im Anschluss im Phänotyp bestätigt wurden. Von diesen wurden saubere Deletionsmutanten erzeugt und in weiteren Wachstumsexperimenten unter Kältestress und auf Lyoner separat, sowie unter einer Kombination beider Bedingungen bestätigt.

Die Ergebnisse zeigen eine wichtige Rolle der Cytosin-spezifischen DNA-Modifikationsmethyltransferase *sau3AIM* und dem Penicillin-bindenden Protein *pbp-B*-Gene bei der Anpassung auf Kältestress. Demgegenüber hat *pur B*, eine zentrale Komponente der Purinbiosynthese, einen spezifischen Fitnesseffekt auf das Wachstum *von L. monocytogens* auf Lyoner.

# VIII. SUMMARY

*L. monocytogenes* is an important food-borne pathogen that causes disease with high hospitalization and mortality rates worldwide. In particular, the ability to grow under refrigerated temperatures and high resilience to typical food-associated stress conditions make this pathogen a priority for food safety. The pathogen can persist in companies for years and get into finished products from the operating environment. Ready-to-eat (RTE) products in particular pose a particular risk as they are usually eaten raw without any further heating step. It is known from challenge tests that different RTE foods offer very different growth conditions and accordingly place diverse demands on the fitness of the pathogen. However, much of the available data on the fitness of *L. monocytogenes* under stress has been determined using laboratory strains and in laboratory media, which only partially reflects the real situation. The present work investigates the interactions of a clinically relevant outbreak strain (LL195) with the Lyoner sausage variety. The fitness effect of individual genes was examined using a transposon mutagenesis library with 2640 individual mutants. In an initial high-throughput screening, promising candidate genes were identified, which were subsequently confirmed in the phenotype. Of these, clean deletion mutants were generated and confirmed in further growth experiments under cold stress and on Lyoner separately, as well as under a combination of both conditions. The results show an important role of the cytosine-specific DNA modification methyltransferase *sau3AIM* and the penicillin-binding protein *pbp-B*-gene in adaptation to cold stress. In contrast, *pur B*, a central component of purine biosynthesis, has a specific fitness effect on the growth of *L. monocytogenes* on Lyoner.

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# X. LIST OF TABLES

# XI. DANKSAGUNG

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