Department for Farm Animals and Veterinary Public Health
University of Veterinary Medicine Vienna
Institute of Food Safety, Food Technology and Veterinary Public Health
(Head: Dipl.ECVPH Univ.-Prof. Dr.med.vet. Martin Wagner)

Recovery of *Listeria monocytogenes* genotypes in challenge tests

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Jessica Khadka

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Supervision: Dr in med.vet. Beatrix Stessl

Unit of Food Microbiology

Institute of Food Safety, Food Technology and Veterinary Public Health

University of Veterinary Medicine Vienna

Veterinärplatz 1, 1210 Vienna; Austria

Reviewer: Dr.rer.nat. Tom Grunert

Unit of Functional Microbiology

Institute of Microbiology

University of Veterinary Medicine Vienna

Veterinärplatz 1, 1210 Vienna; Austria

ABBREVIATIONS AND SYMBOLS

L.	*Listeria*
MLST	multilocus sequence typing
PCR	polymerase chain reaction
g	gram
kb	kilobase
bp	Base-pair
µL	microliter
ml	milliliter
s	seconds
°C	celsius
DNA	deoxyribonucleic acid
rpm	Revolutions per minute
ST	Sequence type
ISO	International Organization for Standardization
pH	Potential of hydrogen a measure of acidity – alkalinity
spp.	Species pluralis
AGES	Austrian Agency for food and health safety
RASFF	Rapid Alert System for Food and Feed
ST	Sequence type
CC	Clonal Complex
%	percentage
WT	Wildtype
EU	European Union
RTE	Ready to eat
EC	European Commission
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
CFU	Colony forming Units
MLST	Multilocus sequence typing
LMO	*Listeria monocytogenes*

TABLES AND FIGURES

1. INTRODUCTION

1.1 *Listeria monocytogenes* background Information

Listeria, a genus of bacteria classified within the family *Listeriaceae*, includes a variety of species. Among them, *Listeria monocytogenes* is a gram-positive, motile, coccoid rod-shaped bacterium characterized by its non-spore-forming nature and catalase-positive activity. *Listeria* is facultatively anaerobic and forms colonies with diameters ranging from 0.5-1.5mm within 24-48 hours of incubation (McLauchlin and Rees, 2015).

The taxonomic classification of the *Listeria* genus includes 30 species and eight subspecies, but only two species, namely *L. monocytogenes* and *L. ivanovii,* are associated with pathogenicity (Euzéby and Parte, 2020; Osek et al., 2022). Today, thirteen *Listeria* serotypes have been identified, categorized into four evolutionary lineages. Following Multilocus sequence typing (MLST), the serotypes are further differentiated into clonal complexes (CCs) and sequence types (STs)(Schoder et al., 2023). The species LMO is diverse, consisting of 3102 STs and 219 CCs, while 880 STs remain unassigned to a CC ("BIGSdb, Institut Pasteur Database," 2022).

Genetic lineages I and II contain most of the human serotypes. Serotypes of 1/2 b and 4b from genetic lineage I contain hypervirulent strains such as CC1, CC4 and CC6 (Raschle et al., 2021). 50% of human outbreaks are traced back to serotype 4b, whereas serotype 1/2a is found more frequently in food isolates (Acciari et al., 2022). Isolates from lineage II, including CC9 and CC121, are associated with food-related cases, while isolates from lineage I containing CC1, CC2 and CC4 are correlated with clinical manifestations (Disson et al., 2021; Fagerlund et al., n.d.).

Many different strains of LMO have successfully adapted to various environmental niches and food matrices. Among these strains are ST121, ST7, ST8, ST9, ST155 and ST204, each demonstrating their ability to persist in different environments. ST5 and ST121 are both known to be more tolerant of disadvantageous environments. Specifically, ST121 isolates have managed to persist in food and food processing environments (Liu et al., 2022; Myintzaw et al., 2023).

In healthy individuals, symptoms of listeriosis may include diarrhea, vomiting, abdominal cramps and nausea, accompanied by febrile gastroenteritis after consuming contaminated food. In immunocompetent individuals, these symptoms typically manifest within 1-2 days of exposure and persist for about two days. The incubation period for listeriosis is approximately 11 days, with 90% of cases manifesting within 28 days (Clauss and Lorber, 2020).

In contrast, listeriosis poses life-threatening consequences in vulnerable populations such as children, newborns, pregnant women, the elderly and immunocompromised patients. This invasive form of listeriosis can result in severe complications, including bacteremia, meningitis, meningoencephalitis, septicemia and miscarriage (Radoshevich and Cossart, 2018).

The *L. monocytogenes* strains belonging to different serotypes exhibit variations in their antigenic determinants, presenting distinct flagellar (H) antigens and somatic (O) antigens on the cell surface. The positive regulatory factor A (PrfA) is the major virulence regulon of LMO. PrfA is encoded in clustered areas known as *Listeria* pathogenicity island 1 (LIPI-1), where many virulence factors are expressed(Quereda et al., 2021). LIPI-1 includes *prfA* itself, along with genes encoding listeriolysin O (*hly*), phospholipase A (*plcA*), phospholipase B (*plcB*), actin assembly inducing protein (*actA*), a zinc metalloproteinase (*mpl*), OrfX (*orfX*) and the internalins A, B, and C (InlA, InlB, InlC) (Quereda et al., 2021).

Internalins such as InlA and InlB support host cell adhesion, binding and internalization. These proteins facilitate the interaction between the bacterium and the cell surface, promoting efficient entry into the host cell. The *Listeria* adhesive protein (LAP) is another important virulence factor that promotes the attachment of the bacteria to the intestinal epithelium, facilitating colonization and infection. The *hly* and *actA* genes encode the toxin LLO, which enables the penetration of listeriolysin into the membrane and the release of LMO from the vacuoles. Phospholipase C, PlcA and PlcB assist LLO during the vacuole lysis process and help the bacteria to exit the host cells. ActA protein activates the actin polymerization in bacterial cells and allows *L. monocytogenes* to move and penetrate neighboring cells by forming an actin tail. Additionally, the OrfX protein promotes the oxidation of macrophages and initiates the infection process (Chlebicz and Śliżewska, 2018).

L. monocytogenes is a ubiquitous pathogen found in water, soil, plants, animals and food processing facilities. On the one hand, *L. monocytogenes* exhibits a saprophytic lifestyle in soil, utilizing decaying organic matter as a nutrient source. However, once ingested by humans, it can become an intracellular pathogen capable of surviving and replicating within host cells. The transition from saprophyte to cytosolic parasite is tightly regulated by the modulation of PrFA. Outside host cells, such as in soil or vegetation, PrfA remains in a state of low activity, resulting in reduced gene expression levels. Once inside the mammalian host, PrfA is activated and gene expression increases, allowing *L. monocytogenes* to enter and invade the host cells (Freitag et al., 2009). Each step of the intracellular infection cycle of listeriosis, from entry, host cell invasion, phagosomal escape, replication, actin-based motility and cell-to-cell spread, is regulated by products of the PrfA regulon (Scortti et al., 2007).

LMO is known for its ability to survive in harsh and changing environments. One such example would be food processing plants where the pathogen is exposed to many environmental stressors including low storage temperatures, high salt concentration, acidic pH, competitive microflora, humidity, osmotic stress and exposure to disinfectants. The alternative sigma factor (SigB) is involved in the stress resistance mechanism of *L. monocytogenes.* The stressosome, a complex supramolecular multiprotein assembly, serves as a sensor for environmental stressors, activating the SigB and triggering a general stress response (GSR). This response results in an up-regulation of a large SigB regulon consisting of more than 300 genes, which allows *L. monocytogenes* to respond to changing environmental conditions (Guerreiro et al., 2020).

Various foods may contain *L. monocytogenes,* such as raw meat, processed meat, sausages, raw milk, fruit, vegetables, mushrooms, soft cheese, fresh cheese, seafood and processed ready-to-eat (RTE) foods (Hof, 2003). In the European Union (EU), the highest prevalence of *L. monocytogenes* was found in fish products, soft and semi-soft cheeses and RTE meat products. Fresh cheese had the lowest mean prevalence, while brined cheese had the highest prevalence rate at 11.8% (Martinez-Rios and Dalgaard, 2018). Since the 1980s, LMO contamination of fresh produce, sprouts and mushrooms has also increased, resulting in fresh produce-associated listeriosis outbreaks. The risk of contamination in fresh RTE produce is high because the foods are consumed raw, without any step to eliminate the microbes (EFSA and ECDC, 2021). Given the ability of *L. monocytogenes* to persist in soil and plants, adhere to surfaces, form biofilms, and exhibit resistance to heat, acidity, and disinfectants, there is concern regarding its presence during pre-harvest, post-harvest and retail stages of food processing (Townsend et al., 2021; Wiktorczyk-Kapischke et al., 2022).

According to the EU One Health Zoonoses Summary Report (EUOHZ), *L. monocytogenes* was the fifth most reported zoonosis in humans in the EU, with 1,876 confirmed cases in 2020 (EFSA and ECDC, 2021; Vasileiadi et al., 2022). The reported cases resulted in 780 hospitalizations and 167 deaths, reflecting a case fatality rate of 13%. Thus, listeriosis stands as one of the most severe foodborne diseases under EU surveillance with the highest mortality rate among all zoonoses (Vasileiadi et al., 2022). In 2020, there were 16 documented foodborne outbreaks in the EU, leading to 120 cases of illness, 83 hospitalizations and 17 fatalities (EFSA and ECDC, 2021). Nine of those outbreaks had strong evidence and were traced back to contamination sources, which were fish products, mixed meat products and cheeses (EFSA and ECDC, 2021). The overall occurrence of *L. monocytogenes* in tested samples of RTE fish and RTE fishery products was 5.3%, RTE meat and meat products were 4.8%, cheeses 0.54% and 2,9% in RTE fruits and vegetables (EFSA and ECDC, 2021).

L. monocytogenes can survive in anaerobic and microaerophilic environments in a temperature range between -0.4 and 45 °C. Although the optimum growth temperature is between 30-37 °C, growth can still be observed at temperatures below 15 °C. This psychrotolerant pathogen is capable of proliferating even under refrigeration temperatures, posing a significant risk for foodborne transmission. Factors such as pH, water activity and the presence of inhibitors can reduce the growth rate of *L. monocytogenes* at lower temperatures by prolonging the lag phase. Although cold shock proteins (CSPs) and cold acclimation proteins (CAPs) have been identified in the organism, their exact function remains unknown (Chan and Wiedmann, 2009). Given its facultative nature, *L. monocytogenes* can adapt and grow in low-oxygen conditions, anaerobiosis, and even aerobic conditions during experiments. Anaerobiosis needs to be considered in experimental designs as it influences stress adaptation, metabolic pathway and survival of the pathogen (Lungu et al., 2009).

The growth of *L. monocytogenes* is influenced by various storage parameters, including temperature, relative humidity and water activity (a_w) Lower relative humidity indicates a decrease in the survival of *L. monocytogenes* (Marik et al., 2020). Furthermore, water activity can limit the growth of the pathogen, with an optimum a_w value equal to or greater than 0.97. According to EC regulation 2073/2005 safety criteria of *L. monocytogenes*, growth is not supported in products with pH ≤ 4,4 or aw ≤ 0,92, as well as products with pH ≤ 5,0 and aw ≤ 0,94 (EC Regulation No 2073/2005, 2005). Relative air humidity (RH) is another factor to consider when looking for approaches to limit microbial growth. RH fluctuation followed by dehydration and rehydration significantly reduced the survival rate of persistent *L. monocytogenes* pathogens by altering the cell envelope. The RH fluctuation could prove beneficial in eliminating pathogens from the surface of food processing equipment (Zoz et al., 2021, 2016).

The pH range for *L. monocytogenes* growth is wide and can range from 4.3 to 9.4. while the optimum pH is 6.0 to 7.0. *L. monocytogenes* is more likely to encounter acidic than alkaline environments. Examples of such acidic environments can be found inside the host's stomach and in the phagosomes of macrophages (Cheng et al., 2015). *L. monocytogenes* activity was observed at a pH of 5.5 with hydrochloric, acetic and lactic acids or neutral pH of 7.3 with benzoic acid (Mastronicolis et al., 2010). The results show that acid adaptation occurs in *L. monocytogenes*, leading to alterations in fatty acid composition by decreasing the ratio of branched chain and saturated straight fatty acids of total lipids (Mastronicolis et al., 2010). Low pH specifically enhanced the antimicrobial activity of the acid. Acid adaptation is also associated with a decrease in total lipid phosphorus in *L. monocytogenes* lipids and an increase in neutral lipid content, which consequently also increases the amount of saturated straight-chain fatty acids in the membrane. The only exception was benzoic acid, where the neutral and polar lipids remained unchanged due to their low antimicrobial activity (Mastronicolis et al., 2010).

Of all the characteristics discussed, the ubiquitous nature of *L. monocytogenes* makes it a serious threat as a foodborne pathogen, as it can easily enter and spread during food production. The 2017 listeriosis outbreak at a meat processing plant in South Africa is an example of how contamination at one facility can lead to a large national outbreak of listeriosis if microbiological and epidemiological surveillance plans are not established (Lecuit, 2020). This particular outbreak resulted in 937 cases of listeriosis affecting vulnerable populations, including pregnant women, neonates and patients with HIV (Thomas et al., 2020).

Understanding the infection process and life cycle helps explain how *L. monocytogene*s infects the host after ingestion. It survives in the acidic environment of the stomach and attempts to cross the intestinal barrier. The pathogen invades the intestinal epithelial barrier, which consists of Peyer's patches, intestinal villi and goblet cells. Invasion occurs when the proteins Internalin- A (inlA) and B (inlB) bind to E-cadherin, allowing the receptors to cluster together. The binding leads to E-cadherin phosphorylation and ubiquitylation, allowing bacteria to enter the cells (Davis et al., 2019; Radoshevich and Cossart, 2018). Once the bacterium infects the intestinal cells, it can transcytose across different cells, such as lymphoid cells and disperse through the body. When the infected vacuole reaches the liver, it can translocate to the gallbladder, replicating extracellularly and allowing reentry into the gastrointestinal tract (Davis et al., 2019). After vacuolar release, the pathogen will polymerize with actin from the cytoplasm, which will help the bacteria propel to the next cell. The ability to spread from cell to cell also occurs when *L. monocytogenes* crosses the placenta or blood-brain barrier (Lecuit, 2005).

Initially, *L. monocytogenes* served as a model in molecular biology to study how bacteria adapt to their host and develop cell-mediated immunity (Rolhion and Cossart, 2017). *Listeria* serves as a model in immunology to investigate the mechanisms that make the bacteria more resistant to host cells (Hof, 2003). Chromatin modification in the cell nucleus is another way in which bacteria alter the expression of host genes. There are several mechanisms by which *L. monocytogenes* can target the host chromatin and epifactors (Bierne and Hamon, 2020). The different epigenetic mechanisms involve bacterial molecular patterns, LLO toxin, an invasion protein or neuromodulin. One mechanism is the recognition of intracellular microbe-associated molecular patterns (MAMPs) by molecular pattern recognition receptors, which activate the mitogen-activated protein kinase (MAPK) signaling pathway. Once the MAPK pathway is activated, histone H3 phosphorylation and histone 3 and 4 acetylation activate pro-inflammatory genes. Another mechanism is the secretion of effectors such as LLO toxins, which activate signaling cascades. This leads to histone modifications by dephosphorylation and deacetylation of histone H3 at serine 10, resulting in K+ efflux at the plasma level and a reduction in the expression of defense genes (Bierne and Hamon, 2020).

1.2 Persistent *Listeria monocytogenes*

There is no standard definition of persistence; however, persistence is commonly defined as the isolation of the same subtype ≥2 times over ≥6 months. *L. monocytogenes* can persist in a "niche" processing environment, such as specific equipment in facilities, making it difficult to eliminate the pathogen. The two most common risk factors for persistent *Listeria* included equipment cleanability and lack of hygienic zoning. Niches, where cleaning and sanitation are difficult, were the same areas where more persistent *Listeria* strains were isolated. A lack of hygiene zoning can increase the spread of *Listeria* from one location to another (Belias et al., 2022). Despite disinfection measures, *L. monocytogenes* can persist on the machines of food processing plants for many years. Unlike sporadic strains, persistent strains isolated from a fish processing plant tolerated higher concentrations of disinfectants, except for iodine-based compounds (Wiktorczyk-Kapischke et al., 2022).

The biofilm-producing ability of *L. monocytogenes* isolates was assessed, and the results showed that stress survival islet 1 (SSI-1) was associated with increased levels of biofilm formation. *L. monocytogenes* strains isolated from meat products produced moderate to solid biofilms compared with dairy samples. The *arsD* stress gene and the truncated inlA protein were both associated with increased biofilm formation (Di Ciccio et al., 2022).

Whole genome sequencing (WGS) was performed to characterize the genetic diversity and determine biomarkers that influence the persistence of *L. monocytogenes* (LMO) isolates in RTE seafood processing plants. LMO isolates from food and food processing environments belonged to five CCs with long-term intra- and interplant persisting clones harboring conserved mobile genetic elements (MGEs), such as plasmids, prophages and transposons (Palma et al., 2020). These MGEs have genes that confer resistance to chemical compounds and biocides used during processing(Palma et al., 2020).

Identical gene sequences were found in plasmids from different *L. monocytogenes* CC isolates, supporting the hypothesis of horizontal gene transfer followed by deletion and homologous recombination to adapt to stressors in food processing plants. Genes found in a 90.8 kbp plasmid were identical to isolates from CC204 and CC155 and highly similar to an 81.6 kbp plasmid from a CC7 isolate. Characterization of the prophage profile showed that most prophages detected in the *L. monocytogenes* genome were integrated into the chromosome, with insertion sites and several prophage-loci being plant-associated. A novel 31.5 kbp *Listeria* genomic island 3 (LGI3) is specific to persistent CC101 strains, is composed of plant-associated loci and chromosomally integrating cadmium resistance determinants called cadA1C (Palma et al., 2020).

Plasmids harbor putative stress response genes and contribute to tolerance to stressors associated with the food processing environment. Three *L. monocytogenes* strains (ST5, ST8, and ST121) were cured of their plasmids and subjected to disinfectant, oxidative, heat, acid and salt stress (Naditz et al., 2019). The wild-type (WT) and plasmid-cured strains were exposed to the stressors and then plated for CFU to examine the pathogen survival. The results showed that WT strains had a significantly higher count of colonies surviving than plasmid-cured strains. Specifically, ST5 WT strains exposed to benzalkonium chloride (BC) and elevated temperatures (50° and 55 °C) had a higher survival rate compared to the plasmid-cured ST5 strain (Naditz et al., 2019).

1.3 *Listeria* incidence and Rapid Alert System for Food and Feed (RASFF) notifications

The European Union (EU), including Austria, has enacted regulations that make listeriosis a notifiable disease (Desai et al., 2019). The European Centre for Disease Prevention and Control (ECDC) maintains a database called the "Surveillance Atlas of Infectious Diseases", which lists 2268 reported cases of listeriosis in the EU and the European Economic Area (EEA) in 2021. France (n= 435) and Germany (n = 560) had the highest reported cases in the ECDC database. The average fatality rate in the EU and EEA was 14.1%. The European Food Safety Authority (EFSA) and ECDC reports within the zoonoses report surveillance data from primary and secondary production and retail levels. The most recent report is from 2020, when 16 listeriosis outbreaks occurred in the EU, resulting in 83 hospitalizations and 17 deaths. Of these, nine were high-confidence outbreaks, with six, two, and one caused by *L. monocytogenes* in fish, meat, and dairy products, respectively. In Austria, there were 38 cases of invasive listeriosis in 2021, resulting in seven deaths, corresponding to a mortality rate of 18.4%. According to AGES data, ten outbreaks of listeriosis were reported in Austria in the last decade (AGES, 2022).

In the search for listeriosis cases in 2021, the Rapid Alert System for Food and Feed (RASFF), an online portal, enables the rapid exchange of information on the safety of products in the European Union. Urgent alerts are disseminated through the portal, enabling food safety authorities to trace products and respond to food safety threats. The database provides information on the type of product, its origin and where it can be traced. Scientific experts and EFSA analyze the risks. The product is classified and notified on the RASFF portal (alert, information, border rejection). This alert system helps to ensure that dangerous products are withdrawn from the market before they pose a risk to public health.

The portal allows filtering of the product category, risk, and hazard category. After selecting the category “fruits and vegetables” along with “pathogenic micro-organism,” the search results showed 68 products contaminated by pathogens. The notification for LMO-contaminated products within EU countries from 16.01.2020 until 03.07.2023 is depicted in Table 1. The most frequently reported products in the fruit and vegetable category were lettuce, mushrooms and ready-to-eat products. The reported cases were classified as serious, and the products were stopped before distribution or recalled from the market.

Table 1: RASFF portal data notifying L. monocytogenes in fruits and vegetables, including ready-to-eat products (2020-2023)

PRODUCTS	COUNTRY	RISK
Potato slices - RTE	UK	not serious
Tomato and cheese – RTE from Poland	France	undecided
Cabbage	Netherlands	serious
Salad	France	serious
RTE salad box	France	serious
Salad	Belgium	serious
Salad	Netherlands	serious
RTE Pasta salad	UK	serious
Frozen sweet corn from Italy	Switzerland	serious
Red beetroot chunks	Netherlands	serious
Cooked beetroots	Netherlands	serious
Shimeji mushrooms	Belgium	not serious
Shredded cabbage	Ireland	undecided
Enoki mushrooms from South Korea	Netherlands	serious
Enoki mushrooms from China	Netherlands	serious
Enoki mushrooms from China	Slovenia	serious
Salad “Mediterranean mixture” from Sweden	Latvia	not serious
Organic vegetarian replacement product for animal product	Germany	serious
Lamb’s lettuce from France	Netherlands	serious
Salad from Netherlands	Belgium	serious
Nem Chua	France	serious
Frozen broccoli from Poland	Finland	serious
Dumplings with meat filling from Poland	Poland	potentially serious
RTE salad from Netherlands	Germany	undecided
Ready meal Cantonese rice	Italy	serious
Pancakes from Belgium	Denmark	serious
RTE product from Slovakia	Poland	serious

1.4 *Listeria monocytogenes* challenge test

According to EC Regulation 2073/2005 on the LMO food safety criterion, foods are classified as those that support or do not support the growth of *L. monocytogenes* (EFSA and ECDC, 2021). Therefore, pH, water activity (aw) and the amount and type of preservatives must be considered for growth promoting products. According to EC 2073/2005, products with a pH ≤ 4.4 or aw ≤ 0.92, products with a pH ≤ 5.0 and aw ≤ 0.94 or products with a shelf-life < 5 days are presumed not to support the growth of *L. monocytogenes*. The latter products must be analyzed according to ISO 11290-2 at the end of the shelf-life and the limit of 100 *L. monocytogenes* colony forming units (CFU) per g must not be exceeded. Products that support the growth of LMO must also be tested for the absence of *L. monocytogenes* in 5x25g prior to retail release. (EC Regulation No 2073/2005, 2005).

The current 2-class presence-absence "zero tolerance" sampling approach leads to the recall of all RTE foods, regardless of the risk profile, as soon as products are tested positive for *L. monocytogenes* (Farber et al., 2021). The 2-class sampling method discourages companies from testing aggressively to find positives. Alternative sampling methods favor a 3-class sampling plan with more stringent testing including a mixture of qualitative (e.g., 0/25g) and quantitative microbiological limits (100cfu/g). This type of sampling plan effectively provides warning signals since the method can distinguish low-frequency, low-level accidental contamination, high-frequency and higher-level contamination (Farber et al., 2021).

Mandatory measures to detect the presence and growth of LMO include food lot testing, environmental monitoring and challenge testing to estimate the growth potential of *L. monocytogenes* in different food matrices. Challenge testing involves artificially contaminating the food being tested with the microorganism of interest, in this case, *L. monocytogenes*. This inoculation process mimics the potential contamination of the food during pre- and postharvest processing, transport, storage and supermarket distribution. The aim was to assess whether the tested food is capable of supporting the growth of *L. monocytogenes* (Spanu et al., 2014).

A revised technical guidance document published in June 2014 by the EU Community Reference Laboratory for *L. monocytogenes (*EURL *Lm)* outlines which foods can and cannot be tested and how to conduct shelf-life studies. Challenge testing is not required for food categories where the food is cooked before consumption, foods that have undergone heat treatment before packaging, fresh, uncut and unprocessed vegetables, bread and biscuits, bottled drinks, sugar, honey, products containing chocolate/cocoa, bivalve mollusks, food grade salt and frozen products (Álvarez-Ordóñez et al., 2015; Bergis et al., 2021). Foods that fall under the EC 2073/2005 pH and a_w requirements are also not tested in challenge tests.

Growth potential is measured in $\log_{10}$ CFU/g at both the highest LMO concentration and the start of the challenge test. A CFU/g difference greater than 0.5 $\log_{10}$ indicates that the product supports the growth of LMO, whereas a difference less than 0.5 $\log_{10}$ indicates that the product is not capable of supporting the growth of LMO. The enumeration of LMO is performed "at least at the beginning of the challenge test and at the end of the shelf life of the product, using the standard method for enumeration of *L. monocytogenes* EN ISO 11290-2" (Álvarez-Ordóñez et al., 2015)

The EURL *Lm* document includes a protocol on how to perform challenge tests. First, if the growth probability and inter-batch variability are high, at least three batches must be selected. The test should be performed with a mixture of at least two *L. monocytogenes* strains. The inoculation should not compromise the physicochemical characteristic of the product, which explains why the inoculum volume is aimed at 100 CFU/g and should not exceed 1% of the mass of the test unit to mimic a realistic contamination scenario. The inoculation can be in-depth (ground food, mixed salads) or at the surface (contamination during slicing). Storage is as long as the shelf-life and should mimic temperatures during the manufacture, arrival at retail and consumer levels (Bergis et al., 2021).

Challenge tests were performed on precut RTE iceberg lettuce to evaluate shelf-life, natural microflora and survival of LMO. Accelerated enzymatic activity, moisture loss and microbial proliferation affect the shelf life and growth of the pathogen (Tucci et al., 2019). The RTE iceberg lettuce was studied six days beyond its shelf-life at storage temperatures of 8 and 12°C. LMO growth was detected at both storage temperatures, but growth was more significant in samples stored at 12°C. The maximum growth rate ($_{\mu}$max) was 0.0104 $\log_{10}$CFU/g/h at 8°C and 0.0183 $\log_{10}$CFU/g/h at 12°C (Tucci et al., 2019). According to the predictive model used, an increase in storage temperature of 6°C would increase the concentration of LMO to more than 6 $\log_{10}$CFU/g on day 10 of the challenge (12th day of shelf life) (Tucci et al., 2019).

As an alternative to conventional cleaning and disinfection, lactic acid bacteria (LAB) strains were tested in challenge tests to determine if they inhibit LMO. Two LAB strains, PS01155 and PS01156, were used in the spot inoculation assay and successfully inhibited LMO growth. However, when LAB was grown with environmental microbiota, the inhibitory effect decreased, highlighting how external factors affect bacterial growth (Sinclair et al., 2022).

Results of an LMO challenge test on RTE salads showed that vegetables do not support the growth of pathogens when <3.4 log CFU/g is low. Gallia melon was the only fruit that supported bacterial growth. Boiled potatoes and pasta products found in RTE salads did not facilitate the growth of LMO in the challenge test (Lokerse et al., 2016).

Another challenge test confirmed that the choice of food matrix, storage temperature and length of storage affect bacterial growth. Different LMO strains from serotypes 1/2a, 1/2b and 4b, CC14, 517 and 6 and ST91, 517 and 6 were isolated from the same facility. Eight RTE salad products with the mentioned strains showed significant growth of LMO. The bacterial strains had higher growth potential at storage temperatures of 8 °C compared to 5 °C. The gas composition was monitored because the RTE salads were packaged under modified atmosphere. In the challenge tests, some of the salad mixes had O2/CO2 values outside the norm range specified by the manufacturer, which may also have affected bacterial growth (Ziegler et al., 2019).

1.5 Aim of Study

The aim of this study was to estimate the growth potential of LMO in lettuce samples in collaboration with a private laboratory. Due to their short shelf life (less than 5 days), salads and mixed salad products are to be assigned to the category LM criterion no. 1.3 "Ready-to-eat foodstuffs that cannot promote the multiplication of LMO" (EC Regulation 2073/2005). The limit value <100 LMO colony forming units (CFU)/g is to be applied here until the end of shelf life.

For this purpose, a strain cocktail of different LMO field isolates (3 persistent genotypes: ST21, ST121, ST2) and an LMO reference strain (ST145) were inoculated into the products and the growth was monitored during the shelf-life. In this study, particular emphasis was placed on the potential growth advantage of individual potentially persistent LMO over others.

The hypothesis was that the in-house isolate, which has persisted in the food environment of the processing plant for several years, would have a growth advantage.

2. MATERIALS AND METHODS

2.1 Materials

The equipment and chemicals used in this study are listed in Supplement Table 1.

2.2 Methods

2.2.1 Experimental Overview

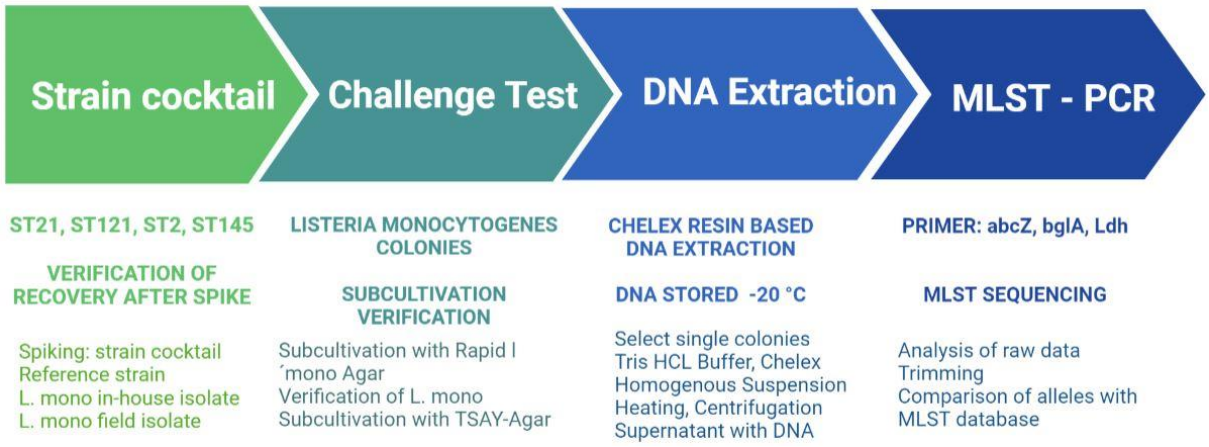

Figure 1: Workflow of the study

Many consumable supermarket products must undergo challenge tests to determine LMO growth and ensure food safety. Challenge tests are used to ensure that LMO that may have been introduced into the product cannot multiply >100 colony forming units (CFU)/g in the product until the end of shelf life.

A private laboratory conducted the challenge tests following the guidelines of ISO 11290-2. Ready-to-eat Venezia and Vogerl salad were the two types of lettuce that were evaluated. The products were inoculated with a LMO strain cocktail, including *L. monocytogenes* reference strain and field isolate strains. The components of the strain cocktail, which are also the field isolates, are ST21, ST121 and ST2. ST145 is the reference strain, also known as ATCC 19115, which belongs to the clonal complex 2 (CC2) and was included in the strain cocktail.

The LMO strain cocktail for the planned challenge trials contained the following in-production field strains (ST2, ST21, ST121), as well as relevant hypervirulent strains (CC4-ST631, ST1) assembled from product and environmental samples. The LMO strain cocktail was cold adapted at 10 °C before each trial to create temperature conditions as realistic as possible for production.

As the producer of salad mono- (Vogerl salad) and mixed products (Venezia salad; Young spinach, Vogerl salad, Lollo verde, rocket salad and Lollo rosso) wanted to test the growth potential under a "worst case scenario", lettuce products were tested in a first series of experiments without the addition of protective gases, at 6 and 8°C storage. The lettuces were inoculated on day 0 with a facility-

specific LMO strain cocktail (genotype ST2-genetic line I, ST21, ST121-both genetic line II) and a reference strain ATCC 19115 (approx. 100 CFU/g).

After inoculation, the lettuce samples were stored and their growth was monitored on days 0, 4, 6 and 8. Challenge tests were performed in triplicate with reference and control samples diluted at 1:3, 1:30 and 1:300. After the examination of the challenge test agar plates, individual colonies were transferred to selective agar plates for subculture and verification. DNA extraction using Chelex resin was used to obtain and purify the DNA. After isolating the DNA, the next step was to perform PCR to amplify the DNA. MLST housekeeping genes were targeted for the PCR ST prescreening (Table 2, Table 5). After confirming the results with agarose gel electrophoresis, the samples were sent to LGC Genomics for multilocus sequence typing (MLST). The raw data from the MLST sequencing were downloaded from the LGC server and analyzed by uploading the sequencing results to the BIGSdb-Pasteur MLST database. A*bcZ, bglA*, *cat, dapE, dat, lhKA* and *ldh* are the seven housekeeping genes found in LMO for MLST (Figure 2).

abcZ	bglA	cat	dapE	dat	ldh	lhkA	ST	CC	
7	7	3	10	5	6	1	21	CC21	Field isolate
7	6	8	8	6	37	1	121	CC121	Field isolate
1	1	11	11	2	1	5	2	CC2	Field isolate
1	1	11	11	2	12	5	145	CC2	Reference ST

***Figure 2:** Field isolates, primers and reference strain in the strain cocktail*

Abbreviations: ST = Sequence type; CC = Clonal complex; ST45 = reference strain (ATCC 19115)

2.2.2 DNA Extraction

Agar plates from the challenge test were examined, labeled, and prepared for DNA extraction. Single colonies were selected from the agar plates and transferred to selective agar plates (Rapid L. mono) using inoculation loops (each ten LMO isolates per subsample (RF, reference sample; EF, control for success and dilution 1:3, 1:30 and 1:300). The replated agar plates were incubated at 37°C for 48 h. DNA extraction was performed according to the protocol for *Listeria* detection provided by the Unit of Food Microbiology (Vetmeduni). In the first step, 2 ml Eppendorf tubes were labeled and 100 µL of 0.01 M Tris HCl buffer was pipetted into the tubes. Single colonies were selected from agar plates using inoculation loops and transferred to Eppendorf tubes. After dissolving the colonies in a Tris HCl buffer, the inoculation loops were discarded and the tubes were vortexed until a homogeneous suspension was obtained. In total 430 isolates were used for DNA extraction.

Next, 400 µL of Chelex® 100 resin (Bio-Rad, Hercules, CA, USA) was added to the suspension. The Chelex solution, which can bind to PCR inhibitors, was mixed before transfer to the tubes. The tubes

were sealed and placed in a heating block at 100°C for 10 minutes. After heating, the samples were centrifuged at 15000 rpm for 5 seconds using a mini centrifuge (Eppendorf AG, Hamburg, Germany). Without shaking the tubes, the supernatant containing DNA was transferred to 1.5 ml maximum recovery tubes (VWR International, Avantor, Radnor, PA, USA). The tubes were labeled and stored in cardboard boxes in a refrigerator at -20°C until further processing.

2.2.3 *Listeria monocytogenes* MLST – PCR

LMO Multilocus sequence typing (MLST) is a method that uses the following seven housekeeping genes listed in table 2 ("BIGSdb, Institut Pasteur Database," 2022).

Table 2: Seven housekeeping genes

abcZ	ABC transporter
bglA	beta glucosidase
cat	catalase
dapE	succinyl diaminopimelate desuccinylase
dat	D-amino acid aminotransferase
ldh	L-lactate dehydrogenase
lhkA	histidine kinase

Table 3 lists the components for the master mix used for the PCR with the final concentration, stock concentration and final amount of each component in microliter per reaction. Table 4 provides information on the conditions and settings in the thermocycler for DNA amplification. Table 5 lists the primers used for the PCR with information regarding the direction and primer sequence. The targets used for the MLST-PCR were *abcZ, bglA* and *ldh.* Using only these primers was sufficient to sequence all genes. The PCR products were sent to LGC Genomics GmbH for multilocus sequencing. The raw data were uploaded to the Bacterial Isolate Genome Sequence Database of the Pasteur Institute (BIGSdb-Pasteur) and later sorted using Excel. By analyzing the results of MLST, we can gain insight into the clonal framework of *L. monocytogenes.* Comparing the different isolates with the reference strain can help explain why specific isolates, such as the in-house isolate, may be more persistent than other strains during recovery.

Table 3: Master mix for Listeria monocytogenes MLST - PCR

COMPONENTS	FINAL CONCENTRATION	STOCK CONCENTRATION	µL PER REACTION
DEPC water			31,3
10x PCR buffer	1x		5
MgCl2	2,5mM	50mM	2,5
PrimerF	200nM	5000nM	2
PrimerR	200nM	5000nM	2
dNTP's	200µM	5000µM	2
Taq pol (Plat.)	1U	5U/µl	0,2
Mastermix			45
Template			5
Reaction volume			50

Table 4: PCR conditions

Initial denaturation	94° for 4 min (1 cycle)
Denaturation	94° for 30 s
Annealing	52° for 30 s* (35 cycles)
Elongation	72° for 2 min
Final Elongation	72° for 10 min (1 cycle), 4° hold

PCR amplification are performed at an annealing temperature of 52°C for all genes except for bglA (45°C).

Table 5: Primers used for PCR

GENE	DIRECTION	PRIMER SEQUENCE
abcZ	*abcZoF*:	GTTTTCCCAGTCACGACGTTGTATCGCTGCTGCCACTTTTATCCA
	abcZoR:	TTGTGAGCGGATAACAATTTCTCAAGGTCGCCGTTTAGAG
bglA	*bglAoF*:	GTTTTCCCAGTCACGACGTTGTAGCCGACTTTTTATGGGGTGGAG
	bglAoR:	TTGTGAGCGGATAACAATTTCCGATTAAATACGGTGCGGACATA
cat	*catoF*:	GTTTTCCCAGTCACGACGTTGTAATTGGCGCATTTTGATAGAGA
	catoR:	TTGTGAGCGGATAACAATTTCAGATTGACGATTCCTGCTTTTG
dapE	*dapEoF*:	GTTTTCCCAGTCACGACGTTGTACGACTAATGGGCATGAAGAACAAG
	dapEoR:	TTGTGAGCGGATAACAATTTCATCGAACTATGGGCATTTTTACC
dat	*datoF*:	GTTTTCCCAGTCACGACGTTGTAGAAAGAGAAGATGCCACAGTTGA
	datoR:	TTGTGAGCGGATAACAATTTCTGCGTCCATAATACACCATCTTT
ldh	*ldhoF*:	GTTTTCCCAGTCACGACGTTGTAGTATGATTGACATAGATAAAGA
	ldhoR:	TTGTGAGCGGATAACAATTTTATAAATGTCGTTCATACCAT
lhkA	*lhkAoF*:	GTTTTCCCAGTCACGACGTTGTAAGAATGCCAACGACGAAACC
	lhkAoR:	TTGTGAGCGGATAACAATTTCTGGGAAACATCAGCAATAAAC

2.2.4 Agarose gel electrophoresis

After PCR, agarose gel electrophoresis was performed to confirm DNA amplification and to check for contamination during DNA isolation. The first step was to prepare the 1.5% agarose gel by transferring 1.5 g of agarose into a flask and diluting the agarose with 100 ml of 1x TAE buffer. The mixture was heated in a microwave until the agarose was diluted in the buffer. 2.5 µL of PeqGreen gel stain (VWR International) was added when the liquid gel had cooled. The flask was gently swirled to dissolve the gel stain in the mixture. The next step was to pour the liquid gel into gel casting trays with the combs inserted. After 30 minutes, the combs were removed and the gel was allowed to set at room temperature. The electrophoresis chamber was set up by pouring in 1x TBE buffer and checking that there were no particles in the buffer. The combs were removed from the solidified gel and the gel tray was placed in the electrophoresis chamber. 10 µL of each sample and the control were mixed with a drop of sample loading buffer (2 µL) on a laboratory film and loaded into the gel wells. However, the first and last wells were loaded with 5 µL of DNA ladder (1kb). After this step, the lid of the electrophoresis chamber was closed, and the power supply was switched on. The gel electrophoresis was run at 120 volts for 30 minutes, separating the DNA fragments. Finally, the gels were photographed using an imaging system that takes pictures under UV light (Gel Doc 2000, Biorad). This technology makes the bands visible and compares how far the DNA fragments have separated. The images of the gels were saved as tiff files for further analysis.

3. RESULTS

The tables in Figures 3, 4 and 5 were produced using the results from the raw data. Figure 3 shows the results of the first and second challenge tests with Venzia and Vogerl salads samples. The table for Experiment 1 shows the growth of ST121 and ST21 in Venezia salad isolates at 6°C and 8°C on days 0, 4, 6 and 8. In the first experiment, no strains were detected in the reference control sample RF3 on day 0. The persistent LMOs (ST21) and another field strain (ST121) showed a clear growth advantage over the reference strain ST145, and ST 2 in the spiked lettuce samples.

Experiment two used Vogerl's salad which also showed similar growth of ST121 and ST21 and no growth of ST2 and ST145 when analyzing the results of the samples. Both experiments one and two supported the growth of the two LMO strains ST121 and ST21 predominantly at dilutions 1:3 and 1:30 and comparatively less at dilution 1:300 on all days of storage. For both products tested, a δ of 0.5 $\log_{10}$ CFU/g was exceeded at both storage temperatures tested, thus promoting the growth of LMO. The shelf life of these products cannot be extended with the given product characteristics.

Figure 4 displays the results of the third experiment with Vogerl salad at 6°C and 8°C on days 0, 4, 6 and 8. The persistent LMO strain ST21 was detected on all days in dilutions 1:3 and 1:30 at 6°C and 8°C. ST2 was detected in the reference control group on day 0 in 1:3 and 1:30 dilutions at both 6°C and 8°C. ST2 is also detectable in 1:3 dilution at 6°C on day 4. However, there was no recovery of the ST2 strain on days 6 and 8 in any dilution at 6°C. In comparison, ST2 recovered on days 0, 4, 6 and 8 in all three dilutions at 8°C. ST121 was detectable on all days at 1:3 and 1:30 dilutions at 6°C. Similarly, ST21 was detectable on all days in all dilutions at 6°C. On day 0, only ST121 and ST21 were detectable at 6°C at a dilution of 1:300.

At 8°C, ST21 is detectable at all dilutions on all days. ST2 was recovered on days 0, 4 and 6 at 1:3 and 1:30 dilutions at 8°C. ST2 was also recovered on day 8 from the sample diluted 1:300 at 8°C. The ST145 reference strain is recovered on days 4 and 8 at 1:3 dilution at 6°C and 8°C. ST121 is also recovered on days 0, 4 and 6 at 1:3 and 1:30 dilutions at both temperatures.

The results of the third experiment with Vogerl salad samples indicated more competition between bacterial strains, with certain strains failing to grow after competition up to dilution 1:300 indicating growth of LMO (growth potential $\delta > 0.5$ $\log_{10}$ CFU/g). Strains ST121 and ST21 were recovered up to day 8 in all three dilutions at 6°C in the third experiment. The results at 8°C in the third experiment showed that ST21 and ST2 were recovered at 1:300 dilution on day 8. ST145 was detectable in the third experiment at 1:3 dilution at both 6°C and 8°C. A summary of all three experiments is shown in Figure 5.

EXPERIMENT 1: VENEZIA

6°C

ID	Sample	Day	1:3		1:30		1:300	
L21/24448/04	RF 1	0	121		121	21		
L21/24448/05	RF 2	0	121	21	121			
L21/24448/06	RF3							
L21/24448/11	EF 1	4	121	21	121	21	121	21
L21/24448/12	EF 2	4	121	21	121	21	121	21
L21/24448/13	EF 3	4	121	21	121	21	121	
L21/24448/25	EF 1	6	121		121		121	
L21/24448/26	EF 2	6		21	121		121	
L21/24448/27	EF 3	6	121	21	121			
L21/24448/39	EF 1	8	121	21	121	21		
L21/24448/40	EF 2	8	121	21	121		121	21
L21/24448/41	EF 3	8	121		121		121	

8°C

ID	1:3		1:30		1:300	
L21/24448/04	121		121	21		
L21/24448/05	121	21	121			
L21/24448/06						
L21/24448/18	121	21		21		21
L21/24448/19	121	21		21		21
L21/24448/20	121	21	121	21	121	21
L21/24448/32	121	21	121	21	121	
L21/24448/33	121	21	121			
L21/24448/34	121	21	121	21	121	
L21/24448/46		21		21	121	
L21/24448/47	121		121	21	121	
L21/24448/48	121	21	121		121	

EXPERIMENT 2: VOGERL

6°C

ID	Sample	Day	1:3		1:30		1:300	
L21/24447/04	RF 1	0	121	21	121			
L21/24447/05	RF 2	0	121	21	121	21		
L21/24447/06	RF 3	0	121	21	121			
L21/24447/11	EF 1	4	121	21		21	121	
L21/24447/12	EF 2	4		21	121			
L21/24447/13	EF 3	4		21	121	21	121	
L21/24447/25	EF 1	6	121		121		121	
L21/24447/26	EF 2	6		21		21	121	
L21/24447/27	EF 3	6	121		121			
L21/24447/39	EF 1	8	121	21	121		121	
L21/24447/40	EF 2	8	121		121			
L21/24447/41	EF 3	8	121		121	21		

8°C

ID	1:3		1:30		1:300	
L21/24447/04	121	21	121			
L21/24447/05	121	21	121	21		
L21/24447/06	121	21	121			
L21/24447/18	121	21	121		121	21
L21/24447/19	121	21	121	21	121	21
L21/24447/20		21	121	21	121	
L21/24447/32	121		121		121	
L21/24447/33		21	121	21		21
L21/24447/34	121	21	121	21		
L21/24447/46	121	21	121	21	121	
L21/24447/47	121		121	21		
L21/24447/48	121		121		121	

Figure 3: Detailed results of experiments 1 and 2: LMO challenge test in Venezia and Vogerl salad.

EXPERIMENT 3: VOGERL

6°C

ID	Sample	Day	1:3				1:30				1:300			
L21/27004/04	RF 1	0		21	2		121	21						
L21/27004/05	RF 2	0		21				21	2					
L21/27004/06	RF 3	0		21				21						
L21/27004/11	EF 1	4		21	2	145	121	21						
L21/27004/12	EF 2	4	121	21		145		21						
L21/27004/13	EF 3	4	121	21		145		21						
L21/27004/25	EF 1	6		21				21						
L21/27004/26	EF 2	6	121	21				21						
L21/27004/27	EF 3	6		21			121							
L21/27004/39	EF 1	8		21				21			121	21		
L21/27004/40	EF 2	8	121	21			121	21				21		
L21/27004/41	EF 3	8				145		21				21		

8°C

ID	Sample	Day	1:3				1:30				1:300			
L21/27004/04	RF 1	0		21	2		121	21						
L21/27004/05	RF 2	0		21				21	2					
L21/27004/06	RF 3	0		21				21						
L21/27004/18	EF 1	4		21	2	145			2					
L21/27004/19	EF 2	4	121	21		145		21						
L21/27004/20	EF 3													
L21/27004/32	EF 1	6	121	21				21						
L21/27004/33	EF 2	6		21	2			21	2					
L21/27004/34	EF 3	6		21			121	21						
L21/27004/46	EF 1	8		21				21						
L21/27004/47	EF 2	8		21				21						
L21/27004/48	EF 3	8		21		145		21				21	2	

Figure 4: Detailed Results of Experiment 3

Experiment 1 6°C
VENENZIA

Sample	Day	1:3		1:30		1:300	
RF 1-2	0	121	21	121	21		
EF 1-3	4	121	21	121	21	121	21
EF 1-3	6	121	21	121		121	
EF 1-3	8	121	21	121	21	121	21

8°C

1:3		1:30		1:300	
121	21	121	21		
121	21	121	21	121	21
121	21	121	21	121	
121	21	121	21	121	

Experiment 2 6°C
VOGERL

Sample	Day	1:3		1:30		1:300	
RF 1-3	0	121	21	121	21		
EF 1-3	4	121	21	121	21	121	
EF 1-3	6	121	21	121	21	121	
EF 1-3	8	121	21	121	21	121	

8°C

1:3		1:30		1:300	
121	21	121	21		
121	21	121	21	121	21
121	21	121	21	121	21
121	21	121	21	121	

Experiment 3 6°C
VOGERL

Sample	Day	1:3				1:30				1:300			
RF 1-3	0		21	2		121	21	2					
EF 1-3	4	121	21	2	145	121	21						
EF 1-3	6	121	21			121	21						
EF 1-3	8	121	21		145	121	21			121	21		

8°C

Sample	Day	1:3				1:30				1:300			
RF 1-3	0		21	2		121	21	2					
EF 1-3	4	121	21	2	145		21	2					
EF 1-3	6	121	21	2		121	21	2					
EF 1-3	8		21		145		21				21	2	

Figure 5: Summary of Results

4. DISCUSSION

The three challenge tests clearly demonstrated the variability of LMO growth in the different experiments (1-3). The persistent strains ST21 and ST121 were the best-adapted spike strains in all three experiments, while the control strains ST145 and ST2 (both CC2 and genetic line I) were only detectable in the third experiment at storage temperatures of 6 and 8°C. ST2, which was detectable in the first dilution, grew slightly better than the reference strain ST145. These results clearly show the importance of using LMO field strains, preferably with persistent properties linked to the food processing environment, in addition to control strains, otherwise, the growth potential of LMO will be underestimated (Álvarez-Ordóñez et al., 2015). When comparing the experiments, it is noticeable that in the first two experiments, the strain fitness of ST21 and ST121 (genetic line II) is significantly better than the other strains in the cocktail (ST2, ST145 genetic lines I). The strains of genetic line I are undetectable in the experiment during storage.

In experiments 1 and 2, ST145 (reference strain) grew significantly worse compared to the other strains. The reason for this could be the cold adaptation ability of the strains, which were adapted at 10°C during cultivation. Genetic line I strains are often involved in food-associated outbreaks and are well adapted to the host (human, animal). LMO was detected more frequently in dairy farms. In particular, the hypervirulent strains CC1 and CC4 are more frequently associated with dairy products (Palacios-Gorba et al., 2021). The PrfA virulence regulon is present in pathogenic *Listeria* spp. strains but unique to LMO is the *Listeria* pathogenicity island 3 *(LIPI-3)*, which is detected in 88% of lineage II isolates. LIPI-3 encodes listeriolysin, which has bactericidal activity and can modify the host gut microbiota during infection, highlighting its importance in the transmission of foodborne listeriosis (Koopmans et al., 2023).

ST121 is a hypovirulent LMO with truncated internalin A gene, most encountered in food production worldwide, no large epidemic outbreaks have been described, only isolated sporadic illnesses in people with multiple diseases, cancer and damaged organs such as liver damage. Food-associated clones CC9 and CC121 are more commonly isolated from severely immunocompromised patients, whereas CC1, CC2, CC4 and CC6 are more commonly found in healthy patients (Maury et al., 2016). When analyzing the clonal complex of LMO, the distribution of InlA truncations was found to be the main feature for the loss of virulence, making ST121 hypovirulent and less invasive during clinical infection (Maury et al., 2016). The adaptability of ST121 and ST21 to the food processing and agricultural environment may favor and explain the growth of these specific strains in lettuce and other vegetables (Félix et al., 2022). Specifically, ST121 was found to be the most prevalent strain in food and food-associated clusters, resulting in 19 non-human cgMLST clusters in 2017 alone. ST21 was detected in food isolates, including

vegetables, meat and food-associated surfaces, but not in clinical isolates (Cabal et al., 2019; Linke et al., 2014).

This study did not examine the microbiome of lettuce, which may have made a significant contribution to the growth/non-growth of LMO. This should also be determined in the context of challenge tests so that the potential competitive flora can also be recorded, and these data can be used for risk assessment. Potential lactic acid bacteria such as *Leuconostoc* and *Weissella* spp. have been identified as anti-listerial indigenous flora in lettuce. Low concentrations of exopolysaccharides secreted by *Weissella* spp. were sufficient to inhibit the growth of LMO, demonstrating its antibacterial ability. The novel bacteriocins *weissellicin D and L* consist of small ribosomal peptides that exhibit antibacterial activity against foodborne pathogens, including LMO. Bacteriocins such as *weissellicin L* could be used in the biopreservation of refrigerated foods due to their thermostable and acid-resistant potential and their strong ability to inhibit LMO growth. (Ahmed et al., 2022).

To ensure food safety during production, there is a need to improve food contact surfaces as they can promote the formation of *L. monocytogenes* biofilms. The goal should be to identify non-fouling materials and coating compositions that can prevent the adherence of pathogens such as LMO and reduce the biofilm formation. Dursan coating in combination with a modified stainless steel surface demonstrated the greatest reduction in LMO biofilm formation, providing a promising approach to improve food sanity design in food processing facilities (Gu et al., 2021).

Soil and water are important niches for the transmission of LMOs to plants, animals and the food chain. Poor hygiene practices can result in traces of contaminated soil and water in the lettuce and can be a vehicle for transmission. Uncultivated soil samples had a higher prevalence of *L. monocytogenes* and were detectable in all seasons. However, in samples collected near agricultural land and urban areas, there was a trend toward detection of LMOs and increased antibiotic resistance. Other microbiota such as *Streptomyces* and *Nocardia* use soil as a reservoir for antibiotics. The increased resistance of LMO to antibiotics may contribute to the survival of LMO in soil. The majority of isolates from food and food processing environments are susceptible to a wide range of antibiotics and only a small number of strains are resistant to antibiotics (Linke et al., 2014).

For the challenge test, the laboratory used 5x 50 g lettuce samples to which the strain cocktail was added and quantitatively homogenized in an initial dilution (e.g., buffered peptone water) without enrichment procedures. The inoculum may not have adhered uniformly to the individual lettuce leaves, and since only a small portion of the liquid (1 ml) was collected at the limit of detection, this may also have affected the recovery of individual strains. Normally in a challenge test, all strains should

be detectable after inoculation with an inoculum of 10 - 100 CFU/g with a target inoculum of 100 CFU/g; this was not the case in all three trials.

To improve the recovery of the LMO strains in future challenge tests, it would be beneficial to use a smaller, more concentrated sample volume (1x25g in a single batch) to ensure that the bacterial strains are detected on day 0 of the challenge test. All lettuce samples must be spiked evenly to ensure that bacterial growth is possible on all parts of the lettuce leaves.

It would be recommended to reduce the number of LMO reference strains used in challenge tests and instead increase the use of field isolates. The use of more field isolates ensures that more realistic, adaptable, persistent, virulent and resistant strains are tested compared to reference strains that are adapted to the controlled laboratory experimental environment, which does not accurately represent the outdoor environment in which bacterial growth occurs. The results also support this theory, as the reference strain ST145 recovered poorly in all three experiments compared to more persistent strains such as ST21 and ST121. ST2 is known for its virulence and, particularly in the third experiment, the results suggest that the strains outcompeted each other, resulting in lower strain recoveries. The less invasive strain ST145 was most likely outcompeted by the more invasive strains such as ST2 and ST21. The individual stress response, virulence potential and differences in strain fitness are factors that influence strain recovery and persistence.

Another aspect to consider is the level of processing, such as whether the tested produce is cut or packaged, as damaged tissue and nutrients on the cut surface can increase LMO colonization and proliferation. Certain fruits and vegetables, such as iceberg lettuce and parsley, have shown greater growth potential than other vegetables such as carrots and corn salad. This variability in growth potential cannot be fully explained by physicochemical properties alone, such as temperature, pH, water activity, CO_2 and acidity. The microbiota, composition and the individual natural defense mechanism of the produce must be considered, as these factors may have influenced the level of *L. monocytogenes* growth after inoculation.

While modeling tools such as Combase can be used to assess LMO growth, they cannot fully replace solid laboratory data in challenge testing for complex food matrices such as RTE salads. Only challenge testing in individual products will allow an accurate representation of microbial growth potential. To better assess growth variability, we agree with Ziegler et al. that in vitro challenge tests in RTE products with abundant microflora are necessary and that pure microbial growth models are not sufficient to assess the risk of LMO growth (Ziegler et al., 2019).

5. SUMMARY

Listeria monocytogenes (LMO), a gram-positive zoonotic pathogen that can be transmitted through contaminated food. *L. monocytogenes* is further subdivided into clonal complexes (CC) and sequence types (ST). LMO strains have adapted to different niches and show growth in different environments such as soil, plants, water, food matrices and food processing facilities. According to EC Regulation 2073/2005 on the food safety criterion of LMOs, food products are classified according to whether LMO growth is possible. Therefore, for products that support growth, there is an additional requirement that *L. monocytogenes* must not be detectable before the product is placed on the market and must not exceed the limit of 100 cfu/g during shelf life. Mandatory measures to detect the presence and growth of *L. monocytogenes* include food lot testing, environmental monitoring and challenge testing to estimate the growth potential of *L. monocytogenes* in different food matrices.

The aim of this study was to estimate the growth potential of *L. monocytogenes* in lettuce samples in collaboration with a private laboratory. An accredited private laboratory prepared the samples for challenge testing and sent the selective agar plates according to ISO 11290-2 for further subtyping. We prepared a strain cocktail of different LMO field isolates (ST121, ST21, ST2) with the reference strain (ST145) available for inoculation of the products. The aim was to determine the potential growth advantage of individual persistent LMO strains over others. Two types of ready-to-eat (RTE) salads were tested for quantitative analysis. Three experiments were performed with the inoculated lettuce samples on days 0, 4, 6 and 8 at three dilutions (1:3, 1:30 and 1:300) at 6°C and 8°C. Experiment 1 was the challenge test with a RTE mixed green salad and experiments 2 and 3 with a monoproduct salad. Reference samples and corresponding success control samples were prepared in triplicates for each sample group. PCR was performed targeting the *abcZ, bglA, cat, dapE, dat, lhkA* and *ldh* alleles, which were sent to LGC Genomics for sequencing. The sequencing results were uploaded to the Multi Locus Sequence Typing (MLST) Institute Pasteur database to determine the CC and ST.

The results confirmed the hypothesis that the in-house persistent isolates had a growth advantage over the reference strain (ST145). ST121 and ST21 showed a clear growth advantage in all three experiments. There was limited or no detection of ST2 and ST145 in experiments 1 and 2. The results of experiment 3 suggest that the virulent ST2 and the other strains ST121 and ST21 outcompeted each other, explaining the overall reduced recovery of the strains. The inoculation method can be improved in future studies to achieve the target inoculum detection limit of 100 CFU/g.

An important factor to consider in challenge testing is the individual plant microbiome, which can influence LMO growth and recovery.

6. ZUSAMMENFASSUNG

Listeria monocytogenes (LMO), ein gram-positiver Zoonoseerreger, der durch kontaminierte Lebensmittel übertragen werden kann. *L. monocytogenes* wird weiter in klonale Komplexe (CC) und Sequenztypen (ST) unterteilt. LMO-Stämme haben sich an verschiedene Nischen angepasst und wachsen in unterschiedlichen Umgebungen wie Boden, Pflanzen, Wasser, Lebensmittelmatrizen und Lebensmittelverarbeitungsanlagen. Gemäß der Verordnung (EG) Nr. 2073/2005 über das Kriterium der Lebensmittelsicherheit von LMOs werden Lebensmittel danach klassifiziert, ob ein LMO-Wachstum möglich ist. Daher gilt für Produkte, die ein Wachstum unterstützen, die zusätzliche Anforderung, dass *L. monocytogenes* nicht nachweisbar sein darf, bevor das Produkt in Verkehr gebracht wird, und dass der Grenzwert von 100 cfu/g während der Haltbarkeitsdauer nicht überschritten werden darf. Maßnahmen zum Nachweisen des Vorhandenseins und Wachstums von *L. monocytogenes* gehören die Untersuchung von Lebensmittelpartien, die Umweltüberwachung und Challenge-Tests zur Abschätzung des Wachstumspotenzials von *L. monocytogenes* in verschiedenen Lebensmittelmatrizen. Ziel dieser Studie war es, das Wachstumspotenzial von *L. monocytogenes* in Salatproben in Zusammenarbeit mit einem privaten Labor zu ermitteln. Ein akkreditiertes privates Labor bereitete die Proben für Challenge-Tests vor und schickte die selektiven Agarplatten gemäß ISO 11290-2 zur weiteren Subtypisierung ein. Wir bereiteten einen Stammcocktail aus verschiedenen LMO-Feldisolaten (ST121, ST21, ST2) vor, wobei der Referenzstamm (ST145) für die Inokulation der Produkte zur Verfügung stand. Ziel war es, den potenziellen Wachstumsvorteil einzelner persistenter LMO-Stämme gegenüber anderen zu ermitteln. Zwei Arten von verzehrfertigen Feldsalaten wurden für die quantitative Analyse getestet. Drei Experimente wurden mit den geimpften Salatproben an den Tagen 0, 4, 6 und 8 in drei Verdünnungen (1:3, 1:30 und 1:300) bei 6°C und 8°C durchgeführt. Versuch 1 war der Challenge-Test mit einem gemischten RTE-Salat und die Versuche 2 und 3 mit einem Monoproduktsalat. Referenzproben und entsprechende Erfolgskontrollproben wurden in dreifacher Ausfertigung für jede Probengruppe hergestellt. Es wurde eine PCR für die Allele *abcZ, bglA, cat, dapE, dat, IhkA* und *ldh* durchgeführt, die zur Sequenzierung an LGC Genomics geschickt wurden. Die Sequenzierungsergebnisse wurden in die Multi Locus Sequence Typing (MLST)-Datenbank des Institute Pasteur hochgeladen, um die CC und ST zu bestimmen. Die Ergebnisse bestätigten die Hypothese, dass die hauseigenen persistenten Isolate einen Wachstumsvorteil gegenüber dem Referenzstamm (ST145) hatten. ST121 und ST21 zeigten in allen drei Versuchen einen deutlichen Wachstumsvorteil. ST2 und ST145 wurden in den Versuchen 1 und 2 nur begrenzt oder gar nicht nachgewiesen. Die Ergebnisse von Versuch 3 deuten darauf hin, dass sich der virulente ST2 und die anderen Stämme ST121 und ST21 gegenseitig verdrängt haben, was die insgesamt geringere Erholung der Stämme erklärt. Die Inokulationsmethode kann in künftigen Studien verbessert werden, um die angestrebte Inokulum - Nachweisgrenze von 100 KBE/g zu erreichen.

Ein wichtiger Faktor, der bei Challenge-Tests zu berücksichtigen ist, ist das individuelle Pflanzenmikrobiom, das das Wachstum und die Erholung von LMO beeinflussen kann.

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8. SUPPLEMENTS

Supplement Table 1: Full list of appliances and consumables used in this study

CATEGORY	PRODUCER/DISTRIBUTOR
I. Technical Equipment	
Workbench and Bunsen burner	Unit of Food Microbiology, VMU Vienna, Austria
Vortex	Scientific Industries Inc., Bohemia, USA
Mini centrifuge	Eppendorf AG, Hamburg, Germany
Big centrifuge	Beckman Coulter Inc., Krefeld, Germany
Heating block 100°C	Kleinfeld Labortechnik GmbH, Gehrden, Germany
Precision scale	Sartorius AG, Göttingen, Germany
Milli-Q Sterile Water Treatment Unit	Merck KGaA, Darmstadt, Germany
Magnet stirring unit	IKA-Werke GmbH & Co. KG, Staufen, Germany
pH-Meter	WTW, Xylem Analytics Inc., Rye Brook, USA
Autoclave	CertoClav Sterilizer GmbH, Leonding, Austria
Incubator 37°C	Ehret GmbH&Co. KG., Emmendingen, Germany
Incubator 30°C	Ehret GmbH&Co. KG., Emmendingen, Germany
Fridge	Liebherr Group, Korneuburg, Austria
Upright freezer -20°C	Liebherr Group, Korneuburg, Austria
Chest freezer -80°C	Sanyo Electric Co. Ltd., Moriguchi, Japan
PCR-Lamina	Gelaire®Laminar Air Flow, Sydney, Australia
PCR-Thermocycler	Bio-Rad Laboratories Inc., Hercules, USA
Microwave	Silva Schneider Electric, Rueil-Malmaison, France
Electrophorese Power Supply	Bio-Rad Laboratories Inc., Hercules, USA
GelDoc 2000 UV-Camera	Bio-Rad Laboratories Inc., Hercules, USA
Thermoprinter	Mitsubishi Corporation, Tokyo, Japan
II. Laboratory instruments	
Pipetting aid	Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, Germany
Pipettes: 10µl, 100µl and 1000µl	Eppendorf AG, Hamburg, Germany
Beaker glasses	Unit of Food Microbiology, VMU Vienna, Austria
Erlenmeyer flasks	Unit of Food Microbiology, VMU Vienna, Austria
Measuring cylinders	Unit of Food Microbiology, VMU Vienna, Austria
Glass bottles: 500ml and 1000ml	Unit of Food Microbiology, VMU Vienna, Austria
Glass funnel	Unit of Food Microbiology, VMU Vienna, Austria
Safe-lock tube racks	Unit of Food Microbiology, VMU Vienna, Austria
Falcon tube rack plastic	Unit of Food Microbiology, VMU Vienna, Austria
Falcon tube rack metal	Unit of Food Microbiology, VMU Vienna, Austria
Cryo block rack	Unit of Food Microbiology, VMU Vienna, Austria
PCR tube rack	Unit of Food Microbiology, VMU Vienna, Austria
Gel molds and combs	Unit of Food Microbiology, VMU Vienna, Austria
III. Expendable items	
Safe-lock tubes: 1.5ml, 2ml, 5ml	Eppendorf AG, Hamburg, Germany
Safe-lock tubes: 5ml	Greiner Bio-One International GmbH, Kremsmünster, Austria
MR safe-lock tubes: 1.5ml	Corning Life Sciences, Tewksbury, USA
PCR Tubes	Greiner Bio-One International GmbH, Kremsmünster, Austria

Red cap falcon tubes: 16ml	Sarstedt AG&Co. KG, Nümbrecht, Germany
Blue cap tubes: 50ml	Greiner Bio-One International GmbH, Kremsmünster, Austria
Disposable Pipettes: 10ml and 25ml	Sarstedt AG&Co. KG, Nümbrecht, Germany
Pipette filter tips: 10µl, 100µl and 1000µl	Greiner Bio-One International GmbH, Kremsmünster, Austria
Inoculation loops: 1µl and 10µl	Sarstedt AG&Co. KG, Nümbrecht, Germany
cotton swabs	Dalian Goodwood Medical Care Ltd., USA
Glass Pasteur pipettes	Brand GmbH & Co. KG, Wertheim, Germany
Stomacher bags	Seward Ltd. Worthing, UK
96-well-plates	Sarstedt AG&Co. KG, Nümbrecht, Germany
96-well-lids	Greiner Bio-One International GmbH, Kremsmünster, Austria
Weighing paper	VWR International LLC, Radnor, USA
Parafilm	Bemis Company Inc. Neenah, USA
Aluminum foil	Unit of Food Microbiology, VMU Vienna, Austria
Autoclave stips	Unit of Food Microbiology, VMU Vienna, Austria
Nitrile gloves	Paul Hartmann GmbH, Wiener Neudorf, Austria
Paper towels	Kimberly-Clark Corporation, Dallas, USA
Disposal bags	Sarstedt AG&Co. KG, Nümbrecht, Germany
IV. Culture media	
PC Plate Count Trypton Soy Agar	Biokar diagnostics, Solabia Group, Cedex, France
Rapid L.mono Agar Plates	Bio-Rad Laboratories Inc., Hercules, USA
60% Glycerol	Sigma-Aldrich Co. LCC, St. Louis, USA
V. Buffers	
Aqua bidest	MilliQ Sterile Water Treatment Unit
DEPC water	Invitrogen - Thermo Fisher Scientific Inc., Waltham, USA
10x PCR buffer (-MgCl2)	Invitrogen - Thermo Fisher Scientific Inc., Waltham, USA
Ringer-Dissolution	B. Braun GmbH, & Co. KG, Melsungen, Germany
Tango Buffer	Sigma Aldrich Co. LCC, St. Louis, MO, USA
Chelex-Dissolution	Unit of Food Microbiology, VMU Vienna, Austria
Sample loading buffer	Unit of Food Microbiology, VMU Vienna, Austria
EC lysis buffer	Unit of Food Microbiology, VMU Vienna, Austria
PIV-buffer	Unit of Food Microbiology, VMU Vienna, Austria
ES buffer	Unit of Food Microbiology, VMU Vienna, Austria
10x TBE buffer	Unit of Food Microbiology, VMU Vienna, Austria
10x TE buffer	Unit of Food Microbiology, VMU Vienna, Austria
Tris-HCl buffer	Unit of Food Microbiology, VMU Vienna, Austria
VI. Enzymes, Primer, Reagents	
H2O2	Unit of Food Microbiology, VMU Vienna, Austria
dNTP's	Invitrogen - Thermo Fisher Scientific Inc., Waltham, USA
MgCl2	Invitrogen - Thermo Fisher Scientific Inc., Waltham, USA
Platinum® Taq DNA Polymerase	Invitrogen - Thermo Fisher Scientific Inc., Waltham, USA
1kb DNA-Marker	Invitrogen - Thermo Fisher Scientific Inc., Waltham, USA
abZ	Unit of Food Microbiology, VMU Vienna, Austria
bglA	Unit of Food Microbiology, VMU Vienna, Austria
Idh	Unit of Food Microbiology, VMU Vienna, Austria
VII. Chemicals	
Sodium deoxycholate	Sigma-Aldrich Co. LCC, St. Louis, USA
NaCl	Merck KGaA, Darmstadt, Germany

Trishydroxymethylaminomethane	Sigma-Aldrich Co. LCC, St. Louis, USA
Chelex	Bio-Rad Laboratories Inc., Hercules, USA
EDTA	Sigma-Aldrich Co. LCC, St. Louis, USA
peqGreen color	Peqlab Biotechnologie GmbH, Erlangen, Germany
NaHCO3-pellets	Merck KGaA, Darmstadt, Germany
Hydrochloric acid	Unit of Food Microbiology, VMU Vienna, Austria
Sodium hypochlorite 1% solution	Colgate-Palmolive, New York City, USA
Mikrozid	Schülke & Mayr GmbH, Norderstedt, Germany
IX. Laboratory protection gear	
Lab coat	Unit of Food Microbiology, VUW, Vienna, Austria
Lab shoes	Unit of Food Microbiology, VUW, Vienna, Austria
Mouth protection mask	LIBOmed - Medizinprodukte, Kleve, Germany
Latex gloves	Unit of Food Microbiology, VUW, Vienna, Austria