Listeria monocytogenes stress resistant variants

Impact on high hydrostatic pressure processing efficacy

Ineke van Boeijen

Propositions

1. Kinetic model-based sampling offers a tool to target the isolation of rare stress-resistant variants of foodborne pathogens.
   (this thesis)

2. Mutants that lost CtsR repressor function are multi-stress resistant, but selection is possible only after exposure to stresses that interfere with protein quality control.
   (this thesis)

3. Boosting sales of second-hand clothes (De Groot, 2022, Brain Science 12, 1526), is all about brainwashing through the nose.

4. Governmental bodies and health insurance companies should include birdwatching when promoting healthy lifestyles.

5. The level of satisfaction of finishing a PhD project and the taste of cheese both prosper from long ripening times.

6. Unique and delicate local dishes are often overrated.

Propositions belonging to the thesis, entitled

Listeria monocytogenes stress resistant variants - Impact on high hydrostatic pressure processing efficacy

Ineke K.H. van Boeijen
Wageningen, 28 March 2023

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Ineke K.H. van Boeijen

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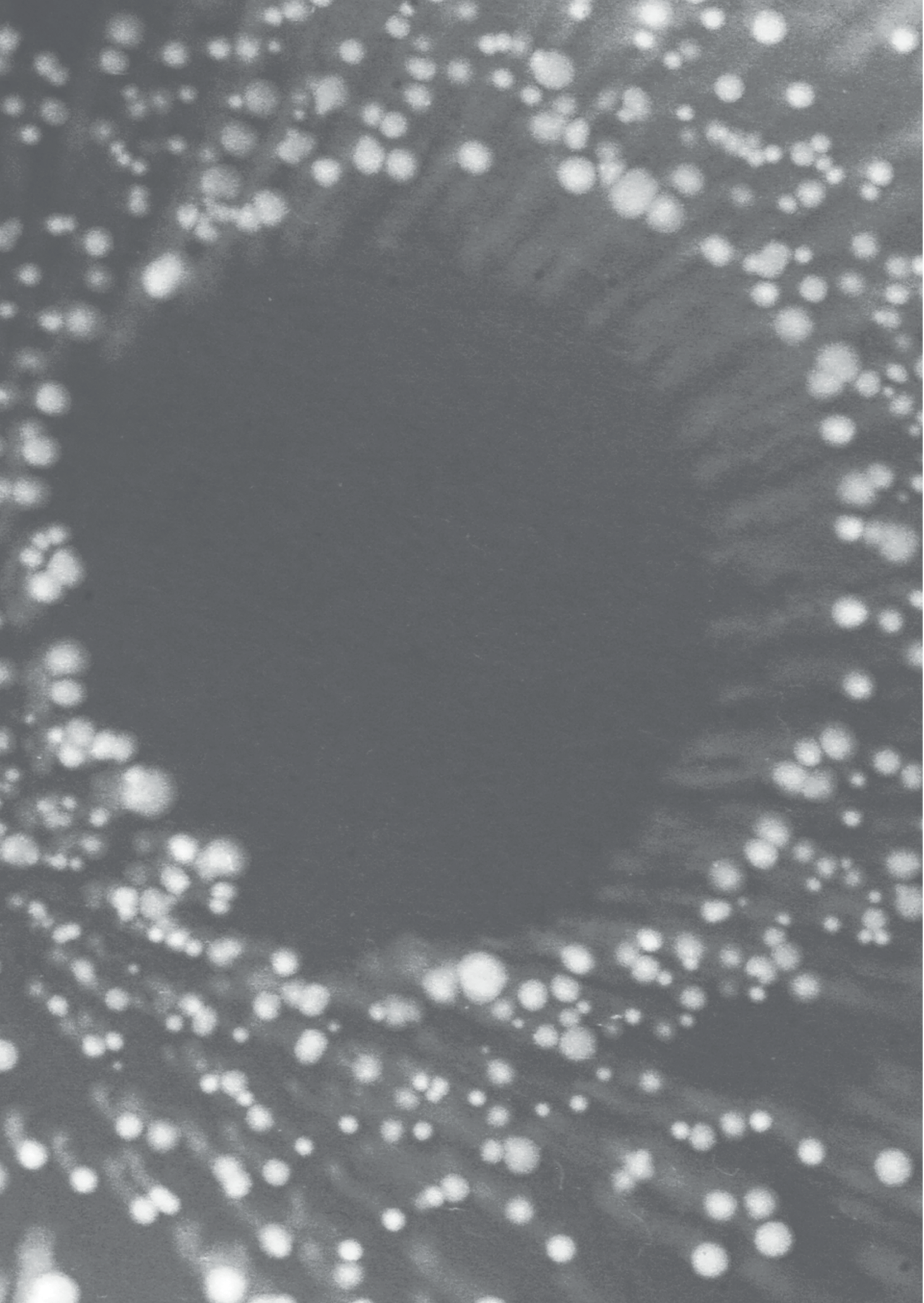
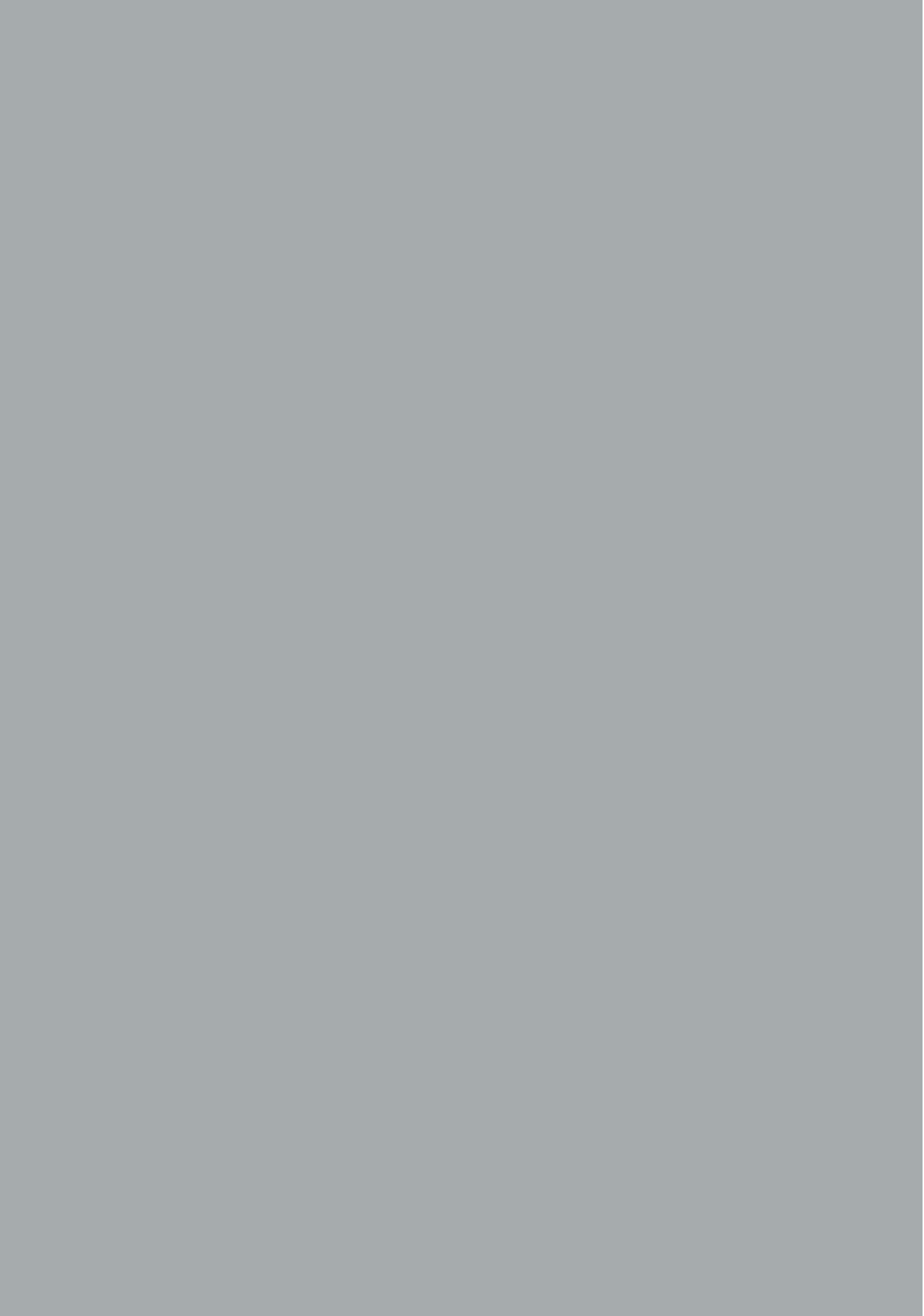


Chapter 1

Introduction and outline of the thesis



Introduction and outline of the thesis

Food is essential to life; hence food safety is a basic human right (106). Billons of people in the world are at risk of unsafe food. An estimated 600 million – almost 1 in 10 people in the world – fall ill after eating contaminated food and 420,000 die every year (36). Quality and safety issues throughout the food chain, i.e., from farm to fork, include a wide range of microbial risks, as microbial contamination can occur at any point along the food chain: during production, distribution, and preparation (36, 47).

Next to food quality and safety, the future challenge includes growth of the global population to at least 9 billion by the year 2050, requiring more food (57). But there are even more challenges, like demographic changes to a population whose immune system is more susceptible to foodborne and opportunistic pathogens, climate changes that will shift where food is produced, and consumers' preferences for raw and minimally processed foods. Hence, improvements to the safety of our foods remains important and will require a shared responsibility of the food industry, scientists, educators, government, and consumers to reduce contamination (26). In food safety, the biggest challenge is microbiological safety. Viruses are responsible for the majority of foodborne illnesses, but hospitalizations and deaths associated with foodborne infections are more often due to bacterial agents. Food by nature is biological and generally capable of supporting growth of microbes that are potential sources of foodborne diseases (36).

Foodborne pathogens

Diseases caused by foodborne pathogens are a serious public health threat. The Centers for Disease Control and Prevention (CDC) estimates that each year 1 in 6 Americans suffer from a foodborne illness resulting in around 48 million cases, including approximately 128,000 hospitalizations, and 3000 deaths (16, 89). In a recent report, the US Department of Agriculture Economic Research Service (99) estimated that the frequency and severity of foodborne illnesses culminate in $17.6 billion of losses annually attributed to medical costs, productivity losses (food recalls), and economic burden due to death, of which around $3.19 billion due to listeriosis. Listeriosis is one of the most severe food-borne infections and is caused by *Listeria monocytogenes*. It is a rare but severe disease with low morbidity (annual incidence around 3 cases per million population) but high hospitalization rate (94%) and lethality (16%) (89). Outbreaks are increasingly recognized, predominantly in upper-income countries where infection is more readily diagnosed and where existing surveillance programs facilitate early recognition. Furthermore, whole-genome sequencing (WGS) is increasingly used by food regulatory and public health agencies to facilitate the detection, investigation, and control of foodborne bacterial outbreaks, and food regulatory and other activities in support of food safety. WGS allows for identification of outbreak-linked cases and definitive attribution of the source, is accessible (Table 1). With this method, even isolates can be detected that differ from each other by >50–100 SNPs/alleles. The presence

of multiple strains on a food production farm or in a facility could indicate insanitary conditions that should be addressed immediately. It is not uncommon to see polyclonal outbreaks, with multiple pathogenic strains causing an outbreak associated with a single food source. A recent example is the *L. monocytogenes* outbreak related to consumption of contaminated ice cream in 2015 in the US in which WGS results indicated two different isolates in these products (7).

Table 1. Recent food-borne outbreaks of listeriosis

Source	Number of cases	Number of deaths	Number of miscarriages	Country	Year	Ref
Cheese (pasteurized milk)	189	27	7	Germany	2006-2007	58
Scalded sausages	16	5		Germany	2006-2007	107
Pasteurized milk	5	3		USA (Massachusetts)	2007-2008	11
Brie and camembert cheese	165	14		Chile	2008	72
Cheese (pasteurized milk)	38	2	3	Canada	2008	39
Jellied pork	12	0		Austria	2008	80
RTE deli meats	57	22		Canada	2008	41
Beef meat	8	2		Denmark	2009	95
Quargel cheese	34	8		Austria, Germany, Czech Republic	2009-2010	35
Cantaloupes	147	33	1	USA (28 states)	2011	60
Cheese (ricotta)	22	4	1	USA (14 states)	2012	49
Smoked fish	20	7	1	Denmark	2013-2015	40
Prepackaged caramel apples	35	7	1	USA (12 states)	2014	87
Ice cream	10	3		USA (4 states)	2015	84
Soft cheeses	30	3	1	USA (10 states)	2015	12
Packaged salads	19	1		USA (9 states)	2016	13
Salmon	4	1		Denmark	2017	33
Processed meat	1060	216	27	South Africa	2017-2018	98
Rockmelons	20	7	1	Australia	2018	105
Ready-to-eat meat	21	3	1	Netherlands, Belgium	2018-2019	30
Bloody sausages	112	2		Germany	2018-2019	48
Chilled roasted pork meat	222	3	5	Spain	2019	43
Enoki mushrooms	36	4	2	USA (17 states)	2020	14
Packaged salads	18	3		USA (13 states)	2021	15
Ice cream	23	1	1	USA (10 states)	2022	17

The largest listeriosis outbreak that has ever been detected worldwide according to the World Health Organization was in South Africa in 2017, in which a staggering total of 1060 cases were reported in a period of 1.5 years (96, 98). During July and August an increase in the number of cases of listeriosis at two public hospitals in Gauteng Province prompted an investigation. Case numbers rapidly increased nationwide, and whole-genome multilocus sequence typing of *L. monocytogenes* isolates from patients identified a single sequence type (sequence type 6 [ST6]) in 93% of the cases. The outcome was known for 806 patients, among whom 216 deaths were reported (case-fatality ratio, 27%). HIV infection was

associated with a more than 50% increased odds of death among patients older than 1 month. Fetal loss occurred in 27 of the 59 pregnant girls and women (98). By early January 2018, food history interviews with patients suggested that "polony" was among the most commonly consumed foodstuff among persons with listeriosis. Polony is a ready-to-eat processed meat product, similar to bologna sausage. Epidemiological and laboratory findings led to the investigation of a large ready-to-eat processed meat production facility in South Africa, named Enterprise Foods. On February 2, 2018, the production facility was inspected, and numerous environmental sampling swabs were collected throughout the facility. *L. monocytogenes* ST6 was isolated from the environment of numerous areas of the production facility, including post-cooking areas. The same ST6 strain was found also in several food products (including polony) manufactured at the facility. A recall of affected food products was initiated (in total over 5800 tons of food was destroyed) and Enterprise Foods' production facilities were shut down. The number of cases decreased dramatically after the recall of the implicated products (96, 98).

Listeria monocytogenes

Listeria monocytogenes was first isolated in 1924 by Murray, Webb, and Swann in England (73). They assigned the name *Bacterium monocytogenes* to this Gram-positive rod-shaped bacterium that was responsible for a lethal disease in rabbits characterized by a marked increase in the number of monocytes circulating in the blood. In 1927, Pirie isolated an identical bacterium from gerbils in South Africa (81). Pirie suggested the genus name *Listeria* in 1940 in honor of the British surgeon Joseph Lister, one of the pioneers in the field of antiseptics and disinfection (82).

The first case of *L. monocytogenes* in humans was reported in 1929 (77). For a long time, *Listeria* was only sporadically isolated from humans. However, in the late 1970s and in the 1980s, the first epidemic outbreaks in humans in North America and Europe established *L. monocytogenes* as an important food-borne pathogen (6, 32, 63, 90). As a result of almost a century of research, *Listeria* is now considered a model pathogen (22).

Lineage and serotyping of *L. monocytogenes*

L. monocytogenes can be divided into distinct evolutionary groups using a range of genotypic (e.g. marker genes: *flaA*, *iap*, and *hly*) and phenotypic (e.g. somatic and flagellar antigens) characteristics (104). This subtyping resulted in four evolutionary lineages (I, II, III, and IV) with different but overlapping ecological niches. Most *L. monocytogenes* isolates belong to lineages I and II, which harbor the serotypes more commonly associated with human clinical cases. Lineage II strains are common in foods, seem to be widespread in the natural and farm environments, and are also commonly isolated from animal listeriosis cases and sporadic human clinical cases. Most human listeriosis outbreaks are associated with lineage

I isolates though. Lineage III and IV strains on the other hand are rare and predominantly isolated from animal sources (59, 78).

The vast majority of human listeriosis cases are caused by three serotypes (1/2a, 1/2b and 4b). The prevalence of these serotypes among clinical and food isolates, clearly points to differences in ability to survive in foods and/or cause disease. However, classifying isolates in only three serotypes makes it difficult to discriminate between different isolates. Recently, whole genome sequencing and multi-locus sequence typing can subdivide isolates according to sequence type (ST) or clonal complex (CC). So far 14 lineage-related serotypes and more than 170 clonal complexes were defined and can be used to identify outbreaks and the source much more accurately and faster (5, 23, 59, 85).

The three strains used in this study represent the common lineages, serotypes, sequence types and clonal complexes (10) (Table 2). *L. monocytogenes* EGDe (serotype 1/2a) is a derivate of the originally EGD strain isolated in 1924 (73). EGDe, one of the most studied strains in many different laboratories around the world, was the first *L. monocytogenes* strain that was genome sequenced (42). Strain LO28 (serotype 1/2c) is a carriage strain recovered from the faeces of a healthy pregnant woman (100). Both these strains belong to lineage II, which are common in foods, but rarely associated with listeriosis outbreaks. *L. monocytogenes* Scott A (serotype 4b) belongs to lineage I and was isolated from a human outbreak in an epidemic in Massachusetts (USA) in 1983 in which pasteurized milk was identified as the source of infection. In this outbreak, 49 patients acquired listeriosis and 14 of these patients (29%) died (32).

Table 2. Origin, lineage, serotype, sequence type (ST), and clonal complex (CC) of *L. monocytogenes* strains used in this thesis

Strain	EGDe	LO28	Scott A
Origin	laboratory strain passaged from an animal isolate from 1924	faeces of a healthy pregnant woman	human outbreak in an epidemic in Massachusetts from pasteurized milk
Reference	42	100	32
Lineage	II	II	I
Serotype	1/2a	1/2c	4b
ST	2	2	2
CC	9	9	2

Infection and disease

L. monocytogenes is a bacterium with two appearances: it is well adapted as a saprophyte for survival in soil and water as well as food processing facilities, but it has a second life as an intracellular bacterial pathogen capable of causing serious infection in humans and in many animal species through several regulatory systems (46, 64). In general, *L. monocytogenes* infects the human host via the oral route through uptake of contaminated food products. After passage of the stomach and by crossing the intestinal barrier, the bacterium is absorbed from the intestinal lumen, and if the immune system does not control the infection, the pathogen disseminates to the bloodstream and mesenteric lymph nodes. *L. monocytogenes*

can then reach the liver, spleen, brain, and fetus in pregnant women (9). Central to the switch between life outside and life inside mammalian hosts is the transcriptional activator PrfA, which regulates the expression of many gene products that are required for bacterial virulence (Fig. 1). Outside a host cell, PrfA exists in a low-activity state, with correspondingly low levels of virulence gene expression. Once inside the host, PrfA becomes activated and induces the expression of gene products that are needed for host cell invasion (34).

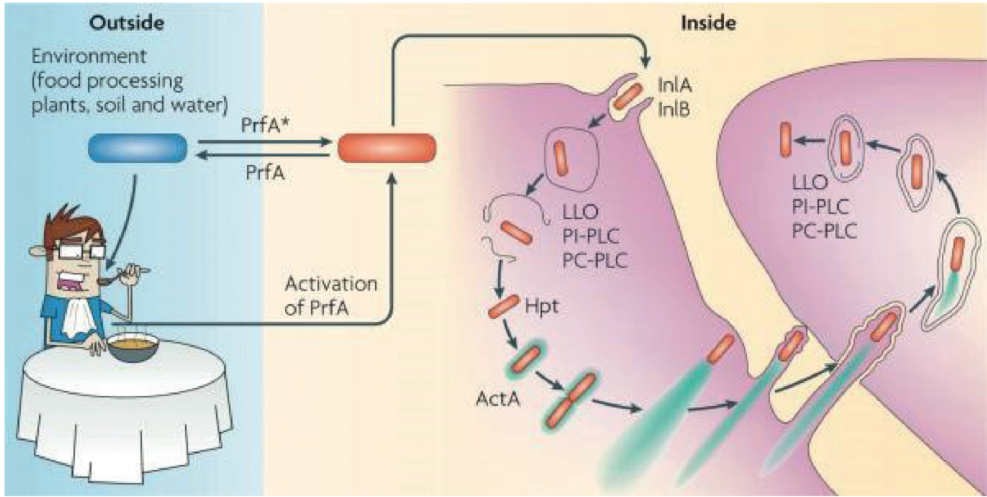


Figure 1. *L. monocytogenes* from saprophyte to intracellular pathogen. *Listeria monocytogenes* survives in a diverse array of environments, in habitats that include soil and water as well as food-processing facilities. Central to the switch between life outside and life inside mammalian hosts is the transcriptional activator PrfA, which regulates the expression of many gene products that are required for bacterial virulence. Outside a host cell, PrfA exists in a low-activity state, with correspondingly low levels of virulence gene expression. Once inside the host, PrfA becomes activated (PrfA*) and induces the expression of gene products that are needed for host cell invasion (internalins InlA and InlB), phagosome lysis (listeriolysin O (LLO), phosphatidylinositol-specific phospholipase C (PI-PLC) and phosphatidylcholine (PC)-PLC), intracellular growth (hexose-6-phosphate transporter (Hpt)), and cell-to-cell spread (actin assembly-inducing protein (ActA); actin polymerization is shown in turquoise). The figure is used with permission from Freitag *et al.* (34).

Two surface proteins mediate entry into cells, internalin A (InlA) and InlB. Internalized bacteria are trapped in a phagosome, from which they escape by expression of listeriolysin O (LLO) and two phospholipases (PC-PLC and PI-PLC). Once in the cytosol, bacteria adapt their metabolism, by synthesizing hexose-6-phosphate transporter (Hpt) that enables bacterial intracellular replication and start polymerizing actin (ActA). Polarized expression of ActA allows *L. monocytogenes* to hijack the host actin polymerization machinery. This prevents recognition by the host autophagic machinery and propels the bacterium in the cytosol. The bacterium invades neighboring cells through the formation of a double-membrane protrusion, resulting in the formation of a so-called secondary vacuole. This compartment is then lysed via LLO and PLCs, allowing a new intracellular infection cycle in adjacent cells (19, 21, 27, 28, 46, 83, 97).

Clinical symptoms of listeriosis range from gastroenteritis to more severe forms of infection, including sepsis and meningitis. Infection during pregnancy may result in mild flulike illness for the mother but a severe outcome for the fetus, such as spontaneous abortion, premature delivery, stillbirth or systemic infection. Immunosuppressed adults, including persons with cancer, organ transplant recipients, or persons with HIV infection, disproportionately experience invasive infection, although, both invasive illness and gastroenteritis can occur in persons with competent immune systems (102).

Transmission of *L. monocytogenes*

L. monocytogenes can survive and multiply in diverse habitats and hosts. The bacterium is well known for its ability to withstand a variety of environmental stresses, including low temperature and high osmolarity, thus making it a hardy environmental organism. *L. monocytogenes* is widespread in the environment, although it is believed to maintain a saprophytic existence (46). In most situations where the organism was found in the environment, for example in soil samples, numbers were low (101). However, *L. monocytogenes* has the ability to persist in soil environments and water and is therefore an important source for contamination of feed for food producing animals, raw food for human consumption, and for contamination of food-processing environments and other environments that may lead to contamination of human foods (Fig. 2) (51, 76).

While the raw materials used in food production could be contaminated with *L. monocytogenes*, most foods are exposed to microbial inactivation treatment at some point during processing. Cross-contamination can, however, occur after processing and generally represents post-processing contamination from environmental sources, including in food-processing plants, retail operations, and household kitchens (51). *L. monocytogenes* can grow at refrigeration temperatures and survive in food for prolonged periods under adverse conditions (4). Various studies have also indicated that certain strains of *L. monocytogenes* survive within the food-processing environment for months to years and keep contaminating food products. The persistence of such strains is of particular concern as they have the potential to act as a continuous source of contamination of the processed product (56, 62, 65, 74). On several occasions, strains that were persistent and prevalent in processing facilities were also associated with human listeriosis (55, 69). It is, however, difficult to correlate adaptive traits directly to persistence. Hence, pheno-genotype association studies are promising approaches to increase our mechanistic understanding of how this pathogen survives along the food chain and infects the human host (59).

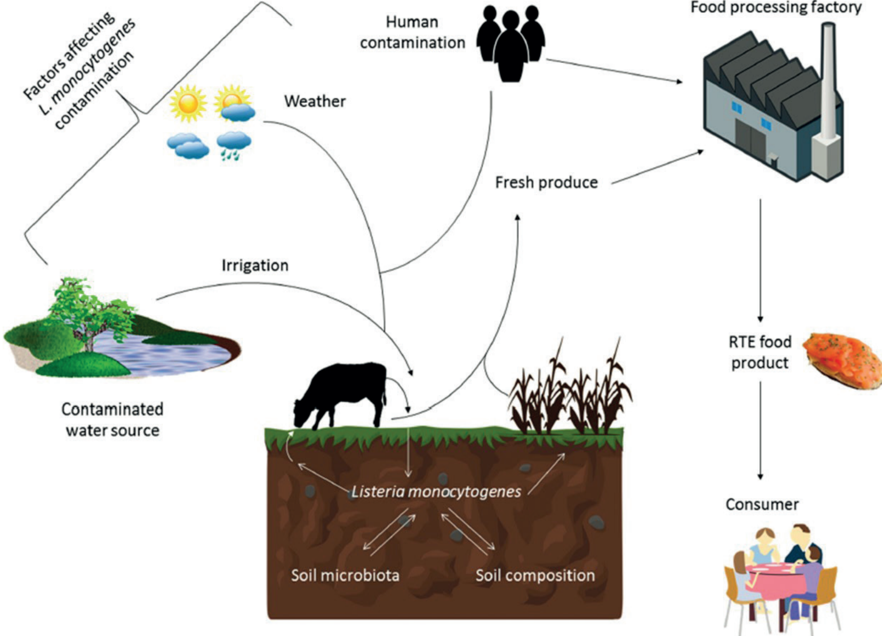


Figure 2. Factors influencing the survival and transmission of *Listeria monocytogenes* in the environment and food chain. The survival of *L. monocytogenes* in the soil is influenced by factors such as the composition of the soil and the competing microbiota present. Its presence in this environment is also influenced by weather events (sunshine and rainfall), irrigation from contaminated sources, as well as human and animal fecal contamination. Therefore, agricultural produce can be contaminated with this pathogen at the point of harvest. This can introduce the pathogen into the food-processing environment, or the produce can become contaminated there if adequate cleaning and decontamination practices are not in place. Ready-to-eat food produce that can support the growth of *L. monocytogenes* is a particular risk to the consumer, especially those that are immunocompromised. The figure is used with permission from NicAogáin *et al.* (76).

A European Commission regulation provides limits for the levels of this bacterium in food. The limit varies according to the type of consumer and characteristics of the food. Absence of *L. monocytogenes* in 25 g is required in some foods, e.g., RTE foods intended for infants and those for special medical purposes (n=10) and other RTE foods that support growth of *L. monocytogenes* before this food has left the immediate control of the food business operator (n=5). While for others, such as those that do not support growth of the organism, or for which shelf-life assessment has been carried out, the higher limit is 100 CFU/g (n=5) at end of shelf-life (31).

Food processing

Foods deteriorate in quality due to a wide range of physical, chemical, enzymatic and microbiological effects. Therefore, foods need preservation to retain their quality for a longer period of time. Food preservation procedures are mostly targeted towards micro-organisms responsible for food spoilage and food poisoning. These preservation techniques can prevent or slow microbial growth or even inactivate micro-organisms (45). One of

these techniques used in the food industry is pasteurization. This process was originally named after Louis Pasteur, who invented the process of heating liquids at a relatively mild temperature for a short time to prevent spoilage. Pasteurization has been widely accepted as an effective preservation method that targets specific pathogens and reduces spoilage organisms that may grow during storage (79). Not only heat treatments fall under the term pasteurization, but also new technologies can satisfy the goals of this preservation method. As a result, the definition of pasteurization allows application of a broad range of (combinations of) technologies, including thermal (like microwave and infrared processing) and nonthermal technologies (for example pulsed electric field, chemical treatments, and high-pressure processing). These new technologies are developed as heat treatment may cause undesirable side effects in the sensory, nutritional, and functional properties of the food (44, 79).

High hydrostatic pressure

One of the food-processing alternatives to classical heat treatment technologies is high hydrostatic pressure (HHP). Already in 1895, it was discovered that high hydrostatic pressure was able to inactivate bacteria and can achieve the same standards of food safety as those of heat pasteurization (86). However, it was not until 1990 that the first industrial high-pressure application for the commercial preservation of food was installed in Japan (24). Applying HHP can inactivate pathogenic and spoilage microorganisms and enzymes, as well as modify structures with little or no effects on the nutritional and sensory quality of foods (103). HHP subjects liquid and solid foods usually to pressures of about 400 to 600 MPa at refrigeration or mild process temperatures (< 45°C). High Pressure (HP) processing is applied mainly to pre-packed juices, sauces, dips, fishery products, meat products and ready-to-eat meals (RTE). There is also an increasing interest for the use of HP processing in the dairy industry as an alternative for pasteurization. Recent instances of commercialization of HPP in dairy industry can include HP treated yogurt and cheese spread (66). The efficacy of HP treatments will be dependent on the pressure applied, the holding time and temperature, the characteristics of the food and the target microorganism. (29). The U.S. Food and Drug Administration has officially approved HP processing as a non-thermal pasteurization technology that can replace traditional pasteurization in the food industry. Clearly defined regulations and specifications will facilitate the development of the application market to improve product quality and consumer trust. The widespread application of HP technology has boosted the development and market demand for HP equipment. Compared with thermal or other nonthermal preservation technologies, HP is considered as relatively expensive technology; therefore, it is particularly applied for high quality foods with the aim of maintaining their fresh and nutritional character, similar to one of an untreated product. In 2019, more than 550 commercial HP machines for food processing were in operation worldwide, 59% of them in North America, 24% in Europe, and 18% in Asia. Despite the high price and high barriers to investment, the specialized original equipment manufacturer

service sector has been gradually increasing, and the annual output value of global HP market has approached $10 billion and is expected to culminate in a market value of $55 billion in 2025 (50).

Primarily, the lethal effects of HP processing on vegetative cells are attributed to enzyme inactivation, cell membrane damage, disintegration of ribosomes and intracellular pH changes (92). Pressure levels of more than 300 MPa can lead to the unfolding and denaturation of proteins, which can also result in enzyme inactivation. At sufficiently high-pressure levels, phase transitions and changes of fluidity of microbial cell membranes are observed, leading to ruptures in the cell membrane and promoting denaturation of membrane proteins. Moreover, disintegration of ribosomes in their subunits and intracellular pH changes are discussed to be the major pressure-induced effects. It can be concluded from the literature that the inactivation of vegetative cells by HP is a complex event, which depends on the interaction of numerous particular effects, finally leading to cell death (2).

Heterogeneity of *L. monocytogenes*

L. monocytogenes is among the most extensively studied bacterial species in terms of HHP inactivation (20). Some of these studies showed that the inactivation of *L. monocytogenes* deviates from linearity, showing curves with pronounced tails (88). Tailing of inactivation curves either indicates experimental artifacts or enhanced survival of resistant subpopulations, reflecting heterogeneity within the population. Phenotypic heterogeneity within microbial populations arises even when the cells are exposed to putatively constant and homogeneous conditions. The outcome of this phenomenon can affect the whole function of the population, resulting in, for example, new “adapted” metabolic strategies and impacting its fitness at given environmental conditions (8). Resistant subpopulations include persister cells as well as cells that are resistant due to mutations. The term persister cells is used for survivors that are temporarily resistant. If these cells start to grow and divide, the resulting population is equally sensitive to stress as the ancestral population. Persistence is a phenotype expressed by almost all bacteria. On the other hand, stable resistance is the result of specific mutations, leading to a higher survival level (1). Interestingly, cells with a stable higher resistance were found in *L. monocytogenes* by the isolation of HHP-resistant and acid stress-resistant variants (52, 53, 70). All these resistant variants appeared to be multi-stress resistant. The frequency of their occurrence within the total population was estimated between 5×10^{-7} and 10^{-5} (54, 71). The origin of resistance of the acid-resistant variants was a mutation in *rpsU*, which encodes ribosomal protein S21. Resistance of the HHP-variants could be linked to a mutation in the *ctsR* gene, which encodes CtsR, the class III heat shock response regulator. CtsR represses the class III stress response genes encoding chaperones and Clp proteases which degrade damaged or misfolded proteins (75). Mutations in *ctsR* can lead to a defect in this repression, which results in transcription of the stress response genes, with concomitant activation of stress defense providing increased robustness (1).

Modeling microbial inactivation

The application of a new technology in food preservation requires a reliable model that accurately describes the inactivation rate of micro-organisms. This model should be able to design appropriate treatment conditions allowing the production of safe foods. Models should be simple, and ideally, they should be built on parameters based on the physiological mechanism of inactivation (61, 67). Thermal processing parameters have generally been calculated through the first-order kinetics model by extrapolating the inactivation curve to the desired level of inactivation. This approach is based on the assumption that the inactivation rate is identical for all the cells of the population (37, 94). Although first-order kinetics model is also commonly used to depict the inactivation rate of microorganisms using high-pressure processing, variations from linear behavior have been identified. After pressure treatment, microbial inactivation curves of different shapes have been described, i.e., curves with shoulders, curves with tails, and sigmoidal curves. Non-linearity of the semi-logarithmic survival curve might be due to the variations in defense and repair mechanism of subpopulations against the lethal agents (91) (Fig. 3). Shoulders have been mainly attributed to the occurrence of sublethal injury, whereas tails are considered to be the reflection of resistance heterogeneity within the population either inherent to the bacterial cells or acquired during the treatment (93). As a result, the suitability of first-order kinetics for the modeling of heat inactivation, as well as for novel technologies is being reconsidered (67).

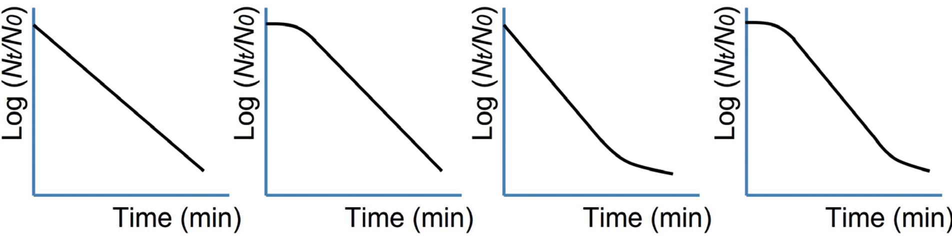


Figure 3. Survival curves according to first-order kinetics (a) with shoulder (b) with tail (c) and with shoulder and tail (d).

In the conventional first-order kinetics approach, when survival curves are not linear, the *D*-value is usually determined by considering the linear segment of the curve, resulting thus in over- or under-estimation of processing times for the commodity. To overcome this problem several other models have been developed for the description of the inactivation data (25). One of these models is the biphasic linear model that is based on the assumption of two populations, a sensitive (fast inactivating) and a resistant (slow inactivating) (18, 29, 38). In current practice, safety margins included in the process conditions are generally sufficient to take moderate tailing effects into account (20). With the increasing interest

in milder preservation, tailing can become a cause of concern in that it can lead to higher numbers of surviving pathogens.

Microbial risk assessment

Modeling inactivation data by itself might not be sufficient to assure the production of safe foods as population heterogeneity affects the survival capacity of a microbial population and consequently, the efficacy of food processing. Population heterogeneity cannot always be detected with population scale methods, as the numerically superior population dominates individual cells with different phenotype, physiological state or even gene expression, which in most cases constitutes a small fraction of the total population. The isolation and characterization of variants from this small fraction makes it possible to unravel and define the extent of bacterial heterogeneity and also to assess residual risk of these subpopulations (3).

Quantitative microbiological risk assessments aim to quantify the risk related to the consumption of food products. They combine the assessment of the severity of the microbiological hazard (i.e., hazard characterization) with the assessment of the prevalence and concentration of the hazard (i.e., exposure assessment) (1). Such an assessment provides us with the ability to estimate the risk to human health by understanding the interaction between specific microorganism, foods and human illness. Information from the overall population including specific subpopulations can be used to compare and evaluate different scenarios, as well as to identify the types of data necessary for mitigating interventions, design production processes, application of control measures and risk management in general (1, 68).

Outline of this thesis

In this research project, the inactivation of various *L. monocytogenes* strains by the minimal processing technology high hydrostatic pressure was examined and described with a kinetic model. Furthermore, several resistant variants were phenotypically and genetically analyzed. This will enhance our understanding of how *L. monocytogenes* survives under HHP and may contribute to improving the safety level of HHP processed food.

Chapter 2 reports the inactivation kinetics of three *L. monocytogenes* strains (EGDe, LO28, and Scott A) under high hydrostatic pressure. The inactivation data of these strains was fitted with a biphasic linear model, indicating the presence of an HHP-sensitive and an HHP-resistant fraction. The resistant fraction of the EGDe population was found to be only temporarily piezotolerant, whereas the resistant fractions of Scott A and LO28 also showed stable piezotolerant subpopulations.

In **Chapter 3**, 24 variants of the stable piezotolerant subpopulation of strain LO28 were characterized for their resistance and growth capacities. This analysis showed all variants to be multi-resistant and their ability to grow under various conditions; however, differences

among variants were observed. Furthermore, nine variants had mutations in their *ctsR* gene or upstream region. Cluster analysis of the variants' characteristics revealed 13 unique variants, exposing diversity within the population.

Chapter 4 provides the virulence aspects of these multi-resistant variants compared with their parental strain LO28. Some variants showed attenuated virulence, whereas other variants performed similar as the wild type. Based on their characteristics, part of the variants has an increased pathogenic potency compared with the parental strain as these variants were not only stress-resistant, but their growth and virulence were not or only slightly attenuated.

In **Chapter 5** kinetic modeling of inactivation curves of two multi-resistant variants and their wild type LO28 revealed that the probability of isolating resistant variants depends on the nature of inactivation and the time of exposure. An optimal strategy and time point could be derived. This new strategy made it possible to isolate for the first-time heat-resistant LO28 as well as heat- and HHP-resistant EGDe variants. The increased resistance of part of these variants again was due to mutations in their *ctsR* genes.

Chapter 6 is a summarizing discussion where the experimental data of the previous chapters is combined with gene-expression profiling data and placed in a broader context concerning the impact of population diversity of *L. monocytogenes* on the efficacy of food-processing and food safety.

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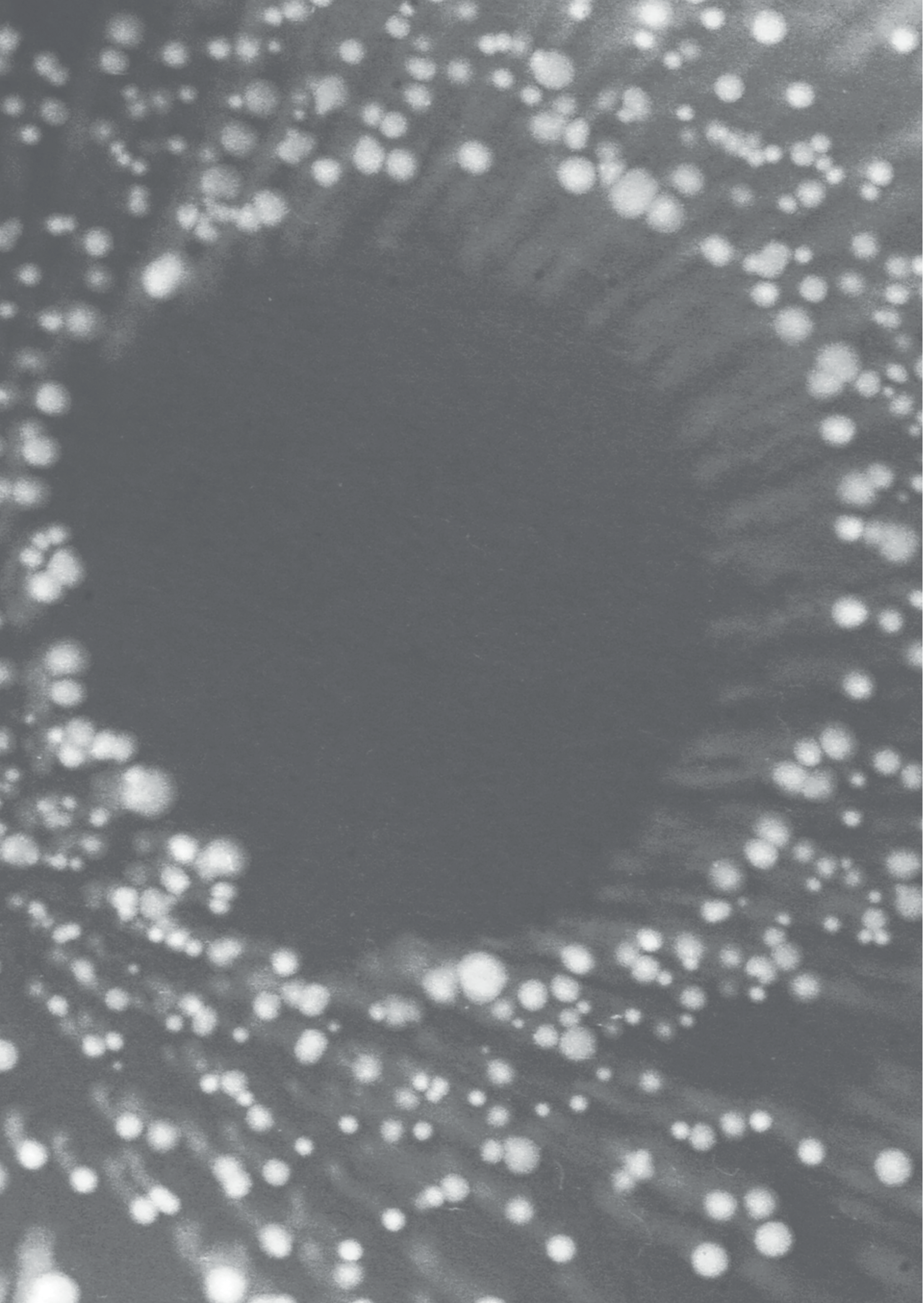

Chapter 2

Inactivation kinetics of three *Listeria monocytogenes* strains under high hydrostatic pressure

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Abstract

High hydrostatic pressure (HHP) inactivation of three *Listeria monocytogenes* strains (EGDe, LO28 and Scott A) subjected to 350 MPa at 20°C in ACES buffer resulted in survival curves with significant tailing for all three strains. A biphasic linear model could be fitted to the inactivation data, indicating the presence of an HHP-sensitive and an HHP-resistant fraction, which both showed inactivation according to first-order kinetics. Inactivation parameters of these subpopulations of the three strains were quantified in detail. EGDe showed the highest *D*-values for the sensitive and resistant fraction, whereas LO28 and Scott A showed lower HHP resistance for both fractions. Survivors isolated from the tail of LO28 and EGDe were analyzed, and it was revealed that the higher resistance of LO28 was a stable feature for 24% (24 of 102) of the resistant fraction. These HHP-resistant variants were 10 to 600,000 times more resistant than wild type when exposed to 350 MPa at 20°C for 20 min. Contrary to these results, no stable HHP-resistant isolates were found for EGDe (0 of 102). The possible effect of HHP survival capacity of stress-resistant genotypic and phenotypic variants of *L. monocytogenes* on the safety of HHP-processed foods is discussed.

Introduction

In general, the intensity and duration of a food preserving treatment are determined on the basis of inactivation curves of selected target microorganisms. In theory, such inactivation curves follow first-order kinetics and the treatment time is usually calculated by extrapolating the curve to the desired level of inactivation. However, in practice, a variety of patterns deviating from first-order kinetics are observed. A commonly observed pattern is that of a gradually decreasing rate of inactivation in time. This phenomenon generally is referred to as tailing (3). In current practice, safety margins included in the process conditions are generally sufficient to take moderate tailing effects into account (7). With the increasing interest in milder preservation, tailing can become a cause of concern in that it can lead to higher numbers of surviving pathogens.

High hydrostatic pressure (HHP) is a minimal processing technology that is applied commercially for a small range of products and is currently evaluated for other applications. HHP enables inactivation of microorganisms with only minimal effects on the product itself. Therefore, products treated with HHP generally have a better sensory quality than products processed in more traditional ways (10, 24).

The use of HHP in the inactivation of microorganisms that can cause spoilage or foodborne illness has been widely studied and recently reviewed by Considine *et al*. (7). A number of studies have measured HHP inactivation of *Listeria monocytogenes* in time, and both first-order inactivation kinetics (21) as well as tailing of inactivation curves have been described (4, 28). Tailing of survival curves of microorganisms can be an artifact of the experimental design but can also be the result of heterogeneity of the population because of physiological or genetic changes (3). Karatzas and Bennik (12) recently demonstrated heterogeneity within *L. monocytogenes* Scott A by isolating a stable HHP-resistant mutant. The frequency of occurrence of stable resistant mutants within the Scott A population was estimated to be greater than 10^{-5} (13). Because the remaining fraction is relatively HHP sensitive, the total population can be divided into a sensitive and a resistant fraction. Notably, detailed kinetic analyses and implementation of quantitative inactivation models have not been described for *L. monocytogenes*.

Therefore, in this study, we investigate heterogeneity within and between populations by quantifying in depth HHP inactivation of *L. monocytogenes* Scott A, LO28 and EGDe. Two models (log-linear and biphasic linear) have been evaluated regarding their ability to describe the inactivation curves. The presence of temporary and stable resistant subpopulations was demonstrated and the effect of these phenomena on safety of HHP-processed foods is discussed.

Materials and methods

Bacterial strains and cell culturing conditions

Three *Listeria monocytogenes* strains have been used in this study: EGDe, Scott A, and LO28 (Department of Agrotechnology and Food Sciences, Wageningen University and Research Centre, the Netherlands). Stock cultures of these strains were kept in 15% (vol/vol) glycerol (Fluka, Buchs, Switzerland) at –80°C, and before the experiments, cells from stock were grown for 2 days at 30°C on BHI agar (Oxoid, Hampshire, UK). Cells from the exponential growth phase were used in this study because *L. monocytogenes* can occur in the growing state in foods, even though conditions might be suboptimal (1, 16). A single colony was used to start a preculture of 10 ml of BHI broth. After 20 h of growth at 30°C in an incubator with shaking at 160 rpm, 0.5% (vol/vol) inoculum was added to 100 ml of BHI broth. After 5 h of growth, when the cells were in the exponential growth phase, the culture was harvested by centrifugation (2,600 x *g*, 20°C, 5 min). The cells (kept at room temperature) were washed twice with 50 mM ACES (N-[2-acetamido]-2-aminoethanesulfonic acid) buffer (Sigma-Aldrich, Steinheim, Germany), pH 7.0 and resuspended in this buffer (6 ml) until a final concentration of approximately 10^{10} CFU/ml was obtained.

HHP inactivation

The kinetics of inactivation were determined at 350 MPa and 20°C. This temperature was selected because it is generally used in industry. Cell suspensions of 700 μl were placed in sterile plastic bags (1.5 by 6 cm) constructed from a stomacher bag (Seward, London, UK). The bags were vacuum sealed and placed in glycol in the six-vessel HHP unit (Resato, Roden, The Netherlands). A thermostat jacket connected to a water bath controlled the temperature of the vessels. Pressure was built up at a rate of 400 MPa/min, and as a result of adiabatic heating, temperature only transiently increased to 35°C. Therefore, the first sample of a time series, which will be used for kinetic modeling, was taken 4 min after starting the pressurization; this time, called t_{eq}, was sufficient to allow the temperature of the vessels to return to 20°C. At regular intervals, samples were taken and serially diluted in 0.1% peptone saline, containing 0.1% (wt/vol) bacteriological peptone (Oxoid) and 0.85% (wt/vol) sodium chloride (Merck, Darmstadt, Germany). Samples of 50 to 200 μl were plated on BHI agar using a spiral plater (Eddy Jet, LabScientific, N.J.). The plates were incubated for 5 days at 30°C to allow all surviving cells to form visible colonies. Survivors were enumerated and this was considered accurate if more than 20 cells were detected. This corresponds to a plate counting detection limit of 2.0 log CFU/ml. Because six data points can be measured within one experiment (six-vessel unit), each experiment contained a sample at t_{eq} and five other time points (ranging from 2.5 to 29 or 64 min, depending on the strain used). Each experiment was reproduced at least two times on different days.

Amplification and sequence analysis of the ctsR gene

Primers for the amplification were designed on the *ctsR* gene of EGDe (forward: GCAGGGATAAACGCTGAAAG; reverse: ACACTCCGGACATCCAACTC). The amplification was performed with PWO Super Yield DNA Polymerase (Roche, Penzberg, Germany) at an annealing temperature of 50°C and with an elongation time of 80 s in a Primus 96 Advanced PCR instrument (Peqlab Biotechnology GmbH, Erlangen, Germany). The PCR products (size, 1.2 kb) were isolated by QIAquick gel extraction (QIAGEN, Venlo, The Netherlands) and sent for sequence analysis (Base Clear B.V., Leiden, The Netherlands).

2

Models used for fitting the data

Two models were used to fit the HHP inactivation data of *L. monocytogenes*: the log-linear model (equations 1 and 2) (6) and the biphasic linear model (equation 3) (3).

The log-linear model assumes first-order inactivation kinetics,

$$N_t = N_{eq} e^{-k\,(t-t_{eq})} \tag{1}$$

where N_t and N_{eq} (CFU/ml) are the microbial populations present at time t and t_{eq} (min), respectively, and k is the specific inactivation rate (min^{-1}). This equation can also be written as:

$$\log\left(\frac{N_t}{N_{eq}}\right) = \log\left(e^{-k\,(t-t_{eq})}\right) = -k(t-t_{eq})\log e = -\left(\frac{(t-t_{eq})}{D}\right) \tag{2}$$

The D-value or the decimal reduction time (min) is the time required for 1-log reduction in the number of cells, and equals ln(10/k).

The biphasic linear, or two-population, model represents the inactivation of two fractions with different resistance (3),

$$\log\left(\frac{N_t}{N_{eq}}\right) = \log\left((1-f_{eq})e^{-k1\,(t-t_{eq})} + f_{eq}\,e^{-k2\,(t-t_{eq})}\right) \tag{3}$$

where $(1 - f_{eq})$ and f_{eq} are the sensitive and the resistant fractions at t_{eq}, respectively. The values k_1 and k_2 are the specific inactivation rates of the sensitive and resistant fractions, respectively, and N_t is the sum of N_{res} and N_{sens}. The inactivation data, starting with t_{eq}, were fitted with the biphasic linear model in addition to the linear model with GInaFiT (9).

Statistical analysis to compare the models

The best fitting model was determined with the root mean square error (RMSE) (equations 4 and 5), the regression coefficient (r^2), and the F-test (equation 6).

The RMSE is the root of the residual sum of squares divided by the degrees of freedom (DF).

$$RMSE_{data} = \sqrt{\frac{RSS}{DF}} = \sqrt{\frac{\sum (average - observed)^2}{(n - m)}} \quad (4)$$

RSS is the sum of squares of the deviation of the observed values from the mean value at one time point and DF is the number of observations (n) minus the number of time points (sampling time, m).

$$RMSE_{model} = \sqrt{\frac{RSS}{DF}} = \sqrt{\frac{\sum (observed - fitted)^2}{(n - p)}} \quad (5)$$

RSS is the sum of squares of the difference between the fitted and observed values and DF is the number of observations (n) minus the number of parameters of the model (p). The model with the RMSE in the same order of magnitude as the RMSE of the data is the best fitting model.

The regression coefficient (r^2) represents the linear relationship between the data and the model predictions. The r^2 of the best fitting model should have a value close to 1. These two criteria do tend, however, to lead often to preference of overparameterized models. Therefore, a better method to compare models (especially if they are “nested”) is to test with an F test if the additional parameters do give a significant improvement in fit, by testing whether the reduction in residual squares is significantly larger than the measuring error (18, 20). The F test provides a measure for the fit of the more complex biphasic model with four parameters compared with the simple log-linear model with only two parameters. With use of the RSS and DF of the two models, the F value is defined as:

$$F = \frac{(RSS_{linear} - RSS_{biphasic}) / (DF_{linear} - DF_{biphasic})}{(RSS_{biphasic} / DF_{biphasic})} \quad (6)$$

The linear model is preferred if the F value is close to 1.0. If the F value is higher, then the biphasic model provides a better fit. If the F value is significant, the more complex biphasic model fits better than the simple linear model ($p < 0.05$) because the error of the linear model is significantly larger than the error of the biphasic model.

Determination of the resistant fraction of the population

The fraction of the population that is resistant to HHP ($f_{0, res}$) was determined by dividing the number of resistant cells before treatment ($N_{0, res}$) by the total number of cells in the population before the treatment (N_0) (equation 7).

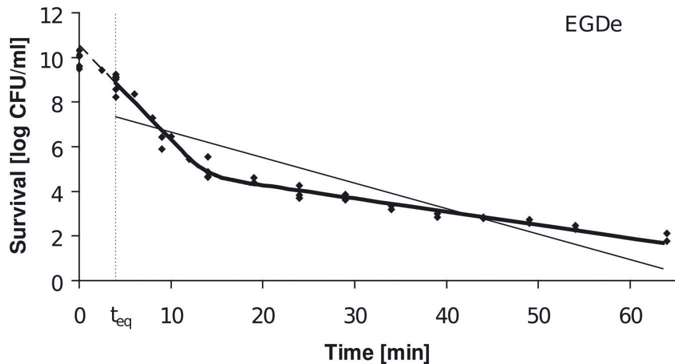
$$f_{0, res} = \frac{N_{0, res}}{N_0} \quad (7)$$

The total number of cells and the number of resistant cells in the population before treatment (at t = 0 min) was calculated by extrapolation of the survival curve on the basis of the fitted values k_1, k_2 and f_{eq}.

Results

HHP inactivation of *Listeria monocytogenes*

A time course study of the inactivation of three *L. monocytogenes* strains by HHP at 350 MPa at 20°C in ACES buffer clearly showed variation in sensitivity to this treatment for the three strains. EGDe showed the highest cell survival compared with LO28 and Scott A (Fig. 1).



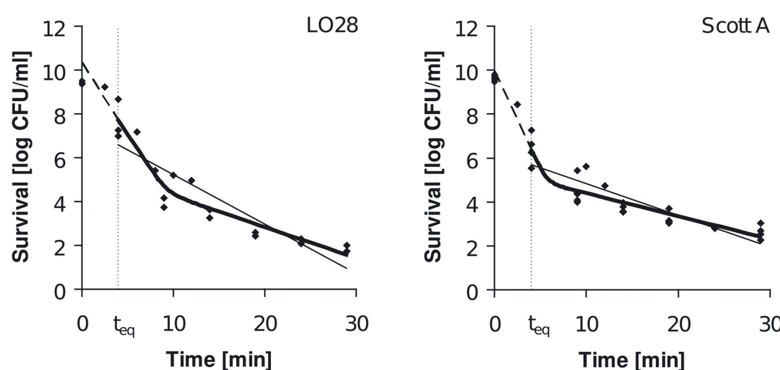


Figure 1. Inactivation kinetics of the linear and biphasic linear model for the survival curves of *Listeria monocytogenes* EGDe, LO28 and Scott A at 350 MPa and 20°C in ACES buffer. Observed values are shown as dots, and the lines represent the two different models. The equilibration time, t_{eq} (4 min), represents the time at which the temperature of the vessel has returned to 20°C after starting the pressurization. This time is represented by the vertical line. The inactivation data, starting with t_{eq}, were used for fitting both models. Extrapolation of the biphasic linear model is represented by a dotted line. The x-axis of the first figure differs from the others by the longer time needed to inactivate EGDe.

Before t_{eq}, the equilibration time, the viability of the cells had already decreased. For all strains, the initial population contained approximately 10^{10} CFU/ml (N_0). At t_{eq}, the initial population of EGDe had decreased by 1.0 log CFU/ml, whereas the initial population of LO28 and Scott A had decreased by 1.8 and 3.2 log CFU/ml, respectively. Because EGDe appeared

to be less sensitive to the pressure treatment compared with LO28 and Scott A, the number of cells at the defined t_{eq} (N_{eq}) was higher for EGDe. For kinetic analysis, the inactivation data starting with t_{eq} were used, in that from this time point, the pressure and temperature were stabilized at 350 MPa and 20°C (29, 30). EGDe was the most resistant strain and could survive 60 min after t_{eq} until the detection limit was reached. LO28 and Scott A were similar in behavior and able to survive for only 25 min after t_{eq}. During the first part of treatment, including the time before t_{eq}, the largest fraction of the initial population was inactivated, and with increasing time, inactivation rates of all three strains decreased. This tailing-off of the inactivation curve indicates the presence of two fractions within the initial population: a sensitive and a more resistant one.

Statistical analysis to compare the models

The inactivation data were fitted with the biphasic linear model in addition to the linear model (Fig. 1 and Table 1) (9).

Table 1. The parameters of the fitted models (linear and biphasic linear) for the survival curves of *Listeria monocytogenes* EGDe, LO28, and Scott A at 350 MPa and 20°C in ACES buffer[a]

		EGDe	LO28	Scott A	
Linear model	N_{eq}	7.3	6.6	5.7	log CFU/ml
	D	8.7	4.4	6.9	min
Biphasic linear model	N_{eq}	8.8	7.7	6.3	log CFU/ml
	D_{sens}	2.3	1.5	1.1	min
	D_{res}	16.8	7.1	9.6	min
	f_{eq}	2.5 x 10^{-4}	2.4 x 10^{-3}	5.0 x 10^{-2}	

[a] N_{eq} is the number of cells at t_{eq}, D is the decimal reduction time, where D_{sens} and D_{res} are the D-values of the sensitive and resistant fractions, respectively; and f_{eq} is the fraction of resistant cells in the total population at t_{eq}.

Although several models can describe tailing, the biphasic linear model was chosen because it contains biological parameters and reflects an inactivation mechanism (8). Statistical analysis was used to compare the fit of the two models for the three strains (Table 2).

Table 2. Statistical analysis of the high hydrostatic pressure inactivation (350MPa at 20°C in ACES buffer) fitted with two models (biphasic linear and linear) of *Listeria monocytogenes* EGDe, LO28, and Scott A[a]

Strain	*n*	RMSEdata log CFU/ml	Model	RMSEmodel log CFU/ml	r^2	RSS	DF	F	*p*
EGDe	37	0.3	Biphasic linear	0.32	0.98	3.1	33	224	< 0.0001
			Linear	1.14	0.78	45.5	35		
LO28	17	0.51	Biphasic linear	0.68	0.92	6.1	13	10	0.0025
			Linear	1.01	0.80	15.2	15		
Scott A	28	0.43	Biphasic linear	0.50	0.88	6.3	24	9	0.0013
			Linear	0.65	0.79	10.9	26		

[a] The data RMSE is the measurement error of the experiments (derived from replicates), *n* is the number of measurements, the model RMSE is calculated from the difference between the observed and fitted data, and r^2 is the regression coefficient. RSS is the residual sum of squares and DF is the degrees of freedom. The F and *p* (probability) values compare the two models in their fitting of the data.

RMSE, on the basis of replicate values for the strains, was between 0.30 and 0.51 log CFU/ml. For all three strains, the model's RMSE, the r^2, and the F test indicated a significantly better fit of the biphasic linear model compared with the linear model. Also for the biphasic linear model, the RMSE of the model is of the same order of magnitude as the RMSE of the data (on basis of replicate values, i.e. the reproduced measuring error). This result demonstrated the presence of two fractions within the initial population with different resistance to HHP. EGDe's sensitive and resistant fractions showed higher *D*-values than the corresponding *D*-values of LO28 and Scott A. For all three strains, the *D*-values of the resistant fractions were 5 to 9 times higher than the *D*-values of the sensitive fractions.

Determination of the resistant fraction of the population

Strain Scott A showed the highest fraction of resistant cells in the initial population, 3.0×10^{-5}, whereas strains EGDe and LO28 contained initial resistant fractions of 8.1×10^{-6} and 2.1×10^{-5}, respectively (Table 3).

Table 3. Calculation of the (stable) resistant fraction of the total population of *Listeria monocytogenes* EGDe, LO28, and Scott A

Strain	$N_{0,\ measured}$ [CFU/ml]	$N_{0,\ extrapolated}$ [CFU/ml]	$N_{0,\ res,\ extrapolated}$ [CFU/ml]	$f_{0,\ res}$	Stable $f_{0,\ res}$
EGDe	7.8×10^9	3.5×10^{10}	2.9×10^5	8.1×10^{-6}	-
LO28	2.6×10^9	2.2×10^{10}	4.5×10^5	2.1×10^{-5}	4.9×10^{-6}
Scott A[a]	4.2×10^9	9.1×10^9	2.7×10^5	3.0×10^{-5}	1.2×10^{-5}

[a] The stable resistant fraction of Scott A was calculated with the results of Karatzas *et al.* (13).

To assess whether the increased resistance is a stable genetic or transient phenotypic feature, a large number of survivors from the tail were isolated, cultured for five consecutive days by starting each day with 0.5% of the culture of the previous day (equivalent to approximately 40 generations), and retested for resistance to HHP at 350 MPa for 20 minutes at 20°C. To determine statistical differences between HHP reduction of survivors and wild type, the *t* test for two samples assuming equal variances was used ($p < 0.05$). Of 102 LO28 survivors, 24 were stable high pressure resistant (Fig. 2).

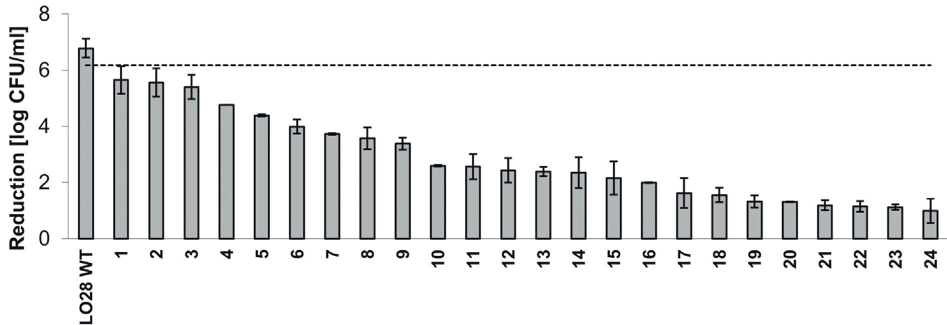


Figure 2. Reduction (log CFU/ml) of *Listeria monocytogenes* LO28 wild type (WT) and 24 HHP-resistant variants after HHP treatment at 350 MPa and 20°C in ACES buffer. Reduction was determined by subtracting the number of surviving cells (log CFU/ml) after 20 min from the number of unpressurized cells (t_0, log CFU/ml). The error bar is 1 times the standard deviation. Significant differences between the log reductions of the HHP-resistant variants and the wild type (p=0.05) are represented by the horizontal line.

These resistant variants differed in their HHP resistance and showed 10 to 600,000 times more survival compared with their wild type. For EGDe, 102 survivors were also tested, but none appeared to be high pressure resistant. Previous experiments with Scott A revealed 33 out of 84 (40%) survivors to be stable, high pressure resistant mutants (13).

Comparative sequence analysis of *ctsR* genes

To explore the possibility that the higher HHP resistance and the apparent absence of stable resistant variants of EGDe is associated with differences in its *ctsR* gene, the *ctsR* genes of EGDe, LO28, and Scott A wild types were amplified and compared. This analysis showed the genes of EGDe and LO28 to be identical, whereas several differences with the *ctsR* gene of Scott A were identified (data not shown). On the basis of these results, the apparent increased HHP resistance of EGDe and the lack of stable stress-resistant variants cannot be attributed to modifications in its *ctsR* gene.

Discussion

In this study, inactivation curves of three *L. monocytogenes* strains under HHP showed significant tailing (Fig. 1). Other research concerning the survival of *L. monocytogenes* at 350 MPa also described tailing (5, 28). However, linear inactivation also was found (21, 26). These differences might be explained by the specific inactivation times and detection ranges used in these studies. A broad detection range together with a long inactivation time makes it possible to visualize the potential presence of a resistant fraction within the population, and consequently tailing. In this study this fraction was calculated to be 10^{-5} to 10^{-6} for the strains LO28, EGDe, and Scott A (Table 3). With a detection limit of 2 log CFU/ml, the initial population should consist of more than 8 log CFU/ml to enable detection of this second inactivation phase.

The measured inactivation data were fitted with two first-order kinetic models: the log-linear and the biphasic linear. The log-linear model has been traditionally used in food processing to calculate, by extrapolating, the desired level of inactivation. However, nonlinear models have been proposed to describe microbial inactivation kinetics for alternative technologies such as high-pressure processing (23). One of these models, the biphasic linear model, has not yet been used to describe HHP inactivation of *L. monocytogenes*, although it has been used in describing HHP inactivation of other microorganisms (17, 25). Statistical analysis of the two models used in our study indicated a significantly better fit of the biphasic linear model compared with the log-linear model (Table 2). Hence, the biphasic model appeared to be a more appropriate model to describe high-pressure inactivation. A good description of inactivation is crucial, especially for the development of minimally processed foods, in that extrapolation of the log-linear model to the desired level of inactivation cannot be relied on for describing high-pressure inactivation of *L. monocytogenes*. Indeed, extrapolation of both models to calculate HHP exposure times required for total inactivation resulted in underestimated treatment times when the linear model was being used compared with the biphasic linear model. In this case the calculated inactivation time would be around 0.8 times too short for the three strains. Hence, around 1 log CFU/ml would still be alive if the linear model would be used for extrapolation to calculate the treatment time necessary to inactivate bacteria in the food below detection levels. Not only the inactivation model, but also the selected strain or strains to be used in food processing studies is of critical importance, as becomes obvious on the basis of the enhanced HHP resistance of *L. monocytogenes* EGDe compared with that of strains LO28 and Scott A. Previously, large variations in resistance of different strains of the same bacteria species to HHP also have been described (27). Therefore, strains to be used in food processing studies should be selected with great care and preferably include stress-resistant variants/strains.

Although tailing inactivation of *L. monocytogenes* has been described before, little research has been performed to unravel the cause of tailing. Tailing can be explained by heterogeneity in apparently clonal populations of cells, resulting in variation in resistance. Cells can be temporary or stable resistant as a result of physiological or genetic changes, which can result in survival because of a better repair mechanism or higher resistance to the treatment (3). In this study, the resistant fractions within the populations that are responsible for the tailing phenomenon have been determined for the three strains. The smallest HHP-resistant fraction, 8.1×10^{-6}, was found for EGDe (Table 3). For this strain, no stable HHP-resistant isolates were found. In addition, the RMSE of the HHP inactivation data of EGDe was much lower than the RMSE of the other strains, indicating less variation within the EGDe strain (Table 2). The LO28 HHP-resistant fraction was 2.1×10^{-5}, of which 24% appeared to be stable HHP resistant. Therefore, the stable HHP-resistant fraction of the initial population of LO28 was estimated at 4.9×10^{-6}. The Scott A HHP-resistant fraction was 3.0×10^{-5}. This value is in accordance with that reported in a previous study with *L. monocytogenes* Scott A, in which 40% of the HHP survivors appeared stable resistant (13).

Taking this value into account, the Scott A stable resistant fraction was estimated at 1.2×10^{-5} (Table 3).

Compared with their respective wild types, LO28 variants showed 10 to 600,000 times and Scott A variants 360 to 6,000 times more survival after 20 min at 350 MPa and 20°C (Fig. 2), indicating that LO28 variants showed more variation in HHP survival compared with Scott A variants. Some LO28 variants were extremely HHP resistant, and even 100 times more resistant than the most resistant Scott A mutant (13).

Genetic analysis revealed two-third of the Scott A piezotolerant isolates to have mutations in the *ctsR* gene (13). CtsR negatively regulates the expression of *clp* genes belonging to the class III heat shock genes. Because of mutations in the *ctsR* gene, the absence of (active) CtsR repressor results in increased expression of the *clp* genes, putatively conferring the high HHP tolerance (14). Notably, high intrinsic HHP resistance of the EGDe strain could not be attributed to the modification of its *ctsR* gene. Other pathogens - *Salmonella*, *Staphylococcus aureus*, and *Escherichia coli* - have also been analyzed for the occurrence of HHP-resistant variants. For *Salmonella* Typhimurium, no stable piezotolerant variants could be isolated (19). However, for *S. aureus* and *E. coli*, resistant HHP variants were isolated after a HHP treatment. The specific genes and regulatory mechanisms causing their HHP resistance are still unknown (11, 15, 22). Recently, Bowman *et al.* (2) investigated expression of *L. monocytogenes* genes during HHP processing. Genes associated with HHP-induced damage involved DNA repair mechanisms and transcription and translation protein complexes, indicating the induction of a generalized repair and maintenance response. Whether similar factors contribute to the HHP resistance of the isolated *L. monocytogenes* LO28 HHP-resistant variants remains to be elucidated.

In conclusion, HHP treatment of all three *L. monocytogenes* strains investigated in this study resulted in tailing inactivation curves that could be successfully described by the biphasic linear model. The resistant fraction of the EGDe population was only temporarily piezotolerant, whereas the resistant fractions of Scott A and LO28 also showed stable piezotolerant subpopulations. The presence of HHP-resistant cells is of importance to the food industry in the design of (new) processes, in that temporary and stable resistant cells might survive HHP processing and affect the safety of products. Furthermore, stable resistant cells might cause problems in the processing line because these cells could survive repeatedly and become “inhouse” flora. Therefore, a HHP process has to be designed to ensure effective inactivation of pressure-resistant strains in foods. Future research will focus on the origin of HHP resistance of *L. monocytogenes*. The 24 LO28 HHP-resistant isolates, as well as the parental strain EGDe, will be characterized further to identify the underlying mechanisms of increased HHP resistance. Such knowledge can contribute to improving the safety level of HHP-treated products.

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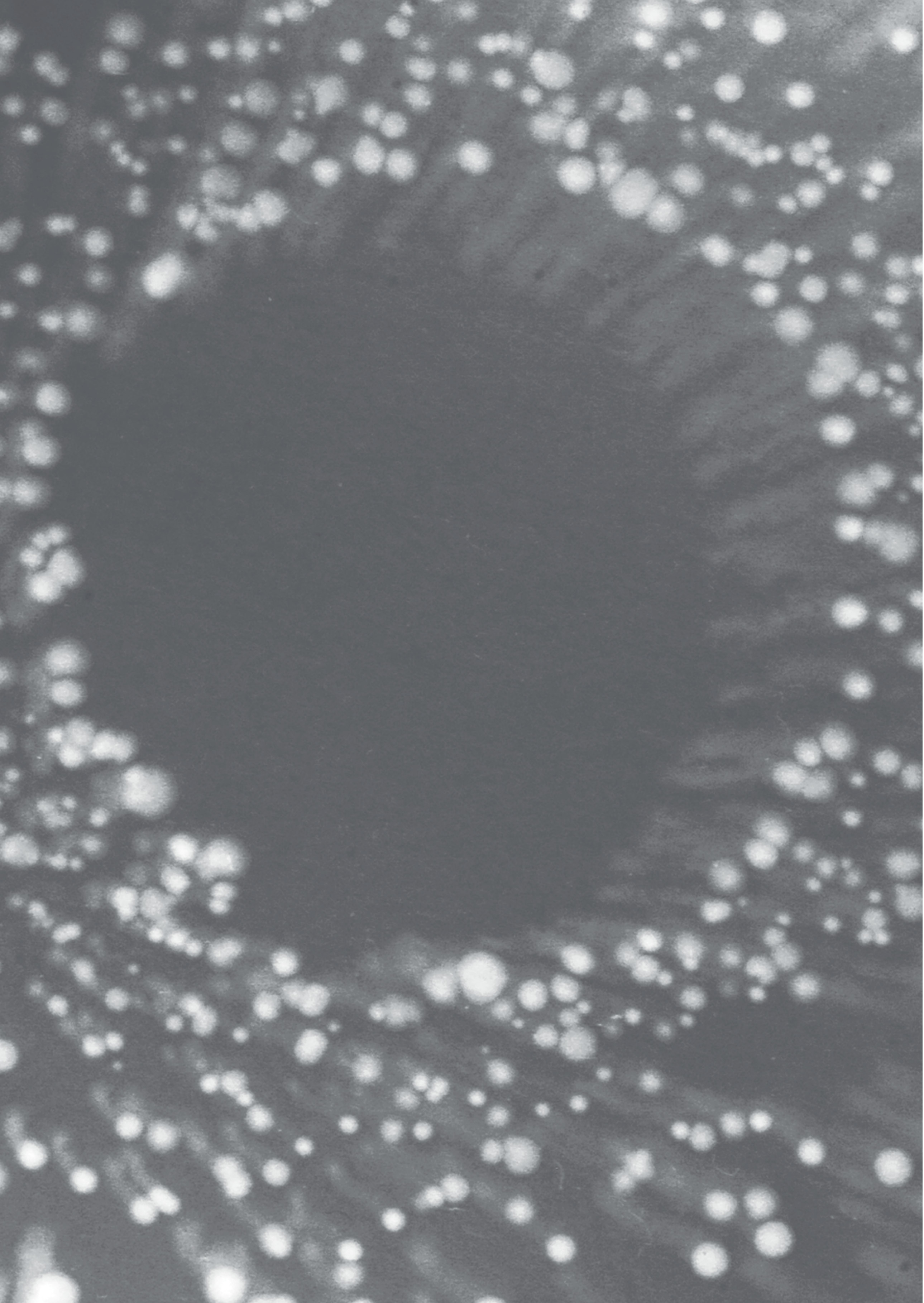

Chapter 3

Population diversity of *Listeria monocytogenes* LO28: phenotypic and genotypic characterization of variants resistant to high hydrostatic pressure

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Abstract

A comparative phenotype analysis of 24 *Listeria monocytogenes* LO28 stress-resistant variants obtained after high-pressure treatment was performed to assess their robustness and growth performance under a range of food-relevant conditions. In addition, genetic analysis was conducted to characterize the promoter regions and open reading frames of the class I and III transcriptional repressors CtsR and HrcA, which control production of specific sets of stress proteins. Analysis of stress survival capacity, motility, biofilm formation, and growth under various conditions showed all variants to be more resistant to pressure and heat than the wild type; however, differences among variants were observed in acid resistance, growth rate, motility, and biofilm-forming capacity. Genetic analysis revealed no variation in the genetic make-up of *hrcA* and its upstream region, but two variants had deletions in the upstream region of *ctsR* and seven variants had mutations in the *ctsR* gene itself. The results of the characterization were cluster analyzed to obtain insight into the diversity of variants. Ten unique variants and three clusters with specific features could be identified: one cluster consisting of seven variants having a mutation in the CtsR regulator gene, one cluster containing two variants with an aerobic biofilm formation capacity similar to that of the wild type, and a cluster composed of five immotile variants. The large population diversity of *L. monocytogenes* stress-resistant variants signifies the organism's genetic flexibility, which in turn may contribute to the survival and persistence of this human pathogen in food-processing environments.

Introduction

The opportunistic pathogen *Listeria monocytogenes* causes listeriosis, a serious infection that most commonly affects newborns, pregnant women, seniors, and immune-compromised patients. Because *L. monocytogenes* is ubiquitous it may be introduced into food-processing plants through many different routes. *L. monocytogenes* has been shown to colonize processing environments and to contaminate products during processing. Certain strains may become persistent in a plant and cause continuously contamination (18, 20, 27). The ability of part of a population to survive in a certain environment because of heterogeneity is called persistence. However, there is a difference between survivors that are phenotypically switching between normal cells and persister cells and survivors that are mutated and therefore genetically different (7). Although the origin of persistence can be different, overall persisters can have specific qualities, such as acid and heat tolerance and adherence to surfaces, contributing to the establishment of house strains. A number of studies have shown persistence of *L. monocytogenes* in various food-processing plants (6, 17, 19, 21, 22). Some of these persistent strains dominated and persisted in a plant or production line for years and caused food contamination and human disease. The generation, occurrence, and selection of these persistent strains can have a significant impact on food processing and safety.

Heterogeneity in a population with an effect on resistance was also observed in the use of the relatively new nonthermal food-processing technology of high hydrostatic pressure (HHP). HHP inactivation of food-borne pathogens has been studied extensively (1, 4, 9). The obtained inactivation curves rarely followed first-order kinetics, as tailing was observed frequently (2, 24, 29). This tailing can indicate heterogeneity in a population with the presence of HHP-sensitive and HHP-resistant fractions. The occurrence of these different fractions has previously been shown for three *L. monocytogenes* strains. The fraction of resistant cells in the initial population of these strains was estimated to be between 8×10^{-6} and 3×10^{-5} (29), and both phenotypic switching and stable piezotolerant variants could be isolated. These stable resistant variants formed 25 to 40% of this fraction of resistant cells for two of the tested strains, LO28 and Scott A (29). Genetic diversity of Scott A stable variants was demonstrated, as over 60% of these variants had a mutation in the *ctsR* gene, which encodes the class III heat shock response regulator. These CtsR variants were nonmotile, resistant to heat and low pH, and displayed reduced growth rates (12, 13). *In vivo* assays with a selected ΔGly-CtsR *L. monocytogenes* Scott A variant (AK01) revealed reduced virulence potential (15). The other Scott A stable variants have unknown mutations (14). Stable HHP-resistant variants of other food-borne pathogens, including *Staphylococcus aureus* (16) and *Escherichia coli* (8, 24) have also been isolated. A few of their phenotypic characteristics have been described, and the studies revealed only diversity in heat resistance among the resistant variants.

The phenotype of stress-resistant variants in previous research was described for only a few characteristics. The current study describes an extensive characterization, as a thorough investigation of the phenotype not only gives more information about the mechanisms playing a role in resistance but also might even reveal the origin of the resistance. Twenty-four *L. monocytogenes* LO28 stable HHP-resistant variants (29) were characterized for a range of phenotypic features, including stress survival capacity, motility, biofilm formation, hemolysis capacity, growth under various conditions, and selected genetic characteristics. Diversity within this population of stable stress-resistant *L. monocytogenes* variants was sorted by cluster analysis, and the impact on safety of HHP-processed foods and production environments is discussed.

Materials and Methods

Bacterial strains and cell culturing conditions

Listeria monocytogenes LO28 (Department of Agrotechnology and Food Sciences, Wageningen University and Research Centre, Netherlands) and 24 LO28 piezotolerant variants (29) were used in this study. These variants had been isolated after three independent HHP treatments at 350 MPa for 20 min at 20°C from an initial population of approximately 3 x 10^9 cells. Stock cultures of all strains were kept in 15% (vol/vol) glycerol (Fluka, Buchs, Switzerland) at -80°C, and before the experiments, cells from stock were grown for 2 days at 30°C on brain heart infusion (BHI) agar (Oxoid, Hampshire, UK). A single colony was used to start a preculture of 10 ml BHI broth. After 20 h of growth at 30°C in an incubator (refrigerated incubator shaker Innova 4335; New Brunswick Scientific, Edison, NJ) with shaking at 160 rpm, 0.5% (vol/vol) inoculum was added to 100 ml of BHI broth. Cultures were used for different inactivation or growth experiments, and each experiment was reproduced at least two times on different days.

High hydrostatic pressure inactivation

High hydrostatic pressure inactivation was performed as described previously by Van Boeijen *et al.* (29). Briefly, cells grown in BHI at 30°C, 160 rpm, from exponential (5 h) or stationary growth (20 h) phase were subjected to 350 MPa at 20°C in 50 mM *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffer (pH 7.0; Sigma-Aldrich, Steinheim, Germany). The time to build up pressure and equilibrate temperature is defined as the t_{eq}. Before and after an HHP treatment of 20 min (from t_{eq}), samples were taken and serially diluted in 0.1 % peptone saline. Samples of 50 to 200 µl were plated on BHI agar using a spiral plater (Eddy Jet; LabScientific, NJ). The plates were incubated for 5 days at 30°C to allow all surviving cells to recover and form visible colonies. Survivors were enumerated, and this was considered accurate if more than 20 cells were detected. This corresponds to a 2-log CFU/ml limit of detection.

Heat inactivation

Cells from the exponential growth phase (5 h of growth at 30°C) were harvested by centrifugation (2,600 x *g*, 20°C, 5 min), washed twice with 50 mM ACES buffer, and resuspended in this buffer until a final concentration of approximately 10^{10} CFU/ml was obtained. For the heat treatment, cell suspensions of 150 µl were placed in sterile glass micropipettes (200 µl, 2-mm inner diameter, 140-mm length; Blaubrand; Brand GmbH, Wertheim, Germany). The pipettes, with the sample in the center of the pipette, were closed by melting the tips and placed in a waterbath (Thermomix ME 4P; B. Braun, Melsungen, Germany) and totally covered by the water. Samples were taken before the treatment and after 1 min at 60°C, serially diluted, and plated, and colonies were enumerated.

Inactivation at low pH

Cells from the exponential growth phase were harvested by centrifugation and added to a tube containing 10 ml BHI, pH 2.5 (pH adjusted with hydrochloric acid [Merck, Darmstadt, Germany]). The medium in the tube, surrounded by water at 37°C, was mixed by using a small magnetic stirrer. Samples were taken directly and after 3 min and then serially diluted in BHI broth instead of 0.1 % peptone saline, to restore the pH. Samples were plated and colonies enumerated.

Colony size

Cells from a preculture were diluted in 0.1 % peptone saline containing 0.1% (wt/vol) bacteriological peptone (Oxoid, Hampshire, UK) and 0.85% (wt/vol) sodium chloride (Merck, Darmstadt, Germany) and plated on BHI agar. The sizes (diameters in mm) of single colonies (average of 20 colonies per plate) were measured after 2 days of growth at 30°C.

Motility test

The motility of the strains was tested in semisolid medium containing 0.25% (wt/vol) agar (Oxoid), 1% (wt/vol) bacteriological peptone (Oxoid), 0.5% (wt/vol) NaCl (Merck, Nottingham, UK), 0.005% (wt/vol) 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich Chemie, Zwijndrecht, Netherlands). A tube, containing 10 ml of motility medium, was inoculated by stabbing a single colony into the medium. After 3 days of incubation at 25°C, strains that were motile, and therefore able to swarm, showed a red cloudy pattern as a result of the reduction of 2,3,5-triphenyltetrazolium chloride to formazan caused by bacterial metabolism.

Hemolysis

Hemolysis tests were performed following the international standard method for detection of *L. monocytogenes*, ISO 11290-1. Single colonies were streaked on blood agar plates, containing 6% of sheep blood (Biotrading, Mijdrecht, Netherlands). *Listeria innocua* was used as a negative control. Plates were examined after incubation at 37°C for 3 days. The

zones of hemolysis were compared between the different variants and the wild type and scored.

Maximum specific growth rate

At 7°C and 30°C, growth was measured for cultures grown in BHI broth in an Erlenmeyer flask based on the optical density (OD). Anaerobic growth was also measured at 30°C in N_2-flushed BHI broth. In time the OD at 660 nm (OD_{660}; measured with a DU 530 Life Science UV/VIS spectrophotometer; Beckman Coulter, Fullerton, CA) of the cultures was measured. The maximum specific growth rate (μ_{max}, in h^{-1}) was calculated from the $\ln(OD_{660})$ data with the modified Gompertz equation (31) in TableCurve 2D software package (version 2.03; Jandel Scientific, San Rafael, CA).

Biofilm formation

Biofilm formation experiments were performed in Hsiang-Ning Tsai medium (HTM), a synthetic minimal defined medium (28). Flatbottom polystyrene microtiter 96-well plates (Greiner Bio-One, Frickenhausen, Germany) were inoculated with 100 µl of HTM containing 1% (vol/vol) inoculum of a preculture in BHI. Plates were incubated at 30°C under aerobic and anaerobic conditions using a BBL GasPak system (Becton Dickinson Microbiology Systems, Cockeysville, MD). After 46 h of incubation the OD_{595} was measured with a microplate reader (Safire; Tecan Benelux BVBA, Giessen, Netherlands), and the number of planktonic cells was determined by plating on BHI agar. Biofilm formation was determined using the method of Djordjevic *et al.* (5). The culture medium was removed from the microtiter plate wells, and the wells were washed with sterile distilled water to remove loosely associated bacteria. Each well was stained with 1% crystal violet solution. After staining, plates were washed and 95% ethanol was added to detach the stained cells. From each well 100 µl was transferred to a new microtiter plate and the amount of crystal violet present in the solution was measured based on the OD at 595 nm.

Amplification and sequence analysis of the *ctsR* gene

Amplification of *ctsR* was performed as described previously by Van Boeijen *et al*. (29). The PCR products (size 1.2 kb) were isolated by QIAquick gel extraction (QIAGEN, Venlo, Netherlands) and sent for sequence analysis (Base Clear B.V., Leiden, Netherlands).

Reverse transcription-PCR (RT-PCR) expression analysis of CtsR-regulated genes

RNA was isolated from cells from the exponential growth phase at 30°C using Tri reagent (Ambion Inc., Austin, TX) and Turbo DNase (Ambion). The quality and quantity of the RNA were checked using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) and the RNA 6000 Nano assay and a 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Synthesis of cDNA was performed with SuperScript III (Invitrogen, Carlsbad, CA). The real-time PCR was carried out with IQ SYBR green Supermix (Bio-Rad, Hercules, CA) in an ICycler

using the following steps: initial denaturation (95°C for 90 s) and amplification (40 cycles of 95°C for 15 s, 58°C for 60 s). Primers, designed based on the genome of strain EGDe, are listed in Table 1.

Table 1. Primers used for determination of expression of *ctsR* and CtsR-regulated genes

Gene	Primer sequence (sense and antisense)
16S-rRNA	5'-GATGCATAGCCGACCTGAGA-3', 5'-TGCTCCGTCAGACTTTCGTC-3'
tpi	5'-AACACGGCATGACACCAATC-3', 5'-CACGGATTTGACCACGTACC-3'
ctsR	5'-GATTAATGGTTGCGGCATTG-3', 5'-CAAAGCAACTAACATCGCCTCT-3'
clpC	5'-AGTCGATGTTTGGCGATGAG-3', 5'-TGGAGGAGCCCCAACTAAAC-3'
clpB	5'-AAAACAGCCATTGTCGAAGG-3', 5'-AAGGGAACCAATGTCGAGTG-3'
clpE	5'-AGCAAACTTTGGGTCGAATG-3', 5'-GTTCACGGTTTGCTTGGTTT-3'
clpP	5'-AGCGGACGTACAAACAATCG-3', 5'-AATTTCAGCGTTTGGCAAGG-3'

The formed products have a size of around 100 bp. Standard curves were derived in order to determine the efficiencies of primer sets corresponding to the different transcripts. Relative expression levels were calculated as described by Pfaffl (25), and C_t values were transformed according to the following equation:

$$\text{ratio} = \frac{E_{\text{target}}^{\Delta\text{Ct(control-sample)}}}{E_{\text{reference}}^{\Delta\text{Ct(control-sample)}}}$$

where *E* is the real-time PCR efficiency, the target is the specific CtsR-repressed gene of interest (*ctsR, clpC, clpB, clpE, or clpP*), references are 16S rRNA and *tpi*, the control is wild type (WT), and samples are the CtsR variants. Significant differences between the expression ratios of samples and controls were calculated with the pairwise fixed reallocation randomization test using the relative expression software tool (REST; version 2; http://www.wzw.tum.de/gene-quantification/).

Statistical analysis

To determine statistical differences between LO28 WT and variants, Student's *t* test for two samples assuming equal variances was used (the limit of significance was set at p=0.05). The relative ratios of the 12 phenotypic characteristics of all HHP-resistant variants compared to LO28 wild type (set at 1) were calculated, and a data matrix was constructed in Excel (for the two parameters motility and hemolysis, the scores ++, +, ± and – were defined as 1, 0.5, 0.25 and 0, respectively). This data matrix was also cluster analyzed using the unweighted pair group method with arithmetic mean (UPGMA) method and Euclidean distances with the GeneMaths XT software (AppliedMaths, St. Martens-Latem, Belgium).

Scanning electron microscopy

Anopore strips cultured with bacteria were glued on a sample holder with conductive carbon cement (Leit-C; Neubauer Chemicalien) and frozen in liquid nitrogen. Samples were

transferred under vacuum to a dedicated cryopreparation chamber (Oxford Cryo-system, CT 1500 HF) onto a sample stage at –90°C. The samples were freeze-dried for 4 min at –90°C in a 3 x 10^{-7} Pa vacuum to remove water vapor contamination. After the sample surface was sputter coated with 10-nm platinum particles, it was transferred to the cold sample stage (–190°C) inside the Cryo-FESEM (JEOL JSM-6300F field emission scanning electron microscope [SEM]) and subsequently analyzed with an accelerating voltage of 5 kV. Approximately 1,000 cells of each sample were examined. Images were digitally recorded (Orion, version 6; ELI sprl, Charleroi, Belgium).

Results

Survival under stress conditions

All 24 LO28 piezotolerant variants, previously isolated after HHP treatments of 20 min at 350 MPa and 20°C (29), were tested for their resistance to HHP, heat and low pH. Detailed analysis of their HHP resistance revealed cells from the exponential growth phase showed 10- to 600,000-fold-higher survival than the WT (Fig. 1a). Tested stationary-phase cells showed similar results for the majority of the variants (Fig. 1b). In most cases stationary-phase cells were more HHP resistant than exponential-phase cells. Exponential-phase cells were also tested for their heat and low-pH survival. After a 1-min exposure at 60°C most HHP-resistant variants showed 2- to 10,000-fold-better survival than the wild type. Only the heat resistance of variant 2 was not statistically significantly different from that of the WT (Fig. 1c). Low-pH survival was tested at pH 2.5 for 3 min at 37°C and revealed for most variants survival 300 to 5,000 times greater than for the wild type. Only variants 15 and 19 showed a reduction similar to that of the WT (Fig. 1d).

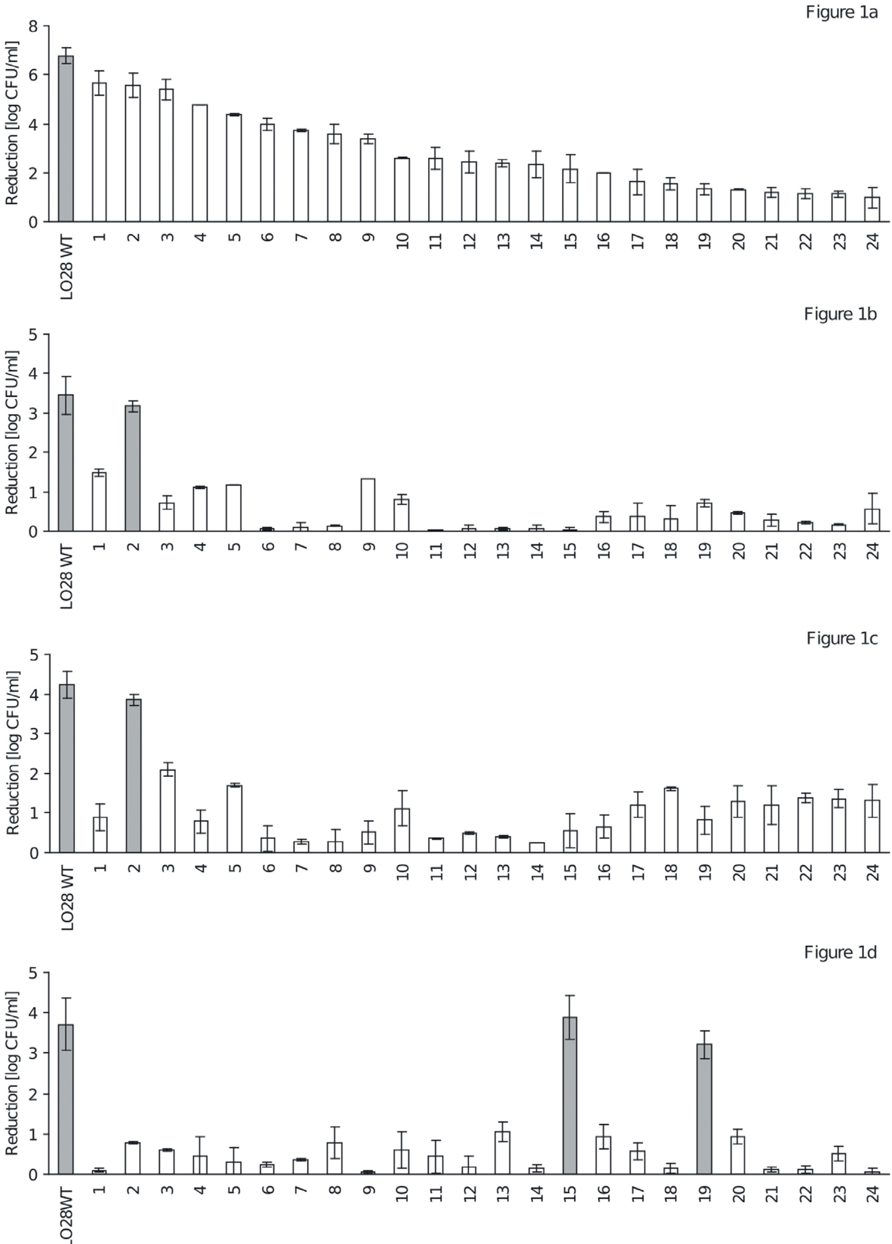

Figure 1. Reduction (in log CFU/ml) of *Listeria monocytogenes* LO28 WT and 24 HHP-resistant variants after HHP treatment of exponentially growing cells at 350 MPa, 20°C for 20 min in ACES buffer (a); HHP treatment of stationary growing cells at 350 MPa, 20°C for 20 min in ACES buffer (b); heat treatment of exponentially growing cells at 60°C for 1 min in ACES buffer (c); and low-pH treatment of exponentially growing cells at pH 2.5, 37°C for 3 min in BHI broth (d). Reduction was determined by subtracting the number of surviving cells (log CFU/ml) after the treatment from the number of unstressed cells (log CFU/ml). Each inactivation experiment was reproduced at least two times on different days. The error bars show 1 standard deviation. Results in gray bars are not statistically different, whereas white bars show significant differences compared to the wild type.

Impact of oxygen and temperature on growth in broth

Analysis of growth performance in BHI under aerobic conditions at 30°C revealed that only 2 of the 24 HHP-resistant variants had maximum specific growth rates (μ_{max}) similar to the wild type. The other variants had a lower μ_{max} (Fig. 2a); however, under anaerobic conditions, 15 variants had μ_{max} values similar to the WT (Fig. 2b). Interestingly, half of the variants had similar μ_{max} values during growth under aerobic and anaerobic conditions, while the wild type and the rest of the variants grew significantly slower under anaerobic conditions. At 7°C all variants were able to grow, of which 17 grew at the same μ_{max} as the WT, with the remaining variants growing more slowly (Fig. 2c).

Small-colony variants

Ten variants showed reduced colony sizes on BHI agar at 30°C (Fig. 2d), and these were classified as so-called small-colony variants (SCVs). The formation of SCVs was independent of maximum growth rate, because SCVs as well as normal-sized colony variants showed similar growth rates in BHI broth at 30°C (Fig. 2a). After replating the SCVs, in addition to small colonies, also normal (WT)-sized colonies were formed at a frequency of approximately 10^{-2} for all SCVs (data not shown). The HHP resistance was tested for these specific normal-sized colonies to determine their stress resistance characteristics. Only 3 of the 10 SCVs (numbers 10, 14, and 15) formed reverted normal-sized colonies that were HHP sensitive. Of these three reverting SCVs, only variant 14 was a CtsR variant, with an insert of 86 bp in the *ctsR* gene. Sequence analysis of the revertant's *ctsR* gene revealed that this *ctsR* gene had regained the WT sequence. On the other hand, the reverted normal-sized colonies of the other seven variants showed similar resistance as their original SCVs. Apparently, the LO28 SCV phenotype is not strictly linked to the stress-resistant phenotype, pointing to different origins of the various phenotypes and their corresponding genotypes.

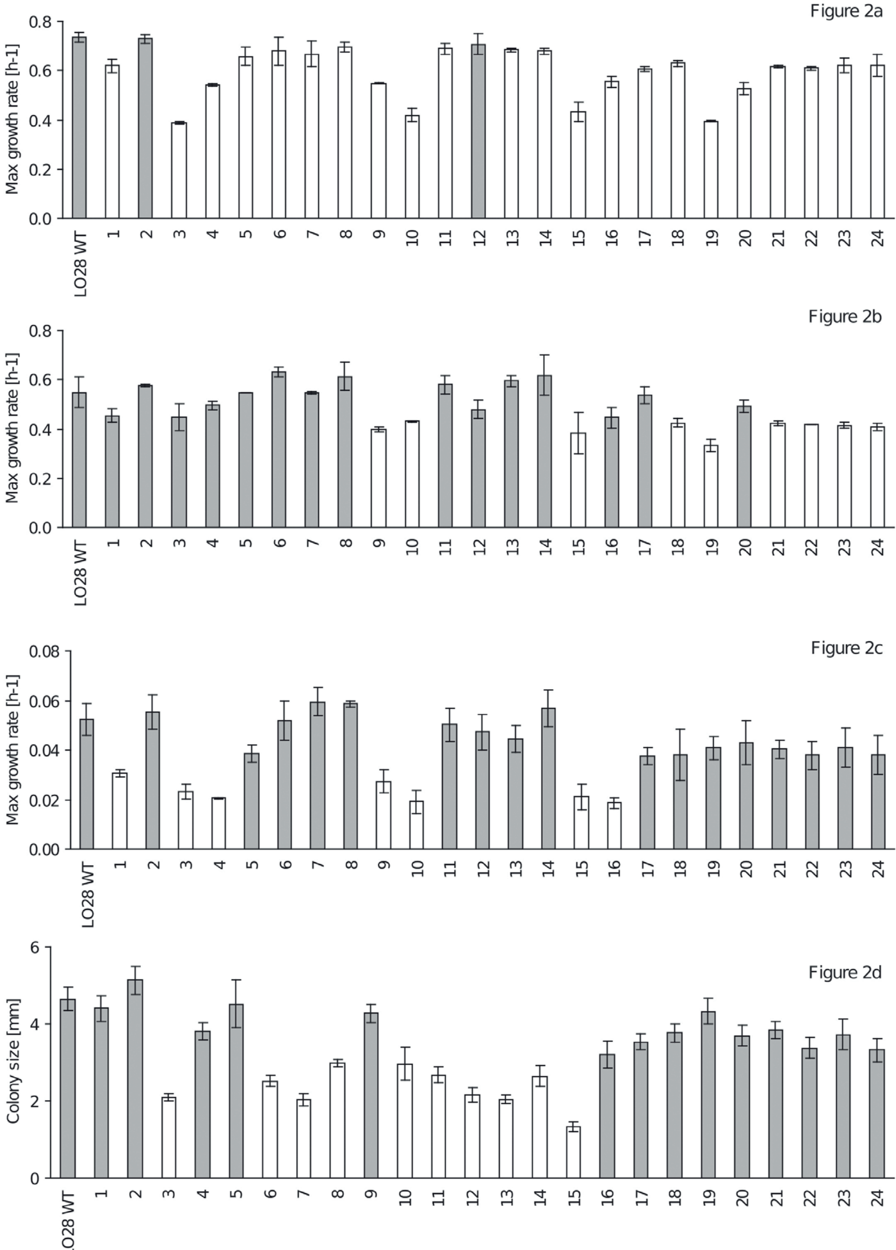

Figure 2. Maximum specific growth rates of *Listeria monocytogenes* LO28 WT and 24 HHP-resistant variants at 30°C in BHI under aerobic conditions (a); at 30°C in BHI under anaerobic conditions (b); or at 7°C in BHI under aerobic conditions (c). (d) Colony size (in mm) was determined by measuring the diameters of colonies after 2 days of growth at 30°C on BHI agar under aerobic conditions. Each growth experiment was reproduced at least two times on different days. The error bars show 1 standard deviation. Results in gray bars are not statistically different, whereas white bars show significant differences compared to the wild type.

Motility, hemolytic activity, and biofilm formation

A large number of variants (13 of 24) had motile behavior similar to the WT, whereas four variants were less motile and seven were nonmotile (Table 2). Fifteen variants showed a similar hemolytic activity as the LO28 WT, whereas the nine other variants showed less hemolysis (Table 2).

Table 2. Differences in *ctsR* genes, motilities, and hemolytic activities of the strains studied

Strain	*ctsR* gene[a]	Motility[b]	Hemolysis[b]
LO28 WT	normal	++	++
1	normal	++	++
2	normal	++	++
3	normal	±	+
4	Δ 268 bp for *ctsR*	-	++
5	normal	++	++
6	Δ GGT	++	++
7	Δ GGT	++	++
8	Δ 55 bp	++	++
9	normal	+	++
10	normal	-	+
11	point mutation aa 38	++	++
12	Δ GGT	++	++
13	Δ 49 bp	++	++
14	+ 86 bp	++	++
15	normal	++	+
16	normal	+	++
17	normal	-	+
18	normal	-	+
19	normal	++	++
20	Δ 198 bp for *ctsR*	++	++
21	normal	-	+
22	normal	-	+
23	normal	+	+
24	normal	-	+

[a] Δ GGT, 3 bp (Gly) deleted in the glycine repeat region; aa, amino acid.

[b] For motility and hemolysis a clear positive result is coded ++ and a clear negative result as -, whereas ± means slightly positive and + indicates a positive response (but less clear than the wild-type response).

Biofilm formation capacity was assayed under both aerobic and anaerobic conditions at 30°C. Under aerobic conditions six variants, and two other variants under anaerobic conditions, produced similar amounts of biofilm as LO28 WT. The other variants formed less biofilm under both growth conditions (Fig. 3a and b).

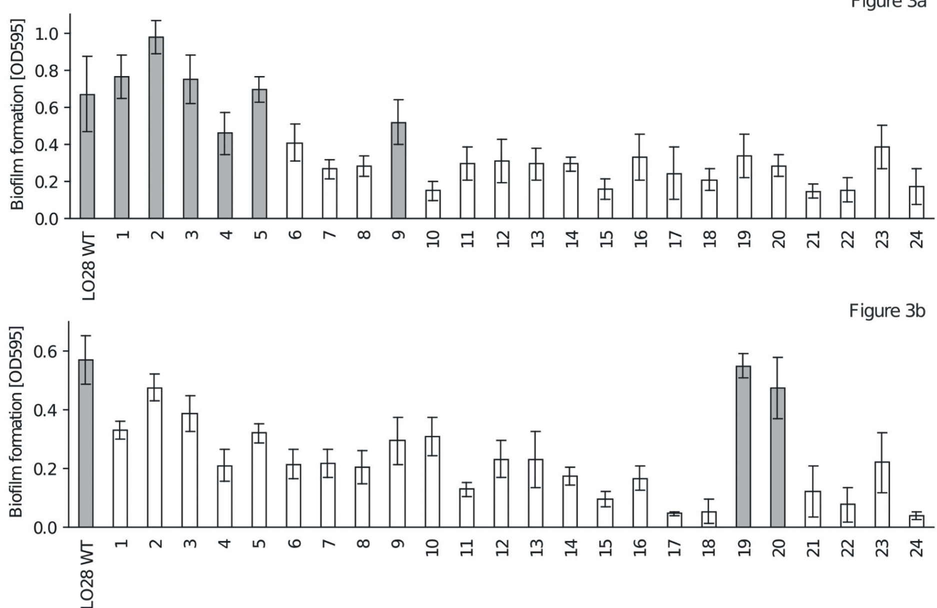

Figure 3. Biofilm formation (measured based on the OD_{595}) after 46 h of incubation in HTM in polystyrene microtiter plates at 30°C under aerobic (a) or anaerobic (b) conditions. Each measurement was reproduced at least three times on different days. The error bars show 1 standard deviation. Results in gray bars are not statistically different, whereas white bars show significant differences compared to the wild type.

ctsR gene sequence analysis and RT-PCR expression of CtsR-regulated genes

Previous studies in *L. monocytogenes* Scott A have shown CtsR to be involved in HHP resistance. Therefore, the *ctsR* gene and promoter region of all variants were analyzed for mutations. From the 24 variants, variants 4 and 20 had a large deletion upstream the *ctsR* gene. Seven other variants had mutations in the *ctsR* gene, including point mutations, insertions, and deletions (Table 2). The *ctsR* genes and promoter regions of the other 15 variants were intact, i.e., similar to the WT. Interestingly, all variants with a mutation in the *ctsR* gene were classified as SCVs (see above).

As CtsR is a negative regulator of class III stress response genes, mutations in or upstream *ctsR* might lead to a decrease in CtsR repression efficiency, resulting in overexpression of CtsR-regulated genes. Therefore, gene expression levels of *ctsR, clpB, clpC, clpE*, and *clpP* of exponentially growing unstressed cells of variants with a deletion in (variant 6) or upstream of (variants 4 and 20) the *ctsR* gene were compared to the WT (Fig. 4).

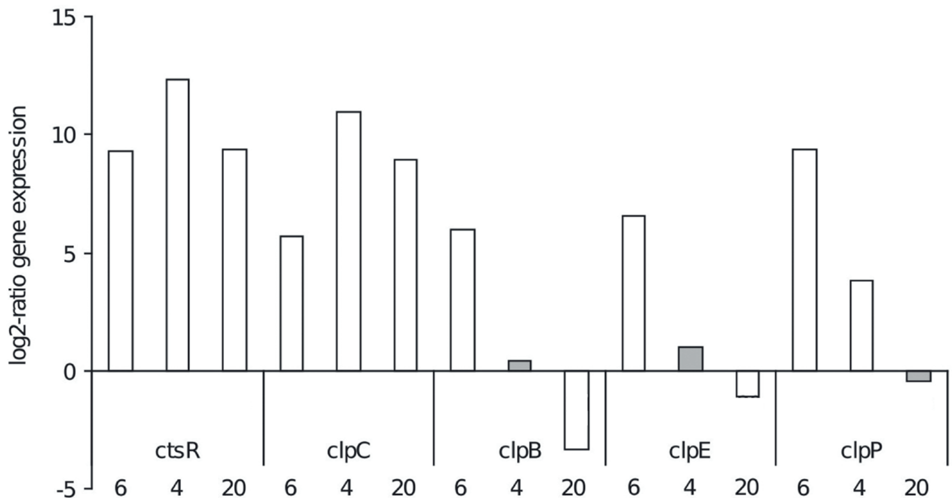

Figure 4. Gene expression ratio (log_2) of *ctsR*, *clpC*, *clpB*, *clpE*, and *clpP* of exponentially growing unstressed cells of variant 6 with a deletion in the *ctsR* gene (Δ*ctsR*) and variants 4 and 20, with a deletion in the promoter region upstream of the *ctsR* gene compared to the wild type. The genes *ctsR* and *clpC* are part of the same operon. Results in white bars show significant differences compared to WT (calculated with pairwise fixed reallocation randomization test, using the relative expression software tool, version 2).

The expression of *clpC* was higher in all tested *ctsR* variants. These variants also showed higher expression of *ctsR* compared to the wild type, which is best explained by the autoregulatory function of CtsR. Variants with a mutation in the *ctsR* gene (represented by variant number 6 in Fig. 4) also showed higher expression levels of the CtsR-regulated genes *clpB, clpE,* and *clpP*, indicating that the repressor function of CtsR is lost. Notably, variants 4 and 20 with a large deletion upstream the starting codon of the *ctsR* gene (positioned 47 and 41 bp upstream ATG, respectively), showed no increased expression of the *clpB*, *clpE*, and *clpP* genes (Fig. 4). This indicated that the repressor function of CtsR is at least partially maintained.

Correlations and cluster analysis

For the cluster analysis the characteristic data (12 phenotypic characteristics) were ordered to extract information concerning similar or unique characteristics of the different HHP-resistant variants. This analysis revealed three clusters formed by 14 of the 24 variants. One cluster was found to consist of the seven variants with a mutation in the CtsR regulator gene. The two other clusters contained two variants with aerobic biofilm formation similar to the wild type and five immotile variants, respectively. The remaining 10 variants did not cluster, signifying their unique characteristics (Fig. 5). The correlation coefficient (R^2) between variant characteristics belonging to one of the three clusters was ≥ 0.9 (p < 0.0002).

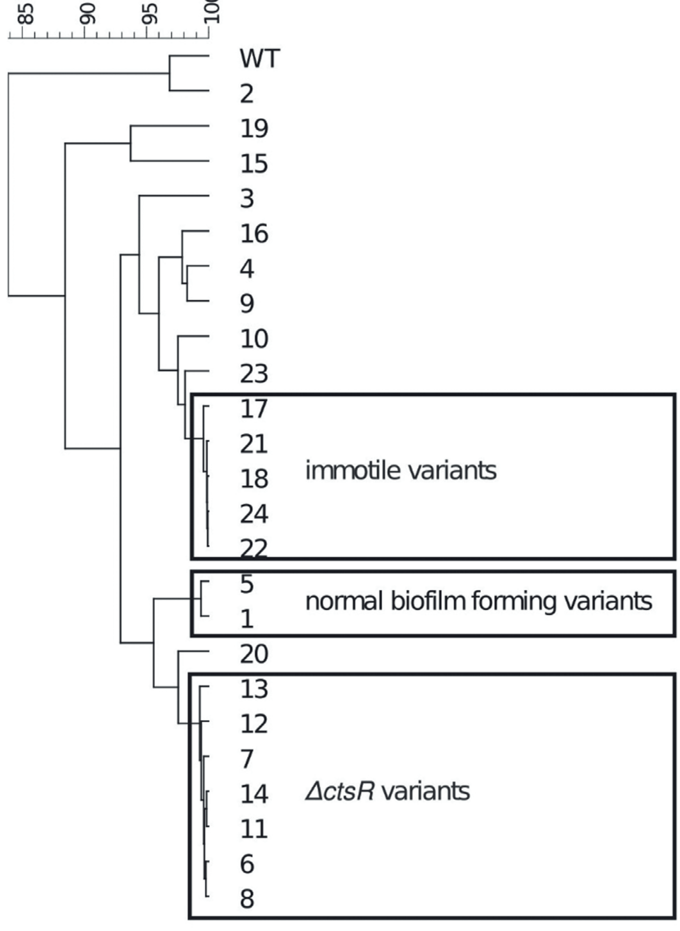

Figure 5. Cluster analysis of phenotypic characteristics of WT and HHP-resistant variants revealed three clusters formed by 14 variants, of which one cluster consisted of 7 variants having a mutation in the CtsR regulator. The two other clusters contained two variants with aerobic biofilm formation similar to the wild type, and five immotile variants, respectively. The remaining 10 variants did not cluster, signifying their unique characteristics.

SEM

Scanning electron microscopy images were made of LO28 wild type and two HHP-resistant variants. These two variants belong to the two most prominent clusters of variants found: variants that have a deletion in the CtsR regulator gene and immotile variants. Cells of both variants showed sizes and morphologies similar to those of the wild type. Flagella were present for the wild type and the motile variant 8 but absent for the immotile variant 17 (Fig. 6).

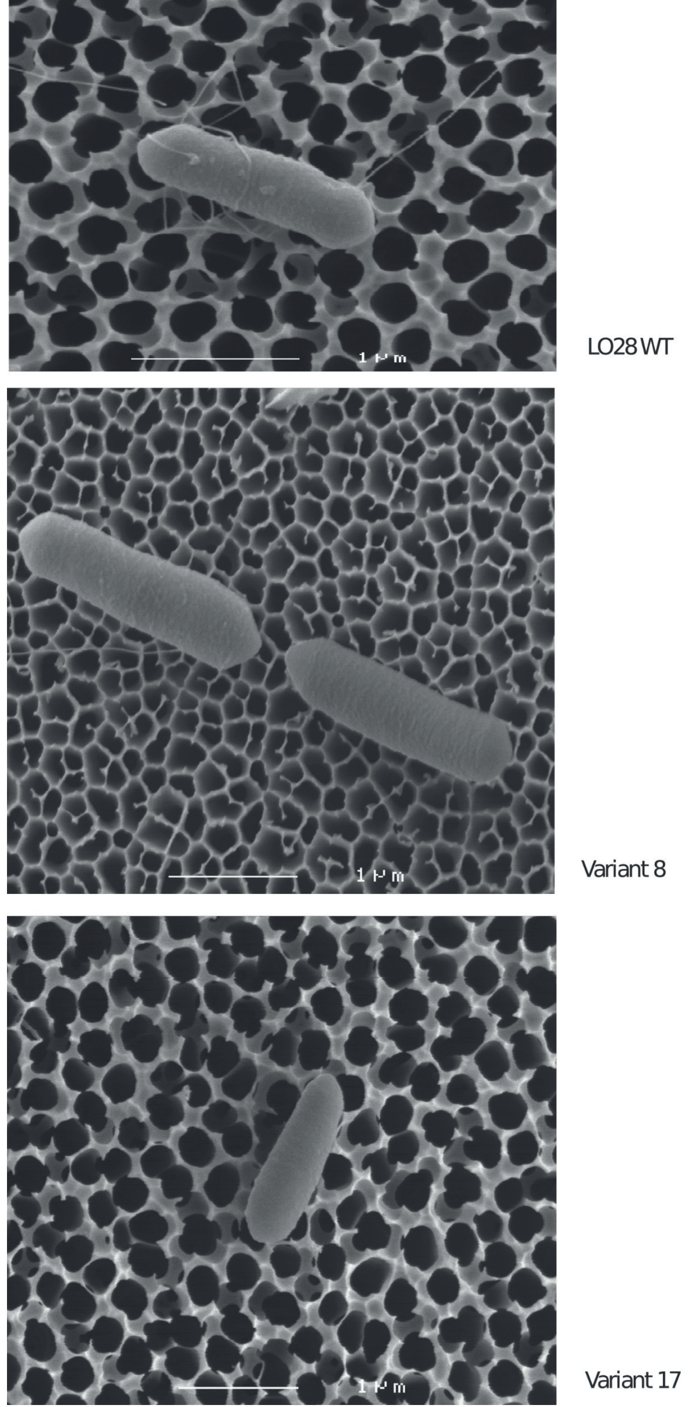

Figure 6. Scanning electron microscopy images of LO28 WT and two HHP-resistant variants (8 and 17). The wild type and variant 8 both showed the presence of flagella, whereas variant 17 showed an absence of flagella. Both variant cell types showed normal size and morphology compared to the wild type.

Discussion

Characterization of 24 resistant variants, which were previously isolated after HHP exposure (29), revealed considerable diversity within this stable resistant fraction. The information obtained in the current study can be combined with that of the previous one (29) and result in the scheme presented in Fig. 7. Starting with five million *L. monocytogenes* LO28 cells, more than 100 cells were able to survive 20 min at 350 MPa. One portion of these HHP survivors was temporarily resistant due to phenotypic switching, whereas the other part was stably resistant because of genotypic heterogeneity. Characterization of 24 of these stable HHP-resistant variants showed most of them to be resistant to other stresses besides HHP and that they were able to grow and form biofilms under various conditions. Ordering all phenotypic characteristics by cluster analysis resulted in 13 clusters of variants with (a combination of) unique characteristics (Fig. 7). This population diversity can be essential to the fitness and persistence of *L. monocytogenes* in a range of environments.

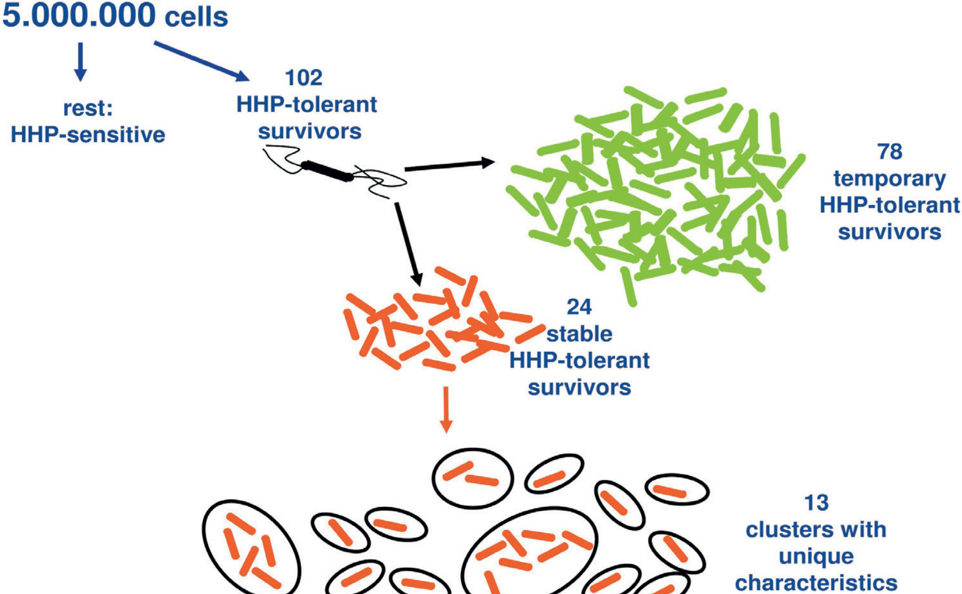

Figure 7. Selection and clustering of stress-resistant *L. monocytogenes* LO28. Of 5 million *L. monocytogenes* LO28 cells, more than 100 cells were able to survive 20 min at 350 MPa. One portion of these HHP survivors was temporarily resistant due to phenotypic switching, whereas the other part was stably resistant because of genotypic heterogeneity (29). Ordering all tested phenotypic characteristics of these stable HHP-resistant variants by cluster analysis resulted in 13 groups of variants with (a combination of) unique characteristics.

Within the 13 different clusters found, three clusters dominated, as they described 14 of the 24 variants. The first cluster contains five immotile variants showing extreme HHP and heat resistance. Resistance to various stresses, as seen for most variants, might be explained by

simultaneous activation through regulatory networks of the different stress mechanisms, as previously described for HrcA, CtsR and σ^B in *L. monocytogenes* (10, 11). The second cluster contains two stress-resistant variants with anaerobic growth and aerobic biofilm formation similar to the wild type (Fig. 2b and 3a). Interestingly, these variants had reduced aerobic growth rates and anaerobic biofilm formation compared to the WT (Fig. 2a and 3b). This outcome confirmed previous findings, showing no correlation between growth rate and biofilm formation under the same environmental conditions (5). The third cluster contains seven motile, small-colony variants, showing normal anaerobic but reduced aerobic growth in broth and hemolytic activity similar to the wild type. The phenotype of this last cluster could be linked to a specific genotype, as only these seven variants have altered *ctsR* genes. CtsR represses the class III stress response genes encoding chaperones and Clp proteases, which degrade damaged or misfolded proteins. Indeed, RT-PCR analysis revealed all seven variants have increased expression of CtsR regulon members, such as *clpB*, *clpE,* and *clpP*. Previously, CtsR was found to be involved in piezotolerance of most *L. monocytogenes* Scott A variants (12, 14).

Comparison of the characteristics of LO28 CtsR variants to the previously isolated Scott A CtsR variant, AK01, revealed similarities as well as notable differences. Both piezotolerant variants showed slightly lower maximum growth rates but increased resistance to heat and acid compared to their wild types (13). Striking differences in the morphology of the LO28 CtsR variants and Scott A AK01 were observed, with AK01 displaying altered morphology, showing not only an absence of flagella but also elongation of cells (13). Scanning electron microscopy revealed wild-type morphology and the presence of flagella in the LO28 CtsR variants. The presence of flagella is in line with the motile characteristics of the LO28 *ctsR* variants. The origin of the differences in morphology of these variants remains to be elucidated. Another difference between AK01 and our LO28 CtsR variants concerned virulence characteristics. The immotile AK01 mutant was less virulent in a mouse infection model compared to its wild type (15). Furthermore, in our study AK01 showed less hemolysis than Scott A and LO28 (data not shown). On the contrary, our LO28 CtsR variants showed similar motility and hemolysis capacity as their wild type, suggesting that virulence factor production capacity was not significantly altered. Virulence of the LO28 HHP-resistant variants will be assessed in more detail in future studies, including *in vivo* experiments using mouse models. Another remarkable observation was made for colony size, as in contrast to AK01, LO28 CtsR variants were SCVs. SCVs have also been described for HHP-resistant staphylococci. These staphylococcal SCVs resulted from a deficiency in aerobic electron transport chain activity, resulting in lower ATP-generating capacity and consequently reduced growth yields under aerobic conditions (16). On the contrary, for the LO28 CtsR SCVs the maximum growth rates under aerobic conditions were similar to that of the wild type at 7°C and similar or only slightly lower at 30°C. This might be the result of reversion of part of the population to normal growing cells, because after replating the LO28 CtsR SCVs normal-sized colonies were found. Retesting revealed that one of the seven

variants had not only reverted to the wild-type phenotype but also to its genotype, by losing the insert in the *ctsR* gene. Next to these seven CtsR SCVs, also three SCVs (number 3, 10 and 15) were found to have an intact *ctsR* gene. These variants showed reduced growth under aerobic conditions, as their maximum growth rates were half the rate of the wild type. Also, these three variants showed reversion to normal (WT)-sized colonies, and two of these reverted variants turned out to be HHP sensitive. Reversion to the wild-type phenotype has also been described for *perR* SCVs of *L. monocytogenes* (26). Deletion of *perR* resulted in an SCV that was slow growing. At a relatively high frequency, large-colony variants arose in the culture. Interestingly, these revertants were *perR* mutants with an unidentified subsequent mutation that showed increased fitness and ultimately dominated the culture. Reversion of SCVs can give rise to persistence because of relatively high-frequency switches between phenotypes and genotypes. Furthermore, in most cases SCVs reverted to wild-type-sized colonies that were still HHP resistant. This would allow *Listeria* to be resistant to different stresses as well as to overcome the fitness disadvantages associated with this resistance by reversion.

So far the only genetic origin of HHP resistance found is alteration of the *ctsR* gene. However, two other LO28 variants (variants 4 and 20), not belonging to the third cluster, have a deletion upstream of the *ctsR* gene. As a result the binding site of CtsR, a heptanucleotide repeat in the promoter region (A/GGTCAAANANA/GGTCAAA) is missing (30). Transcription of the *ctsR* gene in variants 4 and 20 is putatively constitutive, including that of *clpC,* which is located in the same operon. The CtsR produced represses transcription of its regulon members *clpB, clpE,* and *clpP*, whereas increased expression of ClpC may contribute to stress resistance in these variants (Fig. 4). Notably, the two variants did not cluster, and the possible occurrence of an additional mutation(s) cannot be excluded. The underlying mechanisms of their HHP-resistant phenotypes remain to be elucidated.

Recent characterizations of HHP-resistant *S. aureus* variants and pressure-tolerant *L. monocytogenes* strains revealed in both studies no mutations in *ctsR*, suggesting that also differences in other genomic regions are responsible for their phenotype (3, 16). An obvious candidate may be the HrcA repressor, which controls expression of class I stress response genes encoding chaperones (30). All LO28 variants were therefore tested for mutations in this specific repressor gene (*hrcA*), its promoter region, and binding site, but no mutations were found (data not shown). A similar observation was also made in the study with HHP-resistant *S. aureus* isolates (16).

This study showed that *L. monocytogenes* uses population diversity as an insurance policy to guarantee survival when faced with adverse situations. *L. monocytogenes* variants showed not only increased general stress resistance but also the ability to grow under various conditions and to form biofilms, factors that may contribute to persistence of *Listeria* in food-processing environments for long periods of time. For example, the *L. monocytogenes* strain associated with a national outbreak in the United States involving contaminated delicatessen turkey meats was shown to have persisted in a food-processing

facility for more than 10 years (23). To develop strategies to tackle problems associated with diversity and persistence, further research will focus on diversity and the mechanisms involved in diversity generation.

Acknowledgement

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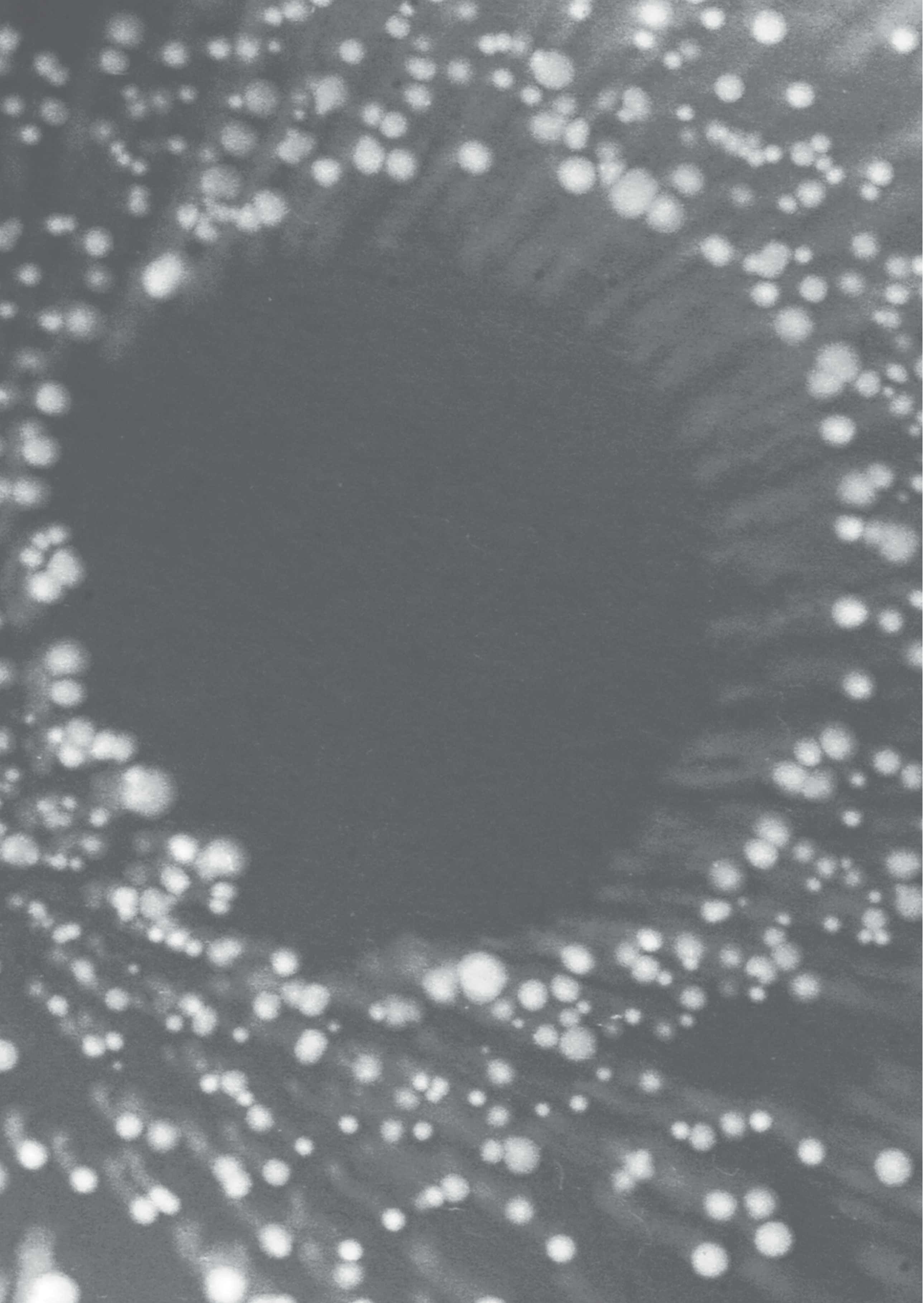

Chapter 4

Virulence aspects of *Listeria monocytogenes* LO28 high pressure-resistant variants

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Abstract

High pressure treatment is a novel food-processing approach for reducing pathogens in foods and food ingredients. However, relatively little is known about the pathogenic potential of organisms that survive the treatment. Twelve previously isolated and characterized variants of *Listeria monocytogenes* LO28 obtained after a high-pressure treatment were assessed for their virulence potential and antibiotic susceptibility. Ten variants showed attenuated virulence while two variants retained full virulence in a mouse model of infection. Seven of the attenuated variants demonstrated a reduction in virulence factor activity. Compared to the wild type, all variants exhibited similar or increased susceptibility to multiple antibiotics commonly used in listeriosis treatment.

Introduction

The pathogen *Listeria monocytogenes* can cause listeriosis, a severe human infection. The estimated annual rate of invasive listeriosis in the United States is approximately 3 cases per million people per year. Although the incidence is low, the high mortality rates (20% - 30%) associated with listeriosis make *L. monocytogenes* one of the most important human foodborne pathogens (23). Most *L. monocytogenes* strains can survive and grow over a wide range of conditions encountered during food processing and storage (8). However, some strains that are associated with human listeriosis are more resistant to food processing conditions (e.g. cold storage, osmotic and acid stress) than strains surveyed from foods or the environment (2,7,22). The increased stress-resistance of these strains may have contributed to survival of food-processing conditions or to the initiation of infection in the host.

High hydrostatic pressure (HHP) is a novel approach to food processing that is becoming more widely used (14). We have previously shown that *L. monocytogenes* cells that survive HHP treatment show a diverse range of phenotypes that impact upon survival of various subsequent stresses and biofilm forming ability (19,21). One frequently isolated type of variant showed mutations in the *ctsR* gene that have previously been associated with survival of HHP processing and a reduction in virulence potential (10,13). However, the pathogenicity of the other surviving isolates is unclear. From a food safety perspective, it is important to appreciate whether HPP survivors display elevated or reduced virulence or enhanced antibiotic resistance. Therefore, we analyzed the virulence characteristics and antibiotic susceptibility of twelve distinct LO28 stress-resistant variants in order to obtain an insight into these characteristics.

4

Materials and methods

Bacterial strains

L. monocytogenes LO28 (Laboratory of Food Microbiology, Wageningen University and Research Centre, Netherlands) and 12 LO28 piezotolerant variants (21) were investigated in this study. These variants were isolated after three independent HHP treatments at 350 MPa for 20 min at 20°C (21). Stock cultures of all strains were kept in 15% (vol/vol) glycerol (Fluka, Buchs, Switzerland) at -80°C.

Determination of virulence-related factors

Primers for the amplification of *inlA* and *inlB* were designed on the internalin genes of EGDe (InlA forward: TTCGGATGCAGGAGAAAATC and reverse: GCAACGTTTGATGTTGATGG; InlB forward: AAGCACAACCCAAGAAGGAA and reverse: CACCTTTGCGCTGCTTAATT). The amplification was performed with REDTaq ReadyMix (Sigma-Aldrich, St. Louis, MO) at an annealing temperature of 53°C and with an elongation time of 135 s in a Primus 96 Advanced PCR instrument (Peqlab Biotechnology GmbH, Erlangen, Germany). The PCR products were

purified by QIAquick gel extraction (QIAGEN, Venlo, Netherlands) and the sequence was analyzed (Base Clear B.V., Leiden, Netherlands).

Phosphatidylcholine phospholipase C (PC-PLC) activity was tested on BHI agar (Oxoid, Hampshire, UK) plates with 5% (vol/vol) egg yolk and 3% NaCl (BioTrading, Mijdrecht, Netherlands) (6).

Virulence assay

Bacterial infection in the primary target organs (liver and spleen) was examined in mice. All experiments were approved by the Animal Experimentation Ethics Committee at University College Cork. Groups of 8- to 10-week-old female BALB/c mice (Harlan, UK) were intraperitoneally inoculated with 6 x 10^5 CFU of *L. monocytogenes* (15). The mice were sacrificed three days post-infection. Organs were homogenized, and serial dilutions were plated onto BHI agar, followed by overnight incubation at 37°C. The resulting colonies were used to calculate the number of bacterial cells per organ and the ratio of survival compared to LO28 wild type. T-test was used to determine statistical differences of results obtained in quadruple in the virulence assay between LO28 WT and variants (limit of significance was set at $P = 0.05$).

Antibiotic assays

The sensitivities of the wild-type strain and resistant variants to a variety of antibiotics were assayed by standard disc diffusion methods (22,23). The filter disks contained 10 µg of the antibiotics: ampicillin, gentamicin, penicillin G or streptomycin or 30 µg of the antibiotics: chloramphenicol, tetracycline or vancomycin. These antibiotics were selected to include antimicrobials used in the clinical treatment of listeriosis (10). Ampicillin, penicillin, and vancomycin target cell wall synthesis, whereas the other tested antimicrobials target protein synthesis (17). For each antibiotic, at least three independent disk diffusion assays were performed.

Results

Gene sequence information for specific virulence factors associated with the *L. monocytogenes* infectious process was determined for the stress-resistant variants and compared to the LO28 wild type (16). Adhesion to and internalization within human epithelial cells is mediated mainly by two surface proteins, internalin A (InlA) and InlB. Both internalin genes *inlA* and *inlB* of the twelve variants showed no alterations compared to the wild type indicating that HPP treatment did not induce mutations in these loci in this experiment. Our work confirmed the presence of a premature stop codon in the gene encoding InlA in LO28, with the stop codon also present in the HPP-surviving clones (11).

Internalized bacteria are trapped in a phagosome, from which they escape by expression of listeriolysin O (LLO) and two phospholipases (16). LLO activity of the stress-resistant

variants was previously determined on blood agar plates (19) and we supplemented this information by assessing phospholipase expression in the current study. Four variants (numbers 2, 5, 6, and 9) showed similar hemolytic (LLO) and phospholipase (PC-PLC) activity compared to the wild type. The remaining variants demonstrated a significant reduction in production of hemolytic and/or phospholipase activity (Table 1).

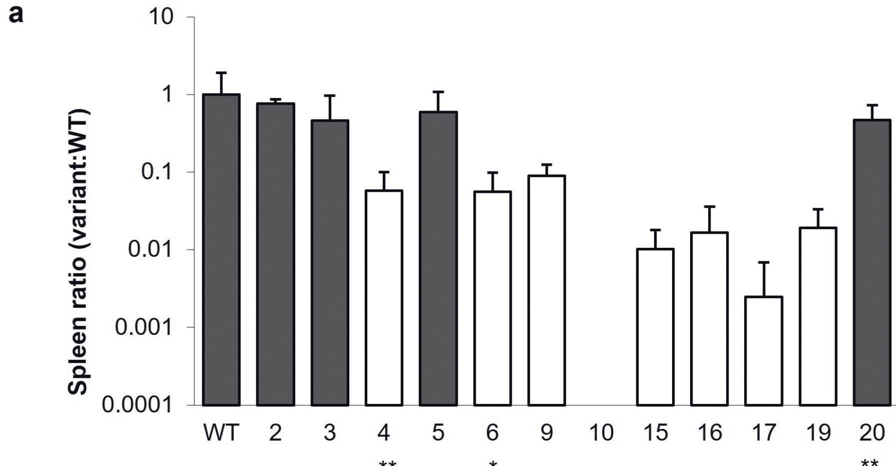
Table 1. Virulence factors of stress-resistant variants compared to their *L. monocytogenes* LO28 wild type.

Strain	WT	2	3	4	5	6	9	10	15	16	17	19	20
LLO[a]	++	++	+	++	++	++	++	+	+	++	+	++	++
PC-PLC[a]	++	++	+	-	++	++	++	-	-	+	-	-	+

[a] Phosphatidylcholine phospholipase C (PC-PLC) and listeriolysin O (LLO) activity (19) of *L. monocytogenes* WT and stress-resistant variants. A clear positive result is coded ++ and a clear negative result –, whereas + means positive (but less clear than the wild type).

Certainly, a profound reduction in both LLO and PC-PLC activity may account for a significant reduction in virulence potential of these variants.

In the mouse model three variants (numbers 2, 4, and 20) showed reduced infection levels in one of the two tested organs (spleen and liver), whereas seven variants showed a significant reduction in the number of cells in both organs compared to their wild type (Fig. 1). The remaining two variants (numbers 3 and 5) retained full virulence in the mouse model.



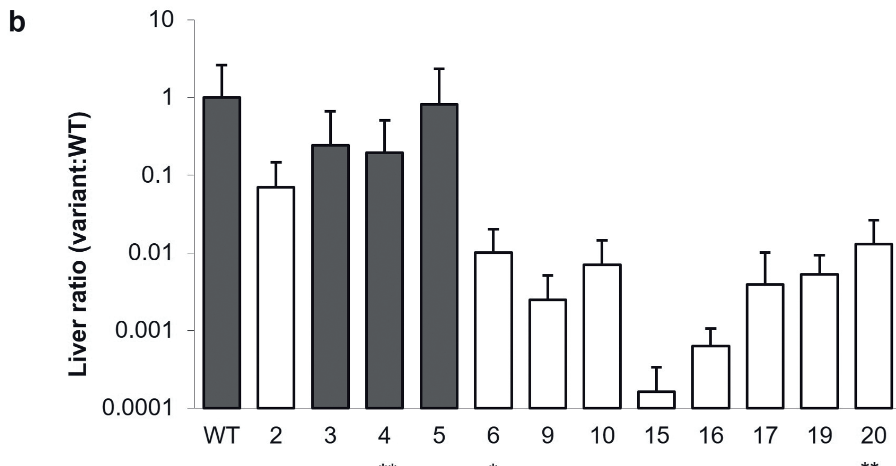


Figure 1. Ratio of colony forming units of *Listeria monocytogenes* LO28 WT and stress-resistant variants in the spleens (a) or livers (b) of infected Balb/c mice after three days compared to WT. Mice were inoculated intraperiteonally with approximately 6 x 10^5 CFU. The average level of cells encountered in the spleen and the liver with infection of LO28 WT cells was 4 x 10^5 CFU and 6 x 10^5 CFU per organ respectively. Data are expressed as means with one standard deviation above the mean of four mice. Results in grey bars are not statistically different, whereas white bars show significant differences compared to the wild type ($P < 0.05$). Variants with * or ** are mutated in *ctsR* or in the upstream region of *ctsR* respectively.

The twelve variants showed either similar or slightly increased susceptibility to the seven antimicrobials tested (Table 2). The most effective antimicrobial in the largest proportion of the variants was tetracycline which inhibits protein synthesis by interacting with the 30S subunit of the ribosome. Seven variants (numbers 3, 4, 10, 15, 16, 17, and 19) were more susceptible to at least three of the antimicrobials tested compared to wild type. Three variants (numbers 2, 9, and 20) were more susceptible to only one of the tested antibiotics, whereas the resistance of two variants (numbers 5 and 6) was similar as that of the wild type for all seven antimicrobials tested.

Table 2. Antibiotic susceptibility of stress-resistant variants compared to their *L. monocytogenes* LO28 wild type expressed by inhibition zone (mm).

strain[a]	amp 10 µg	chl 30 µg	gen 10 µg	pen 10 µg	str 10 µg	tet 30 µg	van 30 µg	more susceptible
WT	32.0	26.3	28.5	32.0	21.3	22.8	24.3	0/7
2	31.7	27.0	27.3	31.0	21.3	26.3*	24.0	1/7
3	n.d.	n.d.	n.d.	n.d.	24.0*	26.0*	29.7*	3/3
4	35.0	29.7	33.3*	35.7*	25.0*	28.0*	27.0*	5/7
5	32.7	24.7	29.3	31.7	22.0	25.0	25.0	0/7
6	33.3	25.7	28.0	32.0	21.3	25.3	23.7	0/7
9	34.7	26.0	30.3	33.0	21.0	28.0*	25.7	1/7
10	35.3	32.0*	29.3	36.7*	24.3*	29.3*	27.3*	5/7
15	36.7	31.7*	31.7	35.3*	24.7*	28.3*	26.7	4/7
16	36.3	30.7*	32.3*	35.3*	24.0*	28.3*	26.3	5/7
17	37.0*	32.3*	32.3*	35.7*	25.0*	30.0*	28.3*	7/7
19	37.0*	32.0*	32.7*	34.7	22.7	29.3*	30.3*	5/7
20	37.0*	28.0	32.0	35.0	23.3	24.7	26.3	1/7
more susceptible	3/11	5/11	4/11	5/11	6/12	9/12	5/12	37/80

[a] The susceptibility of the wild type strain *L. monocytogenes* LO28 and twelve stress-resistant variants to a variety of antibiotics (ampicillin, chloramphenicol, gentamicin, penicillin G, streptomycin, tetracycline, and vancomycin) were assayed by agar diffusion (24 h incubation at 30°C). The diameters of the zones of bacterial growth inhibition surrounding the disks were measured in mm. Each disk diffusion assay was conducted three times on different days. For variant number 3 it was difficult to interpret the antibiotic susceptibility for four of the tested antimicrobials because of the irregular shape of the inhibition zones (n.d. means not detectable). The last column in the table represents for how many of the tested antibiotics the variant is increased susceptible compared to the wild type. The last row in the table represents how many variants are more susceptible to that specific antibiotic compared to the wild type. *Variants are significantly more susceptible compared to wild type to that specific antibiotic.

Discussion

In the present study, we have analyzed the virulence potential and antibiotic susceptibility of *L. monocytogenes* variants surviving high pressure treatment in order to determine the disease risk associated with surviving populations. Overall, the work indicates that the majority of the stress-resistant variants displayed reduced levels of certain virulence factors, reduced virulence potential in a mouse model, and increased susceptibility to various antibiotics.

During this work we verified that stress-resistant variants with a mutation in *ctsR* display significantly reduced virulence potential (13). Variant 6 represents a mutant in the gene encoding CtsR (19) which is similar to the commonly occurring mutations uncovered in previous HHP and heat inactivation studies (12,20). Here we see similar reductions in virulence with this variant reaching around 2 log lower levels than the wild type in the spleens of infected mice. Variants 4 and 20, that both have a large deletion upstream of *ctsR*, exhibited subtle changes in virulence factor expression and displayed significantly reduced infection levels in spleen and liver respectively. The difference in virulence phenotype between these CtsR variants might be related to their different expression of *clp* genes that have been shown to play a role in virulence (3,19). For the remaining variants the nature of

their attenuating mutation(s) is currently unclear. It is possible that some of these variants might have an alteration of the PrfA regulatory network (4) resulting in the diminished virulence gene activity that we identified in phenotypic analyses. ActA is one of the PrfA-regulated gene products in *L. monocytogenes* enabling actin polymerization and thereby promoting its intracellular motility and cell-to-cell spread, and it was recently shown to be critical for bacterial aggregation and biofilm formation (18). Further studies are required to analyze whether expression and/or activity of ActA and other (PrfA-regulated) virulence factors is affected in selected variants.

Two variants (numbers 3 and 5) retained not only full virulence in the mouse model, but were previously characterized as HHP, heat and acid resistant and exhibiting similar anaerobic growth rates and levels of aerobic biofilm growth as the wild type (19). This may indicate a level of fitness that is not evident in the other variants tested. Variant 3 differs from variant 5 as it showed reduced levels of virulence factor activity and is more susceptible to antibiotics. Furthermore, variant 3 is a so-called small colony variant (SCV), displaying smaller colonies on agar plates compared to its wild type (19). Previous research showed reversion of this SCV phenotype (19). Reversion of part of the population to normally growing cells might explain the full virulence phenotype observed in the mouse model compared to the reduced levels of virulence factors for this variant.

Overall, our work demonstrates that the majority of variants surviving HPP treatment demonstrate reduced virulence potential in the murine model reflecting reduced CtsR activity or reduced virulence factor activity (this study, [13,19]). At a lower frequency variants with enhanced stress resistance and normal levels of virulence potential could be isolated. Further work is necessary to determine whether the stress-resistant, virulent survivors may subsequently dominate the surviving population and thereby pose a health risk.

Acknowledgment

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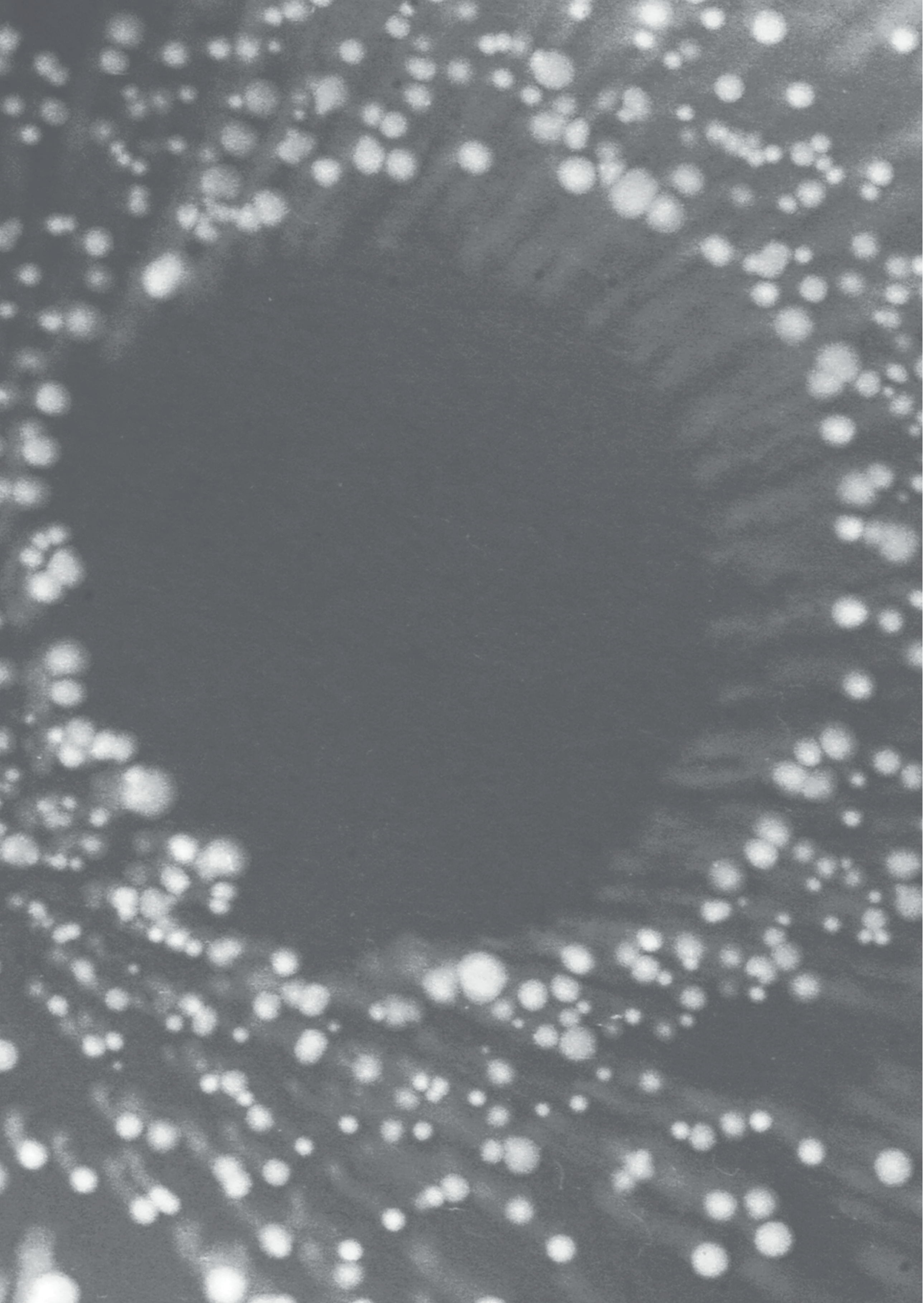

Chapter 5

Isolation of highly heat-resistant *Listeria monocytogenes* variants by use of a kinetic modeling-based sampling scheme

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Abstract

Stable high-hydrostatic-pressure (HHP)-resistant *Listeria monocytogenes* LO28 variants were previously isolated and characterized. These HHP variants were also more resistant to heat. In addition, nonlinear heat inactivation kinetics pointed towards the existence of heat-resistant variants, although these could not be isolated so far. In this study, we used kinetic modeling of inactivation curves of two isolated HHP variants and their wild type, and this revealed that the probability of finding resistant variants should depend on the nature of the inactivation treatment and the time of exposure. At specific heat and HHP conditions, resistant LO28 and EGDe variants were indeed isolated. Resistant LO28 variants were even isolated after a heat inactivation at 72°C in milk, and these variants showed high resistance to standard pasteurization conditions. The increased resistance of part of the isolated LO28 and EGDe variants was due to mutations in their *ctsR* genes. For the variants whose *ctsR* genes and upstream regions were not altered, the mechanisms leading to increased resistance remain to be elucidated. This research showed the strength of kinetic modeling in unraveling the causes of nonlinear inactivation and facilitating the isolation of heat-resistant *L. monocytogenes* variants.

Introduction

Listeria monocytogenes can cause listeriosis in animal and human populations. Human listeriosis is a rare but severe disease and is one of the leading causes of death among patients with food-borne diseases in the United States. The estimated annual rate of invasive listeriosis in the United States is 3 cases and for countries within the European Union is 2 to 10 cases per million people per year (33). Recently, several European countries experienced an apparent increase in the incidence of listeriosis (13).

A specific characteristic of *L. monocytogenes* that appears to be critical to its ability to cause human food-borne illness is its capacity to survive under harsh conditions. The occurrence of variants and generation of population heterogeneity are factors that may contribute to the survival capacity of *L. monocytogenes*. Previous research showed that heterogeneity in *L. monocytogenes* populations (strains EGDe, LO28, and Scott A) affects resistance to high hydrostatic pressure (HHP). Inactivation of such heterogeneous populations resulted in survival curves with significant tailing, indicating the presence of an HHP-sensitive and an HHP-resistant fraction (32). Analysis of the cells that survived such HHP treatments revealed that the higher resistance of LO28 (32) and Scott A (17, 18) was a stable feature for part of the resistant fraction. Contrary to these results, no stable HHP-resistant isolates were obtained for EGDe (32). A significant fraction of the stably resistant variants of both Scott A and LO28 had an altered *ctsR* gene. This gene encodes CtsR, a DNA binding protein that regulates class III heat shock genes (7). The observed alterations in *ctsR* resulted not only in increased resistance to high pressure but also in increased survival to heat (17, 20, 31). Further characterization of the LO28 HHP-resistant variants without mutations in *ctsR* also revealed increased resistance to heat for most of these variants (31). In addition, thermal inactivation of *L. monocytogenes* was previously fitted with a biphasic model, indicating the presence of a heat-resistant fraction (1, 3, 5). However, so far these variants could not be isolated after heat inactivation.

For the present study, we used kinetic modeling of the inactivation of wild-type (WT) LO28 and two HHP-resistant variants as a strategy to determine the probability of detecting resistant variants. With this information, the appropriate conditions to isolate HHP- and heat-resistant variants for LO28 were established. Similar conditions were used to examine the existence of HHP- and heat-resistant variants of strain EGDe. Isolated variants of both strains were checked for mutations in stress regulator CtsR. Finally, the significance of the occurrence of stress-resistant variants of *L. monocytogenes* is discussed.

Materials and methods

Bacterial strains and cell culturing conditions

Listeria monocytogenes LO28 wild type and two stress-resistant variants (*ctsR* variant number 6 and immotile variant number 17) (32) and EGDe wild type (Laboratory of Food

Microbiology, Wageningen University and Research Center, Netherlands) were used in this study. Stock cultures of all strains were kept in 15% (vol/vol) glycerol (Fluka, Buchs, Switzerland) at –80°C, and before the experiments, cells from stock were grown for 2 days at 30°C on brain heart infusion (BHI) agar (Oxoid, Hampshire, UK). A single colony was used to start a preculture of 10 ml BHI broth. After 20 h of growth at 30°C in an incubator (refrigerated incubator shaker Innova 4335; New Brunswick Scientific, Edison, NJ) with shaking at 160 rpm, 0.5% (vol/vol) inoculum was added to 100 ml of BHI broth. Cells grown in BHI at 30°C from exponential growth phase (5 h) were washed twice with 50 mM N-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffer (pH 7.0; Sigma-Aldrich, Steinheim, Germany) and resuspended in this buffer until a final concentration of approximately 10^9 CFU/ml was obtained. The resulting cultures were used for inactivation experiments, and each experiment was conducted at least two times on different days.

High-hydrostatic-pressure inactivation

HHP inactivation was performed as described previously by Van Boeijen *et al.* (32). Cell suspensions were subjected to 350 MPa at 20°C in ACES. Before and after an HHP treatment, samples were taken and serially diluted in 0.1 % peptone saline. Samples of 50 to 200 µl were plated on BHI agar using a spiral plater (Eddy Jet; LabScientific, NJ). The plates were incubated for 5 days at 30°C to allow all surviving cells to recover and form visible colonies. Survivors were enumerated, and this was considered accurate if more than 20 cells were detected. This criterion corresponds to a 2 log CFU/ml limit of quantification. The HHP inactivation data were fitted with the biphasic linear and the linear model using GInaFiT (11).

Heat inactivation

For the heat treatment, cell suspensions of 150 µl were placed in sterile glass micropipettes (200 µl, 2-mm inner-diameter, 140-mm length, Blaubrand; Brand GmbH, Wertheim, Germany). The pipettes, with the sample in the center of the pipette, were closed by melting the tips and placed in a water bath (Thermomix ME 4P; B. Braun, Melsungen, Germany) that covered them totally. The samples were treated at 55°C and then serially diluted and plated, and colonies were enumerated. The heat inactivation data were fitted with the biphasic linear model with shoulder and the linear model with shoulder using GInaFiT (11) and the TableCurve 2D software package (version 2.03; Jandel Scientific, San Rafael, CA).

Estimation of the probability of detecting resistant variants based on kinetic modeling

Kinetic modeling can be used to estimate the probability of detecting resistant variants. For this, the fraction of resistant variants in the population and their inactivation kinetics have to be determined. The two most prominent groups of variants found in the LO28 population comprise immotile and *ctsR* variants. Their specific fraction in the population ($f_{0,var}$) was determined from the HHP inactivation data by dividing the number of specific

variants before the HHP treatment ($N_{0,var}$) (CFU/ml) by the total number of measured cells in the population before the treatment ($N_{0,measured}$) (CFU/ml), as follows:

$$f_{0,\,\mathrm{var}} = \left(\frac{N_{0,\,\mathrm{var}}}{N_{0,\,\mathrm{measured}}} \right) \tag{1}$$

HHP inactivation of variants was linear. Therefore, the initial number of variants was calculated from the fraction of variants that survived HHP and by extrapolation of this fraction's survival curve, as follows:

$$\log N_{0\ \mathrm{var}} = \log N_{t\ \mathrm{var}} + \frac{t}{D_{\mathrm{var}}} \tag{2}$$

5

where $N_{t,var}$ equals the number of variants (log CFU/ml) at time *t* (min) and D_{var} the fitted decimal reduction time or *D*-value (min) of the HHP-inactivated variants (31, 32).

The inactivation data were fitted with an appropriate model. For the wild type, the biphasic linear model (with shoulder) was used, and for both variants, the linear model (with shoulder) was used. The proportions of the variants in relation to the total population were calculated with equations 1 and 2 and the specific inactivation model, assuming that the fractions of these two variants for heat experiments at t=0 were equal to the HHP-inactivated fraction. This proportion of variants is then equal to the probability of isolating variants at a specific time point.

Selection of resistant variants by stress challenge cycles

Previously, survivors were randomly selected and individually cultured before they were subsequently assessed for stable piezotolerant phenotypes (32). Those isolates were individually subcultured five times during five consecutive days using 0.5% (vol/vol) inocula in fresh BHI medium (equivalent to approximately 40 generations) and on day 5 retested for resistance to HHP. In the present study, variants were searched by an optimized series of repetitive stress challenge cycles as described previously for the isolation of resistant variants of *Escherichia coli* and *Staphylococcus aureus* (15, 21). To increase the probability of isolating resistant variants, three challenge treatments were used, and this was shown to be effective. In the stress challenge cycle used for the experiments described herein, *L. monocytogenes* survivors of optimized HHP or heat experiments (ranging from 100 to 10,000 CFU) were harvested from the plates after 5 days of incubation at 30°C. Fresh BHI medium (approximately 5 ml) was added on top of the plate with the surviving colonies, and visible colonies were scraped from the plate with a spatula and resuspended in the added BHI. The cell suspension was added to 5 ml fresh BHI medium by using a pipette and grown for 20 h at 30°C. A 100-ml culture was inoculated with this preculture (0.5% [vol/vol]; 5h at 30°C) and used for a subsequent inactivation experiment. From this second inactivation, survivors were again harvested from the plate, cultured twice, and inactivated. Twenty-four isolates from the third inactivation experiment were obtained and analyzed for their stress-

resistance and their *ctsR* genes and upstream regions. This method makes it possible to examine resistance of a large number of survivors in a relatively easy way. The previous method made it possible to examine the stable resistance of single survivors independently of their growth characteristics, whereas the current method allows the examination of the surviving fraction of the population, consisting of a large number of survivors that will compete in both their growth and resistance.

Statistical analysis

For the wild type and both variants, the selected model for their heat or HHP inactivation was fitted to the independent reproductions individually, and the average decimal reduction times and shoulder lengths were calculated. Student's *t* test for two samples assuming equal variances was used (the limit of significance was set at a *P* value of 0.05) to determine statistical differences between decimal reduction times and shoulder lengths of the wild type and the variants and between the results for resistance of the wild type and the survivors.

Amplification and sequence analysis of the *ctsR* gene

Amplification of *ctsR* was performed as described previously by Van Boeijen *et al.* (32). The sequences of the primers used were 5'-GCAGGGATAAACGCTGAAAG-3' for the forward and 5'-ACACTCCGGACATCCAACTC-3' for the reverse primer. The PCR products (1.2 kb) were isolated by QIAquick gel extraction (Qiagen, Venlo, Netherlands) and sent for sequence analysis (Base Clear B.V., Leiden, Netherlands).

Results

HHP and heat inactivation of *L. monocytogenes* LO28 wild type and resistant variants

In previous research, stable HHP-resistant *L. monocytogenes* LO28 variants were isolated (32). Most of these variants appeared to be heat resistant (31). Furthermore, biphasic inactivation was found not only for HHP but also for heat inactivation of WT LO28. This raised the question of whether stably resistant variants could also be isolated after a heat treatment. We selected a temperature that caused an inactivation profile similar to that found for the HHP inactivation (i.e., biphasic inactivation with a similar *D*-value for the resistant fraction) (Fig. 1a and b and Table 1). One hundred two survivors were selected after 25 min of treatment at 55°C. None of these isolates was stably resistant after culturing and retesting under the same conditions (data not shown). The probability of isolating variants in the tail depends on the characteristics of the survivors. Cells can be temporarily or stably resistant as a result of physiological or genetic changes. For example, after HHP inactivation of *L. monocytogenes* LO28, only 25% of the survivors were stably resistant, whereas the remaining survivors were temporarily resistant (32). Consequently, some of the isolated variants will not be resistant in a subsequent inactivation treatment. Hence, we tried another approach, using the fraction of stably resistant variants in a population combined with their

inactivation kinetics to estimate the probability of detecting these resistant variants during the inactivation. With this information, the best possible conditions can be established for the isolation of resistant variants. We selected representatives of the previously isolated stably resistant HHP variants that showed increased resistance to heat (31). Variant number 6 (a *ctsR* variant) and variant number 17 (an immotile variant) were investigated for their heat and HHP survival compared to the survival of the wild type under these conditions (Fig. 1a and b). The linear model with shoulder was used to fit their heat inactivation data, and the linear model was used to fit their HHP inactivation data (Table 1). For HHP, the application of the linear models starts at the time at which the temperature of the HHP vessel has returned to 20°C. This was the case 4 min after starting the pressurization.

Table 1. The parameters of the fitted models for the survival curves of *Listeria monocytogenes* LO28 wild type and two stress-resistant variants[a]

	HHP			Heat		
	Wild type	***ctsR* variant**	**Immotile variant**	**Wild type**	***ctsR* variant**	**Immotile variant**
Parameters	**(biphasic) linear**			**(biphasic) linear with shoulder**		
$N_{0,measured}$ (log CFU/ml)	9.4			9.7		
$N_{0,extrapolated}$ (log CFU/ml)	10.3	10.0	9.6	9.7	9.6	9.6
D_1 (min)	1.5	7.7*	16.3**	0.86	3.1**	4.5**
D_2 (min)	7.1			7.6		
f_{res}	2.1 x 10^{-5}	6.5 x 10^{-6}	1.0 x 10^{-7}	1.6 x 10^{-4}	6.5 x 10^{-6}	1.0 x 10^{-7}
Sl (min)				3.6	7.8***	5.2

[a]Cells were treated under conditions of 350 MPa, 20°C (HHP) or at 55°C (Heat) in ACES buffer. $N_{0,extrapolated}$ is the model-based fit amount of cells at t_0. $N_{0,measured}$ is the measured amount of cells at t_0. *D* is the decimal reduction time, where D_1 and D_2 are the *D*-values of the first and second part, respectively, of the biphasic linear inactivation curve. f_{res} is the fraction of resistant cells in the population based on $N_{0,measured}$ for the wild type, where the f_{res} given for the wild type is the total fraction of resistant cells (both stably and temporarily resistant) and the f_{res} given for each of the two variants is the fraction of that specific variant in the wild-type population, assuming no shoulder for the two variants (see behavior in Fig. 1b) and taking into account the shoulder in the wild type by using $N_{0,measured}$. *Sl* is the shoulder length. *, statistical analysis ($P < 0.05$) showed that the *ctsR* variant's *D*-value for HHP was only higher than the *D*-value of the sensitive fraction of the population of the wild type. **, the *D*-value for HHP of the immotile variant and both variant's *D*-values for heat were significantly different from the *D*-values of the sensitive and resistant fractions of the wild-type population. ***, the shoulder length of the *ctsR* variant was higher than that of the wild type for heat inactivation.

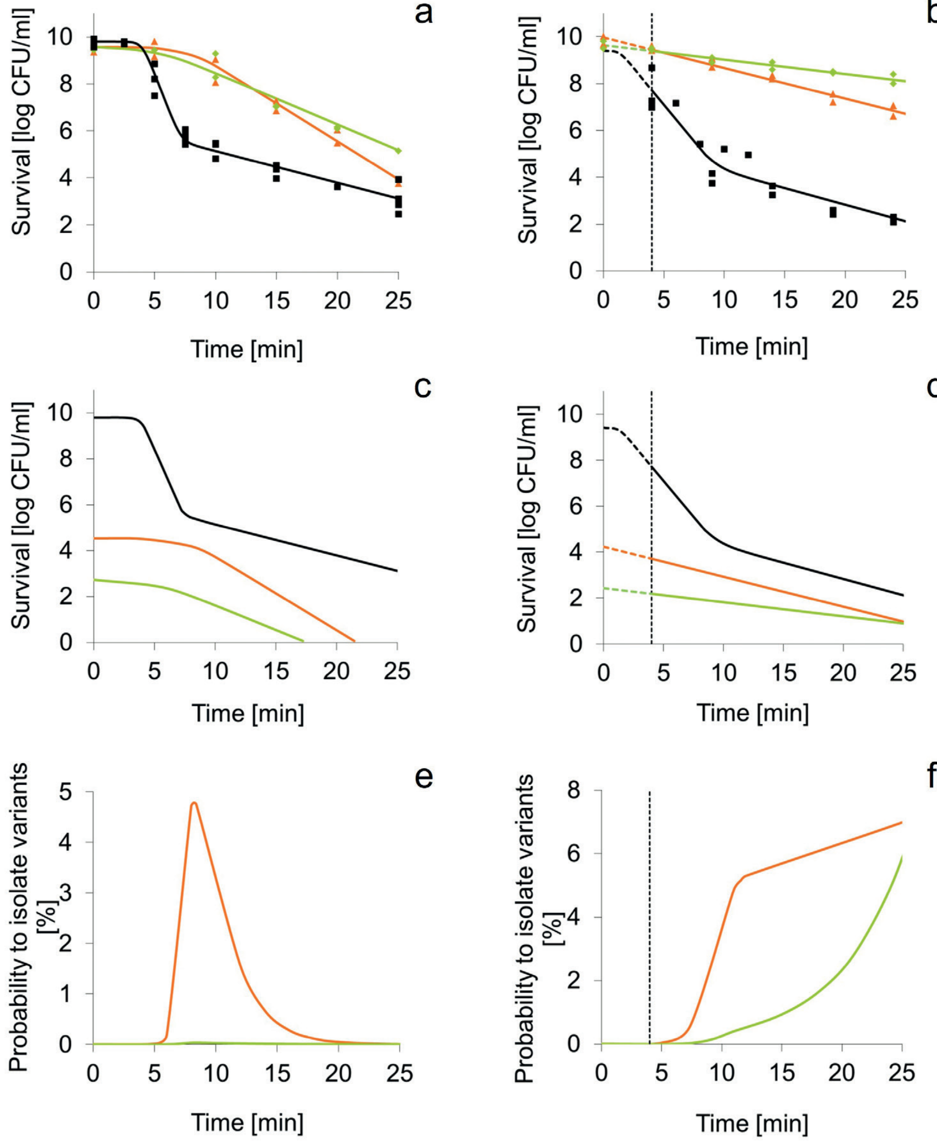

Figure 1. (a and b) Inactivation kinetics of *Listeria monocytogenes* LO28 WT (black), *ctsR* variant number 6 (orange), and immotile variant number 17 (green) after heat treatment of exponentially growing cells at 55°C in ACES buffer (a) or HHP treatment of exponentially growing cells at 350 MPa, 20°C in ACES buffer (b). The observed values are shown as dots, and the lines represent the model-based fit. The vertical line at 4 min, represents the time at which the temperature of the HHP vessel has returned to 20°C after starting the pressurization. Therefore, application of the model starts at 4 min for HHP. At $t = 0$ min, the measured amount of wild-type cells ($N_{0,measured}$) is 9.4 log CFU/ml, whereas the extrapolated amount of wild-type cells ($N_{0,extrapolated}$) is 10.3 log CFU/ml. (c and d) Predicted population inactivation of *Listeria monocytogenes* LO28 wild type, *ctsR* variant number 6, and immotile variant number 17 after heat treatment of exponentially growing cells at 55°C in ACES buffer (c) and HHP treatment of exponentially growing cells at 350 MPa, 20°C in ACES buffer (d). The number of cells at t_0 of both variants is based on their estimated frequency of occurrence in the initial population. (e and f) Probability (%) of isolating resistant *ctsR* or immotile variants in a population of *Listeria monocytogenes* LO28 after heat treatment of exponentially growing cells at 55°C in ACES buffer (e) and HHP treatment of exponentially growing cells at 350 MPa, 20°C in ACES buffer (f).

Estimation of the probability of detecting resistant variants based on kinetic modeling

HHP treatment at 350 MPa of *L. monocytogenes* LO28 inactivated a population of 2.6 x 10^9 CFU/ml to 184 CFU/ml in 24 min (32) (Fig. 1a). Characterization of these cells revealed 4.9 % immotile and 6.9 % stably resistant *ctsR* variants (31, 32). Based on these surviving cell counts (9 immotile and 13 *ctsR* variants per ml present after 24 min at 350 MPa), together with the *D*-values of the inactivation of both variants (Fig. 1a and Table 1) and $N_{0,measured}$, their individual fractions in the initial wild-type population can be calculated (equation 1 and 2). In a wild-type population of LO28, approximately 1 immotile and 65 *ctsR* variants are present per 10 million cells (Fig. 1c, and d, and Table 1). By combining these estimated fractions with the inactivation kinetics during a heat or HHP treatment, the probability of detecting each of these variants was calculated (Table 1 and Fig. 1e and f). The calculations implied that the probability of isolating a *ctsR* or immotile variant after 25 min at 350 MPa is 7 or 5 %, respectively. Using the same fractions of variants at *t*=0 derived from the HHP experiments, the predicted curve for heat inactivation implied that after 25 min at 55°C the probability of isolating one of these variants was close to zero. This fits well with our observation that stable heat-resistant variants were not isolated after treating 102 isolates for 25 min at 55°C.

The data in Fig. 1c and d illustrate the difference in inactivation by the heat and HHP treatments of the variants compared to the results for the wild type. When exposed to HHP, the *ctsR* variant had a higher *D*-value than the sensitive fraction of the wild type, whereas the immotile variant had a higher *D*-value than both the sensitive and the resistant fractions of the wild-type population. On the other hand, for heat inactivation, only the *ctsR* variant had a longer shoulder length than the WT. The variants' *D*-values were significantly higher than that of the sensitive but lower than that of the resistant fraction of the wild-type population. The differences in heat inactivation between the wild type and the variants indicated that the probability of isolating one of the variants from the resistant fraction should be highest after approximately 8 min (5 % for *ctsR* and 0.03% for immotile variants). At this time point, part of the wild-type population would be inactivated, in contrast to only small parts of the variants' populations. Therefore, we performed further research for the isolation of heat-resistant variants at 8 min at 55°C.

Isolation of HHP- and heat-resistant *L. monocytogenes* variants

To increase the chances of isolating resistant variants, the population surviving a treatment was regrown and exposed to heat. Due to the inactivation of sensitive variants, the fraction of resistant variants in this surviving population was increased from 10^{-6} to 10^{-5} to more than 5 x 10^{-2}. With another heat-challenge cycle, the surviving population would contain mostly heat-resistant variants. LO28 survivors of an 8-min treatment at 55°C were harvested, cultured, and retested in a heat challenge cycle. As a control, the LO28 survivors of a 24-min treatment at 350 MPa were harvested, cultured, and retested in an HHP challenge cycle as well. For both HHP and heat, these stress challenge cycles selected for resistant variants

(Fig. 2a and c). The previous isolation of HHP-resistant LO28 variants were confirmed using the stress challenge cycle method. Furthermore, this method also made it possible to isolate for the first time heat-resistant variants of LO28 and HHP- and heat-resistant EGDe variants. Because of this specific selection method, we were able to detect a small fraction of cells with resistant characteristics in a large population (Fig. 2b and d).

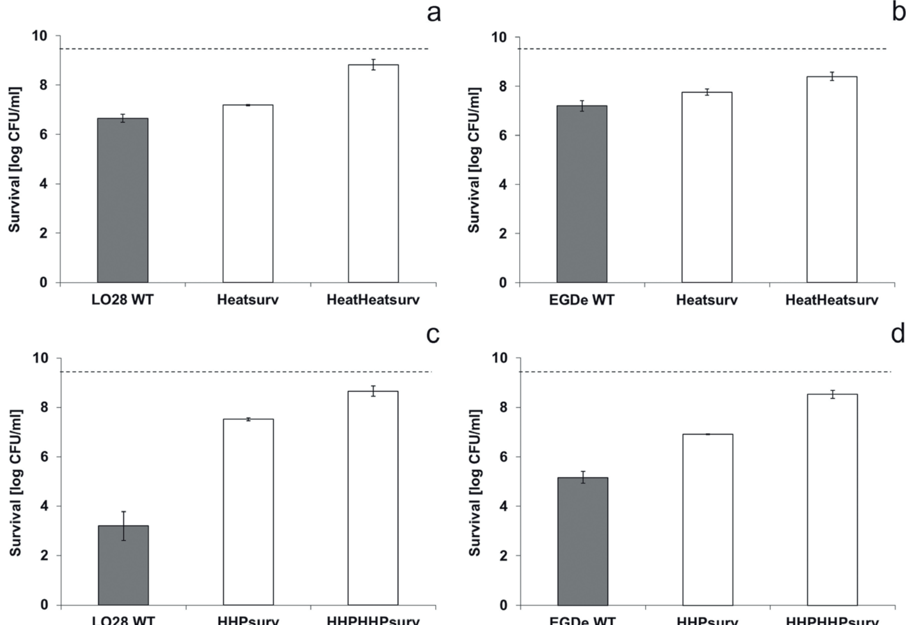

Figure 2. (a and b) Survival of exponentially growing *Listeria monocytogenes* cells in ACES buffer after 8 min at 55ºC for LO28 (a) and EGDe (b) wild type, their recultured survivors of the heat treatment (Heatsurv), and their recultured survivors of a similar subsequent heat treatment (HeatHeatsurv). (c and d) Survival of exponentially growing *L. monocytogenes* cells in ACES buffer after 24 min at 350 MPa for LO28 (c) and EGDe (d) wild type, their recultured survivors of the HHP treatment (HHPsurv), and their recultured survivors of a similar subsequent HHP treatment (HHPHHPsurv). The dotted lines indicate the initial level of cells (9.5 log CFU/ml). Each inactivation experiment was conducted at least two times on different days. The error bars show one standard deviation. Results in white bars are statistically different from the results for the wild type.

Alterations in *ctsR*

Previous research showed that a significant fraction of HHP-resistant variants had mutations in their *ctsR* genes. These *ctsR* variants comprised over 60% of Scott A HHP-resistant variants and almost 30% of LO28 HHP-resistant variants (19, 31). The newly isolated LO28 and EGDe HHP and heat variants were also tested for alterations in their *ctsR* genes and the related upstream regions. In all cases tested (for both strains under both inactivation conditions), *ctsR* mutations were found (Fig. 3). For LO28, 25% of the HHP isolates and 38% of the heat isolates were *ctsR* variants. The fraction of 25% HHP *ctsR* variants is comparable to the fraction of 29% that we found in our previous research (31). For EGDe 79% of the HHP isolates and 33% of the heat isolates were *ctsR* variants. In total 23 different mutations in

ctsR genes were observed: 2 nonsynonymous single nucleotide polymorphisms, 10 inserts (ranging in size from 1 to 912 bp), and 11 deletions (ranging in size from 3 to 590 bp). All inserted DNA appeared to be sequence repeats of *ctsR* at the point of insertion, except for one insert that was a transposase in the C-terminus of the *ctsR* gene. This mobile segment of DNA can insert into nonhomologous target sites, and in this case, it disrupted the *ctsR* gene, resulting in a truncated protein. Almost half of the mutations were found in the heat sensor domain, where a typical well-conserved glycine repeat (GGGG) is located (8, 20). One of these mutations is the CtsRΔGly found for LO28 under heat stress and previously isolated for LO28 and Scott A under HHP stress (20, 31). Karatzas *et al*. indicated in their research that the deletion of the glycine residue in the glycine repeat of CtsR resulted in a loss of the repressor function of this regulator (20). This was indicated by increased expression of CtsRΔGly protein in the mutant concomitantly with increased expression of the *clpP* gene and the *clpC* operon and with increased expression of ClpC and ClpP proteins.

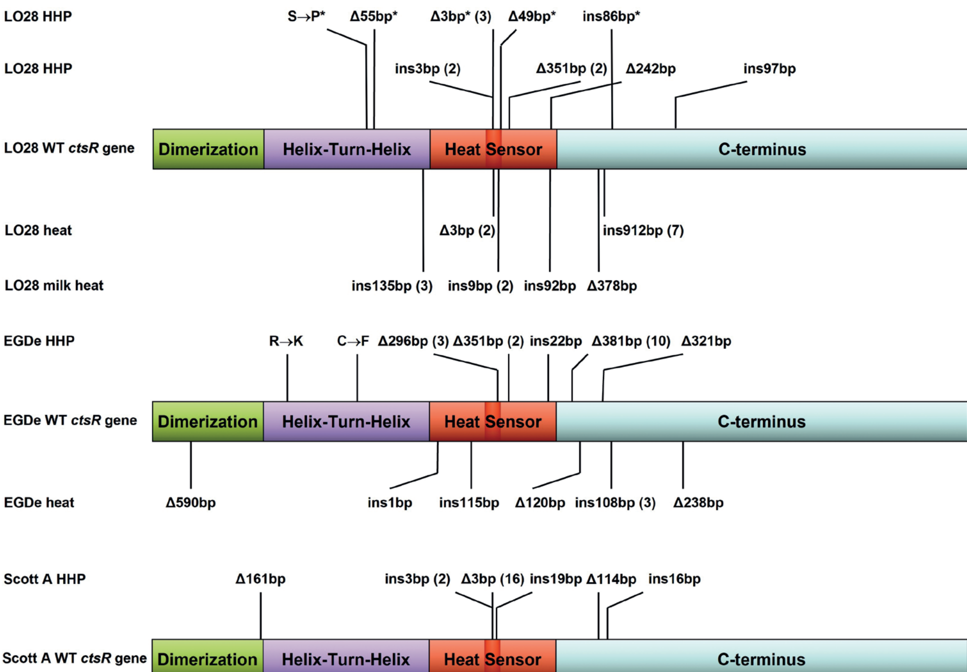

Figure 3. Observed variants of the *ctsR* genes of *Listeria monocytogenes* LO28, EGDe, and Scott A. The four putative functional domains of CtsR comprise an N-terminal dimerization domain (green), followed directly by a helix-turn-helix DNA-binding domain (purple), a central putative heat sensor domain (red), and a supposed C-terminal stabilization domain (7, 8). The glycine repeat is located in the center of the heat sensor. The position and number of base pairs inserted (ins) or deleted (Δ) are shown for all variants and inactivation conditions. *ctsR* variants were isolated after an HHP treatment of 24 min at 350 MPa (HHP), a heat treatment of 8 min at 55°C in ACES buffer (heat), or a heat treatment in UHT-processed whole milk of 6 s at 72°C (milk heat). Previously isolated LO28 *ctsR* variants are marked with an asterisk (31). Scott A *ctsR* variants were previously isolated by Joerger *et al.* (17) (Δ161bp) and Karatzas *et al.* (19) (ins3bp, Δ3bp, ins19bp, Δ114bp, and ins16bp). Some *ctsR* variants were isolated more than once under the same inactivation conditions, which is indicated in parentheses after the specific mutation.

The observed mutations for the LO28 and EGDe strains in the current study represented three major effects at the protein level: (i) in-frame, effecting a change in the glycine repeat; (ii) in-frame, effecting a change in the relative position of the C-terminal domain; and (iii) out-of-frame, effecting a truncation and loss of the C-terminal domain. It is conceivable that the observed changes will affect the function of CtsR and/or the stability of the protein, for example, by an altered interaction of the C-terminus (i and iii) or by modulation and/or loss of temperature sensing (ii). Such a loss of CtsR's repression function would result in induction of its regulon, as previously observed for *L. monocytogenes* Scott A AK01 (20), with concomitant activation of stress defense, resulting in increased stress resistance, as observed in this study.

***L. monocytogenes* heat-resistant variants in milk**

The presence of LO28 heat-resistant variants was also tested under industrially relevant conditions; i.e., by growth in ultra-high-temperature (UHT)-processed whole milk followed by the stress challenge cycle method at 72°C. Survivors of a 6-s treatment at 72°C were harvested, recultured in whole milk, and retested for their heat resistance and survival. The survivors of this second heat treatment were subsequently harvested, recultured in whole milk, and retested for their heat resistance under the same conditions (Fig. 4). From the survivors of the latter heat treatment, 10 isolates were randomly selected and tested for alterations in their *ctsR* genes. For 7 of the 10 variants, 4 different *ctsR* mutations were found (Fig. 3). For the other three variants, the *ctsR* genes and upstream regions were not altered. Analysis of the cultures of all three non-*ctsR* variants showed differences in colony sizes when plated on BHI agar. Only small colonies were found after the heat treatment, whereas both small and large colonies were found before the heat treatment. These small-colony variants appeared to be heat resistant, whereas the large colonies showed sensitivity to the heat treatment that was similar to that of the WT.

All 10 heat-resistant variants were also tested for their survival in whole milk for 15 s at 72°C, as advised by the FDA for pasteurization of whole milk (Fig. 5). The calculated *D*-value of WT LO28 based on linear inactivation at this temperature was 1.01 ± 0.07 s (mean ± standard deviation), whereas the mean *D*-value of the *ctsR* variants was significantly different from that of the wild type and more than 2 times higher, 2.35 ± 0.13 s. Furthermore, the heat-resistant variants without mutations in their *ctsR* genes had a mean *D*-value of 3.02 ± 0.21 s, which was three times higher even than that of the wild type. By using the heat treatment of 15 s at 72°C, the wild type and *ctsR* variants will have a more than 5 log reductions. In contrast, the three non-*ctsR* variants did show reductions close to or even less than 5 log.

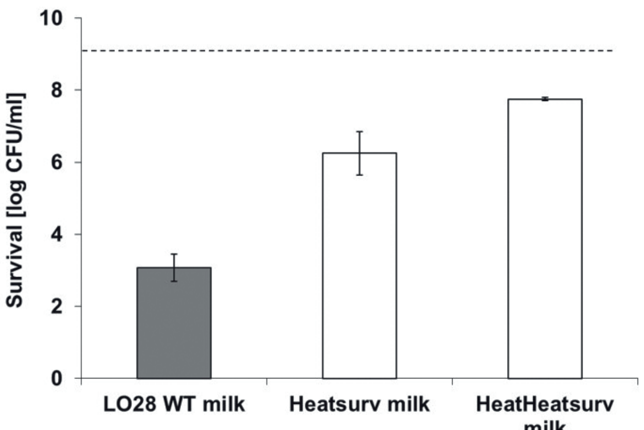

Figure 4. Survival (log CFU/ml) of exponentially growing cells in UHT-processed whole milk after 6 s at 72°C for *Listeria monocytogenes* LO28 wild-type cells, their recultured survivors of the heat treatment (Heatsurv), and their recultured survivors of a similar subsequent heat treatment (HeatHeatsurv). The dotted line indicates the initial level of cells (9 log CFU/ml). Each inactivation experiment was conducted at least two times on different days. The error bars show one standard deviation. Results in white bars are statistically different from the results for the wild type.

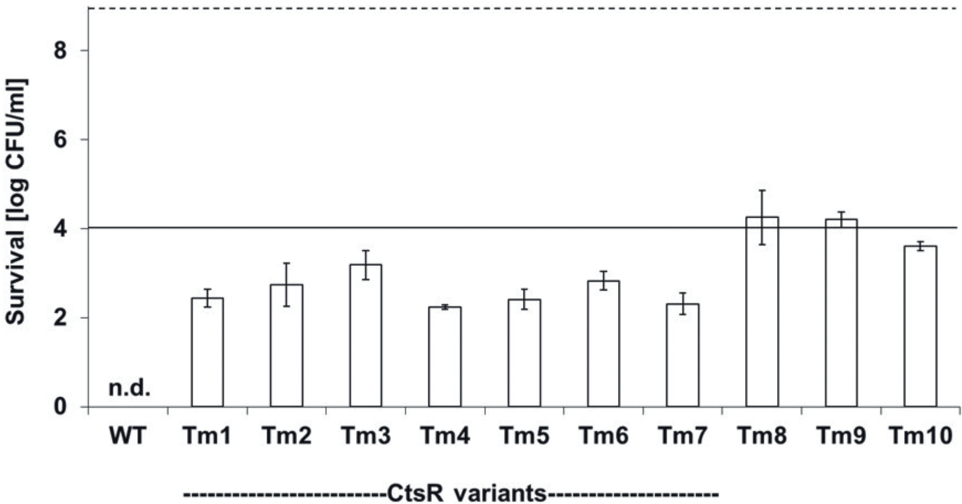

Figure 5. Survival (log CFU/ml) of exponentially growing cells in UHT-processed whole milk after 15 s at 72°C for *Listeria monocytogenes* LO28 wild type and 10 isolated heat-resistant variants. For LO28 WT, no cells could be detected after the heat treatment (n.d.). The first 7 heat-resistant variants are the *ctsR* variants. The dotted line indicates the initial level of cells (9 log CFU/ml). The solid line shows the standard 5 log reduction after 15 s at 72°C as used in industry and advised by the FDA. Each inactivation experiment was conducted at least two times on different days. The error bars show one standard deviation. Results in white bars are statistically different from the results for the wild type.

Discussion

The occurrence of stress-resistant variants in monoclonal populations of bacteria remains an important subject of debate, as some research groups have reported the isolation of such variants while others failed to demonstrate their presence and questioned their existence (1, 3, 5, 16, 17, 19, 24, 29, 31). In this study, we used a new strategy involving a kinetic modeling-based sampling scheme and an optimized stress challenge cycle method for the isolation of stress-resistant variants of *L. monocytogenes*. While our previous strategy led to the isolation of HHP-resistant variants for LO28 only (32), the new strategy allowed us to isolate such variants for EGDe also. In addition, the new strategy revealed the presence of heat-resistant variants within both *L. monocytogenes* LO28 and EGDe populations. By defining specific conditions, resistant variants could be isolated, although they represent only a small fraction (~10^{-7} to 10^{-5}) of the population. The isolation of both HHP- and heat-resistant variants of different *L. monocytogenes* strains illustrates that three factors play an important role in detecting these variants: first, the growth and resistance of the variants compared to their wild type; second, the frequency of revertance of variants to wild type; and finally, the chosen test conditions.

Most models of evolutionary processes imply that new variants establish in populations through mutation and selection. For each new generation, the bacterial DNA is replicated with a spontaneous mutation rate of about 0.0033 per genome. This number, however, is highly variable and depends largely on the circumstances (9). Variants with mutations that confer a competitive advantage in particular environments are selected (9). Taking into account the growth and resistance characteristics of *ctsR* variants, the sizes of the *ctsR* gene and the total genome of *L. monocytogenes*, and the mutation frequency as mentioned above, the chance of finding a mutation in the *ctsR* gene after the first inactivation step (~13 replications) is around 7×10^{-6}. This value is very close to the estimated fraction of approximately 6.5×10^{-6}. By using the optimized stress challenge-cycle method the chances of isolating *ctsR* variants will increase with each cycle based on their growth and stress survival (31). In line with previous observations, variants with mutations in *ctsR* are the most frequently isolated stress-resistant variants, and in the current study, 49 of 106 tested *L. monocytogenes* LO28 and EGDe variants under HHP- and heat-inactivation conditions appeared to fall into this class of variants. Mutations in *ctsR* were previously reported for Scott A and LO28 HHP-resistant variants (17, 18, 32) (Fig. 3). The heat stress regulator CtsR plays an important role in stress survival as repressor of class III heat shock genes. Alterations in CtsR can result in higher stress resistance, conceivably due to increased expression of the *clp* genes in the absence of the (active) CtsR repressor (20). Furthermore, the growth of previously characterized *ctsR* variants was comparable to the growth of the wild-type *L. monocytogenes* LO28 (31). As a result, *ctsR* variants are able to compete with other variants and wild-type cells, which can direct specific selection.

In previous research with *L. monocytogenes* Scott A, six different mutations in *ctsR* were found (17, 19). In this study, using *L. monocytogenes* LO28 and EGDe, 23 different mutations in *ctsR* were observed. Most of these mutations were located in or near the heat sensor domain of the gene (8, 20). The origin of these mutations could be a hot spot mutation sequence, like the *Firmicutes* consensus chi sequence 5'-(A/C)GCG(G/T)-3' (14). Analysis of mutation hot spots in the *ctsR* gene revealed no overrepresentation of this consensus chi sequence (data not shown). Three *ctsR* mutations might result from strand slippage of the DNA polymerase in the GGT repeat region in the heat sensor domain. These three different mutations occurred only in the LO28 strain. None of the EGDe *ctsR* variants resulted from slippage in this repeat region. This contingency locus has previously been shown to be responsible for a high occurrence of mutations in *L. monocytogenes* Scott A (19). Another interesting possibility for the occurrence of *ctsR* variants is revealed by inspecting the genome context of *ctsR*. This context is conserved between *Bacillus* and *Listeria*. The two genes *radA* and *yacK* (lmo0234), which are located immediately downstream of the *clpC* operon (*ctsR* - *mcsA* - *mcsB*- *clpC*), were transcribed simultaneously with this operon under conditions of oxidative stress, indicating a strong relationship (28). Evidence for the involvement of both proteins in DNA repair and competence was obtained by phenotypic studies of mutants with changes in these two genes (23). CtsR seems to be functionally related to RadA, a recombination protein. RadA is required for the efficient repair of certain forms of DNA damage and for genetic recombination and might play a role in the stabilization and/or processing of Holliday junction intermediates (4). The role of these factors in the generation of diversity in *L. monocytogenes* remains to be elucidated and is currently being targeted in our laboratory.

The other half of the 106 *L. monocytogenes* variants were not mutated in their *ctsR* genes and upstream regions. The origin of resistance of these 57 variants is unknown. The underlying mechanisms of increased resistance of selected variants will be investigated in future research by comparative transcriptome analysis and genome sequencing. Three resistant variants belonging to this group were isolated after a heat treatment in milk. These variants showed different colony sizes on BHI agar before the heat treatment, and after heat inactivation, only small colonies could be recovered. Retesting the normal-sized colonies for their heat resistance revealed that these variants had reverted to the wild-type phenotype (data not shown). Future research will not only focus on the origin of mutation but will also include research into the revertance of variants to the wild-type phenotype. Revertance of resistant variants can give rise to persistence because of relatively high-frequency switches between phenotypes and genotypes (12).

The probability of detecting resistant variants depends on their frequency in the population, phenotypic characteristics (growth and resistance) and revertance. We found that the frequency of the *L. monocytogenes* HHP- and heat-resistant variants is below 10^{-5}. Moreover, some previously isolated variants showed slower growth than their wild type (18, 31). Both factors make it difficult to isolate variants directly from the wild-type population.

We showed that kinetic modeling can be used to find the appropriate experimental conditions with the highest probability of detecting resistant variants (Fig. 1). Furthermore, the high frequency of revertance to a wild-type phenotype of some of the isolated resistant variants might explain the difficulty of isolating resistant *L. monocytogenes* variants, as reported before by various research groups (5, 16).

The generation of population diversity and selection of stress-resistant variants will contribute to the survival of *L. monocytogenes* under constantly changing environmental conditions. In fact, resistant variants were even isolated after a heat inactivation at 72°C in milk, and a fraction of these variants showed high resistance to standard pasteurization conditions. The pasteurization of whole milk is specified by the FDA to be 72°C for 15 s and is designed to achieve at least a 5 log reduction of the most heat-resistant nonsporulating pathogen, *Coxiella burnetii*, in whole milk. For *L. monocytogenes*, a 5 log reduction in milk at 72°C in 15 s corresponds to a *D*-value of 3 s. This value is close to the one found for the heat-resistant variants without alterations in their *ctsR* genes and upstream regions. These variants are so resistant that they might become persisters in food industry and, ultimately cause disease. Although outbreaks of listeriosis associated with pasteurized dairy products are rare, in one of the reported outbreaks in Massachusetts pasteurized milk was identified as the vehicle of listeriosis (10). In this case, no mistake in the pasteurization conditions could be identified, and questions were raised about the ability of the standard pasteurization process to eradicate *L. monocytogenes* from contaminated milk (2, 6, 10). In other cases, post-pasteurization contamination of product was identified as the cause of listeriosis (30). Various studies have indicated that certain strains of *L. monocytogenes* survive within the food-processing environment, and the persistence of such strains in a food-processing plant is of particular concern as they have the potential to act as a source of contamination of the processed product (22, 25, 26, 27). The possible generation and establishment of stress-resistant *L. monocytogenes* variants in food-processing environments remains a critical challenge to the food industry.

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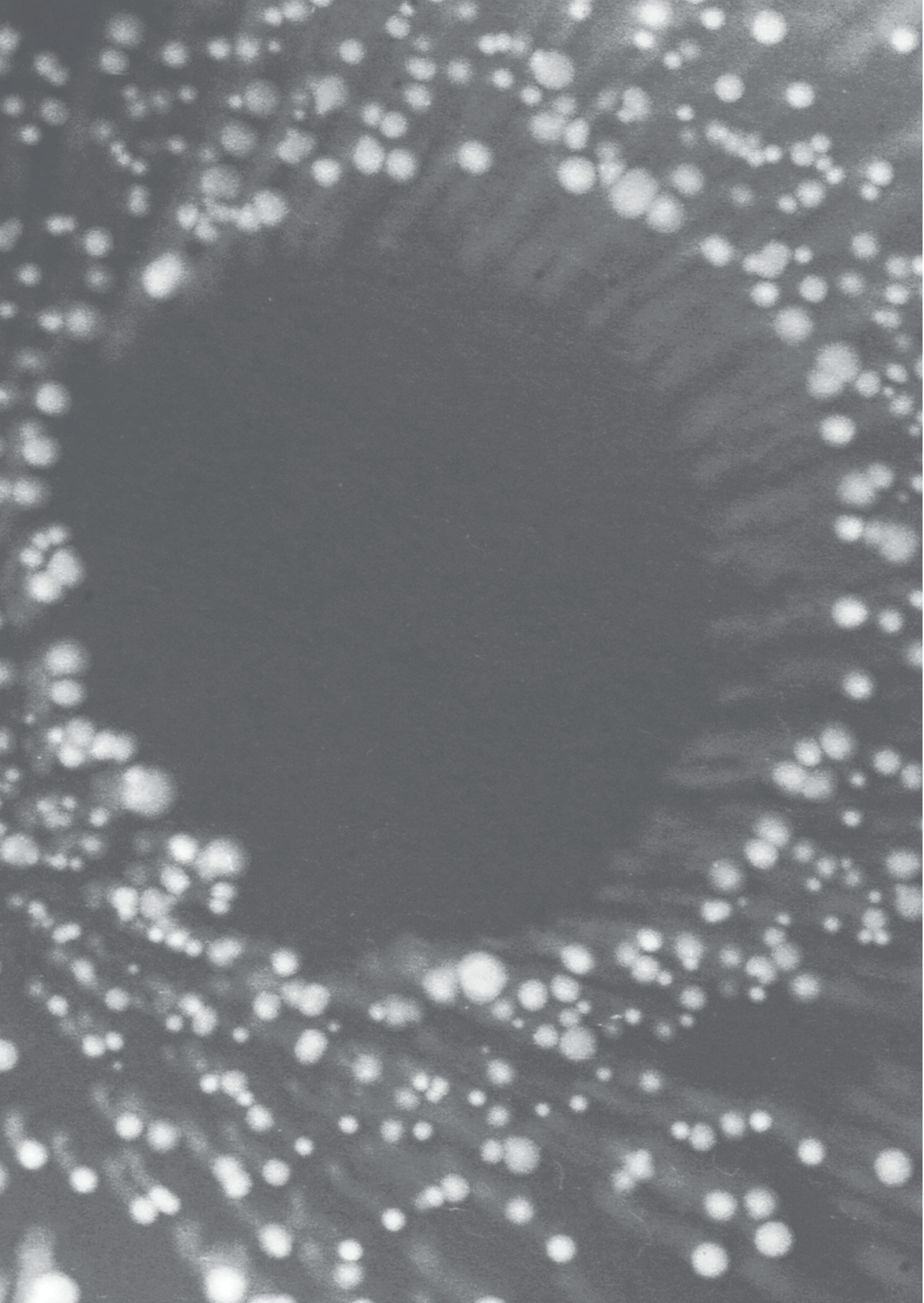
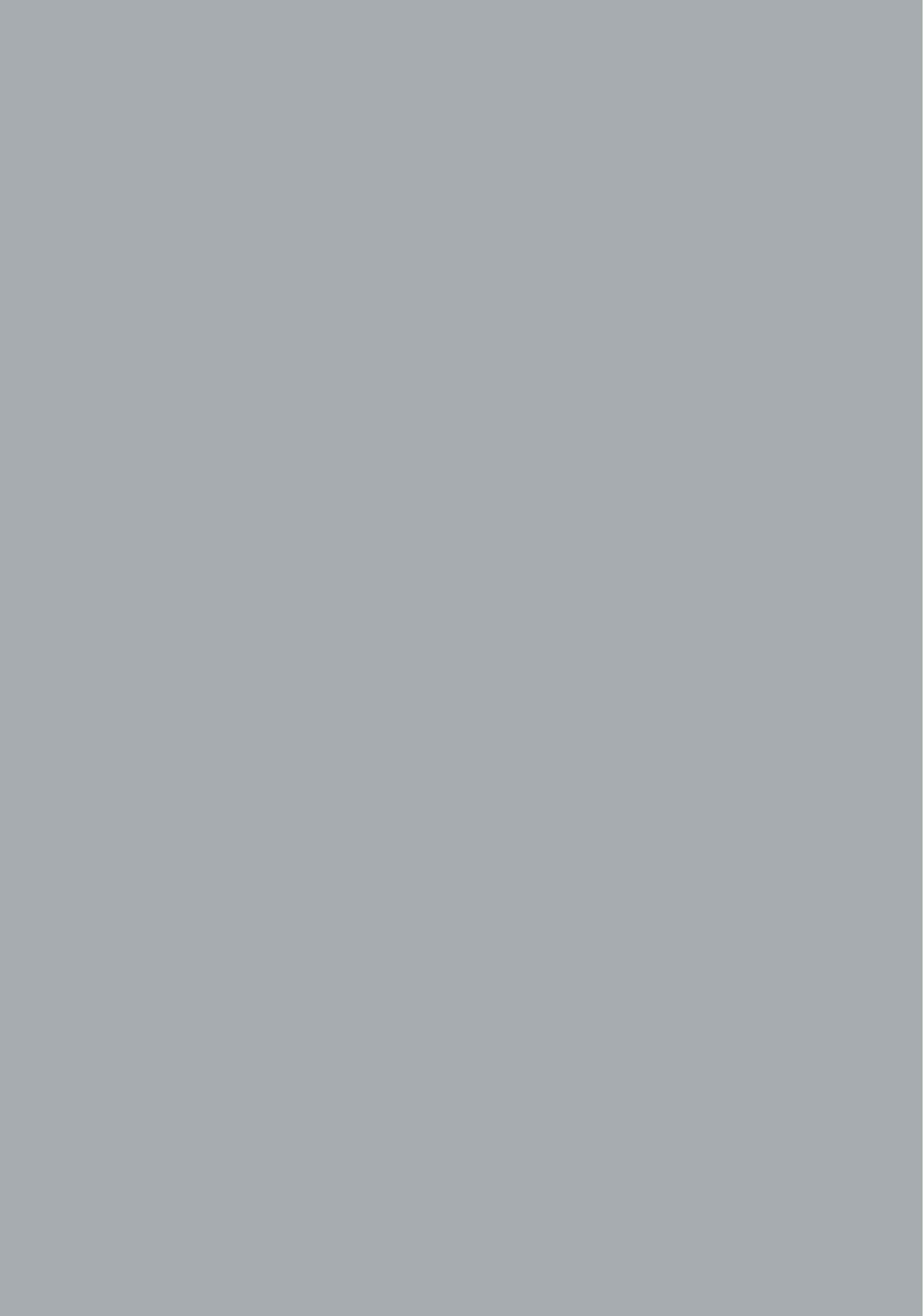


Chapter 6

General discussion and recommendations



General discussion and recommendations

Food preservation methods currently used by the industry rely either on the inhibition of microbial growth and/or on microbial inactivation. Efficient inactivation can be hampered by adaptive stress response and heterogeneity of bacterial populations. Such heterogeneity can provide a selective advantage during changes in environmental conditions encountered during the transmission of foodborne pathogens in the food chain. In some instances, heterogeneity exists at the genetic level, in which significant allelic variation occurs within a population seeded by a single cell resulting in phenotypic heterogeneity. In other cases, heterogeneity exists due to phenotypic differences within a clonal, genetically identical population (17, 44). Phenotypic heterogeneity within microbial populations arises even when the cells are exposed to putatively constant and homogeneous conditions. Microbial populations benefit by the emergence of phenotypic and genetic variants, especially if they have the potential to be better equipped to survive stress and to grow in new niches (5, 9).

6

This thesis describes the isolation and characterization of stable stress resistant variants of the foodborne pathogen *Listeria monocytogenes* following high hydrostatic pressure (HHP) and heat stress and focuses on the largest subcluster composed of *ctsR* mutants. Previously, Karatzas *et al.* (32, 33) and Joerger *et al.* (30) discovered *ctsR* variants in *L. monocytogenes* Scott A after HHP treatment. Recently, also stably stress resistant *L. monocytogenes* variants isolated following lethal acid exposure have been characterized, with the largest subcluster of variants containing mutations in the *rpsU* gene, encoding small ribosomal protein 21 (37, 45, 46). Results obtained in the current study are described in Chapter 2, 3, 4 and 5 and additional results will be presented and discussed in the sections below.

The power of modeling in describing and detecting subpopulations

In this thesis, variant subpopulations were first described by kinetic modeling of HHP inactivation data of actively growing exponential phase cells of the foodborne pathogen *Listeria monocytogenes* (Chapter 2). These data revealed the presence of a sensitive and a resistant fraction within the populations of three different *L. monocytogenes* strains (LO28, EGDe, and Scott A). Further testing of isolated survivors showed that the latter fraction could be divided in a temporarily and a stable resistant population for Scott A and LO28, while for EGDe only temporarily resistant variants were found. Characterization of the isolated HHP stable resistant LO28 variants, showed that these variants were also more resistant to heat (Chapter 3). Using inactivation kinetics data of two of these stable HHP resistant LO28 variants, *ctsR* variant 6 and immotile variant 17, a method was developed to detect and isolate other stress-resistant variants by use of a kinetic modeling-based sampling scheme. With this method it was possible to isolate HHP and heat resistant variants of strain EGDe, for which previously no stable HHP resistant variants could be found. Furthermore, also stable heat resistant LO28 variants were isolated with the kinetic modeling-based sampling

scheme (Chapter 5). This method is built on the probability of finding resistant variants, which depends on the nature of the inactivation treatment and the time of exposure (Fig 1).

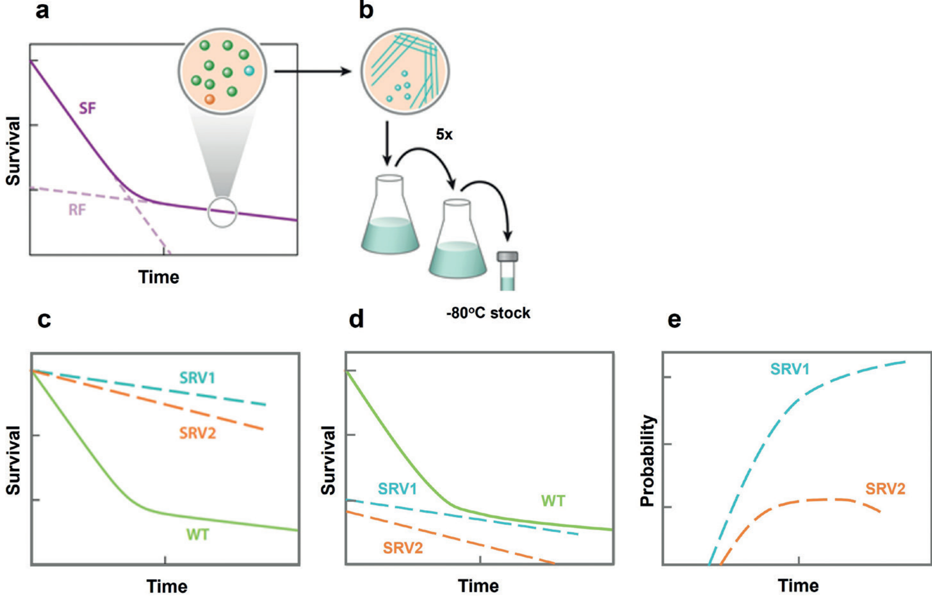


Figure 1. Schematic presentation of the strategy used to isolate stable stress-resistant variants. **a)** Upon exposure to stress, a sensitive wild-type (WT) fraction (SF) and a stress-resistant fraction (RF) can be identified, the latter composed of persister-type WT cells (green) and resistant variants (blue and orange). **b)** Approximately 100 colonies are randomly selected from the tail and inoculated in a fresh medium followed by repeated propagation, which provides stock cultures that are stored in the freezer. **c)** Stress exposure of cultures derived from these 100 stocks enables the identification and quantification of the number of stable stress-resistant variants (SRVs; represented by SRV1 and SRV2) that show enhanced survival compared to WT. **d)** Predicted population inactivation of *L. monocytogenes* WT, SRV1 (e.g. *ctsR* variant number 6), and SRV2 (e.g. immotile variant number 17) after stress treatment, based on their estimated frequency of occurrence in the initial population. **e)** Probability (%) of isolating these specific resistant variants in a population after stress exposure (Adapted from Abee (1)).

In the HHP and heat resistant populations a specific type of variant with mutations in the so-called *ctsR* gene could be isolated after exposure of *L. monocytogenes* LO28, EGDe and Scott A strains to subsequent HHP or heat treatments. This variant was discovered previously in *L. monocytogenes* Scott A after HHP treatment and isolated variants showed different mutations in this gene (30, 32, 33). Of all Scott A HHP resistant variants, over 60% were so-called *ctsR* variants (34). Comparative analysis of HHP resistant variants of *L. monocytogenes* LO28 and EGDe (Chapter 3, 5), showed that *ctsR* variants comprised approximately 25 and 80% of stable stress resistant variants, respectively. Next to HHP, also heat resistant variants could be isolated for LO28 and EGDe after an 8-min treatment at 55°C, and approximately 35% of these variants showed mutations in *ctsR* for both strains. For LO28, stable heat resistant variants were also isolated after 6-s treatment at 72°C in milk, of which 70% were *ctsR* variants (Chapter 3, 5).

Population diversity in *L. monocytogenes* can contribute to survival in adverse situations. In our study, stable HHP-resistant variants were analyzed for phenotypic and specific genetic traits. These results were cluster analyzed to obtain insight in population diversity. Three of the thirteen clusters together contained most variants, with the largest fraction composed of *ctsR* variants (Chapter 3). From each of these clusters one variant (5, *ctsR* variant 6, and 17) was selected for gene expression profiling to understand the mechanisms involved in piezotolerance. In the following sections the phenotypic characteristics of these three variants, as reported in Chapter 3 and 4, will be correlated to their gene expression and compared with micro-array data of the Scott A *ctsR* and LO28 *rpsU* variants.

Phenotypic characteristics

Comparison of the phenotypes of the three variants versus *L. monocytogenes* LO28 wild type, showed reduced growth rates under aerobic conditions at 30°C for all variants, reduced biofilm formation for variant 6 and 17 and reduced growth rates for variants 5 and 17 under aerobic conditions at 7°C. Under anaerobic conditions at 30°C growth rates were comparable to wild type, but biofilm formation was reduced for all variants. Small colony variants were found for variants 6 and 17, whereas variant 5 has similar colony size as wild type (Fig 2a, b). All variants were isolated after HHP treatment, they were not only resistant to HHP, but also to heat and low pH treatments (Fig 2c). Variant 17 was the most HHP resistant variant, whereas variant 6 was the most heat resistant one and both variants 5 and 6 were most resistant against low pH under exponential growth conditions. Overall, increased stress resistance of these variants comes with an impaired growth, either in broth or as biofilm under certain conditions.

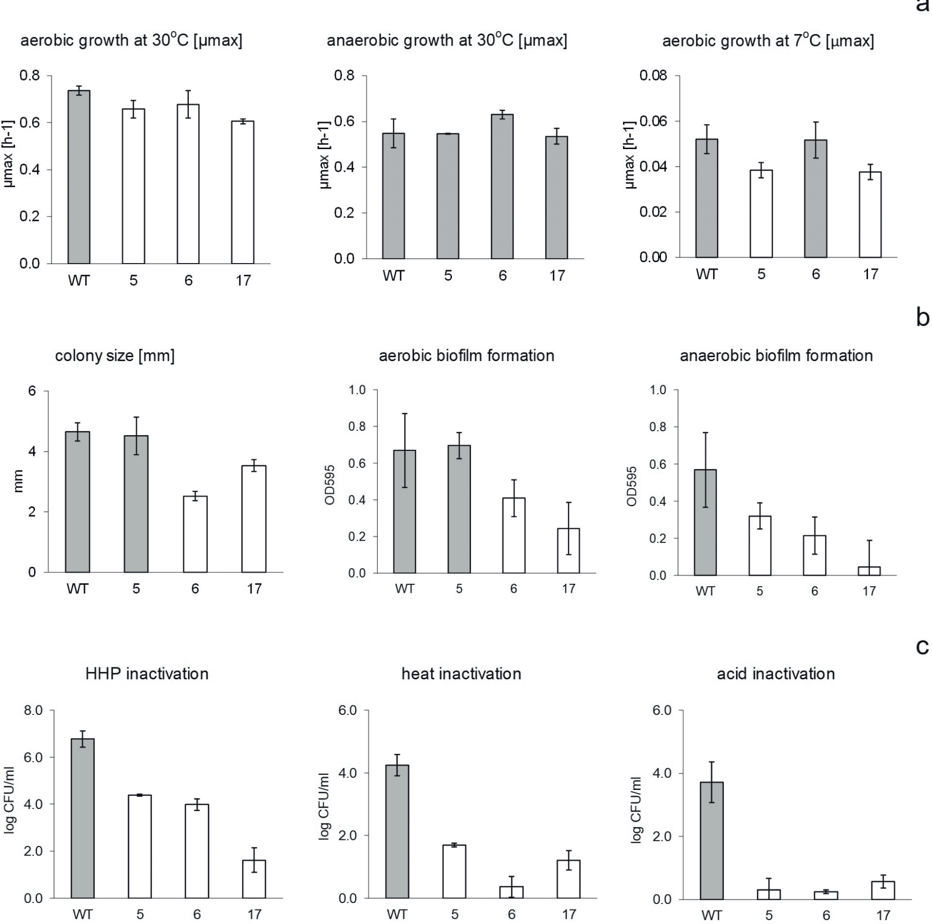


Figure 2. Characteristics of *L. monocytogenes* LO28 WT and variants 5, 6, and 17: **a)** maximum specific growth rate at 30°C under aerobic and anaerobic conditions and at 7°C under aerobic conditions; **b)** colony sizes determined on BHI agar grown at 30°C under aerobic conditions and biofilm formation under aerobic and anaerobic conditions at 30°C; **c)** inactivation of exponential growing cells at 350 MPa (20 min at 20°C), at 60°C for 1 min, and at pH 2.5 (3 min at 37°C). White bars represent significant differences compared to wild type.

Next to growth and resistance performance, also virulence related characteristics were tested: motility, haemolytic activity (phosphatidylcholine phospholipase C and listeriolysin O), and infection of spleen and liver in a mouse-model (Chapter 4). Only variant 17 showed no motility, no PC-PLC activity and reduced LLO activity. The other two variants showed similar *in vitro* virulence performance as the wild type. In the mouse model, both variants 6 and 17 showed reduced infection of spleen and liver, whereas variant 5 achieved similar levels of cells as wild type after intraperitoneally inoculation. Additionally, susceptibility to seven different antibiotics was tested (ampicillin, chloramphenicol, gentamicin, penicillin G, streptomycin, tetracycline, and vancomycin). Only variant 17 was more susceptible to all these antibiotics, the other variants behaved similar as wild type. With all this phenotypic

data a cluster analysis was performed, in which ratios were calculated with a value of 1 for wild type (Fig 3a).

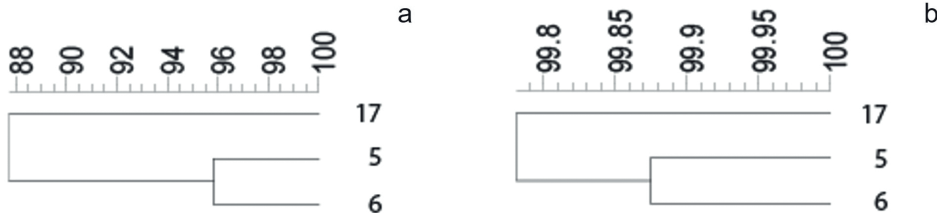


Figure 3. Cluster analysis of **a)** all fifteen phenotypic characteristics and **b)** all gene expression data of the three variants versus wild type.

6

This phenotypic cluster analysis shows a stronger relation between variants 5 and 6 compared to variant 17. To understand the mechanisms involved in multiple stress resistance of these three variants isolated following exposure to lethal HHP (350 mPa) a comparative gene expression analysis was performed (Fig 3b, discussed below).

Gene expression profiling

Gene expression data were obtained for exponentially grown cells of the variants in BHI at 30°C and compared with that of their wild type. These variants, that display a stable resistant phenotype, were isolated earlier under the same growth conditions after HHP treatment. The expression data were analyzed by KEGG, SWISS PROT and GENE ONTOLOGY CATEGORY databases (using FIVA) with cut-off of p <0.05 and >2.0-fold change. All gene expression data were used to perform a cluster analysis (Fig 3b). These data also show a stronger correlation between variants 5 and 6 versus variant 17.

For variant 5, variant 6 and variant 17 respectively 86, 232 and 242 genes were differentially expressed compared to the wild type. Notably, in total 25 genes showed significant differential expression in all three variants, including 12 induced and 13 repressed genes (Fig 4).

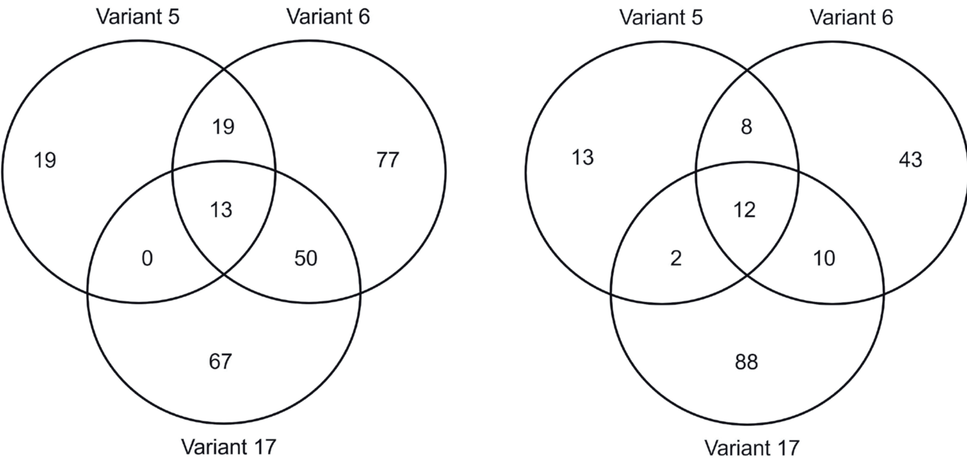


Figure 4. Comparison of differentially expressed genes between the wild type and variants 5, 6 and 17. The number of up- and down-regulated genes is indicated in the circles and the overlapping areas indicate that the same genes were up or down regulated in two or three of the variants.

Upregulated genes in all three variants

Twelve genes were positively regulated in all three variants (Table 1 and S6.1). Of these genes, nine genes are linked to the Class III heat shock genes regulated by CtsR. The other three genes are part of the Class I heat shock genes, regulated by HrcA. Overall, upregulation of CtsR and HrcA regulated genes conceivably underlies the observed HHP, heat and low pH multiple stress resistant phenotype of the three selected variants (Fig 2c).

Table 1. Overlap in differentially expressed genes in all three variants or in two of the three variants in comparison to wild type (blue color in Venn diagram). Upregulation is scored with +, downregulation with – and no change in expression by 0 (see text and supplementary material for details: Tables S6.1 to S6.4).

Venn diagram	5	6	17	Function	# Genes
Variant 5, Variant 6, Variant 17	+	+	+	Class III heat shock genes	9
	+	+	+	Class I heat shock genes	3
	—	—	—	Arginine biosynthesis	9
	—	—	—	Carbohydrate metabolism and transport	4
Variant 5, Variant 6, Variant 17	+	+	0	Various	8
	—	—	+	PTS systems and related genes	8
	—	—	0	PTS systems and related genes	7
	—	—	0	Nucleotide / carbohydrate metabolism	4
Variant 5, Variant 6, Variant 17	+	0	+	Class I heat shock genes	2
Variant 5, Variant 6, Variant 17	0	+	+	Class III heat shock regulator	1
	—	+	+	PTS system	1
	0	+	+	PTS systems	6
	0	+	+	Various	2
	0	—	—	Motility genes	40
	0	—	—	Amino acid metabolism	2
	0	—	—	PTS related systems	2
	0	—	—	Membrane proteins	2
	0	—	—	Various	4

Class III heat shock genes regulated by CtsR

Class III genes encode ATP-dependent proteins including ClpB, ClpC, ClpP, and ClpE that play a role in *L. monocytogenes* adaptive stress response and survival (66). The Clp protease system is a multi-component machinery responsible for protein homoeostasis, protein quality control, and targeted proteolysis of transcriptional regulators in prokaryotic cells (28). *L. monocytogenes* Class III heat shock genes are negatively regulated by CtsR. Hu *et al.* (27) identified at least 10 genes to be directly regulated by CtsR, as they show higher transcript levels in a *ctsR* mutant strain, as well as putative or confirmed CtsR-binding sites upstream of the gene or operon. The *ctsR-mscA-mscB-clpC* operon was confirmed as directly CtsR repressed. In our variants, these four genes were all upregulated, however, the *ctsR* gene itself was only significantly upregulated in variant 6 and 17. Due to a mutation in variant 6 (three bp deletion in the glycine repeat region in *ctsR,* CtsRΔGly) a non-active CtsR is being formed, which is conceivably not able to bind to DNA and to repress the expression of the CtsR regulated genes (Chapter 3, 33, 40). Variants 5 and 17 however do not have a mutation in the *ctsR* gene or promoter region (Chapter 3), still both variants show upregulation of similar genes that are upregulated in *ctsR* variant 6. Next to the *ctsR* operon

genes, also four other traditional class III heat shock genes were upregulated in all variants: *clpB*-lmo2205, *clpP*, and lmo1138, which encodes a protein similar to ClpP (65.4% amino acid similarity to *L. monocytogenes* EGD-e *clpP*). On the contrary, the *tatAC* operon, which is directly repressed by CtsR, encoding a putative twin argenine translocase secretion system, was only significantly upregulated in variant 6.

We also found significant upregulation of two other genes in all three variants: lmo1137 and lmo0997. The gene lmo1137 (with unknown function) is positioned next to gene lmo1138, that encodes a putative ATP-dependent Clp protease proteolytic subunit, of which Hu *et al.* (27) showed direct regulation by CtsR. Our result suggests that the expression of these two genes is co-regulated. The other upregulated gene, lmo0097, is identified as *clpE*, a gene that was previously reported to be CtsR repressed (49).

Class I heat shock genes regulated by HrcA

Genes of class I are overexpressed during the accumulation of denatured proteins in the cytosol and act as intracellular chaperones. The proteins GroEL and GroES (regulation of basic cellular processes) and DnaK, and DnaJ (stabilization of the conformation of unfolded proteins) are the main chaperones protecting *L. monocytogenes* against heat stress (66). HrcA directly represses a total of eight genes located in the two operons *groES-groEL* and *hrcA-grpE-dnaK-dnaJ*-lmo1471-lmo1470. For both operons a putative upstream HrcA binding site was identified following DNA sequence analyses (26). In our variants, only *dnaK*, *grpE* and *hrcA* were significantly upregulated compared to wild type. The operon with the genes *groEL* and *groES* was only upregulated in variant 5 and 17. The obtained results may point to additional levels of control of stress defence proteins, and possible regulatory networks are discussed in the next section.

Regulatory networks in *Listeria monocytogenes*

A number of studies have described considerable overlap among regulons and cross-connections between transcriptional regulators SigB, SigH, SigL, SigC (four alternative sigma factors), PrfA (virulence gene regulator), CtsR, HrcA, AgrA and CodY (12, 20, 22, 26, 27, 42). In the three variants, both CtsR and HrcA regulated genes are differentially expressed compared to the wild type, except for *ctsR* of variant 5. No significant differential expression of the other regulators was observed, except for the upregulation in variant 6 and downregulation in variant 17 of AgrA (Table 4 and S6.5). *L. monocytogenes* contains a complete *agr* locus, comprising the four genes *agrB*, *agrD*, *agrC*, and *agrA.* In variant 17 also the other *agr* genes are downregulated, of which only *agrC* significantly. The two-component system AgrC/AgrA is co-transcribed, in which AgrA is the transcriptional regulator and AgrC the response component (4). Inactivation of the Agr system affects the ability of *L. monocytogenes* to form biofilms at 25°C and lowers its ability to generate infection in a murine model (57). Variant 17 showed the lowest ability to form biofilms under both aerobic and anaerobic

conditions and the lowest infection in the mouse model compared to the wild type and the other two variants (Chapter 3 and 4).

Sigma B is presumed to play a central role in these transcriptional networks. However, in our transcriptome data *sigB* is not significantly up or down regulated, and neither the other alternative sigma factors. SigB is a positive regulator of *hrcA* and both regulators downregulate *groES*. Data of the three variants showed upregulation of *hrcA*, while for variant 5 and 17 also upregulation of *groES* and *groEL* was observed. Besides, SigB is a positive regulator of lmo669 and dnaK, whereas *hrcA* is a negative regulator of these genes. In our variants lmo669 was not differentially expressed, whereas *dnaK* was upregulated in all variants. Based on these data, it is not clear what exact role SigB and HrcA play in the regulation of these genes as they seem to interact both with the Class I heat shock genes. SigB also interacts with Class III heat shock genes, resulting in upregulation of *mcsA*, *clpP* and lmo2205. In our variants these genes were also upregulated, next to other CtsR repressed genes that were upregulated, pointing to a more central role for CtsR versus SigB (Fig 5). Data of expression of our variants were also compared with the list of genes that are part of the SigB operon as described by Liu *et al.* (41). Of the 240 genes in *L. monocytogenes* LO28 that are regulated by SigB, only 11, 17 and 28 of these genes were regulated in variant 5, 6 and 17, representing 5, 7 and 12%, respectively (Table S6.6). This is in strong contrast to the *L. monocytogenes* acid resistant *rpsU* variants of which about 70% of the SigB dependent genes was upregulated, indicating a prominent role of SigB in activation of the multiple stress resistant phenotype of these variants (37). The shared differentially expressed (SigB dependent) genes in the three variants and *rpsU* variants 14 and 15 are presented in Table 2.

Table 2. Overlap in differentially expressed (SigB dependent) genes in variants 5, 6 and 17, and *rpsU* variants 14 and 15 in comparison to LO28 wild type (blue color in Venn diagram). Upregulation is scored with +, downregulation with – (for details see Table S6.6).

Venn diagram	Variants 5, 6, 17	*rpsU* variant	Function	#	Genes
Variants 5, 6, 17 / RpsU variant	+	+	Sigma B regulated genes	3	*gbuA, gbuB, gbuC / opuCA, opuCC, opuCD*
	+	+	Phage shock proteins	2	lmo2484,lmo2485
	—	—	Sigma B regulated genes	2	*gadT2, gadD2*
	—	—	Arginine biosynthesis	5	*argG, argH, arpJ,* lmo2251, lmo2252

Comparative analysis of the induced SigB dependent genes shows upregulation of osmolyte transport systems in all variants. In the three variants 5, 6, and 17 *gbuA*, *gbuB* and *gbuC* are upregulated. The Gbu transporter (glycine betaine porter II) can contribute to stress resistance by the uptake of the respective compatible solutes glycine betaine and carnitine, which have been shown to stimulate *L. monocytogenes* growth at low temperature and functions as osmoprotectants (10). In both *rpsU* variants 14 and 15 *opuCA, opuCC,* and *opuCD* are upregulated. OpuC, the product of the *opuC* operon, is an ABC transporter that has been

shown to transport carnitine in response to osmotic and cold stress (2). Two other genes also regulated by SigB, associated with the glutamate decarboxylase (GAD)-system, *gadT2* and *gadD2*, are both downregulated in *rpsU* variant 15 and the tested variants. The GAD-system exchanges extracellular glutamate for intracellularly produced gamma-aminobutyrate (GABA) under acidic conditions using the *gadT1* and *gadT2* antiporters. Intracellular glutamate can be decarboxylated into GABA by *gadD1*, *gadD2* and *gadD3* with concomitant consumption of a proton, thereby increasing the pH of the cytoplasm. Transcription of antiporter/decarboxylase pair *gadT1D1* and *gadD3* was not different compared to wild type, whereas transcription of *gadT2D2* was downregulated. The decarboxylase/antiporter system encoded by *gadT2D2* plays a central role in allowing survival under extreme acidic conditions; mutants lacking either the GadT2 antiporter or the GadD2 decarboxylase are highly sensitive to low pH (35). Although *gadT2D2* is downregulated, the tested variants still show better survival than their wild type after 3 minutes at pH 2.5 (Fig 2c). For the *rpsU* variants 14 and 15 downregulation was also described for *gadT2D2* and *gadT1D1*, but *gadD3* was upregulated in both variants, conceivably contributing to their acid resistance (37). Interestingly SigB regulated stress related genes lmo2484 and lmo2485 are also induced in *rpsU* and the tested variants. Furthermore, genes lmo2486 and lmo2487 (Table S6.9) are also upregulated, but not in the *rpsU* variants. These genes are part of one operon and code for putative bacterial phage shock proteins (Psp) which help cells to manage the impact of agents impairing cell membrane function (31). Notably, expression of lmo2484-2487 genes is conceivably controlled by the two-component system LiaSR and was previously reported to be induced following exposure of *L. monocytogenes* LO28 cells to the cell wall antibiotic cefuroxime (50). So far little is known about the Psp system in Gram-positive bacteria including *L. monocytogenes*, however in Gram-negative bacteria, i.e., *E. coli*, a range of conditions were identified in which Psp was induced, including alkaline shock, heat shock, and osmotic shock, and in biofilm cells and persister cells (31, 55).

Furthermore, in our transcriptome data *prfA* is not significantly up or down regulated. Genes under the positive control of PrfA comprise the genes in the operon of *prfA: hly, actA, plcA, plcB, mpl,* plus three additional chromosomal loci: the *inlAB* operon, *inclC* and *hpt* (25). Of these genes, only one gene is significantly downregulated (*hly*, coding for Listeriolysin O) in variant 5 and 6, but with ratios below 2 (-1.75 and -1.71 for variant 5 and 6 respectively). These data show no direct connection between the regulators PrfA, CtsR and HrcA.

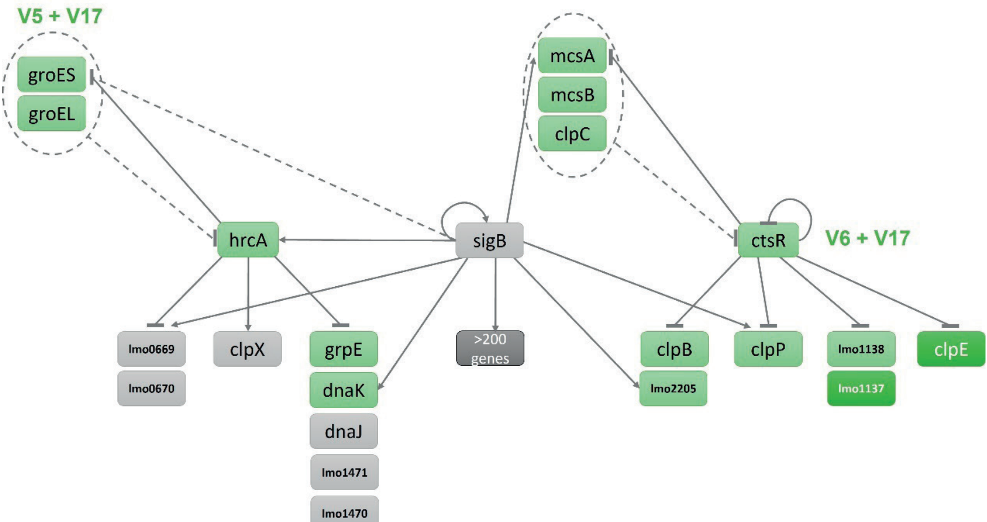


Figure 5. Regulatory networks of HrcA, SigB, and CtsR in *L. monocytogenes*. Green boxes represent genes that are upregulated, with comment means only for these variants, light gray boxed genes are not differentially expressed genes, dark gray box represents all other genes regulated by SigB. Dark green boxes are genes controlled by CtsR, but not shown in the model of Chaturongakul *et al.* (11, 12). Target arrows (↓) indicate positive regulation by a given regulator; target stops (⊥) indicate negative regulation by a given regulator. Loops indicate autoregulation. Solid lines indicate direct regulation of a gene by a given regulator and broken lines indicate indirect regulation. Figure adapted from Chaturongakul *et al.* (11, 12). See text for details.

HrcA is post-transcriptional regulated by GroES and GroEL and CtsR is post-translational regulated by McsA, McsB and ClpC, while GroE regulates HrcA activity (11, 12, 58). Under optimal growth conditions, the interaction of HrcA with GroE inhibits transcription of class I genes. At elevated temperature, unfolded proteins bind GroE contributing to HrcA inactivation and enabling binding of RNA-s32 polymerase with promoters and gene expression. At 37°C, CtsR is in an active confirmation stabilized by arginine phosphatase McsA, which results in class III gene repression. Mutations in *mcsA* would result in loss of repression of class III genes conceivably resulting in a similar phenotype as our *ctsR* variants. In stress conditions, including increased temperature, arginine kinase McsB is activated and phosphorylates CtsR resulting in an inactive conformation, preventing its binding with gene promoters thus interfering with its repressor function. As a result, RNA-s32 polymerase binds with promoters leading to gene expression, and arginine-phosphorylated CtsR is degraded by ClpC (66).

Down regulated genes in all three variants

Next to the 12 genes that were upregulated in the three variants, another 13 genes showed downregulation in the three variants (Table 1 and S6.1). Of the 13 downregulated genes nine genes are involved in the arginine biosynthetic pathway. These genes are regulated by the transcriptional regulator ArgR (lmo1367) by feedback inhibition. In the presence of arginine, ArgR represses transcription and expression of the *argGH* and *argCJBDF* operons

(13). Interestingly, the acid resistant *rpsU* LO28 variants also showed downregulation of five genes in the arginine biosynthetic pathway, *argG*, *argH*, *arpJ*, lmo2251, and lmo2252 (37). Previous studies on the role of ArgR in regulation of arginine synthesis and acid resistance in *L. monocytogenes* pointed to no or limited contribution to acid resistance (13, 59). Down regulation of the *arg* genes has no apparent negative impact on acid resistance in the tested variants and the *rpsU* variants. The exact role of ArgR in regulation of arginine biosynthesis in *L. monocytogenes* and putative effect on stress resistance remains to be elucidated.

WT and variants motility genes expression and phenotypes

Gene expression analysis in mid-exponential cells showed down regulation of genes involved in flagellar assembly and bacterial chemotaxis in variants 6 and 17, whereas variant 5 showed no differences in expression compared to wild type (Table 1 and S6.7). Scanning electron microscopy (SEM) images of liquid cultures at 30°C of LO28 WT and variant 17, showed presence and absence of flagella in WT and variant 17, respectively, in line with the gene expression data. Unfortunately, SEM images are not available for variant 5 and 6, but SEM analysis of another *ctsR* variant 8, showed either absence of flagella or reduced flagellation. Combined with the previous observation that the *L. monocytogenes* Scott A variant AK01 (non-functional CtsRΔGly) and a *ctsR* mutant show decreased flagella protein synthesis and no motility (33), it is conceivable that decreased flagella gene expression in tested *ctsR* variant 6 is indeed linked to loss of motility in the tested condition. Notably, *L. monocytogenes* LO28 *rspU* variants 14 and 15 also show reduced expression of flagella genes, and correspondingly absence or very low levels of Fla proteins and absence of flagella in TEM pictures (37). Analysis of gene expression data provides no direct link to significant differential expression of the putative motility regulator MogR in motile and non-motile variants and WT (table S6.7), as *ctsR* variant showed downregulation of *mogR*, whereas this gene was not differentially expressed in variant 5 and 17. Combined with the previously provided evidence that MogR and other putative regulators of *flaA* expression do not contain CtsR binding sites (33), it is concluded that next to a putative role for MogR, additional mechanisms underlying flagellation status and motility in *L. monocytogenes* variants remain to be elucidated. Impact of presence/absence of motility phenotype in combination with other relevant phenotypes, e.g., biofilm formation and virulence, of tested variants and WT is discussed in the section risk assessment.

Comparison with other isolated *L. monocytogenes* stress resistant variants

The frequently isolated *L. monocytogenes ctsR* variants, contain mutations in the *ctsR* gene or its promotor region (Chapter 3, 5). 29%-79% of HHP-resistant variants in different *L. monocytogenes* strains comprised *ctsR* variants. Almost half of these mutations were found in the heat sensor domain of *ctsR*, where a typical well-conserved glycine repeat (GGGG) is located. One of these CtsRΔGly variants is variant 6. In other research by Liu *et al.* (40) a spontaneous pressure-tolerant *ctsR* variant in Scott A was isolated and further investigated

by micro-array to study gene expression under pressure (450 MPa, 3 min). A total of 73 genes were differentially expressed in this variant compared to its wild type under pressure treatment. Compared to our *ctsR* variant 6, only 38% of gene regulation was similar. Of the 7 genes upregulated in both variants, 5 were CtsR regulated, and 2 genes coded for ABC transporters. Of the downregulated genes 16 of the 19 genes that both variants had in common were regulated by MogR (Table 3 and S6.8).

Table 3. Selection of comparable gene expression of Scott A *ctsR* variant under high pressure (450 MPa, 3 min) and LO28 *ctsR* variant 6 in comparison to their wild types (blue color in Venn diagram). Upregulation is scored with +, downregulation with –. Data for Scott A *ctsR* variant extracted from Liu *et al.* (40), details shown in table S6.8.

Venn diagram	Scott A *ctsR* variant	LO28 Variant 6	Function	#	Genes
Scott A CtsR / LO28 Variant 6	+	+	CtsR regulated genes	5	*ctsR, mcsA, mcsB, clpC, clpE*
	+	+	ABC transporter	2	lmo2214, lmo2215
	–	–	MogR regulated genes	16	*mogR, flgL, flgB, flgC, fliS, fliF, fliG* and others

Notably, the Scott A variant showed no upregulation of HrcA regulated genes, although also this stress mechanism seems to play an important role in the LO28 *ctsR* variant. The Scott A *ctsR* variant was less virulent, immotile, HHP, heat and acid resistant. Furthermore, the cells lacked flagella and were 5-10-fold longer. Our LO28 *ctsR* variant 6 showed similar characteristics, although the cells were motile at 25°C in semi-solid medium, furthermore scanning electron microscopy images of a LO28 *ctsR* variant showed similar cell appearance as wild type but less to no flagella (Chapter 3). Other mutations, than the one in *ctsR*, in both variants and strain differences might explain the variations in phenotype and transcriptome data.

Interestingly, all CtsR repressed genes higher expressed in *ctsR* variants, were also upregulated in variants 5 and 17, that have an intact *ctsR*. Therefore, the gene-expression profile cannot reveal the origin of mutation, as a large number of genes were differentially expressed, either as a primary or as a secondary effect. To get more insight in the genetic differences a full genome sequence of the variants and the wild type might elucidate the (additional) underlaying mutation(s) for the different phenotypes of the isolated variants. Such an approach was used for the acid resistant *L. monocytogenes* variants isolated by Metselaar *et al.* (46), in which whole genome sequencing revealed mutations in *rpsU*, encoding ribosomal protein S21 (RpsU) in the largest phenotypic cluster. Additional studies by Koomen *et al.* (37) showed multiple stress resistance of *rpsU* variants 14 and 15 to be correlated with activation of appr 70% of general stress sigma factor SigB-dependent stress defence proteins. In micro-array data of stress resistant variants 5, 6 and 17, *rpsU* was not differentially expressed compared to the wild type and only a small part of the genes regulated by SigB were differentially expressed. The data of our multi stress resistant variants

pointed to a prominent role of CtsR and HrcA regulated genes in contrast to that of SigB in *rpsU* variants. The use of lethal HHP, heat, or acid stress, apparently selects for variants that deploy other (combinations of) stress defence factors contained in *L. monocytogenes* than general stress sigma factor SigB. Among the other, unidentified variants from our research, Metselaar *et al.* (47) identified also *rpsU* variants. This variant was found at a low abundance in *L. monocytogenes* LO28 after HHP and heat treatments, respectively 8 and 4%. Whereas, after acid treatment of this strain, *rpsU* variants were isolated at a level of 50%, but no *ctsR* variants were found (46, 47). To gain more insight into the impact of the mutation(s) of variants 5, 6, and 17, additional analysis of genes uniquely differentially expressed in these variants will be discussed in the next section.

Specific regulated genes in one variant compared to the other variants and wild type

The unique regulated genes of variants 5, 6, and 17 are listed in Tables S6.9-6.11 and the COG classes of these genes are compared in Figure 6, whereas Table 4 gives an overview of the discussed genes in this section.

Table 4. Gene expression of specific genes in the variants in comparison to wild type (blue color in Venn diagram). Upregulation is scored with +, downregulation with – and no change in expression by 0 (see text and supplementary material for details: Tables S6.9 to S6.11).

Venn diagram	5	6	17	Function	# Genes
Variant 5 (blue), Variant 6, Variant 17	+	0	0	Phage shock proteins	4
	+	0	0	Unknown (*dgt*-lmo2568)	2
Variant 5, Variant 6 (blue), Variant 17	0	+	0	Class III heat shock genes	2
Variant 5, Variant 6 (blue), Variant 17 (blue)	0	+	—	Regulator	1
	0	—	+	Prophage genes	13
	0	—	+	Prophage genes	15
	0	—	+	Maltose/maltodextrin utilization	6
Variant 5, Variant 6, Variant 17 (blue)	0	0	+	Cytochrome aa3-type menaquinol oxidase	4

Variant 5 showed the least number of regulated genes of the three variants, in which most downregulated genes [11] play a role in carbohydrate metabolism and transport (COG class G) and most upregulated genes [6] are related to transcription. In the group of upregulated genes, operon 441 (lmo2484-lmo2487) coding for Phage shock proteins is already discussed in paragraph 'Regulatory networks in *Listeria monocytogenes*', two other interesting genes (no COG class assigned) are lmo2567 and lmo2568 that are both highly upregulated (+8.8 and +7.2 respectively) and regulated by LiaSR. Nielsen *et al.* (50) presented in total 9 LiaSR regulated genes differentially expressed in LO28 wild type after growth in the presence of cefuroxime. Remarkably, all these genes were also significantly upregulated in variant 5. Of the 27 genes known to be regulated by LiaSR in *L. monocytogenes*, 14 genes had significantly higher transcript levels in variant 5, while *liaS* and *liaR* were not differentially expressed (Table S6.9) (19). The gene, lmo2567 (*dgt*) codes for a deoxyguanosine triphosphate triphosphohydrolase-like protein (dGTPase), that hydrolyzes dGTP to deoxyguanosine and tripolyphospate. Deletion of *dgt* in *Escherichia coli* creates a mutator phenotype, indicating that the dGTPase has a fidelity role, possibly by affecting the cellular dNTP pool, whereas overexpression of *dgt* results in a decrease in the dGTP level (63). For *L. monocytogenes* the exact role of *dgt* still remains to be determined. The second gene, lmo2568, codes for a putative histone acetyltransferase (HAT) Hpa2. The expression of the two genes seems to be related, as both genes were downregulated after exposure to ultra-violet blocked pulsed light in *L. monocytogenes* (64). The exact role of both genes, and if they play a role in the specific phenotype of variant 5 is unknown.

Variant 6 with a Gly mutation in *ctsR*, CtsRΔGly, showed a large number of significantly down- and upregulated genes (26 and 9 genes respectively) with putative functions in carbohydrate metabolism and transport (COG class G). Another group showing significant downregulation is the group of genes (33 genes) of which no COG could be assigned, but an apparent 26 classify as prophage genes encoding bacteriophage A118 proteins (43). The impact of prophage carriage and activation on *L. monocytogenes* LO28 environmental transmission remains to be elucidated. Other downregulated genes (lmo2121-lmo2126) code for the maltose/maltodextrin utilization system. Gopal *et al.* (21) constructed insertion mutants of the genes encoding a subunit of the maltodextrin permease (lmo2123), the maltogenic amylase (lmo2126), the transcriptional regulator (lmo2128) and the ATPbinding protein (lmo0278). All mutants showed normal growth on glucose but were unable to utilize maltose or maltodextrin. Two mutants (Ins2123 and Ins2126) were tested for multiplication in Caco-2 cells, which was similar as wild type. In wild type all genes within the cluster lmo2121-lmo2128 were not transcribed in Caco-2 cells, confirming that these genes have no role during the intracellular phase of a *Listeria* infection (21). It is conceivable that downregulation of this cluster of genes is not associated with LO28 and Scott A *ctsR* variant phenotypes including their reduced virulence.

Compared to variant 5 and 6, variant 17 showed most unique regulated genes, in which most upregulated genes [32] play a role in carbohydrate metabolism and transport (COG class G) and most downregulated genes [9] are related to inorganic ion transport and metabolism. Among the upregulated G class genes, next to the maltose/maltodextrin utilization system, also systems with putative roles in transport and metabolism of ribose, mannose, fructose, cellobiose and mannitol were upregulated, which suggests a shift in metabolism to other sugars than glucose. Another interesting operon fully upregulated is cytochrome aa_3-type menaquinol (QoxAB) oxidase (lmo0013-lmo0016). *L. monocytogenes* has two terminal oxidases, a cytochrome *bd*-type (CydAB) and a cytochrome aa_3-type menaquinol (QoxAB) oxidase, and both are used for respiration under different oxygen tensions. CydAB oxidase is essential for aerobic respiration in air and intracellular replication, whereas the QoxAB oxidase is more important for growth under conditions of low oxygen and is not required for intracellular replication (16). All genes of the cytochrome bd-type are downregulated in variant 17, of which only *cydB* is significantly downregulated (-2.20), this is in line as discussed by Corbett *et al.* (16). It is not clear what underlies the activation of the qoxAB oxidase genes and whether this affects variant 17 respiration capacity and/or metabolism resulting in reduced growth performance compared to that of variant 5 and 6 (Fig 2a). Another large group of upregulated genes [18] are not assigned to any COGs, with 15 of these genes encoding putative bacteriophage A118 proteins, while downregulation of A118 genes was observed for variant 6. An induction of prophage gene expression after acid stress exposure has been reported for the *L. monocytogenes* 10403S A118 prophage and *lmaDCBA* operon. Prophage induction in mixed populations might facilitate horizontal gene transfer, allowing the acquisition of novel genetic material. In addition, prophage induction might provide an advantage, mediating bacteria-bacteria competition by killing or inhibiting other strains in food production environments contributing to their persistence (24, 60).

It is noteworthy, that next to the variants described in this study, all other multiple stress resistant *L. monocytogenes* variants isolated so far, show reduced fitness during aerobic growth at 30°C (45). Concerning pathogen transmission from soil to host, a range of parameters including temperature, e.g. refrigeration (7°C), room (20°C) and human body temperature (37°C) are important determinants next to (multiple) stress resistance, biofilm formation capacity and virulence. These aspects will be addressed in the next section on risk assessment.

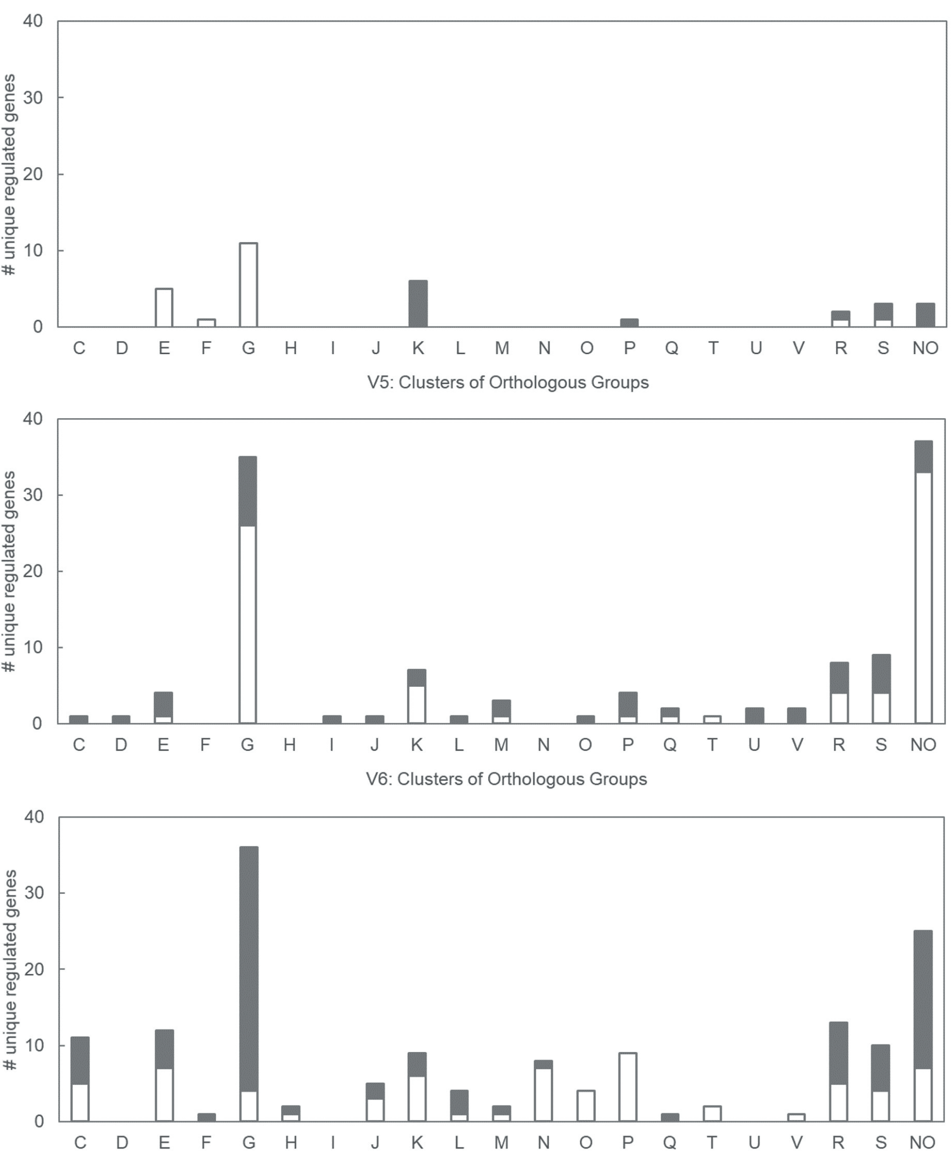


Figure 6. Unique differentially expressed genes in *L. monocytogenes* LO28 variants 5 (V5), 6 (V6) and 17 (V17) compared to wild type. COG (Clusters of Orthologous Groups) assignment of the number of upregulated (closed bars) and downregulated (open bars) genes. C (energy production and conversion), D (cell cycle control and mitosis), E (amino acid metabolism and transport), F (nucleotide metabolism and transport), G (carbohydrate metabolism and transport), H (coenzyme metabolism), I (lipid metabolism), J (translation), K (transcription), L (replication and repair), M (cell wall/membrane/envelope biogenesis), N (cell motility), O (post-translational modification, protein turnover, chaperone functions), P (inorganic ion transport and metabolism), Q (secondary structure), T (signal transduction), U (intracellular trafficking and secretion), V (defense/virulence mechanism), R (general functional prediction only), S (function unknown), and NO (no COGs assigned).

Risk assessment of resistant variants

Listeria monocytogenes is an important foodborne pathogen that frequently causes food recalls and disease outbreaks with significant case numbers and a mortality rate of 20–30% worldwide (8). This organism can adapt, survive, and even grow over a wide range of food production environmental stress conditions such as temperatures, low and high pH, high salt concentration, ultraviolet lights, presence of biocides and heavy metals. Furthermore, this bacterium is also able to form biofilm structures on a variety of surfaces in food production environments which makes it difficult to remove and allows it to persist for a long time. This increases the risk of contamination of food production facilities and finally foods (54).

The characteristics of the three variants can be used to analyse their risk potential related to that of the parental strain and other *L. monocytogenes* strains, as previously reported and described by Abee (1). The risk potential depends on both the probability of exposure and the probability of illness. For the probability of exposure some hurdles encountered in a food processing facility are taken into account: heat survival and growth (in a biofilm and at low temperature) (Chapter 3). For the probability of illness, the parameters: survival at low pH, anaerobic growth, virulence and antimicrobial susceptibility were used (Chapter 3, 4) (Fig 7).

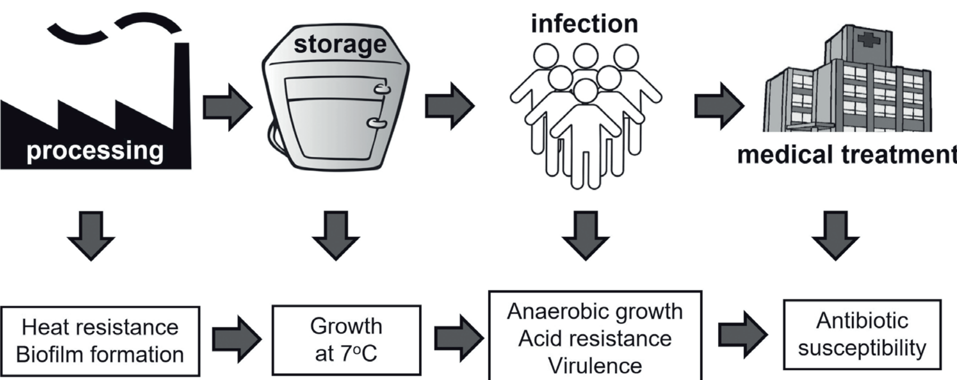


Figure 7. Route of infection of *Listeria monocytogenes*. The route is split up into four steps: processing [1], storage [2], infection [3], and medical treatment [4]. The measured parameters of these four steps are: heat resistance and biofilm formation [1], growth at 7°C [2], acid resistance, (an)aerobic growth (at 30°C), and virulence [3], and antibiotic susceptibility [4].

For each variant, the exposure and illness parameters were scored as similar, increased or decreased compared to the parental strain (Table 5).

Table 5. Analysis of the risk potential of the stress-resistant variants related to that of their parental strain assessed by the probability of exposure (data of survival and growth during and after processing) and the probability of illness (low pH survival, anaerobic growth, virulence model, and antibiotics susceptibility data). Scoring is simplified by using 0 (similar to WT), + (significant increase) or – (significant decrease) of probability for either exposure or illness.

Strain	Probability of exposure			Probability of illness			
	Heat survival	Aerobic biofilm formation	Aerobic growth 7°C	Low pH survival	Anaerobic growth 30°C	Virulence mouse model	Antimicrobial susceptibility
WT	0	0	0	0	0	0	0
5	+	0	-	+	0	0	0
6	+	-	0	+	0	-	0
17	+	-	-	+	0	-	-

Heat survival and low pH survival are the only features in this analysis on which the variants score better than the wild type. Thermal inactivation of pathogens has been studied extensively, which has resulted in a large quantity of *D*-values (n=1027) for various *L. monocytogenes* strains in different products and laboratory media for various circumstances (3, 65). When comparing these overall data, it can be seen that most factors reported to have an effect on the *D*-value are smaller than the variability of all published *D*-values. As shown in Figure 8, the variation in *D*-values can be more than a factor 10 at the same temperature. Only a limited number of factors that did have a significant effect ($p<0.05$) on the *D*-value were identified. The presence of 10% salt or when the water activity is below 0.92 resulted in a higher heat resistance (65). When compared to these datasets, the isolated resistant variants fall within the whole range of strains and are not specific resistant outliers (Fig 8). When compared to *rpsU* variant 14, heat inactivation of late exponential cells exposed to 60°C is similar to that of exponential cells of variants 5 and 17. Besides, heat inactivation of stationary phase cells of *rpsU* variant 14 is comparable to that of exponential cells of our *ctsR* variant (48). Based on the differences shown for *rpsU* variant 14 between late exponential and stationary phase cells, it is expected that survival of stationary phase cells of *ctsR* variant might fall out of the range of *L. monocytogenes* data shown, which types this variant as a very heat resistant variant (Fig 2c). Additionally, when related to the pasteurisation standard of the FDA (High-Temperature Short Time pasteurisation of 15 seconds at 72°C of milk products with fat levels below 10%), *ctsR* and other heat resistant variants with unknown mutations show around 5-log reduction (log *D*-value -1.41 and -1.28 log(min) respectively), which means that their survival is close to what is considered a safe treatment (log *D*-value -1.30 log(min)) (14).

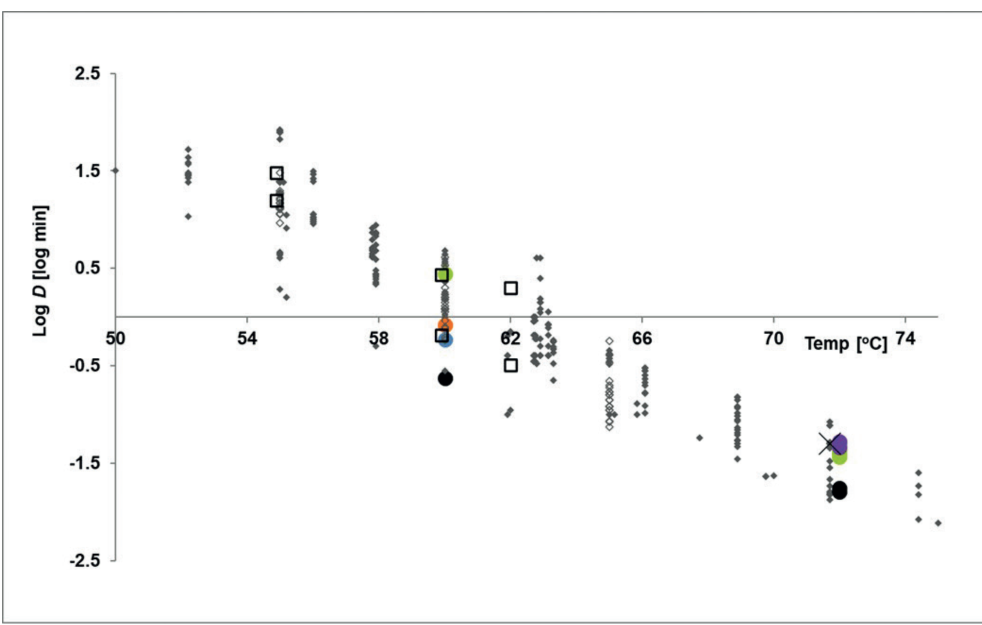


Figure 8. Heat inactivation log *D*-values of *Listeria monocytogenes* strains (grey diamonds: Van Asselt *et al.* (65) and open diamonds: Aryani *et al.* (3) and of *L. monocytogenes* LO28 WT (black circles), variant 5 (blue circle), *ctsR* variant 6 (green circle), variant 17 (orange circle) at 60ºC, and variants isolated after a heat treatment (Chapter 5) *ctsR* isolates (green circles) and other heat-resistant isolates (purple circles). Open squares represent *rpsU* variant 14 at 55, 60 and 62ºC, in which the highest value at each temperature represents stationary phase cells and the other value represents late exponential cells (Metselaar *et al.* (48)). The cross at 72ºC indicates a 5 log reduction at the pasteurisation settings as advised by the FDA (15 seconds, log *D*-value -1.30 log (min)).

Furthermore, the parental strain LO28 is rather heat sensitive compared to the other strains in the dataset. It would therefore be interesting to isolate variants from other, more stress resistant strains and investigate their performance, like for example the stress resistant variants of EGDe (Chapter 5).

The HHP inactivation of the three variants was also compared with a large dataset (74 *D*-values) of various *Listeria monocytogenes* strains in different products and conditions (61) (Fig 9).

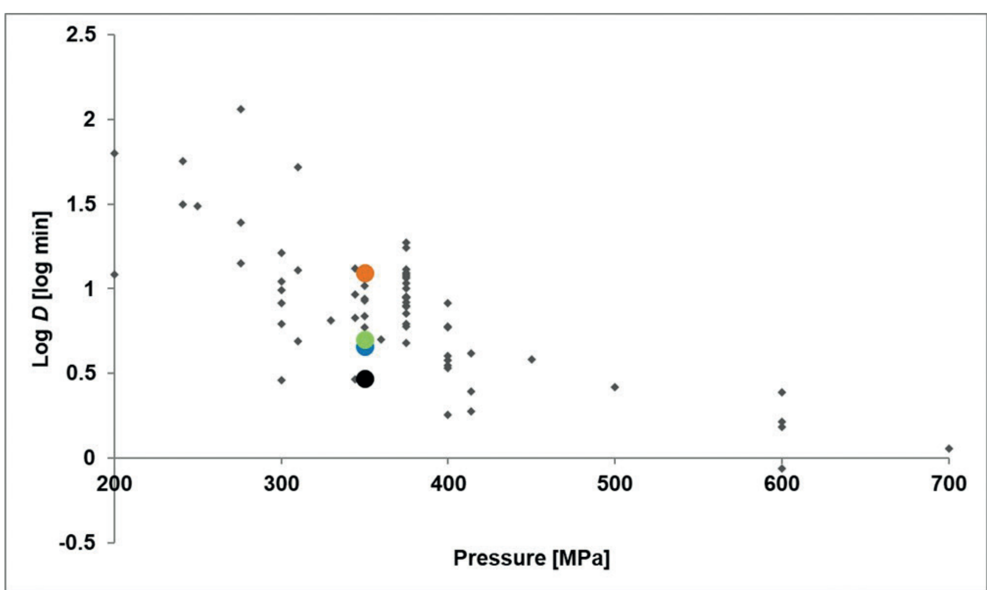


Figure 9. HHP inactivation log *D*-values of *Listeria monocytogenes* strains (Santillana Farakos *et al.* (61)) and of *L. monocytogenes* LO28 WT (black circle), variant 5 (blue circle), *ctsR* variant 6 (green circle), variant 17 (orange circle) at 350MPa.

Although especially variant 17 is very HHP resistant, this variant still falls within the variety of strains used in this dataset. Figure 9 shows that the variation in *D*-values can be more than a factor 10 at the same pressure. A meta-analysis performed by Guillou *et al.* (23), including data of Santillana Farakos *et al.* (61), showed that interstudy variability influenced the variability of reduction values much more than the variability between strains of *L. monocytogenes*. Especially temperature, pH, matrix, initial population, and (de) pressurization rates affect the effeciency of microbial inactivation by HHP (23).

Analysis by Nikparvar *et al.* (51) of gene regulation of *L. monocytogenes* Scott A after exposure to high pressure processing revealed distinct regulatory response modes. Cells were able to survive and recover due to the timely activation of transcription factors associated with an immediate stress response, followed by the expression of genes for repair purposes. They showed that both CtsR and HrcA played an important role in the early cellular response after exposure to HHP. Based on these data the higher survival of our variants can be explained by their already activated stress defense mechanisms, in other words, our variants are actually prepared to cope with multiple stresses.

Although the variants show better survival under stress conditions, the growth at 7°C of variant 5 and 17 is attenuated as well as the biofilm formation at 30°C for variants 6 and 17 (both under aerobic conditions). As a result, cells that might survive the processing step (heat, HHP, low pH), can have reduced growth under further processing or storage conditions which impacts the probability of exposure.

The next step in the risk potential analysis of *L. monocytogenes* is the probability of illness (Table 5). All variants are more resistant to low pH, which can be encountered in the stomach. Compared to the *rpsU* acid resistant variants 14 and 15, our variants are less acid resistant (Fig 2) (45). Although in the method of Metselaar *et al.* 3.5 minutes were used instead of 3 minutes, the late exponential *rpsU* variants were still more resistant, whereas wild type showed a similar reduction compared to our data. It was expected that these *rpsU* variants were more resistant for low pH as they were specifically isolated after acid treatment.

The anaerobic growth of the variants was also assessed, and all variants showed similar growth rates compared to wild type. This anaerobic growth however was tested at 30°C, therefor to better determine the probability of illness it would be recommended to test also anaerobic growth at 37°C. Although the data obtained in the mouse model can also give some information about growth at this temperature as mice and humans have a similar core body temperature (37.0^{o}C in humans and 36.6^{o}C in mice) (56). In the mouse model variants 6 and 17 showed reduced infection levels in both spleen and liver, whereas variant 5 retained full virulence. Interestingly our micro-array data showed also downregulation of flagellum genes in variants 6 and 17 as discussed earlier. As flagellar structures contribute to the virulence of *Listeria monocytogenes* by increasing the efficiency of tissue culture invasion, the downregulation of these genes might explain the lower infection in the mouse model (52). On the contrary, variant 5 showed comparable flagellum genes regulation as wild type and was also able to show similar growth in the mouse model as wild type.

Antibiotics susceptibility of variants versus wild type showed for variants 5 and 6 similar results as wild type, whereas variant 17 was more susceptible. Microarray data showed variation between the variants for the multidrug transporter AnrAB (lmo2114, lmo 2115). In variant 5, *anrA* was significantly upregulated (+1.8), but *anrB* (+1.7) not. In variant 6, *anrA* and *anrB* were both significantly upregulated by 2.4, whereas in variant 17 both genes were not differentially expressed. AnrAB is a multidrug resistance (MDR) ABC transporter that contributes to the innate resistance of *L. monocytogenes* to multiple antibiotics, by removing antimicrobials from the cell envelope (15). This can explain the vulnerability of variant 17 for antibiotics.

Of the three variants, variant 17 showed the least pathogenic potential compared to the wild type due to reduced biofilm formation, reduced growth at 7°C, downregulation of genes involved in motility, reduced virulence in a mouse model and increased antimicrobial susceptibility.

Variant 6, the *ctsR* variant, showed increased heat resistance, but reduced biofilm formation, furthermore this variant is resistant to low pH, but showed reduced virulence in the mouse model. This variant, however, was isolated at the highest frequency after HHP processing in all three strains tested, and even after heat inactivation and might be a rather common variant within a population.

The most noteworthy variant is number 5 as this variant showed both increased probability of exposure as well as increased probability of illness. Compared to the wild type, this variant has similar growth capacity under various conditions (aerobic biofilm, growth on agar plates, and anaerobic growth at 30°C), similar virulence in a mouse model and similar antibiotic resistance next to a 300 times increased resistance to heat as well as to low pH. These characteristics makes variant 5 the variant with the highest pathogenic potential of all three variants.

The combination of stress survival and pathogenic potency of variants can increase the risk in food safety. A high resistance of certain *L. monocytogenes* strains to food-processing conditions may contribute to the particular capability of certain strains to persist and contaminate food, leading to possible food safety issues. While *L. monocytogenes* exists in diverse environments, food product contamination that leads to recalls and outbreaks is often traced back to the processing environment; *L. monocytogenes* may enter the processing environment on raw materials, via employees (e.g., employee boots), via transportation crates or vehicles, etc. (6). Strain subtyping has shown both transient and persistent strains in this environment (36). Persistent strains can become established in a specific facility and isolated repeatedly over several years. Interestingly, the strain involved in the multistate outbreak of listeriosis in the United States in 2000 by contaminated deli turkey meats appeared to have persisted for at least 12 years in the processing plant and may have contaminated food intermittently (53). In general, strain types derived from food processing environments only partially overlap with those implicated in human illness. Many persistent strains are serotype 1/2c (like LO28), which only rarely cause human illness (36). However, it is possible to determine the link between the processing plant, the food and listeriosis as shown by data collected in Sweden of three different sources: processing plants, ready-to-eat food and human listeriosis cases (39). They found that pulsotypes displayed by food isolates were also present among human isolates. Furthermore, most of the food isolates (93%) were found on at least one occasion among the human isolates. Repeated isolation during the whole year of the same pulsotype from food also indicated persistence of one or more specific *L. monocytogenes* strain(s) for some processing plants. Correlations were also found in Denmark. Over 10 years, patient data, clinical outcome and strains isolated showed that 122 cases belonged to just 2 closely related PFGE types. These 2 types were the main cause of a peak in incidence of invasive listeriosis during 2005-2009, possibly representing an outbreak or the presence of a highly prevalent clone (29). To investigate relations between food and illness, the FDA sequences (WGS) all *L. monocytogenes* isolates as they are received when isolated as part of its investigations into foodborne contamination events. For example, in the *L. monocytogenes* ice cream outbreak, two of the outbreak strains differed by up to 29 SNPs. In this outbreak, the outbreak strain also likely persisted in the production environment for many years, thereby enabling its growth and diversification. Since the application of WGS, the average size of outbreaks has become smaller with more

outbreaks being solved, and solved faster than in the past. With early intervention and timely response of regulators and industry, outbreaks may be controlled before they spread (7). Besides using WGS, single cases can be linked to outbreaks by analysing samples of patients in retrospective, like in the case of the multi-country (Netherlands and Belgium) outbreak in which 21 cases of *L. monocytogenes* 4b ST6 could be linked to ready-to-eat (RTE) meat products over a timeframe of three years including the producing company. This information was used to enforce measures taken by the company including a temporal halt of the production and a recall of the involved RTE meat products (18). Furthermore, instead of considering all-hazard strains of a species as equally likely to cause disease or equally likely to survive the food chain, WGS data could give support to rank subtypes with respect to their virulence potential or to group subtypes with respect to their differences in robustness or fitness to reach the consumer stage (38). Our robust variants, although resistant to various stressful conditions, show reduced fitness. These phenotypic characteristics might help to categorize these variants with respect to their virulence potential, although at this moment this is challenging as the actual impact of the analysed parameters is not fully understood and not all relevant information is known. So far, when persisters (colonizers of food-processing facilities) are described, the focus is on tolerance to sanitizers and disinfectants, not on multi-stress resistance, either they are not relevant or not detected up to now. The genomes of our multi-stress resistant variants are very similar to the wild type, small differences might be hard to detect. Therefore, a phenotype profile is needed to detect resistant variants. The multi-resistant variants described in this thesis could be more efficiently isolated after an enrichment step, in which stress resistance-cycles increases the probability to isolate resistant variants as they will compete with cells that are less resistant but can grow faster or better as a biofilm. Therefore, future studies should address the recovery of multi-stress resistant variants and their potential to survive processing, persist and cause illnesses.

In conclusion, this thesis describes the use of modeling to detect and to isolate stress-resistant variants of different *L. monocytogenes* strains. Characterization of these variants showed considerable population diversity within one *L. monocytogenes* strain and revealed that different adverse conditions drive selection for different variants (1). Hence, by diversity *L. monocytogenes* uses bet-hedging strategies to maximize survival. To be able to reduce food contamination and listeriosis it is necessary to control this pathogen by developing approaches that tackle also problems associated with diversity.

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Supplementary material

Table S6.1 Genes significantly up- and down-regulated in all variants compared to wild type

Gene	Gene	Product	Variant 5	Variant 6	Variant 17
lmo0230	*mcsA*	CLPC ATPASE	**2.67**	**8.37**	**5.28**
lmo0231	*mcsB*	Arginine kinase (EC 2.7.3.3)	**2.78**	**8.61**	**4.99**
lmo0232	*clpC*	Negative regulator of genetic competence clpC/mecB / Hemolysin TlyB	**2.74**	**9.06**	**4.83**
lmo0997	*clpE*	ATP-dependent endopeptidase clp ATP-binding subunit clpE	**7.86**	**54.34**	**15.98**
lmo1137	lmo1137	Hypothetical protein	**3.68**	**16.47**	**7.60**
lmo1138	lmo1138	ATP-dependent endopeptidase clp proteolytic subunit clpP (EC 3.4.21.92)	**2.36**	**16.55**	**5.99**
lmo2205	lmo2205	Phosphoglycerate mutase (EC 5.4.2.1)	**2.26**	**19.93**	**6.83**
lmo2206	*clpB*	ClpB protein	**2.38**	**21.91**	**8.80**
lmo2468	*clpP*	ATP-dependent endopeptidase clp proteolytic subunit clpP (EC 3.4.21.92)	**2.32**	**8.46**	**6.22**
lmo1473	*dnaK*	Chaperone protein dnaK	**2.32**	**2.17**	**3.21**
lmo1474	*grpE*	GrpE protein	**2.27**	**2.14**	**3.05**
lmo1475	*hrcA*	Heat-inducible transcription repressor hrcA	**2.69**	**2.37**	**2.55**
Gene	**Gene**	**Product**	**Variant 5**	**Variant 6**	**Variant 17**
lmo1587	*argF*	Ornithine carbamoyltransferase (EC 2.1.3.3)	**-22.82**	**-13.65**	**-11.98**
lmo1588	*argD*	Acetylornithine aminotransferase (EC 2.6.1.11)	**-13.61**	**-6.65**	**-14.72**
lmo1590	*argJ*	Glutamate N-acetyltransferase (EC 2.3.1.35)	**-3.18**	**-2.73**	**-12.15**
lmo1591	*argC*	N-acetyl-gamma-glutamyl-phosphate reductase (EC 1.2.1.38)	**-2.08**	**-2.28**	**-10.63**
lmo1634	lmo1634	Alcohol dehydrogenase (EC 1.1.1.1)	**-4.08**	**-2.74**	**-9.32**
lmo2090	*argG*	Argininosuccinate synthase (EC 6.3.4.5)	**-3.45**	**-4.42**	**-12.51**
lmo2091	*argH*	Argininosuccinate lyase (EC 4.3.2.1)	**-5.43**	**-4.95**	**-18.59**
lmo2250	*arpJ*	Arginine-binding protein	**-2.14**	**-3.30**	**-8.64**
lmo2251	lmo2251	Arginine transport ATP-binding protein artP	**-2.60**	**-3.52**	**-9.31**
lmo2252	lmo2252	Aspartate aminotransferase (EC 2.6.1.1)	**-3.95**	**-4.04**	**-7.01**
lmo2340	lmo2340	Sugar kinases	**-2.99**	**-8.69**	**-6.25**
lmo2341	lmo2341	Ribokinase (EC 2.7.1.15)	**-2.87**	**-12.81**	**-8.01**
lmo2819	lmo2819	Carboxypeptidase, M20(D) family	**-2.10**	**-2.59**	**-2.94**

Table S6.2 Comparable up- and down-regulated genes in variants 5 and 6 (and 17) compared to wild type. Bold values represent significant regulation.

Gene	Gene	Product	Variant 5	Variant 6	Variant 17
lmo0479	lmo0479	Putative secreted protein	**2.29**	**3.38**	*-1.16*
lmo0604	lmo0604	Hypothetical membrane spanning protein	**2.25**	**2.55**	*1.11*
lmo0811	lmo0811	Carbonic anhydrase (EC 4.2.1.1)	**2.94**	**2.52**	*2.50*
lmo0822	lmo0822	Transcriptional regulator, MerR family	**2.08**	**2.09**	*1.32*
lmo1216	lmo1216	Peptidoglycan hydrolase (3.2.1.-)	**2.05**	**3.78**	*1.39*
lmo1245	lmo1245	Hypothetical protein	**2.35**	**2.71**	*1.36*
lmo1945	lmo1945	Riboflavin transporter	**2.23**	**2.01**	*-1.18*
lmo2210	lmo2210	Hypothetical protein	**2.23**	**4.96**	*1.32*
Gene	**Gene**	**Product**	**Variant 5**	**Variant 6**	**Variant 17**
lmo0130	lmo0130	5'-nucleotidase (EC 3.1.3.5)	**-2.24**	**-2.90**	*1.57*
lmo0181	lmo0181	Sugar-binding protein	**-2.05**	**-2.36**	*1.24*
lmo0425	lmo0425	PTS system, mannitol (Cryptic)-specific IIA	**-2.25**	**-4.75**	**5.62**
lmo0471	lmo0471	Hypothetical protein	**-2.01**	**-2.19**	*-1.20*
lmo0914	lmo0914	PTS system, cellobiose-specific IIB component (EC 2.7.1.69)	**-2.48**	**-5.73**	*1.68*
lmo0915	lmo0915	PTS system, lactose-specific IIBC component (EC 2.7.1.69)	**-2.97**	**-8.06**	*2.01*
lmo0916	lmo0916	PTS system, cellobiose-specific IIA component (EC 2.7.1.69)	**-3.00**	**-5.59**	**3.85**
lmo0917	lmo0917	6-phospho-beta-glucosidase (EC 3.2.1.86)	**-3.21**	**-4.67**	**5.49**
lmo1995	*dra*	Deoxyribose-phosphate aldolase (EC 4.1.2.4)	**-2.06**	**-2.84**	*-1.75*
lmo1997	lmo1997	PTS system, mannose-specific IIA component (EC 2.7.1.69)	**-2.60**	**-3.93**	*1.31*
lmo1998	lmo1998	Phosphoaminosugar deglycase family protein	**-2.91**	**-4.83**	*1.40*
lmo1999	lmo1999	Glucosamine--fructose-6-phosphate aminotransferase	**-2.79**	**-4.11**	*1.16*
lmo2000	lmo2000	PTS system, mannose-specific IID component (EC 2.7.1.69)	**-2.40**	**-2.90**	*-1.02*
lmo2001	lmo2001	PTS system, mannose-specific IIC component (EC 2.7.1.69)	**-2.16**	**-2.49**	*-1.15*
lmo2647	lmo2647	Creatinine amidohydrolase family protein	**-4.10**	**-7.25**	**22.98**
lmo2648	lmo2648	Parathion hydrolase (EC 3.1.8.1)	**-4.44**	**-8.78**	**20.14**
lmo2649	lmo2649	Putative transport protein sgaT	**-3.79**	**-5.11**	**14.66**
lmo2650	lmo2650	PTS SYSTEM, IIB COMPONENT (EC 2.7.1.69)	**-5.42**	**-8.69**	**14.82**
lmo2651	lmo2651	PTS system, mannitol-specific IIA component (EC 2.7.1.69)	**-4.04**	**-6.67**	**13.49**

Table S6.3 Comparable up- regulated genes in variants 5 and 17 (and 6) compared to wild type. Bold values represent significant regulation.

Gene	Gene	Product	Variant 5	Variant 6	Variant 17
lmo2068	*groEL*	60 kDa chaperonin GROEL	**2.10**	*1.02*	**4.34**
lmo2069	*groES*	10 kDa chaperonin GROES	**2.35**	*1.09*	**5.43**

Table S6.4 Comparable up- and down-regulated genes in variants 6 and 17 (and 5) compared to wild type, excluded are genes downregulated by MogR as these are listed in table 6.7. Bold values represent significant regulation.

Gene	Gene	Product	Variant 5	Variant 6	Variant 17
lmo0027	lmo0027	PTS system, beta-glucoside-specific IIABC component (EC 2.7.1.69)	**-2.71**	**2.36**	**3.81**
lmo0096	lmo0096	PTS system, mannose-specific IIAB component (EC 2.7.1.69)	*-1.29*	**2.10**	**6.65**
lmo0097	lmo0097	PTS system, mannose-specific IIC component (EC 2.7.1.69)	*-1.15*	**2.27**	**5.16**
lmo0098	lmo0098	PTS system, mannose-specific IID component (EC 2.7.1.69)	*-1.09*	**3.26**	**6.94**
lmo0099	lmo0099	Hypothetical cytosolic protein	*1.01*	**2.11**	**3.81**
lmo0229	*ctsR*	Transcriptional regulator ctsR	*2.33*	**6.24**	**5.16**
lmo0781	lmo0781	PTS system, mannose-specific IID component (EC 2.7.1.69)	*1.50*	**2.03**	**4.21**
lmo1293	*glpD*	Glycerol-3-phosphate dehydrogenase (EC 1.1.99.5)	*1.57*	**3.40**	**6.99**
lmo2522	lmo2522	LysM domain protein / 3D domain protein	*1.63*	**5.52**	**4.03**
lmo2685	lmo2685	PTS system, cellobiose-specific IIA component (EC 2.7.1.69)	*-1.73*	**2.87**	**3.55**
Gene	**Gene**	**Product**	**Variant 5**	**Variant 6**	**Variant 17**
lmo0354	lmo0354	Acetyl-coenzyme A synthetase (EC 6.2.1.1)	*-1.62*	**-2.19**	**-2.40**
lmo0355	lmo0355	Fumarate reductase flavoprotein subunit (EC 1.3.99.1)	*-1.87*	**-2.14**	**-3.85**
lmo0834	lmo0834	Sensor protein fixL (EC 2.7.3.-)	*-1.15*	**-2.73**	**-4.17**
lmo0835	lmo0835	Putative peptidoglycan bound protein (LPXTG motif)	*-1.31*	**-2.23**	**-2.56**
lmo1390	lmo1390	Nucleoside transport system permease protein	*-1.74*	**-2.33**	**-2.07**
lmo1993	*pdp*	Pyrimidine-nucleoside phosphorylase (EC 2.4.2.2)	*-1.46*	**-3.09**	**-3.11**
lmo2335	*fruA*	PTS system, fructose-specific IIABC component (EC 2.7.1.69)	*-1.60*	**-4.74**	**-2.47**
lmo2337	lmo2337	Fructose repressor	*-1.38*	**-3.76**	**-2.10**
lmo2362	*gadT2*	Glutamate/gamma-aminobutyrate antiporter	*-1.43*	**-2.19**	**-5.45**
lmo2469	lmo2469	Amino acid permease	*-1.29*	**-2.21**	**-2.77**

Table S6.5 Expression of regulators in all variants compared to wild type. Bold values represent significant regulation.

Gene	Gene	Product	Variant 5	Variant 6	Variant 17
lmo0051	*agrA*	Accessory gene regulator protein A agrA	1.27	**1.97**	**-3.26**
lmo0200	*prfA*	Listeriolysin regulatory protein	**1.85**	1.35	2.38
lmo0229	*ctsR*	Transcriptional regulator ctsR	2.33	**6.24**	**5.16**
lmo0243	*sigH*	RNA polymerase sigma-H factor	-1.16	1.02	-1.43
lmo0423	*sigC*	RNA polymerase ECF-type sigma factor	-1.09	-1.16	1.43
lmo0895	*sigB*	RNA polymerase sigma-B factor	-1.05	-1.04	-1.18
lmo1280	*codY*	Transcription pleiotropic repressor codY	1.03	1.16	-1.45
lmo1475	*hrcA*	Heat-inducible transcription repressor hrcA	**2.69**	**2.37**	**2.55**
lmo2461	*sigL*	RNA polymerase sigma-54 factor rpoN	-1.10	-1.19	1.03

Table S6.6 Sigma B regulated genes in variants 5, 6 and 17 compared to wild type. Gene *gbuC* was added to the list, although not mentioned in Liu *et al.* (41). Bold values represent significant regulation.

Gene	Gene	Product	Variant 5	Variant 6	Variant 17
lmo0013	*qoxA*	Cytochrome aa3 quinol oxidase polypeptide II (EC 1.9.3.-)	**1.86**	1.26	**4.11**
lmo0014	*qoxB*	Cytochrome aa3 quinol oxidase polypeptide I (EC 1.9.3.-)	1.56	1.54	**3.09**
lmo0015	*qoxC*	Cytochrome aa3 quinol oxidase polypeptide III (EC 1.9.3.-)	1.54	1.35	**2.76**
lmo0016	*qoxD*	Cytochrome aa3 quinol oxidase polypeptide IV (EC 1.9.3.-)	1.58	1.32	**2.51**
lmo0230	lmo0230	CLPC ATPASE	**2.67**	**8.37**	**5.28**
lmo0231	lmo0231	Arginine kinase (EC 2.7.3.3)	**2.78**	**8.61**	**4.99**
lmo0232	*clpC*	Negative regulator of genetic competence clpC/mecB / Hemolysin TlyB	**2.74**	**9.06**	**4.83**
lmo0398	lmo0398	Nitrogen regulatory IIA protein (EC 2.7.1.69)	**-2.11**	**-1.65**	1.62
lmo0400	lmo0400	PTS system, fructose-specific IIBC component (EC 2.7.1.69)	**-2.59**	-1.33	1.59
lmo0401	lmo0401	Alpha-mannosidase (EC 3.2.1.24)	-2.05	1.25	**3.96**
lmo0592	lmo0592	Hypothetical protein	1.02	1.12	**3.04**
lmo0593	lmo0593	Formate/nitrite transporter family protein	1.59	1.12	**-2.93**
lmo0723	lmo0723	Methyl-accepting chemotaxis protein	-1.78	**-10.14**	**-10.10**
lmo0724	lmo0724	Hypothetical protein	**-1.84**	**-9.31**	**-9.94**
lmo0781	lmo0781	PTS system, mannose-specific IID component (EC 2.7.1.69)	1.50	**2.03**	**4.21**
lmo0782	lmo0782	PTS system, mannose-specific IIC component (EC 2.7.1.69)	1.45	**1.96**	**5.45**
lmo0783	lmo0783	PTS system, mannose-specific IIB component (EC 2.7.1.69)	1.37	**1.79**	**5.74**
lmo0784	lmo0784	PTS system, mannose-specific IIA component (EC 2.7.1.69)	1.34	**1.63**	**5.19**
lmo0994	lmo0994	Hypothetical protein	1.28	**3.75**	-1.53
lmo0995	lmo0995	Permease	1.90	**9.79**	1.30
lmo1014	*gbuA*	Glycine betaine transport ATP-binding protein	1.61	**2.14**	1.40
lmo1015	*gbuB*	Glycine betaine transport system permease protein	1.57	**2.19**	1.28
lmo1016	*gbuC*	Glycine betaine-binding protein	1.39	**1.82**	1.44
lmo1140	lmo1140	Hypothetical cytosolic protein	1.26	**2.23**	1.37
lmo1538	lmo1538	Glycerol kinase (EC 2.7.1.30)	1.18	**1.83**	**9.01**
lmo1539	lmo1539	Glycerol uptake facilitator protein	-1.02	1.54	**9.75**
lmo1601	lmo1601	General stress protein	-1.10	-1.09	**2.58**
lmo1602	lmo1602	Hypothetical protein	-1.07	-1.13	**2.52**
lmo2094	lmo2094	L-fuculose phosphate aldolase (EC 4.1.2.17)	-1.65	**2.83**	-1.92
lmo2095	lmo2095	Phosphofructokinase family protein	-1.54	**2.98**	-1.55
lmo2130	lmo2130	Amino acid permease	-1.14	-1.29	**-2.91**
lmo2205	lmo2205	Phosphoglycerate mutase (EC 5.4.2.1)	**2.26**	**19.93**	**6.83**
lmo2362	*gadT2*	Glutamate/gamma-aminobutyrate antiporter	-1.43	**-2.19**	**-5.45**
lmo2363	*gadD2*	Glutamate decarboxylase (EC 4.1.1.15)	-1.23	-1.58	**-3.50**
lmo2391	lmo2391	Putative NAD-dependent dehydrogenase	1.60	**2.05**	1.20
lmo2457	*tpi*	Triosephosphate isomerase (EC 5.3.1.1)	-1.40	-1.36	**-2.05**
lmo2458	*pgk*	Phosphoglycerate kinase (EC 2.7.2.3)	-1.42	-1.43	**-2.24**

lmo2460	lmo2460	Central glycolytic genes regulator	-1.22	1.25	**-5.18**
lmo2468	*clpP*	ATP-dependent endopeptidase clp proteolytic subunit clpP (EC 3.4.21.92)	**2.32**	**8.46**	**6.22**
lmo2484	lmo2484	Integral membrane protein	**3.25**	**1.68**	1.32
lmo2485	lmo2485	Stress-responsive transcriptional regulator PspC	**3.30**	1.54	1.59
lmo2665	lmo2665	PTS system, D-arabitol specific IIC component (EC 2.7.1.69)	**-2.30**	-1.44	1.59
lmo2667	lmo2667	PTS system, D-arabitol-specific IIA component (EC 2.7.1.69)	**-2.23**	**-1.76**	1.99

Table S6.7 Downregulated genes in variants 5, 6 and 17, regulated by MogR compared to wild type. Bold values represent significant regulation. lmo0718 was not listed in the paper of Shen and Higgins (62), but seemed related to gene lmo0717, as their regulation was similar in all variants.

Gene	Gene	Product	Variant 5	Variant 6	Variant 17
lmo0673	lmo0673	Unknown	*-1.37*	*-1.68*	*-1.51*
lmo0674	*mogR*	Motility gene repressor	*-1.39*	**-2.11**	*-1.30*
lmo0675	lmo0675	Flagellar motor switch protein fliN	*-1.13*	*-1.48*	**-5.56**
lmo0676	lmo0676	Flagellar biosynthetic protein fliP	*-1.25*	*-1.40*	**-6.53**
lmo0677	lmo0677	Flagellar biosynthetic protein fliQ	*-1.29*	*-1.33*	**-7.25**
lmo0678	lmo0678	Flagellar biosynthetic protein fliR	*-1.39*	*-1.69*	**-8.60**
lmo0679	lmo0679	Flagellar biosynthetic protein flhB	*-1.46*	*-1.99*	**-7.41**
lmo0680	lmo0680	Flagellar biosynthesis protein flhA	*-1.43*	**-2.19**	**-7.21**
lmo0681	lmo0681	Flagellar biosynthesis protein flhF	*-1.26*	*-1.88*	**-5.51**
lmo0682	lmo0682	Flagellar basal-body rod protein flgG	*-1.23*	*-1.96*	**-5.38**
lmo0683	lmo0683	Chemotaxis protein methyltransferase (EC 2.1.1.80)	*-1.36*	**-2.84**	**-5.16**
lmo0684	lmo0684	Unknown	*-1.31*	**-2.85**	**-5.18**
lmo0685	*motA*	Chemotaxis motA protein	*-1.20*	**-2.19**	**-4.95**
lmo0686	*motB*	Chemotaxis motB protein	*-1.14*	**-2.05**	**-4.67**
lmo0687	lmo0687	Unknown	*-1.21*	**-2.05**	**-4.64**
lmo0688	lmo0688	Glycosyltransferase (EC 2.4.1.-)	*-1.15*	*-1.96*	**-4.40**
lmo0689	lmo0689	Chemotaxis protein cheV (EC 2.7.3.-)	*-1.21*	**-2.07**	**-3.75**
lmo0690	*flaA*	Flagellin	*-1.73*	**-16.05**	**-14.91**
lmo0691	*cheY*	Chemotaxis protein cheY	*-1.12*	**-2.95**	**-4.75**
lmo0692	*cheA*	Chemotaxis protein cheA (EC 2.7.3.-)	*-1.22*	**-3.07**	**-5.26**
lmo0693	lmo0693	Flagellar motor switch protein fliN	*-1.32*	**-3.31**	**-5.37**
lmo0694	lmo0694	Hypothetical cytosolic protein	*-1.39*	**-3.29**	**-6.01**
lmo0695	lmo0695	Unknown	*-1.33*	**-3.35**	**-5.85**
lmo0696	lmo0696	Basal-body rod modification protein flgD	*-1.50*	**-4.28**	**-5.23**
lmo0697	lmo0697	Flagellar hook protein flgE	*-1.38*	**-3.41**	**-5.58**
lmo0698	lmo0698	Flagellar motor switch protein fliN	*-1.36*	**-3.06**	**-6.17**
lmo0699	lmo0699	Flagellar motor switch protein fliM	*-1.28*	**-3.06**	**-6.11**
lmo0700	lmo0700	Chemotaxis protein cheC	*-1.15*	**-2.90**	**-5.89**
lmo0701	lmo0701	Unknown	*-1.10*	**-2.77**	**-5.65**
lmo0702	lmo0702	Unknown	*-1.11*	**-2.84**	**-5.35**
lmo0703	lmo0703	UDP-N-acetylenolpyruvoylglucosamine reductase (EC 1.1.1.158)	*-1.15*	**-3.16**	**-5.39**

Table S6.7 Continued.

Gene	Gene	Product	Variant 5	Variant 6	Variant 17
lmo0704	lmo0704	Unknown	*-1.12*	**-3.16**	**-5.45**
lmo0705	lmo0705	Flagellar hook-associated protein 1	*-1.17*	**-3.31**	**-4.85**
lmo0706	lmo0706	Flagellar hook-associated protein 3	*-1.31*	**-3.68**	**-4.96**
lmo0707	lmo0707	Flagellar hook-associated protein 2	*-1.30*	**-3.62**	**-4.90**
lmo0708	lmo0708	Flagellar protein fliS	*-1.30*	**-3.62**	**-4.67**
lmo0709	lmo0709	Unknown	*-1.38*	**-3.68**	**-4.70**
lmo0710	lmo0710	Flagellar basal-body rod protein flgB	*-1.28*	**-3.56**	**-4.69**
lmo0711	lmo0711	Flagellar basal-body rod protein flgC	*-1.32*	**-3.08**	**-4.41**
lmo0712	lmo0712	Flagellar hook-basal body complex protein fliE	*-1.30*	**-3.20**	**-4.62**
lmo0713	lmo0713	Flagellar M-ring protein fliF	*-1.13*	**-2.64**	**-3.94**
lmo0714	lmo0714	Flagellar motor switch protein fliG	*-1.12*	**-2.18**	**-3.62**
lmo0715	lmo0715	Unknown	*-1.07*	**-2.08**	**-4.37**
lmo0716	lmo0716	Flagellum-specific ATP synthase (EC 3.6.3.14)	*-1.07*	**-2.13**	**-4.35**
lmo0717	lmo0717	Lytic transglycosylase homolog yjbJ	*-1.03*	**-2.16**	**-4.08**
lmo0718	lmo0718	Unknown	*-1.07*	**-2.22**	**-3.98**
lmo0723	lmo0723	Methyl-accepting chemotaxis protein	*-1.78*	**-10.14**	**-10.10**
lmo0724	lmo0724	Hypothetical protein	*-1.84*	**-9.31**	**-9.94**
lmo1699	lmo1699	Pili methyl chemotaxis protein pilJ	*-1.64*	**-5.47**	**-9.07**
lmo1700	lmo1700	Unknown	*-1.89*	**-5.08**	**-12.26**

Table S6.8 Significantly regulated genes in both *ctsR* variants Scott A (as described by Liu *et al.* (40)) and LO28 variant 6 compared to their wild types.

Scott A gene	LO28 gene	Function	Scott A ctsR variant	LO28 variant 6
LMOf2365_lmo0241	lmo0229	Transcriptional regulator CtsR	**6.3**	**6.2**
LMOf2365_lmo0242	lmo0230	UVR domain protein	**10.1**	**8.4**
LMOf2365_lmo0243	lmo0231	ATP:guanido phosphotransferase family protein	**5.2**	**8.6**
LMOf2365_lmo0244	lmo0232	ClpC ATPase	**6.1**	**9.1**
LMOf2365_lmo0442	lmo0426	PTS system; fructose-specific; IIA component	**3.0**	**-4.0**
LMOf2365_lmo0443	lmo0427	PTS system; fructose-specific; IIB component	**3.3**	**-2.9**
LMOf2365_lmo0444	lmo0428	PTS system; fructose-specific; IIC component	**3.1**	**-2.2**
LMOf2365_lmo0445	lmo0429	Glycosyl hydrolase; family 38	**3.7**	**-2.5**
LMOf2365_lmo1018	lmo0997	ATP-dependent Clp protease; ATP-binding subunit ClpE	**39.5**	**54.3**
LMOf2365_lmo2147	lmo2114	ABC transporter; ATP-binding protein	**2.2**	**2.4**
LMOf2365_lmo2148	lmo2115	ABC transporter; permease protein	**2.2**	**2.4**
LMOf2365_lmo2620	lmo2648	Phosphotriesterase family protein	**2.2**	**-8.8**
LMOf2365_lmo0113	lmo0096	PTS system; mannose-specific; IIAB component	**-5.0**	**2.1**
LMOf2365_lmo0114	lmo0097	PTS system; mannose/fructose/sorbose family; IIC component	**-2.0**	**2.3**
LMOf2365_lmo0115	lmo0098	System; mannose/fructose/sorbose family; IID component	**-5.0**	**3.3**
LMOf2365_lmo0143	lmo0125	Hypothetical proteins: conserved	**-2.5**	**-4.0**
LMOf2365_lmo0376	lmo0355	Fumarate reductase; flavoprotein subunit	**-3.3**	**-2.1**
LMOf2365_lmo0710	lmo0674	Hypothetical proteins: conserved	**-2.0**	**-2.1**

LMOf2365_lmo0729	lmo0693	Flagellar motor switch domain protein	**-2.5**	**-3.3**
LMOf2365_lmo0730	lmo0694	Hypothetical proteins: conserved	**-2.5**	**-3.3**
LMOf2365_lmo0731	lmo0695	Hypothetical proteins: conserved	**-3.3**	**-3.3**
LMOf2365_lmo0739	lmo0703	Hypothetical proteins: conserved	**-3.3**	**-3.2**
LMOf2365_lmo0740	lmo0704	Hypothetical proteins: conserved	**-3.3**	**-3.2**
LMOf2365_lmo0742	lmo0706	Putative flagellar hook-associated protein FlgL	**-3.0**	**-3.7**
LMOf2365_lmo0744	lmo0708	Putative flagellar protein FliS	**-5.0**	**-3.6**
LMOf2365_lmo0745	lmo0709	Hypothetical proteins: conserved	**-5.0**	**-3.7**
LMOf2365_lmo0746	lmo0710	Flagellar basal-body rod protein FlgB	**-10.0**	**-3.6**
LMOf2365_lmo0747	lmo0711	Flagellar basal-body rod protein FlgC	**-5.0**	**-3.1**
LMOf2365_lmo0749	lmo0713	Flagellar M-ring protein FliF	**-3.3**	**-2.6**
LMOf2365_lmo0750	lmo0714	Flagellar motor switch protein FliG	**-2.5**	**-2.2**
LMOf2365_lmo0751	lmo0715	Hypothetical proteins: conserved	**-3.3**	**-2.1**
LMOf2365_lmo0753	lmo0717	Transglycosylase; SLT family	**-3.3**	**-2.2**
LMOf2365_lmo0754	lmo0718	Hypothetical proteins: conserved	**-3.3**	**-2.2**
LMOf2365_lmo1365	lmo1348	Glycine cleavage system T protein	**-2.5**	**-3.6**
LMOf2365_lmo2495	lmo2522	LysM domain protein	**-2.0**	**5.5**

Table S6.9 Significantly regulated genes unique in variant 5 compared to wild type and variants 6 and 17. Bold values represent significant regulation.

Gene	Gene	Product	Variant 5	Variant 6	Variant 17
lmo0027	lmo0027	PTS system, beta-glucoside-specific IIABC component (EC 2.7.1.69)	**-2.71**	**2.36**	**3.81**
lmo0279	lmo0279	Anaerobic ribonucleoside-triphosphate reductase (EC 1.17.4.2)	**-2.07**	-1.48	-1.69
lmo0398	lmo0398	Nitrogen regulatory IIA protein (EC 2.7.1.69)	**-2.11**	**-1.65**	1.62
lmo0400	lmo0400	PTS system, fructose-specific IIBC component (EC 2.7.1.69)	**-2.59**	-1.33	1.59
lmo0506	lmo0506	Sorbitol dehydrogenase (EC 1.1.1.14)	**-2.01**	1.10	1.21
lmo0517	lmo0517	Phosphoglycerate mutase family protein	**-2.50**	-1.10	**3.81**
lmo2347	lmo2347	Methionine sulfoxide transport system permease protein	**-2.07**	-1.06	1.80
lmo2348	lmo2348	Methionine sulfoxide transport system permease protein	**-2.06**	-1.08	1.66
lmo2349	lmo2349	Methionine sulfoxide-binding protein	**-2.09**	1.01	2.19
lmo2585	lmo2585	YrhD	**-2.54**	**-1.96**	**4.83**
lmo2586	lmo2586	Formate dehydrogenase alpha chain (EC 1.2.1.2)	**-2.06**	**-1.78**	**3.02**
lmo2664	lmo2664	D-arabinose-5-phosphate 2-dehydrogenase (EC 1.1.1.-)	**-2.16**	-1.44	1.52
lmo2665	lmo2665	PTS system, D-arabitol specific IIC component (EC 2.7.1.69)	**-2.30**	-1.44	1.59
lmo2667	lmo2667	PTS system, D-arabitol-specific IIA component (EC 2.7.1.69)	**-2.23**	**-1.76**	1.99
lmo2683	lmo2683	PTS system, cellobiose-specific IIB component (EC 2.7.1.69)	**-2.31**	1.12	1.39
lmo2708	lmo2708	PTS system, cellobiose-specific IIC component	**-2.56**	**-1.72**	**5.53**
lmo2761	lmo2761	6-phospho-beta-glucosidase (EC 3.2.1.86)	**-2.59**	**-1.94**	2.28
lmo2762	lmo2762	PTS system, cellobiose-specific IIB component (EC 2.7.1.69)	**-2.44**	-1.45	**2.55**

Table S6.9 Continued.

Gene	Gene	Product	Variant 5	Variant 6	Variant 17
lmo2763	lmo2763	PTS system, cellobiose-specific IIC component	**-2.05**	-1.08	**3.03**
Gene	**Gene**	**Product**	**Variant 5**	**Variant 6**	**Variant 17**
lmo0612	lmo0612	Transcriptional regulator, MarR family	**2.16**	**1.81**	1.61
lmo0954	lmo0954	Hypothetical protein	**2.15**	1.18	1.72
lmo1263	lmo1263	Transcriptional regulator, pbsX family	**2.56**	-1.33	1.44
lmo1966	lmo1966	XpaC protein	**2.16**	1.14	1.92
lmo1967	lmo1967	Tellurite resistance protein	**2.14**	1.23	1.68
lmo2088	lmo2088	Transcriptional regulator, TetR family	**2.06**	1.38	-1.24
lmo2484	lmo2484	Integral membrane protein	**3.25**	**1.68**	1.32
lmo2485	lmo2485	Stress-responsive transcriptional regulator PspC	**3.30**	1.54	1.59
lmo2486	lmo2486	Hypothetical protein	**3.18**	1.41	1.70
lmo2487	lmo2487	Hypothetical protein	**3.83**	**1.84**	1.91
lmo2567	lmo2567	Deoxyguanosinetriphosphate triphosphohydrolase-like protein	**8.81**	**1.83**	2.46
lmo2568	lmo2568	Hypothetical: Histone acetyltransferase HPA2 and related acetyltransferases	**7.23**	1.53	2.63
lmo2827	lmo2827	Transcriptional regulator, MarR family	**2.34**	1.73	1.81

Table S6.10 Significantly regulated genes unique in variant 6 compared to wild type and variants 5 and 17. Bold values represent significant regulation.

Gene	Gene	Product	Variant 5	Variant 6	Variant 17
lmo0023	lmo0023	PTS system, mannose-specific IIC component (EC 2.7.1.69)	-1.96	**-2.25**	1.37
lmo0033	lmo0033	Similar to endoglucanase	-2.10	**-2.20**	1.07
lmo0073	lmo0073	Hypothetical protein	-1.50	**-2.14**	-1.06
lmo0074	lmo0074	Hypothetical protein	-1.52	**-2.17**	-1.12
lmo0117	*lmaB*	Antigen B	-1.41	**-4.29**	**5.51**
lmo0118	*lmaA*	Antigen A	-1.47	**-3.55**	**4.57**
lmo0119	lmo0119	Hypothetical protein	-1.47	**-3.70**	**3.43**
lmo0120	lmo0120	Phage protein	-1.47	**-3.09**	**3.37**
lmo0121	lmo0121	Phage protein	-1.59	**-3.69**	**3.80**
lmo0122	lmo0122	Phage protein	-1.47	**-4.26**	**4.40**
lmo0123	lmo0123	Phage protein	-1.38	**-2.01**	**3.27**
lmo0124	lmo0124	Hypothetical protein	-1.38	**-3.85**	**4.84**
lmo0125	lmo0125	Hypothetical protein	-1.41	**-3.97**	**4.63**
lmo0126	lmo0126	Hypothetical protein	-1.44	**-3.30**	**4.50**
lmo0127	lmo0127	Phage related functions	-1.46	**-3.28**	**3.73**
lmo0129	lmo0129	Sporulation-specific N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)	-1.44	**-2.86**	**3.70**
lmo0143	lmo0143	Hypothetical protein	-1.21	**-3.56**	-1.01
lmo0144	lmo0144	Hypothetical protein	-1.12	**-2.28**	-1.02
lmo0153	lmo0153	High-affinity zinc uptake system protein znuA precursor	-1.53	**-2.17**	-1.78
lmo0278	lmo0278	Sugar transport ATP-binding protein	-1.66	**-2.86**	**3.34**
lmo0299	lmo0299	PTS system, cellobiose-specific IIB component (EC 2.7.1.69)	-1.47	**-2.33**	1.88

lmo0300	lmo0300	6-phospho-beta-glucosidase (EC 3.2.1.86)	-1.66	**-3.99**	1.38
lmo0301	lmo0301	PTS system, cellobiose-specific IIA component (EC 2.7.1.69)	-1.68	**-3.31**	-1.06
lmo0344	lmo0344	Short chain dehydrogenase	-1.54	**-2.02**	**5.38**
lmo0346	lmo0346	Triosephosphate isomerase (EC 5.3.1.1)	-1.71	**-2.13**	**4.36**
lmo0347	lmo0347	Dihydroxyacetone kinase (EC 2.7.1.29)	-1.57	**-2.11**	**4.95**
lmo0349	lmo0349	Hypothetical protein	-1.80	**-2.28**	**3.78**
lmo0351	lmo0351	Dihydroxyacetone kinase phosphotransfer protein	-1.53	**-2.13**	**3.31**
lmo0424	lmo0424	Glucose uptake protein homolog	-1.51	**-2.08**	**3.75**
lmo0426	lmo0426	Nitrogen regulatory IIA protein (EC 2.7.1.69)	**-2.00**	**-4.02**	**6.21**
lmo0427	lmo0427	PTS system, fructose-specific IIBC component (EC 2.7.1.69)	**-1.97**	**-2.89**	**4.65**
lmo0428	lmo0428	PTS system, fructose-specific IIBC component (EC 2.7.1.69)	-1.68	**-2.17**	3.50
lmo0429	lmo0429	Alpha-mannosidase (EC 3.2.1.24)	-1.77	**-2.46**	**5.85**
lmo0674	lmo0674	DNA binding domain of the motility gene repressor (MogR)	-1.39	**-2.11**	-1.30
lmo0813	lmo0813	Fructokinase (EC 2.7.1.4)	-1.75	**-2.37**	-1.45
lmo0859	lmo0859	Trehalose/maltose-binding protein	-1.76	**-3.36**	-1.60
lmo0860	lmo0860	Maltose transport system permease protein malF	-1.66	**-2.38**	-2.23
lmo0863	lmo0863	Hypothetical protein	-1.54	**-2.25**	-1.70
lmo1118	lmo1118	Hypothetical protein	-1.48	**-2.27**	1.51
lmo1251	lmo1251	Transcription regulator, crp family	-1.14	**-2.17**	-1.25
lmo1348	lmo1348	Aminomethyltransferase (EC 2.1.2.10)	-1.50	**-3.58**	**4.17**
lmo1389	lmo1389	Nucleoside transport ATP-binding protein	-1.57	**-2.56**	-1.88
lmo1597	lmo1597	Hypothetical protein	-1.13	**-2.50**	-1.00
lmo1994	lmo1994	Transcriptional regulator, LacI family	-1.75	**-2.94**	-2.37
lmo2002	lmo2002	PTS SYSTEM, MANNOSE-SPECIFIC IIAB COMPONENT (EC 2.7.1.69)	-1.67	**-2.22**	-1.11
lmo2003	lmo2003	Transcriptional regulatory protein	-1.44	**-2.12**	-1.05
lmo2079	lmo2079	Hypothetical protein	-1.29	**-2.18**	1.15
lmo2121	lmo2121	maltose phosphorylase (EC 2.4.1.8)	-1.16	**-6.57**	**8.79**
lmo2122	lmo2122	Maltodextrose utilization protein malA	-1.19	**-4.83**	**7.36**
lmo2123	lmo2123	Maltodextrin transport system permease protein malD	-1.28	**-8.59**	**7.98**
lmo2124	lmo2124	Maltodextrin transport system permease protein malC	-1.27	**-8.30**	**7.73**
lmo2125	lmo2125	Maltose/maltodextrin-binding protein	-1.31	**-10.97**	**8.09**
lmo2126	lmo2126	Cyclomaltodextrinase (EC 3.2.1.54) / Maltogenic alpha-amylase (EC 3.2.1.133)	-1.17	**-6.65**	**7.19**
lmo2159	lmo2159	NADH-dependent dehydrogenase	-1.60	**-2.50**	1.56
lmo2160	lmo2160	IolE protein homolog	-1.78	**-2.70**	1.72
lmo2161	lmo2161	ThuA protein	-1.82	**-2.73**	1.84
lmo2162	lmo2162	Xylose isomerase family protein	-1.70	**-2.62**	1.76
lmo2163	lmo2163	NAD-dependent oxidoreductase	**-1.90**	**-3.30**	2.05
lmo2284	lmo2284	Gp19 protein	-1.45	**-7.13**	1.93
lmo2286	lmo2286	Phage protein	-1.48	**-6.31**	1.78
lmo2291	lmo2291	Major tail shaft protein	-1.55	**-6.89**	**2.41**
lmo2293	lmo2293	Minor capsid protein	-1.50	**-4.97**	1.99

Table S6.10 Continued.

Gene	Gene	Product	Variant 5	Variant 6	Variant 17
lmo2294	lmo2294	Gp9 protein	-1.60	**-7.95**	2.18
lmo2295	lmo2295	Gp8 protein	-1.52	**-5.07**	2.09
lmo2296	lmo2296	Major capsid protein	-1.55	**-7.62**	2.25
lmo2297	lmo2297	Minor capsid protein	-1.52	**-5.62**	**2.71**
lmo2298	lmo2298	Minor capsid protein	-1.53	**-5.47**	2.19
lmo2300	lmo2300	Terminase large subunit	-1.57	**-7.79**	2.06
lmo2309	lmo2309	Hypothetical protein	-1.21	**-3.23**	**10.49**
lmo2319	lmo2319	Phage protein	-1.42	**-5.28**	1.79
lmo2320	lmo2320	Similar to arsenate reductase	-1.39	**-12.74**	1.95
lmo2323	lmo2323	Gp43 protein	-1.46	**-8.84**	1.86
lmo2336	*fruB*	1-phosphofructokinase (EC 2.7.1.56)	-1.37	**-4.07**	-2.17
lmo2409	lmo2409	Hypothetical protein	-1.51	**-3.51**	-1.47
lmo2773	lmo2773	Transcription antiterminator, BglG family	-1.37	**-2.02**	-1.09
lmo2803	lmo2803	Hypothetical protein	-1.36	**-2.62**	-1.11
lmo2840	lmo2840	Sucrose phosphorylase (EC 2.4.1.7)	-1.78	**-2.62**	-1.44

Gene	Gene	Product	Variant 5	Variant 6	Variant 17
lmo0186	lmo0186	3D domain protein	**1.72**	**3.30**	-1.10
lmo0319	lmo0319	6-phospho-beta-glucosidase (EC 3.2.1.86)	-1.37	**2.39**	2.40
lmo0361	lmo0361	Sec-independent protein translocase protein tatC	1.17	**2.78**	-1.08
lmo0362	lmo0362	Sec-independent protein translocase protein tatA	1.17	**2.25**	1.06
lmo0365	lmo0365	High-affinity iron permease	1.06	**2.62**	-1.25
lmo0366	lmo0366	Protein ycdO	-1.00	**2.26**	-1.11
lmo0394	lmo0394	SH3 domain protein / NlpC/P60 family protein	1.10	**2.24**	-1.06
lmo0485	lmo0485	Nitroreductase family	1.46	**2.05**	2.10
lmo0486	*rpmF*	LSU ribosomal protein L32P	1.43	**2.02**	1.61
lmo0496	lmo0496	Hypothetical cytosolic protein	**1.78**	**6.82**	1.67
lmo0500	lmo0500	Transaldolase (EC 2.2.1.2)	-1.22	**2.25**	1.30
lmo0588	lmo0588	Deoxyribodipyrimidine photolyase (EC 4.1.99.3)	1.71	**2.05**	1.74
lmo0726	lmo0726	Hypothetical protein	1.64	**2.31**	1.68
lmo0836	lmo0836	Hypothetical membrane spanning protein	1.51	**2.09**	1.23
lmo0868	lmo0868	Hypothetical protein	**1.88**	**2.19**	1.24
lmo0920	lmo0920	Hypothetical membrane spanning protein	1.66	**2.67**	1.24
lmo0976	lmo0976	Acetyltransferase, GNAT family	1.46	**2.07**	1.24
lmo0994	lmo0994	Hypothetical protein	1.28	**3.75**	-1.53
lmo0995	lmo0995	Permease	1.90	**9.79**	1.30
lmo0998	lmo0998	CAAX amino terminal protease family protein	1.53	**2.06**	1.12
lmo1000	lmo1000	Phytoene desaturase (EC 1.14.99.-)	1.42	**2.41**	1.31
lmo1001	lmo1001	Hypothetical protein	1.17	**2.33**	2.19
lmo1014	*gbuA*	Glycine betaine transport ATP-binding protein	1.61	**2.14**	1.40
lmo1015	*gbuB*	Glycine betaine transport system permease protein	1.57	**2.19**	1.28
lmo1140	lmo1140	Hypothetical cytosolic protein	1.26	**2.23**	1.37
lmo1387	lmo1387	Pyrroline-5-carboxylate reductase (EC 1.5.1.2)	**1.80**	**2.34**	2.25
lmo1413	lmo1413	Receptor protein kinase-like protein	**2.00**	**2.15**	-1.62
lmo1482	*comEC*	COME operon protein 3	1.48	**2.42**	-1.16
lmo1690	lmo1690	Hypothetical membrane spanning protein	1.46	**2.69**	1.01

lmo2093	lmo2093	Hypothetical protein	-1.51	**2.98**	-1.62
lmo2094	lmo2094	L-fuculose phosphate aldolase (EC 4.1.2.17)	-1.65	**2.83**	-1.92
lmo2095	lmo2095	Phosphofructokinase family protein	-1.54	**2.98**	-1.55
lmo2096	lmo2096	PTS system, galactitol-specific IIC component (EC 2.7.1.69)	-1.70	**3.45**	-1.78
lmo2097	lmo2097	PTS system, galactitol-specific IIB component (EC 2.7.1.69)	-1.57	**2.18**	-1.69
lmo2098	lmo2098	PTS system, galactitol-specific IIA component (EC 2.7.1.69)	-1.65	**2.48**	-1.71
lmo2114	lmo2114	ABC transporter ATP-binding protein	**1.82**	**2.41**	-1.11
lmo2115	lmo2115	ABC transporter permease protein	1.66	**2.36**	-1.15
lmo2390	lmo2390	Ferredoxin--NAD(P)(+) reductase (EC 1.18.1.-)	1.46	**2.00**	1.02
lmo2391	lmo2391	Putative NAD-dependent dehydrogenase	1.60	**2.05**	1.20
lmo2684	lmo2684	PTS system, cellobiose-specific IIC component	**-1.88**	**2.32**	2.45
lmo2687	lmo2687	Rod shape-determining protein rodA	1.71	**2.68**	1.06
lmo2689	lmo2689	Mg(2+) transport ATPase, P-type (EC 3.6.3.2)	1.68	**2.09**	-1.00
lmo2720	lmo2720	Acetyl-coenzyme A synthetase (EC 6.2.1.1)	1.42	**3.90**	1.14

Table S6.11 Significantly regulated genes unique in variant 17 compared to wild type and variants 5 and 6. Bold values represent significant regulation.

Gene	Gene	Product	Variant 5	Variant 6	Variant 17
lmo0050	lmo0050	Sensory transduction protein kinase (EC 2.7.3.-)	1.26	**1.97**	**-3.59**
lmo0051	lmo0051	Accessory gene regulator protein A AGRA	1.27	**1.97**	**-3.26**
lmo0164	lmo0164	Initiation-control protein	-1.19	-1.09	**-2.07**
lmo0165	lmo0165	Methyltransferase (EC 2.1.1.-)	-1.03	-1.01	**-2.15**
lmo0280	lmo0280	Anaerobic ribonucleoside-triphosphate reductase activating protein (EC 1.97.1.4)	-1.69	**-1.79**	**-3.06**
lmo0283	lmo0283	ABC transporter permease protein	-1.61	-1.38	**-2.28**
lmo0284	lmo0284	ABC transporter ATP-binding protein	-1.72	-1.44	**-2.18**
lmo0412	lmo0412	Hypothetical protein	-1.72	-1.60	**-2.28**
lmo0450	lmo0450	Hypothetical membrane spanning protein	-1.53	-1.13	**-2.82**
lmo0451	lmo0451	Hypothetical protein	-1.34	-1.12	**-2.34**
lmo0519	lmo0519	Lincomycin resistance protein	1.07	1.57	**-2.20**
lmo0523	lmo0523	Hypothetical protein	1.04	-1.03	**-2.04**
lmo0593	lmo0593	Formate/nitrite transporter family protein	1.59	1.12	**-2.93**
lmo0645	lmo0645	Amino acid permease	1.17	**1.73**	**-2.48**
lmo0675	lmo0675	Flagellar motor switch protein fliN	-1.13	-1.48	**-5.56**
lmo0676	lmo0676	Flagellar biosynthetic protein fliP	-1.25	-1.40	**-6.53**
lmo0677	lmo0677	Flagellar biosynthetic protein fliQ	-1.29	-1.33	**-7.25**
lmo0678	lmo0678	Flagellar biosynthetic protein fliR	-1.39	**-1.69**	**-8.60**
lmo0679	lmo0679	Flagellar biosynthetic protein flhB	-1.46	**-1.99**	**-7.41**
lmo0681	lmo0681	Flagellar biosynthesis protein flhF	-1.26	**-1.88**	**-5.51**
lmo0682	lmo0682	Flagellar basal-body rod protein flgG	-1.23	**-1.96**	**-5.38**
lmo0688	lmo0688	Glycosyltransferase (EC 2.4.1.-)	-1.15	**-1.96**	**-4.40**
lmo0809	lmo0809	Spermidine/putrescine transport system permease protein potC	1.16	1.24	**-2.03**
lmo0814	lmo0814	Enoyl-[acyl-carrier protein] reductase (NADH) (EC 1.3.1.9)	-1.28	-1.21	**-2.80**

Table S6.11 Continued.

Gene	Gene	Product	Variant 5	Variant 6	Variant 17
lmo0815	lmo0815	Transcriptional regulator, MarR family	-1.15	-1.24	**-2.45**
lmo0816	lmo0816	Protease synthase and sporulation negative regulatory protein PAI 1	-1.15	-1.13	**-2.01**
lmo0829	*nifJ*	Pyruvate dehydrogenase (ferredoxin/flavodoxin-dependent) (EC 1.2.7.-)	-1.34	-1.20	**-2.87**
lmo0850	lmo0850	Hypothetical protein	-1.17	-1.01	**-2.08**
lmo0897	lmo0897	Sulfate transporter	1.07	-1.21	**-2.93**
lmo0912	lmo0912	Formate/nitrite transporter family protein	-1.38	-1.03	**-3.03**
lmo0960	lmo0960	Peptidase family U32	-1.04	-1.29	**-3.97**
lmo0961	lmo0961	Peptidase family U32	-1.19	-1.39	**-5.27**
lmo1131	lmo1131	ABC transporter ATP-binding protein	-1.37	1.18	**-2.82**
lmo1132	lmo1132	ABC transporter ATP-binding protein	-1.52	-1.01	**-3.91**
lmo1298	*glnR*	Transcriptional regulator, MerR family	**1.81**	1.24	**-4.10**
lmo1299	*glnA*	Glutamine synthetase (EC 6.3.1.2)	1.66	1.06	**-2.53**
lmo1391	lmo1391	Nucleoside transport system permease protein	-1.67	**-1.98**	**-2.25**
lmo1407	*pflC*	Pyruvate formate-lyase activating enzyme (EC 1.97.1.4)	-1.69	-1.26	**-4.63**
lmo1540	*rpmA*	LSU ribosomal protein L27P	1.20	1.05	**-2.49**
lmo1541	lmo1541	hypothetical ribosome-associated protein	1.17	1.01	**-2.00**
lmo1589	*argB*	Acetylglutamate kinase (EC 2.7.2.8)	-4.03	-2.72	**-9.91**
lmo1593	lmo1593	Cysteine desulfurase (EC 2.8.1.7) / Selenocysteine lyase (EC 4.4.1.16)	-1.02	-1.28	**-2.17**
lmo1845	lmo1845	Guanine-hypoxanthine permease	-1.15	-1.14	**-2.20**
lmo1847	lmo1847	Manganese-binding protein	1.24	-1.24	**-2.05**
lmo1848	lmo1848	Manganese transport system membrane protein	1.12	-1.29	**-3.03**
lmo1917	*pflA*	Formate acetyltransferase (EC 2.3.1.54)	-1.99	**-1.90**	**-4.66**
lmo1955	lmo1955	Integrase/recombinase (XerD/RipX family)	-1.24	-1.27	**-2.38**
lmo2063	lmo2063	Hypothetical protein	-1.35	**-1.66**	**-2.00**
lmo2066	lmo2066	Hypothetical protein	-1.66	-1.35	**-2.25**
lmo2105	lmo2105	Ferrous iron transport protein B	-1.68	-1.49	**-4.24**
lmo2130	lmo2130	Amino acid permease	-1.14	-1.29	**-2.91**
lmo2150	lmo2150	Hypothetical protein	-1.22	-1.25	**-2.15**
lmo2180	lmo2180	Phage protein	1.13	1.53	**-2.39**
lmo2354	lmo2354	Metal-dependent hydrolase	-1.22	-1.42	**-2.05**
lmo2355	lmo2355	Multidrug resistance protein	-1.21	-1.27	**-2.23**
lmo2363	lmo2363	Glutamate decarboxylase (EC 4.1.1.15)	-1.23	-1.58	**-3.50**
lmo2457	*tpi*	Triosephosphate isomerase (EC 5.3.1.1)	-1.40	-1.36	**-2.05**
lmo2458	*pgk*	Phosphoglycerate kinase (EC 2.7.2.3)	-1.42	-1.43	**-2.24**
lmo2460	lmo2460	Central glycolytic genes regulator	-1.22	1.25	**-5.18**
lmo2605	*rplQ*	LSU ribosomal protein L17P	1.13	-1.10	**-3.02**
lmo2606	*rpoA*	DNA-directed RNA polymerase alpha chain (EC 2.7.7.6)	1.11	-1.10	**-2.29**
lmo2634	lmo2634	Cobalt transport protein cbiQ	-1.16	-1.04	**-2.05**
lmo2635	lmo2635	1,4-dihydroxy-2-naphthoate polyprenyltransferase (EC 2.5.1.-)	-1.25	-1.13	**-2.20**
lmo2638	lmo2638	NADH dehydrogenase (EC 1.6.99.3)	-1.08	-1.27	**-3.01**
lmo2640	lmo2640	Heptaprenyl diphosphate synthase component I (EC 2.5.1.30)	-1.10	-1.01	**-2.07**

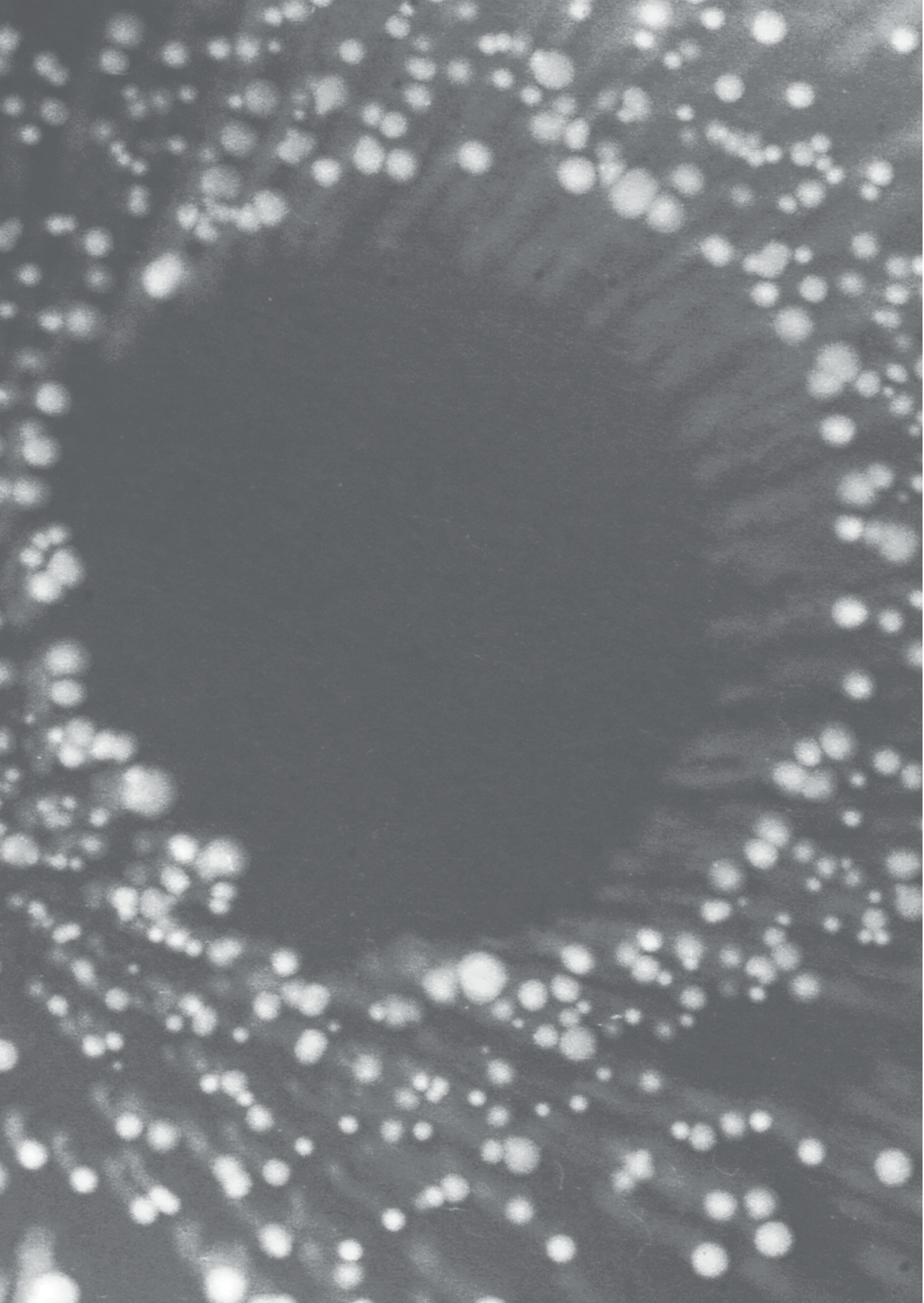
lmo2669	lmo2669	Hypothetical membrane spanning protein	-1.48	1.04	**-2.92**
lmo2717	*cydB*	Cytochrome d ubiquinol oxidase subunit II (EC 1.10.3.-)	-1.01	1.13	**-2.20**

Gene	Gene	Product	Variant 5	Variant 6	Variant 17
lmo0013	*qoxA*	Cytochrome aa3 quinol oxidase polypeptide II (EC 1.9.3.-)	**1.86**	1.26	**4.11**
lmo0014	*qoxB*	Cytochrome aa3 quinol oxidase polypeptide I (EC 1.9.3.-)	1.56	1.54	**3.09**
lmo0015	*qoxC*	Cytochrome aa3 quinol oxidase polypeptide III (EC 1.9.3.-)	1.54	1.35	**2.76**
lmo0016	*qoxD*	Cytochrome aa3 quinol oxidase polypeptide IV (EC 1.9.3.-)	1.58	1.32	**2.51**
lmo0115	*lmaD*	Antigen D	-1.06	-1.70	**2.75**
lmo0117	*lmaB*	Antigen B	-1.41	**-4.29**	**5.51**
lmo0118	*lmaA*	Antigen A	-1.47	**-3.55**	**4.57**
lmo0119	lmo0119	Hypothetical protein	-1.47	**-3.70**	**3.43**
lmo0120	lmo0120	Phage protein	-1.47	**-3.09**	**3.37**
lmo0121	lmo0121	Phage protein	-1.59	**-3.69**	**3.80**
lmo0122	lmo0122	Phage protein	-1.47	**-4.26**	**4.40**
lmo0123	lmo0123	Phage protein	-1.38	**-2.01**	**3.27**
lmo0124	lmo0124	Hypothetical protein	-1.38	**-3.85**	**4.84**
lmo0125	lmo0125	Hypothetical protein	-1.41	**-3.97**	**4.63**
lmo0126	lmo0126	Hypothetical protein	-1.44	**-3.30**	**4.50**
lmo0127	lmo0127	Weakly similar to protein gp20 from Bacteriophage A118	-1.46	**-3.28**	**3.73**
lmo0129	lmo0129	Sporulation-specific N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)	-1.44	**-2.86**	**3.70**
lmo0211	*ctc*	LSU ribosomal protein L25P	1.21	1.60	**2.46**
lmo0278	lmo0278	Sugar transport ATP-binding protein	-1.66	**-2.86**	**3.34**
lmo0342	lmo0342	Transketolase (EC 2.2.1.1)	-1.19	-1.22	**2.99**
lmo0343	lmo0343	Transaldolase (EC 2.2.1.2)	-1.45	-1.73	**4.57**
lmo0344	lmo0344	Short chain dehydrogenase	-1.54	**-2.02**	**5.38**
lmo0345	lmo0345	Ribose 5-phosphate isomerase (EC 5.3.1.6)	-1.31	-1.49	**4.71**
lmo0346	lmo0346	Triosephosphate isomerase (EC 5.3.1.1)	-1.71	**-2.13**	**4.36**
lmo0347	lmo0347	Dihydroxyacetone kinase (EC 2.7.1.29)	-1.57	**-2.11**	**4.95**
lmo0349	lmo0349	Hypothetical protein	-1.80	**-2.28**	**3.78**
lmo0351	lmo0351	Dihydroxyacetone kinase phosphotransfer protein	-1.53	**-2.13**	**3.31**
lmo0401	lmo0401	Alpha-mannosidase (EC 3.2.1.24)	-2.05	1.25	**3.96**
lmo0424	lmo0424	Glucose uptake protein homolog	-1.51	**-2.08**	**3.75**
lmo0425	lmo0425	Transcription antiterminator, BglG family / PTS system, mannitol (Cryptic)-specific IIA	**-2.25**	**-4.75**	**5.62**
lmo0426	lmo0426	Nitrogen regulatory IIA protein (EC 2.7.1.69)	**-2.00**	**-4.02**	**6.21**
lmo0427	lmo0427	PTS system, fructose-specific IIBC component (EC 2.7.1.69)	**-1.97**	**-2.89**	**4.65**
lmo0429	lmo0429	Alpha-mannosidase (EC 3.2.1.24)	-1.77	**-2.46**	**5.85**
lmo0443	lmo0443	Transcriptional regulator, LytR family	-1.11	1.03	**2.74**
lmo0484	lmo0484	Hypothetical cytosolic protein	1.24	1.42	**3.73**
lmo0517	lmo0517	Phosphoglycerate mutase family protein	**-2.50**	-1.10	**3.81**
lmo0592	lmo0592	Hypothetical protein	1.02	1.12	**3.04**

Table S6.11 Continued.

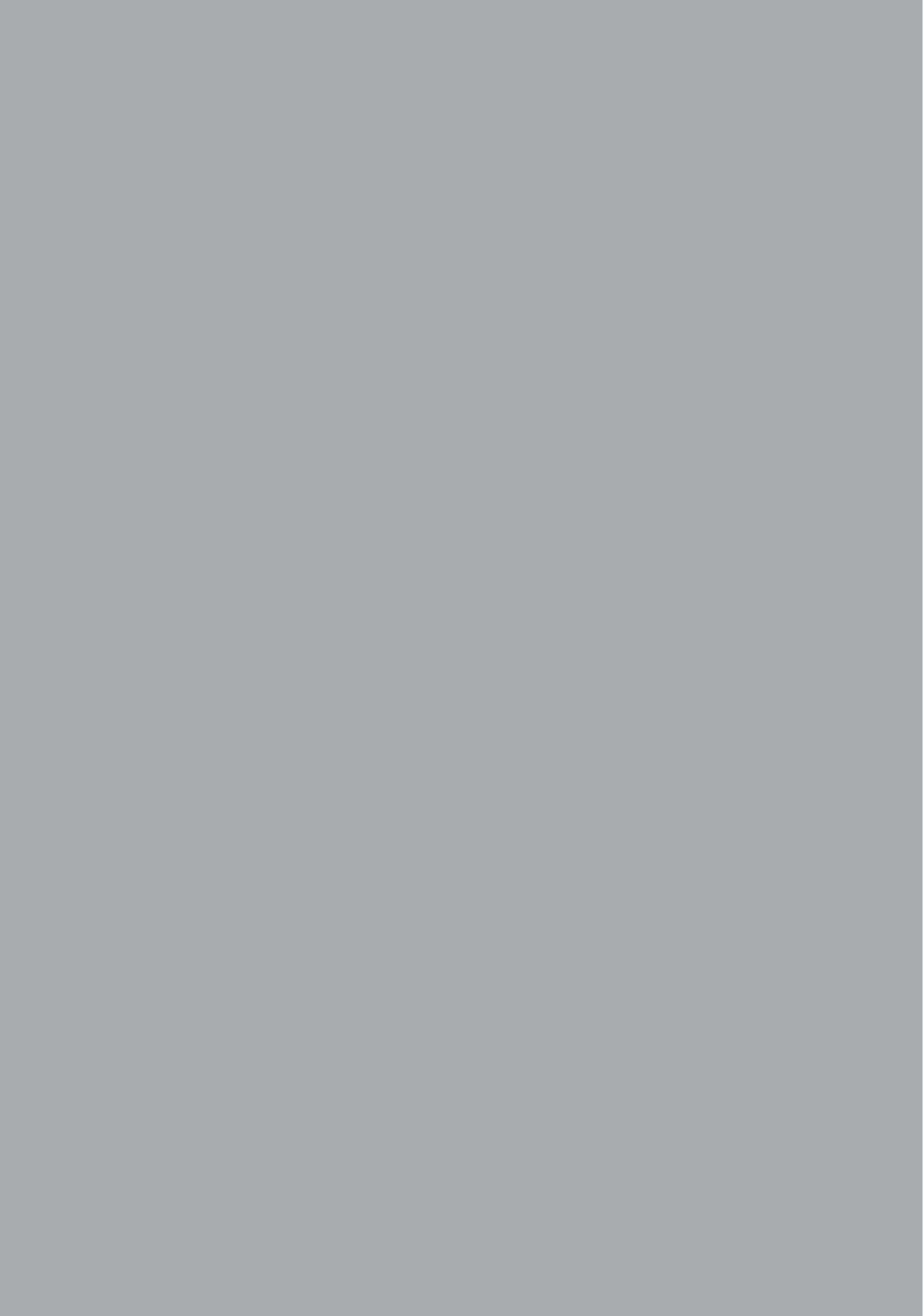
Gene	Gene	Product	Variant 5	Variant 6	Variant 17
lmo0782	lmo0782	PTS system, mannose-specific IIC component (EC 2.7.1.69)	1.45	**1.96**	**5.45**
lmo0783	lmo0783	PTS system, mannose-specific IIB component (EC 2.7.1.69)	1.37	**1.79**	**5.74**
lmo0784	lmo0784	PTS system, mannose-specific IIA component (EC 2.7.1.69)	1.34	**1.63**	**5.19**
lmo0791	lmo0791	Hypothetical protein	-1.22	**-1.65**	**4.48**
lmo0916	lmo0916	PTS system, cellobiose-specific IIA component (EC 2.7.1.69)	**-3.00**	**-5.59**	**3.85**
lmo0917	lmo0917	6-phospho-beta-glucosidase (EC 3.2.1.86)	**-3.21**	**-4.67**	**5.49**
lmo0918	lmo0918	Transcription antiterminator, BglG family	-1.65	-1.83	**5.61**
lmo1348	lmo1348	Aminomethyltransferase (EC 2.1.2.10)	-1.50	**-3.58**	**4.17**
lmo1349	lmo1349	Glycine dehydrogenase [decarboxylating] (EC 1.4.4.2)	-1.08	-1.60	**6.52**
lmo1350	lmo1350	Glycine dehydrogenase [decarboxylating] (EC 1.4.4.2)	1.05	-1.30	**6.66**
lmo1538	lmo1538	Glycerol kinase (EC 2.7.1.30)	1.18	**1.83**	**9.01**
lmo1539	lmo1539	Glycerol uptake facilitator protein	-1.02	1.54	**9.75**
lmo1579	lmo1579	Alanine dehydrogenase (EC 1.4.1.1)	-1.12	1.08	**2.62**
lmo1601	lmo1601	Similar to general stress protein	-1.10	-1.09	**2.58**
lmo1602	lmo1602	Hypothetical protein	-1.07	-1.13	**2.52**
lmo1730	lmo1730	Sugar-binding protein	-1.57	-1.32	**3.10**
lmo1731	lmo1731	Sugar transport system permease protein	-1.54	1.06	**3.15**
lmo1732	lmo1732	Sugar transport system permease protein	-1.52	1.04	**3.35**
lmo1877	lmo1877	Formate--tetrahydrofolate ligase (EC 6.3.4.3)	-1.04	1.08	**2.66**
lmo1975	lmo1975	DNA polymerase IV (EC 2.7.7.7)	1.11	-1.07	**3.17**
lmo1982	lmo1982	Ribosomal-protein-alanine acetyltransferase (EC 2.3.1.128)	1.09	1.17	**2.64**
lmo2121	lmo2121	Maltose phosphorylase (EC 2.4.1.8)	-1.16	**-6.57**	**8.79**
lmo2122	lmo2122	Maltodextrose utilization protein malA	-1.19	**-4.83**	**7.36**
lmo2123	lmo2123	Maltodextrin transport system permease protein malD	-1.28	**-8.59**	**7.98**
lmo2124	lmo2124	Maltodextrin transport system permease protein malC	-1.27	**-8.30**	**7.73**
lmo2125	lmo2125	Maltose/maltodextrin-binding protein	-1.31	**-10.97**	**8.09**
lmo2126	lmo2126	Cyclomaltodextrinase (EC 3.2.1.54) / Maltogenic alpha-amylase (EC 3.2.1.133)	-1.17	**-6.65**	**7.19**
lmo2291	lmo2291	Major tail shaft protein	-1.55	**-6.89**	**2.41**
lmo2297	lmo2297	Minor capsid protein	-1.52	**-5.62**	**2.71**
lmo2309	lmo2309	Hypothetical protein	-1.21	**-3.23**	**10.49**
lmo2310	lmo2310	Hypothetical protein	-1.24	-1.80	**6.78**
lmo2352	lmo2352	Transcriptional regulators, LysR family	1.36	**1.88**	**2.55**
lmo2437	lmo2437	Glyoxalase family protein	1.17	1.23	**3.38**
lmo2489	*uvrB*	Excinuclease ABC subunit B	1.31	1.45	**2.49**
lmo2539	*glyA*	Serine hydroxymethyltransferase (EC 2.1.2.1)	1.19	-1.26	**4.51**
lmo2585	lmo2585	Hypothetical protein similar to YrhD	**-2.54**	**-1.96**	**4.83**
lmo2586	lmo2586	Formate dehydrogenase alpha chain (EC 1.2.1.2)	**-2.06**	**-1.78**	**3.02**
lmo2646	lmo2646	4-Hydroxy-2-oxoglutarate aldolase / 2-dehydro-3-deoxyphosphogluconate aldolase	-2.61	-4.72	**17.86**

lmo2647	lmo2647	Creatinine amidohydrolase family protein	**-4.10**	**-7.25**	**22.98**
lmo2648	lmo2648	Parathion hydrolase (EC 3.1.8.1)	**-4.44**	**-8.78**	**20.14**
lmo2649	lmo2649	Putative transport protein sgaT	**-3.79**	**-5.11**	**14.66**
lmo2650	lmo2650	PTS SYSTEM, IIB COMPONENT (EC 2.7.1.69)	**-5.42**	**-8.69**	**14.82**
lmo2651	lmo2651	PTS system, mannitol-specific IIA component (EC 2.7.1.69)	**-4.04**	**-6.67**	**13.49**
lmo2675	lmo2675	Hypothetical protein	1.31	1.24	**4.61**
lmo2676	lmo2676	ImpB/MucB/SamB family protein	1.31	1.25	**4.20**
lmo2708	lmo2708	PTS system, cellobiose-specific IIC component	**-2.56**	**-1.72**	**5.53**
lmo2743	lmo2743	Transaldolase (EC 2.2.1.2)	1.20	1.23	**2.88**
lmo2762	lmo2762	PTS system, cellobiose-specific IIB component (EC 2.7.1.69)	**-2.44**	-1.45	**2.55**
lmo2763	lmo2763	PTS system, cellobiose-specific IIC component	**-2.05**	-1.08	**3.03**
lmo2824	lmo2824	D-3-phosphoglycerate dehydrogenase (EC 1.1.1.95)	-1.05	1.34	**3.19**
lmo2828	lmo2828	Hypothetical protein	1.12	-1.04	**5.38**



Chapter 7

Summary



A range of so-called minimal processing technologies has been developed that fulfill consumer demand for food products that are safe, healthy, have a good texture and flavor and a long shelf life. One of such processing technologies is high hydrostatic pressure (HHP). HHP utilizes intense pressure, usually about 400 to 600 MPa at chilled or mild process temperatures (<45°C) with common holding times ranging from 1.5 to 6 min. The first commercial HHP-processed foods were launched in 1990 including fruit products such as jams and fruit juices, followed by other products like sliced hams, sausages, oysters and guacamole. To date, HHP has been used as pasteurization process by (sub)lethally inactivating vegetative microorganisms, which needs to be combined with e.g. cooled storage to maintain microbial safety. Nowadays, also higher temperatures (>80°C) at short duration are used in HHP-processing to eliminate spores as well.

The U.S. Department of Agriculture, Food Safety and Inspection Service and the European Union Food Safety Authority recognize HHP-processing as an acceptable food safety intervention for eliminating spoilage and hazardous microorganisms such as *Listeria monocytogenes. L. monocytogenes* is ubiquitous in the ecosystem, and, once introduced in food-processing plants, hard to eradicate as it can grow in biofilms and a wide range of conditions (e.g. temperatures [0 to 45°C] and pH values [4.5 to 9.0] and NaCl concentrations up to 12%). *L. monocytogenes* can cause listeriosis, a severe human infection. The estimated annual rate of invasive listeriosis in the US is approximately 3 cases per million people per year. Although the incidence is low, the high mortality rates (20% - 30%) associated with listeriosis make *L. monocytogenes* one of the most important human foodborne pathogens.

Chapter 1 provides an overall introduction in the infection, disease and transmission of the foodborne pathogen *L. monocytogenes* as well as heterogeneity within the population. The impact of heterogeneity on the food-processing technology HHP is shown with inactivation models. HHP inactivation of *L. monocytogenes* was previously described with first-order inactivation kinetics, but also tailing of inactivation curves has been found. Tailing of survival curves of microorganisms can be an artifact of the experimental design but can also be the result of heterogeneity of the population because of physiological or even genetic changes supporting enhanced survival of the target microbes. Incomplete inactivation of microorganisms that can cause spoilage or foodborne illness can affect the quality and/or safety of products. These inactivation models can be integrated in microbial risk assessments that can help in the design of processes to ensure effective inactivation of pressure-resistant strains in foods. Such knowledge can contribute to improving the safety level of HHP-treated products.

Chapter 2 describes the pasteurization by HHP inactivation of three *L. monocytogenes* strains (EGDe, LO28 and Scott A) resulting in survival curves with significant tailing for all three strains. A biphasic linear model was used to fit this inactivation data, indicating the presence

of an HHP-sensitive and an HHP-resistant fraction. Heterogeneity within Scott A was already demonstrated before by isolation of stable HHP-resistant mutants. Therefore, only survivors isolated from the tail of LO28 and EGDe were analyzed. No stable HHP-resistant isolates were found for EGDe, but for LO28 it was revealed that the higher resistance was a stable feature for 24 of 102 (24%) tested isolates in the resistant fraction. These 24 HHP-resistant variants were 10 to 600,000 times more resistant than wild type when exposed to 350 MPa at 20°C for 20 min.

Chapter 3 describes a comparative phenotypic analysis of these 24 LO28 stress-resistant variants to assess their robustness and growth performance under a range of food-relevant conditions. Analysis of stress survival capacity, motility, biofilm formation, and growth under various conditions showed all variants to be more resistant to HHP and heat than the wild type; however, differences among variants were observed in acid resistance, growth rate, motility, and biofilm-forming capacity. In addition, genetic analysis was conducted focused on the *ctsR* gene and its upstream region, as two-thirds of the Scott A piezotolerant variants showed mutations in this gene. The *ctsR* gene encodes transcriptional repressor CtsR, that negatively regulates the expression of *clp* genes encoding ClpB, ClpP, ClpE and ClpC operon (encompassing *ctsR* itself), belonging to the class III heat shock genes. Because of mutations in the *ctsR* gene, the absence of (active) CtsR repressor results in increased expression of the *clp* genes, putatively conferring the high HHP tolerance. Next, we also performed genetic analysis to characterize the promoter region and open reading frame of the class I transcriptional repressor HrcA, which controls production of an additional set of stress proteins (GroEL, GroES, DnaK and DnaJ). These genetic analyses revealed no variation in the genetic make-up of *hrcA* and its upstream region, but two variants had deletions in the upstream region of *ctsR* and seven variants had mutations in the *ctsR* gene itself. The results of the characterization were cluster analyzed to obtain insight into the diversity of variants. Ten unique variants and three clusters with specific features could be identified: one cluster consisting of seven variants having a mutation in the CtsR regulator gene, one cluster containing two variants with an aerobic biofilm formation capacity like that of the wild type, and a cluster composed of five immotile variants. Notably, two other variants, not belonging to the CtsR regulator cluster, have a deletion upstream of their *ctsR* gene, as a result they lack the CtsR binding site. These two variants not only do not cluster with the CtsR regulator variants, but also do not cluster with each other. The possible occurrence of an additional mutation(s) in these variants cannot be excluded. The large population diversity of *L. monocytogenes* stress-resistant variants signifies the organism's genetic flexibility, which in turn may contribute to the survival and persistence of this human pathogen in food-processing environments.

Twelve representatives of these stress-resistant variants were characterized in Chapter 4 for their virulence potential and antibiotic susceptibility. Ten variants showed attenuated

virulence, whereas the other two variants (small colony variant 3 which can revert to wild type phenotype, and variant 5 capable of similar biofilm formation as wild type) performed comparable to wild type in a mouse model of infection. Seven of the ten attenuated variants demonstrated also a reduction in virulence factor (Phosphatidylcholine phospholipase C [PC-PLC] and listeriolysin O [LLO]) activity. Among the four variants with similar virulence factor activity is the CtsR variant, that showed reduced virulence in the mouse model. The other two variants with a deletion upstream of their *ctsR* gene, displayed only reduced LLO activity and significantly reduced infection levels in spleen or liver respectively. Compared to the wild type, all variants exhibited similar or increased susceptibility to multiple antibiotics commonly used in listeriosis treatment.

In Chapter 5 we aimed to investigate and quantify the occurrence of stable HHP-resistant EGDe variants and heat-resistant variants of both LO28 and EGDe using a so-called kinetic modeling approach. The basis of this approach is the inactivation kinetics of wild type and previously isolated HHP-resistant variants, combined with the estimated frequency of occurrence of these resistant variants in the initial population. From this information the time-point of highest probability of isolating resistant variants in a population during a stress treatment can be assessed. To increase the chances of isolating resistant variants, the population surviving this treatment can be regrown and exposed to the same stress. Due to the inactivation of sensitive variants, the fraction of resistant variants in this surviving population will be increased. With another stress-challenge cycle, the surviving population would contain mostly stress-resistant variants. This approach revealed that the probability of finding resistant variants should depend on the nature of the inactivation treatment and the time of exposure. At specific heat and HHP conditions, resistant LO28 and EGDe variants were indeed isolated at the expected time-points of highest probability. Notably, resistant LO28 variants could even be isolated after a heat inactivation for 6 s at 72°C in milk. These heat-resistant variants showed also high resistance to the pasteurization standard of the FDA: High-Temperature Short Time pasteurization of 15 s at 72°C of milk products with fat levels below 10%. The increased resistance of part of the isolated LO28 (25% of the HHP- and 38% of the heat-isolated) and EGDe (79% of the HHP- and 33% of the heat-isolated) variants was due to mutations in their *ctsR* genes. The other stress-resistant variants were not mutated in their *ctsR* genes and upstream regions, and the origin of resistance of these variants is yet unknown. The underlying mechanisms of increased resistance of these variants can be investigated by comparative transcriptome analysis and whole genome sequencing.

In the discussion session further insight in the three largest multi-resistant LO28 phenotypic clusters (CtsR, immotile, and normal aerobic biofilm forming variants) is obtained by gene-expression profiling of one variant of each group. All three variants showed upregulation of CtsR and HrcA regulated genes and downregulation of ArgR regulated genes. This indicates that both Class I and Class III heat shock genes play a role in multi-stress resistance. The exact

role of downregulation of arginine biosynthesis in stress resistance remains to be elucidated. From this data also a relation was found for the reduced virulence of two of these variants as they both showed downregulation of MogR regulated genes. To get more insight in the genetic differences, whole genome sequencing of selected variants and corresponding wild type(s) might elucidate the underlaying mutation(s) resulting in phenotypic diversity of the tested variants.

In conclusion, this thesis showed the strength of kinetic modeling, phenotypic and genomic analysis in unraveling the causes of nonlinear inactivation and facilitating the isolation of multi-stress resistant *L. monocytogenes* variants, giving biological insights. These insights may be used by food-producers to develop and implement (novel) minimal processing technologies that can control this pathogen and reduce *L. monocytogenes* associated food safety risks.

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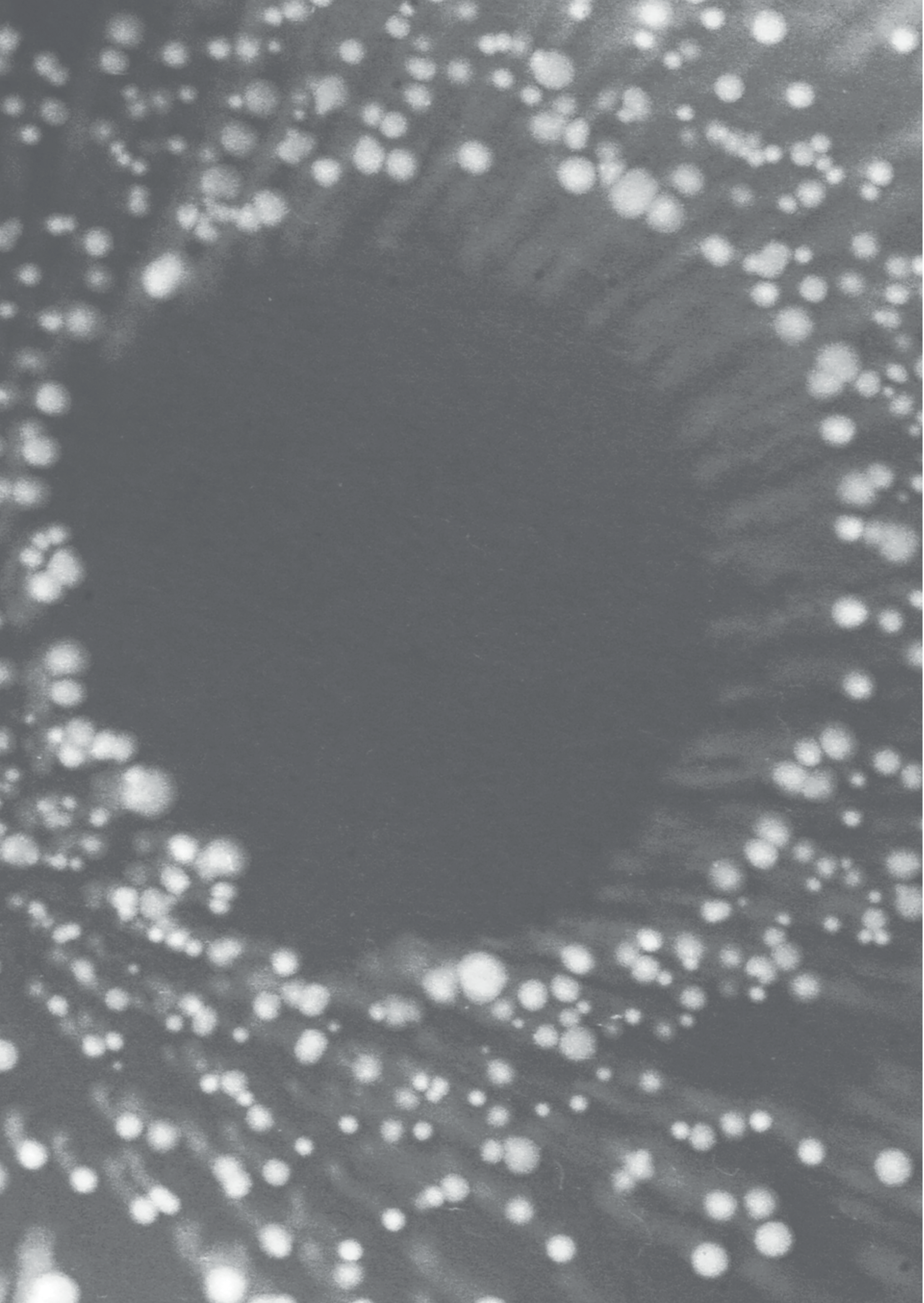
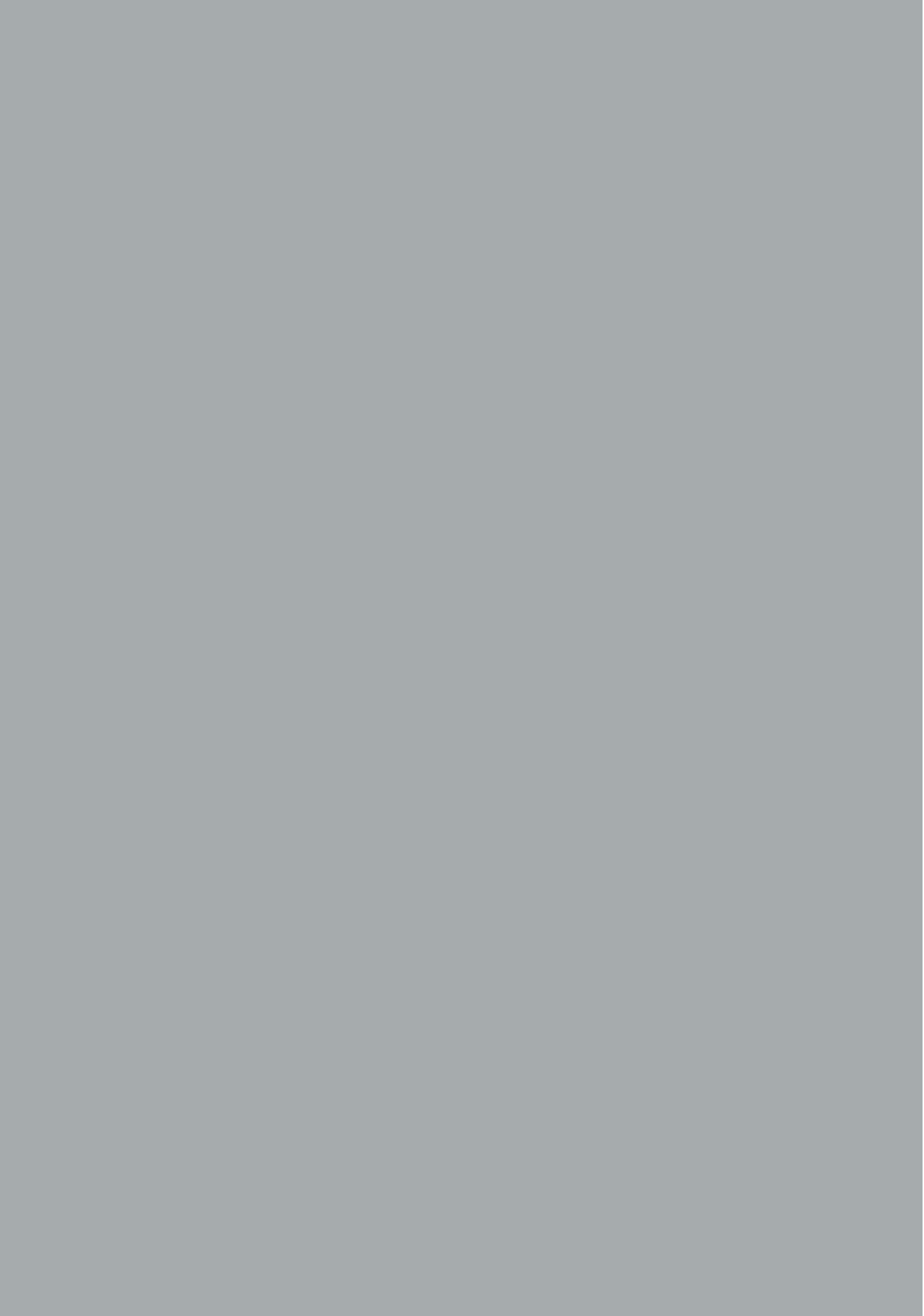


Appendices

Acknowledgements
About the author
List of Publications
Overview of completed training activities



Acknowledgements

“Cranes in Diepholz” October 2022 IvB

After almost two decades this topic will finally be closed. It was quite a flight, starting a PhD with two kids and ending it with a third one was a challenging route, especially knowing the risks of working with *Listeria monocytogenes*. But as most birds travel in groups to reduce risks, also I did not take this path alone. First and foremost, I would like to thank my supervisors Tjakko Abee, Marcel Zwietering and Roy Moezelaar. Tjakko, thanks for being an inspirator, coming up with new ideas and driving my enthusiasm in the practical work. In the past years you have never let me down with the scientific writing, you made time to discuss, and helped me to take the last steps. I am very grateful for that! Marcel, your critical and analytical view challenged me to refine my writing and images and my presentation skills, and you were also there to support me with these last difficult steps. Roy, as you were located closest to my working spot I could drop by any time with my questions and uncertainties. Also, for me, work pleasure is not all about the content, the context needs to be right too. You built a nice team of people within the C009 project, who helped me to feel at home and to enjoy work.

As I was located in the WFBR building, I had more regular contact with the people working there, but also the people of the Food Microbiology group were my much-appreciated colleagues in discussions and meetings. Next to that they became my buddies during WE-sport-days (we even won the first prize…), evenings in the pub and our amazing trips to South-Africa and Canada.

During my thesis I especially enjoyed supervising the practical courses at the University and supervising students. I probably hold a record for the number of students that joined my project. This helped me with the enormous amount of generated data. So, thanks a lot to: Casper, Sabina, Anaïs, Wladir, Parusha, Zheng, Bram, Sjoerd, Thomas and Avelino.

A special thanks to my two paranymphs, Mart and Merel. Mart, I feel proud that you are my son, I am sometimes amazed that part of you is my genetic heritage. Merel, our friendship lasts already longer than this whole project and will last forever. It is my pleasure that you are both next to me during my thesis defense.

It is impossible to do a PhD project without the support of your family. I sometimes made the joke that the whole route would have been shorter if they would not have been there, but actually I could not have done it without them. Willem, thank you for being you and being there when I needed it most. It is great to be able to celebrate this together! Mart, Wieke and Frank, so happy to have you around and make our family complete! Although I miss my parents that I lost during this long route, I am blessed with all the family, friends and colleagues that are here today!

About the author

Ineke van Boeijen was born on 30 March 1973 in Hattem, the Netherlands. In 1991 she graduated from secondary school in Zwolle and continued with Applied Science at Saxion in Deventer, where she finished her BSc in Biomedical chemistry in 1995. As she specialized in Biotechnology, she continued her studies at Wageningen University, but with a new focus: Food Technology. She finished her MSc in 1998 and found her first job at Mars Food Europe in Oud-Beijerland as a product developer. After two years of developing sauces for the brands Uncle Ben's and Dolmio, she decided to return to the field of Microbiology. She moved to Zeist to work as researcher at TNO Food, starting in the detection of GMOs in food. After the birth of her first son, Mart in 2001, she started working in the group of Prof. dr P. J. Punt focusing on molecular work in fungi. Working in this research environment inspired her to make a next step by starting a PhD. She found the perfect position within TI Food and Nutrition, so her family (Willem, Mart and their daughter Wieke, born in 2003) moved to Wageningen. As she worked with specific equipment (High Hydrostatic Pressure), she was located at the Wageningen Food and Biobased Research institute. During the 5 years of research, she especially enjoyed the practical work and the supervision of students. In 2007 another son, Frank, was born. As she worked with *Listeria monocytogenes*, special care was taken to ensure she could still perform her research without any concern for the unborn baby. After her PhD research she changed from the bad bugs to the good bugs with her new position at CSK Food Enrichment in Ede as Scientist Bioprocess-technology for lactic acid bacteria. Currently, after 13 years, she still works in the same area as Managing Senior Scientist Bioprocess Optimization at DSM Food Specialties in Wageningen after they took over the business from CSK. Within these years she finished her publications and the final publication, this thesis, you are reading now.

List of Publications

Van Boeijen, I. K. H., R. Moezelaar, T. Abee, and M. H. Zwietering. 2008. Inactivation kinetics of three *Listeria monocytogenes* strains under high hydrostatic pressure. J. Food Prot. 71:2007-2013.

Van Boeijen, I. K. H., A. A. E. Chavaroche, W. B. Valderrama, R. Moezelaar, M. H. Zwietering, and T. Abee. 2010. Population diversity of *Listeria monocytogenes* L028: phenotypic and genotypic characterization of variants resistant to high hydrostatic pressure. Appl. Environ. Microbiol. 76:2225-2233.

Van Boeijen, I. K. H., C. Francke, R. Moezelaar, T. Abee, and M. H. Zwietering. 2011. Isolation of highly heat-resistant *Listeria monocytogenes* variants by use of a kinetic modeling-based sampling scheme. Appl. Environ. Microbiol. 77:2617-2624.

Van Boeijen, I. K. H., P. G. Casey, C. Hill, R. Moezelaar, M. H. Zwietering, C. G. M. Gahan, and T. Abee. 2013. Virulence aspects of *Listeria monocytogenes* LO28 high pressure-resistant variants. Microb. Pathog. 59-60:48-51.

Overview of completed training activities

Discipline specific activities

Courses

Genetics and Physiology of Food Associated Microorganisms (VLAG, Wageningen)	2004
Systems Biology Course: Principles of ~Omics Data Analysis (VLAG, Wageningen)	2006
Management of Microbiological Hazards in Foods (VLAG, Wageningen)	2006

Meetings and conferences

Safety and Shelf-life symposium (NVvM, SMAS, TNO)	2005
Symposium: Developments in the Area of Food Safety (EFFI)	2005
FoodMicro 2006, Bologna, Italy (oral presentation) (ICFMH)	2006
FoodMicro 2008, Aberdeen, United Kingdom (poster presentation) (ICFMH)	2008

General courses

Oral Presentation (WCFS, COPLA)	2004
VLAG PhD week (Bilthoven)	2004
Project and Time Management (WGS, Valley consult)	2005
END-note Course (WUR)	2005
PhD Scientific English Writing (CENTA)	2005
Professional Communication Strategies (WGS)	2006
Debating Course (WCFS)	2006
Philosophy and Ethics of Food Science and Technology (VLAG, Wageningen)	2008
Training in Writing Press Releases (TIFN)	2009

Teaching activities

Supervisor of Practical BSc and MSc Food Microbiology Courses (WUR)	2005-2006
Supervision of Students (internship, thesis)	2006-2008

Other activities

Preparation of PhD Research Proposal	2004
WCFS/TIFN C009 Project Meetings and Food Microbiology Seminars (WUR)	2004-2008
VLAG PhD Study Trip Laboratory of Food Microbiology, South Africa (organization)	2005
VLAG PhD Study Trip Laboratory of Food Microbiology, Canada	2008

The research described in this thesis was performed within the framework of TI Food and Nutrition.

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Cover SEM photo from Adriaan van Aelst (*Listeria monocytogenes* LO28 *ctsR* variant 8)

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