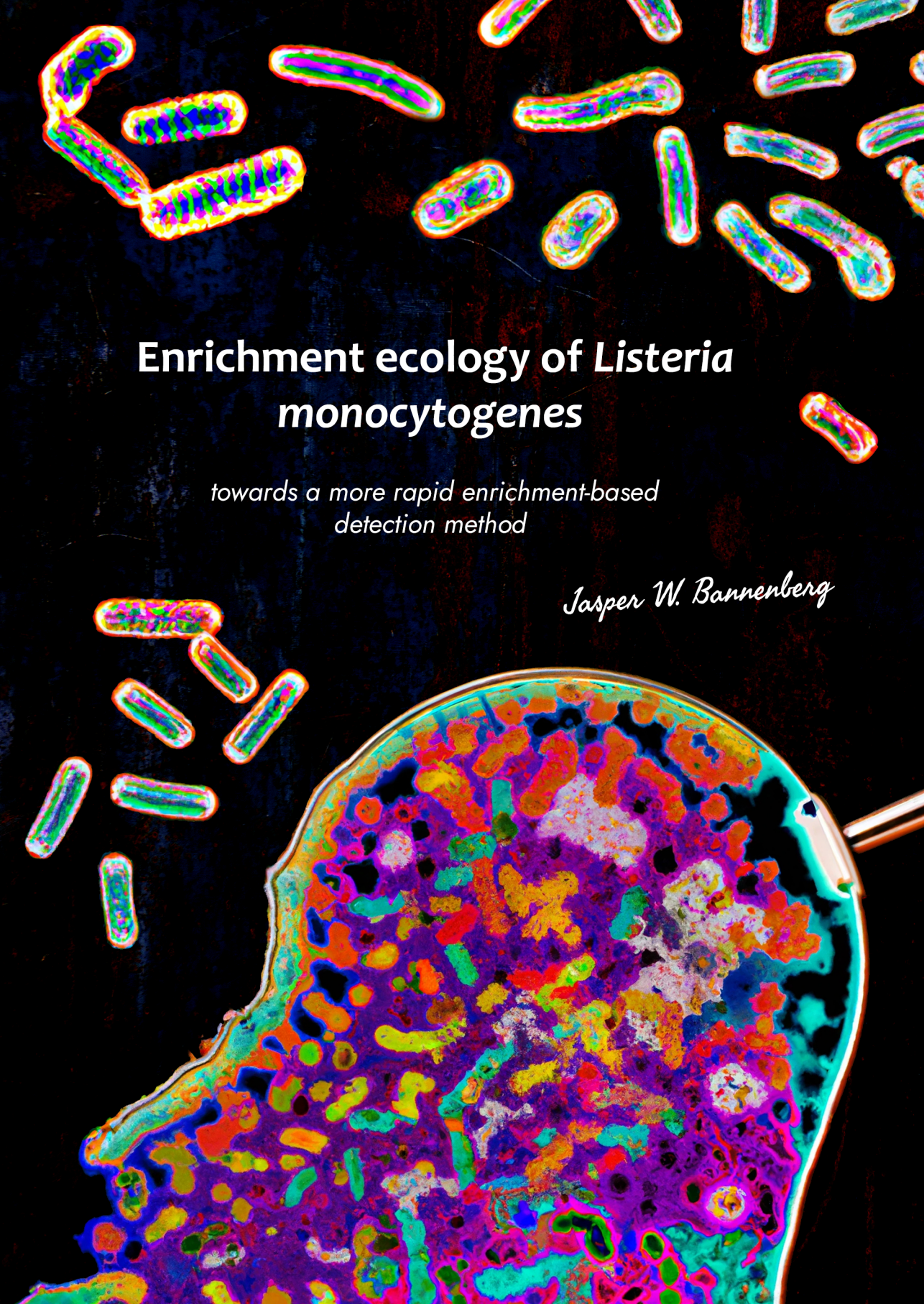

Enrichment ecology of *Listeria monocytogenes*

towards a more rapid enrichment-based detection method

Jasper W. Bannenberg

Propositions

1. Reducing the selectivity of the current ISO 11290-1 enrichment medium is necessary for proper resuscitation of stressed *Listeria monocytogenes* cells.
(this thesis)

2. Rapid detection of *Listeria monocytogenes* is feasible by moving towards a combination of cultural enrichment and molecular detection.
(this thesis)

3. Animal experimentation for scientific progress becomes increasingly harder to justify with the growing knowledge on animal sentience.

4. The performance-oriented culture within academia fosters above average development of mental health problems for researchers.

5. Prioritizing the reduction of meat and dairy agriculture is key in averting disastrous climate change.

6. Measuring economic growth solely by monetary increase undermines the transition to a more sustainable circular economy.

Propositions belonging to the thesis entitled

Enrichment ecology of *Listeria monocytogenes* – towards a more rapid enrichment-based detection method

Jasper W. Bannenberg

Wageningen, 23rd of May, 2023

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Thesis

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General introduction

Jasper W. Bannenberg

1

1.1. Food safety

Safe food is vital for everyone and controlling food safety requires the combined efforts of all stakeholders in the whole food chain, from primary production to food processing and retail to the consumer side. However, inadequate adherence to these combined efforts at any stage of food production can result in unsafe food. The World Health Organization (WHO) estimated that between 420 and 960 million foodborne illnesses are contracted each year, leading to between 310,000 and 600,000 deaths annually (Mehlhorn, 2015). Zooming in to the Netherlands, there were 553,000 estimate cases of foodborne illness in 2020 with 76 fatal cases (Benincà et al., 2021). Both from a public safety as well as an economic perspective, unsafe foods are a significant burden on society.

However, controlling the safety of food is not an easy task and this is complicated by challenges along the food chain. Food products can be contaminated along the food production chain and food products are often able to support the survival and/or growth of microorganisms. Foodborne viruses and bacterial agents are a major cause of foodborne infections and illnesses, ranging from mild gastroenteritis to life-threatening bacterial infections (Cliver & Riemann, 2011; O'Shea et al., 2019). It is therefore extremely important that the presence of foodborne pathogens in foods is under strict control. Foods usually undergo processing steps in which the bacterial load is reduced, such as pasteurization or other (non) thermal treatments and/or the use of preservatives. For the products to remain safe during the rest of manufacturing, adequate environmental hygiene in production facilities is mandatory to significantly reduce the chance of contamination later in the process. This means thorough cleaning and disinfection in production areas, food storage, and transport. Lastly, poor personal hygiene practices of food handlers and consumers pose another risk for unsafe food (Schneider et al., 2010). Simple precautions like hand washing and prevention of cross-contamination in cutting and handling of raw foods is important to reduce the risk of foodborne illness (Hillers et al., 2003).

1.2. *Listeria monocytogenes*: a dangerous foodborne pathogen

The relevance of controlling food pathogens such as *Listeria monocytogenes* came once again to the forefront of worldwide news in June of 2017. The number of cases of patients suffering from *L. monocytogenes* infection started to grow rapidly in South Africa, which would eventually lead to the world's largest outbreak of *L. monocyto-*

genes with 1,060 laboratory-confirmed cases of listeriosis and 216 deaths reported by the National Institute of Communicable Diseases (NICD), in the period between January 2017 to July 17 2018 (Smith et al., 2019). Although outbreaks of *L. monocytogenes* are quite rare, the risk of severe or lethal outcomes is large due to the high mortality rate of 13.0 % in 2020 over the whole EU (European Food Safety Authority, 2021). This makes that this foodborne pathogen is under strict control by food processors and authorities.

Listeria species are Gram-positive, rod-shaped, non-spore forming, and facultative anaerobic bacteria of the order *Bacillales*. As of 2020, the genus *Listeria* consists of 21 recognized species and 6 subspecies (Carlin et al., 2021). The genus can be subdivided into *Listeria* sensu stricto and *Listeria* sensu lato of which two representatives of the former clade are able to colonize mammalian hosts. Pathogenicity of the sensu stricto clade correlates with the presence of intact virulence clusters that can promote colonization, entry into host cells and inter- and intracellular movement (Mishra et al., 2011). Next to *L. monocytogenes*, the only other pathogenic member is *Listeria ivanovii*, which rarely infects humans and is more of a problem in ruminants (Alexander et al., 1992).

1.2.1. Listeriosis and infectivity

Listeriosis is the name for illness in humans caused by invasive infection with *L. monocytogenes*. Although healthy individuals can contract listeriosis, the populations that are most susceptible are the young, the elderly (over 64 years), and the immunocompromised. Pregnant women are also more at risk, where listeriosis can lead to spontaneous abortion, still birth, or foetal death (Rappaport et al., 1960). Although the incidence of invasive infection of *L. monocytogenes* is quite low in comparison to other food pathogens at 0.42 reported cases per 100,000 inhabitants in the European Union (EU), listeriosis has the highest hospitalization rate of all zoonoses under EU surveillance (European Food Safety Authority, 2021). The individual disease burden of listeriosis estimated by disability adjusted life years (DALY) per case is also the highest of all pathogens in the Netherlands with 2 DALY/case and with the cost of illness of 48,000 euro per case (Benincà et al., 2021). Invasive infection is mainly facilitated by strictly regulated pathogenicity clusters. When *L. monocytogenes* enters a host cell, it can escape the vacuole into the cytoplasm by expression of listeriolycin O (Quereda et al., 2018). In the cytoplasmic space it can replicate, and use the host cell actin together with ActA for intracellular motility (Kathariou, 2002). When engulfed by adjacent host cells, bacteria can escape from the double-membrane envi-

ronment by listeriolycin O and phospholipases (Quereda et al., 2018), finally leading to systemic infection in case the host immune system fails to control the infection (D'Orazio, 2019).

There exists quite a wide variation between strains of *L. monocytogenes* and this is exemplified by the classification in 14 serotypes distributed among four different lineages, lineage I to IV (Moura et al., 2016; Yin et al., 2019). Of these 14 serotypes, over 95 % of the strains isolated from humans and food products belong to the serotypes 1/2a, 1/2b, 1/2c, and 4b (Pontello et al., 2012; Swaminathan & Gerner-Smidt, 2007). Strains of these four serotypes are either more likely to survive in the food chain or are better genetically equipped to infect susceptible people. Although serotyping used to be the main method for epidemiological surveillance and outbreak investigation, this is nowadays largely taken over in favour of whole genome sequencing (Buchanan et al., 2017; Lassen et al., 2016). Although the incidence of listeriosis is low – especially in healthy individuals – the disease burden at individual and societal level means that *L. monocytogenes* is considered as an important food pathogen. It is therefore crucial to control this pathogen successfully.

1.2.2. Survival in food processing environments

As a food pathogen, *L. monocytogenes* has quite specific and unique characteristics compared to other food pathogens. Most important of all is its ability to grow at low temperatures. Its wide growth range between 0 °C and 45 °C means it is capable of growing at refrigeration temperatures, which severely limits the use of refrigerators to prevent its growth on foods. Furthermore, it can also tolerate low water activity and is one of the few foodborne pathogens that can grow at water activity levels below 0.93 (Farber et al., 1992; Fontana JR, 2020). The robustness of *L. monocytogenes* is further exemplified by the ability to grow at pH values as low as 4.3 (van der Veen et al., 2008), and salt concentrations of up to 14 % (w/v) (Shabala et al., 2008). Its robustness and ability to grow at a wide range of environmental conditions makes it difficult to control in food processing environments (Lee et al., 2019). Also, the widespread occurrence of the pathogen in nature and especially its niche in soil contributes to its transmission to foods (Vivant et al., 2013). The overall prevalence of *L. monocytogenes* can be up to 19 % in soil (Sauders et al., 2012), which shows that its ubiquitous survival in soil may play a pivotal role in the transfer of this pathogen to cultivated plants and farm animals and subsequently to contamination of food via equipment, food-contact surfaces, and the environment in food processing facilities and by food handlers (Luber et al., 2011). This is facilitated by its ability to activate sigma B-dependent stress

defence and to form biofilms in food processing environments (Møretrø & Langsrud, 2004; NicAogáin & O'Byrne, 2016). Biofilms are composed of surface-attached bacterial cells embedded in an extracellular matrix, and can be very difficult to eradicate from surfaces by cleaning and disinfection. *L. monocytogenes* can adhere and form biofilms on all materials used in food processing environments (Mazaheri et al., 2021) and can persist for years (Martínez-Suárez et al., 2016; Møretrø & Langsrud, 2004; Orgaz et al., 2013). Release of (clumps of) cells from biofilms can result in contamination of food products if not dealt with adequately (Carrasco et al., 2012). However, a single efficient method has not been found to avoid the establishment and propagation of biofilms in food processing environments. Instead, controlling *L. monocytogenes* persistence is best achieved by reducing or eliminating the risk of recontamination due to the presence of biofilms with effective control measures at each entry point (Mazaheri et al., 2021).

1.2.3. Risk products: ready-to-eat foods

Among the most commonly consumed food products during the outbreak in South Africa were ready-to-eat meat sausages called "polony". After the outbreak of listeriosis in South Africa was officially declared, clinical microbiology laboratories in South Africa were formally requested to send all isolates to the Centre of Enteric Diseases (CED) department of the National Institute for Communicable Diseases. All isolates were phenotypically identified and the genomic DNA was sequenced in order to trace the origins of the outbreak (Smith et al., 2019). These laboratory findings eventually led to investigation of a large processed meat production facility called "Enterprise Foods", and a recall of food products was initiated and production was shut down in March 2018 (Smith et al., 2019). Is it not surprising that the source of the largest recorded listeriosis outbreak was caused by processed meat. Ready-to-eat meats and delicatessen (deli) meats are foods which are often associated with listeriosis outbreaks. Other ready-to-eat foods with high risks of transmitting *L. monocytogenes* are smoked fish (e.g. cold-smoked salmon) and soft cheeses (mostly European raw soft cheese) (Lopez-Valladares et al., 2014; Raheem, 2016). Additionally, plant-derived foods and frozen foods (e.g. cantaloupe, ice cream) have been implicated in outbreaks (Laksanalamai et al., 2012; Ottesen et al., 2016; Pouillot et al., 2016). This illustrates that most ready-to-eat foods are risk products for *L. monocytogenes* and have the potential to contribute to disease when consumed by susceptible people (Ricci et al., 2018).

Ready-to-eat food products are foods that will not be cooked or reheated before

consumption (Luber et al., 2011). For ready-to-eat food products the risk of cross contamination can be substantial. Some examples include cutting of deli meats with a contaminated cutter, or the repackaging of soft cheeses on improperly cleaned working surfaces. The risk of cross contamination of ready-to-eat products increases along the supply chain (Acciari et al., 2016; Gwida et al., 2020), and investigations can be difficult to carry out when products are handled by different businesses. The risk of such products seems to grow with the increasing demand for minimally processed foods from consumers and retail, and this puts more pressure on food producers to maintain safe foods.

1.3. How is *L. monocytogenes* controlled?

Food safety management is a combination of requirements at the legal and governmental level and the processes at the food producer side. Requirements of food safety management systems is standardised in ISO 22000:2018 (International Organization for Standardization, 2018) in order to control food safety related hazards. Food safety is a joint responsibility of all participants in the food chain, and as such this standard encompasses all operators involved in production, processing, marketing, and sale of food products. An overview of the control measures along the food chain is given in figure 1.

1.3.1. Safety by design

Food safety starts with Good Agricultural Practices at the primary production location, where procedures are implemented to minimize risks of microbial food safety hazards in the agricultural chain. Further along the food chain, food safety management at the processing stage is adapted. Food safety management relies for a large part on HACCP (hazard analysis and critical control points). HACCP is a focused approach where the aim is to identify, evaluate, and control hazards that are significant for food safety during the food processing process. Here, it is critical that good manufacturing practices and good hygiene practices are taken into account, which provide the minimum requirements that a manufacturer must meet to ensure consistency of its product. A HACCP approach attempts to control hazards rather than relying on inspection of the final products, which provides a more reliable and less costly method than strict end-point testing to ensure safe foods. At any stage in the food production chain hazards can be introduced, and the aim of HACCP is to identify significant hazards and to estimate the likelihood and severity that these hazards have on the safety of a food product. Critical limits need to be established for each critical con-

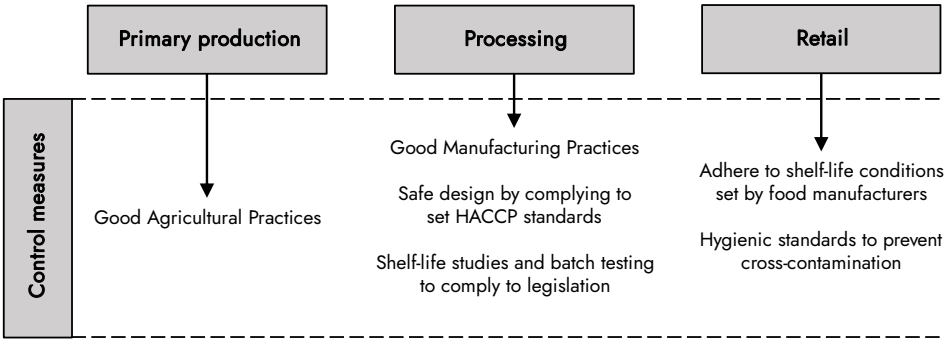

Figure 1: Overview of *L. monocytogenes* control measures along the food chain. Control at primary production is mainly done by implementing Good Agricultural Practices. At the processing stage, Good Manufacturing Practices should be applied and safety should be initiated by setting up HACCP protocols and adhering to critical limits. Shelf-life studies should be done to verify that product quality and safety is valid over the complete shelf-life of the product, and testing of batches is required to comply to legislation. In retail it is necessary to adhere to the shelf-life conditions set by the food manufacturer and to apply hygienic standards to prevent post-production cross-contamination.

trol point to set the maximum deviation that this point is allowed to have, and which corrective actions have to be taken when this maximum deviation is reached. HACCP regulation further requires that records are kept monitoring the validity of the implementation of the hazard protocols. Furthermore, food producers can do shelf-life studies to show that their products adhere to food safety limits laid out in legislation during the complete shelf-life.

The risk associated with *L. monocytogenes* is thus mainly dependent on the effectiveness of the control measures that are implemented by the food operators. In order to verify whether these food operators correctly implement HACCP and adhere to the legal framework, product sampling is still necessary. At the processing stage, sampling can be done by industry during shelf-life studies or to check their own HACCP, but official sampling is done by the competent authorities (European Food Safety Authority, 2021). At retail level, retailers must make sure that they adhere to the shelf-life conditions that are set up by the food manufacturers and that proper hygienic standards are applied to control cross-contamination. In 2020 136,346 sampling units were tested in ready-to-eat products across 24 member states of the EU (European Food Safety Authority, 2021), to test whether these foods adhered to European legislation.

1.3.2. Legislation in food

Because of the serious risks associated with foods contaminated with *L. monocytogenes*, regulations have been set up by the European Commission that apply for the whole European Union (European Commission, 2007). The regulations are laid out in (EC) No. 1441/2007 and they are summarised in figure 2. Here, the distinction is made between ready-to-eat foods that are intended for infants and foods with special medical purposes, and other products which are either able to support the growth of *L. monocytogenes* or not. Next to this, the regulation specifies limits that food producers have to comply to either at the end of processing and during the complete shelf-life of the product. Here, the processing stage encompasses foods before they have left the immediate control of the food producer, and for the product during their shelf-life on the market. For ready-to-eat foods that are not intended for infants of special medical purposes, the food producer has to show that the food product is unable to support the growth of *L. monocytogenes* during its shelf-life. The limit is set at 100 CFU/g during the shelf-life based on enumeration (in 5 samples, n = 5), or absence must be shown in 5 samples (n = 5) of 25 g where not a single cell of *L. monocytogenes* may be detected (c = 0). Furthermore, absence in 10 samples of 25 g also has to be guaranteed for ready-to-eat foods intended for infants or for foods with special medical purposes.

1.4. Current *L. monocytogenes* detection in food

1.4.1. Detection with ISO 11290-1

In order to comply to the legislation set out for *L. monocytogenes* in food products, a reliable detection method is a prerequisite. According to the European Commission, methods to detect presence of *L. monocytogenes* in raw materials, intermediate or end products, include EN ISO 11290-1 or equivalent validated methods according to EN ISO 16140-2. The ISO 11290-1 is a standardised culturing-based method for detection of *L. monocytogenes* and other *Listeria* spp. in the food chain, and was last updated in 2017 (International Organization for Standardization, 2017). According to legislation, absence has to be demonstrated in 5 samples of 25 g food product at processing level for products that support the growth of *L. monocytogenes*. This then means that one cell in 250 ml enrichment broth needs to be detected. It is therefore necessary that samples are enriched to increase the concentration of cells until the detection limit of the specific detection method. For most efficient recovery of *Listeria* cells, a non-selective enrichment medium would be optimal. On the other hand, food products are often contaminated with considerable larger number

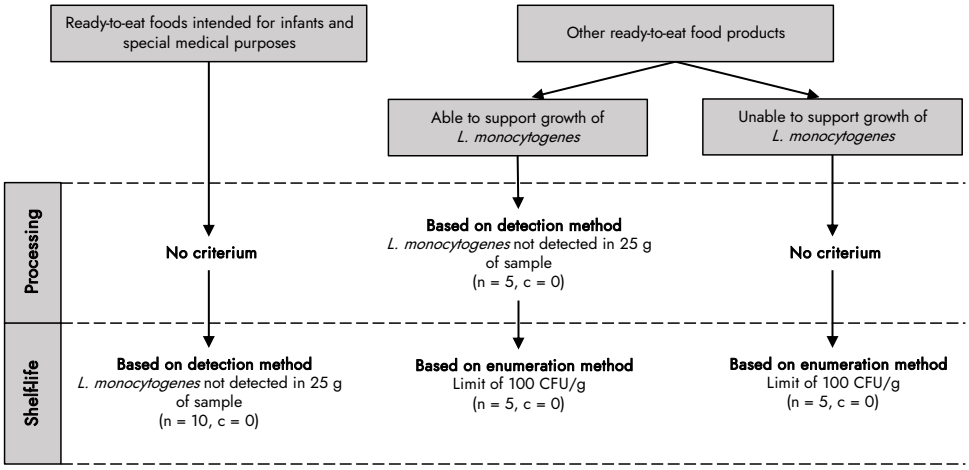

Figure 2: Food safety criteria on *L. monocytogenes* across the ready-to-eat food chain as laid out by the European Commission in (EC) No. 1441/2007 (European Commission, 2007). Ready-to-eat foods are categorised in foods intended for infants and foods with special medical purposes and other foods that are either able to support growth of *L. monocytogenes* or not. Criteria are laid out for limits at the end of the processing stage (before the product has left the immediate control of the food producer), and for the complete shelf-life of the product. The limit of 100 CFU/g only applies if the manufacturer is able to demonstrate to the satisfaction of the competent authority, that the product will not exceed this threshold throughout the complete shelf-life. Figure adapted from the European Union one health 2020 zoonoses report (European Food Safety Authority, 2021).

of other microbiota, and therefore a selective enrichment could be necessary (International Organization for Standardization, 2017). There needs to be a balance between optimal recovery and suppression of competing microbiota, and for this the EN ISO 11290-1 detection method consists of a two-step enrichment (figure 3). The current enrichment medium, Fraser broth (FB), was a modification of the university of Vermont-modified *Listeria* enrichment broth when it was found that *Listeria* species were able to hydrolyse aesculin of which the product can react with ferric ions to blacken the medium (Fraser & Sperber, 1988). Fraser & Sperber (1988) changed this medium by adding lithium chloride, acriflavine, and ferric ammonium citrate. The modification of the enrichment broths to FB reduced the detection time of intact *L. monocytogenes* from two weeks to 48 hours (Fraser & Sperber, 1988). Selectivity of the medium is facilitated mainly by the addition of lithium chloride, acriflavine hydrochloride, nalidixic acid, and sodium chloride (International Organization for Standardization, 2017). Lithium chloride inhibits mainly lactose-fermenting Gram-negative bacteria (Cox et al., 1990), acriflavine hydrochloride is an RNA-synthesis inhibitor (Meyer et al., 1972), nalidixic acid acts as a DNA-gyrase inhibitor (Crumplin &

Smith, 1975), and *L. monocytogenes* is naturally able to adapt and survive in high salt concentrations (Gandhi & Chikindas, 2007). These compounds are used to suppress the growth of other microbiota that may co-occur on foods. However, when *L. monocytogenes* cells are injured during food processing, they can become more sensitive to these selective compounds (Jasson et al., 2007; Miller et al., 2006). Therefore, the primary enrichment is done in a slightly modified version of FB called half Fraser broth (HFB), which due to its halved concentration of nalidixic acid and acriflavine hydrochloride at least partly fulfils the function of recovery of stressed and/or injured *Listeria* cells.

The enrichment starts with the primary enrichment of 25 g or 25 ml of sample in 225 ml of HFB and this mixture is incubated at 30 °C for 24-26 h. The enrichment is continued with transfer of 0.1 ml of HFB to 10 ml FB with a higher amounts of selective components to further suppress competitors during incubation at 37 °C for 24 ± 2 h. Isolation and identification of microorganisms is done by plating on ALOA (agar Listeria according to Ottaviani and Agosti) and a secondary selective medium after both enrichment steps. Colonies of *Listeria* spp. on ALOA can be considered presumptive when the colonies are blue-green. Presumptive colonies for *L. monocytogenes* are blue-green surrounded by an opaque halo due to hydrolysis of a chromogenic compound by the virulence protein phosphatidylinositol-specific phospholipase C (Vlaemynck et al., 2000). Presumptive colonies are confirmed by isolating them on non-selective agar followed by confirmation tests (microscopy, beta-haemolysis test, growth on L-rhamnose, and lack of growth on D-xylose).

1.4.1.1. Enrichment is a black box that needs further elucidation

Although the detection method is the current gold standard, the protocol is labour-intensive and time-consuming, with a required 4-7 days for a fully processed sample. Therefore there is interest in reducing the time of the enrichment-based procedure while still remaining a reliable procedure. With respect to the ISO protocol from 2004, the primary enrichment step has already been halved from 48 h to 24 h without affecting the recovery and selectivity of the method (Gnanou Besse et al., 2016). It is therefore unlikely that further reduction of the enrichment time is possible without impacting the effectivity of the method. Also the shortcoming of identification on agar plates is that it heavily relies on the selectivity of the agar medium. If other microbiota from the food product are able to overcome the selectivity of ALOA plates, they can potentially overgrow colonies of *L. monocytogenes*. If there is a 2-3 $\log_{10}$ difference between competing microbiota and *L. monocytogenes*, there is only a small chance to find presumptive colonies on ALOA. The ISO protocol specifies that

a secondary selective medium of choice must be used to partly remedy this, but this remains a drawback of culture-based detection.

Enrichment medium composition should on one hand support the growth of potential sub-lethally damaged cells, while on the other suppress the growth of food microbiota and competing species (Dailey et al., 2014, 2015; Ottesen et al., 2016; Zitz et al., 2011). These trade-offs need to be carefully balanced, because stressed or injured cells become more sensitive to selective compounds (Kalchayanand et al., 1992; Os-

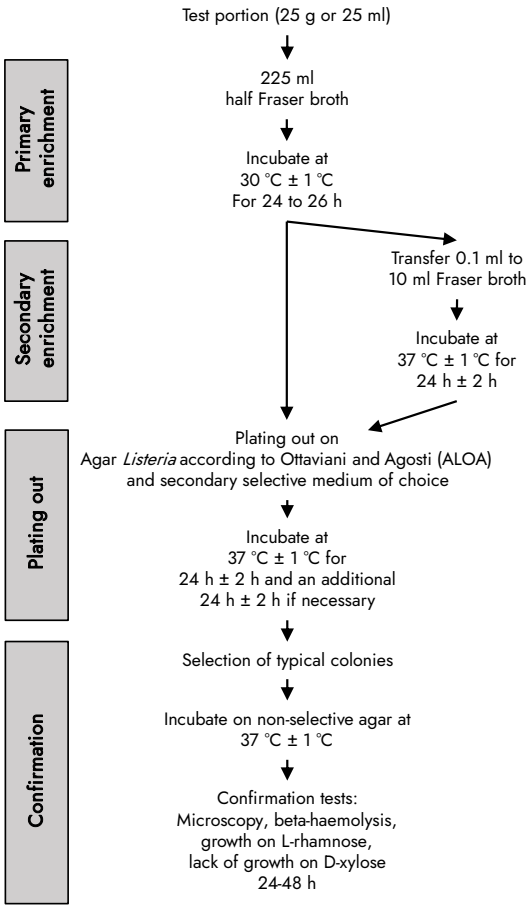

Figure 3: Schematic overview of the detection method of *L. monocytogenes* according to the ISO 11290-1 in the food chain. A two-step enrichment is done in half Fraser broth followed by Fraser broth, and after both steps a loopful (10 µl) of culture is plated on ALOA and a secondary medium of choice. Presumptive colonies (blue-green with an opaque halo for *L. monocytogenes*) are confirmed by isolation on non-selective agar and successfully passing the mandatory confirmation tests. Figure adapted from ISO 11290-1:2017 (International Organization for Standardization, 2017).

manağaoğlu, 2005). Injured cells may show prolonged lag durations when cultured in appropriate selective media that may pose problems for detection, potentially leading to false-negative results (Guillier et al., 2005; Hamill et al., 2020; Lianou et al., 2006; Osborne & Bremer, 2002). In such cases, the lag duration can become so long that the necessary concentration for detection is not reached during enrichment. This means that although a sample might contain *L. monocytogenes*, the pathogen is not detected, hence a false-negative result. Next to this, because of the necessity to detect a single cell in a sample during enrichment, the behaviour of single-cells can play an important role. Such low starting concentrations can be problematic because outgrowth of single cells can be heterogeneous (Aspridou & Koutsoumanis, 2015; Booth, 2002; D'arrigo et al., 2006). The individual cell state can influence outgrowth potential due to heterogeneity in lag duration and growth-rate, something that is rarely studied in the context of enrichments.

The primary enrichment can be considered the most crucial part of the detection procedure, because the recovery of potentially damaged cells is a key step for reliable detection. However, this step remains a black box with regard to the physiology behind lag phase, damage repair, and growth initiation (Rolfe et al., 2012). The trade-off for efficient detection of pathogens from food lies on balancing the required selectivity of the medium with optimal recoverability of potentially stressed cells. This balance is severely complicated by the uncertainties of differing microbiota found on a range of divergent food products, combined with a wide range of stress history that pathogens can have at the start of enrichment. Increasing our knowledge on these uncertainties allows researchers and authorities a better understanding on how to adapt medium choice for optimum recovery of damaged cells. This can tip the balance in favour of a more robust detection of foodborne pathogens. Finally, enrichment-based detection procedures can be made more rapid by combining the traditional culture-based enrichment steps with more rapid detection methods. In this way, detection can be drastically shorter than the 3-5 days of the current culture-based detection that relies on selective plating and confirmation reactions.

1.5. Rapid detection methods

1.5.1. Prerequisites for rapid detection methods

After the enrichment steps when the concentration of cells has increased to higher and detectable levels, the plating out with subsequent confirmation steps takes up a large part of the total procedure. While these morphological and biochemical approaches for identification are simple, inexpensive, and sensitive, they are time- con-

suming. There has been plenty of research into faster detection methods that can bridge the gap towards a more rapid detection method of *L. monocytogenes*.

There are a couple of requirements that rapid detection methods have to fulfil before they can be reliably used in the detection of *L. monocytogenes* in food. The sensitivity (the probability that a positive test is truly positive) has to be high enough to detect *L. monocytogenes* if it is present in the broth after enrichment. Also the specificity (the probability that a negative test is truly negative) has to be high enough that only *L. monocytogenes* is detected and no other non-pathogenic *Listeria* species. Although the detection of other *Listeria* spp. can be useful, in the context of detection a test should only give a positive result when (pathogenic) *L. monocytogenes* is present. Both the sensitivity and specificity describe the accuracy of the rapid detection method, and this has to be validated against the gold standard method, which in this case in the plating out on ALOA and confirmation according to ISO 11290-1. A new method should be validated by ISO 17468:2016 (International Organization for Standardization, 2016) to have at least equal accuracy than the ISO method. Next to this, the cost and speed of the method are also important parameters. Because of the labour-intensive nature of the current ISO, a new detection method has to give an accurate result faster than the 2-3 days that plating out and confirmation currently takes. Next to this, the cost of the method also has to be taken into account. In the EU in 2020, 130,346 samples were tested via official channels only (European Food Safety Authority, 2021), and thus the price of each sample cannot be too high as testing has to be done in high volumes.

1.5.2. Overview of rapid methods

The relative scarce research on the enrichment of *L. monocytogenes* stands in stark contrast with the wealth of research on detection methods. Over the years many different methods have been developed to detect the presence of foodborne pathogens, and an overview of some of the most important of such methods is given in table 1.

1.5.2.1. Immunological methods

Immunoassays rely on antibodies (monoclonal, polyclonal, or recombinant antibodies) to target specific antigens on the surface of *L. monocytogenes*. They usually target structures on the outside of the bacterial cell wall, such as flagella, listeriolysin toxin, or invasion protein P60 (Jantzen et al., 2006; Shamloo et al., 2019). Immunoassays can be easily and rapidly used in enzyme-linked immunosorbent assays (ELISA), but the downside is that sensitivity and specificity depend on the quality of the antibody used. This has been commercialized by multiple companies such as the VIDAS®

LMO2 assay (Vaz-Velho et al., 2000).

1.5.2.2. Biosensors

In biosensors, a biological component – in this case *L. monocytogenes* cells – is coupled with a physicochemical detector (Turner, 2000). Biological responses can be converted into an electric signal, whether this is optical, electrochemical, magnetic or otherwise. As such, many different kinds of biosensors can be used, with the main benefits that the systems are usually portable and easy to handle. There are biosensors available for *L. monocytogenes* detection and these show great promise (Biberoğlu, 2020; Li et al., 2021). Although the sensitivity of most biosensors is not as high as DNA amplification methods (Välimaa et al., 2015), there are promising biosensor-based innovations that could become highly sensitive and accurate methods for detection of pathogens (Schmitz et al., 2020), however this would still require an enrichment.

1.5.2.3. Spectrometry-based methods

Mass spectrometry is a technique that is reliant on the ionization and vaporization of large non-volatile biomolecules to detect intact microorganisms (Fenselau & Demirev, 2001; Seng et al., 2009). The biomolecules are then accelerated through an electrostatic field and the time to reach the detector is dependent on the mass and degree of ionization (Ho & Reddy, 2010; Seng et al., 2009). This can give a spectrum that can be compared to a reference databank to give a numerical score based on the similarity with the observed and stored data. This method is often used in food laboratories due to its fast and accurate species-level identification. However, the downside with regard to high-throughput screening is the high cost of the equipment, and the necessity of pure colonies on non-selective agar plates.

Table 1: Overview of identification methods that can be used for the detection of *L. monocytogenes* after enrichment. Detection methods can be grouped in culture-based methods, immunological methods, biosensors, spectrometry-based methods, and molecular methods. Each method has advantages and limitations and has a detection threshold that has to be reached in order to have efficient detection with said method. Figure adapted from Osek et al. (2022)

detection method	advantages	limitations	detection threshold
Culture-based methods: - Culture-based detection from ALOA plates	- Cost-effective - Only detection of viable cells - No inhibition of matrix components - Approved by regulatory authorities	- Time consuming (3-5 days) - Possible false-negative results - Stressed and injured cells may not be detected	10^1 - 10^2 cells/ml [1]
Immunological methods: - ELISA (enzyme-linked immunosorbent assays)	- Reproducible - Sensitive - Can be automated - Commercial kits available	- Sensitivity and specificity depend on antibody quality - Possible false-negative or false-positive results - Potential cross-reactivity with related antigens	≥ 10^5 cells/ml (Gasanov et al., 2005)
Biosensors: - Optical - Cell-based - Electrochemical	- Highly sensitive and specific - Many systems are portable - Rapid or real-time detection	- High cost - Results may depend on food matrix	≥ 10^3 cells/ml (Ohk et al., 2010)
Spectrometry-based methods: - Mass spectrometry	- Rapid - Accurate - Sensitive	- High cost - Results may depend on environmental conditions	10^1 - 10^2 cells/ml [2]
Molecular methods: - Real-time PCR	- Highly sensitive and specific - Reproducible - Real-time detection - Allows for high-throughput analysis - Commercial kits available	- Relatively costly - Sensitivity depends on PCR inhibitors in food matrix - Identifies both viable and non-viable cells	10^3 - 10^4 cells/ml [3] (Rantsiou et al., 2008)
- LAMP (Loop-mediated isothermal amplification)	- Easy to do - Highly sensitive and specific - Less sensitive to food matrix - Cost-effective	- Complicated primer design - Sensitivity to inhibitors in food products - Potential false-positives - Not as well-developed as real-time PCR	10^2 - 10^3 cells/ml (Wang et al., 2012) (Shan et al., 2012)

[1]Minimum of 1 CFU in a loop of HFB (10 µl) spread on ALOA leads to a detection threshold of 10^1 – 10^2 CFU/ml.

[2]For mass spectrometry an isolated colony is necessary from an agar plate. Therefore the detection threshold is similar to culture-based detection but mass spectrometry gives much faster results.

[3]This detection threshold is straight from an enriched sample, only requiring cell-lysis before real-time PCR can start.

1.5.2.4. Molecular methods

Real-time PCR allows the continuous monitoring of PCR product formation due to the fluorescent emission produced by labelled probes or dyes (Cady et al., 2005; Mackay & Landt, 2007). This has the main convenience with respect to conventional PCR that no gel electrophoresis step is necessary. Different target genes have been proposed and utilized to detect *L. monocytogenes* and to differentiate it from non-pathogenic *Listeria* species in many different food products (Berrada et al., 2006; Garrido-Maestu et al., 2014; Kačániová et al., 2015; Kim & Cho, 2010). Real-time PCR is however sensitive to inhibiting compounds in the food matrix that can interfere with the amplification reaction. Furthermore, amplification of DNA does not distinguish between living cells and dead cells of the target bacterium. However, in enrichment the inhibiting compounds are diluted and live organisms are multiplied which can at least partly negate these limitations (Jantzen et al., 2006). Another molecular method that can be used is loop-mediated isothermal amplification (LAMP) which is a DNA amplification method that can be used under isothermal conditions (60-65 °C). The principle of LAMP is the measurement of increase in turbidity caused by binding of pyrophosphate by-product with magnesium ions (Nagamine et al., 2002). The increase in turbidity corresponds with increased DNA amplification due to four primers targeting six specific DNA regions. LAMP is shown to be less sensitive to the presence of compounds found in the matrix, at least for clinical samples (Kaneko et al., 2007).

1.5.3. Detection threshold of rapid methods

In table 1 the detection threshold of rapid methods is shown. Of the rapid methods, PCR-based methods and biosensors seem to be the most promising, offering rapid, sensitive, and specific techniques that can be analysed with high-throughput. The lowest detection thresholds are for culture-based detection and mass spectrometry (between 10^1 and 10^2 cells/ml), but these methods rely on plating out after enrichment which increases the time until detection. Even these detection thresholds are orders of magnitude higher than the legally required detection set for ready-to-eat foods (not detected in 25 g or 25 ml, meaning not detected in 250 ml enrichment broth). Thus, although the aforementioned rapid methods are promising for reducing the detection time, enrichment of the product samples remains absolutely necessary (Jantzen et al., 2006). Time can be saved by replacing the plating and confirmation steps from the current culture-based detection with more rapid methods that are compared in table 1.

1.6. Objective of thesis

The research that is presented in this thesis aimed at elucidating the behaviour and heterogeneity during primary enrichment on the detection of potentially injured cells of *L. monocytogenes* from food, as well as quantifying the impact of competitors during molecular detection. This knowledge can then be applied to optimize the enrichment-based detection procedure, resulting in more rapid and reliable detection of this important foodborne pathogen. The division of this thesis into chapters is visualized in figure 4.

Contamination of food products can occur with *L. monocytogenes* at different stages of the food production chain. Contamination can happen at primary production or as cross-contamination later during processing or at retail. *L. monocytogenes* can therefore come from different niches with distinct changes in genetic material of a multitude of serotypes. Therefore, the aim of chapter 2 was to evaluate how a diverse set of strains differs in their ability to grow during enrichment with and without a stress history. Variability in lag duration and specific growth rate was quantified to simulate its effect on successful detection by kinetic modelling.

Because according to European legislation no detection of *L. monocytogenes* is allowed in 25-g samples for ready-to-eat foods, the detection limit of enrichment must be one cell per 25 g product. However, behaviour of single-cells can be drastically different than at population level, because the individual cell state becomes more significant. Therefore, in chapter 3 the heterogeneity at single-cell level was measured using flow-cytometry and single-cell sorting. This allows us to study the outgrowth of single-cells and what effect the single-cell state can have on the detectability during enrichment.

The lag duration in general is still quite poorly understood. Therefore, in chapter 4 lag behaviour is studied by using transcriptomics and proteomics to analyse what happens during the lag at gene transcript and protein level. This can give insights into physiological behaviour that can be used for potential modification of enrichment broth to better recover (stressed) cells.

In chapter 5 the effect of competing microbiota on the enrichment of *L. monocytogenes* from ready-to-eat foods was tested when combining an enrichment with rapid molecular real-time PCR detection. The presence of high concentrations of competitors can be problematic for detection as this can stop the growth of *L. monocytogenes* which thereby could fail to reach the detection threshold. In this chapter, growth kinetics of competing strains are quantified to see if competitors are able to outcompete

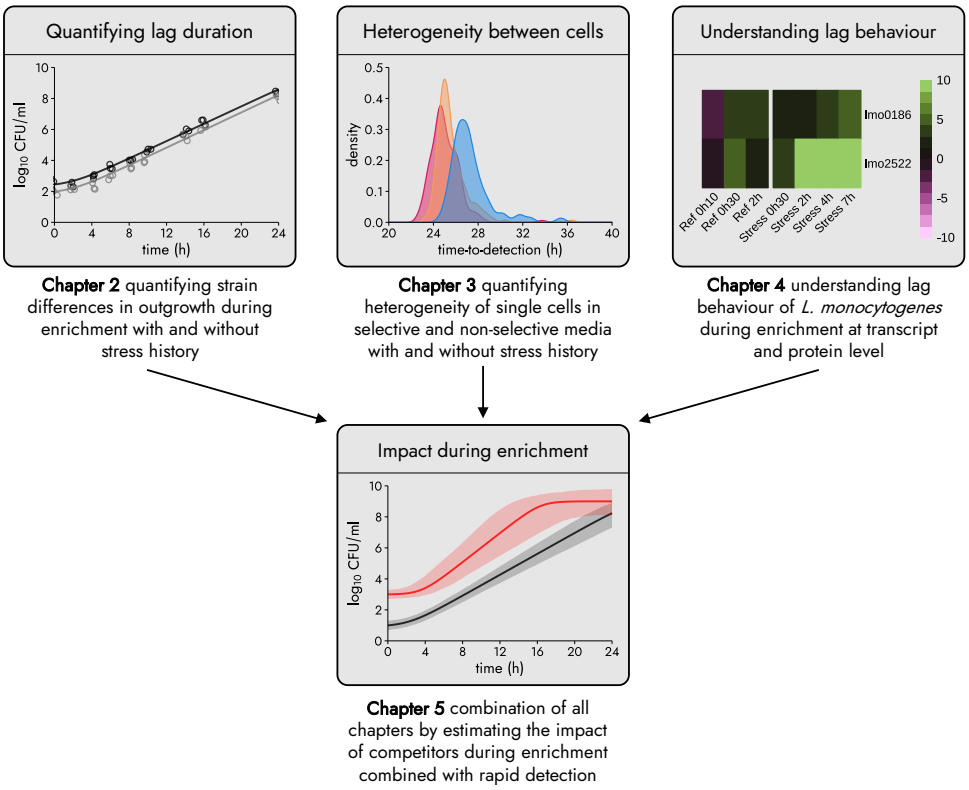

Figure 4: Overview of thesis chapters. In chapter 2 differences between stressed and non-stressed strains with respect of their lag duration were quantified to understand how different strains grow during enrichment. In chapter 3 the heterogeneity was measured at single-cell level. Because contamination levels on food products are often very low, this allows the quantification of difference in outgrowth at very low pathogen concentrations. In chapter 4 the focus was to understand the lag behaviour during enrichment at protein level to find markers that contribute to getting out of lag. And finally in chapter 5, the impact of competitors was assessed in enrichments with ready-to-eat food products combined with detection with a rapid molecular method.

L. monocytogenes during enrichment. With this, the ability to detect *L. monocytogenes* with real-time PCR in the presence of abundant competitors is tested in food products.

Concludingly, the impact and relevance of these chapters on enrichment-based detection of *L. monocytogenes* is discussed in chapter 6. In this chapter the knowledge of this combined research is put into perspective of the current ISO 11290 culture-based detection and touches on ways to redefine the enrichment protocol for more rapid and reliable detection of *L. monocytogenes*.

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2

Variability in lag duration of *Listeria monocytogenes* strains in half Fraser enrichment broth after stress affects the detection efficacy using the ISO 11290-1 method

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Abstract

A collection of 23 *Listeria monocytogenes* strains of clinical and food origin was tested for their ability to recover and grow out in half Fraser enrichment broth (HFB) following the ISO 11290-1:2017 protocol. Recovery of sub-lethally heat-injured cells in HFB was compared to reference cells with no stress pre-treatment. The enrichments were followed over time by plate counts and the growth parameters were estimated with the 3-phase model which described the data best. The reference cells without stress pre-treatment showed a short lag duration, which ranged from 1.4 to 2.7 h. However, significant variation in the ability to recover after 60 °C heat stress was observed among the tested strains and resulted in a lag duration from 4.7 to 15.8 h. A subset of strains was also exposed to low-temperature acid stress, and the lag duration showed to be also stress dependent. Scenario analyses and Monte Carlo simulations were carried out using the growth parameters obtained in the enrichments. This demonstrated that when starting with one cell, the detection threshold for efficient transfer of at least one cell to the secondary enrichment step, i.e. 2 $\log_{10}$ CFU/ml, was not reached by 11 of 23 strains tested (48 %) after exposure to 60 °C heat stress. Increasing the incubation time from 24 to 26 h and the transfer volume from 0.1 to 1.0 ml can increase the average probability to transfer at least one cell to the secondary enrichment step from 79.9 % to 99.0 %. When optimizing enrichment procedures, it is crucial to take strain variability into account as this can have a significant impact on the detection efficacy.

2.1. Introduction

The presence of *Listeria monocytogenes* in (growth supporting) food products is a risk factor for food safety because of the severity of illness that can be caused in vulnerable individuals combined with its ability to grow at refrigeration temperatures. The food safety risk is likely to increase with the rising popularity of ready-to-eat products where no heating step is applied before consumption (Lianou & Sofos, 2007). The European Union laid down a criterion for absence testing in five samples of 25-g portions of ready-to-eat food that can support growth of *L. monocytogenes* after the production stage (European Commission, 2007). For this purpose, standardized microbiological procedures and guidelines have been established so that governments and the food industry can routinely test food samples for the presence of *L. monocytogenes*. In the European Union the analytical reference method is the ISO 11290-1 enrichment protocol for the detection and enumeration of *L. monocytogenes* (International Organization for Standardization, 2017). Testing for pathogen presence is established by culture-based standardized enrichments to allow recovery and an increase in the initial low concentrations of pathogen followed by detection with culturing dependent- or molecular methods. Notably, enrichment media composition should be designed such, that conditions are optimal to support damage repair and growth initiation of potential sublethally injured cells, while at the same time suppressing the growth of competing background microbiota (Dailey et al., 2014, 2015; Ottesen et al., 2016; Zitz et al., 2011). These factors complicate the enrichment steps that are necessary to amplify the pathogen concentration to higher levels in order to support adequate detection. The current ISO 11290-1:2017 protocol for the enrichment of *L. monocytogenes* from food products (International Organization for Standardization, 2017) consists of a 24-h enrichment in half Fraser broth (HFB) followed by a secondary enrichment in full Fraser broth (FB) for 24 h with streaking on selective ALOA-plates and another selective medium of choice for 48 h after both enrichment steps. Afterwards, suspect colonies have to be tested with confirmation reactions. The first enrichment step has to facilitate the recovery of sublethally injured cells that can be present in the product. Hence, the primary enrichment medium contains only half the concentrations of the selective compounds acriflavine and nalidixic acid (International Organization for Standardization, 2017). This is followed by the secondary enrichment with full-strength Fraser broth containing two-fold higher concentrations of selective compounds. These culture-based methods are time-consuming with detection taking up to 7 days while there is still

the possibility of *L. monocytogenes* cells not growing out and giving false-negative results (Gnanou Besse et al., 2016). Furthermore, there can be large differences among different strains of *L. monocytogenes* that can complicate detection. For example, it has been shown that there is a strain bias during selective enrichment of *L. monocytogenes* strains from ham-slices (Zilelidou et al., 2016b). This strain bias has been shown for different enrichment methods (Bruhn et al., 2005; Gorski et al., 2006; Zilelidou et al., 2016a), but none quantified or prioritized the importance of strain variability and variability introduced when experiments are independently reproduced. Therefore, the objective of this research was to quantify the strain variability of *L. monocytogenes* in recovery following the current ISO 11290-1:2017 enrichment protocol. For this, the recovery of 23 outbreak-related strains of *L. monocytogenes* was assessed after heat stress treatment and after acid stress treatment at low temperature. Also, all experiments were independently reproduced in order to quantify and compare the effects of biological diversity and strain diversity on detection efficacy.

2.2. Materials and methods

2.2.1. Bacterial strains and growth conditions

The set of 23 strains of *L. monocytogenes* from different isolation sources and serotypes (table S1) was kept at -80 °C in brain heart infusion (BHI) broth (Becton Dickinson Difco) supplemented with 30 % glycerol (Fluka). Cultures were made by inoculating 10 ml of BHI broth with a single colony from a BHI agar plate (1.5 % agar, Oxoid) obtained from -80 °C freezer stocks. Cultures were grown statically at 30 °C for 16 h to obtain stationary phase cultures. These cultures were subsequently diluted 1:1,000 in fresh BHI broth and incubated at 30 °C for 16 h to obtain a standardized working culture for use in further experiments.

2.2.2. Stress treatment of cells

Working cultures of all strains were stress-treated to reduce the viable counts with one $\log_{10}$ CFU/ml reduction. The D_{60}-values of the strains previously published by Aryani et al. (2015b) were used to determine the heat treatment time for each of the strains. Working cultures were diluted 1:100 in 50 ml BHI broth pre-heated at 60 °C in a water bath (Julabo SW23) for the time of one D_{60}-value reduction (table S1). Afterwards, the cultures were quickly cooled on ice for 15 s and decimally diluted in peptone physiological salt (PPS) solution (Tritium Microbiology) to obtain an initial concentration of approximately 2 $\log_{10}$ CFU/ ml in the enrichment experiments. To determine the pH for the low-temperature acid stress treatments, the working cul-

tures were stressed in acidified BHI broth at 10 °C for 24 h and samples were taken to determine the viable counts. The BHI broth was acidified with 2.5 M HCl until the desired pH value was reached (MeterLab PHM240 pH/ION meter). For each strain, the pH value that gave one $\log_{10}$ reduction after 24 h based on duplicate experiments was chosen for low-temperature acid stress treatment at 10 °C. This low temperature was chosen to simulate the temperature in the cold food chain. Acid stressed cells were subsequently decimally diluted in PPS to obtain an initial concentration of approximately 2 $\log_{10}$ CFU/ml in the enrichment experiments. The working cultures were afterwards incubated for 24 h at 10 °C in plain BHI for subsequent use in enrichments.

2.2.3. Enrichment kinetics in half Fraser broth

Enrichments were carried out in half Fraser enrichment broth, which was made by supplementing Fraser broth base (Oxoid) with half Fraser supplement (Oxoid). Irrespective of their pre-treatment, all enrichments were started with an initial inoculum concentration of 2 $\log_{10}$ CFU/ml. For the reference cells, the working cultures (reaching approximately 9 $\log_{10}$ CFU/ml) were decimally diluted in PPS until a concentration of approximately 3 $\log_{10}$ CFU/ml. This culture was diluted again 1:10 in 45 ml half Fraser enrichment broth in 150 ml Schott flasks resulting in an initial inoculum concentration of 2 $\log_{10}$ CFU/ml. After addition of the cells to the enrichment broth (timepoint 0), samples were taken at 2- h intervals for 10 h and at 24 h to investigate kinetics at 30 °C according to the ISO 11290-1:2017. Samples were spread plated on BHI agar plates and incubated at 30 °C for 24 h before counting. In order to measure the concentration at time-points 14 and 16 h, a parallel enrichment was started later in the day and samples were taken the next morning. Three independent biological reproductions were carried out for reference cells and two for stressed cells, and experiments took place on different days.

2.2.4. Model fitting and statistics

Growth of *L. monocytogenes* strains during primary enrichment for both the reference cells and stressed cells was modelled with the 3-phase model (Buchanan et al., 1997), the modified Gompertz model (Zwietering et al., 1990) and the Baranyi model (Baranyi & Roberts, 1994).

2.2.4.1. Three-phase linear model

Lag phase:

$$\text{For } t \leq \lambda: \quad \log_{10}N_t = \log_{10}N_0 \tag{2.1}$$

Exponential growth phase:

$$\text{For } \lambda < t < t_{max}: \quad \log_{10}N_t = \log_{10}N_0 + \frac{\mu}{\ln(10)}(t-\lambda) \tag{2.2}$$

Stationary phase:

$$\text{For } t \geq t_{max}: \quad \log_{10}N_t = \log_{10}N_{max} \tag{2.3}$$

With $\log_{10}N_t$ the cell concentration at time t ($\log_{10}$ CFU/ml), $\log_{10}N_0$ the initial cell concentration ($\log_{10}$ CFU/ml), $\log_{10}N_{max}$ the maximum cell concentration ($\log_{10}$ CFU/ml), t the elapsed time (h), λ the lag phase duration (h), t_{max} the time when stationary phase is reached (h) and μ the maximum specific growth rate (h^{-1}).

2.2.4.2. Modified Gompertz model

$$\log_{10}N(t) = \log_{10}N_0 + (\log_{10}N_{max} - \log_{10}N_0) \cdot \exp\Bigg(- \exp\left[\frac{\frac{\mu}{\ln(10)} \cdot e}{\log_{10}N_{max} - \log_{10}N_0} \cdot (\lambda - t) + 1\right]\Bigg) \tag{2.4}$$

With $\log_{10}N(t)$ the cell concentration at time t ($\log_{10}$ CFU/ml), $\log_{10}N_0$ the initial cell concentration ($\log_{10}$ CFU/ml), $\log_{10}N_{max}$ the maximum cell concentration ($\log_{10}$ CFU/ml), t the elapsed time (h), λ the lag phase duration (h), and μ the maximum specific growth rate (h^{-1}).

2.2.4.3. Baranyi model

$$\log_{10}N(t) = \log_{10}N_0 + \frac{\mu}{\ln(10)} \cdot A(t) - \frac{1}{\ln(10)} \cdot \ln\left[1 + \frac{\exp[\mu \cdot A(t)] - 1}{10^{[\log_{10}N_{max} - \log_{10}N_0]}}\right] \tag{2.5}$$

$$\text{With } A(t) = t + \frac{1}{\mu} \cdot \ln\left[\exp(-\mu \cdot t) + \exp(-\mu \cdot t_{lag}) - \exp(-\mu \cdot t - \mu \cdot t_{lag})\right] \tag{2.6}$$

With $\log_{10}N(t)$ the cell concentration at time t ($\log_{10}$ CFU/ml), $\log_{10}N_0$ the initial cell concentration ($\log_{10}$ CFU/ml), $\log_{10}N_{max}$ the maximum cell concentration ($\log_{10}$

CFU/ml), t the elapsed time (h), λ the lag phase duration (h), t_{lag} the time when the lag phase ends (h), and μ the maximum specific growth rate (h^{-1}).

Best estimates for the model parameters $\log_{10}N_0$, μ and λ were obtained by the least squares regression analysis using Microsoft Excel's Solver add-in. For each reproduction the significance of the parameter fitting was determined by calculating the 95 % confidence interval by estimating the standard error using the SolverAid add-in for Microsoft Excel. The model fitting performance of the models was compared according to den Besten et al. (2006), where the mean square error of the model describes the fitting performance as follows:

$$MSE_{model} = \frac{RSS}{DF} = \frac{\sum_{i=1}^{n}\left(\log_{10}N^{i}_{observed} - \log_{10}N^{i}_{fitted}\right)^{2}}{n-p} \tag{2.7}$$

Where the mean square error of the model (MSE_{model}) is calculated as the residual sum of squares (RSS) divided by the degrees of freedom (DF). The RSS is the sum of the squared difference between the observed cell concentration $\log_{10}N_{observed}$ ($\log_{10}$ CFU/ml) and the fitted values $\log_{10}N_{fitted}$ ($\log_{10}$ CFU/ml) for each model. DF is the number of data points n minus the number of model parameters p.

The MSE_{model} was calculated for each model for all 23 strains and all conditions. The model that had the lowest MSE_{model} in most cases was deemed to most adequately describe the data.

2.2.4.4. Quantifying biological and strain variability

The biological and strain variabilities of the lag phases for reference cells, low temperature acid stressed cells and 60 °C heat stressed cells was calculated according to the protocol of Aryani et al. (2015a). Here, biological variability is defined as the variability between the independent reproductions and strain variability is defined as the variability between the strains in their recovery from stress history.

Biological variability:

$$MSE_{biological} = \frac{RSS}{DF} = \frac{\sum_{S=1}^{j}\sum_{R=1}^{i}\left(\lambda_{SR} - \lambda_{S}\right)^{2}}{n-p} \tag{2.8}$$

Where the mean square error is calculated from the residual sum of squares divided by the degrees of freedom. The RSS is the sum of squared differences between λ_{SR} and λ_S, where λ_{SR} is the lag duration (h) obtained after enrichment for each reproduction for a certain strain (for reference cells $i = 3$ reproductions and for heat stress $i = 2$ reproductions) and λ_S is the average lag duration (h) from independent

enrichments for each strain (for acid stress $j = 5$ and for heat stress $j = 23$). DF is the number of data points per condition (for reference cells $3 \cdot 23$ and for heat stress $2 \cdot 23$) minus the number of parameters ($p = 1 \cdot 23$). For low-temperature acid stress this was calculated with five strains and two reproductions.

Strain variability:

$$MSE_{strain} = \frac{RSS}{DF} = \frac{\sum_{S=1}^{j} \left(\lambda_S - \lambda_{average}\right)^2}{n - p} \tag{2.9}$$

Where λ_S is the average lag duration (h) from three different enrichments for each strain, $\lambda_{average}$ is the average lag duration (h) of all strains for each condition (for reference cells and heat stress $j = 23$ and for acid stress $j = 5$), DF is the number of data points ($n = 23$ for heat and 5 for acid stress) per condition minus the number of parameters ($p = 1$). An F-test was used to determine statistically significant differences between the mean square error of the biological and strain variability. Data were considered significant with p-values of 0.05 or lower.

Furthermore, it was checked that the model choice did not affect the conclusions drawn by calculating the variabilities based on the growth parameter estimates of all three primary models. However, irrespective of the model choice, the significance of the calculated variabilities did not in fact change.

2.2.5. Modelling the primary enrichment

To model the primary enrichment step in the scenario analysis, a detection threshold after 24 h of 2 $\log_{10}$ CFU/ml was chosen. This concentration was described by Augustin et al. (2016) as the concentration that allows transfer of at least one cell to the secondary enrichment broth with 100 % probability.[1] This detection threshold is used for all scenario analyses in this research. For the scenario analysis the starting concentration is one *L. monocytogenes* cell per 25 g food product that is enriched in 225 ml HFB (-2.4 $\log_{10}$ CFU/ml). The data of the estimated lag durations and maximum specific growth rates of all 23 strains was used to model the growth in the primary enrichment step after stress. Also, the impact of changes in maximum specific growth rate and lag duration was determined on the ability to reach the 2 $\log_{10}$ CFU/ml detection threshold. For this, Monte Carlo simulations were carried out in Microsoft Excel using the add-in @Risk version 7.5 (Palisade Corporation). The maximum specific growth rates and lag durations were fitted to multiple distributions and the growth parameters were a good fit to the normal-distribution. The normal-distribution with

[1]Transfer of at least one cell in 0.1 ml of 2 $\log_{10}$ CFU/ml being Poisson($k > 0, 10$) is 99.995 %

the average and standard deviation was then used for the modelling in @Risk. The probability to transfer at least one cell to the secondary enrichment was determined with the Poisson-distribution in a simulation with 100.000 iterations using Latin Hypercube sampling (Delignette-Muller & Rosso, 2000), together with a Mersenne twister random number generator. The mean probability to transfer at least one cell to the secondary enrichment step was calculated for scenarios with different volumes (0.1 ml and 1 ml) and different incubation times of the primary enrichment (24 h and 26 h).

2.3. Results

2.3.1. Recovery duration after 60 °C heat stress is strain dependent

The recovery of 23 strains of *L. monocytogenes* was tested in half Fraser primary enrichment broth as specified in the first step of ISO 11290-1:2017. The lag duration and growth of reference cells and sublethally injured cells after 60 °C heat stress in HFB was estimated by fitting bacterial growth models to the growth curves. Of the three models, the 3-phase model gave the best fit in 75.7 % of the cases, the Gompertz model in 17.3 % and the Baranyi model in 7.0 % of the cases. Therefore the lag and maximum specific growth rate estimates from the 3-phase model were used in the further analysis of the experimental data. The lag durations for the enrichments in HFB are displayed in figure 1. For the reference condition where no additional stress was applied before enrichment, the strains behaved rather similarly with respect to their lag phase duration. The lag duration of reference condition cells of the 23 tested strains ranged from 1.4 to 2.7 h, with an average lag duration of all 23 strains of 1.9 h (standard deviation of 0.5 h). However, after the 60 °C heat treatment there was a significant increase in lag phase duration, as the heat stressed cells needed more time to recover and also showed larger variability. After heat stress, the lag phase ranged from 4.7 to 15.8 h with the average lag duration of all 23 strains of 10.0 (standard deviation of 2.8 h).

In order to determine a possible correlation between heat resistance and lag duration of individual strains, lag durations were plotted against D_{60}-values after heat stress for all strains tested (figure 2). This showed that there was no clear correlation between heat resistance and lag duration during primary enrichment. The lag duration was also measured with cells after extended exposure time to 60 °C resulting in a 3 $\log_{10}$ reduction (data not shown). Data obtained with the selected strains showed that the lag duration was not significantly different from that of cells after a

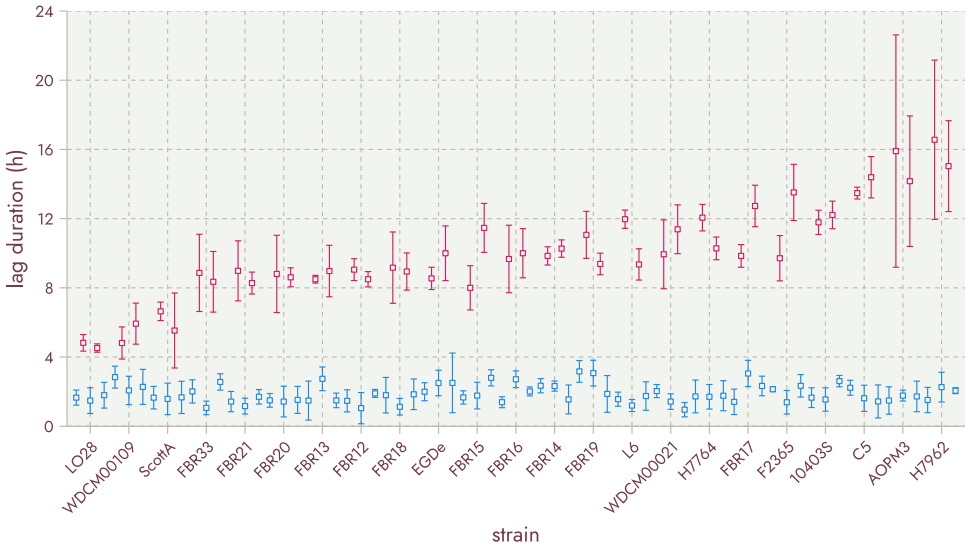

Figure 1: Lag duration of 23 strains of *L. monocytogenes* in half Fraser enrichment broth with reference cells in blue (with no additional stress pre-treatment applied) and 60 °C heat stress pre-treatment in red (aiming for one D_{60}-value reduction). The 3-phase model was used to fit the growth kinetics and the lag duration was estimated for each biological reproduction. The 95 % confidence interval of the fitting was determined for each fitting and displayed as error bars.

one $\log_{10}$ heat stress-induced reduction, indicating that a higher reduction after 60 °C heat stress did not influence the recovery capacity of the smaller and conceivably more severely injured surviving population.

Next to the lag phase, also the maximum specific growth rate was estimated with the 3-phase model for the reference cells and after 60 °C heat stress. The average maximum specific growth rate of all strains in HFB for the reference condition was 0.67 ± 0.05 h^{-1} and for heat-stressed cells 0.68 ± 0.11 h^{-1}. No significant difference was found among the average maximum specific growth rate during enrichments of reference cells and heat stressed cells (p = 0.23), indicating that once cells got out of the lag phase their growth rate was similar. The strain collection contains strains from different serotypes, and the recovery ability among strains was compared (figure S1). In reference condition no significant difference among serotypes was observed. Taking heat stress history into account, serotype 4b (n = 6) seemed to display the highest lag durations although this was not significant. On the other hand, serotype 1/2c strains (n = 3) showed shorter lag durations after heat-stress than the other tested serotypes, though this was neither statistically significant (p = 0.06). Furthermore, differences in recovery among lineages of *L. monocytogenes* and/or their origin (food or clinical

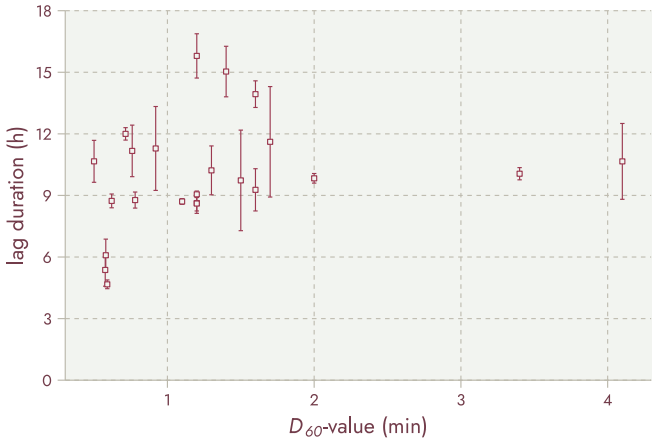

Figure 2: The average lag duration in HFB of each of the 23 tested strains of *L. monocytogenes* after 60 °C heat treatment plotted against the D_{60}-value for each strain. This shows that there is no correlation between the D_{60}-value and the subsequent recovery after heat stress. The error bars depict the standard deviation in lag duration.

isolates) were not found.

2.3.2. Recovery in half Fraser broth is strain and stress dependent

When cells of *L. monocytogenes* were pre-cultured at a lower temperature of 10 °C, there was no significant increase in lag duration observed during primary enrichment when compared to reference cells pre-cultured at 30 °C (data not shown). This led to the hypothesis that a reduction in viable cells was necessary for an increase in lag duration during primary enrichment. Therefore, the recovery after low-temperature (10 °C) acid stress was quantified, in order to test whether the strain recovery is only strain-dependent or also stress-dependent.

For this, a fast recovering strain (ScottA), an intermediate recovering strain (EGDe) and a slow recovering strain (H7962) after heat stress together with the two ISO 11290-1 reference strains (WDCM00021 and WDCM00109 (International Organization for Standardization, 2017)) were tested. To be able to quantify the recovery in the same manner as after heat stress, one $\log_{10}$ CFU/ml reduction was also aimed for during the low-temperature acid stress pre-treatment. Because of strain differences in acid resistance, each strain was stressed at a different pH value to achieve one $\log_{10}$ CFU/ml reduction (table S1). The outgrowth in HFB after low-temperature acid stress pre-treatment was compared to outgrowth following exposure to heat stress

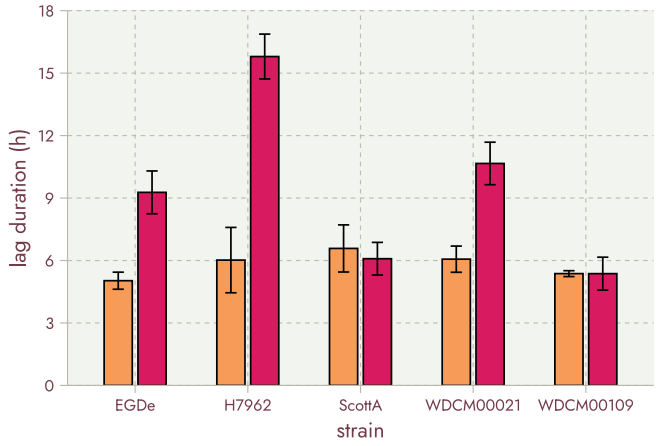

Figure 3: Comparison of the lag duration after 60 °C heat stress (red) and 10 °C low-temperature acid stress (yellow) in half Fraser enrichment broth. Growth kinetics were fitted with the 3-phase model and the average lag duration of two independent reproductions is displayed with the standard deviation as error bars.

(figure 3). Although the strains showed significant differences in lag after one $\log_{10}$ CFU/ml reduction following exposure to heat stress, there were no significant differences among the strains after a one $\log_{10}$ CFU/ml reduction by low-temperature acid stress. This is further exemplified by the biological and strain variabilities in lag duration after exposure to stress (figure 4). For the reference cells as well as for low-temperature acid stressed cells, the biological variability was comparable to the strain variability. In contrast, although heat stress showed a significant increase in the biological variability between experiments ($p = 1.3 \cdot 10^{-5}$), there was a very significant increase in strain variability ($p = 3.8 \cdot 10^{-15}$). Overall, we saw that stressed *L. monocytogenes* cells showed significant strain variability in outgrowth during primary enrichment in HFB.

2.3.3. Slow recovering strains after 60 °C heat stress can fail to reach the detection threshold

The enrichment kinetics of heat-stressed *L. monocytogenes* strains was used to predict the growth during the primary enrichment step. ISO11290-1:2017 specifies an incubation time of 25 h ± 1 h, which means that after a minimum of 24 h the cells should have reached a concentration that is high enough to allow transfer of at least one cell to the secondary enrichment. The minimal initial concentration at the start of the enrichment is one *L. monocytogenes* cell in 25 g of food that is enriched in 250 ml of HFB, meaning a minimum starting concentration of -2.4 $\log_{10}$ CFU/ml. After

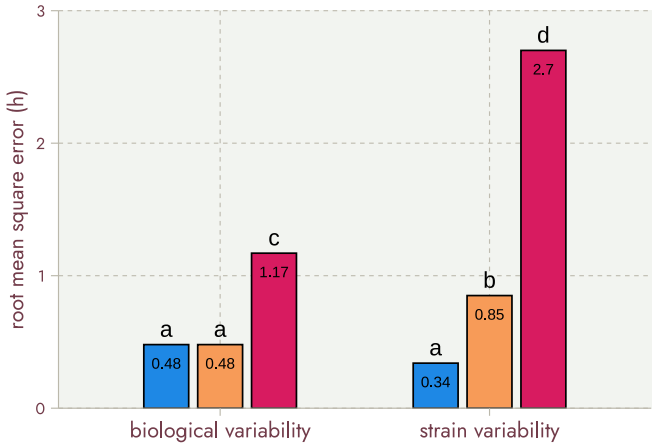

Figure 4: The biological and strain variabilities calculated as the root mean square error of the lag phases of strains of *L. monocytogenes* for the reference cells (blue, *n = 23*), the 10 °C acid pre-treatment (grey, *n = 5*) and the 60 °C heat stressed cells (red, *n = 23*). Bars with different letters indicate significant differences calculated by *F*-test with a *p*-value lower than 0.05.

primary enrichment, 0.1 ml of culture is transferred to the secondary enrichment step, so strains need to reach a concentration of 2 $\log_{10}$ CFU/ml in order to allow transfer of at least one cell to the secondary enrichment broth with 100 % probability[1] (Augustin et al., 2016).

In the scenario analysis the lag duration and maximum specific growth rate was based on the average of the 23 strains, with a variation of two times the standard deviation as a margin. In figure 5 it can be seen that when starting with the lowest possible food contamination levels (i.e. one cell per 25 g food product), the strains that had the lowest measured lag duration after 60 °C heat stress reach the threshold of 2 $\log_{10}$ CFU/ml within 24 h, also including variation in growth rate. The primary enrichment was also modelled for the average measured lag duration and the strains with the longest lag duration (figure 5b/c). Strains with average lag durations after 60 °C heat pre-treatment did not all reach the threshold of 2 $\log_{10}$ CFU/ml within 24 h, and strains with the highest lag duration did not reach this level at all. This corresponds to 11 out of the 23 tested strains not reaching this concentration in the 24 h primary enrichment step (figure 6).

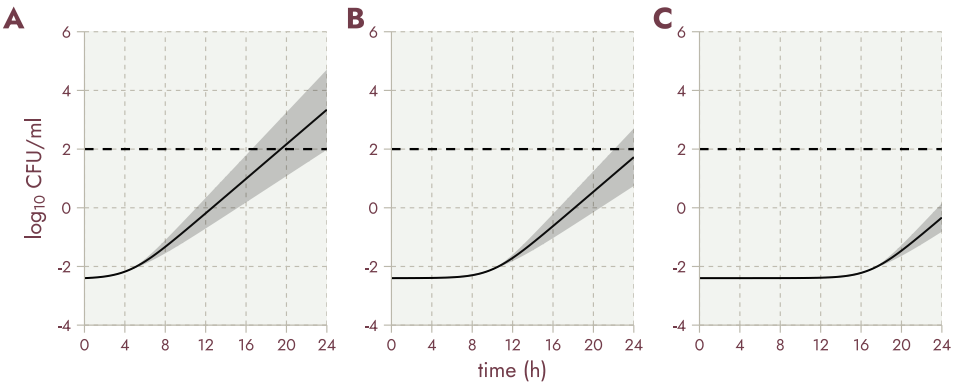

Figure 5: Scenario analyses of the primary enrichment step in HFB starting with one CFU of *L. monocytogenes* per 25 g product. A threshold of 2 $\log_{10}$ CFU/ml needs to be reached in the 24 h of enrichment to transfer at least one cell to the secondary enrichment step with 100 % probability. The inoculum concentration is one CFU that is enriched in 250 ml HFB meaning a concentration of -2.4 $\log_{10}$ CFU/ml. Here, (A) is the minimum lag with the average strain growth rate of 0.67 h^{-1} ± two times standard deviation, (B) is the mean lag with the average strain growth rate of 0.67 h^{-1} ± two times standard deviation and (C) is the maximum lag with the average strain growth rate of 0.67 h^{-1} ± two times standard deviation.

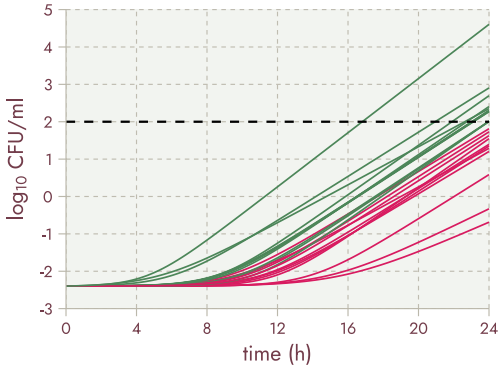

Figure 6: Spaghetti plot with the growth of all 23 tested strains of *L. monocytogenes* after 60 °C heat stress starting with one CFU per 250 ml of HFB. Strains that reach the detection threshold of 2 $\log_{10}$ CFU/ml (black horizontal dotted line) in the 24 h of primary enrichment are shown in green, strains in red do not reach this threshold within the allotted time.

To further illustrate this, Monte Carlo simulations were done out in @Risk to simulate the probability that the threshold of 2 $\log_{10}$ CFU/ml was reached after 24 h (figure S2). This showed that when starting with one cell per 25 g food product, 100 % of the iterations reached the threshold in the reference cells without prior stress history. However, when *L. monocytogenes* cells have been pre-exposed to heat stress, the detection threshold is reached in only 39.1 % of the iterations. In addition, the probability was calculated that at least a single cell would be transferred to the secondary enrichment step, assuming that transfer of cells in the enrichment medium follows a Poisson-distribution (table 1). This showed that the average chance to transfer at least one cell of 60 °C heat-stressed *L. monocytogenes* after 24 h of enrichment is 79.8 %. This probability increases to 90.8 % when enrichment is increased to 26 h and can even reach 99.0 % when 1 ml is transferred instead of the 0.1 ml that is specified by the current ISO11290-1:2017 protocol.

Table 1: Exploration of modifications on the current primary enrichment step on the probability to transfer at least one cell to the secondary enrichment medium. Transfer of cells in 0.1 ml of primary enrichment to full Fraser broth is assumed to follow a Poisson-distribution. The simulated mean probability is calculated as the mean of the Poisson probability curve after 100.000 iterations with normal distributions of the lag duration and the maximum specific growth rate as measured from the 60 °C heat stress experiments

scenario	simulated mean probability
24 h enrichment	0.798
26 h enrichment	0.908
24 h enrichment + 1 ml inoculum	0.965
26 h enrichment + 1 ml inoculum	0.990

2.4. Discussion

In this study the lag duration of a diverse collection of *L. monocytogenes* strains from different isolation sources and of different serotypes was quantified in half Fraser enrichment broth using non-stressed control cells and sub-lethally acid- and heat-damaged cells. This is important as the presence of pathogens in food products can be underestimated because cells sublethally injured during food processing can show extended lag durations, hence minimum cell concentrations are not reached in the 24 h time-span of the primary enrichment specified by the enrichment protocol. It is therefore important to quantify the growth dynamics during enrichments to increase the effectiveness and reliability of the culture-based detection methods for *L. monocytogenes*.

In order to quantify recovery of sublethally injured cells in half Fraser enrichment broth, heat treatment and low-temperature acid stress was used to stress cells. From previous experiments (data not shown) it was observed that a mild stress that does not lead to a reduction in cell concentration, does not affect the subsequent lag duration during enrichment. Therefore, a one $\log_{10}$ inactivation from heat-stress was chosen as a model stress treatment. Heat treatment is a common inactivation technique in food processing, which causes damage by inhibiting intracellular protein- and enzyme activity and by damaging nucleic acids and cell membranes (Wu, 2008). The strains used in this research show a large natural variation in their heat resistance (Aryani et al., 2015b), with a factor 8 difference in D_{60}-value between the least and most resistant strains. Because of this natural variation in heat resistance, the 60 °C pre-treatment was standardized to one $\log_{10}$ CFU/ml reduction resulting in different heat treatment times for each of the strains. Clearly, stress history is not exclusively delineated by the $\log_{10}$ CFU/ml reduction at population level affecting the subsequent cell recovery because heat adaptive responses are strain dependent (Lin & Chou, 2004; Skandamis et al., 2008). It is hypothesized that exposure of the strains to the same heat treatment time would have resulted in a higher strain variability in lag phase, because the heat-induced reduction and subsequent repair capacity will significantly vary for each of the strains. The strains also varied with respect to acid stress robustness and a large difference in acid resistance meant that different pH levels were used to standardize the reduction. A glucose-rich medium was used to culture and stress the strains, and strain variability in acid stress adaptive responses might also have contributed to differences in strain recovery mechanisms.

Extended exposure to 60 °C heat stress to obtain 3 $\log_{10}$ CFU/ml reduction in cell

concentration did not significantly influence the recovery, indicating that the recovery capacity of the injured population remains comparable. In contrast, Bréand et al. (1997) showed for *L. monocytogenes* that an increase in stress duration at 60 °C causes a rapid increase in lag duration that eventually reaches a steady threshold, but these studies were not carried out in HFB but in tryptic soy broth.

In the reference condition there was no significant difference between the biological and strain variabilities. However, significant differences in the ability to recover in half Fraser enrichment broth were found among strains after 60 °C heat stress. Strain variation in recovery suggests that there are differences in repair capacity among strains in the tested conditions. These differences are important to take into account when optimizing the enrichment protocol. Since cells can undergo a multitude of stresses during food processing, we also investigated whether the observed strain difference in recovery after heat stress would also translate to other stresses. After low-temperature acid stress, the lag duration increased compared to reference cells but the strain variability was not significantly different from the biological variability. Thus, for the 5 tested strains no significant inter-strain variation was observed. This is interesting as these tested strains show large differences in recovery from heat stress, which would indicate that recovery in HFB is not only strain-dependent, but also stress-dependent.

In enrichment media there is a balance needed between an optimal recovery capacity for stressed *L. monocytogenes* cells and the suppression of background microbiota. Despite this, the lag phase duration in full Fraser broth was found to be significantly higher than other enrichment broths after heat stress (Silk et al., 2002). Our results showed that the maximum specific growth rate is similar in reference cells and stressed cells in HFB. This indicates that in the conditions tested, once cells of *L. monocytogenes* have recovered, they grew at the same maximum specific growth rate irrespective of their history. This corresponds with the work of Guillier et al. (2005) where they showed that variability in detection time was mainly explained by variation in lag duration following stress exposure and that there was no correlation found with the maximum specific growth rates.

Furthermore, we showed that there were no significant differences in recovery from heat stress based on isolation source, lineage and serotype. This is in agreement with the results of Lianou et al. (2006) where they characterized the growth of 25 *L. monocytogenes* strains after heat and ambient acid stress in tryptic soy broth. They also found extensive variation in growth and stress resistance among tested strains, but could not correlate this to specific serotypes. It should be noted however, that the

statistical power to elucidate serotype effects in the current study was quite low, due to a relative small number of strains per serotype. Strains of serotype 4b did show the highest lag duration in HFB, which could indicate that a detection bias against type 4b strains is present. This can have complications for the detection of serotype 4b as this serotype is common among epidemic outbreaks of *L. monocytogenes* (Kathariou, 2002).

A low inoculum concentration of 2 $\log_{10}$ CFU/ml was used to mimic the low concentrations of *L. monocytogenes* that are found in food products, although cell numbers recovered on plates can be even lower in contaminated samples (Chen et al., 2003). The effect of inoculum level on the lag duration has been studied before for *L. monocytogenes*, where the lag duration has been shown to be unaffected by inoculum size in enrichment broth (Duffy et al., 1994), and under optimal conditions in media containing non-inhibitory salt concentrations (Robinson et al., 2001). Other studies (Gnanou Besse et al., 2006; Stephens et al., 1997) showed an extension and a larger variability in lag duration only at starting concentrations of less than 10 CFU/ml. Dupont & Augustin (2009) studied the effect of stress on lag durations of individual cells in HFB. They stated that injured cells increase in mean and variability of lag and that the physiological state of a cell has a strong impact on its ability to initiate growth. Thus at very low cell concentrations that have to be enriched, the variability in lag duration and the individual cell state becomes increasingly important. Therefore, the performance at the individual cell level in enrichments remains to be further elucidated as this is an important aspect in the optimization of the enrichment protocol.

Obviously, strains that recover the slowest will pose the highest risk of evading detection in the 24 h of the primary enrichment step by not reaching high enough cell concentrations for transfer to the second enrichment step. By combining all heat stress recovery data, the Monte Carlo simulations suggested that when starting with one cell in an enrichment, the threshold of 2 $\log_{10}$ CFU/ml was not reached after 24 h in 61 % of the simulations. Notably, the current ISO-procedure states that primary enrichment broth has to be incubated at 30 °C for 24–26 h. After the minimal 24 h of enrichment, the probability to transfer at least one cell to the secondary enrichment step was calculated to be 79.8 %. Incubating for 2 h more increases this probability to 90.8 %. Because stressed cells with long lag durations may still reach low cell concentrations even after 26 h of incubation, the transfer of only 0.1 ml to the secondary enrichment broth can cause false-negative results. Increasing the transfer volume to 1 ml increases the probability to transfer cells significantly, even up to 99 % when

also incubated for 26 h. Transfer of an even larger volume is not preferred, since this would also increase the levels of competing microbiota that are transferred, as well as decreasing the antibiotics concentration of full Fraser broth in the secondary enrichment.

The detection of *L. monocytogenes* in food products is affected by the effectiveness of the enrichment medium. This can be a problem even in optimized enrichment media because significant differences can be found in the ability to detect especially injured cells (Osborne & Bremer, 2002; Silk et al., 2002). These injured strains can however resuscitate and pose a risk for food safety (Donnelly, 2002). Furthermore there can be large variation among strains in stress resistance and recovery (Cauchon et al., 2017; de Jesús & Whiting, 2003; Francis & O'Beirne, 2005; Lianou et al., 2006; Lundén et al., 2008). Although strain variation in recovery has been shown before for *L. monocytogenes* (de Jesús & Whiting, 2003; Francis & O'Beirne, 2005; Lianou et al., 2006), the current study shows that strain variability can also influence the detection efficacy while using the ISO 11290-1:2017 protocol. For detection of pathogens in food, a reduction of the detection time is favourable but with increased lag durations of stressed and sublethally injured cells it comes with the risk of false-negatives.

2.5. Conclusion

This research shows that strains of *L. monocytogenes* differ significantly in their ability to recover in half Fraser enrichment broth, and that this strain variation should be taken into account when trying to optimize the current enrichment protocol. Our dataset shows that cells with a heat stress history can fail to reach the detection threshold for efficient transfer to the secondary enrichment step. Increasing the incubation time of the primary enrichment from 24 to 26 h, and subsequent transfer volume from 0.1 to 1 ml for the secondary enrichment broth, significantly increases the probability to detect those stressed *L. monocytogenes* strains that have extended lag durations in HFB.

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2.6. Supplementary materials

This section contains supplementary data of this chapter.

Table S1: List of *L. monocytogenes* strains used in this study with their serotype, isolation source, D_{60}-value in minutes and the pH used for one $\log_{10}$ reduction (during 24h at 10 °C)

strain	serotype	source	D_{60} (min)	pH used
WDCM00021	4b	Human isolate meningitidis (used in ISO 11290:2017)	0.5	2.25
ScottA	4b	Human isolate milk outbreak	0.6	2.00
WDCM00109	1/2a	Guinea pig (used in ISO 11290:2017)	0.6	3.35
LO28	1/2c	Healthy pregnant carrier	0.6	-
FBR13	1/2a	Frozen endive a la creme	0.6	-
10403S	1/2a	Human skin isolate	0.7	-
H7764	1/2a	Deli turkey	0.8	-
FBR12	1/2a	Frozen vegetable mix	0.8	-
FBR17	4d	Frozen fried rice	0.9	-
FBR20	1/2a	Frozen vegetables for soup	1.1	-
H7962	4b	Hotdog	1.2	2.25
FBR18	1/2a	Ice cream	1.2	-
FBR21	4d	Fresh yeast	1.2	-
FBR33	1/2c	Pancake	1.2	-
FBR19	1/2a	Frozen meat	1.3	-
AOPM3	4b	Human isolate	1.4	-
FBR15	1/2c	Ice cream packaging machine	1.5	-
EGDe	1/2a	Rabbit	1.6	3.20
C5	4b	Smoked meat	1.6	-
F2365	4b	Jalisco cheese	1.7	-
FBR16	1/2a	Ham (after cutting machine)	2.0	-
FBR14	1/2a	Carrot piece	3.4	-
L6	1/2b	Milk	4.1	-

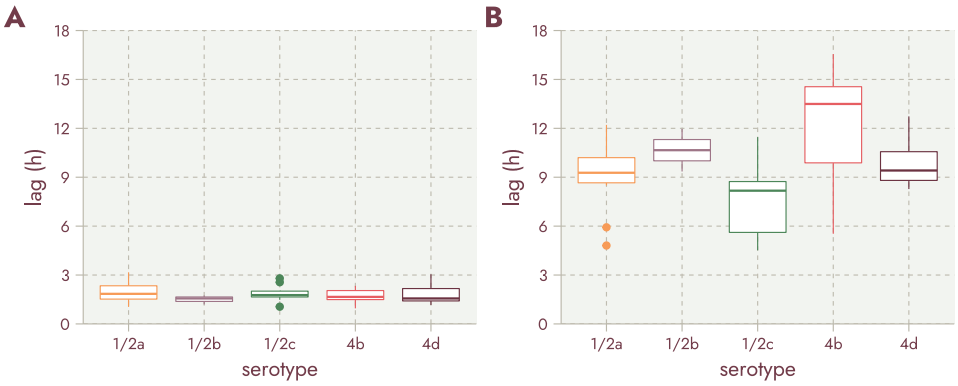

Figure S1: Boxplots of the lag duration in half Fraser primary enrichment broth ordered by strains belonging to serotype 1/2a (*n = 11*), 1/2b (*n = 1*), 1/2c (*n = 3*), 4b (*n = 6*) and 4d (*n = 2*) with (A) in reference cells with no additional pre-treatment and with (B) after 60°C heat pre-treatment. The boxes show the 25 % and 75 % quartiles, the bars show the median, the whiskers show 1.5 times the interquartile range and outliers are shown as points outside the whiskers.

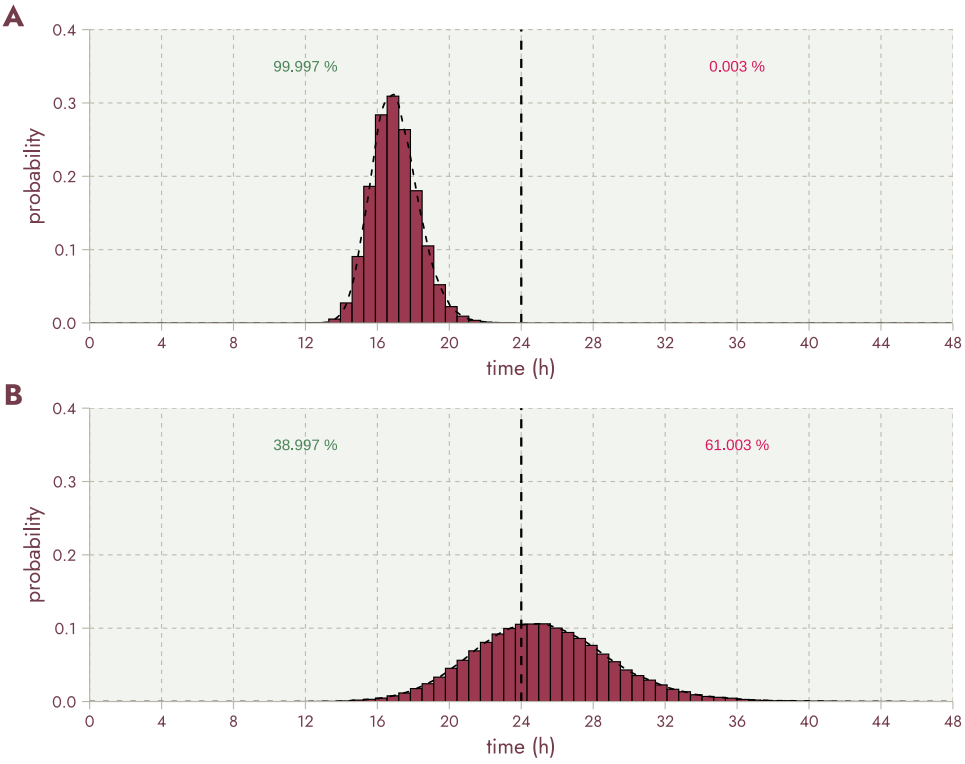

Figure S2: Monte Carlo simulation of 100.000 iterations with the probability of *L. monocytogenes* cells reaching the detection threshold in the 24 hour primary enrichment when starting with one cell per 250 ml of half Fraser. (A) The probability graph of the detection of reference cells, (B) the probability graph of the detection after 60°C heat stress history. The percentage on each side of the vertical 24-h line indicates the percentage of cells that reach the detection threshold within these 24 h.

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3

Heterogeneity in single-cell outgrowth of *Listeria monocytogenes* in half Fraser enrichment broth is affected by strain variability and physiological state

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Abstract

The behaviour of pathogens at the single-cell level can be highly variable and can thus affect the detection efficacy of enrichment-based detection methods. The outgrowth of single cells of three *Listeria monocytogenes* strains was monitored after fluorescence-activated single-cell sorting in non-selective brain heart infusion (BHI) broth and selective half Fraser enrichment broth (HFB) to quantify outgrowth heterogeneity and its effect on the detection probability. Single-cell heterogeneity was higher in HFB compared to non-selective BHI and heterogeneity increased further when cells were heat-stressed. The increase in heterogeneity was also strain-dependent because the fast- recovering strain ScottA showed less outgrowth heterogeneity than the slower-recovering strains EGDe and H7962. Modelling of the outgrowth kinetics during the primary enrichment demonstrated that starting at low cell concentrations could fail detection of *L. monocytogenes*, at least partly due to cell heterogeneity. This highlights that it is important to take single-cell heterogeneity into account when optimizing enrichment formulations and procedures when *L. monocytogenes* contamination levels are low.

3.1. Introduction

Listeria monocytogenes is ubiquitous in nature and can be introduced to food processing environments where it may contaminate products. *L. monocytogenes* infections – called listeriosis – have a high mortality rate of 20–30 % in the susceptible population (young, old, pregnant, and immunocompromised) (Swaminathan & Gerner-Smidt, 2007). Certain food products are routinely tested for the presence of *L. monocytogenes* to verify the effectiveness of a food safety management system. These detection procedures are based on an enrichment step where the pathogen recovers from the stressful conditions during food processing before growing out to detectable levels. Enrichments for *L. monocytogenes* are standardized in the ISO 11290–1:2017 enrichment protocol (International Organization for Standardization, 2017). This protocol consists of two enrichment steps to repair cell damage and selectively increase *L. monocytogenes* cell concentrations to allow subsequent isolation and detection. The enrichment method has to be able to detect very low levels of *L. monocytogenes* in these products as the European Union Food Law states that the absence of *L. monocytogenes* in five samples of 25 g has to be guaranteed in ready-to-eat food products that can facilitate growth after the production process (European Commission, 2007). In positive-tested food products, the contamination level can often be low with *L. monocytogenes* contamination levels in ready-to-eat foods more often containing between 10 and 1,000 CFU/g than more than 1,000 CFU/g (Koskar et al., 2019). The contamination level is also lower than 100 CFU/g in 97.5 % of positive beef and poultry samples (Awaisheh, 2010). The low starting concentrations can be problematic as the outgrowth of single cells can be heterogeneous. Interestingly, it has been shown that growth initiation at the single-cell level is highly heterogeneous due to variability in lag duration (Koutsoumanis, 2008). The heterogeneity in growth initiation is increased even more when cells are recovering from (sub-lethal) injury due to prior food processing (Guillier et al., 2005; Koutsoumanis & Sofos, 2005). Stresses such as freezing, heating, and exposure to low pH, which *L. monocytogenes* may encounter during food production can therefore have a profound effect on single-cell lag durations (Dupont & Augustin, 2009). Also, injured cells can become more sensitive to the selective compounds in the enrichment medium, which can retard their outgrowth potential.

Furthermore, when starting cell concentrations are low, the differences in single-cell outgrowth potential can lead to failure to reach the necessary detection threshold for detection. Such cases would lead to false-negative results where the presence

of *L. monocytogenes* is not detected and thereby posing a risk for food safety. Currently, limited data are available on the influence of single-cell heterogeneity on the outgrowth potential during enrichment with the ISO 11290–1 protocol other than the study by Dupont & Augustin (2009), in which they studied the effect of pre-exposure to stress on lag durations during primary enrichment. All aspects considered, it is conceivable that single-cell heterogeneity in outgrowth efficacy is a significant determinant of the detection rate of *L. monocytogenes* enrichments. Single-cell heterogeneity in growth performance can be effectively studied using flow cytometry combined with sorting of individual cells into multi-well plates and subsequent assessment of their outgrowth kinetics. We previously reported that the variability in *L. monocytogenes* strains during recovery can play an important role in detection at population level (Bannenberg et al., 2021). Therefore, the aim of this study was to quantify the impact of outgrowth heterogeneity of *L. monocytogenes* at single-cell level during primary enrichment following the ISO 11290–1 protocol in half Fraser broth using a selection of slow, medium, and fast-recovering strains of *L. monocytogenes*.

3.2. Materials and methods

3.2.1. Bacterial strains and growth conditions

Three strains of *L. monocytogenes* from different isolation sources and serotypes were kept at -80 °C in brain heart infusion (BHI) broth (Becton, Dickinson and company, USA) supplemented with 30 % glycerol (Fluka, USA). The strains ScottA, EGDe, and H7962 were chosen based on their difference in lag time in half Fraser broth (HFB) after being heat-treated at 60 °C (Bannenberg et al., 2021), with ScottA, EGDe, and H7962 having short, medium, and long lag phases in HFB respectively. Colonies were obtained from -80 °C freezer stocks that were plated out on BHI agar (BHI supplemented with 1.5 % agar, Oxoid, UK). From this, cultures were made by inoculating 10 ml of BHI broth with a single colony. Cultures were grown at 30 °C with 180 rpm shaking for 16 h to obtain stationary phase cultures. These cultures were subsequently diluted 1:1,000 in fresh BHI broth and incubated statically at 30 °C for 16 h to obtain a standardized working culture of around 9.5 $\log_{10}$ CFU/ml for use in further experiments.

3.2.2. Heat treatment of cells

Working cultures of all strains were heat-treated to reduce the viable counts by one $\log_{10}$ CFU/ml. The D_{60}-values of the strains previously published by Aryani et al. (2015) were used to determine the heat treatment time for each of the strains. Work-

ing cultures were diluted 1:100 in 50 ml BHI broth preheated at 60 °C in a water bath (Julabo SW23, Germany) for one D_{60}-value reduction (0.6 min for strain ScottA, 1.6 min for strain EGDe, and 1.2 min for strain H7962). Afterward, the cultures were quickly cooled on ice for 15 s to bring the temperature back to around 35 °C for use in follow-up experiments.

3.2.3. Fluorescence staining

Reference cells (cultures that did not receive any stress treatment) and heat-treated cells were stained with the LIVE/DEAD BacLight bacterial viability kit (Invitrogen, USA). This kit contains the membrane-permeable SYTO 9 dye, which stains all cells green, and the membrane-impermeable propidium iodide (PI) dye, which shows a bright red fluorescence signal upon binding to nucleic acids after entering cells with a permeabilized membrane. Staining of *L. monocytogenes* cells was needed for single-cell sorting to discriminate the small cell size of this bacterium from the background noise. To exclude dye interference on the outgrowth kinetics, SYTO 9 and PI were diluted ten times in DMSO to obtain stock solutions with dye concentrations of 334 µM for SYTO 9 and 2 mM for PI. These concentrations did not affect the growth as measured in additional test experiments using optical density measurements (data not shown). For the staining procedure, 1 ml of cell culture was serially diluted in peptone physiological salt (Tritium Microbiology, Netherlands) until a concentration of around 7 $\log_{10}$ CFU/ml, after which the dyes were added (final concentrations were 500 nM for SYTO 9 and 3 µM for PI) and cultures were incubated at room temperature in the dark for 15 min before use in the flow cytometer.

3.2.4. Flow cytometry and single-cell sorting

Flow cytometry experiments and cell sorting was done with the FACSAria III cell sorter (BD Biosciences, USA) using a 488 nm laser following the protocol of Warda et al. (2016). Briefly, the calibration was done with Cytometer Setup & Tracking beads and Accudrop beads (BD Biosciences, USA). Cells of *L. monocytogenes* were differentiated from electronic background noise using a combination of forward scatter (FSC), side scatter (SSC), propidium iodide red fluorescence (561 nm laser, 600LP with 610/20 nm filter in Texas Red channel), and green Syto 9 (488 nm laser, 502LP with 530/30 nm filter in FITC channel). The gating strategy was based on previous experience in sorting *L. monocytogenes* single cells, and before sorting, this gating strategy was established by collecting 50,000 events to exclude doublet cells. The exclusion of doublet cells was verified by microscopy. Events were randomly sorted

from the FITC green fluorescence channel to ensure the sorting of all cells. Wells of 384-wells plates (Greiner Bio-One, Austria) were filled with 50 µl of either BHI or HFB and a sorted single-cell was added to each well. As a control, PI-positive cells were sorted in a range of concentrations (1, 10, 100, 1,000, 10,000 cells/well), and only late growth was observed at 10,000 cells/well indicating that most PI-positive cells were indeed too damaged to efficiently grow out. The sorting was done mostly aseptically with the plate enclosed during sorting with the cell sorter. Blank wells were taken along as controls, and no growth was measured in these wells. The plates were briefly centrifuged before optical density measurement to remove air bubbles. Two independent reproductions were done on separate days with a total of 192 single-cell events per combination of strain, medium, and condition (a total of 2304 recorded events).

3.2.5. Quantifying single-cell outgrowth

Single-cell outgrowth in BHI broth and HFB was measured in a SpectraMax 384 plus (Molecular Devices, USA) set at 30 °C and at an optical density wavelength of 600 nm (OD_{600}). HFB was chosen as a selective broth following the ISO 11290–1:2017 procedure, and BHI was chosen as a non-selective reference medium. Optical density measurements were taken every 5 min for 48 h with 15 s shaking before each measurement. From the optical density data, the time-to-reach (TTR) of an outgrowing cell was quantified as the time to reach an OD_{600} increase of 0.25, which was chosen because at higher optical densities the HFB will blacken due to esculin hydrolysis, causing a rapid increase in the readings. Wells that did not reach the specified OD_{600} increase of 0.25 within 48 h were plated to verify whether there was outgrowth below the detection limit of the optical density measurements. For this, 10 µl of these wells was spot-plated on BHI agar plates and incubated at 30 °C for 48 h. When colony growth was observed, this indicated that the inoculated cell in the well had grown out but did not reach the concentration needed to cause an optical-density increase within the 48 h time frame. The mean, the standard deviation, and the coefficient of variation (CV, i. e. ratio of the standard deviation to the mean) of the TTR data were calculated for each strain (ScottA, EGDe, and H7962), medium (BHI and HFB), and condition (reference cells and heat-treated cells). All data analyses were done in R (R Core Team, 2021).

3.2.6. Determining the growth rate in wells

The maximum specific growth rate in the wells was determined for reference cells and heat-treated cells for each of the three strains in both BHI broth and HFB. The maximum specific growth rate was estimated using the two-fold dilution method as described by Biesta-Peters et al. (2010). Briefly, reference cells and heat-treated cells were added to 96-wells plates with a starting concentration of around 3 $\log_{10}$ CFU/ml in both BHI broth and HFB. Cells were two-fold diluted in subsequent wells up to the lowest concentration of around 1.8 $\log_{10}$ CFU/ml and the optical density was measured at 600 nm using a SpectraMax 384 plus, set at 30 °C for 48 h. The maximum specific growth rate was then estimated as the negative inverse slope of the TTR plotted against the natural logarithm of the cell concentration. The average maximum specific growth rate for each strain and medium was estimated from the mean of three biologically independent reproductions.

3.2.7. Modelling of the detection probability

To estimate the probability that single-cell outgrowth will lead to a false-negative enrichment outcome, the maximum specific growth rate in HFB and the single-cell lag time were used to estimate the outgrowth kinetics of single cells. To estimate the single-cell lag phase, the cell density was determined when the optical density increased by 0.25, i.e. the threshold that corresponds to the TTR (figure S1a). This allowed determining the minimum TTR taking into account the maximum specific growth rate and assuming no lag phase for a single cell to grow out in the well to reach the optical density threshold. The difference between the observed TTR and the minimum TTR corresponded to the lag time of the individual cell. This lag phase estimation and the maximum specific growth rate of each of the strains was used to simulate the kinetics at lower concentration assuming 1 cell in 250 ml of enrichment, resulting in $\log N_0$ of -2.4 $\log_{10}$ CFU/ml. The concentration that needs to be reached after 24 h to allow subsequent detection (the detection concentration) is around 2 $\log_{10}$ CFU/ml (Augustin et al., 2016) (figure S1b). This detection concentration was used to determine whether the individual cell would be detected after 24 h of enrichment, starting with an initial concentration of -2.4 $\log_{10}$ CFU/ml.

3.3. Results and discussion

3.3.1. Single-cell outgrowth kinetics

The single-cell outgrowth performance was determined for three strains of *L. monocytogenes* in HFB and non-selective BHI (figure 1). When comparing the outgrowth in BHI and HFB, it is clear that outgrowth in BHI is faster than in HFB. It has been shown before that the maximum specific growth rate in HFB is lower than in a non-selective medium such as BHI (Cornu et al., 2002). Moreover, outgrowth in BHI seemed to be more homogeneous than the outgrowth in HFB (figure 1), which suggests that outgrowth medium can impact the heterogeneity. Also, heat-treated cells with a 60 °C heat stress history (figure 1b/d/f) showed an increased outgrowth time when compared to reference cells of the same strain (figure 1a/c/e), most probably caused by an increased lag duration to recover from the sub-lethal injury induced by the heating treatment.

Additionally, the three strains of *L. monocytogenes* showed a clear difference in outgrowth kinetics, which is in line with earlier research where outgrowth kinetics of reference cells and heat-stressed cells were quantified at the population level for 23 strains (Bannenberg et al., 2021). Here, a fast (ScottA), average (EGDe), and slow recovering strain (H7962) was chosen in order to compare the outgrowth capacity at single-cell level. Indeed, the outgrowth kinetics of the individual cells of strain ScottA were faster than those of strains EGDe and H7962. Interestingly, also the outgrowth of single cells was more homogeneous in ScottA with faster outgrowth. Clearly, single-cell outgrowth heterogeneity was influenced by a combination of effects, including recovery medium, (stress) history, and strain characteristics.

3.3.2. Quantifying heterogeneity in outgrowth

For each well the single-cell outgrowth was quantified as the time-to-reach (TTR) to reach an optical density increase of 0.25. The distribution of all single-cell outgrowth events is shown in the density histograms of figure 2. Irrespective of strain and/or history, the distribution of TTR values mostly resembled a lognormal-distribution.

3.3.2.1. Effect of recovery medium on heterogeneity

First of all, the choice of recovery medium had an impact on the single-cell outgrowth in both reference cells and heat-treated cells. For reference cells, the mean TTR in non-selective BHI was 2.7 h shorter than in selective HFB for strain ScottA, 5.1 h shorter for EGDe, and 5.3 h shorter for H7962 (table 1). After heat stress, the

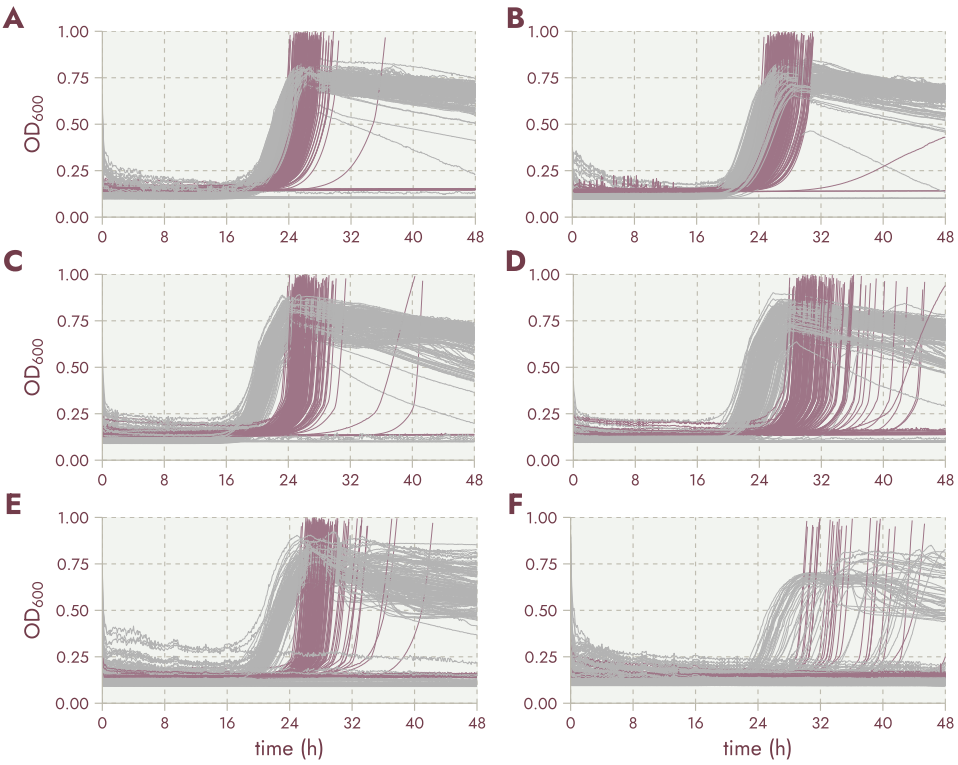

Figure 1: The outgrowth of single cells of *L. monocytogenes* as measured by optical density at 600 nm in BHI (grey lines) and half Fraser enrichment broth (purple lines). The outgrowth in all wells is shown for (A) reference cells of strain ScottA, (B) heat-treated cells of strain ScottA, (C) reference cells of strain EGDe, (D) heat-treated cells of strain EGDe, (E) reference cells of strain H7962 and (F) heat-treated cells of strain H7962. Cells grown in HFB reach much higher optical densities due to the blackening of the medium by the hydrolysis of esculin at higher cell concentrations.

difference in mean TTR between BHI and HFB increased to 2.9 h for ScottA, 7.1 h for EGDe, and 6.0 h for H7962. Recovery in HFB did not only increase in mean TTR and subsequent standard deviation, but also had an increased coefficient of variation (table 1). The higher coefficient of variation in HFB indicates a relative increase in variability in single-cell outgrowth. This observation was tested by comparing the variances with Levene's test of the TTR values. This confirmed that HFB significantly increases the variability in single-cell outgrowth compared to BHI ($p < 0.001$).

A possible factor that could explain the difference in single-cell variability is the presence of antibiotics in HFB. The selective compounds in HFB are acriflavine which inhibits RNA-synthesis (Meyer et al., 1972), nalidixic acid that can inhibit both RNA- and DNA-synthesis (Crumplin & Smith, 1975), and lithium chloride that can com-

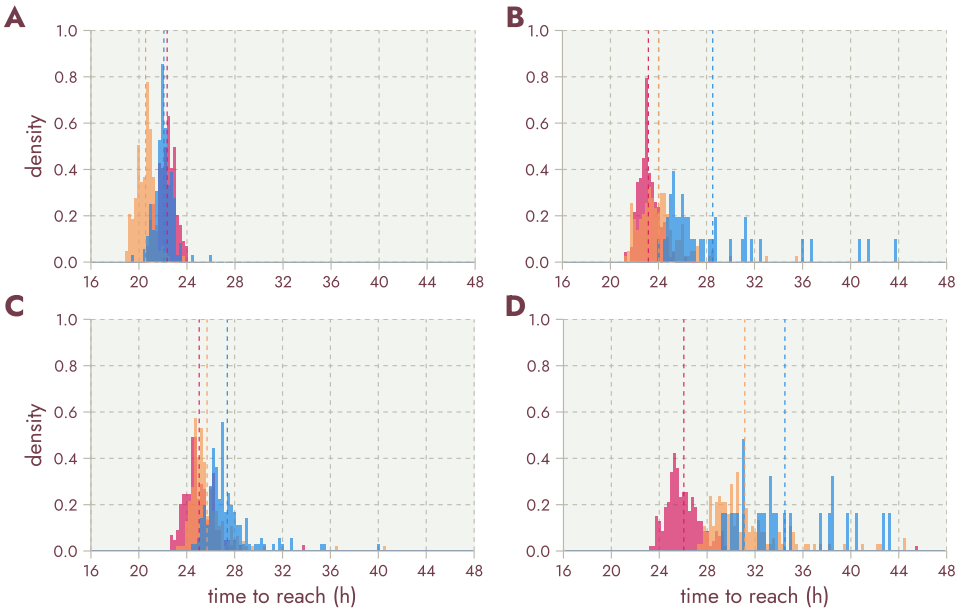

Figure 2: The probability distribution of the time-to-reach for single cells of *L. monocytogenes* in both BHI and HFB for reference cells and heat-treated cells. Time-to-reach was determined as the time to reach an increase in OD_{600} of 0.25. Here, the single-cell outgrowth is shown for reference cells in BHI (A) and in HFB (C), and single-cell outgrowth of heat-treated cells (one $\log_{10}$ CFU/ml reduction) in BHI (B) and in HFB (D). Strain *L. monocytogenes* ScottA is in red, strain EGDe in yellow and strain H7962 in blue. The dotted lines indicate the mean outgrowth for each strain and wells that did not reach the TTR in 48 h were not plotted. The histogram bin size is 15 min and the total area of the histogram is normalized to one for better comparison despite differences in the number of outgrown wells.

pete with divalent cations in susceptible microorganisms (Mendonca & Knabel, 1994). Next to an increased lag duration and reduced growth rates in HFB due to these selective compounds (Beumer et al., 1996), membrane damage or increased permeability caused by heat stress history may facilitate easier entry of selective compounds into the cells. This can explain the increase in heterogeneity as cell-to-cell fluctuations in a selective medium often lead to higher heterogeneity than in a non-selective medium such as in BHI.

3.3.2.2. Effect of heat-stress history on the heterogeneity

Next to the medium effect on outgrowth, figure 2b and d also show that heat treatment increased the average TTR and the heterogeneity in both BHI and HFB (Levene's test $p < 0.001$). When comparing reference cells with heat-treated cells in the same medium, there was a significant increase in average TTR, standard deviation, and in the coefficient of variation (table 1). The addition of a heat-stress history increases the

skewness towards the right, meaning that the variability in TTR increases in favour of longer outgrowth. Here, due to differences at the single-cell level, stress history can increase the range of lag durations and thereby complicate adequate detection during enrichment. This corroborates with previous research in which we found that heat stress can be of significant influence on the subsequent recovery in enrichment broth (Bannenberg et al., 2021). Next to an increase in mean TTR, single-cell outgrowth was also more heterogeneous and this can be of importance during detection. This is in line with earlier research which showed that single-cell growth initiation is also dependent on the stress history (Dupont & Augustin, 2009). In that study, (55 °C) heat treatment was found to be a stress with a significant effect on the single-cell growth initiation in HFB. Furthermore, that study also showed that in injured cells, the single-cell lag times increased in mean and variability. However, in our study single-cells were sorted by flow cytometer and the single-cell behaviour could be compared to the population behaviour during enrichments.

Table 1: Effect of medium and heat-stress treatment on the heterogeneity of single-cell outgrowth. For each strain in BHI and HFB and for reference cells and heat-stressed cells, the number of wells where outgrowth was observed within 48 h is shown. The mean, standard deviation (sd), and coefficient of variation (CV) in the TTR are also given

strain	medium	condition	outgrown wells	mean TTR	sd TTR	CV TTR
ScottA	BHI	Reference	178/192	22.3	0.72	0.032
	HFB		180/192	25.0	1.29	0.052
	BHI	Heat stress	187/192	23.2	0.81	0.035
	HFB		190/192	26.1	1.97	0.076
EGDe	BHI	Reference	175/192	20.6	0.73	0.036
	HFB		189/192	25.7	1.79	0.070
	BHI	Heat stress	174/192	24.0	1.90	0.079
	HFB		153/192	31.1	3.18	0.102
H7962	BHI	Reference	145/192	22.1	0.75	0.034
	HFB		144/192	27.4	2.09	0.076
	BHI	Heat stress	41/192	28.5	4.88	0.171
	HFB		25/192	34.5	4.23	0.123

3.3.2.3. Effect of strain differences on the heterogeneity

Because the selected strains differed in their heat resistance, the D_{60}-value was used to standardize the reduction to one $\log_{10}$ CFU/ml. Even so, the heat treatment affected the tested strains to different extents, with an average increase of 1.0 h for strain ScottA, 4.5 h for strain EGDe, and 6.8 h for strain H7962 after 60 °C heat-stress for both media (table 1). Arguably, this matches with the previously described recovery behaviour of the strains (Bannenberg et al., 2021) because the fast-recovering strain ScottA has the least heterogeneity, the average-recovering strain EGDe has average heterogeneity and the slow-recovering strain H7962 displays the most heterogeneity. This suggests that stress recovery capacity affects heterogeneity at least to some extent, where stressed strains with faster recovery exhibit less heterogeneity in single-cell outgrowth. When outgrowth heterogeneity is dependent on the degree of cell damage that needs to be repaired and the outgrowth characteristics of the strain, this could contribute to a selection bias during enrichment.

Notably, single-cell sorting of heat-treated cells did not always lead to quantifiable outgrowth within 48 h (table 1). This was especially the case for the slow-recovering strain H7962 after heat treatment. To evaluate whether outgrowth below the optical density detection threshold occurred, the contents of the wells were spot-plated on BHI-agar. This showed that even though some single-cell events did not reach the optical density threshold within 48 h, in 21 % of the cases heat-treated cells in HFB still showed colony formation (data not shown). A likely reason is that those single-cells were either too damaged for repair and growth initiation or that they had a considerably increased lag duration. The cell sorting with the flow cytometer might also be a contributing factor, with the conditions during the sorting procedure on already damaged membranes of heated cells giving extra stress on subsequent recovery in the wells. However, cells were sorted using lower flow rates and a sufficiently sized 85 µm nozzle which means that this should have a limited impact on cell integrity. Still, it has been shown previously that the sorting of cells can affect cell resuscitation capabilities, especially for heat-treated cells (Sibanda & Buys, 2017), and even though a 10 times diluted dye concentration was used for sorting a slightly toxic effect cannot be excluded.

3.3.3. Estimation of single-cell probability to miss detection

The often harsh environments that food microbes are exposed to can lead to differences in repairability and subsequently in single-cell outgrowth heterogeneity. Especially, outliers with increased lag durations can become problematic when detection

of low levels of *L. monocytogenes* is required, because differences in outgrowth capacity can lead to failure to detect these cells. To estimate the impact of heterogeneity in single-cell outgrowth on the detection probability, the outgrowth of single cells was modelled. Taking the maximum specific growth rate of each strain into account (table S1), the minimum TTR (i.e. assuming no lag phase) for a single cell to grow out in a well to reach the optical density threshold in HFB could thus be estimated for each strain (figure S1a). The difference between the observed TTR and the minimum TTR is assumed to correspond to the lag time of the individual cell. To estimate the possibility that heterogeneity affects the successful detection probability during enrichment, the maximum lag duration was estimated that still allows efficient transfer of cells to the secondary enrichment step. The concentration that needs to be reached after 24 h of enrichment to transfer at least one cell with a Poisson-chance higher than 99.9 % is a concentration of 1.8 $\log_{10}$ CFU/ml (Augustin et al., 2016; Bannenberg et al., 2021). Again, considering the maximum specific growth rate of each strain, then the estimated maximum lag phase that still allows for subsequent detection is 11.3 h for strain ScottA, 11.6 h for strain EGDe, and 9.8 h for strain H7962 (figure S1b).

Combining this maximum lag duration and the outgrowth in the wells allowed us to set a threshold after which these cells can start to fail the transfer to the secondary enrichment broth and in turn fail detection with the current enrichment protocol. We call this tipping point the 'detection-fail probability' in this context because the probability to fail detection starts to increase after this threshold. This model estimated that the detection-fail probability is reached for sorted single cells with a TTR of higher than 32.9 h for strain ScottA, 30.9 h for strain EGDe, and 33.3 h for strain H7962. The discrete distribution of single-cells that did not reach the detection concentration is shown in figure 3 for reference and heat-stressed cells. Here, single-cells that grew out in the red-shaded areas are at risk of not reaching the detection concentration. The detection-fail probability was then estimated as the number of wells that have a TTR longer than the estimated threshold. In this way, single-cells that did not grow out within 48 h were considered to fail to reach the detection concentration as well. For strain ScottA (figure 3a/b), the estimated detection-fail probability is 0.07 for reference cells and 0.02 for heat-stressed cells. This low probability to not reach the detection concentration corresponds with its fast recovery in HFB as was shown in earlier research (Bannenberg et al., 2021). For strain ScottA the impact on the detection probability is small because even after heat-stress the single-cell heterogeneity in recovery is still within a safe margin for successful detection. On the other hand, the remaining tested strains that already had a longer lag duration af-

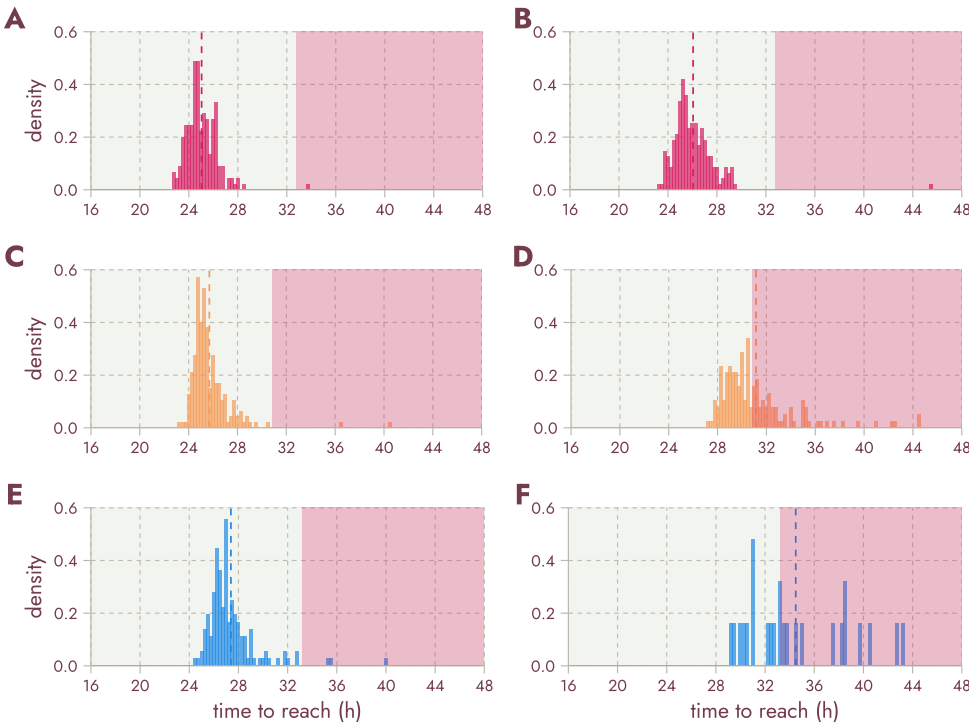

Figure 3: Heterogeneity of the TTR and the predicted detection-fail probability during enrichment in HFB. For each strain and condition, the maximum TTR has been estimated that could lead to a detection-fail outcome, and TTR values falling outside this detection range are shaded in red and are considered to be at risk of failing detection. The single-cell outgrowth of reference cells of strain ScottA with a detection-fail probability of 0.07 (A), heat-treated cells of strain ScottA with a detection-fail probability of 0.02 (B), reference cells of strain EGDe with a detection-fail probability of 0.03 (C), heat-treated cells of strain EGDe with a detection-fail probability of 0.52 (D), reference cells of strain H7962 with a detection-fail probability of 0.27 (E), and heat-treated cells of strain H7962 with a detection-fail probability of 0.93 (F). Cells that did not grow out within the 48 h time period are not plotted but these events are taken into account for the calculation of the detection probability.

ter stress history also had higher probabilities to miss the detection concentration. For the average-recovering strain EGDe and the slow-recovering strain H7962, the detection-fail probability increased significantly after 60 °C heat stress. Detection-fail probabilities for strain EGDe (figure 3c/d) were estimated as 0.03 in reference condition and 0.52 after heat-stress. For H7962 (figure 3e/f) this increased to 0.27 in reference condition and 0.93 for stressed cells in HFB, where most of the single-cell events did not grow out to detectable levels. Strains that can recover faster and have a lower heterogeneity in outgrowth potential during enrichment have a higher probability to be detected than slow recovering strains. In this case, however, it should be

noted that it is a possibility that some single-cells could be too damaged to still facilitate resuscitation and subsequent outgrowth. All in all, variability in single-cell outgrowth in strains of *L. monocytogenes* can lead to failure to detect stressed pathogens in food products.

3.4. Conclusion

Strain differences, history, and single-cell heterogeneity affect the successful detection of *L. monocytogenes* using the ISO 11290–1 enrichment-based detection method. The recovery of stressed cells of *L. monocytogenes* depends not only on strain differences in population behaviour, but the recovery at single-cell level can also vary drastically within a specific strain. Heterogeneity in outgrowth also seems to be higher in strains with larger lag durations, indicating that for strains that already have a hard time recovering in HFB this effect is enlarged at single-cell level. Furthermore, kinetic modelling of the primary enrichment step showed that the aforementioned factors can contribute to false-negative detection outcomes, highlighting the importance to consider this when optimizing the ISO 11290–1 enrichment protocol for the detection of *L. monocytogenes*.

Acknowledgements

The authors would like to thank all partners in this project (Biomérieux, DSM, Innosieve Diagnostics, Merck Darmstadt, NWO, and Wageningen Food Safety Research) for their financial and in-kind contributions. This work is part of the research program “Rapid and reliable enrichment-based detection of foodborne pathogens” with project number 15002, which is partly financed by the Dutch Research Council (NWO).

3.5. Supplementary materials

This section contains supplementary data of this chapter.

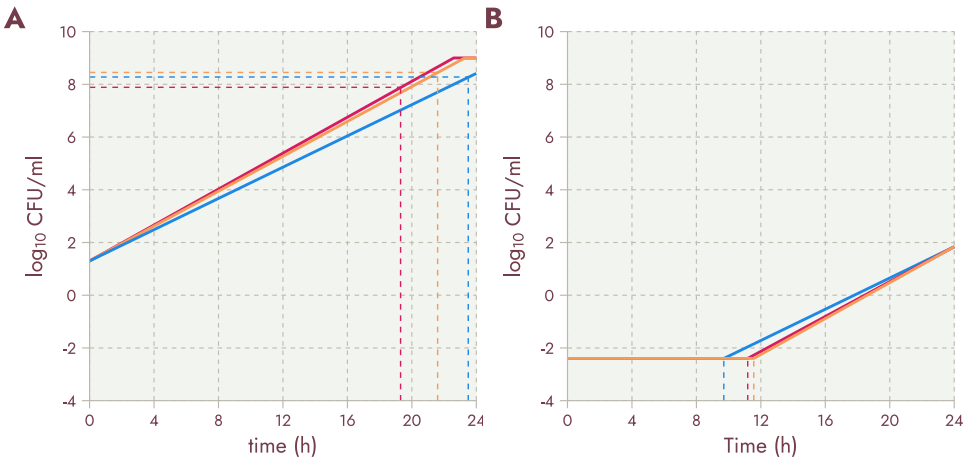

Figure S1: Model of the growth during primary enrichment to estimate the detection-fail chance during enrichment. With (A) the model of the minimum outgrowth in the wells plates assuming no lag duration with ScottA in red, EGDe in yellow, and H7962 in blue (the initial level is 1 cell in 50 µl, which is 1.3 $\log_{10}$ CFU/ml). The TTR in these wells (dotted line) is reached on average in 19.3 h for ScottA, 21.6 h for EGDe, and 23.5 h for H7962. In (B) the primary enrichment step is modeled of the outgrowth of a single cell (in 250 ml HFB, -2.4 $\log_{10}$ with the maximum lag duration (dotted line) that still allows efficient transfer to the secondary enrichment step after 24 h (assuming a Poisson chance to transfer at least one cell with 99.9 % probability). The maximum lag that still allows for this is 11.2 h for strain ScottA, 11.6 h for strain EGDe, and 9.7 h for strain H7962.

Table S1: Parameters used for the modelling of figure S1 for each of the three tested strains of *L. monocytogenes*. For each strain, the concentration is given that needs to be reached for a 0.25 increase in optical density, the maximum specific growth-rate and the maximum lag

strain	concentration ($\log_{10}$ CFU/ml)	µmax (h^{-1})	maximum lag (h)
ScottA	8.5	0.76	11.2
EGDe	7.9	0.78	11.6
H7962	8.3	0.68	9.7

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4

Insight into the lag phase of *Listeria monocytogenes* during enrichment through proteomic and transcriptomic responses

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Manuscript in preparation

Abstract

The dynamics of the enrichment-based detection procedure of the foodborne pathogen *Listeria monocytogenes* from food still remains poorly understood. This enrichment is crucial in the reliable detection of this pathogen and more insight into the recovery mechanism during this step is important to advance our understanding of lag phase behaviour during enrichment. In this study we combined transcriptomics and proteomics to better understand the physiological processes within the lag phase of *L. monocytogenes* during enrichment. Upon transfer of BHI-cultured stationary phase *L. monocytogenes* cells to half-Fraser enrichment broth (HFB), motility-associated genes and proteins were downregulated, while expression of metal uptake transporters, resuscitation-promoting factors that stimulate growth from dormancy, antibiotic efflux pumps and oxidative stress proteins were upregulated. Next to this, when cells with a heat stress history were cultured in enrichment broth, proteins necessary for recovery were upregulated with functions in DNA-damage repair, protein refolding, cell-wall repair, and zinc transport. Proteomic results pointed to possible factors that support shortening the lag duration, including the addition of 10 µM zinc and the addition of spent HFB containing presumed concentrations of resuscitation-promoting factors. However, these interventions did not lead to biologically relevant reduction of lag phase. Also, when cells were enriched in spent HFB, final cell concentrations were similar to enrichments in fresh HFB, indicating that the enrichment broth seems not to lack critical substrates. Concludingly, this study gives insight into the proteomic changes that happen in the lag phase during enrichment and shows that supplementation of HFB is not the best strategy to optimize the current enrichment method.

4.1. Introduction

Listeria monocytogenes is an important food pathogen and thus needs to be adequately detected in food products. A reliable detection method is necessary to verify the control measures that are put in place by food producers to produce safe food products. For this, the current gold standard for detection is the ISO 11290-1:2017 (International Organization for Standardization, 2017), where enrichment of food samples is coupled with culture-based detection of *L. monocytogenes*. However, this enrichment-based procedure is labour-intensive and time-consuming, taking between 4 to 7 days before positive confirmation (International Organization for Standardization, 2017). There have been rapid developments in molecular detection methods to reduce the detection times (Ferone et al., 2020; Law et al., 2014). Rapid methods like real-time PCR and biosensors are fast and are promising alternatives to culturing-based detection. However, these rapid methods have detection limits from 4 $\log_{10}$ CFU/ml (Ferone et al., 2020; Kanayeva et al., 2012), and as such, rely on a preceding enrichment step in order to detect the presence of potentially very low levels of *L. monocytogenes* in a food matrix.

The European Union legislation lays down absence testing of 5 samples of 25 gram for ready-to-eat foods that may support growth (European Commission, 2007). Yet, enrichment of food samples is needed to confirm these low levels. Despite the importance of an enrichment step in the detection procedure, the mechanisms of recovery and outgrowth of cells during enrichment is hardly explored. This knowledge gap on the physiological behaviour during the crucial enrichment step hinders the advancement of the detection method. This is even more important because *L. monocytogenes* cells in food may be damaged by food processing and preservation measures, emphasizing the importance of cell resuscitation during the enrichment step. Also, we demonstrated in earlier research that cells with a stress history can have prolonged lag durations that are dependent on the strain and the type of stress exposure (Bannenberg et al., 2021a,b). The lag phase however, remains quite poorly understood and hard to predict (Hamill et al., 2020; Vermeersch et al., 2019). For this reason, it is important to understand the physiology of bacterial adaptation during the lag phase, and this knowledge can give leads to optimize the enrichment medium to allow for optimal recovery capacity to support detection of *L. monocytogenes*. Therefore, this research aimed to generate insight into the adaptation of *L. monocytogenes* to half-Fraser broth (HFB) which is used as the primary enrichment medium according to the ISO 11290-1. We followed an omics approach where we combined transcriptomics and

proteomics to gain insight into the lag phase during the enrichment of non-stressed and heat-stressed BHI-grown stationary phase cells of *L. monocytogenes*.

4.2. Materials and methods

4.2.1. Bacterial strains and growth conditions

L. monocytogenes strain EGDe was kept at -80 °C in brain heart infusion (BHI) broth (Becton Dickinson Difco) supplemented with 30 % glycerol (Fluka). Cultures were made by inoculating 10 ml of BHI broth with a single colony picked from a BHI agar plate (BHI supplemented with 1.5 % agar, Oxoid) obtained from -80 °C freezer stocks. Cultures were grown at 30 °C with 180 rpm shaking for 16 h to obtain stationary phase cultures. These cultures were subsequently diluted 1:1,000 in fresh BHI broth and incubated statically at 30 °C for 16 h to obtain a standardized working culture for use in further experiments.

4.2.2. Heat treatment of cells

Working cultures of strain EGDe were heat-treated to stress the cells and reduce the viable counts. For that, working cultures were diluted 1:1 in a total of 30 ml BHI broth pre-heated at 60 °C in a water bath (Julabo SW23) for 1.6 min to reach a reduction of around 0.5 $\log_{10}$ CFU/ml for all reproductions. Afterward, the cultures were cooled on ice for 15 s to quickly bring the temperature back to a non-lethal temperature below 42 °C. The heat-stressed working culture was then diluted 10 times by adding 25 ml culture to 225 ml HFB (according to ISO 11290-1) to start the enrichment (starting concentration was around 7.7 $\log_{10}$ CFU/ml). For reference cells, the working cultures were diluted 1:1 in 30 ml BHI and then transferred 1:10 in 250 ml HFB to start the enrichment (starting concentration was around 8.2 $\log_{10}$ CFU/ml). All enrichments were done at 30 °C as specified by ISO 11290-1 for the primary enrichment in HFB (International Organization for Standardization, 2017).

4.2.3. Proteomic analysis

After thee start of reference and heat-stressed enrichments of strain EGDe, samples were collected at 0, 10 min, 30 min, and 2 h for reference cells, and at 0, 30 min, 2h, 4h, and 7h for heat stressed cells (figure S1). Corresponding cell concentrations were determined by plate counting on BHI agar plates. For this, samples were serially diluted in peptone physiological salt (PPS, Tritium Microbiology) and spread plated on agar and incubated at 30 °C for at least 24 h. The lag duration of reference and heat-stressed cells was estimated by fitting the Baranyi model (Baranyi & Roberts, 1994) to

the plate counts data (figure S1) as has been shown before in more detail (Bannenberg et al., 2021a). When a sample was taken, 45 ml of culture was centrifuged at 10,000 g for 2 min, and dissolved in 200 µl 100 mM Tris (pH 8). All samples were washed twice with Tris-buffer before they were snap frozen in liquid nitrogen and stored at -80 °C before further analysis. Samples were taken as three biologically independent reproductions from working cultures propagated from different colonies. Frozen samples were allowed to thaw on ice and were sonicated twice for 30 s to lyse the bacterial cells (MSE Soniprep 150). The protein content was measured with the Bradford assay (Bradford, 1976).

Samples were prepared for proteomic analysis according to the filter-aided sample preparation protocol (FASP) (Wiśniewski et al., 2009) with the following steps: reduction with 15 mM dithiothreitol, alkylation with 20 mM acrylamide, and digestion with sequencing grade trypsin overnight. Each prepared peptide sample was analysed by injecting 5 µl into a nanoLC-MS/MS (Thermo nLC1000 connected to a Q-Exactive HF-X) using a 1 h gradient as previously described by Liu et al. (2021). LCMS data with all MS/MS spectra were analysed with the MaxQuant quantitative proteomics software package (Cox et al., 2014) as described before (Smaczniak et al., 2012). A reference protein database containing all protein sequences of *L. monocytogenes* EGDe (organism ID: 169963, proteome ID: UP000000817) was downloaded from the UniProt database (Apweiler et al., 2004). Filtering and further bioinformatics and statistical analysis of the MaxQuant ProteinGroups file were done with Perseus (Tyanova et al., 2016). Reverse hits and contaminants were filtered out and protein groups were filtered to contain minimally two peptides for protein identification of which at least one is unique and at least one is unmodified. All timepoints were compared against timepoint 0 for both the reference cells and the heat stressed cells. Proteins were considered to be significant differentially expressed with an absolute fold-change increase of 2 (equalling 1 $\log_2$ fold increase) and a *p*-value lower than 0.05. Further data analysis was done in R version 4.1.0 (R Core Team, 2021). Clusters of orthologous groups (COG) were taken from the eggNOG database (Huerta-Cepas et al., 2019), and the KEGG pathway analysis was done with clusterProfiler (Wu et al., 2021).

4.2.4. Transcriptomic analysis

Total RNA was isolated from three biologically independent reproductions for reference cells in HFB. For this, 150 ml of HFB was inoculated with standardized working cultures of strain EGDe (final concentration of 7.5 $\log_{10}$ CFU/ml), and samples were

taken after 0, 5, and 30 min of incubation at 30 °C. For each timepoint, 45 ml of culture was quickly centrifuged for 3 min at 10,000 x g (5804 R, Eppendorf). The supernatant was removed and the pellet was dissolved in 1 ml of TRI-reagent (Ambion), vortexed and allowed to stand for 5 min at room-temperature. The total volume was added to lysing matrix B tubes (0.1 mm silica spheres, MP Biomedicals) and cells were disrupted by beat beating (MP Fast Prep-24, MP Biomedicals) at 6 m/s for 4 times 20 s with 1 min rest in between. To the beads 200 µl chloroform was added, centrifuged for 15 min at 13,300 rpm, and the aqueous phase was added to 500 µl isopropanol in RNase free Eppendorf tubes. The tubes were vortexed for 2 min and centrifuged for 10 min at 13,300 rpm. The supernatant was removed and the pellet was washed with 1 ml of ice cold 70 % ethanol, after which the samples were centrifuged at 4 °C for 10 min at 13,300 rpm. The remaining ethanol was allowed to evaporate in a laminar flow cabinet, and the pellet was dissolved in 90 µl RNase-free water. Remaining DNA was removed by treatment with RNase-free DNase kit (Ambion) using manufacturers specification. RNA quality was measured with NanoDrop One (Thermo Scientific), and stored by adding 10 % of the volume 3 M sodium acetate and 2.5 times the volume of absolute ethanol before storage at -80 °C. RNA was shipped on dry-ice and rRNA was depleted with Ribo-zero depletion and generation of paired-end reads using a MiSeq system by BaseClear (Leiden, Netherlands). Quality control and read mapping was done against the EGDe reference genome using BaseClear in-house methods. Reads were counted using htseq-count (Anders et al., 2015), and differential expression was done using the DEseq2 package (Love et al., 2014). Genes were considered differentially expressed when the absolute $\log_2$ fold change was higher than two, with a Benjamini-Hochberg corrected *p*-value below 0.05. Differentially expressed genes were used to supplement the proteome results.

4.2.5. Compound utilization measurements with HPLC

One ml samples were taken of biologically independent triplicates during enrichment of reference and heat stressed cells at 2 h intervals until 24 h and frozen at -20 °C. To take samples after 12 h, a secondary enrichment was started at the end of the day. In this way samples between 14 and 22 h could be collected. To quantify the organic acids and amino acids, the protocol was followed as described by Lanzl et al. (2022). Briefly, for organic acids measurements, 500 µl sample was thawed and subsequently deproteinized by addition of 250 µl Carrez A and 250 µl Carrez B. The suspension was centrifugated at 13,300 rpm for 2 min and the supernatant was added to HPLC vials and used for injection in the UltiMate 3000 HPLC (Dionex, Germany).

At each timepoint the concentration of the organic acids was measured by referencing the elution peaks with a standard calibration curve containing all measurable compounds that can be detected with this setup (acetate, cellobiose, formate, glucose, glycerol, lactate, malate, pyruvate, succinate, and citrate). The average concentration of each organic acid was then plotted with its standard deviation. For amino acid quantification, 250 µl of thawed sample was derivatized with the AccQ·Tag Ultra derivatization kit (Waters, USA) following the manufacturer's instructions. Derivatized samples were quantified by UPLC and the concentration at each time point was determined by comparing the elution peak with the standard calibration curve (containing alanine, asparagine, aspartate, cysteine, glutamate, glutamine/arginine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine).

4.2.6. Metal supplementation

The effect of metal supplementation on the outgrowth during enrichment was measured by optical density. Reference cells and heat stressed cells were cultured according to subsection 4.2.2. Different concentrations of metal ions were tested and final supplemented concentrations in HFB ranged from 10 µM to 1 mM. Metal-supplemented HFB was added to wells containing a total volume of 200 µl in a 96-wells plate. Working cultures were added to the wells at a starting concentration of around 4 $\log_{10}$ CFU/ml. Plates were incubated at 30 °C in a SpectraMax 384 plus (Molecular Devices), and optical density measurements were taken every 5 min for 48 h with 15 s shaking before each measurement. From the optical density data, the time-to-reach (TTR) of each well was quantified as the time to reach an OD_{600} increase of 0.25. This threshold of 0.25 was chosen because at higher optical densities the HFB will blacken due to the esculin hydrolysis reaction, which will cause a rapid increase in optical density. The TTR was then used to compare the effect of metals on the outgrowth compared to the TTR of standard HFB.

4.2.7. Supplementation with spent medium

To collect spent medium, working cultures were added to HFB and cells were cultured at 30 °C for about 28 h when the medium started to blacken. The cultures were then centrifuged for 5 min at 10,000 g and the supernatant was filter-sterilized with 0.45 µm filters. The spent medium was then kept at 4 °C until the start of experiments. For enrichments, fresh HFB was mixed with spent HFB in different ratios and enrichments were done with 50 ml of medium in 150 ml Schott flasks. Cultures of

L. monocytogenes were added to reach a starting inoculum of 2 $\log_{10}$ CFU/ml and samples were taken at 2 h intervals and plated on brain heart infusion (BHI) agar and incubated at 30 °C for at least 24 h before counting. The experiment was done with four independent biological reproductions. The Baranyi model (Baranyi & Roberts, 1994) was fitted to the plate counts data of each condition according to the protocol described in an earlier manuscript (Bannenberg et al., 2021a). The 95 % confidence interval of each growth curve was estimated by bootstrapping the data 10,000 times using the average and standard errors of the model parameter estimates.

4.2.8. Monte Carlo analysis of growth performance in spent medium

The plate count data in spent media was used to simulate growth kinetics when starting at a very low inoculum level, namely with 1 cell per 250 ml of enrichment (i.e. -2.4 $\log_{10}$ CFU/ml), using Monte Carlo simulations. For this, the mean and standard deviation of the lag duration and the mean and the standard deviation of the maximum specific growth rate was used to simulate 10,000 enrichments. The probability was then estimated at which time the final concentration of 2 $\log_{10}$ CFU/ml is reached that is necessary for the successful propagation of the secondary enrichment step (Augustin et al., 2016; Bannenberg et al., 2021a). This threshold is chosen in order to transfer at least one cell to the secondary enrichment step with a Poisson chance of more than 99.9 % (Augustin et al., 2016).

4.3. Results and discussion

4.3.1. Lag duration at high inoculum

In order to study the proteome and gene expression of *L. monocytogenes* during the lag phase in HFB, robust and reproducible growth conditions need to be applied to generate consistent lag phase behaviour. To accommodate for this, standardized working cultures were grown in BHI at 30 °C and were used to inoculate the enrichment broth according to the ISO 11290-1 standard. High inoculum concentrations had to be used to harvest enough protein material for analysis. To evaluate whether the lag duration in HFB was influenced by the cell density (Augustin et al., 2000; Robinson et al., 2001) , the growth kinetics in HFB were determined for different inoculum concentrations (figure S1). This showed that the lag duration for heat-stressed cells decreases when using an inoculum of 7.5 $\log_{10}$ CFU/ml compared to an inoculum of 2 $\log_{10}$ CFU/ml. Given the duration of the lag for high inocula for reference and heat-stressed cells, time points were taken during the lag and on the transition of lag into exponential phase of heat-stressed cells during enrichment at 0, 0.5, 2, 4, and 7 h. The lag phase of heat-stressed cells was compared to non-stressed reference cells, and reference samples were taken during enrichment at 0, 10 and 30 min, and 2 h when the lag duration is finished and exponential growth starts.

4.3.2. Proteomic response during lag phase

When proteomic expression of reference and heat stress samples was clustered, there was a clear distinction between proteome responses in the two conditions (figure 1a). From this it can also be seen that there was proteome clustering over time with a separation between early lag (0h10 for reference, and 0h30 and 2h for heat stress) and late lag (0h30 and 2h for reference, and 4h and 7h for heat stress). Reference cells and heat-stressed cells reach a distinct proteome pattern, that also changes over time. Furthermore, after heat stress more proteins were significantly expressed ($p < 0.05$ and an absolute $\log_2$ fold-change higher than 1) than in reference condition (figure 1b). This Venn diagram shows that there is a set of 173 (113 plus 60) significantly expressed proteins that make up the proteomic response to HFB.

These lag-phase-associated proteins are necessary for adaptation of BHI-grown stationary phase cells to initiate growth in the selective enrichment broth. Rolfe et al. (2012) showed for *Salmonella enterica* at transcriptional level that adaptation already began within 4 min of inoculation in fresh LB medium. From our data it can be concluded that the rapid adaptations of bacteria to HFB can also be seen at gene expres-

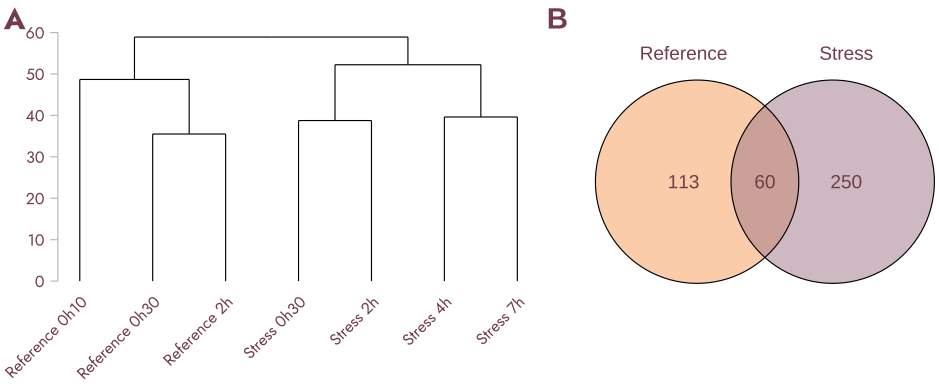

Figure 1: The clustering dendrogram of the proteomics samples (A) shows the hierarchical clustering of reference cells and heat-stressed cells compared to the start of enrichment. The clustering was done with the complete-linkage clustering algorithm. (B) A Venn diagram showing the differentially expressed proteins ($p < 0.05$ and an absolute $\log_2$ fold change of 1 or more) in HFB. The proteins are clustered in 113 unique reference proteins, an overlapping core cluster of 60 proteins, and 250 stress-specific proteins.

sion level within 5 min (data not shown) and at protein level within 10 min or less of transfer to HFB. This points to a fast initiation of an adaptive response with a set of 173 differentially expressed proteins. Interestingly, despite a difference in history for the reference cells and the heat-stressed cells, there was an overlapping core set of 60 proteins expressed in both conditions. Some common patterns are initiated regardless of the different physiological state in which the cells start the enrichment. An additional 250 proteins were uniquely differentially expressed in heat-stressed cells. This would suggest that there is a general proteomic response when adapting to HFB, an elaborated effect due to the effects of stress history, with a core overlapping set of proteins between reference and stressed cells.

In order to look into the differences in protein expression in more detail, the proteins were annotated with clusters of orthologous group-terms in figure 2 (full list of proteins and fold-changes is available in table S1 through table S3). Here it can be seen that the main adaptations for reference cells occurred in carbohydrate and amino acid metabolism, inorganic ion and nucleotide transport, cell wall biosynthesis, and replication, translation, and transcription. The expression of proteins related to nucleotide transport, cell wall biosynthesis, translation and transcription are in accordance with the data of Rolfe et al. (2012) on the lag phase of *Salmonella*, as these patterns and pathways were coherent with the preparations required for initiation of growth. In this study they examined the effect of stationary phase cells grown in LB to fresh LB. Differences in carbohydrate and amino acid metabolism can possibly be attributed to nutritional changes from stationary phase BHI cells to HFB.

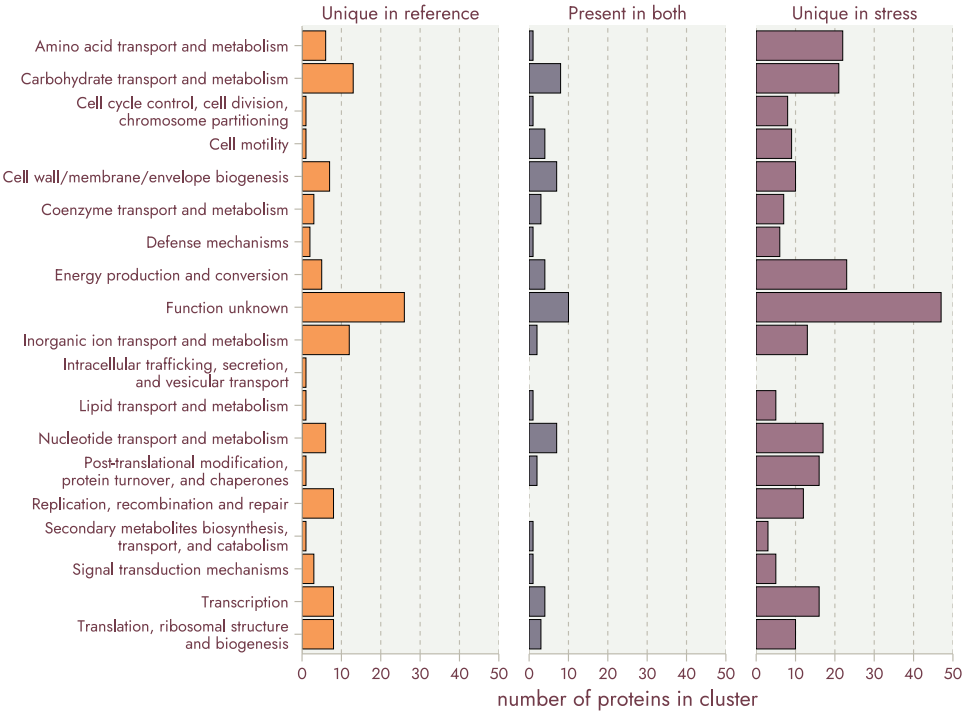

Figure 2: The COG-terms of significant proteins that were clustered into proteins unique for the reference condition, proteins that were found in both reference and heat stressed cells, and proteins that were unique for stressed cells. The number of significantly expressed proteins is shown for each of the 19 COG-terms.

On the other hand, unique proteins after heat stress history had a somewhat different pattern. Next to changes in amino acid and carbohydrate metabolism and replication, recombination and repair as in reference condition, also proteins associated with energy production and conversion, post-translation modification and protein turnover were significantly upregulated. These differences point to the induction of repair mechanisms that were likely induced by damage caused during the heat stress. To provide more insight in the proteome response during the lag phase of *L. monocytogenes* in HFB, representative proteins were divided into relevant themes that are displayed in figure 3.

In figure 3a the expression of significant antibiotic resistance proteins can be seen. HFB contains the antibiotic compounds nalidixic acid (a DNA gyrase inhibitor (Crumplin & Smith, 1975)) and acriflavine (RNA-synthesis inhibitor (Meyer et al., 1972)) to suppress food microbiota during enrichment. Resistance to fluoroquinolones such as nalidixic acid is conferred in *L. monocytogenes* by the *Listeria* drug efflux

pump Lde (Lmo2741 (Godreuil et al., 2003; Jiang et al., 2012). The *lde* gene encodes a 12-transmembrane-segment efflux pump belonging to the major facilitator superfamily of secondary transporters. This protein was not detected during our proteomic analysis, possibly due to the limited capture of membrane proteins with our method due to the hydrophobicity of membrane proteins. The gene was also not significantly upregulated at transcriptional level during the first 30 min of enrichment (data not shown). It could therefore not be validated that this membrane protein was active during enrichment. Another efflux pump system of the MATE family has been shown to recognize and pump out fluoroquinolones (Guérin et al., 2014). Of this MATE-family, Lmo2725 – part of this MepA export protein – was found to be significantly upregulated (figure 3a), indicating that this efflux pump might be involved in the export of either acriflavine or nalidixic acid. Other putative antibiotic resistance proteins Lmo0919, Lmo1309, and Lmo2442 were found to be significantly upregulated. The ABC-transporter Lmo0919 confers lincomycin resistance (Dar et al., 2016), although this antibiotic is structurally not similar to the antibiotics present in HFB. Furthermore, the chromosome partitioning protein Lmo1309 is part of a toxin-antitoxin system uncovered in *L. monocytogenes* (Antonio Agüero et al., 2020). Such a system could play a role under antibiotic stress and thereby cause transient growth arrest (Antonio Agüero et al., 2020), and this protein was highly upregulated at the end of the lag phase. Another significantly upregulated potential antibiotic resistance gene – Lmo2442 – was found to be highly activated upon cefuroxime exposure (Nielsen et al., 2012). Also, in heat stress the upregulation of Lmo2229 is interesting, as this protein has been shown to play a role in beta-lactam resistance (Guinane et al., 2006). HFB does not contain beta-lactam antibiotics, so potentially the effectiveness of this resistance protein may be broader to also include one of the selective compounds in HFB. Gene-knockouts of multiple of such penicillin-binding proteins were made in EGDe and phenotypic studies indicated that Lmo2229 contributes to beta-lactam resistance, virulence potential, and morphogenesis (Guinane et al., 2006). Our data provide evidence for the activation of several antibiotic resistance proteins in reference and stressed cells, conceivably as part of the adaptive response upon exposure to the antibiotics nalidixic acid and acriflavine that are added to the enrichment medium.

As can be seen from figure 3b, two metal transporters show increased expression. Metal ion homeostasis of zinc and magnesium was exemplified here by the significant upregulation of the transcriptional ZurR regulator Lmo1445, and the magnesium-transport protein Lmo2689. Metal ion homeostasis is an important factor for bacte-

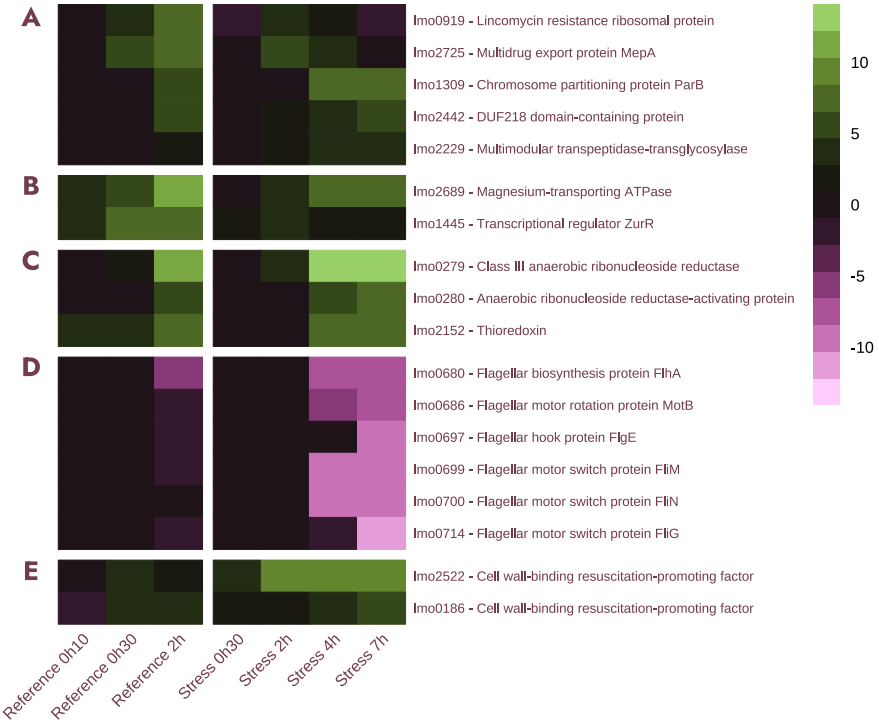

Figure 3: Heatmap of significant proteins of the proteome response expressed in both reference and heat-stressed cells. Example proteins are shown belonging to different groups, with (A) (putative) antibiotic resistance proteins, (B) metal transporters, (C) proteins belonging to oxygen/redox stress, (D) motility-associated proteins, and (E) resuscitation-promoting factors. The colours denote the $\log_2$ fold change of the protein at different timepoints.

rial survival, because metal ions are necessary as cofactors for proper enzyme functionality, but over-supply of metals can lead to inhibition and even toxicity (Sterritt & Lester, 1980). It has been shown before that the lag phase is characterized by the specific but transient uptake of metal ions (Rolfe et al., 2012). In that study, the accumulation of metals as essential micronutrients was measured already after 4 min into the lag. Even though the uptake of different metal ions was observed (mainly zinc and magnesium for *L. monocytogenes* from this study, and manganese, calcium, and iron for *Salmonella* from Rolfe et al. (2012)), it seems that also for *L. monocytogenes* the expression of transporters might provide indication that homeostasis of metal ions plays an important role during lag phase. Rolfe et al. (2012) speculated that increases in cell-associated metal concentrations play an important role in the regulation of metabolic enzymes where they can function as essential co-factors.

In figure 3c it can be seen that three proteins related to oxidative stress were highly

upregulated. First of all, Lmo0279 – a class III anaerobic reductase – was found to be the most highly upregulated protein during enrichment in both conditions, reaching a $\log_2$-fold increase of 14 with respect to the start of enrichment. Together with the anaerobic reductase Lmo2080, and the thioredoxin Lmo2152, this would suggest that *L. monocytogenes* encounters oxidative stress during enrichment. Enrichment samples were cultured in closed Schott flasks during enrichment without supplementation of fresh oxygen. But transfer of stationary phase culture into a fresh and oxygenated environment can cause oxidative stress, and in such a way influence bacterial growth and lag (Cuny et al., 2007). The lag phase of *Salmonella* was also shown to be impacted by oxidative damage when grown statically (Rolfe et al., 2012). Here, almost every gene of the OxyR oxidative stress regulon was activated to protect the cell from oxidative free radicals. Rolfe et al. (2012) also showed that oxidative damage was especially caused by the combination of increased intracellular metal concentrations and the presence of available oxygen. This provides further evidence that the management of oxidative stressful environments is an important part of lag phase.

From figure 3d it can be seen that later on in the lag, proteins that are involved in cell motility and chemotaxis were downregulated. The flagellar motor proteins Lmo0680, Lmo0686, Lmo0697, Lmo0699, Lmo0700, and Lmo0714 were downregulated. Cell motility related proteins were downregulated both in reference condition and after heat stress, however, the downregulation of these proteins was more pronounced in the heat stress cells. This effect can also be clearly observed at the level of pathway analysis where based on the proteomics data the significance of entire KEGG pathways was assessed. The KEGG-pathways for "flagellar assembly" and "bacterial chemotaxis" were found to be significantly downregulated when looking at pathway enrichment analysis (table S4). At the start of the enrichment there is an abundance of nutrient availability that diminishes the need for motile cells in search of nutrients. This is because the flagellar assembly necessary for motility comes with a price as this is one of the most costly bacterial behaviours (Ni et al., 2020). This causes a trade-off between the potential benefits of increased motility and the energetic cost required to maintain this.

The two proteins Lmo2522 and Lmo0186 are described as resuscitation-promoting factors (RPF) (Pinto et al., 2013) and were significantly upregulated in reference and heat-stressed cells (figure 3e). Especially levels of Lmo2522 increase rapidly in heat-stressed cells, reaching a $\log_2$ fold-change of 9.9 after 2 h of enrichment. The gene encoding Lmo2022 also showed significantly increased transcript levels after 30 min of enrichment by transcriptomics. These RPFs are muralytic proteins that are able to

stimulate growth through reduction of lag-phase. Pinto et al. (2013) demonstrated that both RPFs have cell-wall degrading properties that can stimulate growth of *L. monocytogenes*, because their *L. monocytogenes lmo0186 lmo2522* double-mutant showed an increased lag duration. This increased lag duration could be partially undone by the supplementation of His-tagged purified Lmo0185 and Lmo2522. The protein Lmo2522 was also shown to stimulate growth from lag phase in the presence of the antibiotic cefuroxime and high levels of sodium chloride (Nielsen et al., 2012). The high upregulation of the two RPFs seems to indicated that the expression of these proteins is an important factor in getting out of lag for *L. monocytogenes*.

Furthermore, it can be concluded from the transcriptomic data that growth initiation by upregulation of ribosomal proteins already starts within the first 5 min of lag in HFB (data not shown). With the 30S and 50S ribosomal proteins, RpsN (Lmo1882), RplO (Lmo2613), RpmD (Lmo2614), RpsE (Lmo2615), RplR (Lmo2616), RplF (Lmo2617), RpsH (Lmo2618), RpsZ (Lmo2619), RplE (Lmo2620), and finally RplX (Lmo2621) already showing more than 3.5 $\log_2$-fold increase after 5 min and more than 5 $\log_2$-fold increase after 30 min of enrichment, indicating that ribosomal structural proteins are already started to be produced immediately after start of enrichment. This is in accordance with the data on *Salmonella* where the expression of ribosomal genes was already fully upregulated 20 min into lag phase (Rolfe et al., 2012). During lag the amount of ribosomes in the cell has to increase from the low levels at the end of stationary phase up until the cells transition to exponential phase at the end of the lag phase.

Lastly, there is still quite a large part of the EGDe proteome that is not fully annotated. Uncharacterized proteins that had a fold increase of at least 4 $\log_2$ at any timepoint are shown in the supplementary data (figure S2). These uncharacterized proteins were cross-referenced in scientific publications with PaperBLAST (Price & Arkin, 2017), but no hits were found. These proteins also did not show significant functional partners to other known proteins from the string database (Szklarczyk et al., 2021). Although these proteins could play important roles in lag behaviour, their putative roles in getting out of lag and growth in HFB remain to be elucidated.

4.3.3. Getting out of lag response of heat-stressed cells

Next to the general proteome response, the heat stress history affects the proteome significantly with 250 proteins differentially expressed only in stressed cells. The response at protein level of *L. monocytogenes* to heat stress can be divided into different categories; Class I heat shock proteins that function as intracellular chaperones to sta-

bilize and assemble misfolded proteins caused by heat stress (Bucur et al., 2018), and Class III heat shock proteins that encode ATP-dependent proteases that are necessary for the degradation of misfolded proteins (Bucur et al., 2018).

For chaperone proteins, the specific heat stress proteins ClpB (Lmo2206) and especially ClpE (Lmo0997) were found to be upregulated after heat stress (figure 4a). ClpB is a chaperone protein that protects the cell from protein aggregation caused by heat stress. ClpB is involved in thermotolerance where it can allow for increased resistance to lethal temperatures (Chastanet et al., 2004). ClpE is also significantly upregulated at the start of enrichment of heat-stressed *L. monocytogenes*, which exhibits protease activity. The expression of ClpB and ClpE is thermoinducible and is required for thermal resistance and salt stress (Olesen et al., 2009; Ringus et al., 2012). Furthermore, several DNA-repair associated proteins were upregulated, conceivably involved in repair of heat-induced DNA-damage. The DNA helicase Lmo0157 was already upregulated at the start of enrichment. DNA helicase unwinds the double-stranded DNA-helix to allow for translation or repair of DNA damage. As this protein was not significantly upregulated in reference cells, this could indicate that unwinding of the DNA can facilitate heat-induced damage repair. Also, exonuclease Lmo2050 as part of the uvrABC repair system catalyses the recognition and processing of DNA damage as well (Truglio et al., 2006). Also, Lmo1639 – a DNA glycosylase – initiates repair of DNA via base excision repair (Metz et al., 2007). Furthermore, the Holliday junction resolvase Lmo1891 can promote the annealing of single-stranded DNA to double-stranded DNA required for repair (Cañas et al., 2008), and this protein needs magnesium as a subunit (inferred from the presence of magnesium binding sites in the protein). Furthermore, the expression of the cell division protein DivIB (Lmo2034) later during enrichment indicates that the cells are starting dividing because Lmo2034 is involved in stabilizing or promoting the assembly of the division complex (Daniel & Errington, 2000).

Next to this, there is also upregulation of proteins that are involved in cell wall damage repair and cell wall biogenesis, and several cell wall proteins that are only upregulated after heat stress (figure 4b). This subset encompasses proteins that are involved in cell-wall biogenesis, namely, Lmo0927 (involved in polyglycerolphosphate backbone synthesis), Lmo1291 (peptidoglycan O-acetyltransferase), Lmo2505 (peptidoglycan lytic protein P45), Lmo2555 (glycosyltransferase), and Lmo2812 (peptidoglycan synthesis and cell-wall turnover). Even more, Lmo1291 has also been shown to infer resistance to antimicrobial compounds such as lysozyme and beta-lactam antibiotics (Aubry et al., 2011). Upregulation of stress-specific cell-wall proteins might

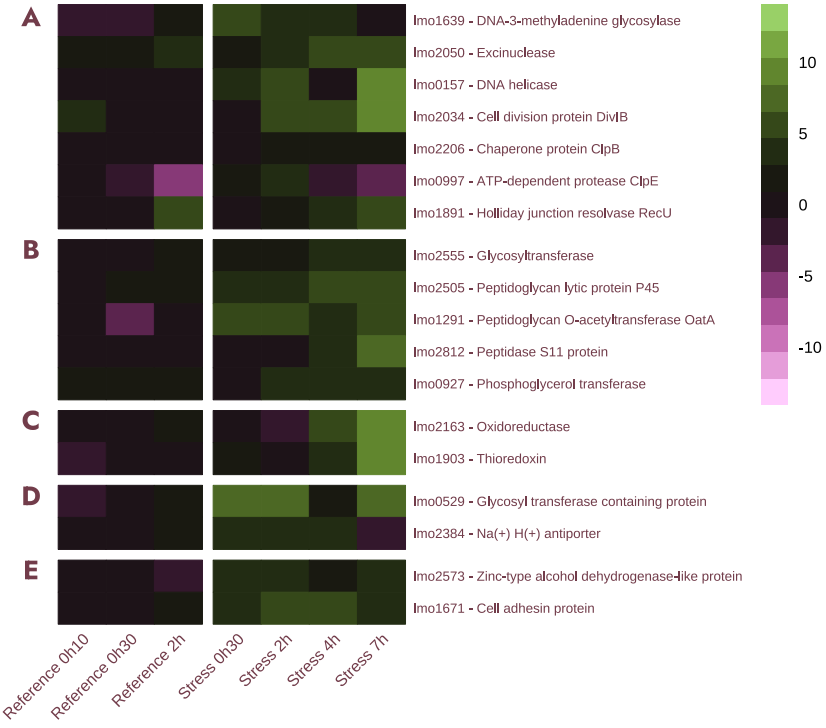

Figure 4: Heatmap of proteins significantly upregulated after heat stress. With (A) proteins belonging to stress response and repair, (B) proteins associated with cell-wall repair and structure, (C) proteins related to oxygen stress, (D) osmotic stress related proteins, and (E) proteins associated with metal transport and homeostasis. The colours denote the $\log_2$ fold change of the protein at different timepoints.

indicate that repair of the bacterial cell-wall after heat stress is necessary. Combined, this would indicate that DNA damage, protein misfolding, and cell wall damage are the important factors for damage repair caused by heat treatment.

According to Kobayashi et al. (2005) heat recovery is often accompanied by oxidative stress. Indeed, next to the general upregulation of proteins related to oxidative stress (see figure 3c), also the oxidoreductase Lmo2163 and thioredoxin-like Lmo1903 were upregulated after heat stress (figure 4c). This could indicate that damaged cell membranes after heat treatment require extra proteins to overcome the oxidative environment during enrichment.

As one of the selective agents in HFB, the concentration of NaCl is set at a high 20 g/l. It has been shown that for stressed cells with potential damaged cell walls, the presence of salt can be repressive (Jasson et al., 2007). This is shown by the significant upregulation of the Na^+/H^+ antiporter Lmo2384 after heat stress but not in

reference cells (figure 4d). Also, Lmo0529 is upregulated in heat-stressed cells and was hypothesized to confer protection against salt stress (Ribeiro et al., 2014). The common way that *L. monocytogenes* tries to maintain osmotic homeostasis is by the uptake of compatible solutes like glycine, betaine and carnitine via active transport (Fraser et al., 2003). Despite this, no significant upregulation of the proteins of the betaine transporter operons BetL and Gbu, and the carnitine uptake operon OpuC was observed in HFB. A possible reason for this could be the underrepresentation of hydrophobic membrane proteins in the proteomic sample preparation. At gene transcript level, the *opuC* and especially *gbu* cluster were significantly upregulated at the start of enrichment. It might also be possible that the osmoprotectants are only present in low concentrations in the enrichment medium, and that *L. monocytogenes* thus has to rely more on active transport of sodium out of the cell.

Furthermore, we have found that a putative ABC transporter/cell adhesin protein Lmo1671 shows significant expression in heat stressed cells (figure 4e). This transporter has been found to be controlled by the zinc uptake regulator-like protein ZurR (Dowd et al., 2012), and forms an operon with the putative high-affinity zinc uptake system (Dalet et al., 1999). The ABC transporter Lmo1671 is thus hypothesized to be a zinc uptake transporter based on its control by ZurR, and this would suggest that during recovery from heat stress an increased uptake of zinc is necessary. Furthermore, Lmo2573, a zinc-type NADPH-dependent quinone reductase, is involved in electron transport and energy generation (Hain et al., 2008). This protein is putative zinc-binding and would further suggest that zinc is an important metal during enrichment.

For uncharacterized proteins where currently no annotation is available, a minimum $\log_2$ fold-change of four was chosen before they were considered to be significantly expressed. In figure S2 a heatmap is visualized with such proteins that are uniquely expressed after heat stress, with most of these active at the end of the lag phase where the cells are transitioning out of lag. A possible role in recover and growth initiation remains to be elucidated.

4.3.4. Linking protein expression to metabolism

At proteomic level we found evidence of changes in carbohydrate and amino acid metabolism during lag (figure 2). *Listeria* cells have to adapt not only to a selective environment, but also to changes in nutritional composition of HFB. In order to look at metabolism in HFB in further detail, HPLC analysis was used to quantify the substrate utilization (figure S3). Notably, no difference in substrate utilization could be

measured between reference cells and heat stressed cells, even though there were differences at proteome level depending on their stress history. In both conditions it can be seen that glucose, glycerol, and malate are fully depleted after 10-14 h of enrichment. Cellobiose utilization starts later on during enrichment and is nearly depleted after 24h. Pyruvate and succinate were not utilized completely, indicating that these are not preferred substrates of *L. monocytogenes* in HFB. However, due to lower cell concentrations, compound utilization during lag cannot be accurately measured.

In oxidative conditions, respiration is most efficient by complete oxidation of an electron donor (e.g. a carbohydrate) to carbon dioxide. However, *L. monocytogenes* lacks a complete TCA-cycle (Trivett & Meyer, 1971), and instead has to rely on the conversion of sugars to different fermentation products (Romick et al., 1996). In aerobic environments, *L. monocytogenes* almost exclusively produces acetate as end-product of its respiration (Rivera-Lugo et al., 2022), of which around 3 mM is generated during a 24 h enrichment (figure S3). Under anaerobic conditions, a mixed acid fermentation is employed by *L. monocytogenes* with lactate as most abundant end-product, and lower levels of ethanol, formate, and acetate (Rivera-Lugo et al., 2022). Based on the abundant conversion into lactate (5.5 mM) and formate (4 mM), it seems that anaerobic respiration during lag phase and the rest of enrichment of *L. monocytogenes* plays an important role.

The proteomic analysis of heat-stressed cells also showed upregulation of amino acid transport and metabolism. Therefore, the concentration of amino acids was quantified in the enrichment medium every 2 h. All essential amino acids except for cysteine were present in HFB, although at differing concentrations ranging from 190 µM of histidine to 2.5 mM of leucine (figure S4). The complete biosynthetic pathways for all amino acids are present in *L. monocytogenes* (Sauer et al., 2019). It is interesting to note, however, that in minimal defined media supplementation of cysteine and methionine is required (Glaser et al., 2001; Tsai & Hodgson, 2003). This is strain dependent, with strain EGDe requiring only cysteine in defined media (Sauer et al., 2019). Nevertheless, it is intriguing that HFB does not contain this essential amino acid, but it is likely that in such a rich medium the necessity of cysteine is less strict. For the rest of the amino acids, the concentration of almost all remained unchanged during enrichment. Only the concentration of glutamate, leucine, and lysine seemed to decrease slightly, and threonine increased during enrichment. Concludingly, it seems that during lag phase and enrichment, *L. monocytogenes* is mainly using anaerobic respiration to mixed fermentation products and with minimal utilization of amino acids.

4.3.5. The importance of zinc on the lag phase

Because the proteomics results pointed to metal and especially zinc regulon activation during enrichment in HFB, the effect of supplementation of metals was assessed. It was hypothesized that zinc and magnesium act as co-factors for lag-related proteins, and that supplementation could support recovery from lag. It has been shown that the lag phase of *Salmonella* involves the transient accumulation of metal ions (Rolfe et al., 2012), and therefore the outgrowth of *L. monocytogenes* in metal-supplemented HFB was measured by optical density. Because the affinity of the up-regulated metal transporter was not well characterized, the effect of addition of zinc, copper, and cobalt was quantified with the time-to-reach (TTR), which was defined as the time necessary for an optical density increase of 0.25. The addition of cobalt and to a lesser extend copper resulted in an increase of the TTR, indicating a negative effect on the growth during enrichment, with for cobalt an apparent inhibition of growth at higher concentrations (table S5). The supplementation of 10 µM zinc reduced the TTR value slightly for the recovery of heat-stressed cells. For reference cells, the addition of 10 µM zinc decreased the TTR from 20.7 ± 0.5 h to 20.1 ± 0.8 h, and for heat-stressed cells the reduction is from 22.8 ± 0.3 h to 21.8 ± 0.6 h. The difference in outgrowth for heat stressed cells was significant (p = 0.03), thus heat stressed *L. monocytogenes* may benefit from the addition of 10 µM zinc. This phenotype is in accordance with the increased expression of ZurR for stressed cells, from which it can be hypothesized that zinc plays a role in the recovery from heat stress.

4.3.6. Supplementation with spent medium can decrease the lag

Both at transcriptome and proteome levels, the two resuscitation-promoting factors Lmo0186 and Lmo2522 were expressed at high levels. Because such proteins have muralytic activity they can stimulate growth from less-metabolically active cells and thereby promote the resuscitation from a dormant state (Mukamolova et al., 1998a,b). The RPF proteins are extracellular and they can be isolated from supernatants of growing cells (Mukamolova et al., 1998a). Because of the high expression of these proteins during enrichment for both reference and heat-stressed cells, it was hypothesized that the medium after culturing contains high levels of RPF that may be functional in reducing the lag duration during enrichment. To test this, fresh HFB was supplemented with spent medium that was collected after enrichment of *L. monocytogenes* in HFB and subsequent filtering of cell mass. HFB was supplemented with either 10 % or 25 % of the volume with spent HFB, and this was subsequently used

to enrich both reference cells as well as heat-stressed cells. Possible reduction of lag duration is especially relevant for stressed cells, as heat-stressed cells can display a long lag phase, potentially resulting in false-negative detection outcomes (Bannenberg et al., 2021a,b).

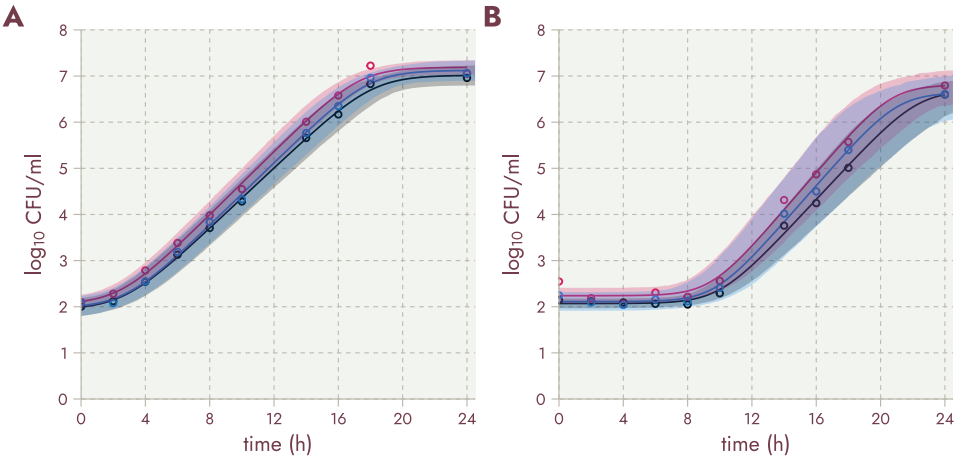

Figure 5: Supplementation with spent medium can decrease enrichment time. In (A) the cell concentration is shown for the enrichment of reference cells. The growth kinetics during enrichment were fitted with the Baranyi model and the kinetic parameters were used to gather 95 % confidence intervals via bootstrapping. Here, the black line denotes HFB, the blue line is HFB that is supplemented with 10 % spent medium, and the red line is HFB supplemented with 25 % spent medium. In (B) the enrichment of heat-stressed cells is shown.

In figure 5a the concentration during a 24 h enrichment is shown for reference cells (data in table S6). No significant difference in growth kinetics was observed between HFB and HFB supplemented with 10 % or 25 % spent medium. Because the lag duration of reference *L. monocytogenes* cells in fresh HFB was only 2.3 h for strain EGDe based on previous research (Bannenberg et al., 2021a), a further reduction of such a lag duration may be hard to quantify. A reduction of lag phase was observed for heat-stressed cells in 10 % or 25 % spent HFB (figure 5b). After heat stress the lag duration decreases from 9.4 ± 0.1 h in HFB to 8.2 ± 0.5 h in HFB with 25 % spent medium (p = 0.04). However, even though there is a significant difference in the lag duration when fitted with a microbial growth model, cells reached a similar final concentration and the 95 %-confidence interval of the growth curve was large due to the variation in growth kinetics. It is therefore unlikely that this will give a biologically interesting effect on the ability to detect *L. monocytogenes* during enrichment. To investigate this, Monte Carlo simulations were done to evaluate the effect of the addition of 25 % spent medium compared to fresh HFB. For this, the lag duration and

the maximum specific growth rate were used to simulate 10,000 enrichments and to quantify the probability that when starting with 1 cell per 250 ml enrichment, the necessary concentration of 2 $\log_{10}$ CFU/ml was reached (figure S5). This threshold needs to be reached during enrichment to transfer at least one cell to the secondary enrichment step with 99.9 % probability (Augustin et al., 2016; Bannenberg et al., 2021a). This threshold of 2 $\log_{10}$ CFU/ml was reached after 30.5 h in HFB and after 29.5 h in HFB supplemented with 25 % spent medium with 99 % probability. This agrees with the fact that although a difference in lag duration is shown, this difference is not biologically relevant to result in enhanced enrichment efficacy of stressed *L. monocytogenes* cells.

Thus, though there is evidence at proteome level that resuscitation-promoting factors play an important role in getting out of lag for *L. monocytogenes*, supplementation does not have a biologically relevant effect on the detection time in HFB. Moreover, it is important to note that even in 100 % spent medium, *L. monocytogenes* was able to grow with a similar lag duration as in fresh HFB, reaching an increase of around 5 $\log_{10}$ CFU/ml to 7.5 $\log_{10}$ CFU/ml within 24 h (data not shown). This highlights that HFB is a nutritious medium and does not lack critical substrates.

4.4. Conclusion

In conclusion, we have shed some light on the black box that is the enrichment of *L. monocytogenes* in this study by investigating the response of the cells during the lag phase (with and without a previous stress). The proteomic approach to study the lag duration has highlighted factors that are potentially important for getting out of lag, and also on the recovery of heat-stressed cells, with an overview of the main factors driving the lag response given in figure 6. Although the addition of 10 µM zinc and the supplementation of spent medium to add resuscitation-promoting factors to the cells had a significant but slight effect on the lag duration, this was not to levels that are biologically relevant to shorten enrichment times considerably. Reduction of the lag of especially stressed cells during enrichment is important to minimize the number of false-positive results due to selectivity of the HFB enrichment medium. Thus, it seems that optimization of the HFB enrichment medium lies not in the supplementation of medium but in other ways to allow optimal resuscitation of (damaged) cells.

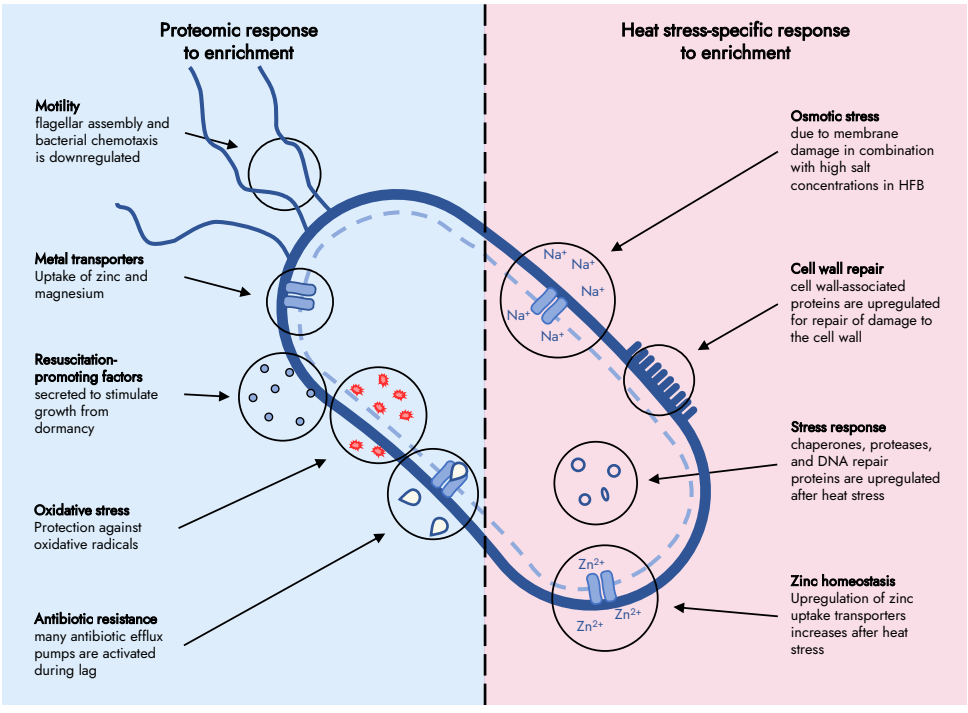

Figure 6: Overview of the lag behaviour of *L. monocytogenes* in enrichment in HFB. The left part of the figure shows the proteomic response as a reaction to transfer to HFB, while on the right part the heat-stress specific response is shown.

Acknowledgements

The authors would like to thank all partners in this project (Biomérieux, DSM, Innosieve Diagnostics, Merck Darmstadt, NWO, and Wageningen Food Safety Research) for their financial and in-kind contributions. This work is part of the research program “Rapid and reliable enrichment-based detection of foodborne pathogens” with project number 15002, which is partly financed by the Dutch Research Council (NWO).

4.5. Supplementary materials

This section contains supplementary data of this chapter.

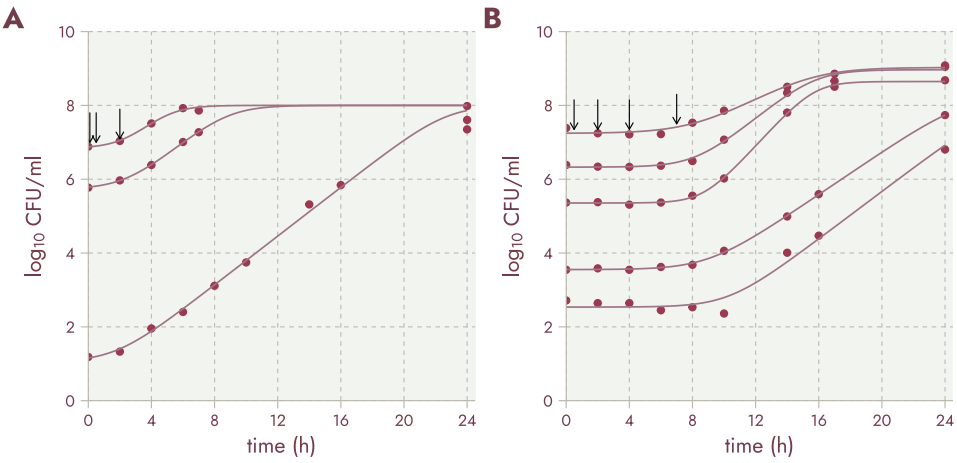

Figure S1: Kinetics of strain EGDe in HFB at different inoculum levels for reference cells (A), and heat-stressed cells (B). Lag duration becomes slightly shorter at higher inoculum levels, and this was taken into account when taking samples for proteomics (black arrows). The Baranyi model was used to fit the microbial growth data and is explained in more detail in Bannenberg et al. (2021a).

Baranyi model:

$$log_{10}N(t) = log_{10}N_0 + \frac{\mu}{ln(10)} \cdot A(t) - \frac{1}{ln(10)} \cdot ln\left[1 + \frac{exp[\mu \cdot A(t)] - 1}{10^{[log_{10}N_{max} - log_{10}N_0]}}\right] \quad (4.1)$$

$$\text{With } A(t) = t + \frac{1}{\mu} \cdot ln\left[exp(-\mu \cdot t) + exp(-\mu \cdot t_{lag}) - exp(-\mu \cdot t - \mu \cdot t_{lag})\right] \quad (4.2)$$

With $log_{10}N(t)$ the cell concentration at time t ($\log_{10}$ CFU/ml), $log_{10}N_0$ the initial cell concentration ($\log_{10}$ CFU/ml), $log_{10}N_{max}$ the maximum cell concentration ($\log_{10}$ CFU/ml), t the elapsed time (h), λ the lag phase duration (h), t_{lag} the time when the lag phase ends (h), and μ the maximum specific growth rate (h^{-1}).

Table S1: Table containing all proteins that are unique for the reference condition with the $\log_2$ fold-change for each timepoint. Bold and underlined values are significant differentially expressed (absolute $\log_2$ fold-change > 1 and *p*-value < 0.05)

uniprot	lmo-code	protein name	COG description	ref 0h10	ref 0h30	ref 2h	stress 0h30	stress 2h	stress 4h	stress 7h
Q8YAU8	lmo0015	Quinol oxidase polypeptide III	Energy production and conversion	**1.62**	0.51	0.31	-0.43	0.90	-0.86	-0.50
Q8YAP0	lmo0077	Uncharacterized protein	Function unknown	2.97	3.49	**3.99**	-0.21	-0.48	-1.94	-1.94
Q8YAL6	lmo0102	ABM domain-containing protein	Function unknown	-0.14	0.13	-1.03	4.09	3.79	0.41	-6.97
Q8YAL5	lmo0103	Nitroreductase domain-containing protein	Energy production and conversion	1.72	1.95	**6.62**	0.20	-1.63	-1.63	-1.63
Q8YAI9	lmo0137	ABC transmembrane type-1 domain-containing protein	Inorganic ion transport and metabolism	0.11	-0.05	-1.21	-0.59	-0.76	-5.99	-3.23
Q8YAE2	lmo0188	Ribosomal RNA small subunit methyltransferase A	Translation, ribosomal structure and biogenesis	0.29	0.57	**1.23**	-0.34	-0.01	0.23	0.50
Q92F78	lmo0189	Lmo0189 protein	Function unknown	0.33	4.56	**4.61**	0.00	0.00	0.00	0.00
Q48793	lmo0199	Ribose-phosphate pyrophosphokinase 1	Nucleotide transport and metabolism	0.11	0.24	**1.19**	-0.05	0.29	0.67	0.91
Q8YAC0	lmo0226	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase	Coenzyme transport and metabolism	-0.18	0.51	**1.48**	-0.68	-0.71	-1.35	0.66
Q8YAB9	lmo0227	tRNA-dihydrouridine synthase	Translation, ribosomal structure and biogenesis	0.40	0.97	**1.23**	0.00	0.35	0.53	0.14
Q8YAB0	lmo0240	Mini-ribonuclease 3	Translation, ribosomal structure and biogenesis	0.15	-0.25	-1.16	2.52	2.53	-2.94	-2.96
Q8YA77	lmo0282	CN hydrolase domain-containing protein	Function unknown	-0.32	-0.02	-1.13	-0.02	-0.60	0.94	0.13
Q8YA68	lmo0291	Lmo0291 protein	Inorganic ion transport and metabolism	0.52	**1.32**	**2.46**	1.88	-0.52	3.89	5.22
Q8YA24	lmo0341	Lmo0341 protein	Function unknown	-2.14	-0.47	-7.07	-0.46	-2.79	-2.79	-2.79
Q8YA01	lmo0366	Efem/EfeO family lipoprotein	Inorganic ion transport and metabolism	-0.30	-0.67	-1.07	2.66	0.28	-2.72	-4.61
Q8Y9T6	lmo0437	Uncharacterized protein	Carbohydrate transport and metabolism	-0.46	-0.33	-1.33	2.86	-1.45	-4.30	-4.30
Q8Y9L2	lmo0515	Universal stress protein family	Signal transduction mechanisms	-0.02	-0.15	-1.18	0.12	-0.03	-1.10	-1.00
Q8Y9I1	lmo0547	Sugar-bind domain-containing protein	Transcription	-0.13	-0.59	-1.05	2.44	2.65	-0.63	2.28
Q8Y9G8	lmo0560	Glutamate dehydrogenase	Amino acid transport and metabolism	0.05	-0.53	-1.38	3.91	4.04	3.97	-6.35
Q8Y9A8	lmo0624	N-acetyltransferase domain-containing protein	Transcription	0.00	4.03	**6.40**	0.00	2.09	0.00	2.18
Q8Y990	lmo0643	DHA Aldolase	Carbohydrate transport and metabolism	-0.08	-0.07	-1.32	2.71	1.98	0.17	2.67
Q8Y980	lmo0653	Lmo0653 protein	Cell wall/membrane/envelope biogenesis	-0.30	-0.55	-1.10	1.07	1.15	0.19	-1.00
Q8Y937	lmo0704	Lmo0704 protein	Function unknown	-0.28	-3.17	-8.51	2.79	2.37	-6.06	-3.54
Q8Y929	lmo0713	Lmo0713 protein	Cell motility	-0.28	-0.55	-1.22	-0.14	0.00	-4.46	-1.02
Q8Y8U3	lmo0800	Fe-S_biosyn domain-containing protein	Function unknown	-0.08	-0.06	-7.31	1.84	-2.49	-4.64	-4.64
Q8Y8R8	lmo0826	Lmo0826 protein	Inorganic ion transport and metabolism	0.30	0.58	**1.07**	2.19	2.83	-2.52	3.09
Q8Y8R5	lmo0830	Fructose-1,6-bisphosphatase class 3	Carbohydrate transport and metabolism	-0.18	-0.48	-1.10	-0.36	-0.44	-3.35	-3.93
Q8Y8Q5	lmo0841	Calcium-transporting ATPase lmo0841	Inorganic ion transport and metabolism	0.32	0.60	**1.51**	0.68	1.50	0.25	2.05
Q8Y8N9	lmo0857	Hydrolase_4 domain-containing protein	Cell wall/membrane/envelope biogenesis	4.62	**6.58**	7.02	4.40	0.11	5.12	0.09
Q8Y8J9	lmo0902	HTH gntR-type domain-containing protein	Transcription	0.24	0.30	**1.07**	-0.86	-1.00	-5.49	-4.94
Q8Y8I8	lmo0914	PTS EIIB type-3 domain-containing protein	Carbohydrate transport and metabolism	0.37	0.53	-7.16	0.32	-2.39	-4.71	-4.71
Q8Y8I3	lmo0919	ABC transporter domain-containing protein	Replication, recombination and repair	0.00	4.44	**7.81**	-1.96	3.43	2.20	-1.46
Q8Y8H6	lmo0927	Sulfatase domain-containing protein	Cell wall/membrane/envelope biogenesis	**1.31**	**2.65**	**1.84**	0.23	4.66	4.65	3.59
Q8Y8G4	lmo0940	Uncharacterized protein	Function unknown	0.15	0.40	**1.47**	-2.36	-0.21	-2.40	0.71
Q8Y8F3	lmo0951	Lmo0951 protein	Function unknown	-2.46	-4.29	-6.42	0.00	0.00	0.00	0.00
Q8Y8F1	lmo0953	Lmo0953 protein	Function unknown	-0.04	-0.37	-1.35	0.13	0.23	0.17	-4.08
Q8Y8A7	lmo1001	Uncharacterized protein	Function unknown	-0.35	0.00	-1.16	-0.47	-0.24	-0.46	-1.24
Q8Y871	lmo1046	Cyclic pyranopterin monophosphate synthase	Coenzyme transport and metabolism	0.16	-0.63	-1.07	3.20	2.99	0.50	2.52

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Table continued.

uniprot	lmo-code	protein name	COG description	ref 0h10	ref 0h30	ref 2h	stress 0h30	stress 2h	stress 4h	stress 7h
Q8Y7Y8	lmo1131	Lmo1131 protein	Defense mechanisms	-1.94	-2.07	-6.27	-0.27	-2.54	-4.70	-4.70
Q8Y7N3	lmo1241	DUF1254 domain-containing protein	Inorganic ion transport and metabolism	-0.07	-0.34	-1.09	0.14	0.23	-0.83	-0.95
Q8Y7M9	lmo1245	Lmo1245 protein	Function unknown	1.65	**5.67**	**5.50**	0.00	0.00	0.00	0.00
Q8Y7L9	lmo1255	Lmo1255 protein	Carbohydrate transport and metabolism	-0.01	-0.39	-1.10	0.43	0.29	-1.77	-0.39
Q8Y7E9	lmo1333	Uncharacterized protein	Nucleotide transport and metabolism	-0.03	0.39	**1.81**	2.50	4.17	5.84	6.55
P64032	lmo1355	Elongation factor P	Translation, ribosomal structure and biogenesis	0.10	0.49	**1.24**	-0.32	0.36	0.76	0.10
Q92BZ6	lmo1358	Alkaline shock protein	Cell wall/membrane/envelope biogenesis	-0.25	-0.31	-1.28	0.27	-2.72	-0.28	-0.16
Q8Y7A4	lmo1384	tRNA uridine(34) hydroxylase	Translation, ribosomal structure and biogenesis	0.21	**1.92**	**1.74**	2.36	2.44	3.35	-2.08
P0A3E6	lmo1445	Transcriptional regulator ZurR	Inorganic ion transport and metabolism	4.48	**6.81**	**7.21**	1.52	4.10	2.62	2.36
Q8Y745	lmo1476	Heme chaperone HemW	Coenzyme transport and metabolism	0.42	0.31	**1.17**	2.35	2.25	2.47	3.24
Q8Y739	lmo1483	CMP/dCMP-type deaminase domain-containing protein	Nucleotide transport and metabolism	-1.34	-1.63	**1.39**	2.20	-0.38	-0.40	-2.03
Q8Y732	lmo1491	CP-type G domain-containing protein	Function unknown	0.21	0.58	**1.01**	-0.23	0.30	0.88	0.02
Q8Y731	lmo1492	Lmo1492 protein	Function unknown	0.08	0.13	**1.19**	-0.22	0.27	1.14	0.39
Q8Y728	lmo1495	Lmo1495 protein	Function unknown	-0.74	-0.12	-1.52	0.44	-2.43	1.19	0.11
Q8Y726	lmo1498	tRNA 5-hydroxyuridine methyltransferase	Amino acid transport and metabolism	0.57	0.67	**1.03**	0.15	0.25	0.33	-4.70
Q92BL2	lmo1501	UPF0473 protein	Function unknown	-0.16	-0.20	-1.79	-0.02	-0.49	-3.56	-5.34
Q8Y723	lmo1502	Putative pre-16S rRNA nuclease	Replication, recombination and repair	0.27	0.24	**1.01**	-0.39	0.53	0.50	0.14
Q8Y6R8	lmo1615	tRNA (guanine-N(7)-)-methyltransferase	Translation, ribosomal structure and biogenesis	0.27	0.46	**1.13**	-0.81	-0.76	0.74	-0.57
Q8Y6Q1	lmo1633	Anthranilate synthase component 1	Amino acid transport and metabolism	-4.71	-2.46	-7.54	0.15	-2.33	0.41	2.73
Q8Y6P5	lmo1639	Lmo1639 protein	Replication, recombination and repair	-2.28	-2.36	**1.49**	5.59	2.88	3.19	0.46
Q8Y6L3	lmo1671	Lmo1671 protein	Inorganic ion transport and metabolism	0.23	0.66	2.33	3.12	4.83	5.51	4.40
Q8Y6L0	lmo1674	Putative 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase	Function unknown	0.00	2.03	**6.49**	0.00	0.00	4.20	2.12
Q8Y6F9	lmo1728	Glyco_hydro_36 domain-containing protein	Carbohydrate transport and metabolism	0.01	-3.32	-9.45	-3.03	-6.13	-3.01	-2.94
Q8Y6D7	lmo1750	Molybdate metabolism regulator	Function unknown	-0.06	-0.19	**1.50**	2.40	2.62	4.44	3.92
Q8Y6C6	lmo1764	Phosphoribosylamine–glycine ligase	Nucleotide transport and metabolism	0.29	0.58	**1.43**	2.86	2.11	2.89	4.82
Q8Y6C2	lmo1768	Amidophosphoribosyltransferase	Nucleotide transport and metabolism	0.11	0.32	**1.21**	2.68	-1.81	0.45	4.51
Q8Y681	lmo1818	Ribulose-phosphate 3-epimerase	Carbohydrate transport and metabolism	0.02	0.34	**1.86**	0.08	0.20	0.97	1.04
Q8Y666	lmo1834	Dihydroorotate dehydrogenase B (NAD(+)), electron transfer subunit	Energy production and conversion	-0.16	-0.35	-1.02	0.09	0.49	-0.43	-6.30
Q8Y633	lmo1867	Pyruvate, phosphate dikinase	Carbohydrate transport and metabolism	-0.18	-0.37	-1.46	2.95	-0.12	-2.54	0.09
Q8Y629	lmo1871	Lmo1871 protein	Carbohydrate transport and metabolism	0.19	0.26	**1.08**	0.04	-0.62	0.63	0.90
Q8Y628	lmo1872	Methyltransf_25 domain-containing protein	Secondary metabolites biosynthesis, transport, and catabolism	-4.98	-0.43	**1.27**	-2.43	-2.04	-2.01	1.62
Q92AD0	lmo1879	CSD domain-containing protein	Transcription	-0.53	-3.65	-1.62	2.44	-3.35	-5.96	-5.96
Q8Y621	lmo1881	53EXOc domain-containing protein	Replication, recombination and repair	-0.10	0.15	**1.19**	-0.24	-0.63	0.14	0.44
Q8Y611	lmo1891	Holliday junction resolvase RecU	Replication, recombination and repair	0.00	0.00	**5.19**	0.00	1.66	4.30	4.73
Q8Y5W3	lmo1942	RecS protein	Replication, recombination and repair	0.00	0.00	**6.22**	-2.17	-0.07	-2.17	2.02
Q8Y5V5	lmo1950	Segregation and condensation protein B	Cell cycle control, cell division, chromosome partitioning	0.36	0.71	**1.00**	2.34	2.05	0.40	-2.11
Q8Y5Q8	lmo1998	Fructosamine deglycase	Cell wall/membrane/envelope biogenesis	-0.14	-0.38	-1.01	3.22	0.13	-2.95	3.72
Q8Y5K9	lmo2050	Lmo2050 protein	Replication, recombination and repair	1.23	2.72	**3.12**	2.64	3.07	4.92	5.16
Q8Y5E5	lmo2119	Lmo2119 protein	Post-translational modification, protein turnover, and chaperones	-0.23	0.09	**1.01**	-3.07	-0.01	0.65	-5.42

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Table continued.

uniprot	lmo-code	protein name	COG description	ref 0h10	ref 0h30	ref 2h	stress 0h30	stress 2h	stress 4h	stress 7h
Q8Y5E4	lmo2120	Diadenylate cyclase	Function unknown	0.17	0.34	**1.22**	-0.17	0.29	0.16	0.78
Q8Y5D3	lmo2132	Cyclic nucleotide-binding domain-containing protein	Signal transduction mechanisms	0.18	-3.62	-5.63	-0.05	-0.12	-4.07	-4.07
Q8Y5B6	lmo2149	MazG domain-containing protein	Nucleotide transport and metabolism	0.05	0.33	**2.20**	0.34	-0.57	4.89	4.78
Q8Y5B4	lmo2153	Flavodoxin-like domain-containing protein	Energy production and conversion	0.60	0.42	**2.84**	-0.49	-2.97	2.54	2.88
Q929L4	lmo2158	UPF0337 protein	Transcription	0.03	0.18	-1.00	0.77	0.69	0.01	-0.67
Q8Y5A9	lmo2159	Lmo2159 protein	Function unknown	0.00	-0.27	**1.01**	2.40	-0.59	6.11	6.19
Q8Y594	lmo2175	Lmo2175 protein	Lipid transport and metabolism	-0.04	-0.41	-1.39	0.25	0.02	-0.10	-6.95
Q7AP52	lmo2196	SBP_bac_5 domain-containing protein	Amino acid transport and metabolism	-0.22	-0.52	-1.65	0.95	1.20	0.35	-0.10
Q8Y546	lmo2230	Arsenate reductase	Signal transduction mechanisms	0.22	-0.30	-1.46	0.09	-0.25	-6.58	-6.48
Q8Y524	lmo2253	Lmo2253 protein	Function unknown	0.39	0.39	**1.49**	3.19	3.60	4.28	4.39
Q8Y4Y8	lmo2291	Major tail shaft protein [Bacteriophage A118]	Function unknown	-3.10	-2.99	-1.09	0.22	0.55	2.86	-2.94
Q8Y4Y3	lmo2296	Lmo2296 protein	Function unknown	-10.72	-3.69	0.98	4.04	4.62	-0.21	3.50
Q8Y4U8	lmo2332	Putative integrase [Bacteriophage A118]	Replication, recombination and repair	-7.15	0.22	0.46	-0.10	-2.51	-4.97	3.20
Q8Y4U4	lmo2337	HTH deoR-type domain-containing protein	Transcription	0.24	-0.01	**1.16**	2.73	3.04	3.57	4.97
Q8Y4U1	lmo2341	HTH crp-type domain-containing protein	Carbohydrate transport and metabolism	-0.27	-0.39	-1.36	0.14	0.14	-0.38	-0.51
Q8Y4U0	lmo2342	Pseudouridine synthase	Translation, ribosomal structure and biogenesis	-0.01	0.81	**1.37**	0.23	-0.40	0.80	1.04
Q8Y4Q2	lmo2382	Lmo2382 protein	Inorganic ion transport and metabolism	5.27	**5.18**	**5.68**	0.15	0.13	-1.62	-1.62
Q8Y4Q1	lmo2384	Lmo2384 protein	Inorganic ion transport and metabolism	0.27	0.77	**1.11**	2.98	3.58	3.93	-2.56
Q8Y4P9	lmo2386	Lmo2386 protein	Function unknown	0.37	-0.08	-1.04	2.34	2.36	-0.38	-5.45
Q8Y4N5	lmo2400	N-acetyltransferase domain-containing protein	Transcription	-2.10	-6.91	-4.48	0.00	-0.10	-2.36	3.00
Q8Y4M0	lmo2417	Lipoprotein	Inorganic ion transport and metabolism	-0.21	-0.04	**2.13**	2.96	2.88	5.23	-1.89
Q8Y4L8	lmo2419	Methionine import ATP-binding protein MetN 2	Inorganic ion transport and metabolism	0.16	0.19	**1.21**	-0.13	0.29	0.50	0.56
Q8Y4J6	lmo2442	DUF218 domain-containing protein	Function unknown	0.00	0.00	**6.52**	0.00	2.19	4.68	4.91
Q8Y4I8	lmo2451	Protein-export membrane protein SecG	Intracellular trafficking, secretion, and vesicular transport	0.27	0.64	**1.06**	2.23	0.11	2.87	-5.16
Q8Y4I4	lmo2456	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	Carbohydrate transport and metabolism	0.01	0.30	**1.97**	-0.33	0.03	0.93	1.03
Q7AP49	lmo2505	Peptidoglycan lytic protein P45	Cell wall/membrane/envelope biogenesis	0.67	**2.40**	**1.87**	3.39	4.68	5.53	5.75
Q8Y4I1	lmo2518	Glyceraldehyde-3-phosphate dehydrogenase	Carbohydrate transport and metabolism	-0.33	0.25	**1.43**	-0.03	0.21	1.34	1.04
Q8Y499	lmo2555	Lmo2555 protein	Cell wall/membrane/envelope biogenesis	-0.13	0.44	**1.80**	2.17	2.53	2.87	4.47
Q8Y482	lmo2573	Zinc-type alcohol dehydrogenase-like protein	Energy production and conversion	0.20	-0.02	-1.09	3.08	2.82	1.64	3.16
Q8Y476	lmo2579	ABM domain-containing protein	Function unknown	-0.74	0.77	**2.16**	-0.92	-1.95	-1.95	-1.95
P65II0	lmo2610	Translation initiation factor IF-1	Translation, ribosomal structure and biogenesis	**2.11**	1.01	1.22	-0.04	0.43	1.45	-1.22
Q927H3	lmo2666	PTS EIIB type-2 domain-containing protein	Carbohydrate transport and metabolism	-0.35	-0.71	-1.37	0.13	-0.30	-3.50	-0.20
Q8Y3V6	lmo2725	Lmo2725 protein	Defense mechanisms	0.43	5.06	**6.75**	-0.19	5.72	3.17	0.38
Q8Y3V5	lmo2726	HTH marR-type domain-containing protein	Transcription	2.46	**8.37**	**9.67**	-5.07	4.09	5.12	5.19
Q8Y3U3	lmo2738	Lmo2738 protein	Amino acid transport and metabolism	0.60	0.41	**1.07**	4.62	0.26	2.60	5.15
Q8Y3L0	lmo2825	Phosphoserine aminotransferase	Amino acid transport and metabolism	0.35	0.94	**1.40**	-0.06	0.46	-2.82	0.27

Table S2: Table containing all proteins that are expressed both in reference condition as well as after heat stress with the $\log_2$ fold-change for each timepoint. Bold and underlined values are significant differentially expressed (absolute $\log_2$ fold-change > 1 and p-value < 0.05)

uniprot	lmo-code	protein name	COG description	ref 0h10	ref 0h30	ref 2h	stress 0h30	stress 2h	stress 4h	stress 7h
Q8YAV4	lmo0009	N-acetyltransferase domain-containing protein	Translation, ribosomal structure and biogenesis	-2.39	-1.54	**1.81**	0.63	0.58	0.71	**1.92**
Q8YAU5	lmo0018	Beta-glucosidase	Carbohydrate transport and metabolism	**1.26**	1.20	**4.23**	-0.79	0.93	**2.41**	**4.83**
Q7AP93	lmo0118	Antigen A	Function unknown	-0.99	-1.96	-0.99	-2.62	-2.45	-5.47	**3.30**
Q8YA94	lmo0261	Lmo0261 protein	Carbohydrate transport and metabolism	0.19	0.35	**1.72**	-0.07	0.92	1.39	**3.87**
Q7AP85	lmo0265	Probable succinyl-diaminopimelate desuccinylase	Amino acid transport and metabolism	-0.05	-0.09	-1.38	-0.17	-0.58	-1.13	-0.86
Q8YA80	lmo0279	Class III anaerobic reductase	Nucleotide transport and metabolism	-0.14	**2.51**	**12.00**	-0.24	4.15	**12.40**	**14.06**
Q8Y9T2	lmo0441	Lmo0441 protein	Cell wall/membrane/envelope biogenesis	0.00	0.21	**1.54**	0.17	0.94	**1.98**	**1.79**
Q8Y9T0	lmo0443	LytR_cpsA_psr domain-containing protein	Transcription	-0.28	-0.16	**1.12**	0.20	**1.37**	**3.08**	**3.22**
Q8Y9P2	lmo0482	Probable dual-specificity RNA methyltransferase RlmN	Translation, ribosomal structure and biogenesis	0.20	-0.14	**1.29**	0.69	0.24	**1.86**	**2.23**
Q92EH3	lmo0484	Heme-degrading monooxygenase	Function unknown	-0.07	-0.31	-1.16	-0.08	-0.42	-0.33	-2.10
Q92EC0	lmo0536	Glyco_hydro_4C domain-containing protein	Carbohydrate transport and metabolism	0.13	0.28	**1.59**	-0.13	0.77	**1.39**	**4.03**
Q7AP81	lmo0686	MotB protein	Cell motility	-0.33	-0.56	-1.73	-0.13	-0.24	-5.36	-7.71
Q8Y948	lmo0689	Lmo0689 protein	Signal transduction mechanisms	-0.21	-0.12	-1.12	-0.36	-0.64	-1.55	-2.12
Q92DV7	lmo0697	Flagellar hook protein FlgE	Cell motility	0.10	-0.08	-1.00	-0.23	0.33	-0.03	-10.28
Q8Y928	lmo0714	Flagellar motor switch protein FliG	Cell motility	-0.05	-0.44	-1.46	-0.09	-0.51	-2.28	-10.36
Q8Y904	lmo0738	Lmo0738 protein	Carbohydrate transport and metabolism	0.02	-0.08	-1.36	0.29	0.68	-1.60	**4.11**
Q8Y8V8	lmo0784	PTS EIIA type-4 domain-containing protein	Carbohydrate transport and metabolism	-0.10	-0.38	-1.15	0.88	1.11	**1.72**	-2.74
Q8Y8V4	lmo0788	Lmo0788 protein	Lipid transport and metabolism	-0.26	-0.52	**1.32**	-0.09	-0.24	**1.11**	0.97
Q8Y8P4	lmo0852	HTH tetR-type domain-containing protein	Transcription	**1.38**	**1.19**	0.13	-2.85	0.97	**1.46**	**1.86**
Q8Y8D4	lmo0974	D-alanine–D-alanyl carrier protein ligase	Secondary metabolites biosynthesis, transport, and catabolism	0.26	0.31	**2.34**	-1.05	-0.92	**1.42**	**1.52**
Q8Y8B1	lmo0997	clpE	Post-translational modification, protein turnover, and chaperones	-0.26	-2.28	-5.81	**2.20**	**3.47**	-1.39	-3.04
Q8Y866	lmo1051	Peptide deformylase	Translation, ribosomal structure and biogenesis	-0.17	0.37	**1.90**	-0.01	0.76	**1.95**	**1.97**
Q8Y7M5	lmo1249	Lmo1249 protein	Function unknown	0.00	0.00	**8.84**	0.00	0.00	0.00	**10.77**
Q8Y7H2	lmo1308	Methyltransf_11 domain-containing protein	Coenzyme transport and metabolism	0.00	0.00	**1.13**	0.18	0.62	1.27	**2.45**
P0A355	lmo1364	Cold shock-like protein CspLA	Transcription	-0.21	-0.17	-1.43	0.23	-0.04	-1.62	-2.58
Q8Y790	lmo1402	Lmo1402 protein	Function unknown	-0.15	0.29	**1.02**	-0.04	-2.61	**1.69**	**1.92**
Q8Y786	lmo1406	Formate acetyltransferase	Energy production and conversion	-0.02	-0.13	**1.53**	0.02	0.35	0.86	**1.75**
Q9XDA6	lmo1447	Zinc uptake system ATP-binding protein ZurA	Inorganic ion transport and metabolism	0.40	0.87	**1.17**	0.06	1.00	**1.68**	-2.21
Q8Y700	lmo1530	Queuine tRNA-ribosyltransferase	Nucleotide transport and metabolism	0.40	0.98	**2.04**	-0.12	0.48	1.02	**1.69**
Q8Y6Y3	lmo1548	Cell shape-determining protein MreB	Cell cycle control, cell division, chromosome partitioning	0.15	0.28	**1.02**	0.02	0.38	**1.35**	0.88
Q8Y6X4	lmo1557	Glutamyl-tRNA reductase	Coenzyme transport and metabolism	2.54	4.07	**8.04**	-2.19	-2.19	0.70	**6.94**
Q8Y6Q0	lmo1634	Aldehyde-alcohol dehydrogenase	Energy production and conversion	0.01	0.02	**2.56**	-0.18	0.65	**4.21**	**5.26**
Q8Y6P6	lmo1638	Lmo1638 protein	Defense mechanisms	0.09	-2.30	**1.67**	0.65	-5.00	**1.83**	**2.26**
Q8Y6I5	lmo1699	Lmo1699 protein	Cell motility	-0.18	-0.31	-1.14	0.28	-0.12	-2.08	-3.34
Q8Y6C5	lmo1765	Bifunctional purine biosynthesis protein PurH	Nucleotide transport and metabolism	0.21	0.68	**1.43**	0.04	0.10	**1.49**	**1.07**
Q8Y6B9	lmo1772	Phosphoribosylaminoimidazole-succinocarboxamide synthase	Nucleotide transport and metabolism	0.61	0.78	**2.12**	0.11	0.77	**1.49**	**2.58**

Continued on the next page

Table continued.

uniprot	lmo-code	protein name	COG description	ref 0h10	ref 0h30	ref 2h	stress 0h30	stress 2h	stress 4h	stress 7h
Q8Y664	lmo1836	Carbamoyl-phosphate synthase small chain	Nucleotide transport and metabolism	-0.24	-0.29	-1.07	0.14	0.16	-0.35	-1.23
Q8Y639	lmo1861	Lmo1861 protein	Function unknown	-0.02	-1.59	**1.33**	-1.89	-2.75	-4.36	**1.76**
Q8Y5Y2	lmo1921	UPF0302 protein	Function unknown	-0.47	-0.43	-1.80	-0.23	-4.32	-1.99	-6.77
P0A357	lmo2016	Cold shock-like protein CspLB	Transcription	-0.07	-0.17	-1.30	0.44	-0.55	-1.03	-2.67
Q8Y5K5	lmo2055	Lmo2055 protein	Function unknown	0.73	0.43	**1.63**	0.45	5.59	3.68	**6.30**
Q8Y5I2	lmo2080	Anaerobic reductase	Function unknown	2.30	4.91	**8.22**	2.22	7.77	**9.59**	**7.69**
Q929L9	lmo2152	Thioredoxin	Energy production and conversion	**3.11**	**4.20**	**8.06**	-0.14	0.04	**7.69**	**7.11**
Q8Y5B3	lmo2154	Ribonucleoside-diphosphate reductase subunit beta	Nucleotide transport and metabolism	0.37	0.40	**1.71**	-0.15	-0.21	**1.04**	**1.88**
Q8Y5B2	lmo2155	Ribonucleoside-diphosphate reductase	Nucleotide transport and metabolism	0.16	0.20	**1.14**	-0.31	-0.25	0.17	**1.17**
Q8Y5A7	lmo2161	Lmo2161 protein	Carbohydrate transport and metabolism	0.00	0.00	**6.75**	0.00	0.00	5.90	**9.50**
Q8Y547	lmo2229	DD-transpeptidase	Cell wall/membrane/envelope biogenesis	-0.05	0.05	**1.00**	0.30	1.38	**2.86**	**3.65**
Q8Y4U6	lmo2335	FruA protein	Carbohydrate transport and metabolism	0.07	0.11	**1.45**	0.37	0.63	0.83	**1.57**
Q8Y4U5	lmo2336	Tagatose-6-phosphate kinase	Coenzyme transport and metabolism	0.16	0.19	**1.36**	-0.16	0.51	**1.01**	**2.33**
Q8Y4I7	lmo2452	Lmo2452 protein	Cell wall/membrane/envelope biogenesis	0.05	**1.07**	**1.07**	0.01	0.22	**1.05**	**1.32**
Q8Y4E2	lmo2504	Peptidase_M23 domain-containing protein	Cell wall/membrane/envelope biogenesis	0.00	**9.16**	**9.11**	0.00	2.97	6.50	**8.42**
Q8Y4C8	lmo2522	Lmo2522 protein	Cell wall/membrane/envelope biogenesis	-0.56	**4.46**	**1.55**	3.83	**9.94**	**9.93**	**9.21**
Q8Y4C4	lmo2526	UDP-N-acetylglucosamine 1-carboxyvinyltransferase 1	Cell wall/membrane/envelope biogenesis	0.60	**1.02**	**1.62**	-0.44	0.32	0.78	**1.28**
Q8Y469	lmo2586	Lmo2586 protein	Energy production and conversion	-0.11	-0.62	-1.00	0.28	-1.01	-9.58	-0.75
Q8Y425	lmo2650	PTS EIIB type-2 domain-containing protein	Carbohydrate transport and metabolism	-0.08	-0.35	-1.09	-0.50	-0.53	-9.65	-7.45
Q8Y403	lmo2675	Lmo2675 protein	Function unknown	0.00	4.22	**7.95**	0.00	0.00	5.11	**9.00**
Q8Y3Z0	lmo2689	Magnesium-transporting ATPase, P-type 1	Inorganic ion transport and metabolism	3.86	**6.22**	**10.46**	0.21	3.44	**7.30**	**8.05**
Q8Y3Y8	lmo2691	Peptidoglycan hydrolase	Cell wall/membrane/envelope biogenesis	-0.29	0.25	**1.13**	0.36	0.39	0.43	**2.24**
Q8Y3V1	lmo2730	Lmo2730 protein	Function unknown	0.00	5.02	**7.73**	0.16	-2.23	0.30	**6.78**
Q8Y3K5	lmo2830	Thioredoxin	Post-translational modification, protein turnover, and chaperones	0.00	-0.23	-1.15	-0.23	-0.42	-1.06	-6.57

Table S3: Table containing all proteins that are unique for the heat stress condition with the $\log_2$ fold-change for each timepoint. Bold and underlined values are significant differentially expressed (absolute $\log_2$ fold-change > 1 and p-value < 0.05)

uniprot	lmo-code	protein name	COG description	ref 0h10	ref 0h30	ref 2h	stress 0h30	stress 2h	stress 4h	stress 7h
Q8YAV0	lmo0013	Quinol oxidase subunit 2	Energy production and conversion	0.13	-0.33	-0.85	-0.02	-0.10	-1.13	-1.38
Q8YAU9	lmo0014	Quinol oxidase polypeptide I	Energy production and conversion	0.02	-0.08	-0.71	-0.19	0.09	-0.64	-1.58
Q8YAT6	lmo0027	Lmo0027 protein	Carbohydrate transport and metabolism	0.31	0.20	-0.19	-0.52	-0.26	-1.43	**1.69**
Q92FQ6	lmo0054	Replicative DNA helicase	Replication, recombination and repair	0.01	0.01	0.43	0.05	-0.09	0.48	**1.25**
Q8YAK8	lmo0110	Abhydrolase_3 domain-containing protein	Lipid transport and metabolism	0.07	0.08	-0.42	-0.22	-0.71	-1.40	-1.54
Q7AP94	lmo0117	Antigen B	Function unknown	-4.51	-4.51	0.14	**6.62**	0.00	0.00	**9.05**
Q7AP92	lmo0119	Lmo0119 protein	Function unknown	0.00	0.00	0.00	0.00	0.00	0.00	**8.97**
Q8YAK3	lmo0121	Lmo0121 protein	Cell motility	0.00	0.00	0.00	0.00	0.00	0.00	**9.00**
Q8YAK2	lmo0122	Lmo0122 protein	Function unknown	-1.98	-1.98	-1.98	0.31	0.66	-1.82	**8.12**
Q8YAK0	lmo0124	Lmo0124 protein	Function unknown	0.00	0.00	0.00	0.00	0.00	0.00	**8.81**
Q8YAJ7	lmo0127	Lmo0127 protein	Function unknown	3.02	1.24	2.55	3.34	-0.01	0.76	**11.22**
Q8YAJ6	lmo0129	MurNAc-LAA domain-containing protein	Cell wall/membrane/envelope biogenesis	-0.52	-0.86	-0.36	0.06	0.44	-1.31	**3.29**
Q8YAJ0	lmo0135	SBP_bac_5 domain-containing protein	Amino acid transport and metabolism	-0.15	-0.15	-0.77	-0.15	-0.21	-1.06	-1.63
Q8YAG9	lmo0157	DNA helicase	Replication, recombination and repair	-0.08	-0.33	0.05	2.89	6.32	0.00	**9.58**
Q8YAG4	lmo0162	Lmo0162 protein	Replication, recombination and repair	-0.17	-0.20	0.41	-0.21	0.22	-2.96	**1.02**
Q8YAD9	lmo0192	Pur operon repressor	Nucleotide transport and metabolism	0.13	-0.04	-0.31	-0.47	-0.29	-0.34	-1.61
Q8YAD5	lmo0196	Putative septation protein SpoVG 1	Cell cycle control, cell division, chromosome partitioning	-0.14	-0.03	0.16	0.37	0.56	**1.97**	0.20
Q92F70	lmo0197	Putative septation protein SpoVG 2	Cell cycle control, cell division, chromosome partitioning	-0.23	0.02	0.19	0.18	0.22	**1.16**	-0.95
P33379	lmo0204	Actin assembly-inducing protein	Function unknown	0.00	0.00	0.00	0.00	0.00	0.00	**8.24**
Q8YAD0	lmo0214	Transcription-repair-coupling factor	Replication, recombination and repair	0.15	0.00	-0.11	-0.46	-0.59	-1.24	-0.85
Q48761	lmo0233	DNA repair protein RadA	Post-translational modification, protein turnover, and chaperones	0.15	0.16	0.83	-0.12	0.14	0.84	**1.19**
Q8YAB1	lmo0239	Cysteine–tRNA ligase	Translation, ribosomal structure and biogenesis	0.00	-0.12	-0.55	0.07	0.09	-0.53	-1.27
Q92F34	lmo0241	SpoU_sub_bind domain-containing protein	Translation, ribosomal structure and biogenesis	-0.22	-0.19	-0.71	-0.18	-0.13	-0.62	-1.32
Q8YA92	lmo0267	Lmo0267 protein	Amino acid transport and metabolism	-0.04	-0.14	-0.66	0.18	-0.12	-0.70	-1.65
Q8YA81	lmo0278	ABC transporter domain-containing protein	Inorganic ion transport and metabolism	-0.06	-0.25	-0.87	-0.29	-0.45	-1.08	-0.32
Q8YA79	lmo0280	Anaerobic ribonucleoside-triphosphate reductase-activating protein	Coenzyme transport and metabolism	0.88	0.03	4.71	0.02	-0.28	4.71	**6.78**
Q8YA75	lmo0284	Methionine import ATP-binding protein MetN 1	Inorganic ion transport and metabolism	0.14	0.26	0.29	-0.25	0.07	-1.22	-5.83
Q8YA74	lmo0285	Lipoprotein	Inorganic ion transport and metabolism	-0.03	-0.27	-0.10	-0.26	0.24	-0.62	-2.64
Q8YA73	lmo0286	Aminotran_1_2 domain-containing protein	Amino acid transport and metabolism	-0.08	-0.05	-0.87	0.10	-0.60	-1.23	-8.79
Q7AP84	lmo0319	Lmo0319 protein	Carbohydrate transport and metabolism	0.18	-0.01	0.44	-0.11	-0.32	-0.88	**1.03**
Q8YA11	lmo0355	FAD_binding_2 domain-containing protein	Energy production and conversion	-0.13	-0.23	0.90	0.02	0.19	**1.56**	**2.14**
Q8Y9Y0	lmo0387	Lmo0387 protein	Function unknown	-0.19	-0.13	-0.49	-0.80	-0.90	-1.61	-1.23
Q8Y9X0	lmo0398	PTS EIIA type-2 domain-containing protein	Carbohydrate transport and metabolism	-0.15	-0.30	-0.93	-0.25	-0.51	-5.12	-1.32
Q8Y9W8	lmo0401	Alpha-mann_mid domain-containing protein	Carbohydrate transport and metabolism	-0.02	-0.28	-0.61	-0.20	-0.95	-2.14	0.02
Q8Y9W7	lmo0402	Putative PTS system EIIA component	Transcription	0.22	0.01	-0.54	-0.43	-0.30	-5.81	-1.07
Q8Y9W2	lmo0407	Lmo0407 protein	Function unknown	-2.15	0.69	0.09	-2.41	-5.74	-8.67	-8.67

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Table continued.

uniprot	lmo-code	protein name	COG description	ref 0h10	ref 0h30	ref 2h	stress 0h30	stress 2h	stress 4h	stress 7h
Q8Y9V5	lmo0415	NodB homology domain-containing protein	Carbohydrate transport and metabolism	-0.31	0.07	0.57	-0.28	0.97	**1.52**	**1.56**
Q8Y9N5	lmo0490	Shikimate dehydrogenase (NADP(+))	Amino acid transport and metabolism	0.12	0.01	0.78	-0.65	0.00	0.67	**1.72**
Q8Y9N4	lmo0491	3-dehydroquinate dehydratase	Amino acid transport and metabolism	-0.14	-0.07	0.67	-0.16	0.09	1.01	**1.27**
Q8Y9N1	lmo0494	SGNH_hydro domain-containing protein	Amino acid transport and metabolism	0.07	0.26	0.98	-0.20	-0.16	0.38	**1.25**
P60073	lmo0496	UPF0291 protein lmo0496	Function unknown	-2.84	-5.11	-5.11	**7.33**	5.30	1.98	-2.47
Q8Y9L0	lmo0517	Lmo0517 protein	Carbohydrate transport and metabolism	0.00	0.00	0.00	0.00	0.00	0.00	**7.06**
Q8Y9J8	lmo0529	Lmo0529 protein	Cell wall/membrane/envelope biogenesis	-1.77	0.80	0.97	**7.20**	**7.16**	1.00	**6.97**
Q8Y968	lmo0552	Lmo0665 protein	Function unknown	-0.51	0.04	0.52	-0.21	-2.60	**1.53**	-3.25
Q8Y9H4	lmo0554	Fe-ADH domain-containing protein	Energy production and conversion	0.06	-0.17	-0.95	-0.12	-0.82	-1.58	-0.51
Q8Y9H1	lmo0557	Lmo0557 protein	Carbohydrate transport and metabolism	0.11	-0.07	0.77	0.36	0.43	1.16	**1.96**
Q8Y9F6	lmo0572	Lmo0572 protein	Function unknown	-0.05	0.49	0.49	-0.39	-10.26	-6.90	-10.26
Q8Y9E6	lmo0584	Lmo0584 protein	Cell cycle control, cell division, chromosome partitioning	0.26	-0.20	-0.27	-0.51	-0.48	-1.32	-0.75
Q8Y9D8	lmo0592	Lmo0592 protein	Function unknown	0.14	0.07	0.04	0.01	-0.23	-0.44	-1.01
Q8Y9D1	lmo0601	Lmo0601 protein	Function unknown	0.07	-0.27	-0.19	0.42	**1.20**	0.60	0.29
Q8Y9D0	lmo0602	N-acetyltransferase domain-containing protein	Transcription	-0.12	-0.35	-0.43	-0.07	-0.18	-2.43	-2.16
Q8Y9C5	lmo0607	Lmo0607 protein	Defense mechanisms	-0.02	-0.22	-0.65	-0.43	0.33	-1.20	-0.77
Q8Y9C4	lmo0608	Lmo0608 protein	Defense mechanisms	0.04	-0.14	-0.87	-0.37	0.03	-1.92	-0.85
Q8Y9C3	lmo0609	Lmo0609 protein	Inorganic ion transport and metabolism	0.39	0.26	-0.12	0.11	0.15	-0.54	-1.09
Q8Y9C1	lmo0611	FMN-dependent NADH-azoreductase 1	Lipid transport and metabolism	-0.03	-0.11	-0.53	-0.22	-0.39	-0.90	-2.02
Q8Y9A6	lmo0626	Lmo0626 protein	Function unknown	-0.11	-0.58	0.08	0.25	**1.25**	0.67	0.71
Q8Y987	lmo0646	Lmo0646 protein	Function unknown	0.13	0.12	-0.20	0.29	-0.12	-2.39	-7.51
Q8Y969	lmo0664	Mac domain-containing protein	Function unknown	0.04	0.01	0.35	0.59	0.56	**1.44**	0.73
Q8Y954	lmo0680	Lmo0680 protein	Cell motility	0.21	-0.22	-4.88	-0.14	0.38	-7.28	-7.28
Q7AP82	lmo0685	MotA_ExbB domain-containing protein	Cell motility	0.03	-0.13	-0.85	-0.38	-0.27	-1.78	-2.95
Q8Y949	lmo0688	Lmo0688 protein	Function unknown	0.12	0.05	-0.59	-0.08	-0.55	-6.04	-8.72
P0A4H5	lmo0691	Chemotaxis protein CheY	Cell motility	0.15	0.16	-0.05	-0.59	-0.53	-0.19	-2.94
Q48768	lmo0692	Chemotaxis protein CheA	Signal transduction mechanisms	0.17	-0.01	-0.80	-0.06	-0.28	-1.17	-2.88
Q8Y942	lmo0699	Flagellar motor switch protein FliM	Cell motility	0.39	0.09	-0.96	-0.21	-0.50	-9.13	-9.13
Q8Y941	lmo0700	Lmo0700 protein	Cell motility	0.30	-0.16	-0.73	-0.17	-0.73	-8.77	-8.77
Q8Y924	lmo0718	Lmo0718 protein	Function unknown	-0.05	0.10	-0.86	-0.03	-0.38	-0.87	-1.94
Q8Y919	lmo0723	Lmo0723 protein	Cell motility	0.10	-0.13	-0.99	0.02	0.07	-1.87	-1.87
Q8Y907	lmo0735	Ribulose-phosphate 3-epimerase	Carbohydrate transport and metabolism	0.00	0.00	0.00	1.78	-0.35	-1.31	**10.25**
Q8Y906	lmo0736	Lmo0736 protein	Carbohydrate transport and metabolism	-0.43	-0.26	-0.29	0.61	0.87	0.65	**4.09**
Q8Y905	lmo0737	Lmo0737 protein	Post-translational modification, protein turnover, and chaperones	2.28	1.29	-4.74	-0.24	-0.71	-4.89	**3.70**
Q8Y903	lmo0739	Lmo0739 protein	Carbohydrate transport and metabolism	-0.05	0.00	-0.73	0.29	-0.33	-3.13	**4.46**
Q8Y8V7	lmo0785	Lmo0785 protein	Transcription	0.06	0.11	0.92	0.07	0.45	0.88	**1.14**
Q8Y8U6	lmo0796	Lmo0796 protein	Post-translational modification, protein turnover, and chaperones	0.22	0.41	-0.21	-0.66	-1.02	-1.60	-2.02
P58724	lmo0799	Blue-light photoreceptor	Signal transduction mechanisms	0.15	-0.04	-0.41	0.23	0.10	-1.21	-0.17
Q8Y8T3	lmo0811	Alpha-carbonic anhydrase domain-containing protein	Inorganic ion transport and metabolism	-0.13	-0.14	-0.79	-0.04	-0.36	-1.89	-2.03
Q8Y8T0	lmo0814	Lmo0814 protein	Function unknown	-0.02	0.14	0.66	0.00	0.43	**1.24**	**1.06**

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Table continued.

uniprot	lmo-code	protein name	COG description	ref 0h10	ref 0h30	ref 2h	stress 0h30	stress 2h	stress 4h	stress 7h
Q8Y8S1	lmo0823	Aldo_ket_red domain-containing protein	Function unknown	0.07	-0.22	-0.63	-0.06	-0.51	-1.70	-1.06
Q8Y8P9	lmo0847	ABC transmembrane type-1 domain-containing protein	Inorganic ion transport and metabolism	0.09	0.04	0.07	-0.02	0.34	-0.04	-1.43
Q8Y8M7	lmo0869	Amidohydro-rel domain-containing protein	Amino acid transport and metabolism	0.04	-0.53	-0.82	-0.39	-1.12	-1.36	-4.44
Q8Y8L2	lmo0885	Holo-[acyl-carrier-protein] synthase	Lipid transport and metabolism	0.38	0.45	0.75	-0.24	0.38	**1.40**	0.76
Q7AP79	lmo0895	RNA polymerase sigma factor	Transcription	0.03	-0.26	0.43	-0.19	-0.01	0.87	**1.03**
Q8Y8J5	lmo0906	Lmo0906 protein	Energy production and conversion	0.05	0.03	-0.25	-0.18	-0.35	-1.12	-0.56
Q8Y8G8	lmo0936	Nitroreductase domain-containing protein	Energy production and conversion	0.04	-0.06	0.26	0.22	0.34	**1.02**	-0.12
Q8Y8G1	lmo0943	DNA protection during starvation protein	Inorganic ion transport and metabolism	0.15	-0.03	-0.91	-0.81	0.03	-1.79	-2.13
Q8Y8F4	lmo0950	Lmo0950 protein	Function unknown	0.03	0.02	0.08	-0.14	0.62	-1.61	**1.72**
Q8Y8E4	lmo0960	Lmo0960 protein	Post-translational modification, protein turnover, and chaperones	-0.37	-0.38	0.57	-0.31	0.02	**1.45**	**1.67**
Q8Y8E3	lmo0961	Lmo0961 protein	Post-translational modification, protein turnover, and chaperones	0.15	0.06	0.91	-0.49	-0.55	0.18	**1.16**
Q8Y8E2	lmo0962	Listeria epitope LemA	Function unknown	0.37	0.38	0.69	0.05	0.19	-0.26	-1.14
P0A438	lmo1002	Phosphocarrier protein HPr	Carbohydrate transport and metabolism	0.14	-0.06	-0.04	-0.11	-0.55	-1.28	-2.27
Q92D14	lmo1008	Lmo1008 protein	Function unknown	-0.22	0.02	0.40	0.07	0.67	**1.74**	0.91
Q8Y899	lmo1013	Lmo1013 protein	Cell wall/membrane/envelope biogenesis	0.02	0.04	0.44	-0.35	-0.05	-0.12	**1.39**
Q7AP75	lmo1015	ABC transmembrane type-1 domain-containing protein GbuB	Inorganic ion transport and metabolism	0.02	-0.05	0.68	-0.09	-2.57	-1.39	-1.30
Q92CZ4	lmo1028	DNA-directed RNA polymerase subunit epsilon	Function unknown	0.04	-0.26	-0.72	0.00	-0.54	-1.87	-1.53
Q8Y875	lmo1042	Molybdopterin molybdenumtransferase	Coenzyme transport and metabolism	0.14	0.07	-0.45	-0.06	-0.67	-1.46	-2.04
Q8Y865	lmo1052	Pyruvate dehydrogenase E1 component subunit alpha	Energy production and conversion	0.08	0.07	-0.42	-0.08	-0.01	-0.56	-1.04
Q8Y864	lmo1053	Transket_pyr domain-containing protein	Energy production and conversion	0.04	0.19	-0.12	-0.34	-0.67	-1.46	-1.49
Q8Y863	lmo1054	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	Energy production and conversion	0.09	0.03	-0.45	-0.15	-0.23	-0.54	-1.12
Q8Y862	lmo1055	Dihydrolipoyl dehydrogenase	Energy production and conversion	-0.05	-0.13	-0.74	0.28	0.19	-0.88	-2.03
P67356	lmo1058	UPF0223 protein	Inorganic ion transport and metabolism	0.02	0.32	0.56	0.84	**1.97**	0.84	0.27
Q8Y853	lmo1065	UPF0637 protein	Function unknown	0.05	-0.08	-0.50	-0.11	-0.61	-1.75	-1.28
Q8Y839	lmo1079	Lmo1079 protein	Cell wall/membrane/envelope biogenesis	0.00	-0.03	0.57	0.24	0.93	**1.22**	**1.43**
Q8Y7Q7	lmo1215	GW domain-containing protein	Cell motility	0.00	0.00	0.00	0.00	0.00	0.00	**7.29**
Q92CI8	lmo1220	Lmo1220 protein	Transcription	0.25	0.79	0.93	0.06	**1.74**	**1.62**	0.71
P0A4L3	lmo1233	Thioredoxin	Post-translational modification, protein turnover, and chaperones	-0.02	0.05	-0.14	-0.13	-0.26	-1.27	-1.37
Q8Y7N4	lmo1240	Phosphoesterase	Function unknown	2.26	2.48	3.57	0.08	0.76	**1.24**	0.58
Q8Y7M0	lmo1254	Aamy domain-containing protein	Carbohydrate transport and metabolism	0.05	-0.09	-0.67	-0.25	-0.83	-2.17	-0.96
Q92CE7	lmo1257	Lmo1257 protein	Function unknown	-0.04	-0.08	0.66	-0.19	0.19	**1.41**	-0.24
Q8Y7L6	lmo1261	GYF_2 domain-containing protein	Cell cycle control, cell division, chromosome partitioning	0.30	0.38	-0.47	0.11	0.25	-0.96	-2.78
Q8Y7I6	lmo1291	Acyl_transf_3 domain-containing protein	Lipid transport and metabolism	-0.12	-2.86	-0.47	4.84	5.34	3.19	**5.96**
Q8Y7I4	lmo1293	Glycerol-3-phosphate dehydrogenase	Energy production and conversion	0.00	-0.09	-0.55	-0.22	-0.25	-1.28	-1.68
Q8Y7H1	lmo1309	ParB domain-containing protein	Transcription	0.00	0.00	4.89	0.00	0.00	**8.26**	**8.24**
Q8Y7G7	lmo1314	Ribosome-recycling factor	Translation, ribosomal structure and biogenesis	-0.20	0.01	0.29	0.50	0.68	**1.35**	0.12
Q8Y7F4	lmo1327	Ribosome-binding factor A	Translation, ribosomal structure and biogenesis	0.12	0.17	0.33	0.14	**1.10**	0.97	0.22
Q8Y7E7	lmo1336	5-formyltetrahydrofolate cyclo-ligase	Coenzyme transport and metabolism	0.35	0.55	0.95	0.13	-0.40	0.81	**1.42**

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Table continued.

uniprot	lmo-code	protein name	COG description	ref 0h10	ref 0h30	ref 2h	stress 0h30	stress 2h	stress 4h	stress 7h
Q8Y7D5	lmo1348	Aminomethyltransferase	Amino acid transport and metabolism	0.05	-0.08	-0.47	-0.32	-0.60	-0.85	-1.71
Q8Y7D3	lmo1350	Probable glycine dehydrogenase (decarboxylating) subunit 2	Amino acid transport and metabolism	0.20	0.10	-0.23	-0.15	-0.18	-0.77	-1.01
Q8Y7B8	lmo1368	DNA repair protein RecN	Replication, recombination and repair	-0.10	-0.29	-0.57	0.08	-0.33	-1.58	-1.00
Q8Y7A2	lmo1387	Lmo1387 protein	Amino acid transport and metabolism	0.24	-0.26	-0.14	-1.00	-1.92	-9.31	-1.21
P0A442	lmo1407	Pyruvate formate-lyase-activating enzyme	Energy production and conversion	-0.38	-0.89	0.58	0.38	0.35	0.90	**1.97**
Q8Y782	lmo1414	Lmo1414 protein	Lipid transport and metabolism	0.19	0.19	0.06	-0.47	-0.66	-0.41	-1.21
Q8Y763	lmo1438	Lmo1438 protein	Cell wall/membrane/envelope biogenesis	-0.10	0.22	0.60	0.34	**1.72**	**1.82**	**2.01**
P28764	lmo1439	Superoxide dismutase [Mn]	Energy production and conversion	-0.26	0.00	-0.77	0.32	-0.36	-0.96	-4.23
Q8Y749	lmo1463	Cytidine deaminase	Nucleotide transport and metabolism	-0.12	-0.01	0.41	0.19	0.44	**1.23**	**1.04**
P0DJM2	lmo1473	Chaperone protein DnaK	Post-translational modification, protein turnover, and chaperones	0.02	-0.14	-0.48	0.29	0.50	0.12	-1.40
Q92BM7	lmo1486	Ribosomal silencing factor RsfS	Function unknown	-0.07	0.05	-0.02	0.18	0.41	**1.47**	0.72
Q8Y734	lmo1489	CRM domain-containing protein	Translation, ribosomal structure and biogenesis	0.12	0.11	0.31	-0.10	0.48	**1.28**	0.76
Q8Y733	lmo1490	Shikimate dehydrogenase (NADP(+))	Amino acid transport and metabolism	-0.17	0.26	0.75	-0.03	-0.05	0.79	**1.58**
P64277	lmo1496	Transcription elongation factor GreA	Transcription	0.02	0.07	0.28	0.11	0.17	**1.13**	-0.78
Q8Y717	lmo1509	ATP-dependent RecD-like DNA helicase	Replication, recombination and repair	-0.63	0.02	0.81	-2.46	-1.95	0.65	**1.06**
Q8Y716	lmo1510	Lmo1510 protein	Function unknown	0.17	-0.01	-0.10	-0.25	-0.61	-2.03	-1.79
Q8Y712	lmo1514	AAA domain-containing protein	Replication, recombination and repair	0.15	0.15	0.32	0.19	0.47	0.88	**1.06**
Q8Y707	lmo1521	Lmo1521 protein	Cell wall/membrane/envelope biogenesis	-0.03	-0.20	0.72	0.30	0.35	**1.79**	**2.56**
P0A2X5	lmo1524	Adenine phosphoribosyltransferase	Nucleotide transport and metabolism	0.52	0.32	0.62	0.11	0.48	0.34	**1.24**
Q8Y6Z2	lmo1538	Glycerol kinase	Energy production and conversion	-0.03	-0.10	-0.70	-0.14	-0.05	-0.57	-1.44
P66125	lmo1540	50S ribosomal protein L27	Translation, ribosomal structure and biogenesis	-0.14	-0.11	0.41	0.04	0.01	1.03	-1.51
Q8Y6X3	lmo1558	Probable GTP-binding protein EngB	Cell cycle control, cell division, chromosome partitioning	0.15	0.28	0.34	-0.58	-1.07	-0.70	-0.02
Q8Y6W2	lmo1569	Lmo1569 protein	Function unknown	1.67	1.73	2.24	-0.39	-0.10	-0.10	-6.39
Q8Y6V1	lmo1580	Universal stress protein	Signal transduction mechanisms	0.07	0.11	-0.40	0.08	-0.09	-0.81	-1.34
Q8Y6T9	lmo1593	Aminotran_5 domain-containing protein	Amino acid transport and metabolism	-0.25	-0.16	0.46	0.13	0.31	0.58	**1.01**
Q8Y6S4	lmo1609	Lmo1609 protein	Energy production and conversion	-0.33	-0.11	-0.60	0.17	0.26	0.68	-1.83
Q8Y6Q7	lmo1627	Tryptophan synthase alpha chain	Amino acid transport and metabolism	0.41	2.12	2.84	-2.50	-5.83	-5.83	-1.40
Q8Y6N8	lmo1646	Nuclease SbcCD subunit D	Replication, recombination and repair	-0.25	1.47	1.65	-2.01	-1.49	1.33	-6.55
Q8Y6L7	lmo1667	L-lactate dehydrogenase	Energy production and conversion	0.06	-0.16	0.52	0.38	0.45	0.35	**1.01**
Q8Y6J0	lmo1694	Lmo1694 protein	Function unknown	0.22	-0.03	-0.99	0.11	-0.48	-2.61	-1.51
Q8Y6I9	lmo1695	Phosphatidylglycerol lysyltransferase	Cell wall/membrane/envelope biogenesis	0.22	-0.10	0.44	0.01	0.44	-1.47	-0.52
Q8Y6H7	lmo1707	UPF0435 protein	Replication, recombination and repair	0.25	0.59	0.88	0.05	0.24	**1.13**	-3.42
Q8Y6G9	lmo1717	Type I site-specific deoxyribonuclease	Cell motility	0.14	-0.23	-0.10	-0.02	-0.74	-1.17	-1.13
Q8Y6G8	lmo1718	Lmo1718 protein	Cell cycle control, cell division, chromosome partitioning	-0.06	-0.01	-0.40	0.24	0.23	0.16	**1.26**
Q92AT9	lmo1719	Lmo1719 protein	Carbohydrate transport and metabolism	-0.14	-0.18	0.03	0.35	0.36	-0.20	**1.57**
Q8Y6D6	lmo1751	Uncharacterized RNA methyltransferase lmo1751	Translation, ribosomal structure and biogenesis	0.07	0.54	0.97	-0.02	0.77	**1.39**	**1.43**
Q8Y6D1	lmo1757	Lmo1757 protein	Function unknown	-0.07	0.04	0.92	0.14	0.64	**1.55**	1.04
Q8Y6D0	lmo1758	DNA ligase	Replication, recombination and repair	0.09	0.13	0.66	0.17	-0.02	0.99	**1.25**
Q8Y6C1	lmo1769	Phosphoribosylformylglycinamidine synthase subunit PurL	Nucleotide transport and metabolism	0.26	0.31	0.38	-0.04	-0.88	0.15	**1.48**
Q8Y6B6	lmo1775	N5-carboxyaminoimidazole ribonucleotide mutase PurE	Nucleotide transport and metabolism	0.06	0.07	0.46	-0.06	0.20	**1.32**	0.76

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Table continued.

uniprot	lmo-code	protein name	COG description	ref 0h10	ref 0h30	ref 2h	stress 0h30	stress 2h	stress 4h	stress 7h
Q8Y6A9	lmo1782	Exodeoxyribonuclease III	Replication, recombination and repair	0.26	0.32	-0.55	-0.41	-0.87	-1.95	-1.29
P0A49I	lmo1784	50S ribosomal protein L35	Translation, ribosomal structure and biogenesis	-0.01	-0.16	0.43	0.38	0.09	-0.15	-2.05
Q8Y668	lmo1831	Orotate phosphoribosyltransferase	Nucleotide transport and metabolism	-0.25	-0.34	-0.92	-0.31	-0.26	-0.45	-1.27
P58641	lmo1832	Orotidine 5'-phosphate decarboxylase	Nucleotide transport and metabolism	0.13	-0.21	-0.72	-0.10	-0.02	-0.86	-1.04
Q8Y667	lmo1833	Dihydroorotate dehydrogenase B (NAD(+)), catalytic subunit	Nucleotide transport and metabolism	-0.11	-0.20	-0.61	0.00	0.41	-0.18	-1.01
Q8Y665	lmo1835	Carbamoyl-phosphate synthase large chain	Nucleotide transport and metabolism	-0.04	-0.25	-0.78	0.05	0.03	-0.58	-1.26
Q8Y663	lmo1837	Dihydroorotase	Nucleotide transport and metabolism	0.33	0.12	-0.61	-0.09	-0.20	-0.30	-1.20
Q8Y662	lmo1838	Aspartate carbamoyltransferase	Nucleotide transport and metabolism	-0.01	0.06	-0.46	-0.36	-0.87	-3.07	-7.29
Q8Y660	lmo1840	Bifunctional protein PyrR	Nucleotide transport and metabolism	-0.06	-0.39	-0.68	0.01	-0.13	0.01	-1.22
Q8Y657	lmo1843	Pseudouridine synthase	Translation, ribosomal structure and biogenesis	0.05	0.08	0.66	-0.21	0.15	**1.08**	0.49
Q8Y652	lmo1848	Manganese transport system membrane protein MntC	Inorganic ion transport and metabolism	0.00	0.00	2.96	-5.81	-2.71	-9.02	-9.02
Q8Y641	lmo1859	Peptide methionine sulfoxide reductase MsrB	Post-translational modification, protein turnover, and chaperones	0.06	0.10	0.32	0.09	0.07	**1.06**	-0.61
Q8Y638	lmo1862	SGNH_hydro domain-containing protein	Amino acid transport and metabolism	0.03	-2.92	-0.24	0.00	**7.63**	6.15	**8.63**
Q8Y624	lmo1877	Formate–tetrahydrofolate ligase	Coenzyme transport and metabolism	0.22	0.29	0.77	0.05	-2.75	**1.36**	1.55
Q8Y610	lmo1892	DD-transpeptidase	Cell wall/membrane/envelope biogenesis	0.14	0.20	0.69	0.63	**2.76**	**3.16**	**3.34**
Q8Y607	lmo1895	DnaD protein	Replication, recombination and repair	0.14	-0.03	-0.06	0.22	0.26	**1.09**	0.78
Q8Y601	lmo1902	3-methyl-2-oxobutanoate hydroxymethyltransferase	Coenzyme transport and metabolism	0.23	-0.35	-0.48	-0.01	0.56	0.81	**1.04**
Q8Y600	lmo1903	Lmo1903 protein	Energy production and conversion	-2.80	0.40	0.76	2.72	0.00	3.02	**9.87**
Q8Y5Y6	lmo1917	Formate acetyltransferase	Energy production and conversion	-0.04	-0.17	0.93	-0.10	-0.91	-0.40	**1.36**
Q8Y5Y1	lmo1922	Lmo1922 protein	Function unknown	0.23	0.12	-0.41	-0.03	-0.23	-1.03	-0.73
Q8Y5X4	lmo1929	Nucleoside diphosphate kinase	Nucleotide transport and metabolism	-0.32	-0.18	0.17	0.15	0.82	**2.20**	**2.44**
Q8Y5U4	lmo1961	Ferredoxin–NADP reductase 1	Energy production and conversion	-0.13	-0.24	-0.68	-0.21	-2.92	-7.80	-7.80
Q8Y5S7	lmo1978	Glucose-6-phosphate 1-dehydrogenase	Carbohydrate transport and metabolism	0.05	-0.21	-0.36	-0.23	0.01	-0.30	-1.08
Q8Y5S6	lmo1979	Lmo1979 protein	Function unknown	-0.23	0.45	0.70	0.15	0.23	**1.67**	-2.19
Q8Y5R3	lmo1993	Pyrimidine-nucleoside phosphorylase	Nucleotide transport and metabolism	0.17	0.05	0.05	-0.08	0.17	-0.23	**1.39**
Q8Y5Q0	lmo2006	AlsS protein	Amino acid transport and metabolism	0.11	-0.18	-0.86	0.01	0.05	-1.11	-1.23
Q8Y5M9	lmo2028	S4 RNA-binding domain-containing protein	Signal transduction mechanisms	0.12	-0.01	-0.22	0.08	-0.76	-7.50	-7.50
Q8Y5M3	lmo2034	Cell division protein DivIB	Cell cycle control, cell division, chromosome partitioning	2.89	0.08	-0.28	0.00	6.05	5.90	**9.47**
Q8Y5L8	lmo2039	PbpB protein	Cell wall/membrane/envelope biogenesis	0.31	0.89	0.54	2.33	3.28	**7.61**	6.37
Q8Y5L1	lmo2048	Lmo2048 protein	Transcription	-0.07	0.07	0.83	0.32	0.75	**1.83**	0.65
Q8Y5K0	lmo2060	MOSC domain-containing protein	Energy production and conversion	2.82	0.12	-0.24	4.92	**7.88**	0.00	2.43
Q9AGE7	lmo2069	10 kDa chaperonin	Post-translational modification, protein turnover, and chaperones	0.05	-0.18	-1.00	0.11	-0.32	-0.75	-1.12
P60384	lmo2072	Redox-sensing transcriptional repressor Rex	Transcription	0.13	0.18	-0.59	-0.03	-0.15	-0.30	-1.17
Q8Y5I4	lmo2078	t(6)A37 threonylcarbamoyladenosine biosynthesis protein TsaE	Function unknown	0.03	0.05	0.85	-0.24	0.22	**1.31**	0.52
Q8Y5I3	lmo2079	Lmo2079 protein	Function unknown	-0.24	-0.18	0.25	0.02	0.19	0.07	**1.08**
Q8Y5G1	lmo2102	Pyridoxal 5'-phosphate synthase subunit PdxT	Coenzyme transport and metabolism	0.37	0.20	-0.59	-0.13	-1.34	-2.83	-1.32
Q8Y5F1	lmo2113	Coproheme decarboxylase	Function unknown	0.07	-0.08	-0.63	-0.37	-0.23	-1.65	-0.93
Q8Y5F0	lmo2114	ABC transporter domain-containing protein	Defense mechanisms	-0.07	-0.14	-0.59	-0.03	-0.18	-1.14	-1.34
Q8Y5D5	lmo2130	Lmo2130 protein	Amino acid transport and metabolism	0.11	1.92	2.39	-4.74	-6.86	-4.80	-2.26
Q8Y5C6	lmo2139	ABC transporter domain-containing protein	Function unknown	0.28	0.27	0.00	-0.31	-0.40	-3.95	-1.21

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Table continued.

uniprot	lmo-code	protein name	COG description	ref 0h10	ref 0h30	ref 2h	stress 0h30	stress 2h	stress 4h	stress 7h
Q8Y5A8	lmo2160	Lmo2160 protein	Carbohydrate transport and metabolism	0.00	0.00	0.00	0.00	0.00	**8.46**	**9.42**
Q8Y5A5	lmo2163	Lmo2163 protein	Function unknown	-0.26	-0.42	1.34	-0.29	-2.63	5.57	**8.64**
Q8Y599	lmo2170	Lmo2170 protein	Function unknown	0.00	0.00	0.00	0.00	0.00	0.00	**8.47**
Q8Y583	lmo2188	Lmo2188 protein	Amino acid transport and metabolism	0.00	-0.11	-0.72	-0.13	-0.48	-1.58	-0.96
Q7AP53	lmo2193	ABC transporter domain-containing protein	Inorganic ion transport and metabolism	0.05	-0.07	-0.06	-0.02	-0.17	-0.40	-1.02
Q8Y576	lmo2199	Lmo2199 protein	Post-translational modification, protein turnover, and chaperones	0.08	-0.28	-0.46	0.70	1.11	**1.78**	-0.63
Q8Y570	lmo2206	Chaperone protein ClpB	Post-translational modification, protein turnover, and chaperones	0.07	0.09	-0.09	0.32	2.00	**1.33**	**1.15**
Q8Y565	lmo2211	Coproporphyrin III ferrochelatase	Coenzyme transport and metabolism	-0.03	-0.05	-0.08	-0.24	-0.52	-1.03	-1.55
Q8Y563	lmo2213	Heme-degrading monooxygenase	Function unknown	0.33	-0.06	-0.19	0.33	0.21	-0.77	-1.58
Q8Y541	lmo2235	Lmo2235 protein	Secondary metabolites biosynthesis, transport, and catabolism	0.00	0.00	0.00	0.00	0.00	0.00	**8.28**
Q8Y529	lmo2247	Aldo_ket_red domain-containing protein	Function unknown	0.00	-0.08	-0.39	-0.25	-0.55	-1.34	0.26
Q929C7	lmo2248	Lmo2248 protein	Inorganic ion transport and metabolism	0.12	-0.03	0.02	-0.29	-0.61	-1.47	0.31
Q8Y526	lmo2251	ABC transporter domain-containing protein	Amino acid transport and metabolism	-0.06	-0.06	-0.50	0.48	1.05	**1.45**	0.20
Q929B9	lmo2256	Lmo2256 protein	Function unknown	0.09	0.22	-0.03	-0.21	-0.36	-0.39	-1.72
Q8Y4V1	lmo2329	HTH cro/CI-type domain-containing protein	Transcription	0.11	0.24	0.45	-0.19	0.50	**1.25**	-0.14
Q8Y4U2	lmo2340	Pseudouridine-5'-phosphate glycosidase	Secondary metabolites biosynthesis, transport, and catabolism	0.09	-0.08	-0.77	-0.21	-0.17	-1.28	-0.93
Q8Y4S2	lmo2360	Transmembrane protein	Defense mechanisms	0.13	0.58	0.61	0.29	**2.06**	**2.52**	1.58
Q8Y4Q8	lmo2376	Peptidyl-prolyl cis-trans isomerase	Post-translational modification, protein turnover, and chaperones	0.12	0.09	-0.05	-0.19	-0.15	-0.12	-1.64
Q8Y4Q0	lmo2385	4HBT domain-containing protein	Secondary metabolites biosynthesis, transport, and catabolism	0.42	0.14	0.37	-0.17	-7.42	-4.78	-4.58
Q8Y4P6	lmo2389	Pyr_redox_2 domain-containing protein	Energy production and conversion	0.10	-0.07	-0.44	0.10	-0.25	-1.41	-1.41
Q8Y4N8	lmo2397	NifU domain-containing protein	Post-translational modification, protein turnover, and chaperones	0.09	-0.31	2.67	2.50	0.00	0.00	**7.70**
Q928M6	lmo2411	Lmo2411 protein	Post-translational modification, protein turnover, and chaperones	-0.04	-0.16	-0.83	-0.06	-0.12	-1.02	-0.84
Q8Y4M4	lmo2413	Cysteine desulfurase	Amino acid transport and metabolism	0.06	0.11	-0.21	-0.39	-0.32	-0.53	-1.91
Q8Y4M3	lmo2414	Lmo2414 protein	Post-translational modification, protein turnover, and chaperones	0.05	-0.09	-0.43	-0.25	-0.32	-1.20	-1.36
Q8Y4M2	lmo2415	ABC transporter domain-containing protein	Post-translational modification, protein turnover, and chaperones	0.14	0.25	-0.16	-0.52	-0.75	-1.17	-1.65
Q8Y4L2	lmo2425	Glycine cleavage system H protein	Amino acid transport and metabolism	0.14	-0.02	-0.66	0.02	-0.24	-1.64	-1.75
Q8Y4L1	lmo2426	Lmo2426 protein	Inorganic ion transport and metabolism	-0.05	-0.08	-0.41	-0.36	-0.10	-0.50	-1.51
Q8Y4K6	lmo2432	Lmo2432 protein	Function unknown	1.66	0.71	-3.01	-3.57	-7.88	-7.88	-6.00
Q8Y4K2	lmo2436	Lmo2436 protein	Transcription	0.15	-0.17	-0.96	0.39	0.24	-8.31	0.16
Q8Y4I3	lmo2457	Triosephosphate isomerase 1	Carbohydrate transport and metabolism	-0.02	-0.04	0.96	0.14	0.34	**1.21**	**1.15**
Q8Y4I2	lmo2458	Phosphoglycerate kinase	Nucleotide transport and metabolism	-0.06	-0.03	0.91	-0.25	0.08	**1.16**	**1.15**
Q8Y4I0	lmo2460	Sugar-bind domain-containing protein	Transcription	0.09	0.04	0.76	-0.16	0.24	**1.32**	**1.52**
Q8Y4H1	lmo2471	NADPH dehydrogenase	Energy production and conversion	0.19	0.08	-0.53	0.22	0.09	-0.37	-1.50

Continued on the next page

Table continued.

uniprot	lmo-code	protein name	COG description	ref 0h10	ref 0h30	ref 2h	stress 0h30	stress 2h	stress 4h	stress 7h
O32823	lmo2478	Thioredoxin reductase	Energy production and conversion	0.03	-0.03	-0.70	-0.03	-0.17	-0.85	-1.03
Q8Y4D2	lmo2518	LytR_cpsA_psr domain-containing protein	Transcription	0.04	0.13	0.02	-0.18	0.75	0.86	**2.04**
Q8Y486	lmo2569	SBP_bac_5 domain-containing protein	Amino acid transport and metabolism	-0.02	-0.01	-0.60	0.00	0.07	-0.95	-1.08
P66290	lmo2609	50S ribosomal protein L36	Translation, ribosomal structure and biogenesis	0.16	0.87	1.00	-0.61	-0.89	-10.74	-0.24
Q8Y449	lmo2611	Adenylate kinase	Nucleotide transport and metabolism	-0.19	0.08	0.36	-0.05	0.20	**1.03**	-0.42
Q8Y420	lmo2657	Deoxyguanosinetriphosphate triphosphohydrolase-like protein	Nucleotide transport and metabolism	0.15	-0.30	-0.11	-0.20	0.06	-1.00	-0.26
Q8Y407	lmo2671	Lmo2671 protein	Amino acid transport and metabolism	0.28	0.70	-0.36	0.07	-0.29	-8.01	0.12
Q8Y405	lmo2673	Lmo2673 protein	Signal transduction mechanisms	0.08	0.00	-0.75	-0.06	0.34	-0.73	-1.22
Q8Y3Z5	lmo2683	PTS EIIB type-3 domain-containing protein	Carbohydrate transport and metabolism	-0.24	-0.10	-0.90	0.02	-0.81	-0.15	**1.71**
Q8Y3U2	lmo2739	NAD-dependent protein deacetylase	Transcription	-2.25	0.45	2.55	-2.58	-2.19	-7.00	-4.63
Q8Y3T8	lmo2743	Probable transaldolase 1	Carbohydrate transport and metabolism	0.12	0.06	-0.42	-0.29	-0.11	-0.78	-1.12
Q8Y3T0	lmo2751	Lmo2751 protein	Defense mechanisms	0.05	0.06	0.44	-0.32	0.12	0.64	**1.85**
Q8Y3S6	lmo2755	PepX_C domain-containing protein	Defense mechanisms	-0.14	-0.21	0.06	-0.49	-0.62	-1.54	-1.13
Q8Y3S1	lmo2761	Lmo2761 protein	Carbohydrate transport and metabolism	-0.15	-0.43	-0.87	-0.17	-0.53	-0.83	-1.15
Q8Y3S0	lmo2762	PTS EIIB type-3 domain-containing protein	Carbohydrate transport and metabolism	-0.11	-0.26	-0.68	0.61	-5.28	-5.49	-8.04
Q8Y3R6	lmo2767	Lmo2767 protein	Function unknown	0.15	-0.13	0.25	0.28	0.71	0.12	**1.54**
Q926W7	lmo2791	Partition protein, ParA homolog	Cell cycle control, cell division, chromosome partitioning	0.26	0.20	0.79	-0.20	0.13	0.60	**1.26**
Q8Y3P3	lmo2792	HTH cro/CI-type domain-containing protein	Transcription	0.17	-0.09	-0.66	-0.07	-0.51	-1.16	-0.30
Q8Y3P1	lmo2794	Nucleoid occlusion protein	Transcription	0.10	0.05	0.49	0.02	0.22	0.52	**1.02**
Q8Y3N4	lmo2801	Putative N-acetylmannosamine-6-phosphate 2-epimerase	Carbohydrate transport and metabolism	-0.01	-0.20	-0.47	1.14	-1.49	-4.58	**2.48**
Q8Y3M7	lmo2808	Lmo2808 protein	Function unknown	-0.02	-0.38	0.44	0.51	-5.51	-2.07	-8.68
Q8Y3M3	lmo2812	Peptidase_S11 domain-containing protein	Cell wall/membrane/envelope biogenesis	0.00	0.00	0.00	0.00	0.00	2.89	**7.51**
Q8Y3K6	lmo2829	Nitroreductase domain-containing protein	Function unknown	0.52	0.45	-0.20	0.60	0.72	0.38	-1.31

Table S4: KEGG pathway enrichment analysis. Proteins that are part of KEGG pathways are clustered and statistical tests were done to see if such pathways are significantly differentially expressed. All significant pathways have a *p*-value < 0.05 and the NES (normalized enrichment score) measures how much a pathway is upregulated (positive NES) or downregulated (negative NES)

KEGG description	NES	*p*-value	sample
Galactose metabolism	1.57	$1.09 \cdot 10^{-2}$	Ref 0h10
DNA replication	-1.55	$2.30 \cdot 10^{-2}$	Ref 0h10
Ribosome	-1.50	$2.32 \cdot 10^{-2}$	Ref 0h10
Galactose metabolism	1.51	$3.84 \cdot 10^{-2}$	Ref 0h30
Bacterial chemotaxis	-1.79	$8.64 \cdot 10^{-4}$	Ref 2h
Phosphotransferase system (PTS)	-1.75	$2.78 \cdot 10^{-3}$	Ref 2h
Purine metabolism	1.66	$4.04 \cdot 10^{-3}$	Ref 2h
Flagellar assembly	-1.64	$2.16 \cdot 10^{-2}$	Ref 2h
Fatty acid biosynthesis	-1.76	$3.78 \cdot 10^{-3}$	Stress 2h
Fatty acid metabolism	-1.67	$1.12 \cdot 10^{-2}$	Stress 2h
Pyruvate metabolism	-1.56	$1.88 \cdot 10^{-2}$	Stress 2h
Glycerolipid metabolism	1.46	$4.29 \cdot 10^{-2}$	Stress 2h
Purine metabolism	1.77	$1.32 \cdot 10^{-3}$	Stress 4h
Phosphotransferase system (PTS)	-1.59	$3.01 \cdot 10^{-3}$	Stress 4h
Peptidoglycan biosynthesis	1.74	$3.39 \cdot 10^{-3}$	Stress 4h
Flagellar assembly	-1.50	$1.86 \cdot 10^{-2}$	Stress 4h
Pyrimidine metabolism	1.49	$2.89 \cdot 10^{-2}$	Stress 4h
Bacterial chemotaxis	-1.46	$4.53 \cdot 10^{-2}$	Stress 4h
Purine metabolism	1.78	$5.60 \cdot 10^{-4}$	Stress 7h
Flagellar assembly	-1.76	$7.81 \cdot 10^{-4}$	Stress 7h
Bacterial chemotaxis	-1.70	$2.06 \cdot 10^{-3}$	Stress 7h

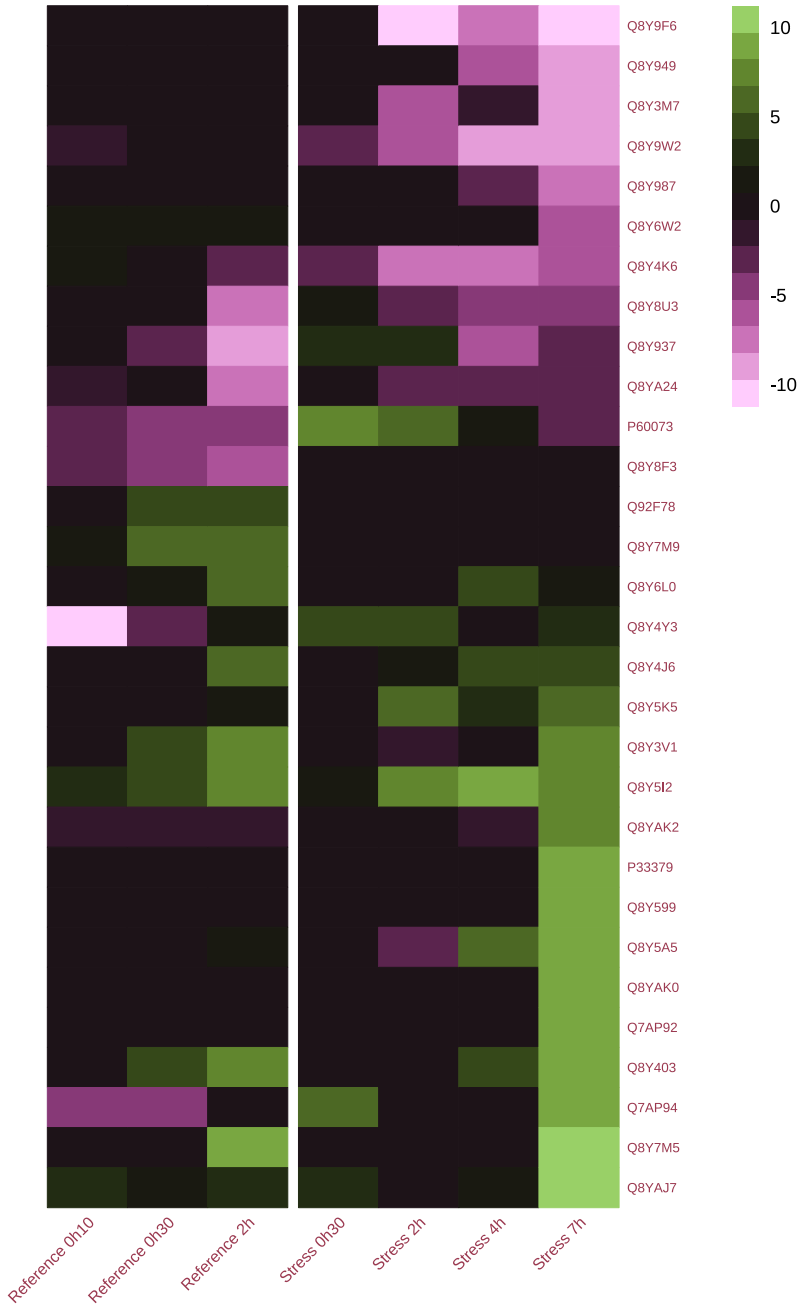

Figure S2: Heatmap of uncharacterized proteins significantly upregulated in reference cells and heat stressed cells with at least an absolute $\log_2$ fold change of four or higher. The colours denote the $\log_2$ fold change of the protein at different timepoints.

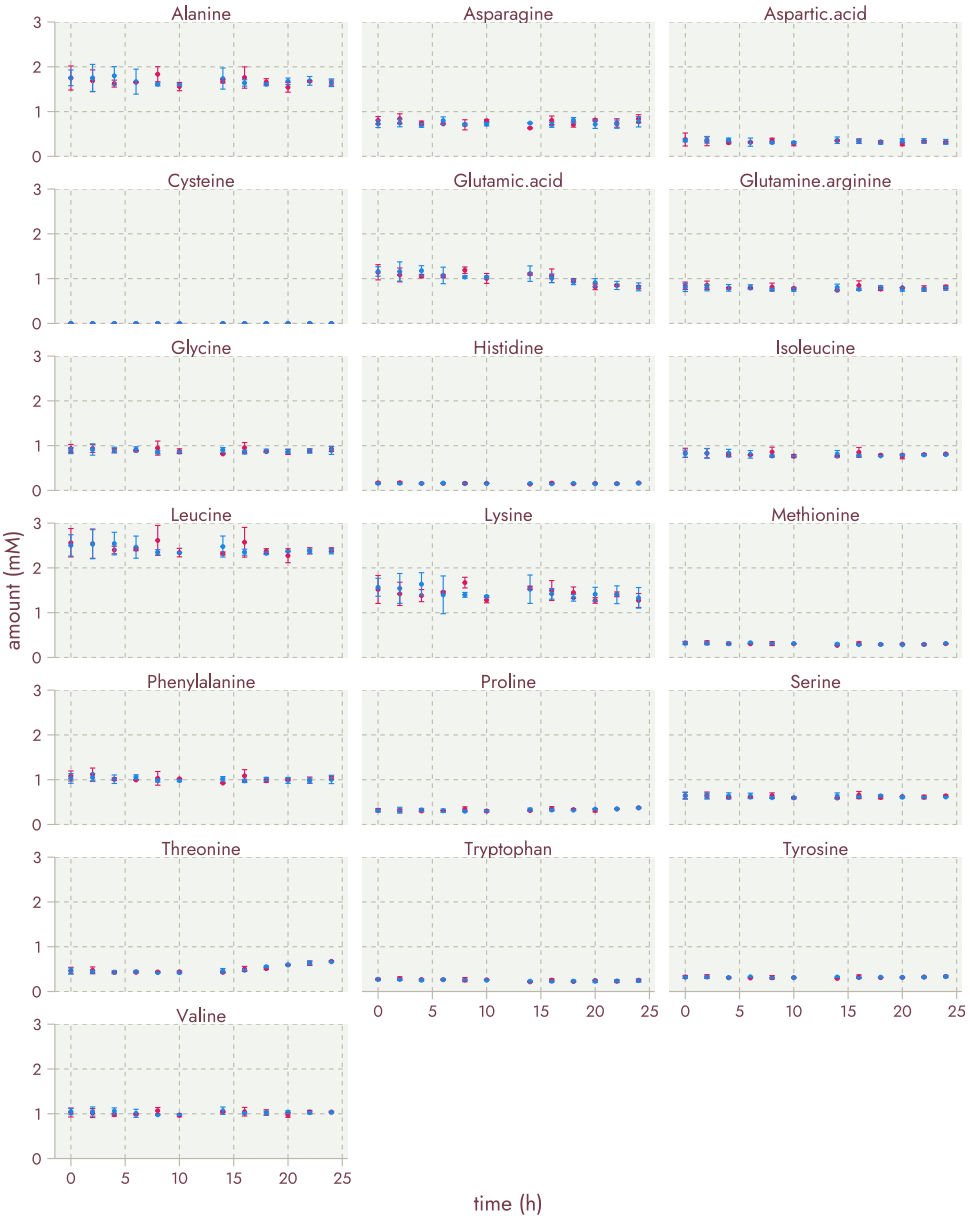

Figure S3: Utilization of organic acids during a 24 h enrichment in HFB. At 2-h intervals samples were taken and analysed by HPLC. The concentration in mM is given for the compounds that were measured using the column setup. Blue dots show the concentration for reference cells, and in red the concentration during enrichment of heat stressed cells.

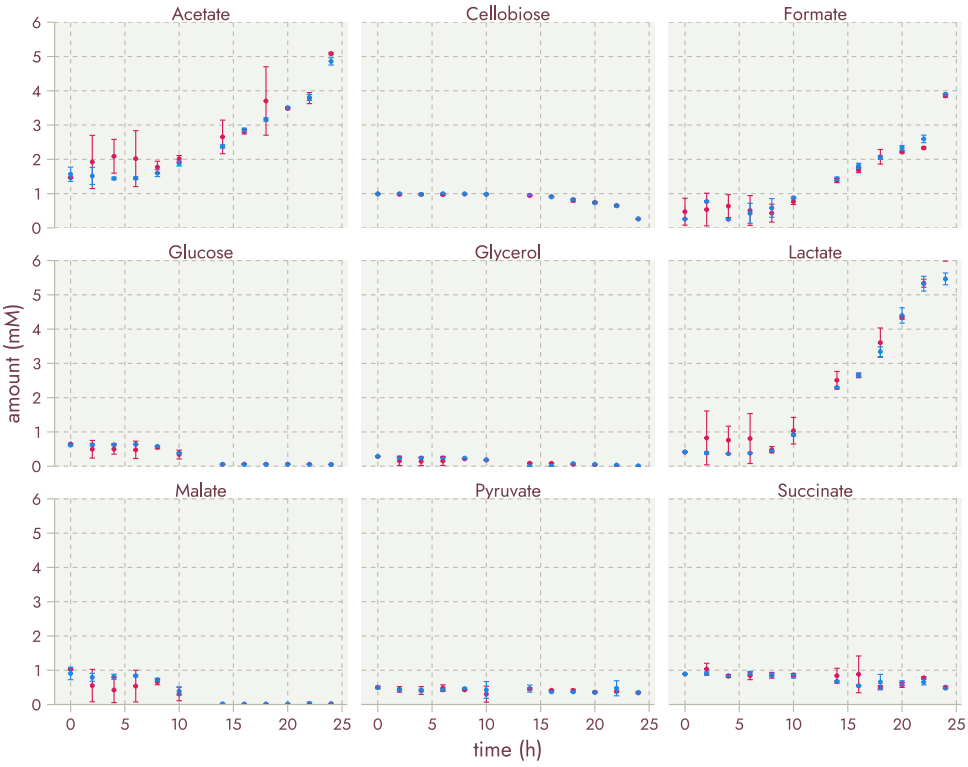

Figure S4: Utilization of amino acids during a 24 h enrichment in HFB. At 2-h intervals samples were taken and analysed by HPLC. The concentration in mM is given for the amino acids that were measured using the column setup. Blue dots show the concentration for reference cells, and in red the concentration during enrichment of heat stressed cells.

Table S5: Effect of metal supplementation on the time-to-reach an optical density threshold. The addition of cobalt, copper, and zinc on the growth of *L. monocytogenes* in HFB was measured by optical density, where the mean TTR is the average outgrowth in this condition. The mean TTR is given with the standard deviation

history	compound	concentration (µM)	mean TTR	sd TTR
reference	none	0	20.7	0.48
reference	cobalt	10	21.6	0.28
reference	cobalt	250	25.4	0.51
reference	cobalt	1000	> 48.0	0.00
reference	copper	10	21.7	0.54
reference	copper	250	22.3	0.50
reference	copper	1000	24.0	0.14
reference	zinc	10	20.1	0.80
reference	zinc	100	20.4	0.99
reference	zinc	250	21.3	0.55
reference	zinc	500	22.6	0.76
reference	zinc	1000	24.1	0.54
heat stress	none	0	22.8	0.28
heat stress	cobalt	10	24.2	0.29
heat stress	cobalt	250	26.9	0.13
heat stress	cobalt	1000	> 48.0	0.00
heat stress	copper	10	23.9	0.43
heat stress	copper	250	24.6	0.30
heat stress	copper	1000	26.6	0.19
heat stress	zinc	10	21.8	0.57
heat stress	zinc	100	22.2	0.70
heat stress	zinc	250	23.9	0.53
heat stress	zinc	500	24.5	0.34
heat stress	zinc	1000	26.0	0.37

Table S6: Effect of supplementation of HFB with spent medium (0 %, 25 % and 10 %) on the lag duration and maximum specific growth rate as fitted with the Baranyi model

history	medium	lag (h)	std. error lag (h)	growth rate (h^{-1})	std. error growth rate (h^{-1})
reference	HFB	2.80	0.52	0.75	0.03
reference	HFB 75 %	2.55	0.37	0.79	0.02
reference	HFB 90 %	2.86	0.52	0.78	0.03
heat stress	HFB	9.98	0.56	0.86	0.08
heat stress	HFB 75 %	9.42	0.72	0.94	0.10
heat stress	HFB 90 %	9.68	0.91	0.92	0.12

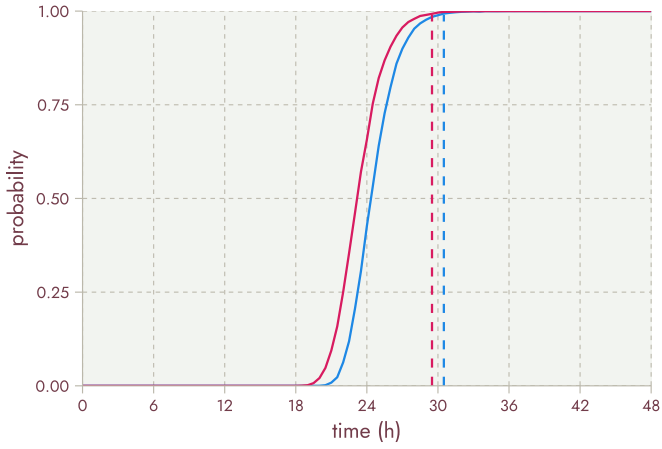

Figure S5: Monte Carlo simulations to estimate the effect of addition of 25 % spent medium on the chance to reach the detection threshold of 2 $\log_{10}$ CFU/ml when starting with 1 cell in 250 ml enrichment broth. After 10,000 simulations, the probability of 0.99 is reached for normal HFB after 30.5 h (blue line) and for the addition of 25 % spent medium after 29.5 h (red line).

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5

Combined enrichment with real-time PCR allows detection of *Listeria monocytogenes* in food samples

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Abstract

Timely detection of *Listeria monocytogenes* from food products is important to verify measures that control this food pathogen. In this study, the effect of competing microbiota on the outgrowth of *L. monocytogenes* during enrichment in half Fraser Broth (HFB) was investigated. This enrichment step was combined with detection by real-time quantitative PCR (qPCR) as alternative procedure to the ISO 11290-1 culture-based detection. For this, competitors were isolated from ALOA agar during routine enrichments by the Dutch Food Safety Authority. Competitors that were able to grow well in HFB had similar growth kinetics to *L. monocytogenes* during enrichment with and without heat stress history, indicating that these competitors are unlikely to have a significant growth advantage in HFB. Furthermore, it was verified whether *L. monocytogenes* could be detected in food samples with artificially high levels of competitive microbiota using enrichment and qPCR based detection. For this, smoked salmon and cured ham in HFB were spiked with 1 $\log_{10}$ CFU/ml of *L. monocytogenes* and a 1,000-fold excess of competitors and enriched in HFB for 24 h. In these enrichments, it was shown that detection of *L. monocytogenes* straight from enrichment broth with the GENE-UP® qPCR was successful in most samples. Complementary simulations demonstrated that starting with one cell of *L. monocytogenes* per enrichment, the qPCR threshold was reached within 48 h. This might pave the way for an alternative and faster enrichment protocol for *L. monocytogenes* whereby a single enrichment step is combined with qPCR detection, hereby drastically shortening the processing time for food samples from up to 7 days to about 2 days.

5.1. Introduction

Listeria monocytogenes is an important foodborne pathogen, and invasive infection with this bacterium had the highest hospitalization rate of all zoonoses in the European Union in 2020 (European Food Safety Authority, 2021). Timely detection of this pathogen in food products is crucial to verify compliance with European regulation. Legislation states that absence of *L. monocytogenes* must be guaranteed in 5 samples of 25 g for ready-to-eat food products that are able to support its growth (European Commission, 2007). Because of the long and labour-intensive nature (4-7 days) of the current gold standard detection method, the ISO 11290-1 (International Organization for Standardization, 2017), there is demand for a faster detection procedure. Various rapid alternative methods are already available and even commercialized for the detection of *L. monocytogenes* (Osek et al., 2022). However, the molecular methods lack the necessary sensitivity to directly detect one or a few cells in 25 g of food without concentration steps and thus an enrichment step is necessary (Jantzen et al., 2006). A commonly used rapid method is real-time quantitative PCR (qPCR), where the presence of target DNA (in the case of *L. monocytogenes* often using virulence genes) is coupled to fluorescent probes to measure the amplification of DNA in real time. This method has high sensitivity and specificity, is rapid and reproducible, and allows for high-throughput analysis. Using qPCR for the detection of *L. monocytogenes* has been demonstrated on many occasions as a good candidate for rapid detection (Cady et al., 2005; Garrido-Maestu et al., 2014; Jantzen et al., 2006; Kačániová et al., 2015; Law et al., 2015), but the effect of competing microbiota on the detectability with qPCR has not been evaluated. Additionally, reaching the necessary cell concentration threshold for efficient detection with qPCR, is also partly affected by the presence of background microbiota in food (Zilelidou & Skandamis, 2018; Zitz et al., 2011). Competition for resources during enrichment can become significant, especially when background microbiota are present in higher concentrations than *L. monocytogenes*. Growth suppression of food microbiota is mainly accomplished in culture-based enrichment by selective components that are added to the enrichment broth (International Organization for Standardization, 2017). This has the downside that stressed or injured *L. monocytogenes* cells can be inhibited by the selective compounds that are added (Bannenberg et al., 2021a; Kalchayanand et al., 1992), and thereby have a longer lag phase before growth is initiated. Therefore, in this research the effect of stressed cells was also taken into account to incorporate the increased lag duration that such stress history can cause. The main aim of this chapter is thus

to measure the effect of background microbiota on the ability to detect *L. monocytogenes* from food samples by combining primary enrichment with qPCR detection.

5.2. Materials and methods

5.2.1. Isolation of competitive strains

The Dutch Food Safety Authority (NVWA) and Wageningen Food Safety Research (WFSR) isolated colonies from ALOA plates during routine analysis in July 2020 when following the ISO procedure 11290-1 for *L. monocytogenes* detection from food products. Non-presumptive colonies (other colonies than the typical blue-green colonies with opaque halo of presumptive *L. monocytogenes*) were taken from ALOA plates that were streaked after either primary enrichment in half Fraser broth (HFB, Fraser broth base (Oxoid) with half Fraser supplement (Oxoid)) or secondary enrichment in Fraser broth (FB, Fraser broth base (Oxoid) with full Fraser supplement (Oxoid)). Single colonies from ALOA were re-streaked on fresh non-selective Columbia blood agar (Oxoid) and incubated 24 h at 37 °C, after which overnight cultures from a single colony were made in brain heart infusion (BHI) broth (Becton Dickinson Difco). Strains were identified by MALDI Biotyper® (Bruker) and strains with a MALDI-score higher than 2.0 were considered correctly identified at species level (Nagy et al., 2012) and were kept at -80 °C in BHI supplemented with 30 % glycerol (Fluka) for further use in mono-culture experiments and co-culture experiments with *L. monocytogenes*.

5.2.2. Bacterial strains and growth conditions

Competitor strains and *L. monocytogenes* strain EGDe were kept at -80 °C and strains were revived by streaking on BHI agar plate (BHI supplemented with 1.5 % agar, Oxoid). Plates were incubated for 24 h at 30 °C, after which a single colony was inoculated in 10 ml of BHI broth. Cultures were grown statically at 30 °C for 16 h to obtain stationary phase cultures. Theses cultures were subsequently diluted 1:1,000 in fresh BHI broth and incubated at 30 °C for 16 h to obtain a standardized working culture for use in further experiments.

5.2.3. Stress treatment of cells

Short heat treatments were applied for stress conditions. Working cultures were diluted 1:100 in 50 ml BHI broth pre-heated at 60 °C in a water bath (Julabo SW23) to obtain approximately one $\log_{10}$ CFU/ml reduction, after which the cultures were quickly cooled on ice for 15 s to reach room temperature. Heat-stressed cultures were

decimally diluted in peptone physiological salt (PPS) solution (Tritium Microbiology) and plated on BHI agar to quantify the reduction due to the heat treatment. The D_{60}-values were determined for each competitor using the protocol described by Aryani et al. (2015). Here, the inactivation curve of working cultures was determined by determining the concentration after different timepoints of exposure to 60 °C and fitting the inactivation curve with the Weibull model.

5.2.4. Measuring outgrowth in BHI and HFB with optical density

Heat-treated cells and reference cells (without prior stress treatment) of the competitor strains were decimally diluted in PPS to a concentration of 4 $\log_{10}$ CFU/ml before being diluted 1:100 in 300 µl of either BHI or HFB in 96-wells plates (starting concentration of 2 $\log_{10}$ CFU/ml). Inoculated wells were closed with parafilm to prevent evaporation, and outgrowth by optical density was measured in a SpectraMax 384 plus (Molecular Devices) set at 30 °C (temperature used for enrichment in HFB as stated in ISO 11290-1) and a wavelength of 600 nm. Optical density measurements were taken every 5 min for 48 h with 15 s shaking before each measurement. The time-to-reach (TTR) was quantified as the time to reach an OD_{600} increase of 0.25. This threshold of 0.25 was chosen because at higher optical densities the HFB will blacken due to the esculin hydrolysis reaction which will cause a rapid increase in optical density. The mean TTR and its standard deviation was calculated from biologically independent triplicate experiments done on different days.

5.2.5. Testing for antimicrobial inhibition

The bactericidal effect of the competitors was tested with the method described by Touré et al. (2003). For this, working cultures of competitor strains were spot-plated (10 µl) on BHI agar and HFB agar plates (HFB supplemented with 1.5 % agar) and grown overnight at 37 °C. After incubation, a top layer of 15 ml agar (sterile water with 1.5 % agar) was poured that was inoculated with a working culture of *L. monocytogenes* strain EGDe with a final concentration in the top layer of around 8 $\log_{10}$ CFU/ml. After pouring the top layer, plates were further incubated for 24 h at 30 °C. Possible inhibition of *L. monocytogenes* by a competitive strain was evaluated by assessing whether there was an inhibition zone around the spot-plated competitor strain.

5.2.6. Growth kinetics in half Fraser broth enrichments

The working cultures of the individual strains (*L. monocytogenes* strain EGDe, one *Micrococcus luteus* strain, one *Corynebacterium testodinoris* strain, and two strains of *Listeria innocua*) were decimally diluted in PPS until a concentration of approximately 4 $\log_{10}$ CFU/ml, and then diluted 1:100 in 50 ml HFB in 150 ml Scott flasks resulting in an initial inoculum concentration of 2 $\log_{10}$ CFU/ml. For the stressed cells, after one $\log_{10}$ CFU/ml reduction at 60 °C, samples were subsequently decimally diluted in PPS also until a final concentration of 2 $\log_{10}$ CFU/ml in HFB. The initial sample point was taken directly after addition of the cells to the enrichment broth (timepoint 0), and samples of reference cells and heat-stressed cells were taken at 2 h intervals for 10 h and at 24 h to investigate kinetics at 30 °C. Samples were spread-plated on BHI agar plates and incubated at 30 °C for 24 h before counting. In order to measure the concentration at time-points 14 and 16 h, a parallel enrichment was started later in the day and samples were taken the next morning. Three independent biological reproductions were carried out for reference cells and heat-stressed cells, and experiments took place on different days. The kinetic parameters lag duration (h) and maximum specific growth-rate (h^{-1}) were estimated by fitting the Baranyi model (Baranyi & Roberts, 1994) to each reproduction, and the mean and standard deviation of the parameters were determined.

Baranyi model:

$$log_{10}N(t) = log_{10}N_0 + \frac{\mu}{ln(10)} \cdot A(t) - \frac{1}{ln(10)} \cdot ln\left[1 + \frac{exp[\mu \cdot A(t)] - 1}{10^{[log_{10}N_{max} - log_{10}N_0]}}\right] \quad (5.1)$$

$$\text{With } A(t) = t + \frac{1}{\mu} \cdot ln\left[exp(-\mu \cdot t) + exp(-\mu \cdot t_{lag}) - exp(-\mu \cdot t - \mu \cdot t_{lag})\right] \quad (5.2)$$

With $log_{10}N(t)$ the cell concentration at time t ($\log_{10}$ CFU/ml), $log_{10}N_0$ the initial cell concentration ($\log_{10}$ CFU/ml), $log_{10}N_{max}$ the maximum cell concentration ($\log_{10}$ CFU/ml), t the elapsed time (h), λ the lag phase duration (h), t_{lag} the time when the lag phase ends (h), and μ the maximum specific growth rate (h^{-1}).

5.2.7. Spiking of food products

Smoked salmon and cured (deli) ham were chosen as model ready-to-eat food products that are risk products for *L. monocytogenes* (European Food Safety Authority, 2021), and the products were purchased at a local supermarket. Before use in experiments, 25 gram of food products were first tested with the ISO 11290-1 method to

verify absence of *L. monocytogenes* in these test samples. Reference cells and heat-stressed cells were cultured as in subsection 5.2.2 and subsection 5.2.3 where strain EGDe was used as the *L. monocytogenes* reference strain and the competitor cocktail consisted of strains *Listeria innocua*, *Listeria welshimeri*, *Corynebacterium testodinoris*, and *Micrococcus luteus*. These strains were selected as competitors because their outgrowth was comparable to *L. monocytogenes* strain EGDe as measured by optical density (subsection 5.2.4). Enrichment was done according to ISO 11290-1. For that, 25 g of food product was added to sterile stomacher bags (Antonides, Netherlands) and the food product was spiked with a maximum of 250 µl of culture such that the inoculum concentration of *L. monocytogenes* in the final enrichment broth was at 1 $\log_{10}$ CFU/ml, and the competitor cocktail was at 4 $\log_{10}$ CFU/ml (2500 CFU/ml of each strain). Afterwards, 225 ml of either HFB or BHI medium was added to the bag and the contents were blended at 260 rpm for 1.5 min with a Stomacher® 400 Circulator (Steward, United Kingdom). Before the start of enrichment, samples were taken and diluted to verify the starting concentrations. The enrichments were incubated for 24 h at 30 °C after which samples were plated on ALOA and used for qPCR analyses.

5.2.8. Detection with GENE-UP®

qPCR detection of the enriched food products was done with the GENE-UP® Thermocycler (bioMérieux, France) using the GENE-UP® *L. monocytogenes* 2 kit (bioMérieux, France) for the detection of *L. monocytogenes*. Cells were first lysed with the GENE-UP® Lysis kit (bioMérieux, France) according to the manufacturer's instructions, with the exception that 10 µl of sample was added to the lysis tubes instead of the recommended 20 µl, because preliminary experiments demonstrated that this reduction in volume improved the performance of the PCR reaction. The GENE-UP® Thermocycler was programmed according to manufacturer's instruction. The detection threshold of the qPCR in HFB was determined by enriching food smoked salmon and cured ham in HFB for 24 h, after which an overnight culture of *L. monocytogenes* EGDe was serially diluted in this enriched broth to obtain a dilution series for *L. monocytogenes* ranging from 8 $\log_{10}$ CFU/ml to 1 $\log_{10}$ CFU/ml. These samples were analysed with the GENE-UP® thermocycler and a calibration curve was made to estimate the minimum cell concentration of *L. monocytogenes* in enrichment broth that allows detection with qPCR.

5.2.9. Enrichment simulations using Monte Carlo analysis

To also estimate the chance that the qPCR detection threshold is reached during the 24 h of enrichment, kinetic growth data was used of 23 food-relevant strains of *L. monocytogenes* based on our previous work (Bannenberg et al., 2021a)(lag of 2.3 ± 0.3 h and maximum specific growth-rate of 0.78 ± 0.05 h^{-1} for reference cells, and a lag of 9.3 ± 1.0 h and a maximum specific growth-rate of 0.66 ± 0.07 h^{-1} for heat-stressed cells). Monte Carlo analysis was done with 100,000 enrichment simulations to estimate the probability reaching the qPCR detection limit in HFB when starting at various initial cell concentrations varying from -2.4 to 2 $\log_{10}$ CFU/ml, simulating contamination levels of 1 to 2.5 · 10^4 cells/25 g of food that is diluted 1:10 in HFB at the start of the enrichment. All data analysis was done in R (R Core Team, 2021).

5.3. Results and discussion

5.3.1. Collecting potential competitors during enrichment

A total of 80 strains were collected from atypical *L. monocytogenes* colonies on ALOA plates during enrichment. Of these strains, almost 50 % of the strains were isolated from ALOA plates after primary enrichment in HFB and the remaining 50 % after secondary enrichment in FB (table 1). As expected, other *Listeriaceae* strains were isolated, namely *L. innocua* and *L. welshimeri* strains. Interestingly, also three *L. monocytogenes* strains were isolated from bluish colonies that did not demonstrate an opaque halo-formation that is necessary for a colony to be presumptive *L. monocytogenes* (International Organization for Standardization, 2017). According to a note in the ISO 11290-1 protocol, some rare *L. monocytogenes* strains can have slow phosphatidyl inositol phospholipase C (PIPC) activity where halo-formation is only seen when ALOA-incubation is extended to four days or more (Leclercq, 2004). As three false-negative atypical *L. monocytogenes* colonies (3.7 %) were observed, this can suggest that the rarity of strains with a slow PIPC-activity might need to be re-evaluated, especially because these strains could still be pathogenic (Leclercq, 2004).

Eight different species of *Staphylococcus* were isolated and represented 75 % of the total number of strains isolated. As the majority of strains were *Staphylococcus*, this could indicate that the specific selectivity in enrichment is low for this genus. Indeed, especially *Staphylococcus sciuri*, *Staphylococcus saprophyticus*, and *Staphylococcus xylosus* have been shown to be able grow under the inhibitory properties of HFB, FB, and ALOA (Angelidis et al., 2015). Furthermore, some other genera can produce similar looking colonies on ALOA with blue turquoise colonies and even with haloes, such as *Bacilli*, and coryneform bacteria (Stessl et al., 2009). In table S1 the colony morphology of all isolated strains can be seen.

Table 1: Overview of the 82 strains isolated by NVWA/WFSR from ALOA plates during routine enrichment after enrichment in HFB or FB, and species identification by MALDI Biotyper®. Indicated is how many strains were collected after primary enrichment in HFB and from secondary enrichment in FB and how many percent of the overall collected competitors this consists of

species	number isolated from HFB	number isolated from FB	total number of strains	percentage of total strains
Bacillus licheniformis	0	1	1	1.2 %
Listeria innocua	2	1	3	3.7 %
Listeria monocytogenes	1	2	3	3.7 %
Listeria welshimeri	3	5	8	9.8 %
Corynebacterium testodinoris	1	0	1	1.2 %
Micrococcus luteus	1	0	1	1.2 %
Macrococcus luteus	1	0	1	1.2 %
Pseudomonas azotoformans	1	0	1	1.2 %
Pseudomonas extremorientalis	1	0	1	1.2 %
Staphylococcus aureus	3	3	6	7.3 %
Staphylococcus carnosus	1	2	3	3.7 %
Staphylococcus epidermidis	0	1	1	1.2 %
Staphylococcus equorum	0	2	2	2.4 %
Staphylococcus saprophyticus	15	13	28	34.1 %
Staphylococcus sciuri	7	4	11	13.4 %
Staphylococcus warneri	2	5	7	8.5 %
Staphylococcus xylosus	3	1	4	4.9 %
total	**42**	**40**	**82**	

5.3.2. Quantifying growth ability of all competitors

Competitors can interfere with the enrichment of *L. monocytogenes* by exhibiting faster growth kinetics and thereby masking *L. monocytogenes* colonies when enrichments are streaked on plates, and this makes selecting presumptive colonies on ALOA more difficult. To evaluate the growth performance of competitive strains in HFB, a representative subset of 16 strains including all species was further characterized by optical density measurements. To compare them to growth of *L. monocytogenes*, reference cells without stress history and heat-treated cells were enriched in HFB, but also non-selective brain heart infusion (BHI) was taken along as non-selective enrichment medium (figure S1).

In reference condition most competitors grew similarly to *L. monocytogenes* in non-selective BHI, with time-to-reach (TTR) of 16.3 ± 0.2 h for *L. monocytogenes* and 18.3 ± 4.8 h for competitors. However, when competitors were grown in the HFB enrichment medium, some competitors – especially *Staphylococci* – grew significantly slower than *L. monocytogenes*. Here, *L. monocytogenes* grew out in 17.7 ± 0.6 h and the competitors on average after 29.9 ± 10.4 h. There was large variation, as some competing strains like other *Listeriaceae*, *M. luteus*, *Macrococcus canis*, and *Bacillus*

licheniformis were able to grow rather similar to *L. monocytogenes* (22.0 ± 3.7 h). The high prevalence of *Staphylococci* found during enrichments could be explained because high contamination levels are common in food products (Crago et al., 2012), and because they are often found in similar ready-to-eat food products as *Listeriaceae* (Chajęcka-Wierzchowska et al., 2015). It is therefore likely that most *Staphylococci* that were found on ALOA plates are due to high contamination levels in the raw food products. A similar trend can be seen for heat-stressed cells, where especially *Staphylococci* and *Pseudomonas* did not reach an optical density increase within 48 h in HFB. This indicates that although these competitors were isolated from ALOA, their growth performance in HFB is severely hindered by the selective compounds.

The interference of the competitors by the production of antimicrobial compounds was also tested, however the competitors did not show inhibition zones, and they were most likely not able to produce antimicrobials against *L. monocytogenes* (data not shown). Previous research demonstrated that some strains of *Staphylococcus equorum* were able to produce the metabolite micrococcin PI, which was shown to reduce the growth of *L. monocytogenes* (Carnio et al., 2000), however, this could not be demonstrated in our assay with the tested strains.

5.3.3. Competitive strains have similar growth kinetics as *L. monocytogenes* in HFB

Strains that showed similar time-to-reach values compared to *L. monocytogenes* in the screening experiments in HFB were chosen for further characterization, namely *L. innocua*, *L. welshimeri*, *M. luteus*, and *C. testodinoris*. Strain *L. monocytogenes* EGDe was used as reference strain, because previous research that characterized the lag duration and the maximum specific growth-rate of 23 strains demonstrated that this strain showed average growth performance in HFB. The growth during primary enrichment (in HFB) of *L. monocytogenes* EGDe and the potential competitors is shown in figure 1. The growth of the competitors was measured for the competitors *L. innocua*, *L. welshimeri*, *C. testodinoris*, *M. luteus*. For mono-culture reference cells (figure 1a), kinetics are similar between competitors and *L. monocytogenes* with no significant growth advantage in HFB when taking the 95 % confidence interval into account. The average maximum specific growth-rate is 0.92 ± 0.2 h^{-1} for competitors and 0.76 ± 0.0 h^{-1} for *L. monocytogenes*. Furthermore, the lag duration is similar with competitors having a lag of 3.2 ± 1.0 h and for *L. monocytogenes* 2.8 ± 0.5 h. With these kinetic parameters, when starting at a concentration of 2 $\log_{10}$ CFU/ml the competitors as well as *L. monocytogenes* reach stationary phase within 24 h.

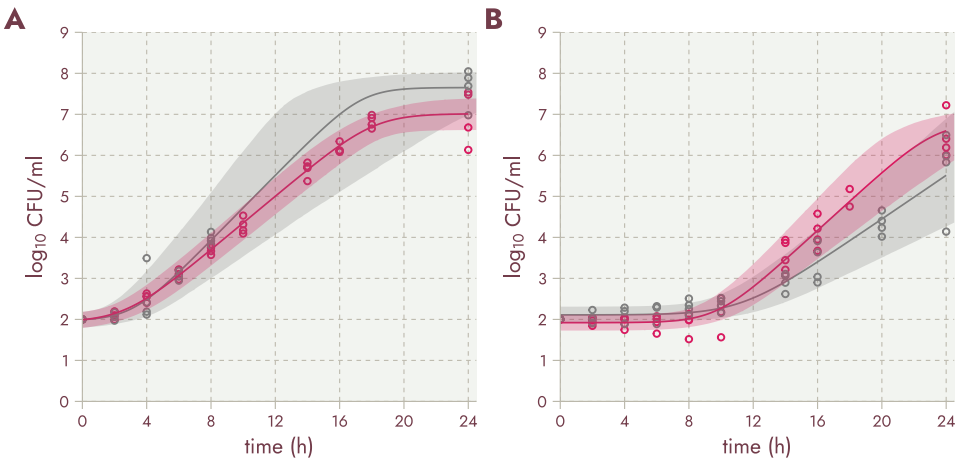

Figure 1: The outgrowth during primary enrichment in HFB for reference cells (A) and for heat-stressed cells (B). Kinetic parameters were determined for *L. monocytogenes* and its competing microbiota by fitting the Baranyi-model to the experimental data. The points are the different reproductions for *L. monocytogenes* and the tested competitor strains. The solid lines depict the average growth during enrichment for *L. monocytogenes* (red) and its competitors (grey). The shaded confidence-intervals were determined by simulating enrichments with the measured mean lag and maximum specific growth-rate and standard deviation, and taking the 95 % confidence-intervals based on 10,000 bootstraps.

When looking at the heat resistance of *L. monocytogenes* and competitors at 60 °C, the D_{60}-value of *C. testodinoris* (1.5 min) and *M. luteus* (1.3 min) are similar to *L. monocytogenes* (1.4 min). However, the heat resistance of *L. welshimeri* (3.7 min) and especially *L. innocua* (8.9 min) is significantly higher. Even so, we have shown before that the heat resistance (i.e. *D*-value) did not correlate with the subsequent lag duration upon enrichment (Bannenberg et al., 2021a). Also for the strains tested in the current study, the effect of heat stress treatment on the lag duration is rather similar for the tested strains, while the D_{60}-values are different (figure 1b), resulting in similar overall kinetics of the tested strains.

Most available studies focus on the interaction between *L. monocytogenes* and *L. innocua* during selective enrichment. A higher maximum specific growth-rate of *L. innocua* compared to *L. monocytogenes* as measured by Cornu et al. (2002) was not found in our tested conditions. Despite this, from our kinetic data it seems unlikely that the competitors are able to outcompete *L. monocytogenes* with faster growth kinetics, at least based on our set of competitive strains. Even when starting with 1 cell of *L. monocytogenes* and a 1,000-fold higher concentration of competitors, the detection threshold of 2 $\log_{10}$ CFU/ml that is necessary for successful transfer to the secondary enrichment step (Augustin et al., 2016) can still be reached for reference

and heat-stressed cells (figure 2). It thus seems likely that non-specific nutritional competition does not play a role in reducing the cell concentrations that *L. monocytogenes* needs to reach during enrichment for successful detection. Even so, competition is often not contributed to a single competitor, but to the interactions of multiple species. On the other hand, seeing as enrichments are done in the relatively defined environment of HFB, growth kinetics of competitors can give an insight in the competition during primary enrichment.

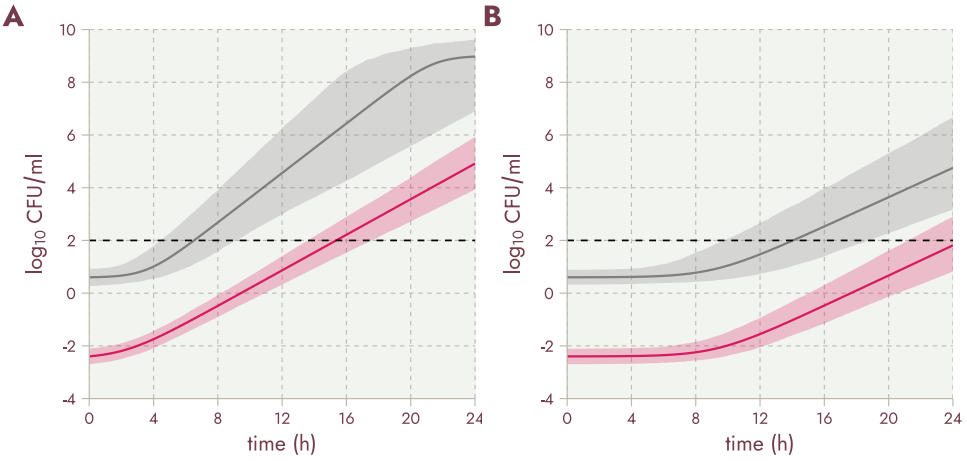

Figure 2: Simulation of the enrichment of *L. monocytogenes* (red) and competitor cocktail (grey) with *L. monocytogenes* starting enrichment at 1 cell in 250 ml enrichment broth, and competitors starting at 1,000-fold higher concentration. In (A) reference cells are simulated and in (B) heat-stressed cells. The detection threshold of 2 $\log_{10}$ CFU/ml can still be reached within 24 h.

5

5.3.4. qPCR detection of *L. monocytogenes*

In order to decrease the time necessary for the detection of *L. monocytogenes* from food products, alternative faster detection methods can be incorporated such as qPCR. To estimate the detection limit of the GENE-UP® qPCR method when contaminated food is enriched in HFB, cured ham and smoked salmon were enriched for 24 h according to ISO 11290-1, and subsequently supplemented with serially diluted *L. monocytogenes* culture. From the calibration curve (figure S2), the concentration that still could be detected in these food products within 35 cycles was 3-4 $\log_{10}$ CFU/ml. To evaluate the sensitivity of the assay, a competitive strain cocktail (containing the four competitors tested in subsection 5.3.3) was inoculated 1,000-fold higher than *L. monocytogenes* in HFB. After 24 h of enrichment, a positive detection outcome for *L. monocytogenes* was obtained (data not shown), and this suggested that even when

L. monocytogenes would be the minority in enrichment broth, detection with qPCR is possible.

The ability to still detect *L. monocytogenes* with a 1,000-fold higher concentration of competitors was validated in food samples. In an effort to simulate these conditions, cured ham and smoked salmon were spiked with 1 $\log_{10}$ CFU/ml of *L. monocytogenes* strain EGDe and 4 $\log_{10}$ CFU/ml of the cocktail consisting of *L. innocua*, *L. welshimeri*, *C. testodinoris*, and *M. luteus*. The spiked food products were enriched according to the ISO 11290-1 protocol for 24 h and then analysed with the GENE-UP® (table 2). When cultures were afterwards plated on ALOA in accordance with the culture-based detection method, almost all plates were completely overgrown by the competitors and halo-formation of *L. monocytogenes* was therefore impossible to see. In contrast, with qPCR, detection was possible for both reference *L. monocytogenes* cells as well as heat stressed cells. Namely, positive identification was obtained in all but one enrichment in cured ham, and all but two enrichments in smoked salmon. In these three cases, an inconclusive results was obtained where both the pathogen-specific probe and the internal amplification control did not reach the set threshold value. Earlier, reduced detectability was reported using automated enzyme-linked fluorescence when co-culturing *L. monocytogenes* with *L. innocua* at very low concentrations (Zitz et al., 2011). But from our combined enrichment with qPCR, we were able to detect the presence of *L. monocytogenes* in most food products even with an excess of *L. innocua*.

Because of earlier evidence that HFB might be too selective to adequately resuscitate damaged cells (Bannenberg et al., 2021a,b), enrichments were also done in non-selective BHI (table S2). Here all smoked salmon enrichments showed the expected positive outcome, but in cured ham in only half of the enrichments. This could indicate that competition in non-selective media such as BHI can play a larger role, because competitors might be able to outcompete *L. monocytogenes* in certain food products and thereby preventing from reaching the necessary pathogen detection threshold. This highlights that there needs to be a balance between reduced selectivity to allow the optimal recovery of damaged cells and thereby shortening their lag duration, but on the other hand enough selectivity to prevent complete overgrowth of competitive microbiota. When using qPCR, the relative abundance of *L. monocytogenes* to the total microbiota is less of a problem as long as the detection threshold is reached during the enrichment. This is underlined by the chance of false-negative detection outcomes when the concentration of competitors is much higher than *L. monocytogenes* during culture-based detection with the current ISO.

Table 2: Smoked salmon and cured ham were spiked with a final concentration of 1 $\log_{10}$ CFU/ml in the enrichment broth of *L. monocytogenes* strain EGDe and 1,000-fold higher concentration of competitor cocktail and enriched in HFB for 24 h before being analysed with the bioMérieux GENE-UP® qPCR. 'Presence' indicates a successful detection of *L. monocytogenes* by the GENE-UP® and 'inhibition' means that either one or both the *L. monocytogenes*-specific probe or the internal amplification control did not give a conclusive result

product	history	result GENE-UP
ham	reference	presence
ham	reference	presence
ham	reference	inhibition
ham	reference	presence
ham	heat stress	presence
ham	heat stress	presence
ham	heat stress	presence
ham	heat stress	presence
salmon	reference	presence
salmon	reference	presence
salmon	reference	presence
salmon	reference	inhibition
salmon	heat stress	presence
salmon	heat stress	presence
salmon	heat stress	presence
salmon	heat stress	inhibition

5.3.5. Proposed alternative enrichment method

The qPCR detection threshold can be used to estimate the chance that *L. monocytogenes* could be detected during enrichment. To assess the effect of competitors on successfully reaching the qPCR threshold, the growth kinetics were used to simulate enrichments with one cell/ml of *L. monocytogenes* and 1,000-fold higher concentrations of competitors (figure S3). In reference as well as heat-stressed cells, *L. monocytogenes* reaches the qPCR detection threshold within 24 h at this initial level, even when Jameson interaction is taken into account. The Jameson effect is a simple interaction model which states that when one microorganism reaches stationary phase, the growth of competing microorganisms is halted (Jameson, 1962). To estimate the effect of lower starting concentration of *L. monocytogenes*, Monte Carlo simulations were done based on experimental growth data (figure 3). Without any stress history, the detection threshold is reached for all inoculum concentration in 24 +/- 2 h (data not shown). In contrast, it can be seen that for 60 °C heat-stressed cells starting at a

concentration of 1 log_{10} CFU/ml the qPCR detection threshold is reached after 26.5 h with 99 % probability. This corresponds with the fact that the spiked food samples with the same inoculum concentration were successfully detected. When food products are contaminated with lower levels of *L. monocytogenes*, the probability to reach the threshold in 24 h rapidly decreases to 72 %, 36 %, and 11 % when starting with 0, -1, and -2 log_{10} CFU/ml respectively. Even with a sensitive molecular method like qPCR, the enrichment medium must be able to facilitate recovery of potentially very low levels of *L. monocytogenes* contamination in food. This is the case for healthy reference cells, but depending on the stress history, that can be difficult to achieve in the 24 h of the primary enrichment step.

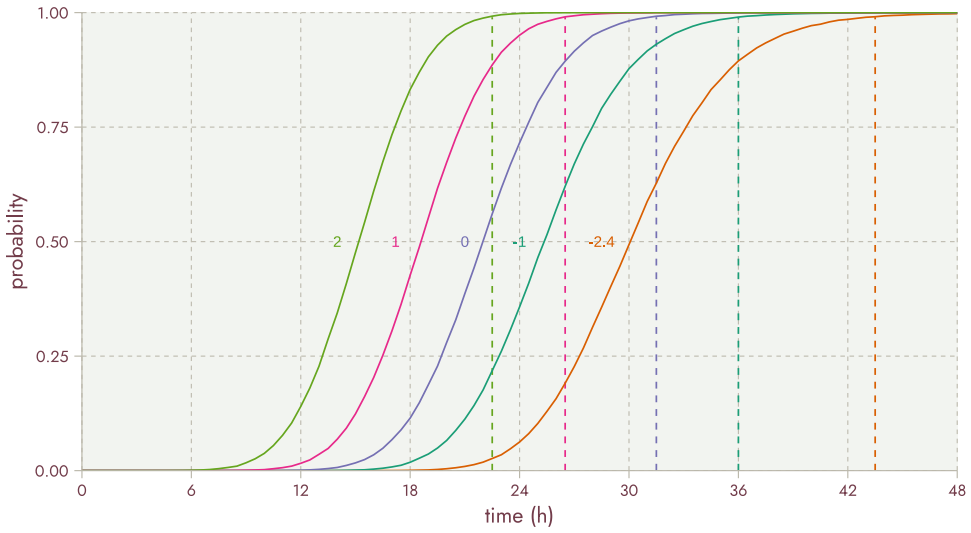

Figure 3: Monte-Carlo simulations were done to estimate when the qPCR-detection limit of 3.5 log_{10} CFU/ml was reached for 60 °C heat-stressed cells starting with different inoculum concentrations from -2.4 (1 cell in 250 ml enrichment) to 2 log_{10} CFU/ml (shown in text next to the lines). The dotted lines indicate at what time a 99 % probability is reached that allows for successful detection by qPCR. These times are 22.5, 26.5, 31.5, 36, and 43.5 h for the inoculum concentrations 2, 1, 0, -1, and -2.4 log_{10} CFU/ml respectively.

Current detection of *L. monocytogenes* using the ISO 11290-1 (figure 4a) consist of a double enrichment of which the primary step is done in HFB for 24-26 h, followed by secondary enrichment in Fraser broth (FB) for 24 h ± 2 h. After both enrichment steps, the culture is plated on ALOA and a secondary selective medium of choice for 24 h ± 2 h up to 48h ± 2 h depending on when presumptive colonies are present. Presumptive colonies are streaked on non-selective agar for 18-24 h after which confirmation tests are done to confirm the presence of *L. monocytogenes* (microscopy, beta-haemolysis, growth on L-rhamnose and lack of growth on D-xylose). A negative

result can be obtained after 4 days, while a positive confirmation is obtained after 5-7 days. However, there might be a bias in selecting presumptive colonies from ALOA, as there is selection for strains that grow well on ALOA. As mentioned before, some strains of *L. monocytogenes* need prolonged incubation before halo-formation is visible (Leclercq, 2004). Also, misinterpretation of the halo-formation of non *L. monocytogenes* strains is a possibility for some *Bacilli* (Serraino et al., 2011), or there could be complete overgrowth of the plate making detection impossible as well.

We evaluated an alternative protocol (figure 4b) after which a single enrichment step is followed by detection with qPCR (either automated with the GENE-UP® or manually with a qPCR machine). From the Monte Carlo simulations in figure 3 it can be concluded that for the recovery of stressed cells, a single enrichment step must be increased from 24 h to 43.5 h if a single cell of *L. monocytogenes* needs to be detected. For cells without damage a 24 h enrichment time should be sufficient, so optionally qPCR could be performed both after 24 h when stressed cells are not expected, and after 48 h of enrichment for slower recovering damaged cells.

A less selective enrichment medium is also preferred to allow optimal resuscitation of damaged cells, but this might also increase competition of microbiota. This therefore needs to be carefully balanced with the growth kinetics of competitors. Detection with qPCR will lead to faster detection that is not as influenced by the amount of competitive background microbiota because there is no culture-based differentiation necessary on ALOA plates. This modified enrichment could shorten the time until detection for negative samples by 2-3 days, given that negative samples with culture-based detection need 4 days (negative ALOA plate after secondary enrichment), while the alternative method needs only 26-50 h. For positive samples, the alternative method can even be 2-5 days shorter because it does not rely on the time-consuming plating and confirmation steps. For this however, there is need for more data on the ability of detection with qPCR in the presence of a wider range of food products, as the ability to effectively detect the presence of *L. monocytogenes* might differ due to the physicochemical composition of the food matrix. Also, the selectivity of enrichment broths needs to be assessed because there is a need for a medium that is selective enough to inhibit competitors to a certain extent, and support outgrowth of damaged *L. monocytogenes* cells.

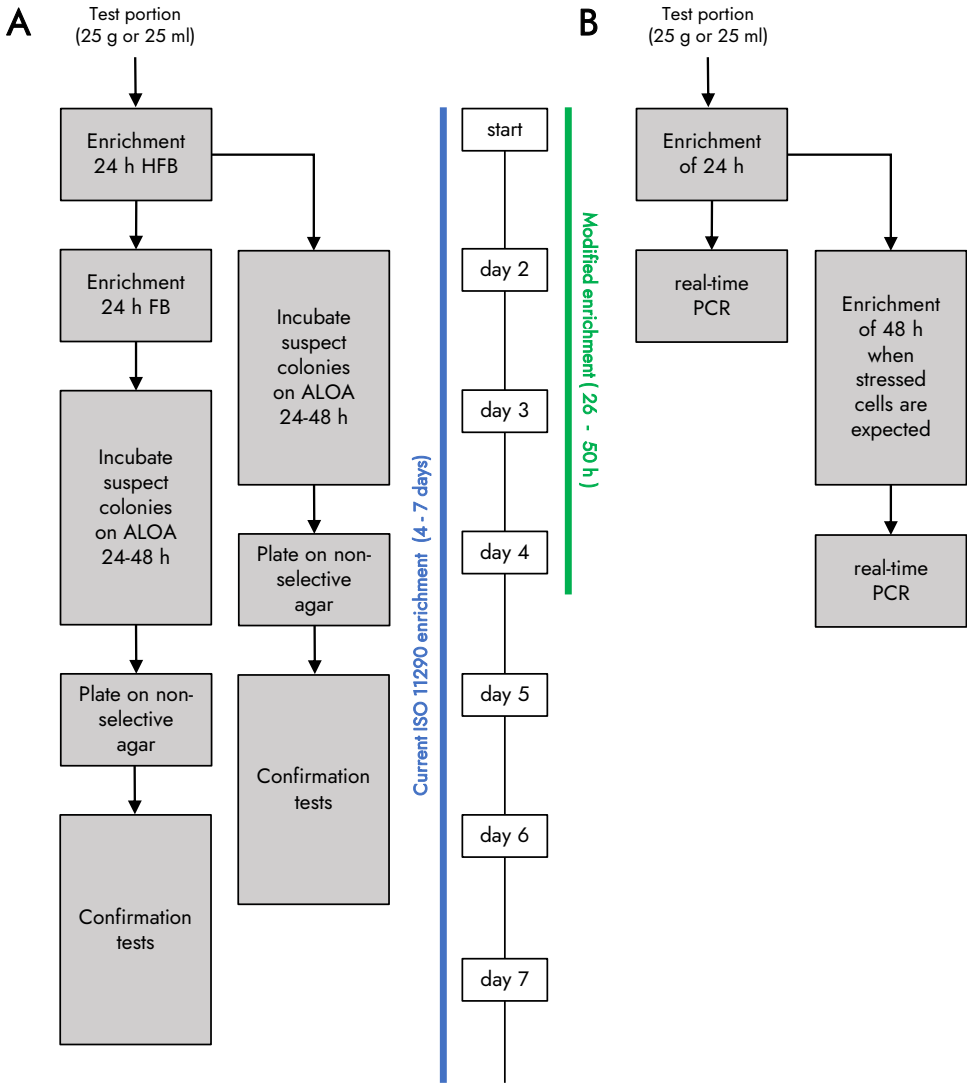

Figure 4: Schematic overview of the current ISO 11290-1 protocol for the enrichment of *L. monocytogenes* from food (A), and a modified protocol that couples a single enrichment step with qPCR detection (B). The current ISO 11290-1 protocol takes between 4-7 days for confirmation. We propose an alternative method in which only a single enrichment step is combined with subsequent detection with qPCR which will take around 50 h. Modification of the enrichment protocol is also advised, hereby favouring recovery of stressed cells over strict suppression of microbiota because of the sensitivity of qPCR as a detection method.

5.4. Conclusion

In this study we showed that competitor strains can have similar growth kinetics in half Fraser broth as *L. monocytogenes*. However, even when competitors are inoculated at 1,000-fold higher concentrations than *L. monocytogenes*, combined enrichment in HFB with detection by qPCR allows its identification in most samples of cured ham and smoked salmon. Detection with qPCR has advantages over the traditional culture-based detection of the ISO 11290-1 method, seeing as it is much faster and less prone to missing *L. monocytogenes* colonies on competitor-overgrown ALOA plates. This alternative enrichment procedure allows detection in positive samples in approximately 50 h instead of the gold standard ISO 11290-1 procedure that can take up to 7 days.

Acknowledgements

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5.5. Supplementary materials

This section contains supplementary data of this chapter.

Table S1: Table of all identified competitor strains, including the enrichment step they were isolated from (either HFB of FB), and the colony morphology

strain	isolation source	colony morphology
Bacillus licheniformis	FB	blue/see-through structure
Corynebacterium testodinoris	HFB	green
Listeria innocua	HFB	blue
Listeria innocua	HFB	blue
Listeria innocua	FB	blue
Listeria monocytogenes	HFB	blue
Listeria monocytogenes	FB	blue
Listeria monocytogenes	FB	blue with dark-blue specks
Listeria welshimeri	HFB	blue
Listeria welshimeri	HFB	blue
Listeria welshimeri	FB	blue
Listeria welshimeri	FB	blue
Listeria welshimeri	FB	blue
Listeria welshimeri	FB	blue
Listeria welshimeri	HFB	blue
Listeria welshimeri	FB	blue
Micrococcus canis	FB	white/yellowish
Micrococcus luteus	HFB	white/yellowish
Pseudomonas azotoformans	HFB	white/see-through
Pseudomonas extremorientalis	HFB	white/see-through
Staphylococcus aureus	HFB	yellow
Staphylococcus aureus	FB	big white/yellowish
Staphylococcus aureus	FB	yellow
Staphylococcus aureus	FB	white/yellowish
Staphylococcus aureus	HFB	yellow with blue dots
Staphylococcus aureus	HFB	yellow
Staphylococcus carnosus	FB	white
Staphylococcus carnosus	HFB	white with wriggly edge
Staphylococcus carnosus	FB	white
Staphylococcus epidermidis	FB	white
Staphylococcus equorum	FB	white
Staphylococcus equorum	FB	white/yellowish
Staphylococcus saprophyticus	HFB	white
Staphylococcus saprophyticus	HFB	white
Staphylococcus saprophyticus	HFB	white
Staphylococcus saprophyticus	HFB	white
Staphylococcus saprophyticus	HFB	white/light-blue
Staphylococcus saprophyticus	HFB	white
Staphylococcus saprophyticus	FB	white
Staphylococcus saprophyticus	HFB	white/yellowish
Staphylococcus saprophyticus	HFB	yellow
Staphylococcus saprophyticus	HFB	white/yellowish
Staphylococcus saprophyticus	HFB	white/yellowish

Continued on the next page

strain	isolation source	colony morphology
Staphylococcus saprophyticus	HFB	white/yellowish
Staphylococcus saprophyticus	HFB	white/yellowish
Staphylococcus saprophyticus	FB	white/yellowish
Staphylococcus saprophyticus	FB	white/yellowish
Staphylococcus saprophyticus	FB	white/yellowish
Staphylococcus saprophyticus	FB	white/yellowish
Staphylococcus saprophyticus	FB	white/yellowish
Staphylococcus saprophyticus	FB	white/yellowish
Staphylococcus saprophyticus	FB	yellow
Staphylococcus saprophyticus	HFB	white
Staphylococcus saprophyticus	FB	white/yellowish
Staphylococcus saprophyticus	FB	white
Staphylococcus saprophyticus	FB	white
Staphylococcus saprophyticus	HFB	white/yellowish
Staphylococcus saprophyticus	FB	white
Staphylococcus saprophyticus	FB	white/yellowish
Staphylococcus saprophyticus	HFB	white
Staphylococcus sciuri	HFB	blue
Staphylococcus sciuri	HFB	blue
Staphylococcus sciuri	HFB	blue
Staphylococcus sciuri	FB	blue
Staphylococcus sciuri	FB	blue
Staphylococcus sciuri	HFB	blue
Staphylococcus sciuri	HFB	blue
Staphylococcus sciuri	HFB	blue/green
Staphylococcus sciuri	HFB	blue with wriggly edge
Staphylococcus sciuri	FB	blue
Staphylococcus sciuri	FB	blue/green
Staphylococcus warneri	FB	yellow
Staphylococcus warneri	HFB	yellow
Staphylococcus warneri	FB	white/yellowish
Staphylococcus warneri	FB	yellow
Staphylococcus warneri	FB	white/yellowish
Staphylococcus warneri	HFB	yellow
Staphylococcus warneri	FB	yellow
Staphylococcus xylosus	HFB	white/yellowish
Staphylococcus xylosus	HFB	white/yellowish
Staphylococcus xylosus	FB	white/yellowish
Staphylococcus xylosus	HFB	white/yellowish

Table S2: Smoked salmon and cured ham were spiked with 1 $\log_{10}$ CFU/ml of *L. monocytogenes* strain EGDe and 1,000-fold higher concentration of competitor cocktail and enriched in non-selective BHI for 24 h before being analysed in the bioMérieux GENE-UP® qPCR machine. "Presence" indicates a successful detection of *L. monocytogenes* by the GENE-UP®, "absence" indicates that no *L. monocytogenes* is present in the sample, and 'inhibition' means that either the *L. monocytogenes*-specific probe or the internal amplification control did not give a conclusive result

product	history	medium	result GENE-UP
ham	reference	BHI	presence
ham	heat stress	BHI	presence
ham	reference	BHI	presence
ham	heat stress	BHI	absence
ham	reference	BHI	presence
ham	heat stress	BHI	absence
ham	reference	BHI	inhibition
ham	heat stress	BHI	absence
salmon	reference	BHI	presence
salmon	heat stress	BHI	presence
salmon	reference	BHI	presence
salmon	heat stress	BHI	presence
salmon	reference	BHI	presence
salmon	heat stress	BHI	presence
salmon	reference	BHI	presence
salmon	heat stress	BHI	presence

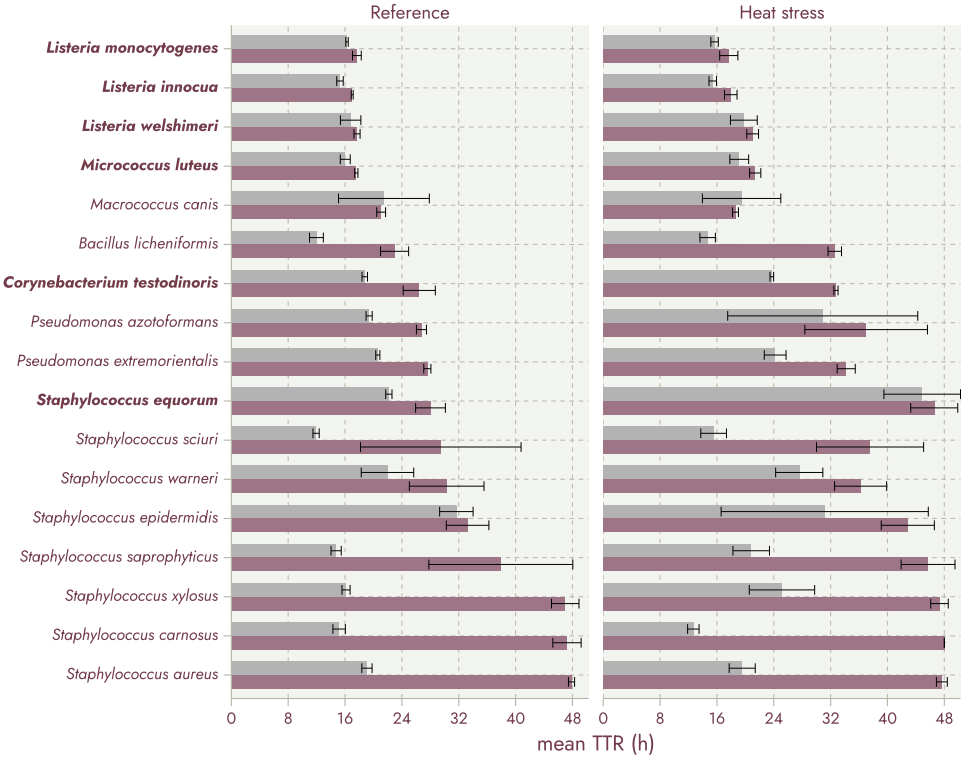

Figure S1: Outgrowth of the strains was measured with optical density (600 nm) in BHI (light-grey) and in HFB (purple) for reference cells (left) and heat-stressed cells (right) for 48 h at 30 °C. Outgrowth was measured as the time to reach an optical density increase of 0.25. Strains highlighted in bold were used in further experiments. Error bars shows the standard deviation of triplicate experiments.

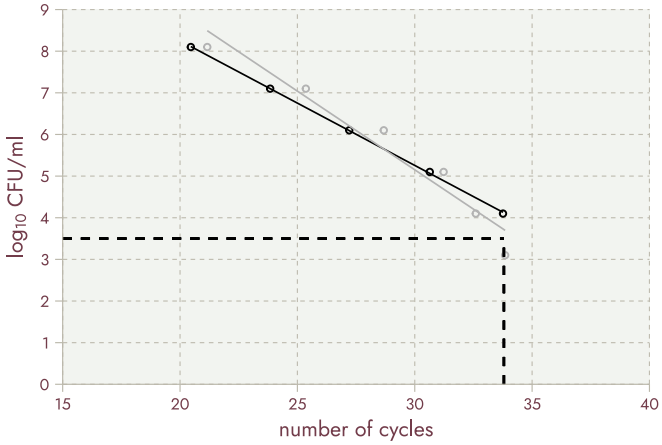

Figure S2: The detection threshold calibration curve was determined by serially diluting *L. monocytogenes* culture in enriched cured ham (black) and smoked salmon (grey). The concentration that can still be detected in HFB for both food products lies around 3.5 $\log_{10}$ CFU/ml.

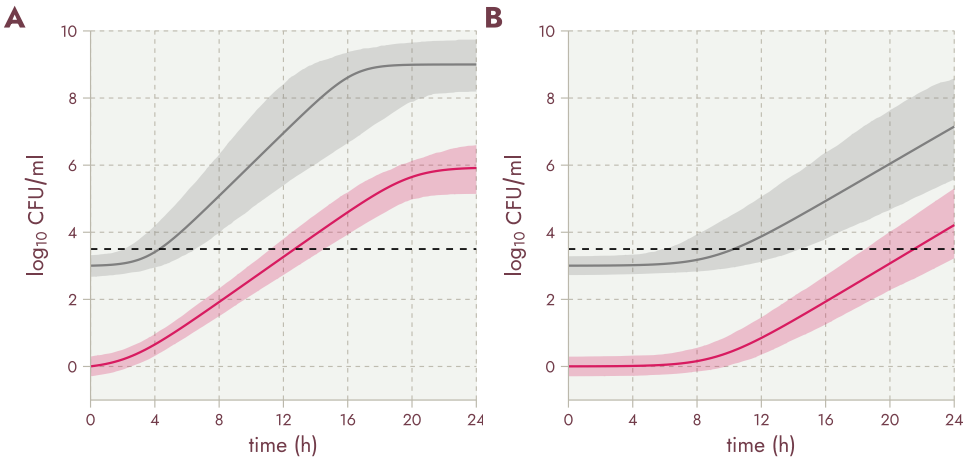

Figure S3: Simulations of the enrichment kinetics between *L. monocytogenes* (red) and a 1,000-fold higher concentration of competitors (grey) when starting with one cell/ml of *L. monocytogenes* in HFB. Jameson-interaction is taken into account such that growth of *L. monocytogenes* halts when competitors reach stationary phase. In both reference condition (A) and after heat stress (B) the 3.5 $\log_{10}$ CFU/ml qPCR detection threshold is reached within 24 h.

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6

General discussion

Jasper W. Bannenberg

6.1. Factors influencing optimal resuscitation

This thesis focused on investigating the enrichment-based detection protocol of *Listeria monocytogenes* from food. Successful resuscitation and subsequent detection of pathogens from food is complex because of the many variables that can influence it. Variation in type and degree of cell damage induced upon food processing (Guillier et al., 2005; Jasson et al., 2007), the physicochemical environment of different food matrices (Wesche et al., 2009), and the influence of a wide range of co-occurring microorganisms (Al-Zeyara et al., 2011; Zilelidou & Skandamis, 2018; Zitz et al., 2011) can have significant effects on the successful resuscitation of *L. monocytogenes* during enrichments. Furthermore, faster detection techniques could be used to reduce the time of the enrichment-based detection protocol. Although there has been extensive research into rapid detection techniques (as illustrated by a recent review paper by Osek et al. (2022)), knowledge about the behaviour of *L. monocytogenes* during enrichment is lacking. This is very relevant because enrichment of *L. monocytogenes* is crucial even with modern rapid detection methods (Jantzen et al., 2006). If the pathogen is present in the product only at a level of a few to a few hundred cells per sample, then the concentration needs to be increased before detection (Osek et al., 2022). Therefore, knowledge on the optimal resuscitation behaviour during enrichments would give directions to make enrichment-based detection procedures shorter and more reliable.

In the chapters of this thesis light has been shed on factors that influence the optimal resuscitation of (damaged) cells. The effect of heterogeneity between strains of *L. monocytogenes* has been discussed in chapter 2, where large variation was found in the lag phase of heat-stressed cells. This indicated that strain differences in lag duration can affect the detection chance during enrichment, with some strains exhibiting long lag durations. Next to this, heterogeneity in growth was also quantified in chapter 3 for single cells. Next to strain variation, the variation between single-cells is also a significant factor for the successful enrichment of *L. monocytogenes*. In chapter 4 the physiology of cells during enrichment was investigated in order to understand more about lag behaviour. Here, it was found that zinc metabolism and the production of resuscitation-promoting factors could decrease the lag duration of *L. monocytogenes* during enrichment. In chapter 5 the use of molecular detection by real-time PCR (qPCR) and the effect of competitors was researched as a more rapid alternative to culture-based detection of *L. monocytogenes*. It was found that the interaction with competing microbiota was not a significant problem for detection because of the low

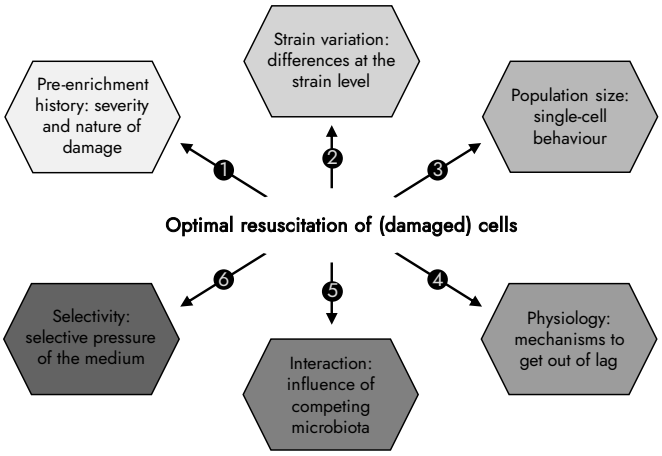

Figure 1: Overview of six contributing factors discussed in this thesis that influence the optimal resuscitation ability of damaged cells during enrichment. Main factors that influence the resuscitation capacity during enrichment are the pre-enrichment (stress) history (1), variation between strains (2), population size (3), physiology of the target microorganism (4), interaction with the competing microbiota (5), and the selective pressure of the enrichment medium.

detection threshold for qPCR that needs to be reached.

The combined chapters of this thesis give insight into the factors that influence resuscitation of damaged *L. monocytogenes* cells from food, as visualized in figure 1. These are the effects of pre-enrichment (stress) history, strain variation, population size, physiology, interaction with competing microbiota, and the selectivity of the enrichment medium. These six main factors and their effect on resuscitation and successful detection of *L. monocytogenes* will be further discussed in this integrating discussion chapter.

6.2. Pre-enrichment history

To study the resuscitation ability of *L. monocytogenes* during enrichment, the main focus in this thesis was on the primary enrichment step in half Fraser broth (HFB). The aim of this step is allowing the cells to recover from their potential damage and grow to higher concentrations. In this thesis the decision was made to focus on heat stress history to study the recovery kinetics of (sublethal) injured cells in HFB. Sublethal injury is reversible damage to bacterial cells that can be repaired in suitable environments (Ray, 1979). The extent of damage within a bacterial population is dependent on the type and intensity of the stress, the cell physiological state at the time, and the nature of the stress whether this is metabolic or structural

(Ray, 1979; Wesche et al., 2009). When cells are injured they can become less tolerant to antimicrobial compounds than their non-damaged counterparts, and subsequently their growth can be inhibited in selective media (Jasson et al., 2007). However, the severity of the stress can influence cells to various degrees and can be thought of as a continuum of injury that ranges from healthy to dead cells (Wesche et al., 2009). Bacterial cells can adapt completely to mild stress with little to no effect on their growth capacity. Low levels of stress can lead to a transient adaptation – an adaptive response – that is accompanied by a temporary physiological change which can result in increased stress tolerance (Wesche et al., 2009). Lethal stress will cause the death of a (large) part of the bacterial population, but not necessarily of all cells. The surviving population may show adaptive gene responses or mutations that can lead to an improved survival of the overall population. How bacterial populations are affected by the range of stressful environments they can encounter depends on the physiological cell state and on the organism. In the case of *L. monocytogenes*, in trial experiments with low pH and low temperature exposure, we found that a reduction in cell counts was necessary to see effects on the subsequent lag duration in an enrichment (data not shown). Due to the robustness and high tolerances of *L. monocytogenes* to stressful conditions, this meant that mild treatments often do not lead to decreases in cell counts. Although cold stress and acid stress can affect the metabolic and structural integrity of membranes (Wesche et al., 2009), in the case of *L. monocytogenes* this did not lead to a significant lag duration increase in HFB.

To see the effect of damage on the lag duration, a more lethal stress has to be applied. We therefore decided to focus on the recovery after reduction by 60 °C heat stress. Thermal treatment is an often-applied control strategy to prevent or limit the contamination of food products by foodborne pathogens (Bucur et al., 2018). Sufficient thermal treatments ensure food safety and can increase shelf-life (van Boekel et al., 2010), but it can also negatively affect food quality and nutritional value (Hardy et al., 1999). Although *L. monocytogenes* is effectively killed by heat treatments used in cooking and commercial food preparation (Chan & Wiedmann, 2008), it has been shown to be able to survive in the heat treatment of meat (Murphy et al., 2003), eggs (Monfort et al., 2012), and vegetables (Mazzotta, 2001). This is even more important when taking into account the fact that *L. monocytogenes* cells can become even more heat resistant when exposed to sublethal stresses prior to heat treatment (Bucur et al., 2018; Shen et al., 2014). Taking all this into account, heat stress history is an interesting and representative condition to study the effects on recovery during enrichment. Due to large variation in D_{60}-values, a decision was made to standardize

the treatment to one $\log_{10}$ CFU/ml reduction for all strains. The effect of simulating a heat treatment for a fixed time for every strain (for example the average D_{60}-value of all strains), would lead to large differences in reduction as shown in figure 2. By using a different heat treatment duration for each strain, the exposure times are not similar between strains but this allows for a more fair evaluation of recovery capacity than using a fixed heat treatment duration for all strains. This approach is therefore more suitable to quantify the strain-specific recovery during enrichment.

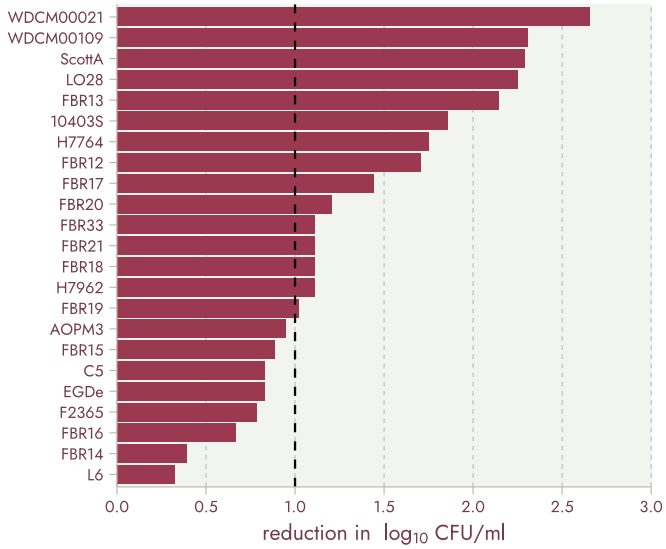

Figure 2: The $\log_{10}$ CFU/ml reduction by 60 °C heat stress if instead of using the specific D_{60}-value for each strain, a set time (the average D_{60}-value in this case) was used for all strains. The bars indicate the $\log_{10}$ CFU/ml reduction when using the same (1.33 min) exposure time for each strain. For this, the heat inactivation curve was simplified as a linear reduction. The black dotted line represents the reduction that was aimed for by using each strain's specific D_{60}-value. Due to the large heat resistance of the strains, this would lead to large differences in reduction.

For the performance testing of the current ISO 11290-1 method, only two strains of *L. monocytogenes* were used, WDCM00021 (serotype 4b) and WDCM00109 (serotype 1/2a) (International Organization for Standardization, 2017a). These strains showed different behaviour with strain WDCM00109 having a fast recovery and WDCM00021 having an average recovery after heat stress (chapter 2 figure 1). It can therefore be argued if this is enough strain variation to accurately assess the ability of the enrichment broths to properly resuscitate cells. Therefore, in this thesis a much larger set of 23 food-related strains was assessed on their recovery after heat stress. Because such large differences in lag duration were found between strains, strain heterogeneity is

something that should be taken into account for future amendments or updates to the ISO protocol. Testing of a larger amount of different stress conditions is advisable to accurately determine whether improvements of the protocol can be extrapolated to the genetic variation between *L. monocytogenes* strains. The survival after food processing and environmental stresses has been shown before to depend on the type and the time of exposure (Faezi-Ghasemi & Kazemi, 2015; Siderakou et al., 2021), as could also be concluded from the data in chapter 2. Extrapolating from the data in this thesis, it could be concluded that harsher stresses that lead to the death of a (large) part of the population are necessary to have significant increase in lag duration in HFB. Here, the increase in lag duration is then the combined effect of the time needed for cell damage repair and the increased susceptibility to the selective compounds in HFB.

6.3. Strain variation on outgrowth

Variability between strains (Aalto-Araneda et al., 2020; Aryani et al., 2015b,a; Lianou & Koutsoumanis, 2011) and variability in the initial concentration (Awaisheh, 2010; Lambertz et al., 2012) can have a significant impact on growth initiation and lag duration during enrichment. This impact has been clearly shown in chapter 2 where significant strain variability has been shown after heat stress during enrichment. Accurate quantification of growth kinetics with plate-counting is a labour-intensive method. To accurately fit a microbial growth model to estimate a lag duration, multiple data points are needed in each growth phase. This becomes even more complex when lag durations increase to more than 10 h when a secondary enrichment is necessary to be able to collect data points during the night. Although accurate estimation of the lag was necessary to compare strain variability, this method is expensive and difficult to do in a more high-throughput way. Although advancements in the knowledge on microbial growth modelling allow for the estimation of lag via high-throughput optical density measurements (Biesta-Peters et al., 2010; Dupont & Augustin, 2009; Guillier et al., 2005; Miled et al., 2011), the level of accuracy may not be enough for some research questions. In contrast, determining the maximum specific growth rate with optical density is more straightforward (Biesta-Peters et al., 2010).

The strains that were used in this study have a factor 10 difference in their D_{60}-value (between 0.5 and 4.1 min). This already shows a wide range of heat resistance between strains of *L. monocytogenes*, even though the heat resistance did not correlate with the lag duration. Even though the individual heat resistances of strains do not have a direct impact on their ability to detect them in an enrichment, it can influ-

ence the strain bias in detection. When a population of multiple strains of *L. monocytogenes* is present on a food product that undergoes a heat treatment step, differences in heat resistance lead to differences in cell reduction. Because of this, heat resistant strains could become more dominant during enrichment.

The kinetics data on strain variability showed that even though no significant differences were observed in the maximum specific growth rate, very significantly large differences in lag durations were seen. This is important in the context of enrichments, seeing as the heterogeneity in lag duration has a direct effect on the ability to detect a specific strain of *L. monocytogenes*. Strain bias during enrichment of *L. monocytogenes* was also reported earlier (Bruhn et al., 2005; Zilelidou et al., 2016b). These studies concluded that certain strains in foods may be missed with the current ISO protocol due to competition between lineages and/or serotypes. Over 95 % of the strains that were isolated from humans and food products belong to serotypes 1/2a, 1/2b, 1/2c, and 4b (Pontello et al., 2012; Shamloo et al., 2019; Swaminathan & Gerner-Smidt, 2007). Even though some serotypes might be more resistant to survive the food production chain, the large prevalence of specific serotypes in foods could lead to the hypothesis that these serotypes may grow faster during enrichment. Such a selection bias in enrichment can skew the statistics towards certain predominant serotypes. However, a serotype predisposition was not found in our data, indicating that such a selection bias can not likely be attributed to serotype differences. Furthermore, the presence of persister cells (Knudsen et al., 2013; Wen et al., 2011) are a burden in food production environments. Cells of *L. monocytogenes* can enter a dormant and non-dividing state with enhanced ability to survive environmental stresses. However, selection bias due to differences in growth kinetics in the detection from food are unavoidable when culture-based enrichment is needed to increase cell numbers. Namely, fast-growing strains during enrichment do not necessarily reflect isolates with high infectivity during infection (Zilelidou et al., 2016a). Concludingly, strain variation in kinetics during enrichment is important to take into account because this can directly influence the chance to detect *L. monocytogenes* from food products.

6.4. Heterogeneity at single-cell level

Next to strain heterogeneity in lag duration, we showed in chapter 3 that there also is a high level of heterogeneity in single-cell recovery behaviour. This single-cell heterogeneity is often underrepresented in microbiological experiments, which are usually done at population levels with hundreds to several millions of cells. This can give a bi-

ased view on the ability to detect small numbers of *L. monocytogenes* from foods. Environmental stresses such as extreme temperatures, pH, or depletion of nutrients can have bacteriostatic or even bactericidal effects on microorganisms. During the food production chain, a substantial proportion of bacterial cells are exposed to stressful environmental conditions that can lead to sublethal injury, which can in turn drastically affect their culturability. Such differences in the physiological state of cells become much more important at close to single-cell level, because the individual probability of growth initiation becomes more significant. It was shown in chapter 3 that outliers with longer lag durations can impact the chance to detect these cells during enrichment. Therefore, by only using the average population lag duration to draw conclusions on the presence of *L. monocytogenes* in food products, you would underestimate the risk due to the extended lag of heterogeneous cells. It is therefore important to not only take into account a wide variety of different strains, but also the effects of single-cell behaviour and this is especially important because routine food samples are expected to have low levels of pathogens.

With our approach we described a high-throughput method for the quantification of single-cell heterogeneity. By sorting single-cell events into wells-plates the optical density can be measured over time to estimate the outgrowth time by a specific increase in optical density. This method can be used to study single-cell heterogeneity for other stresses or different organisms. It is important to increase our knowledge on single-cell (or small populations) heterogeneity and behaviour in order to allow for this in more robust prediction of enrichment of pathogens from food.

As mentioned before, cellular damage can negatively influence the ability to grow in selective environments. The physiological cell state of individual cells can drastically differ and as such influence the outgrowth. The selective pressure in HFB plays a large role in the increased heterogeneity when compared to non-selective BHI where the single-cell variation was much smaller. For optimal resuscitation of damaged cells, a less selective medium is preferred because this can decrease heterogeneity in outgrowth and thereby directly influence the detection. Furthermore, heterogeneity becomes even more problematic for slow-recovering strains. Heterogeneity as well as the amount of extreme outliers increases for these strains with long lag durations which are already at risk of being missed upon detection. This multiplicative effect makes the detection of low levels of slow-recovering strains increasingly difficult in the limited 24 h of the primary enrichment step. As with strain variation, the type of stress is most likely also a contributing factor to single-cell heterogeneity. Exposure to heat can lead to a broad range of injury with damage to DNA/RNA, cytoplasmic

membranes, and enzymes (Métris et al., 2008). Although we accounted for this in our method, the environment during cell sorting can be somewhat harsh for cells with damaged membranes. Membrane damage makes cells more vulnerable to the selective HFB, and therefore stresses that damage the bacterial cell wall and/or membrane are expected to show the largest heterogeneity in single-cell outgrowth.

6.5. Physiology of getting out of lag

The recovery of stressed cells during enrichment is mainly differentiated by the variation in lag duration. Despite lag being an important part of the bacterial growth curve, it remains the most poorly understood growth phase (Bertrand, 2019). Functional genomic studies are an important tool to broaden our knowledge on lag behaviour. However, due to the low concentrations of bacteria during the lag-phase combined with the high amount of genetic material required for such studies, this requires adaptations to overcome this. Although single-cell sequencing and transcriptomics experiments are definitely possible with the current technologies, these are expensive and not routinely used outside of medical studies (Aldridge & Teichmann, 2020). To understand more about the physiology of getting out of lag, larger volumes and cell concentrations had to be used in order to collect enough high-quality RNA and proteins for use in transcriptomics and proteomics. The number of studies on the lag physiology of foodborne pathogens is extremely limited. Rolfe et al. (2012) used a similar experimental setup to study the lag of *Salmonella enterica* in LB broth. In this study, stationary phase cultures grown in LB were added to fresh LB to study lag behaviour. In the experimental setup of chapter 4 the introduction to a different medium (i.e. HFB) from the culture medium (i.e. BHI) led to a larger change in environment. Especially when also taking into account the heat stress history of the cells. To our knowledge, this is the first time that the lag of *L. monocytogenes* has been studied at transcript and proteome level in published scientific literature.

To get some more general insight into the functional genomics of lag behaviour, the main physiological processes of *L. monocytogenes* are compared against the data of the foodborne pathogen *S. enterica* (Rolfe et al., 2012) in figure 3. Although differences in the expression of certain processes are noticeable (which can be due to difference between organism and/or difference between experimental setup), common elements can be observed. It has been shown before that the prokaryotic lag phase response is broadly similar in *Escherichia coli* and *S. enterica* (Pin et al., 2009; Rolfe et al., 2012). Extending that comparison based on transcriptomic data with the proteomic data on *L. monocytogenes* can further increase the understanding of com-

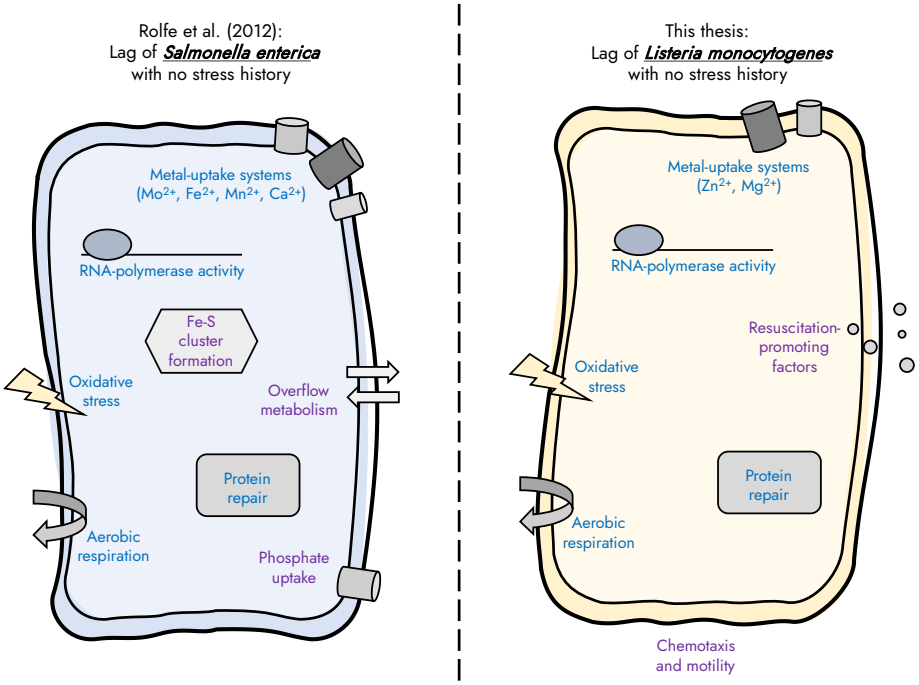

Figure 3: Comparison of the lag behaviour of the foodborne pathogen *S. enterica* (adapted from Rolfe et al. (2012)) with *L. monocytogenes* (this thesis). This shows the similarities in the lag phase adaptations between both pathogens. Expression of similar systems are coloured in blue and differences are coloured in purple. The response of both is without any stress-history and medium-specific elements like antibiotics and salt-stress were not taken into account.

mon lag phase behaviour. Interesting is the (transient) uptake of metals during lag phase. Metal ions play an important role as these essential micronutrients are central in many metabolic processes (Agranoff & Krishna, 2004; Hobman et al., 2005). Even though the uptake differs with respect to specific metals that are taken up, metal import during lag can be an important process that warrants further investigation. The metal uptake is also expected to be influenced by the amount and composition of trace metals in the different growth media that were used. Furthermore, a transient upregulation of oxidative stress proteins seems to be a common process (Cuny et al., 2007). Lastly, protein repair processes are an important part of the lag phase. The accumulation of protein damage during stationary phase combined with oxidative stress management in the new environment indicates that repair processes are important even if these cells were not exposed to other stress beside the stationary phase-associated stresses (Rolfe et al., 2012). Especially, putative repair of aberrant disulfide bonds in cytosolic proteins, conceivably by upregulated thioredoxins was ap-

parent from the *L. monocytogenes* data. Comparative analysis of lag phase behaviour in Gram-negative and Gram-positive representatives, revealed that important processes during lag of *S. enterica* as reported by Rolfe et al. (2012), were also observed during lag of *L. monocytogenes*.

Studying the lag behaviour of *L. monocytogenes* pointed to the importance of metal homeostasis and the secretion of resuscitation-promoting factors. Although supplementation with spent medium containing resuscitation-promoting factors led to a phenotypic response of a shorter lag, the shortening of lag was not large enough to enable more strains reaching the detection threshold. Also, HFB is a very rich medium containing complex enzymatic digests of animal tissue and meat extracts. Supplementation in such a rich medium may not lead to significant improvements in growth kinetics as was shown from our phenotypic data in chapter 4. Supplementation with critical components can have large influences in more minimal medium, as was shown by Pinto et al. (2013) where supplementation with resuscitation-promoting factors decreased the lag in minimal medium. In a rich medium as HFB there is much less risk of missing critical components that are necessary for getting out of lag. This is in accordance with our data that significant shortening of the lag duration is not feasible by supplementation of HFB.

6.6. Competitor interaction

An important conclusion from the chapters of this thesis is that the current enrichment protocol is too selective for the optimal resuscitation of (severely) damaged cells. However, reducing selectivity of the enrichment medium will also have effects on the growth of co-occurring microbiota from the food product. There is a lot of literature describing the co-enrichment of *L. monocytogenes* with competitors. Many focus on the interactions in (liquid) food products with one competitor (as reviewed by Zilelidou & Skandamis (2018)), but microbial communities in foods are often much more complex. Often anti-listerial effects are caused not by individual microorganisms, but by the community as a whole (Imran et al., 2013). The inhibitory effect of a microbial community against *L. monocytogenes* is most probably related to the composition and interactions of the community rather than the number of species (Callon et al., 2014). The effects of microbiota on the growth of *L. monocytogenes* are therefore not easy to predict. Most studies on the growth in HFB therefore focus on the competition with *L. innocua* which is often co-occuring in foods (Stea et al., 2015; Zitz et al., 2011). There are indications that *L. innocua* strains can suppress the growth of *L. monocytogenes* during selective enrichment (Zitz et al., 2011). They showed that

there is a significant decrease in detectability of *L. monocytogenes* in HFB when co-cultured even with lower concentrations of *L. innocua* (Zitz et al., 2011). Engelhardt et al. (2016) agreed with this and also showed that competition between *L. monocytogenes* and *L. innocua* happens in Fraser broth (FB). Overgrowth of *L. innocua* results from a selective growth advantage as well as the production of inhibitory compounds (Cornu et al., 2002). Further studies showed that Gram-negative organisms were inhibited in HFB but that lactic acid bacteria still reached high numbers (Al-Zeyara et al., 2011), but many other organisms might compete with *L. monocytogenes* during enrichment as can be seen in chapter 5. Competitors can negatively interact with the growth of *L. monocytogenes* during enrichment either by suppressing growth with the production of antimicrobials or by growing at a faster rate and thereby depleting essential nutrients. These forms of competition were not found in the experiments with competitors. Even when doing co-culture experiments (data not shown), a competitive growth advantage could not be seen, even though co-cultures were difficult to quantify because of the lack of selective plates for both organisms.

In chapter 5 the growth of competitors was quantified that were isolated from ALOA during routine enrichments. The vast majority of the species collected was *Staphylococci*, with only 3.7 % of the colonies that were collected of *L. innocua*. Most of the strains were unable to grow well in the selective environment of HFB, except for *L. innocua*, *M. luteus*, and *C. testodinoris*. The Gram-positive *M. luteus* can be found in soil, dust, and water and is considered an opportunistic pathogen, while no information on *C. testodinoris* could be found. Excepting *L. innocua*, the competitors that were found are not reported to often co-occur during enrichments. Contrasting from literature data, a growth advantage of *L. innocua* and the other tested competitors could not be found in mono-culture enrichments. Co-cultures are of course much more complex and growth kinetics depend on the intricate microbial interactions that can take place. We simulated the interaction with competitors in spiked food products, and even with 1,000-fold higher concentration of competitors the detection of *L. monocytogenes* could still be facilitated by qPCR. Although the exact growth kinetics during the enrichment are not known, the suppression of *L. monocytogenes* as proposed in literature did not affect the ability to detect this pathogen. As long as the detection threshold for qPCR is reached during the incubation time, the possible overgrowth of competitors is less relevant if you are not relying on culture-based detection methods.

6.7. Selective pressure of the enrichment medium

To balance the need for selectivity to suppress competing microbiota while still allowing for the recovery of (damaged) cells, the ISO protocol splits the enrichment into a double incubation. Here, the primary enrichment contains only half of the selective compounds to allow for recovery of damaged cells. However, the transfer to a secondary medium can have consequences on the detection, as only 0.1 ml is transferred. As has been estimated by Augustin et al. (2016), a threshold of 2 $\log_{10}$ CFU/ml has to be reached during this 24-26 h to efficiently transfer at least one cell with 99.995 % Poisson-probability. This is often not reached by heat-stressed cells (chapter 2). We have shown that increasing the mandatory primary enrichment time to 26 h and increasing the inoculum volume to 1 ml had a positive effect on detecting *L. monocytogenes*. Increasing the inoculum transfer effectively lowers the concentration threshold that needs to be reached, and increasing the enrichment time leads to a higher probability of reaching this threshold, hereby reducing the false-negative chance. This indicates that a 24-h enrichment is not sufficient, and even the lower selective pressure in HFB is likely to significantly affect the growth of cells with a stress history. Out of the 23 tested strains of *L. monocytogenes*, 11 were not able to reach the necessary detection threshold of 2 $\log_{10}$ CFU/ml within the 24 h of the primary enrichment (chapter 2 figure 6). This conclusion was strengthened by the single-cell results where the heterogeneity in outgrowth was also wide enough to be at risk of false-negative results due to increased lag duration after stress. It could therefore be argued that HFB is too selective to allow proper resuscitate and recovery of damaged cells.

6.8. Redefining detection of *L. monocytogenes*

With the current culture-based detection using the ISO protocol, there is a necessity to suppress competing microbiota. This is necessary because after enrichment, the culture is plated on ALOA agar and suspect colonies of *L. monocytogenes* need to be separateable of the competing microbiota. This need for selectivity is a big hurdle, and reducing the concentration of selective compounds in the primary enrichment step is not enough for severely damaged cells. This results in a medium where such damaged cells might not reach the detection threshold in the prescribed time, and the risk in food products can be severely underestimated as a result. In contrast, when relying on the use of a molecular detection technique such as qPCR after enrichment, the overgrowth of competitors is less of an issue (chapter 5). This gives the option to

reduce the selective pressure of the enrichment medium, and hereby focusing on the optimal recovery of *L. monocytogenes*. By utilizing a non-culture-based approach for detection, we can thus focus more on optimal recovery of *L. monocytogenes* during enrichments (figure 4).

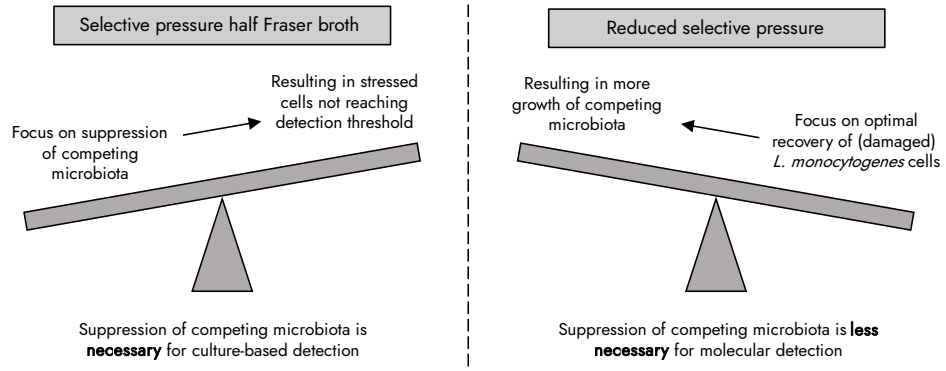

Figure 4: Moving from culture-based detection (left part of the figure) towards molecular detection (right part) allows us to redefine the priorities of enrichments. For the current culture-based detection approach, the suppression of competing microbiota is important to be able to find suspect colonies on ALOA agar. This results in cells with a stress history potentially not reaching the detection threshold. For molecular detection, the suppression of microbiota is less crucial as long as the detection concentration of the method is reached. With reduced selection pressure, the focus can be on the optimal recovery of (damaged) *L. monocytogenes* cells.

6.8.1. Quantifying selective compounds

In order to quantify the effect of selective compounds on the growth in HFB, a factorial experimental design was followed (Gunst & Mason, 2009). In a factorial design, not only the effect of compounds can be quantified, but also the interactions that can have a significant influence on the growth. With this approach, the effect of the selective compounds and the growth-enhancing compounds in HFB could be assessed on the outgrowth (experimental setup is explained in this chapter's supplementary materials). For this, higher and lower concentrations than in HFB were chosen to see its effect on growth of *L. monocytogenes* (exact concentrations are in table S2). The effect of these lower and higher concentrations of these factors can be seen in figure 5. Here the effect of lowering and increasing the concentration can be seen on the outgrowth time measured with optical density. There is a similar trend for reference cells and heat stressed cells, with the exception that the effect on heat stressed cells is larger.

Acriflavine has the biggest effect on the time-to-reach values and this significantly

increases the detection time for *L. monocytogenes*. The effect of nalidixic acid is less severe than acriflavine, but still has a significant effect on the outgrowth. Finally, the effect of lithium chloride on the growth of *L. monocytogenes* is limited. This would suggest that *L. monocytogenes* is tolerant to higher concentrations of lithium chloride, where the addition of double the concentration in HFB (to 6 g/l) does only have

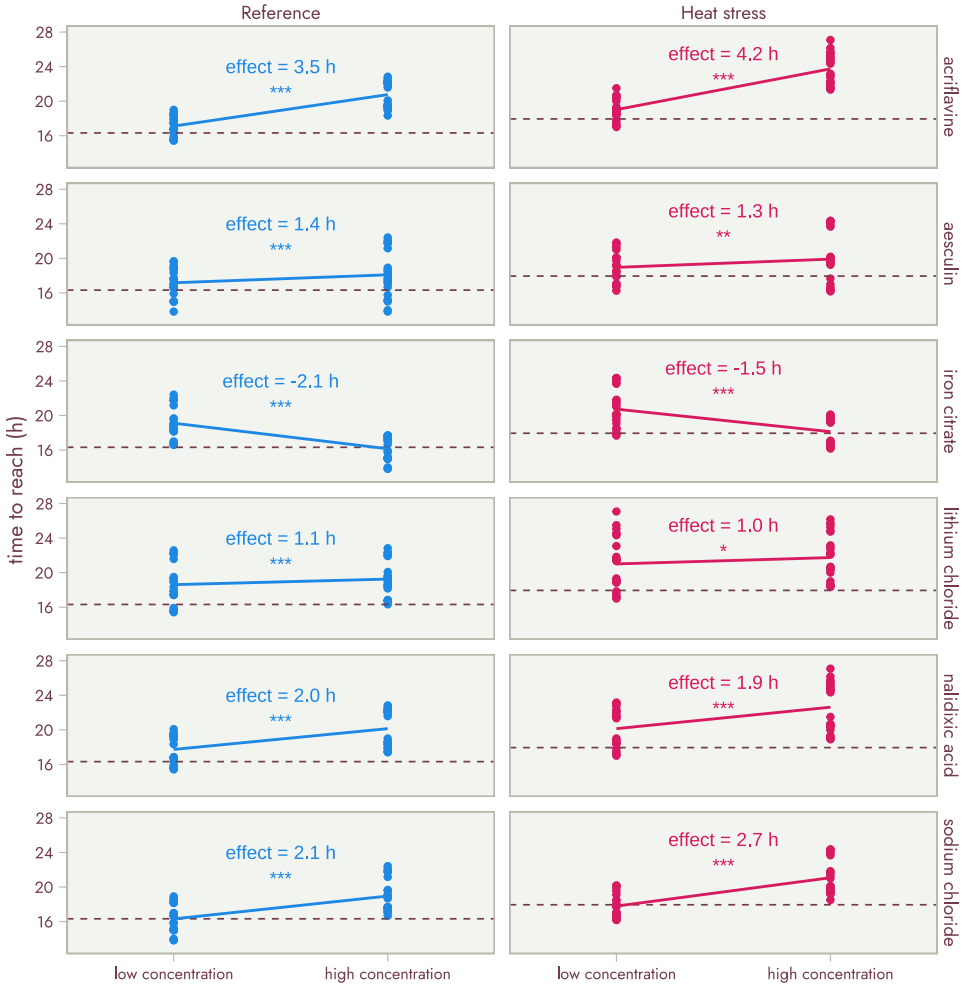

Figure 5: Factorial analysis of selective compounds and supplements on the outgrowth of *L. monocytogenes* in HFB. The effect of using lower and higher concentration of compounds than in HFB was evaluated for reference cells (blue), and for heat-stressed cells (red). The time-to-reach is the time it takes to get an optical density increase of 0.25. For each compound different wells were used, the effect is given in h (the slope of the mean differences), and the significance was tested with linear regression. The dotted line is the mean time-to-reach for HFB without any of the added components.

a marginal effect on the growth. Lithium chloride affects the metabolism in Gram-negative organisms, however the exact effects are not known (Cox et al., 1990). It has been shown before that increasing the lithium chloride concentration to 9 g/l completely inhibited the growth of *Enterococcus faecium* while still allowing recovery and detection of heat-injured *L. monocytogenes* from pasteurized milk (Teo et al., 2001). Furthermore, PALCAM Listeria agar for the selective isolation of Listeria from food and milk products even contains 15 g/l (Power & Johnson, 2009). The concentration of lithium chloride thus could be adjusted in HFB without much effect on the growth of *L. monocytogenes*. In contrast, the concentrations of nalidixic acid and acriflavine have a much larger debilitating effect on *L. monocytogenes* growth. This means that to increase the ability of *L. monocytogenes* cells to recover during enrichment, lowering the concentrations of nalidixic acid and especially acriflavine would be a viable option. Of course, it is hereby important to test whether this still facilitates a necessary level of suppression of competing microbiota.

Next to the selective compounds, also the presence of sodium chloride has a significant influence on the growth. *L. monocytogenes* is known for its tolerance to osmotic conditions of up to 10 % (Duché et al., 2002; Gardan et al., 2003; McClure et al., 1989), and high salt concentrations are used as a selective component in the ISO enrichment media. HFB and FB contain a NaCl concentration of 20 g/l (2 % w/v), but this comes with the price of elongated growth. In chapter 4 we have also shown that on proteomic level the upregulation of salt transporters was also significant, and the salt concentration has a significant effect on the outgrowth in HFB with an added effect for heat-stressed cells.

Furthermore, the addition of aesculin to the medium was originally added to give a blackening reaction for *Listeria* species based on the reaction of iron with the hydrolysed aesculin (Fraser & Sperber, 1988). From figure 5 it seems that this compound has a somewhat negative effect on the growth. Furthermore, the hydrolysis of aesculin is not a good indicator of *Listeria* presence because many *Enterococci* are also able to readily use this substrate (Sangadkit et al., 2016). Contrastingly, the effect of iron citrate seems to have a positive effect on the growth (-2.1 h and -1.5 h for reference cells and stressed cells respectively). Although iron citrate was originally added for the blackening reaction with aesculin hydrolysis (Fraser & Sperber, 1988), it was already suggested then that iron can have a growth enhancing effect (Cowart & Foster, 1985). Since then, the presence of a citrate inducible ferric citrate uptake system was found in *Listeria* (McLaughlin et al., 2011), and the use of iron citrate has positive growth benefits. From the results in figure 5 it can be seen that using higher concentrations of

iron citrate result in faster outgrowth. It is therefore interesting to study if increasing the iron concentration can have additional effects.

Different approaches are taken for the enrichment of other important food pathogens to ensure recovery of stressed cells. For enrichment of *S. enterica* from foods as described in ISO 6579-1:2017 (International Organization for Standardization, 2017c), there is a pre-enrichment step of 18 h (± 2 h) in non-selective buffered peptone water. It has already been shown decades ago that pre-enrichment of *Salmonella* spp. facilitates damage repair caused by food preservation techniques (Edel & Kampelmacher, 1973; Thomason et al., 1977). After pre-enrichment the cells have had time to recover from their (sublethal) injuries and then a selective enrichment is done before plating out and confirmation tests in a similar way as the protocol for *L. monocytogenes*. The ISO 10272-1:2017 protocol for the enrichment of *Campylobacter* from food (International Organization for Standardization, 2017b) takes yet another approach at balancing recovery and selectivity. Here there is a choice between Bolton broth and the more selective Preston broth. Bolton broth is chosen when *Campylobacter* cells are expected to be stressed and/or damaged and when low levels of microbiota are expected. The more selective Preston broth is then used when high number of competitors are expected. This approach would allow for better recovery of stressed cells in a less selective environment, however this choice is heavily dictated by the immunity of extended spectrum beta-lactamase producing bacteria (ESBL's) that are often co-enriched with *Campylobacter* (Hazeleger et al., 2016; Lanzl et al., 2020). The choice of procedure is in this way dependent on the choice of the user, which can be difficult if stressed *Campylobacter* is expected together with high concentrations of ESBL's. The enrichment protocols of other foodborne pathogens use different strategies to balance the need for recovery of the target cells with suppression of co-occurring microbiota. This is of course dependent on the specific needs of the pathogen in question, the food products it is associated with, and the microbiota that may be co-enriched. In the case of *L. monocytogenes*, reducing the selectivity to allow for optimal recovery seems to be a good choice to reduce the chance of false-negative results. This is hopefully taken into account in the revision of the ISO protocol by the current commission ISO/TC 34/SC 9/WG 32 "Improvement of pre-enrichment step in ISO 11290-1" that is starting at the time of writing.

6.8.2. Combining enrichment with molecular detection

In chapter 1 an overview is given of alternative rapid detection methods. Currently, a good reliable and reproducible method to use for detection of pathogens from food is

qPCR. Due to its suitable detection limit, its high sensitivity and specificity, its ease of high-throughput analysis, and the availability of commercial kits, qPCR is already used for the detection of *L. monocytogenes* (Barbau-Piednoir et al., 2013; Berrada et al., 2006; Paul et al., 2015; Rantsiou et al., 2008). An important consideration in the detection of DNA from food samples, is that qPCR does not distinguish between the DNA of live cells or the extracellular DNA of cells that died during food processing. For the detection of foodborne pathogens, only viable cells are of importance and a detection method should be optimized for this. Food products that might contain high amounts of DNA from non-viable cells are first diluted 1:10 in HFB and subsequently the viable cells of *L. monocytogenes* that might be present are multiplied several orders of magnitude. Because there is an enrichment of viable cells, the proportion of DNA from dead cells will become much lower. Therefore, the interference of extracellular DNA of non-viable cells is estimated to not be a significant factor for use of qPCR detection. The disruption of food matrix components on the efficient qPCR reaction might be a bigger hurdle for the widespread adoption of this detection method (Cocolin et al., 2011; Hedman & Rådström, 2013; Sidstedt et al., 2020; Wang & Salazar, 2016). After enrichment, ideally the sample would have to undergo limited steps before detection, besides disruption of cells to free intracellular DNA. For lysis of *L. monocytogenes* cells the use of beads to disrupt the cells is advised due to its hard to lyse cell wall (Hwang et al., 2011). Additional post-enrichment processing steps would increase the chance of cross-contamination between samples, and makes the method more labour-intensive. However, direct lysis from enriched samples can be influenced by food matrix components that interfere with optimal multiplication of target DNA (Hedman & Rådström, 2013; Suther & Moore, 2019). PCR interference can be measured because internal controls are taken along with the samples which indicate if disruption by the food matrix constituents happened. The amount of disruption depends on the food matrix that is analysed, and the results from chapter 5 suggest that with smoked salmon or cured ham some samples showed inhibition and could not give a conclusive result. The inhibition by food matrix components thus remains a large hurdle for efficient use of qPCR as a detection method for pathogens from food samples. However, also adaptations of the enrichment medium could have an influence on this. Components like sodium and lithium chloride can affect the overall ion content, as well as interference by the fluorescent acriflavine, which both have negative effects on the PCR reaction (Hedman & Rådström, 2013). Effort could be invested in the optimization of the enrichment medium with respect to its compatibility with the PCR reaction. For complex food matrices with substantial effect

on the reliable qPCR reaction, an intermediate DNA extraction step could be applied. This DNA extraction step can lead to a more clean sample and this can even lower the detection threshold by concentrating the sample further. DNA isolation after enrichment would lead to a more costly and labour-intensive protocol, but the reliability of the method could increase. Enrichment combined with subsequent DNA extraction and qPCR has been shown to be an efficient method for the detection of *Salmonella* from different food matrices (Siala et al., 2017). Using food matrix clean-up steps or DNA extraction are a possible way to overcome inhibition of qPCR by the food matrix.

6.9. Proposed enrichment protocol

As mentioned before, using qPCR directly on food products in a completely culture-free approach is not possible due to the fact that the detection threshold is far higher than the amount of cells of *L. monocytogenes* that need to be detected. Enrichment combined with rapid molecular detection using qPCR would lead to a drastic shortening of the current detection protocol as already outlined in chapter 5. In figure 6 a concluding overview is given of the kinetics of the current ISO 11290-1 enrichment protocol next to the proposed protocol. When starting at one cell per enrichment many of the tested strains are unable to reach the threshold of 2 $\log_{10}$ CFU/ml necessary for efficient transfer to FB due to variation in recovery of heat-stressed cells. These red-coloured strains in figure 6 might lead to false-negative results when plated on ALOA[1]. The strains with a faster recovery (in green) are able to be either detected on ALOA from HFB, or grow to higher concentrations in FB and be detected from the secondary enrichment step. Subsequent culture-based detection takes between 2-5 days depending on the presence of *L. monocytogenes* in the sample.

For the proposed alternative protocol, a less selective enrichment broth would be preferable to decrease the lag duration and increase the growth-rate of stressed cells. In this case, all the 23 strains tested in this thesis would reach the qPCR detection threshold of around 3.5 $\log_{10}$ CFU/ml in a 48-h enrichment. Even when a competitor is taken into account with 3 $\log_{10}$ CFU/ml higher inoculum concentration (red line in proposed protocol in figure 6), no Jameson-interaction takes place. The proposed alternative protocol would use a single 48-h enrichment instead of splitting this into two 24-h enrichments, because the dilution between these steps increases the chance that slow-growing strains are not transferred to the second incubation. If cells of *L. monocytogenes* have not been subjected to stressful conditions, the detection threshold could be reached much faster than 48 h (data not shown). Therefore, a

[1]With a concentration of 2 $\log_{10}$ CFU/ml (100 cells/ml), a loopful (10 µl) on ALOA results in one cell on a plate.

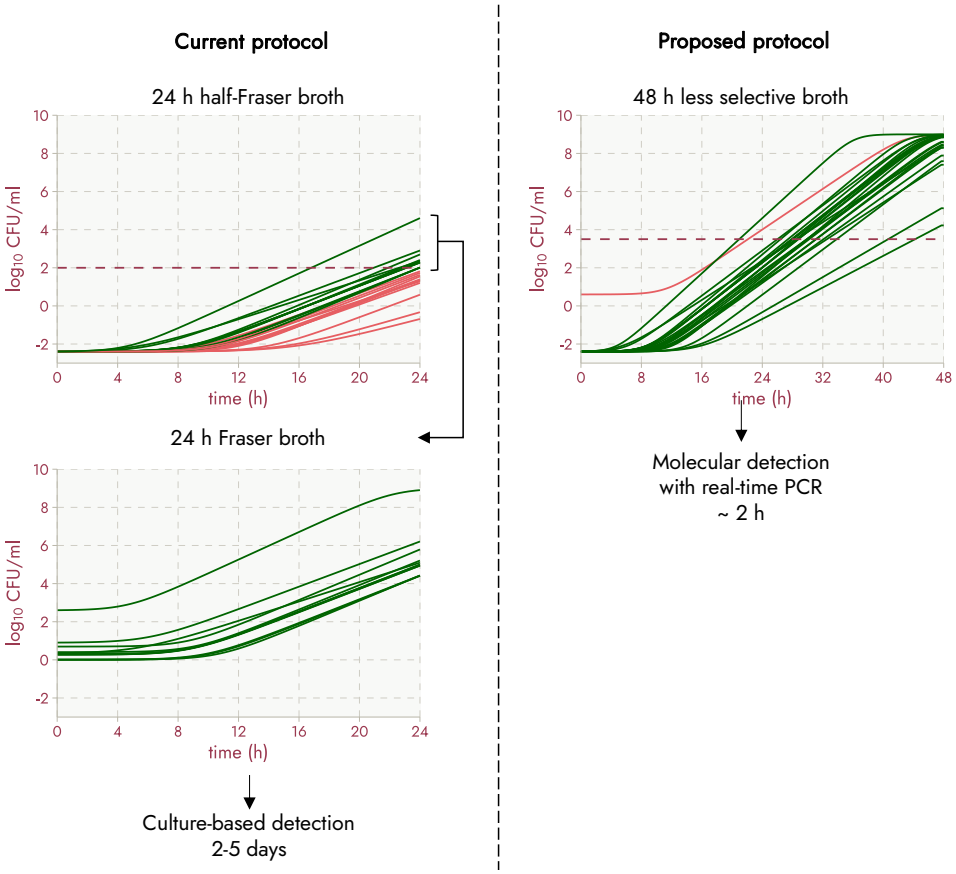

Figure 6: Comparison of the current enrichment protocol with the proposed protocol for the detection of *L. monocytogenes* from food. The lessons learned from this thesis are that when cells of *L. monocytogenes* are damaged they may fail to reach the detection threshold for transfer towards the secondary enrichment step of the current protocol. Based on the kinetics data of heat-stressed strains, with the current protocol 11 out of 23 strains do not reach this threshold and thereby can lead to false-negative results. If instead a one-step enrichment of 48 h is used (preferably with lower selective pressure to allow better resuscitation of damaged cells), *L. monocytogenes* can be detected in a much shorter time period. In the proposed protocol the growth of competitors is given in red and an inoculum concentration of 3 $\log_{10}$ higher than *L. monocytogenes*. Even with this, there is no interaction and all strains can reach the qPCR detection limit.

longer 48-h enrichment would be preferred to reduce the chance of stressed *L. monocytogenes* cells not reaching the detection limit during enrichment.

6.10. Concluding remarks and future perspectives

For the successful detection of *L. monocytogenes* from food, low levels of potentially damaged cells have to be enriched to suitable detection levels. In this thesis multiple factors have been described that can have significant influence on proper resuscitation during enrichment. Notably, these factors can affect the false-negative chance that cells of *L. monocytogenes* present in the product are not detected. For (severely) damaged cells, the selective pressure of the primary enrichment broth is too high to recover these cells during the 24-h time frame. Recommendations have been put forward in this thesis to account for this, such as increasing the primary enrichment time, increasing the transfer volume to the secondary enrichment step, or lowering the selective compounds in the medium. Although such alternatives would increase the probability to detect injured cells, the current culture-based detection method is still a time-consuming lengthy process. The current method heavily relies on labour-intensive cultural laboratory techniques that have been unchanged for the last decades. Therefore, the combination of enrichment with molecular detection techniques provides a more rapid alternative that is also less reliant on suppressing other microbiota that can be present on the food product. Hereby, the focus during enrichment can be shifted towards optimal repair and outgrowth of *L. monocytogenes*. Although molecular methods like qPCR are not without their downsides (detection limits, interference by food matrix components, etc.), this thesis has given the fundamental basis to update the current enrichment protocol to a more rapid and accurate method.

This thesis has also shed more light on the lag phase behaviour of *L. monocytogenes*. Study of the lag phase pointed to specific biomarkers, which could indeed decrease the lag duration in the enrichment medium, although this reduction was not significant. Furthermore, the measurements on single-cell heterogeneity and lag behaviour with proteomics have led to methods that can also be extended to study other (foodborne) microorganisms.

Looking forward, advancements in detection methods can be expected to bring lower detection limits in shorter time frames, but it is conceivable that enrichment remains a crucial part of pathogen detection. The combination of a (less) selective enrichment with rapid detection methods will also be the basis for successful pathogen detection in the future. This thesis provides a deeper understanding of enrichment and can be the basis of further research that will directly benefit food safety.

6.11. Supplementary materials

This section contains supplementary data of this chapter.

Factorial design experiment

To quantify the effect of compounds in HFB on the growth of *L. monocytogenes*, HFB was made using the ingredients list as used in Oxoid's Fraser broth base (table S1). For the compounds to be quantified, lower and higher concentrations were added according to table S2. Of the many different factorial media, 45 µl was added to 384-wells plates and 10 wells were used for each combination of medium and history (reference and heat stressed). Working cultures were made as described earlier in chapter 2, and 5 µl was of inoculum was added to each well for a starting concentration of 2 $\log_{10}$ CFU/ml. The optical density at 600 nm was measured at 30 °C every 5 min. Afterwards, the time-to-reach was determined as the time to reach an optical density increase of 0.25. Linear regression analysis was done to estimate the effect of each of the factors and its significance using R (R Core Team, 2021).

Table S1: Concentration of compounds that make up HFB. The amount of the compounds to be quantified were changed to lower and higher concentrations as stated in table S2

compound	amount (g/l)
Proteose peptone	5.0
Tryptone	5.0
Lab-Lemco powder	5.0
Yeast extract	5.0
NaCl	20.0
Na_2HPO_4	12.0
KH_2PO_4	1.35
Aesculin	1.0
LiCl	3.0
Fe(III) Citrate	0.5
Nalidixic acid	0.020
Acriflavine HCl	0.025

Table S2: Concentrations of compounds in HFB and FB and the low and high concentrations as used in the factorial design experiment. All concentrations are given in g/l

compound	in HFB	in FB	low concentration	high concentration
LiCl	3	3	0	4
Nalidixic acid	0.01	0.02	0	0.04
Acriflavine	0.0125	0.025	0	0.05
NaCl	20	20	10	30
Aesculin	1	1	0	2
Fe(III) citrate	0.5	0.5	0	1

6.12. References

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A

Appendix

Summary

Listeria monocytogenes is an important foodborne pathogen that can cause serious illness in the susceptible population. This pathogen can be found everywhere in the environment, and together with its robustness and ability to grow at refrigeration-temperatures, makes it so that the presence of *L. monocytogenes* in food is strongly regulated. To verify that food products do not contain higher levels of *L. monocytogenes* than is allowed by European law, food products are routinely tested on the presence of this pathogen. The current gold standard for the detection of *L. monocytogenes* from foods in the European Union is set by the International Organisation of Standardisation in ISO 11290-1:2017. This standard consists of a double enrichment where the aim is to resuscitate damaged *L. monocytogenes* cells and to grow them to higher concentrations so that they subsequently can be detected.

However, the detection of *L. monocytogenes* from food can be difficult due to the fact that pathogens are often present in (very) low numbers, and that they need to repair potential damage suffered during food processing. Next to this, the presence of other microbiota on the food product has to be suppressed at the same time as to not interfere with detection. Enrichment is a crucial step in pathogen detection, but there is the careful balance to be considered between optimal ability to resuscitate and the necessary selectivity to reduce the growth of background microorganisms. Although the current enrichment protocol is able to detect the presence of *L. monocytogenes* from food products, it is a time-consuming and labour-intensive process that can take up to 7 days for positive samples. Furthermore, much progress has been made into faster detection methods, however, knowledge on the enrichment ecology of *L. monocytogenes* is limited. The physiological and molecular processes that are necessary for repair and growth initiation during enrichment are poorly understood, and more knowledge on this is important because enrichment forms the basis for all subsequent detection methods. Therefore, knowledge on the optimal resuscitation behaviour during enrichments would give directions to make enrichment-based detection procedures shorter and more reliable. The main aim of this thesis was to get insight into the microbial physiology and ecology of *L. monocytogenes* during enrichment, and to use this knowledge to allow for the optimization the current enrichment-based detection method.

In chapter 2 the variability in lag duration was determined for a large set of food-relevant strains of *L. monocytogenes* to assess the impact of growth kinetics on the detection. For this, the effect of a $\log_{10}$ CFU/ml reduction at 60 °C heat stress was

compared against reference cells that did not have a stress history. The reference cells had a relatively short lag duration, which ranged from 1.4 to 2.7 hours. However, significant variation in the ability to recover after 60 °C heat stress was observed among the tested strains and this resulted in a lag duration ranging from 4.7 to 15.8 hours. Although the application of heat stress increased the biological variability between reproductions, the difference in lag duration was mainly attributed to the variability between strains. When a subset of strains was also exposed to low temperature pH stress, the recovery behaviour was different from heat stress history. This indicated that the variation in lag duration was not only strain-dependent but also stress-dependent. With the growth parameters that were obtained during the enrichments, scenario analyses were done to estimate the effect of strain variability on the chance to detect these strains. These analyses showed that almost half of the strains were not able to reach the necessary concentration for efficient transfer to the secondary step of enrichment after heat stress when starting with one cell. This showed that the increased lag duration of (severely) injured cells can have significant impact on the timely growth during enrichment. Furthermore, we found that increasing the incubation time from 24 to 26 hour and the transfer volume from 0.1 to 1.0 ml can increase the probability to transfer cells to the secondary enrichment step from 79.9% to 99.0%. When optimizing enrichment procedures, it is therefore crucial to take strain variability into account as this can have a significant impact on the detection efficacy.

Because contaminated food products often contain low levels of *L. monocytogenes*, the behaviour at single-cell level can be important to take into account, as this can be highly variable. To measure this effect on the outgrowth of single cells of *L. monocytogenes*, fluorescence-activated cell sorting was used in chapter 3 to sort single cells in wells-plates. The outgrowth of single cells was assessed in half Fraser enrichment broth and compared to non-selective brain heart infusion broth. Single-cell heterogeneity was much higher in enrichment broth than in a non-selective medium, indicating that the presence of selective compounds significantly increases the variation in outgrowth of single cells. The increase in heterogeneity was also found to be strain-dependent because the fast-recovering strain showed less outgrowth heterogeneity than the slower-recovering strains. The outgrowth data was then combined to estimate the effect of heterogeneity on the detection chance. This showed that the heterogeneity between single cells can result in false-negative detection outcomes, thereby highlighting the importance of this in enrichment-based detection.

Even though enrichment is crucial in the reliable detection of *L. monocytogenes*,

the lag behaviour of this pathogen remains poorly understood. Insight into the recovery mechanisms is an important step into understanding the behaviour during enrichment, and in chapter 4 we used an omics approach to shed light onto to physiological processes of lag. Upon transfer of stationary phase *L. monocytogenes* cells to half-Fraser enrichment broth, motility-associated genes and proteins were downregulated, while expression of metal uptake transporters, resuscitation-promoting factors that stimulate growth from dormancy, antibiotic efflux pumps and oxidative stress proteins were upregulated. Next to this, when cells with a heat stress history were cultured in enrichment broth, proteins necessary for recovery were upregulated with functions in DNA-damage repair, protein refolding, cell-wall repair, and zinc transport. Proteomic results pointed to possible factors that support shortening of the lag, although these interventions did not lead to biologically relevant reduction of lag phase in the already rich enrichment medium.

Next, in chapter 5 the effect of competing microbiota was assessed on the ability to detect *L. monocytogenes* with fast molecular detection. For this, competitor strains were collected during routine enrichments by the Dutch food safety authority. We found that competitors had similar growth kinetics to *L. monocytogenes* in half Fraser broth, indicating that these competitors are unlikely to have a significant growth advantage during enrichment. Furthermore, it was shown that *L. monocytogenes* could be detected in food samples with 1,000-fold higher levels of competitive microbiota by combining primary enrichment with qPCR-based detection. Complementary simulations demonstrated that starting with one cell of *L. monocytogenes* per enrichment, the qPCR threshold was reached within 48 h. This might pave the way for an alternative and faster enrichment protocol for *L. monocytogenes* whereby a single enrichment step is combined with qPCR detection, hereby drastically shortening the processing time for food samples from up to 7 days to about 2 days.

In chapter 6 the results of the thesis chapters is integrated into a discussion. Concludingly, although advancements in detection methods can be expected to bring lower detection limits in shorter timeframes, it is conceivable that enrichment remains a crucial part of pathogen detection. This thesis provides a deeper understanding on the enrichment of *L. monocytogenes*, and this can be the basis of a combined enrichment with molecular detection.

Samenvatting

Listeria monocytogenes is een belangrijke voedselpathogeen die ernstige ziekte kan veroorzaken in de daarvoor gevoelige populatie. Deze pathogeen kan overal in het milieu gevonden worden, en samen met haar robuustheid en de mogelijkheid om op koelkasttemperatuur te kunnen groeien, zorgt dit ervoor dat de aanwezigheid van *L. monocytogenes* in voedsel streng gereguleerd is. Om te verifiëren dat voedselproducten geen hogere concentratie van *L. monocytogenes* bevatten dan is toegestaan volgens de Europese wet, worden deze routinematig getest op de aanwezigheid van deze pathogeen. De huidige standaard voor de detectie van *L. monocytogenes* in voedsel in de Europese unie is gestandaardiseerd door de International Organisation of Standardisation in ISO 11290-1:2017. Deze standaard bestaat uit een dubbele ophoping waarbij het doel is om beschadigde cellen van *L. monocytogenes* te laten herstellen zodat ze naar hogere concentraties kunnen groeien waarna ze gedetecteerd kunnen worden.

Echter, de detectie van *L. monocytogenes* uit voedsel wordt moeilijk gemaakt door het feit dat de pathogeen vaak in (zeer) lage aantallen aanwezig is, en dat ze schade moeten herstellen die ze mogelijk hebben opgelopen tijdens het voedselproductieproces. Hiernaast moet de aanwezigheid van andere micro-organismen op het voedselproduct tegelijkertijd onderdrukt worden zodat deze niet het detectieproces beïnvloeden. Ophoping is een cruciale stap in de detectie van pathogenen, maar er moet een voorzichtige balans gevonden worden tussen optimale uitgroeimogelijkheden en de nodige selectiviteit om de groei van andere micro-organismen te kunnen verminderen. En alhoewel het huidige ophopingsprotocol in staat is om de aanwezigheid van *L. monocytogenes* in voedsel aan te kunnen tonen, is dit een tijdrovend en arbeidsintensief proces dat wel 7 dagen kan duren voor positieve monsters. Bovendien is er veel progressie geboekt naar snellere detectiemethoden, alleen is de kennis over de ophopingsecologie van *L. monocytogenes* beperkt. De fysiologische en moleculaire processen die nodig zijn voor herstel en groei tijdens ophoping zijn weinig onderzocht, en meer kennis over deze processen is belangrijk omdat ophoping de basis vormt voor de daaropvolgende detectiemethoden. Daarom zou kennis over het optimale herstelgedrag tijdens ophoping helpen om de detectiemethode korter en betrouwbaarder te kunnen maken. Het hoofddoel van dit proefschrift was om inzicht te krijgen in de microbiële fysiologie en ecologie van *L. monocytogenes* tijdens ophoping, en om deze kennis te gebruiken voor het optimaliseren van de huidige op ophopings-gebaseerde detectiemethode.

In hoofdstuk 2 is de variabiliteit in lagfase (de periode in de microbiologische groeicurve waar nog geen groei plaatsvindt omdat ze zich moeten aanpassen aan de huidige omgeving) bepaald voor een grote set van voedselrelevante stammen van *L. monocytogenes* om de impact van groeikinetiek te bepalen op detectie. Hiervoor is het effect van een $\log_{10}$ reductie in kve/ml (kolonie-vormende eenheden per milliliter) door hitte-stress vergeleken met referentiecellen die geen stress hebben doorstaan. De referentiecellen hadden een relatief korte lagfase die varieerde tussen de 1,4 en 2,7 uur. Daarentegen konden we een significant verschil observeren in de mogelijkheid om te herstellen na 60 °C hitte-stress voor de geteste stammen en dit resulteerde in een lagfase tussen de 4,7 en 15,8 uur. Hoewel het toepassen van hitte-stress de biologische variabiliteit verhoogde tussen reproducties, kon het verschil in lagfase voornamelijk gekenmerkt worden door stamvariabiliteit. Toen een subgroep van de stammen ook werd blootgesteld aan lage-temperatuur pH-stress, was ook dit gedrag anders dan tijdens hitte-stress. Dit gaf aan dat de variatie in lagfase niet alleen stam- maar ook stress-afhankelijk was. Met de groeiparameters die we hebben verzameld tijdens de ophopingsexperimenten, hebben we scenario-analyses gedaan om het effect van stamvariabiliteit te schatten op de detectiekans van de stammen. Deze analyses lieten zien dat bijna de helft van de stammen niet in staat was om de nodige concentratie te bereiken om deze efficiënt naar de tweede ophopingsstap over te zetten als deze een hitte-stress historie hadden en begonnen met maar één cel. Dit liet zien dat de verlengde lagfase van (ernstig) beschadigde cellen een significante impact kan hebben op het tijdig uitgroeien tijdens ophoping. Verder vonden we dat het verlengen van de incubatietijd van 24 naar 26 uur en het verhogen van het overdrachtsvolume van 0,1 naar 1,0 ml de kans kan vergroten om cellen naar de tweede ophopingsstap over te brengen van 79,9% naar 99,0%. Voor het optimaliseren van ophopingsprocedures is het daarom cruciaal om stamvariabiliteit in rekening te nemen, aangezien dit een significante impact kan hebben op de detectie-effectiviteit.

Omdat gecontamineerde voedselproducten vaak maar lage hoeveelheden bevatten van *L. monocytogenes*, is het gedrag van individuele cellen belangrijk om in de gaten te houden, aangezien dit zeer variabel kan zijn. Om het effect van uitgroei van individuele cellen te meten, hebben we cellen gesorteerd op basis van fluorescentie in hoofdstuk 3 om de uitgroei van één enkele cel te meten in elk van de groeiplaten. De uitgroei van individuele cellen werd gemeten in half Fraser ophopingsmedium en dit werd vergeleken met niet-selectief brain heart infusion medium. De heterogeniteit van individuele cellen was veel hoger in ophopingsmedium dan in niet-selectief medium, wat suggereerde dat de aanwezigheid van de selectieve componenten een sig-

nificant effect hebben op de variatie in uitgroei. De verhoging van heterogeniteit was ook stam-afhankelijk, want snelgroeiende stammen lieten minder heterogeniteit zien dan langzaam groeiende stammen. De uitgroeidata werd ook weer gebruikt om het effect van heterogeniteit tussen individuele cellen te simuleren op de detectiekans. Dit liet zien dat heterogeniteit tussen individuele cellen kan resulteren in vals-negatieve detectie, wat ook aantoont dat dit een belangrijk effect is in op ophoping-gebaseerde detectie.

Hoewel ophoping cruciaal is voor betrouwbare detectie van *L. monocytogenes*, is het gedrag tijdens de lagfase van deze pathogeen weinig onderzocht. Inzichten in herstelmechanismen zijn een belangrijke stap in het beter begrijpen van het gedrag tijdens ophoping, en in hoofdstuk 4 hebben we een omics aanpak genomen om licht te werpen op de fysiologische processen tijdens de lagfase. Na het toevoegen van cellen van *L. monocytogenes* uit stationaire fase (de fase in de microbiologische groeifase waar de maximum concentratie bereikt is) aan half Fraser ophopingsmedium, zagen we dat genen en eiwitten met betrekking tot beweging werden onderdrukt, terwijl de expressie van metaalopname pompen, factoren die groei stimuleren, antibiotica pompen, en oxidatieve-stress eiwitten juist werden gestimuleerd. Zodra cellen met een hitte-stress historie werden gegroeid in ophopingsmedium werden eiwitten gestimuleerd voor herstel met functies als DNA-schade herstel, vouwen van beschadigde eiwitten, het repareren van de celwand, en transport van zink. De resultaten van proteomics hintten naar mogelijke factoren die de lagfase kunnen verkorten, alleen waren deze interventies niet genoeg voor een biologisch significante reductie in lagfase in het al voedingsrijke ophopingmedium.

In hoofdstuk 5 is het effect van concurrerende micro-organismen onderzocht op de mogelijkheid om *L. monocytogenes* te detecteren met een snelle moleculaire detectiemethode. Hiervoor zijn er concurrerende stammen verzameld tijdens routine ophopingen van de Nederlandse voedsel- en warenautoriteit. We zagen dat concurrerende micro-organismen gelijkwaardige groeiparameters hadden als *L. monocytogenes* tijdens ophoping, wat aangeeft dat deze concurrenten zeer waarschijnlijk geen significant groeivoordeel hebben tijdens ophoping. Verder lieten we zien dat *L. monocytogenes* gedetecteerd kon worden in voedselmonsters met 1.000-maal hogere concentraties van concurrerende micro-organismen door de eerste ophopingsstap te combineren met moleculaire detectie met qPCR. Complementerende simulaties demonstreerden dat als je een ophoping start met één cel van *L. monocytogenes*, dat de drempel voor succesvolle qPCR detectie gehaald werd binnen 48 uur. Dit biedt de mogelijkheid voor een snellere alternatieve detectiemethode voor *L. monocytogenes*

waar een enkele ophopingsstap gecombineerd wordt met qPCR-detectie, waarbij de verwerkingstijd van voedselmonsters drastisch verkleind kan worden van ten hoogste 7 dagen naar ongeveer 2 dagen.

In hoofdstuk 6 zijn de resultaten van dit proefschrift geïntegreerd in een discussie. Concluderend laat dit zien dat hoewel significante vorderingen op het gebied van detectiemethoden zeer waarschijnlijk steeds lagere detectielimieten met zich mee brengen in steeds kortere tijdsperioden, is het aannemelijk dat ophoping nog altijd een cruciale rol blijft spelen in pathogeendetectie. Dit proefschrift biedt een breder inzicht in de ophoping van *L. monocytogenes*, en dit kan de basis zijn van een combinatie van ophoping met moleculaire detectie.

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About the author

Jasper Wilmer Bannenberg was born on the 13th of May 1993 in Leiden, the Netherlands. After completing his high school at the Bonaventura College in Leiden, he moved to Wageningen in 2011 to start his Biotechnology studies. He chose this direction because of his interest in biology with a focus on application.

During his BSc he gained interest in pathogens and he did a minor in infectious diseases in humans and animals. He finished his BSc with a thesis at the department of host-microbe interactomics where he used zebrafish embryos as an infection model for the pathogen *Streptococcus suis*.

He continued with his MSc in biotechnology, hereby specializing in the cellular and molecular side of biotech. He got interested in the bacterial immune-system CRISPR-Cas, and did his thesis at the department of microbiology to research the priming of this system in *Escherichia coli*. Because he very much liked the combination of medical biotechnology with molecular biology, he did an internship at Intravacc to work on genome modification of the human pathogen *Neisseria meningitidis* for use as a vaccine delivery system.

After his studies, he wanted to continue to become an independent scientist by starting a PhD project at the department of food microbiology. In this application-focused research he could combine research on pathogens with molecular biology. This resulted in the thesis titled "**Enrichment ecology of *Listeria monocytogenes* - towards a more rapid enrichment-based detection method**" that you are now reading.

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LinkedIn: click link or scan QR-code

List of publications

This thesis

Bannenberg, JW, Abee, T, Zwietering, MH, & den Besten, HMW (2021). Variability in lag duration of *Listeria monocytogenes* strains in half Fraser enrichment broth after stress affects the detection efficacy using the ISO 11290-1 method. International Journal of Food Microbiology, 337, 108914. https://doi.org/10.1016/j.ijfoodmicro.2020.108914

Bannenberg, JW, Tempelaars, MH, Zwietering, MH, Abee, T, & den Besten, HMW (2021). Heterogeneity in single-cell outgrowth of *Listeria monocytogenes* in half Fraser enrichment broth is affected by strain variability and physiological state. Food Research International, 150, 110783. https://doi.org/10.1016/j.foodres.2021.110783

Bannenberg, JW, Boeren, S, Zwietering, MH, Abee, T, & den Besten, HMW. Insight in lag phase of *Listeria monocytogenes* during enrichment through proteomic and transcriptomic responses. Manuscript in preparation

Other

Künne, T, Kieper, SN, **Bannenberg, JW**, Vogel, AIM, Miellet, WR, Klein, M, Depken, M, Suarez-Diez, M, & Brouns, SJJ (2016). Cas3-derived target DNA degradation fragments fuel primed CRISPR adaptation. Molecular cell, 63(5), 852-864. https://doi.org/10.1016/j.molcel.2016.07.011

Overview of completed training activities

Discipline specific activities	Organizing institute	Year
Flow cytometry workshop	Thermo Fisher	2018
KNVM Symposium	KNVM	2018
FEMS Europe 2019	FEMS	2019
Intestinal Microbiome of Humans and Animals	VLAG	2019
Proteomics Workshop	Biochemistry	2020
KNVM Symposium	KNVM	2021
HPLC workshop	Food Microbiology (WUR)	2020
KNVM Symposium	KNVM	2021
World Microbe Forum	ASM & FEMS	2021
LAS-ICMSF Webinar	ICMSF - Latin America	2021
IAFP Annual Meeting US	IAFP	2021
KNVM Symposium	KNVM	2022
Food Micro 2022	ICFMH	2022

General courses	Organizing institute	Year
VLAG PhD week	VLAG	2018
Introduction to R	VLAG	2018
Applied statistics	VLAG	2018
Critical thinking and argumentation	WGS	2018
Data visualisation with Adobe Illustrator	WGS	2020
Efficient writing strategies	WGS	2020
Career perspectives	WGS	2021

Other activities	Organizing institute	Year
Preparation of research proposal	Food Microbiology (WUR)	2017
Project partner meetings	Food Microbiology (WUR)	2017-2021
Department friday seminars	Food Microbiology (WUR)	2017-2021
PhD trip to China	Food Microbiology (WUR)	2019
PhD trip to Germany/Switzerland (organisation)	Food Microbiology (WUR)	2022

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Cover design and thesis layout by Jasper W. Bannenberg. The cover illustrates the topic of the thesis by looking at *Listeria monocytogenes* in more detail under a magnifying glass and seeing the wide variety and heterogeneity in size, shape, and physiology that make successful enrichment-based detection a challenge.

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