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In the quest for persistence markers in *Listeria monocytogenes*

By Rui Manuel Santos Meneses

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Thesis presented to *Escola Superior de Biotecnologia of the Universidade Católica Portuguesa* to fulfil the requirements of Master of Science degree in Applied Microbiology

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Abstract

Listeria monocytogenes (*Lm*) is an opportunisticand highly invasive foodborne bacterial pathogen. Although not life-threatening for immunocompetent individuals, *Lm* is deleterious among more susceptible populations. This pathogen's primary transmission route relies on contaminated foods and is often associated with ready-to-eat (RTE) products, such as soft cheeses. Even though considerable efforts are spent in removing *Lm* from processing facilities, due to its prevalence in the environment, this pathogen effortlessly colonizes food processing environments (FPEs), with certain strains more frequently isolated from FPEs than others, with some instances of strains continuously isolated throughout time in the same processing facility. However, no consensus has yet been reached on the causes of persistence. Some attribute it to failure in facility disinfection, while others hypothesize higher intrinsic tolerances from the persistent strains. The objective of the present work was to identify genotypic and phenotypic differences between persistent and sporadic isolates. Seventeen isolates were selected to carry out the proposed tasks, and all were subjected to growth under stressful environments with varying degrees of temperature, pH, and salinity. Moreover, infrared spectra were collected from all isolates grown at 11 and 30 ºC with two objectives: I) To develop classification models that could correctly distinguish isolates grown at both temperatures and persistent and sporadic isolates; II) To evaluate how membrane fluidity in both groups (persistent and sporadic) was affected by the exposure to lower temperatures, providing useful information on phenotypic expression of both groups. Additionally, the accessory genomes of all isolates were characterized for the presence of resistance genes associated with enhanced tolerance to adverse conditions present in FPEs. Growth under the selected stress conditions did not yield significant differences between the growth parameters of persistent and sporadic isolates. Additionally, the accessory genomes of both groups were highly heterogenous and did not contain genetic elements that could account for enhanced tolerance to unfavourable conditions commonly encountered in FPEs. Analysis of the infrared spectra of *Lm* isolates exposed to 30 and 11 ºC yielded promising results. PLSDA prediction models for 11 ºC correctly classified a significant portion of the isolates as persistent or sporadic, possibly implying that the FTIR technique possesses enough discriminatory power to distinguish phenotypical differences between both groups. Thus, in the author's opinion, further research needs to be conducted on the FTIR analysis of isolates grown in various stress conditions to provide reliable and accurate evidence corroborating the claim that persistence may also be attributed to phenotypic characteristics, not only to sanitation deficiencies.

Keywords: *Listeria monocytogene*s, persistence, FTIR, stress exposure, accessory genome.

Resumo

Listeria monocytogenes (*Lm*) é um agente patogénico invasivo. Infeções por este microrganismo são particularmente danosas para as populações mais suscetíveis. É primordialmente disseminado via alimentos, estando associado a produtos prontos a comer, como os queijos. Ainda que se apliquem muitos esforços na sua remoção, devido à sua natureza ubíqua, *Lm* consegue colonizar com grande facilidade ambientes de processamento alimentar (FPEs). Com certas estirpes, sendo frequentemente isoladas nestes ambientes, e outras sendo isoladas esporadicamente. Apesar de não ser totalmente compreendido, alguns autores conjeturam a persistência como decorrência de falhas críticas nos planos de higienização das instalações. Enquanto outros, supõem a presença de mecanismos intrínsecos, responsáveis por este fenómeno. O presente trabalho teve como objetivo a identificação e caracterização de diferenças fenotípicas entre os dois grupos de isolados (persistente e esporádico), comparando também possíveis diferenças nos seus genomas acessórios. Para isso, selecionaram-se 17 isolados, sujeitando-os a crescer sob condições de stress com diferentes parâmetros de temperatura, pH e salinidade. Além disso, recolheram-se espetros de infravermelho (IR) para os 17 isolados crescidos a 11 e 30 ºC com dois propósitos: I) Desenvolver modelos de classificação capazes de distinguir isolados crescidos para ambas as temperaturas, bem como diferenciar entre isolados persistentes e esporádicos; II) Avaliar alterações na fluidez membranar dos dois grupos após a exposição a baixas temperaturas permitindo aferir diferenças na expressão fenotípica de ambos os grupos. Por último, o genoma acessório dos isolados foi caracterizado quanto à presença de genes de resistência que concedessem tolerância a condições adversas presentes em FPEs. Relativamente ao crescimento em condições de stress, não foram identificadas diferenças substanciais entre os parâmetros de crescimento dos isolados persistentes e esporádicos. Adicionalmente, os genomas acessórios de ambos os grupos demonstraram-se intrinsecamente heterogéneos, não contendo elementos genéticos que pudessem conferir tolerância a condições adversas. A análise dos espetros de IR de isolados de *Lm* expostos a 11 e 30 °C foi promissora. Os modelos de predição do PLSDA para 11 °C classificaram corretamente uma porção significativa dos isolados, evidenciando que a técnica de FTIR possuí um poder discriminatório satisfatório para a distinção de diferenças fenotípicas entre os dois grupos. Devem-se conduzir ensaios adicionais, para viabilizar a hipótese de que eventos de persistência podem ser atribuídos não apenas a deficiências na higienização, mas também a características fenotípicas intrínsecas dos isolados.

Palavras-chave: *Listeria monocytogene*s, persistência, FTIR, stress, genoma acessório.

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1. Introduction

1.1 *Listeria monocytogenes* epidemiology & implications for public health

Listeria monocytogenes (Lm), phylum *Firmicutes*, is a Gram-positive, facultative intracellular foodborne pathogen and the causal agent of human listeriosis. In its invasive form, this infection may assume one of three expressions: bacteraemia, neurolisteriosis and maternal-neonatal infection (Charlier et al., 2022; Pägelow et al., 2018; Watanabe et al., 2021).

Non-invasive listeriosis, in immunocompetent individuals, may occur asymptomatically. However, often develops into self-limited gastroenteritis, with symptoms ranging from nausea, headaches, and fever to diarrhoea, vomiting and abdominal pain (Drevets and Bronze, 2008). Due to the ability of *Lm* to cross the intestinal barrier (Lecuit et al., 2001), systemic invasion may follow, leading to the invasion of the bloodstream, in severe cases, the pathogen can cross the blood-brain barrier, manifesting as septicaemia, meningitis, or encephalitis (neurolisteriosis) (Radoshevich and Cossart, 2017). These clinical conditions are more common in elderly and immunocompromised individuals. In the case of pregnant women, *Lm* infections are particularly worrisome. It's one of the few bacteria able to cross the placental barrier (Disson et al., 2008), enabling it to infect the developing foetus, leading to miscarriage or stillbirth, premature delivery, and neonatal infections such as neonatal meningitis (Allerberger and Huhulescu, 2015).

Despite the relatively low incidence rate, listeriosis is known for its high mortality, which even in developing countries ranges from 20 to 30% (Charlier et al., 2017). According to The European Union One Health 2020 Zoonoses Report (EFSA, 2021), in the 27 EU member states, a total of 1,876 confirmed listeriosis cases were reported, a decrease of 7.1% from the previous year. Of these, 780 occurrences led to hospitalizations, with 167 infections resulting in subsequent fatalities. *Listeria monocytogenes* was the fifth most prevalent foodborne pathogen in the EU.

As a psychrotrophic microorganism, *Lm* benefits from a lack of competition in cold environments, allowing it to thrive in refrigeration temperatures (2-4 ºC), growing also at a wide range of temperatures from -0.5 to 45ºC, with an optimal growth temperature of 37 ºC (Allerberger, 2003). Due to acid and osmotic stress response mechanisms, *Lm* also flourishes in a wide variety of unfavourable environments (Michel et al., 2011), growing in pH values as low as 4.6 and water activities down to 0.93 (Nolan et al., 1992; Wijtes et al., 1993). Moreover, *it* is halotolerant, able to grow in environments with salt concentrations up to 13% and survive under 40% NaCl (Liu et al., 2005; Shabala et al., 2008). In humans, as a tribute to

its resilience, it can reside and grow within the gallbladder, exhibiting tremendous endurance towards bile salts (Hardy et al., 2004). This is an indispensable attribute for the pathogenic life phase of *Lm* since as a means of reaching the intestine, it must withstand and survive the gastric passage, where bile salts will be present.

All these qualities enable *Lm* to be a resilient ubiquitous bacterium present in soils, water, decaying vegetation, wild and domesticated animals, rural and urban areas (Vivant et al., 2013), and even transiently in the intestinal tract of healthy individuals (Schlech, 2019). Consequently, the ability to survive and thrive in rapidly changing environments is pivotal to *Lm* transmission, allowing it to effortlessly enter food processing environments (FPEs), either through contaminated raw materials, via fomites such as transportation creates/trolleys and personnel boots (Almeida et al., 2013; Fox et al., 2015; Kabuki et al., 2004), or even via asymptomatic employees transiently carrying the *pathogen* in their intestinal tract (Hafner et al., 2021). From this point on, it may persist in FPEs for months to decades (Harrand et al., 2020), undoubtedly leading to the contamination of foods or animal feeds. Consumption of contaminated foods will be pivotal in the transmission of the pathogen, introducing it to a new host (Figure 1).

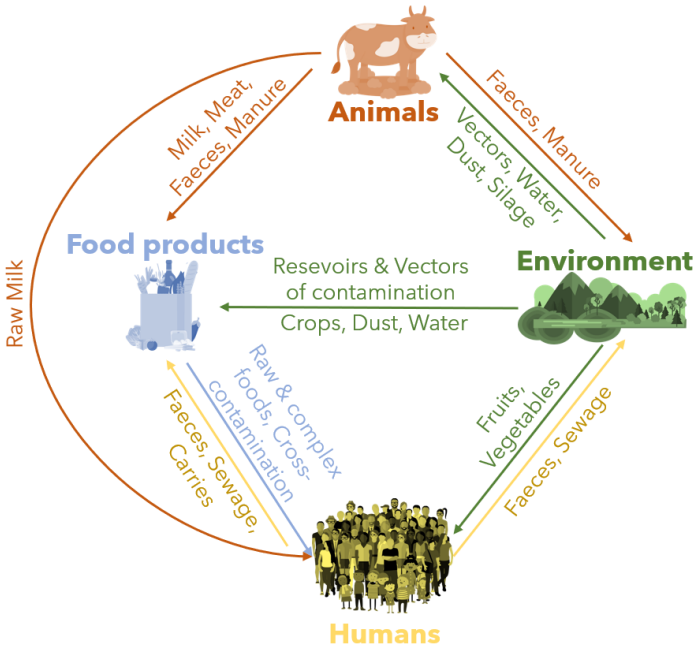

Figure 1. Routes of *Listeria monocytogenes* transmission.

The establishment of *Lm* in FPE is particularly worrisome since it will inevitably lead to its introduction into the food products themselves, leading to their rejection or, if undetected, could cause prolonged outbreaks.

According to the European Commission Regulation No. 2073/2005 (amended by EC No. 1441/2007) presence of *L. monocytogenes* in 25 g of RTE products, such as cheeses, before

leaving manufacturing facilities will lead to the destruction of the product. Furthermore, during their shelf life, *Lm* counts must not surpass 100 CFU/g, or the product will once again be eligible for destruction (European Parliament, 2005). In contrast, the United States policy perceives *Lm* as an adulterant, thus avoidable. As such, a "zero-tolerance" policy is practiced for RTE foods that support *Lm* growth (FDA, 2022; USDA-FSIS, 2014).

Both product rejection and prolonged outbreaks, end up creating a vast economic burden. Estimates point out that in 2018 in the US, the total cost of listeriosis, including morbidity, mortality, and lost revenue, was about $3.1 billion (USDA, 2021). Moreover, from 1994 to 2015 *Lm* was in the top three causative agents for food recall, and destruction, in the US (Gorton and Stasiewicz, 2017). Therefore, by preventing the occurrence of *Lm* in the food processing environment, we would be actively reducing product destruction, leading to a reduction in the industry's carbon footprint, as well as increasing the efficiency and sustainability of production, in line with the twelfth objective of the United Nations' goals for sustainable development (DESA U.N., 2015).

1.2 Listeriosis in cheeses and subsequent outbreaks

Aside from being widely available in the environment, *Lm* is also heavily associated with various food products ranging from vegetables, meats, fish, raw milk, and raw cheeses, including (RTE) foods like cheeses, salads, cold cut meats, and others (Churchill et al., 2019; Luchansky et al., 2017; Mena et al., 2004).

Besides being popular and nutritious, cheese is generally regarded as a safe product due to the sensitivity of many pathogens to the processing conditions involved in the production of most cheeses, particularly cooking and pasteurization temperatures. Nonetheless, in many countries, several foodborne illnesses, especially listeriosis, have been linked to the consumption of cheeses (Table 1). Over 2020, cheese was one of the top three food vehicles for *Lm* transmission, with one outbreak originating from cheese products. Listeria monocytogenes was detected in 0.5% of the 2,532 samples of soft and semi-soft cheeses collected at the processing stage in nine EU member states *at the retail level, 1.3% of all samples tested positive for Lm* (EFSA, 2021).

Due to their high-water activity, salt content and low acidity, fresh and soft-ripened cheeses are more susceptible to bacterial contamination and subsequent growth after the pasteurization process compared to semi-hard or hard cheeses, which have low moisture content (Campagnollo et al., 2018).

Although raw materials act as a source of contamination for FPEs, *Lm* isolated from the final product usually differs from *Lm* present in raw materials, thus suggesting that product contamination during processing is due to *Lm* present in niches or locations adjacent to the processing areas (Ojeniyi et al., 2000).

Table 1- Cheese associated listeriosis outbreaks from 2000-2021.

Country	Year	Serotype	Nº of reported cases (deaths)	Nº of reported maternal cases (fetal losses)	Reference
Mexico	2000	IVb	13 (-)	11 (5)	(MacDonald et al., 2005)
Sweden	2001	-	48 (-)	3 (-)	(Carrique-Mas et al., 2003)
Japan	2001	I/IIa	86 (-)	1 (-)	(Makino et al., 2005)
Canada	2002	IVb	48 (-)	2 (-)	(McIntyre et al., 2015)
USA	2003	IVb	12 (-)	-	(Bell et al., 2005)
Switzerland	2005	I/IIa	14 (3)	4 (2)	(Bille et al., 2006)
Czech Republic	2006	I/IIa	75 (13)	12 (2)	(Vit et al., 2007)
Germany	2006-2007	IVb, I/IIa, I/IIb	189 (26)	11 (7)	(Koch et al., 2010)
Canada	2008	I/IIa	38 (2)	14 (3)	(Gaulin et al., 2012)
Chile	2008	-	165 (14)	-	(Montero et al., 2015)
Austria & Germany	2009-2010	I/IIa	14 (5)	-	(Fretz et al., 2010)
Portugal	2009-2012	IVb	30 (11)	2 (1)	(Magalhães et al., 2015)
Spain	2012	I/IIa	10 (3)	4 (-)	(de Castro et al., 2012)
USA	2012	I/IIa	22 (4)	9 (1)	(Heiman et al., 2016)
USA	2013	-	5 (1)	1 (1)	(Choi et al., 2014)
USA	2015	-	30 (2)	6 (1)	(CDC, 2015)
USA	2017	-	8(2)	1(-)	(CDC, 2017)
USA	2021	-	13 (1)	4 (2)	(CDC, 2021)

1.3 *Listeria monocytogenes* evolutionary lineages, and their differences

In a phylogenetic sense, *Lm* isolates are spread across four distinct evolutionary lineages: I, II, III, and IV (Orsi et al., 2011), with 14 serotypes (Feng et al., 2020) and four PCR serogroups (Doumith et al., 2004), with 300 SLs (Sublineages) and more than 170 CCs (Clonal Complexes) (Moura et al., 2016a).

Lineages I and II encompass most *Lm* isolates. Coincidently both lineages harbour the most frequently clinically associated serotypes I/IIa (lineage II), I/IIb, and IVb (lineage I). Contrastingly lineages III and IV are rarely isolated and are primarily associated with animal isolates (Moura et al., 2016b; Orsi et al., 2011).

Among clinical and food/FPEs isolates, an uneven prevalence of lineages I and II can be observed (Maury et al., 2016a), with lineage II, mainly SL9 and SL121 (CC9 and CC121, respectively), strongly associated with food and FPEs isolates. Meanwhile, isolates of lineage I, SL1, SL2, SL4, and SL6 (CC1, CC2, CC4, CC6, correspondingly), are mainly involved in clinical cases in the Western world (Disson et al., 2021). However, the discrepancy in lineage prevalence between foods/FPEs and clinical isolates may be brought into question, as the

process of isolating *Lm* from clinical and food/FPEs samples differs. In clinical settings, non-selective media are employed, whereas, for food/FPEs, *Lm* selective enrichment procedures are conducted. The problem lies in these enrichment procedures, with some authors reporting a bias of particular enrichment methods towards isolates from lineage II which allows them to outgrow isolates from other lineages when present (Bruhn et al., 2005).

In terms of virulence, lineage I isolate particularly, CC1, CC4, and CC6, are hypervirulent compared with laboratory reference strains, such as EGDe and 10403S. By contrast, isolates from lineage II, more specifically, CC9 and CC121, are hypovirulent compared to the same laboratory reference strains (Maury et al., 2016b). The hypovirulence of isolates from lineage II can be partly attributed to internalin A (InlA) truncation, which is present in most isolates belonging to CC9 and CC121 (Roedel et al., 2019).

InlA, encoded by *inlA*, is a protein belonging to a family of 25 wall-anchored proteins called internalins, which bind to the eukaryotic cell membrane receptor E-cadherin, a receptor of the hepatocyte growth factor (HGF) (Radoshevich and Cossart, 2017). The bond between internalin A and the E-cadherin will induce endocytosis leading to bacterial uptake by the eukaryotic cell (Lecuit et al., 2001). Thus, the *inlA* gene and respective protein play a critical role in *Lm* virulence.

Nonetheless, although some instances of hypovirulence may be explained by InlA truncation due to premature stop codons (PMSC) in the *inlA* gene (Nightingale et al., 2008), lower levels of *inlA* gene expression are also responsible for the hypovirulence of lineage II isolates, while lineage I isolates normally present overexpression of *inlA* (Severino et al., 2007). It should be kept in mind that the hypovirulence of isolates from lineage II should not contribute to the perception of such isolates as avirulent since, even with truncated InlA, they can still invade epithelial tissues leading to infection. For lineage, I isolate InlA truncation is rare (van Stelten et al., 2010).

In addition to virulence properties, premature *inlA* truncation, leading to reduced length InlA, was claimed to enhance biofilm formation to detriment of virulence potential (Franciosa et al., 2009). Even though *inlA* mutations are more frequent in lineage II isolates (Nightingale et al., 2005), no clear-cut correlation between *Lm* serotype and biofilm-forming capabilities was yet established.

Lastly, gene expression profiles between lineages have also been shown to differ, with the main differences linked to genes related to cell wall synthesis, virulence, and the sigma B regulon (Severino et al., 2007).

1.4 Listeria monocytogenes persistence and its intricacies

Although extensively documented, *Lm* persistence in numerous food-associated environments is still not fully understood (Fox et al., 2011; Leong et al., 2017; Melero et al., 2019). By itself, the term persistence possesses a *lato sensu* connotation, describing a multitude of phenomena. For example, in cases where a pathogen resides in the same host for prolonged periods of time, we call it persistent colonization e.g., the case of typhoid Mary, a healthy chronic carrier of *Salmonella Typhi*, and *Staphylococcus aureus* nasal carriers (Soper, 1939; Wertheim et al., 2005). However, persistence is not only constrained to infections. Any clonal population of a microorganism which resides for an extended period of time in a specific environment, matrix, or surface can also be referred to as persistent (Ferreira et al., 2014). This concept of persistence may imply an enhanced ability of the pathogen to migrate and successfully colonize new environments. However, the lack of clear, well-constructed and commonly accepted empirical rules for defining persistence hinders the comparison of results in this field, as the persistent group in one published paper could be considered sporadic by the metrics of another study and vice versa.

Furthermore, the characterization or recognition of a strain as persistent or sporadic is dependent on the subtyping methods employed. Different typing methods are used, and persistence is generally declared upon recurrent isolation of the same typing profile over a sufficiently long span of time (Carpentier and Cerf, 2011). However, the discriminatory power (D) differs between subtyping methods. For example, when using a particular typing method, two isolates may be identified as the same strain and referenced as persistence. However, if a different method, with a higher discriminatory power, is used, these same isolates may be identified as different strains. Thus, when searching for persistent strains, subtyping methods with high discriminatory power should be employed (Ferreira et al., 2014). On the other hand, band-based typing methods such as pulsed-field gel electrophoresis (PFGE) often suffer from over-discrimination. For example, two isolates from the same strain may present different band patterns, usually by a mismatch of three or fewer bands which can be due to prophage or plasmid losses or gains (Stasiewicz et al., 2015). Due to the mismatch of two band patterns, isolates may be incorrectly classified as different strains.

Fortunately, whole-genome-sequencing (WGS) analysis, more specifically, single nucleotide polymorphism (SNP) based phylogeny, can improve the subtyping of foodborne pathogens (Stasiewicz et al., 2015; Taylor and Stasiewicz, 2019).

Another issue lies in the fact that sporadic or transient strains may be repeatedly reintroduced into FPE's, so they are identified as persistent strains when they are not. Some authors refer to these strains as "persistent transient" strains (Belias et al., 2022). With methods other than WGS, differentiating between repeated introduction and true persistence can be challenging, traditionally requiring comprehensive information gathering on facility setup and design, along with a frequent sampling of ingredients, equipment, facility grounds, and surrounding areas. However, WGS has established itself as a useful tool in differentiating persistence from repeated re-introduction (Stasiewicz et al., 2015).

Sampling methodology and techniques used are also other aspects to consider when delving into the topic of persistence. Ideally, we want to disturb as little as possible the environment we intend to study. New sequencing strategies such as 16S rDNA sequencing, metagenomics and metatranscriptomics are three commonly used methods for the taxonomic identification and characterization of bacterial communities and their interactions in food-related biomes (Fanning et al., 2017). These methods allow us to paint the most comprehensible picture of a target environment and its bacterial populations without massively interfering with the observed outcome. It would seem that these approaches are highly desirable in studying *Lm* persistence and its communities in FPEs. However, they are not flawless and ironically face difficulties in heavily cleaned environments frequently exposed to potent disinfectants, since isolating the total DNA from these environments can prove to be quite challenging (Anvarian et al., 2016). Thus, in these environments, we still rely on culture-dependent methods, and as previously mentioned, these methods may introduce some bias to our analysis.

Furthermore, the way sampling is conducted is also very important in avoiding any possible bias. The swabbing of an exposed and easily accessible surface to disinfectants, e.g., the top surface of a workbench, will not yield the same results as swabbing the crevices between floor changes. Hence meticulous and well-thought-out sampling also helps improve the picture of persistence in a particular FPE.

Overall, the only known fact agreed upon is that certain *Lm* strains are more frequently isolated from FPEs than others. However, there is no consensus on why this happens. Some authors search for any survival fitness against exposure to stress conditions in these frequently isolated strains (Lundén et al., 2008; Magalhães et al., 2016), either due to more efficient biofilm formation (Møretrø and Langsrud, 2004) or due to the expression of resistance genes to quaternary ammonium compounds, such as Benzalkonium Chloride which is commonly used in the disinfection of FEPs (Cherifi et al., 2018; Cooper et al., 2021), or due to overexpression

of resistance mechanisms to stress condition exposure (e.g., acidic, osmotic, and cold stresses). However, no direct correlation has yet been found.

Meanwhile, other authors attribute the persistence of *Lm* strains not to the intrinsic characteristics of the strains but to the numerous factors in FPEs. Examples of such factors are failures in food safety procedures due to a lack of proper hygiene and sanitation plans, disinfection of wet surfaces, incorrect dosage of disinfectant concentrations hindering their effectiveness, poor facility and equipment design that hampers cleaning/sanitation, strain prevalence and chance events. All these factors will enable the formation of hard-to-clean contamination niches where pathogens can survive and thrive (Aury et al., 2011; Pouillot et al., 2015).

1.5. Stress response mechanisms, genotypic and phenotypic characteristics of *Listeria monocytogenes*

Lm has several regulatory mechanisms that contribute to its ability to respond to and survive in rapidly changing circumstances. From its well-known ability to grow at refrigeration temperatures to its tolerance to acidic and high osmolarity environments, all attributed to the presence of tolerance systems in *Lm* pangenome. Hence the presence/absence, transcription or regulation of these genes may play a key role in persistence events.

1.5.1 Biocide resistance

Efficient sanitation of FPEs is a crucial step in mitigating possible contaminations on the finished product by *Lm*. When controlling undesirable organisms in FPEs, quaternary ammonium compounds (QACs), and cationic membrane active sanitizing agents capable of disrupting the phospholipid bilayer leading to leakage of the cellular content and subsequent cellular death, are used (Gerba, 2015). Compounds from this group are stable and present low toxicity and non-corrosive properties while virtually not interacting with organic matter making them a perfect choice for the sanitation of FPEs. Of all QACs, the class of benzalkonium chlorides (BCs) is one of the most commonly used, being constituted by an assortment of molecules with varying alkyl chain lengths of C8-C18 (Pereira and Tagkopoulos, 2019).

Tolerance mechanisms to these compounds are diverse, influenced by the environment, and strain-specific. Active efflux pumps are among the most pivotal mechanisms an organism possesses to exert tolerance towards QACs and other biocides (Romanova et al., 2006). Several proteins have been organized into five efflux-pump families: the small multidrug resistance (SMR) family, the multidrug and toxic-compound extrusion (MATE) family, the notorious

ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), and the resistance nodulation division (RND) family (Piddock, 2006). Many of the pumps present in these families are associated with the transport of a wide variety of foreign compounds: antibiotics, disinfectants, toxins, heavy metals, and dyes. Some of these accomplish the transport of more than one class of these substances. (Bolten et al., 2022; Collins et al., 2012; Guérin et al., 2014; Jiang et al., 2019; Romanova et al., 2006).

Concerning *Lm*, endogenously, we may find the operon fepRA, englobing the *fepA* gene coding the FepA efflux pump, from the MATE family, one of three described chromosomally encoded drug resistance efflux pumps in *Lm*, with the other two, MdrL and Lde, both belonging to the MFS. Interestingly, some studies have reported that, upon exposure to QACs, upregulation of endogenous efflux pumps such as FepA and MdrL occurs in strains lacking mobile genetic elements for biocide tolerance (Jiang et al., 2016; Tamburro et al., 2015). Thus, FepA and MdrL play an important role in the tolerance to low concentrations of BCs (Bolten et al., 2022; Jiang et al., 2019).

Regarding mobile genetic elements, we have the transposon located *qacH* gene and the gene cassette *bcrABC*, both of which encode efflux pumps from the SMR family. The QacH efflux pump is associated with the export of BCs from the bacteria cell. Its gene, *qacH*, is located in a chromosomally integrated Tn6188 transposon encompassing three transposase genes (*tnpABC*) along with the *qacH* and the accompanying putative transcriptional regulator (Müller et al., 2014). The *bcrABC* operon, besides conferring cadmium resistance, also encodes one helix-turn-helix (HTH) DNA binding motif *bcrA* and two small multidrug resistance genes, *bcrB* and *bcrC (Dutta et al., 2013; Elhanafi et al., 2010)*.

Some authors linked the presence of these genetic elements for biocide tolerance with the phenomenon of persistence (Cherifi et al., 2018; Cooper et al., 2021), while others have only found limited evidence between the presence of these elements in *Lm* and its persistence in FPEs (Daeschel et al., 2022).

Furthermore, and as previously mentioned, the tolerance to QACs conferred by these mechanisms is only advantageous in scenarios of exposure to low biocide concentrations, well below the level recommended for their use in FPEs (Bolten et al., 2022). Hence, the presence of these genetic markers may not be relevant in the establishment of persistence. Conversely, we could argue that possessing the capability to thrive in environments with low concentrations of QACs may be precisely the edge that persistent strains hold since, although they cannot survive the disinfection process, they can be the first to re-colonize a recently sanitized environment that still bears trace amounts of QACs.

1.5.2 Acid tolerance response

As previously stated, *Lm* frequently encounters acidic conditions in the multitude of environments it colonizes. Foods such as cheeses undergo significant acidification during the conversion of lactose into lactic acid. Moreover, in the FPEs, acidic decontamination is often performed as a sanitization measure. Even during the invasion of the host's intestinal tract, *Lm* will face acidic barriers. Hence, *Lm* had to develop a wide variety of metabolic and homeostatic mechanisms for acid tolerance in uninviting low-pH environments. These mechanisms include the glutamate decarboxylase system, the arginine and agmatine deiminase systems, and the proton pump F_0F_1-ATPase.

The glutamate decarboxylase (GAD) system is one of the major mechanisms for the maintenance of intracellular homeostasis under mild and harsh acidic conditions (Cotter et al., 2001). In *Lm* strains, this system is present in three separate genetic loci encompassing three homologous glutamate decarboxylases namely, GadD1, GadD2, and GadD3, and two glutamate/ γ-aminobutyrate antiporters GadT1 and GadT2. While GadD3 is independently coded by the *gadD3* gene, GadD1 and GadD2, and their respective antiporters, are coded by two operons *gadD1T1*, *gadD2T2* (Cotter et al., 2005). The first operon, *gadD1T1,* is reported to enhance growth under mildly acidic conditions, whereas *gadD2T2* plays a critical role in survival under intensely acidic conditions (Cotter et al., 2005, 2001). Notably, 31.5% of lineage II strains lack the *gadD1* gene (Chen et al., 2012) while the operon *gadD1T1,* appears to be absent in clinical isolates from the serotype IVb. Moreover, *gadD2* and *GadD2* are present in all *Lm* strains (Cotter et al., 2005).

The glutamate decarboxylase enzyme promotes the irreversible conversion of the negatively charge cytosolic amino acid (glutamate) into the neutral compound γ-aminobutyrate (GABA). In this process, intracellular protons (H^+) are expended, leading to the alkalinization of the intracellular environment, thus increasing the pH in the cell's interior. Additionally, GadT1 and GadT2 antiporters will export GABA out of the cell while bringing in extracellular glutamate. This will slightly neutralize the pH in the extracellular environment (Cotter and Hill, 2003).

Two other homeostasis systems involved in the protection of Gram-positive bacteria are the arginine deiminase (ADI) pathway and the lesser-known agmatine deiminase (AgDI) pathway. Relatively to the first system, the ADI pathway encloses three distinct enzymes, arginine deiminase, ornithine carbamoyl-transferase, and carbamate kinase, encoded by the respective *arcA*, *arcB*, and *arcC* genes present in the *arcABC* operon (Cheng et al., 2017).

Through the activities of the enzymes in this pathway, extracellular arginine is transported into the cell in an energy-independent manner by a membrane-bound antiporter encoded by the *arcD* gene, where it ends up catabolized into ornithine, originating carbon dioxide (CO_2), ammonia (NH_3), and ATP (Ryan et al., 2009). The ammonia produced as a byproduct of the system integrates intracellular protons yielding ammonium (NH_4^+), thus protecting the cell from adverse acidic stress (Ryan et al., 2009).

The AgDI pathway, although a lesser-known system, also increases *Lm* stress tolerance and has a similar principle to the ADI pathway yielding the same by-products (CO2, NH3, and ATP). However, the substrate for the pathway will be agmatine which will be catabolized into putrescine (Chen et al., 2011). Even though *Lm* harbours two putative AgDI genes, *aguA1* and *aguA2*, only AguA1 mediates the acid tolerance through the AgDI pathway (Cheng et al., 2013).

The ATP concomitantly synthesized in both pathways can also be expended on the active transport of intracellular protons to the cell's exterior by resorting to proton pumps such as the multi-subunit enzyme system F0F1-ATPase, involved in acid tolerance response to mild acidic stresses (Cotter et al., 2000). This system consists of two distinct domains. Domain F0 is located in the membrane and is incumbent for proton translocation. The cytoplasmatic F1 domain, for the purposes of maintaining homeostasis, conducts the ATP catalysis needed for proton extrusion.

All the three above-described systems act simultaneously to ensure survival and adaptation to acid-stress environments.

Additionally, two other systems have been linked to *Lm* acid tolerance. The thiamine uptake systems, coded by the *thiT* gene, and the two-component LisRK signal transduction system containing the *lisR* and *lisK* genes. Mutants lacking either of these systems were unable to achieve full acid tolerance (Cotter et al., 1999; Cotter and Hill, 2003; Madeo et al., 2012). Moreover, LisRK has also entangled *Lm* stress survival in low-temperature environments (Pöntinen et al., 2015).

Lastly, different *Lm* strains display varying degrees of acid tolerance, implying that strains possess differences in robustness and pathogenicity. Therefore, it is plausible that persistent strains exhibit better tolerance toward acid environments than sporadic isolates (Lundén et al., 2008), either due to the presence of more acid tolerance systems or due to improved upregulation of the systems themselves.

1.5.3 Low temperature survival

From surviving in the environment, where it experiences a wide range of temperatures, to contaminating food products that at some point will be refrigerated (2-4 ºC) or even colonising a host where it will have to grow at a body temperature, Lm faces the need to tolerate a wide range of temperatures. It is, therefore, unsurprising that this pathogen has developed mechanisms that allow growth over a wide range of temperatures (-0.5 to 45º C) (Allerberger, 2003). Regarding cheeses and cheese processing plants, *Lm* will regularly encounter the lower end of the spectrum, facing refrigeration and near refrigeration temperatures. The temperatures in these environments will lead to decrease metabolic rates and loss in membrane fluidity due to transitions from a fluid-like phase to a gel phase, hindering enzyme function and transport across the cell membrane.

To protect itself, *Lm* will resort to physiologically changing the composition of its membrane, increasing the presence of unsaturated fatty acids, employing shorter acyl chains (from C17:0 to C15:0), and altering the ratios of iso and anteiso-branched fatty acids to maintain the normal fluidity encountered at lipid phase temperatures (Chan and Wiedmann, 2008).

Furthermore, *Lm* will also uptake cryoprotectants such as glycine betaine and carnitine. These two major compatible solutes for uptake by *Lm* from the various available ones. Besides providing cryoprotection, they are also involved in the tolerance towards osmotic stress. The chill-activated glycine betaine transporter is encoded by the glycine betaine operon *gbuABC*, with the glycine betaine uptake system heavily reliant on the *betL* gene (Sleator et al., 2000; Wemekamp-Kamphuis et al., 2004).

Regarding carnitine, a two-protein (OpuCB and OpuCD) transmembrane complex encoded on the *opuCABCD* operon is involved in its uptake by the cell (Ko and Smith, 1999). Strains possessing mutations in these genetic units have shown increased sensitivity towards cold and osmotic stresses (Singh et al., 2011).

Other mechanisms used by *Lm* to adapt to cold temperature environments include the expression of the cold shock-domain family proteins (Csps), which are mainly produced in the temperature range of 4-10 ºC. Csps bind to nucleic acids, stabilizing them and acting as molecular chaperones that facilitate replication, transcription, and translation in adverse conditions. Hence Csps regulate the expression of various genes, including stress tolerance genes. Of the identified Csps, three proteins, CspA, CspB, CspD, have been linked with tolerance to low temperatures, although with varying degrees of importance (Schmid et al., 2009). Additionally, they also seem to be involved with resistance to osmotic stress (Schmid et al., 2009).

Ferritin-like protein (Flp), belonging to the cold shock-domain family, is another protein highly induced in response to severe cold conditions, which may suggest its involvement in the cold stress response (Hébraud and Guzzo, 2000).

Finally, yycGF and lisRK are also involved in *Lm* survival in cold environments. YycGF protein is mostly involved in early-stage cell survival to cold, while the proteins from lisRK are rather responsible for cellular acclimatization to prolonged exposure to cold environments (Pöntinen et al., 2015).

1.5.4 Osmotic shock survival

Environments with high osmolarity, due to high concentration in solutes, will present lower levels of water activity. In these conditions, plasmolysis may occur and cell turgor decrease, thus inhibiting cell growth. Furthermore, high solute concentrations will disrupt the electrochemical potential around the cell membrane, thus hindering ATP production through oxidative phosphorylation.

As a foodborne pathogen, *Lm* will regularly face osmotic stresses due to the colonization of foods with high salt or sugar concentrations (e.g., cheese) or foods where salting is the preservation method employed. Therefore, this pathogen has developed responses toward osmotic stresses, also known as osmoadaptation, a two-stage process (Hill et al., 2002). Firstly, after exposure to high salt concentrations, *Lm* will perform physiological changes to maintain its turgor by increasing the intake of potassium ion (K+) and the negatively charged amino acid glutamate. During this first stage, the KdpABC transporter system plays a key role in the transport of K+ crucial for the adaptation to high salt concentrations (Ballal et al., 2007). During the second phase, the cell will proceed with the replacement of the accumulated K+ with compatible solutes or other low-molecular-weight osmolytes (Sleator et al., 2003). This uptake of compatible solutes is crucial in restoring volume and turgor pressure as well as stabilizing the structure and, consequently the function of cell proteins. In the absence of these compatible solutes, *Lm* stress protein Ctc provides osmotolerance (Gardan et al., 2003).

As previously discussed, glycine betaine and carnitine are two major solutes by use *Lm* as cryoprotectants (Chan et al., 2007). These compatible solutes are also used as a defence in environments with high osmolarity.

1.6 Fourier-transform infrared (FTIR) spectroscopy

Fourier-transform infrared (FTIR) spectroscopy is a rapid, non-invasive, inexpensive, easy, and high-throughput physicochemical analytical method that detects chemical bonds based on their vibrational modes (e.g., stretching or bending, wagging scissoring, or twisting) mid-infrared (IR) radiations (Griffiths and de Haseth, 2006). The specificity of the vibrations associated with each functional group of the cellular constituents, including nucleic acids, proteins, polysaccharides, fatty acids, and water (Naumann, 2000), allows for inferring the biochemical composition of microorganisms.

In microbiology, FTIR spectroscopy has been applied in detecting bacteria of interest in specific matrices (Al-Qadiri et al., 2006; Davis et al., 2010; Grewal et al., 2015) or as a phenotyping method that quickly identifies and classifies unknown microorganisms using spectral libraries and or chemometric tools (Campos et al., 2018; Rodrigues et al., 2020). However, the discriminatory power of this technique frequently correlates with the availability of robust and extensive spectral libraries with well-characterized reference specimens. Furthermore, FTIR spectroscopy has also been used to study stress-related metabolic or structural adaptations of organisms when exposed to varying stresses (e.g., temperature, salinity, high pressure) where subtle compositional changes could not be revealed by using other transcriptomics or proteomic methodologies (Alvarez-Ordóñez et al., 2010; Beney et al., 2004; Karatzas and Bennik, 2002; Mille et al., 2002).

1.6.1 Multivariate data analysis

Although the first applications of IR spectroscopy to characterise bacteria can be traced back to the 1950s, the lack of mathematical tools capable of handling the large quantity of data and its multivariate nature made unfeasible the extraction of all of the useful information from the gathered spectra, leading to its low usage throughout the latter half of the twentieth century. Nevertheless, during the 1990s, the emergence of modern software tools with multivariate mathematical models and potent hardware with higher processing power, coupled with developments in chemometrics and quite well-established principles for microbiological applications, paved the way for spectral data analysis aiming bacterial discrimination, classification, and identification at varying taxonomic levels (genera, species, and subspecies level).

To properly extract the maximum reliable information from the acquired IR spectra, a two steps approach is commonly undertaken, which includes (I) the spectral, pre-processing,

and (II) modelling. Regarding spectral pre-processing, the application of corrective methods able to attenuate spectral variations caused by light dispersion phenomena, absorption of atmospheric vapour and carbon dioxide, and differences in the sample amount, among other occurrences, is mandatory. The pre-processing step improves the data quality and boosts the success of the ensuing modelling step.

Among the most common pre-processing methods used to analyse IR spectral data are the (I) normalization, as standard normal variate (SNV) and the multiplicative scatter correction (MSC), and the (II) filtering, as the Savitzky-Golay (SavGol) filter or the orthogonal signal correction (OSC) on the (III) centring ones as mean centering and Pareto scaling. A brief description of those used to analyse the IR data obtained during this work will be provided; namely SNV, the Savitzky-Golay filter and mean centering.

Standard normal variate (SNV) improves the accuracy of prevision without simplifying the model or reducing its interferences. This is achieved by subtracting the row mean from every sample and dividing it by the standard deviation (Zeaiter and Rutledge, 2009). The Savitzky-Golay filter, commonly including spectral derivation, is a smoothing data filter that increases precision without distorting the original signal tendency by removing variations in the simple baseline (Savitzky and Golay, 1964). The SavGol filter parameter (n-smoothing points, x-polynomial order and Y-derivative order) can be optimized toward the higher model robustness. Mean-centering allows the identification and representation of the geographical centre in a set of features, removing the relative intensity of each variable and revealing only data oscillations around the average value, which is of particular relevance to reducing spectral differences that arise from different sample amounts used to obtain the IR spectra.

After the application of the appropriate pre-processing methods, spectra are suitable for the modelling steps. Spectral modelling could be undertaken by supervised or non-supervised chemometric methods, whose main difference relies on the knowledge or not of the sample's classes. Among the most common non-supervised methods are principal component analysis (PCA) and hierarchical cluster analysis (HCA), which can be employed to search for natural clusters among samples. The supervised methods presuppose the knowledge of the sample classes and are frequently used for classification purposes. Partial least-squares discriminant analysis (PLSDA) is an example of this kind of method and is widely used to model infrared spectral data.

This method is a versatile algorithmic tool used for predictive and descriptive modelling, along with combining multivariate dimensionality reduction with discriminative analysis (Lee et al., 2018). Besides its use in reducing dimensionality, it can also be adapted to feature

selection and classification (Ruiz-Perez et al., 2020). In PLSDA, each known sample (xi, bacteria spectra) is assigned a vector of zeros with value one at the position corresponding to its class (yi, in this study, the persistent/sporadic or T=11 ºC/ T=30 ºC). The structure of the PLSDA model is described by Eqs. (2) and (3). Model loadings (P and Q) and corresponding scores (T and U) are obtained by sequentially extracting the components or latent variables (LVs) from matrices X (spectra) and Y (matrix codifying the proteins).

$$X = TPt + E \quad (2)$$

$$Y = UQt + F \quad (3)$$

The prediction (ynew = [ynew,1, ynew,2, … ynew,n]) is then converted into a class assignment (persistent/sporadic or T=11 ºC/ T= 30 ºC) from which confusion matrices are obtained. During the development of PLSDA model, 70% of the samples are randomly selected to obtain the regression matrix B, with 30% used as new samples for prediction. This procedure repeats 100 times per PLSDA model, and confusion matrices present the mean values of correct class assignments for the prediction samples.

1.7 Objectives and thesis outline

Three fundamental ideas lay the groundwork for this thesis:

1. Persistent strains possibly possess different growth kinetics than sporadic strains (higher growth rates, shorter lag phases), allowing them to outgrow sporadic strains, thus explaining their recurrent isolation in food processing environments.

2. Persistent strains and sporadic strains may possess phenotypical differences in their membrane. Furthermore, persistent strains may rearrange their membrane more efficiently than sporadic strains to overcome adverse environmental conditions. Thus, we may detect phenotypic differences between growth at an optimal (30 ºC) and adverse temperature (11 ºC).

3. Persistence may correlate with the acquisition of resistance genes from mobile genetic elements present in the processing environment, which confer enhanced tolerance to stresses present in processing facilities. Thus, persistent strains may display differences in their accessory genome compared to sporadic strains.

With this in mind, seventeen *Lm* isolates were exposed to combined environmental stresses frequently encountered in food processing environments. Furthermore, FTIR spectra from the seventeen isolates grown at 11 and 30 ºC were collected. Lastly, the pangenome of all seventeen isolates was analysed, and their accessory genome was compared (Figure 2).

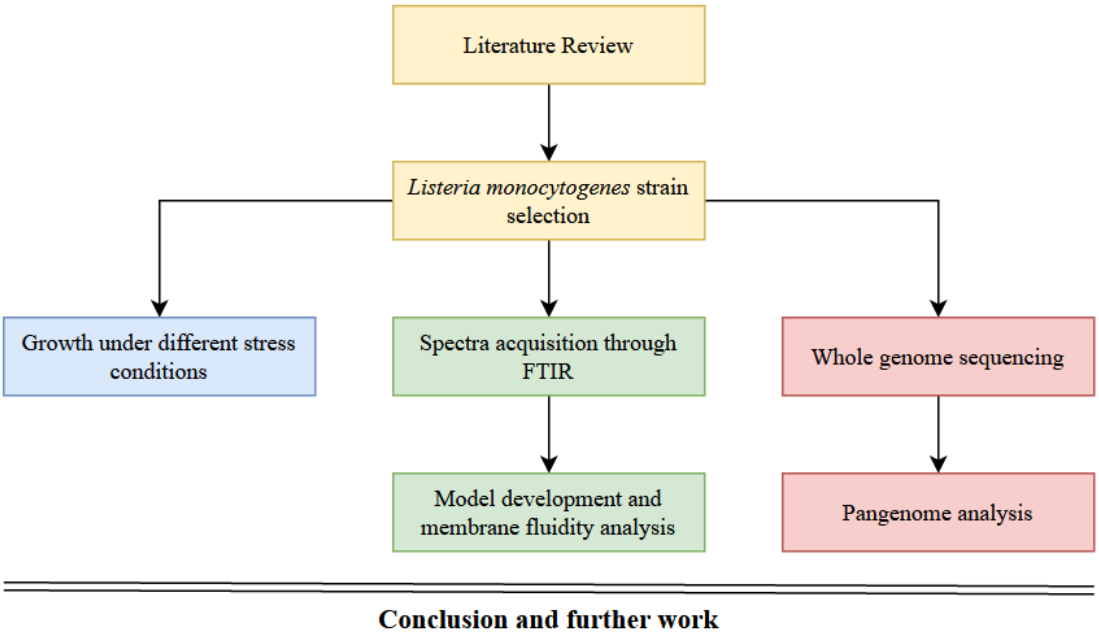

Figure 2. Graphical representation of the thesis outline.

2. Materials and Methods

2.1 Bacterial strains, persistence definition, and bacterial storage conditions

For the purposes of this study, seventeen *Lm* isolates were selected from a broader collection belonging to the *Listeria* Research Center of Escola Superior de Biotecnologia (LRCESB). These isolates were collected as part of a previous longitudinal study by Almeida et al. (2013), being recovered from samples taken across two Portuguese cheese manufacturing facilities (Small scale industrial cheese producer – ISS, Industrial cheese producer – ICP) over a span of 3 to 4 years (Table 2) and identified as persistent or sporadic based on PFGE profiles. Among these seventeen, nine were classified as persistent and eight as sporadic. Isolates with the same PFGE profile recovered ≥ two times over ≥ six months in the same cheese manufacturing facility were classified as persistent.

An attempt was made to ensure that in the isolates from each group, persistent and sporadic, both evolutionary lineages, I and II, were represented in similar numbers. Another selection criterion focused on the place of origin of each isolate. Isolates were selected to comprise a wide array of sample origins, from environmental and raw material samples to

fomites and finished product samples. Furthermore, for the persistent group, an effort was made to encompass several isolates from the same PFGE type collected over a reasonably long period of time.

Stock cultures of *Lm* strains were preserved in Brain Heart Infusion broth (BHI, Biokar Diagnostics, Beauvais, France) supplemented with 20% (v/v) glycerol at -80 ºC.

Table 2. Selected *Listeria monocytogenes* isolates.

Classification	Isolate Code	PFGE profile	Isolate origin	Isolation date		SeroType	Manufacturer	Lineage
Persistent	Lm797	Ka	Environmental, factory floor	17/06/2003	4 Years	I/IIb	SSI	I
	Lm1033	Ka	Environmental, Warehouse floor	20/04/2004		I/IIb	SSI	I
	Lm1592	Ka	Goat cheese	31/05/2005		I/IIb	SSI	I
	Lm1108	Kb	Cheese Mixture	25/05/2004		I/IIb	SSI	I
	Lm2047	Kb	Environmental, factory drain	27/02/2007		I/IIb	SSI	I
	Lm1635	Da	Raw milk	20/07/2005		IVb	APC	I
	Lm1699	E	Environmental, factory floor	09/11/2005	2 Years	I/IIa	SSI	II
	Lm2116	E	Environmental, factory drain	17/07/2007		I/IIa	SSI	II
	Lm2123	E	Environmental, factory drain	25/09/2007		I/IIa	SSI	II
Sporadic	Lm747	Ha	Cow Cheese	21/05/2003		I/IIb	SSI	I
	Lm812	Hb	Environmental, factory sink	29/07/2003		I/IIb	SSI	I
	Lm832	La	Environmental, factory sink	19/08/2003		I/IIb	SSI	I
	Lm929	C	Cheese	10/02/2004		I/IIa	APC	II
	Lm930	F	Cow's raw milk	16/03/2004		I/IIa	SSI	II
	Lm994	G	Goat's raw milk	19/02/2004		I/IIa	SSI	II
	Lm1302	M	Envrionmental, factory drain	26/05/2004		I/IIb	SSI	I
	Lm1712	A	Environmental, factory floor	07/12/2005		I/IIa	APC	II

2.2. Inoculum and stress media preparation

When preparing inoculum for growth curve assays and FTIR spectroscopy, Brain Heart Infusion agar (BHI, Biokar Diagnostics) plates were aseptically streaked with inoculum from the frozen stock and incubated at 37 ºC overnight. Thereafter, single colonies of each *Lm* strain were inoculated separately into 9 ml of BHI broth and incubated at 37 ºC for 17 hours. This cell suspension was sub-cultured (1% v/v) into 9 mL of BHI and incubated under the same conditions as before.

Stress media pH and salt concentration of the media for growth under stress conditions were adjusted with lactic acid ≈ 80% (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and NaCl (≥ 99.0%, Labchem, Zelienople, PA, USA) respectively.

2.3. Experimental Design

For the growth under stress conditions, a three-level, three-variable CCD (Central Composite Design; Table 3) was adopted in this study and required fifteen experiments, which included six factorial points and three central points to provide information about the interior of the experimental region, allowing for the visualization of curvature (Table 4).

Table 3. Variables and levels for Central Composite Design.

Coded variable levels				
Variable	Symbol	-1	0	1
Salt Concentration (%v/w)	NaCl	2.50	4.00	8.00
Temperature (ºC)	Temp	11	22	30
pH	pH	5	6	7

Table 4. Central composite design arrangement for *Listeria monocytogenes* growth under extreme stress conditions.

Variable levels				Responses			
Run	pH	Temp	NaCl	pH	NaCl (% w/v)	Temperature (ºC)	Incubation Period (h)
C1	-1	-1	-1	5	2.5	11	168
C2	-1	-1	1	5	8.0	11	168
C3	-1	1	-1	5	2.5	30	168
C4	-1	1	1	5	8.0	30	168
C5	1	-1	-1	7	2.5	11	72
C6	1	-1	1	7	8.0	11	168
C7	1	1	-1	7	2.5	30	24
C8	1	1	1	7	8.0	30	72
C9	-1	0	0	5	4.0	22	168
C10	1	0	0	7	4.0	22	72
C11	0	-1	0	6	4.0	11	72
C12	0	1	0	6	4.0	30	48
C13	0	0	-1	6	2.5	22	48
C14	0	0	1	6	8.0	22	120
C15	0	0	0	6	4.0	22	72

2.4. Growth responses under stress conditions

All seventeen isolates were grown under fifteen conditions from the CCD. The cultures were prepared as described in section 2.2. For each isolate, cultures were decimally diluted in BHI broth to a level of ≈ 1.0 x 10 -4 CFU/mL, with the last dilution being performed directly into each stress media. 200µL were then transferred to a 96-well microplate in triplicate for each sample. The temperature and duration of the incubation differed accordingly to the test condition (Table 4). Hourly measurements of Optical Density at 600nm (OD600) were performed in a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Data acquisition (lag phase- *lag*, maximal growth rate- *µmáx*, and duplication time - *dt*) and visualization were performed using the proprietary software SkanIt RE (version 6.0.2, Thermo Fisher Scientific). All experiments were carried out in triplicate.

At the beginning and the end of each growth curve, decimal dilutions were performed in Ringer's solution (VWR Chemicals, Poole, UK) and plated on BHI agar plates, in duplicate, by the drop plate technique to determine CFU/ mL values.

2.5. Statistical analysis

For all investigated parameters, Student's t-test was performed to assess whether any statistically significant difference would occur between persistent and sporadic groups. The significance level assumed was 5%. All calculations were carried out using IBM SPSS Statistics software (Version 28.0., IBM Corp, Armonk, NY, USA).

2.6. Infrared spectra acquisition

For all seventeen isolates, IF spectra were acquired from subcultures in BHI broth and inoculated as described in section 2.2. After incubation, for 17h hours at 30 ºC and 72 hours at 11 ºC, cultures were centrifuged for 5 minutes at 10 000 rpm. The cell pellets were washed thrice with Ringer's solution to ensure the removal of any trace elements of the growth media on the final pellet. Throughout the experimental work, the same batch of culture media was used.

Spectra acquisition was carried out on a PerkinElmer Spectrum 100 FTIR spectrometer fitted with a MIR TGS detector and a PIKE Technologies MIRacle Attenuated total reflectance (ATR) accessory according to the following parameters, wavenumber 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1} and 32 scan co-additions. The final cell pellet was spread onto the ATR crystal and allowed to dry on the crystal surface until a thin film formed.

Nine spectra were acquired for each isolate, corresponding to three instrumental replicates x three independently obtained cell cultures (biological replicates). Backgrounds (empty ATR crystal) were obtained between each isolate acquisition.

2.7. Spectral pre-processing and modelling

All acquired FTIR-ATR spectra were pre-processed with, SNV followed by the application of the Savitzky-Golay filter parameters (Savitzky and Golay, 1964) and further mean-centred prior modelling. The SavGol filter parameters (9/12/15- smoothing points, 2^{nd} polynomial order and $1^{st}/2^{nd}$ derivate order) and the spectral region used for modelling were optimized. The spectral region was optimized, according to figure 3, and the PLSDA models were developed considering the four spectral regions alone and in combination. The best pre-

processing conditions were selected based on the higher percentage of correct class assignments obtained from confusion matrices generated.

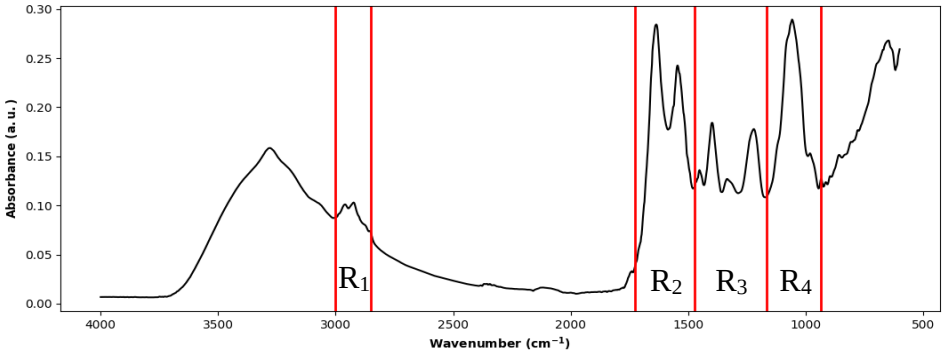

Figure 3. Typical FTIR-ATR spectra with wavenumber regions, (R_1, 3000-2800 cm^{-1}; R_2, 1700-1500 cm^{-1}; R_3, 1500-1200 cm^{-1}; and R_4, 1200 and 900 cm^{-1};), used in the PLSDA model optimization

All data analysis was carried out in Matlab version 7.9 (Math-Works, Natick, MA) and the PLS Toolbox version 5.5.1 for Matlab (Eigenvector Research, Manson, WA).

2.8. Genome assembly, annotation, and accessory genome analysis.

Genomic DNA was extracted using the GRS Genomic DNA Kit – Bacteria (GRISP®, Porto, Portugal), according to the manufacturer's protocols. The concentration of DNA was determined using the Qubit™ Fluorometric dsDNA HS assay kit (Thermo Fisher Scientific). Sample quality was confirmed by NanoDrop (Thermos Fisher Scientific) (260:280-nm and 260:230-nm ratios were between 1.8-2.0 and 2.0-2.2, respectively). The DNA libraries for bacterial genome sequencing were prepared from 200 ng of high-quality genomic DNA with the TruSeq Nano DNA Library Kit (Illumina, San Diego, USA) and sequenced using paired-end (PE) 2x300 bp on the NextSeq™ 550 Illumina® platform at Genoinseq (Cantanhede, Portugal). All procedures were performed according to standard manufacturer's protocols. Sequenced reads were demultiplexed using Bcl2fastq (Illumina, San Diego, USA) and quality filtered with Trimmomatic version 0.32 (Bolgeret al., 2014) using the following parameters: 1) bases with average quality lower than Q15 in a window of 5 bases were trimmed, 2) reads with less than 50 bases were discarded and 3) sequencing adapters on reads were removed.

Draft genome assembly was performed using SPAdes (Species Prediction and Diversity Estimation) software version (2.5.1), which constructs contiguous sequences through the usage of an algorithm that employs multisized De Bruijn graphs with K-mer values of '21, 33, 55, 77' (Bankevich et al., 2012). All draft genomes were annotated using Prokka algorithms (Seemann,

2014). Pan-genome analysis of the seventeen *Lm* isolates was conducted by employing the Anvi'o software (version 7.1) (Eren et al., 2015).

3. Results & Discussion

3.1 Growth responses under stress conditions

After two weeks, no noticeable growth was observed when the pH value of the stress media was 5.0 (C1, C2, C3, C4 & C9). Bacterial enumeration revealed that none of the seventeen isolates survived prolonged exposure to lactic acid at pH 5.0. A confirmatory assay in BHI at pH 5.0 was conducted to evaluate if the outcome would remain the same. Once again, no growth was observed, and *Lm* counts were below the detection limit of the enumeration technique (< 50CFU/mL).

This result is not unsurprising, considering that lactic acid, the acid of choice employed to mimic the cheese processing environment, is a weak organic acid with higher antimicrobial action than its strong acid counterparts. Due to its nature, it is hardly deprotonated under a low environment pH and will, therefore, passively cross the cell membrane. In the cytoplasmatic environment, at a higher pH value, it will deprotonate, lowering the intracellular pH (McLaughlin and Dilger, 1980).

The lack of growth at pH 5.0 made the CCD incomplete and, therefore, invalid. However, the remaining conditions defined in the design were still used to investigate grow kinetics of the isolates under stressful conditions.

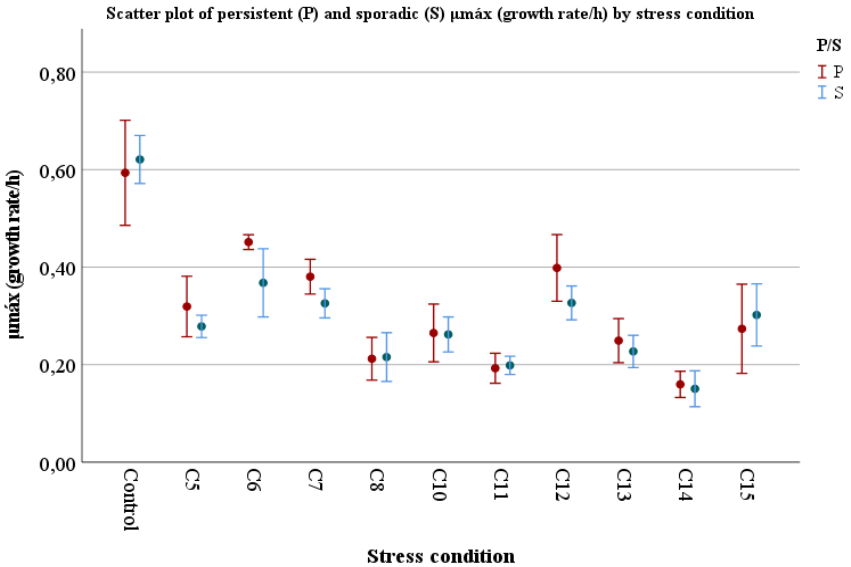

Figure 4. Maximal growth rate. The y axis represents the maximum growth rate (calculated from the maximum slope). In blue and red, respectively, we have represented the average µmáx value for the eight sporadic strains, and the average µmáx value for the nine persistent isolates. For condition C6, C7 and C12, persistent strains demonstrated a significantly higher

The growth characteristics of every tested isolate were evaluated under optimal conditions (BHI at 37 ºC for 24 hours) to develop a baseline to which all other tested conditions would be compared. As expected, the highest values for the specific growth rate were observed under this condition. For persistent and sporadic isolates, the mean $\mu máx$ value was 0.60 h-1 (SD = 0.1) and 0.6 h-1 (SD = 0.06), respectively, with no statistically significant differences ($p>0.05$) between the $\mu máx$ of both groups of isolates. The similarity of the growth kinetics between groups of persistent and sporadic isolates may mean that either the kinetic characteristics of the strains overlap any expression of persistence or, if they exist, kinetic growth characteristics correlated with persistence are not expressed in optimal growth conditions. Altogether, this observation aligns with the reported data, which states the existence of high strain-to-strain variability of *Lm* isolates to acidic and osmotic stresses (Horlbog et al., 2018), which in our study overcame the classification between persistent and sporadic. Furthermore, strain variability also extends to virulence and antimicrobial resistance profiles, as described in Ciolacu et al. (2015), Tang et al. (2013), and Wu et al. (2016).

Isolate Lm1108 (P), an isolate classified as persistent (P), demonstrated the highest growth rate of all the isolates tested in BHI, 0.8h-1 (SD = 0.03). Meanwhile, isolate Lm2123 showed the lowest growth rate, 0.30 h-1 (SD = 0.056). Additionally, during growth, the average lag times for persistent, 10.7 h (SD = 1.3), and sporadic isolates, 10.9 h (SD = 2.0), were highly similar ($p>0.05$). Noticeably, all tested isolates showed uncharacteristically low growth rates and extensive lag phases for *Lm*, with Lm747 (S), an isolate classified as sporadic (S), possessing the most extensive lag phase of 15.0 h (SD = 0.6). The lag phases of Lm1302 (S), Lm1635 (P), and Lm2123 (P) lasted approximately 9.0 h, being the shortest lag phases of all tested isolates. These uncharacteristically long lag times and reduced growth rates might be due to the growth experiments being conducted in 96-well microplates instead of traditional conical flasks, presumably leading to differences in culture aeration, thus reducing growth rates and increasing lag times.

Interestingly, clones of the same strain (Lm797, Lm1033, Lm1108, Lm1592, and Lm2047) showed different growth parameters, possibly indicating that this technique may not be adequate to assess kinetic differences in growth patterns or may indicate that the isolated clones have changed phenotypically throughout the isolation /storage in the frozen state period. Additionally, it could also mean that the presumed clone is not actually clones of each other.

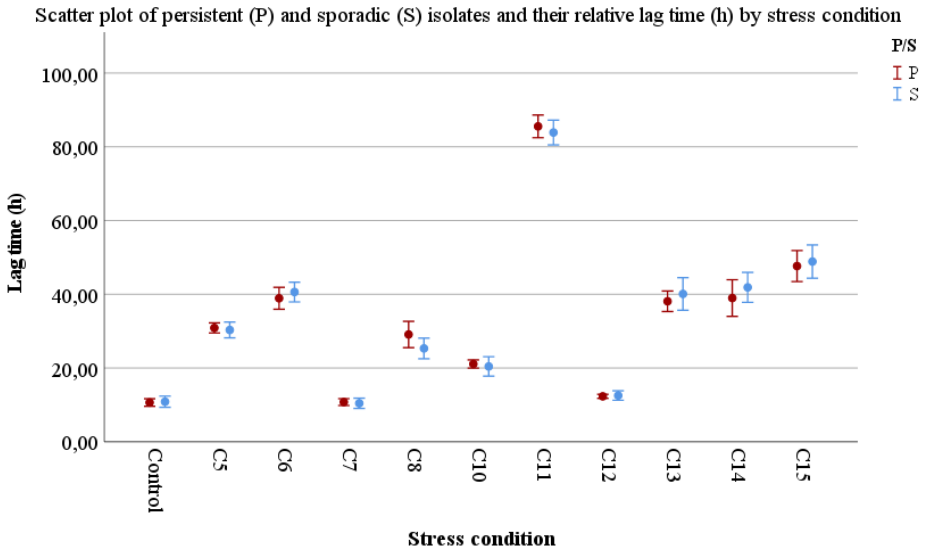

Figure 5. Relative lag time. The y axis represents the lag time (defined as the last time point before the growth curve entered exponential growth). In blue and red, respectively, we have represented the average lag time value for the eight sporadic isolates and nine persistent isolates.

For all isolates, the stress conditions investigated reduced growth rates and increased lag times compared to control conditions ($p < 0.05$). However, all seventeen *Lm* isolates showed exceptional resilience towards environmental stresses managing to survive and grow under stress conditions, including the ones combining low temperatures (11 ºC) and high osmotic stress caused by 8.0% NaCl. Nevertheless, this growth was not astonishing since, as previously mentioned, *Lm* can grow in refrigeration temperatures. Furthermore, Alves et al. (2022) reported the isolation of *Lm* cells in spiked cooking salt, with a water activity (aw) of 0.49, over a hundred and four days after the initial inoculation.

From all stress conditions, C11 proved to be the most challenging condition for *Lm* isolates to adapt and grow, leading to the longest lag phases for both persistent and sporadic strains, 85.6 h (SD = 4.0) and 84.0 h (SD = 4.7) respectively (Figure 5), along with the lowest growth rates, 0.2 h^{-1} (SD = 0.04) and 0.2 h^{-1} (SD = 0.02) accordingly (Figure 4). Concerning lag times, persistent and sporadic strains were overall homogenous, with no significant differences ($p>0.05$) among groups observed across the stress conditions (Figure 5).

Regarding conditions C6, C7, and C12, persistent isolates demonstrated significantly higher growth rates than sporadic isolates ($p>0.05$). However, questions are to be raised about the reliability of this data. The overall tendency for higher growth rates of the persistent group over the sporadic group was not observed in any other tested condition, with both groups generally showing homogenous growth rates ($p<0.05$). Although the environments present in C6 and C12 were among some of the most challenging conditions in the experimental design, which could potentially substantiate why differences between the persistent and sporadic

isolates were only observed in a few selected conditions, in C11, differences between these groups were not observed. This is odd, considering C11 proved to be the most demanding condition for both groups to grow. C7, by contrast, represented a lenient environment and was highly resemblant to the control group, yet persistent/sporadic differences were not noticed when growing in BHI. Additionally, the differences between both groups' growth rates in these three conditions, although statistically significant, are marginally different, presenting erratic patterns contributing to reasonably high standard deviations (Figure 4).

Therefore, it would not be wise to perform the rejection of the null hypothesis regarding growth rates between persistent and sporadic groups, with more confirmatory assays needed to be performed to assure the robustness of the results. An increase in the number of tested isolates would also be desirable since some of the isolates comprehend clones of the same strain, thus reducing the variability of the persistent group. Furthermore, in the matter of lag times, none of the two groups presented significant differences between lag phases which justify the rejection of the null hypothesis once more. No clear pattern of a particular isolate or grouping of isolates consistently overshadowing the remaining isolates across all stress conditions was observed. Isolates with the highest growth rates or lowest lag times varied accordingly with stress conditions. Once again, this may imply that the approach used is not the most effective in discriminating relations of kinetic growth between isolates possible due to low reproducibility.

In summary, given the data collected, it is unlikely that persistent *Lm* isolates inhabiting the same environment as sporadic strains are better adapted, in terms of growth kinetics parameters, to grow in that environment when exposed to normally extrinsic stresses found in that environment. These findings conflict with the works of Lundén et al. (2008) and Magalhães et al. (2016). Both studies provided evidence that persistent strains experienced higher tolerance when exposed to an acidic condition of pH 2.5 for two hours and possessed lower lag times than sporadic strains in 2.5, 4.0 and 8.0% NaCl along with pH 5.0, adjusted with HCl. Nevertheless, it should be highlighted that Lundén et al. (2008) investigated growth under sub-lethal acid stress, which was not attempted in the present study.

Classification methods and sample sizes may also contribute to the discrepancy in results. As in the presented study, both Lundén et al. (2008) and Magalhães et al. (2016) classified persistent and sporadic isolates based on PFGE typing. As previously mentioned, the action of prophages can influence the results of this restriction pattern-based analysis technique, potentially misclassifying persistent/sporadic isolates due to over-discrimination. On top of that, the empirical rule to identify persistent strains varied among studies. In Lundén et al. (2008), persistent strains were defined as strains isolated ≥ five times over a span of ≥ to three

months. By contrast, Magalhães et al. (2016) defined persistence as the repeated isolation of *Lm* with identical molecular profiles on different dates. In other words, strains were isolated ≥ two times over a span of ≥ eight months. The presented study defined persistence as the isolation of the same strain ≥ two times over a span of ≥ six months. Thus, the lack of a well-defined term or empirical rule to define persistence also plays a part in the discrepancies between studies. However, this problem can be circumvented in future works by relying upon WGS-based SNP analysis.

With respect to sample sizes, the presented study included a small sample population of seventeen isolates, while Magalhães et al. (2016) worked with a more comprehensible population of forty-one isolates, some of which were also part of this study. Thus, the question regarding typing methodology and persistence criteria would not represent such a discrepancy. Be that as it may, Magalhães et al. (2016) only exposed the isolates to one stress at a time rather than three simultaneous stresses, as presented here. Additionally, as previously mentioned, Magalhães et al. (2016) used HCl, a strong acid, and as such, with less antimicrobial activity than the lactic acid used in the present study to mimic the conditions present in cheese processing environments. Consequently, the same strains grew at pH 5.

Nonetheless, the results gathered in this study go in accordance with Taylor and Stasiewicz (2019), which used a substantially larger population of ninety-five isolates and noted the lack of significant differences in growth rate between persistent and sporadic isolates when exposed to pH 5.2 (adjusted with HCl), and pH 9.2 (adjusted with NaOH) and 5.0 and 10.0% NaCl. The only criticism that may be posed lies with the temperature used, which at 37 ºC falls under the temperature range for ideal growth of *Lm*. Nevertheless, this criticism may be in question since Magalhães et al. (2016), reported no significant differences in growth kinetics at 37, 22 and 4 ºC between persistent and sporadic strains. Thus, considering the data presented in this study, it may be unlikely that conducting the same experiment with added temperature ranges would alter the outcome of the study by Taylor and Stasiewicz (2019).

3.2. Infrared data analysis

The collected FTIR-ATR spectra displayed the typical shape of pristine bacterial spectra (Fig. 2), comprehending the absorption bands of lipids (3000-2800 cm^{-1}), proteins/amides I and II (1700-1500 cm^{-1}), the mixed region of proteins, fatty acids, phospholipids and other phosphate-containing molecules, DNA and RNA (1500-1200 cm^{-1}) resulting from bending of C-H groups, stretching of C O in COO- groups, amide III groups, amide III groups, and the P-O stretching of phosphodiesters (Helm et al., 1991; Lasch and Naumann, 2015). The region between (1200 and 900 cm^{-1}) is due to the polysaccharide cell components. The bands between (900-600 cm^{-1}) correspond to the fingerprint region, containing weak and unique absorbances characteristic of specific bacteria (Maquelin et al., 2002; Naumann et al., 1991). Strong absorptions were detected in all four spectral regions that characterize the major cellular constituents.

Table 5. Confusion matrix corresponding to the PLSDA model optimized for the discrimination between *Listeria monocytogenes* strains grown at 11°C and 30°C. Values are in %.

FTIR-ATR predection	Temperature	
	Grown at 11 ºC	Grown at 30 ºC
Grown at 11 ºC	49.50	0.50
Grown at 30 ºC	1.88	48.13
Total correct assignments: **97.6**		

To evaluate the ability of FTIR spectroscopy to discriminate *Lm* isolates according to their growth temperature, PLSDA models were developed, with the best classification model coming from the following pre-processing parameters: nine smoothing points, second order polynomial and second derivative in the region 1200-900 cm^{-1}. The best-fitting model presented a high degree of correct assignments, 97.6%, as shown by the confusion matrix in Table 5. Furthermore, this analysis (PLSDA) revealed two well-defined clusters, each containing isolates grown at a single temperature, as shown in the scores map in Fig. 6A. Therefore, with the data gathered through FTIR spectroscopy, the classification model successfully discriminated isolates grown at different temperatures. From a microbiology standpoint, this result would be expected since, for the same isolate, growth at near-refrigeration temperatures would yield constitutional differences from bacterial development at optimal temperatures.

From the data gathered at 30 ºC, a baseline was established to which all results at 11 ºC would be compared in order to assess if any viewed differences were due to the effect of growth temperature and not strain differences. The dissimilarities between the two temperature clusters were clearly provided by the first latent variable (LV1), of which wavenumbers: 1015, 985, 925, and 905 contributed the most (higher loading values) to this defined separation (Fig. 6B). The second latent variable (LV2) was responsible for the segregation of isolate Lm929 from the remaining sixteen isolates grown at 11 ºC, with wavenumber 1025 and 1010 chiefly involved in the viewed division (Fig. 6B). The pattern presented by Lm929 at 11 ºC was not observed during its growth at 30 ºC.

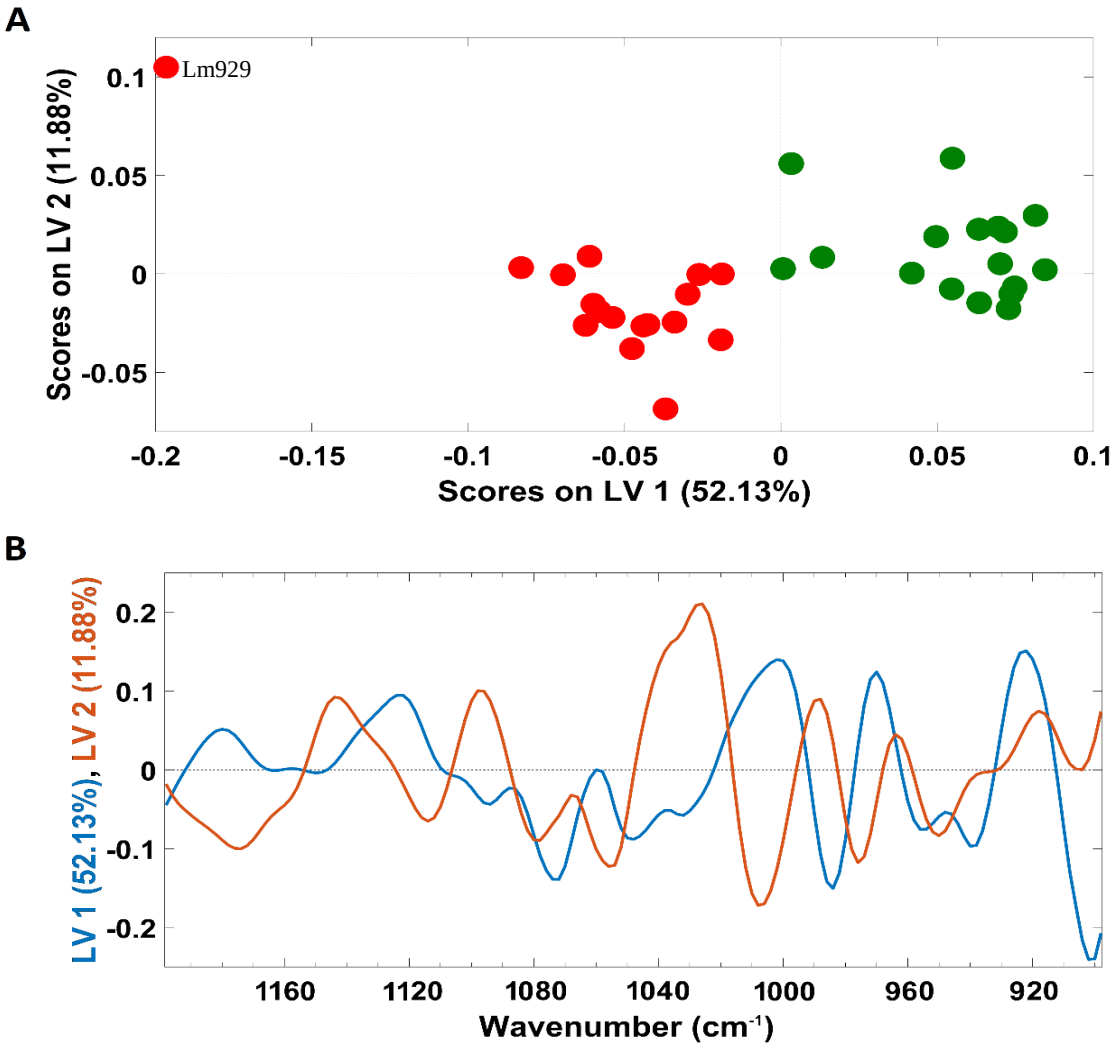

Figure 6. **(A):** Scores map of the PLSDA regression model obtained for the discrimination between *Listeria monocytogenes* strains grown at 11 ˚C (●) and 30 ˚C (●), in the spectral region 1200-900 cm^{-1} and **(B)** the corresponding loadings plot.

As in this analysis, *Lm* isolates were exposed to low temperatures, which promotes the formation of a more rigid lipid bilayer (gel phase), impairing the physiological function of the

biological membrane. The elasticity toward cold environments shown by Lm929 could have been correlated with how this isolate modulates the composition of its cell membrane and wall to achieve the optimal degree of fluidity while ensuring solute transport and other membrane-associated functions during the lipid phase (Mastronicolis et al., 2006; Neunlist et al., 2005). Furthermore, we also set out on evaluating differences in membrane fluidity between persistent and sporadic isolates when exposed to cold environments.

According to the literature (Girardeau et al., 2022), the fluidity of the cell membrane can be evaluated through two different absorption band ratios in the region 3000-2800 cm-1. The first ratio, $asym\,CH_3/asym\,CH_2$, between the CH3 asymmetrical stretching (2958 cm-1), and the CH2 asymmetrical stretching (2925 cm-1) bands, provides information on the acyl chain length, with longer acyl chains leading to reduced membrane fluidity (Pelley, 2007). The second ratio, $asym\,CH_2/sym\,CH_2$, encompassing the CH2 asymmetrical stretching and the CH2 symmetrical stretching (2854 cm-1) bands, allows for the evaluation of the order state in the cell's membrane, with higher disorder correlating with higher liberty of movement and therefore increased membrane fluidity.

According to the two employed ratios, all 17 isolates possess highly similar degrees of fluidity in their membranes, with no significant differences being observed between persistent and sporadic groups nor between Lm929 and the remaining 16 isolates (data not shown). Regarding differences between cell order state and acyl chain length, among persistent and sporadic isolates, no significant differences were observed ($p>0.05$), with both groups apparently possessing the same degrees of membrane fluidity (data not shown). Interestingly, when comparing the membrane fluidity during growth at 11 and 30 ºC, all isolates presented lacklustre differences between both temperatures. This occurrence may indicate that during growth in near-refrigeration temperatures, *Lm* isolates possibly rely upon other low-temperature tolerance systems, with these conditions not evoking the necessity for cell membrane rearrangements, with cell membrane modulation only used by *Lm* when facing harsher refrigeration temperatures.

This result was not unforeseen. The wavenumbers responsible for the separation of Lm929 from the remaining sixteen isolates fall under the mixed region containing phospholipids/DNA/RNA and carbohydrates but not the fatty acids in the cell membrane. Thus, the differences would not be related to membrane fluidity.

Table 6. Confusion matrix corresponding to the PLSDA model optimized for the discrimination between persistent and sporadic isolates. Values are in %.

FTIR-ATR predection	Persistence classification	
	Persistent	Sporadic
Persistent	44.87	9.97
Sporadic	9.48	35.68
Total correct assignments: **80.6**		

IR spectra were additionally modelled (PLSDA) to evaluate the ability of FTIR spectroscopy to discriminate among persistent and sporadic *Lm* isolates. Spectral pre-processing was optimized according to the already-mentioned procedure. The best discriminatory model according to the persistent/sporadic classification was granted by the subsequent pre-processing parameters: twelve smoothing points, second order polynomial, and second derivative in the R1, R2, R3, and R4 regions (Fig. 2), presenting a degree of correct assignments of 80.6%, as summarized in Table 6. In table SX, present in the appendix, the percentage of correct assignments obtained from all de PLSDA models developed during the pre-processing optimization step was presented. It should be noticed that some of these models also achieved reasonably high percentages of total correct assignments in the same relative order of magnitude (≈80%).

Although the results do not plainly validate the developed PLSDA model to discriminate between persistent and sporadic isolates, we should not contemn or discard the potential presented by the FTIR-ATR technique. Even though the total correct prediction percentage of 80.55% does not guarantee the complete robustness of the classification model, it still strongly indicates that the used model and analytical technique appear to have enough discriminatory power to detect phenotypical differences between persistent and sporadic isolates, despite only using two latent variables (LVs) in the analysis.

The capability of distinguishing between persistent and sporadic isolates is very promising since obtaining a robust model that can categorically classify *Lm* isolates will provide a strong argument that persistence may also be due to intrinsic factors. By correctly discriminating an isolate as persistent or sporadic, the model will rely upon specific characteristics of phenotypic nature, present only in one of the groups, hence confirming that

persistence may occur not only due to poor sanitation procedures but also due to strain intrinsic factors, at least during exposure to low temperatures.

However, although the model presented a reasonably high rate of correct assignments, we must keep in mind, as previously stated, the persistent group showed more homogeneity than the sporadic group, with various clones of the same strain present. Therefore, it is plausible that this group underrepresentation compromised the results obtained by the model since, in the persistent group, we only truly had five different strains present, while the sporadic group possessed eight different strains. Thus, an increase in the number of isolates should be considered in future work to robust the herein obtained results.

As mentioned, using the four spectral regions (R1, R2, R3, R4) in the pre-processing gave rise to the best discriminatory model. However, determining, through the FTIR analysis, the exact compounds or group of compounds responsible for the perceived phenotypic differences between persistent and sporadic isolates is unfeasible. In a complex matrix such as a cell pellet, we will encounter various compounds with overlapping absorption regions from which we cannot distinguish the compound of interest. We also cannot claim that the observed differences arise exclusively from the growth at low temperatures. Instead, they may be observed when exposed to any type of stress condition (e.g., pH, salinity, hydrostatic pressure).

Lastly, although FTIR-ATR technique has been used in the past to evaluate structural changes in *Lm* isolates subjected to stress conditions during their growth (Alvarez-Ordóñez et al., 2010; Beney et al., 2004; Karatzas and Bennik, 2002; Mille et al., 2002). To our knowledge, no works have delved into the topic of persistence, with FTIR used as a tool to assess differences between both groups when subjected to stresses.

3.3. Accessory genome analysis

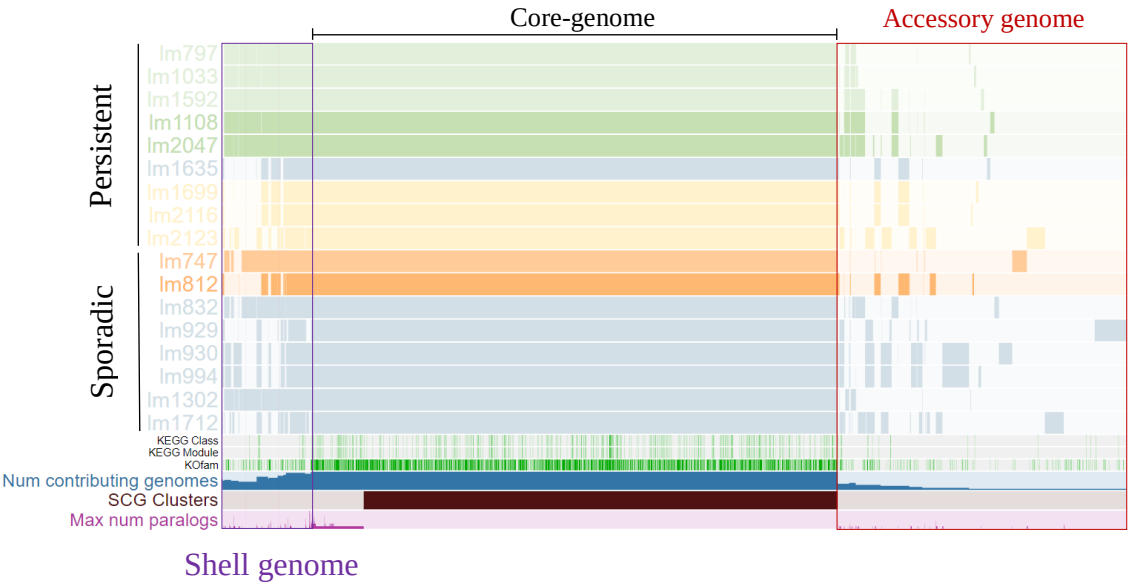

Figure 7. Pangenome analysis of *Listeria monocytogenes* isolates made with Anvi'o software.

Figure 7 displays the pangenome of the seventeen analysed *Lm* strains, including their core, shell, and accessory genomes. Note that the band order in the figure does not correlate with the gene position in the bacterial genome. The core genome encompasses all pangenome genes shared by all isolates in the tested set. Thus, we perceive them as conserved genes (Inglin et al., 2018). In opposition, the accessory genome entails a gene set shared within one or more strains (Segerman, 2012). Relatively to the shell genome, it englobes the group of genes commonly shared by tested isolates. However, there is no clear and universally accepted threshold for defining this part of the pangenome (Snipen and Ussery, 2010). Additionally, the *Lm* pangenome, although highly stable, is an open pangenome, meaning it possesses a relatively small core genome while presenting a large variety of accessory genomes, suggesting an ability to adapt to new niches by generating or including new genetic information (Kuenne et al., 2013).

Looking at both groupings, we notice that persistent isolates do not present a characteristic accessory genome pattern among themselves. If the accessory genomes of persistent isolates were highly similar, it would suggest that the persistence demonstrated by those isolates could be a consequence of newly acquired resistance genes. However, this does not seem to be the case, as persistent isolates possess among themselves varying genes in their accessory genomes.

Intriguingly, between clones of the same strain, the accessory genomes seem to differ, possibly due to the fact that these clones were isolated years apart from each other, which may have led to changes in their genome by newly incorporated genes.

If the accessory genomes of these isolates differ, then there is a chance that the behaviour of these clones may also be different, justifying the different growth patterns observed during growth under stress conditions.

In opposition, the accessory genomes of some isolates are remarkably similar. However, this similarity cannot be explained by the clonal complexes, year, place, or origin of isolation. For instance, Lm1635, Lm1699, and Lm2116 have different PFGE profiles and were recovered from different origins, with Lm1635 being an isolate from raw milk while Lm1699 and Lm2116 are from environmental origin. Moreover, these isolates were also collected in different years, 2005 (Lm1635, Lm1699) and 2007 (Lm2116). Even more perplexing is the fact that isolate Lm2123 which shares a highly similar PFGE profile to Lm2116 and was acquired from the same origin during the same time frame, has a completely different accessory genome from Lm2116 and another clone (Lm1699).

Another occurrence worth mentioning correlates with the benzalkonium chloride resistance cassette *bcrABC*. Fifteen of the chosen isolates, persistent and sporadic, possess the *bcrA* gene, and only this gene, in their accessory genome. However, this is odd considering the fact *that bcrB* and *bcrC* should also be present. The two missing genes could have been a consequence of the sequencing method used, as Illumina sequencing generates shorter reads which leads to more gaps in the sequenced genome, with the possibility of the location of these genes landing on one of those gaps, hence the reason for their absence. However, considering that in Illumina sequencing during library preparation, the genome is fragmented in a random shotgun-like fashion (Bronner et al., 2013), having the same two missing genes in the genome of all fifteen isolates would have been a remarkable coincidence. A more likely explanation is that the perceived presence of the *bcrA* gene is possibly due to an annotation error of the genome, a more frequent occurrence when working with genome technologies that work with shorter reads. Hence, to confirm the validity of the presence of the *bcrABC* cassette in the accessory genome of the fifteen isolates, PCR amplification of said genes must be performed.

Additionally, instead of persistence events being accounted for by the presence or lack thereof in resistance genes, it may instead be explained by the regulation and expression of said resistance genes present in their genome, leading to the differing phenotypic profiles which enable the strain to persist (Muchaamba et al., 2019). Something which we cannot assess with the current approach.

4. Conclusion

The work in this thesis set out to characterize different phenotypic responses of seventeen persistent and sporadic isolates of *L. monocytogenes* recovered from cheese factories over a period of four years, along with evaluating if any differences that could potentially justify the different classification groups were present in the accessory genome of these isolates. We conducted three avenues of approach to fulfil the proposed tasks.

In the first approach, growth in extrinsic stress conditions, as expected, the environmental stresses affected *Lm* ability to proliferate, hindering the growth rate and increasing the lag times. However, regarding the comparison between both groups' growth, no substantial and clear-cut differences were found, suggesting that the persistence of these isolates was not due to strain-specific phenotypic responses to the tested extrinsic stresses (pH, temperature, and salinity).

The second phenotypic approach, resorting to the analysis of the FTIR spectra of *Lm* isolates exposed to 30 and 11 ºC, yielded promising results. The PLSDA prediction models for 11 ºC correctly classified a significant portion of the test set of isolates as persistent and sporadic, possibly implying that the FTIR technique possessed enough discriminatory power to distinguish phenotypical differences between persistent and sporadic isolates. Regarding the study of changes in fluidity between the membrane of persistent and sporadic isolates, no significant differences were observed at the tested temperature of 11 ºC. However, lower temperatures should be employed as the tested temperature was not enough to trigger any changes in membrane composition.

Lastly, in the third approach, the accessory genome analysis, although the seventeen genomes showed high diversity in their accessory genome, persistent and sporadic genomes did not possess recurrent resistance genes that could imply enhanced tolerance to environmental stresses.

Thus, in the author's opinion, further research needs to be conducted on the FTIR analysis of isolates grown in various stress conditions to provide reliable and accurate evidence corroborating the claim that persistence may also be attributed to phenotypic characteristics, not only to sanitation deficiencies.

5. Future work

Some of the results here displayed deserve further research, with some suggestions being:

1. To increase the robustness of the PLSDA model, by including additional *Lm* isolates. Furthermore, introducing new stresses for the model to analyze, such as pH and osmotic stress.
2. To evaluate not the presence of resistance genes in the *Lm* genome, evaluating instead, the differences in their regulation and expression among persistent and sporadic groups.

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8. Appendix

Table SX. Percentage (%) of correct class (discrimination between persistent and sporadic) obtained from the PLSDA models using 2 latent variables (LVs); distinct parameters of the Savitzky-Golay filter and the spectral regions (alone or in combination) specified in figure S1.

SavGol (n,x,y)	R1	R2	R3	R4	R1+R2	R1+R3	R1+R4	R2+R3	R2+R4	R3+R4	R1+R2+R3	R1+R2+R4	R1+R3+R4	R2+R3+R4	R1+R2+R3+R4
(15,2,1)	60.1	76.9	75.3	74.0	78.3	74.1	75.5	77.1	78.2	77.5	77.3	77.8	77.3	78.9	77.5
(12,2,1)	61.2	78.3	75.7	76.5	77.3	75.4	78.8	75.2	78.6	78.1	76.5	78.0	78.5	78.5	79.7
(9,2,1)	61.2	74.7	74.1	78.0	74.5	77.3	79.0	76.1	76.4	77.2	76.4	77.1	79.0	77.7	77.7
(15,2,2)	59.3	75.7	78.3	77.9	75.7	80.0	77.4	78.6	78.3	79.5	80.2	79.1	79.4	79.7	79.8
(12,2,2)	58.1	74.9	77.1	78.2	75.6	78.5	77.4	79.7	76.2	78.2	78.9	76.2	79.8	77.5	**80.5***
(9,2,2)	57.1	73.5	76.6	76.6	69.1	77.7	77.1	79.4	74.9	77.2	79.7	73.9	76.9	76.1	75.8

R1: 3000-2800 cm^{-1}; **R2**: 1750-1500 cm^{-1}; **R3**: 1500-1200 cm^{-1}; **R4**:1200-900 cm^{-1}; ***The higher percentage was obtained using region R1+R2+R3+R4 and SavGol (12,2,2).

Table SY. Growth parameters gathered for each strain in every tested stress condition.

Isolados	Condição	pH	T(ºC)	NaCl%	P/NP	Lag (h)	Td (h)	µMÁX (h-)	ABS(Log(N0/N))
Lm747	S5	7	11	2.5	NP	29	2.18	0.32	5.4
Lm797	S5	7	11	2.5	P	28	2.40	0.29	4.9
Lm812	S5	7	11	2.5	NP	30	2.58	0.27	5.3
Lm832	S5	7	11	2.5	NP	32	2.47	0.28	5.1
Lm929	S5	7	11	2.5	NP	34	2.57	0.27	4.3
Lm930	S5	7	11	2.5	NP	30	2.51	0.28	5.3
Lm994	S5	7	11	2.5	NP	25	2.13	0.33	4.8
Lm1033	S5	7	11	2.5	P	33	2.08	0.33	5.2
Lm1108	S5	7	11	2.5	P	31	2.71	0.26	4.7
Lm1302	S5	7	11	2.5	NP	34	2.99	0.23	4.9
Lm1498	S5	7	11	2.5	NP	29	2.79	0.25	4.7
Lm1592	S5	7	11	2.5	P	30	2.52	0.28	5.2
Lm1635	S5	7	11	2.5	P	29	2.33	0.30	6.5
Lm1699	S5	7	11	2.5	P	32	2.39	0.29	5.1
Lm1712	S5	7	11	2.5	NP	30	2.40	0.29	5.2
Lm2047	S5	7	11	2.5	P	33	2.33	0.30	4.7
Lm2116	S5	7	11	2.5	P	32	2.24	0.31	5.3
Lm2123	S5	7	11	2.5	P	30	1.32	0.53	4.9
Isolados	**Condição**	**pH**	**T(ºC)**	**NaCl%**	**P/NP**	**Lag (h)**	**Td (h)**	**µMÁX (h-)**	**ABS(Log(N0/N))**
Lm747	S6	7	11	8.0	NP	41	1.6	0.43	5.9
Lm797	S6	7	11	8.0	P	43	1.5	0.47	5.7
Lm812	S6	7	11	8.0	NP	41	1.3	0.55	5.8
Lm832	S6	7	11	8.0	NP	39	1.7	0.40	5.0
Lm929	S6	7	11	8.0	NP	43	2.0	0.35	5.3
Lm930	S6	7	11	8.0	NP	43	2.2	0.32	5.8
Lm994	S6	7	11	8.0	NP	45	1.9	0.37	5.4
Lm1033	S6	7	11	8.0	P	47	1.5	0.45	5.6
Lm1108	S6	7	11	8.0	P	37	1.5	0.46	5.7
Lm1302	S6	7	11	8.0	NP	40	2.8	0.25	5.6
Lm1498	S6	7	11	8.0	NP	33	1.8	0.38	5.4
Lm1592	S6	7	11	8.0	P	37	1.5	0.46	5.8
Lm1635	S6	7	11	8.0	P	35	1.6	0.43	6.4
Lm1699	S6	7	11	8.0	P	37	1.5	0.47	5.8
Lm1712	S6	7	11	8.0	NP	41	2.7	0.26	5.6
Lm2047	S6	7	11	8.0	P	41	1.5	0.45	5.5
Lm2116	S6	7	11	8.0	P	39	1.5	0.46	5.8
Lm2123	S6	7	11	8.0	P	36	1.7	0.41	5.7

Table SY. (Continued)

Isolados	Condição	pH	T(ºC)	NaCl%	P/NP	Lag (h)	Td (h)	µMÁX (h-)	ABS(Log(N0/N))
Lm747	S7	7	30	2.5	NP	14	1.81	0.38	5.5
Lm797	S7	7	30	2.5	P	13	1.68	0.41	5.0
Lm812	S7	7	30	2.5	NP	9	2.25	0.31	5.5
Lm832	S7	7	30	2.5	NP	10	2.04	0.34	5.6
Lm929	S7	7	30	2.5	NP	12	2.04	0.34	5.6
Lm930	S7	7	30	2.5	NP	11	2.23	0.31	6.1
Lm994	S7	7	30	2.5	NP	11	1.93	0.36	4.2
Lm1033	S7	7	30	2.5	P	11	1.47	0.47	4.6
Lm1108	S7	7	30	2.5	P	12	1.83	0.38	4.8
Lm1302	S7	7	30	2.5	NP	10	2.72	0.25	5.0
Lm1498	S7	7	30	2.5	NP	8	2.40	0.29	4.5
Lm1592	S7	7	30	2.5	P	11	2.31	0.30	5.8
Lm1635	S7	7	30	2.5	P	10	1.86	0.37	9.8
Lm1699	S7	7	30	2.5	P	10	1.92	0.36	4.9
Lm1712	S7	7	30	2.5	NP	9	1.99	0.35	4.9
Lm2047	S7	7	30	2.5	P	11	1.93	0.36	4.0
Lm2116	S7	7	30	2.5	P	10	1.86	0.37	5.4
Lm2123	S7	7	30	2.5	P	9	1.76	0.39	4.9
Isolados	**Condição**	**pH**	**T(ºC)**	**NaCl%**	**P/NP**	**Lag (h)**	**Td (h)**	**µMÁX (h-)**	**Log(N0/N)**
Lm747	S8	7	30	8.0	NP	32	2.52	0.28	5.2
Lm797	S8	7	30	8.0	P	33	3.26	0.21	4.6
Lm812	S8	7	30	8.0	NP	23	2.75	0.25	4.6
Lm832	S8	7	30	8.0	NP	23	3.13	0.22	4.6
Lm929	S8	7	30	8.0	NP	29	6.74	0.10	3.6
Lm930	S8	7	30	8.0	NP	28	2.76	0.25	4.3
Lm994	S8	7	30	8.0	NP	23	6.60	0.11	4.4
Lm1033	S8	7	30	8.0	P	28	2.93	0.24	4.1
Lm1108	S8	7	30	8.0	P	26	3.55	0.20	4.0
Lm1302	S8	7	30	8.0	NP	26	3.00	0.23	4.7
Lm1498	S8	7	30	8.0	NP	21	2.84	0.24	4.2
Lm1592	S8	7	30	8.0	P	34	6.80	0.10	4.8
Lm1635	S8	7	30	8.0	P	34	3.35	0.21	4.9
Lm1699	S8	7	30	8.0	P	25	2.36	0.29	4.9
Lm1712	S8	7	30	8.0	NP	23	2.69	0.26	5.3
Lm2047	S8	7	30	8.0	P	33	3.95	0.18	4.8
Lm2116	S8	7	30	8.0	P	28	3.38	0.21	4.9
Lm2123	S8	7	30	8.0	P	21	2.45	0.28	4.4
Isolados	**Condição**	**pH**	**T(ºC)**	**NaCl%**	**P/NP**	**Lag (h)**	**Td (h)**	**µMÁX (h-)**	**Log(N0/N)**
Lm747	S10	7	22	4.0	NP	22	2.35	0.29	5.6
Lm797	S10	7	22	4.0	P	23	5.16	0.13	5.3
Lm812	S10	7	22	4.0	NP	22	2.35	0.29	5.8
Lm832	S10	7	22	4.0	NP	15	3.07	0.23	5.1
Lm929	S10	7	22	4.0	NP	17	2.65	0.26	3.8
Lm930	S10	7	22	4.0	NP	22	2.41	0.29	5.6
Lm994	S10	7	22	4.0	NP	24	3.27	0.21	5.7
Lm1033	S10	7	22	4.0	P	20	2.49	0.28	6.8
Lm1108	S10	7	22	4.0	P	19	1.91	0.36	5.3
Lm1302	S10	7	22	4.0	NP	23	2.33	0.30	5.0
Lm1498	S10	7	22	4.0	NP	16	3.93	0.18	5.3
Lm1592	S10	7	22	4.0	P	22	4.56	0.15	5.1
Lm1635	S10	7	22	4.0	P	22	2.42	0.29	4.9
Lm1699	S10	7	22	4.0	P	22	2.66	0.26	5.6
Lm1712	S10	7	22	4.0	NP	23	2.24	0.31	5.4
Lm2047	S10	7	22	4.0	P	22	2.40	0.29	5.3
Lm2116	S10	7	22	4.0	P	21	2.00	0.35	5.6
Lm2123	S10	7	22	4.0	P	19	2.53	0.27	5.5

Table SY. (Continued)

Isolados	Condição	pH	T(ºC)	NaCl%	P/NP	Lag (h)	Td (h)	µMÁX (h-)	ABS(Log(N0/N))
Lm747	S11	6	11	4.0	NP	89	3.25	0.21	6.7
Lm797	S11	6	11	4.0	P	79	4.52	0.15	6.6
Lm812	S11	6	11	4.0	NP	80	3.24	0.21	6.6
Lm832	S11	6	11	4.0	NP	78	4.13	0.17	4.7
Lm929	S11	6	11	4.0	NP	79	3.17	0.22	6.2
Lm930	S11	6	11	4.0	NP	86	2.93	0.24	6.7
Lm994	S11	6	11	4.0	NP	87	4.11	0.17	6.5
Lm1033	S11	6	11	4.0	P	89	2.85	0.24	7.0
Lm1108	S11	6	11	4.0	P	79	3.08	0.23	6.6
Lm1302	S11	6	11	4.0	NP	90	3.50	0.20	6.2
Lm1498	S11	6	11	4.0	NP	83	3.97	0.17	6.4
Lm1592	S11	6	11	4.0	P	85	4.70	0.15	6.4
Lm1635	S11	6	11	4.0	P	86	3.20	0.22	6.2
Lm1699	S11	6	11	4.0	P	88	3.60	0.19	6.5
Lm1712	S11	6	11	4.0	NP	83	3.54	0.20	6.5
Lm2047	S11	6	11	4.0	P	87	5.31	0.13	6.5
Lm2116	S11	6	11	4.0	P	90	3.07	0.23	7.4
Lm2123	S11	6	11	4.0	P	87	3.45	0.20	6.7
Isolados	**Condição**	**pH**	**T(ºC)**	**NaCl%**	**P/NP**	**Lag (h)**	**Td (h)**	**µMÁX (h-)**	**Log(N0/N)**
Lm747	S12	6	30	4.0	NP	14	2.28	0.30	5.2
Lm797	S12	6	30	4.0	P	12	2.14	0.32	5.4
Lm812	S12	6	30	4.0	NP	12	2.41	0.29	5.2
Lm832	S12	6	30	4.0	NP	13	2.14	0.32	5.1
Lm929	S12	6	30	4.0	NP	10	1.76	0.39	4.8
Lm930	S12	6	30	4.0	NP	13	1.93	0.36	5.0
Lm994	S12	6	30	4.0	NP	13	2.09	0.33	5.0
Lm1033	S12	6	30	4.0	P	13	1.34	0.52	4.8
Lm1108	S12	6	30	4.0	P	11	1.78	0.39	6.1
Lm1302	S12	6	30	4.0	NP	13	2.02	0.34	4.8
Lm1498	S12	6	30	4.0	NP	10	1.94	0.36	4.6
Lm1592	S12	6	30	4.0	P	12	1.98	0.35	5.2
Lm1635	S12	6	30	4.0	P	12	2.47	0.28	4.8
Lm1699	S12	6	30	4.0	P	13	2.30	0.30	5.3
Lm1712	S12	6	30	4.0	NP	15	2.89	0.24	4.5
Lm2047	S12	6	30	4.0	P	13	1.43	0.49	5.2
Lm2116	S12	6	30	4.0	P	13	1.45	0.48	4.7
Lm2123	S12	6	30	4.0	P	12	1.51	0.46	5.1
Isolados	**Condição**	**pH**	**T(ºC)**	**NaCl%**	**P/NP**	**Lag (h)**	**Td**	**µMÁX (h-)**	**Log(N0/N)**
Lm747	S13	6	22	2.5	NP	43	2.74	0.25	5.1
Lm797	S13	6	22	2.5	P	43	3.65	0.19	5.0
Lm812	S13	6	22	2.5	NP	39	3.20	0.22	4.6
Lm832	S13	6	22	2.5	NP	29	2.52	0.28	5.3
Lm929	S13	6	22	2.5	NP	45	4.03	0.17	4.6
Lm930	S13	6	22	2.5	NP	47	3.26	0.21	4.8
Lm994	S13	6	22	2.5	NP	43	2.66	0.26	4.5
Lm1033	S13	6	22	2.5	P	43	3.70	0.19	4.8
Lm1108	S13	6	22	2.5	P	37	1.83	0.38	5.1
Lm1302	S13	6	22	2.5	NP	41	4.15	0.17	5.1
Lm1498	S13	6	22	2.5	NP	33	3.39	0.20	5.0
Lm1592	S13	6	22	2.5	P	39	3.34	0.21	5.0
Lm1635	S13	6	22	2.5	P	37	2.43	0.29	5.1
Lm1699	S13	6	22	2.5	P	39	3.05	0.23	5.2
Lm1712	S13	6	22	2.5	NP	41	2.44	0.28	4.4
Lm2047	S13	6	22	2.5	P	37	2.72	0.26	4.8
Lm2116	S13	6	22	2.5	P	37	2.67	0.26	5.2
Lm2123	S13	6	22	2.5	P	31	2.74	0.25	5.2

Table SY. (Continued)

Isolados	Condição	pH	T(ºC)	NaCl%	P/NP	Lag (h)	Td(h)	µMÁX (h-)	Log(N0/N)
Lm747	S14	6	22	8.0	NP	43	4.15	0.17	9.0
Lm797	S14	6	22	8.0	P	43	3.89	0.18	9.1
Lm812	S14	6	22	8.0	NP	42	4.12	0.17	8.9
Lm832	S14	6	22	8.0	NP	44	5.19	0.13	4.4
Lm929	S14	6	22	8.0	NP	40	3.68	0.19	9.3
Lm930	S14	6	22	8.0	NP	44	3.45	0.20	8.9
Lm994	S14	6	22	8.0	NP	51	4.94	0.14	8.7
Lm1033	S14	6	22	8.0	P	51	3.20	0.22	8.7
Lm1108	S14	6	22	8.0	P	39	4.26	0.16	9.0
Lm1302	S14	6	22	8.0	NP	37	4.68	0.15	8.9
Lm1498	S14	6	22	8.0	NP	32	4.01	0.17	8.8
Lm1592	S14	6	22	8.0	P	35	4.83	0.14	9.0
Lm1635	S14	6	22	8.0	P	31	3.98	0.17	8.8
Lm1699	S14	6	22	8.0	P	31	4.55	0.15	8.8
Lm1712	S14	6	22	8.0	NP	44	18.84	0.04	9.2
Lm2047	S14	6	22	8.0	P	44	8.22	0.08	9.1
Lm2116	S14	6	22	8.0	P	40	4.15	0.17	9.2
Lm2123	S14	6	22	8.0	P	37	4.37	0.16	9.1
Isolados	**Condição**	**pH**	**T(ºC)**	**NaCl%**	**P/NP**	**Lag (h)**	**Td(h)**	**µMÁX (h-)**	**Log(N/N0)**
Lm747	S15	6	22	4.0	NP	49	1.61	0.429	5.6
Lm797	S15	6	22	4.0	P	57	5.30	0.131	5.4
Lm812	S15	6	22	4.0	NP	53	1.92	0.361	5.0
Lm832	S15	6	22	4.0	NP	47	2.47	0.280	4.6
Lm929	S15	6	22	4.0	NP	58	2.97	0.234	5.5
Lm930	S15	6	22	4.0	NP	49	2.34	0.296	5.8
Lm994	S15	6	22	4.0	NP	53	2.04	0.339	5.0
Lm1033	S15	6	22	4.0	P	55	2.38	0.291	5.6
Lm1108	S15	6	22	4.0	P	41	1.54	0.449	5.5
Lm1302	S15	6	22	4.0	NP	49	1.82	0.380	4.9
Lm1498	S15	6	22	4.0	NP	37	4.06	0.171	5.0
Lm1592	S15	6	22	4.0	P	45	1.42	0.488	5.1
Lm1635	S15	6	22	4.0	P	45	3.29	0.211	5.0
Lm1699	S15	6	22	4.0	P	51	2.70	0.257	5.1
Lm1712	S15	6	22	4.0	NP	45	3.04	0.228	5.0
Lm2047	S15	6	22	4.0	P	47	3.11	0.223	5.1
Lm2116	S15	6	22	4.0	P	45	3.39	0.204	7.4
Lm2123	S15	6	22	4.0	P	43	3.32	0.209	5.4
Isolados	**Condição**	**pH**	**T(ºC)**	**NaCl%**	**P/NP**	**Lag (h)**	**Td (h)**	**µMÁX (h-)**	**Log(N0/N)**
Lm747	Controlo	7	30	0.5	NP	15	1.09	0.63	4.0
Lm797	Controlo	7	30	0.5	P	13	1.46	0.47	3.9
Lm812	Controlo	7	30	0.5	NP	11	1.12	0.62	4.2
Lm832	Controlo	7	30	0.5	NP	11	1.43	0.48	4.0
Lm929	Controlo	7	30	0.5	NP	10	0.97	0.71	4.2
Lm930	Controlo	7	30	0.5	NP	12	1.16	0.60	4.6
Lm994	Controlo	7	30	0.5	NP	11	1.05	0.66	5.5
Lm1033	Controlo	7	30	0.5	P	11	1.00	0.69	4.3
Lm1108	Controlo	7	30	0.5	P	10	0.90	0.77	4.2
Lm1302	Controlo	7	30	0.5	NP	9	1.18	0.59	4.0
Lm1498	Controlo	7	30	0.5	NP	8	1.11	0.63	3.8
Lm1592	Controlo	7	30	0.5	P	10	1.08	0.64	4.0
Lm1635	Controlo	7	30	0.5	P	9	1.15	0.60	6.0
Lm1699	Controlo	7	30	0.5	P	11	1.21	0.57	4.4
Lm1712	Controlo	7	30	0.5	NP	11	1.04	0.66	3.9
Lm2047	Controlo	7	30	0.5	P	12	1.11	0.62	4.6
Lm2116	Controlo	7	30	0.5	P	11	1.03	0.67	3.8
Lm2123	Controlo	7	30	0.5	P	9	2.38	0.29	4.6