Estimating *Listeria monocytogenes* growth in ready-to-eat chicken salad using a challenge test for quantitative microbial risk assessment

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Thesis to obtain the Master of Science Degree in

Microbiology

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À minha avó

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Abstract

Currently, there is a growing preference for convenience foods, such as ready-to-eat (RTE) foods that are associated to long refrigerated shelf-lives and do not require a heat treatment prior to consumption. Unlike most foodborne pathogens, *Listeria monocytogenes* is able grow at refrigeration temperatures. Inconsistent temperatures during production, distribution and at consumer's household, may allow for the pathogen to thrive, reaching unsafe limits. *L. monocytogenes* is the causative agent of listeriosis, a rare but severe human illness, with high fatality rates, transmitted almost exclusively by food consumption. Therefore, it is of upmost importance to understand the behavior of *L. monocytogenes* in RTE foods. For that this study aimed to develop a challenge test in RTE chicken salads. Salads were inoculated with a three-strain-mixture of cold-adapted *L. monocytogenes* and stored at 4, 12, and 16ºC for 8 days. Results revealed the studied RTE salad was able to support *L. monocytogenes'* growth, even at refrigeration temperatures. Throughout the study, *L. monocytogenes* isolates were detected on blank samples, and molecular characterization by multiplex PCR confirmed that the majority belonged to serogroup IVb, commonly implicated in human disease. Also, pulsed-field gel electrophoresis results suggested a persistent contamination within the assessed RTE chicken salad's producing industry. A PMA-qPCR was used as an alternative enumeration method to the standard ISO 11290-2:2017. The Baranyi primary model was fitted to microbiological data to estimate the pathogen's growth kinetic parameters. Temperature effect on the maximum specific growth rate (μ_{max}) was modelled using a square-root-type model. Storage temperature significantly influenced μ_{max} of *L. monocytogenes* ($p<0.05$). These predicted growth models for *L. monocytogenes* were subsequently used to develop a quantitative microbial risk assessment, estimating an average number of 1.213×10^{-3} listeriosis cases per year linked to the consumption of these RTE salads.

Keywords: *Listeria monocytogenes*, ready-to-eat chicken salad, challenge testing, predictive growth models, genetic typing, quantitative microbial risk assessment.

Resumo

Listeria monocytogenes é o agente causador de listeriose, uma doença rara, mas severa, com elevada taxa de fatalidade, que pode atingir 20% nos grupos de risco. A listeriose é transmitida quase exclusivamente pelo consumo de alimentos contaminados. Esta bactéria, devido ao seu caráter psicrotrófico pode desenvolver-se, caso se observem oscilações térmicas indesejadas durante a produção e distribuição de alimentos e ainda em casa do consumidor, atingindo teores que podem pôr em risco a saúde humana. Devido à crescente preferência por alimentos prontos-a-consumir, associados a uma vida útil refrigerada e ao facto de não necessitarem de tratamento térmico antes do consumo, é de grande importância entender o comportamento de *L. monocytogenes* neste tipo de alimentos. Assim, este estudo teve como objetivo o desenvolvimento de um *challenge test* em saladas de frango prontas-a-consumir. As saladas foram inoculadas com uma mistura de três estirpes de *L. monocytogenes* adaptadas ao frio e armazenadas a 4, 12 e 16ºC durante 8 dias. Os resultados obtidos revelaram que a salada é capaz de suportar o desenvolvimento de *L. monocytogenes*, mesmo a baixas temperaturas. Foi feita uma caracterização molecular por PCR multiplex de isolados de *L. monocytogenes* detetados em amostras de controlo e a maioria pertencia ao serogrupo IVb, comumente associado a doença humana. Além disso, os resultados do PFGE sugerem uma possível contaminação persistente na indústria produtora das saladas de frango prontas-a-consumir. Uma técnica de PCR quantitativo, que considera a viabilidade celular, foi estudada como um método alternativo de enumeração em relação ao método standard (ISO 11290-2:2017). O modelo primário de Baranyi foi ajustado aos resultados microbiológicos de forma a estimar os parâmetros cinéticos de crescimento desta bactéria. O efeito da temperatura na taxa específica de crescimento máximo (μ_{max}) foi modelado através de um modelo secundário. Concluiu-se que a temperatura de conservação influenciou significativamente o valor de μ_{max} ($p<0,05$). Os modelos preditivos de crescimento desenvolvidos para *L. monocytogenes* foram subsequentemente usados para a elaboração de uma avaliação de risco quantitativa microbiológica, estimando um número médio de 1.213×10^{-3} casos de listeriose por ano associados ao consumo destas saladas.

Palavras-chave: *Listeria monocytogenes*, salada de frango pronta-a-consumir, *challege testing*, modelos preditivos de crescimento, tipagem genética, avaliação de risco quantitativa microbiológica.

Communications in congresses and scientific meetings

Some of the experimental results presented in this thesis have already been submitted as follows:

Bernardo, R., Henriques, A. R. (2018) Assessing *Listeria monocytogenes* growth in ready-to-eat chicken salads as a function of temperature. Submitted to Centre for Interdisciplinary Research in Animal Health (CIISA) Congress: Exploring the boundaries of animal, veterinary and biomedical sciences. November 16th-17th, Lisbon, Portugal.

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List of symbols and abbreviations

ActA	Actin assembly-inducing protein
*act*A	ActA encoding gene
ALOA	Agar *Listeria* Ottaviani & Agosti
ANOVA	Analysis of variance
a_w	Water activity
BAC	Benzalkonium chloride
BHI	Brain Heart Infusion
BPW	Buffered Peptone Water
BS	Blank samples (uninoculated)
BS-BPW	Blank samples inoculated with BPW
CAC	*Codex Alimentarius* commission
Caps	Cold acclimation proteins
CDC	Centres for Disease and Control and Prevention
CECT	Colección Española de Cultivos Tipo
cfu	Colony-forming unit
CI	Confidence interval
Csps	Cold shock proteins
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
ECDC	European centre for disease prevention and control
EFSA	European food safety authority
e.g.	*exempli gratia*
et al.	*et alia*
EEA	European Economic Area
EU	European Union
EURL Lm	European Union Reference Laboratory for *Listeria monocytogenes*
FAO	Food and agriculture organization
FBOs	Food business operators
FDA	U.S. Food and Drug Administration
FPE	Food processing environments
FSAI	Food safety authority of Ireland
g	gram
G6PT	glucose-6-phosphate transporter
h	Hour
HIV	Human immunodeficiency virus
HACCP	Hazard analysis and critical control points
hly	Listeriolysin O encoding gene
HPA	Health Protection Agency
Hpt	Hexose Phosphate Transporter
i.e.	*id est*
InlA	Internalin A
InlB	Internalin B
inlA	Internalin A encoding gene
inlB	Internalin B encoding gene
inlC	Internalin C encoding gene
inlJ	Internalin J encoding gene
IS	*L. monocytogenes* inoculated samples
ISO	International standards organization
kb	Kilobase
L.	*Listeria*
LIPI-1	*Listeria* pathogenicity island 1
LLO	Listeriolysin O

log	Logarithm with base 10
min	minute
MPN	Most probable number
mg	milligrams
ml	milliliter
MS	Member states
NaCl	Sodium chloride
nm	nanometres
N_{max}	Maximum population density
No.	Number
OD	Optical density
pH	Potential of hydrogen
PCR	Polymerase chain reaction
PFGE	Pulse-field gel electrophoresis
PlcA	Phospholipase A
PlcB	Phospholipase B
PMA	Propidium monoazide
PrfA	*L. monocytogenes* regulatory protein
prfA	PrfA encoding gene
QACs	Quaternary ammonium compounds
QMRA	Quantitative Microbial Risk Assessment
RNA	Ribonucleic acid
rpm	Rotations per minute
RTE	Ready-to-eat food
RTEMP	Ready-to-eat meat-based food products
RT-qPCR	Real Time quantitative Polymerase chain reaction
R^2	R-squared
s	Seconds
SD	Standard deviation
SE	Standard error
spp.	species
TAM30	Total aerobic microorganisms at 30ºC
TBE	Tris borate EDTA
TE	Tris-EDTA
TGA	Tryptone Glucose Agar
TS	Tryptic soy broth
USA	United States of America
UV	Ultraviolet
VBNC	Viable but non culturable
VCC	Viable cell count
VRBD	Violet Red Bile Dextrose
WGS	Whole genome sequencing
WHO	World Health Organization
ºC	Degrees Celsius
>	Greater than
<	Less than
≥	Equal or greater than
≤	Equal or less than
%	Percent
σB	*L. monocytogenes* alternative sigma B factor
λ	Lag time
µg	microgram
µl	microliter
µm	micrometre
μ_{max}	Maximum specific growth rate

Aims of the study and thesis outline

The research work presented in this thesis aimed to improve the understanding of *L. monocytogenes* in a ready-to-eat (RTE) food. For that, a challenge test was developed in RTE chicken salads produced in an industrial facility with a suspicion of *L. monocytogenes* environmental persistent contamination. The food processing industry has linked the occurrence of *L. monocytogenes* in chicken salad to the processing equipment that is kept in refrigerated rooms (10º to 12ºC). Thus, given the high fatality rate associated to *L. monocytogenes* and the registered increase in the reported cases of listeriosis in Portugal, there was the need to understand the behavior of this pathogen in these salads, considering the processing and storage conditions and the foreseen shelf-life.

Because food products are usually contaminated at low levels, enumeration and detection of *L. monocytogenes* in food may be a challenge using culture-based methods and so, testing alternative molecular-based methods was also considered.

As presumptive *L. monocytogenes* were found on blank samples, confirmation and serogrouping by multiplex PCR and subtyping by PFGE were performed.

There was also the need to develop a quantitative microbial risk assessment model, as there has been no previous study to estimate the risk associated with the consumption of RTE chicken salads for Portuguese population.

This study aimed to improve risk management and strengthen food control by identifying the factors that contribute the most to risk augmentation in the consumption of RTE chicken salads.

In order to achieve the aims above mentioned, the following tasks were identified:

- Investigate the growth of *L. monocytogenes* in defined medium (Brain Heart Infusion broth (BHI)) at 12°C for 288 h and at 37°C for 26 h.

- Understand the correlation of two important methods, optical density (OD) and viable cell counts (VCC), to be used in the subsequent work.

- Confirm RTE chicken salad as a food product able to support the growth of *L. monocytogenes* using physicochemical parameters.

- Test for hygiene indicators (total aerobic microorganisms at 30ºC and Enterobacteriaceae) in blank samples in order to have an indication of their effect on the shelf-life of the product.

- Develop *L. monocytogenes'* predictive growth models, taking into account the processing and storage conditions, and the foreseen shelf-life.

- Estimate the hypothetical maximum concentration of *L. monocytogenes* that could be present at the production stage, in order to comply with the mandatory limit of 100 cfu/g at the end of shelf life.

- Characterize by molecular methods (Multiplex PCR and PFGE) presumptive *L. monocytogenes* isolates collected from blank samples.

- Compare culture-based classical methods (ISO 11290-2:2017) with molecular methods (PMA-RT-qPCR) in quantification of *L. monocytogenes*.

- Establish a quantitative microbial risk assessment of *L. monocytogenes* in RTE chicken salads, to estimate the annual number of listeriosis cases in the Portuguese population, and also detect and rank the most influential factors to the risk of listeriosis by means of a sensitivity analysis.

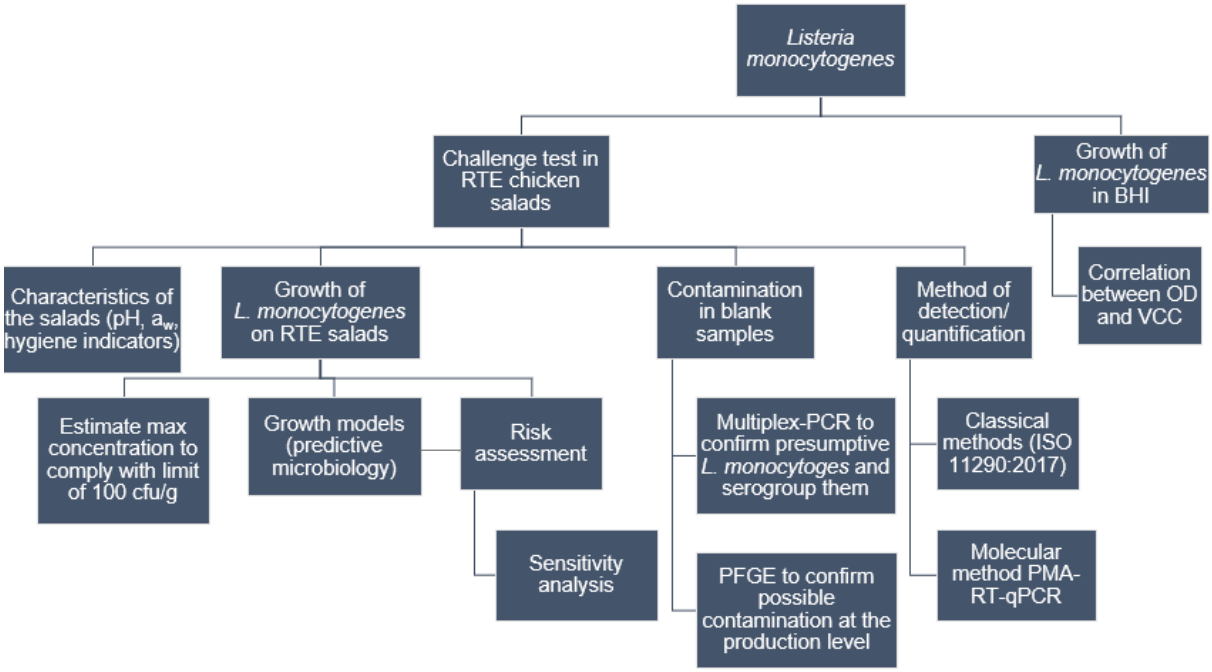


Figure 1. Schematic representation of thesis outline.

1. Literature review

1.1. *Listeria monocytogenes*: Taxonomy and general morphological and physiological characterization

The genus *Listeria* is placed within the Listeriaceae family (McLauchlin, & Rees, 2009) and currently includes 17 recognized species with diverse phenotypic and genotypic characteristics (*Listeria monocytogenes, L. seeligeri, L. ivanovii, L. welshimeri, L. marthii, L. innocua, L. grayi, L. fleischmannii, L. floridensis, L. aquatica, L. newyorkensis, L. cornellensis, L. rocourtiae, L. weihenstephanensis, L. grandensis, L. riparia*, and *L. booriae*) of small rod-shaped Gram-positive bacteria (Orsi, & Wiedmann, 2016; Wang et al., 2017). Amongst the many species of the genus *Listeria,* only *L. monocytogenes* and *L. ivanovii* are recognized pathogens of humans and other animals, but only *L. monocytogenes* is considered an important human pathogen (Guillet et al., 2010; Orsi, & Wiedmann, 2016). *L. ivanovii* predominantly causes disease in animals, especially sheep and cattle, but few cases of listeriosis caused by *L. ivanovii* have been reported in humans. These cases of septicemia have been diagnosed in immunocompromised people, which underlines *L. ivanovii* as a potential opportunistic human pathogen (Guillet et al., 2010; Snapir et al., 2006).

L. monocytogenes is a Gram-positive, non-spore forming rod-like shape bacteria with rounded ends. Cells usually range from 0.5 to 4 micrometers (μm) in diameter and 0.5 to 2 μm in length and are found as single units or arranged in short chains (Meloni, 2014; Reyser, & Marth, 2007). Although *L. monocytogenes* is considered actively motile by means of peritrichous flagella at room temperature (20–25ºC), at 37 °C it does not show motility due to a reduced synthesis and assembly of flagellin (Meloni, 2014; Peel et al., 1988; Reyser, & Marth, 2007). It is a facultative anaerobic bacillus, oxidase negative, and generally catalase positive, since rare catalase-negative isolates have been reported (Donovan, 2015).

The pathogen can survive and multiply from -1.5 to 45ºC, however its optimum growth is around 30 to 37ºC (European Union Reference Laboratory for *Listeria monocytogenes* (EURL Lm), 2014; Meloni, 2014). *L. monocytogenes* can grow within the pH range of 4.2 to 9.5, with an optimum pH value at 7 (Cole et al., 1990; EURL Lm, 2014; George et al., 1988). These bacteria can tolerate low water activity (a_w < 0.93), that would be lethal to other organisms (EURL Lm, 2014; Meloni, 2014). *L. monocytogenes* is able to withstand high salinity conditions, with salt concentrations up to 12% sodium chloride (NaCl), few strains have also been reported to resist even at 20% NaCl content (EURL Lm, 2014; Meloni, 2014; Reyser & Marth, 2007). This ability to adapt to very different and harsh conditions probably explains *L. monocytogenes* survival and proliferation in an extensive variety of environments. This ubiquitous pathogen can be found widely distributed in nature, and it has been isolated from soil, dust, manure, water, human and animal fecal samples and decaying vegetation, including animal feeds and silage, from where it enters the food chain (Ajayeoba et al., 2016; Ferreira et al., 2014; Rodríguez-López et al., 2015; Sahu et al., 2016). Also, *L. monocytogenes* physiological and ecological traits grant the colonization of food environments, such as processing plants, retail establishments, public and household kitchens, enabling its survival to hurdles in processing/storage, and proliferation in a variety of raw, processed and refrigerated food (Buchanan et al., 2017; Ferreira et al., 2014; Li et al., 2016;

Sahu et al., 2016). Moreover, this organism has the capacity to survive, replicate and infect a wide range of host species and host cell types, as intestinal epithelial cells and endothelial cells, following ingestion (Chaturongakul et al., 2008; McGann et al., 2008). Despite its ability to cause infection in host cells, *L. monocytogenes* can also be a transitory resident of the intestinal tract in humans, with 2-10% of the general population being carriers of the microorganism without any apparent health consequences (Buchanan et al., 2017).

1.1.1. *Listeria monocytogenes* evolutionary lineages: ecology and phenotypic differences

L. monocytogenes strains display both genetic and serotypic diversity (Eskhan, & Abu-Lail, 2013). Serotypic diversity arises from combinations of somatic (O) and flagellar (H) antigens present on the cell surface, resulting in 13 recognized serotypes within the species (Eskhan, & Abu-Lail, 2013; EURL Lm, 2014; Kérouanton et al., 2010). In comparison with serotyping, molecular subtyping methods such as ribotyping, pulse-field gel electrophoresis (PFGE), and multilocus sequence typing (MLST), allow for a more sensitive classification of *L. monocytogenes* population structures (Eskhan, & Abu-Lail, 2013; Orsi et al., 2011). Based on subtyping, *L. monocytogenes* strains can be categorized into four major genetic groups or evolutionary lineages (I, II, III, IV) with variable virulence and different but overlapping ecological niches (Eskhan, & Abu-Lail, 2013; Orsi et al., 2011). Lineage I strains are clonal (Eskhan, & Abu-Lail, 2013), include predominantly serogroups IIb and IVb, but also IIc strains (respectively related to serotypes 1/2b and 3b; 4b; and 3c) (Kérouanton et al., 2010; Leclercq et al., 2011). Strains from this lineage are largely adapted to the human host, and due to their significantly higher pathogenic potential compared to strains of lineage II, they are capable of causing disease and are associated with the majority of human listeriosis outbreaks (Eskhan, & Abu-Lail, 2013). Lineage II strains are very diverse due to horizontal gene transfer and include serogroups IIa and IIc (associated with serotypes 1/2a and 3a; and 1/2c, respectively) (Eskhan, & Abu-Lail, 2013; Kérouanton et al., 2010; Leclercq et al., 2011). In comparison with lineage I strains, lineage II strains represent generic heterogeneous strains and are better suited to survive and multiply in the environment, being common in foods and food related-environment, widespread in natural and farm environments and commonly isolated from animal listeriosis cases and sporadic human clinical cases (Eskhan, & Abu-Lail, 2013; Orsi et al., 2011). Two additional lineages (III and IV) have subsequently been identified and are generally rare. These lineages include strains of serogroup IVa (serotypes 4a and 4c), which are predominantly isolated from food-production animals and have been associated with animal listeriosis (Gray et al., 2004; Kérouanton et al., 2010; Orsi et al., 2011), being underrepresented among human clinical cases and foods (Eskhan, & Abu-Lail, 2013). The majority of *L. monocytogenes* isolates appear to belong to lineages I and II, first identified in 1989, which harbor the serotypes more commonly associated with human clinical cases, including serotypes 1/2b and 4b (lineage I) and serotypes 1/2a and 1/2c (lineage II). In fact, at least 95% of human listeriosis cases are attributed to serotypes 1/2a, 1/2b, and 4b. Serotype 4b causes more than 50% of the listeriosis cases worldwide and accounts for nearly all of the outbreaks of human foodborne and perinatal listeriosis (Doumith et al., 2004; Eskhan, & Abu-Lail, 2013; Gray et al., 2004; Orsi et al., 2011).

The variability in distribution of *L. monocytogenes* lineages among human and animal isolates, food and food-associated environments, and natural environments, and the range of virulence characteristics expressed by different strains is important to contribute to improved assessment of the public health risk posed by *L. monocytogenes* (Gray et al., 2004). Phenotypic differences may help explain the apparently different pathogenic potential and ecological and host niches of the different lineages (Orsi et al., 2011). Besides showing higher recombination rates than lineage I isolates, lineage II strains seem to carry more plasmids than lineage I isolates, and these plasmids often confer resistance to toxic metals and possibly other compounds that may be found in the environment. Moreover, lineage II isolates seem to be more resistant to bacteriocins than lineage I isolates, which could provide a selective advantage in food and food-associated samples and environments where bacteriocin-producing organisms are abundant, thus making lineage II isolates more able to persist in foods and food plant environments. Long-term persistence of lineage II strains in food plant environments has been documented, for example, a serotype 1/2a strain that caused a sporadic case of human listeriosis in 1988, was also responsible, in 2000, for a multi-state outbreak, which was associated with consumption of turkey deli meat produced in the same food facility. This strain had persisted 12 years in the same industrial premises (Orsi et al., 2011). Considering their virulence characteristics, the lower pathogenic potential of lineage II strains compared to lineage I strains, might be explained by the considerable proportion of virulence-attenuated isolates (>30%), due to premature stop codons in *inlA*, which encodes for a protein that is critical in *L. monocytogenes* attachment to human host cells (Orsi et al., 2011).

The four *L. monocytogenes* lineages represent distinct ecologic, genetic, and phenotypic characteristics, which appear to affect their ability to be transmitted through foods and to cause human disease. Since the presence of *L. monocytogenes* is of great concern to the food industry, tracing isolates within the food chain and the plant environment and understanding the ecology, evolution, and characteristics of these strains contribute for an improved control of foodborne listeriosis (Kérouanton et al., 2010; Orsi et al., 2011).

1.2. *L. monocytogenes* virulence

1.2.1. Infection cycle and pathogenesis of *L. monocytogenes*

Listeria monocytogenes is a well-known foodborne pathogen that can infect both humans and animals (Freitag et al., 2009). Consumption of contaminated foods by *L. monocytogenes* is considered to be the major source of infection in humans (Vázquez-Boland et al., 2001). The infection occurs due to the ability of *L. monocytogenes* to cross three vital host barriers: the intestinal barrier, the blood-brain barrier and the placental barrier (Camejo et al., 2011; Cossart & Toledo-Arana, 2008). It has also to do with the capacity of this pathogen to endure the highly acidic environment of the host's stomach, proteolytic enzymes, bile salts and to survive and grow within macrophages after phagocytosis and to invade many types of cells which are normally non-phagocytic (Cossart & Toledo-Arana, 2008; Hadjilouka et al., 2015).

Infection typically starts with the ingestion of food contaminated with *L. monocytogenes*, and thus the gastrointestinal tract is the primary site of *L. monocytogenes* entry into the host (Freitag et al., 2009; Vázquez-Boland et al., 2001). After passing through the gastric environment, the pathogen is absorbed from the intestinal lumen, crossing the intestinal epithelium and *lamina propria,* and reaches the bloodstream (Camejo et al. 2011; Freitag et al. 2009; Lecuit, 2007). The pathogen is carried by the lymph or blood to the mesenteric lymph nodes, the spleen, and the liver (Camejo et al., 2011; Vázquez-Boland et al., 2001). *L. monocytogenes* can target, enter and multiply in both phagocytic cells (polymorphonuclear granulocytes, macrophages, dendritic cells, and other cell types (enterocytes, hepatocytes, fibroblasts, epithelial cells and a variety of nerve cells) (Camejo et al., 2011; Orndorff et al., 2006; Vázquez-Boland et al., 2001). Proteins on *L. monocytogenes'* surface (surface ligands) specific to cellular receptors on the surface of these eukaryotic cells allow a close host cell-bacterium interaction and facilitate internalization into the host cell via a "zipper-like mechanism" (Cossart & Toledo-Arana, 2008; Vázquez-Boland et al., 2001).

Once inside the host cell, *L. monocytogenes* can replicate and spread to neighboring cells, causing further infection. During entry, the bacterium becomes engulfed in a phagocytic vacuole, which is disrupted shortly after, allowing *L. monocytogenes* to escape and replicate in the cytosol, where it obtains nutrients from the host cell (Camejo et al., 2011; Vázquez-Boland et al., 2001). Intracytosolic *L. monocytogenes* polymerizes host actin to form actin tails on polar ends of the bacterium. The actin tails are used to propel *L. monocytogenes* intracellularly. This movement is random, so some bacteria eventually reach the cell periphery, come into contact with the membrane, and protrude out to an adjacent cell, forming a pseudopodium-like structure. This results in the formation of a secondary phagosome delimited by a double membrane, that allows the bacterium to spread (Disson, & Lecuit, 2013; Vázquez-Boland et al., 2001). Once it successfully enters the neighboring cell, the now double-membrane vacuole is also dissolved and the bacterium begins a new cycle of infection in contiguous cells (Freitag et al., 2009; Vázquez-Boland et al., 2001).

Unless its replication is restrained by an effective host innate immune response, the bacteria will escape from immune clearance and continue to divide and replicate. Host survival then depends on the development of an effective adaptive immune response, otherwise, the bacteria is able to re-enter the bloodstream, possibly reaching the brain or the placenta, and cause potentially fatal infections (Camejo et al., 2011; Freitag et al., 2009). The ability of *L. monocytogenes* to replicate in the cytoplasm of infected host cells and to spread from cell to cell allows it to evade humoral immune responses (Freitag et al., 2009).

1.2.2. Major virulence factors of *L. monocytogenes*

A versatile arsenal of virulence factors is produced by *L. monocytogenes* in order to facilitate each step of this infection cycle by promoting virulence, evading the host immune response, and adapting to stresses within the host (Cossart, 2011). Two genetic loci, the internalin operon and the virulence gene cluster *Listeria* pathogenicity island 1 (LIPI-1), harbor the key virulence factors in *L. monocytogenes* (de las Heras et al., 2011; Pizarro-Cerdá et al., 2012; Vázquez-Boland et al., 2001). The proteins involved

in cellular invasion and tissue tropism are encoded by the inlAB gene locus. After cell invasion, the factors required for intracellular survival of *L. monocytogenes* are encoded by LIPI-1. They include various virulence proteins, such as pore-forming toxin listeriolysin O (LLO), two phospholipases (PlcA and PlcB) involved in the disruption of phagosomal membranes and bacterial escape to the cytoplasm, sugar phosphate permease - Hpt - that enables cytosolic replication, actin assembly-inducing protein (ActA), a surface protein that mediates the polymerization of cytoplasmic actin and favors cell-to-cell spread and PrfA, the major transcriptional activator of bacterial virulence genes (de las Heras et al., 2011; Pizarro-Cerdá et al., 2012). **Figure 2** outlines the major virulence factors involved at the different stages of *L. monocytogenes* infection cycle.

Internalins

Internalins are the key proteins involved in listerial adherence and invasion of host cells. These surface associated proteins, interact with specific host-cell receptors, triggering phagocytosis into non-phagocytic cells (de las Heras et al., 2011). *L. monocytogenes* strains with null mutations in four internalin genes (*inl*A, *inl*B, *inl*C, and *inl*J) resulted in reduced invasion or virulence in tissue culture or animal models (McGann et al., 2008). The first members of the internalin family to be characterized were internalin A (InlA) and internalin B (InlB), encoded by the *inl*AB operon (Vázquez-Boland et al., 2001). *L. monocytogenes* protein InlA, encoded by *inl*A gene, is involved in invasion of human epithelial cells, by interacting with the adhesion molecule E-cadherin, to promote invasion of enterocytes, translocation across the intestinal barrier and mediation of access to deeper tissues. In contrast, InlB interacts with the hepatocyte growth factor receptor Met, to promote internalization in hepatocyte cells (Hadjilouka et al., 2015; Orsi, & Wiedmann, 2016; Pizarro-Cerdá et al., 2012). InlB may also play a role in placental invasion (Lecuit, 2007; Orsi, & Wiedmann, 2016).

Listeriolysin O

Upon entry in phagocytic or non–phagocytic host cells, *L. monocytogenes* cells first reside in primary vacuoles. Professional phagocytic cells begin almost immediately to destroy bacteria within the vacuoles, and their survival depends on escaping from the vacuole. Listeriolysin O (LLO) is a toxin encoded by *hly,* and is essential for lysing the vacuolar membrane, allowing *L. monocytogenes* to escape from these vacuoles into the cytoplasm of the cell during pathogenesis (Hamon et al., 2012; Vázquez-Boland et al., 2001). LLO is necessary for establishing infection, and its activity is enhanced by the acidic pH in the vacuole, which explains how the pore formation activity is restricted to avoid disruption of the host cell by uncontrolled LLO insertion into the endomembrane system of the host cell (Hamon et al., 2012). The fact that *L. monocytogenes* null mutants for *hly* are able to escape to the cytosol and multiply within certain human epithelial cells (Henle 407 and HeLa cells) suggests that listerial membrane-active products other than LLO may be involved in phagosome disruption (Vázquez-Boland et al. 2001).

Phospholipases

Vacuole escape is assisted by two phospholipases, PlcA and PlcB, two enzymes involved in virulence, that are encoded by *plc*A and *plc*B genes, respectively. Both phospholipases are apparently involved in the invasion and spread of *L. monocytogenes*, since bacteria with mutations in the genes coding for

these enzymes are less virulent to mice than wild-type bacteria (Smith et al., 1995). Experiments with mutant strains for one or both phospholipase encoding genes demonstrated that PlcA has only a minor role in virulence, acting synergistically with PlcB and in conjunction with LLO, to escape from the primary and secondary vacuoles, while PlcB is active during cell-to-cell spread of bacteria, being required for intercellular spread from macrophages to different types of cells (Smith et al., 1995; Vázquez-Boland et al., 2001).

Hexose Phosphate Transporter (Hpt)

L. monocytogenes possesses a hexose phosphate transporter that mediates rapid intracellular replication and is required for cytosolic replication. This transporter, Hpt is a structural and functional homolog of the microsomal glucose-6-phosphate transporter (G6PT), a key element of glucose homeostasis in eukaryotes. The mammalian G6PT is responsible for the uptake of G6P from the cytosol into the endoplasmic reticulum for its conversion into the central fueling metabolite, glucose. The Hpt permease, with an identical function in HP transport, is used by intracytosolic *Listeria* to get fueling metabolites from the host cell cytosol. The role of this virulence mechanism is to optimize the bacterial proliferation rate *in vivo,* by widening the range of carbon sources available for growth within host cells (Chico-Calero et al., 2002).

Actin assembly-inducing protein (ActA)

In order for *L. monocytogenes* to move directly to another cell, a surface protein, ActA, encoded by *act*A on LIPI-1, induces polymerization of globular actin molecules to form a polar actin-based comet tail. Bacterial cells move along these filaments to the cell membrane and cause portions of the membrane to bulge outwards, forming protrusions in the membrane (listeriopods). These protuberances are engulfed by neighboring cells, forming secondary vacuoles, thereby allowing spreading of *L. monocytogenes* without exposure to antibodies or other immunoactive molecules (Disson, & Lecuit, 2013; Kocks et al., 1992; Vázquez-Boland et al., 2001). ActA may also facilitate uptake of *L. monocytogenes* cells that do not produce internalins into certain types of cells (Kocks et al., 1992; Reyser, & Marth, 2007).

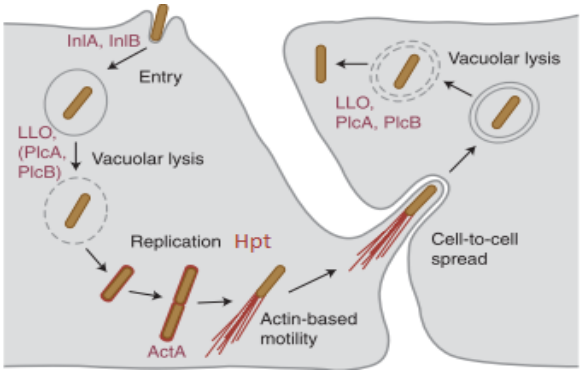


Figure 2. Schematic representation of the intracellular stages of *L. monocytogenes* life-cycle. *L. monocytogenes* binds to epithelial host cells and promotes its own uptake in a process mediated by the two bacterial surface proteins InlA and InlB. The secreted pore-forming toxin LLO (together with the bacterial phospholipases PlcA and PlcB, depending on the cell type) promotes vacuolar rupture and bacterial escape to the cytoplasm, where *L. monocytogenes* can replicate efficiently due to sugar phosphate permease Hpt that enables cytosolic replication. Surface expression of ActA allows intracellular bacteria to polymerize host cell actin and to generate actin comet tails that propel *L. monocytogenes* through the cytoplasm and through membrane protrusions into neighboring cells. There, bacteria localize in a double membrane vacuole, which can be lysed by LLO, PlcA, and PlcB to start a new infection cycle. *Adapted from Pizarro-Cerdá et al. 2012.*

Almost every gene products that are involved in bacterial invasion, cytosolic entry and growth, intracellular motility and spread to neighboring cells, are regulated by the transcriptional regulator PrfA, which positively activates the expression of genes that encodes set of key virulence factors (de las Heras et al., 2011; Freitag et al., 2009). The switch in *L. monocytogenes* lifestyle from saprophytic bacteria to intracellular pathogen requires an increased expression of virulence genes, as these genes are generally expressed at low levels outside the host. This avoids wasteful expression of genes when the bacteria is outside the host, maximizing *L. monocytogenes* fitness, in a mechanism that is still not completely understood (de las Heras et al., 2011). The virulence regulatory protein PrfA is conveniently encoded by *prfA*, located on LIPI-1, which encodes factors required for intracellular survival and cell-to-cell spread (Freitag et al., 2009). PrfA activates transcription of virulence-associated genes by binding to the PrfA box located upstream from the targeted genes (de las Heras et al., 2011; Freitag et al., 2009).

Numerous triggers to express PrfA, and subsequently the set of virulence genes associated with this regulatory protein, have been described. The promoter region of *prfA* contains a thermoswitch, where at temperatures ≤30°C the RNA transcript forms a secondary structure, which masks the ribosome binding region, preventing ribosome binding and translation of *prf*A. At high temperatures (37°C), the transcript is unable to form such an inhibitory secondary structure and translation takes place. In this way, at environmental temperatures below 30°C, the expression of virulence genes is reduced (Freitag et al., 2009; Johansson et al., 2002; Vázquez-Boland et al., 2001). Available carbon sources have also been linked to virulence gene expression. Sugars common in the natural environment as glucose, fructose, mannose or cellobiose, seriously inhibit the expression of PrfA-dependent gene products, while in the presence of sugars commonly utilized during intracellular metabolism, such as glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate and mannose-6-phosphate (hexose phosphate derivatives), the virulence gene expression is not repressed (Freitag et al., 2009).

1.3. Human Listeriosis

Listeria monocytogenes, an opportunistic foodborne pathogen, is the causative agent of a rare but severe human disease named listeriosis (Sahu et al., 2016). The majority (99%) of the infections caused by *L. monocytogenes* are thought to be foodborne (Orsi, & Wiedmann, 2016). Listeriosis has hospitalization rates of more than 92% and a high fatality rate (between 20 and 30%), the highest of any foodborne pathogen, even with antibiotic therapy (Ajayeoba et al., 2016; Doumith et al., 2004; EURL Lm, 2014; Melo et al., 2015; Orndorff et al., 2006). The incidence of listeriosis is low in the general population despite the wide distribution of the pathogen in the environment and the relatively high frequency of isolation in foods (Buchanan et al., 2017).

L. monocytogenes can result in both invasive and non-invasive infections, and their occurrence depends on the host cellular immunity (Donovan, 2015; Khan et al., 2016). In general, non-invasive listeriosis is observed in immunocompetent individuals while invasive listeriosis typically occur in persons with a predisposing condition or diseased state, such as pregnancy, neonates, carcinogenesis, transplantation, alcoholism, immunosuppressive therapy, diabetes, old age, and human immunodeficiency virus (HIV) (Allen et al., 2016; Khan et al., 2016). Non-invasive listeriosis usually

presents as self-limiting febrile gastroenteritis similar to other food-acquired illnesses (Donovan, 2015; Lecuit, 2007). The most severe clinical manifestations of invasive human listeriosis include septicemia, encephalitis, meningitis, mother-to-fetus infection and spontaneous late-term abortion (European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC), 2017; Orsi, & Wiedmann, 2016; Pricope et al., 2013). The inoculum dose of *L. monocytogenes* required to cause infection is thought to be 10^9 bacteria (Donovan, 2015). However, for some authors the dose necessary for infection in susceptible individuals is lower, of only about 10^2-10^3 bacteria (Sahu et al., 2016).

Although listeriosis may be caused by all 13 serotypes of *L. monocytogenes,* only 3 of them, 1/2a, 1/2b, and 4b, account for more than 90% of human and animal cases of listeriosis. And among these listeriosis-associated serotypes, 4b strains cause over 50% of listeriosis cases worldwide (Vázquez-Boland et al., 2001; Yu, & Jiang, 2014).

The prolonged time between the exposure to *L. monocytogenes* and the symptoms' onset can be very long, up to 70 days, which makes it extremely difficult to get accurate dose information and the food source responsible for illness from human epidemiological data, and also delays the detection of a link between cases, and thereof the detection of outbreaks (Buchanan et al., 2017; Luber et al., 2011; Magalhães et al., 2015).

Public health efforts, such as epidemiological surveillance and recommendations regarding food handling and avoidance, and also the development of new and advanced detection methods, are dominant in the efforts to reduce morbidity and mortality secondary to the disease, and they should involve health agencies, food authorities and reference laboratories (Buchanan et al., 2017; Donovan, 2015; Luber et al., 2011; Montero et al., 2015).

1.3.1. Epidemiological Data

The notification of listeriosis in humans is mandatory in most member states (MS), Iceland, Norway and Switzerland, except for four MS, where notification is based on a voluntary system (Belgium and Luxembourg) or other system (Spain and the United Kingdom). The surveillance systems for listeriosis covers the entire population in all MS except in Spain. In the European Union (EU), according to the latest EU summary report on zoonoses, zoonotic agents and foodborne outbreaks of 2016, twenty-eight MS reported 2536 confirmed invasive human cases of listeriosis in 2016. The EU notification rate in 2016 was 0.47 cases per 100 000 population, representing a 9.3% increase compared with 2015. There has been a statistically significant increasing trend of confirmed listeriosis cases in the EU/European Economic Area (EEA) during the overall period 2008–2016, as well as during the period from 2012 to 2016 (**Figure 3**). Half of the MS reported a higher number of listeriosis cases in 2016 compared with 2015. On average, 97.7% of the cases with information on hospitalization status were hospitalized. Listeriosis had the highest proportion of hospitalized cases of all zoonoses under EU surveillance. A total of 247 deaths due to listeriosis were reported by nineteen-member states in 2016, which was the highest number of fatal cases reported since 2008. The overall EU case fatality among cases with known outcome was 16.2%. France reported the highest number of fatal cases (53) followed by Germany (48). *L. monocytogenes* infections were most commonly reported in the age group over 64

years. In the period 2008–2016, a seasonal pattern was observed in the listeriosis cases reported in the EU/EEA, with high summer peaks followed by less high winter peaks. (EFSA & ECDC, 2017). In 2014, seven foodborne outbreaks supported by strong evidence were reported by 5 MS. Implicated food vehicles were: crustaceans, shellfish, mollusks and products thereof (3), followed by cheese (1), meat and meat products (1), pig meat and products thereof (1), vegetables, juices and products thereof (mixed salad) (1) (EFSA & ECDC, 2015).

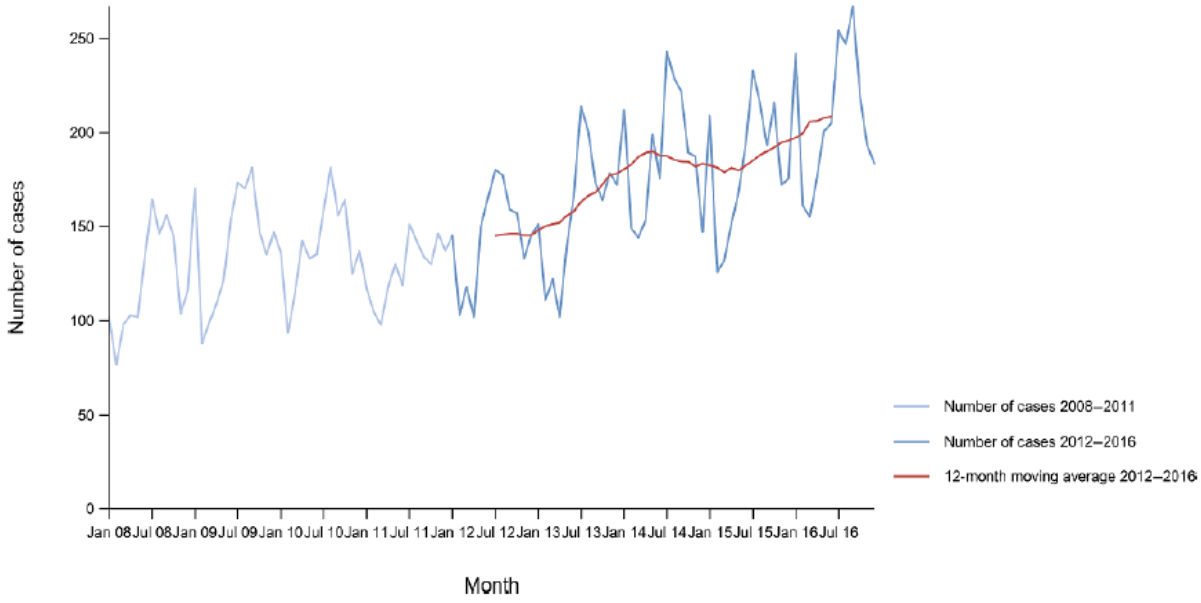


Figure 3. Trend in reported confirmed human cases of listeriosis in the EU/EEA, by month, 2008–2016. Source(s): Austria, Belgium, Cyprus, the Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Malta, the Netherlands, Norway, Poland, Romania, Slovakia, Slovenia, Spain, Sweden and the United Kingdom. Bulgaria, Croatia, Luxembourg and Portugal did not report data to the level of detail required for the analysis. *From EFSA & ECDC, 2017.*

In Portugal, listeriosis has been officially notifiable since April 2014, but until December 2016, there was no active surveillance program, which made the detection of outbreaks an extremely difficult task (Magalhães et al. 2015; Direção Geral de Saúde, 2016). Nevertheless, an increasing trend in the number of human listeriosis cases has been also observed in Portugal. In total, 32 cases of listeriosis with an incidence rate of 0.30 per 100 000 population were reported in Portugal in 2016 (EFSA & ECDC, 2017). With a total of 7 deaths in 2016, the case fatality among cases with known outcome was 22.6%, a higher percentage when comparing with the overall EU case fatality (**Figure 4A)**). The age group with the majority of fatal cases in Portugal was between 45 and 64, followed by the age group over 65 years of age (**Figure 4B)**. In 2016, the highest rates were detected in infants below one year of age (2.34 per 100000 population), people between 45 and 64 years of age (0.52 per 100 000 population) and elderly people over 65 years of age (0.61 per 100 000 population) (**Figure 4C**).

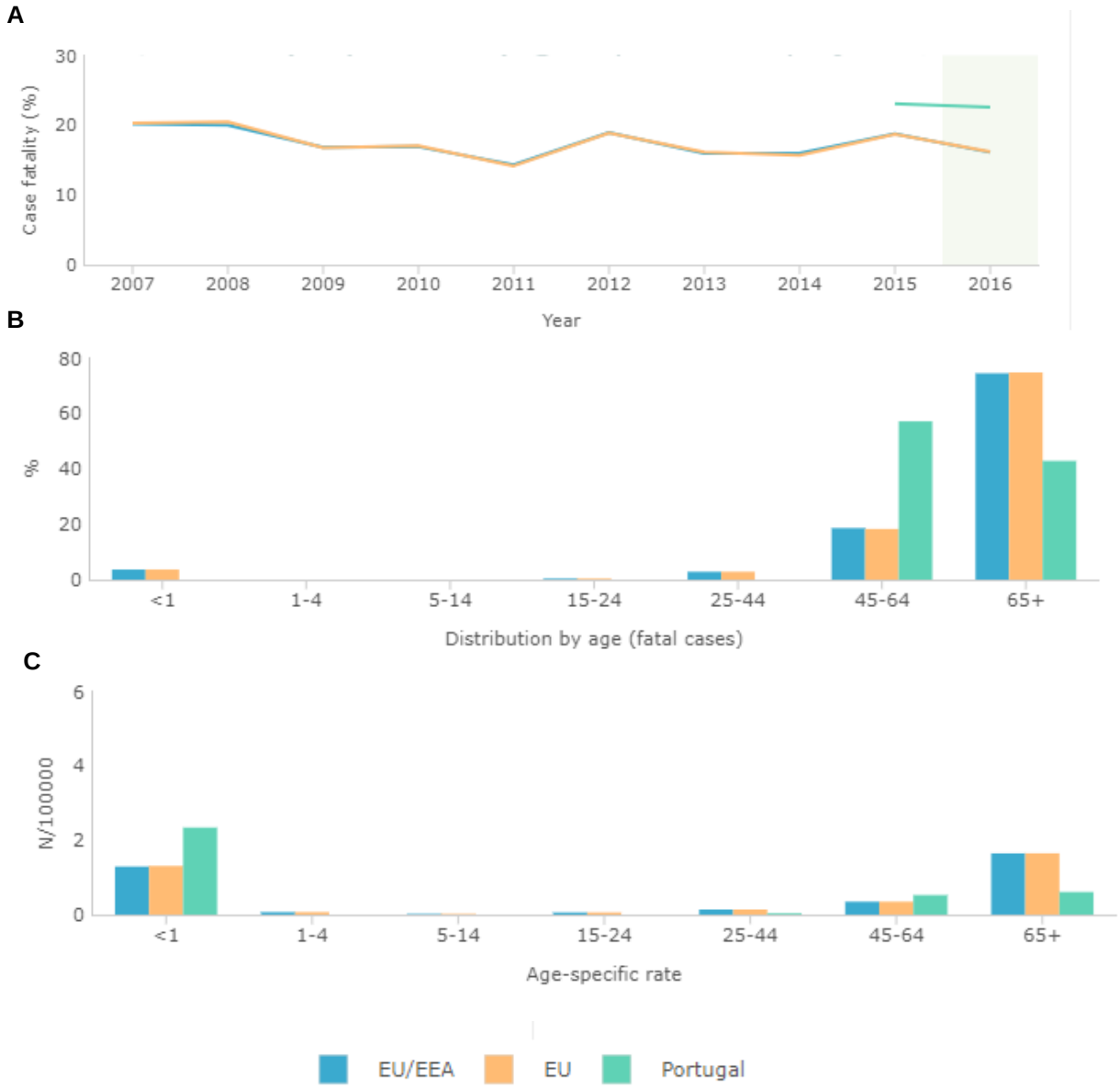


Figure 4. Data on listeriosis reported by the EU/EEA countries. Cases were reported according to the 2012 EU case definition for listeriosis (European Commission, 2012). **(A)** Listeriosis case fatality calculated as proportion of deaths among confirmed cases with known disease outcome (%), for the EU/EEA, EU and Portugal, between 2007 and 2016. **(B)** Listeriosis fatal cases, distributed by age, for the EU/EEA, EU and Portugal, in 2016. **(C)** Age-specific notification rate per 100 000 population for the EU/EEA, EU and Portugal, in 2016. *Obtain with Surveillance ATLAS of Infectious diseases by ECDC.*

1.4. *Listeria monocytogenes* in ready-to-eat (RTE) food products

L. monocytogenes is frequently present in raw foods but can also be found as a result of cross-contamination in ready-to-eat (RTE) products (Sahu et al., 2016; Uyttendaele et al., 2004). *L. monocytogenes* is responsible for many cases of food poisoning through dairy products, processed meats, salads, and other RTE foods, which do not require heating or cooking prior to consumption (Takahashi et al., 2015). In fact, the majority (>99%) of human listeriosis cases are foodborne and

associated with contaminated RTE foods with high levels of *L. monocytogenes* at the time of consumption (Malley et al., 2015). The ability of *L. monocytogenes* to grow in food stored under refrigeration temperatures, allows it, even at low initial numbers, to multiply to a level which potentially threatens consumer health (Szczawiński et al., 2017). This is especially true for RTE foods with a prolonged shelf-life under refrigeration, being categorized as risk products for listeriosis (Uyttendaele et al., 2004).

L. monocytogenes has been isolated from a large variety of RTE foods. In 2016, in EU, among the different RTE food categories and across all sampling stages, *L. monocytogenes* was most frequently detected in "fishery products" (5.6%), "fish" (4.7%), "pork meat products other than fermented sausages" (3.1%) and in "soft and semi-soft cheeses made from raw milk" (2.5%) (EFSA & ECDC, 2017).

The presence of *Listeria monocytogenes* in RTE food products, has been a matter of increased concern, especially nowadays. Contemporary lifestyles have a major influence on food consumption patterns and one of the major trends is the growing preference for convenience foods, to which ready-to-eat foods are well associated (Martins, & Germano, 2011).

The occurrence of *L. monocytogenes* in RTE foods can result from contamination of the raw materials, contamination during processing, or post-processing contamination (Guentert et al., 2006; Guerra et al., 2001). Food processing environments (FPE) have been shown to be contaminated with *L. monocytogenes,* which can spread throughout the facility due to contaminated contact materials, inappropriate personnel movements and food workflow (Muhterem-Uyar et al., 2015). Processing equipment like slicers, dicers, freezers, and conveyors, can be a major source of contamination of the final products; in fact, transfer studies with cut produce have shown continuing transference from contaminated equipment (slicers) to uncontaminated product (Buchanan et al., 2017; Lundén et al., 2003; Uyttendaele et al., 2009). Due to their complex structure, processing equipment are a source of contamination, mainly because of the difficulty in executing sanitizing operations (Lundén et al., 2003).

1.4.1. *L. monocytogenes* in RTE meat-based salads

RTE meat salads consist of diverse ingredients with different abilities to support the growth of *L. monocytogenes*. Salad meat dishes are cold ready-to-eat (RTE) dishes that typically contain raw cuts of vegetables, as well as other cooked and smoked ingredients (Chau et al., 2017). For example, chicken salads usually contain raw ingredients, like lettuce, tomato, carrots, and also cooked ingredients, like chicken, bacon or eggs. These salads endure extensive handling processes during preparation, and thus, are exposed to several opportunities for being contaminated. Since prepacked meat salads lack any heating step before consumption and are maintained under conditions likely to allow the multiplication of psychotropic *L. monocytogenes*, these should be considered as potential vehicles of transmission of human listeriosis (Chau et al., 2017; Little et al., 2007). Controlled raw materials, hygienic manufacture and appropriate storage during shelf-life are factors of extreme importance for RTE meat-based salads safety (Little et al., 2007).

In a United Kingdom study on *Listeria monocytogenes* in retail pre-packaged mixed salads with meat, 1268 samples were tested and 6% were positive (detection in 25 g), among which chicken salad and chicken salad with bacon presented the highest frequencies (Little et al., 2007). In another study on *L. monocytogenes* in pre-packed chicken salads in Singapore, the percentage of positive samples was 6.3%, from a total of 32 samples tested (Chau et al., 2017), a very similar percentage to the latter study. In Iran, from 2010 to 2011, 88 samples of RTE meat products, including poultry salads, sausages and burgers, were tested, and 11.4% were positive for *L. monocytogenes* detection in 25 g of food (Fallah et al., 2012). In all these studies, the predominant serotype recovered from food isolates was 1/2a.

1.4.2. Outbreaks associated with *Listeria monocytogenes* in RTE food products

Increasing evidence suggests that persistence of *L. monocytogenes* in the food processing environment is a major factor in the transmission of this foodborne pathogen and the source of numerous human listeriosis outbreaks (Ferreira et al., 2014)

In Portugal, a retrospective study involving 25 national hospitals led to the detection of an outbreak that occurred from March 2009 to February 2012. The amount of time between the outbreak onset and its detection was 16 months. Of the 30 cases of listeriosis reported, 27 occurred in Lisbon and Vale do Tejo region. Two cases were maternal/neonatal infections and one resulted in fetal loss. The mean age of the non-maternal/neonatal cases was 59 years; 13 cases occurred in individuals with more than 65 years-old. The case fatality rate was 36.7%. All cases were caused by molecular serogroup IVb isolates. Collaborative investigations with the national health and food safety authorities identified *queijo fresco* and a cured cheese as the probable sources of infection, traced to a processing plant. The magnitude of this outbreak, the first reported foodborne listeriosis outbreak in Portugal, highlights the importance of having an effective listeriosis surveillance system in place for early detection and resolution of outbreaks, with the prompt submission of *Listeria monocytogenes* isolates for routine laboratory typing (Magalhães et al., 2015).

In the United States of America (USA), from 1998 to 2016, 68 foodborne listeriosis outbreaks have been reported, with 860 cases of disease, a hospitalization rate of 71.5% and a fatality rate of 15.3% (CDC, 2016).

From 26 October 2013 to 23 April 2014, 32 cases of listeriosis (serotype 4b), were registered in patients from several cantons of Switzerland. Ready-to-eat salads from a food producing company were identified as the outbreak source, since product and environmental samples collected from that company during investigations matched the outbreak strain. The cause for the product contamination was related to a design-inherent hygienic problem of one specific product-feeding belt. Data collected from patient's interviews also identified ready-to-eat green salads bought at one retailer as the likely outbreak source (Stephan et al., 2015).

From July 5, 2015 to January 31, 2016, the CDC and the Food and Drug Administration (FDA) investigated a multistate outbreak of listeriosis. A total of 19 people was reported and hospitalized, among which was a pregnant woman and another person died. Whole genome sequencing (WGS) of

the isolates from all 19 cases revealed a close genetic relation. According to the Public Health Agency of Canada, in 2015-2016 in Canada, several cases of listeriosis revealed the same *L. monocytogenes* strain. Epidemiologic and laboratory evidence indicated that packaged salads produced at the Dole processing facility in Springfield, Ohio, sold under various brand names were the likely source of this outbreak. On January 27, 2016 the company voluntarily recalled all salad mixes (CDC, 2016).

In South Africa, a serious listeriosis outbreak has affected the country, between 1 January 2017 through 24 April 2018, 1024 laboratory-confirmed listeriosis cases have been reported. The outcome of illness is known for 700 patients, of whom 200 (28.6%) of them died. When compared to other reported listeriosis outbreaks worldwide, this outbreak is described as the deadliest recorded outbreak of listeriosis in history. Most of the cases involved individuals with a higher risk for a severe disease outcome, such as neonates, pregnant women, the elderly and immunocompromised persons. In this outbreak, 42% of the cases involved neonates infected during pregnancy or delivery. A ready-to-eat processed meat product was identified as the source of the outbreak, and a country-wide recall of the implicated products was initiated (Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO), 2018).

An overview of confirmed listeriosis outbreaks and associated food vehicles in several locations from 2009 to 2018 can be observed in **Table 1**.

Table 1. Confirmed listeriosis outbreaks from 2009 to 2018 and implicated food vehicles.

Year	Country	Implicated food	No. Cases/ Fatality rate	Reference
2009-2012	Portugal	Fresh cheese (Cured cheese and “queijo fresco”)	30 / 36.7%	(Magalhães et al., 2015)
2010	United States	Celery from chicken salad	10 / 30%	(Gaul et al., 2013)
2011	Belgium	Hard cheese	12 / 16.7%	(Yde et al., 2012)
2011	United States	Cantaloupe	147 / 22%	(MacCollum et al., 2013)
2012	Spain	Fresh cheese	2 / -	(de Castro et al., 2012)
2013-2014	Switzerland	Ready-to-eat-salad	32 / 12.5%	(Stephan et al., 2015)
2014	United States	Pre-packaged caramel apples	35 / 20%	(Glass et al., 2015)
2010-2015	United States	Ice-cream	10 / 30%	(Pouillot et al., 2016)
2015-2016	United States/ Canada	Pre-packaged salads	33 / 12.1%	(CDC, 2016)
2015-2016	Italy	Pork head cheese	24 / 16.7%	(Duranti et al., 2018)
2016-2017	United States	Artisanal soft cheese	8 / 25%	(CDC, 2017)
2017-2018	South Africa	Ready-to-eat processed meat	1024 / 28.6%	(FAO/WHO, 2018)

1.4.3. *L. monocytogenes* control policies and regulations regarding RTE food products

Recommendations and official regulations on *L. monocytogenes* criteria in RTE differ throughout the world. European MS adopted the European Regulation (EC) No. 2073/2005 of 15 November 2005, which lays down the microbiological criteria for certain microorganisms in foodstuffs. For RTE products intended for infants and for special medical purposes, legislation demands the absence of *L. monocytogenes* in 10 samples of 25 g during their entire shelf-life. In RTE products other than those for infants and special medical purposes different microbiological criteria apply depending on the ability of the food product to support growth of *L. monocytogenes*. For RTE products unable to support the growth of *L. monocytogenes*[1] (based on their pH, water activity values and/or other intrinsic factors) and for products with a shelf life of less than 5 days, legislation allows for a maximum of 100 cfu/g for products placed on the market during their shelf-life. In RTE foods that are able to support the growth of the *L. monocytogenes,* the legislation demands the absence of *L. monocytogenes* in 5 samples of 25 g before the product leaves the immediate control of the food business operator. However, the criterion of 100 cfu/g *L. monocytogenes* applies if the manufacturer is able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit of 100 cfu/g throughout the shelf-life, under reasonably foreseeable storage conditions of distribution, storage and use. The manufacturer should base his decision on various types of data and studies, such as food characteristics, challenge tests, durability studies, predictive microbiology, data from scientific literature and risk assessments. In case compliance with the criteria cannot be demonstrated, the absence in 25 g criterion applies (European Commission, 2005; Lokerse et al. 2016; Auvolat & Besse 2016; CAC, 2007). In turn, the Food and Drug Administration (FDA) in the USA applies a "zero tolerance" policy (*i.e.*, non-detection of *L. monocytogenes* in two 25 g samples) regarding *L. monocytogenes*, as it does not allow any level of *L. monocytogenes* in food products to be released onto the market (FDA, 2003).

1.4.4. Microbial challenge testing for *L. monocytogenes* in RTE food

Regulation (EC) No 2073/2005 establishes that food business operators (FBOs) are primarily responsible for the safety of food they place on the market (Álvarez-Ordóñez et al., 2015; EURL Lm, 2014; Spanu et al., 2014). FBOs shall conduct studies to evaluate the growth of *L. monocytogenes* that may be present in the product during its shelf-life under reasonably foreseeable storage conditions of distribution and storage, to assure that their food does not exceed the food safety criteria throughout the defined shelf-life. For this matter, a range of practical guidance documents have been elaborated to assist MS and food industries within the European Union in assessing the growth potential of *L. monocytogenes*. Among these guidance documents, two of them can be highlighted: the first, intended for FBOs, is the Guidance Document on *L. monocytogenes* shelf-life studies for RTE foods, under Regulation (EC) No. 2073/2005 (European Commission, 2008); the second document, complementary to the latter, consists of a technical guidance document directed to laboratories for conducting shelf-life

[1] According to Commission Regulation (EC) No. 2073/2005, products with pH ≤ 4.4 or a_w ≤ 0.92, products with pH ≤ 5.0 and a_w ≤ 0.94, products with a shelf life of less than 5 days shall be automatically considered to belong to this category. Other categories of products can also belong to this category, subject to scientific evidence.

studies on *L. monocytogenes* in RTE foods, prepared by the European Union Reference Laboratory for *Listeria monocytogenes* (EURL Lm, 2014). These guidance documents give guidelines to determine whether tests are necessary and to choose an appropriate challenge test setup, namely the number of samples to test, the design of the shelf-life tests and the development of strains suitable for challenge testing (Buchanan et al., 2017; Lokerse et al., 2016).

A microbial challenge study consists in the artificial contamination of a food product with the target microorganism under controlled experimental conditions and aims at simulating its behavior during processing and distribution under the foreseeable conditions of transportation, and handling and storage, at retail and at consumer level. Challenge tests, as their main objective, intend to determine whether or not a particular RTE food is able to support the growth of *L. monocytogenes* during the designated shelf-life (Álvarez-Ordóñez et al., 2015; Spanu et al., 2014). However, challenge studies an also have other objectives, such as to validate the efficacy of lethality treatment applied to RTE foods intended to reduce or eliminate the pathogen. As proposed by the *Codex Alimentarius* Commission (CAC), growth potential can be estimated from the difference between the log cfu/g at the end of the shelf-life and the log cfu/g at the beginning of the test. A RTE food is considered able to support the growth of *L. monocytogenes* when this difference is greater than 0.5 log cfu/g. Otherwise, when the difference is lower than 0.5 log cfu/g, the food is classified into RTE foods unable to support the growth of *L. monocytogenes* (Álvarez-Ordóñez et al., 2015; Buchanan et al., 2017; Sahu et al., 2016; Spanu et al., 2014). However, food products with pH ≤ 4.4 or a_w ≤ 0.92, products with pH ≤ 5.0 and a_w ≤ 0.94, products with a shelf-life of less than five days are automatically considered to belong to the category of RTE unable to support the growth of *L. monocytogenes* (EURL Lm, 2014; European Commission, 2005). Growth potential depends on various factors, such as inoculated strain(s), the inoculation level, the physiological state of the inoculated cell(s), intrinsic properties of the food (e.g. pH, NaCl content, a_w, associated microflora, antimicrobial constituents) and extrinsic properties (e.g. time-temperature profile, gas atmosphere, moisture). Several factors must be considered when conducting a microbial challenge test, from the number and choice of batches, selection of the strains, preparation and choice of the inoculum level, method of contamination, and storage conditions to the experimental design, microbiological analyses and data interpretation (EURL Lm, 2014; Spanu et al., 2014).

This type of food testing is recognized by the European Commission's Directorate General for health and food safety as a very important tool in providing information about *L. monocytogenes* development in ready-to-eat foods (European Commission, 2008).

1.5. Persistence of *L. monocytogenes* in the food processing environment

Presence of *L. monocytogenes* in the food processing environment is thought to be the principal source of post-processing contamination in processed RTE foods (Buchanan et al., 2017; Ferreira et al., 2014; Hoelzer et al., 2014; Malley et al., 2015). Strains of *L. monocytogenes* are recurrently found on surfaces in the food industry, notably in refrigerated premises, though these are routinely cleaned and disinfected (Carpentier, & Cerf, 2011). *Listeria monocytogenes* strains have been found to persist for years or decades in food processing plants, with specific strains having been repeatedly isolated over the time

from the same food establishments (Buchanan et al., 2017; Doumith et al., 2004; Ferreira et al., 2014). Rørvik et al. (1995), found that all seven *L. monocytogenes* isolates from the finished vacuum-packed smoked salmon represented the same strain that was predominant in the smokehouse, this strain was isolated in the facility during the whole investigation period (6 samplings over 8 months), supporting the hypothesis of persistence in the facility. In a different case, over a period of seven years, one *L. monocytogenes* strain was repeatedly isolated from an ice-cream plant (Miettinen et al., 1999). Elucidating the sources and contamination routes of *L. monocytogenes* in food-processing environments can be done by high resolution molecular typing techniques. Methods such as pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD) analysis and multi-locus enzyme electrophoresis (MEE) have facilitated tracing the routes of *L. monocytogenes* contamination (Ferreira et al., 2014; Møretrø & Langsrud, 2004).

Despite persistence of *L. monocytogenes* in the food-processing environment being well documented, it is still poorly understood. In fact, some authors attribute this lack of understanding to the loose conception of the term persistence, with no standardized criteria to differentiate between persisting and sporadic strains recovered in the food processing environment (Carpentier, & Cerf, 2011). This means that strains surviving and persisting in the food-processing environment, can possible be a result of repeated reintroduction of such strains from the outside sources into food processing facilities over time, for example through raw material, water, animals and personnel (Buchanan et al., 2017; Carpentier, & Cerf, 2011; Møretrø & Langsrud, 2004).

Harborage sites are a key factor in the persistence of *L. monocytogenes.* When used correctly, cleaning and disinfection procedures should be adequate to remove *L. monocytogenes* from the environment. However, failure in properly removing *L. monocytogenes* and its consequent persistence may be due to the survival and growth of certain strains in niches within the food processing environment, such as cracks and crevices of surfaces, seals and gaskets that may be difficult to clean and disinfect, equipment switches, valves, or spaces between close-fitting parts (Buchanan et al., 2017; Carpentier, & Cerf, 2011; Hoelzer et al., 2014).

The persistence of *L. monocytogenes* in the food processing environment can be however linked to some traits which may potentially increase its ability to survive in such environments. These traits include biofilm formation, tolerance to disinfectants commonly used in the food industry and physical adaptation or enhanced tolerance to adverse environmental conditions (Buchanan et al., 2017; Ferreira et al., 2014; Møretrø & Langsrud, 2004).

1.5.1. *L. monocytogenes* surface adherence and biofilm formation

Biofilms can be defined as a sessile microbial structured community of cells embedded in a self-produced polymeric matrix and attached to an inert or living surface (Donlan, & Costerton, 2002; Møretrø & Langsrud, 2004). When bacteria interact with a surface, the first phase is a reversible adhesion of bacteria to the surface, which takes place in a period from minutes to a few hours. Cells ultimately adhere irreversibly to the surface and start multiplying and producing extracellular compounds, forming micro-colonies and subsequently thicker multi-layer and multi-species biofilm (Møretrø & Langsrud,

2004). With time, a more complex three-dimensional network is formed where mature biofilms contain channels for flow of nutrients and waste products (Silva & De Martinis, 2013). In the last stage of biofilm development, microbial cells are able to detach from the biofilm and to disperse into the environment (in their planktonic form), with subsequent colonization of other surfaces (Colagiorgi et al., 2017). True biofilms protect individual cells from environmental stresses and promote interactions between cells in relation to nutrients, toxic metabolites and genetic material that may lead to enhanced survival and growth (Buchanan et al., 2017). An example of that is the improved resistance to both clinical antimicrobials and disinfectants shown by bacteria in biofilms when compared to planktonic bacteria, as they have a barrier which prevents the contact with antimicrobial agents (Allen et al., 2016). Several factors may play a role in biofilm-mediated resistance, such as low diffusion through the extracellular polymeric substances (EPS) matrix, physiological changes of the microorganisms owing to slow growth rate, biofilm heterogeneity, quorum sensing, efflux pumps, starvation responses, induction of attachment-specific drug-resistant physiologies, chemical or enzymatic modification of the antibacterial agent and the presence of persister cells (Allen et al., 2016; Møretrø & Langsrud, 2004).

In industry, the robust nature of biofilms makes them suitable for various biotechnology applications, such as bioremediation processes, wastewater treatments, biocorrosion control, production of biofuels and chemicals pharmaceutical testing (Wood et al., 2011). However, when it comes to pathogenic organisms, like *Listeria monocytogenes*, its ability to form biofilms can represent a threat for public health (Silva, & De Martinis, 2013). *L. monocytogenes* is capable of forming biofilms on several surfaces used in the food industry, such as stainless steel, polystyrene, quartz, marble, granite and glass, representing a major concern for food safety, because it could represent a source of contamination of food products (Colagiorgi et al., 2017; Silva, & De Martinis, 2013). *L. monocytogenes* is able to form monospecies and multispecies biofilms with both Gram-positive and Gram-negative species. The interaction between different species, observed in mixed biofilms, may change biocide tolerance response of every strain involved (Puga et al., 2016). Some authors have also reported *L. monocytogenes* biofilm formation at refrigeration temperatures, as low as 4ºC, on different surfaces (Bonsaglia et al., 2014; Di Bonaventura et al., 2008; Norwood, & Gilmour, 2001). The ability of *L. monocytogenes* to produce biofilms at low temperatures used during food processing and storing increases the likelihood of cross-contamination (Colagiorgi et al., 2017). In biofilms, *L. monocytogenes* is protected from a variety of environmental factors, such as ultraviolet rays, toxic metals, acids, desiccation, salinity, and antimicrobials, and it tolerates better high concentrations of disinfectants and sanitizers making the decontamination of surfaces more difficult to achieve (Silva, & De Martinis, 2013). Thus, surface biofilm, particularly in locations which are problematic to identify and disinfect, through the detaching of *L. monocytogenes* from the biofilm, can act as a persistent source of food product contamination (Borucki et al., 2003).

1.5.2. Tolerance to disinfectants

Disinfection is defined as the treatment of surfaces/equipment using physical or chemical means such that, the amount of microorganisms present is reduced to an acceptable level that does not compromise food safety or suitability. Prior to disinfecting, cleaning of the surface is necessary to remove organic compounds adhered to the surface. Without proper cleaning, disinfection is useless, as remaining product will inactivate the disinfecting agent and microorganisms present will survive the disinfecting treatment (CAC, 2007; Van Asselt, & Te Giffel, 2005). Disinfection may be achieved through physical (thermal, radioactive) and chemical methods. In the food processing environment, chemical disinfection is more frequently used (Gaulin et al., 2011). The disinfectants that are commonly used in the food industries include alcohols, aldehydes, peracetic acid, hypochlorite, organic chlorine-releasing compounds and surface-active agents (surfactants), which include amphoteric, cationic (quaternary ammonium compounds (QACs) such as benzalkonium chloride (BAC)), and biguanide/diguanide compounds (Ortiz et al., 2014). The disinfectants can interact with different bacterial structures, such as outer cellular components, cytoplasmic membrane and cytoplasmic constituents (functional or structural proteins, DNA, RNA) (Morente et al., 2013).

Bacterial response to biocides is determined fundamentally by the nature of the chemical agent and the type of organism involved. To optimize the efficiency of disinfectants it is important to determine the best conditions regarding contact time, temperature, appropriate concentrations, environmental pH and the presence of organic matter (Morente et al., 2013; Silva, & De Martinis, 2013). In fact, several factors can reduce the efficacy of disinfectants in the food processing environment, dilution below the recommended concentration is one of them, most times, dilution is caused by water in places that should be dry but are not; concentration can also be inappropriate when no account is taken of interfering organic matter, for example on insufficiently cleaned surfaces and in the presence of biofilms (Cerf et al., 2010; Soumet et al., 2005). In these conditions, bacteria are exposed to sub-lethal concentrations of disinfectant, which could lead to a selective pressure for acquisition of resistant genes or to adaptive responses leading to increased tolerance for the disinfectants (Møretrø et al., 2017; Soumet et al., 2005). In food processing environment, *L. monocytogenes* is exposed to different disinfectants, sometimes at sub-lethal concentrations, as is the case for disinfectants that are not fully biodegradable, such as QACs, which are only biodegradable at aerobic conditions, persisting in sewage for long periods (Martínez-Suárez et al., 2016). Repeated exposure to sub-lethal concentrations of QACs and long-term environmental persistence of certain strains may facilitate the development of resistance over time (Ortiz et al., 2014). In *L. monocytogenes* some resistance mechanisms have been proposed to explain the increased tolerance to disinfectants. Over-expression of multidrug efflux pumps through mutation or acquisition of mobile genetic elements (plasmids or transposons) by horizontal transfer between different pathogenic and non-pathogenic species of *Listeria* and other species may contribute to disinfectant resistance (Ferreira et al., 2014; Mester et al., 2015; Muenster et al., 2006). This tolerance to disinfectant could explain the capacity of *L. monocytogenes* to survive and persist even after the performance of routine cleaning and disinfection procedures in food processing environments.

1.5.3. *L. monocytogenes* stress adaptation

In the food processing environment, *L. monocytogenes* is continually exposed to environmental stresses such as starvation of nutrients, acid and oxidative stresses, cold temperatures, high osmolarity, desiccation, and competing bacteria (Ferreira et al., 2014; Puga et al., 2016). *L. monocytogenes* stress responses to sublethal conditions result in important changes in gene and protein expression profiles (Ferreira et al., 2014). In *L. monocytogenes* and other Gram-positive bacteria, one of the most fundamental regulatory mechanisms enabling survival to harmful conditions occurring within the food chain involves transcriptional redirection through the association of the alternative sigma factor B (σ^B) with core RNA polymerase (Allen et al., 2016; Ferreira et al., 2014; Gray et al., 2006). σ^B, encoded by *sigB*, has been identified as the general stress responsive alternative sigma factor. In *L. monocytogenes,* σ^B – dependent transcription is induced when the organism enters stationary phase or is subjected to various environmental stresses, including carbon starvation, acid, osmotic and oxidative, and cold stress and contributes to bacterial osmotolerance, detergent stress response, carbon starvation, and survival under acid and oxidative stress. The alternative sigma factor σ^B is also vital to the adaptation of stationary-phase *L. monocytogenes* cells to growth at low temperatures (Allen et al., 2016; Ferreira et al., 2014)

1.5.3.1. Cold temperature stress

Refrigeration is one of the most common procedures used in food processing and distribution to ensure food safety and to prolong shelf-life of food products (Melo et al., 2015; Yousef, & Courtney, 2003). However, the survival and growth of *L. monocytogenes* at refrigeration temperatures (Meloni, 2014), make the control of this pathogen in the food industry a significant challenge (Li et al., 2016). While initial *L. monocytogenes* levels in contaminated foods are usually low, its ability to multiply at refrigeration temperatures seems to be critical in enabling the pathogen to reach high enough levels to cause human disease, particularly if contaminated foods are refrigerated for prolonged times (Chan et al., 2007; Melo et al., 2015). Mechanisms that *L. monocytogenes* may use to adapt to cold stress during exposure to low temperatures in food processing environment and storage include the expression of cold shock proteins (Csps) and cold acclimation proteins (Caps), changes in membrane lipid composition, and the uptake of osmolytes and oligopeptides (Chan et al., 2007; Melo et al., 2015).

Changes in membrane composition include an increase in the proportion of carbon chain $C_{15:0}$ at the expense of $C_{17:0}$, which help to maintain membrane fluidity at a lower temperature. Furthermore, growth at low temperature results in an increase in unsaturated fatty acids, which would help in the survival of *L. monocytogenes* under low temperatures (Beales, 2004).

The cold shock proteins (Csps) are a family of small and highly conserved chaperones, which are thought to bind RNA and DNA, and thus may facilitate the control of processes such as replication, transcription and translation within bacterial cells (Melo et al., 2015; Schmid et al., 2009). Three Csp family proteins (CspA, CspB, and CspD) are found within the sequenced genomes of *L. monocytogenes*. Both cold shock proteins and cold acclimation proteins (Caps) identified in *L. monocytogenes* show increased expression during temperature downshift from its optimal temperature to a lower temperature,

indicating that these proteins aid in physiological adaptation to low temperatures and allow *L. monocytogenes* to survive and persist, despite the stress imposed by the temperature downshift (Chan et al., 2007; Schmid et al., 2009; Yousef, & Courtney, 2003). It is known that Csps are rapidly, but transiently, overexpressed in response to cold stress, while Caps are continuously expressed during growth at cold temperatures, however their functions are not yet clearly understood (Chan et al., 2007; Schmid et al., 2009)

The exposure of *L. monocytogenes* to cold stress conditions, also leads to the uptake and accumulation into the bacterial cell of low-molecular-weight compatible solutes (Chan et al., 2007; Melo et al., 2015). In *L. monocytogenes,* glycine betaine and carnitine are the predominant compatible solutes that accumulate during low-temperature exposure and growth (Bayles, & Wilkinson, 2000; Chan et al., 2007). These osmolytes have a role as cryoprotectants, in fact, at low temperatures, deletions of the osmolyte transporters reduced the growth of *L. monocytogenes* (Melo et al., 2015). Since meat and dairy products are rich in carnitine, and glycine betaine is abundant in plants and shellfish, *L. monocytogenes* has access to these compatible solutes in many different food products.

1.6. *L. monocytogenes* analysis methods

1.6.1. Detection and Quantification methods

Food laboratories currently rely on conventional microbiological methods for quantification of *Listeria monocytogenes*. However, enumeration and detection of *L. monocytogenes* in food faces many difficulties: contamination usually occurs in very low numbers, both in foods and in the processing environment, a great variety of matrices must be considered, and also the eventual presence of other microorganisms. Adequate enumeration methods are essential to provide reliable data for research studies in predictive microbiology, epidemiology, quantitative risk assessment, and for routine analysis or monitoring programs in food processing plants. Therefore, improvement of *L. monocytogenes* enumeration techniques is a topic of great concern in the field of food hygiene (Auvolat, & Besse, 2016).

1.6.1.1. Culture-based techniques

Direct plate colony count methods are quite simple and fast, but are characterized by poor performance in terms of sensitivity, reproducibility, recovery of stressed cells, and sometimes selectivity (Besse, & Colin, 2004; Jasson et al., 2010).

The European and International Standard method for enumeration of *L. monocytogenes* ISO 11290-2 is cited as the reference standard method in the quantitative criteria of EC Regulation No. 2073/2005 for *L. monocytogenes* (European Commission, 2005). However, this method is characterized by a theoretical limit of enumeration of 10 cfu/g in liquid products and 100 cfu/g in solid foods (ISO 11290-2:2017). Consequently, as precision of this standard method is relatively poor, the method still lacks sufficient sensitivity to reliably quantify *L. monocytogenes* and does not seem to be optimal for the examination of food products that are usually contaminated at low levels, less than 10-100 cfu/g (Auvolat, & Besse, 2016; Besse, & Colin, 2004).

Culture-based techniques include different methods besides the reference standard method, such as: most probable number (MPN) technique, culturing methods that include a resuscitation step and culturing methods that include a cell concentration step, all characterized by high variability in precision (Auvolat, & Besse, 2016).

Overall, culture-based techniques are time-consuming and sometimes labor intensive. Also, these methods may require individual biochemical confirmation of the species for some isolated colonies. Moreover, in food, *L. monocytogenes* is often affected by one or several stresses caused by a variety of processing treatments including heating, freezing, drying, exposure to acids, to disinfectants, and to high osmotic pressures. This may lead to loss of their ability to grow on selective media, becoming viable but non culturable (VBNC) bacteria. Recovering stressed *L. monocytogenes* from food is of major importance in food safety, since sublethal injured bacteria may recover in food and regain their pathogenicity (Auvolat, & Besse, 2016; Besse, & Colin, 2004; Brasseur et al., 2015).

1.6.1.2. Molecular biology methods: Real-time quantitative PCR (RT-qPCR)

The development of more sensitive, rapid and specific methods than plate counts for the detection and quantification of viable and VBNC *L. monocytogenes* cells is essential not only for monitoring food quality and for listeriosis prevention, but also for extending the commercial life of short-term food products (Agustí et al., 2018; Cobo Molinos et al., 2010; Postollec et al., 2011).

The RT-qPCR, a modified version of conventional PCR has enabled a quantitative approach of microorganisms, owning to its high sensitivity and accuracy. In qPCR, the PCR products are detected as they accumulate. The amount of generated PCR product is proportional to the increase in a fluorescent signal, which is monitored in real time at every cycle during the exponential phase, with a fluorescent reporter (dye). The number of cells can be estimated using standard regression curves which correlate the cycle threshold (Ct) values of the PCR to genomic copies in the DNA extract, based on samples at known concentrations. Ct value corresponds to the cycle number for which fluorescence signal (i.e. DNA template), which is significantly higher than background signal, is proportionally correlated with initial level of target DNA and serves as a basis for relative to absolute quantification of DNA template. The absence of any essential post-PCR step simplifies the method, making automation and high throughput possible (Auvolat, & Besse, 2016; Chen et al., 2017; Jasson et al., 2010).

Despite RT-qPCR advantages, one of the main problems associated with DNA based methods is their inability to discriminate among viable and dead cells, as DNA of dead cells can persist in the food matrix, leading to a substantial overestimation of target bacteria concentrations. This particular issue has greatly limited the applicability of molecular-based methods for routine monitoring of microorganisms in food products, in which non-viable cells are frequently present after food processing and conservation (D'Urso et al., 2009; Garrido-Maestu et al., 2018; Truchado et al., 2016). Several alternatives have emerged to solve this problem. One of them, the selection of RNA over DNA, due to its lower stability once bacteria die. Another alternative is the use of successive enrichment steps, which by themselves will not eliminate dead microorganisms but, reduce the probability of their detection. Filtration-based protocols, for the selective separation of viable bacteria from dead ones are another alternative. And

finally, the treatment of samples with propidium monoazide (PMA) before DNA extraction, allowing the differentiation between viable and dead cells (D'Urso et al., 2009; Garrido-Maestu et al., 2018; Nocker et al., 2006).

PMA action is based on the presence of an azide group, which after exposure to strong visible light, allows cross-linking of the dye to DNA of dead cells with compromised membrane integrity. This will result in permanent DNA modification, inhibiting amplification in the subsequent PCR reaction, whereas DNA protected by intact membranes of viable cells will be normally detected by qPCR. The dye is cell membrane-impermeable and thus can be used to selectively modify DNA from dead cells with compromised membrane integrity, while leaving DNA from viable cells intact (Li et al., 2014; Nocker et al., 2006; Truchado et al., 2016). The use of PMA has been successfully integrated with qPCR assays for the differentiation of viable and dead *Listeria monocytogenes* cells in different food samples (Agustí et al., 2018; Elizaquível et al., 2012)

Food products are complex matrices that may interfere with the efficacy of the PMA treatment. Factors such as the ratio between viable and dead bacterial cells, pH, and salt concentrations, as well as the natural presence of PMA inhibitors have been highlighted as potential inhibitors for the PMA treatment, DNA extraction and qPCR yield. Moreover, discrepancies on the available literature regarding recommended PMA concentrations make the selection of a PMA-qPCR method difficult. Therefore, optimization and validation of previously developed PMA-qPCR methods, as well as their suitability as monitoring systems in food industry are essential before their application as routine tools in microbial sampling programs (Auvolat, & Besse, 2016; Li et al., 2014; Truchado et al., 2016).

1.6.2. Characterization of Isolates/Subtyping *Listeria monocytogenes*

Since numerous strains of *L. monocytogenes* exist, it is necessary to have a robust, highly reliable and standardized system of subtyping, so that the most effective strategies can be planned for the control and investigation of outbreaks. The presence of *L. monocytogenes* is of great concern to the food industry, tracing the source of contamination within the food chain and the plant environment is of primary importance, making subtyping also very important for developing control strategies within food processing environments. In this way, a subtyping method should ideally be specific, sensitive, fast and reproducible (Jadhav et al., 2012; Kérouanton et al., 2010).

Historically, differentiation of *L. monocytogenes* strains by serotyping is one of the oldest methods of subtyping and is based on the somatic (O) and flagellar (H) antigen differences between strains (Morobe et al., 2012). However, routine analysis of *L. monocytogenes* by conventional serotyping is highly expensive, time consuming, shows low reproducibility and demands good technical practice and expertise. Moreover, the traditional agglutination method is limited by availability and quality of antisera, and typeability of isolates (antigens are shared between *L. monocytogenes* strains which do not allow unambiguous assigning of their subtype). In fact, a multicentric serotyping study conducted by Schönberg et al. (1996), as part of the WHO multicenter international typing study, pointed out some discrepancies in the results and some problems in the quality of antisera, highlighting this method as unreliable (Doumith et al., 2004; Jadhav et al., 2012; Kérouanton et al., 2010).

1.6.2.1. Multiplex PCR

Subtyping by serological tests has remained popular, however, numerous molecular biology methods such as multiplex PCR have come forward in the characterization of *L. monocytogenes* isolates. Multiplex PCR is a method that can be used for rapid separation of *L. monocytogenes* strains (Doumith et al., 2004, 2005; Kérouanton et al., 2010). In contrast to serotyping, the specific serotypes are not detected. The strains are classified in serogroups (IIa, IIb, IIc, IVa and IVb) based on the presence of specific genes. Although several multiplex PCR assays have already been proposed (De Santis et al., 2007; Doumith et al., 2004, 2005), until now, only Kérouanton et al. (2010), described a scheme of improved molecular serotyping strategy, based on two multiplex PCR assays in order to avoid most of the false positive and negative results in the IIa and IIc molecular serogroups (**Table 2**), correctly assigning atypical strains (1/2a, 3a, 1/2c).

Table 2. Gene distribution in the five molecular serogroups. *From Kérouanton et al., 2010.*

Gene	**PCR serogroups**				
	IIa	IIb	IIc	IVa	IVb
lmo0737	+	-	+	-	-
orf2819	-	+	-	-	+
lmo1118	-	-	+	-	-
orf2110	-	-	-	-	+
prs	+	+	+	+	+
prfA	+	+	+	+	+
flaA[a]	+	NT	-	NT	NT

NT=non-tested
[a] Only IIa and IIc strains tested with *flaA* primers

The multiplex PCR is less discriminating than the agglutination method, however, this method distinguishes serotypes most commonly found in food processing environments, belonging to the following serogroups; IIa: 1/2a, IIb: 1/2b, IIc: 1/2c, IVa: 4a and IVb: 4b. Compared to serotyping, serogrouping by multiplex PCR has been considered to have enhanced reproducibility, and less cost-effective and time-consuming, as a result is available within 1 day, instead of 5 days with the agglutination method. Moreover, analysis of PCR patterns is easier than interpretation of agglutinations. Molecular serotyping could be useful as a rapid method for *L. monocytogenes*' characterization and therefore valuable for epidemiological investigations to quickly identify food-associated strains during listeriosis outbreaks, but preferably associated with other molecular subtyping methods such as pulsed-field gel electrophoresis (PFGE) (Doumith et al., 2004, 2005; Kérouanton et al., 2010).

1.6.2.2. Pulsed-field gel electrophoresis (PFGE)

Among the available subtyping techniques, PFGE is regarded as “gold-standard” method for *L. monocytogenes* subtyping, for source tracking and epidemiologic investigation, because of its good discrimination power (Chen et al., 2017; Halpin et al., 2010; Jadhav et al., 2012).

PFGE is an agarose gel electrophoresis technique for separating larger DNA fragments by applying electrical current that periodically changes directions in a gel matrix. The method is based on the use of restriction enzymes that cut the genomic DNA less frequently resulting in 8 to 25 larger fragments ranging between 40 and 600 kb. Genomic DNA is immobilized in agarose plugs/discs for subsequent restriction digestion by specific enzymes, which for *L. monocytogenes* are generally the combination of *Asc*I and *Apa*I. The DNA banding pattern obtained after restriction digestion is then compared for each isolate, to classify them into different pulsotypes (Graves, & Swaminathan, 2001; Halpin et al., 2010; Jadhav et al., 2012). The resulting PFGE profiles can then be analyzed by specialized software in order to accurately compare pulsotypes and the percentage of similarity between strain patterns can be calculated. In this way, recurring strains in the processing environment over time (persistent strains) can be identified. Also, putative routes of transmission and/or sources of contamination may be identified, revealing this method as an important tool for food processing facility safety programs (Stessl et al., 2014; Strydom et al., 2013). The Centre for Disease Control and Prevention (CDC, 2011) of the United States has developed PulseNet (www.cdc.gov/pulsenet/), which is a network of health and food regulatory laboratories that perform standardized PFGE subtyping of foodborne pathogens and this has been used in the event of listeriosis outbreaks (Jadhav et al., 2012). Although PFGE has become a standard subtyping method for detection of listeriosis outbreaks and provides the most sensitive strain discrimination, at the same time it requires skilled labor, specialized equipment, expensive restriction endonucleases, and is laborious and time consuming. It gives results that are difficult to compare between laboratories as they differ with minor changes in the experimental conditions and are highly subjective due to the band marking involved. Thus, inter-laboratory reproducibility analysis has been a problem encountered with PFGE, and thus this typing technique is essentially performed after serotyping (Chen et al., 2017; Jadhav et al., 2012).

1.7. Quantitative Microbial Risk Assessment (QMRA)

Nowadays, modern societies pay great attention to food safety. It is of major importance to identify foods, pathogens, or situations that may lead to foodborne illness (Collado et al., 2011; Lammerding, & Fazil, 2000). During the past several decades, the incidence of foodborne diseases has increased in many parts of the world (CAC/GL, 2007). Recognition of the substantial impact of microbial foodborne disease in terms of human and economic burden to society and industry, combined with an increasing globalization of food markets, has emphasized the need to improve our approaches to manage the food supply safety (CAC/GL, 2007; Lammerding, 1997). Quantitative microbial risk assessment is regarded as a powerful analytical tool to evaluate microbiological risks associated with foodborne microbiological hazards and a possible approach for designing programs to address emerging foodborne disease (Ding et al., 2013; Lammerding, & Paoli, 1997).

According to the *Codex Alimentarius* Commission, which acts internationally to coordinate all food standards to protect consumers' health and ensure fair practices in food trade, risk assessment is included in an overall three-part risk analysis approach (CAC, 2007). Risk analysis is a complex process consisting of three interconnected components: risk assessment (scientific component), risk management (legal component) and risk communication (Collado et al., 2011). The risk assessment component of risk analysis characterizes and estimates the probability of occurrence and severity of known or potential adverse health effects resulting from human exposure to foodborne hazards (Lammerding, 1997). The risk management component is a process of weighing policy alternatives, in consultation with all interested parties, and uses risk assessment's results and other relevant factors to protect consumer's health and to promote fair trade practices, implementing, if required, appropriate control measures (CAC, 2007; Lammerding, A. M., 1997). Risk communication refers to the exchange of information throughout the risk analysis process, considering risk and risk management, among risk assessors, risk managers, consumers, industry, the academic community and other interested parties (CAC, 2007; Lammerding, A. M., 1997).

Risk assessment has a qualitative or quantitative nature depending on data availability (Collado et al., 2011). Qualitative assessment is the most used because of the inexistence or scarcity of data on consumption patterns, dose-response models, initial contamination, and microbial survival after treatment until the time of consumption. In this case, risk is expressed in a categorical/descriptive way: insignificant, low, medium and high risk (Collado et al., 2011; Lammerding, 1997; Membré, 2016). The quantitative microbial risk assessment (QMRA) is more complex and yields a numerical expression of risk. It is based on the availability of specific quantitative data concerning the hazard's prevalence in the studied food product at different steps of the process chain, the necessary dose to produce a host response (dose-response relationship) and the use of mathematical models to characterize that response (Collado et al., 2011; Lammerding, 1997). Ideally, quantitative assessments are desirable (Lammerding, 1997). Ideally, a QMRA should reflect variability of the data (i.e. intrinsic variance which cannot be reduced by increasing sample size, inherent to living or real-world systems) and uncertainty (i.e. variance as a consequence of limited information in the dataset, it might be reduced by increasing sample (Lammerding, & Fazil, 2000; Membré, 2016; Nauta, 2002; Vásquez et al., 2014). Quantitative microbial risk assessment (QMRA) consists of four steps:

Hazard identification, which involves the formulation of the problem, including a systematic identification of the microbial agents or microbial toxins of concern, capable of causing an adverse health effect, and the potential food vehicles involved. Epidemiological and surveillance data, challenge testing, scientific studies of pathogenicity and other pertinent information and expert knowledge are evaluated to ascertain the link between a biological agent in a specific food and consumers' illness (Campagnollo et al., 2018; FAO/WHO, 2008; Lammerding, & Fazil, 2000; Ross, 2014).

Hazard characterization, which is the description and, ideally, the quantification of the effect severity associated with ingestion of the biological agents that may be present in food (Campagnollo et al., 2018; Lammerding, 1997; Ross, 2014). A dose-response assessment should be performed if data are obtainable. Dose-response assessment refers specifically to the determination of the relationship

between the level of the ingested microorganism or the concentration of a microbial toxin (dose) and the probability of infection or probability of illness resulting from ingestion (response) (Lammerding, 1997; Ross, 2014). For microbial agents, elucidation of the nature of the pathogen/host interaction presents several challenges, because of the biological diversity among microorganisms and range of immune status and resistance in human population (Lammerding, & Fazil, 2000; Lammerding, & Paoli, 1997). Quantitative descriptions of dose-response relationships are available for various foodborne pathogens and/or microbial toxins, based on experimental data from human feeding trials, information obtained through animal models and from the analysis of outbreak data collections (Lammerding, 1997; Ross, 2014).

Exposure assessment, which is the evaluation of the probability of consumption of the hazard and the amount likely to be ingested through food at the time of consumption (Campagnollo et al., 2018; Collado et al., 2011; Lammerding, 1997; Ross, 2014). Depending on the scope of the risk assessment, exposure assessment can begin with pathogen prevalence in raw materials ("farm-to-fork" risk assessment), or with the description of the pathogen population at subsequent steps (Lammerding & Paoli, 1997). Exposure assessment takes into account some factors, such as frequency of contamination caused by the pathogen or its level in food during shelf-life, up to the point of consumption. These factors can be influenced by the pathogen's characteristics and its environment, source, frequency, level of contamination, consequences of handling throughout the food chain, consequences of temperature or storage abuse, processing and preservation methods, packaging, distribution, storage and anticipated pre-consumption preparation practices (Collado et al., 2011; Lammerding, 1997). All this results in very dynamic levels of microbial pathogens in foods, and for that, measuring precisely the population of the pathogen present in a food at the time of consumption is very challenging. Therefore, models must be developed to estimate the likely exposure. Predictive modelling techniques describing and quantifying the growth or inactivation of microorganisms are becoming increasingly sophisticated and provide valuable tools for the derivation of probable exposure estimates (Collado et al., 2011; Lammerding, 1997).

Risk characterization, which is essentially a combination of the information gathered in previous steps, to fully explain the nature of the risk in terms of public health, in a defined population, due to a specified hazard in a specified food. It can be helpful in determining the cause of the risk and in providing managers with background information to carry out risk management (Campagnollo et al., 2018; CAC, 2007; Lammerding, & Paoli, 1997; Membré, 2016; Ross, 2014).

The outcome of a quantitative risk assessment is a numerical estimation of risk. For diverse and dynamic biological systems, a probabilistic analytical (stochastic) approach called Monte Carlo simulation can be used to provide frequency distributions of risk, rather than a single value (deterministic approaches), which would have limitations in producing realistic outputs (Lammerding, 1997; Lammerding, & Fazil, 2000).

This approach results in a more realistic risk estimation of the level of human illness by incorporating the inherent variability and uncertainties that exist within the data and the models used to describe the risk situation (Lammerding, 1997). In addition, Monte Carlo simulation also allows the possibility of

carrying out a sensitivity analysis of all parameters involved in the model, providing a valuable means of identifying those factors, between the industry/production and the consumer, that most significantly influence the risk outcome (Collado et al., 2011; Lammerding, 1997). Manipulation of the model by changing the inputs, or the parameters used to describe an input, can be readily performed to simulate the effect of that alteration on the risk outcome. This provides a means of evaluating the effectiveness of risk mitigation options before actual physical implementation (Lammerding, 1997).

1.7.1. Predictive microbiology in exposure assessment

Predictive microbiology is a discipline that combines elements of microbiology, mathematics and statistics to establish models that describe and predict the behavior of microorganisms under specific experimental conditions (Collado et al., 2011). The fundamental principle underlying the concept is the idea that organisms have reproducible behavior and can be described as a function of different variables through a model. Predictive microbiology is essential to perform a quantitative exposure assessment because through it and using a simulation procedure, changes in pathogen levels between various stages in the farm-to-fork continuum, and point of contamination can be estimated providing an assessment of exposure to a particular pathogen, in a particular food product (Collado et al., 2011; Ross, 2014). Knowledge of food-processing operations, food physicochemical properties, time and environmental conditions (particularly temperature) experienced by the food from harvest and during processing are required to use predictive microbiology for estimating hazard levels at the time of consumption (Ross, 2014).

Traditionally, predictive microbiology is based on a two-step modelling approach, including primary and secondary models. The primary models aim at accurately describing bacterial growth kinetics (initial concentration, lag-time, growth rate, maximum population density) with as few parameters as possible, whereas the secondary growth models describe the effect of environmental factors on the bacterial growth kinetics from the primary models (Ross, & McMeekin, 2003).

2. Materials and Methods

2.1. Selection of bacterial strain

To account for variation in growth and survival among strains of *Listeria monocytogenes*, a mixture of three reference strains was used: *L. monocytogenes* CECT 4031, belonging to serogroup IIa, *L. monocytogenes* CECT 935, belonging to serogroup IVb and *L. monocytogenes* CECT 937, belonging to serogroup IIb. Additionally, these strains are representative of the three *L. monocytogenes* serogroups more frequently related to human disease, accounting for more than 95% of listeriosis cases.

2.2. Bacterial strain revival

The strains used in this study were stored at -80°C in a HERAFreeze BASIC cryogenic chamber (Thermo Fisher Scientific, Waltham, United States of America (USA)) in preservation cryotubes containing Brain Heart Infusion (BHI) broth (Scharlab, S.L., Barcelona, Spain) supplemented with 15% glycerol (Merck KGaA, Darmstadt, Germany). All procedures described subsequently were performed in Bio II Advance laminar flow chamber (Telstar Life Science solutions, Terrassa, Spain). For strains' revival, stock cultures were thawed at room temperature and 100 µl of inoculum was transferred into 5 ml of BHI broth. After 24 hours (h) of incubation at 37°C, a loop (10 µl) of inoculum was streaked onto BHI agar (Scharlab, S.L.) and incubated at 37°C for 24 h.

2.3. Isothermal growth in BHI broth

After strains' revival, an isolated *L. monocytogenes* CECT 4031 colony was collected from BHI agar plates and inoculated in 5 ml of BHI broth, and incubated for 26 h at 37°C. Also, in order to induce strains' cold-adaptation, this procedure was repeated using 5 ml of BHI broth prechilled at 12°C that was further incubated for 12 days at 12°C. Optical density (OD) of the bacterial suspensions was measured on a spectrophotometer (Ultrospec 2000 Pharmacia Biotech, Sweden) using a wavelength of 600 nanometers (nm) (OD_{600nm}). Viable cell count (VCC) by incorporation on BHI agar was also performed. The considered sampling time points are shown in **Table 3**. Three independent growth experiments were performed for both temperatures (37°C and 12°C). VCC and OD_{600nm} results were analyzed using simple linear regression of Microsoft Office 2016 *software* (Microsoft Corporation, Redmond, USA).

Table 3. Sampling time points used to assess *L. monocytogenes* CECT 4031 growth in BHI broth at 37ºC and 12ºC.

Sampling time								
Incubation at 37ºC				Incubation at 12ºC				
2 h	10 h	18 h	26 h	4 h	20 h	96 h	192 h	288 h
4 h	12 h	20 h		8 h	24 h	120 h	216 h	
6 h	14 h	22 h		12 h	48 h	144 h	244 h	
8 h	16 h	24 h		16 h	72 h	168 h	264 h	

2.3.1. Curve fitting

Growth curves were fitted to Baranyi and Roberts primary predictive model (Baranyi & Roberts, 1994) (**Equations 1-3**), using DMFit *online* (Quadram Institute, Norwich, United Kingdom), to estimate maximum specific growth rate (μ_{max}), lag time (λ), and the goodness of fit data: R-squared (R^2) and standard error of fit (SE). A fitting method for repeated measures was applied considering the different replicates analyzed in each time point.

$$\mathrm{N(t)} = N_0 + \mu_{max} A(t) - \ln\left[1 + \frac{e^{\mu_{max} A(t)} - 1}{e^{(N_{max} - N_0)}}\right] \quad \textbf{(1)}$$

where

$$A(t) = t + \frac{1}{\mu_{max}} ln\left(\frac{e^{(-\mu_{max} t)} + q_0}{1 + q_0}\right) \quad \textbf{(2)}$$

$$\lambda = \frac{ln\left(1 + \frac{1}{q_0}\right)}{\mu_{max}} \quad \textbf{(3)}$$

Where: N(t) = log of cell concentration (cfu/ml(g)) at time t (h); N_0 = log of initial cell concentration (cfu/ml(g)); μ_{max} = maximum specific growth rate (log cfu/ml(g)/h); N_{max} = log of maximum cell concentration; q_0 = parameter expressing the physiological state of cells when t = t_0; λ = lag time (h). In this work μ_{max} is based on the inflection of the slope of the growth curve in the exponential phase (Baranyi et al., 1993).

The resulting growth curves and parameters were compared to predicted values generated by ComBase Predictor Growth Model (ComBase, Hobart, Australia). ComBase model was run with the following selected parameters: pH = 7.4, a_w = 0.997, temperature = 12ºC/37ºC and initial level = 4.5 log cfu/ml. The pH and a_w values were chosen based on BHI broth data.

2.4. Preparation of *L. monocytogenes* inoculum

In order to determine the inoculum density to be inoculated into the salads using OD_{600nm} measurements, a calibration equation for each strain in study was obtained using three independent calibration curves, in which viable cell counts were plotted against OD_{600nm} data. For this purpose, several dilutions were

made in Tryptic soy broth (TS) (Scharlab, S.L.), and its optical density were measured (dilutions 10^0 to 10^{-3}). At the same time, 10^{-4} to 10^{-9} dilutions were pour plated on BHI agar and incubated at 37°C for 24 h. Colonies were enumerated, and the necessary calculations were performed to express results in colony-forming units per milliliter (cfu/ml).

To recreate an adaptation to the refrigerated food producing-environment, the three selected *L. monocytogenes* strains were incubated separately in BHI for 4 days at 12ºC, to obtain cells in the late exponential growth phase. Cells were then centrifuged in a Centrifuge 5415 R (Eppendorf AG, Hamburg, Germany) at 6000 rpm for 10 min at 4ºC. The harvested cells were resuspended in Buffered Peptone Water (BPW) (Scharlab, S.L.) and centrifuged under the same conditions. The harvested cells of each one of the washed cultures were finally resuspended in 10 ml of BPW, and each of the *L. monocytogenes* strain' suspension was mixed together and diluted, to obtain a suspension containing approximately 10^4 cfu/ml. The inoculum density was confirmed by surface plating onto BHI.

2.5. Chicken salad production process and sample collection

In this study, a chicken salad was used. This salad was produced in a ready-to-eat food-producing industry located in an industrial park of Lisbon's metropolitan region. This industry, officially approved for food production activities, is classified as a medium-sized company, as categorized by the European Commission Recommendation 2003/361/EC. It has an implemented food safety management system based on Hazard Analysis and Critical Control Points method (HACCP) (Regulation No. 852/2004). Chicken salad's technical specification is shown in **Annex I**. This salad is prepared manually in a production line located in a temperature-controlled room (10-12°C). Ingredients are placed in a polyester salad bowl (PET) which is immediately sealed. After production, batches are stored at 5°C and have an attributed 6 days shelf-life. In this study, samples were collected randomly, from February 14 to May 25, 2018 (**Annex II**), from nine different batches produced in different weeks and transported in less than 2 hours to the laboratory in an isothermal box.

2.6. Inoculation and storage of ready-to-eat chicken salad

For the inoculation of ready-to-eat chicken salads, random samples from each batch were chosen, and the protocol of Lokerse et al. (2016) was followed, with some adaptations. For each 100 grams of food, 1 ml of the suspension with 4 log cfu/ml of *L. monocytogenes* was inoculated and uniformly distributed with a pipette throughout the salad in the original package. The protocol of EURL Lm, (2014) recommends an inoculum level of approximately 100 cfu/g. This inoculum level is commonly used in challenge testing because this enables to reduce the effect of measurement uncertainty.

To determine specific physicochemical characteristics, as well as the concentration of commonly assessed ready-to-eat food hygiene indicators, two different blank samples were also prepared: i) blank samples inoculated with BPW, in substitution of the *L. monocytogenes* inoculum, and in the same volume as the inoculum (BS-BPW) to test its role in the salads' microbiota, and ii) blank samples to

which no PBW was added (BS), in order to detect any original contamination of the examined salads. Samples were incubated at 4ºC, 12ºC and 16ºC for 192 hours (8 days).

2.7. Food sampling

L. monocytogenes inoculated test units were analyzed at 0 h, 48 h, 96 h, 144 h and 192 h (corresponding to day 0, 2, 4, 6 and 8) and blank test units (BS and BS-BPW) were analyzed at 0 h, 96 h and 192 h. Three independent replicates (different batches) of the challenge test study were performed for each temperature. **Table 4** resumes the challenge testing experimental determinations, including tested samples and sampling time points.

Table 4. Challenge testing determinations, tested samples and sampling time points.

Determinations (Method)	Tested samples	Sampling time points (hours)				
		0	48	96	144	192
Enumeration of *L. monocytogenes* (ISO 11290-2:2017)	IS	X	X	X	X	X
	BS	X		X		X
Detection of *L. monocytogenes* (ISO 11290-1:2017)	IS	X	X	X	X	X
	BS	X		X		X
Measurement of physicochemical characteristics: pH (NP-3441 (1990)) and a_w (ISO 21807:2004)	BS-BPW BS	X		X		X
Enumeration of total aerobic microorganisms at 30ºC (ISO 4833-1:2013) and Enterobacteriaceae (ISO 21528-2:2017)	BS-BPW BS	X		X		X

IS – *L. monocytogenes* inoculated samples; BS-BPW - BPW inoculated samples; BS - uninoculated blank samples.

2.8. Physicochemical analyses

2.8.1. Potential of hydrogen (pH) determination

For pH determination, blank samples (BS-BPW and BS) were used. Three independent measurements were performed for each homogenized sample in each sampling time point. The evaluation was done according to NP-3441 (1990), using a HI 99163 potentiometer (Hanna Instruments, Rhode Island, USA).

2.8.2. Water activity (a_w) determination

For a_w determination EN ISO 21807:2004 standard was used using a HygroLab C (ROTRONIC Instruments, West Sussex, United Kingdom) water activity meter with AW-40 probe, maintained at 25°C ± 2°C. For this purpose, blank sample salads (BS-BPW and BS) were used, and three independent measurements were performed for each homogenized sample in each sampling time point.

2.9. Microbiological analyses

2.9.1. Food sample preparation

Food samples for microbiological analyses were prepared according to ISO 6887-2:2003. Briefly, 25 g of each sample was aseptically removed from different areas of the salad and homogenized with 225 ml of sterile BPW in 500 ml stomacher bags (Normax, Marinha Grande, Portugal) for 60 seconds in a Stomacher Lab-Blender 400 (Seward Laboratory, Worthing, United Kingdom). From this homogenate, an aliquot of 1 ml was transferred to a tube containing 9 ml of BPW and ten-fold dilution series were prepared.

2.9.2. Enumeration of total aerobic microorganisms at 30ºC

For the enumeration of total aerobic microorganisms at 30ºC (TAM30) the pour plate method technique was used, and 1 ml from suitable dilutions were transferred aseptically into sterile petri dishes. The total number of aerobic mesophilic microorganisms were determined on Tryptone Glucose Agar (TGA agar), (Scharlab, S.L.) and incubated at 30ºC for 48/72h, according to ISO 4833-1:2013. In the enumeration process, all colonies were considered, independently of their morphology. Results were presented as log cfu/g.

2.9.3. Enumeration of Enterobacteriaceae

According to ISO 21528-2:2017 for Enterobacteriaceae enumeration, the pour plate method technique was used. One milliliter of the suitable sample dilutions was cultured on Violet Red Bile Dextrose Agar (VRBD) (Scharlab, S.L.). After homogenization, plates were incubated for 24/48h at 37ºC. The enumeration of characteristic colonies of Enterobacteriaceae was performed according to ISO 21528-2:2004. Results were presented as log cfu/g.

2.9.4. Enumeration of *L. monocytogenes*

Enumeration of *L. monocytogenes* was performed according to ISO 11290-2:2017. For this, from the ten-fold dilution series previously prepared, 0.2 ml was taken from the suitable dilutions and spread plated onto Agar *Listeria* Ottaviani & Agosti (ALOA) medium (BioMérieux, Marcy l'Etoile, France) with a disposable L-shaped cell spreader (Normax), and incubated at 37°C for 24 h. Characteristic bluish-green colonies surrounded by a smooth, round and opaque precipitation zone halo were enumerated, and the necessary calculations were preformed to express results in cfu/g of food.

2.9.5. Detection of *L. monocytogenes*

Detection of *L. monocytogenes* was performed according to ISO 11290-1:2017. Briefly, for the detection of *L. monocytogenes*, 25 g of each sample was aseptically collected from different areas of the salad and homogenized with 225 ml of Fraser I broth (Scharlab, S.L.) in 500 ml stomacher bags (Normax) for 60 seconds in a Stomacher Lab-Blender 400 (Seward Laboratory). This homogenate was incubated at

30°C for 24 h. An aliquot of 1 ml was transferred to tubes containing 10 ml Fraser II broth (Fraser I supplemented with Fraser selective supplement (Scharlab, S.L.) and incubated at 37°C for 24 h. The suspension was streaked onto ALOA plates (BioMérieux) and incubated at 37°C for 24 h. Presumptive *L. monocytogenes* colonies exhibiting a bluish-green coloration surrounded by an opaque, round and smooth halo were considered.

Throughout the RTE chicken salads' challenge test, some BS revealed the presence of characteristic *L. monocytogenes* colonies on ALOA media. Confirmation of *L. monocytogenes* presumptive colonies was done by PCR. The recovered isolates were stored at -80ºC until use.

2.10. *L. monocytogenes* DNA extraction

The isolates recovered from blank samples were revived (section **2.2**), and grown on BHI at 37°C for 16-18 hours, after which 200 µl of cell suspension was taken into a nuclease-free 1.5 ml microcentrifuge tube for DNA isolation, using a High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Briefly, these 200 µl were centrifuged at 3000 × g for 5 min to pellet bacteria. The pellet was resuspended in 200 µl of phosphate-buffered saline (PBS) and 5 µl of lysozyme (10 mg/ml in 10 mM Tris-HCl, pH 8.0) were added, incubating the mixture at 37ºC for 15 min. Afterwards, 200 µl of binding buffer and 100 µl of isopropanol were added and mixed, followed by centrifugation (5 min at 13000 x g) and then pipeted into the upper reservoir of a combined high filter tube collection assembly, and centrifuged at 8000 x g for 1 min. The DNA was retained in the filter tube containing glass fiber and the flow-through was discarded. The filter tube was assembled with a new collection tube and 500 µl of inhibitor removal buffer was added, followed by a centrifugation at 8000 x g for 1 min. The DNA was retained in the filter and the flow-through was discarded. The filter tube was twice washed with 500 µl of the washing buffer and centrifuged at 8000 x g for 1 min to eliminate it. Finally, the filter tube was inserted in a clean, sterile 1.5 ml microcentrifuge tube and 200 µl of pre-warmed (70^0C) elution buffer was added, and centrifuged at 8000 x g for 1 min. All DNA samples were stored at 4ºC. Quantification of extracted DNA was performed spectrophotometrically in a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Fisher Scientific).

2.11. *L. monocytogenes* confirmation and serogrouping

The protocol proposed by Kérouanton et al. (2010) was followed for the multiplex PCR assay, in which *Listeria* genus recognition was ensured by detection of the prs gene, and the prfA gene was targeted to confirm *L. monocytogenes* species-specific recognition. A first PCR assay was conducted in a Thermocycler Doppio 2x48 well block (VWR Int., Radnor, USA). The amplification mix of 25 µl, with 1.5 µl DNA, consisted of 1U of Taq DNA polymerase (NZYTech, Lisboa, Portugal), 1x reaction buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each of the following primer sets: LMO0737; LMO1118; ORF2110; ORF2819 and 0.2 µM of primer sets PRS and LIP (**Table 5**). The cycling program consisted of an initial denaturation for 3 min at 94°C, followed by 35 cycles of denaturation at 94°C (40 s), annealing at 53°C (45 s), extension at 72°C (1 min 15 s) and a final extension at 72°C (7 min).

Table 5. PCR primers used to serotype *L. monocytogenes* strains.

Gene	Primer set	Forward primer sequence	Reverse primer sequence	Amplicon length (bp)
prs	PRS	GCTGAAGAGATTGCGAAAGAAG	CAAAGAAACCTTGGATTTGCGG	370
prfA	LIP	GATACAGAAACATCGGTTGGC	GTGTAATCTTGATGCCATCAGG	274
lmo0737	LMO0737	AGGGCTTCAAGGACTTACCC	ACGATTTCTGCTTGCCATTC	691
lmo1118	LMO1118	AGGGGTCTTAAATCCTGGA	CGGCTTGTTCGGCATACTTA	906
orf2819	ORF2819	AGCAAAATGCCAAAACTCGT	CATCACTAAAGCCTCCCATTG	471
orf2110	ORF2110	AGTGGACAATTGATTGGTGAA	CATCCATCCCTTACTTTGGAC	597
flaA	FLaA	TTACTAGATCAAACTGCTCC	AAGAAAAGCCCCTCGTCC	538

A second PCR assay was performed to detect the presence of the *fla*A gene encoding *L. monocytogenes* flagellar protein, allowing for discrimination of atypical strains belonging to serogroup IIa and IIc. For that, an amplification mix of 25 µl with 0.8 µl DNA containing 1U Taq DNA polymerase (NZYTech), 1x reaction buffer, 4 mM $MgCl_2$, 0.2 mM dNTPs, 0.8 µM *fla*A-F and *fla*A-R primers and 0.02 µM *prs*-1 and *prs*-2 primers. The cycling program consisted of an initial denaturation for 3 min at 94ºC, followed by 40 cycles of denaturation at 94ºC (30 s), annealing at 61ºC (40 s), extension at 72ºC (1 min), and a final extension at 72ºC (7 min). In the reaction, positive controls (CECT 4031 for IIa serogroup, CECT 937 for IIb serogroup, CECT 911 for serogroup IIc, CECT 934 for serogroup IVa, and CECT 935 for serogroup IVb), blank reaction control (water), and negative control (*Escherichia coli* DSMZ 682) were included.

2.11.1. Agarose gel electrophoresis

Amplified PCR fragments were separated using electrophoresis with a 1.5% agarose gel (NZYTech) in 1x Tris borate EDTA (TBE) (Roche Diagnostics), with a 100 bp molecular weight marker (NZYTech) using GelRed (Biotium Inc., Hayward, USA) and visualized under UV light in a ChemiDoc™ XRS+ (Bio-Rad Laboratories, Hercules, USA).

2.12. Pulsed-field Gel Electrophoresis (PFGE) typing

PFGE of the isolates positively confirmed to be *Listeria monocytogenes* (n=10) and the three reference strains (*L. monocytogenes* CECT 4031 (Lm 4031), CECT 935 (Lm 935) and CECT 937 (Lm 937)) was performed according to the Centers for Disease Control and Prevention PulseNet standardized procedure for *L. monocytogenes* typing (Graves, & Swaminathan, 2001).

2.12.1. Disc preparation

Bacterial strains were grown on BHI agar at 37ºC for 16-18 h and then suspended in 4 ml of TE 1X (pH 8). Bacterial suspension's concentration was adjusted to an optical density of 0.5 – 0.7 at a wavelength of 610_{nm} (Ultrospec 2000 Pharmacia Biotech). 400 µl of the cell suspension were then placed in 2 ml *eppendorf* tubes and 20 µl of 20 mg/ml lysozyme in TE 1X was added. The tubes were placed in a 56°C Digital Heatblock Dry Bath for 15/20 min (VWR Int). After that, 20 µl of Proteinase K (20 mg/ml) was added to each tube and mixed gently with a pipet tip. To incorporate the agarose, each cell suspension was gently mixed with 400 µl of molten 1.5% SeaKem Gold agarose (Lonza, Walkersville, USA) and 10% sodium dodecyl sulfate (Roche Diagnostics) solution that had been equilibrated for 15 min in a 60ºC water bath. This mixture was then filled into sterile disposable syringes (KRUUSE, Langeskov, Denmark). The syringes were placed in a straight tray at 4ºC for at least 15 min to allow bacterial genomic DNA in 1.5% agarose to solidify. Once solidified, the tip of the syringe was cut, and with a microscope slide, approximately equal size discs were sliced and placed in falcon tubes (Normax) containing 5 ml cell lysis buffer (50 mM Tris:50 mM EDTA, pH 8 + 1% Sarcosyl) and 25 µl of Proteinase K (20 mg/ml), following an incubation of 2 h in a 54º/55ºC water bath with 100 rpm shaking on a 222DS Benchtop Shaking Incubator (Labnet. Int., Edison, USA). After proteolysis, the lysis buffer solution was removed, and the discs were washed twice with 10 ml of preheated 55ºC sterile distilled water for 10 min each followed by five washes with 10 ml of preheated at 55ºC TE 1X buffer for 10/15 min each in the orbital water bath shaker at 50ºC at 100 rpm. After the final TE wash, the discs were stored in 10 ml TE at 4ºC until ready for restriction.

2.12.2. Restriction Digestion of DNA in agarose discs

The isolates were separately digested with the restriction enzymes *Asc*I (NZYTech) and *Apa*I (NZYTech). The discs were initially incubated in the restriction buffer for 10 min in a 37ºC and 25ºC water bath respectively for *Asc*I and *Apa*I. Then the preincubation buffer was removed and restriction was done at an enzyme concentration of 10 U/µl of *Asc*I for 2 h at 37ºC, and of 50 U/µl of *Apa*I for 2 h at 25ºC.

2.12.3. Electrophoretic Conditions

Once the restriction was completed, electrophoresis of the resulting DNA fragments was performed in 1% SeaKem Gold Agarose (Lonza) gels in 0.5x TBE (NZYTech), with lambda PFG ladder standard (New England Biolabs, Ipswich, USA). The gel was run in a BioRad CHEF DR-III electrophoresis apparatus (Bio-Rad Laboratories) at 14ºC, with 6 V/cm, initial pulsed time of 4 s and final pulsed time of 40 s, included angle of 120º over 19 h.

2.12.4. Gel Staining and Documentation

Gels were stained in a 10 mg/ml ethidium bromide (Merck KGaA) solution for 30 min and destained one to two times with deionized sterile water for 20-30 min, after which it was visualized in a ChemiDoc™ XRS+ (Bio-Rad Laboratories).

2.13. Real Time Quantitative PCR

2.13.1. PMA treatment for q-PCR

For propidium monoazide (PMA) (Biotium Inc.) treatment, samples were treated as described previously with slight modifications (Zhang et al., 2014). Briefly, 500 µl aliquots of salad extracts with BPW were transferred to 2 ml *eppendorfs*. The appropriate volume of PMAxx™ (20 mM stock in H_2O) was added for a final concentration of 80 µM. PMA treated salad extracts were incubated for 5 min in darkness, at room temperature, and shaken with an Orbit™ P4 Digital Shaker (Labnet Int.) at 40 rpm, to promote selective penetration of PMA into dead cells. Then, the tubes were laid on ice, and using an Orbit™ P4 Digital Shaker with brief shaking were exposed to a 1000-W halogen light source (OSRAM Licht AG, Munich, Germany) for 15 min to cause the cross-linking of PMA with DNA and the conversion of unintercalated PMA to hydroxylamino propidium (Josefsen et al., 2010). The distance between microtubes and light source was 40 cm to avoid excessive heating. After the photo-induced cross-linking, bacteria were harvested by centrifugation at 5000 rpm for 10 min.

2.13.2. DNA extraction for RT-qPCR

DNA extraction was performed according to the guanidine thiocyanate method described from Pitcher et al., (1989), with modifications. Briefly, after the PMA treatment, the pellets obtained were resuspended in Tris-EDTA (TE) 1X in 2 mL *eppendorfs,* homogenized with a vortex (Vortex 3, IKA®-Werke GmbH & Co. KG, Staufen, Germany) and centrifuged at 8000 rpm for 10 min, at 4ºC. Samples were pre-treated with lysozyme (10 mg/µl lysozyme in TE buffer) and incubated for 2 hours in a 37ºC water bath. The lysates were mixed with 250 µl of GES reagent (5 M guanidium thiocyanate, 100 mM Ethylenediaminetetraacetic acid (EDTA), 0.5% sarkosyl (Merck KGaA) - 4µl of the internal extraction control DNA was also added in this step (PrimerDesign ™, Ltd, United Kingdom) - and cooled on ice for 10 min. Then, 125 µl of cold 10 M ammonium acetate (Merck KGaA), were added and the samples were held on ice for 10 min. After that, an extraction was performed with 500 µl chloroform-isoamyl alcohol (24:1). The tubes were centrifuged at 13200 rpm for 10 min at 4°C, and the DNA of the subsequent aqueous phase was precipitated with cold isopropanol. After 10 min centrifugation at 13200 rpm, the DNA pellet was washed with 70% ethanol and dried at room temperature overnight. DNA was resuspended in 150 µl TE 1X buffer. Quantification of extracted DNA was performed spectrophotometrically in a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Fisher Scientific). The DNA was stored at 4ºC until use.

2.13.3. RT-qPCR assay

Listeria monocytogenes was quantified using the commercial Genesig real-time RT-PCR (PrimerDesign™, Ltd, United Kingdom), using primers that amplify the invasion-associated protein p60 (iap) gene. Each PCR reaction incorporated 5 µl of template DNAs, ten microliters of PrecisionPLUS 2X qPCR MasterMix (PrimerDesign™, Ltd), 1 µl of *Listeria monocytogenes*-specific primer/probe mix (detected through the FAM channel), 1 µl of internal extraction control primer/probe mix (detected through the VIC channel) and 3 µl of nuclease-free water. Genomic DNA from *L. monocytogenes* served as a positive control for the reaction and a negative control, where template was substituted with nuclease-free PCR grade water, was included in each run. The Applied Biosystem StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, USA) was used for *Listeria monocytogenes* detection and quantification according to the following cycles: first, enzyme activation at 95ºC for 2 min; second, 50 cycles of 95ºC for 10 s (denaturation) and 60ºC for 60 s (data collection). The Ct values obtained by RT- qPCR were quantified by using a relative standard curve generated from positive control DNA at known concentrations.

According to the manufacturer's instructions, the *L. monocytogenes* primers have been designed for the specific and exclusive in vitro quantification of all *L. monocytogenes* and do not detect other *Listeria* species. The primers and probe sequences in this kit have 100% homology with a broad range of clinically relevant reference sequences based on a comprehensive bioinformatics analysis. Under optimal PCR conditions Genesig *L. monocytogenes* detection kits have very high priming efficiencies of >95% and can detect less than 100 copies of target template.

2.14. Modelling growth parameters of *L. monocytogenes* in ready-to-eat chicken salads

2.14.1. Primary model

Growth curves for each temperature were built separately by fitting data to the primary predictive model described by Baranyi and Roberts (Baranyi & Roberts, 1994) (**Equation 1-3**), using Baranyi's DMFit version 3.5 Excel® add-in (www.ifr.ac.uk/safety/DMfit) (Quadram Institute). The predictive primary model was used in order to calculate the growth kinetic parameters of *L. monocytogenes* in the salads. The following parameters were obtained: i) maximum specific growth rate (μ_{max}), ii) lag time (λ), iii) initial cell count (C_0) and iv) maximum population density (N_{max}).

2.14.2. Secondary model for maximum specific growth rate

The predictive secondary model was built using the square-root model described by Ratkowsky et al. (1982) (**Equation 4**) to describe μ_{max} as a function of storage temperature (T).

$$\sqrt{\mu_{max}} = b(T - T_{min}) \quad \textbf{(4)}$$

Where: μ_{max} = maximum specific growth rate (log cfu/g/h); b = regression parameter determined during the modelling process; T = storage temperature (°C); and T_{min} = determined minimum temperature for the growth of microorganisms (°C).

2.15. Statistical analyses

Correlation between methods (OD measurements and VCC) in isothermal growth in BHI broth was statistically analyzed, using Pearson correlation, with GraphPad Prism 5 (GraphPad Software, La Jolla, USA).

All microbiological and physicochemical data were assessed in a database created in GraphPad Prism 5 (GraphPad Software), using a descriptive statistical analysis with average and standard deviation calculation for the three replicates of the assays, corresponding to three batches (replicates) for the three different temperatures. For comparison of blank samples with and without BPW, a t-test for paired samples was performed, for comparisons between results obtained at the three different temperatures, one-way analysis of variance (ANOVA) was used, followed by Tukey's multiple comparison test, and p values of 0.05 or less were considered statistically significant.

For comparison of the two quantification methods (VCC and RT-qPCR), a t-test for paired samples was performed, for each temperature (4°C, 12°C and 16°C), and type of sample (IS, BS and BS-BPW). Comparisons were performed using GraphPad Prism 5 (GraphPad Software).

Dendrogram was constructed based on PFGE patterns of the selected strains using BioNumerics software package version 6.10 (Applied Maths, Sint-Martens-Latem, Belgium). *L. monocytogenes* PFGE patterns were analyzed to determine strain relatedness with an optimization setting and a band-position tolerance of 1.5% for *Asc*I and *Apa*I restriction. Cluster analysis was performed using the unweighted pair group method with arithmetic averages (UPGMA) and band-based Dice correlation coefficient.

The estimated μ_{max} values of *L. monocytogenes* in RTE chicken salads at each isothermal storage condition (4°C, 12°C and 16°C) were compared and checked for significant statistical differences (p-value ≤ 0.05), employing one-factor ANOVA, followed by Tukey's multiple comparison test. P values of 0.05 or less were considered statistically significant. Statistical analyses were carried out in GraphPad Prism 5 (GraphPad Software). Measures of coefficient of determination (R^2) were used to evaluate the performance of the models built in this study. The R^2 was considered as an overall measure of the prediction calculated by the developed model.

2.16. Quantitative microbial risk assessment

2.16.1. Description of the risk assessment model

In this study, a Monte Carlo quantitative microbiological risk assessment (QMRA) of *L. monocytogenes* in RTE chicken salads produced in a Lisbon's RTE food industry was developed. The model estimates the number of listeriosis cases linked to the consumption of RTE chicken salads potentially contaminated with *L. monocytogenes*, taking into account primary data obtained in the exposure assessment step, including: occurrence and levels of *L. monocytogenes* at the point of sale, growth of *L. monocytogenes* from the point of sale to consumption, time and temperature fluctuations (consumer storage), serving size and consumption frequency of salads. The model describes human exposure as a distribution of ingested *L. monocytogenes,* focused on the home storage phase, considering the consumption by different populations with specific susceptibility to the pathogen. The model was built using R programming language (Version 3.5.1, R Development Core Team, 2018). **Figure 5** shows the general scheme of the QMRA model, which was broken down into 3 main modules, described below:

Exposure assessment: **(a)** Prevalence and initial contamination (retail storage): At this initial stage if the product is contaminated, it will have a certain level of pathogen at that point in time. **(b)** Consumer storage: The pathogen could increase during storage depending on the consumer storage conditions, potentially leading to an increase in the final level of *L. monocytogenes* in the product. **(c)** Consumer consumption: the consumers will invariably eat different quantities (population variability) of the product, resulting in varying degrees of exposure.

Dose response: The dose response relates the amount consumed to a clinical outcome, in this study a listeriosis illness (for different risk groups within the population) and is used to translate exposure into a log probability of illness.

Risk characterization: This stage combines the first two steps to characterize the risk of illness for the given simulated exposure level. The model simulated the annual risk of illness for a high risk and low risk population. Each module was modelled with each proceeding module acting as an input into the next. The model provides a baseline description of listeriosis threat by consuming RTE chicken salads contaminated with *L. monocytogenes*.

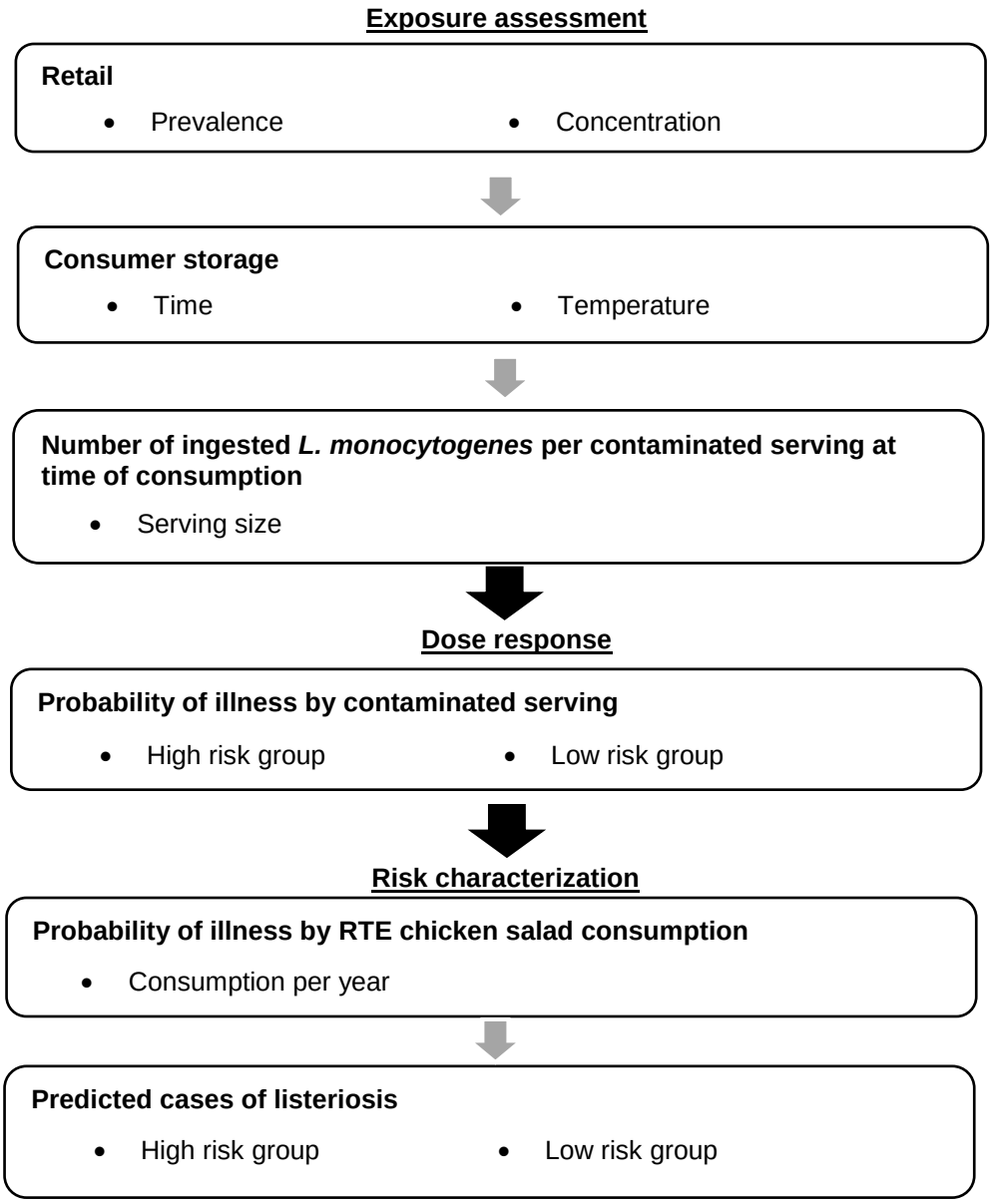


Figure 5. Schematic representation of the three modules used in the development of the baseline model *of L. monocytogenes* in RTE chicken salads.

2.16.2. Exposure assessment

2.16.2.1. Prevalence and initial concentration

The prevalence of *L. monocytogenes* in RTE chicken salads was estimated from the number of positive contaminated uninoculated salads that were found throughout the challenge test (6 out of 27 salads: 22.2%). The prevalence was described by a Beta distribution, assuming α equal to (6 + 1) and β equal to (27 - 6 + 1). As *L. monocytogenes* enumeration of the positive samples was not possible, due to limitations of the method, and also as no information about the level of *L. monocytogenes* in RTE salads was available in Portugal, it was assumed to be equal to the concentration found by Gombas et al. (2003) for bagged pre-cut-leafy salads. And as Carrasco et al. (2010), these concentration values of *L. monocytogenes* were described by the empirical cumulative distribution function.

2.16.2.2. Consumer storage

Temperature during storage in home refrigerators was modelled using a Pert distribution with minimum, most likely and maximum values of 3.2ºC, 6.4ºC and 11ºC, respectively. The data for consumer refrigeration was obtained through the use of calibrated EL-USB-2 data-loggers (Lascar Electronics, Whiteparish, United Kingdom) which monitored the temperature of some households' refrigerators in the region of Lisbon, throughout the year (**Annex II**).

Storage time was modelled by assuming that consumer behavior on storage of foods in the home refrigerator is influenced by shelf-life shown in the label at the moment of purchase. So, a uniform distribution with 0 h as minimum and 192 h (2 days added to the shelf life) as maximum values was used to model storage time at home. The logarithmic growth and level after home storage were calculated by the relationship between growth rate and temperature represented by the square-root model proposed by Ratkowsky et al. (1982), described in section **2.14.2** and from Baranyi & Roberts (1994) adapted model with no lag.

2.16.2.3. Consumption data

Accurate data about individual consumption patterns (typical serving size) of RTE chicken salads by Portugal population were not available. So, it was assumed, since the salads' quantity was of 165 g, 82.5 g (half a salad), 165 g (whole salad) and 247.5 g (a salad and a half), as minimum, most likely and maximum serving sizes, respectively.

2.16.3. Hazard characterization: Dose response model

An exponential dose-response model was applied to each scenario (**Equation 5**):

$$P_{ill}(D;r) = 1 - e^{(-r \times D)} \quad \textbf{(5)}$$

where D represents the ingested dose and r is the dose response parameter, representing the probability of illness. Two different r values were considered: $r = 2.37 \times 10^{-14}$ for the healthy population and $r = 1.06 \times 10^{-12}$ for the susceptible population as suggested by FAO-WHO (2004). The present QMRA was based on the Portuguese population. A distinction between low-risk and high-risk populations was made with the aim of a more accurate assessment of the risk. For this purpose, the fractions reported by FAO/WHO (2004) of the total population corresponding to high-risk individuals (including adults over 65-yold, pregnant women, and individuals with an impaired immune systems and certain medical conditions, such as cancer, HIV, and recent organ transplantation), were applied to the Portuguese population, assuming also that children under 5 years-old, and adults over 69 years-old do not consume RTE salads, the high-risk population size submitted to analysis was 921870 (approximately 9% of the population (**Table 6**). Healthy population was considered the difference between the total population reported in 2018 in Portugal and the sum of susceptible groups.

Table 6. Classification of registered population in Portugal (2018) according to established listeriosis susceptibility groups and available data. *Data obtained from Instituto Nacional de Estatística (INE).*

Group of population	No. inhabitants	(%)
Aged population[a]	619886	6.02
Pregnant women	85289	1.92
Immunocompromised[b]	216695	2.11
Total population at risk	921870	8.96

[a] Aged population considered between 65-69 years old.

[b] Immunocompromised group included only cancer, HIV and transplanted patients (Non-available data for other groups).

2.16.4. Risk characterization

All the information gathered in previous steps was integrated to provide a description of the risk of listeriosis by the ingestion of RTE chicken salad contaminated with *Listeria monocytogenes.* To obtain the expected number of cases, the mean P_{ill} of 100.000 model iterations was multiplied with the number of portions (N_p) produced by the food industry every year (344402) and with the prevalence of contaminated samples. Three possible scenarios were studied regarding the number of listeriosis cases associated with the consumption of RTE chicken salads: one for low-risk population, other for high risk population, and finally a more realistic combination of both.

Overview of the model with its inputs, is shown in **Table 7**.

2.16.5. Sensitivity analysis

Sensitivity analysis is a tool that allows determining the effects that inputs have on model outputs. A sensitivity analysis was performed for the risk of listeriosis per dose. The inputs selected for the analysis were: initial concentration of the pathogen, portion size, storage temperature and time. The sensitivity analysis method was implemented in R software version 3.5.1 (R Development Core Team, 2018), package: “sensitivity” (Saltelli, 2002).

Table 7. Overview of the model and the parameters with their values and/or distributions, and source.

Variable	Definition	Unit	Formula/distribution	Source
P	Prevalence	%	Beta (6 + 1; 27 - 6 + 1)	Calculated from data
C_0	Initial concentration	Log cfu/g	ECDF (c (-1.4, -1, 0, 1, 2), min=1.4, max=3, prob= c (17, 1, 1, 2, 1)	(Carrasco et al., 2010; Gombas et al., 2003)
T	Home storage temperature	ºC	Pert (3.2ºC, 6.4ºC and 11ºC)	Obtained from household monitoring
t	Home storage time	h	Uniform (0, 192)	Assumption based on shelf-life
G	Growth during home storage	Log cfu/g/h	$\sqrt{\mu_{max}} = b(T - T_{min})$	Calculated - Obtained from challenge test (Ratkowsky et al., 1982)
N_{max}	Maximum achievable viable cell count	Log cfu/g	7.8	Calculated
C_f	Concentration after storage	Log cfu/g	$C_f = \log_{10}(10^{N_{max}}) - \log_{10}\left(1 + (10^{\log_{10} 10^{N_{max}}}) - \log_{10}(10^{C_0})\right) - 1 \times e^{(-G \times t)}$	Adapted from Baranyi & Roberts (1994) model with no lag
S	Serving size	g	Pert (82.5, 165, 247.5)	Assumption
D	Ingested dose	cfu	$D = 10^{C_f} \times S$	Calculated
Po_{risk}	Population at risk	%	8.96	Calculated
r	Probability of infection from 1 cell	-	Low risk population: 2.37×10^{-14} High risk population: 1.06×10^{-12}	(FAO/WHO, 2004)
P_{ill}	Probability of infection	-	$P_{ill}(D; r) = 1 - e^{(-r \times D)}$	(FAO/WHO, 2004)
$MP_{ill}H/L$	Mean probability of infection High or Low population	-	Mean (P_{ill})	Calculated
N_p	Number of portions consumed per year	-	344402	Annual production of the factory
N_{cont}	Contaminated portions consumed	-	$N_p \times P$	Calculated
$N_{cont}H$	Contaminated portions consumed by high-risk population	-	$N_{cont} \times \frac{Po_{risk}}{100}$	Calculated
$N_{cont}L$	Contaminated portions consumed by low-risk population	-	$N_{cont} - N_{cont}H$	Calculated
N_cH	Number cases per year in high-risk population	-	$\text{Cont Ns} \times MP_{ill}H$	Calculated
N_cL	Number cases per year low-risk population	-	$\text{Cont Ns} \times \text{MP}_{\text{ill}}\text{L}$	Calculated
N_c	Number cases per year	-	$N_cH + N_cL$	Calculated

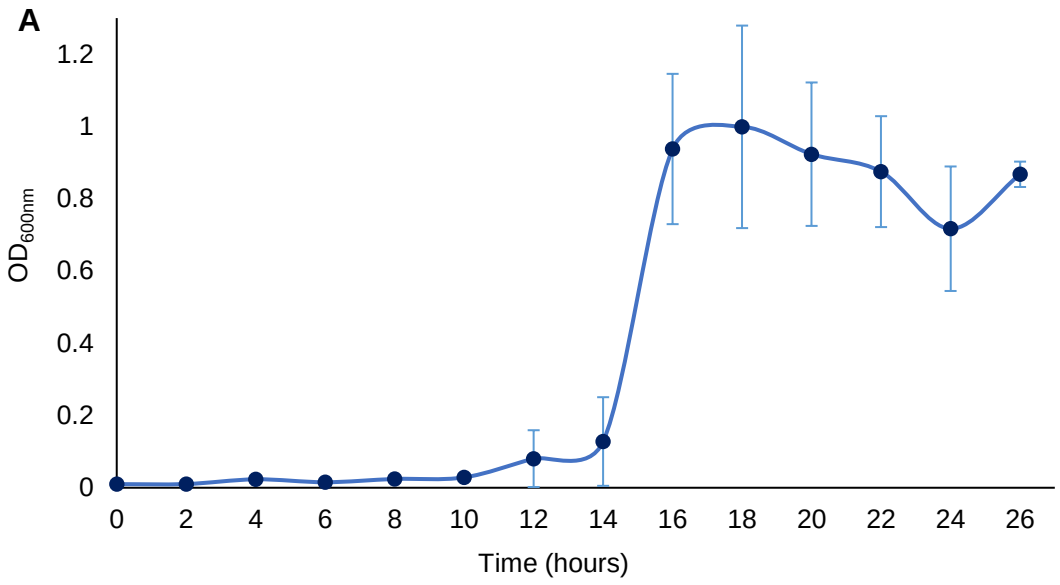
3. Results

3.1. Isothermal growth in BHI broth

As a preliminary step of a challenge test, it was important to understand the pathogen's growth at different temperatures in a defined medium (BHI). For this, *Listeria monocytogenes* CECT 4031 was chosen, since it is the type strain for this specie (Davenport et al., 2014). The chosen temperatures were 37ºC, which is *L. monocytogenes'* optimal growth temperature and 12ºC, which is used in food producing rooms at industrial facilities. *L. monocytogenes* growth curves were built using viable cell counts (VCC) and optical density at 600 nm (OD_{600nm}) measurements.

3.1.1. Optical Density Growth Curves

Figure 6 shows the resulting growth curves based on the average OD values at each sampling time of *Listeria monocytogenes* CECT 4031 in Brain Heart Infusion broth (BHI) incubated for 26 hours at 37°C **(A)** and incubated for 12 days at 12°C **(B)**.



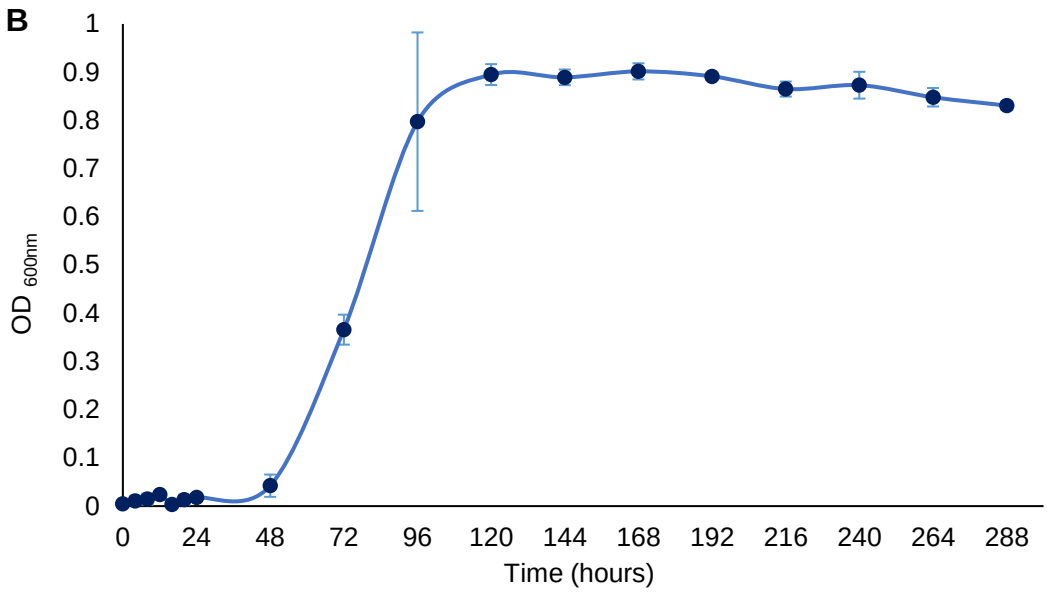


Figure 6. Growth curve obtained from average and standard deviation (SD) (error bars) of *L. monocytogenes* CECT 4031 suspensions OD measurements. **(A)** Incubation for 26 hours at 37°C. **(B)** Incubation for 12 days at 12°C.

Growth occurred at both temperatures, although differences can be observed. The optimal growth temperature of *L. monocytogenes* is 37°C, and at this temperature, a lag phase of approximately 10 hours can be observed followed by an exponential growth phase from 10 h to 18 h. From then on until the end of the incubation time (26 h), stationary phase was observed. The maximum OD value was 0.999 ± 0.280 at 18 h. At 12°C the lag phase lasted approximately 48 hours, and from 48 h to 120 h the exponential growth phase can be observed. Stationary phase seems to be reached at 120 hours. The maximum OD value was 0.902 ± 0.017 at 168 h.

3.1.2. VCC Growth Curve

Figure 7 presents the obtained growth curves for *Listeria monocytogenes* CECT 4031 in BHI considering total viable cell counts (VCC) for the assessed sampling time points, at 37°C **(A)** and at 12°C **(B)**.

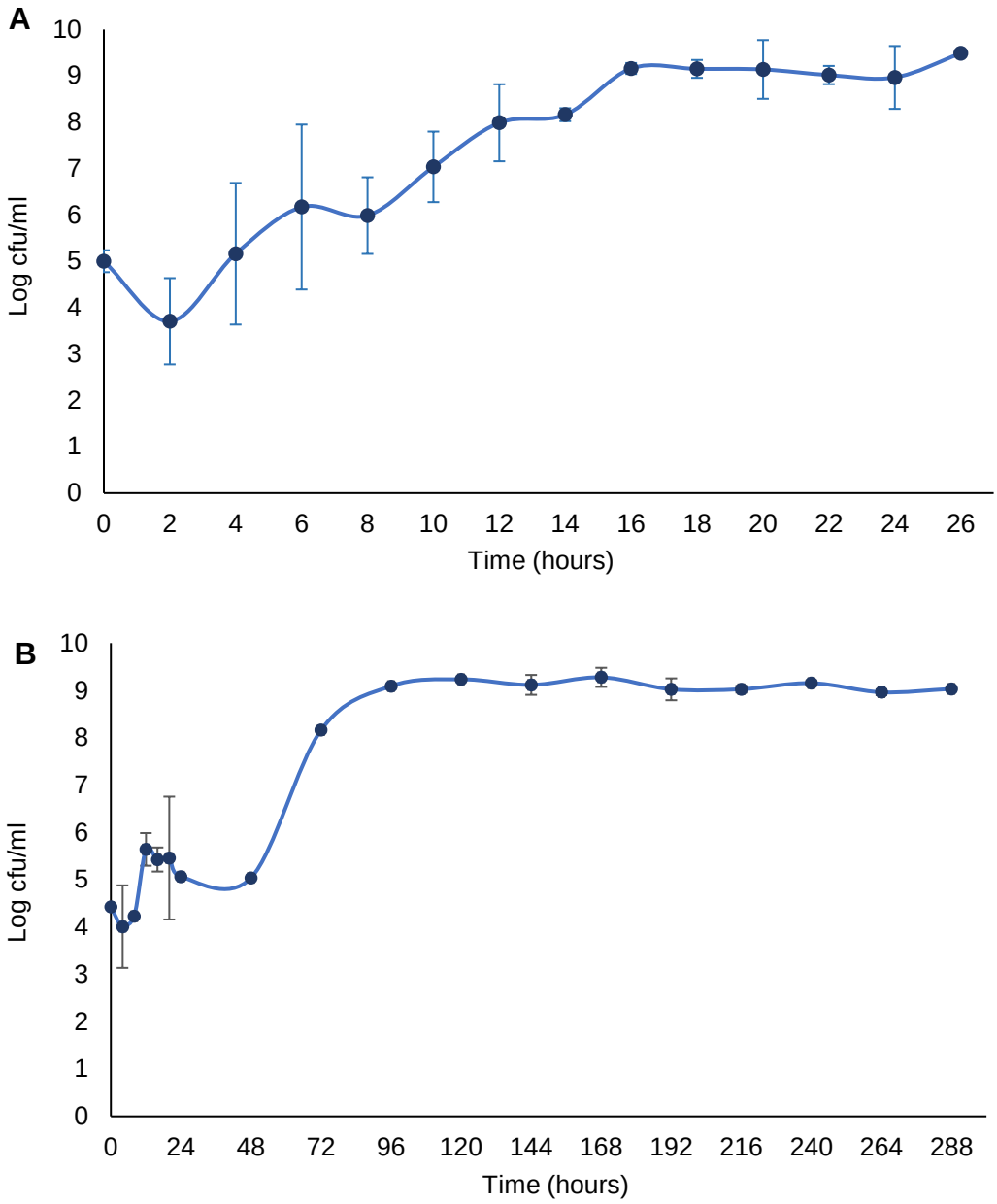


Figure 7. *L. monocytogenes* CECT 4031 growth curve based on viable cell counts (mean and standard deviation (SD) (error bars). **(A)** Incubation for 26 hours at 37°C. **(B)** Incubation for 12 days at 12°C.

Once again, differences in growth can be observed at both temperatures. At 37°C, an exponential phase can be observed in the first 18 h of incubation, however the maximum value of VCC 9.484 ± 0.678 log cfu/ml was obtained at 26h. At 12°C, there seems to be a potential lag phase of approximately 48 hours, and from 48 h to 120 h the exponential growth phase can be observed. Stationary phase seems to be reached at 120 hours after inoculation. The maximum value of VCC reached was 9.277 ± 0.210 log cfu/ml at 168 h.

3.1.3. Comparison of *L. monocytogenes* experimental OD and VCC results

Experimental results of *L. monocytogenes* CECT 4031 OD and VCC were scattered plotted and are shown in **Figure 8A** (37ºC) and in **Figure 8B** (12ºC).

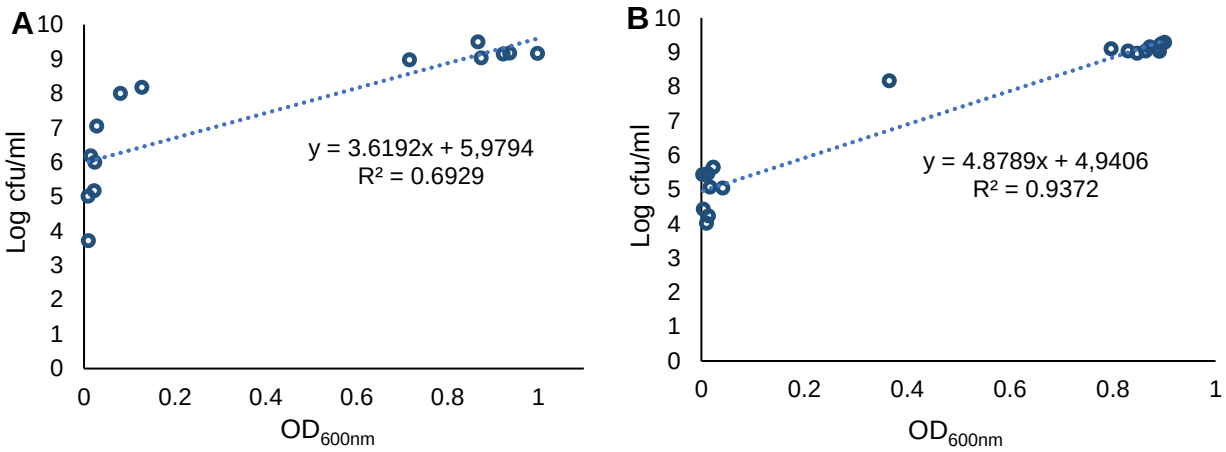


Figure 8. Scattered plot of experimental OD and VCC for *Listeria monocytogenes* CECT 4031. Simple linear regression was used to fit data. **(A)** Data regarding the temperature of 37ºC. **(B)** Data regarding temperature of 12ºC.

Table 8. Correlation analysis between OD measurements and VCC.

Temperature	Pearson correlation	95% confidence interval (IC)	R^2
37ºC	0.8324	0.5404 to 0.9454	0.693
12ºC	0.9681	0.9145 to 0.9883	0.937

In **Table 8**, it is possible to observe that the value of Pearson correlation is, for both temperatures close to 1, indicating that the two methods are moving in agreement. When the values of OD go up the values of VCC also go up, the same happens when the values go down. At 12ºC, the R^2 obtained was 0.937, revealing a good adjustment/fit when using OD to estimate VCC (**Figure 8B** and **Table 8**). However, as can be seen in **Figure 8A** and **Table 8** , R^2 has a lower value (0.693), which reveals a worse adjustment/fit when using OD to estimate VCC at 37ºC, when compared to 12ºC.

3.1.4. Estimated growth parameters

The predictive primary model described by Baranyi and Roberts (1994) was used in order to calculate growth parameters of *Listeria monocytogenes* CECT 4031 on BHI. The growth curves obtained by VCC for each temperature were built by fitting experimental data to the Baranyi's DMFit *online* version. The following parameters were obtained: i) maximum specific growth rate (μ_{max}), ii) lag time (λ), iii) initial and final concentration (C_0 and C_f respectively). R-squared (R^2) and Standard Error of fit (SE) were used to evaluate the performance of the models built in this study.

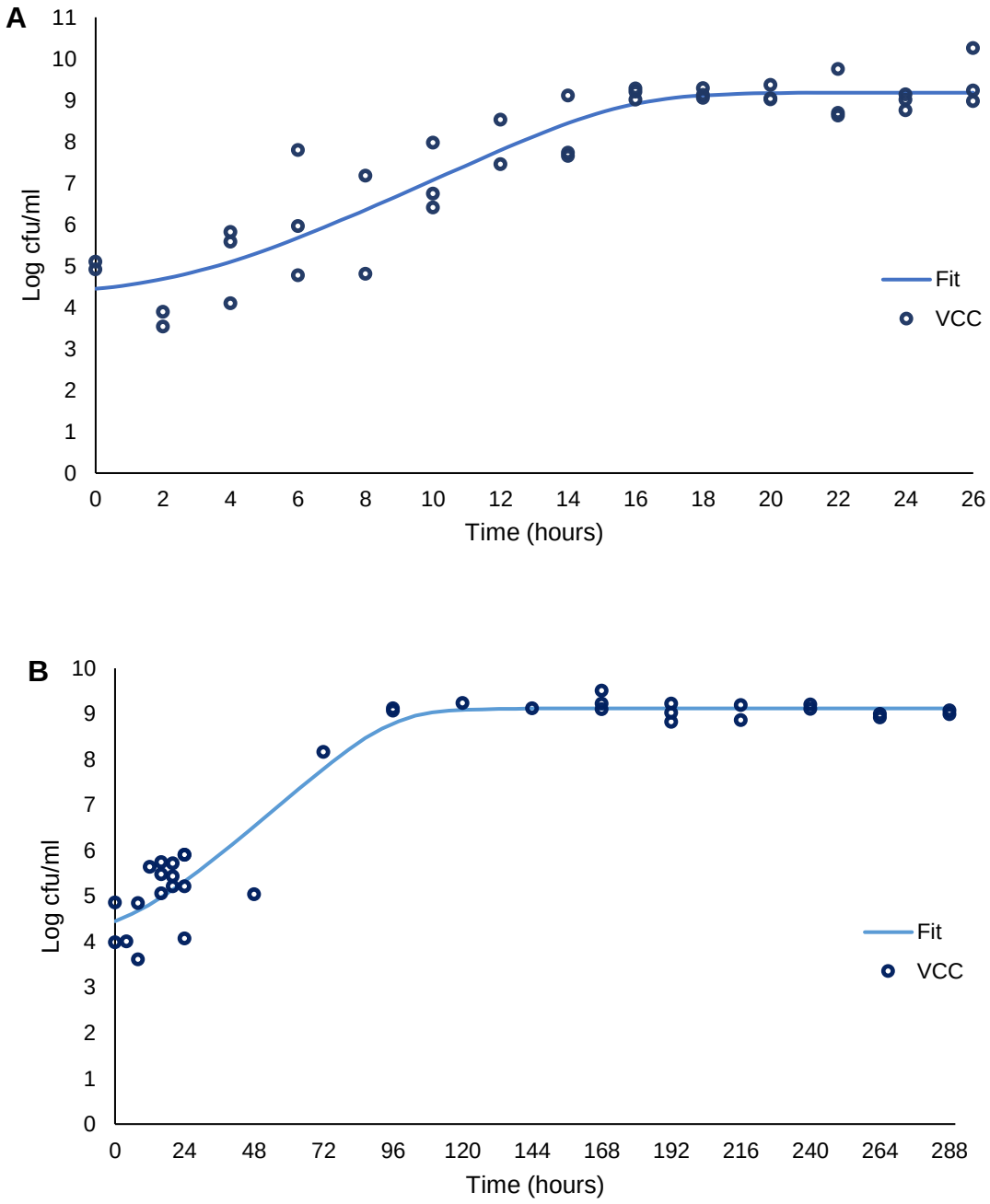


Figure 9. *Listeria monocytogenes* viable cell counts (VCC) (log cfu/ml) fitted with Baranyi and Roberts model. **(A)** Incubation for 26 hours at 37°C (R^2: 0.845; SE: 0.748). **(B)** Incubation for 12 days at 12°C (R^2: 0.937; SE: 0.530).

At 37°C, *Listeria monocytogenes* CECT 4031 concentration peaked at 21 h reaching 9.184 ± 0.204 log cfu/ml and remaining stable until the end of incubation time (stationary phase). At 12°C, *L. monocytogenes* CECT 4031 reached a maximum final concentration of 9.117 ± 0.133 log cfu/ml after 144 h and remained stable until the end of incubation time (stationary phase). These results are shown in **Figure 9**.

The obtained R^2 results for both temperatures revealed a good fit of the used model to experimental data. The resulting parameters obtained by fitting experimental data to the Baranyi's model are shown in **Table 9**.

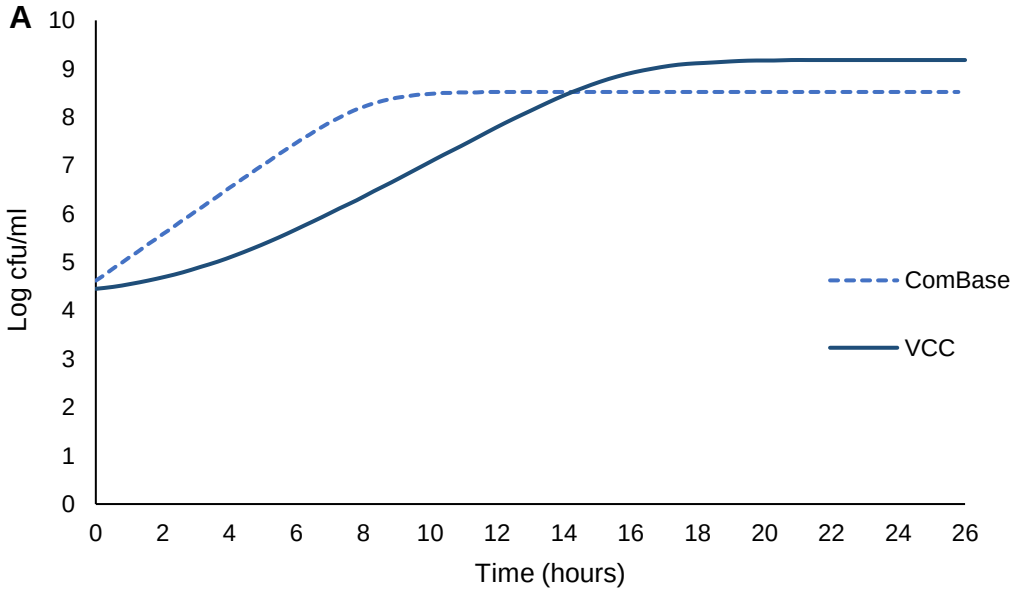
Table 9. Maximum specific growth rate (μ_{max}), lag time (λ), initial and final concentration (C_0 and C_f respectively) (mean ± SD) for *Listeria monocytogenes* CECT 4031 estimated by DMFit Model using VCC results, at 37°C and 12°C.

Temperature	μ_{max} (log cfu/ml/h)	λ (h)	C_0 (log cfu/ml)	C_f (log cfu/ml)
37°C	0.375 ± 0.072	3.026 ± 2.263	4.446 ± 0.436	9.184 ± 0.204
12°C	0.054 ± 0.001	9.856 ± 11.681	4.454 ± 0.289	9.117 ± 0.133

Regarding the estimated growth parameters (**Table 9**), using VCC results, at 37°C a maximum specific growth rate (μ_{max}) of 0.375 ± 0.072 log cfu/ml/h was estimated, higher than μ_{max} at 12ºC with an estimated value of 0.054 ± 0.001 log cfu/ml/h. On the other hand, the lag time at 12°C lasted approximately 9.856 ± 11.681 hours, while at 37ºC it lasted 3.026 ± 2.263 h.

3.1.5. Comparison with ComBase

Predictions obtain from Baranyi and Roberts model using *L. monocytogenes* VCC at both temperatures were compared to the estimated growth using ComBase Predictor Growth model. Results are shown in **Figure 10**.



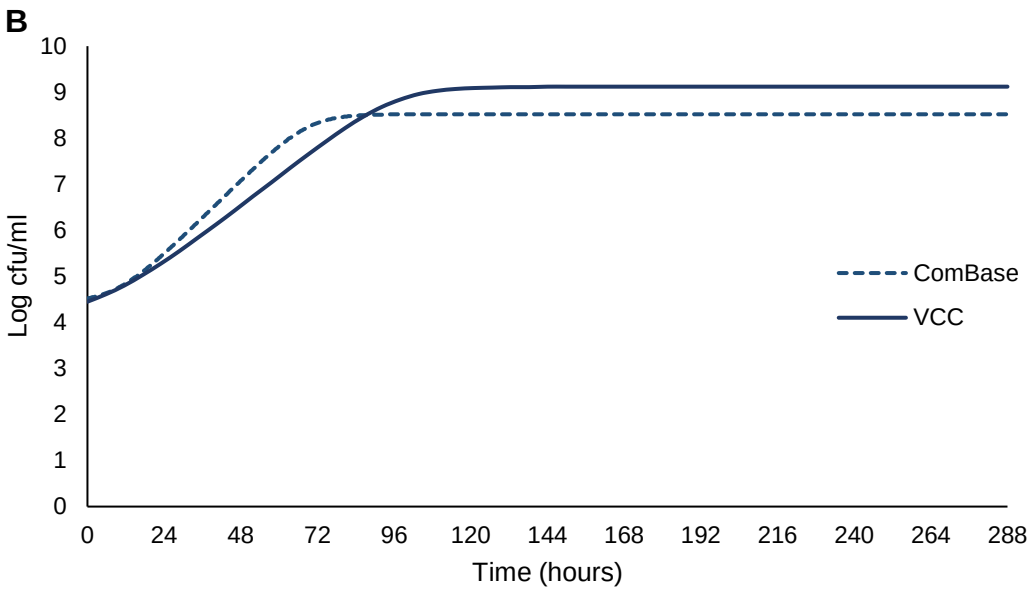


Figure 10. Comparison of *Listeria monocytogenes* fitted growth curves obtained from VCC and the online software ComBase Predictor Growth Model. **(A)** Incubation for 26 hours at 37°C. **(B)** Incubation for 12 days at 12°C.

In general, predictions from ComBase Predictor growth model and this study's results were quite similar. However, when considering the temperature of 37°C, it is possible to see that the growth curve based on VCC presents lower values until 14 h of incubation but higher maximum values when compared with ComBase estimated growth curve. It is also possible to observe that stationary phase is reached sooner in ComBase estimated growth curve. Also, μ_{max} obtained with ComBase predictor was of 0.480 log cfu/ml/h, higher when compared to the experimental data μ_{max} (0.375 ± 0.072 log cfu/ml/h, **Table 9**).

At 12ºC, the stationary phase is reached later in the estimated growth curve based on VCC. ComBase growth curve presents lower maximum log cfu/ml values. Considering the maximum specific growth rate (μ_{max}) obtained with ComBase predictor, at 12°C a μ_{max} of 0.068 log cfu/ml/h was estimated, which is higher than the experimental data μ_{max} (0.054 ± 0.001 log cfu/ml/h, **Table 9**).

3.2. Calibration curves

As said before, in order to determine the inoculum density using OD_{600nm} measurements, a calibration equation for each strain in the study was obtained by performing three independent calibration curves, in which viable cell counts were plotted against OD_{600nm} data, as shown in **Figure 11**.

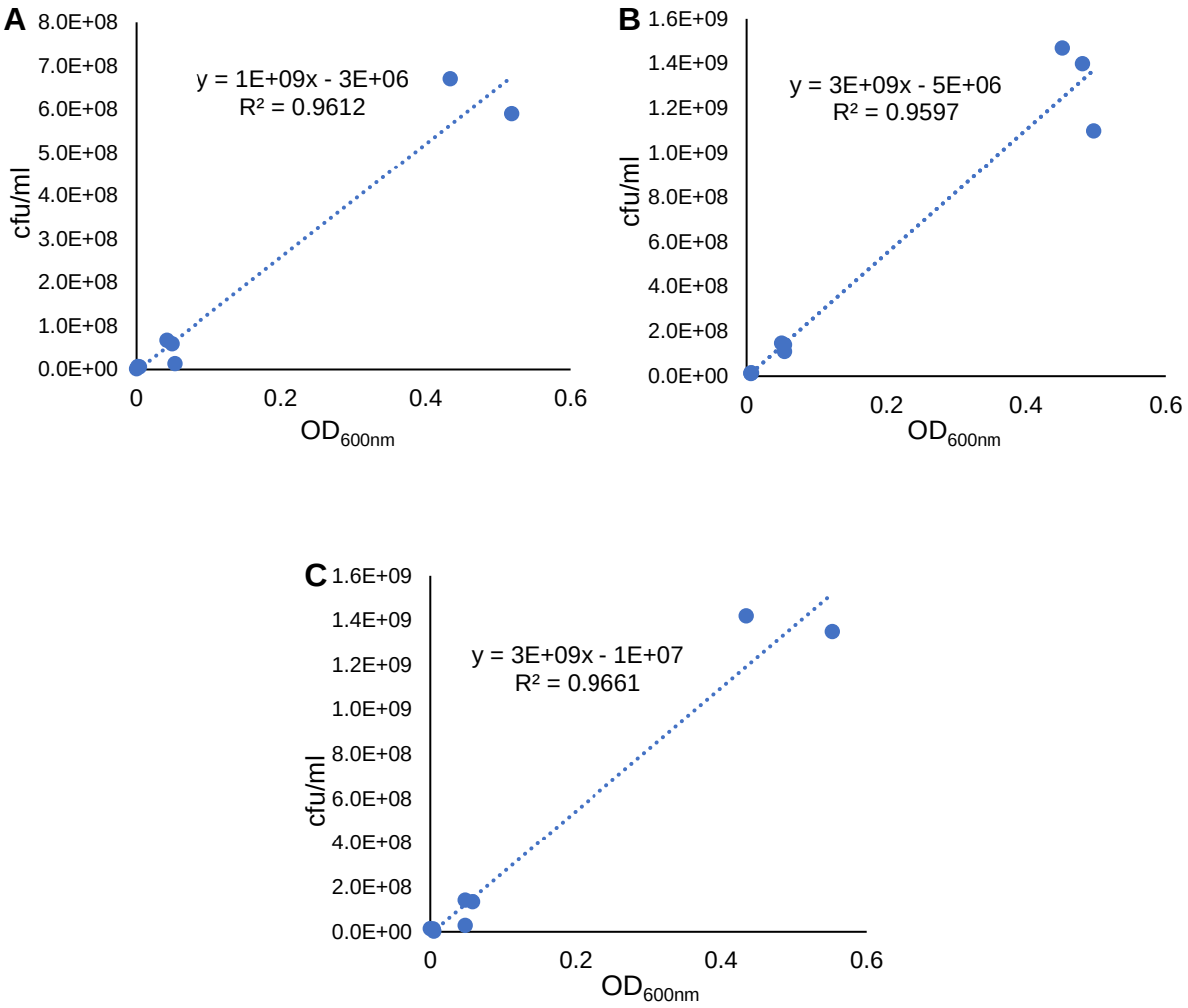


Figure 11. Plot of the observed OD_{600nm} against the VCC (cfu/ml) for **(A)** *Listeria monocytogenes* CECT 4031, **(B)** *Listeria monocytogenes* CECT 935 and **(C)** *Listeria monocytogenes* CECT 937.

A good adjustment between OD measurements and viable cell counts (R^2) can be observed, allowing, with confidence, in further techniques, the use of absorbance values to determine the concentration of microorganisms in the culture, with the use of the calibration equations.

3.3. Challenge testing

3.3.1. pH and a_w measurements

In **Table 10** the obtained pH and a_w values in RTE chicken salad blank samples (BS – no BWP added, and BS-BPW – BWP added) are presented, throughout the 8 days of study at the three tested temperatures: 4°C, 12°C**,** and 16°C.

Table 10. Mean and standard deviation for pH and a_w values obtained from RTE chicken salads at 4º, 12º and 16ºC throughout the challenge test (192 hours).

BS

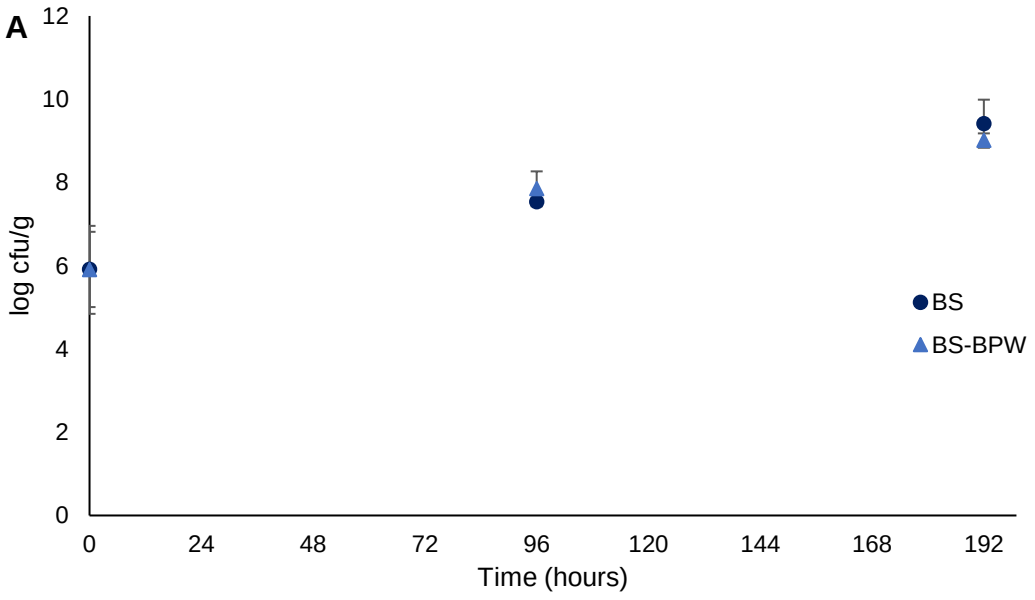
	4°C		12°C		16°C	
Time (h)	**pH**	**a_w**	**pH**	**a_w**	**pH**	**a_w**
0	5.930 ± 0.033	0.955 ± 0.001	6.113 ± 0.191	0.957 ± 0.006	6.251 ± 0.561	0.962 ± 0.009
96	6.021 ± 0.080	0.966 ± 0.001	6.427 ± 0.400	0.957 ± 0.002	6.632 ± 0.554	0.971 ± 0.008
192	6.647 ± 0.491	0.980 ± 0.002	7.309 ± 0.325	0.958 ± 0.001	7.625 ± 0.167	0.963 ± 0.007

BS-BPW

	4°C		12°C		16°C	
Time (h)	**pH**	**a_w**	**pH**	**a_w**	**pH**	**a_w**
0	5.902 ± 0.084	0.958 ± 0.001	6.113 ± 0.151	0.972 ± 0.021	5.890 ± 0.308	0.954 ± 0.002
96	6.027 ± 0.064	0.967 ± 0.009	6.478 ± 0.090	0.957 ± 0.006	6.687 ± 0.528	0.958 ± 0.023
192	6.659 ± 0.256	0.966 ± 0.001	7.208 ± 0.364	0.960 ± 0.005	7.205 ± 0.460	0.961 ± 0.007

3.3.2. Enumeration of total aerobic microorganisms at 30ºC

Figure 12 presents the obtained countings of total aerobic microorganisms at 30ºC (TAM30) in blank samples (BS – no BWP added, and BS-BPW – BWP added), throughout the 192 hours of study at the three tested temperatures: 4°C **(A)**, 12°C **(B)** and 16°C **(C)**.



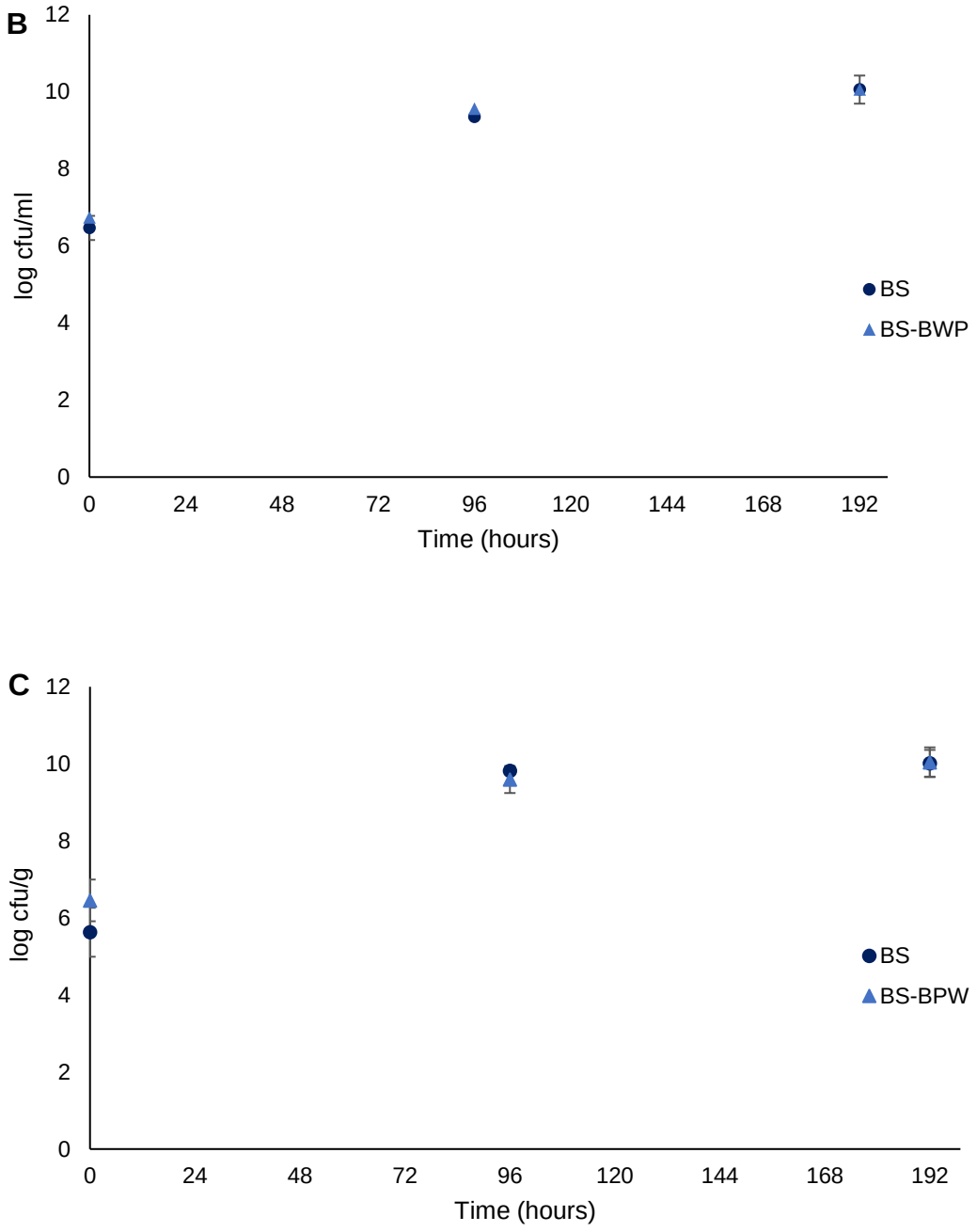
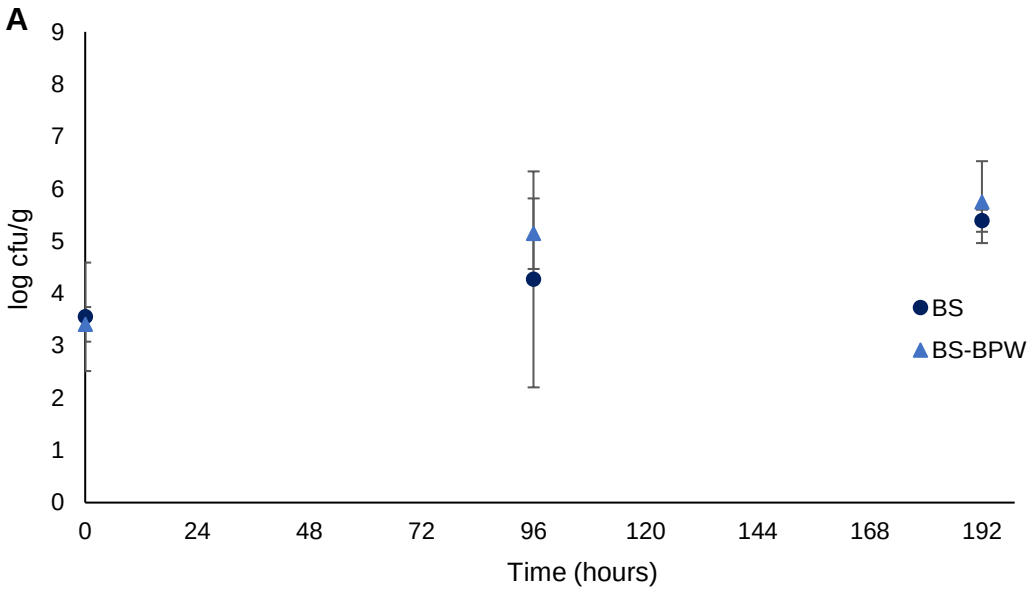


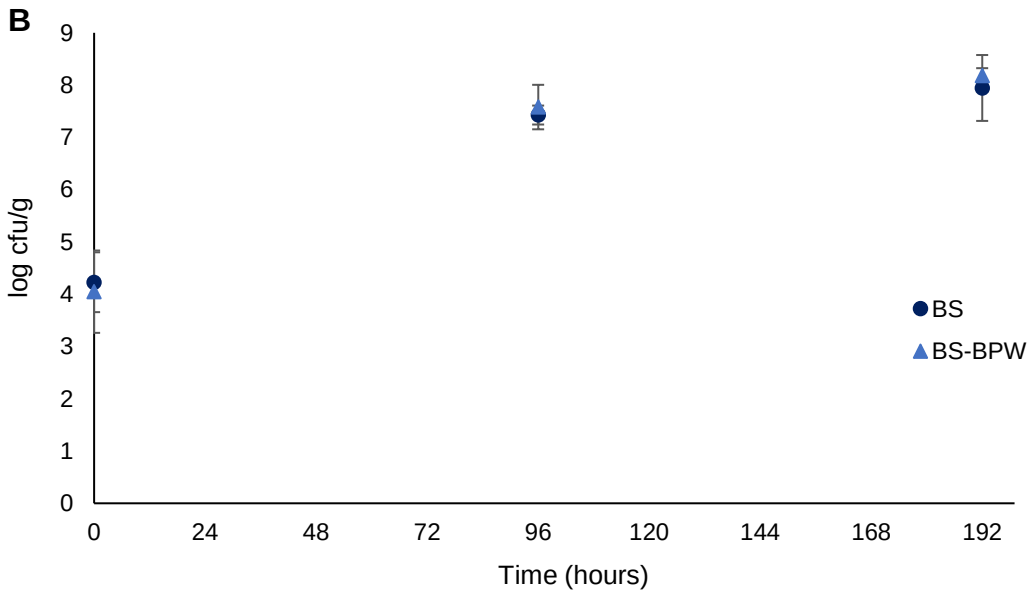
Figure 12. Mean and standard deviation (error bars) of total aerobic microorganisms at 30ºC viable cell counts in blank samples (BS and BS-BPW) throughout the study (192 hours). **(A)** Incubation at 4°C, **(B)** 12°C and **(C)** 16°C.

The evolution in VCC counts of total aerobic microorganisms at 30ºC in RTE chicken salads at 4ºC revealed values ranging from 5.911 ± 0.904 cfu/g to 9.413 ± 0.576 cfu/g for BS and from 5.901 ± 1.057 cfu/g to 9.011 ± 0.168 cfu/g for BS-BPW during 8 days of incubation (**Figure 12A**). At 12ºC the values ranged from 6.467 ± 0.314 cfu/g to 10.059 ± 0.365 cfu/g for BS, and from 6.744 ± 0.350 cfu/g to 10.062 ± 0.369 cfu/g for BS-BPW (**Figure 12B**). Finally, at 16ºC the values ranged from 5.628 ± 0.899 cfu/g to 10.014 ± 0.347 cfu/g for BS, and from 6.454 ± 0.766 cfu/g to 10.041 ± 0.386 cfu/g for BS-BPW (**Figure 12C**).

3.3.3. Enumeration of Enterobacteriaceae

Figure 13 presents the obtained Enterobacteriaceae countings blank samples (BS – no BWP added, and BS-BPW – BWP added), throughout the 192 hours of study at the three tested temperatures: 4°C **(A)**, 12°C **(B)** and 16°C **(C)**.





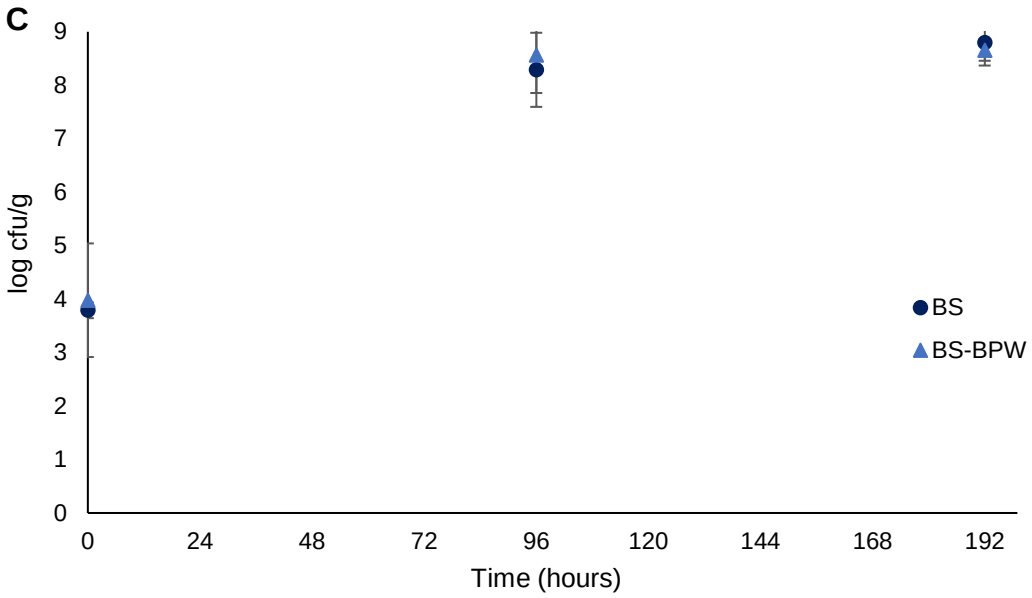


Figure 13. Mean and standard deviation (error bars) of Enterobacteriaceae viable cell countings in blank samples (BS and BS-BPW) throughout the study (192 hours). **(A)** Incubation at 4°C, **(B)** 12°C and **(C)** 16°C.

The evolution in VCC counts of Enterobacteriaceae in RTE chicken salads at 4ºC revealed values ranging from 3.552 ± 1.037 cfu/g to 5.389 ± 0.211 cfu/g for BS and from 3.408 ± 0.331 cfu/g to 5.745 ± 0.783 cfu/ml for BS-BPW during 8 days of incubation (**Figure 13A**). At 12ºC the values ranged from 4.229 ± 0.572 cfu/g to 7.945 ± 0.630 cfu/g for BS, and from 4.048 ± 0.788 cfu/g to 8.180 ± 0.143 cfu/g for BS-BPW (**Figure 13B**). Finally, at 16ºC the values ranged from 3.790 ± 0.146 cfu/g to 8.800 ± 0.432 cfu/g for BS, and from 3.979 ± 1.063 cfu/g to 8.654 ± 0.197 cfu/g for BS-BPW (**Figure 13C**).

3.3.4. Detection and enumeration of *L. monocytogenes* on inoculated samples

L. monocytogenes initial 3-mixed strains' suspension contained approximately 4 log cfu/ml. As can be seen in **Figure 14**, at 0 h, in the beginning of the challenge test, the values of 3-mixed strains *L. monocytogenes* varied between 4.680 ± 0.750 log cfu/g, 4.034 ± 0.021 log cfu/g and 4.184 ± 0.122 log cfu/g at 4ºC, 12ºC and 16ºC respectively, confirming the recovery and viability of the inoculum. It was also possible to observe *L. monocytogenes'* growth in the inoculated samples, at the three studied temperatures.

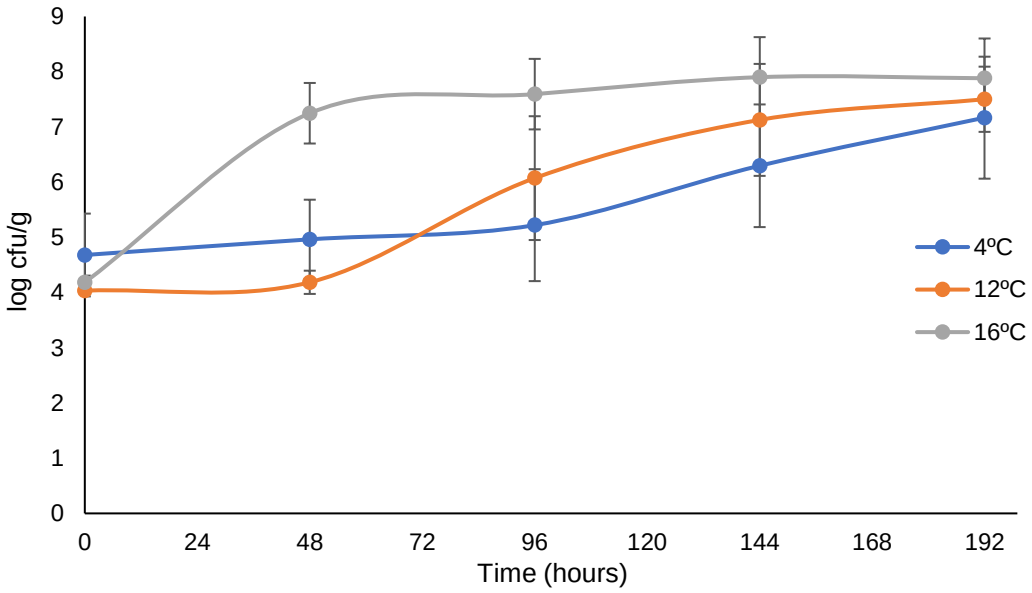


Figure 14. Mean and standard deviation (error bars) of *L. monocytogenes* 3-strain mix viable cell countings throughout the study (192 hours), at 4°C, 12°C and 16°C.

Overall, inoculated 3-mixed strains of *L. monocytogenes* increased by approximately 2.487 log cfu/g, 3.466 log cfu/g and 3.697 log cfu/g throughout the storage time, reaching 7.167 ± 1.105 log cfu/g, 7.501 ± 0.590 log cfu/g and 7.882 ± 0.720 log cfu/g by the end of the challenge test, at respectively 4ºC, 12ºC and 16ºC.

3.3.5. Detection and enumeration of *L. monocytogenes* on blank samples

Throughout the challenge test, it was possible to detect presumptive *L. monocytogenes* colonies in some uninoculated blank samples (BS). **Figure 15** shows an example of those colonies in ALOA plates and the subsequent isolation of some of the suspicious colonies.

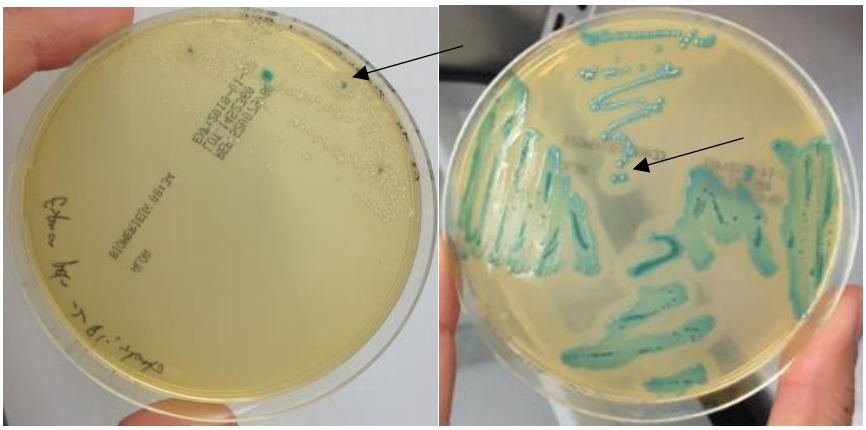


Figure 15. *Listeria monocytogenes* presumptive colonies in ALOA plates with the characteristic bluish-green color with an opaque round halo (black arrow), obtained in non-inoculated samples. The second image corresponds to the isolation of some of those colonies, also presenting the same characteristic (black arrow).

Throughout the study, these presumptive colonies (n=30, **Annex II**) were recovered from 15 blank samples (55.6% of presumptive positive samples) on a routine basis, to be confirmed as *L. monocytogenes* by PCR (section **3.4**).

3.4. Multiplex PCR

Presumptive *L. monocytogenes* isolates' confirmation and serogrouping (n=30) was performed using a multiplex PCR and an additional PCR based on the amplification of the *fla*A gene (Kérouanton et al., 2010). The multiplex PCR enabled the confirmation of presumptive *L. monocytogenes* (n=10), and also allowed strains to be clustered into three molecular serogroups (IIa, IIb and IVb) (**Table 11**) according to the presence of a specific gene distribution (**Table 2** of section **1.6.2.1)**. All five molecular serogroups (IIa, IIb, IIc, IVa and IVb) were also confirmed by *L. monocytogenes* serogroups positive controls.

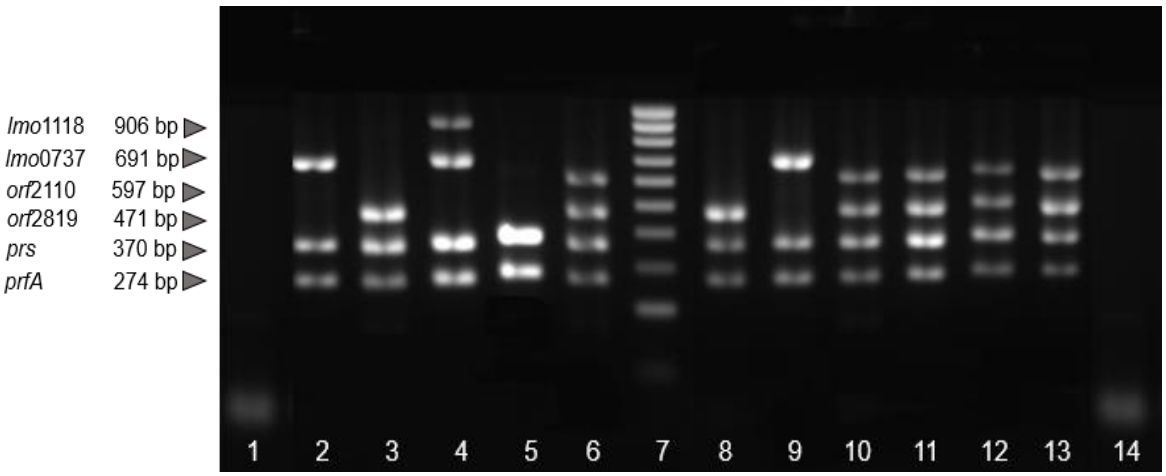


Figure 16. PCR patterns of the five molecular serogroups obtained after agarose gel electrophoresis of DNA products generated by multiplex PCR. Lane 1 - Blank reaction control; Lanes 2 to 6 - *L. monocytogenes* serogroups positive controls: Lane 2 - *L. monocytogenes* CECT 4031 IIa serogroup; Lane 3 - *L. monocytogenes* CECT 937 IIb serogroup; Lane 4 - *L. monocytogenes* CECT 911 IIc serogroup; Lane 5 - *L. monocytogenes* CECT 934 IVa serogroup; Lane 6 - *L. monocytogenes* CECT 935 IVb serogroup; Lane 7 - DNA molecular weight marker (100 bp NZYTech); Lane 8 to 13 - *L. monocytogenes* isolates in test: Lane 8 - CS1/8; Lane 9 - CS1/0; Lane 10- CS3/0-A; Lane 11- CS3/4-A1; Lane 12 - CS5/4-A; Lane 13 - CS7/8-A2; Lane 14 - Negative control sample (*Escherichia coli* DSMZ 682).

Table 11. Serogroups of *Listeria monocytogenes* isolated in RTE chicken salads blank samples.

***L. monocytogenes* serogroup**	**Isolate code**
IIa	CS1/0
IIb	CS1/8
IVb	CS3/0-A, CS3/0-B, CS3/4-A1, CS3/4-A2, CS3/4-A3, CS5/4-A, CS5/4-B, CS7/8-A2

All the isolates not present in **Table 11**, (20 out of the initial 30 isolates) were confirmed to belong to *Listeria* genus but were not from *Listeria monocytogenes* species.

3.5. PFGE typing

The resulting dendrogram obtained from the analysis of the restriction profiles of *L. monocytogenes* isolates with *Apa*I and *Asc*I is shown in **Figure 17**, along with the serogroups. The 10 *L. monocytogenes* isolates from different RTE chicken salads plus the three reference isolates (*L. monocytogenes* CECT 4031, 935 and 937) were diverse, presenting 7 PFGE types. Pulsotypes were considered to be clones when they had 95% or more of similarity.

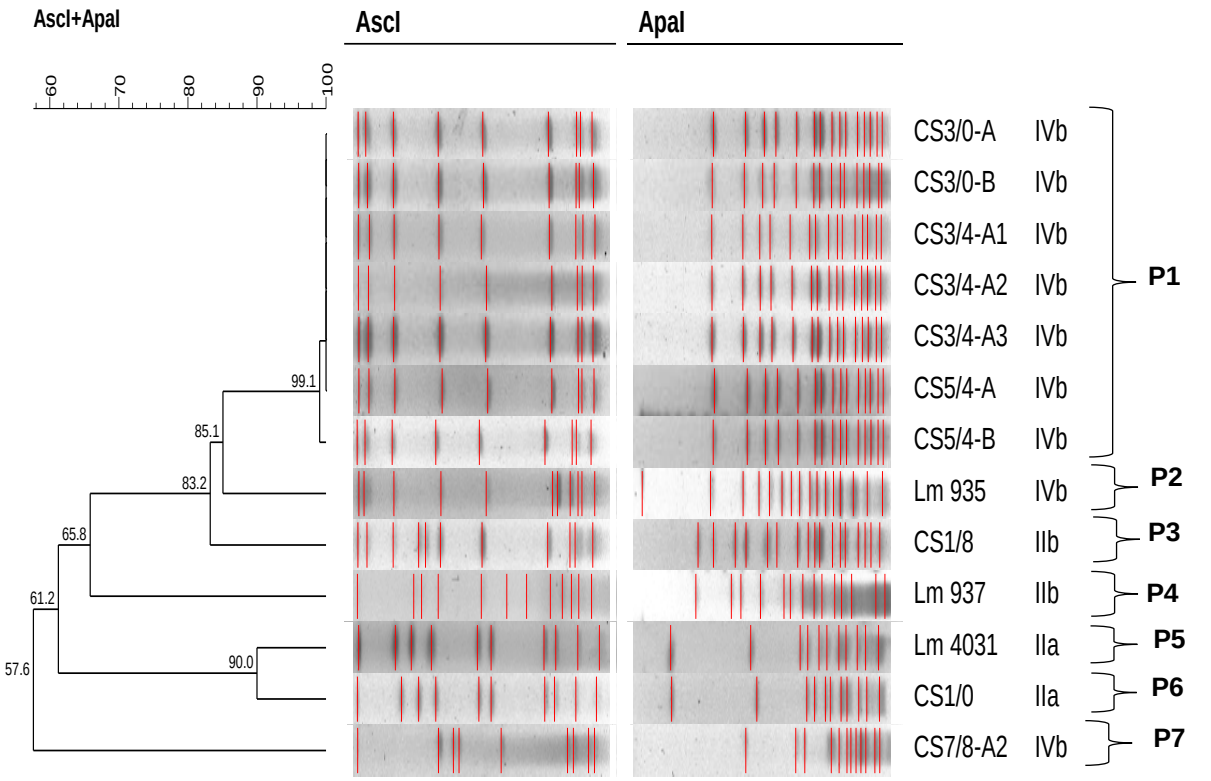


Figure 17. Dendrogram of the *Apa*I-*Asc*I profiles in PFGE and corresponding serogroups for 10 *L. monocytogenes* selected isolates, plus 3 reference isolates (*L. monocytogenes* CECT 4031, 935 and 937). P1 – pulsotype 1, P2 – pulsotype 2, P3 – pulsotype 3, P4 – pulsotype 4, P5 – pulsotype 5, P6 – pulsotype 6, P7 – pulsotype 7.

3.6. Real Time Quantitative PCR

A comparison of the obtained *Listeria monocytogenes* concentrations on the last day (8th day) of the assay, using PMA-qPCR technique and culture-based techniques (viable cell count on ALOA media) is shown in **Figure 18**. The Ct values obtained by RT-qPCR were quantified using a relative standard curve generated from positive control DNA at known concentrations (**Annex III**). Efficiency value (E) of 92% and correlation coefficient (R^2) of 0.995 were obtained.

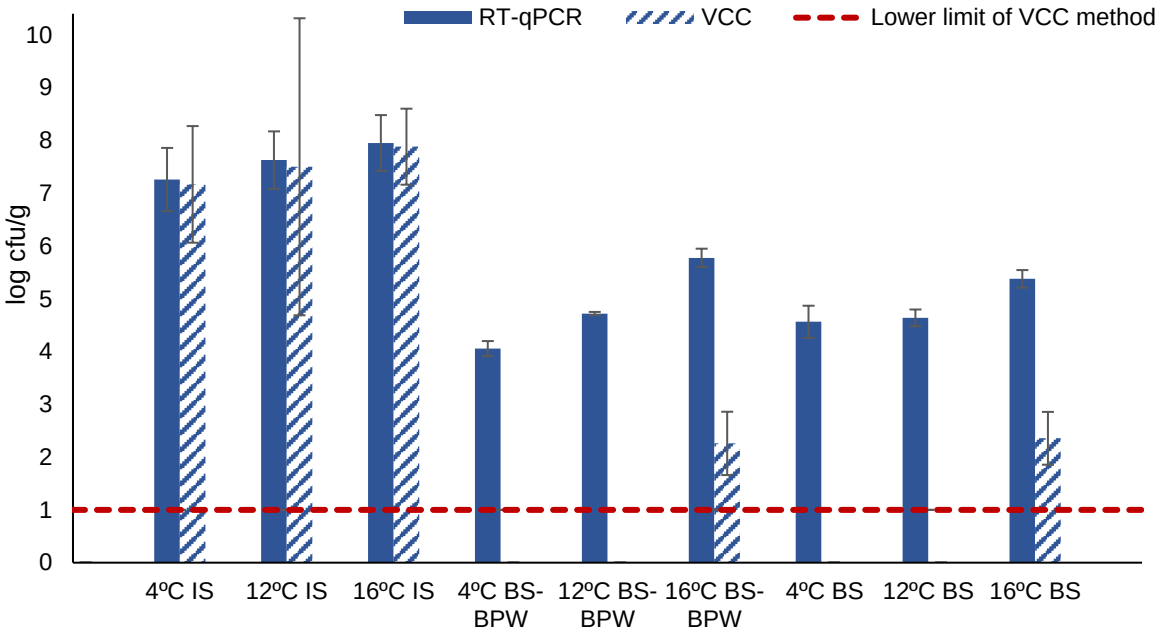


Figure 18. *L. monocytogenes* concentration (log cfu/g) obtained by PMA-qPCR and culture-based techniques (viable cell count in ALOA media), on the final day of each assay (day 8). The average value and SD are presented (qPCR n=6, and VCC n=3). Lower limit of VCC method is represented by the dotted line.

For every assay, the log cfu/g of *L. monocytogenes* obtained by PMA real time quantitative PCR was higher than the ones obtained by VCC in ALOA. For quantification of RTE chicken salads samples inoculated with *L. monocytogenes* (4ºC IS, 12ºC IS and 16ºC IS), no significance difference was detected between the two quantitative methods, for each assay ($p < 0.05$). However, for both quantification of RTE chicken salads blank samples (4ºC, 12ºC and 16ºC BS and BS-BPW), a significance difference was detected between the two quantitative methods.

3.7. Modelling *L. monocytogenes* growth on artificially inoculated salads RTE chicken salads

3.7.1. Primary model and growth parameters for *L. monocytogenes*

L. monocytogenes 3-mixed strains countings, as recovered from inoculated RTE chicken salads, under the considered isothermal conditions (4°C, 12°C and 16°C), are shown in **Figure 19**, **Figure 20** and **Figure 21**, with fitted growth curves generated using the Baranyi and Robert's model (1994).

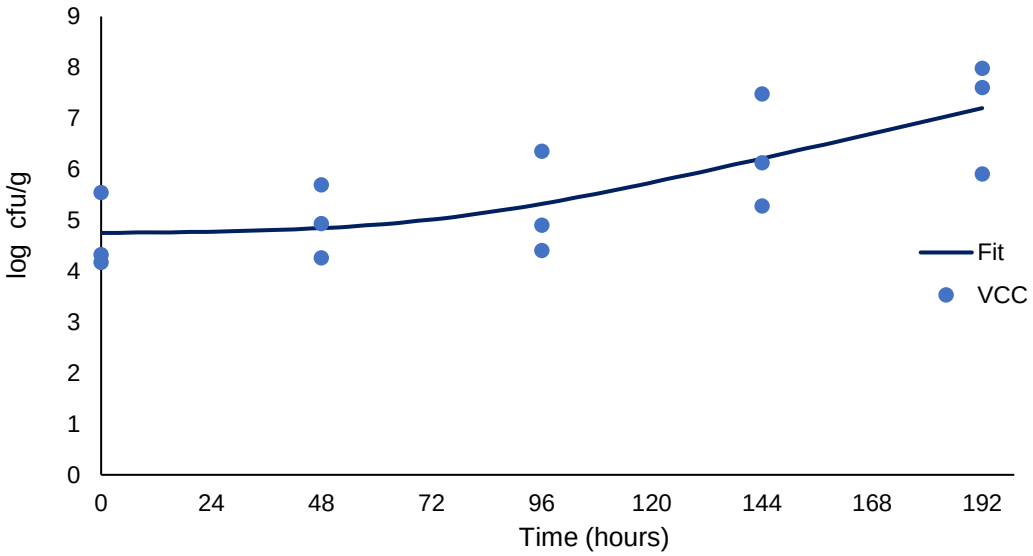


Figure 19. *Listeria monocytogenes* countings obtained from inoculated RTE chicken salad stored at 4°C and growth prediction curves based on the Baranyi's model (Baranyi, & Roberts, 1994).

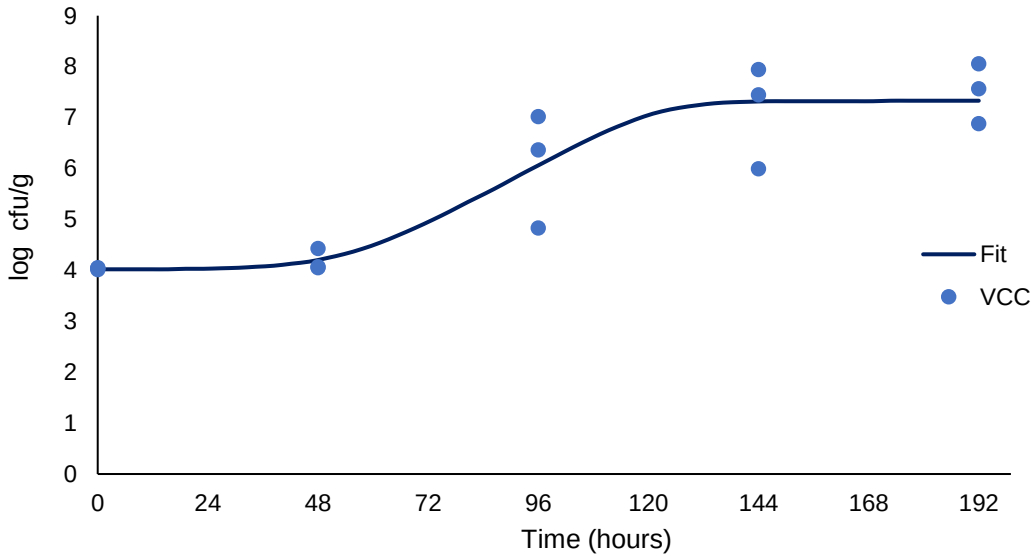


Figure 20. *Listeria monocytogenes* countings obtained from inoculated RTE chicken salad stored at 12°C and growth prediction curves based on the Baranyi's model (Baranyi, & Roberts, 1994).

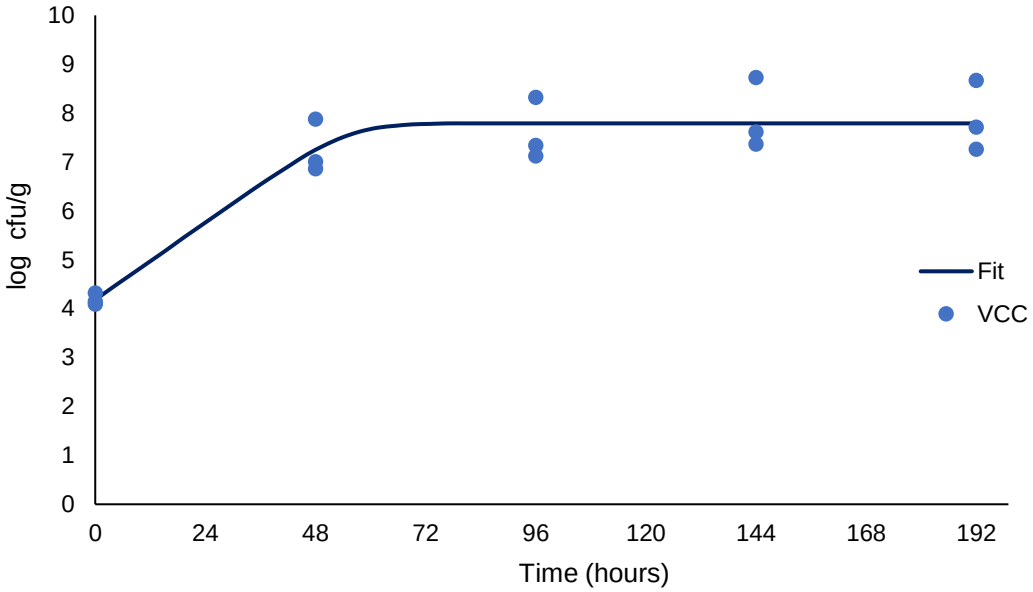


Figure 21. *Listeria monocytogenes* countings obtained from inoculated RTE chicken salad stored at 16°C and growth prediction curves based on the Baranyi's model (Baranyi, & Roberts, 1994).

At 4ºC, the fitted growth curve exhibited a distinct lag phase lasting for 74.4 hours (approximately 3 days), followed by an exponential phase that was observed until the end of the storage time (192 h). It is not possible to observe the settling of a plateau, indicating the beginning of the stationary phase.

At 12ºC, the fitted growth curve exhibited a lag phase of 54.1 hours (approximately 2 and a half days), an exponential phase, and a stationary phase, reaching 7.33 log cfu/g at 172.8 h (7 days of storage).

In the fitted growth curve at 16ºC, the lag phase is not perceptible. After the exponential phase and after approximately 76.8 hours, a stationary phase was reached (3 and half days of storage) peaking at 7.79 log cfu/g.

The growth parameters of *L. monocytogenes* on RTE chicken salads stored at different temperatures predicted by primary model described by Baranyi and Roberts are presented in **Table 12** (maximum specific growth rate (μ_{max}), lag time (λ), initial cell count (C_0), and maximum population density (N_{max}).

Table 12. Growth parameters of *Listeria monocytogenes* in RTE chicken salads, inoculated with the pathogen and stored at different isothermal conditions.

Growth kinetic parameter[a]	Storage temperature		
	4°C	**12°C**	**16°C**
μ_{max} (log cfu/g/h)	0.021 ± 0.008	0.052 ± 0.024	0.066 ± 0.009
λ (h)	74.435 ± 48.466	54.139 ± 31.566	_[b]
C_0 (log cfu/g)	4.751 ± 0.418	4.019 ± 0.410	4.184 ± 0.321
N_{max} (log cfu/g)	_[b]	7.325 ± 0.300	7.792 ± 0.185
R^2	0.512	0.807	0.870

[a] Values are means ± standard deviations (n=3).
[b] No growth kinetic parameter value was estimated.

3.7.2. Secondary model for the maximum specific growth rate (μ_{max}) of *L. monocytogenes* as function of storage temperature RTE chicken salads

Data obtained in the primary model of growth (values of μ_{max} (**Table 12**)) was used to elaborate a secondary model according to the square-root model described by Ratkowsky et al. (1982), which allowed to predict the maximum specific growth rate described on the basis of the temperature variation (**Figure 22**). Model's parameter T_{min} is the intercept of the function with the temperature axis when $\sqrt{\mu_{max}} = 0$.

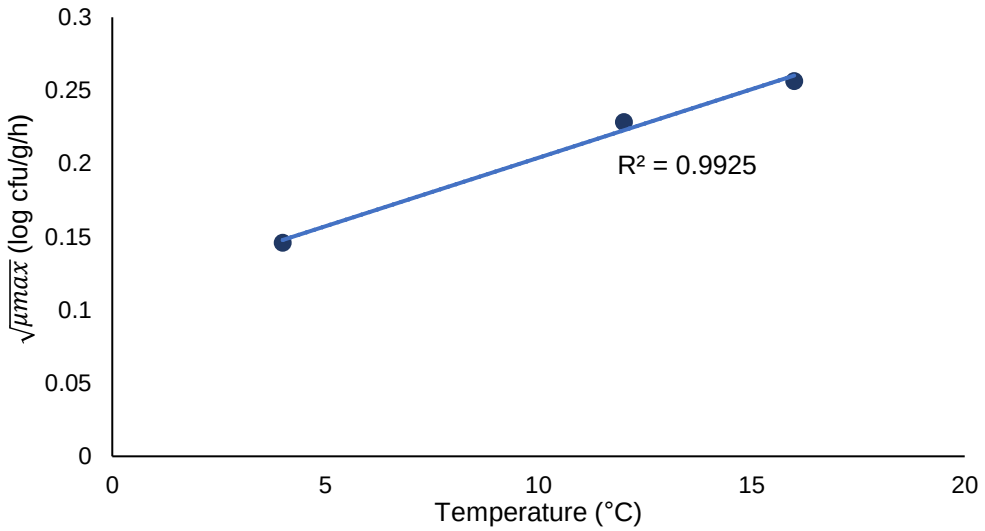


Figure 22. Fitting (line) of the square-root-type model of Ratkowsky et al. (1982) to the estimated values (circles) of the μ_{max} of *L. monocytogenes* in RTE chicken salad, and the fitting value (R^2).

The developed model was able to assess the growth of *Listeria monocytogenes* on RTE chicken salads under sub-optimal temperatures. **Equation 6,** describing the relationship of μ_{max} and temperature for *L. monocytogenes* grown in these RTE chicken salads under suboptimal temperatures is shown above:

$$\sqrt{\mu_{max}} = 0.0094(T + 11.745) \quad \textbf{(6)}$$

3.8. QMRA – Risk characterization

All the information gathered in QMRA steps was integrated to provide a description of the risk of listeriosis by the ingestion of RTE chicken salad contaminated with *L. monocytogenes*.

Figure 23 shows the frequency distribution of the contaminated level of *L. monocytogenes* on RTE chicken salads at the time of consumption at home. The average final contamination level before consumption was 1.142 log cfu/g (CI 95%: -0.985 to 3.694 log cfu/g), with a minimum of -1.396 log cfu/g and a maximum of 5.996 log cfu/g.

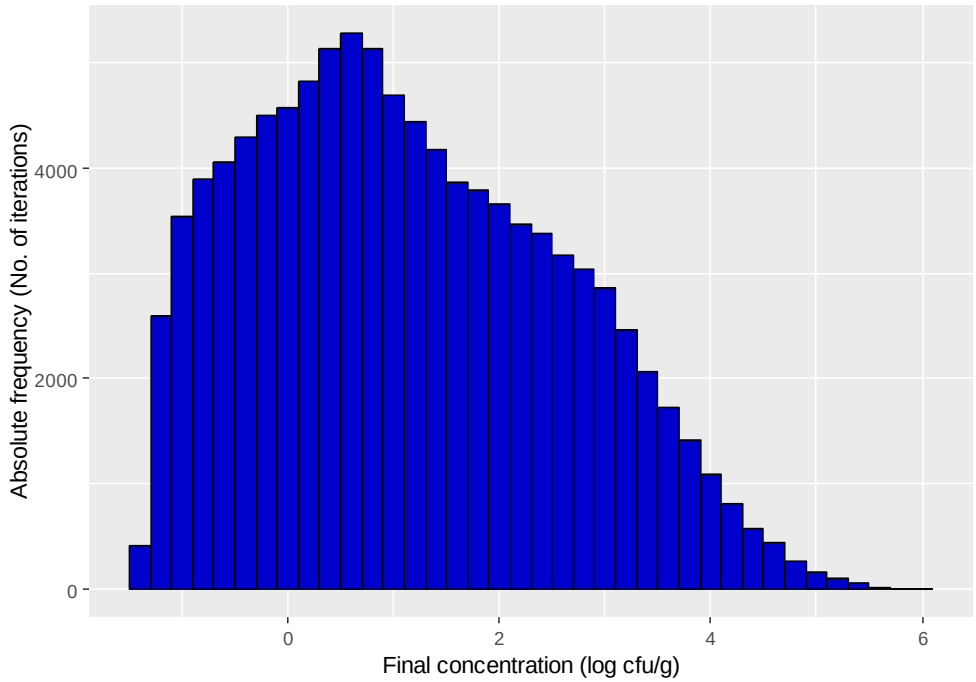


Figure 23. Frequency distribution of *L. monocytogenes* contamination level on lettuce at the time of consumption*. Graphic obtained from ggplot2 (package of R).*

The three possible scenarios regarding the number of listeriosis cases associated with the consumption of RTE chicken salads (high risk, low-risk, and total population (combined) are shown in **Table 13**.

Table 13. Number of listeriosis cases associated with the consumption of RTE chicken salads from the studied industry (Minimum, maximum and mean (CI 95%)).

Population	Minimum	Maximum	Mean (CI 95%)
High-risk	1.900×10^{-4}	5.654×10^{-3}	1.975×10^{-3} (9.311×10^{-4} - 3.242×10^{-3})
Low-risk	4.336×10^{-5}	1.290×10^{-3}	4.509×10^{-4} (2.125×10^{-4} - 7.390×10^{-4})
Total	4.336×10^{-5}	5.654×10^{-3}	1.213×10^{-3} (2.538×10^{-4} - 2.925×10^{-3})

The more realistic scenario (combination of high risk, low-risk population) of the annual number of listeriosis due to the consumption of RTE chicken salads is shown in **Figure 24** by means of a frequency distribution**.**

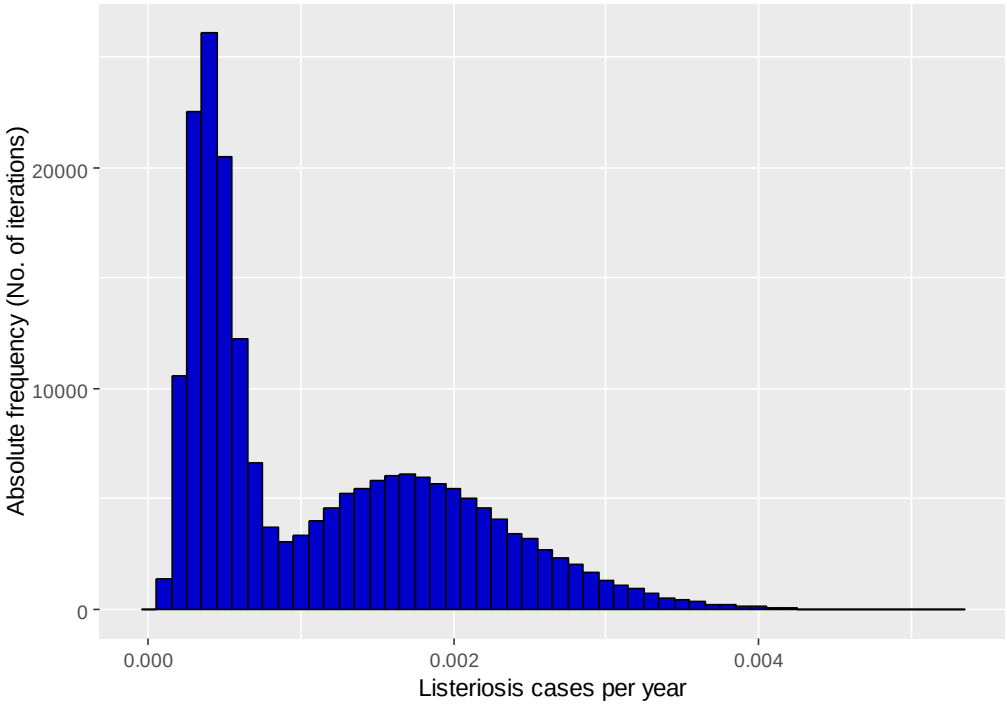


Figure 24. Frequency distribution of annual number of listeriosis cases due to the consumption of RTE chicken salads produced on the industry of the study. *Graphic obtained from ggplot2 (package of R).*

3.8.1. Sensitivity analysis

Sensitivity analysis indicates that the variability of storage time and consumer refrigerator temperature are the main contributors to risk variability, followed by initial concentration of *L. monocytogenes*. The consumers' variability in portion size had no significant contribution to the total effect (**Figure 25**).

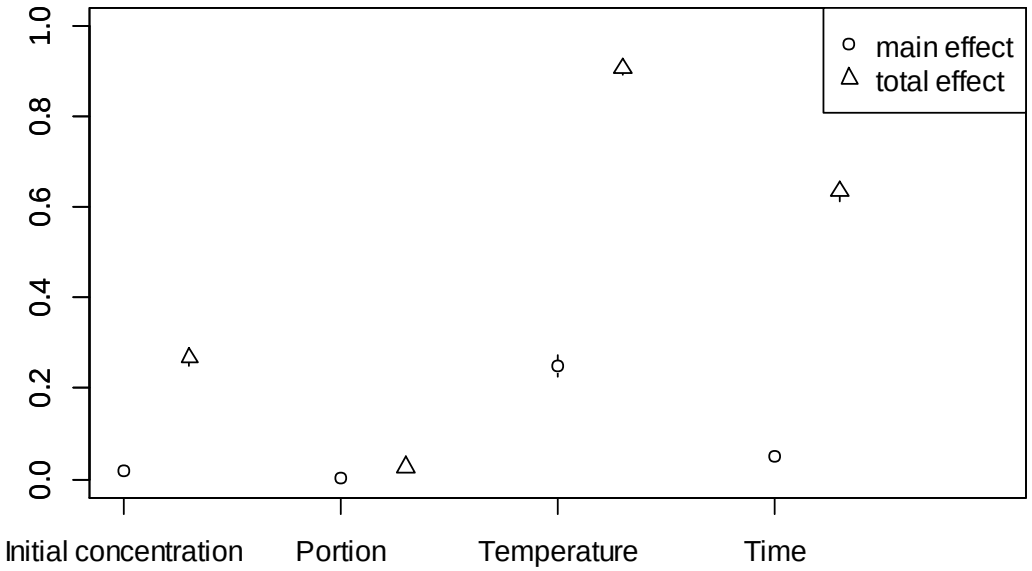


Figure 25. Sensitivity risk factors affecting the risk of listeriosis per dose, due to the consumption RTE chicken salads. *Graphic obtained from R.*

4. Discussion

4.1. *Listeria monocytogenes* isothermal growth in BHI broth

When studying the growth of *L. monocytogenes* in BHI broth, at different temperatures (optimal: 37°C and non-optimal: 12°C), it can be observed that temperature had a considerable influence on *L. monocytogenes* growth, because although initial and final concentrations are similar for both temperatures, the time needed to reach final concentration was higher for the lower temperature (12ºC). In order to reach approximate maximum concentrations, around 18/20 hours were needed at 37ºC and 5/6 days at 12ºC. This was observed when applying both quantification methods, OD_{600nm} measurements and viable cell counts.

Considering the obtained estimated growth parameters (**Table 9**), μ_{max} at 37ºC was higher than μ_{max} at 12ºC, and a longer lag phase was observed at this temperature (12ºC). The longer lag phase at 12°C may be due to an adaptation period to lower temperatures. Similar growth parameters were obtained by Pla et al. (2015), when assessing *L. monocytogenes* CECT 4031 growth in Tryptic soy broth supplemented with 0.6% yeast extract at 37ºC (μ_{max} = 0.447 and λ = 1.86) and by Wang et al. (2015) when studying the growth of *L. monocytogenes* in BHI at 10ºC (μ_{max} = 0.066), and a longer lag phase was also observed (λ = 17 h).

More time is needed for *L. monocytogenes* to grow at 12ºC and reach the same concentrations as those obtained at 37ºC. However, final concentrations of the pathogen were very similar, emphasizing the ability of *L. monocytogenes* to grow at refrigerated temperatures, as the ones used in food producing rooms at industrial facilities. In fact, as referred before at 12ºC, after 5/6 days of incubation, the levels of *L. monocytogenes* are similar to the ones reached at 37ºC. Duh & Schaffner (1993), in an article in which they study and model the effect of temperature on the growth rate and lag time of *L. monocytogenes* even refer: “Refrigerated storage alone cannot assure that the growth of *L. monocytogenes* will not occur. Predictive microbiology is a potentially powerful tool which the food microbiologist can use to support the traditional microbiological methods used to evaluate the potential for *Listeria* growth in foods, and the subsequent possibility of foodborne disease”. In this study, VCC results fitted to Baranyi and Roberts growth model were compared to those estimated by ComBase that revealed to be a useful tool to predict how *L. monocytogenes* survives and grows under a variety of (primarily food-related) conditions.

4.1.1. Comparison of *L. monocytogenes* experimental OD and VCC results

When comparing experimental OD and VCC results (**Table 8**), although a good correlation was observed between both methods, the Pearson correlation and R^2 were higher at 12ºC. At 37ºC, the R^2 value (0.693) revealed a low adjustment/fit when using OD to estimate VCC. This difference may be related to the fact that optical density measures the turbidity of a suspension, and because of that its relationship with cell concentration may not be linear (deposits of non-viable cells in suspension are also measured as total number of cells). Differences between both methods are often discussed in the literature (Baty, & Flandrois, 2002; Begot et al., 1997). However, the better correlation between methods

at 12ºC than at 37ºC obtained was not expected. Some authors defend that the difference between both methods is especially true for assessing growth parameters for isolates in stressful conditions, as morphological changes in the cell may result in optical density values that do not reflect the actual cell numbers (Bereksi et al., 2002; Francois et al., 2005). Jones, Gill, & McMullen, (2003) showed that cold adaptation can sometimes cause cell elongation, as cells further increase in cell length before dividing to normal cell length, strongly affecting the relationship between the OD levels reached and the estimated log cfu/ml.

Nonetheless, the correlation between both methods was high, indicating that for further experiments, VCC values can be inferred through OD measurements with the use of calibration equations.

4.2. Challenge test

In this work, the growth of *L. monocytogenes* in artificially contaminated RTE chicken salad was studied during storage under reasonably foreseen temperature abuse at 12ºC and 16ºC, and at the recommended storage temperature (4ºC), throughout 8 days, representing also storage time abuse, since the recommended commercial shelf life is 6 days.

4.2.1. pH and a_w

pH and a_w results confirmed this study's RTE chicken salad as a food product able to support the growth of *L. monocytogenes*, allowing for a precise use of the microbiological criteria limits established in both European regulation 2073/2005 and in Technical guidance document for conducting shelf-life studies on *L. monocytogenes* in foods (EURL Lm, 2014).

The variation in pH and a_w values measured on BS and BS-BPW samples throughout the study, at each temperature, as can be seen in **Table 10**, revealed no significant differences ($p > 0.05$).

For both blank RTE chicken salads samples (with and without BPW), a_w measurements revealed a slight variation (less than 0.05 units) during the 8 days, at all the tested temperatures (**Table 10**). No significant differences ($p > 0.05$) were found in a_w measurements during the assessed shelf life at all the studied temperatures. Moreover, none of the registered a_w values for all samples and all temperatures reached the lower and the upper growth limit for *L. monocytogenes*, which are 0.93 and >0.99 respectively (EURL Lm, 2014).

Considering the obtained pH values in RTE chicken salad stored at 16ºC, an increase was registered throughout the storage period for BS and BS-BPW, and the same was observed at 12ºC, revealing significant differences ($p < 0.05$) in pH measurements during the challenge test, for both temperatures.

However, at 4ºC, a slight increase was observed in pH values, but no significant differences ($p > 0.05$) were found, for BS and BS-BPW. Nevertheless, none of the registered pH values for all samples and all temperatures reached the lower and the upper growth limit for *L. monocytogenes*, which are 4.2 and 9.5, respectively (EURL Lm, 2014).

4.2.2. Hygiene indicators

Direct testing of pathogens is not always possible or practical. The presence of indicator bacteria in ready-to-eat food, although not inherently a hazard, can be a useful and cost-effective means of assessing the microbiological status of food in a relatively rapid way. Indicator microorganisms tend to be present in higher levels than most pathogens and may be associated with an increased likelihood of the presence of pathogens. They can also be indicative of the effectiveness of hygiene and process controls, such as poor quality of raw materials or food ingredients, undercooking, cross-contamination, poor cleaning, poor temperature and time control (Food Safety Authority of Ireland (FSAI), 2016; Health Protection Agency (HPA), 2009).

4.2.2.1. Total aerobic microorganisms at 30ºC

At 4ºC, for both BS and BS-BPW, on day 0, the total aerobic microorganisms at 30ºC (TAM30) countings were approximately 6 log cfu/g, reaching approximately 9 log cfu/g by the end of the storage period (day 8). At 12ºC and 16ºC initial values (day 0) were around 6.5 log cfu/ml for both BS and BS-BPW (except for BS at 12ºC, which was around 5.5 log cfu/g), reaching values of nearly 10 cfu/g by the end of storage time (**Figure 12**). These findings were in agreement with a previous study by Omac et al. (2018), where total aerobic microorganism' growth on fresh spinach leaves inoculated with *L. monocytogenes*, at 3ºC, 5ºC and 8ºC was also reported, after 16 days of storage. Skalina, & Nikolajeva (2010) also found a significant increase in total aerobic microorganisms on RTE mixed salads artificially inoculated with *L. monocytogenes*, throughout 48 hours of storage at 3ºC and 7ºC.

No significant differences on the evolution of the total aerobic counts at 30ºC were found ($p > 0.05$) between BS and BS-BPW samples for all temperatures.

TAM30, is an indicator of quality, not safety, and cannot directly contribute towards a safety assessment of ready-to-eat foods. Nevertheless, TAM30 can provide useful information to assess the quality of a food, and should be used as part of a shelf-life testing program (HPA, 2009; FSAI, 2016).

Microbes are inevitably introduced during slicing, packaging, portioning and other manipulations but this should be minimized by good hygiene, both of personnel and of equipment. There are many factors contributing to the rate of microbial growth, including the type of food product and the processing it has received, the type of packaging or the storage temperature throughout shelf-life (Food Standards Australia New Zealand (FSANZ), 2016). In fact, in our study, mesophilic aerobic colony counts presented the lower values by the end of the challenge test at 4ºC.

For raw, ready-to-eat food commodities such as salad vegetables, TAM30 are likely to be much higher, between 10^6 and 10^8 cfu/g. This will tend to limit their shelf-life as spoilage may occur relatively rapidly and will usually be visible (HPA 2009). That was the case of the studied RTE chicken salads that have as ingredients not only fresh vegetables, but also cheese, both belonging to food categories that tend to yield higher levels of microorganisms. In fact, by the end of the salad's commercial shelf life (6 days), when stored at 12ºC and 16ºC, there was a lack of acceptability based on appearance, smell and texture, revealing the inadequacy of those temperatures as storage temperatures for these salads.

4.2.2.2. Enterobacteriaceae

At 4ºC, for both BS and BS-BPW, on day 0, Enterobacteriaceae counts were approximately 3.5 log cfu/g, reaching 5.5 log cfu/g by the end of the studied storage period (day 8). At 12ºC and 16ºC, initial values (day 0) were around 4 log cfu/g for both BS and BS-BPW, reaching values of nearly 8 log cfu/g and 9 log cfu/g for 12ºC and 16ºC, respectively, at the end of storage time (**Figure 13**). These findings were in agreement with a previous study by Manios et al. (2013), where an increase in Enterobacteriaceae at 8ºC was observed on vegetable salads artificially inoculated with *L. monocytogenes*, after 10 to 12 days of storage.

No significant differences on the evolution of Enterobacteriaceae counts were found ($p > 0.05$) between BS and BS-BPW samples for all temperatures.

The Enterobacteriaceae family is commonly used to assess adequacy of food processing and hygiene practices. This group includes species that originate from the intestinal tract of animals and humans, as well as plants and the environment (Baylis et al., 2011; HPA, 2009). As all Enterobacteriaceae are destroyed by the heat processing used in food production, their presence in pasteurized or cooked foods can indicate inadequate processing or post-process contamination. These microorganisms should be readily removed from the factory, equipment and surfaces by appropriate cleaning procedures. Their presence in heat treated foods therefore signifies inadequate cooking or post-processing contamination (Craven et al. 2003; HPA 2009).

According to the Health Protection Agency (2009), Enterobacteriaceae counts higher than 4 log cfu/g would be unsatisfactory in terms of hygiene when assessing RTE foods. However, the significance of testing Enterobacteriaceae will depend on the type of food under analysis. High countings of Enterobacteriaceae are expected in some food commodities such as raw salads and vegetables, since some bacteria of this family are natural colonizers of fresh vegetables. Therefore, the high values of Enterobacteriaceae observed in the studied RTE salads, with fresh vegetables in its constitution, may not indicate lack of hygiene practices. The use of sanitizing rinses may reduce but not entirely remove these organisms (FSAI, 2016; HPA, 2009).

Also, significant differences ($p < 0.05$) were detected on Enterobacteriaceae countings on the first and last day of the challenge test, at 12ºC and 16ºC, indicating the presence of psychrotrophic Enterobacteriaceae that are able to multiply in chilled foods. These are widely distributed and can be found in a variety of foods (Baylis et al., 2011).

4.2.3. *Listeria monocytogenes* growth on artificially inoculated RTE chicken salads

In **Figure 14** it is possible to see the exhibited growth of *L. monocytogenes* on the artificially inoculated RTE chicken salads stored at 4ºC, 12ºC and 16ºC. A significant difference ($p < 0.05$) was obtained when comparing *Listeria monocytogenes* concentrations at the beginning and by the end of the challenge test, for all temperatures. As expected, *L. monocytogenes* had the ability to survive and grow in this type of RTE salads throughout refrigerated storage, as has been also confirmed by the obtained pH and a_w values (EURL Lm, 2014). However, some factors could presumably hamper the growth capacity of this

pathogen on these salads, such as the potential competition exerted by other microorganisms eventually present or the antibacterial effects of carrot (which is one of the salad's ingredient) on *Listeria* species due to the antilisterial activity of carrot tissue's intrinsic factors (Beuchat, & Brackett, 1990; Francis & Beirne, 2001; Nguyen-the, & Lund, 1992; Noriega et al., 2010). Nevertheless, results obtained in these RTE chicken salads sustain what many authors have already determined: the ability of *L. monocytogenes* to develop in this kind of RTE food (Chau et al., 2017; De Cesare et al., 2018; Fallah et al., 2012; Little et al., 2007; Sahu et al., 2016; Söderqvist, 2017; Takahashi et al., 2015).

As mentioned before, for the three assessed temperatures, *L. monocytogenes* countings at 0 h (in the beginning of the challenge test) ranged between 4.680 ± 0.750 log cfu/g, 4.034 ± 0.021 log cfu/g and 4.184 ± 0.122 log cfu/g, at 4ºC, 12ºC and 16ºC respectively and no significant differences ($p > 0.05$) were found. The same happened ($p > 0.05$) with the final population density, reaching 7.167 ± 1.105 log cfu/g, 7.501 ± 0.590 log cfu/g and 7.882 ± 0.720 log cfu/g by the end of the challenge test, at respectively 4ºC, 12ºC and 16ºC. However, although initial and final population densities are very similar among storage temperatures during challenge testing (8 days), *L. monocytogenes* growth behavior during that period was different when considering the assessed temperatures.

As temperature increased, the lag phase duration shortened, reaching values of 74 h at 4ºC, and of 56 h at 12ºC. No lag phase could be perceived at 16°C. Also, as the temperature increased the time needed to reach maximum population densities (N_{max}) decreased. At 16ºC, only three and a half days of storage were needed to reach N_{max}. At 12ºC, 7 days of storage were necessary to reach N_{max}, but at 4ºC, it seems that N_{max} was not reached during the 8 days of the study, and *L. monocytogenes* seemed to be at an exponential phase of growth. Some authors defend that quantity and nature of background microbiota are known to affect the growth of *L. monocytogenes* (Francis & O'Beirne, 1998, 2001; Sahu et al., 2016), and since growth and multiplication of natural microbiota are expected to be restricted at low temperatures that might pose less competition for *L. monocytogenes* growth (Fouladkhah et al., 2013; Sahu et al., 2016). As can be observed in **Figure 12A** and **Figure 13A**, at 4ºC the levels of the assessed hygiene indicators were lower than the ones observed at other storage temperatures (**Figure 12B/C** and **Figure 13B/C**), which might explain the fact that at this temperature, by the end of the challenge test, a stationary phase could not be observed and instead, the pathogen was still in a growing phase.

These results evidenced that time and temperature abuse during shelf-life may influence the growth potential of *L. monocytogenes* in these RTE chicken salads. Although a reduction in the storage temperature might extend the lag phase and reduce the growth rate, *L. monocytogenes* will still be able to grow at low refrigeration temperatures and reach unsafe concentrations, if there is storage time abuse.

For the blank salads samples (BS), as shown in **Figure 15**, it was possible to detect the presence of some bluish-green colonies with a clear and round halo (*Listeria monocytogenes* characteristic colonies on ALOA media), more specifically 30 isolates. Since these salads were not inoculated and were expected to be negative for *L. monocytogenes* presence, it had to be confirmed i) if the colonies were true *L. monocytogenes* and not some other species that could display similar morphology on ALOA

plates: ii) if previous results were positive, it was necessary to confirm that it was not an experimental contamination, and to check if the salads might have been originally contaminated in the producing industry. For that, Multiplex PCR and PFGE were performed, and the obtained results are discussed later in sections **4.3** and **4.4**.

4.3. Multiplex PCR

A total of 30 isolates were tested and all of them were identified as *Listeria* spp. Of these, 10 (33.3%) were classified as *L. monocytogenes* (CS5/4-A, CS5/4-B, CS1/8, CS1/0, CS3/0-A, CS3/0-B, CS3/4-A1, CS3/4-A2, CS3/4-A3, CS7/8-A2). Because ISO 11290-2:2017 highlights that some strains of *L. monocytogenes* may show a very weak halo (or even no halo), colony's collection was performed having this in mind, and so a total of 30 presumptive isolates were initially collected. Moreover, ISO 11290-2:2017 also mentions that *L. ivanovii* colonies may have the same morphological aspect as *L. monocytogenes,* i.e., blue green colonies surrounded by an opaque halo. These facts might explain the confirmation of only 10 isolates as *L. monocytogenes* by PCR.

Nevertheless, these results are not surprising, as the industrial unit has a suspicion of *L. monocytogenes* environmental persistent contamination.

The presence of *Listeria* spp., other than *L. monocytogenes,* in these RTE salads, is also important, because it can be used as an indicator to assess the hygienic status of a food product. Although other *Listeria* species are not pathogenic to humans, with very rare exceptions (Guillet et al., 2010; Snapir et al., 2006), its detection might be worrisome, especially in foods able to support its growth. Preventive and corrective actions should be considered, especially for foods likely to be consumed by vulnerable groups, for whom the risk of listeriosis is increased (HPA 2009).

Three different serogroups were detected: IVb (CS3/0-A, CS3/0-B, CS3/4-A1, CS3/4-A2, CS3/4-A3, CS5/4-A, CS5/4-B and CS7/8-A2), IIa (CS1/0) and IIb (CS1/8), representing 80%, 10% and 10% respectively (**Table 11**). It is noteworthy that *L. monocytogenes* isolates in food samples presented the 3 serogroups more implicated in human disease, namely IIa, IIb and IVb. The majority of *L. monocytogenes* isolates belonged to serogroup IVb, associated with Lineage I. Serogroup IVb isolates have been associated to the majority of clinical strains causing severe human infections (Maury et al., 2016). Moreover, during 2010 and 2012, most of human listeriosis deadly cases in Europe were linked to serogroups IIa and IVb (ECDC, 2015), and according to "*Listeria monocytogenes* contamination of ready-to-eat foods and the risk for human health in the EU" (EFSA and ECDC, 2017) the number of serogroup IVb reported cases appears to be increasing.

In Tetouan, North-Western of Morocco, a total of 1096 food samples, including dairy products (n = 404), bovine meat products (n = 258), pastry (n = 162), salads (n = 143), poultry meat products (n = 103), chickpea flour cooked with eggs sold in the street (n = 20) and mayonnaises (n = 6), were collected from January 2009 to August 2015, to examine the presence of *Listeria* spp (Amajoud et al., 2018). Eighty (7.3%) of the tested samples were found positive for the presence of *Listeria* spp., while *L. monocytogenes* was detected in 16 (1.5%) samples. *L. monocytogenes* isolates belonged to serogroup

IVb (87.5%), and IIa (12.5%). Serogroup IVb was predominant, although *L. monocytogenes* was only found in pastries, bovine and dairy products (Amajoud et al., 2018).

In a study aiming to characterize *L. monocytogenes* strains isolated from RTE products collected as part of official food control and monitoring in Poland, a total of 105 *L. monocytogenes* isolates from RTE products (54 cakes and 51 delicatessen products) were examined. Serogroup distribution revealed a higher percentage of isolates belonging to molecular group IVb (serotypes 4ab-4b, 4d-4e) which comprised 33 strains (31.4%). However, the isolation rate of molecular group IIa (serotype 1/2a-3a) was at a similar level as molecular group IIb (serotypes 1/2b-3b-7), respectively 21.9% and 24.8%. Molecular group IIc (serotypes 1/2c-3c) was relatively rare in RTE (2.9%) and there were no representatives of molecular group IVa (serotype 4a-4c) (Maćkiw et al., 2016).

Both studies mentioned above are in agreement with this study's results in terms of serogroup IVb, as it was the predominant serogroup found in RTE foods. The presence serogroup IVb among such food items, indicates that these foods may pose a potential public health risk, due to their significantly higher pathogenic potential.

However, in other works assessing *L. monocytogenes* serogroups in RTE foods, and contrarily to this study, the predominant serogroup was not IVb, but others, such as IIa serogroup. IIa strains are believed to be better suited to survive and multiply in the environment, being common in foods and food related-environment (Eskhan, & Abu-Lail, 2013; Orsi et al., 2011). In a study investigating the occurrence and diversity of *L. monocytogenes* in three ewes' milk processing factories in Slovakia, a total of 639 samples from the cheese production chain were collected from 2011 to 2014. Twenty (3.1%) samples were found to be positive for *L. monocytogenes*. Sporadic *L. monocytogenes* contamination was observed in all three factories with 10% positive samples being the products, while 90% of positive samples were associated with the production environment. By molecular serotyping using multiplex PCR, *L. monocytogenes* isolates were classified in three serogroups – 80% in serogroup IIa, 10% in serogroup IIc and 10% in serogroup IVb (Véghová et al., 2015). The results obtained in this study are in agreement with another work, by Véghová et al. (2016) aimed at determining the occurrence and diversity of *Listeria monocytogenes* in a traditional meat-processing facility to reveal persistent contamination. A total of 268 samples, including 196 environmental samples and 72 meat samples were collected during a four-year period, and 70 were found to be *L. monocytogenes* positive. Molecular serotyping by multiplex PCR classified the 77 *L. monocytogenes* isolates into four different serogroups, with the majority of 34 (44.1%) strains in serogroup IIa, followed by 22 (28.6%) strains in serogroup IVb, 15 (19.5%) strains in serogroup IIc and 6 (7.8%) strains in serogroup IIb (Véghová, et al., 2016). *Listeria monocytogenes* isolates collected from final products and food contact surfaces of 10 ready-to-eat meat-based food products (RTEMP) producing industries were analyzed, and a collection of 62 isolates was obtained from the 14 *L. monocytogenes* positive samples; in some cases, more than one *L. monocytogenes* serogroup was identified in the same sample. Serogroup IIb was represented in 36% of the positive samples and in 26% of the obtained isolates, followed by serogroup IIa with 29% and 19%, respectively (Henriques et al., 2017).

The two multiplex PCR assays used in this study provided a rapid and reproducible alternative method for *L. monocytogenes* characterization (Kérouanton et al., 2010). Moreover, to effectively control this pathogen, it is necessary to have a method that can detect and differentiate *L. monocytogenes* from other *Listeria* species, in this specific case from food samples, but also from environmental and from clinical samples (Liu et al., 2015).

4.4. PFGE typing

The confirmed *L. monocytogenes* isolates were subjected to PFGE typing, to check for strain relatedness and discard any experimental contamination of the blank salad samples. Simultaneously, it intended to assess if there was a common source of contamination of the RTE chicken salads in the producing industry.

The obtained 10 *L. monocytogenes* isolates together with three reference strains (*L. monocytogenes* CECT 4031, 935 and 937) were assigned to 7 different pulsotypes (P1 to P7, **Figure 17**) with more than 95% of similarity.

Pulsotypes 2, 4 and 5 correspond to *L. monocytogenes* CECT 935 (serogroup IVb) and CECT 937 (serogroup IIb), and CECT 4031 (serogroup IIa), respectively. These reference strains were the ones used in the inoculation mix and were included in the PFGE analysis to discard any experimental contamination event. As can be seen in **Figure 17**, the inoculated reference strain *L. monocytogenes* CECT 4031 shares 90% of similarity with isolate CS1/0, while the other reference strains relate distantly (< 86% of similarity) with the other recovered isolates from blank salad samples. These results discard experimental contamination of the blank samples, pointing towards a contamination event in the producing industry.

Pulsotype 1 includes the majority of the assessed isolates (70%), all belonging to serogroup IVb. These isolates were all recovered from RTE chicken salad batches 3 and 5 (collected on March 7, 2018 and April 18, 2018, respectively). These results seem to indicate that pulsotype 1 isolates may represent a persistent contamination within the assessed food industry and might point out to a common source of contamination. However, as this pulsotype was only observed in two batches, it is difficult to conclude about a persistent contamination and more batches would have to be analyzed during a prolonged period of time. Nevertheless, it is important to highlight that this possible persistent contamination involves *L. monocytogenes* serogroup IVb isolates, commonly associated to human disease, present in a RTE chicken salad that will not undergo any heat-treatment prior to consumption (Maury et al., 2016; Montero et al., 2015).

Pulsotypes 3, 6 and 7, belonging to serogroups IIb, IIa and IVb respectively, display distinct profiles. Yu, & Jiang (2014) also found distinct profiles in approximately 30% of the studied PFGE profiles, when assessing *L. monocytogenes* isolates collected from retailed foods in Henan, China.

Nevertheless, a thorough sampling plan should be considered during a prolonged time frame, in order to conclude on the persistence of *L. monocytogenes* strains in the assessed food industry. For that

purpose, food related environment and raw materials should also be considered in the sampling scheme.

4.5. RT-qPCR

L. monocytogenes quantification presented distinct results when assessed by different methods (**Figure 18**). RT-qPCR results presented higher values of *L. monocytogenes* log cfu/g for all the assessed samples when compared to VCC results obtained by using ISO 11290-2:2017 quantification method.

However, two different scenarios were observed when comparing different types of samples.

No significant differences ($p > 0.05$) were found for RT-qPCR and VCC results when comparing inoculated samples with *L. monocytogenes* (4ºC IS, 12ºC IS and 16ºC IS). Considering these results, RT-qPCR coupled with PMA, to access bacterial viability, is a powerful approach. This approach allows for an easier, sensitive, specific and time-saving *L. monocytogenes* quantification. This is especially important when considering RTE foods due to their short commercial shelf-life (Agustí et al., 2018; Muhterem-Uyar et al., 2015; Postollec et al., 2011; Scariot et al., 2018).

Contrarily, when comparing quantification by both methods in blank samples (BS and BS-BPW), significant differences were found ($p < 0.05$) with an underestimation of *L. monocytogenes* quantification by traditional microbiological analysis. In most of the studied blank samples, the difference reached 3 log cfu/g, already considering the VCC method's lower limit. Similar results were reported by other authors when comparing both methods for quantification of bacteria in food and food processing environments (Scariot et al., 2018; Truchado et al., 2016). A few reasons for this underestimation were outlined. Firstly, the occurrence of VBNC bacteria in food is very common and, while RT-qPCR is able to detect VBNC bacteria, traditional VCC's methods lack the sensibility to do so. In a study by Truchado et al. (2016) with *Escherichia coli,* concentrations using PMA-qPCR assay yielded values around 1.4 orders of magnitude higher than that obtained by culture-based techniques. These authors concluded that VBNC organisms might have an important role in this discrepancy. In fact, in food, *L. monocytogenes* is often affected by one or several stresses caused by a variety of processing treatments, leading to a loss of bacterial cultivability while viability remains unaltered (Auvolat, & Besse, 2016; Brasseur et al., 2015). Another reason that might explain the obtained discrepancy of results might be due to the quantification of DNA from dead cells. Although the use of PMA had the purpose of distinguishing dead from viable cells, allowing only the quantification of these last ones, this procedure may not have been successful. In fact, discrepancies on the available literature regarding the most suitable PMA concentrations can hamper the selection of a PMA-qPCR method. Moreover, some studies demonstrated that viable and dead cell mixtures containing high density of dead cells might reduce the performance of the PMA treatment (Contreras et al., 2011; Elizaquível et al., 2012; Løvdal et al., 2011; Truchado et al., 2016). In this way, it is necessary to be cautious and critical when using PMA-qPCR. Simultaneously, when using solely traditional enumeration methods, false negative results might be expected, risking the presence on the market of food products contaminated with *L. monocytogenes*.

Moreover, the altered picture obtained by using only conventional methods, does not allow the FBO to really understand the level of the contamination, thereby not permitting the implementation of strategies able to eliminate the pathogen from the processing plant (Agustí et al., 2018; Alessandria et al., 2010; Postollec et al., 2011). Therefore, RT-qPCR should not be dissociated from other classical techniques, but rather regarded as a complementary tool. In the future, this promising technique may become a reliable and accurate method to be transferred from expert research to routine laboratories in the food industry (Auvolat, & Besse, 2016; Postollec et al., 2011).

4.6. Development of growth models for *L. monocytogenes*

Predictive models in this study were developed using the Baranyi and Roberts model (Baranyi, & Roberts, 1994) based on experimental data (**Figure 19**, **Figure 20** and **Figure 21**) and the predicted growth parameters are shown in **Table 12**. At 12ºC and 16ºC, R^2 (> 0.8) reveals a good fit of the experimental data to the primary model; however, at 4ºC, R^2 (0.512) indicated a poor fit to Baranyi's model. Nevertheless, the decision to adapt all experimental data to the same model, even with different temperatures, seemed to be more appropriate.

As storage temperature increased, the lag time (λ) in this study decreased, with values of 74.435 ± 48.466 hours (4°C) and of 56.139 ± 31.566 hours (12°C). The lag time was not evident at 16°C. In contrast, the maximum specific growth rate (μ_{max}) increased gradually as the storage temperature increased, with values of 0.021 ± 0.008, 0.052 ± 0.024, and 0.066 ± 0.009 log cfu/g/h at 4º, 12º, and 16ºC, respectively. Significant differences ($p < 0.05$) were detected in μ_{max} for the three different temperatures, using one-way ANOVA statistical analysis, revealing that temperature has influence on the pathogen's growth in these RTE salads. However, when applying Tukey's multiple comparison test, significant differences ($p < 0.05$) were only observed between 4º and 16ºC.

Similar observations under chilling conditions have been made with regard to fresh vegetables and mixed salads. Omac et al. (2018) reported that cold-adapted *L. monocytogenes* inoculated on fresh spinach leaves grew from an initial inoculation level of 2.33 log cfu/g to 4.54 log cfu/g at 16 days of storage at 5°C, with a μ_{max} of 0.024 log cfu/g/h, and a λ of 38.75 h. These authors also reported that for a temperature of 8ºC a final concentration of 5.85 log cfu/g of *Listeria monocytogenes* was registered with a μ_{max} of 0.037 log cfu/g/h and a λ of 17 h. In a *Listeria monocytogenes*' growth study on iceberg lettuce (Koseki, & Isobe, 2005), the authors obtained a μ_{max} of 0.021, 0.047 and 0.090 cfu/g and a λ of 60.1, 45.6 and 10.2 h, respectively, at 5º,10º and 15ºC. When studying the growth potential of *Listeria monocytogenes* in artificially contaminated chicken salad, Sahu et al (2016), obtained a μ_{max} of 0.013-0.016 log cfu/g/h during 30 days of storage at 5°C, a μ_{max} of 0.040-0.049 log cfu/g/h during 12 days of storage at 10°C, and a μ_{max} of 0.133-0.182 log cfu/g/h during 7 days of storage at 25°C. In a study modelling the growth kinetics of *Listeria monocytogenes* in pasta salads (containing cheese, chicken, smoked ham, vegetables, among others) at different storage temperatures, a μ_{max} of 0.054 was obtained, at 12ºC, and a μ_{max} of 0.010 at 4ºC (De Cesare et al., 2018).

With the μ_{max} obtained at 4ºC, it is possible to determine the maximum concentration of *L. monocytogenes* that may be present at the production stage in order to comply with the mandatory limit of 100 cfu/g at the end of shelf life, considering that the consumer respects the recommended commercial shelf life (144h – 6 days) and storage temperature. At 4ºC, the predicted μ_{max} using the primary model described by Baranyi and Roberts is 0.021 log cfu/g/h, corresponding to 0.504 log cfu/g/day, meaning that, by the end of the sixth day *L. monocytogenes* expected concentration would be of 3.024 log cfu/g. This means that the salad had to leave the industry with approximately -1 log cfu/g (approximately 0.1 cfu/g). With the obtained predicted μ_{max}, in order to comply with the mandatory limit of 100 cfu/g at the end of shelf life and allowing the product to leave the production stage with 1 log cfu/g, the shelf-life of these salads would have to be shortened to only 2 days, instead of the recommended 6 days.

The fitting of the secondary square-root-type model to the estimated μ_{max} at each of the tested isothermal conditions (**Figure 22**) resulted in the estimation of the theoretical minimum temperature that allows microbial growth (T_{min}). The estimated value of T_{min} for RTE chicken salad was -11.745°C, with R^2 = 0.993. However, the European Union Reference Laboratory for *Listeria monocytogenes* (EURL Lm, 2014), indicates -1.5ºC, as minimum growth temperature for *L. monocytogenes*. Such difference may relay on the fact that this value was based on research carried out primarily in lab media under optimum conditions and may vary depending on the strain and food matrix. In fact, in other studies, different minimal growth temperatures for *L. monocytogenes* were determined, for different food matrixes (**Table 14**).

Table 14. Minimal growth temperatures for *L. monocytogenes* for different food matrixes.

Reference	Food matrix	T_{min} (ºC)
(Lianou et al., 2017)	Vanilla cream pudding	-2.54ºC
(Lianou et al., 2017)	Vanilla cream pudding with cinnamon extract	-0.39ºC
(Li et al., 2016)	Salmon roe	-0.5ºC
(Wanget al., 2013)	White cabbage	-2.02ºC
(Posada-Izquierdo et al., 2013)	Fresh-cut leafy green vegetables	-4.26ºC
(Sant'Ana et al., 2012)	Ready-to-eat lettuce	-1.96ºC

Moreover, it is noteworthy that the model's parameter T_{min} is the intercept of the function with the temperature axis when $\sqrt{\mu_{\max}}$=0. It represents only theoretically the minimum temperature for bacterial growth o and it has been suggested that the estimated T_{min} value can be considerably lower (i.e. 5 to 10 °C) than the lowest temperature at which microbial growth is actually observed (Ross, & Dalgaard, 2004).

4.7. Quantitative microbial risk assessment

To evaluate the potential risk associated with this particularly RTE chicken salad in study, experimental data, predictive microbiology, consumption data and statistical data was combined with probabilistic modelling (Monte Carlo simulations) to estimate the annual number of listeriosis in the population, considering its diversity in terms of health condition (**Figure 24**).

This study had some limitations that need to be considered. First, the number of blank uninoculated samples analyzed (n=27) was relatively small, and the data may not reflect the overall contamination rate of *L. monocytogenes* in these salads. Second, the enumeration was not registered, and the levels had to be assumed equal to the concentration found by Gombas et al. (2003) for bagged pre-cut-leafy salads. And finally, due to lack of statistical data, the number of portions (Np) consumed in a year were assumed to be equal to the produced by the food industry every year.

The average final contamination level of *L. monocytogenes* on RTE chicken salads at the time of consumption was 1.142 log cfu/g **(Figure 23)**, which is lower than the mandatory limit of 100 cfu/g (2 log cfu/g) at the end of shelf life, however with a large CI of 95%: from -0.985 to 3.694 log cfu/g, and with a maximum level of 5.996 log cfu/g.

As shown in **Table 13**, based on Monte Carlo simulations, the average number of listeriosis cases per year linked to the consumption of these RTE chicken salads was 1.213×10^{-3} (CI 95%: 2.538×10^{-4} - 2.925×10^{-3}). Moreover, when comparing the estimated number of listeriosis cases in low- and high-risk subpopulations (represented by both peaks in **Figure 24**), it is possible to observe that high risk population has a risk of listeriosis of four times higher. Thus, particular preventive tips should be given to high risk population.

In Portugal in 2016, a total, 32 cases of listeriosis were reported, with the highest rates detected in infants below one year of age (2.34 per 100000 population), people between 45 and 64 years of age (0.52 per 100 000 population) and elderly people over 65 years of age (0.61 per 100 000 population) (EFSA & ECDC, 2017). The average number of cases of listeriosis per year linked to the consumption of these RTE chicken salads is low when compared with the total number of cases in Portugal in 2016, which can be linked to the fact that infants and elderly people, with high notification rates are not the typical consumers of these types of salads (and by that reason were not considered high risk population in the study).

Moreover, even the number of cases of listeriosis attributed to high-risk groups (especially immunocompromised patients) may be overestimated, since most of these people are often advised to avoid the consumption of these type of products, exactly because of the potential risk that they represent to health (CDC, 2018; Chau et al., 2017).

The sensitivity analysis (**Figure 25**) allowed to determine that household' storage temperature and duration of storage are the most influential factors for the risk of listeriosis derived from the consumption of RTE chicken salads. Thus, consumers should be educated and informed about good conservation practices, and provided sufficient information regarding risk issues, being this a responsibility shared by food suppliers, educators and governments. Similar results in terms of the most influential factors for

the risk of listeriosis were obtained by Ding et al. (2013), Sant'Ana et al. (2014) and Tromp et al. (2010), when assessing the risk of listeriosis derived from the consumption of leafy greens intended to be eaten raw.

Initial *L. monocytogenes* concentration also had an average contribution to the risk of listeriosis, thus an intervention to be applied would be the improvement of hygienic practices during RTE chicken salads production.

Although microbiological risk assessment in Portugal for RTE food are rare, there are some available QMRA models for the risk of *L. monocytogenes* in RTE food products in other countries. Franz et al. (2010), assessed the risk of *Escherichia coli 0157, Salmonella*, and *L. monocytogenes* in leafy green vegetables consumed at salad bars in Netherlands. Based on first-order Monte Carlo simulations, the average number of cases per year linked to the consumption of leafy greens at salad bars was 166, 187, and 0.3 for *Escherichia coli 0157:H7, Salmonella,* and *L. monocytogenes,* respectively.

Carrasco et al. (2010) determined the risk of *L. monocytogenes* in ready-to-eat lettuce salads from farm to table, in Spain. In this study, Monte Carlo simulations of the model were run to estimate the number of cases in low-risk and high-risk populations. The estimated number of listeriosis cases was 0.04 and 244 in low- and high-risk subpopulations, respectively. Moreover, according to this study, modified atmosphere packaging was a very effective method to decrease the number of cases.

Ding et al. (2013) determined the risk of *L. monocytogenes* on lettuce from farm to table in Korea. The authors of this study showed that the final contamination levels of *L. monocytogenes* at restaurant and household level were -1.50 log cfu/g and -0.146 log cfu/g, respectively. The average number of annual listeriosis cases estimated by the quantitative risk assessment model ranged from 559 to 817, which means the incidence of listeriosis ranged from 11.9 to 17.4 cases per million person.

5. Conclusion and future perspectives

Listeria monocytogenes is a ubiquitous, psychrotrophic bacteria, known as the causative agent of human listeriosis, an important foodborne disease with a high fatality rate particularly in high-risk population such as the new-born infants, pregnant woman, elderly and immunocompromised patients. Although continuously improved applied control measures have been applied and studied, an increasing trend of listeriosis has been registered.

This study examined the growth of cold adapted 3-mixed strains of *L. monocytogenes* artificially inoculated in RTE chicken salads stored at refrigerated temperatures (4ºC, 12ºC and 16ºC), for 8 days, through a challenge test. The pH and a_w results confirmed this study's RTE chicken salad as a food product able to support the growth of *L. monocytogenes*. Also, the results highlighted that temperature influenced the growth of *L. monocytogenes* in these RTE chicken salads. This work underlined the importance of strict temperature control from processing to consumption. Refrigerated temperatures must be maintained during transportation, distribution, storage or handling in supermarkets and by consumers, however, it is noteworthy to emphasize that results showed that *L. monocytogenes* will still be able to grow at low refrigeration temperatures and reach unsafe concentrations, if there is storage time abuse.

Experimental data on *L. monocytogenes*' growth at different temperatures in this study, was used to develop predictive growth models, providing insights into predictive microbiology, which may be applied by the food industry and regulatory agencies to estimate the growth of *L. monocytogenes* in similar types of RTE foods, with comparable pH, shelf life and storage conditions. This can provide a fast and cost-effective alternative to laboratory studies to estimate the effects of storage temperature on *L. monocytogene*s behavior on similar food products.

During the challenge test, 30 presumptive *L. monocytogenes* isolates were detected on blank samples, on ALOA plates. The confirmation of only 10 isolates (33.3%) as *L. monocytogenes* by a multiplex PCR, showed that ISO 11290-2:2017 was not very specific. From the 10 confirmed positive isolates of *L. monocytogenes*, three different serogroups were detected: IVb (80%), IIa (10%) and IIb (10%), with the predominant serogroup, IVb, the most implicated in human disease and associated to the majority of clinical strains causing severe human infections. Moreover, PFGE results indicate that some of these isolates from serogroup IVb might represent a persistent contamination within the assessed food industry and might point out to a common source of contamination. The results were not surprising, as the industrial unit has a suspicion of *L. monocytogenes* persistent environmental contamination.

Considering the results obtained for RT-qPCR coupled with PMA, it can be concluded that it is a powerful approach, which allows for an easier, sensitive, specific and time-saving *L. monocytogenes* quantification. Nonetheless, due to high discrepancies when comparing the levels of *L. monocytogenes* on blank samples with the classical method (ISO 11290-2:2017), optimization and validation of the developed PMA-qPCR are essential before its application as a routine tool in microbial sampling programs, in food industry.

The quantitative microbial risk assessment performed estimated an average number of listeriosis cases per year linked to the consumption of these RTE chicken salads of 1.213×10^{-3}. The sensitive analysis for the risk of listeriosis per dose indicates the need for strict adherence to time-temperature recommendations, as these were determining factors to the increase of the risk of listeriosis linked to the consumption of these RTE salads. For this reason, it is important to highlight that the best risk management of listeriosis is to improve food safety educational programs. These formative campaigns should inform consumers on how to store RTE products, by controlling refrigerator temperature and reducing time of storage. Due to the rare development of microbiological risk assessment in Portugal, this study may contribute to a better understanding and prediction of listeriosis cases by consumption of contaminated RTE products, and subsequently improve risk management and strengthen food control. In this case, the goal was focused into using more realistic data, which at the same time was important for identifying the gaps or limited information needed for this type of analysis. The quantitative risk assessment model developed in this study might be further improved, however, for this and other studies, it implies a further investment in collecting scientific information.

In future works, in terms of optimizations, a broader range of temperatures could be assessed. Temperatures other than sub-optimal ones for the organism are important to improve predictive models and, consequently, improving risk assessment. Also, growth data during non-isothermal storage conditions (dynamic temperature models) should be done in order to validate the developed models.

Furthermore, it would be interesting, in the future, to make an extended characterization of the collection of *L. monocytogenes* isolates found on RTE chicken salads blank samples, given the fact that these isolates may represent a persistent contamination in a food industry. Such characterization could include a study on their biofilm formation, resistance to industrial disinfectants and to the most common antibiotics used in listeriosis therapy. Furthermore, besides serogrouping, other methods of subtyping could be done, such as serotyping, multiple locus variable-number tandem repeat analysis (MLVA) and whole genome sequencing (WGS).

A thorough sampling plan should be considered during a prolonged time frame, in order to conclude not only on the persistence of *L. monocytogenes* strains in the assessed food industry, but also the source of contamination. For that purpose, food related environment and raw materials should also be considered in the sampling scheme. Moreover, this sampling plan could provide information about the contamination/prevalence status of the producing industry, contributing to fill the gaps in the QMRA, regarding prevalence and concentration of *L. monocytogenes* in these salads.

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Annex I. Chicken salad's technical specification.

FICHA TÉCNICA DE PRODUTO ACABADO
"SALADA DE FRANGO"

FT.10510.04
Data: 2016/08/01

Denominação legal:

Salada com mistura de vegetais, frango, queijo italiano, bacon.

Lista de ingredientes:

Ingredientes: Mistura de Vegetais (44,1%) (Alface iceberg, Lollo Rosso, Apollo Green e Cenoura ripada, em proporções variáveis), Peito de Frango (30,2%) (carne de peito de frango, óleo de girassol, fécula de batata, aroma natural, estabilizador (E407a), sal), Queijo Caesar (8,2%) (**LEITE**) (queijo duro, lisozima de OVO) e Bacon (8,2%) (entremeada de porco, água, sal, antioxidante (E301) e conservante (E250)).

PODE CONTER VESTÍGIOS DE CRUSTÁCEOS, PEIXE, AMENDOIM, SOJA, FRUTOS DE CASCA RIJA, AIPO, MOSTARDA, SEMENTES DE SÉSAMO, SULFITOS, TREMOÇO, MOLUSCOS.

Alergénios:

Alergénios:	Sim	Não	Pode Conter Vestígios
Glúten	☐	☒	☐
Crustáceos	☐	☐	☒
Ovo	☒	☐	☐
Peixe	☐	☐	☒
Amendoim	☐	☐	☒
Soja	☐	☐	☒
Leite	☒	☐	☐
Frutos de casca rija	☐	☐	☒
Aipo	☐	☐	☒
Mostarda	☐	☐	☒
Sementes de sésamo	☐	☐	☒
Sulfitos	☐	☐	☒
Tremoço	☐	☐	☒
Moluscos	☐	☐	☒

Prazo de validade:

Consumir até:
Dia de produção + 6 dias.

Lote:

Lote: aaddd.

Condições de conservação:

Conservar entre 1°C e 4°C.

Peso líquido:

Peso Líquido: 165g ℮

Características nutricionais:

DECLARAÇAO NUTRICIONAL – valores médios	Por 100g
Energia	552 kJ / 132 kcal
Lípidos	6g
dos quais saturados	3,4g
Hidratos Carbono	2g
dos quais açúcares	0,1g
Fibra	1,2g
Proteínas	16g
Sal	0,6g

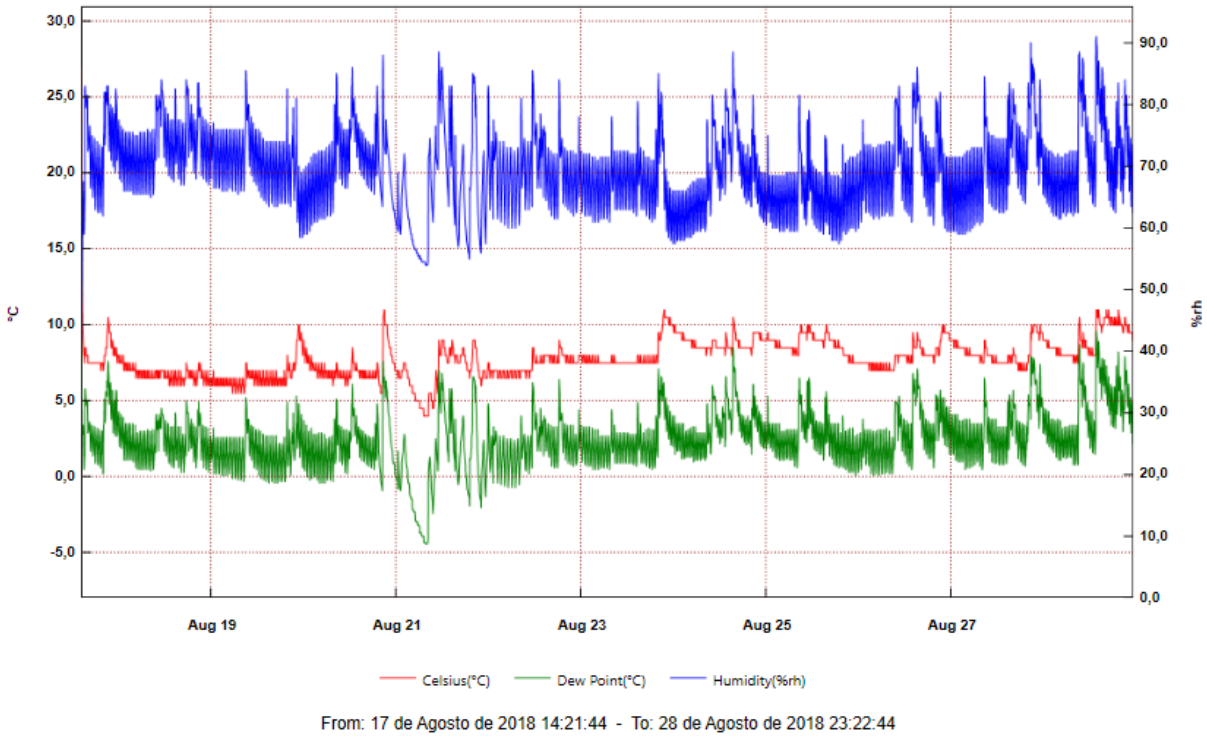
Instruções de utilização:

Temperar a gosto e esta pronto a consumir.

Annex II. Salad samples date of collection in the producing industry of each isolate code and description. Assays 1, 2 and 3 correspond to tested temperature of 4ºC, assays 4, 5 and 6 correspond to 12ºC, and assays 7, 8 and 9 correspond to 16ºC.

Salad samples date of collection in the producing industry (or batch production date)	**Isolate code**	**Isolate collection description**
February 14, 2018	CS1/0	Detection on BS: assay 1, 0 h, dilution 10^{-2}
February 14, 2018	CS1/8	Detection on BS: assay 1, 192 h, dilution 10^{-1}
February 27, 2018	CS2/0-A	Detection on BS: assay 2, 0 h, dilution 10^{-1}
February 27, 2018	CS2/0-B	Detection on BS: assay 2, 0 h, dilution 10^{-2}
March 7, 2018	CS3/0-A	Detection on BS: assay 3, 0 h, dilution 10^{-1}
March 7, 2018	CS3/0-B	Detection on BS: assay 3, 0 h, dilution 10^{-2}
March 7, 2018	CS3/4-A1	Detection on BS: assay 3, 96 h, dilution 10^{-1}
March 7, 2018	CS3/4-A2	Detection on BS: assay 3, 96 h, dilution 10^{-1}
March 7, 2018	CS3/4-A3	Detection on BS: assay 3, 96 h, dilution 10^{-1}
March 7, 2018	CS3/4-B	Detection on BS: assay 3, 96 h, dilution 10^{-2}
March, 13 2018	CS4/0-A	Detection on BS: assay 4, 0 h, dilution 10^{-1}
March, 13 2018	CS4/0-B	Detection on BS: assay 4, 0 h, dilution 10^{-2}
April 18, 2018	CS5/0-A	Detection on BS: assay 5, 0 h, dilution 10^{-1}
April 18, 2018	CS5/0-B	Detection on BS: assay 5, 0 h, dilution 10^{-2}
April 18, 2018	CS5/4-A	Detection on BS: assay 5, 96 h, dilution 10^{-1}
April 18, 2018	CS5/4-B	Detection on BS: assay 5, 96 h, dilution 10^{-2}
April 30, 2018	CS6/0	Detection on BS: assay 6, 0 h, dilution 10^{-2}
May 10, 2018	CS7/0-A	Detection on BS: assay 7, 0 h, dilution 10^{-1}
May 10, 2018	CS7/0-B	Detection on BS: assay 7, 0 h, dilution 10^{-2}
May 10, 2018	CS7/8-A1	Detection on BS: assay 7, 192 h, dilution 10^{-1}
May 10, 2018	CS7/8-A2	Detection on BS: assay 7, 192 h, dilution 10^{-1}
May 10, 2018	CS7/8-B	Detection on BS: assay 7, 192 h, dilution 10^{-2}
May 21, 2018	CS8/0 - A	Detection on BS: assay 8, 0 h, dilution 10^{-1}
May 21, 2018	CS8/0 -B	Detection on BS: assay 8, 0 h, dilution 10^{-2}
May 21, 2018	CS8/8-A1	Detection on BS: assay 8, 192 h, dilution 10^{-2}
May 21, 2018	CS8/8-A2	Detection on BS: assay 8, 192 h, dilution 10^{-2}
May 25, 2018	CS9/0-A1	Detection on BS: assay 9, 0 h, dilution 10^{-2}
May 25, 2018	CS9/0-A2	Detection on BS: assay 9, 0 h, dilution 10^{-2}
May 25, 2018	CS9/8-A	Detection on BS: assay 9, 192 h, dilution 10^{-1}
May 25, 2018	CS9/8-B	Detection on BS: assay 9, 192 h, dilution 10^{-2}

Annex II. Example of an output of the data from households' refrigerators obtained with EL-USB-2 data-loggers (Lascar Electronics, Whiteparish, United Kingdom).



Annex III. DNA standard curve of *Listeria monocytogenes*. Standard curve obtained with cycle threshold (Ct) plotted against the logarithmic concentration of the serial dilutions.