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**Final Degree Project**

# *Listeria monocytogenes* in ready-to-eat cooked meat products: data and tools to assess its growth

Cristina Serra Castelló

**Degree in Biotechnology**

**Directors:** Dra. Sara Bover Cid and Dra. Anna Jofré Fradera
(Programa de Seguretat Alimentària, IRTA-Monells)
**Tutors:** Anna M. Dalmau Roda (UVic-UCC)

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# Resum

**Títol:** *Listeria monocytogenes* en productes carnis llestos per consumir: dades i eines per avaluar el seu creixement.

**Paraules clau:** Microbiologia predictiva, *Listeria monocytogenes,* productes carnis llestos per consumir, "PMP", "ComBase", "FSSP", taxa de creixement, seguretat alimentària.

**Autora:** Cristina Serra Castelló

**Tutores:** Dra. Sara Bover Cid, Dra. Anna Jofré Fradera i Anna M. Dalmau Roda

**Data:** Gener, 2016.

Aquest document descriu el treball de final de grau desenvolupat en el marc del Programa de Seguretat Alimentària de l'IRTA (Monells). L'objectiu principal d'aquest projecte va ser avaluar i comparar l'ús de diferents tipus de dades i eines per avaluar la capacitat de creixement de *L. monocytogenes* en productes carnis cuits llestos per al consum.

Aquest treball de final de grau descriu els resultats de l'anàlisi estadística de factors fisicoquímics de productes carnis llestos per al consum i de les taxes de creixement de *L. monocytogenes* obtingudes a partir de l'ús de models predictius del comportament microbià i a partir de bases de dades bibliogràfiques que contenen resultats de "challenge tests" en aquests tipus de productes. S'han utilitzat tres programes diferents amb models predictius ("PMP", "ComBase Predictor" i "FSSP") per obtenir la taxa de creixement de *L. monocytogenes* en 47 productes caracteritzats, utilitzant els resultats de la determinació analítica dels factors fisicoquímics com a "inputs" del model. S'han obtingut les taxes de creixement de *L. monocytogenes* dels "challenge tests" disponibles a les bases de dades bibliogràfiques, a partir de "ComBase Browser" (n=659) i dels articles científics descarregats de la plataforma "Science Direct" (n=204). Els resultats de l'anàlisi estadística dels factors fisicoquímics de productes comercials carnis llestos per al consum van indicar que el pH, l'àcid acètic i l'àcid làctic són factors fisicoquímics que afecten significativament el creixement de *L. monocytogenes*. També van suggerir que FSSP és el model predictiu que prediu unes taxes de creixement del bacteri més acurades, ja que és el model que té en compte un nombre més elevat de factors ambientals com a "inputs". Quan es comparen les taxes de creixement obtingudes amb els models predictius amb aquelles obtingudes a partir de bases de dades bibliogràfiques, s'ha de tenir en compte la quantitat de cada factor fisicoquímic en el producte així com la temperatura d'emmagatzematge, ja que són factors que afecten significativament el creixement de *L. monocytogenes*.

La darrera conclusió derivada de l'estudi és que els models predictius són una eina molt útil ja que permet a l'usuari (p.e. indústria alimentaria) predir la vida útil segura del producte, alternant "inputs" (factors fisicoquímics) del model.

# Summary

**Title:** *Listeria monocytogenes* in ready-to-eat cooked meat products: data and tools to assess its growth.

**Keywords:** Predictive microbiology, *Listeria monocytogenes,* ready-to-eat cooked meat products, PMP, ComBase, FSSP, growth rate, food safety.

**Author:** Cristina Serra Castelló

**Tutors:** Dr. Sara Bover Cid, Dr. Anna Jofré Fradera and Dr. Anna M. Dalmau Roda

**Date:** January, 2016.

The present document describes the work carried out within the framework of the final degree project, which has been performed at the Food Safety Program of the IRTA (Monells). The main objective of this project was to evaluate and compare the use of different type of data and tools to assess the growth capability of *L. monocytogenes* in RTE cooked meat products.

This final degree project describes the results of the statistical analysis of the physicochemical factors of ready-to-eat cooked meat products and of the *L. monocytogenes* growth rates obtained with microbiological predictive models and bibliographic databases that contain results from challenge tests in that type of products.

Three different software have been used for obtaining *L. monocytogenes* growth rates, from 47 characterized products, with microbiological predictive models: PMP, ComBase Predictor and FSSP using the analytical determination results of physicochemical factors as model inputs. *L. monocytogenes* growth rates from challenge test experiments available at bibliographic databases has been obtained from ComBase Browser (n=659) and scientific articles downloaded from the Science Direct portal (n=204). Results from the statistical analysis of the physicochemical factors of commercial cooked meat products indicate that pH, acetic and lactic acids are physicochemical factors that significantly affect *L. monocytogenes* growth. They also suggest, that FSSP is the predictive model which provides the most accurate *L. monocytogenes* growth rates as it is the most complex model taking into account a high number of environmental factors as inputs. When comparing growth rates obtained with predictive models from those obtained with bibliographic databases, it has to be taken into account the amount of the physicochemical factors in products as well as the temperature storage, as they are factors that significantly affect *L. monocytogenes* growth rates.

Last conclusion derived from the study is that microbiological predictive models are a useful tool due to they allow to user (e.g. food industry) to predict the safe shelf-life of product by toggling inputs (physicochemical factors) of the model.

# 1. Introduction

In this final degree project, it is presented a non-experimental study, aimed at assessing the growth behaviour of *L. monocytogenes* in ready to eat cooked meat products. Different methodological approaches were used, all of them being procedures included in the EU regulation 2073/2005 to evaluate the microbiological safety of food, that is the use of microbiological predictive models and results of challenge tests from bibliographic databases, in order to determinate the safe shelf-life of this type of products.

The election of this theme is due to the high risk of listeriosis associated with ready to eat cooked meat products. Therefore, the need to estimate from a quantitative point of view the safe shelf life of this type of products, mainly determined by the growth rate of *L. monocytogenes*.

The main objective of the present work was to evaluate and compare the use of different type of data and tools to assess the growth capability of *L. monocytogenes* in RTE cooked meat products.

This final degree project described in this report is structured in 5 main sections.

First section, to start with, is a background about *L. monocytogenes* and listeriosis. It is also detailed, how *L. monocytogenes* grows in food products and why ready-to-eat cooked meat products have a higher risk to be associated with listeriosis outbreaks. It is also exposed the existent regulation on the microbiological criteria allowed for *L. monocytogenes* in ready-to-eat cooked meat products. Lastly, within background, the different types of studies that can be carried out to assess the risk of *L. monocytogenes* are described.

In second section, it is exposed the main objective of the final degree project and which specific objectives are proposed to achieve the main objective.

The third section, includes the methodology used and which procedure has been followed when working with it.

In fourth section, is focused on the description and discussion of the results obtained, including the statistical study of the results.

Finally, in fifth section, it is presented the conclusions derived from the analysis done in previous sections.

# 2. Background

## 2.1. *Listeria monocytogenes* and listeriosis.

*Listeria* consist of short (0.5 µm in diameter by 1 to 2 µm long) Gram-positive, non-spore-forming rods. *Listeria monocytogenes* is one of six species are currently recognized within the genus and it is the responsible for listeriosis. It is a facultative anaerobic bacterium, capable of surviving in the presence or absence of oxygen. It can grow and reproduce inside the host cells and is one of the most virulent foodborne pathogens, with 20 to 30% of clinical infections resulting in death (Lomonaco, Nucera, & Filipello, 2015). It can be isolated from numerous species of domestic and wild animals, as well as from soil, silage, and other environmental sources.

Although rare when compared to many other foodborne diseases, listeriosis often leads to severe consequences, particularly in susceptible populations groups (elderly, fetuses, neonates, and immunocompromised individuals) (Lomonaco et al., 2015). A person with listeriosis usually has fever and muscles aches, often preceded by diarrhea or other gastrointestinal symptoms.

Centers for Disease Control and Prevention (CDC) have estimated that approximately 2500 cases of listeriosis occur annually in United States. The overall annual incidence of listeriosis in the United States has been estimated to range from 3.4 per million to 4.4 per million. The incidence of listeriosis reported from other countries vary substantially, for example in 2013, the EU notification rate was 0.44 cases per 100,000 population which was an 8.6% increase compared with 2012. The highest specific notification rates have been observed in Finland, Spain, Sweden and Denmark (1.12, 1.00, 0.97 and 0.91 cases per 100,000 population, respectively) (European Food Safety Authority (EFSA), 2006).

## 2.2. Ecological characteristics of *Listeria monocytogenes* in food products.

The growth and survival of *L. monocytogenes* is influenced by a variety of factors. In food these include extrinsic factors as temperature and $CO_2$ (packaging atmosphere), and intrinsic factors as pH, water activity, salt and preservatives.

*L. monocytogenes* can grow under both anaerobic and aerobic conditions, although it grows better in an anaerobic environment.

The temperature range for its growth is between -1.5 and 45 °C, being 30-37°C the optimal temperature. Freezing can also lead to a reduction in *L. monocytogenes* numbers (Lou & Yousef,

1999). It is important to take into account that *L. monocytogenes* can grow at temperatures as low as 0°C and thus it has the potential to growth in food during refrigerated storage.

This bacteria can grow in a broad pH range of 4.0 – 9.6. Although growth at pH < 4.0 has not been documented, *L. monocytogenes* seems to be relatively tolerant (i.e. survive without growing) to acidic conditions but its sensitivity to acidic conditions increases at higher temperatures (Lou & Yousef, 1999).

The type of acid used to acidify the material and the storage temperature have a marked effect on the ability of *Listeria* to survive and grow at low pH. Week organic acids, such as citric, acetic, and lactic acids seem to have antibacterial properties, which are related not only to pH but also to their degree of dissociation, with the un-dissociated form being the inhibitoriest.

The effect of preservatives on the growth of *L. monocytogenes* is influenced by the combined effects of temperature, pH, salt concentration and water activity (i.e. hurdle technology defined by Leistner).

Like most bacterial species, *L. monocytogenes* grows optimally at a water activity of 0.97 (Farber & Peterkin, 1991). *L. monocytogenes* can survive for extended periods of time at a water activity value of 0.81. It is reasonably tolerant to salt and has been reported to grow in 13-14% sodium chloride (Farber & Peterkin, 1991). Survival in the presence of salt is influenced by the storage temperature. Studies have indicated that in concentrated salt solutions, the survival rate of *L. monocytogenes* is higher when the temperature is lower (Lou & Yousef, 1999).

## 2.3. Scientific Assessment and Management (decision-making) of the Microbiological Risk associated with *L. monocytogenes*.

A series of illness outbreaks associated with the consumption of coleslaw, pasteurized milk, and fresh soft cheese in the early 1980s led to the recognition of *Listeria monocytogenes* as a foodborne pathogen. In 1991, NACMCF (National Advisory Committee on Microbiological Criteria for Foods) recommended control strategies to minimize the presence, survival, and multiplication of *Listeria monocytogenes* in foods. These control strategies included the development of an effective surveillance system for listeriosis, targeted efforts on specific foods, and the use of HACCP-based (Hazard Analysis and Critical Control Points) programs to ensure the safety of foods from processing o consumption (Jofré & Garriga, 2011).

During the 1990s, due to the implementation of these control strategies, the incidence of listeriosis was reduced up to 50% approximately. However, further reduction in illness are increasingly difficult. Several barriers to its control include that the microorganism is commonly found in the environment, including food processing, distribution, and retail environments, in foods and in the home. It primarily affects a small segment of the population that has heightened susceptibility. It can grow slowly in many foods during refrigerated storage. It is more resistant than most bacteria to the conditions and treatments used to control foodborne pathogens.

*Listeria monocytogenes* risk assessment was initiated as an evaluation tool in order to achieve the reduction of the incidence of infection to a level of 0.25 cases per 100,000 people.

The structure of risk assessment consists of four components (Richard Whiting, 2003):

- Hazard identification: identification of known or potential health effects associated with a particular biological, chemical or physical agent.
- Exposure assessment: qualitative and/or quantitative evaluation of the degree of intake likely to occur.
- Hazard characterization: qualitative and/or quantitative evaluation of the nature of the adverse effects associated with biological, chemical, and physical agents that may be present in food (Dose-response relationship).
- Risk characterization: integration of hazard identification, hazard characterization and exposure assessment into an estimation of the adverse effects likely to occur in a given subpopulation, including attendant uncertainties.

Based on the known characteristics of this microorganism and the disease, FDA maintains a policy of “zero-tolerance” for *Listeria monocytogenes* in ready to eat foods. This means that in 25 gram’s sample of a food it has to be absence of *Listeria monocytogenes*. Most of European countries have different policies for dealing with *Listeria monocytogenes* contamination. Some of them, have a “non-zero tolerance” for some classes of food, but in all cases, no sample can exceed 100 organisms of *Listeria monocytogenes* per gram at time of consumption.

EU legislation (Regulation 2073/2005) established microbiological criteria for RTE food product categories depending on their capability to enable or not *L. monocytogenes* growth (Table 1).

**Table 1**. Food safety criterion for *L. monocytogenes* in RTE cooked meat products.

| RTE food category | Limits | Phase where the criterion is applied |
|---|---|---|
| **RTE products intended for infants and for special medical purposes[(1)]** | Absence in 25 g of sample | Products placed on the market during their shelf-life |
| **RTE products that are able to support the growth of the *L. monocytogenes* and that are not intended for infants and for medical purposes** | 100 cfu/g[(2)] | Products placed on the market during their shelf-life |
| | Absence in 25 g of sample[(3)] | Before the food has left the immediate control of the food business operator, who has produce it |
| **RTE products that are not able to support the growth of the *L. monocytogenes* and that are not intended for infants and for medical purposes[(1)(4)]** | 100 cfu/g | Products placed on the market during their shelf-life |

(1) In normal circumstances, regular testing related to this criterion is not required for ready-to-eat foods which have received heat treatment or other effective process to eliminate *L. monocytogenes*, when recontamination after this treatment is not possible.

(2) This criterion is applied if the producer can demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit of 100 cfu/g throughout its shelf-life.

(3) This criterion is applied to products before leaving the immediate control of the food business operator when it cannot be demonstrated, to the satisfaction of the competent authority, that the product will not exceed the limit of 100 cfu/g during its shelf-life.

(4) Automatically, products with a pH ≤ 4.4 or $a_w$ ≤ 0.92, those with a pH ≤ 5.0 and $a_w$ ≤ 0.94, and products with a shelf-life lower than 5 days, belong to this category. Other food categories also can belong to this category, when it is scientifically justified.

### 2.3.1. Products that can promote the growth of *Listeria monocytogenes.*

The infectious dose of the pathogen has been reported to be high, particularly compared with other foodborne pathogens. However, it may depends on the type of food product, strain virulence, and especially on the host susceptibility; therefore, relative low doses may still cause infection in high risk populations.

Depending on the type of the product (raw or processed) and its intended use (e.g. requires further heating or is ready to eat), the stage in the food chain where contamination occurs may be associated with the level of the organism to which consumers will be exposed. Epidemiological data indicate that certain food products are more likely to be associated with listeriosis outbreaks than others (Figure 1); such foods are products consisting of raw ingredients or that are eaten raw, products not subjected to listericidal process, and products susceptible to post-lethality contamination that have an extended shelf-life under refrigeration and allow

pathogen growth, particularly when intended to be consumed without further cooking by vulnerable populations (Hereu, 2014). The demands of modern life and modern eating habits promote development and availability of convenience products, i.e. products that require little or no preparation before being consumed (RTE products), with longer shelf-life and minimally processed. From a legal standpoint, a ready to eat product is defined according to European Commission 2073/2005, as a food destined for direct human consumption without the need to apply it cooking or any other effective processing to eliminate or reduce dangerous microorganisms to an acceptable level (Hereu, 2014).

The risk very high (Figure 1) includes two food categories, deli meats and frankfurters, not reheated. Deli meats are also known as lunch meats, cold cuts, luncheon meats, sandwich meats, sliced meats, cooked meats and cold meats (for instance cooked ham, mortadella…).

These are food categories that have a high predicted relative risk rankings on both a per serving and per annum basis, reflecting the fact that they have relatively high rates of contamination, support the relatively rapid growth of *Listeria monocytogenes* under refrigerated storage, are stored for extended periods of time, and also are consumed extensively. These products have also been directly linked to outbreaks of listeriosis.

This risk designation is one that is consistent with the need for immediate attention in relation to the national goal for reducing the incidence of foodborne listeriosis. Likely activities include the development of new control strategies and/or consumer education programs suitable for these products (Richard Whiting, 2003).

The development of such products is a challenge for microbiological safety because it requires the implementation of post-processing operations such as chopping, dosage and packaging among others, which posed a real cross-contamination risk for spoilage microorganisms and pathogens (such as *L. monocytogenes*) from the environment, tools and manipulators.

The following sections deal with bacterial growth and, in particular, *L. monocytogenes* growth, as it is one of the most relevant factors determining the risk of listeriosis in a contaminated food product.

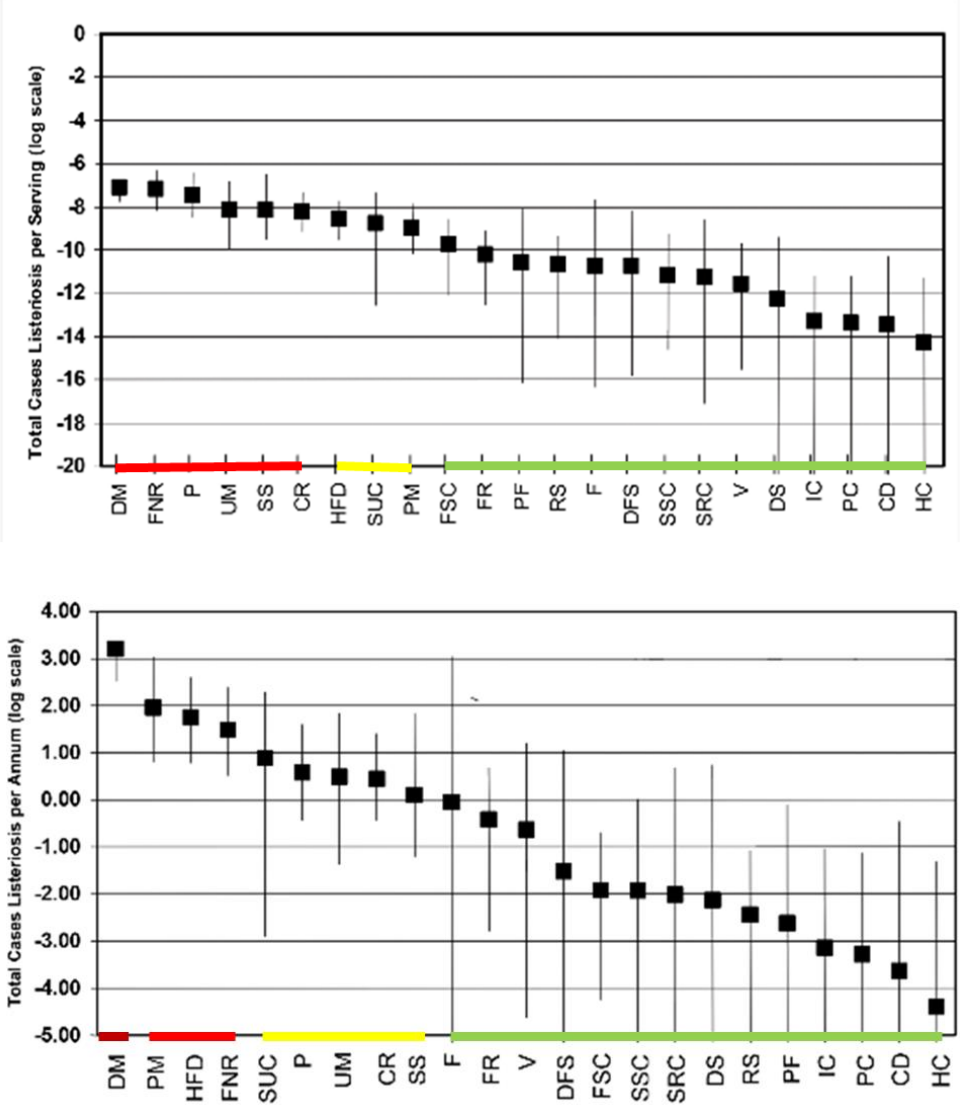


**Figure 1**. **A)** Predicted causes of listeriosis associated with food categories for the total U.S. population on a per serving basis. **B)** Predicted causes of listeriosis associated with a food categories for the total U.S. population on a per annum basis. The box indicates the median predicted number of listeriosis and the bar indicates the lower and upper bounds. DM, deli meats; PM, pasteurized fluid milk; HFD, high fat and other dairy products; FNR, frankfurters (not reheated); SUC, soft unripened cheese; P, pâté and meat spreads; CR, cooked ready-to-eat crustaceans; UM, unpasteurized fluid milk; SS, smoked seafood; F, fruits; FR, frankfurters (reheated); V, vegetables; DFS, fry and semidry fermented sausages, FSC, fresh soft cheese; SSC, semisoft cheese; SRC, soft ripened cheese; DS, deli-type salads; RS, raw seafood; PF, preserved fish; IC, ice cream and frozen dairy products; PC, processed cheese; CD, cultured milk products; HC, hard cheese. High risk products are indicated in red, moderate risk products in yellow and low risk products in green. Modified from (Richard Whiting, 2003).

### 2.3.2. Studies to investigate compliance with microbiological criteria along the all products shelf-life.

These studies are part of the exposure assessment. Its purpose is to quantitative determine the growth or survival of bacteria under different conditions and thus, to determine the product's safe shelf life (Ce, 2005). It is important to take into account that the growth curve of *L. monocytogenes* includes three phases: latency, exponential growth and stationary phase (Monod, 1949), which can be describe by the kinetic parameters λ, $\mu_{max}$ and $N_{max}$, respectively (Figure 2).

Latency phase or lag time (λ) is characterized for being the phase where the microorganisms adapt themselves in environment without being duplicated. The duration of this phase is determined by the physiological state of microorganisms, i.e. the previous history, as well as environmental conditions where they are (Ross, Dalgaard, & Tienungoon, 2000).

During the exponential growth phase, microorganisms are duplicated at a constant rate, which is called the specific growth rate ($\mu_{max}$), and it is determined by environmental conditions (e.g. temperature, pH, etc…) but not for the previous physiological state of microorganism.

Stationary phase arrives when microorganisms approach to the maximum population density ($N_{max}$) due, among other reasons, to the nutrients exhaustion and the metabolites accumulation that make the environment unfavourable for their growth.

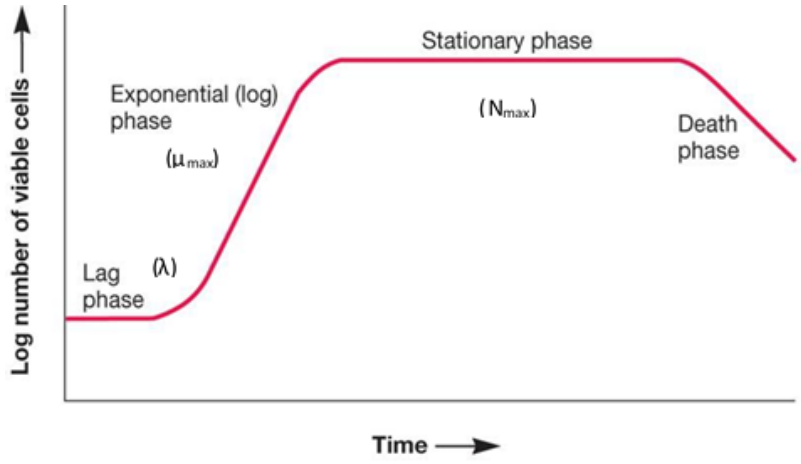


**Figure 2**. Bacterial growth curve. Modified from (Willey, Sherwood, & Woolverton, 2008).

In accordance with regulation EC 2073/2005 it is necessary to carry out studies to investigate the compliance of the microbiologic criterions throughout the products shelf-life. On one hand, it is necessary to carry out studies with the aim of determining the specific physicochemical characteristics of the product. On the other hand, it is necessary to consult bibliographic databases, to carry out challenge and durability tests and to use predictive microbiological models in order to determine the *L. monocytogenes* behaviour in the product. These studies must take into account the inherent variability of the product, the microorganism studied and the transformation and storage conditions.

#### 2.3.2.1. Characterization of the product.

When determining the shelf-life of a ready to eat food, it is important to consider whether the food is capable of supporting the survival or growth of *L. monocytogenes*. The survival and growth of this pathogen in ready to eat foods is a function of the characteristics of this food and the conditions under which that ready to eat food is produced, packaged and stored. These characteristics are sometimes referred to as the intrinsic and extrinsic properties of the ready to eat food.

The most important product characteristics influencing the survival and growth of *L. monocytogenes* in ready to eat products are its pH, $a_w$ and the temperature and time under which the food is stored. Moreover, the preservatives and microflora, including starter cultures or spoilage microorganisms, may have a significant impact on the survival and growth of *L. monocytogenes* in the product.

Regulation EC 2073/2005 notes that products are not considered to support the growth of *L.monocytogenes* if they have one of these requirements:

- pH is equal or lower than 4.4
- $a_w$ is equal or lower than 0.92
- pH is equal or lower than 5.0 and $a_w$ is equal or lower than 0.94
- Shelf life is less than 5 days

#### 2.3.2.2. Characterization of the pathogen's behaviour in the product.

##### 2.3.2.2.1 Scientific bibliography and historical data.

A wide resource of data on *L. monocytogenes* growth is available from various books, scientific journals, universities or technical institutions. Furthermore, national, European and international bodies have available data.

Scientific bibliography is composed for many *L. monocytogenes* kinetic parameters obtained from challenge tests collated in research establishments and from publications.

Historical data generated over a period of time for comparable ready to eat foods and which continued to be generated can be used to establish the trend followed by *L. monocytogenes*. Where levels of bacteria in these foods at the end of shelf-life are consistently low or absent and no results have been obtained which exceed 100 cfu/g, such data can be used in combination with data from sampling and processing areas and equipment, and on quality of raw materials to give a sufficient level of confidence that such ready to eat foods will not pose a risk to public health (Bover-Cid & Garriga, 2010). The level of confidence increases with the amount of data available. The more product units are tested the more reliable the historical data becomes.

##### 2.3.2.2.2 Challenge tests (inoculation tests).

A microbiological challenge test assessing a growth potential is a laboratory-based study that measures the growth of *L. monocytogenes* in artificially contaminated food stored under foreseeable conditions of transportation, storage at retail and at consumer levels. A microbiological challenge test has to reflect the foreseeable conditions that might be expected to occur throughout the cold chain, including storage conditions between production and consumption. The test period starts the day of contamination and finishes at the end of the shelf-life.

Microbiological challenge testing is a useful tool for determining the ability of a food to support the growth of microorganisms. The design, implementation and assessment of challenge studies is a complex task that depends on factors related to how the product is formulated, manufactured, packaged, distributed, prepared and consumed.

The protocols to develop inoculation tests have common aspects (number of batches, strain selection criteria, products sample preparation) and others which are specific (preparation and level of the inoculum, storage conditions of the inoculated products, calculations and interpretation of the results) depending on whether we assess the growth potential or the growth rate (Bover-Cid & Garriga, 2010).

Tests to determine the growth potential of the pathogen are specific for the product and conditions tested. Generally, they have been used as a preliminary investigations to demonstrate the product's capacity to promote or no, the pathogen's growth, to quantify the pathogen's behaviour in the tested conditions and finally, to fix compatible intermedium limits in compliance with the criterion of 100 cfu/g to the end of product's shelf life. Although the results have a direct application, they cannot be used to estimate the behaviour of the pathogen in case of changes in product, process, storage time and temperatures profiles.

Tests to determine the growth rate combine the experimental results of inoculation tests with predictive models in order to interpret and apply the obtained results (Richard Whiting, 2003). The results obtained allow to quantify the pathogen's behaviour at a given time of the product's shelf-life in function of time and conservation temperature and to fix compatible intermediate limits in compliance with the criterion of 100 cfu/g to the end of product's shelf life (Bover-Cid & Garriga, 2010).

#### 2.3.2.2.3 Durability tests.

Durability studies allow an evaluation of the growth of a given microorganism in a naturally contaminated food during its storage according to reasonably foreseeable conditions. These could be considered more realistic than a challenge test for individual foods since the contamination is naturally occurring.

Through widely use to assess microbiological spoilage, when dealing with pathogens the interpretation of the results of the durability tests may be difficult as there is likely to be a relatively low prevalence of food product units contaminated with *L. monocytogenes*, very low numbers initially present and heterogeneous distribution in food.

A history of the durability studies conducted for the same product under the same process, representative of the variability of the manufacturing conditions, will allow the levels of *L. monocytogenes* in the food to be evaluated at the end of the test. It may be used to assess the proportion (with its associated confidence interval) of units (commercial units) exceeding the limit value 100 cfu/g at the end of the shelf-life, after a storage period reflecting the foreseeable conditions of distribution and storage (Bover-Cid & Garriga, 2010).

The level of confidence increases with the amount of data available. Therefore the more product units that are tested the more reliable the shelf-life study becomes.

#### 2.3.2.2.4 Predictive microbiology.

Anticipating the behaviour of microbial pathogens in food is an important goal of food safety managers. Predictive microbiology (also called quantitative microbial ecology) use mathematical functions to quantify microorganism's behaviour through growth, survival or inhibition parameters as well as biochemical processes related with metabolites production.

McMeekin et al., 2002, defined it as a quantitative science that enables users to evaluate objectively the effect of processing, distribution and storage operations on the microbial safety and quality of foods.

Predictive microbiology is based on the premise that microbial population's responses on environmental factors are reproducible and thus, it's possible to predict, by interpolation of previous observations, how it's going to behave in a given environment without being explicitly tested (McMeekin, Olley, Ratkowsky, & Ross, 2002).

Predictive microbiological models are developed using laboratory data from one set of experimental conditions an can be used to predict the likely responses under new set of conditions not previously tested. Modifications of new or existing product's formulation can be evaluated on the computer using a predictive model very quickly and easily, before embarking on expensive laboratory experiments or pilot-scale production runs. Although that, models do not replace experimental (laboratory) work or bibliographic research. Availability of databases among microorganism's behaviour in different conditions and environments it's considered to be a critical point to offset the uncertainty of predictions done with mathematic models. They are not the solution at food safety and quality problems but they can be contemplated as a useful tool to face the problems although their limitations, which the most important ones are the workload required to develop the model, the difficulty on handling mathematical functions and statistical tests, the error obtained through the uncertainty and variability of microbial responses and heterogeneity of food systems, and finally, the empirical naturalness of the models in which are included a reduced number of environmental factors that can affect the relevance of the model in real situations (Bover-Cid & Garriga, 2010).

Tools provided by predictive microbiology are keystones for microbial risk assessment, from the institutional (public health authorities) and operational (food producers) standpoint. It allows to assess the consequences on food handling and processing operations on growth, survival and inactivation pathogens and thus support the decision-making process of food safety managers.

Successful development and implementation of predictive models involves a series of steps that include experimental design, model development, model validation and production of an effective interface between the model and the end-user.

There are different types of models in the predictive microbiology. The selection of the model depends on the objective pursued, the microorganism studied and the number of environmental factors to be considered. Depending on the mathematical function they can be differentiated into probabilistic models or growth, survival and inactivation models. The first ones allow the user to estimate the limits of growth and non-growth or production and non-production of toxins. The second ones determine the number of microorganisms in function of time.

Sometimes the differentiation between the two models is confusing, for this reason, it is usually used a system classification consisting in three levels within the predictive modeling process: primary, secondary and tertiary models (Bover-Cid & Garriga, 2008).

**Primary models** describe changes in the number of microorganisms in function of time under certain and constant environmental conditions. Fitting mathematical functions to microbial growth curves, using for example Baranyi and Roberts equation, Gompertz equation or lineal model in three phases, allows us to determinate microbial parameters such as the specific maximum growth rate ($\mu_{max}$), the generation time (GT) or the lag time ($\lambda$) of the microorganism studied (Bover-Cid & Garriga, 2008).

**Secondary models** describe the parameters obtained in primary models as a function of the environment conditions such as temperature, pH or $a_w$, observing the interaction between two or more factors with microbial growth. They include, for example, the root square model, the Arrhenius equation or gamma functions (Bover-Cid & Garriga, 2008).

**Tertiary models** consist in the integration of one or more primary and secondary models for direct application in order to achieve predictive purposes.

They are interactive computer packages. The final user of these applications does not need to understand about modelling and what kind of models have been used to develop them. These models allow the user to simulate microorganisms behaviour as function of some factors as temperature, pH, $a_w$ and eventually, other components, regardless of the food.

Among the most widely used computer interfaces with predictive purposes, it is noteworthy the ones describe below:

- Pathogen Modeling Program (PMP) was the first available program, developed by USDA Agricultural Research Service of EEUU. It currently contains numerous growth and inactivation models for different pathogens.
- ComBase is an international database developed through a joint initiative of the Institute of Food Research (UK) with the Ministry of Agriculture Fisheries and Food, and with USDA Agricultural Research Service of EEUU, with the support of numerous institutes, universities and European companies. In addition of including different

growth, survival and inactivation models for different conditions and organisms, it also includes the possibility to realize predictions at dynamic temperature conditions (temperature fluctuation among time) (Baranyi & Tamplin, 2004).

- Food Spoilage and Safety Predictor was developed for Danish Institute for Fisheries Research to predict product's shelf-life based on sensory evaluation of specific spoilage microorganism and *Listeria monocytogenes* growth in different products, at constant temperature or at fluctuations temperature conditions (Dalgaard, Buch, & Silberg, 2002).

**Practical application of predictive microbiology**

Within the risk assessment process, predictive models are keystones in the exposure assessment module, as they allow to estimate the changes in hazard (microbial pathogen) throughout the food chain until the point of consumption.

From the operational food production point of view, predictive microbiology may be useful for many applications, which the main ones would be to predict bacterial growth in various conditions, to predict the growth probability of microorganisms in foods, to evaluate the compliance microbiological criteria, to estimate the contamination level at a given day of the shelf-life, to test the variability between batches, to optimise formulation (additives, pH, salt) to assure the best stability, to evaluate the impact of cold chain breaks, to test different storage scenarios and to help to identify Critical Control Points in a process (Bover-Cid & Garriga, 2008).

# 3. Objectives

The main objective of the present work was to evaluate and compare the use of different type of data and tools to assess the growth capability of *L. monocytogenes* in RTE cooked meat products, including:

1) Physicochemical factors of RTE cooked meat products obtained from analytical determination and predictive models to estimate *L. monocytogenes* growth parameters (mainly the growth rate μ).
2) *L. monocytogenes* growth parameters (mainly μ) obtained from challenge tests in RTE cooked meat products available from scientific literature, i.e.:
    2.1) Scientific papers (peer-review articles downloaded from Science Direct).
    2.2) ComBase Browser.

The following specific objectives were purposed in order to achieve this general objective:

A) To analyse data available from the Food Safety Programme of IRTA about the physicochemical factors of RTE cooked meat products from the retail market.
B) To estimate *L. monocytogenes* growth rates in RTE cooked meat products (from retail market) using different predictive models and analyse the results obtained.
C) To obtain *L. monocytogenes* growth rates in RTE cooked meat products from challenge test available in literature and compare them with the results obtained in B).
D) To estimate the safe shelf-life of RTE cooked meat products on the basis of the results of *L. monocytogenes* growth rates.

# 4. Materials and methods

## 4.1. Product characterization data.

The physicochemical factors of 47 commercial cooked meat products obtained from the retail market (Girona and surroundings) were studied. These products included cooked ham, mortadella, frankfurters, catalan sausage, bacon, chopped pork, breast, foie gras and cold cuts and all are elaborated of pork, turkey or chicken. All were commercialized in a convenient RTE format (e.g. Sliced and MAP/vacuum packaged).

Up to seven physicochemical factors were analysed by the research group of the Food Safety Programme of IRTA, including pH, NaCl in water phase, $a_w$, lactic and acetic acid, nitrite and $CO_2$ in the head space of MAP packages.

Raw data from analytical determinations was given in an excel file to be used for the present work. Products were identified with a product's number as listed below:

- Cooked ham → Product's number: 2, 3, 4, 6, 8, 10, 12, 14, 19, 24, 20, 26, 30, 35, 36, 37, 39, 40, 43, 44, 45 and 46.
- Turkey breast → Product's number: 1, 7, 9, 15, 21, 25, 27, 28, 29, 31, 32, 33, 34, 38, 41 and 42.
- Catalan sausage, Mortadella, Frankfurters, Bacon, Foie gras, Chooped pork and Cold cuts → Product's number: 5, 11, 13, 16, 17, 18, 22, 23 and 47.

## 4.2. Tools used to assess *Listeria monocytogenes* growth.

### 4.2.1. Microbiologic predictive models used to estimate *L. monocytogenes* growth rate.

Pathogen Modeling Program (PMP), ComBase and Food Spoilage and Safety Program (FSSP) were the predictive microbiological applications used in this work. These applications have different predictive models for *L. monocytogenes* growth and the characteristics of the ones used here are described below.

- **Pathogen Modeling Program (PMP)**

Of all the anaerobic growth models in the PMP, it has been worked with two different predictive models:

- Growth Model: *Listeria monocytogenes* (Broth Culture, Anaerobic) (PMP Broth culture). Inputs allowed and outputs obtained in this model are detailed in Table 2.

**Table 2.** Inputs and outputs of *Listeria monocytogenes* (Broth Culture, Anaerobic) model of PMP software program.

| Model Inputs | Range of applicability | Units |
|---|---|---|
| **Initial Level** | 3 – 5.9 | Log (cfu/g) |
| **Temperature** | 4 – 37 | °C |
| **pH** | 4.5 – 8.0 | |
| **Sodium Chloride** | 0.5 – 5.0 | % (g/dL) |
| **Sodium Nitrite** | 0 – 150 | ppm |
| **Model Outputs** | | **Units** |
| **Lag Phase Duration** | | h |
| **Generation Time** | | h |
| **Growth Rate** | | Log (cfu/ml)/h |
| **Max Population Density** | | Log (cfu/ml) |

- Growth Model: Growth of *Listeria monocytogenes* in Ground Ham (PMP_GH). Inputs allowed and outputs obtained in this model are detailed in Table 3:

**Table 3.** Inputs and outputs of *Listeria monocytogenes* in Ground Ham model of PMP software program.

| Model Inputs | Range of applicability | Units |
|---|---|---|
| **Initial Level** | 1.0 – 3.0 | Log (cfu/g) |
| **Temperature** | 6 -36 | °C |
| **Sodium diacetate**[a] | 0.05 – 0.20 | % |
| **Sodium Lactate** | 1.0 – 4.2 | % |
| **Model Outputs** | | **Units** |
| **Lag Phase Duration** | | h |
| **Generation Time** | | h |
| **Growth Rate** | | Log (cfu/g)/h |

a. The reported data from product's characterization only contains the acetic acid's concentration. The factor used to calculate sodium diacetate by acetic acid's concentration is:

$$2\ CH_3CO_2H + NaOH \rightarrow Na^+[(CH_3CO_2)_2H]^- + H_2O$$

$$\frac{g\ acetic\ acid}{Kg\ product}\ x\ \frac{1\ mol\ acetic\ acid}{60.0211\ g\ acetic\ acid}\ x\ \frac{1\ mol\ sodium\ diacetate}{2\ mol\ acetic\ acid}\ x\ \frac{142.09\ g\ sodium\ diacetate}{1\ mol\ sodium\ diacetate}\ x\ \frac{1\ Kg\ product}{1000\ g\ product} = \%\ sodium\ diacetate$$

- **ComBase Predictor**

Predictive models for ComBase Predictor are based on results from laboratory experiments carried out in culture media under well controlled laboratory conditions.

In order to study *L. monocytogenes* behaviour, all the models available for *Listeria monocytogenes* were used: *Listeria monocytogenes* / *innocua*, *Listeria monocytogenes*/ *innocua* (acetic), *Listeria monocytogenes* / *innocua* ($CO_2$), *Listeria monocytogenes* / *innocua* (lactic) and *Listeria monocytogenes* / *innocua* (nitrite). The inputs allowed in each model and the outputs obtained are described in Table 4.

In the ComBase Predictor models only one of the two closely related factors $a_w$/NaCl can be used by toggling between NaCl and $a_w$ buttons. It is assumed that salt is in aqueous phase. The % NaCl values are transformed into $a_w$ values by the following formula:

$$Aw = 1 - \%\ NaCl\ x\frac{(5.2471+0.12206\ x\ \%\ NaCl)}{1000}$$ (Chirife & Resnik, 1984)

In order to analyse if there are significant microbial differences between using %NaCl or $a_w$, each model was performed twice, one using %NaCl and another using $a_w$.

**Table 4**. Inputs and outputs of ComBase Predictor models.

| Model inputs | Range of applicability | Units | Model |
|---|---|---|---|
| **Initial level** | 0 – 7 | Log (cfu/ml) | All models |
| **Physiological state** | 0 – 1 | - | All models |
| **Temperature** | 1 – 40 | °C | All models |
| **pH** | 4.4 – 7.5 | - | All models |
| **$a_w$** | 0.934 – 1 | - | All models |
| **NaCl** | 0 – 10.2 | % | All models |
| **Acetic** | 0 – 10000 | ppm | *Listeria monocytogenes* / *innocua* (acetic) (ComBase_acetic) |
| **$CO_2$** | 0 – 100 | % | *Listeria monocytogenes* / *innocua* ($CO_2$) (ComBase_$CO_2$) |
| **Lactic** | 0 – 20000 | ppm | *Listeria monocytogenes* / *innocua* (lactic) (ComBase_lactic) |
| **Nitrite** | 0 – 200 | ppm | *Listeria monocytogenes* / *innocua* (nitrite) (ComBase_nitrite) |
| **Model outputs** | | **Units** | |
| **Max. rate** | | Log (conc/h) | |
| **Dbl. time** | | h | |

The "physiological state" is a dimensionless number between 0 and 1 expressing the physical suitability of the cells to their environment. If its value is 0, the growth will not occur (infinite lag); if the value is 1, then growth will commence immediately, without lag. A typical value is used as default (e.g. For *L. monocytogenes* physical value = 2.1 $e^{-2}$).

- **Food Spoilage and Safety Predictor (FSSP)**

The model which fits with our product characteristics is: *Listeria monocytogenes* in chilled seafood and meat products. *Growth of L. monocytogenes (FSSP)*.

Inputs allowed and outputs obtained in this model are detailed in Table 5.

Some specific clarifications in relation to the assumptions taken to perform the simulations need to be done:

- NaCl concentration used as input was always expressed in water phase.
- $CO_2$ concentration for vacuum package regarding products was assumed to be 30%.
- To develop this study, *Listeria monocytogenes* behaviour has only been studied through the use of growth predictive models at static temperature conditions. All simulations were performed at 5°C as this value is close to the average temperature of consumer

refrigerators (Jofré, Belletti, Latorre-moratalla, & Garriga, 2005). Not inactivation models or fluctuations temperatures were used.

- For growth models, the "No Lag" prediction option was chosen to get a more conservative prediction. This option assumes that the microorganism is adapted to the product and storage conditions and does not add a Lag Time to the growth scenario. It has to be taken into account that models that were developed in a sterile broth system usually provide fail-safe predictions. Typically, the growth rate observed in broth media under optimum conditions for NaCl /water activity and pH would be equal to or greater than that occurring in food containing other chemical components, microorganisms and have a solid structure.

**Table 5**. Inputs and outputs of *Listeria monocytogenes* in chilled seafood and meat products model of FSSP software program.

| Model inputs | Range of applicability | Units |
|---|---|---|
| ***L. monocytogenes* initial cell level** | | cfu/g |
| **Temperature** | 2.0 -25.0 | °C |
| **NaCl in water phase** | 0.7 – 9.0 | % |
| **pH** | 5.6 – 7.7 | |
| **Smoke components – phenol** | 0.0 - 20.0 | ppm |
| **$CO_2$ in headspace gas at equilibrium** | 0.0 – 100.0 | % |
| **Nitrite** | 0.0 – 150.0 | mg/kg |
| **Storage period** | 1.0 – 90.0 | days |
| **Acetic acid** | 0.0 – 11000 | ppm |
| **Benzoic acid** | 0.0 – 1800 | ppm |
| **Citric acid** | 0.0 – 6500 | ppm |
| **Diacetate** | 0.0 – 3800 | ppm |
| **Lactic acid** | 0.0 – 60000 | ppm |
| **Sorbic acid** | 0.0 – 1300 | ppm |
| **Model outputs** | | **Units** |
| **Growth rate** | | 1/h |
| **Lag time** | | days |
| **Ψ (growth boundary parameter)[a]** | | - |
| **Time for 100 – fold increase *L. monocytogenes*** | | days |

a- Ψ is a dimensionless number that provides a measure of the distance between a given set of environmental conditions and the growth boundary (Ψ = 1.0). The psi-value describes how far specific product characteristics and storage conditions are from the predicted growth boundary (Ψ=1.0). Predicted growth and no-growth responses has Psi-values lower and higher than 1.0, respectively (Mejlholm et al., 2010).

### 4.2.2. Scientific literature (results from challenge tests).

- **ComBase Browser**

Dataset contained within this database consists of numerous data reported from experiments about the behaviour of foodborne bacteria obtained from scientific publications and/or provided by research institutions.

Data about the growth of *L. monocytogenes* in RTE cooked meat products were obtained from the search of *Listeria monocytogenes* / *innocua* in four different matrixes; Pork, Poultry, Sausage and Beef at static temperatures between 0 °C to 25 °C. These four matrixes were selected as they are the ones which possibly contained products matching with ours. This range of temperature enables the use of the secondary root square predictive model describing the impact of storage temperature on the growth of *L. monocytogenes*.

Records in ComBase Browser can be extracted automatically in a format suitable to use the DMFit program to fit growth curves and to obtain the growth rate of *Listeria monocytogenes*.

The study has the objective to study the behaviour of *Listeria monocytogenes* at 5°C, but many growth rates obtained were from experiments done at different temperatures. Knowing the growth rate value (rate $_{ref}$) at a tested temperature ($T_{ref}$), it is possible to calculate the growth rate (Rate) at another temperature (T).

The calculation of rate in the same food (with the same physicochemical characteristics) at another temperature T can be obtained using the root square secondary model. If T and $T_{ref}$ are both lower than 25°C, the followed simplified formula is suggested (Baranyi & Tamplin, 2004):

$$Rate = rate_{ref} \; x \; \frac{(T - T_{\min})^2}{(T_{ref} - \; T_{min})^2}$$

(With $T_{min}$ = minimal growth temperature for *L. monocytogenes* = -1.5°C) (EURL-Lm, 2014)

$Rate_{ref}$ and rate are expressed in log (cfu/g) per time unit.

- **Peer-review articles from Science Direct**

Science Direct is a world's leading platform for scientific, technical, and medical research. It has been gone through many articles in order to obtain growth rates of *Listeria monocytogenes* in

ready to eat products. If temperature was not equal to 5°C, the simplified formula of root square secondary model described above was used.

Up to 204 *L. monocytogenes* growth rates in RTE cooked meat products were collected from 18 articles of Science Direct platform for the Food Safety Program of IRTA. Most of the *L. monocytogenes* growth rates are results of challenge test assessing the growth inhibition capability of a particular combination of factors.

Raw data of this collected articles was given in an excel file to be used for the present work.

## 4.3. Statistical analysis.

Statistical analysis was carried out using the R program.

Descriptive analysis of physicochemical factors and growth rates was carried out to obtain the statistical parameters describing the distribution of values as well as a graphic representation of frequency histograms. Numerical summary provides the minimum, maximum, interquartile range (IQR), mean, standard deviation and percentiles of the dataset.

Correlation matrix is any statistical relationship between two random variables or two sets of data. Correlation refers to any of a broad class of statistical relationships involving dependence. To develop the correlation matrix between the studied variables (physicochemical factors), the Pearson product-moment correlation coefficient was used. It is a measure of the linear correlation between two variables X and Y, giving a value between +1 and -1 inclusive, where 1 is total positive correlation, 0 is no correlation, and -1 is total negative correlation.

Cluster analysis was applied as an exploratory data analysis tool, aiming to sorting data into groups in a way that the degree of association between two objects is maximal if they belong to the same group and minimal otherwise.

The dendrogram plot was built to illustrate the arrangement of the clusters produced by hierarchical clustering, its vertical axis denoting the linkage distance. Thus, for each node in dendrogram, it can be read off the criterion distance at which the respective elements were linked together into a new single cluster. When each object represents its own cluster, the distances between those objects are defined by Euclidean distance measure which it is simply the geometric distance in the multidimensional space. Ward's method was used for determining

which clusters are sufficiently similar to be linked together. This method uses an analysis of variance approach to evaluate the distances between clusters.

To study if the dataset followed or not a normal distribution the Shapiro-Wilk test was applied. Its null and its alternative hypothesis being:

$$H_0 = data\ is\ normally\ distributed$$
$$H_1 = data\ is\ not\ normally\ distributed$$

Alpha level or significance level is the probability of rejecting the null hypothesis given that it is true. Alpha level chosen for all the tests was set to 0.05 (5%), implying that it is acceptable to have a 5% probability of incorrectly rejecting the null hypothesis.

As non-normal distribution of data was generally obtained non-parametric (i.e. distribution-free) tests were applied:

- Mann-Whitney-Wilcoxon test in order to compare the mean of two independent samples. Its null and its alternative hypothesis being:

$$H_0 = two\ samples\ come\ from\ the\ same\ population$$
$$H_1 = two\ samples\ do\ not\ come\ from\ the\ same\ population$$

- Kruskall Wallis test in order to compare the means of the samples. Its null and its alternative hypothesis being:

$$H_0 = means\ of\ samples\ are\ considered\ to\ be\ equal$$

$$H_1 = means\ of\ samples\ are\ not\ considered\ to\ be\ equal$$

In some cases, parametric tests (for normal distribution of data) were also performed:

- T-test in order to compare the mean of two independent samples. Its null and its alternative hypothesis being:

$$H_0 = two\ samples\ come\ from\ the\ same\ population$$
$$H_1 = two\ samples\ do\ not\ come\ from\ the\ same\ population$$

- ANOVA (Analysis of variance) to compare the means of more than two independent samples. Its null and its alternative hypothesis being:

$$H_0 = means\ of\ samples\ are\ considered\ to\ be\ equal$$
$$H_1 = means\ of\ samples\ are\ not\ considered\ to\ be\ equal$$

# 5. Results and Discussion

## 5.1. Physicochemical characterization of the RTE cooked meat products.

Table 6 shows a numerical summary of 7 physicochemical factors obtained from the analysis of 47 commercial ready to eat cooked meat products. Figure 3 shows the histograms of frequencies for the same variables. These factors are relevant for the microbial behaviour and thus they usually are inputs of predictive models.

Separately, averages of pH, $a_w$ and NaCl in water phase (WPS) were optimum for *Listeria monocytogenes* growth. However, it has to be taken into account the combined effect of these factors with other preservative factors (nitrite, $CO_2$ in the environment, lactic and acetic acids) which could result in an inhibition of its growth as it will be discussed later.

**Table 6**. Numerical summary of the quantification of seven factors studied (pH, $a_w$, NaCl in water phase, nitrite, lactic acid, acetic acid and $CO_2$) in the 47 commercial ready-to-eat cooked meat products.

| Parameter | pH | $a_w$ | NaCl in water phase (%) | Nitrite (ppm) | Lactic acid (ppm) | Acetic acid (ppm) | $CO_2$ (%) |
|---|---|---|---|---|---|---|---|
| **Mean** | 6.08 | 0.975 | 3.9 | 5.4 | 11530 | 641 | 20.53 |
| **Sd** | 0.24 | 0.004 | 0.3 | 5.8 | 7796 | 1353 | 9.67 |
| **IQR** | 0.27 | 0.005 | 0.4 | 7.0 | 9951 | 65 | 18.65 |
| **Minimum** | 5.26 | 0.959 | 3.3 | 0 | 969 | 0 | 5.90 |
| **$P_{25}$** | 5.98 | 0.973 | 3.7 | 0 | 5560 | 0 | 11.35 |
| **$P_{50}$** | 6.12 | 0.976 | 3.9 | 5.0 | 8864 | 0 | 19.60 |
| **$P_{75}$** | 6.25 | 0.978 | 4.1 | 7.0 | 15511 | 65 | 30.00 |
| **Maximum** | 6.46 | 0.982 | 4.9 | 23.0 | 32148 | 5411 | 35.10 |
| **P-value Shapiro-Wilk test** | 0.0067 | 0.00054 | 0.026 | $5.193 \times 10^{-6}$ | 0.0021 | $8.70 \times 10^{-11}$ | $2.31 \times 10^{-6}$ |

Cases where the standard deviation's value was greater than the medium suggested the presence of one or more extreme values, indicating a bias in the values of the same parameter for the different products. This phenomenon was reflected in acetic acid (mean = 641 ppm and standard deviation = 1353 ppm) and in nitrite (mean = 5.4 ppm and standard deviation = 5.8 ppm). In the case of these two factors a considerable number of values were below the detection/quantification limit of the analytical determination, which explain the strong skewness of data distribution.

According to the Shapiro Wilks tests none of the factors were normally distributed (p-value < 0.05). WPS was the parameter that had a greater p- value (0.026) and thus the one which was closer to follow a normal distribution. By contrast, acetic acid and nitrite were the ones which had the lower p-value.

CO2 also showed a very low p-value, the non-normal distribution was explained, at least partially, by the fix value (30%) assumed for the vacuum packaged products, which represented the 44% of the analysed products.

Aiming to graphically see the distribution and frequency of the data analysed, a histogram for each factor was developed as shown in figure 3.

Although the p-value of Shapiro Wilk test indicated that data did not follow a normal distribution, it is sensed a certain normality in the values distribution on the right hand in the cases of pH and $a_w$. 9/47 values of pH and 6/47 values of $a_w$ which were on the left hand, were lower and they fostered a greater difficulty in *L. monocytogenes* growth.

Method used for the detection of nitrite had a detection/quantification limit of 5 ppm. When processing data, it has been assumed that these values were equal to zero, though probably, that was not exactly the true. The added nitrite reacts with the factors within the product and as a result, the quantified residual nitrite is very low. For this reason many times it was not detected in analysis.

The variability of the data obtained in the analysis of lactic acid was quite large. To identify the amount of endogenous lactate present in RTE cooked meat products in comparison to the amount derived from the addition of preservatives (e.g. E-325, E-326, E-261, E-262…) the data was analysed separately (Table 7, Figure 4) for:

A) Products without added lactate
B) Products with added lactate.

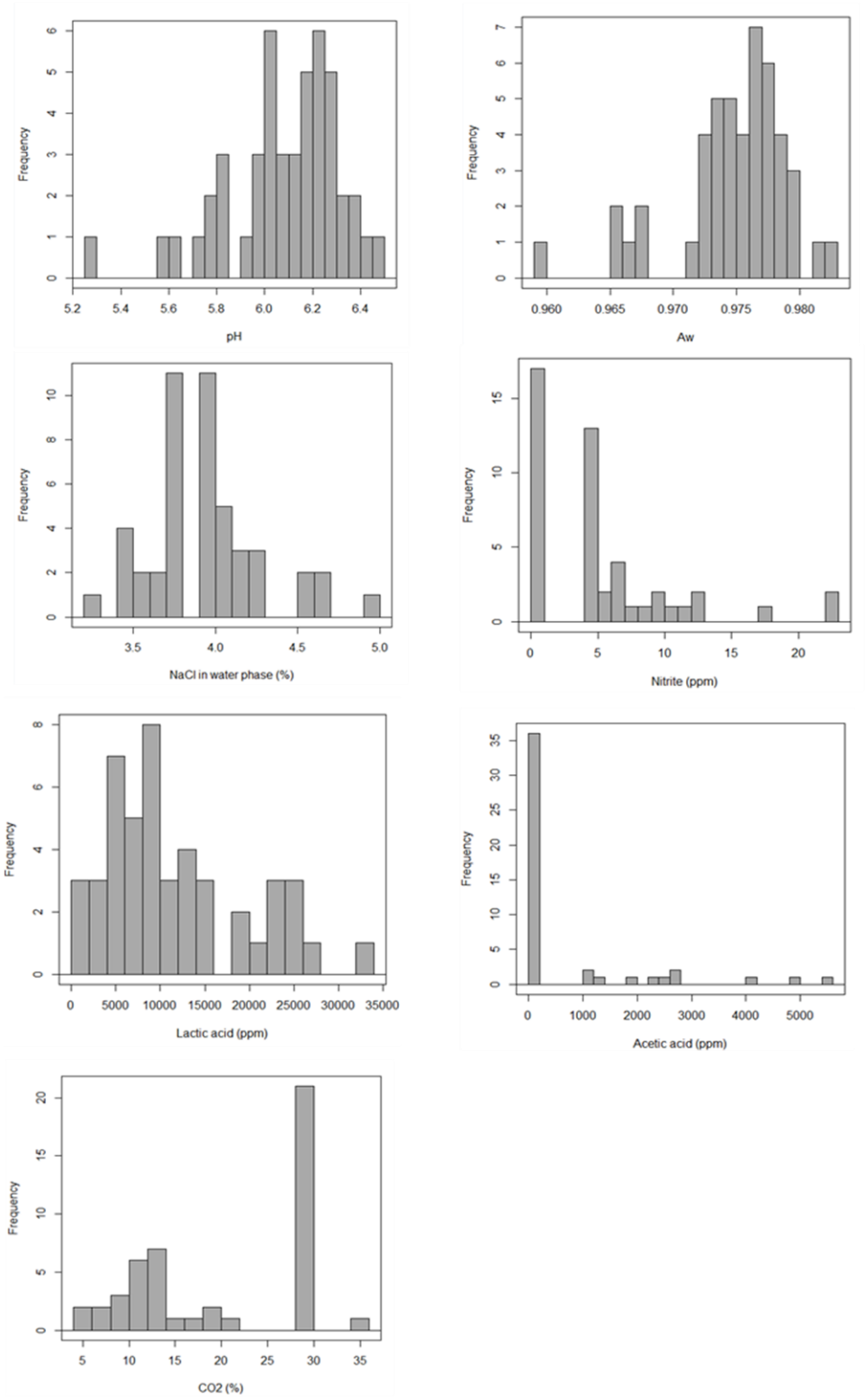


**Figure 3.** Graphical representation (histogram) of the absolute frequency of the analytical quantification of 7 factors studied (pH, $a_w$, NaCl in water phase, nitrite, lactic acid, acetic acid and $CO_2$) in the 47 commercial ready-to-eat cooked meat products.

**Table 7**. Numeric summary of the quantification of lactic acid in local ready-to-eat cooked meat products with and without added lactate in their formulation.

| | Without added lactate (ppm) | With added lactate (ppm) |
|---|---|---|
| **Mean** | 6988 | 19515 |
| **Sd** | 3920 | 6422 |
| **IQR** | 3872 | 9650 |
| **Minimum** | 949 | 9399 |
| **$P_{25}$** | 4757 | 14509 |
| **$P_{50}$** | 6617 | 20891 |
| **$P_{75}$** | 8629 | 24158 |
| **Maximum** | 19339 | 32244 |
| **n** | 30 | 17 |
| **P-value Shapiro-Wilk test** | 0.0676 | 0.5603 |
| **P-value T test** | $1.992 \times 10^{-7}$ | |
| **P-value WIlcoxon-sum-rank test** | $8.843 \times 10^{-10}$ | |

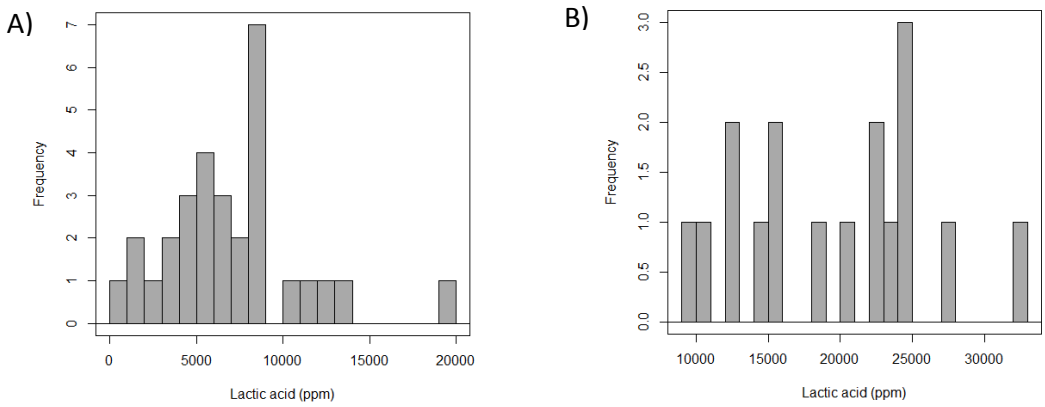


**Figure 4.** Graphical representation of absolute frequency of the analytical quantification of lactic acid in local ready-to-eat cooked meat products. A**)** Quantification of lactic acid in products without added lactate in their formulation. B**)** Quantification of lactic acid in products with added lactate in their formulation.

As it could be seen in the numerical summary and in histograms, products which did not have an addition of lactate in their formulation had 6988 ppm as an average value for lactic acid. This amount was an agreement with the 0.7% usually reported in the literature (Mejlholm et al., 2010). When separating between products with and without addition of lactate in their formulation, this factor followed a normal distribution. Because it followed a normal distribution, but the number of values were small, it was carried out a T-test and a Wilcoxon test. According to both tests, there were significant statistical differences between the two averages.

Method used for the detection of acetic acid was not accurate and sensitive enough. When processing data, it has been assumed that values in products where acetic acid was not detected were equal to zero, though probably, that was not exactly the true. This assumption was what produces the non-normal distribution of the data.

Most of the products have a $CO_2$ value of 30%. This was because it was assumed that products which were vacuum packed had a 30% value of $CO_2$ (Garriga & Hugas, 1996). This assumption was what produces the non-normal distribution of the data. The rest of values reflect products which were under a modified atmosphere packaging.

A correlation matrix was conducted using the Pearson correlation with the aim of determining if there was a linear dependence between the physicochemical factors. The correlation coefficients between the variables (Table 8) showed a very small and in some cases a moderate magnitude (0.53, -0.55 and -0.57).

The correlation was particularly significant between $a_w$ and WPS concentration, which could be explained because $a_w$ is highly dependent on the solute concentration (including mainly NaCl) (Rahman & Perera, 2007). Through NaCl is usually the main solute contributing to the slope of $a_w$, other compounds can also contribute. For instance, $a_w$ was also significantly correlated, though with a much lower coefficient, with lactic acid. Therefore it would be reasonable to get a similar result in the microbiological predictive model regardless of which one of the two inputs is used. As the correlation matrix indicated, it was true that there was a negative linear correlation between these two variables, i.e. when one increases, the other decreases. However, this correlation (-0.55) indicated a moderate dependency, not higher, and therefore, was not equivalent to use one variable instead of the other.

A similar correlation coefficient was obtained for pH and $CO_2$ (-0.57). This relationship could be explained by the fact that a higher concentration of $CO_2$ in atmosphere results in an acidification (lower pH) on the surface of the product (Yam, 2009).

It is also known that a lower pH induces nitrite - nitric oxide reaction (Massol Deya, 1994) and thus, it reduces the amount of residual nitrite in the product. This fact was reflected in the correlation (0.53) between these two factors; a lower pH resulted in a lower concentration of nitrite.

**Table 8**. Pearson's correlation between the studied factors (pH, $a_w$, NaCl in water phase, nitrite, lactic acid, acetic acid and $CO_2$) obtained from the 47 commercial ready-to-eat cooked meat products. Significance level: *0.09 **0.01 ***0.0001

| pH | $a_w$ | NaCl in water phase (%) | Nitrite (ppm) | Lactic acid (ppm) | Acetic acid (ppm) | $CO_2$ (%) |
|---|---|---|---|---|---|---|
| pH | 0.07 | -0.07 | 0.53** | 0.09* | -0.25* | -0.57*** |
| | $a_w$ | -0.55*** | 0.16 | -0.36** | -0.21 | -0.25* |
| | | NaCl in water phase (%) | 0.02 | -0.18** | 0.34** | 0.34** |
| | | | Nitrite (ppm) | -0.06 | 0.06 | -0.22 |
| | | | | Lactic acid (ppm) | -0.13 | -0.28 |
| | | | | | Acetic acid (ppm) | 0.23 |
| | | | | | | $CO_2$ (%) |

A hierarchical cluster analysis was carried out between the products using seven different physicochemical factors as variables (pH, $a_w$, WPS, nitrite, lactic acid, acetic acid and $CO_2$) in order to determine if any of these factors were statistical significant different between the clusters obtained (Figure 5).

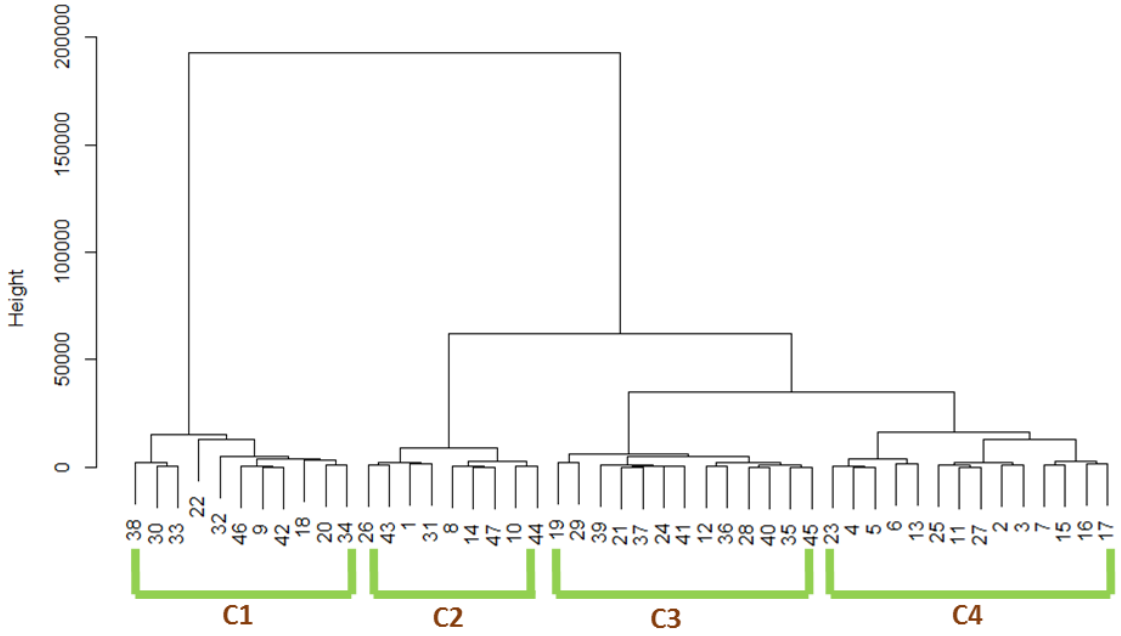


**Figure 5.** Cluster dendrogram of 47 commercial ready-to-eat cooked meat products using as variables 7 physicochemical factors (pH, $a_w$, NaCl in water phase, nitrite, lactic acid, acetic acid and $CO_2$).

The first relevant observation was the four distinct clusters obtained through the analysis. Within each of the four clusters, no differences regarding the type of the raw product were found in all clusters; there were products made from pork, chicken and/or turkey. What was observed is that most of the fattiest cooked ready to eat meat products (mortadella, catalan sausage, foie gras and sausages) were within cluster number fourth (Table 9).

**Table 9.** Numerical summary of the factors of clusters obtained through the clustering analysis of 47 local ready-to-eat cooked meat products using as variables 7 physicochemical factors (pH, aw, NaCl in water phase, nitrite, lactic acid, acetic acid and $CO_2$).

| Cluster | Products | pH | $a_w$ | Nacl in water phase (%) | Nitrite (ppm) | Lactic acid (ppm) | Acetic acid (ppm) | $CO_2$ (%) |
|---|---|---|---|---|---|---|---|---|
| C1 | 33,38,30,22, 32,34,20,18, 46,42,9 | 5.97 | 0.977 | 3.7 | 5.8 | 13204 | 136 | 17.07 |
| C2 | 44,10,47,14, 8,43,26,31, 1 | 6.07 | 0.974 | 4.2 | 7.4 | 3970 | 1396 | 24.00 |
| C3 | 29,19,39,41, 45,24,37,21, 35,40,28,36, 12 | 6.18 | 0.971 | 3.9 | 5.3 | 23643 | 430 | 18.59 |
| C4 | 13,6,23,5, 4,17,16,15, 7,25,27,11, 3,2 | 6.10 | 0.977 | 3.9 | 3.2 | 8261 | 355 | 20.82 |
| p-value (kruskal) | | 0.3731 | 0.006 | 0.01418 | 0.3632 | $2.562 \times 10^{-9}$ | 0.2707 | 0.1818 |
| p-value (ANOVA) | | 0.281 | 0.00223 | 0.00623 | 0.229 | $<2 \times 10^{-16}$ | 0.0866 | 0.339 |

We studied which of the physicochemical factors significantly differed between different clusters by the non-parametric Kruskal-Wallis test as factors did not follow a normal distribution. The result of the test indicated that the mean of lactic acid, $a_w$ and WPS differed significantly (p-value < 0.05) between clusters. Of these three variables, lactic acid was the one which had a lower p-value indicating a greater significance between the clusters means.

An ANOVA was carried out only to check whether or not we obtained the same result as the one obtained with the Kruskal-Wallis test. ANOVA, although being a parametric method which implies to work with data that follows a normal distribution, it is very robust. ANOVA's result confirmed the result obtained by Kruskal-Wallis test; lactic acid, $a_w$ and WPS were significantly different between clusters.

The relevance of the studied physicochemical factors for the growth of *L. monocytogenes* in RTE cooked meat products was assessed through the Ψ (psi) parameter provided by the FSSP model (Dalgaard et al., 2002).

Psi provides a measure of the distance between a given set of environmental conditions and the combination of growth/no growth, i.e. growth boundary (Ψ = 1). The higher the value psi (Ψ>1), the greater the distance and therefore it will be harder for *Listeria* to grow. On the contrary, the lower the psi value (Ψ<1) the more favourable is the growth of *L. monocytogenes*. The difference of Ψ (|ΔΨ|) between two different conditions provides a measure of the magnitude of the influence of the change of the environmental conditions.

The difference between the Ψ values obtained within the range of each physicochemical factor, while the other factors are kept to the median ($P_{50}$) values (|ΔΨ|) provides an indication of the magnitude of the impact of the particular factor of the ready to eat cooked meat products on the growth potential of *L. monocytogenes* between two different conditions (Table 10).

Two types of ranges of the factors were tested: max-min as well as interquartile range ($P_{75}$-$P_{25}$) of the distribution of values.

**Table 10**. Numerical summary of the psi values obtained within each factor studied (pH, NaCl in water phase, nitrite, lactic acid, acetic acid and $CO_2$) while others are kept to the median ($P_{50}$).

| Parameter | pH | NaCl | Nitrite | Lactic | Acetic | $CO_2$ |
|---|---|---|---|---|---|---|
| **Psi at minimum value** | 1.0901 | 0.4824 | 0.4973 | 0.3174 | 0.4976 | 0.4952 |
| **Psi at $P_{25}$** | 0.5764 | 0.4928 | 0.4973 | 0.4207 | 0.4976 | 0.4958 |
| **Psi at $P_{50}$** | 0.4976 | 0.4976 | 0.4976 | 0.4976 | 0.4976 | 0.4976 |
| **Psi at $P_{75}$** | 0.4439 | 0.5008 | 0.498 | 0.6611 | 0.5468 | 0.5017 |
| **Psi at maximum value** | 0.385 | 0.5299 | 0.5048 | 1.1664 | 1.2976 | 0.5046 |
| **Distance percentiles \|ΔΨ\|** | 0.1325 | 0.008 | 0.0007 | 0.2404 | 0.0492 | 0.0059 |
| **Distance max-min \|ΔΨ\|** | 0.7051 | 0.0475 | 0.0075 | 0.849 | 0.8 | 0.0094 |

*$A_w$'s factor was not studied as it is not an input of the FSSP model.

Looking at Table 10, the greater differences between maximum and minimum values were in lactic and acetic acids. The higher the concentration of acetic and lactic acids, the more inhibited the growth of *Listeria monocytogenes*.

We have to take into account that the values of nitrite used to calculate the psi were very low; due to the values were the ones that products contained. To see more clearly the effect of this factor over the growth of *Listeria monocytogenes* in these products, psi was calculated for 150 ppm of nitrite which is the maximum ingoing amount allowed for this factor in cooked meat products. The psi value obtained was 0.8373, therefore the difference between the maximum and minimum nitrite values amounted to 0.34, which is not a high value compared to the ΔΨ for pH and lactic acid, and thus claimed that nitrite at the levels used by the manufacture of cooked meat products is not the most relevant factor affecting the *L. monocytogenes* growth potential.

After organic acids, pH was the physicochemical factor with higher influence on *L. monocytogenes* growth. Table 10 showed how Ψ for pH at minimum value (pH =5.6) was greater than Ψ=1, indicating that *L. monocytogenes* growth was inhibited. *L. monocytogenes* is able to grow in a range of pH [5 – 9.6] if all others factors are optimal (e.g. $a_w \geq 0.98$, no lactate, no nitrite). However, RTE cooked meat products did not show the optimal values for growth, therefore those having the lower pH even if ≥ 5, could inhibit its growth if other product's factors were not optimal.

$CO_2$ and WPS were factors that affect, in the range observed in the cooked meat studied products, the growth of *Listeria monocytogenes* very less compared to others. The difference between their values of psi of the maximum and minimum was not so high.

## 5.2. Assessment of *Listeria monocytogenes* growth in cooked meat products by predictive models.

Table 11 presents a numerical summary of the growth rates of *Listeria monocytogenes* of the 47 ready to eat cooked meat products analysed obtained from the different microbiological predictive models used.

The normal distribution of the growth rates was studied by using the Shapiro-Wilk test. The results showed that there were four models that provided growth rates that follow a normal distribution (p-value > 0.05) and the rest of the models did not provide it (p-value < 0.05).

Due to the limited number of growth rates obtained from each model (n= 47) and the possible presence of outliers, it was used non parametric tests when doing statistical analysis, although some variables followed a normal distribution.

Graphically, through histograms, and together with the numerical summary (Table 11, Figure 6) it could be clearly seen the differences between growth rates obtained with different models and whether or not there were outliers among its dataset.

**Table 11**. Numerical summary of *L. monocytogenes* growth rates obtained from 47 commercial ready-to eat cooked meat products through using 13 different microbiological predictive models.

| Model | Mean | sd | IQR | Min | $P_{25}$ | $P_{50}$ | $P_{75}$ | Max | Shapiro – Wilk test |
|---|---|---|---|---|---|---|---|---|---|
| **PMP_GH** | 0.0129 | 0.0140 | 0.017 | -0.0018 | 0.004 | 0.0072 | 0.02035 | 0.0409 | 0.07686 |
| **PMP broth culture** | 0.0267 | 0.0023 | 0.002 | 0.018 | 0.026 | 0.028 | 0.028 | 0.029 | $7.40 \times 10^{-7}$ |
| **Combase $a_w$** | 0.0133 | 0.0020 | 0.003 | 0.008 | 0.012 | 0.014 | 0.015 | 0.016 | 0.008259 |
| **Combase _NaCl** | 0.0140 | 0.0018 | 0.002 | 0.008 | 0.013 | 0.014 | 0.015 | 0.017 | 0.003201 |
| **Combase_nitrite_$a_w$** | 0.013 | 0.0020 | 0.002 | 0.007 | 0.012 | 0.013 | 0.014 | 0.016 | 0.004968 |
| **Combase_nitrite_nacl** | 0.0137 | 0.0017 | 0.002 | 0.008 | 0.013 | 0.014 | 0.015 | 0.016 | 0.0004566 |
| **Combase_lactic_$a_w$** | 0.0118 | 0.0022 | 0.003 | 0.006 | 0.01 | 0.012 | 0.013 | 0.016 | 0.1472 |
| **Combase_lactic_nacl** | 0.0122 | 0.0021 | 0.003 | 0.006 | 0.011 | 0.013 | 0.014 | 0.016 | 0.007446 |
| **Combase_acetic_$a_w$** | 0.0004 | 0.0014 | 0 | 0 | 0 | 0 | 0 | 0.005 | $1.2 \times 10^{-6}$ |
| **Combase_acetic_NaCl** | 0.0004 | 0.0014 | 0 | 0 | 0 | 0 | 0 | 0.005 | $1.2 \times 10^{-6}$ |
| **Combase _$CO_2$_$a_w$** | 0.0107 | 0.0024 | 0.004 | 0.006 | 0.009 | 0.01 | 0.013 | 0.015 | 0.1077 |
| **Combase _$Co_2$_nacl** | 0.0113 | 0.0023 | 0.004 | 0.006 | 0.010 | 0.011 | 0.013 | 0.016 | 0.1252 |
| **FSSP** | 0.0105 | 0.0072 | 0.013 | 0 | 0.004 | 0.0119 | 0.017 | 0.02 | 0.000211 |

*Growth rates were estimated by each product using the vales of the physicochemical factors as input. For all simulations the storage time was 5°C (see material and methods).

PMP_GH predictions provided a nearly uniform distribution of growth rates. This could be explained because very few relevant physicochemical factors were taken into account as inputs.

Most of the growth rates obtained with ComBase models whose input is acetic acid had a value closer or equal to zero as acetic acid has a strong effect in reducing the growth potential of *L. monocytogenes*. Compared with other models were those that provide the lowest growth rates. Most of the growth rates obtained with PMP_GH model also had a value close or equal to zero but unlike ComBase models whose input is acetic acid, it also had the greatest growth rates (growth rates>0.04) providing extreme values.

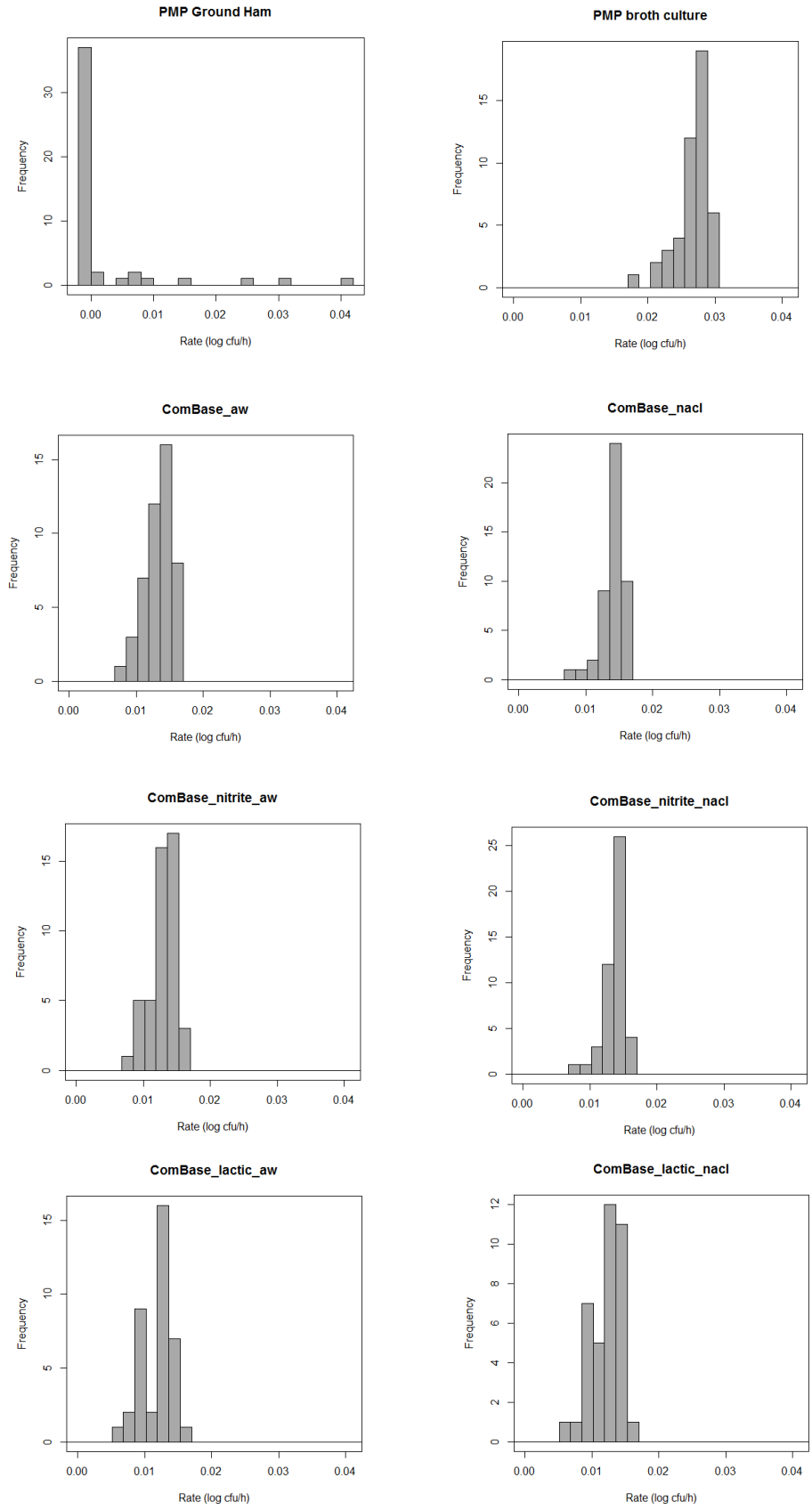


**Figure 6.** Graphical representation of the absolute frequency of the *L. monocytogenes* growth rates obtained from 47 local ready-to eat cooked meat products in each microbiological predictive model. Simulations were carried out at a temperature storage of 5°C.

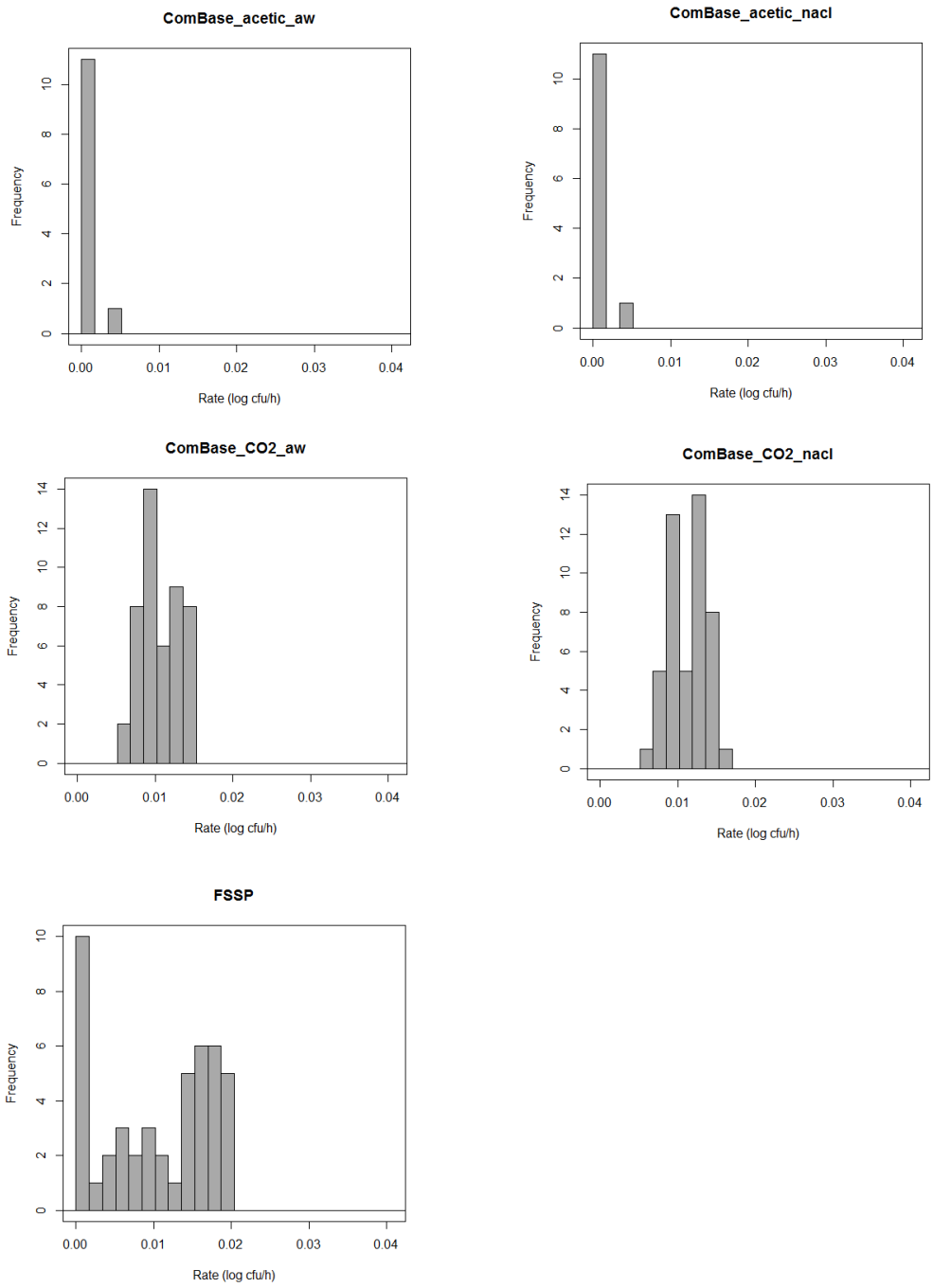


**Figure 6.** Graphical representation of the absolute frequency of the *L. monocytogenes* growth rates obtained from 47 local ready-to eat cooked meat products in each microbiological predictive model. Simulations were carried out at a temperature storage of 5°C. (Continuation)

PMP broth culture and ComBase models were built on results obtained in laboratory (broth) media, and thus they usually predict greater growth rates than those models developed with experiments done in other more complex matrixes.

Although ComBase models also include the growth of *Listeria monocytogenes* in broth culture media, growth rates obtained were not as high as in the PMP broth culture model. This could be because as the number of inputs allowed in ComBase models is higher than in the PMP broth culture model and therefore there could be more factors, like organic acids, that could limit the growth rates.

As in the physicochemical factors analysis were found that $a_w$ and WPS factors did not have a higher, but a moderate lineal dependence, ComBase models were carried out twice, once using $a_w$ as an input an another with WPS in order to determine whether the obtained growth rates differed depending on which of these two inputs was used.

In ComBase models, when using WPS as an input instead of $a_w$, growth rates had always been slightly higher than that of its counterpart. One possible explanation for this result would be described for the mathematical formula used to transform the WPS values into $a_w$ which do not give a result equal to the $a_w$ obtained analytically. Though the solute contributing to $a_w$ in cooked meat products is mainly WPS, other ingredients (e.g salts of organic acids, sugars…) can also reduce $a_w$ values.

However, Wilcoxon test results (Table 12) determinate that there were no differences (p-value > 0.05), in growth rates obtained with ComBase models, when using $a_w$ instead of WPS as an input. ComBase models whose input is nitrite and those which do not have any inputs as $CO_2$, nitrite and organic acids had their respectively p-values (0.06539 and 0.05889) so close to the p-value decision, thus, in these cases, the affirmation of no differences in the growth rates obtained by using one of this inputs in these models was not so clear.

**Table 12**. P-values of Wilcoxon test obtained when comparing *L.monocytogenes* growth rates from ComBase models when using $a_w$ or NaCl in water phase as inputs.

| Models compared | P-value Wilcoxon test |
|---|---|
| **ComBase_$a_w$ *vs* ComBase_NaCl** | 0.06539 |
| **ComBase_nitrite_$a_w$ *vs* ComBase_nitrite_NaCl** | 0.05889 |
| **ComBase_lactic_$a_w$ *vs* ComBase_lactic_NaCl** | 0.3165 |
| **ComBase_acetic_$a_w$ *vs* ComBase_acetic_NaCl** | 1 |
| **ComBase_$CO_2$_$a_w$ *vs* ComBase_$CO_2$_NaCl** | 0.2706 |

FSSP is the model that offers the greatest variability among the obtained growth rates, but it is also the model that incorporates more inputs for its development. So, probably it will be the

model which is going to provide the most accurate growth rates, as it has as inputs; pH, acetic and lactic acid which are the studied factors that have the highest impact on bacteria's growth.

In order to compare the growth rates obtained with the predictive models, a hierarchical cluster analysis was performed to the results provided by the different microbiological predictive models used to predict the growth rate of *Listeria monocytogenes* using as variables the forty-seven ready to eat cooked meat products with their respectively predicted growth rates. Four distinct clusters were obtained from the clustering analysis (Figure 7).

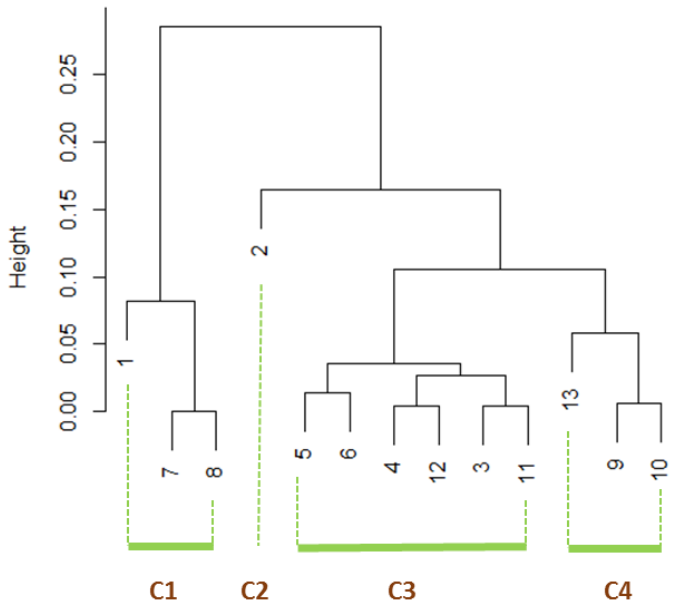


**Figure 7**. Cluster dendrogram of *L. monocytogenes* growth rates obtained with the microbiological predictive models. 1, PMP_GH; 2, PMP broth culture; 3, ComBase. *Listeria monocytogenes/innocua* ($a_w$); 4, Combase. *Listeria monocytogenes/innocua* (NaCl); 5, Combase. *Listeria monocytogenes/innocua* ($CO_2$) ($a_w$); 6, Combase. *Listeria monocytogenes/innocua* ($CO_2$) (NaCl); 7, Combase. *Listeria monocytogenes/innocua* (acetic) ($a_w$); 8, Combase. *Listeria monocytogenes/innocua* (acetic) (NaCl); 9, Combase. *Listeria monocytogenes/innocua* (lactic) ($a_w$); 10, Combase. *Listeria monocytogenes/innocua* (lactic) (NaCl); 11, Combase. *Listeria monocytogenes/innocua* (nitrite) ($a_w$); 12, Combase. *Listeria monocytogenes/innocua* (nitrite) (NaCl); 13, FSSP.

The first cluster involved the PMP_GH model and the ComBase models which have acetic acid as an input.

PMP_GH model do not have the pH as an input whereas all other predictive models have. Growth rates obtained with this model would not be as accurate as other predictive models due to pH is a factor that affects significantly *L. monocytogenes* growth.

As mentioned, most of the growth rates obtained with these models were zero or negative. It has to be taken into account that the acetic acid quantification was not so sensitive and all values not quantified were assumed to be equal to zero, thus, the presence of extreme values.

What was concluded is that acetic acid was an important physicochemical parameter in the estimation of the growth of *Listeria monocytogenes* due to it significantly affected its growth.

The second cluster comprised only the PMP culture broth model which had as a relevant input the pH but not organic acids. This could be the reason for being one of the models which provided greater growth rates as the inhibitory effect of organic acids was not taken into account.

The third cluster was made up for ComBase models whose input is $CO_2$, ComBase models whose input is nitrite and for those ComBase models which do not consider nitrite, organic acids and $CO_2$ as inputs. This cluster demonstrated that the different growth rates of *L. monocytogenes* obtained from these models whose input is $CO_2$ and nitrite are not significantly different to those models which do not consider nitrite, organic acids and $CO_2$ as an inputs. It has to be taken into account, that the nitrite values used in the models were the residual values which are very low and it was logic not to find so much differences due their poor significance.

Finally, the last cluster included the ComBase models whose input is lactic acid and the FSSP model which also takes lactic acid into account. As seen in the analysis of physicochemical factors, lactic acid is an organic acid that strongly affects the growth of *Listeria monocytogenes*. Therefore, it was logic to get different growth rates with the models that have lactic acid as an input. It is true that acetic acid was a relevant factor in *L. monocytogenes* growth, but due to the quantification of acetic acid was not accurate enough, differences between growth rates obtained through predictive models which have acetic acid and lactic acid as input in front of those which not, could not be properly studied.

A numerical summary of the different clusters of the models is detailed below (Table 13). Cluster 1 had the lowest growth rates mean but also had the maximum value within its dataset compared to others clusters. Its standard deviation was greater than the average of the group, this reflected the presence of extreme values due to most of the values within the cluster were less or equal to zero.

**Table 13**. Numerical summary of the clusters obtained through the clustering analysis of the *L. monocytogenes* growth rates obtained with the microbiological predictive models.

| Cluster | Models | Mean | sd | IQR | Min | $P_{25}$ | $P_{50}$ | $P_{75}$ | Max |
|---|---|---|---|---|---|---|---|---|---|
| **1** | 1, 7, 8 | 0.0043 | 0.0097 | 0.005 | -0.0018 | 0.000 | 0.000 | 0.005 | 0.0409 |
| **2** | 2 | 0.0267 | 0.0023 | 0.002 | 0.0180 | 0.026 | 0.0280 | 0.028 | 0.0290 |
| **3** | 5, 6, 4, 12, 3, 11 | 0.0127 | 0.0024 | 0.003 | 0.0060 | 0.011 | 0.0130 | 0.014 | 0.0170 |
| **4** | 13, 9, 10 | 0.0114 | 0.0048 | 0.004 | 0.000 | 0.010 | 0.0123 | 0.014 | 0.0201 |

According to the results obtained by the study of the physicochemical factors and the microbiology predictive models we could conclude that acid organics (acetic and lactic acids) and pH were the most important inputs to be taken into account when you are using a predictive model to assess the growth potential of *L. monocytogenes* in ready-to-eat cooked meat products due to their higher affectation on *L. monocytogenes* growth. Models which contemplate these factors as inputs will provide a more accurate growth rate of *L. monocytogenes* for these products.

As a consequence, FSSP would be the model which would provide more accurate growth rates due to it is the only model which has all the relevant factors as inputs. For this reason, FSSP is the model used to carry out the work of objective B and C, the results of which are described and discussed in the next sections.

## 5.3. Comparison of *L. monocytogenes* growth rates obtained with the FSSP predictive model and those obtained through bibliographic databases.

In Table 14, a numerical summary of the dataset obtained from bibliographic research is presented. Bibliographic databases contain many growth rates in a wide variety of environmental conditions and therefore the variability of the data is very high. In many articles, it is checked whether adding or removing certain components affects the growth of the bacteria.

Mean of growth rates obtained with the records retrieved from ComBase Browser database was higher than the one obtained with Science Direct database. Results showed that standard deviation was greater than the average in Browser database and not greater but it approached to the average of the group in Science Direct database, indicating a huge variation in their dataset.

**Table 14**. Numerical summary from *L. monocytogenes* growth rates of bibliographic databases; ComBase Browser and Science Direct.

| | **Combase Browser** | **Science _Direct** |
|---|---|---|
| **Mean** | 0.0205 | 0.0152 |
| **sd** | 0.0603 | 0.0101 |
| **IQR** | 0.0095 | 0.0127 |
| **minimum** | $1.73 \times 10^{-6}$ | $4.95 \times 10^{-5}$ |
| **$p_{25}$** | 0.004 | 0.0079 |
| **$p_{50}$** | 0.0073 | 0.0141 |
| **$p_{75}$** | 0.0135 | 0.0205 |
| **maximum** | 0.6062 | 0.0471 |
| **N** | 659 | 204 |
| **p-value Shapiro-Wilk test** | $2.2 \times 10^{-16}$ | $5.32 \times 10^{-6}$ |

A non-normal distribution was followed by dataset (p-value < 0.05). Most of the growth rates obtained through these databases were closer or equal to zero. This could be explained because these records showed the results of challenge tests assessing the growth inhibition capability of a particular combination of factors. There were also some high growth rates which were associated with the experiments assessing the *L. monocytogenes* growth in RTE cooked meat products with no added growth inhibitors.

Graphically (Figure 8) and numerically with the summary Table 14, it was observed how growth rates obtained from the Science Direct database were smaller and had less variability than the ones obtained with ComBase Browser database. The presence of extreme values in ComBase Browser was what causes a greater standard deviation compared to the average of its dataset.

In order to see if there were significant differences between doing a risk assessment through using microbiological predictive models or bibliographic databases, a Wilcoxon test was carried to compare the growth rates obtained from these three sources (Table 15). As seen before, FSSP was the microbiological predictive model which provided the most accurate growth rates, thus it was the predictive model chosen to be compared with.

Results obtained by the analysis showed how growth rates obtained with the FSSP model were statistically significant different (p-value<0.05) from those provided for Science Direct but not (p-value>0.05) from those provided for ComBase Browser. Also, there were statistical significant differences between growth rates obtained from both bibliographic databases.

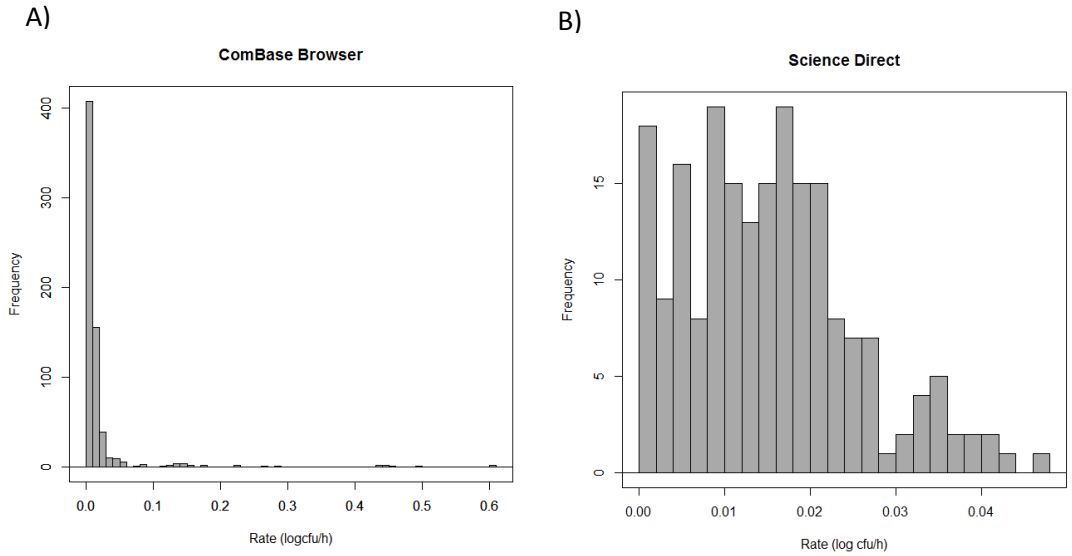


**Figure 8.** A) Graphical representation of the absolute frequency of the *L. monocytogenes* growth rates from ComBase Browser database. B) Graphical representation of the frequency of the *L. monocytogenes* growth rates from Science Direct database.

**Table 15.** P-values of Wilcoxon test when comparing *L. monocytogenes* growth rates obtained with bibliographic databases and the FSSP model.

| Methods compared | p-value Wilcoxon test |
|---|---|
| **Browser vs Science Direct** | $4.442 \times 10^{-11}$ |
| **Browser vs FSSP** | 0.345 |
| **Science Direct vs FSSP** | 0.00676 |

The number of growth rates provided for ComBase Browser was very high (n=659), which makes it contained a great variability within its dataset. Growth rates obtained with the predictive model FSSP and those from Science Direct may be included in this variability. Therefore, a more exhaustive analysis of the dataset was made.

Given the fact that the physicochemical studied factors that affect the growth rate of *L. monocytogenes* were pH and organic acids, and taking also into account, the non-analytical detection of acetic acid in some products, the addition of lactic acid was used to make this deeper analysis.

Products were grouped in function of having or not added lactate in their formulation. It has to be kept in mind that all cooked meat products have a percentage of endogenous lactate (about 7000 ppm). Based on this grouping, growth rates of the products were analysed (Table 16).

**Table 16**. P-values of Wilcoxon test when comparing *L. monocytogenes* growth rates between products with and without added lactate in their formulation obtained within each method (bibliographic databases and FSSP model).

| Added lactate vs without added lactate | P-value Wilcoxon test |
|---|---|
| **FSSP** | 0.0846 |
| **ComBase Browser** | $2.714 \times 10^{-11}$ |
| **Science Direct** | $1.09 \times 10^{-4}$ |

The first result obtained was that there were significant differences (p-value<0.05), within each bibliographic database, in growth rates between those provided for products with added lactate and those which not. On other hand, these differences were not significant (p-value>0.05) in growth rates obtained within the FSSP dataset. It has to be said that the number of growth rates obtained by FSSP (n=47) was lower compared to the ones provided for bibliographic databases (n=659 and n=204). Probably, if it had been obtained more growth rates through analysing more products with FSSP, a significance between the two groups of products would be found.

Once the difference between growth rates for products with and without added lactate in their formulation has been demonstrated, it was studied whether there were significant differences between the three methods used to assess the microbiological risk in products with and without added lactate.

When evaluating *L. monocytogenes* growth rates obtained from products which did not have added lactate in their formulation (only have endogenous lactate) (Table17, Figure 9), no significant differences were found between growth rates provided for bibliographic databases and those obtained through the FSSP model (Table 18). Although that, significant differences between the growth rates of the two bibliographic databases were present. As it can be seen, ComBase Browser is the database which provides the higher growth rates compared to others, and as a counterpart, Science Direct is the one which provides the lowest (Table 17).

**Table 17**. Numerical summary of *L. monocytogenes* growth rates of products, without added lactate in their formulation, obtained with bibliographic databases and the FSSP model.

| Without added Lactate | FSSP | ComBase Browser | Science Direct |
|---|---|---|---|
| **Mean** | 0.0118 | 0.0214 | 0.0157 |
| **Sd** | 0.0074 | 0.0617 | 0.0100 |
| **IQR** | 0.0120 | 0.0096 | 0.0121 |
| **Minimum** | 0 | $1.73 \times 10^{-6}$ | $6.24 \times 10^{-5}$ |
| **$P_{25}$** | 0.0063 | 0.0044 | 0.0087 |
| **$P_{50}$** | 0.0150 | 0.0077 | 0.0155 |
| **$P_{75}$** | 0.0183 | 0.0140 | 0.0208 |
| **Maximum** | 0.0201 | 0.6062 | 0.0471 |
| **n** | 30 | 628 | 194 |
| **P-value Shapiro-Wilk test** | 0.0002951 | $2.2 \times 10^{-16}$ | $2.265 \times 10^{-5}$ |
| **P-value Kruskal-Wallis test** | | $1.15 \times 10^{-11}$ | |

**Table 18**. P-values of Wilcoxon test when comparing *L. monocytogenes* growth rates, in products without added lactate in their formulation, obtained from bibliographic databases and FSSP model.

| Without added lactate | P-value Wilcoxon test |
|---|---|
| **FSSP vs Browser** | 0.1729 |
| **FSSP vs Science Direct** | 0.08044 |
| **Browser vs Science Direct** | $1.892 \times 10^{-11}$ |

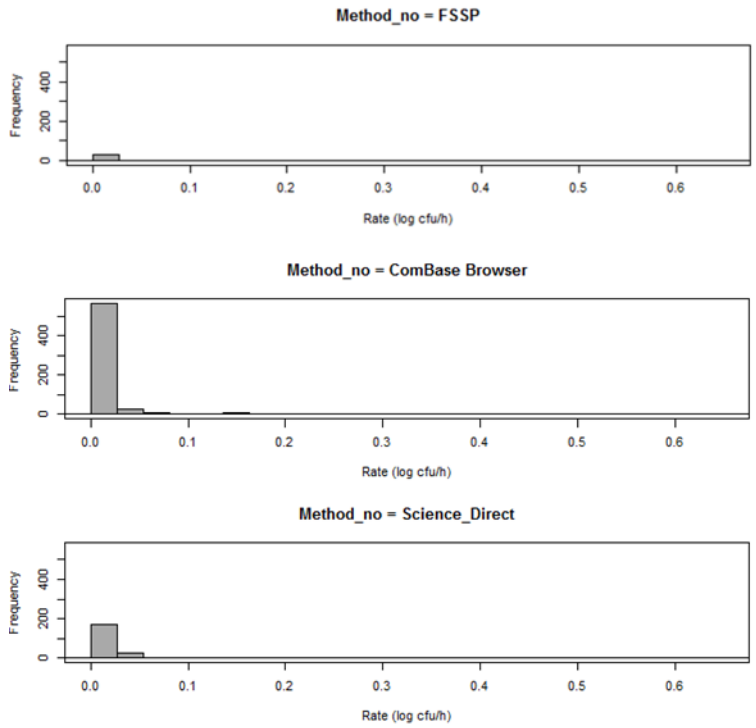


**Figure 9**. Graphical representation of the absolute frequency of the *L. monocytogenes* growth rates, in products without added lactate in their formulation, obtained from bibliographic databases and FSSP model.

When evaluating *L. monocytogenes* growth rates obtained from products which have added lactate in their formulation (Table 19, Figure 10), no significant differences were found between the two bibliographic databases, and between FSSP and Science Direct (Table 20). ComBase Browser is the database with the lowest growth rate values which means that probably products tested in this database contain a higher amount of lactate than those used in the other microbiological risk assessment sources. In counterpart, FSSP is the method with the highest growth rates meaning that products tested (i.e. the ones obtained from retail market) have lower amount of lactate than the others (Table 19). Because of these differences, growth rates of these two models were statistical significant different (Table 20).

**Table 19**. Numerical summary of *L. monocytogenes* growth rates of products, with added lactate in their formulation, obtained with bibliographic databases and the FSSP model.

| With added lactate | FSSP | ComBase Browser | Science Direct |
|---|---|---|---|
| **Mean** | 0.0084 | 0.0022 | 0.0043 |
| **Sd** | 0.0063 | 0.0027 | 0.0041 |
| **IQR** | 0.0099 | 0.0017 | 0.0039 |
| **Minimum** | 0 | $5.7794 \times 10^{-6}$ | $4.9500 \times 10^{-5}$ |
| **$P_{25}$** | 0.0038 | 0.0008 | 0.0019 |
| **$P_{50}$** | 0.0073 | 0.0015 | 0.0033 |
| **$P_{75}$** | 0.0137 | 0.0025 | 0.0059 |
| **Maximum** | 0.0182 | 0.0106 | 0.0134 |
| **n** | 17 | 31 | 10 |
| **P-value Shapiro-Wilk test** | 0.2498 | $2.229 \times 10^{-6}$ | 0.2029 |
| **P-value Kruskal-Wallis test** | | 0.01116 | |

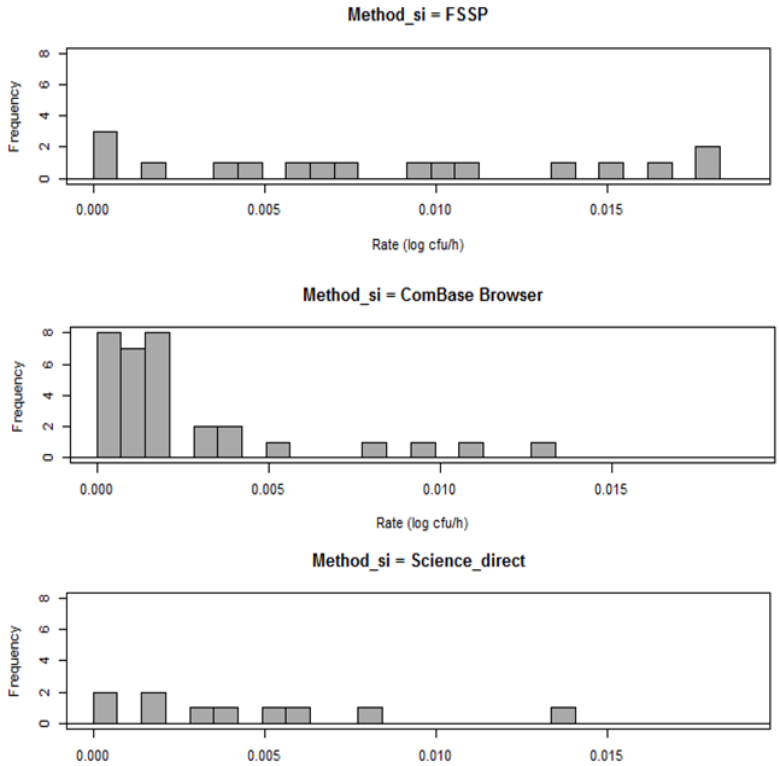


**Figure 10**. Graphical representation of the absolute frequency of the *L. monocytogenes* growth rates, in products with added lactate in their formulation, obtained from the FSSP model and bibliographic databases.

**Table 20**. P-values of Wilcoxon test when comparing *L. monocytogenes* growth rates, in products with added lactate in their formulation, obtained from bibliographic databases and FSSP model.

| With added lactate | P-value Wilcoxon test |
|---|---|
| **FSSP vs Combase Browser** | 0.00538 |
| **FSSP vs Science Direct** | 0.1595 |
| **ComBase Browser vs Science Direct** | 0.1298 |

It was studied the amount of lactic acid in products with added lactate in their formulation in order to contrast the hypothesis that a greater concentration of lactic acid implies a lower *L. monocytogenes* growth rate (Figure 11).

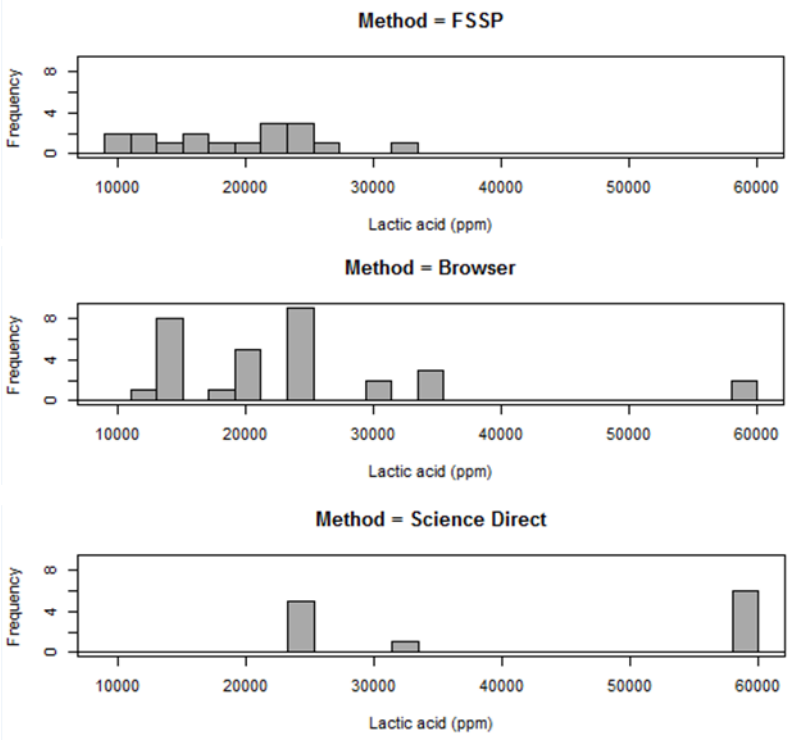


**Figure 11.** Graphical representation of the absolute frequency of the quantity of lactic acid contained in products analyzed by the FSSP model and products of bibliographic databases (ComBase Browser and Science Direct) with added lactate in their formulation.

Indeed, compared to products of bibliographic databases, products analysed with FSSP had less amount of lactic acid in their formulation (Figure 11). These products were obtained from the retail market. Bibliographic databases contain products in which had been tested the effect of the addition of some preservatives or factors on *Listeria monocytogenes* growth, most in order to inhibit its growth. Therefore, they were products manufactured ad hoc for the particular experiment not necessarily reflecting the reality of the commercially available cooked meat products. For this reason, it was normal to find a greater amount of added lactic acid in these products and thus, to obtain lower growth rate of this bacteria.

Science Direct contained more products with a higher amount of lactic acid and had less variability than the other models. The growth rates mean obtained from ComBase Browser was lower than in Science Direct although having less products with a higher amount of lactic acid. On the other hand, the mean of the growth rates of *Listeria monocytogenes*, obtained from products with a less amount of lactic acid compared to other models, had the highest value in FSSP.

Results obtained from the analysis of the growth rates of *L. monocytogenes* in products without added lactate in their formulation and by that obtained in products with added lactate

emphasise the fact that lactic acid is a factor that has a major effect on growth rates of *L. monocytogenes*. However, lactic acid is not the only determinant factor, as a product with a higher amount of lactic acid not necessarily implies a lower growth rate. The effect of preservatives on the *Listeria monocytogenes* growth is influenced by the combined effects of extrinsic and intrinsic factors of the product.

Taking into account all the results obtained through the comparison of those three methods used in *L. monocytogenes* risk assessment, there are not such a big differences between the rates obtained using predictive models and bibliographic databases as it can be seen when a large quantity of growth rates are taken from bibliographic databases, as in case of ComBase Browser.

It is important to be aware that some physicochemical factors, mainly pH, acetic and lactic acids, significantly affect the growth rate of *Listeria*, and thus, growth rates should be studied in function of the concentration of these factors on the studied products when doing the *L. monocytogenes* risk assessment in ready-to-eat cooked meat products. Therefore, the source of information (data and tools) to be used to conduct a risk assessment about the growth of *L. monocytogenes* in food (e.g. ready to eat cooked meat products) should be chosen depending on the particular aim and the scope of the study.

## 5.4. Use of *L. monocytogenes* growth rates to estimate the safe shelf-life of RTE cooked meat products.

As regulation EC 2073/2005 indicates, the maximum level allowed for *L. monocytogenes* is 100 cfu/ml. In order to quantify the effect of lactic acid in product's safe shelf life, it has been calculated, through the growth rate, the time that takes for *L. monocytogenes* to achieve these 100 cfu/ml, assuming an initial cell number of 1 cfu/g, for a temperature storage of 5°C.

This analysis has been carried out through the growth rates obtained with FSSP from those 47 ready to eat cooked meat products due to are products that are found in the market and their physicochemical factors levels were analytically quantified.

The average of *L. monocytogenes* growth rates for products with added lactate in their formulation at a storage temperature of 5°C was 0.0084 $h^{-1}$ and thus the time for *L. monocytogenes* to achieve 100 cfu/g in product was 22.84 days. In the other hand, growth rates average for products without added lactate in their formulation at a storage temperature of 5°C

was 0.0118 $h^{-1}$ and thus, the time for *L. monocytogenes* to achieve 100 cfu/g in product was 16.26 days.

Shelf-life of ready-to-eat cooked meat products is incremented with the factor 1.41 when adding lactate in their formulation and when the storage temperature is 5°C. It has to be said that shelf-life of these products was calculated for conservative conditions, i.e. without taking into account the possible lag time in which bacteria does not grow but also does not decrease.

In all study, it has been worked with growth rates at 5°C but it has to be contemplated that many home refrigerators could have another temperature storage. Moreover, predictive models allow you, in microbiological safety terms, to predict the product's safe shelf-life by testing physicochemical factors involved in *L. monocytogenes* growth and by using different temperatures storage.

# 6. Conclusions

- Physicochemical factors that mostly affect the *Listeria monocytogenes* growth in RTE cooked meat products are organic acids, such as lactic and acetic acid (their quantitative amount depending on the addition of preservative additives) as well as pH.
- There are significant differences between the growth rates of *Listeria monocytogenes* obtained with the different predictive models. Therefore, the selection of the most appropriate predictive model must be done according to the objective of the study, the environmental factors taken into account (type and range) and the product characteristics.
- FSSP is the model which could provide the most accurate growth rates of *Listeria monocytogenes* as it is the one taking more physicochemical factors into account as model inputs.
- Big differences are not seen in growth rates obtained from the FSSP predictive model and from bibliographic databases (ComBase Browser and Science Direct) when they are clustered in function of the physicochemical factors of the products from which have been calculated.
- Bibliographic databases (ComBase Browser and Science Direct) and the microbiological predictive models (FSSP) provide *L. monocytogenes* growth rates that can be used to assess the safe shelf-life of RTE cooked meat products.

- At a storage temperature of 5°C, safe shelf-life of RTE cooked meat products, calculated from the average of the growth rate values, is incremented with the factor 1.41 when lactate is added as antimicrobial preservative in their formulation.

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