UNIVERSIDAD DE CÓRDOBA

Facultad de Veterinaria

Departamento de Bromatología y Tecnología de los Alimentos

Programa de Doctorado en Biociencias y Ciencias Agroalimentarias

Application of Predictive Microbiology to investigate *Listeria monocytogenes* kinetics and bioprotective strategies on fishery products

Aplicación de la Microbiología Predictiva para investigar la cinética de *Listeria monocytogenes* y estrategias bioprotectoras en productos pesqueros

Tesis Doctoral presentada por

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Córdoba, Mayo 2021

TITULO: *Application of Predictive Microbiology to investigate Listeria monocytogenes kinetics and bioprotective strategies on fishery products*

AUTOR: *Araceli Bolívar Carrillo*

TÍTULO DE LA TESIS: Application of Predictive Microbiology to investigate Listeria monocytogenes kinetics and bioprotective strategies on fishery products

DOCTORANDO/A: Araceli Bolívar Carrillo

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

La doctoranda Dña. Araceli Bolívar Carrillo ha realizado satisfactoriamente el trabajo de investigación presentado en esta memoria de tesis habiendo alcanzado todos los objetivos planteados en la memoria inicial y en los plazos previstos dentro de su beca FPU del Ministerio. El trabajo ha abordado un análisis del potencial de crecimiento/inhibición de *Listeria monocytogenes* frente a cultivos bioprotectores, constituidos por bacterias ácido lácticas, en productos pesqueros (principalmente del Mediterráneo), En base a esto, la doctoranda ha desarrollado modelos cuantificando las interacciones microbianas subyacentes mediante la aplicación de la microbiología predictiva, centrando también su investigación en el efecto de las propiedades y microestructuras de la matriz alimentaria sobre el crecimiento microbiano. El inicio de las actividades de la doctoranda consistió en identificar los géneros y especies bacterianas de mayor importancia sobre el deterioro y la transmisión de enfermedades en productos pesqueros (Capítulo 1). Una vez conocida la ecología microbiana de productos pesqueros del Mediterráneo, el siguiente paso fue la modelización del crecimiento de *L. monocytogenes* en condiciones in vitro, a través de un medio de crecimiento obtenido de extracto de pescado donde se desarrolló y validó los modelos predictivos del comportamiento de este patógeno (Capítulo 2). El siguiente trabajo consistió en investigar el efecto del contenido de grasa de la matriz alimentaria en el comportamiento de *L. monocytogenes* para lo cual se diseñó un sistema modelo basado en las propiedades fisicoquímicas y microestructurales que simularán a los productos pesqueros (Capítulo 3). Esta experiencia fue llevada a cabo a través de 2 estancias de investigación de 3 meses cada una en la Universidad de Gante (Bélgica). En relación con el estudio de las interacciones microbianas, se llevó acabo la evaluación de una cepa productora de bacteriocina con actividad frente a *L. monocytogenes para su* potencial uso bioprotector en diferentes productos pesqueros listos para su consumo y del mismo modo frente a diferentes condiciones de almacenamiento, generando un nuevo tipo de modelo para la describir la interacción microbiana en alimentos (Capítulo 4 y 5).

Adicionalmente, realizó una estancia de investigación en la Universidad de Turín de 3 meses sobre la Aplicación de la Secuenciación de Próxima Generación en el estudio de comunidades microbianas en alimentos. Esta estancia, unida a las anteriormente señalas, le permitirán optar al título de doctor, con mención internacional.

La doctoranda ha sido también beneficiaria de una Ayuda para la formación de profesorado universitario "FPU del Ministerio de Educación" con las correspondientes actividades de formación y de iniciación a la docencia. En el ámbito formativo, tal y como se puede comprobar en la relación de actividades desarrolladas, la doctoranda no solo ha completado los ítem propios del programa sino que ha realizado numerosos cursos, ha participado en contratos con empresas y ha realizado el curso de formación del profesorado novel impartido por esta universidad para mejorar así su formación no solo como investigador sino también como docente. Así mismo, se ha integrado en el equipo de trabajo de un proyecto nacional. Y de las actividades propias del grupo de investigación (HIBRO, PAIDI AGR-170).

Todos los desarrollos anteriores expuestos son prueba de la calidad e impacto científico a nivel internacional, de las investigaciones realizadas por la Doctoranda, siendo más que evidente gracias a la publicación de la totalidad de los trabajos que se han derivado de la tesis en 4 publicaciones científicas en revistas Q1 como primera autora y un capítulo de libro en una editorial de reconocido prestigio dentro del campo de estudio, también como primera autora. A esto hay que añadirle un total de 8 publicaciones científicas de alto impacto, y al menos 10 comunicaciones en eventos científicos. Por todo ello, a nuestro juicio cumpliendo con los requisitos necesarios para optar al grado de doctor, se autoriza la presentación de la tesis doctoral.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 21 de mayo de 2021

Firma del/de los director/es

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"Para trascender en la vida, para ser de verdad,

hay que ampliar horizontes, hay que cambiar de lugar"

(María Zambrano)

A mis padres,

por apoyarme siempre

Agradecimientos

En todo proyecto o trabajo de investigación participan, de manera directa o indirecta, numerosas personas que contribuyen a que las metas sean alcanzadas, y esta Tesis Doctoral no es una excepción. Por ello, agradezco de la manera más especial y sincera:

En primer lugar, al Profesor Dr. D. Fernando Pérez, por su infinita capacidad de trabajo, por ayudarme de forma incondicional, por su confianza depositada en mí, y sobre todo por permitirme aprender a su lado día a día lo apasionante y lo complejo de la ciencia. Gracias también por las oportunidades ofrecidas más allá de esta tesis, porque me han hecho crecer tanto a nivel profesional como personal. Me siento enormemente orgullosa de haber sido su doctoranda.

A la Profesora Dra. Dña. Guiomar Denisse Posada, por enseñarme la destreza en el laboratorio.

Al Profesor Dr. D. Gonzalo Zurera, por sus valiosos consejos y palabras de ánimo que desde el inicio siempre me han acompañado en este camino.

Al grupo de microbiología predictiva BioTeC+ de la KU Leuven, en especial al Dr. D. Jan Van Impe y la Dra. Dña Maria Baka, por dedicarme su tiempo, amabilidad y acogerme en Gante como si fuera de ellos. Y muy especialmente a mis amigos Satya y Valeria de BioTeC+, por haber hecho de mis estancias en Gante unas de las mejores experiencias en mi vida. Gracias por las increíbles aventuras vividas, por las conversaciones infinitas, siempre con este toque científico divertido, y por demostrar lo que significa la verdadera amistad, aunque estemos lejos.

A todos mis amigos, compañeros y profesores del grupo Hibro de la Universidad de Córdoba, Jean, Isa, Ari, Mónica, el Profesor Antonio Valero, la Profesora Elena Carrasco y un largo etcétera…por su ayuda, sabios consejos, las salidas fuera de Rabanales…en definitiva, por hacer que el día a día haya sido más fácil y alegre.

A mis amigas de siempre, por quererme y respetarme como soy.

A mi familia, en especial a mis padres Francisco y Araceli, por su inagotable generosidad y apoyo incondicional en todas las decisiones que he tomado siempre. Gracias por ser y estar, por guiarme a utilizar mis alas, por volar conmigo. GRACIAS, por dármelo todo sin pedir nada a cambio.

A Juan, por hacerme feliz, por escucharme y entenderme, por llevarme de la mano.

SUMMARY

The production and consumption of fish and fishery products has notably increased during the last years. Many of these products are designed as ready-to-eat (RTE) and/or lightly preserved food and are usually consumed without prior cooking. These food commodities, and especially those requiring refrigeration, are predominantly associated with the foodborne pathogen *Listeria monocytogenes*, the causative agent of human listeriosis. The present work aims at assessing the performance of bioprotective cultures, consisting of lactic acid bacteria, on the growth/inhibition of *L. monocytogenes* on relevant fish products from the Mediterranean Sea, quantifying the underlying microbial interactions through the use of predictive models while considering the effect of the food matrix properties and microstructures on the pathogen growth and inhibitory capacity. To this end, in the first instance, an overview of the microbial ecology of Mediterranean fish and fishery products was carried out, describing the most relevant bacterial genera and species causing spoilage and human foodborne diseases (Chapter 1). A stepwise modelling approach from broth to food was designed to develop and validate predictive growth models of *L. monocytogenes* using data obtained in relevant Mediterranean fishery products (Chapter 2). A new fish-based model system was developed with stable physicochemical and microstructural properties generating a reliable simulation environment of fish products to investigate the isolated food matrix fat content effect on *L. monocytogenes* growth dynamics (Chapter 3). The performance of the bioprotective *Latilactobacillus sakei* CTC494 (a bacteriocin-producing strain with recognized antilisterial activity) against *L. monocytogenes* was investigated in different RTE fish products and storage conditions (Chapter 4 and 5). Based on the generated experimental data, microbial interaction models were deployed to describe the competitive growth of *L. sakei* CTC494 and *L. monocytogenes* in RTE fish products (Chapter 4 and 5). A new proposed expanded Jameson-effect model, which included and additional interaction coefficient (β) for lag time, allowed for description of not only antagonistic but also mutualism-based interactions based on their influence on lag time (λ) (Chapter 5). Results of this Thesis demonstrate that mathematical models can be deployed as reliable and efficient tools to assess the performance of bioprotective culture-based strategies and set the optimal application conditions to reduce the risk of listeriosis associated with the consumption of fishery products.

RESUMEN

La producción y el consumo de pescado y productos pesqueros ha aumentado notablemente durante los últimos años. Muchos de estos productos se comercializan como alimentos listos para el consumo (RTE) y/o mínimamente procesados, y, por lo general, se consumen sin un cocinado previo. Estos productos alimenticios, y especialmente los que requieren refrigeración, se encuentran asociados frecuentemente al riesgo de contaminación por el patógeno *Listeria monocytogenes*, el agente causante de la listeriosis humana. El presente trabajo tiene como objetivo evaluar el potencial de cultivos bioprotectores, constituidos por bacterias ácido lácticas, sobre el crecimiento/inhibición de *L. monocytogenes* en productos pesqueros de especial relevancia del mar Mediterráneo, cuantificando las interacciones microbianas subyacentes mediante el uso de modelos predictivos teniendo en cuenta el efecto de las propiedades y microestructuras de la matriz alimentaria sobre el crecimiento microbiano. Con este fin, en primer lugar, se llevó a cabo una descripción general de la ecología microbiana de productos pesqueros del Mediterráneo, presentando los géneros y especies bacterianas de mayor importancia asociadas con la alteración y con enfermedades transmitidas por los alimentos (Capítulo 1). Mediante un enfoque de modelización cuantitativa, se desarrollaron modelos predictivos de crecimiento para *L. monocytogenes* utilizando datos obtenidos en productos pesqueros del Mediterráneo (Capítulo 2). El desarrollo de un nuevo sistema modelo con propiedades fisicoquímicas y microestructurales estables permitió generar un entorno de simulación de productos pesqueros apropiado para investigar el efecto aislado del contenido de grasa de la matriz alimentaria en la dinámica de crecimiento de *L. monocytogenes* (Capítulo 3). El potencial bioprotector de *Latilactobacillus sakei* CTC494 (una cepa productora de bacteriocina con actividad listericida reconocida) se evaluó frente a *L. monocytogenes* en diferentes productos pesqueros RTE y condiciones de almacenamiento (Capítulo 4 y 5). En base a los datos experimentales generados, se implementaron modelos de interacción microbiana para describir el crecimiento competitivo de *L. sakei* CTC494 y *L. monocytogenes* en productos pesqueros RTE (Capítulo 4 y 5). La ampliación de un modelo de interacción microbiana basado en el efecto Jameson mediante la incorporación de un coeficiente de interacción adicional (β) para el parámetro cinético tiempo de latencia (λ) permitió la descripción de interacciones no solo basadas en el antagonismo sino también en el mutualismo (Capítulo 5). Los resultados de esta Tesis demuestran que modelos de microbiología predictiva pueden implementarse como herramientas fiables y eficientes para evaluar el uso de estrategias basadas en cultivos bioprotectores y establecer las condiciones óptimas de aplicación para reducir el riesgo de listeriosis asociado con el consumo de productos pesqueros.

Palabras clave: biopreservación; productos pesqueros; *Listeria monocytogenes*; bacterias productoras de bacteriocinas; microestructura; cinética de crecimiento; microbiología predictiva; interacción microbiana; biología computacional

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LIST OF ABBREVIATIONS

AICc	Akaike Information Criterion
ANOVA	Analysis Of Variance
APROMAR	Spanish Aquaculture Producers Association
ASZ	Acceptable Simulation Zone
BCCM	Belgian Coordinated Collections of Microorganisms
BHI	Brain Heart Infusion
CCP	Critical Control Point
CECT	Spanish Type Culture Collection
CFU	Colony Forming Unit
CPD	Critical Population Density
CTAQUA	Andalusian Aquaculture Technology Centre
EC	European Commission
EFSA	European Food Safety Authority
EU	European Union
EUMOFA	European Market Observatory for Fisheries and Aquaculture Products
FAO	Food and Agriculture Organization of the United Nations
FDA	United States Food and Drug Administration
FSIS	Food Safety and Inspection Service (United States Department of Agriculture)
GRAS	Generally Recognized As Safe
HACCP	Hazard Analysis and Critical Control Points
ICMSF	International Commission on Microbiological Specifications for Foods
IRTA	Institute of Agrifood Research and Technology
ISO	International Standard Organization
LAB	Lactic Acid Bacteria
LMG	Laboratory of Microbiology of Ghent University
LSD	Least Significant Difference
MPN	Most Probably Number
MRS	Man, Rogosa and Sharpe broth
MVC	Mesophilic Viable Count
NPN	Non-Protein Nitrogen
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
QPS	Qualified Presumption of Safety
RMSE	Root Mean Squared Error

RTE   Ready-To-Eat

SD   Standard Deviation

TVBN   Total Volatile Basic Nitrogen

I. INTRODUCTION

Introduction

The world human population is expected to exceed 9 billion by 5050, according to the Food and Agricultural Organization of the United Nations (FAO). To support this, food production will have to increase by 70%. Aquaculture is nowadays the fastest-growing food production system and its position is projected to be maintained for the next two decades. The European Union (EU) is the first and most relevant world market for aquatic products (APROMAR, 2020). In particular, Spain is home to the largest fish processing industry in the EU (EUMOFA, 2019).

The aquaculture sector faces important challenges such as the environmental sustainability of the production systems or the post-harvest quality and economic loss management. In relation to the latter, at least 30% of aquatic products are discarded during post-harvest due to microbial spoilage (Odeyemi et al., 2020). Foodborne diseases are also a prime concern for the fish industry. According to the last EFSA report on zoonoses, 'fish and fishery products' is the food group most frequently implicated in strong-evidence outbreaks in the EU, accounting for 27% total outbreaks (EFSA, 2021). *Listeria monocytogenes*, the causative agent of human listeriosis, is nowadays one of the most serious foodborne pathogens under EU surveillance due to its high fatality rate (17.6%) (EFSA, 2021). This pathogenic bacterium represents an important microbial risk in ready-to-eat (RTE) foods due to its psychrotrophic and ubiquitous nature as well as the absence of a listericidal process prior to consumption (Buchanan et al., 2017). In 2018, 'fish and fish products' was the second food category causing strong-evidence foodborne listeriosis outbreaks in the EU (EFSA, 2019). Although the numbers of *L. monocytogenes* in this food category at retail are generally low, there are several studies demonstrating significant growth potential of the pathogen in different fish matrices during product shelf life (Aymerich et al., 2019; Eicher et al., 2020; Mejlholm and Dalgaard, 2009).

In this context, biopreservation, also called bioprotection or biocontrol, is an alternative and sustainable strategy to extend food shelf life and enhance product safety which is in line with the current consumer demands for chemical-free foods. It comprises the deliberated use of (live) microorganisms or their metabolites without modifying food sensory characteristics (Wiernasz et al., 2017). Lactic acid bacteria (LAB) are considered a new generation of food additives and generally recognized as safe (GRAS) for this purpose. They are selected for their antimicrobial properties based on a combination of different mechanisms such as displacement/exclusion (regarded as the ability of bacteria cells to adhere strongly to surfaces), production of antimicrobial compounds and competition for nutrients and space (Said et al., 2019).

Foods represent for complex biological and physicochemical ecosystems in which different microbial populations coexist and interact. In this regard, particular rheological, compositional and microstructural characteristics of a food product may locally affect the microecological

conditions within the food matrix and thus the ability of microorganisms to proliferate (Verheyen et al., 2018). Therefore, microbial ecology and food microstructure may play, under certain conditions, an important role in determining the safety of a food product. Predictive Microbiology, also known as quantitative microbial ecology, is a useful approach to characterize and quantify the behaviour of microorganisms in food as a function of extrinsic and intrinsic factors (Serra-Castelló et al., *in press*). In addition, predictive models are considered as scientifically validated tools to comply with legal requirements for food safety.

Over the last decade, the importance of considering the effect of microbial interactions during predictive (growth) model development has been highlighted (Cornu et al., 2011). In this regard, a number of mathematical models have been developed to describe the competitive growth of indigenous microbiota (mainly LAB) and pathogenic bacteria assuming that microbial interactions in foods only limit (reduce) the maximum population density. This has been traditionally based on a simple competition hypothesis, the so-called Jameson-effect, that describes the simultaneous deceleration of all microbial populations that may be result from non-specific competition for a common limiting resource (Jameson 1962). To model this interaction, a generic primary growth model coupled with a logistic deceleration function can be used (Giménez and Dalgaard, 2004). However, the development of such models becomes more complex due to the presence of indigenous or intentionally added cultures with specific antagonistic activities (e.g., bacteriocinogenic or acidophilic LAB cultures) based on the modification of the medium where both microorganisms coexist by modifying their physicochemical properties or producing target specific antimicrobial compounds like bacteriocins (Cadavez et al., 2019). As stated by Cornu et al. (2011), "To circumvent cases in which the simplistic hypothesis of simultaneous deceleration is not applicable, variants of the Jameson-effect models have been proposed". Another modelling approach which has been notably applied to describe microbial interactions in food is based on the predator-prey (Lotka-Volterra) equations (Fujikawa et al., 2014; Giuffrida et al., 2007). This approach relies on the logistic growth model and includes two empirical parameters measuring the overall competitive effect of species A on species B and vice-versa.

In recent years, studies have been conducted to mathematically characterize and describe the interaction between LAB and *L. monocytogenes* in fish species mostly consumed in Nordic European countries (e.g., salmon, halibut or shrimp) (Hwang and Sheen, 2009; Mejlholm et al., 2015; Mejlholm and Dalgaard, 2015, 2009, 2007). Other fish species are of special interest since their relevant consumption patterns and added value in Mediterranean countries, such as sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*). Interestingly, it has recently been demonstrated that predictive modelling approaches can be used to assess and optimize the

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application of bioprotective cultures in order to reduce the risk of listeriosis associated with the consumption of fishery products (Costa et al., 2019).

The present work aims at assessing the performance of bioprotective cultures, consisting of LAB, on the growth/inhibition of *L. monocytogenes* on relevant fish products from the Mediterranean Sea, quantifying the underlying microbial interactions through the use of predictive models while considering the effect of the food matrix properties and microstructures on the pathogen growth and inhibitory capacity. To this end, different experimental and review works were performed, which are presented herein as specific Thesis chapters.

First, an overview of the microbial ecology of Mediterranean fish and fishery products was carried out, describing the most relevant bacterial genera and species causing spoilage and human foodborne diseases, and presenting the molecular methods often used for pathogen detection in fishery products. This work is presented in **Chapter 1** of this thesis.

Considering the utmost importance of *L. monocytogenes* in fish products identified in **Chapter 1**, this pathogen was subject of a specific quantitative analysis by using Predictive Microbiology. Thus, a stepwise modelling approach from broth to food was designed by developing and applying growth models of *L. monocytogenes* in fish-based juice and later validated using growth data obtained in two relevant Mediterranean fishery products (filleted sea bream and sea bass). In this approach, the effect of different atmospheric conditions and storage temperatures was evaluated. The experimental setup and modelling approach as well results of this work are presented in **Chapter 2.**

Food matrix affects microbial behaviour, and therefore, the inclusion of food microstructural factors describing the impact on growth dynamics is needed for the development of accurate predictive models. In **Chapter 3**, the isolated effect of fat content on *L. monocytogenes* growth kinetics was investigated in two fish-based systems by means of predictive growth models.

In **Chapter 4**, the inhibitory effect of a selected bacteriocinogenic LAB culture (i.e., *Latilactobacillus sakei* strain CTC494) on *L. monocytogenes* was evaluated on RTE hot-smoked sea bream and microbial competition models were deployed to describe the interaction of both populations underpinned on predictive models and kinetic parameters generated in previous works.

Understanding the role of food-related factors on the efficacy of protective cultures is essential to attain optimal results for designing bioprotective culture-based strategies. **Chapter 5** presents a first attempt to deep into the quantitative characterisation of microbial interaction considering the significance of two different RTE fish products on the efficacy of *L. sakei* CTC494 against *L. monocytogenes* by using microbial interaction models. A new expanded version of an existing

interaction model was proposed allowing for description of different microbial competition patterns.

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II. OBJECTIVES

Objectives

1) To provide an overview of the microbial ecology of Mediterranean fish and fishery products, presenting the most significant spoilage and pathogen microorganisms as well as molecular methods used for pathogen detection (*Chapter 1*).

2) To develop and validate predictive growth models of *L. monocytogenes* in Mediterranean fish species from aquaculture production (*Chapter 2*).

3) To investigate the influence of food matrix by isolating the effect of fat content on *L. monocytogenes* growth kinetics in fish product models (*Chapter 3*).

4) To quantitatively characterize the bioprotective effect of *L. sakei* strain CTC494 against *L. monocytogenes* under constant and dynamic storage temperatures in ready-to-eat fish products by using microbial interaction models (*Chapter 4*).

5) To evaluate exiting microbial interaction models and propose new modelling approaches to describe the competitive growth of *L. sakei* CTC494 and *L. monocytogenes* in ready-to-eat fish products (*Chapter 5*).

III. RESULTS

Chapter 1: Characterization of foodborne pathogens and spoilage bacteria in Mediterranean fish species and seafood products

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1.1. Fish quality assurance

Traditionally, fish processors have regarded quality assurance as the responsibility of the regulatory governmental agency, and the means used by these agencies have been the formulation of food laws and regulations, inspection of facilities, and processes and final product testing. The processors' own efforts have in many cases been based entirely on final product testing. Such a system is costly and ineffective; it provides no guarantee of quality but merely a false sense of safety. At this point, quality assurance (QA), according to International Standards (ISO 8402), can be defined by "all those planned and systematic actions necessary to provide adequate confidence that a product or service will satisfy given requirements for quality". In other words, QA is a strategic management function, which establishes policies, adapts programs to meet established goals, and provides confidence that these measures are being effectively applied.

1.2. Microbiological standards to be accomplished

Microbiological hazards in foodstuffs form a major source of foodborne diseases in humans. From January 1, 2006, the European Union (EU) health conditions for the production and placing on the market of fishery products are laid down in the consolidated Hygiene Regulations 852/2004, 853/2004, and 854/2004 and Official Feed and Food Control Regulation 882/2004. Seafood production in third countries (not EU member states) has to match EU standards in terms of hygiene and food safety. That means that it is covered by the same general principles of food law and food safety as exist in the EU. These general principles are laid out in Council Regulation 178/2002. The EU hygiene legislation also applies Regulation (EC) No. 2073/2005, which establishes microbiological criteria for a range of foods. The aim of this legislation is to complement food hygiene requirements, ensuring that foods being placed on the market do not pose a risk to human health, and the legislation applies to all businesses involved in food production and handling. Chapter 1 of the regulation focuses on food safety criteria that cover foods such as ready to eat foods, fishery products, and live bivalve molluscs. Chapter 2 focuses on process hygiene criteria, with Chapter 2.4 referring to fishery products.

Rules relating to visual inspections for the detection of parasites in fishery products are stated in Annex II (obligations on the competent authorities) of Regulation (EC) No. 2074/2005. This covers total volatile basic nitrogen (TVBN) limits and how to determine them.

A number of microbiological tests in fishery products are used by authorities to check their microbiological status. The purpose of these tests is to detect pathogenic bacteria (Salmonella, Staphylococcus aureus, and E. coli), indicator organisms of fecal pollution or other types of general contamination or poor handling practices (coliform bacteria, faecal streptococci, and total

viable count) (Huss, 1995a). The most widely accepted microbiological criteria for chilled and frozen raw fish are those set for aerobic plate counts (APC) at 25°C and E. coli proposed by the International Com- mission on Microbiological Specifications for Foods (ICMSF).

The following regulations about fish designations need to be considered:

- Regulation (EC) No 104/2000 on the common organisation of the markets in fishery and aquaculture products.
- Regulation (EC) No 2065/2001 laying down detailed rules for the application of Council Regulation (EC) No 104/2000 as regards informing consumers about fishery and aquaculture products.

International requirements are based upon having a risk-based, preventive management system in place at all stages of the supply chain. The requirements for this preventive approach is described as follows:

- Fish and fishery products should be prepared in plants certified by the local competent authority. All certified plants should comply with the good hygienic practices (GHP).
- The fisheries industry should take responsibility, implement, and maintain safety management systems based upon HACCP.
- The national competent authority is responsible for the certification of fish processing and manufacturing plants, verification of effective systems, and issuing of certificates of compliance for export products. This includes auditing and inspection programs.
- National surveillance and monitoring pro- grams should be in place to demonstrate that all identified hazards are under control, for example, biotoxins, and to identify potentially emerging hazards.

The (International) Codex Committee has published a Code of Practice for Fish and Fishery Products. In the United States, the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) has published a number of recommendations on the safety of modified-atmosphere packaging and vacuum packaging refrigerated raw fishery products.

The U.S. Food and Drug Administration (FDA) operates a mandatory safety program for all fish and fishery products under the provisions of the Federal Food, Drug and Cosmetic (FD&C) Act, the Public Health Service Act, and related regulations. The FDA program includes research, inspection, compliance, enforcement, outreach, and the development of regulations and guidance. As a cornerstone of that program, FDA publishes the Fish and Fisheries Products Hazards and Controls Guidance, an extensive compilation of the most up-to-date science and policy on the hazards that affect fish and fishery products and effective controls to prevent their occurrence.

The fourth edition of this guidance document, which has become the foundation of fish and fishery product regulatory programs around the world, is now available.

1.3. Hazard Analysis and Critical Control Points (HACCP) implemented in the fishery industry

The global legislative requirement for controlling the safety (and quality) of fish and seafood products is to apply a preventive, risk-based system based upon the seven principles of Hazard Analysis and Critical Control Point (HACCP) supported by pre-requisite or good practices programs at all stages of the food chain, from farm to fork. Therefore any microbiological criteria need to be part of this HACCP-based system to verify that the system is under control. One reason for this development is that a number of national food legislations today are placing full responsibility for food quality on the producer (e.g., EEC Council Directive no. 91/493/EEC) and the use of the HACCP system is required.

The HACCP team should ideally have access to expertise on the appropriate fisheries, including practices on board fishing vessels, or in aquaculture operations. These factors are likely to have a significant effect on some potential hazards, for example, contamination by foodborne pathogens as a result of time/temperature abuse or unsatisfactory handling practice. In addition, there may be species-specific hazards related to certain fish and shellfish, such as enteric viruses in shellfish, marine toxins associated with particular fish and shellfish species, aquaculture drug residues, and potentially pathogenic *Vibrio* spp. naturally present on fish from warm waters ($> 15 \, °C$). Expertise on such inherent hazards, challenge testing, and inoculation studies for evaluation of safety aspects are therefore essential for an effective HACCP team.

The hazard analysis for seafood products is fairly straightforward and uncomplicated. The live animals are caught in the sea, handled and processed without any use of additives or chemical preservatives, and finally distributed with icing or freezing as the only means of preservation. Contamination with pathogenic bacteria from the human/animal reservoir can occur when the landing place is unhygienic or when the fish is washed with contaminated water. Certain products may be contaminated or carry pathogenic organisms as a part of the natural flora. If the processing does not include a kill step, the only critical control point (CCP) that can render the product safe from pathogenic organisms is adequate heat treatment during preparation. However, it must be noted here that certain toxins (algal and shellfish) are not destroyed by heat treatments; contaminated fish and shellfish should not be used.

The term "fish and seafood" includes an extremely varied group of products, and there are equally varied ranges of potential hazards associated with them. For example, there are particular hazards

associated with contamination of shellfish by microorganisms from human sewage, and the potential growth of *Listeria monocytogenes* on smoked fish.

Many of the microbiological hazards associated with fish and seafood products are derived from the raw materials. Pathogens may be part of the resident microbiota of the living animal (e.g., *Vibrio* spp.), or may originate from polluted water or from post-capture contamination (e.g., *Salmonella* spp. and viruses). The incidence of psychrotrophic, non-proteolytic *Clostridium botulinum* on fresh fish is also sufficiently high that its presence may be assumed. There may also be inherent hazards from parasites such as the roundworm *Anisakis simplex* and from marine toxins such as ciguatera in reef fish (derived from microalgae) and scombrotoxin (biogenic amines) development in fish containing high levels of histidine.

Hazards introduced during processing of fish products depend very much on the characteristics of the process. For example, modified atmosphere packed of chilled raw fish may provide conditions suitable for the growth of psychrotrophic *C. botulinum*, or poor control of batter mixes in frozen battered fish portions may allow growth of *Staphylococcus aureus* and production of enterotoxin. Therefore, it is not possible, or desirable, to generalize about expected hazards and the reader is once again referred to the appropriate product chapter for additional advice on specific hazards.

It can be said that effective control measures are likely to include the following:

- Careful selection of sources for raw materials
- Adequate temperature control
- Effective sanitation
- Food handler hygiene
- Prevention of cross-contamination

Critical limits separate acceptable from unacceptable products. Where possible, critical limits should be specified and validated for each CCP. More than one critical limit may be defined for a single step. For example, it is necessary to specify both time and temperature for a thermal process. Criteria used to set critical limits must be measurable and may include physical, chemical, biological, or sensory parameters. Some examples relevant to fish products are:

- Time and temperature limits for pasteurized and cooked products
- Temperature limits for chilled fish
- Brine concentration in cured fish
- Water activity (aw) values for dried fish
- pH values in fermented fish
- Microbiological quality of water in shellfish production areas

1.4. Microbial ecology of Mediterranean fishery products

1.4.1. Chilled and frozen fish

Fresh fish is a food that undergoes biochemical and microbiological changes over storage from capture until consumption. It is a product highly susceptible to rapid spoilage that leads to a short shelf life. Fish spoilage is mainly caused by oxidation of lipids, denaturation of proteins by enzymatic activity of fish enzymes, as well as microbial enzymes, and production of off-odor metabolites.

The microbial population of fish is a mixture of subpopulations present on the live animal and those microorganisms contaminating the product during processing and handling. Fish micro-biota largely varies with the pollution level and temperature of the water (Gram, 2009). Bacteria from many groups, as well as viruses, parasites, and protozoa, can be present in the raw materials. Each processing operation has its own unique microbiota reflecting the raw materials and the preservation parameters used (BaggeRavn et al., 2003). Among these microbial groups, only a few parts will be able to tolerate the product specific conditions (i.e., temperature) and proliferate during subsequent storage.

Fish muscle is initially sterile, but scales, gills, and intestines harbor high microbial loads. Fresh or warm-water fish tend to have a biota that is composed of more mesophilic gram-positive bacteria than cold-water fish, which tends to be largely gram-negative (Jay et al., 2005). Fish can have around 10^3 to 10^8 bacterial cells/g. In general, the microorganisms that are part of the microbial ecology of Mediterranean fish can highlight genera as total mesophilic aerobic, psychrotrophic, sulphite-reducing clostridia (SRC), *Aeromonas*, *Enterobacter, Escherichia coli*, *Lactobacillus*, *Listeria*, *Salmonella*, *Pseudomonas*, *Photobacterium*, *Shewanella*, *Vibrio*, yeasts, and some molds (Carrascosa et al., 2015; Esteve et al., 2012; Koutsoumanis and Nychas, 2000).

The effects of chilling and freezing on fish are well documented (Yin et al., 2014; Ladip et al., 2013; Viegas et al., 2013). Bacteriological spoilage largely depends on type of fish microbiota, thus influencing on product shelf life. The effect of biochemical changes induced by the bacterial growth on fish spoilage is not pronounced until the specific spoilage organisms have increased to a certain level (Gram and Huss, 1996). In addition, as microbial counts increase, enzymes secreted by microorganisms may also cause additional softening of the fish (Nielsen et al., 2001).

During storage at cooling temperatures, death and sub-lethal injury is initially more pronounced (about 7 days) and decrease during storage afterward. Temperature fluctuations are the most significant factors affecting the quality of both chilled and frozen fish (Flemming et al., 2014). The effect is more pronounced with gram-negative organisms such as *E. coli*, *Pseudomonas aeruginosa*, and *Salmonella* Typhimurium. The lethal effect of freezing on microorganisms in

fishery products varies depending on storage duration, rate of cooling and thawing, and storage temperatures (IIR, 1986). Incidence of pathogens, including *Salmonella*, *Vibrio*, and *Listeria* spp. in frozen fishery products has been of great concern in the international trade of the commodities.

During freezing, as water temperature is being reduced, a large proportion of water becomes frozen, thus growth of most microbial species is inhibited, apart from some psychrophilic bacteria, yeasts, and molds. At –20 °C most cells have lethal or sub-lethal injury. The freezing rate dictates the extent of microbial damage resulting from the formation of ice crystals. Bacteria viability of often lost at abrupt temperature decreases (i.e., from 37 to 0 °C) (Venugopal, 2006).

1.4.2. Molluscan/crustacean shellfish

The microbiota of molluscan/crustacean shellfish is more variable than that of fish. The number and type of microorganisms present in the aquatic habitat, from which the animals were harvested, depend various factors such as salinity, environmental conditions, water tempera- ture, feeding regime, fish capturing, and chilling conditions (Vernocchi et al., 2007; Cao et al., 2009). The fundamental differences in spoilage of these foods are referred, generally, to the way in which they are handled, and to their specific chemical composition (Jay et al., 2005).

Molluscan shellfish are filter feeders and can concentrate toxic substances and microorganisms. Several studies have reported that contaminants, such as heavy metals (Copat et al., 2013), viruses such as human noroviruses or hepatitis A virus (HAV) (Campos and Lees, 2014; Suffredini et al., 2012), bacteria (Iwamoto et al., 2010), and marine toxins produced by algae (USFDA, 2012) can be accumulated in shellfish. Therefore, bivalves can act as carriers of food contaminants and under certain circumstances, they can produce human diseases.

Molluscs usually have a resident bacterial population that in the case of oysters fluctuates between 10^4 and 10^6 cfu/g of tissue, being the higher counts present when water temperatures are high (ICMSF, 2005). The dominant groups of bacteria found in shellfish are gram-negative of the genera *Vibrio*, *Pseudomonas*, *Acinetobacter*, *Moraxella*, *Aeromonas*, *Flavobacterium*, and *Alcaligenes*. Lower numbers of gram-positive bacteria may also be present such as species of *Bacillus*, *Corynebacterium*, and *Micrococcus*. Bacterial genera such as *Escherichia*, *Enterobacter*, and *Lactobacillus* were isolated in spoiled oysters (Jay et al., 2005).

Regarding crustacean species, microbial spoilage of shrimps is more prevalent than that of crabs and lobsters. Whereas crabs and lobsters remain alive until they are processed, shrimps die during harvest. The flesh of crustacean is rich in NPN compounds (amino acids, especially arginine and trimethylamine oxide), contains ca 0.5% glycogen, and has a pH above 6.0 (Ray, 2005). These factors allow growth of spoilage microorganisms such as *Morganella* spp., *Proteus* spp., or *Pseudomonas* spp. (Matches, 1982).

The bacterial profile of freshly caught crustaceans should be expected to reflect the waters from which these foods are caught, and contaminants from the deck, handlers, and washing waters. Some representative species causing spoilage in crustaceans are *Pseudomonas*, *Acinetobacter*, *Moraxella*, and yeasts (Jay et al., 2005). *Pseudomonas fragi* and *Shewanella putrefaciens* have been identified as the primary spoilage agents of chill-stored shrimps, with *P. fragi* spoiling iced-stored shrimp and *S. putrefaciens* being the dominant microorganism in shrimp stored in ice slurry (Chinivasagam et al., 1996). Microbial spoilage of shrimp is dominated by odor changes due to production of volatile metabolites of NPN compounds (from decay and putrefaction), slime production, and loss of texture (soft) and color. If the shrimps are processed and frozen rapidly, spoilage can be minimized. Spoilage in other crustacean species such as prawns, are characterized by the formation of amines, sulphides, and esters associated to the growth of *P. fragi* and *S. putrefaciens* (Chinivasagam et al., 1998). Lobsters are frozen following processing or sold live and thus are not generally exposed to spoilage conditions. Crabs, lobsters, and shrimps are also cooked to extend their shelf life. Blue crabs are steamed under pressure, and the meat is picked and marketed as fresh crabmeat. To extend shelf life (and safety), the meat is also heat processed (85 °C for 1min) and stored at refrigerated temperature. Under refrigerated conditions, they have a limited shelf life because of growth of surviving bacteria and post heat contaminants (Ray, 2005).

1.4.3. Cured, smoked, dried, and fermented seafood

Preservation methods like salt-curing and drying have been used for centuries to obtain fully preserved products and access to good, safe, and nutritious food in all seasons and areas where the availability of fresh food is limited (Leroi et al., 2006). Processing methods include drying, salting, smoking, pickling, and marinating of fish. There can be also different combinations of these methods and preservation of fish by fermentation (Food and Agriculture Organization (FAO), 1983; Jarvis, 1988).

The fish curing industry has flourished through the ages and has not been affected to any great extent by modern fish preservation and processing techniques. This is because cured fish is a highly appreciated and traditional product in many countries, mainly due to its excellent storage stability, special organoleptic characteristics, and nutritional value (Lauritzsen et al., 2004).

Traditionally, ground fish species (cod, haddock, ling, blue ling, and tusk) are used for salting processes, including light salting and heavy salting, mainly because fish muscle has a low lipid content. Pelagic species (herring, sardine, capelin, blue whiting, and mackerel) and salmonids (salmon, trout, and arctic char) with a higher lipid content are more suited to other curing processes (smoking, marinating). Salt-cured cod, the precursor to klipfish, and known as the traditional product *bacalao* in Spain and *bachalau* in Portugal, has had this position for centuries,

but today salt-cured cod is popular due to its sensory properties rather than lack of availability of other foods (Leroi et al., 2006).

Salt-cured and dried fish products are generally regarded as safe, even though they are produced in relatively open houses with limited possibilities to regulate temperature and maintain good hygienic conditions. It is considered that salt curing is an effective barrier against bacteria. However, rehydrated salt-cured cod spoils rapidly, and it is found that this is due to growth of *Psychrobacter* spp. These bacteria are present on the skin of fresh fish, survive in a non-growing mode during salt curing, but recover and grow during and after rehydration (Bjorkevoll et al., 2003). A number of other bacteria have also been found to survive the salt- curing step (Barat et al., 2006). Listeria spp. and Staphylococcus spp. are occasionally found in salt-cured cod products but it has not been clear whether these bacteria survive in the fish if introduced to the fish prior to salt curing or only when they are introduced directly to the salt- cured cod shortly before the sample is taken (Pedro et al., 2004).

Smoking is one of the oldest methods used to process and preserve fish and meat. Smoking introduces flavor, taste, and preservative ingredients into fish muscle by exposing fish to the smoke produced by burning or smoldering plant materials, most often wood. The volatile compounds in smoke penetrate into the fish muscle. Smoking usually extends the shelf-life of fish due to the combination effects of (a) salting, which lowers water activity resulting microbial growth, (b) elevated temperature drying, which provides a physical surface barrier to the passage of microorganisms, and c) deposition of antimicrobial and antioxidant compounds, such as aldehydes, carboxylic acid, and phenols, which delays microbial growth and rancidity development (Efiuvwevwere and Ajiboye, 1996; Leroi and Joffraud, 2000).

Moreover, fish muscle exposed to smoke in combination with a high temperature can effectively limit harmful enzymatic reactions (FAO, 1992). The smoking process is usually characterized by an integrated combination of salting, drying, heating, and smoking steps in smoking chamber/smokehouses (Alcicek and Atar, 2010). Although the general operations in all smoked fish processing plants are similar, the specific processing procedures can vary considerably. This variability relates to differences in equipment, regional and ethnic consumer preferences, raw materials, and tradition (Flick and Kuhn, 2012). Smoked fish is a perishable food, so to maintain its good quality and to prevent foodborne illnesses it must be preserved after smoking by processing techniques. Bacteria, yeasts, and molds are microorganisms associated with smoked fish. The microbiota isolated from smoked fish include *Lactobacillus curvatus*, *L. sakei*, *L. plantarum*, *Carnobacterium* spp., *Leuconostoc* spp., *Serratia liquefaciens*, *S. grimesii*, *Enterobacter agglomerans*, *Hafnia alvei*, *Photobacterium phosphoreum*, *Brochothrix thermosphacta*, *Aeromonas* spp., *Micrococcus luteus*, *Pseudomonas* spp., *Alcaligenes* spp.,

Staphylococcus sciuri, *S. xylosus*, *Bacillus cereus*, *Raoultella ornithinolytica*, *B. thuringiensis*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *E. aerogenes*, *E. cloacae*, *Psychrobacter immobilis*, and *Shewanella putrefaciens* (Lakshmanan et al., 2002; Bjorkevoll et al., 2003; Hsu et al., 2009).

Drying is one of the thermal treatments applied in many food industries, and the use of dried foods is expanding rapidly (Senadeera et al., 2005). Drying processes can be broadly classified as (Nguyen et al., 2014):

- Thermal drying: a gaseous or void medium is used to remove water from the material, thus thermal drying can be divided into three types: (a) air drying, (b) low air environment drying, and (c) modified atmosphere drying (Rahman and Perera, 1999).
- Osmotic dehydration: solvent or solution is applied to remove water, whereas in mechanical dewatering, physical force is used to remove water. Consideration should be given to many factors before selecting a drying. These factors are (a) the type of product to be dried, (b) properties of the finished product desired, (c) the products susceptibility to head, (d) pretreatments required, (e) capital and processing cost, and (f) environmental factors. There is no one best technique for all products (Rahman and Perera, 1999).
- Mechanical and chemical dewatering. Drying is the removal of water content to safe levels that can slow down the actions of the enzymes, bacteria, yeasts, and molds. The effects of heating on the activity of microorganisms and enzymes are also important when food is dried (Rahman, 2006).

Fishes are prone to rapid microbial spoilage, thus adequate care must be taken in drying these products. Microbial standards are usually based on the total number of indicator organisms or number of pathogens. The microbial load and its change during drying and storage are important information for establishing a standard that will ensure food safety. Poor processing, handling, and storage practices often results in a limited storage life of the dried salted fish (Rahman, 2006).

Fermentation plays an important role in food technology. It is one of the oldest and most widely used food preservation methods in households and small-scale food industries as well as in large companies. Fermented foods are those in which the action of microorganisms or enzymes is involved, causing desirable bio- chemical changes and significant modifications in comparison to the fresh product (Tanasupawat and Visessanguan, 2012).

The main components of the fermentation ecosystem include microbes (yeasts, molds, and bacteria), organic materials to be fermented, a solution in which the fermentation takes place, a vessel with a controlled gate, and various tools that may be used to develop and monitor the fermentation (Scott and Sullivan, 2008).

The highest production of fermented food occurs in Europe, North America, and Africa. In Europe, traditional pickled fish products such as rakeorret in Norway, maatjes in Netherlands, and gravad fish in Scandinavia and Scotland are made with a low salt content, whereas surstromming in Sweden is produced with a medium salt content. Traditional Mediterranean products are fermented anchovies, where the fish is dry-salted and fermented at 15–20 °C. After a few days, further salt is added. After 6 months of storage, the fish is chilled, washed in brine, filleted by hand, and the fillets are blotted dry. The product is canned in vegetal oil (Venugopal, 2006).

The term "fermented fish products" is used to describe the products of freshwater and marine finfish, shellfish, and crustaceans that are processed by the combined action of fish enzymes and bacterial enzymes with salt to cause fermentation and thereby to prevent putrefaction (Ruddle and Ishige, 2010). Fish fermentation produces three different types of products: those that retain the fish in their original form, those that reduce fish to paste or structured products, and those that reduce fish to a liquid sauce (Joshi and Petricorena, 2012). Fermentation is an energy-neutral process that extends the shelf life of fish and fish contents products and increases their palatability and nutritional value. In the fermentation in fish products the muscle proteins are degraded into smaller peptides and amino acids that are nutrients for microorganisms. Hence, this process is often combined with the addition of salt or drying to reduce water activity and eliminate proteolytic and putrefying microorganisms (Salampessy et al., 2010). The basic aim of the fermentation process is to transform the highly perishable substrate muscle into a stable and safe product maintaining optimum nutritive values and sensory quality. The nature of the raw material and activity of the microorganism affects the process of fermentation (Peredes- Lopez and Harry, 1988).

In fermentation, raw materials are converted into metabolites through the activity of endogenous enzymes or microorganisms (bacteria, yeasts, and molds). The increase of bacteria with time corresponds to a decrease of pH that can be attributed to the production of acid by lactic acid bacteria (LAB) (Taira et al., 2007). Such behavior occurs in the production of both fish sauce and fish paste (Thapa et al., 2004).

The microbial species used for fermentation generally belong to *Lactobacillus*, *Streptococcus*, *Pediococcus*, or *Leuconostoc* spp. (Owens and Mendoza, 1985). Generally, the microbial population experiences a slight reduction in the first 10 days, and the first high initial microbial increase is probably due to fermentable sugar that promotes the growth of acid-forming bacteria (Kasankala et al., 2011). Xu et al. (2010) reported an increase of lactic acid bacteria (LAB) within 24 hours of fermentation and a decrease in other microbes, which can be explained by the rapid

drop in pH and an increased stress against the growth of other microorganisms such as gram-negative bacteria (Xu et al., 2008).

1.5. Fish and seafood spoilage: characterization of spoilage microorganisms during capture, manufacture, and distribution of fishery products

The fisheries and aquaculture sector has achieved to blur the outlines of the geographical borders evolving to a global market. In this way, the manufacturing and distribution chain is by road, rail, air, and sea. Because of during these stages there are a big number of intermediaries who handle these products, it is necessary to identify where and which are the microbiological agents responsible for the fishery product's damage.

For this reason, spoilage microorganisms can occur during capture, manufacture, and distribution steps.

During capture, fishery products are very susceptible to changes caused by microorganisms and to autolytic and oxidative processes. Natural spoilage processes that take place post-mortem, include not only microbiological changes, but physical, chemical, and biochemical changes affecting the initial characteristics of the product from the time of capture (Ólafsdóttir et al., 1997). These types of products, which have high non-protein nitrogen content and a high pH (>6), allow the proliferation of many microorganisms (Gram and Huss, 1996).

In order to reduce the incidence of deterioration factors and also to ensure a proper conservation of organoleptic properties, it is very important to take into account the time between the moment of capture and the reduction of temperature in the distribution chain (Ólafsdóttir et al., 2004).

There are both extrinsic and intrinsic factors that influence the intensity and rate of fish deterioration; like the species and fish physiology, age, sex, seasonality, and capture area (Huss, 1995b; Nazrul and Razzaq, 2005).

Temperature fluctuations can favor the loss of quality and especially the safety of fishery products, allowing the proliferation of spoilage microorganisms (Koutsoumanis et al., 2002; Mejlholm et al., 2010).

During fish manufacture good handling practices are essential to prevent tissue dam- age, such as cuts and wounds, which facilitate bacteria internalization and in turn, lead to cross-contamination in gutting and filleting operations, for example. By contrast, in the packaging step the deterioration rate can be reduced by using modified atmosphere or vacuum packaging, which hinder the ratio of initial microbial load and final product.

Finally, in the distribution step, autolytic processes and microbial growth on fish products can occur if abuse storage conditions are applied. The control of temperature is the most important factor to ensure the preservation of the product (Simpson et al., 2003). The multi- plication of bacterial load is responsible for the loss of quality due to the progressive deterioration during storage (Huss, 1995).

The most representative spoilage microorganisms in fishery products and their associated effects are represented in Table 1.1.

Table 1.1. Representative microbial species of spoilage bacteria in fishery products.

Spoilage microorganism	Characteristics and growth requirements	Effect on fish products
Pseudomonas spp.	Strict aerobic bacilli, gram negative, catalase and oxidase positive, able to grow in ice storage	Natural microbiota, spoilage during storage, responsible for unpleasant odors, mucus, and tissue degradation. Present in packaged products
Shewanella spp.	Facultative anaerobic bacilli, gram negative, catalase/ oxydase positive, able to grow on ice storage	Responsible for the production of unpleasant odors from (L-cisteine)
Photobacterium phosphoreum	Facultative anaerobic bacilli, gram negative, it is in the intestinal tract, able to grow at low temperatures	Responsible for the deterioration of packaged fillets, through the production of trimethylamine
Lactic acid bacteria	Able to grow in a wide range of temperature, generally psychrotrophic	Responsible for the deterioration in freshwater fish in vacuum packed, brine, and modified atmosphere products
Enterobacteriaceae	Facultative anaerobic bacilli, gram negative, mesophilic and psychrotrophic	Responsible for the deterioration of packaged products. Production of unpleasant odours and flavors
Brochothrix thermosphacta	Gram-positive bacilli, part of the dominant flora from the Mediterranean Sea	Responsible for the deterioration of packaged products in modified atmosphere and smoked vacuum-packed products

1.6. Foodborne pathogens in Mediterranean fishery products

Fishery products can be contaminated with foodborne pathogens that could be present in the water, at the processing factories or within the intestinal tract of living species.

The most representative species are summarized below:

1.6.1. *Aeromonas* spp.

It is a gram-negative, non-sporulated rod and facultative anaerobic bacterium. *Aeromonas* spp. have many similarities to the family Enterobacteriaceae. The genus is divided into two groups: psychrophilic *Aeromonas* (*A. salmonicida*) and mesophilic *Aeromonas* (single polar flagellum),

considered potentially hazardous to human health (*A. hydrophila*, *A. caviae*, *A. veronii* subsp. *sober*, *A. jandaei*, *A. veronii* subsp. *Veronii*, and *A. schubertii*).

This bacterium is ubiquitous in all freshwater environments and in brackish water. Some strains of *A. hydrophila* are able of causing gastroenteritis and other infections in humans. It is believed that disease-causing strains are only a fraction of the diversity of strains present in the environment.

Traditional techniques for its detection in foods consist on a pre-enrichment in alkaline peptone water (incubation at 30 °C for 24h). Then, Base Phenol Red Agar is used adding 10% of starch and ampicillin (10 mg/L); incubating at 30 °C for 24h. Presumptive colonies appear with a yellow halo after addition of lugol solution.

Further biochemical confirmation includes tests such as Gram stain, cytochrome c oxidase activity, catalase activity, and oxidation-fermentation of glucose. Differentiation from *Plesiomonas* spp. or *Vibrio* spp. can be achieved through the resistance to vibriostatic compound O-129. Further confirmation tests include Sulphide-Indole-Mobility (SIM), hydrolysis of aesculin, gas production from D-glucose, methylred test Voges-Proskauer, decarboxylation of L-lysine and L-ornithine and hydrolysis of L-arginine, acid production from D-mannitol, salicin, L-arabinose, inositol, and sucrose using traditional protocols in each case.

1.6.2. *Clostridium perfringes*

It is an anaerobic, but aero tolerant, bacterium. It is a gram-positive and spore-forming rod that produces enterotoxin. This bacterium is relatively psychrophilic, and its spores are heat- resistant. *C. perfringens* has been traditionally isolated from freshwater fish products, being frequently found in the intestinal tract of animal species. This is also found in improperly sterilized canned foods. *C. perfringens* has many isotopes, including the isotope A, which contains CPE gene associated to exterotoxin production. Also the isotopes B, C, D, and E might contain this gene. The infective dose is >106 vegetative cells or spores/g of food. Toxin production in the digestive tract is associated with sporulation.

The horizontal method for the enumeration of *C. perfringens* is referred to the ISO 7937: 2004, using TSC medium (Tryptose Sulfite Cycloserine) and incubating at 37 °C for 20 h under anaerobic conditions. Black colonies could be presumptive of *C. perfringens*. Biochemical confirmation is carried out in Usp Thioglycollate medium, incubated for 18-24 h at 37 °C under anaerobic conditions. Final confirmation is achieved in half Lactose Thioglycolate Sulphite Broth at 46 °C for 18–24 h. The presence of >1/4 gas production together with black colonies is confirmatory of *C. perfringens*.

1.6.3. *Clostridium botulinum*

C. botulinum is a gram-positive rod-shaped, obligate anaerobe, spore-forming, and toxin-producing bacterium (botulinum toxin, the causative agent of botulism). *C. botulinum* is classified into proteolytic (protein digestion performed and H_2S production) and non-proteolytic. There are eight types of botulinum toxins, namely A, B, C, D, E, F, G, and H. The spores can survive in most environments and they are difficult to be destroyed even at the boiling point of water. They have a special importance in canning, both animal and vegetable origin.

The bacteria growth can be prevented with acid pH (<4.5), high sugar concentration, high levels of oxygen, and low humidity. The high toxicity of botulinum toxins requires detection methods capable of toxin measurement in the low to sub pg/mL range. Polymerase chain reaction (PCR) methods can readily detect the presence of low levels of *C. botulinum* DNA but do not detect the presence or absence of the toxin. Current methods for toxin detection rely on 1) the gold standard mouse bioassay, or 2) in vitro tests such as molecular tests, immunoassays, and/or activity-based assays. Activity-based assays can detect active toxin but generally have poorer detection limits than immunoassays. New assays must also be carefully validated in individual food matrices or for as many toxin subtypes as possible, in order to establish assay performance standards.

1.6.4. *Listeria monocytogenes*

It is a gram-positive, rod-shaped, facultative bacterium, motile by means of flagella, that is among the leading causes of death from foodborne illness. It has 13 serotypes, including 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7. Among them, serotypes 1/2a, 1/2b, and 4b have been associated with the vast majority of foodborne infections. *L. monocytogenes* is recognized as salt-tolerant, surviving and growing at refrigeration temperatures, unlike many other pathogens. It has been isolated in fresh fish, processed fish, and sea- food products. Fatality rates associated to *L. monocytogenes* infection is around 15 to 30%. The infective dose of *L. monocytogenes* is undetermined, but is believed to vary with the strain and susceptibility of the host, and the food matrix involved. The enumeration and detection method for *L. monocytogenes* is based on the ISO 11290-1: 1996 and ISO 11290-2: 1998.

These methods are based on the use of Half-Fraser broth (incubation at 30 °C for 24h) and Fraser broth (incubation at 37 °C for 48 h). Then, the final culture is streaked on Oxford, Palcam and chromogenic (ALOA) selective media. Presumptive positives are finally submitted to biochemical confirmation using haemolysis, acid production (rhamnose and xylose), and cAMP assays (control strains: *S. aureus* and *Rhodococcus equi*).

1.6.5. *Salmonella*

Salmonella is a genus of rod shaped, gram-positive, non-spore-forming, and facultative anaerobic bacteria included in Enterobacteriacea family. *Salmonella* possesses peritrichous flagellas. There

are two species of *Salmonella*, *Salmonella bongori*, and *Salmonella enterica*. *Salmonella enterica* comprises six subspecies and around 2500 serovars (Wingstrand and Aabo, 2014). This microorganism exists in the intestinal tract of warm-blooded animals, though most of which are subclinical excretors. Besides that, *Salmonella* can survive in other environments such as moist soil, water, fecal material, animal feeds, and foods. Infection by Salmonella enterica is a zoonosis that can be transmitted by foods, this microorganism being of the most relevant foodborne pathogens.

For enumeration purposes, when detectable levels are expected, homogenized samples are directly cultured onto selective agar media, such as Xylose-Lysine-Desoxycholate (XLD) agar, and incubated at 37 °C for 18–24 hours. The microbiological method most widely used by food industry is that proposed by ISO corresponding to the standard ISO 6579:2002. It is an agar-based method for detection of *Salmonella* spp. in products intended for human consumption and feeding of animals and in environmental samples in the area of food production and food handling.

Basically, this method, in a first stage, develops a pre-enrichment process by using buffered peptone water incubated at 37 °C for 18 h. Then, pre-enriched culture is subcultured into two selective enrichment media, that is, Rappaport Vassiliadis Soy Broth (RVS and Muller-Kauffmann Tetrathionate-Novobiocin, and incubated for 24 h at 45.5 °C and at 37 °C, respectively. Then, selective enrichment culture showing growth are inoculated onto two selective agar media and incubated at 37°C for 24h. At least, one of the agars should correspond to Xylose-Lysine-Desoxycholate (XLD) and other optional selective agar. There are several alternatives such as Bismuth Sulphite agar, Brilliant Green agar, and Hektoen Enteric agar. A number of selective chromogenic agar media specifically designed for the differentiation of *Salmonella* colonies are commercially available. Typical *Salmonella* colonies on selective agar are sub cultured onto non-selective media prior to confirmatory testing (Dykes, 2016).

1.6.6. *Vibrio*

Vibrio is a genus of curve rod-shaped, facultative anaerobic, non-spore forming and gram-negative bacteria included in the group of Proteobacteria where *Salmonella* spp. and *E. coli* are also included. *Vibrio* spp. are important components of the coastal microbial communities, thus, most members of this genus are halophilic but sensitive to acid pH and tolerant to basic pH values. These microorganisms are oxidase positive and motile due to the presence of a flagellum. Some species are opportunistic human pathogens associated with the ingestion of contaminated water or contaminated (undercooked) seafood products and also can cause septicemia through open wounds. The most known pathogenic vibrio species are *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*.

Conventional methods to detect and enumerate pathogenic *Vibrio* spp. are based on the use of selective agar media and Most Probable Number (MPN). The most widely selective agar media for *Vibrio* isolation and enumeration is Thiosulfate-citrate-bile salts sucrose (TCBS) (Mandal and Mandal, 2014). The basis of this selective agar is the formulation of sucrose and the presence of a pH indicator. Generally, *Vibrio* spp. are not able to ferment sucrose yielding green colonies, while those fermenting sucrose results in areas of reduced pH with yellow colonies. However, the capacity of discrimination of TCBS is not adequate for some bacterial species. For example, *V. cholerae* is sucrose positive and other non-vibrio species growing on TCBS such as *Aeromonas* and *Shewanella* are sucrose negative. The medium CHROMagar Vibrio (CaV) allows to select *Vibrio* spp. using an alkaline pH = 9 and based on the formulation of different chromogenic substrates, it can discriminate between *V. parahaemolyticus* and *V. vulnificus* but not between *V. vulnificus* and *V. cholerae* (Dick et al., 2012). Conversely, TCBS can differentiate between *V. vulnificus* and *V. cholerae*. Based on the thin-agar layer method, some researchers have developed a double-layer agar plate (DLAP) method for direct enumeration of *V. parahaemolyticus* (Wang et al., 2015). This method is prepared using a chromogenic selective agar, such as Bo-chrome Vibrio medium (BCVM), as bottom layer over laid with a nonselective medium (e.g., tryptone soy agar [TSA]) supplemented with 3% NaCl. Results demonstrated that DLAP was as efficient as NMP method, showing better recovery of heat-injured *V. parahaemolyticus* (Wang et al., 2015).

The Most Probable Number (MPN) technique described in Bacteriological Analytical Manual Online is based on using ASPW, followed by plating onto selective agar, generally TCBS. In this method, results can be obtained after 5–7 days.

The method ISO/TS 21872–1 is intended to detect enteropathogenic *Vibrio* spp. particularly, *V. parahaemolyticus* and *V. cholerae*. To this end, a first selective enrichment with Alkaline Saline Peptone Water (ASPW) is used to perform the initial suspension, which should be incubated at 37 °C for 6 h for deep-frozen products or at 41.5 °C for 6 h for fresh, dried, or salted products. The second selective enrichment is also performed with ASPW with incubation at 41.5 °C for 18 h. After, incubation cultures from ASPW are used to inoculate TCBS agar plate. Likewise, a second optional selective agar medium is required such as CHROMagar Vibrio. Typical colonies will be isolated to test presumptive identification by oxidase test, microscopic examination, halotolerance test, and biochemical tests (detection of ornithine decarboxylase, L-Lysine decarboxylase, arginine dihydrolase, B-galactosidase, indole). To confirm pathogenicity, serology test, and presence of toxin production or toxigenicity genes for *V. cholerae* as well as thermostable direct haemolysin or TDH-related haemolysin gene for *V. parahaemolyticus* should be performed.

1.7. Molecular methods for pathogen detection in fishery products

1.7.1. Polymerase chain reactions

In Europe, there is possibility of using alternative methods, if they are properly validated. One of the major challenges still lies on the use of real-time quantitative polymerase chain reaction (RT-qPCR). The main advantages of using qPCR are related to the differentiation of viable and non-culturable cells and rapid detection of pathogens (*Listeria* can be detected in 48h, as shown by Boyer and Combrisson, 2013). In fishery products, qPCR can used for simultaneously detection of *Salmonella* and *Listeria* in salmon (Amagliani et al., 2010).

A number of PCR assays have been used to amplify Vibrio spp. from food, blood, or environmental samples. Pathogenicity of *V. parahaemolyticus* is strongly linked to production of the thermostable direct hemolysin (*tdh*) and/or the thermostable direct-related hemolysin (*trh*), although other virulence factors are likely involved (Yeung and Boor, 2004). Therefore, many molecular detection assays have targeted the genes responsible for production of the *tdh* and *trh* hemolysins (Bej et al., 1999; Nemoto et al., 2009; Nordstrom et al., 2007; Tada et al., 1992). Multiplex PCR has also been widely used for the detection of *Vibrio* spp. (*V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus*) in raw and cooked seafood and fish samples (Messelhäusser et al., 2010). Espiñeira et al. (2010) validated a method for the detection of five *Vibrio* species using multiplex PCR in seafood. They concluded that the method is sufficiently robust and adaptable to identify the serogroup, biotype, and /or virulence factors.

1.7.2. DNA microarrays and biosensors

One limitation of traditional PCR-based methods is that they are normally limited to the analysis of a single pathogen, a small group of related pathogens, or a small number of relevant genes. Microarray technology enables a significant expansion of the capability of DNA-based methods in terms of the number of DNA sequences that can be analyzed simultaneously. This enables molecular identification and characterization of multiple pathogens and genes in a single array assay. Microarray analysis of microbial pathogens has potential uses in research, food safety, medical, agricultural, regulatory, public health, and industrial settings. DNA microarrays have been used to detect fish pathogens including *A. hydrophila*, *V. alginolyticus*, *V. anguillarum*, or *V. harveyi*, corroborating the results of parallel molecular methods (plate culturing and 16S rNA gene sequencing) (Shi et al., 2012).

Biosensor-based methods rely on the recognition of antigen targets or receptors by antibodies, aptamers, or high-affinity ligands. The captured antigens may be then directly or indirectly detected through an antibody or high-affinity and high-specificity recognition molecule. The possibility to use antigens in combination with a transducer such as a piezo- electric crystal

detector offers the ability to develop a group-specific piezo-immunosensor for the detection of pathogenic bacteria in fish products (Guilbault and Luong, 1991).

1.7.3. Antibody-based tests

A variety of antibody-based tests and molecular tests have been developed to detect mainly bacterial and viral fish pathogens, although tests have also recently been reported for parasites and fungal agents. The antibody-based tests include slide agglutination, co-agglutination/latex agglutination, immunodiffusion, direct and indirect fluorescent antibody tests (FAT and IFAT), immunohistochemistry (IHC), and enzyme-linked immunosorbent assay (ELISA), dot blot/dip stick, and western blot (WB) (reviewed by Adams, 1999). The antibody-based test selected for the identification of pathogens depends on a variety of factors since each method has its merits and disadvantages. Although such methods are useful for the detection of pathogens in pure culture or/and in infected fish tissue, their sensitivity thresholds limit use in environmental samples, especially where pathogen levels are extremely low. Studies performed in seafood samples used monoclonal antibodies (mAbs) for detection of *V. parahaemolyticus*.

Normally, *trh* recombinant proteins of pathogenic *V. parahaemolyticus* can be applied with sandwich enzyme-linked immunosorbent assays (ELISA). Advantages include performance simplicity, high throughput, speed, and cost (Krishna Kumar et al., 2011). Alternatives are the use of monoclonal antibody based immunochromatographic strip tests for direct detection of *V. cholerae* O1 (Chaivisuthangkura et al., 2013). However, the detection sensitivities were also depending on the content of the other bacteria that might inhibit the growth of *V. cholerae* during pre-enrichment step.

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Chapter 2: Modelling the growth of *Listeria monocytogenes* in Mediterranean fish species from aquaculture production

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2.1. Abstract

Over the last couple of decades, several studies have evaluated growth dynamics of *L. monocytogenes* in lightly processed and ready-to-eat (RTE) fishery products mostly consumed in Nordic European countries. Other fish species from aquaculture production are of special interest since their relevant consumption patterns and added value in Mediterranean countries, such as sea bream and sea bass. In the present study, the growth of *L. monocytogenes* was evaluated in fish-based juice (FBJ) by means of optical density (OD) measurements in a temperature range 2–20 °C under different atmosphere conditions (i.e., reduced oxygen and aerobic). The Baranyi and Roberts model was used to estimate the maximum growth rate (μ_{max}) from the observed growth curves. The effect of storage temperature on μ_{max} was modelled using the Ratkowsky square root model. The developed models were validated using experimental growth data for *L. monocytogenes* in sea bream and sea bass fillets stored under static and dynamic temperature conditions. Overall, models developed in FBJ provided fail-safe predictions for *L. monocytogenes* growth. For the model generated under reduced oxygen conditions, bias and accuracy factor for growth rate predictions were 1.15 and 1.25, respectively, showing good performance to adequately predict *L. monocytogenes* growth in Mediterranean fish products. The present study provides validated predictive models for *L. monocytogenes* growth in Mediterranean fish species to be used in microbial risk assessment and shelf-life studies.

Keywords: Foodborne pathogens; Sea bream; Sea bass; Predictive microbiology; Model validation

2.2. Introduction

World supply of fish for human consumption has steadily grown over the last 5 decades at an annual rate of 3.2% (FAO, 2016). Spain is the EU member state with the highest aquaculture production volume, constituting 22.3% EU production in 2015 (APROMAR, 2017). Sea bream is one of the most farmed species in Mediterranean countries, being for many years the fish species with the largest production volume in Spain with 16,230 t in 2014 (9.4%), followed by rainbow trout and sea bass (APROMAR, 2015). The consumption of seafood and fishery products has increased recently, among other reasons, because of rising consumer awareness on the health benefits of fish as well as the variety of species available in the market. The global trend towards the consumption of minimally processed and/or ready-to-eat (RTE) food products, while natural and safe, has led to new food safety challenges, bringing more efficient food processing and preservation technologies.

It is recommended that fresh fin fish should be cooked at an internal temperature of 63 °C before consumption (FDA, 2016). However, mild processing and/or culinary practices (i.e. cold-smoking, salting, marinating, etc.) may lead to lower treatment temperatures enabling bacterial survival during cooking. In recent years, fresh sea bream and sea bass have been introduced as main ingredient in some of the most popular non-heated RTE seafood products, like *sushi* and its varieties (*nigiri*, *sashimi*), *carpaccio* and other fishery preparations. The lack of an effective lethal treatment in this type of products can pose a major risk of contamination by foodborne pathogens such as *Listeria monocytogenes*. According to the EFSA report on *L. monocytogenes* prevalence in certain RTE foods at retail (EFSA, 2013) the proportion of positive samples at retail was higher for fishery products in comparison to other RTE foods such as meat and cheese products. In addition, the physi-cochemical properties of many fishery products allow for *L. monocytogenes* growth during refrigerated storage, which can lead to pro- ducts exceeding the criterion of < 100 CFU/g throughout their shelf-life. Therefore, it is of interest for the fishery industry and governments to develop effective control measures aiming at reducing *Listeria* levels in fish products. In this sense, predictive models are proposed from different organizations as an important tool to improve microbial food safety and quality (ICMSF, 2011).

Moreover, several mathematical models have been developed to predict behaviour of spoilage and pathogenic microorganisms in seafood and fishery products. However, most of these models were based on RTE products from fish species mostly consumed in Nordic European countries, such as salmon, halibut or shrimp (Hwang and Sheen, 2009; Mejlholm et al., 2015; Mejlholm and Dalgaard, 2015, 2009). Other fish species from aquaculture production are of special interest since their relevant consumption patterns and added value in Mediterranean countries. Some studies were published in scienti!c literature regarding microbial quality and safety of sea bream and sea

bass (Andres-Bello et al., 2015; Provincial et al., 2013b, 2010; Tsironi and Taoukis, 2010). However, these studies focused on the effectiveness of preservation techniques, such as modified atmosphere packaging (MAP), to prevent growth of spoilage and/or pathogenic bacteria during shelf-life and at present, there is a lack of information about *L. monocytogenes* dynamics on Mediterranean fish products.

In the present study, the growth of *L. monocytogenes* was evaluated in fish-based juice under two environmental conditions (reduced oxygen and aerobic atmosphere) by means of Optical Density (OD) measurements in the range 2–20 °C. Secondary models were generated describing the effect of temperature on *L. monocytogenes* growth. The developed models were validated through challenge tests with sea bream and sea bass fillets under constant and dynamic storage conditions.

2.3. Materials and methods

2.3.1. Generation of predictive models in fish-based juice

2.3.1.1. Sterile fish-based juice preparation

Fish-based juice (FBJ) obtained from fish muscle of fresh sea bream was used to evaluate microbial growth dynamics in a substrate reproducing the fish muscle composition. To prepare the juice, a modified methodology proposed by Dalgaard (1995) was applied. This variation included a filtration step through successive bacteriological filters with decreasing pore sizes (1.8, 1.2, 0.8, 0.45 and 0.22 μm Ø, Sartorius, Germany) to obtain a clarified juice containing only soluble matter. Then, generated juice was filter-sterilized through a step-by-0.22μ membrane (Millipore filter unitExpress Plus PES, USA) and was kept at –20 °C until its use.

Water activity (a_w) was measured using an Aqualab model 4TE water activity meter (Decagon Devices, USA). The measured a_w value corresponded to 0.997 ± 0.003.

2.3.1.2. Bacterial strain and preculture conditions

In a previous assay, monoculture experiments were carried out in FBJ to evaluate the growth of 5 different *L. monocytogenes* strains (NCTC 11994, ATCC 13932, ATCC 19111, ATCC 19115, ATCC 19116) at 20 °C under aerobic conditions. After transforming OD data into cell counts by means of calibration curves (Section 2.1.4), the average maximum growth rate values from the obtained growth curves ranged from 0.185 to 0.204 log CFU/h (Suppl. Table 1). The strain exhibiting the fastest growth (NCTC 11994) was used for further experiments in this study.

Stock culture, acquired from the Spanish Type Culture Collection (CECT), was stored in cryovials (MicrobankTM, Pro-Lab Diagnostics, USA) at –20 °C. For the inoculum preparation, a bead of the strain was transferred to a tube containing 9 mL Brain Heart Infusion (BHI) broth (Oxoid, U.K.) and incubated at 37 °C for 24 h. After two consecutive cultures, the final tube was incubated

at 37 °C for 16–18 h and broth culture was removed from cell suspensions through three consecutive washing steps in 0.85% Saline Solution (SS) (Scharlau, Spain) by centrifugation at 4100 rpm for 10 min (Jouan C4i, Thermo Electron Corporation, France). A target inoculum level of 10^6 CFU/mL was set for the inoculation of FBJ.

2.3.1.3. Growth curve experiments

The Bioscreen C analyser (Labsystems, Helsinki, Finland) was used to monitor bacterial growth based on OD measurements at aerobic and reduced oxygen (RO) conditions. A wideband filter at 420–580 nm was used and micro-plates (10×10 wells) were utilised to perform the growth experiments in Bioscreen C. For aerobic conditions, 400 μL of inoculated FBJ were transferred into each well of the Bioscreen C plates. To simulate a RO environment, wells were covered with liquid parafin. The plates for aerobic and RO conditions were incubated at different temperatures (2, 4, 8, 11, 15, 20 °C) during a period from 2 to 28 days and OD was measured until the microorganism reached the stationary growth phase. A total of 10 replicates were carried out for each combination of temperature and atmosphere.

2.3.1.4. Calibration curves

OD measurements were transformed to viable count data through the building of calibration curves. They were performed following the procedure described by Metris et al. (2006) in which samples for OD and viable count were collected during microbial growth experiments. For that, Bioscreen C plates were filled for each atmosphere condition with inoculated FBJ as described above and incubated at 6 and 18 °C. At regular time intervals throughout the incubation period, samples (100 μL) were removed from wells into 9.9 mL SS, further diluted, and plated onto BHI Agar (Oxoid) for viable counts. The sampled Bioscreen C plate wells were discarded for subsequent sampling and analyses. Bioscreen C was set-up as explained above and the OD of the well was recorded immediately before the sample was taken. Measurements were corrected by a blank containing only FBJ without inoculum. A logarithmic transformation was performed on both OD values and viable count data to stabilize the variance between data points. These transformed data were used to fit a linear regression in MS-Excel software (Microsoft Corp., Redmond, WA, USA).

OD growth curves recorded in the range 2–8 °C were transformed into cell densities (CFU/mL) by using calibration curves performed at 6 °C. For OD curves obtained in the range 11–20 °C, calibration curves performed at 18 °C were applied.

2.3.1.5. Primary and secondary modelling

Log-absorbance data were transformed into cell counts (log CFU/mL) using the calibration curves obtained previously. Then, the Baranyi model (Baranyi and Roberts, 1994) was fitted to cell count

data using DMFit version 3.5 Excel Add-in (IFR, Norwich, UK) to estimate the kinetic parameter maximum growth rate (μ_{max}) A one-way analysis of variance (ANOVA) was performed using SPSS 20.0 (SPSS Inc., Chicago, Illinois, USA) to determine the significance of the effect of the atmospheric environment on μ_{max} (P < 0.05). The obtained μ_{max} values were used to develop a square root type model (Eq. (1)) (Ratkowsky et al., 1982) describing the influence of the temperature for each atmospheric condition. The fitting of the model was performed by using MS-Excel.

$$\sqrt{\mu_{max}} = b(T - T_{min}) \tag{2.1}$$

where T is the storage temperature (°C), T_{min} is the minimum temperature for growth (°C) and b is a regression parameter.

The ability of primary and secondary models to describe the experimental data was analysed by statistical indices: coefficient of determination (R^2), adjusted R-square (Adj. R^2) and standard error of fit (SE).

2.3.2. Models validation: challenge tests with *L. monocytogenes* on fish fillets

2.3.2.1. Collection of fish samples

Aquacultured fresh gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) were supplied by a fishery industry located in Ayamonte (Huelva, Spain) on the southwest Spanish coast. Fishes were killed by a cold-thermal shock immediately after capture. Then, fishes were manually gutted and filleted, washed with cold ozonized water for 20 min, inoculated and packaged individually in plastic trays. Fish fillets were MAP packaged in polyethylene terephthalate (PET) trays sealed with a biaxially-oriented PET laminate film with a water vapour permeability of 2.0 g/m²/day at 23 °C and an oxygen permeability of 5.0cm³/m²/day at 23 °C (Wipak, Bomlitz, Germany) in a packaging machine (BELCA, Guipúzcoa, Spain). Headspace gas composition was measured throughout the storage time with a gas headspace analyser (Gaspace 2, Systech Instruments, Oxon, UK) by determining the CO_2 and O_2 concentrations. The average weight of fish fillets was 77.4 ± 19.6 g and the initial pH was 6.4 and 6.6 for sea bream and sea bass, respectively.

2.3.2.2. Inoculation and storage of fish samples

The selected *L. monocytogenes* strain was reconstituted as described above and diluted in SS to obtain an initial concentration on the fish fillet of 10^4 CFU/g approx. Before packaging, 10 µL of the diluted culture was injected on the caudal region of the fish fillet's muscle using a 50 µL pipet. This part of the fillet (of 25 g approx.) was considered the analytical fish sample.

Samples were divided into two batches for each fish species: inoculated and control batch (non-inoculated). Experiments were carried out at three constant storage temperatures (4, 9 and 16 °C). Inoculated fish fillets were also stored under dynamic refrigerated conditions using a temperature profile from 2.6 to 8.9 °C. Incubation temperatures were measured continuously throughout the experiments by data loggers (MicroLite, Fourier Technologies, Israel). In each sampling point, two different fish samples were analysed for each assayed condition. The experiments were repeated twice in different days.

2.3.2.3. Microbial enumeration

To monitor microbial growth over time, 25g fish sample was aseptically cut, mixed with 0.1% peptone water (Oxoid) and homogenized for 60 s using a Stomacher (IUL Instruments, Barcelona, Spain). For non-inoculated (control) samples, the growth of aerobic mesophilic viable count (MVC) and *Listeria* investigation (presence/absence) was studied during storage at the tested temperatures. MVC counts were determined by pour plating decimal dilutions of homogenized samples in Plate Count Agar (PCA, Oxoid) incubated 48 h at 37 °C. The presence/absence of *L. monocytogenes* was carried out following the method EN ISO 11290-1. For samples inoculated with *L. monocytogenes*, appropriate decimal dilutions of homogenized samples were plated on Oxford agar (CM0856, Oxoid) supplemented with Oxford selective supplement (SR0140, Oxoid). Counts were performed after incubating the plates during 24–48 h at 37 °C. Endogenous lactic acid bacteria (LAB) in inoculated samples was enumerated on de Man, Rogosa, Sharpe agar (MRS, Oxoid), which was incubated 48 h at 33 °C with 10% CO_2.

2.3.2.4. Curve fitting and model simulation under static and dynamic temperature conditions

To determine μ_{max} of *L. monocytogenes* in challenge tests with fish fillets under constant temperatures, the Baranyi model was fitted to log (CFU/g) data (DMFit Excel Add-in). This model was also used to estimate the lag phase parameter (λ, h) for the pathogen in challenge tests. The prediction of *L. monocytogenes* growth under dynamic storage temperature was based on the time-temperature profile of the fish fillets, T(t), in conjunction with the square root model (Eq. (1)) and the differential equations of the Baranyi model (Eqs. (2.2) and (2.3)), which were numerically integrated in MS-Excel.

$$\frac{dy}{dt} = [b(T(t) - T_{min})]^2 \left(\frac{q}{q+1}\right) \left(1 - \frac{y}{y_{max}}\right) y \qquad (2.2)$$

$$\frac{dq}{dt} = [b(T(t) - T_{min})]^2 q \qquad (2.3)$$

where *t* is time, *y* is the concentration of the microbial population at time *t*; y_{max} is the maximum population concentration and *q* is a variable related to the physiological state of the cells.

2.3.3. Model evaluations

Observed and predicted μ_{max} values of *L. monocytogenes* obtained at constant storage temperatures were compared by calculation of the validation indices Bias (B_f) and Accuracy (A_f) factor proposed by Ross (1996). For pathogenic bacteria, a B_f of 0.87–0.95 or 1.11–1.43 indicate acceptable model performance (Baranyi et al., 1999; Mejlholm et al., 2010), while A_f should be < 2 (Pérez-Rodríguez and Valero, 2013). The capacity of prediction was also assessed by the Root Mean Square Error (RMSE). For experiments under dynamic storage temperatures, the acceptable simulation zone (ASZ) approach was used, with ASZ defined as ± 0.5 log-units from the simulated *L. monocytogenes* counts (Velugoti et al., 2011). To determine the acceptability of the model, at least 70% of the observed log CFU/g values should be inside this zone (Oscar, 2005).

The Food Spoilage and Safety Predictor (FSSP, version 4.0) software was used to estimate μ_{max} for further comparison with the obtained data in this study at constant temperature conditions. Predictions were performed based on the values of pH, aw, storage temperature and atmospheric conditions of the fish species used in challenge tests. The selected FSSP growth model was "*L. monocytogenes* in chilled seafood and meat products" (Mejlholm and Dalgaard, 2015; Mejlholm et al., 2010; Mejlholm and Dalgaard, 2009).

2.4. Results and discussion

2.4.1. Predictive models in fish-based juice

In the present study, a FBJ broth was generated from fresh fish and used to assess *L. monocytogenes* growth based on OD measurements. The limitation of turbidimetry to evaluate microbial growth is mainly at- tributed to the high detection thresholds of spectrophotometric devices (10^6–10^7 CFU/mL). OD methods can be used to predict viable counts only when some attempt at calibration is made (McKellar and Lu, 2004). To this sense, the importance of calibration curves should be taken into account especially when environmental stresses are present, as they affect cell morphology and viability, and this can have an influence on OD data (Baka et al., 2015; Valero et al., 2006). Thus, calibration curves were performed in our study at low (6 °C) and high (18 °C) temperatures for each atmospheric composition (Fig. 2.1). The validity region of the calibration curves used to relate OD and viable count data started with the first OD value above the quantification limit of Bioscreen C, which corresponded to 7 log CFU/mL approx., as shown in Fig. 2.1. The regression parameters and the coefficients of determination obtained for each calibration curve are summarized in Table 2.1. In general,

regression lines remained parallel (similar slope) at the studied temperatures, apart from the calibration curve performed at 6 °C under RO conditions where a lower slope was obtained. This shift in the performance of calibration curves was also observed by Valero et al. (2006) at low temperatures (4 and 7 °C) compared to higher temperatures (10, 15 and 30 °C) at different pH levels.

The estimated μ_{max} of *L. monocytogenes* in FBJ by the fitted Baranyi model at the tested experimental conditions are provided in Table 2.2. Results showed that the pathogen grew in FBJ at all studied conditions. Overall, it is demonstrated that *L. monocytogenes* grew faster as storage temperature increased. The effect of temperature on microbial growth kinetics has been extensively studied in the past (Ratkowsky et al., 1982). The statistical analysis indicated that there was a significant effect of the atmosphere (P < 0.05) on μ_{max} for temperatures > 2 °C, showing lower μ_{max} values for RO than for aerobic atmosphere. A greater effect of the atmospheric environment was observed when increasing the storage temperature. At 20 °C, *L. monocytogenes* exhibited the highest μ_{max} in FBJ under aerobic conditions (0.204 log CFU/h). High R^2 values (> 0.99) were obtained for the fitted Baranyi model.

Noteworthy, the simulated environment in microtiter plates by using liquid 44araffin do not lead to a strict anaerobic atmosphere due to the presence of residual oxygen concentration in the generated FBJ. Thus, results under these conditions could be extrapolated to micro-aerophilic environments, but they should be considered carefully when extrapolating to strict anaerobic atmospheres. The facultative anaerobic nature of *L. monocytogenes* enables it to grow in reduced oxygen atmospheres as shown in Table 2.2. In general, aerobic conditions are more favourable for the growth of this microorganism (Romick et al., 1996), producing higher growth rates as shown in our results in FBJ (Table 2.2).

The use of food model systems to evaluate microbial behaviour allows the collection of large number of data with great advantages over real food environments. Particularly, the use of fish-based systems to obtain growth data could be proposed as suitable means to resemble microbial growth in fish products, due to the similarities in the chemical composition (e.g. low carbohydrate content). In Baka et al. (2017), the growth dynamics of *L. monocytogenes* in model systems mimicking the composition of fish pâtés was assessed. In that study, planktonic growth occurred in liquid and emulsions systems and the reported μ_{max} values (e.g. 0.029 log CFU/h in liquids and 0.025 log CFU/h in emulsions at 8 °C) are generally lower than those obtained in FBJ (Table 2.2). Growth rate differences among studies could be mainly attributed to variability in *L. monocytogenes* strains and compositional aspects of the growth media used in the experiments.

The predicted μ_{max} values in FBJ were used to develop a secondary model for each atmosphere condition. The effect of temperature on *L. monocytogenes* μ_{max} was modelled by using the square

root type model. Fig. 2.2 illustrates the graphical version of the fitted models and the parameters of the generated mathematical equations are shown in Table 2.3. The relationship between μ_{max} and temperature was linear with a high R^2 value (> 0.97) for both models, indicating that the square root model adequately described this temperature dependence.
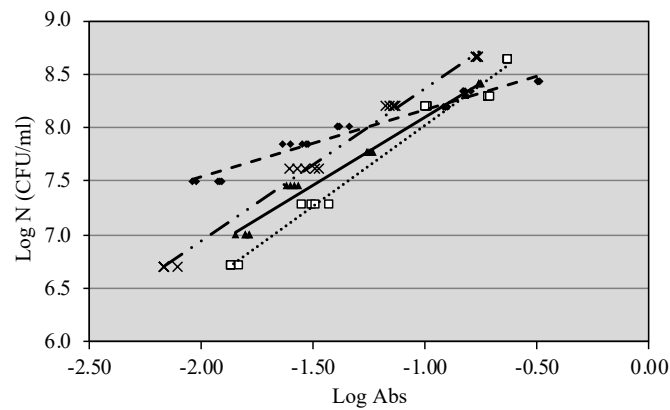
Figure 2.1. Calibration curves performed at different temperatures and atmosphere conditions. Reduced oxygen atmosphere: 6 ºC (♦) and 18 ºC (×). Aerobic atmosphere: 6 ºC (▲) and 18 ºC (□).
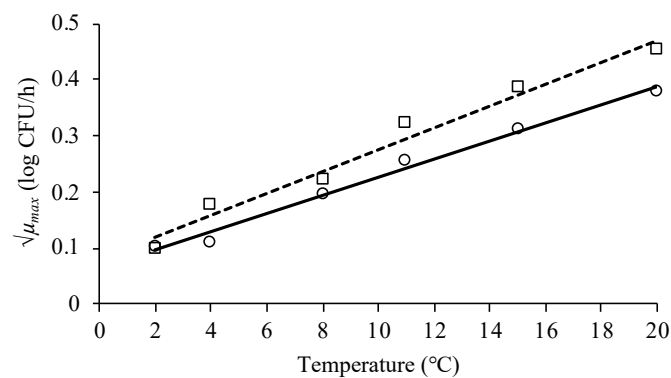
Figure 2.2. Representation of the square root Ratkowsky models developed for *L. monocytogenes* growth in fish-based juice under reduced oxygen (solid line) and aerobic (dashed line) atmosphere.

Table 2.1. Growth conditions employed for the calibration curves with the main statistical parameters for each regression curve.

Temp (ºC)	Atmosphere	Slope	Intercept	$R^{2,a}$	SE^b	n^c
6	Reduced oxygen	0.631	8.803	0.970	0.058	33
6	Aerobic	1.283	9.387	0.986	0.066	29
18	Reduced oxygen	1.440	9.821	0.995	0.054	24
18	Aerobic	1.514	9.537	0.977	0.115	28

[a] Coefficient of determination.
[b] Standard error of the estimate.
[c] Number of data used to construct the calibration curve.

Table 2.2. Estimated maximum growth rate (μ_{max}) of *L. monocytogenes* in fish-based juice for the different temperatures and atmosphere conditions employed for experiments in Bioscreen C (n = 10 for each experimental condition).

Temp (ºC)	Atmosphere	μ_{max} (log CFU/h)	Adj. $R^{2,b}$	SE^c
2	Reduced oxygen	0.010 ± 0.001^a	0.990	0.113
	Aerobic	0.009 ± 0.002	0.995	0.082
4	Reduced oxygen	0.012 ± 0.001	0.993	0.042
	Aerobic	0.031 ± 0.003	0.990	0.129
8	Reduced oxygen	0.038 ± 0.003	0.992	0.066
	Aerobic	0.050 ± 0.003	0.999	0.032
11	Reduced oxygen	0.065 ± 0.004	0.998	0.035
	Aerobic	0.105 ± 0.003	0.998	0.055
15	Reduced oxygen	0.097 ± 0.002	0.992	0.070
	Aerobic	0.149 ± 0.006	0.993	0.091
20	Reduced oxygen	0.143 ± 0.017	0.995	0.052
	Aerobic	0.204 ± 0.010	0.992	0.070

[a] Mean ± standard deviation.
[b] Adjusted R-square of the fitted Baranyi and Roberts model.
[c] Standard error of fit.

Table 2.3. Estimated parameters and their standard error (SE) of the square root Ratkowsky model developed for *L. monocytogenes* growth in fish-based juice under different atmospheric conditions.

Atmosphere	$b^a \pm SE$	$T_{min}^b \pm SE$	$R^{2,c}$
Reduced oxygen	0.016 ± 0.001	-3.83 ± 0.034	0.987
Aerobic	0.020 ± 0.002	-4.16 ± 0.054	0.974

[a] Regression coefficient.
[b] Minimum temperature for growth (ºC).
[c] Coefficient of determination.

2.4.1.1. Growth modelling of L. monocytogenes on fish fillets at constant temperatures

Growth data of *L. monocytogenes* on sea bream and sea bass fillets stored under MAP were collected at different constant temperatures and fitted to Baranyi model (Fig. 2.3). Table 2.4 shows the estimated μ_{max} values for the tested temperatures. Overall, the pathogen showed the same trend for both fish species, obtaining similar μ_{max} values. The population of *L. monocytogenes* increased by *ca*. 4 log throughout the storage time at all tested temperatures, reaching values close to 8 log CFU/g in both fish species. An adaptation period (λ) of 26 ± 10 h and 19 ± 17 h was observed for the pathogen at 4 °C in sea bream and sea bass, respectively.

Despite the well documented occurrence of *L. monocytogenes* in RTE fish products (EFSA, 2013) only two studies have focused on evaluating the growth/survival of pathogens in Mediterranean fish species (Provincial et al., 2013a, 2013b). The study conducted by Provincial et al. (2013b) showed that the growth of *L. monocytogenes* in fresh sea bream fillets was slower in the presence of CO_2 at the tested concentrations (60–80%). In our study, the initial CO_2 and O_2 concentration in the headspace of trays was $10 \pm 2\%$ and $15 \pm 3\%$, respectively. In general, an increase in CO_2 (%) and a reduction of O_2 (%) was observed after the second day of analysis at the tested temperatures. Particularly, O_2 concentrations dropped up to levels below 7% (data not shown) because of O_2 consumption by aerobic bacteria present in the microbiota of the fish fillets. The only comparable result by Provincial et al., (2013b), with our study, was that obtained at 4 °C under normal atmosphere (i.e. air) packaging due to the presence of high oxygen levels in both atmospheres. Comparing results in sea bream fillets, our observed growth rates were slightly lower (Table 2.4) than that found by Provincial et al., (2013b) (0.3 log CFU/g). The lower oxygen levels used in our study, together with the type of strain and microbiota composition, may explain for the differences between studies.

In our study, no samples were positive for *Listeria* in non-inoculated fish fillets. LAB and MVC showed low initial counts in both fish species, with an average value of 1.5 and 2.0 log CFU/g, respectively (Fig. 2.3). Low numbers of LAB and MVC in the initial microbiota of sea bream and sea bass fillets have been also reported by other researchers (Cakli et al., 2006; Kyrana and Lougovois, 2002; Parlapani et al., 2014; Provincial et al., 2013b, 2010; Tsironi and Taoukis, 2010). The growth of endogenous LAB, monitored in inoculated samples, was noticeably lower than MVC, with final levels below 6 log CFU/g after the storage period. In turn, the growth of MVC was much faster in both fish species, which was especially evident at 16 °C (Fig. 2.3), reaching high population densities (8.4–9.2 log CFU/g). The presence of a significant headspace O_2 concentration in trays could be the cause for the fast growth observed for MVC as compared to LAB, which is a microbial group whose presence and relevance is mostly related to RO atmospheres (Françoise, 2010). This is in agreement with the results presented in Parlapani et al.

(2014), where MAP containing 60% CO_2 and 10% O_2 enhanced the growth of LAB in sea bream fillets compared to aerobic storage. The presence of LAB (although at low levels) in the fish fillets along with the faster growth of MVC, could affect the growth rate of the *L. monocytogenes*, especially at high temperatures (16 °C), producing an important growth inhibition as an effect of competitive microbiota. These assumptions could explain that μ_{max} obtained at 16 °C in the fish fillets were not as high as expected, since values were close to those obtained at 9 °C (Table 2.4).
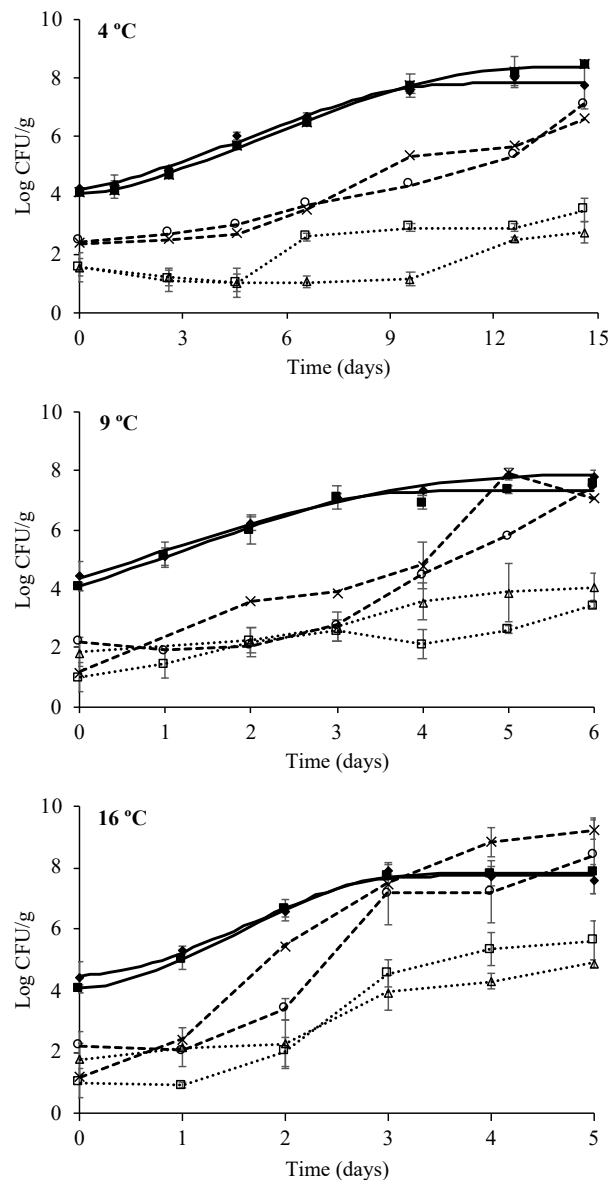
Figure 2.3. Microbial counts obtained from challenge tests at 4 ± 0.3 °C (upper graph), 9 ± 0.3 °C (medium graph) and 16 ± 0.3 °C (low graph) in sea bream and sea bass fillets. *L. monocytogenes* NCTC 11994 count in inoculated sea bream (■) and sea bass (♦) samples fitted to the Baranyi and Roberts model (—). Evolution of growth of endogenous LAB in inoculated sea bream (□) and sea bass samples (△) and MVC in non-inoculated sea bream (○) and sea bass (×) samples. Represented growth data are mean values from duplicate trials with vertical lines representing for ± standard deviation.

Table 2.4. Estimated maximum growth rate (μ_{max}, log CFU/h) of *L. monocytogenes* inoculated on sea bream and sea bass fillets by the fitted Baranyi and Roberts model in this study and calculated from Food Spoilage and Safety Predictor (FSSP).

	This study		FSSP	
	Sea bream	Sea bass	Sea bream	Sea bass
$\mu_{max}\_4\,^\circ$C	0.0189	0.0183	0.0089	0.0091
$\mu_{max}\_9\,^\circ$C	0.0423	0.0392	0.0272	0.0276
$\mu_{max}\_16\,^\circ$C	0.0684	0.0618	0.0700	0.0710

2.4.2. Validation under constant temperature conditions

Predictions by the generated FBJ models were validated by comparing them to growth data obtained in challenge tests in fish fillets under static conditions. The validation results are presented in Table 2.5. In general, it is demonstrated that models developed in FBJ predicted faster growth than that observed in fish samples ($B_f > 1$), providing fail-safe predictions. In aerobic conditions, the model significantly overestimated growth rates of *L. monocytogenes* on the studied fish species, as shown by average B_f and A_f of 1.74. The performance of the model generated under RO conditions resulted in a B_f of 1.15, demonstrating that predictions were closer to observations. In addition, the calculated A_f for the latter model ($A_f = 1.25$) was within the range of acceptability ($A_f < 2$). The presence of indigenous microbiota and CO_2 in the fish fillets, together with differences in (micro)structural properties between the FBJ and fresh fish (i.e. matrix effect), may be the main causes for deviations between the model predictions and the observed growth data. In relation with CO_2, it has been reported that atmospheric CO_2 can be absorbed in muscle tissue of the fish fillet (Sivertsvik et al., 2002), thereby reducing pH in the product. This fact can explain a more reduced growth in MAP fish fillets in comparison to FBJ under RO conditions as observed in our study. In order to improve accuracy of predictions, some authors have developed mathematical models considering the inhibitory effect of natural microbiota on *L. monocytogenes* growth in fish products (Gimenez and Dalgaard, 2004; Mejlholm and Dalgaard, 2015; Mejlholm and Dalgaard, 2007; Vermeulen et al., 2011). In these studies, LAB was selected as the primary group responsible for microbial interaction. This is mainly because, as mentioned above, most of these models were performed on lightly preserved seafoods, where LAB often predominate. However, other microbial groups should be considered when modelling microbial interaction on Mediterranean fresh fish, specially under air or non-enriched CO_2 atmospheres. In fish packed aerobically, competition occurs between members of an aerobic Gram-negative microbiota (mainly pseudomonas and H_2S producing bacteria) (Koutsoumanis et al., 2000). These bacteria have been reported to be the dominant spoilage microbiota in sea bream fillets packaged under aerobic conditions, with Enterobacteriaceae being also co-dominant at high

temperatures (Parlapani et al., 2014). Thus, the influence of the atmosphere environment on *L. monocytogenes* growth rate when considering Mediterranean fish products should be carefully evaluated and quantified in predictive models by considering its influences on the development of specific spoilage bacteria and microbial interactions. In this sense, the obtained *L. monocytogenes* growth parameters (μ_{max}) from monoculture in FBJ would be very useful information to develop interspecific competition models on Mediterranean fish species, since the effect of bacterial competition can be isolated and quantified by comparing the experimental data obtained in FBJ and on the actual fish product. As it is demonstrated in the study of Mejlholm et al. (2010), predictions with good precision can be obtained when complexity of the growth model matches the complexity of foods of interest. Based on our results, composition of the atmosphere packaging and accompanying microbiota as well as interactive effects between them should be considered in models' development when evaluating minimally processed Mediterranean fish products.

Growth data obtained, in this study, for both Mediterranean species (i.e. challenge tests) were compared with predictions provided by the abovementioned growth model for *L. monocytogenes* included in FSSP. This tertiary model is probably the most well-known software package to predict growth of microorganisms in seafood and fishery products. The selected FSSP model was originally developed for processed and RTE seafood (Mejlholm and Dalgaard, 2009). When evaluated for Mediterranean fresh fish products in the present study, average B_f and A_f of 0.71 and 1.49 were obtained, which means that FSSP model produced fail-dangerous predictions ($B_f < 1$). However, the values of RMSE (Table 2.5) confirmed that, overall, the FSSP model produced closer predictions to observations. In the case of pathogenic bacteria, conservative predictions are preferred from a public health perspective (Pérez-Rodríguez and Valero, 2013). The fact that FSSP models have been developed and extensively validated on processed foods, especially cold-smoked salmon or brined shrimp, by including growth inhibitors and preservatives (phenolic compounds, organic acid, etc.) may explain the underestimations obtained for growth in Mediterranean fresh fish products. These results clearly reinforce the importance of performing validation processes by challenging the model against actual growth observation obtained under different conditions or food products, thereby conforming or not its validity for a specific food application.

Table 2.5. Evaluation of the μ_{max} (log CFU/h) prediction of *L. monocytogenes* by the growth models generated in fish-based juice (FBJ) and by Food Spoilage and Safety Predictor (FSSP) based on the growth data obtained from challenge tests in this study.

Growth model	A_f[a]	B_f[b]	RMSE[c]
FBJ (reduced oxygen)	1.25	1.15	0.033
FBJ (aerobic)	1.74	1.74	0.099
FSSP	1.49	0.71	0.014

[a] Bias factor.
[b] Accuracy factor.
[c] Root Mean Square Error.

2.4.3. Validation under dynamic temperature conditions

The generated FBJ models were validated against observed growth at fluctuating conditions using a dynamic temperature profile simulating cold-chain distribution and storage of fishery products in Spain (ranging from 2.6 to 8.9 °C). The performance of the model generated under RO and aerobic atmosphere at dynamic temperature conditions is graphically presented in Fig. 2.4A and B, respectively. Predictions were based on Eqs. (2.2) and (2.3), which were solved numerically for q(t) and y(t). The maximum population density of *L. monocytogenes* in fish fillets was found to be independent of temperature and it was therefore taken as the average of the values estimated during testing at static conditions (y_{max} = 8.0 log CFU/g). For initial concentration (y_0), the value determined by the plate count method in challenge tests was used, which corresponded to 4 log CFU/g approx. Predictions were based on a fixed value for the h_0 parameter ("work to be done" during the lag phase) of 0.42, which was calculated as the product μ_{max} x λ based on the average of the respective values observed at 4 °C in challenge tests. A reduction of the pathogen population was observed at the end of the storage period (17 days), especially in sea bass. This final decline might be observed since experiments were carried out up to 17 days of storage, while the actual shelf-life for MAP fish fillets is about 11–15 days under refrigerated conditions (DeWitt and Oliveira, 2016). Thus, the performance of the developed models using the Acceptable Simulation Zone (ASZ) approach was conducted by considering *L. monocytogenes* counts up to 15 days of storage and excluding the last observed point (corresponding to 17 days) in the studied fish products. Taking into account the observed *L. monocytogenes* growth data in both fish species, 85.7% count values were within the AZS for the model generated under RO conditions. These results demonstrate that the model provided reasonable predictions for *L. monocytogenes* growth at changing temperatures in the evaluated Mediterranean fish species. On the contrary, for the model generated under aerobic conditions, < 70% of the relative errors (i.e. 42.9%) fell within the ASZ (Fig. 2.4B), which indicates a non-successful validation of the model at the tested temperature profile.

A

B
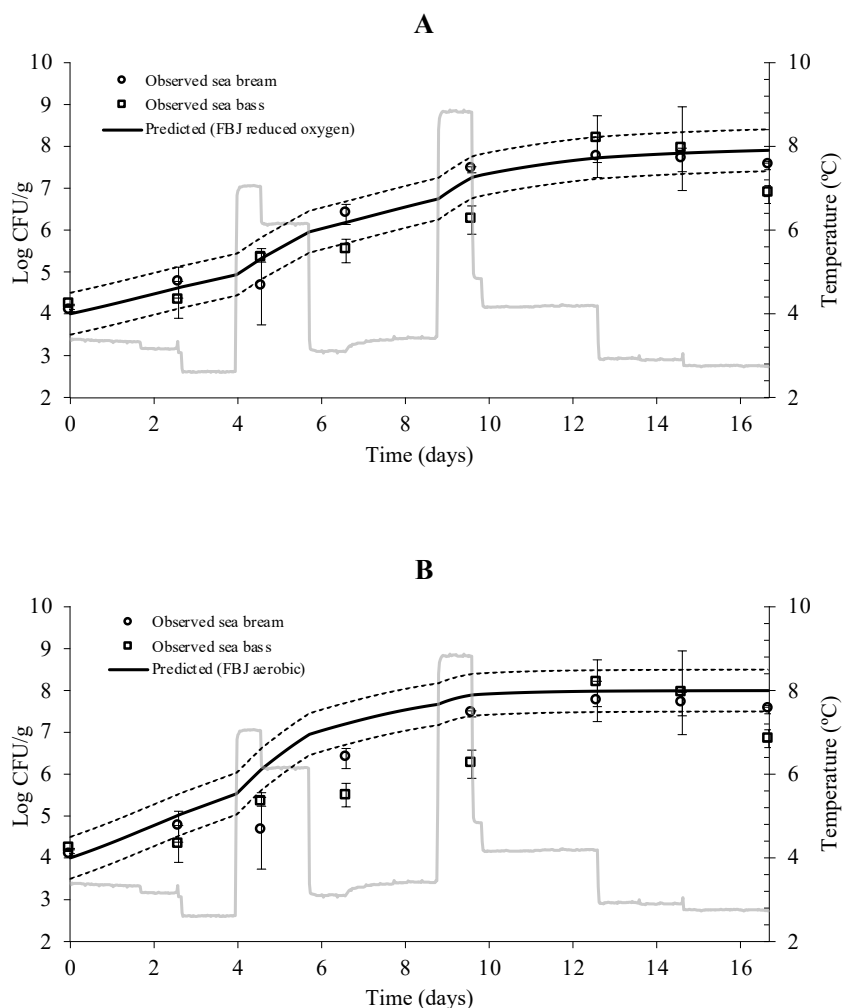
Figure 2.4. Comparison between observed (points) and predicted (lines) growth of *L. monocytogenes* in sea bream and sea bass fillets during dynamic refrigerated storage. Graphs include ± 0.5 log CFU/g (dashed lines) defining the acceptable simulation zone (ASZ). Growth kinetics for *L. monocytogenes* was simulated using the differential equations (Eq. (2.2) and (2.3)) of the Baranyi model. Represented growth data are mean values from duplicate trials with vertical lines representing for ± standard deviation. The temperature profile is shown as grey line in (A) and (B).

In our study, differences in physicochemical characteristics and gaseous composition between the FBJ and fish fillet product could explain the average overestimation of growth rates by the generated models. Nevertheless, the validation study of the model developed in FBJ under RO conditions concluded that the model could adequately describe *L. monocytogenes* growth in Mediterranean fish species under static and dynamic storage temperatures. The models generated in this study can be valuable and suitable tools to be applied in quantitative risk assessment studies and shelf-life determination.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.02.005

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Chapter 3: Isolating the effect of fat content on *Listeria monocytogenes* growth dynamics in fish-based emulsion and gelled emulsion systems

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3.1. Abstract

The influence of food matrix fat content on growth kinetics of bacteria is quite complex and, thus far, not fully elucidated, with different studies reporting contradictory results. Since results in studies involving real food products are possibly influenced by variations in compositional and physicochemical properties, there is a need for systematic studies in artificial food model systems among which the influence of fat content is effectively isolated. In this study, the isolated effect of a gradual increase in fat content, in the range of 1–20% (w/w), on the growth dynamics of *Listeria monocytogenes* in fish-based emulsion and gelled emulsion model systems was investigated at 4, 7 and 10 °C. Growth parameters estimated by the Baranyi and Roberts model were compared among the different model systems. Overall, an increase from 1 to 5% fat resulted in a significant reduction of the lag phase duration λ in both model systems at all studied temperatures, while a further increase in fat content did not significantly affect λ. The relationship between the fat content (%) and the maximum specific growth rate μ_{max} was more complex, following the same trends for both emulsions and gelled emulsions and tested temperatures, i.e., (i) μ_{max} was higher at 5% than at 1% fat, (ii) μ_{max} was lower at 10% than at 5% fat, (iii) μ_{max} at 20% fat was higher than or equal to "max at 10% fat, and (iv) μ_{max} was the highest at 5% fat. Based on these experiments, fundamental knowledge was provided which could lead to the development of food matrix-related factors describing the influence of fat content in future predictive modeling tools which include food micro- structural elements. Such models could increase the accuracy of the shelf-life estimation for fat-containing foods, in turn resulting in improved food safety.

Keywords: Food safety; Microstructure; Fat; Model fish products; Growth kinetics

3.2. Introduction

The transformation of fish by-products into ready-to-eat (RTE) products or partially processed foods constitutes an important opportunity for the food industry to produce sustainable and value-added products for human consumption. Several RTE and semi-RTE products have recently been developed and introduced into the market, such as fish burgers, sausages, surimi and pâtés (Palmeira, Mársico, Monteiro, Lemos, & Conte Junior, 2016). Most of these products are "manufactured" emulsions where the manufacturer controls the product formulation. In this context, it is of utmost importance for the food industry to get insight into how specific matrices (i.e., structured foods) and formulations influence the survival and growth of microorganisms, and so the impact on food safety.

In biphasic foods (e.g., oil-in-water or water-in-oil emulsions), the lipid phase imposes structure, which can affect the form of bacterial growth, its growth rate, and the habitat domain in which growth occurs (Brocklehurst & Wilson, 2000). Due to the opacity of the fat droplets, visualisation of microorganisms within complex foods is challenging, but non-invasive *in situ* imaging techniques (e.g., fluorescence and confocal laser scanning microscopy) have contributed widely to increase the knowledge concerning growth morphology and spatial colony distribution (Lobete, Fernandez, & Van Impe, 2015). Many commercial emulsion-type fish products may be characterised as oil-in- water emulsions or the composite form of gelled emulsions (i.e., an oil-in-water emulsion with a gelled aqueous phase), with fat contents generally varying from 10 to 40% for pâtés and < 10% for surimi-type sausages (Aquerreta, Astiasarán, Mohino, & Bello, 2002; Santana, Huda, & Yang, 2015). In oil-in-water food emulsions, the oil phase is present as polydisperse droplets with a mean diameter ranging from 1 to 8 µm (Brocklehurst & Wilson, 2000). As water is necessary for the synthesis of cellular materials, microbial growth solely occurs in the aqueous phase (Wilson et al., 2002). In addition, the interface between the fat and the water phase, together with food rheology and microstructure may locally affect the microecological conditions within the food matrix and thus the ability of microorganisms to proliferate (Buchanan, Gorris, Hayman, Jackson, & Whiting, 2017).

International regulations and guidelines propose Predictive Microbiology as a scientifically validated approach to attain legal requirements for food safety (Pérez-Rodríguez et al., 2019). Predictive models quantitatively express the effect of environmental factors on microbial dynamics through mathematical equations. A major shortcoming in predictive modelling is the limited inclusion of food micro- structure in terms of quantifiable (metric) variables, which should be considered for the development of accurate predictive models (Skandamis & Jeanson, 2015). The implementation of such model parameters requires a better understanding of the impact of the underlying factors on microbial responses. To tackle this, a number of studies have recently

aimed at identifying the most relevant microstructural aspects influencing growth dynamics, focussing on foodborne pathogens like *Listeria monocytogenes* or *Salmonella* Typhimurium (Baka, Vercruyssen, Cornette, & Van Impe, 2017a, Baka, Verheyen, Cornette, Vercruyssen, & Van Impe, 2017b, Baka, Noriega, Van Langendonck, & Van Impe, 2016; Aspridou, Moschakis, Biliaderis, & Koutsoumanis, 2014; Theys et al., 2008; Verheyen et al., 2018b). These works have revealed that predictive models will benefit from the inclusion of food microstructural factors describing, among others, the presence of fat droplets in the food matrix.

The presence of fat droplets in the food matrix is generally assumed to suppress microbial growth if the fat content is sufficiently high, mainly due to a decrease in water activity and available growth space. More specifically, bacteria become immobilised between the fat droplets, resulting in a shrinkage of the habitat domain which limits the availability of water, space and nutrients, in turn leading to a reduction in growth rate (Baka et al., 2017b; Samapundo et al., 2015; Wilson et al., 2002). However, recent studies have also shown that the presence of fat in the food matrix can also enhance microbial growth under certain conditions (Baka et al., 2017a; Hauerlandová et al., 2014; Lorenzo, Pateiro, García Fontán, & Carballo, 2014; Verheyen et al., 2018b). In some studies, a cryoprotectant influence of the fat on the physiology of the cells at low temperatures has been put forward as a possible explanation of these growth-enhancing effects (Baka et al., 2017a, b; Knechtges, 2011, chap. 5). Nevertheless, this cryoprotectant influence cannot be the sole explanation for the enhanced growth, which has also been reported at higher temperatures of up to 37 °C (Gutierrez, Barry-Ryan, & Bourke, 2008). In addition, growth-enhancing effects caused by the presence of fat in the food matrix during cold storage (i.e., 3–15 °C) were absent in other studies (Aryani, Zwietering, & den Besten, 2016; Lobacz & Kowalik, 2015). The ambiguity of the results in the different aforementioned studies could have been caused by the use of different (model) food products (e.g., milk, cream, cheese, fish-based model systems), entailing differences in compositional and physicochemical factors. The use of artificial food model systems, among which the influence of fat content is isolated, could hence be beneficial to further elucidate the influence of food matrix fat content on microbial growth dynamics (Verheyen et al., 2018a).

The aim of this work was to systematically investigate the isolated influence of fat content on microbial growth dynamics. *L. monocytogenes* was selected as the target pathogen, since it is highly associated with contamination of fish products in the EU (Verheyen et al., 2018b). Emulsions and gelled emulsions with various fat contents were stored at temperatures of 4, 7 and 10 °C, representative for fish product storage (i.e., cold, domestic refrigerator, and mild abuse temperature, respectively). Growth parameters estimated by the Baranyi and Roberts (2000) model were compared for the different model systems. In addition, the pH and a_w of the model

systems were measured to confirm that significant differences in microbial kinetics were not caused by possible differences in physicochemical factors among the model systems.

3.3. Materials and methods

3.3.1. Microorganisms and preculture conditions

A *L. monocytogenes* strain cocktail consisting of strains LMG 23773, LMG 23774 (both isolated from smoked salmon), and LMG 26484 (isolated from tuna salad), acquired from the BCCM/LMG bacteria collection of Ghent University in Belgium, was used for all growth experiments. Microorganisms were individually prepared by two consecutive 24h-precultures at 30 °C as described in Verheyen et al. (2018b). The mixture of equal volume aliquots of each strain culture resulted in a *L. monocytogenes* strain cocktail with a cell density of approximately 10^9 CFU/mL.

3.3.2. Model system preparation

Oil-in-water model emulsions and gelled emulsions with different fat content (1, 5, 10, and 20%) were prepared as described in Verheyen et al. (2018a). Model systems consisted of hydrolysed salmon fish protein (ProGoTM, Hofseth Biocare ASA, Ålesund, Norway), sodium alginate (Sigma-Aldrich, MO, USA), NaCl (Sigma-Aldrich, MO, USA), CaCO3 (Sigma-Aldrich, MO, USA), D-(+)-gluconic acid δ-lactone (GDL, Sigma-Aldrich, MO, USA), sunflower oil (Eldorado, local supermarket, Stavanger, Norway), Tween 80 (Sigma-Aldrich, MO, USA), Span 80 (Sigma-Aldrich, MO, USA), Xanthan gum (Sigma-Aldrich, MO, USA), and distilled H_2O. A detailed overview of the composition of the model systems is provided in Table 3.1.

Table 3.1. Composition of the emulsion and gelled emulsion model systems with different fat contents (i.e., 1, 5, 10, and 20%).

Ingredients (% w/w)	Emulsion				Gelled emulsion			
	1%[a]	5%	10%	20%	1%[a]	5%	10%	20%
Fish protein	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Alginate	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
NaCl	0.95	0.90	0.84	0.74	0.94	0.89	0.84	0.73
$CaCO_3$	/	/	/	/	0.40	0.38	0.36	0.31
GDL[b]	/	/	/	/	0.95	0.90	0.84	0.74
Sunflower oil	1.00	5.00	10.00	20.00	1.00	5.00	10.00	20.00
Tween 80	0.10	0.35	0.35	0.35	0.10	0.35	0.35	0.35
Span 80	0.20	0.65	0.65	0.65	0.20	0.65	0.65	0.65
Xanthan gum	0.50	0.50	0.50	0.50	/	/	/	/
Distilled H_2O	89.25	84.6	79.66	69.76	88.41	83.83	78.96	69.22

[a] Taken from Verheyen et al. (2018a).
[b] D-(+)-gluconic acid δ-lactone.

3.3.3. Growth experiments

Emulsion and gelled emulsion model systems were homogeneously inoculated with the *L. monocytogenes* strain cocktail to a cell density of 10^2 CFU/mL as described in Verheyen et al. (2018b). Model systems were then incubated under static conditions at three different temperatures, i.e., 4, 7, and 10 °C. At appropriate time intervals, samples were taken in duplicate and enumerated by viable plate counts on Brain Heart Infusion Broth (BHI, VWR International, Leuven, Belgium) supplemented with 1.4% (w/v) agar (Technical No3, Oxoid Ltd., Basing- stoke, UK), following the protocols employed by Verheyen et al. (2018b) for viscous and gelled systems. Prior to enumeration, the plates were incubated at 30 °C for approximately 30 h. For each set of growth conditions, experiments were independently performed in triplicate. Data for emulsion and gelled emulsion systems containing 1% fat at 4 and 10 °C has already been previously published in Verheyen et al. (2018b).

3.3.4. Mathematical modelling and parameter estimation

The primary growth model of Baranyi and Roberts (2000) was fitted to the obtained microbiological data. Since the experiments were conducted at static temperature conditions, the explicit form of the model equations was used, as represented by Equations (3.1) and (3.2) (Baranyi & Roberts, 2000).

$$\ln\big(N(t)\big) = \ln(N(0)) + \mu_{max}\, A(t) - \frac{N_{max} - N(0)}{M} \ln\left(1 - e^{-M} + e^{-M\frac{N_{max} - N(0) - \mu_{max}A(t)}{N_{max} - N(0)}}\right) \qquad (3.1.)$$

$$A(t) = t - \lambda\left(1 - \frac{1}{h_0}\ln\left(1 - e^{-h_0\frac{t}{\lambda}} + e^{-h_0\left(\frac{t}{\lambda}-1\right)}\right)\right) \qquad (3.2.)$$

with N(t) [ln CFU/mL], the cell density at time t; N(0) [ln CFU/mL], the initial cell density at time 0; μ_{max} [1/h], the maximum specific growth rate; N_{max} [ln CFU/mL], the maximum cell density; M [-], a dimensionless curvature parameter characterising the transition from the exponential to the stationary phase; h_0 [-], a dimensionless curvature parameter characterising the transition from the lag to the exponential phase; and λ [h], the lag phase duration of the cells.

In the fitting of bacterial counts by the Baranyi and Roberts model, it is generally recommended to keep the curvature parameters as fixed number with default values of $M = 10$ and $h_0 = 10$ (Baranyi & Roberts, 2000). In the current study, these values were set to an average of 8 and 4 for M and h_0, respectively. This resulted in a better model performance, especially for the estimation of the lag phase λ parameter. With those optimal parameter values, the Baranyi and Roberts model was fitted to the experimental growth curves by using Equations (3.1) and (3.2). Model parameters (except for the curvature parameters M and h_0) were estimated from the set of experimental data via the minimisation of the sum of squared errors, using the lsqnonlin routine

of the Optimisation Toolbox of Matlab version R2016b (The Mathworks Inc.). Standard errors of parameter estimates were calculated from the Jacobian matrix.

3.3.5. Physicochemical model system characterisation

pH was measured using a FiveEasy Plus FEP20 pH meter (Mettler Toledo, Greifensee, Switzerland). Emulsion model systems were analysed using a pH Electrode LE438 (Mettler Toledo, Greifensee, Switzerland), while gelled emulsion model systems were analysed using an InLab Surface electrode (Mettler Toledo, Greifensee, Switzerland).

Water activity (a_w) of emulsion and gelled emulsion model systems was measured using an AquaLab 4 TE a_w meter (Decagon Devices Inc., WA, USA).

3.3.6. Statistical analysis

The statistical analysis was performed separately for each set of model systems (emulsions and gelled emulsions) and storage temperature. Significant differences among parameter values or physicochemical properties were determined using analysis of variance (ANOVA, single variance) test. Fisher's Least Significant Difference (LSD) test was used to distinguish which means were significantly different from others. The standardised skewness and standardised kurtosis were used to assess if data sets came from normal distributions. The analyses were performed using Statgraphics Centurion 17 Package (Statistical Graphics, Washington, USA). The significance level for the statistical tests was $P \leq 0.05$.

3.4. Results and discussion

Independent of the fat content and storage temperature, both emulsions and gelled emulsions were shown to be structurally stable over the course of the growth experiments, as also confirmed by Verheyen et al. (2018a). Similarly, fat droplet size among all model systems remained evenly distributed around approximately 1 μm. The growth of *L. monocytogenes* at 4, 7, and 10 °C in the emulsion and gelled emulsion model system with different fat content is illustrated in Fig. 3.1 and Fig. 3.2, respectively. A short lag phase, an exponential growth phase, and a stationary phase can be distinguished at each temperature for all fat contents. The stationary phase was reached after approximately 600, 300, and 200 h at 4, 7, and 10 °C, respectively. The different fat levels under study did not have a significant influence on the maximum cell density N_{max} of *L. monocytogenes*, with an average of 20.8 ± 0.3, 20.5 ± 0.1 and 20.1 ± 0.1 ln CFU/mL for emulsions and 20.6 ± 0.1, 20.4 ± 0.2 and 20.3 ± 0.2 ln CFU/mL for gelled emulsions at 4, 7, and 10 °C, respectively. Thus, results and related discussion presented hereafter will refer only to the kinetics parameters λ and μ_{max}. A statistical analysis comparing these parameters among emulsion and gelled emulsion model systems with equal fat content at each temperature (results not shown) revealed that λ in the gelled emulsion was always longer than or equal to λ in the corresponding emulsion. The

value of μ_{max} in emulsion was always higher than or equal to μ_{max} in the corresponding gelled emulsion. This phenomenon is probably caused by a reduction in nutrient, oxygen and metabolite diffusion induced by the gelled matrix (Antwi et al., 2006; Brocklehurst, Mitchell, & Smith, 1997; Pajic-Lijakovic et al., 2017; Skandamis & Jeanson, 2015; Verheyen et al., 2018b; Wilson et al., 2002; Wimpenny, Coombs, Lovitt, & Whittaker, 1981, Wimpenny, Leistner, Thomas, Mitchell, Katsaras, & Peetz, Wimpenny, Leistner, Thomas, Mitchell, Katsaras, & Peetz, 1995).
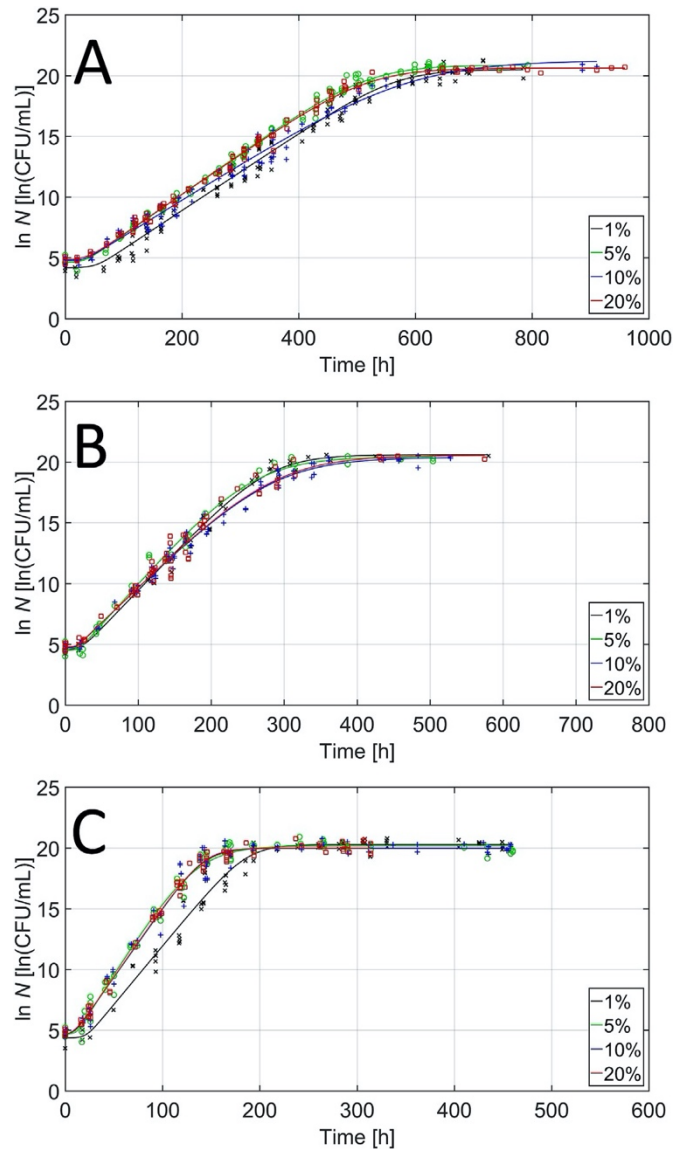
Figure 3.1. *L. monocytogenes* growth in the emulsion model systems containing 1, 5, 10, and 20% fat at 4 (A), 7 (B), and 10°C (C). Symbols (×, ○, +, □, for 1, 5, 10, and 20% fat, respectively) correspond to the experimental data and lines correspond to the global fit of the growth model of Baranyi and Roberts (2000).
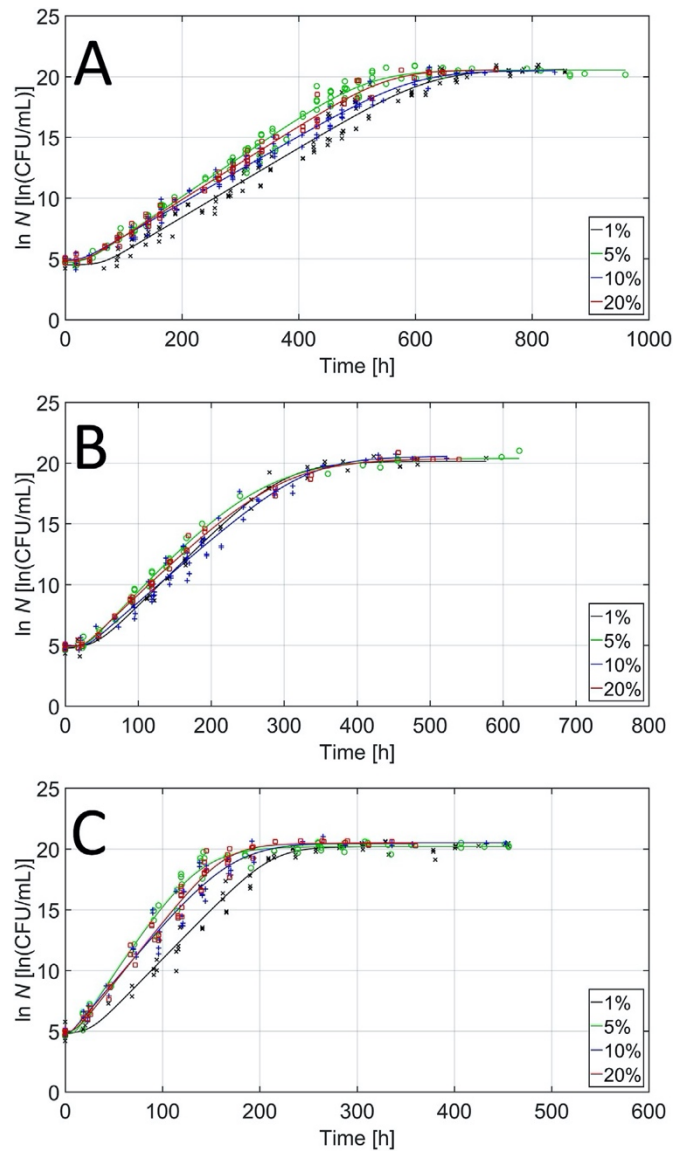
Figure 3.2. *L. monocytogenes* growth in the gelled emulsion model systems containing 1, 5, 10, and 20% fat at 4 (A), 7 (B), and 10°C (C). Symbols (×, ○, +, □, for 1, 5, 10, and 20% fat, respectively) correspond to the experimental data and lines correspond to the global fit of the growth model of Baranyi and Roberts (2000).

3.4.1.   Effect of fat content on lag phase duration

The statistical analysis of the lag phase duration λ of *L. monocytogenes* for the different fat contents at each temperature is shown in Table 3.2 for the emulsions and gelled emulsions. In general, the lag phase λ was significantly longer in the emulsion and gelled emulsion with 1% fat than in the systems with higher fat content. The exception to this trend was seen at 7 °C, where no significant differences were observed among the different fat contents, but the lag phase λ was also longer in the emulsion containing 1% fat than in the emulsion with higher fat levels.

Verheyen et al. (2018b) investigated the isolated effect of a small amount of fat droplets on *L. monocytogenes* growth dynamics at 4 and 10 °C by comparing growth parameters for the

emulsion containing 1% fat and a similar liquid system (i.e., the same composition as the emulsion containing 1% fat, except for the absence of 1.0% sunflower oil and 0.3% emulsifiers) on the one hand, and the gelled emulsion containing 1% fat and a similar aqueous gel without fat on the other hand. The lag phase λ was significantly shorter in the systems containing 1% fat than in the systems without fat, although only at 4 °C. It was suggested that this phenomenon was caused by a cryoprotective effect of the fat droplets, as already proposed by Baka et al. (2017a). In the current study, it can be observed that the lag-reducing effect increased when the fat content was increased to 5%, but a further increment in the fat level resulted in no additional reduction of λ. This correlation can possibly be explained by the Confocal Laser Scanning Microscopy images which were obtained following the growth of a fluorescent *L. monocytogenes* strain at 10 °C for 14 days in the relevant model systems (Verheyen et al., 2019), as illustrated in Fig. 3.3. In the emulsion containing 1% fat (Fig. 3.3A), cells mainly grew as single cells or micro-colonies in the spaces between the fat droplets, but there was also micro-colony growth on the fat-water interface (i.e., around the fat droplets). A similar behaviour was observed in the gelled emulsion containing 1% fat (Fig. 3.3E), where micro-colonies grew in the spaces among the fat droplets, but also on the fat-water interface. As the fat level increased to 5, 10, and 20% (Fig. 3.3B-3.3D and 3.3F-3.3H, for emulsions and gelled emulsions, respectively) and the available space between the fat droplets decreased, *L. monocytogenes* almost exclusively grew as micro-colonies on the fat-water interface around the fat droplets, both in the emulsions and gelled emulsions (Verheyen et al., 2019). In relation to this, microbial hydrophobicity may play an important role in food emulsions due to interactions between the cells and food components such as lipids and proteins (Krasowska & Sigler, 2014). *L. monocytogenes* is generally characterised as a hydrophobic bacterium, and thus interfacial energies may explain its affinity to grow around the fat droplets, especially at higher fat levels. It should, however, be mentioned that the affinity of the cells for the fat-water interface might be dependent on growth temperature and on the specific *L. monocytogenes* strain. Hence, growth behaviour of the *L. monocytogenes* strain cocktail used in the current study and the *L. monocytogenes* fluorescent strain might be different, taking also into account that confocal images were obtained after growth at 10 °C and not at 4 or 7 °C. The possibility for the cells to also grow on the fat-water interface might enhance their growth in the systems containing 1% fat, possibly due to a cryoprotective effect of the fat droplets, resulting in a reduced lag phase at 4 °C. The increased affinity for the fat-water interface at higher fat levels (i.e., 5, 10 and 20% in the current study) might result in improved growth conditions compared to the systems with only 1% fat, although not only at 4 °C, but also at 7 °C (only statistical differences for the gelled emulsions) and 10 °C. Consequently, the lag phase reduction is not only caused by a cryoprotective effect. Possibly, *L. monocytogenes* colonies start growing faster when there is an interface to which the cells can adhere. In this regard, bacterial growth at particular interfaces may not only be influenced by electrostatic cell surface properties but also by the potential ability of

microorganisms to generate physicochemical interactions between both the biphasic matrix and the diffusing solutes (e.g., free amino acids and peptides) (Floury et al., 2015). Since there was no significant lag phase reduction observed when the fat content was increased from 5 to 10 and 20% in the current study, the full extent of this lag-reducing effect was already reached at a fat level of 5%, both in the emulsions and gelled emulsions.

To the best knowledge of the authors, studies that have systematically investigated the influence of fat content on the growth dynamics of *L. monocytogenes* in detail (i.e., by focussing on the effect of growth parameters such as λ and μ_{max} in representative food model systems) are scarce. Gutierrez et al. (2008) conducted growth experiments in emulsion model systems containing 0 (i.e., as a control), 1, 5, and 10% of sunflower oil. They reported a shorter λ when increasing the fat content from 0 to 1%, but no further reduction at higher fat content. Consequently, they found a λ-reducing effect at low fat content, similar to results of the current study, although a lower minimum fat content was necessary to acquire this effect. This lower minimum fat content could be related to the specific compositional, physicochemical and rheological properties of the model systems used by Gutierrez et al. (2008). Results of the current study were also compared with a few other works which involved growth of *L. monocytogenes* in real food products of the gelled emulsion category. Baka, Noriega, Tsakali, and Van Impe (2015) found that there were no significant differences in the lag phase of *L. monocytogenes* in Frankfurter sausages containing 15 and 20% fat at 4, 8 and 12 °C, which is in accordance with the findings obtained in the current study. In turn, in the study of Samapundo et al. (2015), the lag phase λ of *L. monocytogenes* at 7 °C was shorter in low fat pork liver pâté containing 13.5% fat than in normal pork liver pâté containing 40% fat. Hence, fat levels higher than 20%, although not investigated in the current study, could result in a longer lag phase. However, since the food products in the studies of Samapundo et al. (2015) and Baka et al. (2015) also varied in compositional and physicochemical factors, no clear conclusions may be drawn from the comparison with the current study.

Table 3.2. Lag phase duration (λ) $\pm$ standard error of *L. monocytogenes* in the emulsion and gelled emulsion model systems with different fat contents (i.e., 1, 5, 10, and 20%), retrieved from the growth model of Baranyi and Roberts (2000) for growth experiments at 4, 7, and 10 °C. For the different fat contents at the same temperature, values bearing different lowercase letters are significantly different (P $\leq$ 0.05).

Fat content (% w/w)	4ºC		7ºC		10ºC	
	Emulsion	Gelled emulsion	Emulsion	Gelled emulsion	Emulsion	Gelled emulsion
1	51.86 ± 8.27[Ba]	63.12 ± 11.72[Ba]	21.71 ± 4.69[A]	41.066 ± 4.84[B]	21.41 ± 5.94[Ba]	25.29 ± 6.55[Ba]
5	33.17 ± 5.26[A]	34.82 ± 7.97[A]	18.30 ± 4.86[A]	28.891 ± 3.69[A]	11.57 ± 2.24[A]	9.63 ± 3.08[A]
10	33.03 ± 10.54[A]	29.22 ± 9.41[A]	13.82 ± 4.17[A]	31.562 ± 6.69[A]	8.84 ± 3.66[A]	5.54 ± 5.59[A]
20	36.83 ± 4.91[A]	33.89 ± 7.49[A]	15.60 ± 6.39[A]	25.125 ± 4.19[A]	9.36 ± 2.03[A]	10.10 ± 4.32[A]

[a] Published by Verheyen et al. (2018b).

Table 3.3. Maximum specific growth rate (μ_{max}) $\pm$ standard error of *L. monocytogenes* in the emulsion and gelled emulsion model systems with different fat contents (i.e., 1, 5, 10, and 20%), retrieved from the growth model of Baranyi and Roberts (2000) for growth experiments at 4, 7, and 10 °C. For the different fat contents at the same temperature, values bearing different lowercase letters are significantly different (P $\leq$ 0.05).

Fat content (% w/w)	4ºC		7ºC		10ºC	
	Emulsion	Gelled emulsion	Emulsion	Gelled emulsion	Emulsion	Gelled emulsion
1	0.0317 ± 0.0006[Ba]	0.0286 ± 0.0006[Ba]	0.0625 ± 0.0015[B]	0.0595 ± 0.0017[B]	0.0964 ± 0.0044[Aa]	0.0819 ± 0.0038[Aa]
5	0.0335 ± 0.0003[C]	0.0328 ± 0.0005[D]	0.0678 ± 0.0021[C]	0.0656 ± 0.0020[C]	0.1285 ± 0.0033[C]	0.1228 ± 0.0036[C]
10	0.0299 ± 0.0007[A]	0.0276 ± 0.0004[A]	0.0584 ± 0.0012[A]	0.0522 ± 0.0017[A]	0.1139 ± 0.0037[B]	0.0951 ± 0.0041[B]
20	0.0329 ± 0.0004[C]	0.0303 ± 0.0005[C]	0.0587 ± 0.0020[A]	0.0578 ± 0.0016[B]	0.1148 ± 0.0019[B]	0.1015 ± 0.0033[B]

[a] Published by Verheyen et al. (2018b).
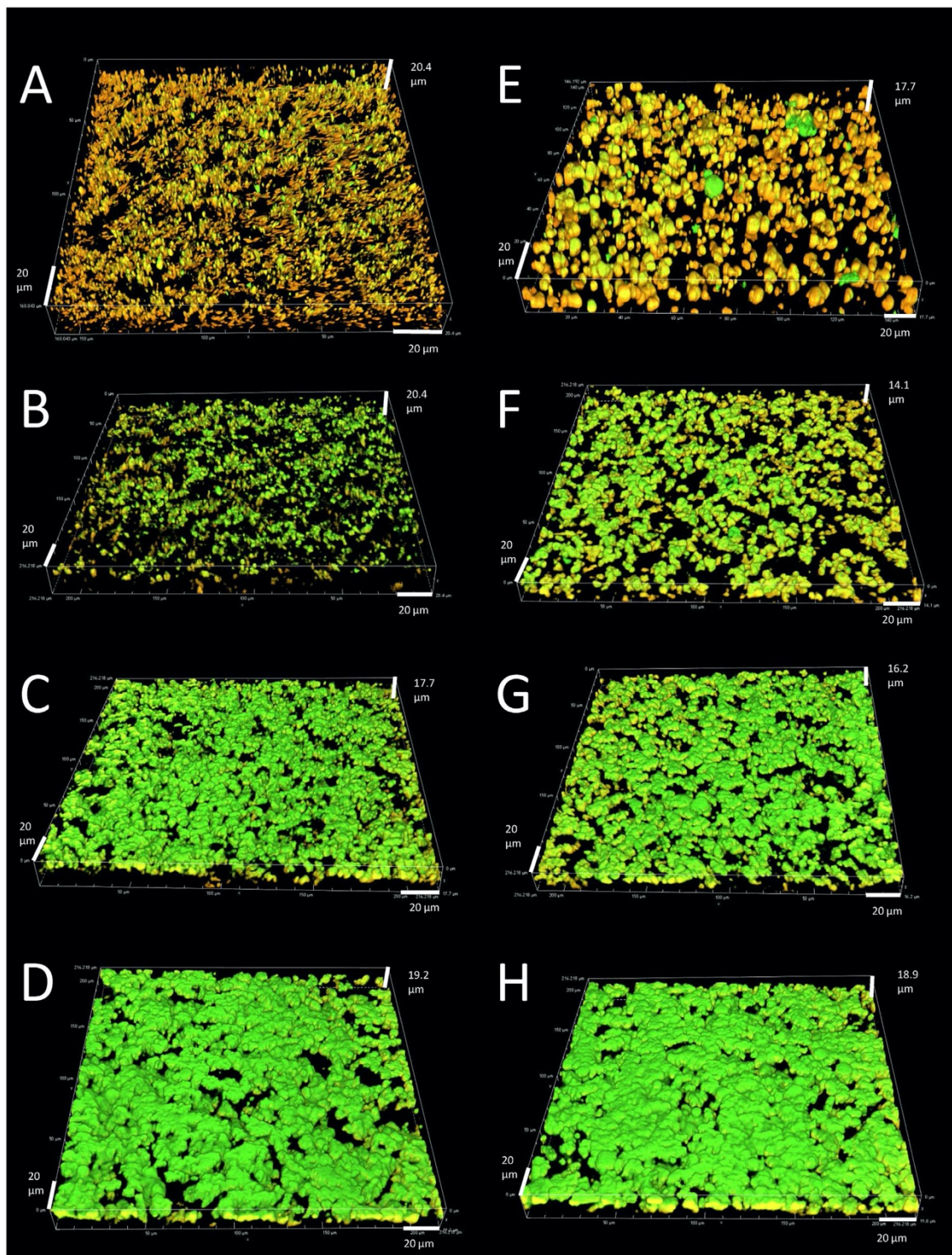
Figure 3.3. *L. monocytogenes* (green fluorescent protein strain) growth in the emulsion and gelled emulsion model systems with various fat content, i.e., emulsions containing 1 (A), 5 (B), 10 (C) and 20% (D) fat, and gelled emulsions containing 1 (E), 5 (F), 10 (G) and 20% (H) fat. Bacterial cells and fat droplets are depicted in green and orange, respectively. Yellow areas represent *L. monocytogenes* growth on the fat-water interphase (adapted from Verheyen et al., 2019). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4.2. Effect of fat content on maximum specific growth rate

Table 3.3 shows an overview of the statistical analysis of μ_{max} of *L. monocytogenes* for the different fat contents at each temperature for the emulsions and gelled emulsions. The relationship between the different fat contents differed slightly for the different conditions, although a common trend can be observed for both model systems and tested temperatures, i.e., (i) μ_{max} was always higher in the systems with 5% fat than in the systems with 1% fat, (ii) μ_{max} was lower in the systems with 10% fat than in those with 5% fat, (iii) μ_{max} in the systems with 20% fat was higher than or equal to μ_{max} in those with 10% fat, and (iv) μ_{max} was the highest in the emulsion and gelled emulsion containing 5% fat. Hence, the relation between fat content and μ_{max} was more complex than the relation between fat content and λ.

Verheyen et al. (2018b) demonstrated that the presence of a small amount of fat droplets (i.e., 1% fat content) resulted in a significantly higher μ_{max} of *L. monocytogenes* at 4 °C in comparison with the systems without fat (i.e., emulsion vs. similar liquid system and gelled emulsion vs. aqueous gel). Similar to the lag phase λ, this growth-promoting effect was suggested to be caused by a cryoprotective effect of the fat droplets. To explain the higher μ_{max} in the systems with 5% fat in comparison with the systems with 1% fat at all temperatures in the current study, the same reasoning as for λ can also be followed, i.e., a combination of a cryoprotective effect and easier colony formation/ growth when the cells can adhere to an interface. The lower μ_{max} in systems with 10% fat than in systems with 5% fat is not analogue to what was observed for λ. This phenomenon is possibly caused by the reduced amount of water present and/or the reduction in space available for bacterial growth in the model system as the fat content increases (Brocklehurst, Parker, Gunning, Coleman, & Robin, 1995; Skandamis & Jeanson, 2015; Wilson et al., 2002). The increase in μ_{max} for some conditions when the fat content is further increased towards 20%, could be due to the increased proximity of the fat droplets at this fat content (as illustrated in Fig. 3.3D and 3.3H) and the resulting properties of the biphasic system.

In the emulsions of Gutierrez et al. (2008), containing 0 (i.e., as a control), 1, 5, and 10% of sunflower oil, the maximum specific growth rate μ_{max} decreased with increasing fat content. Hence, their observed trend was less complex than that of the current study, a difference which was probably also caused by the specific compositional, physicochemical and rheological properties of the systems. In this regard, the lower viscosity of the emulsions of Gutierrez et al., due to the absence of thickening agents in their systems, probably resulted in an increased mobility of the cells in comparison to the systems used in the current study (with xanthan gum and sodium alginate). This increased mobility might have enabled a larger portion of the cells to grow in planktonic form (Wilson et al., 2002), resulting in the absence of a possible growth-promoting effect caused by the adherence of the cells to the fat-water interface. Similar to the

discussion of the effect of fat content on λ, results of the current study were also compared to studies conducted in real food products (i.e., emulsion and gelled emulsion category). Baka et al. (2015) found that μ_{max} of *L. monocytogenes* was higher in Frankfurter sausages containing 20% fat than in those containing 15% fat, again corresponding to the results of the gelled emulsion in the current study. A similar trend was found by Lobacz and Kowalik (2015) in other emulsion-type foods with various fat contents (2, 12 and 30%) during storage at 3-15 °C, although compositionally and structurally different (UHT milk and cream). Even though they indicated that the fat content did not have any statistically significant impact on the growth of *L. monocytogenes*, the obtained μ_{max} values in the products with 12 and 30% fat were generally higher than those obtained at 2% fat. For a similar product (i.e., milk), Aryani et al. (2016) reported no significant differences among μ_{max} values of *L. monocytogenes* in systems containing 1.5 and 3.0% fat at 7 °C. These results indicate that the fat content necessary to increase μ_{max} is higher than 3% (e.g., 5% in the current study). In another food product (i.e., pork liver pâté), Samapundo et al. (2015) observed a higher μ_{max} in pâté containing 13.5% fat than in pâté containing 40% fat, suggesting that fat levels higher than 20% could result in a reduced μ_{max}. Finally, although less comparable due to the use of different target microorganisms (e.g., *Aspergillus flavus*, *Bacillus cereus*, *Clostridium sporogenes*), growth-promoting effects at specific relatively low fat contents, similar to results from the current study, were also reported in other studies (Hauerlandová et al., 2014; Kosegarten, Ramírez-Corona, Mani-López, Palou, & López-Malo, 2017; Lorenzo et al., 2014).

In general, the relation between the fat content of emulsion and gelled emulsion systems and the maximum specific growth rate μ_{max} is rather complex. There seems to be an equilibrium between growth-promoting (i.e., a combination of a cryoprotective effect and an easier colony formation/growth when the cells can attach to an interface) and growth-inhibiting forces (i.e., a reduced water and growth space availability) which is dependent on the fat content. The comparison to results from previous studies implies that the influence of food matrix fat content on μ_{max} is also intertwined with the rheological properties of the food matrix. This phenomenon may be related to the dependence of the preferred phase for cell growth (i.e., aqueous phase or fat-water interface) on the rheological properties of the food matrix. This effect should be further elucidated in future studies, especially since rheological properties possibly exert an important influence on microbial growth dynamics in increased-viscosity liquid and gelled food products (Aspridou et al., 2014). In addition, the adherence of bacteria to fat droplets depends on their ability to develop specific adherence features such as pili, fimbriae, and flagella (Ron, 2000). Therefore, future studies could also investigate the influence of the presence of such traits, related bacterial mobility, and gene expression on *L. monocytogenes* at various temperatures in systems with different fat content.

3.4.3. Physicochemical model system characterisation

The pH and aw of all model systems was measured in order to investigated whether differences in growth dynamics of *L. monocytogenes* among the different model systems could also have been caused by differences in physicochemical properties. Table 3.4 provides an overview of the pH and a_w values of the emulsion and gelled emulsion model system with different fat content. pH and a_w values for the emulsion and gelled emulsion model systems containing 1% have already been previously published in Verheyen et al. (2018a). Physicochemical properties of the model systems were relevant for real processed fish products, both concerning pH (i.e., mostly ranging from 5.5 to 6.8) and a_w (i.e., higher than 0.938) (Jay, Loessner, & Golden, 2005, chap. 3; Schmidt and Fontana., 2007), and also similar to the properties of fish-based model systems used in other studies (Baka et al., 2017a, b).

It can be observed that the pH of the emulsion containing 1% fat (6.35 ± 0.01) (Verheyen et al., 2018a) is significantly higher in comparison to the emulsions of this study with higher fat percentages. The pH of the gelled emulsions with 1% fat (6.34 ± 0.11) (Verheyen et al., 2018a) and 20% fat is significantly higher than the pH of the gelled emulsions with 5 and 10% fat. While it is generally accepted that pH exerts an effect on the growth dynamics of *L. monocytogenes* (Hwang & Tamplin, 2005; Vermeulen et al., 2007), the effect on and μ_{max} is limited at pH values greater than 5.5 (McKellar, Lu, & Knight, 2002). Furthermore, there was no clear correlation between the small differences in pH and the observed differences in growth dynamics at the different fat contents in the current study. Therefore, the effects on λ and μ_{max} which were observed, as discussed in Section 3.4.1 and 3.4.2, were most likely not caused by the aforementioned pH differences.

Concerning a_w, no significant differences were observed among the different emulsions, including the emulsion containing 1% fat (a_w of 0.988 ± 0.001) (Verheyen et al., 2018a). For the gelled emulsions, no significant differences were observed between the a_w of the gelled emulsion with 1% fat (0.985 ± 0.003) (Verheyen et al., 2018a) and the gelled emulsions with higher fat content. The a_w of the model system with 20% fat was significantly lower than for the system with 5% and 10% fat. These differences in a_w were, however, rather small (i.e., 0.004–0.008, taking into account the standard deviation), being very unlikely to exert a significant influence on *L. monocytogenes* growth (Baka et al., 2016). In addition, the significant differences in a_w were not clearly correlated to the differences in growth dynamics. Hence, the observed effects on λ and μ_{max} were most likely also not caused by the differences in a_w.

Table 3.4. pH and water activity (a_w) ± standard deviation of the emulsion and gelled emulsion model systems with different fat contents (i.e., 5, 10, and 20%). For the different fat contents, values bearing different lowercase letters are significantly different (P ≤ 0.05).

Fat content (% w/w)	Emulsion		Gelled emulsion	
	pH (–)	a_w (–)	pH (–)	a_w (–)
5	6.13 ± 0.07[a]	0.989 ± 0.003[a]	6.01 ± 0.05[a]	0.988 ± 0.001[b]
10	6.15 ± 0.06[a]	0.990 ± 0.003[a]	6.02 ± 0.11[a]	0.987 ± 0.004[b]
20	6.19 ± 0.08a	0.989 ± 0.002[a]	6.32 ± 0.02[b]	0.982 ± 0.001[a]

3.5. Conclusions

This work was one of the first attempts to systematically study the growth kinetics of *Listeria monocytogenes* as affected by the isolated effect of fat content. It was demonstrated that the combination of a fat content increase from 1 to 5% and the particular rheological, compositional and microstructural characteristics of the studied model systems may have created a local micro-environment at the fat-water interface that supports faster growth of *L. monocytogenes* (i.e., a shorter lag phase duration λ and a higher maximum specific growth rate μ_{max}). An increase in fat content from 5% to 10 or 20% did not result in a further reduction of λ. On the other hand, a complex relationship between fat content and μ_{max} was observed with varying fat content, suggesting the existence of both a growth-promoting micro-environment at the fat-water interface and a growth-inhibiting effect caused by the reduced water content and growth space availability.

Overall, it was demonstrated that fat content and the resulting microstructural and rheological properties significantly affect *L. monocytogenes* growth dynamics in fish-based food model systems. Therefore, the consideration of these factors during predictive (growth) model development would be beneficial to more accurately estimate food safety in fat-containing RTE food products which are susceptible to *Listeria* contamination. In this regard, findings should be validated in real emulsion and gelled emulsion type foods, including fish-based food products such as fish pâté, surimi, and fish sausage.

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Chapter 4: Quantifying the bioprotective effect of *Lactobacillus sakei* CTC494 against *Listeria monocytogenes* on vacuum packaged hot-smoked sea bream

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Manuscript published in:

4.1. Abstract

In this study, the bioprotective potential of *Lactobacillus sakei* CTC494 against *Listeria monocytogenes* CTC1034 was evaluated on vacuum packaged hot-smoked sea bream at 5 ºC and dynamic temperatures ranging from 3 to 12 ºC. The capacity of three microbial competition interaction models to describe the inhibitory effect of *L. sakei* CTC494 on *L. monocytogenes* was assessed based on the Jameson effect and Lotka-Volterra approaches. A sensory analysis was performed to evaluate the spoiling capacity of *L. sakei* CTC494 on the smoked fish product at 5 ºC. Based on the sensory results, the bioprotection strategy against the pathogen was established by inoculating the product at a 1:2 ratio (pathogen:bioprotector, log CFU/g). The kinetic growth parameters of both microorganisms were estimated in mono-culture at constant storage (5 ºC). In addition, the inhibition function parameters of the tested interaction models were estimated in co-culture at constant and dynamic temperature storage using as input the mono-culture kinetic parameters. The growth potential (δ log) of *L. monocytogenes*, in mono-culture, was 3.5 log on smoked sea bream during the experimental period (20 days). In co-culture, *L. sakei* CTC494 significantly reduced the capability of *L. monocytogenes* to grow, although its effectiveness was temperature dependent. The LAB strain limited the growth of the pathogen under storage at 5 ºC (< 1 log increase) and at dynamic profile 2 (< 2 log increase). Besides, under storage at dynamic profile 1, the growth of *L. monocytogenes* was inhibited (< 0.5 log increase). These results confirmed the efficacy of *L. sakei* CTC494 for controlling the pathogen growth on the studied fish product. The Lotka-Volterra competition model showed slightly better fit to the observed *L. monocytogenes* growth response than the Jameson-based models according to the statistical performance. The proposed modelling approach could support the assessment and establishment of bioprotective culture-based strategies aimed at reducing the risk of listeriosis linked to the consumption of RTE hot-smoked sea bream.

Keywords: Biopreservation; Ready-to-eat fish products; Food safety; Microbial interaction model; Predictive microbiology; Bacteriocin-producing bacteria

4.2. Introduction

Smoked fish products are sold as ready-to-eat (RTE) foods characterized by a relatively long refrigerated shelf-life when packaged under vacuum (Hwang, 2007). These seafood commodities are popular, but they are also considered among the top risk foodstuffs since they can be contaminated with foodborne pathogens and no cooking is applied before consumption (Ghanbari et al., 2013). At present, the microbiological concerns in the EU associated with extended shelf-life refrigerated RTE foods are focused on psychrotrophic foodborne pathogens such as *Listeria monocytogenes* (EFSA BIOHAZ, 2018).

Biopreservation, also called bioprotection, is a biocontrol approach to enhance product safety and shelf-life using microorganisms selected for their antimicrobial properties, so called protective cultures (Leroi et al., 2015). Lactic-acid bacteria (LAB) are considered a new generation of food additives and the basis of food biopreservation (Said et al., 2019). Protective cultures are considered by the regulatory agencies as 'new' food additives, meaning that they require market authorization for their technological use in foods. However, most LAB are Generally Recognized as Safe (GRAS) and many LAB species (including *Lactobacillus sakei*) have been granted by EFSA with the Qualified Presumption of Safety (QPS) status (EFSA (European Food Safety Authority), 2018). In the EU, microorganisms with the latter food-grade standard do not need to undergo a further safety assessment other than to provide evidence of efficacy and to satisfy the specified qualifications, if applicable, for its market approval. Two recent studies have proved that the antilisterial sakacin k-producing *Lactobacillus sakei* strain CTC494 (from meat origin) is effective to inhibit *L. monocytogenes* in filleted sea bream and cold-smoked salmon under refrigerated storage (Aymerich et al., 2019; Costa et al., 2019). Nevertheless, the inhibitory capacity of this bioprotective LAB strain has not been tested in other fish products where the differences in product's characteristics and formulations might either favor its inhibition thanks to the antimicrobial hurdle combinations (Leistner, 2000) or hinder the ability of the strain to inhibit *L. monocytogenes* (Tahiri et al., 2009; Vasilopoulos et al., 2010).

Quantifying microbial interaction in food can be highly complex and often overlooked in predictive microbiology studies (Powell et al., 2004). Most of the competitive growth models available in literature are based on two approaches: one based on the Jameson effect phenomenon (i.e. nutrient competition) (Jameson, 1962) and the other using the general Lotka-Volterra competition model (i.e. predator-prey model) (Powell et al., 2004; Valenti et al., 2013). Both mathematical models represent a simultaneous deceleration of bacterial populations. The inhibition of *L. monocytogenes* by endogenous LAB usually responsible for spoilage has been studied and modelled in minimally processed fish products (Mejlholm et al., 2015; Mejlholm and Dalgaard, 2015, 2007). In this regard, most of the published microbial interaction models aim at

describing competition between background microbiota and microbial pathogens, rather than to characterize the performance of bioprotective bacteria with specific antagonistic activities, that are normally added at higher levels than the natural background (spoilage) microbiota (Cornu et al., 2011). To the best author's knowledge, studies having quantified the bioprotective effect of bacteriocin-producing LAB cultures through the development and implementation of predictive models are scarce. The first attempt to model the inhibitory effect of a bacteriocinogenic LAB strain against *Listeria* in fish was made by Costa et al. (2019), in which model parameters were derived from experiments in a fish-based broth and then validated on fresh filleted sea bream.

The objective of this study was (i) to evaluate the bioprotective potential of *L. sakei* CTC494 against *L. monocytogenes* on hot-smoked sea bream under constant and dynamic storage temperature conditions and (ii) to evaluate the capacity of three microbial interaction models based on the Jameson effect and Lotka-Volterra approaches to describe the inhibitory effect of *L. sakei* CTC494 on *L. monocytogenes*.

4.3. Materials and methods

4.3.1. Bacterial strains

The selected bacterial strains used in this work were the bioprotective culture *L. sakei* CTC494 and the target pathogen *L. monocytogenes* CTC1034. This pathogenic strain was used in a previous work as a reference strain to study the antagonism of bacteriocin-producing LAB, including *L. sakei* CTC494 (Garriga et al., 2002). Both microorganisms were stored at −80 ºC in the appropriate culture broth with 20% (v/v) glycerol. Before inoculation, a fresh culture was prepared for each strain and a well-isolated colony was used to perform two consecutive 24 h-subcultures, which were grown in de Man, Rogosa and Sharpe broth (MRS, Oxoid, UK) for *L. sakei* at 30 ºC with 10% CO_2 and Brain Heart Infusion (BHI, Oxoid, UK) for *L. monocytogenes* at 30 ºC. This resulted in a cell density of *ca.* 10^8 CFU/mL and 10^9 CFU/mL for *L. sakei* and *L. monocytogenes*, respectively.

4.3.2. Preparation of hot-smoked sea bream

Gilthead sea bream fishes (*Sparus aurata*) from marine aquaculture were collected and processed by the Andalusian Aquaculture Technology Centre (CTAQUA, Cádiz, Spain) following an industrial hot smoking process. First, fish samples were manually scaled, gutted, filleted and bled in ice-water. Then, fillets were brined in a NaCl/sugar solution (ratio 3:1) for 2 h. After that, fish fillets were removed from the brine solution, washed with water and introduced in a smoking oven (till the fish core temperature reached 65 ºC during 30 min). After cooling at room temperature for 15 min, smoked fillets were vacuum-packed and transferred to the laboratory the day after processing in polystyrene boxes under cold conditions.

4.3.3. Sensory assessment

A sensory analysis was carried out to evaluate the impact of the bioprotective culture on the organoleptic characteristics of hot-smoked sea bream during refrigerated storage based on an acceptance test. For that, 10 g-fish portions were surface inoculated with the *L. sakei* CTC494 as described below (section 4.3.4) at three initial cell densities (*ca*. 2 log CFU/g, 4 log CFU/g and 6 log CFU/g) and stored at 5 ± 0.5 ºC under vacuum packaging conditions for 18 days. A control batch was prepared without inoculating bacteria.

The sensory evaluation was performed based on the work by Yanar et al. (2006), who evaluated the shelf-life of hot-smoked tilapia stored at 4 ºC. For that, 5 panelists were trained according to the standard EN ISO 8586:2012 method. The descriptors were generated by open discussion and consensus in a previous session using fish samples stored under the same experimental condition applied in this study. The descriptors retained were general appearance (score of the overall appearance), intensity of odour (score of the overall odour) and texture to the touch. The assessors scored control and inoculated samples for the appearance, odour and texture characteristics using a 9-point hedonic scale. A score of 7–9 denoted "very good" quality, a score of 4.0–6.9 "good" quality, and a score of 1.0–3.9 indicated "unacceptable" quality. The sensory assessment was performed on the storage days 4, 7, 11, 14 and 18. At each evaluation point, one fish portion from each assayed condition was individually served under white light on conventional petri dishes, labeled with three-digit random numbers.

The obtained sensory scores for each attribute were plotted against time and sensory deterioration rates were obtained by fitting a linear model. Deterioration rates (slopes) for inoculated and control samples were statistically compared by performing *t*-test ($p \leq 0.05$) using the statistical software package SPSS 25.0 (Chicago, Illinois, USA).

4.3.4. Challenge tests

In the first trial, the growth of mono-cultured and co-cultured *L. sakei* CTC494 and *L. monocytogenes* CTC1034 on smoked sea bream was evaluated under constant refrigerated storage (5 ± 0.5 ºC) for 20 days. Four batches were prepared: (1) non-inoculated product (control); (2) inoculated with *ca*. 2 log CFU/g *L. monocytogenes*; (3) inoculated with *ca*. 4 log CFU/g *L. sakei* and (4) inoculated with a mixture of *L. monocytogenes* and *L. sakei* at a ratio 1:2 (i.e. 2 log CFU/g *L. monocytogenes* and 4 log CFU/g *L. sakei*). The inoculation ratio pathogen:bioprotector used in batch 4 was selected based on the results previously obtained from the sensory analysis (section 2.3).

Prior to inoculation, smoked fish fillets were cut into portions of *ca*. 5 cm^2 and 10 g using a sterile scalpel. This fish portion was considered the analytical fish sample. The caudal region of the fish

fillet was discarded from the analysis to avoid experimental variability. Smoked sea bream portions were surface inoculated on the flesh side at 1% (v/w) from the appropriate decimal dilution using a L-shaped sterile spreader. For experiments in co-culture (batch 4), the product was first inoculated with the pathogen and left for 10 min in the safety cabinet to allow cell attachment. The LAB strain was then spread, followed by a cell attachment period as the pathogen case. This inoculation procedure (Aymerich et al., 2019) was used to mimic a post-processing contamination scenario in which the fish product is contaminated by the pathogen after the hot-smoking process and then the bioprotective culture is applied.

After inoculation, fish portions were individually vacuum packaged in polyamide-polyethylene plastic bags (Sacoliva, Barcelona, Spain) and stored as described above.

In the second trial (approx. 2 months later) with a new batch of hot-smoked sea bream, the bioprotective effect of *L. sakei* CTC494 on *L. monocytogenes* was evaluated in co-culture at two different dynamic temperature profiles, which were designed to simulate the fluctuating conditions of the cold-chain distribution and storage of smoked fish products in Spain. Profile 1 had a mean temperature of 6.3 ºC and range between 3.6 and 12.8 ºC while profile 2 had 7.6 ºC of mean temperature and range between 3.3 and 11.8 ºC. Microorganisms were co-inoculated on the sample's surface and vacuum packaged as described before for batch 4. An additional non-inoculated (control) batch was also studied.

The challenge tests were conducted in Hot-Cold incubators containing programmable time-temperature profiles (Selecta, Barcelona, Spain). Incubation temperatures were measured continuously throughout the experiments by data loggers (MicroLite, Fourier Tech- nologies, Israel). For experiments, 2 samples (duplicate) were analysed for microbiological and/or physicochemical determinations at different sampling points. The experiments were repeated twice in different days.

4.3.5. Microbiological analyses

Fish portions of 10 g were aseptically transferred into a sterile stomacher bag and homogenized for 60 s (masticator, IUL Instruments, Barcelona, Spain) with 0.1% sterile peptone water (Oxoid, UK).

For non-inoculated (control) samples, the growth of aerobic mesophilic viable count (MVC), endogenous LAB counts and *Listeria* investigation (presence/absence) was evaluated during storage at the tested temperatures. MVC counts were determined by pour plating decimal dilutions from homogenized samples in Plate Count Agar (PCA, Oxoid) incubated 48 h at 37 ºC. For LAB enumeration, de Man, Rogosa, Sharpe agar (MRS, Oxoid) was used, which was incubated 48 h

at 33 ºC with 10% CO_2. The presence/absence of *L. monocytogenes* was carried out following the method EN ISO 11290-1.

For inoculated samples, the enumeration of *L. monocytogenes* was carried out using Oxford agar (Oxoid) containing Oxford selective supplement (SR0140, Oxoid). Counts were obtained after incubating the plates for 24–48 h at 37 ºC. LAB were enumerated as for non-inoculated samples.

4.3.6. Physicochemical analyses

The physiochemical characteristics of hot-smoked sea bream (i.e. pH and a_w) were determined by analysis of 2 fish portions (10 g each). pH measurement was performed for samples from batch 1 (non-inoculated) and batch 3 (inoculated with *ca*. 4 log CFU/g *L. sakei*) on the days of the sensory analysis (section 4.3.3) using the pH meter Edge HI2020 (HI11310 electrode, Hanna Instruments, USA) by homogenizing the fish portion with distilled water at ratio 1:1.

The determination of aw was performed with the AquaLab 4 TE aw meter (Decagon Devices Inc., WA, USA) for non-inoculated samples at the start of the challenge tests.

4.3.7. Modelling microbial interaction

The inhibition of *L. monocytogenes* by the bioprotective *L. sakei* CTC494 was modelled following the mathematical approach proposed by Costa et al. (2019). This consists of (i) the estimation of the growth parameters lag phase, maximum specific growth rate and maximum population density of *L. sakei* CTC494 and *L. monocytogenes* CTC1034 in mono-culture, (ii) the estimation of the inhibition function parameters of three existing microbial competition interaction models using as input the mono-culture kinetic parameters and (iii) the statistical analysis of the performance of the tested models to describe experimental observations.

4.3.7.1. Primary growth kinetic parameters and secondary model

The observed growth data for mono-cultured *L. sakei* CTC494 and *L. monocytogenes* CTC1034 on hot-smoked sea bream obtained at constant storage temperature (5 ºC) were used to fit the primary growth model of Baranyi and Roberts (1994) and the kinetic parameters lag phase (λ, h), maximum specific growth rate (μ_{max}, 1/h) and maximum population density (N_{max}, log CFU/g) were determined for each microorganism. The model was fitted to the experimental data using the program DMFit for Excel v.3.5 (IFR, Norwich, UK).

Growth rate at different temperatures was predicted using the secondary model for μ_{max} as a function of temperature published by Costa et al. (2019). This model was developed in fish juice of sea bream for the same bacterial strains used in this study and was validated on fresh filleted sea bream stored under isothermal and non-isothermal conditions. The model corresponded to a square root type equation (Ratkowsky et al., 1982) with parameter values *b* and T_{min} being 0.028

and –4.5 ºC, respectively for *L. sakei* CTC494, and 0.026 and –3.4 ºC, respectively for *L. monocytogenes* CTC1034. Since an effect of the fish matrix and vacuum conditions on μ_{max} was expected, an adjustment factor was applied to simulate the microbial interaction on smoked sea bream. The adjustment factor for μ_{max} of each microorganism was calculated as the ratio between the μ_{max} values obtained in this study from mono-culture experiments at 5 ºC in smoked sea bream and in fish juice by Costa et al. (2019).

4.3.7.2. Microbial interaction models

Three existing microbial competition models were evaluated to assess their ability to describe the growth response of the bioprotective effect of *L. sakei* CTC494 on *L. monocytogenes* at isothermal and non-isothermal storage conditions: the Jameson effect model, a modified version of the Jameson effect model and the Lotka-Volterra model.

The generic system of equations (Eq. (4.1)) of the tested interaction models, applied in their implicit form to co-cultured *L. sakei* CTC494 (subscript '1') and *L. monocytogenes* (subscript '2'), can be expressed as:

$$\frac{dN_1(t)}{dt} = N_1 \cdot \mu_{max1} \cdot \left(\frac{Q_1}{1+Q_1}\right) \cdot f_1(t)$$

$$\frac{dN_2(t)}{dt} = N_2 \cdot \mu_{max2} \cdot \left(\frac{Q_2}{1+Q_2}\right) \cdot f_2(t)$$

$$\frac{dQ_1}{dt} = Q_{1\,t-1} \cdot \mu_{max1} \qquad\qquad (4.1)$$

$$\frac{dQ_2}{dt} = Q_{2\,t-1} \cdot \mu_{max2}$$

$$Q_0 = \frac{1}{e^{(\mu_{max} \cdot \lambda)} - 1}$$

where N is the cell concentration (CFU/g) at time t, μ_{max} is the maximum specific growth rate (1/h), Q and Q_0 is a measure of the physiological state of cells at time t and $t = 0$, respectively, $f(t)$ is an inhibition function and λ (h) the lag phase duration of the cells.

The different microbial competition models were tested based on the generic system of equations in Eq. (4.1) and using the corresponding inhibition function $f(t)$ described in either Eq. (4.2), or (4.3) or (4.4) for each type of model.

The empirical Jameson effect model is based on the assumption that all bacterial populations stop growing when the dominant culture reaches its maximum population density (Giménez and Dalgaard, 2004). In this case, the inhibition function $f(t)$ is defined as:

$$f_1(t) = \left(1 - \frac{N_1}{N_{max1}}\right) \cdot \left(1 - \frac{N_2}{N_{max2}}\right) \qquad\qquad (4.2)$$

$$f_2(t) = \left(1 - \frac{N_2}{N_{max2}}\right) \cdot \left(1 - \frac{N_1}{N_{max1}}\right)$$

where N_{max} is the maximum cell density (CFU/g) for each population with subscript '1' and '2' for *L. sakei* CTC494 and *L. monocytogenes*, respectively, and other parameters are as indicated in Eq. (1).

The modified Jameson effect model was based on a modification of the inhibition function f_2(t) in Eq. (2), in which the maximum density of population 1 (i.e. *L. sakei* CTC494; N_{max1}) is replaced by a critical population density, being typically lower than its N_{max1} (Cornu et al., 2011; Le Marc et al., 2009). This parameter describes the concentration value of the population 1 that results in the stop of the growth of population 2. This critical concentration value can be related to the production of an inhibitory substance, at a certain level, able to inhibit growth of the other population. In addition, if $N_{CPD1} < N_1 < N_{max1}$, then f_2(t) < 0, which describes a decline of population 2.

$$f_2(t) = \left(1 - \frac{N_2}{N_{max2}}\right) \cdot \left(1 - \frac{N_1}{N_{CPD1}}\right) \qquad (4.3)$$

where N_{CPD1} is the critical population density of population 1 (i.e. *L. sakei* CTC494; CFU/g) and other parameters are as indicated in Eqs. (4.1) and (4.2).

Finally, it was used the classical predator-prey (Lotka-Volterra) model for interspecific bacterial competition (Dens et al., 1999; Powell et al., 2004), which includes two coefficients of interaction (α_{12} and α_{21}) measuring the competitive effect of species 1 on species 2 and vice-versa. In our study, the competition term of *L. monocytogenes* on *L. sakei* (α_{21}) was fixed to zero since we assumed that the pathogen did not influence growth of *L. sakei* CTC494 due to the higher concentration, shorter lag time and faster growth of the LAB strain (Mejlholm and Dalgaard, 2015; Møller et al., 2013). Therefore, the tested model includes one inhibition function f_1(t) formulated as:

$$f_1(t) = \left(1 - \frac{N_1 + \alpha_{12} \cdot N_2}{N_{max1}}\right) \qquad (4.4)$$

where α_{12} is the competition term of *L. sakei* CTC494 on *L. monocytogenes* and other parameters are as indicated in Eqs. (4.1) and (4.2).

Simultaneous growth of *L. sakei* CTC494 and *L. monocytogenes* was described by estimating the inhibition parameter for each tested model, using as input the kinetic parameters estimated by the Baranyi and Roberts primary model from mono-culture data (λ, N_{max}), together with the square root function for μ_{max} reported by Costa et al. (2019) to account for the temperature effect. The parameter estimations were performed in Microsoft Excel with Solver add-in tool using numerical integration with a time step of 0.5 h (Microsoft Corp., Redmond, WA, USA). The interaction factors (CPD_1, α_{12}) were estimated by regression analysis (MS Excel) using kinetic parameters from mono-culture experiments and the secondary model by Costa et al. (2019).

4.3.7.3. Interaction models prediction performance

The capacity of the prediction of the tested models under constant and dynamic temperature storage conditions was assessed by the statistical indexes Root Mean Squared Error (RMSE) and corrected Akaike Information Criterion (AICc) calculated as shown in Eqs. (4.5) and (4.6), respectively.

$$RMSE = \sqrt{\frac{\sum(fitted - observed)^2}{n-p}} \tag{4.5}$$

$$AICc = n \cdot ln\left(\frac{RSS}{n}\right) + 2 \cdot (p+1) + \frac{2 \cdot (p+1) \cdot (p+2)}{n-p-2} \tag{4.6}$$

where *n* is the number of observations and *p* is the number of model parameters to be estimated in Eqs. (4.5) and (4.6) and *RSS* is the residual sum of squares in Eq. (4.6).

In addition to the statistical performance described above, the Acceptable Simulation Zone (ASZ) method was used to evaluate the prediction capacity of the interaction models at dynamic storage temperatures. The acceptable interval was defined as ± 0.5 log-units from the simulated growth of *L. sakei* or *L. monocytogenes*. The simulations were considered acceptable when at least 70% of the observed counts were within the ASZ (Oscar, 2005).

4.4.   Results and discussion

4.4.1.   Sensory evaluation of the bioprotective *L. sakei* CTC494 on smoked sea bream

The average sensory scores and sensory deterioration rates obtained for the three evaluated attributes during storage at 5 ºC for non-inoculated (control) and inoculated samples are showed in Fig. 4.1 and Table 4.1, respectively. Overall, no significant differences (p > 0.05) were found between the spoilage rates obtained for control and inoculated samples with 2 and 4 log CFU/g of *L. sakei* CTC494 throughout the storage period, with sensory scores denoting, in general, "very good" or "good" quality (4–9). On the contrary, sensory scores were < 4 at 11 days for samples inoculated with *L. sakei* CTC494 at 6 log CFU/g for the attribute appearance (Fig. 4.1A). Moreover, spoilage rates were significantly higher (p ≤ 0.05) at inoculation level of 6 log CFU/g, indicating that such a high inoculation level may cause undesired changes to the hot-smoked sea bream as compared to the samples of lower inoculum sizes. Although the smoked fish product was not tested for its taste, it can be assumed that the product was not significantly acidified due to the addition of the LAB strain at levels ≤ 4 log CFU/g, as indicated by the recorded pH values at the end of the storage period for control and inoculated samples at 4 log CFU/g *L. sakei*, which corresponded to 6.0 ± 0.1 and 5.9 ± 0.1, respectively.

Our results are consistent with those reported by Costa et al. (2019), who also sensory validated the application of *L. sakei* CTC494 on fresh fish fillets under modified atmosphere packaging (MAP) and found that at levels of 2 and 4 log CFU/g the sensory characteristics were statistically similar to the non-inoculated product. Based on these results, the bioprotection strategy against the pathogen was established in this study by inoculating the product with 4 log CFU/g of *L. sakei* CTC494 at the beginning of the experiments.

Table 4.1. Sensory deterioration rates (slope ± standard error) obtained for vacuum packaged hot-smoked sea bream samples non-inoculated (control) and inoculated at different initial levels of *L. sakei* CTC494 (2, 4 and 6 log CFU/g) under storage at 5 ºC. Values for the same attribute with different uppercase letters are significantly different (p ≤ 0.05).

Initial level (log CFU/g)	Appearance	Odour	Texture
Control	-0.237 ± 0.03^A	-0.195 ± 0.03^A	-0.150 ± 0.04^A
2	-0.220 ± 0.05^A	-0.213 ± 0.03^A	-0.145 ± 0.03^A
4	-0.182 ± 0.03^A	-0.186 ± 0.03^A	-0.136 ± 0.03^A
6	-0.337 ± 0.04^B	-0.331 ± 0.02^B	-0.311 ± 0.01^B
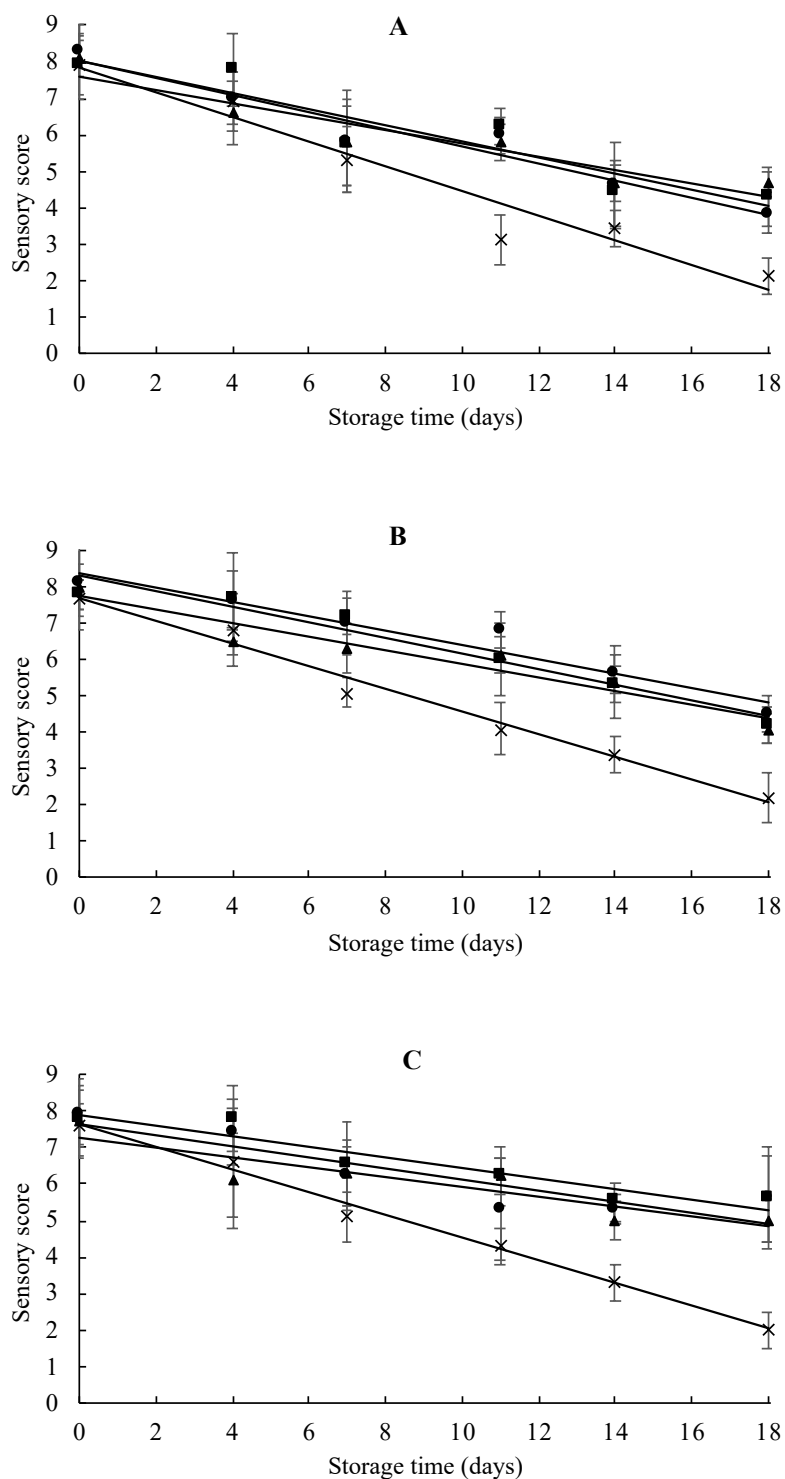
Figure 4.1. Sensory scores obtained for the attribute appearance (A), odour (B) and texture (C) for vacuum packaged hot-smoked sea bream samples non-inoculated (●) and inoculated at different initial levels of *L. sakei* CTC494: 2 log CFU/g (■), 4 log CFU/g (▲) and 6 log CFU/g (×) under storage at 5 ºC. Data point are the mean values from five panelists with vertical lines denoting ± the standard deviation.

4.4.2. Evaluating the bioprotective effect of *L. sakei* CTC494 against *L. monoctogenes*

4.4.2.1. Mono-culture growth on hot-smoked sea bream

Regarding the microbiological analysis of endogenous microbiota of hot-smoked sea bream, the concentration of endogenous LAB and MVC obtained at the starting day for control samples were below the detection level (< 1 log CFU/g) and remained below this level for LAB and < 2 log CFU/g for MVC during the evaluated storage period at both constant and dynamic temperature conditions (data not shown). In addition, *L. monocytogenes* was not detected in any control sample. The initial aw and pH of non-inoculated (control) samples corresponded to 0.96 ± 0.007 and 6.0 ± 0.1, respectively.

The kinetic parameters and their standard errors estimated by the Baranyi and Roberts model for experiments in mono-cultures at 5 ºC are showed in Table 4.2. A long lag phase λ and an exponential growth rate μ_{max} was distinguished for each studied microorganism. *L. sakei* CTC494 reached the stationary phase after approximately 13 days of storage. However, no stationary phase was observed for *L. monocytogenes* CTC1034 at the end of the experimental period (20 days), thus the Baranyi and Roberts model (no asymptote) was used to describe the growth behaviour of *L. monocytogenes* on hot-smoked sea bream. Despite this, the growth potential (δ log) of *L. monocytogenes* was 3.5 log during the storage period, which demonstrates that refrigeration at 5 ºC does not prevent the pathogen growth on the studied fish product. *L. monocytogenes* growth was also observed in other vacuum-packed hot-smoked fish products at refrigeration temperatures (Branciari et al., 2016; Mahmoud et al., 2012; Tosun and Özden, 2014).

The parameter μ_{max} in mono-culture experiments was much higher for *L. sakei* (0.062 1/h) than for *L. monocytogenes* (0.024 1/h). The μ_{max} value obtained for the latter microorganism was lower than that estimated by Costa et al. (2019) for the same strain in mono-culture in fish juice at 5 ºC (0.0477 1/h), corresponding to a reduction of 50% with respect to the value reported by Costa et al. (2019). These authors found that the μ_{max} value of *L. monocytogenes* in the fish product (i.e. MAP fresh filleted sea bream) presented a reduction of 68% with respect to that observed in fish juice. Differences in reduction rate could be due to the specific characteristics of the product studied herein. In the work by Costa et al. (2019), the presence of indigenous microbiota in fresh fish fillets that was higher can explain a more reduced pathogen growth when compared to smoked sea bream in our study, where MVC and LAB were in low numbers as a consequence of the hot smoking process. In addition, the presence of CO_2 in MAP fish fillets has been described as an inhibiting factor influencing *L. monocytogenes* growth (Bolívar et al., 2018; Provincial et al., 2013). Regarding this, Tosun and Özden (2014) reported that MAP was most effective in controlling the growth of *L. monocytogenes* in hot-smoked rainbow trout fish fillets at 2 ºC while vacuum packaging had no effect.

Table 4.2. Estimated lag time (λ), maximum specific growth rate (μ_{max}), maximum population density (N_{max}) ± associated standard error for the individual growth curves of *L. sakei* CTC494 and *L. monocytogenes* CTC1034 on vacuum packaged hot-smoked sea bream at 5 ºC.

	λ (hours)	μ_{max} (1/h)	N_{max} (log CFU/g)	Adj. $R^{2,a}$
L. sakei CTC494	133.1 ± 33.7	0.0620 ± 0.006	8.59 ± 0.17	0.977
L. monocytogenes CTC1034	160.3 ± 39.5	0.0240 ± 0.001	ne[b]	0.954

[a] Adjusted coefficient of determination of the fitted Baranyi and Roberts model (Adj. R^2).

[b] The Baranyi and Roberts model (no asymptote) was fitted to *L. monocytogenes* growth data (ne).

4.4.2.2. Co-culture growth on smoked sea bream

Growth curves of co-cultured *L. sakei* CTC494 and *L. monocytogenes* CTC1034 on smoked sea bream are showed in Fig. 4.2. The LAB strain exhibited a similar lag phase at 5 ºC and under dynamic temperature profile 1 (around 5 days), which also corresponded to that estimated in mono-culture (Table 4.2). *L. sakei* CTC494 showed a shorter lag phase under profile 2, probably due to the abrupt temperature rise at 4 storage days. In addition, the microorganism reached similar *Nmax* values (*ca*. 8.6 log CFU/g) at all storage temperatures and both (mono and co-) culture conditions. These results suggest, as expected, that the growth of *L. sakei* CTC494 in co-culture was not affected by the presence of *L. monocytogenes*.

On the contrary, *L. sakei* CTC494 significantly reduced the capability of *L. monocytogenes* to grow at the tested inoculation ratio, although its effectiveness was temperature dependent. In this regard, *L. sakei* CTC494 limited the growth of the pathogen under storage at 5 ºC (< 1 log increase) and at dynamic profile 2 (< 2 log increase) throughout the evaluated period (Fig. 4.2A and C, respectively). Besides, under storage at dynamic profile 1 (Fig. 4.2B), the growth of *L. monocytogenes* was inhibited (< 0.5 log increase).

The competition pattern was similar for all temperature conditions, showcasing a slight *L. monocytogenes* increase, which ceased when the *L. sakei* population approached to its N_{max} (> 8 log CFU/g). This phenomenon would reflect a non-specific interaction described by a potential Jameson effect between populations. At 5 ºC and profile 1, *L. monocytogenes* population showed a remarkable decline after *L. sakei* had reached its N_{max}, but at profile 2 growth of the pathogen slightly continued.

The efficacy of *L. sakei* CTC494 against *L. monocytogenes* has already been proved on fish. Aymerich et al. (2019) reported that the growth of *L. monocytogenes* on three types of cold-smoked salmon inoculated with *L. sakei* CTC494 was completely inhibited after 21 days at 8 ºC under vacuum packaging. In other study, the increase of the pathogen was less than 1 log units on fresh filleted sea bream in the presence of the LAB strain after storage under MAP at isothermal

and non-isothermal storage conditions (Costa et al., 2019). The degree of inhibition of *L. monocytogenes* exerted by *L. sakei* CTC494 in different types of fish products illustrates the capacity of this bioprotective culture as a potential antimicrobial agent (AMA) to be used as part of the *L. monocytogenes* control alternatives for RTE food defined by the US Food Safety Inspection Service Listeria rule (FSIS, 2014).
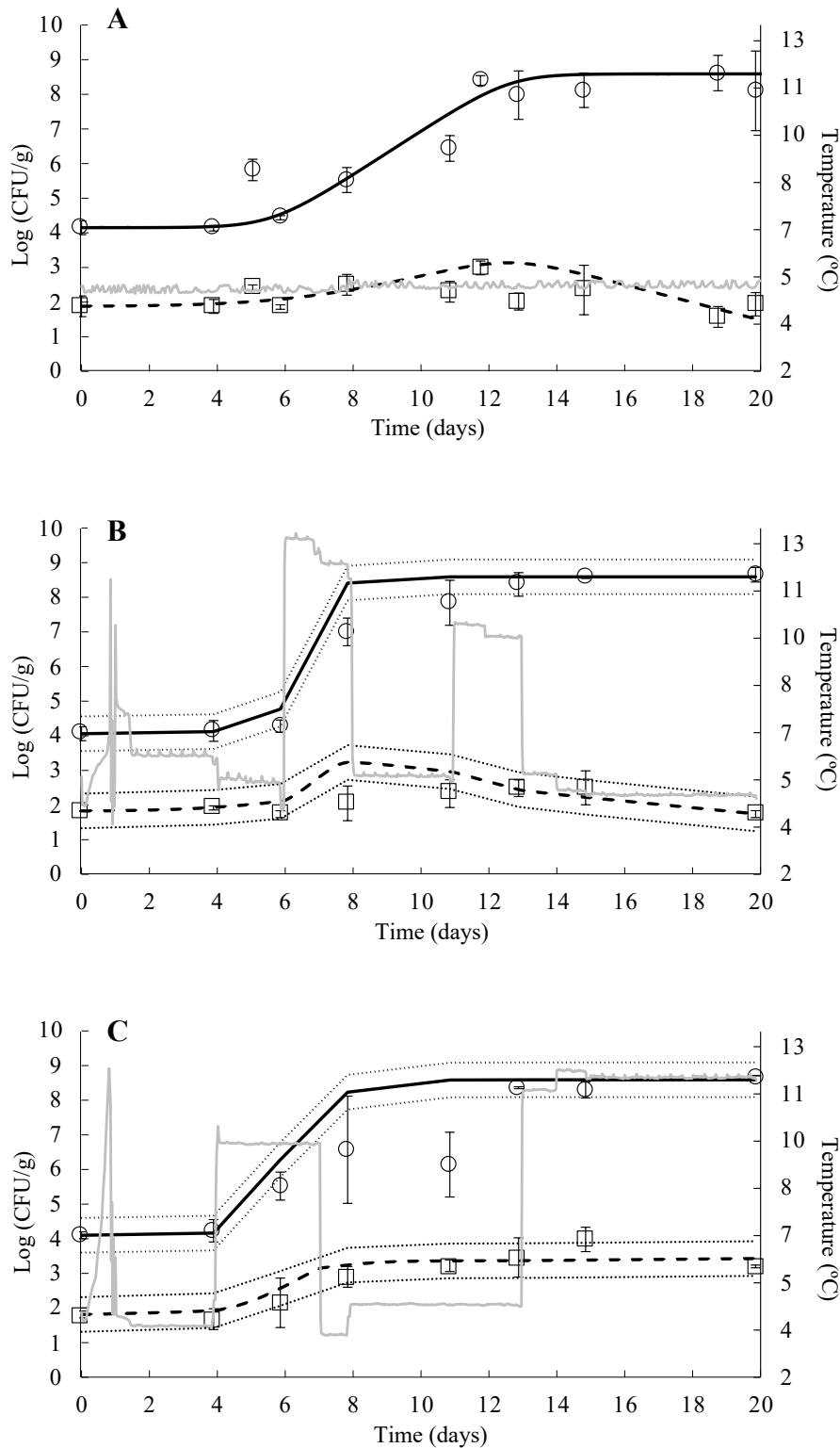
Figure 4.2. Experimental growth data of *Listeria monocytogenes* CTC1034 (□) and *Lactobacillus sakei* CTC494 (○) on vacuum packaged hot-smoked sea bream during storage at 5 ºC (A) and under dynamic profile 1 (B) and 2 (C). Dashed and solid lines stand for the fit by the Lotka-Volterra model for *L. monocytogenes* and *L. sakei*, respectively. Dotted fine lines define the acceptable simulation zone (ASZ) in (B) and (C). The recorded storage temperature is shown as grey solid line. Growth data points are the mean values from two independent trials with vertical lines showing ± the standard deviation.

4.4.3.   Microbial interaction on smoked sea bream

The three microbial interaction models were fitted to the co-culture experimental results with Eq. (1) estimating the corresponding inhibition function parameters (Eq. (4.2), (4.3) or (4.4)). The fitted inhibition parameters (CPD_1 and α_{12}) and the goodness-of-fit indexes are presented in Table 4.3, together with the ASZ values (%) obtained from the prediction capacity evaluation for dynamic storage temperatures.

To consider the effect of food matrix on μ_{max} on the growth simulation at dynamic temperature conditions, the μ_{max} for *L. monocytogenes* estimated from the secondary model (based on fish juice) was adjusted by applying an adjustment factor of 0.50 (section 4.4.2.1) in the tested microbial interaction models. For *L. monocytogenes*, the Lotka-Volterra model showed, in general, the best fitting according to the statistical indices RMSE and AICc, with values varying from 0.334 to 0.536 and −12.19 to −4.65, respectively. The modified Jameson effect model presented better fitting than the Jameson effect model, except for dynamic profile 2 in which the lowest RMSE value was obtained for the latter model (0.314).

For *L. sakei* CTC494, the three interaction models provided similar fitting, with RMSE values being slightly lower for the Jameson effect model (Table 4.3). For all tested models, the worst fitting was obtained for profile 2 as indicated by higher RMSE and AICc values (Table 4.3). In profile 2, it can be also observed that the bioprotector strain exhibited an adaptation delay after the initial lag phase (Fig. 4.2C). This phenomenon has been previously described as intermediate lag periods induced by abrupt temperature shifts in which cells need to adjust to the changing temperature environment (Swinnen et al., 2005). Intermediate lag periods of significant duration are expected to be present when the temperature profiles are below the optimal temperature of growth, thus the predictive ability of the secondary models is affected negatively (Longhi et al., 2013). This phenomenon has been previously observed for the same microorganism in fresh fish (Costa et al., 2019) and also for different *Lactobacillus* strains in other food products stored at dynamic temperatures (Longhi et al., 2013; Silva et al., 2017).

Owing to the LAB strain used in our study is a sakacin K producing species, a specific antagonistic effect would be expected due to this potentially inhibitory substance on *L. monocytogenes*. Although the bacteriocin was not quantified to confirm this effect, the fish product and storage temperatures under study might constitute a suitable bacteriocin production-supporting environment. In this regard, Hugas et al. (1998) quantified sakacin K produced by *L. sakei* CTC494 in MRS broth and observed bacteriocin activity at initial pH of 6.0 and 6.5 and higher production at low temperatures (4, 10 and 15 ºC) compared to abuse storage (20, 25 and 30 ºC). In addition, Leroy and De Vuyst (2001) suggested that extremely rich environments (e.g. MRS broth) will not necessarily increase specific bacteriocin production compared to nutrient-depleted

environments, as in the present work (i.e. sugar limitation). Apparently, bacteriocin production is stimulated by less favorable growth conditions, such as low temperatures and competing microbiota (Delboni and Yang, 2017).

Regarding the prediction capacity of the tested models under dynamic profile 1, all models were able to describe satisfactory the simultaneous microbial growth, with 75% of the observations within the ASZ, excepting for the Jameson model, for which the value for *L. monocytogenes* was 37.5%. Under dynamic profile 2, none of the models provided reasonable predictions for co-cultured *L. sakei* CTC494 (< 70%). This lack of prediction could be explained by the existence of an intermediate lag phase as mentioned in above lines. However, all models represented adequately the growth response of *L. monocytogenes*, as confirmed by ASZ values equal to 87.5%.

The estimated value of the CPD_1 parameter included in the modified Jameson effect model remained similar for the different temperatures (Table 4.3). The value of the competition factor of *L. sakei* CTC494 on *L. monocytogenes* (α_{12}) estimated by the Lotka-Volterra model was different depending on the temperature conditions. At 5 ºC and profile 1, the obtained α_{12} were above 1, which properly describes the observed *L. monocytogenes* population decline when *L. sakei* CTC494 reached its N_{max}. In contrast, at profile 2, α_{12} was < 1, which means that *L. monocytogenes* growth was slowed down as *L. sakei* CTC494 approached to its N_{max}, as can be seen in Fig. 4.2C. This reflects the different effect of non-isothermal conditions on the interaction between both microorganisms. The Lotka-Volterra's coefficient of interaction (α) of different microbial species in diverse food matrices has also been reported in the literature as a temperature-dependent parameter. Some examples are the values of α estimated at different temperatures for the growth of *L. monocytogenes* in fish juice (α-average = 1.4 between 2.2 and 5 ºC and α-average = 1.6 and 1.8 at 8 and 12 ºC, respectively) (Costa et al., 2019), or the polynomial model developed by Møller et al. (2013) which described the effect of temperature on α of the natural microbiota on growth of *Salmonella* spp. in ground pork during storage between 9 ºC to 24 ºC.

Most of the studies dealing with microbial interaction responses in fish products have been performed on mixed cultures based on a pathogen and non-bacteriocinogenic microorganisms, being either endogenous or artificially inoculated (Giménez and Dalgaard, 2004; Koseki et al., 2011; Mejlholm and Dalgaard, 2015, 2007). These studies aimed to describe the maximum population density of the pathogen, for which the classical Jameson effect model is used. Other studies have used the Lotka-Volterra model as in the case of the work by Giuffrida et al. (2007) that analysed the interaction between *Aeromonas hydrophila* and aerobic natural microbiota on sea bream surfaces considering the complexity of fluctuating environmental conditions and the interspecific bacterial interactions.

Table 4.3. Estimated inhibition parameters (CPD_1, α_{12}) and goodness-of-fit of the three microbial interaction models used to describe the bioprotective capacity of *L. sakei* CTC494 against *L. monocytogenes* CTC1034 on vacuum packaged hot-smoked sea bream stored at different temperatures.

Microorganism	Temp. (ºC)	n[a]	Jameson model		Modified Jameson model				Lotka-Volterra model			
			RMSE[b]	ASZ (%)[c]	CPD_1[d]	RMSE	AICc[e]	ASZ (%)	α_{12}[f]	RMSE	AICc	ASZ (%)
L. sakei CTC494	5.0	11	0.611	–	8.28	0.676	-1.39	–	2.019	0.642	-5.32	–
	Profile 1[g]	8	0.595	75.0	8.44	0.688	3.72	75.0	1.418	0.638	-1.87	75.0
	Profile 2[h]	8	1.087	62.5	8.59	1.256	13.34	62.5	0.989	1.163	7.74	62.5
L. monocytogenes CTC1034	5.0	11	0.879	–	–	0.506	-7.78	–	–	0.480	-11.71	–
	Profile 1	8	0.932	37.5	–	0.579	0.95	75.0	–	0.536	-4.65	75.0
	Profile 2	8	0.314	87.5	–	0.361	-6.59	87.5	–	0.334	-12.19	87.5

[a] Number of observations (n).
[b] Root Mean Squared Error (RMSE).
[c] Percentage (%) of observed values falling within the acceptable simulation zone (ASZ), defined as the simulated value ± 0.5 log-units.
[d] Critical population density for *L. sakei* (CPD_1, log CFU/g) estimated by the modified Jameson effect model fitting Eqs. (4.1) and (4.3).
[e] Corrected Akaike Information Criterion (AICc).
[f] Competition factor of *L. sakei* on *L. monocytogenes* (α_{12}) estimated by the Lotka-Volterra model fitting Eqs. (4.1) and (4.4).
[g] Dynamic temperature profile ranging from 3.6 to 12.8 ºC (average of 6.3 ºC).
[h] Dynamic temperature profile ranging from 3.3 to 11.8 ºC (average of 7.6 ºC).

4.5. Conclusions

The results from this study contribute to extend the application of the bacteriocinogenic strain *L. sakei* CTC494 for controlling growth of *L. monocytogenes* in hot-smoked fish products from Mediterranean aquaculture during refrigerated storage. Results from the challenge tests demonstrated the potential of *L. sakei* CTC494 applied at a dose of 4 log CFU/g to limit or inhibit the growth of *L. monocytogenes* on hot-smoked sea bream under different storage temperatures. Nevertheless, further research is still needed to confirm this inhibitory effect under other possible scenarios such as different temperature profiles (e.g. abrupt temperature changes and abuse temperatures) and process parameters as well as considering batch variability. The proposed modelling approach, based on a validation process in food and the application of adjustment factors to kinetic parameters from the modified Jameson and Lotka-Volterra models, was able to satisfactorily describe the bioprotective effect of *L. sakei* CTC494 on *L. monocytogenes* in the target fish product. This predictive tool could support the assessment and establishment of bioprotective culture-based strategies aimed at reducing the risk of listeriosis linked to the consumption of RTE hot-smoked sea bream.

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Chapter 5: A new expanded modelling approach for investigating the bioprotective capacity of *Latilactobacillus sakei* CTC494 against *Listeria monocytogenes* in ready-to-eat fish products

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5.1. Abstract

Understanding the role of food-related factors on the efficacy of protective cultures is essential to attain optimal results for developing biopreservation-based strategies. The aim of this work was to assess and model growth of *Latilactobacillus sakei* CTC494 and *Listeria monocytogenes* CTC1034, and their interaction, in two different ready-to-eat fish products (i.e., surimi-based product and tuna pâté) at 2 and 12 ºC. The existing expanded Jameson-effect and a new expanded Jameson-effect model proposed in this study were evaluated to quantitatively describe the effect of microbial interaction. The inhibiting effect of the selected LAB on the pathogen growth was product dependent. In surimi product, a reduction of lag time of both strains was observed when growing in coculture at 2 ºC, followed by the inhibition of the pathogen when the bioprotective *L. sakei* CTC494 reached the maximum population density, suggesting a mutualism-antagonism continuum phenomenon between populations. In tuna pâté, *L. sakei* CTC494 exerted a strong inhibition of *L. monocytogenes* at 2 ºC (< 0.5 log increase) and limited the growth at 12 ºC (< 2 log increase). The goodness-of-fit indexes indicated that the new expanded Jameson-effect model performed better and appropriately described the different competition patterns observed in the tested fish products. The proposed expanded competition model allowed for description of not only antagonistic but also mutualism-based interactions based on their influence on lag time.

Keywords: Computational biology; Predictive microbiology; Microbial ecology; Microbial interaction; Mutualism; Antagonism; Jameson effect; bacteriocin-producing bacteria

5.2. Introduction

The healthy aspect of fish, the accessibility of products and prices together with the "fashion" of different varieties of raw fish or meals based on fish is changing the habits in the European Union (EU) from more traditional meals, especially among the younger consumers (EUMOFA, 2017). Many of these products are designed as ready-to-eat (RTE) and/or lightly preserved food since they are produced from fresh seafood and processed using mild technologies (e.g. sushi and its varieties, marinated or smoked fish, seafood salads or fish pâtés). These food commodities are usually consumed without prior cooking and have been accompanied by various safety concerns due to the risk of microbial contamination during handling and processing (Sheng & Wang, 2021).

RTE foods, and especially those requiring refrigeration, are predominantly associated with the foodborne pathogen *Listeria monocytogenes*, the causative agent of human listeriosis (EFSA BIOHAZ, 2018). The high public health impact of this pathogen can be highlighted by the severity of listeriosis. In 2019 in EU, 92% listeriosis cases required hospitalization associated with a fatality rate of 17.6% which accounts for more than half of the deaths associated with foodborne diseases (EFSA, 2021). During the last two decades, several studies have documented the occurrence and persistence of this pathogen in RTE fish products and fish processing environments (Elson et al., 2019; Fonnesbech Vogel, Huss, Ojeniyi, Ahrens, & Gram, 2001; Thimothe, Nightingale, Gall, Scott, & Wiedmann, 2004; Tocmo et al., 2014). A wide survey conducted during the period 2010-2011, reported prevalence of *L. monocytogenes* in RTE foods at the EU level was highest in 'RTE fish' (10.3%), followed by 'RTE meat' (2.07%) and 'RTE cheese' (0.47%) at the end of shelf-life (EFSA, 2013). In addition, it has been reported that the growth of *L. monocytogenes* in RTE food at the consumer phase during the shelf-life could account for about one third of the listeriosis cases in the EU (EFSA BIOHAZ, 2018).

Biopreservation technology is an interesting and cost-effective alternative for shelf-life extension and improvement of food safety (Singh, 2018). It comprises the deliberated use of selected microorganisms and/or their natural metabolites with minimal impact on intrinsic sensory properties of food. Lactic acid bacteria (LAB) are the quintessential microbial group used as protective cultures due to their generally recognized as safe (GRAS) status, as well as their capacity to produce a wide range of antimicrobial compounds (Gao et al., 2019). Besides, many LAB species are included in the qualified presumption of safety (QPS) list (EFSA BIOHAZ, 2021). In recent years, different studies have focused on developing and testing protective cultures for their application in fish product preservation (Cifuentes Bachmann & Leroy, 2015; Ghanbari, Jami, Domig, & Kneifel, 2013; Gómez-Sala et al., 2016; Leroi et al., 2015; Wiernasz et al., 2017; 2020). In particular, the bioprotective potential of the strain *Latilactobacillus sakei* CTC494, producer of the bacteriocin sakacin K, against *L. monocytogenes* has been successfully proved in

fresh fish (Costa et al., 2019) and smoked fish (Aymerich, Rodríguez, Garriga, & Bover-Cid, 2019; Bolívar et al., 2021) under dynamic and constant refrigeration temperatures. In this regard, factors influencing growth and bacteriocin production are of most importance when using bacteriocinogenic cultures (Gálvez, Abriouel, López, & Omar, 2007). In fact, specific food systems can affect the performance of bacteriocin-producing LAB and/or cause potential loss of bacteriocin activity due to, for instance, physicochemical and food-related factors (pH, temperature, a_w, O_2, CO_2, food (micro)structure, buffering capacity…) as well as interaction with food additives or ingredients such as proteins, fat, emulsifiers and nitrites (Gálvez et al., 2007; Said, Ne Gaudreau, Dallaire, Le Tessier, & Fliss, 2019). Thereby, understanding the role of the food-related factors on the efficacy of protective cultures is necessary to attain optimal results for developing biopreservation-based strategies.

Studies considering the significance of different food products on microbial interaction between food-borne pathogens and bacteriocinogenic LAB cultures are scarce. Therefore, the aims of this work were i) to evaluate the growth dynamics of *L. sakei* CTC494 and *L. monocytogenes* in mono and coculture conditions in two different fish products (i.e., surimi-based product and tuna pâté) and ii) to quantitatively describe the effect of microbial interaction using microbial competition models.

5.3. Material and methods

5.3.1. Fish products

Two commercial processed RTE fish products (surimi-based product and tuna pâté) were provided by food producing companies to be used for the challenge tests. The surimi-based product consisted of individual plastic trays containing eight slices packaged under normal (aerobic) atmosphere with an average weight of 13.5 ± 0.5 g per slice. Its ingredient and additives' composition was surimi 49% (with 45% of fish), water, starch, egg white, sunflower oil, salt, sugar, smoke aroma, monosodium glutamate, potassium sorbate and food colouring (carmine and natural candy). The other product corresponded to tuna pâté packaged under normal (aerobic) atmosphere conditions in 25g-single-dose containers made of semi-rigid aluminium foil. The composition was tuna (35%), sunflower oil, cow milk, potato flakes, water, salt, carrageenan, monosodium glutamate, disodium ribonucleotides, citric acid, acacia gum and aroma. Sliced surimi product and tuna pâté were obtained directly from the producer and kept at refrigeration (2 ºC), and room temperature, respectively, up to a maximum of 2 days before the experiments.

5.3.2. Bacterial strains and culture conditions

L. sakei strain CTC494 with recognized antilisterial activity was selected as the bioprotective culture in this study (Hugas, Garriga, Aymerich, & Monfort, 1995). The pathogenic species was

L. monocytogenes strain CTC1034 (serotype 4b) from the culture collection of the Food Safety and Functionality Programme of IRTA, previously used as reference strain to evaluate the antagonism produced by bacteriocins (including sakacin K) (Garriga, Aymerich, Costa, Monfort, & Hugas, 2002). Stock cultures were maintained at -80 °C in cryovials (MicrobankTM, Pro-LAB Diagnostic, USA) in 20% glycerol (v/v) as cryoprotectant.

A bead of *L. sakei* CTC494 was pre-cultured separately at static conditions for 24 h in de Man Rogosa and Sharp broth (MRS, Oxoid, UK) at 33 °C with 10% CO_2. For *L. monocytogenes*, a bead of the stock culture was pre-cultured at static conditions for 24 h in Brain Heart Infusion broth (BHI, Oxoid, UK) at 37 °C. Afterwards, a 24 h-subculture was made for each microorganism in the same incubation conditions, followed by a third subculture incubated for 18-20 h (Costa et al., 2019). This process resulted in early stationary phase cultures, with a cell density of ca. 10^8 CFU/mL and 10^9 CFU/mL for *L. sakei* CTC494 and *L. monocytogenes* CTC1034, respectively.

5.3.3. Challenge tests

Four batches were prepared for each fish product: (1) non-inoculated product (control); (2) inoculated with ca. 10^2 CFU/g *L. monocytogenes*; (3) inoculated with ca. 10^2 CFU/g *L. sakei* CTC494 and (4) inoculated with *L. monocytogenes* and *L. sakei* CTC494 of ca. 10^2 and 10^4 CFU/g, respectively, generating the ratio 1:2 expressed in logarithmic scale. This inoculum ratio was selected based on previous sensory assessments for *L. sakei* CTC494 in fresh fish (Costa et al., 2019) and hot-smoked fish (Bolívar et al., 2021). In both studies, it was demonstrated that the LAB culture did not increase sensory spoilage rates at an initial concentration $\leq 10^4$ CFU/g when compared to non-bioprotected samples.

Monoculture experiments were carried out to individually assess the growth of each microorganism in both RTE fish products. For that, batches 2 and 3 were used. Slices of surimi-based product were surface inoculated at 1% (v/w) from the appropriate decimal dilution using a L-shaped sterile spreader. Slices were maintained in the safety cabinet for 1 min to allow inoculum absorption. Afterward, surimi slices were individually vacuum packaged (AUDIONVAC, 151HG) using plastic bags (PA/PE 150 microns, 140 x 300 mm, Sacoliva, Barcelona, Spain). Single-dose containers of tuna pâté were inoculated with aliquots of 50 µL (2% v/w) from the appropriate decimal dilution using a sterile syringe with needle (BD Plastipak, Spain) inserted through an adhesive septum (ø 15 mm, PBI Dansensor, Denmark) which was previously placed on the upper central part of the aluminum foil lid.

Coculture experiments were performed to examine the impact of microbial interaction on growth kinetics using batch 4. For surimi samples, *L. sakei* CTC494 was first surface inoculated and maintained in the safety cabinet as described before. *L. monocytogenes* was then spread, followed

by a cell attachment period as the LAB strain case. For tuna pâté samples, the inoculation was performed by preparing a previous mixture of *L. sakei* and *L. monocytogenes* at the ratio described before for batch 4 and inoculated as mentioned for monoculture experiments.

Both RTE fish products were stored at two constant temperatures targeted at 2 and 12 °C, representative for fish product distribution and storage (i.e., cold and mild abuse temperature, respectively). Depending on the microorganism and condition, the storage period ranged from 15 to 50 days at 2 ºC and from 6 to 10 days at 12 ºC to ensure the stationary growth phase was reached. The storage temperature of all experimental conditions was regularly recorded by data loggers (Fourtec, MiniLitE5032L, USA), with average values of 2.2 ($\pm$ 0.21) and 12.2 ($\pm$ 0.61) ºC. Growth experiments were carried out in duplicate.

5.3.4. Microbiological analyses

Individual slices of surimi product and single-dose containers of tuna pâté were considered the analytical samples. The growth of *L. sakei* CTC494 and *L. monocytogenes* CTC1034 was enumerated by plate count methods. For that, two samples were aseptically taken at each sampling point, homogenized 1:10 for 60 s (1500 rpm) in a stomacher bag (Masticator, IUL Instruments, Spain) and subsequently diluted 10-fold in PSW (0.85% NaCl). *L. sakei* was enumerated by pour plating in MRS agar (Oxoid) supplemented with bromocresol purple (BP, 0.12 g/L, Sigma-Aldrich, USA) and incubating with overlay at 33 °C under 10% CO_2 for 48 h. BP is a pH indicator used for the enumeration of LAB in foods that indicates the production of lactic acid by changing the MRS colour from purple to yellow (Sobrun, Bhaw-Luximon, Jhurry, & Puchooa, 2012). *L. monocytogenes* was enumerated by surface plating on *Listeria* selective agar base (Oxford formulation; Oxoid) containing selective supplement (SR140E; Oxoid) and incubating at 37 °C for 48 h (method ISO 11290-2).

5.3.5. Physicochemical analyses

Water activity (a_w) of surimi product and tuna pâté was determined in triplicate from non-inoculated samples (batch 1) using an a_w-meter AquaLab 4 (Decagon Devices Inc. Pullman, WA, USA). Lactic acid concentration and pH were determined in the studied fish products from batch 4 (coculture) on the initial and final experimental day. The pH of the fish products was measured using a portable pH-meter (Hanna Edge, HI2020, USA) with a penetration probe (HI10530). The analysis of lactic acid was performed in duplicate from 5 g of fish sample, after the extraction with 0.15 M perchloric acid, by HPLC using an ion exclusion column (Transgenomic ICSepICE-ORH-801, Chrom Tech. Inc., MN, USA) with a refractive index (RI) detector (Hereu, Dalgaard, Garriga, Aymerich, & Bover-Cid, 2014). An ANOVA ($p \leq 0.05$) was performed in MS-Excel software (Microsoft Corp., Redmond, WA, USA) to determine significant differences on lactic acid concentration between the starting and final day of the storage period.

5.3.6. Mathematical modelling of microbial interaction

The mathematical approach used, in this study, to investigate the interaction effect between *L. sakei* CTC494 and *L. monocytogenes* in two fish products and describe inter-species competition was based on (i) the determination of the kinetic parameters of the two microbial populations from experimental growth curves obtained in monoculture and coculture (ii) the simulation of simultaneous growth based on microbial competition models using the monoculture kinetic parameters and (iii) the assessment of the performance of the tested microbial competition models to describe the observed microbial data by suitable statistical indexes.

5.3.6.1. Primary model fitting

The Baranyi & Roberts (1994) primary growth model was fitted to microbial growth curves obtained in mono and coculture conditions for *L. sakei* CTC494 and *L. monocytogenes* at the studied storage temperatures (2 and 12 ºC). This model was used to obtain the kinetic growth parameters lag time (λ, h), maximum specific growth rate (μ_{max}, h^{-1}) and maximum population density (N_{max}, log CFU/g). Model parameters were estimated using the lsqcurvefit routine of the Optimisation Toolbox of Matlab version R2020b (The MathWorksInc®, Natick, USA). Standard errors of parameter estimates were calculated from the Jacobian matrix obtained in the fitting process.

In addition, an F-Test ($p \leq 0.05$) was performed in MS-Excel to prove model adequacy in data fitting for mono and coculture conditions. An F-value inferior to the critical percentile of the F distribution indicates that the model was not statistically accepted to fit the data set (Gil, Miller, Brandão, & Silva, 2017).

To assess the influence of product type on the obtained growth kinetic parameters (λ, μ_{max}, N_{max}), the Student's T-Test ($p \leq 0.05$) was used to compare the two means (independent samples for surimi product vs. tuna pâté) in MS-Excel. Then, the T-Test was also applied to assess differences between growing conditions (monoculture vs. coculture) on the kinetic parameters.

5.3.6.2. Modelling microbial interaction in RTE fish products

The modelling approach used to describe the simultaneous growth of *L. sakei* CTC494 and *L. monocytogenes* in the fish products was primarily built on the Jameson-effect interaction. This phenomenon determines that two microbial populations in mixed culture simultaneously stop growing when the maximum population density of the total population is achieved (Jameson, 1962), which may be result from non-specific competition between populations for a common limiting resource. To model this interaction, a generic primary growth model coupled with the logistic deceleration function can be used, that is the so-called Jameson-effect model. The logistic

deceleration describes an empirical self-limiting growth process that represents the exhaustion of critical resources and the accumulation of growth inhibitory by-products (Cadavez et al., 2019). Giménez & Dalgaard (2004) modified the deceleration function that describes interaction between populations by including the maximum population density of each competing population. Similarly, this approach considers that one population stops growing when the other has reached its maximum population density. It can be written as represented by Eq. (5.1), standing subscript '1' for the population of *L. sakei* and subscript '2' for the population of *L. monocytogenes*.

$$\begin{cases} \frac{dN_1}{dt} = \mu_{max1}N_1\left(1 - \frac{N_1}{N_{max1}}\right)\left(1 - \frac{N_2}{N_{max2}}\right)\frac{Q_1}{1+Q_1} \\[2mm] \frac{dQ_1}{dt} = \mu_{max1}Q_{1\ t-1} \\[2mm] \frac{dN_2}{dt} = \mu_{max2}N_2\left(1 - \frac{N_2}{N_{max2}}\right)\left(1 - \frac{N_1}{N_{max1}}\right)\frac{Q_2}{1+Q_2} \\[2mm] \frac{dQ_2}{dt} = \mu_{max2}Q_{2\ t-1} \end{cases} \qquad (5.1)$$

where N is the cell concentration (CFU/g) at time t, μ_{max} is the maximum specific growth rate (h^{-1}), N_{max} is the maximum population density (CFU/g); Q quantifies the physiological state of cells at time t allowing for description of the lag time.

The value of Q at $t = 0$ (Q_0) was calculated for both microorganisms as follows (Baranyi & Roberts, 1994):

$$Q_0 = \frac{1}{e^{(\mu_{max}\lambda)} - 1} \qquad (5.2)$$

where λ (h) is the lag time duration of the bacterial population.

The Jameson-effect hypothesis is, of course, not applicable to every interaction between two microbial populations. As stated by Cornu et al. (2011), "To circumvent cases in which the simplistic hypothesis of simultaneous deceleration is not applicable, variants of the Jameson-effect models have been proposed". The observations in our study demonstrate that microbial populations showed different competition patterns depending on the fish product and storage temperatures. Therefore, the expanded version of the Jameson-effect model as proposed by Møller et al. (2013) was used in this study (Eq. (5.3)).

$$\begin{cases} \frac{dN_1}{dt} = \mu_{max1}N_1\left(1 - \frac{N_1}{N_{max1}}\right)\left(1 - \frac{\gamma_{12}\times N_2}{N_{max2}}\right)\frac{Q_1}{1+Q_1} \\[2mm] \frac{dQ_1}{dt} = \mu_{max1}Q_{1\ t-1} \\[2mm] \frac{dN_2}{dt} = \mu_{max2}N_2\left(1 - \frac{N_2}{N_{max2}}\right)\left(1 - \frac{\gamma_{21}\times N_1}{N_{max1}}\right)\frac{Q_2}{1+Q_2} \\[2mm] \frac{dQ_2}{dt} = \mu_{max2}Q_{2\ t-1} \end{cases} \qquad (5.3)$$

where γ_{12} and γ_{21} are the interaction coefficients measuring the effect of *L. sakei* CTC494 on *L. monocytogenes* and vice-versa, respectively; other parameters are as indicated for Eq. (5.1).

This approach is more flexible than the classical Jameson-effect model since it includes an interaction parameter (γ) that must be estimated from microbial growth curves in coculture. The parameter γ defines the inhibiting effect of the dominant population on the other population which may differ among environmental conditions. In this way, Eq. (5.3) allows to describe the concentration of *L. monocytogenes* either when (i) it increases after *L. sakei* has reached its N_{max} ($\gamma_{12} < 1$) or (ii) it decreases after *L. sakei* has reached its N_{max} ($\gamma_{12} > 1$). With an γ-value = 1.0, Eq. (5.3) and the classical Jameson-effect model (Giménez & Dalgaard, 2004) are equivalent (i.e. where the concentration of *L. monocytogenes* stabilizes to the maximum population density value after *L. sakei* has reached its N_{max}).

The Jameson-effect hypothesis has been traditionally based on microbial interactions in foods that only limit the maximum population density, without any significant effect on the lag time or maximum growth rate (Cauchie et al., 2020). When mutual interaction occurs, microbial lag time may be affected, resulting in earlier entry into the exponential growth phase (Yang et al., 2017). To reflect the effect of microbial interaction on lag time, we propose a modification of the adjustment function $Q(t)$ by including an empirical parameter (β) that needs to be fitted from coculture experiments. In this way, if $\beta_{12} < 0$, population 1 shortens the lag time of population 2 and vice-versa, describing a mutualism-based interaction between the two populations. The new expanded Jameson-effect model can be expressed as follows:

$$\begin{cases} \frac{dN_1}{dt} = \mu_{max1} N_1 \left(1 - \frac{N_1}{N_{max1}}\right) \left(1 - \frac{\gamma_{12} \times N_2}{N_{max2}}\right) \frac{Q_1}{1+Q_1} \\[2mm] \frac{dQ_1}{dt} = \mu_{max1} Q_{1\,t-1} \left(1 - \beta_{12} \frac{N_2}{N_1}\right) \\[2mm] \frac{dN_2}{dt} = \mu_{max2} N_2 \left(1 - \frac{N_2}{N_{max2}}\right) \left(1 - \frac{\gamma_{21} \times N_1}{N_{max1}}\right) \frac{Q_2}{1+Q_2} \\[2mm] \frac{dQ_2}{dt} = \mu_{max2} Q_{2\,t-1} \left(1 - \beta_{21} \frac{N_1}{N_2}\right) \end{cases} \quad (5.4)$$

where β_{12} and β_{21} are the interaction coefficients describing the effect of population 1 on the lag time of population 2 and vice-versa, respectively; other parameters are as indicated for Eq. (5.1) and (5.3).

5.3.7. Evaluation and interaction models fitting

The performance of the microbial interaction models to describe the experimental data was assessed by the goodness-of-fit measure root mean squared error (RMSE) and corrected Akaike Information Criterion (AICc).

$$RMSE = \sqrt{\frac{\sum(logN_{obs}-logN_{est})^2}{n-p}} \qquad (5.5)$$

$$AICc = n \cdot ln\left(\frac{\sum(logN_{obs}-logN_{est})^2}{n}\right) + 2 \cdot (p+1) + \frac{2 \cdot (p+1) \cdot (p+2)}{n-p-2} \qquad (5.6)$$

where $logN_{obs}$ and $logN_{est}$ is the log-transformed cell concentrations observed and estimated with the model, respectively, n is the number of observations, and p is the number of model parameters.

The fitting process of microbial interaction was performed in Matlab using the previous estimated kinetic parameters in monoculture conditions and optimizing the interaction parameters (γ and β). For that, the Runge-Kutta method was used to numerically solve the differential equations through the *ode45* function in Matlab. This method was combined with the *fminsearch* function to estimate the interaction parameters by least-squares optimization. The initial guess selection for the parameter optimization algorithm was based on an iterative process, by choosing a wide range of values for each parameter, at the outset, and narrowing them according to the improvement in RMSE. Thus, the initial guess of the expanded Jameson model parameters was γ_{12} and $\gamma_{21} = 0$ for surimi product and $\gamma_{12} = 2$ and $\gamma_{21} = 0$ for tuna pâté. Whereas for the new expanded Jameson model fitting, the chosen starting values of γ_{12}, γ_{21}, β_{12} and β_{21} corresponded to 2, 1, 0 and 0, respectively, for surimi product, and 2, 0, 0 and 0, respectively, for tuna pâté. The confidence intervals of the interaction parameters were calculated by bootstrap method.

5.4. Results and discussion

5.4.1. Physicochemical characterization

Surimi-based product and tuna pâté exhibited an initial a_w of 0.988 ($\pm$ 0.002) and 0.987 ($\pm$ 0.002), respectively. The pH and lactic acid concentration values obtained in batch 4 (coculture) of the studied fish products are presented in Table 5.1. No relevant changes in pH were detected during growth experiments in both fish products and storage temperatures.

Regarding the concentration of lactic acid, levels obtained in the tested fish products on the initial experimental day were within the lower range values reported for endogenous lactic acid in other processed and RTE meat and seafood products (Mejlholm et al., 2010). The concentration of lactic acid did not significantly increase (p > 0.05) at the end of the experimental period compared to the initial day with the exception of tuna pâté at 2 ºC (Table 5.1).

Table 5.1. Lactic acid concentration and pH values (± standard deviation) obtained in the ready-to-eat (RTE) fish products under study.

Product	Batch	T (ºC)	Storage time	Lactic acid (%)	pH
Sliced surimi	4 (Co-culture)	N/A[a]	Initial	2.41 ± 0.01	6.76 ± 0.32
		2	Final	1.45 ± 0.04	5.89 ± 0.50
		12	Final	1.80 ± 0.10	6.10 ± 0.33
Tuna pâté	4 (Co-culture)	N/A	Initial	0.75 ± 0.01	6.04 ± 0.01
		2	Final	0.95 ± 0.11[*]	6.13 ± 0.07
		12	Final	0.78 ± 0.02	5.76 ± 0.06

[a] Not applicable (N/A).
[*] This value was significantly higher compared to the initial storage day ($p \leq 0.05$).

5.4.2. Microbial growth in monoculture

Figure 5.1 presents the experimental growth data of *L. sakei* CTC494 and *L. monocytogenes* obtained in monoculture at 2 and 12 °C in sliced surimi product (upper graphs) and tuna pâté (low graphs). The initial concentration of both microorganisms in the fish products ($t = 0$ h) was around 2.5 log CFU/g except for *L. sakei* CTC494 at 12 ºC in tuna pâté which was a bit lower (1.6 ± 0.5 log CFU/g), though quantifiable by plate count methods.

Table 5.2 summarizes the kinetic parameters estimated from Baranyi & Roberts (1994) model for the four sets of experimental growth curves. The primary growth model adequately described the experimental growth data in monoculture according to the F-test and the goodness-of-fit indexes, with low RMSE values (< 0.32).

Comparing microbial kinetics in the studied fish products, the parameter lag time λ in monoculture was significantly shorter ($p \leq 0.05$) in tuna pâté than in surimi product for both microorganisms and storage temperatures, although this trend was much remarkable at 2 ºC. The longer lag time in surimi product may be related to the fact that this product was vacuum packaged as well as the presence of potassium sorbate in the formulation, both known to delay the growth of *L. monocytogenes* compared with normal (air) atmosphere packaging (Pal, Labuza, & Diez-Gonzalez, 2008) and the absence of sorbate (González-Fandos & Dominguez, 2007) in tuna pâté. In addition, other compositional differences between the tested fish products include the fat content (i.e., 26% fat in tuna pâté and 0.4% fat in surimi product). In relation to this, a recent study investigated the isolated effect of fat content on *L. monocytogenes* growth dynamics in fish-based emulsions and gelled emulsions systems (ranging from 1 to 20% fat) and observed a lag-reducing effect in systems containing > 1% fat at temperatures of 4-10 ºC (Verheyen et al., 2020). These authors suggested that this phenomenon was caused by a protective effect of the fat droplets at chilling temperatures.

Regarding the parameter maximum specific growth rate (μ_{max}), there were no significant differences (p > 0.05) comparing the fish products for *L. sakei* CTC494 and *L. monoctogenes*. In addition, the two studied microorganisms exhibited similar μ_{max} values in each fish product and storage temperature (Table 5.2). Interestingly, the obtained μ_{max} values for *L. monocytogenes* at 2 ºC in surimi product and tuna pâté are in accordance with those reported by Verheyen et al. (2020) at 4 ºC in fish-based gelled emulsions systems containing 1% fat (0.029 h^{-1}) and 20 % fat (0.030 h^{-1}). In that study, it was also observed that the μ_{max} of the pathogen was not significantly affected by the different fat contents. The fish matrices under study did not have a significant influence (p > 0.05) on the maximum population density N_{max} of the LAB strain or the pathogen.

Table 5.2. Kinetic parameters and their standard errors estimated from the model of Baranyi & Roberts (1994) for mono and coculture experiments.

Product	Microorganism	T (°C)	λ (hours)		μ_{max} (h⁻¹)		N_{max} (log CFU/g)		RMSE[a]	
			Mono	*Co*	*Mono*	*Co*	*Mono*	*Co*	*Mono*	*Co*
Sliced surimi	*L. sakei* CTC494	2	259.62 (0.62)	171.12 (0.67)	0.021 (0.008)	0.050 (0.057)	8.30 (0.08)	8.42 (0.28)	0.096	0.275
		12	32.08 (0.22)	63.93 (0.18)	0.151 (0.127)	0.257 (0.458)	8.52 (0.16)	8.41 (0.12)	0.192	0.144
	L. monocytogenes	2	200.64 (0.96)	78.54 (0.89)	0.025 (0.019)	0.037 (0.045)	8.26 (0.14)	6.61 (0.25)	0.191	0.244
		12	13.38 (0.29)	31.83 (0.26)	0.144 (0.135)	0.167 (0.240)	9.01 (0.20)	6.72 (0.23)	0.234	0.278
Tuna pâté	*L. sakei* CTC494	2	48.03 (0.80)	75.14 (0.76)	0.033 (0.026)	0.053 (0.060)	7.69 (0.31)	7.69 (0.34)	0.071	0.165
		12	18.25 (0.34)	15.32 (0.12)	0.141 (0.180)	0.150 (0.128)	8.23 (0.38)	8.06 (0.39)	0.311	0.234
	L. monocytogenes	2	108.86 (1.37)	N/A[b]	0.029 (0.051)	N/A	7.36 (0.61)	N/A	0.310	N/A
		12	0.55 (0.2)	N/A	0.138 (0.055)	N/A	8.52 (0.20)	N/A	0.189	N/A

[a] Root Mean Squared Error (RMSE).
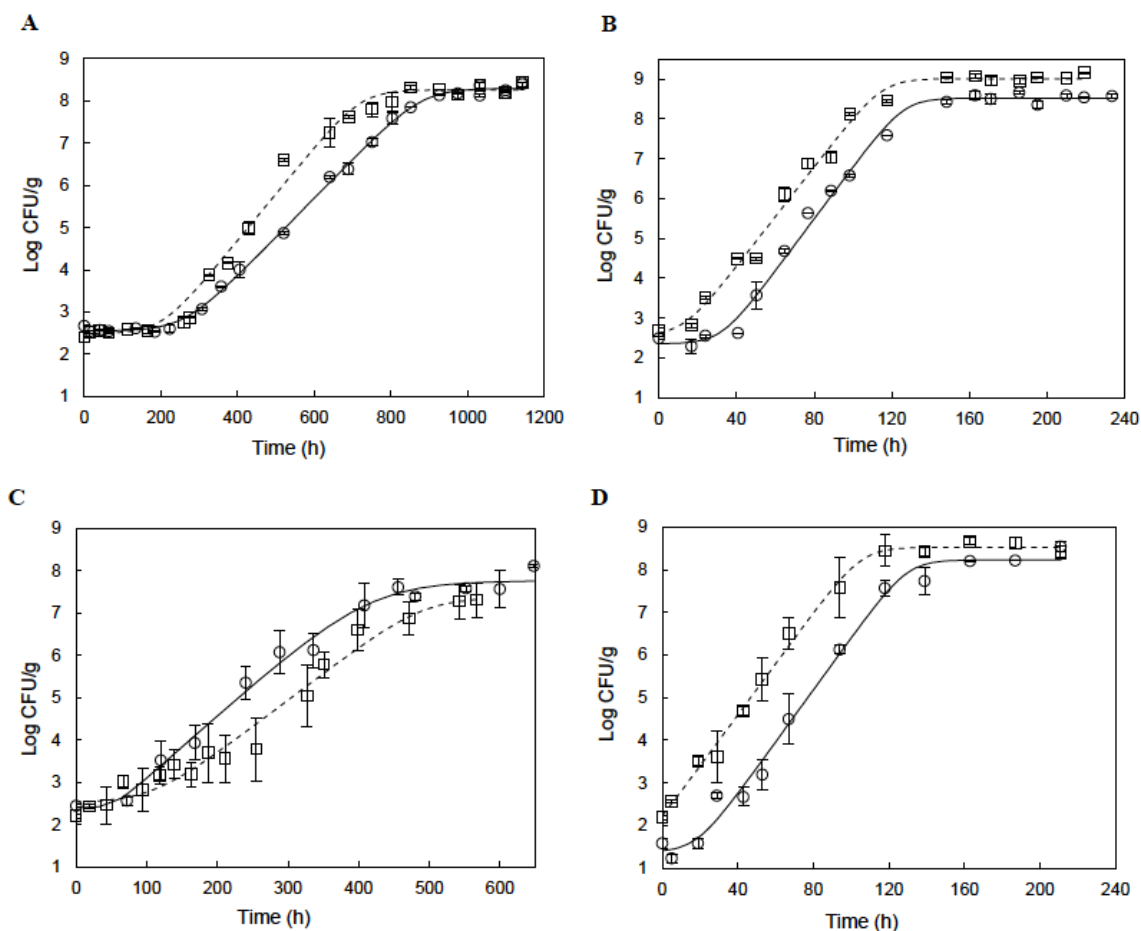[b] Not applicable (N/A).

Figure 5.1. Experimental growth data of *Latilactobacillus sakei* CTC494 (○) and *Listeria monocytogenes* CTC1034 (□), in monoculture, on sliced surimi product (A and B) and tuna pâté (C and D). A and C refer to temperatures of 2 ºC and B and D refer to temperatures of 12 ºC. Solid and dashed lines correspond to the fit of the Baranyi and Roberts model for *L. sakei* and *L. monocytogenes*, respectively. Error bars represent the standard deviation of two independent experiments with duplicate samples.

5.4.3. Microbial growth in coculture

The obtained growth data in coculture and the estimated parameters resulting from the fitting of the Baranyi & Roberts (1994) model are shown in Figure 5.2 and Table 5.2, respectively. According to results from the F-Test, the Baranyi model was not statistically adequate to fit microbial data obtained for *L. monocytogenes* in tuna pâté at both storage temperatures as limited increase (< 1 to 2 log units at 2 and 12 ºC, respectively) occurred due to the antagonistic activity of the *L. sakei* CTC494.

A shorter λ (p ≤ 0.05) was observed in tuna pâté compared to sliced surimi product for both microorganisms, in coculture, and storage temperatures, which could be explained, as mentioned in the case of monoculture experiments, by differences in the packaging atmospheres as well as in the food composition, i.e. sorbate and fat content of the studied fish products, causing the latter

a possible protective (lag-reducing) effect of the fat droplets in the product with higher fat content (i.e., tuna pâté). The values of μ_{max} and N_{max} obtained for *L. sakei* CTC494 in coculture were similar (p > 0.05) to those observed in monoculture experiments in both fish products and storage temperatures. By contrast, the parameter λ at 2 ºC for cocultured *L. sakei* and *L. monocytogenes* obtained in sliced surimi product presented a reduction compared to that observed in monoculture. This fact was also observed by Costa et al. (2019) for the same strains in fish juice at 2 ºC, who suggested that the differences were rather produced by the fitting process together with relatively λ short duration (i.e. low value) than a hypothetical interaction between microorganisms. However, in our study, in which actual fish products were used, a clear and long λ was observed and estimated with low fitting errors (Table 5.2). By contrast, no significant differences were obtained for the parameter μ_{max} in sliced surimi comparing mono and coculture experiments, while the parameter N_{max} for *L. monocytogenes* was significantly lower (p ≤ 0.05) in coculture at both assayed temperatures, with a mean drop of 2 log units. In fact, the inhibiting effect of *L. sakei* CTC494 on the pathogen growth in surimi was temperature dependent, i.e., at 2 ºC growth of *L. monocytogenes* slightly continued after the LAB strain reached its N_{max}, whereas at 12 ºC growth of *L. monocytogenes* stopped when *L. sakei* reached its N_{max}. The latest phenomenon corresponds to the classical Jameson-effect, which describes a non-specific interaction between microbial populations that are similarly inhibited by the depletion of the same nutrient, or by the production of the same end-product (Giménez & Dalgaard, 2004; Jameson, 1962; Le Marc, Valík, & Medveďová, 2009).

The observations in sliced surimi at 2 ºC (i.e., lag-reducing effect in coculture together with the reduced pathogen N_{max}) suggest that at the early stages of the storage period the type of microbial interaction could be categorized as mutualistic since both interacting species gained fitness (Smid & Lacroix, 2013). This relationship gradually evolved into amensalism (-/0 interaction) as *L. sakei* dominated the food system. Notice that amensalism can be considered as a form of asymmetrical antagonistic (competitive) interaction between species (Mougi, 2016). These phenomena could be explained by the fact that microorganisms can lie on a continuum between mutualism and antagonism which can shift with environmental changes (i.e., catabolism of carbon sources, production of by-products, …) (Roossinck, 2015). Thus, a mutualism-antagonism continuum could be hypothesized in sliced surimi at 2 ºC. In this regard, Yang et al. (2017) evidenced a mutual growth-promoting effect between *Bifidobacterium bifidum* and *L. monocytogenes*, and its molecular mechanism was investigated. They observed that the expression of proteins related to biosynthesis and cell reproduction was upregulated after growth in coculture, promoting the growth of the two bacteria, which led to an earlier entry into the exponential growth phase. Ačai et al. (2016) reported a positive effect of *Escherichia coli* on the growth of a starter LAB culture in milk and mentioned that "the LAB generally requiring complex media were supported by the

metabolites produced by *E. coli* population". These examples demonstrate the complex web of interactions between species in mixed cultures.

In tuna pâté, growth kinetics of *L. sakei* CTC494 in coculture were comparable to that observed in monoculture, reaching similar N_{max} values (7.69 log CFU/g). In contrast, the growth of *L. monocytogenes* was significantly affected by the presence of *L. sakei* CTC494 at both storage temperatures. As can be observed in Figure 5.2C, the growth of the pathogen at 2ºC slightly increased up to 10 storage days, reaching maximum count values of 4.07 ± 0.12 log CFU/g. At 12 ºC (Figure 5.2D), *L. sakei* CTC494 limited *L. monocytogenes* growth (< 2 log increase) even before *L. sakei* approached to its N_{max}, which suggests a specific inhibition activity by the LAB strain rather than the classical Jameson-effect. In this regard, *L. sakei* CTC494 is a low acidifying culture, according to the measured pH and lactic acid values (section 3.1); thus, the influence of by-products such as organic acids would be discarded as potential inhibitors. The pathogen suppression could be chiefly attributed to the bacteriocinogenic capacity of *L. sakei* CTC494, specifically, to the production of the antilisterial bacteriocin sakacin K (Hugas et al., 1995; Leroy, Lievens, & De Vuyst, 2005). In fact, Hugas et al. (1998) confirmed sakacin K production by *L. sakei* CTC494 in MRS broth at a range of temperatures from 4 ºC to 30 ºC and at initial pH from 5 to 6.5, reporting higher bacteriocin production at low temperatures (4 and 10 ºC). Recent studies have reported different degrees of inhibition exerted by *L. sakei* CTC494 against *L. monocytogenes* in fish products. For instance, Aymerich et al. (2019) demonstrated the total suppression of the pathogen growth (< 0.5 log increase) in cold-smoked salmon during storage at 8 ºC, while Bolívar et al. (2021) stated the temperature dependency of the bioprotective efficacy in hot-smoked sea bream, i.e., by limiting (< 2 log increase) or inhibiting (< 1 log increase) the pathogen growth under different temperature conditions.

5.4.4. Modelling microbial interaction in RTE fish products

The competition coefficients estimated by the tested microbial interaction models are shown in Table 5.3. Figure 5.2 presents the fitting with the new expanded Jameson-effect model (Eq. 5.4) for simultaneous growth of *L. sakei* CTC494 and *L. monocytogenes* in RTE fish products. In general, the expanded Jameson model (Eq. 5.3) could not sufficiently describe the competitive growth curves in the studied fish products. That is, this model showed higher RMSE and AICc values for simultaneous growth curves than that of the new expanded Jameson model (notice that the lower the values of RMSE and AICc, better the model fit).

In surimi product, the interaction coefficient γ_{12} estimated by fitting the expanded Jameson model indicated no microbial interaction between *L. sakei* and *L. monocytogenes* at 2 ºC ($\gamma_{12} = 0$) while a γ_{12} value of *ca.* 1.0 was determined at 12 ºC, describing the classical Jameson-effect. Notice that the γ-values estimated by the new expanded Jameson model should not be equally interpreted

than the expanded Jameson model due to the use of additional competition parameters. Although the aforementioned γ_{12} values are in agreement with the observed competition pattern (see growth data in Figure 5.2A and B), the expanded Jameson-effect model produced a poor representation of the experimental growth curves in surimi product as evidenced by the goodness-of-fit measures RMSE and AICc. However, the new expanded Jameson model, that included an additional coefficient of interaction (*β*), satisfactorily simulated the simultaneous growth of both microorganisms as demonstrated in Figure 5.2, yielding much lower RMSE and AICc values (Table 5.3). In surimi product at 2 ºC, the effect of microbial interaction on lag time was showcased by the estimated interaction coefficient values (β_{12} and β_{21} < 0) indicating a *λ* reduction of both microorganisms and consequently a mutualism effect between populations. The *L. monocytogenes* interaction coefficient β_{21} was *ca.* zero for other temperature and food matrix, indicating no effect by the pathogen on the *λ* of *L. sakei* CTC494.

In tuna pâté, both the existing Jameson and the new expanded Jameson models reflected the bacteriostatic/inhibitory effect of *L. sakei* CTC494 on *L. monocytogenes* growth, providing the latest slightly better fit according to the RMSE values (Table 5.3). In fact, *L. sakei* competition coefficient (γ_{12}) fitted by expanded Jameson model was > 1 at both storage temperatures, indicating that *L. monocytogenes* growth was inhibited by the LAB strain after reaching its N_{max}, as observed in Figure 5.2C. In particular, the new expanded Jameson-effect equation appropriately described the pathogen growth inhibition (< 2 log increase) at 12 ºC as shown in Figure 5.2D and RMSE value of 0.343. Indeed, the values of interaction coefficient (*β*), close to zero, confirmed the convergence of both models since no strong interaction was observed for lag time in this specific product.

Studies dealing with modelling of microbial competition in foods have been focussed on antagonistic interactions in which the main underlying mechanism is the consumption of the limiting substrate and/or the production of active inhibitory metabolites. This type of inter-species competition and its effect on the maximum population density (N_{max}) has been successfully described by the classical Jameson-effect model or its variants in a widely range of foods (Cadavez et al., 2019; Cornu et al., 2011; Mejlholm & Dalgaard, 2015; Ye et al., 2014). In our study, the introduction of an additional parameter (*β*) in an existing Jameson-effect model allowed for description of not only antagonistic but also mutualism-based interactions based on their influence on lag time (*λ*).

Table 5.3. Interaction coefficients with 95% confidence intervals (lower limit, upper limit) as estimated by the expanded Jameson-effect model (Eq. 5.3) and the new expanded Jameson-effect model (Eq. 5.4) used to describe the simultaneous growth of *L. sakei* CTC494 and *L. monocytogenes* in ready-to-eat (RTE) fish products.

		Expanded Jameson model				New expanded Jameson model					
Product	T (ºC)	γ_{12}[a]	γ_{21}[b]	RMSE[c]	AICc[d]	γ_{12}	γ_{21}	β_{12}[e]	β_{21}[f]	RMSE	AICc
Sliced surimi	2	0.057 [-0.10, 0.25]	$-3.527*10^5$ [$-1.25*10^6$, $-1.85*10^4$]	0.988	6.79	0.651 [0.36, 0.87]	$-1.797*10^3$ [$-2.11*10^4$, $-5.10*10^2$]	-15.969 [-32.64, 0.90]	-0.034 [-1.06, -0.01]	0.348	-19.62
	12	1.034 [0.92, 1.54]	-18.531 [-169.93, 240.83]	0.866	1.26	0.968 [0.86, 1.63]	$-3.604*10^3$ [$-2.84*10^3$, 295.87][*]	20.356 [-0.29, 27.50]	0.003 [0.00, 0.02]	0.557	-7.32
Tuna pâté	2	2.012 [1.57, 2.45]	$-2.374*10^3$ [$-7.72*10^3$, -733.92]	0.554	-7.37	27.948 [-139.95, 113.89]	$-6.402*10^3$ [$-2.26*10^4$, $-3.48*10^3$]	2.224 [-30.59, 27.24]	0.030 [0.00, 0.07]	0.454	-3.84
	12	1.395 [1.26, 1.58]	$-1.820*10^4$ [$-6.48*10^4$, $-4.50*10^3$]	0.696	0.52	0.001 [0.00, 0.00]	-0.005 [-0.01, 0.00]	-0.005 [-0.01, 0.00]	0.008 [0.00, 0.02]	0.343	-1.49

[a] Competition coefficient describing the effect of *L. sakei* CTC494 on *L. monocytogenes* growth (γ_{12}).
[b] Competition coefficient describing the effect of *L. monocytogenes* on *L. sakei* CTC494 growth (γ_{21}).
[c] Root Mean Squared Error (RMSE).
[d] Corrected Akaike Information Criterion (AICc).
[e] Competition coefficient describing the effect of *L. sakei* CTC494 on the lag time of *L. monocytogenes* (β_{12}).
[f] Competition coefficient describing the effect of *L. monocytogenes* on the lag time of *L. sakei* CTC494 (β_{21}).
[*] The estimated value fell outside the 95% confidence interval probably as result of an asymmetric distribution of the parameter error.

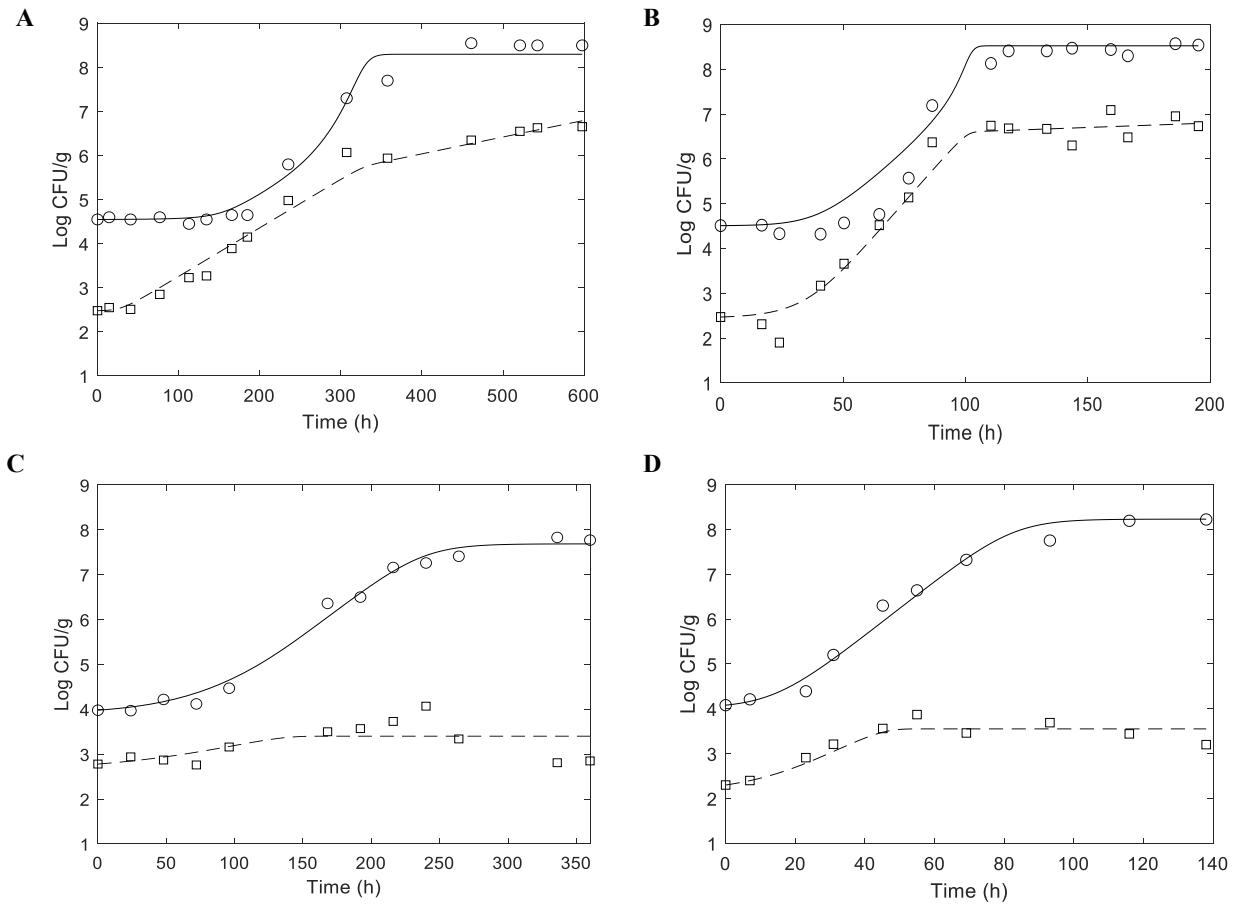**Figure 5.2.** Observed and predicted growth of *Latilactobacillus sakei* CTC494 (○) and *Listeria monocytogenes* CTC1034 (□), in coculture, on sliced surimi product (A and B) and tuna pâté (C and D). A and C refer to temperatures of 2 ºC and B and D refer to temperatures of 12 ºC. Growth of *L. sakei* (solid line) and *L. monocytogenes* (dashed line) was described by the new expanded Jameson model of the present study (Eq. (4)).

5.5. Conclusions

This work made a first attempt to deep into the quantitative characterisation of microbial interaction considering the significance of different food matrices on the efficacy of bacteriocinogenic LAB cultures against *L. monocytogenes* by using microbial interaction models. The different competition patterns obtained in the tested fish products suggest that the inhibiting effect of *L. sakei* CTC494 on growth of *L. monocytogenes* was food product dependent. Interestingly, a mutualism-antagonism continuum of both populations was observed at 2 ºC in surimi product with a mutualistic effect on lag time (λ). The application of an additional interaction coefficient (β) in the expanded Jameson-effect model offered the possibility to quantitatively characterize the different competition patterns. Further research based on omics approaches (e.g. proteomics, transcriptomics) should be applied to elucidate the underlying mechanisms of the different microbial interactions observed in the studied fish products.

5.6. References

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IV. CONCLUSIONS

Conclusions

FIRST: According to the reviewed scientific literature, spoilage bacteria being part of the microbial ecology of Mediterranean fish and fishery products encompass species such as *Enterobacter*, *Lactobacillus*, *Pseudomonas* spp., *Photobacterium* and *Shewanella* spp. (*Chapter 1*).

SECOND: Fishery products can be contaminated with foodborne pathogens that could be present in the aquatic environment, at the processing factories or within the intestinal tract of living species, being the most representative species *Aeromonas* spp., *C. perfringens*, *C. botulinum*, *L. monocytogenes*, *Salmonella* spp. and *Vibrio* spp. A variety of antibody-based tests and molecular tests, such as polymerase chain reactions and DNA microarrays and biosensors, have been developed for the detection of pathogenic species in fishery products (*Chapter 1*).

THIRD: The use of a fish-based juice generated from fresh fish allowed to satisfactorily assess *L. monocytogenes* growth under reduced oxygen and aerobic conditions in a wide temperature range (2–20 ºC). The Ratkowsky square root model properly described the effect of storage temperature on the pathogen growth rates ($R^2 > 0.97$) (*Chapter 2*).

FOURTH: The validation study using experimental growth data of *L. monocytogenes* in filleted sea bream and sea bass proved that the model generated in fish-based juice under reduced oxygen conditions was suitable to describe the growth of pathogen in Mediterranean fishery products, with bias factor and accuracy factor for growth rate predictions of 1.15 and 1.25, respectively. The validated model can be deployed as a valuable and reliable tool to perform quantitative risk assessment studies and shelf-life determination of Mediterranean fishery products (*Chapter 2*).

FIFTH: A new fish-based model system was developed based on an optimized and reproducible formulation including compounds such as fish proteins, alginate, sunflower oil, etc., with stable physicochemical and microstructural properties generating a reliable simulation environment of fish products to investigate the isolated food matrix fat content effect, in the range of 1–20%, on *L. monocytogenes* growth dynamics (*Chapter 3*).

SIXTH: According to the test performed in the new developed fish-based model system, an increase from 1 to 5% fat resulted in a significant reduction of *L. monocytogenes* lag phase duration λ at all studied temperatures, while a further increase in fat content did not significantly affect λ. On the contrary, the relationship between the fat content (%) and the maximum specific growth rate μ_{max} was less clearly evidenced for the fish-based model system and tested temperatures (*Chapter 3*).

SEVENTH: The findings obtained with the fish-based model demonstrated that fat content and the resulting microstructural and rheological properties significantly affect *L. monocytogenes*

growth dynamics in fish-based food model systems. Therefore, the consideration of these factors during predictive (growth) model development would be beneficial to more accurately estimate food safety in fat-containing ready-to-eat (RTE) food products which are susceptible to *Listeria* contamination (*Chapter 3*).

EIGHTH: With the inoculation experiments performed on hot-smoked sea bream formulated with the bacteriocinogenic *L. sakei* CTC494 isolated from fermented meat product, it was demonstrated that the application of this bioprotective culture at an initial level of 4 log CFU/g can significantly reduce the capability of *L. monocytogenes* to grow under different storage temperatures while maintaining the product sensory properties (*Chapter 4*).

NINETH: The effectiveness of the selected *L. sakei* CTC494 for controlling *L. monocytogenes* growth on hot-smoked sea bream was temperature dependent. The LAB strain limited the growth of the pathogen under storage at 5 ºC (< 1 log increase) and at a dynamic profile averaging 7.6 ºC (range 3.3–11.8 ºC) (< 2 log increase). Furthermore, with a dynamic profile averaging 6.3 ºC (range 3.6–12.8 ºC), the growth of *L. monocytogenes* was more strongly inhibited (< 0.5 log increase) (*Chapter 4*).

TENTH: The present work developed an innovative quantitative modelling approach, based on a validation process in food and the application of adjustment factors to kinetic parameters from the modified Jameson-effect and Lotka-Volterra competition models. This mathematical method was able to satisfactorily describe the bioprotective effect of *L. sakei* CTC494 on *L. monocytogenes* in hot-smoked sea bream. According to the statistical performance, the Lotka-Volterra model showed slightly better fit to the observed simultaneous growth than the Jameson-effect based models (*Chapter 4*).

ELEVENTH: The coculture studies in two different fish matrices, namely surimi-based product and tuna pâté evidenced different competition patterns for the simultaneous growth of *L. sakei* CTC494 and *L. monocytogenes*. In tuna pâté, *L. sakei* CTC494 exerted a strong inhibition on *L. monocytogenes* at 2 ºC (< 0.5 log increase) and limited the growth at 12 ºC (< 2 log increase). Interestingly, in surimi product, a reduction of lag time (λ) of both strains was observed when growing in coculture at 2 ºC, followed by the inhibition of the pathogen when the *L. sakei* CTC494 reached the maximum population density, suggesting a mutualism-antagonism continuum phenomenon between populations (*Chapter 5*).

TWELFTH: The new proposed expanded Jameson-effect model, which included and additional interaction coefficient (β) for lag time, allowed for description of not only antagonistic but also mutualism-based interactions based on their influence on lag time (λ). The root mean squared error (RMSE) of the new expanded model presented a reduction of 35% with respect to the RMSE

value of the existing expanded model, demonstrating that the former model performed better and appropriately described the different competition patterns (*Chapter 5*).

THIRTEENTH: Overall, outcomes from this Thesis illustrate a successful proof of application of Predictive Microbiology, demonstrating that mathematical models can be deployed as reliable and efficient tools to investigate microbial dynamics considering the effect of food-matrix related factors, and to quantitatively characterize microbial interaction phenomena, assessing the performance of bioprotective culture-based strategies and setting the optimal application conditions in order to reducing the risk of listeriosis associated with the consumption of fishery products.

CURRICULUM VITAE

Publications

Articles

1) **Bolívar, A.**; Tarlak, F.; Costa, J.C.C.P.; Cejudo-Gómez, M.; Bover-Cid, S., Zurera, G., Pérez-Rodríguez, F. A new expanded modelling approach for investigating the bioprotective capacity of *Latilactobacillus sakei* CTC494 against *Listeria monocytogenes* in ready-to-eat fish products. *Submitted to* Food Research International.

2) **Bolívar, A.**; Costa, J.C.C.P., Posada-Izquierdo, G.D., Bover-Cid, S., Zurera, G., Pérez-Rodríguez, F., 2021. Quantifying the bioprotective effect of *Lactobacillus sakei* CTC494 against *Listeria monocytogenes* on vacuum packaged hot-smoked sea bream. Food Microbiology, 94:103649.

3) Křepelka, P., **Bolívar, A.,** Pérez-Rodríguez, F., 2021. Two-dimensional mid and near-infrared correlation spectroscopy for bacterial identification. Journal of Near Infrared Spectroscopy, 29(2), 63-72.

4) Costa, J.C.C.P., **Bolívar, A.;** Valero, A., Carrasco E., Zurera, G., Pérez-Rodríguez, F., 2020. Evaluation of the effect of *Lactobacillus sakei* strain L115 on *Listeria monocytogenes* at different conditions of temperature by using predictive interaction models. Food Research International, 131, 108928.

5) Verheyen, D.*; **Bolívar, A.***; Pérez-Rodríguez, F.; Baka, M.; Skåra, T.; Van Impe, J.; 2020. Isolating the effect of fat content on *Listeria monocytogenes* growth dynamics in fish-based emulsions and gelled emulsion systems. Food Control, 108, 106874. (*Both authors contributed equally to this work).

6) Tarlak, F., Johannessen, G., Bascón Villegas, I., **Bolívar, A.,** Posada-Izquierdo, G.D., Pérez-Rodríguez, F., 2020. Modelling of the behaviour of *Salmonella enterica* serovar Reading on commercial fresh-cut iceberg lettuce stored at different temperatures. Foods, 9, 946.

7) Costa, J.C.C.P.; Bover-Cid, S.; **Bolívar, S.**; Zurera, G.; Pérez-Rodríguez, F.; 2019. Modelling the interaction of the sakacin-producing *Lactobacillus sakei* CTC494 and *Listeria monocytogenes* in filleted gilthead sea bream (*Sparus aurata*) under modified atmosphere packaging at isothermal and non-isothermal conditions. International Journal of Food Microbiology, 297, 72-84.

8) Pérez-Rodríguez, F.; Kwon, J.; **Bolívar, A.**; Sauer, K.; Ryu, D.; Todd, E.; 2019. Probabilistic risk model of norovirus transmission during handling and preparation of fresh produce in school foodservice operations. International Journal of Food Microbiology, 290, 159-169.

9) Cubero González, S.; Possas, A.; Carrasco, E.; Valero, A.; **Bolívar, A.**; Posada-Izquierdo, G.D.; García-Gimeno, R.M.; Zurera, G.; Pérez-Rodríguez, F.; 2019. 'MicroHibro': A software tool for predictive microbiology and microbial risk assessment in foods. International Journal of Food Microbiology, 290, 226-236.

10) **Bolívar, A.**; Costa, J.C.C.P., Posada-Izquierdo, G.D., Valero, A., Zurera, G., Pérez-Rodríguez, F., 2018. Modelling the growth of *Listeria monocytogenes* in Mediterranean fish species from aquaculture production. International Journal of Food Microbiology, 270, 14-21.

11) Verheyen, D.; **Bolívar, A.;** Pérez-Rodríguez, F.; Baka, M.; Skåra, T.; Van Impe, J., 2018. Effect of food microstructure on growth dynamics of *Listeria monocytogenes* in fish-based model systems. International Journal of Food Microbiology, 283, 7-13.

Book chapters

1) **Bolívar, A.,** Pérez-Rodríguez, F., 2021. Application of sensitivity analysis methods in quantitative risk assessment. In *Risk assessment methods for biological and chemical hazards in food*. CRC Press, Taylor & Francis Group. ISBN: 9781498762021.

2) **Bolívar, A.,** Costa, J., Posada-Izquierdo, G.D., Pérez-Rodríguez, F., Bascón, I., Zurera, G., Valero, A., 2017. Characterization of foodborne pathogens and spoilage bacteria in Mediterranean fish species and seafood products. In *Foodborne pathogens and antibiotic resistance.* Wiley-Blackwell. ISBN: 9781119139188.

Scientific contributions in conferences

1) Oral communication. Probabilistic risk model of norovirus transmission during handling and preparation of fresh produce in school foodservice operations.11th International Conference on Predictive Modelling in Food, 17-20 September, 2019, Braganza (Portugal). ISBN: 978-972-745-261-3

2) Oral communication. Development and validation of growth models for *Listeria monocytogenes* in Mediterranean fish species from aquaculture production. IAFP'S European Symposium on Food Safety, 25-27 April, 2018, Stockholm (Sweden).

3) Oral communication. Development of a software tool for risk assessment of *Listeria monocytogenes* in selected ready-to-eat food categories in the European Union. IAFP'S European Symposium on Food Safety, 25-27 April, 2018, Stockholm (Sweden).

4) Oral communication. Sistema experto para la evaluación del riesgo microbiológico y estimación de la vida comercial de alimentos. 7º Foro Europeo Transfiere, 14-15 Febrero, 2018, Málaga (Spain).

5) Poster. Inhibitory effect of *Lactobacillus sakei* against *Listeria monocytogenes* growth in smoked Mediterranean fish products from marine aquaculture. 10th International Conference on Predictive Modelling in Food, 26-29 September, 2017, Córdoba (Spain). ISBN: 1-5275-3705-6

6) Poster. Effect of food (micro)structure on growth dynamics of *Listeria monocytogenes* in fish based model systems. 10th International Conference on Predictive Modelling in Food, 26-29 September, 2017, Córdoba (Spain). ISBN: 1-5275-3705-6

7) Poster. Assessing the growth of *Listeria monocytogenes* in Mediterranean fish products from marine aquaculture. IAFP'S European Symposium on Food Safety, 29-31 March, 2017, Brussels (Belgium).

8) Poster. Evaluation of methodologies for the isolation of bioprotectives cultures of lactic acid bacteria from aquaculture, Mediterranean fish species. IAFP'S European Symposium on Food Safety, 29-31 March, 2017, Brussels (Belgium).

9) Poster. Modelling the growth of *Lactobacillus plantarum*, *Listeria monocytogenes* and *Salmonella* spp. in Mediterranean fish-based extract. 25th International ICFMH Conference Food Micro, 19-22 July, 2016, Dublin (Ireland).

10) Poster. Evaluation of microbial distribution and survival of *Escherichia coli* O157:H7 in soybean sprouts and seeds. 25th International ICFMH Conference Food Micro, 19-22 July,

2016, Dublin (Ireland).

Research stays

• 2019. Department of Agricultural, Forest and Food Science. Università degli Studi di Torino (Turin, Italy). Topic: Application of next generation sequencing to study microbial communities in food. 01/05/2019-25/07/2019.

• 2018. BioTeC+ Group, KU Leuven (Gent, Belgium). Topic: Predictive modeling of microbial interactions in structured food (seafood/fish and fishery products as a case study). 17/06/2018-30/09/2018.

• 2017. BioTeC+ Group, KU Leuven (Gent, Belgium). Topic: Predictive modeling of microbial interactions in structured food (seafood/fish and fishery products as a case study). 11/01/2017-30/03/2017.

Additional training

1) Título de Experto en Docencia Universitaria (2019/2020). University of Cordoba (Spain).

2) International School on Modeling and Simulation in Food and Bio Processes (125 hours; 5 ECTS). Training School of COST action CA15118 FoodMC. 3-7 February, 2020, Novi Sad (Serbia).