FACULTY OF VETERINARY
UNIVERSITY OF CÓRDOBA
Department of Food Science and Technology
International Doctorate School in Agri-food
Doctoral Program in Biosciences and Agri-food Sciences

UNIVERSIDAD D CÓRDOBA

PhD Thesis

Jean Carlos Correia Peres Costa

Development of microbial interaction predictive models for *Listeria monocytogenes* and lactic acid bacteria and their application for the optimization of bio-protective cultures in fishery products

Desarrollo de modelos predictivos de interacción microbiana para *Listeria monocytogenes* y bacterias del ácido láctico y su aplicación para la optimización de cultivos bio-protectores en productos pesqueros

Academic advisor: Fernando Pérez-Rodríguez

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TITULO: *Development of microbial interaction predictive models for Listeria monocytogenes and lactic acid bacteria and their application for the optimization of bio-protective cultures in fishery products*

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TÍTULO DE LA TESIS: Development of microbial interaction predictive models for *Listeria monocytogenes* and lactic acid bacteria and their application for the optimization of bio-protective cultures in fishery products

DOCTORANDO/A: Jean Carlos Correia Peres Costa

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

La presente tesis se enmarca dentro de una iniciativa de colaboración entre la Universidad de Córdoba, y el Gobierno Brasileño, mediante la financiación de la estancia y estudios de doctorando del Jean Correia. El plan de tesis se sustenta en las temáticas de un proyecto de excelencia del Plan Andaluz de Investigación (AGR2014-1906), donde se abordan aspectos relacionados con la bioconservación y su aplicación en la extensión y mejora de la vida útil de productos de la acuicultura de la región andaluza. Por ello, el desarrollo de la tesis se ha basado en dos ámbitos, la microbiología predictiva, que pertenece al *Know-how* del grupo de investigación que respalda al doctorando, y su aplicación en el desarrollo de cultivos bioprotectores para productos pesqueros, que supone el comienzo de una nueva línea de trabajo.

La tesis comienza con un estudio prospectivo, abordando la caracterización microbiológica de los productos pesqueros de la acuicultura andaluza. Como aspecto novedoso, estos se realizaron en zonas de estuario, pertenecientes a los ríos Guadiana y Guadalquivir; por tanto, no solo caracterizan un producto fresco, sino que también abordan aspectos relacionados con el ecosistema en el que se encuentran. Adicionalmente, se evaluó el impacto de la calidad microbiológica de estos productos utilizando modelos de microbiología predictiva. Este trabajo resultó en la siguiente publicación:

-Publicación I: Study of the microbiological quality, prevalence of foodborne pathogens and product shelf-life of Gilthead sea bream (*Sparus aurata*) and Sea bass (*Dicentrarchus labrax*) from Aquaculture in Estuarine Ecosystems of Andalusia (Spain). *Food Microbiology*, 90, 103498, 2020. **Índice de impacto: 4.155 (Q1).**

Una vez decidido el producto y los microorganismos de interés, en especial *Listeria monocytogenes*, se procedió a la selección de cultivos bioprotectores. Estos, inicialmente se intentaron aislar del mismo ecosistema de estuario, sin embargo, no se encontraron especies con una capacidad bioprotectora suficiente para una utilización comercial óptima. Por ello, se optó por utilizar otros existentes, ya validados, en estudios científicos o disponibles en colecciones de cultivos tipo. Se probaron cepas aisladas por investigadores españoles, y para ello se desarrolló un estudio en caldo de cultivo donde se enfrentó una cepa productora de bacteriocina, y potencialmente bioprotectora, al patógeno alimentario *L. monocytogenes*. Los resultados confirmaron

este efecto, aunque en menor medida de la esperado. Además, los datos fueron utilizados para generar modelos matemáticos que demostraron una gran capacidad predictiva, captando, satisfactoriamente, los fenómenos de interacción entre ambas especies bacterianas. Esta investigación dio lugar a la siguiente publicación:

-Publicación II: 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, 2020. **Índice de impacto: 4.972 (Q1).**

En un siguiente paso, se estableció contacto con el Instituto de Investigación de Tecnología Alimentaria (IRTA), dependiente del gobierno autonómico catalán, que nos suministró la cepa de *Lactobacillus sakei* CTC494, productora de sakacina, una bacteriociona, con potencial bioprotector sobre *L. monocytogenes*, habiendo sido validada principalmente en productos cárnicos. Para su aplicación, se abordó un estudio de gran calado, donde se implicó a una empresa del sector de acuicultura, "Esteros de Calidad", procedente de su participación en el proyecto de Excelencia. Así se ideó un diseño que permitió probar y desarrollar cultivos bioprotectores apoyados en estudios in vitro, alimento, y en simulaciones matemáticas con modelos interacción microbiana. Este trabajo es de una gran relevancia, no solo por el experimental desarrollado, tanto en el laboratorio como en los procesos tecnológicos en planta de fabricación, sino también por las contribuciones en el campo de la microbiología predictiva y utilización de cultivos bioprotectores en el sector pesquero. En el primer caso, como resultado de este trabajo se ha desarrollado una nueva estrategia sistematizada para la obtención de modelos de interacción, que describen, de manera adecuada, los fenómenos de interacción y de bioprotección observados frente a *L. monocytogenes*. En segundo lugar, la demostración de que un diseño adecuado, apoyado en modelos, puede resultar en una optimización en las formulaciones de cultivos bioprotectores contra el patógeno, permitiendo una mayor vida útil del producto y mejorando la calidad y seguridad microbiológica de este. En el estudio, se logró reducir o inhibir el crecimiento de *L. monocytogenes*, utilizando un cultivo bioprotector de la cepa CTC494 adicionándolo a niveles sin efecto en la calidad sensorial del alimento y bajo condiciones reales de proceso para un producto de dorada fileteada envasada en atmosfera modificada. Por tanto, podemos considerar este trabajo como uno de los primeros estudios científicos que realizan un proceso completo de diseño, desarrollo y aplicación de cultivos bioprotectores en productos mínimamente procesados procedentes de la agricultura, y ofrece a industriales y administración sanitaria con instrumentos de predicción para el control y mejora de esta tecnología de bioconservación. La publicación que recoge este trabajo corresponde se cita a continuación:

-Publicación III: 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*, 16, 72-84, 2019. **Índice de impacto: 4.187 (Q1).**

En paralelo a estos estudios de investigación, se elaboró un capítulo de libro donde se abordan los conceptos y fundamentos que rigen los estudios de microbiología predictiva, incidiendo en aquellos modelos que han sido base para los estudios experimentales y de modelado en los ensayos de interacción microbiana y de diseño y aplicación de cultivos bioprotectores. Este capítulo forma parte de un libro de referencia en el área, citado más abajo, del que se espera tenga una gran repercusión en el campo de la microbiología predictiva y evaluación de riesgos microbiológicos en alimentos.

-Publicación IV – Capítulo de libro: Predictive microbiology tools for exposure assessment. Chapter 11 of the book “Risk Assessment Methods for Biological and Chemical Hazards in Food”, edited by Fernando Pérez-Rodríguez, published by Taylor & Francis, in 2020. ISBN: 978-1-4987-6202-1.

Es imprescindible y obligado señalar las aptitudes y buen quehacer del doctorando en todas las fases de los estudios de doctorado. Soy consciente, a nivel personal y profesional, de las dificultades que entrañan este tipo de estudios, que combinan una fase experimental y un tratamiento matemático avanzado de los datos, con el agravante de la lejanía patria, que, bajo mi opinión personal, se ha de considerar siempre en estos casos, aunque estemos en ámbitos donde lo conciso y objetivo prevalece sobre lo emocional. Debo destacar, además, su gran capacidad para abordar los grandes objetivos experimentales impuestos, que han requerido un sacrificio y esfuerzo extraordinario, demostrando una gran solvencia en todas las facetas presentes, y una capacidad de adaptación a los limitados recursos y condicionantes que se han presentado a lo largo de la historia de su tesis. También quiero poner en valor sus habilidades para trasladar lo observado a lo numérico, en contextos de cierta complejidad, donde se requiere unos claros fundamentos matemáticos, y una abstracción estadística de los fenómenos bajo estudio. Jean Correia ha demostrado durante este tiempo su “madera” y “corazón” de científico e investigador, con una perseverancia y tesón encomiable; por ello, le auguro una gran carrera, de éxitos y logros, en el campo de la microbiología predictiva. Esta tesis, en el final, es solo el comienzo.

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

Firmado por PEREZ RODRIGUEZ FERNANDO - 30825948Z el día 22/10/2020 con un certificado emitido por AC FNMT Usuarios

Table of Contents

Preface

This Ph.D. thesis has been accomplished within the Doctoral programme in Biosciences and Agri-food Sciences, at the Department of Food Science and Technology, as fulfilment of requirements for the Ph.D. degree from the University of Córdoba, Spain.

The Research Group "Higiene Bromatológica" (HIBRO, PAIDI AGR-170) belonging to the Department of Food Science and Technology at the University of Córdoba, is formed by a group of highly specialized professors and researchers in the area of predictive microbiology and quantitative microbial risk assessment in food. HIBRO has extensive experience (> 25 years) in the field of predictive microbiology, which currently has great relevance in the establishment of Hazard Analysis and Critical Control (HAPPC) systems and provides scientific support for estimation of shelf-life and microbial risk assessment. Currently, the Group has consolidated itself through extensive research activity in field of microbiological risk assessment in regional (> 15), national (> 20) and international (> 5) projects, as well as extensive training activities and knowledge transfer to agri-food-sector companies.

The present work was conducted under the supervision of Professor Fernando Pérez-Rodríguez (UCO, Spain) and carried from November 2015 to November 2020, mainly at the Department of Food Science and Technology, University of Córdoba (UCO, Spain), and partly at the Food Safety Programme, Institute of Agri-food Research and Technology (IRTA, Spain) and Research Group for Microbiology and Hygiene, National Food Institute, Technical University of Denmark (DTU-Food).

This Ph.D. thesis was partly supported by the project entitled: "*Desarrollo y aplicación de modelos predictivos para la mejora de la calidad y seguridad de productos de la acuicultura marina mínimamente procesados*" (AGR201-1906) funded by the Andalusian Government (Spain), research group AGR-170 HIBRO and the Brazilian National Council for Scientific and Technological Development (CNPq) (grant Ph.D. Full Abroad-Proc. GDE 229638/2013-9).

The Ph.D. thesis focused on the application of bio-protective cultures as a suitable preservation technique for assuring food safety and quality and enhancing shelf-life of Ready to Eat (RTE) fish products from the Andalusian aquaculture. For that, a predictive microbiology approach was chosen, by developing and applying interaction models reflecting the inhibitory effect of selected bio-protective culture on the growth of *Listeria*

monocytogenes, a well-known foodborne pathogen, which is a prime concern for governments and the food industry.

The thesis is presented as a *Compendium of Publications*, elaborated in accordance with the objectives outlined in this study. The work is introduced by a first chapter, addressing an extensive review of the main aspects and concepts covered by the present thesis, including, among others, the aquaculture sector in Andalusia, pathogenic and spoilage microorganisms in fishery products, listeriosis incidence, the use of bio-protective cultures or microbial risk assessment (**Chapter 1– General Introduction**). The introductory chapter is followed by four publications, from Chapter 2 to 5, corresponding to a book chapter on the fundamentals of predictive microbiology (**Chapter 2 –Chapter Book**), and three research publications that undertake jointly the different objectives proposed in the present thesis. The Chapter 3 is a prospective work, in which the microbiota of two relevant fish species produced in Andalusian estuaries is assessed and the pathogen incidence is tested using culture-based and molecular methods. In addition, in this work, a first approach to applying predictive microbiology models for shelf-life estimation is taken (**Chapter 3 – Manuscript I**). The fourth chapter makes an attempt to assess the bio-protective capacity of a selected lactic bacteria species on *L. monocytogenes*, using a laboratory culture medium. Results are interpreted and compared based on the application of predictive microbiology models. Interestingly, for the first time in the thesis, interaction models are developed and applied to simulate the interaction of both populations **(Chapter 4 – Manuscript II**). In the last chapter, the knowledge and expertise gained from previous works and research collaborations in the different research missions carried out are deployed to design an innovative predictive modelling approach able to simulate microbial interaction, based on a stepwise system, from data to predictions and from broth to food. In this work, models generated are applied to a commercial aquaculture fish product in order to assess and optimize the use of bio-protective cultures to minimize the risk by *L. monocytogenes* during the product shelf-life (**Chapter 5 – Manuscript III**). The thesis finally presents the most outstanding conclusions drawn from the different works and research included in the present work. The chapter titles and related publications are showed as follows:

Chapter 2 – Chapter Book: Predictive microbiology tools for exposure assessment. *Chapter 11 of the book "Risk Assessment Methods for Biological and Chemical Hazards*

in Food", edited by Fernando Pérez-Rodríguez, that will be published by Taylor & Francis, in 2020. ISBN: 978-1-4987-6202-1.

Chapter 3 – Manuscript I: Study of the microbiological quality, prevalence of foodborne pathogens and product shelf-life of Gilthead sea bream (*Sparus aurata*) and Sea bass (*Dicentrarchus labrax*) from Aquaculture in Estuarine Ecosystems of Andalusia (Spain). *Food Microbiology*, 90, 103498, 2020. **Impact Factor: 4.155 (Q1).**

Chapter 4 – Manuscript II: 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, 2020. **Impact Factor: 4.972 (Q1).**

Chapter 5 – Manuscript III: 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*, 16, 72-84, 2019. **Impact Factor: 4.187 (Q1).**

Córdoba, October 2020.

Agradecimientos

Como todo lo que comienza tiene que terminar, pues esto llegó a su fin, una etapa más que finalizo del escalón de mi formación profesional. Si echo la vista atrás, aún recuerdo el día, que recibí el comunicado del Consejo Nacional de Desarrollo Científico y Tecnológico de Brasil (CNPq) que había sido seleccionado con una beca de investigación predoctoral en España. Confieso que jamás, me esperaba tal hecho.

Tras haber embarcado en este viaje, hoy, son muchas personas que me vienen a la mente y a la que me gustaría expresar mi gratitud, aun a riesgo de omitir algunos nombres. Qué difícil es agradecer con palabras a tod@s los que se cruzaron en mi camino durante estos años de doctorado. Sois muy especiales.

En primer lugar, me gustaría agradecer a mi tutor Fernando Pérez-Rodríguez, por la confianza, paciencia e, incentivo. Le agradezco también a su tiempo, interés, dedicación y, en particular, que haya estado siempre disponible, en cualquier momento que le necesitaba. Al largo de estos años de doctorado estoy seguro de que lo que comenzó como una relación tutor-alumno se convirtió en una amistad. Eres un estímulo científico y personal constante. Tienes una forma de entender la vida de la manera más sencilla posible y que eso motiva a todos a tu alrededor. Muchas gracias por orientarme y aconsejarme siempre. Ha sido un honor para mí trabajar contigo en este proyecto y en los muchos que tenemos por delante. ¡Gracias Fer!

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En definitiva, quiero dar mi más sincero agradecimiento a todo el personal del Grupo de Investigación Higiene Bromatológica (Hibro, PAIDI AGR-170), en especial a los profesores doctores Antonio Valero y Elena Carrasco, por el constante apoyo, atención y gran disposición en integrarme al grupo y permitirme iniciar esta gran experiencia.

A mi profesora y tutora de Máster, Glaúcia Maria de Aragão, por haber sido el puente en hacer el primer contacto con Fernando. Gracias por todos los conocimientos de microbiología predictiva y sobre todo por haber creído en mí. Has sido muy importante en mi crecimiento personal y profesional.

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Al Programa de Seguretat Alimentària del Institut de Recerca i Tecnologia Agroalimentàries (IRTA-Monells), por el periodo de estancia para el desarrollo de mi tesis doctoral. En especial a doctora Sara Bover-Cid, por los conocimientos transmitidos a respecto de los cultivos bio-protectores y gran incentivo a la investigación. Eres un ejemplo de profesional a seguir. Admiro mucho todos los roles que desarrollas, cada uno de ellos con mucho éxito. He aprendido mucho con esa oportunidad. A todos los compañeros de laboratorio de donde he conseguido hacer nuevas amistades: Margarita, Cristina, Loli, Mario y Carlos. Moltes mercès!

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Al fantástico mundo de los microorganismos. ¡Sois increíbles!

Por último, no mucho menos importante, no podría dejar de agradecer a Córdoba, esta ciudad que me acogió con su encanto, historia y, que hoy, me tiene enamorado.

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Dedicated to:

To my three mothers, Adélia (who has the title of fact), Erotides (grandma "Nenzinha") and Anilda (aunt).

Dedicado para:

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"La vida es aquello que te va sucediendo mientras tú te empeñas en hacer otros planes"
(John Lennon).

Y yo contento con todo lo que me ha sucedido hasta ahora.

"O sonho é que leva a gente para frente. Se a gente for seguir a razão, fica aquietado, acomodado"
(Ariano Suassuna).

Summary

Minimally processed and ready-to-eat (RTE) fish products that have not undergone any lethal treatment prior to consumption represents for a potential risk of pathogenic microorganisms, such as *Vibrio* spp. and *Listeria monocytogenes*. These pathogens cause foodborne infections resulting from consumption of contaminated raw fish or partially cooked fish or products contaminated during food handling and preparation. The genus *Vibrio* are environmentally ubiquitous in estuarine or marine water, able to reach consumers when products are not properly cooked, while the ability of *L. monocytogenes* to re-contaminate RTE products, during food handling and processing, and multiply at refrigeration temperatures poses a significant risk to human health. The microbial behaviour in food can be described using predictive mathematical models, taking into account the processing, distribution and home conditions along the food chain. In the first section of this work, an extensive review was elaborated, including the main aspects and concepts covered by the present thesis, which comprise of, among others, information on the aquaculture sector in Andalusia, relevant pathogenic and spoilage microorganisms in fishery products, listeriosis incidence, the use of bioprotective cultures or microbial risk assessment (Chapter 1). In addition, predictive microbiology tools were reviewed, providing an overview of the predictive modelling process, in particular, focused on microbial interaction models, and its application to quantitative microbial risk assessment (QMRA) (Chapter 2). In the experimental and investigation part, the microbiota and foodborne pathogens of Gilthead sea bream (*Sparus aurata*) and Sea bass (*Dicentrarchus labrax*) in two estuarine ecosystems were characterized and the shelf-life of both products was estimated using predictive models. Noteworthy, *Vibrio parahaemolyticus* was isolated from estuarine water and the initial microbiological quality of fish species and estuarine water was demonstrated to impact on product shelf-life (Chapter 3). For the study of biopreservation technology, lactic acid bacteria (LAB) strains were used to inhibit the growth of *L. monocytogenes*. The data obtained from this study were used to develop predictive microbiology models, able to describe the microbial interaction of both microorganisms in culture media (Chapter 4) and filleted Gilthead sea bream under modified atmosphere packaging at isothermal and non-isothermal conditions (Chapter 5). As result of these studies, an innovative stepwise modelling process was designed to generate predictive models describing interaction of BAL and *L. monocytogenes* based on the combination of mono and co-culture growth data. In the case of the assay with

Lactobacillus sakei CTC494, recognized as a bacteriocin-producing strain, it was evidenced that this bioprotective culture induced an early stationary phase of *L. monocytogenes* (Chapter 5), inhibiting the pathogen growth or reducing its concentration (Chapter 5). These experiments also confirmed that *L. sakei* CTC494 could be used, at a level of 4 log cfu/g, as bioprotective culture against *L. monocytogenes* in the above-mentioned Gilthead sea bream product, without negatively affecting the sensory quality of the product. Furthermore, the interaction models generated for this bioprotective culture demonstrated an optimal prediction capacity of the inhibitory effect of *L. sakei* CTC494 on the pathogen in the fish product (Chapter 5).

Overall, results of this work demonstrated the potential of using bioprotective cultures to control *L. monocytogenes* growth in Mediterranean fish products and enhance product shelf-life. In addition, the approach based on predictive microbiology was shown to be a suitable and effective method to simulate the simultaneous growth of bacteriocin-producing LAB strains and *L. monocytogenes* in culture media and fish products, presenting itself as a tool for supporting the design and optimization of preservation strategies based on the use bioprotective cultures in minimally processed and RTE fish products.

Keywords: foodborne pathogen, fish farm, minimally processed fish, Gilthead sea bream, Sea bass, shelf-life, bioprotective culture, CTC494, *Lactobacillus sakei,* sakacin, biopreservation, bacteriocin, predictive microbiology, microbial risk assessment, microbial interaction, competition model Lotka-Volterra model.

Resumen (Summary in Spanish)

Los productos pesqueros mínimamente procesados y listos para el consumo (RTE) sin tratamiento letal previo al consumo representan un riesgo potencial de presencia de microorganismos patógenos de transmisión alimentaria, como podrían ser *Vibrio* spp. y *Listeria monocytogenes*. Esos patógenos pueden estar presentes en el pescado crudo contaminado o pescado parcialmente cocido o bien recontaminarlo durante su manipulación y preparación. Los patógenos del género *Vibrio* tienen su origen en aguas de estuario o marinas, y pueden llegar hasta el consumidor, si el alimento no ha sido cocinado adecuadamente, mientras que *L. monocytogenes* puede contaminar los productos RTE, por contaminación cruzada, y multiplicarse a temperaturas de refrigeración, representando, por ello, un riesgo significativo para la salud humana. El comportamiento de los microorganismos en los alimentos puede describirse utilizando modelos matemáticos de microbiología predictiva, considerando las condiciones de procesado, de distribución y de la fase de consumo, cubriendo toda la cadena alimentaria. El propósito de la presente tesis doctoral fue estudiar y cuantificar el efecto bio-protector de cultivos bacterianos seleccionados sobre patógenos alimentarios y optimizar su diseño y uso en productos pesqueros RTE, mediante un enfoque de microbiología predictiva. Para ello, en primer lugar, se abordó una extensa revisión sobre los principales aspectos y conceptos objeto del presente trabajo, incluyendo información sobre el sector de la acuicultura en Andalucía, los tipos de microorganismos patógenos y alterantes de relevancia en productos pesqueros, la incidencia de listeriosis, el uso de cultivos bioprotectores y la evaluación del riesgo microbiano (Capítulo 1). A su vez, se realizó un análisis de las herramientas de microbiología predictiva, proporcionando una descripción general de los procesos de generación de modelos matemáticos, en particular, de modelos de interacción microbiana y de su aplicación en la evaluación cuantitativa del riesgo microbiano (ECRM) (Capítulo 2). En la parte experimental y de investigación del trabajo, se realizó un estudio de caracterización de la microbiota y patógenos alimentarios de la Dorada (*Sparus aurata*) y Lubina (*Dicentrarchus labrax*) en dos ecosistemas de estuario, estimándose la vida útil de los productos pesqueros mediante modelos predictivos. Es destacable la presencia de *Vibrio parahaemolyticus*, que se aisló de agua de estuario, además del impacto demostrado de la calidad microbiológica de las especies pesqueras y del agua en el que se encuentran sobre la vida útil del producto (Capítulo 3). En la parte que estudió y aplicó las tecnologías de bioconservación, se utilizaron cepas de bacterias

ácido-lácticas (BAL) para inhibir el crecimiento de *L. monocytogenes*. Los datos obtenidos sirvieron para generar modelos de microbiología predictiva que permitieron describir, adecuadamente, la interacción microbiana entre ambos microrganismos en medios de cultivo (Capítulo 4) y en productos de filetes de Dorada envasados en atmósfera modificada bajo condiciones isotérmicas y no isotérmicas (Capítulo 5). Como resultado de estos estudios, se diseñó un proceso sistematizado, basado en etapas, para la generación de modelos de interacción entre BAL y *L. monocytogenes*, combinando datos de crecimiento de mono y cocultivo en caldo y en alimento. En el caso de los experimentos con la cepa de *Lactobacillus sakei* CTC494, reconocida como productora de bacteriocina, se pudo observar que esta indujo una fase estacionaria temprana sobre el crecimiento de *L. monocytogenes* (Capítulo 5), reduciendo su crecimiento o, incluso, disminuyendo su concentración. Estos experimentos también confirmaron que *L. sakei* CTC494 podría aplicarse, a un nivel de 4 log ufc/g, como cultivo bioprotector contra *L. monocytogenes* en el producto de Dorada previamente mencionado, sin afectar negativamente la calidad sensorial del producto. Además, los modelos de interacción generados para este cultivo mostraron una buena capacidad de predicción del efecto bioprotector de *L. sakei* CTC494 sobre el patógeno en el producto de Dorada fileteada (Capítulo 5).

En general, los resultados de la tesis doctoral demostraron el gran potencial de los cultivos bioprotectores para controlar el crecimiento de *L. monocytogenes* en productos pesqueros del mediterráneo y mejorar su vida útil. Se comprobó, además, que el enfoque, diseñado en el presente trabajo, basado en la utilización de modelos de interacción microbiana, fue un método valido y efectivo para simular el comportamiento simultáneo de diferentes cepas de BAL, productoras de bacteriocina, y *L. monocytogenes* en medios de cultivo y productos pesqueros, proponiéndose, por tanto, como una herramienta de gran valor para el diseño y optimización de estrategias de conservación basadas en el uso de cultivos bioprotectores en pescados mínimamente procesados y RTE productos.

Palabras clave: patógeno alimentario, piscifactoría, pescado mínimamente procesado, dorada, lubina, vida útil, cultivo bioprotector, CTC494, *Lactobacillus sakei*, sakacina, biopreservación, bacteriocina, microbiología predictiva, evaluación del riesgo microbiano, interacción microbiana, modelo de competencia, modelo Lotka-Volterra..

Hypothesis

The rise in consumer demand for fish products has led to a considerable increase in aquaculture production in Spain, with Andalusia being one of the main producing regions of fishery products. For the aquaculture sector, food quality and safety are a prime concern, but also criteria to improve competitiveness and access to more profitable retailing channels. The application of traditional preservation methods offers the fishery industry an effective instrument, in line with the current consumer demands for more natural products, able to ensure food optimal conditions, meeting the strictest food safety standards. This work will underpin on the use of bio-protective microorganisms as a renovated traditional preservation technique suitable to control and improve the food safety and quality of aquaculture food products, considering their benefits for enhancing and extending product shelf-life. In this context, predictive microbiology models are proposed, as in-silico tools, able to provide a rapid and accurate response to microbial food safety issues. Mathematical models are introduced, in this field, to investigate the microbial interaction phenomena governing the bio-protective effect of certain microbial cultures (e.g. lactic acid bacteria). Models might be, in addition, validated and adapted for the practical application in the fishery industry, which would allow to design effective bio-protective strategies by considering the relevant technological factors and defining the most suitable food and process parameters. To introduce a predictive microbiology approach in the field of biopreservation, a relevant microbiological problem and context should be first identified and then, scientific data should be generated *in vitro* and subsequently, translated into real environments, for which valid mathematical models should be developed and deployed. The present thesis will develop this hypothesis focused on *Listeria monocytogenes*, as foodborne pathogen, in Ready to Eat fishery products from the Andalusian aquaculture, and investigate the use of bio-protective cultures to minimize its risk, leveraging the potential of the predictive microbiology. Results from the present thesis could be used as decision support tools to improve food safety and quality and enhance shelf-life of Andalusian aquaculture fishery products.

Objectives

The overall goal of this thesis was to investigate and develop mathematical models for predicting microbial interaction and their application to the use of bio-protective cultures, based on lactic acid bacteria, against the foodborne pathogen, *Listeria monocytogenes* in fishery products. To achieve this general objective, several specific objectives were proposed, which were accomplished in the different works of the present thesis.

First: To provide an overview of Predictive Microbiology, presenting the main concepts and types of models and describe the modelling process from data generation to food application.

Second: To assess the microbiological quality and prevalence of foodborne pathogens in Gilthead sea bream (*Spaus aurata*) and Sea bass (*Dicentrarchus labrax*) raised in estuarine ecosystems in Andalusia (Spain).

Third: To determine the antimicrobial capacity of specific lactic acid bacteria strains on the foodborne pathogen, *L. monocytogenes*, in laboratory culture media and fish juice from Mediterranean fish species.

Fourth: To develop growth models, in monoculture, for specific lactic acid bacteria strains, with bio-protective potential and *L. monocytogenes* in simulated food systems and actual fish products.

Fifth: To design a suitable mathematical modelling approach to simulate microbial interaction of lactic acid bacteria, as bio-protective cultures, and *L. monocytogenes* in foods.

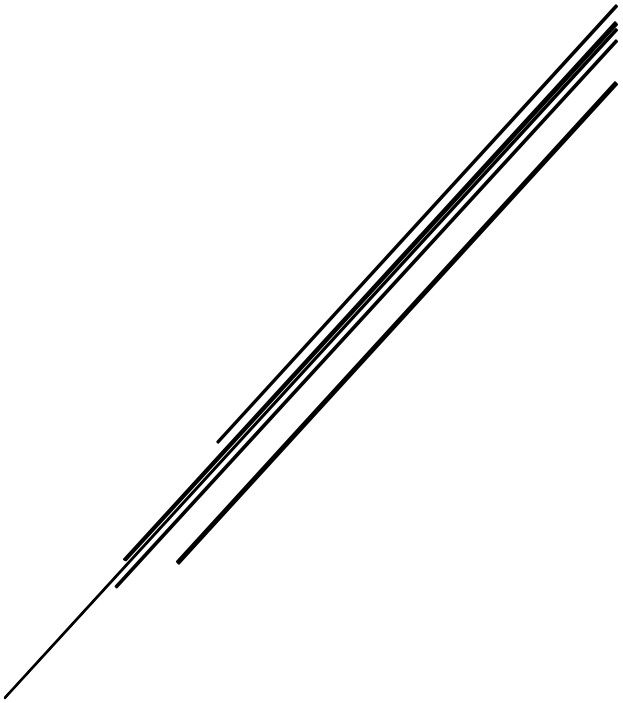
Sixth: To quantify the inhibitory effect of lactic acid bacteria on *L. monocytogenes* in fish product models and Mediterranean fishery products by using predictive microbiology models.

Seven: To validate the developed microbial interaction models simulating the inhibitory effect of a lactic acid bacterium strain CTC494 on *L. monocytogenes* in a commercial product of filleted Gilthead sea bream under different refrigeration temperature profiles.

Eighth: To assess the shelf-life of Mediterranean fish products from the aquaculture taking in consideration sensory and microbial aspects, together with the impact of bio-protective cultures, based on the use of predictive microbiology models.

Chapter 1

General introduction



General Introduction

This section introduces the background on the main themes addressed in this present thesis. Relevant topics related to the proposed objectives and thesis contents will be described in specific subsections.

1.1. Aquaculture production in the Mediterranean

Worldwide production of fish from aquaculture have had a significant growth over the past 50 years, especially in the last two decades. In per capita terms, this production resulted in a record-high consumption grew from 9.9 kg in the 1960s to 20.3 kg in 2016 (FAO, 2018a). Since 1961, the average annual increase in global fish consumptions (3.2%) outpaced population growth (1.6%) and exceeded the augment of consumption of meat products (e.g. bovine and pork) (2.8%), excepting for poultry (4.9%) (FAO, 2018a; FAO, 2018b). In 2019, out of 177.8 million tonnes of fish produced, 89% was used for human consumption, which, turned into global per capita consumption, corresponding to 20.5 kg/year (FAO, 2019). The increased production and consumption of fish and fishery products have been driven by a combination of different factors, linked to population growth, new consumer demands of diversified diets and nutritional foods, improved distribution channels, wastage reduction, higher incomes and urbanization (Belton et al., 2011; Vannuccini et al., 2018). Fish and fish products are an important component of human diet, with a significant nutritional value being a noticeable source high quality protein, omega-3 fatty acids, minerals, vitamins and antioxidants (Sarojnalini and Hei, 2019).

The European Union (EU), including the United Kingdom, has a coastline of approximately 68,000 km and offers environmental physical and oceanographic conditions suitable for aquaculture (EC, 2006; Markus, 2019). In this sense, European aquaculture has demonstrated to have the know-how, experience and technical resource to be a sustainable activity from an environmental and economic point of view, offering employment opportunities and safe healthy and quality food (APROMAR, 2018). Thus, aquaculture has become an important economic activity in the EU, being the major supplier of fisheries products. In 2017, the EU produced 1,353,201 tons in aquaculture products is valued at 4,147 million EUR, an increase of 4.8%, related to the market in 2016. Furthermore, aquaculture represents 19.2% the volume of total aquatic production

(i.e. aquaculture and fisheries) in the EU. The remaining 80.8% of production came from extractive fishing, that is, 5,680,902 tons (APROMAR, 2019).

Contemporary Mediterranean aquaculture started in the 1980s, mainly with the introduction of species fish of Gilthead sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), together with shellfish species (Grigorakis and Rigos, 2011). Although the Mediterranean region has a long history of fish farming, aquaculture has experienced a dramatic expansion from 1990s, with the introduction of new aquaculture Mediterranean species, such as Bluefin tuna (*Thunnus thynnus*), Meagre (*Argyrosomous regious*) and Greater amberjack (*Seriola dumerili*), with the latter two species being considered the most promising ones concerning the improved production systems and market prospects (Barazi-Yeroulanos, 2010). Notwithstanding foregoing, the most important commercial species in the Mediterranean are still represented by Gilthead sea bream and European sea bass. This increasing trend has been boosted by an overall decline in catchable wild fish stocks and an increase in consumer demand for fish and shellfish resources. This situation also promoted the development of a more competitive aquaculture industry (Fernandes et al., 2000).

Aquaculture production in Spain is one the most diverse in Europe due to, among other aspects, the longitude of its coastline, varied availability of water resources on which it is feasible to develop aquaculture, both in the marine and continental areas (i.e. fresh waters) and the diversity of ecosystems (APROMAR, 2014). For these reasons, Spain ranks first in EU aquaculture production, with 311,032 tons in 2017, accounting doe 23.0% the Union production, followed by the United Kingdom with 222,434 tons (16.4%) and France with 166,000 tons (12.3%). However, when considering the value of production, Spain occupies the fourth position, with 466.6 million EUR (12.2%), while United Kingdom is obtaining the highest economic value with 1,160.8 million EUR (28% of the total value).

In Andalusia, aquaculture is an activity that can become a strategic economic sector for both fish and shellfish products, given the wealthy resources in coastal areas. The success of its development relies on suitable marketing strategies, culture diversification and improved productions methods, as well as the economic management. Moreover, the aquaculture must demonstrate knowledge and means to be an environmentally viable and sustainable activity, offering healthy, safe and quality products (Junta de Andalucía, 2014). In Andalusia, a significant volume of fish production is linked to aquaculture in estuaries, located in the provinces of Cádiz, Huelva and Seville. These estuarine farms

constitute a unique and specific ecosystem in natural enclaves composed of brackish and/or intertidal waters that can be exploited as marine environments for the commercial cultivation of fish, molluscans and crustaceans (Marti, 2018). At national level, Andalusia is the main producer of Gilthead sea bream and European sea bass. In 2018, the Andalusian aquaculture production for these two species, were 4,726 tons and 37,328 million EUR. In the same period, Gilthead sea bream and European sea bass were sold mainly whole chilled, corresponding to 99.7% of production of fish, which is the main marketing channel for these products (AGAPA, 2019).

1.1.1. Microbial ecology of Mediterranean fish products: Spoilage and pathogenic microorganisms

The fish muscle is sterile at the time of catch, but the skin, mucus, gills and intestines contain significant amounts of bacteria. The number and diversity of the natural microbiota and the presence of pathogens in fish can be related to many factors such as temperature and salinity of the water, level of dissolved oxygen and degree of pollution, etc. (Françoise, 2010; Gram, 2009). In general, the natural fish microflora tends to reflect the microbiota of the production waters. Moreover, contamination with pathogenic bacteria can occur because the unhygienic conditions of the landing place in fish boats or when the fish is washed with contaminated water (Mokrani et al., 2018).

Fish has a high amount of non-protein nitrogenous (NPN) compounds and a low acidity (pH > 6), which support the fast growth of microorganisms that are the main cause of spoilage. The microorganisms are found on outer surfaces (i.e. skin and gills) and in the intestines of fish. The total number of organisms is quite variable. On skin surface, fish can show concentrations between 10^2 and 10^7 CFU/cm^2, while in gills and intestines the values can vary from 10^3 to 10^9 CFU/g (Boziaris, 2014; Huss, 1995; Sivertsvik et al., 2002). The microbial ecology of Mediterranean fish can include diverse microbial groups and genera, which are dominated by Gram-negative, rodshaped bacteria such as *Aeromonas, Moraxella, Pseudomonas, Photobacterium, Shewanella* and *Vibrio*. In turn, lactic acid bacteria (LAB), *Bacillus*, *Clostridium* are the most common Gram-positive genera of the indigenous microbiota. Furthermore, *Enterobacter, Escherichia coli, Listeria, Salmonella, Shigella*, *Staphylococcus aureus,* yeasts and some molds can be found in the initial microbial population mainly as a result from contamination events (Carrascosa et al., 2015; Huss et al., 2000; Parlapani et al., 2015).

1.1.1.1. Spoilage microorganisms in fish products

Microbiological spoilage is the main cause of the loss of quality in fresh fish. Although fish microbiota initially encompasses a wide range of bacterial genera, only a small number of these microorganisms are able to give rise spoilage, in which processing and storage conditions play an important role (e.g. temperature and atmosphere) (Boziaris and Parlapani, 2017). The spoilage microorganisms are so called specific spoilage organisms (SSOs), that are related to the production of metabolites in quantities that result in off-flavours leading to the sensorial rejection of the product (Gram and Dalgaard, 2002; Gram and Huss, 1996). In fish from Mediterranean Sea waters (e.g. Gilthead sea bream), *Pseudomonas* spp. and *Shewanella* spp. are the main SSOs involved in the spoilage of unpackaged fish at low temperature, while LAB and *Photobacterium phosphoreum* are more related to spoilage of packaged fish products under modified atmosphere, due to the tolerance of this microorganism to relatively high CO_2 levels (Françoise, 2010; Koutsoumanis et al., 2000; Parlapani et al., 2014; Tryfinopoulou et al., 2002). The SSOs for Mediterranean fish species, including Gilthead sea bream and Sea bass are constituted by *Pseudomonas* spp., *Brochothrix thermosphacta*, H_2S-producing *Shewanella* bacteria and lactic acid bacteria (Koutsoumanis et al., 1999; Odeyemi et al., 2018; Parlapani et al., 2013, 2014; Tryfinopoulou et al., 2002; Zaragozá et al., 2013).

1.1.1.2. Food hygiene indicator microorganisms and pathogens in fish products

Food hygiene indicator organisms comprise is a group of microorganisms that are used to reflect the microbiological quality and safety of products. Aerobic mesophilic bacteria and aerobic plate count (APC) are widely used to monitor the quality of the entire production process while *Enterobacteriaceae*, coliforms and *E. coli*, are employed to assess poor hygiene practice and enteric contamination (Anihouvi et al., 2019; Armani et al., 2016; Popovic et al., 2010).

Human pathogens can also be part of the microbiota (e.g. intestinal tracts and gills) of fish and can naturally be found in estuarine and coastal waters (Givens et al., 2014; Jammal et al., 2017). Pathogenic *Vibrio* species represents an increasing concern for public health systems due to the rise in prevalence and number of cases. The new consumption trends for fish products and ocean warming are probable contributing factors to the Vibrio incidence (Feldhusen, 2000). The genus *Vibrio* includes a large number of species naturally distributed in seawater and also in estuaries located in the Mediterranean Sea (Esteves et al., 2015). Factors like salinity and water temperature can

affect the prevalence of *Vibrio* species, being present in higher concentrations between May and October, when the water temperature is warmer (CDC, 2019). The bacterial species *V. parahaemolyticus* and *V. vulnificus* are pathogenic for humans and the most common non-cholera *Vibrios* associated with foodborne diseases worldwide (Feldhusen, 2000; Su and Liu, 2007). The disease is linked to raw or undercooked fish or fish products and seafood, particularly shellfish, harvested in contaminated water or that have been improperly preserved after harvest (Baffone et al., 2000; Iwamoto et al., 2010; Tsironi et al., 2017). The consumption of these foods without adequate previous heat treatment can cause acute gastroenteritis, so-called Vibriosis, which is characterized by diarrhea, headache, vomiting, nausea, abdominal cramps, low fever. In more severe cases, complications such as septicemia can lead to death in infected patients (Callol et al., 2015; de Magny et al., 2009; Gauthier, 2015).

Another important pathogen associated with the consumption of fish and fish products is *Listeria monocytogenes*, responsible for a foodborne disease so-called Listeriosis (Nørrung, 2000). Although this pathogen is not part the natural microbiota of fish, *L. monocytogenes* can be introduced at different stages of fish production chain (Miettinen and Wirtanen, 2005). Besides, *L. monocytogenes* can be present in aquatic environments, which correlates with the degree of human activity. Its wide distribution in the environment allows this microorganism to enter into the food chain through raw fish material, which can be considered an important source of contamination in the processing facilities, resulting in the subsequent contamination of the final product due to cross-contamination during handling and preparation and then grow during distribution and storage due to its ability to grow at low refrigeration temperatures (Miettinen and Wirtanen, 2005; Soultos et al., 2007; Yücel and Balci, 2010).

The microbiological quality and the presence of pathogenic microorganisms in fish products from fish produced in estuarine ecosystems located in Mediterranean Sea has been reported in few studies. Alexopoulos et al. (2011) evaluated the microbiological quality of water for aquaculture and the species Gilthead sea bream and European sea bass from different fish farms in Greece. In this study, total and faecal coliforms were detected in almost all water samples. For microbiological analysis of fish (n = 75), the results showed presence of *Staphylococcus* sp. (29.3%), *E. coli* (30.6%), *Salmonella* sp. (1.3%), *Pseudomonas* sp. (13.3%), *Vibrio* sp. (18.7%), *Vibrio alginolyticus* (2.5%) and *Vibrio anguillarum* (9%). Recently, Costa et al. (2020) evaluated the microbiological

quality and prevalence of foodborne pathogens of Gilthead sea bream and Sea bass from aquaculture in estuarine ecosystems of Andalusian (Spain). This is the first study on the estuaries in Andalusia, one of the first producers in the Mediterranean of Gilthead sea bream and Sea bass. The results of this study are unique, and novel and improve the knowledge basis on the fish species and type of production from a food safety and quality perspective.

1.2. Ready-to-eat (RTE) fish products

The increasing demand for convenience and easy to prepare products has led the food industry to gear its production to ready-to-eat (RTE) and minimally processed foods (Hierro et al., 2014). According to EC Regulation 2073/2005, RTE food corresponds to "food intended, by the producer or the manufacturer, for direct human consumption without need for cooking or other effective processing to eliminate or reduce to an acceptable level of microorganism of health concern" (EC, 2005).

Fish products are attracting an increase interest by consumers given their high food quality and nutritional value (Ghanbari et al., 2013). In recent years, fresh Mediterranean fish (e.g. Gilthead sea bream and European sea bass) among others has been introduced as main ingredient in some culinary trends, like products eaten raw (sushi and carpaccio), lightly preserved (smoked and salted fish) or ready-to-eat (fish sausage, surimi and pâtés) and convenience foods (fish or seafood packed under vacuum or modified atmosphere) (Bilgin et al., 2008; Bolívar et al., 2018; Chuapoehuk et al., 2001; Parlapani et al., 2015).

The combination of lipid oxidation, autolytic (biochemical) reactions, the presence of trimethylamine oxide (TMAO) and physico-chemical characteristics makes fish products highly perishable foods. Moreover, TMAO can stimulate the growth and activity of spoilage microorganisms and foodborne pathogens, naturally present in the marine environment or originated from recontamination processes (Dalgaard et al., 2006). In vacuum-packed or modified atmosphere fish products, microbial species such as Enterobacteriaceae, *Shewanella* and *Vibrio* are able to reduce TMAO to trimethylamine (TMA). This reduction is the cause of the typical ammonia like and fish off-odours in spoiled fish products, particularly in products with pH above ~6.5 (FAO, 2014). Nonetheless, several processing treatments or combination of treatments are successfully applied to retard food spoilage and enhance shelf-life of fresh fish and fish products while satisfying the increasing consumer demands for safe, fresh-tasting, ready-to-eat, minimally processed fish (Gálvez et al., 2007).

Lightly preserved fish products (LPFP) and semi-preserved fish products (SPFP) are products that have not undergone a lethal heat treatment or mild heat treatment (e.g. pasteurization), which can be formulated with chemical preservatives (salt, organic acids or smoke) and combined with packaging under vacuum (VP) or under modified atmosphere (MAP) in order to extend the shelf-life of these products (Hoffman et al., 2002). The LPFP group includes chilled fish products stored at < 5 °C packed under vacuum conditions, which are characterized by having pH > 5.0 and < 6% NaCl in the water phase of the product. For some products, the addition of preservatives (benzoate, sorbate or smoke) plays a paramount role for controlling microbial growth. This group is a category of high value products and delicatessen products and includes cold-smoked, salted, marinated, pickled (gravad) and brined seafood that are typically consumed as ready-to-eat products, without any additional heat treatment (Françoise, 2010; Ghanbari et al., 2013) The second group, SPFP, with a recommended storage temperature of < 10 °C, are usually packed in aluminium cans, glass or plastic containers. They are characterized by having pH < 5 and > 6% NaCl in the water phase and to which preservatives are added (benzoate, sorbate and nitrite). Typically, the European products of this category comprises salted and/or marinated fish or caviar, anchovies, fermented fish (Mejlholm and Dalgaard, 2007; Mejlholm et al., 2008). The fermented fish are also considered to be RTE fish products, with pH changing from neutral to acid and NaCl percentages being < 8%. This products, typically, are stored at room temperature (Huss et al., 2004).

The microbial risk associated with the consumption of LPFP is related to the handling and preparation tasks that these products usually involves (e.g. slicing), which can result in product recontamination and/or the product conditions might support the growth of pathogenic bacteria. *L. monocytogenes* is a foodborne pathogen can be present in raw material or contaminate the post-processing product through handling operations (e.g. slicing, peeling, packaging and reusing brine for several days) (EFSA, 2013). Any post-processing process is susceptible to cause cross-contamination of the pathogen between the contaminated product and environmental surfaces such as equipment, utensils and personal and then these can contaminate new uncontaminated products (Huang and Hwang, 2012). Effective hygiene, cleaning and sanitization practices applied during the production of fish products followed by the use of control programs (e.g. GHP and HACCP), risk assessment, and mathematical models (e.g. predictive microbiology)

represent key factors in reducing the *L. monocytogenes* in fresh fish and RTE fish products (Rotariu et al., 2014).

1.3. *Listeria monocytogenes*

Listeria monocytogenes is a foodborne pathogen that is especially relevant in refrigerated RTE products. The genus *Listeria* is currently made up of 20 species, which are subdivided in two major groups. The first group, represented by the *Listeria sensu stricto*, defines a distinct group of 6 species that shares common phenotypic characteristics (e.g. ability to grow at low temperature and flagelar motility). This group is organised by the species *Listeria monocytogenes*, *Listeria innocua*, *Listeria welshimeri*, *Listeria seeligeri*, *Listeria ivanovii* and *Listeria marthii*. The second group, *Listeria sensu lato*, presents several phenotypic characteristics that differentiate them from the first group. This group consists of 14 species including *Listeria grayi*, *Listeria rocourtiae*, *Listeria fleischmannii*, *Listeria weihenstephanensis*, *Listeria floridensis*, *Listeria aquatica*, *Listeria cornellensis*, *Listeria riparia*, *Listeria grandensis*, *Listeria booriae*, *Listeroa newyorkensis*, *Listeria costaricensis, Listeria goaensis* and *Listeria thailandensis*. The last three species of this group being recently isolated and identified from Costa Rica, India and Thailand, respectively (Chiara et al., 2015; Doijad et al., 2018; Leclercq et al., 2019; Núñez-Montero et al., 2018; Orsi and Wiedmann, 2016).

Among these species, only two are pathogenic, *L. monocytogenes*, which infects humans and animals and represents a public health problem worldwide by causing a severe disease that, in some cases, can be lethal for specific groups of humans (e.g. immunocompromised, pregnant, elderly population), which makes it a major concern for the food industry (Allerberger and Wagner, 2010; Jordan et al., 2018; Vázquez-Boland et al., 2001). *L. ivanovii* which mainly infects warm-blooded ruminants, causing economic losses (Chen et al., 2017). Nevertheless, there are some reports showing that this species could also cause disease in humans (Guillet et al., 2010; Snapir et al., 2006).

Listeria monocytogenes are short, rod-shaped bacteria cells, with a typical size ranging of 0.4-0.5 μm in diameter by 1-2 μm in length, have rounded ends and are not encapsulated. The cells can be found as individual units or in short chains and often arranged in V and Y forms or in palisades (Meloni, 2014; McLauchlin and Rees, 2015). This bacterium is Gram-positive, has aerobic and facultative anaerobic metabolism (ability to grow in the presence and absence of oxygen), non-spore-former, catalase-positive and oxidase negative. L(+)- lactic acid, acetic acid and some other end products

are formed by homofermentative anaerobic catabolism of glucose, but gas is not produced (Wagner and McLauchlin, 2008). *L. monocytogenes* possesses peritrichous flagella which give the bacterium a motility at 20-25 °C, while they become non-motile at 37 °C due to a lack of flagellin expression at this temperature (Farber et al., 1991; Wang and Orsi, 2013; Way et al., 2004). It is also able to grow or survive in different types of environments, even in stressing conditions, such as dry environments and mild preservation treatments (e.g. pasteurization, vacuum and CO_2 packed food), at a wide pH range (4.4 to 9.2), at high salt concentrations (10 to 16% w/v), water activity above 0.92 and, most importantly, at refrigeration temperature (-0.4 to 9.3 °C) (Chan and Wiedmann, 2009; Farber and Peterkin, 1999; Ferreira et al., 2014; Liu et al., 2005; ICMSF, 1996). The ability of *L. monocytogenes* to multiply at refrigeration temperature conditions is strongly pH dependent (Martinez-Rios et al., 2019; Tienungoon et al., 2000).

Owning to these capabilities, *L. monocytogenes* is ubiquitously distributed in a variety of environments, which can easily result in contamination of a wide variety of food processing environments and, ultimately, in processed food products (Cartwright et al., 2013; Norton et al., 2001). In fact, *L. monocytogenes* has been isolated from natural environment (soil, water, and vegetation), livestock manure, animal feed (silage) and effluents (Gram, 2001; Ivanek et al., 2006; Jordan et al., 2018).

Different areas and facilities within the food processing environment can serve as reservoir or source of *L. monocytogenes* contamination, which makes it extremely difficult to determine the specific origin of contamination of the pathogen (Jami et al., 2014). In the fish processing environment, different niches for the pathogen have been reported directly and indirectly, which include mainly conveyors, spiral/blast freezers, equipment and smoking area, injection brines and other solution, drains and floors/gangways (Chen et al., 2010; Gudbjörnsdóttir et al., 2004; Hansen et al., 2006; Nakamura et al., 2006; Pagadala et al., 2012; Skowron et al., 2019). Another concern of the fishery industry is the high persistence of *L. monocytogenes* strains in processing environments, where the pathogen can remain in specific sites despite regular cleaning and disinfection regimes (Wulff et al., 2006). This emphasizes the risk of colonization of strains capable of adhering to many materials found in fish processing environment, especially on sites difficult to access. Thus, the long-term persistence of these strains in different locations can result in biofilms formation on abiotic surfaces, leading to continuous contamination of fish products, which poses a risk to public health (Beresford

et al., 2001; Carpentier and Cerf, 2011; Doijad et al., 2015; Ferreira et al., 2014). The potential contamination routes of fish products by *L. monocytogenes* are schematically depicted in Figure 1.1.

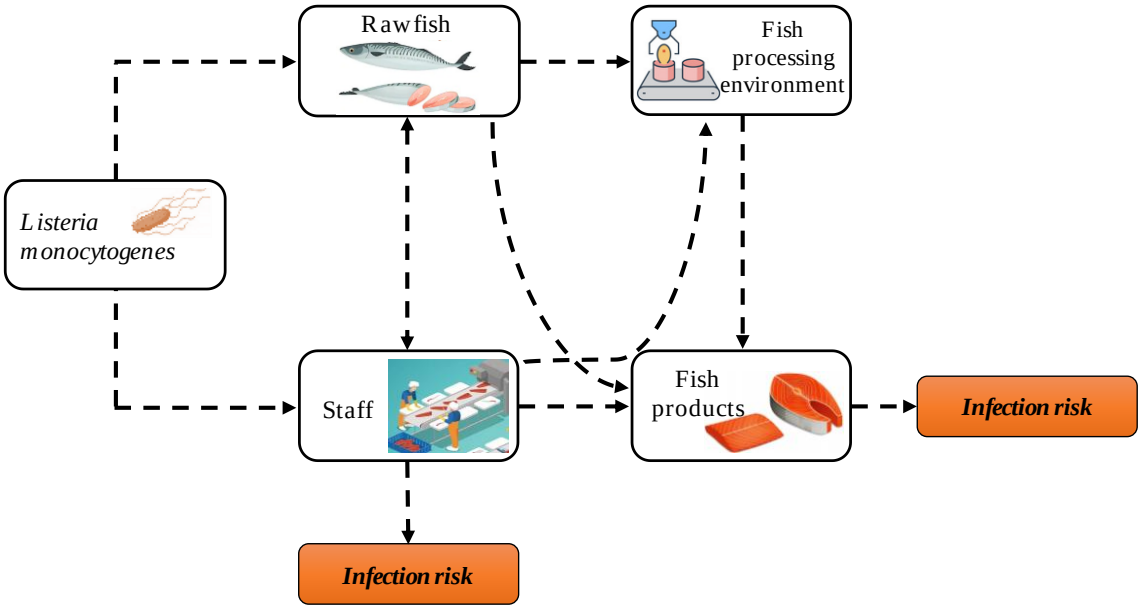


Figure 1.1. Flow-chart of contamination of fish products with *L. monocytogenes* (Source: adapted from Larsen et al., 2014).

The resistance of *L. monocytogenes* strains to different environmental, microbiological and processing factors has been studied including different types of stress such as disinfectant and desiccation (Møretrø et al., 2017; Ratani et al., 2012; Vogel et al., 2010), the capacity of biofilm formation (Colagiorgi et al., 2017; Kadam et al., 2013) and salt concentration Ribeiro and Destro, 2014). Several studies have tried to relate *L. monocytogenes* serotypes with their abilities to persist in food processing environment, however, certain results remain contradictory or inconclusive (Doijad et al., 2015; Lee et al., 2019; Norwood and Agilmour, 2001). Notwithstanding aforementioned, studies have suggested that the persistence of *L. monocytogenes* strains, in food processing environment (e.g. desiccation resistance and biofilm formation) is associated with serotype, number of cells and gene expression (Djordjevic et al., 2002; Kadam et al., 2013; Kragh and Truelstrup Hansen, 2020; Takahashi et al., 2009; Zoz et al., 2017). In this respect, persistent *L. monocytogenes* have been reported in listeriosis infections and recalls of RTE fish products (EFSA, 2019a; FDA, 2019; Schjørring et al., 2017; Vongkamjan et al., 2013; Wulff et al., 2006).

1.4. Listeriosis

Listeriosis is a foodborne disease caused by the consumption of foods contaminated by *L. monocytogenes* exhibiting several clinical symptoms, depending on the dose ingested, the host and the pathogen strain. The incubation period of listeriosis ranges from 1 to 90 days, up to the onset of disease. This long incubation period hinders determining the source infection, resulting in unnotified listeriosis cases (Rees et al., 2017; Vázquez-Boland et al., 2001).

Although relatively few frequent, listeriosis can produce severe symptoms with high rate hospitalization (> 90%) and high mortality (20 - 30%), especially in vulnerable groups such as pregnant women and their newborns, elderly people and individuals with weakened immune systems, including people with liver or kidney disease, diabetes, cancer, AIDS, transplant recipients and alcoholism (CDC, 2017; Choi et al., 2018; de Noordhout et al., 2014; Pontello et al., 2012). The clinical symptoms of listeriosis are vomiting, diarrhoea, fever, muscle aches, gastroenteritis and convulsions. In cases of invasive listeriosis, in which *L. monocytogenes* has the ability to cross the intestinal barrier, the blood–brain barrier, and the fetoplacental barrier, infecting organs such as the brain or uterus, meningitis, encephalitis, septicemia are the most common forms of disease that might lead to death or cause abortion and stillbirth in pregnant women (Aygun and Pehlivanlar, 2006; Buchanan et al., 2017). Non-invasive listeriosis usually occurs in non-immunocompromised people and generally causes a mild form of disease hence, in these cases, *L. monocytogenes* is not considered a pathogen of concern (Allerberger and Wagner, 2010; Angelidis and Koutsoumanis, 2006).

The EU has undergone an increase in notifications of listeriosis outbreaks over the last years. European Food Safety Authority (EFSA) reported that in 2018, a total of 2,549 confirmed cases of invasive listeriosis were reported in 28 member states, corresponding to an EU notification rate of 0.47 cases per 100,000 individuals (an 2.7% higher compared with 2017). Most listeriosis cases were reported to be domestically acquired. The highest notification rates in reported cases of listeriosis were observed for Estonia, Finland, Sweden and Denmark with 2.05, 1.45, 0.88 and 0.85 cases per 100,000 individuals, respectively. Overall, in 2018, the reported cases of hospitalisations and deaths were high, of 1,049 (97%) and 229 (15.6%), respectively, which makes listeriosis one of the most serious foodborne diseases under EU surveillance (EFSA, 2019b). In 2019, two cases of listeriosis were reported in EU. The first in Denmark, with 9 cases and 2 deaths linked to

the consumption of cold-smoked fish products and the second in Spain, with 217 reported cases, 3 deaths and 6 women that had miscarriages associated with the consumption of a chilled roasted pork meat product (known as "carne mechada") (EFSA, 2019a; CCAES, 2019).

Outbreaks of listeriosis infection is also related to the serotype of the *L. monocytogenes* strains and can be determined by unique combinations of their somatic (O) and flagellar (H) antigens (Liu, 2006). Differentiation between serotype is necessary for epidemiological studies, which also contributes to the monitoring of sources of contamination in food processing environment (Alonso-Calleja et al., 2019). Fifteen serotypes have been identified and are classified into Division I (1/2b, 3b, 4b, 4d and 4e), Division II (1/2a, 1/2c, 3a and 3c), Division III (4a and 4c) and Division IV (4a, 4c and atypical 4b) (Chen et al., 2017; Orsi et al., 2011; Ward et al., 2008). Out of these 15 serotypes described, only 1/2a, 1/2b and 1/2c are frequently isolated from food products. However, serotypes 1/2a, 1/2b and 4b are responsible for approximately 95% human listeriosis cases (Montero et al., 2015; Swaminathan and Gerner-Smidt, 2007). In particular, serotype 4b is linked to most cases and outbreaks of human listeriosis (Kathariou et al., 2006; McLauchlin et al., 2004).

1.4.1. Outbreaks caused by L. monocytogenes *in raw fish and RTE fish products*

The first reported case of listeriosis linked to consumption of fish was reported in Italy in 1989, when a 54-year-old woman contracted meningitis 4 days after consuming steamed fish. Two *L. monocytogenes* isolates were obtained, on from the patient's cerebrospinal fluid and the other from a leftover portion of the fish. Both strains were serotype 4 (specific serotype not determined), identical in terms of phage type and restriction analysis of chromosomal DNA, indicating that the fish was most likely vehicle of infection (Facinelli et al., 1989; Jinneman et al., 2007). Although in 1980, a previous outbreak of listeriosis occurred in New Zealand with 22 perinatal infections and 7 foetal deaths, the cause has not been confirmed, an epidemiological survey suggested that the outbreak could be associated with the consumption of shellfish and raw fish (Lennon et al., 1984). During 2014-2019, 22 multi-country outbreak cases, including 5 deaths, were reported in five countries EU: Denmark, Estonia, Finland, France and Sweden linked to consumption of cold-smoked fish products (i.e. salmon and trout). The first case had symptom onset in July 2014 in Estonia, and the most recent case occurred in Denmark in February 2019 (EFSA, 2019a).

Although *L. monocytogenes* has been observed in a wide variety of foods, its psychrotolerant nature represents a risk factor for refrigerated RTE foods products that are generally eaten with little or no prior heating and exhibit a relative long shelf-life (Bremer et al., 2003). Raw fish and fishery products are on the top among these high risk RTE products (EFSA, 2018a; Jelena et al., 2011). An overview of the listeriosis outbreaks occurred worldwide between 1989 and 2019 associated with fish and fish-based products is shown in Table 1.1. According to this data review, the total number of cases corresponded to 1,795 cases, from which 36 were deaths.

Table 1.1. Overview of the listeriosis outbreaks between 1989 and 2019 associated with the consumption of fish and fishery products.

Product	Country	Year	Number of cases (n° deaths)	Serotype	Reference
Shellfish and raw fish	New Zealand	1980	22 (7)	1/2a	Lennon et al. (1984)
Gravad salmon	Sweden	1987-1989	3 (ND)[a]	1/2a	Loncarevic et al. (1998)
Fish	Italy	1989	1 (0)	4	Facinelli et al. (1989)
Cooked shrimp	USA	1989	10 (1)[b]	4b	Riedo et al. (1994)
Smoked cod roe	Denmark	1989	1 (0)	NR[c]	Jensen et al. (1994)
Smoked mussels	Tasmania	1991	4 (0)	1/2a	Mitchell et al. (1991)
Smoked mussels	New Zealand	1992	3[d] (0)	1/2a	Brett et al. (1998)
Smoked trout /gravad salmon	Sweden	1993-1994	2 (ND)	1/2a	Loncarevic et al. (1998)
Cold-smoked rainbow trout	Sweden	1994-1995	9 (2)	4b	Ericsson et al. (1997)
Surimi-based crabmeat	Canada	1996	2 (0)	1/2b	Farber et al. (2000)
Tuna-corn salad	Italy	1997	1566 (0)	4b	Aureli et al. (2000)
Cold-smoked rainbow trout	Finland	1998	5 (0)	1/2a	Miettinen et al. (1999)
Vacuum-packed fish	Finland	1999-2000	10 (4)	1/2	Hatakka et al. (2000)
Sandwiches[e]	United Kingdom	2003	2 (0)	1/2a	Little et al. (2012)
Tuna salad	USA	2008	5 (3)	1/2a	Cokes et al. (2011)
Sandwiches[e]	United Kingdom	2008	3 (2)	4b	Little et al. (2012)
Sandwiches[e]	United Kingdom	2010	5 (1)	4	Little et al. (2012)

Table 1.1 continued

Product	Country	Year	Number of cases (n° deaths)	Serotype	Reference
Sushi	USA	2010	2 (0)	NR	MMWR (2013)
Gravad or cold-smoked fish	Finland	2010	54 (?)[f]	1/2a and 4b	Nakari et al. (2014)
Herring cutlet marinated oil	Germany	2010	8 (1)	NR	Aichinger (2010)
Sandwiches and salads	United Kingdom	2011	3 (0)	4[g]	Coetzee et al. (2011)
Cold-smoked salmon	Denmark	2013-2015	10 (4)	2a	Lassen et al. (2016)
Gravad salmon/frozen halibut	Denmark	2013-2015	10 (3)	4b	Lassen et al. (2016)
Gravad/smoked fish	Sweden	2013-2015	27 (ND)	1/2a	Lopez-Valladares G., pers. comm.
Chilled and frozen products from salmon and trout	Estonia	2014-2019	6 (2)	NR	EFSA (2019a)
Cold gravad salmon/cold-smoked salmon	Sweden	2015-2016	4 (?)[f]	NR	EFSA (2019a)
Chilled and frozen products from salmon and trout	Finland	2016-2017	2 (?)[f]	NR	EFSA (2019a)
Cold-smoked salmon[h]	France	2016	1 (1)	NR	Schjørring et al. (2017)
Cold-smoked trout and/or cured salmon	Denmark	2017	6 (1)	NR	Schjørring et al. (2017); EFSA (2019a)
Salmon products	Germany	2017-2018	5 (3)	NR	EFSA (2018b)

Table 1.1 continued

Product	Country	Year	Number of cases (n° deaths)	Serotype	Reference
Chilled cold-smoked trout	France	2018	1 (?)[f]	NR	EFSA (2019a)
Cold-smoked salmon or cold-smoked trout	Denmark	2018-2019	3 (1)	NR	EFSA (2019a)
Total of listeriosis outbreaks and (number of deaths)	-	-	**1795 (36)**	-	-

[a] ND, not determined.
[b] Foetal demise.
[c] NR, serotype not reported.
[d] Perinatal case.
[e] Sandwiches prepared with salmon, tuna or tuna salad.
[f] Fatalities uncertain.
[g] Isolated from the blood cultures of the three patients.
[h] Inconclusive (history of food consumption not available at the time of diagnosis, as the person had died).

The Listeriosis cases linked to RTE fish products are often result of the combination of different elements, which comprise the presence of *L. monocytogenes* at low level, physicochemical characteristics of the product supporting listeria growth (i.e. a_w, pH, etc.) and inadequate storage conditions (Cornu et al., 2006; Jørgensen and Huss, 1998). Related to the latter factor, it is reported that inadequate consumer knowledge on the storage temperature of RTE food at home can enable *Listeria* growth until infective doses (Gambarin et al., 2012; Markinder et al., 2004). Moreover, the epidemiological patterns of human listeriosis suggest that cross-contamination in homes and in food-service establishments could be the major contributing factor to sporadic cases and occasional outbreaks (Beumer and Kusumaningrum, 2003; Chen et al., 2001; Pérez-Rodríguez et al., 2008).

Compared to listeriosis outbreaks associated with other foods, a low number of cases have been linked to RTE fish products (Table 1.1). Different reasons can be put forward to explained the low occurrence of listeriosis outbreaks transmitted by fish products: i) the low number of *L. monocytogenes* generally present in raw fish; ii) deficient epidemiological tracking due to the long incubation period of listeriosis, which makes it difficult to determine the food source; iii) the relatively low volumes of production and reduced distribution chains; iv) increased consumer awareness that RTE fish products should be refrigerated compared to other products; and v) lower consumption of high-risk products, such as RTE fish products, by immunocompromised individuals and susceptible population (i.e. pregnant women are recommended to avoid smoked fish). A thorough study of these factors together with the analysis the *L. monocytogenes* epidemiological patterns linked to RTE fish products and fish processing environments is needed for understanding and managing this microbial risk (Jami et al., 2014; Rocourt et al., 2000; Tompkin, 2002).

1.5. Legislation: Microbiological criteria for *L. monocytogenes* in RTE foods

The potential of *L. monocytogenes* to grow in a particular food during storage and distribution period has been a determining factor in the level of consumer exposure and the basis for the risk categorization by food regulatory agencies and associated microbiological criteria (Farber et al., 2011). In EU, the Regulation (EC) 2073/2005 (amended by Regulation (EC) 1441/2007) on microbiological criteria lays down the food safety criteria for *L. monocytogenes* in RTE foods. The food business operator (FBO) must ensure that food complies with these microbiological criteria and, when necessary,

conduct studies to investigate compliance with the criteria over the shelf-life of these products (EC, 2005).

The food safety criteria differentiate RTE foods in three categories based on their properties to support *L. monocytogenes* growth. These criteria are laid down in EC 2073/2005 as follows:

i) Category 1.1: RTE foods intended for infants and for special medical purposes, which are able to support the growth of *L. monocytogenes*; the criterion is the absence in 25 g in 10 sample units during the declared shelf-life on the market. The EU regulation does not differentiate between RTE foods intended for infants and special medicinal use, as *L. monocytogenes* should not be detected in these products, whether growth is prevented or not;

ii) Category 1.2: RTE food that supports growth, the FBO must demonstrate, to the satisfaction of the Competent Authority (CA), that if contaminated, *L. monocytogenes* must not be present in levels exceeding 100 (CFU/g) during the shelf-life in 5 sample units;

iii) Category 1.3: RTE foods with a pH of ≤ 4.4 or $a_w \leq 0.92$, or with a pH of ≤ 5.0 and $a_w \leq 0.94$ or with a shelf life < 5 days are considered to be unable to support a significant growth of *L. monocytogenes*. Furthermore, other products may also belong to this group subject to scientific justification that no-growth of *L. monocytogenes* is possible throughout the declared shelf-life. Most RTE fish products exhibit physico-chemical characteristics (pH > 5.0 and < 6% WPS and $a_w > 0.96$) which are not compatible with Category 1.3. For this type of products, additional studies should be conducted to provide scientific data demonstrating that the pathogen is not able to grow in the specific fish product, otherwise, the product should be included in Category 1.2.

In contrast to the EU Regulation 2073/2005, some countries like Australia, Japan, United States and New Zealand, the microbiological criterion is tighter and set a policy of regulatory action at the limit of detection (zero tolerance) which means that *L. monocytogenes* should not be detected in RTE foods (i.e. 0 CFU/25 g). Although there is a difference in the criterion, several countries also use different sampling plans and methodologies, which make the issues more complex (Archer, 2018; FAO, 1999; Shimojima et al., 2016). Predictive microbiology (i.e. MicroHibro and FSSP), among other supporting tools (i.e. challenge testing and shelf-life or durability studies) has been

used following the regulations of these countries as a scientific justification to demonstrate that *L. monocytogenes* does not grow in a specific RTE food (EC, 2005).

Microbiological risk assessment studies estimated that most cases of listeriosis are linked to consumption of foods with higher level of *L. monocytogenes*, that would exceed the zero tolerance limit (0 CFU/25 g) and even the EU legal limit of 100 CFU/g. The risk assessment also suggests that effective control measures should be put in place to reduce the frequency of contamination and the occurrence of high levels of contamination at consumption, thus preventing the listeriosis cases. For RTE foods supporting *L. monocytogenes* growth (categories 1.1 and 1.2), better temperature control or limiting prolonged storage would reduce risk, while in RTE foods not supporting *Listeria* growth (category 1.3), reducing the occurrences at manufacture/retail would improve public health (Buchanan et al., 2017; FAO/WHO, 2004).

In the last EFSA report, the food category of "fish and fishery products" showed non-satisfactory which was consistently higher at the processing stage compared to retail. Considering the occurrence data of *L. monocytogenes* originating from all sampling stages (e.g. single units and batches), for the category of "fish and fish products", the occurrence of the pathogen showed the highest levels (6.0%) of non-compliance with the food safety microbiological criteria for *L. monocytogenes* laid down by EC Regulation 2073/2005, when compared with other categories of RTE food (EFSA, 2018).

1.6. Prevalence of *L. monocytogenes* in raw fish and RTE fish products

Contamination of raw fish and RTE fish products with *L. monocytogenes* can occur at all stages of the production consumption chain. Although *L. monocytogenes* is not considered a marine microorganism, their presence in fish can occur by water runoff from contaminated agricultural areas that can increase the abundance of the pathogen in aquatic systems (Gram, 2001; Lyautey et al., 2007). The contamination level of *L. monocytogenes* in raw fresh fish varies between 0% and 30% depending on the type of product and production context (Jami et al., 2014). Once the pathogen enters in the processing plant, there is a high probability of cross contamination for those that are submitted to post-process operations (e.g. slicing and packaging) and are not heat-treated before consumption such as hot-smoked fish and surimi (Gombas et al., 2003; Miettinen and Wirtanen, 2005).The prevalence of *L. monocytogenes* is higher in cold-smoked fish ranging from 34-60%, while for heat-treatment and cured fish the prevalence is lower, varying in the range 4-12% (Jørgensen and Huss, 1998).

The prevalence and the positive number of *L. monocytogenes* in raw fish and RTE fish products has been reported in different studies (Cabedo et al., 2008; Hamidiyan et al., 2018; Kramarenko et al., 2013; Lambertz et al., 2012). Fallah et al. (2013) reported a total prevalence of *L. monocytogenes* of 11.4% (*n* = 105 samples) and 14.5% (*n* = 131 samples) in raw fish and RTE fish products, respectively obtained from markets. In fish processing plants, these authors reported a high prevalence of 29.3% (*n* = 12/41 samples) for fish fillets, which indicates that contamination by *L. monocytogenes* might be associated with the processing operations and 9.75% (*n* = 4/41 samples) for the final RTE fish products. Chen et al. (2010) reported a prevalence of 43.3% (*n* = 13/30 samples) of *L. monocytogenes* in fresh unchilled catfish fillets, while a high prevalence of 76.7% (*n* = 23/30 samples) was observed in fillet after chilling in chiller water (chilled). The high prevalence of *L. monocytogenes* found in this study suggests that an important source of contamination on chilled catfish fillets may originate from processing plants and chiller water. In Spain, Garrido et al. (2009) reported the prevalence of *L. monocytogenes* in RTE smoked fish (e.g. salmon and trout) collected from different supermarkets and retail establishments. The prevalence observed in smoked salmon and trout was of 10.8% (*n* = 11/102 samples) and 25.0% (*n* = 10/40 samples), respectively. These authors attribute the presence and high levels of *L. monocytogenes* in these products to the manufacturing and slicing processes in the smoked fish industry.

In 2010-2011, an EU-wide baseline survey (BLS) found that the prevalence and concentration of *L. monocytogenes* in RTE fish products was 10.3% with a confidence interval (CI): 9.1-11%, in which 1.7% (CI: 1.3-2.3%) could exceed the limit of 100 CFU/g at the end of shelf-life (EFSA, 2013). The extensive literature search performed by Jofré et al. (2016) reported the prevalence of *L. monocytogenes* in RTE fish products was of 13% for cold-smoked fish and 12% for smoked fish and cured/salted fish. In 2017, *L. monocytogenes* isolations were reported in 365 RTE fish (i.e. marinated, gravad/slightly salted, hot and cold-smoked) and 30 unspecified RTE fishery products sample units (i.e. cooked-chilled, RTE-chilled and frozen), obtained from a total of 5,255 and 1,423 analyzed sample units, respectively. The positive units for *L. monocytogenes* were sampled mainly at the retail and processing environments and corresponded to 11% (*n* = 203) and 4% (*n* = 175) of the total samples, respectively while only a few isolations were obtained at catering facilities, which corresponded to 1% samples (*n* = 1). Most of the reported isolations were smoked fish, with 8% (*n* = 363), including hot and cold-smoked fish (EFSA, 2019a).

1.7. Biopreservation

Over the last years, consumers are becoming increasingly aware of the risk to human health caused, not only by foodborne diseases, but also by the excessive use of chemical preservatives in foods, which has led the agri-food sector to look for alternative technologies able to reduce additive formulations while ensuring food safety and quality. In this sense, the chemical preservatives can be replaced by the application of more natural alternatives, such as those based on biopreservation (Silva et al., 2018; Skariyachan and Govindarajan, 2019).

Biopreservation is a method of preserving food using microorganisms, called bio-protective cultures, and/or their metabolites (Leroi et al., 2015). This method is able to meet the necessary safety standards using traditional methods of preservation and modern demand of food safety and quality (Singh, 2018). Biopreservation can be described as an "ecological control", in which the food microbiota is enhanced by bacteria that are generally recognized as safe (GRAS), with the aim of inhibiting growth of undesirable microorganisms, namely pathogenic and spoilage microorganisms (Khassehkhan and Eberl, 2016). With biopreservation, product shelf-life is improved through the use of microbiota naturally present in food or by intentionally inoculating non-pathogenic antagonistic microorganisms and/or their metabolic products (Paul Ross et al., 2002; Stiles, 1996).

In fresh fish and RTE fish products, biopreservation is an alternative to comply with safety standards, hygienic quality and to control microbial spoilage, minimizing the negative impact on the sensory and nutritional quality of the product (Ghanbari et al., 2013). Lactic acid bacteria (LAB) is the bacterial group, mostly used in biopreservation. LAB are safe for consumption and, during storage, they naturally dominate the microbiota of different products, for instance, vacuum-packed fish (Castellano et al., 2008; Emborg et al., 2002; Gancel et al., 1997). Although LAB has been demonstrated to be efficient biopreservation tools, they should be combined with other measures such as good manufacturing, processing, storage and distribution practices in order to ensure food safety standards and hygienic quality (Holzapfel et al., 1995).

1.7.1. Lactic acid bacteria (LAB) as bio-protective cultures

Lactic acid bacteria (LAB) encompass a heterogeneous group of bacteria, in which many species are used as microbial food cultures (MFC) in food production (Bourdichon et al., 2012; Herody et al., 2010). In the EU, there is no specific regulation regarding MFC; but with a long history of safe use, they are considered food additives, which means that they are legally permitted without premarket approval (Costa et al., 2019). The LAB group comprises Gram-positive, non-sporulating, anaerobic or facultative aerobic bacilli and cocci. This group is composed of genera *Lactobacillus*, *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Pediococcus* and *Streptococcus*, which having as a common metabolic property the production of lactic acid as the majority end-product from the fermentation of carbohydrates (Khalid, 2011; Mayo et al., 2010).

LAB play a significant role in the food industry in terms of fermentation and biopreservation processes, acting as starter and bio-protective cultures, respectively (Ramírez-Chavarín et al., 2010). Their importance is associated mainly with their safe metabolic activity, while growing in the foods, they use available sugar to produce organic acids and other metabolites. The biopreservation depends mainly on the level of activity of the biological systems of LAB, which can contribute to enhance microbial food safety or offer one or more sensorial, technological, nutritional or health benefits (Bintsis, 2018; Leroy and De Vuyst, 2004; Singh, 2018). LAB as bio-protective cultures has been generally regarded as safe, and recognized as non-hazardous to human health, which contributes to their natural acceptance as GRAS by the FDA (2018) and have the criteria of QPS (Qualified Presumption of Safety) established by EFSA (EFSA, 2018). In addition, the EFSA's "Panel on Biological Hazards (BIOHAZ)" has concluded that the fermenting and bio-protective cultures associated with food, regardless of resistance to antibiotics, except for enterococci, do not pose a clinical problem (EFSA, 2008).

In terms of food safety, technological effectiveness and economics, the desirable properties of bio-protective cultures include: i) non-pathogenic and legally approved as safe (GRAS and QPS); ii) known metabolic products under a given set of parameters (e.g. organic acids or bacteriocins production/no gas); iii) ability to survive and remain active during the manufacture and distribution of the product; iv) being easily available and feasible from the economic point view; v) possibility of being preserved by freezing or lyophilisation with few practical loss of activity; vi) ability to grow on food during storage

at refrigerator temperature; vii) not affecting the sensory characteristics of the product (flavor and texture) and other intrinsic characteristics; and viii) not being applied for medicinal purposes to prevent or treat diseases in humans and animals (Ben Said et al., 2019; Buckenhüskes, 1993).

Although LAB are not considered as naturally microbiota of the aquatic environment, certain genera, including *Lactobacillus*, *Carnobacterium*, *Lactococcus* and *Enterococcus*, have been isolated from fresh and sea water fresh fish (Ghanbari et al., 2009; González et al., 2000; Ringø, 2004; Ringø et al., 2018) and has also been isolated from LPFP (e.g. cold-smoked, gravad and marinated fish) and SPFP (e.g. salted and/or marinated fish or caviar and fermented fish)(Gelman et al., 2000; Ida Muryany et al., 2017; Mejlholm and Dalgaard, 2007; Tomé et al., 2006). In fact, the use of bio-protective LAB isolated from fish products entails advantages due to its reported ability to grow even at refrigerated temperature and ease of adaptation to the RTE fish products environments (i.e. vacuum-packed, modified atmosphere packaging, smoked, high salt concentration, low pH and presence of additives like lactic acid or acetic acid) (Ghanbari et al., 2013). In RTE fish products, LAB was considered the dominant group of bacteria.

The application of selected LAB strains in fresh fish and RTE fish products as bio-protective cultures has demonstrated a high potential to control undesirable spoilage and pathogenic microorganisms, including *L. monocytogenes* (Anacarso et al., 2014; Aras Hisar et al., 2005; Axelsson et al., 2020; Aymerich et al., 2019; Brillet et al., 2005; Duffes et al., 1999a, 1999b; Richard et al., 2004; Tahiri et al., 2009). Therefore, LAB are generally recognized as efficient bio-protective cultures to improve food quality and enhance shelf-life of fresh fish and RTE fish products (Calo-Mata et al., 2008; Katikou et al., 2007).

1.7.2. Antimicrobial mechanisms of LAB

Despite the paramount importance of knowing how LAB can interact with pathogens and spoilage microorganisms, the mechanism still it is not well understood. Probably, the antibacterial activity of LAB is due to a combination of several factors that could act synergistically (Gao et al., 2019). In the case of LAB and *L. monocytogenes*, it is put forward that bactericidal or bacteriostatic activity could be caused by i) displacement/exclusion, which is summed in the ability of planktonic cells of some strains of LAB to adhere strongly to surfaces and survive on them for long periods of time impeding or reducing biofilm formation by *L. monocytogenes* (Ben Said et al., 2019;

Woo and Ahn, 2013) ii) competition for nutrients and space (Nilsson et al., 2005; Vermeiren et al., 2006); and iii) production of one or more antimicrobial metabolites such as bacteriocins (Tomé et al., 2006; Weiss and Hammes, 2006), organic acids (Amézquita and Brashears, 2002), hydrogen peroxide (Ito et al., 2003) and reuterin (Montiel et al., 2014).

1.8. Microbial interaction

Food matrices are considered complex microbial ecosystems, where various sets of heterogeneous microbial population can coexist and interact with each other and with their environment. Microorganisms in these communities often engage in complex interactions of multicellular and intercellular behaviours that can affect the presence and persistence of pathogens in foods (Orihuel et al., 2018). These interactions occur through environmental recognition followed by transfer of molecular and genetic information that includes many mechanisms, classes of molecules and categories of interaction, which can result in different types of interaction, positive, negative or neutral (Braga et al., 2016). The mutual effects of these outcomes allow to classify the interaction between two microbial populations in five different categories as shown in Table 1.2 (Faust and Raes, 2012; Sieuwerts et al., 2008). However, determining the exact nature of these interactions can be challenging due to different factors such as the types of microbial species present in the food, manifold of interactions they can engage in, the factors inherent in food and its preservation conditions (Zuñiga et al., 2017).

In foods, microbial interactions, can either be non-specific (direct) or specific (indirect), may have a great influence on the fate of pathogenic species that contaminate foods (Haruta et al., 2009; Zilelidou and Skandamis, 2018). Non-specific interaction requires physical contact or quorum-sensing in order to induce microbial interactions. This type of interaction is also caused by competition for space (i.e. niches) and nutrient limitation due to growth of the numerically dominant species (Mellefont et al., 2008). On the other hand, the specific interactions are when physical contact is not required, that is, derived from changes in the growth environment with the production of antimicrobial metabolites, as it is the case of bacteriocins and production of other metabolites, such as organic acids with a concurrent decrease in pH (Fredrickson, 1977; Martens et al., 1999). However, for some authors, competition for nutrients is also considered a specific interaction, and that most of the studies on microbial interactions were qualitative in nature (Alves et al., 2005; Malakar et al., 1999).

Studying microbial interaction in foods products is extremely difficult because this usually requires to know a priori the potential interaction factors between microbial population. Traditionally, microbiological and analytical approaches are used to monitor, from a macroscopic point of view, for instance, changes in populations size over time and concentrations of growth-limiting nutrients. However, sometimes this monitoring is not sufficient to capture the interaction phenomenon, due to the interactions do not only occur between microbial species, but also between microorganisms and food environments (Leroy and De Vuyst, 2007).

Nowadays, omics technologies have opened a new era enabling to obtain huge amounts of multidimensional information of bacterial populations in actual environments (transcriptomic, genomic, proteomic, etc.) (O´Donnell et al., 2020; Papadimitriou et al., 2016; Zhang et al., 2010). Omics are, therefore, a very powerful tool to elucidate the mechanism of interaction in food ecosystems, aiding to identify dominant population(s) and determine those traits and processes conferring dominance to these populations (den Besten et al., 2018). Several studies have already applied omics technologies to determine the interaction of LAB in foods (Lahtvee et al., 2011; McLeod et al., 2010; Nyquist et al., 2011). Benson et al. (2014) used metagenetics to understand the ecology of complex microbial communities in real food matrices. An interesting study evaluated the interaction between *Lactobacillus plantarum* and *L. monocytogenes* in "Alheira de Vitela" (traditional fermented smoked meat sausage from North of Portugal) during storage at 4 °C using 16S rRNA metagenomics analysis. The main result of this study demonstrated the anti-listerial activity of *L. plantarum* until the end of the storage of the product, in which counts of *L. monocytogenes* decreased, while counts of LAB remained stable (Macieira et al., 2019).

Table 1.2. The five categories of microbial specific interaction and the mutual effect, positive (+), negative (-) and neutral (0) on the interaction of two populations.

Interaction categories[a]	Effect on population A	Effect on population B	Example	Reference
Amensalism	0	-	Bacteriocin produced by population A.	Aymerich et al. (2019)
Competition	-	-	Competition for nutrients.	Nilsson et al.(2005)
Commensalism	+	0	Production of metabolites by population B which can be used by population A, while the population B is not affected.	Jørgensen et al. (2000)
Mutualism or synergism	+	+	Exchange of growth factors.	Sieuwerts et al. (2010)
Parasitism	+	-	Bacteriophages (population A) attach bacteria (population B).	Sturino and Klaenhammer, (2004)

[a] For each interaction, there are three possible outcomes: positive **(+)**, negative **(-)** and neutral **(0)**. For instance, in competition, the population A is affected **(-)**, whereas the population B is also affect **(-)**, that is, both populations are negatively affected by competition for the resource (nutrients, space and etc.); then, this interaction is thus represented by the symbol pair **(- -)**.

1.8.1. Amensalism

Amensalism is an interaction between microorganisms of two different populations, in which one negatively affects the growth of other population without being affected itself (Sieuwerts et al., 2008). This can occur though the production of antimicrobial metabolites as part of their metabolism. For instance, the production of organic acids by LAB, from glucose consumption, decreases pH of the environment. Both lower pH and increasing organic acid concentrations affect generally pathogens and spoilage microorganisms, while LAB itself is much more tolerant to these environmental changes (Khassehkhan and Eberl, 2016). Another example of antimicrobial metabolites are bacteriocins produced by LAB, which play an important role in food biopreservation.

1.8.1.1. Antimicrobial metabolites: organic acids and bacteriocins

LAB species are able to reduce and prevent growth of *L. monocytogenes* in different products, including RTE fish products, due to the production of antimicrobial metabolites, as previously mentioned. The fermentation of carbohydrates (e.g. glucose) by LAB

species belonging to homo-and heterofermentative *lactobacilli,* through the glycolysis and phosphoketolase pathways, results in the production of different organic acids where lactic acid is the major end product, followed by acetic acid and other acids such as formic, succinic, citric, propionic and butyric (Lauret et al., 1996; McLeod et al., 2010; Özcelik et al., 2016). *L. sakei*, as a bacteria well adapted to fermented meat and fish processing environments, exhibits alternative pathways for the production of organic acids (McLeod et al., 2011). The amounts and types of organic acids produced depend on LAB species and strains, culture composition and growth conditions (Lindgren and Dobrogosz, 1990). The antimicrobial activities of organic acids are primarily associated with the undissociated forms of the acids that permeates inside microbial cells, producing cytoplasm acidification causing a decrease in intracellular pH, which has significant impacts on cell metabolism, resulting in reduced growth (Kashket, 1987; Stasiewicz et al., 2011). For this reason, the inhibitory activities of organic acids are affected by pH values of foods and their effects increase with decreasing pH (Yost, 2014). The ratio between the dissociated and the undissociated form can be calculated from the pK_a-value of organic acid and the pH value of the specific foods by the Henderson–Hasselbalch equation (Ross and Dalgaard, 2004). This equation was initially developed for aqueous solutions and later modified by Mejlholm and Dalgaard (2009) for lightly preserved seafood products.

The pH of fresh Mediterranean fish (e.g. Gilthead sea bream and European sea bass) and LPFP (e.g. cold-smoked salmon) during storage is relatively stable around 6.0-6.4 and is slightly affected by the presence of LAB (Abbas et al., 2008; Tomé et al., 2006). This is due to the low amount of carbohydrates in the fresh fish and LPFP, which is often less than 1%, and the buffering capacity of fish matrices, result from their high protein content (Paludan-Müller et al., 2002). Tomé et al. (2006) isolated two strains of *L. plantarum* from vacuum packed cold-smoked salmon and suggested that their inhibitory activity against *L. monocytogenes* was due to the production of organic acids. However, these authors did not measure the amount of produced organic acids.

Another important metabolite produced by LAB, which has been gained increased attention in food biopreservation, are bacteriocins. Bacteriocins are ribosomal-synthesized, extracellularly released low-molecular-mass peptides or proteins (usually 30–60 amino acids), heat stable with bactericidal or bacteriostatic activity that inhibits a spectrum of bacteria, including *L. monocytogenes* (Cotter et al., 2005; Zacharof and

Lovitt, 2012). Bacteriocin-producing LAB must protect themselves from the toxic effect of their own bacteriocins. For that, LAB express cognitive immunity genes that make them immune to their own bacteriocin and possibly also to other bacteriocins (Deegan et al., 2006). One LAB strain may produce one or several different bacteriocins. For instance, *L. sakei* subsp. *sakei* 2A isolated from a Brazilian meat product was able to produce three different bacteriocins (de Carvalho et al., 2010).

Different bacteriocins produced by LAB have been isolated from fish (carnocin), fish viscera (divercin and carnobacteriocin), RTE fish products (divergicin, piscicocin and sakacin), fermented fish product (weissellicin) and seafood (enterocin and pediocin). Some of these bacteriocins have been purified, characterized and tested against *L. monocytogenes* and other microorganisms with potential application to enhance the safety and quality of RTE fish products (Bhugaloo-Vial et al., 1996; Leisner et al., 2007; Métivier et al., 1998; Pinto et al., 2009; Srionnual et al., 2007; Stoffels et al., 1992; Tahiri et al., 2009; Todorov et al., 2011; Yamazaki et al., 2003). Bacteriocinogenic LAB and their bacteriocins, such as pediocin, nisin and sakacin isolated from meat and fermented meat products, have been used to inhibit the growth of *L. monocytogenes* and extend the shelf-life of fish and RTE fish products (Aymerich et al., 2019; Behnam et al., 2015; Papagianni and Anastasiadou, 2009).

LAB bacteriocins constitute a large and heterogeneous group. They are classified according to features such as size, homology, structure, post-translational, target microorganisms and mode of action (Nes et al., 2016). Alvarez-Sieiro et al. (2016) proposed a classification scheme based on the biosynthesis mechanism and biological activity. These authors propose three main classes, although other researches include a fourth class for bacteriocins (Cotter et al., 2005; Klaenhammer, 1993). The division of bacteriocins classes is as follows:

i) Class I: includes post-translationally modified bacteriocins, known as lantibiotics, which constitute the antimicrobials of the lanthipeptides groups (lanthionine and methyllathionine). Lantibiotics are thermostable peptides of very low molecular weight (< 5 kDa) that can either bind to Lipid II (a precursor peptidoglycan in the synthesis of the cell wall of bacteria) and prevent proper cell wall synthesis or can make pores in the membrane by using Lipid II as a docking molecule (Cotter et al., 2005). Nisin, lactocin and mersacidin are the main bacteriocins representing this group; and *Lactobacillus lactis* subsp. *lactis* is the typical producer bacteria (Parada et al., 2007; Zacharof and Lovitt, 2012).

ii) Class II: corresponds to bacteriocins that do not contain unusual modifications. This class involve a large and heterogeneous group of small thermostable (< 10 kDa) and non-lantibiotic peptides. Based on structural and function characteristics, the bacteriocins are divided into three subclass: IIa (anti-listerial peptides, such as pediocin, sakacin and enterocin), IIb (bacteriocins that require synergy of two peptides for activity where Lactacin F and lactococcin G are examples of this group) and IIc (cyclic bacteriocins that require reduced cysteine residues for activity, e.g. reuterin 6). This bacteriocins' class affect the membrane permeability and cell wall formation of target bacteria. The class IIa bacteriocins produced by LAB are most studied due to their potent anti-listerial activity and antimicrobial properties against spoilage microorganisms and other foodborne pathogens. Among the typical producing bacteria in this group are *Enterococcus faecium, L. sakei* and *Lactobacillus acidophilus* (Ringø et al., 2018; Sahoo et al., 2016). Piscicocin and divergicin, produced by *Carnobacterium piscicola* and *Carnobacterium divergens*, respectively, isolated from RTE fish and seafood products were classified as class IIa bacteriocins showing a noteworthy antimicrobial activity against *L. monocytogenes* (Tahiri et al., 2004; Yamazaki et al., 2005).

iii) Class III: this group consists of unmodified bacteriocins of large molecular weight (< 30 kDa), heat labile with a bacteriolytic and non-lytic mechanisms of action, capable of directly break down the cell wall of Gram positive bacteria (Alvarez-Sieiro et al., 2016; Cotter et al., 2005). This group has not been extensively investigated and has limited prospects for use in food biopreservation. Bacteriocins included in this group are helveticin I and enterolysin produced by *Lactobacillus helveticus* and *E. faecium*, respectively (Zacharof and Lovitt, 2012).

iv) Class IV: comprises a large complex group of bacteriocins, which require carbohydrates or lipids in their structure to show antimicrobial activity. They are circular and heat stable peptides. Gassericin A produced by *Lactobacillus gasseri* is an example of class IV bacteriocin and has been considered an important bacteriocin in the food biopreservation, owing to its activity at high pH and temperature tolerance (Fraqueza et al., 2017; Pandey et al., 2013).

Bacteriocins have many mechanisms of action that differ from those of antibiotics. The bacteriocin mechanisms can be divided into those that act mainly on the bacterial envelope and those exert their action inside cells, affecting gene expression and protein production. Furthermore, the bacteriocins produced by LAB produces their antimicrobial

action by targeting cell envelope and its synthesis, which is higher at the end of the exponential growth phase (Cotter et al., 2013; Yamazaki et al., 2003). A decrease in the bacteriocin concentration may happen during the stationary phase which cause an increase in the number of pathogen (e.g. *L. monocytogenes*). Environmental conditions are a major determinant of the antimicrobial activity of bacteriocins since the amount of produced bacteriocins is affected mainly by the temperature, pH, specific nutrients for each strain or species and NaCl (Himelbloom et al., 2001).

The application of bacteriocins has been shown to be less effective in food compared to laboratory culture media. This could be due to organic compounds present in food (e.g. fat, NaCl and microbial enzymes)(Leroy and De Vuyst, 1999). In cold-smoked salmon, the strain of *C. piscicola* was able to suppress growth of *L. monocytogenes*, while in cold-smoked salmon juice the pathogen did not only stop growing but also decreased (Nilsson et al., 1999). Thus, the application of bacteriocins in food should be tested to confirm its effectiveness in each type of food matrix (Silva et al., 2018). The application of bacteriocins can be introduced in the food biopreservation in three different ways: i) inoculation of food with the bacteriocin-producing strains; ii) addition of semi or totally purified bacteriocins as food additive (if authorized); and iii) use of a product previously fermented with a bacteriocin-producing strains as an ingredient of the target food (Schillinger et al., 1996). These three methods have been evaluated by Tahiri et al. (2009). The antimicrobial action of bacteriocins can be improved when combining with other preservation methods (e.g. MAP, vacuum packed, smoke, organic acids, high hydrostatic pressure and, etc.). This combined effect, known as hurdle technology, has been widely studied to improve food safety and quality, including fish products (Blázquez et al., 2018; Leistner and Gorris, 1995; Tsironi et al., 2020). Recently, Bolívar et al. (2020) evaluated the synergistic effect of sakacin-producing *L. sakei* CTC494 and smoke in the reduction of *L. monocytogenes* in vacuum-packed hot-smoked Gilthead sea bream fillets. The optimum combination of hurdles will depend greatly on the type of food and its initial microbial concentration. This must be carefully assessed, since different hurdles often have different effects on members of a microbial population (Gálvez et al., 2007).

The production of one or more antimicrobials active metabolites is part of the complex mechanism by which LAB strains becomes dominant in the presence of other competing bacteria. These antimicrobial metabolites, such as bacteriocins, are often regulated by a quorum-sensing mechanisms (Hibbing et al., 2010). Table 1.3 includes, among others, a

summary of studies applying bacteriocin-producing LAB against *L. monocytogenes* in laboratory culture media, fish and RTE fish products.

1.8.2. Competition

Competition is the second category of antagonistic interaction, which also represents a negative interaction between two microbial populations, since both population are affected with respect to their survival and growth (Sieuwerts et al., 2008). In this category, competition occurs when both populations use the same resources (e.g. nutrients and niches) for the growth, and some of the populations will be compromised, in terms of reduction the maximum population density and or/growth rate (Barton and Northup, 2011; Mellefont et al., 2008). Also, microbial populations may release, into the environment, metabolites that are toxic or inhibitory to their competitors (Fredrickson and Stephanopoulos, 1981). Ultimately, one of the populations will be excluded from the environment and this effect is an example of competitive exclusion.

1.8.2.1. Competition for nutrients

Nutritional resources play a significant role in understanding microbial interaction by competition. Microbial population present in a given common environment compete for nutrients, space and other resources. Despite foods are unlimited sources of nutrients, microbial growth in them may be halted by limited amounts of specific molecules including different types of carbohydrates, vitamins, amino acids and mineral (Gram et al., 2002; Hibbing et al., 2010). In this type of interaction, competition between two microbial populations, when grown together, tends to eliminate one of the populations, from their common environment. For instance, LAB or background microbiota and pathogens bacteria (e.g. *L. monocytogenes*).

In Table 1.3, the studies of Buchanan and Bagi (1997) and Nilsson et al. (1999) suggested that the inhibition of *L. monocytogenes* in brain heart infusion (BHI broth) and cold-smoked salmon juice by *Carnobacterium piscicola*, respectively, was due to competition for nutrients and not for production of anti-listerial compounds. These studies concluded that the anti-listerial capacity of *C. piscicola* was not caused by any known antimicrobial compounds, which were below the limit of detection. In this sense, authors hypothesized that given that *C. piscicola* is a fast-growing bacteria, its growth could produce a depletion of some necessary nutrients for the growth of the pathogen. Little been investigated on which nutrients are responsible for the inhibitory effect. However,

Nilsson et al. (2005) found that exhaustion glucose was a limiting nutrient for competition, inhibiting the growth of *L. monocytogenes* by non-bacteriocinogenic LAB in BHI broth.

Competition of LAB and *L. monocytogenes* has been also reported, where the growth ability and initially numerical dominance of LAB populations are the main factors in the reduction of the pathogen growth by nutrient competition. In this respect, different studies have reported a significant effect of inoculum size on the inhibition of *L. monocytogenes* in RTE fish products and liquid laboratory media (Mellefont et al., 2008; Nilsson et al., 2004; Quinto et al., 2016; Saraoui et al., 2018; Szigeti, 2001). From an application standpoint, selecting LAB species with better growth ability, at low temperatures, and inoculating them at higher concentration can result in a better competition capacity improving the inhibitory activity on the pathogen (Mellefont et al., 2008).

In 1962, Jameson demonstrated for the first time the phenomenon of co-culture inhibition of two intestinal organisms, *Salmonella* Litchfield and *E. coli*, grown in laboratory culture media (Jameson, 1962). In brief, the study showed that *S.* Litchfield and *E. coli*, inoculated simultaneously, grew without affecting each other´s latency time and growth rate until *S.* Litchfield reached its stationary phase, decreasing the maximum population density (N_{max}) of *E. coli*. This led to a simultaneous stop in the growth of both populations. In another study, Buchanan and Bagi (1999) demonstrated that *L. monocytogenes* grown in co-culture with *Pseudomonas fluorescens* can reach a low, high, or equal N_{max} compared to the levels of the pathogen in mono-culture. In this study, growth was dependent on the temperature, acidity and a_w of the environment in which both bacteria were grown. In fact, these studies provide a phenomenological description of the behaviour of one bacterial population, in the presence of other population (i.e. competitors), in which, due to microbial interaction, a lower N_{max} is observed for one of the populations or both, without providing an explanation for why such lower values are found (Giuffrida et al., 2009). Afterwards, this phenomenon of microbial interaction was introduced to the field of predictive microbiology by Ross et al. (2000) termed the "Jameson-effect". To quote the fine comparison of Mellefont et al. (2008), Jameson effect "can be described as a race between species to use the resources of the environment (e.g. nutrients) to maximise their growth and population numbers. When these resources are depleted, the race is over, and the species that reached N_{max} first deplete the available growth nutrients required by other species, inhibiting their N_{max}, then the growth of each species in the population stops."

This phenomenon has also been reported in several studies of microbial growth in co-culture, such as LAB and *L. monocytogenes* or natural microbiota and other pathogenic bacteria (Al-Zeyara et al., 2011; Beaufort et al., 2007; Cauchie et al., 2017; Mejlholm and Dalgaard, 2015; Mellefont et al., 2008; Møller et al., 2013; Quinto et al., 2016; Speranza et al., 2010).

RTE fish products (e.g. LPFP) generally contain considerable concentrations of LAB that may be present as a natural microbiota due to their ability to adapt to the conditions prevailing in those products (e.g. VP and MAP packaging) or added intentionally as bio-protective cultures. In this sense, the "Jameson-effect" is a phenomenon that can be exploited for biopreservation based on certain LAB groups naturally occurring in fish products able to compete and minimize the pathogen growth (Giménez and Dalgaard, 2004; Ross et al., 2000).

Table 1.3. Studies reporting the mechanisms of interaction of Lactic Acid Bacteria (LAB) species and *Listeria monocytogenes* in laboratory culture media, fish and RTE fish products.

Competing microorganisms	Growth environment	Effect on *L. monocytogenes*	Suggested mechanism	Reference
Lactobacillus plantarum	TSB-agarose solid surface and laboratory culture media	Dissipation of the proton gradient across the cell membrane	Bacteriocin and organic acids production[a,b]	Nielsen et al. (2010)
Carnobacterium divergens and *Carnobacterium piscicola*	Vacuum-packed cold-smoked salmon	Growth inhibition	Bacteriocin production[c, d]	Duffes et al. (1999a)
Carnobacterium piscicola	Laboratory culture media	Growth inhibition	Competition for nutrients and organic acid production[e,f]	Nilsson et al. (2005)
Lactobacillus spp.	Cold-smoke fish system	Growth inhibition	Acid production[b]	Duffes et al. (1999b)
Leuconostoc spp. *and Lactobacillus plantarum*	Model fish product	Reduction of maximum population density	Hydrogen peroxide and proteinaceous substance	Jeppesen and Huss, (1993)
Carnobacterium spp.	Cold-smoked salmon	Growth inhibition	Bacteriocin production[c]	Brillet et al. (2004)
Lactobacillus spp.	Vacuum-packed cold-smoked salmon	Growth inhibition	Organic acids production or competition for nutrients[b,g]	Tomé et al. (2006)
Carnobacterium maltaromaticum, Leuconostoc gelidum and *Lactococcus piscium*	Salmon Gravlax	Growth inhibition	Cell-to-cell and bacteriocin production[h]	Wiernasz et al. (2020)

Table 1.3 continued

Competing microorganisms	Growth environment	Effect on *L. monocytogenes*	Suggested mechanism	Reference
Lactobacillus sakei	Cold-smoked salmon	Growth inhibition	Bacteriocin production[i]	Katla et al. (2001)
Lactobacillus sakei, Lactobacillus curvatus and *Carnobacterium maltaromaticum*	Vacuum-packed cold-smoked salmon	Growth inhibition	Bacteriocin production[j]	Aymerich et al. (2019)
Carnobacterium piscicola	Model fish systems	Growth inhibition and reduction of maximum population density	Bacteriocin production and competition for nutrients[g,h]	Alves et al. (2005)
Carnobacterium maltaromaticum	Alginate film on smoked salmon	Growth inhibition and reduction of maximum population density	Bacteriocin production[h]	Concha-Meyer et al. (2011)
Carnobacterium piscicola	Cold-smoked salmon juice and cold-smoked salmon	Growth restriction and influence of growth kinetic parameters	Bacteriocin production, potential ecological niches[h]	Nilsson et al. (1999)
Carnobacterium piscicola	Smoked salmon juice	Growth inhibition, reduction of maximum population density	Bacteriocin production and competition for nutrients[k,g]	Nilsson et al. (2004)
Lactobacillus platarum	Laboratory culture media	Growth inhibition	Competition for nutrients, pH reduction, organic acids and inoculum size[b,g,l]	Mellefont et al. (2008)

Table 1.3 continued

Competing microorganisms	Growth environment	Effect on *L. monocytogenes*	Suggested mechanism	Reference
Lactococcus piscium	Chemically defined medium based on shrimp composition	Reduction of maximum population density	Mechanism unknown	Saraoui et al. (2017)
Enterococcus durans, *Lactococcus lactis* subsp. *lactis* and *Lactobacillus plantarum*	Laboratory culture media	Inhibition of planktonic growth	Production of inhibitory metabolites[m]	Zhao et al. (2004)
Carnobacterium piscicola	Laboratory culture media and cold-smoked salmon	Population decline and growth inhibition	Bacteriocin production and inoculum size[n,o]	Yamazaki et al. (2003)
Enterococcus faecium	Laboratory culture media	Population decline and growth inhibition	Bacteriocin production, competition for nutrients, cell-to-cell contact[p,g]	Huang et al. (2016)
Carnobacterium divergens	Laboratory culture media	Growth inhibition	Bacteriocin production[c]	Richard et al. (2003)
Lactococcus piscium	Peeled tropical shrimp	Reduction of maximum population density	Unidentified mechanism of action	Matamoros et al.(2009a)
Carnobacterium piscicola	Laboratory culture media	Reduction of maximum population density	Competition for nutrients[g]	Buchanan and Bagi, (1997)
Lactobacillus fuchuensis and Lactococcus piscium	Laboratory culture media	Growth inhibition	Competition for nutrients[g]	Matamoros et al.(2009b)

Table 1.3 continued

Competing microorganisms	Growth environment	Effect on *L. monocytogenes*	Suggested mechanism	Reference
Lactococcus piscium	Chemically defined medium based on shrimp composition	Growth inhibition	Cell-to-cell contact and competition for nutrients[q]	Saraoui et al. (2016)
Lactobacillus lactis	Semi-synthetic media	Growth inhibition and Reduction of maximum population density	Bacteriocin production and inoculum size[r,s]	Szigeti, (2001)
Lactobacillus sakei	Cold-smoked salmon juice	Reduction growth	Bacteriocin production[h]	Weiss and Hammes, (2006)
Lactococcus piscium	Tropical cooked peeled shrimp	Growth inhibition	Competition for nutrients[g]	Fall et al. (2010)
Lactobacullus plantarum	Laboratory culture media	Growth inhibition	Acid production[t]	Wilson et al. (2005)
Lactic acid bacteria	Lightly preserved seafood	Reduction of maximum population density	Unidentified mechanism of action[u]	Mejlholm and Dalgaard, (2007)
Lactobacillus sakei	Laboratory culture media	Growth inhibition and inactivation	Bactercion production[v]	Pleasants et al. (2001)
Psychrotolerant lactic acid bacteria	Processed seafood and mayonnaise-based seafood salads	Reduction of maximum population density	Unidentified mechanism of action[u]	Mejlholm and Dalgaard, (2015)
Lactic acid bacteria	Italian marinated seafood salad	Growth inhibition	Competition for nutrients[g]	Andrighetto et al., (2009)

Table 1.3 continued

Competing microorganisms	Growth environment	Effect on *L. monocytogenes*	Suggested mechanism	Reference
Lactobacillus sakei	Laboratory culture media	Reduction of maximum population density	Competition for nutrients[g]	Blanco-Lizarazo et al. (2016)
Weissela viridecens	Laboratory culture media	Growth inhibition	Hydrogen peroxide, proteinaceous substance and inoculum size[x]	Ye et al., (2018)

[a] Plantaricin production.
[b] Unspecified organic acids.
[c] Divercin production.
[d] Piscicocin production.
[e] Glucose competition.
[f] Acetate production.
[g] Unspecified nutrient competition.
[h] Unspecified bacteriocin production.
[i] Sakacin P production.
[j] In this study, only sakacin K, produced by *L. sakei* CTC494 was reported.
[k] Carnobacteriocin B2 production.
[l] The inoculum size of the *L. plantarum* used to evaluate growth in co-culture with *L. monocytogenes* was 10^3, 10^4 and 10^6 CFU/mL.
[m] Production of inhibitory metabolites reported only for *L. lactis* subsp. *lactis*. The isolate had nisin A and B genes. For *E. durans* the authors suggested enterocins as an inhibitory metabolite.
[n] The inoculum size of *C. piscicola* used to inhibit the growth of L. monocytogenes was 10^4 and 10^6 CFU/g.
[o] Bacteriocin characterized as Pisciconcin (Yamazaki et al., 2005).
[p] Presence of the enterocin A, B and P genes (*ent*A, *ent*B and *ent*P) in the *E. faecium* strain.
[q] Adenina, guanine and uracil competition.
[r] Nisin production.
[s] The inoculum size of *L. lactis* used to inhibit the growth of *L. monocytogenes* was 10^5 and 10^6 CFU/mL.
[t] Lactic acid production.
[u] Mechanism suggested as Jameson effect.
[v] Sakacin A production.
[x] The inoculum size of the *W. viridecens* used to evaluate growth in co-culture with *L. monocytogenes* was 10^3 and 10^6 CFU/mL..

1.8.3. Mutualism

Mutualism, also so-called symbiosis, is the category in which both participating populations are totally dependent on one another, which can be developed in different ways (Table 1.2) (Ivey et al., 2013). One of these ways is the production and release in the food matrix of a metabolite or set of metabolites, called coupling substance(s), produced by each population and necessary for another, but cannot produced by itself. The interaction does not have to be specific; all that is needed is that metabolism of both populations are complementary (Fredrickson, 1977).

Interestingly, Yang et al. (2017) observed a mutual growth between *Bifidobacterium bifidum* and *L. monocytogenes* in laboratory culture media (PBS, MRS or BHI) at 37 °C for 24 h. These authors found that for these conditions, the results showed that culturing the two bacteria together could promote the growth of each other, resulting in earlier entry into the logarithmic phase. However, to best of our knowledge, there is no specific data on mutualism observed between foodborne pathogens (e.g. *L. monocytogenes*) and other microbial population in foods and specifically fish products.

1.9. Theoretical approach for interaction modelling: Jameson-effect phenomenon and predator-prey approaches

In most studies of microbial interaction in food products, the results indicate that fully considering the complexity of microbial community dynamics would require detailed knowledge of food systems, its microbial composition and inoculum levels, control over the production and release of bacteriocins or other inhibitory compounds by benign microorganisms that compete with pathogens, the factors that affect the interactions and how the food is handled during transportation, storage, distribution, and use (Buchanan and Bagi, 1997; Powell et al., 2004). However, all this knowledge has the cost of an intensive and exhaustive work, which requires a large amount material and makes them weakly eligible for more realistic applications for microbial interactions (Cornu, 2001; Cornu et al., 2011). Moreover, some of this information is not considered in mathematical models and, of the existing classic models for microbial interaction, only include intra-species interaction and not inter-species interaction, due to the extensive data sets for mixed culture growth are not available (Imran et al., 2012; Vereecken et al., 2000).

1.10. Predictive Microbiology

Predictive microbiology, or the quantitative microbial ecology of foods, is an area of research in food microbiology that integrates, in addition to traditional knowledge of microbiology, different disciplines including mathematics, engineering, computer science and statistics (Whiting, 1995). It is a promising field that applies mathematical models to describe the behavior of microbial populations in food environments allowing to predict growth, microbial interaction, inactivation and transfer in response to certain specific environmental conditions. Models usually take into account intrinsic factors to food (e.g. pH, a_w, competing microbiota etc.), extrinsic ones (temperature, atmosphere, moisture, etc.) and/or processing conditions in food matrix or food model systems (UV-radiation, high pressure processing) (Pérez-Rodríguez and Valero, 2013; Valdramidis, 2016). Thus, predictive microbiology has become a powerful tool for microbial food safety quality, shelf-life assessment, HACCP programs and QMRA (Huang, 2014).

In predictive microbiology, models can be classified according to different criteria: i) modelling approach: empirical and mechanistic models; ii) and their purpose: probabilistic and kinetic models. Empirical (phenomenological) models are equations that describe the data in a convenient mathematical relationship through a fitting procedure. Polynomial equations are a type of empirical models, which are generally represented as quadratic response surfaces describing the environment dependence of a kinetic parameter of the microbial population (McMeekin et al., 1993). Mechanistic (deterministic) models are more flexible and built on a theoretical basis allowing the interpretation of the response in terms of known phenomena and processes that govern microbial kinetics. These models are preferable over empirical ones although they are scarce in literature due to the limited information and data concerning the biological processes. For their development, an extensive knowledge of the underlying biological mechanisms is required (Ferrer et al., 2009). However, mechanistic models are preferable to empirical ones, as they usually contain fewer parameters, fit the data better and extrapolated more sensibly (Draper, 1988). Baranyi and Roberts model (Baranyi and Roberts, 1994) is an example of a semi-mechanistic models, which focuses on the lag phase described on the basis of certain parameters defining of physiological state of cells (Ferrer et al., 2009; Jagannath and Tsuchido, 2003).

Probabilistic models, known as growth/no growth models, predict the likelihood of the microbial response (e.g. toxin production, metabolic activity, etc.) under specific food

environmental conditions within a certain period of time. However, they cannot explain the rates (Baker and Genigeorgis, 1990; McMeekin et al., 2002). Kinetic models are used to describe the microbial response (growth or death) over time, in which extrinsic variables affecting growth (e.g. temperature and pH) can be considered dynamic, i.e. time-dependent variables. When the dynamic variable is temperature, the models are called non-isothermal models (Corradini and Peleg, 2005; Van Impe et al., 1992). The use of non-isothermal models to predict microbial population behavior under varying conditions, can be an alternative to understand the changes that occurred during the production chain of chilled food, such as fresh fish and RTE fish products minimally processed (Costa et al., 2019; Koutsoumanis, 2001). Among the various non-isothermal that used to predict microbial growth in varying conditions, there are the Baranyi and Roberts (Baranyi and Roberts, 1994), Gompertz (Van Impe et al., 1992) and Corradini and Peleg (Corradini and Peleg, 2005) non-isothermal models. This type of models constitutes a fundamental model category in predictive microbiology, since they can be used to assess the level exposure of consumers to foodborne pathogenic bacteria at any time along the food chain (Skandamis and Jeanson, 2015).

Whiting and Buchanan (1993) proposed another classification for models in predictive microbiology splitting them into three levels: primary, secondary and tertiary. The primary models describe the change of microbial concentration as a function of time, using a limited number of kinetics parameters (initial concentration, lag time, growth rate and maximum population density) (McKellar and Lu, 2004; Dalgaard and Mejlholm, 2019). The secondary models represent for the effect of environmental conditions (e.g. temperature, pH, NaCl, ect.) on the kinetic parameters of the microbial curve estimated by primary models (Ross and Dalgaard, 2004). Finally, the tertiary models are computer applications integrating primary and secondary models to enable predictions on microbial behaviour (e.g. growth and inactivation) in foods under different environmental conditions using an user-friendly interface (Baranyi and Tamplin, 2004; Whiting and Buchanan, 1994). There are several tertiary models, however, the software Combase (Baranyi and Tamplin, 2004), MicroHibro (González et al., 2019) and FSSP(Dalgaard, 2014) deserve to be mentioned given their relevance and type of application.

The complexity of microbial interactions and the implications of competitive growth in foods are often overlooked in predictive microbiology, which can lead to a significant discrepancy between predictions and reality (Leroy and De Vuyst, 2007; Pérez-Rodríguez

and Valero, 2013). The two most employed modelling approaches for microbial interaction are based on the Jameson-effect and Lotka-Volterra, which are intended to provide a general representation for the interaction dynamics observed in a specific microbial community (Cornu et al., 2011; Powell et al., 2004). The Jameson-effect-based models is a set of mathematical derivations, from the logistic and Baranyi´s models that are built on the observational phenomenon of the Jameson-effect, described above. The Predator-prey theory is another alternative approach to the study of microbial interactions. This theory was proposed independently by Alfred James Lotka in 1925 and Vito Volterra in 1928 (Lotka, 1925; Volterra, 1928), also known as Lotka-Volterra predator-prey system (Becker and Leopold-Wildburger, 2020). The generalized Lotka-Volterra approach is able to describe the time-dependent population dynamics and predict ecological relationships (i.e. amensalism, competition and mutualism) between members of different microbial species (Gao et al., 2018). Lotka-Volterra approach, historically proposed in ecology, was introduced in predictive microbiology by Dens et al. (1999) and Vereecken et al. (2000). Different studies have investigated microbial interaction using different Lotka-Volterra approaches for LAB and *L. monocytogenes* and interactions involving other bacteria in fresh fish and RTE fish products (Bolívar et al., 2020; Costa et al., 2019; Giuffrida et al., 2007; Valenti et al., 2013), laboratory culture media (Fujikawa and Sakha, 2014) and other foods (Liu et al., 2006; Mounier et al., 2008; Ye et al., 2014).

In general, these two modelling approaches assume that the interaction between two microbial populations (intra-and inter-specific interactions) are linear, describing different situations, either the reduction in maximum population density (N_{max}) or the decline of one or both populations (Ayala et al., 1973; Berryman, 1992). These effects are related to the bacterial concentration, which, however, depends only on the environmental conditions in which they are being grown, since the interaction does not affect the maximum growth rate (Giuffrida et al., 2009). Determining the kinetic parameters under mono-culture conditions can help to better assess the interaction level between populations. The quantitative analysis of these values, obtained in co and mono-culture, can be also used to construct more comprehensive and representative interactions models to be applied or validated in different situations or food matrices (Giuffrida et al., 2009; Powell et al., 2004).

1.11. Microbial Risk Assessment

The Risk Analysis process for food safety represents a structured decision-making and have been used to address the challenges of globalized trade in food, in terms of setting international standards and guidelines, and for national regulations, which include the transmission of pathogenic bacteria with increase resistance along the food chain and between different countries (Codex Alimentarius, 2007; Possas et al., 2020). The process of Risk Analysis, as defined Codex Alimentarius, integrates three components as independent concepts: Risk Assessment, Risk Management and Risk Communication (Figure 1.2).

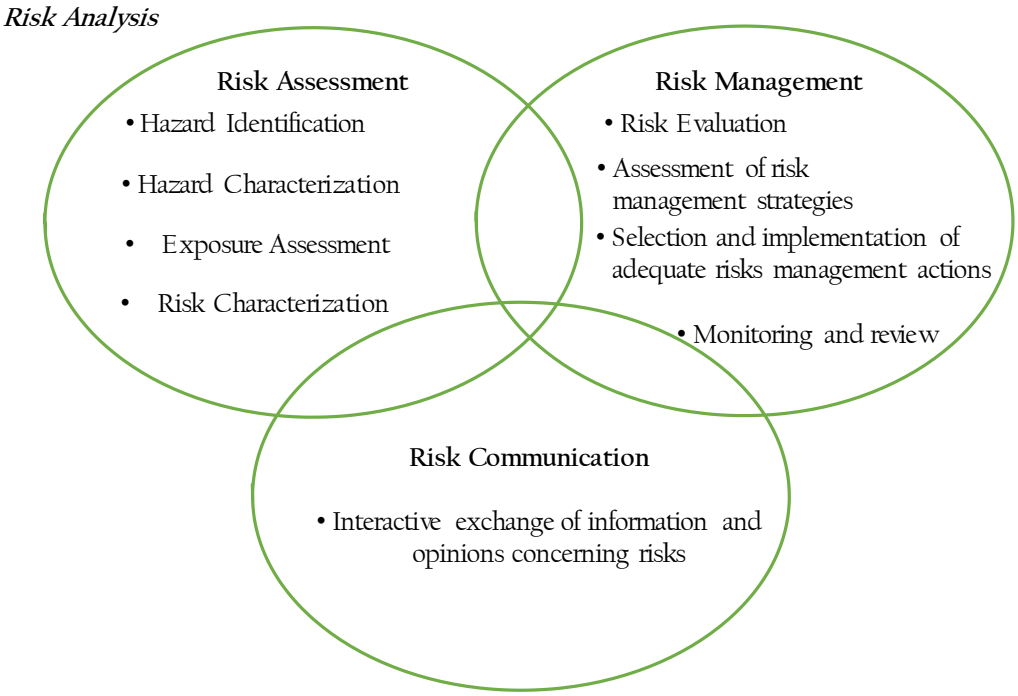


Figure 1.2. Interaction between the three components of risk assessment (FAO/WHO, 2006).

Risk Assessment, the central specific component of the Risk Analysis, consists of a systematic process which aims at better understanding and managing the nature of food safety risks (i.e. biological, chemical and physical), which can be qualitative (descriptive or categorical treatments of information) or quantitative (mathematical analyses of numerical data, typically regarding with the probability of disease or death) (Kavlock et al., 2018; Lammerding and Fazil, 2000).

A Microbial Risk Assessment (MRA) is a systematic approach and one of the most relevant topics that has received attention over the last decades to address the risk of

pathogens in foods and/or and processes and to aid in the quest for a better means of ensuring the production of food safe (Bassett, et al., 2012). The process of conducting a MRA is a scientifically based framework for understand the level of risk in a given food/pathogen scenario, consisting in four successive key steps:

i) Hazard Identification: "The identification of biological agents capable of causing adverse health effects and which may be present in a particular food or group of food."
ii) Hazard Characterization: "The qualitative and/or quantitative evaluation of the nature of the adverse health associated with hazard."
iii) Exposure Assessment: "The qualitative and/or quantitative evaluation of the likely intake of a biological agent via food, as well as exposure form other sources if relevant."
iv) Risk Characterization: "The process of determining the qualitative and/or quantitative estimation, including attended uncertainties, of the probability of occurrence and severity of known or potential adverse health effects in a given population based on Hazard Identification, Hazard Characterization and Exposure Assessment."

These steps involve estimating the magnitude of public health risks in terms of likelihood of exposure to foodborne pathogens and the likelihood and impact of any adverse health effects after exposure, which may cover the complete "farm-to-fork" pathway or focus only on one part (i.e. specific step) considered relevant regarding to food safety (Lammerding, 1997; Miranda and Schaffner, 2018).

Risk Assessments for *L. monocytogenes* in the consumption of RTE fish products has been carried out by different authors (Garrido et al., 2010; Pouillot et al., 2007, 2009). Pasonen et al. (2019) evaluated the exposure to *L. monocytogenes* in cold smoked and salt-cured fishery salmon products in Finland for consumers in two age groups, the elderly populations as a risk group and the working-age population as baseline. Incidence was assessed by estimating the growth of *L. monocytogenes* in the products at three temperatures. Furthermore, the risk estimated was based on pathogen occurrence and product consumption data and epidemiological data. On this basis, the number of cases in Finland could be estimated. The results showed that elderly population greater risk of acquiring listeriosis, around 55%, whereas healthy adults 27%, even if products have been stored at recommend temperature (between 0 and 3 °C). In other study, Grønlund (2010) carried out a risk assessment in Denmark for the consumption of vacuum-packed cold-salmon. The groups included pregnant women, elderly over 60 years old and immunocompromised people. The results showed that immunocompromised group

accounted 66.6% of the case of listeriosis. It has also been shown that the concentration of *L. monocytogenes* could be reduced, if an initial concentration of LAB of 10^2 CFU/g was present in the product, therefore, the number of cases of listeriosis would also be reduced.

More specific Risk assessment studies should be carried out to elucidate the public health impact of the use of bio-protective cultures (Popovic et al., 2011; WHO/FAO, 2004). To this end, suitable predictive microbiology models are to be developed and incorporated into the risk assessment process (Pérez-Rodríguez and Valero, 2013). For instance, the interaction between LAB and *L. monocytogenes* that can described using different mathematical models. Mejlholm and Dalgaard (2007) estimated changes in the concentration of *L. monocytogenes* in presence of LAB from predictions using predictive tools (FSSP) that can be used as parameters in the risk assessment and, thus, estimate the risk of eating of a certain fish product (e.g. fresh fish under MAP or vacuum-packed cold-smoked fish) stored at isothermal and non-isothermal conditions throughout the shelf-life.

1.12. References

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Chapter 2

Predictive microbiology tools for exposure assessment

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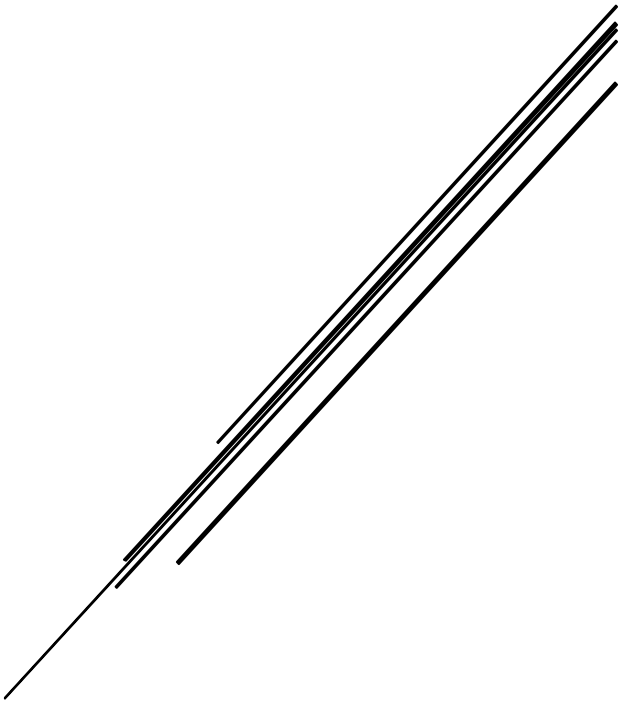

2.1. Predictive Microbiology for Quantitative Microbiological Risk Assessment

In the food chain from farm-to-fork, microorganisms can encounter a series of food processing and preservation steps. The area of predictive microbiology makes use of a number of mathematical models to describe the behaviour of bacterial population in these steps. Predictive microbiology models are of great importance for quantitative microbiological risk assessments (QMRA), as QMRA should explicitly consider the dynamics of microbiological growth, survival, and death in foods (Codex 1999).

In recent years, predictive microbiology has proved to be a promising tool to estimate the changes in microbial concentration in foods over the farm-to-fork chain (Messens et al. 2018). This occurred mainly due to successfully validated models and active software development in the area (Koutsoumanis et al. 2016, Mejlholm et al. 2015, Mejlholm et al. 2010). Predictive microbial models can help to understand the microbial behaviour in food systems depending on environmental conditions, being a powerful tool to evaluate the microbial exposure in the exposure assessments step within a QMRA (Koutsoumanis et al. 2016).

The application of predictive models in exposure assessments is not always straightforward, especially for QMRA aiming to evaluate the status of public health concerning a specific hazard and/or food product. While QMRA studies assess probabilities and therefore need to use stochastic models, preferably second order Monte Carlo models, predictive growth are generally deterministic models, i.e. they are developed and validated to produce point estimates outputs (Nauta 2002). This deficiency highlighted the need for the development of models expressing populations of microorganisms in terms of probability and drove the start of the so-called “stochastic predictive microbiology” (Koutsoumanis et al. 2016; Nicolai and Van Impe 1996). In the last 20 years, a number of stochastic predictive modelling approaches, aimed at quantifying and integrating different types of variability, have been reported (Augustin et al 2011; Couvert et al. 2010; Delignette-Muller et al. 2006; Koutsoumanis et al. 2007; Koutsoumanis et al 2010; Mejlholm et al. 2015; Membré et al. 2005; Pouillot et al. 2003). Besides, transfer, mixing, partitioning and some growth/no growth boundary models apply a probabilistic modelling approach.

The farm-to-fork food chain has large variety of processes that require different models. The variety of models that can be used is large, and a description of the food chain may be difficult (Nauta 2008). Nauta (2001) introduced the use of modular process

risk models (MPRMs) as a tool for QMRA. This approach splits the food pathway into processing steps that describe one of six basic processes: growth, inactivation, partitioning, mixing, removal and cross-contamination. In theory, once the modelling of these basic processes is established, any food pathway can be modelled when it is described as a sequence of consecutive basic processes. Traditionally, models developed in the area of predictive microbiology describe microbial growth and inactivation. These models together with cross-contamination (transfer), mixing, partitioning and removal are discussed in this chapter.

2.2. Predictive Microbiology Model Types

The literature on predictive microbiology presents several classifications of mathematical models. Predictive models can be classified as (i) primary, secondary and tertiary, (ii) kinetic or probabilistic, and (iii) empirical or mechanistic (McDonald and Sun 1999). These proposed classifications are based on modelling levels, the way of obtaining the experimental data and construction form of the mathematical model, respectively.

The classification of models by modelling level was proposed by Whiting and Buchanan (1993). Primary models measure the response of the microorganism with time to a single set of conditions, in which each population versus time curve can be described by a set of specific values for each of the parameters in the model (e.g. lag phase, growth rate and *D*-value). Secondary models describe the response of one or more parameters of a primary model to changes in one or more of the environmental conditions. The environmental conditions can be intrinsic to the food product, such as pH, water activity (a_w), organic acids concentration, and/or extrinsic, such as temperature, pressure, air composition of the packaging, among others. Tertiary models are applications of one or more secondary models to generate systems for providing predictions to non-modellers (e.g. user-friendly or applications software and expert systems) (Whiting and Buchanan 1993).

Primary and secondary models are based on experimental data at constant conditions. However, environmental conditions (e.g. temperature) can change during distribution and storage. Thus, dynamic models have been developed to predict the behaviour of microorganisms under conditions that vary with time, especially under non-isothermal conditions (Haberbeck et al. 2012; Longhi et al. 2013, Silva et al. 2017). Dynamic models can be categorized as tertiary models, which are widely used to describe microbial growth

from information obtained with the primary and secondary models for varying environmental conditions. Some of these tertiary models are available in user-friendly software, such as ComBase, MicroHibro, PMP, FSSP, among others. These software may include algorithms for calculating changes in environmental conditions, comparing microbial behaviour under different conditions or constructing growth curves of more than one microorganism simultaneously.

Baty and Delignette-Muller (2004) presented the classification of the microbial growth models by their mathematical form of construction. The models can be characterized as sigmoid models, models with an adjustment function or compartmental models. Sigmoid models (e.g. logistic and Gompertz models) were historically used to describe the increase in the logarithm of the bacterial cell density with time. Models with an adjustment function (e.g. Baranyi and Roberts model) are less empirical and based on differential equations. Compartmental models (e.g. logistic model with delay and Buchanan three-phase linear model) were developed in order to model the lag phase (Baty and Delignette-Muller 2004). These models will be discussed later.

McMeekin and Ross (2002) reported the classification of the models by the construction form. Predictive models can be classified as mechanistic, which are models that present physical, chemical and/or biological explanations for their parameters, or as empirical, which are models without explanations for their parameters and are usually proposed based only on the observation of the format of common mathematical functions (McMeekin and Ross 2002). Most of the models used in predictive microbiology are not purely mechanistic, and some of them are simply empirical models with simple mathematical function adjustments. For Zwietering and Den Besten (2011), the use of models for the description of microbial growth kinetics does not assume that the mechanism has been fully understood, and the acceptable performance of model fit is not a guarantee that its mechanism is right. The development of fully mechanistic microbial growth models has been limited by the inability to provide quantitative values for all model parameters (McMeekin 1993). The investigation of correlations between results of different experiments is important because biological principles can be found and mechanisms can be inferred. On the other hand, Corradini and Peleg (2005) stated that a completely different approach consists in abandoning the attempt to find a universal growth model and the environmental conditions. The format of the model can be chosen through convenient mathematical considerations using the principle of parsimony.

2.3. Primary Models: Growth, Interaction and Inactivation Models

2.3.1. Growth Models

Growth models have been a major area of development in predictive microbiology over the past 25 years. Traditionally, these models rely on the generation of kinetic data, under defined environmental conditions, allowing the description of microbial curves in food. These microbial curves present four phases: lag, exponential growth, stationary and decline (see Figure 2.1). In practice, microbial growth models in foods assume a sigmoidal growth function, i.e. the decline phase is ignored (Amézquita, et al. 2011; Buchanan, et al. 1997).

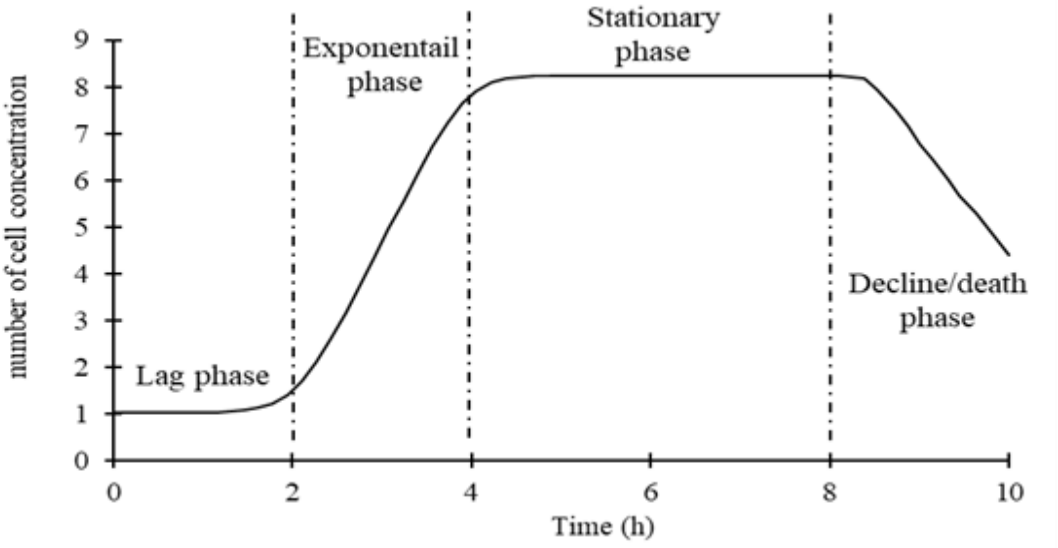

Figure 2.1. Typical microbial growth curve as a function of time representing four phases: lag, exponential growth, stationary and decline.

2.3.1.1. Gompertz and Logistic Models

Gompertz and logistic models were introduced by Gibson et al. (1987) and have been successfully used to describe nonlinear microbial responses, making possible to express the growth in log colony forming units (cfu)/(g or mL) as a function of time. These models are the most common sigmoidal functions used to fit to microbial growth data, because they consist of three phases, similar to the microbial growth curve (Pérez-Rodríguez and Valero 2013).

The Gompertz equation (Equation 2.1) is a function based on the limitation of space and/or nutrients as well as the production of toxic metabolites, where the growth rate is not constant. Typically, growth rate would increase to a maximum, and then it would

decrease. In this way, the maximum growth rate is determined at the point of inflection in the microbial curve (Devlieghere et al. 2009; McKellar and Lu 2003).

$$log\ N(t) = A + C\,exp\left\{-exp\left[-B\left(t-M\right)\right]\right\} \tag{2.1}$$

where *N(t)* is the microbial concentration (cfu/[g or mL]) at time t (h), A (log cfu/[g or mL]) is the lower asymptotic value as t decrease to zero, C (log cfu/[g or mL]) is the difference between the upper and lower asymptotic value, M (h) is the time when the absolute growth rate is maximum, and B (h^{-1}) is the relative growth rate at M.

The regression empirical parameters of the Gompertz model can be replaced by parameters with biological meaning (e.g. lag time, maximum growth rate, initial and maximum microbial population) through adequate mathematical expressions, as proposed by Zwietering et al. (1990). Thus, the lag time duration (λ) (h) (Equation 2.2) and the maximum growth rate (μ) (log [cfu/g or mL/h]) (Equation 2.3) can be calculated:

$$\lambda = M - \frac{1}{B} \tag{2.2}$$

$$\mu = \frac{B \cdot C}{e} \tag{2.3}$$

where e is equal to 2.7182.

In order to simplify the fitting process, the reparametrized of Gompertz model (Equation 2.4) proposed by Zwietering et al. (1990) can be written:

$$y = D\,exp\left\{-exp\left[\frac{\mu\, e}{A}\left(\lambda - t\right)+1\right]\right\} \tag{2.4}$$

where y is the logarithm of the relative population size ($y = \ln[N/N_0]$), N_0 is the microbial concentration (cfu/[g or mL]), D is the maximum increase of population attained in the stationary phase.

Table 2.1 summarizes all variables presented in the different equations of this chapter.

Table 2.1. Summary of all variables presented in the different equations.

Symbol	Description	Unit
A	Lower asymptotic value as time decrease to zero	log cfu/(g or mL)
$A(t)$	An adjustment function for the modified Baranyi and Roberts model	h
a_f and A_f	Accuracy factor	unitless
B	Relative growth rate at M	h^{-1}
b	Regression coefficient	$(h^{-0.5}\ °C^{-1})$
b_f and B_f	Bias factor	unitless
C	Difference between the upper and lower asymptotic value	log cfu/(g or mL)
c	Proportionality constant	$°C^{-1}$
D	Maximum increase of population attained in the stationary phase	log cfu/(g or mL)
D_{ref}	The decimal reduction time at a reference temperature T_{ref} (°C)	s or min
D-value	The decimal reduction time	s or min
E_a	Activation energy of the system	J/mol
H	Removal function	log cfu/(g or mL)
h_0	Physiological state of the cells at $t = t_0$	unitless
K	First-order rate constant	s^{-1} or min^{-1}
k_{max}	Maximum specific decay rate	min^{-1} or h^{-1}
M	Time when the absolute growth rate is maximum	h
m	Curvature factor	unitless
n	Number of observations	unitless
$N(t)$, N_t or N	Microbial concentration at time t	log cfu/(g or mL)
N_0	Microbial concentration at time zero	log cfu/(g or mL)
N_{max}	Maximum microbial concentration	log cfu/(g or mL)
N_{res}	Residual microbial concentration	log cfu/(g or mL)
N_{cri}	Maximum critical concentration	log cfu/(g or mL)
N_{out}	Microbial concentration at the end of the process step	log cfu/(g or mL)
N_{in}	Microbial concentration at the beginning of the process step	log cfu/(g or mL)
Q	Measure of the physiological state of cells at time t	unitless

Table 2.1 continued

Symbol	Description	Unit
R	Universal gas constant	J/mol.K
R^2	Coefficient of determination	unitless
r_0	Fraction of the growing initial subpopulation	unitless
$RMSE$	Root-mean-square error	unitless
$S(t)$	Momentary ("instantaneous") survival ratio at time t	unitless
t	Time	h, min or s
T	Temperature	°C
t_L	Time before inactivation	h, min or s
t_{lag}	Time when the lag phase ends	h
v	Rate of increase of the limiting substrate, generally assumed to be equal to μ_{max}	unitless
t_{max}	Time when N_{max} is reached	h
T_{max}	Temperature in the upper part of the range over which growth is not possible	°C
T_{min}	Minimum theoretical temperature at which growth is detected	°C
T_{ref}	Reference temperature at which the shelf-life is known	°C
y	Logarithm of the relative population size ($y = \ln[N/N_0]$)	unitless
Y	Natural logarithm of cell concentration at time t ($Y = \ln[N]$)	ln cfu (g or mL)
y_0	Natural logarithm of the cell concentration at $t = t_0$	log cfu (g or mL)
y_{max}	Natural logarithm of the maximum cell concentration	log cfu (g or mL)
$y_{observed}$	Data observed experimentally	according to the data
$y_{predicted}$	Data predicted by the model	according to the data
z-value	The temperature increase required to reduce the D-value by a factor of 10	°C
α	Scale parameter	s or min
α_{AB}	The competition factor parameter of population A on population B	unitless
α_{BA}	The competition factor parameter of population B on population A	unitless
β	Shape parameter	unitless
λ	Lag time duration	h
μ_{max}	Maximum specific growth rate	h^{-1}
μ	Specific growth rate	log cfu/(g or mL)/h
μ_G	Maximum specific growth	h^{-1}
μ_D	Maximum specific death	h^{-1}
$\bar{y}_{observed}$	Arithmetic mean of the observed data	according to the data

Several studies have been using the deterministic Gompertz models to describe the counting microbial growth in foods (Dabadé et al. 2015; Kim et al. 2018; Slongo et al. 2009; Tarlak et al. 2018) and growth curves based on turbidity data (Chatterjee et al. 2015; Mytilinaios et al. 2012; Perni et al. 2005). However, some authors (Dalgaard et al. 1994; Membré et al. 1999) reported some limitations associated with the use of the Gompertz model, for instance, overestimated growth rate compared with the usual definition of the maximum growth rate (Pérez-Rodríguez and Valero 2013). An additional limitation is that experimental data are required over the whole growth range in order to get a good model fit (McMeekin et al. 2013; Peleg 1997).

Another widely applied primary growth model to describe the microbial growth is the logistic model (Equation 2.5), also proposed by Gibson et al. (1987). The difference between logistic and Gompertz models is that the former is described with a symmetric growth pattern.

$$log\,N(t) = A + \frac{C}{1 + exp\left[-B\left(t - M\right)\right]} \tag{2.5}$$

The parameters λ and μ can be defined by Equations 2.6 and 2.7, respectively.

$$\lambda = M - \frac{2}{B} \tag{2.6}$$

$$\mu = \frac{B \cdot C}{4} \tag{2.7}$$

To correct a failure in the logistic equation, several modifications were proposed accounting for asymmetric growth curves and improving its accuracy (Augustin and Carlier 2000a; Fujikawa et al. 2003; Zwietering et al. 1990). Rosso et al. (1996) proposed a modification to the logistic model (Equation 2.8) with delay ($\lambda > 0$) and without delay ($\lambda = 0$).

$$\begin{cases} If \quad t < \lambda & Log\left(N_t\right) = Log\left(N_0\right) \\ If \quad t \geq \lambda & Log\left(N_t\right) = Log\left(\dfrac{N_{max}}{1 + \left(\dfrac{N_{max}}{N_0} - 1\right) \cdot \left(exp\left(-\mu_{max} \cdot \left(t - \lambda\right)\right)\right)}\right) \end{cases} \tag{2.8}$$

where N_{max} is the maximum microbial concentration (cfu/[g or mL]), and μ_{max} is the maximum specific growth rate (h^{-1}).

The application of logistic model with and without delay has provide good accuracy to describe deterministic microbial growth curves (Bover-Cid et al. 2019; Hereu et al. 2014; Wang et al. 2012). Ancelet et al. (2012) applied the logistic model with delay in a farm-to-fork QMRA model to determine the growth of *Bacillus cereus* in courgette purée.

2.3.1.2. Baranyi and Roberts Model

Baranyi and Roberts (1994) developed a mechanistic model for bacterial growth (Equation 2.9), including an exponential linear growth phase and a lag phase calculated by an adjustment function *A(t)* (Equation 2.10). In this model, the lag phase extension depends on the cell's physiologic state and its adaptation to the new environment. For instance, lag phase is longer if the cells are not adapted to the new environment. Once the cells have adjusted to the new environment, they grow exponentially until reach the stationary phase, limited by restrictions dictated by the growth medium (Baranyi and Roberts 1994; Pérez-Rodríguez 2013; McKellar and Lu 2003).

$$Y = y_0 + \mu_{max} \cdot A(t) - \frac{1}{m} \cdot ln\left(1 + \frac{e^{m \cdot \mu_{max} \cdot A(t)} - 1}{e^{m(y_{max} - y_0)}}\right) \tag{2.9}$$

$$A(t) = t + \frac{ln\left(e^{-\mu_{max} t} + e^{-h_0} - e^{vt - h_0}\right)}{\mu_{max}} \tag{2.10}$$

where *m* is a curvature factor and *A(t)* represents an adjustment function for the model.

The Baranyi and Roberts model has been used extensively to describe the growth of pathogenic and spoilage bacteria (Carrasco et al. 2006; Silva et al. 2018; Tarlak et al. 2018), including the increasing colony diameter of heat-resistant fungi (Tremarin et al. 2015). The model has shown advantages when compared with other growth models: (i) it is availability on two user-friendly software programs: DMFit and MicroFit; (ii) it has good fitting capacity; and (iii) most of the model parameters are biologically interpretable. Another advantage of the Baranyi and Roberts model is that it is available as differential equations that allow microbial growth to be modelled in a dynamic environment, generally resulting from non-isothermal conditions (Costa et al. 2016; Gospavic et al. 2008; McKellar and Lu 2003; Xanthiakos et al. 2006).

2.3.1.3. Buchanan Three-Phase Linear Model

The three-phase linear model (Equation 2.11) proposed by Buchanan et al. (1997) was developed to describe the three bacterial growth phases (lag, exponential and stationary) by applying straight lines. The authors elaborated a biological basis for justifying the suitability of the linear model. During the lag phase, it is assumed that the cells do not divide due to adaptation in the new environment. For this reason, the growth rate is zero. During the exponential phase, it is assumed that the growth rate is a constant, in which the logarithm of the cellular concentration increases linearly with time. Once the stationary phase is reached, there is no increase in the cells, and the growth rate returns to zero.

$$N(t)\begin{cases} For\ t \le t_{lag}, log\ N_t = log\ N_0 & -lag\ phase \\ For\ t_{lag} < t < t_{max}, log\ N_t = log\ N_0 + \mu_{max}\left(t - t_{lag}\right) & -exponential\ growth\ phase \\ For\ t \ge t_{max}, log\ N_t = log\ N_{max} & -stationary\ phase \end{cases} \quad (2.11)$$

where t_{lag} is the time when the lag phase ends (h) and t_{max} is the time when the N_{max} is reached (h).

Buchanan et al. (1997) compared this model with the Gompertz and Baranyi and Robert models. The curves predicted by the three models presented good-fit to the data, and the growth kinetic parameters were similar. The three-phase linear model was more robust than the other models, especially when there were few experimental data. The QMRA developed by Koutsoumanis et al. (2010) to evaluate the growth of *Listeria monocytogenes* in pasteurized milk from production to the time of consumption used the three-phase linear model to calculate the pathogen's growth.

2.3.2. Interaction Models

The growth of microorganisms in foods is a complex system where different microbial populations can coexist and interact. The interaction between two different microorganisms can be direct or indirect and the effect of the interaction may be positive, neutral or negative. Direct interactions occur when two populations use *quorum sensing* or physical contact, while indirect interactions occur through a change of the environment. Indirect interactions can be specific, due for example to the production of bacteriocin and organic acids and competition for nutrients (Casla et al. 1996; Greer and Dilts 1995; Fredrickson, 1977). Table 2.2 describes the five types of indirect interaction between two populations (Fredrickson, 1977; Sieuwerts et al. 2008).

Table 2.2. The five types of microbial indirect interactions. The effect of coexistence of two populations is shown with an example of the interaction.

Interaction types	Effect on population A	Effect on population B	Example
Amensalism	Neutral	Negative	Bacteriocin produced by population A.
Competition	Negative	Negative	Nutrient competition.
Commensalism	Neutral	Positive	Production of lactic acid by lactic acid bacteria (population A) is metabolized by yeast and fungi species (population B) (Mounier et al. 2005).
Mutualism	Positive	Positive	Exchange of growth factors.
Parasitism	Negative	Positive	Bacteriophages (population B) attach bacteria (population A).

Many studies have observed that natural food microbiota can inhibit or reduce growth of pathogenic bacteria (Buchanan and Bagi 1999; Brillet et al. 2004; Hwang and Sheen 2011; Lardeux et al. 2015). Nevertheless, microbial interaction is often not considered in predictive microbiology and QMRA, due to its complexity (Malakar et al. 2003). Interaction models are usually intended to quantify how much the growth of one population is reduced by the growth of other populations (Cornu et al. 2011; Pérez-Rodríguez and Valero 2013). Two model approaches can be used to describe the interaction between microorganisms. One approach considers the Jameson effect, which describes the simultaneous stopping of growth of all bacterial species at the time when the dominant bacteria population reaches its stationary phase (Giménez and Dalgaard 2004; Jameson 1962; Mellefont et al. 2008). The other approach considers the Lotka-Volterra competition model, which describes the dynamics of two competing bacterial populations in food products by incorporating an additional reduction term in the population growth rate that is proportional to the population density of another competing population (Powell et al. 2004; Valenti et al. 2013; Vereecken et al. 2000).

2.3.2.1. Jameson Effect Model

Jameson effect model is based on Equations 2.12 through 2.15, which assumes that the growth of the pathogen halts when the dominant microbial population reaches its N_{max} (Cornu et al. 2011; Jameson 1962).

$$\frac{dN_A}{dt} = N_A \cdot \mu_{max\,A} \cdot \left(1 - \frac{N_A}{N_{max\,A}}\right) \cdot \left(1 - \frac{N_B}{N_{max\,B}}\right) \cdot \left(\frac{Q_A}{1+Q_A}\right) \tag{2.12}$$

$$\frac{dN_B}{dt} = N_B \cdot \mu_{max\,B} \cdot \left(1 - \frac{N_B}{N_{max\,B}}\right) \cdot \left(1 - \frac{N_A}{N_{max\,A}}\right) \cdot \left(\frac{Q_B}{1+Q_B}\right) \quad (2.13)$$

$$\frac{dQ_A}{dt} = Q_{A\,t-1} \cdot \mu_{maxA} \quad (2.14)$$

$$\frac{dQ_B}{dt} = Q_{B\,t-1} \cdot \mu_{maxB} \quad (2.15)$$

where subscript A or B represents the different microbial populations and Q is a measure of the physiological state of cells at time t.

The value of Q at $t = 0$ (Q_0) can be calculated for both microorganisms (Equation 2.16).

$$Q_0 = \frac{1}{e^{(\mu_{max} \cdot \lambda)} - 1} \quad (2.16)$$

where e is the Euler´s number and λ is the lag time estimated from co-culture experiments.

Different variants of the Jameson model have been proposed to evaluate the interaction between two microbial populations (Cornu et al. 2011; Giménez and Dalgaard 2004; Møller et al. 2013). Costa et al. (2019) proposed a modification of the Jameson effect model that includes two parameters (N_{criA} and N_{criB}) describing the maximum critical concentration that one population should reach to inhibit the growth of the other population. Mejlholm et al. (2015) developed a stochastic model for *Listeria monocytogenes* growth in naturally contaminated lightly preserved seafood applying the Jameson effect. The quantitative assessment of the exposure to *L. monocytogenes* from cold-smoked salmon consumption developed by Pouillot et al. (2007) considered the competitive bacterial growth between the pathogen and the background flora using the Jameson effect model principle.

2.3.2.2. Lotka-Volterra Model

The classic Lotka-Volterra (Equations 2.17 through 2.20), also called predator-prey model, describes the interaction of two competing bacteria in a co-culture. This model is based on the logistic growth model and includes two empirical parameters reflecting the degree of interaction between microbial species (α_{AB} and α_{BA}) (Giuffrida et al. 2007; Fujikawa et al. 2014; Lotka, 1956). Depending on the empirical parameter value for population A (α_A), the growth of population B (α_B) can be affected in three different ways: i) if $0 < \alpha_{AB} < 1$, population B grows with reduced μ_{max} after population A reaches N_{max}; ii)

if $\alpha_{AB} = 1$, population B stops growing when the population A reaches its N_{max} and; iii) if $\alpha_{AB} > 1$, population B declines when population A reaches its N_{max}.

$$\frac{dN_A}{dt} = N_A \cdot \mu_{max\,A} \cdot \left(1 - \frac{N_A + \alpha_{AB} \cdot N_B}{N_{max\,A}}\right) \cdot \left(\frac{Q_A}{1+Q_A}\right) \tag{2.17}$$

$$\frac{dN_B}{dt} = N_B \cdot \mu_{max\,B} \cdot \left(1 - \frac{N_B + \alpha_{AB} \cdot N_A}{N_{max\,B}}\right) \cdot \left(\frac{Q_B}{1+Q_B}\right) \tag{2.18}$$

$$\frac{dQ_A}{dt} = Q_{A\,t-1} \cdot \mu_{maxA} \tag{2.19}$$

$$\frac{dQ_B}{dt} = Q_{B\,t-1} \cdot \mu_{maxB} \tag{2.20}$$

where α_{AB} and α_{BA} are, respectively, the competition factor parameters of population A on population B and vice-versa.

A number of mathematical models have been developed to predict microbial interaction, mainly between lactic acid bacteria and *L. monocytogenes* in various food matrices (Blanco-Lizarazo et al. 2016; Mejlholm and Dalgaard 2007, Mejlholm and Dalgaard 2015; Quinto et al. 2016; Ye et al. 2014; Østergaard et al. 2014) and between natural microbiota and pathogenic bacteria (Buchanan and Bagi 1999; Guillier et al. 2008; Koseki et al. 2011; Le Marc et al. 2009).

2.3.3. Phoenix Phenomenon Model

The experimental observation that some bacteria, after exposition to stress conditions (osmotic or thermal, for example) at constant temperature, present a kinetic growth characterized by a decrease in the initial cell counts followed by an exponential increase in the level of the count is known as Phoenix phenomenon (Amézquita et al, 2005). This term was probably applied because Phoenix, in ancient times, symbolized immortality through "death and resurrection" (Shoemaker and Pierson, 1977). Colle et al. (1961) and Shoemaker and Pierson (1977) described this microbiological phenomenon in *Clostridium perfringens*, Kelly et al. (2003) in *Campylobacter jejuni*, and several authors in *Salmonella* (Airoldi and Zottola 1988; Aspridou et al. 2018; Zhou et al. 2011; Paganini et al. submitted).

The Phoenix phenomenon was observed by Shoemaker and Pierson (1977) in log count curves for *C. perfringens* after thermal stress. The authors explain this behaviour considering three phases: injury, recovery and growth. Mellefont et al. (2005), working

with *Salmonella* Typhimurium M48 under osmotic stress, thought that the phenomenon was due to an initial inactivation of a portion of the population followed by growth. However, they considered that the Phoenix phenomenon represented the responses of different sub-populations, in which some cells are dying but both recovery of culturability and exponential growth were occurring simultaneously. By studying single cell of *Salmonella enterica* serotype Agona, Aspridou et al (2018) concluded that the Phoenix phenomenon occurred under severe osmotic stress (5.7% and 6.75% NaCl) as result of simultaneous growth, survival and death of cells.

The modified Baranyi and Roberts (1994) model was suggested by Zhou et al (2011) to model the Phoenix phenomenon (Equations 2.21 and 2.22) based on the assumption that the log count curve of the total population was the sum of a dying and a surviving-then-growing subpopulation.

$$ln(N) = ln\left\{exp\left[(1-r_0)ln(N_0) - \mu_D t\right] + \frac{exp\left[r_0\, ln(N_0) + \mu_G A(t)\right]}{\left[1 + \frac{e^{\mu_G A(t)} - 1}{e^{ln(N_{max}) - r_0\, ln(N_0)}}\right]}\right\} \quad (2.21)$$

where μ_G and μ_D are the maximum specific growth and death rates (h^{-1}), respectively, r_0 is the fraction of the growing initial subpopulation ($0 \leq r_0 < 1$).

$$A(t) = t - \lambda + \frac{ln\left[1 - e^{-\mu_G t} + e^{-\mu_G (t-\lambda)}\right]}{\mu_G} \quad (2.22)$$

with λ being the lag time of the growing subpopulation (therefore, as per definition, of the whole population).

Paganini et al. (submitted*)* applied the modified Baranyi and Roberts model similar to Zhou et al. (2011) to describe curves of four serotypes of *Salmonella enterica* Typhimurium under osmotic stress (a_w 0.95) for different inoculum conditions. The predictive ability of the model was assessed through statistical indexes, with good results ($R^2 > 0.973$ and RMSE < 0.288).

The modified Baranyi and Roberts model can be applied to studies about the Phoenix phenomenon, which show the different cellular responses and the complexity in the behaviour of microbial populations in conditions close to the boundary of growth.

2.3.4. Inactivation Models

The kinetics of microbial inactivation has been receiving attention since the 1920s and constitute one of the earliest forms of predictive microbiology. Inactivation models describe the decrease in a microbial population over time when it is exposed to a lethal process or agent, such as thermal treatments, non-thermal technologies and drying/dehydration processes. The distinction between inactivation and survival models is not always clear, but survival processes are usually associated with slowly declining patterns, while inactivation refers to lethal process showing a rapid decrease of microbial population (Pérez-Rodríguez 2013; Peleg 2006).

Traditionally, microbial inactivation has been assumed to show first-order kinetics (McKellar and Lu 2003; Peleg 2003). However, non-log-linear microbial inactivation models have been frequently presented in the literature and are used to describe the kinetic of inactivation of a wide variety of microorganisms with the most varied behaviour (Bevilacqua et al. 2015; Peleg and Cole 1998). The most commonly models used to describe the microbial inactivation in foods are presented in Figure 2.2, and a brief description of the various models is provided next.

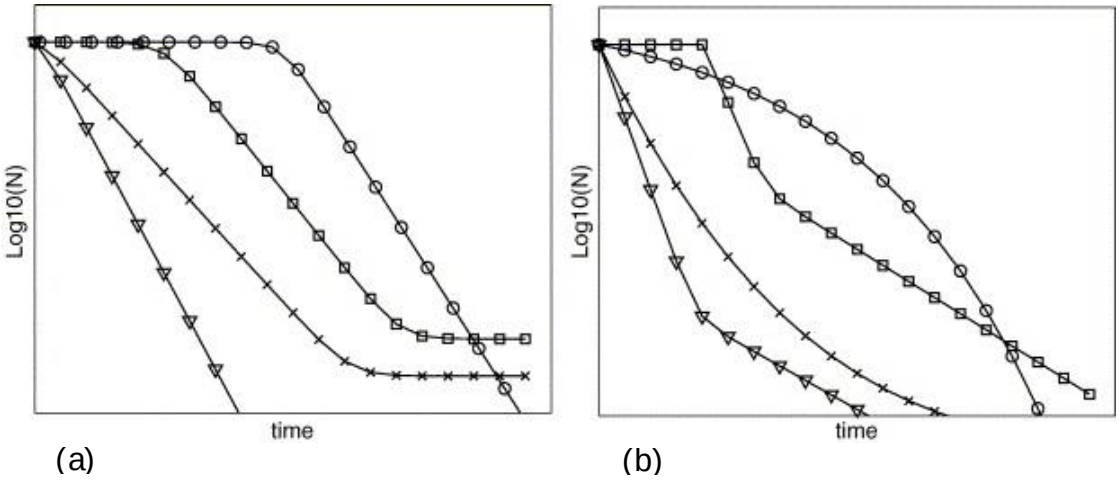

Figure 2.2. Representation of eight different shapes of inactivation curves. (a) Linear (▽, shape I), linear with tailing (×, shape II), sigmoidal-like (□, shape III), linear with a preceding shoulder (○, shape IV). (b) Biphasic (▽, shape V), concave (×, shape VI), biphasic with a shoulder (□, shape VII), and convex (○, shape VIII) (From Geeraerd et al. 2005 with permission of the authors).

2.3.4.1. Bigelow Model

The Bigelow or linear model (Equation 2.23) was first proposed to describe the inactivation of microorganisms and enzymes in the canning industry, in particular to establish the *D*-value (decimal reduction time) as a function of temperature (Bigelow and Esty 1920; Bigelow 1921). This model assumes that the inactivation kinetics is first-order and that all cells or spores of a microbial population have identical resistance to lethal treatments (Whiting and Buchanan 1997).

$$ln(N) = ln\ N_0 - kt \tag{2.23}$$

where k is the first-order rate constant (s^{-1} or min^{-1}).

By rearranging Equation 2.23, Equation 2.24 can be obtained.

$$log\ \frac{N}{N_0} = log\ S(t) = -\frac{t}{D_{value}} \tag{2.24}$$

where *D*-value is the decimal reduction time (*D*-value = 2.303/k, units in minutes or seconds) and *S(t)* is the momentary ("instantaneous") survival ratio at time *t* (Peleg 2006).

The *D-value* is a measure of the thermal resistance of a microorganism at a given temperature required to destroy one log cycle (90%) of the target microorganism. When the log *D*-values are plotted versus the treatment exposure temperature, the reciprocal of the slope is equal to the *z*-value, which is the temperature increase required to reduce the *D*-value by a factor of 10 in order to increase the rate of destruction by a factor of 10 (Bigelow and Esty 1920).

2.3.4.2. Weibull Model

The Weibull model (Equation 2.25) has been used to describe the inactivation of microorganism due to its mathematical simplicity and great flexibility (Peleg and Cole 1998). This model is based on the different distribution of resistance or sensitivity between the individuals in a microbial population (Van Boekel 2002; Van Derlinden et al. 2012).

$$log\ S(t) = -\frac{1}{2.303}\left(\frac{t}{\alpha}\right)^{\beta} \tag{2.25}$$

where α and β are parameters related to the scale and shape of the inactivation curve, respectively.

The shape parameter accounts for upward concavity of a survival curve ($\beta < 1$), a linear survival curve ($\beta = 1$), and downward concavity ($\beta > 1$). Therefore, when $\beta = 1$, no biological variation is assumed (each cell is equally susceptible to be destroyed), and the survivor curve is linear with first-order kinetics. Although the Weibull model is of an empirical nature, a link can be made with physiological effects. Curves with upward concavity ($\beta < 1$) forming a tail indicate that the population of microorganisms contains members that die rapidly. However, as the destruction process occurs, the survivors are most resistant, leading to a longer inactivation time, while curves with downward concavity ($\beta > 1$) indicate that the population progressively decreases, and the time required to destroy the same fraction of microorganisms decreases over time (Aragao et al. 2007; Peleg 2006). This model has successfully described survival and inactivation curves of *B. pumilus*, *B. coagulans, L. monocytogenes*, *C. jejuni*, *S. enterica*, *Yersinia enterocolitica* (Chun et al. 2009; Couvert et al. 2005; Haberbeck et al. 2012; Patil et al. 2009; Virto et al. 2005).

2.3.4.3. Shoulder/Tail Model (Geeraerd Model)

The shoulder/tail model (Equation 2.26) developed by Geeraerd et al. (2000) is based on the physiological state of cells and the residual population density on the basis of the existence of shoulder (or lag time) and tail region. The shoulder describes the initial segment of the inactivation curve, in which microbial population remains in similar levels, and the tail region corresponds to the final segment of the inactivation curve, representing the residual population more resistant to the lethal treatment.

$$N(t) = \left[\left(N_0 - N_{res}\right) exp\left(-k_{max} t\right) \frac{exp\left(-k_{max} t_L\right)}{1 + exp\left(\left(-k_{max} t_L\right) - 1\right) exp\left(-k_{max} t\right)} + N_{res} \right] \tag{2.26}$$

where k_{max} is the maximum specific decay rate (min^{-1} or h^{-1}), t_L is the time before inactivation (min^{-1} or h^{-1}) and N_{res} is the residual microbial population concentration (cfu/g or ml).

Geeraerd et al. (2005) created GInaFiT, a freeware add-in for Microsoft Excel. This tool includes nine different types of primary inactivation models, including shoulder/tail models.

2.4. Secondary Models

2.4.1. Ratkowsky or Square Root model

The Ratkowsky model (Ratkowsky et al. 1982), known as square root model (Equation 2.27), is one of the most popular models to describe the effect of temperature on maximum specific growth rate. This model has the advantage of homogenizing the data variance and providing a linear response in the region of temperatures below the optimum growth temperature (McMeekin et al. 2013).

$$\sqrt{\mu_{max}} = b \cdot \left(T - T_{min}\right) \tag{2.27}$$

where, b represents the regression coefficient ($h^{-0.5}\,°C^{-1}$), T is the temperature (°C) and T_{min} is the minimum theoretical temperature at which growth is detected (°C).

Ratkowsky et al. (1983) extended the square root model to describe effect on the growth rate in the whole region ranging from the minimum to the maximum temperature for growth (Equation 2.28).

$$\sqrt{\mu} = b \cdot \left(T - T_{min}\right) \cdot \left(1 - exp\left(c\left(T - T_{max}\right)\right)\right) \tag{2.28}$$

where in the expanded model, the parameters c and T_{max} are the proportionality constant ($°C^{-1}$) and the temperature (°C) in the upper part of the range over which growth is not possible, respectively.

Other adaptations include the effect of alternative environmental factors, such as pH (Adams et al. 1991; Wijtzes et al. 2001), a_w (McMeekin et al. 1987; Miles et al. 1997), carbon dioxide and phenol (Dalgaard et al. 1997; Giménez and Dalgaard 2004) and lactic acid (Ross et al. 2003). In some cases, the square root model has been applied also to describe lag time (Mataragas et al. 2006; Sant'Ana et al. 2012). For the quantitative assessment of the exposure to *L. monocytogenes* from cold-smoked salmon consumption, Pouillot et al. (2007) applied the square root model to described the effect of temperature on the specific growth rate.

Square root models are widely applied in predictive microbiology due to their simplicity and easy interpretability of the model parameters (e.g. T_{min}, pH_{max} and a_{wmin}) (Ross and Dalgaard 2004). Another advantage is that these models can be easily adapted to encompass the whole biokinetic range of environmental factors, making them more attractive to predictive microbiology practitioners (Pérez-Rodríguez and Valero, 2013).

2.4.2. Arrhenius-Type Model

Arrhenius-type model is considered fundamental in different scientific fields. This model is derived empirically based on thermodynamics, to describe chemical reaction kinetics and/or biological processes (Labuza and Riboh, 1982). In predictive microbiology, this model is used to describe the relationship between the maximum specific growth rate (μ_{max}) of a microorganism and the growth temperature (Equation 2.29) (Gonzales-Barron 2012; McMeekin et al. 2013).

$$ln(\mu) = \mu_0 \cdot \left(\frac{\Delta E_a}{R \cdot T} \right) \tag{2.29}$$

where μ_0 is a constant, T is the absolute temperature (K), R is the universal gas constant (8.314 J/mol.K), and E_a is the so-called activation energy of the system (J/mol).

An Arrhenius-type model was used to predict moulds growth (Longhi et al. 2014; Silva et al. 2010), bacteria growth (Silva et al. 2018; Huang et al. 2011; Koutsoumanis et al. 2000) and inactivation (Amos et al. 2001; Cerf et al. 1996). This model was extended by Davey (1989) to represent the relationship between the maximum growth rate or death rate of microorganisms under additional processing conditions, such as pH and/or a_w. The model has also been used to calculate relative rate of spoilage (RRS), which is defined as the shelf-life (determined by sensory evaluation) at a reference temperature (T_{ref}) divided by the shelf-life observed at the actual storage temperature (Equation 2.30) (Dalgaard 2002).

$$RRS = \frac{Shelf-life\ at\ T_{ref}}{Shelf-life\ at\ T} = exp\left[\frac{E_a}{R} \cdot \left(\frac{1}{T} - \frac{1}{T_{ref}} \right) \right] \tag{2.30}$$

where T_{ref} is a reference temperature (°C) at which the shelf-life is known.

RRS models are interesting because they enable shelf-life to be predicted at different temperatures and for products where the specific spoilage organisms or the type of reaction responsible for spoilage are not known (McKellar and Lu 2003).

2.4.3. Polynomial or Response Surface Models

Polynomial or response surface are the most commonly used models to describe the relationship between environmental conditions and microbial growth parameters, as well as to determine optimal process conditions (Dalcanton et al. 2018; Devlieghere et al. 2000). Generally, second-order polynomial equations (Equation 2.31) are used, including

three terms: first-order, second-order (quadratic) and interaction terms (Pérez-Rodríguez and Valero 2013).

$$y_i = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum_{j \neq l}^{k} \beta_{jl} X_j X_l + \varepsilon \quad (2.31)$$

where y_i is the dependent variable (e.g. growth rate or lag phase), β_0, β_j, β_{jj} and β_{jl} are the estimated regression coefficients, X_j and X_l are the independent variables (environmental factors), and ε is the error term.

Polynomial models are characterized by a high number of parameters, which increases exponentially when the number of factor included in the model is increased. Response surface models were evaluated to predicted the growth parameters of *Leuconostoc mesenteroides*, cultivated under different combinations of temperature, pH, NaCl and $NaNO_2$, and the optimal composition of culture media for production of biosurfactants by probiotic bacteria *Lactococcus lactis* and *Streptococcus thermophiles* (Rodrigues et al. 2006; Zurera-Cosano et al. 2006).

2.4.4. Bigelow Model

A secondary model equivalent to Bigelow (1921) was developed to describe the effect of temperature on the microbial inactivation rate (Equation 2.32).

$$k_{max} = \frac{ln10}{D_{ref}} = exp\left(\frac{ln10}{z} \cdot \left(T - T_{ref} \right) \right) \quad (2.32)$$

where k_{max} (min^{-1} or h^{-1}) is the maximum inactivation rate, D_{ref} (min or h) the decimal reduction time (D-value) at a reference temperature T_{ref} (°C). Different Bigelow-type model approaches have been developed, including additional physicochemical factors as well as temperature (e.g. pH, a_w, high pressure and etc.) (Adekunte et al. 2010; Gaillard et al. 1998; Mafart and Leguerinal 1998).

2.5. Transfer Models

Cross-contamination is one of the most important factors linked to foodborne outbreaks and food spoilage. According to Pérez-Rodríguez et al. (2008), the cross-contamination phenomenon is defined as a general term that refers to the transfer, direct or indirect, of microorganisms from a contaminated product to a non-contaminated product or due to the environmental conditions where the foods are processed, air, poor hygiene of handlers and contaminated equipment.

In general, the microbial transfer can be classified into three specific types: i) air-to-food transfer (AF); ii) surface-to-food in fluids (SFF); and iii) surface-to-food contact (SFC). The last is the most frequent type, given its high incidence in domestic consumption phase (Pérez-Rodríguez et al. 2008). Although microbial transference phenomena have always been understood as an important cause of food contamination, together with the pronounced need to incorporate them into risk assessment studies, transfer models have only in recent years been implemented in predictive microbiology. Møller et al. (2012) developed a model to describe the transfer and survival of *S.* Typhimurium during the grinding of pork. The model satisfactorily predicted the observed concentrations of *S.* Typhimurium during grinding of meat pork. In other study, Møller et al. (2016) evaluated the robustness of the model obtained before to predict the transfer and survival of *Salmonella* spp. and *L. monocytogenes* during the grinding of meat pork and beef, using two different grinders different sizes and different numbers of pieces of meats to be ground. The parameters obtained under different conditions may not be applied to describe cross-contamination. However, the risk estimates showed that the risk of foodborne disease can be reduced when meat is ground in a grinder made of stainless steel using a well-sharpened knife in cooling room with temperature below 4 °C. Through the application of transfer models, it is possible to identify routes and risk factors associated with microbial transfer using quantitative approach and to predict the number microorganisms transferred from one surface to another (Den Aantrekker et al. 2003; Reij and Den Aantrekker 2004). Cross-contamination models are discussed further on Chapter 12 of the book *"Risk Assessment Methods for Biological and Chemical Hazards in Food*."

2.6. Mixing, Partitioning and Others

Mixing, partitioning and removal are among the main types of unit operations in the food industry. These processes will influence the microbial status of the product in terms of both likelihood of contamination and numbers. Thus, descriptive data of these processes should be collected.

2.6.1. Mixing

Mixing is one the commonest unit operations in the food processing industries with a primary objective of achieving a homogenous mixture. Generally, this means attaining a nearly uniform distribution of the ingredients for improving food quality, such as texture and colour development. Food mixtures involve many ingredients, including liquids,

powders and gases. Some important ingredients are contained only in minor quantities, which should be dispersed evenly and efficiently in the final mixture (Levine and Boehmer 1997). Nowadays, a large majority of processed ready-to-eat products are indeed multiphase dispersions such as: solid dispersed in liquids (e.g. canned foods), emulsions (e.g. soups, margarines and spreads) or bubbly dispersions (e.g. merengue, ice cream and sponge cake, among others) (Niranjan 2009).

In mixing process, food units are combined into new large unit, as show in Figure 2.3.

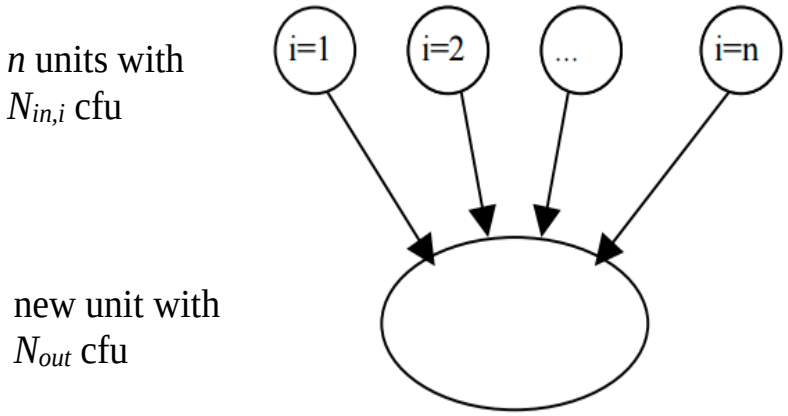

Figure 2.3. Mixing: n small units, containing $N_{in,i}$ cells (particles and spores) in the units i ($i = 1 \ldots n$) are put together to form a new larger unit with N_{out} cells. The objective is to describe the probability distribution of N_{out}, given a distribution of the $N_{in,i}$ (From Nauta 2005 with permission of the authors).

This process affects the food matrix and causes physical or chemical change in the materials being processed. As a consequence the microorganisms can be redistributed, resulting in an increase in prevalence and a decrease in the mean concentration in contaminated food units (Bassett et al., 2012; Nauta 2005). The consequence of mixing process on public health risk is uncertain, because it depends on the mean individual risk, which will be affected in various ways according to the total number of microbial cells reallocated and the pathogenicity of the foodborne pathogen of concern (Augustin et al. 2017). This basic mixing process is commonly encountered in many food production scenarios, such as ground beef manufacturing (Smith et al. 2013), milk collection from multiple farm tanks (Albert et al. 2005), shredded lettuce processing (Danyluk and Schaffner 2011), or processed food manufacturing (Daelman et al. 2013).

There is little published information on the effect of mixing operations on microorganisms. It is unlikely that the shearing conditions or temperature in a mixer would reduce the number of containing microorganisms, and hence mixing does not have

a preservative effect. In some instances, especially where the temperature of the food is allowed to rise during mixing, there may be an increase in number of microbial contaminants, caused in part by the greater availability of nutrients as a result of the mixing action (Fellows 2009). Although these processes do not result in an increase or decrease in the number of microorganisms in the total amount of food produced, they change the distribution of microorganisms among food items. This has an impact on the variability of doses between servings and therefore may have an impact on the risk assessment (Bassett et al. 2012).

The modelling approach for mixing suggested by Nauta (2005; 2008) assumes that if the numbers of cells on or in all small units are known, summation can be used to model the effect of mixing on the number of cells per unit: if n units are combined, with unit i containing $N_{in,i}$ cells ($i = 1 \ldots n$), the larger unit (N_{out}) will contain the sum of all, as in Equation 2.33:

$$N_{in,i}cells = N_{out} = \sum_n N_{in,i} \tag{2.33}$$

Hence, the total number of cells in the system remains the same. The fraction of contaminated units (prevalence) will increase unit size. Assuming random homogeneous mixing and equally size small units, the increased prevalence after mixing of n small units can be estimated as show in Equation 2.34:

$$P_{out} = 1 - \left(1 - P_{in}\right)^n \tag{2.34}$$

Nauta (2005) discussess complications related to the efficient and correct modelling of mixing process and some practical solutions for food chain risk assessment modelling. In model simulations, mixing can be modelled by application of the Dirichlet distribution, a counterpart of the multinomial distribution as applied for partitioning (Nauta 2008). Mathematical methodologies relevant to modelling mixing and partitioning are presented in Nauta (2001; 2008) and Nauta et al. (2001).

2.6.2. Partitioning

Partitioning (fractionation) is a process used in the food industry when a large volume (e.g. an industrial batch) is split up into several small units (e.g. consumer packages), as shown in Figure 2.4; for example, when milk from different cows is placed in the same milk tank and distributed in bottles, or cuts of different carcasses are joined for grinding

and then distributed in packages. Here, the redistribution of microorganisms could result in a lower prevalence and a higher concentration in contaminated food units (Bassett et al. 2012).

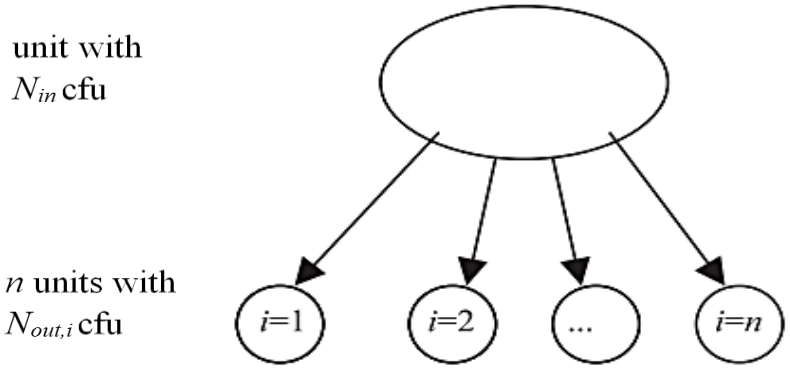

Figure 2.4. Partitioning: a major unit containing N_{in} cells (particles and spores) is split up in n smaller units i ($i = 1 \ldots n$) that contain $N_{out,i}$ cells. The objective is to describe the distribution of the $N_{out,i}$ over the small units given the values of N_{in} and n (From Nauta 2005 with permission of the authors).

For the partitioning model, it is possible to describe the reallocation of the N cells (or spores, particles, colony forming units [cfu]) present in a large unit over n small units. Basically, the N_{in} cells are distributed into n portions $N_{out,i}$ ($i = 1 \ldots n$). The challenge is to find the appropriate model to describe this distribution, which ultimately results in a distribution of N_{out} resulting from a distribution of N_{in}. As the number of units in the food chain increases with portioning, and the total number of cells in the system remains equal, the prevalence is likely to decrease. In the literature, several partitioning models have been proposed and discussed by Nauta (2005).

2.6.3. Removal

Removal process during food processing can be classified into two categories (Nauta 2008). First, removal can be considered as a process whereby some units (or parts of units) are selectively removed from the production. Examples are the rejection of carcasses by veterinary inspectors in the slaughterhouse or the visual discarding of vegetables or fruits due to, for instance, injuries. Thus, in this category, the removal of units is not a random process as removal is performed because there is a presumed relation with microbial contamination and visual appearance. Second, removal can occur during the process of washing, peeling, cutting and filtering, for instance. In this category, the

removal process in principle aims at all units, yielding a similar model as an inactivation model, with a decrease in the number of cells that can be described by Equation 2.35.

$$log\left(N_{out}\right) = log\left(N_{in}\right) - h(.) \quad (2.35)$$

where N_{out} and N_{in} are the microbial concentration (cfu/[g or mL]) at the end and beginning of the process step, respectively, and $h(.)$ is a positive "removal" function. For instance, if the removal process is a washing process, $h(.)$ can be assumed constant or variable for a process step. More complex models can be developed if the removal mechanistic are incorporated in the model (Nauta 2008).

The basic process of removal of *Y. enterocolitica* was considered during derinding of pork belly cuts to produce minced meat (Van Damme et al. 2017). The number of *Y. enterocolitica* after derinding was described through a binomial distribution with the number of *Y. enterocolitica* per belly cut after cutting (process occurring before derinding) and the proportion of the pathogen remaining on the bely cut after derinding as parameters.

2.7. Growth Probability Models (Growth/No Growth)

Growth probability models, also known as growth/no growth, are employed to predict the probability that growth of a microorganism of concern could occur in a food product as a function of intrinsic and extrinsic factors. Growth is not always the only feature of interest for food producers and scientists; in many occasions, the possibility of growth is the most import issue of concern (Carrasco et al. 2012). This approach is often used to predict suitable combinations of hurdles making microbial growth highly unlikely in a specific food product during, for instance, the storage time (Masana and Baranyi 2000). Those models were first applied concerning the prediction of the probability of formation of staphylococcal enterotoxin or botulinum toxin within a specific period of time under conditions of storage and product composition (Genigeorgis 1981; Gibson et al. 1987). Knowledge of these growth/no growth limits under different environmental conditions is important since it enable for a better quality and safety management of foods (McMeekin et al. 2000).

Studies on growth probability test combinations of hurdles used for food preservation under different levels. Typically, a number of repetitions are tested for each combination. After a certain time of incubation, each repetition is examined whether growth occurred or not; 100% growth is considered when growth is confirmed for all repetitions and 0%

when no growth is observed for all repetitions. The classical graphical representation of growth probabilities is illustrated in Figure 2.5. The hypothetical data in Figure 2.5 show the combined effect of pH and temperature on the probability of growth of a microorganism over a certain time period. In this example, from a food safety point of view, a food product with a pH equal to 4.5 should be held at temperatures lower than 20 °C to prevent microbial growth.

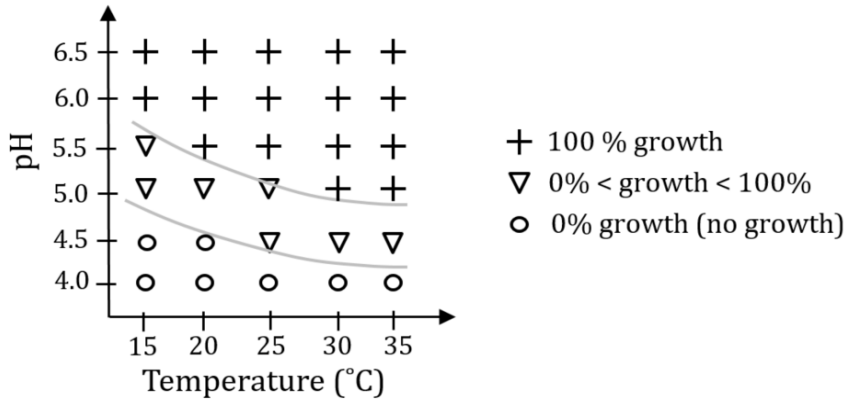

Figure 2.5. Example of a classical graphical representation for growth probabilities according to two environmental factors: in this example, temperature and pH.

The transition from the growth zone, where the probability of growth is 100%, to the no growth zone, where the probability of growth is 0%, can occur gradually, as depicted in Figure 2.5 and as in Haberbeck et al. (2015), Belessi et al. (2011), Mertens et al. (2011) and Vermeulen et al. (2009). Consequently, a growth boundary zone where the probability of growth can be between 0% and 100% exists. For instance, if 5 out of 10 biological repetitions grew, the estimated growth probability equals 50%. This behaviour reflects the heterogeneity in phenotypic response within a population (Sumner and Avery 2002). The observation that some cells of a microbial population are sensitive to an environmental stress, such as low pH and temperature, whereas others are resistant is a readily observed manifestation of heterogeneity (Avery 2006). On other cases, this transition can occur abruptly, and in this case, the zone with different growth probabilities would not be present in the graphical representation, as in López et al. (2007), McKellar and Lu (2001), Presser et al. (1998) and Skandamis et al. (2007). Importantly, the growth/no growth zone is time dependent. Thus, the longer the incubation period prior the verification of growth, the wider the growth zone may become, since the cells would have more chances to initiate growth. Consequently, there is a greater chance that the transition zone will occur abruptly.

Various approaches have been suggested to model the growth/no growth boundary: empirical deterministic approaches (Augustin and Carlier 2000b; Le Marc et al. 2002; Membré et al. 2001), logistic regression (Haberbeck et al. 2015; Kakagianni et al. 2018) and artificial neural networks (Hajmeer and Basheer 2002, 2003). Koutsoumanis and Angelidis (2007), Koutsoumanis et al. (2010) and Sanaa et al. (2004) are examples of studies applying growth/no growth models and concepts into QMRA.

2.8. Model Generation Process

2.8.1. Data Generation: Experimental Design, Data Acquisition and Data Process

The generation of experimental data to describe and to predict microbial behaviour in foods should be structured in some basic steps, starting with the experimental design, followed by the data collection and finally, the data processing.

2.8.1.1. Experimental Design

A premise that must be considered in the experimental design is about the factors, which influence the microbial behaviour. All intrinsic (e.g. pH, water activity and salt concentration) and extrinsic (e.g. temperature and gas concentration) factors related to the food that may vary during the process under study must be carefully checked to incorporate such variations in the predictive model. For refrigerated perishable foods, for instance, the impact of temperature variation on microbial behaviour is commonly analysed, since temperature is a factor that has a great influence on microbial growth, and it can vary greatly throughout the food production chain from manufacturing to consumption. Factors with very small or no variations during the production chain are generally considered constant and are not incorporated into the experimental design. The incorporation of many factors in the experimental design increases the complexity of experiments execution and the proposition of a predictive mathematical model.

The definition of the main factors to be investigated allows one to proceed to the definition of the levels to be considered in the study. The levels depend directly on the range of expected values in each factor to be reached by the product throughout the processes. In the case of storage of perishable products, for instance, the Brazilian legislation allows refrigeration up to 10 °C (Brasil 1984), while in the United Kingdom it is recommended that temperatures should be ≤5 °C (FSA 2015) and in the United State, ≤4.4 °C (USA FDA 2014). However, in practice, if some problem occurs in the cold chain (during transportation of the product or exposure for sale in supermarkets), the product

temperature can exceed the recommended maximum. If experiments are designed and performed to describe the microbial behaviour only in the range from 4 °C to 10 °C (e.g. 4 °C, 6 °C, 8 °C and 10 °C), the predictions by the mathematical model will be valid only within that range of values. Thus, the extent of the model predictions at temperatures above or below the tested range are treated as extrapolation and should be avoided (as depicted in Figure 2.6).

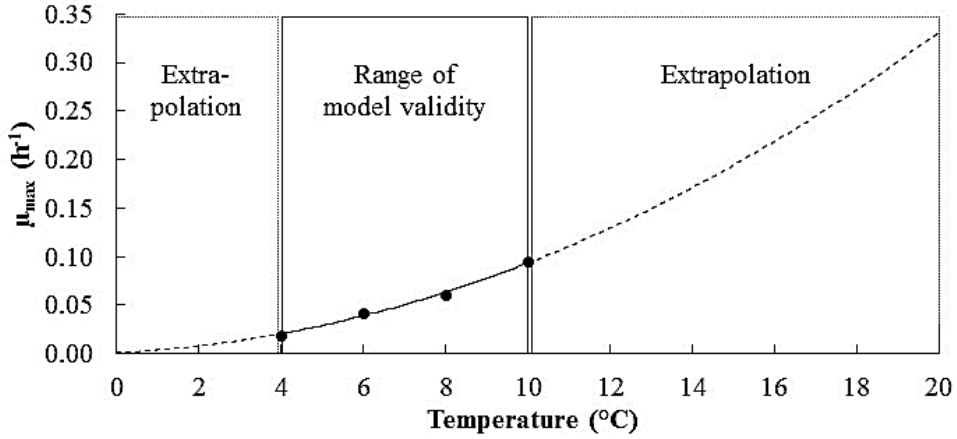

Figure 2.6. Graphical representation of the range of validity of a mathematical model (temperature between 4 °C and 10 °C) and extrapolation.

In this context, many studies involving the prediction of microbial behaviour in perishable foods consider higher than actual temperature ranges to avoid extrapolation, e.g. the growth of *Pseudomonas* spp. in poultry from 2 °C to 20 °C (Gospavic et al. 2008), *L. monocytogenes* in pasteurized milk from 1.5 °C to 16 °C (Xanthiakos et al. 2006), *S. enteritidis* SE86 on homemade mayonnaise from 7 °C to 37 °C (Elias et al. 2016) and *Lactobacillus viridescens* in vacuum-packed sliced ham from 4 °C to 30 °C (Silva et al. 2017).

The appropriate levels for the study can be defined by different strategies of experimental design. Traditional statistical designs, e.g. full and factional factorial designs, in many cases are not suitable in predictive microbiology, since the responses (dependent variables) usually do not present linear and/or parabolic dependence in relation to the factors (as described by the quadratic equation of the response surface). The responses usually present other nonlinear behaviours (secondary models to describe different nonlinear responses were covered Section 2.4). In this context, the optimal experimental design is an interesting alternative, for which some applications in predictive microbiology has been proposed since the late 1990s (Balsa-Canto et al. 2008; Cunha et al. 1997; Longhi et al. 2018; Stamati et al. 2016; Van Derlinden et al. 2008;

Versyck et al. 1999). If appropriate criteria and parameters are used in the design and the experiments are correctly performed, the optimization results in time and resource savings, ensuring parameter estimation with great accuracy. One advantage of the optimal experimental design is the execution of some experiments with optimal variation in the levels within the desired range, instead of performing several experiments under fixed levels within the desired range. A graphical representation of four kinetic growth curves under fixed levels and one optimally designed experiment, both in the range from 4 °C to 10 °C, is shown in Figure 2.7.

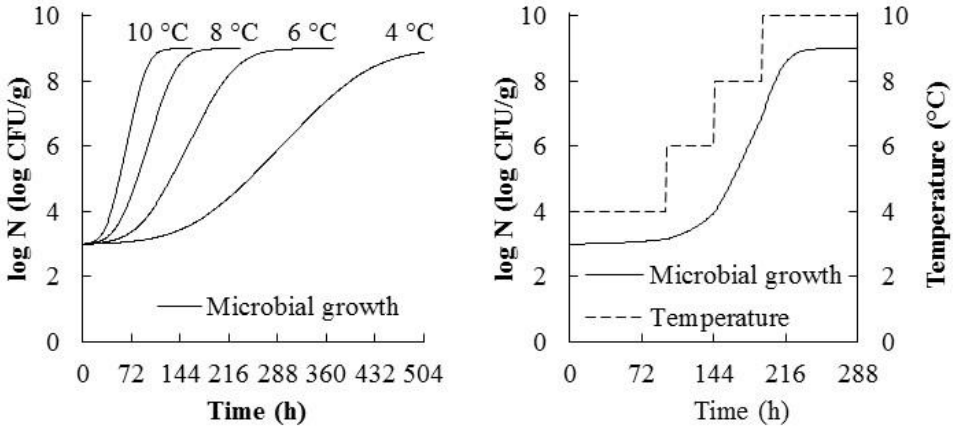

Figure 2.7. Graphical representation of kinetic growth curves under fixed levels (isothermal experiments at 4 °C, 6 °C, 8 °C and 10 °C) and under optimal variation of the levels (non-isothermal experiment between 4 °C and 10 °C) with the respective temperature profiles.

2.8.1.2. Data Collection

A widely used technique for obtaining experimental data in food microbiology is plate counting, which is used to determine the number of viable cells in a sample. The method is usually associated with high cost of analysis and a long time to obtain the responses and often presents moderate uncertainty in the responses. Modern methods, many of them with sophisticated equipment, have been proposed in recent years to obtain microbiological experimental data. These methods are based on molecular biology techniques (e.g. polymerase chain reaction [PCR], restriction fragment length polymorphism [RFLP] and DNA microarray assay), immunological techniques (e.g. enzyme-linked immunosorbent [ELISA]), biophysical and biochemical principles with the application of biosensors (e.g. bioluminescence sensor, bio-analytical sensors utilizing enzymes, electrical impedometry and flow cytometry) (Mandal et al., 2011).

They often depend on high initial investment for equipment acquisition and laboratory intensive work to validate the responses obtained.

The U.S. Food and Drug Administration Bacterial Analytical Manual recommends counting in the range from 25 to 250 cfu (based on 1 mL of sample) on one plate (Maturin and Peeler 2001). As microbiological concentration in a food can be higher than this range, dilutions of the sample are necessary, which may propagate experimental errors and generate uncertainty in the experimental measurements. The number of samples analysed at each time in the experiment is a decisive aspect to minimize this problem; in which is recommended that, at least, two samples should be collected and analysed to generate a duplicate of the experimental data. Then, the result is expressed by the average of the measurements at that time, also allowing the measurement error to be calculated. Depending on the characteristics of the experiment and the available resources, three data points can be obtained, providing triplicate results, which increasing confidence in the experimental data. Another important recommendation in the data collection is the repetition of the experiments aiming to verify its reproducibility. The results of the initial experiment and their repetitions under the same experimental conditions should be as close as possible to prove the reproducibility. The treatment of experimental data for replicates and repetitions will be presented in a next section.

When performing experiments, monitoring and recording data of intrinsic and extrinsic factors is extremely important. Preferably, this should be done by appropriate and calibrated equipment connected to information storage equipment (usually computers). This monitoring ensures that the experiment occurred at the designed levels and that the mathematical model incorporates the correct information.

In the case of kinetic experiments, time is the independent variable and deserves special attention. The establishment of the times required to obtain experimental kinetic data is essential, and for this, the different phases of the microbial behaviour should be remembered. For microbial growth, the lag, exponential and stationary phases (in most cases) can be observed (Figure 2.1), while for microbial death, the inactivation (at constant or variable rate), shoulder and/or tail phases can be observed (Figure 2.2). The total experiment time should be long enough to ensure that all phases of microbial behaviour were detected, but it should be short enough to optimize the response and avoid wasting time. In practice, the experimental time is difficult to determine and usually depends on the researcher's experience and ability to predict the responses to be obtained.

The number of experimental data point and the time for collecting each experimental data point should be delineated in order to optimize the cost of the experiments and later, to ensure that the parameters of the microbial behaviour can be estimated with accuracy. For the parameter estimation of the mathematical models, the minimum number of experimental data points required is equal to the number of parameters of the model plus one. Since most mathematical models have four parameters, the minimum number of experimental data to be collected is five, but for an accurate estimation of the parameters, these five data must be strategically collected to represent all phases of microbial behaviour and phase transitions (e.g. experiment A of Figure 2.8), which is a very difficult task even for experienced researchers. In turn, less experienced researchers tend to perform experiments with some additional data that could be avoided (e.g. experiment C of Figure 2.8). Therefore, a general recommendation is the collection of at least eight experimental data points for a sufficient representation of the complete kinetic curve (e.g. experiment B of Figure 2.8), allowing the three phases of the microbial behaviour, in addition to the phase transitions, to be clearly identified, guaranteeing an accurate estimation of the parameters of the mathematical model.

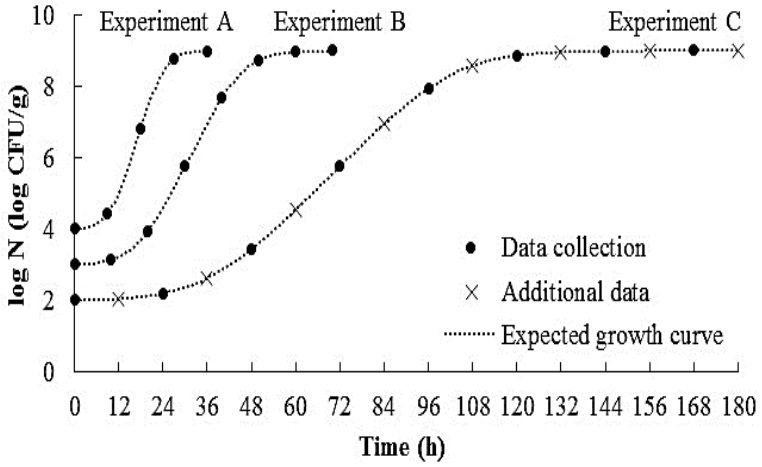

Figure 2.8. Representation of complete growth kinetics model (lag, exponential and stationary phases) with minimal (experiment A), sufficient (experiment B) and additional (experiment C) data collection and experimental time.

2.8.1.3. Data Processing

The initial recommendation for data processing is the organization of information in spreadsheets, especially in some software that will be used later to fit the mathematical models. Among the software available for this task, there are some free software options (no need to pay for a license), such as R, Scilab and Open Office. The feeding of the experimental data in the spreadsheet should be performed in an organized way to carry out the first data processing.

In the case of kinetic studies, it is recommended to dedicate one column to the experimental time and successive columns for the responses obtained from the microbial behaviour. As mentioned in the data collection section, the mean values of each point of the experiment (replicates) should be calculated, as well as the mean errors of each point. Software tools can be used to generate the spread of the experimental data, which contributes to a better presentation and interpretation of the microbial behaviour in relation to the data presentation in tables. In each set of experimental data, it is recommended to check whether there is any very discrepant kinetic data, a so-called outlier. In general, this discrepant data is the result of some experimental error and can be discarded. Figure 2.9 shows a schematic representation of kinetic growth curves with experimental data collected in duplicate (data 1 and 2) and with repeated experiments (experiments A and B).

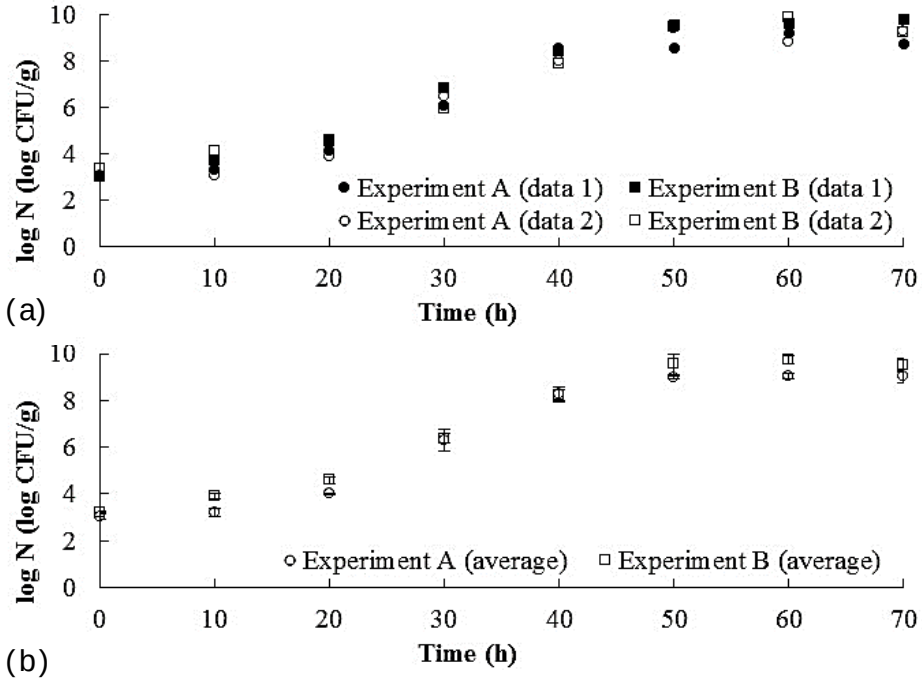

Figure 2.9. (a) Representation of microbial growth kinetics with duplicates of experimental data (data 1 and 2) and repetitions of experiments (experiments A and B). (b) treatment of experimental data with average experimental data and error bars.

2.8.2. Model Fitting and Goodness-of-Fit Indexes

2.8.2.1. Model Fitting

The logarithmic transformation of the experimental data is a statistical strategy for the linearization of the exponential phase of the microbial curve. Similarly, the square root or logarithm transformation of the maximum specific growth rate values in the Square Root and Arrhenius models, respectively, have the same purpose of linearization of the curves. The transformation usually also helps to reduce the variation among the data, which is very important when analysing the impact of the residuals in the step of fitting the models to the data and in the estimation of more precise parameters.

Let us analyse a hypothetical example of fitting a model to the growth data of a microorganism in a food with initial and final concentrations of 10^3 cfu/g and 10^9 cfu/g, respectively. If the mean relative residuals of the fit are equal to 1% in both sets of data, the absolute residuals are of very different order of magnitude, of 10^1 cfu/g and 10^7 cfu/g in the measurements of the initial and final concentrations, respectively. As the procedure of fitting the model to the experimental data is based on the minimization of the residuals between observed data and predicted by the model, the residual of the final concentration data is much more impacting than the residue of the initial concentration data. With the logarithmic transformation of the data, the initial and final concentrations become 3 log cfu/g and 9 log cfu/g, respectively, and the absolute residuals of the measurements become equal (0.00436 log cfu/g).

The fitting of a mathematical model to the experimental data is carried out using software, for which there are several options available today. One can choose to use specific predictive microbiology software available in the internet (e.g. ComBase, GInaFiT, among others, as presented by Plaza-Rodríguez et al. 2017 and Tenenhaus-Aziza and Ellouze 2015) or program the fitting procedure manually using commercial or free software (e.g. Matlab or R). The chosen software should not be considered as a decisive aspect, since it is only auxiliary in the procedure of fitting the model to the data. The mathematical method of fitting the model to the data is also not usually decisive, and different methods of fitting should lead to solutions equivalent to the estimated

parameters. The most decisive criterion is to select the model that best describes the experimental data, and this can be done by analysing statistical indexes of goodness-of-fit of the model to the data.

2.8.2.2. Goodness-of-Fit Indexes

The main statistical indices used to verify the goodness-of-fit in predictive microbiology are the coefficient of determination (R^2), root-mean-square error (*RMSE*), bias and accuracy factors, bias and accuracy discrepancy. The R^2 is the proportion of the variance in the dependent variable that is predictable from the independent variables, and its calculation is defined by Equation 2.36. The *RMSE* measures the differences between data predicted by a model and the experimental data observed, and its calculation is defined by Equation 2.37.

$$R^2 = \frac{\sum_{i=1}^{n}\left(y_{predicted,i} - \bar{y}_{observed,i}\right)^2}{\sum_{i=1}^{n}\left(y_{observed,i} - \bar{y}_{observed,i}\right)^2} \tag{2.36}$$

$$RMSE = \sqrt{\frac{\sum_{i=i}^{n}\left(y_{predicted,i} - y_{observed,i}\right)^2}{n}} \tag{2.37}$$

where $y_{predicted}$ is the data predicted by the model, $y_{observed}$ is the observed data, $\bar{y}_{observed}$ is the arithmetic mean of the observed data and n is the number of observations.

The accuracy factor (a_f) and bias factor (b_f) were proposed by Ross (1996) to evaluate the performance of models in predictive microbiology. According to the author, the indices assess the level of confidence one can have in the predictions of the model and whether the model displays any bias that could lead to fail-dangerous predictions. The accuracy factor is defined by Equation 2.38 and the bias factor is defined by Equation 2.39. In addition to being quantitative, the bias factor is also qualitative, since a bias factor greater than one indicates that the model is "fail-dangerous" because it predicts greater data than the observed, and a bias factor lower than one indicates that a model is "fail-safe" because it predicts lower data than the observed.

$$a_f = 10^{\left(\frac{\sum_{i=1}^{n}\left|\log\left(y_{predicted,i}/y_{observed,i}\right)\right|}{n}\right)} \tag{2.38}$$

$$b_f = 10^{\left(\frac{\sum_{i=1}^{n} log\left(y_{predicted,i} / y_{observed,i}\right)}{n}\right)} \tag{2.39}$$

Baranyi et al. (1999) proposed a different formulation for new accuracy factor (A_f) and bias factor (B_f), as defined by Equations 2.40 and 2.41, respectively. According to the authors, the advantage of the modified definition is that it is consistent with the least squares algorithm of fitting models to observed values. The new factors allow the calculation of "*per cent* discrepancy" (%D_f) and "per cent bias" (%B_f) between model and observations, as defined by Equations 2.42 and 2.43, respectively. Equation 2.44 indicates whether the overall bias is negative or positive, in which if $\%B_f > 0$, on average, model predicts higher data than the observations and if $\%B_f < 0$, on average, model predicts lower data than the observations.

$$A_f = exp\left(\sqrt{\frac{\sum_{i=1}^{n}\left(ln\, y_{predicted,i} - ln\, y_{observed,i}\right)^2}{n}}\right) \tag{2.40}$$

$$B_f = exp\left(\frac{\sum_{i=1}^{n}\left(ln\, y_{predicted,i} - ln\, y_{observed,i}\right)}{n}\right) \tag{2.41}$$

$$\%D_f = \left(1 - A_f\right)\cdot 100\% \tag{2.42}$$

$$sgn\left(ln\, B_f\right) = \left\{\begin{array}{l} +1,\ if\ ln\, B_f > 0 \\ 0,\ if\ ln\, B_f = 0 \\ -1, if\ ln\, B_f < 0 \end{array}\right\} \tag{2.43}$$

$$\%B_f = sgn\left(ln\, B_f\right)\cdot\left(exp\left(ln\, B_f\right) - 1\right)\cdot 100\% \tag{2.44}$$

Confidence on the estimated parameters is another important criterion of analysis. Many models have great goodness-of-fit but parameters with greater uncertainty. This characteristic can be verified by analysing the confidence intervals of the parameters.

2.8.3. Model Validation

Model validation is a procedure that aims to assess the performance of fitted models and to determine whether they can be used to aid decision-making (Haberbeck et al. 2018). It consists of comparing model predictions with independent

experimental/observational data though indices of model performance such as bias factor, accuracy factor and the acceptable simulation zone approach in combination with graphical methods (Mejlholm et al. 2010; Oscar 2005; Østergaard et al. 2014).

Although some predictive models were constructed in real foods, the vast majority was constructed from experiments performed in laboratory culture media. Ideally, for both cases the validation process should include comparisons with the behaviour of microorganisms in real foods or during real food processes. However, due often to cost but also other factors, validation can be done in model systems or using previously published data (Brocklehurst, 2004).

2.9. Conclusions

Modelling the microbial behaviour on a QMRA through the entire farm-to-fork chain is a complex process, but the increasing number of validated predictive models in the literature facilitates this task. The aim of this chapter was to give an overview of predictive models and of the process to generate these models. Deterministic predictive models have been developed and successfully validated for more than 20 years. However, more research the so-called 'stochastic predictive microbiology' should improve the use of predictive models into QMRA. Finally, a generation process and validation of robust predictive models are of great importance, since the final value of the model is significantly influenced by the experimenter's choices. In addition, these predictive models can be used in food processing industries as powerful tools to support and develop food safety standards.

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Chapter 3

Study of the microbiological quality, prevalence of foodborne pathogens and product shelf-life of Gilthead sea bream (*Sparus aurata*) and Sea bass (*Dicentrarchus labrax*) from Aquaculture in Estuarine Ecosystems of Andalusia (Spain)

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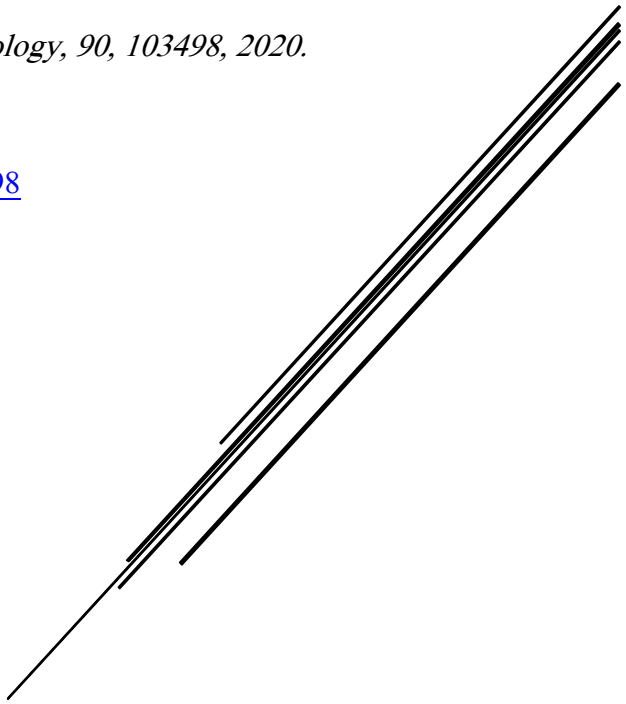

HIGHLIGHTS

- Microbial counts were statistically similar in Gilthead sea bream and Sea bass.
- *Vibrio parahaemolyticus* was isolated from estuarine water.
- Neither *Listeria monocytogenes* nor *Salmonella* spp. were detected in fish and water
- High microbial levels were found in fish viscera in comparison with skin.
- The initial microbiological quality could have impact on the product shelf-life.

Abstract

This study was aimed at characterizing microbiologically Gilthead sea bream (*Sparus aurata*) and Sea bass (*Dicentrarchus labrax*) produced in two estuarine ecosystems in Andalusia (Spain): the estuary of the river *Guadalquivir* (La Puebla del Río, Sevilla) (A), and the estuary of the river *Guadiana* (Ayamonte, Huelva) (B). The collected fish individuals and water were analysed for hygiene indicator microorganisms and pathogens. The statistical analysis of results revealed that microbial counts for the different microbiological parameters were not statistically different for fish type. On the contrary, considering anatomic part, viscera showed significantly higher concentrations for *Enterobacteriaceae*, total coliforms and for *Staphylococcus* spp. coagulase +. Furthermore, location A showed in water and fish higher levels for lactic acid bacteria, aerobic mesophilic bacteria, *Enterobacteriaceae*, total coliforms and *Staphylococcus* spp. coagulase +. Neither *Listeria monocytogenes*, nor *Salmonella* spp. were detected, though *Vibrio parahaemolyticus* was identified, molecularly, in estuarine water in location B. The predictive analysis demonstrated that the initial microbiological quality could have an impact on product shelf-life, being longer for location B, with better microbiological quality. Results stress the relevance of preventing the microbiological contamination of water in estuary production systems in order to assure the quality and safety of Gilthead sea bream and Sea bass.

Keywords: *Vibrio parahaemolyticus*; Shelf-life; 16S ribosomal RNA sequence; Molecular identification; Predictive microbiology; Fish farm.

3.1. Introduction

Aquaculture is an important economic activity in the European Union (EU), being the major source of aquatics products of the market. In 2016, the EU produced 1.3 million tons in aquaculture products valued in 3,729 million EUR, an increase of 3.9%, related to the market in 2015. Spain is the first EU aquaculture producer according to the volume of aquaculture production, 283,831 tons in 2016 (22.1% of the total Union production), valued in 449.4 million EUR (10.2% of the total UE value) (APROMAR, 2018). Aquaculture production in Spain is the most diverse in Europe due to, among other aspects, the longitude of its coastline and the diversity of environments (APROMAR, 2014). At national level, Andalusia is the main producer of Gilthead sea bream (*Sparus aurata*) and Sea bass (*Dicentrarchus labrax*).

The consumption of fishery products has increased recently because of the rising consumer awareness on the health benefits of fish as well as the variety of species available in the market (Domingo, 2007).

Muscle tissue of fish is usually free of microorganisms at the moment of capture while bacteria are normally present in skin, mucus, digestive tract and gills (ICMSF, 2005). Composition of the natural microbiota and the presence of pathogens in fish can be related to different factors such as production methods, cultivation techniques, capture system, water environmental conditions or the intestinal tract of living species (Gram, 2009; ICMSF, 2005; Painter et al., 2013; Svanevik et al., 2015). Natural microbiota in fish can also change due to the different levels of tolerance of microorganisms to preservation conditions (Gram and Dalgaard, 2002). Moreover, contamination with pathogenic bacteria can occur because the unhygienic conditions of the landing place in fish boats or when the fish is washed with contaminated water (Bolívar et al., 2017; Mokrani et al., 2018).

In general, the microbiota that represents the microbial ecology of Mediterranean fish can include diverse microbial groups and genera such as sulphite-reducing clostridia (SRC), *Aeromonas, Enterobacter, Escherichia coli, Lactobacillus, Listeria, Salmonella, Pseudomonas, Photobacterium, Shewanella, Vibrio,* yeasts and some molds (Bolívar et al., 2017; Carrascosa et al., 2015; Esteve et al., 2012; Koutsoumanis and Nychas, 2000). These include microbial species that can cause product deterioration (i.e. spoilage microorganisms) such as Lactic Acid Bacteria (LAB) and *Pseudomonas* spp. (Françoise, 2010; Parlapani et al., 2014; Ringø, 2008). The latter microorganism is considered the

specific spoilage organism (SSO) of unpackaged fresh Mediterranean fish (e.g. Gilthead sea bream) while LAB are more related to spoilage of reduced oxygen packaged fish products (Ghaly et al., 2010; Koutsoumanis, 2001; Tryfinopoulou et al., 2002). Some of the genera present in fish and water microbiota can include pathogenic microorganisms. The presence of enteric pathogens such as *Salmonella* spp. in marine environments may arise from the contamination by animal faeces or discharge of untreated sewage (Lee et al., 2008). Another important pathogen associated with foodborne infections via seafood is *Listeria monocytogenes*, which can contaminate fish due to cross-contamination during handling and preparation and then grow during distribution and storage due to its psychrotrophic properties (Huss et al., 2000; Jami et al., 2014). Furthermore, some species of *Vibrio*, adapted to marine and estuarine water conditions, are pathogenic, and can cause foodborne diseases, being a relevant pathogen of this type of marine ecosystems (Chakraborty et al., 1997).

Vibrio spp. are Gram-negative, rod-shaped bacteria and mostly halophilic (Thompson et al., 2004) naturally found in estuarine or marine environments throughout the world. *Vibrio vulnificus* and *Vibrio parahaemolyticus* are the most common *Vibrio* species associated with human foodborne diseases resulting from consumption of raw or partially cooked fish (Tsironi et al., 2017). Infection caused by *Vibrio* spp. (foodborne disease) is characterised by haemorrhagic septicaemia and produces symptoms such as diarrhoea, nausea, vomiting, fever, chills or other extra intestinal infections (Austin, 2010; Dong et al., 2016). Infection outbreaks specifically caused by *V. vulnificus* and *V. parahaemolyticus*, have been reported in Spain, mainly due to the consumption of oysters (Lozano-León et al., 2003; Martinez-Urtaza et al., 2004, 2005, 2016), and other countries that are part of the Mediterranean Sea (Bisharat et al., 1999; Hervio-Heath et al., 2005; Ottaviani et al., 2010).

Natural microbiota and pathogenic bacteria of wild Gilthead sea bream and Sea bass have been characterised by several research groups (Floris et al., 2013; Haldar et al., 2010; Kahla-Nakbi et al., 2006; Parlapani and Boziaris, 2016). However, only few works have jointly included the study of food hygiene indicator microorganisms and pathogenic bacteria in Gilthead sea bream and Sea bass from aquaculture (Balebona et al., 1998). Food hygiene indicator microorganisms are usually employed to assess overall hygienic or environmental conditions that can be associated with an increased risk of exposure to a pathogen (Ghafir et al., 2008; Tortorello, 2003) and/or a reduced product shelf-life due

to microbial spoilage (Koutsoumanis and Nychas, 2000). The most representative and widely used food hygiene indicator microorganisms in fish products comprise aerobic mesophilic bacteria or aerobic plate count (APC), which is used as general indicators of microbiological quality, and *Enterobacteriaceae*, coliforms and *E. coli*, which are mostly used to assess poor hygienic practice and enteric contamination (Anihouvi et al., 2019; Eizenberga et al., 2015; Papadopoulou et al., 2007; Pierson et al., 2007; Popovic et al., 2010).

The objectives of this work were i) to investigate and quantify the presence of pathogenic bacteria and hygiene indicator microorganisms in Gilthead sea bream (*Sparus aurata*) and Sea bass (*Dicentrarchus labrax*) produced in two estuarine fish farms, corresponding with two different estuarine ecosystems in Andalusia (Spain) and ii) to assess the potential impact of the microbial load found in the captured fish species on the product shelf-life.

3.2. Material and Methods

3.2.1 Samples and transport

The Gilthead sea bream and Sea bass specimens were collected over a 6-month period (November–April) from two estuarine fish farms, located in different estuarine ecosystems. For the first location (A), fish was bred and reared in tanks containing water from the estuary of the river *Guadalquivir* (La Puebla del Río, Sevilla, Spain) while in the second location (B), fish was breed in natural ponds located in the estuary of the river *Guadiana* (Ayamonte, Huelva, Spain). A total of 95 individuals were collected for both production locations and fish species, corresponding to 20 and 25 individuals for Gilthead sea bream and Sea bass, respectively, for location A and 25 individuals for each species for location B. For sampling, fish specimens showing a suitable commercial size were harvested and slaughtered in liquid ice. Samples of water from both production locations were taken at the time of capture of both fish species. Sterile bottles with a volume of 100 mL were submerged to the bottom of the tank, collecting the samples homogeneously. Then, samples were transported under refrigeration temperatures in thermal boxes with ice (<4 °C) up to laboratory (Department of Science and Food Technology, University of Córdoba, Spain) and immediately analysed.

3.2.2. Sample preparation

The fish samples were prepared without previous manipulation. Firstly, the skin was removed with scalpel and the ventral belly surfaces of the fish were opened to expose the peritoneal cavity to extract all viscera. The process was carried out under aseptic conditions. Tools and instruments used to collect skin and viscera samples were previously sterilized in autoclave (121 °C; 15 min) and between samples and specimens, tools were flamed in ethanol to avoid cross contamination. Skin and viscera were weighted and introduced in stomacher bags containing the corresponding diluent according to the type of microorganism(s) to be analysed (*section 3.2.3*), to obtain a 1:10 dilution. The same for water samples. Diluents used were peptone water (PW, Oxoid, UK), *Listeria* fraser broth (Oxoid) or alkaline saline peptone water (ASPW, Oxoid). Then, all samples were homogenized during 60 s at 1500 rpm using a stomacher (Masticator, IUL Instruments, Spain).

3.2.3. Microbial analysis

Samples (skin, viscera and water) were microbiologically analysed for lactic acid bacteria (LAB), aerobic mesophilic bacteria (AMB) (ISO 4833–2:2003), *Enterobacteriaceae* (ENT) (ISO 7402:1993), total coliforms (TC) (ISO 9308-2:2013), *Staphylococcus* spp. coagulase + (STAP) (ISO 6888-2:1999), *Clostridium perfringens* (ISO 7937:2005), *Escherichia coli* (ISO 16649-1:2003), *Listeria monocytogenes* (ISO 11290-1:1997/A1:2005), *Salmonella* spp. (ISO 6579:2003), *Vibrio* spp. (ISO 21872-1:2007) and Bacillus cereus (ISO 7932:2005).

3.2.4. Water quality parameters

The water quality parameters of salinity, temperature, pH and total nitrogen for location A and B were taken from the monitoring system developed by the Department of Environmental Health of the Regional Government of Andalusia (i.e. Consejería de Medio Ambiente, Junta de Andalucía, Spain) available at the website http://laboratorioredíam.cica.es/Visor_DMA/. This on-line application shows information on the water quality in the intra-community hydrological demarcations of Andalusia. Sampling stations can be localized for a specific geographical area based on a Web Map Service (WMS), i.e. georeferenced map images, allowing for downloading water quality parameters measured for a specific location within a defined period. Data

for location A and B were taken from the closest sampling stations located in the surrounding area for the periods of time when samples were obtained.

3.2.5. 16S RNA amplification and sequencing for molecular identification for bacterial isolates

For molecular identification of bacterial isolates, gene coding for 16S ribosomal RNA was amplified by PCR combining oligonucleotide rrn-f27 with rrn-519, 1406 R or pH depending on the length of the expected fragment. These oligonucleotides plus those used for DNA sequencing are listed in Table 3.1. For PCR, one colony was resuspended in 100 µL of sterile deionized water, boiled for 10 min and centrifuged prior use. When necessary, total DNA was extracted following the procedure described by Ruiz-Barba et al. (2005). Briefly, a single colony was resuspended in 100 µL of sterile deionized water and 100 µL of chloroform/isoamyl alcohol (24:1) was added. After vortexing, the mix was centrifuged at 16.000 g for 5 min at 4 °C and 10 µL of the upper aqueous phase was used as template for PCR reactions. To standardise, Illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare) were used for PCR reactions. Bands were purified from gel using GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). Sequencing was carried out by Secugen (Madrid). Candidates were identified by comparing the 16S gene DNA sequences to sequences from type material at the databases using Blastn (Altschul et al., 1990) and selecting the best score with default parameters.

Table 3.1. Oligonucleotides used to amplify and sequence the 16S ribosomal RNA gene.

Oligonucleotide	Sequence 5'-3'[a]	Position[b]	Reference
rrn-f27	AGAGTTTGATCMTGGCTCAG	8-23	Hugenholtz et al. (1998)
rrn-r519	GTATTACCGCGGCTGCTG	536-519	Hugenholtz et al. (1998)
1406R	ACGGGCGGTGTGTMC	1406-1392	Olsen et al. (1986)
pH	AAGAGGTGATCCAGCCGCA	1542-1522	Edwards et al. (1989)
green_JP	CCTAGGTGGGATTAGCTA		This work
purple_JP	AACATTTCACAACACGAG		This work
yellow_JP	ATCTCTACGCATTTCACC		This work
red_JP	TTTCAGTCGTGAGGAAGG		This work

[a] M= A or C.
[b] Numbering is based upon the *E. coli* sequence.

3.2.6. Statistical analysis

Microbial counts obtained in the collected samples were transformed logarithmically (log cfu/g). Data were then analysed statistically using the non-parametric test of Kruskal-Wallis for multiple comparisons as the assumptions of the Analysis of Variance

(ANOVA) were not met. The statistical test assessed whether factors like production location (A and B), species (Gilthead sea bream and Sea bass) and anatomical part (skin and viscera) exerted a significant effect ($p < 0.05$) on the microbiological parameters. The data analysis was performed in IMB SPSS Statistics 22.0 (IMB Corp. Released 2013. Version 22.0. Armonk, NY).

3.2.7. Fish shelf-life assessment

With the aim of illustrating the importance of the initial microbial concentration at the harvest point on the shelf-life of fresh fish (i.e. Gilthead sea bream and Sea bass), an example was developed based on the use of Predictive Microbiology models. Shelf-life was determined considering aerobic mesophilic bacteria, enumerated in the present study, as spoilage microorganisms, and simulating their growth under aerobic conditions at 4 and 8 °C, which represent for the maximum refrigeration temperature recommended by manufacturers and a mild abuse temperature, respectively. For that, the initial concentrations found in location A and B were used. To estimate microbial growth, a predictive microbiology model for *Pseudomonas* spp. developed in Gilthead sea bream was used (Koutsoumanis and Nychas, 2000).

The application of the models was performed on the predictive software, MicroHibro (www.microhibro.com) (González et al., 2019), which is a scientifically validated online tool able to estimate microbial kinetics in foods and food processing environments under specific conditions defined by users.

3.3. Results and Discussion

3.3.1. Hygiene indicator microorganisms

Table 3.2 shows the microbiological results obtained for skin and viscera of Gilthead sea bream (*S. aurata*) and Sea bass (*D. labrax*), treated jointly (i.e. mean of both fish species) as well as for water of two different production locations. Since data showed a heteroscedastic character (i.e. non-homogeneous variance) that could not be removed by variable transformation, the application of parametric tests based on the General Linear Model (GLM) were not appropriate. Therefore, a non-parametric test, Kruskal Wallis test for independent samples, was employed to determine the statistical effect of the different factors considered in the study (location, species and anatomic parts).

Table 3.2. The mean microbial counts from Gilthead sea bream (*S. aurata*) and Sea bass (*D. labrax*) (treated jointly) and water considering as factors the two different production locations and anatomical parts.

		Lactic acid bacteria	Aerobic mesophilic bacteria	*Enterobacteriaceae*	Total coliforms	*Staphylococcus* coagulase +
Anatomic part	Skin	2.44 ± 0.84[a]	2.95 ± 0.94	1.73 ± 0.67	1.97 ± 0.73	2.79 ± 0.63
	Viscera	2.31 ± 0.44	3.08 ± 0.61	2.32 ± 0.83	2.41 ± 0.91	2.40 ± 0.38
Location[b]	Location A	2.99 ± 0.36	3.74 ± 0.33	2.75 ± 0.35	2.99 ± 0.32	3.08 ± 0.35
	Location B	1.77 ± 0.18	2.28 ± 0.29	1.30 ± 0.37	1.39 ± 0.31	2.11 ± 0.18
	Total	2.38 ± 0.43*	3.01 ± 0.52*	2.03 ± 0.55**	2.19 ± 0.59**	2.60 ± 0.37**
Water	Location A	2.48 ± 0.59	3.48 ± 0.42	3.48 ± 0.62	3.38 ± 0.32	2.98 ± 0.46
	Location B	1.75 ± 0.41	2.25 ± 0.85	0.97 ± 0.23	2.18 ± 1.24	2.13 ± 0.36
	Total	2.12 ± 0.36	2.87 ± 0.61*	2.23 ± 1.26*	2.78 ± 0.60	2.56 ± 0.43

One and two asterisks in columns indicate the existence of statistical effect of location and anatomic part, respectively ($p < 0.05$) on the different microbiological parameters.
[a] mean± standard deviation.
[b] Location: A (estuary of the river Guadalquivir, Sevilla, Spain) and B (estuary of the river Guadiana, Huelva, Spain).

According to the statistical analysis, no significant differences were found for the type of fish species ($p > 0.05$). On the contrary, concentration levels for *Enterobacteriaceae*, total coliforms and *Staphylococcus* spp. coagulase + were statistically different for the factor anatomic part ($p < 0.05$), with higher levels in viscera for the two first microbiological groups, and lower for *Staphylococcus* spp. coagulase +. Similarly, the statistical test reported significantly higher concentrations, by more than 1 log cfu/g, in LAB, AMB, *Enterobacteriaceae*, TC and *Staphylococcus* spp. coagulase + in location A ($p < 0.05$). The mixture of fresh water and sea water and a possible failure in water treatment systems are the main causes that might explain the higher microbial levels in location A (Grigoryan et al., 2014). Temperature and salinity of water may also play a relevant role in the microbiota profile in fish (Alexopoulos et al., 2011; Lesel, 1990). In our case, salinity values did not show noticeable difference between both locations (Table 3.3). On the contrary, temperature values measured in location A were, on average, 3 °C higher than those obtained in location B. These higher temperature values could increase, in general, the growth potential of mesophilic bacteria, and in particular, for those that are more sensitive to low temperatures (i.e. high minimum growth temperature), in which slight temperature increases, close to the growth limits, could lead to a significant growth. The latter case could result in higher counts of microorganisms like *E. coli* and *Staphylococcus aureus,* that present a minimum growth temperature >7 °C (Schmitt et al., 1990; Shaw et al., 1971).

Table 3.3. Water quality parameters recorded in location A (river *Guadalquivir*) and location B (river *Guadiana*).

Parameters	Location A	Location B
Salinity (g/kg)	2.9 ± 0.9[a]	3.3 ± 2.9
Temperature (°C)	15.4 ± 2.5	11.9 ± 0.9
pH	8.3 ± 0.1	8.1 ± 0.1
Total nitrogen (mg/L)	0.3 ± 0.1	6.7 ± 1.6

[a] Mean± standard deviation.

Interestingly, total nitrogen concentration was much lower in location A (<1 mg/L) in comparison with values in location B, which were 6.7 mg/L. The presence of *Staphylococcus* spp. coagulase + (2.6 log cfu/g), which is not part of the natural microbiota of fresh fish (Al Shabeeb et al., 2016; Huss, 1988), can also support the hypothesis that the high content of organic matter in water as well as poor hygiene or sanitation during capture could favour higher counts for this microorganism in location A (Abrahim et al., 2010; Herrera et al., 2006; Simon and Sanjeev, 2007). The relevance of the microbiological quality of water in the

microbiological load and profile found in fresh fish was also confirmed in our study (Table 3.2). The highest contamination levels were found in water from location A, in concordance with the high counts obtained in fish samples from this location. Furthermore, greater concentration values were detected for *Enterobacteriaceae* and total coliforms in water (> 3 log cfu/mL) than in fish samples (Table 3.2).

For *C. perfringens* and *E. coli*, no counts were obtained in some conditions, producing an unbalanced data matrix and thus impeding the statistical analysis of data. Table 3.4 shows counts (log ufc/g), percentages (%) of positive anatomical part samples of each species and percentage (%) of positive specimen (individual) samples (considering jointly both species) for *C. perfringens, E. coli* and *B. cereus*, expressed per location. Although results were not statistically validated due to the unbalanced data matrix, they suggest a higher prevalence of *C. perfringens* in location A (i.e. 71% individuals). Sabry et al. (2016) also found a high occurrence of *C. perfringens* in fresh fish derived from aquaculture (55%), which contrasts with the values obtained from canned fish collected from supermarkets (18%), where a thermal treatment is applied. Our results highlight the importance of the external surface of fish as a potential source of contamination of *C. perfringens*.

Table 3.4. Microbial counts and percentages of prevalence of presumptive *Clostridium perfringens*, *Escherichia coli* and *Bacillus cereus* isolated from skin and viscera of Gilthead sea bream (*S. aurata*) and Sea bass (*D. labrax*) and water collected from two production locations.

Production location	Type of sample	Anatomical Part	*C. perfringens* (log cfu/g)	(%)[d]	*E. coli* (log cfu/g)	(%)	(%) *B. cereus*
A[a]	Gilthead sea bream (n = 20)	Skin	ND[c]	-	ND	-	100.0
		Viscera	1.70	35	2.23	5.0	100.0
	Sea bass (n = 25)	Skin	2.15	100	ND	-	100.0
		Viscera	2.10	100	2.92	4.0	100.0
	Total[b]		1.98±0.20	71.1	2.58±0.34	4.4	100
B	Gilthead sea bream (n = 25)	Skin	1.76	30	ND	-	44.0
		Viscera	1.70	35	2.06	4.0	20.0
	Sea bass (n = 25)	Skin	2.0	8	ND	-	68.0
		Viscera	ND	-	ND	-	60.0
	Total		1.82±0.13	18.3	2.06±0.01	2.0	66.0
A	Water (n = 10)	-	ND	-	ND	-	100.0
B			ND	-	ND		60.0
	Total		-	-	-	-	80.0

[a] Location: A (estuary of the river Guadalquivir, Sevilla, Spain) and B (estuary of the river Guadiana, Huelva, Spain).
[b] The percentages given in the rows of Total are referred to the of positive individuals of both species per location.
[c] ND, below limit of detection (<10 cfu/g).
[d] The percentage values provided in the rows for anatomical part are referred to the positive anatomical part samples per fish species and location.

E. coli was detected, with a low prevalence (< 5% individuals), in viscera of both species from location A and only of Sea bream from location B (Table 3.4). This result confirmed that *E. coli* could be present in fish viscera, probably, as part of the normal intestinal microbiota. Alexopoulos et al. (2011) evaluated the microbial ecology of Sea bream and Sea bass and found 30.7% positive samples for *E. coli* (n = 75). Their presence could result in cross contamination of fillets during fish preparation or handling during processing (Noor Uddin et al., 2013; Pao et al., 2008).

Overall, presumptive *B. cereus* was detected (>10,000 cfu/g) in 80% total analysed individuals (Table 3.4). The total of individuals in location A was contaminated with this microorganism including skin and viscera of both fish species, yielding 100% of prevalence (n =45); while for location B, the total prevalence was 66% for both body parts. In water, the trend was similar with 100% samples contaminated in location A, and 60.0% in location B. No molecular characterisation was performed for this microorganism; therefore, no pathogenicity could be assessed in this study. Nonetheless, the fact that the microorganism was found in fish and water could suggest that the main source of contamination was the water.

3.3.2. Foodborne pathogens

Neither *Salmonella* spp. nor *L. monocytogenes* were detected in any fish or water sample in this study. Although *Listeria* is not considered a marine microorganism, there are three possibilities for its presence in fish: (1) water runoff from contaminated agricultural areas or other sources of contamination, such as animal feces that can increase the abundance of the pathogen in aquatic systems (Lyautey et al., 2007); (2) the spread of *Listeria* from the intestinal contents to other fish tissues, especially if the period between death and removal of viscera exceeds 2 h (Jami et al., 2014) and; (3) cross-contamination due to fish manipulation using contaminated equipment and inappropriate transport (De Souza et al., 2008; Gudmundsdóttir et al., 2008). The contamination level by *L. monocytogenes* in raw fish tends to be low and varies between 0% and 30% (Jami et al., 2014; Miettinen et al., 2003; Thomas et al., 2012). Since microbiological determinations were performed on fish just harvested, the absence of positive samples for *L. monocytogenes* and *Salmonella* spp. shows that natural contamination by both pathogens was an unlikely event in the production locations sampled. Nonetheless, it cannot be discarded that the pathogens could be present in water or fish at levels below the detection limit of the used techniques, or that products could be later contaminated in

other relevant points along their production chain (i.e. evisceration, slicing, preparation and handling).

3.3.3. Characterization and molecular identification of Vibrio *spp.*

All samples were positive for presumptive *Vibrio* spp. (specimens and water from location A and B). To confirm positive results obtained on selective agars, a non-exhaustive molecular identification of *Vibrio* spp. isolates was carried out by PCR amplification and sequencing of the 16S RNA gene (Supplementary Table 3.1). Most of the identifications were carried out using the almost complete 16S gene sequence but in some cases, only a *ca.* 500 bp fragment obtained with oligos rrn-f27 and rrn-r519 that includes the variable regions V1, V2 and V3 could be amplified and sequenced.

According to the molecular characterisation, 82 isolates out of the total number of presumptive *Vibrio* ssp. analysed ($n = 92$) were confirmed as *Vibrio* spp. which represented for 89.1% isolates, whereas the rest (10.9%) corresponded to *Shewanella* spp. The latter results corresponded to isolates obtained from the Thiosulfate-citrate-bile salts-sucrose (TCBS) agar used for the isolation of *Vibrio* spp. These isolates belonged to samples of Sea bass from location B in both skin ($n = 8/10$) and viscera ($n = 2/10$). Although TCBS agar is an effective and differential medium for the isolation of *Vibrio* spp. from estuarine and marine waters (Bolinches et al., 1988; Oliver, 2011), a number of non-vibrios (i.e. *Acinobacter*, *Enterobacter*, *Flavobacterium*, *Shewanella* and *Pseudomonas* spp.) are known to grow on TCBS (Lotz et al., 1983; Thompson et al., 2004). Despite *Shewanella* species usually produce black colonies in TCBS due to the ability of FeS precipitation as a result of H_2S production from thiols (Oliver, 2011), in our study, the isolates identified as *Shewanella* spp. exhibited green colonies, similar to the characteristic colonies of *Vibrio* spp. These findings underline the importance of performing a molecular identification in order to reduce false positive of *Vibrio* spp.

From the confirmed *Vibrio* spp., 89.0% was isolated from skin and viscera from both fish species corresponding to a total of 29 and 17 individuals from location A and B, respectively; whereas 11.0% isolates were obtained from water samples collected in both production locations. Scarano et al. (2014) detected *Vibrio* spp. in Gilthead sea bream obtained from mariculture farms located in three different Italian regions. The prevalence was of 58.7% in skin samples and 41.7% in viscera.

Table 3.5 shows the identified *Vibrio* species for Gilthead sea bream and Sea bass, including water for both production locations. *Vibrio anguillarum* (previously referred

as *Listonella anguillarum*) was the species most identified in our study, comprising 69.5% isolates (*n* = 57/82). Canak and Akayli (2018) also isolated, as predominant species, *V. anguillarum* in addition to V. scophthalmi from the visceral organs of Gilthead sea bream samples. In general, *V. anguillarum* is considered as a pathogen of fish, including seafood, causing considerable economic losses in the fishing and aquaculture industries due to its high morbidity and mortality rates (Frans et al., 2011; Hickey and Lee, 2017). *Vibrio* spp. tends to be more common in warmer water when temperature above 15 °C (Osunla and Okoh, 2017). Recently, the first case of *V. anguillarum* associated with a human case resulting in death was reported. Based on the epidemiological investigation, the exposure of a small wound or the fly bite to contaminated water and/or the consumption of seafood contaminated with *Vibrio* could be considered as the source and route of infection (Sinatra and Colby, 2018).

Table 3.5. Molecular identification of *Vibrio* spp. isolated from skin and viscera of Gilthead sea bream (*S. aurata*) and Sea bass (*D. labrax*) and water collected from two production locations ($n = 82$).

Species	Anatomical part	*V. aestuarianus subsp. francensis* 02/041	*V. alfacsensis* CAIM 1831	*V. alginolyticus*	*V. anguillarum*	*V. pacinii* LMG 19999	*V. neocaledonicus* NC 470	*V. parahaemolyticus* ATCC 17802	*V. ponticus* 69
Gilthead sea bream	Skin ($n = 13$[a])	0	0	0	13[d]	0	0	0	0
	Viscera ($n = 13$)	1	0	1[b]	11[d]	0	0	0	0
Sea bass	Skin ($n = 26$)	3	0	1[b]	14[d]/1[e]	7	0	0	0
	Víscera ($n = 21$)	3	1	0	17[d]	0	0	0	0
Water	($n = 9$)	1	0	2[b]/2[c]	1[d]	0	1	1	1

[a] number of confirmed *Vibrio* spp isolates.
[b] *Vibrio alginolyticus* NBRC 15630.
[c] *Vibrio alginolyticus* ATCC 17749.
[d] *Vibrio anguillarum* NBRC 13266.
[e] *Vibrio anguillarum* DSM 21597.

Vibrio aestuarianus subsp. *francensis* was identified, corresponding to 9.8% isolates (*n* = 8/82). *V. aestuarianus* was first isolated and reported by Tison and Seidler (1983) as a new species from estuary waters and shellfish and was described as showing close similarity with *Vibrio anguillarum*, *Vibrio ordalii* and *Vibrio pelagius* (Goudenège et al., 2015; Pillidge et al., 1987) and therefore included in the Anguillarum clade on the basis of multilocus sequence analysis (MLSA) (Sawabe et al., 2007). This subspecies is associated with the syndrome known as "summer mortality" of the oyster (*Crassostrea gigas*) in the French coast (Garnier et al., 2008). There are few studies that characterise *V. aestuarianus* species in fish, in particular in Sea bass and Sea bream (Scarano et al., 2014), since this species shows major prevalence and high rate of mortality in mollusks and oysters (Romero et al., 2014; Travers et al., 2017).

Vibrio alginolyticus and *Vibrio pacinii* had a percentage of isolation of 7.3% (*n* = 6/82) and 8.5% (*n* = 7/82), respectively (Table 3.5). In our study, *V. alginolyticus* species were found in water (i.e. 4 isolates), viscera and skin (i.e. one isolate for each anatomic part) of Gilthead sea bream and Sea bass, respectively, from location B. Saad et al. (2015) found similar results, 2 isolates for *V. alginolyticus* from farm water cultivated with *Tilapia nilotica*. Abdel-Aziz et al. (2013) also detected one isolated of *V. alginolyticus* on skin of Sea bream while for Sea bass, it was not detected. This *Vibrio* species is a natural host of estuarine and marine water (Baffone et al., 2000) and is considered one of the most dangerous pathogens causing severe economic losses in aquaculture throughout the Mediterranean area and worldwide (Kahla-Nakbi et al., 2006). In humans, *V. alginolyticus* has been considered an emerging pathogen of foodborne disease (Mustapha et al., 2013). Studies have reported human infection with *V. alginolyticus* causing serious symptoms especially among vulnerable groups such as elderly people and mortality in immunocompromised patients (Campanelli et al., 2008; Horii et al., 2005; Reilly et al., 2011). In addition, Sabir et al. (2011) related the load of *V. alginolyticus* and sea surface temperature with a clear distinction between the seasons of the year (i.e. cold and warm seasons) and suggested that the number of infections in humans may increase with the coastal warming attributed to climate change.

Other less frequent isolated species were *V. alfacsensis* (sea bass viscera), *V. neocaledonicus*, *V. ponticus* and *V. parahaemolyticus* (strains isolated from water). These four species were only found in location B. The two first species are rather related to fish pathogens and the third species is a common environmental organism found in seawater. Whereas *V. parahaemolyticus* is a widely recognised food-borne pathogen.

The isolates identified as *V. alfacsesis* corresponds to a new strain, named *Vibrio alfacsensis* sp. nov., which was proposed previously based on the phenotypic and genotypic analyses of five strains isolated from marine organisms, being two of them isolated from two regions of Spain (Chimetto et al., 2009; Gomez-Gil et al., 2007, 2012). The 16S rRNA gene sequences of the *V. alfacsensis* isolates showed similarity to *V. ponticus*, *Vibrio furnissii* and *Vibrio fluvialis*.

V. ponticus was also isolated from water, yielding 1.2% (n = 1/82) of isolates (Table 3.5). Macián et al. (2004) isolated *V. ponticus* from cultured mussel in Spain. Xie et al. (2007) characterised the pathogenic potential of the strain RP30 *V* in cultured Japanese sea bass (*Lateolabrax japonicas*) which was identified as *V. ponticus* based on the similarity values of 16S rRNA gene sequences shown by the strain (99.3%). The strain identified in our study as *V. neocaledonicus* was also isolated by other authors from aquacultured *Chanos chanos* and *Oreochromis niloticus* in Philippine (Langaoen et al., 2018). However, to the best of our knowledge, there are no studies identifying this *Vibrio* strain in Mediterranean fish species.

V. parahaemolyticus is recognised as a relevant foodborne human pathogen associated with seafood products. *V. parahaemolyticus* is part of the natural and estuarine microbiota and costal marine waters (DePaola et al., 2003) and is one of the species of *Vibrio* that causes human infections. The foodborne disease is characterised by diarrhoea, severe abdominal pain and fever after the consumption of contaminated fish and shellfish (Daniels et al., 2000; Slayton et al., 2014) and has been also linked to Mediterranean fish species such as Sea Bass and Gilthead sea bream (Korun and Timur, 2008). Data obtained from clinical journals and unreported cases of *V. parahaemolyticus* infections identified in hospitals have shown that infections by this species of *Vibrio* in Spain are more frequent than usual due to the characteristics of the water where they proliferate (i.e. pH, salinity and temperature) and the season (i.e. summer) (Martinez-Urtaza et al., 2004, 2005).

In our study, the pathogen was only isolated from a sample of water, accounting for 1.2% (n = 1/82) isolates (Table 3.5). This isolate was identified as the strain ATCC 17802 (99%), which is considered as a pathogenic strain (https://www.ncbi.nlm.nih.gov/nuccore/CP014046.2). Scarano et al. (2014) also found low prevalence of *V. parahaemolyticus* but in that case, it was isolated from Gilthead sea bream reared in Italian mariculture. Khouadja et al. (2013) isolated six strains of *V.*

parahaemolyticus from the internal organs of diseased Sea bass (*Dicentrarchus labrax*) on two fish farms located in Tunisia. Davies et al. (2001) studied the incidence of *V. parahaemolyticus* in fresh fish purchased from commercial outlets from several European locations. Although these authors did not observe presence of *V. parahaemolyticus* in Gilthead sea bream, this species was detected in 35.0% (n = 7/20) and 14.0% (n = 14/101) of fresh fish samples from Portugal and Greece, respectively. In addition, several studies have reported outbreaks caused by *V. parahaemolyticus* in Europe countries (Huehn et al., 2014; Martinez-Urtaza et al., 2004; Ottaviani et al., 2009; Quilici et al., 2005) and foodborne vibriosis cases (Lozano-León et al., 2003; Martinez-Urtaza et al., 2008).

3.3.4. Fish shelf-life assessment

The average level of AMB after harvesting in both fish species was 3.01 log cfu/g. These values are in line with those reported by other authors for these species, in which counts for fresh fish ranged from 3 to 4 log cfu/g (Özden et al., 2007; Papadopoulos et al., 2003; Parlapani et al., 2013). The concentration levels based on the type of location corresponded to 3.74 and 2.28 log cfu/g for location A and B, respectively. These values were inputted into the selected predictive microbiology model to predict *Pseudomonas* growth as a representative spoilage microorganism of fresh Mediterranean fishes at two relevant temperatures (4 and 8 °C). *Pseudomonas* spp. was appraised as suitable microorganism to predict shelf-life, since it is usually predominant in fish products and is equally considered a relevant spoilage microorganism for chilled stored Mediterranean fish, under air packaging as it was demonstrated by several studies (Paleologos et al., 2004; Papadopoulos et al., 2003; Parlapani et al., 2013, 2014; Taliadourou et al., 2003; Tryfinopoulou et al., 2002). The simulated curves and kinetic parameters are presented in Figure 3.1 and Table 3.6, respectively.

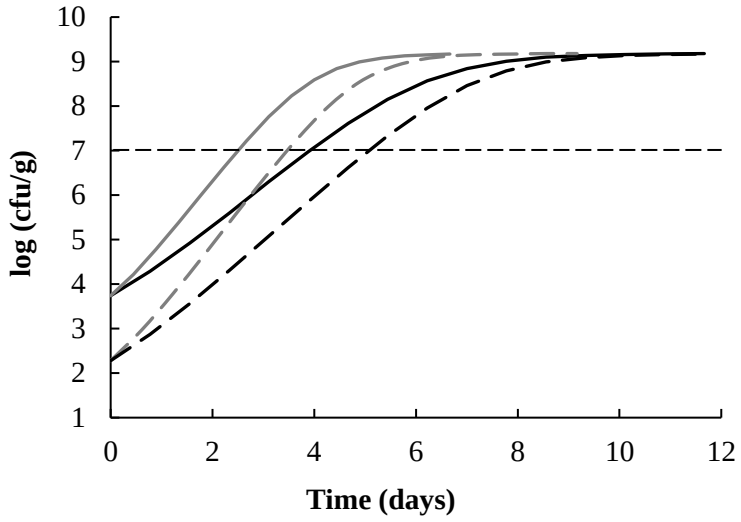

Figure 3.1. Growth curves for *Pseudomonas* spp. predicted with the model by Koutsoumanis and Nychas (2000) at 4 and 8 °C for the aquaculture Mediterranean fish species. The concentration levels found for aerobic mesophilic bacteria in our study of 3.74 and 2.28 log (cfu/g) for locations A and B, respectively were extrapolated as initial values of *Pseudomonas* spp. The black and grey solid lines correspond with concentration data estimated for location A at 4 and 8 °C, respectively, while the black and grey dashed lines represent for the growth estimates obtained for location B at 4 and 8 °C, respectively. The horizontal dashed line located at the concentration of 7.0 log cfu/g defines the end of shelf-life for fresh fish based on sensory quality criteria. Locations: A (estuary of the river Guadalquivir, Sevilla, Spain) and B (estuary of the river Guadiana, Huelva, Spain).

Table 3.6. Prediction parameters for the growth of *Pseudomonas* spp. and shelf-life estimation at two storage temperatures (4 and 8 °C) based on microbial data obtained in this study for Gilthead sea bream and Sea bass produced in two different aquaculture locations.

Location	Temperature (°C)	λ (h)	μ_{max} log (cfu/h)	Maximum population log(cfu/g)	Shelf-life time (d)[b]
A[a]	4	10.68	0.040	9.18	4
	8	6.97	0.063	9.17	3
B	4	10.68	0.04	9.17	5
	8	6.97	0.063	9.18	4

[a] Location: A (estuary of the river Guadalquivir, Sevilla, Spain) and B (estuary of the river Guadiana, Huelva, Spain).
[b] Day when the microbial species reached the concentration of 7 log cfu/g defining the end of shelf-life for fresh fish.

The end of shelf-life on fresh fish was reached when the predominant microorganism (i.e. *Pseudomonas* spp.) exceeded the upper limit of acceptability of 7 log cfu/g which is usually linked to sensory deterioration in fresh fish (ICMSF, 1986; Olafsdóttir et al., 1997). The shelf-life predictions for both fish species were 4 and 3 days at 4 and 8 °C, respectively, for location A, while for location B, the shelf-life was estimated in 5 and 4 days at the same temperatures. The difference in shelf-life between the two production location systems (A and B) was of 1 day, which, in our case, accounts for the 20% and 25% of the total shelf-life at 4 and 8 °C, respectively. This result proves the relevant impact of the hygienic conditions at primary production (i.e., fish farming) on the microbiological quality of harvested fish, and hence on the food quality along the food production-distribution chain and shelf-life duration. In our example, fish product obtained in location A, with worse microbiological quality, showed shorter shelf-life, increasing the probability of non-conformities, the generation of food waste, and consumer complaints (e.g. sensory rejection). As mentioned above, the use of adequate facilities for water treatment and a better hygienic control on water management systems seem to be key to ensure a good microbiological quality in harvested fish, which is expected to affect the next steps along the food chain.

3.4. Conclusion

Results in our study suggest that the location and the associated physico-chemical parameters and organic load of water could significantly affect microbiological quality of Gilthead sea bream and Sea bass. The indicator microorganisms, lactic acid bacteria, aerobic mesophilic bacteria, *Enterobacteriaceae*, total coliforms and *Staphylococcus* spp. coagulase + were demonstrated to be influenced by the location, showing higher concentrations in location A. On the other hand, neither *L. monocytogenes* nor *Salmonella* spp. were present in samples of both fish species and estuary water. Importantly, the predictive analysis demonstrated that the initial microbiological quality could have a remarkable effect on the product shelf-life, being longer for location B, where samples showed better microbiological quality. Notwithstanding of foregoing, the pathogenic species *V. parahaemolyticus* was only found in water of location B, which showed lower levels of hygiene indicator microorganisms. Therefore, no relation could be stablished between hygiene indicators and presence of pathogenic *Vibrio* spp. Apart from the isolated *Vibrio* spp. identified as fish pathogens, attention should be given to other *Vibrio* spp. that are not traditionally

considered as human pathogens but in the last few years have been linked with illness cases in humans. Summing up, results stress the relevance of controlling the microbiological quality of water and fishes in estuary production systems in order to ensure the quality and safety of Gilthead sea bream and Sea bass and along the food chain.

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Appendix A. Supplementary Table 3.1

The following is the Supplementary data to this article:

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Chapter 4

Evaluation of the effect of *Lactobacillus sakei* strain L115 on *Listeria monocytogenes* at different conditions of temperature by using predictive interaction models

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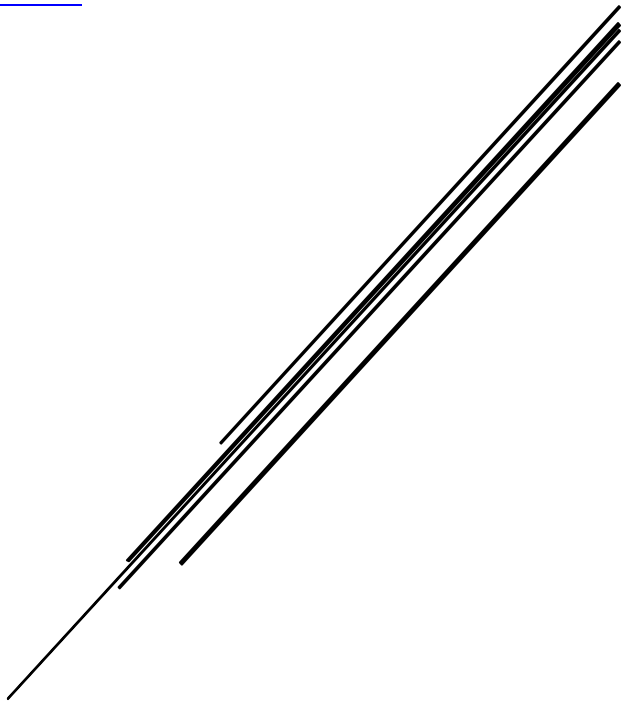


HIGHLIGHTS

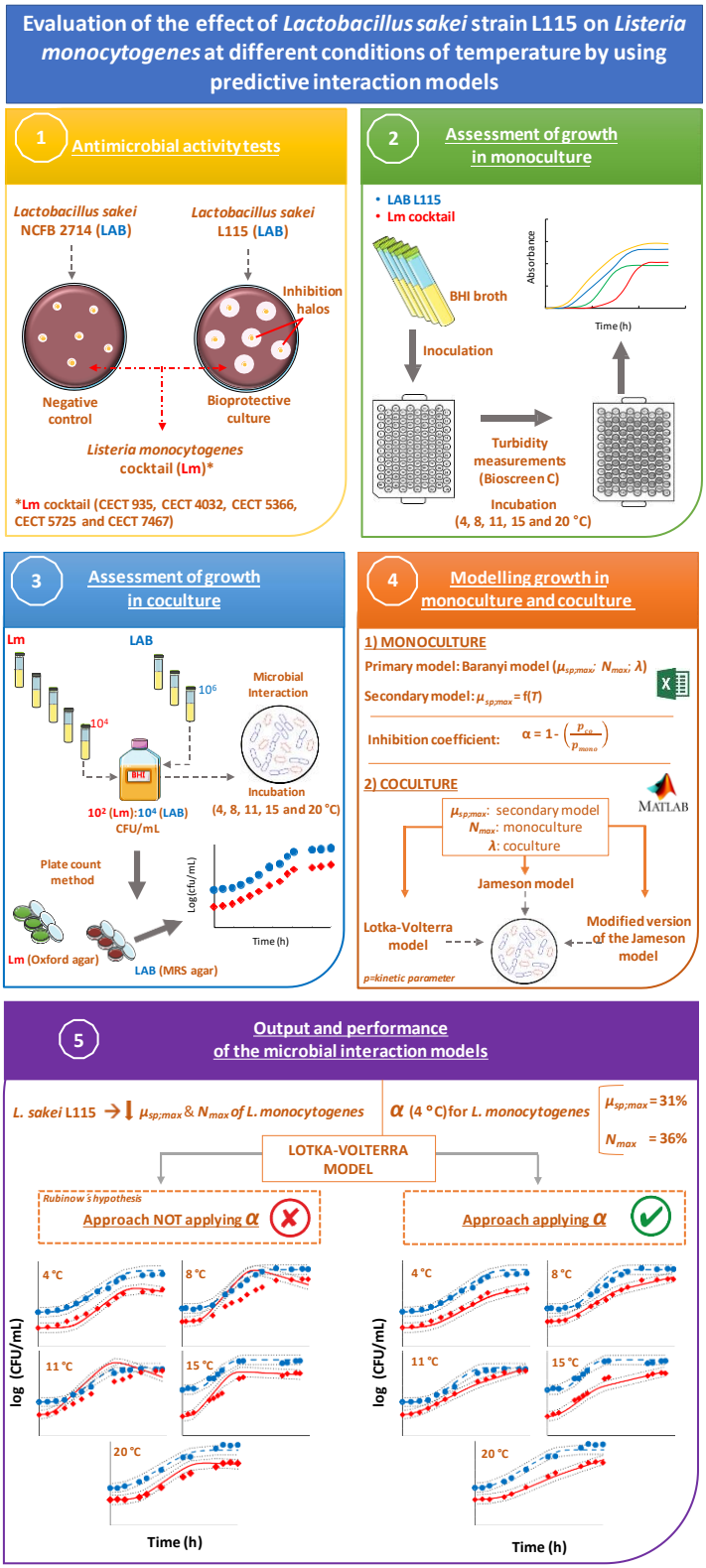
- *Lactobacillus sakei* strain L115 induced an early stationary phase of *Listeria monocytogenes*.
- *Lb. sakei* L115 produced a reduction of the maximum growth rate of *L. monocytogenes* of 31-48%.
- Maximum population density of *L. monocytogenes* in coculture with *L. sakei* L115 decreased by 36% at 4 °C.
- Square-root-model-described properly the growth rate of both microorganisms in monoculture.
- Modified Jameson and Lotka-Volterra models, using the inhibition coefficient α (%), could predict microbial interaction.

Abstract

In this study, the inhibitory capacity of *Lactobacillus sakei* strain L115 against *Listeria monocytogenes* has been assayed at 4, 8, 11, 15 and 20 °C in broth culture. Besides, the use of predictive microbiology models for describing growth of both microorganisms in monoculture and coculture has been proposed. A preliminary inhibitory test confirmed the ability of *Lb. sakei* strain L115 to prevent the growth of a five-strain cocktail of *L. monocytogenes*. Next, the growth of microorganisms in isolation, i.e. in monoculture, was monitored and kinetic parameters maximum specific growth rate ($\mu_{sp;max}$) and maximum population density (N_{max}) were estimated by fitting the Baranyi model to recorded data. Inhibition coefficients (α) were calculated for the two kinetic parameters tested ($\mu_{sp;max}$ and N_{max}) to quantify the percentage of reduction of growth when the microorganisms were in coculture in comparison with monoculture. The kinetic parameters were input into three interaction models, developed based on modifications of the Baranyi growth model, namely Jameson effect, new modified version of the Jameson effect and Lotka-Volterra models. Two approaches were utilized for simulation, one using the monoculture $\mu_{sp;max}$, under the hypothesis that the growth potential is similar under monoculture and coculture conditions provided the environmental conditions are not modified, and the other one, based on adjusting the monoculture kinetic parameter by applying the corresponding α to reproduce the observed $\mu_{sp;max}$ under coculture conditions, assuming, in this approach, that the existence of a heterogeneous population can change the growth potential of each microbial population. It was observed that in coculture, $\mu_{sp;max}$ of *L. monocytogenes* decreased (e.g., α= 31% at 4 °C) and the N_{max} was much lower than that of monoculture (e.g., α= 36% at 4 °C). The best simulation performance was achieved applying α to adjust the estimated monoculture growth rate, with the modified Jameson and Lotka-Volterra models showing better fit to the observed microbial interaction data as demonstrated by the fact that 100% data points fell within the acceptable simulation zone (± 0.5 log CFU/mL from the simulated data). More research is needed to clarify the mechanisms of interaction between the microorganisms as well as the role of temperature.

Keywords: microbial interactions; Lotka-Volterra model; Jameson effect; bacteriocins; predictive microbiology; bioprotective cultures; lactic acid bacteria.

Graphical abstract



4.1. Introduction

Listeria monocytogenes is a foodborne pathogen posing a significant risk to human health because of the fatal consequences of the disease, i.e. listeriosis, in immunocompromised individuals including meningitis, septicemia, miscarriages in pregnant women, etc. (CDC, 2017). In non-immunocompromised people, *L. monocytogenes* is not considered a pathogen of concern, as it generally causes a mild form of illness (Angelidis & Koutsoumanis, 2006). Foodborne outbreaks investigations have pointed out that ready-to-eat foods constitute the main vehicle for *L. monocytogenes* in Asia, Europe and USA (EFSA, 2015; Lomonaco et al., 2013; Makino et al., 2005). In this sense, in the summer of 2019, a large outbreak of listeriosis linked to the consumption of a chilled roasted pork meat product (known as "carne mechada") was reported in Spain, with a total of 217 reported cases, 3 deaths and 6 women that had miscarriages linked to this outbreak (CCAES, 2019).

Biopreservation is a preservation technique based on the use of natural antimicrobial agents produced by microorganisms, endowed with high potential to reduce microbial and organoleptic deterioration of foods, thus extending their shelf-life. This technique, used in combination with other preservation methods, can result in an improvement of food safety and quality, while it satisfies the increasing consumer demand for more natural products (Singh, 2018; Yusuf, 2018). Regarding food safety, the inhibitory effect of biopreservative cultures against foodborne pathogens has been widely used, e.g. through the addition of starter bioprotective cultures. In this sense, lactic acid bacteria (LAB) are regarded as ideal antagonists of pathogens in food-associated microbial communities (Vereecken et al., 2003). A well-known LAB, *Lactobacillus sakei*, is widely used in France and Western Europe in association with micrococci and yeasts as a starter for the manufacture of fermented sausages (Champomier-Verges et al., 2002). Besides that, this species has also been used with the aim of inhibiting the development of pathogens in foods (Bredholt et al., 2001; Chaillou et al., 2014; Ruiz Martinez et al., 2015). Additionally, *Lb. sakei* is considered one of the most psychrotrophic species among the LAB genera since some strains can grow at 2-4 °C (Champomier-Verges et al., 2002) highlighting its potential application for improving the quality and safety of refrigerated ready-to-eat foods. Katikou et al. (2005) demonstrated that *Lb. sakei* strain L115 (CECT 4808) produced bacteriocin-like inhibitory substances against *L. monocytogenes* growth. In the same study, this strain also reduced significantly the levels

of spoilage microorganisms in beef slices during refrigerated storage. In a later study by the same group (Katikou et al., 2007), the above-mentioned strain was shown to improve the shelf-life of vacuum-packaged trout fillets stored at 4 °C. Although these results have shown promise for its application in food biopreservation, the spectrum of action and the level of inhibition under different temperature conditions (e.g. refrigeration and temperature abuse) requires further research.

Predictive microbial interaction models may help to clarify how specific conditions that prevail in the food environment influence the effectiveness of LAB growth and/or of their metabolites production (Leroy & De Vuyst, 2003). In this sense, the development and application of microbial interaction models for the effect of LAB on pathogenic (or spoilage) bacteria continues to be a strategic research line in the field of predictive microbiology (Quinto et al., 2016). Modelling approaches have been focused on the well-known Jameson and Lotka-Volterra interaction models (Giménez & Dalgaard, 2004; Vereecken et al., 2000). However, the modified Jameson model (Le Marc et al., 2009), based on the estimation of a critical concentration of both microorganisms that inhibit the growth of each other could be a promising strategy to model microbial interaction of *Lb. sakei* against the *L. monocytogenes* cocktail.

Previous studies have attempted to model the inhibitory effect of *Lb. sakei* against single strains of pathogens such as *Yersinia enterocolitica* IP 1105 O:8 (Janssen et al., 2006), *Listeria innocua* LMG 13568 (Leroy et al., 2005) and *L. monocytogenes* Scott A (Quinto et al., 2016) in liquid laboratory media. However, its inhibition kinetics has not been evaluated against pathogenic strain mixtures, which can be found in real food systems. The aim of this study was to describe and evaluate the potential interaction between *Lb. sakei* strain L115 (CECT 4808) and a five-strain cocktail of *L. monocytogenes* in mono- and coculture within a temperature range from 4 to 20 °C by using predictive microbial interaction models.

4.2. Material and Methods

4.2.1. Inoculum preparation

Bacterial lyophilized cultures of *Lb. sakei* and *L. monocytogenes* were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain) and reconstituted following the manufacturer's recommendations. The strains used for the assay were *Lb. sakei* L115 (CECT 4808), with demonstrated anti-listerial activity (Katikou et al., 2005),

and a cocktail of five strains of *L. monocytogenes*, namely CIP 59.53, NCTC 11994, Li2, CIP 78.39 and KCTC 3569 (CECT 935, CECT 4032, CECT 5366, CECT 5725 and CECT 7467, respectively). The *L. monocytogenes* strains used for the experiments were selected on the basis of the isolation source, i.e. clinical samples in humans and food samples of animal origin (poultry and chicken). Also, as negative control, a non-bacteriocin producing strain, i.e. *Lb. sakei* strain NCFB 2714 (CECT 906T), was acquired.

Strains of *Lb. sakei* and *L. monocytogenes* were resuscitated in individually in Brain Heart Infusion broth (BHI, Oxoid, Basingstoke, U.K), incubating at 30 and 37 °C for 24 h, respectively. Afterwards, the strains were subcultured twice followed by incubation at the same temperatures until the early stationary phase was reached (18-20 h). Prior to inoculation in test media, cultures were washed twice with phosphate-buffered saline (PBS) (Medicago AB, Uppsala, Sweden) by centrifugation at 4.100 rpm (Jouan C4i, Thermo Electron Corporation, France) for 10 min and finally re-suspended in BHI. The cocktail of *L. monocytogenes* strains was prepared by mixing volumes of 1 mL from each *Listeria* strain suspension (5×10^9 CFU/mL approx.) in a sterile test tube.

4.2.2. Antimicrobial activity test

The anti-listerial capacity of *Lb. sakei* L115 was tested against the prepared cocktail of *L. monocytogenes* strains. To this aim, the procedure of Benkerroum et al. (2000) was adapted for the inhibitory test, based on the agar spot method. An overnight grown culture of the test organism in De Man, Rogosa and Sharpe broth (MRS, Oxoid) was serially diluted in 0.85% saline solution and 0.1 mL aliquots were surface-plated on MRS agar (Oxoid) to obtain separate colonies, spaced 3 cm apart approx. measured with a caliper (Powerfix Profi, Germany). Plates were incubated at 33 °C for 24 h under 10% CO_2. Then, the MRS agar plates were carefully overlaid with 5 mL of Tryptone Soya Agar (0.7%) (TSA, Oxoid) inoculated with 0.1 mL of an 18-h culture cocktail of the 5 *L. monocytogenes* strains. The concentration of the cocktail was $\approx 5 \times 10^7$ CFU/ mL. Plates were incubated at 37 °C for 24 h. The bactericidal activity was demonstrated by the formation of clear zones around colonies of the putative producers (i.e. inhibition halos). Five replicates were performed following the same protocol and the size of *Lb. sakei* colonies and inhibition zones were measured using a digital caliper.

4.2.3. Growth assessment of Lb. sakei *and* L. monocytogenes *cocktail in monoculture*

The growth of *Lb. sakei* and *L. monocytogenes* cocktail was individually assessed through absorbance measurements using the Bioscreen C (Labsystems, Finland). Microtiter plates (10 × 10 wells) were filled with 400 µL of inoculated BHI at a concentration of *ca.* 10^5 CFU/mL for each bacterial culture. Absorbance measurements were taken with a wideband filter (420–580 nm) using a total of sixteen replicates and four blanks (non-inoculated BHI medium) per culture. Microtiter plates were incubated at five different temperatures (4, 8, 11, 15 and 20 °C) for a period from 2 to 21 days. Absorbance measurements expressed in logarithmic scale were transformed into viable cell counts (log CFU/mL) by performing calibration curves (Supplementary File 4.1) as described by Bolívar et al. (2018). To check for calibration curves performance, enumeration tests were carried out at different time points during the growth of the monoculture experiments in Bioscreen.

4.2.4. Assessment of Lb. sakei and L. monocytogenes growth in coculture

Growth experiments in coculture were monitored by plate counts. In these experiments, suspensions of ≈ 10^4 and 10^2 CFU/mL of *Lb. sakei* and *L. monocytogenes* respectively, were prepared in flasks of 100 mL BHI. After inoculation, flasks were incubated at the five studied temperatures (4, 8, 11, 15 and 20 °C).

For enumeration analysis of *Lb. sakei*, 0.1 mL of adequate dilutions were spread, in duplicate, onto MRS agar which were incubated at 33 °C for 48 h under 10% CO_2. *L. monocytogenes* counts were determined using Oxford agar (Oxoid) incubated at 37 °C for 24 h. Cultures flasks were periodically sampled until the stationary phase was reached. Two independent trials per experiment were performed. pH was measured throughout the storage period at regular time intervals using a pHmeter (Crison Basic 20, Alella, Spain).

4.2.5 Modelling Lb. sakei *and* L. monocytogenes *growth under different temperature conditions*

4.2.5.1. Primary model

The Baranyi and Roberts primary model (Baranyi & Roberts, 1994), as described in Eq. 4.1 and 4.2, was fitted to growth data obtained from mono- and coculture experiments. The growth curves were adjusted using the DMFit Excel add-in (http://www.combase.cc) (Baranyi & Tamplin, 2004). The kinetic parameter maximum specific growth rate ($\mu_{sp;max}$,

h^{-1}) from monoculture experiments were further used to develop secondary models (see *Section 4.2.5.2*) and introduced as inputs in the microbial interaction model (see *Section 4.2.5.4*). The parameters estimated from coculture conditions maximum specific growth rate (μ_{co}, h^{-1}) and maximum population density ($N_{max;co}$, log CFU/mL) were used for comparison purposes by estimating the inhibition coefficient (see *Section 4.2.5.3*).

$$log\ N_t = log\ N_0 + \mu_{sp;max} \cdot \frac{F(t)}{ln(10)} - \frac{1}{m \cdot ln(10)} \cdot ln\left(1 + \frac{e^{m \cdot \mu_{sp;max} \cdot F(t)} - 1}{10^{m(log\ N_{max} - log\ N_0)}}\right) \quad (4.1)$$

$$F(t) = t + \frac{1}{\mu_{sp;max}} \cdot ln\left(e^{(-\mu_{sp;max} \cdot t)} + e^{(-\mu_{sp;max} \cdot t_{lag})} - e^{\left[(-\mu_{sp;max} \cdot t) - \mu_{sp;max} \cdot t_{lag}\right]}\right) \quad (4.2)$$

where N_t is the cell concentration (log (CFU/mL)) at time t (h), N_0 is the cell concentration (CFU/mL) at time zero, $\mu_{sp;max}$ is the maximum specific growth rate (h^{-1}), t_{lag} is the duration of the lag time of the growth curve (h), N_{max} is the maximum population density (log (CFU/mL)), m is a curvature factor (m = 1), $F(t)$ represents an adjustment function for the model (Baranyi & Roberts, 1994).

4.2.5.2. Secondary model

The primary growth parameter $\mu_{sp;max}$ (h^{-1}) from monoculture experiments, were used to fit the square root model (Eq.(4.3)) (Ratkowsky et al., 1982) in order to describe the influence of the storage temperature. The model was fitted by a linear regression using Microsoft Excel® (Redmond, USA).

$$\sqrt{\mu_{sp;\max}} = b \cdot (T - T_{\min}) \quad (4.3)$$

where $\mu_{sp;max}$ was described previously (Eqs.(4.1) and (4.2)); b is a constant, T (°C) is the temperature and T_{min} is the theoretical minimum temperature for growth. The values of $\mu_{sp;max}$ predicted by the secondary models in monoculture were used for the simulation of the interaction models (see *Section 4.2.5.4*).

The ability of primary and secondary models to describe the experimental data was analyzed by the statistical indices Standard Error (SE), Adjusted R-square (R^2-Adj.) and Root Mean Squared Error (RMSE) (Eqs.(4.4)–(4.6)) (Hervas et al., 2001).

$$SE = \sqrt{\frac{\sum_{i=1}^{n}\left(y_i - \overline{y_i}\right)^2}{n-k}} \quad (4.4)$$

where y_i and $\bar{y}_i$ is the observed and predicted values, respectively, n is the number of observations in the growth curve and k is the number of fitted parameters of the model.

$$Adjusted\ R^2 = 1 - \left(\frac{n-1}{n-s}\right) \cdot \left(\frac{SS_{residuals}}{SS_{total}}\right) \tag{4.5}$$

where n is the number of observations in the growth curve, s is the number of fitted parameters of the model, $SS_{residuals}$ is the residual sum of squares and SS_{total} is the total sum of squares.

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}\left(y_i - \overline{y_i}\right)^2}{n}} \tag{4.6}$$

where y_i and $\bar{y}_i$ is the observed and predicted values, respectively, and n is the number of observations in the growth curve.

4.2.5.3. Inhibition coefficient for Lb. sakei *and* L. monocytogenes

The inhibition effect between the selected *Lb. sakei* strain and *L. monocytogenes* cocktail was described by an inhibition coefficient (α) for $\mu_{sp;max}$ and N_{max}. This was defined as the complement of the fraction between the kinetic parameter in coculture (μ_{co} or $N_{max,co}$) and monoculture (μ_{mono} or $N_{max,mono}$) (Eqs. (4.7) and (4.8)). The standard deviation was calculated using the values (in percentage) of the results and the mean value of the inhibition coefficient.

$$\alpha_{\mu} = 1 - \left(\frac{\mu_{co}}{\mu_{mono}}\right) \tag{4.7}$$

$$\alpha_{N_{max}} = 1 - \left(\frac{N_{max_{co}}}{N_{max_{mono}}}\right) \tag{4.8}$$

4.2.5.4. Simulating microbial interaction of Lb. sakei *and* L. monocytogenes

To describe the interaction between *Lb. sakei* and *L. monocytogenes* cocktail at different temperature conditions three different predictive models of microbial interaction were applied: the Jameson effect model, a new modified version of the Jameson effect model and the Lotka-Volterra model. Maximum specific growth rate was defined, in abovementioned equations, following two approaches. In the first approach, it was assumed that maximum specific growth rate was an intrinsic characteristic of each microbial population and therefore, invariant under the same environmental conditions,

regardless of the presence of another microbial population. Thus, the kinetic parameters obtained in monoculture for *L. monocytogenes* and *Lb. sakei* were used to input models to simulate microbial interaction (Rubinow, 1984). On the contrary, for the second approach, growth potential of one population was considered to be affected by the existence of the other microbial population. In this case, maximum specific growth rate under microbial interaction was not equal to the one observed in monoculture, and therefore, it was defined based on the growth capacity exhibited in coculture experiments. In the first hypothesis, maximum specific growth rate ($\mu_{sp;max}$; h^{-1}) was described based on the outcome of the secondary model of monoculture experiments. Whereas, for the second one, the inhibition coefficient (α) was applied to the secondary model outcome to consider the interaction effect of the other microbial population. In both approaches, N_{max}, understood as the maximum carrying capacity of the population, was considered to be equal in coculture and monoculture, so that the secondary model for this parameter obtained under coculture conditions was used to define this parameter in both approaches.

The traditional Jameson effect model (Giménez & Dalgaard, 2004; Jameson, 1962), based on Eqs. (4.9)–(4.12), describes the microbial interaction as the growth suppression of the minority of the population when the dominant bacterial population reaches their N_{max} (Giménez & Dalgaard, 2004; Irlinger & Mounier, 2009; Jameson, 1962; Ross et al., 2000).

$$\frac{dN_{Ls}}{dt} = N_{Ls} \cdot \mu_{sp;max\,Ls} \cdot \left(1 - \frac{N_{Ls}}{N_{max\,Ls}}\right) \cdot \left(1 - \frac{N_{Lm}}{N_{max\,Lm}}\right) \cdot \left(\frac{Q_{Ls}}{1+Q_{Ls}}\right) \tag{4.9}$$

$$\frac{dN_{Lm}}{dt} = N_{Lm} \cdot \mu_{sp;max\,Lm} \cdot \left(1 - \frac{N_{Lm}}{N_{max\,Lm}}\right) \cdot \left(1 - \frac{N_{Ls}}{N_{max\,Ls}}\right) \cdot \left(\frac{Q_{Lm}}{1+Q_{Lm}}\right) \tag{4.10}$$

$$\frac{dQ_{Ls}}{dt} = Q_{Lst-1} \cdot \mu_{sp;max\,Ls} \tag{4.11}$$

$$\frac{dQ_{Lm}}{dt} = Q_{Lmt-1} \cdot \mu_{sp;max\,Lm} \tag{4.12}$$

where N is the cell concentration (CFU/mL) at time t, $\mu_{sp;max}$ is the maximum specific growth rate (h^{-1}), N_{max} is the maximum population density (CFU/mL) and Q is a measure of the physiological state of cells at time t. The subscript letters Ls and Lm in the parameters stand for *Lb. sakei* and *L. monocytogenes*, respectively.

The value of Q at t=0 (Q_0) was calculated for both microorganisms as follows:

$$Q_0 = \frac{1}{e^{\left(\mu_{sp;max} \cdot \lambda\right)} - 1} \tag{4.13}$$

where e is the Euler´s number, $\mu_{sp;max}$ is as above, and t_{lag} is the lag time estimated from coculture experiments.

A new modified version of the Jameson effect model (Costa et al., 2019), represented by Eqs. (4.14)–(4.17), replace N_{maxLs} and N_{maxLm} by the parameters N_{criLs} and N_{criLm}, that describe the maximum critical concentration that a microbial population should reach to inhibit the growth of the other microbial population. The N_{criLs} and N_{criLm} parameters are lower than $N_{maxLs}$and N_{maxLm}respectively, and this assumption was demonstrated to enhance the performance of the model for *Staphylococcus aureus* in milk in presence of a starter culture, providing more accurate predictions (Le Marc et al., 2009).

$$\frac{dN_{Ls}}{dt} = N_{Ls} \cdot \mu_{sp;maxLs} \cdot \left(1 - \frac{N_{Ls}}{N_{maxLs}}\right) \cdot \left(1 - \frac{N_{Lm}}{N_{criLm}}\right) \cdot \left(\frac{Q_{Ls}}{1+Q_{Ls}}\right) \tag{4.14}$$

$$\frac{dN_{Lm}}{dt} = N_{Lm} \cdot \mu_{sp;maxLm} \cdot \left(1 - \frac{N_{Lm}}{N_{maxLm}}\right) \cdot \left(1 - \frac{N_{Ls}}{N_{criLs}}\right) \cdot \left(\frac{Q_{Lm}}{1+Q_{Lm}}\right) \tag{4.15}$$

$$\frac{dQ_{Ls}}{dt} = Q_{Lst-1} \cdot \mu_{sp;maxLs} \tag{4.16}$$

$$\frac{dQ_{Lm}}{dt} = Q_{Lmt-1} \cdot \mu_{sp;maxLm} \tag{4.17}$$

where N_{cri} is the maximum critical concentration (CFU/mL) of *Lb. sakei* that inhibits the growth of *L. monocytogenes* and vice-versa. The subscript letters $_{Ls}$ and $_{Lm}$ in the parameters are as above. The rest of model parameters are as described in Eqs. (4.9)–(4.13).

Additionally, the Lotka-Volterra model was used to describe the interaction between *Lb. sakei* and *L. monocytogenes* cocktail according to Eqs. (4.18)–(4.21) (Cornu et al., 2011; Dens et al., 1999; Lotka, 1956; Vereecken et al., 2000). This model includes two empirical parameters (the so-called competition factors), reflecting the degree of interaction or inhibition between both populations (F_{LsLm} and F_{LmLs}). The growth of *L. monocytogenes* can be affected depending on the empirical parameter value for *Lb. sakei* (F_{LsLm}), as follows:

1) If $0 < F_{LsLm} < 1$, *L. monocytogenes* grows with reduced $\mu_{sp;max}$ after *Lb. sakei* reaches N_{max}.

2) If $F_{LsLm} = 1$, *L. monocytogenes* stops growing when *Lb. sakei* reaches its N_{max}.

3) If $F_{LsLm} > 1$, *L. monocytogenes* population declines when *Lb. sakei* reaches its N_{max}.

$$\frac{dN_{Ls}}{dt} = N_{Ls} \cdot \mu_{sp;max\,Ls} \cdot \left(1 - \frac{N_{Ls} + F_{LsLm} \cdot N_{Lm}}{N_{max\,Ls}}\right) \cdot \left(\frac{Q_{Ls}}{1 + Q_{Ls}}\right) \quad (4.18)$$

$$\frac{dN_{Lm}}{dt} = N_{Lm} \cdot \mu_{sp;max\,Lm} \cdot \left(1 - \frac{N_{Lm} + F_{LmLs} \cdot N_{Ls}}{N_{max\,Lm}}\right) \cdot \left(\frac{Q_{Lm}}{1 + Q_{Lm}}\right) \quad (4.19)$$

$$\frac{dQ_{Ls}}{dt} = Q_{Lst-1} \cdot \mu_{sp;max\,Ls} \quad (4.20)$$

$$\frac{dQ_{Lm}}{dt} = Q_{Lmt-1} \cdot \mu_{sp;max\,Lm} \quad (4.21)$$

where F_{LsLm} and F_{LmLs} are, respectively, the competition factor parameters of *Lb. sakei* on *L. monocytogenes* cocktail and vice-versa. The other parameters are as indicated in Eqs. (4.9)–(4.13).

The three interaction models were computed in MATLAB version R2015b (The MathWorkInc®, Natick, USA) using the functions *fmincon* and o*de45*, intended to optimize non-linear problems and perform numerical integrations based on the 4th order Runge Kutta algorithms, respectively, in order to simulate the simultaneous growth of both microorganisms. The prediction performance of the tested interaction models was assessed by calculating corrected Akaike information criterion (AICCc) and RMSE. In order to compare the observed and predicted growth in coculture, acceptable simulation zone (ASZ) was used and defined as ±0.5 log CFU/mL from the simulated *L. monocytogenes* and *Lb. sakei* counts. The simulations were considered acceptable when at least 70% of the observed values (log CFU/mL) were inside the corresponding zone (Oscar, 2005; Møller et al., 2013).

4.3. Results and Discussion

4.3.1. Assessing Lb. sakei *and* L. monocytogenes *growth in mono and coculture at different temperature conditions*

4.3.1.1. Microbial growth in monoculture

Lb. sakei and *L. monocytogenes* cocktail grew at all temperatures studied. Calibration curves have been extensively used to express microbial counts from absorbance measurements. Francois et al. (2005) and Valero et al. (2006) found that calibration curves may deviate in their slope when performed at different temperatures and/or pH values. In our study, these potential deviations were taken into account in relation to the applied temperature range (4-20 °C). The high μ_{max} values of *L. monocytogenes* found in our study in comparison with those obtained by Valero et al. (2006) may be attributed to

the strains used. These authors used a single strain while in this study, a cocktail was assayed. It is well reported that differences in phenotypic responses among strains of the same microbial species constitute an important source of variability in microbiological studies (Lianou & Koutsoumanis, 2013). When using cocktails, some strains are generally dominant on the others (Pal et al., 2008), thus presenting faster adaptation and growth. Further works should focus on the study of extrinsic factors (i.e. temperature) on the dominance of certain individual strains over the others within a cocktail, since this fact could introduce additional variability in the estimated μ_{max} values.

The kinetic parameters $\mu_{sp;max}$ and N_{max} for both microorganisms were estimated by fitting the Baranyi and Roberts model to data. For the sake of clarity, growth rate was also expressed as maximum growth rate (μ_{max}, log CFU/h) hereinafter. Parameters are shown in Table 4.1.

Table 4.1. Kinetic parameters ± standard error of *Lactobacillus sakei* strain L115 and *Listeria monocytogenes* cocktail obtained by the fitted Baranyi and Roberts model for experiments in monoculture.

Temp. (°C)	Microorganisms in monoculture	μ_{max} (log CFU/h)	N_{max} (log CFU/mL)	RMSE [b] (log CFU/mL)	R^2-Adj. [c]
4	*Lb. sakei*	0.021±0.001 [a]	8.05±0.021	0.119	0.987
	L. monocytogenes	0.037±0.001	9.26±0.025	0.116	0.992
8	*Lb. sakei*	0.091±0.005	7.69±0.029	0.128	0.989
	L. monocytogenes	0.072±0.003	9.21±0.024	0.084	0.994
11	*Lb. sakei*	0.108±0.002	8.11±0.013	0.037	0.999
	L. monocytogenes	0.128±0.003	9.26±0.012	0.037	0.999
15	*Lb. sakei*	0.144±0.005	8.10±0.035	0.129	0.997
	L. monocytogenes	0.211±0.019	9.16±0.083	0.198	0.989
20	*Lb. sakei*	0.275±0.018	8.46±0.038	0.070	0.996
	L. monocytogenes	0.328±0.028	9.28±0.079	0.153	0.994

[a] Standard error (SE).
[b] Root mean squared error (RMSE).
[c] Adjusted coefficient of determination.

The model showed an excellent adjustment, with a R^2-Adj. ≥ 0.98 and low values of RMSE (< 0.20 log CFU/mL). As expected, an increase of temperature from 4 to 20 °C caused an increment in μ_{max} of *Lb. sakei* and *L. monocytogenes* from 0.021±0.001 and 0.037±0.001 log CFU/h to 0.0275±0.018 and 0.328±0.028 log CFU/h, respectively. However, this effect was not observed in the case of N_{max}, since final values ranged between 7.69 and 9.28 log CFU/mL, for *Lb. sakei* and *L. monocytogenes*, respectively, without showing any trend.

Growth of *L. monocytogenes* matched the results found by Bolívar et al. (2018) in fish-based juice at 4 °C (0.031 log CFU/h) but they were different at higher temperatures (20 °C = 0.204 log CFU/h) probably due to the use of a food matrix based medium instead of BHI, as in the present study. In relation to *Lb. sakei*, it was capable to grow at all assayed temperatures, including refrigeration temperatures. It is noteworthy that at 4 °C *Lb. sakei* grew slower than *L. monocytogenes* as denoted by the estimated growth rates (Table 4.1). Matamoros et al. (2009) and Boulares et al. (2012) highlighted that LAB species having good ability to grow at low temperatures should be considered as good candidates for biopreservation of refrigerated foods.

The effect of temperature on growth rate of the microorganisms grown in monoculture was studied by fitting square root model to growth data (Fig. 4.1).

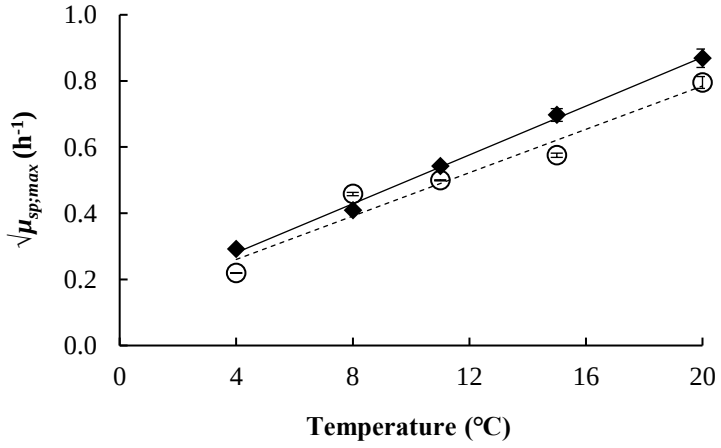


Fig. 4.1. Temperature effect on the maximum specific growth rate ($\mu_{sp;max}$) of *Lactobacillus sakei* strain L115 (o) and *Listeria monocytogenes* cocktail (♦) in monoculture. The lines represent the fitted model of the secondary model for *Lb. sakei* (- - -) and *L. monocytogenes* cocktail (—).

The model satisfactorily described the effect of temperature on the growth rate of *Lb. sakei* strain L115 and *L. monocytogenes* cocktail showing low RMSE values (<0.27 $h^{-0.5}$)

and acceptable R^2-Adj. values (≥0.84) for each microorganism. The best-fit parameter *b* from the square root model was 0.033 and 0.037 for *Lb. sakei* and *L. monocytogenes*, respectively, as shown in Table 4.2. Giménez & Dalgaard (2004) and Koseki et al. (2011) agreed on the suitability of the square root model to assess the effect of temperature on μ_{max} of *L. monocytogenes* in microbial interaction models with spoilage microorganisms.

Table 4.2. Estimated parameters ± standard error (SE) and statistical indices of the square root model for the effect of temperature on the maximum specific growth rate ($\mu_{sp;max}$; h^{-1}) of *Lactobacillus sakei* strain L115 and *Listeria monocytogenes* cocktail in monoculture on BHI broth.

Microorganisms	b ($h^{-0.5}$ °C^{-1})	T_{min} (°C)	RMSE[b] ($h^{-0.5}$)	R^2-Adj.[c]
Lb. sakei	0.033±0.004[a]	-3.94±2.152	0.223	0.859
L. monocytogenes	0.037±0.001	-3.61±0.513	0.271	0.842

[a] Standard error (SE).
[b] Root mean squared error (RMSE).
[c] Adjusted coefficient of determination (R^2-Adj).

4.3.2.1. Microbial growth in coculture

As a previous step, an inhibitory test was performed, showing that the non-bacteriocin producing strain, *Lb. sakei* NCFB 2714, produced inhibition halos of 2.87 ± 0.49ø mm, while *Lb. sakei* strain L115 presented inhibition zones of 5.81 ± 0.67ø mm, thus confirming the anti-listerial activity reported by Katikou et al. (2005).

The growth of *Lb. sakei* L155 and *L. monocytogenes* cocktail in coculture and the parameters of the Baranyi primary models at different temperature are presented in Fig. 4.2 and Table 4.3, respectively. As expected, the increase of temperature led to shorter t_{lag} and higher μ_{max} values for both microorganisms. Both visual and statistical analyses showed that the Baranyi model properly described the growth of the two microorganisms at all temperatures studied; the values for the R^2-Adj. were around 0.99 and the RMSE values were as low as 0.07-0.13 log CFU/mL. Quinto et al. (2016) obtained similar values of R^2 when evaluating the effect of competitive growth of *Lb. sakei* MN on the growth kinetics of *L. monocytogenes* Scott A in meat gravy.

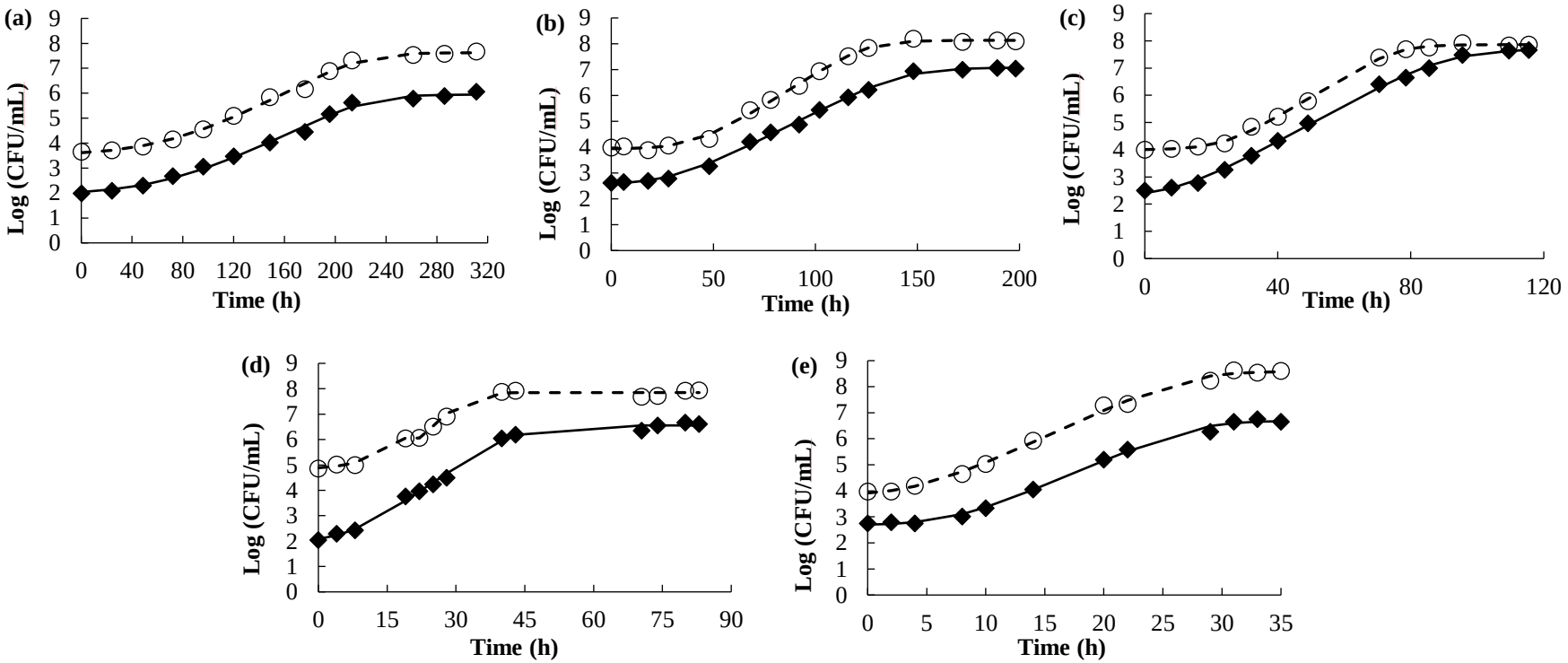


Fig. 4.2. Observed growth data of *Lactobacillus sakei* strain L115 (o) and *Listeria monocytogenes* cocktail (♦) in coculture at (a) 4 °C, (b) 8 °C, (c) 11 °C, (d) 15 °C and (e) 20 °C. The lines represent the Baranyi model fitted to *Lb. sakei* (- - -) and *L. monocytogenes* cocktail (—) growth.

Table 4.3. Average ± standard error (SE) of kinetic parameters and statistical indices obtained by fitting the Baranyi and Roberts model to growth data of *Lactobacillus sakei* strain L115 and *Listeria monocytogenes* cocktail in coculture experiments at 4, 8, 11, 15 and 20 °C.

Temp. (°C)	Microorganisms in coculture	t_{lag} (h)	μ_{max} (log CFU/h)	N_{max} (log CFU/mL)	RMSE[b] (log CFU/mL)	R^2-Adj.[c]	Time to N_{max} (h)[d]
4	*Lb. sakei*	73.2 ± 13.9[a]	0.0269 ± 0.003	7.62 ± 0.063	0.103	0.996	261.0
	L. monocytogenes	73.1 ± 19.4	0.0255 ± 0.004	5.95 ± 0.078	0.123	0.994	286.0
8	*Lb. sakei*	41.0 ± 2.8	0.0495 ± 0.002	8.13 ± 0.043	0.085	0.998	172.0
	L. monocytogenes	30.6 ± 3.5	0.0397 ± 0.002	7.07 ± 0.051	0.086	0.998	172.0
11	*Lb. sakei*	23.8 ± 1.5	0.0740 ± 0.003	7.86 ± 0.037	0.069	0.998	95.50
	L. monocytogenes	11.3 ± 2.4	0.0662 ± 0.003	7.68 ± 0.071	0.087	0.998	115.5
15	*Lb. sakei*	12.8 ± 5.1	0.1322 ± 0.042	7.85 ± 0.053	0.102	0.990	70.5
	L. monocytogenes	8.0 ± 3.7	0.1280 ± 0.016	6.55 ± 0.059	0.122	0.995	70.5
20	*Lb. sakei*	4.5 ± 1.2	0.2056 ± 0.016	8.59 ± 0.084	0.126	0.996	31.0
	L. monocytogenes	7.1 ± 1.1	0.1895 ± 0.014	6.69 ± 0.074	0.103	0.996	35.0

[a] Standard error (SE).
[b] Root mean squared error (RMSE).
[c] Adjusted coefficient of determination (R^2-Adj.).
[d] Time to reach N_{max}, assuming that this time is when $N = 100 - 99\%\ N_{max}$, was calculated by the bisection algorithm (iterations= 5000; tolerance= 0.01%) applied to the fitted growth model.

The values of μ_{max} obtained for *L. monocytogenes* in coculture with *Lb. sakei* were noticeably lower than those in monoculture. The inhibition ratios for μ_{max} of *L. monocytogenes* cocktail and *Lb. sakei* for the different temperatures can be observed in Table 4.4. The largest inhibition levels of *L. monocytogenes* were observed at intermediate temperatures, corresponding to 8 and 11 °C, as denoted by the calculated inhibition ratio (α), which indicates that growth rate in coculture was reduced by 45 and 48%, respectively. The lowest inhibition was observed at 4 °C (α= 31%). On the other hand, μ_{max} for *Lb. sakei* was also affected by *L. monocytogenes* especially at 8 and 11 °C, with α= 46 and 31%, respectively. These results point out a possible interaction due to competition for nutrients, which was dependent on temperature conditions. This temperature-dependent effect has also been reported by other authors for the interaction between *L. monocytogenes* and *Lb. sakei* (Aguilar & Klotz, 2010). No recognizable pattern for the temperature effect can be identified based on the values of Table 4.4. The lack of mathematical correlation between temperature and the reduction of growth rate of *L. monocytogenes* in coculture (i.e. growth inhibition) was also observed by Quinto et al. (2016). However, these authors reported lower μ_{max} values for *L. monocytogenes* in coculture with *Lb. sakei* MN than those reported in our study (Table 4.3). These differences could be attributed to the strain used; a single strain, Scott A, in the study of Quinto et al. (2016), while a cocktail was tested in this study, in which the expected higher between-cell variability could lead to the strain(s) more resistant to the inhibition by *Lb. sakei* (i.e. competitive) became dominant in the cocktail populations.

Table 4.4. Inhibition ratio (α), expressed in percentage (%), ± standard deviation of the maximum specific growth rate ($\mu_{sp;max}$; h^{-1}) and maximum population density (N_{max}; log CFU/mL) for *Listeria monocytogenes* cocktail and *Lactobacillus sakei* in coculture at the five temperatures studied.

Temperature (°C)	*L. monocytogenes*		*Lb. sakei*	
	α ($\mu_{sp;max}$)[a]	α (N_{max})[b]	α ($\mu_{sp;max}$)	α (N_{max})
4	31±0.16	36±0.01	-28±0.22*	5±0.01
8	45±0.05	23±0.01	46±0.05	-6±0.01*
11	48±0.04	17±0.01	31±0.04	3±0.01
15	39±0.14	28±0.01	8±0.42	3±0.01
20	42±0.09	28±0.02	25±0.11	-2±0.02*

[a] Inhibition ratio (%) between the maximum specific growth rate in coculture (μ_{co}) and monoculture (μ_{mono}) of *L. monocytogenes* and *Lb. sakei*, respectively, using Eq. (6).
[b] Inhibition ratio (%) between the maximum population density in coculture (N_{maxco}) and monoculture ($N_{maxmono}$) of *L. monocytogenes* and *Lb. sakei*, respectively, using Eq. (7).
* The negative α values corresponded to an increase of the kinetic parameter in coculture.

Lb. sakei did not show a noticeable reduction on N_{max} in coculture as shown by the values obtained for α, which were lower than 5% at all studied temperatures (Table 4.4). In turn, for *L. monocytogenes*, N_{max} was drastically affected in coculture, decreasing from 9.26 log CFU/mL, observed in monoculture at all temperature, to 5.95 log CFU/mL in coculture at 4 °C. For this temperature, the inhibitory effect (α) on this parameter resulted in a reduction of 36% with respect to the values obtained in monoculture. At 11 °C, N_{max} was 7.68 log CFU/mL while at 15-20 °C, values were around 6.67 log CFU/mL. According to the growth curves (Fig. 4.2) and the time to reach N_{max} shown in Table 4.3, at 8, 15 and 20 °C, *L. monocytogenes* growth ceased when *Lb. sakei* reached its N_{max}, whereas at 4 and 11 °C, the pathogen stopped growing around 20-25 h after *Lb sakei* achieved N_{max}.

The differences between N_{max} of *L. monocytogenes* in coculture and monoculture indicate that *Lb. sakei* strain induced an early stationary phase of *L. monocytogenes* cocktail, corroborating that the depletion of nutritive substance (s) and/or generation of metabolic products of *Lb. sakei* may affect the growth of *L. monocytogenes* cocktail (Leroy & De Vuyst, 2003). Other authors have observed a decrease in N_{max} values of *L. monocytogenes* when cocultured with LAB being attributed to competition for nutrients or cell-to-cell contact (Buchanan & Bagi, 1997; Huang et al., 2016). Similar results were obtained by Quinto et al. (2016), although with more dramatic reductions of *L. monocytogenes* in coculture at the lower temperatures (N_{max} around 3 log CFU/mL at 4

and 10 °C). The use of a cocktail of *L. monocytogenes* allows the presence of more adapted strains to the environment, being able to reach a higher N_{max} than those cases where a single strain is used. Other authors have reported that, when *Lactobacillus* spp. grew in coculture with *L. monocytogenes* at 4 °C up to 7.38 log CFU/mL, the N_{max} of *L. monocytogenes* was only 5.07 log CFU/mL (Ye et al., 2014). Mataragas et al. (2003) studied the antagonism of LAB against *L. monocytogenes* in sliced cooked cured pork shoulder stored under vacuum or modified atmosphere at 4±2 °C. In their study, LAB had an antagonistic effect on *L. monocytogenes* growth in the pork product under vacuum conditions. Values of N_{max} of *L. monocytogenes* decreased from 7.3 log CFU/g in monoculture to 3.3-3.6 log CFU/g in coculture experiments. A similar trend was obtained in our study, when N_{max} for *Lb. sakei* and *L. monocytogenes* in coculture were 7.62 and 5.95 log CFU/mL, respectively, at 4 °C, thus confirming the inhibitory effect of the LAB used.

Regarding the medium pH, a slight decrease was observed in this parameter, starting when the bacteria entered in the exponential phase, and reaching final values of 6.4-6.7 at 4, 8 and 11 °C, 6.2 at 15 °C and 5.9 at 20 °C at the stationary phase of the microorganisms. This slight decrease in the pH may be related to the low concentration of glucose (0.2% w/v) present in the culture medium (BHI). As the level of production of organic acids greatly depends on the concentration of carbon source like glucose, the inhibition by organic acids in the medium might be discarded. This fact was also corroborated by previous studies. For instance, Vereecken & Van Impe (2002) demonstrated that the initial glucose concentration present in culture media largely influenced the production of lactic acid and other metabolites by LAB and thus, their antimicrobial activity. Al-Zeyara et al. (2011) investigated the interaction between the natural microbiota of food, including LAB, on the growth of *L. monocytogenes* in TSB, a medium with 2 g of glucose. The study concluded that the acid production was not the inhibition factor since the pH slightly decreased from 7.2 to 6.3 once *L. monocytogenes* had stopped growing. As expected, the pH decrease observed in our study was more evident as temperature increases, likely due to the higher microbial metabolic activity at higher temperatures, thus increasing production of organic acids.

Overall, the *Listeria* inhibition pattern observed in our study can be mostly explained by nutrient competition and depletion, affecting *Listeria* kinetic parameters μ_{max} and N_{max}. Moreover, the role of the potential bacteriocin production should be considered in the inhibitory effect on *L. monocytogenes*. Other authors have found a major inhibition by

other bacteriocin-producing microorganisms (Leroy et al., 2002), possibly due to differences in the tested bacterial strain and experimental set-up. In this respect, further studies considering molecular aspects are needed to elucidate the role of bacteriocin production by *Lb. sakei* L115 on the inhibition of *Listeria* growth.

4.3.2. Performance of microbial interaction models for Lb. sakei *and* L. monocytogenes *cocktail growth in coculture*

Three different interaction models, that is, the Jameson-effect based model, a modified version of the Jameson model (Giménez & Dalgaard, 2004) and the Lotka-Volterra model (Dens et al., 1999; Fujikawa, 2016) were used to simulate the effect of *Lb. sakei* growth on *Listeria* behaviour in BHI. The models were applied following the two approaches described in *Section 4.2.5.4*, consisting of not applying and applying the inhibition coefficient (α). In general, a deceleration of *L. monocytogenes* growth and the induction of early stationary phase when the *Lb. sakei* reached the stationary phase were observed. The adequacy of the above models to simulate the observations was evaluated statistically through AICc and RMSE and their prediction capacity, with ASZ. Their values can be consulted in Tables 4.5 and 4.6, for both approaches, together with the fitted interaction model parameters. For the former approach, where monoculture maximum specific growth rates were used (i.e. not applying α), the best fitting was obtained with the Lotka-Volterra model according to the statistical indexes (Table 4.5) and visual analysis (Figure 4.3 a.1-e.1). The ASZ for *L. monocytogenes* was equal to or above 70% for models at 4 and 20 °C (Table 4.5). However, no model was able to describe, satisfactorily, the simultaneous growth of *Lb. sakei* and *L. monocytogenes* cocktail at 8 and 11 (ASZ ≤40%), overestimating, in both cases, *Listeria* growth (Figure 4.3 b.1-c.1 and Table 4.5). The best results were obtained with Lotka-Volterra model for 4 and 20 °C, with ASZ values > 80% and RMSE<0.5. In the second approach, in which μ_{mono} values from the secondary models were adjusted using α, fit results showed better performance, and all temperatures could be represented adequately by the tested models as shown by Figure 4.3 a.2-e.2, especially by the modified Jameson model and Lotka-Volterra model. These models exhibited ASZ levels for *L. monocytogenes* equal to 100%, excepting for 20 °C, for which it was 85%, while for *Lb. sakei,* values were always above 85%. AICc and RMSE results, shown in Table 4.6, also confirmed the adequacy of the above models. There are other studies in the literature that have evaluated the modified

version approaches of the Jameson and Lotka-Volterra models demonstrating a good performance to describe the dynamics of the interaction between microbial species in different systems (culture broth and foods) (Blanco-Lizarazo et al., 2016; Cornu et al., 2011; Costa et al., 2019; Giménez & Dalgaard, 2004; Le Marc et al., 2009; Vereecken et al., 2000; Ye et al., 2014). Despite the Lotka-Volterra model presented worse fitting with the first approach, F_{LsLm} values, slightly above 1, were in line with the fact that the cease of the *L. monocytogenes* growth was observed when *Lb sakei* reached its N_{max} (Table 4.5). For the second approach, with much better fitting, the parameter was lower than 1, indicating that there was inhibition but, in less extent than what was shown in the first approach (Table 4.6). According to these results, it is likely that the use of a monoculture maximum specific growth rate adjusted for coculture conditions renders this interaction factor less meaningful or representative for the observed microbial interaction phenomenon. This trend could be similarly observed for $Ls_{cri.}$ from the modified Jameson model, showing lower concentrations for the first approach. Therefore, in these cases, where maximum specific growth rate observed at monoculture conditions is adjusted (reduced) to represent coculture conditions, both F_{LsLm} and α should be considered jointly to obtain a more reliable (correct) mathematical interpretation of the microbial interaction observed.

Table 4.5. Estimated interaction parameters and goodness-of-fit indexes obtained from the simulation and fitting of different interaction models considering the approach in which the monoculture maximum specific growth rate obtained from the secondary models is used to define this kinetic parameter in the model equations. The models describe the simultaneous growth of *Lactobacillus sakei* strain L155 and *Listeria monocytogenes* cocktail in BHI broth stored at 4, 8, 11, 15 and 20 °C.

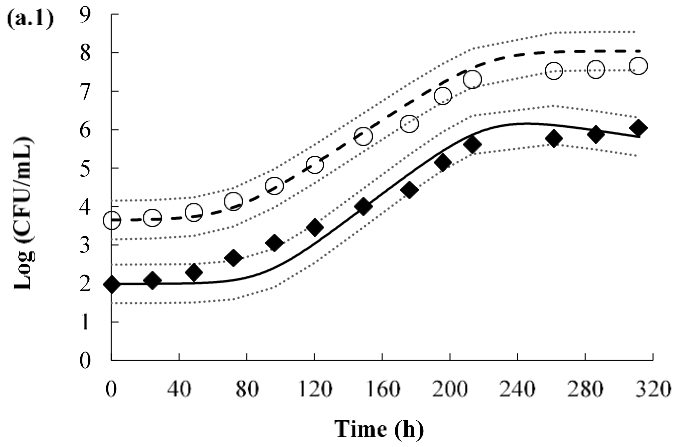
Temp. (°C)	n[a]	Jameson model				Modified version of Jameson model								Lotka-Volterra model							
		Lb. sakei		*L. monocytogenes* cocktail		*Lb. sakei*				*L. monocytogenes* cocktail				*Lb. sakei*				*L. monocytogenes* cocktail			
		RMSE[b]	ASZ (%)[c]	RMSE	ASZ (%)	RMSE	AICc[d]	ASZ (%)	Lm_{cri}[e] (CFU/mL)	RMSE	AICc	ASZ (%)	Ls_{cri}[f] (CFU/mL)	RMSE	AICc	ASZ (%)	F_{LmLs}[g]	RMSE	AICc	ASZ (%)	F_{LsLm}[h]
4	13	0.304	92.3	0.426	69.2	0.350	-23.42	92.3	$1.00x10^{8}$±5.10x10^{6}	0.415	-18.98	86.4	$9.23x10^{7}$±7.07x10^{6}	0.304	-23.37	92.3	0.00	0.359	-13.78	86.4	1.20±0.15
8	15	0.338	86.7	1.522	26.7	0.377	-25.75	86.7	$7.00x10^{8}$±3.89x10^{8}	1.519	16.07	33.3	$8.12x10^{7}$±1.36x10^{7}	0.342	-25.07	86.7	0.00	1.293	14.85	40.0	1.25±0.21
11	13	0.230	100	1.584	23.1	0.301	-27.36	92.3	$7.03x10^{8}$±9.11x10^{7}	2.265	25.14	15.4	$6.48x10^{7}$±1.13x10^{7}	0.258	-27.62	100	0.00	1.246	13.35	38.5	1.36±0.25
15	13	0.194	100	0.549	61.5	0.223	-35.07	100	$9.99x10^{7}$±6.24x10^{6}	0.600	-9.38	61.5	$1.08x10^{8}$±9.89x10^{6}	0.194	-35.00	100	0.00	0.516	-9.56	61.5	1.04±0.09
20	12	0.339	83.3	0.411	83.3	0.397	-18.02	83.3	$9.26x10^{7}$±1.03x107	0.468	-13.99	83.3	$1.05x10^{8}$±8.67x10^{6}	0.339	-17.98	83.3	0.00	0.399	-14.11	83.3	1.05±0.08

[a] Number of samples for *Lb. sakei* and *L. monocytogenes* cocktail (n).
[b] Root mean squared error (RMSE).
[c] Acceptable simulation zone (ASZ).
[d] Corrected akaike information criterion (AICc).
[e] Maximum critical concentration for *L. monocytogenes* cocktail obtained from the modified version of the Jameson model (Lm_{cri}).
[f] Maximum critical concentration for *Lb. sakei* obtained from the modified version of the Jameson model (Ls_{cri}).
[g] Competition factor of *L. monoctytogenes* cocktail in *Lb. sakei* obtained from Lotka-Volterra model (F_{LmLs}).
[h] Competition factor of *Lb. sakei* in *L. monoctytogenes* cocktail obtained from Lotka-Volterra model (F_{LsLm}).

Table 4.6. Estimated interaction parameters and goodness-of-fit indexes obtained from the simulation and fitting of different interaction models considering the approach in which the monoculture maximum specific growth rate based on the secondary models, which is used to define this kinetic parameter in the model equations, is previously adjusted with the inhibition factor (α). The models describe the simultaneous growth of *Lactobacillus sakei* strain L155 and *Listeria monocytogenes* cocktail in BHI broth stored at 4, 8, 11, 15 and 20 °C.

Temp. (°C)	n[a]	Jameson model				Modified version of Jameson model										Lotka-Volterra model							
		Lb. sakei		*L. monocytogenes* cocktail		*Lb. sakei*				*L. monocytogenes* cocktail						*Lb. sakei*				*L. monocytogenes* cocktail			
		RMSE[b]	ASZ (%)[c]	RMSE	ASZ (%)	RMSE	AICc[d]	ASZ (%)	Lm_{cri}[e] (CFU/mL)	RMSE	AICc	ASZ (%)	Ls_{cri}[f] (CFU/mL)	RMSE	AICc	ASZ (%)	F_{LmLs}[g]	RMSE	AICc	ASZ (%)	F_{LsLm}[h]
4	13	0.304	92.3	0.380	86.4	0.350	-23.38	92.3	$1.30x10^{8}$±$8.58x10^{6}$	0.331	-24.82	100	$1.61x10^{8}$±$1.09x10^{7}$	0.304	-23.37	92.3	0.00	0.287	-24.82	100	0.69±0.04
8	15	0.342	86.7	0.650	73.3	0.385	-25.08	86.7	$1.10x10^{9}$±$7.10x10^{7}$	0.211	-43.18	100	$1.73x10^{8}$±$1.01x10^{7}$	0.342	-25.07	86.7	0.00	0.187	-43.18	100	0.64±0.04
11	13	0.258	100	0.847	61.5	0.342	-23.99	84.6	$7.43x10^{8}$±$9.48x10^{7}$	0.221	-35.37	100	$3.15x10^{8}$±$4.08x10^{7}$	0.258	-27.62	100	0.00	0.181	-36.86	100	0.38±0.05
15	13	0.194	100	0.966	53.8	0.224	-35.01	100	$9.89x10^{7}$±$7.06x10^{6}$	0.439	-17.51	84.6	$1.57x10^{8}$±$1.07x10^{7}$	0.194	-35.00	100	0.00	0.380	-17.50	84.6	0.71±0.05
20	12	0.339	83.3	0.810	66.7	0.398	-17.98	83.3	$9.77x10^{7}$±$1.02x10^{7}$	0.221	-32.14	100	$3.90x10^{8}$±$4.29x10^{7}$	0.339	-17.98	83.3	0.00	0.188	-32.16	100	0.27±0.03

[a] Number of samples for *Lb. sakei* and *L. monocytogenes* cocktail (n).
[b] Root mean squared error (RMSE).
[c] Acceptable simulation zone (ASZ).
[d] Corrected akaike information criterion (AICc).
[e] Maximum critical concentration for *L. monocytogenes* cocktail obtained from the modified version of the Jameson model (Lm_{cri}).
[f] Maximum critical concentration for *Lb. sakei* obtained from the modified version of the Jameson model (Ls_{cri}).
[g] Competition factor of *L. monoctytogenes* cocktail in *Lb. sakei* obtained from Lotka-Volterra model (F_{LmLs}).
[h] Competition factor of *Lb. sakei* in *L. monoctytogenes* cocktail obtained from Lotka-Volterra model (F_{LsLm}).


(a.1)
Log (CFU/mL)
Time (h)
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2
3
4
5
6
7
8
9
0
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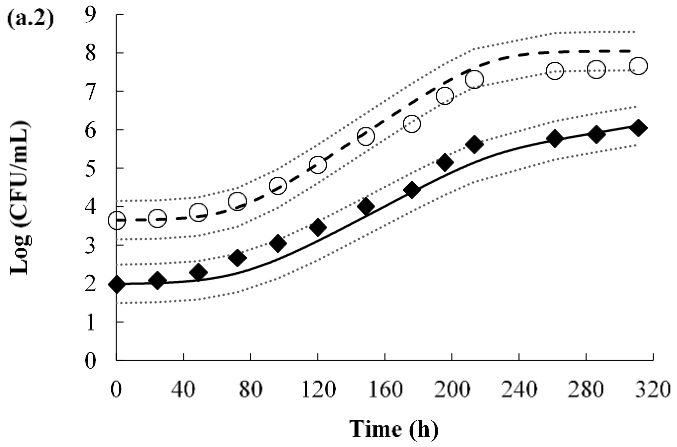
80
120
160
200
240
280
320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Log (CFU/mL)
Time (h)
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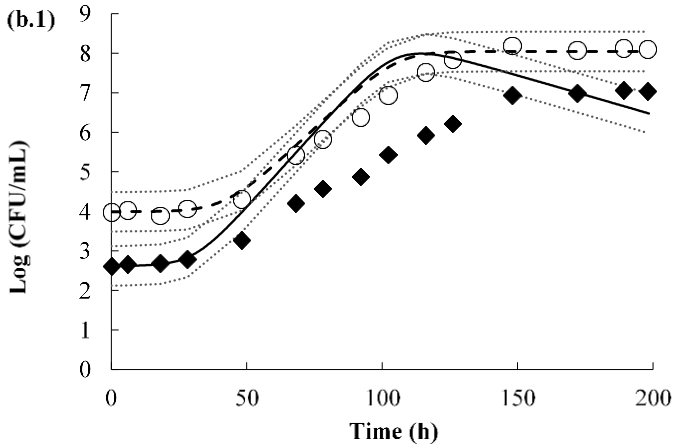
280
320


(b.1)
Log (CFU/mL)
Time (h)
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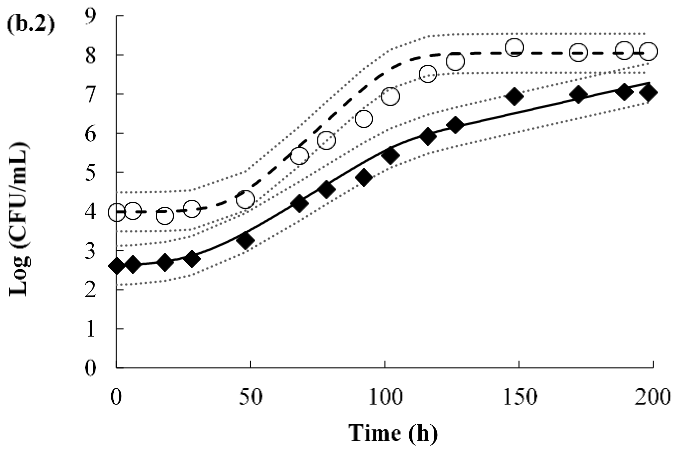
1
2
3
4
5
6
7
8
9
0
50
100
150
200


(b.2)
Log (CFU/mL)
Time (h)
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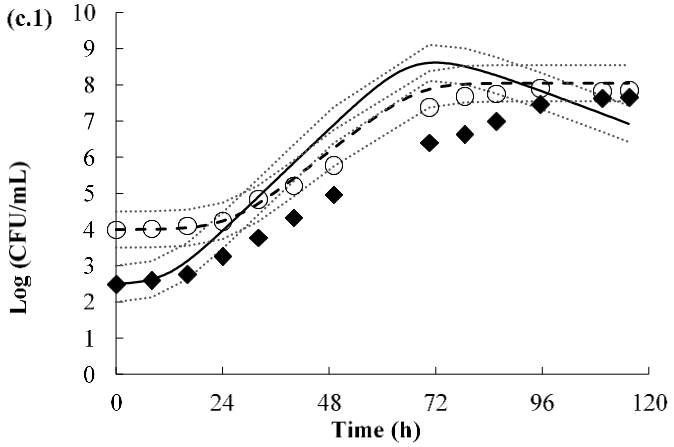
1
2
3
4
5
6
7
8
9
0
50
100
150
200


(c.1)
Log (CFU/mL)
Time (h)
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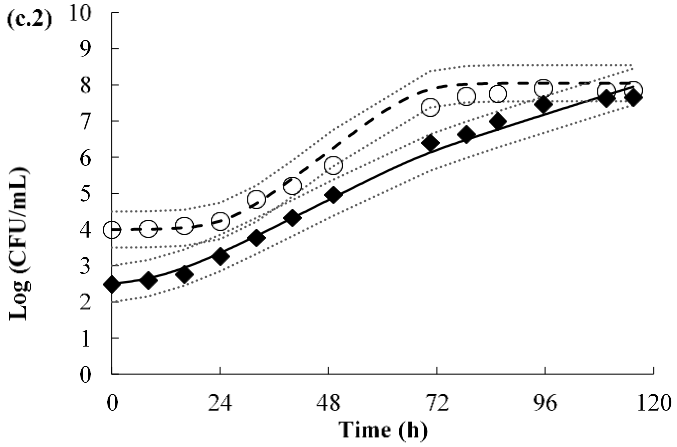
1
2
3
4
5
6
7
8
9
10
0
24
48
72
96
120


(c.2)
Log (CFU/mL)
Time (h)
0
1
2
3
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5
6
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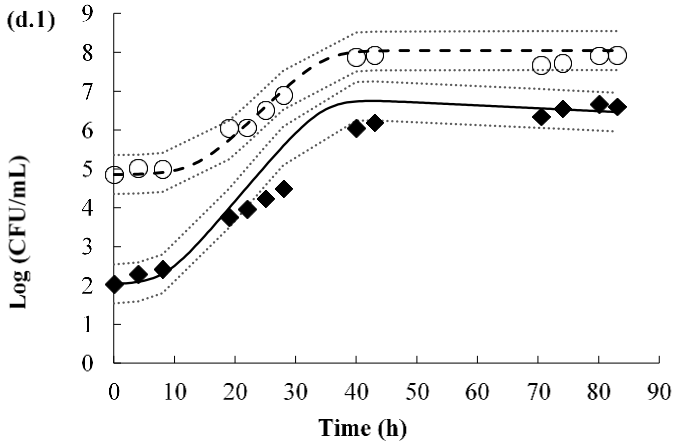
8
9
10
0
24
48
72
96
120


(d.1)
Log (CFU/mL)
Time (h)
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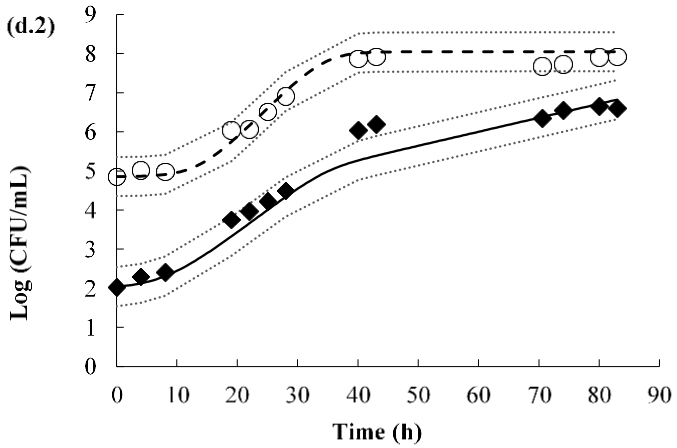
1
2
3
4
5
6
7
8
9
0
10
20
30
40
50
60
70
80
90


(d.2)
Log (CFU/mL)
Time (h)
0
1
2
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40
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60
70
80
90

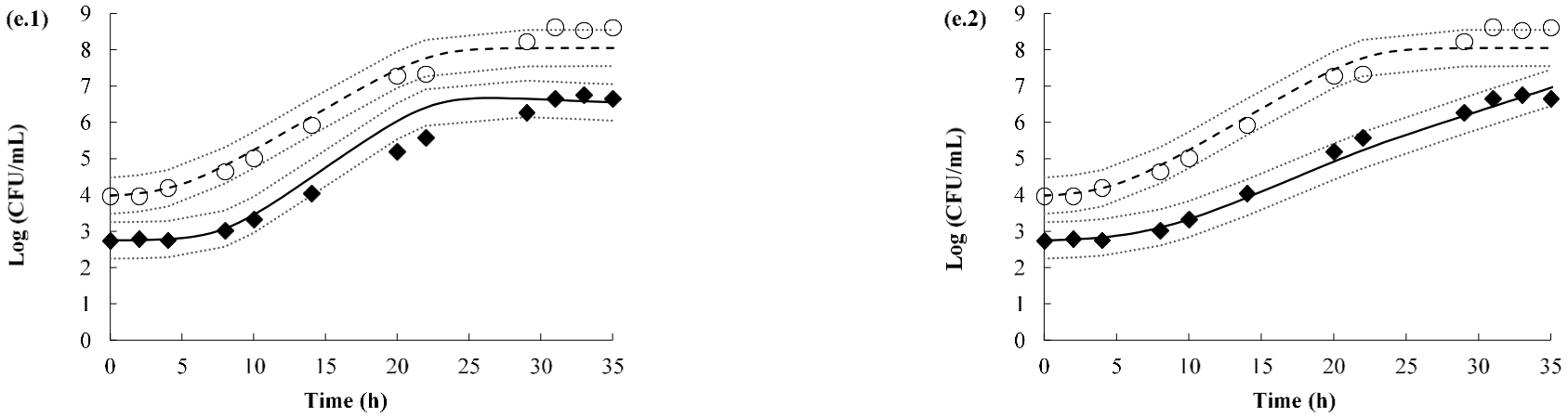

Fig. 4.3. Observed growth data of *Lactobacillus sakei* strain L115 (o) and *Listeria monocytogenes* cocktail (♦) in coculture at (a) 4 °C, (b) 8 °C, (c) 11 °C, (d) 15 °C and (e) 20 °C. The lines represent the predictions for *Lb. sakei* (- - -), *L. monocytogenes* cocktail (—) growth obtained from the Lotka-Volterra model considering two different approaches: 1) using the monoculture maximum growth rate obtained from the secondary model and 2) adjusting the estimated monoculture maximum growth rate by applying the inhibition factor (α) (2). The acceptable simulation zone (ASZ) defined as ± 0.5 log CFU/mL from the observed counts is represented by a dotted line (·····).

The failure of the first approach to describe the microbial competition in some of the temperatures (8 and 11 °C) could bring in question the statement by Rubinow (1984) claiming that the growth potential is characteristic to the species and the environment. The Rubinow´s hypothesis assumes that the growth rate for a specific microbial population would be invariant under the same environmental conditions regardless of whether or not there is another microbial population in the same substrate. This hypothesis is supported by several scientific studies, that have been able to obtain reliable growth predictions for coculture conditions, assuming that the maximum growth rate of the mixed culture is the same one as the estimated in pure culture (Cornu et al., 2002; Cornu et al., 2011). However, the results, in our work, suggest that coculture conditions could influence growth potential in some cases (i.e. temperature conditions). A possible explanation for this finding could be that environment can change dynamically overgrowth since the microbial activity can modify the physico-chemical properties (i.e., pH, lactate, etc.) of the substrate due to the production of metabolites (e.g. bacteriocins) or degradation of chemical compounds. This would lead to different growth potentials of the microbial populations along incubation period. Besides, it should not be discarded that the mere presence of a heterogenous microbial population can influence the capacity of each population to exploit the environmental resources required to grow. In our study, no conclusive data could be provided concerning what (molecular and biological) phenomena could be behind the fact that the growth potential in coculture conditions was reduced in spite of the physico-chemical factors were similar between experiments. These results and gaps suggest that a more biological knowledge should be developed for a deeper understanding of the mechanisms of microbial interaction and coexistence as well as how the interaction depends on environmental conditions (Vandermeer & Goldber, 2003). In any case, from a practical point of view, this study highlights the importance of assessing the impact of the microbial ecology on the microbial kinetic parameters in order to develop more accurate microbial interaction models.

4.4. Conclusion

The present study demonstrated that the growth rate and maximum population density of *L. monocytogenes* noticeably decreased when cocultured in presence of *Lb. sakei* L115, thus the inhibitory effect of *Lb. sakei* L115 against a number of mixed strains of the pathogen was confirmed. The combination of a high concentration of *Lb. sakei* L115, low temperatures, depletion for nutrients and bacteriocin production can be hypothesized as the main causes for inhibiting the growth of *L. monocytogenes*. In addition, the simultaneous growth of *Lb. sakei*

and *L. monocytogenes* cocktail could be better simulated with the Lotka-Volterra model and modified Jameson model under the hypothesis that the growth potential of one population is affected (reduced) in presence of another population rather than assuming that this potential growth could be constant at monoculture and coculture conditions as represented by the first approach in this study. Notwithstanding, more research is needed to clarify the inhibitory role of *Lb. sakei* on *Listeria* growth capacity at a biomolecular level. As experiments were carried out in culture broth medium, the impact of food matrix on the interaction phenomenon should be also assessed. The findings of this study could be the first step to develop more mechanistic models able to describe different microbial interaction patterns, including those observed in the present study.

CrediT authorship contribution statement

J.C.C.P. Costa: Methodology, Investigation, Visualization, Writing - original draft. **A. Bolívar:** Writing - review & editing, Validation. **A. Valero:** Writing - review & editing. **E. Carrasco:** Writing - review & editing. **G. Zurera:** Validation, Project administration. **F. Pérez Rodríguez:** Conceptualization, Writing - review & editing, Supervision.

Declarations of Competing Interest

None.

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Appendix B. Supplementary material

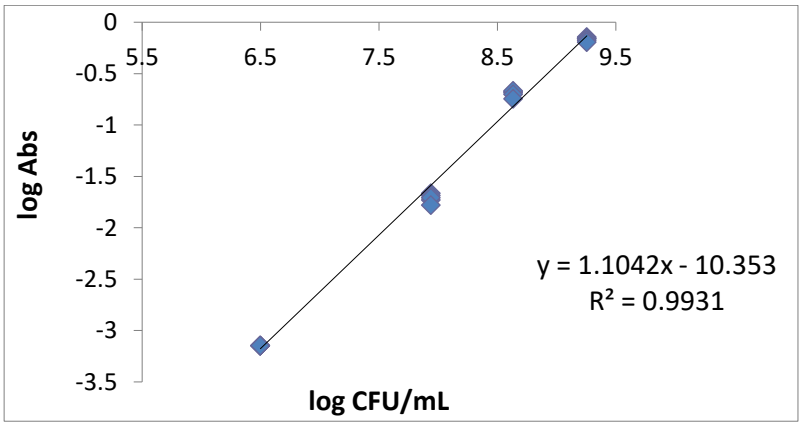
Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2019.108928.

Appendix B. Supplementary File 4.1

Absorbance measurements of *L. monocytogenes* at 6 °C expressed in logarithmic scale were transformed into viable cell counts (log CFU/mL) by performing calibration curves as described by Bolívar et al. (2018).

CFU/ml	Absorbance	Absorbance-Control	log Absorbance	log CFU/ml	Control (non-inoculated BHI)	
1.80E+09	0.998	0.71771429	-0.144048409	9.255272505	0.28	
1.80E+09	0.975	0.69471429	-0.15819377	9.255272505	0.278	
1.80E+09	0.979	0.69871429	-0.155700377	9.255272505	0.282	
1.80E+09	0.951	0.67071429	-0.173462443	9.255272505	0.281	
1.80E+09	0.92	0.63971429	-0.194013951	9.255272505	0.28	
4.30E+08	0.487	0.20671429	-0.684629509	8.633468456	0.28	
4.30E+08	0.491	0.21071429	-0.67630602	8.633468456	0.281	
4.30E+08	0.496	0.21571429	-0.666121093	8.633468456		
4.30E+08	0.48	0.19971429	-0.699590869	8.633468456	0.280285714	AVERAGE
4.30E+08	0.461	0.18071429	-0.743007515	8.633468456	0.001253566	ST. DEV
8.65E+07	0.302	0.02171429	-1.663254452	7.937016107		
8.65E+07	0.301	0.02071429	-1.683730038	7.937016107		
8.65E+07	0.299	0.01871429	-1.727826744	7.937016107		
8.65E+07	0.3	0.01971429	-1.705218954	7.937016107		
8.65E+07	0.297	0.01671429	-1.776912178	7.937016107		
3.15E+06	0.281	0.00071429	-3.146128036	6.498310554		
3.15E+06	0.281	0.00071429	-3.146128036	6.498310554		
3.15E+06	0.281	0.00071429	-3.146128036	6.498310554		
3.15E+06	0.281	0.00071429	-3.146128036	6.498310554		
3.15E+06	0.281	0.00071429	-3.146128036	6.498310554		

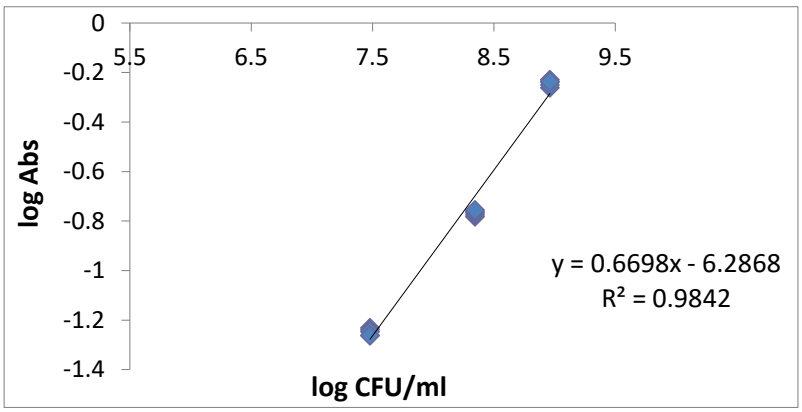
Calibration curve for *L. monocytogenes* at 6 °C.



Absorbance measurements of *L. monocytogenes* at 25 °C expressed in logarithmic scale were transformed into viable cell counts (log CFU/mL).

CFU/ml	Absorbance	Absorbance-Control	log Absorbance	log CFU/ml	Control (non-inoculated BHI)	
1.41E+09	1.431	1.19866667	0.078698428	9.149219113		
1.41E+09	1.391	1.15866667	0.063958513	9.149219113		
1.41E+09	1.397	1.16466667	0.066201646	9.149219113		
1.41E+09	1.391	1.15866667	0.063958513	9.149219113	0.232	
1.41E+09	1.407	1.17466667	0.069914645	9.149219113	0.234	
9.15E+08	0.822	0.58966667	-0.229393422	8.961421094	0.232	
9.15E+08	0.81	0.57766667	-0.238322692	8.961421094	0.23	
9.15E+08	0.78	0.54766667	-0.261483691	8.961421094	0.234	
9.15E+08	0.795	0.56266667	-0.249748812	8.961421094	0.232	
9.15E+08	0.811	0.57866667	-0.237571534	8.961421094		
2.21E+08	0.397	0.16466667	-0.783394306	8.344392274	0.232333333	AVERAGE
2.21E+08	0.4	0.16766667	-0.77555327	8.344392274	0.001505545	ST. DEV
2.21E+08	0.403	0.17066667	-0.767851294	8.344392274		
2.21E+08	0.405	0.17266667	-0.762791495	8.344392274		
2.21E+08	0.408	0.17566667	-0.75531064	8.344392274		
3.01E+07	0.291	0.05866667	-1.231608587	7.478566496		
3.01E+07	0.29	0.05766667	-1.239075152	7.478566496		
3.01E+07	0.289	0.05666667	-1.246672333	7.478566496		
3.01E+07	0.287	0.05466667	-1.262277407	7.478566496		
3.01E+07	0.287	0.05466667	-1.262277407	7.478566496		
1.50E+06	0.264	0.03166667	-1.499397649	6.176091259		
1.50E+06	0.266	0.03366667	-1.472799881	6.176091259		
1.50E+06	0.264	0.03166667	-1.499397649	6.176091259		
1.50E+06	0.263	0.03066667	-1.513333427	6.176091259		
1.50E+06	0.266	0.03366667	-1.472799881	6.176091259		

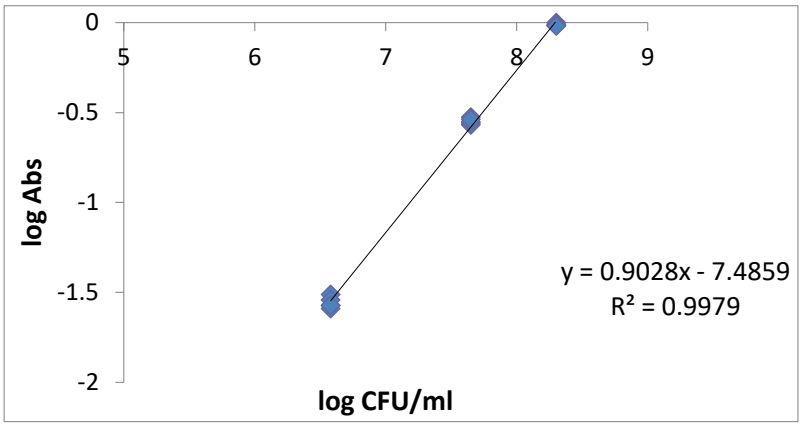
Calibration curve for *L. monocytogenes* at 25 °C.



Absorbance measurements of *Lb. sakei* L115 at 6 °C expressed in logarithmic scale were transformed into viable cell counts (log CFU/mL).

CFU/ml	Absorbance	Absorbance-Control	log Absorbance	log CFU/ml	Control (non-inoculated BHI)	
2.00E+08	1.246	0.96571429	-0.015151344	8.301029996		
2.00E+08	1.277	0.99671429	-0.001429317	8.301029996		
2.00E+08	1.255	0.97471429	-0.011122669	8.301029996		
2.00E+08	1.248	0.96771429	-0.014252848	8.301029996		
2.00E+08	1.238	0.95771429	-0.018764034	8.301029996	0.28	
4.45E+07	0.557	0.27671429	-0.557968419	7.648360011	0.278	
4.45E+07	0.578	0.29771429	-0.526200325	7.648360011	0.282	
4.45E+07	0.55	0.26971429	-0.56909605	7.648360011	0.281	
4.45E+07	0.561	0.28071429	-0.551735485	7.648360011	0.28	
4.45E+07	0.57	0.28971429	-0.538030089	7.648360011	0.28	
3.80E+06	0.306	0.02571429	-1.589825535	6.579783597	0.281	
3.80E+06	0.311	0.03071429	-1.51265958	6.579783597		
3.80E+06	0.307	0.02671429	-1.573256433	6.579783597		
3.80E+06	0.309	0.02871429	-1.541901983	6.579783597	0.280285714	AVERAGE
3.80E+06	0.307	0.02671429	-1.573256433	6.579783597	0.001253566	ST. DEV
9.45E+05	0.286	0.00571429	-2.243038049	5.975431809		
9.45E+05	0.286	0.00571429	-2.243038049	5.975431809		
9.45E+05	0.286	0.00571429	-2.243038049	5.975431809		
9.45E+05	0.286	0.00571429	-2.243038049	5.975431809		
9.45E+05	0.286	0.00571429	-2.243038049	5.975431809		

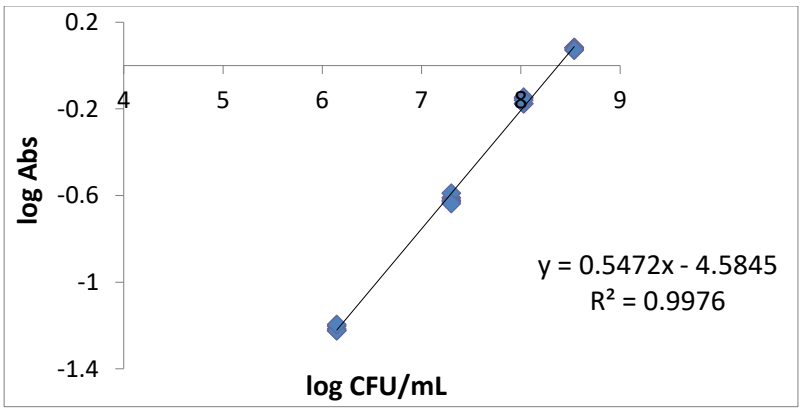
Calibration curve for Lb. sakei L115 at 6 °C.



Absorbance measurements of *Lb. sakei* L115 at 25 °C expressed in logarithmic scale were transformed into viable cell counts (log CFU/mL).

CFU/ml	Absorbance	Absorbance-Control	log Absorbance	log CFU/ml	Control (non-inoculated BHI)	
3.45E+08	1.441	1.208666667	0.082306545	8.537819095		
3.45E+08	1.443	1.210666667	0.083024585	8.537819095		
3.45E+08	1.439	1.206666667	0.081587316	8.537819095	0.232	
3.45E+08	1.409	1.176666667	0.070653451	8.537819095	0.234	
3.45E+08	1.417	1.184666667	0.073596169	8.537819095	0.232	
1.07E+08	0.94	0.707666667	-0.150171261	8.029383778	0.23	
1.07E+08	0.899	0.666666667	-0.176091259	8.029383778	0.234	
1.07E+08	0.9	0.667666667	-0.175440305	8.029383778	0.232	
1.07E+08	0.946	0.713666667	-0.146504587	8.029383778		
1.07E+08	0.924	0.691666667	-0.160103154	8.029383778	0.232333333	AVERAGE
2.00E+07	0.478	0.245666667	-0.609653767	7.301029996	0.001505545	ST. DEV
2.00E+07	0.471	0.238666667	-0.622208232	7.301029996		
2.00E+07	0.468	0.235666667	-0.627701841	7.301029996		
2.00E+07	0.49	0.257666667	-0.588941761	7.301029996		
2.00E+07	0.463	0.230666667	-0.63701516	7.301029996		
1.40E+06	0.295	0.062666667	-1.202963405	6.146128036		
1.40E+06	0.292	0.059666667	-1.224268224	6.146128036		
1.40E+06	0.293	0.060666667	-1.217049867	6.146128036		
1.40E+06	0.292	0.059666667	-1.224268224	6.146128036		
1.40E+06	0.296	0.063666667	-1.196087887	6.146128036		
5.20E+05	0.269	0.036666667	-1.43572857	5.716003344		
5.20E+05	0.269	0.036666667	-1.43572857	5.716003344		
5.20E+05	0.268	0.035666667	-1.447737477	5.716003344		
5.20E+05	0.267	0.034666667	-1.460087915	5.716003344		
5.20E+05	0.269	0.036666667	-1.43572857	5.716003344		

Calibration curve for *Lb. sakei* L115 at 25 °C.



Chapter 5

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

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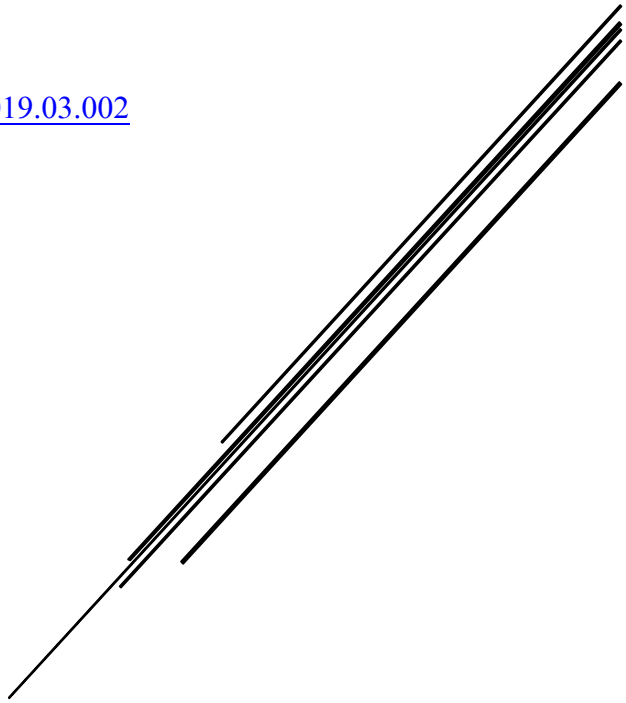

HIGHLIGHTS

- *Lactobacillus sakei* CTC494 inhibited *Listeria monocytogenes* in sea bream fillets during chilled and moderated abuse temperature storage.
- *L. sakei* CTC494 did not increase deterioration of fillets sea bream at an initial level of ≤ 4 log cfu/g.
- *L. sakei* CTC494 showed potential as bioprotective culture for fish products.
- An approach from both to food was developed for modelling microbial interaction.
- Model simulation the bioprotective effect of *L. sakei* CTC494 on *L. monocytogenes* in sea bream.

Abstract

The objective of this work was to quantitatively evaluate the effect of *Lactobacillus sakei* CTC494 (sakacin-producing bioprotective strain) against *Listeria monocytogenes* in fish juice and to apply and validate three microbial interaction models (Jameson, modified Jameson and Lotka Volterra models) through challenge tests with gilthead sea bream (*Sparus aurata*) fillets under modified atmosphere packaging stored at isothermal and non-isothermal conditions. *L. sakei* CTC494 inhibited *L. monocytogenes* growth when simultaneously present in the matrix (fish juice and fish fillets) at different inoculation ratios pathogen:bioprotector (i.e. 1:1, 1:2 and 1:3). The higher the inoculation ratio, the stronger the inhibition of *L. monocytogenes* growth, with the ratio 1:3 yielding no growth of the pathogen. The maximum population density (N_{max}) was the most affected parameter for *L. monocytogenes* at all inoculation ratios. According to the microbiological and sensory analysis outcomes, an initial inoculation level of 4 log cfu/g for *L. sakei* CTC494 would be a suitable bioprotective strategy without compromising the sensory quality of the fish product. The performance of the tested interaction models was evaluated using the Acceptable Simulation Zone approach. The Lotka Volterra model showed slightly better fit than the Jameson-based models with 75- 92 % out of the observed counts falling into the Acceptable Simulation Zone, indicating a satisfactory model performance. The evaluated interaction models could be used as predictive modelling tool to simulate the simultaneous behaviour of bacteriocin-producing *Lactobacillus* strains and *L. monocytogenes*; thus, supporting the design and optimization of bioprotective culture-based strategies against *L. monocytogenes* in minimally processed fish products.

Keywords: biopreservation, food-borne pathogen, lactic acid bacteria, competition model, minimally processed fish, predictive microbiology.

5.1. Introduction

Global consumption of fresh and minimally processed fish has grown rapidly in recent decades. In this regard, aquaculture has been responsible for the extraordinary growth in the supply of fish for human consumption, which resulted in a record-high per capita consumption of 20.3 kg in 2016 (FAO, 2018). The combination of chemical oxidation of lipids, autolytic biochemical reactions and physico-chemical characteristics make fish a highly perishable product, but also an ideal environment for growth of spoilage microorganisms and food-borne pathogens (Dalgaard et al., 2006; Parlapani et al., 2014). Among the pathogenic bacteria, *Listeria monocytogenes* stands out because of its ability to tolerate salty environments and multiply in refrigerated foods, coupled with the high mortality rates in humans (CDC, 2017). The pathogen has been isolated from a variety of raw fish and processed fish products (Abdollahzadeh et al., 2016; Lennox et al., 2017; Rožman et al., 2016), and according to the last report of the European Food Safety Authority (EFSA), "fish and fishery products" showed the highest levels of non-compliance with the food safety microbiological criteria for *L. monocytogenes* laid down by Regulation (CE) 2073/2005 (EFSA, 2017).

Lactic acid bacteria (LAB), and lactobacillus in particular, constitute the dominant microbiota in several types of foods and many LAB species are used as microbial food cultures (MFC) in food production. In the European Union (EU), there is no specific regulation regarding MFC; but with a long history of safe use, they are considered traditional food ingredients and are legally permitted without premarket approval. Thus, MFC defined as characteristic food ingredients must be listed on the ingredient labels of the final food in agreement with the Regulation (EU) 1169/2011. In addition, when added to a food, MFC must comply with the requirements established in the General Food Law (Regulation (EC) 178/2002), i.e. they must be safe for their intended use (Herody et al., 2010; Laulund et al., 2017). Many LAB genera and species are generally recognized as safe (GRAS) by the FDA (2018) and have the qualified presumption of safety (QPS) status established by EFSA. Among LAB, *Lactobacillus* is the genus including a high number of GRAS species, and particularly, *Lactobacillus sakei* is included in the QPS list (EFSA BIOHAZ, 2017), thus not requiring the full safety assessment (antibioresistance, virulence, and biogenic amine characterization) for its market authorisation in the EU. The application of selected LAB strains as bioprotective cultures has demonstrated a high potential to inhibit undesirable spoilage and pathogenic bacteria in fresh fish and RTE fish products, including *L. monocytogenes* (Anacarso et al.,

2014; Brillet et al., 2005). The inhibitory mechanism of LAB includes microbial growth competition as well as microbial antagonism associated with the production of antimicrobial metabolites such as organic acids (lactic acid, acetic acid, etc.), hydrogen peroxide and more specifically, bacteriocins active against specific bacteria such as *L. monocytogenes* (Gómez-Sala et al., 2016). In relation to the latter, sakacins, being produced by certain *L. sakei* strains, belong to subclass IIa of bacteriocins which are generally known to have a strong anti-listerial activity (Leroy and De Vuyst, 2000). The lethal action of these bacteriocins results from membrane pore formation of the target cell causing depletion of vital components as well as dissipation of the proton motive force (Héchard and Sahl, 2002).

Microbial interaction has been addressed in the predictive microbiology field mainly focused on the inhibitory effect of endogenous LAB on *L. monocytogenes* behaviour (Mejlholm and Dalgaard, 2007). Interaction models are usually intended to quantify how much the growth one population is reduced by the growth of other populations (Cornu et al., 2011; Pérez-Rodríguez and Valero, 2013). Thus, two model approaches are generally used to describe the interaction of LAB and *L. monocytogenes*: i) those based on the Jameson effect phenomenon (Jameson, 1962) that describes the simultaneous stop of growth of all bacterial populations at the time when the dominant bacteria population reaches its stationary phase (Giménez and Dalgaard, 2004; Mellefont et al., 2008; Møller et al., 2013) and ii) the predator-prey models based on the Lotka Volterra equation, which allow to describe the dynamics of two competing bacterial populations by incorporating an additional term describing the reduction of the growth rate of a given population, this being proportional to the population density of other competing population (Powell et al., 2004; Valenti et al., 2013; Vereecken et al., 2000).

Predictive models dealing with the interaction between the pathogen *Listeria* and bacteriocin-producing LAB strains in foods other than fermented meat products (Drosinos et al., 2006; Leroy et al., 2005) are, to the best knowledge of the authors, not available in literature. Their development would provide the food industry with valuable tools to evaluate the effect of potential bioprotective cultures against *L. monocytogenes* in specific food matrices, thereby enhancing food safety. In this respect, minimally processed and RTE fish products made of raw fish, which are consumed without applying any lethal treatment, could pose a serious risk in relation to *L. monocytogenes* (Jami et al., 2014; Miettinen and Wirtanen, 2005; Rožman et al., 2016). Sea bream, considered a valuable fish species in Mediterranean EU countries, has been

included over the last years as main ingredient in popular non-heated RTE fish products, such as sushi, carpaccio and other products (Bolívar et al., 2018). This fish species is mostly commercialized fresh as whole fish and in several supermarket chains as filleted fish under modified atmosphere packaging (MAP).

Therefore, the objective of this work was i) to quantitatively evaluate the effect of the sakacin-producing bioprotective strain *Lactobacillus sakei* CTC494 against *L. monocytogenes* CTC1034 in a fish model system and ii) to apply and validate microbial interaction models to simulate the simultaneous growth of both microorganisms in gilthead sea bream (*Sparus aurata*) fillets under MAP at isothermal and non-isothermal conditions.

5.2. Material and Methods

5.2.1. A step-wise approach for interaction model development

A step-wise approach was followed to develop interaction models simulating the growth of the bioprotective *L. sakei* CTC494 and *L. monocytogenes* CTC1034 in fish fillets under MAP during isothermal and dynamic storage temperature. A schematic overview of the step-wise method is shown in Figure. 5.1.

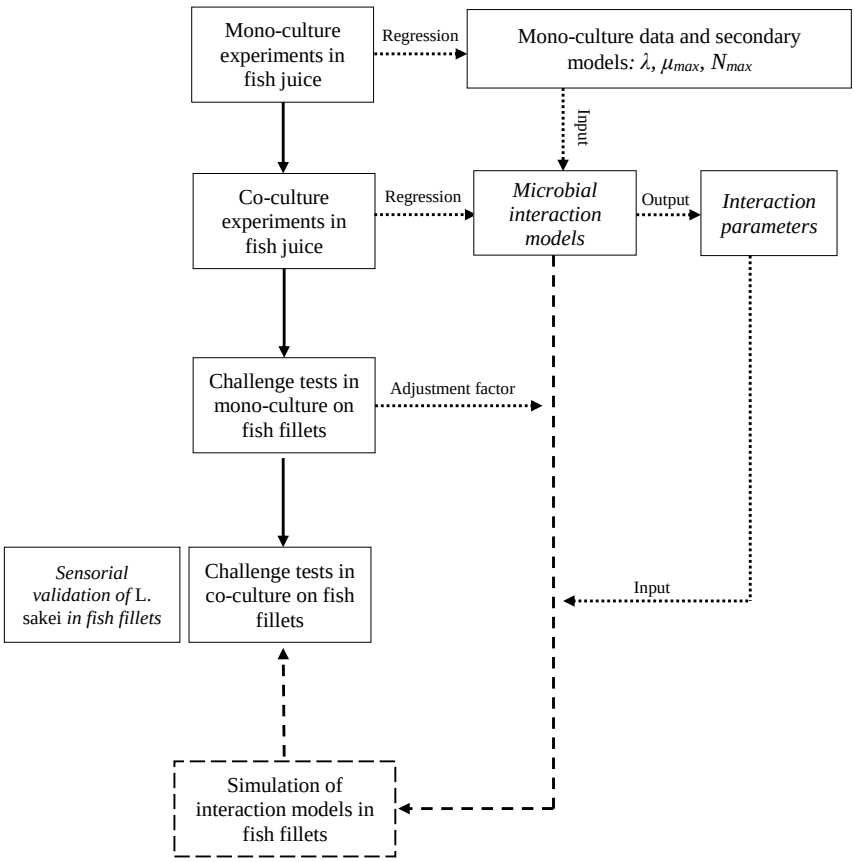

Figure. 5.1. A schematic overview of the modelling approach used in this study. Solid lines represent the experiments carried out for data generation, while dotted and dashed lines stand for the model building process and interaction model simulation, respectively. Lag phase duration: λ, maximum specific growth rate: μ_{max} and maximum population density: N_{max}.

In the first step, the primary kinetic parameters lag time (λ), maximum specific growth rate (μ_{max}) and maximum population density (N_{max}) were obtained for each microorganism from experimental data in mono-culture in fish (sea bream) juice at different temperature conditions (*Section 5.2.3*) and based on those, secondary models were generated (*Section 5.2.7.2*). Secondly, experimental data obtained in fish juice in co-culture were used (*Section 5.2.3*) to estimate competition parameters in interaction models by means of a regression process (*Section 5.2.7.4*). In a third step, the parameters from the secondary models and estimated interaction parameters for the model showing the best performance were used to simulate microbial interaction on fish fillets stored under MAP at isothermal and non-isothermal conditions *(Section 5.2.5.2*). The values for interaction parameters were assumed to be constant in the tested ratios for both microorganisms, hence the average from all assayed temperatures was used to define these parameters. Since an effect of the fish matrix and MAP conditions on kinetic parameters was expected, the maximum specific growth rate obtained in fish juice was adjusted to consider such effects. To determine the adjustment factor, data from experiments made with fish fillets (*Section 5.2.5.1*) were used, in which both microorganisms were inoculated separately at the same level and monitored under the same temperature conditions used in the fish juice experiments. The adjustment factor for μ_{max} of each microorganism was calculated as the ratio between the μ_{max} values obtained in fish product and in fish juice and were assumed to be constant for the range of temperatures tested. Therefore, the same adjustment factor was applied to simulate the microbial interaction on fresh fish fillets at isothermal and non-isothermal temperature conditions.

5.2.2. Bacterial strains and inoculum preparation

The bacteriocin-producing *L. sakei* CTC494 strain was selected as bioprotective culture in this study. This strain is a producer of bacteriocin, sakacin K, being able to inhibit the growth of spoilage bacteria and *Listeria* (Hugas et al., 1993). The strain *L. monocytogenes* CTC1034 previously used as indicator to study the antagonism the LAB produced bacteriocins (Garriga et al., 2002) was used in the present study as target pathogen. This strain has the same serotype

(i.e. 4b) as the clinical isolate Scott A. Stock cultures were stored at −80 °C in de Man Rogosa and Sharpe (MRS, Oxoid, UK) broth for the LAB strain and in Brain Heart Infusion (BHI, Oxoid) for the pathogen, both with 20% glycerol as cryoprotectant.

Before experiments, *L. sakei* CTC494 and *L. monocytogenes* CTC1034 were pre-cultured separately at static conditions in MRS (Oxoid, UK) at 33 °C with 10% CO_2 and BHI broth (BHI, Oxoid) at 37 °C, respectively. Two consecutive 24 h-subcultures were made for each microorganism by transferring 0.1 mL to tubes containing 9 mL of fresh respective media and incubating at the same above-mentioned temperatures. Then, a third subculture was prepared, and tubes were incubated for 18-20 h at the appropriate temperature resulting 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.

5.2.3. Experiments with L. sakei *CTC494 and* L. monocytogenes *CTC1034 in mono- and co-culture in fish juice*

Sterile fish juice was prepared from fresh muscle of gilthead sea bream following the protocol described by Bolívar et al. (2018). The prepared cultures (*Section 5.2.2*) were twice-washed in phosphate buffered saline solution (PBS) (Medicago AB, Uppsala, Sweden) by centrifugation at 4100 rpm (Jouan C4i, Thermo Electron Corporation, France) for 10 min and cells were re-suspended in fish juice. The suspensions of *L. monocytogenes* and *L. sakei* were serially diluted ten-fold in fish juice to obtain the desired concentration to be inoculated to fish juice at 1% (v/v).

Growth experiments were carried out at static conditions in sterile 250-mL Schott bottles containing fish juice. In the mono-culture experiments, the inoculum concentration of each microorganism was set to ca. 10^2 cfu/mL. For the co-culture experiments, the inoculum concentration of *L. monocytogenes* was always 10^2 cfu/mL, while for *L. sakei* CTC494, three different concentrations were investigated, (10^2, 10^4 and 10^6 cfu/mL), thus generating three (initial) inoculation ratios *L. monocytogenes*: *L. sakei* that corresponded to 1:1, 1:2 and 1:3 when bacterial concentrations were expressed in logarithmic scale. After inoculation, flasks were stored at four constant temperatures targeted at 2, 5, 8 and 12 °C during a period from 5 to 46 days. Storage temperature was recorded at regular time intervals using data loggers (Fourtec, MiniLitE5032L, USA) and the mean of registered temperatures (i.e. 2.2, 5.0, 8.1 and 12.1 °C) was used for modelling purposes. Each experiment was performed in duplicate.

5.2.4. Quality deterioration assessment of fresh fish sea bream fillets under MAP

5.2.4.1. Fish fillet product description

Individual plastic trays containing two fresh gilthead sea bream fillets packed under MAP were supplied by a private company (Zaragoza, Spain). Fish trays were received at the laboratory 18–24 h after processing in expanded polystyrene boxes with flake ice. The average weight of the fish fillets was 332.2 ± 12.1 g with an initial pH of 6.11 ± 0.05 (Hanna Edge, HI2020, USA). The initial headspace gas composition in the trays was measured using a O_2/CO_2 gas analyser (Gaspace 2, Systech Instruments, U.K.) and the obtained values corresponded to37.4 ± 0.7% for O_2 and 27.0 ± 1.0% for CO_2.

5.2.4.2. Inoculation of fish fillets

Bacterial suspensions prepared as described in *Section 5.2.2* were serially diluted ten-fold with physiological saline water (PSW, 0.85 % w/v NaCl). For inoculation, aliquots of 0.01 mL were taken from the appropriate decimal dilution and deposited on the caudal region of the fish fillet. Inoculation was performed using a 1-mL syringe with needle (BD Plastipak, Spain) inserted through an adhesive septum (ø 15 mm, PBI Dansensor, Denmark) which was previously placed on the laminate film of the plastic tray.

5.2.4.3. Sensory analysis

A preliminary sensory analysis was conducted to assess the effect of the initial level of *L. sakei* CTC494 on fish quality deterioration. In that aim, fish fillets were inoculated with *L. sakei* CTC494 as described in the previous section at three initial concentrations of 10^2, 10^4 and 10^6 cfu/g (n = 14, 14 and 10, respectively). A control batch was prepared without added bacteria (n = 14). All trays were stored at 5.0 ± 0.12 °C.

A semi-trained sensory panel made up of five members from the Faculty of Veterinary (University of Cordoba, Spain) was required in order to evaluate the quality changes of the fish fillets using the Quality Index Method (QIM) (Bremner, 1985). This method is based on the use of significant sensory parameters and characteristic attributes for raw fish with a scoring system of demerit points (≤ 3), which is in direct proportion to their importance in the deterioration pattern of the species (Huidobro et al., 2000). The scores for all the characteristics are summed-up to give an overall sensory score, the so-called Quality Index (QI) (Botta, 1995).

A QI of 0 indicates a very fresh fish and score increases as the freshness's characteristics deteriorate (Campus et al., 2011).

In our study, the QIM was adapted from the scheme proposed by Lougovois et al. (2003) and Campus et al. (2011) to evaluate freshness in gilthead sea bream fillets under MAP. The attributes scored by the sensory panel are shown in Supplementary Table S5.1. A linear correlation was established for each experimental condition (i.e. control and inoculated batches) between the freshness expressed by the QI and storage time (Microsoft Excel, Redmond, USA). The QI scores obtained by the five panellists in each evaluation day for inoculated and control fillets were statistically compared by a t-Student test ($p = 0.05$) using the statistical software package SPSS 24.0 (Chicago, Illinois, USA).

Sensory results demonstrated that the rate of freshness loss was similar for fillets inoculated with 10^2 and 10^4 cfu/g of *L. sakei* compared to control fillets (data not shown). Hence, a level of 10^2 and 10^4 cfu/g of *L. monocytogenes* and *L. sakei*, respectively (ratio 1:2 in log scale) was defined for co-inoculation experiments in fish fillets (*Section 5.2.5.2*).

The application of *L. sakei* CTC494 as protective culture was sensory validated on fish fillets inoculated with both microorganisms at a ratio 1:2, which corresponded to, in arithmetic scale, ca. 10^2 cfu/g *L. monocytogenes* CTC1034 and ca. 10^4 cfu/g *L. sakei* CTC494. Inoculated fish and control (i.e. non-inoculated) fillets were stored at 5 ± 0.12 °C for 8 days. Sensory assessment was performed on days 0, 4, 6 and 8.

5.2.5. Experiments with L. sakei *CTC494 and* L. monocytogenes *CTC1034 on fresh gilthead sea bream fillets*

5.2.5.1. Mono-culture experiments

The effect of food matrix on the growth of *L. sakei* CTC494 and *L. monocytogenes* CTC1034 was evaluated by inoculating both microorganisms independently in fresh fish fillets. For that, fish fillets were acquired and inoculated (n = 36) as described in *Sections 5.2.4.1* and 5.*2.4.2*. An additional control batch (n = 22) with non-inoculated fish fillets was prepared. Experiments were carried out at a target temperature of 5 °C (measured mean temperature of 4.8 ± 0.14 °C) for 25 days until microorganisms reached the stationary phase.

5.2.5.2. Co-culture experiments

The interaction between *L. sakei* and *L. monocytogenes* on fish fillets was evaluated by co-inoculation at the selected 1:2 ratio (i.e. 2 log cfu/g *L. monocytogenes* and 4 log cfu/g *L. sakei*),

which was previously defined according to results from *Section 5.2.4.3*. Before inoculation, bacterial suspensions were serially diluted ten-fold in PSW to obtain the desired concentration and mixed at equal volumes. Control (n = 56) and inoculated (n = 106) fillets were stored at two isothermal conditions with a mean of 4.8 ± 0.14 and 8.2 ± 0.10 °C for to 14 and 10 days, respectively. For the experiments at non-isothermal conditions, fillets were stored at two dynamic temperature profiles, ranging from 4 to 8 °C (profile 1) and from 2.5 to 12 °C (profile 2), for a total period of 12 and 10 days, respectively. The storage temperature was recorded at regular time intervals using data loggers (Fourtec, MiniLitE5032L, USA).

5.2.6. Microbiological analyses

For experiments in fish juice, at each sampling point, 1 mL sample was aseptically taken from each flask and serially diluted ten-fold in PSW. For experiments with fish product, a 25-g portion of the (inoculated) fish fillet's caudal region, considered as the analytical sample, was taken aseptically and transferred to a stomacher bag containing 225 mL PSW. Samples were homogenized for 60 s (1500 rpm) in a stomacher (Masticator, IUL Instruments, Spain). MRS agar supplemented with bromocresol purple (BP, 0.12 g/L, Sigma-Aldrich, USA) and *Listeria* selective agar base (Oxoid) containing selective supplement (SR140E; Oxoid) were used for the enumeration of *L. sakei* and *L. monocytogenes*, respectively. 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 et al., 2012). Plates were incubated for approx. 48 h at 33 °C under 10% CO_2 for *L. sakei* and at 37 °C for *L. monocytogenes*.

5.2.7. Development of predictive models

5.2.7.1. Primary model fitting to mono-culture data

Plate counts for *L. sakei* and *L. monocytogenes* were transformed into decimal logarithmic values (i.e. log cfu/g or mL). The growth parameters λ, μ_{max} and N_{max} obtained from each storage temperature for mono and co-culture experiments were estimated by fitting the Baranyi and Roberts (1994) defined by Eqs. (5.1) and (5.2) to the observed data (mean of duplicates at each sampling point) using DMFit Excel Add-in v. 3.5.

$$log\ N_t = log\ N_0 + \frac{\mu_{max}}{ln(10)} \cdot F(t) - \frac{1}{m \cdot ln(10)} \cdot ln\left(1 + \frac{e^{m \cdot \mu_{max} \cdot F(t)} - 1}{10^{m(log\ N_{max} - log\ N_0)}}\right) \quad (5.1)$$

$$F(t) = t - \lambda + \frac{1}{\mu_{max}} \cdot ln\left(1 - e^{-\mu_{max} \cdot t} + e^{-\mu_{max} \cdot (t-\lambda)}\right) \quad (5.2)$$

where N_t is the cellular concentration (cfu/g or mL) at time t, N_0 is the initial concentration (cfu/g or mL), μ_{max} is the specific maximum growth rate (h^{-1}), λ is the lag time (h), N_{max} is the maximum population density (cfu/g or mL), m is a curvature factor, $F(t)$ represents an adjustment function for the model.

5.2.7.2. Secondary models for mono-culture experiments

The influence of temperature on the primary growth parameters of *L. sakei* and *L. monocytogenes* in fish juice was estimated using the square-root model (Eq. 5.3) (Ratkowsky et al., 1982) which was fitted in MS-Excel (Microsoft, Redmond, USA).

$$\sqrt{p} = b \cdot (T - T_{min}) \quad (5.3)$$

where p is the kinetic parameter (i.e. λ and μ_{max}), b is a constant, T (°C) is temperature and T_{min} is the theoretical minimum temperature for growth.

5.2.7.3. Effect of microbial interaction on kinetics parameters

To quantify the reduction on *L. monocytogenes* growth by the bioprotective *L. sakei* CTC494 in fish juice, a reduction ratio (α) was calculated based on the fraction between the parameters obtained in co-culture (p_{co}) and mono-culture (p_{mono}) as shown by Eq. (5.4). To that aim, the parameters from co-culture experiments were also obtained by the Baranyi model (see *Section 5.2.7.1*).

$$\alpha = 1 - \left(\frac{p_{co}}{p_{mono}}\right) \quad (5.4)$$

where α is the reduction ratio and p_{co} and p_{mono} the kinetic parameters (i.e. λ and μ_{max}) in co-culture and mono-culture, respectively.

5.2.7.4. Modelling microbial interaction between L. sakei *CTC494 and* L. monocytogenes *CTC1034*

To predict the simultaneous growth between the bioprotective *L. sakei* strain (at different initial concentrations) and *L. monocytogenes* in fish juice stored at 2.2 ± 0.08, 5.0 ± 0.33, 8.1 ± 0.33 and 12.1 ± 0.12 °C, three different microbial interactions models were tested.

Firstly, the Jameson effect model based on Eqs. (5.5) and (5.6), which assumes that the growth of the pathogen halts when the dominant microbial population reaches its N_{max} (Cornu et al., 2011; Jameson, 1962).

$$\frac{dN_{Ls}}{dt} = N_{Ls} \cdot \mu_{max\,Ls} \cdot \left(1 - \frac{N_{Ls}}{N_{max\,Ls}}\right) \cdot \left(1 - \frac{N_{Lm}}{N_{max\,Lm}}\right) \cdot \left(\frac{Q_{Ls}}{1+Q_{Ls}}\right) \tag{5.5}$$

$$\frac{dN_{Lm}}{dt} = N_{Lm} \cdot \mu_{max\,Lm} \cdot \left(1 - \frac{N_{Lm}}{N_{max\,Lm}}\right) \cdot \left(1 - \frac{N_{Ls}}{N_{max\,Ls}}\right) \cdot \left(\frac{Q_{Lm}}{1+Q_{Lm}}\right) \tag{5.6}$$

$$\frac{dQ_{Ls}}{dt} = Q_{Ls\,t-1} \cdot \mu_{max\,Ls} \tag{5.7}$$

$$\frac{dQ_{Lm}}{dt} = Q_{Lm\,t-1} \cdot \mu_{max\,Lm} \tag{5.8}$$

where N is the cell concentration (cfu/mL) at time t (h), μ_{max} is the maximum specific growth rate (h−1), N_{max} is the maximum population density (cfu/mL) and Q is a measure of the physiological state of cells at time t (h), for *L. sakei* (Ls) or *L. monocytogenes* (Lm).

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

$$Q_0 = \frac{1}{e^{(\mu_{max} \cdot \lambda)} - 1} \tag{5.9}$$

In our study, a modification of the Jameson effect model was also used, represented by Eqs. (5.10) and (5.11). This modification includes the parameters $Ncri_{Ls}$ and $Ncri_{Lm}$ that describe the maximum critical concentration that a population should reach to inhibit the growth of the other population (Jameson, 1962; Le Marc et al., 2009; Vasilopoulos et al., 2010).

$$\frac{dN_{Ls}}{dt} = N_{Ls} \cdot \mu_{max\,Ls} \cdot \left(1 - \frac{N_{Ls}}{N_{max\,Ls}}\right) \cdot \left(1 - \frac{N_{Lm}}{N_{criLm}}\right) \cdot \left(\frac{Q_{Ls}}{1+Q_{Ls}}\right) \tag{5.10}$$

$$\frac{dN_{Lm}}{dt} = N_{Lm} \cdot \mu_{max\,Lm} \cdot \left(1 - \frac{N_{Lm}}{N_{max\,Lm}}\right) \cdot \left(1 - \frac{N_{Ls}}{N_{criLs}}\right) \cdot \left(\frac{Q_{Lm}}{1+Q_{Lm}}\right) \tag{5.11}$$

$$\frac{dQ_{Ls}}{dt} = Q_{Ls\,t-1} \cdot \mu_{max\,Ls} \tag{5.12}$$

$$\frac{dQ_{Lm}}{dt} = Q_{Lm\,t-1} \cdot \mu_{max\,Lm} \tag{5.13}$$

where N_{cri} is the maximum critical concentration (cfu/mL) of *L. sakei* (Ls) on *L. monocytogenes* (Lm) and vice-versa. The rest of model parameters are described in Eqs. (5.5) to (5.9).

Finally, the traditional Lotka Volterra model, also referred to as predator-prey model, was used according to Eqs. (5.14) and (5.15). This model includes two empirical parameters reflecting the degree of interaction between microbial species (F_{LsLm} and F_{LmLs}) (Cornu et al., 2011; Fujikawa et al., 2014; Giuffrida et al., 2008). Depending on the empirical parameter value for *L. sakei* (F_{LsLm}), the growth of *L. monocytogenes* can be affected in three different ways:

1) If $0 < F_{LsLm} < 1$, *L. monocytogenes* grows with reduced μ_{max} after *L. sakei* reaches N_{max}.
2) If $F_{LsLm} = 1$, *L. monocytogenes* stops growing when *L. sakei* reaches its N_{max}.
3) If $F_{LsLm} > 1$, *L. monocytogenes* population declines when *L. sakei* reaches its N_{max}.

$$\frac{dN_{Ls}}{dt} = N_{Ls} \cdot \mu_{max\,Ls} \cdot \left(1 - \frac{N_{Ls} + F_{LsLm} \cdot N_{Lm}}{N_{max\,Ls}}\right) \cdot \left(\frac{Q_{Ls}}{1 + Q_{Ls}}\right) \tag{5.14}$$

$$\frac{dN_{Lm}}{dt} = N_{Lm} \cdot \mu_{max\,Lm} \cdot \left(1 - \frac{N_{Lm} + F_{LmLs} \cdot N_{Ls}}{N_{max\,Lm}}\right) \cdot \left(\frac{Q_{Lm}}{1 + Q_{Lm}}\right) \tag{5.15}$$

$$\frac{dQ_{Ls}}{dt} = Q_{Ls\,t-1} \cdot \mu_{max\,Ls} \tag{5.16}$$

$$\frac{dQ_{Lm}}{dt} = Q_{Lm\,t-1} \cdot \mu_{max\,Lm} \tag{5.17}$$

where F_{LsLm} and F_{LmLs} are, respectively, the competition factor parameters of *L. sakei* CTC494 on *L. monocytogenes* CTC1034 and vice-versa. The other parameters are as indicated in Eqs. (5.5-5.9).

The interaction parameters N_{cri} (maximum critical concentration of one population) and F_{LsLm} and F_{LmLs} (competition factors of one species on the other) were estimated by regression using kinetic parameters derived from mono-culture data (see *Sections 5.2.7.1* and *5.2.7.2*). To estimate the best-fit values of interaction parameters, an optimization procedure was implemented in MATLAB version R2015b using the functions *fmincon* and *ode45* (The MathWorksInc®, Natick, USA).

5.7.2.5. Goodness-of-fit indexes and predictive model performance

The goodness-of-fit of the primary and secondary models was assessed by root mean square error (RMSE) and coefficient of determination (R^2).

The performance of the developed interaction models to predict the behaviour of the bioprotective *L. sakei* CTC494 and *L. monocytogenes* CTC1034 in MAP-fish fillets under isothermal and non-isothermal temperature conditions was evaluated by the acceptable simulation zone (ASZ) approach. Model performance is considered acceptable when at least 70% of the observed log counts values are within the ASZ, defined as ± 0.5 log-units from the simulated concentration in log units (Mejlholm and Dalgaard, 2015; Møller et al., 2013).

5.3. Results

5.3.1. Primary growth parameters of L. sakei *CTC494 and* L. monocytogenes *CTC1034 in mono-culture in fish juice and fish fillets*

The two studied microorganisms were able to grow in sterile fish juice when stored at 2.2 ± 0.08, 5.0 ± 0.33, 8.1 ± 0.33 and 12.1 ± 0.12 °C and on fish fillets at 4.8 ± 0.14 °C. The growth curves obtained from the fit of the Baranyi and Roberts model provided a good description of the observed data (Supplementary Fig. S5.1). The parameters λ and μ_{max} varied with temperature, while N_{max} was not affected, with average values of 7.92 and 8.74 log cfu/mL for *L. sakei* CTC494 and *L. monocytogenes* CTC1034, respectively. The parameters estimated by the Baranyi and Roberts model are shown in Table 5.1. For both fish matrices (juice and fillets) the model showed good fit to data ($R^2 > 0.98$) (Supplementary Table S5.2). A minimum of 7 and a maximum of 23 sampling points were taken for each microorganism depending on the storage temperature. In summary, results in mono-culture confirmed that the bioprotective strain *L. sakei* CTC494 presented better ability to grow in fish juice at low temperatures, which was also observed on fish fillets.

Table 5.1. Estimated lag time (λ), maximum specific growth rate (μ_{max}) and N_{max} (maximum population density) and associated standard errors for *L. sakei* CTC494 and *L. monocytogenes* CTC1034 in mono-culture obtained from the Baranyi and Roberts model in sterile fish juice of sea bream and sea bream fillets under modified atmosphere packaging.

Matrix	Temp. (°C)	*Lactobacillus sakei* CTC494				*Listeria monocytogenes* CTC1034			
		log N_0 (Ls) (log cfu/mL or g)	λ (h)	μ_{max} (h^{-1})	log N_{max} (log cfu/mL or g)	log N_0 (Lm) (log cfu/mL or g)	λ (h)	μ_{max} (h^{-1})	log N_{max} (log cfu/mL or g)
Fish juice[a]	2.2	2.36	92.4 ± 7.55	0.0351 ± 0.0004	7.70 ± 0.03	2.59	166.7 ± 23.20	0.0226 ± 0.0004	8.92 ± 0.10
	5.0	2.04	43.1 ± 6.72	0.0697 ± 0.0005	7.85 ± 0.05	1.53	36.1 ± 7.13	0.0477 ± 0.0005	8.65 ± 0.05
	8.1	2.67	18.7 ± 5.04	0.1273 ± 0.0039	7.94 ± 0.05	2.29	15.1 ± 6.26	0.0892 ± 0.0019	8.68 ± 0.07
	12.1	2.48	5.3 ± 2.68	0.2140 ± 0.0052	8.17 ± 0.07	2.39	2.0 ± 2.01	0.1685 ± 0.0020	8.70 ± 0.06
Fresh fish fillets[b]	4.8	1.49	33.8 ± 11.39	0.0806 ± 0.0036	7.08 ± 0.13	2.71	56.1 ± 35.23	0.0154 ± 0.0006	5.68 ± 0.13

[a] Experiments in sterile fish juice of gilthead sea bream inoculated with *ca.* 10^2 cfu/mL of *L. sakei* or *L. monocytogenes*.
[b] Experiments on gilthead sea bream fillets under modified atmosphere packing inoculated with *ca.* 10^2 cfu/g of *L. sakei* or *L. monocytogenes*.

5.3.2. Secondary model

The parameters λ and μ_{max} obtained from the Baranyi and Roberts model were used to fit a square-root model (Eq. (5.3)). The ability of the secondary models to describe the influence of temperature on the growth parameters was proven to be satisfactory according to the values from RMSE and R^2, whose values were in the ranges 0.064–0.086 and 0.874–0.999, respectively. A summary of results from the fitting of the square-root model for both microorganisms is shown in Table 5.2.

Table 5.2. Coefficients of the square-root model describing the effect of temperature on lag time (λ) and maximum specific growth rate (μ_{max}) of *L. sakei* CTC494 and *L. monocytogenes* CTC1034 in sterile fish juice of sea bream.

Parameters	Microorganisms	*b*	T_{min} (°C)	RMSE[a]	R^2
λ	*L. sakei* CTC494	-0.7269	14.69	7.365	0.9695[b]
	L. monocytogenes CTC1034	-1.0868	12.42	30.332	0.8737
μ_{max}	*L. sakei* CTC494	0.0280	-4.50	0.086	0.9994
	L. monocytogenes CTC1034	0.0263	-3.40	0.064	0.9990

[a] RMSE, Root mean square error.
[b] R^2, Coefficient of determination.

5.3.3. Interaction of L. sakei CTC494 and L. monocytogenes CTC1034 in fish juice ate different temperatures and inoculation ratios

The influence of storage temperature and the inoculation ratio (1:1, 1:2 and 1:3) on the interaction of *L. sakei* CTC494 and *L. monocytogenes* CTC1034 was assessed. To allow a comparison with kinetic parameters in mono-culture, the Baranyi and Roberts model without considering interaction was fitted to experimental data in co-culture (Table 5.3). The statistical indexes for the fitted model presented satisfactory RMSE and R^2 values (Supplementary Table S5.3).

Table 5.3. Estimated lag time (λ), maximum specific growth rate (μ_{max}), N_{max} (maximum population density) and associated standard error from the Baranyi and Roberts model without interaction fitted to the growth of *L. sakei* CTC494 and *L. monocytogenes* CTC1034 in co-culture in sterile fish juice of sea bream.

Ratio[a]	Temp. (°C)	*Lactobacillus sakei* CTC494				*Listeria monocytogenes* CTC1034			
		log N_0 (Ls) log cfu/mL	λ (h)	μ_{max} (h^{-1})	log N_{max} (log cfu/mL)	log N_0(Lm) log cfu/mL	λ (h)	μ_{max} (h^{-1})	log N_{max} (log cfu/mL)
1:1	2.2	2.37	53.3 ± 11.72	0.0350 ± 0.0007	7.74 ± 0.05	2.52	75.4 ± 16.32	0.0223 ± 0.0006	5.77 ± 0.04
1:2		4.58	59.5 ± 8.08	0.0355 ± 0.0097	7.63 ± 0.04	2.60	106.1 ± 6.45	0.0268 ± 0.0008	4.28 ± 0.01
1:3		6.77	39.4 ± 14.39	0.0284 ± 0.0027	7.99 ± 0.03	2.57		NG[c]	
1:1	5.0	2.84	30.0 ± 4.72	0.0675 ± 0.0012	7.94 ± 0.07	2.42	36.9 ± 6.87	0.0490 ± 0.0012	5.94 ± 0.03
1:2		4.59	23.0 ± 7.52	0.0625 ± 0.0026	7.86 ± 0.05	2.49	14.5 ± 18.80	0.0334 ± 0.0053	4.22 ± 7.35
1:3		6.62	11.8 ± 3.13	0.0294 ± 0.0007	7.68 ± 0.01	2.46[b]		NG	
1:1	8.1	2.42	20.3 ± 3.50	0.1373 ± 0.0029	8.11 ± 0.06	2.27	12.3 ± 19.73	0.1085 ± 0.0131	6.07 ± 0.18
1:2		4.34	15.2 ± 2.15	0.1266 ± 0.0023	8.14 ± 0.03	1.92	0.0 ± 0.00	0.0977 ± 0.0058	4.03 ± 0.11
1:3		6.37	0.0 ± 0.00	0.0703 ± 0.0027	7.96 ± 0.03	2.24[b]		NG	
1:1	12.1	2.49	6.4 ± 1.62	0.2292 ± 0.0035	8.17 ± 0.06	2.33	1.3 ± 1.86	0.1959 ± 0.0035	6.81 ± 0.08
1:2		4.40	5.1 ± 1.16	0.2273 ± 0.0046	8.20 ± 0.03	2.47	0.0 ± 0.00	0.1733 ± 0.0065	4.86 ± 0.12
1:3		6.37	0.0 ± 0.00	0.1716 ± 0.0073	8.06 ± 0.03	2.47[b]		NG	

[a] Ratio of inoculation of *L. monocytogenes* CTC1034 and *L. sakei* CTC494 in fish juice of sea bream where the ratios 1:1, 1:2 or 1:3 represent the initial concentrations of 2 log cfu/mL for the *L. monocytogenes* strain and 2, 4, 6 log cfu/mL for the *L. sakei* strain, respectively.
[b] Observed initial concentration of *L. monocytogenes*.
[c] NG, no growth.

The most evident outcome from these experiments was that higher ratios produced stronger inhibition of *L. monocytogenes* growth, with the ratio 1:3 yielding no apparent growth for the pathogen. The parameter μ_{max} was little influenced, even though values obtained in co-culture were generally lower than those obtained in mono-culture. Fig. 5.2 represents, through a bar diagram, a comparison of λ and N_{max} obtained from mono-culture and co-culture at the different conditions by using the reduction ratio (α) calculated according to Eq. (5.4). From this figure, it can be observed that α for λ varied for *L. monocytogenes* among the different inoculation ratios, but in all co-culture experiments, λ presented a reduction with respect to that observed in mono-culture. However, further analysis of data confirmed that differences were rather produced by the fitting process (i.e. prediction error) affected by the relatively λ short duration (≥5 °C; $\lambda \leq 36$ h) than a hypothetical interaction between microorganisms.

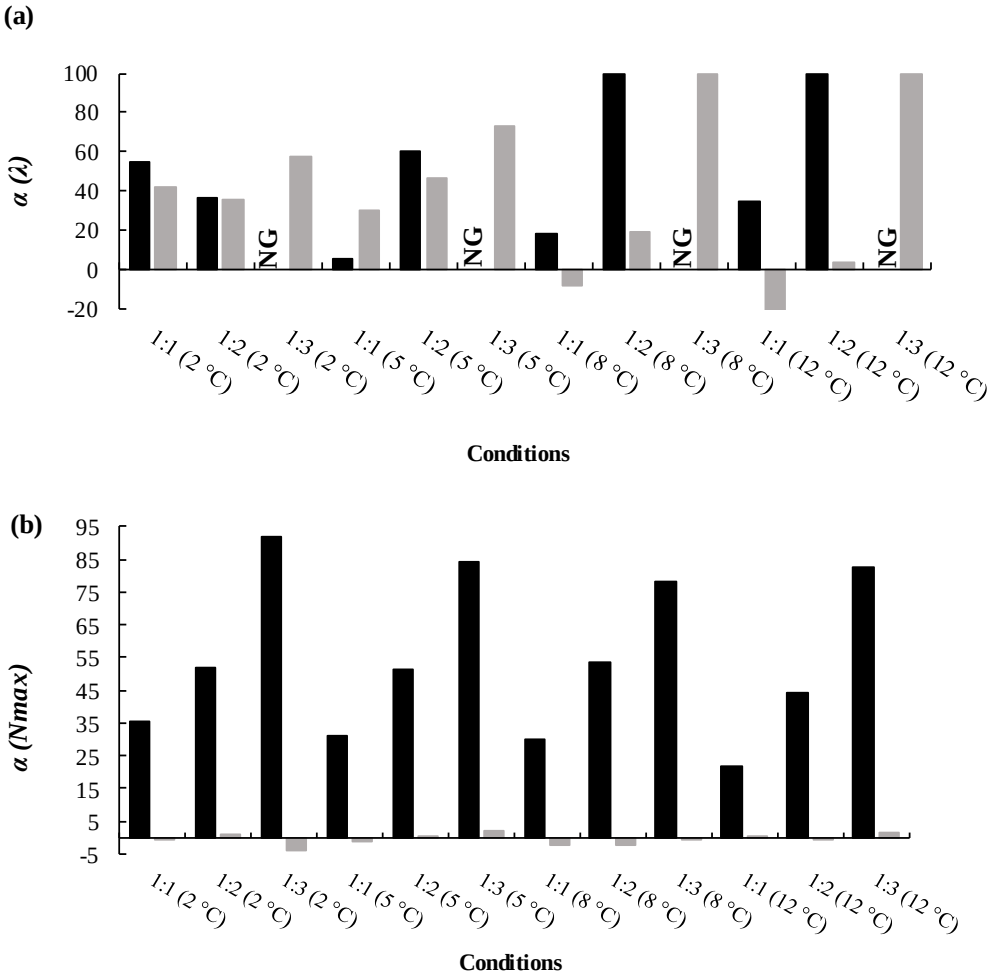

Fig.5.2. Reduction ratio(α), in %, of the parameters (a) lag time (λ) and (b) N_{max} for *Listeria monocytogenes* CTC1034 (black bars) and *Lactobacillus sakei* CTC494 (greys bars) in co-culture on sterile juice fish of sea bream at different storage temperatures with three

inoculation ratios of *L. monocytogenes*: *L. sakei*. The negative bars represent an increase in co-culture for the specific parameter. No growth of *L. monocytogenes* was observed at the ratio 1:3 (NG).

On the other hand, N_{max} was the most affected parameter for *L. monocytogenes* at all concentration ratios. For instance, in mono-culture experiments at 5.0 °C (Table 5.1), log N_{max} was 8.65 log cfu/mL while for co-culture experiments, the parameter was gradually decreasing to 5.94 (α = 31%), 4.22 (α = 51%) and 1.37 (α = 84%) log cfu/mL for inoculation ratios 1:1, 1:2 and 1:3, respectively. For the latter, the putative "N_{max}" was taken from observations since the Baranyi and Roberts model could not be fitted to data at ratio 1:3 as no growth was observed. Similar inhibition patterns were observed for the other assayed temperatures (Fig. 5.2).

5.3.4. Sensorial analysis

The sensory evaluation results obtained for sea bream fillets under MAP conditions stored at 5 °C are presented in Table 5.4. The QI scores obtained for fish samples inoculated at a ratio 1:2 (*L. monocytogenes: L. sakei*) were compared to control samples (i.e. non-inoculated). In general, QI scores increased linearly during storage with a correlation coefficient (R^2) of 0.82 and 0.67 for control and inoculated batches, respectively. The statistical analysis of QI scores showed that *L. sakei* CTC494 did not significantly affect the sensory properties of fish fillets ($p > 0.05$) during the evaluated storage time (8 days). Though the deterioration rate was slightly lower for control (slope = 0.47) than for inoculated samples (slope = 0.55), the differences were not statistically significant ($p > 0.05$). Therefore, from the sensory perspective, the addition level of 10^4 cfu/g of *L. sakei* CTC494 would be suitable as bioprotective strategy without modifying the spoilage rate in comparison with a control (non-bioprotected) product.

Table 5.4. Quality Index values obtained from the sensory analysis of sea bream fillets packaged under modified atmosphere and stored under refrigerated conditions (5 °C, 8 days) for samples inoculated at a ratio 1:2 (*Listeria monocytogenes*: *Lactobacillus sakei*) (i.e., 2 log cfu/g and 4 log cfu/g, respectively) and control fillets (non-inoculated).

Storage time (days)[a]	Quality Index	
	Inoculated fillets	Control fillets
0	0.3 ± 0.5[b]	0.0 ± 0.0
4	0.6 ± 1.3	1.2 ± 1.6
6	2.0 ± 1.7	2.0 ± 2.0
8	6.3 ± 0.5	4.8 ± 1.9

[a] Storage under modified atmosphere packaging at 5 °C.
[b] Mean ± standard deviation (n = 5 panellists).

5.3.5. Modelling interaction of L. sakei *CTC494 and* L. monocytogenes *CTC1034 in fish juice*

The three interaction models (Fig. 5.3) were tested using the kinetic parameters (λ, μ_{max} and N_{max}) obtained from the Baranyi and Roberts model fitted to mono-culture experiment data and estimating the respective interaction factors by regression analysis. The statistical performance of the models was evaluated by RMSE whose values are shown in Table 5.5 together with the estimated parameters.

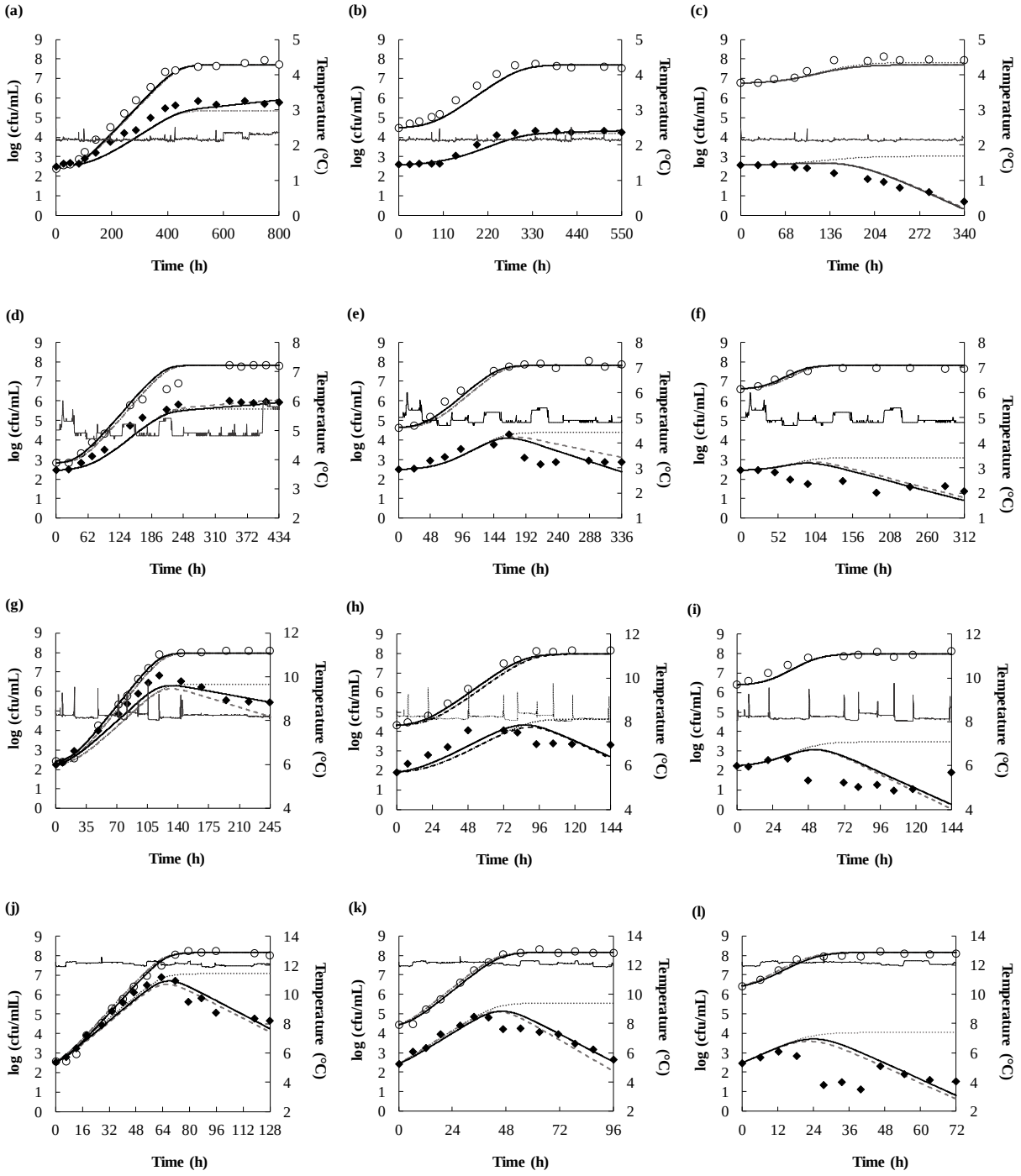

Fig. 5.3. Experimental observed data and fitted Jameson (dotted line), modified Jameson (dashed line) and Lotka Volterra (solid line) models for *Lactobacillus sakei* CTC494 (○) and *Listeria monocytogenes* CTC1034 (♦) in sterile fish juice of sea bream stored at (a, b, c) 2.2, (d, e, f) 5.0, (g, h, i) 8.1 and (j, k, l) 12.1 °C for the inoculation ratios of *L. monocytogenes*: *L. sakei*, 1:1, 1:2 and 1:3, respectively. The grey dotted line stands for the storage temperature recorded.

Table 5.5. Estimated maximum critical concentration (N_{cri}) of the modified Jameson effect model and competition factors (F_{LsLm} and F_{LmLs}) of the Lotka Volterra model and goodness-of-fit index (RMSE) for *L. sakei* CTC494 and *L. monocytogenes* CTC1034 in co-colture in fish juice of sea bream.

Ratios[a]	Temp. (°C)	n[b]	Jameson model		Modified Jameson model				Lotka Volterra model			
			Lactobacillus sakei CTC494	*Listeria monocytogenes* CTC1034	*Lactobacillus sakei* CTC494		*Listeria monocytogenes* CTC1034		*Lactobacillus sakei* CTC494		*Listeria monocytogenes* CTC1034	
			RMSE[c]	RMSE	RMSE	Lm_{cri}[d] (cfu/mL)	RMSE	Ls_{cri}[e] (cfu/mL)	RMSE	F_{LmLs}[f]	RMSE	F_{LsLm}[g]
1:1	2.2	17	0.314	0.423	0.349	$1.00*10^8$	0.469	$5.00*10^7$	0.314	0.00	0.370	0.84
1:2		14	0.293	0.189	0.335	$1.00*10^8$	0.215	$5.00*10^7$	0.293	0.00	0.181	0.94
1:3		11	0.224	1.214	0.350	$1.00*10^8$	0.369	$1.90*10^7$	0.294	0.00	0.308	2.67
1:1	5.0	14	0.370	0.387	0.371	$1.00*10^8$	0.316	$7.49*10^7$	0.371	0.00	0.316	0.90
1:2		13	0.279	1.058	0.322	$1.00*10^8$	0.687	$5.00*10^7$	0.274	0.00	0.481	1.54
1:3		10	0.139	1.240	0.139	$1.00*10^8$	0.612	$4.62*10^7$	0.139	0.00	0.612	1.46
1:1	8.1	14	0.216	0.666	0.217	$1.00*10^8$	0.592	$7.16*10^7$	0.216	0.00	0.530	1.20
1:2		11	0.300	0.930	0.300	$1.00*10^8$	0.609	$5.00*10^7$	0.301	0.00	0.606	1.87
1:3		11	0.190	1.796	0.190	$1.00*10^8$	0.978	$5.00*10^7$	0.190	0.00	0.973	1.86
1:1	12.1	16	0.213	1.138	0.210	$9.99*10^7$	0.328	$8.92*10^7$	0.214	0.00	0.312	1.63
1:2		14	0.108	1.424	0.108	$1.00*10^8$	0.382	$7.57*10^7$	0.124	0.00	0.341	1.81
1:3		11	0.105	2.050	0.105	$1.00*10^8$	1.089	$7.51*10^7$	0.105	0.00	1.089	1.95

[a] Ratio of inoculation of *L. monocytogenes* CTC1034 and *L. sakei* CTC494 in sterile fish juice where the ratios 1:1, 1:2 or 1:3 represent the initial concentrations of 2 log cfu/mL for the *L. monocytogenes* strain and 2, 4, 6 log cfu/mL for the *L. sakei* strain, respectively.
[b] n, number of data (sampling points) for *L. sakei* CTC494 and *L. monocytogenes*.
[c] RMSE, Root mean square error.
[d] Lm_{cri} maximum critical concentration for *L. monocytogenes* CTC1034 obtained from the Jameson´s modified model.
[e] Ls_{cri} maximum critical concentration for *L. sakei* CTC494 obtained from the Jameson´s modified model
[f] F_{LmLs} competition factor of *L. monoctytogenes* CTC1034 in *L. sakei* CTC494 obtained from the Lotka Volterra model.
[g] F_{LsLm} competition factor of *L. sakei* CTC494 in *L. monoctytogenes* CTC1034 obtained from the Lotka Volterra model.

The Jameson effect model presented the worst fitting to data, showing the highest RMSE values. This result suggests that the interaction between both microorganisms could not be exclusively explained by the Jameson effect, where growth inhibition is the result from a depletion in nutrient bioavailability and toxicity increase when the dominant population reaches N_{max}. The modified Jameson effect model including the parameter N_{cri} showed better performance, with RMSE lower values. For both microorganisms, N_{cri} remained in the same order of magnitude for the different temperatures and inoculation ratios, with average values, in log scale, of 7.7 and 8 log cfu/mL for *L. sakei* CTC494 and *L. monocytogenes* CTC1034, respectively (Table 5.5).

The Lotka Volterra interaction model showed slightly better fit to data than the above models according to RMSE (Table 5.5) and visual analysis of growth curves (Fig. 5.3). In the case of the ratio 1:3, a poor fitting was observed for *L. monocytogenes* although this condition also yielded unsatisfactory fitting results for the Jameson effect-based models. This could be due to the difficulty of the models to suitably describe the large decline of *L. monocytogenes* population at this ratio.

As regards the inhibitory effect of *L. sakei* CTC494 on *L. monocytogenes* CTC1034 growth, competition factors (F_{LsLm}) at the lowest temperature (2.2 °C) were below 1 for inoculation ratios 1:1 and 1:2, with values of 0.84 and 0.94, respectively. However, for the same temperature at ratio 1:3, the competition factor was equal to 2.67. This higher value reflected the noticeable decline of *L. monocytogenes* CTC1034 population (down to 0.70 log cfu/mL). For inoculation ratios 1:2 and 1:3, at 5 °C, the competition factors increased up to 1.54 and 1.46, respectively. For higher temperatures (8-12 °C), this increasing trend in the competition factor was minimized showing a rather variable pattern, and therefore, no mathematical model could be derived for such a relationship. Thus, for modelling purposes, this parameter was fixed to the average value observed at different temperatures for the corresponding inoculation ratio.

L. monocytogenes CTC1034 did not exert any inhibitory effect on *L. sakei* CTC494 as demonstrated by the competition factor (F_{LmLs}) being equal to 0 (Table 5.5).

5.3.6. Simulation of growth interaction of L. sakei CTC494 and L. monocytogenes CTC1034 on fish fillets

The simultaneous growth of *L. sakei* CTC494 and *L. monocytogenes* CTC1034 was evaluated in sea bream fillets under MAP at two isothermal (4.8 and 8.2 °C) as well as at

two non-isothermal conditions (profile 1: 4-8 °C and profile 2: 2.5-12 °C) at an initial inoculation ratio 1:2 based on the sensory analysis outcome (Fig. 5.4).

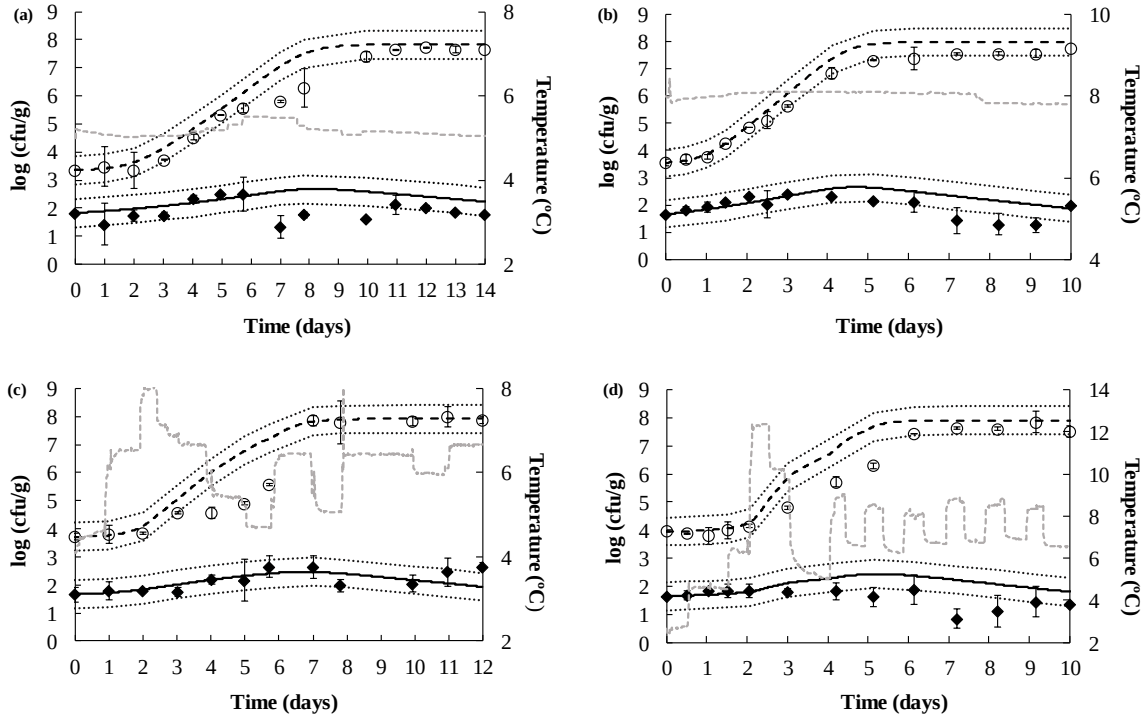

Fig. 5.4. Experimental observed data (mean and standard deviation of 3 replicates) and simulations provided by the predictive model based on the Lotka Volterra equation for *Latobacillus sakei* CTC494 (○) and *Listeria monocytogenes* CTC1034 (♦) on sea bream fillets under modified atmosphere packaging at isothermal conditions: (a) 4.8 °C, (b) 8.2 °C; and dynamic temperature conditions (c) profile 1 (4-8 °C) and (d) profile 2 (2.5-12.0 °C). Dashed and solid line represent the simulations for *L. sakei* and *L. monocytogenes* strains, respectively. Dotted lines show the acceptable simulation zone (ASZ) used to compare observations versus predictions of the interaction between *L. sakei* CTC494 and *L. monocytogenes* CTC1034. Grey dashed line stands for the storage temperature recorded.

For isothermal conditions, *L. sakei* CTC494 reached the stationary phase with 10 and 5 days of storage at 4.8 °C and 8.2 °C, respectively, with an average log N_{max} of 7.91 log cfu/g, while for *L. monocytogenes* under the same conditions, log N_{max} was 2.23 and 1.87 log cfu/g respectively. The value obtained at 4.8 °C represented for a reduction of 67% compared with log N_{max} estimated in mono-culture in fish fillets (5.68 log cfu/g).

The average pH values for fish fillets remained constant throughout the storage time (6.15 ± 0.02) and the gas concentration in the packaging at the end of storage was 31.2% ± 0.85 and 29.0% ± 0.19 for O_2 and CO_2, respectively.

The kinetic parameters, in the model, for both microorganisms were estimated by using the secondary models (λ and μ_{max}) derived from mono-culture experiments except for log N_{max} which was not temperature dependent and therefore, the average value was used instead (i.e., in log scale, 7.92 and 8.74 log cfu/mL for *L. sakei* CTC494 and *L. monocytogenes* CTC1034, respectively). To consider the effect of food matrix on μ_{max} (h^{-1}), the reduction of this parameter on the fresh fish product (4.8 °C) in relation to that observed in fish juice in mono-culture was estimated (5.0 °C), which corresponded to 0.68. Thus, for simulating growth, the specific growth rate for *L. monocytogenes* was adjusted applying the above reduction rate in the Lotka Volterra model. Due to the difficulty to set an equation describing the temperature effect on the competition factor, this was fixed to the average of the values obtained at the different temperatures at the ratio 1:2, which corresponded to 1.54. It was deemed that the value was representative for the assayed temperatures, considering that most temperatures in challenge tests were in the range 4-12 °C, where the competition factor was similar. The same reduction rate and competition factor were used for the experiments at isothermal conditions (4.8 and 8.2 °C) as well as for the two dynamic time-temperature profiles.

Table 5.6 shows RMSE and ASZ values for the growth interaction of *L. sakei* CTC494 and *L. monocytogenes* CTC1034 on fish fillets predicted by Lotka Volterra model. The RMSE values for experiments under isothermal conditions varied between 0.378 to 0.555 and 0.452 to 0.593 for *L. sakei* CTC494 and *L. monocytogenes* CTC1034, respectively. The visual inspection of the simulated line also confirmed that good performance of models, demonstrating that the model was able to simulate the observed slight *Listeria* increase and subsequent decline, though at 8.2 °C, observations showed a more prominent decline than the one predicted by the model simulation (i.e. fail-safe prediction). Furthermore, values for ASZ considering as criterion ± 0.5 log.units showed that models can mostly accounted for the counts recorded during the interaction experiments, with values of 79% (Table 5.6).

Table 5.6. Predictive performance of the Lotka Volterra model when applied to simulate the simultaneous growth of *L. sakei* CTC494 and *L. monocytogenes* CTC1034 in sea bream fillets under modified atmosphere packaging stored under isothermal and non-isothermal conditions.

Temp. (°C)	n[a]	*Lactobacillus sakei*			*Listeria monocytogenes*		
		N_0 (LAB) cfu/g	RMSE[b]	ASZ[c]	N_0 (Lm) cfu/g	RMSE	ASZ
4.8	14	3.36	0.555	79%	1.83	0.593	79%
8.2	14	3.55	0.378	79%	1.65	0.452	79%
Profile 1 (4-8)	12	3.71	0.894	75%	1.66	0.309	92%
Profile 2 (2.5-12)	13	3.96	0.645	77%	1.65	0.615	77%

[a] n, number of data (sampling points) for *L. sakei* CTC494 and *L. monocytogenes* CTC1034.
[b] RMSE, Root mean square error.
[c] ASZ, acceptable simulation zone defined as ± 0.5 log-units from the simulated log cfu/g values (Møller et al., 2013).

For non-isothermal temperature conditions, RMSE values ranged from 0.64 to 0.894 and 0.309 to 0.615 for *L. sakei* and *L. monocytogenes*, respectively. Lotka Volterra model showed closer predictions to experimental data in fish fillets for *L. monocytogenes* under profile 1. The percentages for the ASZ corresponded to 92% (11/12) and 77% (10/13) for profile 1 and 2, respectively. For *L. sakei*, ASZ values varied between 75 (9/12) and 77% (10/13) for both profiles. Lotka Volterra model overestimated the exponential phase of *L. sakei* CTC494, while for *L. monocytogenes* the same was observed only for profile 2. The overestimation in profiles for *L. monocytogenes* could be considered as a "fail-safe" prediction since growth was predicted when no-growth was actually observed (i.e. profile 2).

5.4. Discussion

5.4.1. L. monocytogenes *growth in mono-culture*

For *L. monocytogenes* in mono-culture at 5 °C, μ_{max} values obtained in our study were 16 % higher than those found by Verheyen et al. (2018) for in fish-protein based emulsions at 4 °C used as food model system for fish. On the contrary, the μ_{max} observed by Bolívar et al. (2018) in fish juice within the interval 5-11 °C were higher (30-57%) than those found in our study in the range 4-12 °C. Differences in growth rates could be mainly attributed to strain variability and experimental conditions. By the contrary, the predictions provided by Combase Predictor (https://www.combase.cc/index.php/en/)

considering the same physico-chemical characteristics as those obtained for fish juice (pH = 6.66; a_w = 0.997) were similar in all temperatures studied. Furthermore, the obtained μ_{max} for *L. monocytogenes* in our fish juice were in the range of values reported for other fish matrices (i.e. 0.0329-0.2075 at 4-12 °C) such as smoked salmon, raw tuna, vacuum-packed rainbow trout fillets and sea bream fillets under MAP conditions (Faber, 1991; Hisar et al., 2005; Hwang, 2007; Liu et al., 2016; Provincial et al., 2013).

5.4.2. Growth interaction of L. sakei *CTC494 and* L. monocytogenes *CTC1034*

In general, observations in our study showed that the suppression of *Listeria* growth occurred when the dominant population, i.e. *L. sakei* CTC494 reached their N_{max}. This result would signal a potential Jameson effect between populations. Several studies have considered the Jameson effect in the simultaneous growth of microorganisms and *L. monocytogenes* on fish products (Beaufort et al., 2007; Giménez and Dalgaard, 2004; Koseki et al., 2011; Mejlholm and Dalgaard, 2007).

According to results, the inhibitory effect was influenced by the inoculation ratio and temperature, which has been also reported in other works (Quinto et al., 2016; Yamazaki et al., 2003). Differences in inoculum level is key to determine the dominant microorganism in the microbial interaction and thus, the level of inhibition between microbial populations (Mellefont et al., 2008). Despite this fact, we observed that *L. sakei* CTC494 exerted a slight inhibition on N_{max} of *L. monocytogenes* even when both microorganisms were inoculated the same level (ratio 1:1). This inhibition at equal inoculum level could be associated with production of bacteriocin since *L. sakei* CTC494 produces sakacin K (Hugas et al., 1995; De Vuyst and Leroy, 2007; Leroy et al., 2005; Ravyts et al., 2008) and the influence of other metabolites such as organic acids was discarded as potential inhibitors because of no relevant changes in pH were detected during growth experiments in fish juice and fish samples.

In summary, the interaction between *L. sakei* CTC494 and *L. monocytogenes* CTC1034 presented in our study could be understood by a combination of two mechanisms: i) a non-specific interaction involving the Jameson effect on the inhibition of *L. monocytogenes,* occurring when *L. sakei* CTC494 is present at an initial concentration higher than *L. monocytogenes* together with the fact that the bioprotective strain grows faster than the pathogen (Mellefont et al., 2008; Jameson, 1962) and ii) specific interaction caused by modification of the medium where both microorganisms coexist, resulting in an specific antagonistic effect on the growth of *L. monocytogenes*

due to bacteriocin production (i.e. sakacin K) by the bioprotective strain (Aguilar and Klotz, 2010; Vescovo et al., 2006). However, the production of bacteriocin was not quantified in our study, thus no conclusion can be drawn about which interaction phenomenon was more relevant. Nor could mechanistic models be applied due to the lack of biological insight into the metabolic and genetic phenomena arising from the simultaneous growth of two microbial populations.

5.4.3. Lotka Volterra's competition fator

The competition factors for *L. monocytogenes* (F_{LsLm}) in fish juice were slightly temperature dependent for all ratios (Table 5.5). The largest increase in the competition factor took place at low temperatures for ratios 1:2 and 1:3 (i.e. 2.2-5 °C) while for ratio 1:1, higher temperatures (8-12 °C) were responsible for a higher rise of this factor. No mathematical expression could be derived from data because of the limited number of observations, reduced temperature range and the lack of a clear pattern in data. Møller et al. (2013) estimated the competition factors for natural microbiota on growth of *Salmonella* spp. in fresh pork using the Lotka Volterra model and expanded Jameson effect model and found dependency on range of storage temperature assayed. By the contrary, Mejholm and Dalgaard (2015) using the model proposed by Giménez and Dalgaard (2004) did not find that the competition factor was temperature dependent. Furthermore, the traditional Jameson effect model or its modification suggested by Le Marc et al. (2009) have been used to predict growth of microorganisms in food at different storage temperature (Giménez and Dalgaard, 2004; Le Marc et al., 2009; Mejholm and Dalgaard, 2007; Vermeulen et al., 2011). In those studies, however, the effect of microbial interaction on growth patterns was independent of the studied storage temperatures. The divergence between studies to correlate interaction factors with temperature can be related to the different conditions used in experiments (i.e. type of microorganism, food matrix and inoculum concentration).

Competition factors, in our study, were also under the influence of the inoculation ratio. Thus, the lowest values were obtained for ratios 1:1 and 1:2 (Table 5.5). Baka et al. (2014) estimated low competition factors for the interaction between *Leuconostoc carnosum* and *L. monocytogenes* in vacuum packed Frankfurter sausages stored at 4 °C for the ratio 1:1. At intermediate temperatures (8.1-12.1 °C), the competition factors decreased with the initial increase of concentration of *Leuconostoc carnosum*. Fujikawa et al. (2014) found that the values for the competition factors did not vary with the

combinations of the initial populations of *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* spp. at 28 °C.

According to Baka et al. (2014), differences in values of the competition factor can be attributed to the combination of different variables, such as temperature, nutrient depletion, pH, bacteriocin production, organic acid, MAP conditions, etc., which can be considered as part of the hurdle concept (Leistner, 1995). When these variables are identified, the Lotka Volterra model can be modified for more realistic microbial interaction descriptions, for instance, the effect of environmental conditions (i.e. temperature), the influence of inhibitory substances on lag phase duration of pathogenic organisms or whether bacteriocin production is dependent quorum sensing (Dens et al., 1999, Powell et al., 2004).

5.4.4 Simulating growth inhibition and bioprotective activity of L. sakei *CTC494 on* L. monocytogenes *CTC1034 on fish fillets*

A challenge test on gilthead sea bream fillets under MAP inoculated with *L. monocytogenes* CTC1034 and *L. sakei* CTC494 in co-culture at ratio 1:2 was carried out under isothermal and non-isothermal conditions. The Lotka Volterra model slightly overestimated the experimental observations of *L. sakei* in the exponential phase for the profiles 1 and 2. These discrepancies can be partly explained by the fact that the performance of a dynamic model depends on the performance of the primary and secondary models, and the sudden temperature changes can cause an intermediate lag time that cannot be predicted by the models (Longhi et al., 2013). Nevertheless, this fact did not affect predictions for *Listeria* growth, providing a reliable estimate for this pathogen for two dynamic conditions, according to the ASZ approach.

The control of pathogenic bacteria using LAB as bioprotective cultures in fish products is widely reported in literature (Bernardi et al., 2011; Chowdhury et al., 2012; Ghanbari et al., 2013; Hisar et al., 2005; Matamoros et al., 2009; Nath et al., 2014; Tahiri et al., 2009; Tomé et al., 2008; Weiss and Hammes, 2006), thus showing that live microbiological cultures can be a more effective alternative to the use of bacteriocins (Pilet and Leroy, 2011), which in addition are not permitted by most of the food additive regulations. However, the selection of candidates as bioprotective cultures to improve food quality and extend shelf-life has been attributed to the capacity not to produce undesirable organoleptic changes in foods. In this sense, *L. sakei* CTC494 is reported in literature as a starter culture providing good organoleptic and sensory properties in

fermented meat products and as bioprotective (not spoiling) culture in cooked ham (Aymerich et al., 2002; Bover-Cid et al., 2001; Hugas et al., 1995; Hugas et al., 1998; Hugas et al., 2002). Our study proposed the extension of the use the bacteriocinogenic *L. sakei* CTC494 in raw fish and other minimally processed fish products demonstrating that its availability to grow in a different food matrix and its application as a suitable approach for controlling *L. monocytogenes* growth in packaged sea bream fillets stored under isothermal and non-isothermal conditions including moderate abuse.

5.5. Conclusion

Results demonstrated that the use of the bacteriocinogenic strain *L. sakei* CTC494, as bioprotective culture is a suitable strategy for controlling *L. monocytogenes* CTC1034 growth in minimally processed fresh fish products (i.e. filleted gilthead sea bream) under refrigerated storage. Furthermore, the modelling approach, developed herein, based on a step-wise scheme from mono-culture experiments in fish juice under isothermal conditions to experiments performed in co-culture in actual fish product under dynamic temperature profiles was proved to be effective to derive reliable microbial interaction models. These mathematical models could be used as a predictive tool to simulate the simultaneous behaviour of bioprotective lactobacillus strain and *L. monocytogenes*. Thus, these tools can support the design and optimization of bioprotective culture based strategies against *L. monocytogenes* in minimally processed fish products.

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Declarations of interest

The authors declare that there is no conflict of interest in the publication of this paper.

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Appendix C. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.03.002.

Appendix C. Supplementary Table S5.1

Supplementary Table S5.1. Quality Index Method (QIM) scheme applied to the sensory analysis of filleted sea bream under modified atmosphere packaging adapted from Lougovois et al. (2003) and Campus et al. (2011).

	Parameter	Attributes	Demerit points
Appearance	Skin	Bright, shining, iridescent	0
		Less bright, some loss of iridescence	1
		Pale, dull	2
	Slime/Mucus	Clear-transparent	0
		Slightly cloudy/cloudy	1
Flesh	Colour	Fresh, translucent	0
		Waxy, milky	1
		Dull, slightly discoloured, yellowish	2
	Stiffness	Firm	0
		Some softening	1
		Soft	2
Odour	Odour	Fresh	0
		Neutral	1
		Slight off-odours	2
		Spoiled	3
Quality Index (QI, as the sum of assigned demerit points)			0-10

Appendix C. Supplementary Table S5.2

Supplementary Table S5.2. Goodness-of-fit indexes for the fit of the Baranyi and Roberts model to the growth data of *L. sakei* CTC494 and *L. monocytogenes* CTC1034 in mono-culture in sterile fish juice of sea bream and sea bream fillets under modified atmosphere packaging at different temperatures.

Matrix	Temp. (°C)	n (Ls)[c]	n (Lm)[d]	*Lactobacillus sakei* CTC494		*Listeria monocytogenes* CTC1034	
				RMSE[e]	R^2	RMSE	R^2
Fish juice[a]	2.2	12	19	0.061	0.9991[f]	0.154	0.9955
	5.0	18	23	0.107	0.9972	0.091	0.9985
	8.1	14	14	0.120	0.9968	0.122	0.9965
	12.1	14	17	0.126	0.9965	0.105	0.9977
Fresh fish fillets[b]	4.8	7	11	0.191	0.9930	0.151	0.9840

[a] Experiments in sterile fish juice of gilthead sea bream inoculated with *ca.* 10^2 cfu/mL of *L. sakei* or *L. monocytogenes*.
[b] Experiments on gilthead sea bream fillets under modified atmosphere packing inoculated with *ca.* 10^2 cfu/g of *L. sakei* or *L. monocytogenes*.
[c] n (Ls), number of data (sampling points) for *L. sakei* CTC494.
[d] n (Lm), number of data (sampling points) for *L. monocytogenes* CTC1034.
[e] RMSE, Root mean square error.
[f] R^2, Coefficient of determination.

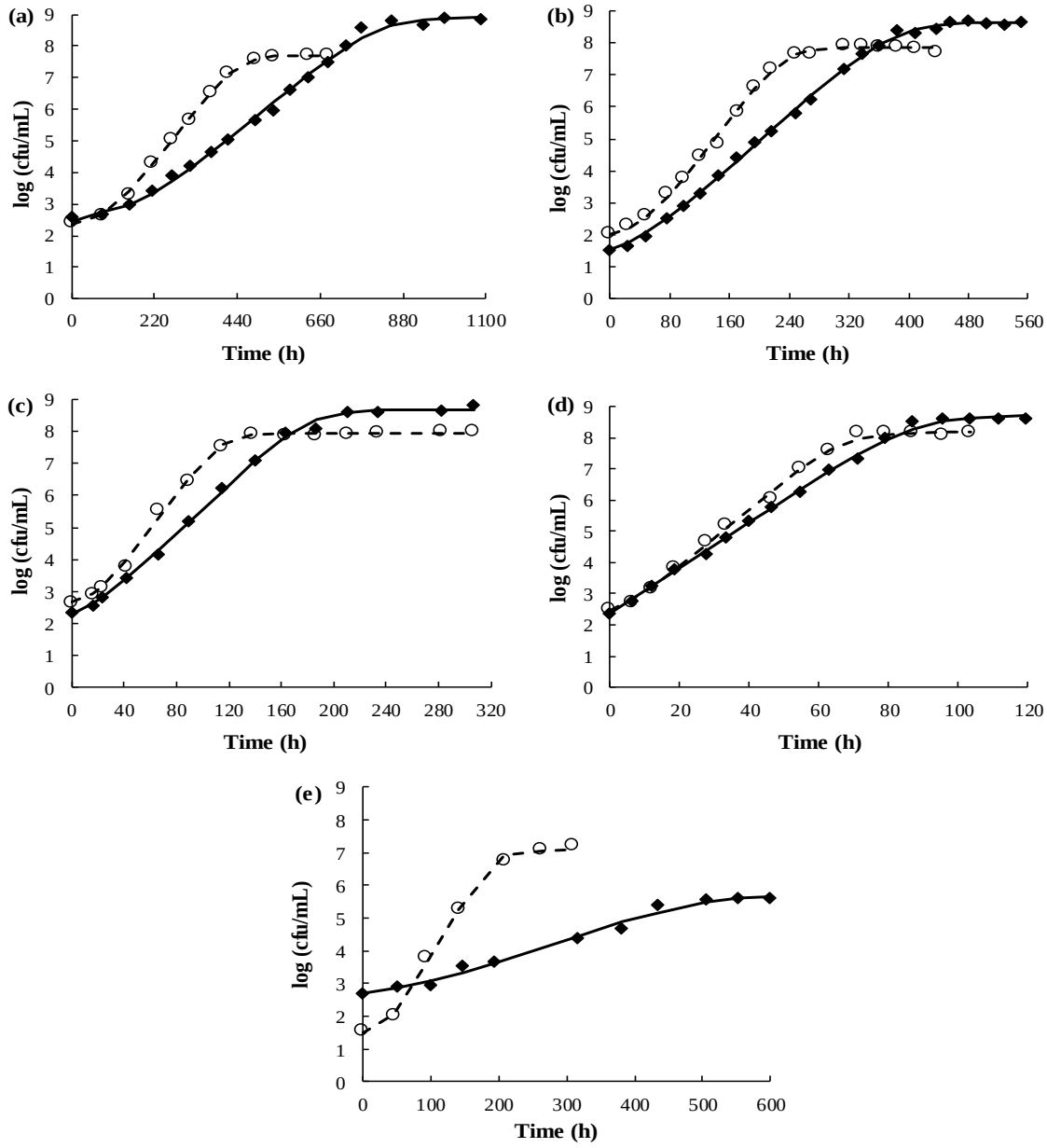
Appendix C. Supplementary Table S5.3

Supplementary Table S5.3. Goodness-of-fit indexes for the Baranyi and Roberts model without interaction fitted to co-culture growth data of *L. sakei* CTC494 and *L. monocytogenes* CTC1034 in sterile fish juice of sea bream and sea bream fillets under modified atmosphere packaging at different temperature.

Ratio[a]	Temp. (°C)	n (Ls)[b]	n (Lm)[c]	*Lactobacillus sakei* CTC494		*Listeria monocytogenes* CTC1034	
				RMSE[d]	R^2	RMSE	R^2
1:1	2.2	17	17	0.100	0.9979[e]	0.092	0.9954
1:2		14	14	0.081	0.9963	0.037	0.9976
1:3		11	11	0.071	0.9818	NF[f]	
1:1	5.0	14	14	0.113	0.9968	0.074	0.9974
1:2		13	7	0.117	0.9924	0.157	0.9432
1:3		10	10	0.023	0.9969	NF	
1:1	8.1	14	14	0.135	0.9965	0.472	0.9090
1:2		11	7	0.060	0.9986	0.180	0.9684
1:3		11	11	0.092	0.9800	NF	
1:1	12.1	16	11	0.128	0.9965	0.122	0.9970
1:2		11	7	0.081	0.9970	0.143	0.9823
1:3		11	11	0.100	0.9761	NF	

[a] Ratio of inoculation of *L. monocytogenes* CTC1034 and *L. sakei* CTC494 in sterile fish juice of gilthead sea bream where the ratios 1:1, 1:2 or 1:3 represent the initial concentrations of 2 log cfu/mL for the *L. monocytogenes* strain and 2, 4, 6 log cfu/mL for the *L. sakei* strain, respectively.
[b] n (Ls), number of data (sampling points) for *L. sakei* CTC494.
[c] n (Lm), number of data (sampling points) for *L. monocytogenes* CTC1034.
[d] RMSE, Root mean square error.
[e] R^2, Coefficient of determination.
[f] NF, no fit as no growth was observed.

Appendix C. Supplementary Figure S5.1



Supplementary Figure S5.1. Growth curves of *Lactobacillus sakei* CTC494 (○) and *Listeria monocytogenes* CTC1034 (♦) in mono-culture obtained in sterile fish juice of gilthead sea bream at (a) 2.2, (b) 5.0, (c) 8.1 and (d) 12.1 °C and (e) on sea bream fillets at 4.8 °C. Dashed line and solid line represent the fittings for the *L. sakei* and *L. monocytogenes* strains, respectively, obtained with the Baranyi and Roberts model.

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Conclusions

First: The review of the fundamentals and mathematical models developed in the area of Predictive Microbiology over the last 20 years shows that, despite the great advances in the field, there are still microbial phenomena requiring a deeper mathematical understanding. One of these phenomena is the interaction of microbial communities in food, for which there is a variety of models. These models are suitable to be deployed in different food contexts like the use of bioprotective cultures. For their optimal application, these models should be developed or/and adjusted considering the underlying mechanisms governing interaction and applying tailor-made data generation and modelling approaches (***Chapter 2***).

Second: In the study of the microbial quality of two Mediterranean fish species produced in different Andalusian estuaries, the anatomic part was identified as a factor influencing microbial contamination levels, with counts of *Enterobacteriaceae*, total coliforms and *Staphylococcus* spp. coagulase+ being consistently high in viscera as compared to skin. In addition, no significant differences were found for the type of fish species, showing, both Gilthead sea bream and Sea bass, a similar microbiological contamination profile. *Escherichia coli* was detected with low prevalence (< 5% individuals) in viscera of Gilthead sea bream and Sea bass from location A and only in Sea bass from location B. This result confirmed that *E. coli* could be present in fish viscera, probably, as part of the normal intestinal microbiota (***Chapter 3***).

Third: The location of estuarine fish farms and the associated physical-chemical parameters and organic load of water can significantly affect the microbiological quality of Gilthead sea bream and Sea bass. Higher levels of lactic acid bacteria, aerobic mesophilic bacteria, *Enterobacteriaceae*, total coliforms and *Staphylococcus* spp. coagulase+ were found in location A. This fact suggests that the control of microbiological quality of water in estuaries production systems could be key to improve the microbiological quality of harvested fish, and consequently, to enhance the quality and safety of Gilthead sea bream and Sea bass along the chain food. (***Chapter 3***).

Fourth: Neither *Listeria monocytogenes* nor *Salmonella* spp. were detected in samples of both fish species and estuary water. However, the presence of *Vibrio parahaemolyticus*, being considered a foodborne pathogen, was detected in one water

sample (n = 1/9), which demonstrates the potential incidence of this pathogen in fish products derived from estuary production systems (***Chapter 3***).

Fifth: Model predictions using prospective data from fish farming demonstrated longer shelf-life of 5 and 4 days at 4 and 8 °C, respectively, for fresh fish products (i.e. Gilthead sea bream and Sea bass) from location B, where the fish samples showed better microbiological quality, demonstrating that the initial microbiological quality could have a remarkable effect on the fish product shelf-life (***Chapter 3***).

Sixth: The growth in monoculture of *Lactobacillus sakei* L115 and CTC494 and *L. monocytogenes* was satisfactorily described by using the Baranyi and Roberts model. Likewise, empirical models, based on the Bělehrádek approach, could be generated to describe the effect of temperature on the main kinetic parameters of the three microorganisms (***Chapter 4***).

Seventh: *In-vitro* studies demonstrated that *Lb. sakei* L115 presented inhibition against a *L. monocytogenes* cocktail, confirming its anti-listerial activity. The maximum specific growth rate and maximum population density of the *L. monocytogenes* cocktail, obtained from the Baranyi and Roberts model, noticeably decreased from 0.037 to 0.026 log CFU/h (i.e. 31 %) and from 9.26 to 5.95 log CFU/mL (i.e. 37 %), respectively, when the pathogen was co-cultured in presence of *Lb. sakei* L115. The combination of a high concentration of *Lb. sakei* L115, low temperature, high competition for nutrients and bacteriocin production can be hypothesized as the main causes of the growth inhibition of *L. monocytogenes* (***Chapter 4***).

Eighth: The simultaneous growth of *Lb. sakei* L115 and *L. monocytogenes* cocktail in culture broth could be better simulated with the Lotka-Volterra model and a new modified version of the Jameson-effect model, in which, the inhibitory effect was represented by applying the inhibition coefficients (α) to adjust the estimated monoculture maximum specific growth rate to reflect interaction under the hypothesis that the growth potential of one population is not affected in presence of another population, provided the environmental conditions are not modified. For this approach, fitted models showed better performance, and all temperatures could be represented adequately for which the acceptable simulation zone (ASZ) levels for *L. monocytogenes* cocktail was 100%, except for 20 °C, in which it was 85%, while *Lb. sakei* L115, values were always above 85% (***Chapter 4***).

Nineth: A stepwise approach based on kinetic parameters from monoculture studies was proved to be effective to simulate microbial interaction of *Lactobacillus sakei* CTC494 and *L. monocytogenes* in actual fish product, by incorporating, as parameters, a competition factor and food adjustment factor estimated from *in-vitro* experiments (i.e. sterile fish juice of Gilthead sea bream) (***Chapter 5***).

Tenth: The application of different inoculum levels (2, 4 and 6 CFU/mL) of the sakacin-producing *L. sakei* CTC494 inhibited growth of *L. monocytogenes* inoculated at 2 log CFU/mL in sterile fish juice of Gilthead sea bream in the temperature range from 2 to 12 °C. Maximum population density (N_{max}) was the most affected parameter for *L. monocytogenes*. For instance, at 5 °C, the N_{max} of the pathogen in mono-culture gradually decreased from 8.65 log CFU/mL to 5.94 (α = 31%), 4.22 (α = 51%) and 1.37 (α = 84%) when co-cultured with *L. sakei* CTC494 at the inoculum of 2, 4 and 6 log CFU/mL, respectively (***Chapter 5***).

Eleventh: Validation experiments with actual food samples demonstrated that the use of the bioprotective culture at a level of 4 log CFU/g was a suitable strategy to reduce and inhibit *L. monocytogenes* growth (≤ 2 log CFU/g) in Gilthead sea bream fillets packaged under modified atmosphere during chilled and moderate abuse temperature storage. *L. sakei* CTC494, did not show any significant sensory impact on the product, as compared to a control batch (***Chapter 5***).

Twelfth: The present work demonstrated that microbial interaction can be used as reliable tools to simulate the simultaneous behavior of bioprotective *lactobacillus* strains and *L. monocytogenes*. Thus, these tools can support the design and optimization of bioprotective culture-based strategies against *L. monocytogenes* in ready-to-eat fish products (***Chapter 5***)

Curricular Summary

1) Education

Years	Title	Institute
2015-2020	PhD candidate in Biosciences and Agri-food Sciences (Grant PhD Full Abroad-Proc. GDE 229638/2013-9 - CNPq Brazil) "Development of microbial interaction predictive models for *Listeria monocytogenes* and lactic acid bacteria and their application for the optimization of bio-protective cultures in fishery products"	Department of Food Science and Technology, University of Córdoba (UCO), Córdoba, Spain
2011-2013	Master's degree in Food Engineering (CAPES Scholarship – Brazil)	Department of Chemical Engineering and Food Engineering, Federal University of Santa Catarina, Santa Catarina, Brazil
2006-2010	Degree in Food Engineering	School of Engineering, Pontifical Catholic University of Goias, Goiânia, Brazil

2) Career

- **Since 2013:** Department of Food Engineering, Federal University of Rondônia (Brazil).

  Assistant Professor. Teaches the subjects of General and Food Microbiology, Biochemical Engineering, Food Industry Processes and Industrial Refrigeration for the Food Engineering Course (Campus Ariquemes). (www.engalimentos.unir.br)

- **Since 2015:** Food Engineering Research Group. Department of Food Engineering, Federal University of Rondônia (Brazil).

  Researcher. Participation in the research project titled "Modelling the growth of *Salmonella* Thyphimurium during the shelf-life in minimally processed and ready-to-eat fish products from Amazonia region treated with essential oils stored under isothermal and dynamic conditions". Project leader: Tania Maria Alberte. (http://dgp.cnpq.br/dgp/espelhogrupo/191797).

- **01/2015 – 01/2018:** HIBRO Research Group (PAIDI AGR-170), University of Córdoba (Spain).

  Researcher. Participation in the research project titled "Development and application of predictive models to improve the quality and safety of minimally processed marine aquaculture products" (AGR201-1906). Financial support: Andalusian Government (Spain). Project leader: Gonzalo Zurera. Supervisors: Fernando Pérez-Rodríguez and Gonzalo Zurera. (http://www.uco.es/hibro/es/home).

- **05/2017 – 11/207:** International Stay - Short Term Scientific Mission (STSM).

  Scientific visit to the National Food Institute (DTU Food) at the Technical University of Denmark (Denmark), in the framework of "*Doctorado hacia la Excelencia. Becas de Movilidad Internacional 2016/2017*" (BOUCO-2016/00529). STSM title: Microbial interaction between lactic acid bacteria and *Listeria monocytogenes* in seafood products. Financial support: University of Córdoba. Supervisor: Paw Dalgaard. (www.food.dtu.dk)

- **04/2016 – 07/2017:** National Stay - Short Term Scientific Mission (STSM).

  Scientific visit to the Institute of Agrifood Research and Technology (IRTA) (Spain), in the framework of grant PhD Full Abroad-Proc. Scholarship GDE 229638/2013-9. STSM title: Modelling the interaction *Lactobacillus sakei* CTC494 and *Listeria monocytogenes* in fish juice. Supervisor: Sara Bover-Cid. (www.irta.cat/es/).

- **01/2009 – 01/2011:** Krion Biotecnologia (Brazil).

  Research Assistant in the field Microbiology. Collaboration in the research project titled "Selection and application of agro-industrial waste decomposing microorganisms (sugarcane bagasse) in the production of organic additives for agriculture. Financial support: Krion Agroscience. Supervisor: Luis Antonio Nascimento Louza Umbelino. (www.krion.com.br).

3) Publications

Articles

Bolívar, A., **Costa, J.C.C.P.,** Posada-Izquierdo, G.D., Bover-Cid, S., Zurera, G., Pérez-Rodríguez, F. Quantifying the bioprotective effect of *Lactobacillus sakei* CTC494 against *Listeria monocytogenes* on vacuum packaged hot-smoked sea bream. *Food Microbiology*, v. 94, 103649, 2021. **Impact Factor: 4.155 (Q1).**

Costa, J.C.C.P., Bolívar, A., Valero, A., Carrasco, E., Zurera, G., Pérez-Rodríguez, F. 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, v. 131, 108928, 2020. **Impact Factor: 4.972 (Q1).**

Costa, J.C.C.P., Floriano, B., Bascon, I., Rodríguez-Ruiz, J.P., Izquierdo, G.D.P., Zurera, G., Pérez-Rodríguez, F. Study of the microbiological quality, prevalence of foodborne pathogens and product shelf-life of Gilthead sea bream (*Sparus aurata*) and Sea bass (*Dicentrarchus labrax*) from aquaculture in estuarine ecosystems of Andalusian (Spain). *Food Microbiology*, v. 90, 103498, 2020. **Impact Factor: 4.155 (Q1).**

Costa, J.C.C.P., Bover-Cid, S., Bolívar, A., Zurera, G., Pérez-Rodríguez, F. 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, v. 97, p. 72-84, 2019. **Impact Factor: 4.006 (Q1).**

Bolívar, A., **Costa, J.C.CP.,** Posada-Izquierdo, G.D., Valero, A., Zurera, G., Pérez-Rodríguez, F. Modelling the growth of Listeria monocytogenes in Mediterranean fish species from aquaculture production. International Journal of Food Microbiology, v. 270, p. 14-21, 2018. **Impact Factor: 4.006 (Q1).**

Costa, J.C.C.P., Tremarin, A., Longhi, D.A., Silva, A.P.R., Carciofi, B.A.M., Laurindo, J.B., Aragão, G.M.F. Predictive modeling of the growth of *Lactobacillus viridescens* under non-isothermal conditions. Procedia Food Scince, v. 7, p. 29-32, 2016.

Book Chapter

Costa, J.C.C.P., Longhi, D.A., Haberbeck, L.U., Aragão, G.M.F. Predictive microbiology tools for exposure assessment. In: F. Pérez-Rodríguez (Ed.) Risk Assessment Methods for Biological and Chemical Hazards in Food. Boca Raton: Taylor and Francis, 2020, v.1, p. 301-354. ISBN: 978-1-4987-6202-1.

Bolívar, A., **Costa, J.C.C.P.,** Posada-Izquierdo, G.D., Bascón, I., Zurera, G., Valero, A. Characterization of foodborne pathogens and spoilage bacteria in Mediterranean fish species and seafood products. In: O.V. Singh (Ed.) Foodborne pathogens and antibiotic resistance. New York: Wiley-Blackwell, 2017, v. 1, p. 21-34. Print ISBN:9781119139157 |Online ISBN:9781119139188 |DOI:10.1002/9781119139188.

Costa, J.C.C.P., Posada-Izquierdo, G.D., Pérez-Rodríguez, F., García-Gimeno, R.M. Impact of forming and mixing operations on the microbial ecology of foods: focus on pathogenic microorganisms. In: A.S. Sant'Ana (Ed.) Quantitative microbiology in food processing: modelling the microbial ecology. New York: Wiley-Blackwell, 2016, v. 1, p. 241-249. Print ISBN:9781118756423 |Online ISBN:9781118823071 |DOI:10.1002/9781118823071.

Oral communication

Costa, J.C.C.P., Talark, F., Vargas, M., Cejudo, M., Bover-Cid, S., Bolívar, A., Zurera, G., Pérez-Rodríguez, F. A mathematical approach for investigating the effect of matrix on the bio-protective capacity of *Lactobacillus sakei* CTC494 against *Listeria monocytogenes* in ready-to-eat fish products. In: 11th International Conference of Predicitve Modelling in Food, Braganza, Portugal, 2019.

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Costa, J.C.C.P., Lausern L., Pedersen, R., Mejlholm, O., Dalgaard, P., Carrasco, E., Pérez-Rodríguez, F. Mathematical model of the bio-protector effect of *Lactobacillus sakei* LA-5 against *Listeria monocytogenes* in minimally processed shrimp products. In: 13th Spanish Network of Lactic Bacteria (RedBAL), Madrid, Spain, 2019.

Costa, J.C.C.P., Bover-Cid, S., Zurera, G., Pérez-Rodríguez, F. Modelling interaction of a sakacin-producing *Lactobacillus sakei* (CTC494) and *Listeria monocytogenes* (CTC1034) in gilt-head seabream (*Sparus aurata*) under isothermal and non-isothermal conditions. In: 10th International Conference of Predictive Modelling in Food, Córdoba, Spain, 2017.

Costa, J.C.C.P., Pérez-Rodríguez. Modelo matemático de interacción entre *Lactobacillus sakei* y *Listeria monocytogenes* en diferentes condiciones de temperatura. In: 5ª Congreso Científico de Investigadores de Formación de la Universidad de Córdoba, Córdoba-España, 2016.

Posters

Oliveira, T.S., Ortiz, V.A., Alberte, T.M., Bicca, G.B., **Costa, J.C.C.P.** Modelling thermal inactivation of *Salmonella* Typhimurum in rice milk. In: 13th Latin American Symposium of Food Science, Campinas, Brazil, 2019.

Bragra, D.J.N., Alberte, T.M., Chaves, M., **Costa, J.C.C.P.,** Alvarenga, V.O., Bicca, G.B. Senso Excel: A tool in Excel-VBA for sensory food analysis. In: 13th Latin American Symposium of Food Science, Campinas, Brazil, 2019.

Bolívar, Izquierdo, G.D.P., Valero, A., **Costa, J.C.C.P.,** Zurera, G., Pérez-Rodríguez, F. Assessing the growth of *Listeria monocytogenes* in Mediterranean fish products from marine aquaculture. In: IAFP's European Symposium of Food Safety, Brussels, Belgium, 2017.

Izquierdo, G. D. P., Alguacil, J. M., **Costa, J.C.C.P.,** Bascon, I., Valero, A., Garcia-Gimeno, R.M., Zurera, G. Evaluation of methodologies for the isolation of bioprotectives cultures of lactic-acid bacteria from aquaculture Mediterranean fish species. In: IAFP's European Symposium on Food Safety, 2017, Brussels, Belgium, 2017.

Bolívar, A., **Costa, J.C.C.P.,** Izquierdo, G.D.P., Zurera, G., Valero, A., Pérez-Rodríguez, F. Inhibitory effect of *Lactobacillus Sakei* against *Listeria Monocytogenes* growth in smoked Mediterranean fish products from marine aquaculture. In: 10th International Conference of Predictive Modelling in Food, 2017, Córdoba, Spain, 2017.

Costa, J.C.C.P., Capurso, M., Pugliese, E., Pérez-Rodriguez, F., Gobetti, M., Valero, A., Bolivar, A., Possas, A., Carrasco, E., Garcia-Gimeno, R. M., Zurera, G., Izquierdo, G.D.P. Study and modeling of the behavior of *Listeria Monocytogenes* in vegetable infusions alone and in emulsion with olive oil at different temperatures. In: 25th International ICFMH Conference – Foodmicro, Dublin, Ireland, 2016.

Costa, J.C.C.P., Rodriguez-Ruiz, J.P., Floriano, B., Bolívar, A., Valero, A., Zurera, G., García-Gimeno, R.M., Possas, A., Carrasco, E., Pérez-Rodríguez, F. Identification of pathogenic bacteria in Sea bream (*Sparus aurata*) and Sea bass (*Dicentrarchus labrax*) of aquaculture in Andalusia (Spain). In: 25th International ICFMH Conference – FoodMicro, Dublin, Ireland, 2016.

Bolívar, A., **Costa, J.C.C.P.,** Valero, A., Pérez-Rodríguez, F., Zurera, G., Izquierdo, G.D.P. Modelling the growth of *Lactobacillus plantarum*, *Listeria monocytogenes* and *Salmonella* spp. in Mediterranean fish-based extract. In: 25th International ICFMH Conference – FoodMicro, Dublin, Ireland, 2016.

Costa, J.C.C.P., Longhi, D., Zandonai, S., Tribuzi, G., Carciofi, B.A.M., Laurindo, J.B., Aragão, G.M.F. Modeling the non-isothermal growth of spoilage bacteria in mussels (*Perna perna*) treated with oregano essential oil and heat. In: 12th International Congress on Engineering and Food, Quebec, Canada, 2015.

Tremarin. A., Longhi, D., **Costa, J.C.C.P.,** Salomao, B.C.M., Aragão, G.M.F. Comparasion of ergosterol content and radial colonies measurements for *Byssochlamys fulva* and *Neosartorya fischeri* growth under isothermal conditions: In 8th Internarional Conference on Predictive Modelling in Food, Paris, France, 2013.

Costa, J.C.C.P., Tribuzi, G., Souza, C.K., Tremarin, A., Laurindo, J.B., Aragão, G.M.F. Modeling of microbial growth during the shelf-life of mussels (*Perna perna*) treated with oregano essential oil (*Origanum vulgare*). In: 1st Sao Paulo School of Advanced Sciences – Advances in Predictive Modeling and Quantitaive Microbiological Risk Assessment of Food, Sao Paulo, Brazil, 2013.

Academic advisory

Marina Sáiz Vargas. Application of predictive models for the study of the bio-protector effect *Lactobacillus sakei* CTC494 on *Listeria monocytogenes* in a surimi product. Work of completion for graduation in Food Science and Technology, University of Córdoba, 2019.

Regiani Zanon Rosa. Elaboration of rosé wine from Niáguara Rosada (*Vitislabrusca*) grapes produced in the State of Rondônia (Brazil): physical-chemical, microbiological and sensory parameters. Work of completion for graduation in Food Engineering, Federal University of Rondônia, 2017.

Alciléia Costa. Determination of the kinetic growth parameters of *Bacillus coagullans* under isothermal conditions. Work of completion for graduation in Food Engineering, Federal University of Rondônia, 2014.

Silamara Zandonai. Modelling of the thermo-chemical effect on shelf-life of mussels (*Perna perna*) stored under non-isothermal conditions. Work of completion for graduation in Food Engineering, Federal University of Santa Catarina, 2014.

4) Complementary Education

2018: Multivariant Analysis Techniques. *Centro de Estudios Andaluces* (Seville, Spain) 16 h.

2017: EFSA Symposium on Quantitative Microbial Risk Assessment in Food. University of Córdoba (Córdoba, Spain) 4 h.

2017: Toward a new era in Predictive Microbiology: Next-generation omics in model. University of Córdoba (Córdoba, Spain) 4 h.

2015: Isolation, Characterization and Identification of Microorganisms in Foods. *Instituto de la Grasa*, Pablo Olavide University (Seville, Spain) 24 h.

2014: Optimal experiments design of predictive models. 20th Brazilian Chemical Engineering Congress, Federal University of Santa Catarina (Florianopolis, Brazil) 6 h.

2014: Application of predictive microbiology to assure f. Federal University of Santa Catarina (Florianopolis, Brazil) 9 h.

2013: PCR real time. LAB Trade Brazil 8 h.

2011: Introduction to using MATLAB: concepts and applications. Federal University of Santa Catarina (Florianopolis, Brazil) 16 h.

5) Language

Portuguese: Native speaker
Spanish: Advanced level
English: Intermediate level

6) Link for OCIRD and Google Scholar

https://orcid.org/0000-0001-6839-8746
https://scholar.google.es/citations?user=Zf3Kuq8AAAAJ&hl=en

7) Otras informaciones relevantes

Knowledge and experience in Food Science and Technology, focusing mainly on food safety, biopreservation, predictive microbiology, mathematical modelling and emerging food preservation technologies and statistical analyses (MATLAB, R studio, ComBase, FSSP, MicroHibro, etc.).