Memoria de Tesis Doctoral

DEVELOPMENT OF ACTIVE EDIBLE COATINGS TO IMPROVE THE MICROBIOLOGICAL QUALITY AND SAFETY OF FISH AND SEAFOOD PRODUCTS

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Pamplona, octubre de 2015

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INFORMAN:

Que la presente memoria de tesis Doctoral titulada "Development of Active Edible Coatings to Improve the Microbiological Quality and Safety of Fish and Seafood Products" elaborada por Dña. Ximena Carrión Granda ha sido realizada bajo nuestra dirección, y que cumple con las condiciones exigidas por la legislación vigente para optar por el grado de Doctor con Mención Internacional.

Y para que así conste, firman la presente en Pamplona, octubre de 2015

Dn. Juan Maté C. Dn. Jordi Rovira C.

Para la realización de esta tesis doctoral, Ximena Carrión Granda obtuvo una ayuda predoctoral del Programa para la Formación del Personal Investigador de la Universidad Pública de Navarra.

DEDICATORIA

A mis padres, que me enseñaron a volar.

“Caminante no hay camino, se hace camino al andar” (A. Machado)

AGRADECIMIENTOS

A Juan Maté Caballero y Jordi Rovira Carballido, mis directores de tesis, quienes supieron guiarme a lo largo de este proceso y compartieron su conocimiento conmigo. Gracias por todo su apoyo y confianza, especialmente en momentos claves de mi "travesía particular" en España.

Al Departamento de Tecnología de Alimentos de la UPNA y Departamento de Biotecnología y Ciencias de los Alimentos de la UBU, por su acogida en el programa de doctorado y en diferentes proyectos de los cuales fui parte. Además gracias a Juanjo y Gustavo, el equipo técnico del Departamento, por su ayuda en todo momento.

To Glenn Young and his team, thank you for giving me the opportunity to make an internship at UC Davis and guiding my work there.

A mis compañeros de batalla tanto del laboratorio de la UPNA, de la UBU y de Idoki, gracias por compartir conmigo muchas experiencias y brindarme su apoyo cuando más lo necesitaba. Siempre estarán en mi mente y ocuparán un lugar especial en mi corazón.

A Idoya por su apoyo desinteresado y por compartir sus conocimientos conmigo desde el primer momento en que llegué al laboratorio. Ido, gracias por ayudarme a salir de todos esos momentos "crisis" que viviste conmigo. Sin tu apoyo esto no hubiese llegado a buen fin!!

A mis padres por apoyarme y acompañarme en esta aventura que empezó 10 años atrás cuando dejé mi casa y emprendí vuelo tratando de descubrir algo más. Como siempre papi y mami, mis logros son sus logros, porque sin sus enseñanzas, su amor y dedicación nunca hubiese llegado hasta aquí.

A mis hermanas, por estar siempre junto a mí, a pesar del océano que nos separa.

A mis sobrinos y cuñado, por su apoyo y cariño siempre.

A mis amigos…..de aquí y de allá…...los antiguos y los recientes, por hacer mi vida lejos de mi familia mucho más fácil y llevadera. Gracias por aguantar estos últimos meses mi "monotema" de conversación llamado tesis.

Gracias a todos!!!

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ABSTRACT

Edible films and coatings (EFCs) made from polysaccharides, proteins and lipids are becoming a new, innovative and promising technology used to improve food quality and safety. EFCs can be used as carriers of different antimicrobials and be applied to fish and seafood products in order to enhance their microbial quality and increase their shelf-life.

The objective of this work was to design edible films and coatings incorporated with different antimicrobial materials: supercritical fluid extracts (SCFEs), essential oils (EOs) and lactic acid bacteria (LAB). The *in vitro* antimicrobial activity of the developed films was tested and their use as potential preservation technology to extend the shelf-life of fish and seafood products was assessed.

In order to select the antimicrobial compounds for our application, four SCFEs and two EOs were tested against 14 different strains related with the spoilage and pathogenicity of fish products. Rosemary SCFE and oregano and thyme EO showed the highest antimicrobial activity, inhibiting the growth of most of bacteria tested. These compounds were incorporated at different concentrations into whey protein edible films (WPI) and chitosan and their antimicrobial activity was tested again. Results showed that active films were able to inhibit the growth of the different strains, being this activity totally related with the concentration of SCFE/EO used and the target bacteria tested.

The best film formulations were used to evaluate their effectiveness on the microbial growth of fresh tuna steaks, fresh hake fillets and peeled shrimp tails. Different lots of products were prepared, packaged under presence of air or modified atmosphere (MAP) and stored in refrigeration for different periods of time. In all cases

the use of antimicrobial edible films retarded the microbial growth and increase significantly their shelf-life.

Finally, bioactive films based on WPI containing lactic acid bacteria were successfully tested against *L. monocytogenes.* The experiments showed that LAB in the films were kept viable during chilled storage.

RESUMEN

Las películas y recubrimientos comestibles (PRCs) obtenidos a partir de polisacáridos, proteínas y lípidos se han convertido en una tecnología innovadora y prometedora con el objetivo de mejorar la calidad y seguridad alimentaria PRCs pueden ser usados como portadores de diferentes sustancias antimicrobianas y ser aplicados a pescado y marisco con la finalidad de mejorar su calidad microbiológica y extender su vida útil.

El objetivo de este trabajo fue diseñar films y recubrimientos comestibles incorporando diferentes materiales antimicrobianos: extractos supercríticos (SCFEs), aceites esenciales (EOs) y bacterias ácido lácticas (LAB). La actividad antimicrobiana *in vitro* de los films desarrollados y su uso como una tecnología potencial de conservación para extender la vida útil de pescado y marisco fue evaluada.

Con el propósito de seleccionar los agentes antimicrobianos para ser usados en nuestras aplicaciones, cuatro SCFEs y dos EOs fueron evaluados frente a 14 bacterias relacionadas con el deterioro y patogenicidad del pescado y marisco. El extracto de romero y los aceites esenciales de orégano y tomillo mostraron la mayor actividad antimicrobiana, inhibiendo el crecimiento de la mayoría de las bacterias evaluadas. Estos compuestos fueron incorporados a diferentes concentraciones en films de proteína de suero de leche (WPI) y quitosano y su actividad antimicrobiana fue evaluada. Los resultados mostraron que los films activos fueron capaces de inhibir el crecimiento de las diferentes bacterias, siendo esta actividad totalmente relacionada con la concentración de compuesto activo y de la bacteria objetivo.

Las mejores formulaciones fueron usadas para evaluar su efectividad sobre el desarrollo microbiano de filetes de atún fresco, filetes de merluza fresca y colas peladas

de langostinos. Se prepararon diferentes lotes de productos, envasados en presencia de aire o de atmósfera modificada (MAP) y almacenados en refrigeración durante diferentes periodos de tiempo. En todos los casos el uso de los recubrimientos antimicrobianos retardó el crecimiento microbiano e incrementó la vida útil de los productos tratados.

Finalmente, films bioactivos en base a WPI que contenían bacterias ácido lácticas fueron evaluados exitosamente *in vitro* frente a *L. monocytogenes*. Los experimentos mostraron que las LAB se mantuvieron viables dentro de los films durante el almacenamiento bajo condiciones de refrigeración.

INTRODUCTION

Fish and seafood products are a very popular food product in many countries but they are very perishable, even more than red meat or chicken, due to its large content of free aminoacids and volatile nitrogen bases compared with other meats. Throughout processes like handling and/or storage, fresh fish and seafood deterioration takes place rapidly and limits their shelf-life. The spoilage process of fish usually starts with the production of off-odours and flavours, generated basically by the growth of spoilage microorganisms. Bacteria like *Shewanella* spp., *Pseudomonas* spp., *Aeromonas* spp., *Vibrio* spp., Enterobacteriaceae, Lactic acid bacteria, are common spoilers of fresh and chilled fish stored either under air or modified atmosphere conditions.

The current changes in consumer's lifestyle that include an increasing demand of fresh and minimally processed foodstuffs with no synthetic additives, have made the food industry search into new and innovative preservation technologies. Edible films and coatings is an innovative and promising alternative. They can be used as water, aroma and oxygen barriers and also as carriers of different compounds like antioxidant, antimicrobials and other preservatives in order to improve food quality and safety. Essential oils (EOs) and plant extracts are aromatic oily liquids obtained from medicinal herbs and plants. They are complex mixtures of different compounds and have been studied as one of the "natural" alternatives to extend the shelf-life of food products. The antimicrobial activity of essential oils is associated with the content of different substances like terpenes, alcohols, aldehydes, phenolic compounds and others. Lactic Acid Bacteria are another "natural" option to preserve food products. They are normally part of the microbiota of fresh and packed food and are able to produce different

metabolic products such as organic acids, diacetyl, acetoin, hydrogen peroxide, reuterin, reutericyclin, antifungal peptides and bacteriocins, with a proved antibacterial effect.

The objective of this work was to design edible films and coatings incorporated with different antimicrobial materials: supercritical fluid extracts (SCFEs), essential oils (EOs) and lactic acid bacteria (LAB). The *in vitro* antimicrobial activity of the developed films was tested and their use as potential preservation technology to extend the shelf-life of fish and seafood products was assessed.

Hake (*Merluccius merluccius*) and tuna (*Thunnus alalunga*) fish were chosen to be treated with the antimicrobial coatings. Hake is one of the most consumed fish species in Spain. Tuna is one of the most nutritive fish species and it is an important part of the Mediterranean diet. Besides, peeled shrimp tails were chosen as example of seafood due to its high consumption also in the Spanish market.

To do so, the present thesis was divided in 6 chapters. In the first one a careful bibliographic review is presented, in which edible films and coatings are introduced. Different antimicrobial compounds currently used for film formulations are presented and their application in different food products is discussed.

In Chapters 2 and 3, a study of the development of whey protein edible films and coatings as carriers of supercritical fluid extracts (SCFE) and essential oils (EOs) is presented. Firstly, the *in vitro* antimicrobial activity of four SCFEs (sage, oregano, garlic and rosemary) and two EOs (oregano and thyme) was tested against 14 different strains related to the spoilage and pathogenicity of fish and seafood. Rosemary SCFE and oregano and thyme EOs effectively inhibiting the growth of all strains tested showing different inhibition areas depending of the target strain. These aforementioned materials were incorporated into WPI films and their antimicrobial activity was tested. Results

showed that WPI-enriched films were able to inhibit the growth of the strains, showing higher inhibition areas films incorporated with oregano EO.

Besides, in Chapter 2, and based on the results of the *in vitro* evaluation of WPI films, the effect of WPI coatings incorporated with rosemary SCFE on the microbiological deterioration fresh tuna steaks was evaluated. Results indicated that coatings were effective in reducing the microbial load especially of aerobic mesophilic bacteria and Enterobacteriaceae.

In Chapter 4 the effect of the best WPI-EO formulation on the shelf-life of hake fillets was studied. In order to do that, fresh hake fillets were treated with WPI coatings containing two different concentrations of oregano and thyme EO. The effect of the different treatments on the evolution of the microbial quality of hake fillets packaged under air or modified atmosphere during chilled storage was studied. It was found that the shelf-life of fresh hake fillets treated with WPI+3 % oregano EO+MAP was twice as long as control samples.

In Chapter 5, chitosan edible films were used as carrier of essential oils. Different concentrations of oregano and thyme EO were incorporated in chitosan films and their *in vitro* antimicrobial activity was tested. Chitosan films containing oregano EO inhibited most of the bacteria tested. Once the effectiveness of the films was proven, peeled shrimp tails were coated with chitosan coatings containing the aforementioned EOs and packaged under modified atmosphere. Chitosan coatings containing 0.5 % of thyme EO were effective in reducing the microbial growth, keeping the final counts of total viable microorganisms under the microbiological limit suggested by legislation.

Finally, Chapter 6 shows the results of the development of bioactive edible films containing three different lactic acid bacteria. Whey protein isolate was used as effective carrier of lactic acid bacteria and their antilisterial activity was demonstrated. The

viability of the LAB incorporated into WPI films under chilled storage was proven. Results showed that LAB were able to keep viable within the WPI films during the storage period.

The results of this work show that antimicrobial edible films and coatings can be effectively used to improve the microbiological quality and extend the shelf-life of fish and seafood products. Optimizing formulations and application procedure could be a needed step before the final industrial use.

CHAPTER 1. ANTIMICROBIAL FILMS & COATINGS

Published as:

CARRIÓN-GRANDA, X., FERNÁNDEZ-PAN, I. and MATÉ, J.I. (2016). **"Antimicrobial Films and Coatings".** in "*Edible Films and Coatings: Fundamentals and Applications*", Montero, P., Gómez-Guillén, C., López-Caballero, ME., Barbosa-Cánovas, G. (Ed), CRC Press, Taylor & Francis Group, LLC, Boca Raton, Fl, USA. ISBN: 978-1-4822-1831-2 (*In press*).

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CHAPTER 2. DEVELOPMENT OF WHEY PROTEIN ISOLATE FILMS INCORPORATED WITH SUPERCRITICAL FLUID EXTRACTS TO IMPROVE THE MICROBIOLOGICAL QUALITY OF FRESH TUNA

CARRIÓN-GRANDA, X., FERNÁNDEZ-PAN, I., IÑARRA, B., MEZQUITA, S., ROVIRA, J. and MATÉ, J.I. (2016). Development of whey protein isolate films incorporated with supercritical fluid extracts to improve the microbiological quality of fresh tuna

Submitted to the International Journal of Food Science and Technology

CHAPTER 2. DEVELOPMENT OF WHEY PROTEIN ISOLATE FILMS INCORPORATED WITH SUPERCRITICAL FLUID EXTRACTS TO IMPROVE THE MICROBIOLOGICAL QUALITY OF FRESH TUNA

2.1 Abstract

The antibacterial activity of supercritical fluid extracts (SCFEs) of sage (*Salvia officinalis*), oregano (*Origanus vulgaris*), garlic (*Allium sativum*) and rosemary (*Rosmarinus officinalis*) was tested against 12 different bacteria related with the spoilage of fish and seafood. Results show that rosemary SCFE was the most effective followed by garlic extract. Bacteria like *S. epidermidis* or *A. caviae* were the most sensitive. In addition, rosemary SCFE was included in whey protein isolate (WPI) films at three different concentrations and their antibacterial activity was also tested. WPI-rosemary films showed different activity clearly dependent on the concentration of extract and bacteria strain. *S. putrefaciens* was the most sensitive strain showing zones of inhibition even at the lowest concentration tested (1 %). On the other hand, *P. fragi* was not inhibited by any of the films tested. When fresh tuna steaks (*Thunnus alalunga*) stored at refrigeration conditions were coated with WPI incorporated with 2 different concentrations of rosemary SCFE, the growth of microorganisms was inhibited especially aerobic mesophilic and Enterobacteriaceae. Whey protein edible films could be proposed as a preservation technology to improve the microbiological quality and food safety of fish products.

2.2 Introduction

Fish has been an important part of the human diet in many countries for centuries. Fish and shellfish are excellent protein, vitamin, minerals and polyunsaturated fatty acids (PUFAs) sources. Among fish species, tuna (*Thunnus alalunga*) is highly appreciated due to its high content of omega-3 fatty acids. It is an essential component of the well-known Mediterranean diet. In Spain, approximately 27.95 million kg of fresh tuna were consumed in 2014 representing 5 % of the total fresh fish consumed (Ministerio de Agricultura, 2014).

Fish, though, is one of the most highly perishable food products. Its spoilage process begins immediately after catching. The average losses related to fish spoilage is estimated in 25 % (Adams and Moss, 2008). The major cause of food spoilage is microbial growth (Gram and Dalgaard, 2002) and can be manifested as changes in the sensory characteristics like production of off-odours and flavours, slime formation, production of gas, discolouration and/or large visible pigmented or non-pigmented colonies (Gram and Huss, 1996).

Bacteria dominated by psychrotrophic Gram-negative bacteria belonging to the genera *Pseudomonas, Moraxella, Acinetobacter, Shewanella, Flavobacterium, Vibrionaceae* and *Aeromonadaceae* are normally part of the biota of fresh fish and seafood. But Gram-positive organisms such as *Bacillus, Micrococcus, Clostridium, Lactobacillus* and *Corynebacterium* can also be found in varying proportions (Forsythe, 2000; Jay, 2000).

In western societies consumers increasingly demand fresh refrigerated foods with extended shelf life and with restrictions in the use of additives especially chemical ones. This fact has made the food industry search for innovative preservation

technologies to extend the shelf-life, while ensuring safety of fresh foods. Therefore prevention of spoilage has become an important challenge for the food industry.

On the other hand, to avoid the use of preservatives or chemical additives, the use of natural extracts has been studied in the last years. The use of plants, herbs and their essential oils in medicine, cosmetics and the food industry is a practice registered since ancient times. In the past, it was very common to use herbs for improving organoleptic characteristics of foods or to preserve food. Essential oils from plants such clove (Yano et al., 2006), sage (Menaker et al., 2004), rosemary, tea tree (Alvarez et al., 2013), oregano and laurel (Muñoz et al., 2009) among others, have been described as antimicrobial substances and suggested as an alternative to chemical preservatives. Many authors have related the antibacterial activity of these substances with their high content of phenolic compounds such as carvacrol, thymol, 1-8-cinoleo (Lambert et al., 2001), eugenol, borneol, thujone, alfa-pinene (Tajkarimi et al., 2010). Oregano, rosemary, sage and garlic are herbs and plants commonly used in Spanish cuisine and characterised by their high content in the aforementioned active compounds, making these essential oils a valid alternative to synthetic preservatives against microorganisms associated with food spoilage (Bozin et al., 2007; Burt, 2004).

The supercritical fluid extracts (SCFEs) used in the present study were selected after a careful bibliographic review where antibacterial activity of such substances against different bacteria strains was reported (Bozin et al., 2007; Burt, 2004; Sokovic et al., 2010). Besides, those extracts were also selected taking into account the possible application in the preservation of fish and fish products since they have organoleptic properties that could be compatible with these food products.

In recent years, supercritical fluid extraction (SFE), an environmental friendly and efficient extraction technique, has become an alternative to conventional extraction

processes such as organic solvent extraction or steam distillation. SFE makes use of supercritical fluids to perform the extraction process. A fluid is considered to be in a supercritical state when the system pressure and temperature are above its critical point (Rosa et al., 2009). At this region the gas and liquid phases become indistinguishable and the fluid has the unique ability to penetrate through solids like a gas and dissolve materials like a liquid (McHugh and Krukonis, 1994).

Among others, CO_2 is the most popular supercritical solvent because it is odourless, colourless, safe, readily available, non-toxic, non-flammable, recyclable, easy to remove from extracted products and has a low cost (Rosa et al., 2009).

Most of the substances related to the antimicrobial activity of essential oils, like carvacrol, thymol, camphor or borneol, are thermolabile. The use of SFE as an extraction technique would theoretically reduce the deterioration of such compounds, because the extraction process normally is performed at low temperatures.

Currently, active edible coatings can be considered as an innovative technology to extend and guarantee the shelf life of especially fresh products. Many researchers are working in combining, for example, edible coatings (acting as a carrier) with antimicrobial/antioxidant essential oils to reduce the growth of spoilage or pathogen microorganisms and extend the shelf life of food products (Atarés et al., 2010; Chana-Thaworn et al., 2011; Min and Oh, 2009) . Many different biopolymers can form films including polysaccharides and proteins (Han and Gennadios, 2005). In this study whey protein isolate (WPI), a biopolymer which forms flexible, odourless, tasteless and transparent films was used as carrier of natural antimicrobials.

The main objective of this work was to develop antimicrobial edible films to be applied to fish products. Therefore, first we developed and evaluated the antibacterial activity of different supercritical fluid extracts on the growth of microorganisms related

with the spoilage and pathogenicity of fish and seafood. The most effective SCFE were incorporated into whey protein isolated films and their antimicrobial activity was tested. Finally, the effect of the WPI films incorporated with two concentrations of rosemary SCFE on the microbial deterioration of tuna fish steaks under refrigerated storage was also evaluated.

2.3 Materials and methods

2.3.1 Supercritical fluid extracts

Supercritical fluid extracts (SCFEs) of sage (*Salvia officinalis*) (10 g/100 g), oregano (*Origanus vulgaris*) (10 g/100 g), garlic (*Allium sativum*) and rosemary (*Rosmarinus officinalis*) (10 g/100 g) were provided by Idoki SCF Technologies S.L. (Derio-Spain).

2.3.2 Bacteria strains

Twelve bacterial strains (Table 2.1) were used to evaluate the antimicrobial activity of extracts. All strains were supplied by the Spanish Type Culture Collection (CECT, Universidad de Valencia-Spain). After recuperation, bacterial strains were kept either under freezing storage in Eppendorf tubes containing a mix of Brain Heart Infusion broth with 30 g/100 mL of glycerol or in cryovials with porous beads at -80 ºC.

Table 2.1: Bacteria strains tested and growth conditions

No. CECT	BACTERIA	Gram	Culture medium	Incubation Temp (ºC)
838	*Aeromonas caviae*	-	Nutrient Broth I	30
5173	*Aeromonas hydrophila*	-	Tripticase Soy Agar	30
194	*Enterobacter cloacae*	-	Nutrient Broth I	30
173	*Morganella morganii*	-	Nutrient Broth I	37
378	*Pseudomonas fluorescens*	-	Nutrient Broth II	26
446	*Pseudomonas fragi*	-	Nutrient Broth II	26
5346	*Shewanella putrefaciens*	-	Nutrient Broth II	26
115	*Stenotrophomonas maltophila*	-	Nutrient Broth II	30
521	*Vibrio alginolyticus*	-	Nutrient Broth I + 3%NaCl	26
481	*Enterococcus faecalis*	+	Brain Heart Infusion	37
232	*Staphylococcus epidermidis*	+	Nutrient Broth II	37
236	*Staphylococcus warneri*	+	Nutrient Broth II	37

CECT: Colección Española de Cultivos Tipo

2.3.3 Other materials

WPI was provided by Davisco Food International (Le Seur, MN, USA). Glycerol was provided by Panreac Química S.A. (Barcelona-Spain). Filter paper discs, Nutrient Broth I and Nutrient Broth II were provided by Oxoid (Barcelona-Spain), Trypticase Soy Broth and Trypticase Soy Agar, Brain Heart Infusion and Sodium Chlorine were provided by Merck (Madrid-Spain).

2.3.4 Antibacterial activity of supercritical fluid extracts

The disc diffusion method (NCCLS, 2003) was used for screening the antibacterial activity of SCFEs against the 12 selected bacteria strains. Overnight cultures grown in the specific media for each bacteria were used. A filter paper disk (6 mm diameter) soaked with 10 µl of SCFE was placed on top of the agar plate inoculated with a loan of 10^7 cfu/ml of each strain. Plates were incubated at the corresponding conditions (Table 2.1) for 24 hours. All tests were performed in triplicate. The diameters of zones of inhibition were measured using a calliper and expressed as areas of inhibition in mm^2 excluding the disc diameter.

2.3.5 Formulation of the Whey Protein Isolate films

WPI films were prepared as follows: Whey protein isolate (10 % w/w) was dissolved in distilled water and 5 % (w/w) of glycerol was added as plasticizer. Then, film forming solutions (FFS) were heated using a thermostatic bath at 90 ºC during 30 minutes under constant stirring. The selected extracts were added at 1, 3 and 5 % (w/w) once FFS were cooled at room temperature. The FFS were homogenised by sonication (UP 400S Hielscher Ultrasound Technology, Germany) using a 7 mm diameter tip for 5 minutes at 100 % of amplitude and immersing FFS in an ice-water bath in order to avoid temperature raises over 30 ºC. FFS were casted pouring 14 g onto glass dishes (14 cm diameter) and dried overnight at room temperature and relative humidity.

2.3.6 Antibacterial activity of WPI films enriched with SCFE

Same as for the case of SCFEs, the disc diffusion method was used to evaluate the antibacterial activity of WPI films against four bacteria strains: *Aeromonas hydrophila, Shewanella putrefaciens, Vibrio alginolyticus* and *Pseudomonas fragi*. In this case, discs of 17 mm diameter of every film formulation were cut using a punch and placed onto agar plates inoculated with an inoculum of 10^7 cfu/ml of each strain. Plates were incubated under the conditions showed in Table 2.1 for 24 hours. All tests were performed in triplicate. The inhibition zone diameters were measured using a calliper and expressed as areas of inhibition in mm^2 excluding the disc diameter.

2.3.7 Fish sample preparation

Fresh raw tuna steaks were provided by a local supplier and transported to the laboratory in a cooler filled with ice. Under sterile conditions, steaks were cut into pieces of 5 x 3 x 2 cm (length x width x height) and 30 g weight approximately. Cut steaks

were randomly separated into 3 groups: the first one without any treatment used as control (CONTROL: C), and the other two groups were coated with the respectively FFS (WPI-Ros-3 for WPI + Rosemary 3 % and WPI-Ros-4 for WPI + Rosemary 4 %). For coating, every piece was dipped in 100 ml of FFS during 2 minutes; then, the FFS excess was allowed to drip off for 30 seconds and finally, pieces were dried under cool air stream for 45 seconds each side. Both, control and coated samples were packaged in polypropylene trays, sealed with PE/PP/EVOH/PP film without modified atmosphere and stored at 4 ºC during 8 days.

2.3.8 Microbiological analysis

25 g of sample was aseptically weighed, placed in a sterile plastic bag (Seward, UK) and homogenized with 225 ml of buffered peptone water (Cultimed, Spain) using a stomacher (Stomacher 400, England) for 2 minutes. Decimal dilutions were prepared as needed and seeded in the correspondent media in order to perform the following determinations: a) total viable counts (TVC) on pour plates of Plate Count Agar (PCA, Merck-Germany) incubated for 48 hours at 30 °C; b) total psychrotrophic bacteria on pour plates of Plate Count Agar (PCA, Merck-Germany) incubated for 7-10 days at 4 °C; c) Enterobacteriaceae on double-layered pour plates of Violet Red Bile Glucose (VRBG, Cultimed-Spain) incubated for 24 hours at 37 °C; d) *Pseudomonas* spp. on spread plates of Pseudomonas Agar Base (Oxoid-Spain) supplemented with Cetrimide-Fucidine-Cephalosporine (CFC, Oxoid-Spain) incubated for 48 hour at 30 °C; and, e) lactic acid bacteria (LAB) on spread plates of de Man, Rogosa and Sharpe Agar (MRS, CONDA-Spain) incubated for 5 days at 30 °C. All plates were examined visually for typical colonies associated with each medium. Microbiological analyses were conducted at days 0, 1, 3, 6 and 8 of the storage period. All microbiological results are

expressed as the log of the colony forming units (CFU) per gram of sample. All analyses were done in triplicate.

2.3.9 Statistical analysis

All tests were performed in triplicate. Statistical analyses were performed using SPSS 21.0 (IBM, USA) software. Significant differences among extracts were determined using ANOVA and Duncan's multiple range post hoc test (confidence level of 95 %).

2.4. Results and discussion

2.4.1 Antimicrobial activity of SCFEs

The antimicrobial activity of SCFEs against the 12 bacteria strains related with the spoilage and pathogenicity of fish and seafood is presented in Table 2.2. In general, rosemary SCFE showed the largest zone of inhibition, followed by garlic SCFE; sage SCFE showed little or no activity and oregano SCFE showed the lowest inhibition zones. Regarding strains the Gram-negative *P. fluorescens* and *E.cloacae* were the most resistant bacteria (with the lowest inhibition area) and on the other hand *A. caviae, M. morganii, S. epidermidis* and *V. alginolyticus* the most sensitive. It is remarkable to mention that every bacteria strain showed different degrees of susceptibility for every SCFE tested.

Table 2.2: Inhibitory areas (mm^2, excluding disc area) of SCFE against different bacteria strains

Letters a-c show significant differences among SCFEs. Letters A-E show significant differences among strains ($p < 0.05$).

	S-SCFE	Or-SCFE	Gar-SCFE	Rg-SCFE
Aeromonas caviae (-)	1.31^{aA}	0.26^{aA}	35.85^{bB}	99.56^{cE}
Aeromonas hydrophila (-)	0.13^{aA}	1.83^{aC}	25.91^{bB}	50.76^{cBC}
Enterobacter cloacae (-)	0.13^{aA}	0.92^{aABC}	8.90^{bA}	0.26^{aA}
Morganella morganii (-)	0.26^{aA}	1.44^{aBC}	66.86^{cD}	48.80^{bBC}
Pseudomonas fluorescens (-)	3.27^{aA}	0.26^{aA}	35.85^{bB}	0.65^{A}
Pseudomonas fragi (-)				37.03^{aB}
Shewanella putrefaciens (-)	46.58^{bC}	0.7^{aABC}	37.29^{bB}	71.57^{cD}
Stenotr. maltophilia (-)	3.01^{aA}	0.13^{aA}	37.81^{bB}	3.53^{aA}
Vibrio alginolyticus (-)	3.01^{aA}	1.05^{aABC}	53.25^{bC}	76.28^{cD}
Enterococcus faecalis (+)	0.39^{aA}	0.39^{aAB}	28.52^{bB}	64.37^{cCD}
Staphylococcus epidermidis (+)	27.61^{bB}	0.13^{aA}	25.91^{bB}	130.70^{cE}
Staphylococcus warneri (+)	1.44^{aA}	0.13^{aA}	26.43^{bB}	77.06^{cD}

S-SCFE: Sage SCFE (10 %) dissolved in glycerine; Or-SCFE: Oregano (10 %) SCFE dissolved in glycerine; Gar-SCFE: Garlic SCFE; Rg-SCFE: Rosemary SCFE (10 %) dissolved in glycerine

Regarding rosemary extract, *S. epidermidis* was the most sensitive strain showed the largest inhibition area and on the other hand *P. fluorescens* and *E. cloacae* were the less susceptible strains (showed the smallest inhibition areas). Many researchers have shown the effectiveness of rosemary essential oil (EO) or extract in the growth inhibition of different bacteria and are in accordance with our results, despite the absolute values of inhibition areas are not the same. Ivanovic et al. (2012) tested rosemary SCFE against bacteria like *B. cereus, E. faecium, S. enteritidis* or *E. coli* and found that the extract was more active against the Gram-positive bacteria than against the Gram-negative ones. In the same line, Muñoz et al. (2009) determined the effect of different plant SCFEs on the growth of *L. monocytogenes* and found that rosemary extract was the most effective (compared to oregano and laurel SCFE), reducing counts even below the detection limit. Same as our results, Silva et al. (2013) found that rosemary EO did not inhibit the growth of *E. cloacae* and *P. aeruginosa* but it was effective in the inhibition of other strains like

E. coli, L., monocytogenes or *S. epidermidis*. The activity or rosemary SCFE has been related with the presence of active compounds like carnosic acid, camphor, carnosol, borenol, 1,8-cineole, alfa-pinene (Ivanovic et al., 2012; Santoyo et al., 2005). In their work Ramírez et al. (2006) evaluated the antimicrobial activity of rosemary SCFE and the three different fractions obtained by SCF fractionation process; besides, the composition of the extract and every fraction collected was analysed by gas chromatography. They associated the activity of the extract with the carnosic acid content as they found that the fraction that was richer in (mainly composed by) carnosic acid presented the highest MIC against *S. aureus* and *E. coli*. Jordán et al. (2012) showed in their research that high content of carnosol in relation to carnosic acid improved the antimicrobial activity of rosemary methanolic extracts against *L. monocytogenes* and *S. aureus*.

Garlic SCFE was also able to inhibit the growth of all strains tested. Bacteria like *M. morganii* or *V. alginolyticus* showed clear inhibition areas of 66,86 and 53,25 mm^2 respectively. Some investigations have shown that extracts from garlic bulbs inhibit the growth of *V. paraemolitycus, E. coli* and other bacteria belonging to the *Enterobacteriacea* genera (Mahmoud et al., 2004; Yano et al., 2006). Mahmoud et al. (2004) found that garlic oil at 1 % and 2 % inhibited different strains isolated from carp fillets. For example strains of *Flavobacterium* sp. or *Moraxella* sp. isolated from the carp's skin and gill were inhibited and zones of clear growth were reported. Little inhibition was found in the bacteria *Enterobacteriaceae* sp, which is similar to our results where *E. cloacae* presented the lowest inhibition area. Such activity has been attributed to the presence of thiosulfinates compounds including allicin, the main component of garlic (Harris et al., 2001; Kyung, 2012). In their work, Zalepugin et al. (2010) tested different active compounds obtained from garlic SCFE and found that

allicin showed weak antimicrobial activity against *P. aurantiaca* and *E. coli* and no activity against *B. cereus*. The active compound diallyl disulfide and trisulfide showed little activity against *B. cereus* and no activity against the two Gram-negative bacteria. Besides, they also tested synthetic disulfide and found that it had higher antimicrobial activity against *B. cereus* compared to the natural one. This could be explained by the thermal instability of all compounds present in garlic extracts.

Oregano SCFE showed very little or no activity against all bacteria strains tested. In the study presented by Mith et al. (2014), oregano EO at different concentrations (100 %, 50 %, 10 %, 5 % and 2,5 %) was tested against *L. monocytogenes*. The EO at 100 % inhibited the growth of the bacteria while when the concentration decreased at 50 % or 10 %, the EO did not inhibit the growth of the aforementioned bacteria. In our study the concentration of the oregano SCFE was 10 % and the poor activity of oregano SCFE could be attributed to the very low concentration tested. On the other hand, and despite some previous works have described the antibacterial activity of oregano SCFE against many bacteria (Fornari et al., 2012; Menaker et al., 2004; Stamenic et al., 2014), the lack of activity of oregano SCFE could be also related to the extraction process and the minimum concentration of active compounds like carvacrol or thymol. SCFE can contain, besides the active principles, some additional compounds like waxes, which could encapsulate these principles and diminish or even remove such activity (Rosa et al., 2009). Furthermore, the extraction yield, and the percentage of active compounds present in the extract, could be affected by the particle size and the moisture content of the raw material (Capuzzo et al., 2013).

In the case of sage SCFE, there was no antimicrobial activity against most of the tested bacteria strains, except for *S. putrefaciens* and *S. epidermidis,* which were

inhibited. On contrary, some previous studies have reported strong antibacterial activity for sage EO (Bozin et al., 2007; Ozkan et al., 2010).

As it was mentioned before *P. fluorescens* was one of the most resistant bacteria against all extracts except garlic SCFE. The results of the present study are in agreement with previous works in which the resistance of *Pseudomonas* spp. is documented (Bozin et al., 2007; Gómez-Estaca et al., 2010; Lambert et al., 2001). Such resistance is related to the nature of *Pseudomonas* spp.: they are Gram-negative bacteria and besides the cytoplasmic membrane, they have an outer membrane which is known to restrict the entrance of hydrophobic compounds acting therefore as a barrier against hydrophobic antimicrobial substances like EOs. For example, the high resistance of *P. aeruginosa* to antimicrobial compounds is attributed to the high Mg^{2+} content in the outer membrane, helping to produce strong lipopolysaccharide-lipopolysaccharide links and the small size of pores limit general diffusion (Russel, 2005). Despite *S. putrefaciens, A. hydrophila* and *V. alginolyticus* are Gram-negative bacteria, rosemary and garlic SCFE caused inhibition in their growth. Similar results have been shown by Gómez-Estaca et al. (2010) and Iturriaga et al. (2012) who found that these strains were totally or partially inhibited by clove, thyme or rosemary, among others EOs.

Despite literature attributing less resistance for the Gram-positive rather than Gram-negative bacteria against antimicrobial compounds, this research found a significant susceptibility of some of the tested Gram-negative strains. Same as our results, Sharififar et al. (2007) also reported that the EO of *Zataria multiflora* Boiss inhibited the growth of some Gram-negative strains like *S. typhi* or *S. flexneri*. In her work, Burt (2004) reported that such difference in susceptibility between Gram-positive and Gram-negative strains could be related to possible variations in composition of different batches of essential oils or extracts.

2.4.2 Antibacterial activity of WPI films

Rosemary SCFE was selected to be incorporated into the film formulation in order to test its antibacterial activity once included inside a film matrix. Such extract was selected due to its high antibacterial activity, compared with the other three extracts, as shown in the screening previously performed (Table 2.2).

A. hydrophila, S. putrefaciens, V. alginolyticus and *P. fragi* were selected in order to test the antimicrobial activity of WPI films. These bacteria have been largely studied and related with the spoilage and pathogenicity of fish and seafood products (Gram and Dalgaard, 2002; Gram and Huss, 1996).

As it was expected, the higher the concentration of rosemary SCFE included in the WPI film, the greater the inhibition areas of the tested films. *S. putrefaciens* was the most sensitive bacteria, showing areas of inhibition even at 1 % concentration of SCFE. *A. hydrophila* presented inhibition just at 5 % concentration of rosemary SCFE. On the other hand and *P. fragi* was again the most resistant bacteria and WPI-enriched films did not inhibit its growth (Figure 2.1). Gómez-Estaca et al. (2010) also found that *P. fragi* was the most resistant strain when tested gelatin-chitosan films with thyme EO.

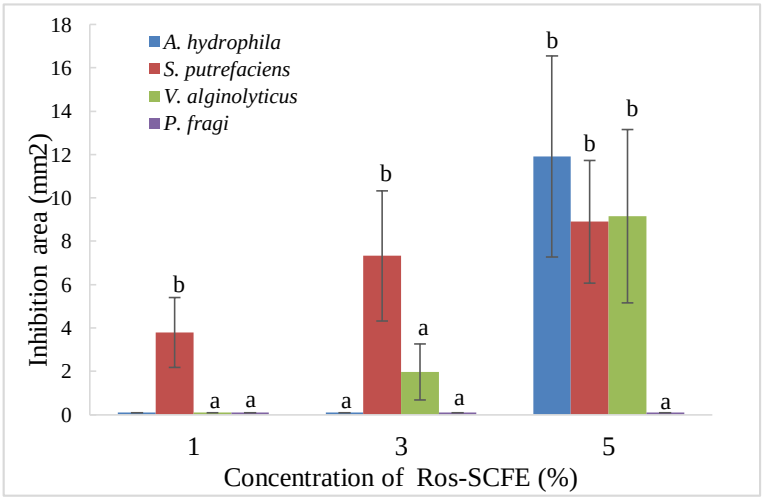

Figure 2.1: Inhibitory areas (mm^2, excluding film area) of WPI films enriched with rosemary SCFE against *A.hydrophila, S. putrefaciens, V. alginolyticus*, and *P. fragi*
Different letters mean significant differences among concentration of SCFE ($p < 0.05$).

The results of the antibacterial activity of WPI-rosemary SCFE enriched films are consistent with the literature. Ponce et al. (2008) formulated chitosan and carboxymethyl cellulose films and included in the formulation 1 % of rosemary EO and found that such films inhibited the growth of *L. monocytogenes* and squash native microbiota. Morsy et al. (2014) tested pullulan films enriched with 1 % and 2 % of rosemary EO and concluded that films inhibited the growth of *S. Typhimurium, L. monocytogenes, E. coli* O157:H7 and *S. aureus* at both concentrations. Same as our results the higher the concentration of SCFE, the stronger the antimicrobial activity.

Tested as extract, rosemary exerted antimicrobial activity against *A. hydrophila, S. putrefaciens, V. alginolyticus* and *P. fragi* (see Table 2.2), but when rosemary was included in WPI films the inhibition areas decreased or even were absent. This reduction in activity could be associated with the degree of diffusivity of extracts when included into the matrix. In their research Royo et al. (2010) tested sage EO added to filter paper discs and WPI and found that the EO had antimicrobial activity against *L. innocua, S. enteritidis* and *S. aureus* when included in filter paper discs but they did not have any activity when included in the biopolymer. They stated that this difference of activity could be related with the low diffusivity of the active compounds through the WPI matrix. They concluded that the antimicrobial effect depend on the matrix used and its ability to release active compounds. In the same line Fernández-Pan et al. (2012) tested different essential oils as such and incorporated in WPI films and concluded that the lack or poor activity of WPI-EO enriched films could be attributed to the fact that the essential oils were not released from the matrix at the concentration needed to inhibit the growth of the bacteria.

2.4.3 Microbiological changes on tuna steaks

The changes in the microbiota of tuna steaks coated with FFS enriched with different concentrations of rosemary SCFE and stored at 4 ºC are shown in Figure 2.2A to E. The initial value of TVC were low (around 3 log cfu/g) implying acceptable quality and correct handling practices during processing of steaks. Similar low counts were observed in previous experiments with other fish species (Pyrgotou et al., 2010; Zinoviadou et al., 2009). In any case, TV final counts did not exceed 7 log cfu/g during the storage period, which is the threshold value recommended by ICMSF (2002) for the commercialization of fresh water and marine species. As it was expected, counts in control samples significantly increased during the storage period. Same as for TVC, the initial population of Enterobacteriaceae was low (1.65 log cfu/g) highlighting the good hygiene of tuna steaks.

Since day 3 for all microbial group analyzed, samples treated with WPI-Ros-3 and WPI-Ros-4 showed significant differences ($p < 0.05$) compared with control samples. In the case of *Pseudomonas,* none of the treatments inhibited the growth of this bacteria. Between the two treatments, significant differences were observed depending mainly of type of microorganism analyzed, concentration of SCFE and the storage day.

Edible coating containing 4 % of rosemary SCFE was more effective in inhibiting the growth of TV microorganisms than the other treatments, as it can be observed in Fig. 2.2A. At the end of the storage period a difference in counts of 1 log cfu/g was observed when compared with C samples. In the case of WPI-Ros-3 smaller but significant difference of 0.5 log cfu/g was observed, always compared with the C samples. WPI-Ros-3 and WPI-Ros-4 also reduced significantly ($p < 0.05$) the TV counts at day 6 of storage. In their study, Gómez-Estaca et al. (2007) coated cold-smoked

sardine with gelatin coatings incorporated with rosemary EO and stored for 20 days at 5 °C. At day 16 of storage TVC were also reduced in about 1.5 log cfu/g, though at the end of the storage period no reductions were observed.

Regarding LAB, edible coatings exerted their antimicrobial effect from day 3 of storage and on (Fig. 2.2B). Reduction of about 1 log cfu/g was observed for both treatments when compared with control samples. At the end of storage period (day 8) just WPI-Ros-4 significantly ($p < 0.05$) inhibited the growth of this microorganisms. Final counts of LAB for C and WPI-Ros-3 were around 6 log cfu/g.

Counts of Enterobacteriaceae are shown in Fig. 2.2C. As it was mentioned before, low initial counts of this bacteria (1.65 log cfu/g) indicates adequate practices during degutting, filleting and handling of tuna steaks. Data showed significant differences ($p < 0.05$) between treated and control samples. Differences in counts (compared with control samples) of 1 and 0.5 log cfu/g were observed for WPI-Ros-4 and WPI-Ros-3, respectively, at the end of the storage period. Similar behavior was observed at day 3 of storage. During the rest of the storage period, no inhibition was observed.

The evolution of psychrotrophic bacteria during the storage period is showed in Figure 2.2D. Both treatments inhibited the growth of this bacteria observing significant ($p < 0.05$) reductions in counts of 1.5 log cfu/g at day 8 of analysis, compared with control samples. During the storage, no significant differences were observed between WPI-Ros-3 and WPI-Ros-4 until day 8 when WPI-Ros-4 was more effective in inhibited the growth of psychrotrophic bacteria. Same as our results, Abdollahi et al. (2014) reported reductions of 1.5 log cfu/g of psychrotrophic bacteria at the end of the refrigerated storage period (16 days) in silver carp fillets coated with chitosan/clay biopolymer incorporated with rosemary essential oil.

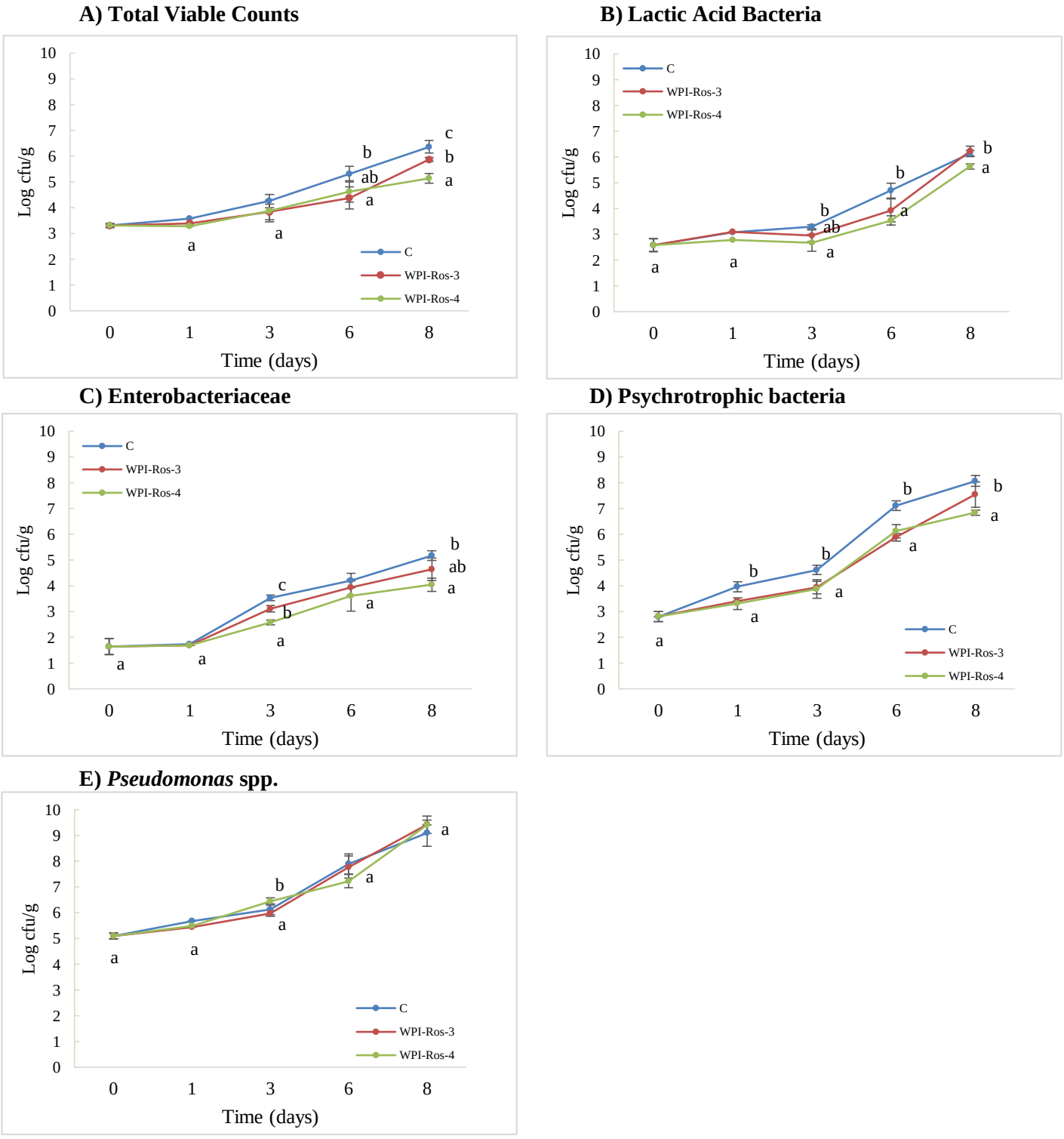

Figure 2.2: Evolution of the natural microbiota of WPI-ROS coated tuna steaks. C: Control; WPI-Ros-3: WPI + Rosemary SCFE 3 %; WPI-Ros-4: WPI + Rosemary SCFE 4 %. Different letters mean differences among treatments in the same day and bacterial group ($p < 0.05$).

Counts of Enterobacteriaceae are shown in Fig. 2.2C. As it was mentioned before, low initial counts of this bacteria (1.65 log cfu/g) indicates adequate practices during degutting, filleting and handling of tuna steaks. Data showed significant differences ($p < 0.05$) between treated and control samples. Differences in counts (compared with control samples) of 1 and 0.5 log cfu/g were observed for WPI-Ros-4 and WPI-Ros-3, respectively, at the end of the storage period. Similar behavior was observed at day 3 of storage. During the rest of the storage period, no inhibition was observed.

The evolution of psychrotrophic bacteria during the storage period is showed in Figure 2.2D. Both treatments inhibited the growth of this bacteria observing significant ($p < 0.05$) reductions in counts of 1.5 log cfu/g at day 8 of analysis, compared with control samples. During the storage, no significant differences were observed between WPI-Ros-3 and WPI-Ros-4 until day 8 when WPI-Ros-4 was more effective in inhibited the growth of psychrotrophic bacteria. Same as our results, Abdollahi et al. (2014) reported reductions of 1.5 log cfu/g of psychrotrophic bacteria at the end of the refrigerated storage period (16 days) in silver carp fillets coated with chitosan/clay biopolymer incorporated with rosemary essential oil.

As it was mentioned before, none of the treatments could inhibit the growth of *Pseudomonas.* High initial counts (5 log cfu/g) were registered (Fig. 2.2E). At the end of the storage period *Pseudomonas* spp. became the dominant group in tuna steaks with final counts of 9 log cfu/g. Fernández-Saiz et al. (2013) also found *Pseudomonas* as a prevalent group in sole fillets stored under refrigeration. This lack of activity of the treatments was expected since films incorporated with rosemary SCFE did not show any inhibitory effect against *P. fragi* when they were tested *in vitro* (Fig. 2.1). It is well known the resistance of *Pseudomonas* spp. to the action of natural antimicrobial

compounds due to the outer membrane which restricts the entrance of hydrophobic compounds (Emiroglu et al., 2010). Besides, this lack of activity of edible coatings over this bacteria could be related with high counts since the beginning of the experiment. High numbers of *Pseudomonas* spp. could require higher concentration of rosemary SCFE to be inhibited.

Some previous studies have also pointed out the effectiveness of rosemary essential oil or extract included in different edible coatings in inhibiting the growth of natural microbiota present in food products. Morsy et al. (2014) found reductions in counts of *L. monocytogenes* and *S. aureus* inoculated in RTE turkey deli meat coated with pullulan films enriched with 2 % of rosemary EO, stored at 4 °C and vacuum packed. Liu et al. (2012) found that CMC films enriched with rosemary extract inhibited the growth of TVC on fresh beef refrigerated at 4 ºC. Contrary to our results, Giménez et al. (2004) did not observe any reduction in TV counts when treated sea bream fillets with rosemary EO.

The antimicrobial activity of rosemary extract has been discussed before. It is related with the presence of carnosic acid, carnosol and rosmarinic acid, which are phenolic diterpenes containing a phenolic group in their structure. The mechanism associated at the antimicrobial activity of diterpenes is still not clear. It has been suggested that the ability of the hydrophobic structure of diterpenes enables them to partition the lipids of the bacterial cell membrane, making it more permeable (Cowan, 1999).

2.5 Conclusions

After performing the antimicrobial screening of the different supercritical fluid extracts, rosemary SCFE showed the highest antibacterial activity against the 12 spoilage and pathogenic bacteria strains tested, followed by garlic SCFE. *P. fluorescens* and *E. cloacae* were the most resistant strains. Rosemary SCFE was successfully incorporated in the WPI formulations at three different concentration and films showed different antimicrobial activity that was clearly dependant of the concentration of extract included and the susceptibility of the bacteria strain tested. *S. putrefaciens* was the most sensitive bacteria strain while the most resistant was *P. fragi* which could not be inhibited for any of the formulated films. Then, the resistance of *Pseudomonas* spp. to the action of plant extracts was confirmed. WPI coatings incorporated with 4 % rosemary SCFE have proven to help the preservation of fresh tuna steaks, inhibiting the growth of the natural microbiota present in the product. Therefore, the use of WPI films as carriers of natural compounds could control fish and seafood spoilers and pathogens and extend the shelf-life of fresh fish stored under refrigeration conditions.

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CHAPTER 3. WHEY PROTEIN ISOLATE FILMS INCORPORATED WITH ESSENTIAL OILS AS AGENTS OF FISH AND SEAFOOD PRESERVATION

CARRIÓN-GRANDA, X., FERNÁNDEZ-PAN, I., ROVIRA, J. and MATÉ, J.I. (2016). Whey protein isolate films incorporated with essential oils as agents of fish and seafood preservation.

Under review in Journal of Food Science

CHAPTER 3. WHEY PROTEIN ISOLATE FILMS INCORPORATED WITH ESSENTIAL OILS AS AGENTS OF FISH AND SEAFOOD PRESERVATION

3.1 Abstract

Essential oils (EO) of thymol thyme (*Thymus zygis*) and oregano (*Origanum vulgaris*) and carvacrol and thymol (as main active compounds of these 2 EOs) were tested to evaluate their antimicrobial activity against 14 bacteria responsible for the spoilage and pathogenicity of fish products. Thyme essential oil showed significantly higher activity, inhibiting the growth of both, gram-positive and gram-negative strains. The two active principles showed lower inhibitory areas than the EOs, implying a synergistic effect. Carvacrol was more effective compared with thymol. Then, films made of whey protein isolate (WPI) were incorporated with both EOs at three different concentrations and their antimicrobial activity was tested against six selected microorganisms: *Aeromonas hydrophila, Shewanella putrefaciens, Vibrio alginolyticus, Pseudomonas fragi, Listeria monocytogenes* and *Salmonella Typhimurium.* Films containing any of the studied EOs at 3 and 5 % concentration successfully inhibited the growth of all these microorganisms regardless of type of microorganisms. *L. monocytogenes* was the most sensitive bacteria and *P. fragi* was the most resistant. *S. putrefaciens* and *A. hydrophila,* two bacteria highly related with the spoilage and pathogenicity of fish products were inhibited by the films tested, indicating WPI-EO edible films as viable technology for fish preservation.

3.2 Introduction

Traditionally, medicinal plants, herbs and spices have been used for food flavouring and preservation. Essential oils (EOs) are aromatic oily liquids obtained from these plants. They are complex mixtures of different compounds and among other qualities they possess various biological properties. They have been studied as one of the "natural" alternatives to extend the shelf life of food products due to their proven antibacterial properties (Gul and Bakht, 2015; Ouattara et al., 1997; Santoyo et al., 2005). Essential oils of clove, tea tree, laurel, sage (Fernández-Pan et al., 2012), , oregano (Gutierrez et al., 2009), rosemary and orange (Fernández-López et al., 2005), thyme (Mejlholm and Dalgaard, 2002) among others have been studied and their antibacterial activity against a variety of spoilage and pathogen bacteria has been effectively demonstrated.

In general, the antimicrobial activity of essential oils is associated with the content of different substances like terpenes, alcohols, aldehydes, phenolic compounds and others. Carvacrol and thymol, present mainly in the essential oils of oregano and thyme, are considered among the most active components of essential oils (Pei et al., 2009). The antimicrobial activity of such compounds against different strains have been reported in several studies (Bagamboula et al., 2004; Ultee et al., 1999) and their use is preferred above essential oils since the impact on the organoleptic characteristics of the food product could be drastically minimized.

Fish and seafood products are a very popular food product in many countries but they are also some of the most perishable ones. Its spoilage process begins immediately after death, when fish is in contact with different microflora. Fish is even more perishable than red meat or chicken due to its large content of free aminoacids and

volatile nitrogen bases compared with other meats (Mexis et al., 2009). Throughout processes like handling and/or storage, fresh fish deterioration takes place rapidly and limits its shelf life. Fish harvested from both fresh and saltwater are susceptible to spoilage through autolytic enzyme actions, oxidation of unsaturated fatty acids, and microbial growth (Ray, 2004). Spoilage is not only due to the visible growth of microorganisms (formation of slime or colonies or loos of texture), but also to the production of end metabolites which result in off-odours and flavours, gas, changing the sensory characteristics of the product and making fish products unacceptable for human consumption. Enzymatic and chemical reactions are usually responsible for the initial loss of freshness whereas microbial activity is responsible for the obvious spoilage and thereby establishes product shelf life (Gram and Huss, 1996).

The current changes in consumer's lifestyle, where they are demanding fresh and minimally processed foodstuffs with no synthetic additives, have made the food industry put high efforts and search for new and innovative preservation technologies to reduce the growth of both spoilage and pathogen microorganisms and guarantee the shelf life of such products.

Edible films and coatings made from polysaccharides, proteins and lipids are becoming a new, convenient, innovative and promising technology used to extend the shelf-life of food and to increase their quality. In general, food technology can use films as barrier to moisture, water, aroma and oxygen and also as carriers of different compounds like antioxidant, antimicrobials and other preservatives in order to improve food quality and safety (Krochta, 2002). Several studies have proved the effectiveness of edible films and coatings as carriers of natural antimicrobial substances (Emiroglu et al., 2010; Fernández-Pan et al., 2012; Ruiz-Navajas et al., 2015). Whey protein isolate (WPI) has been used as carrier of natural preservatives (like essential oils) due to its

ability to form flexible, odourless and tasteless films. Its antimicrobial activity, when combined with, for example, essential oils, has been demonstrated and it is well documented. Shakeri et al. (2011) tested WPI films incorporated with *Zataria multiflora* Boiss essential oil, a plant which grows manly in Iran, Pakistan and Afghanistan, and found that the formulated films inhibited the growth of some lactic acid bacteria strains named *L. acidophilus, L. rhammosus, L. casei* and *L. plantarum*.

The aims of this work were: 1) to evaluate the antibacterial activity of oregano (*Origanum vulgaris*) and thyme (*Thymus zygis*) essential oils and their main active compounds (carvacrol and thymol) against 14 bacteria responsible for the spoilage and pathogenicity of fish and seafood; and 2) to evaluate the antibacterial activity of whey protein isolated films incorporated with essential oils.

3.3 Materials and methods

3.3.1 Essential oils and active principles

Essential oils (EOs) of thymol thyme (*Thymus zygis*) (49.25 % thymol, 18.99 % p-cymene) and oregano (*Origanum vulgaris*) (71.70 % carvacrol, 4.28 % thymol) were provided by Esencias Martínez Lozano (Murcia-Spain). Carvacrol and thymol main active principles (AP) of oregano and thyme essential oils were also tested and were provided by Sigma-Aldrich (Madrid-Spain). Thymol (10 % w/v) was dissolved in a hydroalcoholic solution. Both, essential oils and active principles are considered food grade.

3.3.2 Bacteria strains

Fourteen different bacterial strains were used to evaluate the antimicrobial activity of essential oils and active principles (Table 3.1). All strains, except *L. monocytogenes* and *S. Typhimurium,* were supplied by the Spanish Type Culture Collection (CECT, Universidad de Valencia-Spain). The two aforementioned bacteria were kindly provided by Prof. Glenn Young from the Food Science and Technology Department, University of California – Davis. After recuperation, bacterial strains were kept either under freezing storage in Eppendorf tubes containing a mix of Brain Heart Infusion broth with 30 % (v/v) of glycerol or in porous beads at -80 ºC.

Table 3.1: Bacteria strains tested and growth conditions

No. CECT	BACTERIA	Gram	Culture medium	Incubation Temp (ºC)
481	*Enterococcus faecalis*	+	Brain Heart Infusion	37
	Listeria monocytogenes	+	Brain Heart Infusion	37
232	*Staphylococcus epidermidis*	+	Nutrient Broth II	37
236	*Staphylococcus warneri*	+	Nutrient Broth II	37
838	*Aeromonas caviae*	-	Nutrient Broth I	30
5173	*Aeromonas hydrophila*	-	Tripticase Soy Agar	30
194	*Enterobacter cloacae*	-	Nutrient Broth I	30
173	*Morganella morganii*	-	Nutrient Broth I	37
378	*Pseudomonas fluorescens*	-	Nutrient Broth II	26
446	*Pseudomonas fragi*	-	Nutrient Broth II	26
	Salmonella Typhimurium	-	Brain Heart Infusion	37
5346	*Shewanella putrefaciens*	-	Nutrient Broth II	26
115	*Stenotrophomonas maltophila*	-	Nutrient Broth II	30
521	*Vibrio alginolyticus*	-	Nutrient Broth I + 3%NaCl	26

CECT: Colección Española de Cultivos Tipo

3.3.3 Other materials

WPI was provided by Davisco Food International (Le Seur, MN, USA). Glycerol was provided by Panreac Química S.A. (Barcelona-Spain). Filter paper discs, Nutrient Broth I and Nutrient Broth II were provided by Oxoid (Barcelona-Spain), Trypticase

Soy Broth and Trypticase Soy Agar, Brain Heart Infusion and Sodium Chlorine were provided by Merck (Madrid-Spain).

3.3.4 Antibacterial activity of Essentials Oils and Active Principles

The disc diffusion method (NCCLS, 2003) was used for screening the antibacterial activity of EOs and AP against the 14 selected bacteria strains. Overnight cultures grown in the specific media for each bacteria were used. A filter paper disk (6 mm diameter) soaked with 10 µl of EO/AP (depending of the case) was placed on top of the agar plate inoculated with a loan of 10^7 cfu/ml of each strain. Plates were incubated at the corresponding conditions (Table 3.1) for 24 hours. All tests were performed in triplicate. The diameters of zones of inhibition were measured using a calliper and expressed as areas of inhibition in mm^2 excluding the disc diameter.

3.3.5 Formulation of the Whey Protein Isolate films

WPI films were prepared as follows: Whey protein isolate (10 % w/w) was dissolved in distilled water and 5 % (w/w) of glycerol was added as plasticizer. Then, film forming solutions (FFS) were heated using a thermostatic bath at 90 ºC during 30 minutes under constant stirring. The essential oils were added at 1, 3 and 5 % (w/w) once FFS were cooled at room temperature. The FFS were homogenised by sonication (UP 400S Hielscher Ultrasound Technology, Germany) using a 7 mm diameter tip for 5 minutes at 100 % of amplitude and immersing FFS in an ice-water bath in order to avoid temperature raises over 30 ºC. FFS were casted pouring 14 g onto glass dishes (14 cm diameter) and dried overnight at room temperature.

3.3.6 Antibacterial activity of WPI films enriched with EOs

Same as for the case of EOs, the disc diffusion method was used to evaluate the antibacterial activity of WPI films against six bacteria: *Aeromonas hydrophila, Shewanella putrefaciens, Vibrio alginolyticus, Pseudomonas fragi, Listeria monocytogenes* and *Salmonella Typhimurium.* In this case, discs of 17 mm diameter of every film formulation were cut using a punch and placed onto agar plates inoculated with an inoculum of 10^7 cfu/ml of each strain. Plates were incubated under the conditions showed in Table 3.1 for 24 hours. The inhibition zone diameters were measured using a calliper and expressed as areas of inhibition in mm^2 excluding the disc diameter. All tests were performed in triplicate.

3.3.7 Statistical analysis

All tests were performed in triplicate. Statistical analyses were conducted using SPSS 21.0 (IBM, USA) software. Significant differences among extracts were determined using ANOVA and Duncan´s multiple range post hoc test (confidence level of 95 %).

3.4 Results and Discussion

3.4.1 Antimicrobial activity of Essential Oils

The antimicrobial activity of EOs against 14 strains related with the pathogenicity and spoilage of fish and seafood is presented in Table 3.2. Both, oregano and thyme EOs caused inhibition against all tested strains (both Gram-positive and Gram-negative). For all the tested bacteria, except for *P. fluorescens*, thyme EO showed the highest inhibitory effect. Regarding strains, *P. fluorescens* and *P. fragi* were the most

resistant bacteria (with the lowest inhibition area) and *A. caviae* and *M. morganii* were the most sensitive to both essential oils tested. It should be noticed that every strain showed different resistance to the antibacterial action of both essential oils.

Table 3.2 Inhibitory areas (mm^2, excluding disc area) of EO against different bacteria strains.
Letters a-h in the same column show significant differences ($p < 0.05$)

	OR-EO	**THY-EO**
Gram-positive strain		
Enterococcus faecalis	843±158^{d}	1723±356^{h}
Listeria monocytogenes	929±130^{d}	1147±136^{f}
Staphylococcus epidermidis	428±30^{a}	1503±204^{g}
Staphylococcus warneri	347±42^{a}	703±123^{c}
Gram-negative strain		
Aeromonas caviae	1167±132^{e}	1739±204^{h}
Aeromonas hydrophila	371±68^{a}	486±58^{b}
Enterobacter cloacae	566±42^{bc}	1137±140^{ef}
Morganella morganii	1289±97^{f}	1502±183^{g}
Pseudomonas fluorescens	679±104^{c}	275±41^{a}
Pseudomonas fragi	341±33^{a}	675±158^{bc}
Salmonella Typhimurium	539±66^{b}	567±66^{bc}
Shewanella putrefaciens	584±110^{bc}	975±142de
Stenotr. maltophilia	909±76^{d}	1677±188^{gh}
Vibrio alginolyticus	574±77^{bc}	891±54^{d}

OR-EO: Oregano essential oil; THY-EO: Thyme essential oil

The antimicrobial activity shown by oregano and thyme EOs against both Gram-positive and Gram-negative strains is in good agreement with the literature (Baydar, H, et. al., 2004 Castilho, P. C. et.al., 2012). Such activity has been related to the presence of mainly phenolic compounds (Chorianopoulos et al., 2004; Pei et al., 2009). Therefore the mechanisms of action of these EOs could be the same as phenolic compounds. These compounds disrupt the cell membrane, causing an increased permeability (Ultee et al., 1999). They could also interact with membrane proteins, causing a deformation in structure and functionality (Viuda-Martos et al., 2008).

Thyme EO showed the highest inhibitory effects, with inhibition areas ranging from 275 to 1720 mm^2 and this results are similar to works previously done (Bagamboula et al., 2004; Yano et al., 2006). Our results showed that *P. fluorescens* was the most resistant bacteria having the smallest inhibition area. Other strains tested like *A. hydrophila, P. fragi, S. thypimurium* and *S. warneri* were also resistant to the action of thyme EO. On the other hand, *S. epidermidis, M. morganii, Stenotr. maltophilia, E. faecalis* and *A. caviae* showed the largest inhibition zones. In their research, Silva et al. (2013) evaluated the antibacterial activity of eight essential oils against 10 foodborne and spoilage bacteria and reported that, similar to our results, thyme and oregano were the most active EOs inhibiting in different degree the growth of all bacteria including *L. monocytogenes* and *P. aeruginosa*.

Oregano EO also exhibited intense antibacterial activity and such results are in accordance with previous researches (Castilho et al., 2012; Viuda-Martos et al., 2008). Bacteria like *S. epidermidis, S. warneri, A. hydrophila, P. fragi* were the less susceptible to the action of oregano EO showing the lowest inhibition areas. On the other hand, *E. faecalis, L. monocytogenes, Sternotr. maltophilia, A. caviae* and *M. morganii* were the most sensitive bacteria having the greatest zones of inhibition. Similar to our work, Chorianopoulos et al. (2004) evaluated the *in vitro* antibacterial activity of oregano EO against five foodborne bacteria including *L. monocytogenes* and *S. enteritidis,* and demonstrated that all strains were inhibited showing different degree of susceptibility. In our study *L. monocoytogenes* and *S. Typhimurium*, among others, were also successfully inhibited by oregano EO. On the other hand, Gutierrez et al. (2009) concluded that *P. fluorescens* and *P. putida* were the most resistant bacteria strains to the action of oregano EO showing the lowest inhibition areas.

L. monocytogenes, a pathogen of concern within the food industry, was one of the tested strain which was effectively inhibited by oregano and thyme EOs. In line with our results, Mith et al. (2014) and Gutierrez et al. (2009) found that oregano EO effectively inhibited the growth of *L. monocytogenes* among others.

Several studies have demonstrated the resistance of *Pseudomonas* spp. to the action of natural antibacterial substances (Fernández-Pan et al., 2012; Sokovic et al., 2010). Our results also confirmed such resistance because *P. fragi* and *P. fluorescens* were the two less susceptible strains showing the lowest inhibition areas. *Pseudomonas* spp. are Gram-negative bacteria and as it is known this bacteria genus are less sensitive to the action of antimicrobial substances because of the presence of the lipopolysaccharide outer membrane which restricts the diffusion of hydrophobic compounds like essential oils (Cox and Markham, 2007). Nevertheless, the inhibitory effect produced by both, thyme and oregano EOs against *Pseudomonas* spp. is not negligible.

In general, Gram-negative bacteria are usually considered to be less sensitive to the action of essential oils than Gram-positive ones (Burt, 2004; Gutiérrez-Larraínzar et al., 2012). But in our study Gram-negative bacteria like *M. morganii, A. caviae, Enterobacter cloacae, S. Typhimurium* or *Stenotr. maltophilia* were even more sensitive than some Gram-positive strains. These results are consistent with other studies concluding that essential oils did not exert different antimicrobial activity regarding different Gram-genus (Viuda-Martos et al., 2008; Wilkinson et al., 2003). In their study Ouattara et al. (1997) found variability among tested strains and therefore difference in resistance of Gram-positive bacteria to the effects of essential oils. Burt (2004) made an extensive review about the antibacterial activity of essential oils and reported that possible variation in composition between different batches of EOs could influence the degree of susceptibility of Gram-negative and Gram-positive bacteria.

Salmonella Typhimurium is another pathogen of concern within the food industry. It belongs to the Gram-negative group and in our study it presented more resistance to the action of EOs than Gram-positive strains. In our study *S. Typhimurium* presented an intermediate susceptibility to the action of the EOS tested showing inhibition areas of 539 and 566 mm^2 for oregano and thyme respectively. These results are in agreement with other works where the EOs tested were less active to this strain than other tested (Mazzarrino et al., 2015; Thanissery et al., 2014).

The Gram-negative *Aeromonas hydrophila* was another strain which presented low areas of inhibition. This result is in agreement with other works previously done which showed that this strain was poorly inhibited or even not inhibited by essential oils like *Satureja subspitica* or .*Salvia pisidica* (Ozkan et al., 2010; Skočibušić et al., 2006). On the other hand Longaray Delamare et al. (2007) found that essential oil of *Salvia triloba* was effective in inhibiting the growth of *A. hydrophila.* Difference in results of antibacterial activity could be attributed to the bacterial strains used and the composition of the essential oil used in each study.

Despite *M. morganii* and *A. caviae* are Gram-negative strains, they were successfully inhibited by thyme and oregano EOs. In fact they showed the highest inhibition area among all tested bacteria ($p < 0.05$). Similar results are presented by Iturriaga et al. (2012) who tested different EOs against *A. caviae* and found that oregano and thyme EO were the most effective in inhibiting the growth of this strain. Similarly, Pandey and Mishra (2010) also found *Aloe barbadensis* extract successfully inhibited the growth of *M. morganii*.

In order to know the contribution of phenolic compounds to the antimicrobial activity of EOs, the antimicrobial activity of carvacrol and thymol, the main constituents

of oregano and thyme EO (according to the technical data sheet provided by the EO supplier) was tested.

The disc diffusion method was also used to test the antibacterial activity of AP against all the bacteria strains used for the determination of the antimicrobial activity of EOs except for *L. monocytogenes* and *S. Typhimurium*.

As it can be seen in Table 3.3, both, carvacrol and thymol successfully inhibiting the growth of all bacteria tested. *P. fluorescens, P.fragi* and *S. warneri* were the most resistant bacteria showing the lowest inhibition zones and on the other hand, *S. putrefaciens, Stenotr. maltophilia, A. caviae* and *M. morganii* were the most sensitive to both active principles. These results were consistent with what was observed for oregano and thyme EOs (Table 3.2) where the aforementioned bacteria strains were also the most resistant and sensitive respectively.

Same as our results, Friedman et al. (2002) and Bagamboula et al. (2004) reported growth inhibition caused by the action of carvacrol and thymol. Lambert et al. (2001) attributed the antibacterial activity of carvacrol and thymol to the ability to make the cell membrane permeable, disintegrating the outer membrane of Gram-negative bacteria. Cox and Markham (2007) also concluded that the toxicity for carvacrol could be related to the capability of this substance to damage the bacterial cytoplasmic membrane of bacteria like *P. aeruginosa.*

Table 3.3: Inhibitory areas (mm^2, excluding disc area) of carvacrol and thymol against different bacteria strains
Letters a-d in the same column show significant differences ($p < 0.05$)

	CARVACROL	THYMOL
Gram-positive strain		
Enterococcus faecalis	289±36^{c}	64±13^{a}
Staphylococcus epidermidis	304±26^{c}	59±11^{a}
Staphylococcus warneri	2312±20^{b}	55±10.^{a}
Gram-negative strain		
Aeromonas caviae	602±72^{f}	289±23^{c}
Aeromonas hydrophila	289±29^{c}	342±38^{d}
Enterobacter cloacae	358±41^{d}	117±14^{b}
Morganella morganii	654±67^{g}	478±40^{e}
Pseudomonas fluorescens	587±52^{f}	39±10^{a}
Pseudomonas fragi	158±23^{a}	45±9^{a}
Shewanella putrefaciens	310±24^{c}	699±35^{f}
Stenotr. maltophilia	616±39^{fg}	317±63^{cd}
Vibrio alginolyticus	447±37^{e}	294±37^{c}

The mode of action of phenolic compounds is generally thought to involve interference with functions of the cytoplasmic membrane (López-Malo Vigil et al., 2005; Thanissery et al., 2014). Phenolics exert antimicrobial activity by attaching to the lipid bilayer of the cytoplasmic membrane, injuring lipid-containing membranes, which results in leakage of cellular contents. They alter the permeability of microbial cell, permitting the loss of macromolecules from the interior. They could also interact with membrane proteins, causing a deformation in its structure and functionality (Russel, 2005). Carvacrol and thymol, two hydrophobic compounds, cause functional damage to plasma membrane becoming it permeable (Lambert et al., 2001).

Despite quantitative comparison between the antibacterial activity of EOs and APs was not done, it is worthy to mention that the EOs showed higher inhibition areas than the APs. Then it can be assumed a synergistic effect between carvacrol, thymol and other minor compounds present in the composition of both, oregano and thyme EO. In other words, the main components like carvacrol and thymol (present in high

proportions) are not necessarily singularly responsible for the total antimicrobial activity of essential oils. Other minor components like *p*-cymene, borneol, myrcene could have a synergistic effect in the antimicrobial activity of the essential oil.

This result is in line with Pei et al. (2009) who used the chequerboard method to test the combined effects of such compounds. They concluded that a combination of 200 ppm of carvacrol and thymol had higher antimicrobial activity than the single compounds. Lambert et al. (2001) also found than the inhibitory effect of oregano EO is mainly due to the mixture of carvacrol and thymol. Castilho et al. (2012) also attributed the antimicrobial activity of oregano EO to the content of carvacrol and thymol. After their study, Longaray Delamare et al. (2007) also concluded that synergistic effects of major and minor components of essential oils should be taken into account when talking about antimicrobial activity of EOs.

3.4.2 Antibacterial activity of WPI films

Oregano and thyme EOs were incorporated into the WPI film formulation at three different concentrations (1, 3 and 5 % w/w) in order to test their antibacterial activity once included in a film matrix. Films without the presence of EOs were also tested and served as control. As it was expected, lack of antimicrobial activity of WPI control films was shown. Overall, films containing oregano (WPI-OR) and thyme (WPI-THY) EOs showed antibacterial activity against all bacteria tested regardless of the concentration (Table 3.4). Even at 1 % of EOs, inhibition areas were observed against bacteria like *L. monocytogenes, V. alginolyticus* or *S. Typhimurium*. Similar results were found by Emiroglu et al. (2010) and Zivanovic et al. (2005) who tested oregano and thyme EOs incorporated in different polymeric matrices and showed antimicrobial activity against organisms like *E. coli, S. aureus* or *L. monocytogenes.*

In general, WPI films containing oregano EO showed larger inhibition areas compared to the areas produced by WPI-THY films. As it was expected, films with the higher concentration of EOs presented the higher inhibition areas. Among strains, *L. monocytogenes* and *V. alginolyticus* were the most sensitive. In fact, *L. monocytogenes* showed the largest inhibition areas for both tested EOs and for all three different concentrations. On the other hand, *P. fragi* and *A. hydrophilas* were the most resistant strains, which were inhibited just by films containing 3 and 5 % of EOs (no inhibition with films containing 1 % of EOs). *S. putrefaciens* was also poorly inhibited by the tested films. *S. Typhimurium* showed an intermediate susceptibility compared to the other strains.

As it was mentioned before, *L. monocytogenes* was the most sensitive strain showing inhibition areas from 492 to 1085 mm^2 ($p < 0.05$). Similar results were found by Seydim and Sarikus (2006) who developed WPI films enriched with oregano EO and also inhibited the growth of *L. monocytogenes* and *S. enteritidis* at concentrations of 2 to 4 %. (Teixeira et al. (2014)) also found that *L. monocytogenes* and *L. innocua* were sensitive to the action of fish protein films incorporated with origanum EO. Contrary to our results *S. Typhimurium* was not inhibit.

S. Typhimurium, another pathogen of concern within the food industry, was also inhibited by WPI-OR and WPI-THY films. Our results are in agreement with Morsy et al. (2014) who tested pullulan films incorporated with oregano and rosemary EOs at 1 and 2 %. They demonstrated that films at 2 % concentration were effective in inhibiting the growth of bacteria like *S. Typhimurium* or *S. aureus*. Fernández-Pan et al. (2012) tested WPI enriched films and showed that films containing oregano and clove EOs inhibited the growth of *S. enteritidis.*

Shewanella putrefaciens is a strain associated with the spoilage of fresh fish and it is responsible for the off-odours of spoiled fish. In our study this strain was poorly inhibited by the WPI-OR and WPI-THY films. However, other results from previous works showed that *S. putrefaciens* was the most sensitive bacteria when tested different biopolymer matrices incorporated with thyme and oregano EO (Jouki, Mortazavi, et al., 2014; Jouki, Yazdi, et al., 2014).

A. hydrophila and *P. fragi*, two Gram-negative bacteria were, as expected, the most resistant strains. The resistance of Gram-negative bacteria to the action of essential oils is well known and documented (Burt, 2004; Cox and Markham, 2007; Gutiérrez-Larraínzar et al., 2012) and it has been discussed before in this paper. Other works previously performed also report the low inhibition or no inhibition of the aforementioned strains (Emiroglu et al., 2010; Fernández-Pan et al., 2012; Iturriaga et al., 2012).

Table 3.4: Inhibitory areas (mm^2, excluding disc area) of WPI-films against different bacteria strains

Letters a-d in the same column show significant differences among different WPI-films. Letters A-F in the same row show significant differences among strains ($p < 0.05$)

	Aeromonas hydrophila	***Shewanella putrefaciens***	***Vibrio alginolyticus***	***Pseudomonas fragi***	***Listeria monocytogenes***	***Salmonella Typhimurium***
WPI-OR 1%	NA^{aA}	5 ± 2^{aA}	26 ± 9^{aB}	NA^{aA}	185 ± 25^{bC}	24 ± 5^{aB}
WPI-OR 3%	33 ± 12^{bA}	66 ± 24^{bB}	144 ± 18^{bC}	93 ± 17^{cB}	821 ± 41^{dD}	130 ± 15^{bC}
WPI-OR 5%	104 ± 10^{cA}	238 ± 48^{dB}	479 ± 52^{dE}	294 ± 31^{dC}	1085 ± 49^{eF}	381 ± 43^{cD}
WPI-THY 1%	NA^{aA}	5 ± 2^{aA}	90 ± 17^{aC}	6 ± 2^{aA}	49 ± 9^{aB}	NA^{aA}
WPI-THY 3%	26 ± 8^{bA}	162 ± 27^{cBC}	166 ± 24^{bC}	16 ± 4^{aA}	219 ± 27^{bD}	140 ± 11^{bB}
WPI-THY 5%	133 ± 13^{dB}	159 ± 30^{cB}	348 ± 47^{cD}	35 ± 9^{bA}	460 ± 51^{cE}	279 ± 22^{dC}

NA: no inhibition area registered

When tested alone, oregano and thyme EOs showed higher inhibition effects than when included into the WPI matrix. This reduction in activity could be related to the degree of diffusivity of EO from the biopolymer matrix used. The release rate of EO

from filter paper discs is likely higher than from WPI films, which means higher availability of EO to inhibit the growth of bacteria. When working with pullulan films enriched with caraway EO, Gniewosz et al. (2013) stated that part of the oils could be retained in the film, therefore the action of films was significantly lower than the pure EO. Royo et al. (2010) and Shakeri et al. (2011) concluded that it is important to note that different film matrices have different levels of diffusivity and could affect the migration mechanism of the EO. Another possible reason has been exposed by Zivanovic et al. (2005) who attributed this change in activity to the partial loss of volatile compounds of EOs during the process of film formulation. It is also important to take into account that a minimum inhibitory concentration is needed in order to inhibit the growth of bacteria which could not be reached by the EO incorporated into the WPI films.

The different degree of antimicrobial susceptibility of microorganisms against WPI enriched films observed in this work could depend of the source of EOs, the different concentration of active compounds, the release of the active groups and the interaction between EOs and the film material.

3.5 Conclusions

Both, oregano and thyme essential oils exerted a wide-ranging antimicrobial spectrum suggesting their use as natural preservatives. Thyme essential oil showed higher antibacterial activity against the 14 studied bacteria associated with the spoilage and pathogenicity of fish products.

The contribution of carvacrol and thymol to the antimicrobial activity of oregano and thyme EOs was observed. Both active principles could also be used as an alternative

for food preservation. However, since a synergistic effect could be implied, the use of EOs could be recommended.

Both thyme and oregano EOs were successfully incorporated into the WPI films, working as carrier of the EOs. The antibacterial activity of thyme and oregano was maintained after being included into WPI formulations. Such activity was clearly dependant on type and concentration of EO included, bacteria strain tested and diffusivity of EO within the matrix. WPI-Or films showed in most of the cases, higher antibacterial activity than the WPI-Thy films.

The use of WPI films as carriers of natural compounds is a very promising preservation technology for controlling food spoilers and pathogens on fresh fish and seafood products. Bacteria like *L. monocytogenes* or *S. Typhimurum*, which can grow under refrigeration temperatures (around 4 °C), were inhibited by WPI-enriched films, being *L. monocytogenes* the most sensitive one. These results support the alternative use of essential oils and edible films as an emerging fish preservation technology.

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CHAPTER 4. EFFECT OF WHEY PROTEIN COATINGS AND MODIFIED ATMOSPHERE PACKAGING ON THE SHELF-LIFE OF HAKE (*Merluccius merluccius)* FILLETS

CARRIÓN-GRANDA, X., FERNÁNDEZ-PAN, I., ROVIRA, J. and MATÉ, J.I. (2016) Effect of whey protein coatings and modified atmosphere packaging on the shelf-life of hake (*Merluccius merluccius)* fillets

Partially presented in:

VIII Congreso Ciencia y Tecnología de los Alimentos (CYTA)/ Ingeniería de los Alimentos (CESIA), Badajoz, Spain. 2015.

Submitted to Food Control

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4.1 Abstract

The effect of whey protein isolate (WPI) coatings incorporated with oregano and thyme essential oils (EO) and combined with modified atmosphere packaging (MAP) on the shelf-life of fresh hake (*Merluccius merluccius)* fillets was evaluated when storage at 4 °C. Fresh hake fillets were coated with different formulations of WPI-EO coatings and packaged under air and MAP conditions (50% CO_2/45% N_2/5% O_2). When WPI-enriched films were applied with or without the presence of MAP over hake fillets with a high initial microbial population, a limited but significant effect on the microbial growth was observed. This effect was more intense on Enterobacteriaceae and H_2S-producing bacteria. When hake fillets with lower initial microbial counts were treated a more intense antimicrobial effect was observed on all studied microbial groups. In addition a synergistic effect between WPI-EO coatings and MAP was observed. A significant extension of the lag phase and reduction, primarily, on the total viable counts and H_2S-producing bacteria was detected, doubling the shelf-life of hake fillets compared with control samples. The initial microbial load of the samples is a key factor influencing the effectiveness of the treatment. The obtained results demonstrated the effectiveness of this combined strategy as a promising alternative for enhancing the quality of fish and seafood products during storage at refrigeration temperatures.

4.2 Introduction

Fresh fish is consumed all over the world as one of the most nutritive food products. However fish is highly perishable, even more than red meat or chicken, due to its large content of free aminoacids and volatile nitrogen bases compared with other meats (Mexis et al., 2009). The spoilage process of fish usually starts with the production of off-odours and flavours, generated basically by the growth of typical spoilage microorganisms. Besides, loss of texture and presence of slime could be detected. As a result of all these changes, the shelf-life of unpacked and packed fresh fish is very limited (Gram and Dalgaard, 2002; Ray, 2004). Bacteria like *Shewanella* spp., *Pseudomonas* spp., *Aeromonas* spp., *Vibrio* spp., Enterobacteriaceae, Lactic acid bacteria, are common spoilers of fresh and chilled fish stored either under air or modified atmosphere conditions (Nychas and Drosinos, 2009).

Hake (*Merluccius merluccius*) is one of the most consumed fish species in Spain, either fresh or frozen. In 2011, the 26 % of consumed fresh fish in the Spanish market was hake. According to the Spanish Agricultural Ministry during 2013 the consumption per capita of fresh hake was 2,63 kg (Ministerio de Agricultura, 2014). Therefore any new strategy developed to preserve or extend the shelf-life of this fish species is considered important for this economic area.

Modified atmosphere packaging (MAP) has been extensively applied by the food industry as one of the most effective preservation techniques. MAP of fresh produce relies on modification of the gas composition inside the package, based on the interactions between two processes, the respiration of product and the transfer of gases through the packaging (Arvanitoyannis, 2012). According to results of previous works, modified atmosphere in combination with refrigeration seems to be a suitable technology to extend

the shelf-life of fresh fish and seafood (Arvanitoyannis et al., 2011; Pantazi et al., 2008; Parlapani et al., 2015). In a previous study, swordfish fillets were packaged under different atmospheres and stored under refrigeration. Results showed that MAP and vacuum packaging inhibiting the growth of bacteria until day 9 of storage and the shelf-life was extended in 11-12 days (Pantazi et al., 2008). In addition, Parlapani et al. (2015) studied the microbiological changes of sea bass fillets stored under refrigeration and packed under air and commercial MAP. They found that fish samples stored under MAP had a shelf-life extension 4 days longer that samples stored under air. These two technologies retard the microbial activity but they do not fully control the deterioration of fish.

In addition to traditional methods used to extend the shelf-life of fish products, like MAP or chilling storage, there is an increasing interest in the use of edible films and coatings with antimicrobial properties in order to reduce, inhibit or delay the growth of microorganism on the surface of foods. Edible films and coatings can be made of polysaccharides, proteins and lipids and can act as carriers of different compounds like antioxidant, antimicrobials and other preservatives in order to improve food quality and safety. Whey protein isolate (WPI) has been used as carrier of different natural preservatives (like essential oils) or synthetic one due to its ability to form flexible, odorless and tasteless films (Krochta, 2002). Its effectiveness, when combined with essential oils (EOs), in reducing or inhibiting the growth of microorganisms in food products other than fish, has been demonstrated and it is well documented. Fernández-Pan et al. (2014) treated chicken breast fillets with WPI coatings incorporated with oregano and clove EOs and evaluated their effect against the microbiota developed during storage at refrigeration temperatures. They concluded that WPI-oregano coating duplicated the shelf-life of chicken samples. Zinoviadou et al. (2009) wrapped beef cuts

with WPI-oregano and thyme films, resulting in a significant reduction of total viable counts and a complete inhibition of lactic acid bacteria.

Oregano and thyme EOs have been widely tested *in vitro* as antimicrobial substances and effectively inhibited the growth of an extensive range of bacteria (Gutiérrez-Larraínzar et al., 2012; Mazzarrino et al., 2015; Mith et al., 2014; Stamenic et al., 2014). Phenolic compounds are the main active principles of EOs and the antibacterial activity of the latest substance are totally related with them. The mode of action of phenolic compounds and in consequence of essential oils is associated with the ability of these compounds to harm and disintegrate the outer membrane of cells (Lambert et al., 2001). Thus, the use of EO could be considered as part of a hurdle approach in combination with other preservation technologies such as MAP or edible coatings.

Multiple barrier technology, also known as hurdle technology, is a promising preservation strategy to give consumers safe products while keeping quality, freshness, organoleptic aspects and extending their shelf-life. Hurdle technology makes use of existing or new different preservation techniques (also known as hurdles) effectively combined in order to achieve a multi-target and reliable microbial spoilage control (Leistner and Gorris, 1995). Bacteria are stressed by the combination of hurdles, and synergistic or additive effects will be expected (Lee, 2004). Therefore, the combined use of whey protein coatings, essential oils, refrigeration and MAP, as different preservation technologies, could be a option for extending the shelf-life of hake fillets due to the potential synergistic effect.

Then, the objectives of this work were 1) to evaluate the effectiveness of whey protein isolate edible coatings enriched with essential oils on the shelf-life extension of hake fillets; and, 2) to assess the combined effects of edible coatings and MAP on the shelf-life of hake fillets stored under refrigeration conditions. Besides, the influence of

initial fish microbial loads in the effectiveness of edible coatings and MAP over the shelf-life of hake fillets was analyzed.

4.3 Materials and methods

4.3.1 Film forming solution preparation

WPI (10 % w/w) (Davisco Food International, USA) was dissolved in distilled water and 5 % (w/w) of glycerol (Panreac Química, Spain) was added as plasticizer. Then, film forming solutions (FFS) were heated using a thermostatic bath at 90 ºC during 30 minutes under constant agitation. Food grade oregano (OR) and thyme (THY) EOs (Esencias Martínez Lozano, Spain) were added at 1 and 3 % (w/w) once FFS were cooled at room temperature. The FFS were homogenised by sonication (UP 400S Hielscher Ultrasound Technology, Germany) using a 7 mm diameter tip for 5 minutes at 100 % of amplitude. During sonication FFS were maintained in an ice-water bath in order to avoid temperature raises over 40 ºC.

4.3.2 Fish samples preparation

Fresh boneless and skin-off fish fillets were obtained from a local store and transported to the laboratory in a cooler filled with ice. Under sterile conditions, fillets were cut into pieces of 5 x 3 x 2 cm (length x width x height) and 30 g weight approximately. Cut fillets were randomly separated into 6 groups: the first one without any treatment used as control (CONTROL: C), and the other 5 groups were coated with the corresponding FFS. For coating, every piece was dipped in 150 ml of FFS for 1 minute; then the FFS excess was allowed to drip off for 45 seconds and finally pieces were dried for 5 minutes under air stream. After all this process, the pieces were dipped

for a second time for 1 minute, drained for 45 seconds and dried for 30 minutes under air stream. This two cycle coating process guaranteed the formation of a homogenous, uniform and continuous coating in every fillet (Fig 4.1). Samples were stored at two packaging conditions: air packaging (Exp. 1) and modified atmosphere (50 % CO_2/45 % N_2/5 % O_2) (Exp. 2) at 4 °C during 8 days, in the case of air packaging and 16 days in the case of modified atmosphere packaging samples. Both, control and coated samples were packaged in polypropylene trays, sealed with PE/PP/EVOH/PP film. The specific gas mixture was chosen based on previous studies found in literature (Kostaki et al., 2009; Kykkidou et al., 2009). In order to evaluate if the initial microbial population of hake fillets affected the effectiveness of the treatments, a third experiment was prepared. Hake fillets were obtained from a local store which has daily provision of fresh fish direct from the main supplier. A special care with the hygiene of the fillets during cleaning and deboning processes was asked to the new store. Samples were coated with WPI coatings containing 3 % of oregano EO, packed under MAP and stored at 4 ºC during 12 days. The coating procedure was the same used for experiment 1 and 2.

Figure 4.1: Coated hake fillet

4.3.3 Microbiological analysis

25 g of sample was aseptically weighed, placed in a sterile plastic bag (Seward, UK) and homogenized with 225 ml of buffered peptone water (Cultimed, Spain) using a stomacher (Stomacher 400, England) for 2 minutes. Decimal dilutions were prepared as needed and seeded in the correspondent media in order to perform the following determinations: a) total viable counts (TVC) on pour plates of Plate Count Agar (PCA, Merck-Germany) incubated for 48 hours at 30 °C; b) H_2S-producing bacteria (black colonies) on pour plates of Iron Agar (CONDA-Spain) incubated for 48 hours at 30 °C; c) total psychrotrophic bacteria on pour plates of Plate Count Agar (PCA, Merck-Germany) incubated for 7-10 days at 4 °C; d) Enterobacteriaceae on double-layered pour plates of Violet Red Bile Glucose (VRBG, Cultimed-Spain) incubated for 24 hours at 37 °C; e) *Pseudomonas* spp. on spread plates of Pseudomonas Agar Base (Oxoid-Spain) supplemented with Cetrimide-Fucidine-Cephalosporine (CFC, Oxoid-Spain) incubated for 48 hour at 30 °C; and, f) lactic acid bacteria (LAB) on spread plates of de Man, Rogosa and Sharpe Agar (MRS, CONDA-Spain) incubated for 5 days at 30 °C. All plates were examined visually for typical colonies associated with each medium. Microbiological analysis were done at days 0, 4, 8, 12 and 16 of the storage period. All microbiological results are expressed as the log of the colony forming units (cfu) per gram of sample. All analyses were done in triplicate.

4.3.4 Statistical analysis

All tests were performed in triplicate. Statistical analyses were conducted using SPSS 21.0 (IBM, USA) software. Significant differences among treatments were determined using ANOVA and Duncan's multiple range post hoc test (confidence level of 95 %).

4.4 Results and discussion

4.4.1 Microbiological changes in samples packed under air conditions (Experiment 1)

In order to verify the effectiveness of WPI coatings enriched with EOs over the microbial quality of fresh hake fillets packed under air conditions, the following samples were prepared:

CONTROL (C: no coating)

Control WPI (C-WPI)

WPI-OR 1 % (WPI-OR-1)

WPI-OR 3 % (WPI-OR-3)

WPI-THY 1 % (WPI-THY-1)

WPI-THY 3 % (WPI-THY-1)

The microbial counts of samples packed under air conditions are shown in Fig. 4.2 and 4.3. The initial value of TVC of hake fillets were higher (around 5 log cfu/g) compared with previous experiments performed with other fish species (Albertos et al., 2015; Pantazi et al., 2008). This high load of bacteria could be related to poor handling practices during processing of fish fillets. On day 4 of analysis most of the samples reached 7 log cfu/g which is the threshold value defined by ICMSF (2002) for the commercialization of fresh water and marine species.

Result showed that WPI-OR or WPI-THY samples with both concentrations of EOs did not show significant differences ($p < 0.05$) in TVC compared with the control samples at the end of the storage period (Fig. 4.2A and 4.3A). Just a slight reduction of less than 1 log cfu/g was observed in WPI-THY-1 and WPI-THY-3 samples on day 4 of storage. No significant differences were also observed between C and C-WPI samples,

indicating that WPI coatings did not have any effect (neither positive nor negative) on the microbiota of hake fillets.

LAB counts are shown in Fig. 4.2B and 4.3B. Comparing the final counts, no differences were observed either for WPI-THY-1 and WPI-THY-3 samples but a reduction of 1.5 log cfu/g was observed for WPI-OR-3 samples. Besides, significant reductions ($p < 0.05$) were observed in day 4 of treatment (2 and 1.5 log cfu/g respectively for WPI-OR-3 and WPI-THY-1 and WPI-THY-3).

Results of the evolution of Enterobacteriaceae are shown in Fig 4.2C and 4.3C. Significant differences were observed among the 4 treatments (WPI+EOs at 2 concentrations) during the whole storage period. WPI-OR-3 and WPI-THY-3 reduced the bacteria load in 2 and 1 log cfu/g respectively at the end of the storage period (day 8). Similar reductions were observed also in the day 4 of storage. Smaller but still significant differences were found in WPI-OR-1 samples.

The evolution of psychrotrophic bacteria during storage are shown in Fig. 4.2D and 4.3D. Similar to the case of TVC, none of the treatments significantly affected ($p < 0.05$) the growth of psychrotrophic bacteria. After day 4 of analysis, all counts reached 7 log cfu/g, the limit for microbiological quality of fish and seafood products advised by ICMSF (2002).

H_2-S producing bacteria (including *S. putrefaciens*) were significantly reduced for the treatments (Fig. 4.2E and 4.3E). Reductions of about 2 log cfu/g were found in WPI-OR-3 samples. Reductions of 2 and 2.5 log cfu/g showed the WPI-THY-1 and WPI-THY-3 samples at the end of the storage period. WPI-OR-1 was not effective in reducing this bacteria.

Fig. 4.2F and 4.3F showed the counts of *Pseudomonas* spp. This bacteria was not inhibited by WPI+OR treatment. On the other hand, a slight reduction in counts (0.5 log

cfu/g) was observed in WPI-THY-3 samples at day 8 of storage. At the end of the storage period, final counts were > 7.5 log cfu/g, showing that *Pseudomonas* spp. was the dominant spoiler of hake fillets, with counts almost similar to the TVC or psychrotrophic bacteria. In previous experiments done in our laboratory testing *in vitro* the antimicrobial activity of WPI films enriched with oregano and thyme EOs (results not shown) the high resistance of *Pseudomonas* spp. had been observed. It could be attributed to the high tolerance of this Gram-negative bacteria to the action of EOs (Castilho et al., 2012; Fernández-Pan et al., 2012).

Mastromatteo et al. (2010) tested sodium alginate films incorporated with thymol (one of the main constituents of oregano and thyme EO) on peeled shrimps and found, similar to our results, slight but significant reductions in counts of TV and psychrotrophic bacteria when using 1000 ppm of the compound. No reductions were found with coatings incorporated 500 ppm of thymol. Similar results have also been reported by Giatrakou et al. (2008) with slight reductions in counts of TVC, *Pseudomonas* spp., H_2S-producing bacteria, LAB and high reductions in Enterobacteriaceae when treated fresh swordfish with oregano EO. In a similar experiment, Zinoviadou et al. (2009) treated fresh beef with WPI coatings incorporated with oregano EO and also found significant reductions in counts of TV and LAB during refrigerated storage.

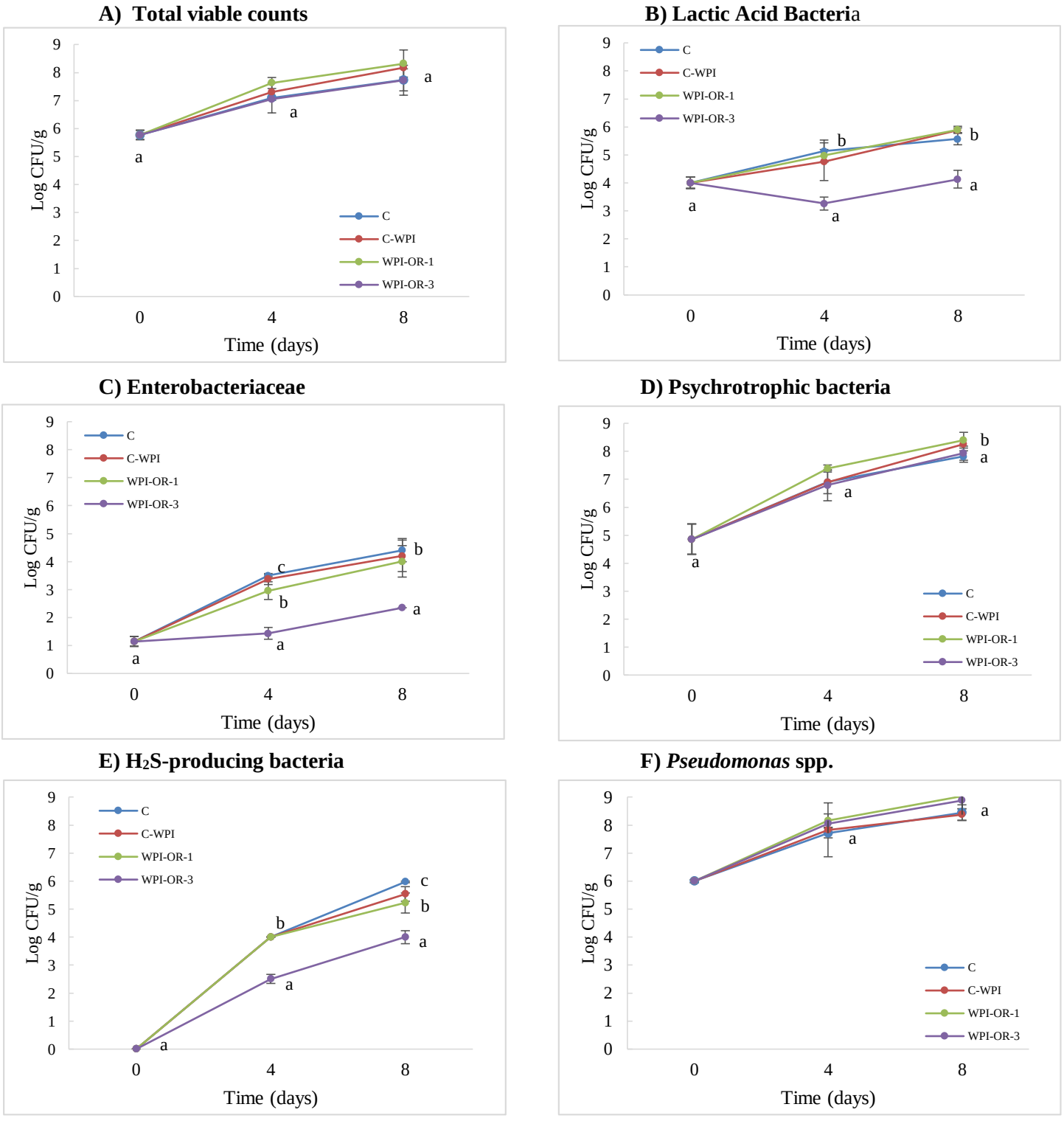

Figure 4.2 (Experiment 1): Evolution of the natural microbiota of WPI-OR coated hake fillets stored under air conditions. C: control; C-WPI: control-WPI; WPI-OR-1: WPI+OR 1 %; WPI-OR-3: WPI-OR 3 %. Different letters mean differences among treatments in the same day and bacterial group ($p < 0.05$)

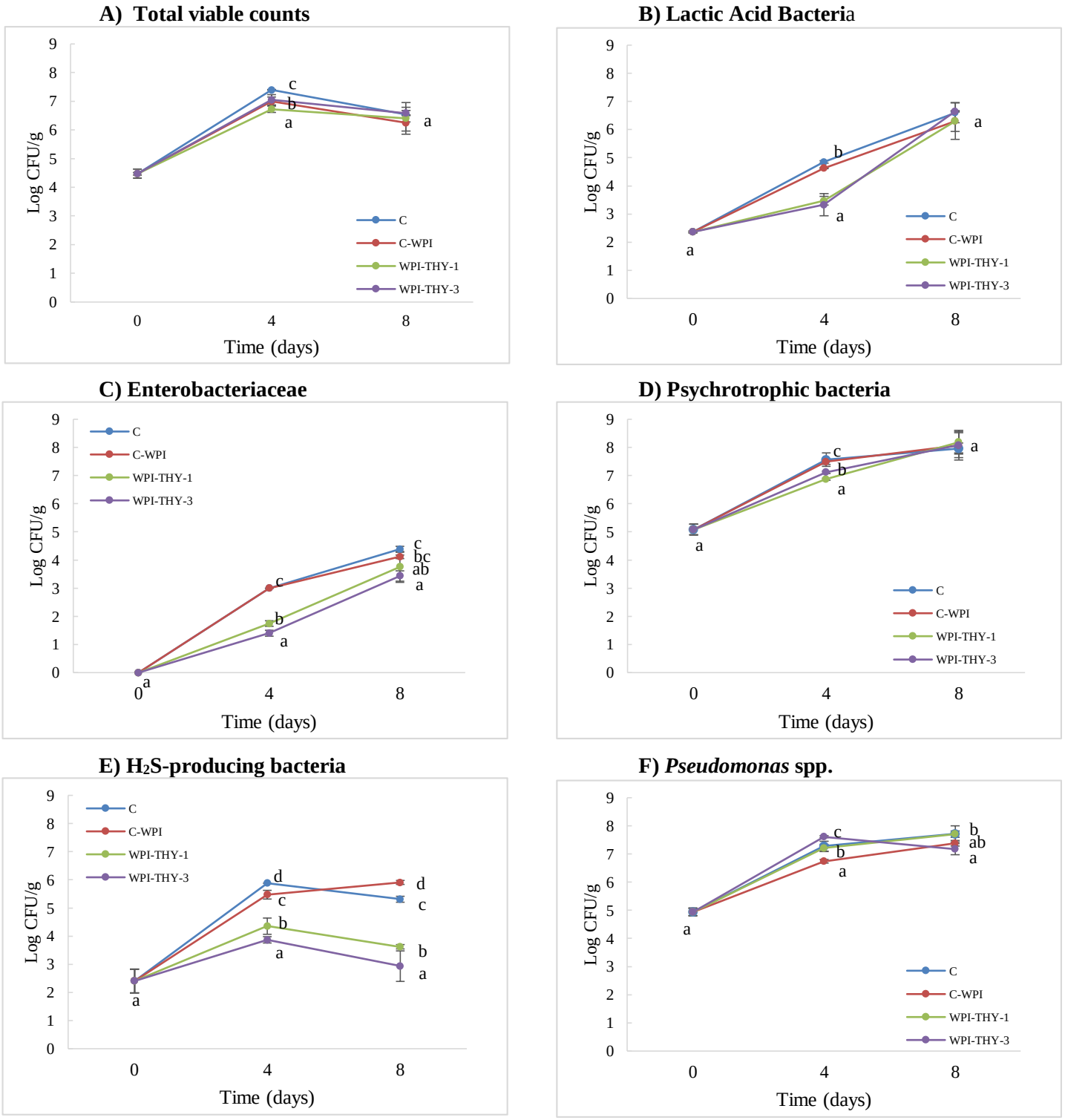

Figure 4.3 (Experiment 1): Evolution of the natural microbiota of WPI-THY coated hake fillets stored under air conditions. C: control; C-WPI: control-WPI; WPI-THY-1: WPI+THY 1 %; WPI-THY-3: WPI-THY 3 %. Different letters mean differences among treatments in the same day and bacterial group ($p < 0.05$)

4.4.2 Microbiological changes in samples packed under MAP conditions (Experiment 2)

In order to verify the effectiveness of WPI coatings enriched with EOs over the microbial quality of fresh hake fillets packed under MAP conditions, the following samples were prepared:

CONTROL (C-MAP: no coating + MAP)

Control WPI + MAP (C-WPI-MAP)

WPI-OR 1 % + MAP (WPI-OR-1-MAP)

WPI-OR 3 % + MAP (WPI-OR-3-MAP)

WPI-THY 1 % + MAP (WPI-THY-1-MAP)

WPI-THY 3 % + MAP (WPI-THY-3-MAP)

The evolution of TVC during the storage period for all treated samples and stored under MAP conditions is shown in Fig 4.4A and 4.5A. Initial counts were lower than the previous experiment (around 4 log cfu/g) which demonstrated a better quality of raw material (Albertos et al., 2015; Pyrgotou et al., 2010). None of the samples examined reached the recommended value for TVC of 7 log cfu/g. No significant differences were observed between any of the investigated coatings ($p < 0.05$) with respect to control samples at the end of storage period, even though significant differences were observed at days 4 and 8 of storage, when reductions between 0.8 and 2 log cfu/g were found for WPI-OR-3-MAP and WPI-THY-3-MAP respectively. Under this packaging condition, lower counts were observed compared to the values obtained for air packaged samples. This could be related either with the action of CO_2 as bacteriostatic agent or with lower initial counts in hake samples.

Counts of LAB are shown Fig. 4.4B and 4.5B. No reductions were found at day 16 of storage period, but significant reductions were observed during the 12 first days of storage. For example > 2.5 log cfu/g reductions were observed in WPI-OR-3-MAP

samples at day 8 of storage. Higher reductions were observed in WPI-OR-MAP samples when compared with WPI-THY-MAP samples in which in some cases (day 12 and 16 of storage) even not reductions were observed.

Enterobacteriaceae were successfully inhibited by all treatments (except WPI-OR-1-MAP) with significant reductions of 2 and 1 log cfu/g for WPI-OR-3-MAP and WPI-THY-3-MAP respectively at the end of the storage period. Slower growth of population of these microorganisms was observed during the 16 days of storage (Fig. 4.4C and 4.5C). Even a decrease in counts in WPI-THY-1-MAP and WPI-THY-3-MAP samples was observed, indicating a clear inhibition of this bacterial group.

The same behavior as previous microorganisms was observed for psychrotrophic bacteria. No reductions in counts at day 16 were found for all treatments except for WPI-OR-3-MAP in which 1 log cfu/g reductions was observed. Significant differences in counts ($p < 0.05$) were observed during the storage period when comparing with the control samples, with reductions of 1.5 to 2.5 log cfu/g for both EOs (Fig. 4.4D and 4.5D).

H_2-S producing bacteria were also affected by the coating treatments. In this case, reductions of 1 and 2 log cfu/g were found at day 16 (WPI-OR-3-MAP and WPI-THY-3-MAP respectively). Also during the whole storage period, significant and similar reductions were observed for the aforementioned treatments (Fig. 4.4E and 4.5E).

In the case of *Pseudomonas* spp. the final population was slightly reduced for WPI-OR-1-MAP and WPI-THY-1-MAP, while the treatments effectively decreased the counts at days 4, 8 and 12 of storage, with reductions ranging from 1 to 2 log cfu/g. Final counts of *Pseudomonas* spp. were < 5.5 log cfu/g, which are very low values compared with previous studies (and also with Exp. 1), in which counts used reached values higher than 7 or 8 log cfu/g (Fernández-Saiz et al., 2013; Pantazi et al., 2008).

Similar to our results, reductions in TVC, H_2-S producing bacteria and *Pseudomonas* spp. have been reported by Kykkidou et al. (2009) who treated swordfish fillets with 0.1 % thyme EO and stored the samples under MAP conditions. A combination of oregano EO and MAP was also tested by Pyrgotou et al. (2010) on the shelf-life of rainbow trout fillets for a period of 21 days. Significant reductions were found in TVC, *Pseudomonas* spp., H_2-S producing bacteria, LAB and Enterobacteriaceae resulting in a shelf-life extension of 8 days.

For all the microorganisms tested a synergistic effect could be observed until the day 12 of storage. Samples treated with WPI+EOs+MAP were significantly different when compared with control samples (just MAP packed) and sample treated just with WPI coating without the presence of EOs+MAP. In all the cases higher reductions were observed in samples packaged under MAP conditions and coated. Furthermore, the effectiveness of the treatments is clearly dependent of the microbial group analyzed. Although in some cases the counts for all the treatments were similar after 16 days of storage and no differences were detected, the microorganisms were effectively inhibited within the first 8 or 12 days of storage. This could be because a) the EOs included in the coatings lost activity during the storage period due to evaporation from the coating; b) the microorganisms were higher in number and the EOs were not able to exert their mode of action; or, c) the amount of CO_2 remained in the packaged could be lower than the initial value, then it was not enough to act as a preservative.

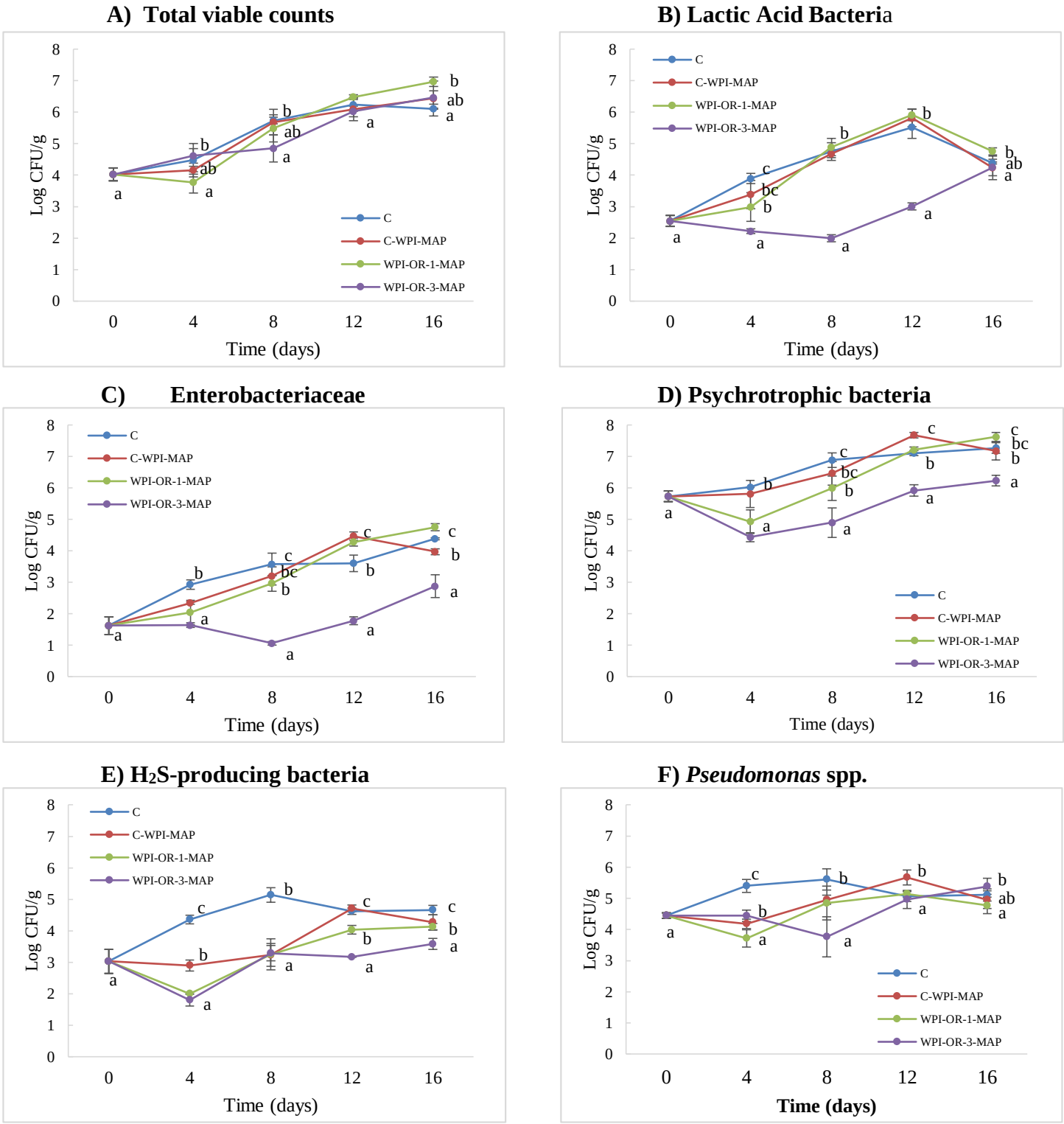

Figure 4.4 (Experiment 2): Evolution of the natural microbiota of WPI-OR coated hake fillets stored under MAP conditions. C: control; C-WPI-MAP: control-WPI; WPI-OR-1-MAP: WPI-OR 1 %+MAP; WPI-OR-3-MAP: WPI-OR 3 %+MAP.
Different letters mean differences among treatments in the same day and bacterial group ($p < 0.05$)

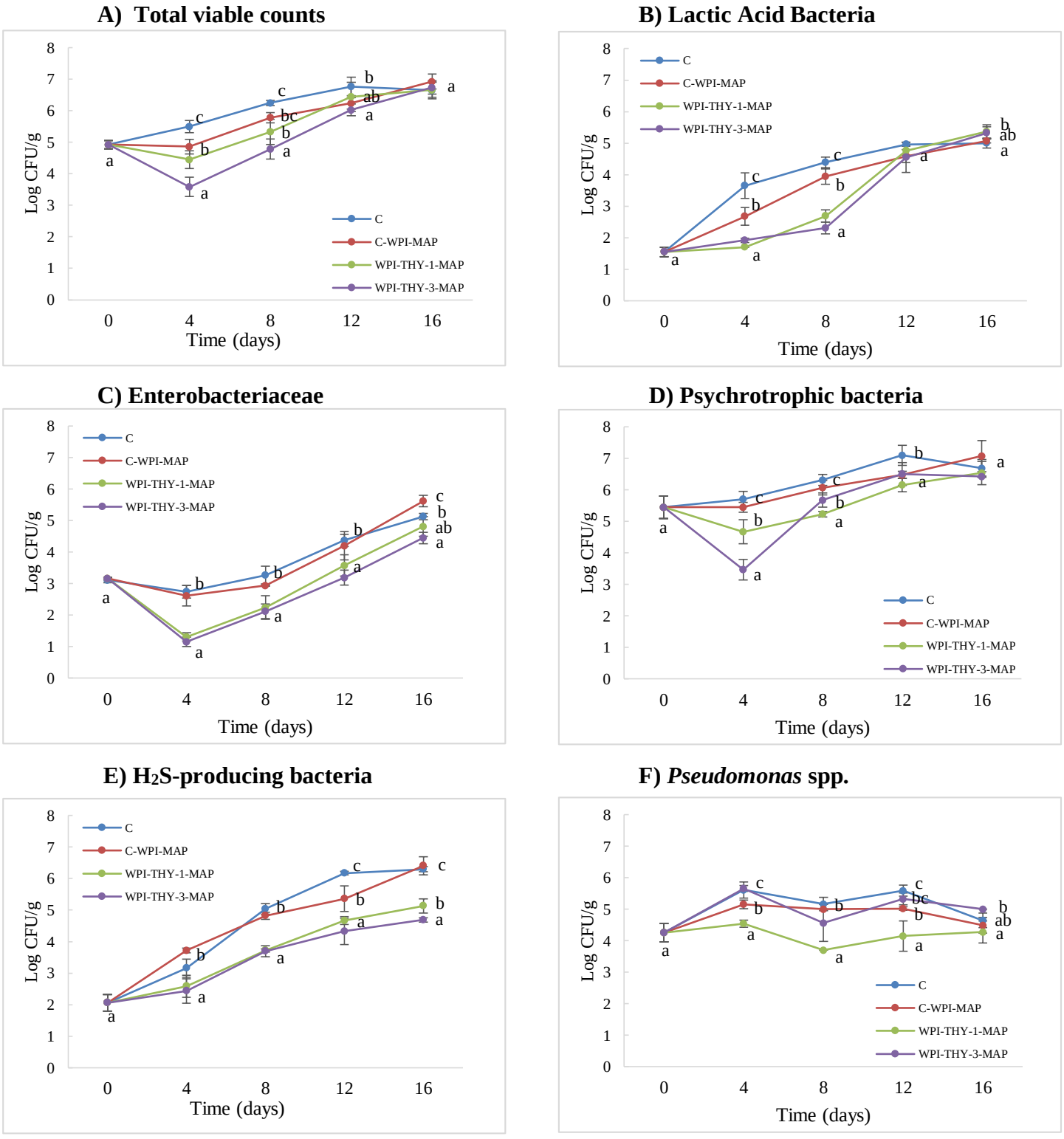

Figure 4.5 (Experiment 2): Evolution of the natural microbiota of WPI-THY coated hake fillets stored under MAP conditions. C: control; C-WPI-MAP: control-WPI; WPI-THY-1-MAP: WPI-THY 1 %+MAP; WPI-THY-3-MAP: WPI-THY 3 %+MAP.
Different letters mean differences among treatments in the same day and bacterial group ($p < 0.05$)

4.4.3 Microbiological changes in samples with low initial microbial load (Experiment 3)

Comparing the initial counts of fresh hake fillets for Exp. 1 and Exp. 2, differences can be observed. TV and psychrotrophic counts in Exp. 1 are, respectively, 1.5 and 1 log cfu/g higher than in Exp. 2. Besides, when comparing final counts (in samples stored under air) for the same bacterial groups at day 8 of storage, differences in counts remained as high as the initial one. Then, the poor effectiveness of WPI coating treatments found in Exp. 1 could be attributed to the high initial microbial population. Therefore, in order to verify if the initial bacterial counts of samples could affect the effectiveness of WPI coatings, a new experiment was performed. 4 lots of new samples obtained from a new supplier (with low initial counts) were coated as before, having the following treatments:

CONTROL (C: no coating, no MAP)

Control-MAP (C-MAP: no coating + MAP)

WPI-OR 3 % (WPI-OR-3)

WPI-OR 3 % + MAP (WPI-OR-3-MAP)

Microbiological analyses were done at days 0, 4, 8 and 12 of the storage period. Besides, the possible, but still not clear, synergistic effect between WPI coatings and MAP, observed in the Exp. 2 was also verified.

Low microbial counts were found in day 0 of analysis (Fig. 4.6). Around 4 log cfu/g were registered for TVC and psychrotrophic bacteria. *Pseudomonas* spp. were around 3 log cfu/g. Enterobacteriaceae, LAB and H_2S-producing bacteria were undetectable (< 1 log cfu/g). Other authors have reported also low counts at the beginning of the experiment, showing better and acceptable microbiological quality (Fernández-Saiz et al., 2013; Ordóñez et al., 2000)

Changes in TVC of treated hake fillet samples during storage are shown in Fig. 4.6A. Significant differences ($p < 0.05$) were found in the counts of all treated samples.

C, C-MAP and WPI-OR-3 samples exceeded the threshold recommended for commercialization of fresh marine species (ICMSF, 2002) at days 8 and 12 of storage, respectively. On the other hand, WPI-OR-3-MAP samples did not reach the recommended value at any point of the 12 days of storage (final counts of 5.64 log cfu/g). This results indicated that the combination of coating and MAP technology were much more effective together. When WPI+OR coating was used in combination with MAP, a significant inhibition of TV counts was observed, with > 3 log cfu/g reduction at day 12 of storage. Similar results have been reported in previous works (Fernández-Segovia et al., 2007; Giatrakou et al., 2008; Gómez-Estaca et al., 2010). Despite the final values of C-MAP and WPI-OR-3 samples exceeded the threshold, significant reductions were found during the whole storage period, having higher reductions in C-MAP samples compared with WPI-OR-3 samples. From Fig. 6A, an extension of the lag phase could be observed in all samples except the control. Counts of C samples increased steadily from day 4 whilst the TVC growth rate was lower in the other cases.

LAB are part of natural microbiota of hake fillets and as facultative anaerobic bacteria can grow under high concentration of CO_2, thus constitute an important part of the microbiota of fresh fish packed under MAP conditions. In the current study the initial LAB counts were not detectable (Fig. 4.6B) and at the end of the storage period counts reached 6 log cfu/g for C samples. Significant differences were observed when compared treatments ($p < 0.05$). At day 12, C-MAP treatment was unable to inhibit the growth of LAB while WPI-OR-3 decreased the counts in 1 log cfu/g compared with C. The highest inhibition was observed in WPI-OR-3-MAP samples with 2.5 log cfu/g counts lower than the control samples. An extension of the lag phase it is also observed in Fig 4.6B. The same figure also showed that LAB growth rate in WPI-OR-3-MAP samples during the storage was lower than the other samples. Gram-positive microorganisms like LAB are

generally more resistant to frozen storage, salt, low water activity and high carbon dioxide levels than Gram-negative microorganisms (Dalgaard, 2003). On the other hand, it is well known that these group of microorganisms are sensitive to the action of EOs (Chorianopoulos et al., 2004; Fernández-Pan et al., 2012; Longaray Delamare et al., 2007). For these reasons, a higher and a synergistic effect of the combined use of EO incorporated in edible films and MAP conditions are expected.

Enterobacteriaceae were undetectable at day 0 and 4 of analysis (Fig. 4.6C) indicating a good hygiene and acceptable handling practices of the hake fillets. WPI-OR-3 and WPI-OR-3-MAP treatments had a significant effect on the samples since completely inhibition of this bacteria was observed through the experiment. A reduction of around 1 log cfu/g was observed in the case of C-MAP samples when compared with the final counts of C samples. The population of Enterobacteriaceae was lower compared with other microorganisms groups analyzed in this study; however the contribution of this bacteria to the final microbiological quality has to be taken into account.

There is a clear and significant effect of WPI-OR coatings alone or in combination with MAP on the growth of LAB. Similar results were found by Gómez-Estaca et al. (2010) who treated cod fillets with chitosan-clove films and found total inhibition of this bacteria at the end of the storage period. Pantazi et al. (2008) also found reductions in Enterobacteriaceae counts when analyzing fresh swordfish packed under different packaging conditions. MAP samples had lower counts compared with samples stored under air conditions.

With a similar behavior as TVC, significant differences ($p < 0.05$) among samples were shown for Psychrotrophic bacteria counts. Reductions of 3, 1.5 and 0.5 log cfu/g were observed for WPI-OR-3-MAP, WPI-OR-3 and C-MAP samples respectively (comparing with C), having the higher inhibitory effect, once again, in samples coated

and packaged under MAP conditions (Fig. 4.6D). Similar results were found by López de Lacey et al. (2014) who showed that agar films containing green tea extract delayed the growth of psychrotrophic bacteria, including *Pseudomonas* spp. and H_2-S microorganisms. In their study Mastromatteo et al. (2010) also found differences in psychrotrophic bacteria counts when comparing peeled shrimp treated with alginate coatings enriched with thymol and packed under MAP and stored under air conditions.

H_2-S producing bacteria including *S. putrefaciens* like bacteria are normally used as a good spoilage indicator of fish and seafood products (Gram and Huss, 1996). At the beginning of the experiment H_2-S producing bacteria counts were not detected but later exponential growth was observed in all samples. Counts for WPI-OR-3-MAP, C-MAP and WPI-OR-3 samples were significantly ($p < 0.05$) lower than for C samples during the whole storage period (Fig. 4.6E). Differences in counts of 3, 2.5 and 2 log cfu/g, respectively, were detected at day 12, when compared with control samples. Similarly Boskou and Debevere (1998) reported that *S. putrefaciens* was unable to develop when high concentration of CO_2 (> 50 %) was applied on cod fillets. Ravn Jørgensen et al. (1988) concluded that the growth of *S. putrefaciens* is inversely related to the shelf-life of iced cod, meaning higher counts shorter shelf-life. In addition Gram et al. (1987) reported that counts of *S. putrefaciens* higher than 6 log cfu/g start producing sulfur compounds and spoilage of fish takes place. In our case, lower values of H_2-S producing bacteria (4 log cfu/g) were observed for WPI-OR-3-MAP samples and are related with the lower values obtained for TVC (5.64 log cfu/g) and psychrotrophic bacteria (5.80 log cfu/g), confirming the shelf-life extension of hake fillets. Final counts of 7.25 log cfu/g for H_2-S producing bacteria in C samples were registered.

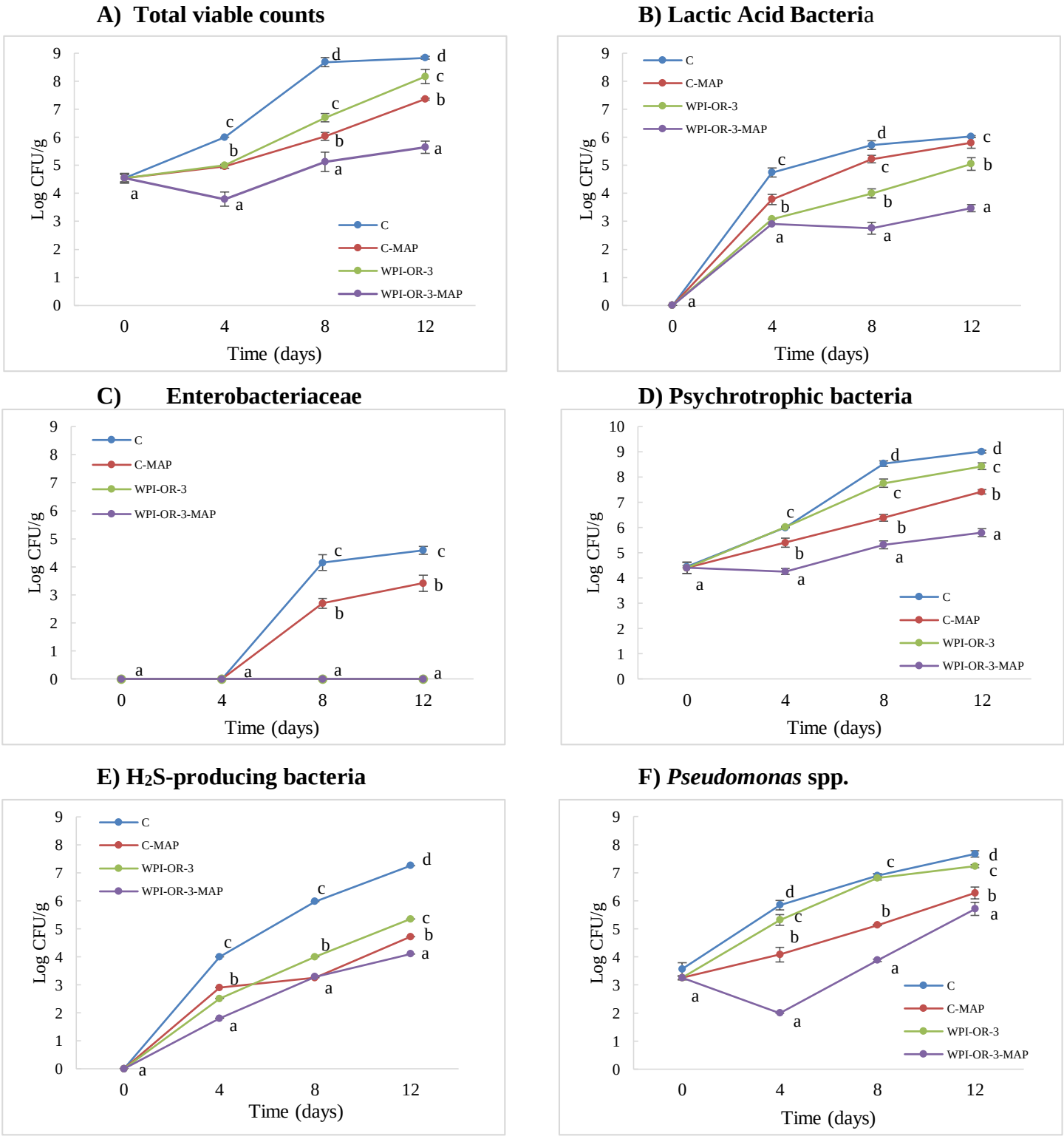

Figure 4.6 (Experiment 3) Evolution of the natural microbiota of WPI-OR coated hake fillets stored under air and MAP conditions. C: control; C-MAP: control+MAP; WPI-OR-3: WPI+OR 3 %; WPI-OR-3-MAP: WPI-OR 3 %+MAP.
Different letters mean differences among treatments in the same day and bacterial group ($p < 0.05$)

Pseudomonas spp. together with *Shewanella* spp. are common spoilers of chilled fish and seafood products packed under aerobic conditions (Dalgaard, 2003). They have also been reported as specific spoilage microorganisms or fish from temperate water (Gram and Huss, 1996). WPI-OR-3-MAP samples presented the lowest counts (5.71 log cfu/g) when compared with the other treatments and C samples. Fig. 4.6F shows the evolution of *Pseudomonas* spp. during the storage period. At the end of the storage time the final counts reached 7.67 log cfu/g for C samples. *Pseudomonas* spp. presented at the end of the storage period higher counts for all samples than H_2-S producing bacteria or other bacteria group, therefore constituting the major flora of hake fillets. Similar results were found in previous experiments with different fish species like cod (Gómez-Estaca et al., 2010), swordfish (Pantazi et al., 2008) or sole (Fernández-Saiz et al., 2013) where *Pseudomonas* spp. were also the predominant bacteria.

According to Spanish law (RD 135/2010 B.O.E. 25/02/2010) the limit for commercialization of fresh fish is 7 log cfu/g for TVC and 4 log cfu/g for Enterobacteriaceae. Comparing these two data for control and treated samples, a shelf-life extension of 4 days for C-MAP samples and > 4 days for WPI-OR-3-MAP hake samples were achieved, attributed to the combination of the antimicrobial effect exerted by the WPI-oregano EO coating and the MAP conditions. Besides, the effect of low initial counts was also observed, being a key parameter for the potential application of edible coatings.

4.5 Conclusions

In the Exp. 1, when samples with high initial bacteria load were used, WPI coatings incorporated with oregano and thyme EOs at different concentrations did not affect the microbial growth of most of the microorganisms developed in fresh hake fillets packed under air and stored for 8 days at refrigeration conditions. Just Enterobacteriaceae and H_2-S producing bacteria were inhibited for these treatments. In Exp. 2 when active coatings were combined with MAP conditions (but still high initial counts in samples), same bacterial group as before were inhibited but higher antimicrobial effect was observed during the storage period. Then, an effect of MAP on the samples treated could be deduced.

Results of Exp. 3 showed that the combination of EO-enriched coatings with MAP and low initial microbial counts was effective in extending the shelf-life of fresh hake fillets. While in the Exp. 1 coatings did not extend the shelf-life of the samples, in experiment 3 (working with lower initial microbial population) the shelf-life of hake fillets coated with WPI-OR-3 and stored under air was extended in around 4 days. The shelf-life of samples treated with WPI+OR 3 % +MAP was doubled compared with the control samples (from the microbiological point of view). The growth of all microorganisms analyzed was delayed, especially H_2-S producing bacteria, considered as one of the main spoilers of fresh fish. The antimicrobial effectiveness was clearly dependent of the initial microbial population, the concentration of EO, the presence of MAP and the bacteria genera.

The synergistic effect of this combined strategy was observed. WPI coating could act as barrier against oxygen availability on the fillet surface, CO_2 exerted its known inhibitory effect and oregano and thyme could decrease the growth rate of bacteria by

disrupting the membrane cell. Applying both, EO, incorporated into WPI coatings and high concentration of CO_2 were more effective on maintaining the quality of hake fillets than applying only WPI-EO or MAP. Such effect was characterized by lower microbial counts and extension of the lag phase.

Summarizing, the application of WPI-enriched films combined with MAP conditions delayed the growth of microorganism in hake fillets; therefore the shelf-life was extended. The low initial microbial load of samples seems a key parameter to take into consideration. The combination of WPI edible coatings with oregano and thyme EO and a packaging atmosphere rich in CO_2 could be a valid alternative to preserve fish and fish products.

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CHAPTER 5. IMPROVEMENT OF THE MICROBIOLOGICAL QUALITY OF READY-TO-EAT PEELED SHRIMPS BY THE USE OF CHITOSAN COATINGS

CARRIÓN-GRANDA, X., FERNÁNDEZ-PAN, I., JAIME, I., ROVIRA, J. and MATÉ, J.I. (2016). Improvement of the microbiological quality of ready-to-eat peeled shrimps by the use of chitosan coatings

Partially presented in:

23rd International ICFMH Symposium, Food Micro 2012. Global Issues in Food Microbiology. Istambul, Turquey. 2012.

Submitted to the International Journal of Food Microbiology

CHAPTER 5. IMPROVEMENT OF THE MICROBIOLOGICAL QUALITY OF READY-TO-EAT PEELED SHRIMPS BY THE USE OF CHITOSAN COATINGS

5.1Abstract

Chitosan films incorporating oregano (*Origanum vulgaris*) and thyme (*Thymus zygis*) essential oils (EO) at three different concentrations were developed and their antimicrobial activity was tested against 8 different common pathogens and spoilers of fish and seafood products. Films containing oregano EO were more effective in inhibiting the growth of the bacteria tested showing the higher inhibition areas. Such activity was related to the strain tested and the concentration used. Then, chitosan coatings incorporated with 0.5 % of oregano and thyme EO were applied onto ready-to-eat peeled shrimp tails and packed under MAP conditions. The growth of naturally present spoilage microorganisms was evaluated during chilled storage (4 ºC). Coatings containing thyme EO were more effective in inhibiting the microbial species studied, specially lactic acid and psychrotrophic bacteria. As carrier of EOs, chitosan was more effective in inhibiting the growth of bacteria present in peeled shrimps than the direct application of an O/W emulsion. On the other hand, results from sensory analysis showed that the sensorial quality was affected by the chitosan-thyme coatings despite firmness and colour were kept. The present work demonstrates the effectiveness of chitosan enriched coatings, offering a promising alternative to control the growth of spoilage microorganisms on peeled shrimps during refrigeration storage.

5.2 Introduction

Shrimps are an excellent source of protein, non-protein nitrogen compounds (aminoacids), omega-3-fatty acids among other nutrients. However, this rich biological composition makes shrimps highly susceptible to microbiological spoilage, limiting their shelf-life (Gram and Dalgaard, 2002). During storage, the quality of seafood degrades due to a complex process, where biochemical, microbiological or physical changes occurs. Microbial activity is responsible for spoilage of most fresh and lightly preserved seafood. This process is dominated by slime production, loss of texture (soft) and colour and odour changes due to production of volatile metabolites of non-protein nitrogenous compounds (from decay and putrefaction) (Ray, 2004).

There has been an increasing interest in extending the shelf-life of shrimps due to a growing demand for fresh, less preserved and ready-to-eat (RTE) foods. Seafood is commercialised mainly under refrigeration or freezing conditions, but these technologies are not effective enough in retarding the microbial deterioration of seafood. Then, prevention of spoilage is an important challenge for the food industry.

Nowadays, there is a growing interest in the use of edible films and coatings with antimicrobial properties, which help improving the shelf-life of food products. Chitosan is a natural polymer obtained by deacetylation of chitin, the main component of the crustaceans' shells and the second most abundant polysaccharide found in nature. Chitosan has received a significant attention as antimicrobial agent due to its biodegradability, biocompatibility, cytotoxicity and antimicrobial activity (Dutta et al., 2009). Besides, chitosan possess cationic properties allowing the slow release of active components incorporated in it (Lacroix and Le Tien, 2005). Several studies have proven the antimicrobial activity of chitosan in different food products like minimally processed

fruits and vegetables (Devlieghere et al., 2004), cheese (Di Pierro et al., 2011), meat products (Beverlya et al., 2008), fish (Jeon et al., 2002).

In order to increase the antimicrobial efficiency of edible films and coatings, the inclusion of different preservative substances (natural or synthetic) into the films are an excellent option. In the last decades, several investigations have focused on the use of natural ingredients, like essential oils (EOs), for fish and seafood preservation. Antioxidant and antibacterial properties, among others, have been attributed to different essential oils (Del Nobile et al., 2009; Yano et al., 2006). Oregano and thyme EOs have been extensively tested *in vitro* as antimicrobial substances and effectively inhibited the growth of several bacteria (Castilho et al., 2012; Dorman and Deans, 2000; Fabio et al., 2003). The antimicrobial activity of both, oregano and thyme EOs, is mainly related with the content of phenolic compounds (Friedman et al., 2002). The mode of action of phenolic compounds and in consequence of essential oils is associated to the ability to damage and disintegrate the outer membrane. (Lambert et al., 2001).

Modified atmosphere packaging (MAP) is not a novel technology, it has been widely studied and used successfully in combination with refrigeration for the shelf-life extension of fish and seafood products (Dalgaard et al., 1993; Mejlholm et al., 2005). MAP inhibits the normal spoilage of fish and seafood products by the action of different gasses like CO_2, N_2 and O_2 used individually or in combination (Arvanitoyannis et al., 2011; Sivertsvik et al., 2002) depending of the type of product, temperature and packaging system to be used.

Taking into account that antimicrobial edible films are a novel technology and can be used in combination with MAP to help preservation of seafood products, the aim of this work was to determine the effect of chitosan-essential oil enriched coatings on the shelf-life of ready-to-eat peeled shrimp tails by monitoring microbiological changes

during storage at 4 ºC. To the best of our knowledge, little work has been done in preservation of peeled shrimp tails using chitosan coatings.

5.3 Materials and Methods

5.3.1 Essential oils

Food grade essential oils (EOs) of oregano (*Origanum vulgaris*) and thyme (*Thymus zygis*) were provided by Esencias Martínez Lozano (Murcia-Spain).

5.3.2 Bacteria strains

Eight different bacterial strains responsible for the spoilage and pathogenicity of shrimps were used to evaluate the antimicrobial activity of chitosan films (Table 5.1). All strains were supplied by the Spanish Type Culture Collection (CECT, Universidad de Valencia-Spain). After recuperation, bacterial strains were kept either under freezing storage in Eppendorf tubes containing a mix of Brain Heart Infusion broth with 30 % (v/v) of glycerol or in porous beads at -80 ºC.

Table 5.1: Bacteria strains tested and growth conditions

No. CECT	BACTERIA	Gram	Culture medium	Incubation Temp (ºC)
481	*Enterococcus faecalis*	+	Brain Heart Infusion	37
	Lactobacillus sakei	+	De Man Rogosa and Sharp Agar	30
232	*Staphylococcus epidermidis*	+	Nutrient Broth II	37
5173	*Aeromonas hydrophila*	-	Tripticase Soy Agar	30
378	*Pseudomonas fluorescens*	-	Nutrient Broth II	26
446	*Pseudomonas fragi*	-	Nutrient Broth II	26
5346	*Shewanella putrefaciens*	-	Nutrient Broth II	26
521	*Vibrio alginolyticus*	-	Nutrient Broth I + 3%NaCl	26

CECT: Spanish Type Culture Collection

5.3.3 Film forming solution preparation

The method developed by Gómez-Estaca et al. (2011) with slightly modifications was used to obtain the chitosan film forming solutions (FFS). As so, to prepare 100 ml of FFS, 3 g of chitosan (95 % deacetylation degree; Guinama, Valencia-Spain) was dissolved in a solution of acetic acid 0.15 M (Chromanorm, Barcelona-Spain). The mixture was stirred at 45 °C for 2 hours to obtain a homogeneous blend. After, keeping the same conditions for 5 more minutes, 0.9 g of glycerol (Panreac, Barcelona-Spain) and 0.15 g of Tween 80 (Scharlab, Barcelona-Spain) were added as plasticizer and emulsifying agents, respectively. Once cooled at room temperature, the essential oils of oregano and thyme were incorporated to the FFS at concentrations of 0.5, 1 and 2 % (w/w). The FFS were homogenized using an Ultraturrax blender (T-25 basic, IKA-WERKE, Germany) during 5 minutes at high speed. After all the process, FFS were kept overnight at room temperature. In order to obtain the films, 30 g of every FFS were poured onto plastic dishes (14 cm diameter) and dried for 5 h at 45 °C in a forced-air oven. Prior to analyses, the films were conditioned in a desiccator at 75 % RH during 48 h. Different resulting films were CH-OR 0.5, CH-OR 1, CH-OR 2, CH-THY 0.5 and CH-THY 1. Films with 2 % of thyme EO were not developed because the film forming solution was not stable during the time.

5.3.4 Antimicrobial activity of chitosan films incorporated with EOs

The disc diffusion method (NCCLS, 2003) was used to evaluate the antibacterial activity of films against the selected strains. Discs of 13 mm diameter of every film formulation were cut using a punch and placed onto agar plates inoculated with an inoculum of 10^7 cfu/ml of each strain. Plates were incubated under the conditions showed in Table 5.1 for 24 hours. Diameters of inhibition halos were measured using a calliper

and expressed as inhibition areas in mm^2 excluding the disc diameter. All tests were performed in triplicate.

5.3.5 Shrimp samples preparation

Cooked and peeled shrimp tails were provided by a local producer and transported to the laboratory in a cooler. Under sterile conditions the shrimps were randomly separated in 4 groups: the first one without any treatment used as control (CONTROL), the second one coated with chitosan (CONTROL-CH), the third and fourth one coated with chitosan with oregano or thyme at 0.5 % (CH-OR and CH-THY). For coating, every piece was dipped in 200 ml of FFS for 1 minute, after the FFS excess was allowed to drip off for 2 minutes and finally pieces were dried for 30 minutes under air stream. Both, control and coated samples were packaged in polystyrene trays, sealed with PET/PVdC/PE film with modified atmosphere (MAP) (40 % CO_2/60 % N_2) and stored at 4 °C during 12 days. The specific gas mixture was provided by the shrimp supplier. The gas/shrimp ratio was 3/1 in the final packed product.

In order to analyse the effectiveness of the chitosan as structural matrix and carrier of EOs, the same concentration of EOs was tested but in a form of an oil/water (O/W) emulsion. So, 0.7 g of tween 80 was added to warm distilled water (40 ºC) and mixed for 2 minutes at 6000 rpm using an overhead stirrer (RZR 1 Heidolph, Germany). After, 0.5 g of soy lecithin were added and mixed for 4 minutes at the same speed. Finally 0.5 % of oregano or thyme EO (depending on the case) was added and mixed for 4 more minutes at 6000 rpm. The application of the emulsion on the shrimp tails was done simulating the procedure previously described for coating application. Samples were packed and stored under the same conditions as for the samples treated with chitosan coatings. Final samples were CONTROL, OR-0.5 and THY-0.5.

5.3.6 Microbiological analysis

25 g of sample was aseptically weighed, placed in a sterile plastic bag (BagPage, Interscience-France) and homogenized with 225 ml of Ringer water (Oxoid, Barcelona-Spain) using a stomacher (Stomacher 400, London-UK) for 2 minutes. Decimal dilutions were prepared as needed and seeded in the correspondent media in order to perform the following determinations: a) total viable counts (TVC) on pour plates of Plate Count Agar (PCA, Pronadisa, Madrid-Spain) incubated for 48 to 72 hours at 30 °C; b) lactic acid bacteria (LAB) on spread plates of de Man, Rogosa and Sharpe Agar (MRS, Oxoid, Barcelona-Spain) incubated for 72 hours at 30 °C in an oven with 6 % CO_2; c) Enterobacteriaceae on double-layered pour plates of Violet Red Bile Glucose (VRBG, Pronadisa, Madrid-Spain) incubated for 24 hours at 37 °C, and, d) total psychrotrophic bacteria on pour plates of Plate Count Agar (PCA, Pronadisa, Madrid-Spain) incubated for 7-10 days at 4 °C. Microbiological analyses were performed at days 0, 4, 8, 10 and 12 of the storage period. All plates were examined visually for typical colony types and morphological characteristics associated with each growth medium. All microbiological results were expressed as the log of the colony forming units (cfu) per gram of sample. All analyses were done in triplicate. Three packs from each sub-batch were analysed at the set day. Besides, two replicates of at least three appropriate dilutions were enumerated.

5.3.7 Intralaboratory sensory test

Samples treated with CH-THY were evaluated by five trained panellists on every day of sampling. Every sample was coded and given to the panellists in a random order. Judges were asked to rate intensity of colour, brightness, characteristic odour, unusual odour, characteristic taste, unusual taste, firmness and juiciness. All evaluation was done

using a 1-5 scale: 1, absence of such parameter and 5, maximum intensity. Sensory evaluation was carried out in individual booths under known conditions of relative humidity, temperature and light.

5.3.8 Statistical analysis

All tests were performed in triplicate. Statistical analyses were conducted using SPSS 21.0 (IBM, USA) software. Significant differences among extracts were determined using ANOVA and Duncan's multiple range post hoc test (confidence level of 95 %).

5.4 Results and discussion

5.4.1Antimicrobial activity of chitosan films incorporated with EOs

The results of the antimicrobial activity of chitosan films incorporated with oregano and thyme EOs at three different concentrations are shown in Table 5.2 The target bacteria used for this study are related with the spoilage and pathogenicity of fish and seafood products and in particular with shrimp (Dalgaard, 2003; Nychas and Drosinos, 2009).

Control films of chitosan did not show inhibitory effect against any of the studied bacteria. On the other hand, when oregano and thyme EOs were included into films, different inhibition areas were observed. Films enriched with oregano EO showed in the most of the cases higher inhibitory areas than films with thyme EO. The inhibitory effect of Ch-OR films against the microorganisms tested is expressed in the following order: *A. hydrophila* > *S. putrefaciens* > *V. alginolyticus* > *S. epidermidis* > *E. faecalis* > *P. fragi* > *L. sakei*. For *A. hydrophila* and *S. putrefaciens* clear halos of inhibition were observed

even at 0.5 % concentration of EO. *P. fluorescens* was not inhibited for any of the films tested. Significant differences ($p < 0.05$) were found when comparing concentration of EO. As so, the higher the concentration, the greater the inhibition halo. Azevedo et al. (2014) estimated the effects of different parameters on the antimicrobial activity of edible chitosan coatings and concluded that essential oil concentration was the most significant parameter for the antimicrobial activity of coatings against *S. aureus* and *B. subtilis*.

As it was mentioned before higher inhibitory areas were observed for films enriched with OR EO for all bacteria and all three concentration tested, ranging for example from 101 to 1734 mm^2 (in the case of CH-OR 2 %). The antimicrobial activity of oregano and thyme EOs is related with the presence of carvacrol and thymol in their composition These substances are known to have the ability to disintegrate the outer membrane of bacteria, making the cell membrane permeable and allowing the action of EOs (Lambert et al., 2001). The higher antimicrobial activity of Ch-OR films could be related with the higher content of carvacrol than in thyme EO. According to the technical information provided by the essential oil supplier, oregano EO has 71.7 % of carvacrol compared to 2.96 % present in thyme EO. Some studies previously done have reported that carvacrol is associated with higher antimicrobial activity than thymol (Bagamboula et al., 2004; Friedman et al., 2002). Other reason for the difference in antibacterial activity could be related with the chemical structure of carvacrol. In general phenolic compounds, which are the responsible of the antimicrobial activity of EOs, have in their structure a hydroxyl group. The relative position of this group in carvacrol is such that its antimicrobial power is higher than the other phenolic compounds like thymol (Zinoviadou et al., 2009).

Table 5.2: Inhibitory areas (mm^2, excluding disc area) of CH-films against different bacteria strains

	Aeromonas hydrophila	*Shewanella putrefaciens*	*Vibrio alginolyticus*	*Pseudomonas fragi*	*Pseudomonas fluorescens*	*Lactobacillus sakei*	*Staphylococcus epidermidis*	*Enterococcus faecalis*
CH-OR 0.5	285 ± 43^{bE}	255 ± 33^{aD}	79 ± 13^{aC}	NA^{aA}	NA^{aA}	NA^{aA}	NA^{aA}	39 ± 9^{bB}
CH-OR 1.0	760 ± 86^{cF}	617 ± 61^{cE}	255 ± 33^{cD}	41 ± 6^{bAB}	NA^{aA}	NA^{aA}	128 ± 20^{bC}	91 ± 17^{cBC}
CH-OR 2,0	1734 ± 0^{eG}	1075 ± 0^{dF}	805 ± 82^{dE}	104 ± 17^{cB}	NA^{aA}	101 ± 10^{bB}	533 ± 86^{cD}	291 ± 29^{dC}
CH-THY 0.5	51 ± 10^{aB}	301 ± 57^{aC}	29 ± 8^{aAB}	NA^{aA}	NA^{aA}	NA^{aA}	NA^{aA}	NA^{aA}
CH-THY 1.0	805 ± 82^{dE}	443 ± 36^{bD}	160 ± 21^{bC}	NA^{aA}	NA^{aA}	NA^{aA}	51 ± 19^{aB}	9 ± 3^{aAB}

Letters a-c in the same column show significant differences among different CH-films. Letters A-G in the same row show significant differences among strains ($p < 0.05$)

Despite the fact that chitosan has been pointed out as antimicrobial substance (Fernandez-Saiz et al., 2009), in this work chitosan films did not show any clear halos of inhibition. This could be related with the lack of diffusivity of chitosan. According to Coma et al. (2002) chitosan does not have the ability to diffuse through the agar media. In the same line, Fernández-Pan et al. (2015) also concluded that chitosan films only inhibited the growth of bacteria by direct contact and not by diffusivity of chitosan.

A. hydrophila, *S. putrefaciens* and *V. alginolyticus* are, among others, the main microorganisms responsible for the spoilage of seafood products (Dalgaard, 2003) and were successfully inhibited by chitosan-enriched films. *A. hydrophila* showed the highest inhibitory areas for both oregano and thyme films at all concentrations. Fernández-Pan et al. (2015) tested chitosan films enriched with carvacrol, the main active compound of oregano EO, and they were effective in inhibiting the growth of *A. hydrophila* and *S. putrefaciens.* Similar to our results, *S. putrefaciens* was also inhibited by quince seed mucilage films enriched with oregano and thyme EO, showing the highest sensitivity among 9 tested strains (Jouki, Mortazavi, et al., 2014; Jouki, Yazdi, et al., 2014). On the other hand, Teixeira et al. (2014) included oregano, clove and garlic into fish protein films and tested against *S. putrefaciens*. The bacteria was not inhibited by fish protein + origanum films.

L. sakei was poorly inhibited by the tested films. The strain was just inhibited by chitosan film enriched with oregano EO at 2 % concentration. Such resistance could be related with: a) *L. sakei* possesses a cell envelope less permeable that the other bacteria or, b) the bacteria possess a resistance mechanism which allows it to inactivate EOs (Gill and Holley, 2004).

The formulated films were not effective in inhibiting the growth of *P. fluorescens*. In the case of *P. fragi*, just chitosan films enriched with 1 and 2 % of oregano EO inhibited

its growth and produced clear zones of inhibition. Films containing thyme EO did not inhibit the aforementioned bacteria. The resistance of *Pseudomonas* spp. to the action of essential oils and biopolymers enriched with EO has been studied before (Fernández-Pan et al., 2012; Gómez-Estaca et al., 2010). They are Gram-negative bacteria and they have cytoplasmic membrane plus an outer membrane which is known to restrict the entrance of hydrophobic compounds acting therefore as a barrier against hydrophobic antimicrobial substances like EOs (Cox and Markham, 2007; Emiroglu et al., 2010).

In general, literature attributed higher sensitivity to the action of antimicrobials to Gram-positive bacteria than Gram-negative ones (Azevedo et al., 2014; Burt, 2004; Pranoto et al., 2005). But our results showed a different trend. The three most sensitive bacteria to the action of the tested films were Gram-negative ones, followed by two Gram-positive. Same as our results, Mith et al. (2014) concluded that oregano EO was more active against Gram-negative than Gram-positive bacteria. On contrary to our results, Pranoto et al. (2005) found that Gram-positive bacteria were more sensitive to the action of chitosan films enriched with garlic EO than Gram-negative strains. The differences in results of the antimicrobial activity of films could be attributed to factors like stage of growth of the strain, genus, species and previous exposure to stress and injury of the strains (Davidson, 2000). Another study has concluded that EOs did not exert different antimicrobial activity regarding different Gram-genus (Viuda-Martos et al., 2008).

5.4.2 Application of chitosan-EO enriched coatings to RTE shrimps

The microbiota of ready to eat seafood products is dominated by Lactic acid bacteria, Enterobacteriaceae, *P. phosphoreum* and other marine *Vibrio* spp. (Dalgaard, 2003; Gram and Huss, 1996). The evolution of the natural microbiota (aerobic mesophilic bacteria, lactic acid bacteria, Enterobacteriaceae and psychrotrophic bacteria) of peeled

shrimp tails coated with chitosan-OR and chitosan-THY and packaged under MAP and stored at 4 ºC was registered and is shown in Fig. 5.1.

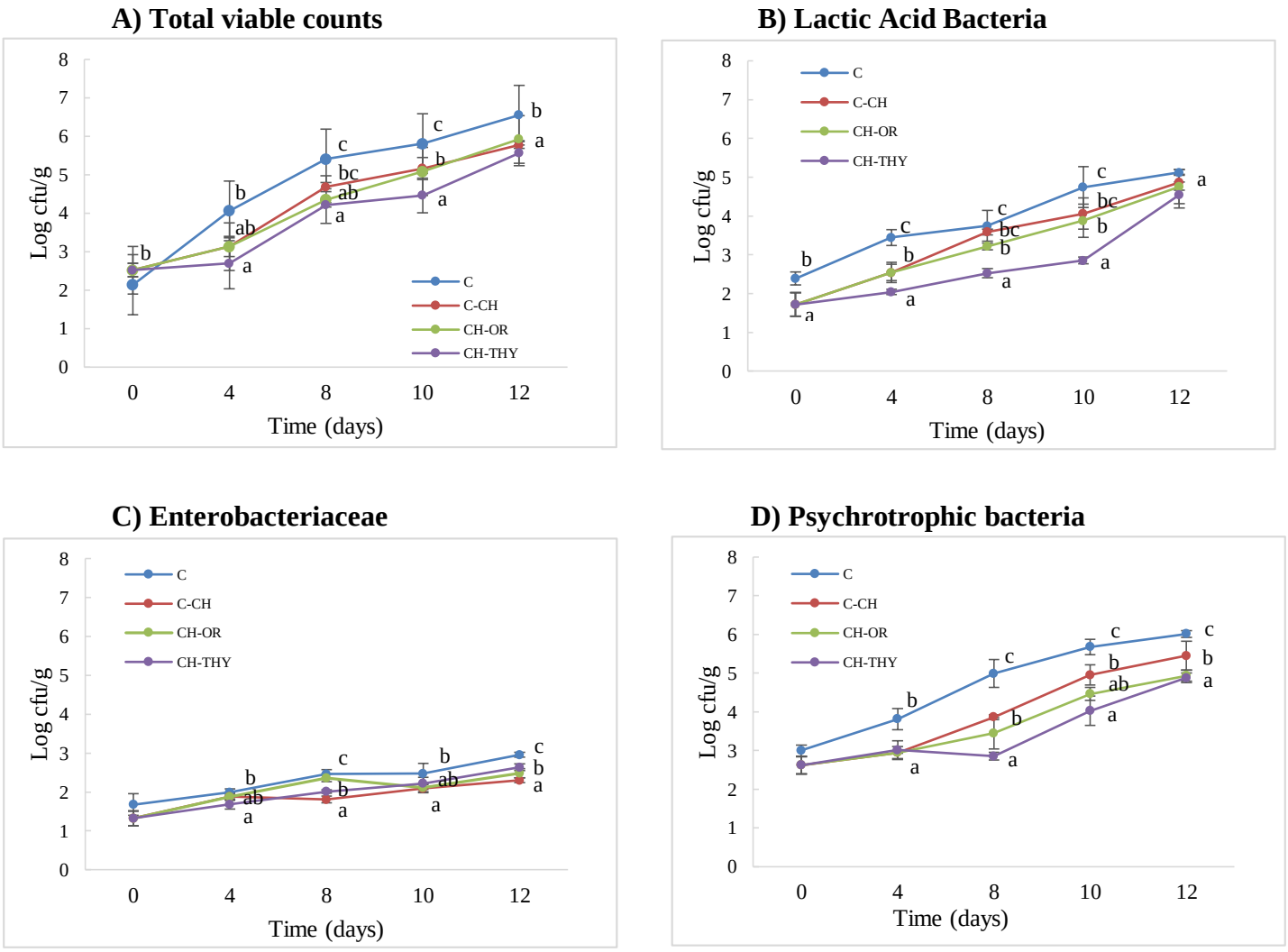

Figure 5.1: Evolution of the natural microbiota of RTE coated peeled shrimps stored at 4 ºC under MAP. C: Control, no coated; C-CH: CH coated; CH-OR: CH + OR 0.5 %; CH-THY: CH + THY 0.5 %
Different letters mean differences among treatments in the same day and bacterial group ($p < 0.05$)

The initial counts of total viable bacteria of shrimps was low (2.50 log cfu/g) implying acceptable quality and correct manipulation of the product. Similar low initial counts were reported in previous studies performed with fish and seafood products (Del

Nobile et al., 2009; Mastromatteo, Danza, et al., 2010). The final counts did not exceed 6 log cfu/g (except for TVC), which is the threshold value for cooked shrimp recommended by the FSANZ (2001). As it should be expected, control samples (without coating) showed in all microorganisms evaluated the highest counts (Fig. 5.1).

Results showed that samples treated with CH-THY and CH-OR showed significant differences ($p < 0.05$) compared with samples treated just with chitosan or the control samples. Between the two coating-enriched treatments, Ch-THY samples showed lower counts and reductions of about 1 log cfu/g were observed at the end of storage period. It is worthy to mention that higher reductions (meaning higher effectiveness of treatments) were observed at days 4, 8 and 10 of storage. This could be due to chitosan coatings were more effective when the microbial load was low. Samples coated just with chitosan also showed significant differences when compared with the control samples, but reduction in final counts was very low. Differences in microbial load reductions during the storage period have been also reported by other authors, who also found higher reductions along the storage period but lower at the end of such period (Ouattara et al., 2001; Weerakkody et al., 2011).

The combination of chitosan-thyme coatings with MAP inhibited the growth of aerobic mesophilic bacteria (Figure 5.1A). Reductions around of 1.5 log cfu/g were observed at days 4, 8 and 10 of storage period. At the end of the experiment, a reduction of 1 log cfu/g was observed always compared with the control samples. Lower reductions, but still statistical significant ($p < 0.05$) were observed when CH and CH-OR were applied (compared against the control samples). Other works support our results, reporting reductions when samples were coated with soy protein isolate or chitosan respectively (Ouattara et al., 2001; Vásconez et al., 2009). In the same line, Mastromatteo, Danza, et al. (2010) concluded that sodium alginate coatings incorporated with thyme EO reduced

mesophilic and psychrotrophic bacteria load of coated peeled shrimps packed under MAP.

LAB are facultative anaerobic bacteria and can grow under high concentrations of CO_2 (higher than 50 %), therefore are part of the natural microbiota of MAP products (Nychas and Drosinos, 2009). Initial counts of these bacteria were not higher (2.39 log cfu/g). At the end of the storage period counts reached 5.12 log cfu/g in the case of control samples and 4.54 log cfu/g for CH-THY samples. Significant differences ($p < 0.05$) were observed in LAB counts during the storage period in all groups of samples (Figure 5.1B). Again, similar to the mesophilic bacteria, higher reductions were observed at days 4, 8 and 10, being day 10 reduction around 2 log cfu/g. Samples treated with CH-THY shower higher reductions compared with the ones treated with CH-OR. Similar to our results, significant reductions in LAB counts were reported by Fernández-Saiz et al. (2013) who treated fresh fish fillets with chitosan coatings and stored under aerobic conditions. In the same line, Albertos et al. (2015) also found significant differences in counts when trout fillets were coated with chitosan films incorporating essential oils. Normally, LAB are the predominant microbiota presented in MAP packed products (Dalgaard, 2003), then it is important to notice that chitosan-EO enriched films could control the growth of this bacteria.

Enterobacteriaceae is a good indicator of hygiene. According to FSANZ (2001) guidelines RTE products are considered satisfactory when Enterobacteriaceae are below 2 log cfu/g. In this study initial counts were low in all samples analysed (1.68 log cfu/g for control samples and 1.32 log cfu/g for treated samples) indicating good hygiene of the samples. Data showed (Figure 5.1C) significant differences ($p < 0.05$) among counts for all different treatments. Samples treated with CH and CH-THY showed lower microbial counts. At the end of the storage period a reduction of just half of log cfu/g was observed.

In their study, Gómez-Estaca et al. (2010) coated cod fish with chitosan-gelatine films incorporated with clove EO and significant reductions in the number of Enterobacteriaceae were also found. Contrary to our results, Fernández-Saiz et al. (2013) observed that chitosan films were not able to reduce this bacteria in sole fillet samples when packed under aerobic or vacuum conditions.

Figure 5.1D showed the counts for psychrotrophic bacteria during the storage period. Similar behaviour as for mesophilic bacteria was observed. Samples treated with CH-THY showed the higher reductions in microbial counts (around 2 log cfu/g in day 8 of storage) but no significant differences ($p < 0.05$) were observed when comparing CH-OR with CH samples. Same as our results, Tsai et al. (2002) found that chitosan films reduced in about 1 log cfu/g the final load of this bacteria in freeze-thawed salmon fillets stored at 4 ºC. Other previous works also reported reductions in the microbial load of psychrotrophic bacteria when chitosan films incorporated with different essential oils were applied to fish and seafood products (Gómez-Estaca et al., 2010; Jeon et al., 2002; Vásconez et al., 2009).

The effect of chitosan coating enriched with essential oils against the microbiota of RTE peeled shrimp could be associated with: *a)* the positively charged aminoacid group that possesses chitosan, which interacts with negatively charged microbial cell membranes, leading to the leakage of proteinaceous and other intracellular constituents of the microorganisms (Pranoto et al., 2005); and, *b)* the hydrophobic nature of essential oils that are able to partition the bacteria cell membrane disturbing the structure and make it more permeable (Lambert et al., 2001).

When tested *in vitro*, films containing oregano EO were more effective reducing the growth of seafood spoilage bacteria. However, when tested over peeled shrimps, coatings containing thyme were more effective. This change in activity could be related

to *1)* the fact that films act different when tested against pure strains than when exposed to a mix of microorganisms present in a food matrix; and/or, *2)* different interactions between food components and the two essential oils tested (Burt, 2004).

In order to analyse the effectiveness of chitosan as structural matrix and carrier of EOs, the same concentration of EOs was used to prepare oil/water (O/W) emulsions and treat RTE peeled shrimps. The evolution of the microbiological quality was registered and compared with the data obtained from samples coated with chitosan + oregano and thyme EOs.

Same as for the case of the samples treated with chitosan, the final counts did not exceed the threshold recommended by the FSANZ (2001) for ready-to-eat shrimps. Control samples showed the highest counts (Figure 5.2). For all the microorganisms evaluated significant differences ($p < 0.05$) were found at the end of the storage period. Slight but statistical significant reductions in final counts in all microorganisms analysed were observed. Counts in samples treated with OR and THY O/W were different ($p < 0.05$) compared to the control ones. But no significant differences were observed between the two O/W treatments.

Regarding TVC, significant differences were observed in the samples treated with O/W. Reductions of approximately 1.50 log cfu/g were observed at the end of the storage period for both treatments. The same behaviour was observed for LAB, Enterobacteriaceae and psychrotrophic bacteria in which a reduction of around 1 log cfu/g was quantified. Similar results were found by Kykkidou et al. (2009) who treated swordfish fillets with thyme EO (0.1 %) and packed under MAP conditions. They reported no significant reductions for LAB and 1.7 log cfu/g reduction for Enterobacteriaceae. Higher reductions though (about 3 log cfu/g) were reported for TVC. Giatrakou et al. (2008) observed that dipping swordfish fillets in a solution 0.1 % of

oregano EO and packed under MAP reduced in about 1 log cfu/g the final counts of TVC, LAB and Enterobacteriaceae.

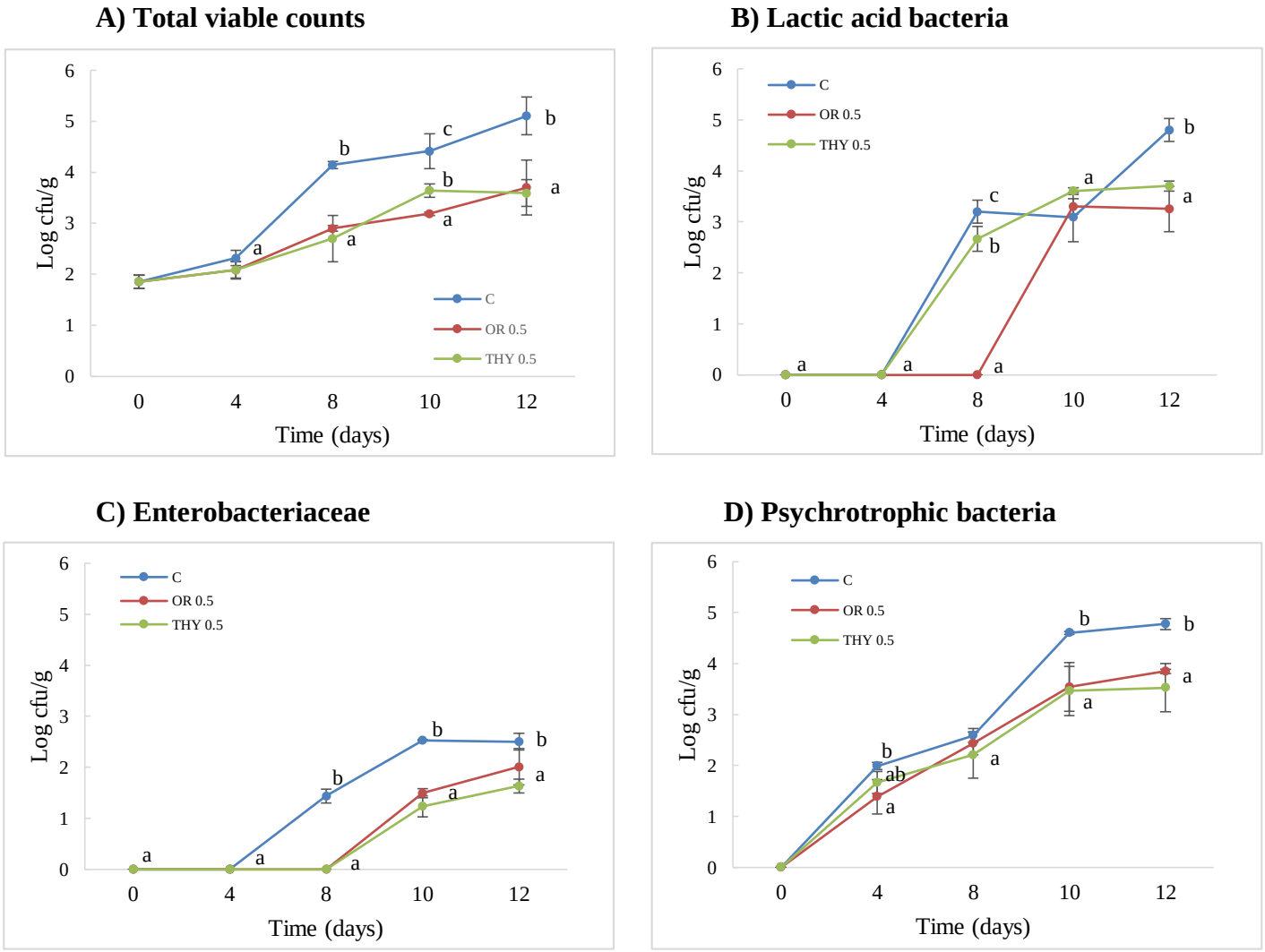

Figure 5.2: Evolution of the natural microbiota of RTE shrimp treated with O/W emulsion and stored at 4 ºC under MAP. C: Control; OR-0.5: OR 0.5 %; THY-0.5: OR 0.5 %.
Different letters mean differences among treatments in the same day and bacterial group ($p < 0.05$)

Since the experiments (with CH-coatings and with O/W emulsions) were performed using different samples and in different time period, an increment (Δ) log cfu/g between samples treated with chitosan coatings and samples treated with emulsions were calculated in order to make the comparison viable (Table 5.3).

Significant differences ($p < 0.05$) were found in TVC and psychrotrophic bacteria when comparing samples coated with chitosan + EO against samples treated with O/W emulsions. Higher reductions were observed in samples treated with CH-THY. On the other hand, for LAB and Enterobacteriaceae the treatment with the emulsion was more effective, with higher reductions in final counts. For example in day 8 of storage, CH-THY was more effective showing higher reductions in TVC, LAB and psychrotrophic bacteria, but was unable to reduce Enterobacteriaceae counts. Although similar concentrations of essential oils were used in both experiments, in general higher inhibitory effects (meaning higher reduction in microbial loads) were observed when EOs were incorporated into chitosan films. Similar results were found by Cutter and Siragusa (1996) when tested alginate gels + nisin and observed higher reductions in microbial counts on samples treated with the coating compared with the direct application of nisin on beef carcass.

Table 5.3: Δ in counts of microbial population between samples treated with CH and O/W emulsions (log cfu/g)

Letters a-c show significant differences between treatments for each day of analysis ($p < 0.05$)

	TOTAL VIABLE COUNTS					LACTIC ACID BACTERIA					ENTEROBACTERIACEAE					PSYCHROTROPHIC				
DAY	0	4	8	10	12	0	4	8	10	12	0	4	8	10	12	0	4	8	10	12
CH-OR	0.00^{a}	0.89^{ab}	1.06^{a}	0.72^{a}	0.63^{a}	0.67^{a}	0.92^{b}	0.53^{a}	0.91^{a}	0.42^{a}	0.21^{a}	0.18^{ab}	0.14^{a}	0.36^{a}	0.47^{ab}	0.38^{a}	0.65^{a}	1.54^{b}	1.22^{a}	1.08^{a}
CH-THY	0.00^{a}	1.37^{b}	2.60^{b}	1.35^{a}	0.99^{a}	0.67^{a}	1.40^{c}	1.22^{b}	1.89^{b}	0.58^{a}	0.21^{a}	0.31^{b}	0.45^{b}	0.25^{a}	0.32^{a}	0.38^{a}	0.80^{a}	2.14^{b}	1.65^{a}	1.13^{a}
OR	0.00^{a}	0.23^{a}	1.24^{a}	1.23^{a}	1.41^{a}	0.00^{a}	0.00^{a}	3.20^{c}	0.05^{a}	1.55^{b}	0.00^{a}	0.00^{a}	1.44^{c}	1.03^{b}	0.49^{ab}	0.00^{a}	0.60^{a}	0.19^{a}	1.06^{a}	0.93^{a}
THY	0.00^{a}	0.22^{a}	1.45^{a}	0.77^{a}	1.51^{a}	0.00^{a}	0.00^{a}	0.53^{a}	0.03^{a}	1.10^{ab}	0.00^{a}	0.00^{a}	1.44^{c}	1.30^{b}	$0,.87^{b}$	0.00^{a}	0.32^{a}	0.38^{a}	1.14^{a}	1.25^{a}

Table 5.4: Effect of CH-THY on sensory properties of RTE peeled shrimps stored at 4 ºC under MAP

	Control				CH-THY			
Parameter	**Day 4**	**Day 8**	**Day 10**	**Day 12**	**Day 4**	**Day 8**	**Day 10**	**Day 12**
Colour	2.8 ± 0.84^{ab}	2.4 ± 0.55^{a}	3.0 ± 0.82^{ab}	3.2 ± 0.84^{ab}	3.0 ± 1.00^{ab}	3.4 ± 0.89^{ab}	2.8 ± 1.09^{ab}	3.6 ± 1.14^{b}
Brightness	2.8 ± 1.48^{abc}	3.0 ± 1.00^{bc}	3.0 ± 1.00^{bc}	3.6 ± 1.14^{c}	2.0 ± 0.70^{ab}	1.8 ± 0.44^{ab}	1.6 ± 0.89^{a}	1.8 ± 0.84^{ab}
Characteristic odour	4.0 ± 0.71^{a}	4.0 ± 0.00^{a}	4.0 ± 0.00^{a}	3.8 ± 1.09^{a}	1.6 ± 0.55^{b}	1.2 ± 0.45^{b}	1.4 ± 0.55^{b}	2.0 ± 1.73^{b}
Unusual odour	1.0 ± 0.00^{a}	1.3 ± 0.50^{a}	1.5 ± 0.58^{a}	1.8 ± 1.30^{a}	4.2 ± 0.84^{b}	4.6 ± 0.55^{b}	3.8 ± 1.26^{b}	3.8 ± 1.64^{b}
Characteristic taste	3.8 ± 0.45^{b}	4.3 ± 0.57^{b}	4.0 ± 0.00^{b}	3.0 ± 1.73^{ab}	1.6 ± 0.89^{a}	1.75 ± 0.96^{a}	1.5 ± 0.71^{a}	2.0 ± 1.41^{a}
Unusual taste	1.2 ± 0.45^{a}	1.0 ± 0.00^{a}	1.0 ± 0.00^{a}	3.0 ± 2.83^{abc}	4.6 ± 0.55^{c}	4.5 ± 0.58^{c}	4.5 ± 0.71^{bc}	$2.3\pm2,31^{ab}$
Firmness	3.8 ± 0.45^{b}	3.8 ± 0.50^{b}	2.3 ± 1.15^{a}	3.3 ± 0.96^{ab}	3.8 ± 0.44^{b}	4.0 ± 1.15^{b}	4.0 ± 1.00^{b}	3.8 ± 0.50^{b}
Juiciness	3.0 ± 0.71^{a}	4.0 ± 1.00^{a}	3.5 ± 0.71^{a}	3.33 ± 1.15^{a}	3.2 ± 0.84^{a}	2.7 ± 0.58^{a}	2.5 ± 0.71^{a}	3.0 ± 0.00^{a}

Letters a-c show significant differences between treatments for each day of analysis ($p < 0.05$)

As it can be seen in the results, the use of chitosan as carrier of essential oils is advantageous for the shelf-life of RTE peeled shrimps, inhibiting in a greater extent the microbial growth of different microorganisms. Chitosan itself has antimicrobial properties and, in this study it has been boosted by the presence of essential oils. Besides, acting as carrier, chitosan coatings are able to control the release of the EO, keeping high concentrations of these active compounds on the food surface, make them available for longer time in order to inhibit the growth of bacteria. Furthermore, chitosan as structural matrix helps to uniformly distribute the EO over the whole surface of the product, where most of the microorganism growth is happening.

5.4.3 Sensory evaluation

Since the treatment CH-THY was more effective in reducing the microbial load, it was chosen in order to perform the sensory evaluation. Table 5.4 presents the results of comparison between control samples and samples treated with CH-THY at different days of storage period (4, 8 10 and 12 day). Chitosan coating did not affect the colour and juiciness of shrimps, but significantly affected ($p < 0.05$) brightness, odour, taste and firmness.

Shrimp colour was not significantly ($p < 0.05$) affected by coating, despite that slight differences in colour intensity were observed in coated samples at the end of the storage period. In control samples, brightness values were significantly different compared with coated samples, in which lack of brightness was reported by the judges. As it was expected odour and taste were significantly ($p < 0.05$) affected by CH-THY treatment. While for the parameters “characteristic odour” and “characteristic taste” control samples scored higher than the coated ones, on the other hand for “unusual odour” and “unusual taste” treated samples scored higher. Judges clearly identified the presence

of a "spice" in the treated samples. It is worthy to mention that the intensity of unusual/aromatic odour and taste changed during the storage period, registering higher scores for day 4 (4.2 and 4.6 respectively) and lower ones at day 12 (3.8 and 2.3 respectively). At day 12 control samples reported a high value of "unusual taste" assuming presence of off-odours and flavours implying the beginning of decomposition stage. Coating significantly ($p < 0.05$) affected firmness of samples. Control samples showed loss of firmness from day 10 of analysis while treated samples kept values for the whole storage period. Juiciness was not affected by coating.

In their experiment, Huang et al. (2012) evaluated whiteleg shrimp coated with chitosan and stored at aerobic conditions at 0 °C. Similar to our results, they found a significant effect on discoloration when comparing with control samples. In chitosan treated samples the colour was kept. Also all batches (including control samples) did not develop putrid odours. Mastromatteo, Conte, et al. (2010) coated shrimps with sodium alginate solution incorporated with thymol (1000 ppm) and packed under MAP and stored at 4 °C. After sensory evaluation they concluded that coated samples with thymol have a higher overall quality (base on evaluation of colour, odour and firmness) compared with samples coated without thymol. Besides, significant differences were also observed between samples stored in air or using MAP conditions. Maneesin et al. (2014) evaluated shrimp samples treated with garlic EO and packed in MAP and, similar to our results, control samples developed at the end of the storage period (7 days) discoloration, fishy odours and loss of firmness.

It is known that the use of high concentration of CO_2 in packaging atmospheres could negatively affect some sensorial parameters like increasing exudation, loss of texture (softening), discoloration, etc. (Sivertsvik et al., 2002). Since CH-THY kept firmness and colour in treated samples, the combination of MAP and chitosan coatings

could be a valid alternative for keeping good sensory characteristics in MAP-packed peeled shrimp.

5.4 Conclusion

The potential of chitosan as an antimicrobial has been boosted by the incorporation of oregano and thyme EOs. Such activity was clearly dependent on type and concentration of EO included and bacteria strain tested, being CH-OR films more effective than CH-THY. On the other hand, chitosan coatings incorporated with 0.5 % thyme EO effectively reduced the bacterial growth of RTE peeled shrimps, keeping final counts of TVC under the microbiological limit suggested by legislation. The antimicrobial effect was different depending of the microbial group analysed, being more effective in reducing TVC and LAB. As it was expected, sensory attributes were affected by the addition of EO into the chitosan coatings. However, the positive effect of coatings on colour and firmness should be taken into account.

The use of antimicrobial chitosan coating seems an effective way to reduce the growth of microorganisms in RTE peeled shrimps rather than the O/W emulsion. By the immobilisation of EOs in the matrix, the availability of essential oils is improved, the release is controlled and the microbial quality of shrimps is extended. Chitosan edible coatings can be used as a promising emerging technology in order to maintain the microbiological quality of RTE peeled shrimps during refrigerated storage.

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CHAPTER 6. DESIGN OF BIOACTIVE FILMS BASED ON WHEY PROTEIN ISOLATE CONTAINING LACTIC ACID BACTERIA TO CONTROL FOOD PATHOGENS

CARRIÓN-GRANDA, X., FERNÁNDEZ-PAN, I., ROVIRA, J., YOUNG, G. and MATÉ, J.I. (2016) Design of bioactive films based on whey protein isolate containing lactic acid bacteria to control food pathogens.

Submitted to Journal of Food Science and Technology

CHAPTER 6. DESIGN OF BIOACTIVE FILMS BASED ON WHEY PROTEIN ISOLATE CONTAINING LACTIC ACID BACTERIA TO CONTROL FOOD PATHOGENS

6.1 Abstract

The antimicrobial activity of the Lactic Acid Bacteria (LAB) *Lactobacillus plantarum* (WCFS1), *Lactococcus lactis* (F20) and *Weisella confusa* (TW1) against two pathogens of major concern for the food industry *Listeria monocytogenes* and *Salmonella Typhimurium* was investigated. Agar well diffusion and spot on the lawn methods were compared when evaluating the antibacterial activity of LAB. *L. monocytogenes* was effectively inhibited by the three LAB tested. On the other hand, the LAB did not inhibit the growth of *S. Typhimurium*. After, bioactive edible films were prepared by the incorporation of the three aforementioned LAB into whey protein isolate (WPI) film forming solutions. Bioactive films were obtaining by casting and the survival of LAB during storage of films and antibacterial potential was evaluated. *L. lactis* presented the higher inhibition areas. *L. lactis*-containing films were the most effective in inhibiting the growth of *L. monocytogenes*. LAB kept viable along the whole storage period (15 days) showing that WPI is a good carrier of these bacteria. The present study demonstrated the potential use of WPI as carrier of LAB and their potential use as preservation tool.

6.2 Introduction

In the last decades, consumer food habits have changed considerably. They invest less time in cooking and prefer to consume fresh-cut and ready-to-eat food products. Besides, consumers are more interested in minimally or even non-processed food, with a long shelf-life, with few preservatives from a natural source. But these kinds of products are very prone to the growth of pathogen microorganisms that could lead to foodborne outbreaks. Therefore, food safety has become a key issue within the food industry. Novel technologies have emerged in order to satisfy these new consumer demands, to ensure food safety and to extend the shelf-life of food products. Examples of new preservation technologies could be high hydrostatic pressure, pulsed electric fields, modified atmosphere packaging, active packaging and biopreservation.

Among these new preservation technologies, biopreservation is gaining more and more interest within the food industry. The main objective of biopreservation is the use of the antimicrobial potential of naturally occurring organisms in food and/or their metabolites with the main purpose of extending the shelf-life and improving the hygienic quality, minimising the impact on the nutritional and organoleptic characteristics of perishable food products (García et al., 2010). The most important bacterial group used so far in biopreservation have been lactic acid bacteria and/or their antibacterial metabolites.

Lactic Acid Bacteria (LAB) are normally part of the microbiota of fresh and packed food and are able to produce different metabolic products, with a proved antibacterial effect, such as organic acids, diacetyl, acetoin, hydrogen peroxide, reuterin, reutericyclin, antifungal peptides and bacteriocins (Ghanbari, Jami, Domig, et al., 2013; Muñoz-Atienza et al., 2013).The mode of action of LAB as antimicrobials is related to

one or the synergistic effect of several mechanisms: *i)* competition for nutrients with spoilage and pathogen microbiota, *ii)* reduction of pH (with the production of organic acids), *iii)* change in gas composition of atmosphere and *iv)* the production of the aforementioned metabolites (Nes et al., 2012; Stiles, 1996). Most LAB have a GRAS status (generally recognised as safe), what makes them an appropriate alternative for natural preservatives.

Bacteriocins are antibacterial peptides produced by LAB. These antimicrobial agents are generally heat-stable, apparently hypoallergenic and readily degraded by proteolitic enzymes in the human intestinal tract (Cleveland et al., 2001; García et al., 2010). Numerous bacteriocins have been characterized showing different mechanisms of action, antimicrobial spectra and chemical properties. Although some bacteriocins, such as pediocin PA-1 and lacticin 3147, have been developed for approval and use, the most well-known bacteriocin is nisin (E-234), which is recognized as GRAS by the US Food and Drug Administration and it is commonly used as an additive in the conservation of cheese. Foods like meat and dairy products are a regular source for isolation of bacteriocins (Cleveland et al., 2001).

As it was mentioned before, active packaging, and within it, active edible coatings can be considered an innovative technology to extend and guarantee the shelf-life of fresh products. Edible films and coatings can be obtained from different sources like polysaccharides or proteins and act as oxygen and moisture barriers, retaining moisture, slowing the oxidation of products, carriers of active substances, therefore enhancing food product quality and extending the shelf-life (Cagri et al., 2004; Falguera et al., 2011). A range of different antimicrobial substances have been incorporated into edible coatings and films and tested either *in vitro* or onto food products with different results (Aşik and Candoğan, 2014; Di Pierro et al., 2011; Teixeira et al., 2014; Volpe et al., 2015). Whey

protein isolate (WPI) has been used as carrier of natural preservatives due to its ability to form flexible, odorless and tasteless films, beside the good mechanical properties that owes (Krochta, 2002).

Generally antimicrobials have shown higher effectiveness in retarding the growth of bacteria when they are incorporated into films and coatings and not added directly to food products. Antimicrobials are immobilized into the matrix, their release rate is controlled, make them available for longer periods of time (Fernández-Pan et al., 2014) (Cutter and Siragusa, 1996; Kristo et al., 2008). Then, the inclusion of natural preservatives into edible films and coatings formulation is the utmost importance.

Listeria monocytogenes and *Salmonella Typhimurium* are two of the most common foodborne pathogens found in fresh refrigerated food products. The contamination of food products with those bacteria can occur in any step of the food processing line due to mishandling (Adams and Moss, 2008). Both bacteria can be found in a wide range of different environments, grow easily under refrigeration conditions and tolerate lower pH and high salt content, which make them very difficult to control and a serious risk for the food industry and consumers (Hoffman et al., 2003). Several recalls of food products due to pathogen contamination occurred during the last years, have made food industry very compromised in finding a valid strategy to inhibit or kill the grow of these particular microorganisms.

Therefore, the aims of the present work was *i)* to describe the inhibitory effect of three lactic acid bacteria *Lactobacillus plantarum* (WCFS1), *Lactococcus lactis* (F20) and *Weisella confusa* (TW1) against two pathogens of concern of the food industry; *ii)* to formulate bioactive edible coatings based on whey protein isolate incorporated with Lactic Acid Bacteria and their effectiveness as antimicrobial systems; and, *iii)* to analyse

the viability and the anti-*Listeria* effect of LAB cells incorporated in those films during storage under refrigeration conditions.

6.3 Materials and methods

6.3.1 Bacteria strains

The Lactic Acid Bacteria *Lactobacillus plantarum* (WCFS1), *Lactococcus lactis* (F20) and *Weisella confusa* (TW1) strains (kindly provided by Dr. Maria Marco, Food Science and Technology Department, University of California – Davis) were used throughout this research. The stock cultures were kept at -80 ºC in de Man, Rogosa and Sharpe (MRS)/glycerol broth (70:30) and were regenerated, streaking them onto MRS agar plates and incubated at 30 ºC for 24 h. After the incubation period, one single colony was added to 5 ml of MRS broth and incubated at 30 ºC for 24 h under constant agitation.

Stock cultures of *Listeria monocytogenes* (strain 1343 Library Dan Portroy) and *Salmonella Typhimurium* (strain SVM 679) used as target bacteria, were kept at -80 ºC in Brain Hearth Infusion (BHI) supplemented with 30 % of glycerol. Cultures were regenerated streaking a loopful onto a petri dish containing BHI agar and incubated at 37 ºC for 24 h. Then, one single colony was added to 5 ml of BHI broth and incubated again at 37 ºC for 18 h under constant agitation, in order to reach the exponential phase of growth. Appropriate dilutions were prepared with the purpose of inoculate in the plates a final concentration of 10^7 cfu/ml.

6.3.2 Preparation of the LAB resuspended pellet

Once the bacteria has grown in the MRS broth, cells were harvested by centrifugation (6000 rpm/20 min/5 °C) and washed twice with 1 ml of phosphate buffered saline (PBS) solution. The pellet was finally resuspended in 1 ml of MRS.

6.3.3 Antimicrobial activity of Lactic Acid Bacteria against Listeria monocytogenes and Salmonella Typhimurium

The antimicrobial activity of the three LAB (whole cell culture (WCC) and the resuspended pellet (RP)) was tested using two methods: agar well diffusion and spot-on-lawn test.

6.3.1.1 Agar well diffusion test: Tempered soft BHI agar was prepared and 1 % (v/v) of the overnight culture of the target bacteria was added in order to get a final concentration of 10^7 cfu/ml. 5 ml of the inoculated soft agar were poured over a petri dish containing a first solidified layer of BHI agar. The inoculated plates were dried for about 30 minutes. After, wells of 6 mm diameter were done using a sterile punch. 40 µl of the LAB (either WCC or RP) to be tested was placed in every well. Then, plates were incubated for 24 h at 37 °C. After the incubation period, zones of inhibition around the well were checked and measured using a calliper and registered in mm.

6.3.1.2 Spot-on-lawn test: This assay was conducted using a methodology previously described by Milillo et al. (2013) introducing slight modifications. Tempered soft BHI agar was prepared and 1 % (v/v) of the overnight culture of the target bacteria was inoculated in order to get a final concentration of 10^7 cfu/ml. 5 ml of the inoculated soft agar were poured over a petri dish containing a first solidified layer of BHI agar. The inoculated plates were allowed to solidify for 30 minutes under a laminar flow hood. 10

µl aliquots of the LAB (WCC or RP) to be tested were spotted. Then plates were incubated for 24 h at 37 °C. After the incubation period, zones of inhibition around the spot were checked and measured using a calliper and registered in mm.

6.3.4 Formulation of the Whey Protein Isolate films

WPI films were prepared as follows: Whey protein isolate (10 % w/w) (Davisco Food International, Le Seur, MN, USA) was dissolved in distilled water and 5 % (w/w) of glycerol (Sigma, USA) was added as plasticizer. Then, film forming solutions (FFS) were heated using a thermostatic bath at 90 ºC during 30 minutes under constant agitation. Once FFS were cooled at room temperature, 10 g of FFS were casted onto glass petri dishes (90 mm diameter) and dried overnight at room temperature. Film thickness was measured using a micrometre in 5 different points of films.

6.3.5 Preparation of the bioactive films

In order to obtain the bioactive films, 1 ml of the whole cell culture was added in 99 ml of FFS, and mix thoroughly for 10 min. By that way the final concentration of the LAB in the FFS was 10^7 cfu/ml. After, 10 g of FFS were casted onto glass petri dishes (90 mm diameter) and dried overnight at room temperature.

6.3.6 Viability of LAB during the storage of the films

The viability of LAB in the prepared films was analysed using the method adapted from Gialamas et al. (2010). Films were stored inside a desiccator at 4 ºC and 75 % RH in such a way to simulate the most common storage conditions of fresh food. Stored films were analysed at day 0, 7 and 15 of the storage period. In all cases, films were removed aseptically from the dishes and placed in a sterile bag containing 100 ml of buffered

peptone water and stomached for 2 minutes. Decimal dilution were prepared, spread in MRS agar plates and incubated at 30 °C for 48-72 h.

6.3.7 Antimicrobial activity of bioactive films

The disc diffusion method was used for testing the antimicrobial activity of films formulated with the three LAB. Overnight cultures of *Listeria monocytogenes* prepared as mentioned before, were used.

Tempered soft BHI agar was prepared and 1 % (v/v) of the overnight culture of the target bacteria was added in order to get a final concentration of 10^7 cfu/ml. 5ml of the soft agar were poured over a petri dish containing a first solidified layer of BHI agar. The inoculated plates were allowed to solidify for 30 minutes under a laminar flow hood. Using a punch, discs of 17 mm diameter of every film formulation were cut and placed onto the inoculated plates. Films without the presence of LAB were also tested and used as control. Finally, plates were incubated at 37 ºC for 24 h. The diameters of zones of inhibition were measured using a calliper and expressed as areas of inhibition in mm^2 excluding the disc diameter.

The antimicrobial activity of the films was also tested during storage (4 ºC and 75 % RH) at day 7 and 15. Films were removed from petri dishes and analysed following the procedure mentioned before.

6.3.8 Statistical analysis

All tests were performed in triplicate. Statistical analyses were conducted using SPSS 21.0 (IBM, USA) software. Significant differences among extracts were determined using ANOVA and Tukey's multiple comparison post hoc test (confidence level of 95 %).

6.4 Results and discussion

6.4.1 Antimicrobial activity of Lactic Acid Bacteria against Listeria monocytogenes and Salmonella Typhimurium

The antimicrobial activity of *L. plantarum, L. lactis* and *W. confusa* against these two pathogens of main concern in the food industry was evaluated. The three tested LAB effectively inhibited the growth of the Gram-positive *L. monocytogenes* (Fig. 6.1). In the case of *S. Typhimurium*, which is a Gram-negative bacteria, no inhibition was detected. As it can be seen in Fig. 6.2, in general *L. lactis* showed the highest inhibition areas (> 300 mm^2) followed by *W. confusa* and *L. plantarum*, which showed similar inhibition activity ($p < 0.05$). Moreover, there was no significant difference between the activity observed for the LAB whole cell culture or the resuspended pellet (Fig. 6.2A and B). Similar antimicrobial activity could be related with the fact that metabolites (organic acids, bacteriocin and bacteriocin-like substances) responsible for the antimicrobial activity are suspended in the growth media. Then, they are present in the whole cell culture. Whereas in the case of the resuspended pellet, the obtained cells are resuspended in MRS broth, allowing the harvested living cells to growth again and produce new metabolites, inhibiting the growth of the target bacteria. In their study Concha-Meyer et al. (2011) resuspended the obtained LAB cell pellet in a solution of phosphate buffer and MRS broth prior incorporation into alginate films and tested against *L. monocytogenes.* Antilisterial activity was registered mainly due to the production of bacteriocin in the newly prepared cell suspension. Léonard et al. (2014) also used LAB cell resuspended in MRS broth and incorporated them into alginate-caseinate films. The growth of *L. monocytogenes* was effectively controlled. Gialamas et al. (2010) formulated sodium

caseinate films incorporated with a LAB cell suspension and tested in beef fillets. Again the growth of *L. monocytogenes* was inhibited by the bioactive films.

a) Agar well diffusion method method **b) Spot-on-the-lawn**

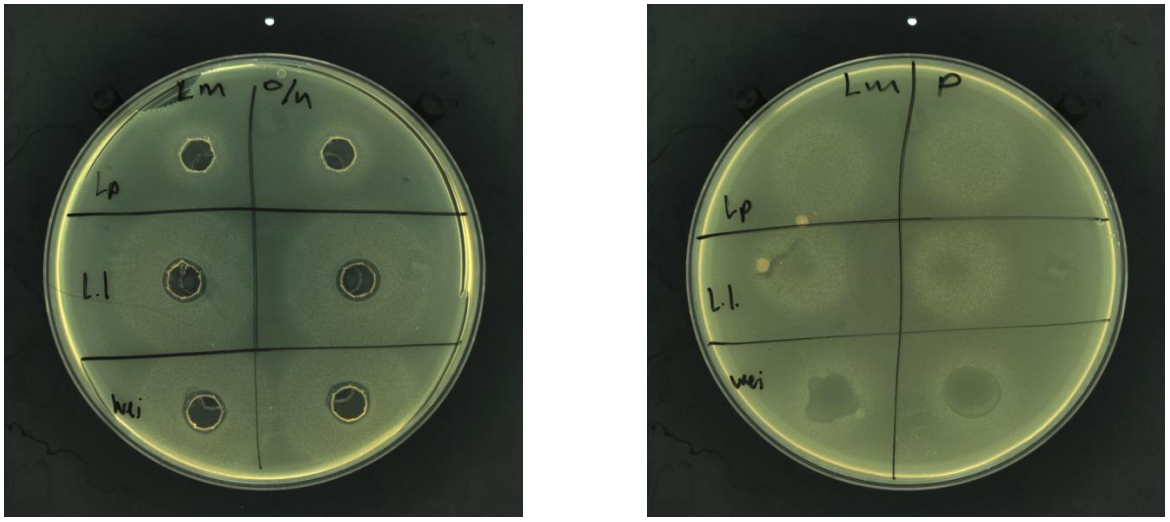

Figure 6.1: Images of antimicrobial activity assay against *L. monocytogenes*. Lp: *L. plantarum*, Ll: *L. lactis*, Wei: *W. confusa*

Regarding used methods, no significant difference was observed between the agar well diffusion method and the spot on the lawn method (Fig. 2A and B) in any of the studied microorganisms. This result was expected since both methods are based on diffusivity of the active compound (in this case different metabolites) through the inoculated agar.

LAB has been reported as effective antimicrobial organisms due to, among others, the production of different metabolites such as bacteriocins, organic acids, and hydrogen peroxide. Bacteriocins are defined as bacterial ribosomally peptides or proteins that inhibit or kill other microorganisms (Balciunas et al., 2013; García et al., 2010). One of the most studied and known bacteriocin is nisin, which is mainly produced by some species of *Lactococcus lactis* during fermentation. Bacteriocins are generally considered to act at the cytoplasmic membrane of sensitive cells, disrupting the proton motive force

through the formation of pores in the phospholipid bilayer resulting in cell death (Deegan et al., 2006; O'Keeffe and Hill, 2000). They are more effective in inhibiting Gram-positive bacteria than Gram-negative ones (Alison and Delves-Broughton, 2000). In our experiment, this theory was confirmed since *S. Typhimurium*, a Gram-negative strain, was not inhibited for any of the LAB tested. It is well known that Gram-negative bacteria are intrinsically more resistant to the action of antimicrobial compounds. They have, besides the cytoplasmic membrane, the lipopolysaccharide outer membrane which acts as a protective barrier against antimicrobial compounds (Russel, 2005).

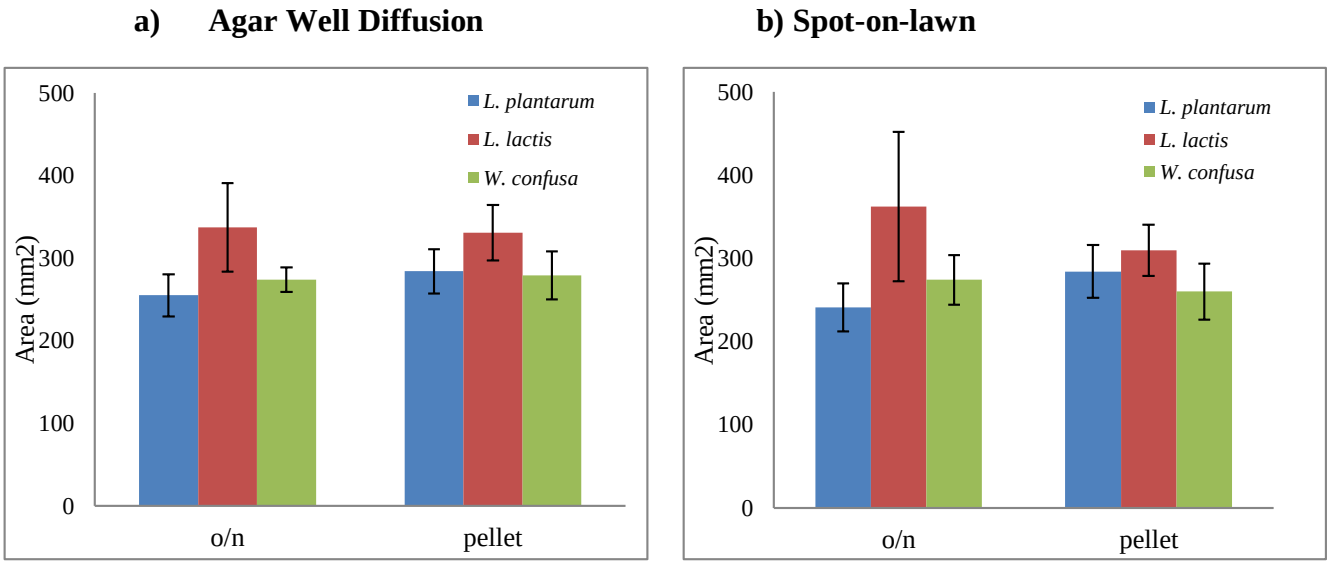

Figure 6.2: Antibacterial activity of LAB against *L. monocytogenes* using two methods (results expressed as inhibition area in mm^2) o/n: whole cell culture; pellet: resuspended pellet

Similar to our results and using the spot on the lawn method, Koo et al. (2012) found that a strain of *L. monocytogenes* was inhibited by the action of a combination of three LAB (*L. animalis*, *L. amylovorus* and *P. acidilactici*). In their study (Ghanbari, Jami, Kneifel, et al. (2013)) isolated 84 strains of *Lactobacillus* from fish and found that *L. casei* AP8 and *L. plantarum* H5 successfully inhibited the growth of *L. monocytogenes* and showed the highest inhibition areas compared to the other LAB isolated. Significant

inhibition of *Listeria* spp. and *L. monocytogenes* growth have been also reported in similar studies for LAB strains producing bacteriocins (Engelhardt et al., 2015; Martinez et al., 2015). All the aforementioned works have related the antibacterial activity of LAB with the presence of bacteriocins. On the other hand, and contrary to our results, Cizeikiene et al. (2013) evaluated the antimicrobial activity of 5 different LAB strains and found that *P. pentosaceus* and *L. sakei* inhibited the growth of *S. Typhimurium*. They concluded that the antibacterial activity of LAB were dependent of the type of LAB strain and the target microorganism. The lack of activity of the LAB strains used in the present work against *S. Typhimurium* is likely related with the specific bacteriocins produced for these strains and their concentration. According to Cleveland et al. (2001) bacteriocins could present some affinity to specific strains. The phospholipid composition of the target strain influences the minimum amount of bacteriocin needed to inhibit the growth of bacteria. Furthermore, this lack of activity could also be associated with the cell initial load. It could be suggested that *S. Typhimurium* needs a higher initial cell concentration to effectively be inhibited. In their study Léonard et al. (2014) found that the antimicrobial activity of *L. lactis* incorporated in alginate-caseinate films was clearly concentration dependant.

Another antimicrobial metabolite produced by LAB is organic acids, especially lactic acid. During growth, sugars are mostly transformed to lactic acid, the pH decreases and organic acids start to act. They neutralize the electrochemical potential of cytoplasmic membrane, penetrate into it, affecting the metabolic processes, consequently inhibiting the growth of target bacteria (Nes et al., 2012). The antimicrobial effectiveness of lactic acid, tested alone or included in different polymeric matrixes has been studied. Both, Gram-positive and negative bacteria have been successfully inhibited by lactic acid (Pintado et al., 2009; Ramos et al., 2012). In their work Siroli et al. (2015) found that

none of the LAB strains isolated from lamb's lettuce and sliced apples produced bacteriocins; nevertheless they were able to inhibit the growth of *L. monocytogenes*, *S. enteritidis* and *E. coli*. Such activity could be related with the presence of organic acids or other metabolites produced by LAB.

6.4.2 Antimicrobial activity of films against Listeria monocytogenes

The results of the antimicrobial activity of WPI films incorporated with Lactic Acid Bacteria are shown in Figure 6.3. Since the three LAB tested in the previous experiment did not show any inhibitory effect against *S. Typhimurium*, WPI films were not tested against this strain. WPI films without LAB were used as control films. As expected, films without the presence of LAB were not effective at inhibiting the growth of *L. monocytogenes*. Overall, films containing the LAB tested showed antimicrobial activity against the target strain. Significant differences ($p < 0.05$) were found when comparing inhibition between *L. lactis* films and *L. plantarum* and *W. confusa* films. Similar to the results obtained in the previous experiment, WPI films containing *L. lactis* showed the highest inhibition areas at D0 of analysis and also during the whole storage period (15 days). The difference in activity among lactic acid bacteria tested could be related with different levels of *in situ* production of active metabolites and difference in the diffusivity of such products into the culture media.

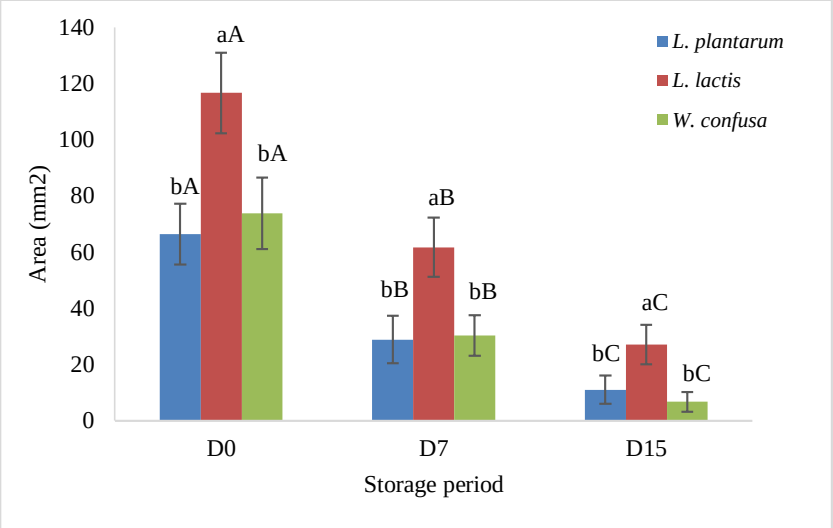

Figure 6.3: Inhibitory areas (mm^2 excluding film diameter) of WPI films incorporated with LAB and stored for 15 days at 4 ºC. Lower case letters mean differences among strains. Capital letters mean differences among storage day ($p < 0.05$)

During storage, the antilisterial activity of the formulated films gradually decreased (Fig. 6.3). A significant difference ($p < 0.05$) in the film activity was observed when comparing inhibition areas for the three formulated films at day 0, 7 and 15. The higher antibacterial activity of films incorporated with *L. lactis* was maintained during storage. Significant differences were not observed when comparing the antilisterial activity of films incorporated with *L. plantarum* and *W. confusa* at day 0. The decrease in activity during the refrigerated storage of films could be attributed to the death of LAB within the film and consequently less production of antimicrobial metabolites. WPI films or any other polymeric matrix represent a stressful environment for LAB growth because of the less availability of nutrients. Despite a decrease in antilisterial activity was observed, the remained activity is not negligible.

Similar results were found by Concha-Meyer et al. (2011) who developed alginate films as carriers of LAB isolated from cold-smoked salmon and stored under refrigeration for 20 days. They observed also a gradually decrease in the inhibition of *L.*

monocytogenes which was higher between day 5 and day 10 of storage. In their study Sánchez-González et al. (2013) found that methylcellulose films incorporated with *L. plantarum* were able to inhibit the growth of *L. innocua* on TSA medium until day 8 of refrigerated storage. After that, no inhibition was observed. Kristo et al. (2008) formulated sodium caseinate films with 0.5 mg of nisin and found no increase in population of *L. monocytogenes* until day 5 of storage period (10 ºC). They also compared the effectiveness of nisin added directly onto agar (without the presence of any matrix) and found lesser efficacy in the inhibition of the target strain.

6.4.3 Viability of lactic acid bacteria strains within the WPI films

As it was mentioned before, the viability of the three LAB was studied during a 15 days storage period under refrigeration conditions (4 °C). This temperature was selected in order to simulate the usual distribution and storage temperature of fresh food. The viability of the immobilized LAB in the WPI films are shown in Fig. 6.4. As it can be observed, the three LAB remained viable until day 15 of analysis. However, differences in counts are observed during the storage period. A 2 log cfu/g reduction in total counts is registered for the 3 LAB. Besides, a difference is observed when compared the initial concentration incorporated into the film forming solution (10^7 cfu/ml) with the concentration of the D0 of analysis, when a decrease of 1 log cfu/g was registered.

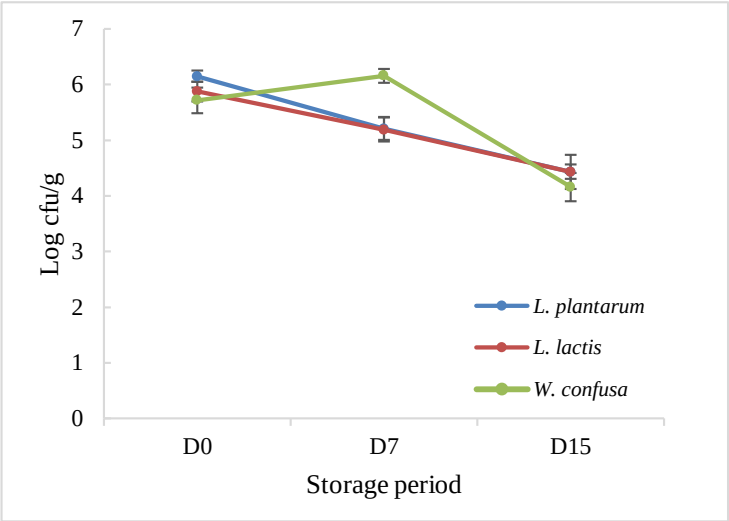

Figure 6.4: Viability of WPI-LAB during film storage period at 4 ºC.

LAB are subjected to stress conditions when they are included into biopolymeric matrix due to the casting process, therefore the difference observed in the viability of LAB throughout the storage period could be related with this fact. Even though, research shows LAB, like other bacteria, have developed defence mechanisms against stress that allow them to resist environmental changes and severe conditions (Ghanbari, Jami, Domig, et al., 2013), it seems that in some cases these mechanisms are not good enough to maintain the viability of the cells.

Sánchez-González et al. (2013) also observed a decrease in the viability of *L. plantarum* of films incorporated into sodium caseinate, pea protein or HPMC films stored at 5 °C during 30 days. The decrease in viability was less severe for protein films. López De Lacey et al. (2012) also found that *L. acidophilus* and *B. bifidum* incorporated into gelatin films remained viable during the storage period but observing a slight reduction in the counts at the end of the storage period (10 days). Similar, Gialamas et al. (2010) developed sodium caseinate films incorporated with *L. sakei* and found that the bacteria remained viable during the 30 days storage period at 4 °C. However, some reduction in

viability was observed in films which the addition of *L. sakei* was done by spraying the surface of the film.

The LAB cell survival during storage of films could also be related with the nature of the polymeric matrix used. Whey protein isolate, which has 90 % protein in its composition, can act as a protein source, stimulating the growth of LAB due to availability of nutrients needed. In this sense Léonard et al. (2014) tested alginate and alginate-caseinate films and found that the viability of LAB during storage was higher in the second matrix compared with alginate films. They concluded that the presence of caseinate exert a positive effect in the viability of LAB in the films and promote the release of antimicrobial metabolites. In the same line Sánchez-González et al. (2013) concluded that the nature of the matrix used is a determining factor for bacteria viability. Survival of LAB during cold storage was higher in pea protein films compared with hydroxypropylmethylcellulose films.

Despite reductions of 2 log cfu/g of LAB at the end of the refrigeration storage was observed, a high amount (10^5 cfu/g) of LAB remained viable (Fig. 4), showing they can grow under temperatures below their optimal growth temperature. Then, an acclimation to cold temperature could be inferred. Adaptation to cold temperatures of some LAB could be a real advantage in the use of biopreservation of fresh products. According to Van de Guchte et al. (2002) LAB have developed a cold-shock response which is based on the on the synthesis of different cold-induced proteins. This mechanism allows them to adapt to a temperature downshift and continue growing at a reduced rate.

The reduction in antilisterial activity (Figure 6.2) of the formulated films during the storage period is totally related with the decrease in viability of the LAB. As less LAB viable are present in films, less antimicrobial activity (small inhibition areas) should be expected, due to less production of metabolites or less availability of antimicrobial cells.

These results demonstrate that it is possible to maintain LAB cells and the production of metabolites into the WPI films during refrigeration storage.

Further experiments need to be carry out in order to determine precisely whether the antimicrobial activity of these specific LAB strains correspond to the production of bacteriocin or bacteriocin-like substances, organic acids or peroxide hydrogen.

6.4 Conclusions

Lactic acid bacteria were successfully incorporated into whey protein isolate films. *L. monocytogenes* was partially inhibited by the three LAB strains, L. *lactis* being the most effective showing the highest inhibition areas. On the other hand *S. Typhimurium* was not inhibited by any of the 3 LABs tested. No difference was found when tested the antimicrobial activity of the whole cell culture or the resuspended pellet. The results demonstrate that it is possible to incorporate and keep viable lactic acid bacteria in WPI films during 15 days of storage at 4 °C, being the matrix a possible source of nutrients for the LAB. Films with *L. lactis* showed higher effectiveness in inhibiting the growth of *L. monocytogenes*. LAB remained viable during the refrigerated storage period, showing that LAB can adapt to cold conditions and unfavourable environments, constituting a real advantage against pathogens which grow under refrigeration, like *L. monocytogenes*. These results demonstrated the potential of WPI films as carriers of lactic acid bacteria and that it is possible to improve the safety of fresh food by preventing or inhibiting the growth of pathogen bacteria with the use of LAB. Biopreservation could be used as an additional preservation tool to reduce the incidence of foodborne outbreaks.

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GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

The antimicrobial activity of EFCs depends on the nature and type of hydrocolloid used in the structural matrix, the type and concentration of the antimicrobial agent employed and the interactions with the target bacteria:

- Rosemary SCFE, oregano and thyme EOs were found to have the highest antimicrobial efficiency against all the tested bacteria. *A. caviae* and *S. putrefaciens* were the most sensitive bacteria, while *P. fluorescens* and *P. fragi* were the most resistant ones.
- Rosemary SCFE, oregano and thyme EOs were successfully incorporated into WPI and chitosan films and presented antimicrobial activity which was dependent on concentration of active compound and bacteria strain.
- The lactic acid bacteria *L. lactis, L. plantarum* and *W. confusa* inhibited the growth of *L. monocytogenes*. *L. lactis* was the most effective one.
- Lactic acid bacteria were successfully incorporated into WPI films. LAB remained viable in the films during 15 days of storage at 4 °C, showing that LAB can adapt to cold conditions and unfavourable environments within the protein matrix. WPI films containing *L. lactis* showed the highest effectiveness in inhibiting the growth of *L. monocytogenes*.
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The usefulness of antimicrobial edible coatings as an emerging technology to improve the microbiological quality and to extend the shelf-life of fish and seafood products packaged under different conditions is well established:

- WPI coatings enriched with rosemary SCFE and applied on fresh tuna steaks inhibited the growth of most of the natural microbiota present in the product.

Higher inhibitions were observed in aerobic mesophilic and Enterobacteriaceae groups.

- WPI coatings incorporated with oregano and thyme EOs and applied over fresh hake fillets (with high initial bacteria load) reduced the microbial growth of Enterobacteriaceae and H_2-S producing bacteria developed in the fillets stored at cold temperature and packed under air and MAP conditions. Higher reductions were observed though in samples packed under MAP.
- When hake fillets with low initial microbial load were treated, the combination of WPI coatings incorporated with oregano EO and MAP was effective in extending the shelf-life of samples. The growth of all microorganisms analyzed was delayed, especially H_2-S producing bacteria, resulting in a shelf-life of hake fillets twice as long as control samples.
- The antimicrobial effectiveness of WPI coatings enriched with oregano and thyme EOs was clearly dependent on the initial microbial population, the concentration of EO, the presence of MAP and the bacteria genera.
- Chitosan coatings incorporated with 0.5 % thyme EO effectively reduced the bacterial growth of ready-to-eat peeled shrimps, keeping final counts of aerobic mesophilic microorganisms under the microbiological limit suggested by legislation. Chitosan coatings helped maintain the sensory attributes of samples, keeping the color and firmness of shrimp tails during storage.

Finally, we can state that the use of antimicrobial edible coatings could be considered as an emerging technology with the aim of improving the microbiological quality and extending the shelf-life of fresh fish and seafood products. Edible films and coatings can

be used as carriers of different kinds of antimicrobials and can served as complement to the present preservation procedures employed nowadays in the fish and seafood industry.

CONCLUSIONES GENERALES

La actividad antimicrobiana de las PRCs depende de la naturaleza y tipo de hidrocoloide usado en la matriz estructural, del tipo y concentración del agente antimicrobiano empleado y de las interacciones con la bacteria objetivo:

- El extracto supercrítico de romero, y los aceites esenciales de orégano y tomillo demostraron la actividad antimicrobiana más grande frente a todas las bacterias probadas. *A. caviae* y *S. putrefaciens* fueron las bacterias más sensibles, mientras que *P. fluorescens* y *P. fragi* fueron las más resistentes.
- El extracto supercrítico de romero, y los aceites esenciales de orégano y tomillo fueron incorporados exitosamente en films de WPI y quitosano y presentaron actividad antimicrobiana la cual dependió de la concentración del compuesto activo y de la bacteria objetivo.
- Las bacterias ácido lácticas *L. lactis, L. plantarum y W. confusa* inhibieron el crecimiento de *L. monocytogenes*. *L. lactis* fue la más efectiva mostrando la mayor inhibición.
- Las bacterias ácido lácticas fueron incorporadas en films de WPI. Todas las LAB se mantuvieron viables dentro de los films durante los 15 días de almacenamiento a 4 ºC, demostrando que las LAB pueden adaptarse a condiciones de refrigeración y a ambientes desfavorables dentro de la matriz de proteína. Films que contenían *L. lactis* mostraron más efectividad inhibiendo el crecimiento de *L. monocytogenes*.

La eficacia de los recubrimientos comestibles antimicrobianos como una tecnología emergente para mejorar la calidad microbiológica y alargar la vida útil de pescado fresco y marisco envasado bajo diferentes condiciones de envasado ha sido determinada:

- Los recubrimientos de WPI enriquecidos con extracto supercrítico de romero y aplicado sobre filetes de atún fresco inhibieron el crecimiento de la mayoría de la microbiota naturalmente presente en el producto. Las mayores inhibiciones fueron observadas en el recuento de aerobios mesófilos y Enterobacterias.
- Los recubrimientos de WPI enriquecidos con aceite esencial de orégano y tomillo y aplicados sobre filetes de merluza fresca (con alta carga microbiana inicial) redujeron el crecimiento microbiano de Enterobacterias y bacterias sulfito reductoras desarrolladas en los filetes almacenados en refrigeración y envasados en aire y en atmósfera modificada. Se observaron mayores reducciones en las muestras envasadas con MAP.
- Al tratar filetes de merluza con baja población microbiana inicial, la combinación de recubrimiento comestible con aceite esencial de orégano y MAP fue efectiva, extendiendo la vida útil de las muestras. El crecimiento de todos los microorganismos analizados fue retardado, especialmente las bacterias sulfito reductoras, duplicándose la vida útil de los filetes de merluza fresca comparada con las muestras control.
- La efectividad de los recubrimientos de WPI enriquecidos con EO de orégano y tomillo fue claramente dependiente de la población microbiana inicial de las muestras, de la concentración de aceite esencial usado, de la presencia de MAP y del género de bacteria analizado.

- Los recubrimientos de quitosano que contenían 0.5 % de aceite esencial de tomillo redujeron de manera efectiva el crecimiento bacteriano de colas de langostino peladas, manteniendo los recuentos finales de aerobios mesófilos bajo el límite microbiológico sugerido por la legislación. Dichos recubrimientos ayudaron a mantener el color y la firmeza de las muestras durante el almacenamiento.

Finalmente, podemos afirmar que el uso de recubrimientos antimicrobianos comestibles puede ser considerado como una tecnología emergente capaz de mejorar la calidad microbiológica y extender la vida útil de pescado fresco y marisco. Las películas y recubrimientos comestibles pueden ser usados como portadores de diferentes agentes antimicrobianos y servir como una tecnología complementaria a los procedimientos de conservación actuales empleados en la industria del pescado y marisco.