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ESCOLA SUPERIOR DE BIOTECNOLOGIA

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DEVELOPMENT AND CHARACTERIZATION OF BIOACTIVE EDIBLE FILMS AND COATINGS BY INCORPORATION OF FUNCTIONAL BACTERIA

Thesis submitted to the Universidade Católica Portuguesa to attain the degree of PhD in Biotechnology – with specialization in Food Science and Engineering

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Joana Odila Mendes Sá Pereira

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Under the supervision of Professor Maria Manuela Estevez Pintado

Under the co-supervison of Professor Ana Maria Pereira Gomes

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Resumo

A inovação no mercado alimentar tem sido associada à procura de alimentos seguros e nutritivos que previnam ou controlem doenças, promovendo a saúde e a qualidade de vida. Portanto, é imperativo que as empresas alimentares criem novos produtos baseados em ingredientes e formulações inovadoras. Revestimentos e filmes comestíveis elaborados a partir de proteínas do soro surgem como uma alternativa às embalagens convencionais, sendo uma valorização sustentável de subprodutos da indústria alimentar. Neste sentido, o principal objetivo deste programa de doutoramento foi o desenvolvimento e caracterização de revestimentos e filmes bioativos comestíveis, feitos a partir de proteína de isolada de soro (PIS) ou alginato (ALG), incorporados com bactérias probióticas e prebióticos para melhorar o valor funcional, qualidade e segurança dos alimentos.

Assim, na primeira secção, foi realizada a incorporação e avaliada a estabilidade de *Bifidobacterium animalis* subsp. *lactis* BB-12 e *Lactobacillus casei* 01 em filmes comestíveis de PIS. Os filmes foram avaliados ao longo de 60 dias a 23 e 4 °C, e os resultados mostraram que as células viáveis permaneceram elevadas, atingindo 10^6 unidades formadoras de colonias (UFC)/g. A *B. animalis* subsp. *lactis* BB-12 permitiu maiores contagens viáveis durante o período de armazenamento. Além disso, as propriedades físicas cor, atividade da água, espessura, textura e estrutura molecular foram avaliadas e permaneceram estáveis durante todo o período de armazenamento em ambas as temperaturas. Para corroborar esta afirmação, os filmes foram usados para revestir fatias de fiambre, para entender se estes eram capazes de manter a viabilidade probiótica e conferir eficiência antimicrobiana, promover a preservação de fiambre e, eventualmente, aumentar o seu prazo de validade. Estes revestimentos comestíveis inibiram o crescimento detetável de *Staphylococcus* spp., *Pseudomonas* spp., *Enterobacteriaceae* e leveduras/bolores durante 45 dias de armazenamento, a 4 °C. As fatias de fiambre revestidas perderam menos água e não mostraram alteração de cor em comparação com fatias não revestidas. Uma avaliação sensorial revelou uma preferência pelo fiambre revestido em comparação com o fiambre tradicional. No final do

armazenamento, o número de células viáveis foi mantido a 10^8 UFC/g sugerindo que o fiambre fatiado é um transportador adequado para as estirpes probióticas até após 45 dias de armazenamento.

Na segunda secção, foi avaliada a viabilidade de *B. animalis* subsp. *lactis* BB-12 em filmes de PIS e ALG incorporando prebióticos (inulina e fructooligosacarídeos (FOS)), a 23 ºC durante 60 dias. A incorporação do prebiótico melhorou a sobrevivência de *B. animalis* subsp. *lactis* BB-12, mantendo-o a ca. 10^6 UFC/g até ao final do armazenamento, com a inulina a apresentar os melhores resultados. A análise estrutural não demonstrou diferenças entre os filmes sem e com prebióticos e probióticos. Além disso, o teor de humidade e a solubilidade em água diminuíram com a incorporação de prebióticos.

Como prova de conceito, uma barra de cereais foi desenvolvida com os revestimentos comestíveis com *B. animalis* subsp. *lactis* BB-12 e inulina, e a aceitabilidade do consumidor, as propriedades físico-químicas e microbiológicas foram avaliadas durante 90 dias de armazenamento. A PIS foi a melhor solução de revestimento mantendo a viabilidade celular em barras de cereais durante o armazenamento e após a digestão *in vitro*. Durante todo o período de armazenamento, as propriedades físico-químicas de todos os tipos de barras não mudaram. No entanto, as barras revestidas apresentaram valores mais altos de atividade da água e teor de humidade comparativamente às barras não revestidas. Cor e textura não foram afetadas após o revestimento das barras. Os parâmetros sensoriais mostraram que as barras revestidas foram tão bem aceites como as barras controlo. Além disso, os consumidores apreciaram mais o odor e o sabor das barras revestidas com PIS do que as barras revestidas com ALG.

No geral, com base neste projeto, conseguimos obter revestimentos comestíveis e filmes à base de proteínas de soro com probióticos, que também podem conter prebióticos na sua composição, para garantir a estabilidade dos probióticos, a segurança dos alimentos e o aumento do nível nutricional e funcional dos mesmos. Além disso, a indústria de embalagens pode rever estratégias para vender embalagens comestíveis e biodegradáveis, assim como a indústria alimentar pode aumentar a vida útil dos seus alimentos, mantendo-os seguros e tornando-os funcionais.

Abstract

Innovation in food industry has been linked to the search for safe and nutritional foods that prevent or control disease, promoting health and quality of life. So, it is imperative that the food industry creates novel products based on innovative ingredients and formulations. Edible coatings and films made from whey proteins appear as an alternative answer to the consumer's demand and represent a sustainable valorization of the food industry by-products.

In this sense, the main objective of this doctoral program was the development and characterization of bioactive edible coatings and films, made from whey protein isolate (WPI) or alginate (ALG), incorporated with functional bacteria and prebiotics to improve the functional value, quality and safety of food products.

In the first section, the incorporation and stability of *Bifidobacterium animalis* subsp. *lactis* BB-12 and *Lactobacillus casei* 01 in WPI edible films was evaluated. Films were evaluated throughout 60 days at 23 and 4 °C, showing that the viable cells remained high, reaching 10^6 colony forming units (CFU)/g. *B. animalis* subsp. *lactis* BB-12 was found in higher viable counts during the storage period. In addition, the films physical properties like color, water activity, thickness, texture and molecular structure were evaluated and remained stable throughout the storage period, at both temperatures. To corroborate this assertion, the films were used to coat slices of ham to understand if they were able to maintain probiotic viability and confer antimicrobial efficiency, promote sliced ham preservation and eventually increase its shelf-life. These edible coatings inhibited the detectable growth of *Staphylococcus* spp., *Pseudomonas* spp., *Enterobacteriaceae* and yeast/molds during 45 days of storage at 4 °C. Coated sliced ham lost less water and exhibited no color change compared to uncoated slices. A sensory evaluation revealed a preference for the coated ham compared to the traditional, uncoated one. By the end of storage, viable cell numbers were maintained at 10^8 CFU/g suggesting that the sliced ham is a suitable carrier for probiotic strains even after 45 days of storage.

In the second section, the viability of *B. animalis* subsp. *lactis* BB-12 in WPI and ALG films incorporated with prebiotics (inulin and fructooligosaccharides (FOS), at 23 ºC for 60 days was assessed. The incorporation of prebiotic compounds improved the survival of *B. animalis* subsp. *lactis* BB-12 viability, with inulin showing the best performance, as it maintained the viability at 10^7 CFU/g. The structural analyzes demonstrated no differences between films without and with prebiotics and probiotics. Furthermore, moisture content and water solubility decreased with prebiotics incorporation.

As proof of concept, a cereal bar was developed with an edible coating which included *B. animalis* subsp. *lactis* BB-12 and inulin, and the consumer's acceptability and physicochemical and microbiological properties were evaluated during 90 days of storage. WPI was the best coating solution maintaining cell viability in cereal bars during storage and after *in vitro* digestion. Throughout the storage period, physicochemical properties of uncoated, WPI and ALG bars did not change. However, coated bars showed higher values of water activity and moisture content comparatively to uncoated bars. Color and texture were not affected after coating the bars. The sensory parameters showed that the coated bars were as well accepted as the control ones. In addition, consumers appreciated more the odor and flavor of WPI coated bars than ALG coated bars.

Overall, based on this project, we were able to obtain whey proteins based edible coatings and films with probiotics, which may also contain prebiotics in their composition that assure the stability of probiotics, the safety of food, and increasing nutritional and functional capacity of the food. Additionally, the packaging industry can review their strategies for selling edible and biodegradable packaging, as well as the food industry can increase food shelf-life of their food, keeping safety and turning them functional.

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Keywords

Alginate

Antimicrobial

Bifidobacterium

Cereal bars

Consumer acceptance

Edible coatings

Edible films

Fructooligosaccharides

Functional bacteria

Gastrointestinal system

Inulin

Lactobacillus

Milk proteins

Packaging

Prebiotics

Probiotics

Shelf-life

Sliced ham

Viability

Whey protein isolate

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Scope and outline

Preliminary note: The core of this thesis is composed of one book chapter and four papers (two published and two submitted) in international scientific periodicals with referee's scientific journals.

This thesis is organised into four parts, which include seven chapters. The different chapters describe how the research/project evolved throughout time.

The main objective of the thesis was to develop and characterize WPI-based edible films and coatings by incorporation of probiotic strains (*Lactobacillus* and *Bifidobacteria*) and prebiotics and to study their direct application in two distinctive food matrices - sliced ham and a cereal bar. This information was integrated and optimized to those films formulations that could maximize the viability of bacteria aiming the development of functional food with specific properties. The thesis was organized in different parts and chapters in order to systematize the sequence and content of the studies.

Part I includes chapter 1 in which a general revision of the literature search on edible packaging as a vehicle for functional compounds was performed.

Part II encompasses chapters 2 and 3. The chapter 2 describes studies of the viability and stability of probiotic microorganisms in WPI-based films, taking to account the physicochemical characteristics of films. In chapter 3, previously developed edible coatings incorporating functional strains were evaluated directly on packed sliced ham foreseeing extension of shelf-life and as a potential carrier for viable probiotic cells and simultaneously physicochemical properties and consumer acceptability were evaluated.

Part III is divided in chapter 4 and 5 where after selecting the best probiotic strain that remained more stable over time, we observed that films for being stored at ambient temperatures should be improved. In chapter 4, sodium alginate and whey protein-based edible films incorporating *B. animalis* subsp. *lactis* BB-12 and combined with prebiotic compounds were developed and the microbiological and physicochemical characteristics were studied. In chapter 5, a cereal bar functionalized with edible film that showed the best performance in previous studies was developed and characterized in terms of functional value, physicochemical properties and consumer acceptability.

In Part IV, the concluding research findings were presented in chapter 6 and future perspectives in chapter 7.

PART I: Bibliographic survey

CHAPTER 1
Literature survey

PART II: Study of WPI-based films and coatings incorporating probiotic strains

CHAPTER 2

Edible films as carriers for lactic acid bacteria

CHAPTER 3

Impact of whey protein coatings incorporating *Bifidobacterium* and *Lactobacillus* on sliced ham properties

PART III: Study of WPI and ALG-based films and coatings incorporating *Bifidobacterium animalis* subsp. *lactis* BB-12 and prebiotics

CHAPTER 4
Characterization of edible films based on alginate or whey protein incorporated with *Bifidobacterium animalis* subsp. *lactis* BB-12 and prebiotics

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PART IV: Conclusions and Future Perspectives

CHAPTER 6

Conclusions

CHAPTER 7

Future Perspectives

Most information presented in the seven chapters that constitute this dissertation have been already submitted to international peer reviewing, via publication in international scientific journals – according to the following list:

Chapter 1:

Pereira, Joana O. and M. Manuela Pintado. "Edible Packaging: A Vehicle for Functional Compounds." Edible Food Packaging: Materials and Processing Technologies (2016). Eds: Miquel Angelo Parente Ribeiro Cerqueira, Ricardo Nuno Correia Pereira, Oscar Leandro da Silva Ramos, Jose Antonio Couto Teixeira, Antonio Augusto Vicente. CRC Press. 215-242 ISBN: 978-989-97478-6-9

https://www.crcpress.com/Edible-Food-Packaging-Materials-and-Processing-Technologies/Cerqueira-Pereira-Ramos-Teixeira-Vicente/p/book/9781482234169

Chapter 2:

Odila Pereira, J., Soares, J., Sousa, S., Madureira, A. R., Gomes, A., & Pintado, M. (2016). Edible films as carrier for lactic acid bacteria. LWT - Food Science and Technology, 73, 543-550.

https://doi.org/10.1016/j.lwt.2016.06.060

Chapter 3:

Odila Pereira, J., Soares, J., Monteiro, M. J. P., Gomes, A. & Pintado, M. (2018). Impact of whey protein coating incorporated with *Bifidobacterium* and *Lactobacillus* on sliced ham properties. Meat Science, 139, 125-133.

https://doi.org/10.1016/j.meatsci.2018.01.016

Chapter 4:

Odila Pereira, J., Soares, J., Gomes, A., & Pintado, M. (2018). Characterization of edible films based on alginate or whey protein incorporated with *Bifidobacterium animalis* subsp. *lactis* BB-12 and prebiotics. Submitted to Journal of Food Hydrocolloids.

Chapter 5:

Odila Pereira, J., Soares, J., Monteiro, M. J. P., Gomes, A. & Pintado, M. (2018). Cereal bars functionalized through *Bifidobacterium animalis* subsp. *lactis* BB-12 and inulin incorporated in edible coatings of whey protein isolate and alginate. Submitted to Journal of Functional Foods.

PART I - Bibliographic Survey

CHAPTER 1 - Literature survey

1.1. Introduction

Although active coatings have been developed to play an active role in food preservation, the bioactive coating is a new concept of technology to assist in the production of functional foods, whose bioactive principles are designed to be contained within coatings or coating materials.

Advances in this area imply that diet and/or its components should contribute to reduce the risk of diseases and improving of well-being and quality of life. These new concepts have led to the introduction of a new category of health-promoting food, or functional food (Korhonen, 2002).

The term “Functional foods”, was first used in 1984 in Japan, however, there still is no unique definition of what a functional food actually is (Siro et al., 2008). To date, a number of national authorities, academic bodies and the industry have proposed definitions for functional food (Roberfroid, 2002). Thus, can be checked several definitions for functional foods (Bigliardi and Galati, 2013, Granato et al., 2017), one the recent definitions of functional foods in literature are “foods that have a potentially positive effect on health beyond basic nutrition, helping the promotion of optimal health conditions and reducing the risk of non-communicable diseases” (Granato et al., 2017).

The main difference between the known technology of edible packaging itself and bioactive coatings/films is that while edible packaging primarily deals with maintaining or increasing quality and safety of packaged foods, extending their shelf life, the bioactive

packaging has a direct impact on consumer health by generating healthier packaged foods by a specific bioactive attribute (Han, 2005).

Therefore, there is a new conceptual approach towards the development of functional foods using a new packaging technology in which to a food package or coating is given a distinctive role in increasing the impact of food on the health of the consumer.

In general, edible packaging can provide several functions that do not exist in conventional packaging systems. Edible coatings and films, directly consumed with the food, were initially developed in order to increase the shelf life of food, thereby controlling food spoilage reactions and microbial contamination, through the control of some relevant properties such as moisture, gas exchange and oxidative reaction rate (Kester and Fennema, 1986, Naushad and Stading, 2007, Cuq et al., 1995, Han 2000). Currently, the edible coatings/films exhibit most interesting features. Bioactive edible coating and film is defined as a protective coating/film applied to the surface of a food and furthermore may possess other purposes e.g. adding high-value to food products through the addition of functional compounds such as antioxidants, colors, flavors, nutraceuticals, nutrients, probiotics, prebiotics and antimicrobials that increase the functionality of the coating and add extra functions to food products, as represent the Figure 1.1 (Pranoto et al., 2005b, Salmieri and Lacroix, 2006, Min et al., 2005).

Edible coatings and films may be used as carriers for functional compounds, allowing their protection and controlled release strategy by which functional compounds are provided in the desired site and at desired time and rate (Pothakamury and Barbosa-Cánovas, 1995, Ansorena et al., 2018).

These functional compounds can be incorporated to produce new functional foods, increasing its useful life and enhancing the nutritional quality and consumer acceptance.

In general, active food packaging can provide several functions that do not exist in conventional packaging systems. The selection of functional compounds for incorporation is restricted to edible coatings/films, because they have to be consumed with the coating layers and foods together with guarantee of being safe for the consumer. Functional compounds are components “extra-nutrient” found in plants, animals, microorganisms and marine organisms that normally occur in food in small quantities and can be obtained by extraction and biotechnological methods (Kris-Etherton et al., 2002). The components that enable the functionality of the functional foods may be present naturally in products, but need appropriate strategies for maintaining bioactivity during application, processing and storage of the formulated product and control the release of the functional component to the desired target (Lopez-Rubio et al., 2006, Ozimek et al., 2010).

A direct surface application of functional compounds has limited benefits, because the active substances can be neutralized on contact or diffused rapidly into the bulk of food (Siragusa and Dickson, 1992, Torres et al., 1985, Min and Krochta, 2005).

For development of functional foods, the incorporation of functional compounds into edible coating and films provides some advantages, like conveying substances that bring some benefits not only for food itself but also for the consumer (Falguera et al., 2011). Additionally, as functional compounds may have certain disadvantages such as off flavors and an early loss of functionality (Silva-Weiss et al., 2013), the utilization of edible coatings/films as a carrier is a promising technique that can help solving these drawbacks. Depending on the nature of the functional compound and coating materials, different techniques are used to coat the food. The techniques used for edible coatings include classic methodology of coating: spray fluidization, falling and pan coatings, spraying,

dipping and brushing. These processes are usually followed by drying in the case of aqueous products, or by cooling for coatings based on lipids (Debeaufort et al., 1998).

For edible films, the used techniques are similar to those for manufacturing flexible plastic films: extrusion (or co-extrusion of multilayer films), lamination, molding and roll-drying to remove solvent (Debeaufort et al., 1998, Guilbert et al., 1996, Hernandez-Izquierdo and Krochta, 2008, Hernandez-Izquierdo and Krochta, 2009).

These techniques are more efficient than a direct application of functional agents on the food surface, because edible coatings/films delay the migration of the agents away from the surface, helping to maintain a high concentration of functional compounds where it is needed.

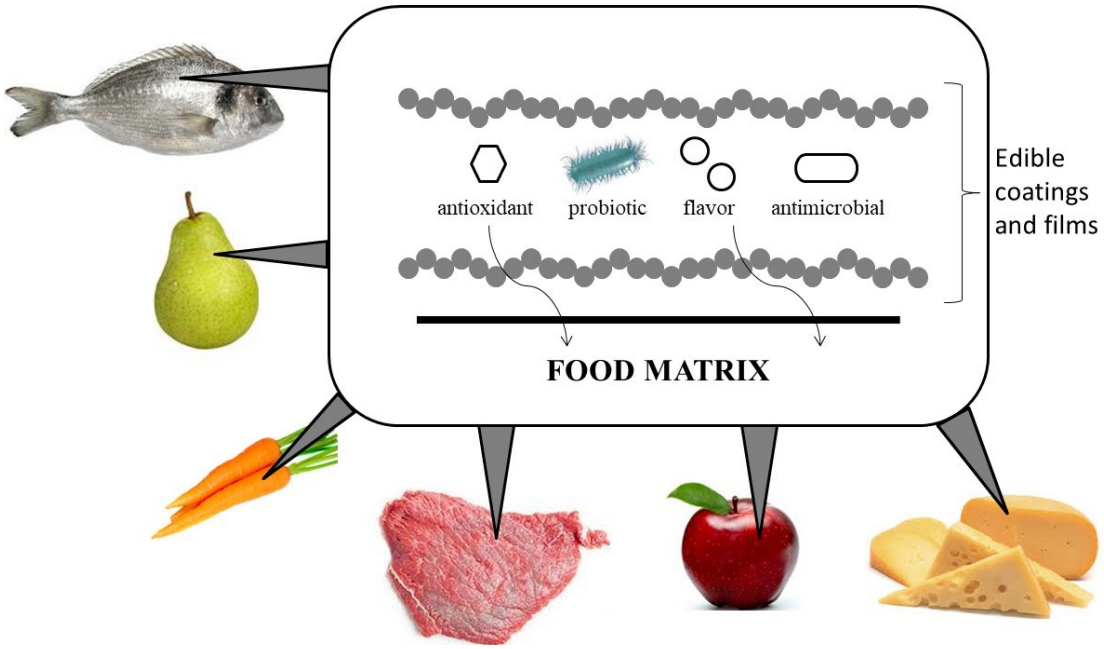

Figure 1.1 - Representation of edible bioactive coatings and films including the most used bioactive compounds (e.g. antioxidants, antimicrobials, probiotics and flavors) and more frequent applications into food matrix (e.g. fruits, fish, meat, vegetables and cheeses).

1.2. Incorporating functional compounds: Why?

There are several functional compounds appropriate and necessary to promote our health and well-being. The most commonly incorporated into edible packaging are phytochemicals, flavors and probiotics.

All these compounds have various functions that make them much requested and appreciated.

Phytochemicals are chemicals of non-nutritious plants that contain protective compounds (antioxidants and antimicrobials) that prevent microbial growth and certain diseases. They are mainly associated with the prevention and/or control of certain chronic diseases, such as cancer, diabetes, cardiovascular disease and hypertension (Traka and Mithen, 2011). These compounds help prevent cell damage, replication of malignant cells and reduce cholesterol. In addition, several of these compounds are phenolics with antioxidant capacity for direct activity in elimination of free radicals and an indirect effect due to chelation of ions of pro-oxidant metals (Shahidi, 2000, Wettasinghe and Shahidi, 1999, Flora, 2009, Rahman, 2007).

These compounds can be found in plants/seeds, but during certain processing steps they are removed or lose their activity, failing in promoting health or preventing disease (Mattila-Sandholm et al., 2002).

As edible coatings and films, in general, are consumed with product, therefore, the incorporation of these should not adversely affect consumer acceptance (Rojas-Graü et al., 2009).

So, the flavors are usually incorporated with the main aim of increasing customer satisfaction and promote consumption and consumer acceptance.

Probiotics are considered “live microorganisms which when administered in adequate amounts, not be less than 10^6 CFU/g, confer a health benefit on the host” (FAO/WHO,

2002). Among other effects attributed to the probiotics, increased digestibility, nutritional contribution for the production of conjugated linoleic acid, vitamins and short chain fatty acids, antagonistic activities against enteric pathogens and modulation of intestinal flora and possible protection against colon cancer are the most prominent (Gomes and Malcata, 1999, Vasiljevic and Shah, 2008, Reid, 2008, Zubillaga et al., 2001, Shida and Nanno, 2008, Chen and Walker, 2005, Harzallah and Belhadj, 2013, Maleki et al., 2015).

Additionally, probiotics can produce antimicrobial compounds, such as bacteriocins, organic acids, which may inhibit pathogens, not only in the gut but in the matrices where they are incorporated, and this may also constitute an antimicrobial strategy in foods and/or packaging (Linares et al., 2018).

1.3. Advantages and limitations of functional compounds

There are several functional compounds that can be added in our diet, through coated food, such as phenolic acids, carotenoids, vitamins, dietary fibers and some specific molecules such as ascorbic acid (Gorinstein et al., 2011, Lanciotti et al., 2004, Ajila et al., 2010, Gonzalez-Aguilar et al., 2008, Soong and Barlow, 2004).

Nowadays, there is a growing demand for these food additives by consumers due to the attributes that have demonstrated several benefits to human health.

The use of functional compounds has previously been directed to pharmaceutical or food products. In the latter area, the compounds are developed to create functional foods with some features such as antioxidants, antimicrobials, probiotics and flavors (Pranoto et al., 2005b, Salmieri and Lacroix, 2006, Min et al., 2005).

However, functional compounds may have certain constrains such as off flavors, an early loss of functionality or interaction with other components of the food matrix causing the loss of quality of functional food products (Silva-Weiss et al., 2013). These adverse

effects occur since the manufacture of these foods implies a series of limitations and difficulties, namely:

a) The compound introduced is not compatible with the food matrix (e.g.: lipophilic compounds in foods with extensive aqueous phase) interacting with food compounds leading to loss of functionality and/or quality of functional food (McClements et al., 2009);

b) The need to adapt the production line for incorporation of bioactive compounds in food. Usually this process involves considerable expenses that are accessible only to large companies (Homayouni et al., 2012);

c) Due to their sensitivity during the treatment, several bioactive compounds, as lipids, vitamins, peptides, fatty acids, antioxidants, minerals but also living cells such as probiotics, can be modified or even inactivated; some relevant treatment factors include temperature, pH, pressure or stirring (de Vos et al., 2010);

d) The loss of functionality and quality of the product during storage time (Fogliano and Vitaglione, 2005), since changes resulting from interactions between food and bioactive compounds or modifications of specific compounds during storage conditions may result in significant losses;

e) The induction of undesirable flavors and aromas in foods through reactions of oxidation, Maillard among others, which if extensive may generate toxic compounds (Sun Pan et al., 2006).

So, the utilization of edible coatings/films as a carrier is a promising technique to overcome some of the limitations of functional compounds when applied as food additives. Specific advantages and limitations for the main bioactive compound groups used in edible coatings/films are presented below.

1.3.1. Antioxidants and Antimicrobials

The harmful effects of free radicals and other reactive oxygen compounds can be neutralized by antioxidants that promote human health and have beneficial effects on functional food technology. Tissues are under constant oxidative stress from free radicals, reactive oxygen species, and pro-oxidants generated both exogenous (heat and light) and endogenously (transition metals and H_2O_2). For this reason, the human organism developed antioxidant systems to control free radicals, lipid oxidation catalysts, oxidation intermediates and secondary degradation products (Nakatani, 2003, Agati et al., 2007, Brown and Kelly, 2007, Chen, 2008, Iacopini et al., 2008).

Potential sources of antioxidants can be plants, fruits, vegetables, cereals and herbs.

These antioxidants include flavonoids, phenolic acids, carotenoids, tocopherols among others and can inhibit induced oxidation, scavenge free radicals and act as reducers (Khanduja and Bhardwaj, 2003, Ozsoy et al., 2009).

In addition to some advantages, the use of these compounds involves certain drawbacks, such as vulnerability to high temperature and light, high volatility, limited solubility and unpleasant taste; these characteristics result in a loss of functionality, which limits its application (Fang and Bhandari, 2010).

Some flavonoids (e.g. isoflavones) and phenols have limited solubility in lipophilic systems (Viskupicova et al., 2010). Many polyphenols (quercetin, kaempferol, taxifolin, procyanidines, salicin, thymol and eugenol), terpenes and carotenoids are astringent and have an unpleasant taste. Ferulic acid is easily volatilized therefore cannot inhibit the oxidation at elevated temperatures for extended periods of time (Nyström et al., 2007). Furthermore, various vitamins (e.g. C, E and K) and some phytochemicals (e.g. phenol

and flavonoids) are sensitive to UV-B and UV-C light radiation, limiting its use in food when they are exposed to such conditions (Durand et al., 2010).

Antimicrobials are compounds that perform the function of protection against microorganisms (Rauha et al., 2000).

For this reason, it makes sense to describe these compounds as functional additives in foods. Natural antimicrobials most commonly used to increase food safety and lifetime are enzymes (e.g. lysozyme and lactoperoxidase), polysaccharides (e.g. chitosan), bacteriocins (e.g. nisin) and more recently herbs and spices (e.g. oregano, thyme, cinnamon) and essential oils including terpenes, alcohols, ketones, phenols, acids, and aldehydes esters (Tajkarimi et al., 2010).

For their antioxidant and antimicrobial activities, essential oils have been widely studied. These are recognized as safe for health and have shown effectiveness against a wide range of bacteria and molds (Fisher and Phillips, 2008).

Despite the advantages of essential oils derived from plant extracts, their high volatility, reactivity and unpleasant aroma may limit its potential use (Del Toro-Sánchez et al., 2010).

Moreover, these oils due to its hydrophobic character can represent a problem due to the possible interactions between them and the food, essentially if the foods have high water content (Holley and Patel, 2005). Therefore, some packaging technologies can effectively reduce these constrains.

1.3.2. Flavors

Natural aromatic compounds are small molecules responsible for flavor and aroma in foods.

Among the most important sensory attributes on food, flavor and aroma are included, and are often key indicators of lifetime, from the point of view of consumers and a determining factor in the purchase decision (Beaulieu and Lea, 2003).

In the universe of flavor compounds the most important are the essential oils. These aromatic compounds are mostly constituted of short hydrocarbon chains, complemented with oxygen, nitrogen and sulfur atoms attached at various points of the chain (Braca et al., 2008).

The loss of quality of food products can be related to the loss of aroma compounds, causing a reduction of flavor intensity and change in the typical food flavor.

These metabolic changes in the flavor are the result of synthesis or catabolism of either flavor compounds or compounds responsible for off-flavors (Beaulieu and Lancaster, 2007).

There is a difficulty in keeping these compounds in foods, as they are generally sensitive aroma, volatile and hydrophobic, which is a disadvantage (Reineccius, 2009).

However, such deleterious reactions can be decreased with various preserving technologies, such as cooling and/or freezing, modified atmosphere and edible coatings/films, with the aim of accomplish consumer demands of a high-quality produce.

1.3.3. Probiotics

Probiotics are microorganisms intended to modify intestinal microbiota, as defined earlier. There is increasing evidence that the maintenance of healthy intestinal microbiota may provide protection against gastrointestinal disorders, including gastrointestinal infections and intestinal diseases (Reid, 2008; Vasiljevic and Shah, 2008(Maleki et al.,

2015)). For this reason, the incorporation of probiotics in food products has been increasing, to assure safe and healthy products.

Though a wide variety of genera and species of microorganisms are considered as potential probiotics (Holzapfel et al., 1998, Shah and Ravla, 2004), the most common bacteria commercially available are from the genera *Lactobacillus* and *Bifidobacterium*.

Some works have studied the effect of the incorporation of probiotics in food and have found some opportunities.

Probiotic food products made out of fermentation of milk, cereals, fruits and vegetables and meat are receiving great attention from the scientific world, as well as from general consumers (Gupta and Abu-Ghannam, 2012, Rouhi et al., 2013, Kołożyn-Krajewska and Dolatowski, 2012, Rößle et al., 2010, Min et al., 2018, Maleki et al., 2015).

The main challenge to the incorporation of multifunctional bacteria in food matrices is ensuring their viability, which cannot be less than 10^6 CFU/ml (Madureira et al., 2011b).

The survival of probiotics during gastrointestinal transit is affected by the physical and chemical properties of food carriers, which may represent advantages or disadvantage, depending on the food matrices.

Low pH value foods such as fruit juices, salads and condiments present a problem for probiotic survival, as well as foods prepared or stored at high temperatures (Rodgers, 2007).

Thus, the viability and growth of beneficial bacteria can be enhanced by proteins or polysaccharides - in particular, prebiotics that are film-forming biopolymers, such as whey proteins, or polymers that may be embedded in the film's matrix, such as inulin or fructooligosaccharides.

1.3.4. Prebiotics

By introducing the concept of prebiotics, it was assumed that, through modulation of the structure of the gut microbiota by selectively stimulating the growth of health-promoting bacteria and suppressing, or at least reducing the number of, potentially harmful microorganisms, food ingredients might be developed to improve colonic health and, indirectly, health of the host as well as the host's ability to reduce the risk of various diseases (Gibson and Roberfroid, 1995, Gibson et al., 2004, Gibson et al., 2017a). However, the most recent definition of prebiotic was reported by Gibson et al. (2017a) ''a substrate that is selectively utilized by host microorganisms conferring a health benefit''.

A food or a food ingredient to be considered as prebiotic involve some specific scientific requirements, such as resistance to gastric acidity, hydrolysis by mammalian enzymes, and gastrointestinal absorption; fermentation by intestinal microflora; and selective stimulation of the growth and/or activity of gastrointestinal bacteria associated with health and well-being. In addition, fermentation of a prebiotic food ingredient must be directed toward health promoting bacteria, with lactobacilli and bifidobacteria presently being the most common chosen targets (Gibson and Roberfroid, 1995, Gibson et al., 2004, Gibson et al., 2017a).

Inulin and oligofructose are the most considered and well-established prebiotics. They resist digestion in the upper gastrointestinal tract and reach the large intestine practically intact, where they are fermented and exert their function. Furthermore, a differential stimulation of growth of the beneficial flora, namely bifidobacteria, and to a lesser extent lactobacilli, and possibly other species. The main physiological effects of these prebiotics

on colonic functions are well-studied and have been extensively reviewed (Gibson et al., 2017a).

1.4. Structural matrix of edible packaging for functional compounds

Edible coatings and films offer some advantages such as edibility, biocompatibility, an esthetic appearance (color, transparent, low opacity, brightness), barrier properties to solute or gas, moisture, being non-toxic, non-polluting, etc. (Baldwin et al., 2012, Dhall, 2013, Han 2000, Kester and Fennema, 1986). In addition, edible coatings and films, by themselves acting as carriers of functional compounds, have been particularly considered in food preservation because of their ability to extend the shelf life, reduce the risk of pathogen growth on food surfaces, as well as provide a functional product with health benefits to the consumer. The incorporation of functional compounds into edible coatings and films is a way to protect these additives against severe environmental factors assuring that they can exert the desired effect on the expected target (Franssen et al., 2003, Donhowe and Fennema, 1994, Guilbert and Biquet, 1996, Oliveira et al., 2012, Rojas-Graü et al., 2009, Franssen et al., 2004).

For the selection of a film-forming biopolymer for a specific functional compound, it must be considered the efficiency of both and possible interactions between them and others food components, since these interactions can modify their activity, as well as the characteristics of the film (Campos et al., 2010).

The evaluation of the sensory attributes of foods with edible packaging is usually performed by means of descriptive analysis (Eswaranandam et al., 2006) or consumer and free-choice profiling panels (Han et al., 2005). In some cases, especially when lipids are incorporated into coatings, consumers may reject the samples because of their artificial color and waxy appearance (Han et al., 2005, Tanada-Palmu and Grosso, 2005).

On the other hand, the compatibility of the various constituents is very important, especially to withstand stress factors caused by e.g. high pressures, electric fields, ultrasound, microwave radiation, gamma radiation, temperature and light or to improve solubility and controlled release of bioactive compounds (Campos et al., 2010).

In edible coating/film formulation to incorporate functional compounds, different structural materials (Al-Hassan and Norziah, 2012) have been used such as proteins, lipids, polysaccharide and composite. In the last 10 years several reviews have focused on such edible coatings and films based on lipids (Hambleton et al., 2009, Hassan et al., 2017), protein (Ramos et al., 2012b, Hassan et al., 2017) and polysaccharides (Jiménez et al., 2013, Jridi et al., 2014, Hassan et al., 2017) regarding the development and application of edible coatings/films thereof. The production of biodegradable coatings/films by combining various polysaccharides, lipids and proteins is considered with the aim of taking advantage of the properties of each compound and the synergy between them. The mechanical and barrier properties of these films not only depend on the compounds used in their formulation, but also on their compatibility (Falguera et al., 2011).

The optimization of edible coatings/films composition is of great importance in the research of this field, since they must be formulated according to the properties of the raw material to which they have to be applied (Falguera et al., 2011).

Polysaccharides and proteins are the most widely investigated biopolymers and are great materials for the formation of edible packaging, as they demonstrate excellent mechanical and structural properties, but they have generally a poor barrier capacity against moisture transfer (Iwata et al., 2000, Falguera et al., 2011). This problem is not generally found in lipids due to their hydrophobic properties. To overcome the poor mechanical strength of

lipids compounds, they can be employed in combination with hydrophilic materials by means of the formation of an emulsion or through a lamination with an hydrocolloid film lipid layer (Falguera et al., 2011). The efficiency of an edible coating/film against moisture transfer cannot be simply improved with the addition of hydrophobic materials in the formulation, unless the formation of a homogeneous and continuous lipid layer inside the hydrocolloid matrix is achieved (Falguera et al., 2011).

Polysaccharides used for edible coatings or films include cellulose, starch derivatives, pectin derivatives, seaweed extracts, exudate gums, microbial fermentation gums and chitosan (Krochta and De Mulder-Johnston, 1997). Several studies investigated polysaccharides-based films and its derivatives regarding their physical, chemical, and biological properties (Arnon et al., 2015, Oms-Oliu et al., 2008, Rojas-Graü et al., 2008, Bravin et al., 2006, Carneiro-da-Cunha et al., 2009, Brasil et al., 2012).

Among polysaccharides, chitosan and its derivatives showed a great number of applications focused on active coating systems (Azevedo et al., 2014, Arnon et al., 2014, Gol et al., 2013, Suseno et al., 2014, Vásconez et al., 2009). Chitosan has a vast potential that can be applied in the food industry because of its particular physicochemical properties such as biodegradability, biocompatibility with human tissues, null toxicity and especially its antimicrobial and antifungal activity (Aider, 2010). Chitosan is obtained by deacetylation of chitin, which is extracted from the exoskeleton of crustaceans and fungal cell walls. It has been extensively used in films and coatings due to its ability to inhibit the growth of various bacterial and fungal pathogens (Romanazzi et al., 2002). Chitosan has also been studied in combination with other biopolymers. New research and reviews on the use of chitosan gather some information on the effect of the deacetylation degree on its antimicrobial activity, use in active coating and its interaction with other

components of food matrices (Aider, 2010, Devlieghere et al., 2004, Martínez-Camacho et al., 2010, No et al., 2002).

Another important polysaccharide is alginate, an appealing film-forming compound because of its non-toxicity, biodegradability, biocompatibility, and low price (Vu and Won, 2013). Its functional properties have been well studied, such as, thickening, stabilizing, suspending, film-forming, gel-producing, and emulsion-stabilizing (Tavassoli-Kafrani et al., 2016).

Alginate is a linear polysaccharide that is abundant in nature and is synthesized by brown seaweeds (e.g. *Laminaria digitata* and *Ascophyllum nodosum*) and some soil bacteria. It has an anionic character and is water-soluble, consisting of monomeric units of 1-4-linked α-d-mannuronate (M blocks) and β-l-guluronate (G blocks), as well as segments of alternating mannuronic and glucuronic acids (MG blocks). The physical properties of alginates depend on the relative proportion of these three blocks, which are directly related with extraction source (Pawar and Edgar, 2012).

Sodium alginate is the most used in industry and was the first by-product from algal purification. Having an efficient brown seaweed extraction would be interesting for producing an environmentally friendly biopolymer-rich extract for industrial applications, such as food packaging material, release agents, paper, pharmaceutical and medical uses, among others (Tavassoli-Kafrani et al., 2016). Due to the linear structure of alginate, the membranes are strong, with adequate fibrous structures in solid state, being considered a good filmogenic material (Blanco-Pascual et al., 2014).

Protein-based edible coatings/films have received considerable attention in recent years because of their advantages, including their use as edible packaging materials, over the synthetic films. In addition, those films can supplement the nutritional value of the food

(Gennadios and Weller, 1990). The mechanical properties of protein-based films are generally better than polysaccharide or lipids-based films, since proteins have a distinctive structure which confers a wider range of functional properties, mainly a high intermolecular binding potential (Cuq et al., 1995, Khwaldia et al., 2004, Hassan et al., 2017). Nevertheless, the poor water vapor resistance of protein films and lower mechanical strength in comparison with synthetic polymers limit their application in food packaging. Many approaches exist to improve the barrier properties of edible protein coatings/films, such as by modifying properties of protein by chemical and enzymatic methods, combining with hydrophobic material or some polymers, or by using a physical method. The methods primarily focus on improving the mechanical strength and moisture barrier properties. Several proteins, including collagen, wheat gluten, corn zein, soy protein, whey protein and bean protein, have been investigated for their film properties (Bourtoom, 2009). Protein edible coatings and films are usually produced from solutions of the protein as the solvent evaporates. The solvent is normally limited to water, ethanol or ethanol-water mixtures. Generally, proteins must be denatured by heat, acid, bases, and/or solvents in sequence to form the more extended structures that are required for film formation. Once extended, protein chains can associate through hydrogen, ionic, hydrophobic and covalent bonding. The chain-to-chain interaction that produces cohesive films is affected by the degree of chain extension and the nature and sequence of amino acid residues. The uniform distribution of polar, hydrophobic, and/or thiol groups along the polymer chain increase the likelihood of the respective interactions. The promotion of polymer chain-to-chain interaction results in films that are stronger but less flexible and less permeable to gases, liquids and vapors. Polymers containing groups that can be associated through hydrogen or ionic bounding result in films that are excellent oxygen

barriers but susceptible to moisture. Thus, protein films are expected to be good oxygen barriers at low relative humidity (Wittaya, 2012).

Protein-based edible coatings and films can find application for the individual packaging of small portions of food, applied inside heterogeneous foods at the interfaces between different layers of components; they can be tailored to prevent the deterioration of inter-component moisture and solute migration in foods. Moreover, protein-based edible coatings/films can function as carriers for antimicrobial and antioxidant agents and they also can be used at the surface of food to control the diffusion rate of preservative substances from the surface to the interior of the food. Another possible application could be their use in multilayer food packaging materials together with non-edible coatings and films (Wittaya, 2012, Hassan et al., 2017).

Edible barriers based on hydrophobic substances such as lipids were developed specifically for limiting moisture migration within foods. These hydrophobic substances are effective barriers against moisture migration because of their apolar nature (Morillon et al., 2002).

Lipids commonly used in the coating formulation to preserve minimally processed products are stearic acid, palmitic acid and some vegetable oils, such as soybean and sunflower (Colla et al., 2006, Garcia et al., 2000, Martin–Belloso et al., 2005). Natural and synthetic waxes are also used, showing good gas barrier and better moisture barrier properties than coatings containing only fatty acids (Rhim and Shellhammer, 2005, Rojas-Argudo et al., 2009, Talens and Krochta, 2005). The emulsion stability and lipid particle size affect barrier properties of emulsified coatings, which make the study of physical stability important. Nevertheless, physical stability of composite edible coatings/films is rarely presented in literature.

The mainly function of a lipid coating is to block transport of moisture due to their relative low polarity. In contrast, the hydrophobic characteristic of lipid forms thicker and more brittle films. Consequently, they must be associated with film forming agents such as proteins or cellulose derivatives (Debeaufort et al., 1993). Generally, water vapor permeability decreases when the concentration of hydrophobicity phase increases. Lipid-based films are often supported on a polymer structure matrix, usually a polysaccharide, to provide mechanical strength (Bourtoom, 2008).

1.5. Functionalities and applications of functional edible packaging

The concept of functional edible packaging has been designed to respond to the current barriers and limitations in the production of functional foods and in some cases, additionally to improve quality or extend the shelf life of the food product through the bioactive compounds.

The development of food packaging systems will provide an alternative, more efficient and, in some cases, unique means for providing industrial foods with better impact on human health in consumption (Lagaron, 2005).

1.5.1. Antioxidants and Antimicrobials

The antioxidant capacity is often combined with antimicrobial property, so in this context some studies including both properties are described.

The incorporation of antioxidants in edible packaging may be primarily a carrier of antioxidants with bioactive properties with benefits for the consumer, but also an interesting alternative to food preservation, since oxidation is one of the major problems affecting food quality. Antioxidants can be added into the edible packaging to protect

against oxidative rancidity, degradation, and discoloration of certain foods (Baldwin et al., 1995, Vargas et al., 2008).

Thus, extensive research has been conducted to adopt some natural antioxidants as mentioned above in place of synthetic antioxidants used in functional packaging (Moore et al., 2003, Wu et al., 2001).

Similar to biopreservatives and edible packaging materials, the natural antioxidants are also readily accepted by the consumers and they are not considered as chemicals.

There are some studies (summarized in Table 1.1) that demonstrate the antioxidant capacity of various compounds when incorporated into edible packaging.

Güçbilmez et al. (2007) reported that the incorporation of partially purified lysozyme into zein films in combination with chickpea albumin extract (CPAE), bovine serum albumin (BSA) and disodium EDTA. The retained antioxidant activity in the zein film with 2019 U/cm^2 of lysozyme and 530 μg/cm^2 of CPAE was almost 84 % (6.8 nmol vit. C/cm^2) higher than that retained at the control film surfaces (12.5 nmol vit. C/cm^2). This study clearly showed the benefits of using CPAE to improve antioxidant activity in zein films.

Oussalah et al. (2004) developed films containing oregano, pimento, or oregano-pimento essential oils mix based in milk protein and they were applied on beef muscle slices. This oregano-based film stabilized lipid oxidation and was the most effective against *Escherichia coli* O157:H7 and *Pseudomonas spp*. in beef muscle samples, whereas pimento-based films presented the highest antioxidant activity.

Another study reported that the incorporation of specific phenolic compounds such as catechin (CAT), gallic acid (GA), p-hydroxy benzoic acid and ferulic acid in zein films possesses antioxidant and antimicrobial potential. The Trolox equivalent antioxidant capacity (TEAC) of different phenolic compounds was determined by area under the curve (AUC) method using ABTS free radical and the TEACs for the values most

significant antioxidant were 21.0 for CAT and 86.2 μmol Trolox/cm^2 for GA (Arcan and Yemenicioğlu, 2011).

Other authors have shown that the antioxidants ascorbic acid, citric acid and oxalic acids incorporated into the carrageenan and whey protein coatings exhibited antioxidant activity and was advantageous in maintaining color during 2 weeks and effectively prolonged the shelf life of apple slices (Lee et al., 2003).

Similarly, the incorporation of ascorbic and sorbic acids into methylcellulose-based edible coatings were able to retard browning and to enhance texture of cut-pear wedges (Olivas et al., 2003). Ayranci and Tunc (2004) also developed methylcellulose-based edible coatings, but with polyethylene glycol and incorporating citric, ascorbic and stearic acids in order to control and reduce oxygen, water permeability and vitamin C losses. It was observed that coatings with stearic acid, are effective in reducing the water loss of fresh apricots and green peppers, and the coatings with citric and ascorbic acids reduces the vitamin C loss of these fresh foods.

Han et al. (2004) observed that the incorporation of Gluconal® CAL and DL-α-tocopheryl acetate as antioxidants into chitosan-based coating, significantly delayed the color change of fresh and frozen strawberries.

Rojas-Graü et al. (2007) proved that antioxidant agents such as cysteine or glutathione incorporated into edible coatings based on alginate and gellan can be used to protect the surface of fresh-cut apples.

The same group, observed that the antioxidant N-acetylcysteine containing into both alginate and gellan edible coatings prevented apple wedges from browning (Rojas-Graü et al., 2008).

Table 1.1 - Functionality of edible coatings and films with antioxidants and antimicrobials as bioactive compounds.

ANTIOXIDANTS AND ANTIMICROBIALS	*Edible packaging material*	*Assigned feature*	*Application*	*Reference*
Ascorbic, citric and oxalic acids	Carrageenan and whey protein coatings	Maintain color and prolong the shelf life	Apple slices	Lee et al. (2003)
Ascorbic acid and sorbic acid	Methylcellulose-based edible coatings	Retard browning and enhance texture	Cut-pear wedges	Olivas et al. (2003)
Gluconal® CAL and DL-α-tocopheryl acetate	Chitosan-based coating	Delay the color change	Fresh and frozen strawberries	Han et al. (2004)
Oregano, pimento and oregano-pimento essencial oils	Milk protein-based films	Antioxidant and antimicrobial (against *Escherichia coli* O157:H7 and *Pseudomonas spp*.) activities	-	Oussalah et al. (2004)
Stearic, citric and ascorbic acids	Methylcellulose-based and polyethylene glycol edible coatings	Control and reduce oxygen, water permeability and Vitamin C losses	Apricots and green peppers	Ayranci and Tunc (2004)
Cysteine or glutathione	Alginate and gellan edible coatings	Antioxidant activity	Fresh-cut apples	Rojas-Graü et al. (2007)
Purified lysosyme in combination with chickpea albumin extract (CPAE), bovine serum albumin (BSA) and disodium EDTA	Zein films	Improve antioxidant (lysosyme and CPAE – 6.8 nmol vit. C/cm^2) and antimicrobial (against *E. coli* and *Bacillus subtilis*) activities	-	Güçbilmez et al. (2007)
N-acetylcysteine	Alginate and gellan edible coatings	Prevent browning	Apple wedges	Rojas-Graü et al. (2008)
Vitamin C and tea polyphenols	Alginate-based edible coating	Increase the sensory quality, retard chemical spoilage and water loss	Fish	Song et al. (2011)
Catechin (CAT), gallic acid (GA), p-hydroxy benzoic and ferulic acids	Zein films	Antioxidant (21.0 for CAT and 86.2 μmol trolox/cm^2 for GA) and antimicrobial potential	-	Arcan and Yemenicioğlu (2011)
Ascorbic and citric acids	Alginate-based edible coating	Preserve color and increase the antioxidant potential	Fresh-cut mangoes	Robles-Sánchez et al. (2013)

Note: (-) not found

1.5.2. Flavors

The addition of flavors in edible packaging represents an effective method for adding flavors to foods, which allows control flavor loss and release.

Surface flavoring by edible coating/film permits to reduce flavor compounds in the food to be flavored assuring the improvement of taste. The flavors are concentrated at the surface in a very thin layer; it can be release very fast in the mouth and then provide a higher sensory impact. This can also be used for masking bitterness. Some studies have reported successful results incorporating flavors in edible coatings and films, which were summarized in Table 1.2.

Kaushik and Roos (2007) observed that the food matrix to incorporate the flavor should have good solubility, emulsifying properties and low viscosity at high solid concentration. The efficiency of the film or coating to retain volatile compounds also depends on the nature of the medium matrix in contact.

Marcuzzo et al. (2010) demonstrated the incorporation of ten different compounds in carrageenan films. The films prepared with carrageenan may release aroma compounds and thereby maintain the sensory characteristics such as aroma and taste for certain periods of time.

Hambleton et al. (2009) proved that the aroma compound n-hexanal incorporated into packaging made of polysaccharides such as alginate are well protected due to its low oxygen permeability. Fabra et al. (2012) analyzed the release of n-hexanal and D-limonene from edible coatings. Aroma compounds were released easily in water medium, being D-limonene released quickly at higher temperatures. Therefore, these coating technologies represent a promising approach for improving food aroma or flavor.

Monedero et al. (2010) observed that it was necessary to add beeswax to improve the capacity of soy protein-based films to retain n-hexanal, owing to the affinity of n-hexanal with the non-polar lipids as beeswax, which contribute to link the aroma compound and also the hydrophilic properties of soy protein.

Coated non-aromatic milled rice was reported by Laohakunjit and Kerdchoechuen (2007) with sorbitol-plasticized rice starch containing natural pandan leaf extract (*Pandanus amaryllifolius Roxb*.). The rice starch coating containing natural pandan extract produced rice with aroma compounds similar to that of aromatic rice.

Thus, these studies demonstrate the ability of edible packaging as carrier of flavorings, to enhance the sensory properties of the food, making it most appealing and acceptable by the consumers.

Table 1.2 - Functionality of edible coatings and films with flavors as bioactive compounds.

FLAVORS	*Edible packaging material*	*Assigned feature*	*Application*	*Reference*
Pandan leaf extract	Sorbitol-plasticized rice starch	Produce rice with aroma compounds. Flavor holding after 6 months of storage	Rice	Laohakunjit and Kerdchoechuen (2007)
n-hexanal	Alginate emulsion-based films	Protect aroma compounds	-	Hambleton et al. (2009)
Ethyl acetate, ethyl butyrate, ethyl iso-butyrate, ethyl hexanoate, ethyl octanoate, 2-pentanone, 2-heptanone, 2-octanone, 2-nonanone, 1-hexanol	Iota-carrageenan emulsion based edible film	Controlled release of aroma compounds and maintain aroma and taste	-	Marcuzzo et al. (2010)
n-hexanal	Soy protein based films	Capable of retaining and maintaining the n-hexanal with beeswax	-	Monedero et al. (2010)
n-hexanal and D-limonene	Iota-carrageenan films (with and without lipid)	Quickly release of aroma compounds at higher temperatures (25 and 37 °C)	-	Fabra et al. (2012)

Note: (-) not found

1.5.3. Probiotics

The probiotics are important for human well-being but are sensitive to several external factors and for that reason it is important to protect and incorporate them in food matrixes. In this context of edible films production containing probiotics, is imperative that the film has the ability to provide viable numbers of bacteria to gastrointestinal tract. The viability, and stability of the probiotic strains under various environments have been well studied, whereas only some studies are dedicated to the incorporation of probiotic strains in films in order to assess their suitability and viability in those carriers. The edible films and coatings that have been used for probiotics incorporation are summarized in Table 1.3.

The first study reported the incorporation of *Bifidobacterium animalis* subsp. *lactis* BB-12, in alginate-gellan coatings and films for fresh-cut apple and papaya. The authors maintained for 10 days of storage values higher than 10^6 CFU/g, thus demonstrating the objective of maintaining the viability of probiotics in films applied to fresh fruit (Tapia et al., 2007). Gialamas et al. (2010) tested the survival of *Lactobacillus sakei* added to sodium caseinate films via spraying or direct incorporation. They used sorbitol as plasticizer in the film-forming solution and two temperatures (4 and 25ºC) for storage. Direct incorporation of probiotic strains into the film-forming solution improved viability for both temperatures used. Concha-Meyer et al. (2011) studied the incorporation of lactic acid bacteria into alginate films which were used to cover salmon inoculated with *Listeria monocytogenes*. It was demonstrated that lactic acid bacteria viability and growth within food matrix was enhanced after contact with the salmon, possibly because of nutrient diffusion and buffering capacity of salmon juice since the pH of the film increased from 4.4 to 5.6 after the salmon was wrapped during 24 h.

The incorporation of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* into gelatin edible coatings applied to fish and the assessment of its effects during chilled storage was performed by López de Lacey et al. (2012). During the storage period, the bacteria remained viable and the H_2S producing microorganisms were reduced in 2 log cycles.

The same authors applied a high-pressure treatment to fish coated with gelatin films and incorporated with bifidobacteria, which resulted after 13 days of storage in a reduction of total viable counts. The application of gelatin edible packaging incorporated with bacteria can be promising for fish preservation, especially when combined with other technologies such as a high-pressure (López de Lacey et al., 2012).

Kanmani and Lim (2013) reported that the viability of probiotic strains, viz. *Lactobacillus reuteri* ATCC 55730, *Lactobacillus plantarum* GG ATCC 53103 and *Lactobacillus acidophilus* DSM 20079 was maintained in starch-pullulan based edible films.

However, the viability of bacteria was influenced by the pullulan to starch ratio and storage temperature. Pure pullulan and pullulan/potato starch films presented the highest relative cell viabilities, followed by pullulan/tapioca starch films, while pure starch films exhibited lower cell viabilities after thirty days of storage at 4 ºC. An experiment by Soukoulis et al. (2014b), demonstrated the development of probiotic pan bread by the application of edible coatings based in sodium alginate or sodium alginate/whey protein concentrate incorporated with *Lactobacillus rhamnosus* GG, followed by different drying steps.

The presence of whey protein concentrate improved significantly the viability of *L. rhamnosus* GG throughout air drying and room temperature storage and the use of film based exclusive on sodium alginate improved the viability throughout the simulated gastrointestinal conditions (Soukoulis et al., 2014b). Also, Soukoulis et al. (2016) investigated starch-based films from corn and rice with incorporation of *Lactobacillus*

rhamnosus GG and also protein additives. The incorporation of sodium caseinate improved the probiotic viability compared to others protein used. In corn starch films, the protein type had a significant effect on probiotic viability during storage and was also starch- and temperature-dependent. This effect was not observed in rice starch films, when proteins acted independently of storage temperature on probiotic survival.

The same author investigated the viability of the probiotic *Lactobacillus rhamnosus GG* by incorporation in films of different polymers (pectin, sodium alginate and locust bean gum/k-carrageenan) with the addition of whey protein concentrate. It was found that the highest viability was obtained when whey protein concentrate was incorporated in the film forming solution. This might be explained because of the low pH of the polymer film solutions without whey protein concentrate (Soukoulis et al., 2017).

Pavli et al. (2017) considered the viability of *Lactobacillus plantarum* and *Lactobacillus pentosus* strains included into sodium alginate edible films. The viability of incorporated strains was examined in contact with ham slices at 4 ºC for 66 days, at 8 ºC for 47 days, and at 12 ºC for 40 days. The storage temperature had no effect on the cell number viability of the inoculated strains, nevertheless, it was strain-dependent.

These studies represent promising advances in the search for new applications of edible coatings and films as carriers of diverse probiotics, and open new possibilities for the development of novel food with probiotic products.

Table 1.3 - Functionality of edible coatings and films with probiotics as bioactive compounds.

PROBIOTICS	***Edible packaging material***	***Assigned feature***	***Application***	***Reference***
B. animalis subsp. *lactis* BB-12	Alginate-gellan coatings and films	Maintain the viability of probiotics in films	Fresh-cut apple and papaya	Tapia et al. (2007)
Lactobacillus sakei	Sodium caseinate films	Direct incorporation of probiotic strains into the film-forming solution improved viability	-	Gialamas et al. (2010)
Lactic acid bacteria	Alginate films	Lactic acid bacteria viability and growth within food matrix was enhanced after contact with the salmon	Salmon	Concha-Meyer et al. (2011)
Lactobacillus acidophilus and *Bifidobacterium bifidum*	Gelatin edible coatings	Maintain the viability of bacteria and decrease the H_2S producing microorganisms. Preservation, especially when combined with high-pressure	Fish	López de Lacey et al. (2012)
Lactobacillus reuteri ATCC 55730, *Lactobacillus plantarum* GG ATCC 53103 and *Lactobacillus acidophilus* DSM 20079	Starch-pullulan based edible films	Effective delivery and carrier systems for probiotics; maintaining the viability during the storage	-	Kanmani and Lim (2013)
Lactobacillus rhamnosus GG	Sodium alginate or sodium alginate/whey protein concentrate based edible coatings	Improved significantly the viability of bacteria throughout the simulated gastrointestinal conditions and throughout air drying and room temperature storage	Pan bread	Soukoulis et al. (2014b)
Lactobacillus rhamnosus GG	Starch-based films from corn and rice and other proteins	The incorporation of sodium caseinate improved the probiotic viability compared to others protein used.	-	Soukoulis et al. (2016)
Lactobacillus plantarum and *Lactobacillus pentosus*	Sodium alginate edible films	In ham slices with or without HPP treatment, probiotic bacteria were enumerated above 10^6 CFU/g during storage at all temperatures.	Sliced ham	Pavli et al. (2017)
Lactobacillus rhamnosus GG	Pectin, sodium alginate and locust beangum/k-carrageenan films with the addition of whey protein concentrate	The highest viability was obtained when whey protein concentrate was incorporated in the film forming solution.	-	Soukoulis et al. (2017)

Note: (-) not found

1.5.4. Prebiotics

In this section we will consider only to prebiotics compounds that selectively favors probiotic viability. It is recognized that the synergetic blend of prebiotics with probiotics promotes colonization in the intestinal tract as well as preventing several forms of cancer. The chemical nature of some oligosaccharides makes them resistant to digestive enzymes and thus, they became available in the large intestine where they can be fermented by saccharolytic bacteria. In this way, adding prebiotics is a promising technology for an effective probiotic fortification (Pavli et al., 2018a). There are only few studies involving prebiotics co-addition to probiotics in edible films (Table 1.4).

The viability of the probiotic *Lactobacillus rhamnosus* GG incorporated in gelatine films with addition of several prebiotics such as inulin, polydextrose, wheat dextrin and glucose-oligosaccharides were studied by Soukoulis et al. (2014a). The results showed that addition of polydextrose and glucose-oligosaccharides demonstrated better results in terms of viability of the probiotic strain, while inulin and wheat dextrin had an adverse effect on the survival rates. At 4 ºC the survival rates of the probiotic strain were improved, compared to films stored at room temperature (25 ºC). In general terms, the presence of prebiotics in the matrices improved probiotic storage stability, with exception of polydextrose films stored at 25 ºC. Inulin gave the best results, followed by wheat dextrin, glucose-oligosaccharides and polydextrose at both temperatures tested. Moreover, the shelf life of the edible films, respecting to probiotic survival, range from 63-100 days and 17-30 days at 4 and 25 ºC, respectively, with inulin films having the longest shelf life (Soukoulis et al., 2014a).

The addition of fructo-oligosaccharides as prebiotics and *Lactobacillus delbrueckii* subsp. *bulgaricus* CIDCA 333 and *Lactobacillus plantarum* CIDCA 83114 as probiotics in

methylcellulose edible films were studied by Romano et al. (2014). The drying step of the film-forming solution led to a significant decrease in the *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 probiotic strain when fructo-oligosaccharides were not included in the film forming solution. Increasing fructo-oligosaccharide concentration in film forming solution had a strong positive effect up to 3 % (w/v). *L. plantarum* CIDCA 83114, demonstrated a greater resistance towards the drying process and the addition of fructo-oligosaccharides up to 5 % (w/v) did not significantly enhanced its viability (Romano et al., 2014)

Table 1.4 - Functionality of edible coatings and films with prebiotics as bioactive compounds.

PREBIOTICS	*Edible packaging material*	*Assigned feature*	*Application*	*Reference*
Inulin, polydextrose, wheat dextrin or glucose-oligosaccharides	Gelatin films	The presence of prebiotics in the matrices improved probiotic storage stability. Inulin had the best results.	-	Soukoulis et al. (2014a)
Fructo-oligosaccharides	Methylcellulose edible films	Increasing fructo-oligosaccharide concentration in film forming solution had a strong positive effect in viability of studied strains.	-	Romano et al. (2014)

Note: (-) not found

1.5.5. Other functional compounds

Edible packaging is an excellent carrier to enhance the nutritional value of foods by carrying basic nutrients and/or nutraceuticals that lacking or are present in only low amounts in foods (Park and Zhao, 2004, Bourbon et al., 2011, Mei et al., 2002).

Nutrients and nutraceuticals can be incorporated into the formulation of edible coatings and films, providing an alternative way to fortify unprocessed foods, such as fresh fruits, and encouraging their consumption. The edible coatings and films promotes the delivery of these compounds to foods mainly by preventing their interaction with other food components, e.g. iron bioavailability is commonly affected by interactions with food ingredients (Lynch, 1997, Sandström, 2001, Thankachan et al., 2008).

Some studies have reported the effect of the addition of active compounds in the functionality of edible coatings and films, summarized in Table 1.5.

Therefore, Mei and Zhao (2003) indicated that a successful development of edible packaging containing high concentration of nutraceuticals strongly depends on the type of coating materials and the type and concentration of nutraceuticals incorporated into the coating formulations.

Mei et al. (2002) developed xanthan gum-based coating incorporated with high concentration of calcium and vitamin E not only preventing moisture loss and surface whitening, but also significantly increasing the calcium and vitamin E contents of peeled baby carrots.

Park and Zhao (2004) reported the development of chitosan coatings containing high concentrations of calcium, zinc or vitamin E providing alternative ways to fortify fresh fruits and vegetables. This application has been successfully demonstrated on fresh and frozen strawberries and red raspberries (Han et al., 2004).

Similarly, Hernández-Muñoz et al. (2006) observed that chitosan-coated strawberries retained more calcium gluconate than strawberries dipped into calcium solutions.

Souza et al. (2011) incorporated different lipid fractions: fish and vegetable oils, stearic and oleic acids into chitosan films. Results showed that incorporation decreased the water vapor permeability (WVP) (1.3–1.8 g mm m^{-2} day^{-1} kPa^{-1}) as compared with pure chitosan film (3.8 g mm m^{-2} day^{-1} kPa^{-1}). A higher reduction in WVP (65 %) was found with the addition of refined fish oil to the continuous matrix of the films than with the addition of refined rice oil, oleic or stearic acid (50–60 %).

A similar study, incorporated sodium caseinate, glycerol and lipids (oleic acid and/or α-tocopherol) into films based on starch. After storage, films containing lipid were more stretchable. Lipid addition did not induce a notable decreased in WVP, but oxygen permeability highly increased when they contained oleic acid. The incorporation of α-tocopherol greatly increased the antioxidant capacity of the films which affected oxygen permeability (Jiménez et al., 2013).

Peptides can also act as functional compounds. Bourbon et al. (2011) produced a packaging films containing different functional compounds, a peptide fraction from whey protein concentrate hydrolysate, glycomacropeptide and lactoferrin based on chitosan and evaluate their mechanical properties. Therefore, the results demonstrate a high potential to use these packaging to carrier functional compounds and to enhance the bioactivity in foods.

Thus, it was observed that other functional compounds can also be incorporated into edible packaging in order to achieve the desired effect and increase the functionality of foods.

Table 1.5 - Functionality of edible coatings and films with other specific bioactive compounds.

OTHER BIOACTIVE COMPOUNDS	*Edible packaging material*	*Assigned feature*	*Application*	*Reference*
Calcium and vitamin E	Xanthan gum based coating	Increase the calcium and vitamin E contents. Prevent moisture loss and surface whitening	Peeled baby carrots	Mei et al. (2002)
Calcium, zinc or vitamin E	Chitosan coatings	Fortify fresh fruits and vegetables	Fresh and frozen strawberries and red raspberries	Park and Zhao (2004), Han et al. (2004)
Calcium gluconate	Chitosan coatings	Increased nutritional value	Strawberries	Hernández-Muñoz et al. (2006)
Peptide fraction from whey protein concentrate hydrolysate, glycomacropeptide and lactoferrin	Chitosan packaging films	Carrier of functional compounds and enhance the bioactivity in foods	-	Bourbon et al. (2011)
Fish and vegetable oils, stearic and oleic acid	Chitosan films	Decrease water vapor permeability. Higher reduction on chitosan with refined fish oil (1.3–1.8 g mm m^{-2} day^{-1} kPa^{-1})	-	Souza et al. (2011)
Sodium caseinate, glycerol and lipids (oleic acid and/or α-Tocopherol)	Starch films	Films containing lipid were more stretchable. Oxygen permeability highly increased in films with oleic acid. α-Tocopherol greatly increased the antioxidant capacity	-	Jiménez et al. (2013)

Note: (-) not found

As an alternative to conventional packages, these new edible packaging has been recognized by several authors as having great potential for protecting and add functionality to the food.

In regard to the inherent attributes of edible coatings and films it may be also important to refer their ability to incorporate functional compounds as antioxidants and antimicrobials, flavors (reinforcing the pre-existent flavor or adding new flavors), probiotics and nutraceuticals, without compromise their initial structure and functionality or even the consumer acceptability. These films incorporating bioactive compounds intended to be a healthy way to ingest functional compounds that positively influence the health of the consumer and may even be a way of acquiring substances that otherwise the consumer would have restricted access. In this perspective, it is predicted that they may play an important role in human health and disease prevention. Also, the addition of these compounds is intended to achieve an improvement of the nutritional, sensory and shelf life of food characteristics. Furthermore, all these compounds are safe for health and the vast majorities are natural and can also contribute to a valorization of a by-product.

Functional compounds are often perishable and very sensitive and can lose their activity or even be degraded, so the edible packaging provides protection to these compounds, allowing them to be stable and in some cases released under controlled conditions.

However, when the bioactive ingredients are added to the edible coatings and films, these may be affecting, for example, the mechanical, sensory and functional properties.

Therefore, further studies with the aim of developing new application processes to improve the functionality of the films and coating and even food compounds are required.

So, the main purpose of this work was the development and characterization of bioactive edible films and coatings by incorporation of functional bacteria (*Lactobacillus* and

Bifidobacteria probiotic strains) and prebiotics, and study their application in two distinctive food matrices - sliced ham and a cereal bar.

PART II - Study of WPI-based films and coatings incorporating probiotic strains

CHAPTER 2 - Edible films as carrier for lactic acid bacteria

Joana Odila Pereira, José Soares, Sérgio Sousa, Ana Raquel Madureira, Ana Gomes & Manuela Pintado

CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Porto, Rua Arquiteto Lobão Vital, Apartado 2511, 4202-401 Porto, Portugal

2.1. Abstract

The use of edible coatings and films formulated with bioactive compounds in food products in order to convey new functionalities or extend shelf-life opens new possibilities as a carrier for functional lactic acid bacteria. In this work the main objective was to study the stability of probiotic microorganisms, viz. *Bifidobacterium animalis* subsp. *lactis* BB-12 and *Lactobacillus casei* 01, in edible film formulations based on WPI. The results demonstrated a loss of bacterial cell viability of ca. 3 log cycles (reaching 10^6 CFU/g film) until 60 d at both 23 and 4 ºC, noting that the most marked decrease was at 23 °C for both strains. *Bifidobacterium animalis* subsp. *lactis* BB-12 remained viable for a longer period of time and with less decrease in its cell numbers (10^8 CFU/g film).

Physical properties, namely color, water activity, thickness, young´s modulus, tensile strength, elongation at break and the molecular structure of WPI films were maintained stable throughout the storage period at both temperatures tested.

Edible films incorporated with probiotics can be good carriers for these to be ingested together with food products.

2.2. Introduction

Edible coatings and films are natural polymers used to retain the appearance and physicochemical properties of foods during storage. When adsorbed on the surface of the food, they may promote protection against moisture migration or oxidation and control of microbial growth (Han 2000, Naushad and Stading, 2007).

Several reviews have demonstrated that edible films can be prepared from different structural materials such as lipids (Hambleton et al., 2009), polysaccharides (Jiménez et al., 2013, Jridi et al., 2014) and proteins (Ramos et al., 2011, Ramos et al., 2012, Ramos et al., 2013) or by combining two or several of these compounds. Protein based films have received considerable attention because they have advantages over others, in particular, due to their mechanical properties that are generally better since proteins have a distinctive structure, which confers a wider range of functional properties, (Cuq et al., 1995, Wittaya, 2012, Gennadios and Weller, 1990).

Edible packaging systems mainly deal with maintaining or increasing the quality and safety of packaged foods, extending their shelf life. On the other hand, bioactive packaging systems (coating/films) are a new technology concept to assist in the production of functional foods, where bioactive principles are designed to be contained within coatings or coating materials (Korhonen, 2002) in order to have a direct impact on consumer health, creating healthy foods packed for a specific bioactive attribute (Han et al., 2005). A bioactive edible coating/film is defined as a protective coating applied to the surface of a food with addition of functional compounds such as antioxidants, color agents, flavors, nutrients, probiotics, prebiotics and antimicrobial agents that increase the

functionality of the coating/film (Min et al., 2005, Pranoto et al., 2005a, Salmieri and Lacroix, 2006). These coatings/films have the function of protecting and controlling the release rate at which functional compounds are provided in the desired location (Pothakamury and Barbosa-Cánovas, 1995). To promote these characteristics, the coating must be prepared in accordance with the incorporated compound and the nature of the food. In this sense, the development of protein-based films is considered suitable for use as the carrier of functional ingredients and covers a wide range of foods.

Various studies have pointed out the beneficial effects of probiotics (Daoud and Hani, 2013, Vasiljevic and Shah, 2008, Nagpal et al., 2012). Probiotics have key functionalities including: relief from lactose intolerance, increased resistance to intestinal invasion by pathogenic bacterial species, stimulation of the immune system and possible protection against colon cancer (Zubillaga et al., 2001). Another functionality is the antimicrobial capacity, through bacteriocins, or by competition *in situ* (Messaoudi et al., 2013). For these reasons, the incorporation of probiotics into food products has been increasing, to assure safe and healthy products.

Though a large number of genera and species of microorganisms are considered as potential probiotics (Holzapfel et al., 1998, Shah and Ravla, 2004), the most common bacteria commercially available are from the genera *Lactobacillus* and *Bifidobacterium*. Probiotics incorporated into food products made by fermentation of milk, cereals, fruits and vegetables and meat are currently receiving great attention (Rößle et al., 2010, Gupta and Abu-Ghannam, 2012, Kołożyn-Krajewska and Dolatowski, 2012, Rouhi et al., 2013). However, the main challenge for their successful incorporation is ensuring their viability (Madureira et al., 2011a).

The survival of probiotics during the production of foods can lead to significant losses of viability due to mechanical and/or heat treatments, presence of oxygen and osmotic stress

mechanisms (Fu and Chen, 2011, Bustos and Bórquez, 2013), hence, it is important to protect and incorporate them in coated foods. Preparation of such bioactive coatings is a very innovative field in the food industry and therefore only a small number of studies on this topic are available (Tapia et al., 2007, López de Lacey et al., 2012, Kanmani and Lim, 2013, Soukoulis et al., 2014b).

The first study describes the incorporation of *Bifidobacterium animalis* subsp. *lactis* BB-12, in alginate-gellan coatings and films for coating of fresh-cut apple and papaya. Storage values of probiotic viable cell numbers higher than 10^6 CFU/g were maintained for 10 d, thus maintaining the viability of probiotics in films applied to fresh fruit (Tapia et al., 2007). López de Lacey et al. (2012) studied the incorporation of *Lactobacillus acidophilus* and *B. bifidum* into gelatin edible coatings applied to fish and assessed its effect during storage. Lactic acid bacteria remained viable (above 10^8 CFU/g) and the H_2S producing microorganisms were reduced in 2 log cycles. Additionally, this coated fish was suited to treatment with high pressure (200 MPa/10 min/20 °C) which resulted in a reduction of total viable counts (< 2 log cycles) after 13 d of storage (López de Lacey et al., 2012). Kanmani and Lim (2013) reported that starch-pullulan based edible films incorporated with probiotic strains, *L. reuteri* ATCC 55730, *L. plantarum* GG ATCC 53103 and *L. acidophilus* DSM 20079 maintained the viability of the selected strains after 30 d of storage at 4 ºC. More recently, Soukoulis et al. (2014b) demonstrated the development of a probiotic pan bread by application of edible coatings based on sodium alginate or sodium alginate/whey protein concentrate incorporated with *L. rhamnosus* GG. *Lactobacillus rhamnosus* GG viability was improved significantly by the presence of whey protein concentrate throughout 7 d storage (Soukoulis et al., 2014b).

All the above studies represent innovations of great interest and of promising nature when it comes to using edible coatings and films as carriers of probiotics, opening new

possibilities for the development of novel functional foods. However, no study has considered until now, the characteristics and bacterial cell viability of stable dried edible whey protein films incorporated with probiotics.

So, considering that there are very few studies on this topic, in particular using protein-based films, the objective of this study was to evaluate the stability of two probiotic microorganisms in edible films based on whey protein formulations opening new possibilities for coating food products.

2.3. Materials and Methods

2.3.1. Bacterial strains, media and growth conditions

Probiotic strains, *Bifidobacterium animalis* subsp. *lactis* BB-12 and *Lactobacillus casei* 01 obtained from Christian Hansen (Denmark) were stored at -80 ºC in de Man–Rogosa–Sharpe (MRS) broth (Biokar Diagnostics, France) supplemented with 30 % (v/v) sterile glycerol. The aforementioned microorganisms were reactivated, and pre-cultures were prepared in MRS medium supplemented with filter-sterilized 0.05 % (w/v) L-cysteine·HCl (Fluka, St. Gallen, Switzerland), in order to lower the redox potential, and incubated at 37 °C during 24 h under anaerobic conditions, in a plastic anaerobic jar with an AnaeroGen sachet (an atmosphere generation system, Oxoid, Basingstoke, England). Subsequently, grown cells were harvested by centrifugation at 4000 rpm for 30 min, at 4 °C. The supernatant was discarded, and the pellet was resuspended in a 0.9 % (w/v) NaCl solution.

2.3.2. Formulation of the films

The film-forming solutions, one for each probiotic strain, were prepared by slowly dissolving 10 % (w/w) whey protein isolate (WPI) powder (Armor Proteines, Saint Brice en Coglés, France) in deionized water, according to Pérez-Gago and Krochta (2002). Glycerol was added at 5 % (w/w), as plasticizer, and the resulting solutions were magnetically stirred for approximately 2 h. Subsequently, the solutions were heated in a water bath at 80 ºC, for 20 min under continuous agitation and cooled to room temperature for 1.5 h.

Afterwards, 5 % (w/w) inoculum of each probiotic strain was added to each film solution to attain a final concentration of 10^9 CFU/ml. To prepare the films, the same amount (300 ml) of each solution was poured onto level Teflon plates (38 x 34 cm), so as to control the film thickness. The film solutions were allowed to dry at room conditions under a sterile environment (in a vertical laminar-flow cabinet, ca. 23 ºC and 50 % relative humidity, RH) for 24 h, according to the procedure of Gounga et al. (2007) and Oses et al. (2009). Once formed, the films were peeled off and conditioned at 23 ± 2 ºC and 50 ± 2 % RH, in a controlled temperature and humidity storage room (Packaging Center, CBQF, Porto Portugal), for at least 72 h prior to testing (ASTM, 2000). All film physical measurements described below were conducted also at 23 ± 2 ºC and 50 ± 2 % RH.

2.3.3. Enumeration of bacteria in the films throughout storage

The viability of incorporated bacteria was studied in the films during 60 d of storage under vacuum at different temperature conditions, i.e. 23 and 4 ºC.

Three films were studied, without bacteria (control F_0), with *B. animalis* subsp. *lactis* BB-12 (F_{ba}) and with *L. casei* 01 (F_{lc}).

Each film was cut into circular discs with 1 cm of diameter and stored in plastic bags for 60 d under abovementioned conditions and sampled at 0, 3, 5, 10, 40 and 60 d. At each sampling point, the disks were put in a sterile flask and 2 ml of sterile peptone water (1 g/L) were added and homogenized by vortexing during 1 min to completely dissolve the film and extract the bacteria.

Appropriate sequential 10-fold dilutions were done in sterile peptone water and plated onto MRS and incubated under anaerobic conditions in a plastic anaerobic jar with an AnaeroGen sachet (an atmosphere generation system, Oxoid, Basingstoke, England) at 37 °C during 48 h.

2.3.4. Film characterization

2.3.4.1. Thickness

The film thickness was measured using a micrometer Model m120 (from Adamel Lhomargy, Roissy en Brie, France), to the nearest 0.001 mm. The mean thickness was calculated from five independent measurements, taken randomly at different locations on a disk film sample, at each sampling time.

2.3.4.2. Water activity

The water activity (a_w) was measured using a HygroLab 2 (from Rotronic, Bassersdrof, Germany). Pieces of films (ca. 0.5 g) were placed on the sample holder of the water activity device; a sealed system was formed by placing the water activity probe on top of the sample holder. The probe was equipped with a small fan to circulate air inside the

sample container, a thin film capacitance sensor able to measure RH from 0 to 100 ± 1.5 %, and a platinum resistance temperature detector with a precision of ± 0.3 °C. When a_w became constant (which usually took less than 1 h), its value was recorded. Calibration resorted to six saturated solutions of known a_w (viz. LiCl = 0.114, $MgCl_2$ = 0.329, K_2CO_3 = 0.443, Mg $(NO_3)_2$ = 0.536, NaBr = 0.653 and KCl = 0.821). The tests were run in quadruplicate.

2.3.4.3. Color of films

The color was evaluated using a portable Chroma meter CR-400 (from Minolta Chroma, Osaka, Japan). A CIELab color scale was employed to measure the degree of lightness (L), redness ($+a$) or greenness ($-a$), and yellowness ($+b$) or blueness ($-b$) of the films, under $D65$ (daylight). Film disks were measured, on the surface of the white standard plate, with color coordinates $L_{standard}$ = 97.7, $a_{standard}$ = 0.04 and $b_{standard}$ = 1.47. The color of the films was expressed as the total difference in color (ΔE), calculated according to:

$$\Delta E = [(L_{film} - L_{standard})^2 + (a_{film} - a_{standard})^2 + (b_{film} - b_{standard})^2]^{1/2}$$

For each condition, four samples were measured – and, on each film disk, four readings were made on each side.

2.3.4.4. Texture analysis

The preparation of film specimens and measurements were done according to the ASTM D-882-02 standard (ASTM, 2002). Texture analysis was performed using a texturometer

(TA. XT plus Texture Analyser, Stable Micro Systems, Cardiff, UK). Force calibration was performed with a weight of 5 Kg and height calibration was performed for Mini Tensile Grips (Stable Micro Systems). Evaluated parameters were young's modulus (Eq. (1)), tensile strength, and elongation at break (%). Tensile strength (MPa) stands for the maximum tensile stress that the test sample was capable of carrying. Elongation at break (%) was determined as the strain at the fracture point which corresponds to the ratio of the change of length of the specimen to initial length. Each sample was cut into rectangular film probes (100 x 15 mm). All measurements were performed in five films for each formulation.

$$Young's\ modulus\ (MPa) = \frac{Force\ at\ corresponding\ strain}{Cross\text{-}sectional\ area\ of\ the\ film \times Corresponding\ strain} \quad (1)$$

2.3.4.5. FTIR-ATR analysis

The spectra of the whey protein films were obtained with a Fourier transform infrared spectrometer (FTIR), model ABB MB3000 (ABB, Zürich, Switzerland), with a horizontal attenuated total reflectance (ATR) accessory (PIKE Technologies, Madison, WI, USA) with a diamond/ZnSe crystal. All spectra were acquired with 32 scans and 4 cm^{-1} resolution, in the region of 4000-600 cm^{-1}. Three replicates were collected for each film surface sample.

2.3.5. Scanning Electron Microscopy

Film morphology was evaluated by Scanning Electron Microscopy (SEM) using a JSM-5600 Lv microscope (from JEOL, Tokyo, Japan). The samples were coated with gold/palladium using a Sputter Coater (Polaron, Bad Schwalbach, Germany). The cold-

stage (-20 ºC) method was used to examine the samples. SEM was operated at the low vacuum mode, using a spot size of 25–29 and a potential of 16–23 kV.

2.3.6. Statistical analyses

Statistical analyses were performed using the Statistical Package for Social Sciences, v. 17.0 (SPSS, Chicago IL, USA), via one-way analysis of variance. The difference of means between pairs was resolved via confidence intervals, using a Tukey test. The significance level was set at $P < 0.05$.

2.4. Results and Discussion

2.4.1. Viability of B. animalis subsp. *lactis* BB-12 and *L. casei* 01 *in films during storage*

Enumeration of viable cell numbers was attained in films stored for 60 d under vacuum at two different temperatures, 23 and 4 ºC, in order to verify the viability and stability of the probiotic microorganisms tested as shown in Table 2.1.

Table 2.1 - Viable cell numbers (Log CFU/g) of *B. animalis* subsp. *lactis* BB-12 and *L. casei* 01 incorporated in whey protein-based films stored under vacuum for 60 days at 23 ± 1 ºC and 4 ± 1 ºC.

	23 °C		4 °C	
	B. animalis subsp. *lactis* BB-12	*L. casei* 01	*B. animalis* subsp. *lactis* BB-12	*L. casei* 01
Film-forming solutions	9,06 ± 0,03	9,07 ± 0,03	9,06 ± 0,03	9,07 ± 0,03
0 days	8,98 ± 0,01[a]	8,93 ± 0,04[a]	8,94 ± 0,03[a]	8,93 ± 0,04[a]
3 days	8,81 ± 0,04[b]	8,53 ± 0,04[b]	8,64 ± 0,03[b]	8,55 ± 0,03[b]
5 days	7,90 ± 0,01[c]	7,43 ± 0,04[c]	8,56 ± 0,03[c]	8,34± 0,02[c]
10 days	7,17 ± 0,01[d]	6,53 ± 0,05[d]	8,41 ± 0,03[d]	8,75 ± 0,02[d]
40 days	6,66 ± 0,01[e]	6,40 ± 0,02[e]	8,07 ± 0,04[e]	7,17 ± 0,03[e]
60 days	6,09 ± 0,02[f]	5,98 ± 0,01[f]	7,90 ± 0,01[f]	6,95 ± 0,01[f]

Note: [a, b, c, d, e, f] Means within the same columns, labeled with the same letter, do not statistically differ from each other ($P > 0.05$).

According to Rodrigues et al. (2011), the high relative humidity, high temperature and long storage periods are harmful to probiotics survival. Viability of both *B. animalis B. animalis* subsp. *lactis* BB-12 and *L. casei* 01 was well correlated with storage temperature ($P < 0.05$). Viable cell numbers of both probiotic species decreased, as expected, at significantly higher rates ($P < 0.001$) in the film systems stored at 23 ºC. Extrinsic factors such as a_w, temperature and presence of oxygen are known to adversely influence the viability of encapsulated probiotic viable cells (Soukoulis et al., 2014a).

In this way, from an initial concentration of ca. 10^9 CFU/g film, a loss of viability of ca. 3 log cycles (reaching 10^6 CFU/g film) was observed within 10 d of storage at 23 ºC for both probiotics tested and was maintained steady thereafter, with a very slight further decrease upon 40 d of storage. This loss of viability is due to storage temperature. Some studies demonstrate that probiotic food products should preferably be stored at a temperature of 4–5 °C (Mortazavian et al., 2007, Tripathi and Giri, 2014, Rodrigues et al., 2011, Sousa et al., 2012). Indeed, at 4 ºC the loss of viability was less pronounced,

with ca. 1 and 2 log cycles reduction for *B. animalis* subsp. *lactis* BB-12 and *L. casei* 01, respectively, at the end of the storage period. In addition, the viability of both species of probiotics remained constant for 10 d. This is in agreement with Soukoulis et al. (2014a), which confirm that storage at 4 ºC is better to maintain the viability of a *L. rhamnosus* probiotic strain in prebiotic edible films. Mortazavian et al. (2007) also confirmed highest viability of *L. acidophilus* LA-5 in yogurt for up to 20 d when stored at 2 °C, whereas for *Bifidobacterium animalis* subsp. *lactis* BB-12, the optimum storage temperature was 8 °C (Mortazavian et al., 2007, Tripathi and Giri, 2014, Mortazavian et al., 2007). Storage temperature of 20 °C resulted in significant reductions in viable counts of this species in the dried products.

These results suggest that the stability of probiotics increases with low temperatures. This phenomenon occurs as the microorganisms are kept in a latent state avoiding rearrangements in the wall material, preventing thus inadequate exposure of microorganisms (Albertini et al., 2010). Temperatures near to 0 °C improve the cell viability rate, because low temperatures reduce chemical reaction rates that are harmful for microorganisms, such as, oxidation (Nag et al., 2011).

Moreover, addition of whey protein and glycerol in the film solutions which are known as protectants could help in protecting the viability of probiotic cells (Tripathi and Giri, 2014).

Furthermore, whey protein has significant positive effects on the survival of probiotic microorganisms during storage (Mohammadi et al., 2011) by providing nutrition for the cells, reducing redox potential of the medium as well as increasing buffering capacity of the medium that results in a smaller decrease in pH (Soukoulis et al., 2014a, Mortazavian et al., 2007).

Nevertheless, the viable cell numbers at the end of the storage period for both temperatures and probiotics tested are still within the minimum threshold necessary for intended biological function in the human body, although the storage at 23 ºC after 10 d would slightly compromise the current most accepted value of 10^7 CFU/g film (FAO/WHO, 2002).

In similarity to the studies mentioned above, the results of this study were the most promising to maintain the viability of probiotics.

Table 2.2 - Evolution of thickness (mm) and water activity (a_w) of control and probiotic films incorporated with *B. animalis* subsp. *lactis* BB-12 and *L. casei* 01 during storage for 60 days at 23 ± 1 ºC (a) and 4 ± 1 ºC (b).

	Control		*B. animalis* subsp. *lactis* BB-12		*L. casei* 01	
	Thickness (mm)	a_w	Thickness (mm)	a_w	Thickness (mm)	a_w
a)						
0 days	0,396 ± 0.006[a]	0,578 ± 0,004[a]	0,408 ± 0.006[a]	0,637 ± 0,001[b]	0,395 ± 0.001[a]	0,738 ± 0,001[c]
3 days	0,395 ± 0.003[a]	0,577 ± 0,003[a]	0,407 ± 0.001[a]	0,637 ± 0,001[b]	0,395 ± 0.001[a]	0,738 ± 0,002[c]
5 days	0,400 ± 0.007[a]	0,575 ± 0,001[a]	0,408 ± 0.001[a]	0,638 ± 0,001[b]	0,396 ± 0.001[a]	0,737 ± 0,001[c]
10 days	0,399 ± 0.008[a]	0,576 ± 0,001[a]	0,405 ± 0.002[a]	0,637 ± 0,002[b]	0,396 ± 0.002[a]	0,736 ± 0,003[c]
40 days	0,397 ± 0.008[a]	0,577 ± 0,001[a]	0,409 ± 0.001[a]	0,637 ± 0,001[b]	0,397 ± 0.007[a]	0,738 ± 0,001[c]
60 days	0,397 ± 0.001[a]	0,576 ± 0,001[a]	0,408 ± 0.003[a]	0,637 ± 0,001[b]	0,395 ± 0.002[a]	0,737 ± 0,001[c]
b)						
0 days	0,405 ± 0.001[a]	0,589 ± 0,002[a]	0,410 ± 0.001[a]	0,551 ± 0,001[b]	0,399 ± 0.001[a]	0,570 ± 0,001[c]
3 days	0,406 ± 0.001[a]	0,588 ± 0,002[a]	0,408 ± 0.000[a]	0,550 ± 0,001[b]	0,400 ± 0.002[a]	0,570 ± 0,002[c]
5 days	0,404 ± 0.000[a]	0,590 ± 0,003[a]	0,410 ± 0.001[a]	0,550 ± 0,002[b]	0,400 ± 0.001[a]	0,571 ± 0,001[c]
10 days	0,404 ± 0.001[a]	0,588 ± 0,001[a]	0,409 ± 0.001[a]	0,551 ± 0,001[b]	0,398 ± 0.001[a]	0,570 ± 0,001[c]
40 days	0,402 ± 0.000[a]	0,589 ± 0,001[a]	0,410 ± 0.002[a]	0,551 ± 0,001[b]	0,399 ± 0.000[a]	0,570 ± 0,001[c]
60 days	0,403 ± 0.001[a]	0,589 ± 0,001[a]	0,410 ± 0.001[a]	0,549 ± 0,003[b]	0,397 ± 0.002[a]	0,570 ± 0,000[c]

Note: [a, b, c], Means within the same columns, labeled with the same letter, do not statistically differ from each other ($P > 0.05$).

2.4.2. Film properties

The effect microorganism's incorporation on the thickness of those films was evaluated. The film thickness was measured at 0, 3, 5, 10, 40 and 60 d.

The thickness control is an important variable in studies of barrier properties, and in this case to protect microorganisms. With the increased thickness, there is a reduction in the water vapor transfer resistance and low WVP (McHugh et al., 1996).

The results presented in Table 2.2 show that all developed films, with or without microorganisms, showed thickness of ca. 0.400 ± 0.010 mm (0.395 – 0.410 mm) and no effects ($P > 0.05$) of the probiotics strains on the film thickness were observed.

Furthermore, the film thickness, during the storage period, at both temperatures, did not change significantly in both control films (without microorganisms) and films with probiotic strains ($P > 0.05$), probably because all films were made with the same amount of solution, as described by Rubilar et al. (2015) and the microorganisms did not impact on this property.

The films were also evaluated for their a_w during storage. The a_w ranged from 0.549 to 0.738, as shown in Table 2.2. No differences ($P > 0.05$) in terms of a_w were observed during the storage period in the films produced, probably due to the fact that the films are sealed in vacuum high barrier plastic bags, without contact with the environment thus minimizing any unpredictable loss.

The visual aspect is related to the color and transparency of the whey protein films and was evaluated by color variation between the three types of film. The color characteristics of the films are given in Table 2.3.

Table 2.3 - Color characteristics of control and probiotic films incorporated with *B. animalis* subsp. *lactis* BB-12 and *L. casei* 01 during storage for 60 days at 23 ± 1 ºC (a) and 4 ± 1 ºC (b), viz. L* (black–white), a* (green–red), b* (blue–yellow) and ΔE (color difference).

	Control				*B. animalis* subsp. *lactis* BB-12				*B. animalis* subsp. *lactis* BB-12			
	L^*	a^*	b^*	ΔE	L^*	a^*	b^*	ΔE	L^*	a^*	b^*	ΔE
a)												
0 days	91.11 ± 0.16^{a}	-0.94 ± 0.01^{a}	19.31 ± 0.08^{a}	19.05 ± 0.02^{a}	93.66 ± 0.04^{b}	-0.75 ± 0.02^{b}	16.56 ± 0.02^{b}	15.65 ± 0.01^{b}	93.57 ± 0.03^{b}	-0.68 ± 0.01^{c}	16.17 ± 0.00^{b}	15.28 ± 0.01^{b}
3 days	91.33 ± 0.18^{a}	-0.94 ± 0.01^{a}	19.35 ± 0.26^{a}	19.04 ± 0.18^{a}	93.63 ± 0.03^{b}	-0.76 ± 0.01^{b}	16.60 ± 0.03^{b}	15.69 ± 0.02^{b}	93.58 ± 0.09^{b}	-0.66 ± 0.01^{c}	16.17 ± 0.05^{b}	15.29 ± 0.02^{b}
5 days	91.38 ± 0.09^{a}	-0.96 ± 0.00^{a}	19.36 ± 0.06^{a}	19.00 ± 0.09^{a}	93.64 ± 0.02^{b}	-0.71 ± 0.01^{b}	16.59 ± 0.01^{b}	15.67 ± 0.02^{b}	93.61 ± 0.01^{b}	-0.63 ± 0.01^{c}	16.21 ± 0.01^{b}	15.31 ± 0.01^{b}
10 days	91.34 ± 0.11^{a}	-0.94 ± 0.01^{a}	19.36 ± 0.06^{a}	19.03 ± 0.01^{a}	93.63 ± 0.03^{b}	-0.73 ± 0.02^{b}	16.61 ± 0.02^{b}	15.70 ± 0.03^{b}	93.59 ± 0.01^{b}	-0.65 ± 0.01^{c}	16.19 ± 0.01^{b}	15.30 ± 0.01^{b}
40 days	91.34 ± 0.03^{a}	-0.94 ± 0.01^{a}	19.39 ± 0.03^{a}	19.04 ± 0.01^{a}	93.62 ± 0.01^{b}	-0.73 ± 0.00^{b}	16.61 ± 0.01^{b}	15.70 ± 0.01^{b}	93.57 ± 0.03^{b}	-0.66 ± 0.01^{c}	16.18 ± 0.02^{b}	15.30 ± 0.01^{b}
60 days	91.36 ± 0.01^{a}	-0.92 ± 0.00^{a}	19.51 ± 0.02^{a}	19.15 ± 0.01^{a}	93.61 ± 0.01^{b}	-0.75 ± 0.00^{b}	16.59 ± 0.01^{b}	15.69 ± 0.00^{b}	93.55 ± 0.02^{b}	-0.64 ± 0.01^{c}	16.16 ± 0.01^{b}	15.30 ± 0.01^{b}
b)												
0 days	91,78 ± 0,02^{a}	-0,75 ± 0,00^{a}	17,39 ± 0,01^{a}	17,00 ± 0,00^{a}	90,93 ± 0,06^{b}	-0,61 ± 0,02^{b}	18,97 ± 0,04^{b}	18,77 ± 0,01^{b}	92,74 ± 0,01^{c}	-0,53 ± 0,01^{c}	17,45 ± 0,05^{a}	16,75 ± 0,05^{a}
3 days	91,78 ± 0,03^{a}	-0,76 ± 0,00^{a}	17,38 ± 0,03^{a}	17,00 ± 0,04^{a}	90,92 ± 0,12^{b}	-0,59 ± 0,01^{b}	19,10 ± 0,21^{b}	18,90 ± 0,15^{b}	92,75 ± 0,01^{c}	-0,53 ± 0,01^{c}	17,44 ± 0,06^{a}	16,73 ± 0,05^{a}
5 days	91,78 ± 0,01^{a}	-0,75 ± 0,01^{a}	17,38 ± 0,01^{a}	16,99 ± 0,01^{a}	90,88 ± 0,01^{b}	-0,59 ± 0,01^{b}	19,11 ± 0,14^{b}	18,92 ± 0,14^{b}	92,75 ± 0,01^{c}	-0,53 ± 0,01^{c}	17,46 ± 0,01^{a}	16,75 ± 0,01^{a}
10 days	91,78 ± 0,02^{a}	-0,76 ± 0,01^{a}	17,39 ± 0,01^{a}	17,00 ± 0,00^{a}	90,93 ± 0,01^{b}	-0,59 ± 0,01^{b}	19,05 ± 0,08^{b}	18,85 ± 0,08^{b}	92,75 ± 0,01^{c}	-0,51 ± 0,01^{c}	17,43 ± 0,02^{a}	16,72 ± 0,02^{a}
40 days	91,79 ± 0,01^{a}	-0,76 ± 0,01^{a}	17,39 ± 0,01^{a}	17,00 ± 0,00^{a}	90,95 ± 0,02^{b}	-0,59 ± 0,01^{b}	19,04 ± 0,04^{b}	18,83 ± 0,05^{b}	92,75 ± 0,01^{c}	-0,53 ± 0,01^{c}	17,43 ± 0,02^{a}	16,72 ± 0,02^{a}
60 days	91,77 ± 0,03^{a}	-0,76 ± 0,01^{a}	17,39 ± 0,01^{a}	17,01 ± 0,00^{a}	90,97 ± 0,03^{b}	-0,59 ± 0,01^{b}	19,10 ± 0,03^{b}	18,88 ± 0,04^{b}	92,75 ± 0,01^{c}	-0,53 ± 0,00^{c}	17,42 ± 0,01^{a}	16,71 ± 0,00^{a}

Note: $^{a, b, c}$, Means within the same columns, labeled with the same letter, do not statistically differ from each other ($P > 0.05$).

All films showed high brightness values ($L^* \geq 90.88$) showing that they are light-colored. The results are in agreement with Rhim et al. (1998) who obtained values of $L^* \geq 89.80$ in films made by in films made by soy protein isolate. Brindle and Krochta (2008) also obtained translucent films with glycerol, whey protein isolate and hydroxypropyl methylcellulose blended films. According to Ramos et al. (2013), whey proteins are important polymers used in the preparation of transparent and flexible biodegradable films.

All the samples maintained stable values of lightness (L^*), redness (a^*) and yellowness (b^*) throughout the storage period ($P > 0.05$).

Many authors have reported WPI films to be tasteless, colorless, transparent, odorless and flexible (Fairley et al., 1996, Perez-Gago et al., 2003, Perez-Gago et al., 2005), which is in agreement with our results and no changes in color were observed during the storage period highlighting the high stability of the films.

Mechanical properties of whey protein films are important to maintain their integrity during storage and handling. Young´s modulus (YM), tensile strength (TS) and elongation at break (EB) are parameters that describe the film behavior under different conditions and expose changes in the film microstructure.

Data pertaining to the tensile properties of WPI films, with different probiotic bacteria, are shown in Table 2.4.

Table 2.4 - Texture characteristics of control and probiotic films incorporated with *B. animalis* subsp. *lactis* BB-12 and *L.* casei 01 during storage for 60 days at 23 ± 1 ºC (a) and 4 ± 1 ºC (b), viz. Young's Modulus (MPa), Tensile Strength (MPa) and Elongation at Break (%).

	Control			*B. animalis* subsp. *lactis* BB-12			*L.* casei 01		
	Young's Modulus (Mpa)	Tensile Strength (Mpa)	Elongation at Break (%)	Young's Modulus (Mpa)	Tensile Strength (Mpa)	Elongation at Break (%)	Young's Modulus (Mpa)	Tensile Strength (Mpa)	Elongation at Break (%)
a)									
0 days	0,311 ± 0.000[a]	0,770 ± 0,004[a]	65,500 ± 1,803[a]	0,309 ± 0.000[a]	0,675 ± 0,025[b]	64,667 ± 0,577[a]	0,310 ± 0.002[a]	0,615 ± 0,020[c]	65,000 ± 3,500[a]
3 days	0,312 ± 0.001[a]	0,767 ± 0,007[a]	65,500 ± 1,803[a]	0,309 ± 0.000[a]	0,676 ± 0,025[b]	64,671 ± 0,570[a]	0,310 ± 0.002[a]	0,615 ± 0,020[c]	65,018 ± 3,500[a]
5 days	0,311 ± 0.001[a]	0,771 ± 0,003[a]	65,534 ± 1,859[a]	0,309 ± 0.000[a]	0,676 ± 0,025[b]	64,700 ± 0,608[a]	0,310 ± 0.002[a]	0,616 ± 0,020[c]	65,037 ± 3,505[a]
10 days	0,311 ± 0.001[a]	0,777 ± 0,003[a]	65,533 ± 1,790[a]	0,310 ± 0.001[a]	0,675 ± 0,025[b]	64,737 ± 0,553[a]	0,311 ± 0.002[a]	0,615 ± 0,026[c]	65,004 ± 3,505[a]
40 days	0,311 ± 0.000[a]	0,769 ± 0,010[a]	65,521 ± 1,782[a]	0,313 ± 0.006[a]	0,679 ± 0,023[b]	64,677 ± 0,586[a]	0,312 ± 0.003[a]	0,610 ± 0,020[c]	64,968 ± 3,449[a]
60 days	0,312 ± 0.001[a]	0,767 ± 0,010[a]	65,500 ± 1,803[a]	0,310 ± 0.000[a]	0,679 ± 0,025[b]	64,684 ± 0,547[a]	0,311 ± 0.003[a]	0,615 ± 0,023[c]	65,001 ± 3,499[a]
b)									
0 days	0,310 ± 0.002[a]	0,771 ± 0,005[a]	65,502 ± 1,807[a]	0,310 ± 0.001[a]	0,676 ± 0,024[b]	64,700 ± 0,578[a]	0,311 ± 0.000[a]	0,615 ± 0,020[c]	65,020 ± 3,498[a]
3 days	0,312 ± 0.001[a]	0,770 ± 0,004[a]	65,510 ± 1,810[a]	0,310 ± 0.002[a]	0,677 ± 0,023[b]	64,685 ± 0,547[a]	0,10 ± 0.002[a]	0,612 ± 0,020[c]	65,018 ± 3,497[a]
5 days	0,311 ± 0.001[a]	0,771 ± 0,003[a]	65,527 ± 1,850[a]	0,308 ± 0.000[a]	0,676 ± 0,025[b]	64,720 ± 0,609[a]	0,311 ± 0.003[a]	0,615 ± 0,020[c]	65,021 ± 3,500[a]
10 days	0,310 ± 0.001[a]	0,775 ± 0,006[a]	65,530 ± 1,795[a]	0,311 ± 0.002[a]	0,679 ± 0,024[b]	64,733 ± 0,554[a]	0,312 ± 0.002[a]	0,613 ± 0,025[c]	65,020 ± 3,500[a]
40 days	0,311 ± 0.002[a]	0,771 ± 0,009[a]	65,525 ± 1,804[a]	0,310 ± 0.002[a]	0,679 ± 0,025[b]	64,635 ± 0,580[a]	0,312 ± 0.003[a]	0,610 ± 0,021[c]	65,027± 3,486[a]
60 days	0,312 ± 0.001[a]	0,767 ± 0,012[a]	65,507 ± 1,806[a]	0,310 ± 0.000[a]	0,678 ± 0,025[b]	64,690 ± 0,548[a]	0,310 ± 0.000[a]	0,615 ± 0,022[c]	65,011 ± 3,489[a]

Note: [a, b, c], Means within the same columns, labeled with the same letter, do not statistically differ from each other ($P > 0.05$).

No significant differences ($P > 0.05$) during the storage period for all the parameters studied, viz. YM, TS and EB, were observed in the films with or without bacteria.

Young´s modulus was around 0.310 ± 0.002 MPa (Table 2.4) for all the different films tested and were similar to those reported for similar whey protein based films (Ramos et al., 2013). The addition of probiotics did not affect the YM ($P > 0.05$) values. A similar trend was observed by Sánchez-González et al. (2014) with incorporation of *L. acidophilus* in glycerol plasticized sodium caseinate films. In addition, Piermaria et al. (2015) studied glycerol plasticized films with inclusion of both, lactobacilli and yeasts, and demonstrated that no apparent changes in the YM value were observed (Piermaria et al., 2015).

WPI films showed TS ranging between 0,777 ± 0,003 and 0,610 ± 0,020 MPa (Table 2.4). These results are in agreement with Ramos et al. (2013) who obtained similar values in edible WPI films.

The incorporation of probiotic cells in films lead to a slight decrease in TS parameter value ($P < 0.05$). Similar results were achieved by Kanmani and Lim (2013), through addition of probiotic cells into the pullulan/starch film which resulted in a reduction of TS.

The results for EB of WPI films ranging between 65.534 ± 1,859 and 64,968 ± 3,449 % are depicted in Table 2.4. These findings are in agreement with those of Ramos et al. (2013), who obtained analogous values in edible WPI films. No significant differences were observed in EB values throughout the storage period for both probiotic bacteria at each of the temperatures tested ($P > 0.05$). Both temperature and the microorganism's addition appeared to not affect the elasticity of the films. In agreement with these results, no change in this parameter was reported by Sánchez-González et al. (2014) for methylcellulose and hydroxypropyl methylcellulose films.

The FTIR spectra of WPI films and the potential changes in the molecular structure of WPI films upon incorporation of probiotics cells were studied using FTIR-ATR.

The spectra of WPI control, with *B. animalis* subsp. *lactis* BB-12 at 0 days of storage at 23 ± 1 ºC (a), with *B. animalis* subsp. *lactis* BB-12 at 60 days of storage at 23 ± 1 ºC (b), with *B. animalis* subsp. *lactis* BB-12 at 0 days of storage at 4 ± 1 ºC (c) and with *B. animalis* subsp. *lactis* BB-12 at 60 days of storage at 4 ± 1 ºC (d) are displayed together in Fig. 2.1.

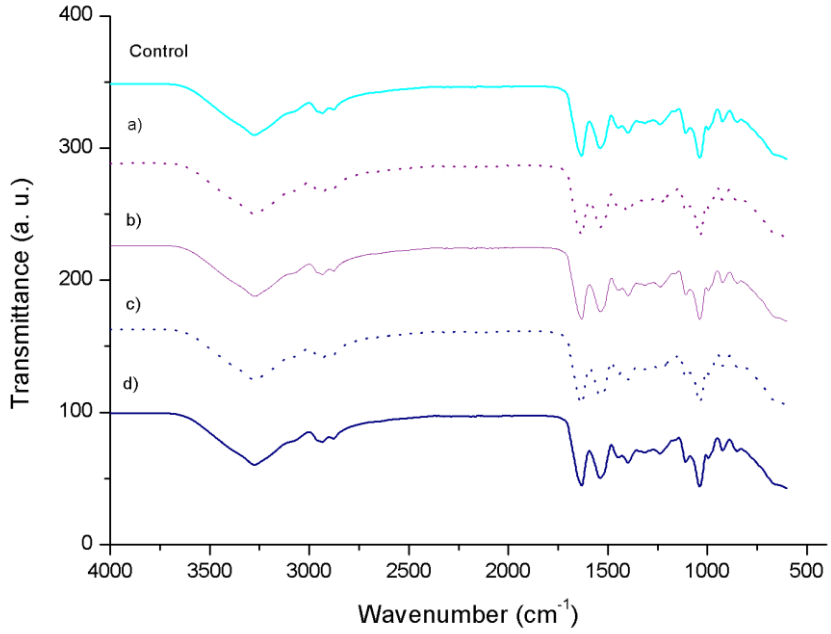

Figure 2.1 - FTIR absorbance spectra of whey protein based films without microorganism (control), with *B. animalis* subsp. *lactis* BB-12 at 0 days of storage at 23 ± 1 ºC (a), with *B. animalis* subsp. *lactis* BB-12 at 60 days of storage at 23 ± 1 ºC (b), with *B. animalis* subsp. *lactis* BB-12 at 0 days of storage at 4 ± 1 ºC (c) and with *B. animalis* subsp. *lactis* BB-12 at 60 days of storage at 4 ± 1 ºC (d).

In the spectrum of WPI (Fig. 2.1 control) the main transmittance peaks were located in the spectral range: (i) 800-1150 cm^{-1} - transmittance bands of glycerol; (ii) 1200-1350 cm^{-1} - combination of N-H in-plane bending with C-N stretching vibrations (amide III); (iii)

1400-1550 cm^{-1} - N-H bending (amide II); (iv) 1600-1700 cm^{-1} - stretching vibration of C=O and C-N groups (amide I); (v) 2850-2980 cm^{-1} - C-H stretching; and (vi) 3000-3600 cm^{-1} - free and bound O-H and N-H groups (Ramos et al., 2013, Bagheri et al., 2013).

The temperature and the storage time did not affect the molecular structure of the films. Moreover, the incorporation of microorganisms did not modify the infrared spectra, as observed in Fig. 2.1 (a, b, c and d), which may indicate that there are no interactions between the probiotics and the carry material.

Some studies have also demonstrated that microorganisms did not affect the FTIR spectra of WPI and denatured WPI microcapsules (Rajam and Anandharamakrishnan, 2014) and the WPC capsules (López-Rubio et al., 2012).

The WPI films containing microorganisms revealed a very slight difference compared to WPI films without probiotics only among TS values. The bacteria once integrated into the film structure, may make the film slightly less brittle and more malleable. Nevertheless, these small differences are further confirmed by FTIR-ATR results where no changes in the films structures are reported.

2.4.3. Morphology of films by SEM

A scanning electron microscopy analysis of the WPI based films allowed visualization of *B. animalis* subsp. *lactis* BB-12 and *L. casei* 01 (Fig. 2.2b, c and e). The incorporation of probiotic strains in the edible film did not confer any noticeable modification to the structural conformation of the films (Fig. 2.2a), apart from the presence of the bacterial cells embedded (tiny rod-like shapes as indicated by the arrows) in the plasticized protein matrix. This incorporation of the cells within the protein matrix can have a protective

effect upon cell viability, has it has been shown in studies where protein matrices were utilized to produce microcapsules with probiotics, enhancing probiotic resistance to environmental conditions (Tripathi and Giri, 2014, Rodrigues et al., 2011). The different temperatures did not result in detectable changes in the microstructure of the probiotic films (results not shown). In all cases, the films retained their structure, having non-uniform and reticular characteristics, these images are in agreement with previous studies, in which similar results are observed (Shakila et al., 2012).

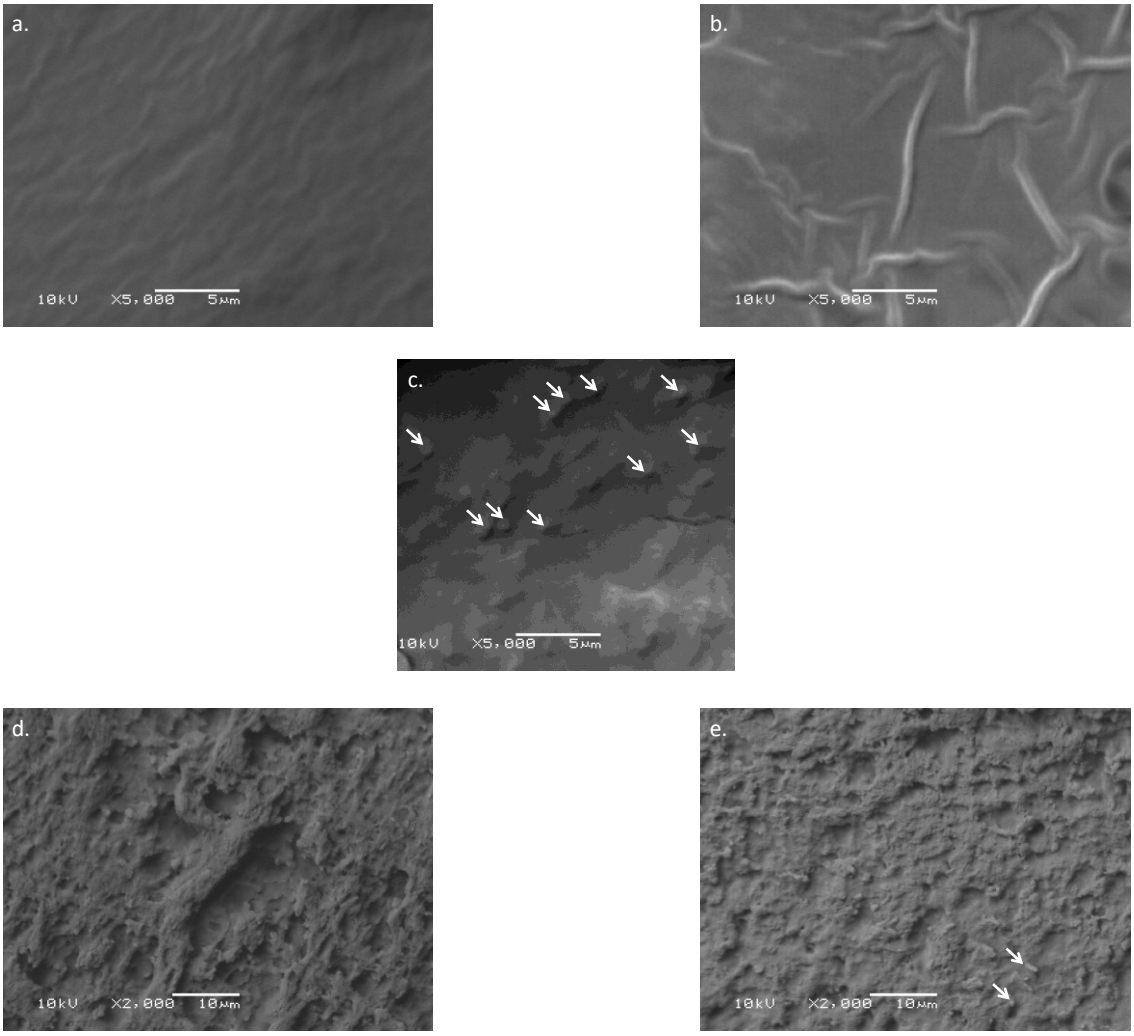

Figure 2.2 - Scanning electron microscopy visualization of the surface (a, b and c) and cross-section (d and e) of whey protein-based films without microorganism (a and d) and with *B. animalis* subsp. *lactis* BB-12 or *L. casei* 01 (b, c and e). Arrows indicate bacterial cells.

2.5. Conclusions

Taking into account the results, we conclude that WPI edible films could act as a suitable matrix to incorporate probiotic lactic acid bacteria and support their viability throughout shelf-life. The probiotics remained viable for 60 days maintaining high viable cell number levels.

Storage of the edible films under refrigerated temperature was the best condition to maintain cellular viability of *B. animalis* subsp. *lactis* BB-12 and *L. casei* 01. It is also noteworthy that no structural changes in the film during the study period were observed, providing these films with high durability and with a high shelf-life allowing good solutions for food applications.

Thus, incorporation of probiotics in edible films could be a good carrier for these bacteria to be ingested together with food and simultaneously exert specific activities in food. Further investigations are warranted to demonstrate these potential applications.

CHAPTER 3 - Impact of whey protein coating incorporated with *Bifidobacterium* and *Lactobacillus* on sliced ham properties

Joana Odila Pereira, José Soares, Maria J. P. Monteiro, Ana Gomes & Manuela Pintado

CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Porto, Rua Arquiteto Lobão Vital, 172, 4200-374 Porto, Portugal

3.1. Abstract

Edible coatings/films with functional ingredients may be a solution to consumers' demands for high-quality food products and an extended shelf-life.

The aim of this work was to evaluate the antimicrobial efficiency of edible coatings incorporated with probiotics on sliced ham preservation. Coatings was developed based on whey protein isolates with incorporation of *Bifidobacterium animalis* subsp. *lactis* BB-12 or *Lactobacillus casei* 01.

The physicochemical analyses showed that coating decreased water and weight loss on the ham. Furthermore, color analysis showed that coated sliced ham, exhibited no color change, comparatively to uncoated slices.

The edible coatings incorporating the probiotic strains inhibited detectable growth of *Staphylococcus spp*., *Pseudomonas spp*., *Enterobacteriaceae* and yeasts/molds, at least, for 45 days of storage at 4 ºC. The sensory evaluation demonstrated that there was a

preference for the sliced coated ham. Probiotic bacteria viable cell numbers were maintained at ca. 10^8 CFU/g throughout storage time, enabling the slice of ham to act as a suitable carrier for the beneficial bacteria.

3.2. Introduction

The growing demand for ready-to-eat (RTE) and convenient food products is creating some challenges for the control of food quality and safety. Furthermore, today's consumers are greatly concerned about food value and determining their preferences based on sensory and nutritional characteristics (Norton and Sun, 2008).

Food products, such as sliced ham, that have been processed by heat-treatment are generally recognized as safe (GRAS). Nevertheless, contamination with foodborne pathogens during post-processing, such as peeling, slicing and repackaging, may be the cause of outbreaks of foodborne diseases (Reij et al., 2004). On the other hand, deleterious microorganisms growing on the product may cause changes on its quality that may compromise its shelf-life and subsequent consumer acceptance. Such rationale has occasioned the search for new ways to inhibit microbial growth in RTE products, while maintaining their quality, freshness, safety and nutritional value. A new tendency in food technology preservation consists in using active packaging, which is a system where product, packaging and environment interact, and may contribute to enlarge this safety border (Appendini and Hotchkiss, 2002, Biji et al., 2015).

Antimicrobial packaging is an innovative approach within the framework of the active packaging concept, owing their favorable interaction with the food to the possibility of increasing shelf life and improving safety and even organoleptic properties. This technology has been developed to delay, reduce and/or inhibit the growth of microorganisms on the surface of the foods, thus satisfying the consumer's demands for

safe, nutritious, convenient and ready-to-use foods (Durango et al., 2006, Seol et al., 2009, Biji et al., 2015).

Traditionally, the antimicrobial effect of edible coatings has been obtained by incorporating in their structure antimicrobial agents, such as enzymes, polysaccharides, bacteriocins and more recently herbs, spices and essential oils (Appendini and Hotchkiss, 2002, Pranoto et al., 2005a, Tajkarimi et al., 2010). More recently, the possibility of including bacteria capable of producing antimicrobial substances *in situ* has begun to be explored. The first reports published over the last few years (Gialamas et al., 2010, Concha-Meyer et al., 2011, Léonard et al., 2014, Leonard et al., 2015, Sánchez-González et al., 2013, Sánchez-González et al., 2014) include only lactic acid bacteria (LAB) with main inhibitory effect upon *L. monocytogenes*; none of these studies have envisaged studying the effect of beneficial probiotic bacteria for such purpose.

The potential of applying antimicrobial coatings to prevent post-processing-surface contamination of foodborne pathogens on ham products has been demonstrated in a few studies (Lee and Min, 2013, Moradi et al., 2011, Santiago-Silva et al., 2009, Theinsathid et al., 2012).

Despite the existence of these studies none have attempted the incorporation of probiotic strains capable of generating antimicrobial compounds *in situ* (Tapia et al., 2007, Kanmani and Lim, 2013, Soukoulis et al., 2014b). Therefore, this study was the first encompassing the application of whey edible films containing probiotics, to exert antimicrobial effect, against contaminant microorganisms in sliced ham. The incorporation of these microorganisms in coatings/films will be particularly innovative, by combining the antimicrobial effect of the coatings (antimicrobial substances synthesis or by competition *in situ*) with health benefits (as a carriers of probiotic bacteria).

Both, *Lactobacillus* spp. and *Bifidobacterium* spp. produce antimicrobial substances (acids and bactericides) that can help protect food, as well as other health benefits, since many strains therein have been proven as probiotics. Probiotic bacteria are defined as "live microorganisms, that when administered in adequate amounts not less than 10^6 CFU/g (preferentially at least 10^7 CFU/g), confer a health benefit on the host" (FAO/WHO, 2002).

Despite its promising application, the main challenge to the incorporation of multifunctional bacteria into food matrices is ensuring their viability (Madureira et al., 2011a). However, there have been several studies in which this objective has been achieved (Tapia et al., 2007, López de Lacey et al., 2012, Kanmani and Lim, 2013, Soukoulis et al., 2014b).

In chapter 2 it was reported the successful incorporation of *B. animalis* subsp. *lactis* BB-12 and *L. casei* 01 in whey protein dried edible films reaching high cell viable numbers (10^8 CFU/g film) throughout storage time. Although the results are preliminary evidences, it was demonstrated that the study of bacteria with inhibitory properties incorporated in edible coatings is a promising field and may have great interest; this enables the production of functional coatings for foods to assure a protective antimicrobial environment *in situ*, working as a carrier for stable and viable microorganisms, endowed with beneficial properties to human intestinal flora.

The employment of this type of whey protein film may be limiting in products where the absence of lactose is a required priority. However, processed meat products use mostly whey protein to modify the overall technological and sensorial characteristics of a meat system such as water holding capacity, fat holding capacity and texture properties (Petracci et al., 2013), so it was a justified matrix to use.

Thus, the objective of this study was to evaluate the effect of edible whey protein coatings incorporating functional *Lactobacillus* and *Bifidobacterium* strains on the quality and safety of packed sliced ham foreseeing extension of shelf-life and at the same time analyzing the potential to be used as a carrier for viable probiotics.

3.3. Materials and Methods

3.3.1. Bacterial strains, media and growth conditions

Bifidobacterium animalis subsp. *lactis* BB-12 and *Lactobacillus casei* 01 kindly donated by Christian Hansen (Denmark) were stored at -80 ºC in de Man–Rogosa–Sharpe (MRS) broth (Biokar Diagnostics, France) supplemented with 30 % (v/v) sterile glycerol. The microorganisms were reactivated, and pre-cultures were prepared in MRS medium supplemented with filter-sterilized 0.05 % (w/v) L-cysteine·HCl (Fluka, St. Gallen, Switzerland) and incubated at 37 °C during 24 h under anaerobic conditions. Subsequently, grown cells were harvested by centrifugation at 4000 rpm for 30 min, at 4 °C. The supernatant was discarded, and the pellet was resuspended in a 0.9 % (w/v) NaCl sterile solution for posterior incorporation in the coatings.

3.3.2. Formulation of the coatings

The coatings solutions, one for each probiotic strain, were prepared by dissolving 10 % (w/v) whey protein isolate (WPI) powder (Armor Proteins, Saint Brice en Coglés, France) in deionized water, according to Pérez-Gago and Krochta (2002). Glycerol was added at 5 % (w/w), as a plasticizer, and the resulting solutions were homogenized for 2 h.

Subsequently, the solutions were heated in a water bath at 80 ºC, for 20 min and cooled to room temperature.

Afterwards, 5 % (w/w) of centrifuged solution of 0.9 % NaCl inoculum of either *B. animalis* subsp. *lactis* BB-12 or *L. casei* 01 (15 ml) was added to each 300 ml of film solution to attain a final concentration of 10^9 CFU/ml, as described in chapter 2.

3.3.3. Coating the sliced ham

The ham samples were composed of: poultry meat (turkey breast) (60 %), water, starch, salt, dextrose, milk proteins, flavoring, emulsifier (sodium tripolyphosphate), gelling agent (processed Eucheuma seaweed), chloride potassium, vegetable fiber, antioxidants (trisodium citrate and sodium ascorbate), spices, preservative (sodium nitrite), flavor enhancer (monosodium glutamate) and smoke flavor (contains soy and milk (including lactose)). The sliced ham was coated in the production chain of the Primor Charcutaria-Prima, S.A. facility, by slice immersion, for 2 min, in each of the previous WPI probiotic coating solutions. The samples were prepared in three conditions, one without bacteria (control ham), one with *B. animalis* subsp. *lactis* BB-12 (C_{ba}) and one with *L. casei* 01 (C_{lc}). Therefore, 21 packages per condition were prepared, containing 3 slices each (in total 63 slices were allocated to each condition).

The excess of liquid in the sliced ham was drained for around 30 s, and thereafter it was packaged (three slices of ham in each package) under usual conditions, i.e. modified atmosphere (70 % of nitrogen and 30 % of carbon dioxide). Packages were stored at 4 ºC for 45 days and at each sampling point (0, 5, 10, 15, 25, 35, 45 days), three packages of each condition were evaluated (total of 21 packages per condition).

3.3.4. Enumeration of bacteria

The viability of incorporated probiotic bacteria was studied in the sliced ham during 45 days of storage at 4 ºC. The enumeration of microbial contamination was also monitored during the same period through the determination of total mesophilic aerobic bacteria *Staphylococcus* spp., *Pseudomonas* spp., *Enterobacteriaceae* and yeasts and molds.

At each sampling point (0, 5, 10, 15, 25, 35, 45 days) 10 g of ham slice sample were diluted to 1:10 (w/v) in sterile 1 % (w/v) sodium citrate (Merck, Darmstadt, Germany) solution into a Stomacher (Seward, West Sussex, UK) bag and homogenized in a Stomacher 400 Circulator (Seward) for 3 min at 260 rpm.

Appropriate sequential 10-fold dilutions were done in sterile peptone water and plated, in triplicate, onto the respective medium and incubated under the preferential conditions for the different microorganisms.

The medium for *Lactobacillus* was MRS and the plates were incubated under anaerobic conditions in a plastic anaerobic jar with an AnaeroGen sachet (an atmosphere generation system, Oxoid, Basingstoke, England) at 37 °C during 48 h. The medium for *Bifidobacterium* was MRS supplemented with filter-sterilized 0.05 % (w/v) L-cysteine·HCl (Fluka, St. Gallen, Switzerland) and incubated at 37 °C during 48 h under anaerobic conditions in a plastic anaerobic jar with an AnaeroGen sachet (an atmosphere generation system, Oxoid, Basingstoke, England). Total mesophilic aerobic bacteria were enumerated on plate count agar (PCA) (Biokar Diagnostics, France). *Staphylococcus* spp. were enumerated on Baird-Parker agar (BPA) (Lab M, Bury, UK), supplemented with egg-yolk and tellurite emulsion (Biokar Diagnostics, France), as proposed by Baird-Parker (1969) and *Pseudomonas* spp. were enumerated on Pseudomonas agar base (Lab M, Bury, UK). Plates from PCA, BPA and Pseudomonas media were incubated

aerobically at 37 °C for 24 h. *Enterobacteriaceae* were counted on violet red bile glucose agar (VRBGA) (Lab M, Bury, UK), incubated aerobically at 37 °C for 24 h. The yeasts and molds were determined on rose bengal agar (Lab M, Bury, UK), with 0.1 g/L chloramphenicol (Fluka, Buchs, Switzerland), incubated aerobically at 30 °C for 3 d. The surface plating technique described by Miles et al. (1938) was followed for all samples and growth media, except with violet red bile glucose agar, for which the pour-plate technique was used.

3.3.5. Sliced ham characterization

3.3.5.1. Water activity

The water activity (a_w) was measured using a HygroLab 2 (from Rotronic, Bassersdrof, Germany). Samples (ca. 0.5 g) were placed on the sample holder; a sealed system was formed by placing the water activity probe on top of the sample holder. The probe was equipped with a small fan to circulate air inside the sample container, a thin film capacitance sensor able to measure RH from 0 to 100 ± 1.5 %, and a platinum resistance temperature detector with a precision of ± 0.3 ºC. When a_w became constant (which usually took less than 1 h), its value was recorded. Calibration resorted to six saturated solutions of known a_w (viz. LiCl = 0.114, $MgCl_2$ = 0.329, K_2CO_3 = 0.443, Mg $(NO_3)_2$ = 0.536, NaBr = 0.653 and KCl = 0.821). The assays were run in quadruplicate.

3.3.5.2. Color

To measure the color a portable Chroma meter CR-400 (from Minolta Chroma, Osaka, Japan) with a *C D65 illuminant, with a light source of pulsed xenon lamp, an aperture size of 8 mm, a closed cone and a standard observer of 2 ° Closely matches CIE 1931 ($\bar{x}2\lambda$, $\bar{y}\lambda$, $\bar{z}\lambda$), was used. A CIELab color scale was employed to measure the degree of lightness (L), redness ($+a$) or greenness ($-a$), and yellowness ($+b$) or blueness ($-b$) of the films. A white standard plate, with color coordinates $L_{standard}$ = 97.7, $a_{standard}$ = 0.04 and $b_{standard}$ = 1.47, was used to calibrate the equipment. The color of the ham slices was expressed as the total difference in color (ΔE), calculated according to:

$$\Delta E = [(L - L_0)^2 + (a - a_0)^2 + (b - b_0)^2]^{1/2}$$

where L_0, a_0 and b_0 are the initial values obtained for slices of ham under each experimental condition, and L, a and b were the values measured throughout the storage period.

For each condition, four samples were measured – and, on each slice, four readings were made on each side.

3.3.5.3. Weight loss

Each ham slice was individually weighed on an automatic electro-balance, with a precision of 0.001 g, on the first day, upon coating, and during the storage period at each sampling point. The relative weight loss, ΔW, was calculated as

$$\Delta W = ((w_0 - w)/w_0) \times 100$$

where w_0 is the initial weight and w is the final weight at each sampling point. Three readings of each slice were assessed.

3.3.5.4. pH measurement

The pH value was measured using a pH meter (Micro pH 2002, Crison, Barcelona, Spain), equipped with a probe for surface in contact directly with the ham sample. The instrument was calibrated prior to and immediately after each session using pH 4 and pH 9 standards as per the manufacturer's instructions. Three readings of each ham sample were taken.

3.3.6. Consumer study

The study was conducted in accordance with the Declaration of Helsinki. Participants were informed about the general aim and procedures for handling personal data and gave written informed consent prior to participation. All samples were prepared using food ingredients obtained via commercial suppliers and all additives were food-grade. Preparation prior to testing was performed in a dedicated preparation kitchen and samples were produced and prepared according to good hygiene and manufacturing practices.

Seventeen subjects participated in the consumer test. All participants consumed turkey sliced ham at least once a week and 72 % consumed it daily, their age ranged between 24 and 49 years old (31 ± 7) and 59 % were female. Overall liking (OL) was evaluated using a 9-point hedonic scale (Jones et al., 1955, Peryam and Pilgrim, 1957, Gaze et al., 2015). The appropriateness of the intensities of five sensory attributes – brightness, odor, flavor, acid taste, and texture was evaluated by ratings provided on a 5-point, just-about-right scale, where 1 and 2 corresponded to too weak (TW) evaluations, 3 to just about-right (JAR) and 4 and 5 to too strong (TS) evaluations (Popper, 2014).

Samples (one slice of each ham) were assigned 3-digit codes and were presented to consumers following an incomplete balanced design. No information about the samples was provided to participants, except for safety and hygiene considerations related to their production and preparation. Water was provided to clean the palate between tastings. Tasting sessions took place in the ISO 8589 (ISO, 2007) compliant sensory facilities of Escola Superior de Biotecnologia.

3.3.7. Statistical analyses

Analysis of variance was performed to determine whether probiotic bacteria (i.e. *B. animalis* subsp. *lactis* BB-12 or *L. casei* 01), coating (presence of bacteria or absence of bacteria) or storage time (0-45 d) were statistically significant sources of variation, at the 0.05 level of significance. Homoscedasticity requirements were met, i.e. experimental errors were independently and normally distributed and possess a constant variance. A paired Tukey's test was used to test for significant differences in the different microbiological and physicochemical parameters between packaging systems and controls. The significance level was set at $P < 0.05$.

All tests were performed to a 5 % significance level, using Statistical Package for Social Sciences, v. 17.0 (SPSS, Chicago IL, USA).

Data collected for the sensory analysis was performed with XLSTAT software V. 2015 (Addinsoft, Paris, France).

Overall liking ratings of C_{ba} and C_{lc} were compared pairwise with the control sample using the Friedman's test. For each sample and for each sensory attribute, the frequencies of intensity ratings (TW, JAR, TS) were determined and the corresponding proportions calculated and compared using z-test. A weighted penalty analysis was conducted to relate attribute intensity ratings to OL (Popper, 2014). Weighted penalties corresponding

to less than 20 % of respondents and to mean drops under 1.0 were considered negligible (Popper, 2014).

3.4. Results and Discussion

The physicochemical and microbiological analyses of sliced ham coated with the previously developed edible formulation (i.e. 10 % (w/v) WPI with 5 % (w/w) glycerol), as well as, that further incorporated with two probiotic bacteria, throughout 45 days of storage at 4 ºC.

3.4.1. Microbiological characterization of sliced ham

Enumeration of viable cell numbers was performed during 45 days of storage period under modified atmosphere at 4 ºC with a dual purpose: i) to monitor the microbial contamination occurring over a 45 days storage period and the role the incorporated probiotic microorganisms may play thereon; and ii) to assess the viability of the selected probiotic microorganisms throughout storage. Results obtained are shown in Figures 3.1,3.2,3.3 and 3.4.

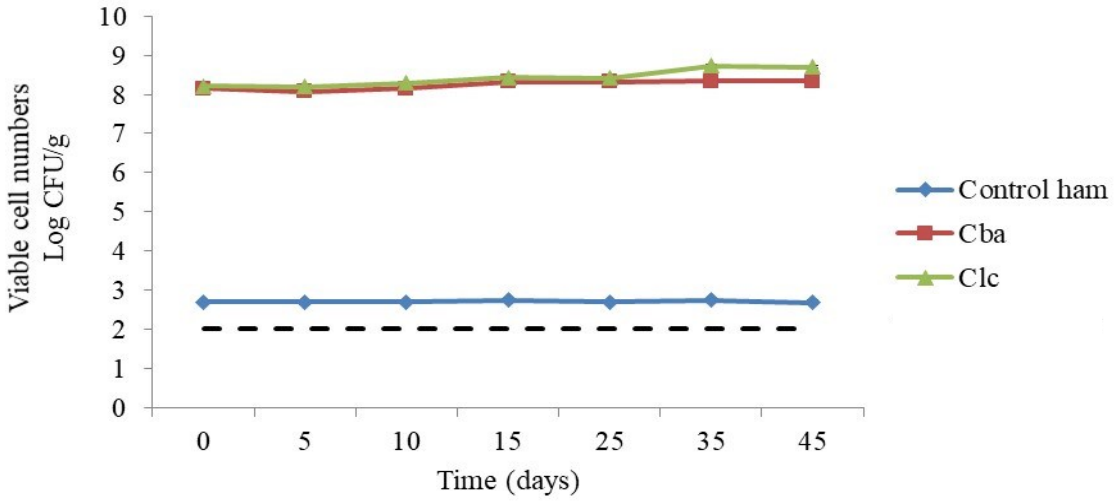

Figure 3.1 - Viable cell numbers (Log CFU/g) of *L. casei* 01 and *B. animalis* subsp. *lactis* BB-12 in uncoated ham (Control ham), coated ham with *B. animalis* subsp. *lactis* BB-12 (Cba) and coated ham with *L. casei* 01 (Clc) stored under modified atmosphere for 45 days at 4 ± 1 ºC.
Dashed line - detection limit of growth (10^2 CFU/g).

Overall, results showed an extension of the shelf-life, at least to 45 days, of the sliced modified atmosphere-packed ham, stored at 4°C, when edible films with functional bacteria were used, which is greater than that described in literature for sliced modified atmosphere-packed ham, namely 15-28 days at a storage temperature of 4°C (Ahvenainen et al., 1989). Indeed, the most widely used packaging technique for cooked meat products is modified atmosphere packaging. Several studies, performed so as to improve the shelf-life and preserve the desirable quality attributes of meat products, have shown that low-oxygen atmospheres are the most favorable to increase the associated shelf-life (Kotzekidou and Bloukas, 1996). Nevertheless, none of the published studies have enabled a doubling of the shelf-life as the edible film tested herein.

The Portuguese legislation (Regulamento(CE)n.º1441/2007, Santos et al., 2005), regulates for sliced ham kept under 0 ± 1 to 5 ± 1 ºC: maximum viable cell numbers of 10^2 CFU/g *Staphylococcus* spp. and yeasts and molds; maximum viable cell numbers of

10 CFU/g for *Enterobacteriaceae* and absence of *Pseudomonas* spp. in 25 g of the product.

Thus, considering these microbiological parameters the main microorganisms that are present in the sliced ham throughout sampling were evaluated.

As far as the incorporated probiotic strains are concerned, *Lactobacillus* and *Bifidobacterium* are considered as "GRAS" microorganisms that are safe to consume, have benefits for the consumer and nowadays are commonly used in food (Bredholt et al., 2001). These bacteria, besides other benefits, have the potential to be used in biopreservation, due their ability to produce several antimicrobial substances including lactic acid and bacteriocins (De Martinis and Freitas, 2003).

The viable cell numbers of both *L. casei* 01 and *B. animalis* subsp. *lactis* BB-12 were maintained at high and constant levels, around $10^8 \pm 0.06$ CFU/g of coated ham (above the minimum threshold of 10^7 CFU/g commonly required for probiotic claim of a food) throughout storage as observed in Fig. 3.1. Such high viable cell numbers are quite promising from both microbiological and nutritional points of view, since a minimum ingestion of 10-20 g of coated sliced ham will already provide the required amounts of viable probiotic cells (ca. 10^9 CFU/g) to enable a probiotic effect on a daily basis – such a portion size is also aligned with nutritional recommendations for such a processed food product, , demonstrating the feasibility of WPI edible film to carry and support stable and viable probiotics in ham slices.

As previously mentioned, the addition of probiotics to obtain functional edible films and coatings has been only recently studied, and in particular for meat products scant information is available (Muthukumarasamy and Holley, 2006, Tassou, 2016). The first edible film incorporating probiotics was produced by Tapia et al. (2007), but in relation to meat products, to our knowledge, the work of Muthukumarasamy and Holley (2006)

was the first study investigating the potential use of microencapsulation technology to protect probiotics in meat products, since unencapsulated *L. reuteri* viable cell numbers dropped from 7.12 ± 0.12 to 4.54 ± 0.18 log CFU/g during sausage fermentation while microencapsulated *L. reuteri* viable cell numbers only decreased by ≤ 0.5 log, so the viability of encapsulated probiotic cells was improved compared to free cells.

In this study sliced ham was coated with whey protein isolate, and the probiotic viability therein is in agreement with results obtained by Tassou (2016) who used Na-alginate based edible films to deliver probiotic bacteria in a high pressure processed meat product, ensuring viable cells above 10^6 CFU/g in all sliced ham samples during their shelf life at 4, 8 and 12 ºC. Recall that the WPI edible film tested in this study enabled the presence of viable cell numbers above 10^8 ± 0.06 CFU/g throughout a 45 days storage period.

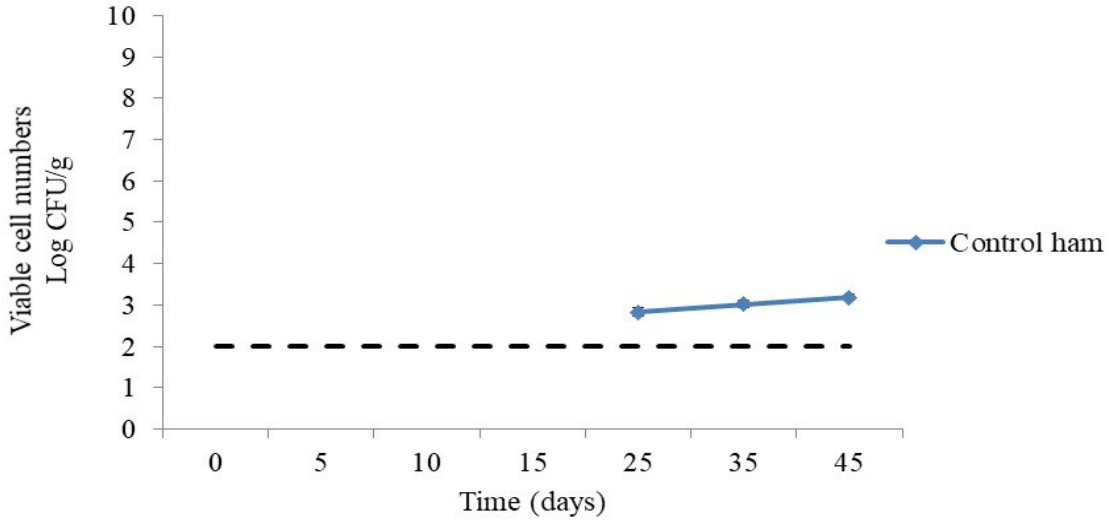

Figure 3.2 - Viable cell numbers (Log CFU/g) of *Enterobacteriaceae* in uncoated ham (Control ham) stored under modified atmosphere for 45 days at 4 ± 1 ºC.
Dashed line - detection limit of growth (10^2 CFU/g).

Viable cell numbers of enterobacteria (Fig. 3.2) reached values of 3 ± 0.10 Log CFU/g after 25 days storage in the control hams whereas in coated hams no growth of those microorganisms was detected at least up to 45 days (under the detection limit of growth);

this significant difference may be attributed to the presence of the two probiotic strains that may be exerting a protective role against these microorganisms.

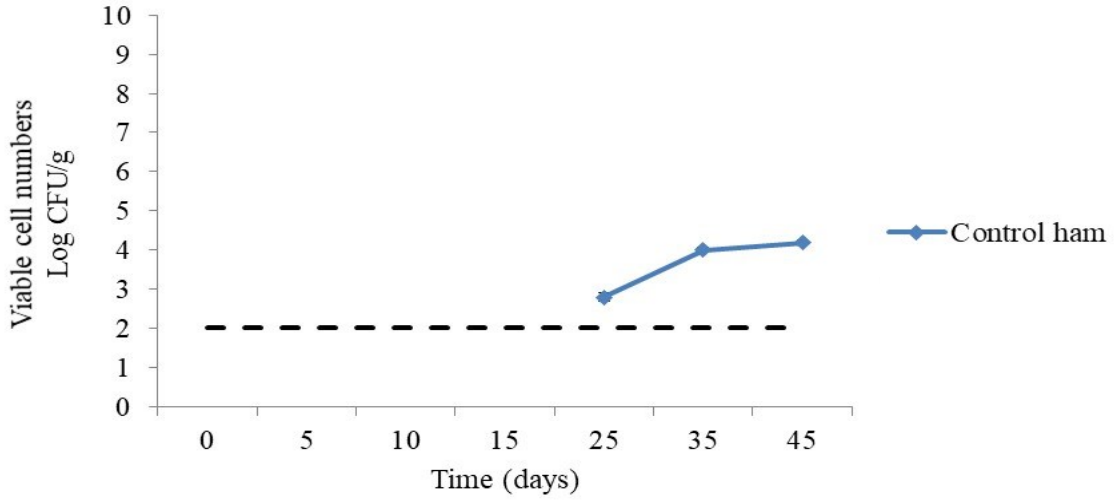

Figure 3.3 - Viable cell numbers (Log CFU/g) of *Staphylococcus* spp. in uncoated ham (Control ham) stored under modified atmosphere for 45 days at 4 ± 1 ºC.
Dashed line - detection limit of growth (10^2 CFU/g).

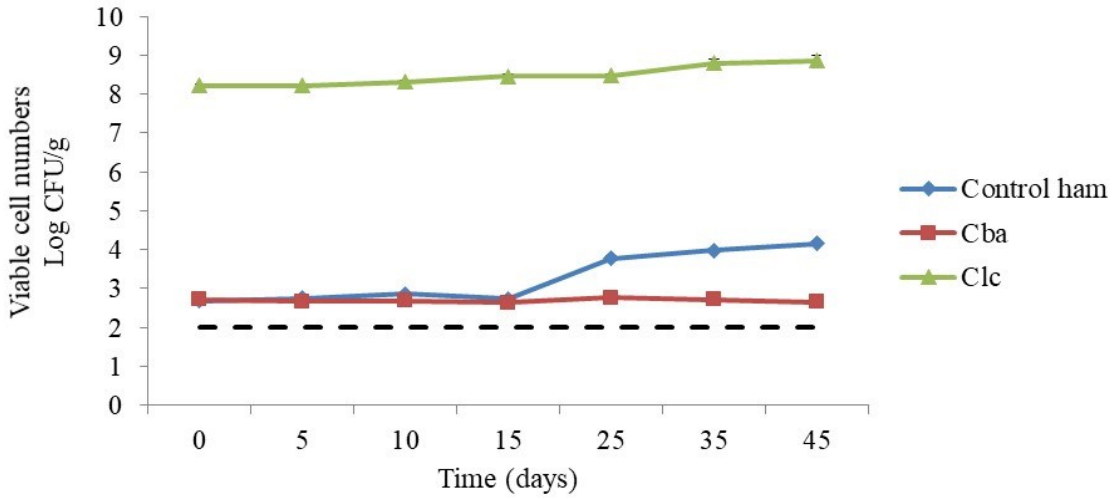

Figure 3.4 - Viable cell numbers (Log CFU/g) of total mesophilic aerobic bacteria in uncoated ham (Control ham), coated ham with *B. animalis* subsp. *lactis* BB-12 (Cba) and coated ham with *L. casei* 01 (Clc) stored under modified atmosphere for 45 days at 4±1 ºC.
Dashed line - detection limit of growth (10^2 CFU/g).

Staphylococcus spp. (Fig. 3.3) were detected in uncoated hams at 4 ± 0.05 Log CFU/g starting at 15 days and maintaining this value until the end of storage, 45 d, yet no detectable growth thereof was observed in both coated hams. Once again, this inhibition may indicate that either of the two probiotic strains tested may be exerting a protective role against these microorganisms, at least up to 45 days.

The total mesophilic aerobic bacteria counts (Fig. 3.4) were generally maintained constant and low (ca. 3 ± 0.11 log CFU/g) until 15 days of storage, after which a notorious increase in growth (up to ca. 4 ± 0.14 log CFU/g) was observed in control ham, probably due to the growth of the *Enterobacteriaceae* and *Staphylococcus* spp. Enumeration of mesophilic aerobic bacteria of the sliced ham coated with *Lactobacillus casei* 01 showed numbers ca. 8 ± 0.12 log CFU/g, since as they are aerobic-anaerobic facultative bacteria they could grow on PCA under aerobic conditions, with similar numbers registered on MRS. On the other hand, for sliced ham coated with WPI incorporated with *B. animalis* subsp. *lactis* BB-12 this trend was not evident, since these bacteria are strictly anaerobic, and the growth conditions were aerobic besides the fact that PCA does not includes specific nutrients required by these bacteria and present in MRS.

Pseudomonas and yeasts and molds were not detected in this study for all the coated samples tested (results not shown) as well as in the control sliced ham samples (results not shown).

Overall, uncoated sliced ham samples revealed a low stability to spoilage by contaminant bacteria such as *B. animalis* subsp. *lactis* BB-12 and *Staphylococcus* spp. In fact, viable cell numbers detected upon 15 d of storage increased steadily thereafter (up to 45 days) and were beyond the limit permitted by the Portuguese legislation for sliced ham (Regulamento(CE)n.º1441/2007, Santos et al., 2005). During the same storage period, no detectable growth of these contaminant bacteria was reported for sliced ham coated with

our edible WPI film containing *L. casei* 01 or *B. animalis* subsp. *lactis* BB-12 demonstrating the importance of our coating in the prevention of spoilage microorganisms. Such observed inhibition may be explained either by the possibility of competition mechanisms between probiotic and spoilage bacteria or eventually via the production of antimicrobial substances by the probiotic strains, such as lactic acid or bacteriocins.

Although most studies deal with control of spoilage microorganisms by metabolic products produced by protective starter cultures (namely bacteriocins), in meat products, such as sliced ham (Enan, 2006, Jofré et al., 2008a, Jofré et al., 2008b, Santiago-Silva et al., 2009), some attention has been given to the use of the protective LAB cultures themselves for control against contaminant and pathogenic microorganisms in meat products (Bredholt et al., 2001, Jacobsen et al., 2003, Maragkoudakis et al., 2009, Gao et al., 2015). For example, Enan (2006) observed an anti-listerial effect with a plantaricin producing *L. plantarum* strain in uncooked and cooked chicken meat. Moreover, similar results were achieved by Yildirim et al. (2007) against *L. monocytogenes*, when *Bifidobacterium bifidum* and *L. lactis* bacteriocins were applied in raw chicken meat under refrigeration conditions. Jofré et al. (2008a) and Jofré et al. (2008b) used nisin and enterocins A and B in combination with high hydrostatic pressure treatment against *Salmonella* in refrigerated sliced cooked ham and sausages. Furthermore, Santiago-Silva et al. (2009) demonstrated that pediocin-coated package film reduces *Salmonella* growth in sliced ham. In all these studies it is the presence of added bacteriocins (metabolic end products) that has been related with the observed antimicrobial effect, whereas in our study, the *Lactobacillus* and *Bifidobacterium* strains themselves demonstrated the antimicrobial activity *in situ* against the studied spoilage microorganisms. Such a trend has also been observed with the use of other lactic acid

bacteria directly applied to meat products. Bredholt et al. (2001) demonstrated that *Lactobacillus sakei* can inhibit the growth (decrease of 10^3 CFU/g) of a cocktail of three rifampicin resistant mutant *L. monocytogenes* strains both at 8 and 4°C in cooked ham and of servelat sausage, while Jacobsen et al. (2003) showed that using *Leuconostoc carnosum* added to the sliced meat product was more effective in preventing growth of *L. monocytogenes* than the use of the partially purified leucocins 4010 or bacteriocin produced during fermentation before heat treatment of the saveloy.

Another study, demonstrated that *Enterococcus faecium* PCD71 and *Lactobacillus fermentum* ACA-DC179 applied in chicken meat against *L. monocytogenes* and *S. enteritidis,* respectively, could reduce growth of these pathogenic bacteria (Maragkoudakis et al., 2009). Gao et al. (2015) showed that *L. sakei* C2 and its bacteriocin have potential to inhibit *L. monocytogenes* CMCC 54002 in meat products, and bring about the entire elimination of *L. monocytogenes* CMCC 54002 when they were used in combination.

All these studies demonstrate the antimicrobial activity against spoilage microorganisms, yet none of these have used a carrier strategy such as a whey edible coating for the effective immobilization and functionality of protective cultures.

Although the nature of the observed antimicrobial activity against contaminant and pathogenic food microorganism in sliced ham needs to be further investigated, this study is, to the best of our knowledge, the first report of an application of a live protective culture with *Lactobacillus* or *Bifidobacterium* strains, incorporated in edible coating against microorganisms in sliced ham enabling a two-fold increase in the product shelf-life without affecting the quality attributes Furthermore, besides a safety issue, such coated sliced ham may constitute an interesting alternative carrier for probiotic strains. Since reported survival of *L. casei* 01 and *B. animalis* subsp. *lactis* BB-12 in the sliced

ham product was high and passage through gastrointestinal tract is anticipated to be effective based on previous preliminary results (data not shown), a 10-20 gram slice of ham (preferably low-fat ham important from a nutritional point of view) may enable bacteria to reach gut target sites in sufficiently high numbers to promote a beneficial effect. Despite preliminary results this property needs to be further investigated.

3.4.2. Physicochemical characterization of sliced ham

Weight loss, water activity and pH of sliced ham and coated sliced ham was monitored throughout the storage period.

The a_w ranged from 0.714 ± 0.003 to 0.838 ± 0.001 ($P < 0.001$) in the control sliced ham and 0.932 ± 0.001 to 0.938 ± 0.001 ($P > 0.05$) in both coated sliced hams.

The pH ranged from 5.80 ± 0.01 to 6.12 ± 0.01 ($P < 0.001$) in the control sliced ham and 6.03 ± 0.01 to 6.08 ± 0.01 ($P > 0.05$) in both coated sliced hams.

These parameters have varied significantly ($P < 0.001$) in the control sliced ham during the 45 days storage period (Table 3.1), except for pH between 0- and 5-days storage. In the control sliced ham, the observed decrease in water activity is related with weight loss as a consequence of the decrease in water content.

Table 3.1 - Evolution of weight loss (g), water activity (a_w) and pH of uncoated ham (Control ham), coated ham with *B. animalis* subsp. *lactis* BB-12 (Cba) and coated ham with *L. casei* 01 (Clc) stored under modified atmosphere for 45 days at 4 ± 1 ºC.

	Control			*B. animalis* subsp. *lactis* BB-12			*L. casei 01*		
	Weight loss (g)	a_w	pH	Weight loss (g)	a_w	pH	Weight loss (g)	a_w	pH
0 days	12.468 ± 0.007^{a}	0.838 ± 0.001^{a}	6.12 ± 0.01^{a}	14.207 ± 0.005^{b}	0.937 ± 0.001^{b}	6.07 ± 0.01^{b}	14.211 ± 0.001^{b}	0.938 ± 0.001^{c}	6.05 ± 0.01^{c}
5 days	12.391 ± 0.009^{b}	0.825 ± 0.001^{b}	6.11 ± 0.01^{a}	14.204 ± 0.004^{b}	0.937 ± 0.001^{b}	6.08 ± 0.01^{b}	14.209 ± 0.001^{b}	0.938 ± 0.001^{c}	6.05 ± 0.01^{c}
15 days	11.511 ± 0.014^{c}	0.806 ± 0.001^{c}	6.03 ± 0.01^{b}	14.203 ± 0.002^{b}	0.935 ± 0.003^{b}	6.07 ± 0.01^{b}	14.209 ± 0.006^{b}	0.937 ± 0.001^{c}	6.04 ± 0.01^{c}
25 days	11.408 ± 0.004^{d}	0.785 ± 0.001^{d}	5.96 ± 0.01^{c}	14.202 ± 0.005^{b}	0.934 ± 0.001^{b}	6.05 ± 0.02^{b}	14.205 ± 0.007^{b}	0.935 ± 0.001^{c}	6.04 ± 0.01^{c}
35 days	11.103 ± 0.002^{e}	0.737 ± 0.002^{e}	5.90 ± 0.01^{d}	14.200 ± 0.002^{b}	0.932 ± 0.002^{b}	6.04 ± 0.01^{b}	14.204 ± 0.001^{b}	0.934 ± 0.001^{c}	6.03 ± 0.01^{c}
45 days	11.090 ± 0.006^{f}	0.714 ± 0.003^{f}	5.80 ± 0.01^{e}	14.200 ± 0.001^{b}	0.932 ± 0.001^{b}	6.04 ± 0.01^{b}	14.203 ± 0.000^{b}	0.933 ± 0.001^{c}	6.03 ± 0.01^{c}

Note: $^{a, b, c, d, f}$ Means ± standard error, within the same columns, labeled with the same letter, do not statistically differ from each other ($P > 0.05$).

Our results are generally in agreement with the findings of Glass et al. (2007) who reported that the results for ham pH and water activity are 6.39 ± 0.02 and 0.967 ± 0.000, respectively and with those presented by Bredholt et al. (2001) who described that the ham had pH and water activity values of approximately 6.2 and 0.97, respectively.

It is noteworthy that the coating protects the sliced ham from water loss and maintained the weight and pH during the storage period.

All samples investigated in this study were manufactured entirely from turkey breasts and were all taken from the same piece of ham, to minimize differences in the visual appearance at the beginning of the study (Fig. 3.5).

Control ham Cba Clc

Figure 3.5 - Appearance of sliced uncoated ham (Control ham), coated ham with *B. animalis* subsp. *lactis* BB-12 (Cba) and coated ham *L. casei* 01 (Clc) stored under modified atmosphere at 4 ± 1 ºC with 15 days of storage.

Table 3.2 - Color characteristics of uncoated ham (Control ham), coated ham with *B. animalis* subsp. *lactis* BB-12 (Cba) and coated ham with *L. casei* 01 (Clc) stored under modified atmosphere for 45 days at 4 ± 1 ºC, viz. L* (black–white), a* (green–red), b* (blue–yellow) and ΔE (color difference).

	Control				*B. animalis* subsp. *lactis* BB-12				*L. casei 01*			
	*L**	*a**	*b**	Δ*E*	*L**	*a**	*b**	Δ*E*	*L**	*a**	*b**	Δ*E*
0 days	73.23 ± 0.01[a]	5.31 ± 0.02[a]	9.72 ± 0.00[a]	26.35 ± 0.00[a]	73.24 ± 0.01[a]	5.26 ± 0.01[a]	9.68 ± 0.01[a]	26.32 ± 0.01[a]	73.23 ± 0.01[a]	5.29 ± 0.03[a]	9.67 ± 0.01[a]	26.33 ± 0.01[a]
5 days	73.23 ± 0.02[a]	5.29 ± 0.01[a]	9.70 ± 0.02[a]	26.34 ± 0.03[a]	73.21 ± 0.02[a]	5.30 ± 0.01[a]	9.67 ± 0.01[a]	26.35 ± 0.02[a]	73.24 ± 0.01[a]	5.31 ± 0.01[a]	9.71 ± 0.01[a]	26.34 ± 0.01[a]
15 days	73.22 ± 0.01[a]	5.28 ± 0.03[a]	9.70 ± 0.01[a]	26.34 ± 0.00[a]	73.22 ± 0.02[a]	5.28 ± 0.01[a]	9.70 ± 0.02[a]	26.35 ± 0.01[a]	73.24 ± 0.00[a]	5.31 ± 0.01[a]	9.70 ± 0.02[a]	26.34 ± 0.01[a]
25 days	73.20 ± 0.00[a]	5.27 ± 0.02[a]	9.70 ± 0.02[a]	26.37 ± 0.01[a]	73.22 ± 0.01[a]	5.30 ± 0.01[a]	9.70 ± 0.04[a]	26.35 ± 0.00[a]	73.22 ± 0.01[a]	5.31 ± 0.03[a]	9.69 ± 0.01[a]	26.35 ± 0.01[a]
35 days	73.22 ± 0.01[a]	5.27 ± 0.01[a]	9.67 ± 0.01[a]	26.34 ± 0.02[a]	73.20 ± 0.00[a]	5.28 ± 0.01[a]	9.69 ± 0.01[a]	26.37 ± 0.00[a]	73.22 ± 0.01[a]	5.29 ± 0.01[a]	9.69 ± 0.04[a]	26.35 ± 0.02[a]
45 days	73.20 ± 0.00[a]	5.27 ± 0.00[a]	9.68 ± 0.02[a]	26.36 ± 0.01[a]	73.23 ± 0.02[a]	5.30 ± 0.00[a]	9.69 ± 0.02[a]	26.34 ± 0.01[a]	73.21 ± 0.01[a]	5.31 ± 0.01[a]	9.69 ± 0.01[a]	26.36 ± 0.01[a]

Note: [a] Means ± standard error, within the same columns, labeled with the same letter, do not statistically differ from each other ($P > 0.05$).

In Table 3.2 are displayed the color characteristics reported for all samples of both uncoated and coated sliced ham. All samples showed values around 73.20 ± 0.00 to 73.24 ± 0.01 for lightness (L^*), 5.26 ± 0.01 to 5.31 ± 0.03 for redness (a^*) and 9.67 ± 0.01 to 9.72 ± 0.00 for yellowness (b^*). The results are in agreement with Iqbal et al. (2013) who obtained values of value L^* (70.14-77.60), a^* value (3.49-6.02) and b^* (3.72-9.60) in cooked, pre-sliced turkey hams.

The presence of the edible film with either *Lactobacillus casei* 01 or *B. animalis* subsp. *lactis* BB-12 did not affect significantly ($P > 0.05$) the color of the sliced ham (L^*, a^*, b^* and ΔE). The latter parameter might be of particular interest, not only because ΔE values greater than 3 have been reported as a visual threshold for the distinction of color changes by the human eye, but also because of the potential formation of products with free radical scavenging activities that could potentially protect entrapped bacteria from free radical-driven oxidative damage (Martínez-Cervera et al., 2011).

Slices of ham coated with *B. animalis* subsp. *lactis* BB-12 and *L. casei* 01 solutions exhibited a similar appearance.

3.4.3. Consumer acceptance of sliced ham

A consumer study was performed, as was done by Santos et al. (2015), a consumer acceptability using a 9-point hedonic scale to evaluate OL of the samples and subsequently complemented with the JAR scales to provide insights on improvements. Results of consumer assessments showed that hams coated with *B. animalis* subsp. *lactis* BB-12 (7.25 ± 1.13) and with *L. casei* 01 (7.00 ± 1.37) were liked moderately and were better accepted than the control sample (5.59 ± 2.15) ($P < 0.05$).

Figure 3.6 shows the frequencies of TW, JAR and TS intensity ratings for each sample and for all sensory attributes evaluated.

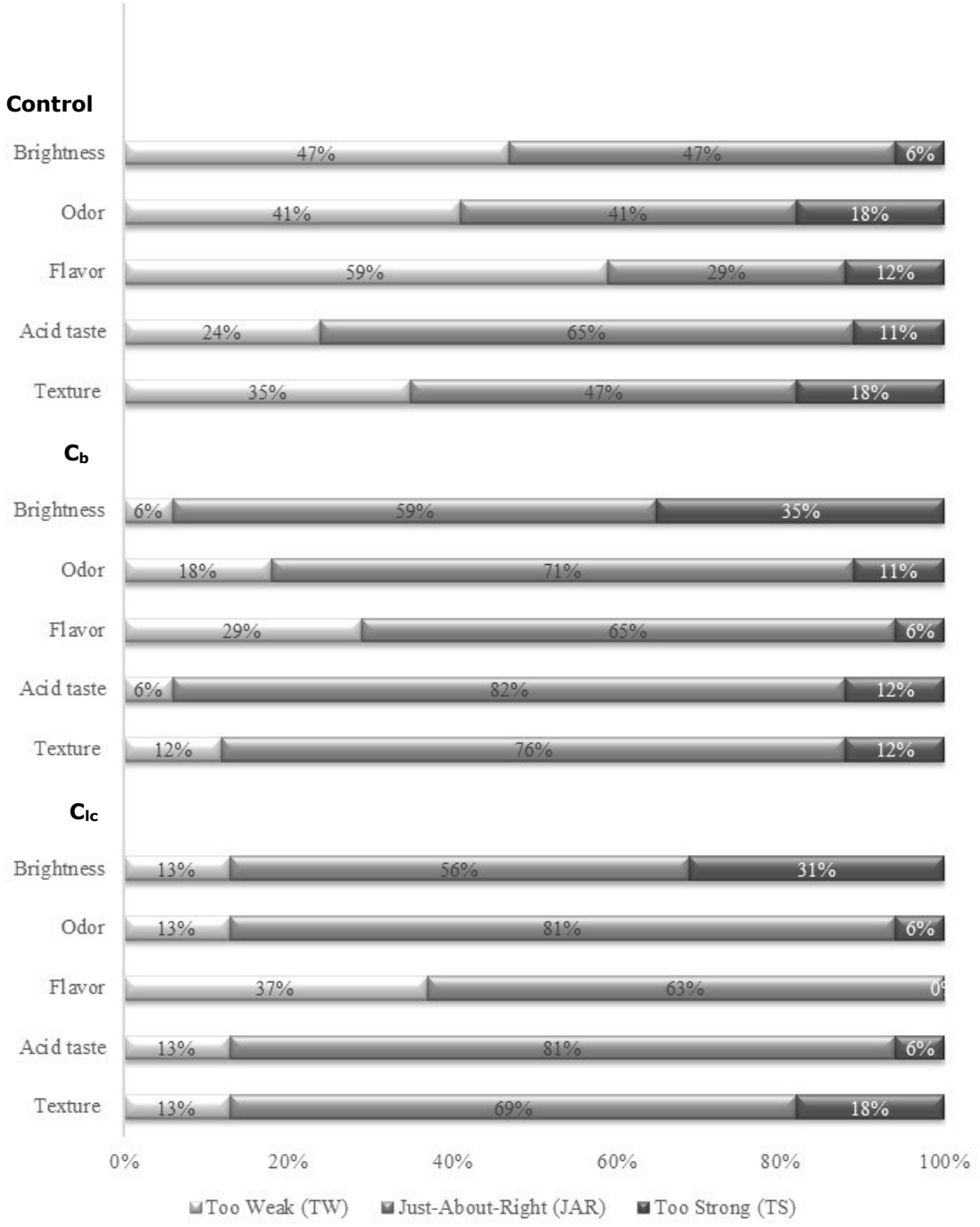

Figure 3.6 - JAR evaluations (%) for sliced ham - uncoated ham (Control ham), coated ham with *B. animalis* subsp. *lactis* BB-12 (Cba) and coated ham with *L. casei* 01 (Clc).

A preponderance of JAR ratings was observed for C_{ba} and C_{lc} for the five attributes evaluated, with their frequencies ranging from 56 to 82 %. For the control sample, however, lower proportions of JAR ratings ($P < 0.05$) were observed for odor and flavor intensities, than for C_{ba}, whereas higher proportions of TW ratings for brightness were found ($P < 0.05$). Also, for the control sample, lower proportions of JAR ratings than for ham coated with C_{lc} were observed for flavor and texture intensities ($P < 0.05$) and a higher proportions of TW rating for brightness intensities. Moreover, penalty analysis showed only negligible penalties for C_{ba} and C_{lc} for all attributes, except for TW flavor evaluations of the last. For the control sample non negligible penalties were observed relatively to TW evaluations of brightness, odor and flavor intensities.

Since significant differences were found in this study, we should, in the future, increase the number of consumers and elaborate a sensory profile of the coated samples obtained by Polarized Projective Mapping (PPM), such was done by Horita et al. (2017).

3.5. Conclusions

Based on reported results, WPI edible coatings may act as a suitable matrix to incorporate probiotic *L. casei* 01 or *B. animalis* subsp. *lactis* BB-12 and such coating is easily applied on sliced ham.

Application of a probiotic edible coating to sliced ham enables decreased water and weight loss on the ham surface throughout storage contributing to product freshness. Furthermore, no color changes or differences in pH values, between uncoated and coated slices of ham were detected, assuring the expected quality until 45 days.

The antimicrobial edible coatings successfully inhibited detectable growth of spoilage bacteria, at least up to 45 days of storage, more 28 days than the normal recommended shelf-life.

Furthermore, probiotic bacteria numbers were maintained at high and constant levels of ca. 10^8 CFU/g throughout storage time, assuring required levels to promote functionality associated to a probiotic and thus providing the associated positive effects to the consumers, requiring an ingestion of a portion size of 10-20 g to assure the recommended daily dose of 10^9 CFU. The sensory results demonstrated that the coated slices of ham were the most appreciated by the consumers, compared to the uncoated slices, suggesting a potential consumer market for coated sliced ham.

PART III - Study of WPI and ALG-based films and coatings incorporating *Bifidobacterium animalis* subsp. *lactis* BB-12 and prebiotics

CHAPTER 4 - Characterization of edible films based on alginate or whey protein incorporated with *Bifidobacterium animalis* subsp. *lactis* BB-12 and prebiotics

Joana Odila Pereira, José Soares, Eduardo Costa, Sara Silva, Ana Gomes & Manuela Pintado

CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Porto, Rua Arquiteto Lobão Vital, 172, 4200-374 Porto, Portugal

4.1. Abstract

Recently, edible films showed to be an effective strategy for the delivery of functional ingredients, such as probiotics and prebiotics. With that in mind two soluble fibers (inulin and fructooligosaccharides) were selected as prebiotic elements, in whey protein isolate (WPI) and alginate (ALG) matrices plasticized with glycerol and used for the incorporation of *Bifidobacterium animalis* subsp. *lactis* BB-12.

The results obtained showed that the viability of the *B. animalis* subsp. *lactis* BB-12 probiotic strain was maintained within the minimum threshold (10^6 CFU/g) necessary to act as a probiotic throughout 60 days of storage at 23 ºC. The incorporation of prebiotic compounds improved *B. animalis* subsp. *lactis* BB-12 viability, with inulin showing the best performance, as it maintained the viability at 7.34 log CFU/g. The compositional characteristics (biopolymer type and prebiotics addition) of the film forming solutions

had no significant impact upon the viability of the probiotic strain. The incorporation of probiotics and prebiotics did not modify the infrared spectra, revealing that the molecular structure of the films was not modified. The moisture content and water solubility decreased positively in WPI- and ALG-based films with the addition of prebiotics compounds.

Overall, the results obtained in this work support the use of WPI films containing inulin as a good strategy to immobilize *B. animalis* subsp. *lactis* BB-12, with potential applications in the development of functional foods.

4.2. Introduction

Environmental problems associated with non-natural products used in food packaging, such as synthetic plastics and other materials, and the demand for high food quality have led to the development of innovative food packaging systems. Food coatings and films have specific characteristics, such as renewability, degradability and edibility, that make such materials suitable for food packaging applications, which are essential to preserve the physical, organoleptic and nutritional value of food during storage, transportation and distribution (Espitia et al., 2014, Baldwin et al., 2012). Furthermore, edible films incorporated with bioactive compounds promote new functionalities or extend the shelf life of food products and open new possibilities as a carrying material for functional bacteria and prebiotics, beyond basic nutrition (Soukoulis et al., 2014a, Tapia et al., 2007).

Edible films can be prepared from different structural materials such as lipids (Hambleton et al., 2009), polysaccharides (Jiménez et al., 2013, Jridi et al., 2014) and proteins (Ramos et al., 2011, Ramos et al., 2012, Ramos et al., 2013) or by combining two or several of these compounds.

Among biopolymers used to produce edible films, proteins have received considerable interest since it provides a film with distinct and valuable properties. Edible films from whey protein isolate (WPI) possess relevant sensorial, optical and mechanical barrier properties, besides its positive transparency, tasteless and odorless, which can make them a favorable carrier for functional compounds (Cuq et al., 1995, Wittaya, 2012, Gennadios and Weller, 1990, Fang et al., 2002, Hernandez-Izquierdo and Krochta, 2008, Hernandez-Izquierdo and Krochta, 2009, Ramos et al., 2012a, Schmid et al., 2012).

In addition to protein films, ALG films are also widely used today. An ALG film can act as a semipermeable barrier to moisture, gases, and aromatics, while maintaining structural integrity and handling characteristics, and holding the capacity to retain volatile aromatic compounds from food products. Additionally, it can be a carrier for important additives, such as, anti-browning agents, colorants, flavors, nutrients, spices and antimicrobial compounds (that can extend product shelf-life and reduce the risk of pathogen growth on food surfaces) and probiotics (Rhim, 2004, Rojas-Graü et al., 2009, Lin and Zhao, 2007). According to some authors, probiotics have a large number of benefits, among which are the regulation of the gastro-intestinal tract, stimulation of the immune system, reduction of serum cholesterol levels, prevention of cardiovascular disease and several forms of cancer (Enujiugha and Badejo, 2017, Sarao and Arora, 2017). Probiotics can be incorporated into the food matrices through the previously described edible films (Soukoulis et al., 2014a, Soukoulis et al., 2014b, Rößle et al., 2011, López de Lacey et al., 2014), since it is important to protect them from the damage induced by environmental conditions generated in and outside the matrices, or by food processing and storage (Fu and Chen, 2011).

Considering that probiotics viability and microbial load are significant constraints for their efficacy the addition of a prebiotic element may be of key importance, as prebiotics

have the potential to improve probiotic cell numbers, its survival in the gastrointestinal tract and its further attachment and growth in the intestine (Burgain et al., 2011). The definition of prebiotics was recently reviewed and considered a substrate that is selectively utilized by host microorganisms conferring a health benefit (Gibson et al., 2017b). It has been reported that the ingestion of prebiotics prevents several forms of cancer (Roberfroid, 2007, Saad et al., 2013) and some intestinal disorders, such as ulcerative colitis and irritable bowel disease (Bosscher et al., 2006). Furthermore, prebiotics can be added successfully as co-components for microencapsulation conferring a beneficial effect on probiotics cell viability in a dried format (Fritzen-Freire et al., 2012). In addition, the symbiotic combination of prebiotics and probiotics promotes the inhibiting of human or animal pathogens and promote bifidogenicity (Mugambi et al., 2012).

The products containing both probiotics and prebiotics are commonly defined as "symbiotic", attributed to products in which the prebiotic selectively favors the probiotics strains (Schrezenmeir and de Vrese, 2001). Recently, some authors suggested the inclusion of prebiotics was a suitable strategy to preserve probiotics in films (Romano et al., 2014, Tavera-Quiroz et al., 2015, Soukoulis et al., 2014a).

Although the above studies represent innovations of great interest, there work regarding the combination of these compounds in edible films is, to the best of our knowledge, still quite limited. So, considering this, the main objective of this study was to compare the microbiological and physicochemical characteristics of sodium alginate-based and whey protein-based edible films incorporated with *B. animalis* subsp. *lactis* BB-12 combined with prebiotic compounds, namely inulin and FOS.

4.3. Materials and Methods

4.3.1. Bacterial strain, media and growth conditions

Bifidobacterium animalis subsp. *lactis* BB-12 was stored at -80 ºC in de Man Rogosa and Sharpe (MRS) broth (Biokar Diagnostics, France) supplemented with 30 % (v/v) glycerol. Afterwards, the probiotic strain was reactivated in MRS broth supplemented with filter-sterilized L-cysteine·HCl at 0.05 % (w/v) (Fluka, St. Gallen, Switzerland) and incubated at 37 °C during 24 h under anaerobic conditions. Subsequently, grown cells were harvested by centrifugation at 4000 rpm for 30 min, at 4 °C. The pellet was re-suspended in a 0.9 % (w/v) NaCl sterile solution for future film incorporation.

4.3.2. Film formulations

WPI-based film forming solutions were prepared by dissolving WPI at 10 % (w/v) (Armor Proteins, Saint Brice en Coglés, France) in deionized water, according to Pérez-Gago and Krochta (2002). Glycerol (Panreac, Barcelona, Spain) was added at 5 % (w/w), as a plasticizer, and solutions were homogenized for 2 h.

ALG-based film forming solutions were prepared by dissolving sodium-ALG at 2 % (w/v) (FMC Biopolymer, Ireland) and glycerol at 1.2 % (w/w) in deionized water and homogenized for 2 h. Then, both solutions were heated in a water bath at 80 ºC, for 20 min and cooled at room temperature. The prebiotics were included in the film formulation by adding high soluble inulin at 2 % (w/v) (Orafti®HIS, BENEO, Germany) or Fructooligosaccharides (FOS) at 2 % (w/v) (Orafti®, BENEO, Germany), and the probiotic strain (5 %, v/v), was incorporated to reach a final concentration of 10^9 CFU/ml.

Films were according to the procedure of Gounga et al. (2007) and Oses et al. (2009). Briefly, 300 ml of the final solution were casted in sterile Teflon plates which were then dried at room conditions for 24 h in a ventilated incubator. Subsequently, the films were peeled off and conditioned in a controlled storage room (Packaging Center, CBQF, Porto Portugal) at 23 ± 2 ºC and 50 ± 2 % RH, for at least 72 h prior to testing (ASTM, 2002). Films with only probiotics were prepared as control. All films produced are described in detail in Table 4.1.

Table 4.1 - Compositional aspects of the film forming solutions

Edible film	Biopolymer	Prebiotic	Probiotic
WCBA	WPI	-	*B. animalis* subsp. *lactis* BB-12
WIBA	WPI	Inulin	*B. animalis* subsp. *lactis* BB-12
WFBA	WPI	FOS	*B. animalis* subsp. *lactis* BB-12
ACBA	ALG	-	*B. animalis* subsp. *lactis* BB-12
AIBA	ALG	Inulin	*B. animalis* subsp. *lactis* BB-12
AFBA	ALG	FOS	*B. animalis* subsp. *lactis* BB-12

4.3.3. Enumeration of bacteria and storage stability

Each film was cut into 1 cm diameter circular discs, stored in plastic bags under vacuum conditions and sampled at 0, 3, 5, 10, 40 and 60 d of storage at 23 ºC. At each sampling point, the disks were put in a sterile flask, 2 ml of sterile peptone water (1 g/L) were added and the samples were homogenized by vortexing for 1 min to completely dissolve the film and extract the bacteria. Sequential 10-fold dilutions were carried out in sterile peptone water, plated in quadruplicate onto MRS and incubated under anaerobic conditions in a plastic anaerobic jar with an AnaeroGen sachet (an atmosphere generation system, Oxoid, Basingstoke, England) at 37 °C during 48 h.

4.3.4. Film characterization

4.3.4.1. Thickness

The thickness of each film was measured using a micrometer Model m120 (from Adamel Lhomargy, Roissy en Brie, France), to the nearest 0.001 mm. The mean thickness was calculated from five independent measurements, taken randomly at different locations on each film sample.

4.3.4.2. Water activity

The water activity (a_w) of films was measured using a HygroLab 2 (Bassersdrof, Germany). Pieces of film (ca. 0.5 g) were placed on the sample holder of the water activity device and a sealed system was formed by placing the water activity probe on top of the sample holder. The probe was equipped with a small fan to circulate air inside the sample container, a thin film capacitance sensor able to measure RH from 0 to 100 ± 1.5 %, and a platinum resistance temperature detector with a precision of ± 0.3 °C. When a_w became constant (which usually took less than 1 h), its value was recorded. Calibration curves were drawn using six saturated solutions of known a_w (viz. LiCl = 0.114, $MgCl_2$ = 0.329, K_2CO_3 = 0.443, Mg $(NO_3)_2$ = 0.536, NaBr = 0.653 and KCl = 0.821). The tests were performed in quadruplicate.

4.3.4.3. Moisture content

Film samples (0.5 g) were weighed into glass crucibles and dried at 105 °C in an oven for 24 h. Moisture content was determined as a percentage of the initial film weight loss after drying and reported on a wet basis. The analysis was performed in quadruplicate for each film.

4.3.4.4. Water solubility

The previously dried film samples, used to determine the initial dry matter, were immersed in 50 ml distilled water and placed in a stirred water bath at 25 °C. After 24 h, the samples were filtered through Whatman No. 1 filter paper. The filters containing any insolubilized film were then dried at 105 °C for 24 h. The water solubility (WS) of the film was calculated using the following equation described by Norajit et al. (2010):

$$WS\ (\%) = [(W_o - W_f) / W_0] \times 100$$

where W_o and W_f are initial and insoluble dry matter, respectively. All tests were carried out quadruplicate.

4.3.4.5. Film color

Film color was evaluated using a portable Chroma meter CR-400 (from Minolta Chroma, Osaka, Japan) with a *C D65 illuminant, a light source of pulsed xenon lamp, an aperture size of 8 mm, a closed cone and a standard observer of 2 ° Closely matches CIE 1931 ($\bar{x}2\lambda$, $\bar{y}\lambda$, $\bar{z}\lambda$). A CIELab color scale was employed to measure the degree of lightness (*L*), redness (+*a*) or greenness (−*a*), and yellowness (+*b*) or blueness (−*b*) of the films. Film

disks were measured, on the surface of the white standard plate, with color coordinates $L_{standard} = 97.7$, $a_{standard} = 0.04$ and $b_{standard} = 1.47$. The color of the films was expressed as the total difference in color (ΔE), calculated according to the equation bellow.

$$\Delta E = [(L_{film} - L_{standard})^2 + (a_{film} - a_{standard})^2 + (b_{film} - b_{standard})^2]^{1/2}$$

For each condition, four samples were measured and on each film disk, four readings were made on each side.

4.3.4.6. Texture analysis

Texture analysis was performed using a texturometer (TA.XT plus Texture Analyser, Stable Micro Systems, Cardiff, UK) according to the ASTM D-882-02 standard (ASTM, 2002). Each sample was cut into rectangular film probes (100 x 15 mm). Force calibration was performed with a weight of 5 Kg and height calibration was performed for Mini Tensile Grips (Stable Micro Systems). Evaluated parameters were Young's modulus (Eq. (1)), Tensile strength, and Elongation at break. Tensile strength (MPa) stands for the maximum tensile stress that the test sample can carry. Elongation at break (%) was determined as the strain at the fracture point, which corresponds to the ratio of the change of length of the specimen to initial length. All measurements were performed using five films of each formulation.

$$\text{Young's modulus (MPa)} = \frac{Force\ at\ corresponding\ strain}{Cross\text{-}sectional\ area\ of\ the\ film \times Corresponding\ strain} \quad (1)$$

4.3.4.7. FTIR-ATR analysis

The spectra of films were obtained with a FTIR, model ABB MB3000 (ABB, Zürich, Switzerland), with a horizontal attenuated total reflectance (ATR) accessory (PIKE Technologies, Madison, WI, USA) with a diamond/ZnSe crystal. All spectra were acquired with 32 scans and 4 cm^{-1} resolution, in the region of 4000-600 cm^{-1}. Three replicates were collected for each film surface sample.

4.3.5. Statistical analyses

Statistical analyses were performed using the Statistical Package for Social Sciences, v. 17.0 (SPSS, IBM, Chicago IL, USA), via two-way analysis of variance, at the 0.05 level of significance.

Homoscedasticity requirements were met, i.e. experimental errors were independently and normally distributed and possess a constant variance. A Fischer's LSD test was used to test for significant differences in the different microbiological and physicochemical properties.

4.4. Results and Discussion

In this work, we have studied the physicochemical and microbiological properties of WPI- and ALG-based films with incorporation of two types of prebiotics to evaluate the possible synergisms of probiotics strains with those prebiotics. It has been recognized that a synergistic mixture of probiotic bacteria and prebiotics encourages intestinal colonization and has been associated with a reduction of the risk of developing several

forms of cancer. The structure of some oligosaccharides makes them resilient to digestive enzymes and thus, they can reach the large intestine where they become available to be fermented by some beneficial bacteria (Pavli et al., 2018b). Thus, prebiotics addition to bioactive edible films and coatings represents an appealing technological solution for the protection of probiotic bacteria embedded with edible films. In this way, the film's chemistry and the film forming procedure is crucial for microbial survival during the storage period and their resistance to the digestive process present imperative parameters that affect the films performance.

4.4.1. Viability of B. animalis subsp. *lactis* BB-12 *during the drying process*

The changes in viable counts of *B. animalis* subsp. *lactis* BB-12 during the drying process are displayed in Fig. 4.1.

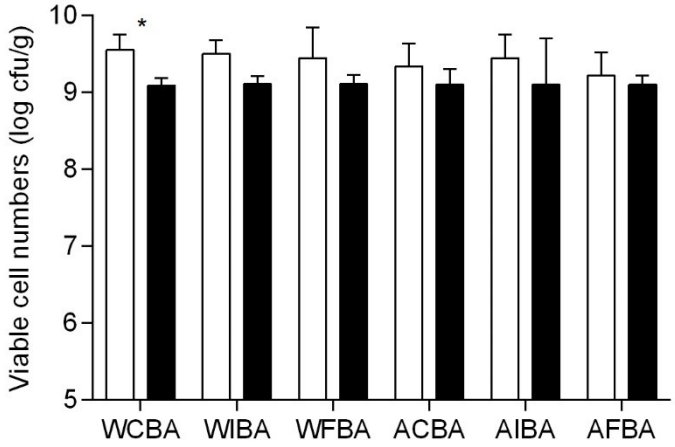

Figure 4.1 - *B. animalis* subsp. *lactis* BB-12 total viable counts during air drying for each film composition (as described in Table1). Each bar represents the standard error of the difference between treatment. Asterisk indicate significant difference between the drying process. *, $P < 0.05$; white bar = start of drying, and black bar = end of drying.

According to the ANOVA results, the type of film forming solution had no significant impact ($P > 0.05$) upon the inactivation of the probiotic strain. Overall, only a mean reduction of 0.40 and 0.24 log CFU/g were detected in WPI and ALG-based films, respectively. Therefore, no severe toxic effects were observed upon the survival of *B. animalis* subsp. *lactis* BB-12 in the film forming solutions. Furthermore, viability losses due to heat induced injuries should were considered as non-significant due to the low drying temperatures used (Soukoulis et al., 2014a). In fact, during the drying process (23 ºC, 50 % RH, 24 h), no significant ($P > 0.05$) decrease was observed in *B. animalis* subsp. *lactis* BB-12 viability, with the only exception being for WCBA films in which case a significant ($P < 0.05$) reduction in viable counts was verifies.

4.4.2. Viability of B. animalis subsp. *lactis* BB-12 *in films during storage*

Storage conditions are one of the most important factors when considering the stability of probiotics. Fig 4.2 shows the survival of the probiotic strain incorporated (stored for 60 d at 23 ºC) into the biopolymer-based films with or without prebiotics addition.

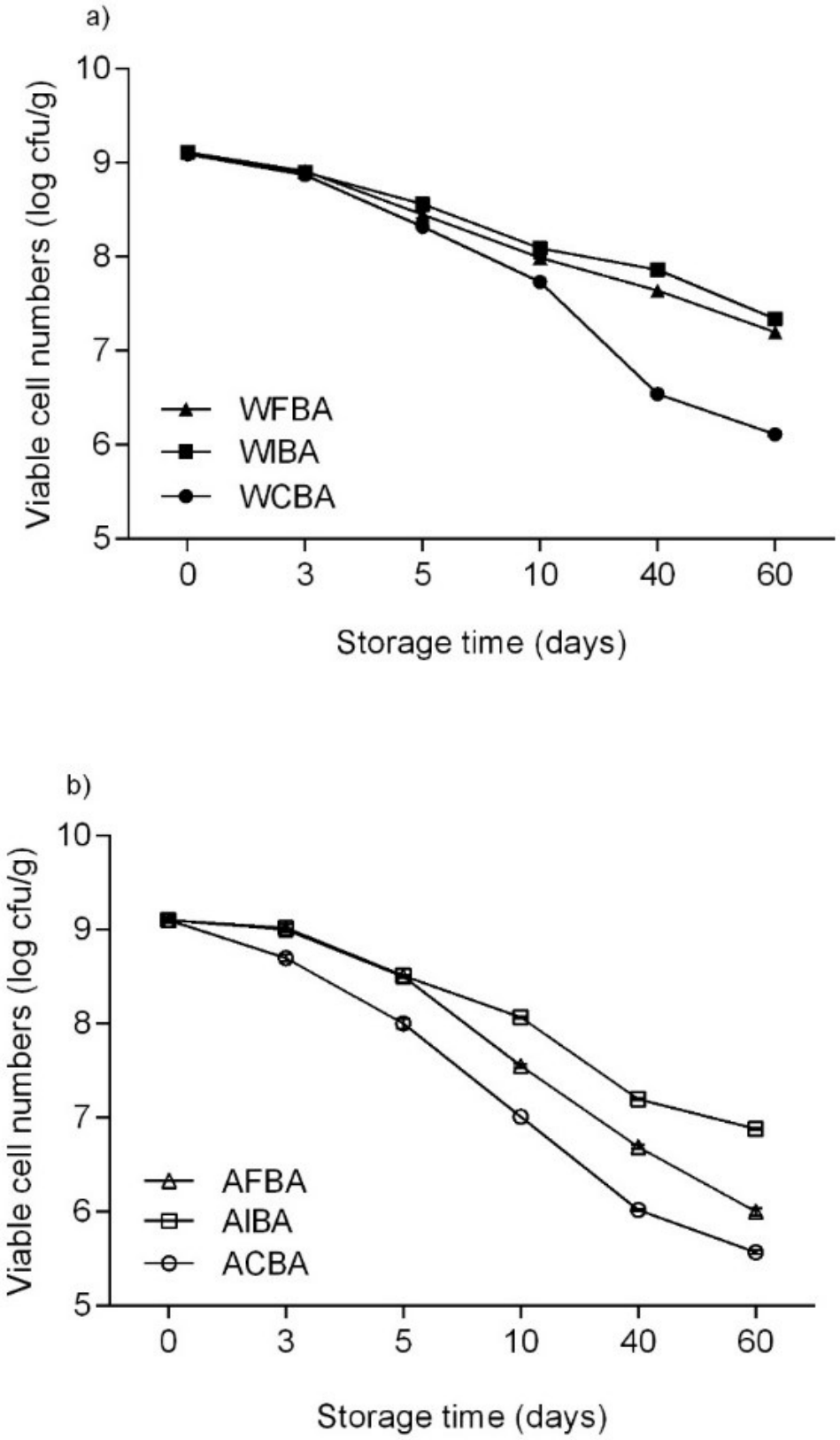

Figure 4.2 - Survival of *B. animalis* subsp. *lactis* BB-12 during storage (60 days) at room (23 ºC) temperature in a) WPI-based and b) ALG-based films with or without prebiotic incorporation.

The viability of *B. animalis* subsp. *lactis* BB-12 demonstrated a negative correlation with the storage time ($P < 0.0001$), i.e. the number of viable cells of *B. animalis* subsp. *lactis* BB-12 dropped from an initial population of 10^9 CFU/g to 10^5-10^6 in WCBA and ACBA (controls) films, and to 10^6-10^7 CFU/g in films with containing prebiotics after 60 days of storage. Moreover, the difference between prebiotic containing films and the controls was statistically significant ($P < 0.0001$) for all biopolymer-based films assayed. Overall, the highest viability loss was observed in ACBA at the end of the 60 days storage period, with the viable cells counts reaching only 10^5 CFU/g film.

Regarding the effect of prebiotics' incorporation, films with inulin showed a significantly higher survival rate of *B. animalis* subsp. *lactis* BB-12 in either WPI- and ALG-based coatings until after 60 days of storage. Considering that, in control films (WCBA and ACBA) showed a ca. 3.3 mean log reduction was observed after 60 days of storage, while for the films with inulin (WIBA and AIBA) or FOS (WFBA and AFBA) the reduction levels were of ca. 2.0 and 2.5 mean log reduction, respectively, our results suggest that prebiotics addition may play an important role in improving the viability of *B. animalis* subsp. *lactis* BB-12 strain when incorporated into edible films or coatings. Overall, there are few studies focusing on the incorporation of prebiotics in probiotic containing edible films, in an attempt to improve the stability of the incorporated probiotic strains. Similar findings were described by Soukoulis et al. (2014a) when studying the stability of *L. rhamnosus* GG in prebiotic containing films. They observed that the supplementation of edible films with prebiotics improved the storage stability of the probiotic strain considered with inulin being the most effective prebiotic (based on its capacity to maintain the survival of *L. rhamnosus* GG), followed by wheat dextrin, glucose oligosaccharides and polydextrose. Similarly, Romano et al. (2014) demonstrated that the

incorporation of 3 % (w/v) FOS into methylcellulose-based films also improved the viability of *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 after film preparation.

Overall, WPI-based films experienced a 2.2 mean log reduction while ALG-based film registered a 3.0 mean log reduction of viable cells. These results suggest that the stability of probiotics was promoted by the addition of whey protein to the films solutions, by providing nutrients to the cells, reducing redox potential of the medium as well as through the increase of the buffering capacity of the medium (Shori, 2017, Soukoulis et al., 2014a, Soukoulis et al., 2017). Soukoulis et al. (2014b) studied the development of probiotic baked cereal products (with *L. rhamnosus* GG) through the application of film solutions comprised of either 1 % (w/w) sodium ALG or binary blends of 0.5 % (w/w) sodium ALG and 2 % (w/w) whey protein concentrate containing with the samples with whey protein exhibiting an improved survival of *L. rhamnosus* GG throughout room temperature storage.

Although, the hereby described results indicated a significant reduction in the viability of the probiotic strains, the levels of viable cells still assure the (FAO/WHO, 2002) recommended viable cell counts for probiotic bacteria to be delivered to the humans, since the commonly accepted concentration of 10^6 viable CFU/g was mantained until the end of storage at room temperature (Mohammadi et al., 2011, Burgain et al., 2011).

4.4.3. Edible films physical properties

To be used as food coating materials edible films should be sufficiently resilient to external factors while also being elastic and remaining strong during packaging and storage (Ebrahimi et al., 2018). Thus, the physical properties of protein- and polysaccharide-based films (containing prebiotics as possible carriers for functional

bacteria) were studied during 60 days of storage. In Table 4.2 the thickness, $a_{w,}$ moisture content, water solubility and color properties of those films can be found.

Table 4.2 - Physicochemical and color properties of edible films containing *B. animalis* subsp. *lactis* BB-12 and different types of prebiotics fibers.

Edible film	Thickness (mm)	a_w	Moisture content (%, dry basis)	Water solubility (%)	L^*	a^*	b^*	ΔE
WCBA	0.40 ± 0.01^{a}	0.57 ± 0.01^{a}	30.95 ± 1.33^{a}	-	93.13 ± 0.55^{a}	-1.58 ± 0.01^{a}	19.36 ± 0.21^{a}	18.54 ± 0.17^{a}
WIBA	0.40 ± 0.01^{a}	0.58 ± 0.01^{a}	28.32 ± 0.39^{b}	-	93.35 ± 0.10^{a}	-1.76 ± 0.02^{b}	18.48 ± 0.28^{b}	17.65 ± 0.31^{b}
WFBA	0.40 ± 0.01^{a}	0.57 ± 0.02^{a}	28.18 ± 0.87^{b}	-	93.53 ± 0.45^{a}	-1.73 ± 0.01^{b}	16.39 ± 0.28^{c}	15.59 ± 0.31^{c}
ACBA	0.13 ± 0.01^{b}	0.57 ± 0.01^{a}	30.95 ± 1.35^{a}	70.35 ± 1.50^{a}	95.72 ± 0.10^{b}	-0.42 ± 0.05^{c}	4.14 ± 0.17^{d}	3.36 ± 0.15^{d}
AIBA	0.12 ± 0.01^{b}	0.57 ± 0.01^{a}	26.72 ± 1.50^{b}	71.43 ± 1.73^{a}	95.92 ± 0.08^{b}	-0.49 ± 0.06^{d}	$4.41 \pm 0.16^{d, e}$	3.47 ± 0.19^{d}
AFBA	0.12 ± 0.01^{b}	0.57 ± 0.01^{a}	27.94 ± 2.29^{b}	71.21 ± 1.98^{a}	95.63 ± 0.04^{b}	-0.49 ± 0.01^{d}	4.47 ± 0.07^{e}	$3.69 \pm 0.04^{d, e}$

Note: Means ± standard error within the same column labeled with the same letter, do not statistically differ from each other ($P > 0.05$). (-) The film was completely dissolved under the tested conditions.

Thickness is a critical parameter that influences, among others, the mechanical properties of the films and also contribute to improve the mechanical integrity of food products (Kanmani and Lim, 2013). The thickness ranged from 0.117 to 0.400 mm among protein and polysaccharide-based films. Prebiotics incorporation was not a statistically significant ($P > 0.05$) factor influencing films' thickness. Similar results were previously observed in chapter 2 during 60 days of storage (at 23 and 4 ºC)of edible films incorporated with lactic acid bacteria. Soukoulis et al. (2014a) also reported no significant modifications of film thickness due to the addition of prebiotic fibers to probiotic films. Furthermore, ALG-based films were significantly thinner than WPI films ($P < 0.0001$) with similar findings being reported by Soukoulis et al. (2014b) whom showed that ALG-based films with probiotics had a significantly lower thickness than WPC-based films.

Moisture content, an important parameter for measuring mouth melting of edible films also affects probiotics viability during long term storage (Kanmani and Lim, 2013). Inulin or FOS incorporation was associated with a decrease ($P < 0.001$) in the moisture content of edible films. Therefore, the highest moisture content was exhibited in WCBA and ACBA films. According to the ANOVA results, the biopolymer type had no significant differences in moisture content ($P > 0.05$). Similarly. the impact of prebiotic addition or biopolymer type upon a_w was also not significant ($P > 0.05$).

Generally, food applications may require low water solubility to improve the product integrity and water resistance but, in some cases such as food coatings, a high water solubility might be beneficial (Moreira et al., 2016). All WPI-based films dissolved in water after 24 h, whereas the solubility among ALG films was of around 70 %. The incorporation of prebiotics into edible films had no significant impact upon the film's solubility ($P > 0.05$).

To ensure the acceptability of food coatings, color is a crucial parameter not only from the consumer's standpoint but also for the packaging of light-sensitive materials (Goksu et al., 2007). Both biopolymer-based films showed high brightness values ($L^* \geq 93.13$) demonstrating that films appeared clear and transparent. The color values for WPI films are in agreement with those reported in chapter 2 and color values for ALG films were similar to the values obtained by Moreira et al. (2016). The ALG-based films exhibited higher L^* values than WPI- based films which could be attributed to their lower solid contents and subsequent lower thickness (Table 4.2). The addition of prebiotics was not associated with differences in L^* values ($P > 0.05$); which is in accordance with the observations of Soukoulis et al. (2014a). According to ANOVA results, film type (WPI vs ALG) had a significant effect ($P < 0.0001$) upon a* and b* values. With WPI-based films exhibiting the highest ($P < 0.0001$) scores for green and yellow hue color components, which confirms previous findings (Soukoulis et al., 2016). In terms of color difference (ΔE*), WPI-based films had the highest ΔE value and the type of prebiotic (Inulin vs FOS) incorporation had a significant ($P < 0.0001$) effect on ΔE among the edible films produced.

4.4.4. Mechanical properties of films

Typically, edible films must have good mechanical properties in order to resist the external factors involved in processing, management and storage of the food products (Soukoulis et al., 2017). The mechanical aspects of the different types of films can be found in Table 4.3.

Table 4.3 - Mechanical properties of edible films containing *B. animalis* subsp. *lactis* BB-12 and different types of prebiotics fibers

Edible film	Young's Modulus (Mpa)	Tensile Strength (Mpa)	Elongation at Break (%)
WCBA	0.310 ± 0.001^{a}	0.771 ± 0.010^{a}	65.526 ± 1.813^{a}
WIBA	0.312 ± 0.001^{a}	0.652 ± 0.019^{a}	64.700 ± 1.608^{a}
WFBA	0.311 ± 0.005^{a}	0.652 ± 0.012^{a}	64.900 ± 1.654^{a}
ACBA	7.310 ± 0.012^{b}	33.772 ± 0.810^{b}	14.535 ± 0.422^{b}
AIBA	7.310 ± 0.014^{b}	31.021 ± 0.804^{c}	14.505 ± 0.453^{b}
AFBA	7.310 ± 0.011^{b}	31.023 ± 0.804^{c}	14.504 ± 0.452^{b}

Note: Means ± standard error within the same column, labeled with the same letter do not statistically differ from each other ($P > 0.05$).

To best of our knowledge, this is the first report evaluating the combined effect of prebiotics and probiotics upon texture parameters of WPY- and ALG-based films. The addition of the plasticizer (glycerol) facilitated the development of flexible and extensible films. The polysaccharide-based films exhibited similar mechanical profiles i.e. higher stiffness and tensile strength and lower elongation properties compared to protein-based films. Films containing WPI were more extensible which may be due to its protein network. Regarding the addition of prebiotic compounds to WPI- and ALG-based films, significant differences were found when comparing the tensile strength of the films ($P < 0.0001$) while no effects on elongation at break ($P > 0.05$) or on Young's modulus ($P > 0.05$) were observed.

4.4.5. Fourier transform infrared spectroscopy (FTIR) measurements

FTIR was performed in order to consider potential changes in the molecular structure of biopolymer-based (WPI or ALG) films incorporated, or not, with Inulin or FOS. FTIR spectra of the different films can be observed in Fig 4.3.

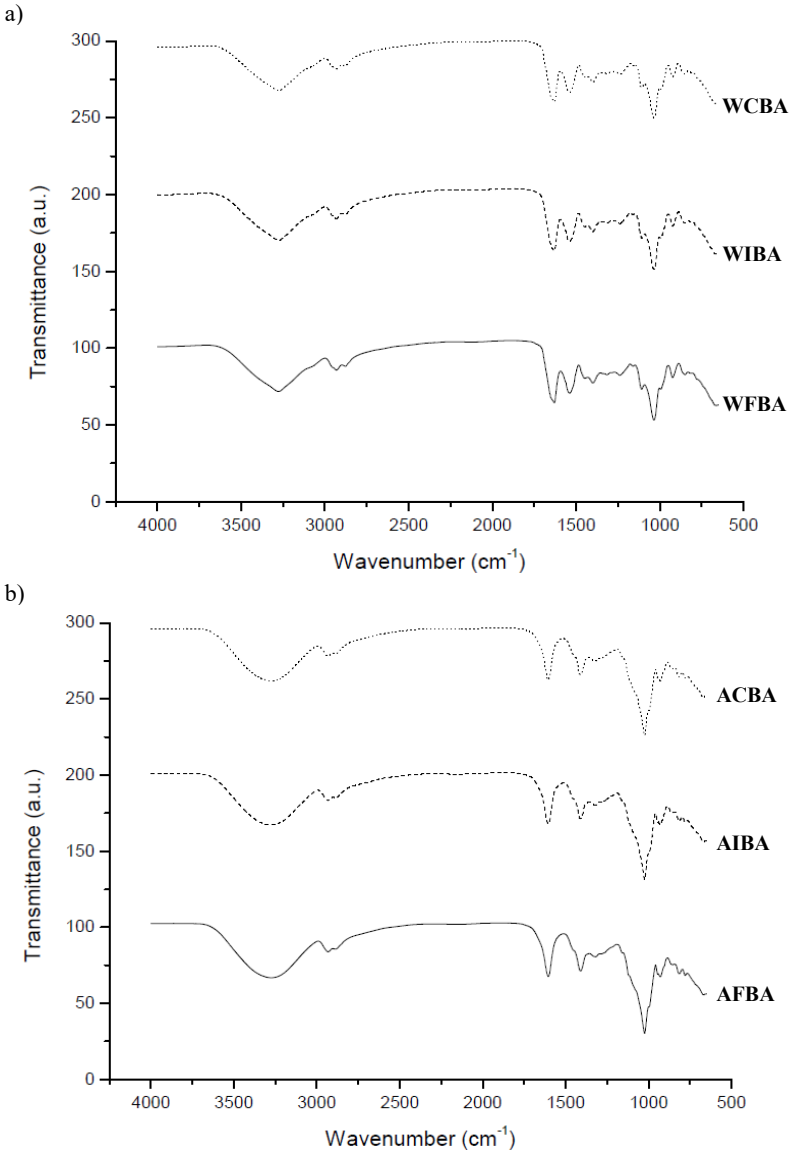

Figure 4.3 - FTIR spectra of: a) WPI-based and b) ALG-based films containing *B. animalis* subsp. *lactis* BB-12 with or without prebiotic incorporation at 0 days of storage.

The most relevant peaks were found between 3600-3000 cm^{-1}, 3000-2800cm^{-1}, and 1700 and 800cm^{-1}. The broad band 3600-3000 cm^{-1} was attributed to a stretching vibration of -OH and -NH groups (Tonyali et al., 2018). The range between 3000-2800 cm^{-1} was assigned to the C-H stretching vibrations of the carbonyl groups of triglycerides. The peaks observed at 2924 cm^{-1} and 2854 cm^{-1} were identified as related to the fat present in dairy products (Botelho et al., 2015). The area of these peaks is greater in WPI-based

films which might be due to the larger amounts of fat found in WPI-based films. Between 1700 cm^{-1} and 1500 cm^{-1} highest peaks were observed with significant differences being found among the samples. Two major peaks are clearly evidenced in WPI-based-films (amide I (1640 cm^{-1}) and amide II (1550 cm^{-1}) which are related to peptide bonds (CO-NH). These peaks are closely associated with the sample's protein concentration. As expected, WPI-based films exhibited the highest spectral intensity height due to the higher amount of protein. Consequently, in Fig. 4.3 the decrease in intensity of the amide I peak, and lack of amide II was observed in ALG-based films. Finally, the region between 1150-800 cm^{-1}, the absorption bands observed were attributed to glycerol (previously described in chapter 2). This spectrum region remained practically unchanged with prebiotics incorporation. However, a slight decrease in band intensity was observed when comparing WPI- and ALG-based films which could be related to the migration of glycerol (Piccirilli et al., 2019).

Overall, the hereby described results are in accordance with the results of chapter 2, in terms of FTIR spectra in regards to the spectra of WPI-based film formulations. Augusto et al. (2018), who made ALG edible films with *Codium tomentosum* seaweed extract, also reported no significant differences between the FTIR spectra of an ALG film with and without seaweed extract in terms of wavenumber absorbance.

4.5. Conclusions

Overall, WPI films were more effective in preserving *Bifidobacterium animalis* subsp. *lactis* BB-12 viability than ALG films. The prebiotic compounds added to probiotic edible films effectively protected the functional bacteria throughout storage and, while both, Inulin and FOS, improved the storage stability of the probiotic strain, the most effective results were obtained in films incorporated with inulin.

ALG-based films were thinner and presented a lower solubility in water. FTIR spectroscopy provided structural information about WPI and ALG films loaded with prebiotics with no structural changes being found in the films with the addiction of probiotics and prebiotics. Both WPI- and ALG-based edible films containing inulin or FOS developed in this work may be used as a good carrier for functional bacteria, alone or combined with bioactive compounds, to be ingested together with food and simultaneously exert specific biological activities upon the human organism.

CHAPTER 5 - Cereal bars functionalized through *Bifidobacterium animalis* subsp. *lactis* BB-12 and inulin incorporated in edible coatings of whey protein isolate or alginate

Joana Odila Pereira, José Soares, Maria J. P. Monteiro, Ana Amaro, Ana Gomes & Manuela Pintado

CBQF – Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Porto, Rua Arquiteto Lobão Vital, 172, 4200-374 Porto, Portugal

5.1. Abstract

Currently, consumption of ready to eat products, namely snacks, has increased widely due to changes in people's lifestyles. Among the different types of snacks available, cereal bars are gaining interest globally because of their nutritionally balanced and convenient nature. One healthy strategy is to add probiotics to cereals bars, to make them a functional product.

So, in this study we developed a cereal bar functionalized with edible coatings of whey protein isolate (WPI) and alginate (ALG) incorporated with *Bifidobacterium animalis* subsp. *lactis* BB-12 and inulin and evaluated the consumer acceptability and physicochemical and microbiological properties, throughout 90 days of storage.

WPI-coated cereal bars showed to be the solution that better maintained the level of incorporated probiotic strain when compared to the ones coated with ALG, although both were able to maintain values above 10^6 log CFU/g throughout storage and after de *in vitro* gastrointestinal digestion.

The physicochemical properties of the bars, namely a_w, moisture content, color and texture were not altered over storage time. However, the coated bars exhibited higher a_w and moisture content values relative to the control bars. Color and texture were not affected upon addition of the coating. The sensory evaluation showed that coated bars were well accepted as control bars. Moreover, the consumers appreciated more the odor and flavor of WPI coated bars rather than ALG coated bars.

5.2. Introduction

The best a new food product responds to consumers' needs, the higher the chance to be successfully accepted in the market. So, innovation in the food market has been linked to the search for safe and nutritional foods that prevent or control disease, promoting health and better life quality.

In recent years, the consumption of cereal bars has increased, which has promoted a 20 % production growth, per year (Silva et al., 2016).It is a very convenient food product, adapted to current life style, and has been associated to nutritional and healthier properties. So, it's imperative that manufacturers create novel products based on innovative ingredients and formulations.

Cereal bars are obtained by combination of dry ingredients and binder components, which complement each other by conferring the correct sensory properties to the bars (Garcia et al., 2018).

The tendency to consume healthier foods instead of sweets has led to the emergence of different types of bars, including formats using chocolate coatings or incorporation of different fruits and nuts. Once the consumption of cereals has been expanding to any moment of the day, these products have become an excellent carrier for delivering functional ingredients to foods in the marketplace (Silva et al., 2016).

Probiotics and prebiotics can be added to different food matrices with cereal bars representing a healthy matrix to incorporate them, even because their consumption is a well-established habit all around the world (Tavera-Quiroz et al., 2015).

Although the addition of probiotics to food faces some challenges, namely bacterial injuries and loss of viability derived from processing conditions (Bustos and Bórquez, 2013), antimicrobial components, and reduced anaerobic conditions, these shall be overcome. In addition, the properties of cereal bars (color, flavor, texture) cannot be disregarded, since their loss may lead to a non-acceptance by the consumers.

Therefore, the development of cereal bars with probiotics and prebiotics requires a strict control of these and other aspects, which still require deeper studies, considering the limited information in the literature.

Immobilization of probiotics in prebiotics in edible films appears as a promising way to protect them and coat food systems when probiotics cannot be incorporated by direct inoculation (Soukoulis et al., 2014a, Romano et al., 2014). Moreover, the film matrices generally protect the microorganisms against food storage and gastrointestinal conditions (Soukoulis et al., 2014b).

The matrices to incorporate probiotics and prebiotics can be based on proteins, polysaccharides, etc. (Soukoulis et al., 2014a, Romano et al., 2014).

Soukoulis et al. (2014a) studied the viability of the probiotic *Lactobacillus rhamnosus* GG incorporated in gelatine films with addition of several prebiotics such as inulin,

polydextrose, wheat dextrin and glucose-oligosaccharides. The addition of polydextrose and glucose-oligosaccharides demonstrated better results in concern of viability of the probiotic strain, while inulin and wheat dextrin had an adverse effect on the survival rates. In brief, the presence of prebiotics in the matrices improved probiotic storage stability, with exception of polydextrose films stored at 25 ºC. Inulin showed the best results, followed by wheat dextrin, glucose-oligosaccharides and polydextrose, at both temperatures tested (Soukoulis et al., 2014a).

In another study, Romano et al. (2014) developed methylcelullose edible films incorporated with fructo-oligosaccharides as prebiotics and *Lactobacillus delbrueckii* subsp. *bulgaricus* CIDCA 333 and *Lactobacillus plantarum* CIDCA 83114 as probiotics. The drying step of the film-forming solution led to a significant decrease in the *L. delbrueckii* subsp. *bulgaricus* CIDCA 333 strain when fructo-oligosaccharides were not included in the film-forming solution. Increasing fructo-oligosaccharide concentration in film-forming solution had a strong positive effect up to 3 % (w/v). *L. plantarum* CIDCA 83114 demonstrated a greater resistance towards the drying process and the addition of fructo-oligosaccharides up to 5 % (w/v) did not significantly enhanced its viability (Romano et al., 2014).

There are only few studies involving the simultaneous prebiotics and probiotics incorporation in edible films, and those studies combine positively the addition of prebiotics that selectively preserve probiotic viability. However, to the best of our knowledge, there is no study in which these types of edible films are integrated into cereal bar, in order to make it a functional food.

Within this context, the objective of this study was to formulate a cereal bar functionalized with whey protein isolate and alginate edible coatings through the addition of *Bifidobacterium animalis* subsp. *lactis* BB-12 and inulin and, characterize the formulation in terms of

functional value (probiotic viability), physicochemical properties, consumer acceptability and stability.

5.3. Materials and Methods

5.3.1. Bacterial strains, media and growth conditions

The microorganism *Bifidobacterium animalis* subsp. *lactis* BB-12, kindly donated by Christian Hansen (Denmark) was stored at -80 ºC in de Man–Rogosa–Sharpe (MRS) broth (Biokar Diagnostics, France) supplemented with 30 % (v/v) sterile glycerol. *B. animalis* subsp. *lactis* BB-12 were reactivated and pre-cultures were prepared in MRS medium supplemented with filter-sterilized 0.05 % (w/v) L-cysteine·HCl (Fluka, St. Gallen, Switzerland) and incubated at 37 °C during 24 h, under anaerobic conditions. To obtain the required inoculum, 300 ml of inoculum were grown, reaching 10^9 CFU/ml after 24 h.

Subsequently, grown cells were harvested by centrifugation at 4000 rpm, for 30 min, at 4 °C. To incorporate in the coatings, the supernatant was discarded, and the pellet was resuspended in a 0.9 % (w/v) NaCl sterile solution, in total of 10 ml with 10^{12} CFU/ml of viable cells.

5.3.2. Formulation of the coatings

The whey protein isolate (WPI) coating solution was prepared by dissolving 10 % (w/v) WPI powder (Armor Proteins, Saint Brice en Coglés, France) in deionized water, according to Pérez-Gago and Krochta (2002). Glycerol was added at 5 % (w/w), as a plasticizer, and the resulting solutions were homogenized for 2 h.

The alginate (ALG) coating solution was prepared by dissolving 2 % of sodium alginate (w/v) (FMC Biopolymer, Ireland) and glycerol (Panreac, Barcelona, Spain), as a plasticizer, at 1.2 % (w/w), in deionized water and the resulting solutions were homogenized for 2 h.

Subsequently, the solutions were heated in a water bath at 80 ºC, for 20 min, and cooled to room temperature.

The edible coating solutions were prepared by adding 2 % of high soluble inulin (w/v) (Orafti®HSI, BENEO, Germany) sterilized by 0.22 µm filter to each of the WPI and ALG coatings solutions.

Afterwards, 5 % (w/w) of centrifuged solution of 0.9 % NaCl inoculum of *B. animalis* subsp. *lactis* BB-12 was added to each 300 ml of coating solution to attain a final concentration of 10^9 CFU/ml, as described in chapter 2.

5.3.3. Production of the cereal bars

The cereal bars were developed with food grade and local ingredients (Porto, Portugal). The cereal bars recipe is described in Table 5.1.

Table 5.1 - Formulation of cereal bars.

Dry ingredients (45%)	%	g/1000g product
Oatmeal	24	108
Rice flakes	28	126
Corn flakes	11	49.5
Nuts	15	67.5
Raisins	22	99
Binder ingredients (55%)		
Glucose syrup	50	275
Palm oil	5	27.5
Soy lecithin	1	5.5
Inverted sugar	10	55
Salt	1,5	8.25
Sucrose	17	93.5
Water	15,5	85.25

Initially, binder ingredients were solubilized following the order showed in Table 5.1 and the mixture were subjected to a temperature of 80 °C, to solubilize all the sugar. The mixture was allowed to cool to 50 °C and dry ingredients were added and well homogenized.

The mix was placed on foil-covered trays and pressed flat to approximately 1 cm thickness. All the trays were placed into a cold place for 2 h. After cooling, the bars were cut into uniform pieces of ca. 25 g. Afterwards, the bars were coated, by bars immersion, for 2 min, in each probiotic coating solution.

The samples were prepared in three conditions, one without coating, one with WPI coating incorporated with *B. animalis* subsp. *lactis* BB-12 and inulin and one with ALG coating incorporated *B. animalis* subsp. *lactis* BB-12 and inulin, and codes were attributed:

1. Cereal bars non-coated (CBC)
2. Cereal bars coated with WPI with *B. animalis* subsp. *lactis* BB-12 and inulin (CBWPI)

3. Cereal bars coated with ALG with *B. animalis* subsp. *lactis* BB-12 and inulin (CBALG)

The excess of liquid in the cereal bars was drained for around 30 s and after that the cereal bars were dried at room conditions under a sterile environment (in a vertical laminar-flow cabinet, ca. 23 ºC and 50 % relative humidity, RH) for 24 h.

Thereafter, 120 bars were packaged under vacuum. Packages were stored at 23 ºC for 90 days and at each sampling point (0, 10, 40, 90 days), ten packages of each condition were evaluated.

5.3.4. Enumeration of viable cell bacteria

The viability of incorporated probiotic bacteria was studied in the cereal bars during 90 days of storage, at 23 ºC.

At each sampling point (0, 10, 40, 90 days) 1 cereal bar, ca. 25 g, was diluted to 1:10 (w/v) in sterile 1 % (w/v) sodium citrate (Merck, Darmstadt, Germany) solution in a Stomacher bag (Seward, West Sussex, UK) and homogenized in a Stomacher 400 Circulator (Seward), for 3 min at 260 rpm.

Appropriate sequential 10-fold dilutions were performed, using sterile peptone water, and plated, in triplicate, by the surface plating technique described by Miles et al. (1938).

The medium for *Bifidobacterium* was MRS supplemented with filter-sterilized 0.05 % (w/v) L-cysteine·HCl (Fluka, St. Gallen, Switzerland) and incubated at 37 °C, during 48 h, under anaerobic conditions, in a plastic anaerobic jar with an AnaeroGen sachet (an atmosphere generation system, Oxoid, Basingstoke, England).

5.3.5. In vitro simulated gastrointestinal digestion

To monitor the viability of *Bifidobacterium* throughout the simulated gastrointestinal digestion, the cereal bars were evaluated immediately after their production (time equal to 0) and during the storage period, at 10, 40 and 90 days.

The method was adapted from Oliveira and Pintado (2015) with minor modifications. Mouth digestion was performed by addition of 0.6 ml of α–amylase solution (100 U/ml) to the samples and incubation for 1 min, at 37 ºC and 200 rpm. The pH value was adjusted to 2.0 with 1 N HCl, to simulate gastric digestion, and the mixture was incubated with pepsin (25 mg/ml) (from porcine stomach mucosa, pepsin A), at a rate of 0.05 ml/ml of sample, in a shaking bath (130 rpm), for 1 h at 37 ºC. After, the pH value was adjusted to 6.0 with 1 M $NaHCO_3$, to simulate intestinal digestion, before the addition of pancreatin (from porcine pancreas, 2 g/l) and bile salts (12 g/l), at a ratio of 0.25 ml/ml of sample, and then the mixture was incubated for 2 h at 37 ºC, in a shaking bath (100 rpm). After that, aliquots of 1 ml of digesta were taken and the viable cells enumerated, as described in the section 2.4.

The survival rate of probiotic bacteria throughout the simulated gastrointestinal digestion was calculated according to the following equation:

$$\%\ viability = 100\ \times \frac{N}{N_0}$$

Where: N_0, N represent the number of viable probiotic bacteria prior and after the simulated gastrointestinal digestion.

5.3.6. Cereal bar characterization

5.3.6.1. Water activity

A HygroLab 2 (from Rotronic, Bassersdrof, Germany) was used to measure the water activity (a_w). Samples (ca. 0.5 g) were placed on the sample holder; a sealed system was formed by placing the water activity probe on top of the sample holder. The probe was equipped with a small fan to circulate air inside the sample container, a thin film capacitance sensor able to measure RH from 0 to 100 ± 1.5 %, and a platinum resistance temperature detector with a precision of ± 0.3 ºC. When a_w became constant (which usually took less than 1 h), its value was recorded. Calibration resorted to six saturated solutions of known a_w (viz. LiCl = 0.114, $MgCl_2$ = 0.329, K_2CO_3 = 0.443, Mg $(NO_3)_2$ = 0.536, NaBr = 0.653 and KCl = 0.821). The assays were run in quadruplicate.

5.3.6.2 Moisture content

Bar samples (5 g) were weighed into glass crucibles and dried at 105 °C in an oven, for 24 h. Moisture content was determined as the percentage of the initial film weight loss during drying and was reported on a wet basis. The analysis was performed in triplicate for each bar.

5.3.6.3. Color

To measure color, a portable Chroma meter CR-400 (from Minolta Chroma, Osaka, Japan) with a *C D65 illuminant, with a light source of pulsed xenon lamp, an aperture size of 8 mm, a closed cone and a standard observer of 2 ° Closely matches CIE 1931

($\bar{x}2\lambda$, $\bar{y}\lambda$, $\bar{z}\lambda$), was used. A CIELab color scale was employed to measure the degree of lightness (L), redness ($+a$) or greenness ($-a$), and yellowness ($+b$) or blueness ($-b$) of the films. A white standard plate, with color coordinates $L_{standard}$ = 97.7, $a_{standard}$ = 0.04 and $b_{standard}$ = 1.47, was used to calibrate the equipment.

For each condition, four samples were measured – and, on each bar, four readings were made (two on each side).

5.3.6.4. Texture analysis

Texture analysis was performed using a texturometer (TA. XT plus Texture Analyser, Stable Micro Systems, Cardiff, UK). Force calibration was performed with a weight of 5 Kg and height calibration was performed for Ottawa cell with a 5-blade Kramer shear cell. The five-bladed Kramer shear cell was used to perform a "multiple cutting" test, which gives a measure of the bite force that a consumer would experience. The hardness (Kg) of the bars was indicated by the maximum peak force required to break the bars.

All measurements were performed in quadruplicate, for each condition during storage time.

5.3.7. Consumer study

Participants were informed about the general aim of the work, procedures for handling personal data and gave written informed consent prior to participation. The study was conducted in accordance with the Declaration of Helsinki. All samples were prepared using food ingredients obtained via commercial suppliers and all additives were food-grade. Preparation prior to testing was performed in a dedicated preparation kitchen and

samples were produced and prepared according to good hygiene and manufacturing practices.

Seventeen subjects participated in the consumer test. All the participants consumed cereal bars at least once a week and 53 % consumed them daily. Their age ranged between 21 and 50 years old (30 ± 7) and 71 % were female. Overall liking (OL) was evaluated using a 9-point hedonic scale (Jones et al., 1955, Peryam and Pilgrim, 1957, Gaze et al., 2015). The appropriateness of the intensities of seven sensory attributes – appearance, color, odor, flavor, texture, crunchiness and adhesiveness - was evaluated by ratings provided on a 5-point, just-about-right scale, where 1 and 2 corresponded to "too weak" (TW) evaluations, 3 to "just about-right" (JAR) and 4 and 5 to "too strong" (TS) evaluations (Popper, 2014).

Samples (one bar of each condition) were assigned 3-digit codes and were presented to consumers following an incomplete balanced design. No information about the samples was provided to participants, except for safety and hygiene considerations related to their production and preparation. Water was provided to clean the palate between tastings. Tasting sessions took place in the ISO 8589 (ISO, 2007) compliant sensory facilities of Escola Superior de Biotecnologia.

5.3.8. Statistical analyses

Analysis of variance was performed to determine whether coating (non-coated, WPI or ALG) or storage time (0-90 d) were statistically significant sources of variation, at the 0.05 level of significance. Homoscedasticity requirements were met, i.e. experimental errors were independently and normally distributed and possess a constant variance. A paired Tukey's test was used to test for significant differences in the different

microbiological and physicochemical parameters between coated cereal bars and controls. The significance level was set at $P < 0.05$.

All tests were performed to a 5 % significance level, using Statistical Package for Social Sciences, v. 17.0 (SPSS, Chicago IL, USA).

Data collected for the sensory analysis was performed with XLSTAT software V. 2015 (Addinsoft, Paris, France).

Overall liking ratings of WPI and ALG samples were compared pairwise with the control sample using the Friedman's test. For each sample and for each sensory attribute, the frequencies of intensity ratings (TW, JAR, TS) were determined and the corresponding proportions calculated and compared using z-test. A weighted penalty analysis was conducted to relate attribute intensity ratings to OL (Popper, 2014). Weighted penalties corresponding to less than 20 % of respondents and to mean drops under 1.0 were considered negligible (Popper, 2014).

5.4. Results and Discussion

Microbiological and physicochemical analyses were performed to evaluate and compare the control and coated cereal bars (with WPI or ALG) with *Bifidobacterium animalis* subsp. *lactis* BB-12 and inulin, throughout 90 days of storage, at 23 ºC.

5.4.1. Microbiological properties of cereal bars during storage

The probiotic and prebiotic coated cereal bar samples were microbiologically tested for 90 days storage in order to evaluate the probiotic viability within the cereal bars whilst exposed to the extrinsic and intrinsic stresses that occur in typical storage conditions.

The initial concentrations in each bar were 9.21 ± 0.03 log CFU/g and 9.18 ± 0.07 log CFU/g for the CBWPI and CBALG, respectively.

The microbial viability showed a slight decrease over storage time ($P < 0.0001$), for both cereal bars coated with the two solutions, with the exception of WPI coated bar between days 10 and 40, in which viability was maintained ($P > 0.05$), as displayed in Fig. 5.1.

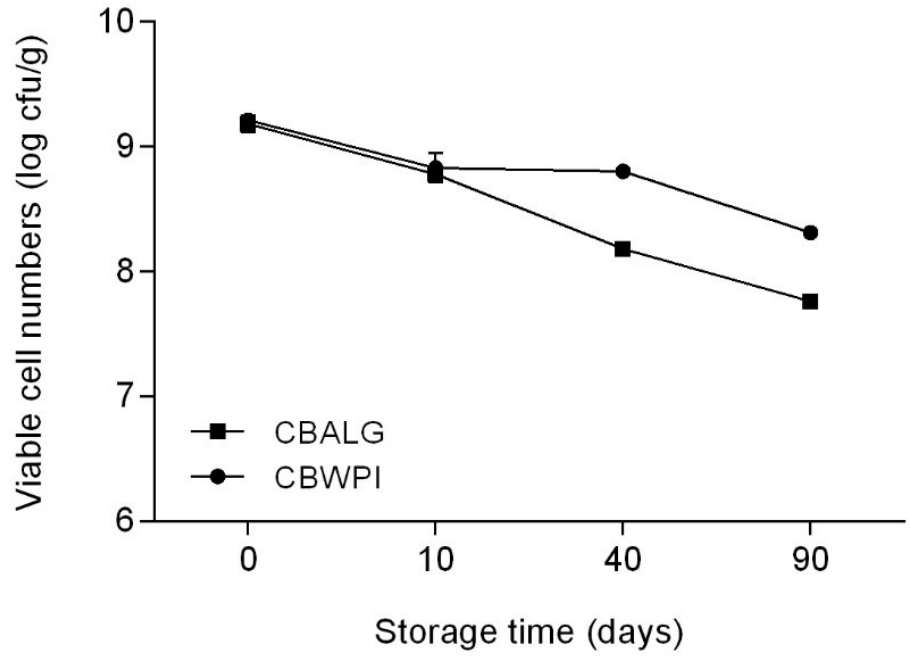

Figure 5.1 - Survival of *B. animalis* subsp. *lactis* BB-12 during storage (90 days) at room (23 ºC) temperature in cereal bars coated with WPI-based (CBWPI) and ALG-based (CBALG) with inulin incorporation.

Drying of the films has already been studied in chapter 2 and is well established that it induces a significant viability loss, and that it may be due to changes in cell structure, i.e. the phase transitions of the membranes lipid bilayers may lead to its rupture and to cytoplasmic material leakage (Fu and Chen, 2011). However, the values of viable cells, at the end of storage, were 8.31 ± 0.01 log CFU/g and 7.76 ± 0.02 log CFU/g for CBWPI and CBALG, respectively, guaranteeing the recommended number of *Bifidobacterium* in

the cereal bar to exert a probiotic effect, since the level commonly accepted is 10^6-10^7 viable cells/g (Burgain et al., 2011, Mohammadi et al., 2011, Tripathi and Giri, 2014). WPI-coated cereal bars appear as those that contribute to the better viability maintenance of the incorporated strain ($P < 0.0001$) when compared to cereal bars coated with ALG. As can be seen in Fig. 5.1, the viability of the bacteria during storage is significantly influenced ($P < 0.0001$) by the composition of the film into which they were incorporated. More specifically, a decrease in viability in ALG-coated cereal bars (8.18 ± 0.02 log CFU/g) compared to WPI coated bars (8.80 ± 0.02 log CFU/g), was observed after 40 days of storage ($P < 0.0001$).

Thus, the type of material used to produce the coatings may also negatively influence cell viability. For example, polysaccharides such as pectin or alginates have been reported to impact the viability of bacterial cells both throughout the drying process and storage period (Bustos and Bórquez, 2013, Yonekura et al., 2014).

In addition, Burgain et al. (2013) investigated the interaction between probiotic bacteria and milk proteins (micellar casein, or native whey proteins) and observed that *Lactobacillus* exhibited the ability to interact through their adhesive features with whey proteins improving their viability rates in dairy-based food matrices.

Similar to our work, Bastos et al. (2014) developed a potentially probiotic cereal bar, however, they incorporated lyophilized *L. acidophilus* LA-14 FloraFIT, directly with other ingredients during the preparation of the bar. After 6 weeks they obtained a cellular concentration of 7.33 log CFU/g when stored at room temperature. Considering the extensive shelf life of commercially available cereal bars, other approaches of incorporation of microorganisms in these products should be studied. Thus, our work has demonstrated that the method of incorporation is a critical and relevant step, showing that

the edible films represent good carriers for probiotics protection achieving a cell viability of 8.31 ± 0.01 log CFU/g after 90 days.

This result was expected because the microorganisms inside the edible film were protected from the presence of oxygen, water, and oxidative stress and remained in a latent state (Bampi et al., 2016).

5.4.2. Microbiological properties of cereal bars after in vitro digestion

As well as the conditions and time of storage, the passage through the gastrointestinal tract can lead to damage of microorganisms, and then lead to a decrease of cells viability (Soukoulis et al., 2014b).

Taking in account this affirmation, we evaluated the effect of simulated gastrointestinal conditions, not only after preparing the cereal bars (0 days), but also during storage up to 90 days.

Results for viable cells during storage followed by exposure to simulated gastrointestinal conditions are displayed in Fig. 5.2.

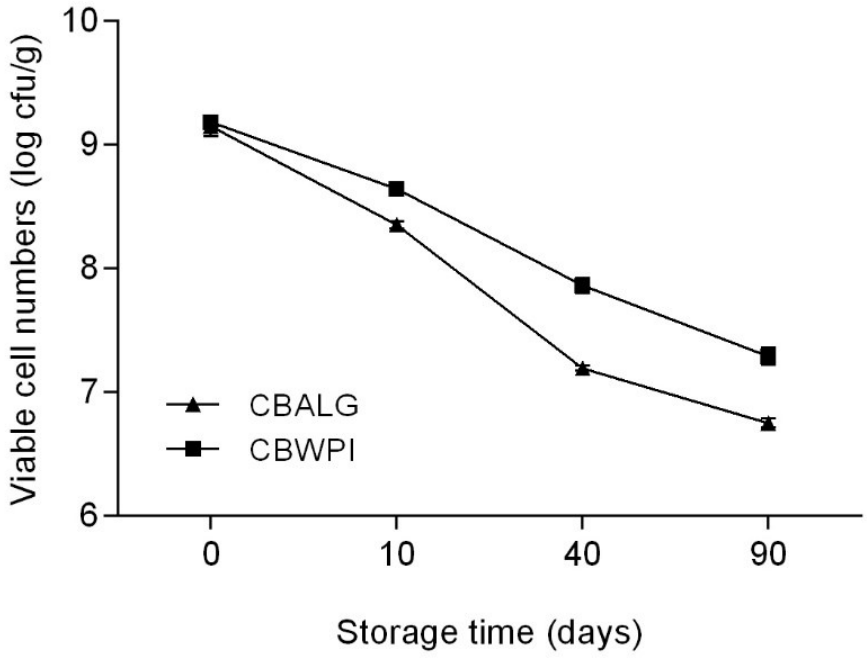

Figure 5.2 - Survival of *B. animalis* subsp. *lactis* BB-12 after gastrointestinal simulated tract during storage (90 days) at room (23 ºC) temperature in cereal bars coated with WPI-based (CBWPI) and ALG-based (CBALG) with inulin incorporation.

In order to probiotics to be fully effective, arrive the colon and colonize, multiply and exert their beneficial effects, the microorganisms should maintain their viability throughout the gastrointestinal digestion and exert noticeable probiotic effect (Flach et al., 2017).

To achieve this goal, the probiotics must overcome the harsh gastric and intestinal conditions. Therefore, it should be assessed earlier to predict if a probiotic (functional) food possesses any probiotic potential.

In this sense, only few authors evaluated the effect of simulated gastrointestinal tract conditions on the viability of probiotics in food during storage (Buriti et al., 2010, Bedani et al., 2014, Tavera-Quiroz et al., 2015).

In this study, we can conclude that at day 0 of storage there are no significant differences comparing coated cereal bars before passing through the simulated gastrointestinal tract ($P > 0.05$), perhaps because the microorganisms still maintain preserved structure.

After 10 days of storage, significant differences were observed after the gastrointestinal tract in vitro ($P < 0.05$).

This decrease in viability suggests that probiotics may have suffered some damage during storage, affecting their stability, as described by Tavera-Quiroz et al. (2015).

It should be noted that even after passage through the *in vitro* gastrointestinal system, probiotic strain remains within the viable cell values (10^6-10^7) to exert a beneficial effect and the foods are assumed to be a potential functional probiotic food.

In this study worst case, a cellular viability of 6.75 log CFU/g (for CBALG) was obtained, after 90 days of storage, and after passage throughout the *in vitro* gastrointestinal system. Thus, the edible films and coatings may be considered a relevant technological strategy to address some limitations (low pH and bile salts) and enhance viability.

5.4.3. Physicochemical characterization of cereal bars

The water activity (a_w), moisture content, texture and color of cereal bars were evaluated during the storage period and the visual appearance of cereal bars is showed in Fig. 5.3.

CBC CBWPI CBALG

Figure 5.3 - Appearance of uncoated cereal bar (CBC), coated cereal bar with WPI incorporated with *B. animalis* subsp. *lactis* BB-12 and inulin (CBWPI) and coated cereal bar with ALG incorporated with *B. animalis* subsp. *lactis* BB-12 and inulin (CBALG) at 23 ± 1 ºC with 90 days of storage.

As can be seen in Table 5.2 and Table 5.3, the values of the various parameters analyzed for each cereal bar, a_w, moisture content, color and texture (force), did not change significantly over the storage time ($P > 0.05$). The non-alteration of these parameters may be due to the storage conditions of the bars (sealed in vacuo), which reduces the possibility of physiochemical properties change.

Table 5.2 - Evolution of water activity (a_w), moisture content (%, wet basis) and hardness (Kg) of uncoated control cereal bars (CBC) and coated cereal bars with WPI incorporated with *B. animalis* subsp. *lactis* BB-12 and inulin (CBWPI) or ALG incorporated with *B. animalis* subsp. *lactis* BB-12 and inulin (CBALG) during storage for 90 days at 23 ± 1 ºC.

	CBC			CBWPI			CBALG		
	a_w	Moisture content (%, wet basis)	Hardness (Kg)	a_w	Moisture content (%, wet basis)	Hardness (Kg)	a_w	Moisture content (%, wet basis)	Hardness (Kg)
0 days	$0.520 \pm 0.026^{a, A}$	$6.6114 \pm 0.0650^{a, A}$	$29.32 \pm 2.94^{a, A}$	$0.555 \pm 0.013^{a, B}$	$9.5436 \pm 0.1037^{a, B}$	$28.56 \pm 4.81^{a, A}$	$0.560 \pm 0.028^{a, B}$	$9.4259 \pm 0.1856^{a, B}$	$30.44 \pm 5.65^{a, A}$
10 days	$0.517 \pm 0.016^{a, A}$	$6.6722 \pm 0.1735^{a, A}$	$29.07 \pm 3.92^{a, A}$	$0.545 \pm 0.021^{a, B}$	$9.5479 \pm 0.3842^{a, B}$	$29.08 \pm 5.07^{a, A}$	$0.548 \pm 0.011^{a, B}$	$9.4288 \pm 0.2402^{a, B}$	$31.77 \pm 5.77^{a, A}$
40 days	$0.523 \pm 0.015^{a, A}$	$6.6381 \pm 0.1537^{a, A}$	$28.09 \pm 3.93^{a, A}$	$0.548 \pm 0.032^{a, B}$	$9.5033 \pm 0.3842^{a, B}$	$30.00 \pm 4.97^{a, A}$	$0.554 \pm 0.016^{a, B}$	$9.5136 \pm 0.2331^{a, B}$	$29.81 \pm 4.86^{a, A}$
90 days	$0.522 \pm 0.015^{a, A}$	$6.6491 \pm 0.2010^{a, A}$	$29.81 \pm 2.72^{a, A}$	$0.546 \pm 0.027^{a, B}$	$9.6481 \pm 0.2083^{a, B}$	$29.69 \pm 5.29^{a, A}$	$0.559 \pm 0.034^{a, B}$	$9.4348 \pm 0.3354^{a, B}$	$29.31 \pm 4.73^{a, A}$

Note: [a] Means ± standard error within the same columns, labeled with the same letter, do not statistically differ from each other ($p > 0.05$).
[A, B] Means ± standard error within the same rows, labeled with the same letter, do not statistically differ from each other ($p > 0.05$).

The a_w values are shown in Table 5.2, and the results ranged from 0.517 ± 0.016 to 0.523 ± 0.015, for the uncoated bars, and these values are significantly lower ($P < 0.05$) than the values obtained for the coated bars. The WPI and ALG coated bars exhibited slightly higher, but closer values than the control bars and were similar between them ($P > 0.05$), 0.545 ± 0.021-0.555 ± 0.013 and 0.560 ± 0.028-0.548 ± 0.011, for WPI and ALG coated bars, respectively. These results may have been influenced by the WPI or ALG coating. Water activity is influenced by various factors, such as moisturizing agents, interaction between the sugars and salts, affinity between solute and solvent and nutritional composition (Pereira de Souza et al., 2014). The cereal bars exhibited a a_w of less than 0.60, which favors the stability of the incorporated microorganisms. When less water is available for biochemical reactions, microorganisms remain in the latent state, which prolongs their shelf life (Bampi et al., 2016). Water availability (a_w) determines both the vitality and functionality of living systems. The majority of microorganisms cannot multiply below 0.900 and for the most extremophilic species, cell division has only been observed down to 0.610 (Stevenson et al., 2015b, Stevenson et al., 2015a). In this sense, the microbiological control was not studied, because microorganisms hardly grow in the range of a_w of this study cereal bars (< 0.600).

Our results are in agreement with Aigster et al. (2011) that showed that a 0.450-0.570 a_w is suitable for a cereal bar, but were higher than bars made by Pereira de Souza et al. (2014), with whole flour of pseudo-cereals (0.430-0.470).

Besides water activity, moisture content is a crucial factor for the stability and acceptability of cereal bars during and after storage.

The control cereal bar prepared showed a moisture content (Table 5.2) between 6.6114 ± 0.0650 to 6.6722 ± 0.1735 % and did not change during the storage time ($P > 0.05$). However, moisture content increased when we applied the coating ($P < 0.05$). Moisture

content is related to water activity, thus removing of water the water activity reduces and vice-versa (Loveday et al., 2009).

The cereals bars coated with WPI and ALG showed higher values than the control bars, but similar between them ($P > 0.05$), 9.4259 ± 0.1856 - 9.6481 ± 0.2083 %, respectively for WPI and ALG bars.

The moisture content of CBC was lower than the one from whole flour pseudo-cereals bars (7.19–8.24 %) reported by Pereira de Souza et al. (2014) and much lower than cereal bars made with jackfruit seed and jenipapo (21.0 %), developed by Torres et al. (2011). The CBWPI and CBALG were in the range of the snack bars (5.6–11.5 %) produced by Sun-Waterhouse et al. (2010), but also much lower than cereal bars made with jackfruit seed and jenipapo (21.0 %) (Torres et al., 2011).

Color is one of the most crucial parameters for the finished product. The cereal bars are composed by many different ingredients, resulting in an heterogeneous surface, so color has a large variation, depending very much on constitution, for instance the use of raisins. In this study, the coatings had no significant influence on the color of the cereal bars ($P > 0.05$), as displayed in Table 5.3.

Table 5.3 - Evolution of color characteristics, L* (black–white), a* (green–red) and b* (blue–yellow) of uncoated control cereal bars (CBC) and coated cereal bars with WPI incorporated with *B. animalis* subsp. *lactis* BB-12 and inulin (CBWPI) or ALG incorporated with *B. animalis* subsp. *lactis* BB-12 and inulin (CBALG) during storage for 90 days at 23 ± 1 ºC.

	CBC			CBWPI			CBALG		
	L^*	a^*	b^*	L^*	a^*	b^*	L^*	a^*	b^*
0 days	57.97 ± 0.95[a]	11.48 ± 0.17[a]	29.51 ± 0.35[a]	57.35 ± 1.46[a]	11.41 ± 0.45[a]	30.48 ± 0.63[a]	57.05 ± 1.53[a]	11.57 ± 0.04[a]	30.15 ± 0.73[a]
10 days	57.45 ± 0.84[a]	11.45 ± 0.13[a]	30.36 ± 0.26[a]	57.33 ± 0.78[a]	11.21 ± 0.20[a]	30.24 ± 0.81[a]	56.26 ± 1.51[a]	11.54 ± 0.36[a]	29.95 ± 0.71[a]
40 days	57.32 ± 0.82[a]	11.57 ± 0.92[a]	30.13 ± 0.91[a]	56.83 ± 0.92[a]	11.35 ± 0.24[a]	30.01 ± 1.37[a]	57.15 ± 0.37[a]	11.37 ± 0.52[a]	30.33 ± 0.64[a]
90 days	57.46 ± 0.78[a]	11.24 ± 0.23[a]	29.38 ± 0.45[a]	57.22 ± 0.86[a]	11.46 ± 0.21[a]	29.57 ± 0.77[a]	56.06 ± 1.27[a]	11.42 ± 0.28[a]	30.17 ± 0.47[a]

Note: [a] Means ± standard error within the same columns and rows, labeled with the same letter, do not statistically differ from each other ($p > 0.05$).

Therefore, the values of CBC, CBWPI and CBALG were similar ($P > 0.05$), exhibiting values of L^* between 56.06 ± 1.27 and 57.97 ± 0.95, values of a^* between 11.21 ± 0.20 and 11.57 ± 0.92 and b^* values between 29.38 ± 0.45 and 30.36 ± 0.26.

Simial results were obtained by Aigster et al. (2011) for granola bars (L^* 48.9-59.7, a^* 6.99-11.1, b^* 30.3-32.5).

Regarding texture, shown in Table 5.2, over time, there were no significant differences ($P > 0.05$) in the force required to break the cereal bars, as well as, between control bars and coated bars ($P > 0.05$). It should be noted that the coating did not affect the hardness of the bars. Similar results were reported by Soukoulis et al. (2014b), who reported that there was no significant impact ($P > 0.05$) of the film composition used on textural parameters of pan bread.

5.4.4. Sensorial analysis of cereal bars

In order to complete the study, a consumer study was performed. The consumer study was conducted with 17 subjects and the consumer acceptability was performed using a 9-point hedonic scale to evaluate OL of the cereal bars and subsequently complemented with the JAR scales to provide insights on improvements (Aigster et al., 2011).

Figure 5.4 displayed the frequencies of TW, JAR and TS intensity ratings for each sample and for all sensory atributes evaluated.

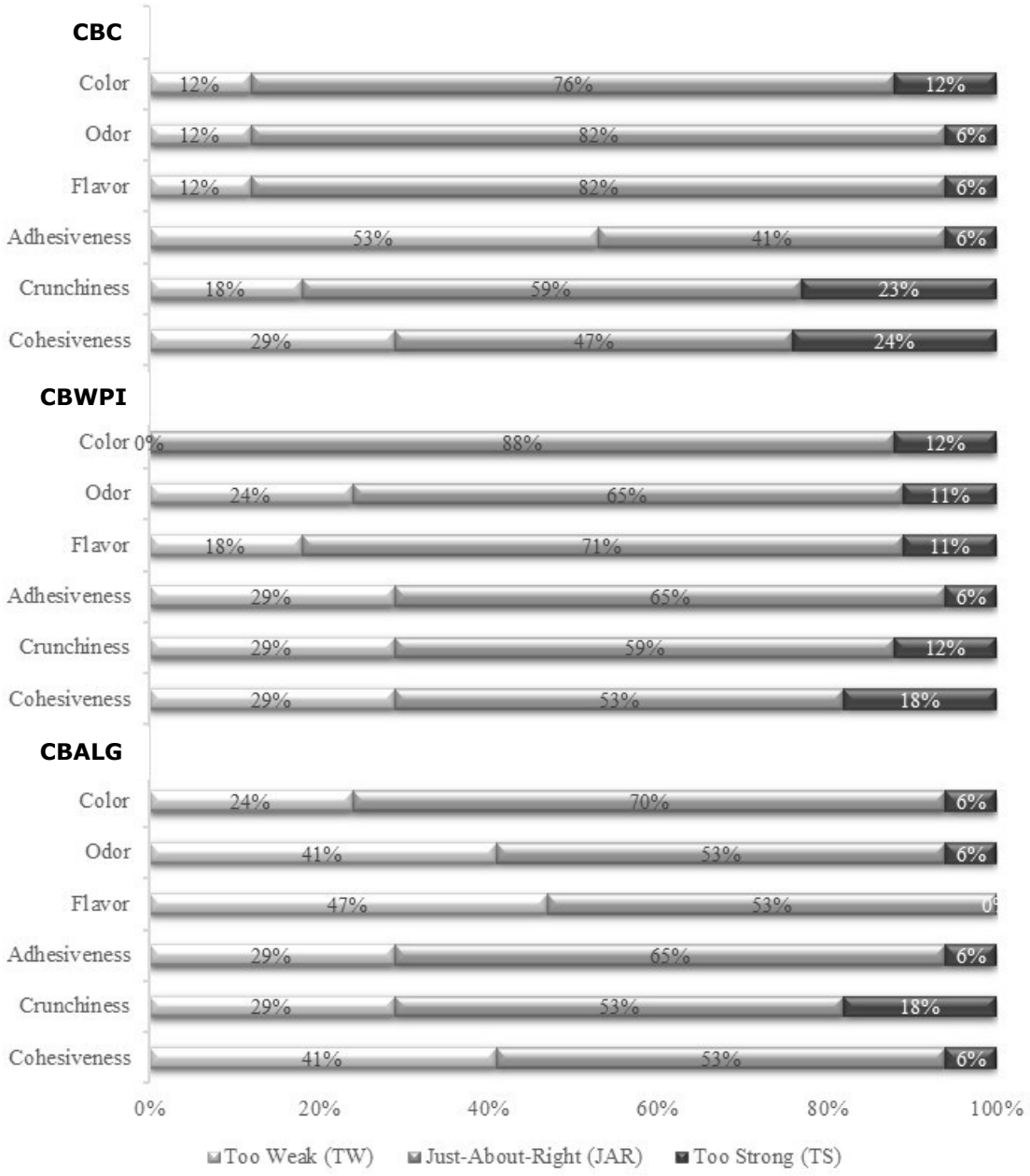

Figure 5.4 - JAR evaluations (%) for cereal bars - uncoated cereal bar (CBC), coated bar with WPI incorporated *B. animalis* subsp. *lactis* BB-12 and inulin (CBWPI) and coated bar with ALG incorporated *B. animalis* subsp. *lactis* BB-12 and inulin (CBALG).

Consumers liked all the samples with no significant differences ($P > 0.05$) in hedonic ratings being observed among them. For the CBC samples, the median of the evaluations corresponded to "liked very much"and for CBWPI and CBALG it corresponded to "liked moderately".

A preponderance of just-about-right ratings were observed for all samples for color (70 % to 88 %), with no significant differences being observed among these, which corroborates the results of color properties in the cereal bars. For odor and flavor intensities, a higher proportion ($P < 0.05$) of just-about-right ratings were observed, for the CBC (82 % for both attributes) and CBALG (53 % for both attributes) but not for CBWPI (65 % for odor intensity and 71 % for flavor intensity). Penalty analysis showed that for CBALG, the "too weak" odor intensity evaluations by consumers (41 %) led to a decrease in acceptance of this sample. For texture evaluation, consumers were asked to evaluate the cohesiveness, the crunchiness and the adhesiveness of the samples. For all these attributes no significant differences among the samples were observed for the proportion of just-about-ratings, varying between 41 % (adhesiveness of the CBC sample) and 65 % (adhesivness of samples CBWPI and CBALG). These results corroborate those obtained for texture properties in each cereal bar.

Bampi et al. (2016) evaluated the viability of microencapsulating *Lactobacillus acidophilus* and *Bifidobacterium animalis subsp. lactis* using the spray chilling technique to add them to savory cereal bars and observed that control and products were highly acceptable to consumers and did not affect the bars sensory and structural qualities, corroborating the results of this study, even though different methods and matrices were used.

5.5. Conclusions

For the first time, functionalized probiotic cereal bars were successfully obtained through the use of WPI and ALG coatings incorporating *Bifidobacterium animalis* subsp. *lactis* BB-12 and inulin, proving to be a feasible approach to obtain a healthy snack with high

sensory acceptability and at the same time provide the level of probiotic (*Bifidobacterium animalis* subsp. *lactis* BB-12) and prebiotic (inulin) required to exert health benefits.

The application of the coatings on the cereal bars did not appear to affect color or texture parameters, however, coated cereal bars presented slightly higher a_w and moisture content when compared to the uncoated cereal bars, but not constraining sensory or safety levels. Throughout the 90 days of storage the cereal bars physicochemical properties remained unchanged, thus guaranteeing a high-quality product.

WPI-coated cereal bars are better at maintaining *Bifidobacterium animalis* subsp. *lactis* BB-12 cellular viability. However, both coated bars, before and after the *in vitro* gastrointestinal tract simulation, have high and constant levels of ca. 10^7 CFU/g throughout the storage time, assuring required levels to providing the associated positive effects to the consumers.

Sensory evaluation showed that coated bars were well accepted as control bars, and it should be noted that consumers appreciated more the odor and flavor of WPI coated bars rather than ALG coated bars. These results suggest a great potential for the application of WPI coating with probiotics and prebiotics in cereal bars.

PART IV - Conclusions and Future Perspectives

CHAPTER 6 - Conclusions

It was our intention, with this doctoral program, to develop WPI-based films with probiotics and prebiotics incorporation, creating an opportunity for valorization of dairy industry by-products-based products.

Based on main results we can conclude that it is possible to produce functional edible films using an affordable approach, creating a product with high protein content and exhibiting some relevant functional properties when applied to food products – probiotics and prebiotics vehicle, able to provide antimicrobial properties to some of them, extending their shelf-life.

In a more detailed perspective, from chapter 2 we can conclude that WPI edible films could act as a suitable matrix to incorporate probiotic bacteria and support their viability throughout 60 days, at 23 and 4 ºC. Refrigerated temperature (4 ºC) was the best condition for maintaining cellular viability of *B. animalis* subsp. *lactis* BB-12 and *L. casei* 01, maintaining high viable cell number levels (10^7 CFU/g of film). In addition, during the storage period, no structural changes in the film were observed, providing high durability and high shelf-life, which can be considered good solutions for food application.

In chapter 3, it was our intention to demonstrate the practical application of these formulations in food matrices. Therefore, slices of ham coated with edible films, developed previously in chapter 2, were produced and stored at 4 ºC.

The coatings inhibited detectable growth of spoilage bacteria, until 45 days, thus extending the recommended shelf-life of sliced ham. Furthermore, the viability of probiotic bacteria was preserved at high levels, ca. 10^8 CFU/g, throughout 45 days, assuring the required levels to promote functionality, and thus providing positive effects

to the consumers, requiring an ingestion of a portion size of 10–20 g to assure the recommended daily dose of 10^9 CFU. The sensory evaluation demonstrated that the coated slices of ham were the most appreciated by the consumers, suggesting a potential market for coated sliced ham.

Thus, at the end of this stage, we also concluded that *B. animalis* subsp. *lactis* BB-12 is the most stable strain and the temperature of 4 ºC was the most favorable for probiotics stabilization, as expected. However, we also considered to demonstrate the potential of our functional films in food products to be stored at 23 °C. For this purpose, in Chapter 4, we produced WPI and ALG-based coatings incorporating *B. animalis* subsp. *lactis* BB-12 probiotic strain and *B. animalis* subsp. *lactis* BB-12 compounds. WPI-based films assured better results in terms of probiotic survival and prebiotic compounds added to edible films effectively protected functional bacteria throughout storage, particularly inulin.

In chapter 5 the main objective was to coat a cereal bar based on the best solution obtained from chapter 4, namely, WPI and ALG edible films with inulin and *B. animalis* subsp. *lactis* BB-12.

Cereal bars were successfully obtained and proved to be a feasible solution to obtain a healthy snack with high sensory acceptability, and at same time to provide the level *B. animalis* subsp. *lactis* BB-12 to be a probiotic food product, i.e. to exert health benefits. During the storage period (90 days) the physicochemical properties remained unchanged, thus guaranteeing a high-quality product.

WPI-coated cereal bars assured better results in terms of cellular viability of *B. animalis* subsp. *lactis* BB-12; however, both coated bars after the *in vitro* gastrointestinal tract exhibited high and constant levels of ca. 10^7 CFU/g

The sensory evaluation showed that coated bars were well accepted as control bars, and it should be noted that consumers appreciated more the odor and flavor of WPI coated bars rather than ALG coated bars.

The progress of novel functional foods is a major task to address the expectation of consumers who look for healthy and beneficial food products to promote their health. As an overall conclusion, the results suggest a great potential for the application of WPI edible films in delivering probiotics for human consumption, with additional potential to extend shelf-life of some foods. In addition, the use of prebiotics to protect probiotic cells has gained increasing interest and revealed to be a good strategy to improve probiotics survival.

Besides the contribution towards the preservation of probiotic strains, this strategy also supports potential applications of the immobilized microorganisms in the development of functional products containing both probiotics and prebiotics.

CHAPTER 7 - Future Perspectives

This doctoral thesis developed novelty and brought more information and knowledge to the scientific community and to the food and packaging industry, permitting extension of the shelf-life of food products with the addition of functionality.

In this section, our purpose is to highlight some points that might be interesting and fruitful to explore and complete in the future.

Since the main purpose is to turn edible coatings into a commercially available product, there are some modifications that can be made in the structure of edible packaging without compromising the safety of the food product, and consequently the consumer. In this regard, polymer's crosslinking, generation of composites with different biofillers, addition of new hydrophilic biopolymers blended with lipids, may be valuable solutions to add innovative properties to our developed polymer-based coatings. Similarly, generation of a multilayer structure can reduce film's degradation, and at the same time a low diffusion, gradual release, and acceptable bioavailability of the added compound/probiotic can be achieved. However, the possible interactions of such compounds with films and coatings with whey protein should be studied in greater depth.

Considering the renewable nature of the matrix involved, whey-based edible films may be certainly the future of some food packaging materials. However, such innovative materials are not expected to completely replace conventional packaging materials in the near future, since there are obstacles such as resistance and higher cost of film production. Thus, actual applications are still limited to special niches like those with environmental considerations. In this context, the presence of WPI in edible films and coatings make them attractive to the consumers due to the additional sensory and nutritional appeal

conveyed by the whey. Films and coatings containing whey are not merely barriers to the food but have their own market appeal. Additionally, the use of inexpensive food processing by-products is a promising strategy for developing films that still present the sensory and physicochemical characteristics that distinguish them from films made up of conventional polymers.

In the future it will be necessary to further deepen this issue in a systematic way and to determine the magnitude of the benefit/cost ratio for each edible coating or film developed. Another challenge to the use of edible coatings and films is their compatibility with other emerging stress factors, for instance combinations of other technologies such as high pressure, electric fields, ultrasound, microwave radiation and gamma radiation. Also, it is expected that these edible and biodegradable packages will also be applicable with modified atmosphere, considerably extending its range of use.

Although we advanced in evaluating cell viability upon simulated gastrointestinal conditions, *in vivo* studies are required to evaluate the effect of these films in the modulation of gastrointestinal microbiota and to study if the probiotic strains may reach the intestine at adequate levels to exert their function.

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