**Universidade de Aveiro** Departamento de Química
**Ano 2014**

**Maria Inês**

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# EDIBLE FILMS AND COATINGS FOR CHEESE

# FILMES E REVESTIMENTOS COMESTÍVEIS PARA QUEIJOS

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo Biotecnologia Alimentar, realizada sob a orientação científica do Doutor José António T. Lopes da Silva, professor auxiliar do Departamento de Química da Universidade de Aveiro.

## o júri

| | |
|---|---|
| Presidente | Doutor Jorge Manuel Alexandre Saraiva<br>Investigador auxiliar do Departamento de Química da Universidade de Aveiro |
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| Orientador | Doutor José António Teixeira Lopes da Silva<br>Professor auxiliar do Departamento de Química da Universidade de Aveiro |

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**Palavras-chave**

Filmes comestíveis, proteína de ervilha, proteína de soja, queijo, propriedades mecânicas, propriedades de barreira, atividade antioxidante, oligoquitosanos, óleos essenciais

**Resumo**

O interesse na substituição de materiais sintéticos por biodegradáveis tem vindo a aumentar devido aos problemas ecológicos. Filmes comestíveis e biodegradáveis podem ser produzidos utilizando polissacarídeos, lípidos, proteínas e compósitos e atuar como embalagens, sem danificar o meio ambiente. Ao escolher uma composição adequada para um revestimento é possível preservar várias propriedades desejáveis de um produto alimentar. Propriedades importantes como mecânicas, funcionais e de barreira devem ser consideradas. O principal objetivo deste estudo foi avaliar filmes e revestimentos comestíveis de proteínas vegetais (ervilha, soja), com agentes antimicrobianos e antioxidantes naturais incorporados, para proteger queijo de deterioração físico-química e microbiana e para preservar as características organolépticas, especialmente de queijos cortados/fatiados. O trabalho realizado foca-se principalmente na preparação e caracterização de filmes de proteína de ervilha, com a adição de oligoquitosanos (OQ) (0,5%, 1% e 2%) e dois tipos de óleos essenciais (1%), óleos de louro e tomilho. Filmes com 0,5% de OQs apresentaram valores mais elevados de módulo de Young, tensão de rutura e alongamento. Em relação às propriedades de barreira, o filme com 1% de OQs mostrou o valor de permeabilidade mais baixa. A adição de pequenas quantidades de OQs pode ser vantajosa para melhorar as propriedades mecânicas dos filmes de proteína de ervilha, além dos esperados efeitos antimicrobianos. Uma concentração OQs intermediária (1%) poderia ser vantajosa para reduzir a permeabilidade ao vapor de água, mas também resultaria em efeitos prejudiciais sobre as propriedades mecânicas. A hidrofobicidade dos filmes foi dependente da quantidade de OQs e óleos essenciais adicionados. Para os filmes com OQs, a presença dos óleos essenciais aumentou a hidrofobicidade dos filmes, um efeito dependente do tipo de óleo adicionado. Os efeitos observados parecem complexos e provavelmente dependem das interações entre os diferentes componentes do filme; Estes aspetos merecem mais estudos a fim de melhorar e compreender as interações / aderência do revestimento sobre a superfície do queijo. Os filmes de proteínas por si só mostraram alguma atividade antioxidante, e os resultados da adição de OQs ou óleos essenciais mostram uma taxa mais elevada deste efeito (diminuição do tempo de reação para observar os efeitos antioxidantes). Os filmes com óleo de louro revelaram uma maior atividade antioxidante, podendo ser útil e complementar aos efeitos esperados sobre as propriedades organolépticas de amostras de queijo revestidas.

**Keywords** Edible films, pea proteins, soy proteins, cheese, mechanical properties, barrier properties, antioxidant activity. chitooligosaccharides, essential oils

**Abstract**

Over the last years there has been an increasing interest to replace synthetic materials by biodegradable ones, due to the ecological problems. Edible and biodegradable films can be produced using polysaccharides, lipids, proteins and composites, and act as a package without damaging the environment. By choosing a suitable coating composition it is possible to preserve several desired properties of a certain food product. Important properties should be considered, such as mechanical, functional and barrier properties. The main goal of this study was to evaluate edible films and coatings from plant proteins (pea, soy), with incorporated natural antimicrobial and antioxidant agents, to potentially protect cheese from physico-chemical and microbial deterioration and to preserve the organoleptic characteristics, especially of sliced cheeses. The work performed focused mainly on the preparation and characterization of pea protein films, with added chitooligosaccharides (COs) (0.5%, 1% and 2%) and two types of essential oils at 1%, bay and thyme oils. Films with 0.5% of COs showed the highest values of Young's modulus, tensile strength and elongation. Regarding the barrier properties, the film with 1% of COs showed the lower permeability value. Addition of small amounts of COs may be advantageous to improve the mechanical properties of the PPI films, besides the expected antimicrobial effects. An intermediate COs concentration (1%) could be advantageous to reduce the water vapor permeability, but it will also result in detrimental effects on the mechanical properties. Film's hydrophobicity was also dependent on the amount of added COs and essential oils. For the films with COs, the presence of the essential oils increased the film's hydrophobicity, an effect dependent on the type of added oil. The observed effects seem complex and they are probably dependent on the interactions among film components; certainly these aspects deserve further studies in order to improve and better understand the interactions/adhesion of the coating onto the cheese surface. The protein films by their own showed already some antioxidant activity, and the addition of COs or the essential oils results mainly on a higher rate of this effect (lower times to observe the antioxidant effects). Even so the films prepared with the bay oil revealed a higher antioxidant activity, which can be useful and complement the expected effects on the organoleptic properties of cheese samples treated with these films.

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## Abbreviations

| | |
|---|---|
| ABTS | 2,2'-azino-bis (3-ethilbenzotiazolina-6-sulfonic) |
| AW | Water Activity |
| B | Bay oil |
| BSA | Bovine Serum Albumin |
| COs | Chitooligosaccharides |
| E | Elongation |
| EFC | Edible Films and Coatings |
| GLY | Glycerol |
| PPC | Pea protein concentrate |
| PPI | Pea protein isolate |
| T | Thyme oil |
| TS | Tensile Strength |
| SP | Soy Protein |
| SPC | Soy Protein Concentrates |
| SPI | Soy Protein Isolates |
| WP | Whey Protein |
| WPI | Whey Protein Isolates |
| WVP | Water Vapor Permeability |
| WVTR | Water Vapor Transmission Rate |

# 1. Objectives of this study

The main goal of this study was to evaluate edible films and coatings from plant proteins (pea, soy), with incorporated natural antimicrobial and antioxidant agents, to potentially protect cheese from physico-chemical and microbial deterioration and to preserve or improve the organoleptic characteristics, especially of sliced cheeses. For that, pea protein films were prepared and evaluated regarding mechanical and barrier properties, antioxidant and antimicrobial properties. Soy protein films were also studied for comparison. The properties of the pea protein films were tentatively improved by adding chitooligosaccharides and essential oils. The performance of the optimized films for coating sliced cheese was also studied and preliminary results obtained.

# 2. Bibliographic Review

## 2.1 Films and Coatings

### 2.1.1 Origin and definition

Over the years there has been a growing need to protect food from external environment, namely through adequate packaging strategies. Starting with packages made of leaf and animals skins, then waxes and pig fat that was used to protect fruits and other foods [1]. However, the first plastic appears on 1856, a polymer made of celluloses. In 1907 it was discovered a plastic resulting from the junction of phenol and formaldeide [2]. Since then, different materials have been discovered giving a large range of synthetic polymers used in packaging. These polymers are excellent barriers for aromatic composts, gas and water vapor. However they are not biodegradable [1] and certainly cause a high environmental impact. In this context, alternatives to replace these materials were studied, for example, using biopolymers due to the fact that they are biodegradable. Nevertheless, the use of bio-based materials from renewable sources still has not achieve enough credit in the industry, mainly due to the lack of incentive, the higher commercial costs and because synthetic polymers have generally better properties.

In the food area, relevant studies of edible films and coatings (EFC) have increased in the last decade. Edible and biodegradable films are produced from biopolymers and are used as packaging materials. For a film to be considered biodegradable, it has to be completely degradable by microorganisms during the composting, originating only natural compost, like carbon dioxide and water.

Edible films and coatings are thin layers of edible materials that create a modified overhead atmosphere, that act as a barrier between the product and the exterior environment to increase the shelf life, to reduce the moisture and gas exchanges, respiration rate, oxidative reactions and solute migration. They can serve as carrier of food additives like anti-browning and antimicrobial agents, antioxidants, colorants, flavors, nutrients and spices [3].

The prerequisites of a good packaging film are [4, 5]:

- Allow for a slow but controlled respiration (reduced $O_2$ absorption) of the product;
- Allow for a selective barrier to gases ($CO_2$) and water vapor;

- Creation of a modified atmosphere with respect to internal gas composition, thus regulating the ripening process (if it applies) and leading to shelf-life extension;
- Should reduce the migration of lipids - of use in confectionery industry;
- Should maintain structural integrity (delay the loss of chlorophyll) and improve mechanical handling;
- Serve as a vehicle to incorporate food additives (flavour, colors, antioxidants, or antimicrobial agents);
- Prevent (or reduce) microbial spoilage during extended storage.

All the above prerequisites can be met with polymer composites, whose composition and formulation vary from commodity to commodity.

### 2.1.2 Formulation

Edible films can be produced using various products, such as polysaccharides, lipids, proteins with the addition of plasticizers and surfactants. They can be divided into three categories:

- Hydrocolloid: that includes proteins (soy, pea, whey, corn, etc) and polysaccharides (starch, alginates, cellulose, chitosan, etc);
- Lipids: that includes waxes, fatty acids and acylglycerols;
- Composites: that includes both hydrocolloid and lipids.

The various naturally occurring biopolymeric materials, used in the formulation of films and coatings are shown in Figure 1. Generally, hydrocolloids and lipids are used in combination for the preparation of biodegradable packaging films or composites, due to the fact that individually they lack structural integrity and characteristic functionality. For example, hydrocolloids being hydrophilic are poor moisture barriers, a property compensated by adding lipids, which are very good moisture barriers. Composite films are a mixture of these and other ingredients in varying proportions, which determine their barrier (to $H_2O$, $O_2$, $CO_2$ and aroma compounds), mechanical, surface and other properties.

Sometimes a composite film formulation can be tailor made to suit to the needs of a specific commodity or farm produce [1].

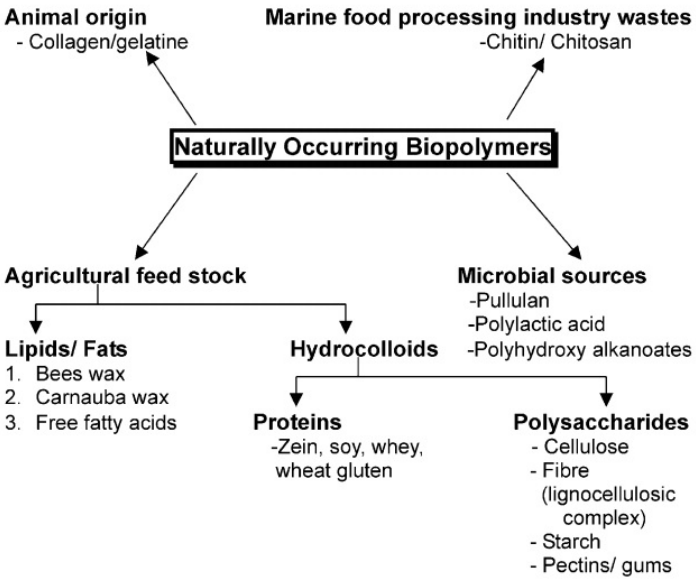


Figure 1 - Naturally occurring biopolymers used in biodegradable packaging films and composites [1]

Depending on the film composition, the formation process and application method, films and coating can have different mechanical and transport properties that will affect the functionality and behavior of the film/coating. It is important to point that films and coatings are two different terms. The film is a thin layer formed from a solution of biopolymers that is prepared separately from the food and then applied to the product. The coating can be a suspension or emulsion that is applied directly to the surface of the food, by diving the product into the coating solution/emulsion or by spraying, which after drying forms the film.

Usually EFC are prepared through solvent casting, in which the biopolymer solution is placed on a support and the solvent evaporation is promoted by air exposure at a certain temperature, and then removing the film formed by detachment from the support [5].

The formation of these films is due to the establishment of inter- and intra-molecular interaction or lathing of the polymeric chains, resulting on a semi-rigid three-dimensional network that mobilizes the solvent. Protein-based films consist of continuous, low moisture, more-or-less ordered macromolecular networks and interactions among proteins

need to be numerous and uniform. To form a macromolecular network from proteins is required three steps: rupture of low-energy intermolecular bonds that stabilize polymers in their native state, arrangement and orientation of polymer chains, and formation of a three-dimensional network stabilized by new interactions and bonds after the agent that ruptured intermolecular bonds is removed[6, 7]. The degree of cohesion depends on the structure of the polymer, the solvent, the temperature used to evaporate the solvent and the presence of other molecules such as plasticizers [5]. A plasticizer is a small molecule with low volatility, that when added to polymeric materials can modify the three-dimensional structure, decreases attractive intermolecular forces, and increases free volumes and chain mobility. Plasticizers act by entering between polymeric molecular chains, physicochemically associating with polymer, by increasing extensibility, distensibility, and flexibility and by decreasing cohesion, elasticity, and rigidity [6, 8].

The use of plasticizers, such as glycerol, sorbitol or polyethylene glycol in films formulation is advantageous, since it increases their flexibility. Consequently, the resulted films are easier to handle and typically have higher elongation rates. The presence of plasticizer decreases the firmness of the film, because this type of low molecular weight compounds complicates the establishment of hydrogen bonds between the biopolymer chains, increasing mobility of the same. It should be noted that the presence of the plasticizer in films not only influences the mechanical properties, but also the barrier properties by reducing the permeability to water vapor and other gases, due to its hydrophobic nature [2].

To choose a suitable coating composition for a particular type of food product, there are a number of criteria which must be considered. The effectiveness of edible coatings for food preservation depends on the control of wettability of the coating to ensure a uniformly coated surface and other factors, such as mechanical and transport properties, solubility and color.

### 2.1.3 Properties

The most important properties of edible films are mechanical, barrier and appearance, because they determine under what conditions they can be applied and used. As with traditional plastic film packaging, the most significant mechanical properties of interest are tensile strength, yield strength, Young’s modulus and percent elongation. The

most important barrier properties are determined as film oxygen permeability and water vapor permeability. Carbon dioxide, oil and aroma permeability properties are also of interest, but the information is of value for more specific applications. The most important appearance properties are transparency, color and gloss.

#### 2.1.3.1 Functional properties

Biodegradable films protect the food from the external environment. Typically the shelf life and quality of the food is reduced when food experience an increase or decrease on the moister, resulting from interactions with the environment, and also when they are contaminated by microorganisms and exposed to oxygen, occurring lipid oxidation.

The primal cause of food deterioration is the development of microorganisms on the surface of food. The application of antimicrobial agents in films and coatings avoid or prevent this from happening, leading to an increase on shelf life making food safer [9]. The antimicrobial agents most often used in food industry are chemical compounds, including organic acids, fungicides, antibiotics and alcohols. These agents have antiseptic properties under certain conditions and are known as food preservatives. Food preservatives are generally stable substances, and it is unlikely that they decompose for a certain period of time [9]. There are some natural polymers that exhibit antimicrobial activity, especially those derived from chitin. Also, essential plant oils rich in phenolic compounds have been reported to have a wide spectrum of antimicrobial activity. Among these, oregano has been found to be one of the most effective. Its antimicrobial properties have been demonstrated in numerous studies [10]. Carvacrol, thymol, c-therpinene and p-cymene are the principal constituents of oregano essential oil [11]. Its antimicrobial properties have been demonstrated in numerous studies [10, 12, 13]. In this work we have tentatively changed the properties of pea protein films by adding both chitooligosaccharides and selected essential oils.

#### 2.1.3.2 Barrier properties

As previously mentioned, one of the main functions of the films is to protect food from the possible effects of the environment, such as water, water vapor, gases, aromas, microorganisms and mechanical shocks. Development of edible films requires knowledge of their barrier properties. Thus, it is necessary to consider the permeability of the films to water vapor, aromas, solutes and lipids [14].

The permeability of a membrane is calculated from the combination of the 1st Fick's law of diffusion and Henry's law of solubility and is determined by the flow of permeate through a non-porous membrane, assuming that has no imperfections [14].

The water vapor transmission rate (WVTR) is defined as the steady water vapor flow in unit time through unit area of a body, normal to specific parallel surface, under specific conditions of temperature and humidity at each surface. The water vapor permeance is the time rate of water vapor transmission through unit area of flat material or construction induced by unit vapor pressure difference between two specific surfaces, under specific temperature and humidity conditions [15, 16]. The permeance is therefore a measure of flow which does not consider the film thickness and is used to evaluate the performance of this film, instead of describing an intrinsic property of the material. The water vapor permeability (WVP) is defined as the permeance through a membrane of unitary thickness. Therefore, it is the product of permeance and thickness of the membrane [14, 15].

The hydrophilic nature of the polymers constituting the edible films limits their protective action as a barrier to water vapor. Thus, for EFC to be used for this purpose, it is necessary to add materials with hydrophobic character, such as waxes and edible fatty acids. However, since these films show some hydrophilic character, thereby establishing hydrogen bonding interactions between the polymers, they are excellent barriers to nonpolar substances, such as oxygen, lipids and some aromatic compounds with a controlled amount of humidity. It should also be noted that the increase of interactions between polymers leads to a decreased permeability of the films.

According to Krochta and Mulder-Johnson [17], films can be considered weak barriers, moderate barriers or good barriers to water vapor according to the WVP values

showed on Table 1. Table 2 shows WVP values corresponding to different types of films, as examples.

Table 1 - Type of barriers according to the WVP values [17]

| Type of barriers | WVP (g.mm/ $m^2$.h.kPa) |
|---|---|
| **Weak barriers** | 0.4-4.2 |
| **Moderate barriers** | 0.004-0.4 |
| **Good barriers** | 0.0004-0.004 |

Table 2 - Permeability to water vapor for different types of films

| Type of film | WVP (g.mm/ $m^2$.h.kPa) | Reference |
|---|---|---|
| **WPI: Glycerol (2:1)** | 12.12 | Chen [18] |
| **WPI: Glycerol (1:1)** | 6.40 | Ramos *et al*. [19] |
| **WPI: Sorbitol (1:1)** | 0.90 | Ramos *et al*. [19] |
| **Chitosan** | 0.07-0.17 | Butler *et al*. [20] |
| **Low density polyethylene (LDPE)** | 0.0013 | Shellhammer and Krochta [21] |

Considering the classification mentioned above, we can state that biodegradable films are weak barriers to water vapor, comparing with synthetic polymers. It is also noted that the amount of plasticizer added to the polymer affects the WVP. The higher the quantity of plasticizer added to the polymer, the lower the WVP, giving the best protection against water vapor. On the other hand, the presence of the plasticizer causes a decreased on the ability of the films to act as effective barriers to oxygen, flavors and lipids [22].

2.1.3.3 Mechanical properties

The mechanical properties of the EFC are as important as the barrier properties. These properties are important to protect the food during the process and handling. Edible films and coatings with satisfactory mechanical properties and good appearance are potential alternatives to the use of synthetic films. Some of the tests used in commercial synthetic films are also used to characterize edible films. In this type of test, the films are subjected to an increasing deformation, by uniaxial tension tests, and the parameters obtain are recorded during the test. Thus, we can obtain curves of stress *versus* strain. An example of a tensile test curve of an edible film is shown on Figure 2.

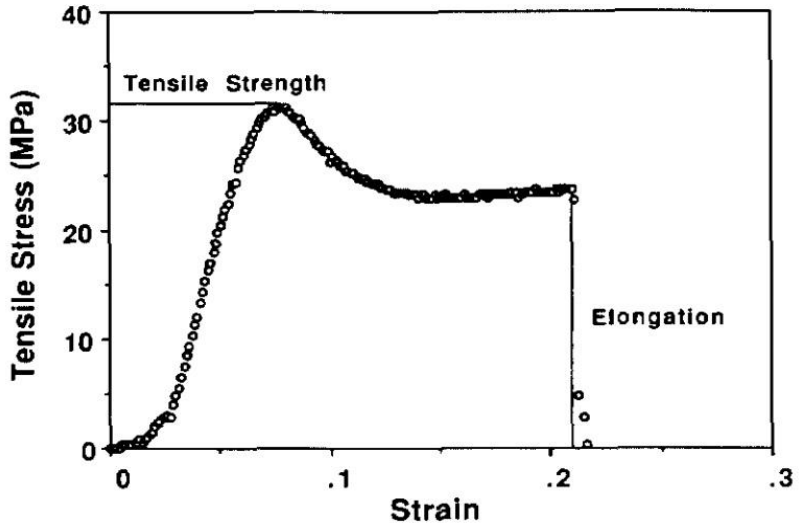


Figure 2 - A typical tensile curve for edible film based on protein [18].

From that curves, we can determine the following mechanical parameters: yield strength or stress at break, tensile strength or stress at maximum force, Young's modulus and percentage elongation or strain at break. Tensile strength is the maximum tensile stress that a film can sustain. Elongation is usually taken at the point of break and is expressed as the percentage of change of the original gauge length of the specimen. Yield strength is the tensile stress at which the first sign of nonelastic deformation occurs. Yield strength is a critical parameter in the interaction between packaging machine and film when a sudden snatch by the machine could cause a permanent distortion. Modulus of elasticity, or Young's modulus, is the ratio of stress to strain over the linear range and measures the

intrinsic stiffness of the film [18]. The tensile properties can be adjusted to make more flexible, stretchable, resilient films by changing the state of the protein or by the addition of plasticizers. Plasticizer efficiency, or how well a plasticizer adjusts tensile properties, is dependent on the size, shape and compatibility of the plasticizer with the protein [23]. In these tests the results are greatly influenced by the experimental conditions such as: storage conditions, sample size, temperature, relative humidity, the magnitude of the applied deformation and application rate [18].

According to Gennadios and Han [24] a film can be considerate adequate for packaging or coating food products if they have elongations between 10-100% and a tensile strength between 10-100 MPa.

## 2.2 Cheese application

Milk, cream, fermented milk products, and processed cheese all require low oxygen permeability to avoid oxidation and microbial growth. Dairy products should be protected against light-induced oxidation (which causes, for example discoloration, off-flavor formation, and nutrient loss), and against water evaporation [25]. Cheese is a complex food product consisting mainly of casein, fat and water [26]. The complex cheese composition along with environmental conditions during handling and storage often promote extensive mould and bacteria development at cheese surface, which considerably reduces its quality. Edible coatings can act as effective carriers of antimicrobials for treating cheese surfaces which are a likely location of microbial contamination. In addition, edible coatings can enhance food quality by acting as a semipermeable barrier to oxygen, carbon dioxide and water vapor exchange, leading to weight loss reduction and respiratory rate modification [27].

Several researchers have recommended that fresh cheeses (e.g. cream cheese, decorated cream cheese, soft cheese, and cottage cheese) should be packaged under modified atmospheres with $N_2$ and/or $CO_2$ replacing the $O_2$ in the package [26, 28]. However, spoilage caused by yeast and especially bacteria may still occur even at very low $O_2$ and elevated $CO_2$ levels [29]. Semisoft and hard cheeses (whole, sliced or shredded) have a relatively high respiration rate, which require a packaging material somewhat

permeable to $CO_2$ to avoid blowing of the packaging. Meanwhile, oxygen must be kept out to avoid fungal spoilage and oxidation of the cheese. The primary spoilage organism on these cheeses is *Penicillium commune* [30]. Mould ripened cheeses, such as white cheeses (Brie/ Camembert) and blue-veined cheeses (Danablue and Roquefort), contain active fungal cultures. As a consequence, the oxygen content should not be too low as this may cause anaerobic respiration and production of off-flavors. Additionally, a change in atmospheric composition can cause a change in the microbiota. Instead these products require a balanced oxygen and carbon dioxide atmosphere to prolong shelf-life [31].

A great variety of microbial species are involved in the ripening of cheese, the total population generally exceeding $10^9$ organisms per gram. The principal bacterial groups involved in ripening are the lactic bacteria *Steptococci*, *Leuconoctoc*, *Lactobacolli* and *Propionibacteria* species. *Micrococci* and *Corynebacteria* species may also be involved, because they are aerobic and salt tolerant and thus they can grow especially on the surface of cheeses. Yeasts are widely distributed in nature and are found in raw milk and some cheeses. In the majority of cheeses the basic flora are species of the genus *Kluyveromyces*. Yeasts produce enzymes that are capable of degrading the constituents of the curd and contribute to modify the texture of the cheese and the development of flavor and aroma. They are capable of converting lactose into $CO_2$ and may also take part in lipid degradation. Fermentation of lactose to lactic acid during manufacture and the metabolism of residual lactose during the initial stage of ripening reduce the pH of cheese to around 5, depending on variety. At this pH, the growth of many pathogenic bacteria is inhibited [32].

Pathogens such as *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli* have all been involved in cheese-associated outbreaks, resulting in severe illness and some deaths [33-36]. Undesirable microorganisms such as *Pseudomonas fluorescens*, yeast, and molds may cause defects in flavor, texture, and appearance of cheese and result in economic losses [37, 38]. One of the potential approaches is to pack or coat cheese surfaces using materials with antimicrobial functionalities. For example, chitosan has shown antimicrobial activities against different groups of microorganisms, including bacteria and fungi [39].

The use of packaging films based on antimicrobial polymers could be proved more efficient, by maintaining high concentrations of the active substance on food surface while

preventing its migration, thereby maintaining a critical concentration for an extended period of time [40].

The most important factors that affect cheese stability are water activity and pH. Water activity, $a_w$, depends mainly on moisture and salt contents. During ripening, $a_w$ is not constant but decreases until the cheese surface is in equilibrium with the surrounding atmosphere. During cheese manufacture, the pH decreases inhibiting the growth of many pathogenic bacteria as mentioned before. While the packaging does not have influence on the pH of the cheese, the water vapor transmission rate through the packaging material is crucial for controlling $a_w$. Additional environmental factors which must be considered in selecting a material for cheese coating are light and oxygen. Light promotes fat oxidation, which in turn is responsible of off-flavor. The oxygen in contact with the cheese contributes to the oxidation of fats and to the growth of undesirable microorganisms [32]. All these factors affect not only cheese physical characteristics but also its flavor during storage. In fact many different compounds contribute to cheese flavor and most of them form during cheese ripening. The breakdown of milk proteins, fats, lactose and citrate during ripening gives rise to a series of volatile and non volatile compounds which may be related to total flavor. Sensory experiments confirm the contribution given by fat-derived compounds to cheese flavor [41-43].

Some studies have been already performed related to the use of coatings to preserve the characteristics of cheeses.

Mei *et al* studied the mechanical, physicochemical, optical and structural properties of films with different amylose content made with starch-chitosan, with the addition of hydrophilic glycerol and hydrophobic perilla oil. They conclude that with the addition of perilla oil, there was a decrease on mechanical properties and the addition of glycerol lead to higher water vapor permeability. The results showed that the cheese coated with chestnut starch-chitosan with perilla oil had the best results according to microbial growth and shelf life length [44].

Duan *et al* studied the antimicrobial activity of chitosan-lysozyme films against microorganisms inoculated onto the surface of Mozzarella cheese. The microorganisms tested were *E. coli*, *P. fluorescens*, *L. monocytogenes*, mold and yeast. Films with different

proportion of Chitosan and lysozyme were tested and all significantly reduced the growth of these bacteria and mold in Mozzarella cheese, although they had lesser antimicrobial effect on yeast [45]

Di Pierro *et al* studied cheese microbiological and physicochemical changes throughout a 30 days storage period, under modified atmosphere packaging. They conclude that films with chitosan/whey protein had about three times higher water vapor permeability that films prepared with chitosan alone. The viable numbers of lactic acid bacteria and mesophilic and psychrotrophic microorganisms were significantly lower in the chitosan/whey protein coated cheese than in the control (uncoated) [46]

Concluding, main problems occurring during cheese ripening and later on, throughout the distribution chain, are contamination by molds and the loss of water. Usually, this problem is solved by the use of synthetic coatings where an antimicrobial agent is introduced. More deep knowledge on the relationships between coating composition and functionality and further studies on alternative materials are still needed. The objective of this work was to study the ability of proteins to be used as edible films and coatings, with incorporated natural antimicrobial agents, taking into account the final application in the preservation of sliced cheese.

## 2.3 Plant based protein films

Proteins and polysaccharides from plant and animal sources are broadly used in human food because of their nutritional benefits and functional properties. Substantial studies have covered proteins from animal origin while plant proteins have received lesser attention. As a result, challenges to replace animal proteins with plant proteins in novel food products have increased. Moreover, there is an economical reason that increases the use of plant proteins worldwide, such as less energy usage for the production of plant proteins[47-49].

At the present time, the worldwide market of vegetable protein is dominated by soy protein. Low price, quality, and versatile applications make them difficult to compete with. Consequently, a large part of the literature is focused on soy protein-based films. There are

a number of studies of edible film based on legume and vegetable proteins [50-54], such as corn, soybean, wheat, cottonseed, and other crops, but only few studies deal with properties of films from pea protein [55-58].

Price comparison made by Choi and Han on whey protein isolate (WPI) ($13.5–27/kg), soy protein isolate (SPI) ($3–3.8/kg), corn zein ($23–35/kg), and pea protein isolate (PPI) ($2.5–2.8/kg) [55] indicates that utilization of pea protein in food production, including manufacture of edible films and coatings could contribute to economic benefits. The lack of genetic modification in commercially available pea species makes pea protein a great alternative to soy protein preparates, which are mostly obtained from transgenic plants.

Zein is found in corn endosperm accounting for 50% or more of total endosperm protein [59]. Zein films are easily cast from alcohol solutions [60]. The most common plasticizer for zein films is glycerol, however it tends to migrate to the food surface due to its weak interaction with protein molecules [61]. Initially the films are transparent, but glycerol causes cloudiness leading to loss of flexibility. These films have tensile strength and elongation at break similar to wheat gluten films. With the addition of the plasticizer, the tensile strength decreases and the elongation at break increase. Tensile strength depends on relative humidity and temperature conditions, decreasing with the increase of these parameters [60]. Zein films have low vapor-barrier ability, however that could be considered an advantage, as it allow excessive water vapor movement across the film, thus preventing water condensation inside the package leading to microbial spoilage [62, 63]. Studies have reported that zein/ soy protein and zein/ wheat gluten protein films have lower permeability than single zein films [64].

Wheat gluten is the cohesive and elastic mass that is left over after starch is washed away from wheat flour dough [65]. These proteins are water insoluble, so their solubilization and films preparation require a complex solvent system, for example basic or acid conditions in the presence of alcohol and disulfide bond-reducing agents [66]. Glycerol and ethanolamine are the plasticizers preferred for these proteins [65]. Wheat gluten films show low tensile strength and high elongation at break compared to other filmd [67]. The mechanical properties of these proteins depend on the processing conditions, the addition of plasticizer, lipids and cross-linking agents, and external

conditions, such as relative humidity and temperature. The water vapor permeability of these films is similar to those from other protein or polysaccharide based films, but is relatively high compared to synthetic polymer films [68]. The barrier properties of these films could be particularly interesting for applications in the field of active coating, active packaging, drug delivery systems and modified atmosphere packaging [65].

Cotton is cultivated mainly for fiber production, but it also produces seeds which is an important source of proteins for human and animal consumption. Cottonseed protein films are obtained directly from cottonseed flour using a casting process [16]. The conditions for these proteins to form films are difficult to determine due to the complexity of the raw material [16]. Many studies have established that the optimal conditions to obtain cottonseed films are pH between 8 and 12, range of temperature from 20 to 60ºC, concentration from 10% to 50% (w/v), plasticizer content of 10% to 50% (w/w) (dry basis), and the use of dispersive agents [69]. The mechanical properties of these films depend on the source of the flour, the chemical cross-linking agents, temperature, relative humidity, and glycerol level [16]. Using cross-linking agents such as formaldehyde, glyosal or glutaraldehyde improve the mechanical properties of these films. Formaldehyde produce more resisting films than other agents [16]. The tensile strength is five to tenfold weaker than films made from synthetic materials, however if the cottonseed flour contains cotton fibers, the resulting films may be as strong as synthetic materials. The water vapor permeability of these films is similar to that of other protein films such as zein or soy, but higher than synthetic materials. These films may be suitable for application in non-food packaging, because good mechanical resistance and insolubility in water are required [69].

Peanut protein concentrates and isolates are commercially produced from defatted peanut flour. There is a proven allergenicity associated with peanut proteins, what is an important limitation when these proteins are added to foods for nutritional purposes [70]. Two different methods are used to prepare peanut protein films. The first involves the formation of peanut-protein-lipid films on the surface on heated peanut milk [71-73]. The second method involves casting peanut protein concentrates or isolates solutions. Jangchud and Chinnan [52, 53] have studied peanut protein films at different pH (6, 7.5 and 9), dried temperature (70, 80 and 90 ºC), and with different plasticizers. They conclude that films formed at higher pH (7.5 or 9) and higher drying temperature (80 or 90 ºC) were less

humid and sticky at the surface than the films formed at the remaining conditions. Glycerol was determined to be the best performing plasticizer. With higher drying temperature, the water vapor permeability decreased, whereas the pH had no significant effect. Tensile strength and elongation increased when film drying temperature increased from 70 to 90 ºC. This was attributed to increased protein denaturation at higher temperature, resulting in a tighter, more compact film structure.

### 2.3.1 Soy proteins

Since the preparation and study of soy protein films was an initial purpose of this work, we present below a more detailed review of current knowledge about the functionality of soy proteins.

Soybeans are widely grown in the world. Soy proteins are a co-product from soybean oil industry. One of the new uses for these protein are as base material for biodegradable edible films and coatings [74].

#### 2.3.1.1 Composition

Soy protein have been commercially produced and supplied in forms of soy flour, soy protein concentrates (SPC) and soy protein isolates (SPI). The SPI has a higher protein contents (>90%) than SPC (≈80%). Films made from SPI are more common than SPC, due to the fact that SPC have non-protein components that affect the film-forming ability [75].

Most of the protein in soybeans can be classifies as globulins. A widely used nomenclature system for soy protein is based on relative sedimentation rates of the different protein fractions under a centrifugal force, being then separated and designated as 2S, 7S, 11S and 15S (Table 3) [76, 77]. The 7S and 11S are the main fractions of the total extractable protein as shown on Table 3 [78].

Table 3 - Approximate distribution of the major components of soy proteins, adapted from Kinsella [75].

| Fraction | Content | Principal components |
|---|---|---|
| **2S** | 8% | Trypsin inhibitor, Cytochrome |
| **7S** | 35% | Lipoxygenase, Amylase, Globulins |
| **11S** | 52% | Globulins |
| **15S** | 5% | Polymers |

The 7S protein is extensively glycosylated and can assume as many as seven different forms due to combinations of its three peptide subunits. On the other hand, the 11S protein does not contain an appreciable amount of carbohydrate, and its subunits differ in charge and molecular weight. These structural differences contribute to variations in the functional properties of 7S and 11S fractions. For instance, the 11S fraction has a more significant impact on gelation characteristics of soy protein than does of the 7S fraction. Kunte *et al.* compared films made from commercial soy protein isolate to those of films formed from laboratory-prepared crude 7S, crude 11S, and soy protein isolate. They conclude that the use of 11S soy globulin fraction alone can give stronger films than commercial soy protein isolate. However, this improvement in film strength would come at the additional costs of further purifying soy protein isolate or separating the 11S protein fraction[79].

#### 2.3.1.2 Soy protein films

Within the biopolymers used on edible films and coatings, soy protein produces more elastic, soft, smooth and clear films compared to other films from plants sources and they have impressive gas barrier properties compared with those films prepared from lipids and polysaccharides [74, 80]. Because of these properties, they have achieved considerable attention.

The film-forming ability of soy proteins has traditionally been utilized in the Far East for production of soy protein-lipid films called yuba films [81, 82]. The process of yuba film formation consists of boiling soy milk in shallow pans, collecting the films formed, due to surface dehydration, by means of rods, and hanging the films to air-dry [83, 84].

Similarly to other protein-based films, the formation of films from soy protein has been described as a process involving the denaturation of proteins followed by surface dehydration. SPI has limited solubility due to the fact that acid precipitation or industrial heat treatments decrease the nitrogen solubility of soy proteins by denaturation and aggregation. As a result, soy protein solubility increases under alkaline conditions [80, 85].

However due to the hydrophilic nature of these proteins, the films obtained present two major disadvantages: fragility in the wet state and poor properties of moisture barrier. These effects can be minimized by using physical, chemical or enzymatic treatments including the addition of hydrophobic additives like neutral lipids, fatty acids or waxes [74].They have low water vapor permeability, because of the presence of free hydroxyl groups which interact strongly with migrating water molecules [75].

To make a 'good' film, addition of a plasticizer is also essential. Glycerol is the most often used plasticizer in 3–5% w/v concentrations for soy protein-based films. Glycerol also affects the WVP as glycerol is hydrophilic and thus favors water adsorption and migration through the film.

The natural pH of soy milk is about 6.7, but film formation is favored in more alkaline environment. At pH values close to the isoelectric range of soy proteins, films formation is negatively affected due to protein aggregation/coagulation.

Mauri and Añón [86] investigated the changes in solubility and molecular properties of soy protein isolates films prepared at different pH values (2, 8, and 11). During film formation, proteins retained their native conformation at pH 8, while they were partially or extensively denatured at pH 11 and 2. The proteins at extreme pH values readily established chain-to-chain associations by a combination of covalent and noncovalent interactions, and films obtained at pH 2 and 11 showed denser microstructures than those formed at pH 8. In addition, SPI films prepared at pH 6 to 11 were found to have significantly higher tensile strength, higher percentage elongation at break, and lower WVP than those obtained at pH from 1 to 3. Gennadios *et al* reported similar results to those of Mauri and Añón [82]. In many studies, the solution is made at alkaline pH, for example pH 10, to unfold the protein [8] and to improve the final film properties.

Heating above 60ºC and under alkaline condition (below pH 10.5) promote soy protein polymerization by altering the three-dimensional structure through the unfolding of polypeptide chains, thus exposing sulphydryl and hydrophobic groups and promoting the establishment of new intermolecular interactions [87]. Upon drying the cast solutions, unfolded macromolecules approach each other and are linked by disulfide bonds and hydrophobic interactions. SPI films from solutions heated at 95 ºC have greater tensile strength and elongation values than films from solution heated at 75 ºC [88]. Heating and alkaline conditions are accepted to facilitate soy protein denaturation, thus promoting the formation of disulfide bonds within the structure of the dried films [67].

In general, soy protein films are similar to other protein films, they have only moderate mechanical properties compared to commonly used plastic films [17, 82]. They have poor moisture resistance and water vapor permeability due to the natural hydrophilicity of the protein and the substantial amount of hydrophilic plasticizers used to impart the film flexibility. Compared to synthetic films, cast SPI films have higher water vapor permeability values by roughly four orders of magnitude. On the contrary, soy protein films are effective oxygen barriers, at least at low relative humidity environments.

It has been reported that films formed in alkaline conditions leads to lower water vapor permeability. Films thermally treated also have lower WVP [67, 89]. Wan *et al* [90] studied the effect of a mixture of glycerol and another plasticizer (propylene glycol, polyethylene glycol, sorbitol, or sucrose) on water vapor permeability and mechanical properties. They conclude that a mixture of glycerol and sorbitol (50:50) was the best combination because of its low WVP value and relatively high flexibility and strength [90].

#### 2.3.1.3 Applications of soy-protein films

Although soy protein films are poor water vapor barriers, they have been found to be very effective oxygen barriers [84, 91]. The good oxygen barrier ability of soy protein films could be utilized in the manufacture of multilayer packaging where soy protein films would function as the oxygen barrier-providing layer. In another potential application, soy protein coatings on precooked meat products could control lipid oxidation and limit surface moisture loss. Incorporation of antioxidants and flavoring agents in soy protein coatings

could improve overall quality characteristics of food products [79]. Also, soy protein films may find applications as microencapsulating agents of flavors and pharmaceuticals, or in coatings of fruits, vegetables, and cheese [25]. Protective soy protein coatings could also be used on certain food products, such as meat pies and high-moisture-low-sugar cakes, which require films that are highly permeable to water vapor [91].

### 2.3.2 Pea Proteins

Pea is a cool-season pulse crop with an annual worldwide production around 10-20 million tones [92]. They were traditionally used for animal feed, especially for swine, due to their high nutrition value [93]. Now they are manly purchased for human consumption or high-value feeds, as the price of peas increased [94]. Pea proteins are similar to proteins isolated from soy beans; they have a wide range of applications in variety of food materials. They have a high protein content, a relatively low price, a lack of genetic alterations, and pea protein is not a common allergen (unlike other proteins such as soy, peanuts, egg, milk, wheat gluten and other nuts, which account for almost 90% of food allergies) [95]. They could be a potential component for edible films.

#### 2.3.2.1 Composition

Pea protein can be processed into pea flour, pea protein concentrate (PPC), and pea protein isolates (PPI) [96, 97]. The PPI has a higher protein content (85%) than PPC (70,6%) [98]. Since PPI have high nutritional value and non-allergic character, they are manly used as an additive to enrich the protein content in the food industry. The number of technical applications of pea protein is very limited including surfactants, films and microspheres in cosmetics [99].

Pea protein consists mainly of globulins (>80%) and a small fraction of albumins [100]. The globulin fraction contains legumin, the major fraction, vicilin, the second major, and small quantities of convialin, that are storage proteins [96, 101, 102]. Albumins, which compose 13-14% of the total proteins, are cytoplasmic proteins, consisting of many kinds of subunits and containing more sulfur amino acid residues than the globulins [103].

#### 2.3.2.2 Pea protein films

Few studies can be found in the literature about the ability of pea protein to form films. *Choi and Han* reported that pea protein based films showed similar physicochemical properties to those found for soy protein, whey protein or zein, but at a considerably lower price. They can be utilized to prepare edible films with water vapor permeability (WVP) and physical characteristics similar to films obtained from those proteins [55, 56]. Increasing the concentration of glycerol as a plasticizer in the film, decreases the tensile strength and Young's modulus, but increases the elongation and water vapor permeability [55]. Heat treatment of PPI solution for 5 minutes at 90ºC, induces denaturation and increases the films' tensile strength and elongation, while decreasing their water vapor permeability and Young's modulus [56]. Choi and Han also reported that comparing the non-heated and heated-denatured films, FTIR spectroscopy showed more water in the non-heated, and electrophoresis suggested that intermolecular disulfide bonds are created during the heat denaturation, which lead to a greater integrity of the films compared to the non-heated ones.

#### 2.3.2.3 Applications of pea-protein films

Reports on the application of pea protein films are scarce. Kowalczyk and Baraniak studied films made with 10% of pea protein and found that the films resisted UV light transmission, wich resulted from the presence of UV absorbing chromophores induced by disulfide bonds and amino acids. The authors suggested that pea protein films could be used as packaging material to prevent the degradation of UV-sensative food ingredients. This could potentially lead to applications in food packaging [104].

## 2.4 Chitooligosaccharides

### 2.4.1 Composition

Chitin is a natural non-toxic N-acetyl polysaccharide extracted from the shells of crustaceans (e.g., crabs, lobsters and shrimps), the exoskeletons of insects and the cell walls of fungi. Chitosan is obtained by deacetylating chitin. The molecular weight of chitosan is very large, which limits its physiological activity [105].

Chitosan and its oligosaccharides, which are known to possess multiple functional properties, have attracted considerable interest due to their biological activities and potential applications in the food, pharmaceutical, agricultural and environmental industries [106-109]. These biopolymers present a wide range of biological properties such as biocompatibility, biodegradability, hemostatic, analgesic, anti-inflammatory, antimicrobial, antioxidant, anticolesterolemics, antitumoural and immunostimulatory [110-116]. The water solubility of chitooligosaccharide is higher than the parent polysaccharides and are thus easily absorbed by living bodies, so their bioactivity can be brought into full play.

Chitooligosaccharides (COs) are the degraded products of chitosan or chitin, which have recently been produced by several methods such as enzymatic and acidic hydrolysis. Enzymatic preparation methods have received great interest due to their safety and ease of control. Many nonspecific enzymes, such as cellulases, lipases and proteases as well as chitosanases, have been used to prepare COs [117, 118]. Generally, these oligosaccharides have molecular weights of 10 kDa or less, with a degree of polymerization of 2-20 glucosamine monomers, connected by β-1,4-glycosidic bonds.

### 2.4.2 Antimicrobial Activity

Antimicrobial activity of chitooligosaccharide can be affected by several factors such as degree of polymerization, degree of deacetylation, type microorganism [110], molecular weight and concentration [119]. Some authors report that oligosaccharides showed antibacterial activity and a 0.5% concentration completely inhibited the growth of *Escherichia coli*[120].

In contrast to chitin, chitosan and chitooligosaccharide have in their structures an higher amount of primary amino groups. The amount of amine groups plays a critical role in the antimicrobial activity, and several mechanisms to describe this type of activity have been proposed [58]. The most accepted mechanism consists in the fact that the primary amine group causes alteration on cell membrane permeability of the microorganism causing its rupture and subsequent release of multiple intracellular components leading to death of the microorganism [121].

### 2.4.3 Antioxidant activity

In the past years, natural antioxidants have gained a lot of attention due to their capability to protect human body from free radicals, delaying the appearance of chronic diseases [122]. An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation reactions can produce free radicals that can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. This can be inhibit by the addition of antioxidants, due to the fact that they can retard the free radical effect, by reacting with them, preventing them to react with lipids [123] or other sensitive compounds.

Lipid oxidation is an important issue to both consumer and food industry, because these reactions give rise to potentially toxic products and products with an undesirable flavor [123]. Synthetic antioxidants can be added to food products to prevent lipid oxidation. However, regulation restrictions and the growing consumer demand for food devoid of synthetic antioxidants has focused research on the development of new natural preservatives [124].

Many biological compounds such as carbohydrates, peptides and certain phenolic compounds were found to be very effective antioxidants. More recently, chitosan and its derivatives have been the subject of many studies, due to their antioxidant activity [110]. The chitooligosaccharides have a high capacity to capture hydroxyl, superoxide and hydrogen peroxide radicals [125]. Their antioxidant activity depends on the degree of deacetylation and molecular weight [110].

These oligosaccharides have been used in this work to tentatively improve the antimicrobial and antioxidant of the protein-based films.

## 2.5 Essential Oils

Essential oils (EOs) are aromatic oily liquids obtained from plant material such as flowers, seeds, leaves, herbs, wood, fruits, and roots. The common method to obtain these oils is by steam distillation of plants [11]. Their chemical composition is complex and strongly dependent on the part of the plant considered (for example seed or leaves), the moment of the harvest (before, during, or after flowering), the harvesting season and the geographical sources [126, 127].

The relatively recent appreciation in "green" consumerism has lead to a renewal of scientific interest in these substances due to their properties. They are rich in phenolic compounds, such as flavonoids and phenolic acids, which exhibit a wide range of biological effects such as antimicrobial, antioxidant, antifungal, and antiviral [11, 128-132]. These characteristics are possibly related to the function of these compounds in plants [133]. Examples of such plants are cassia, clove, garlic, sage, oregano, pimento, thyme and rosemary. However, it has been reported that other minor components in these oils have a critical influence on those properties acting synergistically with other components [131, 134, 135].

Most of the EOs are classified as Generally Recognized as Safe (GRAS), but their use is limited due to its flavouring. In order to act as an effective antimicrobial, doses required can modify the organoleptic properties to an unacceptable level [136]. An alternative to avoid this problem is the incorporation of EOs within edible films. Edible films can reduce the diffusion of antimicrobial compounds into the product since the EO forms part of the chemical structure of the film and interacts with the polymer and the plasticizer. Compared with direct application, smaller amounts of antimicrobial agents would be needed when edible films are used [132]. Antimicrobial compounds release from the edible films depends on many factors, including electrostatic interactions between the antimicrobial agent and the polymer chains, osmosis, structural changes induced by the presence of antimicrobial, and environmental conditions [137].

These films can act as a carrier for antimicrobial and antioxidants compounds in order to maintain high concentrations of preservatives on the food surfaces [138, 139]. The use of EOs can also be an improvement on barrier properties due to their hydrophobic compounds [140].

Studies concluded that the antimicrobial activity of these oils is related to the disruption of the microbial cytoplasmic mebrane [11]. Among the most effective essential oils, thyme and oregano essential oils have been pointed out to possess better antimicrobial potential for some applications, which could be attributed to the presence of phenolic compounds, particularly thymol and carvacrol [138, 141, 142].

These essential oils have also been reported to possess antioxidant properties [132, 143]. As mentioned before the use of edible films incorporated with these oils are of interest to decrease the amount of oils needed to act effectively without significantly changes in the flavour. The antioxidant efficiency of edible films with EO has been tested using different approaches [144]. In some studies, the film was disintegrated and different tests (such as radical scavenging assays) were performed to the resulting formulation. In these cases, the disintegration procedure depended on the material and its solubility properties. Methods like DPPH, ABTS, FRAP, amongst others, measure the ability of an antioxidant agent to intercept free radicals [145].

The antioxidant activity of edible films and coatings is greatly influenced by the water availability, which is affected by the moisture of the product and the ambient relative humidity. In dry conditions the network structure of the film is tightly packed and its oxygen permeability is very limited. The reduced oxygen availability in the coated product leads to a positive effect on preservation of quality. In some cases, the addition of antioxidants can entail further protection by enhancing the oxygen barrier properties [146, 147]. However, in these conditions of reduced molecular mobility no chemical activity of the antioxidant agents can be observed and the only antioxidant effect is due to the oxygen barrier effect [148]. On the other hand, in wet systems the coating network is plasticized and mass transference is favored. In this context, the oxygen permeability of the film or coating is dramatically increased and the specific activity of antioxidant agents could become more relevant, including those of EOs.

These compounds have also been used in this work to tentatively improve the antimicrobial and antioxidant activity of the protein-based films and the potential improvement of the organoleptic properties of the food products where they can be applied as coatings.

# 3. Materials and methods

## 3.1. Materials

The sample of pea protein isolates used in this study was from Cosucra Groupe Warcoing, Pisane® M9, 88% of protein. The glycerol utilized as a plasticizer agent was obtain from Sigma-Aldrich, with a 99,5% of purity. Potassium persulfate (≥99,0%) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) (≥98,0 %) were purchased from Sigma-Aldrich Chemical Company. Ethanol (99.9%) was from Scharlau. The magnesium nitrate hexahydrate was purchase from VWR Chemicals (99%). The chitosan oligosaccharides were purchase from Yaizu Suisankagaku Industry. The thyme oil and Bay oil samples were from Sigma-Aldrich Chemical Company. Soy protein was purchase from Solbar Plant Extract.

## 3.2. Preparation of soy protein films

Film forming solutions were prepared by slowly dissolving 8% (w/w) of SPI powder in deionized water and then homogenized by using the Ultra Turrax T25 basic, IKA-Werke, at 9500 rotations per minute for 10 minutes. The pH was adjusted to 9 with sodium hydroxide 0.25 M. The glycerol (GLY) was added to the solution at SPI: GLY ratio of 2:1. Then the solution was heated in a water bath at 90 °C for 10 minutes under stirring. This step is very important for the formation of intermolecular bonds which will in turn assist the establishment of a cross-linked polymeric network structure. After filtration through a G2 porous filter, and degasification under vacuum, different amounts of the mixture were placed in acrylic plates over an area of 139 $cm^2$. Finally the solvent was allowed to evaporate at room temperature for 2 days.

## 3.3. Formulation of pea protein films

Film forming solutions were prepared by slowly dissolving 10% (w/w) of PPI powder in deionized water and then the pH adjusted to 9 with sodium hydroxide 0.25 M. The glycerol (GLY) was added to the solution at PPI: GLY ratio of 2:1. Then the solution was heated in a water bath at 90 °C for 20 minutes under stirring. Then, further steps to obtain the PPI films were performed as described above (§3.2).

### 3.3.1. Formulation of pea protein films with chitooligosaccharides

Film forming solutions were prepared by slowly dissolving 10% (w/w) of PPI powder and chitooligosaccharides in deionized water and then the pH adjusted to 9 with sodium hydroxide 2 M. The COs was added to the solution at a COs concentration of 0,5%, 1%, 2% and 5% (w/w). The glycerol (GLY) was added to the solution at PPI: GLY ratio of 2:1. Then, further steps to obtain the PPI/COs films were performed as described above (§3.3).

### 3.3.2. Formulation of pea protein films with essential oils

Incorporation of essential oils in the PPI films was tested for two oils: Thyme and Bay oil. Films were prepared as described above (§3.3), except for the addition of the essential oil before the filtration step. The essential oil was added to the solution at a concentration of 1% (w/w). We prepare films without chitooligosaccharides and with 0,5% and 2% (w/w) of COs with the essential oil.

## 3.4. Film characterization

### 3.4.1. Mechanical properties

To evaluate the tensile properties we used a texture analyzer equipment (TA-Hdi, Stable Micro Systems) as mention by Santos *et al* [149]. This analyzer is equipped with fixed grips lined with thin rubber on the ends. The thickness of the samples was measured by using a digital micrometer (Mitutoyo Corporation). The films prepared were cut into rectangular samples with dimensions of 100 mm long and 10 mm wide. The films were placed in a chamber with a saturated solution of magnesium nitrate under controlled temperature and humidity conditions (50 ± 5% relative humidity and a temperature of 22 ± 5 ° C) for about 48 hours. The initial grip separation was set at 50 mm, and the crosshead speed was 0.5 mm/s. Young's modulus, percentage elongation or strain at break, yield strength or stress at maximum force and tensile strength or stress at break, were determined

from stress–strain curves obtained from uniaxial tensile tests to membrane failure. All experiments were conducted at 23°C (±2°C) and 50% (±2%) of relatively humidity. Eight samples were tested for each type of films. Figure 3 illustrates the set up used to test the mechanical properties of the films.

Figure 3 - Photograph of a tensile test.

### 3.4.2. Water vapor permeability

Water vapor permeability was determined based on the ASTM standard method 96-95, following the ‘‘desiccant method’’. The film specimen (diameter 6 cm) was sealed to the open mouth of a test cup containing a desiccant, anhydrous calcium chloride pre-dried at 200°C for 2 hours, using a silicon sealant and four screws symmetrically located around the cup circumference (Figure 4). The cylindrical test cups are made of polymethylmethacrylate. The assembly was placed in a test chamber maintained at 23 ± 2°C and at 53% relative humidity using a saturated aqueous magnesium nitrate solution (Santos *et al*. [149]). Air was continuously circulated throughout the chamber with a fan (air velocity ≈ 160 m/min). Periodic weighings were performed in order to determine the rate of water vapor movement through the specimen into the desiccant. Steady state conditions were assumed to be reached when the rate of change in weight of the cup

became constant. This constant rate of weight increase was obtained by linear regression. At least five samples of each film type were tested.

Figure 4 - Photograph of the cup used to evaluate the water vapor permeability.

### 3.4.3. Contact angle

The drop method, based on the optical contact angle, was used to estimate the surface hydrophobicity of the films. The shape of the sessile drop was studied after 45 seconds with a Video-Based Contact Angle Meter model OCA 20. Image analyses were carried out using SCA20 software. The liquid used for the test was ultra pure water. A drop of 3 µL was placed, automatically dosed by a syringe connected to system, on the film surface. The evolution of the drop shape was recorded with a camera associated with the system. Calculation of contact angles was performed using software image analysis by the method of the ellipse. The resulting contact angle between the surface edge of the drop and the film surface is called the angle θ being defined as the angle between the plane tangent to the water droplet at the contact point of balance in the interface film / water / vapor and plane in which the droplet is deposited in accordance with Figure 5. The final values are the average of nine determinations (9 drops of ultra pure water at different places on the film) [3, 150].

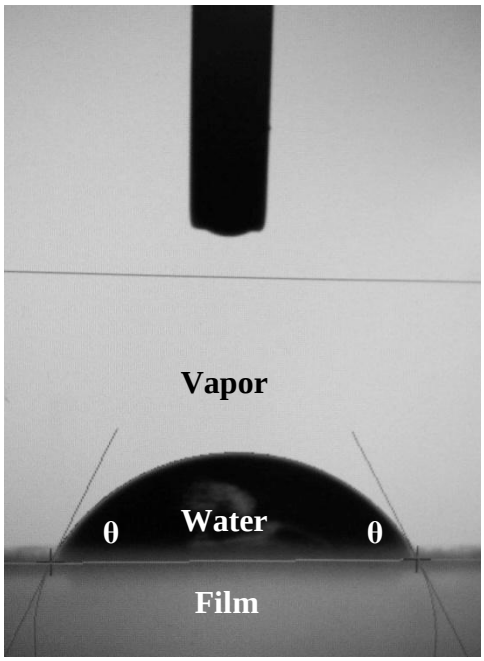

Figure 5 - Photograph of the drop in the film.

### 3.4.4. Antioxidant activity

The antioxidant activity of the produced films was determined by an adaptation of the method for removing radicals of the acid 2,2'-azino-bis (3-ethilbenzotiazolina-6-sulfonic), ABTS, described by Re et al. [151].

ABTS 7 mM dissolved in potassium persulphate 2.45 mM was allowed to react in the dark at room temperature for 12-16 hours for the formation of $ABTS^{\bullet +}$. 1 mL of this solution was diluted in 80 mL of ethanol and the absorbance at 734 nm was measured in a spectrophotometer (Jenway 6405 UV / Vis) to adjust the concentration of the solution to obtain absorbance values between 0, 7 and 0.8.

A square film with 1 $cm^2$ was placed in 3 mL of $ABTS^{\bullet +}$ and allowed to react in the dark. The absorbance at 734 nm of the solution $ABTS^{\bullet +}$ was measured over time, as well as the absorbance of ABTS solution without film.

The antioxidant activity of the films was determined by the percent inhibition calculated as follows:

$$\%\ of\ inibition\ = \frac{Abs\ (control) - \ Abs\ (sample)}{Abs\ (control)} \times 100$$

Where Abs (control) is the value of the absorbance of $ABTS^{+\bullet}$ (without film) and Abs (sample) is the value of the absorbance of $ABTS^{+\bullet}$ with the sample (film).

### 3.4.5. Cheese treatments and analysis

Preliminary tests were performed to optimize film deposition on the surface of sliced cheese and to evaluate some properties of the treated samples. *Flamengo* cheese was sliced in cubes of about 1 $cm^3$, and the coatings were applied by dipping the cheese samples into the film solution during 1 minute, two times to assure that all the film was covered. After the first and second dipping the samples were dried during 5 and 12 hours, respectively, at room temperature (≈21 ºC) and humidity. The dried samples were then storage in a cool room at 5 ºC. Samples were evaluated for microbial growth over time by a qualitative analysis.

## 3.5. Statistical analysis

The results for antioxidant activity, hydrophobicity, mechanical properties, permeability and contact angle were statistically evaluated in order to check which ones were significantly different.Statistical analysis was performed using SPSS (SPSS 20.0 for Windows, SPSS Inc., USA). The existence of significant differences among the different conditions was assessed by one-way analysis of variance (ANOVA) model. A value of $p < 0.05$ was considered to be statistically significant.

# 4. Results and discussion

## 4.1 Preliminary assays – Addition of Chitooligosaccharides

To obtain films with COs it was important to test the best time for their addition, during the preparation process, due to their ability to precipitate with proteins. COs were added at three different steps during the procedure described in § 3.1.: (1) When the PPI was dissolved in deionized water before the adjustment of the pH to 9; (2) After adjusting the pH to 9; (3) After the solution was heated. The results obtained, regarding precipitate formation, are shown in Table 4. The isoelectric point of pea protein is around 4.5. The increase in pH leads to a higher density of negative charges, a greater repulsion between chains, and probably a higher availability of the proteins to interact with other compounds, in particular with the amino groups of COs.

Table 4 - Precipitate formation in the final filmogenic solutions, with 1% COs added at three different steps of the preparation procedure.

| Step | Observation |
|---|---|
| **Before pH adjustment** | Didn't precipitate |
| **After pH adjustment** | Precipitate |
| **After Heating** | Precipitate |

Based on these results, the addition of COs was performed before adjusting the pH to 9. When COs was added before the adjustment of the pH, the initial ph was 6.3, while without COs the initial pH was 7.5. Results shown in Table 4 were obtained for 1% COs. For the PPI dispersions prepared with 0.5%, 2% and 5% of COs the initial pH was 7.1, 6.2 and 5.8, respectively. The addition of COs decreased the initial pH, and more sodium hydroxide was needed to adjust the pH. The amount of sodium hydroxide solution needed to adjust the pH of the dispersion with lower initial pH (5% COs) was taken as the reference to ensure that all the filmogenic dispersions underwent a similar dilution and thus at the end would have the same final protein concentration, . For the dispersions with lower amount of COs , the amount of sodium hydroxide was also measured and deionized water was added to complete the same volume added in the dispersion with 5% COs. The solution with 5% COs wasn't able to form a proper film, so no tests were further done for this dispersion.

## 4.2 Preliminary assays – Effect of the pH

Preliminary experiments were performed to define the better pH to form films, based essentially on their final mechanical properties and easy to handle. Mechanical testes were made for PPI films produced at pH 7 and 9. Although the Young's modulus was not significantly different (Figure 8), for the tensile strength and elongation (Figure 6 and 7) it is clear that films made at pH 9 had significantly higher values of these mechanical parameters than the films prepared at pH 7 (ANOVA, $p<0,05$). This may be due to the fact that as mentioned before, the increase in pH leads to a higher availability of the proteins to interact with other compounds. From these results it was decided to use this pH to form films.

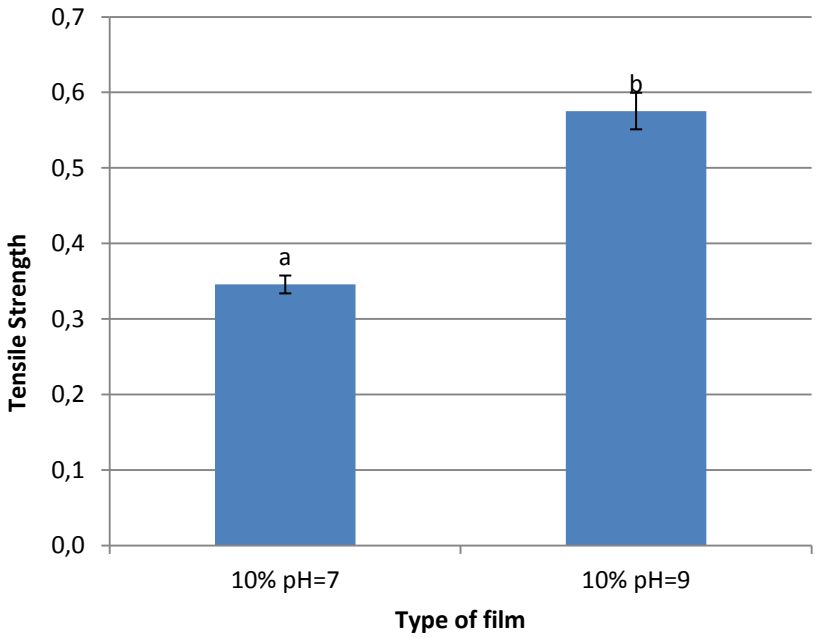


Figure 6 - Tensile strength of PPI films at different pH values. Different letters denote for statistically significant differences ($p<0.05$).

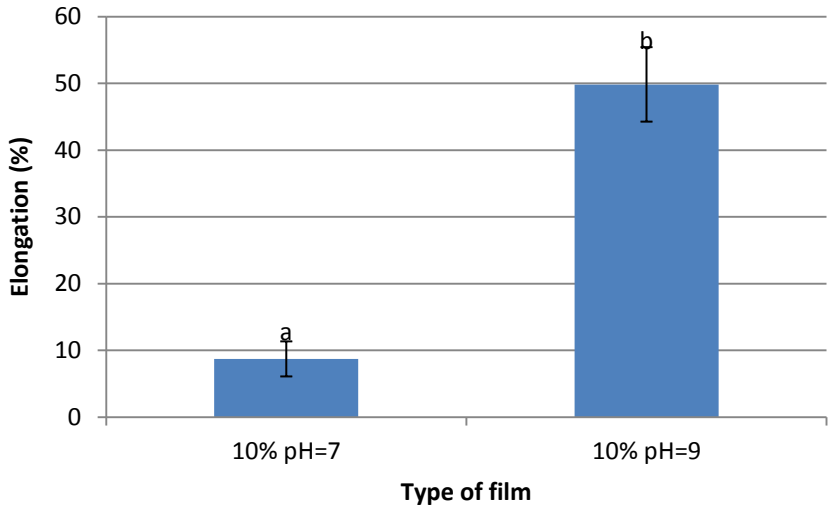


Figure 7 - Elongation of PPI films at different pH values. Different letters denote for statistically significant differences ($p<0.05$).

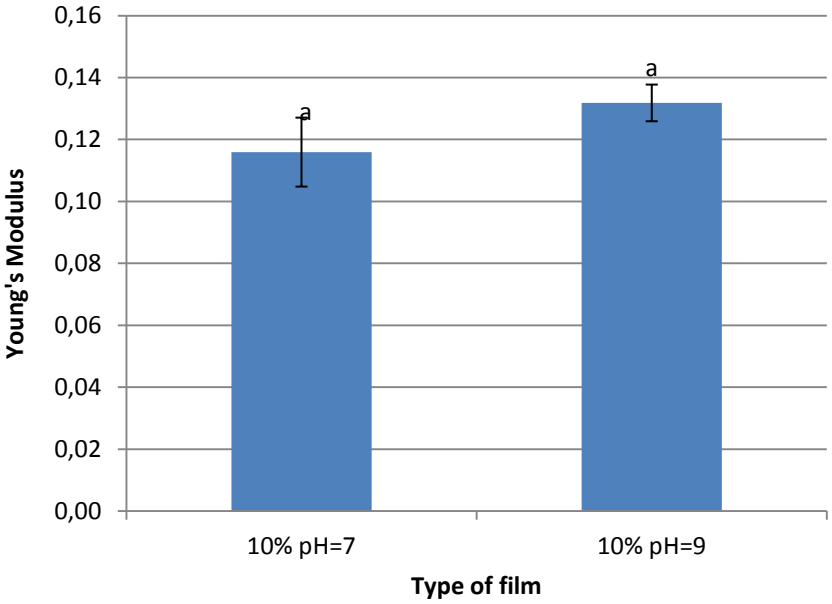


Figure 8 - Young's Modulus of PPI films at different pH values. Different letters denote for statistically significant differences ($p<0.05$).

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## 4.3 Water vapor permeability

The moisture transfer is one of the most important factors contributing to the change in food quality during transportation and storage. Critical values of water activity must be maintained in order to ensure the quality and safety of food products. Therefore analysis of the films prepared was made for the permeability to water vapor in order to check whether the use of such films as food coating would be advantageous. These tests were made on both surfaces of the films. The external surface (exposed to atmospheric conditions of temperature and humidity during drying) and internal (in contact with the acrylic plate during the process of drying), but no significantly differences were observed (ANOVA, $p>0,05$). The results shown in Figure 9 are the average from both surfaces of the film. Films without COs, with 0,5% and 2% of COs aren't significantly different from each other (ANOVA, $p>0,05$). The film with 1% COs have significantly lower water vapor permeability (ANOVA, $p<0,05$).

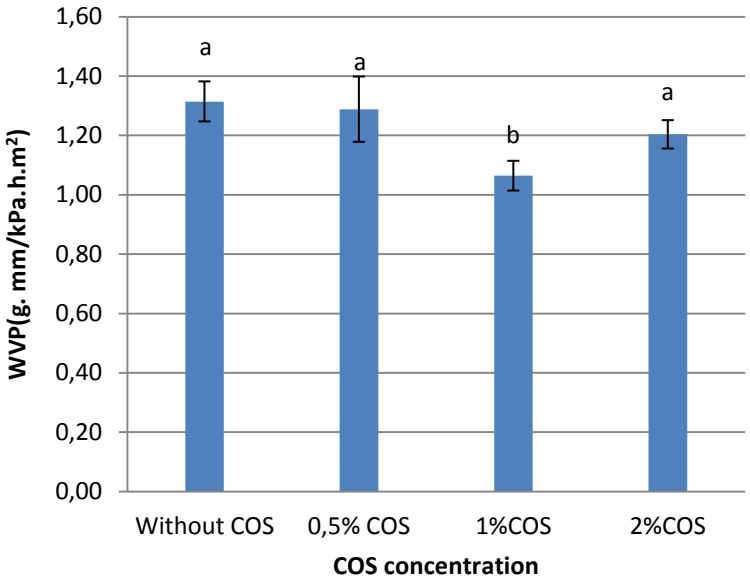


Figure 9 - Vapor permeability of PPI films with different COs concentrations. Different letters denote for statistically significant differences ($p<0.05$).

In Figure 10 is shown the water vapor permeability of soy protein and pea protein. Although the protein content is different, PPI has a significantly lower water vapor

permeability than SPI (ANOVA, p<0,05). The protein content differs only in 2% from each other, leading to conclude that the decreased in the WVP is due to the protein itself. Films made with pea protein have lower water vapor permeability than films based on soy protein.

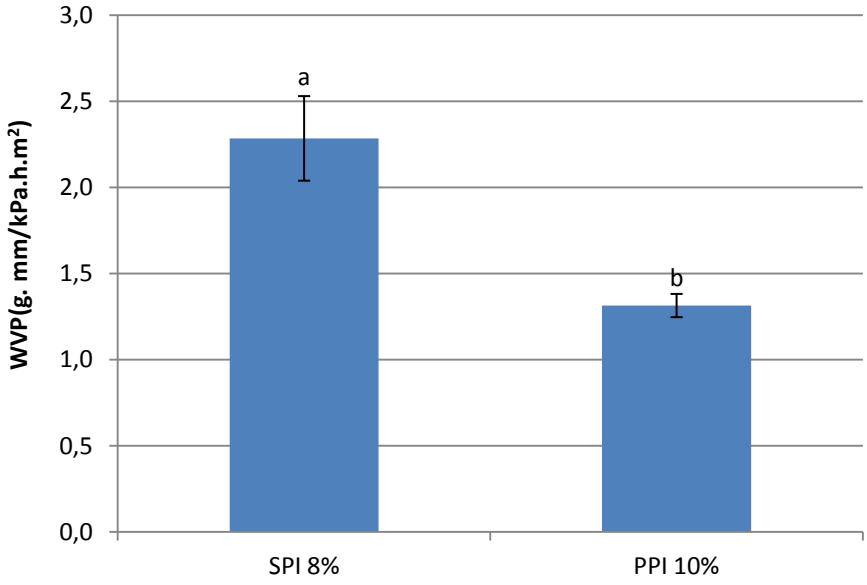


Figure 10 - Water vapor permeability for different proteins. Different letters denote for statistically significant differences (p<0.05).

Choi and Han studied the water vapor permeability for films with 10% of pea protein concentrate with a ratio of 70:30 of pea protein:glycerol, and have described WVP of 4.1 g. mm/kPa.h.m$^2$ [55].

The films in this study have lower water vapor permeability than the film mentioned in the study mentioned above.

## 4.4 Mechanical properties

For edible films and coatings, the mechanical properties are typically tested using tension assays. As mentioned before, in these tests the films are subjected to an increasing deformation, by uniaxial tension, and the parameters obtain are recorded during the test. Thus, we can obtain curves of stress *versus* strain, as far as we know well-defined

dimensions for the sample. From this curve we can determinate the tensile strength, Young's modulus and percentage elongation. Figure 11 shows examples of curves associated with PPI films prepared with different COs concentrations.

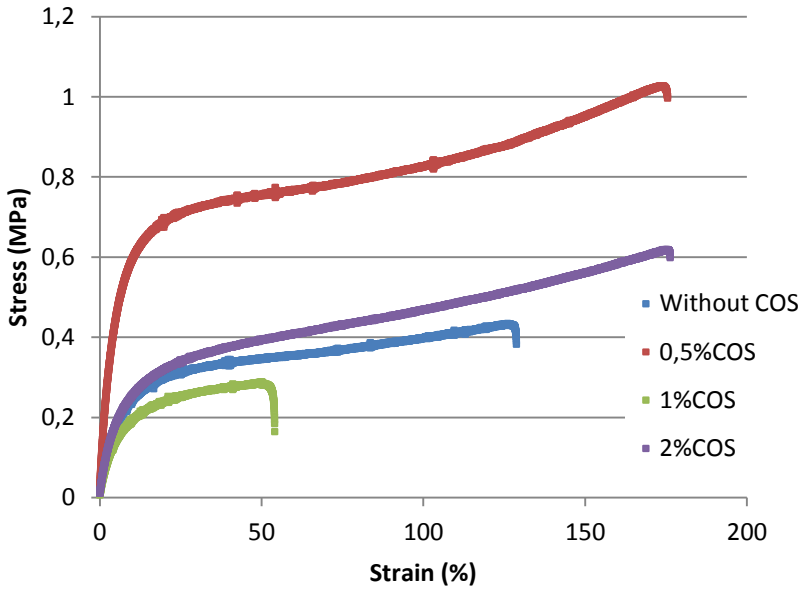


Figure 11 - Stress *vs*. Strain curve profile for PPI films with different COs concentrations. Different letters denote for statistically significant differences ($p<0.05$).

Films made with 0.5% and 2% of COs are resilient and stretchable compared to those with 1% of COs or without COs. In Figure 12 we can notice that films with 1% COs have lower elongation and the films with 0.5% and 2% have the highest and aren't significantly different (ANOVA, $p>0,05$) among them. Regarding to the tensile strength of the films, the values obtained were significantly different from each other (ANOVA, $p<0,05$). The higher tensile strength was observed for the PPI film with 0.5% of COs, and the film with 1% was the one with the lowest (Figure 13).

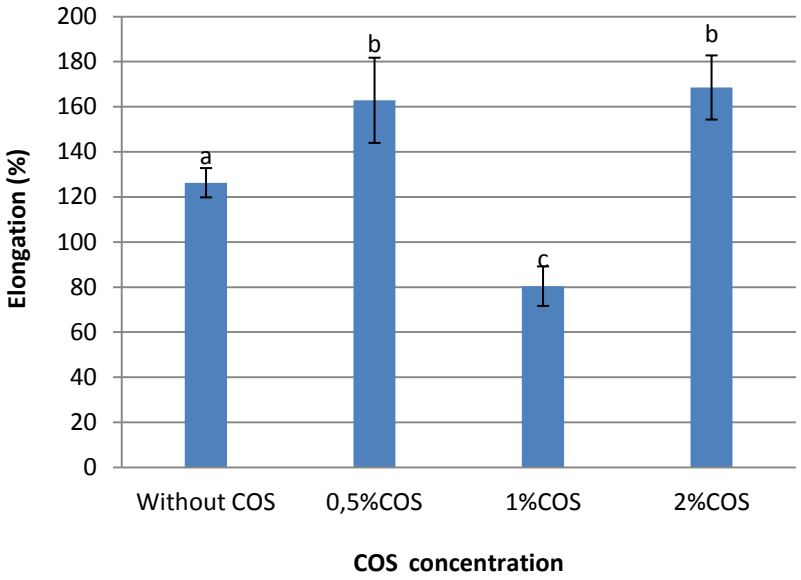


Figure 12 - Elongation for different PPI films, as a function of COs concentration. Different letters denote for statistically significant differences ($p<0.05$).

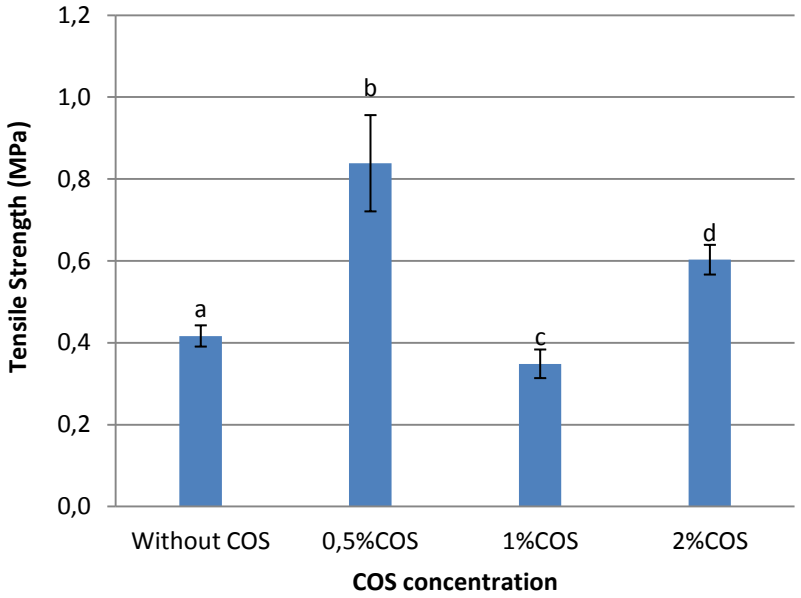


Figure 13 - Tensile strength for different PPI films, as a function of COs concentration. Different letters denote for statistically significant differences ($p<0.05$).

The film with 0.5% COs had the highest Young's modulus. The remaining films aren't significantly different from each other (ANOVA, $p>0,05$), as shown in Figure 14.

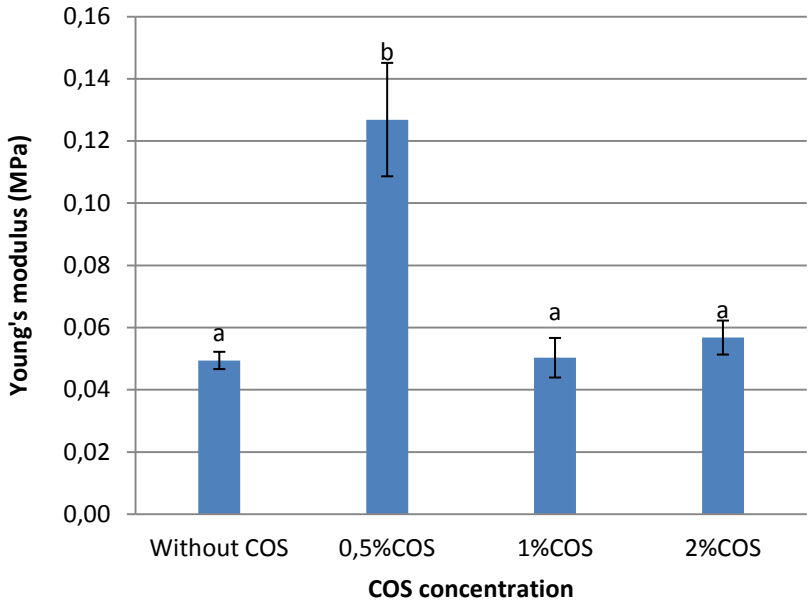


Figure 14 - Young's modulus for different PPI films, as a function of COs concentration. Different letters denote for statistically significant differences ($p<0.05$).

A film can be considerate a good package according to its mechanical properties, if they have an elongation and a tensile strength between 10-100 % and 10-100 MPa, respectively [17]. The tensile strength of the films, in general, was lower than 1 MPa. The film with 0,5% COs have an elongation of 160% witch make him potential attractive for packaging applications. This film have the highest values for the three parameters analyzed, being the film with the best mechanical properties.

Kowalczyk *et al.* studied the mechanical properties of films with 10% of PPI with a ratio of 2:1 of PPI:glycerol, and have described tensile strength values around 2 MPa and elongation around 100% [152]. Choi and Han studied PPI/glycerol films with similar composition but obtained a tensile strength of 20 MPa and elongation of 20% [56].

The films in this study have a higher elongation and a lower tensile strength comparing with the studies mentioned above. The different results are likely related to the origin and composition of the protein sample and most likely to the preparation procedures used to obtain the films.

## 4.5 Contact Angle

Figure 15 represents the results for the contact angle measured using the drop method for PPI films with different amounts of COs and essential oils, to determine their relative hydrophobicity. A correlation between the amount of COs and the hydrophobicity was observed from the results: as the amount of COs increases, the hydrophobicity also increases. The films with Bay oil showed the highest contact angle values, meaning higher hydrophobicity. For the films with no added COs, the effect of the two essential oils on the films' hydrophobicity was not significant (ANOVA, $p>0,05$). For the films with COs, the presence of the essential oils increases the film's hydrophobicity, an effect dependent on the type of added oil. The film with the highest hydrophobicity is the one with 2% of COs and Bay oil. The observed effects seem complex and they are probably dependent on the interactions among film components.

The results obtained here are in agreement with those obtained by Gueguen et al. [58] for PPI/glycerol [1:1] films, showing a contact angle around 40 º, despite the PPI concentration and plasticizer ratio being different.

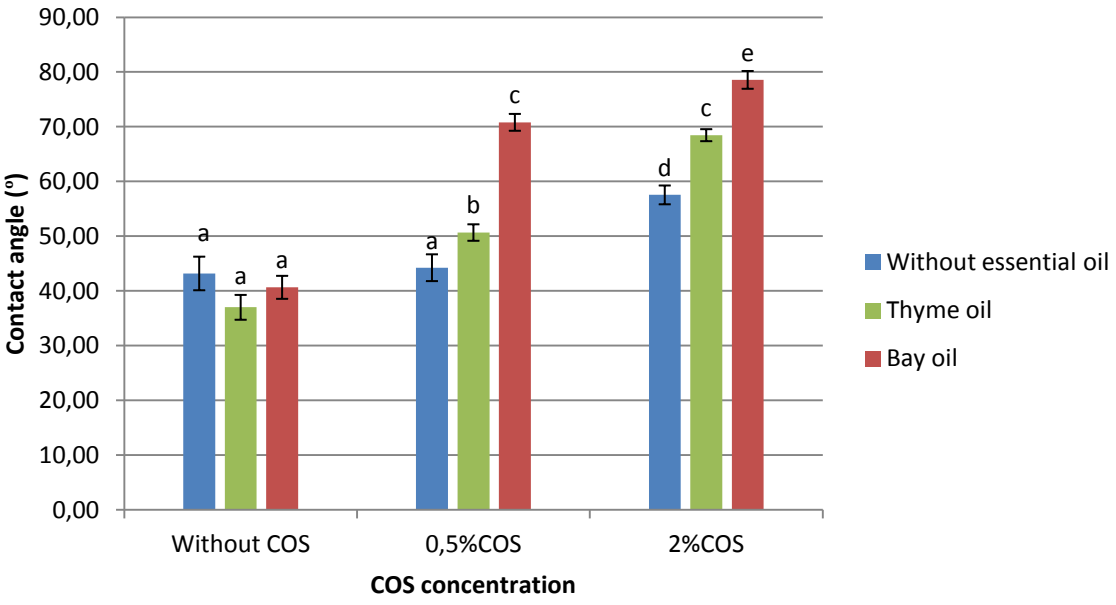


Figure 15 - Contact angle for different COs concentrations with different Bay and Thyme oil. Different letters denote for statistically significant differences ($p<0.05$).

## 4.6 Antioxidant activity

The antioxidant activity was tested for films with different concentrations (0.5%, 1% and 2%) of chitooligosaccharides during 192 hours. It was also tested for films with two different types of essential oil, Bay (B) and Thyme (T), and different COs concentration (0,5% and 2%) during 120 hours.

Figure 16 shows the % of inhibition obtained at different reaction times using the ABTS method, for PPI films with Bay and Thyme oil without COs. Films with Bay oil have reacted fully in the first test, after 0,5 hours, while the film with Thyme oil only achieve the same % of inhibition after 48 hours. This shows that Bay oil, at the tested concentration and within this particular matrix, has an higher antioxidant activity than Thyme oil.

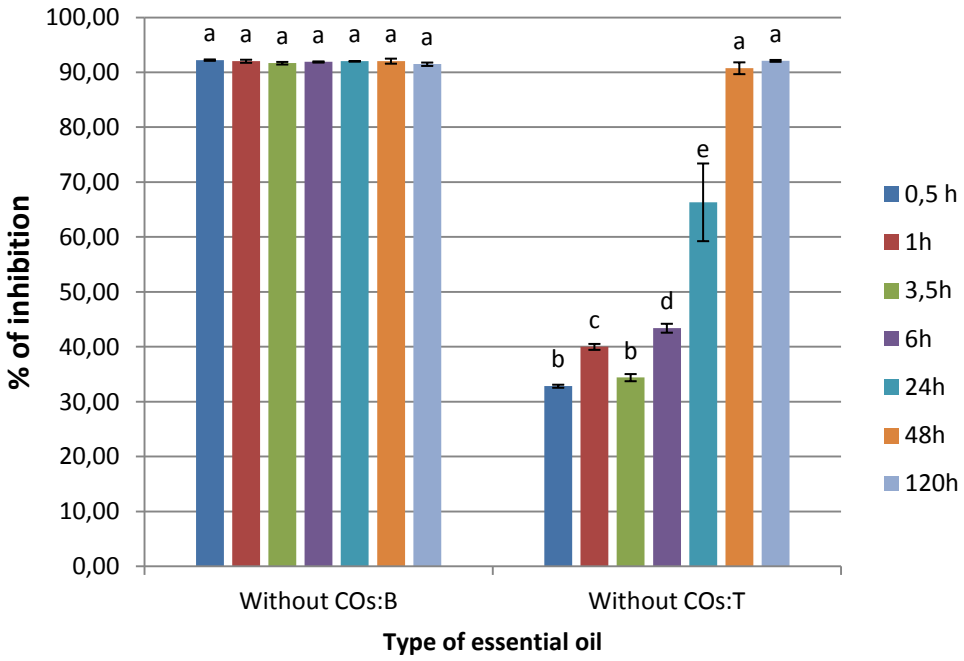


Figure 16 - % of inhibition against reaction time for films with Bay (B) and Thyme (T) oil without COs. Different letters denote for statistically significant differences ($p<0.05$).

In Figure 17 represents the % of inhibition obtained at different reaction times for PPI films with for Bay and Thyme oil with 0,5% of COs. It shows, as mentioned before, that Bay oil has higher antioxidant activity than Thyme oil. However the film with Bay oil only reacts fully after 24 hours, in contrast to the films without COs, that fully reacted after 0.5 hours. This may be due to the fact that the essential oil reacts with COs, and the

amount of essential oil left to react with the free radical is limited. For Thyme oil, comparing to the film without COs, the % of inhibition was significantly higher until 24 hours, (ANOVA, $p<0,05$).

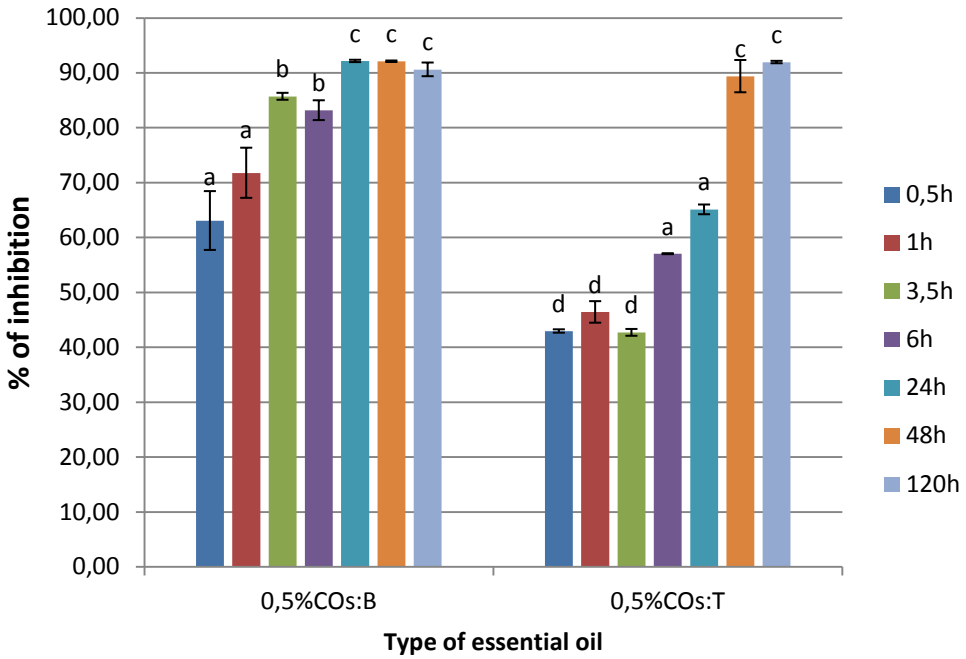


Figure 17 - % of inhibition against reaction time for films with Bay (B) and Thyme (T) oil with 0,5% of COs. Different letters denote for statistically significant differences ($p<0.05$).

In Figure 18 represents the % of inhibition for PPI films with Bay and Thyme oil and with 2% of COs. For the Bay oil, after 0.5 hours, the films reacted to its maximum capability, while the Thyme oil only achieved the same value after 48 hours. For these films (2% COs:B), the percent of inhibition is substantially equal to the films without COs: both react completely after 0.5 hours with a percent inhibition of approximately 91%. However for 0.5% COs: B, there was a decrease until 24 hours. This may have to do with the binding of essential oils with the COs within the three-dimensional matrix of the film, interfering with the ability of any of them to react with free radicals. With increasing concentration of COs, although the essential oils are linked to them, the detrimental effects are probably minimized due to the inherent antioxidant activity of COs.For Thyme oil, in the first measures, the % of inhibition was significantly higher than without COs and with 0,5%COs. This may be due to the influence of COs concentration.

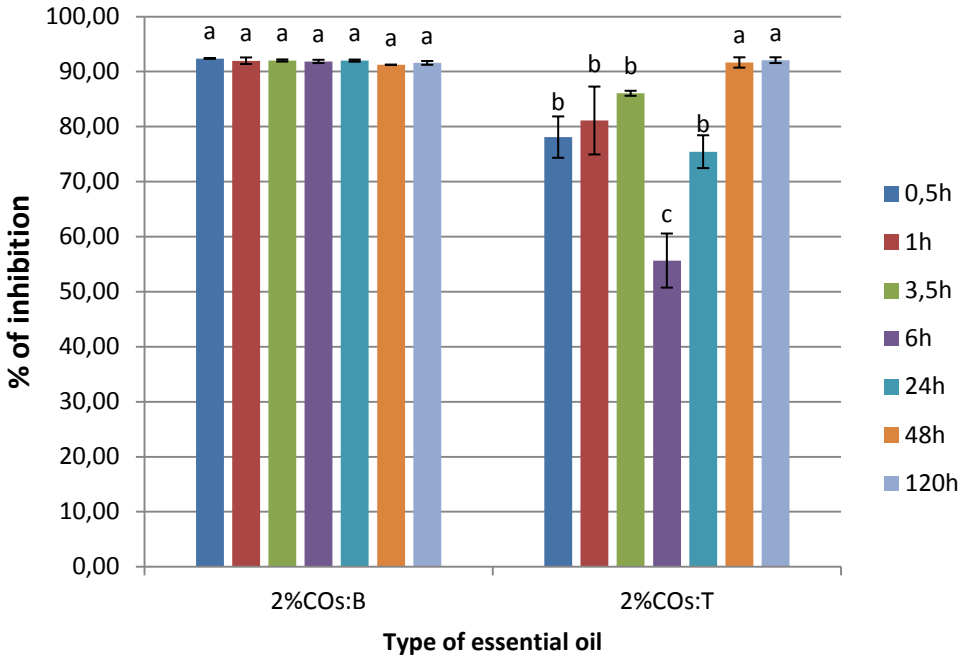


Figure 18 - % of inhibition against time for films with Bay (B) and Thyme (T) oil with 2% of COs. Different letters denote for statistically significant differences ($p<0.05$).

Figure 19 shows the percentage of inhibition for different COs concentration. In general, the presence of COs increased the films' antioxidant activity, with the PPI film with 0.5% COs showing higher antioxidant activity After 120 h the effect of the COs is only residual and PPI films with or without COs behave similarly.

These results also suggest that pea protein isolates have, by their own, some significant antioxidant activity.

Comparing to the films with essential oils, one can see an increase in the % of inhibition at each time, suggesting that the essence oils, manly Bay oil, have higher antioxidant activity than COs. At 48h, all the films with essential oil have approximately 90% of inhibition, while without essential oils, at 48h they have only 70% and lower, leading to conclude that film with Bay oil are the ones with better antioxidant activity.

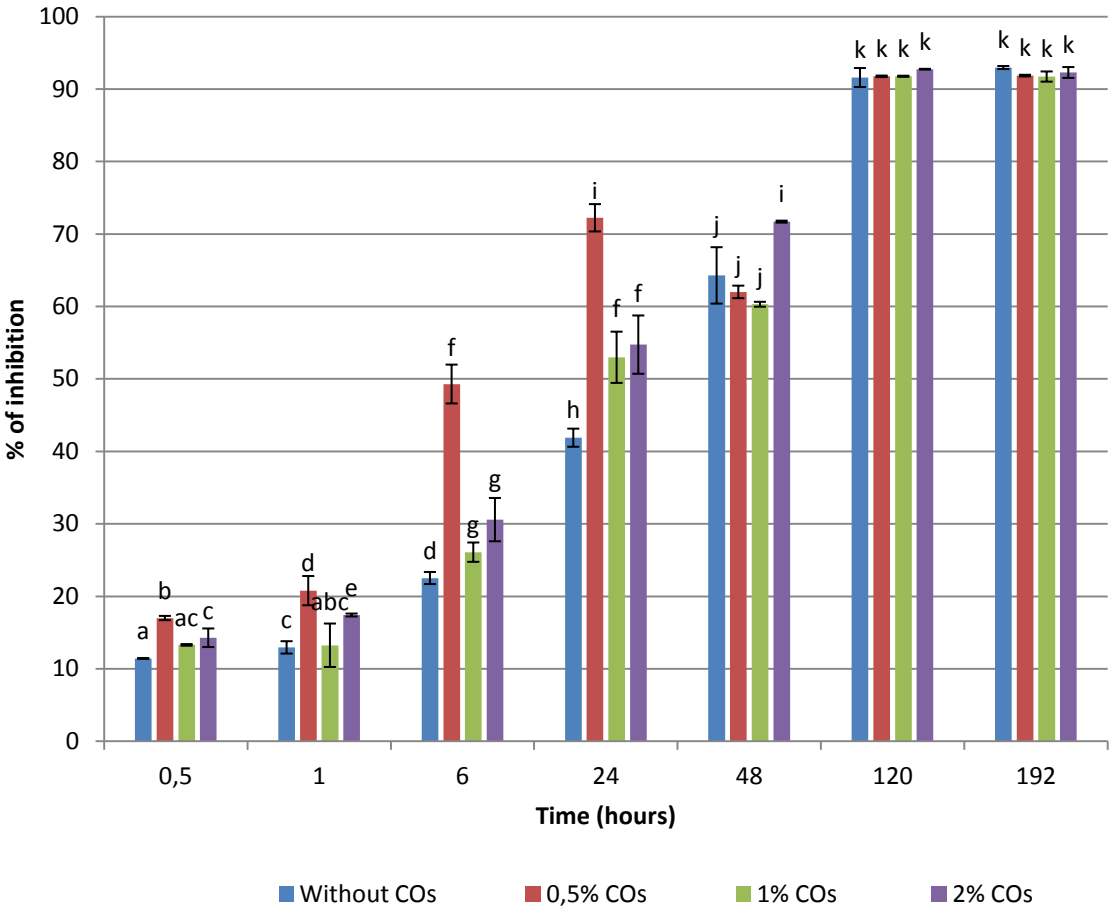


Figure 19 - % of inhibition against time for different COs concentrations. Different letters denote for statistically significant differences ($p<0.05$).

## 4.7 Cheese treatment

The coated and uncoated cheese cubes were stored in a cold room to evaluate the microbial growth over 35 days. However, after 35 days no microbial growth was observed in any of the samples, treated with the coating or uncoated. The plan was to obtain some preliminary data on cheese preservation, and the tests were performed for only one type of cheese and one lot of samples. It was observed that the cheese samples dried extensively, what could contribute to a very low water activity what, in some way, probably preclude to observe the effects of the tested coatings, namely microbial grow even for the untreated cheese samples. These observations mean also that probably the relatively high water vapor permeability of the coatings could not avoid the extensive water loss.

Other factor that could cause this is the quality of the cheese because the control cheeses (without the film) became also hard and dry and no microbial growth was observed. It is important to further study different types of cheese, to evaluate differences that could occur. If not, this film could be appropriate for hard cheeses to tentatively manipulate their organoleptic properties. Photographs of the coated and uncoated cheese over time are shown in Figures 20 to 24.

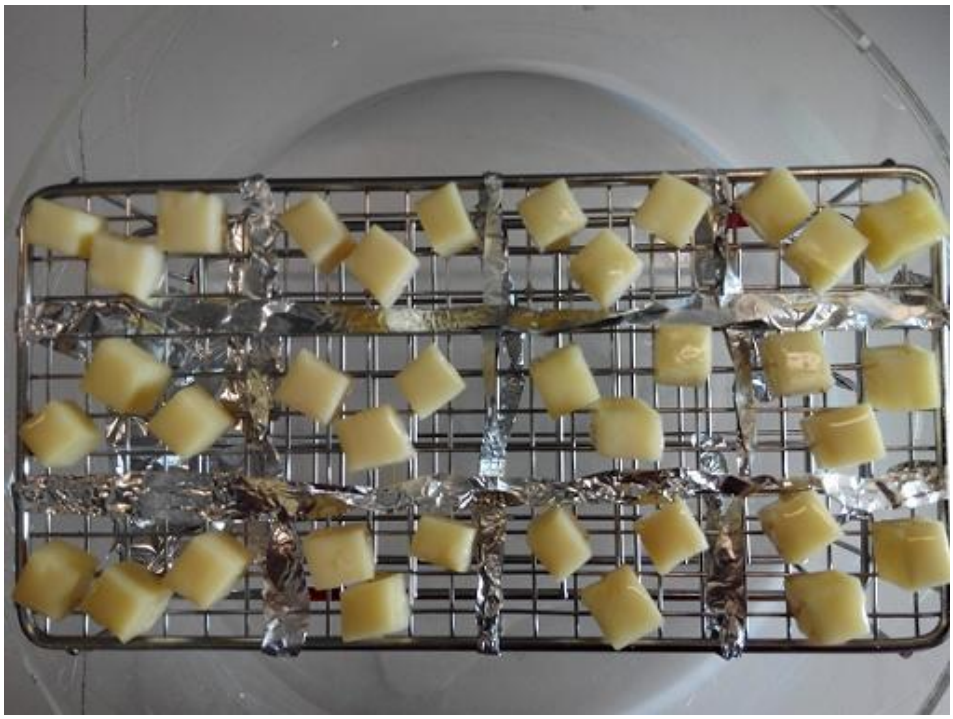

Figure 20 - Photograph of the coated cheese on day 0.

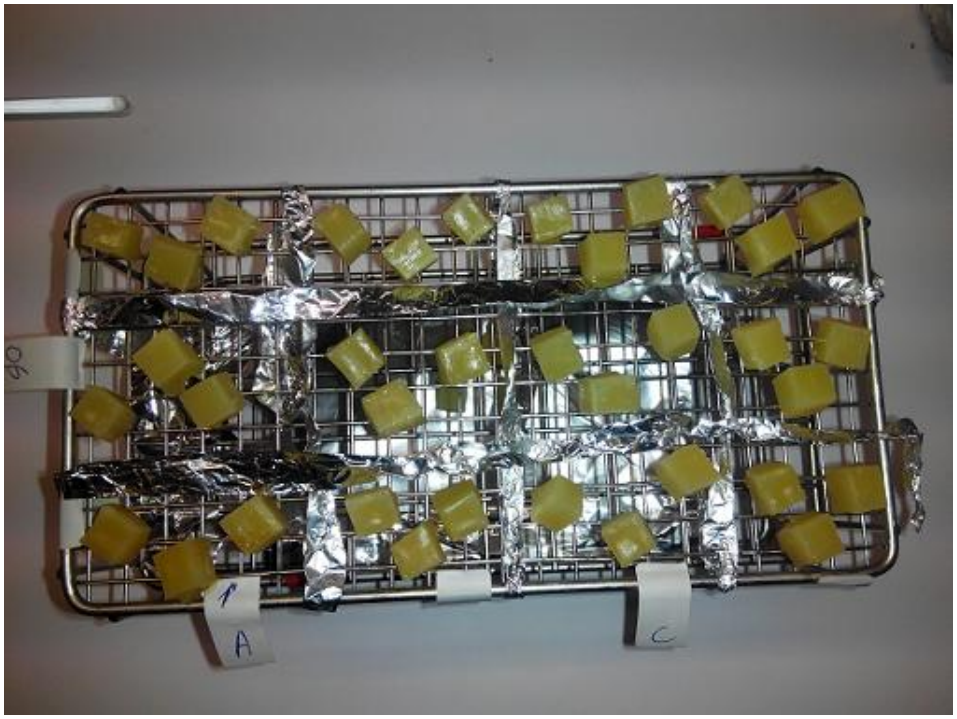


Figure 21 - Photograph of the coated cheese on day 11.


Figure 22 - Photograph of the coated cheese on day 35.

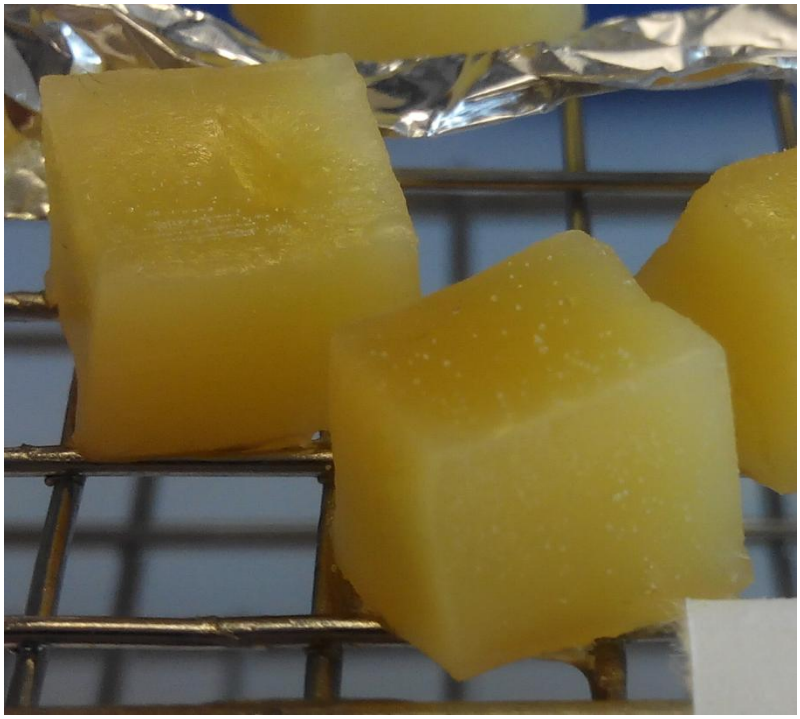

Figure 23 - Photograph of one coated cheese on day 35.

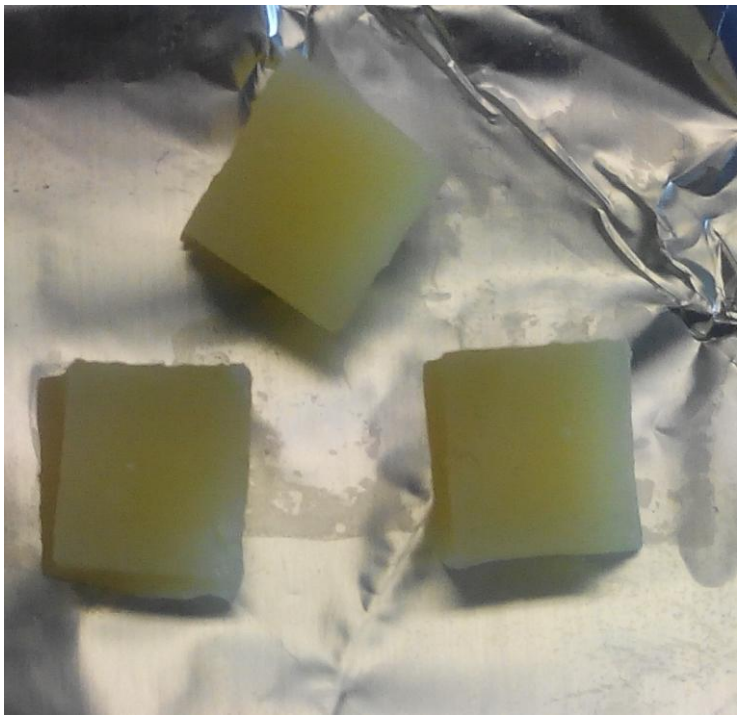

Figure 24 - Photograph of the uncoated cheese on day 35

# 5. Conclusions

It is important to replace synthetic polymers by biopolymers to contribute to environment preservation. Renewable sources of materials for packaging will create a steady and reliably supply. Edible films are "green" alternatives to traditional plastics. They can avoid migration of moisture, oxygen, or carbon dioxide, or of any other solutes, and serve as carrier of food additives such as antioxidants, antimicrobials, or specific nutrients while increasing the shelf-life of the product.

Despite pea protein isolate high protein content, relatively low price, lack of genetic alterations and availability, there are few studies about edible films with this protein. In this study, the antioxidant activity, mechanical and barrier properties of films based on this protein source, with the addition of antimicrobial and antioxidant agents were evaluated.

It was tested various concentrations of chitooligosaccharides (0,5%, 1% and 2%) and two types of essential oils, Bay and Thyme, with a concentration of 1%. For the mechanical properties the film with 0,5% of COs has the highest values for the three parameters analyzed. For the barrier properties, the film with 1% of COs has the lower value.

Addition of small amounts of COs may be advantageous to improve the mechanical properties of the PPI films, besides the expected antimicrobial effects. An intermediate COs concentration (1%) could be advantageous to reduce the water vapor permeability, but it will also result in detrimental effects on the mechanical properties.

The hydrophobicity of the film's was also dependent on the amount of added COs and essential oils. For the films with COs, the presence of the essential oils increased the film's hydrophobicity, an effect dependent on the type of added oil. The observed effects seem complex and they are probably dependent on the interactions among film components. Certainly these aspects deserve further studies in order to improve and better understand the interactions/adhesion of the coating onto the cheese surface.

The protein films by their own show already some antioxidant activity, and the addition of COs or essential oils results mainly on a higher rate of this effect (lower reaction time to observe the antioxidant effects). Even so the films prepared with the Bay oil revealed a higher antioxidant activity, which can be useful and complement the expected effects on the organoleptic properties of cheese samples treated with these films.

After film optimization, coatings based on PPI and COs and/or essential oils were made to protect cheese from microbial deterioration and to preserve or deliberately modify

the organoleptic characteristics. Though no microbial growth was observed, that doesn't mean that there are no microorganisms, due to decreased water activity. Further testes needed to be done, like antimicrobial growth of the coated cheese through time and the evaluation of the microbial activity of the films themselves.

Time limitations prevented to achieve some of the initial objectives regarding the application on the surface of sliced cheeses and the evaluation of the resulting organoleptic properties (sensory analysis) and microbial contamination (microbiologic analysis).

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