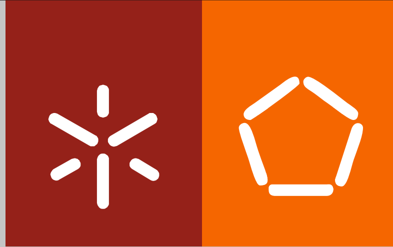

Universidade do Minho
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María Liliana Flores López

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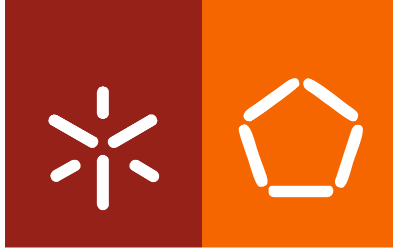
July 2016



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Doctoral Dissertation for PhD degree in Chemical and Biological Engineering

Supervisors of the thesis

Professor António Augusto Martins de Oliveira Soares Vicente

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July 2016

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Título da tese
Development and characterization of edible and biodegradable nano-laminate coatings as vehicle of incorporation of functional and antimicrobial agents from *Aloe vera*, for extending shelf life of *Lycopersicum esculentum* Mill. (tomato)

Orientadores

Professor António Augusto Martins de Oliveira Soares Vicente

Professor Diana Jasso de Rodríguez

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É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE/TRABALHO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE

Universidade do Minho, 28 de Julho de 2016

STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.

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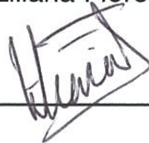

No te rindas que la vida es eso,
continuar el viaje,
perseguir tus sueños,
destrabar el tiempo,
correr los escombros y destapar el cielo.

Mario Benedetti

Con amor,

A ti Mamá, a ti Papá

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ABSTRACT

During the last decades, there has been an increasing demand for fresh fruits and vegetables forcing the food industry to develop new and better methods for maintaining food quality and extending shelf life. Great losses (from 20% to 80%) in the quality of fresh fruits have been reported; such losses occur from harvesting to final consumption. In this context, edible coatings offer a beneficial impact on the produce quality (e.g. acting as a selective gas barrier and presenting an antimicrobial effect) and also to the environment, by means of reducing food waste.

Currently, the utilization of nanotechnology represents a good tool to overcome problems regarding the application of edible coatings, such as high water vapour permeability and poor mechanical properties in comparison with synthetic materials. Some advantages when these coatings are at nanoscale are: higher stability at the substrate surface, facility of preparation and lower concentration of materials are required. Also, these systems can provide additional protection for the produce, and can incorporate antimicrobial, antifungal and antioxidant substances into their structure.

Based in these ideas, the main purpose of this thesis was the development and characterization of nano-laminate coatings using bioactive components of *Aloe vera* for extending the shelf life of tomato fruit (*Lycopersicum esculentum* Mill.). Tomato was utilized here as model, being of course possible to apply these systems to other fruits.

Aloe vera fractions (gel and liquid) evidenced both antifungal and antioxidant activities, representing a good alternative to extend the shelf life of tomato fruit. Therefore, polyelectrolyte solutions based on alginate and chitosan and their optimal concentrations were selected to build nano-laminate coatings; also, the effect of surfactant and plasticizer concentrations was determined. The best formulations were selected based on their wettability on tomato fruit surface.

On the other hand, nano-laminate coatings with carvacrol (directly applied or encapsulated into zein) also showed (*in vitro*) their potential to be a good candidate

to improve the shelf-life of foodstuffs due to the improved barrier properties and antifungal activity.

Nano-laminate films functionalized with bioactive fractions of *A. vera* (gel and liquid fraction) have shown good barrier properties and evidenced *in vitro* antifungal activity. The system based on alginate/chitosan functionalized with *A. vera* liquid fraction was selected in order to evaluate its effectiveness *in vivo*. Tomatoes coated with this system exhibited a lower weight loss and microbial contamination was delayed (mainly in terms of yeasts and molds), together with a reduction in the gas transfer rate and ethylene production during cold (11 °C and 90% relative humidity, *RH*) and room temperature (20 °C and 85% *RH*) storage of tomato fruits. It should be noted that nano-laminate coating technology is transferable to other fruit models (both climacteric and non-climacteric), being an attractive postharvest tool for extending the quality and shelf life of fruits and vegetables.

RESUMO

Durante as últimas décadas, verificou-se uma crescente procura de frutas e vegetais frescos, o que obrigou a indústria alimentar a desenvolver novos e melhores métodos para manter a qualidade dos alimentos e prolongar a sua de vida de prateleira. Elevadas perdas (de 20% a 80%) na qualidade de frutos frescos foram reportadas; tais perdas ocorrem desde a colheita até o seu consumo final. Neste contexto, os revestimentos comestíveis oferecem um impacto benéfico sobre a qualidade do produto (por exemplo, atuando como uma barreira seletiva a gases e apresentando efeito antimicrobiano) e também para o meio ambiente, através da redução do desperdício de alimentos.

Atualmente, o uso da nanotecnologia representa uma boa ferramenta para superar os problemas relacionados com a aplicação de revestimentos comestíveis, tais como a elevada permeabilidade ao vapor de água e fracas propriedades mecânicas em comparação com materiais sintéticos. Algumas vantagens quando estes revestimentos são à escala manométrica são: maior estabilidade na superfície do substrato, facilidade de preparação e menor concentração de materiais necessários. Adicionalmente, estes sistemas podem oferecer uma proteção adicional para o produto, e podem incorporar substâncias antimicrobianas, antifúngicas e antioxidantes na sua estrutura.

Com base nestas ideias, o principal propósito desta tese foi o desenvolvimento e caracterização de revestimentos nano-laminados utilizando componentes bioativos de *Aloe vera* para prolongar a vida de prateleira do fruto de tomate (*Lycopersicum esculentum* Mill.). O tomate foi utilizado como modelo, sendo, naturalmente, possível aplicar estes sistemas a outros frutos.

Ambas as frações de *Aloe vera* (gel e líquido) evidenciaram atividades antifúngicas e antioxidantes, o que representa uma boa alternativa para prolongar a vida de prateleira do fruto de tomate. Deste modo, soluções de polieletrólitos baseadas em alginato e quitosano e as suas concentrações ótimas foram selecionadas para construir revestimentos nano-laminados; além disso, determinou-se o efeito das concentrações de surfactante e plasticizante. As

melhores formulações foram selecionadas com base na sua capacidade molhante da superfície do fruto de tomate.

Por outro lado, os revestimentos nano-laminados com carvacrol (aplicado diretamente ou encapsulado em zeína) também demonstraram (*in vitro*) o seu potencial para serem bons candidatos para melhorar a vida de prateleira dos produtos alimentares devido às propriedades de barreira melhoradas e à atividade antifúngica.

Os filmes nano-laminados funcionalizados com frações bioativas de *A. vera* (frações gel e líquida) demonstraram boas propriedades de barreira e evidenciaram atividade antifúngica *in vitro*. Selecionou-se o sistema baseado em alginato/quitosano funcionalizado com fração líquida de *A. vera* com a finalidade de avaliar a sua eficácia *in vivo*. Tomates revestidos com este sistema registaram a menor perda de peso e a contaminação microbiana foi retardada (principalmente em termos de leveduras e bolores), juntamente com uma redução da taxa de transferência de gás e produção de etileno durante armazenamento sob refrigeração (11 °C e 90% de humidade relativa, *HR*) e a temperatura ambiente (20 °C e 85% *HR*) de frutos de tomate. Finalmente, é importante referir que a tecnologia de revestimentos nano-laminados é transferível para outros frutos (climatéricos e não climatéricos), sendo uma ferramenta de pós-colheira atrativa para prolongar a qualidade e vida de prateleira de frutos e vegetais.

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LIST OF GENERAL NOMENCLATURE

Symbol

WVP – water vapour permeability

TS – tensile strength

O_2P – oxygen permeability

TPC – total phenolic content

RSA – radical scavenging activity

R^2 – coefficient of determination

IC_{50} – concentration of the compounds that caused a 50% inhibition of the radical scavenging activity

W_s – spreading coefficient

W_a – work of adhesion

W_c – work of cohesion

WVTR – water vapour transmission rate

EE – encapsulation efficiency

O_2TR – oxygen transmission rate

W_i – initial sample weight

W_f – final sample weight

a/b** – degree of redness

R_{O_2} – O_2 consumption rate

R_{CO_2} – CO_2 production rate

$R_{C_2H_4}$ – C_2H_4 production rate

V_f – free volume of the container

V_ρ – total volume of the container

Greek symbol

ζ-potential – zeta potential

γ_L – surface tension

θ – contact angle

ρ – density

Abbreviations

PHSL – postharvest handling and storage losses

RH – relative humidity

EPA – Environmental Protection Agency

EOs – essential oils

GRAS – generally recognized as safe

EU – European Union

LPOS – lactoperoxidase system

BO – bergamot oil

LO – lemongrass oil

DHP – dynamic high pressure

O_2 – oxygen

CO_2 – carbon dioxide

LbL – Layer-by-Layer

SEM – Scanning Electron Microscopy

CP – cut pears

WP – whole pears

DPPH – 2,2-Diphenyl-1-picrylhydrazyl

BHA – buthylatedhydroxyanisole

Na_2CO_3 – sodium carbonate

FC – Folin-Ciocalteau

PDA – potato dextrose agar

PDB – potato dextrose broth

AE-B – aqueous extract from bagasse

EE-B – ethanolic extract from bagasse

AOAC – Official Method of Analysis

HLPC – High-Performance Liquid Chromatography

H_2SO_4 – sulfuric acid

MUM – Micoteca da Universidade do Minho

OD – optical density

ANOVA – Analysis of variance

LSD – Least Significance Difference

CAM – crassulacean acid metabolism

n.d. – not detected

M – mannuronic acid

G – α-L-guluronic acid

Gly – glycerol

Alg – alginate

CH – chitosan

$–NH^{3+}$ – amine groups

$-COO^-$ – carboxylic group

FDA – United States Food and Drugs Administration

PET – polyethylene terephthalate

HCl – Hydrochloric acid

A/C PET – aminolyzed/charged polyethylene terephthalate

C – carvacrol oil

Z/C – nanocapsules zein-carvacrol

CH-C – chitosan-carvacrol

N_2 – nitrogen

log CFU cm^{-2} – log colony forming unit per cm^2

TEM – Transmission Electronic Microscopy

AL – *Aloe vera* liquid fraction

AG – *Aloe vera* gel

NL-Av – chitosan/alginate nano-laminate coating with *A. vera* liquid fraction

NL – chitosan/alginate nano-laminate coating

DRBC – dichloran-rose Bengal-chloramphenicol

DCPIP – 2,6-dichlorophenol-indolphenol

PCA – Plate count agar

CH-Av – chitosan with *Aloe vera* liquid fraction

Alg-Av – sodium alginate with *Aloe vera* liquid fraction

TA – titratable acidity

SSC – soluble solid content

AA – ascorbic acid

log CFU g^{-1} – log colony forming units per gram

TCD – thermal conductivity detector

FID – flame ionization detector

C_2H_4 – ethylene

Chapter 1

MOTIVATION AND OUTLINE

The motivation, outline and research aims of this work are approached in this chapter, where a general overview of the thesis is provided.

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1.1 THESIS MOTIVATION

During the last decades, there has been an increasing demand for fresh fruits and vegetables forcing the food industry to develop new and better methods for maintaining food quality and extending shelf life. Great losses (from 20% to 80%) were reported in the quality of fresh fruits, occurring from harvesting to final consumption. The short shelf life of fruits is thus a major drawback in the distribution chains (Kitinoja, 2010).

The increasing consumers' demand for fresh fruits and vegetables of higher quality and more nutritious has encouraged the food industry to develop new and better methods for maintaining quality and extending shelf life (Barry-Ryan et al., 2007). In this context, the development of edible coatings from biodegradable materials is receiving a growing interest from the food industry. Edible coatings offer beneficial impacts on produce quality and environment, since they provide a gas barrier, reduce the metabolic rate and diminish the use of disposable and non-degradable packaging materials, among others (Falguera et al., 2011; Moreira et al., 2011; Tapia et al., 2008). Recent works have shown that nano-laminate coatings improve transport properties, being suggested that these materials can present improved functionality when used at the nanoscale (Jang et al., 2008). In addition, they have the ability to incorporate functional compounds (e.g. in nanoparticles) into their structure, which presumably have greater chemical reactivity and can be more bioactive than larger particles as their size allows a better access to any structure (Lugo-Medina et al., 2010).

The application of nano-laminate coatings has been scarcely explored for fruits and vegetables. Many works have focused on the use of edible coatings at micro-scale dimension (conventional edible coatings) (Brasil et al. 2012; Fajardo et al. 2010; Hambleton et al. 2008; Garcia et al. 2006). However, few works have investigated the use of nano-laminate coatings to extend the shelf life of fruits and vegetables (Souza et al., 2015; Medeiros et al., 2012a, 2012b).

Tomato (*Lycopersicum esculentum* Mill.) is a climacteric fruit and continues to ripen after harvest (Athmaselvi et al., 2013). It has been reported that over 30% of tomato harvested is lost due to postharvest diseases before it reaches the consumer (Boyette et al., 1994). The major problem is the inefficient postharvest processing and preservation techniques causing fruit spoilage at a very early stage. Fungicides are the primary means of controlling postharvest fungal diseases on fruits and vegetables; however, many fungicides are not used in postharvest treatment or have been removed from the market because of potential toxic hazards (Eshel et al., 2009; Calvo et al., 2007). The application of nano-laminate coatings can provide an additional protective coating for fresh tomato fruit, extending its shelf life.

1.2 RESEARCH AIMS

The main objective of this thesis was the development and characterization of nano-laminate coatings using bioactive components of *Aloe vera* for extending the shelf life of tomato fruits (*Lycopersicum esculentum* Mill.). The main focus areas were:

- Chemical characterization of *Aloe vera* fractions and evaluation of their antifungal activity against postharvest occurring fungi;
- Selection of the best nano-laminate coatings and optimal application conditions in tomato fruits;
- Characterization of carvacrol-based nano-laminate coatings in terms of barrier properties and antifungal activity;
- Characterization of *Aloe vera*-based nano-laminate coatings in terms of barrier properties and antifungal activity;
- Study of the effectiveness of functionalized nano-laminate coatings with *Aloe vera* during cold and room temperature storage of tomato fruits.

1.3 THESIS OUTLINE

The thesis is organized in eight chapters. This first chapter describes the motivation, research aims and thesis outline. Chapter 2 presents an overview on the utilization of conventional edible coatings and the introduction of nano-laminate coatings for extending the shelf life of fruits and vegetables. Chapters 3-7 present the main experimental results, distributed as follows:

Chapter 3 contains the characterization of *Aloe vera* fraccions (gel, liquid fraction and bagasse extracts). The antifungal and antioxidant activities of each fraction were discussed and compared.

Chapter 4 is dedicated to the selection of the formulations for the construction of nano-laminate coatings. This selection was based on their affinity to the tomato surface. Polyelectrolyte solutions of sodium alginate and chitosan evidenced their ability to bind to each other by electrostatic interactions, being able to form multilayer systems.

Chapter 5 presents the characterization of nano-laminate films with carvacrol, incorporated either as an emulsion or encapsulated into zein. The gas barrier properties and *in vitro* antifungal activity were evaluated.

Chapter 6 shows the characterization of nano-laminate films with *A. vera* fractions (gel and liquid fraction), and their gas barrier properties and *in vitro* antifungal activity were evaluated.

In Chapter 7 the effectiveness of a nano-laminate coating with *A. vera* liquid fraction was evaluated in terms of physicochemical parameters, microbiological analysis, respiration rate and ethylene production under cold and room temperature storage of tomato fruits.

Finally, Chapter 8 exhibits the overall conclusions, recommendations and suggestions for future work.

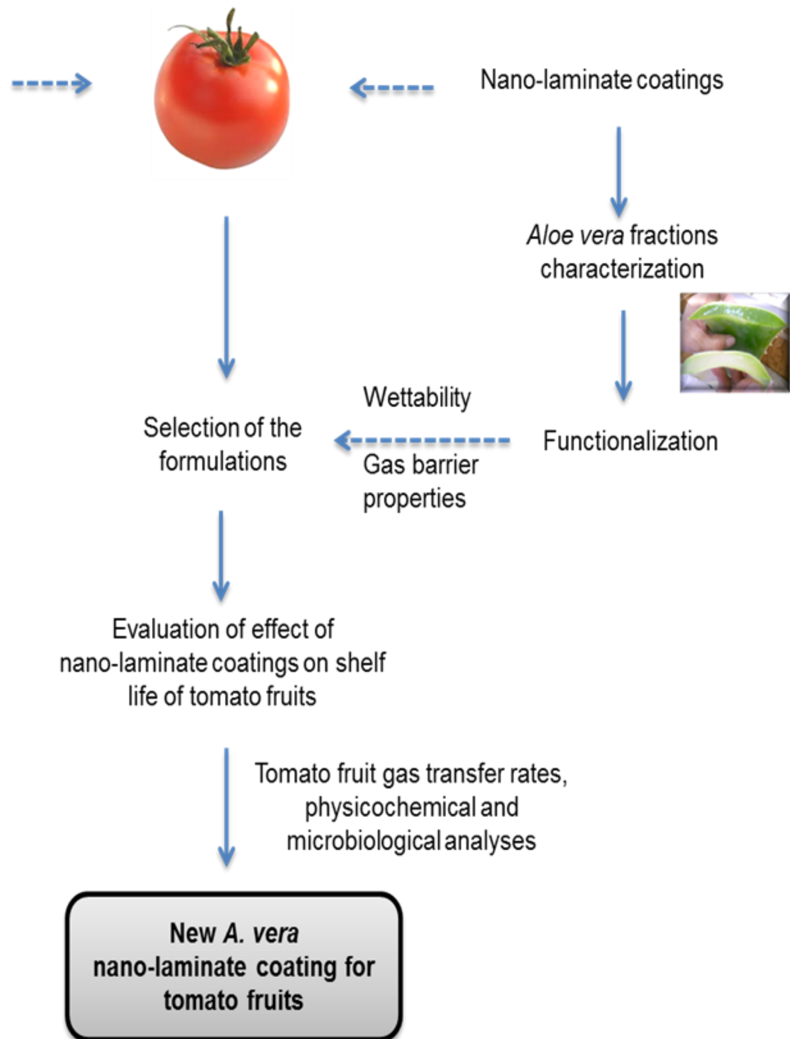

Figure 1-1. Flow chart of the outline and the main ideas of this thesis.

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Chapter 2

EDIBLE COATINGS AND NANO-LAMINATE COATINGS FOR EXTENSION OF POSTHARVEST STORAGE OF FRUITS AND VEGETABLES

This chapter presents a general overview on the utilization of conventional edible coatings and the introduction of nano-laminate coatings for extending the shelf life of fruits and vegetables.

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2.1 INTRODUCTION

Around the world, agriculture and food industry suffer significant product losses from harvest to consumer, due to different factors involved. Such losses depend firstly on the management conditions existing in each region as well as on its economic resources. Thus in industrialized countries more than 40% of the food losses (including cereals, roots and tubers, oilcrops and pulses, fruits and vegetables, meat, fish and dairy) occur at retail and consumer levels; while in developing countries, more than 40% of the food losses occur at postharvest and processing levels (Gustavsson et al., 2011). In the year 2011, Latin America presented the highest percentages of postharvest handling and storage losses (PHSL) in crops (28%); while in industrialized countries (Europe and North America and Oceania) the percentages of PHSL were considerably lower (18% and 16%, respectively) (Table 2-1).

Moreover, the largest postharvest losses in fruit and vegetable crops are due to deterioration caused by microorganisms after harvest and during cold storage. Fruits, due to their low pH, higher moisture content and nutrient composition, are very susceptible to the attack of fungi, while vegetables are generally less acidic, and their spoilage is usually by bacteria (Tripati and Dubey, 2004; James and Kuipers, 2003). Although it is very difficult to determine the full extent of postharvest losses due to decay (i.e. attack by microorganisms and physical damages), it is well known that these losses are significant (McCollum, 2002).

The use of simple postharvest practices (e.g. selection of suitable harvest timing by maturity indices, cleaning of the product, sorting, packaging, quick cooling and good refrigerated storage and appropriate transportation and distribution) have been successful for small farmers when they are correctly applied. However, these practices do not always guarantee produce integrity forcing producers to apply several treatments during postharvest preservation of food crops (Yahia et al., 2004). Chemical treatment is one of the postharvest techniques that are normally used before and after harvest to prolong shelf life and reduce food spoilage (Eshel et al., 2009). However, the lack of regulation in less developed countries has

generated the indiscriminate use of pesticides in fruits and vegetables allowing the improvement of resistance of plagues to the most acceptable pesticides, while also affecting human health. Many of those pesticides have thus been removed from the market (Calvo et al., 2010), consequently reducing the options for convenient and safe treatment of crops. Physical and quality losses are also due to deficient storage conditions, use of poor quality packages, rough handling, and a lack of suitable tools for postharvest management. These are the main reasons for losses of crops' market value and food safety, thus leading to low incomes for producers (Kitinoja et al., 2011).

Table 2-1. Estimated/assumed postharvest handling and storage losses by region. Adapted from Gustavsson et al. (2011)

Crop group*	Postharvest handling and storage losses by region (%)		
	Europe (incl. Russia)	North America and Oceania	Latin America
Cereals	4	2	4
Fruits and vegetables	5	4	10
Roots and tubers	9	10	14

*Cereals (excluding beer): wheat, rice (milled), barley, maize, rye, oats, millet, sorghum, other cereals.

Fruits and vegetables: oranges and mandarins, lemons and limes, grapefruit, other citrus, bananas, plantains, apples, pineapples, dates, grapes, other fruit, tomatoes, onions, other vegetables.

Roots and tubers: potatoes, sweet potatoes, cassava, yams, other roots.

The increasing consumers' demand for fresh fruits and vegetables of higher quality and more nutritious has encouraged the food industry to develop new and better methods for maintaining food quality and extending shelf life (Barry-Ryan et al., 2007). Recent studies of postharvest treatments, particularly the use of edible coatings and nano-laminate coatings, are receiving a growing interest from food industry. It is known that producers in developing countries are largely small farmers, rarely associated into formal organizations; therefore, the access to technical training, and in general, new postharvest technologies, is limited; also the scarcity of information about costs and financial benefits of using these new

technologies is a problem. The implementation of a technology from the laboratory to the field represents an area of opportunity (Kitinoja et al., 2011; Kitinoja, 2010).

This literature review presents a new insight on the application of edible coatings and nano-laminate coatings as potential postharvest technologies for fruits and vegetables storage.

2.2 PARAMETERS INVOLVED IN FRUITS AND VEGETABLES POSTHARVEST LOSSES

During harvesting, fruits and vegetables continue living despite being separated from their natural source of organic and mineral nutrients and water. The energy used to conduct these activities results from the respiration process; it involves the breakdown of carbohydrates to produce carbon dioxide, water and heat (Fig. 2-1). Also, the transpiration process takes place moving water vapour from the plant organs' surface to the surrounding air (Fig. 2-1) (Van Hung et al., 2011; Datta, 2003). These processes do not continue indefinitely, causing initial shrinkage and subsequent weight loss of the tissues (about 5-10% of their weight) (FAO, 1989). Both respiration and transpiration processes are considered as the major causes of postharvest losses and poor quality in produce, and their control is important in order to extend produce shelf life (Ben-Yehoshua and Rodov, 2003).

The control of relative humidity (*RH*) and temperature of storage is also important, since these are factors that play an important role in maintaining produce quality due to the direct influence they have on transpiration and respiration processes, as well as on the vapour gradient between the produce and the storage atmosphere.

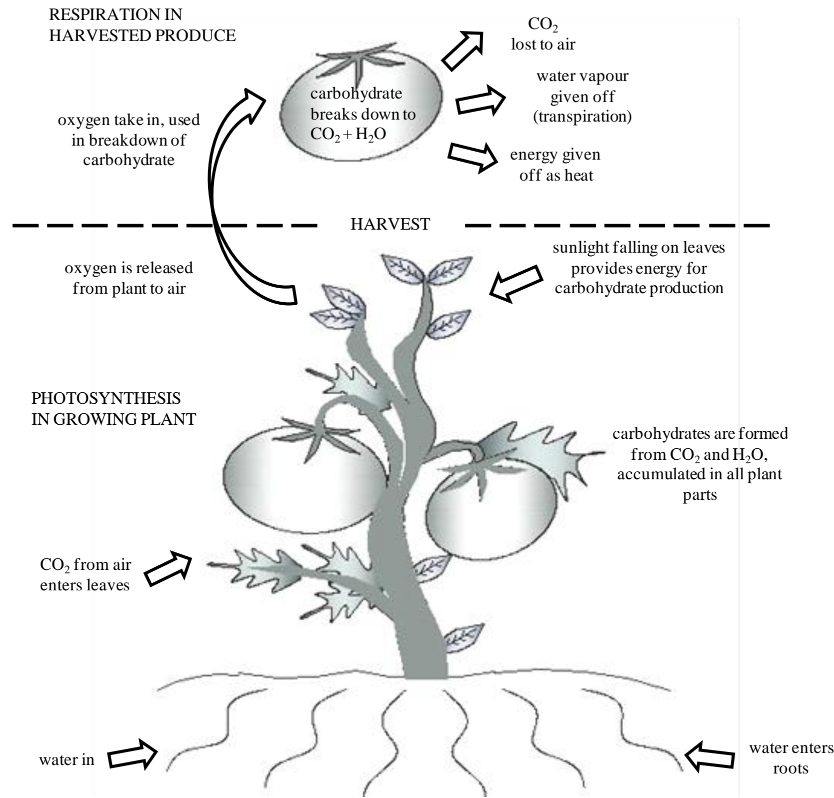

Figure 2-1. Processes involved in the respiration of harvested produce.

Then, when the produce is kept at a temperature similar to that of the storage environment, transpiration rate is highly correlated with the *RH* during storage (Van Hung et al., 2011). It has been shown that high *RH* values during storage can reduce moisture losses and subsequently maintain fruit firmness by decreasing the transpiration rate of fruits or sub-cuticle evaporation, mainly under reduced air velocities and low temperature (Henriod, 2006). In addition, low temperature (4-8 °C) can reduce respiration rate, increase tissue resistance to ethylene action, delay compositional breakdown of macromolecules, retard senescence, and control the development of rot microorganisms (Yahia et al., 2004). However, at such temperatures some tropical native fruits and vegetables can present chilling injuries. Due to the influence of these factors (i.e. *RH*, temperature), each produce has its own ideal set of conditions that allows a successful storing for the maximum length of time, although *RH* levels around of 85-95% are commonly recommended for the storage of fresh fruits and vegetables (Van Hung et al., 2011).

On the other hand, decreases in yield and quality of fruits and vegetables caused by pest damages (especially by fungi) during storage can be even higher than losses occurring in the field, and these are favoured when the produce is not rapidly cooled or is not transported and stored in appropriate conditions (Batta, 2007). Commonly, chemical treatment is a postharvest technique used before and after harvest to prolong shelf life and reduce food spoilage (Eshel et al., 2009).

The use of biopesticides has emerged as one alternative substitute for chemical pesticides. Biopesticides are certain types of pesticides manufactured from living microorganisms (e.g. bacteria, fungi or viruses) or plant extracts (including secondary metabolites and essential oils) and other biochemicals (e.g. insect sex pheromones) (Chandler et al., 2011; Bailey et al., 2010). The increasing use of this kind of biocontrol was demonstrated with the recent approval of more than 430 biopesticides active ingredients and 1320 active products on the list of EPA (Environmental Protection Agency, 2014).

Within microbial-based biopesticides, the use of antagonistic microorganisms, mostly bacteria and fungi, has shown its efficiency to control different postharvest rot pathogens of sweet cherries and table grapes (Schena et al., 2003), banana (De Costa and Erabadupitiya 2005), citrus (Cañamás et al., 2008), pineapple (Wijesinghe et al., 2008), apple (Mari et al., 2012; Spadaro et al., 2002), peach (Qin et al., 2012), potato (Recep et al., 2009), tomato (Li et al., 2012), and mandarin (Hong et al., 2014), among others. However, this type of biopesticides presents some disadvantages according to tests conducted under commercial or semi-commercial conditions. More in detail, the use of formulated biopesticide preparations lead to inconsistency and variability in disease control level, being this one the most significant barriers preventing widespread implementation of biocontrol technology (Droby et al., 2009; Eshel et al., 2009). Indeed, simple application of antagonistic microorganisms does not provide comparable control results to chemical pesticides (Droby et al., 2003); although it has been proven that the combination of antagonistic agents with innocuous exogenous substances, such as chitosan, aminoacids, antibiotics, calcium or bicarbonate salts has

increased the level of protection against *Penicillium digitatum* and *P. italicum* on oranges (Montesinos et al., 2006).

Another promising alternative to chemical pesticides is the use of biopesticides based on plant extracts and essential oils (EOs) of aromatic plants (Kotan et al., 2014). These are denominated green pesticides since they are obtained manly employing organic solvents (e.g. water, ethanol, methanol and hexane). It is known that the antimicrobial activity depends on plant species as well as on the nature of the solvent extract used; in this order, several works have been focused in obtaining new plant extracts and essential oils with acceptable antimicrobial activity (Castillo et al., 2011). Recently, methanolic extracts from nine wild edible herbaceous species showed the highest efficacy (*in vitro* and *in vivo*) against some important postharvest pathogens, i.e. *Botrytis cinerea*, *Monilinia laxa*, *P. digitatum*, *P*. *expansum*, *P*. *italicum*, *Aspergillus carbonarius*, and *A*. *niger*; the inhibition efficacy of these extracts was associated to the presence of some caffeic acid derivatives and/or flavonoids (Gatto et al., 2011). Moreover, Jasso de Rodríguez et al. (2011) reported effective antifungal activity (*in vitro*) of hexanic and ethanolic extracts of Mexican semi-desert plants against *Rhizopus stolonifer*, *Colletotricum gloesporoides* and *P. digitatum*; the authors reported that the effectiveness depends on the nature of extracting solvent used.

Biopesticides are accepted worldwide, however their utilization still faces some important challenges such as: 1) poor stability - this is the major drawback of these products, which need improvement of their formulations for a better market acceptance; 2) packaging - it should be designed in such a way that the stability of the packaged products can be maintained during storage (e.g. no container swelling due to the growth of spoilage microorganisms); 3) shelf-life - biopesticides shelf life is often low; 4) highly specific activity - causing that biopesticides will be niche products (thus with significantly lower sales) when compared to chemical products, with a broad spectrum of activity; and 5) distribution - being the major obstacle due to higher cost, leading to lower margins and limited training for sellers, distributors and farmers (Villaverde et al., 2014).

An attractive alternative to overcome these disadvantages and generate new postharvest technologies can be the incorporation of active agents used in formulation of biopesticides into edible coatings. The use of edible coatings can increase biopesticides' stability and shelf-life and at the same time add new functionalities to the final product (e.g. decrease packaging gas transfer rates).

At this moment there are only a few biopesticide-based products applicable at the postharvest stage, since most of them are aimed at controlling preharvest pests. Also, it is known that most of the biopesticide producing companies are medium and small enterprises, therefore having limited resources for R&D, product registration and promotion (Chandler et al., 2011); this highlights the importance of developing new and inexpensive technologies such as coating-based technologies.

2.3 EDIBLE COATINGS TO INCREASE QUALITY AND SHELF LIFE OF FRUITS AND VEGETABLES

Currently, edible coatings have been successfully introduced in food processing due to the beneficial impact on the produce quality and environment, since they preserve the organoleptic properties of foods, retard moisture loss, create a barrier for gas exchange between the fresh fruit and the surrounding atmosphere, and reduce the use of disposable and non-degradable packaging materials, maintaining their organoleptic properties (Souza et al., 2010; Valverde et al., 2005). The major advantage of edible coatings is that can they be consumed with the packaged products (Bourtoom, 2008); therefore all components used in their formulation should be classified and recognized GRAS (generally recognized as safe) and should have been approved to be consumed with the food products. Most edible coatings are based on polysaccharides, proteins and lipids, being used alone or in blends (Lin and Zhao, 2007); their mechanical and barrier properties depend strongly of the physical and chemical characteristics of their constituents (Pavlath and Orts, 2009).

2.3.1 Lipids

Lipid-based coatings are commonly made from waxes (e.g. carnauba wax, beeswax and paraffin wax), oils (e.g. mineral and vegetable oil) and resins (e.g. shellac wood resin, coumarone-indene resin) (Lin and Zhao, 2007). These coatings have low polarity and because of that are effective for reducing water transmission (Trezza and Krochta, 2000). Moreover, they provide protection on chilling injury and improve the appearance of the produce (Dou, 2004). These coatings have been extensively used on whole fruits and vegetables; however, they show some disadvantages such as formation of cracks, lack of homogeneity, sensorial alterations, poor adhesion to the produce, and in some cases, the high gas barrier they establish leads to anaerobic conditions (Debeaufort and Voilley, 2009; Baldwin, 1995). Their combination with polysaccharides or proteins may interact favourably, resulting in edible coatings with a good mechanical strength and controlled barrier characteristics (Chiumarelli and Hubinger, 2014; Valenzuela et al., 2013).

2.3.2 Proteins

A variety of proteins from natural sources have been used for edible coatings production, some examples are: casein, whey protein, collagen, gelatin, keratin, wheat gluten, soy protein, peanut protein, corn-zein and cotton seed protein (Dhall, 2013). These coatings usually exhibit good mechanical properties since they are structured by 20 different monomers (amino-acids), allowing high potential for forming numerous linkages via disulfide (S-S) covalent bonding, electrostatic forces, hydrogen bonding and hydrophobic interactions. Protein-based coatings also present good oxygen barrier properties at low *RH*; however most of them are poor barriers against water vapour due to their hydrophilic nature (Bourtoom, 2009). Several procedures, including chemical and enzymatic modification of protein properties, combination with hydrophobic materials, and physical methods, have been performed in order to improve their barrier and mechanical properties (Bourtoom, 2009).

2.3.3 Polysaccharides

Polysaccharide-based coatings have been the most commonly used to coat fruits and vegetables due to their appropriate adhesion and flexibility properties on the produce surface (Ramos-García et al., 2010). There is a great variety of polysaccharides from diverse sources used for elaboration of edible coatings; among the most common are: chitosan (Martins et al., 2012), galactomannans (Cerqueira et al., 2014), pectin (Zimet and Livney, 2009), alginate (Galus and Lenart, 2013), carrageenan (Hamzah et al., 2013), and starch (Sánchez-González et al., 2010). Depending on their chemical composition they are able to: 1) regulate mass transfer processes involving oxygen (Ayrancy and Tunc, 2003; Miller and Krochata, 1997), carbon dioxide (Galietta et al., 1998b), water vapour (Avena-Bustillos and Krochta, 1993), ethylene (Galietta et al., 1998b) and other volatile compounds (Miller and Krochata, 1997); and 2) have an effect on the mechanical properties of the food (Galietta et al., 1998a). Polysaccharide-based coatings generally exhibit poor water vapour resistance due to their hydrophilic nature; despite of that characteristic some polysaccharides, applied in the form of high moisture viscous coatings, are able to retard water loss from coated foods (Kester and Fennema, 1986).

2.3.4 Composites

The blend of more than one material can lead to the development of composite edible coatings with interesting properties. The usual objective is to take advantage of the maximum possible performance of the blend without changing drastically the properties of their components. Mixtures between different polysaccharides, polysaccharides and proteins and polysaccharides and lipids and waxes are the most studied blends (Kurek et al., 2014; Ruiz et al., 2013; Galus et al., 2013; Cerqueira et al., 2012; Lima et al., 2010; Fabra et al., 2009).

2.3.5 Plasticizers

Within the study of coatings, improvement of mechanical and transport properties through the incorporation of other compounds (i.e. plasticizers and lipids) has been a constant subject of interest (Bergo and Sobral, 2007). Plasticizers have been incorporated to enhance flexibility and resilience of coatings (Sothornvita and Krochtab, 2005) and decrease the presence of cracks and pores (Garcia et al., 2000). As the plasticizer acts by decreasing the intermolecular attraction between polymeric chains, allowing the penetration of polar water vapour molecules (Kester and Fennema, 1986), highly influencing the final coating permeability. Water, oligosaccharides, polyols, and lipids are different types of plasticizers used in hydrocolloid-based coatings (Suyatma et al., 2005). Glycerol is one of the most used plasticizers; it is a hydrophilic molecule (polar) and increasing its concentration causes an increase of water vapour mass transfer. Cerqueira et al. (2012) evaluated the influence of glycerol and corn oil on physicochemical properties of galactomannan from *Gleditsia triacanthos* and chitosan-based coatings, and confirmed that the presence of glycerol and corn oil originated a more hydrophilic structure and a decreased affinity of the coating matrix to water in both polysaccharides, respectively. Olivas and Barbosa-Cánovas (2008) carried out a similar study, where the effect of four plasticizers (fructose, glycerol, sorbitol and, polyethylene glycol) was evaluated on the mechanical properties and water vapour permeability (*WVP*) of alginate coatings. These authors reported that the use of plasticizers modified the mechanical properties of alginate coatings, decreasing tensile strength (*TS*), being this effect more pronounced when *RH* increases; also, results showed that water acts as a plasticizer in hydrophilic coatings.

2.3.6 Edible coatings as carriers of bioactive molecules

The favourable effects of edible coatings on fruits and vegetables (i.e. gas barrier and reduction of metabolic rate) have been extensively proven (Falguera et al., 2011; Moreira et al., 2011; Tapia et al., 2008). Edible coatings have the particularity to act as carriers for a wide range of food additives such as

antioxidants, nutraceuticals, flavouring agents and antimicrobials (Rojas-Graü et al., 2007; Pérez-Pérez et al., 2006). Several antimicrobials can be incorporated into edible coatings, including organic acids (e.g. citric, lactic, acetic, benzoic, tartaric, propionic, and sorbic acid), polypeptides (e.g. lysozyme, lactoferrin, natamycin, nisin, and peroxidase), plant extracts and essential oils (e.g. cinnamon, capsicum, garlic, carvacrol, oregano, and lemongrass), mineral salts (e.g. sodium bicarbonate, ammonium bicarbonate, and sodium carbonate), parabenes, oligosaccharides (chitooligosaccharides), among others (Paladines et al., 2014; Ramos et al., 2012; Valencia-Chamorro et al., 2011; Raybaudi-Massilia et al., 2008). These compounds must be considered as GRAS by the corresponding international regulatory agencies in order to be incorporated into edible coatings. Antimicrobials are regulated in the European Union (EU) by the European Commission Framework Directive 1130 (EC, 2011), whilst in the United States (US) by the part 21CFR172 (FDA, 2009).

Several authors observed through *in vitro* studies that the inclusion of antimicrobials into edible coatings enhances the control of rots that cause spoilage in fruits and vegetables. However, more studies of incorporation are necessary to understand how to maintain stable coating properties after bioactive incorporation (e.g. gases barrier, mechanical properties and appearance) (Sánchez-González et al., 2010). For example, Mohamed et al. (2013) evaluated the incorporation of lactoperoxidase system (LPOS), an antimicrobial of broad spectrum, into chitosan coatings at different concentrations (0.5, 1 and 1.5%); the addition of LPOS showed no significant effect on mechanical properties of the coatings, but led to a bacterial and fungal inhibitory effect depending on chitosan concentration and the strain on *Xanthomonas campestris* pv. *Mangifera indica*, *Colletotrichum gloeosporioides* and *Lasiodiplodia theobromae*. Meanwhile, Ahmad et al. (2012) reported that properties of gelatin films from skin of unicorn leatherjacket were affected by the incorporation of bergamot (BO) and lemongrass oil (LO), resulting in decreases in tensile performance (i.e. tensile strength and elongation-at-break), film solubility and transparency, being *WVP* also decreased when LO was added. The authors reported higher antimicrobial activity in films incorporated with LO that

those with BO, being more effective against Gram-positive bacteria (*Staphylococcus aureus* and *Listeria monocytogenes*) than Gram-negative bacteria (*Escherichia coli* and *Salmonella typhimurium*), but showing no inhibition towards *Pseudomonas aeruginosa*.

Recent works addressed the incorporation of nanoemulsions into edible coatings as a method to disperse lipophilic active ingredients in lower doses and with increased effectiveness. In this context, Acevedo-Fani et al. (2015) reported the suitability of nanoemulsions loaded with of EOs (thyme, lemongrass and sage oil) for formation of edible films by microfluidization. The results indicated that physical properties (colour, barrier and mechanical) of resulting edible films were influenced by the droplet size and ζ-potential, and were improved for those films including EOs when compared with pure alginate films; furthermore, authors mentioned that antimicrobial activity depends on the composition of EOs and the susceptibility of each particular microorganism to the antimicrobial agent. In that work, edible coatings containing thyme evidenced higher antimicrobial activity against *Escherichia coli*, while films formed from sage oil presented higher transparency, *WVP* and flexibility than those formed from thyme and LO. Also, Kim et al. (2014) demonstrated the stability of emulsions based on carnauba wax and LO was enhanced by forming nanoemulsions using dynamic high pressure (DHP) process. The coatings were applied on grape berries, showing antimicrobial activity against *Salmonella typhimurium* and *E. coli* O157:H7 during storage at 4 and 25 °C for 28 days. The coatings allowed reducing loss of weight, total anthocyanin concentration, antioxidant activity and firmness, and also avoided the degradation of phenolic compounds; whilst they did not significantly change the flavour of the berries. Salvia-Trujillo et al. (2015) evaluated another coating with nanoemulsions based on alginate and LO (0.1, 0.5 and 2% v/v) and compared its effect with conventional coatings on the safety and quality attributes of fresh-cut *Fuji* apples during cold storage. Edible coatings with LO nano sized droplets showed a better inactivation of *E. coli* that conventional emulsions. Higher LO concentration (0.5 or 1% v/v) allowed significant browning, but not on those coated with 0.1% (v/v) of LO. Also, the respiration of fresh-cut apples was reduced when increasing

concentration of LO, but droplet size showed no significant influence on the quality parameters.

Incorporation of antimicrobial agents into edible films allows using small antimicrobial concentrations and low diffusion rates; then their activity can be prolonged during produce distribution, transport and storage. However, it is important to modulate the release rate and migration of antimicrobial compounds from the edible coating matrix. The use of release kinetics models allows estimating optimal active agent concentrations during postharvest storage periods; an example is reported by Del Nobile et al. (2008), which determined that Fick's Second Law properly describes the release kinetics of thymol from zein films at 5, 10, 20 and 35% (weight of thymol/weight of dry polymer), and that thymol diffusion coefficient is independent from thymol concentration. Some examples of antimicrobial edible coatings showing efficiency on the control of rot pathogens of several fruits and vegetables are presented in Table 2-2.

Table 2-2. Examples of applications of antimicrobial edible coatings in fruits and vegetables.

Matrix	Antimicrobial agent	Microorganisms target	Fruit / vegetable	Reference
Hydroxypropyl methylcellulose and beewax	Ammonium carbonate	*Botrytis cinerea*	Cherry tomatoes	Fagundes et al., 2014
Pullulan	Sweet basil extract	*Rhizopus arrhizus*	Apple	Synowiec et al., 2014
Chitosan	Lemon essential oil	*Botrytis cinerea*	Strawberry	Perdones et al., 2012
Gum arabic	Cinnamon oil	*Colletotrichum musae* and *Colletotrichum gloeosporioides*	Banana and papaya	Maqbool et al., 2011
Mesquite-based gum	Thyme and Mexican lime essential oils	*Colletotrichum* gloeosporioides and *Rhizopus stolonifer*	Papaya	Bosquez-Molina et al., 2010
Chitosan	Grapefruit seed extract	*Botrytis cinerea*	Redglobe table grapes	Xu et al., 2007
Chitosan	Calcium chloride	Decreases the microbial growth rate (fungi and bacteria)	Strawberries	Ribeiro et al., 2007

2.3.7 Edible coating selection and application

Successful application of coatings depends on the selection of the adequate method, which can be chosen between dipping, brushing, spraying, and panning (Zhao, 2012). These procedures can be selected based on surface characteristics

of the produce and the main purpose of the coating. The most common coating procedure implies wetting the produce by the coating mixture followed by an adhesion process, where the penetration of the solution into the produce's skin occurs (Hershko et al., 1996). The wetting phase (governed by the surface's spreadability) is crucial, because if the affinity of the coating for the produce is optimal, the time required for this operation is minimal allowing virtually spontaneous spreading of the coating solution (Mittal, 1997).

Before deciding on coating application it is necessary to take into account the two ripening patterns of the produce (climacteric and non-climacteric), in order to select the optimal coating in each case. Climacteric fruits (e.g. tomato, banana, avocado, and apple) are characterized by increased respiration and ethylene production rates during ripening. The harvest of this type of produce is recommended as soon as possible, once its physiological maturity is reached. Nevertheless, they ripen rapidly during transport and storage, thus some of the challenges are to prevent ripening by slowing down respiration and preventing dehydration. Application of coatings able to reduce the ethylene production rate and to control gas exchange (CO_2/O_2) is a possibility for postharvest control of climacteric fruits, in such a way that they can delay the maturing process (Baldwin, 2001). Adequate coatings for this kind of fruits are those based on blends of polysaccharides, proteins and/or lipids, since blends can allow overcoming deficiencies of particular components. For example, blends of polysaccharides and additives (e.g. glycerol and lipids) can improve the permeability to gases and water vapour transfer when compared to polysaccharides alone (Cerqueira et al., 2012). Lima et al. (2010) reported the effectiveness of galactomannan-collagen blends in reducing O_2 consumption rate by 28% and CO_2 production rate by 11% when compared with uncoated mangoes, and both rates by 50% when compared with uncoated apples, respectively.

In non-climacteric fruits (e.g. citrus, pineapples, strawberry and grapes), respiration shows no dramatic change and ethylene is not required for fruit ripening (Giovannoni, 2001), being the losses mostly related to weight loss during transportation. For non-climacteric fruits it is a common practice to apply lipid-based coatings (e.g. waxes and resins) where the low permeability to CO_2, O_2, and

water vapour allows reducing metabolic rates and water loss, while also providing an attractive appearance to the produce (Baldwin, 2001). Nevertheless, excessive restriction of gas exchange sometimes occurs in waxed fruits, leading to undesirable flavour changes (Tietel et al., 2010; Hagenmaier, 2002; Baldwin, 1995). Blends of lipids and polysaccharides can be used instead to provide appropriate gas and moisture barrier (Perez-Gago et al., 2002). Furthermore, it has been demonstrated that when polysaccharides solutions are applied at higher concentrations (e.g. chitosan), respiration can be reduced together with changes in weight loss, firmness and external colour in strawberry fruits (Hernández-Muñoz et al., 2008).

In addition to the issues mentioned above, in both cases (climacteric and non-climacteric fruits) it is important to take into account temperature control, due to the impact that it shows in fruits' respiration rate. In fact, respiration rate significantly increases or decreases when temperatures are increased or decreased, respectively. This temperature effect must be taken into consideration since even coatings built for ideal storage temperatures can cause anaerobic fermentation and physiological disorders (Baldwin, 2001) if respiration rates are significantly changed.

Different formulations of edible coatings are available commercially; examples of products well-known in the market are:

1) NatureSeal® (Mantrose-Haeuser, Co., Inc., Westport, CT, USA). Based in ascorbic acid, calcium chloride, hydroxypropyl methylcellulose, it inhibits enzymatic browning, maintains taste, texture, and colour of fresh-cut fruits and vegetables;

2) Pro-long™ or TAL Pro-long™ (Courtaulds Ltd., Derby, United Kingdom) is an aqueous dispersion of sucrose polyesters of fatty acids and sodium salt of carboxymethylcellulose; it modifies the internal atmosphere of the fruit and maintains its natural colour;

3) Semperfresh™ (Agricoat Industries Ltd., Seattle, WA, USA) is a mixture of sucrose esters of short-chain unsaturated fatty acids and sodium salts of carboxymethylcellulose; it is a coating developed for the postharvest protection of

fruits like melons, pears, pineapples or cherries, it allows reduction of the respiration rate, ripening, weight loss and conserves the natural colour of fruits (Bai and Plotto, 2012).

New promising natural products have been recently introduced such as:

1) Clarity Citrus (Fagro Post Harvest Solutions S.A. DE C.V., Ramos Arizpe, Mexico), composed of polyethylene, shellac and carnauba; it is specially formulated for citrus fruits at postharvest stage and acts reducing gas exchange, the ripening process and water loss;

2) Naturcover (Decco Ibérica Post Cosecha S.A.U., Valencia, Spain), based on sucrose esters of fatty acids and other additives; it is an edible coating that reduces weight loss and chilling injury in stone fruit, and delays ripening in apples and pears. It also reduces stains of scratches on pears, and maintains freshness in citrus fruits;

3) Foodcoat Fr Drencher DMC (Domca S.A.U., Granada Spain) is formulated from oil acids derivates; it acts reducing the respiration rates of some fruits and vegetables, diminishes fruit weight loss and retards ripening. It also helps enhancing natural brightness and maintaining fruit consistence (Namesney and Delgado, 2014).

2.4 EMERGING TECHNOLOGIES - DEVELOPMENT OF NANO-LAMINATE COATINGS

Edible coatings can be considered an effective postharvest technology for extending shelf life of fruits and vegetables. However, their application still faces a number of disadvantages since: 1) they can impart off-flavours associated to the flavour of coating materials and to their deterioration (e.g. rancidity of lipids); 2) they may have their own colour and be possibly unattractive for consumers; 3) they can provide an undesirable tacky consistence; 4) it is difficult to obtain an adequate homogeneity for each produce surface being necessary to optimize the application and the drying step conditions; and 5) despite being good carriers of bioactive

agents, coatings can require large amounts of those compounds in order to reach optimal effectiveness, and sometimes this incorporation presents difficulties.

All of these problems have been studied in the last years, being the solutions presented in most of the cases based in the use of new emerging technologies. One of the examples is the use of nanotechnology. Nanotechnology uses materials at nanoscale (≤100 nm), exploiting differences in physicochemical properties exhibited by these materials when compared to those at a larger scale (Granda-Valdés et al., 2009). It represents a new tool for food technologists in the food packing area by promising packaging materials that will guarantee food products with a longer shelf life, maintaining their safety and quality (Neethirajan and Jayas, 2011).

On the other hand, one technique that explores the nanoscale advantages is the Layer-by-Layer (LbL) deposition which can be used for nano-laminate coatings formation. It consists in the use of two or more layers of e.g. oppositely charged materials with nanometer dimension (1–100 nm per layer) that are physically or chemically bound to each other and are assembled layer-wise on core materials (Labouta and Schneider, 2010; Zhang et al., 2007; Decher and Schlenoff, 2002).

The LbL technique is quite simple and enables using a wide range of materials (e.g. proteins, polysaccharides, lipids, and nanoparticles). These materials are able to interact either by electrostatic interactions, hydrogen bonding, covalent bonds, complementary base pairing and hydrophobic bonding. Moreover, depending on the template used (e.g. planar or colloidal) it is possible to design a variety of nano-laminate systems including nanoemulsions, nanofilms and nanocapsules (Cerqueira et al., 2014).

The resulting properties of nano-laminate coatings such as mechanical properties, gas permeability and swelling and wetting characteristics, are influenced by the kind of adsorbing materials utilized and also by the sequence, the total number of layers, and the conditions used for preparation (e.g. temperature, pH and ionic strength) (Weiss et al., 2006). This leads to a great number of possibilities, thus

allowing tailoring the final properties of the coating in order to ensure the desired functionality.

One of the advantages of these nano-systems is their gas barrier properties when compared with conventional edible coatings. Table 2-3 shows the permeabilities to oxygen and water vapour of conventional and nano-laminate coatings. It is suggested that barrier properties of nano-laminate coatings are improved due to their nano-structure, which has an increased tortuosity resulting from the electrostatic interactions between the nano-laminate's components and also from the interpenetration of the successively deposited layers that hampers gas molecules migration through the structure (Pinheiro et al., 2012; Medeiros et al., 2012a; Jang et al., 2008). The application of LbL technique in fruits and vegetables is very recent and few studies showed its effect on shelf-life parameters. One of the first steps in the application of LbL technique in produce is to prove its success (by means of microscopy techniques and/or contact angle measurements). Figure 2-2b shows a nano-laminate coating on mangoes surface by means of scanning electron microscopy (SEM) (Figure 2-2) where it is clear the alternate deposition of alginate and chitosan on mangoes' surface when compared with mango without nano-laminate coating (Figure 2-2a).

Application of coatings or waxes at industrial level is typically conducted by micro-spraying using specific nozzles with a bed of propylene brushes or by direct immersion of the food products. The products go through a washing and disinfection step (most cases), being perfectly dried before the coating step. For the application by spraying the products are rotating while the coating/wax is adhered to the surface. Drying steps can be performed in a tunnel with strong ventilation (40-45 °C) between 1.5-2.0 min or at room temperature. As far as we know, nano-laminate coatings have not been applied at industrial level. In our opinion for a successful application of nano-laminate coatings the immersion method should be used with washing (in water) and drying (at temperatures around 30 °C with strong ventilation) steps between layer applications. The times for this process as evaluated at laboratory scale are around 10-20 min, but a re-evaluation/adaptation is necessary in order to be applied at industrial scale. It is

important to mention that there are studies referring the possibility of using spraying in the development of multilayers (Schlenoff et al., 2000).

Table 2-3. Water vapour (*WVP*) and O_2 permeabilities (O_2P) values of conventional edible coatings and nano-laminate coatings

Composition	Type	$WVP \times 10^{-11}$ ($gm^{-1}s^{-1}Pa^{-1}$)	$O_2P \times 10^{-14}$ ($gm^{-1}s^{-1}Pa^{-1}$)	Thickness (μm)	Reference
Starch	Coating	17.7	N.D.	69.2	Garcia et al., 2006
L-carrageenan	Coating	11.80-235*	720	50	Hambleton et al., 2008
Chitosan	Coating	8.60	0.71	50	Fajardo et al., 2010
Alginate and chitosan	Nano-laminate	0.85	N.D.	0.12	Carneiro-da-Cunha et al., 2012
κ-carrageenan and chitosan	Nano-laminate	0.020	0.043	0.342	Pinheiro et al., 2012
Pectin and chitosan	Nano-laminate	0.019	0.069	0.266	Medeiros et al., 2012a

*Depending on temperature and humidity gradient
n.d.: not determined

Recent works showed successful applications of nano-laminate coatings in commodities describing the application conditions, such as number of layers, immersion time, washing and drying steps. Medeiros et al. (2012a) evaluated a nano-laminate coating based on five layers of pectin and chitosan (at a concentration of 0.2%, w/v) on whole 'Tommy Atkins' mangoes applied by immersion of 15 min into each polyelectrolyte solution and a washing procedure with distilled water at pH 7.0 and 3.0 for pectin and chitosan, respectively. After 45 days the coated mangoes presented better appearance, reduction in water loss,

and absence of fungal growth that uncoated mangoes; also, reduction of gas flow was observed, as result the shelf life of mangoes was increased. Moreover, Medeiros et al. (2012b) reported the positive effect on shelf life extension of Rocha' fresh-cut pears (CP) and whole pears (WP) upon application of a nano-laminate coating composed of five layers of κ-carrageenan and lysozyme (each at concentrations of 0.2%, w/v). The immersion time into each polyelectrolyte solution was 5 and 15 min for CP and WP, respectively; and subsequently rinsed with deionized water with pH 7.0 (κ-carrageenan) and pH 3.8 (lysozyme). The coating avoided mass loss of CP, proving the efficiency of the nano-laminate as water loss barrier. Total soluble solids values were lower for both coated CP and coated WP during the experimental period (7 and 45 days, respectively), whilst low values of titratable acidity for coated CP and WP were an indicative of the delay in maturation process associated to the reduction of gas exchange (O_2 and CO_2) by the application of the coating. More recently, Souza et al. (2015) studied a nano-laminate coating based on five alternate layers of alginate and chitosan (each at concentrations of 0.2%, w/v) to extend the shelf life of fresh-cut mangoes stored under refrigeration (8 °C) for 14 days. Polyelectrolyte solutions were applied by immersion for 15 min and subsequently rinsed with deionized water with pH 7.0 and 3.0 for alginate and chitosan, respectively. An additional drying step with flow of nitrogen at 25°C for 15 min was used between layers. Lower values of soluble solids, mass loss, and higher titratable acidity were observed on coated fresh-cut mangoes. Moreover, the nano-laminate allowed the reduction of malondialdehyde content (an indication that the coating application prevents senescence). According to microbial analyses the shelf life of fresh-cut mangoes was increased up to 8 days at 8 °C when compared with uncoated fresh-cut mangoes (<2 days).

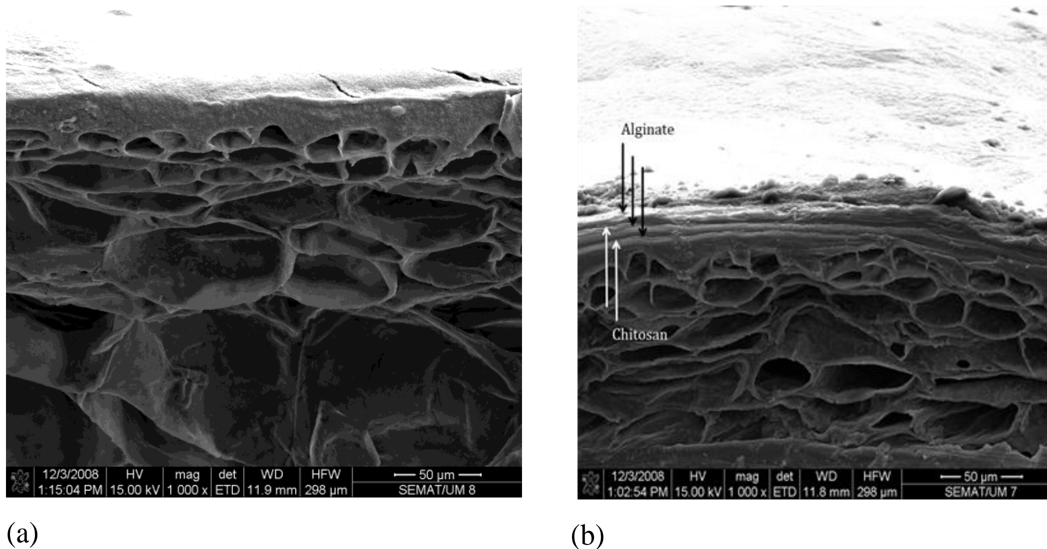

Figure 2-2. Scanning electron microscopy images of the mango surface (a) and of nano-laminate coating on mango surface (Alginate/ chitosan/ alginate/ chitosan/ alginate) (b). Adapted from Flores-López et al. (2015).

Nano-laminate coatings are able to incorporate functional compounds under the form of nanoparticles, which presumably have greater chemical reactivity and can be more bioactive than larger particles as, their size has better access to any structure (Lugo-Medina et al., 2010). Furthermore, nanoparticles can have a dual purpose: besides acting as carriers of additives, they may also provide improvements in the mechanical and barrier properties of the structures where they can be incorporated. However, the efficiency of nanolayer systems with a variety of features (e.g. antioxidant, antimicrobial and reduction of gas exchange) still remains little studied.

The use of LbL technique has also been studied at micro-scale; some examples are reported by Brasil et al. (2012). In this work, a microencapsulated beta-cyclodextrin and *trans*-cinnamaldehyde complex (2 g/100 g) was incorporated into a laminate coating made of chitosan and pectin; the quality of fresh-cut papaya was extended to 15 days at 4 °C while uncoated fruits could not reach this far (< 7 days). The coating reduced the losses of vitamin C and total carotenoids content; in addition, the encapsulation of *trans*-cinnamaldehyde was successful, since it had no negative impact on the fruit's flavour. In another work, Mantilla et al. (2013) evaluated the efficacy of a microencapsulated antimicrobial complex (beta-

cyclodextrin and trans-cinnamaldehyde) incorporated into a laminate coating composed of pectin-alginate on fresh-cut pineapples. The system showed microbial growth inhibition, while the original qualities (colour, texture and pH) of pineapples were kept and the shelf-life was extended to 15 days at 4 °C.

2.5 CONCLUSION

One of the major causes of postharvest losses in fruits and vegetables worldwide is the lack of postharvest technology solutions in developing countries. One of the solutions is the application of edible coatings, where nano-laminate coatings showed in the last years to be one of the promising technologies to increase fruits shelf life. Despite the promising results, is still needed an appropriate optimization and implementation of these technologies, in order to be effectively used in the processing chain of fruits and vegetables.

The use of nanotechnology promises a great impact in food and agriculture industries. Nanotechnology advanced not only in packaging technologies, through the development of nano-laminate and bioactive nano-laminate coatings for application on fruits and vegetables, but also in the design of biosensors to identify and quantify diseases, residuals of agrochemicals, modification of food composition, and in the nanoformulation of agrochemicals to control pests and application of fertilizers. However, optimization and implementation of these technologies still face some challenges e.g. difficulty to measure the nano-laminte coatings' thickness (nanoscale) and industry viability, due to the changes needed in packing-houses for the application of nano-laminate coatings.

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Chapter 3

EVALUATION OF WHOLE FRACTIONS DERIVED FROM *ALOE VERA* PROCESSING: CHEMICAL CHARACTERIZATION AND ANTIOXIDANT AND ANTIFUNGAL POTENTIAL

The characterization of *Aloe vera* fraccions (gel, liquid fraction and bagasse extracts) was conducted in this chapter. Carbohydrates (mainly glucose and mannose) were the major components of *A. vera* fractions. Hallmarks: for gel, higher amount of malic and uronic acid; for bagasse, the occurrence of lignin; and for the ethanolic bagasse extract (EE-B), the higher total polyphenol content (*TPC*). The antifungal and antioxidant activities of each fraction were discussed and compared.

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3.1 INTRODUCTION

For many years, plants from different ecosystems have been collected and studied as a source of new bioactive compounds for a huge range of applications, such as antioxidants (Kuppusamy et al., 2016), drugs (Zengin et al., 2015), pesticides (Jasso de Rodríguez et al., 2011), among others.

Postharvest damages of fruits and vegetables are often caused by colonization of various microorganisms, reducing their shelf life as well as their market value. In developing countries postharvest losses reach more than 40%, being these losses even higher in the storage stage than those occurring in the field (Flores-López et al., 2015). The use of synthetic chemicals, such as pesticides, is the most common approach for disease control in different crops; however, the application of such chemicals has caused severe damage to the health and environment, and frequently their application is only allowed during preharvest (Jasso de Rodríguez et al., 2011). Their indiscriminate use has developed microorganism resistance to the most widely used synthetic pesticides, causing their exit of the market (Flores-López et al., 2015). Thence, the need for new pesticides with enhanced performance and having a low impact on the environment. Natural products represent an eco-friendly alternative to the use of chemicals for the management of diseases of fruits and vegetables.

Aloe vera, from the Liliaceae family, is a perennial plant with lance shaped leaves formed by a thick epidermis (skin). It has traditionally been consumed as whole leaf in folk medicine for its beneficial health effects (Grindland and Reynolds, 1986). Its biological activity is broadly accepted and it is used for several medical, nutraceutical and cosmetic applications (Boudreau and Beland, 2006). The plant is divided in two components: a colourless mucilaginous pulp (gel) and a bitter yellow sap (exudate) (Grindland and Reynolds, 1986). The gel is the most studied and used part of *A. vera* due to its complex chemical composition. It is composed by carbohydrates being mostly acemannans polysaccharides (Lee et al., 2001), but also soluble sugars, organic acids, proteins, phenolic compounds, vitamins, minerals and aminoacids are present (Boudreau and Beland, 2006). The

effectiveness of *A. vera* gel to control fungal growth has been extensively proven against *Penicillium digitatum*, *P. expansum*, *Botrytis cinerea*, and *Alternaria alternata*, among others (Castillo et al., 2010). Also, it has been incorporated into edible coatings (neat or in combination with other components) to extend the postharvest storage of strawberries (Sogvar et al., 2016) and apple slices (Chauhan et al., 2011).

The conventional methods for the extraction of *A. vera* gel are: (1) the traditional hand filleted pulp method, in which the entire gel is blended; and (2) the mechanical procedure characterized by a mechanical filleting followed by pressing, where the resulting gel can also be liquidized and filtered. The mechanical procedure also allows obtaining a liquid fraction (Jasso de Rodríguez et al. 2005). Recently, the interest for the liquid fraction has arisen, since it has shown to possess antifungal activity (Jasso de Rodríguez et al., 2005) and beneficial effects such as increasing the shelf life of blueberries has been reported (Vieira et al., 2016). However, there is limited information about the chemical composition and biological activities of *A. vera* liquid fraction.

The production process of *A. vera* fractions generates a large amount of solid wastes. These residues (bagasse) include the spikes, bases and tips removed from the leaves, and the skin resulting from the separation of the gel. Thus far, the bagasse has not been given any added value. Bioactive compounds can be extracted from the bagasse using organic solvents which are safe/less toxic (Cann, 2009), allowing an integral exploitation of *A. vera*. Therefore, the aims of this work were to (1) characterize the gel, liquid and bagasse of *Aloe vera*, (2) obtain extracts from bagasse, and (3) evaluate biological activity of gel, liquid and bagasse extracts in terms of antioxidant and antifungal activities on phytopathogenic fungi.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Malic acid was supplied from Riedel-de Haën (Germany), citric acid anhydrous from J. T. Baker (USA), formic acid from Merck (Sweden), acetic acid from Sigma (USA) and lactic acid was supplied by Acros organics (USA). Galactose and mannose were obtained from Acros organics (USA), glucose from Fisher Scientific (USA), arabinose from Sigma (USA), galacturonic acid and xylose were supplied from Fluka (Slovakia). Sulfuric acid (95-98%) and barium carbonate were purchased from Sigma (USA). Aloin of purity >97% from *Aloe barbadensis* Miller leaves, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), buthylatedhydroxyanisole (BHA), sodium carbonate (Na_2CO_3) and Folin-Ciocalteau reagent (FC) were purchased from Sigma (USA). Ethanol absolute and methanol were obtained from Fisher chemical (UK). Potato dextrose agar (PDA) was purchased from Difco (France) and potato dextrose broth (PDB) from Liofilchem (Italy). All samples, standards and eluents were prepared using demineralized Milli-Q water from Millipore, USA.

3.2.2 Plant material and sample preparation

Fresh whole *Aloe vera* leaves (four years old), supplied by Aloe Vera Ecológico (Alicante, Spain), were washed with water, immersed in a 2.0% sodium hypochlorite solution, and rinsed with distilled water. The leaves were weighed (g), and measured for their length (cm), thickness (cm) and width (cm). For each leaf the spikes, inferior and superior parts were removed before longitudinally slicing to separate the epidermis from the parenchyma (fillet). The fillet was pressed by means of a laboratory manual roll processor desinged by our group (Annexes 1 and 2), and filtered in order to separate the liquid fraction from the gel and the bagasse. The yields were determined and expressed as percentage of either the obtained gel or liquid fractions with respect to the entire leaf weight. The gel and liquid fractions were pasteurized by heating at 65 °C for 30 min and cooled immediately; this step was repeated three times (Jasso de Rodríguez et al., 2005).

Afterwards, one part of the samples was lyophilized and another was stored at -20 °C until further analyses were performed.

3.2.2.1 Preparation of the bagasse extracts

The bagasse resulting from the separation of the gel and liquid fraction was dried at 40 °C, then ground to a particle size equivalent to mesh No. 50 prior to extraction. Approximately 5 g of dried bagasse was thoroughly extracted in a Soxhlet apparatus during 48 h with absolute ethanol or distilled water (ratio 1:20) at 99.4 °C and 78.4 °C for aqueous (AE-B) and ethanolic extract (EE-B), respectively. The crude extracts were subsequently filtered (Nº 1 Whatman filter paper) and concentrated in a rotary evaporator. The extracts were stored in the dark at 5 °C until further use.

3.2.3 Physico-chemical characterization of Aloe vera fractions

All methodologies were conducted following the recommendations of the Official Method of Analysis (AOAC, 1990). The lipid content was determined gravimetrically by means of Soxhlet extraction (AOAC 960.39). The crude protein level was calculated by the Kjeldahl method with a conversion factor of 6.25 (AOAC 960.52). The ash content was evaluated by incineration in a muffle at 550 °C (AOAC 923.03). Moisture content was determined using the method AOAC 934.06. The pH value was determined using a pH meter (Metrohm, Swiss). All measurements were carried out in triplicate.

3.2.3.1 Organic acid analysis

The extraction of organic acids from lyophilized gel and liquid fraction was carried out with water (30 min at 60 °C), following the method described by Bozzi et al. (2007). After the extraction process, solutions were filtered through a 0.45 µm cellulose acetate membrane and organic acids (malic, citric, acetic and lactic acid) were determined by High-Performance Liquid Chromatography (HPLC). Chromatographic separation was performed using a Metacarb 87H column (300 × 7.8 mm, Varian, USA) under the following conditions: mobile phase 0.005 mol L^{-1}

H_2SO_4, flow rate 0.7 mL min^{-1}, and column temperature 60 ºC. The equipment used was a UV detector set at 210 nm (Jasco, Tokyo, Japan) and a Jasco AS-2057 Plus intelligent auto sampler (Jasco, Tokyo, Japan). The volume injected was 20 µL per sample. The peaks obtained from each sample were identified and quantified through standard calibration curves.

3.2.3.2 Polysaccharide analysis after hydrolysis

Bagasse and lyophilized gel and liquid fraction were hydrolyzed via a two-step acid hydrolysis for polysaccharides quantification. Samples (100 mg) were pre-hydrolyzed in H_2SO_4 72% by continuously stirring at 30 °C during 1 h; then post-hydrolysis was continued in H_2SO_4 4.0% by autoclaving at 121 °C for 1 h. Samples were neutralized with barium carbonate, filtered through 0.45 µm cellulose acetate membranes and analyzed by HPLC for glucose, xylose, galactose, arabinose and mannose using a 1100 series Hewlett-Packard chromatograph fitted with a refractive index detector operated at 50 ºC and 300 × 7.8 mm CARBOsep CHO 682 column (Transgenomic, Glasgow, UK) operating at 80 ºC. Distilled water was used as the mobile phase (flow rate 0.4 mL min^{-1}). Uronic acids were colorimetrically determined using hydrolyzed samples and reported as total uronic acid (Ahmed and Labavitch, 1978).

3.2.3.3 Analysis of free sugars

Free sugars were analyzed by HPLC. Firstly, water-soluble monosaccharides and disaccharides were extracted with water (30 min at 70 °C) (Bozzi et al., 2007). Samples were subsequently filtered through a 0.45 µm cellulose acetate membrane and analyzed using the CARBOsep CHO 682 column and the HPLC conditions already described in Section 3.2.3.2.

3.2.4 Biological activity and phenolic composition

3.2.4.1 Phenolic composition

Total phenolic content (*TPC*) was determined using a 96-well microplate-adapted colorimetric assay using FC reagent as described by Meneses et al. (2013). Briefly, lyophilized gel and liquid fractions (0.01 g) were homogenized in 0.5 mL of methanol; the mixture was mixed using a vortex and extracted for 48 h at room temperature in darkness. To determine *TPC* from EE-B and AE-B, the extracts (0.01 g) were re-suspended in ethanol and distillated water (5 mL), respectively. After homogenization, all samples were centrifuged at 12,000 *g* for 5 min. Subsequently, 5 µL of supernatant was mixed with 60 µL of Na_2CO_3 solution (7.5%, w/v) and 15 µL of reactive FC. Then 200 µL of distilled water were added and solutions were mixed. Absorbance was measured using a spectrophotometric microplate reader (Synergy HT, Biotek, USA) at 700 nm after 5 min incubation at 60°C. A calibration curve was prepared using a standard solution of aloin (0.2, 0.4, 0.6, 0.8, 1.0 mg mL^{-1}, R^2 = 0.9905). All experiments were performed in triplicate. The total phenolic content was determined as aloin equivalents and values are expressed as mg of aloin per g of extract.

3.2.4.2 Antioxidant activity

Free radical scavenging activity of gel, liquid and bagasse extracts (EE-B and AE-B) was determined using the DPPH method with some modifications (Pinheiro et al., 2015). BHA was used as antioxidant of reference and ethanol as control. Briefly, 0.2 mL of ethanol and 0.3 mL of the sample dissolved in ethanol (concentrations ranging from 0.05 to 30 mg mL^{-1}) were mixed with 2.5 mL of DPPH (60 µmol L^{-1} in ethanol) to achieve a final volume of 3.0 mL. The solution was mixed in a vortex and kept at room temperature for 30 min in the dark. Then, 0.2 mL of each sample was transferred into a 96-well microplate to measure absorbance at 515 nm (BiotekSinergy II, USA) and antioxidant activity was expressed as percentage DPPH-scavenging activity relative to the control, using the following equation:

$$\% \, Radical\ scavenging\ (RSA) = \left\lfloor \frac{(A_{control} - A_{sample})}{A_{control}} \right\rfloor \cdot 100 \qquad \text{Eq. 3-1}$$

where $A_{control}$ represents the absorbance value of the control sample and A_{sample} represents the absorbance value of the analyzed sample. The IC_{50} value was calculated as the concentration required to obtain a 50% of inhibition of radical scavenging activity (*RSA*). IC_{50} was determined from a graph of *RSA* (%) against sample concentration (mg mL^{-1}). All experiments were performed in triplicate.

3.2.4.3 Antifungal activity

Penicillium expansum (MUM 02.14) and *Botrytis cinerea* (MUM 10.138) were obtained from MUM (Micoteca da Universidade do Minho, Braga, Portugal). The fungi were routinely cultured on PDA at 25 °C for 7-14 d; the spores were collected and diluted with sterile water until suspensions reached a spore concentration of 10^4 mL^{-1}.

Antifungal activity of gel and liquid fractions at 3 doses (0.1, 1.0 and 50%, v/v) and bagasse extracts (EE-B and AE-B) at 3 doses (50, 100 and 500 ppm, w/v) were evaluated following the procedure reported by Kouassi et al. (2012), with some modifications. 100 µL of each concentration were pipetted into a sterile 96-well microplate. Each well was inoculated with a 100 µL aliquot of fungal inoculum to reach a final volume of 200 µL. A positive control was carried out by mixing 100 µL of sterile PDB with 100 µL of each fungal suspension. The negative control of each group of replicates was a non-inoculated medium. In order to ensure that the solvent did not interfere with the test, controls only with water (for AE-B) and absolute ethanol (for EE-B) were carried out. Fungal growth was monitored spectrophotometrically at 530 nm (BiotekSinergy II, USA) by measuring optical density (OD) during 72 h (at 24 h intervals) and incubation at 25 ± 2 °C. Percentage of growth inhibition was determined using the following equation:

$$Inhibition\ (\%) = \left\lfloor \frac{(OD_{control} - OD_{sample})}{OD_{control}} \right\rfloor \cdot 100 \qquad \text{Eq. 3-2}$$

where OD_{sample} represents the optical density of the each treatment and $OD_{control}$ represents the optical density of the control. Experiments were replicated three times per treatment.

3.2.5 Statistical analyses

Data analyses were subjected to analysis of variance (ANOVA) using FAUANL software (Olivares, 1994). Fisher's Least Significance Difference (LSD) multiple comparison test was performed to detect significant differences ($p<0.05$) between treatments.

3.3 RESULTS AND DISCUSSION

3.3.1 Leaf characteristics and yields

The characteristics of the leaves utilized in this work were found quite in agreement with the leaf dimensions (30-60 cm in length, 5-12 cm wide at the base and 0.8-3 cm thick) and weight (364-455 g) reported for *A. vera* (Rodríguez-García et al., 2007; Añez and Vásquez, 2005) (Table 3-1). The gel and bagasse were separated from the liquid fraction, obtaining extraction yields of 15.76%, 33.00%, and 51.20%, respectively. The extraction yield of *A. vera* gel is generally around *ca.* 60% (Zapata et al., 2013), although these yields are considered without separation of liquid fraction and are directly influenced by the water content of the leaves during planting (Rodríguez-García et al., 2007) and the method of extraction. The yields obtained are in the range reported by Hernández-Cruz et al. (2002), which performed the separation of gel and liquid fractions using a laboratory roll processor. The authors reported extraction yields of 20% and 40% for gel and liquid fractions, respectively. The pH values found in the gel and liquid fractions (pH of 4.3 and 4.9, respectively) are within the range reported previously for *A. vera* gel (pH of 4.58-5.30) (Zapata et al., 2013).

Table 3-1. Leaf dimensions, weight and extraction yield of *Aloe vera* for gel, liquid and bagasse

Leaf dimensions (cm)				Weight (g)	Yield (%)		
Length	Width at base	Width at half	Thickness		Gel	Liquid	Bagasse
36.80 ± 6.14	10.15 ± 0.63	8.55 ± 0.72	2.63 ± 0.40	484.34 ± 46.10	15.76 ± 4.00	51.20 ± 5.20	33.00 ± 5.00

Values reported are the mean ± standard deviation (*n*=10).

3.3.2 Chemical composition

The mean values and standard deviations of the composition results obtained for *A. vera* fractions in an oven-dry basis are presented in Table 3-2. The major feature of both gel and liquid fraction is their high water content with 98.62% and 99.35%, respectively. In contrast, the bagasse is constituted by higher content of solids. Lipids were a minor component for all *A. vera* fractions and are within the range reported previously for *A. vera* gel (0.08 - 0.19%) (Zapata et al., 2013) and skin (2.71%) (Femenia et al., 1999). Also, proteins were in agreement with the values reported for gel (3.72%) (Vega-Gálvez et al., 2011a) and skin (6.33%) (Femenia et al., 1999). Ashes were a minor fraction for gel and liquid fraction with less than 1.5%, whilst bagasse shown relative higher ash (13.92%) and lipids (2.08%) contents. Previous works have reported higher ashes content for gel (17.64- 23.61%) than those found in this work (Vega-Gálvez et al., 2011a; Femenia et al., 1999), whilst for bagasse the results are in agreement with the values reported by Femenia et al. (1999) for skin of *A. vera* (13.46%). The presence of minerals in *A. vera* is essential for the proper functioning of various enzymes systems in different metabolic pathways and few are antioxidants; also, minerals such as potassium have been associated with the regulation of the healing properties of *A. vera* (Surjushe et al., 2008). Thence, it appears reasonable to speculate that higher content of minerals can be concentrated in the skin with the aim of improving the resistance of the plant to biotic and abiotic stresses (i.e. attack by microorganisms and high water stress).

The sum of carbohydrates and lignin found in the gel, liquid fraction and bagasse represented 57.45%, 40.09% and 56.86% of the total components, respectively (Table 3-2). Lignin was only detected in the bagasse, since the occurrence of secondary lignified walls leads to cross-linking of cell wall polysaccharides causing an increase in the hardening of that tissue (Femenia et al., 1999). Also, it is known that lignin is an important source of polyphenolic compounds available from natural biomass feedstocks (Jung et al., 2015).

As can be seen in Table 3-2 for all *Aloe* fractions, glucose and mannose were found as the major constituents in a ratio of *ca* 1:1. These sugars have been reported in various ratios as components of polysaccharides occurring in the *Aloe* gel, e.g. acetyled glucomannans (Lee et al., 2001). It has been reported that *A. vera* gel is formed by linear polymers with no branching and having 1,4 glycosidic linkages with glucose and mannose (Lee et al., 2001). The presence of higher amounts of uronic acids followed by lower amounts of galactose confirms the occurrence of pectic polysaccharides in gel and bagasse. Rodríguez-González et al. (2011) reported that the large presence of galacturonic acid units and the lower amounts of galactose and arabinose are associated to the presence of homogalacturonans, and minor amounts of rhamnogaluctoronans with a low degree of branching. This was confirmed by Gentilini et al. (2014), who extracted pectin from *A. vera* gel with high content of galacturonic acid and a low degree of esterification. The occurrence of relatively small amounts of xylose in the gel and bagasse fractions can be related to the presence of hemicellulosic xyloglucans (Femenia et al., 2003). On the other hand, the liquid fraction only presented traces of galactose and arabinose (no xylose was detected) and had the lowest values of uronic acid units (2.75%), indicating a lower concentration of pectic polysaccharides in this fraction. This fact shows than one of the most important differences between *Aloe* gel and liquid fraction is the occurrence of different concentrations of pectin, which in presence of calcium can form an intra-cellular “cement” that provides firmness to the tissues and can thus be related to the gel-like behaviour of the gel fraction (Alonso et al., 1995).

On the other hand, the main soluble sugar detected in *A. vera* fractions was glucose, being the values higher in the liquid fraction (10.37%) when compared with those obtained for gel (7.71%). Bozzi et al. (2007) detected also other free sugars in *A. vera* fresh gel such as fructose (5.30%), sucrose (0.16%) and galactose (0.05%), but in lower concentrations than glucose (11.85%).

The measurement of organic acids (e.g. malic acid) is used as a quality parameter in *A. vera* processing; however, their concentrations in *Aloe vera* fractions can vary depending on biological variability and the manufacturing process (Bozzi et al., 2007). The organic acid profile detected for *Aloe vera* gel was characterized mainly by the presence of high amounts of malic acid (18.17%), whilst in the liquid fraction, lactic acid was found in higher concentration (19.53%) (Table 3-2). Malic acid is considered as an indicator of gel freshness and quality (Rodríguez et al., 2010) and is formed in *Aloe vera* gel as a result of crassulacean acid metabolism (CAM), being present usually in the range of 11.1 and 40.4% (Jiao et al., 2010). In contrast, the presence of other organic acids can suggest possible microbial and enzymatic degradation (e.g. lactic, fumaric, formic, succinic, and acetic acids) (Rodríguez et al., 2010). Since malic acid is susceptible to bacterial degradation into lactic acid (Garcia et al., 1992), the lower value of malic acid and highest values of lactic acid in liquid fraction are indicators of bacterial degradation of the sample.

Table 3-2. Chemical characterization of *Aloe vera* fractions (results are expressed as percentages on dry matter basis)

	Gel	Liquid	Bagasse
Total solids	1.38 ± 0.36	0.65 ± 0.01	92.33 ± 1.31
Soluble sugars (Glucose)	7.71 ± 0.14	10.37 ± 1.46	n.d.
Proteins	3.17 ± 0.12	3.28 ± 0.11	4.78 ± 0.10
Lipids	0.66 ± 0.03	0.53 ± 0.10	2.08 ± 0.11
Ashes	0.43 ± 0.06	0.70 ± 0.00	13.92 ± 0.39
Uronic acids	15.80 ± 0.78	2.75 ± 0.55	19.81 ± 2.25
Polysaccharides			
Glucose	16.78 ± 0.40	13.01 ± 0.11	14.77 ± 0.52
Mannose	14.90 ± 0.35	13.03 ± 0.07	12.09 ± 1.47
Galactose	0.92 ± 0.52	0.36 ± 0.00	2.40 ± 0.95
Xylose	0.57 ± 0.14	n.d.	0.40 ± 0.07
Arabinose	0.77 ± 0.32	0.57 ± 0.00	0.84 ± 0.16
Lignin	n.d.	n.d.	8.16 ± 0.33
Organic acids			
Acetic	3.65 ± 0.32	2.99 ± 0.07	2.23 ± 0.08
Malic	18.17 ± 2.85	4.07 ± 1.11	n.d.
Citric	0.36 ± 0.10	0.92 ± 0.32	n.d.
Lactic	n.d.	19.53 ± 1.04	n.d.

n.d.: not detected.

Values reported are the mean ± standard deviation.

3.3.3 Phenolic compounds and antioxidant activity

The antioxidant activity of *A. vera* gel, liquid fraction and bagasse extracts (EE-B and AE-B) is shown in terms of IC_{50}, which corresponds to the concentration required to achieve 50% of inhibition of the oxidation (Table 3-3). In general, EE-B had the lowest value of IC_{50} (0.34 mg mL^{-1}) when compared with the gel and liquid fraction ($p<0.05$). However, there are no significant differences ($p>0.05$) between

the IC_{50} of EE-B and AE-B, whose recovery yields were of 9.62 ± 0.45% and 47.41± 5.10%, respectively.

Previous works have reported the relation between the *TPC*, *RSA* and IC_{50} values, showing a positive correlation of higher values of *TPC* with the antioxidant activity (Cerqueira et al., 2010). In this work, such tendency was also observed since EE-B, that exhibited higher antioxidant activity, also presented a higher *TPC* content (454.10 ± 4.51 mg aloin g^{-1} extract) than the other samples. These polyphenolic compounds could be extracted from bagasse due to the affinity that exists between ethanol (polar solvent) and polar structures (Jasso de Rodríguez et al., 2011).

The liquid fraction had a significantly lower IC_{50} value than the gel ($p<0.05$), being these results comparable with those reported by Vieira et al. (2016) for liquid and gel (7.76 and 22.37 mg mL^{-1}, respectively). This can be associated with the fact that liquid fraction presented values of *TPC* 2-fold higher (43.30 ± 1.66 mg aloin g^{-1} extract) than gel (19.11 ± 0.91 mg aloin g^{-1} extract).

It has been demonstrated that the amount of phenolic compounds can vary among different forms of *A. vera* (e.g. aloe exudate, gel, skin and flowers) (López et al., 2013) and the age of the plant (Rodríguez et al., 2010), therefore exhibiting different antioxidant activities. Previous studies regarding the content of phenolic compounds in the liquid fraction were not found; however, the antioxidant activity of gel found in the present work is in agreement with the work of Vega-Gálvez et al. (2011b). For the gel, the antioxidant activity has been attributed to *Aloe* polysaccharides (Chun-hui et al., 2007) and vitamins C and E (Rodríguez et al., 2010).

Table 3-3. Total phenolic content (*TPC*), radical scavenging activity (*RSA*) and IC_{50} of gel, liquid fraction and bagasse extracts of *Aloe vera*; *RSA* and IC_{50} values for BHA are given for comparison.

Sample	*TPC* (mg aloin g^{-1} extract)	*RSA* (%)	IC_{50} (mg mL^{-1})
Gel	19.11 ± 0.91^{d}	58.33 ± 2.26^{c}	17.01 ± 0.77^{a}
Liquid	43.30 ± 1.66^{c}	61.41 ± 0.13^{c}	7.66 ± 0.71^{b}
AE-B	88.37 ± 4.41^{b}	61.20 ± 0.83^{c}	0.40 ± 0.01^{c}
EE-B	454.10 ± 4.51^{a}	69.26 ± 0.98^{b}	0.34 ± 0.01^{c}
BHA	0.62 ± 0.04^{e}	82.26 ± 1.56^{a}	0.07 ± 0.01^{c}

Values in the same column followed by different letters are statistically different (Tukey test, $p<0.05$).
Values reported are the mean ± standard deviation.

3.3.4 Antifungal activity

The inhibition effect of *A. vera* gel and liquid fraction as a function of concentration is presented in Figure 3-1. For both fungi, the antifungal activity was concentration-dependent, being higher (100% of mean inhibition) when the highest concentration (50%) was evaluated. A higher inhibition effect was observed on *P. expansum* than *B. cinerea* at lower concentrations of gel and liquid fraction (0.1 and 1.0%). The results are in agreement with previous reports in which the antifungal effect of *A. vera* gel is related with the fungal genera, as reported by Nabigol and Asghari (2013) that described a higher antifungal activity of *A. vera* gel against *P. digitatum* than against *Aspergillus niger*, whereas Castillo et al. (2010) reported higher growth inhibition for *P. digitatum* than for *B. cinerea*.

Otherwise, it was observed a greater effect of gel when it is compared with liquid fraction after 48 and 72 h on *P. expansum*. It can be associated with the fact that the gel presented a higher content of malic acid (Table 3-2) than the liquid fraction; this compound has been demonstrated to have antimicrobial activity (Raybaudi-Massilia et al., 2008). For *B. cinerea* a similar behaviour of the gel and liquid fraction was observed; although the liquid fraction presented a higher inhibition ($p<0.05$) against *B. cinerea* at 24 h for a lower dose when compared with the gel, this effect was not observed at 72 h (Figure 3-1b). Jasso de Rodríguez et al.

(2005) reported a higher inhibitory effect of *A. vera* liquid fraction against *Colletotrichum coccodes* and *Rhizoctonia solani* (22 and 28%, respectively) than that observed for the gel fraction; the *A. vera* gel showed a higher growth inhibition (53%) of *Fusarium oxysporum* than the liquid fraction (38%). In the literature, the antifungal activity of gel of *Aloe* species has been attributed to diverse bioactive compounds, such as aloe-emodin and aleonin, showing control of growth of *A. niger*, *Cladosporium herbarum* and *F. moniliforme* (Ali et al., 1999). Meanwhile, Zapata et al. (2013) evidenced the positive correlation of antifungal activity with the aloin content in gel of *A. ferox*, *A. mitriformis*, *A. sapononaria* and *A. vera*. However, as far as we are aware, no reports about liquid fraction composition and its relation with its antifungal activity were found. According to our results, the antifungal activity of liquid fraction could be related with the higher ($p<0.05$) amount of *TPC* when compared to the gel (Table 3-3).

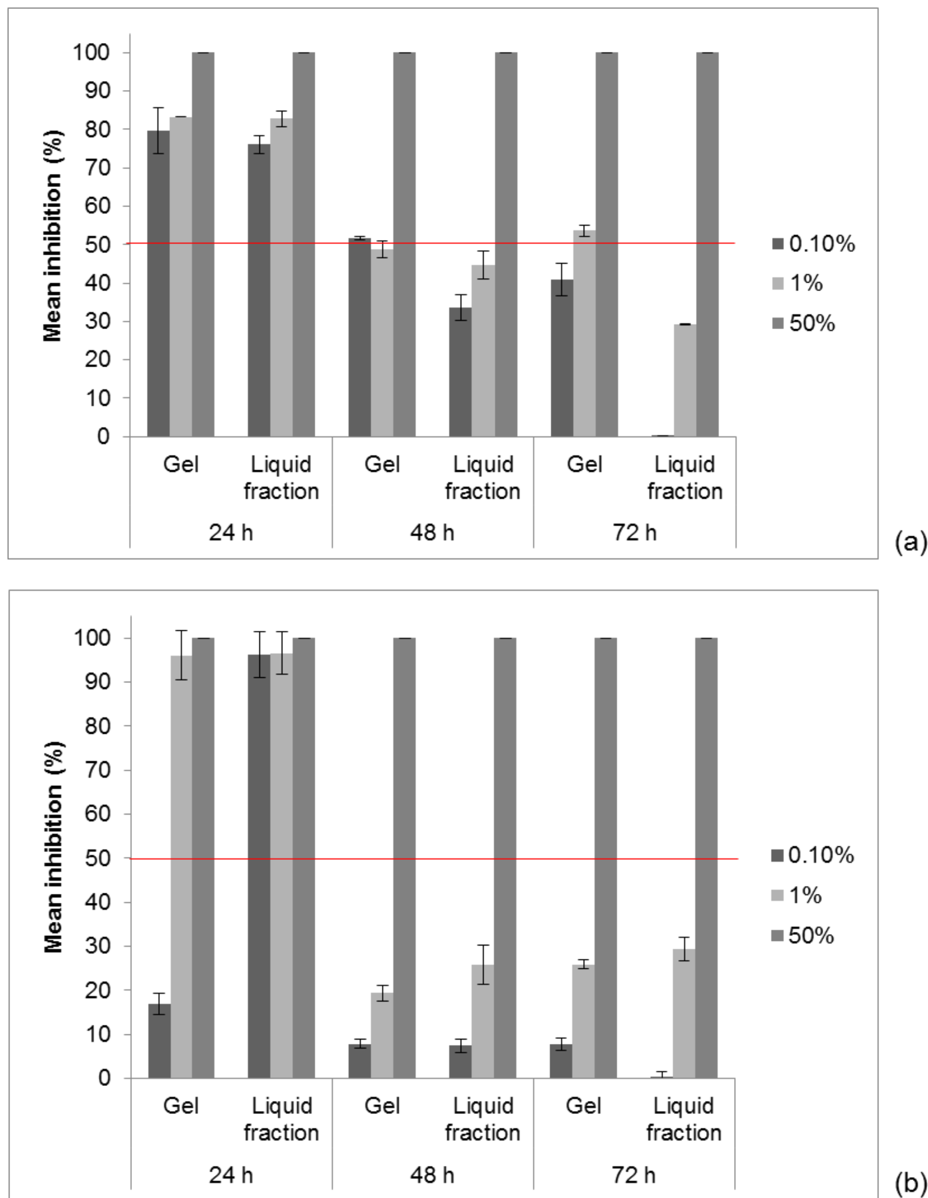

Figure 3-1. Mean inhibition effect (%) of gel and liquid fraction on a) *Penicillium expansum* and b) *Botrytis cinerea* for 24, 48 and 72 h of incubation.

The antifungal effect of ethanolic and aqueous extracts of *A. vera* bagasse (EE-B and AE-B, respectively) is presented in Figure 3-2. Both extracts have shown concentration-dependent antifungal activity. The aqueous extract has shown an inhibition effect of up to 50% at 24 and 48 h on both fungi, but no effect was observed at 72 h on *B. cinerea*; whereas on *P. expansum* the inhibition decreased at 72 h (40 – 50%). The AE-B extract showed a lower inhibitory effect, which might be associated with the extracting capacity of the solvent (water) and the

concentration of phytochemicals recovered in the extract. Several studies have reported that aqueous extracts do not have large inhibition against fungi, since most of the active phytochemicals are better dissolved in alcoholic solvents than in water (Moorthy et al., 2013). EE-B presented higher inhibition (up to 50%) than AE-B and this effect was maintained during 72 h, suggesting a better stability of this extract. EE-B showed also a similar effect on both phytopathogenic fungi. It has been demonstrated that the effectiveness of the extracts depends on the nature of the solvent, as well as on the plant species and on the fungus evaluated (Jasso de Rodríguez et al., 2011; Ali et al., 1999). The results obtained can point to the conclusion that the use of ethanol as solvent allows higher recovery of polyphenolic molecules (Table 3-3) with strong antifungal activity from the bagasse of *A. vera*.

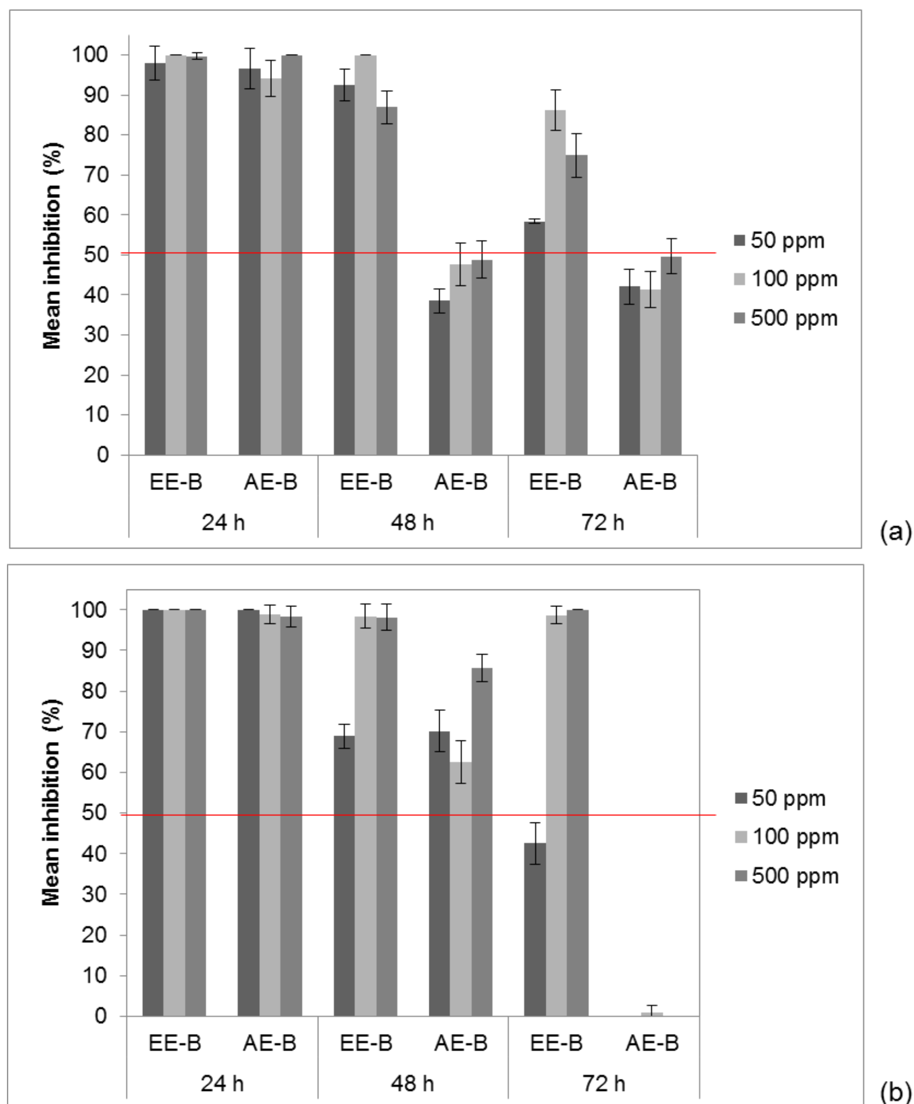

Figure 3-2. Mean inhibition effect (%) of ethanolic (EE-B) and aqueous (AE-B) bagasse extracts on a) *Penicillium expansum* and b) *Botrytis cinerea* for 24, 48 and 72 h of incubation.

3.4 CONCLUSION

Gel and liquid fraction from *A. vera* and the resulting bagasse of the separation process were separately characterized and their antifungal and antioxidant properties were evaluated. Also, ethanolic and aqueous extracts from bagasse were obtained. Glucose and mannose were the main sugars present in the three *A. vera* fractions, in a relation of *ca.* 1:1. Bagasse was characterized by the presence of lignin and higher content of ashes; in addition, it presented uronic acids related with pectic polysaccharides. The main difference between gel and liquid fraction

was the occurrence of higher amounts of uronic acids and malic acid in the gel. The liquid fraction presented a significantly better IC_{50} than the gel, and this can be attributed to the higher amounts of the *TPC*. In general the gel, liquid fraction and bagasse extracts presented high antioxidant activity, being that the ethanolic extract of bagasse reported the highest activity among all the extracts tested. Antifungal activities against *P. expansum* and *B. cinerea* were exhibited for both *A. vera* fractions (gel and liquid), being concentration-dependent and varying according to the fungus genera. For bagasse extracts the inhibition effect also was concentration-dependent, where EE-B has shown a better antifungal activity than AE-B, which may be related with the higher amount of *TPC* detected in EE-B.

A. vera fractions can represent an interesting natural alternative for formulations aiming at controlling phytopathogenic fungi in industrial crops during pre- and postharvest stages. This is the first scientific report of the phenolic composition and antioxidant activity of the liquid fraction of *A. vera* and antioxidant and antifungal activity of bagasse.

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Chapter 4

NANO-LAMINATE COATINGS SELECTION AND EVALUATION OF THEIR APPLICATION CONDITIONS ON TOMATO FRUITS

The composition of nano-laminate coatings and the application conditions were defined in this chapter. The influence of glycerol, Tween 80 and polysaccharide concentration on sodium alginate and chitosan coatings properties was evaluated by determination of their wettability on tomato surface. Subsequently the coatings were functionalized by incorporation of *Aloe vera* liquid fraction, and their properties were evaluated in terms of wettability and zeta potential (ζ-potential). The application of coatings and their deposition on tomato surface were evaluated by means of contact angle measurements.

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4.1 INTRODUCTION

Tomato fruit (*Lycopersicon esculentum* Mill.) is a climacteric fruit, thus the hormone ethylene is required for normal fruit ripening being a trigger of a wide range of physical, physiological and biochemical changes that make tomato fruit attractive for consumption (Zapata et al., 2008). The conversion of tomato fruit from the mature green to the fully ripe stage involves these changes that subsequently affect the quality parameters such as colour, texture and flavour; such changes can occur both during development on plant and after harvesting (Athmaselvi et al., 2013).

After harvest, ripening continues and tomato fruits can became overripe very rapidly, affecting their quality and restricting their shelf life (Batu, 2004). The major limiting factors in the storage of tomato fruits are transpiration, fungal infection, acceleration of the ripening process and senescence. The use of low temperature storage is effective in delaying and/or reducing ethylene production; however, tomato fruits are sensitive to chilling injury which causes damages in their quality; this occurs below 12.5 °C and leads to great losses in the fruit quality (Ali et al., 2010; Zapata et al., 2008). Since the production of ethylene accelerates biochemical and physiological changes during ripening, leading to senescence, any tool that prevents ethylene biosynthesis and/or action would delay the quality losses and in turn increase the tomato postharvest shelf life. Besides the use of low temperature storage, very few postharvest technologies have been introduced to maintain tomato quality parameters. Some treatments include the use of modified atmosphere packing and 1-methylcyclopropene (Domínguez et al., 2016).

Recently, the development of nano-laminate coating systems based on polysaccharides has brought new alternatives to conventional films and coatings to extend the shelf life of fruits and vegetables. The use of nanoscale could improve the performance of edible films and coatings, introducing new properties, particularly the possibility of combining different layers in order to confer diverse functionalities (e.g. antimicrobial and antioxidant) with improved and suitable gas barrier characteristics (Flores-López et al., 2015; Medeiros et al., 2012b).

The use of the layer-by-layer (LbL) deposition technique allows the development of nano-laminate coatings with functional thin films (<1 μm thick). These thin films are built by alternately exposing a substrate (e.g. a produce) with molecules or particles with positive and negative charges (Zhong et al., 2006). One of the most used polysaccharides in multilayer construction is chitosan, which is a very interesting polymer for numerous applications due to its biocompatibility, biodegradability and nontoxicity, as well as its antimicrobial activity. As a cationic polymer, chitosan may be associated with anionic polyelectrolytes such as sodium alginate, leading to the formation of a polyelectrolyte multilayer. Sodium alginate is a linear block of copolymers composed of varying proportions of 1,4-linked β-D mannuronic acid (M) and α-L-guluronic acid (G), forming regions of M-blocks, G-blocks and blocks of alternating sequence (MG-blocks). They have the ability to form gels in the presence of multivalent counterions, which is a direct consequence of the fact that alginates are polyelectrolytes (Draget et al., 1998).

The effectiveness of the nano-laminate coatings for fruits and vegetables, as well as for conventional edible coatings, depends mainly on the control of the wettability of the coating solutions. It affects the coating thickness, and also its permeability and mechanical resistance (Park, 1999). Also, the surface energy or surface tension is a controlling factor in the process involving wetting and coating of surfaces (Karbowiak et al., 2006). The determination of the surface tension typically comprises the measurement of the contact angles that several standard liquids make with that surface. The energy of the solid surface is then related to the surface tensions of the liquids and the contact angles. This method allows an estimation of the critical surface tension of the studied surface, by extrapolation from the Zisman plot (Zisman, 1964).

The application of the layers forming the nano-laminate coating involves wetting of the produce to be coated by the coating solutions, possible penetration of the solution into the skin, followed by a possible interaction between the produce and the successive layers. The wetting stage is very important, because if the suitability of the coating for the object to be coated is optimal, the time required for this

operation is minimal, which may cause spontaneous spreading of the coating solution (Flores-López et al., 2015).

As far as we know, nano-laminate coatings have not yet been applied at industrial scale, although recent studies reported by Souza et al. (2015) and Medeiros et al. (2012a and 2012b) showed a successful application on cheese and fruits at laboratory scale. Nevertheless, the application conditions (e.g. immersion time, number of layers, drying conditions) of nano-laminate coatings have not been evaluated in foods.

The aim of this chapter was to define the adequate formulations for the construction of a nano-laminate coating, obtained by means of the LbL deposition technique, assembled directly on the tomato fruit skin with alternate layers of alginate and chitosan. The wettability of the coating solutions was evaluated for different concentrations of polysaccharide (alginate and chitosan), plasticizer (glycerol) and surfactant (Tween 80). Then the functionalization of the nano-laminate coating was conducted by the incorporation of *Aloe vera* liquid fraction in the selected coating solutions.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Sodium alginate was obtained from Manutex RSX (Kelco International, Ltd., Portugal) and chitosan (91.23% deacetylation degree and high molecular weight) was purchased from Golden-Shell Biochemical Co., Ltd (China). Lactic acid with 90% of purity was obtained from Sigma-Aldrich (Germany). Glycerol was supplied by Panreac (Spain) and Tween 80 was purchased from Acros Organics (Belgium).

Tomatoes (*Lycopersicum esculentum* Mill.) at turning-pink stage of ripening according the USDA standard tomato colour classification chart (USDA, 1991) were purchased from a local supermarket in Braga, Portugal. The fruits were visually selected for uniformity in size, colour and absence of fungal infection, and were kept at 6 °C until use.

4.2.2 Selection of nano-laminate coatings

4.2.2.1 Materials coatings forming solutions and preparation

Coating formulations were based on a three-level factorial design as displayed in Table 4-1. Alginate solutions were prepared by dissolving alginate in distilled water through stirring at room temperature (20 °C), until complete dissolution. Afterwards, glycerol and Tween 80 were added to the solution and stirred more 2 h. The pH of the forming solution was adjusted to 7.0 with a solution of 1 mol L^{-1} NaOH. Chitosan, at the respective concentration, was dispersed in an aqueous solution of lactic acid (1.0%, v/v) and left under magnetic stirring overnight at room temperature (20 °C). Finally, the respective glycerol and Tween 80 concentrations were added to the solution and stirred during 2 h at room temperature (20 °C), and the chitosan solution was adjusted to pH 3.0 with a 1 mol L^{-1} lactic acid solution.

Table 4-1. Factors and levels used to define the concentrations of coating formulations.

Factors	Levels (%)		
Polysaccharide*	0.2	0.4	0.6
Glycerol	0.0	0.05	0.1
Tween 80	0.0	0.05	0.1

*Polysaccharide: alginate and chitosan

4.2.2.2 Preparation of specimens for contact angle measurements

Tomatoes were selected for their uniformity, size, colour and absence of damage and fungal infection. Before testing, tomatoes were immersed into sodium hypochlorite solution at 0.05% (v/v) for 3 min, and then were left to dry at room temperature (20 °C). Thin portions of the outer surface (skin) of tomato were cut with a sharp knife and adhered to a glass plate (8 cm diameter).

4.2.2.3 Wettability and surface tension

In order to obtain a uniform spreading on tomato surface, the spreading coefficient (W_s) (Eq. 4-1) needed to be determined. The wettability of the tomato surface was

determined for each coating formulation, expressed in terms of W_s, and thus the best formulation was selected.

$$W_s = W_a - W_c \quad \text{Eq. 4-1}$$

While the forces of adhesion (expressed as the work of adhesion, W_a) (Eq. 4-2) favour the spreading of the liquid (i.e. alginate- or chitosan-based formulations) on the solid surface (i.e. tomato), the forces of cohesion (expressed as the work of cohesion, W_c) (Eq. 4-3) promote its contraction, thus acting against spreading.

$$W_a = \gamma_L (1+ \cos(\theta)) \quad \text{Eq. 4-2}$$

$$W_c = 2\ \gamma_L \quad \text{Eq. 4-3}$$

W_s was obtained by measuring the contact angle (θ) between the coating solutions and the fruits' surface and the surface tension (γ_L) of the coating solutions. The contact angle at the tomato surface was measured by the sessile drop method, and observed with a contact angle meter (OCA 20, Dataphysics, Germany). Each coating solution was taken with an automatic piston 500 µL syringe (Hamilton, Switzerland) with a 0.75 mm diameter needle. The contact angle at the tomato surface was measured with the help of computer aided image processing using a digital camera. To avoid changes on the tomato surface, measurements were made in less than 45 seconds. Fifteen replicates of contact angle were obtained at 20 (±1) °C, for each formulation.

Surface tension values (γ_L) of each coating solution were determined according to the Ring method described by Gudiña et al. (2012). A Krüss K6 tensiometer (Krüss GmbH, Germany) equipped with a 1.9 cm De Noüy platinum ring was used. All the measurements were performed in triplicate at room temperature (20 °C).

4.2.2.4 Functionalization of nano-laminate coatings with Aloe vera

The functionalization of the best coating solutions (selected based on wettability results) composed by chitosan and alginate was conducted by adding the liquid fraction of *A. vera*. In order to clarify, the concentration of *A. vera* liquid fraction

used was selected according to, on the one hand, its range of bioactivity (antifungal and antioxidant; please see Chapter 3 (Sections 3.3.3 and 3.3.4) and, on the other hand, the amount of alginate and chitosan used in the solutions in order to obtain a polysaccharide:*A. vera* liquid fraction ratio of 1:1.

The effect of addition of *A. vera* liquid fraction on the W_s of the coating solutions was determined by measuring the contact angle and surface tension of the functionalized solutions as described in Section 4.2.2.3.

4.2.2.4.1 Zeta potential

The zeta potential (ζ-potential) of each coating solution was determined using a particle micro-electrophoresis instrument (Zetasizer Nano ZS-90, Malvern Instruments, UK). Each sample was placing into disposable capillary cells (DTS 1060, Malvern Instruments) for the measurements at room temperature (Silva et al., 2015). Also, the effect of addition of *A. vera* liquid fraction on the coating solutions was evaluated. Three replicates were conducted.

4.2.3 Application of nano-laminate coating on tomato fruits

The hydrophobicity/hydrophilicity of each layer were measured by means of contact angle analysis. For the application of coating solutions, alginate (anionic) or chitosan (cationic), tomatoes were immersed during 10 s in each coating solution. This time was selected based on previous works (Singh et al., 2010; Olivas et al., 2007) and the suitability to be applied in industry (i.e. lower times of application are preferred). Subsequently, tomatoes were rinsed with distillated water at pH = 7.0 or 3.0 after alginate or chitosan layer deposition, respectively. A drying step was conducted between each layer at 30 °C for 20 min in an oven with air circulation (Binder, USA). Also, the drying time was considered based on previous works (Olivas et al., 2007).

Contact angle was measured after application of each layer as described previously in Section 4.2.2.3 with some modifications. A 2 µL droplet of ultra pure water was placed on the horizontal tomato surface with a 500 µL syringe.

Measurements were made after 15 s. Fifteen replicates of contact angle measurements were performed at 20 (±1) °C.

4.2.4 Statistical analyses

Data analyses were performed using Statistica software (release 7, edition 2004, Statsoft, Tulsa, OK, USA). Pareto charts were drawn to express virtually the statistical significance of each factor.

4.3 RESULTS AND DISCUSSION

4.3.1 Alginate and chitosan coating forming solutions

4.3.1.1 Wettability

According to Zisman (1964), systems having a surface tension lower than 100 mN m^{-1} are low-energy surfaces. Tomato is a produce with reported values of polar and dispersive components of 3.04 and 25.67 mN m^{-1}; the surface tension corresponds to the sum of the two components (28.71 mN m^{-1}). Therefore, tomato has a low-energy surface able to interact with liquids mainly through dispersion forces (Casariego et al., 2008). The compatibility between tomato surface polarity and coatings has an important role in the wettability of the surface, because after drying, coating should have an appropriate adhesion, cohesion and durability.

The wettability of the coating solutions was evaluated by measuring the values of spreading coefficient (W_s). It is a fact that the closer the values of W_s are to zero, the better the surface will be coated (Martins et al., 2010b). Figures 4-1a and 4-1b show the Pareto charts of effects for polysaccharide, surfactant and plasticizer concentrations on the W_s of the coating forming solutions on tomato surface. Polysaccharide concentration was the parameter that most influenced W_s values. For the cationic layer, higher chitosan concentrations allow a better spreadability on the tomato surface; meanwhile for alginate the behaviour was opposite, since lower alginate concentrations favoured the spreadability, in both cases, also the plasticizer and surfactant concentration influenced W_s values (Figure 4-2).

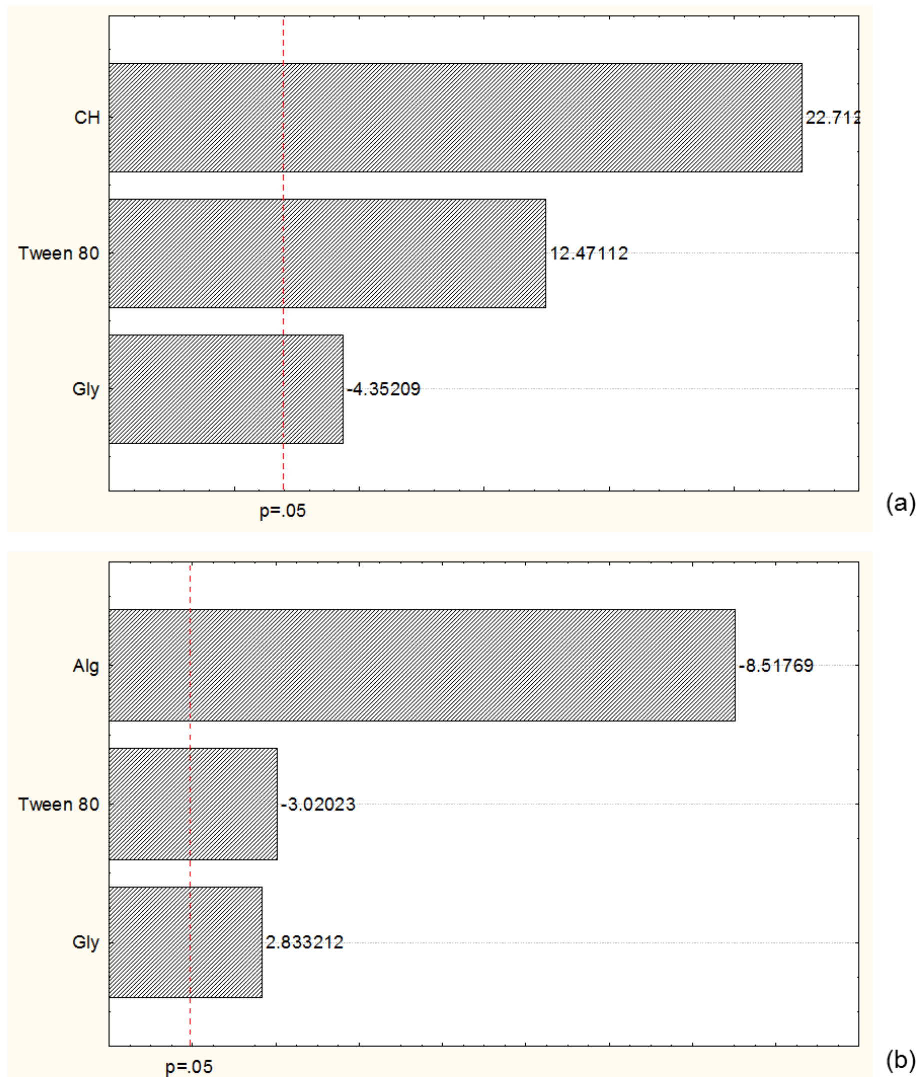

Figure 4-1. Pareto charts of the effect of the surfactant (Tween 80), plasticizer (glycerol, Gly) and polysaccharide concentration (a: chitosan, b: alginate) for spreading coefficient (W_s).

The pH of the solutions plays an important role in the interaction between the coating solutions and produce surface, as well as with the sequentially adsorbed layers, being one of the most influencing parameters on the thickness of the formed film (Martins et al., 2010a). Lower pH values (below pH 5.0) of chitosan solution led to an increase of the charge density due to the protonation of the amine groups ($–NH_3^+$); presumably this fact favoured the interaction between the chitosan solution (pH 3.0) and tomato surface, once a greater number of amine groups are available in the solution with higher chitosan concentration evaluated (0.6% w/v) (Figure 4-1a). Conventional coatings such as those reported by

Casariego et al. (2008), used an optimal chitosan concentration to coat tomato and carrot of 1.5% (w/v), presenting W_s values ranging between –57.64 and –48.97 mN m^{-1}; whilst Vieira et al. (2016) used a concentration of 0.5% (w/v) to coat blueberries with W_s values of –46.61 mN m^{-1}.

The protonation of alginate has been reported as being stable at pH values ranging from 5 to 8 (Harnsilawat et al., 2006); therefore the anionic carboxylic ($–COO^-$) groups present in the alginate-based coating solution evaluated here (pH 7.0) are able to interact with opposite charges present on tomato's surface. It has been reported that the tomato surface is mostly composed by cuticular waxes, which are a complex mixture of linear hydrocarbons of long-chain aldehydes, alcohols, acids and esters as well as of sterols and triterpenes with the same polar groups (Bauer et al., 2004; Vogg et al., 2004). The results obtained have shown that the lower alginate concentration used (0.2%, w/v) allows better values of W_s (closer to zero), possibly due to a lower presence of positive charged groups on tomato surface (Figure 4-1b).

The incorporation of glycerol and Tween 80 in both coating solutions had a positive effect on the values of W_s (Figure 4-2 and 4-3). The highest concentrations of Tween 80 and glycerol evaluated (0.1%, w/v) lead to an increase of W_s in chitosan solutions on tomato surface (Figure 4-2). The best values of W_s for chitosan solutions with the highest chitosan and Tween 80 concentrations can be explained by the "high" ratio between Tween 80 (which acts by reducing the superficial tension of the liquid) and chitosan.

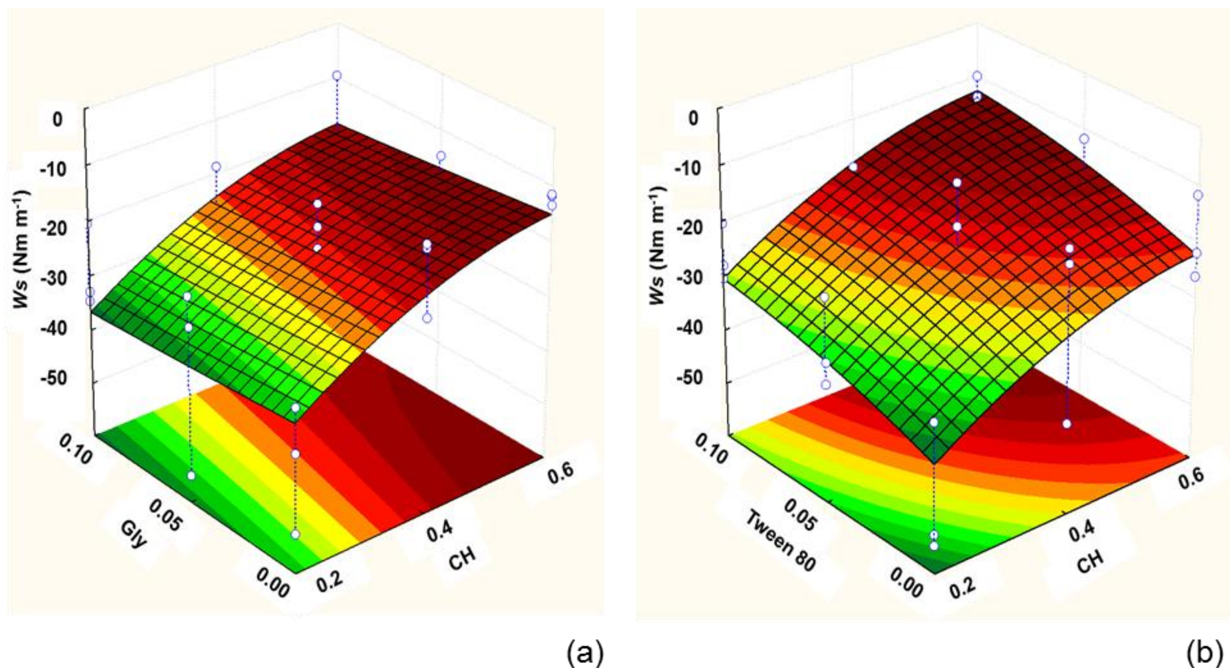

Figure 4-2. Spreading coefficient (W_s) versus chitosan (CH) and (a) glycerol (Gly) and (b) Tween 80 concentrations on tomato fruits.

For alginate solutions, central concentrations of both additives (0.05%, w/v) have shown a better affinity to the tomato surface (Figure 4-3). The objective of the addition of plasticizers to a coating is to improve its mechanical properties once it is formed; this is because the plasticizer decreases the attractive intermolecular forces in the molecular tridimensional organization, and increases the chain mobility, thus imparting to the coating a more flexible structure when dried.

The incorporation of glycerol into the chitosan and alginate coating solutions, in the presence of Tween 80, probably will form micellar structures, thus the interaction between the polyelectrolyte and glycerol is made through the hydrophilic and hydrophobic parts of the Tween 80 molecule, respectively; this will contribute to the increase of the superficial tension of the liquid once Tween 80 molecules are occupied in the micelles and are no longer available to reduce the superficial tension of the liquid (Cerqueira et al., 2009). As result an improved compatibility between the solutions and the tomato surface can occur (Martins et al., 2010b).

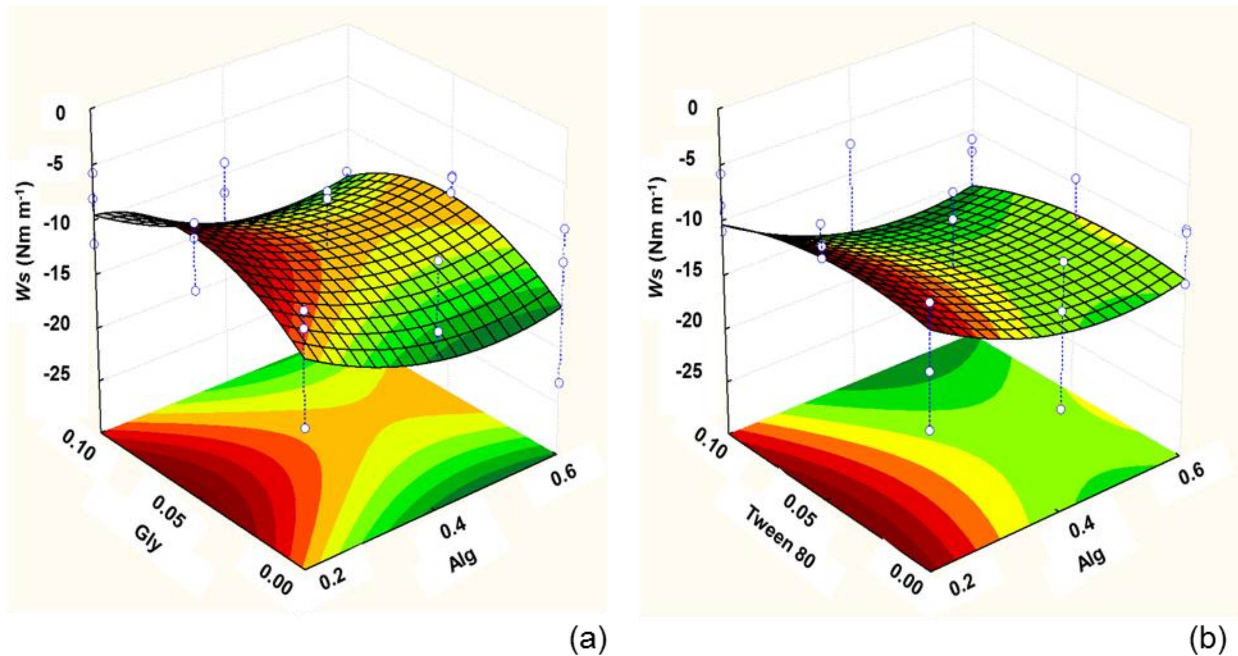

Figure 4-3. Spreading coefficient (W_s) versus alginate (Alg) and (a) glycerol (Gly) and (b) Tween 80 concentrations on tomato fruits.

In order to select the best coating forming solutions statistical analyses of W_s values were conducted. From a total of 27 chitosan coating solutions and 27 alginate coatings solutions (Annexes 3 and 4), we selected solution 27: chitosan, 0.6% (w/v), Tween 80, 0.1% (w/v) and glycerol, 0.1% (w/v) with a value of W_s of –9.75 ± 2.48; and solution 32: alginate, 0.2% (w/v), Tween 80, 0.05% (w/v) and glycerol, 0.05% (w/v) with value of W_s of –4.98 ± 0.64 (Table 4-2).

4.3.2 Functionalization of nano-laminate coatings with Aloe vera

In order to evaluate the suitability of the selected coating forming solutions to interact through electrostatic interactions, the charge of each solution was confirmed by the determination of ζ-potential (Table 4-2). The ζ-potential value for alginate solutions was found to be –60.40 ± 4.20 mV at pH = 7.0, lower and oppositely charged in comparison to that found for chitosan solution (65.40 ± 3.70 mV) at pH = 3.0. This condition was enough to support the electrostatic self-assembly between chitosan and alginate.

The functionalization of the coating was conducted by adding the *A. vera* liquid fraction to the coating forming solutions. In order to confirm that its incorporation did not change coating properties, the W_s and ζ-potential were measured and compared with the original coating forming solutions. Table 4-2 presents the values for the coating forming solutions without and with the presence of the *A. vera* liquid fraction. Results showed that there are no statistically significant differences ($p>0.05$) between the W_s values of coating solutions with and without *A. vera* (Table 4-2). The ζ-potential of alginate coating solution with *A. vera* liquid fraction has shown an increase of the charge ($p<0.05$), probably due to partial neutralization of the carboxylic groups of alginate with some positive charged groups of *A. vera* components such as proteins (Vieira et al., 2016); however, this does not compromise the electrostatic interaction with opposite charges. No significant differences ($p>0.05$) were found in the ζ-potential of chitosan coating solution with the incorporation of *A. vera*.

Table 4-2. Effect of addition of *Aloe vera* liquid fraction on spreading coefficient (W_s) and ζ-potential values of each polyelectrolyte solutions

Coating forming solution	Spreading coefficient (W_s)	ζ-potential (mV)
Solution CH 27	-9.75 ± 2.48^a	65.40 ± 3.70^a
Solution CH 27 – liquid fraction	-9.25 ± 2.35^a	72.20 ± 4.50^a
Solution Alg 32	-4.98 ± 0.64^a	-60.40 ± 4.20^a
Solution Alg 32 – liquid fraction	-4.72 ± 0.60^a	-45.50 ± 3.30^b

[a-b]Different superscript letters indicate a significantly significant difference (Tukey test, $p<0.05$).

4.3.3 Application of nano-laminate coating on tomato fruits

Polysaccharides such as alginate and chitosan provide the possibility of film formation by the breakage of polymer segments and subsequent reforming of the polymer chain into a film matrix or gel (Cerqueira et al., 2012). This process can be achieved by evaporating a solvent permitting the formation of hydrophilic and hydrogen bonding and/or electrolytic and ionic bonds (Butler et al., 1996). This

capability allowed the interaction of both coating solutions (i.e. alginate and chitosan) with the tomato surface. The conditions of application of coatings depend on the surface to be coated, being the hydration and subsequent drying step, parameters that influence the construction of polyelectrolyte multilayer films. Hydration impacts film thickness, swellability and diffusion of the film's components; meanwhile, the drying step between layers can significantly impact on the swelling of the film between dried and wet states (Crouzier et al., 2010).

The time of immersion (10 s) and drying between layers (20 min), as well as the temperature of drying (30 °C) used in this work were established based on preliminary tests (data not shown). These conditions were selected in order to facilitate the future scale-up of the process and application at the industrial level. The deposition of successive layers of functionalized alginate and chitosan on tomato surface was evaluated by means of contact angle measurement. Figure 4-4 shows the values of contact angle, where high values represent a hydrophobic surface and low values indicate a hydrophilic surface (Medeiros et al., 2012b). The contact angles with significantly ($p<0.05$) smaller magnitude were obtained for the layers assembled at pH = 7.0 (Alg) being the higher contact angles obtained for the layers assembled with the chitosan solution at pH = 3.0 (CH) (Figure 4-4). Results clearly showed the variation of the contact angles with the alternate deposition of Alg and CH on tomato surface, confirming that the film was progressively assembled by alternate deposition of these two polyelectrolytes. A significant decrease ($p<0.05$) in the contact angles of layer 1 and layer 3 can be observed when compared to layer 5 (alginate layers). A possible explanation could be the interaction between previously deposited layers of Alg and CH with the tomato fruit surface, in such a way that the hydrophilicity of alginate can be favoured.

Medeiros et al. (2012a) reported that in a nano-laminate coating composed by two weak polyelectrolytes, the highly charged polymer chains (pectin) tended to be absorbed as thin layers, while the less charged polymer (chitosan) chains tended to be absorbed as thicker layers. In this work, we confirmed the adsorption of alginate solution, a highly charged hydrophilic polymer, on tomato surface since the contact angle of tomato was significantly reduced ($p<0.05$) with its application.

In turn, the adsorption of chitosan solution was possible due its hydrophobicity, confirmed with the increase of contact angle when it is applied on top of the layer of alginate (Figure 4-5).

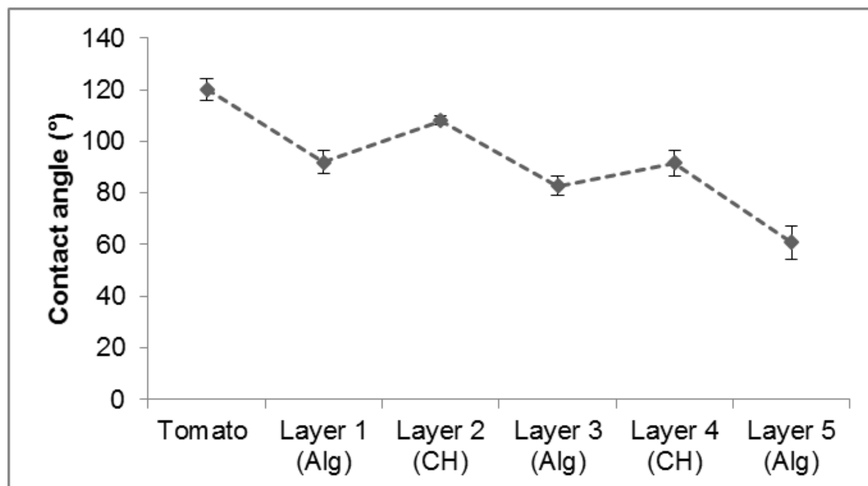

Figure 4-4. Contact angles measured after 15 s of droplet application. Results on "Tomato" correspond to contact angles measured on tomato surface and the subsequent values correspond to contact angles measured on the five successive layers applied on tomato surface containing alginate (Alg) and chitosan (CH). Each data point is the average of fifteen measurements and the *error* bars show the standard deviation.

4.4 CONCLUSION

In this chapter the best formulations for the construction of a nano-laminate coating on tomato surface were selected, based on their affinity (measured by W_s) to tomato surface. It was guaranteed the electrostatic interaction between the coating forming solutions. The functionalization with *A. vera* liquid fraction did not alter the properties of the film forming solution of both polyelectrolytes (i.e. alginate and chitosan). Also, the successful deposition of polyelectrolyte layers on tomato surface was confirmed.

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Chapter 5

CHARACTERIZATION OF NANO-LAMINATE FILMS CONTAINING CARVACROL

In this chapter, nano-laminate films with carvacrol and zein nanocapsules as bioactive compontens were built through an alternate dipping procedure and subsequently characterized. These systems have shown significantly decreased water vapour transmission rate (*WVTR*) when compared to those prepared with alginate and chitosan. In addition, the oxygen barrier properties were improved in films containing encapsulated carvacrol, while also evidencing the most significant antifungal activity against *Alternaria* sp.

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5.1 INTRODUCTION

Nanotechnology provides food scientists with a great number of ways to create novel laminate films suitable for use in the food industry. A nano-laminate consists of two or more layers of material with nanometer dimensions that are physically or chemically bonded to each other. One of the most powerful methods is based on the layer-by-layer (LbL) deposition technique, in which the charged surfaces are coated with interfacial films consisting of multiple nanolaminated films of different materials (Weiss et al., 2006; Decher and Schlenoff, 2003).

The sequential buildup of polymers via the LbL technique provides an efficient and versatile means for depositing functional polymer coatings on surfaces (Pinheiro et al., 2012; Weiss et al*.,* 2006). Thus, a variety of functional thin films can be produced using the LbL assembly technique. Thin films, typically <1 μm thick, are created by alternately exposing a substrate to positively- and negatively-charged molecules or particles. Each individual layer may be 1 – 100 nm thick depending on the linear charge density and molecular weight of the adsorbing polymers, extent of film hydration and ionic strength, temperature, deposition time, counter ion and pH of the species being deposited (Zhong et al., 2006). Some advantages when these coatings are at the nano-scale are: high stability on the substrate surface, facility of preparation (Peng et al*.,* 2001) and lower concentration of materials required (Hinrichsen et al*.,* 2003). Chitosan is a cationic polymer that has the ability to associate with anionic polyelectrolytes, such as alginate, allowing the formation of a polyelectrolyte multilayer (Fabra et al., 2016).

In addition, LbL technique could be used to encapsulate various substances (hydrophilic, amphiphilic, or lipophilic) within the nano-laminate films by incorporating them, for example, in oil droplets or association colloids (such as micelles or liposomes). Thus, it would be possible to incorporate active functional agents such as antimicrobials, antibrowning agents, antioxidants, enzymes, flavours, and colours into the films. These functional agents would increase the shelf life and quality of coated foods (Weiss et al., 2006).

Essential oils show a great potential to be used as antimicrobial compounds both when directly added to foods and via their use in the form of vapour. Many essential oils, which are hydrophobic and volatile compounds derived from plants, have shown natural antifungal, insecticidal, antimicrobial, and antioxidant properties. The inhibitory effect of carvacrol on the growth of various microorganisms is well documented and described extensively (Nostro and Papalia, 2012; Ben et al., 2006; Burt, 2004,). In fact, Zabka and Pavela (2013) have recently demonstrated that carvacrol is one of the most effective antifungal essential oils. Carvacrol is classified as Generally Recognized as Safe (GRAS) by the United States Food and Drugs Administration (FDA: Lambert et al., 2001), being one of the main components of thyme. Carvacrol might be incorporated within biopolymer materials and the efficiency of this system is determined by its (controlled) diffusion and release properties and if concentrations are kept high enough for the desired antimicrobial impact. However, the inherent volatility of essential oils could imply that the amount of carvacrol required to reach the inhibitory effect will be higher. Thus, nanoencapsulation can be applied to avoid this problem, favouring a controlled release of the active compound which could prolong the antimicrobial effect during storage.

Zein, a corn prolamine protein, is a GRAS food-grade ingredient. It has three quarters of lipophilic and one quarter of hydrophilic amino acid residues. Because of its high hydrophobicity, zein has been studied in food and pharmaceutical industries for encapsulation and sustained release of hydrophobic bioactives, such as fish oil (Zhong et al., 2009), α-tocopherol (Luo et al., 2011), vitamin D (Luo et al., 2012), curcumin (Patel et al., 2010) and thymol (Zhang et al., 2014).

In a previous work, Carneiro-da-Cunha et al. (2010) reported the ability of chitosan and alginate polyelectrolyte solutions to form nano-laminate films. The present work is a follow up of that study, where results were presented on the physical and thermal properties of a chitosan/alginate nano-laminate polyethylene terephthalate (PET) film. The aim of the present work is to analyze the effect of adding carvacrol on antifungal properties of nano-laminate films assembled through LbL technique. The effect of encapsulating carvacrol into zein nanocapsules was also compared

with those obtained by direct addition into a chitosan matrix. Thus, nano-laminate films with sodium alginate and aqueous solutions of zein nanocapsules containing carvacrol were also obtained by LbL assembly and characterized.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Films of polyethylene terephthalate (PET) were obtained from Canson (France). Sodium alginate was obtained from Manutex RSX (Kelco International, Ltd., Portugal) and chitosan (91.23% deacetylation degree and high molecular weight) was purchased from Golden-Shell Biochemical Co., Ltd (China). The 1,6-hexanediamine (> 98% of purity) and carvacrol (> 95.5% of purity) were purchased from Sigma-Aldrich (Germany). Lactic acid with 90% of purity and hydrochloric acid with 37% of purity were obtained from Sigma-Aldrich (Germany). Glycerol was supplied by Panreac (Spain). Purified zein and Tween 80 were purchased from Acros Organics (Belgium). Ethanol (> 99.8% of purity) and sodium hydroxide were obtained from Riedel-de Haën (Germany).

5.2.2 Preparation of nano-laminate film assembly

The nano-laminate films were built on aminolyzed/charged PET support films. Five alternative polysaccharide layers (alginate and chitosan) or zein/carvacrol nanocapsules were adsorbed onto the support with the sequence described below (Table 5-1).

Table 5-1. Description of the five successive layers of each multilayer system

Nano-laminate film	Support film	1st layer	2nd layer	3rd layer	4th layer	5th layer
A	A/C PET	Alg*	CH	Alg	CH	Alg
B			Z/C*		CH	
C			Z/C		Z/C	
D			CH-0.09C*		CH-0.09C*	
E			CH-0.6C*		CH-0.6C*	

A/C PET: aminolyzed/charged PET; Z/C: nanocapsules zein-carvacrol; Alg: alginate; CH: Chitosan; C: carvacrol; (*)Carvacrol concentration in the CH emulsion, 0.09 and 0.6%, respectively.

5.2.2.1 Aminolysis of polyethylene terepthalate surface

Polyethylene terephthalate (PET) films were cut into rectangular pieces of 0.8 × 5.0 cm and circular pieces of 5.0 cm of diameter and were aminolyzed as described by Fu et al. (2005). Briefly, PET films were cleaned in ethanol/water (1:1, v/v) solution for 3 h, followed by a thorough rinsing with distilled water, and dried at 30 ºC for 24 h. Afterwards, the films were immersed into 0.06 $g{\cdot}mL^{-1}$ 1,6-hexanediamine/propanol solution at 37 ºC for 4 h, thoroughly washed with distilled water to remove free 1,6-hexadiamine and finally dried at 37 ºC for 24 h. The aminolyzed PET films were treated with 0.1 HCl solution for 3 h at room temperature (20 ºC), and then washed with a large amount of distilled water, dried at 30 ºC for 24 h and termed “aminolyzed/charged PET” (A/C PET). This procedure was needed to positively charge the otherwise natural PET surface, aiming at a stronger interaction with the negatively charged alginate.

5.2.2.2 Preparation of polyelectrolyte solutions

Sodium alginate solution was prepared dissolving 0.2% (w/v) sodium alginate in distilled water and stirred, at room temperature, until sodium alginate was completely dissolved. Based on screening studies related to the spreading coefficient of these alginate solutions onto tomato surface (Chapter 4, Section 4.3.1.1), glycerol at 0.05% (w/v) and Tween 80 at 0.05% (w/v) were added as a

plasticizer and as a surfactant, respectively. This issue was considered because the affinity between the food surface and coating formulation is fundamental in the coating design, considering that the effective spreading of a coating on a food is greatly influenced by the wettability of the surface by the coating formulation. The pH of the film forming solution was adjusted to 7.0 with a solution of 1 mol L^{-1} sodium hydroxide.

Chitosan (0.6%, w/v) solution was dispersed in an aqueous solution of lactic acid (1.0%, v/v) under magnetic stirring until it was completely dissolved at room temperature. Based on the above-mentioned screening studies (Chapter 4, Section 4.3.1.1), glycerol at 0.1% (w/v) and Tween 80 at 0.1% (w/v) were added as plasticizer and surfactant, respectively. Finally, chitosan solution was adjusted to pH 3.0 with a 1 mol L^{-1} lactic acid solution.

Chitosan-carvacrol (CH-C) solutions were prepared by adding the active compound to chitosan solutions to reach a final concentration of 0.09 or 0.6% (w/v). CH-C mixtures were emulsified at room temperature using a rotor-stator homogenizer (Ultraturrax, Jankeand Kunkel, Staufen, Germany) at 10 000 rpm for 2 min. Solution nomenclature was CH-nC, being *n* the carvacrol concentration in the chitosan solution.

To clarify, the carvacrol essential oil concentrations used were selected according to, on the one hand, the encapsulation efficiency of zein-carvacrol systems (0.09%, w/v) and, on the other hand, the amount of chitosan used in the solution (0.6%, w/v) in order to obtain a chitosan:carvacrol ratio of 1:1.

5.2.2.2.1 Zein-nanocapsules development

Solutions of zein-carvacrol nanocapsules were prepared as follows: zein (2 mg mL^{-1}) was dissolved in ethanol/water binary solvent (75:25, v/v) and carvacrol was added to zein solution at a zein-carvacrol ratio of 1:0.5. After stirring overnight, zein-carvacrol nanocapsules were obtained by pumping 10 mL of zein-carvacrol solution into 50 mL of distilled water which was continuously stirred at 600 rpm. Finally, aqueous solution of zein-carvacrol nanocapsules was adjusted to pH 3.0 with 1 mol L^{-1} lactic acid solution.

5.2.2.3 *Coating procedure of A/C PET*

A/C PET pieces were immersed into the alginate solution for 20 min and subsequently rinsed with deionized water with the same pH (7.0). The samples were dried by hanging them inside a chamber with a nitrogen flow in order to speed up the process. This procedure was repeated with the other polyelectrolyte solutions (chitosan, chitosan/carvacrol) and charged nanocapsules´ suspensions based on zein/carvacrol) but, in this case, samples were rinsed with an aqueous solution at pH 3.0. This procedure was repeated with the alternate deposition of a total of five nanolayers with different sequences, as described in Table 5-1. As a result, five types of nano-laminate coatings on A/C PET films were produced and maintained at 20 ± 2 ^{o}C and 50 ± 5% of relative humidity (*RH*) provided by the laboratory air conditioning system before analysis.

5.2.3 *Physicochemical characterization*

5.2.3.1 *Zeta potential and particle size*

The zeta potential (ζ-potential) and particle size of each polyelectrolyte solution was determined as explained in Chapter 4 (Section 4.2.2.4.1).

5.2.3.2 *Contact angle measurements*

Contact angles (θ^{o}) of the original PET, A/C PET and the subsequent nano-laminate coatings´ surface were measured by the sessile drop method (Newman and Kwok, 1999), in which a droplet of ultra-pure water was placed on a horizontal surface using a contact angle meter (OCA 20, Dataphysics, Germany), equipped with an image analysis software. The water droplet (2 μL) was placed on the horizontal surface with a 500 μL syringe (Hamilton, Switzerland) with a needle of 0.75 mm diameter. Measurements were made at 0, 15 and 30 s and, for each type of surface, three film samples were used. For each film sample, seven contact angle measurements were carried out at 20.5 ± 0.3 ^{o}C.

5.2.3.3 Encapsulation efficiency

Encapsulation efficiency (*EE%*) was determined after the separation of the zein-carvacrol nanocapsules from the supernatant with free carvacrol. The separation was performed using an Amicon®Ultra-0.5 centrifugal filter device (Amicon®Ultra – 0.5 mL 3 K device, Millipore Corp., Ireland). Briefly, 0.5 mL of sample was added to the Amicon® and was centrifuged at 14,000 × *g* during 10 min. After centrifugation a filtrate with free carvacrol and a concentrate with zein-carvacrol nanocapsules were obtained. The filtrate was then assayed spectrophotometrically at 271 nm and the amount of free vitamin was determined. *EE%* was determined as follows:

$$EE\% = \frac{[Carvacrol]_{total} - [Carvacrol]_{free}}{[Carvacrol]_{total}} \cdot 100 \qquad \text{Eq. 5-1}$$

where $[\text{Carvacrol}]_{\text{total}}$ represents the total amount of carvacrol added during nanoparticles production and $[\text{Carvacrol}]_{\text{free}}$ the free carvacrol in the filtrate.

5.2.3.4 Barrier properties

*5.2.3.4.1 Water vapour transmission rate (*WVTR*)*

The water vapour transmission rate (*WVTR*) of the nano-laminate films was measured according to the ASTM E96 (ASTM, 2011) gravimetric method (Casariego et al., 2009; McHugh et al., 1993). Measurements were taken in triplicate. *WVP* was determined experimentally for the sustaining layer (A/C PET) and for the obtained nano-laminate films (multilayer A, B, C, D and E). The *WVTR* of the polysaccharide or polysaccharide/protein nano-laminate coating (without A/C PET) was determined by the following equation (Cooksey et al., 1999).

$$WVTR_B = \frac{1}{\frac{1}{WVTR_T} - \frac{1}{WVTR_A}} \qquad \text{Eq. 5-2}$$

where A, B and T refer to the sustaining layer (A/C PET), the polysaccharide-protein nano-laminate coating and the resulting nano-laminate film (A, B, C, D and E), respectively.

5.2.3.4.2 Oxygen transmission rate (O_2TR*)*

Oxygen transmission rate was determined based on ASTM (2002) method as previously described by Cerqueira et al. (2012). Briefly, the permeation cell was composed by two chambers divided by the film to be studied: either the A/C PET (used as support film) or each type of multilayer mounted on A/C PET. The permeation gas (oxygen) flowed continuously through the lower chamber, and nitrogen (as a carrier gas) was passed through the upper chamber. The flow rate of oxygen and nitrogen was maintained constant and controlled by a gas flow meter (J and W Scientific, ADM 2000, USA) to keep its pressure constant in the compartment. The permeant gas passing across the film was dragged by the carrier gas and analyzed by gas chromatography (Chrompack 9001, Middelburg, Netherlands) at 110 ºC with a column Porapak Q80/100 mesh 2m×1/8 in. × 2 mm SS equipped with a hydrogen flame ion detector. Helium (at 23 mL min^{-1}) was used as carrier gas in the chromatograph. Calibration was done with a standard mixture containing 10% CO_2, 20% O_2 and 70% N_2. The oxygen permeation rate was determined when the steady state was reached and three replicates for oxygen permeability measurements were obtained for each sample.

The O_2TR of the polysaccharide or polysaccharide/protein nano-laminate coating (without A/C PET) was determined by the following equation (Cooksey et al., 1999).

$$\mathrm{O_2TR}_B = \frac{1}{\frac{1}{O_2TR_T} - \frac{1}{O_2TR_A}} \qquad \text{Eq. 5-3}$$

where A, B and T refer to the sustaining layer (A/C PET), the polysaccharide-protein nano-laminate coating and the resulting nano-laminate film (A, B, C, D and E), respectively.

5.2.4 Microbiological analysis

5.2.4.1 Fungal strain

Stock cultures of *Alternaria* sp. (MUM 02.42) and *Rhizopus stolonifer* (MUM 10.260) were supplied by Micoteca da Universidade do Minho (MUM, Braga, Portugal). Fungus were inoculated on Potato Dextrose Agar (PDA) and incubated at 25 ºC until sporulation. The concentration of each inoculum was adjusted by means of a haemocytometer at 10^4 spores per mL.

5.2.4.2 Antifungal effectiveness of nano-laminate films

Antimicrobial effectiveness of nano-laminate films was determined by adapting the methodology used by Kristo et al*.,* 2008.

Aliquots of PDA (7 mL) infected by the fungus were poured into 50 mm Petri dishes. After solidification of the culture medium, the support films (A/C PET) and each multilayer system (A, B, C, D, E) of the same diameter as the Petri dishes (5 cm^2) were placed on the surface. Inoculated uncoated PDA was used as control. Petri dishes were then covered with parafilm to avoid dehydration and stored at 25 ºC for 30 days. *Alternaria* sp. and *Rhizopus stolonifer* counts on PDA plates were examined immediately after inoculation and periodically during the storage period. In order to do this, each sample with the agar was removed aseptically from Petri dishes and placed in a sterile plastic bag with 50 mL of tryptone phosphate water (Becton, Dickinson and Company Sparks, Le Pont de Claix, France) and homogenized for 5 min in a Stomacher blender (3500, Seward Medical, U.K.). Serial dilutions were made and then poured onto PDA-containing Petri dishes. These Petri dishes were incubated for four days at 25 ºC for evaluation of *Alternaria* sp. and *R. stolonifer* growth and colonies were counted. Antifungal properties analyses were run in duplicate.

5.2.5 *Statistical analysis*

Statistical analysis of data was performed through analysis of variance (ANOVA) using Statgraphics Plus for Windows 5.1 (Manugistics Corp., Rockville, MD). Fisher's least significant difference (LSD) procedure was also used.

5.3 RESULTS AND DISCUSSION

5.3.1 *Preparation of nano-laminate films*

5.3.1.1 *Characterization of polyelectrolyte solutions*

PET samples were previously aminolyzed and treated with HCl to charge positively the support surface (A/C PET). Thus, the first negatively charged surface layer (alginate layer) will interact with the support surface by electrostatic forces and then with the subsequent positively charged layer. In order to guarantee the electrostatic interaction with the A/C PET surface, the charge of each solution was confirmed by the determination of ζ-potential (Table 5-2). The ζ-potential value for alginate was found to be −60.40 ± 4.20 mV at pH 7.0, lower and oppositely charged in comparison to that found for chitosan (65.43 ± 3.70 mV), chitosan-carvacrol emulsions (67.60 ± 2.20 and 53.20 ± 4.50 mV) and zein-carvacrol nanocapsules (44.60 ± 1.00 mV), all of them at pH 3.0. This condition was enough to support the electrostatic self-assembly and to build the five layers, made as described in Table 5-1. The negative ζ-potential value for alginate can be explained by the free carboxyl groups present in its structure at pH 7.0 (higher that its *p*Ka that stands at 3.5) (Saravanan and Rao, 2010; Harnsilawat et al*.,* 2006). These values of ζ-potential were similar to those found by Carneiro-da-Cunha et al. (2010) for alginate (−62.13 mV at pH 7.0). At pH 3.0, the amino groups of chitosan are positively charged and the reduction of the electrical net charge (decrease in ζ-potential) with the increase of carvacrol content (only noticeable at carvacrol contents of 0.6%) could be explained by the electrostatic interactions between chitosan and the carvacrol compounds at the tested pH (pH 3.0) (Sánchez-González et al., 2011). In fact, the ζ-potential of carvacrol dispersed in distilled water (in the absence of chitosan) was −21.50 ± 1.20 mV and this negative charge

was associated with the presence of dissociable compounds in the carvacrol and with the adsorption of negative ions on the droplet surface. At this pH, the adsorption of chitosan on the droplets of carvacrol results in positively charged dispersed particles. The positive charge of amino groups of chitosan could be partially neutralized through the interaction with some negatively charged groups of the carvacrol components when adsorbed on the oil droplet surface. This charge in the surface of dispersed particles was also found by incorporation of chitosan to oil-in-water emulsions with different types of surfactants (Hou et al., 2010). It is worthwhile to note that the high value of ζ-potential (higher than + 30 mV) implied a strong surface charge of the particles, guaranteeing the action of the repulsive forces among these and thus, the stability of chitosan-carvacrol emulsions was ensured by electrostatic stabilization. Aqueous solutions containing zein-carvacrol nanocapsules were also positively charged (44.60 ± 1.00 mV), which also guaranteed the construction of nano-laminate films. This ζ-potential value was higher than that reported by Zhang et al. (2014) for zein/thymol nanocapsules obtained by freeze-drying and redispersed in deionized water (28.10 ± 0.14 mV). The encapsulation efficiency of carvacrol in zein nanoparticles was 90.0 ± 1.50% and is in agreement with results presented elsewhere (Luo et al., 2012; Luo et al., 2011).

The particle size distribution was also characterized and it was observed that chitosan-carvacrol solutions provided larger particle size diameters (2.0 ± 0.20 and 4.50 ± 0.10 μm for those CH-0.09C and CH-0.6C, respectively) than aqueous solutions of zein-carvacrol nanocapsules (115 ± 10 nm). The differences in particle size can be explained by the solubility of each protein in the aqueous solution and thus, by polymer-solvent interactions. It is well-known that the use of a solvent for a particular polymer in which it is highly soluble (which is the case of chitosan in the aqueous-acid media, pH=3), makes the polymer chains to swell and expand, favouring polymer/solvent interactions and thus, the particle size will increase. However, when a solvent of poor solubility for a particular polymer is used, polymer-polymer self-interactions preferentially occur (Luo et al., 2012). Therefore, the addition of zein-carvacrol in the antisolvent aqueous phase, which is a well-

known procedure for nanoencapsulation (Li et al., 2012), will favour intermolecular interactions and the formation of the nanoparticles.

Table 5-2. ζ-potential values of each polyelectrolyte solution used for multilayer assembly

Layer	ζ-potential (mV)
Carvacrol oil (C)	–21.50 ± 1.20[a]
Alg	–60.40 ± 4.20[b]
CH	65.43 ± 3.70[c]
CH-0.09C	67.60 ± 2.20[c]
CH-0.6C	53.20 ± 4.50[d]
Z/C	44.60 ± 1.00[e]

a-d: Different superscripts within the same column indicate significant differences among polyelectrolyte solutions (Tukey test, $p<0.05$).

5.3.1.2 Characterization of the coating procedure of A/C PET

Contact angle measurements are a useful tool to determine the hydrophobic or hydrophilic nature of film´s surface: lower values ($\theta<20^o$) are closely related with wettable surfaces, on the contrary, the hydrophobic surfaces show high values ($\theta>70^o$) of the contact angle. Thus, the deposition of successive layers onto A/C PET can be followed by contact angle measurements due to the different wettability properties of the electrolyte solutions used for nano-laminate films formation. Figure 5-1 displays contact angle values observed in A/C PET and when successive layers were added. Each data point is an average of seven determinations and the error bars represent the standard deviation. The original PET surface showed a contact angle value of 83.2 ± 1.5º which was significantly higher ($p<0.05$) than the value of 77.0 ± 1.0º found for A/C PET surface, indicating the hydrophobic nature of both ($\theta>70^o$). This difference in the contact angle values confirmed the effectiveness of aminolysis of the original PET surface. Similar results were observed by Medeiros et al. (2012 and 2013), Carneiro-da-Cunha et al. (2010) and Xu et al., (2008) for aminolyzed PET. The successful assembly of the polyelectrolytes on A/C PET films was verified by the alternating values of

contact angles. It was observed that contact angles of alternate assembling of polycation layers were higher than those of polyanion layers (e.g. alginate), illustrating that alginate was more hydrophilic than both chitosan and zein/carvacrol solutions. Comparing the polycation layers, the contact angles of adsorbed chitosan layers were much higher than adsorbed chitosan-carvacrol layers which implied a more hydrophobic structure of chitosan than the resulting chitosan-carvacrol layers. A similar behaviour was observed by Kurek et al. (2014) in chitosan-carvacrol films.

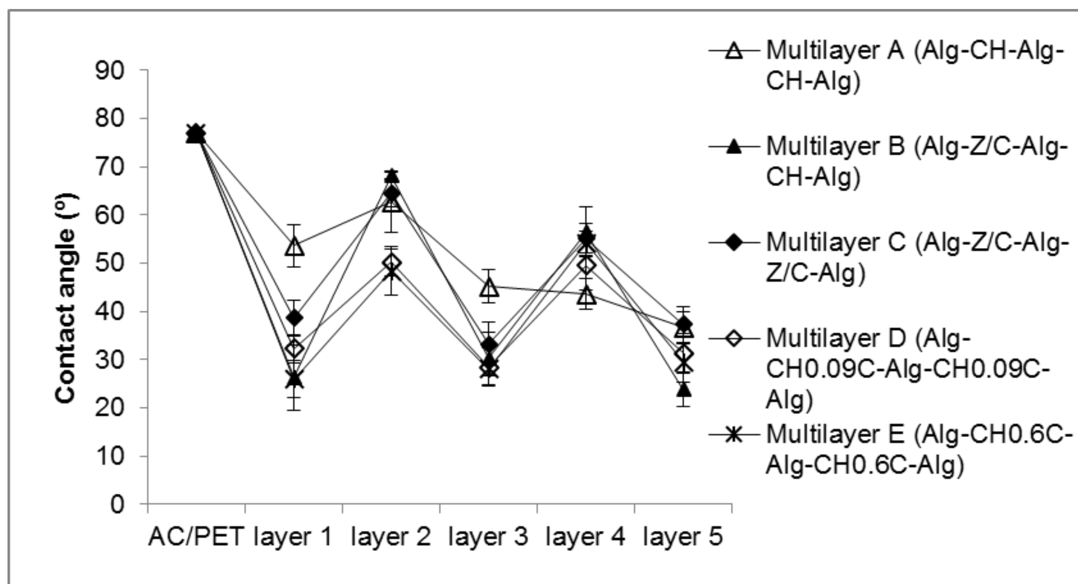

Figure 5-1. Contact angle measured on original A/C PET and on the five successive layers of each nano-laminate film system.

5.3.1.3 Water vapour and oxygen transmission rates of multilayer films

Barrier properties are one of the most important issues to be considered in coating materials since the presence of oxygen could lead to the decrease in food shelf life and quality (Li et al., 2013). Water vapour (*WVTR*) and oxygen transmission rates (O_2TR) of nano-laminate films were measured and the results are shown in Table 5-3. The *WVTR* values for the developed multilayer films were lower than those previously reported for neat alginate or chitosan films, 17 and 15 × 10^{-3} g m^{-2} s^{-1}, respectively (Jost et al., 2014; Rodríguez-Núñez et al., 2014). The decrease in *WVTR* for multilayer films could be attributed to the strong interactions established between successive alginate and chitosan layers (Jang et al., 2008). The presence

of carvacrol and zein nanocapsules greatly decreased the value of *WVTR* as compared to the multilayer system prepared with alginate and chitosan which may be due, on the one hand, to the hydrophobic nature of carvacrol and, on the other hand, to the better barrier properties of zein as compared to other proteins and polysaccharides (Fabra et al., 2014; Cho et al., 2002).

Table 5-3. Water vapour (*WVTR*) and oxygen transmission (O_2TR) rate of nano-laminate films and polysaccharide/protein multilayer

Sample	$WVTR \cdot 10^{-3}$ (g m^{-2} s^{-1})		$O_2TR \cdot 10^{-7}$ (g m^{-2} s^{-1})	
	Nano-laminate film	Polysaccharide/ protein multilayer	Nano-laminate film	Polysaccharide/ protein multilayer
AC/PET	1.31 ± 0.01^{a}		11.71 ± 0.40^{a}	
A	1.07 ± 0.17^{ab}	5.84	6.50 ± 0.48^{b}	14.61
B	0.92 ± 0.09^{b}	3.09	6.51 ± 0.65^{b}	14.66
C	0.85 ± 0.06^{b}	2.42	7.50 ± 0.60^{b}	20.76
D	0.79 ± 0.07^{b}	1.99	10.35 ± 0.70^{a}	89.12
E	0.79 ± 0.07^{b}	1.99	10.75 ± 0.81^{a}	131.13

a-b: Different superscripts within the same column indicate significant differences among samples (Tukey test, $p<0.05$).

The oxygen transmission rate behaved differently. The addition of carvacrol to the chitosan emulsion increased the value of O_2TR which can be mainly attributed to its liquid state at room temperature, favouring molecular mobility and diffusion phenomena through the lipid phase. Thus, O_2 can permeate easily through the films containing a higher amount of carvacrol (Multilayer "E"). On the other hand, previous works (Fabra et al., 2012) reported that liquid lipids such as oleic acid had a plasticizing effect on hydrocolloids matrices, which also helped to promote diffusion phenomena through the matrix, decreasing gas barrier efficiency. However, this did not occur when carvacrol was encapsulated within the zein matrix probably due to zein matrix acting as an oxygen barrier. In fact, zein has excellent potential as gas barrier in dry state (Fabra et al., 2014, 2013).

5.3.2 Antifungal activity of nano-laminate films

The mechanism of the antifungal effect depends predominantly on the ability to affect the function of cellular lipoprotein membranes, causing an impairment of cellular ionic homeostasis, acidification of vacuolar and cytosolic pH, and even the destruction of structural cellular integrity (Rao et al., 2010; Xu et al., 2008). In this work, the effect of active nano-laminate films against *Alternaria* sp. and *R. stolonifer* was analyzed. The antifungal effect of nano-laminate films against *Alternaria* sp. was determined on PDA medium and is shown in Figure 5-2. It is worth to note that, although some evaporation of carvacrol can occur during the preparation of chitosan-carvacrol polyelectrolyte solutions and nano-laminate film formation, the initial carvacrol concentration in the film-forming solution was considered for the discussion. Results obtained for *R. stolonifer* cannot be shown since the growth of this fungus is highly invasive (Odeniyi et al., 2009) and the CFU could not be counted. In a recent work, García-Rincón et al. (2010) demonstrated that chitosan application to *R. stolonifer* also affected potassium efflux, pH of the media and H+ATP-ase of the plasma membrane activity and, thus, *R. stolonifer* survival.

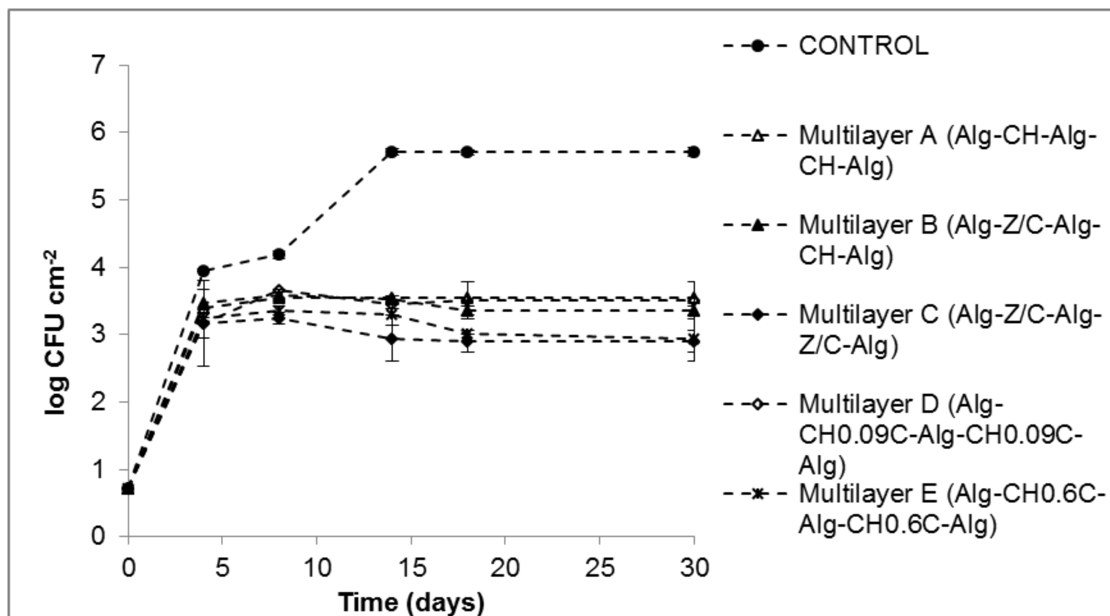

Figure 5-2. Antifungal activity of the developed nano-laminate films against *Alternaria* sp.

Nano-laminate films were shown to have antifungal properties against *Alternaria* sp.; this happened even with those obtained without carvacrol, in this case most probably due to the presence of chitosan. Similarly, Bhaskara et al. (1998) reported that chitosan affected the spore viability of *Alternaria alternata* (a fungus typically found in tomato fruit), whilst in other findings the degree of inhibition of the growth and sporulation of mycelia from this fungus isolated from tomato fruits relied on the molecular weight of chitosan (Sánchez-Dominguez et al., 2007). From SEM (Scanning Electronic Microscopy) images, these authors also observed that chitosan modified the mycelia morphology of *Alternaria altenata* causing deformation, swollenness and nodulations. In a more recent work, Sanchez-Dominguez et al. (2011) confirmed that *A. alternata* is quite susceptible to chitosan treatments, demonstrating through TEM (Transmission Electronic Microscopy) analysis that the cell wall of the fungal hyphae and conidia was seriously damaged. However, some fungi have been shown to be insensitive to chitosan activity. In this sense, Roller and Covill (1999) found, in similar studies, that the growth of *Aspergillus flavus, Cladosporium cladosporioides* and *Penicillium aurantiogriseum* was not affected by the presence of chitosan. In a more recent work, Sánchez-González et al. (2010) also showed that chitosan did not affect the growth of *P. italicum*. Thus, the effectiveness of the antifungal activity of chitosan depends on the fungus.

As commented above, nano-laminate films containing carvacrol were prepared due to their antifungal properties. Ahmad et al. (2011) and Rao et al. (2010) reported that the molecular structure and relative position of the functional groups of carvacrol are responsible for the strong ability to dissolve and accumulate in the cell membrane, resulting in cell membrane destabilization which can be attributed to a more efficient disruption of proton transfer. In fact, Ahmad et al. (2011) and Ultee et al. (2002) pointed out that the antifungal properties of these compounds are connected simply to their ability to block ATP and ergosterol synthesis.

As can be observed in Figure 5-2, the antifungal activity of nano-laminate films prepared by alternating layers of alginate with layers of nanocapsules of zein-carvacrol (Multilayer C) was in the same range ($p>0.05$) as nano-laminate films

prepared with the highest content of carvacrol (Multilayer E), probably due to the controlled release of the active agent (carvacrol) in the case of the Multilayer C which delivers more efficiently the antifungal effect, avoiding the volatilization of this compound. No significant differences (p>0.05) were found between nano-laminate films prepared with the lower amount of carvacrol (0.09%, w/w) and those containing one layer of nanocapsules of zein-carvacrol (multilayer B). The volatile nature of carvacrol could contribute to the fact that the amount remaining in the multilayer with 0.09% (w/w) carvacrol will be too low to have an antifungal effect, showing no significant differences (p>0.05) with those prepared without carvacrol (multilayer A).

Similar results were observed by Zabka and Pavela (2013), showing that carvacrol and thymol are the most promising antifungal agents against *Fusarium oxysporum, F. verticilioides, P. brevicompactum, P. expansum* and *A. flavus*.

5.4 CONCLUSION

Nano-laminate coatings on an A/C PET support were successful built-up through an alternate dipping procedure. The *WVTR* and O_2TR values for the developed multilayer films were lower than those previously reported for neat alginate or chitosan-based films. The presence of carvacrol and zein nanocapsules significantly decreased *WVTR* (up to 40%) of nano-laminate films as compared to those prepared with alginate and chitosan. However, the oxygen barrier properties of nano-laminate films containing carvacrol were only improved (up to 45%) when it was nanoencapsulated. Nano-laminate films prepared by alternating alginate with layers of nanocapsules of zein-carvacrol (Multilayer C) showed the highest antifungal activity against *Alternaria* sp., which did not significantly differ from nano-laminate films obtained with the highest amount of carvacrol (Multilayer E), probably due to the controlled release of the active agent (carvacrol) in the case of the Multilayer C which delivers more efficiently the active compound, avoiding its volatilization. Taking into account the overall properties of the developed nano-laminate films, those prepared with alternating layers of alginate and zein

nanocapsules can be proposed as a good candidate to improve the shelf life of foodstuffs due to the improved barrier properties and antifungal activity.

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Chapter 6

CHARACTERIZATION OF NANO-LAMINATE FILMS CONTAINING *ALOE VERA*

This chapter aims at the construction of chitosan/alginate nano-laminate films functionalized with bioactive fractions of *Aloe vera* and their physicochemical characterization. The nano-laminate coatings have shown good barrier properties (water vapour and oxygen transmission rate, *WVTR* and O_2TR, respectively) when compared with conventional films. The functionalized nano-laminate films evidenced antifungal activity against the growth of *Alternaria* sp.

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6.1 INTRODUCTION

The use of innovative technologies for reducing food losses is required by the agro-food industry. The application of edible coatings on food surface is a promising way to achieve this goal. Edible coatings are composed by natural polymers that provide a selective gas barrier and antimicrobial effect, among others functions, in order to improve the quality and safety of foods (Vieira et al., 2016; Lima et al., 2010). Currently, the utilization of nanotechnology represents a good tool to overcome some problems regarding the application of edible coatings, such as high water vapour permeability (*WVP*) and poor mechanical properties in comparison with synthetic materials (Medeiros et al., 2014). These coatings have advantages when they are at the nanoscale: higher stability on the substrate surface, facility of preparation and lower concentration of materials required (Fabra et al., 2016; Souza et al., 2015). Furthermore, nano-laminate coatings may incorporate functional ingredients (e.g. vitamins, antimicrobials, antioxidants, among others) at nanoscale dimension, which presumably have higher reactivity, solubility and bioavailability that the larger particles since has better access to any structure (Cerqueira et al., 2014). However, few studies have reported the application of edible coatings on foods at nanoscale level.

The construction of nano-laminate coatings can be achieved by the layer-by-layer (LbL) deposition technique, consisting in the electrostatic self-assembly deposition of successive layers on a substrate surface (Medeiros et al., 2014). This technique allows obtaining nano-laminate films based on natural polymers such as alginate/chitosan (Fabra et al., 2016) and alginate/lysozyme (Medeiros et al., 2014). The materials used for the construction of a nano-laminate film should be electrostatically charged, being suggested that those materials could have functional properties (e.g. antimicrobial and antioxidant). For this purpose, alginate and chitosan, and also, gel and liquid fraction of *A. vera* were used in the present work.

Alginate is a natural anionic polysaccharide that consists of a linear block of copolymers composed of varying proportions of 1,4-linked β-D mannuronic acid

(M) and α-L-guluronic acid (G), forming regions of M-blocks, G-blocks and blocks of alternating sequence (MG-blocks) (Olivas and Barbosa-Cánovas, 2008). Chitosan, is a cationic polysaccharide obtained from deacetylation of chitin, which is the major constituent of exoskeleton of crustaceous animals. Chitosan is nontoxic, biodegradable, biocompatible and presents antimicrobial activity (Pinheiro et al., 2015).

Recently, the potential antioxidant and antifungal properties of gel and liquid fraction of *A. vera* was reported, showing to be good alternatives to be used as chemical fungicides for phytopathogenic fungi control in industrial crops during pre- and postharvest stages (Vieira et al., 2016). The incorporation of these materials into nano-laminate coatings or films as bioactive ingredients has not been studied. The aim of this study was to develop nano-laminate films based on chitosan and alginate polyelectrolytes functionalized with *A. vera* fractions (gel and liquid fraction) and evaluate their effect on barrier (water vapour and oxygen transmission rate, *WVTR* and O_2TR, respectively) and antifungal properties.

6.2 MATERIALS AND METHODS

6.2.1 Materials

Films of polyethylene terephthalate (PET) were obtained from Canson (France). Sodium alginate was obtained from Manutex RSX (Kelco International, Ltd., Portugal) and chitosan (91.23% deacetylation degree and high molecular weight) was purchased from Golden-Shell Biochemical Co., Ltd (China). The 1,6-hexanediamine (>98% of purity) was purchased from Sigma-Aldrich (Germany). Lactic acid with 90% of purity and hydrochloric acid with 37% of purity were obtained from Sigma-Aldrich (Germany). Glycerol was supplied by Panreac (Spain). Tween 80 was purchased from Acros Organics (Belgium). Ethanol (>99.8% of purity) and sodium hydroxide were obtained from Riedel-de Haën (Germany).

6.2.2 Preparation of nano-laminate film assembly

The nano-laminate films were built on aminolyzed/charged PET support films. Five alternative polysaccharide layers (alginate and chitosan) functionalized with *A. vera* fractions (i.e. gel and liquid fraction) were adsorbed onto the support with the sequence described below (Table 6-1).

Table 6-1. Description of the five successive layers of each multilayer system

Nano-laminate film	Support film	1st layer	2nd layer	3rd layer	4th layer	5th layer
A	A/C PET	Alg	CH	Alg	CH	Alg
F		Alg/AG	Alg/AG	Alg/AG	CH/AG	Alg/AG
G		Alg/AL	CH/AL	Alg/AL	CH/AL	Alg/AL

A/C PET: aminolyzed/charged PET; Alg: alginate; CH: Chitosan; AL: *Aloe vera* liquid fraction; AG: *Aloe vera* gel

6.2.2.1 Aminolysis of polyethylene terepthalate surface

Aminolysis of polyethylene terephthalate (PET) films was performed as explained in Chapter 5 (Section 5.2.2.1).

6.2.2.2 Preparation of polyelectrolyte solutions

Polyelectrolyte solutions based on alginate and chitosan were prepared as explained in Chapter 5 (Section 5.2.2.2). The concentrations of each polysaccharide, surfactant (Tween 80) and plasticizer (glycerol) were defined in Chapter 4 according to the determination of the spreading coefficient (W_s) of the coating solutions on tomato surface.

The nano-laminate films functionalized with *A. vera* gel and liquid fraction were prepared by adding the bioactive fraction to either alginate or chitosan solutions to reach a final concentration of 0.2 or 0.6% (w/v), respectively. The mixtures were stirred during 2 h at room temperature (20 °C).

The concentration of *A. vera* fractions (gel and liquid fraction) utilized was selected according to their range of bioactivity (antifungal and antioxidant; please see Chapter 3, Sections 3.3.3 and 3.3.4) and also the amount of alginate and chitosan used in the solutions (0.2% and 0.6%, w/v, respectively) in order to obtain a polysaccharide:*A. vera* ratio of 1:1.

6.2.2.3 Coating procedure of A/C PET

A/C PET pieces were immersed into the alginate (with and without *A. vera* fractions) solution for 20 min and subsequently rinsed with deionized water with the same pH (7.0). The samples were dried by hanging them inside a chamber with a nitrogen flow in order to speed up the process. This procedure was repeated with chitosan (with and without *A. vera* fraction), but in this case the samples were rinsed with an aqueous solution at pH 3.0. This procedure was repeated with the alternate deposition of a total of five layers with different sequences, as described in Table 6-1. As result, three types of nanolaminates were produced on A/C PET films and maintained at 20 ± 2 ºC and 50 ± 5% of relative humidity (*RH*) provided by the laboratory air conditioning system before analysis.

6.2.3 Physicochemical characterization

6.2.3.1 Zeta potential, contact angle measurements, water vapour transmission rate (WVTR) and oxygen transmission rate (O_2TR)

Zeta potential and contact angle measurements were performed as described in Chapter 5 (Sections 5.2.3.1, and 5.2.3.2, respectively); *WVTR* and O_2TR were performed as described in Chapter 5 (Sections 5.2.3.4.1 and 5.2.3.4.2).

6.2.4 Microbiological analysis

6.2.4.1 Fungal strain

Alternaria sp. (MUM 02.42) was supplied by Micoteca da Universidade do Minho (MUM, Braga, Portugal). The fungus was inoculated on PDA and incubated at

25 ºC until sporulation. The concentration of inoculum was adjusted by means of a haemocytometer at 10^4 spores per mL.

6.2.4.2 Antifungal effectiveness of nano-laminate films

Antifungal effectiveness of nano-laminate films was determined by adapting the methodology used by Kristo et al. (2008). Aliquots of PDA (7 mL) infected by the fungus were poured into 50 mm Petri dishes. After solidification of the culture medium, the support films (A/C PET) and each multilayer system (A, F, G) of the same diameter as the Petri dishes (5 cm^2) were placed on the surface. Inoculated uncoated PDA was used as control. Petri dishes were then covered with parafilm to avoid dehydration and stored at 25 ºC for 14 days. *Alternaria* sp. counts on PDA plates were examined immediately after inoculation and periodically (0, 4, 8 and 14 days) during the storage period. The counts were carried out by asseptically removing the agar from Petri dishes and placing it in a sterile plastic bag with 50 mL of tryptone phosphate water (Becton, Dickinson and Company Sparks, Le Pont de Claix, France), being this mixture subsequently homogenized for 5 min in a Stomacher blender (3500, Seward Medical, U.K.). Serial dilutions were made and then poured onto PDA-containing Petri dishes. The Petri dishes were incubated for four days at 25 ºC for evaluation of *Alternaria* sp. growth and colonies were counted. The analyses were perfomed in duplicate.

6.2.5 Statistical analysis

Data analyses were subjected to analysis of variance (ANOVA) using FAUANL software (Olivares, 1994) and Statistica software (release 7, edition 2004, Statsoft, Tulsa, OK, USA).

6.3 RESULTS AND DISCUSSION

6.3.1 Preparation of nano-laminate films

6.3.1.1 Characterization of polyelectrolyte solutions

The aminolysis of PET was conducted in order to charge positively the support surface (A/C PET), promoting the interaction of the PET surface with the first layer (alginate layer). To guarantee that the interactions were possible through electrostatic forces, the charge of each of the multilayer components was confirmed by means of determination of ζ-potential value (Table 6-2). The ζ-potential value for neat alginate was found to be –60.40 ± 4.2 mV at pH 7.0, meanwhile the incorporation of gel and liquid fraction of *A. vera* to the alginate solution, significantly increases ($p<0.05$) its charge to –44.70 ± 5.30 and –45.53 ± 3.30 mV, respectively, also at pH 7.0. Positive charges present in *A. vera* fractions seem to partially neutralize the negative carboxylic groups ($–COO^-$) present in the structure of alginate at pH 7.0 (Fabra et al., 2016; Vieira et al., 2016). The electrostatic interaction was guaranteed, since neat and functionalized chitosan with gel and liquid fraction presented charges of +65.43 ± 3.70, +69.50 ± 7.30 and +72.20 ± 4.50 mV at pH 3.0, respectively, being oppositely charged to alginate solutions. The positive charges of chitosan are due to the protonation of the amine groups ($–NH_3^+$) present in its structure at pH 3.0 (Martins et al., 2010). This condition was enough to support the electrostatic interaction and promote the self-assembly to build the five layers of the nano-laminate coatings, as described in Table 6-1.

Table 6-2. ζ-potential values of each polyelectrolyte solution used for multilayer assembly

Layer / Active compound	ζ-potential (mV)
Alg	–60.40 ± 4.20[c]
CH	65.43 ± 3.70[a]
Alg-AL	–45.53 ± 3.30[b]
Alg-AG	–44.70 ± 5.30[b]
CH-AL	72.20 ± 4.50[a]
CH-AG	69.50 ± 7.30[a]

a-c: Different superscripts within the same column indicate significant differences among polyelectrolyte solutions (Tukey test, $p<0.05$).

6.3.1.2 Characterization of the coating procedure of A/C PET

Figure 6-1 shows the water contact angle of the nano-laminate films after each layer deposition. The hydrophobic character of the original PET substrate was confirmed by the high value obtained for its contact angle (83.2 ± 1.5º). This value is in good agreement with previous studies (Pinheiro et al., 2012; Carneiro-da-Cunha et al., 2010). After aminolysis, the PET surface exhibited a significantly ($p<0.05$) lower contact angle (77.0 ± 1.0º) which means that the A/C PET is more hydrophilic than the original PET. Other authors (Pinheiro et al., 2012) reported a decrease in contact angle from 78.37 to 63.21º after the aminolysis of PET. In addition, the difference obtained in contact angle between original and A/C PET confirms that the aminolysis process was successfully performed. Figure 6-2 shows the A/C PET (Figure 6-2a) and multilayer G (Figure 6-2b) films, where a slightly tubidity can be observed in multilayer G, evidencing the deposition of the layers; a scanning electron microscopy (SEM) image of multilayer G film (Figure 6-2c) is also shown, where part of the successive layers are observable at the border of the film.

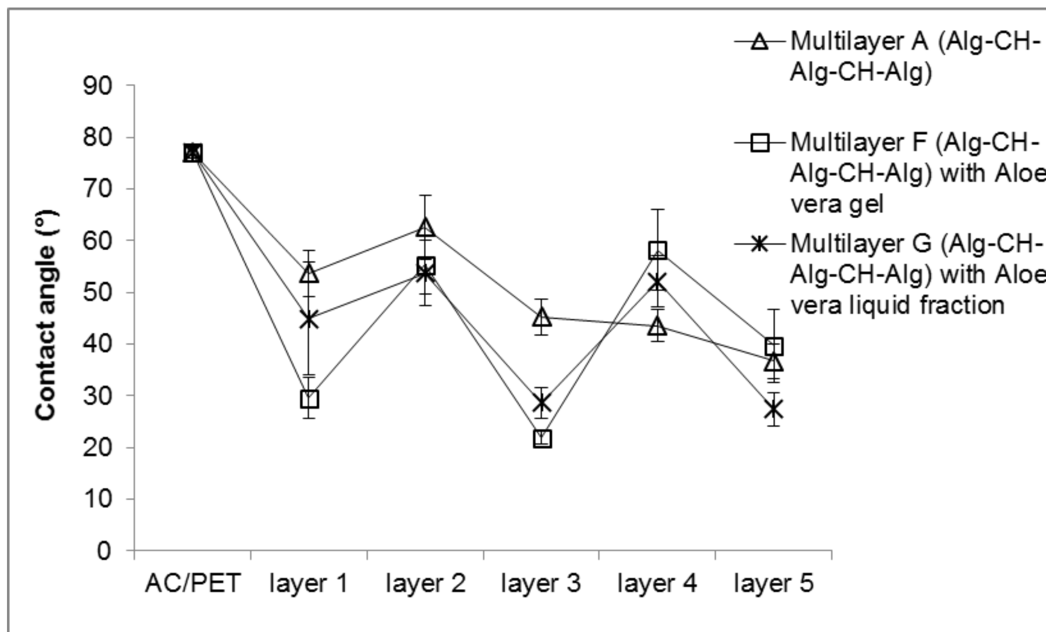

Figure 6-1. Water contact angle measured on original A/C PET and on the five successive layers of each nano-laminate film system.

The contact angle results also exhibit a distinct behaviour when the outermost layer was changed between alginate and chitosan (either functionalized or not), suggesting the alternate construction of the layers by the deposition of the nanolayers. It was observed that contact angles of alternate assembly of polycation layers were higher than those of polyanion layers (e.g. alginate), proving that alginate was more hydrophilic than both neat chitosan and functionalized chitosan solutions. These results can be explained by the higher hydrophobicity of chitosan when compared to alginate (Medeiros et al., 2012). The incorporation of *A. vera* fractions (i.e. gel and liquid fraction) did not interfere with the either hydrophilic or hydrophobic nature of alginate and chitosan, respectively. As the outermost layer is alginate, the produced nano-laminate film exhibits a contact angle of approximately 30-40°, which makes it a relatively hydrophilic coating.

Also, the differences found regarding the water contact angle on alginate and chitosan layers may also be related to other factors besides the hydrophilicity/hydrophobicity of the functional groups of the adsorbed layer. These factors may include its chemical composition, the level of interpenetration of the

outermost layer by segments of the previously adsorbed polymer layer and the swelling of the layers when in contact to water droplet (Pinheiro et al., 2012).

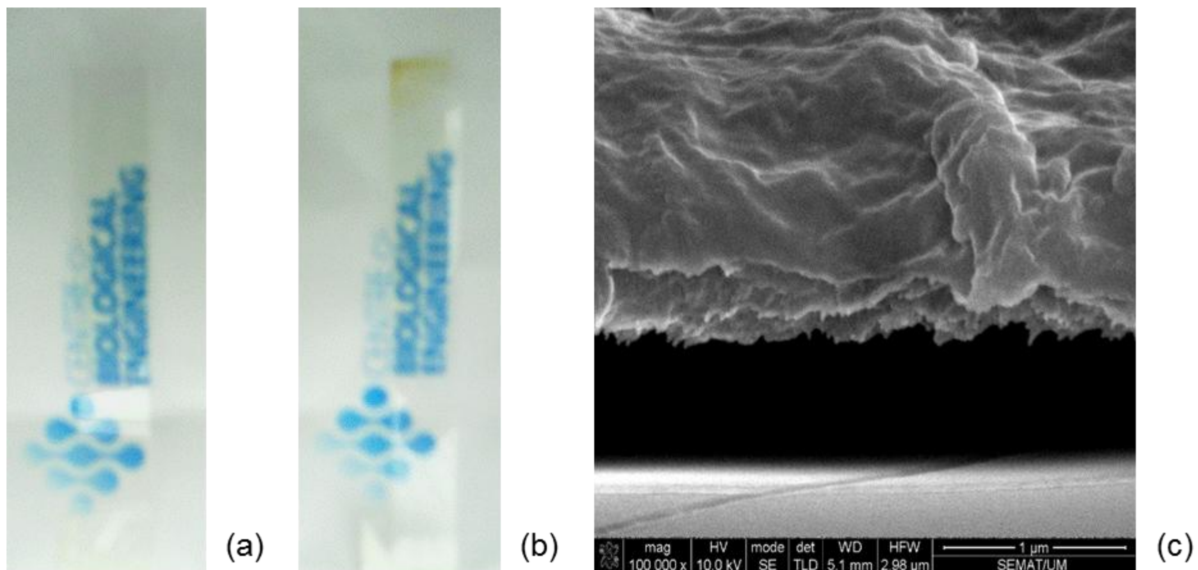

Figure 6-2. AC/PET (a), multilayer G supported on PET film (b) and SEM image of multilayer G supported on PET film (c).

6.3.1.3 Water vapour and oxygen transmission rates of multilayer films

Gas barrier properties of the nano-laminate films were evaluated, using the A/C PET film as reference. Water vapour (*WVTR*) and oxygen (O_2TR) transmission rates of nano-laminate films were measured and the results are shown in Table 6-3. One of the main functions of food packaging is to avoid or decrease water loss, therefore the *WVTR* should be as low as possible (Pinheiro et al., 2012).

The produced multilayers (A, F and G) exhibited a lower *WVTR* when compared with the values reported for conventional edible films composed of neat alginate and chitosan 17 and 15 × 10^{-3} g m^{-2} s^{-1}, respectively (Jost et al., 2014; Rodríguez-Núñez et al., 2014). *WVTR* values for the nano-laminate coatings obtained ranged between 0.87 and 1.70 × 10^{-3} g m^{-2} s^{-1}, and do not present statistical significant differences (p>0.05) when *A. vera* fractions (gel and liquid fraction) were incorporated into each system. These results are in agreement with those reported by Medeiros et al. (2014) and Pinheiro et al. (2012), 1.03 × 10^{-3} and 1.37 × 10^{-3} g m^{-2} s^{-1}, respectively, both works also reporting the case of five nanolayers' systems. The *WVTR* is strongly governed by the interaction between polymer and

water molecules. The hydrophobic character of chitosan nanolayers, supported by contact angle measurements (Figure 6-1), could explain the lower *WVTR* values of the nanolayers in comparison with the *WVTR* value of A/C PET, although these differences were not found to be statistically significant (p>0.05).

The oxygen transmission rate (O_2TR) values obtained for the nano-laminate films were found in the range between 5.85 and 7.97 × 10^{-7} g m^{-2} s^{-1} (Table 6-3), that are quite similar with the values reported by Tihminlioglu et al. (2010) that present values of 6.86 × 10^{-7} and 3.31 × 10^{-5} for corn zein and polypropylene films, respectively. The multilayer systems obtained (A, F and G) have shown significantly lower (p<0.05) O_2TR values when compared with the AC/PET films; however, the presence of *A. vera* fractions did not induce differences when compared with the multilayer used as reference (multilayer A).

WVTR and O_2TR results may also be related to the interactions existing between the five nanolayers that exhibit a compactly packed network structure formed by the polymeric matrix, which possibly increases tortuosity and thus restricts the water and oxygen molecules' transport (Medeiros et al., 2014; Medeiros et al., 2012). Also, this restriction can be related to the fact that both water and oxygen molecules must pass through the several interfaces established between the multilayer systems (Medeiros et al., 2012; Pinheiro et al., 2012).

Table 6-3. Water vapour (*WVTR*) and oxygen transmission (O_2TR) rate of nano-laminate films and polysaccharide multilayer

Sample	*WVTR* · 10^{-3} (g m^{-2} s^{-1})		O_2TR · 10^{-7} (g m^{-2} s^{-1})	
	Nano-laminate film	Polysaccharide/ Multilayer	Nano-laminate film	Polysaccharide/ multilayer
AC/PET	1.31 ± 0.01^a		11.71 ± 0.40^a	
A	1.07 ± 0.17^a	5.84	6.50 ± 0.48^b	14.61
F	0.87 ± 0.16^a	2.60	7.97 ± 0.84^b	24.93
G	1.70 ± 0.40^a	n/a	5.85 ± 0.50^b	11.69

a-b: Different superscripts within the same column indicate significant differences among samples (Tukey test, p<0.05).

6.3.2 Antifungal activity of nano-laminate films

The effect of functionalized nano-laminate films against *Alternaria* sp. was analyzed and compared with a system of reference (multilayer A) and an absolute control (Figure 6-3). Nano-laminate films were shown to have antifungal properties against *Alternaria* sp. The multilayer systems with gel and liquid fraction of *A. vera* (multilayer F and G, respectively) shown a significant inhibition (p<0.05) of *Alternaria* sp. at the fourth and eighth day, respectively, when compared with the multilayer of reference (A) and the control. The antifungal activity of both *A. vera* fractions has been reported by Vieira et al. (2016) and Jasso de Rodríguez et al. (2005). This antifungal activity is maintained, although this behaviour becomes similar to the multilayer A at the end of the test; presumably the antifungal activity of the *A. vera* fractions is not sustained after the eight day of the test. As shown in Chapter 3, the antifungal activity of *A. vera* fractions is associated with the phenolic content, being also concentration-dependent, and can vary according to the fungal genera.

On the other hand, a possible synergistic effect between chitosan and *A. vera* fractions can be expected. The antifungal effect of chitosan has been extensively reported in the literature and depends on fungal genera (Sánchez-González et al., 2011). Recently, Vieira et al. (2016) reported the decrease of contamination of blueberries by the application of a conventional chitosan coating with the incorporation of *A. vera* liquid fraction. This inhibition effect was explained by the authors as being the result of the combination of chitosan and *A. vera* liquid fraction.

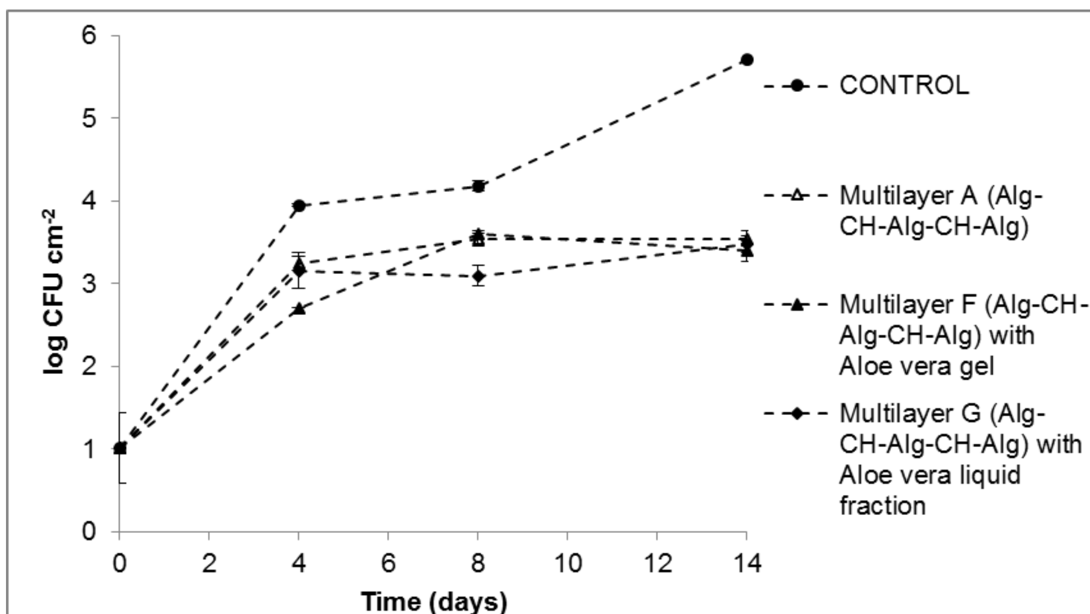

Figure 6-3. Antifungal activity of the developed nano-laminate films against *Alternaria* sp.

6.4 CONCLUSION

Nano-laminate coatings on an A/C PET support were successful built through the LbL deposition technique. The *WVTR* and *O_2TR* values for the developed multilayer films were lower than those previously reported for neat alginate or chitosan-based films. The incorporation of *A. vera* fractions (gel and liquid fraction) did not show significant differences of the *WVTR* values of nano-laminate films when compared to those prepared with alginate and chitosan and AC/PET. The oxygen barrier properties of nano-laminate films containing *A. vera* fractions were improved (up to 30%). Nano-laminate films prepared by incorporation of gel and liquid fraction of *A. vera* (Multilayers F and G) showed the highest antifungal activity against *Alternaria* sp. at the fourth and eight day of test, respectively. These systems can be recommended as an alternative to the conventional coatings with good barrier and potential antifungal properties.

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Chapter 7

EFFECT OF *ALOE VERA* NANO-LAMINATE COATING ON THE SHELF LIFE PARAMETERS OF TOMATO FRUITS (*Lycopersicum esculentum* Mill)

In this chapter the effectiveness of nano-laminate coatings with and without the incorporation of *Aloe vera* liquid fraction (NL-Av and NL, respectively) was evaluated on the shelf life parameters of tomato fruits and compared with uncoated tomato fruit. The evaluations were conducted under two conditions: cold storage (11 °C and 90% relative humidity, *RH*) and room temperature (20 °C and 85% *RH*), used to simulate the distribution and consumption chain of tomato fruits. The application of NL-Av allowed lower weight loss and lower mold and yeasts counts, as well as a reduction in the gas transfer rate and ethylene production during cold and room temperature storage. Therefore, the use of NL-Av is an attractive postharvest alternative to extend the quality of tomato fruits during storage.

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7.1 INTRODUCTION

Tomato fruits (*Lycopersicon esculentum* Mill.) are the second most important vegetable crop in the world after potato, with an annual production of around 163.4 million tonnes of fresh weight (FAO, 2016). Being a climacteric fruit, tomato ripening is accompanied by an increase in respiration rate and ethylene production, thus having a relatively short postharvest shelf life (Domínguez et al., 2016).

The plant hormone ethylene is required for normal fruit ripening being considered as a trigger of a wide range of physical, physiological and biochemical changes that make tomato fruits attractive for consumption. The conversion of tomato fruits from the mature green to fully ripe stage involves the above changes affecting quality parameters such as colour, texture, flavour, which can occur during development on plant and after harvesting (Zapata et al., 2008).

Respiration also brings less interesting physiological consequences to the fruit such as senescence, decay, degradation of chlorophyll and subsequently deterioration in the normal course of time. Respiration process involves the consumption of oxygen (O_2) for oxidative break-down of carbohydrates to produce carbon dioxide (CO_2), water, with concurrent release of energy (Kandasamy et al., 2015). Meanwhile, the occurrence of the transpiration causes initial shrinkage and subsequent weight loss of the produce by moving water vapour from the produce surface to the surrounding air (Flores-López et al., 2015).

On the other hand, tomatoes are susceptible to microbial attack during field production, mainly by the use of contaminated irrigation water, soil, field tools and containers. Furthermore, the fruits can receive injuries during harvest and handing operation that promote new sites for colonization of microorganisms. Fungi (e.g. *Penicillium* spp., *Alternaria alternata*, *Rhizopus stolonifer)* can spread to adjacent fruits causing the loss of the entire box within a few days. Moreover, bacteria may also induce serious ailments in humans when contaminated fruit is consumed (Ramos-García et al., 2012).

Thus, the major limiting factors in the storage of tomato are the respiration and transpiration along with microbial contamination, and thus the control of these

factors is primordial to extend its shelf life. The use of low temperature storage is effective in delaying and/or reducing ethylene production, but tomato is sensitive to chilling injury and temperatures over 11 °C are advisable for postharvest storage (Zapata et al., 2008). Besides the storage at low temperatures, the use of postharvest pesticides and sanitizers are the most frequently used methods to reduce pathogens levels in fruits; however, their application leads to fruit pesticide residues that may exceed the maximal level allowed, thus representing a serious problem to human health (Dorais et al., 2008).

The high production of tomato fruit during harvest time demands the use of better and more efficient postharvest processing and preservation techniques. The application of edible coatings represents an alternative for extending postharvest life of fruits. Coatings can be used to modify the internal atmosphere since they provide a semi-permeable barrier against O_2, CO_2 and moisture while also avoiding volatiles loss, therefore reducing respiration, water loss and oxidation reactions (Souza et al., 2015). Polysaccharide-based coatings have been used to extend the shelf life of fruits and vegetables, and e.g. sodium alginate, chitosan, galactomannans were used as main components (Vieira et al., 2016; Lima et al., 2010; Olivas et al., 2007). Recently, some works suggested that these materials can have improved functionality when they are used at the nanoscale (Fabra et al., 2016; Medeiros et al., 2012a, 2012b).

In this context, nano-laminate coatings composed by the alternate deposition of biopolymers have been designed in order to combine their bioactivity and barrier properties, and they have shown an enhanced efficiency and better gas barrier when compared with conventional coatings (Flores-López et al., 2015). These systems have been produced using polyelectrolytes with opposite charges (e.g. chitosan/sodium alginate, pectin/chitosan, among others) by means of the layer-by-layer (LbL) deposition technique (Souza et al., 2015; Medeiros et al., 2012a, 2012b). It also been shown their ability to incorporate bioactive compounds (directly or encapsulated) with a controlled release of the active agent and thus prolonging its bioactivity during storage time (Fabra et al., 2016).

Currently, there is an increasing interest in the use of *Aloe vera* liquid fraction, which has been separated and characterized in Chapter 3, evidencing potential antioxidant and antifungal properties against *Penicillium expansum* and *Botrytis cinerea*, fungi of economic importance for tomato fruit postharvest. Therefore, the aims of this study were evaluating the effect of *A. vera* liquid fraction applied as a nano-laminate coating on physicochemical parameters related to fruit quality during cold and room temperature storage in tomato fruits, as well as determining its role in controlling microbial spoilage and its effect on the respiration process of the tomato fruits.

7.2 MATERIALS AND METHODS

7.2.1 Materials

Sodium alginate was obtained from Manutex RSX (Kelco International, Ltd., Portugal) and chitosan (91.23% deacetylation degree and high molecular weight) was purchased from Golden-Shell Biochemical Co., Ltd (China). Lactic acid with 90% of purity and oxalic acid dehydrate were purchased from Merck (Germany). Tween 80 was purchased from Acros Organics (Belgium). Sodium hydroxide was obtained from Riedel-de Haën (Germany) and ascorbic acid from VWR (USA). Dichloran-rose Bengal-chloramphenicol (DRBC), glycerol, sodium chloride and phenolphthalein were supplied by Panreac (Spain). The dye 2,6-dichlorophenol-indolphenol (DCPIP) was obtained from Sigma (USA). Plate count agar (PCA) and peptone bacteriological were purchased from HiMedia Laboratories (India).

Tomatoes (*Lycopersicum esculentum* Mill.) at turning-pink stage of ripening according the USDA standard tomato colour classification chart (USDA, 1991) were purchased from a local supermarket in Braga, Portugal. The fruits were visually selected for uniformity in size, colour and absence of fungal infection, and were kept at 6 °C until use. Before treatments were applied, fruits were washed with a solution of sodium hypochlorite (0.05%) for 3 min, and air-dried at room temperature.

7.2.2 Experimental design

The shelf life analyses were performed in two main sets of experiments; these conditions were selected in order to simulate the cold storage (11 °C and 90% relative humidity, *RH*) and market operations (room temperature) (20 °C/85% *RH*) conditions of tomato fruits. Three different treatments: uncoated fruit (control), nano-laminate coating (NL) and nano-laminate coating with liquid fraction of *A. vera* (NL-Av) were evaluated as presented in Table 7-1. The physicochemical and microbiological analyses were conducted at regular intervals during 20 days for cold storage (0, 5, 10, 15 and 20 days) and during 15 days (0, 3, 6, 9, 12 and 15 days) for room temperature. Respiration rate was evaluated daily for 15 and 8 days for cold and room temperature storage, respectively.

7.2.3 Coating preparation

Polyelectrolyte solutions based on sodium alginate (Alg) and chitosan (CH) were prepared as explained in Chapter 5 (Section 5.2.2.2). The concentrations of each polysaccharide, surfactant (Tween 80) and plasticizer (glycerol) were defined in Chapter 4 according to studies of spreading coefficient (W_s) on tomato surface. Subsequently, the liquid fraction of *A. vera* was added to both chitosan (CH-Av) and sodium alginate (Alg-Av) coating solutions to reach a final concentration of 0.6 or 0.2% (w/v), respectively, and mixed during 2 h at room temperature for homogenization.

7.2.4 Coating application on tomato fruits

The coatings were applied on the test groups by the LbL deposition technique as presented in Table 7-1; no coating was applied to the control group (uncoated). Following the methodology described previously in Chapter 4 (Section 4.2.3), tomatoes were immersed into a 0.2% (w/v) Alg solution at pH 7.0 for 10 s and subsequently rinsed with distilled water with the same pH (7.0). The samples were dried at 30 °C for 20 min in an oven with air circulation (Binder, USA) and the procedure was repeated using a CH solution (0.6%, w/v) at pH 3.0 and rising with

distilled water at the same pH. This process was repeated with the alternate deposition of a total of five nanolayers (Alg-CH-Alg-CH-Alg). The samples of each group were placed in trays which were left inside of a controlled temperature and humidity chamber (Binder, USA) under the conditions described for cold and room temperature storage. During the shelf life and respiration testes, temperature and relative humidity were recorded with an iButton data logger (Thermochron, USA).

Table 7-1. Treatment description of nano-laminate coatings applied to tomato fruit

Treatment	1st layer	2nd layer	3rd layer	4th layer	5th layer
Uncoated					
Nano-laminate coating (NL)	Alg	CH	Alg	CH	Alg
Nano-laminate coating/ *Aloe vera* liquid fraction (NL-Av)	Alg/Av*	CH/Av**	Alg/Av*	CH/Av**	Alg/Av*

Liquid fraction of *Aloe vera* at concentration of 0.2% (w/v) for alginate* and 0.6% (w/v) for chitosan**.

7.2.5 Physicochemical analyses

7.2.5.1 Weight loss

Weight loss was evaluated by weighing all samples with a precision balance (Mettler AE200, Germany) at the beginning of storage (day 0) and during the experimental storage time. The percentage of weight loss was determined by the following equation:

$$\text{Weight loss } (\%) = \frac{W_i - W_f}{Wi} \times 100 \qquad \text{Eq. 7-1}$$

where W_i is the initial sample weight and W_f is the final sample weight.

7.2.5.2 Titratable acidity (TA), pH, soluble solid content (SSC)

Tomato fruits from each treatment (50 g) were ground in a blender and filtered through filter paper Whatman No. 1 under vacuum. Titratable acidity (TA) was determined using 942.15 AOAC methods (AOAC, 1997), specific for fruit

derivatives, by measuring the amount of 0.1 mol L^{-1} NaOH. Results were expressed as percent (%) in grams of citric acid equivalent per 100 g of tomato.

Determination of pH was conducted using a pH meter (Hanna Instruments Inc., Romania). After the homogenization of the samples, pH was measured by direct immersion of the electrode.

The soluble solid content (SSC) is an index of soluble solids concentration. The juice from the fruit (test and control groups) was used to determine the SSC according to 932.12 AOAC method (AOAC, 1997) by using a refractometer (HI 96801, Hanna Instruments Inc., Romania) previously standardized with water. Results were expressed as percentage (%).

At each sampling time, and for all physicochemical tests, three samples per treatment were analyzed.

7.2.5.3 Ascorbic acid (AA) determination

Ascorbic acid (AA) content was estimated using the dye 2,6-dichlorophenol-indolphenol titration (DCPIP) method of Ranggana (1977) with some modifications. Briefly, the juice from the fruit previously obtained was centrifuged (Sigma 4K15, UK) for 5 min at 12,000 *g* at room temperature. 2 mL of the supernatant were mixed with 5 mL of oxalic acid (4%, w/v) and 2 mL of distilled water. The volume required to provoke a change of colour of DCPIP (0.024 g mL^{-1}) was registered. A standard of ascorbic acid at 0.02 g mL^{-1} was used as reference. The results were expressed as mg of ascorbic acid content per 100 g. All the determinations were performed in triplicate.

7.2.6 Colour

Tomato skin colour values were measured using a Minolta colorimeter (CR 400; Minolta, Japan) and average readings at three points on the circumference of the fruits was recorded. The instrument was calibrated against a standard white colour plate (Y = 93.5, x = 0.3114, y =0.3190). In a Minolta colorimeter the a^* value corresponds to the degree of redness whereas the b^* value represents yellowness.

In this study the results were reported as redness values according to the scale proposed by Batu (2004) (Table 7-2) for tomato fruits. The redness was calculated by the following equation:

$$Redness = \frac{a^*}{b^*} \quad \text{Eq. 7-2}$$

Table 7-2. Classification of mature stages of tomato fruit according to redness values. Adapted from Batu (2004)

Redness values (a^*/b^*)*	USDA tomato colour stages
–0.59 to –0.47	Green
–0.47 to –0.27	Breaker
–0.27 to 0.08	Turning
0.08 to 0.60	Pink
0.60 to 0.95	Light red
0.95 to 1.21	Red

7.2.7 Firmness

Fruit firmness was determined using a texture analyzer (TA.XT, Stable Micro Systems, UK). The tomato was placed at the center of the platform and the force (*N*) required to penetrate 2.0 cm in the fruit was measured in the break point with a 6 mm flat head stainless steel cylindrical probe with a test speed of 5 mm s^{-1}. Firmness was determined at the start and end of each test and the results were the mean ± SE (*n*=10) and expressed in Newton (*N*).

7.2.8 Microbiological analyses

Microbiological analyses were conducted by counting the total aerobic mesophilic microorganisms and molds and yeasts for cold and room temperature storage, according to Olivas et al. (2007). 10 g of sample was placed in a sterilized filter stomacher bag (VWR Scientific, USA) containing 90 mL of sterilized peptone water (0.1%). This was blended for 120 s using a blender Stomacher (3500, UK). Serial decimal dilutions of the filtrate in 0.1% peptone were pour-plated in duplicate on

plate count agar (PCA) and incubated at 37 °C for 2 days to count aerobic mesophilic microorganisms. The same decimal dilutions were spread-plated on dichloran-rose Bengal-chloramphenicol (DRBC) agar and incubated for 5 days at 25 °C to count molds and yeasts. All analyses were performed with two replicates. The results were expressed in log colony forming units per gram (log CFU g^{-1}).

7.2.9 Gas transfer rate and ethylene production

The closed system method was used for measurement of the gas exchange (O_2 and CO_2) and ethylene production of tomato fruits. Acrylic air-tight cylindrical containers with a top lid fitted with a septum for gas sampling were used for each fruit and measured on a daily basis. A whole intact fruit sample was placed in each container, which was then placed in a controlled temperature and humidity chamber (Binder, USA) in order to maintain the desired storage conditions. The cold storage was conducted at 11 °C and 90% *RH* and evaluations were made on a daily basis during 15 days; meanwhile for room temperature storage was conducted at 20 °C and 85% *RH* and evaluated daily during 8 days. Temperature and *RH* (%) were recorded using an iButton data logger (Thermochron, USA) placed inside the container.

The O_2 and CO_2 content was determined using a gas chromatograph (Bruker Scion 456, Germany) connected with two thermal conductivity detectors (TCD). The gas chromatograph was equipped with two columns. The O_2 determination was carried out with a column SS MolSieve 13 × (80/100), 2 m × 2 mm × 1/8'' and argon was used as carrier gas at 30 mL min^{-1} at 32 psi. The injector and the detector were set at 100 °C and 130 °C, respectively. For CO_2 measurement a BR Q PLOT, 30 m × 0.53 mm column and helium as carrier gas at 15 mL min^{-1} at 32 psi were used. The injector and the detector were set at 100 °C and 130 °C, respectively. A mixture containing 10% CO_2, 20% O_2 and 70% N_2 was used as standard for calibration. The ethylene production was evaluated using a Varian model 3400 gas chromatograph, coupled with flame ionization detector (FID) (USA) and equipped with a vf-5 ms 30 m × 0.25 mm, 0.25 µm (film thickness) column. Helium (1 mL min^{-1}), nitrogen (30 mL min^{-1}), air (250 min^{-1}) and hydrogen

(30 mL min^{-1}) were utilized as carrier gases. The injector was set at split/splitless mode (ratio 1:7) at 250 °C and the detector was set at 280 °C. Ethylene at 500 ppm (Calgaz, UK) was used as a standard. For the determination of gas transfer rate three replicates were performed for each group of samples.

Three full replicates were performed for each of the control group and for each of the coated fruit groups.

The O_2 consumption and CO_2 and ethylene (C_2H_4) production rates were determined through Eqs. 7-3, 7-4 and 7-5 according with Cerqueira et al. (2009), with some modifications.

$$\mathrm{dy}_{O_2} = -R_{O_2} \frac{w}{V_f} \mathrm{d}t \qquad \text{Eq. 7-3}$$

$$\mathrm{dy}_{CO_2} = R_{CO_2} \frac{w}{V_f} \mathrm{d}t \qquad \text{Eq. 7-4}$$

$$\mathrm{dy}_{C_2H_4} = R_{C_2H_4} \frac{w}{V_f} \mathrm{d}t \qquad \text{Eq. 7-5}$$

where, $R_{O_2}$is the O_2 consumption rate, mL [O_2] kg^{-1} h^{-1}, $R_{CO_2}$is the CO_2 production rate, mL [CO_2] kg^{-1} h^{-1}, $R_{C_2H_4}$is the ethylene production rate, μL [C_2H_4] kg^{-1} h^{-1}, w (kg) is the weight of the tomato fruit, and V_f(mL) is the free volume of the container. The free volume V_f was calculated by:

$$V_f = V_\rho - \frac{w}{\rho_{tomato}} \qquad \text{Eq. 7-6}$$

where, V_ρ(mL) is the total volume of the container, w (kg) is the weight of the tomato fruit, and ρ_{tomato} is the true density of the tomato fruit, in this case 1.0 × 10^{-3} kg mL^{-1}, obtained experimentally following the method described by Owolarafe et al. (2007). The graphs of O_2 consumed versus time or CO_2 and ethylene produced versus time were used to calculate the slopes, which correspond to the derivatives, $\mathrm{dy}_{O_2}/\mathrm{d}t$, $\mathrm{dy}_{CO_2}/\mathrm{d}t$ or $\mathrm{dy}_{C_2H_4}/\mathrm{d}t$.

7.2.10 Statistical analyses

Data analyses were subjected to analysis of variance (ANOVA) using FAUANL software (Olivares, 1994) and Statistica software (release 7, edition 2004, Statsoft, Tulsa, OK, USA).

7.3 RESULTS AND DISCUSSION

7.3.1 Physicochemical analyses

7.3.1.1 Weight loss

A weight loss above 5% is a limiting factor for the postharvest life of fruit crops, making the fruit unsuitable for consumption and marketing; and it is known the relation between this parameter with the temperature and storage time (Aktas et al., 2012). Figure 7-1 shows the weight loss of uncoated and coated tomatoes with conditions simulating the cold (Figure 7-1a) and room temperature storage (Figure 7-1b). In both conditions, a weight loss was observed, being more remarkable the loss due to storage at 20 °C and 85% *RH*. According to previous works, weight loss corresponds nearly exclusively to water loss since other components that can be lost (e.g. aromas, flavours and gaseous products of respiration) are practically undetectable in terms of weight loss (Souza et al., 2015).

These experiments show that from the fifth day of cold storage onwards there is a significant difference ($p<0.05$) between the weight loss values of coated and uncoated tomato fruits; however, when analyzing only coated fruits no significant differences ($p>0.05$) between the two coatings tested were found (Figure 7-1a). In contrast, when analyzing the behaviour of samples during room temperature storage (Figure 7-1b) it was found a significant difference ($p<0.05$) between samples coated with NL-Av and those coated with NL (without *A. vera*) and uncoated, being this difference higher at the day 15, with values of 5.22 ± 1.20% for samples coated with NL-Av, and 8.50 ± 0.58% and 16.24 ± 1.24%, for those coated with NL and uncoated, respectively. The incorporation of *A. vera* thus seems to have retarded the moisture loss. A similar behaviour was reported by

Vieira et al. (2016) when blueberries coated with a chitosan-based coating containing liquid fraction of *A. vera* saw their weight loss significantly reduced after being stored at 5 °C and 90% *RH* during 25 days.

These results are in agreement with the barrier capacity detected for both coating systems when mounted on polyethylene terephthalate (PET) films, developed and characterized in Chapter 6 (Table 6-3), where multilayer A corresponds to NL and multilayer G to NL-Av coating. Therefore, both systems showed lower values of water vapour transmission rate (*WVTR*) than conventional coatings, being *WVTR* equal to $1.07 \pm 0.17 \times 10^{-3}$ g m^{-2} s^{-1} and $1.70 \pm 0.40 \times 10^{-3}$ g m^{-2} s^{-1} for NL (multilayer A) and NL-Av (multilayer G), respectively. The improved barrier properties of nano-laminate coatings can be explained by the electrostatic interactions between adjacent layers of alginate and chitosan, which allows an increase of tortuosity of the matrix thus decreasing the diffusion of molecules through the matrix materials (Souza et al., 2015). In addition, the incorporation of *A. vera* liquid fraction can further improve the barrier properties of the chitosan-alginate nano-laminate coating due to the reported capacity of *A. vera* of diminishing the interaction between the hydrophilic groups of chitosan with water, thus improving its barrier properties towards water vapour (Khoshgozaran-Abras et al., 2012).

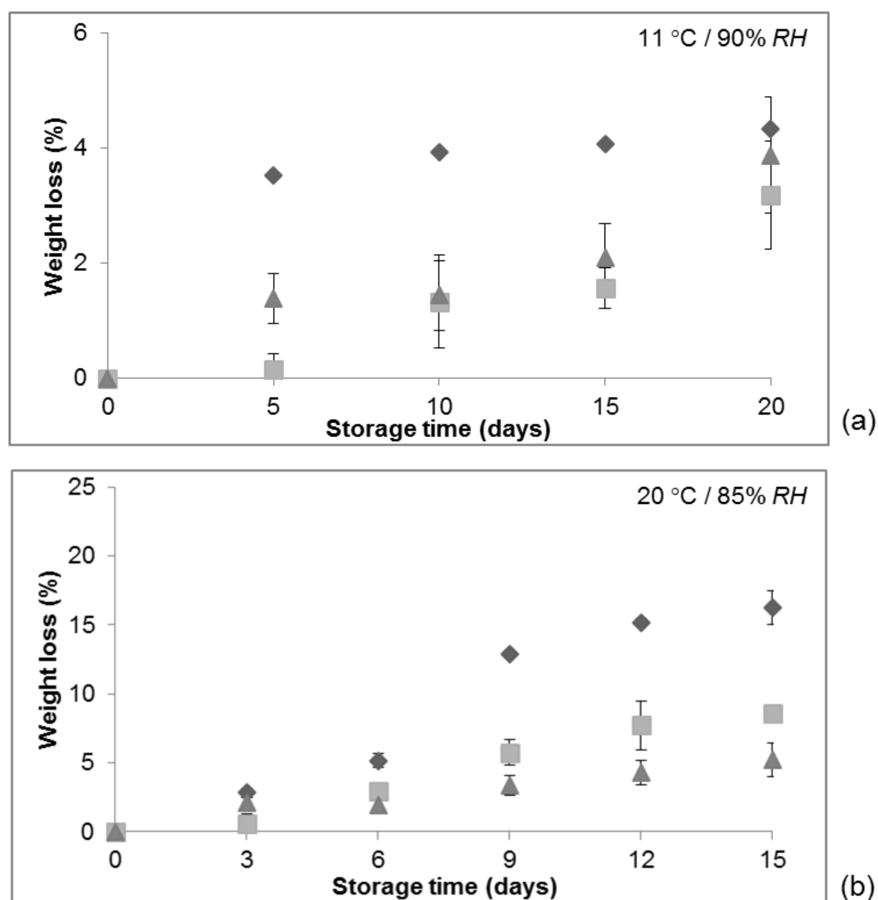

Figure 7-1. Weight loss (%) of tomato fruits uncoated (◆), coated with NL (■) and NL-Av (▲) coatings and stored (a) 20 days at 11 °C/90% *RH*, and (b) 15 days at 20 °C/85% *RH*. Each data point is the average of five determinations and the *error bars* show the standard deviation.

7.3.1.2 Titratable acidity (TA), pH, soluble solid content (SSC)

Tables 7-3 and 7-4 present the pH, acidity and SSC values for tomato fruits under the storage conditions evaluated. The range for SCC in tomato fruits for both storage conditions was 3.6-5.1%, being in agreement with the values reported by Zapata et al. (2008) for tomato fruits. During the treatment at 11 °C, the SSC was maintained for tomato fruits coated with both coatings, whereas for uncoated samples SCC significantly increased ($p<0.05$) on day 20 (5.1 ± 0.4%) (Table 7-3). Generally, the increase of SCC in harvested fruits takes place when upon the start of the ripening process, due to the degradation of polysaccharides to simple sugars and also due to the conversion of organic acids, and is inhibited by lowering temperature (Liplap et al., 2013). The values of pH and TA were maintained

throughout cold storage in all the samples, probably explained by the lower temperature that prevents alteration of both parameters; however prolonged cold storage is not recommendable because it can cause chilling injury in tomato fruits (Biswas et al., 2012).

Table 7-3. Physicochemical properties of tomato fruits, uncoated and treated with different coatings during storage at 11 °C/90% *RH*

Storage time (days)		0	5	10	15	20
Uncoated	TA	0.3 ± 0.0^{Aa}	0.3 ± 0.0^{Aa}	0.2 ± 0.0^{Aa}	0.3 ± 0.0^{Aa}	0.3 ± 0.0^{Aa}
	pH	4.5 ± 0.1^{Aa}	4.4 ± 0.1^{Aa}	4.4 ± 0.1^{Aa}	4.8 ± 0.1^{Aa}	4.4 ± 0.2^{Aa}
	SSC	3.9 ± 0.2^{Aa}	4.0 ± 0.2^{Aa}	3.7 ± 0.1^{Aa}	4.0 ± 0.2^{Aa}	5.1 ± 0.4^{Ba}
NL	TA	0.3 ± 0.0^{Aa}	0.2 ± 0.0^{Aa}	0.2 ± 0.0^{Aa}	0.2 ± 0.0^{Aa}	0.4 ± 0.0^{Ab}
	pH	4.5 ± 0.1^{Aa}	4.4 ± 0.0^{Aa}	4.4 ± 0.1^{Aa}	4.5 ± 0.1^{Aa}	4.4 ± 0.1^{Aa}
	SSC	3.9 ± 0.2^{Aa}	4.4 ± 0.3^{Aa}	3.7 ± 0.3^{Aa}	3.8 ± 0.5^{Aa}	3.7 ± 0.4^{Ab}
NL-Av	TA	0.3 ± 0.0^{Aa}	0.3 ± 0.0^{Aa}	0.2 ± 0.0^{Aa}	0.3 ± 0.0^{Aa}	0.2 ± 0.0^{Aa}
	pH	4.5 ± 0.1^{Aa}	4.6 ± 0.1^{Aa}	4.4 ± 0.0^{Aa}	4.6 ± 0.3^{Aa}	4.6 ± 0.2^{Aa}
	SSC	3.9 ± 0.2^{Aa}	4.3 ± 0.1^{Ba}	3.7 ± 0.2^{Aa}	3.6 ± 0.3^{Aa}	3.6 ± 0.2^{Ab}

TA = Titratable acidity (%, in grams of citric acid equivalent per 100 g of tomato).

SSC = soluble solid content (%)

Means followed by the same lowercase letters in the columns and uppercase letters in rows did not show a statistically significant difference by Tukey's test ($p<0.05$).

Meanwhile, no significant changes ($p>0.05$) were detected in the SCC, neither between treatments nor with respect to the day 0 under storage at 20 °C. The same trend has been reported by Javanmardi and Kubota (2006) for tomato fruits stored at room temperature (25-27 °C) and at low temperature (5-12 °C). Also, TA values were stable for uncoated tomato during the storage; a significant ($p<0.05$) decrease was only observed on the third day (0.2 ± 0.0) with respect to day 0, possibly related with the variability of the samples. The fruits coated with NL exhibited a significant decrease ($p<0.05$) in TA values from day 6 onwards, and those coated with NL-Av from day 9 to 12, both with respect to day 0 (Table 7-4). The acidity of tomato fruit is important because it imparts taste to the fruit, and its reduction is associated with the maturation process. Even though, acidity does not change linearly with time since for some authors malic acid concentration falls

during ripening and citric acid increases up to turning stage, whereas other authors reported that malic acid increased gradually thru maturation (Athmaselvi et al., 2013).

Table 7-4. Physicochemical properties on tomato fruit uncoated and treated with different coatings during storage at 20 °C/85% *RH*

Storage time (days)		0	3	6	9	12	15
Uncoated	TA	0.3 ± 0.0^{Aa}	0.2 ± 0.0^{Ba}	0.2 ± 0.1^{Aa}	0.3 ± 0.0^{Aa}	0.2 ± 0.1^{Aa}	0.2 ± 0.0^{Aa}
	pH	4.5 ± 0.1^{Aa}	4.5 ± 0.2^{Aa}	4.5 ± 0.2^{Aa}	4.5 ± 0.2^{Aa}	4.5 ± 0.1^{Aa}	4.6 ± 0.1^{Aa}
	SSC	3.9 ± 0.2^{Aa}	4.3 ± 0.2^{Aa}	4.1 ± 0.2^{Aa}	4.0 ± 0.4^{Aa}	4.0 ± 0.3^{Aa}	3.6 ± 0.1^{Aa}
NL	TA	0.3 ± 0.0^{Aa}	0.2 ± 0.0^{Aa}	0.2 ± 0.0^{Ba}	0.2 ± 0.0^{Bb}	0.2 ± 0.0^{Ba}	0.2 ± 0.0^{Ba}
	pH	4.5 ± 0.1^{Aa}	4.5 ± 0.2^{Aa}	4.6 ± 0.1^{Aa}	4.7 ± 0.1^{Aa}	4.6 ± 0.0^{Aa}	4.8 ± 0.0^{Bb}
	SSC	3.9 ± 0.2^{Aa}	4.6 ± 0.0^{Ba}	3.9 ± 0.4^{Aa}	3.9 ± 0.1^{Aa}	4.0 ± 0.2^{Aa}	3.8 ± 0.1^{Aa}
NL-Av	TA	0.3 ± 0.0^{Aa}	0.3 ± 0.0^{Aa}	0.3 ± 0.1^{Aa}	0.2 ± 0.0^{Bb}	0.2 ± 0.0^{Ba}	0.2 ± 0.1^{Aa}
	pH	4.5 ± 0.1^{Aa}	4.5 ± 0.2^{Aa}	4.4 ± 0.2^{Aa}	4.6 ± 0.2^{Aa}	4.7 ± 0.1^{Aa}	4.9 ± 0.1^{Bb}
	SSC	3.9 ± 0.2^{Aa}	4.0 ± 0.3^{Aa}	4.0 ± 0.3^{Aa}	4.0 ± 0.2^{Aa}	3.9 ± 0.1^{Aa}	4.5 ± 0.1^{Ab}

TA = Titratable acidity (%, in grams of citric acid equivalent per 100 g of tomato).

SSC = soluble solid content (%)

Means followed by the same lowercase letters in the columns and uppercase letters in rows did not show a statistically significant difference by Tukey's test (p<0.05).

The pH of tomato fruit at 20 °C was maintained during the storage period for uncoated fruits, while higher values of pH were observed only in tomato fruits with either of the coatings on day 15 when compared with day 0 (Table 7-4). The diminishing and increasing values of TA and pH, respectively, of coated tomatoes stored at 20 °C is an indication that the fruits can continue their maturation process, while being protected in terms of weight loss as shown in Figure 7-1b.

7.3.1.3 Ascorbic acid

Fruits are a natural source of ascorbic acid (AA) being known that its level decreases during processing and ripening. Due to its sensitivity, it is often used as an indicator of the severity of postharvest fruit damage (Souza et al., 2015). Figures 7-2a and 7-2b show the concentration of AA of uncoated and coated tomato fruits. The AA contents at the beginning of the tests are in the range of

published data (Abushita et al., 1997) for tomato harvested at turning-pink stage (~16 mg 100 g^{-1} fruit). A reduction in the levels of AA on the second day of analysis can be noticed in both experiments, which is in agreement with previous data and related with the fact that AA is used either as a substrate or converted into sugar during ripening (Domínguez et al., 2016).

During cold storage, no significant differences (p>0.05) were found between NL and uncoated tomato fruits until day 10 (Figure 7-2a). Also, at 20 °C the AA was maintained through storage time in tomato fruits coated with either of the coatings showing statistically significant differences (p<0.05) with the uncoated fruits from day 12 onwards (Figure 7-2b). This drastic reduction of AA in uncoated fruits for both storage conditions is associated to the advanced ripeness of such fruits, probably due to its antioxidant function when the ripening cells absorb high amounts of oxygen as a result of increasing respiration rate, which is a distinctive physiological change of climacteric fruits and vegetables at ripeness (Abushita et al., 1997). The use of either of the nano-laminate coatings helped reducing the AA loss. However, in both storage conditions, the incorporation of liquid fraction of *A. vera* did not have effect on the retention of AA, since no significant differences (p>0.05) were found between the two coatings. The use of multilayer systems such as those reported by Brasil et al. (2012), a chitosan-(β-cyclodextrin + trans-cinnamaldehyde complex)-pectin-based multilayer edible coating (thickness = 300 ± 1μm), has also allowed retaining higher values of AA than in uncoated papaya during cold storage (Brasil et al., 2012).

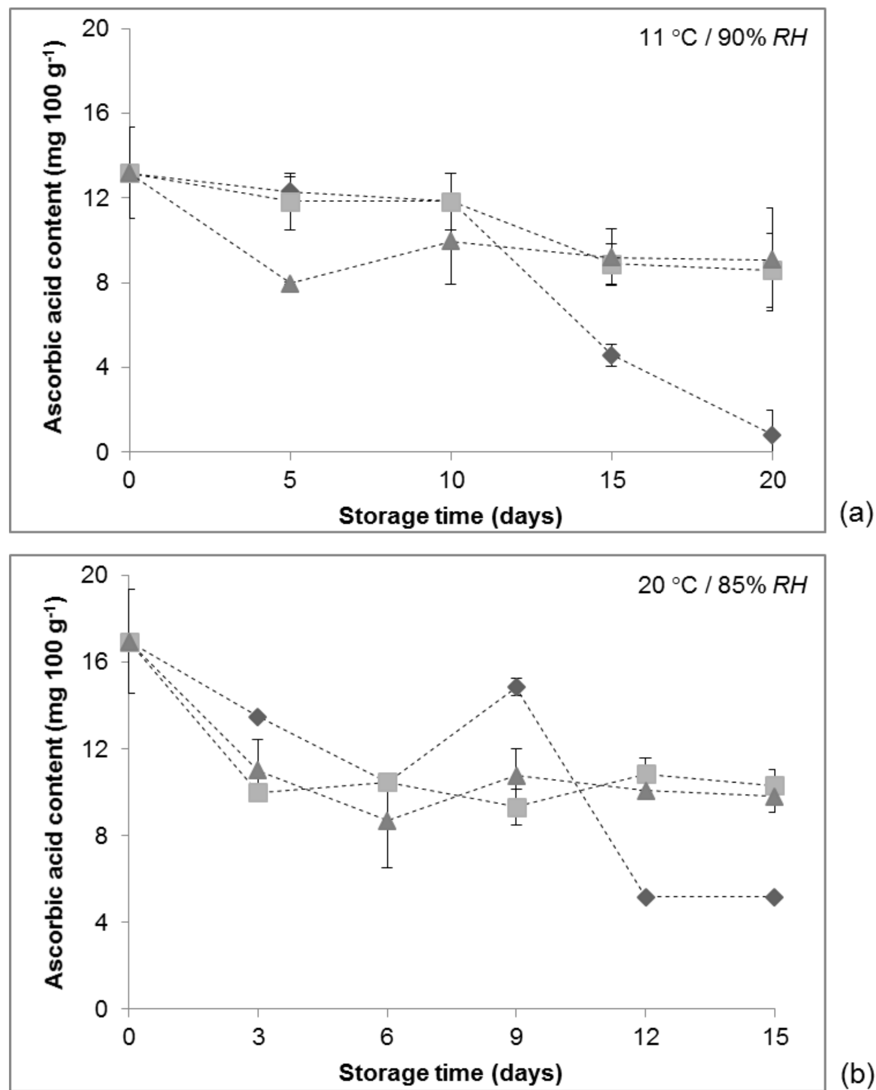

Figure 7-2. Ascorbic acid content in tomato fruits uncoated (◆), coated with NL (■) and NL-Av (▲) coatings and stored (a) 20 days at 11 °C/90% *RH*, and (b) 15 days at 20 °C/85% *RH*. Each data point is the average of three determinations and the *error bars* show the standard deviation.

7.3.2 Colour and firmness

Colour is an indicator of ripeness and the most obvious indicator of quality of tomato fruits (Ali et al., 2010). The a^* value is a good indicator to monitor red colour developement and the degree of ripening in tomato fruit, whilst b^* shows yellow discoloration; based on this fact, Batu (2004) reported a scale of a^*/b^* values for expressing the redness and its relation with maturation stage of tomato fruit (Table

7-1). As can be observed in Figures 7-3a and 7-3b, the colour development was significantly ($p<0.05$) more pronounced in uncoated tomato fruits over storage time in both conditions, whereas application of either of the nano-laminate coatings helped preserving ($p<0.05$) the colour attributes of the fruits. Tomato fruits coated with NL and NL-Av maintained the pink stage until day 10 and later the light red stage was retained throughout the cold storage period, whilst for room temperature storage the light red stage was maintained throughout the test. No significant differences ($p>0.05$) were found between NL and NL-Av coatings until day 20 under cold storage, time at which both uncoated and coated with NL samples changed to red stage (a^*/b^* values up to 0.95), while those coated with NL-Av kept at the pink stage. The beneficial effect of *A. vera*-based coatings in decreasing the development of colour of table grapes and mushrooms has been reported by other authors (Mohebbi et al., 2012; Valverde et al., 2005).

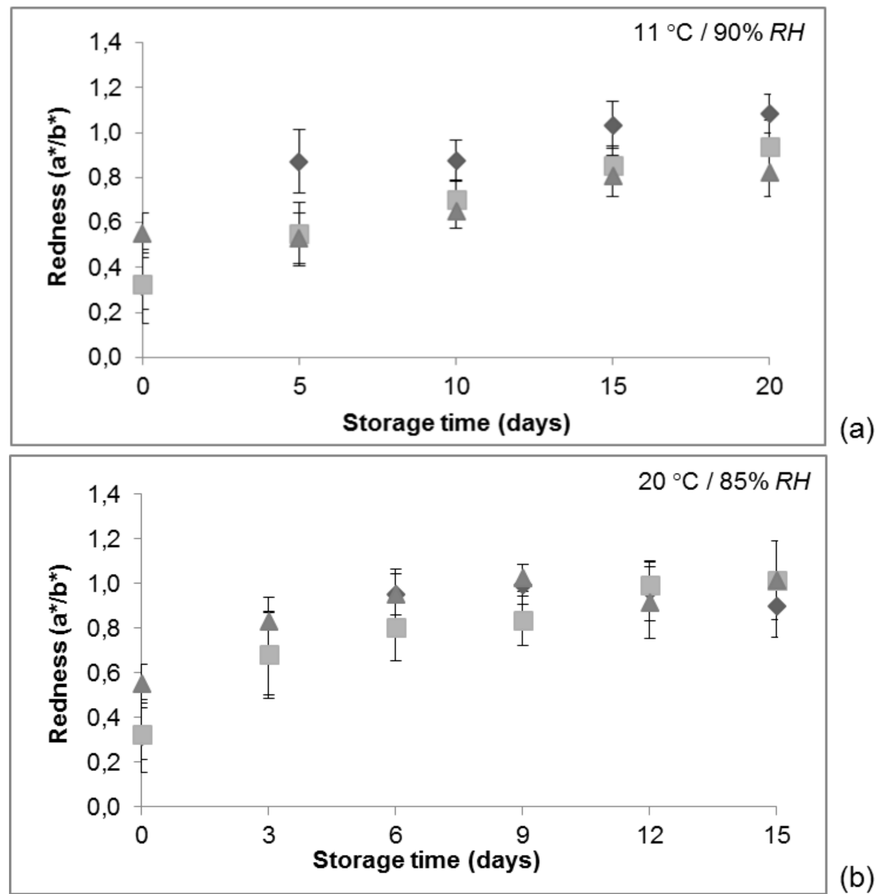

Figure 7-3. Redness (a^*/b^*) of tomato fruits uncoated (◆), coated with NL (■) and NL-Av (▲) coatings and stored (a) 20 days at 11 °C/90% *RH*, and (b) 15 days at 20 °C/85% *RH*. Values are the mean and the *error bars* show the standard deviation.

Regarding fruit firmness, the application of NL-Av at day 0 significantly ($p<0.05$) increases the firmness of tomato fruits when compared to uncoated and NL coated fruits, probably due to this coating has a greater thickness; also Athmaselvi et al. (2013) reported higher firmness in tomato fruits coated with a *A. vera* based coating. A significant ($p<0.05$) decrease occurred at the end of storage time, being the uncoated fruits those which clearly showed the lowest firmness under both storage conditions. The maximum firmness was kept by coated tomato fruits, but no significant ($p>0.05$) differences were found between the two types of nano-laminate coatings under consideration. In agreement with these results, Ali et al. (2010) reported lower loss of firmness in tomatoes coated with gum Arabic-based coating throughout storage at 20 °C and, 80-90% *RH*. The softening of the fruits

results of degradation of cell structure and internal composition of cell wall by the action of enzymes (e.g. hydrolases) on pectin and starch being these actions closely linked to the fruit ripening progress (Ali et al., 2010). The effect of coatings on delaying the softening of fruits is associated to their capability to act as a barrier for O_2 uptake, and the results of gas transfer rate (please see Section 7.3.4) showed that the tomato fruits coated exhibited a significant (p<0.05) lower O_2 consumption than uncoated fruits, thus slowing the metabolic activity and consequently the ripening process (Sogvar et al., 2016).

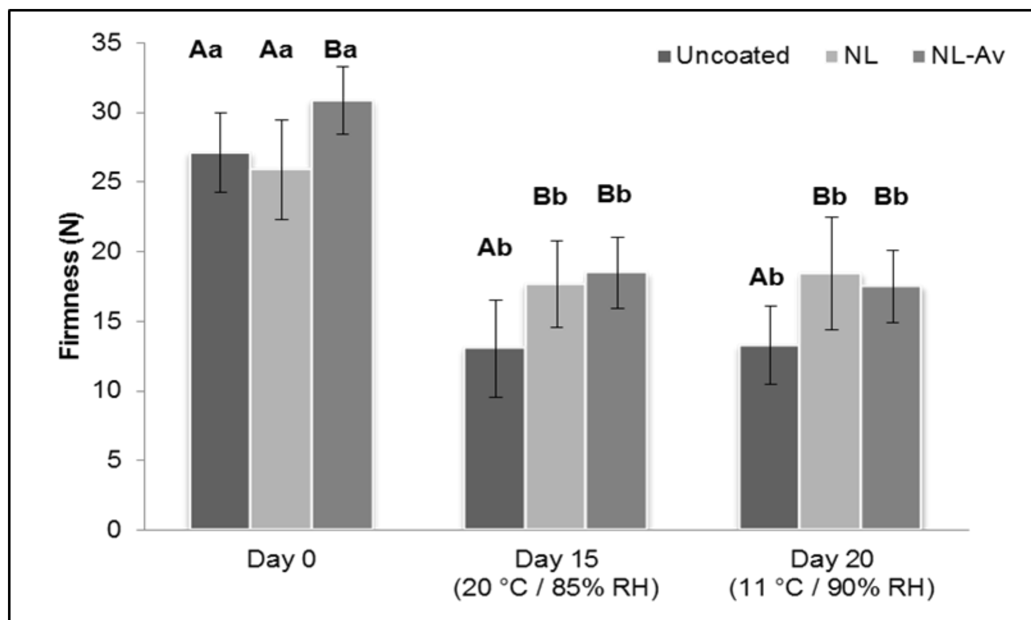

Figure 7-4. Fruit firmness (*N*) of tomato fruits uncoated and coated with NL and NL-Av during storage at 11 °C/90% *RH* and 20 °C/85% *RH*. Values are the mean and the *error bars* show the standard deviation. Uppercase letters are used to compare means inside the same group (same storage time); lowercase letters are used to compare means obtained by a given sample at different storage times. Means with the same uppercase and lowercase letter did not differ by Tukey's test (p<0.05).

7.3.3 Microbiological analysis

Following 20 days of cold storage, mold and yeast populations of coated tomato fruits were significantly (p<0.05) reduced (3.5 – 4.0 log UFC g^{-1}) when compared with uncoated samples (8.0 ± 0.0 log UFC g^{-1}). The reduction was slightly (p<0.05) more effective for fruits coated with NL-Av than for those coated with NL, although the effect was less evident and even comparable to NL treatment after day 15

(Figure 7-5a). A similar behaviour was observed during room temperature storage, during which NL-Av exhibited a better inhibition until day 9 and after day 12 no significant ($p>0.05$) differences were found between the two nano-laminate coatings under analysis. However, these treatments have shown reduced ($p<0.05$) mold and yeast populations (4.0 ± 0.0 log CFU g^{-1}) with respect to those of uncoated fruits (5.3 ± 0.01 log CFU g^{-1}) (Figure 7-5b). The results obtained *in vivo* are in agreement with those found in Chapter 6 (Section 6.3.2), in which the nano-laminate film with *A. vera* liquid fraction (multilayer G) showed a significant increase ($p<0.05$) of antifungal activity until the eight day when compared to the film based on chitosan/alginate (multilayer A) and to the control. After this time, the activity of the multilayer G was also comparable with that of multilayer A.

The antifungal activity of *A. vera* liquid fraction has been associated to the suppression of germination and inhibition of mycelial growth of fungi such as *R. solani*, *Fusarium oxysporum, Colletotrichum coccodes*, *P. expansum* and *B. cinerea* (Vieira et al. 2016; Jasso de Rodríguez et al. 2005). These activities can be attributed to the presence of more than one active compound, although the specific action mechanism is still unknown (Valverde et al., 2005). Recently, Vieira et al. (2016) reported significantly low levels in yeast and molds counts on blueberry fruit coated with a chitosan and chitosan-liquid fraction of *A. vera*-based coatings after 25 days of storage at 5 °C and 90% *RH*; such activity was higher when *A. vera* was incorporated into the coating, although the authors also signaled a combination of the effects of chitosan and *A. vera*.

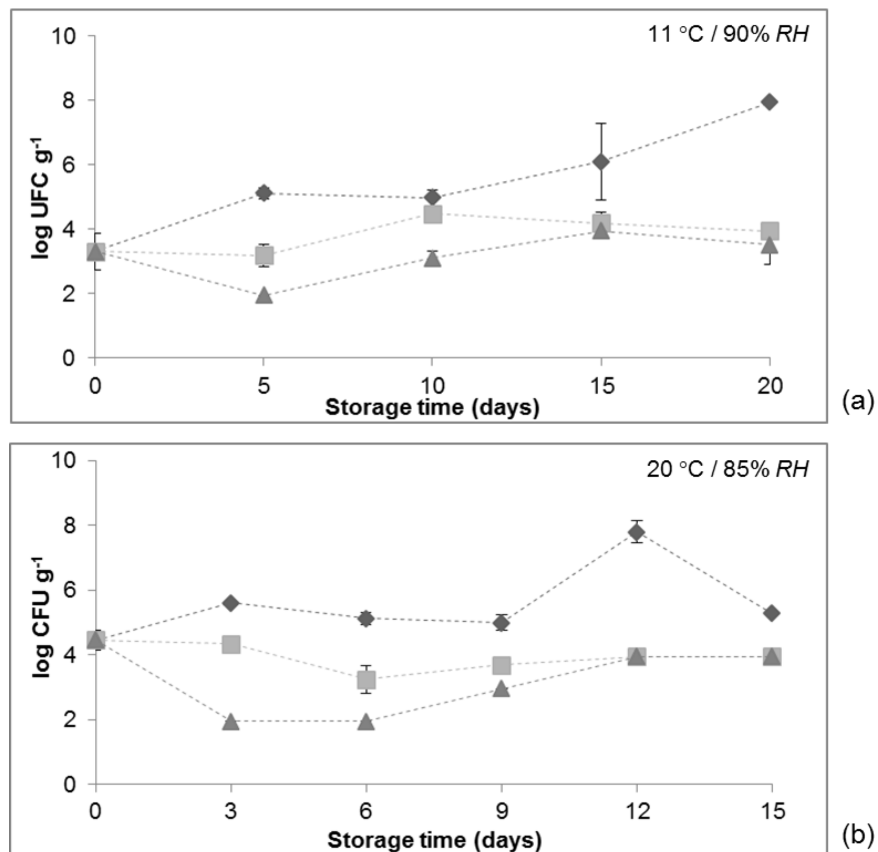

Figure 7-5. Microbiological counting of molds and yeasts throughout storage time of tomato fruits uncoated (◆), coated with NL (■) and NL-Av (▲) coatings at (a) 11 °C/90% *RH* and (b) 20 °C/85% *RH*. Each data point is the mean and the *error bars* show the standard deviation.

Regarding mesophilic microorganisms, the initial count was 4.0 ± 0.7 log CFU g^{-1}, and increased during storage (Figure 7-6). Under cold storage, a significant reduction (p<0.05) in mesophilic count was observed in tomato fruits coated with NL-Av (3.3 ± 0.4 log UFC g^{-1}) from day 15 onwards, but this effect was similar to that exhibited by tomato coated with NL at day 20 (5.7 ± 0.1 log UFC g^{-1}); whereas uncoated fruit has shown counts of up to 7.0 ± 1.0 log CFU g^{-1} from day 15 onwards (Figure 7-6a). During room temperature storage, no significant differences (p>0.05) in mesophilic counts were found between coated and uncoated tomato fruits, but a slight reduction (p<0.05) was observed at day 12 when compared to uncoated samples. Such activity was not sustained until the end of the test (Figure 7-6b).

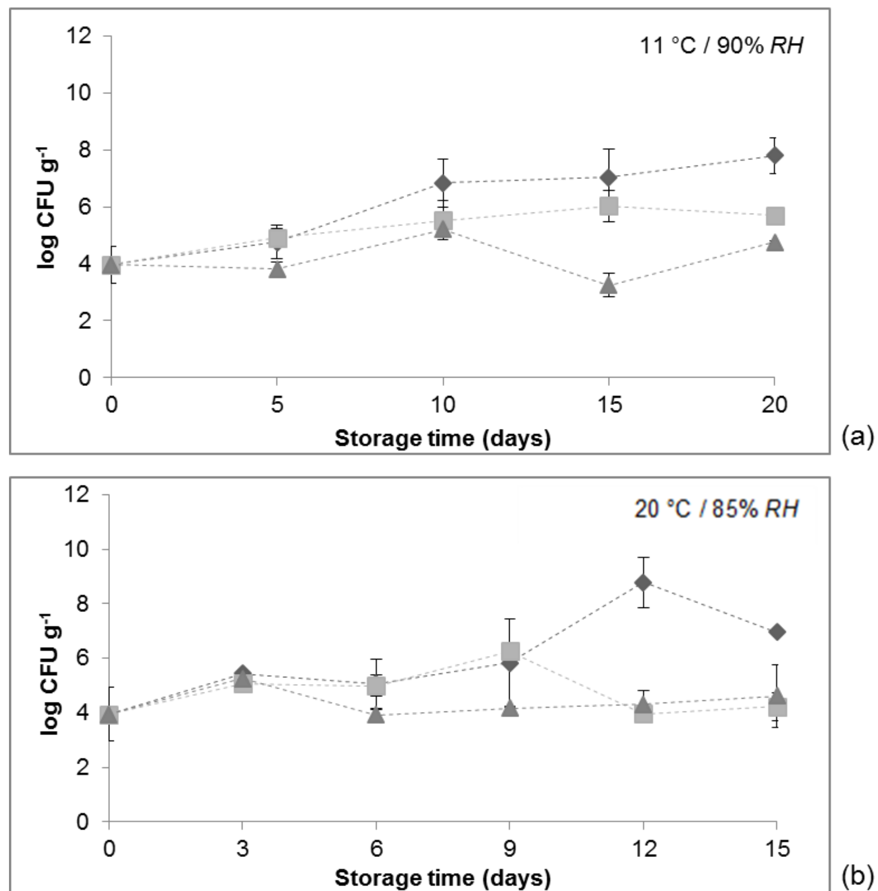

Figure 7-6. Microbiological counting of mesophilic throughout storage time of tomato fruits uncoated (◆), coated with NL (■) and NL-Av (▲) coatings at (a) 11 °C/90% *RH* and (b) 20 °C/85% *RH*. Each data point is the mean and the *error bars* show the standard deviation.

The effectiveness of the evaluated nano-laminate coatings was higher for yeast and molds than for mesophilic microorganisms; Valverde et al. (2005) also found the same behaviour on table grapes coated with *A. vera* gel. It is supposed that antimicrobial activity of *A. vera* cannot be sustained throughout storage due, probably, to the stability of its bioactive compounds related with their antimicrobial activity, mainly phenolic compounds and organic acids as can be reported in Chapter 3. Even though, visual evaluation confirmed that the uncoated tomato fruits had extensive spoilage on the surface after 15 and 20 days of room temperature and cold storage, respectively (Figure 7-7).

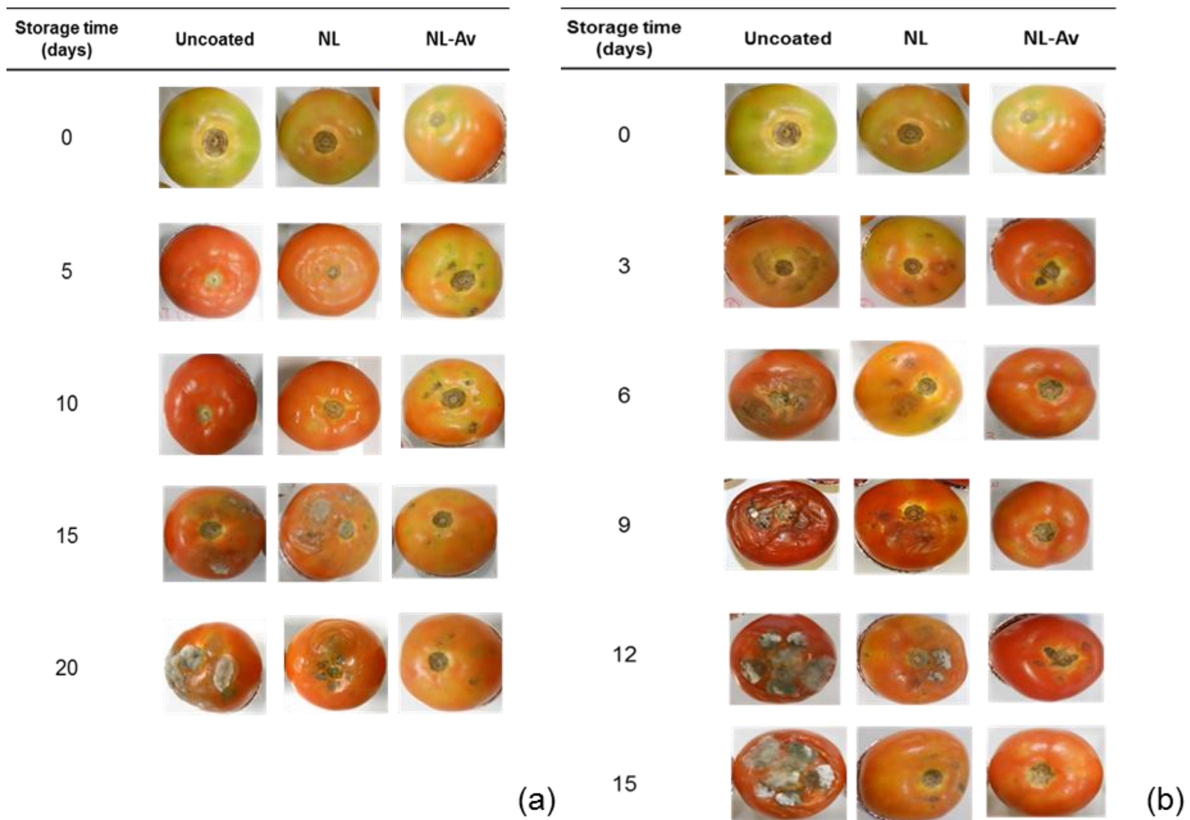

Figure 7-7. Tomato fruits' gradual change throughout storage time at (a) 11 °C/90% *RH* and (b) 20 °C/85% *RH*.

7.3.4 Gas transfer rate and ethylene production

The CO_2 production rate in uncoated tomato fruits significantly increased (p<0.05) over cold storage, whereas that rate was maintained for NL- and NL-Av-coated samples (Figure 7-8a). After 15 days of cold storage, respiration rates were 4.8 ± 0.2, 1.8 ± 0.1 and 1.5 ± 0.2 mL CO_2 kg^{-1} h^{-1} for uncoated, NL and NL-Av coated fruits, respectively. During room temperature storage, the reduction of the respiration rate was statistical significant (p<0.05) for tomato fruits coated with NL-Av when compared to uncoated and NL coated fruits (Figure 7-8b).

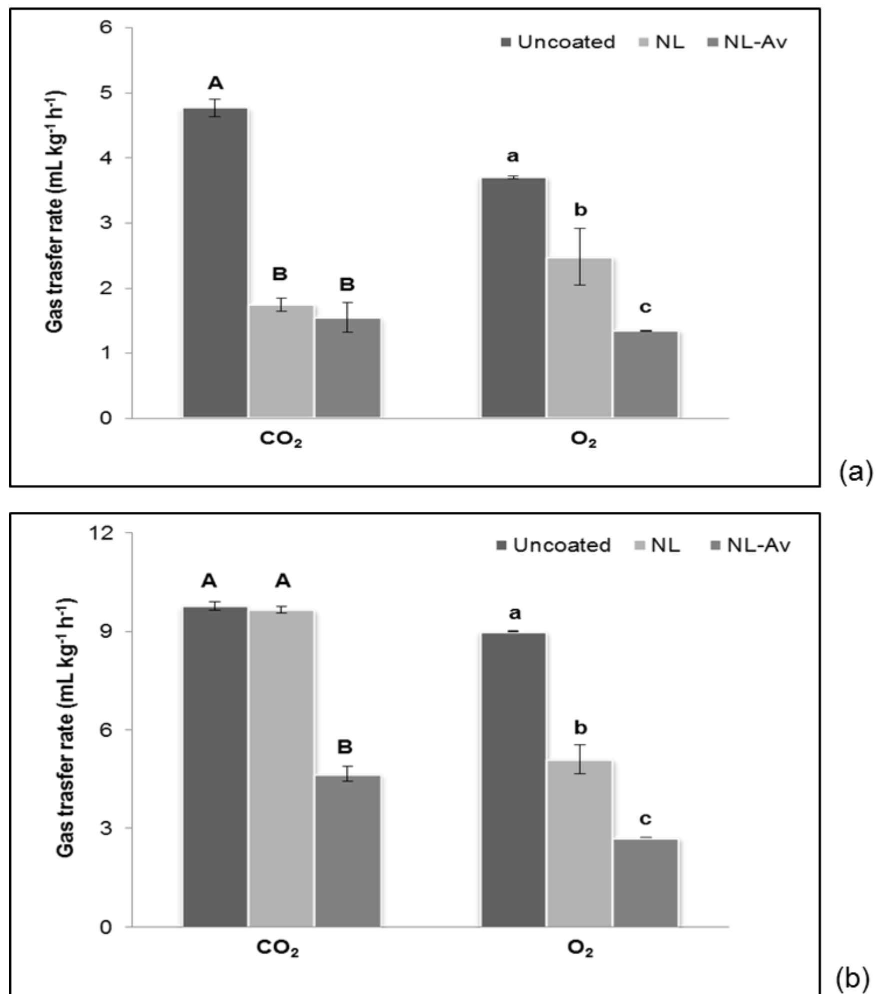

Figure 7-8. O_2 and CO_2 transfer rates of tomato fruits uncoated, coated with NL and NL-Av coatings and stored (a) 15 days at 11 °C/90% *RH*, and (b) 8 days at 20 °C/85% *RH*. Each data point is the mean and the *error bars* show the standard deviation. Equal uppercase letters mean that there was no statistically significant difference between CO_2 transfer rates and equal lowercase letters mean that there was no statistically significant difference between O_2 transfer rates in the Tukey test ($\rho<0.05$).

Ethylene production rate showed an increase with respect to the storage temperature, being more remarkable during room temperature storage (Figure 7-9). No significant differences ($p>0.05$) were found between the two nano-laminate coatings under evaluation, both showing restricted ($p<0.05$) ethylene synthesis in tomato fruits compared to uncoated fruits at cold storage. In room temperature storage experiments, tomato fruits coated with NL-Av exhibited significant ($p<0.05$) ethylene restriction (0.11 ± 0.0 μL $kg^{-1}h^{-1}$) when compared with NL-coated (2.4 ± 0.1 μL kg^{-1} h^{-1}) and uncoated (2.3 ± 0.7 μL $kg^{-1}h^{-1}$) tomato fruits. The reduction in

the metabolic rates of the fruits due to the application of coating on their surface restricts permeation of respiratory gases. Also, the lower yeast and molds counts can influence the lower ethylene production in tomato fruit coated with NL-Av, which could indicate that the accumulated ethylene in uncoated fruit proceeds from the fungal metabolism rather than from the tomato fruit (Valverde et al., 2005).

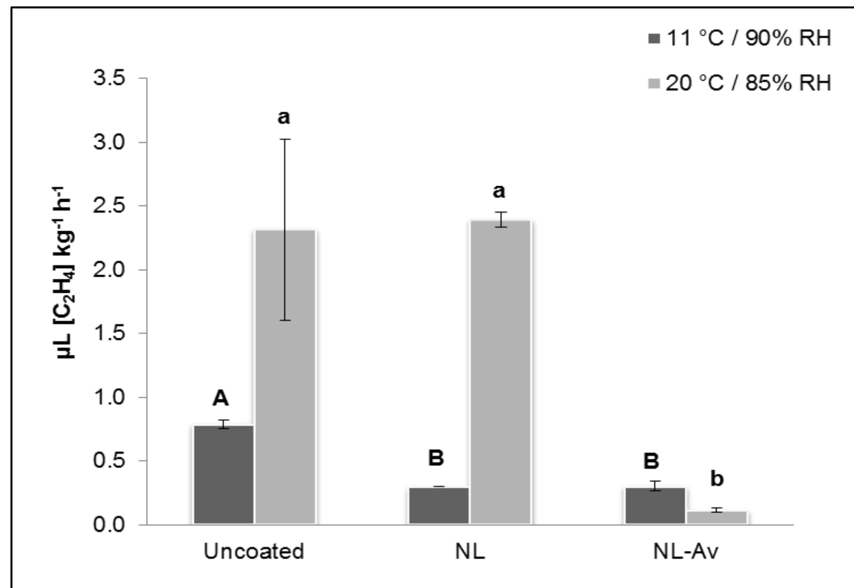

Figure 7-9. Ethylene production of tomato fruits uncoated and coating with NL and NL-Av coatings during storage at 11 °C/90% *RH* and 20 °C/85% *RH*. Values are the mean and the *error bars* show the standard deviation. Equal uppercase letters mean that there was no statistically significant difference between storage treatments at 11 °C/90% *RH* and equal lowercase letters mean that there was no statistically significant difference between 20 °C/85% *RH* in the Tukey test ($\rho<0.05$).

NL-Av exhibited a lower gas transfer rate, since a 64% less O_2 consumption and 68% less CO_2 production and 52% less O_2 consumption and 70% less CO_2 production were observed when compared to uncoated samples, after cold and room temperature storage, respectively. These results suggest that the NL-Av coating possibly could provide a barrier against ethylene production and gas exchange between inner and outer environments. This fact results in the delay of the maturation process and the consequent extension of the shelf life of fruits.

7.4 CONCLUSION

The effectiveness of nano-laminate coatings containing *A. vera* liquid fraction was evaluated and compared with an alginate/chitosan coating as reference and

uncoated tomato fruits. The use of nano-laminate coatings was effective to control the maturation process in both cold and room temperature storage, since a reduction in the gas transfer rate was shown in the coated tomato fruits. In addition, nano-laminate coatings containing *A. vera* liquid fraction showed to be a better protection against weight loss and to the control the gas transfer rate, and also, restricted ethylene production. Furthermore, microbial spoilage was reduced during cold and room temperature storage, impacting the quality of tomato fruits.

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Chapter 8

GENERAL CONCLUSIONS

This chapter presents the major conclusions of this thesis and advances, recommendations/suggestions for further research in this field.

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8.2 RECOMMENDATIONS 180

8.1 CONCLUSIONS

The main objective of this thesis was the development and characterization of nano-laminate coatings using bioactive components of *Aloe vera* for extending the shelf life of tomato (*Lycopersicum esculentum* Mill.). In order to cover successfully the thesis aims, several subjects were studied and strategies were implemented. More in detail, the work involved the characterization of *Aloe vera* fractions (liquid and gel) and evaluation of their biological activity and optimal formulations of nano-laminate coatings were defined based on their wettability on tomato fruit surface, and their physical properties. Also, nano-laminate coatings with direct and encapsulating carvacrol into zein were built and evaluated their characteristics and antifungal properties. The effectiveness of nano-laminate coatings with *A. vera* liquid fraction incorporated as bioactive compound was evaluated on the room temperature and cold storage of tomato fruits. The main contributions of this thesis were the following:

- Separation and characterization of *A. vera* fractions, since typically the liquid fraction is not recover. The method of separation was simple and guaranteed good yields. Also, it was possible to define that liquid fraction shows highest antioxidant activity and acceptable antifungal control on *Alternaria* sp.;
- Polyelectrolyte solutions based on alginate and chitosan and their optimal concentrations were selected to construction of nano-laminate coatings; also, the effect of surfactant and plasticizer concentrations was determined. The best formulations were selected based on their wettability on tomato surface;
- Nano-laminate coatings with carvacrol and zein nanocapsules significantly decreased the water vapour transmission rate of nano-laminate films when as compared to those prepared with alginate and chitosan. Their oxygen barrier properties were improved (up to 45%) when it was nanoencapsulated and evidenced the highest antifungal activity against *Alternaria* sp.

- Nano-laminate films functionalized with bioactive fractions of *A. vera* shown good barrier properties when compared with conventional films. The functionalized nano-laminate films evidenced antifungal activity against the growth of *Alternaria* sp;
- The effectiveness of nano-laminate coating with incorporation of *A. vera* liquid fraction was proven on tomato fruit. This exhibited a lower weight loss and minor microbial contamination, mainly yeasts and molds microorganisms. The coatings showed a reduction in the gas transfer rate and ethylene production during cold and room temperature storage of tomato fruits.

Briefly, the use of nano-laminate coatings functionalized with *A. vera* liquid fraction represents an attractive postharvest tool for extending the quality and shelf life of tomato fruits. This work may also be a guide for the study of future fruit models with different maturation patterns for similar purposed.

8.2 RECOMMENDATIONS

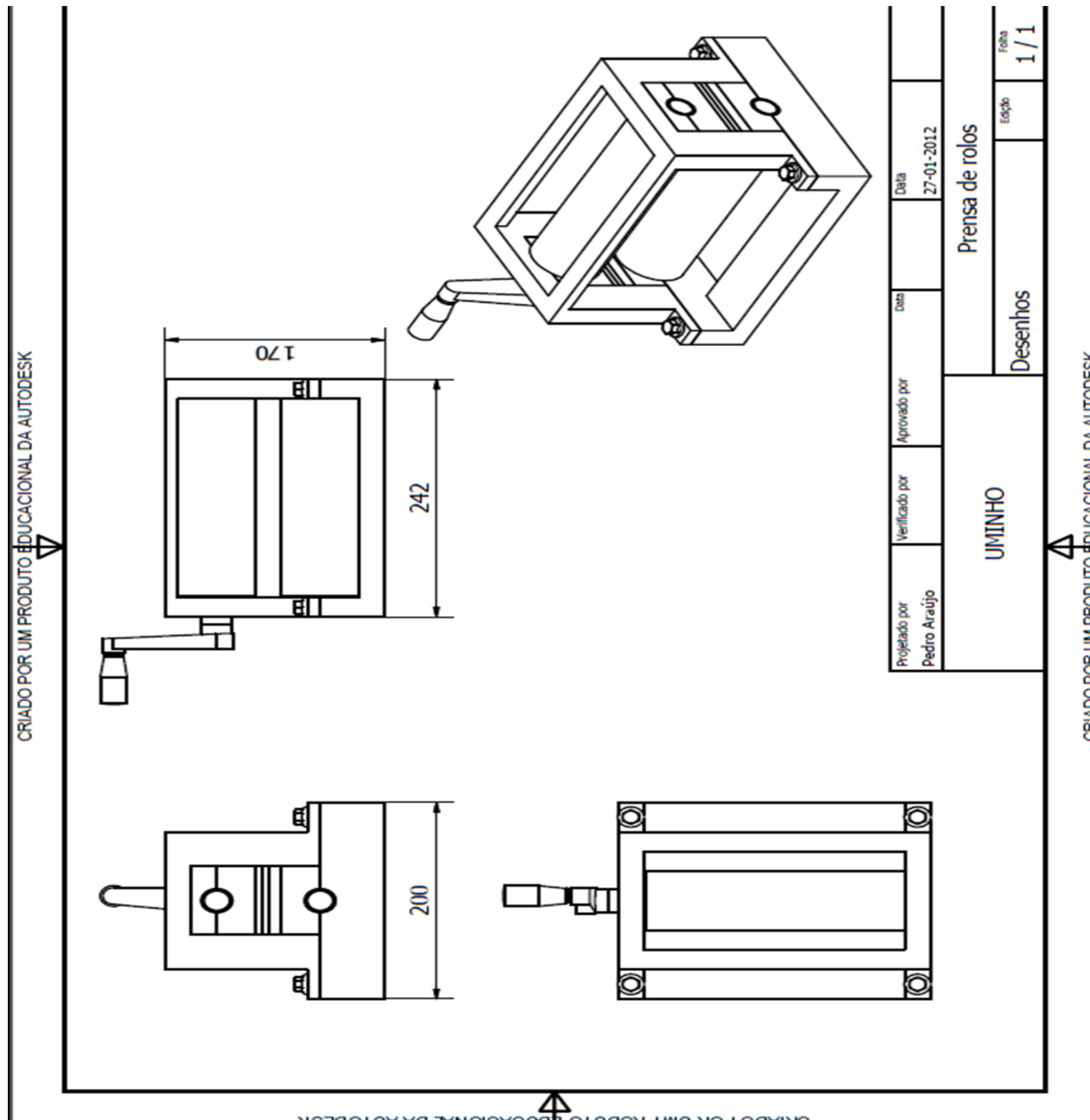
Despite the main objectives have been achieved, some work still stays to be done in order to better understand the mechanisms of action of nano-laminate coatings, as well as optimization of the application conditions (e.g. time of applications, drying step, number of layers). Therefore, some recommendations for enhancement of the present work and guidelines for future work in this field can be advanced:

- Characterization of *A. vera* fractions (liquid fraction, gel and bagasse extracts) was conducted and also their antifungal and antioxidant activity have been shown; however, more studies regarding identification of the main compounds responsible for such activities should be performed;
- Study of different fruit respiration patterns (climacteric and non-climacteric) in order to design specific systems according to specific necessities of fruits and vegetables;

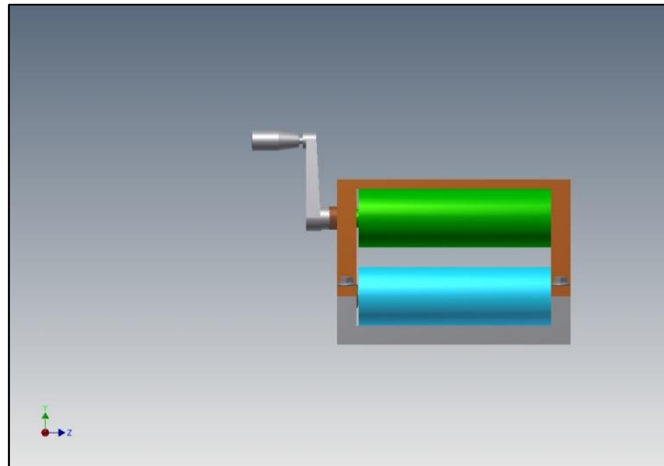
- Optimization of application conditions of functionalized nano-laminate coatings (e.g. number of layers, drying conditions, time of application);
- Finally, the evaluation of the selected formulation at an industrial level, in order to understand the behaviour, bioactivity and factibility of these nano-laminate coatings during an industrial scale application and subsequently postharvest shelf life.

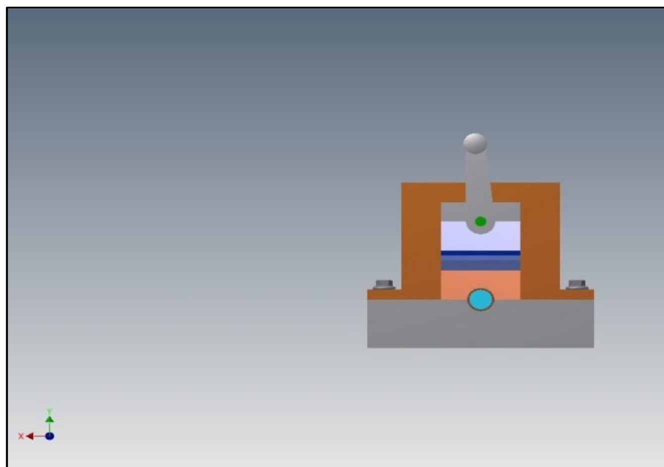
ANNEXES

Annex 1. Design of laboratory manual roll processor used to separate the liquid fraction from gel of *Aloe vera*.



Annex 2. 3D Three-dimensional visualization of laboratory manual roll processor used to separate the liquid fraction from gel of *Aloe vera*.





Annex 3. Spreading coefficient (W_s) achieved for the tested chitosan solutions on tomato fruits surface

Formulation	Chitosan (%, w/v)	Glycerol (%, w/v)	Tween 80 (%, w/v)	Spreading coefficient W_s (mN m^{-1})
1	0.2	0.0	0.0	-52.84
2	0.2	0.05	0.0	-54.93
3	0.2	0.1	0.0	-32.85
4	0.2	0.0	0.05	-38.48
5	0.2	0.05	0.05	-22.68
6	0.2	0.1	0.05	-34.53
7	0.2	0.0	0.1	-30.34
8	0.2	0.05	0.1	-28.01
9	0.2	0.1	0.1	-20.41
10	0.4	0.0	0.0	-12.36
11	0.4	0.05	0.0	-15.08
12	0.4	0.1	0.0	-43.40
13	0.4	0.0	0.05	-11.51
14	0.4	0.05	0.05	-19.25
15	0.4	0.1	0.05	-33.73
16	0.4	0.0	0.1	-24.48
17	0.4	0.05	0.1	-23.18
18	0.4	0.1	0.1	-18.55
19	0.6	0.0	0.0	-11.88
20	0.6	0.05	0.0	-26.51
21	0.6	0.1	0.0	-22.30
22	0.6	0.0	0.05	-11.76
23	0.6	0.05	0.05	-19.75
24	0.6	0.1	0.05	-23.25
25	0.6	0.0	0.1	-13.64
26	0.6	0.05	0.1	-14.88
27	0.6	0.1	0.1	-9.75

Annex 4. Spreading coefficient (W_s) achieved for the tested sodium alginate solutions on tomato fruits surface

Formulation	Alginate (%, w/v)	Glycerol (%, w/v)	Tween 80 (%, w/v)	Spreading coefficient W_s (mN m^{-1})
28	0.2	0.0	0.0	-17.30
29	0.2	0.05	0.0	-6.28
30	0.2	0.1	0.0	-12.18
31	0.2	0.0	0.05	-7.03
32	0.2	0.05	0.05	-4.98
33	0.2	0.1	0.05	-8.01
34	0.2	0.0	0.1	-8.59
35	0.2	0.05	0.1	-10.93
36	0.2	0.1	0.1	-5.76
37	0.4	0.0	0.0	-7.40
38	0.4	0.05	0.0	-20.55
39	0.4	0.1	0.0	-11.76
40	0.4	0.0	0.05	-13.62
41	0.4	0.05	0.05	-6.54
42	0.4	0.1	0.05	-8.97
43	0.4	0.0	0.1	-32.79
44	0.4	0.05	0.1	-7.17
45	0.4	0.1	0.1	-18.01
46	0.6	0.0	0.0	-9.07
47	0.6	0.05	0.0	-9.35
48	0.6	0.1	0.0	-14.02
49	0.6	0.0	0.05	-23.19
50	0.6	0.05	0.05	-9.58
51	0.6	0.1	0.05	-19.99
52	0.6	0.0	0.1	-12.07
53	0.6	0.05	0.1	-10.88
54	0.6	0.1	0.1	-17.53