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Development of Chitosan-based Edible Coatings containing *Aloe vera* for Blueberries Application

Dissertação de Mestrado em Segurança Alimentar, orientada pela Professora Doutora Maria do Céu R. Sousa e co-orientada pela Doutora Joana Teresa Rodrigues Martins e apresentada à Faculdade de Farmácia da Universidade de Coimbra

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ABSTRACT

Common problems related with food such as foodborne outbreaks, precocious decaying and economic losses related with fresh food retention have led to the development of novel technologies and systems for the protection of food, such as edible coatings based on natural compounds.

The increasing rate of blueberries consumption allied to the excellent harvesting conditions that Portugal can offer, have created a remarkable opportunity to increase the production competitiveness of this product while offering a product with high added value that can better resist to the challenges of fruit processing, transport and refrigeration. Thus, this novel approach could be further applied on blueberries, often affected by problems, such as fungal infections and losses of water and nutritional properties, in order to extend their shelf life.

One of the most widely used natural compounds in the edible coating development is chitosan. This biopolymer possesses several valuable characteristics: high antimicrobial activity, biocompatibility, biodegradability and non-toxic profile.

The innovative character of this project is based on the potential incorporation of bioactive ingredients, *Aloe vera*, in chitosan coating solutions for application in blueberries. The synergy between these two components may be useful to avoid a chain of biochemical and nutritional changes that could lead to blueberries spoilage.

The objectives of this work were 1) to choose the best chitosan-based formulation to be applied on blueberries and 2) to evaluate antifungal and antioxidant activities *in vitro* of different concentrations of *Aloe vera* fractions (i.e. gel and juice) to incorporate in chitosan-based formulation previously chosen. The best composition of the chitosan-based coating solution in terms of the improved adhesion of edible coating on blueberries surface was 0.5% Chitosan (w/v), 0.5% *Aloe vera* (w/v), 0.5% Glycerol (v/v) (plasticizer) and 0.1% Tween 80 (w/v) (surfactant).

To assess the antioxidant activity of the *Aloe vera* gel and juice fractions (0.1 %, 0.5%, 1%, 5% and 10% (w/v)), DPPH radical scavenging activity assay was conducted. It was noticed that both juice and gel offer considerable antioxidant potential. For values of concentration

of 0.1% of gel and juice, the values of radical scavenging activity were 71.8% and 78.9%, respectively.

Quantity of 0.5%, 5%, 20% and 100% of each *Aloe vera* fractions were tested against 3 fungal species typically present in blueberries: *Botytris cinerea*, *Penincullium expansum* and *Aspergillus niger*. The antifungal activity results obtained show that the behavior of the three molds when exposed to *Aloe vera* fractions was different. For *Botytris cinerea*, the main mold causing blueberry deterioration, showed a percentage of inhibition above 80%, for 0.5% of gel and juice concentrations, after 72 h of growth; on the other hand, 0.5% *Aloe vera* gel and juice fractions inhibited 50% of *P. expansum* and about 18% of *A. niger* after 72 h of growth.

Chitosan-based coating incorporated with the fraction of *Aloe vera* chosen (juice 0.5%) was tested *in vivo*, to study their potential as a protective barrier to blueberry. Uncoated and coated blueberries samples were monitored over a period of 25 days in a controlled environment (temperature (5.5 ± 0.6 °C and relative humidity (90 ± 3%)), without forced contamination, and for a period of 18 days with forced contamination (15 storage days under the conditions described, followed by 3 days at room temperature (25 ± 0,5 °C; relative humidity of 58 ± 5%)). During this storage time was analyzed titratable acidity, pH, soluble solids content, weight loss, color and growth of molds and yeasts. It was verified that weight loss of the coated blueberries were lower after 25 days compared to uncoated blueberries. In the tests without forced contamination, weight loss after 25 days was 6.2% (B), 5.1% (BC) and 3.7% (BCA). In tests with forced contamination, weight loss after 18 days was 6.9% (BF) and 5.6% (BAF). The pH of the coated blueberries remained lower throughout the period of storage, and these improved results in microbiological level, blueberries coated with *Aloe vera* juice, and yeast fungi presented contamination only on day 9 of storage (1.3 log (CFU)/g).

The results obtained show that *Aloe vera* gel and juice fractions may have high potential to be incorporated in chitosan coatings and could successfully improve shelf life stability and retard postharvest deterioration of blueberries.

Keywords: Edible coatings, chitosan, *Aloe vera*, blueberries.

RESUMO

Os problemas associados aos alimentos mais comuns, nomeadamente surtos de origem alimentar, deterioração precoce e as perdas económicas relacionadas com o não escoamento de alimentos frescos têm levado ao desenvolvimento de novas tecnologias e sistemas de protecção de alimentos, como por exemplo, os revestimentos edíveis à base de compostos naturais.

O aumento da taxa de consumo de mirtilos aliado às excelentes condições de colheita que Portugal pode oferecer, criaram uma oportunidade notável para o desenvolvimento e inserção deste tipo de revestimento no mercado, de forma a aumentar a competitividade entre produtores, uma vez que este fruto de elevado valor acrescentado poderá resistir melhor aos desafios do armazenamento, transporte e refrigeração. Assim, os revestimentos edíveis, com os constituintes certos poderiam ser aplicados em mirtilos, frequentemente afectados por diversos problemas, tais como infecções por fungos e perdas de água e propriedades nutritivas, a fim de aumentar a sua vida de prateleira.

Um dos compostos naturais mais utilizados e com grande potencial para inserção no revestimento comestível devido às suas características, é o quitosano. Este biopolímero possui diversas particularidades importantes: alta actividade antimicrobiana, biocompatibilidade, biodegradabilidade e perfil não-tóxico.

O carácter inovador deste projecto baseia-se na possível incorporação de compostos bioactivos com potenciais propriedades de barreira dos alimentos contra factores externos, como as fracções de *Aloe vera* (gel e sumo), em soluções de revestimento de quitosano. A sinergia obtida entre estes dois componentes pode ser útil para evitar uma cadeia de alterações aos níveis bioquímico e nutricional que podem levar à deterioração dos mirtilos.

Os objectivos deste trabalho foram: 1) escolher a melhor formulação à base de quitosano para ser aplicado em mirtilos e 2) avaliar as actividades antifúngica e antioxidante *in vitro* de diferentes concentrações de fracções de *Aloe vera* (ou seja, gel e suco) para incorporar na melhor formulação à base de quitosano. A melhor composição da solução de revestimento à base de quitosano, em termos de aderência e coesão do revestimento comestível à superfície dos mirtilos foi 0,5% de quitosano, 0,5% de *Aloe vera* (sumo), 0,5% de glicerol (plastificante) e 0,1% de Tween 80 (surfactante).

Para avaliação da actividade antioxidante das fracções da *Aloe vera* gel e sumo (0,1%, 0,5%, 1%, 5% e 10%), a actividade sequestradora de radicais livres foi avaliada através da

técnica de DPPH. Constatou-se que o sumo, assim como o gel, apresentaram um potencial antioxidante elevado. Para os valores de concentração de 0,1% de gel e suco, os valores de actividade de eliminação de radicais livres foram 71,8% e 78,9%, respectivamente.

Quantidades de 0,5%, 5%, 20% e 100% de cada uma das fracções da *Aloe vera* foram testadas contra três espécies de fungos tipicamente presentes em mirtilos: *Botytris cinerea, Penincullium expansum* e *Aspergillus niger*. Os resultados obtidos para *B. cinerea*, principal fungo causador da deterioração mirtilo, mostraram uma percentagem de inibição superior a 80%, para 0,5% da concentração do gel e do sumo, após 72 h de crescimento. Por outro lado, a mesma concentração destas fracções de *Aloe vera* inibiu 50% o crescimento de *P. expansum* e cerca de 20% o crescimento de *A. niger*, após 72 h.

Realizados os testes *in vitro*, o revestimento foi testado *in vivo*, através das análises de shelf-life, confirmando assim a sua potencial barreira de protecção para o mirtilo. As amostras foram monitorizadas ao longo de um período de 25 dias num ambiente controlado (temperatura de 5.5 ± 0.6 °C e humidade relativa de 90 ± 3%), sem contaminação forçada, onde mirtilos revestidos e não revestidos foram comparados. Foram também realizados testes com contaminação forçada de uma quantidade e concentração conhecida de *Botrytis cinerea,* ao longo de um período de 18 dias e em ambiente controlado (15 dias nas condições de armazenamento descritas anteriormente, seguido de 3 dias à temperatura ambiente de 25 ± 0,5 °C e humidade relativa de 58 ± 5%) Durante o tempo de armazenamento foram analisadas a acidez titulável, o pH, teor de sólidos solúveis, a perda de peso, cor e o crescimento de fungos e leveduras. Verificou-se que a perda de peso dos mirtilos revestidos foi menor em relação aos mirtilos não revestidos. Nos testes sem contaminação forçada a perda de peso após 25 dias foi de 6.2% (B), 5.1% (BC) e 3.7% (BCA). Nos testes com contaminação forçada, a perda após 18 dias foi de 6.9% (BF) e 5.6% (BAF). O pH dos mirtilos revestidos manteve-se mais baixo ao longo do tempo de armazenamento, tendo estes menos contaminações microbiológicas (fungos e leveduras). De facto, os mirtilos revestidos com sumo da *Aloe vera* (sem contaminação forçada) apenas apresentaram fungos e leveduras no dia 9 do período de armazenamento (1.3 log (UFC)/g).

Os resultados obtidos mostram que a fracção do sumo da *Aloe vera* pode ser incorporada em revestimentos de quitosano, de modo a prolongar a vida útil do mirtilo, retardando a sua deterioração.´

Palavras-chave: Revestimentos edíveis, Quitosano, *Aloe vera*, Mirtilo.

TABLE OF CONTENTS, LIST OF FIGURES, LIST OF TABLES AND LIST OF ABBREVIATIONS

TABLE OF CONTENTS

AGRADECIMENTOS III
ABSTRACT V
RESUMO VII
TABLE OF CONTENTS XI
LIST OF FIGURES XV
LIST OF TABLES XIX
LIST OF ABBREVIATIONS XXI

1. THESIS MOTIVATION, OBJECTIVE AND OUTLINE 1
   1.1 Thesis Motivation 3
   1.2 Research Aims 5
   1.3 Outline 6

2. GENERAL INTRODUCTION 7
   2.1 Introduction 9
   2.2 Blueberry 9
      2.2.1 Taxonomic Classification and Morphology 10
      2.2.2 Chemical Composition 12
      2.2.3 Blueberry Production – Considerations 13
      2.2.4 Postharvest Physicochemical Changes 14
      2.2.5 Postharvest Microbiological Changes 15
   2.3 Edible Coatings 16
      2.3.1 Polysaccharides 18
         2.3.1.1 Chitosan 19
      2.3.2 Plasticizer and Surfactant Additives 20
      2.3.3 Bioactive compounds 21
      2.3.4 *Aloe vera* 22
         2.3.4.1 Taxonomic Classification 23
         2.3.4.2 Chemical Composition 23

2.3.4.3 *Aloe vera* in the Context of Coatings....24

3. MATERIALS AND METHODS.... 25
3.1 Blueberry Samples....27
3.2 Preparation of coating-forming solutions....27
3.2.1 Materials....27
3.2.2 Methods....27
3.3 Coating/Film characterization....29
3.3.1 Surface Tension and Wettability....29
3.3.2 Water Vapour Permeability....30
3.4 *Aloe vera* gel and juice Extration....31
3.5 Antifungal activity....32
3.5.1 Fungal strains....32
3.5.2 *In vitro* Antifungal Assay....32
3.6 Antioxidant activity....33
3.6.1 Materials....32
3.6.2 Metodology....32
3.7 *Shelf-life* Analyses – Experimental design....34
3.7.1 Coating application and blueberry inoculation....35
3.7.2 Preparation of storage conditions for blueberries....36
3.7.3 Titratable acidity (TA)....37
3.7.4 pH....38
3.7.5 Soluble solid content (SSC)....38
3.7.6 Weight loss....39
3.7.7 Colour....39
3.7.8 Microbiological analysis....41
3.8. Statistical Analyses....41

4. RESULTS AND DISCUSSION....43
4.1 Surface Tension and Wettability....45
4.2 Water Vapor Permeability (WVP)....47
4.3 Antifungal Activity of *Aloe vera*....48
4.4 Analysis of Antioxidant Capacity....51

4.5 *Shelf-life* Analysis 52
4.5.1 Titratable acidity (TA) 52
4.5.2 pH 53
4.5.3 Soluble Solid Content (SSC) 55
4.5.4 Weight loss 56
4.5.5 Microbiological Analysis 58
4.5.6 Colour 60

5. CONCLUSIONS 63
5.1 Conclusions 65
5.2 Recommendations and Future Projects 66

6. BIBLIOGRAPHY 67

ANNEXS 75
ANNEX I 87
ANNEX II 90
ANNEX III 92

LIST OF FIGURES

Figure 1 | Blueberry production in Portugal from 2004 to 2010 according to FAO data.....10

Figure 2 | Fruitful Blueberry branch: a) Axiliary bud; b) terminal aborted bud; c) fruits.....12

Figure 3 | Leaf infection (a) and berry infected in the field (b), due to *Alternaria tenuissima*..15

Figure 4 | Main functions of edible films and coatings for food application to extend it shelf-life……...……..16

Figure 5 | Molecular structure of chitin and chitosan.....20

Figure 6 | Chemical composition of aloe vera gel, on dry weight basis.....24

Figure 7 |Chronological Order of the Extraction of Aloe vera gel and juice fractions. a) Reception of Aloe vera; b) Aloe vera leaves were washed with distilled water and 2% sodium hypochlorite to remove dirt from the surface; c) Collect a sample for analysis; d) Weighing the whole leaf; e) Measurement of width, length and thickness; f) Cutting the base of the leaves to remove aloin; g) The skin was carefully separated from the parenchyma using a scalpel-shaped knife; h) The epidermis was then separated from the gel and the gel was separated from the juice using a laboratory roll processor; i) Separating gel and juice with a strainer; j) Gel, Juice and bagasse, from left to right. 31

Figure 8 | Scheme of 96-well elisa microplate to evaluate the antifungal activity of Aloe vera against different fungal species. 33

Figure 9 | Scheme of 96-well elisa microplate to evaluate the antioxidant capacity of of different concentrations of the Aloe vera fractions (0.05%, 0.1%, 0.5%, 1% and 5%) and their respective calibration curve. The pink and gray pits are the controls. 34

Figure 10 | Diagram representing the five different treatments applied in blueberries. Formulation of Chitosan coating is 0.5% Chitosan + 0.5% Glycerol + 0.1% Tween 80 and the formulations of Chitosan-Aloe vera (juice) coating is 0.5% Aloe vera + 0.5% Chitosan + 0.5% Glycerol + 0.1% Tween 80. 35

Figure 11 | Processing steps representative of the application of chitosan coating to blueberries. 35

Figure 12 | Application of the coating by immersion, and subsequent drying with ventilation at 20 °C, during 4-5 hours. 36

Figure 13 | Incorporation of 10 µl of *Botrytis cinerea* at blueberries furrows (about 2000 blueberries). 36

Figure 14 | Images of the adapted greenhouses to create desired atmosphere conditions (temperature and relative humidity) inside the cold room..... 37

Figure 15 | Refractometer used in the measurement of °Brix of the different blueberry treatments. 38

Figure 16 | Chromatic coordinates L* (ranging from black to white), a* (ranging from - a*: greenness to + a*: redness) and b* (ranging from - b*: blueness to + b*: yellowness). 40

Figure 17 | Colorimeter used for color characterization of blueberry pulp, to the different treatments over time. 40

Figure 18 | Contact angle of water liquid drop on blueberry and three interfacial tensions: solid-liquid (γ_{SL}), solid-vapor (γ_{SV}), liquid-vapor (γ_{L}). 45

Figure 19 | Water vapor permeability of different formulations of chitosan edible films. 47

Figure 20 | Spreading Coefficient (Ws) obtained for chitosan solutions with different concentrations of Aloe vera gel and juice (0.5% and 5%) on blueberry. 48

Figure 21 | Inhibition effect of *Aloe vera* liquid fraction (juice) at different concentrations on *Botrytis cinerea* (a), *Penicillium expansum* (b) and *Aspergillus niger* (c). 50

Figure 22 | Inhibition effect of *Aloe vera* gel fraction at different concentrations on *Botrytis cinerea* (a), *Penicillium expansum* (b) and *Aspergillus niger* (c). 50

Figure 23 | Tritatable acidity expressed in g citric acid per 100 g blueberry, over time, for the different treatments. 52

Figure 24 | pH values of different blueberry treatments during 25 days of storage at 5.5 ± 0.6 °C(without forced contamination) and for treatments with forced contamination during 15 days storage at 5.5 ± 0.6 °C followed by 3 days at 25 ± 0.5 °C(from 15th to 18th days). 54

Figure 25 | Soluble solids contents (SSC) of blueberry samples during the 25 days of storage (5.5 ± 0.6 °C and 90 ± 3% of HR) (without forced contamination) and during 15 days at 5.5 ± 0.6 °C and relative humidity of 90 ± 3%, followed by 3 days at 25 ± 0.5 °C and humidity of 58 ± 5% (with forced contamination). 55

Figure 26 | Percentage of weight loss during 25 days storage at 5.5 ± 0,6 °C (without forced contamination) and for treatments with forced contamination during 15 days storage at 5.5 ± 0,6 °C and 3 days at 25 ± 0,5 °C (from 15th to 18th days). 57

Figure 27 | Total yeasts and molds plate count (log CFU/g) for control and coated samples of blueberries without forced contamination during storage (25 days) at 5.5 ± 0.6 °C. The maximum acceptable limit for consumption is 3.0 log CFU/g, represented by the horizontal orange line. 58

Figure 28 | Total yeasts and molds plate count (log CFU/g) for control coated sample of blueberries with forced contamination (Botrytis cinerea) during storage (18 days) at 5.5 ± 0.6 °C and 3 days at 25 ± 0,5 °C (from 15th to 18th days). The maximum acceptable limit for consumption is 3.0 log CFU/g, represented by the horizontal orange line. 59

Figure 29 | ΔE values for blueberries samples during 25 days of storage. B - blueberry control, BC - blueberry with chitosan coating, BCA - blueberry coated with chitosan-Aloe vera coating, BF - blueberry with forced contamination and BAF - blueberry coated with chitosan-Aloe vera coating and with forced contamination.. 66

Figure 30 | Representing the final results of the different treatments (B, BC and BCA) after 25 days of storage at 5.5 ± 0.6 °C and with an average relative humidity of 90 ± 3%..... 66

LIST OF TABLES

Table 1 | Taxonomic classification of blueberry, used for research in this thesis. 11

Table 2 | Nutritional Profile of Blueberries in 100 g of fruit (Sousa, Curado et al. 2007)… 13

Table 3 | Issues to considered when developing edible coatings (Soliva-Fortuny, Rojas-Graü et al. 2012; Poša, Ćirin et al. 2013)..... 17

Table 4 | Food applications of polysaccharide-based coatings and their functions 18

Table 5 | Taxonomic classification of *Aloe vera* (USDA, 2011)..... 23

Table 6 | Tested chitosan-based solutions..... 28

Table 7 | Spreading Coefficient (Ws) achieved for the tested chitosan solutions on blueberry..... 46

Table 8 | Percentage of Radical Scavenging Activity of *Aloe vera* fractions. 51

Table 9 | L*,a*and b*coordinates values of blueberries samples during 25 days of storage. 61

LIST OF ABBREVIATIONS

A_{transf}	Film area
ANOVA	Analysis of variance
B	Blueberry (control) without forced contamination
BAF	Blueberry coated with chitosan-*Aloe vera* coating with forced contamination
BC	Blueberry with chitosan coating without forced contamination
BCA	Blueberry coated with chitosan-*Aloe vera* coating without forced contamination
BF	Blueberry with forced contamination
BHA	Buthylated Hydroxyanisole
CFU	Colony forming units
Chi	Chitosan (1,4-linked 2-amino-2-deoxy-β-D-glucan)
CO_2	Carbon dioxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
FAO	Food Agriculture Organization
FDA	Food and Drug Administration
Fig.	Figure
G	Gram
Gly	Glycerol
Kg	Kilogram
$Mg(NO_3)_2 \cdot 6H_2O$	Magnesium nitrate
mN/m	miliNewton per metre
O_2	Oxygen
PDA	Potato Dextrose Agar
RH	Relative Humidity
RSA	Radical scavenging activity
SSC	Soluble Solids Content
TA	Titrable acidity

Twe80	Tween 80
USDA	United States Department of Agriculture
UV	Ultraviolet
X	film thickness
Wa	Work of adhesion
Wc	Work of cohesion
WL	Weight loss
Ws	Spreading coefficient
WVP	Water vapour permeability
WVTR	Water vapour transmission rate
ΔE	Distance metric
Δm	Mass change over time
Δ**P**	partial vapor pressure difference across the two sides of the film
Θ	Contact angle
γ_L	Surface tension

1. Thesis Motivation, Objective and Outline

1.1 Thesis Motivation

The increase consumption of fresh fruits is notorious in recent years, especially in developed countries. This is due not only to advances in technology and skilled labor in agriculture, allowing greater production and lower prices, but also due to population awareness about the importance of fresh fruit in a healthy diet. To answer to this pattern, food industry has shown a clear need to develop new technologies and several significant innovations in food packaging, with the purpose of create a more efficient system for food storage and to extend shelf-life of fresh food [1].

Food packaging serve a number of important functions, including protection of food against contamination maintaining the sensory quality and safety of food, as well as conferring convenience and acting as a vehicle for information about the product to the consumers. However, the environmental impact of non-biodegradable plastic material wastes is of increasing global concern. Thus, there is an urgent need to develop alternative renewable and biodegradable packaging to ensure food safety. In recent years, one of the most promising upgrades within food packaging field is the development of environmental friendly and active bio-based packages that may contribute to microbiological safety and protection from external factors [2].

The principles of this type of packaging are based on intrinsic properties of the polymer, used as packaging material, or on active agents incorporated therein. An active agent may be incorporated into the packaging material in multilayer structures, especially elements associated with packaging material or even adsorbed into their surface. This type of packaging system can provide several advantages such as controlled migration of active compounds, in order to keep the concentration of agent at the surface rather than directly adding the additive in the food product preventing loss of efficacy and/or the use of high concentrations of the additive [2].

Edible coatings are applied to food surface so that the final product is fitted for consumption. To some, coatings that are edible are those that are legal and safe to use on food products, to others, edibility of coatings implies that they have nutritional value. However, the aim is to keep food safety and its sensory qualities for the longest period of time as possible, without compromising environmental health, since one of the advantages of

this type of packaging is the use of biopolymers, biodegradable materials, thereby avoiding the accumulation of synthetic materials in nature [3].

The main food application of edible coatings is fruits and vegetables, to reduce moisture loss, to avoid subsequent softening and shriveling due to loss of turgor, and also to improve appearance. Coatings can also be used as carriers of functional ingredients, for example, fungicides, antibacterials, preservatives and antioxidants agents. These ingredients have been added to coatings to improve microbial stability, appearance, and texture of the food product [4].

Blueberries are currently one of the most valuable fruits worldwide, not only for its organoleptic characteristics, but also for their nutritional properties. However, these fruits are highly perishable and their qualitative properties diminish after a short period of time (about 2 weeks).

The fruit industry producers seek to improve preservation techniques of the fruit during postharvest period, for example, improving storage conditions and controlling the surrounding atmosphere through containers design, which limit losses due to physical damage [5]. Despite these efforts, losses associated with commercialization of blueberry remain a problem, especially due to infections by fungi, such as *Botrytis cinerea*, limiting the export of fruit and/or storage.

Therefore, edible coatings can be an alternative to common synthetic packaging or modified atmosphere packaging since they create a protective barrier against microbial agents, and a semi-permeable barrier to gases and water vapor, as well as be a vehicle of functional ingredients, in order to achieve a more efficient preservation of blueberry in postharvest stages.

1.2 Research Aims

The main objective of this thesis was to develop chitosan-based edible coating/film, in which *Aloe vera* was incorporated, to extend the shelf life of blueberry.

To reach this goal, several evaluations have to be performed:

- Define optimal concentration of biopolymer (chitosan) to use directly in blueberry (through spreading coefficient measurements);
- Determine the need of incorporation of plasticizers (glycerol) and surfactants (Tween 80) in chitosan-based solution, and respective concentrations;
- Study the antifungal and antioxidant capacities of different *Aloe vera* fractions concentrations (gel and juice);
- Evaluate the effect of *Aloe vera* juice incorporated in chitosan film to minimize blueberry physico-chemical and microbiological changes during the storage time.

1.3 Outline

The present thesis is organized in six chapters, being the present chapter the Chapter I, which described the motivation, research aims and outline of the thesis. The following Scheme shows how chapters 2 to 6 are organized.

Chapter II

- "INTRODUCTION" This chapter presents a review information about thesis theme and its topics.

Chapter III

- MATERIALS AND METHODS" Chapter III presented materials and methodologies used for edible coatings preparation, characterization, and blueberry application. *Aloe vera* juice/gel extraction was also described.

Chapter IV

- "RESULTS AND DISCUSSION": In this chapter, the influence of *Aloe vera* on physicochemical properties of chitosan-based coatings/films was presented. The impact of this coating in the shelf life extension of blueberry fruit was also discussed.

Chapter V

- "CONCLUSIONS": General conclusions and recommendations for future work were presented in this chapter.

Chapter VI

- "BIBLIOGRAPHY"

2. General Introduction

2.1 Introduction

In recent years, edible films and coatings have been considered as one of the technologies with greater potential to improve microbiological safety of food and to protect it from the influence of external environmental factors, thus increasing shelf life of food [6].

One of the major potential in the development of coatings/films is the use of materials derived from renewable sources such as hydrocolloids of biological origin, and incorporation of functional ingredients that can be used, for example, as antioxidants and/or antimicrobial agents [4]. A comprehensive study of several parameters such as functional compounds concentration incorporated within coatings, their stability, chemical structure, degree of dispersion, and interaction with the polymer-based coating is of high importance [2].

Naturally, this category of active packaging may be an important alternative to protect fresh food, particularly those with added value such as berries.

The production of small fruits, such as blueberry, a fruit of high commercial and nutritional value, is reportedly growing worldwide including in Portugal. The growing demand and the nutritional characteristics and value added of blueberry make it a target for this type of coating.

In the Nordic countries, for instance, the consumption of blueberry and other small fruit has increased by 300 percent. Portugal has perfect environmental conditions to place on the market high quality products, such as blueberries. Considering its increasing consumption trend, edible coatings can potential prevent precocious blueberry spoilage and increase shelf life of this fruit, avoiding a depreciation of the product [7].

2.2 Blueberry

Blueberry is a fruit that awakening general interest from producers to consumers and researchers given to its attractive economic return, their nutritional properties and exotic flavor.

In Portugal, it grows spontaneously in North (Minho and Trás-os-Montes) and Serra da Estrela regions. In 2014, blueberry crop is quite widespread throughout Portugal, having strong potential to become a major share to country agricultural economy (FAO database).

According to FAO (Food and Agriculture Organization), Portugal is, since 2004, one of the 20 main blueberry producers in the world, occupying the 17th place, a rank led by USA. As stated in Figure 1, blueberry production is set to increase sharply from year to year.

Blueberry is a climacteric fruit, i.e., maturation and ripening processes are continuous with increase ethylene production rates and respiration until senescence stage. They are juicy and possess a bittersweet taste [8]. Known for their rich antioxidant compounds content, including flavonoids, phenolic acids, tannins, and anthocyanins, it is believed that these antioxidants, individually or synergistically, combat free radicals, and help protect against cardiovascular disease, cancer, inflammation, obesity, diabetes, and other chronic diseases [9, 10]. Due to blueberry low glycemic index, this fruit is ideal for diabetics and anyone seeking to avoid foods with high sugar content [11]. It is also commonly used in combination with cranberries to treat urinary tract disorders, due to their high vitamin C content, minerals (e.g. magnesium, potassium, calcium, phosphorus, iron, or manganese) and other compounds (e.g. pectin, tannin, citric, malic and tartaric acids) [12, 13].

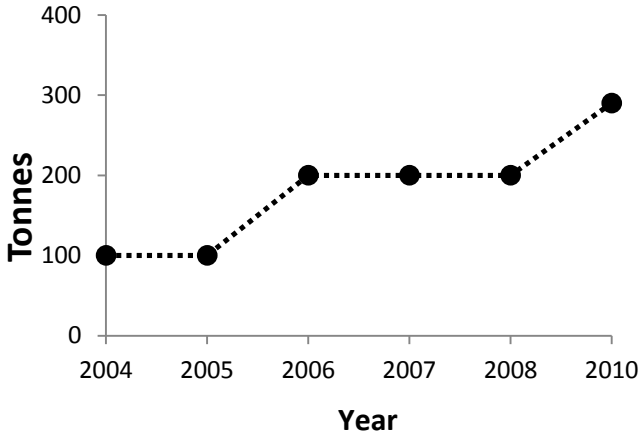


Figure 1 | Blueberry production in Portugal from 2004 to 2010 according to FAO.

2.2.1 Taxonomic Classification and Morphology

The blueberry plant, *Vaccinium spp*, is a genus of shrubs or dwarf shrubs in the plant family Ericaceae (Tab. 1), which includes more than 450 plants that grown around the world. Taxonomically there is a variety of species of blueberry. The predominant cultivated blueberry species are *Vaccinium corymbosum L., Vaccinium ashei* Reade and *Vaccinium angustifolium* Ait. [14] . It is a perennial, consisting of a shallow root system and woody canes

that originate from the crown. The root system is very fibrous with many fine feeder roots but no root hairs. A mature cultivated blueberry bush usually has 15 to 18 canes [15]. The plants included in this family have a huge disparity in appearance and dimensions, ranging from just a few inches tall, as *V. macrocarpon*, (a plant that produces branches that can reach two meters length), passing by *V. myrtillus* and *V. corymbosum* from Europe (herbaceous stems does not exceed one and half meters), up to ten meters height shrub (e.g. *V. ashei* from USA southern).

Table I | Taxonomic classification of blueberry, used for research in this thesis.

Kingdom	Plantae
Phylum	Magnoliophyta
Class	Magnoliopsida
Order	Ericales
Family	Ericaceae
Genus	*Vaccinium*
Species	*Vaccinium corymbosum*

Growth habit varies among cultivars; some bushes grow very upright, while others have a more spreading growth habit. Fruit is born on buds formed on previous growing [16].

Blueberry fruits are berries formed from the development of an inferior ovary. The fruits ripen, usually around 2-3 months after flowering, depending on the cultivar and weather conditions, particularly temperature and plant vigor (Fig. 2).

Their size varies, usually between 7 and 20 millimeters in diameter. Depending on the species, they have dark blue or red tint when total ripened, and are covered by a wax layer called bloom. This wax layer may present a potential barrier to water loss, mainly after few days of harvest, thus preventing a rapid leak [17].

However, berry has a furrow, diametrically opposite to apex varying in size and shape, depending on the species and cultivar. The furrow of the blueberry, when wet and large, can

be a focus of microbial contamination and may also cause postharvest depreciation, loss of moisture that bloom cannot prevent by itself [18].

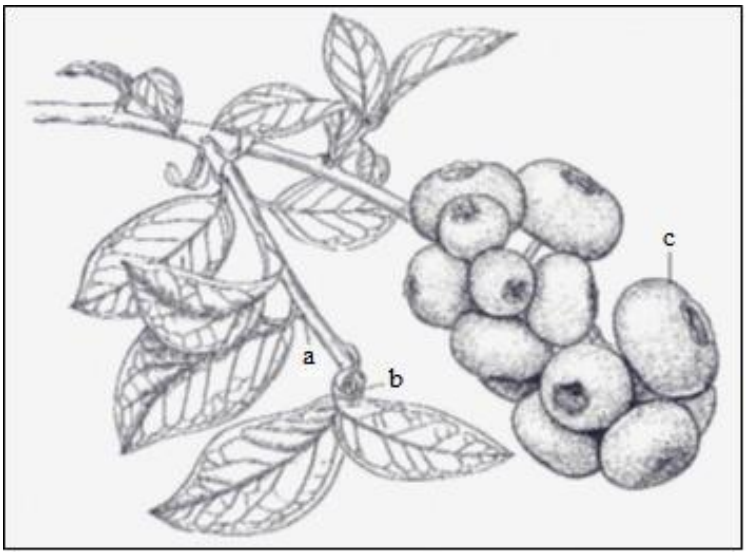


Figure 2 | Fruitful Blueberry branch: a) Axiliary bud; b) terminal aborted bud; c) fruits [17].

2.2.2 Chemical Composition

Chemical composition of fruits is conditioned both by genetic (cultivar) and environmental factors (weather, agronomic practices).

The blueberry has, on average, about 85% water. The water content of the tissues depends, among other effects, of soil water availability at the time of harvest. Sugars are the main soluble components and represent about 80% of dry matter. Glucose and fructose are the main sugars in blueberry and its content influence its taste (Tab. 2). It contains also, organic acids at high levels, the most common being citric, malic and quinic acids [19, 20].

Blueberries contain multiple nutrients, phytonutrients, polyphenols, salicylic acid, carotenoids, fiber, folate, vitamin C, vitamin E, manganese, iron, riboflavin, niacin and phytoestrogens. They are also rich sources of antioxidant, with higher levels of phytochemical content, being mainly represented by anthocyanins, procyanidins, chlorogenic acid and other flavonoid compounds [21]. Anthocyanins with different aglycones and sugar moieties have different bioavailability and potential health effects. Recent studies have shown that antioxidant compounds can neutralize free radicals, which are unstable molecules that cause the development of a number of diseases such as cancers, heart, cardiovascular and

neurodegenerative diseases [22]. They have also shown the ability to slow the aging process [23].

Table 2 | Nutritional Profile of Blueberries in 100 g of fruit [15].

Nutrients		Minerals		Vitamins and other componentes	
Humidity	83-87 g	**Calcium**	11.4-12.2 mg	**Vitamin C**	22-62 mg
Energy value	51-62 kcal	**Iron**	0.6 mg	**Tannins**	270-550 mg
Proteins	0.4-0.7 g	**Magnesium**	5.8-8.4 mg	**Pectins**	300-600 mg
Lipids	0.5 g	**Phosphorus**	14-47 mg	**Anthocyanins**	300-725 mg
Glucose	5-7 g	**Potassium**	48-112 mg		
Fructose	5-7 g	**Sodium**	3.4-4.3 mg		
Saccharose	Nd	**Zinc**	0.1 mg		
Fiber	1-1.5 g	**Copper**	0.1 mg		
Ash	0.19-0.25 g	**Manganese**	0.4-1.2 mg		

2.2.3 Blueberry Production – Considerations

The responsibility of ensuring food products safety is of all those involved at any stage of the food chain. Trade globalization has led to creation of common rules to be adopted by all those who interact with food. Therefore, in regard to the agricultural sector, this is an important stage in the food chain, it can be say that agricultural practices should have a good relationship with food security.

The harvest periods differ according to the blueberry plant, altitude and region. In Portugal, crops run from mid-April to early September. In the case of Sever do Vouga, one of the biggest location of blueberry production in Portugal, harvest runs from mid-May to mid-August.

During berries growth and maturation three different phases can be distinguished. The first stage is characterized by a rapid increase on the berry, a result of a rapid cell division

and increased cell size, which lasts about a month. During the second stage, berry size increases slightly but the embryos within the seed develop and become mature. In the third stage, berry begins to mature and undergoes a rapid increase in volume resulting from a large increase in the volume of cells; this phase lasts approximately 16 to 26 days [15]. The first growth phase occupies 60% of time, whereas second and third growth phases occupy 30% to about 10% of time.

Maturation occurs in the period corresponding to the third phase during which tissues soften, chlorophyll content decreases and anthocyanins content increases, consequently, berries passed from green to blue color. Likewise, the sugars and other soluble components increase in this phase, acidity decreases slowly and breathing decreases after a quick change [15].

The blueberry bush, like most plants need soil to hold the roots and to supply water and minerals necessary for growth and fruit formation. The physical and chemical soil characteristics are important; blueberry cultivars require a soil with acidic pH. The blueberry prefers sandy soils, sandy loam or moderately loamy, not very deep, with good aeration and drainage, ensuring that fine and fibrous roots can spread [24].

Climatic factors act differently upon development stages in which plant is found, i.e. during the dormant or vegetative phase, determining production potential. During dormant phase, cold is the most important factor, so, plant must pass at least 700 hours at 7 °C. Most cultivars are unaffected by winter temperatures of -18 °C. In vegetative stage, plants are vulnerable to cold winds in late spring that might occur after the opening of the flowers. Temperatures above 30 °C in summer can lead to death of the leaves, especially in cultivars of rapid vegetative growth that are fully exposed to the sun. At these temperatures the roots cannot absorb water enough to offset the losses by transpiration carried out by the leaves [15].

2.2.4 Postharvest Physicochemical Changes

From the moment that blueberry is harvest, the fruit no longer receives nutrients from the mother plant, provoking changes at structural, chemical, nutritional and biochemical level, until it is no longer edible.

Postharvest physiological disorders are normally caused by nonpathological factors such as inappropriate temperature, humidity, and gas storage conditions, which affect the functioning of plant system [8]. However, changes in post-harvest period can be accelerated by action of microorganisms, mostly by fungal outbreaks [25].

Some of the major changes in blueberries during ripening are pH, acidity, water loss and soluble solids content that influence fruit organoleptic characteristics; they are important parameters to determine potential blueberry deterioration [15].

2.2.5 Postharvest Microbiological Changes

Fungi growth is especially difficult to control because of their ability to metabolize many substances. Postharvest deterioration, caused by fungal activity, is an important concern in commercial blueberry production. Thus, it is necessary to find new ways to reverse this scenario [26]. Bio-based coatings may be a potential ecofriendly barrier for fungal occurrence, avoiding qualitative losses of blueberries. [27].

Water loss leads to fruit wilting and mass loss, but high moisture content makes them generally susceptible to deterioration, increasing the possibility of contamination by microbial growth.

Blueberries deterioration is, normally, caused by fungi, such as *Colletotrichum acutatum* (Anthracnose), *Botrytis cinerea* (a haploid necrotrophic fungal pathogen of numerous plants even its low temperature resistance, it can survive and even grow slowly at temperatures as low as 1 °C) and *Alternaria spp*. (Alternaria rot) [26, 27, 28]. Other potentially dangerous fungi to blueberries are *Alternaria tenuissima*, *Aspergillus niger* and *Penincillium expansum* [29] (Fig. 3).

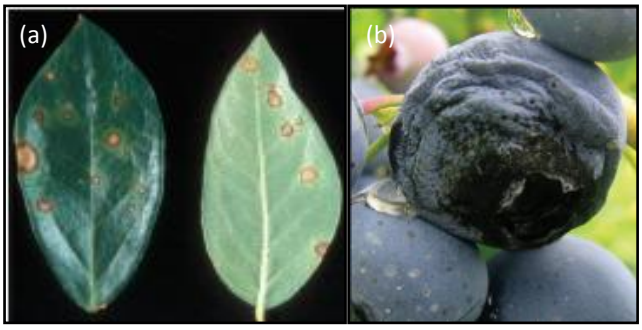


Figure 3 | Leaf infection (a) and berry infected in the field (b), due to *Alternaria tenuissima*.

2.3 Edible Coatings

Edible coatings have been used for centuries to protect foods and prevent moisture loss. Natural cuticle waxes on surface of fruits and vegetables help to protect against excessive transpiration and consequently, water loss. In cleaning fresh fruit or simply with time, this natural barrier is disturbed or washed away and needs to be replaced [4].

Coatings comprise the formation of a thin layer directly on the surface of a food product. They intend to protect and produce a modified atmosphere on coated fruits by isolating the coated product from the environment (Fig. 4) [5].

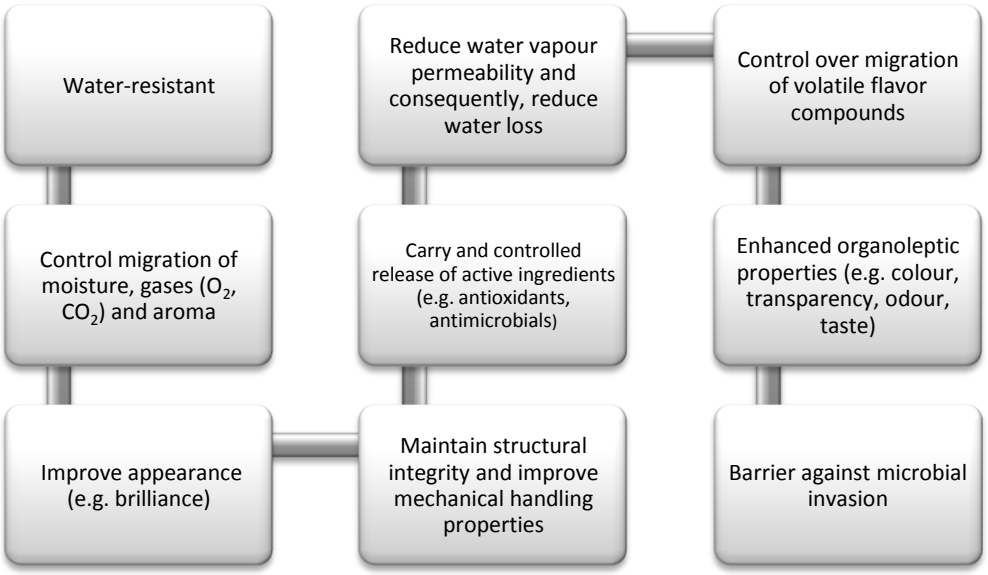


Figure 4 | Main functions of edible films and coatings for food application to extend shelf-life.

The immersion method is generally used to coat fruits, cheeses, vegetables, fish and meat. Here, food product is directly immersed within coating formulation (in aqueous medium) following by excess removal. The coating is then dried forming a film on food surface. A coating must present several demands to achieve validity, safety, and performance capability, as we can see in Table 3. During storage, it must not ferment, coagulate, separate, and develop off-flavors. Once applied, it should not crack, discolor, or peel during handling

and storage, including when in contact with condensate. Finally, it should not react adversely with the food, and not impair food quality [8].

Table 3 | Issues to considered when developing edible coatings [4, 8].

Chemical safety	As with all food ingredients and additives, safety is a fundamental requirement. However, much is still unknown about the safety of all food additives, including coating ingredients.
Cost	Ingredients and method of application.
Barrier properties	Ideal coatings form an acceptable barrier for gas exchange between food and atmosphere, or between two phases of the same food item, neither too restrictive nor too permeable.
Food quality	Coatings tend to change appearance, flavor, and mouthfeel, and efforts are needed to achieve changes that are good, not harmful.
Nutritive value	Some coatings are so thick that they change the nutritional value (e.g., frosting increases the caloric value of cake).
Environment	Volatile organic compounds (usually alcohol) are sometimes released when edible coatings dry.

In order to improve the efficiency and stability of edible coatings/films it is essential to find adequate materials. In the particular case of the food sector it is also important to replace non-food-grade materials by bio-based and biodegradable food-grade materials. Coatings/films can be produced using a wide variety of products such as polysaccharides, proteins, lipids or resins, alone or, more often, in combination. The development and use of new coatings produced from naturally-occurring materials represent several potential applications in different fields as pharmaceutical, food and biotechnology. Biopolymers, the main ingredient of edible coatings [4], may constitute an alternative source to synthetic packaging due its biodegradability, preventing environment-related problems.

These coatings may be improved using some compounds such as plasticizers and surfactants to improve structural properties and coating adhesion to food surface [30].

The properties of the coatings can be enhanced using functional ingredients incorporated in the coatings such as antibrowning and antimicrobial agents, nutraceuticals, volatile precursors, and colors [5]. Other ingredients, such as preservatives, antioxidants, and firming agents have all been added to coatings to improve microbial stability, appearance, and texture of coated product. Often, essential oils, acids, or natural plant extracts that have antimicrobial activity are incorporated into coatings [4].

2.3.1 Polysaccharides

Polysaccharides are natural polymers composed by monosaccharide units that are linked by glycosidic bonds. The polysaccharides are present in plants, fungi, bacteria, algae and animals. Environmental conditions and monomer sequence can be responsible for molecular structure of polysaccharides [31]. Each polysaccharide has different chemical structures, molecular weight, degree of branching, flexibility, and electrical charge [32]. Besides, depending on ionic groups presented in polysaccharide chain, some polysaccharides can be neutral (e.g. starch and cellulose), anionic (e.g. alginate, xanthan gum), or cationic (e.g. chitosan). These differences allow polysaccharides to present different functional properties such as: solubility, thickening, gelation, water holding capacity, surface activity, emulsification and digestibility [31].

Table 4 | Food applications of polysaccharide-based coatings and their functions [4].

Foods	Coatings	Function of coating	Reference
Fresh-cut fruits: Apple, pear, strawberry, papaya, mango, red pitaya	Carrageenan, alginate, gellan gum, apple puree + pectin, maltodextrin, cellulose derivatives, chitosan	Antioxidant, antimicrobial, and nutraceutical carrier; gases and moisture barrier; antifungal protection; color, flavor, and texture improvement	Assis and Pessoa, 2004; Baldwin et al., 1996; Brancoli and Barbosa-Cánovas, 2000; Chien et al., 2007a, 2007b; Del-Valle et al., 2005; Lee et al., 2003; McHugh and Senesi, 2000; Olivas et al., 2003; Rojas-Graü et al., 2007a, 2007b; Tapia et al., 2007
Whole fruits and vegetables: Apple, grape, cherry, plum, strawberry, baby carrot, mango, banana, kiwi, zucchini	Cellulose derivatives, chitosan, cactus-mucilage, xanthan gum	Gases, lipid and moisture barrier; antifungal protection; color, flavor, and texture improvement; retention of functional compounds; antimicrobial and functional compounds carrier	Baldwin et al., 1999; Chien et al., 2006, 2007c; Conforti and Totty, 2007; Diab et al., 2001; Han et al., 2004; Kaynas and Ozelkok, 1999; Martínez-Romero et al., 2006; Mei et al., 2002; Serrano et al., 2006; Valverde et al., 2005; Yaman and Bayoindirli, 2002
Meats, poultry, fish and seafood	Carrageenan, alginate, cellulose derivatives, chitosan, pullulan	Oxygen, lipid and moisture barrier; texture improvement; antioxidant and antimicrobials carrier; bacterial growth prevention	Hargens-Madsen, 1995; Holownia et al., 2000; Jeon et al., 2002; Ouattara et al., 2000; Oussalah et al., 2006; Sathivel, 2005; Wu et al., 2001
Nuts and cereals	Starch, dextrin, cellulose derivatives	Oxygen and moisture barrier	Laohakunjit and Kerdchoechuen, 2007; Noznick and Bundus, 1976; Roudaut et al., 2002

Table 4 shows some food applications of polysaccharides as edible coatings, such as cellulose derivatives, starch derivatives, chitosan, pectin, carrageenan, alginate and gums.

Polysaccharides coatings are widely available and nontoxic. They are also selectively permeable to CO_2 and O_2, and thus delaying ripening and respiration of many fruits and vegetables by limiting O_2 availability [33]. Polysaccharide coatings are hydrophilic and are poor barriers to moisture. To improve this property, other compounds may be added, such as surfactants, plasticizes, oils and plant extracts [4].

2.3.1.1 Chitosan

Chitosan (1,4-linked 2-amino-2-deoxy-β-D-glucan) (Fig. 5) is one of the most abundant polysaccharide in nature, resulting from the deacetylation of chitin by alkaline hydrolysis at high temperature. Chitin is the principal constituent of exoskeleton of approximately 1 million species of arthropods, such as insects, crabs and lobsters. It is a linear polysaccharide (Fig. 5) composed of N-acetyl D-glucosamine units in β bond [34]. Use of chitin in many industrial processes generate solids residues. Thus, use of chitosan would be an alternative to minimize these residues. Chitosan presents potential reactive functional groups, amine groups, and a number of primary and secondary hydroxyl groups [35].

Chitosan is not soluble in water, but it forms viscous solutions in various organic acids. For example, lactic acid has been used to dissolve chitosan [36].

Due to chitosan characteristics such as biodegradability, antimicrobial activity, biocompatibility , coating ability and non-toxicity, this polysaccharide has been studied for application in different areas, with primary emphasis on food and pharmaceutical industries (such as in cosmetics and drug formulation), but also in medicine, agriculture, and environmental [37]. Chitosan's safety can be evaluated by its lethal doses (1.6 g/kg of body weight in rats), being comparable to those of sugar and even less toxic than salt. For all these reasons, chitosan has been accepted as a dietary supplement or food additive in many countries, such as Italy, France, Norway, Poland, United States of America, Argentine and Japan [21].

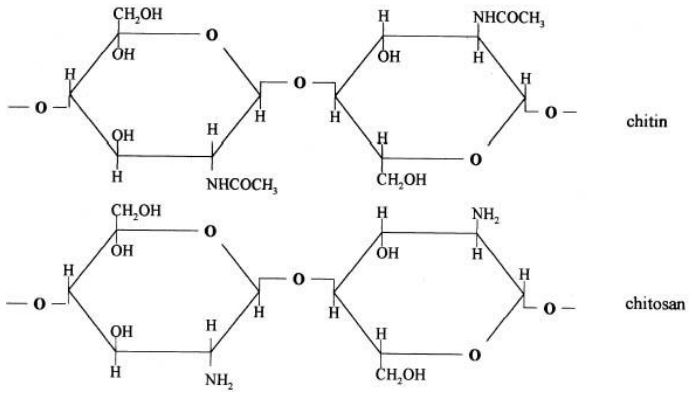


Figure 5 | Molecular structure of chitin and chitosan.

Chitosan may also be used to produce edible films and coatings due to their viscoelastic properties, yielding tough, durable and flexible films. Thus, it can form semipermeable coatings, thereby delaying ripening and decreasing water migration. It also presents some positive effects against fungi and phytopathogens [8]. Several studies placed chitosan as a reference polysaccharide for fresh fruit coatings. For instance, Got et al. have been successfully tested chitosan as an edible coating to extend the shelf-life and maintain quality of strawberry fruit [38]. Most mechanical properties of chitosan films are comparable to many commercial polymers [39]. Chitosan films may improve food conservation and quality; their properties depend on several parameters such as chitosan molecular weight and degree of deacetylation. To improving their functionality, plasticizers may be added to improved mechanical properties, or surfactants for increased stability and better adhesion [8].

2.3.2 Plasticizer and Surfactant Additives

Generally, edible films and coatings are formed by hydrocolloids, proteins and polysaccharides, serving as a structural matrix. However, sometimes it is necessary to incorporate into hydrocolloid matrix other compounds such as surfactants and plasticizers to improve flexibility, stability and improved barrier properties [40].

A plasticizer, in most cases, is required for manufacturing biopolymer films, because films structure is often brittle and stiff due to extensive interactions between polymer molecules. Plasticizers decrease intermolecular forces between polymer chains, thus increasing flexibility and extensibility. Generally, plasticizers act by entering between

polymeric molecular chains, physicochemical associating with the polymer, reducing cohesion within the film network, and effectively softening the film structure [41, 42].

Main non-volatile plasticizers are glycerol, sorbitol, propylene glycol or polyethylene glycol [43]. Glycerol is the most used plasticizer due to its good plasticization efficiency, suitable, large availability and low exudation [41].

Surfactant addition to edible coatings/films is often used to emulsify and lower surface tension, improving wettability and adhesion [41]. It may decreases hygroscopicity due to increase in hydrophobic portions in film, forming a homogenous film [4, 8, 44]. With the addition of these compounds, such as Tween 80, is expected a decrease in water vapor permeability values, due to the incorporation of components with some hydrophobic characteristic [41]. Tween 80 is an aliphatic, nonionic surfactant approved by U.S. Food and Drug Administration (FDA) for parenteral, oral and topical application. It is used as an excipient in pharmaceutical formulations to stabilize aqueous formulations of hydrophobic medications [8].

2.3.3 Bioactive compounds

Chemical fungicides have been intensively used to control fruits diseases' in postharvest conditions, nevertheless these actions have developed resistance in pathogens, led to atmosphere contamination, and affected people's health through residual toxic compounds present in food [27, 45, 46]. In order to avoid use of these chemicals in postharvest period, different bioactive compounds may be incorporated into chitosan films, and thus form a more effective barrier against food spoilage by microbial action.

To enhance efficacy of friendly bio-based coating, such as chitosan coating, against pathogens and expand the scope of its application without resorting to chemicals compounds, natural occurring antimicrobial agents incorporation could be an option. For example, essential oils (thymol and carvacrol), have been incorporated into chitosan coating to fight several foodborne pathogenic bacteria [47].

Bioactive compounds from plant extracts(e.g. *Aloe vera* extract) have been studied in order to increase antimicrobial and antioxidant coating capacity and consequently, to improve quality of coated food [48, 49, 50].

2.3.4 *Aloe vera*

Aloe vera (Aloe barbadensis Miller), a perennial plant with turgid green leaves, tropical or sub-tropical herb, monocot and almost sessile is one of the most biologically active plant, since it is a rich source of antimicrobial and antioxidant agents [51].

The plant contains two separate juice materials: (1) yellow exudate (designated latex), known for its laxative capacity, extracted from vascular bundles at junction between rind and fillets and, (2) a transparent mucilaginous gel, extruded from the inner pulp. Chemicals constituents in higher quantities and more active are phenolic compounds, particularly hydroxyanthraquinone derivatives, such as aloin [52].

Recent studies have demonstrated that extracts from *Aloe vera* leaves are widely used against numerous forms of diseases in fruits and vegetables, caused by fungi [27, 49].

Plant genus *Aloe* has a history of economic and medicinal use that spans thousands of years and it's a source of some of the oldest known herbal medicines. Its name is probably derived from the Arabic word "Alloeh" which means bright and bitter substance [53]. Their applications were recorded in ancient cultures of India, Egypt, Greece, Rome, Japan and China [53]. Egyptians bathed with *Aloe vera* to ensure beauty and power. Alexander the Great, used this plant to treat wounds of his soldiers. To the present days, "harmonious remedy" or "elixir of immortality", as it is treated in China, *Aloe vera* has been considered over time an appropriate medical plan for all situations [54].

Thereby, in this thesis, *Aloe vera* was selected as a source of bioactive compounds to be incorporated into chitosan coating with a potential capacity of preventing/minimizing blueberry postharvest deterioration process.

The pulp of field-grown *Aloe vera* is reported to have a pH of 4.4–4.7 and a soluble solids content of 0.56–0.66%; however, have a seasonal fluctuations due to water availability [53].

According to European Medicines Agency (2006) there are no recents systematic preclinical tests for aloes or preparations thereof.

Tests in rats were carried out and the results demonstrated that no teratogenic or foetotoxic effects were seen after oral treatment with aloes extract (up to 1,000 mg/kg) or aloin A (up to 200 mg/kg) and no specific toxicity was observed in mice when aloes extract was orally administered up to 50 mg/kg daily for 12 weeks and aloin was orally administered up to 60 mg/kg daily for 20 weeks.

2.3.4.1 Taxonomic Classification

Aloe vera is a member of the family Liliaceae (Tab. 5), which comprises more than 360 different species in the world. It is considered a promising plant due to wide range of medicinal and nutritional attributes, and it is one of major industrial crops used in food, pharmaceutical and cosmetic industries [55, 56].

Table 5 | Taxonomic classification of *Aloe vera* (USDA, 2011).

Kingdom	Plantae
Phylum	Magnoliophyta
Class	Liliopsida
Order	Liliales
Family	Liliaceae (Aloeacea)
Genus	*Aloe L.*
Species	*Aloe vera* (L.) Burm. f. (*Aloe Barbadensis* Miller)

2.3.4.2 Chemical Composition

A. vera is known for their nutraceutical and cosmeceutical properties, such as, anti-viral, anti-bacterial, laxative, anti-oxidant, anti-inflammation, anti-cancer, anti-diabetic, anti-allergic, immuno-stimulation, UV protecting activities [57]. This specie is rich in vitamins A, C and E, especially B complex (fundamental for brain function, neuro-psychic and energy supply); minerals, such as selenium, calcium, potassium, sodium; enzimes (such as peroxidase, cellulase, carboxypeptidase, amylase, alkaline phosphatase); unsaturated fatty acids or sterol (anti-inflammatory agents); polysaccharides, responsible for quite a few healing abilities of this plant; essential and nonessential amino acids, important in tissue repair; and saponins, salicylic acid (anti-inflammatory and analgesic properties) and lignin (a substance that facilitates absorption of other substances) [54](Fig. 6).

As for general precautions, *Aloe vera* gel/juice fractions are contraindicated in cases of known allergy to plants of family Liliaceae (WHO 1999).

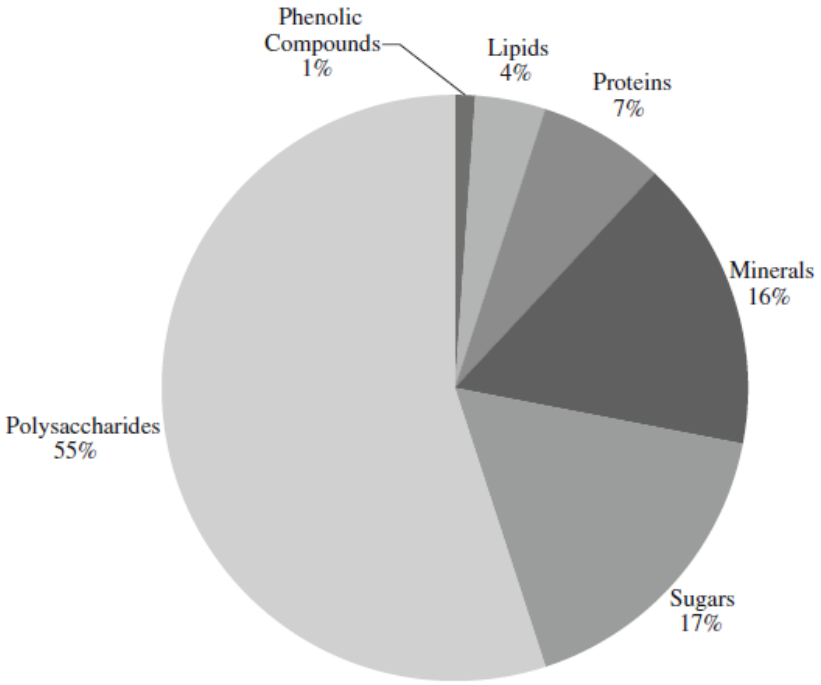


Figure 6 | Chemical composition of *Aloe vera* gel, on dry weight basis [54].

2.3.4.3 *Aloe vera* in the Context of Coatings

Although *A. vera* gel is best known for its therapeutic effect, nowadays, *A. vera* is used as an additive to fruits and vegetables products (as an ingredient, coating, etc.) [58].

A. vera gel as edible coating showed efficacy on maintaining several fruits postharvest quality such as sweet or sour cherry [59], nectarine [60], table grape [27, 61], strawberry [62], peach and plum [63]. In addition, preharvest application of *A. vera* gel showed benefits in terms of delaying postharvest ripening of table grape [27, 64].

At this time, Portuguese market has a wide variety of dietary supplements containing *Aloe vera*, since legislation allows products derived from herbs/plants to be marketed in Europe as both medicinal and food products [53].

Thus, *Aloe vera* incorporation in chitosan-based coatings results in a potential antifungal and antimicrobial barrier, as well as water loss and gas exchange control, avoiding premature blueberry deterioration. This can bring many benefits to blueberry, prolonging its shelf life and maintain its nutritional quality.

3. MATERIALS AND METHODS

3.1 Blueberry Samples

In this study, the blueberry specie tested was *Vaccinium corymbosum*, produced and harvested in Sever do Vouga (Mirtilusa). The Mirtilusa is one of the leading companies in Portugal in terms of blueberries exportation. Thus, samples of blueberries provided have guaranteed quality and homogeneity of the fruit samples, needed for the development of this research.

The entire process of postharvest period was followed, from harvesting (July, 2014) to the packaging and transportation, in order to maintain the integrity of the fruit.

3.2 Preparation of coating-forming solutions

3.2.1 Materials

Chitosan (AquaPremier CO., Thailand), glycerol (86-89%, SigmaAldrich Co., Louis, MO, USA), Tween 80 (Acros Organics, Belgium), L(+)-Lactic Acid 90 % (Acros Organics) and distilled water.

3.2.2 Methods

The coating formulations were based in a three-level factorial design with chitosan concentrations of: 0.5%, 1.0% and 1.5% (w/v); glycerol concentrations of: 0.5%, 1.0% and 1.5% (v/v); and Tween 80 concentrations of: 0%, 0.1% and 0.2% (w/v) (Tab. 6).

Table 6 | Tested chitosan-based solutions.

Formulation	Chitosan (w/v)	Glycerol (v/v)	Tween 80 (w/v)
1	0.5	0.5	0
2	0.5	0.5	0.1
3	0.5	0.5	0.2
4	0.5	1.0	0
5	0.5	1.0	0.1
6	0.5	1.0	0.2
7	0.5	1.5	0
8	0.5	1.5	0.1
9	0.5	1.5	0.2
10	1.0	0.5	0
11	1.0	0.5	0.1
12	1.0	0.5	0.2
13	1.0	1.0	0
14	1.0	1.0	0.1
15	1.0	1.0	0.2
16	1.0	1.5	0
17	1.0	1.5	0.1
18	1.0	1.5	0.2
19	1.5	0.5	0
20	1.5	0.5	0.1
21	1.5	0.5	0.2
22	1.5	1.0	0
23	1.5	1.0	0.1
24	1.5	1.0	0.2
25	1.5	1.5	0
26	1.5	1.5	0.1
27	1.5	1.5	0.2

The concentrations were chosen based on preliminary tests, where it was determined that 1) chitosan dissolution was difficult for contents above 2% (w/v), 2) previous studies indicated that a glycerol maximum concentration of 1.5% (v/v) would be necessary to keep the resistance of the film, while for values lower than 0.5% the film would be too brittle 3) and chitosan films produced with Tween 80 above 0.2% (w/v) presented excessive oiliness.

Under these circumstances, coating solutions were prepared, dissolving the chitosan (concentrations of 0.5, 1.0 or 1.5% w/v) in a lactic acid (1.0% (v/v)) aqueous solution under agitation using a magnetic stirrer during 10 hours at room temperature (20 °C), to obtain a homogeneous solution. Then, Tween 80 was added as a surfactant (concentrations of 0.1% or 0.2% (w/v)) and glycerol was added as plasticizer (concentrations of 0.5, 1.0 or 1.5% v/v), with agitation during 3 hours at room temperature, to reach complete interaction.

To prepare the films, a constant amount (28 mL) of coating-forming solution was cast in a Petri dish, with 9.6 cm diameter, to maintain film thickness. The Petri dishes wereplaced in an oven at 35 °C during 24 hours.

Films were subsequently stored in a desiccator containing a saturated solution of $Mg(NO_3)_2 \cdot 6H_2O$ at 20 °C and 50% RH at least 48 hours, in order to perform water vapor permeability and thickness measurements. Five thickness measurements were randomly taken on each testing sample at different points with a digital micrometer (No. 293-5, Mitutoyo, Japan). Mean values were used to calculate water vapor permeability (WVP).

3.3 Coating/Film characterization

3.3.1 Surface Tension and Wettability

Determination of wettability, the study of the adhesion and cohesion coefficients, as well as the study of surface properties of the food products, is crucial, once the correct formulation choice is essential for the maintenance and durability of the coating on the food products. The formulations of edible coatings must wet and spread evenly on the surface of the fruit. After drying, the coating must have an adhesion, cohesion and suitable strength [42].

In order to obtain a uniform spreading on blueberry surface, spreading coefficient (Ws) (Eq. 1) and the works of adhesion (Wa) and cohesion (Wc) values need to be determined. The wettability of the blueberry surface was determined for each formulation of chitosan, glycerol and Tween 80, and thus the best formulation was selected.

$$Ws = Wa - Wc \quad \textbf{(Eq. 1)}$$

While the forces of adhesion (Wa) (Eq. 2) favor the spreading of liquid (chitosan based formulations) on the solid surface (blueberry), the forces of cohesion (Wc) (Eq. 3) promote their contraction.

$$Wa = \gamma_L (1 + \cos(\theta)) \quad \textbf{(Eq. 2)}$$

$$Wc = 2\,\gamma_L \quad \textbf{(Eq. 3)}$$

To obtain Ws, contact angle (θ) and surface tension (γ_L) were determined. The contact angle at the blueberry surface was measured by the sessile drop method, and

observed with a face contact angle meter (OCA 20, Dataphysics, Germany). The samples of the coating solution with different concentrations of their constituents (chitosan, glycerol and Tween 80) were taken with an automatic piston 500 µL syringe (Hamilton, Switzerland) with a needle of 0.75 mm of diameter, The contact angle at the solid surfaces was measured, using computer aided image processing using a digital camera. To avoid changes on the blueberry, measurements were made in less than 45 seconds and stored in a refrigerated place.

The surface tension of the coating solution was measured by the pendant drop method using the Laplace-Young approximation, in which a droplet of the tested liquid was placed on a horizontal surface and observed [65].

Twenty replicates of contact angle measurements and four replicates of surface tension measurements were obtained at room temperature, for each formulation.

3.3.2 Water Vapour Permeability

The water vapor permeability (WVP) of chitosan films with or without *Aloe vera* was determined gravimetrically, using ASTM E96-92 procedure, with some modifications [66]. The film was sealed on the top of a permeation cells. The permeation cell was filled with 50 ml of distilled water in order to generate a 100% RH and covered by the film (2337 Pa vapor pressure at 20 °C). Then, the cells were weighted using an analytical balance (Mettler AE200) and placed inside a desiccator containing silica (0% RH; 0 Pa water vapor pressure; the air circulation kept constant by using a fan inside the desiccator). The tests were conducted in triplicate and changes in the weight of the cells were recorded at intervals of 2 hours during 10 hours (in triplicate). The WVP of the films tested was determined by the following equations:

$$\text{WVTR} = \frac{slope(\Delta m_{over\ time\ (h)})}{A_{transf}} \quad \textbf{(Eq.4)}$$

$$\text{WVP}\left(g.Pa^{-1}.m^{-1}.s^{-1}\right) = \frac{WVTR \times X}{\Delta P} \quad \textbf{(Eq.5)}$$

where, WVTR = water vapor transmission rate; Δm = mass change over time (g); X = film thickness (mm); A_{transf} = film area (m^2); ΔP = partial vapor pressure difference across the two sides of the film (2337 Pa, at 22 ◦C) (Annex I).

The WVP of edible films depends on several factors: integrity of the film, hydrophilic/hydrophobic ratio, polymeric chain mobility and ratio between crystalline and amorphous zones [67]. WVP should be as low as possible, since one of the main objectives of edible films is to decrease moisture transfer between food and surrounding atmosphere.

3.4 *Aloe vera* gel and juice Extration

Aloe vera leaves (*Aloe barbadensis* Miller) were provided by the "*Aloe vera* Ecologico" company, in the city of Badajoz, Spain (January, 2014). Homogenous leaves were selected according to size, ripeness, colour, and freshness.

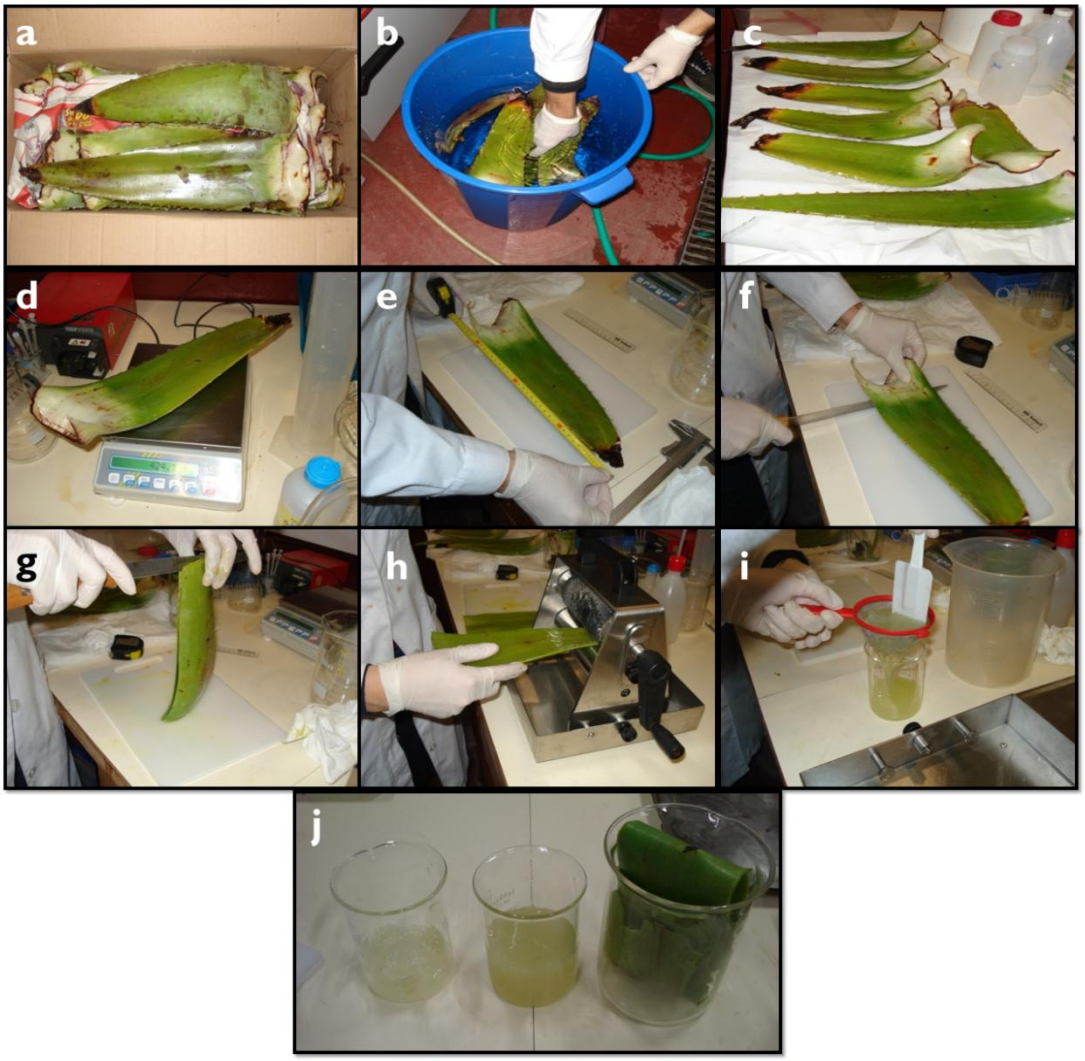


Figure 7| Chronological Order of the Extraction of *Aloe vera* gel and juice fractions. a) Reception of *Aloe vera*; b) *Aloe vera* leaves were washed with distilled water and 2% sodium hypochlorite to remove dirt from the surface; c) Collect a sample for analysis; d) Weighing the whole leaf; e) Measurement of width, length and thickness; f) Cutting the base of the leaves to remove aloin; g) The skin was carefully separated from the parenchyma using a scalpel-shaped knife; h) The epidermis was then separated from the gel and the gel was separated from the juice using a laboratory roll processor; i) Separating gel and juice with a strainer; j) Gel, Juice and bagasse, from left to right.

Figure 7 shows the chronological order of *Aloe vera* juice and gel fractions extraction. Aloin (a yellow-coloured liquid) was extracted by cutting the base of the leaves and allowing them to drain vertically for 30 minutes [49].

All the material used for the extraction was disinfected and/or sterilized. The results of the extraction of the leaves of *Aloe vera* are shown in annex II.

After gel and juice extracted from the *Aloe vera*, these fractions were pasteurized and stored in a freezer.

3.5 Antifungal activity

3.5.1 Fungal strains

Botrytis cinerea (MUM 10.138), *Penicillium expansum* (MUM 02.14) and *Aspergillus níger* (MUM 92.13), used to test the antifungal activity of fractions from *Aloe vera*, were obtained from MUM(Micoteca da Universidade do Minho, Braga, Portugal). All fungi were routinely cultured on potato dextrose agar (PDA) (Difco™, France).

3.5.2 *In vitro* Antifungal Assay

B. cinerea, *P. expansum* and *A. niger* spores were collected and diluted with sterile water until suspensions reached 10^4 spores per mL counts and stocked for further use (Neubauer chamber).

Antifungal activity was evaluated following a modification of the procedure reported by Kouassi et al. (2012)[68].100 µL of fresh gel and liquid fraction at concentrations of: 0.5%, 5%, 20% and 100% was pipetted into wells of a sterile 96-well microplate (Fig. 8). Each well was inoculated with a 100 µL aliquot of fungal inoculum (*B. cinerea*, *P. expansum* and *A. niger*) to reach a final volume of 200 µL. A positive control was carried out by mixing 100 µL of sterile potato dextrose broth (PDB) (Liofilchem, Italy) with 100 µL of each fungal suspension. The negative control of each group of replicates was a well containing non-infected medium. Fungal growth was monitored spectrophotometrically at 530 nm (Biotek Sinergy II, USA, Software GEN5) by measuring optical density (OD) of each well for 96 h (at 24-h intervals) of incubation at 25 °C. Experiments were replicated three times for each mold.

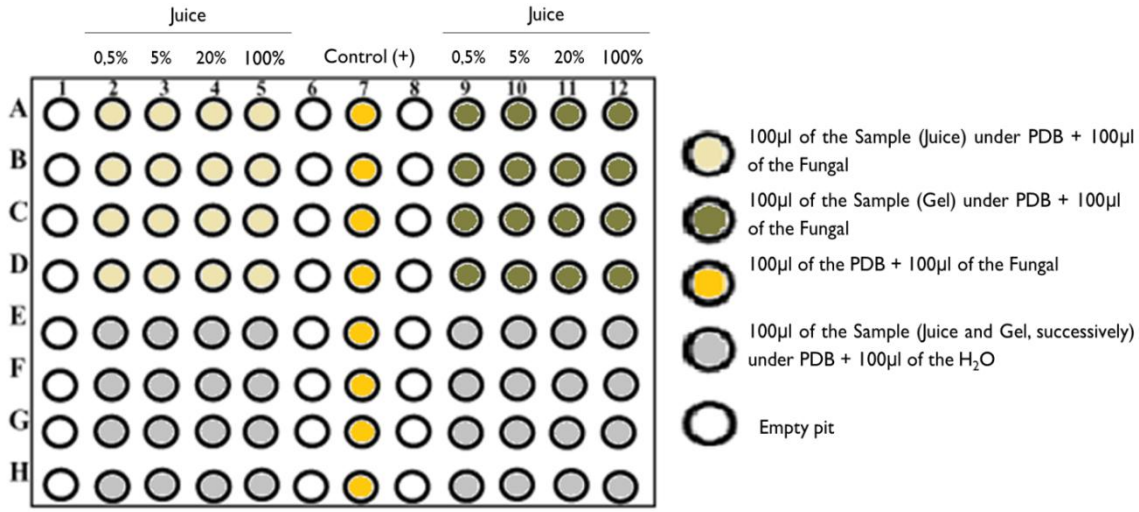


Figure 8 | Scheme of 96-well elisa microplate to evaluate the antifungal activity of *Aloe vera* against different fungal species.

3.6 Antioxidant activity

3.6.1 Materials

The radical scavenging activity of *Aloe vera* fractions was measured using the stable radical 2,2-Diphenyl-1-picrylhydrazyl (DPPH), methanol, and buthylated hydroxyanisole (BHA). All reactants were purchased from Sigma (Louis, MO, USA).

3.6.2 Methodology

Free radical scavenging activity of the *Aloe vera* fractions was determined using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method, in accordance with Williams et al. (1958)[69] with modifications. The DPPH-scavenging activity of phenolic compounds, which are present in the *Aloe vera*, responsible for much of its antioxidant activity, has been studied in many works [67].

To evaluate the antioxidant activity of the *Aloe vera* gel and juice fractions (0.1 %, 0.5%, 1%, 5% and 10%). An aliquot of 150 µl of each sample was added into a well containing 50 µl of 0.1 mM DPPH radical in a proportion of 1:1 (methanol:H_2O). The reaction mixture was mixed in the 96-well microplate (Fig. 9) and kept at room temperature in the dark for 30 minutes. The absorbance was measured at 517 nm, using a spectrophotometric microplate reader (Synergy HT, Biotek, USA) and antioxidant activity was expressed as percentage radical scavening activity (RSA) relative to the control.

The antioxidant activity of the fractions was calculated according to the equation:

$$\text{RSA}(\%) = \frac{(Abs_{control} - Abs_{sample})}{(Abs_{control})} \times 100 \qquad \textbf{(Eq. 6)}$$

Abs_{sample} - represents the absorbance of the sample solution;
$Abs_{control}$ - represents the absorbance of DPPH solution without the addition of the *Aloe vera*.

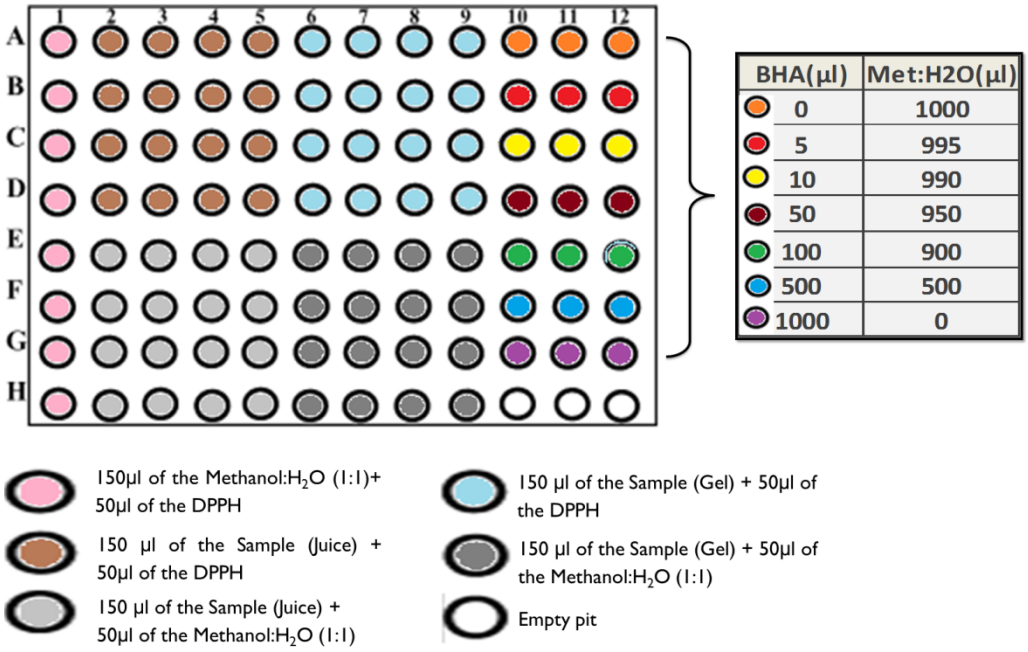


Figure 9 | Scheme of 96-well elisa microplate to evaluate the antioxidant capacity of of different concentrations of the *Aloe vera* fractions (0.05%, 0.1%, 0.5%, 1% and 5%) and their respective calibration curve. The pink and gray pits are the controls.

3.7 *Shelf-life* Analyses – Experimental design

By definition [70], in general, *shelf-life* is the time during which the food product will:

- Remain safe;
- Be certain to retain desired sensory, chemical, physical and microbiological characteristics.

The objective of the shelf life analysis is the simulation, as close as possible, to the reality, i.e., evaluate the effectiveness of edible coatings on blueberries conservation from harvest to retail chains and consumption.

The shelf-life analyzes were performed in two sets of experiments with the same storage conditions, one of them with blueberries forced contamination and the other one

without, at the same time, to avoid cross-contamination (Fig. 10). *Botrytis cinerea*, was chosen for forced contamination, since this is one of the most representative fungi responsible for decay of blueberries.

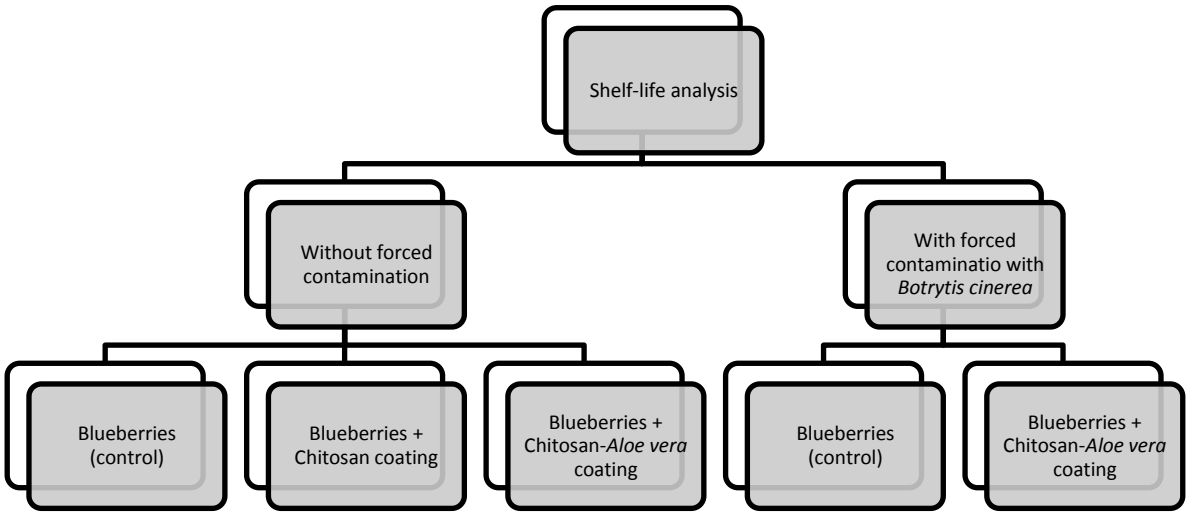


Figure 10 | Diagram representing the five different treatments applied in blueberries. Formulation of Chitosan coating is 0.5% Chitosan + 0.5% Glycerol + 0.1% Tween 80 and the formulations of Chitosan-*Aloe vera* (juice) coating is 0.5% Aloe vera + 0.5% Chitosan + 0.5% Glycerol + 0.1% Tween 80.

3.7.1 Coating application and blueberry inoculation

In the figure 11 are shown the steps that blueberries followed until the required storage conditions.

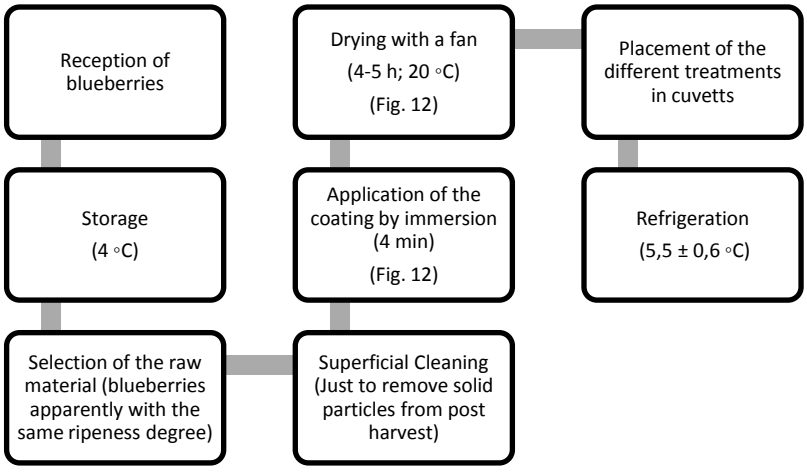


Figure 11 | Processing steps representative of the application of chitosan coating to blueberries.

As mentioned above, *B. cinerea* was used for inoculation (treatment with forced contamination), to test antifungal activity of edible coating with *Aloe vera*. Thus, blueberries without coating (control) and blueberries with chitosan-*Aloe vera* coating were inoculated in

the furrow (diametrically opposite to apex), with 10 µl of *B. cinerea* at a concentration of 10^4 spores/ml (Figure 13).

The other set treatment, with no forced contamination, will be used to understand the differences between the blueberries with applied coatings and blueberries without coatings.

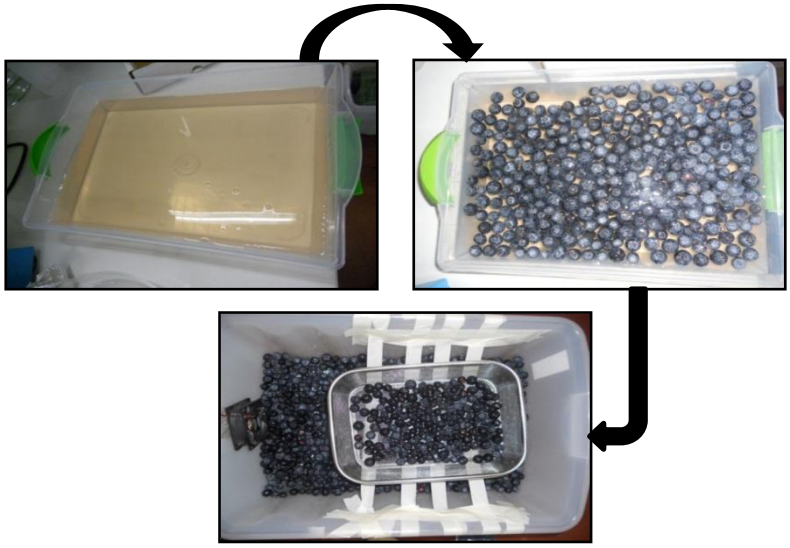


Figure 12 | Application of the coating by immersion, and subsequent drying with ventilation at 20 °C, during 4-5 hours.

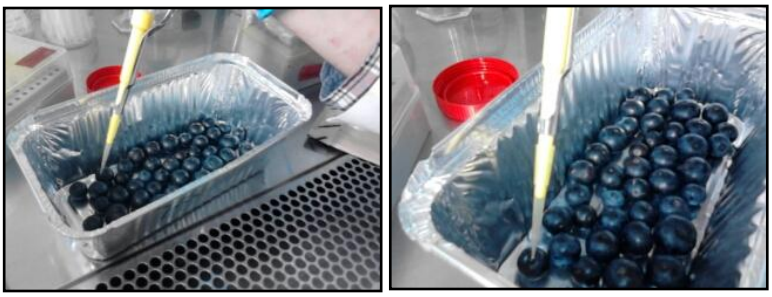


Figure 13 | Incorporation of 10 µl of *Botrytis cinerea* at blueberries furrows (about 2000 blueberries).

3.7.2 Preparation of storage conditions for blueberries

After drying the coating, blueberries were divided by treatment and placed in aluminum cuvettes, previously disinfected and identified. Each treatment had a cuvette containing ≈ 100 grams to register blueberry weight loss during storage, and a cuvette with ≈ 130 grams per day of analysis. Thereafter, cuvettes samples with and without forced contamination were stored at two different greenhouses (suitable for creating the desired atmosphere), both

placed in a cold room with the same temperature and relative humidity (Fig. 14). During food shelf-life studies, temperature and relative humidity was recorded with an ibutton. Under refrigerated storage conditions, temperature (5.5 ± 0.6 °C) and relative humidity (90 ± 3%) were recorded. Blueberries samples without forced contamination were weighted, and pH, titratable acidity (TA), soluble solid content (SSC) and microbiological analyses were conducted at 0, 2, 4, 6, 9, 12, 15, 18, 21 and 25 storage days under the conditions described.

Blueberries samples with forced contamination, were weighted, and pH, titratable acidity (TA), soluble solid content (SSC) and microbiological analyses were conducted at 0, 2, 4, 6, 9, 12 and 15 storage days under the conditions described, followed by 3 days at room temperature (25 ± 0,5 °C; relative humidity of 58 ± 5%), to observe the behavior of the coating at room temperature after a period of refrigeration.

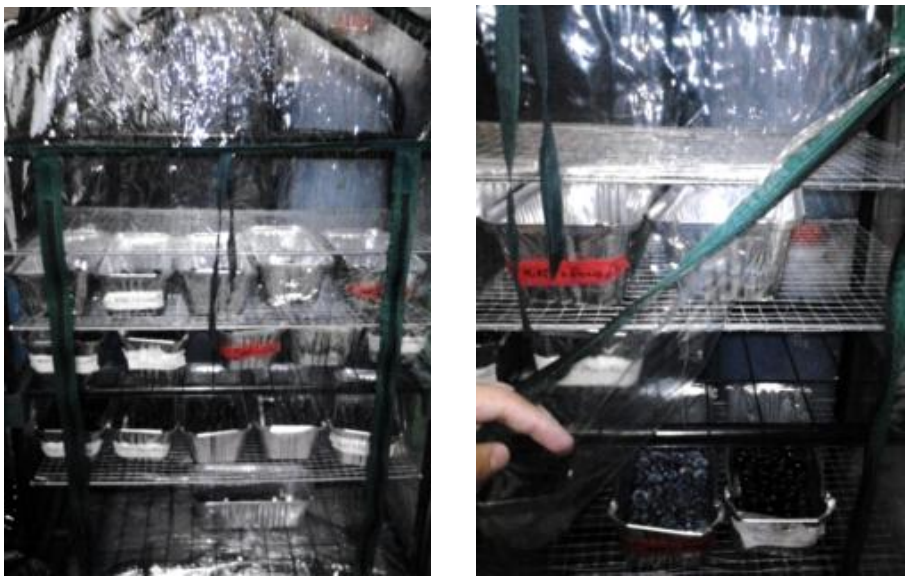


Figure 1 | Images of the adapted greenhouses to create desired atmosphere conditions (temperature and relative humidity) inside the cold room.

3.7.3 Titratable acidity (TA)

The official analytical method of the Association of Official Agricultural Chemists 942.15, specific for fruit derivatives (AOAC International, 1995), was chosen for determining titratable acidity (TA) of blueberry samples. TA was determined (in triplicate) from pulp obtained after grinding (2 minutes) and centrifugation (5 minutes) (Sigma Laboratory Centrifuges, 4K15) of the blueberry samples for each treatment. The liquid used for all

analyzes except for the colour and microbiological analyzes, is acquired through pulp centrifugation, for 5 minutes at a temperature of 20 °C and a speed of 9000 rpm.

Results were expressed as grams of citric acid equivalent per 100 g of fresh weight.

3.7.4 pH

The pH value was determined using a pH meter (Hanna Instruments Inc., Romania). After the homogenization of the samples in a commercial blender, juice and solid parts were placed into a container where the pH was measured by direct immersion of the electrode. At each sampling time, 3 samples per treatment were analyzed. It is an important parameter in determining potential microbial growth that could cause deterioration.

3.7.5 Soluble solid content (SSC)

The method used to quantify the soluble solids content was AOAC (932.12) specifically for fruits (AOAC International, 1995).

SSC was determined from the juice obtained from blueberry samples grinded (during 2 minutes) and centrifuged (during 5 minutes), using a refractometer RHB-32ATC (Fig. 15), previously standardized with water (Hanna Instruments Inc. (96801), Romania).

Three measurements were taken for each sample at 20 °C and results were expressed as °Brix.

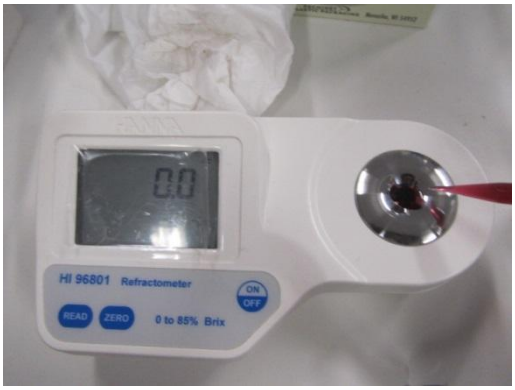


Figure 15 | Refractometer used in the measurement of °Brix of the different blueberry treatments.

3.7.6 Weight loss

Weight loss was evaluated by weighting all samples with a precision balance (Mettler AE200) at the initial of storage (day 0) and at all sampling days. The difference between the two values was considered as the weight loss. The percentage of weight loss was determined by the following equation:

$$Weight\ loss\ (\%) = \frac{Wi - Wf}{Wi} \times 100 \quad \textbf{(Eq. 7)}$$

Wi – Initial weight
Wf – Weight at the time x

3.7.7 Colour

The CIE L* a* b* model was chosen to specifying a three-dimensional space, and can be represented (Fig. 16) on a graphic whose coordinates x, y and z are related to the a *, b * and L *, respectively. Which are based on the fact that the human eye has three types of color sensors which are sensitive to the colors red, green, and blue and that all colors are seen as a mixture of these three colors [5].

In this model, L * defines lightness that varies between 0 (black) to 100 (white)., a * and b * are chromatic components that range between -60 and +60 which indicate, respectively, red/green and yellow/blue values.

Several authors determined the surface color of fruit [59, 71]; however, for blueberries this process may have some shortcomings, since the blueberry surface is very heterogeneous due to surrounding wax which quickly changes overtime. Thus, blueberries were triturated in a commercial blender for 5 minutes and color parameters were measures.

Color measurements were performed in a Minolta colorimeter (Minolta CR 400, Tokyo, Japan), appropriately calibrated (Y=93.5, x=0.3114, y=0.3190) with a standard white tile (UE certificated) to determine L*, a* and b* parameters. After the homogenization of the samples, the mixture were placed in a test tube and placed in an appropriate support for the color measurements (Fig. 17).

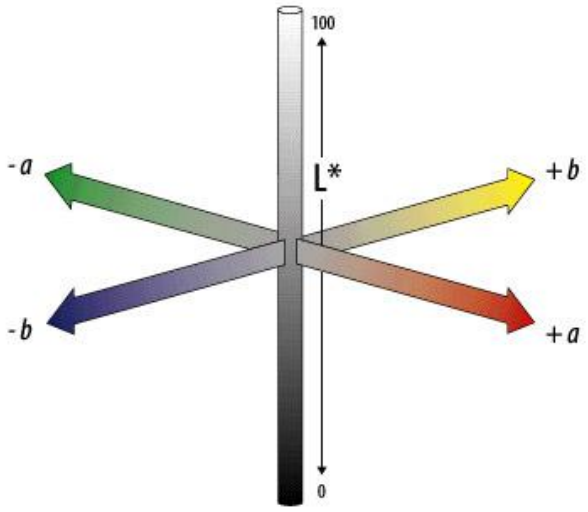


Figure 16 | Chromatic coordinates L* (ranging from black to white), a* (ranging from - a*: greenness to + a*: redness) and b* (ranging from - b*: blueness to + b*: yellowness).

In order to get a measure of the magnitude of colour difference, in the CIE L*a*b* colour space, total colour difference (ΔE) can be determined. ΔE was determined according to the following equation:

$$\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2} \qquad \textbf{(Eq. 8)}$$

ΔL - $(L^* - L_0^*)$;
Δa - $(a^* - a_0^*)$;
Δb - $(b^* - b_0^*)$;
L_0^*, a_0^* and b_0^* are the initial colour values and L*, a* and b* are the colour values during the experiment

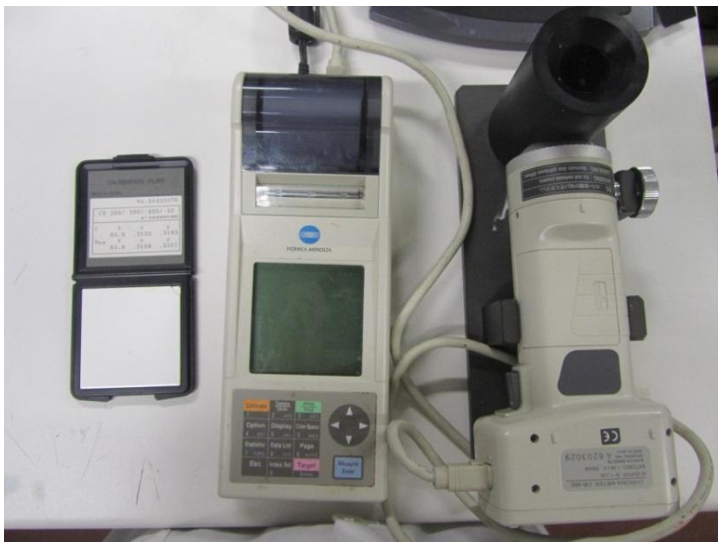


Figure 17| Colorimeter used for color characterization of blueberry pulp, to the different treatments over time.

3.7.8 Microbiological analysis

20 g samples from each treatment were weighed and processed under sterile conditions (vertical laminar air flow chamber). Then, samples were transferred to individual sterile stomacher bags with 180 mL of sterile 0.1% peptone water (Becton, Dickinson and Company, France), which were homogenized in a blender Stomacher 3500 (Seward Medical, London, U.K.) for 2 minutes.

Serial dilutions were carried out, and 100 µl was spreaded potato dextrose agar agar (PDA) (Liofilchem, Italy). Plates were incubated during 5 days at 25 °C and total molds/yeast colony-forming units (CFU) were determined.

Samples were prepared in triplicate to dilutions of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}, and only counts of 15-300 colonies were considered.

Microbiological counts were converted to log CFU $\cdot g^{-1}$, and the means and standard deviations were calculated.

3.8. Statistical Analyses

The data of this thesis were analyzed using the program SigmaPlot and using Microsoft Windows Excel 2010. Data from analytical determinations were subjected to analysis of variance (ANOVA) and the mean comparisons were performed using the high significant difference (HSD) Tukey's test to examine if differences between treatments and storage time were significant at $p < 0.05$.

4. Results and Discussion

4.1 Surface Tension and Wettability

As can be seen in Chapter III the Zisman method is applicable only to low energy surfaces (lower than 100 mN/m). Thus it was necessary to know the surface tension of blueberry to ensure the applicability of this method. Skurtys et al. (2011) [72] demonstrated that surface energy of blueberry is 29.1 mN/m (without wax is 38.3 mN/m) [72].

This compatibility between blueberry surface polarity and coating has an important role in the wettability of the surface, because after drying, coating should have an appropriate adhesion, cohesion and durability.

Wettability can be used as a measurement of the effectiveness of edible coatings to coat a food surface, in this case, chitosan formulations on blueberry surface. Wettability of a solid by a liquid results from intermolecular interactions. The degree of wetting is determined by balance forces of adhesion and cohesion [73].

The contact angle of the liquid droplet on the solid surface is set by mechanical equilibrium drop under the action of three interfacial tensions: solid-liquid (γ_{SL}), solid-vapor (γ_{SV}), liquid-vapor (γ_L) (Fig. 18). Young's equation shows the relationship between these equilibrium [74]. The surface energy or surface tension of the food product is also a controlling factor in the adhesion of the coatings on the surfaces of food [2, 75].

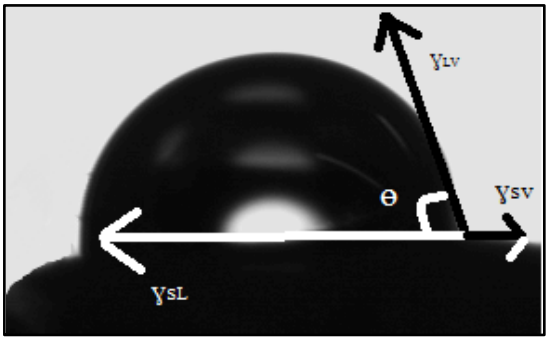


Figure 18 | Contact angle of water liquid drop on blueberry and three interfacial tensions: solid-liquid (γ_{SL}), solid-vapor (γ_{SV}), liquid-vapor (γ_L).

Through the contact angle measurements was possible to evaluate the effect of different concentrations of chitosan, glycerol and Tween 80 on the blueberry superficies. Influence of Tween 80 in chitosan coating was determined by contact angle measurements, and could be observed through spreading coefficient (Ws) on the blueberry surface. The addition of this surfactant reduced the cohesion forces, therefore reducing the surface tension and increasing the wettability, thus, improved compatibility between the solution and the fruit skin surface. Experimentally, the closer the Ws values are to zero, the better a

surface will be coated [73]. The results show that depending on the amount of chitosan, glycerol and Tween 80 added, Ws values are statistically different ($p < 0.05$). Considering the solutions tested, coating solutions that presented best values of Ws were solutions 2, 3, 5 and 6, however, there is no significant differences ($p > 0.05$) between these four coating formulations.

Analyzing the results obtained (Tab. 7), it is clear that this surfactant improves the wetting properties, since the value of the Ws with Tween 80 at different concentrations (0.1% and 0.2%) are the nearest from 0, probably due to the reduction of surface tension of the liquid. The improvement of Ws with addition of Tween 80 was also shown by Cerqueira et al (2009)[42], when studying wetting ability of chitosan-based coating on the surface of cheese.

Table 7 | Spreading Coefficient (Ws) achieved for the tested chitosan solutions on blueberry.

Formulation	Chitosan (w/v)	Glycerol (v/v)	Tween 80 (w/v)	Spreading Coefficient (Ws)
1	0.5	0.5	0	-89.79 ± 2.02 f
2	0.5	0.5	0.1	**-46.61 ± 4.27** a
3	0.5	0.5	0.2	**-46.12 ± 4.88** a
4	0.5	1.0	0	-63,22 ± 4,56 d
5	0.5	1.0	0.1	**-46.71 ± 4.42** a
6	0.5	1.0	0.2	**-46.92 ± 4.44** a
7	0.5	1.5	0	-84.93 ± 4.90 e, f
8	0.5	1.5	0.1	-53.05 ± 4.13 b, c
9	0.5	1.5	0.2	-52.08 ± 2.96 b, c
10	1.0	0.5	0	-81.15 ± 5.25 e
11	1.0	0.5	0.1	-51.85 ± 3.75 b
12	1.0	0.5	0.2	-50.63 ± 3.80 b
13	1.0	1.0	0	-80.63 ± 3.19 e
14	1.0	1.0	0.1	-55.73 ± 2.96 c
15	1.0	1.0	0.2	-53.71 ± 3.28 b, c
16	1.0	1.5	0	-82.97 ± 5.99 e, f
17	1.0	1.5	0.1	-55.08 ± 3.98 b, c
18	1.0	1.5	0.2	-53.62 ± 3.28 b, c
19	1.5	0.5	0	-65.04 ± 6.14 d
20	1.5	0.5	0.1	-50.41 ± 4.76 a, b
21	1.5	0.5	0.2	-52.04 ± 4.91 b, c
22	1.5	1.0	0	-82.07 ± 2.94 e
23	1.5	1.0	0.1	-62.59 ± 2.26 d
24	1.5	1.0	0.2	-60.02 ± 1.97 c, d
25	1.5	1.5	0	-80.32 ± 5.06 e
26	1.5	1.5	0.1	-52.70 ± 4.20 b, c
27	1.5	1.5	0.2	-52.91 ± 4.21 b, c

a-f: Different letters in the same column correspond to statistically different samples for a 95% confidence level

The better values of Ws were the formulations with lower chitosan concentrations (0.5% chitosan) and glycerol (0.5% glycerol) (1:1), in the presence of Tween 80.

Once there were no statistically significant differences between chitosan formulations (2, 3, 5, 6), it has been assumed that their differentiation must be made on the basis of other criteria, such as water vapor permeability, since, water loss is a major problem in the deterioration of the fruit.

4.2 Water Vapor Permeability (WVP)

WVP is considered one of the main parameters to have in consideration when developing films and coatings, primarily because of the importance of water in deteriorative reactions and dryness of fruits.

The four coatings formulations with best wettability values were chosen for the WVP evaluation. The incorporation of glycerol (Gly) and Tween 80 (Twe80) at different concentrations into chitosan (Chi) films did not change significant WVP values ($p > 0.05$). Solution 6 (0.5% Chi + 1.0% Gly + 0.2% Twe80) presented higher WVP values ($p > 0.05$) than the others (2, 3, 5) which means that presented a poor barrier to water vapour.

The WVP values of the films were between 5.1×10^{-10} (g/(m.s.Pa)) and 5.8×10^{-10} (g/(m.s.Pa)) (Fig. 19). Pranoto et al. (2005)[76] obtained values of WVP for chitosan-based films incorporated with an antimicrobial (nisin) between $1.5x10^{-11}$ and $2.2x10^{-11}$ (g/(m.s.Pa)) [76].

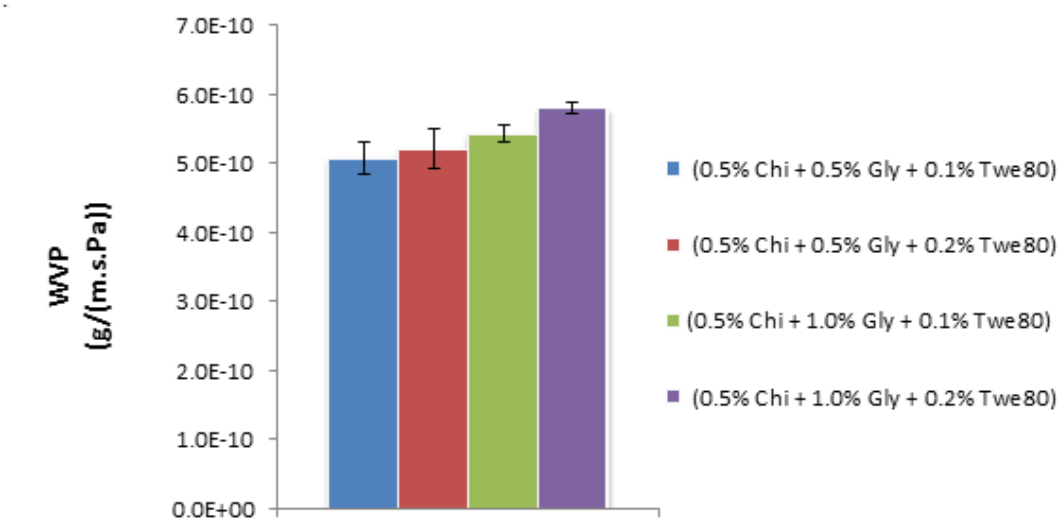


Figure 19 | Water vapor permeability of different formulations of chitosan edible films.

Since no significant differences were found between solutions 2, 3 and 5, the formulation with lower concentrations of each constituent was chosen, thereby benefiting the use of less compounds concentration, reducing the costs of this packaging. Once selected the best formulation according to the wettability and WVP (solution 2 - 0.5% Chi + 0.5% Gly + 0.1% Tween 80), the following step was the incorporation of *Aloe vera* juice and gel, with the concentration previously defined, according to antioxidant and antifungal tests (sections 4.4 and 4.3, respectively). The *Aloe vera* fractions concentrations tested were 0.5% and 5% because only these concentrations were capable of forming a coating with appropriate features to produce a uniform coating on blueberry surface. Then, wettability test and WVP were conducted with the chosen *Aloe vera* fraction.

After repeating the tests, it was concluded that the addition of *Aloe vera* fractions (gel or juice) reduced WVP values (data not shown). Khoshgozaran-Abras et al. (2012)[77] concluded that *Aloe vera* gel incorporation enhanced chitosan film barrier to water vapor [77]. Addition of gel or juice to chitosan solutions did not significantly change ($p > 0.05$) wettability values, as can be seen in Figure 20.

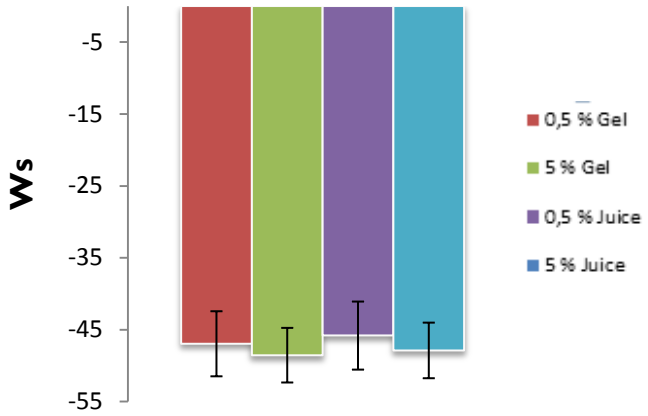


Figure 20 | Spreading Coefficient (Ws) obtained for chitosan solutions with different concentrations of *Aloe vera* gel and juice (0.5% and 5%) on blueberry.

4.3 Antifungal Activity of *Aloe vera*

The antifungal potential of *Aloe vera* gel and juice fractions was tested *in vitro* against three fungi (*Botrytis cinerea*, *Penicillium expansum* and *Aspergillus niger*), that potential cause disease of blueberry.

One of the reasons to separate the *Aloe vera* fractions was the limited and contradictory results reported in the literature about the antimicrobial activity of *Aloe* extracts. According Jasso de Rodriguez et al. (2005)[49] the differences were due to the use of different compounds in each fraction and their antimicrobial activity.

The concentrations of *Aloe vera* fractions used for the test were: 0.5%; 5%; 20% and 100% (v/v). The fungal inhibition, as a function of *Aloe* concentration is shown in Figure 21 and 22 for the three fungi. As can be seen in these figures, the addition of increasing *Aloe vera* gel and juice concentrations led to lower mold growth. However, the inhibitory effect is dependent on fungi species. It may be due to the presence of compounds/substances in *Aloe vera*, such as quinones and phenol compounds (flavonoids), more active for some fungi than others [78, 79].

Antifungal activity of the gel and juice exhibit similar inhibitory results for the three fungi. *Botrytis cinerea* and *Penicillium expansum* presented more growth inhibition in the presence of *Aloe vera* gel or juice than *A. niger*. After 72 h of incubation with 0.5% *Aloe vera* juice, an inhibition of ≈ 85% was observed for *B. cinerea* (Figure 21) while *A. niger* presented ≈ 15% of growth inhibition. With 0.5% *Aloe vera* gel, an inhibition of ≈ 80% was observed for *B. cinerea*, an inhibition of ≈ 55% for *P. expansum* and ≈ 15% of growth inhibition for *A. niger*. For the 100% of each fraction, according to the figures 21 and 22, it can be seen that *Aloe vera* does not work as a power supply for the three fungi during 72 h. Saks and Barkai-Golan (1995)[48] found that blueberry pulp presented antifungal activity against some fungi, such as *B. cinerea* and *P. expansum*, and the inhibitory results obtained presents some resemblance to those presented in Figures 21 and 22. Also Castillo et al (2010)[27], reported that *Aloe vera* inhibited mycelium growth of *Penicillium digitatum* and *Botrytis cinerea*, however, *Penicillium digitatum* presented higher growth inhibition than *Botrytis cinerea.*

In accordance with the results obtained, the choice of the *Aloe vera* fraction to incorporate into the chitosan film was delayed, since the difference in the inhibition of all three fungi was not significant. Quantity of 20% of gel or juice show, in general, better inhibition over time, when compared with other percentages (0.5% and 5%). However, this amount, when incorporated into the chitosan-based film, formed an inconsistent film. The proportions of 0.5% and 5%, present results very similar, so, the best option would be 0.5%, because with less quantity, high inhibition was obtained, as can seen in the two following figures. However, to confirm the percentage to use, antioxidant activity tests were performed.

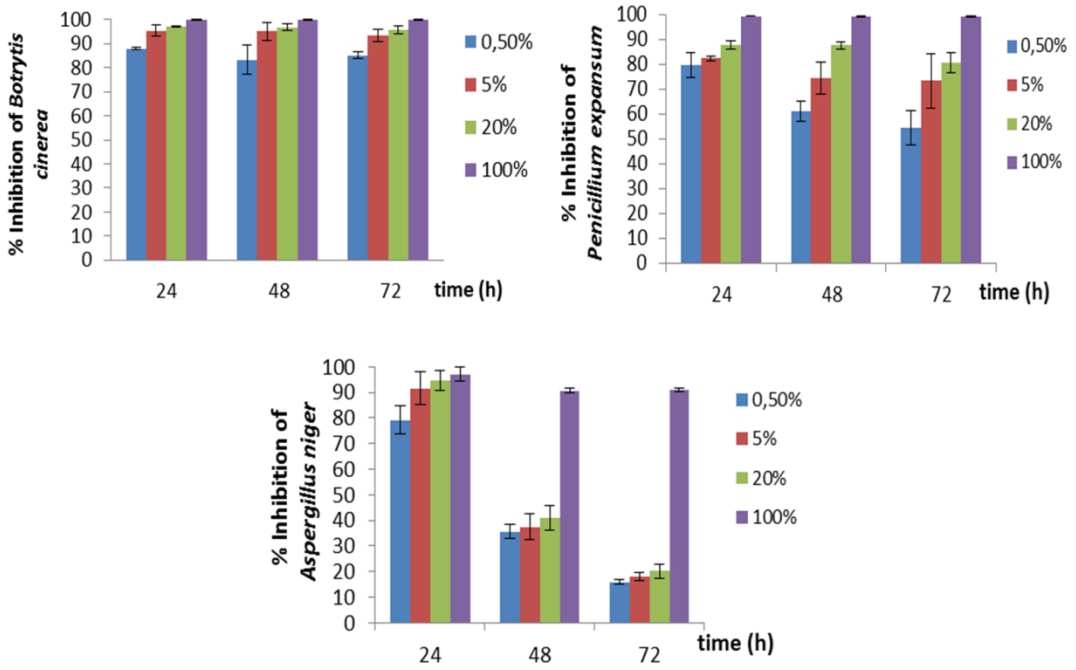


Figure 21 | Inhibition effect of *Aloe vera* liquid fraction (juice) at different concentrations on *Botrytis cinerea* (a), *Penicillium expansum* (b) and *Aspergillus niger* (c).

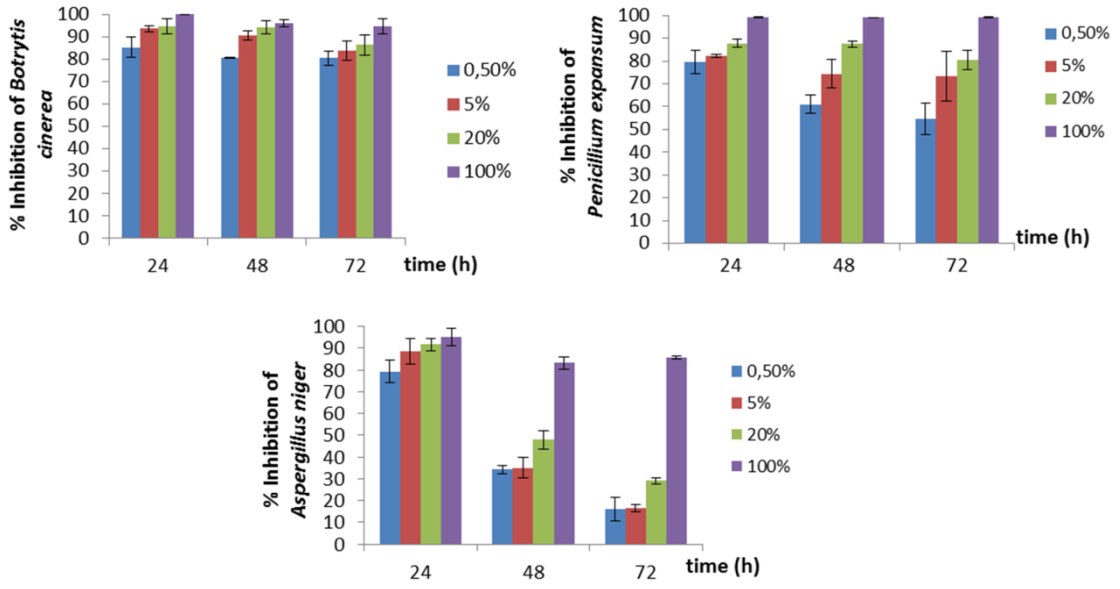


Figure 22 | Inhibition effect of *Aloe vera* gel fraction at different concentrations on *Botrytis cinerea* (a), *Penicillium expansum* (b) and *Aspergillus niger* (c).

4.4 Analysis of Antioxidant Capacity

The DPPH radical is often used to evaluate the free-radical scavenging capacity of antioxidants. The radical scavenging activity (RSA) of *Aloe vera* fractions is reported in Table 8. The high % RSA was observed to *Aloe vera* juice fraction. . This result may be related to vitamins C and E or total phenolic content present in higher content on juice fraction, since those compounds act as scavengers of free radicals produced during oxidation reactions [25, 80].

Aloe vera fractions concentrations studied in this work were selected according to their potential, ie, below 0.1%, the antioxidant capacity was below 50% for both fractions and for concentration value higher than 10%, antioxidant capacity was very satisfactory, 91.5 ± 2.35% and 87,7 ± 1.54% (Juice and Gel, respectively).

As presented in Table 8, all *Aloe vera* fractions samples exhibited antioxidant activity, although it slightly decreases as concentration decreases. On juice fraction, between 0.1% and 0.5% and between 5% and 10% there were no significant differences ($p < 0.05$). Regarding to gel, no significant differences between 0.1% and 1% were observed. Many authors have reported that *Aloe vera* has a high antioxidant capacity. According to Wu et al. (2010)[9] , *Aloe vera* has a high antioxidant capacity due to various active compounds at different stages of development with different degrees of antioxidant capacity.

Table 8 | Percentage of Radical Scavenging Activity of *Aloe vera* fractions.

% Radical Scavenging Activity (RSA)					
% Juice	**0.1**	**0.5**	**1**	**5**	**10**
	78.9 ± 1.23a	80.8 ± 1.65[a]	85.1 ± 2.09b	89.2 ± 0.60c	91.5 ± 2.35c
% Gel	**0.1**	**0.5**	**1**	**5**	**10**
	71.8 ± 0.77a	71.9 ± 2.67[a]	72.7 ± 0.77b	74.5 ± 0.78b	89.5 ± 1.54c

a-c: Different letters in the same line correspond to statistically different samples for a 95% confidence level

According to these results (Tab. 8), it would be more accurate to choose 5% of juice fraction to be incorporated in chitosan coating. However, since the target food is a fruit, the coating should be as transparent and unnoticed as possible, in order to avoid the rejection by the consumer. Once 0.5% of juice also provides excellent antioxidant capacity, this concentration was selected to be incorporated into chitosan coating. The choice for a lower

concentration is economically advantageous once it minimizes costs when comparing to the use of higher concentrations and, as shown, does not compromise the performance.

According to wettability, antifungal and antioxidant activity results, juice fraction was chosen to be incorporated into chitosan-based films.

4.5 *Shelf-life* Analysis

Five different treatments were analyzed: Blueberry control (B), blueberry with chitosan coating (BC), blueberry coated with chitosan-*Aloe vera* coating (BCA), blueberry with forced contamination (BF) and blueberry coated with chitosan-*Aloe vera* coating and with forced contamination (BAF).

4.5.1 Titratable acidity (TA)

Figure 23 shows the evolution of titratable acidity for the different blueberry treatments, throughout storage.

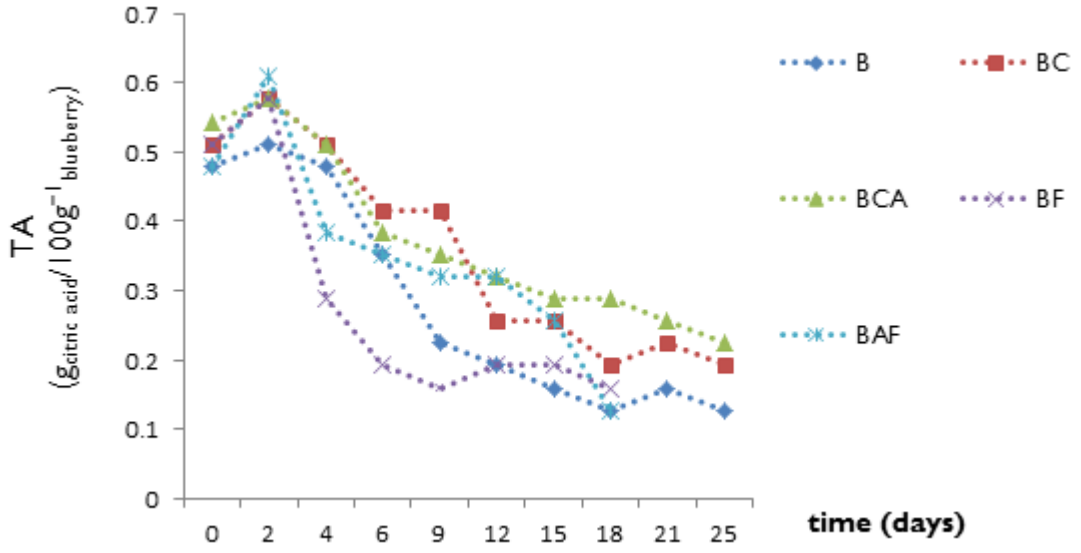


Figure 23 | Titratable acidity expressed in g citric acid per 100 g blueberry, over time, for the different treatments. B - blueberry control, BC - blueberry with chitosan coating, BCA - blueberry coated with chitosan-Aloe vera coating, BF - blueberry with forced contamination and BAF - blueberry coated with chitosan-Aloe vera coating and with forced contamination.

With this analysis it is intended to observe the differences in loss of citric acid in blueberry over time for the different treatments. As expected [5, 38], in general, the citric acid concentration decreased over time, probably due to the use of organic acids (such as citric acid) in the breathing process.

TA was significantly affected by various treatments during storage ($p < 0.05$) (Fig. 23). B samples had significantly lower TA values than BC and BCA during storage, thus, coating helped retain TA of blueberries ($p > 0.05$).

There was no difference ($p > 0.05$) in the TA values of BF and BAF during the period between 15th and 18th days of storage at 25 ± 0.5 °C. However, BF titratable acidity values fall sharply (0.576 to 0.16 $g_{citric\ acid}$/100 $g_{blueberry}$), when compared to other treatments, this can be explained by the increased microbiological, since fungi can be accelerated loss of citric acid, it can be considered as a power supply for them. Which may indicate that BAF treatment may be preventing rapid TA decrease from 0 to 9 days of storage, and remained constant in the remaining storage time.

The acid content of fruits tends to decrease with fruit maturity, unlike the soluble solids (e.g. sugars) which tend to increase [5].

These results are in agreement with Benítez et al. (2013)[81] results. These authors demonstrated that *Aloe vera* coating application in the Kiwi, reduced TA values over storage time regardless of the treatment used, but was higher in treatment with the highest *Aloe vera* concentration.

4.5.2 pH

The initial pH values (0 day) were different for the five treatments, as shown in the Figure 24. The pH of BC, BCA and BAF samples presents more acidic values, probably resulting from low pH of coating solutions (chitosan and chitosan-*Aloe vera* coating solutions presented pH=4.18 and 3.0, respectively). However, no statistically significant differences ($p > 0.05$) were observed for initial pH values of blueberries treated with different coatings (pH_{BC} =3.12; pH_{BCA} = 3.15; pH_{BAF} = 3.09).

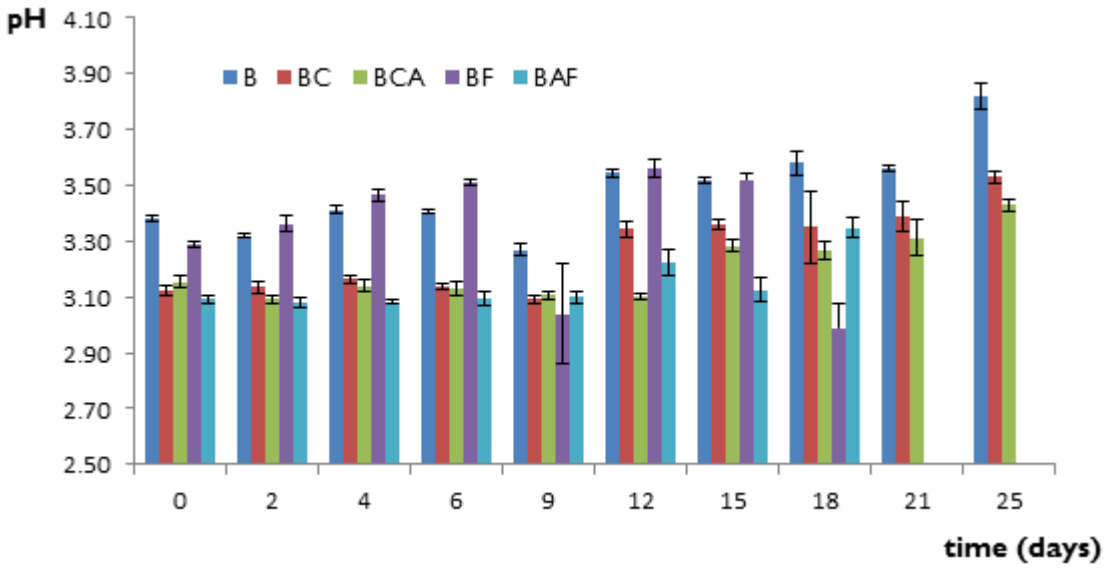


Figure 24 | pH values of different blueberry treatments during 25 days of storage at 5.5 ± 0.6 °C (without forced contamination) and for treatments with forced contamination during 15 days storage at 5.5 ± 0.6 °C followed by 3 days at 25 ± 0.5 °C (from 15th to 18th days). B - blueberry control, BC - blueberry with chitosan coating, BCA - blueberry coated with chitosan-Aloe vera coating, BF - blueberry with forced contamination and BAF - blueberry coated with chitosan-Aloe vera coating and with forced contamination.

The coatings maintain the pH of blueberries at lower levels compared to the uncoated fruit, with and without contamination forced over 9 days. *Aloe vera* coating (BCA) maintained lower pH (3.15) value during 12 days of refrigeration (5.5 ± 0.6 °C).

As we can observe in Figure 24, after 12 days of storage at 5.5 ± 0.6 °C, pH value of blueberries without coating was significantly ($p < 0.05$) higher than initial pH (0 day) (B pH increased from 3.38 to 3.54; BF pH increased from 3.29 to 3.56). pH of BC blueberries, only after 12 days of storage reached initial pH (at 0 day) of B blueberries. In general, increase of pH values may be related to blueberry spoilage, with formation of alkaline autolysis compounds [82] (for example, nitrogenous compounds) and production of fungal metabolites on blueberries surface, once the pH changes when the CFU/g values increase.

According to the results it can be seen that the BAF treatment was able to maintain for approximately 15 days, the initial pH value, while the BF treatment had higher pH. Thus, with *Aloe vera* coating the pH change was attenuated, despite the forced contamination of blueberries.

In accordance with Benítez et al. (2013)[81], kiwifruit coated with *Aloe vera* (1%) slightly lower the initial pH of the fruit, and keeps the pH values over 12 days (3.43 ± 0.08 to 3.48 ± 0.06).

4.5.3 Soluble Solid Content (SSC)

The objective of determining the soluble solids, pH and titratable acidity, is to observe its relationship with the degree of maturation of the blueberry.

Figure 25 shows the evolution of the amount of Soluble Solids Content (SSC) registered for different blueberry treatments during storage.

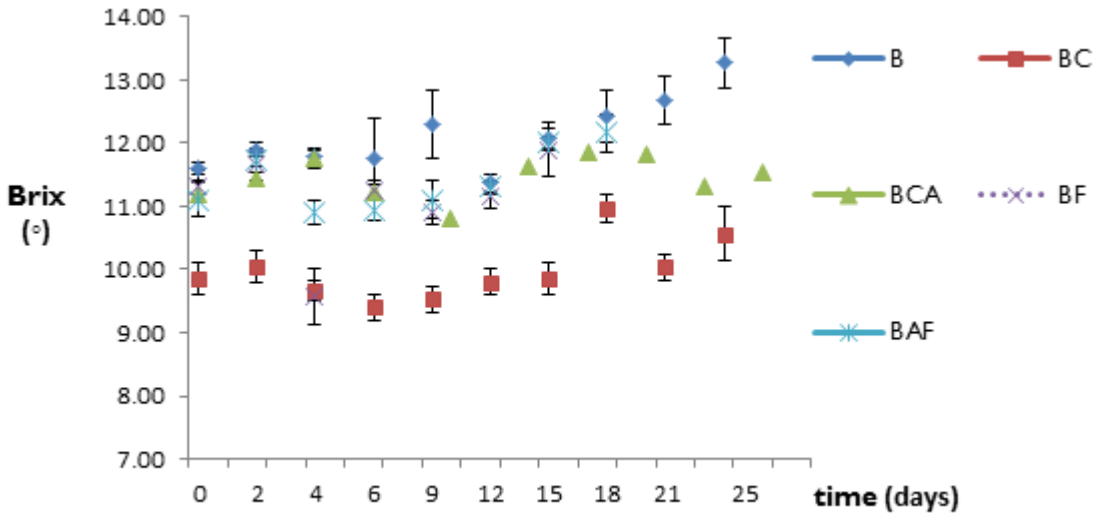


Figure 25 | Soluble solids contents (SSC) of blueberry samples during the 25 days of storage (5.5 ± 0.6 °C and 90 ± 3% of HR) (without forced contamination) and during 15 days at 5.5 ± 0.6 °C and relative humidity of 90 ± 3%, followed by 3 days at 25 ± 0.5 °C and humidity of 58 ± 5% (with forced contamination). B - blueberry control, BC - blueberry with chitosan coating, BCA - blueberry coated with chitosan-Aloe vera coating, BF - blueberry with forced contamination and BAF - blueberry coated with chitosan-Aloe vera coating and with forced contamination.

SSC was relatively stable during the storage but some differences can be seen between different treatments. Blueberries coated, without forced contamination (BC and BCA), were those with the minor variation on SSC compared to uncoated fruit.

For uncoated fruit without forced contamination (B) it can be observed an increase of SSC from day 15 until day 25, most probably due to the water loss, which caused increase of sugar concentration.

BC presented a low level of the initial total soluble solids (9.87 °Brix), and maintains this value constant until the end of the 25 days (10.57 °Brix), having a more significant increase in 15th to 18th days, which is probably related to greater weight loss, as can be seen in Figure 25.

BCA samples showed constant SSC values over time, compared to other samples, such as BC. This behavior can be translated by lower water loss relative to the uncoated sample and to the forced contamination samples, while maintaining quality properties of blueberry preserved for longer.

Concerning to the contaminated samples, while BAF treatment maintains SSC values constant until day 12, reasonably increased until day 18 (11.30 to 12.17 °Brix), the uncoated sample (BF) shows SSC values with abrupt changes over every day (ranging from 9.57 and 13.27 °Brix), probably due to the fact that the fungus had consumed more glucose at day 4, in this sample.

Water loss (Fig. 26) causes an apparent increase on the concentration of SSC that may be incorrectly interpreted as a true change in the amount of acids or sugars present on fruits [5].

These results are in agreement with some studies already carried out in other fruits. Martinez-Romero et al. (2006) [59] showed that *Aloe vera* coated cherries maintains soluble solids content over time.

4.5.4 Weight loss

Fruit weight changes were monitored during storage period to determine how effective the coating is as a moisture barrier. It can be considered that weight loss corresponds nearly exclusively to water loss since other components that can be lost (aromas or flavors, and gases product of respiration) are basically nonexistent in terms of weight [5].

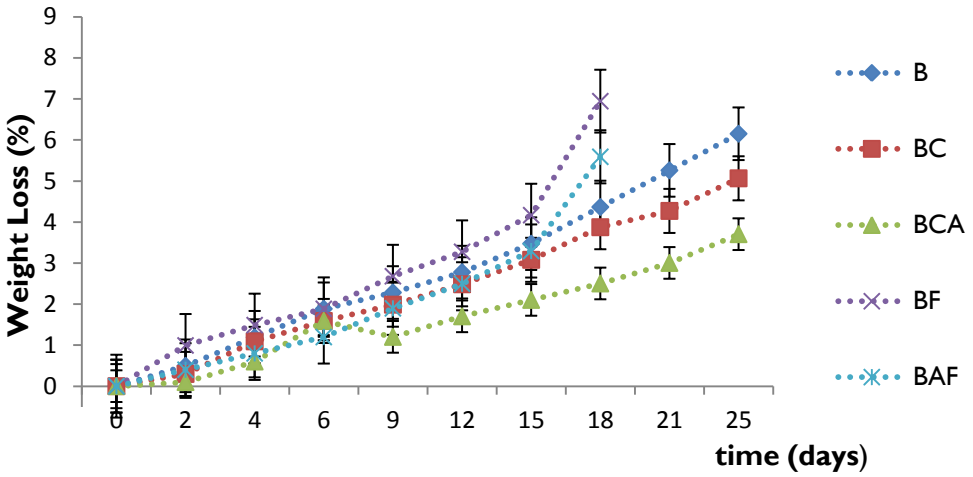


Figure 26 | Percentage of weight loss during 25 days storage at 5.5 ± 0,6 °C (without forced contamination) and for treatments with forced contamination during 15 days storage at 5.5 ± 0,6 °C and 3 days at 25 ± 0,5 °C (from 15th to 18th days). B - blueberry control, BC - blueberry with chitosan coating, BCA - blueberry coated with chitosan-Aloe vera coating, BF - blueberry with forced contamination and BAF - blueberry coated with chitosan-Aloe vera coating and with forced contamination.

Several authors reported that chitosan-based coatings, such as those based on *Aloe vera* plus chitosan, retard water loss from fruits over time [25, 27].

BCA treatment delayed dehydration of fruit, since the lowest weight loss was obtained with this treatment, with a weight loss of 3.7% after 25 days of storage. At 25th day, control (B) weight loss is two times higher than BCA treatment.

As to BC, it was observed that weight loss decreases by 1% comparing to control (B), at 25th day of storage.

For treatments with forced contamination (BF and BAF), the percentage of weight loss were more marked from 15thday, when exposed to room temperature (25 ± 0.6 °C).

In sum, at the end of storage, weight losses of B was 6.2%, BC was 5.1%, BCA was 3.7% (25th day, without forced contamination), BF was 6.9% and BAF was 5.6% (18th day, with forced contamination).

The results obtained for coatings with chitosan and with *Aloe vera* are in accordance with results presented by Hernandez-Munoz et al. (2008)[83] and Vargas et al. (2006)[84]. These authors showed that chitosan-based films promotes retention of water. Martinez-Romero et al. (2006)[59] showed that the films based on *Aloe vera* retard water loss and also controlled

gas exchange, thereby reducing respiration and oxidation reactions, during the storage period.

Hereupon, these results shown that higher SSC observed in B samples (Fig. 25), could be due to sugar concentration as a result of water loss by dehydration. Also, the lower weight loss found in Aloe-treated blueberries, can be translated by the maintenance of soluble solids over time. Thus, it was inferred that during postharvest storage, acid metabolism converted starch and acid to sugar, thus resulting in the decrease of TA (Fig. 23) and the increase of SSC [85].

4.5.5 Microbiological Analysis

Incorporation of an antifungal compound, such as *Aloe vera*, within edible chitosan-based coatings, it is intended to minimize problems that causes greatest damage in blueberry, in order to maintain CFU/g values, at least, within acceptable values in fruit products ($>10^2 \leq 10^5$ for yeast and $>10^2 \leq 10^3$ for mold) (Annex III).

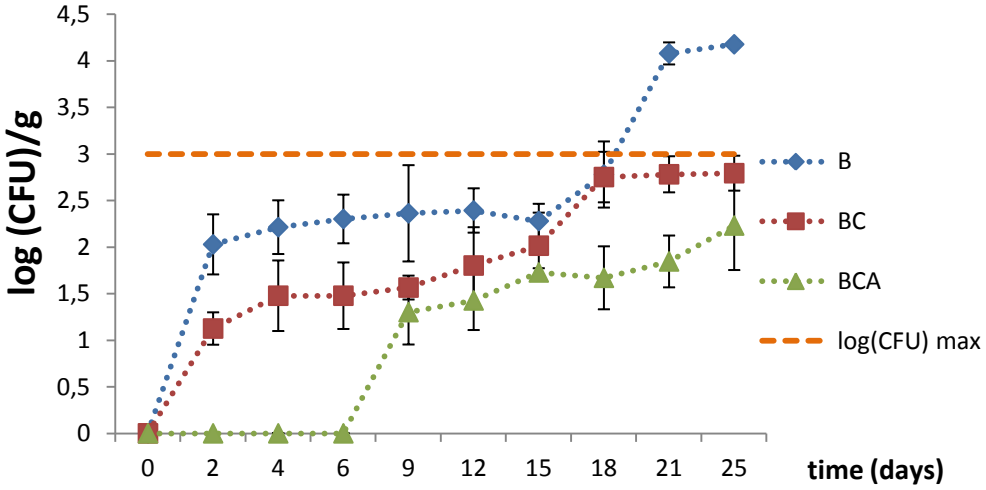


Figure 27 | Total yeasts and molds plate count (log CFU/g) for control and coated samples of blueberries without forced contamination during storage (25 days) at 5.5 ± 0.6 °C. The maximum acceptable limit for consumption is 3.0 log CFU/g, represented by the horizontal orange line. B - blueberry control, BC - blueberry with chitosan coating, BCA - blueberry coated with chitosan-Aloe vera coating, BF - blueberry with forced contamination and BAF - blueberry coated with chitosan-Aloe vera coating and with forced contamination.

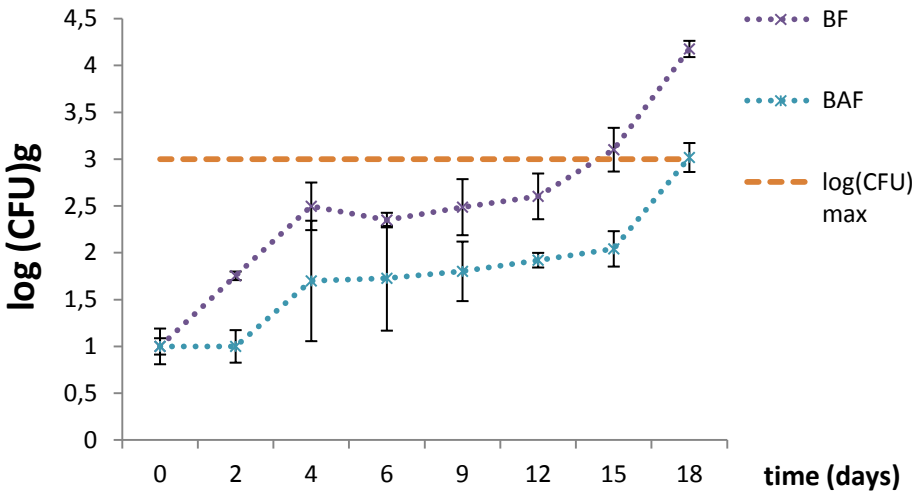


Figure 28 | Total yeasts and molds plate count (log CFU/g) for control coated sample of blueberries with forced contamination (*Botrytis cinerea*) during storage (18 days) at 5.5 ± 0.6 °C and 3 days at 25 ± 0,5 °C (from 15th to 18th days). The maximum acceptable limit for consumption is 3.0 log CFU/g, represented by the horizontal orange line. B - blueberry control, BC - blueberry with chitosan coating, BCA - blueberry coated with chitosan-Aloe vera coating, BF - blueberry with forced contamination and BAF - blueberry coated with chitosan-Aloe vera coating and with forced contamination.

By the statistical analysis of the data is relevant to mention that the different treatments over time there were significant differences in the growth of molds and yeasts ($p < 0.05$). Except for the 15th day for the B and BC treatments.

According to Figure 27, BCA presented contamination only on day 9 of storage (1.3 log (CFU)/g). These results showed that antifungal activity of *Aloe vera* juice demonstrated *in vitro* was also manifested *in vivo*.

Additionally, BCA presented log CFU/g values below the observed to uncoated fruit (B) and BC samples during 25 days of storage ($p < 0.05$). These results may be associated with fungicide action of the *Aloe vera* [49, 59]. BC showed lower CFU/g values (< 3 log CFU/g) when compared to B during 15 days of storage, a fact that may be associated with antifungal action of chitosan [30, 86, 87].

Regarding blueberries with forced contamination, results showed fungistatic effect of BAF during the storage period (Fig. 28). This coating (chitosan + *Aloe vera*) may improve blueberry safety by inhibition or delay of the growth of microorganisms, giving a further step to the concept of active packaging.

Through these results it can be concluded that chitosan coating and chitosan-*Aloe vera* coating were effective on extending blueberry shelf life, as can be seen in the Figure 27, since at day 18, B treatment exceeds 3 log (CFU)/g (maximum acceptable limit for consumption). The differences between BC and BCA ($p<0.05$) at the end of 25 days (2.8 log (CFU)/g and 2.2 log (CFU)/g, respectively) may be due to synergy between chitosan and *Aloe vera*, resulting in better antifungal capacity.

Castillo et al. (2010)[27], also obtained results that prove that *Aloe vera* gel could be applied as a postharvest treatment to inhibit microbial (yeasts and molds) spoilage and reduce decay incidence during storage of table grapes.

4.5.6 Colour

It is noteworthy that blueberries with coating presented an attractive natural-looking sheen. This is important because this is a key factor for consumers when purchasing fresh produce.

Colour parameters (L*, a* and b*) of coated and uncoated blueberries samples during 25 days of storage were studied. In sum, and observing Table 9, can be observed that the variation of color parameters is smaller in pulp of blueberries coated than in controls.

Results indicated that the use of edible coatings (with and without *Aloe vera*) had a significant ($p < 0.05$) effect in color parameter b* of blueberries.

Looking at the data of the Table 9, in general, L* values of the samples were slightly affected by the coating and storage time, however B treatment tend to reach a peak (32.41 ± 0.90) at 9th day and decrease to 31.11 ± 0.20 at 12th day. This occurrence may be explained by the fact that the *Aloe vera* being an antioxidant compound, helps to maintain stability and conservation of compounds present in blueberry, and consequently, retaining blueberry color. On the other hand, pulp darkening (decrease of L* values) may be associated with a net increase of total anthocyanins content, due to fruit ripening [59], thus BC, BCA and BAF retarded anthocyanins loss. All the treatments of blueberries became lighter during storage and the chromatic a* value increased significantly faster in the BF and BAF samples than in the samples without forces contamination ($p < 0.05$), getting results more close to red colour.

Table 9 | Colour parameters. L*, a* and b* coordinates values of blueberries samples during 25 days of storage.

		Storage time (days)									
		0	**2**	**4**	**6**	**9**	**12**	**15**	**18**	**21**	**25**
B	**L***	27.08 ± 0.09a	27.24 ± 0.26a	27.62 ± 0.36a	30.02 ± 0.55b	32.41 ± 0.90c	31.11 ± 0.22d	29.80 ± 0.60b,d	30.97 ± 0.52b,d	30.97 ± 0.46b,d	29.26 ± 0.61b,d
	a*	5.75 ± 0.32a	5,67 ± 0,10a	5.66 ± 0.26ª	7.30 ± 0.05b	7.92 ± 0.72b	7.00 ± 0.06c	6.58 ± 0.17d	8.37 ± 0.76b	8.12 ± 0.38b	6.34 ± 0.81c,d
	b*	0.75 ± 0.24a	1,07 ± 0,14a	0.97 ± 0.23ª	1.98 ± 0.24b	2.08 ± 0.75b	1.27 ± 0.11a	0.79 ± 0.16a	1.10 ± 0.22a	1.45 ± 0.28b	1.13 ± 0.29a
BC	**L***	26.92 ± 0.19a	27.06 ± 0.08a	26.57 ± 0.36ª	29.62 ± 0.76b	29.35 ± 0.37b	30.60 ± 0.23b	29.20 ± 0.30b	30.84 ± 0.37c	30.14 ± 0.38b	29.80 ± 0.58b
	a*	5.40 ± 0.34a	5.60 ± 0.47a	5.79 ± 0.26ª	7.27 ± 0.38b	7.27 ± 0.60b	7.67 ± 0.93b	8.49 ± 0.32c	8.23 ± 0.03c	9.12 ± 0.88c	7.84 ± 0.80b,c
	b*	0.93 ± 0.11a	0.99 ± 0.08a	1.08 ± 0.13ª	2.28 ± 0.45b	1.63 ± 0.30c	1.70 ± 0.30c	1.20 ± 0.14a,c	1.22 ± 0.11a,c	1.65 ± 0.47c	1.28 ± 0.15a,c
BCA	**L***	26.83 ± 0.27a	26.85 ± 0.18a	26.70 ± 0.03ª	28.29 ± 0.21b	29.97 ± 0.27c	30.14 ± 0.08c	29.96 ± 0.08c	30.50 ± 0.48c	30.10 ± 0.62c	31.41 ± 0.79d
	a*	7.59 ± 0.41a	7.75 ± 0.38a	7.89 ± 0.29ª	7.03 ± 0.75ª	7.88 ± 0.29a	8.92 ± 0.70b	8.61 ± 0.34b	10.40 ± 0.56c	8.77 ± 1.13a,b	9.93 ± 0.77b
	b*	0.90 ± 0.15a	0.89 ± 0.04a	0.92 ± 0.11ª	1.57 ± 0.50b	2.35 ± 0.29b	1.76 ± 0.28b	1.35 ± 0.10b	2.39 ± 0.43b	0.85 ± 0.35a,b	1.95 ± 0.37b
BF	**L***	26.93 ± 0.08a	27.04 ± 0.11a	27.49 ± 0.23b	29.37 ± 0.48c	29.65 ± 0.30c	30.43 ± 1.61c	33.16 ± 0.86d	29.08 ± 0.25c		
	a*	6.95 ± 0.21a	7.13 ± 0.15a	4.80 ± 0.17b	6.16 ± 0.49c	11.07 ± 0.26d	7.62 ± 0.99a,c	9.07 ± 0.85e	9.16 ± 0.68e		
	b*	0.97 ± 0.11a	0.93 ± 0.09a	1.47 ± 0.43ª	1.45 ± 0.22b	2.66 ± 0.10c	1.19 ± 0.55a	2.22 ± 1.01b,c	2.23 ± 0.25c		
BAF	**L***	26.86 ± 0.06a	26.96 ± 0.10a	27.68 ± 0.39b	27.90 ± 0.16b	31.27 ± 0.20c	30.97 ± 0.85c	29.75 ± 0.54d	30.68 ± 0.45c,d		
	a*	5.16 ± 0.27a	5.39 ± 0.30a	5.13 ± 0.37ª	5.53 ± 1.02ª	11.05 ± 0.68b	10.31 ± 0.08b	10.63 ± 0.77b	10.34 ± 1.11b		
	b*	0.89 ± 0.08a	0.97 ± 0.05a	1.40 ± 0.10b	1.70 ± 0.17c	2.82 ± 0.20d	2.48 ± 0.39d	2.47± 0.34d	1.93 ± 0.40d		

a-e: Different letters in the same line correspond to statistically different samples for a 95% confidence level

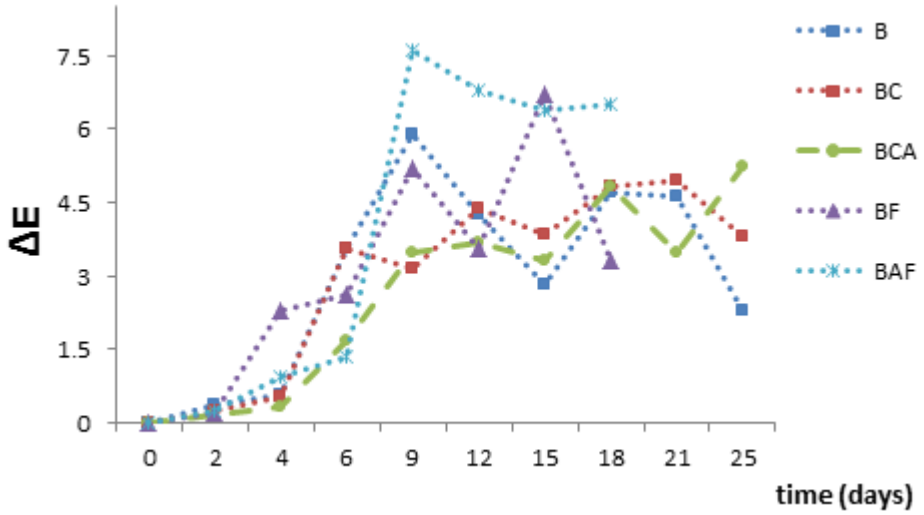


Figure 29 | ΔE values for blueberries samples during 25 days of storage. B - blueberry control, BC - blueberry with chitosan coating, BCA - blueberry coated with chitosan-Aloe vera coating, BF - blueberry with forced contamination and BAF - blueberry coated with chitosan-Aloe vera coating and with forced contamination.

As can be seen in Figure 29, the ΔE values are quite low (ΔE <16) [5], concluding that the colors are very similar for the different treatments over time. The coatings do not significantly change the standard pulp color of blueberries. However, it appears that minor changes of color are in the BC and BCA treatments when compared with B treatment (control).

5. CONCLUSIONS

5.1 Conclusions

Edible coatings coupled with storage conditions (temperature and relative humidity) can improve fruit quality and extend its shelf life by providing the fruit with a barrier to moisture and gases. *Aloe vera* added to the coating improves its performance against fungal contamination.

As expected, in the analysis of different treatments, ripening parameters such as colour, weight loss, SSC, TA and especially microbiological analyzes, showed significant changes from the initial levels to the final values after 25 days at 5.5 ± 0.6 °C (with no forced contamination) and 15 days at 5.5 ± 0.6 °C followed by 3 days at 25 ± 0.5 °C for blueberries with fungal contamination.

Thus, by maintaining a lower pH, the coated sample showed higher microbial inhibition and potentially contributes to prolong blueberry shelf-life. Use of chitosan coatings with *Aloe vera* reduce ripening index according to maintenance of TA while the content of SSC was unchanged along storage.

The incorporation of antifungal compounds, such as *Aloe vera*, into edible films or coatings provides a novel way to improve safety and shelf-life of blueberries, without using synthetic compounds.

The synergy between storage conditions and application of edible coatings based on chitosan and *Aloe vera* in blueberries has great potential in expanding the shelf-life of the fruit. This is explained and demonstrated in this research through the antifungal tests, in vitro and in vivo, and also by reducing rate of water loss level results, the two main factors of deterioration in the postharvest blueberry. Based on the tests performed, it is concluded that use of chitosan edible coating with *Aloe vera*, may extend the shelf life for about 5 days, which may represent a significant commercial value to blueberries producers.

For the other hand, *Aloe vera* fractions, could be an attractive natural alternative against fungi that attack fruits and vegetables, avoiding application and excessive use of chemicals, and thereby preventing the occurrence of health and environmental problems.

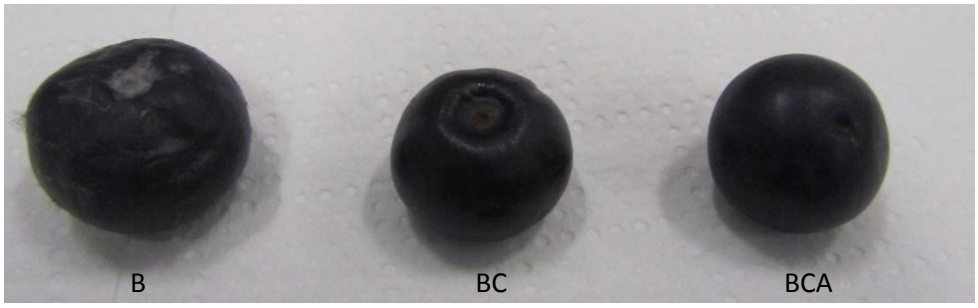


Figure II0 | Representing the final results of the different treatments (B, BC and BCA) after 25 days of storage at 5.5 ± 0.6 °C and with an average relative humidity of 90 ± 3%.

5.2 Recommendations and Future Projects

The main objectives of this thesis were, in general, achieved. However, some work can be improved and done to better understand the properties of the edible coating developed and how they can be used in the food industry. Thus, some recommendations for the improvement of this work and future projects can be advanced:

- Test concentrations of the juice of *Aloe vera* in the interval between 0.5% and 5%, since the results obtained in this thesis suggest a greater success in terms of water loss and microbial control when applied in blueberries.
- In order to confirm the mechanism of action of this coating preservation blueberry, it would be important to analyze other essays, such as, the texture of the fruit, sensorial analysis, determining the respiration rate throughout the storage time.
- As a future project, it would be interesting to use this coating solution at concentrations of *Aloe vera* superior, ideally 20% to avoid pathological diseases that may arise during the **preharvest** of the blueberries, replacing or minimizing the use of fungicides. Until now there are almost no publications on preharvest treatments with *Aloe vera* on fruit. These applications could reduce losses in blueberries before harvest, which is very important both economically and scientifically. Preharvest treatments also contribute to the maintenance of postharvest quality, with additional benefits of cost reduction and production of fruits with greater security, since infections in the field can be controlled.

6. BIBLIOGRAPHY

1 - P. K. Dutta, Shipra Tripathi, G. K. Mehrotra and Joydeep Dutta, **Perspectives for chitosan based antimicrobial films in food applications.** Food Chemistry 2009; 114 (4): 1173-1182.

2 - Pinheiro, A.C.; Cerqueira, M.A.; Souza, B.W.S.; Martins, J.T.; Teixeira, J.A.; Vicente, A.A., **Utilização de revestimentos/filmes edíveis para aplicações alimentares.** Boletim de Biotecnologia (2010) 18-28.

3 - C. Bourlieu, V. Guillard, B. Vallès-Pamiès, S. Guilbert and N. Gontard, **Edible Moisture Barriers: How to Assess of their Potential and Limits in Food Products Shelf-Life Extension?** Critical review in Food Science and Nutricion (2009) 49:5: 474-499.

4 - Elizabeth A. Baldwin, Robert Hagenmaier and Jinhe Bai, **Edible Coatings and Films to Improve Food Quality**, 2nd Edition, (2012) Taylor & Francis Group.

5 - G. I. Olivas and G. V. Barbosa-Cánovas, **Edible Coatings for Fresh-Cut Fruits**. Critical Reviews in Food Science and Nutrition, 2005; (45): 657-670.

6 - Carneiro-da-Cunha, M.G.; Cerqueira, M.A.; Souza, B.W.S.; Souza, M.P.; Teixeira, J.A. and Vicente, A.A., **Physical properties of edible coatings and films made with a polysaccharide from Anacardium occidentale L.** Journal of Food Engineering, 2009; 95(3): 379-385.

7 - AgroTec, **BRAGA: PRODUÇÃO DE MIRTILO E OUTROS PEQUENOS FRUTOS VISTA COMO SAÍDA PARA A CRISE.** AgroTec - Revista técnico-cientifica agrícola, 21 de Março de 2012.

8 - Mihalj Poša, Dejan Ćirin, Veljko Krstonošić, **Physico-chemical properties of bile salt-Tween 80 mixed micelles in the viewpoint of regular solution theory.** Chemical Engineering Science, 2013; 98(0): 195-202.

9 - Ruyi Wu, Balz Frei, James A. Kennedy, I, Yanyun Zhao, **Effects of refrigerated storage and processing technologies on the bioactive compounds and antioxidant capacities of 'Marion' and 'Evergreen' blackberries.** LWT - Food Science and Technology, 2010; 43(8): 1253-1264.

10 - Celia M. Cantín, Ioannis S. Minas, Vlasios Goulas, Manuel Jiménez, George A. Manganaris, Themis J. Michailides, Carlos H. Crisosto, **Sulfur dioxide fumigation alone or in combination with CO2-enriched atmosphere extends the market life of highbush blueberry fruit.** Postharvest Biology and Technology, 2012; 67(0): 84-91.

11 - Brewer GJ, Torricelli JR, Lindsey AL, Kunz EZ, Neuman A, Fisher DR, Joseph JA., **Age-related toxicity of amyloid-beta associated with increased pERK and pCREB in primary hippocampal neurons: reversal by blueberry extract.** The Journal of Nutritional Biochemistry, 2010; 21(10): 991-998.

12 – Lynn Stothers, **Chapter 10 - Cranberry and Other Dietary Supplements for the Treatment of Urinary Tract Infections in Aging Women. Complementary and Alternative Therapies and the Aging Population.** R. R. Watson. San Diego, Academic Press (2009): 179-191.

13 - Afshar K1, Stothers L, Scott H, MacNeily AE., **Cranberry Juice for the Prevention of Pediatric Urinary Tract Infection: A Randomized Controlled Trial.** The Journal of Urology, 2012; 188(4, Supplement): 1584-1587.

14 - J. B. Retamales and J. F. Hancock, **Blueberries.** Cambridge University Press, 2012; 48(4): 336

15 - Maria Beatriz Sousa, Teresa Curado, Fernando Negrão e Vasconcellos and Maria João Trigo, **Mirtilo - Qualidade Pós Colheita.** Divulgação Agro 556, Novembro 2007; nº8.

16 - Kathleen Demchak, **The Mid - Atlantic Berry Guide - for commercial berry growers (2013 - 2014).** Pennstate. AGRS-097. Chapter 7 - Blueberries: 115-135.

17 - Luís Lopes da Fonseca and Pedro Brás de Oliveira, **A planta de mirtilo - Morfologia e fisiologia.** Agro 556. Divulgação AGRO 556, Novembro 2007; nº2.

18 - Robert E Gough, **The Highbush Blueberry and Its Management.** CRC Press; 1 edition, 1991; 288.

19 - Nicoletta Sinelli, Anna Spinardi, Valentina Di Egidio, Ilaria Mignani, Ernestina Casiraghi, **Evaluation of quality and nutraceutical content of blueberries (Vaccinium corymbosum L.) by near and mid-infrared spectroscopy.** Postharvest Biology and Technology, 2008; 50(1): 31-36.

20 - Jamil Harb, Basel Khraiwesh, Josef Streif, Ralf Reski, Wolfgang Frank, **Characterization of blueberry monodehydroascorbate reductase gene and changes in levels of ascorbic acid and the antioxidative capacity of water soluble antioxidants upon storage of fruits under various conditions.** Scientia Horticulturae, 2010; 125(3): 390-395.

21 - Bunea A, Rugină D, Sconța Z, Pop RM, Pintea A, Socaciu C, Tăbăran F, Grootaert C, Struijs K, VanCamp J., **Anthocyanin determination in blueberry extracts from various cultivars and their antiproliferative and apoptotic properties in B16-F10 metastatic murine melanoma cells.** Phytochemistry, 2013; 95(0): 436-444.

22 - Joseph JA, Denisova N, Fisher D, Bickford P, Prior R, Cao G., **AGE-RELATED NEURODEGENERATION AND OXIDATIVE STRESS: Putative Nutritional Intervention.** Neurologic Clinics, 1998; 16(3): 747-755.

23 - Gad G. Yousef, Allan F. Brown, Yayoi Funakoshi, Flaubert Mbeunkui, Mary H. Grace, James R. Ballington , Ann Loraine, and Mary A. Lila, **Efficient Quantification of the Health Relevant Anthocyanin and Phenolic Acid Profiles in Commercial Cultivars and Breeding Selections of Blueberries (Vaccinium spp.).** Journal of Agricultural and Food Chemistry, 2013; 61: 4806-4815.

24 - Filomena Serrado, Marisa Pereira, Sofia Freitas, Susana Martins, Teresa Dias, **Mirtilos - Guia de boas práticas para a produção, promoção e comercialização.** ADRIMAG – Associação de Desenvolvimento Rural das Serras de Montemuro, Arada e Gralheira, em parceria com a AGIM – Associação para a Gestão, Inovação e Modernização do Centro Urbano de Sever do Vouga e a Mirtilusa – Sociedade de produtores hortofrutícolas, Lda, 2008.

25 - Guiyun Yang, Jin Yue, Xincheng Gong, Bingjun Qian, Huajun Wang, Yun Deng, Yanyun Zhao, **Blueberry leaf extracts incorporated chitosan coatings for preserving postharvest quality of fresh blueberries.** Postharvest Biology and Technology, 2014; 92(0): 46-53.

26 - L.K. Mehra, D.D. MacLean, R.L. Shewfelt, K.C. Smith, H. Scherm, **Effect of postharvest biofumigation on fungal decay, sensory quality, and antioxidant levels of blueberry fruit.** Postharvest Biology and Technology, 2013; 85(0): 109-115.

27 - S. Castillo, D. Navarro, P.J. Zapata, F. Guillén, D. Valero, M. Serrano, D. Martínez-Romero, **Antifungal efficacy of Aloe vera in vitro and its use as a preharvest treatment to maintain postharvest table grape quality.** Postharvest Biology and Technology, 2010; 57(3): 183-188.

28 - Shiow Y. Wang, Chi-Tsun Chen, Jun-Jie Yin, **Effect of allyl isothiocyanate on antioxidants and fruit decay of blueberries.** Food Chem., 2010; 120(1): 199-204.

29 - Mary Anne Roshni Amalaradjou, Kumar Venkitanarayanan, **Mycotoxins in Fruits and Vegetables.** Chapter 10 - Detection of Penicillium, Aspergillus and Alternaria Species in Fruits and Vegetables. Edited by: Rivka Barkai-Golan and Nachman Paster, Academic Press, 2008; 225-247.

30 - M.A. Cerqueira, A.I. Bourbon, A.C. Pinheiro, J.T. Martins, B.W.S. Souzaa, J.A. Teixeira, A.A. Vicente, **Galactomannans use in the development of edible films/coatings for food applications.** Trends in Food Science & Technology, 2011; 22(12): 662-671.

31 - Liqun Yang, Li-Ming Zhang, **Chemical structural and chain conformational characterization of some bioactive polysaccharides isolated from natural sources.** Carbohydrate Polymers, 2009; 76(3): 349-361.

32 - Alison Matalanis, Owen Griffith Jones, David Julian McClements, **Structured biopolymer-based delivery systems for encapsulation, protection, and release of lipophilic compounds.** Food Hydrocolloids, 2011; 25(8): 1865-1880.

33 - Cha, D. S. and Chinnan, M. S., **Biopolymer-Based Antimicrobial Packaging: A Review.** Critical Reviews in Food Science and Nutrition, 2004; 44(4):223-37.

34 - David L. Nelson and Michael M. Cox, **Lehninger Principles of Biochemistry.** 4th edition, New York, Worth Publishers, 2000.
Web site at: **www.whfreeman.com/lehninger4e**

35 - Fereidoon Shahidi, Janak Kamil Vidana Arachchi, You-Jin Jeon, **Food applications of chitin and chitosans.** Trends Food Sci. Technology, 1999; 10(2): 37-51.

36 - Yu Zhong, Xiaoyong Song, Yunfei Li, **Antimicrobial, physical and mechanical properties of kudzu starch–chitosan composite films as a function of acid solvent types.** Carbohydrate Polymers, 2011; 84(1): 335-342.

37 - Jiang T, Deng M, James R, Nair LS, Laurencin CT, **Micro- and nanofabrication of chitosan structures for regenerative engineering.** Acta Biomater, 2014; 10(4): 1632-1645.

38 - Neeta B. Gol, Pooja R. Patel, T.V. Ramana Rao, **Improvement of quality and shelf-life of strawberries with edible coatings enriched with chitosan.** Postharvest Biology and Technology, 2013; 85(0): 185-195.

39 - B.L. Butler, P.J. Vergano, R.F. Testin, J.M. Bunn and J.L. Wiles, **Mechanical and barrier properties of edible chitosan films as affected by composition and storage.** Journal of Food Science, 1996; 61(5): 952-956.

40 - Ricardo Villalobos, Pilar Hernández-Muñoz, Amparo Chiralt, **Effect of surfactants on water sorption and barrier properties of hydroxypropyl methylcellulose films.** Food Hydrocolloids, 2006 20(4): 502-509.

41 - Khalid Ziani, Javier Oses, Veronique Coma, Juan I. Maté, **Effect of the presence of glycerol and Tween 20 on the chemical and physical properties of films based on chitosan with different degree of deacetylation.** LWT - Food Science and Technology, 2008; 41(10): 2159-2165.

42 - Miguel A. Cerqueira, Álvaro M. Lima, José A. Teixeira, Renato A. Moreira, António A. Vicente, **Suitability of novel galactomannans as edible coatings for tropical fruits.** Journal of Food Engineering, 2009; 94(3-4): 372-378.

43 - Virginia Epure, Marie Griffon, Eric Pollet, Luc Avérous, **Structure and properties of glycerol-plasticized chitosan obtained by mechanical kneading.** Carbohydrate Polymers, 2011; 83(2): 947-952.

44 - J. Prakash Maran, V. Sivakumar, K. Thirugnanasambandham, R. Sridhar, **Response surface modeling and analysis of barrier and optical properties of maize starch edible films.** International Journal of Biological Macromolecules, 2013; 60(0): 412-421.

45 - D. Jasso de Rodríguez, R. Rodríguez García, F.D. Hernández Castillo, C.N. Aguilar González, A. Sáenz Galindo, J.A. Villarreal Quintanilla, L.E. Moreno Zuccolotto, **In vitro antifungal activity of extracts of Mexican Chihuahuan Desert plants against postharvest fruit fungi.** Industrial Crops and Products, 2011; 34(1): 960-966.

46 - A. Nabigol and A. Asghari, **Antifungal activity of *Aloe vera* gel on quality of minimally processed pomegranate arils.** International Journal of Agronomy and Plant Production, 2013; 4: 833-838.

47 - Alana Novaes Azevedo, Paula Ribeiro Buarque, Elizângela Mércia Oliveira Cruz, Arie Fitzgerald Blank, Péricles Barreto Alves, Maria Lúcia Nunes, Luciana Cristina Lins de Aquino Santana, **Response surface methodology for optimisation of edible chitosan coating formulations incorporating essential oil against several foodborne pathogenic bacteria.** Food Control, 2014; 43(0): 1-9.

48 - Yolanta Saks, Rivka Barkai-Golan, ***Aloe vera* gel activity against plant pathogenic fungi.** Postharvest Biology and Technology, 1995; 6(1-2): 159-165.

49 - D. Jasso de Rodríguez, D. Hernández-Castillo, R. Rodríguez-García, J.L. Angulo-Sánchez, **Antifungal activity in vitro of *Aloe vera* pulp and liquid fraction against plant pathogenic fungi.** Industrial Crops and Products, 2005; 21(1): 81-87.

50 - Ana I. Bourbon, Ana C. Pinheiro, Miguel A. Cerqueira, Cristina M.R. Rocha, Maria C. Avides, Mafalda A.C. Quintas, António A. Vicente, **Physico-chemical characterization of chitosan-based edible films incorporating bioactive compounds of different molecular weight.** Journal of Food Engineering, 2011; 106(2): 111-118.

51 - Antonio Vega-Gálveza, Margarita Miranda, Mario Aranda, Karem Henriquez, Judith Vergara, Gipsy Tabilo-Munizaga, Mario Pérez-Won**, Effect of high hydrostatic pressure on functional properties and quality characteristics of *Aloe vera* gel (*Aloe barbadensis* Miller).** Food Chemistry, 2011; 129(3): 1060-1065.

52 - A. Bozzi, C. Perrin, S. Austin, F. Arce Vera, **Quality and authenticity of commercial *Aloe vera* gel powders.** Food Chemistry, 2007; 103(1): 22-30.

53 - Boudreau, M. D. and Beland, F. A., **An Evaluation of the Biological and Toxicological Properties of *Aloe Barbadensis* (Miller), *Aloe Vera*.** Journal of Environmental Science and Health Part C Environ Carcinog Ecotoxicol Rev., 2006; 24: 103-154.

54 - Ahlawat, K. S. and B. S. Khatkar , **Processing, food applications and safety of *A. vera* product: a review.** J Food Sci Technol, 2011; 48(5): 525-533.

55 - Choi, S. and Chung, M., **A review on the relationship between *Aloe vera* components and their biologic effects.** Seminars in Integrative Medicine, 2003; 1(1): 53-62.

56 – Rodriguez, E.R; Martin, J.D and Romero, C.D., ***Aloe vera* as a functional ingrediente in Foods.** Critical Reviews in Food science and Nutrition, 2010; 50:305-326.

57 - Anirban Ray and S. Dutta Gupta, **A panoptic study of antioxidant potential of foliar gel at different harvesting regimens of *Aloe vera* L..** Industrial Crops and Products, 2013; 51: 130-137.

58 - Margarita Miranda, Antonio Vega-Gálvez, Purificación García, Karina Di Scala, John Shi, Sophia Xue, Elsa Uribe, **Effect of temperature on structural properties of *Aloe vera* (*Aloe barbadensis* Miller) gel and Weibull distribution for modelling drying process.** Food and Bioproducts Processing, 2010; 88: 138–144.

59 - D. Martínez-Romero, N. Alburquerque, J.M. Valverde, F. Guillén, S. Castillo, D. Valero, M. Serrano, **Postharvest sweet cherry quality and safety maintenance by *Aloe vera* treatment: A new edible coating.** Postharvest Biology and Technology, 2006; 39(1): 93-100.

60 - Diana Navarro, Huertas M. Díaz-Mula, Fabián Guillén, Pedro J. Zapata, Salvador Castillo, María Serrano, Daniel Valero, Domingo Martínez-Romero, **Reduction of nectarine decay caused by Rhizopus stolonifer, Botrytis cinerea and Penicillium digitatum with *Aloe vera* gel alone or with the addition of thymol.** International Journal of Food Microbiology, 2011; 151(2): 241-246.

61 - Diego Paladines, Daniel Valero, Juan Miguel Valverde, Huertas Díaz-Mula, María Serrano, Domingo Martínez-Romero, **The addition of rosehip oil improves the beneficial effect of *Aloe vera* gel on delaying ripening and maintaining postharvest quality of several stonefruit.** Postharvest Biology and Technology, 2014; 92(0): 23-28.

62 - Gemma Oms-Oliu, Mª Alejandra Rojas-Graü, Laura Alandes González, Paula Varela, Robert Soliva-Fortuny, Mª Isabel Hernando Hernando, Isabel Pérez Munuera, Susana Fiszman, Olga Martín-Belloso, **Recent approaches using chemical treatments to preserve quality of fresh-cut fruit: A review.** Postharvest Biology and Technology, 2010; 57(3): 139-148.

63 - Fabián Guillén, Huertas M. Díaz-Mula, Pedro J. Zapata, Daniel Valero, María Serrano, Salvador Castillo, Domingo Martínez-Romero, ***Aloe arborescens* and *Aloe vera* gels as coatings in delaying postharvest ripening in peach and plum fruit.** Postharvest Biology and Technology, 2013; 83(0): 54-57.

64 - Juan Miguel Valverde, Daniel Valero, Domingo Martínez-Romero, Fabián Guillén, Salvador Castillo, María Serrano, **Novel Edible Coating Based on *Aloe vera* Gel To Maintain Table Grape Quality and Safety.** J. Agric. Food Chem., 2005; 53: 7807-7813.

65 - A. Casariego, B.W.S. Souza, A.A. Vicente, J.A. Teixeira, L. Cruz, R. Díaz, **Chitosan coating surface properties as affected by plasticizer, surfactant and polymer concentrations in relation to the surface properties of tomato and carrot.** Food Hydrocolloids, 2008; 22(8): 1452-1459.

66 - Thawien Bourtooma and Manjeet S. Chinnan, **Preparation and properties of rice starch–chitosan blend biodegradable film.** LWT - Food Science and Technology, 2008; 41(9): 1633-1641.

67 - Miguel A. Cerqueira, Bartolomeu W.S. Souza, Joana T. Martins, José A. Teixeira, António A. Vicente, **Seed extracts of Gleditsia triacanthos: Functional properties evaluation and incorporation into galactomannan films.** Food Research International, 2010; 43: 2032-2038.

68 - K. Konan, N. J. David, M. Souleymane, Y. Ahoua, Y. H. Félix, D. A. Joseph, ***In Vitro* Antioxidant Activity and Phenolic Contents of the Leaves of Olax subscorpioidea and Distemonanthus benthamianus.** Research Journal of Pharmaceutical, Biological and Chemical Sciences, 2013, 4: 1419-1429.

69 - IFST (1993). **Shelf Life of Foods – Guidelines for its Determination and Prediction.** London: Institute of Food Science & Technology.

70 - W. B. Williams, M. Cuvelier, C. Berset, **Use of a free radical method to evaluate antioxidant activity**. Lebensm-Wiss Technol, 1995; 28: 25-30.

71 - V. Del-Valle, P. Hernández-Muñoz, A. Guarda, M.J. Galotto, **Development of a cactus-mucilage edible coating (Opuntia ficus indica) and its application to extend strawberry (Fragaria ananassa) shelf-life.** Food Chemistry, 2005; 91(4): 751-756.

72 - O. Skurtys, P. Velásquez, O. Henriquez, S. Matiacevich, J. Enrione, F. Osorio, **Wetting behavior of chitosan solutions on blueberry epicarp with or without epicuticular waxes.** LWT - Food Science and Technology, 2011; 44(6): 1449-1457.

73 - Joana T. Martins, Miguel A. Cerqueira, Bartolomeu W.S. Souza, M.d. Carmo Avides, Antonio A. Vicente, **Shelf Life Extension of Ricotta Cheese Using Coatings of Galactomannans from Nonconventional Sources Incorporating Nisin against Listeria monocytogenes.** J. Agric. Food Chem., 2010; 58: 1884-1891.

74 - Rulon E. Johnson Jr. and Robert H. Dettre, **The wettability of low-energy liquid surfaces.** Journal of Colloid and Interface Science, 1996; 21(6): 610-622.

75 - Tapashi Sengupta, Jung H. Han, **Chapter 4 – Surface chemistry of food, packaging and biopolymer materials.** Innovations in Food Packaging (Second Edition), 2014; 51-86.

76 - Y. Pranoto, S.K. Rakshit, , V.M. Salokhe, **Enhancing antimicrobial activity of chitosan films by incorporating garlic oil, potassium sorbate and nisin.** LWT - Food Science and Technology, 2005; 38(8): 859-865.

77 - Sadegh Khoshgozaran-Abras, Mohammad Hossein Azizi, Zohreh Hamidy, Niloofar Bagheripoor-Fallah, **Mechanical, physicochemical and color properties of chitosan based-films as a function of Aloe vera gel incorporation.** Carbohydrate Polymers, 2012; 87(3): 2058-2062.

78 - Jean Bruneton, **Pharmacognosy, phytochemistry, medicinal plants.** Lavoisier, 2nd Edition, 1995; 1136.

79 - Dr. Jorge Enrique León Sarabia, Dra. Vivian del Pilar Rosales Clares, Lic. Reinaldo Alberto Rosales Clares and Dr. Vital Pavón Hernández, **Actividad Antiflamatoria y Cicatrizante del Unguento Rectal de Aloe vera L. (SÁBILA).** REV CUBANA PLANT MED., 1999; 4: 106-109.

80 - Margarita Miranda, Héctor Maureira, Katia Rodríguez, Antonio Vega-Gálvez, **Influence of temperature on the drying kinetics, physicochemical properties, and antioxidant capacity of Aloe Vera (Aloe Barbadensis Miller) gel.** Journal of Food Engineering, 2009; 91(2): 297-304.

81 - S. Benítez, I. Achaerandio, F. Sepulcre, M. Pujolà, ***Aloe vera* based edible coatings improve the quality of minimally processed 'Hayward' kiwifruit.** Postharvest Biology and Technology, 2013; 81: 29-36.

82 - Nuno M. Soares, Tânia S. Mendes, António A. Vicente, **Effect of chitosan-based solutions applied as edible coatings and water glazing on frozen salmon preservation – A pilot-scale study.** Journal of Food Engineering, 2013; 119(2): 316-323.

83 - P. Hernandez-Munoz, E. Almenar, V. Del Vale, D. Velez and R. Gavara, **Effect of chitosan coating combined with postharvest calcium treatment on strawberry (Fragaria × ananassa)** quality during refrigered storage. Food Chemistry, 2008; 110: 428-435.

84 - M. Vargas, A. Albors, A. Chiralt and C. Gonzalez-Martinez, **Quality of cold-stored strawberries as affected by chitosan-oleic acid edible coatings.** Postharvest Biology and Technology, 2006; 41: 164-171.

85 - Jingyun Duan, Ruyi Wu, Bernadine C. Strik, Yanyun Zhao, **Effect of edible coatings on the quality of fresh blueberries (Duke and Elliott) under commercial storage conditions.** Postharvest Biology and Technology, 2011; 59(1): 71-79.

86 - Hui Ai, Furong Wang, Yuqian Xia, Xiaomin Chen, Chaoliang Lei, **Antioxidant, antifungal and antiviral activities of chitosan from the larvae of housefly, Musca domestica L.** Food Chemistry, 2012; 132(1): 493-498.

87 - Qiu M., Wu C., Ren G., Liang X., Wang X., Huang J., **Effect of chitosan and its derivatives as antifungal and preservative agents on postharvest green asparagus.** Food Chemistry, 2014; 155(0): 105-111.

Annexs

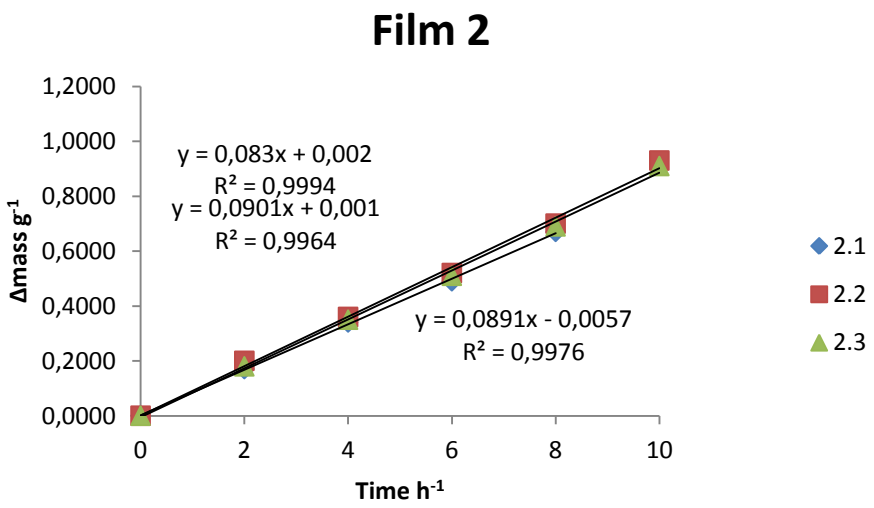
ANNEX I

The results presented are related to the data used to determine the WVTR, and consequently will obtain the relative values of permeability of the four films selected formulations (2, 3, 5, 6) from the scattering coefficient (WC).

Formulation 2 (0.5% Chi + 0.5% Gly + 0.1% Twe80)

	Mass g^{-1}			Δmass g^{-1}		
Time h^{-1}	**2.1**	**2.2**	**2.3**	**2.1**	**2.2**	**2.3**
0	196,53	195,69	189,03	0,0000	0,0000	0,0000
2	196,36	195,49	188,85	0,1700	0,2000	0,1800
4	196,19	195,33	188,68	0,3400	0,3600	0,3500
6	196,04	195,17	188,52	0,4900	0,5200	0,5100
8	195,86	194,99	188,34	0,6700	0,7000	0,6900
10	195,64	194,76	188,12	0,8900	0,9300	0,9100

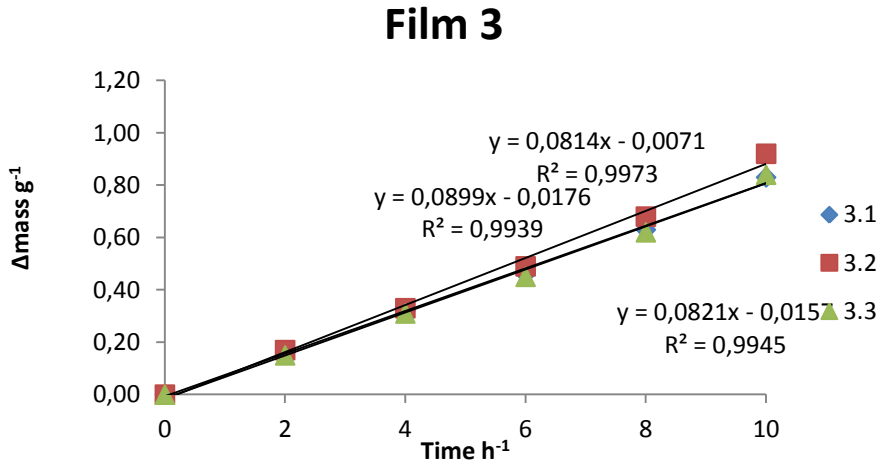
*GLY – GLYCEROL
*CHI – CHITOSAN
*TWE80 – TWEEN 80



Formulation 3 (0.5% Chi + 0.5% Gly + 0.2% Twe80)

	Mass g^{-1}			Δmass g^{-1}		
Time h^{-1}	**3.1**	**3.2**	**3.3**	**3.1**	**3.2**	**3.3**
0	199,26	179,08	197,01	0,0000	0,0000	0,0000
2	199,10	178,91	196,86	0,1600	0,1700	0,1500
4	198,94	178,75	196,70	0,3200	0,3300	0,3100
6	198,80	178,59	196,56	0,4600	0,4900	0,4500
8	198,63	178,40	196,39	0,6300	0,6800	0,6200
10	198,43	178,16	196,17	0,8300	0,9200	0,8400

*GLY – GLYCEROL
*CHI – CHITOSAN
*TWE80 – TWEEN 80

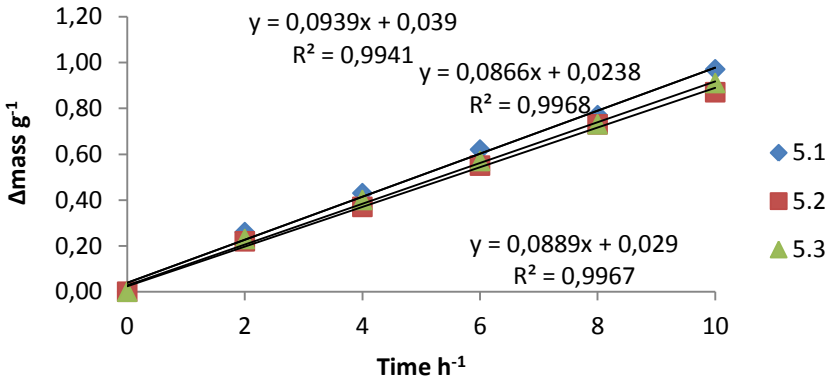


Formulation 5 (0.5% Chi + 1.0% Gly + 0.1% Twe80)

	Mass g^{-1}			Δmass g^{-1}		
Time h^{-1}	**5.1**	**5.2**	**5.3**	**5.1**	**5.2**	**5.3**
0	196,86	195,46	189,22	0,0000	0,0000	0,0000
2	196,6	195,24	188,99	0,2600	0,2200	0,2300
4	196,43	195,09	188,82	0,4300	0,3700	0,4000
6	196,24	194,91	188,65	0,6200	0,5500	0,5700
8	196,09	194,76	188,49	0,7700	0,7300	0,7300
10	195,89	194,59	188,31	0,9700	0,8700	0,9100

*GLY – GLYCEROL
*CHI – CHITOSAN
*TWE80 – TWEEN 80

Film 5

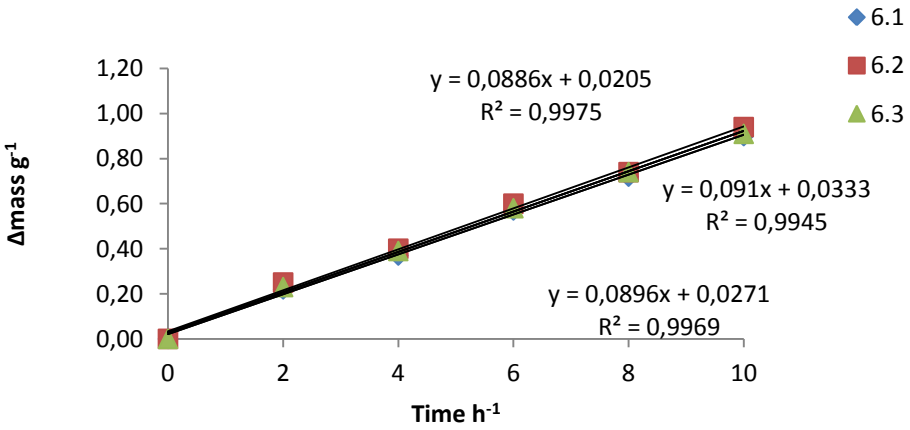


Formulation 6 (0.5% Chi + 1.0% Gly + 0.2% Twe80)

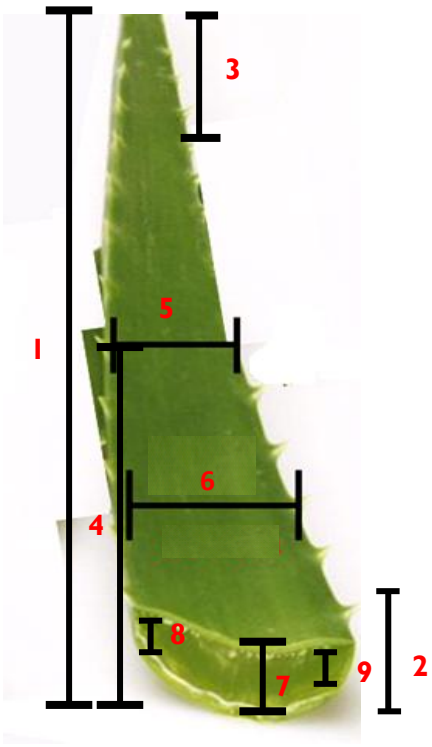
	Mass g^{-1}			Δmass g^{-1}		
Time h^{-1}	**6.1**	**6.2**	**6.3**	**6.1**	**6.2**	**6.3**
0	188,89	198,91	197,13	0,0000	0,0000	0,0000
2	188,67	198,66	196,90	0,2200	0,2500	0,2300
4	188,52	198,51	196,74	0,3700	0,4000	0,3900
6	188,32	198,31	196,55	0,5700	0,6000	0,5800
8	188,17	198,15	196,39	0,7200	0,7400	0,7400
10	187,99	197,97	196,22	0,9000	0,9400	0,9100

*GLY – GLYCEROL
*CHI – CHITOSAN
*TWE80 – TWEEN 80

Film 6



ANNEX II



1 – Comprimento total
2 – Parte inferior
3 – Parte superior
4 – Distância desde o início da folha
5 – Diâmetro médio
6 – Diâmetro inferior
7 – Espessura central
8 – Espessura lateral direita
9 – Espessura lateral esquerda

Amostra	Peso folha (g)	Peso resíduo (g)	Comprimento total (cm)	Parte inferior (cm)	Dm inferior (cm)*	Dm médio (cm)**	Distância desde início da folha** (cm)	Espessura central (cm)
1	424.1	75.7	35.0	6.0	10.4	9.2	16.0	2.0
2	384.3	81.1	39.5	5.5	10.0	8.5	16.0	2.6
3	398.4	93.0	37.5	6.0	9.6	8.7	16.0	2.3
4	401.4	115.1	50.0	6.0	9.3	8.0	16.0	2.2
5	371.4	111.0	37.0	6.5	9.6	8.4	15.0	2.9
6	2825	105.5	31.0	6.5	10.0	7.6	14.0	2.5
Media	377.02	96.90	38.33	6.08	9.82	8.40	15.50	2.42
Desvio	49.57	16.24	6.40	0.38	0.39	0.55	0.84	0.32

Amostra	Espessura direito (cm)	Espessura esquerdo (cm)	Parte superior (cm)	Peso bagaço (g)	Peso Gel (g)	Peso Sumo (g)	Volume do sumo (mL)
1	1.7	1.6	5.0	103.2	58.4	130.0	131.0
2	1.8	1.3	6.5	111.3	49.8	151.8	158.0
3	1.8	1.8	5.5	84.7	57.0	122.4	132.0
4	1.5	1.6	7.5	73.9	33.0	155.2	157.0
5	2.0	1.7	7.5	90.1	37.8	103.0	110.0
6	1.5	2.3	8.0	55.5	39.4	67.6	78.0
Media	1.72	1.72	6.67	86.45	45.90	121.67	127.70
Desvio	0.19	0.33	1.21	20.15	10.67	32.78	30.30

Dm – Diâmetro

	Rendimento (%)		
Amostra	**Bagaço**	**Gel**	**Sumo**
1	35.3909465	20.0274348	44.5816187
2	35.5704698	15.915628	48.5139022
3	32.0711852	21.5827338	46.346081
4	28.1953453	12.5906143	59.2140404
5	39.0212213	16.3707233	44.6080554
6	34.1538462	24.2461538	41.6
Media	34.07	18.46	47.48
Desvio	3.34	3.89	5.65

Fracção	Volume (mL)	pH	°Brix
Sumo	2820	4,72	0,9
Gel	1300	4,45	0,8

ANNEX III

Values Guide for total microorganisms in food products.

Microrganismo	Grupo de alimentos	Qualidade Microbiológica (ufc/g quando não indicado)			
		Satisfatório	Aceitável	Não satisfatório	Inaceitável / potencialmente perigoso
Microrganismos a 30ºC	1 2 3	$\leq 10^2$ $\leq 10^3$ $\leq 10^4$	$>10^2 \leq 10^4$ $>10^3 \leq 10^5$ $>10^4 \leq 10^6$	$>10^4$ $>10^5$ $>10^6$	NA NA NA
Leveduras	1* e 2 3	$\leq 10^2$ $\leq 10^2$	$>10^2 \leq 10^4$ $>10^2 \leq 10^5$	$>10^4$ $>10^5$	NA NA
Bolores	1* e 2 3	≤ 10 $\leq 10^2$	$>10 \leq 10^2$ $>10^2 \leq 10^3$	$>10^2$ $>10^3$	# #
Coliformes totais	1 2 3	≤ 10 ≤ 10 $\leq 10^2$	$>10 \leq 10^2$ $>10 \leq 10^3$ $>10^2 \leq 10^4$	$>10^2$ $>10^3$ $>10^4$	NA NA NA
E. coli	1, 2 3	<10 ≤ 10	NA $>10<10^2$	≥ 10 $\geq 10^2$	NA NA
Listeria spp.	1, 2 e 3	$<10^2$	NA	$\geq 10^2$	NA
Anaeróbios sulfito redutores	1, 2 e 3	≤ 10	$>10 \leq 10^3$	$>10^3<10^4$	$\geq 10^4$#
Patogénios					
Staphylococcus coagulase positiva	1, 2 e 3	$<10^2$	NA	$\geq 10^2 \leq 10^4$	$>10^4$
Bacillus cereus	1, 2 e 3	$\geq 10^2$	$>10^2 \leq 10^3$	$>10^3<10^5$	$\geq 10^5$
Clostridium perfringens	1, 2 e 3	<10	$\geq 10 \leq 10^3$	$>10^3<10^4$	$\geq 10^4$
Salmonella spp.	1, 2 e 3	Ausente em 25g			Presente em 25g
Listeria monocytogenes	1, 2 e 3	Ausente em 25g	Presente em 25g $<10^2$#	-	$\geq 10^2$
Campylobacter spp.	1, 2 e 3	Ausente em 25g			Presente em 25g
Vibrio parahaemolyticus	1, 2 e 3	Ausente em 25g			Presente em 25g
Yersinia enterocolitica	1, 2 e 3	Ausente em 25g			Presente em 25g

NA - Not acceptable

Food group:

1 - Meals; sandwiches; cakes; Sweet desserts with ingredients fully cooked, or added spices, herbs, dried, dehydrated or treated with ionizing radiation, UHT products and mayonnaise.

2 - Meals; sandwiches; cakes; Cooked candy desserts added with raw ingredients and/or with specific flora.

3 - Salads; vegetables; Raw fruits.