Universidade de Lisboa

Faculdade de Farmácia

Development of cranberry extract films for the enhancement of food packaging antimicrobial properties

Catarina Monteiro Severo

Dissertation supervised by Professor Isabel Alexandra Caldeira Ribeiro and co-supervised by Professor Ana Francisca Campos Simão Bettencourt

Food Quality and Health

2019

Universidade de Lisboa

Faculdade de Farmácia

Development of cranberry extract films for the enhancement of food packaging antimicrobial properties

Catarina Monteiro Severo

Dissertation supervised by Professor Isabel Alexandra Caldeira Ribeiro and co-supervised by Professor Ana Francisca Campos Simão Bettencourt

Food Quality and Health

2019

Abstract

Numerous outbreaks of foodborne pathogens have been found to be associated with biofilms, which poses a concern to the food industry since foodborne pathogens and their biofilm can adhere to a wide range of food contact materials. There are current strategies to control this problem such as chemical treatments to prevent biofilm formation, however, there has been an arising problem of resistant strains that decreased the effectiveness of these conventional methods. Thus, there is a need for the development of novel strategies that can control biofilm formation, especially regarding smart and intelligent packaging systems able to incorporate antimicrobial agents into the packaging material. Berries are suggested to have numerous health benefits for humans due to its high content in phenolic compounds, especially flavonoids. These polyphenolic antioxidants prove to have various biological activities such as anti-inflammatory and antimicrobial properties. Thus, the main aim of this research was to develop a biodegradable film incorporated with cranberry extracts intended for food packaging and to study the film antimicrobial impact against some biofilm forming bacteria.

The study started by evaluating the antimicrobial properties (Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Minimum Biofilm Inhibitory Concentration (MBIC)), the total phenolic content and the antioxidant capacity (DPPH radical scavenging activity) of cranberry extracts from three different cranberry sources, respectively fresh, powdered and dried cranberries. Then, chitosan based films were developed, optimized and their physical and mechanical properties were evaluated through several analysis such as Total Soluble Matter (TSM), moisture content, swelling degree, surface wettability degree, light-barrier properties, hardness values, water vapour permeability and oxygen transmission rate. After cranberry extracts incorporation, films antimicrobial properties were tested through a disk diffusion assay and films antibiofilm properties were tested by the plate counting method and by scanning electron microscopy.

The results showed that all cranberry extracts presented antimicrobial properties against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 and antioxidant properties. Fresh and powdered cranberries extracts showed the highest antimicrobial properties. Both extracts had a MIC against *S. aureus* and *E. coli* of 3.1 and 6.3 mg/mL, respectively. These extracts also demonstrated antibiofilm properties against both strains, having both extracts a MBIC of 6.3 and 25 mg/mL against *S. aureus* and *E. coli*, respectively. The total phenolic content from cranberry extracts ranged from 1.4 ± 0.2 to 106.3 ± 13.2 mg Gallic Acid eq/g cranberry extract with powdered and fresh cranberry extracts having the higher total phenolic content results. From the DPPH radical scavenging method, it was possible to determine that dried cranberry extracts had the lowest scavenging capacity and powdered cranberry revealed the highest scavenging capacity. Additionally, in fresh cranberry extracts, it was possible to identify by LC-MS/MS the presence of different phenolic compounds (i.e. isorhamnetin 3-glucoside, phloridzin, p-hydroxybenzoic acid, epicatechin and chlorogenic acid) and to assume the presence of A-type procyanidin dimers, widely associated to anti-adhesion properties. Thus, fresh and powdered cranberry extracts were considered to be the more suitable cranberry extracts for incorporation into the developing films.

After films optimization, evaluation of the films physical properties demonstrated similar results between the three different chosen chitosan mixtures, however, films prepared with 0.5% medium molecular weight chitosan and 2% high molecular weight chitosan showed a higher potential to resist to water. After cranberry extracts incorporation, films wettability degree increased, films light-barrier properties improved and films showed antimicrobial properties against both *S. aureus* and *E. coli*. Furthermore, in films with cranberry extracts incorporation, it was also observed a reduction of *E. coli* and *S. aureus* biofilm formation on the tested surface. The antibiofilm properties also showed to improve with the increase of cranberry extract concentration in the chitosan films and a reduction higher than 2 log units could be observed with cranberry film samples when compared to the positive control.

Overall, the investigation revealed the potential of cranberry extract films for the improvement of food packaging material antimicrobial properties.

Keywords: *natural antimicrobials; biofilm prevention; food packaging material; cranberries; chitosan.*

Resumo

A formação de biofilmes por bactérias patogénicas é motivo de grande preocupação para a indústria alimentar visto que várias bactérias têm a capacidade de aderir a superfícies que entram em contacto com alimentos e formar biofilmes. A formação de biofilmes faculta a estas bactérias uma maior resistência a condições ambientais e tratamentos, o que compromete a segurança e qualidade dos alimentos. Atualmente existem algumas estratégias de controlo desde problema, tais como tratamentos químicos para prevenir a formação de biofilmes. No entanto, há um problema crescente de resistência de estirpes que diminuem a eficácia dos métodos convencionais. Assim, nos últimos anos, tem existido um maior atenção para o desenvolvimento de novas estratégias de prevenção e controlo de biofilmes, particularmente, no desenvolvimento de embalagens inteligentes e ativas capazes de incorporar agentes antimicrobianos no material da embalagem tais como nano materiais, biossurfactantes e inibidores de *quorum-sensing*. Nos últimos tempos, os frutos vermelhos têm ganho uma relevância particular devido ao seu alto conteúdo em compostos bioativos. O fruto arando, especificamente, é associado a vários efeitos benéficos para a saúde humana tais como proteção de doenças cardiovasculares, diminuição de infeções do trato urinário e prevenção de danos oxidativos devido ao seu alto conteúdo em compostos fenólicos que revelam ter várias propriedades bioativas, tais como propriedades anti-inflamatórias, antioxidantes e antimicrobianas.

Por parte dos consumidores, existe também uma maior preocupação para o desenvolvimento de embalagens alimentares sustentáveis, que possam substituir os plásticos sintéticos, caracterizados por grandes períodos de degradação. Deste modo, os bioplásticos, em especial os biodegradáveis, surgem como uma alternativa. O quitosano, é um polímero que tem tido grande destaque pelo seu potencial de aplicação em embalagens alimentares devido à sua atividade antimicrobiana e capacidade de formação de filmes. Filmes à base de quitosano têm sido produzidos especialmente incorporados com outros compostos antimicrobianos e têm comprovado melhorarem o tempo de vida e reduzir a contaminação dos alimentos sobre os quais são aplicados. A produção de embalagens biodegradáveis incorporadas com agentes antimicrobianos apresenta-se assim como uma solução para a melhoria da qualidade e segurança dos alimentos.

Assim, o principal objetivo deste trabalho foi desenvolver um filme incorporado com extratos de arando destinado à aplicação em embalagens alimentares e estudar os seus efeitos antimicrobianos contra a formação de biofilme bacteriano. Desta forma, o estudo foi principalmente distribuído em três partes fundamentais: i) caracterização e extração de arandos sob diferentes formas de apresentação; ii) desenvolvimento e otimização de filmes de quitosano; iii) funcionalização de filmes de quitosano com incorporação de extratos de arando. Foram assim utilizadas três fontes diferentes de arando, nomeadamente arando fresco, arando em pó e arando desidratado, e com base na determinação de compostos fenólicos totais e análises de cromatografia líquida de alta eficiência foram escolhidas as melhores condições para a extração de compostos bioativos. De seguida, foram avaliadas as propriedades antimicrobianas (Concentração inibitória mínima (CIM), Concentração bactericida mínima (CBM), Concentração inibitória mínima de formação de biofilme (CIMB)), o conteúdo fenólico total e a capacidade antioxidante (método de sequestro de radicais livres DPPH) dos extratos

de arando. Os filmes à base de quitosano foram desenvolvidos, otimizados e as suas propriedades físicas e mecânicas foram avaliadas através de vários ensaios, tais como solubilidade total, conteúdo em humidade, capacidade de absorção de água, grau de molhabilidade, transmissão de luz, transparência, dureza, permeabilidade ao vapor de água e taxa de transmissão de oxigénio. Após incorporação dos extratos de arando, as propriedades antimicrobianas dos filmes foram também avaliadas através do método de difusão em agar e as propriedades antibiofilme foram avaliadas através do método de contagem de colónias e por observação de imagens ao microscópio eletrónico de varrimento.

Os resultados demonstraram que todos os extratos de arando apresentaram capacidade antimicrobiana contra *Escherichia coli* ATCC 25922 e *Staphylococcus aureus* ATCC 25923 e também propriedades antioxidantes, particularmente os extratos de arando fresco e arando em pó. Ambos os extratos de arando fresco e arando em pó apresentam uma CIM de 3,1 mg/mL para *S. aureus* e 6,3 mg/mL para *E. coli*. Adicionalmente, os extratos de arando fresco e arando em pó demonstraram propriedades antibiofilme contra ambas as bactérias, obtendo-se valores de CIMB contra *S. aureus* e *E. coli*, em ambos os extratos, de respetivamente 6,3 mg/mL e de 25 mg/mL. Foi importante testar a capacidade antimicrobiana dos extratos contra estas duas bactérias, visto serem duas bactérias patogénicas frequentemente associadas a surtos de origem alimentar, com capacidade de aderir a várias superfícies da indústria alimentar e formar biofilmes. O conteúdo total em compostos fenólicos dos extratos de arando variou entre 1,4 ± 0,2 a 106,3 ± 13,2 mg Ácido Gálico/ g extrato de arando, sendo que os extratos de arando em pó e arando fresco obtiveram os valores mais altos de conteúdo em compostos fenólicos. Relativamente à atividade antioxidante, foi possível determinar que os extratos em arando desidratado obtiveram os valores mais baixos de capacidade de sequestro de radicais livres DPPH enquanto o extrato de arando em pó demonstrou uma maior de atividade antioxidante, relativamente aos restantes. Adicionalmente, os compostos fenólicos presentes no extrato de arando fresco foram determinados através de LC-MS/MS e foi possível identificar a presença de nomeadamente isoramnetina-3-glucósido, florizina, ácido hidroxibenzóico, epicatequina e ácido clorogénico. Foi também possível assumir a presença de dímeros de procianidinas A, frequentemente associadas a propriedades antimicrobianas. Deste modo, o extrato arando fresco e arando em pó foram considerados os mais adequados para incorporação nos filmes em desenvolvimento.

Inicialmente, foi observado que os filmes desenvolvidos com uma combinação de quitosano de alto peso molecular e quitosano de médio peso molecular obtinham melhores resultados relativamente aos filmes apenas desenvolvidos com um tipo de massa molecular de quitosano, os quais se apresentavam mais frágeis. Desta forma, foram selecionados para os seguintes ensaios, os filmes 1% + 2%, 0,5% + 2% e 1,5% + 1,5% de quitosano de médio peso molecular e quitosano de alto peso molecular, respetivamente, combinados com o plastificante polietilenoglicol (PEG) ou com PEG e glicerol. As propriedades físicas dos filmes selecionados apresentaram resultados semelhantes, no entanto, foi verificado que os filmes 0,5% + 2% apresentaram um conteúdo mais baixo em humidade, menor solubilidade e menor grau de molhabilidade. Com base nestes resultados, filmes preparados com 0,5% + 2% de quitosano de médio peso molecular e de alto peso molecular respetivamente (com e sem adição de glicerol) foram selecionados para ensaios com incorporação de extrato de arando visto que não se observaram diferenças evidentes nos filmes nos restantes ensaios, respetivamente propriedade

de transmissão de luz, transparência e ensaios antimicrobianos. O grau de molhabilidade dos filmes aumentou após incorporação dos extratos de arando, foi observado um aumento da opacidade e redução de transmissão de luz e os filmes mantiveram as suas propriedades antimicrobianas. Nos filmes incorporados com extratos de arando, foi observado uma redução da formação de biofilme de *E. coli* e *S. aureus* na superfície testada e esta redução aumentou com o aumento da concentração de extrato de arando presente no filme. Através do método de contagem de colónias também foi possível observar que os filmes preparados à base de quitosano levaram a uma diminuição da formação de biofilme na superfície testada, tanto para *S. aureus* como *E. coli*, e ainda que essa redução foi superior nos filmes preparados com extrato de arando. Nos filmes com extrato de arando observou-se uma redução superior a unidades 2 log comparativamente com o controlo. Foi demonstrado também que uma maior concentração de extrato de arando nos filmes leva a uma maior redução da formação de biofilme. As imagens obtidas por microscopia eletrónica permitiram observar que nas superfícies dos filmes sem extrato de arando há uma formação significativa de colónias de *S. aureus* enquanto nos filmes com extrato de arando, tanto com 100 mg/mL de extrato como 200 mg/mL, há uma ausência de formação de colónias.

De um modo geral, os resultados apresentados neste estudo revelam a capacidade de desenvolvimento de filmes de extrato de arando com propriedades antimicrobianas e antibiofilme, demonstrando deste modo, um potencial de aplicação em embalagens alimentares. O desenvolvimento de embalagens ativas torna-se cada vez mais importante na indústria alimentar, para assegurar a segurança do consumidor e a qualidade do produto prevenindo a contaminação dos produtos através da redução e inibição do crescimento de microrganismos. Deste modo, os resultados deste estudo apontam para uma estratégia que pode ser explorada para utilização em embalagens alimentares, demonstrando também a capacidade dos compostos bioativos de arando no controlo da propagação de microrganismos.

Mais estudos devem ser realizados para aperfeiçoamento das propriedades dos filmes, nomeadamente a melhoria das propriedades físicas e potenciação da atividade antimicrobiana através de uma combinação com outros compostos antimicrobianos. Novos estudos devem igualmente ser feitos relativamente à estabilidade em condições de conservação alimentar e observação do efeito e segurança destes filmes no contacto direto com produtos alimentares.

Palavras-Chave: *agentes antimicrobianos naturais; prevenção de biofilme; embalagens alimentares; arando; quitosano.*

Acknowledgements

I would like to express my gratitude to my supervisors Professor Isabel Ribeiro and Professor Ana Bettencourt from Faculdade de Farmácia da Universidade de Lisboa for all the support and guidance during my work. I want to thank all knowledge, dedication and availability given over the last year, which allow me to successfully complete this thesis.

I would like to thank PhD Victor Souza, Professor Isabel Coelhoso and Professor Ana Luísa Fernando for the help in the water vapour permeability and oxygen transmission rate assays required for films properties characterization.

I would also like to thank Professor Lídia Gonçalves, Professor Maria Rosário Bronze and research technician Ana Bárbara Pereira for all the help and assistance in the sequence of my work.

To Professor Judite Costa for allowing the use of the rotary evaporator device.

To Postdoctoral researcher Joana Carrola and Msc Andreia Silva, responsible for the LC-MS equipment, for the time invested in the required analyses of my samples.

To Professor Sofia Arantes e Oliveira from Faculdade de Medicina Dentária da Universidade de Lisboa, for providing the hardness tester equipment.

To my colleague, Rita Mendes, that accompanied me in many steps of this work.

To my parents for always being present, believing in me and supporting me through this journey.

To all my family and friends for the motivation and support given.

Financial support:

Portuguese government, Fundação para a Ciência e Tecnologia (FCT), for financial support, Projects: Pest-UID/DTP/04138/2014.

Index

Index of figures

Index of tables

Abbreviations

BHI	Brain Heart Infusion
CS_H	High molecular weight chitosan
CS_M	Medium molecular weight chitosan
DB	Dried Cranberries
DPPH	2,2-diphenyl-1-picrylhydrazyl
EPS	Extracellular polymeric substance
ESI	Electrospray ionisation
EU	European Union
FB	Fresh Cranberries
GA	Gallic Acid
GLY	Glycerol
HDPE	High-density polyethylene
HPLC	High-performance liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LDPE	Low-density polyethylene
m/z	Mass-to-charge ratio
MBC	Minimum Bactericidal Concentration
MBIC	Minimum Biofilm Inhibitory Concentration
MH	Mueller-Hinton
MIC	Minimum Inhibitory Concentration
MRM	Multiple Reaction Monitoring
PB	Powdered Cranberries
PBS	Phosphate Buffered Saline
PEG	Polyethylene glycol 400
PET	Polyethylene terephthalate
PLA	Polylactic acid
PP	Polypropylene
PVA	Polyvinyl alcohol
PVC	Polyvinyl chloride
QAC	Quaternary Ammonium Compounds
QS	Quorum Sensing
ROS	Reactive Oxygen Species
SD	Standard Deviation
SEM	Scanning Electron Microscope
STEC	Shiga toxin-producing *Escherichia coli*
TPP	Pentasodium Tripolyphosphate
Trolox	6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
TSA	Trypticase Soy Agar medium
TSM	Total Soluble Matter
WVP	Water Vapour Permeability

Chapter 1

Introduction

1.1. Biofilms in the food industry

Biofilms are formed by a community of microorganisms immersed in a self-produced extracellular polymeric substance (EPS) matrix. The intercellular interaction between microbial cells provides distinct properties to the sessile cells (biofilm) when comparing to individual bacterial cells since it enables the attachment of bacteria to a surface and leads to a persistent contamination problem (1). Biofilm structure and development depends mainly on the bacterial surface properties, the attachment surface and the environmental conditions (2).

Conditions that affect biofilm formation

Several bacterial strains can be presented in two forms: i) in a planktonic form, where the cell growth and development is higher; ii) in a sessile form, where the cells become more resistant, enabling the cell resistance to stress conditions. Biofilms are initially formed by cells attachment to a surface, and this initial attachment is dependent on bacterial surface structures (2). Several studies suggested that, depending on the type of bacteria, flagellar motility can be important for cells to reach the surfaces and forwardly spread and develop the biofilm. Surface appendages such as fimbriae and pili seem to play an important role in bacteria initial adhesion to surfaces and biofilm development stimulation (3). Cells attachment to a surface may also be conditioned by phytochemical properties of the attachment surface, since molecules accumulation on the films surface can influence surface factors such as free energy, hydrophobicity and electrostatic charges. Factors such as pH and temperature of the surrounding medium can also play a role in the surface properties (4).

Biofilms are generally formed in four stages: initial attachment, microcolony formation, biofilm maturation and dispersion (Figure 1). After initial attachment, cells start to replicate and form microcolonies. EPS, produced mostly by the cells, plays a crucial role in immobilizing biofilm and allowing cell to cell communication. This matrix constitutes the cells immediate environment and is responsible for conditioning the development of the bacterial community structure and architecture. EPS is mainly a mixture of polysaccharides, proteins, nucleic acids and lipids but its composition is dependent on the surrounding conditions. Finally, after biofilm maturation, due to several factors unfavourable for biofilm formation, such as nutrient availability, the dispersion stage occurs, where planktonic bacteria are release from the biofilm structure. (2,5,6)

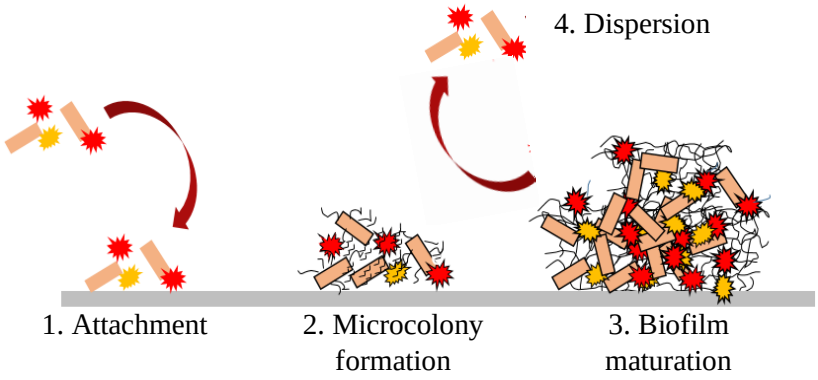

Figure 1. Illustration of four stages of biofilm formation.

Problems related to biofilm formation

Biofilm formation presents a particular concern to the food industry since the pathogenic and spoilage biofilm-forming species are able to develop in several food factory surfaces (e.g. packaging materials, tables, liquid pipelines) and have a higher resistance to environmental conditions and treatments within a biofilm form which compromises food safety and quality (7). One of the main concerns for the food industry is the formation of biofilm by human pathogenic species such as *Listeria monocytogenes*, *Salmonella enterica*, Enterohemorrhagic *Escherichia coli*, *Staphylococcus aureus* and *Vibrio* species that are often related to outbreaks of foodborne diseases (8). Only in 2017, an evaluation in 37 European countries, reported a total of 5.079 foodborne outbreaks, including waterborne outbreaks, being *Campylobacter* and *Salmonella* the most frequently detected causative agents (9).

That year, in the European Union (EU), *Salmonella* was reported to lead 91.662 human salmonellosis cases with meat and meat products being the major food categories of contamination (9). Studies show the ability of *Salmonella* cells to form biofilms in food surfaces depending on the type of surface and temperature. Plastic surfaces showed to be more susceptible to biofilm formation than stainless steel surfaces. Furthermore, studies also demonstrated a higher resistance to sanitizers of biofilm cells comparatively with planktonic cells. *Salmonella* biofilms present difficult to be removed after stabilization in food surfaces which can lead to cross-contamination (10,11). According to Borges and co-workers (12), both *Salmonella* Enteritidis and *Salmonella* Typhimurium, were considered to be among the most prevalent serotypes associated to salmonellosis outbreaks, and being able to form biofilms on a polystyrene surface at 37 ºC and 28 ºC.

Regarding *L. monocytogenes* cases reported in 2017, 2.480 human listeriosis occurrences were observed and the predominant contaminated food products were fish, meat, cheeses, fruits, vegetables and salads (9). *L. monocytogenes* cells are shown to adhere to different surfaces, such as stainless steel, marble, granite, glass and especially polypropylene (PP) (13). *L. monocytogenes* is a highly concerning bacteria since its infection has high fatality rate in spite of being a more rare disease when comparing with other foodborne diseases. This pathogen affects mostly immunocompromised individuals, pregnant women, infants and elderly (14). Currently, the control of *L. monocytogenes* contamination in food processing environments is challenging since this bacteria is able to multiply at low temperatures and its

biofilm forming capacity allows the persistence in this type of environments (15). Sanitiser's lack of efficiency against this bacteria is a concern for the food industry. *L. monocytogenes* biofilms seem to resist several sanitizers such as peroxide sanitizer, quaternary ammonium compounds (QAC) and chlorine. Studies suggest that this resistance is associated to the EPS in the matrix rather than the cell present in the biofilm (16). Teichoic acids are one of the most important components in the bacteria biofilm matrix, and interestingly, this soluble carbohydrates were found to be similar to the ones present in the bacteria cell wall (17). The key to *L. monocytogenes* biofilm prevention maybe linked to antimicrobial compounds that can interfere with teichoic acid biosynthesis inhibiting (18).

In 2017, 6.073 human cases of Shiga toxin-producing *E. coli* (STEC) infections were reported in the EU. STEC is known to cause haemorrhagic colitis and haemolytic uremic syndrome in humans and the major contamination sources are predominantly linked to meat, specially bovine, and associated products. STEC O157:H7, specifically, has been reported to cause the most severe outbreaks worldwide (9,19). *E. coli* O157:H7 has shown to be able to colonize several food-contact and equipment surfaces in food processing. In a study, the ability of *E. coli* O157:H7 to adhere and colonize beef-contact surfaces, respectively stainless steel and high-density polyethylene, was demonstrated. This attachment was influenced by soiling substrate and temperature. Findings showed that at both 15 ºC and 4 ºC, *E. coli* O157:H7 population was able to increase with the storage time (20). Even though *E. coli* O157:H7 is the predominantly serotype to cause the most severe outbreaks, other serotypes are increasingly being associated to outbreaks (21). Additionally, several studies state that some of these serogroups are also capable of adhere and form biofilms in food contact surfaces. Studies show that biofilm formation is dependent on the strain and environmental conditions (22). Environmental factors such as temperature play an important role in cells attachment to a surface. For instance, a study done with ten different *E. coli* serogroups, demonstrated that only five of this, respectively O113, O145, O91, O157, and O121 were capable of forming biofilms on stainless steel surfaces at 22 ºC (23).

S. aureus is a Gram-positive opportunistic pathogen able to cause a wide variety of infection diseases (24). This bacteria is able to survive in dry and stressful environments such as human skin, clothes and different surfaces (25). This bacteria is also able to produce enterotoxins in a wide range of food products. Bacterial toxins were considered in 2017 the third most important causative agent of foodborne outbreaks in Europe. Bacterial toxins outbreaks were reported in 20 European Member States mostly associated with toxins by *Staphylococcus* specie (9). Biofilm-associated infections are widely associated to *S. aureus* and this bacteria is able to form biofilms on both abiotic and biotic surfaces which is a critical problem to the food industry (26). Furthermore, potential treatment strategies such as disinfectants are reported to promote biofilm formation and increase virulence (27). The *S. aureus* capacity to form biofilms is dependent on the bacteria strains and environmental conditions such as temperature, nutrient availability and surface type (28). In Ciccio and co-workers study, it was tested the *S. aureus* ability to form biofilms on polystyrene and stainless steel surfaces at different temperatures (12 and 37 ºC). The study was performed with 67 *S. aureus* isolates and 3 reference strains (S. aureus ATCC 35556, S. aureus ATCC 12600 and S. epidermidis ATCC 12228). Results showed that, at 37 ºC, *S. aureus* was able to form biofilm

on polystyrene (65.7%) and stainless steel surfaces (63.1%). At 12 ºC, one *S. aureus* isolated strain was also able to form biofilms (29). In another study, it was tested the capacity of 26 *S. aureus* isolates from seafood to form biofilms on polystyrene surfaces at different environmental conditions. The results also revealed a high ability of *S. aureus* to adhere to polystyrene surfaces particularly at 37 ºC and also that the type of strain affect biofilm formation capacity (30).

Since several biofilm forming bacterial species are able to adhere to food contact surfaces, multispecies biofilms are usually formed. These interactions influence biofilms architecture and provide emergent properties to biofilms that contribute for biofilms enhanced resistance to environmental stress (31). Interactions between different bacterial species can lead to a negative impact in some strains due to competitive interactions for a particular source or by production of compounds that cause inhibition of one of the species (32). However, several studies also show the beneficial impact of mixed-biofilms formation for cells. Reports revealed that multispecies biofilms formation can lead to a higher resistance to antimicrobial agents. A study that investigated the influence of *E. coli* O157:H7 and *S.* Typhimurium strains mixed biofilm formation showed that, in comparison with single-strain biofilms, mixed biofilms had developed enhanced sanitizer resistance. Results showed that EPS components formed by EPS-producing strains were also able to protect non-producing EPS strains from other species (33). In another study, a mixed biofilm formed by *L. monocytogenes* and *Lactobacillus plantarum* showed more resistance to the disinfectants benzalkonium chloride and peracetic acid than each single species under biofilm and planktonic cells (34). Interactions between different species can have an influence in spatial distribution, biomass shaping and mainly it can modulate gene expression (31).

In addition to causing a risk to consumer's health, it can also cause a financial problem to the food industry as well as environmental problem by reducing the shelf-life of food products in contact with these biofilms and, therefore, increasing the food waste levels. *Pseudomonas fluorescens*, for instance, a bacteria frequently isolated from dairy products, is widely known to be responsible for food spoilage. A study conducted in 2017 showed that, from 72 strains of *Pseudomonas* species, isolated from milk and dairy products, 80% had the ability to form biofilm *in vitro* on polystyrene microplates. This percentage was influenced by variations in time and temperature of incubation (35). In another study, five *Enterobacteriaceae* strains, highly associated with meat spoilage, were study to evaluate their attachment and biofilm formation on stainless steel surfaces at a short (5 h) and a long term assay (7 days). *Proteus mirabilis* and *Citrobacter freundii* showed the higher adhesion rates. During the short term assay, more than 4.5 log CFU/cm^2 cells were transferred to the stainless steel surface and at the end of the 7 days, more than 8 log CFU/cm^2 of biofilm formation was found on stainless steel surface (36).

With such a large public concern, the efforts in the last few years have been directed to the investigation of solutions for biofilm prevention and methods that can control this problem.

Prevention and control methods

Cleaning and disinfection daily procedures are one of the principal steps to prevent the propagation of microorganisms in the food industry. Chlorine dioxide, for instance, is a commonly used sanitizing agent, used both in aqueous and gaseous form. It has been associated with disinfection of ready-to-eat fresh products presenting more stability than Chlorine (37,38). However, it is demonstrated that after cleaning and disinfection procedures residual microorganism can still be present in food facilities (39). Furthermore, there is an arising problem of resistant strains that decrease the effectiveness of these chemical treatments, as briefly described above. For instance, QAC are antibacterial agents widely used as disinfectants in the food industry but many studies have already demonstrated the resistance of some *L. monocytogenes* strains to this disinfectant. A study conducted in several *L. monocytogenes* isolates collected from different meat and salmon processing plants overall showed that strains containing *qacH* and *bcrABC* genes revealed increased tolerance to benzalkonium chloride, showing that *L. monocytogenes* with this QAC type of resistance genes are prevalent in the food industry (40). Thus, it is clear that conventional control methods, like chemical treatments, are not enough to control the biofilm formation problem. As a result, in the last few years several novel strategies have been developed to mitigate the formation of biofilms, especially related to the investigation, production and application of antimicrobial compounds, like nanomaterials, bio-surfactants, quorum-sensing inhibitors and others, in active packaging materials (Figure 2) (40–43).

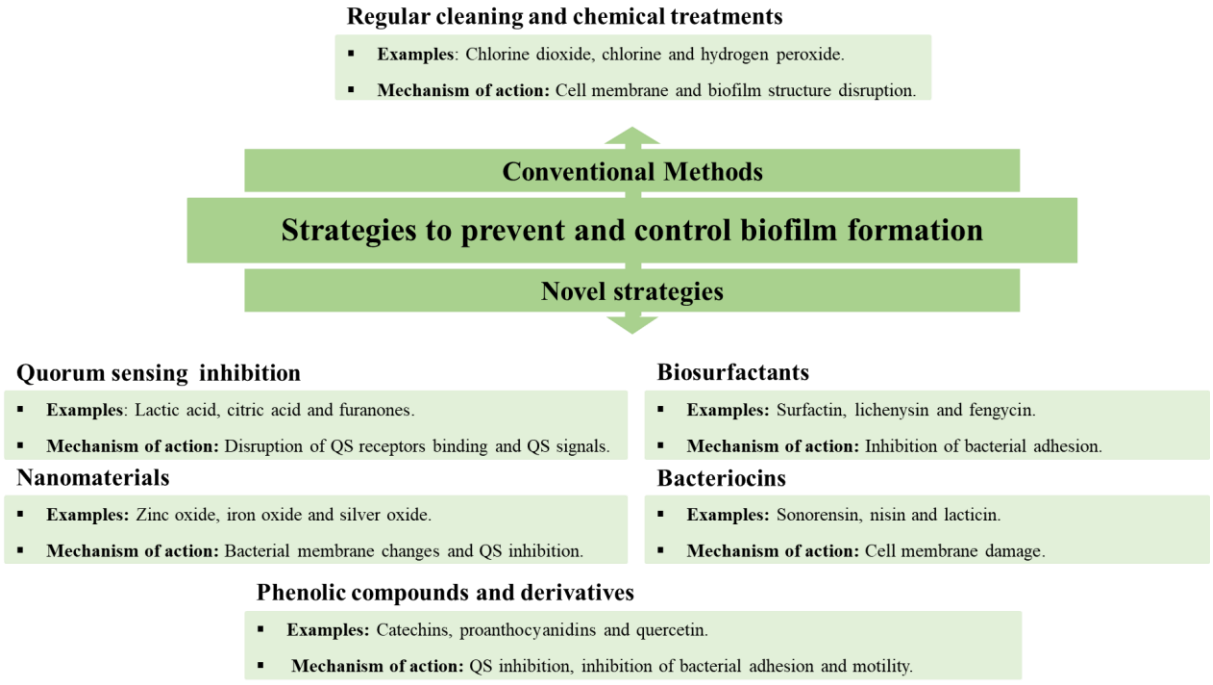

Figure 2. Representation of conventional and novel strategies used to prevent and control biofilm formation. Examples and overall mechanism of action of each method.

Quorum sensing inhibitors

One approach employed by the food industry is the inhibition of quorum sensing (QS) activity. QS is an intercellular signalling mechanism used by some cells to regulate biofilm formation. This mechanism allows for the mediation of bacterial gene expression, by chemical auto-inducer molecules through a cell density dependent signalling, allowing bacteria to have a unified response adapted to the surrounding environment (44).

Organic acids have proven to detain QS inhibition capacity and are currently being studied as a safer alternative to other chemical treatments more commonly used. Several studies have already demonstrated the antimicrobial effects of organic acids, and an example can be the effect of acetic acid, citric acid and lactic acid on the inactivation of *E. coli* and *Salmonella* species on the surface of a cucumber (43). Another study also showed the effect of lactic and acetic acids as inhibitors of auto-inducer 2, a QS molecule. Some QS bacteria's are known to respond to this molecule and its inhibition can affect the QS mechanism. In this study, *E. coli* O157:H7 and *S.* Typhimurium strains present QS ability and that was inhibited by lactic and malic acids (45).

Natural compounds, like essential oils, have also revealed to interfere with QS systems and are currently being used in the food industry as antimicrobial agents in packaging materials. Ten essential oil components (carvone, hexanal, carvacrol, citral, geraniol, salicylic acid, cinnamic acid, thymol, eugenol and cinnamaldehyde) revealed QS inhibition for the vegetable spoilage bacteria *Erwinia carotovora* and *Pseudomonas fluorescens*. The results showed that all components inhibited biofilm formation by inhibiting bacteria mobility and exopolysaccharide production (46). Many other studies also showed the QS inhibitory effects of terpene compounds from plant extracts in bacteria, for example, exposure to carvacrol on stainless steel surfaces was reported to inhibit biofilm formation of *Pseudomonas aeruginosa* with evidence related to QS interruption (47). *Thymus vulgaris* essential oil and its major components have also showed QS inhibition in relation to *P. fluorescens* biofilm formation on stainless steel surfaces trough inhibiting the production of QS auto-inductors (48).

Nanomaterials

Nanomaterials are a promising approach to prevention of biofilms formation in the food industry. A lot of research is currently being conducted in this subject to investigate strategies towards incorporating nanoparticles in food packaging material. A number of studies are currently focused in metal nanoparticles due to their potent antimicrobial effect. Zinc oxide nanoparticles from *Nigella sativa* plant extract were studied to have QS biofilm-related inhibitory effects in *L. monocytogenes, P. aeruginosa, and E. coli* and *Chromobacterium violaceum* food pathogens. The results showed a decreased QS mechanism of *C. violaceum* and *P. aeruginosa* and a reduction of biofilm formation in the four food pathogens as well as in the swarming motility and EPS production (41). There are many other widely studied metal nanoparticles that have also shown evident antimicrobial properties, like silver, titanium oxide and iron oxide. Different studies on silver nanoparticles, for instance, are focused on the nanoparticles ability to prevent microbial infections (49,50). Titanium oxide presents photocatalytic properties that enables the creation of self-cleaning surfaces and there are being

developed several studies in this subject. Upon photoactivation, the antimicrobial effects of titanium oxide nanoparticles are associated with the destruction of bacterial cells membrane due to the generation of reactive oxygen species (ROS), alterations of Coenzyme A-dependent enzyme activities and damage to DNA through hydroxyl radical (51). According to Nica and co-workers, two types of titanium oxide nanoparticles co-doped with iron and nitrogen, were synthesized in hydrothermal conditions and the assessment of microbial and cytotoxic activity showed significant antimicrobial and antibiofilm activity. However, titanium oxide nanoparticles antimicrobial mechanism is not yet fully understood (52).

Other findings on nanoparticles also preview nanoparticles ability to inhibit bacterial attachment and subsequent prevention of biofilm formation. Organosilane based products, for instance, were demonstrated to reduce bacterial adhesion of *Salmonella* and *E. coli* in nanocoating glass surfaces. The results also showed a reduction of *Salmonella* and *Yersinia* biofilm cells on stainless steel coupons for coated surfaces (53).

These antimicrobial nanocompounds demonstrate high antimicrobial efficacy at very low levels and are presumably a very promising approach to food contact packaging material development, however, there's a need for more research in this subject and a more regulatory framework in nanotechnology application since the toxicity effects of these nanoparticles are not still well documented (54,55).

Biosurfactants

Biosurfactants are natural compounds that have the advantage of being eco-friendly and have low toxicity (56). Their effect is mediated by the modification of bacterial surface hydrophobicity, which reduces bacterial adherence to any surface. One study exhibited the potential of lichenysin, a surfactant produced by *Bacillus licheniformis*, to prevent the adhesion of food pathogens and reduce biofilm formation (57). Others, like surfactin, iturin and fengycin are widely studied biosurfactants. These biosurfactants, extracted from *Bacillus* species, demonstrate to have good antifungal properties against many phytopathogens like *Fusarium moniliforme*, *Fusarium oxysporum*, *Fusarium solani* and *Trichoderma atroviride* (58). Trehalose lipid biosurfactants, produced by *Rhodococcus ruber*, exhibited effects on bacterial adhesion and biofilm formation on polystyrene surfaces, revealing that the surfaces treated with the biosurfactant were more hydrophilic (59). A novel lipopeptide biosurfactant, extract from a new marine bacterium identified as *Pontibacter korlensis* strain SBK-47, also revealed promising antibiofilm properties (42).

All these results seems to show that biosurfactants can be an efficiently strategy to prevent the initial attachment of foodborne pathogens to food contact surfaces disrupting biofilm formation, however, more studies should be made to better understand biosurfactants disrupting mechanisms.

Bacteriocins

Another solution to antibiotic resistant bacteria that is presently being studied as antibiofilm agents are bioactive peptides produce by bacteria, called bacteriocins. Bacteriocins are ribosomal synthesized antimicrobial peptides that have been used in the last years as food preservatives (60). There are a lot of studies reporting bacteriocin antimicrobial effects. Sonorensin, for instance, is a bacteriocin that showed growth inhibition of spoilage bacteria in chicken meat and tomato samples by damaging bacterial membrane (61). Combination of nisin, lacticin and plantaricin also reported antimicrobial control of food pathogens (62). Likewise, pyocins showed to be an effective biofilm-forming bacteria inhibitor agent (63). Other bacteriocins, such as lactocin from lactic acid showed to have beneficial effects when applied in meat and meat products. The novel bacteriocin BMP11, produced by *Lactobacillus crustorum*, showed to promote cell lysis of food pathogens in dairy products and also antibiofilm formation activity (64,65).

Despite nisin and pediocin remaining the only bacteriocins approved for food commercial application, there are currently a larger number of researches exhibiting the effect of novel bacteriocins against foodborne pathogens, implying its promising future application as antimicrobial agents in food (66).

Phenolic compounds and derivatives

Phenolic compounds are associated with health benefits mainly due to their antioxidant activity. These compounds demonstrate to have antimicrobial and antibiofilm properties against food spoilage and pathogenic microorganisms. The advantage of using these compounds as antibiofilm agents, in food packaging materials, is the positive outlook that consumers have on them since they are extracted from natural sources. In nature, polyphenols represent a large class scale of metabolites such as flavonols, proanthocyanidins, coumarins, stilbenes, lignans and lignins (67). Flavan-3-ols, flavonols and tannins are particularly interesting to the food industry since they were verified to have higher antimicrobial and antibiofilm properties in contrast with other polyphenols (68).

Catechins, the major polyphenols in green tea, have been associated over the years with inhibition of several bacteria growth. Studies show that these molecules are able to supress virulence factors as well as reverse some pathogen bacteria resistance (69). Studies observed that catechins, especially epigallocatechin-3-gallate, from green tea, inhibited bacteria's adherence to surfaces and were responsible for influence biofilm formation by interfering with bacteria QS mechanism and influence expression of virulence factors from bacteria such as enterohemorrhagic *E. coli* and Staphylococcal enterotoxin B (70). Other phenolic compounds have also been associated to inhibition of growth and biofilm formation of several bacteria species. Quercetin and tannic acid, for instance, were the main responsible for *S. aureus* biofilm formation inhibition by influencing gene expression (71). Other phenolic compounds such as gallic acid, also showed to be effective against *E. coli* and *Streptococcus mutans* under different environmental conditions. Polyphenolic extracts of cherry and blackurrant leaves, rich in gallic acid, coumaric acid, catechins and quercetin, have also been shown to have antimicrobial effects in meat products, enhancing the shelf-life of vacuum-packed sausages (72). Additionally,

synergy between multiple phenolic compounds seems to enhance antibacterial and antibiofilm properties. For instance, polyphenols extracted from muscadine grape (*Vitis rotundifolia* Michx.) skin and seed showed higher antimicrobial activity against *S. aureus* planktonic cells as well as a higher inhibition of biofilm formation in comparison with commercial phenolic standards such as gallic acid, caffeic acid, catechin and quercetin represented in Figure 3 (73).

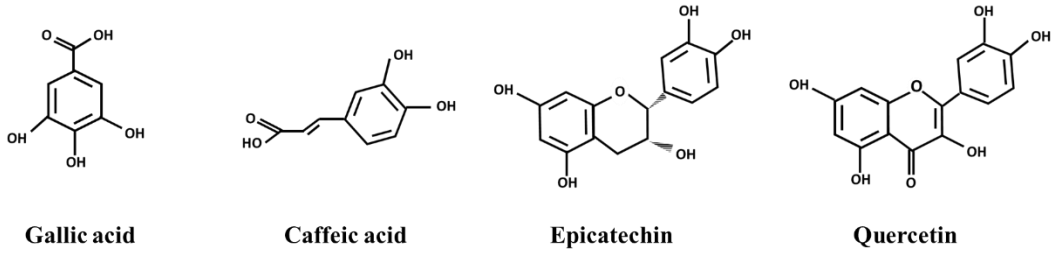

Figure 3. Example of phenolic compounds with antimicrobial activity: gallic acid, caffeic acid, catechin and quercetin.

Among phenolic compounds present in large concentrations in wild fruits, are anthocyanins and proanthocyanidins, especially in cranberries that have gain a big interest from the food industry. These bioactive compounds have been studied for their wide range of health benefits, particularly for their antioxidant and antimicrobial properties in combination with other phenolic compounds (74,75). Antimicrobial properties of four American cranberry varieties extracts rich in anthocyanins, for instance, were measured against several Gram-negative and Gram-positive bacteria. All the extracts showed antimicrobial activity, particularly towards *Bacillus cereus* and *Micrococcus luteus* that were observed to be more sensitive to the extracts action (76). In another study, antiadhesion and antibiofilm formation properties of anthocyanin-rich ethanolic blueberry extract were evaluated against several pathogens (*i.e. P. aeruginosa, E. coli, Proteus mirabilis, Acinetobacter baumannii* and *S. aureus*). In general, the results showed that the anthocyanin-rich blueberry extract was capable of inhibiting biofilm formation and bacterial adhesion for all microorganism evaluated (77). Proanthocyanidins have been studied to inhibit the adherence and growth of a wide variety of bacteria and particularly pathogenic bacteria. Cranberry proanthocyanidins are widely associated with antiadhesion activity of p-fimbriated uropathogenic *E. coli* to cells, a pathogen related to urinary tract infections (78). This property was also observed in biomaterials where proanthocyanidins extracted from North American cranberries demonstrated to be able to reduce initial adherence of *E. coli* and *Enterococcus faecalis* to biomaterials polyvinyl chloride (PVC) and polytetrafluoroethylene (79). Other studies also show proanthocyanidins influence in biofilm reduction (80). For instance, Ulrey and co-workers (81), demonstrated that the exposure of *P. aeruginosa* biofilm to increasing proanthocyanidins concentrations lead to a higher decrease of the produced biofilm.

The use of phenolic compounds in food packaging has been widely studied by the food industry. The use of these compounds, for instance incorporated in food packaging has shown not only to promote packaging antimicrobial properties and preventing antibiofilm formation as well as preventing lipid oxidation, enhancing food products quality, shelf-life and safety (82,83).

1.2. Cranberries

Potential health effects

In the last couple of years berry fruits such as blueberries, strawberries, cranberries and raspberries, have gain more attention due to their bioactivity and relation to human health benefits. Cranberries, specifically, have had a great public health interest over the past years as a result of their association with urinary tract infections prevention. Several studies have demonstrated the relation between cranberry juice consumption and decrease in urinary tract infections. (84) Furthermore, in the last years, cranberries have also been widely associated with other health benefits such as oxidative damage prevention, cardiovascular diseases protection and a potential role in anti-cancer mechanisms (85,86). With the research development, studies started to show an association between cranberry health properties and its high content in polyphenols, specifically, the association between cranberry polyphenols and *in vitro* properties such as antiviral, antibacterial, anti-inflammatory, anti-mutagenic and antioxidant properties (87).

Bioactive compounds constitution

Cranberries are constituted by a wide source of phytochemicals, over 150 phytochemicals compounds have already been identified. Some of the principal bioactive compounds with relevance for cranberries properties are proanthocyanidins, anthocyanidins, flavonols, terpene and pectin, and some will be referred below (88).

Anthocyanins are present in high quantities in cranberries being responsible for the cranberries colour. Anthocyanins are constituted by one anthocyanindin molecule (cyanidin, delphinidin, malvidin, perlagonidin, petunidin, or peonidin) bound to one or more sugar structures (Figure 4). It is known that cranberries content on these compounds increases according to the fruit maturation (87,89). Anthocyanins are associated with a high antioxidant and anti-inflammatory potential which can be associated to some cranberry consumption human health benefits. According to Huang and co-workers (90), anthocyanins show the protection capacity towards human retinal capillary endothelial cells when submitted to glucose-induced injury. Several studies also show anthocyanins antibacterial properties, as described briefly above. A study demonstrated that anthocyanins extracts from European cranberries were responsible for the inhibition of growth of different pathogenic bacteria, correspondingly *E. coli*, *Salmonella*, *L. monocytogenes*, *S. aureus*, *Bacillus subtilis* and *E. faecalis* (74). Properties such as anti-viral, anti-proliferative, anti-mutagenic, anti-carcinogenic, protection from cardiovascular damage and allergy prevention have also been demonstrated by these compounds (91).

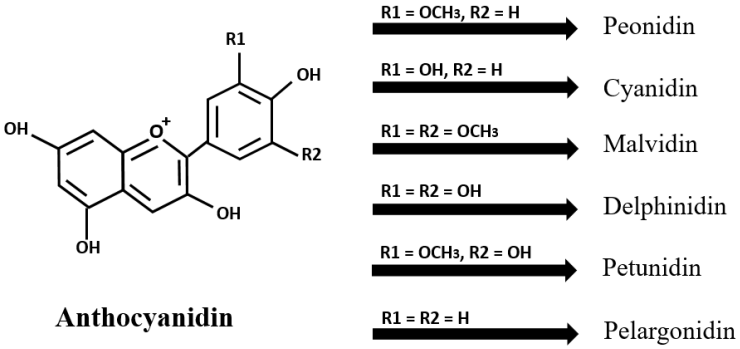

Figure 4. Anthocyanidins basic chemical structure. R differs with the type of anthocyanidin.

Cranberries also have a high content in proanthocyanidins, compounds that are also referred as condensed tannins. Proanthocyanidins are oligomers or polymers of flavan-3-ols, like catechins and epicatechins. The most commonly two types of proanthocyanidins dimers are ‘’A-type’’ and ‘’B-type’’ (Figure 5) and they occur in plants. In B-type proanthocyanidins, an interflavan bond is formed between the started and the extension unit (C4–C6 or C4–C8) and in the A-type proanthocyanidins linkage occurs in two positions, an interflavan bond between the started and the extension unit (C4-C8), identical to the B-type proanthocyanidins, and an additional ether linkage between the C7 of the started unit and the C7 of the extended unit. Cranberry proanthocyanidins groups, however, vary according to their units, linkage type and degree of polymerization (92–94). As previously described above, proanthocyanidins show to have significant antibacterial and antibiofilm properties, particularly A-type proanthocyanidins are showed to have a significant activity against pathogenic bacteria (78,79). Proanthocyanidins were demonstrated to act as iron chelator in *E. coli* limiting bacteria normal function which could be related to proanthocyanidins anti-adhesive properties (95). Other mechanism are also proposed to be associated with proanthocyanidins antibacterial properties such as disruption of bacteria cell membrane and influencing bacterial metabolism (68). Furthermore, proanthocyanidins have shown to present beneficial health effects to humans such as protection against cardiovascular risk factors as well as association with cancer prevention, demonstrating to have therapeutic potential (96,97).

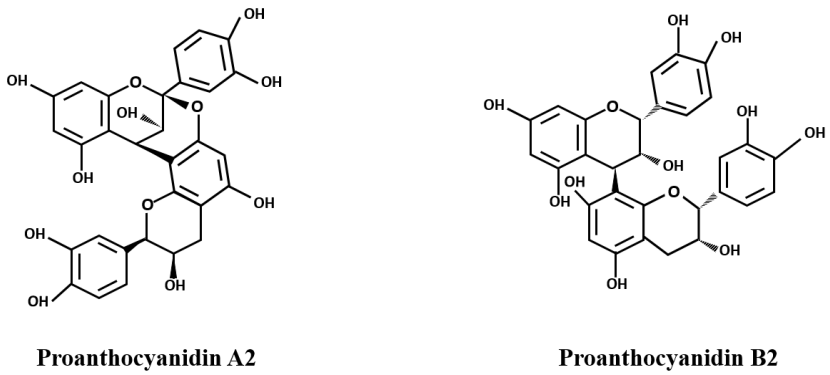

Figure 5. Chemical structures of proanthocyanidins A2 and B2.

Terpenes are important for cranberries flavour and aroma. One of the most commonly studied compound is the ursolic acid due to its association with antiproliferative properties (88,98). Studies demonstrated the inhibition of *E*. coli induced inflammation mechanisms by cranberries extracts where ursolic acid and there derivatives were identified as active constituents (99). Ursolic acid and its esters are proposed to be responsible for cranberry anticancer properties since studies show the capacity of these compounds to inhibit tumour cells proliferation, migration and metastasis (100).

1.3. Antimicrobial active packaging materials

A strategy developed in the last years to control food products contamination and extend food products shelf-life is the development of antimicrobial active packaging. The incorporation of active compounds in synthetic or bio-based packaging materials enables the reduction of growth of spoilage and pathogenic microorganisms (101,102).

Synthetic polymers

The compounds generally used in the production of synthetic food packaging include polyethylene terephthalate (PET), PVC, low-density polyethylene (LDPE), high-density polyethylene (HDPE), PP and polystyrene. These compounds are largely used as packaging material due to its high availability, cheapness and easy manufacturing process. PVC, for instance, is the most commonly used food packaging material since it presents some advantages such as good flexibility, toughness and chemical resistance. PET as good mechanical and transparency properties and adequate gas barrier properties. Comparing with other materials, HDPE presents the advantage of having superior water vapour and gas barrier properties (103,104). These synthetic polymers present the advantage of being thermally processed which enables them to be converted in packaging with different forms. Furthermore, these processing techniques can influence materials morphological properties which can be an advantage to control the incorporation and release of active compounds. However, this type of thermal processing can lead to a decrease of antimicrobial activity of compounds that are directly incorporated into these materials (102).

Nevertheless, the overproduction of non-biodegradable food packaging materials, respectively petroleum-based plastics, has become a big environmental concern since these materials have long degradation periods. Consequently, there is a present interest in the production of more eco-friendly and non-toxic alternatives to reduce the use of such materials.

Bio-based polymers

Biopolymers based packaging has been an alternative to synthetic polymers since they can be obtained from renewable sources and most are biodegradable (Figure 6). Presently, several biopolymers and their food packaging potential have already been studied such as starch, alginate, pectin, carrageenan, agar and chitosan (102). In general, bio-based polymers are hydrophilic demonstrating poor water vapour and moisture barrier properties. However these polymers show good gas barrier properties (105).

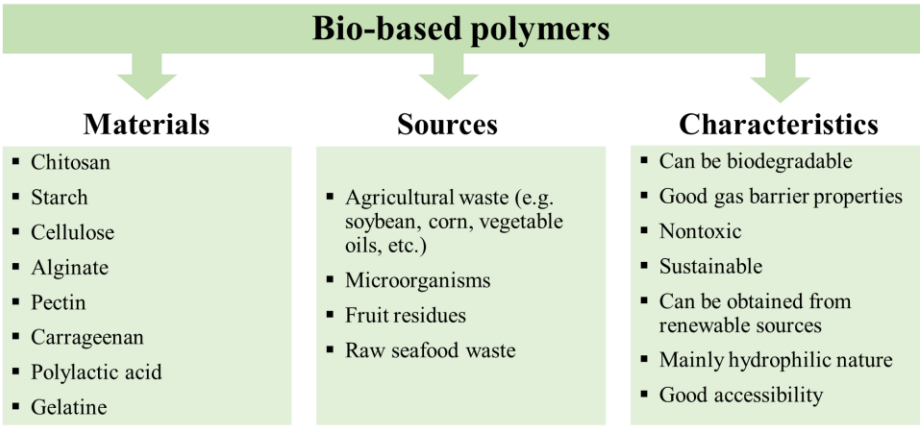

Figure 6. Examples of bio-based polymers, sources and overall characteristics.

Several researchers have been evaluating starch films properties obtained from different sources. Food packaging industry has given great attention to this component due to its biodegradability, easy accessibility and cheapness. However, starch presents some limited mechanical and barrier properties, therefore, its raw use in film manufacturing is limited and its application is normally associated with other hydrophobic components. For instance, the incorporation of clay minerals into starch films by thermally processing techniques showed to improve films mechanical properties (106–108).

Polylactic acid (PLA) is a bio-based degradable polymer, derived from renewable sources, that has also been emerging as a possible alternative to synthetic polymers (102). PLA is known to have good mechanical and barrier properties comparable to synthetic polymers such as LDPE or HDPE, however, presents the disadvantage of having high production costs (109).

Gelatin is a natural water soluble protein that has also been widely used in the food packaging industry, in the production of edible films. Gelatin-based films have an excellent film forming capacity, good gas barrier properties and is a cheap and easy obtainable protein which makes it suitable for films manufacturing. Due to its hydrophilic properties, gelatin has been widely study conjugated with other compounds such as crosslinkers, plasticizers or additives in order to improve the film functional properties (110).

Also, the formation of cohesive films from a blend between pectin, obtained from citrus fruit residues, and carrageenan, obtained from natural resources, has been a particular interest to the food packaging industry. This blend demonstrates increased mechanical properties, water permeability and hydrophilic properties that increase with the increase of carrageenan content. Moreover, the addition of mica flakes into the polymer matrix has shown to decrease films water permeability (111).

Another used polymer is chitosan that presents food safety application, and has easy film forming capacity. Different bio-based chitosan films have been produced and applied in the food packaging field, especially in combination with other microbial compounds like nanocomposites and natural antimicrobial agents (112,113). Chitosan is a natural biodegradable polysaccharide, derived from partial deacetylation of chitin (Figure 7), which is reported to

bring a lot of advantages to the food industry, mainly regarding its antimicrobial activity and ability to form protective films. For instance, chitosan edible coatings have demonstrated to have the ability to enhance shelf-life of fishery products and showing the capacity to reduce microbial counting (*Pseudomonas* species, H_2S forming bacteria and *Enterobacteriaceae*) (114). In another study, application of chitosan based-coating on tangerines demonstrated to extend shelf-life, delaying weight loss and yellowness of the fruit during storage, at the same time as it kept soluble solids, vitamin C and titratable acidity (115). The mode of action of chitosan antimicrobial activity may be due to its positively charged NH_3^+ group of glucosamine which enables interaction with negatively charged microbial cells surface. Other characteristics that might be linked to chitosan effects are its high solubility, its ability to chelate metals allowing the removal of toxins and capacity to interact with DNA. However chitosan shows to only have antimicrobial activity in acidic medium (116–119). Chitosan is frequently combined with other biodegradable polysaccharides, like starch and Polyvinyl alcohol (PVA) to develop more functional films and even with non-degradable biopolymers like polyethylene (120). For example, application of an edible coating developed using a combination of 0.5% of cassava starch and 0.25% of chitosan on post-harvest mangoes demonstrated to prolong the fruit shelf-life for more 3 days than the control fruits and also showed to decrease CO_2 production rates (121). In another study, preparation of a mixture combining chitosan and PVA films showed significant antimicrobial activity against *P. aeruginosa* PAO1 inhibiting the adhesion and biofilm formation of this bacteria. Incorporation of PVA into chitosan films seems to improve chitosan mechanical properties (122). Furthermore, many researchers have already studied the application of chitosan-based films with other antimicrobial compounds to enhance chitosan antimicrobial properties as well as mechanical properties in food packaging material. These studies show promising results of the chitosan biofilm application in a variety of food products. For instance, application of chitosan-starch films against various bacteria and fungi demonstrated to have greater antimicrobial results when the films were combined with natural extracts (112).

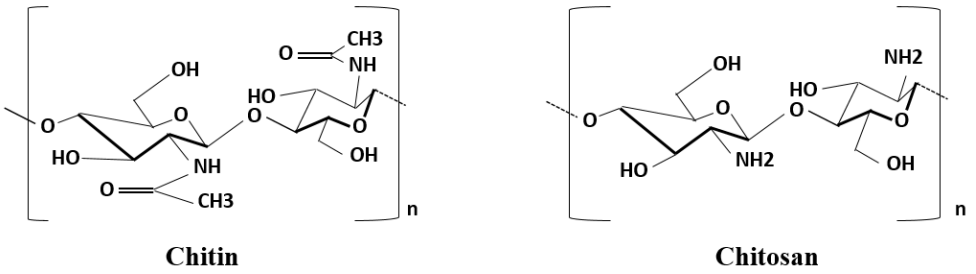

Figure 7. Chemical structure of chitin and chitosan.

There has been an emergent application of chitosan-based films as antibacterial films. Since these compounds already present antimicrobial properties, the incorporation of natural compounds in these type of films have presented a serious interest to the food industry for the production of biodegradable films capable of enhancing food shelf-life and quality (123). A number of natural antimicrobial compounds incorporation in food packaging has already been investigated, such as essential oils, bacteriocins, organic acids and plant extracts due to their

wide antimicrobial capacity, as seen above (103). A study where chitosan films were prepared with maqui berry extracts revealed that films with extracts incorporation had significant enhanced antibacterial properties against several Gram-negative and Gram-positive bacteria in comparison with films without the extract (124). Chitosan films incorporated with thinned young apple polyphenols also showed potential as bioactive food packaging material since films presented significant antimicrobial properties against *E. coli, L. monocytogenes, S. aureus* and three variety of moulds and also good mechanical and physical properties (83). Additionally, natural extract incorporation into food packaging also presents the advantage of increasing films antioxidant properties. Chitosan films, for instance, developed with tea extracts demonstrated to improve films antioxidant property (125). Incorporation of 10% micro-particles of olive residue flour into chitosan matrix showed to improve antioxidant properties of the film. In this study the films that were incorporated with olive residues show a protective oxidation effect on nuts storage for a period of 31 days (126).

Active food packaging with natural antimicrobials represents a great potential for food industry. Since consumers are also demanding for more eco-friendly alternatives to synthetic packaging the production of bio-degradable packaging with natural antimicrobials incorporation presents a great solution that allows for an enhancement in food quality, safety and shelf-life duration. However, further studies of these novel food packaging strategies should be done to assess packaging efficiency, stability and toxicity in order to be used implement in the food industry.

Chapter 2

Thesis Aim and outline

Biofilm formation presents a current concern to the food industry, therefore, there is a necessity for the development of innovative strategies able to control this problem. One solution is directed to the improvement of packaging systems by incorporation of antimicrobial agents into the packaging material. Berries, especially cranberries, have had through the years a great public interest due their rich polyphenolic content and respective association with interesting bioactive properties, including antimicrobial activity. As a result, the main goal of this research was to study the functionalization of surfaces intended for food packaging with cranberry extracts incorporation. Therefore, the specific goals of this study were:

i. To extract the bioactive compounds from three different cranberry sources (powdered, fresh and dried cranberry);
ii. To evaluate the antimicrobial and biofilm inhibition properties of the cranberry extracts
iii. To characterize the produced biodegradable films;
iv. To evaluate the functionalization of the material intended for food contact with the cranberry extracts incorporation;

This thesis is organized in 5 key chapters. In chapter 1 is presented the introduction, where biofilms problem in food industry are addressed, as well as cranberries bioactive compounds and a small review on antimicrobial active packaging. Chapter 2 covers the research aims of this thesis. In chapter 3, the materials and methods are exhibited, regarding cranberry extraction and characterization, films preparation, optimization and characterization and finally films antibiofilm properties. Results and discussion are presented in chapter 4: the optimal cranberry extraction method is determined (Section 4.1), cranberry extracts antimicrobial activity, antioxidant properties and total phenolic content is determined (Section 4.2), films preparation methodology is defined (Section 4.3), films are characterized according to several analysis such as total soluble matter, moisture content, swelling degree, surface wettability degree, light-barrier properties, water vapour permeability and oxygen transmission rate (Section 4.4) and films antibiofilm properties are exposed (Section 4.5). Lastly, in chapter 5 the thesis conclusions are revealed as well as future perspectives for this work.

Chapter 3

Materials and Methods

3.1. Chemicals

The following chemicals were used: acetic acid 100%, gallic acid (anhydrous) from Merk Millipore (Darmstadt, Germany), high molecular weight chitosan (310000 to 375000 Da, >75% deacetylated), medium molecular weight chitosan (75-85% deacetylated), resazurin sodium salt (molecular weight 251.17), trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid [molecular weight 250.29]), DPPH (2,2-diphenyl-1-picrylhydrazyl [molecular weight 394.32]), *Folin & Ciocalteu's* phenol reagent, sodium carbonate anhydrous, crystal violet (medium molecular weight 407.98), sodium phosphate dibasic ACS reagent (ACS reagent ≥ 99.0%), potassium chloride (ACS reagent, 99.0-1.5%), sodium chloride (ACS reagent ≥ 99.0%), monobasic potassium phosphate (ACS reagent ≥99.0%, molecular weight 136.09) from Sigma Aldrich (St. Louis, USA), methanol (ACS, ISO, Reag. Ph Eur) from EMSURE (Darmstadt, Germany), sodium chloride from Panreac (Milan, Italy), potassium acetate 99% and pentasodium tripolyphosphate (TPP) from Alfa Aesar (Massachusetts, United States), Agar Bacteriologique type, Brain Heart Broth, Müeller-Hinton (MH) broth, Trypto-casein soy broth (TSB) from Bioakar Diagnostics (Allonne, France), glycerol (GLY) from Labonal-Equipamentos Técnicos de Laboratório, Lda. (Lisbon, Portugal), ethanol absolute anhydrous (for analysis-ACS-Reag. Ph.Eur.-Reag. USP) from Carlo Erba reagents (Rodano, Italy), polyethylene glycol 400 (PEG) from Vaz Pereira (Lisbon, Portugal), levofloxacin from (Honeywell, Fluka) and water was purified by Milli-Q purification system.

3.2. Cranberry samples

Cranberries from three different sources were used as samples in the present work: i) fresh cranberries collected from a local market; ii) dried cranberries obtained from NATIVA (United States of America) and iii) powdered cranberries obtained from SOLGAR vegetable capsules (United States of America).

3.3. Cranberry extraction

3.3.1. Sample preparation

Fresh, dried and powdered cranberries were used as samples. Fresh cranberries (FB), after being washed with distilled water, and dried cranberries (DB), were milled and lyophilized. Powdered cranberries (PB) were isolated from the vegetable capsules. All samples were stored at -23ºC.

3.3.2. Extraction optimization

In order to evaluate the best extraction method, the FB samples were used as a model for the remaining. The FB sample (0.3 g) was extracted with 1 mL of acidified ethanol (0.01% HCl V/V) and vortexed for 1 min (77) .Subsequently, the samples were sonicated (J.P. Selecta) for 20, 40, and 60 min respectively and then vortexed for 2 min. The suspension was centrifuged at 12000 rpm (Heraeus Biofuge from ThermoFisher Scientific) for 10 min. The samples were extracted twice and the remaining supernatant was used for total phenolic content determination and High Performance Liquid Chromatography (HPLC) analysis. Analyses were performed in triplicate.

3.3.2.1. HPLC analysis for bioactive content determination

To assess the extracts suitable extraction method, regarding sonication time, samples were analysed by HPLC. The samples used for the analysis were from FB extracts dissolved in ethanol, (0.003 mg/mL). Since samples were extracted twice (1º extraction and 2º extraction), both extracted samples from each extraction time were analysed (Figure 8).

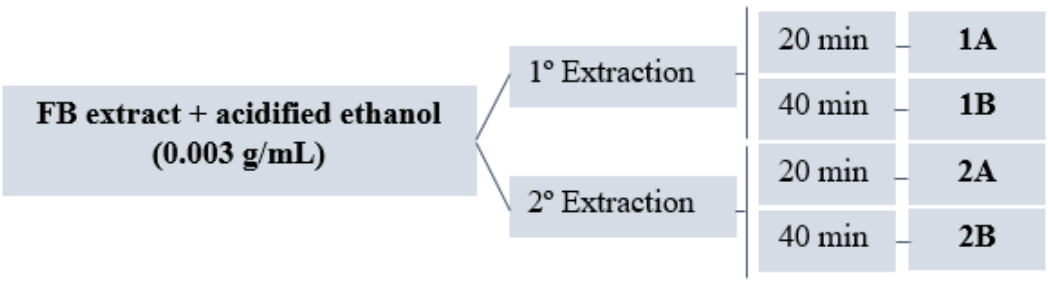

Figure 8. Samples analysed by HPLC.

The HPLC method used has been previously described (127). HPLC system from Thermo Finnigan (Surveyor model) equipped with a diode-array detector (Thermo Finnigan-Surveyor) and an electrochemical detector (Dionex, ED40). Chromatographic separation was carried out on a LiChrospher RP18 column (250 x 4 mm, 5 µm, Merck). The column was kept at 35ºC, an injection volume of 20 µL and a flow rate of 0.3 mL/min were used. The mobile phase consisted of a formic acid aqueous solution (0.5% V/V) as eluent A and an acetonitrile aqueous solution (90% V/V) added of formic acid (0.5% V/V) as eluent B. The following gradient elution program was applied: gradient for 15 min from 5.6% to 16.7% of eluent B; gradient for 5 min until 22.2% of eluent B; 10 min isocratic at 22.2% of eluent B; gradient for 25 min till 33.3 of eluent B; gradient for 25 min till 55.6% of eluent B; gradient for 40 min until 100% of eluent B, 15 min

isocratic at 100% of eluent B; gradient for 5 mins until 5.6% of eluent B, isocratic 20 min at 5.6% of eluent B. The chromatographic profiles of samples were compared to determine which of the sonication times lead to a higher extraction. Eluted peaks were monitored in four different wavelengths, respectively 280, 320, 360 and 520 nm. The equipment was controlled by Waters Empower Software.

3.3.3. Extraction method

After extraction optimization all cranberry samples were extracted according to the following method. The DB, FB and PB samples (3 g) were extracted with 10, 30 and 10 mL of acidified ethanol (0.01% V/V HCl) respectively and homogenize for 1 min .Then, the samples were sonicated (J.P. Selecta) for 40 min with occasional shaking and vortexed for 2 min .The resulting suspensions were centrifuged at 40000 rpm (Heraeus Biofuge from ThermoFisher Scientific) for 8 min .Samples were extracted twice and the solvent was evaporated from the extracts in a rotary evaporator (IKA HB 10 digital) at 40 ºC. Finally, the resulting concentrates were dried overnight using a vacuum pump (Vacuubrand) and stored at -20ºC until use for analysis.

3.4. Cranberry extracts characterization

3.4.1. Extraction Yield

The extraction yield of the cranberry extraction method used was determined according to the following formula:

$$\text{Extraction Yield (\%)} = \frac{\text{Dried sample extract mass}}{\text{Initial sample mass}} \times 100$$

3.4.2. Microbiological assays

Escherichia coli ATCC 25922 and *Staphylococcus aureus* ATCC 25923 obtained from the American Type Culture Collection (ATCC) and frozen at -80 ºC. Aliquots from the frozen stocks were used to culture the microorganisms in Tryptic Soy Agar (TSA) for 24 h at 37 ºC.

3.4.2.1. Minimum Inhibitory Concentration

The antibacterial activity of the berry samples (FB, PB and DB) and respective extracts was tested against *E. coli* and *S. aureus* trough the evaluation of the minimum inhibitory concentration (MIC) previously described by Clinical and Laboratory Standards Institute (128). For each sample a stock solution was prepared in Mueller-Hinton (MH) medium. This solution was homogenized and sonicated (J.P. Selecta) for 10 min .Samples were two-folded diluted in MH medium and the final assay concentrations ranged from 0.1 to 50 mg/mL. For inoculum preparation, each strain was first seeded in a TSA plate and incubated for 24 h at 37 ºC. To obtain a concentration of $1x10^8$ UFC/mL, a 3 mL direct colony suspension was prepared in MH and dilutions were performed until reaching an absorbance (Anthos Zenyth 3100) corresponding to

0.5 MacFarland units. Dilutions of the 1×10^8 UFC/mL suspension were performed in order to obtain a final assay concentration of 5×10^5 UFC/mL in each well of the microtiter plate. The microplate was incubated for 24 h at 37 ºC and the absorbance was read at 600 nm in a microplate multimode detector (Anthos Zenyth 3100). A positive control was prepared using inoculated culture media and a negative control was prepared using non-inoculated culture medium. Non-inoculated sample dilutions were also tested and levofloxacin two-fold dilutions was also used as assay control. All assays were done in triplicated.

3.4.2.2. Minimum Biofilm Inhibitory Concentration

For the Minimum Biofilm Inhibitory Concentration (MBIC) assay, the content of the plate was discarded from the previous MIC assay, and the wells were washed twice with sterile Phosphate Buffered Saline (PBS). After fixation of the biofilm with ethanol 96% (V/V) for 60 min, a crystal violet 0.1% (V/V) staining solution was added for 10 min. The wells were washed three times with distilled water and each well content was suspended in an acetic acid 1% (V/V) solution. The absorbance was then read with a microplate multimode detector (Anthos, Zenyth 3100) and the results were presented according to the following formula:

$$\text{Adhesion inhibition (\%)} = 100 - \frac{(100 \times \mathrm{A_{sample}})}{\mathrm{A_{control}}}$$

3.4.2.3. Minimum Bactericidal Concentration

After confirmation of the berry extracts samples antimicrobial activity against *E. coli* and *S. aureus*, the Minimum Bactericidal Concentration (MBC) was determined by alamarBlue® assay. In this test, the blue coloured and weakly fluorescent resazurin is reduced by viable cells into resorufin that is a pink coloured and highly fluorescent product, which allows the quantification of cranberry extracts cytotoxicity (2). Thus, after the MIC assay, 20 µL of each well that showed inhibition and positive controls, were transferred to a new 96-well plate with 180 µL of MH medium. After 24 h incubation at 37 ºC the absorbance (Anthos Zenyth 3100) was read at 600 nm and 20 µL of resazurin (25 µg/mL) were added to each well. Fluorescence was measured (Anthos Zenyth 3100) in a suitable 96-well microplate, with an excitation wavelength of 535 nm and an emission wavelength of 590 nm. Results were expressed as fluorescence units. The data is expressed as mean and standard deviation (mean ± SD) of at least 3 independent experiments.

3.4.3. Total phenolic content determination and antioxidant activity

3.4.3.1. Total phenolic content determination

The total phenolic content was determined based on a previously described method with slightly modifications (129). A Gallic acid (GA) stock solution (1 mg/mL) was first prepared by dissolving 10 mg of GA in 10 mL of methanol/distilled water (8:2 V/V). A 0.1 mg/mL GA solution was prepared by dilution from the stock solution in methanol/distilled water (8:2 V/V), and used to obtain different GA solutions for a calibration curve with concentrations ranging from 0.002 to 0.01 mg/mL. These solutions were prepared by adding 0.2, 0.4, 0.6, 0.8 and 1 mL of the 0.1 mg/mL GA solution to 10 mL of distilled water. To a 96-well microtiter plate, it was added

262.5 µL of each calibration curve solution and of each extracted samples. For the reaction, 7 µL of *Folin & Ciocalteu's* reagent were added and after 3 min, wells were supplemented with 30 µL sodium carbonate aqueous solution (35% w/V). The microplate remained in the dark for further 60 min and then the absorbance was measured at 725 nm with a microplate absorbance reader (SPECTROstar *Omega* from BMG Labtech). For this analysis a blank was also prepared by replacing samples by distilled water. All the measurements were carried out in triplicate. The total phenolic content was calculated with the linear function obtained from the calibration curve and the results were expressed as mg of Gallic acid equivalents (GA eq)/ g of cranberry extract.

3.4.3.2. DPPH radical scavenging activity

The free radical scavenging activity was measured against the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. In the presence of hydrogen donors DPPH protonates, according to the Figure 9, changing its purple colour to yellow and allowing to quantity the reaction by measuring the absorbance (129).

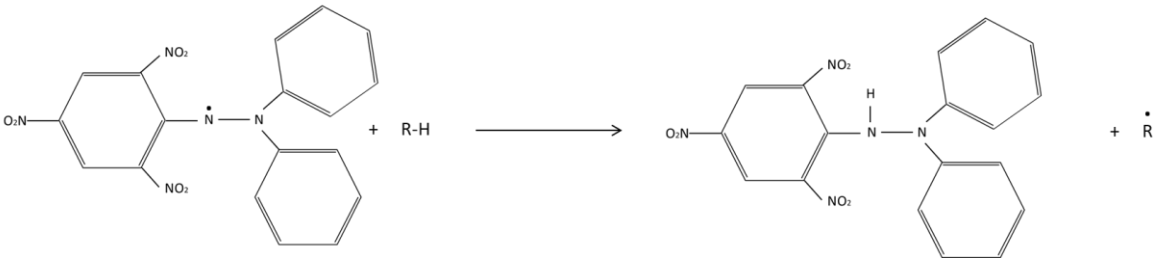

Figure 9. Reaction of DPPH radical with a hydrogen donor.

In a 96-well plate, 180 µL of a DPPH solution (0.06 mg/mL) were added to 20 µL of each sample dilution. The plate was shaken for 60 s at 240 rpm mim^{-1} (IKA KS 130 basic) and after incubation in the dark for 40 min at room temperature, the absorbance was measured at 517 nm (*SPECTROSTAR Omega* microplate reader). Trolox was used as positive control and different Trolox solutions with concentrations ranging from 0.013 to 0.13 mg/mL were prepared from a trolox stock solution (0.13 mg/mL). The negative control was prepared with 200 µL of the solvent and the blank with 20 µL of the solvent and 180 µL of the DPPH solution. All solutions were prepared in methanol/distilled water (8:2 V/V) and the assays were performed in triplicate. Samples were also measured in the absence of DPPH radical and the value deducted from the absorbance measured with the DPPH radical. The following equation was used to calculate the % DPPH quenched:

$$\%\ \text{DPPH quenched} = \left(\frac{A_{blank} - A_{sample}}{A_{blank}}\right) \times 100$$

A_{blank} represents the absorbance of the blank and A_{sample} represents the absorbance of the sample.

3.4.4. Cranberry extracts content identification

In order to identify compounds present in cranberry extracts, these were analyzed by High Performance Liquid Chromatography coupled to Mass Spectrometry (HPLC-MS). A LiChrospher®100 RP-18 column (250 × 4.0 mm, 5 μm; Waters™ Atlantis) was used in a HPLC equipment (Waters Alliance 2695 Separation Module, Ireland) coupled to a Tadem Quadrupole Mass Spectrometer (MicroMass Quattromicro API, Ireland) with an electrospray ionisation (ESI) source. The flow rate was 0.3 mL/min, the injection volume was 20 μL and samples were kept at 10 ºC. The mobile phase consisted of a formic acid aqueous solution (0.5% V/V) as eluent A and acetonitrile added of formic acid (0.5% V/V) as eluent B. The following gradient elution program was applied: isocratic 10 min at 5% of eluent B; gradient for 20 min until 15% of eluent B; 15 min gradient until 20% of eluent B; isocratic 20 min at 20% of eluent B; gradient for 30 min until 5% of eluent B; gradient for 15 min until 63% of eluent B, 5 min gradient until 5% of eluent B; isocratic for 20 mins at 5% of eluent B. The software data module used for data processing was MassLynx 4.1.The equipment was controlled by Waters Empower Software.

Samples and standards, described in Table 1, were analysed in both ESI positive and negative ion mode and mass spectra were recorded in the range *m/z* 60-1100. ESI cone voltage was set on 30 V and capillary voltage on 2.50 kV. Source temperature was adjusted to 120 ºC and desolvation temperature to 350 ºC. The cone gas flow and desolvation gas flow were respectively 50 and 750 L/Hr. A mixture of standards described in Table 1 was used in this analysis.

The identification of phenolic compounds was performed through the selective Multiple Reaction Monitoring (MRM). MRM parameters were optimized for each standard with a cone voltage ranging from 10 to 60 V and a collision energy ranging from 10 to 40 V. Additionally, to identified procyanidin A-type, MS/MS analyses were also conducted. The molecular ion (precursor) was selected and the characteristic products fragments were identified in the ESI negative ion mode. In these experiments, the collision energy ranged from 10 to 40 V.

Table 1. Mixture of standards used in the LC-MS analysis.

Compounds		
Apigenin	5-Hydroxymethylfurfural	p-Hydroxybenzoic Acid
Benzoic Acid	Hyperoside	Pelargonidin
Caffeic Acid	Isoliquiritigenin	Phloretin
Catechin	Isoquercetin	Phloridzin
Chlorogenic Acid	Isorhamnetin	Protocatechuic Acid
Cinnamic Acid	Isorhamnetin 3-glucoside	Quercetin
Cyanidin 3-o-glucoside	Isorhamnetin 3-rutinoside	Quinic Acid
Cyanidin 3-o-rutinoside	Kaempferol	Resveratrol
Ellagic Acid	Luteolin	Rosmarinic Acid
Epicatechin	Luteolin 7-glucoside	Rutin
Epicatechin Gallate	m-Coumaric Acid	Salicylic Acid
Eriodictyol	Malic Acid	Spiraeoside
Ethyl Caffeate	Myricetin	Syringaldehyde
Ethyl Gallate	Naringenin	Tartaric Acid
Ferulic Acid	Oleuropein	Trimethoxycinnamic Acid
Gallic Acid	o-Coumaric Acid	Tyrosol
Hesperidin	p-Coumaric Acid	Vanillin

3.5. Films preparation and optimization

3.5.1. Chitosan films preparation

Chitosan films were prepared according to previous studies with some modifications (130,131). Initially, to select the optimal chitosan concentration and mixtures for film preparation, different formulations were prepared as listed in Table 2. Chitosan solutions were prepared directly in a 24 well microplate (Sarstedt, Germany) by dissolving the chitosan (w/V), medium and high molecular weight in 0.5 mL of an aqueous solution of acetic acid 1% (V/V). The solutions were then magnetic stirred for 4 h at 1000 rpm (VWR Hotplate/Stirrer). Then to remove air bubbles samples were sonicated (J.P. Selecta) for 1 h. After sonication, samples were dried for 4 h at 50 ºC. For chitosan films cross linkage 0.5 mL of a Pentasodium Tripolyphosphate (TPP) 0.5% (w/V) aqueous solution were added to each well for 10 min (130). The crosslink solution excess was discarded and samples were washed tree times with distilled water. Finally, chitosan films were dried for 30 min at 50 ºC.

Table 2. Chitosan concentrations (w/V) in prepared film samples.

Film description	Medium molecular weight chitosan (%)	High molecular weight chitosan (%)
CS_M3%	3	-
CS_M2.5%	2.5	-
CS_M2%	2	-
CS_H3%	-	3
CS_H2.5%	-	2.5
CS_H2%	-	2
CS_M1%-CS_H2%	1	2
CS_M0.5%-CS_H2%	0.5	2
CS_M1.5%-CS_H1.5%	1.5	1.5

Note: CS represents chitosan, CS_M represents medium molecular weight chitosan and CS_H represents high molecular weight chitosan.

3.5.2. Chitosan films preparation with plasticizers incorporation

After selecting the chitosan concentration and mixtures as explained in results section 4.3., films were then prepared with plasticizers incorporation (i.e. polyethylene glycol 400 (PEG) and glycerol (GLY)) according to the following (Table 3): i) 20% of PEG; ii) 20% of PEG combined with 20% of GLY (131). After stirring chitosan solutions for 4 h at 1000 rpm, the plasticizers were incorporated and the mixture was stirred for additional 30 min at 1000 rpm. The following steps were performed as previously described. Samples were prepared in triplicate.

Table 3. Chitosan concentrations (w/V) and plasticizer incorporation (%) used to produce different chitosan films.

Film description	Chitosan medium molecular weight (%)	Chitosan high molecular weight (%)	PEG (%)	GLY (%)
$CS_M1\%\text{-}CS_H2\%^{PEG}$	1	2	20	-
$CS_M0.5\%\text{-}CS_H2\%^{PEG}$	0.5	2	20	-
$CS_M1.5\%\text{-}CS_H1.5\%^{PEG}$	1.5	1.5	20	-
$CS_M1\%\text{-}CS_H2\%^{PEG+GLY}$	1	2	20	20
$CS_M0.5\%\text{-}CS_H2\%^{PEG+GLY}$	0.5	2	20	20
$CS_M1.5\%\text{-}CS_H1.5\%^{PEG+GLY}$	1.5	1.5	20	20

Note: CS represents chitosan, CS_M represents medium molecular weight chitosan and CS_H represents high molecular weight chitosan; plasticizer concentration expressed as % of total chitosan mass.

3.5.3. Drying technique

The optimal drying temperature for the films preparation was estimated comparing three different temperatures, respectively 25 ºC, 36 ºC and 50 ºC based on temperatures used in several studies (130–132). Following the preparation method described above (section 3.5.1. and 3.5.2.) each film was prepared with 0.5 mL of different chitosan solutions (i.e. $CS_M1\%\text{-}CS_H2\%$, $CS_M0.5\%\text{-}CS_H2\%$ and $CS_M1.5\%\text{-}CS_H1.5\%$) to obtain films with a 1.8 × 1.8 cm circle dimension. Formulations containing PEG combined with and without GLY were obtained. One microplate was left to dry at 25 ºC for 24 h, another microplate at 36 ºC for 5 h and the last plate for 4 h at 50 ºC. After crosslinker addition, the first was left to dry at 25 ºC for 24 h, the second at 36 ºC for 3 h and 20 min and the third for 30 mins at 50 ºC.

3.5.4. Films preparation with cranberry incorporation

For cranberry incorporation, films were first prepared in 96-well microplates. The prepared chitosan solution (100 µL) was transferred with a syringe to the respective wells and leave to dry at 36 ºC for 36 h. Then to each well it was added 40 µL of a TPP aqueous solution 0.5% (w/V) combined with cranberry extract in two concentrations, respectively 100 mg/mL and 200 mg/mL. Finally, films were left to dry 36 ºC for 36 h (124).

3.6. Films characterization

3.6.1. Thickness and density

The films thickness was measured with a manual micrometre and the films density was calculated according to the following formula (83):

$$\text{Film density } (\text{g/cm}^{-3}) = \frac{\text{film weight}}{\text{film area } \times \text{film thickness}}$$

Films diameter was measured in three different points with a Vernier calliper (Powerfix) and the mean was used for area calculation.

3.6.2. Antimicrobial properties

To test the enhancement in antimicrobial activity of films after cranberry extracts incorporation, films activity was evaluated through the Kirby-Bauer assay against *E. coli* and *S. aureus*. Films were prepared in a 96-well microplate, as described above, with and without cranberry incorporation. The concentrations tested were CS_M1%-CS_H2%, CS_M0.5%-CS_H2% and CS_M1.5%-CS_H1.5% incorporating PEG with and without GLY addition.

For inoculum preparation, strains were first seeded in a TSA plate and incubated for 24 h at 37 ºC. Then, a direct colony suspension in MH medium was prepared to obtain a final inoculum concentration of 1×10^8 CFU/mL. The inoculum was swapped in a MHA plate and film samples to be tested were placed on top of the inoculated plate. The plate was incubated for 24 h at 37 ºC and afterwards the inhibition zone diameter of each film was measured with a Vernier calliper (Powerfix). Films incorporating the berry extract were prepared with 100 mg/mL of FB and PB extract. A levofloxacin (5 µg) disk was used as control. Each test was done in triplicate.

3.6.3. Total soluble matter and moisture content

For evaluation of films total soluble matter (TSM), previously weighted films (1.8 cm ×1.8 cm) were immerse in 10 mL of distilled water for 4 h at 25 ºC. Films were then removed and dried at 105 ºC for 24 h to obtain a dry mass (Mf). The TSM was calculated using the following formula:

$$\text{TSM}\ (\%) = \frac{\text{Mi} - \text{Mf}}{\text{Mi}} \times 100$$

To determine the moisture content films were weighted (Mi) and subsequently dried at 105 ºC for 24 h. Films dried mass was weighted (Mf) and then the moisture content was calculated as follows:

$$\text{Moisture content}\ (\%) = \frac{\text{Mi} - \text{Mf}}{\text{Mi}} \times 100$$

These tests were done in triplicate for each film and both methods were based in previous studies (133).

3.6.4. Swelling degree

For evaluation of films swelling degree, previously weighted films (1.8 cm ×1.8 cm) were immerse in 10 mL of distilled water for 4 h at 25 ºC (131). Films were then dried superficially with filter paper and the final film mass was obtained (Mf). The swelling degree was calculated using the following formula:

$$\text{Swelling degree (\%)} = \frac{\text{Mf} - \text{Mi}}{\text{Mi}} \times 100$$

The test was done in triplicate for each film.

3.6.5. Surface wettability degree

Surface hydrophobicity was analysed by measuring the contact angle of water on the films surface (134). A drop of deionized water (2µL) was placed on the film surface and the drop was recorded using a digital microscope for 5 min .The image was acquired every min with a video capture application (AMCap - 9.016). An image processing software (ImageJ) was used to measure the contact angles recorded. There were taken 6 different measurements in different points of the film. Four measures were performed per film at room temperature.

3.6.6. Optical properties

Light-barrier properties were determined by measuring the films absorbance from 220 to 800 nm (SPECTROstar *Omega* from BMG Labtech). Films transparency was determined by A_{600}/T, where A_{600} is the film absorbance at 600 nm and T represents the film thickness (mm).

3.6.7. Hardness values

The mechanical properties of the films were characterized by determination of films hardness values trough nanoindentation tests by a hardness tester (Struers Duramin-5). The nanoindentation measurements were performed using a diamond tip and a press load of 98.12 mN for 5 s. Three indentations were performed in each film and IBM SPSS Statistics program was used for statistical analysis.

3.6.8. Water vapour permeability

The water vapour permeability (WVP) of the films was measured gravimetrically at 30º C according to a previous study (135). The films were sealed in glass cells that contained a saturated salt solution (76.5% relative humidity) and then placed inside a desiccator with a saturated potassium acetate solution (22.5% relative humidity). For air circulation the desiccator was equipped with a fan. The room temperature and humidity were monitored for 24 h and the glass cells weigh was measured at regular time intervals during that period. The WVP (mol.m/m^2 sPa) was calculated according to the following formula:

$$WVP = \frac{N_w \times \delta}{\Delta P_{w,eff}}$$

where N_w (mol/m^2·s) represents the water vapour flux, δ (m) the film thickness and $P_{w,eff}$ (Pa) the effective driving force.

Results presented as mean ± standard deviation of the three replicates analysed. For the test, films were prepared with a 1.8 cm × 1.8 cm circle dimension and the films tested were CS_M0.5%-CS_H2% ^{PEG} and CS_M0.5%-CS_H2% $^{PEG+GLY}$ with and without cranberry extracts incorporation. The test was done in triplicate.

3.6.9. Oxygen transmission rate

According to previous studies (135), this assay was developed in a stainless steel cell with a permeation area of 33.18 cm^2 and two identical chambers separated by the film being tested. Previously, the films were equilibrated at a constant relative humidity in a desiccator containing a saturated $MgCl_2 \cdot 6H2O$ solution. The permeability was evaluated by measurement of the pressure change in both chambers after pressuring one of the chambers up to 0.4 bar with oxygen. Independent measurements were made at constant temperature with a thermostatic bath. The permeability was calculated by the following equation:

$$OP = \frac{\Delta V \delta}{A \Delta P}$$

where ΔV represents the volume of the permeated oxygen, δ (m) is the film thickness, A (m^2) is the area of the membrane and ΔP is the pressure difference between the feed and the permeating film sides.

3.7. Films antibiofilm properties

3.7.1. Plate colony counting method

Inhibition of *E. coli* and *S. aureus* biofilm was assessed through plate count colony forming units according to previous studies (136). Antimicrobial films were prepared in a 96-well plate on the top of expanded polystyrene foam disks. The sterilized expanded polystyrene foam was fixed at the bottom each well with and the films were prepared on top of the expanded polystyrene foam using the same method described above (section 3.5.4). Inoculum was obtained from direct colony suspension, in Brain Heart Infusion (BHI) medium supplemented with glucose at 1% (w/V) and transferred to each well of the microtiter plate to obtain a final inoculum concentration of 3×10^6 CFU/mL. The microtiter was then incubated at 36 ºC for 24 h. After incubation each well was twice washed with PBS to remove non-adherent cells. Each expanded polystyrene foam covered with film was transferred to a 2 mL tube containing 1 mL of MH medium and to detach biofilm samples were scraped, sonicated (J.P. Selecta) for 4 min and vortexed for an additional 4 min .The biofilm cells suspensions were diluted in MH medium and 20 µL of each solution was platted on TSA medium. Plates were incubated at 36 ºC for 24 h to determine the CFU/mL. Expanded polystyrene foam disks were used as positive control and Expanded polystyrene foam disks covered with films with cranberry extracts (i.e. 100 (CE100) and 200 (CE200) mg/mL) and without extracts were tested. Assays were performed in triplicate.

3.7.2. Scanning electron microscopy analysis

S. aureus biofilm inhibition when using cranberry films as described in section 3.5.4., was observed by scanning electron microscopy (SEM) (136). Sterilized expanded polystyrene foam was fixed at the bottom each well with silicone and the films were prepared on top of the expanded polystyrene foam. Inoculum was obtained from direct colony suspension, in BHI medium supplemented with glucose at 1% (w/V) and transferred to each well of the microtiter plate to obtain a final inoculum concentration of 3×10^6 CFU/mL After 24 h of incubation at 36 ºC each well was washed twice with PBS to remove non-adherent cells. Samples were then fixed with different ethanol aqueous solutions for 40 min and the biofilm inhibition was observed by a scanning electron microscope (FEG-SEM, model JSM7001F [JEOL, Japan] operated at 10 kV). To increase the conductivity of the specimens they were coated with a thin layer of conductive gold film, under vacuum in an argon atmosphere (QuorumTechnologies, Polaron E5100). Expanded polystyrene foam disks were used as positive control and expanded polystyrene foam disks covered with films with cranberry extracts (i.e. 100 and 200 mg/mL) and without extracts were tested.

Chapter 4

Results and discussion

4.1. Cranberry extraction

The first step in the present work was to optimize the extraction conditions. Although extraction of bioactive compounds from berries has been widely studied, to date there is not a completely acceptable technique able to extract all the phenolic compounds from these fruits (137). More commonly, polyphenolic compounds are extracted with resource to methanol or acetone which studies show to be effective extraction solvents (138). In Moore and co-workers study, tea polyphenols extracts were successfully obtained by extraction with 80% (V/V) methanol for 3 h and then twice with 80% (V/V) methanol containing 0.15% HCl (138,139). For the present work, ethanol was selected as the extraction solvent since it is not toxic in comparison with solvents that are efficient, like methanol, but present high toxicity, which is unfavourable to the food industry (140). Moreover, a study comparing different solvents for the extraction of the phenolic compounds from American cranberry press residues, namely acetonitrile, water, acetone, methanol and ethanol, demonstrated that although methanol had the highest extraction content of total polyphenols (4.80 g/100 g of berry powder), ethanol also demonstrated a high polyphenolic extraction with a total polyphenol content of 3.43 g/100g of the berry powder, assuring that ethanol can be an ideal solvent for phenolic compounds extraction (141).

In order to obtain a suitable extraction yield of phenolic compounds from cranberry samples, the influence of ultrasound time on the extraction efficiency was studied. Thus, FB samples were extracted at three different sonication times, respectively 20, 40 and 60 min, and their total phenolic content was evaluated (Table 4). These results showed that samples extracted with 40 min sonication time had a higher total phenolic content and samples extracted with a 20 min sonication time had a lower total phenolic content.

Table 4. Total phenolic content as Gallic Acid equivalents (mg GA eq/g FB extract) in fresh cranberry (FB) extracts sonicated for 20, 40 and 60 min.

Ultrasound time (min)	Total phenolic content (mg GA eq/g FB extract)
20	4.27
40	7.65
60	6.79

To further confirm these results samples sonicated for 20 and 40 min were also analysed by HPLC and the chromatographic profiles compared. Figure 10 shows the chromatographic profiles for samples extracts (Figure 10), obtained by sonicating with ethanol during 20 min (Samples 1A and 2A) and during 40 min (Samples 1B and 2B). In red (1A) and blue (1B) is presented the chromatographic profiles from the first time samples were extracted with ethanol and in pink (2A) and black (2B) is present the chromatographic profiles from the second time samples were extracted with ethanol. The chromatographic profiles are displayed at four different wavelengths, respectively 280 nm (Figure 10.(a)), 320 nm (Figure 10.(b)), 360 nm (Figure 10.(c)) and 520 nm (Figure 10.(d)).

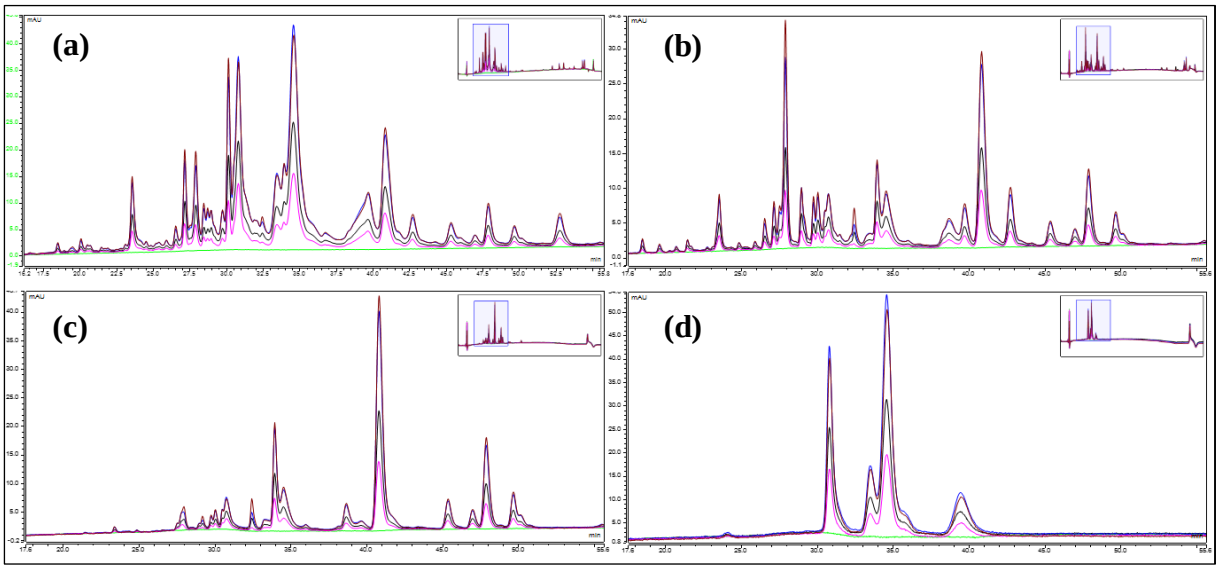

Figure 10. Chromatographic profiles comparison between samples sonicated for 20 and 40 min at different wavelengths: (a) 280 nm, (b) 320, (c) 360 and (d) 520 nm; Chromatograms were amplified between 16 and 56 min. and samples response after each extraction can be observed: first 20 min in red (1A); second 20 min in pink (2A); first 40 min in blue (1B); second 40 min in black (2B).

As expected, it was possible to observe that samples from the first extraction 1A and 1B (red and blue) had higher peaks comparatively with samples from the second extraction 2A and 2B (pink and black). In the samples from the first extraction, 1A and 1B, peaks presented to be very similar in all four chromatographic profiles but regarding the second extraction it is possible to observe that in the four chromatographic profiles (Figure 10), the area of peaks corresponding to the samples sonicated for 40 min (2B) was higher than the peaks from the 2A samples, sonicated for 20 min. As a result, it was possible to determine that extracting cranberry samples with a 40 min sonication time would improve the extraction method comparatively with the remaining times.

Recent studies have shown the increase in extraction efficiency of targeted compounds with the application of an ultrasound-assisted extraction (142–144). An ultrasound-assisted extraction is an efficient process to optimize and reduce extraction time. Furthermore, it presents the advantage of being less destructive comparatively with conventional solvent methods such as thermal extraction and also it allows for an increase in contact between solvent and the targeted compounds (145). Therefore, the implementation of this process to the

extraction methodology, specifically applying a 40 min sonication time, allowed for an improvement in the extraction efficiency.

4.2. Cranberry extracts characterization

After determination of the suitable sonication extraction time the cranberry extracts properties were evaluated to assess the best sample for film incorporation.

4.2.1. Yield of extractable solids

The extraction yield for the ethanol extraction was assessed (Table 5). Results show that DB had the highest yield of extractable solids and FB had the lowest extraction yield. Extraction yield is dependent on several factor such as sample phytochemical content, extraction method used, solvent used (146). In this study, since the applied method was the same for all three cranberry sources the differences between the extracts can be explained by the differences in the content of the extractable compounds between the different sources.

Table 5. Mass extraction yield % (mass of the sample/mass of the dried extracted sample) from fresh (FB), dried (DB) and powdered (PB) cranberry extracts.

Cranberry extract	Extraction yield % (w/w)
FB	20.8 ± 2.1
DB	40.2 ± 2.5
PB	34.4 ± 5.9

4.2.2. Extracts antimicrobial activity

The antimicrobial activity of cranberries and cranberries extracts was tested against *E. coli* and *S. aureus* by assessing the MIC, MBC and additionally MBIC associated to the biofilm inhibition capacity (Figure 11). Non-extracted samples of FB, PB and DB (Samples dissolved directly on MH medium without an extraction procedure) did not demonstrated antimicrobial activity against both tested strains. On the contrary, the obtained results (Figure 11.(a)) revealed that overall cranberry extracts presented antimicrobial activity, specifically FB and PB extracts. The results also showed that *S. aureus* strains are more susceptible to berries activity than *E. coli*. strains. Regarding each strain FB and DB presented the same MIC and MBC as well as the same MBIC, while DB did not displayed antibiofilm activity.

Regarding the MBC, both FB and PB exhibited a bactericide activity at 6.3 mg/mL against *S. aureus* and at 25 mg/mL against *E.coli*. In both FB and PB extracts, the biofilm inhibition concentration against the two strains was equal to the bactericide concentration. Additionally, DB did not revealed bactericide activity.

(a)

Cranberry extracts (mg/mL)		Microorganism	
		S. aureus	*E. coli*
MIC	FB	3.1	6.3
	PB	3.1	6.3
	DB	50	50
MBC	FB	6.3	25
	PB	6.3	25
	DB	-	-
MBIC	FB	6.3	25
	PB	6.3	25
	DB	-	-

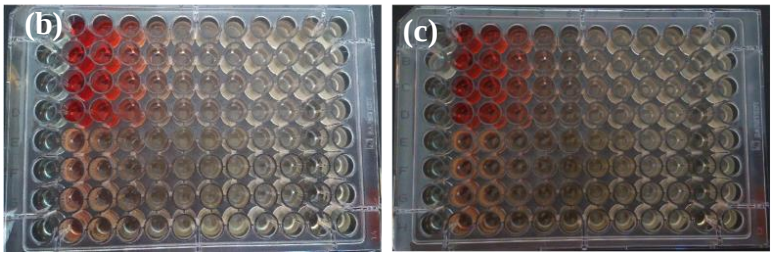

Figure 11. (a) Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Minimum Biofilm Inhibitory Concentration (MBIC) results (mg/mL) from fresh (FB), dried (DB) and powdered (PB) cranberry extracts and example of 96-well plates prepared for MIC assessment of the different extracts against (b) S. aureus and (c) E. coli.

The results are in agreement with other studies that have also proved the antimicrobial activity of extracted cranberries against the tested bacteria (*S. aureus* and *E. coli*). In LaPlante and co-workers study (147), three different cranberry extracts, two powder extracts (Cran A and Cran B) and one liquid extract (Cran C), were proved to have antimicrobial activity and biofilm inhibition capacity against two different *S. aureus* strains (*S. aureus* ATCC 35556 and *S. aureus* L4278). However, at the highest tested concentration of 20 mg/mL (Cran A and Cran B) and 10 mg/mL (Cran C) cranberry extracts were inactive against *E. coli* (ATCC 25922). In another study, the antimicrobial effects of several cranberry constituents, respectively sugar, anthocyanins and phenolics at natural pH showed antimicrobial activity against *E. coli* O157:H7. Phenolics had a MIC and MBC of 2.7 mg/mL and anthocyanins a MIC and MBC of 0.0148 mg/mL (148).

Microorganism are shown to have different sensitivities to berry extracts antimicrobial activity depending on the strain and type of berry tested (149). Although cranberry extracts mechanism of action is still not certain, antimicrobial activity of cranberries may be linked to multiple mechanisms since several studies have proven that the high content of polyphenolic compounds in cranberries is associated to its antimicrobial activity (68). Cell wall structural differences between Gram-positive and Gram-negative bacteria may be the cause of the higher susceptibility of *S. aureus* to cranberry extracts in comparison to *E. coli*, since Gram-negative bacteria have a lipopolysaccharide membrane surrounding the cell wall that decreases permeability to cranberry phenolic compounds (150). Some studies suggest that cranberry concentrate antimicrobial mechanism not only involves the disruption of the cell outer membrane but also the inhibition of gene transcription preventing the synthesis of proteins essential for bacterial growth (151). QS inhibition mechanisms is also a proposed mechanism of action. In Ferldman and co-workers study (13), cranberry constituents have shown ability to

inhibit bacterial signalling system of *Vibrio harveyi* by interfering with the autoinducer-receptor binding process.

These results confirm that cranberry compounds can be used as antimicrobial agents in the food industry, especially FB and PB extracts, which present a high antimicrobial activity against both strains in comparison to DB extracts.

4.2.3. Extracts total phenolic content determination and antioxidant activity

Since there is a strong relationship between phenolic compounds and antioxidant activity, cranberries extracts total phenolic content was determined (Figure 12). Observing the results (Figure 12.(a) and Figure 12.(b)) it can be seen that PB extract presented an evident higher phenolic content, 106.3 ± 13.2 mg GA eq/g BE, in comparison with the FB and DB extracts that presented 13.2 ± 1.6 and 1.4 ± 0.2 mg GA eq/g BE respectively . Studies demonstrated that total phenolic content may be influenced by cranberry maturity, variety and processing. For example, a study with four different *Vaccinium* species cultivants demonstrated that an increased in berries maturity at harvest, increased total phenolic content (152)**.** In another study, different dehydration methods, respectively air-drying, vacuum microwave drying and freeze drying, were applied to Saskatoon berries. Results exhibited that all berries had a lower content in total phenolic compounds and total anthocyanins in comparison with unprocessed samples (153) . Thus, a hypothesis for DB low phenolic content could be related to cranberry processing that led to the loss of more bioactive compounds.

Results from FB extracts total phenolic content are in agreement with the study of Sariburun and co-workers, who determined the total phenolic content of 5 raspberry and 4 blackberry cultivants in water and methanol extracts. Results exhibited that the total phenol content of the cultivars ranged from 10.4 ± 0.2 to 27.9 ± 0.2 mg GA eq/ g fresh weight in which blackberry cultivants present the higher total phenolic content (154).

(a)

Cranberry extract	Total phenolic content (mg GA eq/ g BE)
FB	13.2 ± 1.6
DB	1.4 ± 0.2
PB	106.3 ± 13.2

(b)

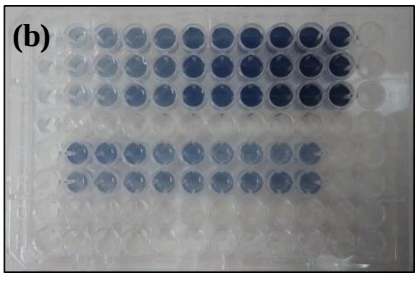

Figure 12. (a) Total phenolic content (mg Gallic Acid equivalents/g cranberry extract) for fresh (FB), dried (DB) and powdered (PB) cranberry extracts; (b) Example of a 96 well-plate prepared for the total phenolic content evaluation by Folin & Ciocalteu's method after 1 h in the dark at room temperature.

Since phenolic compounds are known for their antioxidant activity, the different extracts antioxidant activity was assessed through the DPPH radical scavenging method (Figure 13). The DPPH radical scavenging activity assay allows the assessment of bioactive compounds capacity to scavenge the radical DPPH. The results (Figure 13) showed that all extracts

presented DPPH radical inhibition activity at different concentrations. It was observed that the free radical scavenging activity was higher for PB. For a 50% scavenging activity the extracts concentration employed for PB, FB and DB was 0.5, 4.8 and 64 mg/mL, respectively. The PB used in this study contained Vitamin C, a known antioxidant compound (17), and that could be an explanation for the higher antioxidant capacity results regarding PB extracts. In a study, the DPPH radical scavenging of myrtle (*Myrtus communis*), a perennial shrub, methanolic, ethanolic and aqueous extracts was measured to assess extracts antioxidant activity. Results from that study demonstrated that compared to berry extracts, ascorbic acid exhibited one of the highest radical scavenging activity (155). The difference between the results can also be due to differences in the cranberry species used in each sample source.

Since phenolic compounds are described to have antioxidant activity, the results from this work are in agreement with the previously determined total phenolic content, where PB extracts presented a higher phenolic content. In many studies, phenolic content is exhibited to correlate with the DPPH radical scavenging assay (155). In this study, DB extracts, which had lower phenolic content, demonstrated a lower scavenging capacity, whereas, PB, with a higher phenolic content from the three extracts, revealed the highest scavenging capacity. Therefore, it is suggested that the phenolic content determined in the cranberry extracts is related to extracts antioxidant capacity, determined through the DPPH assay.

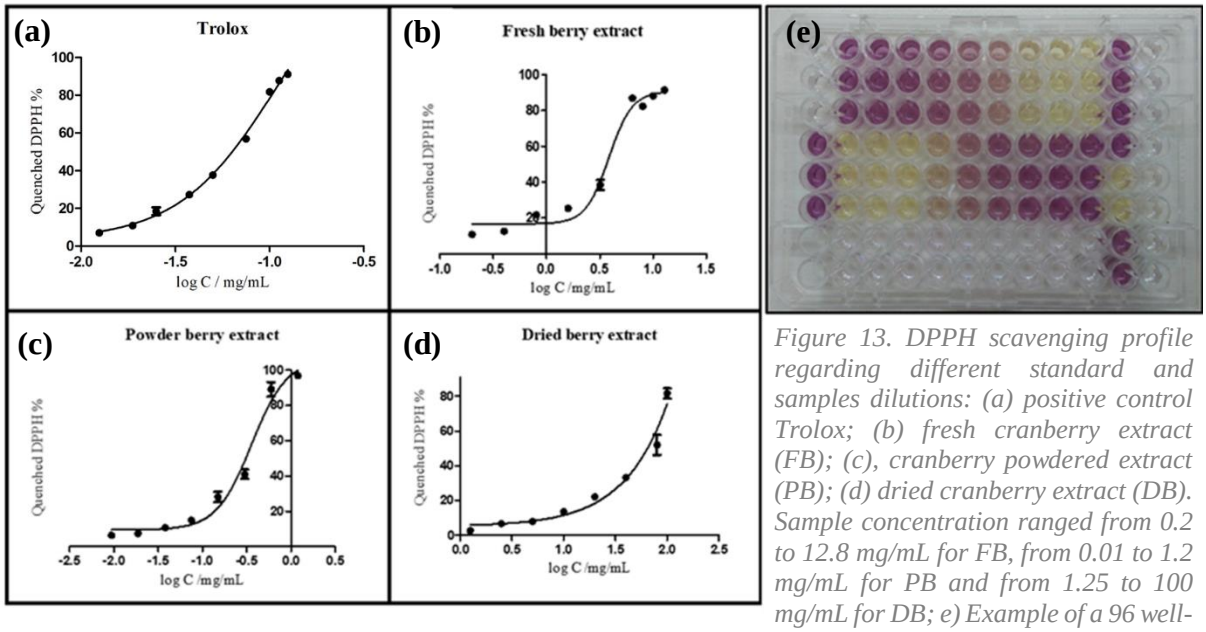

Figure 13. DPPH scavenging profile regarding different standard and samples dilutions: (a) positive control Trolox; (b) fresh cranberry extract (FB); (c), cranberry powdered extract (PB); (d) dried cranberry extract (DB). Sample concentration ranged from 0.2 to 12.8 mg/mL for FB, from 0.01 to 1.2 mg/mL for PB and from 1.25 to 100 mg/mL for DB; e) Example of a 96 well-plate prepared for the DPPH assay after 40 min. in the dark at room temperature.

4.2.4. Cranberry extracts content identification

Phenolic compounds present in cranberry extracts samples were identified through MRM mode. The selection of precursor ions in the first quadrupole originated specific product ions in the third quadrupole allowing the comparison between standards and samples

transitions. Among the variety of standards tested, it was possible to identify the following compounds (Table 6): Isorhamnetin 3-glucoside, phloridzin, p-hydroxybenzoic acid, epicatechin and chlorogenic acid.

Table 6. Phenolic compounds present in cranberry extracts samples identified by LC-MS/MS through the MRM mode.

Compounds	Ionization mode	Cone Voltage (V)	Collision energy (V)	Specific transition	Retention time (min)	FB extract	PB extract
Isorhamnetin 3-glucoside	ES+	20	10	479 > 317	79.73	+	-
p-Hydroxybenzoic Acid	ES-	30	35	137 > 93	40.55	+	-
Phloridzin	ES-	30	15	435 > 273	81.81	+	+
Pelargonidin	ES+	60	30	272 > 121	42.53	+	-
Chlorogenic acid	ES-	40	15	353 > 191	42.02	+	+
Epicatechin	ES-	40	25	289 > 109	47.13	+	+

Note: (+) represents de presence of the compound in the samples and (-) represents the absence of the compound in the sample

Flavonols are usually found in glycosylated forms in cranberries (88). Isorhamnetin-3-glucoside is reported in the literature, mainly present in red grapes and wines (156). The identification of isorhamnetin-3-glucoside in this study is in agreement with other studies that also reported the presence of isorhamnetin-3-glucoside in cranberries (157). The presence of hydroxybenzoic acid has also been reported in cranberries (98,158) as well as phloridzin. Although phloridzin itself may not have bioactive properties, its combination with other compounds is described to possibly have a synergic effect (159). Moreover, pelargonidin is one of the anthocyanins usually present in high quantities in cranberries. Wu and co-workers demonstrated that from 18 fruits rich in anthocyanin, only cranberries and concord grapes had all six anthocyanins, including pelargonidin (160). Chlorogenic acid, commonly found in berries and identified in this study in FB extracts, is described to contribute to the antioxidant properties demonstrated by cranberries (161,162). The flavan-3-ols (catechin and epicatechin), highly found in cranberries, constitute the monomers of proanthocyanidins. So, the presence of epicatechin in FB extracts is correspondingly as expected and has also been observed in other studies (163).

The presence in samples of A-type procyanidin (Table 7) was also searched through MS/MS analyses under ESI negative ion mode. Based on the applied collision energy, different characteristic product fragments, previously described in Rue and co-workers study (164), were observed . Additionally, when searching the precursor ion and their correspondent fragments it was possible to detect their presence around the retention time of 64 and 65 min.

Table 7. A-type procyanidin identification at different collision energies based on characteristic fragments reported in the literature (164) using negative ionisation mode.

Compound	MW (g/mol)	Collision energy (V)	Retention time (min)	MS^- (m/z)
A-type procyanidin	576.51	10	65.0	285
		20	63.9	285,289
			65.0	423,449
		25	63.9	289,285
			65.0	449,423
		30	63.9	285,423,289
		35	63.9	285,449
		40	65.0	285,289,423

The *m/z* 285 and 289 fragments identified correspond to quinone methide ions formation by fragmentation of two catechin or epicatechin subunits. The *m/z* 423 fragment is probably related to retro-Diels–Alder fission. Additionally, the identification of the *m/z* 449 fragment allows for a possible association to A-type procyanidins loss of a phloroglucinol molecule (164).

Since A-type proanthocyanidins have a wide structure heterogeneity, it is difficult to proper identify all the compounds in the sample. However, based on the results is possible to assume the presence of A-type proanthocyanidins dimers present in the FB extract sample. Proanthocyanidins are present in several common food products, especially berries and fruits (165). Cranberries are reported to be particularly rich in proanthocyanidins and principally cranberry proanthocyanidins A-type linkages are widely associated to in vitro anti-adhesion properties (78,88). As a result, the FB extracts content in A-type procyanidins can be responsible for the antimicrobial activity previously demonstrated by the cranberry extracts (section 4.2.1).

4.3. Film preparation

Different chitosan-based films have been used in the development of food packaging, due to their physicochemical properties and film forming capacity (166,167). Chitosan films have been demonstrated to improve food products quality characteristics and shelf-life (168,169). In order to develop the optimal chitosan films for further extract incorporation it was important to select the type and concentration of chitosan for film development and drying conditions.

For the primary film preparation, films formed only with high molecular weight chitosan revealed to be more difficult to homogenize. Additionally, both films produced only with high molecular weight chitosan (CS_H3%; CS_H2.5%; CS_H2%) or medium molecular weight chitosan (CS_M3%; CS_M2.5%; CS_M2%) exhibited to be more easily broken than the chitosan mixtures. As a result, the chitosan concentrations selected to be applied in the further analysis were the chitosan mixtures, respectively CS_M1%-CS_H2%, CS_M0.5%-CS_H2% and CS_M1.5%-CS_H1.5% with plasticizers addition (20% PEG or 20% PEG + 20% GLY).

The films drying technique was assessed with three different temperatures and times namely 25 ºC for 24 h, 36 ºC for 5 h and 50 ºC for 4 h. After films preparation (Figure 14) it was perceptible, in all six different chitosan compositions, that films left to dry at 25 ºC, appeared to have overall better results than the films prepared with the other two experimental conditions. Films dried at 25 ºC were more flexible and less stiff than the remaining, providing a better option for a future use as packaging material. Additionally, it can also be observed that films prepared with the chitosan mixture CS_M0.5%-CS_H2% were visually more homogenised than the other two mixtures, especially in films left to dry at 25 ºC and 36 ºC. As it can be seen, all films left to dry at 50 ºC for 4 h, were not suitable for further analysis since the films were more shrunken, hard and had lower flexibility.

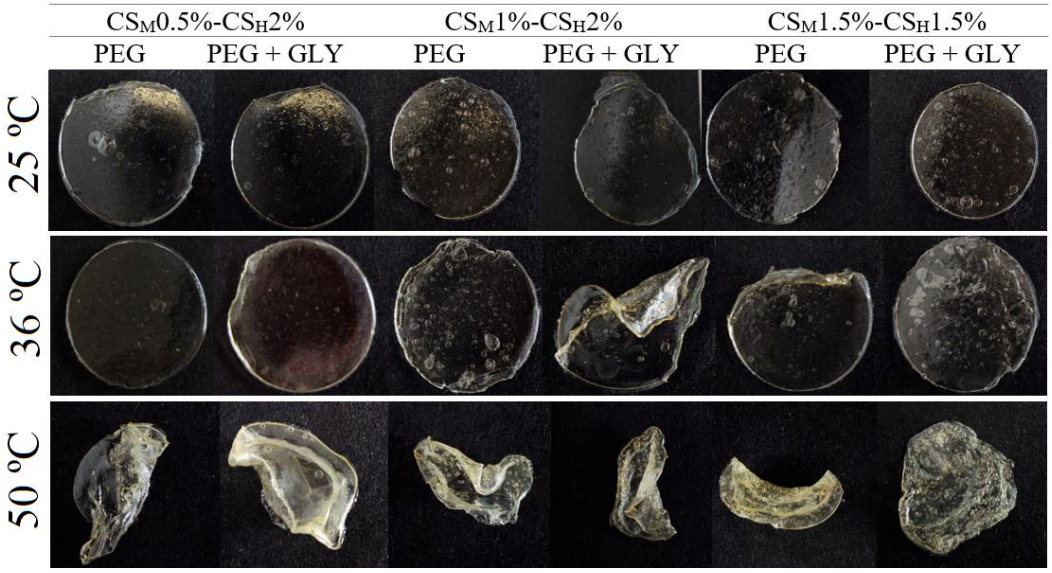

Figure 14. Pictures of films prepared on 24-well plate lid (1.8 × 1.8 cm) with three different concentrations of CS_M1%-CS_H2%, CS_M0.5%-CS_H2% and CS_M1.5%-CS_H1.5% with and without glycerol (GLY) addition dried at three different temperatures: 25ºC for 24h, 36ºC for 5 h and 50ºC for 4 h. CS_M represents medium molecular weight chitosan and CS_H represents high molecular weight chitosan..

Films drying methods and conditions are described to have a distinct effect on films physical and thermal properties. In Mayachiew and co-workers study, the effects of drying methods and temperature on the chitosan films properties were investigated and films were exposed to different drying techniques (ambient, hot air, vacuum and low-pressure superheat steam) at different conditions. In that study, low-pressure superheat steam drying at 70 ºC exhibited the best film forming conditions since films physical properties were improved (170). In another study, from the three different evaluated temperatures (35, 40, 45 °C), 40 ºC for 48 h demonstrated to produce films with the better physical properties regarding thickness, penetrability, transmittance and water vapour transmission rate. Additionally, chitosan concentrations also revealed to affect films properties (171).

In conformity with the obtained results, it was concluded that, the best drying method for films preparation with cranberry incorporation was at 25 ºC for 24 h. This temperature was also considered to be the less harmful for cranberry extracts bioactive compounds, since thermal processing shows to decrease berry phenolic content (172).

4.4. Film characterization

Water sensitivity is an important property to be assessed on chitosan-based films and can be evaluated by different techniques such as moisture content, solubility, contact angles and the water vapour permeability (173). Therefore, the effect of the chosen chitosan formulations (CS_M1%-CS_H2%, CS_M0.5%-CS_H2% and CS_M1.5%-CS_H1.5%) added of plasticizers (PEG and GLY) on films water sensitivity expressed as total soluble matter, swelling degree, moisture content and wettability degree was evaluated.

Regarding thickness and density values of prepared films (Table 8), results showed to be overall very similar between the tested formulations. Addition of GLY seemed to slightly lower films thickness while raising the density. Films prepared only with PEG were found to have a lower moisture content than films with the same chitosan mixture but prepared with PEG and GLY (Table 8). The film CS_M0.5%-CS_H2% ^{PEG} demonstrated the lowest moisture content values.

Table 8. Thickness (mm), density (g/cm^3) and moisture content (%) values of chitosan films. Results are expressed as mean ± SD and the evaluations on done in triplicate.

Film Sample	Thickness (mm)	Density (g/cm^3)	Moisture Content (%)
CS_M0.5%-CS_H2%^{PEG}	0.06 ± 0.01	0.08 ± 0.01	17.2 ± 0.7
CS_M0.5%-CS_H2% $^{PEG+GLY}$	0.04 ± 0.01	0.16 ± 0.02	22.6 ± 2.0
CS_M1%-CS_H2% ^{PEG}	0.08 ± 0.01	0.11 ± 0.01	18.0 ± 1.4
CS_M1%-CS_H2% $^{PEG+GLY}$	0.06 ± 0.00	0.15 ± 0.02	21.1 ± 2.4
CS_M1.5%-CS_H1.5% ^{PEG}	0.09 ± 0.01	0.10 ± 0.01	20.8 ± 0.5
CS_M1.5%-CS_H1.5% $^{PEG+GLY}$	0.08 ± 0.01	0.12 ± 0.01	20.1 ± 2.3

Note: CS_M represents medium molecular weight chitosan and CS_H represents high molecular weight chitosan.

Concerning the TSM evaluation (Figure 15.(a)), of the three different chitosan mixtures, films CS_M0.5%-CS_H2% have the lowest solubility values, showing that chitosan molecular weight influence solubility. Additionally, differences in plasticizers were not distinctive, only films CS_M1.5%-CS_H1.5% showed to have higher solubility with GLY addition. Other studies show that an increase in plasticizers concentration leads to an increase in films solubility (174). Due to plasticizers hydrophilic nature, they weaken polymer molecular chains promoting water diffusion into the film matrix which leads to an increase in films solubility in water (175). In tapioca starch based films, for instance, a study showed that a higher GLY concentration in the films increased their solubility, moisture content and swelling degree (176).

Analysing the swelling evaluation results (Figure 15.(b)) it was possible to verify that films prepared with PEG and GLY had a lower swelling degree and that, from the three chitosan mixtures films CS_M0.5%-CS_H2% also demonstrated a lower swelling degree. It is important to determine biodegradable films solubility and swelling degree in order to understand the water resistance of the films and its potential as a packaging material. Generally, films applied in food packaging are required to have good water insolubility in order to preserve food products, especially in humid conditions (135).

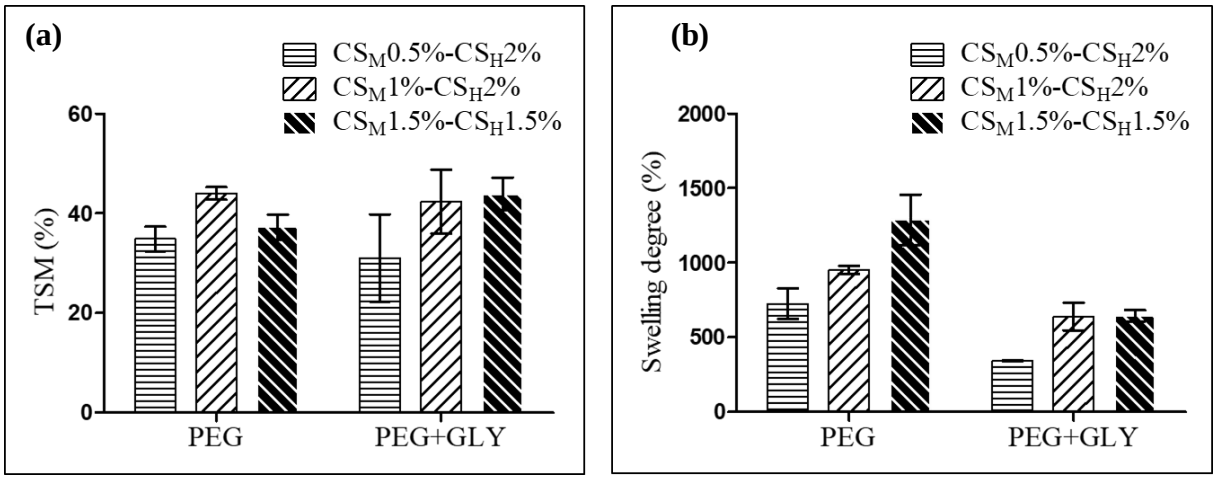

Figure 15. (a) Total Soluble Matter (%) of chitosan films; (b) Swelling degree (%) of chitosan films. Experiments were performed in triplicate.

Water contact angle is a good indicator of films wettability degree since it is known that a higher water contact angle corresponds to a lower wettability degree. In Figure 16 it is possible to observe the water contact angle measurements obtained from the different film preparations. It is possible to observe that films only prepared with PEG have slightly higher contact angle values, while films with both plasticizers ($CS_M1\%$-$CS_H2\%$ $^{PEG+GLY}$ and $CS_M1.5\%$-$CS_H1.5\%$ $^{PEG+GLY}$) exhibit lower contact angle values. Other studies have also shown an effect of GLY on the wettability due to its hydrophilic nature (133). These results were also in agreement with the moisture content evaluation since, an addition of GLY to films led to an increase in their moisture content and a decrease in contact angles showing a lower potential for films to resist water and therefore to be more hydrophilic.

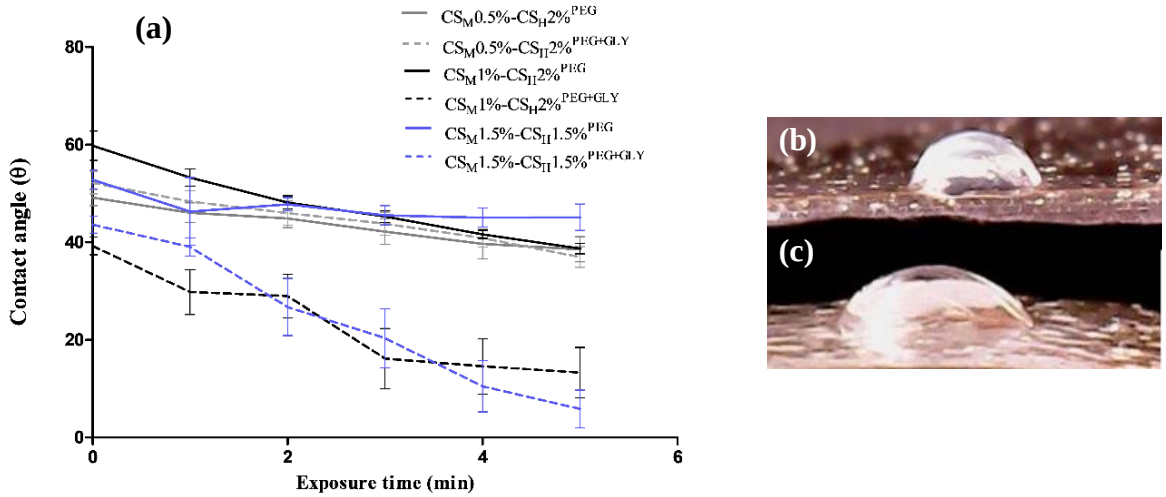

Figure 16. (a) Water contact angle (Θ) from 6 different chitosan films recorded every minute for 5 min. All results are presented as average of 8 replicate experiments and respective standard deviation (SD); Images of the water drops in films without fresh cranberry (FB) extracts incorporations at t=0 min:(b) $CS_M1.5\%$-$CS_H1.5\%^{PEG}$; (c) $CS_M0.5\%$-$CS_H2\%^{PEG+GLY}$.

Once the antimicrobial activity of cranberry extracts was confirmed, it was necessary to assess if this property was still present after extracts incorporation into films. Since FB and PB extracts presented the higher antimicrobial results, films incorporated with these two cranberry

sources (FB and PB) were evaluated trough agar disk diffusion method. All film chitosan mixtures (CS_M1%-CS_H2%, CS_M0.5%-CS_H2% and CS_M1.5%-CS_H1.5%) with both plasticizers combinations (PEG or PEG and GLY) were assessed with and without extracts incorporation in order to observe if there was any influence in films antimicrobial activity. In Figure 17 and Table 9 are shown the inhibitory effects of cranberry films against two different bacteria (*S. aureus* and *E. coli*). The results showed that all samples tested inhibited activity of both bacteria. It was also possible to observe that there was not loss of antimicrobial activity after cranberry extracts incorporation.

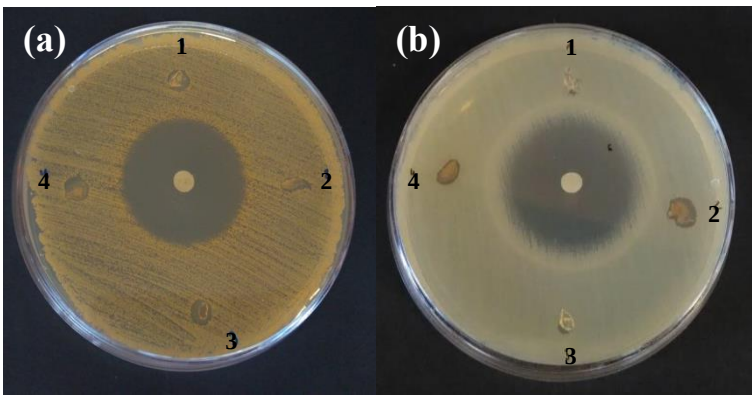

Figure 17. Disk diffusion test with (a) S. aureus and (b) E. coli; 1-film sample with PEG and without extract, 2-film sample with PEG and cranberry extract (100 mg/mL), 3-film sample with PEG+GLY and without extract, 4-film sample with PEG+GLY and fresh cranberry extract (100 mg/mL), 5- 5µg levofloxacin (positive control).

Table 9. Diameter of inhibition zone (mm) of chitosan films prepared with and without fresh (FB) and powdered (PB) cranberry extract (100mg/mL) against S. aureus and E. coli. Results are expressed as mean ± SD and the evaluations on done in triplicate.

Cranberry incorporation	Plasticizer	Chitosan mixture (CS_M% + CS_H%)	Diameter of inhibition zone (mm)	
			S. aureus	*E. coli*
Without	PEG	2+0.5	5.5±0.4	4.1±1.3
		2+1	7.3±0.8	4.8±0.8
		1.5+1.5	5.9±0.7	5.0±0.9
	PEG + GLY	2+0.5	5.0±1.0	4.5±0.8
		2+1	6.8±1.2	5.7±0.6
		1.5+1.5	6.3±0.7	6.5±0.5
PB extract	PEG	2+0.5	6.3±0.9	5.0±0.1
		2+1	6.6±0.3	7.3±0.4
		1.5+1.5	7.0±0.7	7.4±0.4
	PEG + GLY	2+0.5	7.7±1.0	6.5±1.1
		2+1	7.0±0.2	7.1±0.5
		1.5+1.5	7.8±0.6	7.1±1.1
FB extract	PEG	2+0.5	7.3±0.5	4.9±0.8
		2+1	5.2±0.3	7.0±0.1
		1.5+1.5	6.1±0.5	7.5±0.6
	PEG + GLY	2+0.5	6.1±0.1	6.3±1.3
		2+1	5.7±0.1	7.1±0.2
		1.5+1.5	5.7±0.8	6.9±0.7

Films without cranberry extract incorporation showed inhibition halos against both strains. These results can be explained by the presence of chitosan, since pure chitosan films have been shown to have antibacterial activity against several bacteria (177). Noo H. and co-workers (178) studied the antibacterial activity of six chitosan and six chitosan oligomers against four gram-negative and seven gram-positive bacteria and the study demonstrated that chitosan had antimicrobial activity against most tested bacteria including *E. coli* and *S. aureus*. Additionally, chitosan showed stronger bacterial effects against gram-positive bacteria. This higher susceptibility of gram-positive to chitosan films was not observed in the obtained results where the same films demonstrated to formed similar inhibition halos against both *S. aureus* and *E. coli*. Although antimicrobial activity of films incorporated with cranberry extracts have not been studied until now, other studies have already analysed the antibacterial activity of chitosan films incorporated with bioactive compounds. Maqui berries extracts incorporation into chitosan films was demonstrated to improve films antibacterial properties and to delay the oxidation. In this study, from the 8 bacteria tested (*Listeria innocua, Serratia marcescens, Aeromonas hydrophila, Achromobacter denitrificans, Alcaligenes faecalis, P. fluorescens, C. freundii and Shewanella putrefaciens*), films with maqui berry extracts were able to show a inhibitory effect against 7 of the bacteria, whereas, chitosan films without the extracts only showed inhibitory effects against 2 (*S. putrefaciens and P. fluorescens*) (124). In another study, chitosan films were developed with thinned young apple polyphenols and the results revealed that an increase in thinned young apple polyphenols addition to the films increased films antimicrobial properties against the tested bacteria (*E. coli, L. monocytogenes* and *S. aureus)* and moulds (*Colletotrichum fructicola, Botryosphaerial dothidea and Alternariatenuissima*) (83). Results also showed that there were no distinct differences between the plasticizers, since both films prepared with and without GLY presented similar inhibition halos. Likewise, chitosan concentrations did not influence films antimicrobial properties.

It was also interesting to evaluate if differences in chitosan concentrations and plasticizers affected films optical properties. Thus, chitosan films light transmission and opacity were assessed. In Figure 18 are displayed the results of the light transmission (Figure 18.(a)) and opacity values (Figure 18.(b)) from the chitosan films without cranberry extracts. Observing the results it is possible to determine that, for the same chitosan concentration, films with GLY presented a lower light transmission percentage and higher opacity comparatively with films without GLY. Suggesting that GLY slightly decreases films transparency.

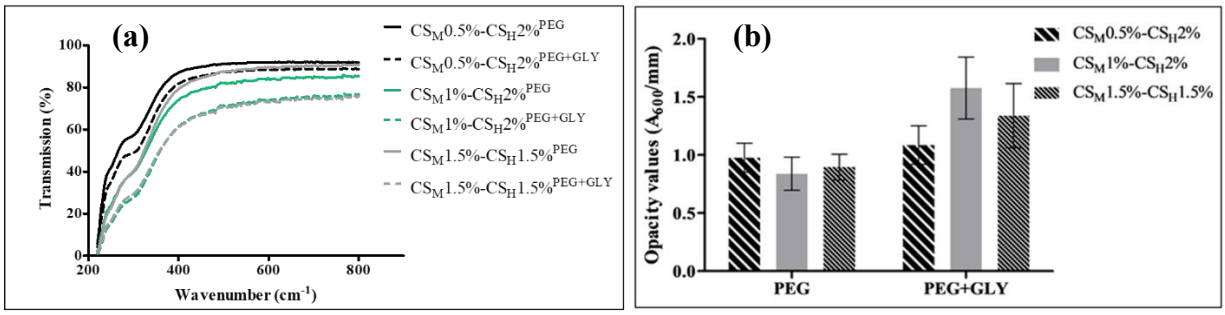

Figure 18. (a) Light transmission (%) of chitosan films without cranberry extracts; (b) Opacity values of films prepared without cranberry extract.

Thus, analysing the total film characterization assays, it is possible to determined that from the three different tested chitosan mixtures (CS_M0.5%-CS_H2%, CS_M1%-CS_H2% and CS_M1.5%-CS_H1.5%) the films CS_M0.5%-CS_H2% demonstrated to be the most satisfactory for use in food packaging due to presenting the lower values of moisture content, solubility and swelling degree, properties essential for preservation of food products. Additionally, as there were no distinct differences between films chitosan concentrations in the antimicrobial activity and optical properties results films CS_M0.5%-CS_H2% ^{PEG} and CS_M0.5%-CS_H2% $^{PEG+GLY}$ were selected for further analysis with cranberry extract incorporation.

Experiments proceeded with films CS_M0.5%-CS_H2% ^{PEG} and CS_M0.5%-CS_H2% $^{PEG+GLY}$ with FB extract incorporation and properties such as wettability degree, WVP, oxygen transmission rate and hardness, were evaluated.

The results from contact angle measurements are presented in Figure 19.(a), Figure 19.(b) and Figure 19.(c) .Observing the results, it is noticeable that there were some clear physical changes with cranberry incorporation. Water contact angles decrease distinctly after cranberry incorporation, which was also observed from the water drops images (Figure 16.(b), Figure 16.(c), Figure 19.(a) and Figure 19.(b)). Meaning that the wettability degree is higher for films with cranberry extracts**.** These results are in agreement with other studies which hypothesised that some hydrophilic groups of polyphenols interact with water molecules and improve film matrix ability to bind with water, making films more hydrophilic (83). The decrease in surface hydrophobicity by cranberry extracts incorporation can affect the initial bacteria attachment and consequently interfere with further biofilm formation.

After cranberry extracts incorporation, the films exhibited a red colouring and the visual observation was confirmed by the significantly increase in the opacity values (Figure 19.(d)) compared to the values from films without the extract. A reduction in the light transmission was also observed once the extracts were incorporated (Figure 19.(e)). This property is valuable in food packaging, since it protects foods from visible light and UV radiation, allowing for a protection against oxidative deterioration preventing films from nutrition loss, discoloration and flavour alterations (167,173).

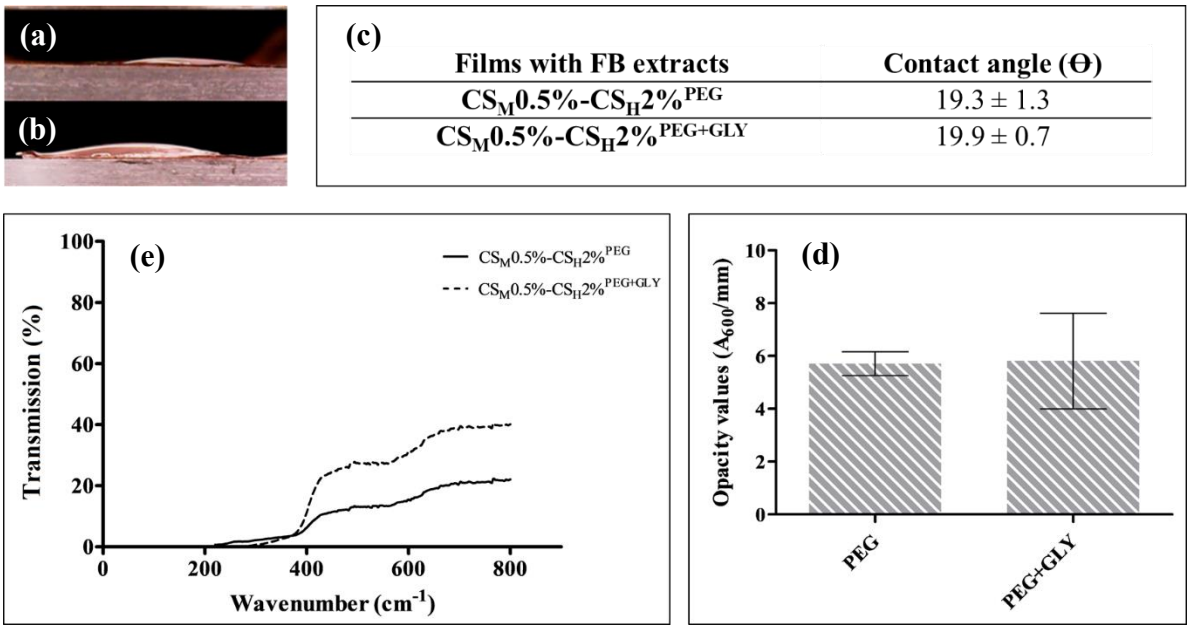

Figure 19. Images of the water drops in films with fresh cranberry (FB) extracts incorporation (100 mg/mL) at t=0 min: (a) CS_M1.5%-CS_H1.5%^{PEG} and (b) CS_M0.5%-CS_H2%$^{PEG+GLY}$; (c) Water contact angle (Θ) from chitosan films incorporated with FB extracts (100mg/mL); (d) Opacity values of films prepared with FB extract (100mg/mL); (e) Light transmission (%) of chitosan films with FB extracts (100mg/mL).

WVP and oxygen transmission rate are important properties in food packaging materials to determine food product shelf-life. It is important for a food packaging material to control moisture transfer. In Table 10 and Table 11 are shown respectively the WVP and the oxygen transmission results from the selected films (CS_M0.5%-CS_H2% ^{PEG} and CS_M0.5%-CS_H2% $^{PEG+GLY}$). From the VWP results it is possible to observe that films with and without GLY presented similar results and that after FB extracts incorporation, permeability did not considerably differ from films without the extract. Some studies suggest that polyphenolic incorporation in biopolymer films decreases films WVP since polyphenolic compounds may interact with the chitosan network limiting availability of hydrogen groups to bond with water (125,180). Siripatrawan and co-workers (181) also reported that films prepared with propolis, a natural resinous substance rich in polyphenols, had lower WVP results comparatively with chitosan films without the substance.

Thus, in this work, it was expected a decrease in WVP since extracts are composed by various phenolic compounds. However, although it is possible to observe a slightly decrease in WVP values for CS_M0.5%-CS_H2%^{PEG} films after cranberry extracts, the same was not observed for films CS_M0.5%-CS_H2% $^{PEG+GLY}$. This can possibly be explained by the low concentration of FB extracts in the films that will not cause any change in the film matrix. The influence of concentration of the extracts on WVP of chitosan films was also observed in another study (37) where the decrease in WVP was only observed at high concentrations of a green tea extract. Specifically, as the concentration of green tea extract increased from 0 to 20% (w/V), the permeability coefficient decreased from 0.256 ± 0.023 to 0.087 ± 0.012 g.mm.m^{-2}.d^{-1}.kPa^{-1} (180). However, the chitosan films in this study reported higher water vapour barrier properties comparatively with results demonstrated in other studies (4.13 × 10^{-11} ± 0.13 mol.m/m^{2}s.Pa.),

which can derived from differences in plasticizers concentration, chitosan molecular weight and concentration and other aspects (135).

Table 10. Water vapour permeability (WVP) (mol.m/m^{2}s.Pa) for selected films, with and without fresh cranberry extract (100 mg/mL). Results are expressed as mean and the evaluations on done in triplicate.

FB extract	Film sample	WVP 10^{-12} (mol.m/m^{2}s.Pa)
Without	$CS_M0.5\%$-$CS_H2\%^{PEG}$	4.87
	$CS_M0.5\%$-$CS_H2\%^{PEG+GLY}$	3.33
With (100 mg/mL)	$CS_M0.5\%$-$CS_H2\%^{PEG}$	3.71
	$CS_M0.5\%$-$CS_H2\%^{PEG+GLY}$	4.00

Concerning the oxygen transmission results the films with FB extracts that were added with GLY had a slightly higher transmission rate (Table 11). This increase in oxygen transmission rate by GLY addition has also been reported in other studies as GLY has the capacity to diffuse oxygen molecules trough the film by promoting chain mobility (133). Usually, polysaccharide films have good gas barrier properties. In Ferreira and co-workers study, for instance, chitosan films revealed a permeability of $2.35 \times 10^{-16} \pm 0.48$ mol.m/m^{2}s.Pa (135). Comparatively with other studies, chitosan films prepared in this work showed lower oxygen permeability barriers, which can have been affected by FB extracts incorporation into the film matrix. A proper oxygen barrier is important for prevention of oxidation, allowing for an improved food quality and extended shelf-life (182). Comparing the chitosan films oxygen permeability with synthetic polymers commonly used in packaging material, such as LPDE (10.03×10^{-16} mol.m/m^{2}s.Pa), the values indicate to be similar (183). Although the presence of FB extracts in the chitosan film can be responsible for affecting films oxygen permeability, the advantage of developing chitosan films with cranberry incorporation is that the prevention of oxidation is still possible since FB extracts were revealed to have antioxidant activity (section 4.2.3.), therefore generating films with better antioxidants properties in comparison with common synthetic materials such as LPDE.

Table 11. Oxygen transmission rate (mol.m/m^{2}s.Pa) for selected films with fresh cranberry extract incorporation (100 mg/mL). Results are expressed as mean ± SD and the evaluations on done in triplicate.

Film sample	Oxygen transmission rate 10^{-16} (mol.m/m^{2}s.Pa)
$CS_M0.5\%$-$CS_H2\%^{PEG}$	9.38 ± 0.36
$CS_M0.5\%$-$CS_H2\%^{PEG+GLY}$	10.37 ± 0.53

Films mechanical properties were also analysed through nanoindentation hardness testing. The aim was to assess if there were significant differences after cranberry extract incorporation into the films.

Results (Figure 20.(a)) demonstrate that GLY addition decreased films hardness for films without cranberry extract. When cranberry extract was incorporated into the films,

hardness values reduced considerably in both chitosan preparations. Between the films incorporated with cranberry extract, however, results seem to be very similar. These results are in conformity with the visual observation that films with cranberry extracts incorporation presented more elasticity and were less brittle. Figure 20.(b) shows the image of a nanoidentation on film $CS_M0.5\%\text{-}CS_H2\%^{PEG+GLY}$ with cranberry extract incorporation. The hardness decrease values of chitosan films after GLY addition are also in agreement with other studies. Plasticizers, such as GLY, are generally added to increase films flexibility and decrease films stiffness, since it reduces chain-to-chain intermolecular interactions in the polymers (184). However, there are not considerable studies regarding the hardness values of chitosan films after berry extracts incorporation to appropriately compare the results. In a study with cross-linked chitosan films, the hardness values from nanoindentation were 1.6 GPa (185). These values are similar to the results obtained in this work for films incorporated with FB extracts, respectively 1.14 ± 0.55 GPa for films without GLY and 1.22 ± 0.24 GPa for films with GLY. However, mechanical properties can differ by the type and concentration of the antimicrobial compound incorporated into the film as well as the polymer used. For instance, Fahim and co-workers reported that the concentration of nanofillers addition to chitosan films (graphene and fullerene) enhanced directly the hardness of non-crossed-linked and crosslinked chitosan films (186).

(a)

FB extract	Film sample	Hardness values (GPa)
Without	$CS_M0.5\%\text{-}CS_H2\%^{PEG}$	7.81 ± 1.58
	$CS_M0.5\%\text{-}CS_H2\%^{PEG+GLY}$	3.98 ± 0.87
With (100 mg/mL)	$CS_M0.5\%\text{-}CS_H2\%^{PEG}$	1.14 ± 0.55
	$CS_M0.5\%\text{-}CS_H2\%^{PEG+GLY}$	1.22 ± 0.24

(b)

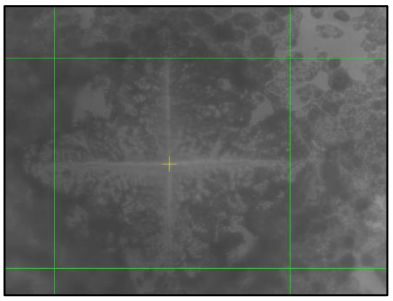

Figure 20. (a) Hardness values (GPa) for selected films, with and without fresh cranberry extract (100mg/mL); (b) Image of a residual imprint on film $CS_M0.5\%\text{-}CS_H2\%^{PEG+GLY}$ with fresh cranberry extract produced by a nanoidenter. Results are expressed as mean ± SD and the evaluations on done in triplicate.

Films mechanical properties are important in food packaging for food integrity protection. Films need to be resistant and flexible to completely protect food during storage. However, there are still some challenges in the development of bio-based materials with good mechanical and physicochemical properties. With the development of this study, chitosan films properties, namely films permeability, could be further developed with the addition of supplementary compounds and with the addition of a higher concentration of cranberry extracts to test a possible decrease in WVP as observed in other studies.

4.5. Antibiofilm properties of films

The biofilm inhibition of the films was evaluated by plate colony counting method against *E. coli* and *S. aureus.* In Figure 21 are exhibited the results from colony growth of *S. aureus* (Figure 21.(a)) and *E. coli* (Figure 21.(b)) on films prepared without cranberry extracts and with incorporation of 100 mg/mL and 200 mg/mL of cranberry extract.

In both *E. coli* and *S. aureus* results, there is an evident decrease in colonies growth after cranberry extract incorporation, especially at 200 mg/mL concentration of the extract. Additionally, at 200 mg/mL the films prepared with GLY showed lower growth. Interestingly, films without cranberry incorporation also inhibit colony formation comparatively with the control group for both strains, which was expected since pure chitosan films also showed antimicrobial properties in the previous described microbiological analysis (section 4.4.). In both strains a reduction of more than 2 log units could be observed with cranberry film samples when compared to the positive control.

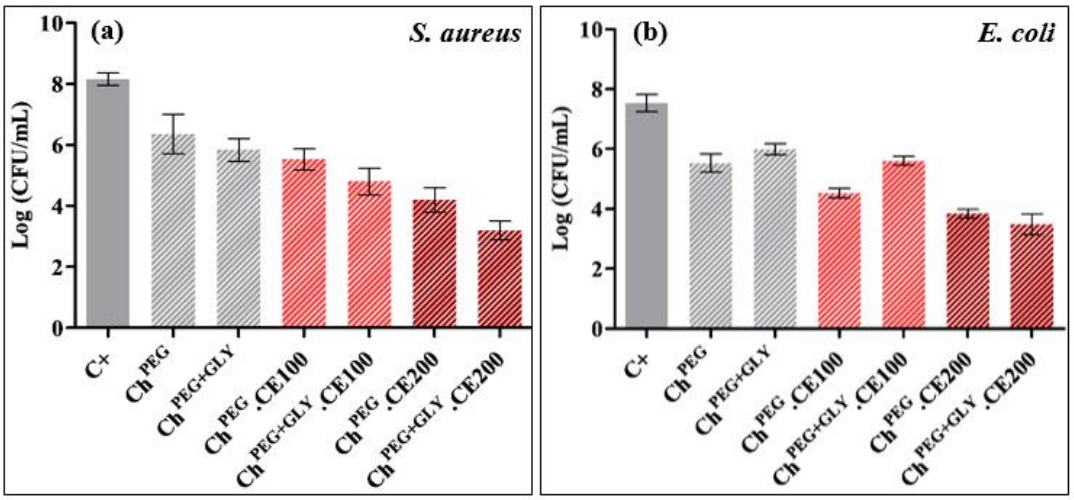

Figure 21. (a) S. aureus and (b) E. coli colonies formation units (CFU) counting for growth on films without cranberry extract (Ch), with 100 mg/mL of cranberry extract (Ch.CE100) and with 200 mg/mL of cranberry extract (Ch.CE200). Ch^{PEG} represents chitosan films (CS_M0.5%-CS_H2%) prepared only with PEG addition as plasticizer and $Ch^{PEG+GLY}$ represents chitosan films with PEG and GLY as plasticizers. C^+ represents the colony formation of S. aureus or E. coli in the tested surface without the film presence.

Cranberry extract biofilm inhibition had already been demonstrated trough the MBIC analysis (section 4.2.2.), however, it was important to understand if that property was maintained after cranberry extracts incorporation into the films. Additionally, GLY may also contribute for the biofilm inhibition, especially against *S. aureus*, which can be associated to the plasticizers hydrophilic nature that increased films wettability degree. From the contact angle measurements exhibited above (Figure 19), cranberry extracts incorporation also increased films surface wettability degree, this property combined with the proved cranberry extracts antimicrobial activity could have led to decrease in bacteria initial attachment and therefore limited biofilm formation, as described in other studies done in polymeric films with natural antimicrobial compounds addition (187). Some studies have already demonstrated the antibiofilm properties of berries extracts against several strains, such as *E. coli, P. aeruginosa,*

S. aureus, *P. mirabilis* and *A. baumannii* (77,81). Proanthocyanidins, phenolic compounds present in the cranberry fruit, have been commonly implicated in cranberry antibiofilm properties, since proanthocyanidins demonstrated to prevent bacterial adhesion (188).

To further confirm these results, *S. aureus* biofilm inhibition was also assessed by SEM (Figure 22). It was observed that films prepared without berry extract (a) and (d), presented a high *S. aureus* colonies formation. The images also showed that the *S. aureus* colonies formation decreased in films incorporated with berry extracts, especially in films with higher berry extracts concentration, (Figure 22.(c) and Figure 22.(f)) that presented absence of colonies formation. Comparing SEM images of films (a), (b) and (c), prepared without GLY, with films (d), (e) and (f) prepared with GLY it was possible to observe that there were no distinctly differences between them.

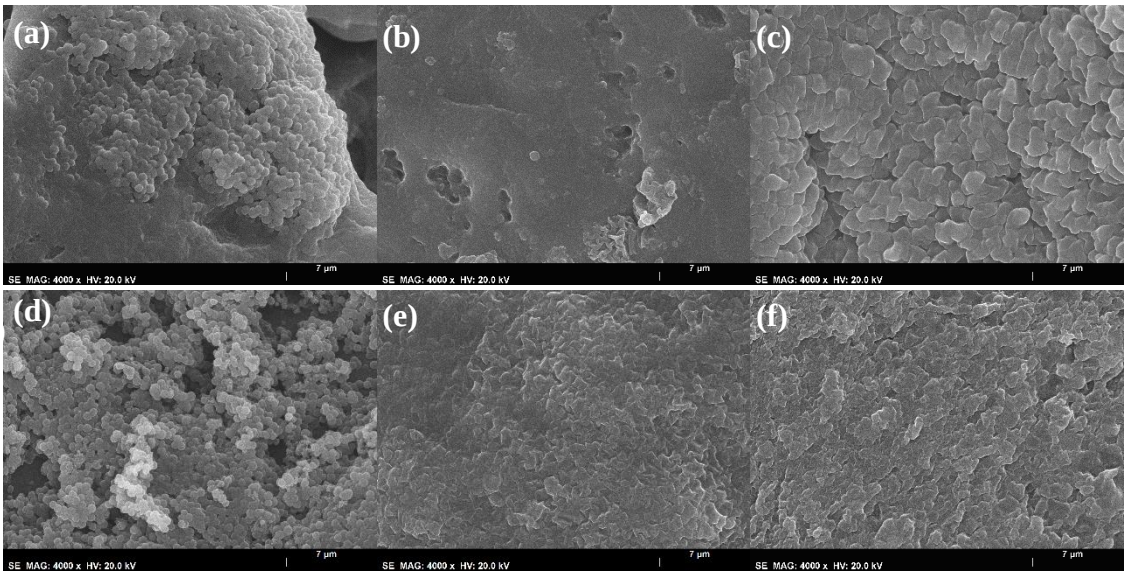

Figure 22. Scanning Electron Microscopy (SEM) images of biofilm formation by S. aureus on films prepared: (a) and (d)- without fresh cranberry extract; (b) and (c) - films with 100 mg/mL of fresh cranberry extract; (c) and (f) – films with 200 mg/mL of fresh cranberry extract. (a), (b) and (c) - films prepared without glycerol; (d), (e) and (f) - films prepared with glycerol. Magnification of 4000 x.

These results are in agreement with the ones from the plate colony counting analysis where an incorporation of cranberry extracts into the films promoted a higher inhibition of biofilm formation (Figure 22.(a)). Bioactive phenolic compounds from fruit extracts have also been tested in other studies against several strains. In Riaz and co-workers study, chitosan films were incorporated with different concentrations of apple peel polyphenols ranging from 0.25 to 1%. In this study, chitosan films also had inhibition effects, however, with polyphenols incorporation films demonstrate a higher concentration dependent antimicrobial activity against the tested bacteria (*B. cereus, E. coli, S. typhimurium and S. aureus*) in comparison with the control films (189). In Yuan and co-workers studies chitosan-based films antimicrobial activity was tested with carvacrol incorporation (10 mg/mL), with pomegranate peel extract incorporation (10 mg/mL) and with a combination of both (10 mg/mL each). Interestingly, all films demonstrated antimicrobial activity against *E. coli* and *S. aureus* except films prepared only with pomegranate peel extract incorporation. Additionally, films with incorporation of both compounds had a synergic action, demonstrating a higher inhibition activity against *S.*

aureus (132). Other studies have also reported antibacterial effects of chitosan based films with polyphenols, such as turmeric extracts (190) and maqui berry (124). Furthermore, reduction of biofilm formation have also been observed by incorporation of antimicrobial agents in polymeric films. In Nostro and co-workers study, polyethylene-co-vinylacetate films with different concentrations of essential oil constituents, carvacrol or cinnamaldehyde, showed to reduce considerably biofilm formation on polymeric film surface in comparison with the pure control against *E. coli* and *S. aureus* biomass (187).

In this study, it was not only possible to demonstrated films antimicrobial properties against the tested strains but it was also possible to prove that films prepared with cranberry extracts incorporation inhibited biofilm formation on films surface. This study brings a new alternative for the development of antimicrobial packaging material that had not yet been observed in previous studies, by exploiting cranberry extracts antimicrobial properties.

Chapter 5

Conclusion and future perspectives

Currently, the formation of biofilms in the food industry by spoilage and especially with pathogenic bacteria continues to be a problem that conducts to food waste and puts in risk the consumer's health. Several microorganism species are able to adhere to food contact surfaces, multiply and form biofilm with improved resistance to environmental conditions and treatments. The use of sanitizers seems to not be enough to control this problem, thus, there is a need for the development of novel strategies that enable the prevention and control of biofilm formation. The search for efficient food packaging is important for food protection against bacteria and since consumers demand is now directed for the use of more natural and sustainable alternatives the development of food packaging from biodegradable polymers incorporated with natural antimicrobials poses an interesting potential strategy for the food industry. Due to its high content in bioactive compounds, cranberries have already proven to have antioxidant and antimicrobial properties against pathogenic bacteria showing a potential to act as food preservatives (191). The aim of this research was the functionalization of a chitosan film, intended for food packaging, with cranberry extracts incorporation. The research was therefore mainly focused in three parts: i) Cranberry extraction and characterization; ii) Development of chitosan films and iii) Functionalization of chitosan films with cranberry incorporation.

Three different sources of cranberries were tested, respectively powdered (PB), fresh (FB) and dried (DB). After determination of the best extraction methodology to enhance cranberries bioactivity, with application of a 40 mins sonication time, cranberries and cranberries extracts bioactive properties were characterized. Results demonstrated that all three sources of cranberry extracts showed antimicrobial activity against *E. coli* and *S. aureus*. Particularly PB and FB, revealed a bactericidal effect against both species. Moreover, these two samples of cranberries extracts had also antibiofilm activity against both species, demonstrating both a MBIC of 6.3 and 25 mg/mL against *S. aureus* and *E. coli*, respectively. Following, the total phenolic content of extracts was determined. The results revealed that FB and PB had a total phenolic content of respectively 13.2 ± 1.6 and 106.3 mg GA/g BE ± 13.2. DB had the lowest total phenolic content. Additionally, extracts antioxidant activity was tested trough DPPH radical scavenging activity assay and it was possible to determine that all extracts had antioxidant activity in the following order: PB extracts > FB extracts > DB extracts in which PB and FB demonstrated a 50% scavenge capacity respectively at 0.5 and 4.8 mg/mL. Additionally, it was possible to identify the presence of phenolic compounds by LC-MS/MS, specifically isorhamnetin 3-glucoside,

phloridzin, p-hydroxybenzoic acid, epicatechin and chlorogenic acid. It was also detected the possible presence of A-type proacyanidins dimers. As a result, FB and PB extracts were selected for incorporation into the developing films.

After the characterization of cranberry extracts, chitosan-based films were then successfully developed with similar thickness and density between them. It was observed that the chitosan mixtures, respectively CS_M1%-CS_H2%, CS_M0.5%-CS_H2% and CS_M1.5%-CS_H1.5% combined only with plasticizer PEG or PEG and GLY, were most efficient for film formation than chitosan from only one type of molecular weight. Additionally, it was also concluded that from three different tested temperatures, respectively 25 ºC, 36 ºC and 50 ºC, the best drying method that kept films integrity was 25 ºC for 24 h. Regarding the physical properties analysis, from the three tested mixtures, respectively CS_M0.5%-CS_H2%, CS_M1%-CS_H2% and CS_M1.5%-CS_H1.5%, films CS_M0.5%-CS_H2% were considered to be more suitable for implementation in food packaging with lower moisture content, solubility values and swelling degree. Therefore, CS_M0.5%-CS_H2% ^{PEG} and CS_M0.5%-CS_H2% $^{PEG+GLY}$ were selected for further analysis since reduction of water exchange between the product and the environment is an important characteristic of food packaging.

The incorporation of cranberry extracts into chitosan films altered films properties. After cranberry incorporation films demonstrated to develop a more hydrophilic surface. Furthermore, from the agar disk diffusion evaluation it was possible to prove that films prepared with the cranberry extracts maintained the antimicrobial activity against *E. coli* and *S. aureus,* improved films light-barrier properties and decreased chitosan films hardness. From the antibiofilm evaluations, it was also possible to demonstrate that films with cranberry extracts presented a reduction of biofilm formation on the tested surface against *E. coli* and *S. aureus* and that antibiofilm properties improved with the increase in the cranberry extract concentration in the chitosan films. A reduction of more than 2 log units could be observed when comparing cranberry extract films with the positive control.

To conclude, the results presented in this study, revealed that it was possible to develop chitosan films incorporated with cranberry extracts with antimicrobial properties and antibiofilm properties against *E. coli* and *S. aureus,* both pathogens commonly related to foodborne outbreaks. Furthermore, studies show that these properties may be associated to the bioactive compounds present in cranberry extracts, especially proanthocyanidins. The development of chitosan films with cranberry extracts may present a contribution for the food packaging industry intended for the development of more packaging options that enhance food products safety, quality and shelf-life extension.

For future work, it would be interesting to combine cranberry extracts with other antimicrobial compounds, such as biosurfactants, in order to potentiate the antimicrobial properties of the cranberry films. Furthermore, to potentiate the antimicrobial properties, it would also be interesting to isolate the compounds with antimicrobial activity previously identified in cranberry extracts and to directly incorporate these compounds into the chitosan films. Films WVP and gas barrier properties should also be improved so that films can be used as efficient food packaging materials. This could be achieved, for instance, by combination of chitosan films with nanoparticles that have been demonstrated to improve water barrier and gas

properties of the materials. Mechanical properties of chitosan films could also be enhanced by blending chitosan with other polysaccharides. Antioxidants are used as food additives in the food industry to prolong products shelf-life, therefore, the antioxidant capacity of the extracts is an important property since, when incorporated into films, can lead to film degradation prevention and preserve food products preservation, therefore, additional studies of films with cranberry extracts incorporation should be made to test this concept. Moreover, to also support the antibiofilm and antimicrobial results presented in this study, films direct influence on food products should also be tested. In the future, more studies should similarly be done regarding films stability, biodegradability, biocompatibility and safety regarding application as food packaging material.

References

1. Flemming HC, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S. Biofilms: An emergent form of bacterial life. Nat Rev Microbiol. 2016;14(9):563–75. doi: http://dx.doi.org/10.1038/nrmicro.2016.94

2. Rabin N, Zheng Y, Opoku-Temeng C, Du Y, Bonsu E, Sintim HO. Biofilm formation mechanisms and targets for developing antibiofilm agents. Future Med Chem. 2015;7(4):493–512. doi: 10.4155/fmc.15.6

3. Van Houdt R, Michiels CW. Biofilm formation and the food industry, a focus on the bacterial outer surface. J Appl Microbiol. 2010;109(4):1117–31. doi: 10.1111/j.1365-2672.2010.04756.x

4. Palmer J, Flint S, Brooks J. Bacterial cell attachment, the beginning of a biofilm. J Ind Microbiol Biotechnol. 2007;34(9):577–88. doi: 10.4155/fmc.15.6

5. Flemming HC, Wingender J. The biofilm matrix. Nat Rev Microbiol 2010;8(9):623–33. doi: http://dx.doi.org/10.1038/nrmicro2415

6. Vu B, Chen M, Crawford RJ, Ivanova EP. Bacterial extracellular polysaccharides involved in biofilm formation. Molecules. 2009;14(7):2535–54. doi: 10.3390/molecules14072535

7. Mah TFC, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 2001;9(1):34–9. doi: 10.1016/S0966-842X(00)01913-2

8. Giaouris E, Heir E, Desvaux M, Hébraud M, Møretrø T, Langsrud S. Intra- and inter-species interactions within biofilms of important foodborne bacterial pathogens. Front Microbiol. 2015;6:1–26. doi: 10.3389/fmicb.2015.00841

9. European Food Safety Authority. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017. EFSA J . 2018;16(12).

10. Joseph B, Otta SK, Karunasagar I, Karunasagar I. Biofilm formation by Salmonella spp. On food contact surfaces and their sensitivity to sanitizers. Int J Food Microbiol. 2001;64(3):367–72. doi: 10.1016/S0168-1605(00)00466-9

11. Chylkova T, Cadena M, Ferreiro A, Pitesky M. Susceptibility of salmonella biofilm and planktonic bacteria to common disinfectant agents used in poultry processing. J Food Prot. 2017;80(7):1072–9. doi: 10.4315/0362-028X.JFP-16-393

12. Borges KA, Furian TQ, de Souza SN, Menezes R, de Lima DA, Fortes FBB. Biofilm formation by Salmonella Enteritidis and Salmonella Typhimurium isolated from avian sources is partially related with their in vivo pathogenicity. Microb Pathog. 2018;118:238–41. doi: 10.1016/S0168-1605(00)00466-9

13. Silva S, Teixeira P, Oliveira R, Azeredo J. Adhesion to and viability of Listeria monocytogenes on food contact surfaces. J Food Prot. 2008;71(7):1379–85. doi: 10.4315/0362-028X-71.7.1379

14. Newell DG, Koopmans M, Verhoef L, Duizer E, Aidara-Kane A, Sprong H. Food-borne diseases — The challenges of 20 years ago still persist while new ones continue to emerge. Int J Food Microbiol. 2010;139:S3–15. doi: 10.1016/j.ijfoodmicro.2010.01.021

15. Carpentier B, Cerf O. Review - Persistence of Listeria monocytogenes in food industry equipment and premises. Int J Food Microbiol. 2011;145(1):1–8. doi: 10.1016/j.ijfoodmicro.2011.01.005

16. Pan Y, Breidt F, Kathariou S. Resistance of Listeria monocytogenes biofilms to sanitizing agents in a simulated food processing environment. Appl Environ Microbiol. 2006;72(12):7711–7. doi: 10.1128/AEM.01065-06

17. Brauge T, Sadovskaya I, Faille C, Benezech T, Maes E, Guerardel Y. Teichoic acid is the major polysaccharide present in the Listeria monocytogenes biofilm matrix. FEMS Microbiol Lett. 2015;363(2):1–7. doi: 10.1093/femsle/fnv229

18. Zhu X, Liu D, Singh AK, Drolia R, Bai X, Tenguria S. Tunicamycin mediated inhibition of wall teichoic acid affects Staphylococcus aureus and Listeria monocytogenes cell morphology, biofilm formation and virulence. Front Microbiol. 2018;9:1–18. doi: 10.3389/fmicb.2018.01352

19. Etcheverría AI, Padola NL. Shiga toxin-producing Escherichia coli: Factors involved in virulence and cattle colonization. Virulence. 2013;4(5):366–72. doi: 10.4161/viru.24642

20. Dourou D, Beauchamp CS, Yoon Y, Geornaras I, Belk KE, Smith GC. Attachment and biofilm formation by Escherichia coli O157:H7 at different temperatures, on various food-contact surfaces encountered in beef processing. Int J Food Microbiol. 2011;149(3):262–8. doi: 10.1016/j.ijfoodmicro.2011.07.004

21. Luna-Gierke RE, Griffin PM, Gould LH, Herman K, Bopp CA, Strockbine N. Outbreaks of non-O157 Shiga toxin-producing Escherichia coli infection: USA. Epidemiol Infect. 2014;142(11):2270–80. doi: 10.1017/S0950268813003233

22. Wang J, Stanford K, McAllister TA, Johnson RP, Chen J, Hou H. Biofilm Formation, Virulence Gene Profiles, and Antimicrobial Resistance of Nine Serogroups of Non-O157 Shiga Toxin-Producing Escherichia coli. Foodborne Pathog Dis. 2016;13(6):316–24. doi: 10.1089/fpd.2015.2099

23. Ma Z, Bumunang EW, Stanford K, Bie X, Niu YD, McAllister TA. Biofilm Formation by Shiga Toxin-Producing Escherichia coli on Stainless Steel Coupons as Affected by Temperature and Incubation Time. Microorganisms. 2019;7(4):95. doi: 10.3390/microorganisms7040095

24. Otto M. Staphylococcal Infections: Mechanisms of Biofilm Maturation and Detachment as Critical Determinants of Pathogenicity. Annu Rev Med. 2013;64(1):175–88. doi: 10.1146/annurev-med-042711-140023

25. Chaibenjawong P, Foster SJ. Desiccation tolerance in Staphylococcus aureus. Arch Microbiol. 2011;193(2):125–35. doi: 10.1007/s00203-010-0653-x

26. Heilmann C. Adhesion Mechanisms of Staphylococci.. 2011. p. 105–23. doi: 10.1007/978-94-007-0940-9_7

27. Slany M, Oppelt J, Cincarova L. Formation of Staphylococcus aureus biofilm in the presence of sublethal concentrations of disinfectants studied via a transcriptomic analysis using transcriptome sequencing (RNA-seq). Appl Environ Microbiol. 2017;83(24):1–13. doi: 10.1128/AEM.01643-17

28. Oulahal N, Brice W, Martial A, Degraeve P. Quantitative analysis of survival of Staphylococcus aureus or Listeria innocua on two types of surfaces: Polypropylene and

stainless steel in contact with three different dairy products. Food Control. 2008;19(2):178–85. doi: 10.1016/j.foodcont.2007.03.006

29. Di Ciccio P, Vergara A, Festino AR, Paludi D, Zanardi E, Ghidini S. Biofilm formation by Staphylococcus aureus on food contact surfaces: Relationship with temperature and cell surface hydrophobicity. Food Control. 2015;50:930–6. doi: 10.1016/j.foodcont.2014.10.048

30. Vázquez-Sánchez D, Habimana O, Holck A. Impact of food-related environmental factors on the adherence and biofilm formation of natural staphylococcus aureus isolates. Curr Microbiol. 2013;66(2):110–21. doi: 10.1007/s00284-012-0247-8

31. Liu W, Røder HL, Madsen JS, Bjarnsholt T, Sørensen SJ, Burmølle M. Interspecific bacterial interactions are reflected in multispecies biofilm spatial organization. Front Microbiol. 2016;7:1–8. doi: 10.3389/fmicb.2016.01366

32. Yuan L, Hansen MF, Røder HL, Wang N, Burmølle M, He G. Mixed-species biofilms in the food industry: Current knowledge and novel control strategies. Crit Rev Food Sci Nutr. 2019;0(0):1–17.doi: 10.1080/10408398.2019.1632790

33. Wang R, Kalchayanand N, Schmidt JW, Harhay DM. Mixed biofilm formation by Shiga toxin-producing Escherichia coli and Salmonella enterica serovar typhimurium enhanced bacterial resistance to sanitization due to extracellular polymeric substances. J Food Prot. 2013;76(9):1513–22. doi: 10.4315/0362-028X.JFP-13-077

34. Van der Veen S, Abee T. Mixed species biofilms of Listeria monocytogenes and Lactobacillus plantarum show enhanced resistance to benzalkonium chloride and peracetic acid. Int J Food Microbiol. 2011;144(3):421–31. doi: 10.1016/j.ijfoodmicro.2010.10.029

35. Rossi C, Serio A, Chaves-López C, Anniballi F, Auricchio B, Goffredo E, Cenci-Goga B, Lista F, Fillo S, Paparella A . Biofilm formation, pigment production and motility in Pseudomonas spp. isolated from the dairy industry. Food Control. 2018;86:241–8. doi: 10.1016/j.foodcont.2017.11.018

36. Wang H, Qi J, Dong Y, Li Y, Xu X, Zhou G. Characterization of attachment and biofilm formation by meat-borne Enterobacteriaceae strains associated with spoilage. Lwt. 2017;86:399–407. doi: 10.1016/j.lwt.2017.08.025

37. Chen Z, Zhu C, Han Z. Effects of aqueous chlorine dioxide treatment on nutritional components and shelf-life of mulberry fruit (Morus alba L.). J Biosci Bioeng. 2011;111(6):675–81. doi: 10.1016/j.jbiosc.2011.01.010

38. Van Haute S, Tryland I, Escudero C, Vanneste M, Sampers I. Chlorine dioxide as water disinfectant during fresh-cut iceberg lettuce washing: Disinfectant demand, disinfection efficiency, and chlorite formation. LWT - Food Sci Technol. 2017;75:301–4. doi: 10.1016/j.lwt.2016.09.002

39. Maes S, Heyndrickx M, Vackier T, Steenackers H, Verplaetse A, De Reu K. Identification and spoilage potential of the remaining dominant microbiota on food contact surfaces after cleaning and disinfection in different food industries. J Food Prot. 2019;82(2):262–75. doi: 10.4315/0362-028X.JFP-18-226

40. Møretrø T, Schirmer BCT, Heir E, Fagerlund A, Hjemli P, Langsrud S. Tolerance to quaternary ammonium compound disinfectants may enhance growth of Listeria

monocytogenes in the food industry. Int J Food Microbiol. 2017;241:215–24. doi: 10.1016/j.ijfoodmicro.2016.10.025

41. Al-Shabib NA, Husain FM, Ahmed F, Khan RA, Ahmad I, Alsharaeh E, Khan M, Hussain A, Rehman M,Yusuf M, Hassan I, Khan J, Ashraf G, Alsalme A, Al-Ajmi M, Tarasov V, Aliev Gj. Biogenic synthesis of Zinc oxide nanostructures from Nigella sativa seed: Prospective role as food packaging material inhibiting broad-spectrum quorum sensing and biofilm. Sci Rep. 2016;6:1–16. doi: 10.1038/srep36761

42. Balan SS, Kumar CG, Jayalakshmi S. Pontifactin, a new lipopeptide biosurfactant produced by a marine Pontibacter korlensis strain SBK-47: Purification, characterization and its biological evaluation. Process Biochem. 2016;51(12):2198–207. doi: 10.1016/j.procbio.2016.09.009

43. Amrutha B, Sundar K, Shetty PH. Effect of organic acids on biofilm formation and quorum signaling of pathogens from fresh fruits and vegetables. Microb Pathog. 2017;111:156–62. doi: 10.1016/j.micpath.2017.08.042

44. Annous BA, Fratamico PM, Smith JL. Quorum sensing in biofilms: Why bacteria behave the way they do. J Food Sci. 2009;74(1). doi: 10.1111/j.1750-3841.2008.01022.x

45. Almasoud A, Hettiarachchy N, Rayaprolu S, Babu D, Kwon YM, Mauromoustakos A. Inhibitory effects of lactic and malic organic acids on autoinducer type 2 (AI-2) quorum sensing of Escherichia coli O157:H7 and Salmonella Typhimurium. LWT - Food Sci Technol. 2016;66:560–4. doi: 10.1016/j.lwt.2015.11.013

46. Zhang Y, Kong J, Xie Y, Guo Y, Cheng Y, Qian H, Yao W. Essential oil components inhibit biofilm formation in Erwinia carotovora and Pseudomonas fluorescens via anti-quorum sensing activity. LWT - Food Sci Technol. 2018;92(1800):133–9. doi: 10.1016/j.lwt.2018.02.027

47. Tapia-Rodriguez MR, Hernandez-Mendoza A, Gonzalez-Aguilar GA, Martinez-Tellez MA, Martins CM, Ayala-Zavala JF. Carvacrol as potential quorum sensing inhibitor of Pseudomonas aeruginosa and biofilm production on stainless steel surfaces. Food Control. 2017;75:255–61. doi: 10.1016/j.foodcont.2016.12.014

48. Myszka K, Schmidt MT, Majcher M, Juzwa W, Olkowicz M, Czaczyk K. Inhibition of quorum sensing-related biofilm of Pseudomonas fluorescens KM121 by Thymus vulgare essential oil and its major bioactive compounds. Int Biodeterior Biodegrad. 2016;114:252–9. doi: 10.1016/j.ibiod.2016.07.006

49. Martinez-Gutierrez F, Boegli L, Agostinho A, Sánchez EM, Bach H, Ruiz F, James G. Anti-biofilm activity of silver nanoparticles against different microorganisms. Biofouling. 2013;29(6):651–60. doi: 10.1080/08927014.2013.794225

50. Velázquez-Velázquez JL, Santos-Flores A, Araujo-Meléndez J, Sánchez-Sánchez R, Velasquillo C, González C, Martínez-Castañon G, Martinez-Gutierrez F. Anti-biofilm and cytotoxicity activity of impregnated dressings with silver nanoparticles. Materials Science and Engineering. 2015; 49: 604-611. doi: 10.1016/j.msec.2014.12.084

51. Kubacka A, Diez MS, Rojo D, Bargiela R, Ciordia S, Zapico I, Albar, Juan P. Barbas C, Martins dos Santos Vi, Fernández-García M, Ferrer M.Understanding the antimicrobial mechanism of TiO 2 -based nanocomposite films in a pathogenic bacterium. Sci Rep. 2014;4:1–9. doi: 10.1038/srep04134

52. Nica IC, Stan MS, Popa M, Chifiriuc MC, Lazar V, Pircalabioru GG, Dumitrescu I. Ignat M, Feder M, Tanase L, Mercioniu I, Diamandescu L, Dinischiotu A. Interaction of new-developed tio2-based photocatalytic nanoparticles with pathogenic microorganisms and human dermal and pulmonary fibroblasts. Int J Mol Sci. 2017;18(2). doi: 10.3390/ijms18020249

53. Gkana EN, Doulgeraki AI, Chorianopoulos NG, Nychas GJE. Anti-adhesion and anti-biofilm potential of organosilane nanoparticles against foodborne pathogens. Front Microbiol. 2017;8:1–9. doi: 10.3389/fmicb.2017.01295

54. Dimitrijevic M, Karabasil N, Boskovic M, Teodorovic V, Vasilev D, Djordjevic V. Safety Aspects of Nanotechnology Applications in Food Packaging. Procedia Food Sci . 2015;5:57–60. doi: http://dx.doi.org/10.1016/j.profoo.2015.09.015

55. Hoseinnejad M, Jafari SM, Katouzian I. Inorganic and metal nanoparticles and their antimicrobial activity in food packaging applications. Crit Rev Microbioal. 2018;44(2):161–81. doi: 10.1080/1040841X.2017.1332001

56. Cameotra SS, Makkar RS. Recent applications of biosurfactants as biological and immunological molecules. Curr Opin Microbiol. 2004;7(3):262–6. doi: 10.1016/j.mib.2004.04.006

57. Coronel-León J, Marqués AM, Bastida J, Manresa A. Optimizing the production of the biosurfactant lichenysin and its application in biofilm control. J Appl Microbiol. 2016;120(1):99–111. doi: 10.1111/jam.12992

58. Sarwar A, Brader G, Corretto E, Aleti G, Abaidullah M, Sessitsch A, Hafeez G. Qualitative analysis of biosurfactants from Bacillus species exhibiting antifungal activity. PLoS One. 2018;13(6):1–15. doi: 10.1371/journal.pone.0198107

59. Kuyukina MS, Ivshina IB, Korshunova IO, Stukova GI, Krivoruchko A V. Diverse effects of a biosurfactant from Rhodococcus ruber IEGM 231 on the adhesion of resting and growing bacteria to polystyrene. AMB Express. 2016;6(1):1–12. doi: 10.1186/s13568-016-0186-z

60. Chikindas ML, Weeks R, Drider D, Chistyakov VA, Dicks LM. Functions and emerging applications of bacteriocins. Curr Opin Biotechnol. 2018;49:23–8. doi: 10.1016/j.copbio.2017.07.011

61. Chopra L, Singh G, Kumar Jena K, Sahoo DK. Sonorensin: A new bacteriocin with potential of an anti-biofilm agent and a food biopreservative. Sci Rep. 2015;5:1–13. doi: 10.1038/srep13412

62. Mills S, Griffin C, O'Connor PM, Serrano LM, Meijer WC, Hill C, Ross R. A Multibacteriocin Cheese Starter System, Comprising Nisin and Lacticin 3147 in Lactococcus lactis, in Combination with Plantaricin from Lactobacillus plantarum. Björkroth J, editor. Appl Environ Microbiol. 2017;83(14):1–17. doi: 10.1128/AEM.00799-17

63. Paškevičius Š, Starkevič U, Misiūnas A, Vitkauskienė A, Gleba Y, Ražanskienė A. Plant-expressed pyocins for control of Pseudomonas aeruginosa. PLoS One. 2017;12(10):1–17. doi: 10.1371/journal.pone.0185782

64. Woraprayote W, Malila Y, Sorapukdee S, Swetwiwathana A, Benjakul S, Visessanguan W. Bacteriocins from lactic acid bacteria and their applications in meat and meat

products. Meat Sci. 2016;120:118–32. doi: 10.1080/07388551.2016.1262323

65. Yi L, Li X, Luo L, Lu Y, Yan H, Qiao Z, Lu X. A novel bacteriocin BMP11 and its antibacterial mechanism on cell envelope of Listeria monocytogenes and Cronobacter sakazakii. Food Control. 2018;91:160–9. doi: 10.1016/j.foodcont.2018.03.038

66. Barbosa AAT, Mantovani HC, Jain S. Bacteriocins from lactic acid bacteria and their potential in the preservation of fruit products. Crit Rev Biotechnol. 2017;37(7):852–64. doi: 10.1080/07388551.2016.1262323

67. Pereira DM, Valentão P, Pereira JA, Andrade PB. Phenolics: From chemistry to biology. Molecules. 2009;14(6):2202–11. doi: 10.3390/molecules14062202

68. Daglia M. Polyphenols as antimicrobial agents. Curr Opin Biotechnol. 2012 Apr;23(2):174–81. doi: 10.1016/j.copbio.2011.08.007

69. Taylor PW, Hamilton-Miller JMT, Stapleton PD. Antimicrobial properties of green tea catechins. Food Sci Technol Bull. 2005;2(7):71–81. doi: 10.1616/1476-2137.14184

70. Hengge R. Targeting bacterial biofilms by the green tea polyphenol EGCG. Molecules. 2019;24(13):15–7. doi: 10.3390/molecules24132403

71. Lee JH, Park JH, Cho HS, Joo SW, Cho MH, Lee J. Anti-biofilm activities of quercetin and tannic acid against Staphylococcus aureus. Biofouling. 2013;29(5):491–9. doi: 10.1080/08927014.2013.788692

72. Nowak A, Czyzowska A, Efenberger M, Krala L. Polyphenolic extracts of cherry (Prunus cerasus L.) and blackcurrant (Ribes nigrum L.) leaves as natural preservatives in meat products. Food Microbiol. 2016;59:142–9. doi: 10.1016/j.fm.2016.06.004

73. Xu C, Yagiz Y, Hsu WY, Simonne A, Lu J, Marshall MR. Antioxidant, antibacterial, and antibiofilm properties of polyphenols from muscadine grape (Vitis rotundifolia Michx.) Pomace against selected foodborne pathogens. J Agric Food Chem. 2014;62(28):6640–9. doi: 10.1021/jf501073q

74. Česonienė L, Jasutienė I, Šarkinas A. Phenolics and anthocyanins in berries of European cranberry and their antimicrobial activity. Medicina (B Aires). 2009;45(12):992. doi: 10.3390/medicina45120127

75. Li Y, Zhang J-J, Xu D-P, Zhou T, Zhou Y, Li S. Bioactivities and Health Benefits of Wild Fruits. Int J Mol Sci. 2016;17(8):1258. doi: 10.3390/ijms17081258

76. Viskelis P, Rubinskienė M, Jasutienė I, Šarkinas A, Daubaras R, Česonienė L. Anthocyanins, Antioxidative, and Antimicrobial Properties of American Cranberry (Vaccinium macrocarpon Ait.) and their Press Cakes. J Food Sci. 2009;74(2):C157–61. doi: 10.1111/j.1750-3841.2009.01066.x

77. Silva S, Costa EM, Mendes M, Morais RM, Calhau C, Pintado MM. Antimicrobial, antiadhesive and antibiofilm activity of an ethanolic, anthocyanin-rich blueberry extract purified by solid phase extraction. J Appl Microbiol . 2016;121(3):693–703. doi: 10.1111/jam.13215

78. Howell AB, Reed JD, Krueger CG, Winterbottom R, Cunningham DG, Leahy M. A-type cranberry proanthocyanidins and uropathogenic bacterial anti-adhesion activity. Phytochemistry. 2005;66:2281–91. doi: 10.1016/j.phytochem.2005.05.022

79. Eydelnant IA, Tufenkji N. Cranberry derived proanthocyanidins reduce bacterial

adhesion to selected biomaterials. Langmuir. 2008;24(18):10273–81. doi: 10.1021/la801525d

80. Koo H, Duarte S, Murata RM, Scott-Anne K, Gregoire S, Watson GE, Singh,AP, Vorsa N.. Influence of cranberry proanthocyanidins on formation of biofilms by streptococcus mutans on saliva-coated apatitic surface and on dental caries development in vivo. Caries Res. 2010;44(2):116–26. doi: 10.1159/000296306

81. Ulrey RK, Barksdale SM, Zhou W, Van Hoek ML. Cranberry proanthocyanidins have anti-biofilm properties against pseudomonas aeruginosa. BMC Complement Altern Med. 2014;14(1):1–12. doi: 10.1186/1472-6882-14-499

82. Barbosa-Pereira L, Aurrekoetxea GP, Angulo I, Paseiro-Losada P, Cruz JM. Development of new active packaging films coated with natural phenolic compounds to improve the oxidative stability of beef. Meat Sci. 2014;97(2):249–54. doi: 10.1016/j.ijbiomac.2013.04.019

83. Sun L, Sun J, Chen L, Niu P, Yang X, Guo Y. Preparation and characterization of chitosan film incorporated with thinned young apple polyphenols as an active packaging material. Carbohydr Polym. 2017;163:81–91. doi: 10.1016/j.carbpol.2017.01.016

84. Kontiokari T, Sundqvist K, Nuutinen M, Pokka T, Koskela M, Uhari M. Randomised trial of cranberry-lingonberry juice and Lactobacillus GG drink for the prevention of urinary tract infections in women. BMJ. 2001;322(7302):1571–1571. doi: 10.1136/bmj.322.7302.1571

85. Neto CC. Cranberry and Its Phytochemicals: A Review of In Vitro Anticancer Studies. J Nutr. 2007;137(1):186S–193S. doi: 10.1093/jn/137.1.186s

86. Dohadwala MM, Holbrook M, Hamburg NM, Shenouda SM, Chung WB, Titas M. Effects of cranberry juice consumption on vascular function in patients with coronary artery disease. Am J Clin Nutr. 2011;93(5):934–40. doi: 10.3945/ajcn.110.004242

87. Côté J, Caillet S, Doyon G, Sylvain JF, Lacroix M. Bioactive compounds in cranberries and their biological properties. Crit Rev Food Sci Nutr. 2010;50(7):666–79. doi: 10.1080/10408390903044107

88. Pappas E, Schaich KM. Phytochemicals of cranberries and cranberry products: Characterization, potential health effects, and processing stability. Crit Rev Food Sci Nutr. 2009;49(9):741–81. doi: 10.1080/10408390802145377

89. Çelik H, Özgen M, Serçe S, Kaya C. Phytochemical accumulation and antioxidant capacity at four maturity stages of cranberry fruit. Sci Hortic. 2008;117(4):345–8. doi: 10.1016/j.scienta.2008.05.005

90. Huang W, Yan Z, Li D, Ma Y, Zhou J, Sui Z. Antioxidant and anti-inflammatory effects of blueberry anthocyanins on high glucose-induced human retinal capillary endothelial cells. Oxid Med Cell Longev. 2018;2018. doi: 10.1155/2018/1862462

91. Ghosh D, Konishi T. Anthocyanins and anthocyanin-rich extracts.pdf. Asia Pac J Clin Nutr. 2007;16(2):200–8. doi: 10.3390/ijms13022472

92. Krueger CG, Reed JD, Feliciano RP, Howell AB. Quantifying and characterizing proanthocyanidins in cranberries in relation to urinary tract health. Anal Bioanal Chem. 2013;405(13):4385–95. doi: 10.1007/s00216-013-6750-3

93. Yuzuak S, Ballington J, Xie DY. HPLC-qTOF-MS/MS-based profiling of flavan-3-ols and dimeric proanthocyanidins in berries of two muscadine grape hybrids FLH 13-11 and FLH 17-66. Metabolites. 2018;8(4). doi: 10.3390/metabo8040057

94. Foo LY, Lu Y, Howell AB, Vorsa N. The structure of cranberry proanthocyanidins which inhibit adherence of uropathogenic P-fimbriated Escherichia coli in vitro. Phytochemistry. 2000;54(2):173–81. doi: 10.1016/S0031-9422(99)00573-7

95. Hidalgo G, Ponton A, Fatisson J, O'May C, Asadishad B, Schinner T, Tufenkji N. Induction of a state of iron limitation in Uropathogenic Escherichia coli CFT073 by Cranberry-derived proanthocyanidins as revealed by microarray analysis. Appl Environ Microbiol. 2011;77(4):1532–5. doi: 10.1128/AEM.02201-10

96. Kruger MJ, Davies N, Myburgh KH, Lecour S. Proanthocyanidins, anthocyanins and cardiovascular diseases. Food Res Int. 2014;59:41–52. doi: 10.1016/j.foodres.2014.01.046

97. Xu Z, Du P, Meiser P, Claus J. Proanthocyanidins: Oligomeric structures with unique biochemical properties and great therapeutic promise. Nat Prod Commun. 2012;7(3):381–8.

98. He X, Rui HL. Cranberry phytochemicals: Isolation, structure elucidation, and their antiproliferative and antioxidant activities. J Agric Food Chem. 2006;54(19):7069–74. doi: 10.1021/jf061058l

99. Huang Y, Nikolic D, Pendland S, Doyle BJ, Locklear TD, Mahady GB. Effects of cranberry extracts and ursolic acid derivatives on P-fimbriated Escherichia coli , COX-2 activity, pro-inflammatory cytokine release and the NF-κβ transcriptional response in vitro. Pharm Biol. 2009;47(1):18–25. doi: 10.1080/13880200802397996

100. Kondo M, Mackinnon SL, Craft CC, Matchett MD, Hurta RAR, Neto CC. Ursolic acid and its esters: Occurrence in cranberries and other Vaccinium fruit and effects on matrix metalloproteinase activity in DU145 prostate tumor cells. J Sci Food Agric. 2011;91(5):789–96. doi: 10.1002/jsfa.4330

101. Otoni CG, Espitia PJP, Avena-Bustillos RJ, McHugh TH. Trends in antimicrobial food packaging systems: Emitting sachets and absorbent pads. Food Res Int. 2016;83:60–73. doi: 10.1016/j.foodres.2016.02.018

102. Mousavi Khaneghah A, Hashemi SMB, Limbo S. Antimicrobial agents and packaging systems in antimicrobial active food packaging: An overview of approaches and interactions. Food Bioprod Process. 2018;111:1–19. doi: 10.1016/j.fbp.2018.05.001

103. Huang T, Qian Y, Wei J, Zhou C. Polymeric Antimicrobial food packaging and its applications. Polymers (Basel). 2019;11(3). doi: 10.3390/polym11030560

104. Mangaraj S, Goswami TK, Mahajan P V. Applications of Plastic Films for Modified Atmosphere Packaging of Fruits and Vegetables: A Review. Food Eng Rev. 2009;1(2):133–58. doi: 10.1007/s12393-009-9007-3

105. Katiyar V. Bio-based Plastics for Food Packaging Applications. Smithers P. 2017. 298 p.

106. Avella M, De Vlieger JJ, Errico ME, Fischer S, Vacca P, Volpe MG. Biodegradable starch/clay nanocomposite films for food packaging applications. Food Chem. 2005;93(3):467–74. doi: 10.1016/j.foodchem.2004.10.024

107. Mohammadi Nafchi A, Cheng LH, Karim AA. Effects of plasticizers on thermal properties and heat sealability of sago starch films. Food Hydrocoll. 2011;25(1):56–60. doi: 10.1016/j.foodhyd.2010.05.005

108. Galdeano MC, Mali S, Grossmann MVE, Yamashita F, García MA. Effects of plasticizers on the properties of oat starch films. Mater Sci Eng C. 2009;29(2):532–8. doi: 10.1016/j.msec.2008.09.034

109. Rhim JW, Mohanty AK, Singh SP, Ng PKW. Effect of the processing methods on the performance of polylactide films: Thermocompression versus solvent casting. J Appl Polym Sci. 2006;101(6):3736–42. doi: 10.1002/app.23403

110. Ramos M, Valdés A, Beltrán A, Garrigós M. Gelatin-Based Films and Coatings for Food Packaging Applications. Coatings. 2016;6(4):41. doi: 10.3390/coatings6040041

111. Alves V, Costa N, Hilliou L, Larotonda F, Gonçalves M, Sereno A, Coelhoso I. Design of biodegradable composite films for food packaging. Desalination. 2006;199(1–3):331–3. doi: 10.1016/j.desal.2006.03.078

112. Lozano-Navarro JI, Díaz-Zavala NP, Velasco-Santos C, Martínez-Hernández AL, Tijerina-Ramos BI, García-Hernández M, Rivera-Armenta J, Páramo-García U, Reyes-de la Torre AI. Antimicrobial, optical and mechanical properties of Chitosan–Starch films with natural extracts. Int J Mol Sci. 2017;18(5):1–18. doi: 10.3390/ijms18050997

113. Wang H, Qian J, Ding F. Emerging Chitosan-Based Films for Food Packaging Applications. J Agric Food Chem. 2018;66(2):395–413. doi: 10.1021/acs.jafc.7b04528

114. Renuka V, Mohan CO, Kriplani Y, Sivaraman GK, Ravishankar CN. Effect of Chitosan Edible Coating on the Microbial Quality of Ribbonfish , Lepturacanthus savala (Cuvier , 1929) Steaks. Fish Technol. 2016;53:146–50.

115. Plácido GR, Silva RM da, Cagnin C, Cavalcante MD, Silva MAP da, Caliari M, Luis M, Nascimento E. Effect of chitosan-based coating on postharvest quality of tangerines (Citrus deliciosa Tenore): Identification of physical, chemical, and kinetic parameters during storage. African J Agric Res. 2016 Jun 16;11(24):2185–92. doi: 10.5897/AJAR2014.9355

116. Helander IM, Nurmiaho-Lassila EL, Ahvenainen R, Rhoades J, Roller S. Chitosan disrupts the barrier properties of the outer membrane of Gram-negative bacteria. Int J Food Microbiol. 2001;71(2–3):235–44. doi: 10.1016/S0168-1605(01)00609-2

117. Je JY, Kim SK. Chitosan derivatives killed bacteria by disrupting the outer and inner membrane. J Agric Food Chem. 2006;54(18):6629–33. doi: 10.1021/jf061310p

118. Qin C, Li H, Xiao Q, Liu Y, Zhu J, Du Y. Water-solubility of chitosan and its antimicrobial activity. Carbohydr Polym. 2006;63(3):367–74. doi: 10.1016/j.carbpol.2005.09.023

119. Tayel AA, Moussa S, Opwis K, Knittel D, Schollmeyer E, Nickisch-Hartfiel A. Inhibition of microbial pathogens by fungal chitosan. Int J Biol Macromol. 2010;47(1):10–4. doi: doi:10.1016/j.ijbiomac.2010.04.005

120. Reesha K V., Satyen Kumar P, Bindu J, Varghese TO. Development and characterization of an LDPE/chitosan composite antimicrobial film for chilled fish storage. Int J Biol Macromol. 2015;79:934–42. doi: 10.1016/j.ijbiomac.2015.06.016

121. Camataro FDDS, Santana LCLDA, Carneldsso MAG, Alexandre APS, Nunes ML, Gdulart MDF, Naraon N, Solva M, Silva M. Impact of edible coatings based on cassava starch and chitosan on the post-harvest shelf life of mango (Mangifera indica) 'tommy atkins' fruits. Food Sci Technol. 2018;38:86–95. doi: 10.1590/1678-457x.16417

122. Wu Y, Ying Y, Liu Y, Zhang H, Huang J. Preparation of chitosan/poly vinyl alcohol films and their inhibition of biofilm formation against Pseudomonas aeruginosa PAO1. Int J Biol Macromol. 2018;118:2131–7. doi: 10.1016/j.ijbiomac.2018.07.061

123. Rojas-Graü MA, Soliva-Fortuny R, Martín-Belloso O. Edible coatings to incorporate active ingredients to fresh-cut fruits: a review. Trends Food Sci Technol. 2009;20(10):438–47. doi: 10.1016/j.tifs.2009.05.002

124. Genskowsky E, Puente LA, Pérez-Álvarez JA, Fernandez-Lopez J, Muñoz LA, Viuda-Martos M. Assessment of antibacterial and antioxidant properties of chitosan edible films incorporated with maqui berry (Aristotelia chilensis). LWT - Food Sci Technol. 2015;64(2):1057–62. doi: 10.1016/j.lwt.2015.07.026

125. Peng Y, Wu Y, Li Y. Development of tea extracts and chitosan composite films for active packaging materials. Int J Biol Macromol. 2013;59:282–9. doi: 10.1016/j.ijbiomac.2013.04.019

126. de Moraes Crizel T, de Oliveira Rios A, D. Alves V, Bandarra N, Moldão-Martins M, Hickmann Flôres S. Active food packaging prepared with chitosan and olive pomace. Food Hydrocoll. 2018;74:139–50. doi: 10.1016/j.foodhyd.2017.08.007

127. Ribeiro IA, Bronze MR, Castro MF, Ribeiro MHL. Optimization and correlation of HPLC-ELSD and HPLC-MS/MS methods for identification and characterization of sophorolipids. J Chromatogr B Anal Technol Biomed Life Sci. 2012;899:72–80. doi: 10.1016/j.jchromb.2012.04.037

128. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. M100 Performance Standards for Antimicrobial Susceptibility Testing. 2016;100–25.

129. Sarikurkcu C, Arisoy K, Tepe B, Cakir A, Abali G, Mete E. Studies on the antioxidant activity of essential oil and different solvent extracts of Vitex agnus castus L. fruits from Turkey. Food Chem Toxicol. 2009;47(10):2479–83. doi: 10.1016/j.fct.2009.07.005

130. Velickova E, Winkelhausen E, Kuzmanova S, Moldão-Martins M, Alves VD. Characterization of multilayered and composite edible films from chitosan and beeswax. Food Sci Technol Int. 2015;21(2):83–93. doi: 10.1016/j.carbpol.2010.10.010

131. Conzatti G, Chamary S, De Geyter N, Cavalie S, Morent R, Tourrette A. Surface functionalization of plasticized chitosan films through PNIPAM grafting via UV and plasma graft polymerization. Eur Polym J. 2018;105:434–41. doi: 10.1016/j.eurpolymj.2018.06.020

132. Yuan G, Lv H, Yang B, Chen X, Sun H. Physical properties, antioxidant and antimicrobial activity of chitosan films containing carvacrol and pomegranate peel extract. Molecules. 2015;20(6):11034–45. doi: 10.3390/molecules200611034

133. Leceta I, Guerrero P, De La Caba K. Functional properties of chitosan-based films. Carbohydr Polym. 2013;93(1):339–46. doi: 10.1016/j.carbpol.2012.04.031

134. Suyatma NE, Tighzert L, Copinet A, Coma V. Effects of hydrophilic plasticizers on

mechanical, thermal, and surface properties of chitosan films. J Agric Food Chem. 2005;53(10):3950–7. doi: 10.1021/jf048790

135. Ferreira ARV, Torres CAV, Freitas F, Sevrin C, Grandfils C, Reis MAM. Development and characterization of bilayer films of FucoPol and chitosan. Carbohydr Polym. 2016;147:8–15. doi: 10.1016/j.carbpol.2016.03.089

136. Pontes C, Alves M, Santos C, Ribeiro MH, Gonçalves L, Bettencourt AF, Ribeiro I. Can Sophorolipids prevent biofilm formation on silicone catheter tubes? Int J Pharm. 2016;513(1–2):697–708. doi: 10.1016/j.ijpharm.2016.09.074

137. Naczk M, Shahidi F. Extraction and analysis of phenolics in food. J Chromatogr A. 2004;1054(1–2):95–111. doi: 10.1002/jssc.200700261

138. Antolovich M, Prenzler P, Robards K, Ryan D. Sample preparation in the determination of phenolic compounds in fruits. Analyst. 2000;125(5):989–1009. doi: 10.1039/b000080i

139. Cabrera C, Giménez R, López MC. Determination of tea components with antioxidant activity. J Agric Food Chem. 2003;51(15):4427–35. doi: 10.1021/jf0300801

140. Dailey A, Vuong Q V. Effect of extraction solvents on recovery of bioactive compounds and antioxidant properties from macadamia (Macadamia tetraphylla) skin waste. Cogent Food Agric. 2015;1(1):1–10. doi: 10.1080/23311932.2015.1115646

141. Klavins M. Comparison of methods of extraction of phenolic compounds from american cranberry (Vaccinium macrocarpon L .) press residues Comparison of methods of extraction of phenolic compounds from American cranberry (Vaccinium macrocarpon L .) press residues. Agron Res. 2017;15:1316–1329.

142. Pradal D, Vauchel P, Decossin S, Dhulster P, Dimitrov K. Kinetics of ultrasound-assisted extraction of antioxidant polyphenols from food by-products: Extraction and energy consumption optimization. Ultrason Sonochem. 2016;32:137–46. doi: 10.1016/j.ultsonch.2016.03.001

143. Ivanovic J, Tadic V, Dimitrijevic S, Stamenic M, Petrovic S, Zizovic I. Antioxidant properties of the anthocyanin-containing ultrasonic extract from blackberry cultivar "Čačanska Bestrna." Ind Crops Prod. 2014;53:274–81. doi: 10.1016/j.indcrop.2013.12.048

144. Chen M, Zhao Y, Yu S. Optimisation of ultrasonic-assisted extraction of phenolic compounds, antioxidants, and anthocyanins from sugar beet molasses. Food Chem . 2015;172:543–50. doi: 10.1016/j.foodchem.2014.09.110

145. Chen F, Sun Y, Zhao G, Liao X, Hu X, Wu J, Wang Z. Optimization of ultrasound-assisted extraction of anthocyanins in red raspberries and identification of anthocyanins in extract using high-performance liquid chromatography – mass spectrometry. Ultrason Sonochem. 2007;14:767–78. doi: 10.1016/j.ultsonch.2006.12.011

146. Stalikas CD. Extraction, separation, and detection methods for phenolic acids and flavonoids. J Sep Sci. 2007;30(18):3268–95. doi: 10.1002/jssc.200700261

147. LaPlante KL, Sarkisian SA, Woodmansee S, Rowley DC, Seeram NP. Effects of Cranberry Extracts on Growth and Biofilm Production of Escherichia coli and Staphylococcus species. Phyther Res. 2012;26(9):1371–4. doi: 10.1002/ptr.4592

148. Lacombe A, Wu VCH, Tyler S, Edwards K. Antimicrobial action of the American cranberry constituents; phenolics, anthocyanins, and organic acids, against Escherichia coli O157:H7. Int J Food Microbiol. 2010;139(1–2):102–7. doi: 10.1016/j.ijfoodmicro.2010.01.035

149. Nohynek LJ, Alakomi H-L, Kähkönen MP, Heinonen M, Helander IM, Oksman-Caldentey K-M, Puupponen-Pimiä R. Berry Phenolics: Antimicrobial Properties and Mechanisms of Action Against Severe Human Pathogens. Nutr Cancer. 2006;54(1):18–32. doi: 10.1207/s15327914nc5401_4

150. Tian F, Li B, Ji B, Yang J, Zhang G, Chen Y, Luo Y. Antioxidant and antimicrobial activities of consecutive extracts from Galla chinensis: The polarity affects the bioactivities. Food Chem. 2009;113(1):173–9. doi: 10.1016/j.foodchem.2008.07.062

151. Wu VCH, Qiu X, de los Reyes BG, Lin CS, Pan Y. Application of cranberry concentrate (Vaccinium macrocarpon) to control Escherichia coli O157:H7 in ground beef and its antimicrobial mechanism related to the downregulated slp, hdeA and cfa. Food Microbiol . 2009;26(1):32–8. doi: 10.1016/j.fm.2008.07.014

152. Prior RL, Cao G, Martin A, Sofic E, McEwen J, O'Brien C, Lischner, Neal Ehlenfeldt M, Kalt W, Krewer G, Mainland C. Antioxidant Capacity as Influenced by Total Phenolic and Anthocyanin Content, Maturity, and Variety of Vaccinium Species. J Agric Food Chem. 1998;46(7):2686–93. doi: 10.1021/jf980145d

153. Kwok B, Hu C, Durance T, Kitts Dd. Dehydration Techniques Affect Phytochemical Contents and Free Radical Scavenging Activities of Saskatoon berries (Amelanchier alnifolia Nutt.). J Food Sci. 2006;69(3):122-126. doi: 10.1111/j.1365-2621.2004.tb13381.x

154. Sariburun E, Şahin S, Demir C, Türkben C, Uylaşer V. Phenolic content and antioxidant activity of raspberry and blackberry cultivars. J Food Sci. 2010;75(4). doi: 10.1111/j.1750-3841.2010.01571.x

155. MahassineAmensour, Esther Sendra, Jamal Abrini, Samira Bouhdid JAP-A and JF-L. Total Phenolic Content and Antioxidant Activity of Myrtle. Nat Prod Commun. 2009;4(6):819–24.

156. Liang NN, He F, Bi HQ, Duan CQ, Reeves MJ, Wang J. Evolution of flavonols in berry skins of different grape cultivars during ripening and a comparison of two vintages. Eur Food Res Technol. 2012;235(6):1187–97. doi: 10.1007/s00217-012-1850-4

157. Yan X, Murphy BT, Hammond GB, Vinson JA, Neto CC. Antioxidant activities and antitumor screening of extracts from Cranberry fruit (Vaccinium macrocarpon). J Agric Food Chem. 2002;50(21):5844–9. doi: 10.1021/jf0202234

158. Zuo Y, Wang C, Zhan J. Separation, characterization, and quantitation of benzoic and phenolic antioxidants in American cranberry fruit by GC-MS. J Agric Food Chem. 2002;50(13):3789–94. doi: 10.1021/jf020055f

159. Turner A, Chen SN, Joike MK, Pendland SL, Pauli GF, Farnsworth NR. Inhibition of uropathogenic Escherichia coli by cranberry juice: A new antiadherence assay. J Agric Food Chem. 2005;53(23):8940–7. doi: 10.1021/jf052035u

160. Wu X, Prior RL. Systematic identification and characterization of anthocyanins by HPLC-ESI-MS/MS in common foods in the United States: Fruits and berries. J Agric

Food Chem. 2005;53(7):2589–99. doi: 10.1021/jf048068b

161. Chen H, Zuo Y, Deng Y. Separation and determination of flavonoids and other phenolic compounds in cranberry juice by high-performance liquid chromatography. J Chromatogr A. 2001;913(1–2):387–95. doi: 10.1016/S0021-9673(00)01030-X

162. Zheng W, Wang SY. Oxygen radical absorbing capacity of phenolics in blueberries, cranberries, chokeberries, and lingonberries. J Agric Food Chem. 2003;51(2):502–9. doi: 10.1021/jf020728u

163. Arts ICW, Van De Putte B, Hollman PCH. Catechin contents of foods commonly consumed in The Netherlands. 1. Fruits, vegetables, staple foods, and processed foods. J Agric Food Chem. 2000;48(5):1746–51. doi: 10.1021/jf000025h

164. Rue EA, Rush MD, van Breemen RB. Procyanidins: a comprehensive review encompassing structure elucidation via mass spectrometry. Phytochem Rev. 2018;17(1):1–16. doi: 10.1007/s11101-017-9507-3

165. Hellström JK, Törrönen AR, Mattila PH. Proanthocyanidins in common food products of plant origin. J Agric Food Chem. 2009;57(17):7899–906. doi: 10.1021/jf901434d

166. Talón E, Trifkovic KT, Nedovic VA, Bugarski BM, Vargas M, Chiralt A, González-Martínez C. Antioxidant edible films based on chitosan and starch containing polyphenols from thyme extracts. Carbohydr Polym. 2017;157:1153–61. doi: 10.1016/j.carbpol.2016.10.080

167. Rubilar JF, Cruz RMS, Silva HD, Vicente AA, Khmelinskii I, Vieira MC. Physico-mechanical properties of chitosan films with carvacrol and grape seed extract. J Food Eng . 2013;115(4):466–74. doi: 10.1016/j.jfoodeng.2012.07.009

168. Kanatt SR, Rao MS, Chawla SP, Sharma A. Effects of chitosan coating on shelf-life of ready-to-cook meat products during chilled storage. LWT - Food Sci Technol. 2013;53(1):321–6. doi: 10.1016/j.lwt.2013.01.019

169. Ribeiro C, Vicente AA, Teixeira JA, Miranda C. Optimization of edible coating composition to retard strawberry fruit senescence. Postharvest Biol Technol. 2007;44(1):63–70. doi: 10.1016/j.postharvbio.2006.11.015

170. Mayachiew P, Devahastin S. Comparative evaluation of physical properties of edible chitosan films prepared by different drying methods. Dry Technol. 2008;26(2):176–85. doi: 10.1080/07373930701831309

171. Singh TP, Chatli MK, Sahoo J. Development of chitosan based edible films: process optimization using response surface methodology. J Food Sci Technol. 2015;52(5):2530–43. doi: 10.1007/s13197-014-1318-6

172. Hager TJ, Howard LR, Prior RL. Processing and storage effects on monomeric anthocyanins, percent polymeric color, and antioxidant capacity of processed blackberry products. J Agric Food Chem. 2008;56(3):689–95. doi: 10.1111/j.1750-3841.2008.00761.x

173. Martins JT, Cerqueira MA, Vicente AA. Influence of α-tocopherol on physicochemical properties of chitosan-based films. Food Hydrocoll. 2012;27(1):220–7. doi: 10.1111/j.1750-3841.2008.00761.x

174. Müller CMO, Yamashita F, Laurindo JB. Evaluation of the effects of glycerol and

sorbitol concentration and water activity on the water barrier properties of cassava starch films through a solubility approach. Carbohydr Polym. 2008;72(1):82–7. doi: 10.1016/j.carbpol.2007.07.026

175. Sanyang ML, Sapuan SM, Jawaid M, Ishak MR, Sahari J. Effect of plasticizer type and concentration on physical properties of biodegradable films based on sugar palm (arenga pinnata) starch for food packaging. J Food Sci Technol. 2016;53(1):326–36. doi: 10.1007/s13197-015-2009-7

176. Maran JP, Sivakumar V, Sridhar R, Thirugnanasambandham K. Development of model for barrier and optical properties of tapioca starch based edible films. Carbohydr Polym. 2013;92(2):1335–47. doi: 10.1016/j.carbpol.2012.09.069

177. Chung YC, Wang HL, Chen YM, Li SL. Effect of abiotic factors on the antibacterial activity of chitosan against waterborne pathogens. Bioresour Technol. 2003;88(3):179–84. doi: 10.1016/S0960-8524(03)00002-6

178. No HK, Young Park N, Ho Lee S, Meyers SP. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. Int J Food Microbiol. 2002;74(1–2):65–72. doi: 10.1016/S0168-1605(01)00717-6

179. Elsabee MZ, Abdou ES. Chitosan based edible films and coatings: A review. Materials Science and Engineering C.2013;33(4):1819–41. doi: 10.1016/j.msec.2013.01.010

180. Siripatrawan U, Harte BR. Physical properties and antioxidant activity of an active film from chitosan incorporated with green tea extract. Food Hydrocoll. 2010;24(8):770–5. doi: 10.1016/j.foodhyd.2010.04.003

181. Siripatrawan U, Vitchayakitti W. Improving functional properties of chitosan films as active food packaging by incorporating with propolis. Food Hydrocoll. 2016;61:695–702. doi: 10.1016/j.foodhyd.2016.06.001

182. Sothornvit R, Pitak N. Oxygen permeability and mechanical properties of banana films. Food Res Int. 2007;40(3):365–70. doi: 10.1016/j.foodres.2006.10.010

183. Gontard N, Thibault R, Cuq B, Guilbert S. Influence of relative humidity and film composition on oxygen and carbon dioxide permeabilities of edible films. J Agric Food Chem. 1996;44(4):1064–9. doi: 10.1021/jf9504327

184. Zhong QP, Xia WS. Physicochemical Properties of Edible and Preservative Films from Chitosan/Cassava Starch/Gelatin Blend Plasticized with Glycerol. Food Technol Biotechnol. 2008;46(3):262–9.

185. Aryaei A, Jayatissa AH, Jayasuriya AC. Nano and micro mechanical properties of uncross-linked and cross-linked chitosan films. J Mech Behav Biomed Mater. 2012;5(1):82–9. doi: 10.1016/j.jmbbm.2011.08.006

186. Fahim IS, Aboulkhair N, Everitt NM. Nanoindentation Investigation on Chitosan Thin Films with Different Types of Nano Fillers. J Mater Sci Res. 2018;7(2):11. doi: 10.5539/jmsr.v7n2p11

187. Nostro A, Scaffaro R, D'Arrigo M, Botta L, Filocamo A, Marino A, Bisignano G. Study on carvacrol and cinnamaldehyde polymeric films: Mechanical properties, release kinetics and antibacterial and antibiofilm activities. Appl Microbiol Biotechnol. 2012;96(4):1029–38. doi: 10.1007/s00253-012-4091-3

188. Howell AB. Bioactive compounds in cranberries and their role in prevention of urinary tract infections. Mol Nutr Food Res. 2007;51(6):732–7. doi: 10.1002/mnfr.200700038

189. Riaz A, Lei S, Akhtar HMS, Wan P, Chen D, Jabbar S, Abid M, Hashim M, Zeng X. Preparation and characterization of chitosan-based antimicrobial active food packaging film incorporated with apple peel polyphenols. Int J Biol Macromol. 2018;114:547–55. doi: 10.1016/j.ijbiomac.2018.03.126

190. Kalaycıoğlu Z, Torlak E, Akın-Evingür G, Özen İ, Erim FB. Antimicrobial and physical properties of chitosan films incorporated with turmeric extract. Int J Biol Macromol. 2017;101:882–8. doi: 10.1016/j.ijbiomac.2017.03.174

191. Wu VCH, Qiu X, Bushway A, Harper L. Antibacterial effects of American cranberry (Vaccinium macrocarpon) concentrate on foodborne pathogens. LWT - Food Sci Technol . 2008;41(10):1834–41. doi: 10.1016/j.lwt.2008.01.001