UNIVERSIDADE
BEIRA INTERIOR

Development of k-carrageenan films incorporating *Cymbopogon winterianus* and *Lavandula viridis* essential oils for food packaging applications

Catarina Filipa Cunha Santos

Dissertação para obtenção do Grau de Mestre em
Química Industrial
(2º ciclo de estudos)

Orientador: Prof. Dra. Maria Emília da Costa Cabral Amaral
Co-orientador: Prof. Dr. Ângelo Filipe Santos Luís

outubro de 2023

Folha em branco

Declaração de Integridade

Eu, Catarina Filipa Cunha Santos, abaixo assinado, estudante com o número de inscrição M11990 do Mestrado em Química Industrial da Faculdade de Ciências, declaro ter desenvolvido o presente trabalho e elaborado o presente texto em total consonância com o **Código de Integridade da Universidade da Beira Interior**.

Mais concretamente afirmo não ter incorrido em quaisquer variedades de Fraude Académica, e que aqui declaro conhecer, que em particular atendi à exigida referenciação de frases, extratos, imagens e outras formas de trabalho intelectual, e assumindo assim na íntegra as responsabilidades da autoria.

Universidade da Beira Interior, Covilhã 09/10/2023

Catarina Santos

Folha em branco

Acknowledgments

At the end of this thesis, I would like to express my gratitude to everyone who contributed to this stage of my life and provided me with moments of education, reflection, and happiness.

First, I would like to thank my advisor, Professor Maria Emilia Amaral, for her friendship and persistent concern along the way. Already having been guided by her in the past, there was no way for me to alter it in my master's thesis. We worked well together. She was always available to assist and shared numerous scientific insights with me.

Next, I'd like to acknowledge my co-supervisor, Professor Ângelo Luís, who, from the start of my degree, sparked my interest in the world of biodegradable films and eco-friendly alternatives. He provided tremendous support at this point in time, with unremitting effort and availability at all times.

I'd like to thank Professor Ana Ramos for her time and persistent determination in conducting the contact angle testing. I would also like to thank Dr. Ana Paula Gomes from the optics center at the University of Beira Interior for always being so accessible to us and for conducting both the SEM and DSC tests on our behalf.

To my laboratory companion, Alexandra Simões, who always provided a cheerful atmosphere and made us giggle, with whom I shared tasks, and missed so much when she returned to her hometown.

I would also like to thank my closest friend, Mariana Martins, and my boyfriend, João Vidal, who were always there for me, in good times and bad, and who always believed in me. I would not have been able to conclude this phase of my life without their support. They were the strength I lacked in many instances, and for that, I can only offer a heartfelt, enormous thank you.

Finally, I would like to thank my family for all their support.

Thank you very much!

Folha em branco

Resumo

A partir do século XX, a inovação dos plásticos trouxe uma revolução global, desempenhando um papel fundamental ao proporcionar conforto, praticidade e a redução de desperdício alimentar. No entanto, essa inovação também se revelou acompanhada de desvantagens significativas, em particular, a poluição ambiental generalizada. O crescente reconhecimento dos danos causados pelos plásticos no ecossistema, impulsionou a investigação científica e a exploração de alternativas sustentáveis, com o foco particular nas embalagens destinadas aos produtos alimentares. No contexto desta problemática complexa e multifacetada, o objetivo da presente dissertação é desenvolver e caracterizar filmes de k-carragenina contendo os óleos essenciais de *Cymbopogon winterianus* e *Lavandula viridis*. O intuito é a utilização destes filmes para produzir um material biodegradável com a capacidade de prolongar o tempo de prateleira dos alimentos embalados. Os óleos essenciais foram caracterizados quimicamente, para identificar e quantificar os compostos químicos totais e predominantes. As propriedades antioxidantes e antimicrobianas dos óleos essenciais foram também estudadas. O composto maioritário do óleo essencial *C. winterianus* é o citronelal, que possui atividade antioxidante significativa, como demonstrado pelo ensaio de DPPH• e o teste do branqueamento do β-caroteno. Este óleo essencial teve um bom desempenho contra *Listeria monocytogenes* LMG 16779, o que sugere ter propriedades antibacterianas. O composto maioritário do óleo essencial de *L. viridis* é o 1,8-cineol, que teve um bom desempenho na avaliação do DPPH• e apresentou valores inferiores ao óleo essencial anterior no teste do branqueamento do β-caroteno. Para o teste antibacteriano, este óleo essencial apresentou um resultado similar ao anterior contra a mesma estirpe. Para os filmes produzidos, estudaram-se as suas propriedades antioxidantes, antibacterianas, de barreira e as propriedades físicas. As mudanças na composição dos filmes foram avaliadas através de FTIR.

A realização dos testes de tração nas amostras permitiu inferir que, os filmes incorporados com os óleos essenciais perderam, de modo geral, parte da sua resistência, quando comparados com o filme de controlo. Em termos de testes antioxidantes, os resultados para DPPH• foram satisfatórios, mas os resultados para *C. winterianus* foram mais promissores em termos de β-caroteno, pois é necessária menor quantidade deste para exercer efeito antioxidante. Nos testes antibacterianos, a afinidade de ação pela *L. monocytogenes* LMG 16779 é evidente para ambos os filmes. Este resultado foi validado com o teste de anti-biofilme, através da formação de biofilmes na superfície dos filmes, posteriormente visualizados no SEM, mostrando a clara redução do número de bactérias.

Por fim, realizou-se um ensaio num modelo alimentar, no qual amostras de queijo fatiado foram embaladas com os filmes produzidos, mantidas a 4°C durante 15 dias, sendo depois observadas. Os resultados mostraram que não foram encontrados sinais visíveis de podridão ou mofo no queijo.

Palavras-chave

k-carragenina; *Cymbopogon winterianus*; *Lavandula viridis*; filmes; embalagem alimentar

Folha em branco

Abstract

From the XX century onwards, the innovation of plastics brought a global revolution, playing a fundamental role in improving comfort and practicality and minimizing food waste. However, this breakthrough had substantial drawbacks, most notably widespread pollution. The growing recognition of the damage caused by plastics to the ecosystem has driven scientific research and the exploration of sustainable alternatives, with a particular focus on food packaging. In the context of this complicated and diverse challenge, the purpose of this investigation was to create and characterize k-carrageenan films containing essential oils of *Cymbopogon winterianus* and *Lavandula viridis*. The aim is to use these films to produce a biodegradable material that can extend the shelf life of packaged foods. The essential oils were chemically characterized to identify and quantify the total and predominant chemical compounds. The antioxidant and antimicrobial properties of the essential oils have also been studied. The major compound in *C. winterianus* essential oil is citronellal, which has significant antioxidant activity, as demonstrated by the DPPH• assay and the β-carotene bleaching test. This essential oil performed well against *Listeria monocytogenes* LMG 16779, which suggests it has antibacterial properties. The main composition of *L. viridis* essential oil is 1,8-cineole, which performed well in the DPPH• evaluation and had lower values than the previous essential oil in the β-carotene bleaching test. This essential oil showed a similar result to the previous one against the same strain for the antibacterial test. The antioxidant, antibacterial, barrier and physical properties were studied for the films produced. Changes in the composition of the films were evaluated using FTIR.

Carrying out tensile tests on the samples allowed us to infer that the films incorporated with essential oils lost, in general, part of their resistance when compared to the control film. In terms of antioxidant tests, the results for DPPH• were satisfactory, but the results for *C. winterianus* were more promising in terms of β-carotene, as less of it is needed to exert an antioxidant effect. In antibacterial tests, the layer of action by *L. monocytogenes* LMG 16779 is evident for both films. This result was validated with the anti-biofilm test, through the formation of biofilms on the surface of the films, subsequently visualized in the SEM, showing a clear reduction in the number of bacteria. Finally, a test was carried out in a food model, in which sliced cheese samples were packaged with the films produced, kept at 4°C for 15 days, and then observed. The results showed that no visible signs of rot or mold were found in the cheese.

Keywords

k-carrageenan; *Cymbopogon winterianus*; *Lavandula viridis*; films; food packaging

Folha em branco

Index

Folha em branco

List of Figures

Folha em branco

List of Tables

Folha em branco

List of Acronyms

AAI	*Antioxidant Activity Index*
ASLT	*Accelerated shelf-life tests*
CW	*Cymbopogon winterianus* Jowitt
DPPH•	*2,2-difenil-1-picrilhidrazil*
DSC	*Differential Scanning Calorimetry*
EB	*Elongation at break*
EM	*Elastic modulus*
EO	*Essential oil*
FTIR	*Fourier-Transform Infrared Spectroscopy*
GC-FID	*Gas Chromatography coupled to Flame Ionization Detector*
GC-MS	*Gas Chromatography couples to Mass Spectrometry*
HACCP	*Hazard Analysis and Critical Control Points*
LB	*Luria-Bertani*
LV	*Lavandula viridis* L.Her
MHB	*Müeller-Hinton broth*
MIC	*Minimum inhibitory concentration*
OP	*Oil permeability*
OW	*Owens and Wendt*
PCR	*Polymerase Chain Reaction*
PET	*Polyethylene terephthalate*
RH	*Relative humidity*
SD	*Standard deviation*
SEM	*Scanning Electron Microscopy*
Tg	*Glass transition temperature*
TI	*Tensile Index*
TS	*Tensile Strength*
TSB	*Tryptic Soy Broth*
WPV	*Water Vapor Permeability*
WVTR	*Water Vapor Transmission Rate*

Folha em branco

Chapter 1
General Introduction

1 - General Introduction

Plastic-based pollutants have had a significant negative impact on Earth, severely exposing all biotic and abiotic elements. Each year, 19 to 23 million tons of plastic waste are dumped into the environment [1, 2]. Recycling plastic presents challenges, especially for single-use plastic. A third of the world's pollution deposits come from it, which harms the environment, costs a lot to clean up, and frequently ends up in landfills [2]. In addition, plastic degrades into microplastics, which, when reach the food chain, become highly toxic to all animals [2–5]. Almost every ecosystem on the planet is negatively impacted by plastic waste, it is a growing environmental issue. According to several studies [1, 6], the world's sustainability is being threatened by plastic pollution.
Companies still rely on plastic packaging because of its low production costs, toughness, and other particular properties. Millions of tons of single-use plastic packaging make up a significant portion of the plastic waste that escapes collection systems and enters waterways yearly [7, 8].

Plastic waste has been recorded at every location and domain, from the rivers to the deep-sea deposits. This harms oceans, animals, human health, and economic activities dependent on thriving aquatic ecosystems because of its accumulation and prolonged degradation [7]. Therefore, it is essential to conduct research in order to develop materials that have the potential to serve as suitable alternatives to plastics. As a result, biodegradable polymers have attracted much attention as prospective substitutes for petroleum-based packaging [9].

With the development of k-carrageenan films that incorporate essential oils (EOs), obtaining a more environmentally friendly alternative to plastic packaging parts such as cling films is now possible. This helps to contribute not only to the reduction of pollution but also to improve, extend, or maintain the shelf-life of the packaged food, which in turn reduces the amount of wasted food [10]. Several species of linear sulphated polysaccharides known as carrageenans, which are found in the *Rhodophyceae* family of red seaweeds, have been exploited to create innovative food packaging materials [8].

Chapter 2

Literature Review

2.1 - The Evolution of Food Packaging

Going back in history (Figure 1), people who lived as nomads would consume whatever they could find and gather from their surroundings without thinking about preserving and storing their food. However, as people began to live in groups, the necessity to contain and store food and adequate methods of preservation and packaging became imperative [11].

To hold and carry food quickly after harvest, the earliest packaging methods included leaves, shells, pumpkins, animal skins and bladders, and even human skulls [12]; however, food storage containers became necessary as the society transitioned from a nomadic to a settled lifestyle [13].

Pottery, paper, and glass were the first materials that could be molded into food containers [11, 13]. The Industrial Revolution saw the development of novel materials and industrial techniques. The first produced metal cans were for tobacco storage because they offered a significant barrier to keep the product's moisture. Later, they were employed in the canning process that Nicholas Appert found after Napoleon Bonaparte challenged him (1809) to devise a way to preserve food for his army. Due to their fragility, glass bottles could be replaced by metal cans, which made it easier to heat process products to increase their shelf-life and avoid spoiling [13, 14].

In the 1800s, paper underwent a major technological evolution, going from simple paper wrapping without prints, to the first paper bag production machine, made by Francis Wolle in 1852, to the device that performed the folds on cereal boxes [11, 12, 14].

Hard rubber must be considered the first instance of a plastic substance and, in 1832, natural rubber was heated with sulfur and crosslinked or vulcanized, in order to make natural rubber less tacky. This development was significant since it involved the production of the first thermosetting plastic and the first to apply a straightforward chemical modification of a natural substance [11]. In the first decade of the 20th century, synthetic plastic was created (1907). This thermoset made of phenol formaldehyde was known as "Bakelite".

The 1860s are typically thought to be the beginning of the plastics industry's development; however, large production of plastics started at the end of World War II, with yearly production reaching over 5 million tons in the 1950s.

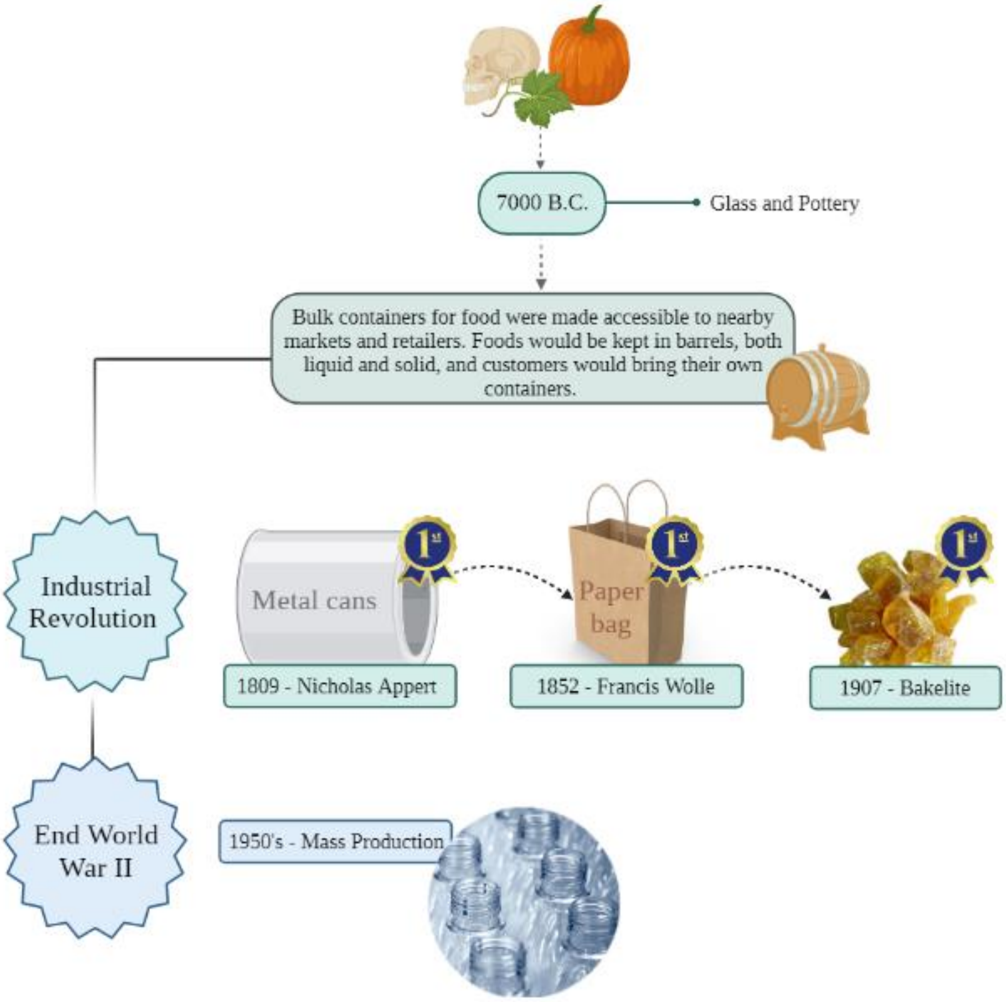


Figure 1. The evolution of food packaging throughout history [12–14].

Plastics' advantages immediately became apparent due to their lightweight, robust, affordable, long-lasting, and corrosion-resistant properties. Plastics are incredibly adaptable materials that can be used to create a variety of goods, including flexible and stiff objects, adhesives, foams, and fibers [11, 15]. The development of polyethylene, polyvinyl chloride, and polyvinylidene chloride was accelerated by World War II [12].

The second half of the 20th century saw advancements in the food industry, including the development of extrusion techniques, metalized films to create multilayer barriers, the aseptic processing of processed foods among others presented in Table 1. Many aspects of food packaging have seen significant transformations, from using polyethylene terephthalate (PET) bottles for bottling, to producing polypropylene and PET packaging that can be microwaved. When compared to the Stone Age, it can be said that the dependence that was created on plastics was so exponential that we are currently living in the Plastic Age [12, 15].

Table 1. Developments in food packaging trends (adapted from [12, 16]).

Period	*Assignments and problems*
1960s	**Convenience and advertising,** e.g.: boil-in-bags, cheese in Polypropylene film.
1970s	**Energy savings, source reduction, and lightweight,** e.g.: bag-in-box for wine, PET soft-drink bottles.
1980s	**Security,** e.g.: microwaveable polymers, modified atmosphere packaging.
1990s	**Impact on the environment,** e.g.: resealable aluminum bottles.
2000s	**Security and safety,** e.g.: active packaging; intelligent packaging.

2.2 – Food Storage and Assigning Shelf-Life

For the regular consumer, shelf-life refers to the time before a product is deemed unfit for human ingestion, since it has already been acknowledged by the European Union and received legal recognition for this concept [17, 18].

When foods are preserved, their qualities are always altered. Microbiological, nonmicrobiological, biochemical, chemical, physical, and temperature-related changes have been identified. By controlling these factors, these changes that will culminate in food spoilage can be minimized [17, 18].

Microbial proliferation depends on several well-known factors, which have been summarized as the intrinsic characteristics of the food, external processing factors, and implicit factors [19]. Because food is constituted by chemicals, biochemical and/or chemical transformations are inevitable. These changes can result from reactions within the food, between its elements, and external organisms or influences such as oxygen or light. For example, the use of pigmented glass vessels (green or brown) prevents certain wavelengths of light from penetrating the food and accelerating the lipid oxidation process [12, 18].

In packaged food, interactions between the packaging and the food can occur, many of which are chemical. Oxidative rancidity (rancidity of fatty foods and food products), hydrolysis of aspartame (sweetener) (reduction in the flavor of calorie-free/low-calorie soft beverages), enzymatic browning (browning in precut vegetables and fresh fruit salads), are few examples [17]. Some physical and chemical alterations may have food safety implications, such as spreading chemical substances from the packaging material into the food, particularly when the shelf-life is extended [12]. Modifications with temperature are among the most significant external elements, influencing all the variations in several ways. For instance, temperature determines the microorganisms that can sustain and thrive in a refrigerator [17].

Some examples of microorganisms and their minimum growth temperature are *Salmonella* at 4°C; *Staphylococcus aureus* at 5.2°C; *Escherichia coli* at 7-8°C; *Listeria monocytogenes* at -0.4°C; *Clostridium botulinum* (nonproteolytic B, E, F) at 3°C, *Pseudomonas aeruginosa* at 4ºC and *Enterococcus faecalis* at 8-16ºC [17, 20, 21].

The primary goal of a storage trial for shelf-life is to determine whether the product satisfies its shelf-life objectives and to determine as precisely as possible, under specified storage conditions, the period at which the product has either become unsafe or undesirable to the target consumers [19].

There are two ways to carry out shelf-life tests: either real or accelerated shelf-life tests (ASLT) [22]. As the name implies, the test is conducted under typical circumstances, where the food's preservation conditions are maintained, and time is permitted to pass until indications of deterioration appear. It is usually used ASLT, for foods whose decay occurs slowly, such as those that undergo oxidation [23]. In these experiments, food degradation is sped up by manipulating the environment, the oxidation kinetics of the food is determined, and the calculation is extrapolated to the actual damage time [22]. As a result of microbial growth degrading a product’s overall quality and safety, organoleptic properties such as off-odors, color, texture, and aroma are altered.

Regarding food safety, microbial growth can cause significant outbreaks of infectious illness [24]. Cross-contamination is one of the ways that contamination of food can show [25]. This may prove to be extremely troublesome in the case of *L. monocytogenes*. *Listeria monocytogenes* is an adaptable, Gram-positive, facultatively aerobic bacillus, that can grow in a wide range of conditions and settings (Figure 2), including dry environments, high temperatures or refrigeration (4ºC), low pH, high salt concentrations, and even sublethal amounts of biocides [26].

In the food industry, it is difficult to control the presence of *L. monocytogenes*, and its ability to form biofilms exacerbates the problem. Biofilms are significant in this industry because they create microbial structures that challenge cleaning processes [27]. Depending on the individual’s health, food contaminated with this bacterium can cause mild to severe symptoms, ranging from muscle pain to meningitis (Figure 2) [28]. Therefore, it is essential to control the presence of *L. monocytogenes*, through the ministry of agriculture, livestock, and supply (MAPA) normative instruction [29], and the most effective method to do so is to employ an effective sanitation, and sterilize (quaternary ammonium compounds - QAC'S, sodium hypochlorite (NaClO)) [28].

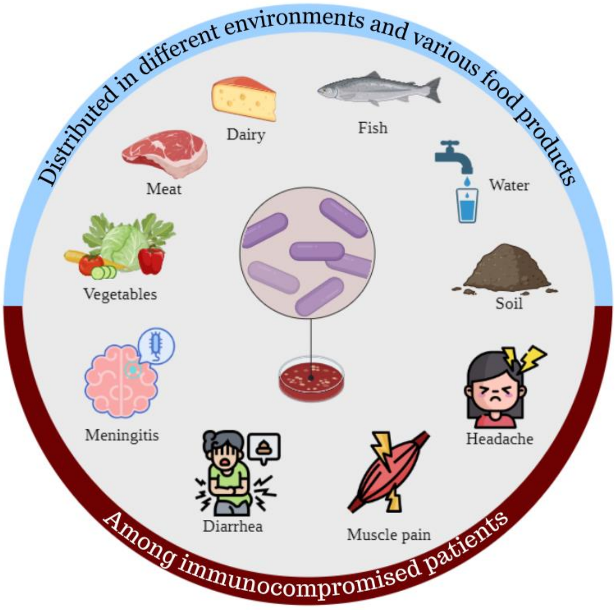


Figure 2. *Listeria monocytogenes'* habitats and immunocompromised patients' symptoms [27, 28].

Regarding periodic control for the presence of *L. monocytogenes*, both the Food and Drug Administration (FDA) bacteriological and analytical method (BAM) and the International Organization for Standardization (ISO) 11290, can be implemented.

To assess the presence of *L. monocytogenes*, it needs the suppression of competitive microflora, since it has a slow growth rate. Bacteriostatic agents (acriflavin and nalidixic acid) are added to enriched media or selective agar to achieve this outcome [30]. *Listeria monocytogenes* is confirmed when the following characteristics are observed: Gram-positive bacilli, non-sporulated, oxidase-negative, aerobic, and facultative anaerobic, ferments sugars, and produces acid without forming gas. Due to the time-consuming nature of these tests and the constant pressure on the food industry to assess its products, there was a need to develop alternative methods for detecting *L. monocytogenes* [tests targeting ribonucleic acid (RNA); Polymerase Chain Reaction (PCR), among others]. The current consensus holds that Enzyme-linked immunosorbent (ELISA) based tests or conventional PCR are the most effective methods [30].

There are several assessments for microbiological stability and safety depending on the type of food, whether it is ready to consume, and whether it was developed by independent food research associations [17]. Hazard Analysis and Critical Control Points (HACCP), good manufacturing practices (GMP), sanitation standard operating procedures (SSOP), and evaluations are currently used to produce products of high quality. In addition to HACCP systems, a wide variety of ready-to-eat foods also use modified atmosphere packaging to maintain or enhance the quality and safety of the final products [24]. Nonmicrobiological parameters used for determining shelf-life are often more tailored to the individual characteristics of the product, in the best-case scenario, these criteria are either contained in the original marketing brief or can be developed from it. When the product safety is successfully established, sensory evaluation is the most common method for determining the end point of shelf-life [17].

2.3 – Active Packaging

Active packaging consists of packaging that provides a function other than supplying an inert barrier against external conditions, in this way, it interacts with the food and in some cases responds to changes making it possible to preserve the chemical and nutritional components of food [31, 32]. With the evolution of food packaging and the escalating demand for ready-to-eat meals and/or the preservation of quickly perishable foods, there was a need for food packaging to no longer have a passive effect on products, but rather to begin having an active effect [33]. Mass transfer (such as oxygen, carbon dioxide, and water vapor) between internal and exterior environments occurs constantly in a packaged food system. Therefore, it is inevitable that packaged foods will degrade due to processes like lipid oxidation and dehydration [34]. The domain of active packaging is incredibly vast, including modified atmosphere packaging (e.g., oxygen, carbon dioxide and equilibrium-modified atmosphere), and intelligent packaging (e.g., sensors and bar codes) [31, 33].

According to their purpose, active packaging systems can be divided into two groups (Figure 3): (1) Emitters, release active substances whether directly in the food product, in the packaging materials or in the internal atmosphere of the packaging to bring beneficial effects in the package, and (2) Scavengers, which minimize undesirable substances like ethylene, oxygen, carbon dioxide, off-odors, and moisture in the packaging environment [35].

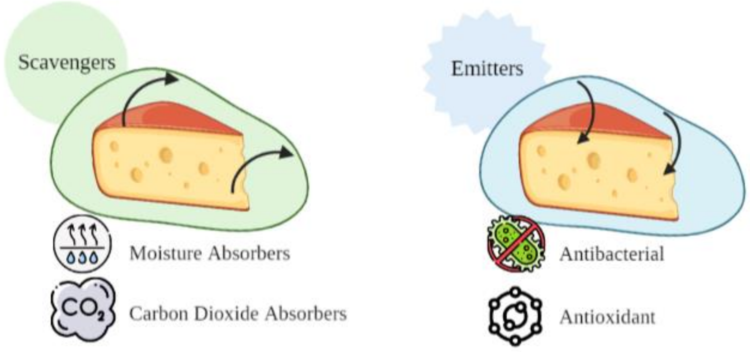


Figure 3. The two groups of active packaging systems [36].

As a result, packages for all sorts of food and diverse functionality exist (Figure 4), such as moisture absorbers, odor and/or taste absorbers, carbon dioxide absorbers, and even ethylene absorbers. There are two elements of emission systems: (a) antibacterial; synthetic agents can be manufactured (organic acids) or natural (EOs, enzymes); (b) antioxidant, in which oxygen absorbers (iron oxide, enzymes) or free radical scavengers are used, it is possible to follow the synthetic (selenium nanoparticles) or natural EOs side [33, 36, 37].

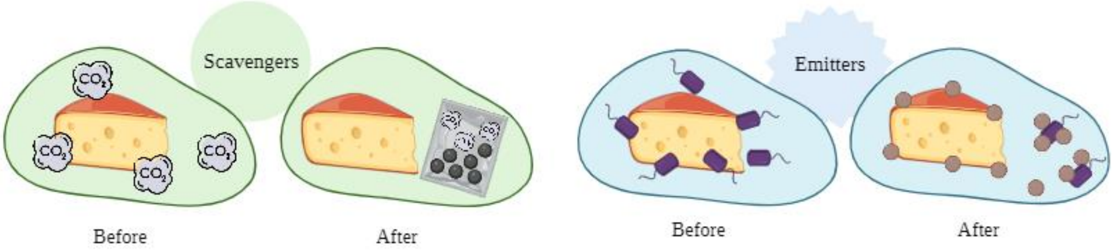


Figure 4. Before *vs* After (active packaging): (Scavengers) Carbon Dioxide scavenger and (Emitters) Antibacterial packaging (adapted from[38]).

The term modified atmosphere packaging (MAP) refers to a method of preserving food by enclosing it in a container with an environment designed to prolong its freshness and maintain its quality, usually a combination of an inert gas (typically nitrogen) and an antimicrobial active gas (typically carbon dioxide). It has low production costs, can be applied quickly and easily, and may be used in various packaging and manufacturing settings. [12, 39]. Atmospheric modification may be accomplished in two ways: actively and passively. The active change gas-flushing involves replacing the air with a carefully calibrated gas combination. Due to respiration and/or the metabolism of microorganisms associated with the food, passive change occurs; the package system typically incorporates a polymeric film, and the permeability of gases through the film varies based on the properties of the film and its storage temperature, additionally impacts the atmosphere's composition [11].

A synchronized food packaging system that allows consumers to obtain data regarding the food supply chain’s quality by monitoring the packaged food’s storage conditions is how the term "intelligent packaging" is often described [40]. This procedure may be carried out with the assistance of various sensors and indicators connected to the packaging system, or it can be carried out with the assistance of colorimetric changes. While the indicators keep track of time, temperature, and pH, the sensors detect, record, and send any changes or information on the product's quality [41, 42].

Generally, active, and intelligent packaging are an exciting breakthrough that allows the food industry to combine the preservation effects of antimicrobials with the protective capabilities of an existing packaging concept, demonstrating the use of smart packaging technology, categorization, and functionalities to enhance food quality control and safety, as illustrated in Figure 5 [43].

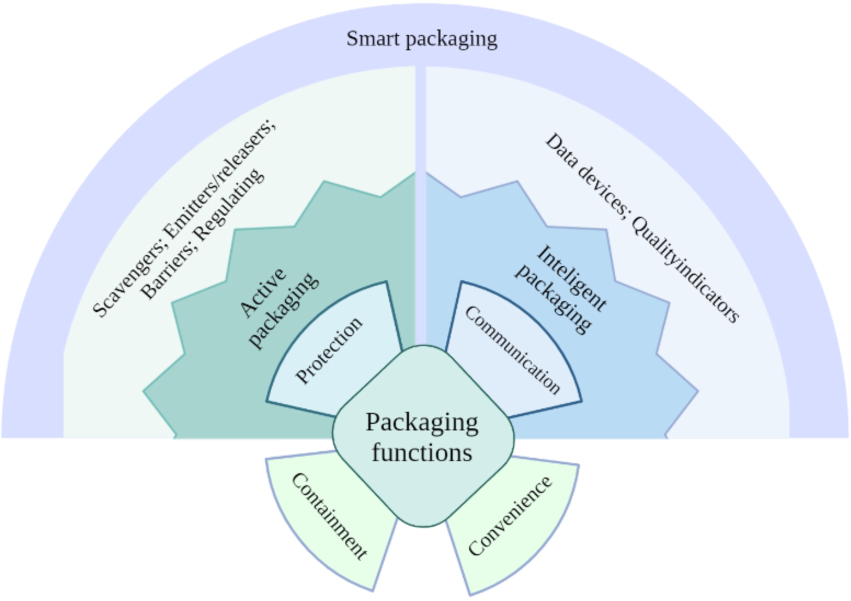


Figure 5. Smart packaging, grouping, and functionality for improving food packaging functions [36].

2.4 – Natural Polysaccharides

Nature presents a great diversity, whether on land, under the sea, or in the air. Each location is home to living and nonliving creatures, and humans have explored every inch to its own advantage [44]. Natural polysaccharides are long-chain monomeric units linked primarily by glycosidic bonds to form polymeric carbohydrate molecules. These macromolecules are employed to create the basic building blocks of life. They fall into one of two categories: homopolysaccharides, made of just one type of monomeric unit, like cellulose, starch, glycogen, and dextran; and heteropolysaccharides, made of two or more types of monomeric units, such as pectin, hyaluronic acid, and polygalacturonides [45, 46].

Nature produces polysaccharides continuously and are frequently regenerable or derived from renewable resources. A resurgence of natural polymers as raw materials across a wide range of applications is being noticed [47]. Additionally, since they can be derived from several sources, including plants (starch, cellulose, pectins), animals (glycogen, collagen, hyaluronic acid), microbes (xanthan, gellan, dextran), seaweeds (carrageenan, alginates), crustaceans and insects (chitin), natural polysaccharides are generally regarded as nontoxic, environmentally friendly, sustainable, and economically advantageous [44].

The principal constituent of cell-wall structures of seaweeds and exoskeletons of crustaceans are marine polysaccharides. Several kinds of edible red seaweed belonging to the *Rhodophyceae* family are used to produce carrageenans, which are linear sulphated anionic polysaccharides [48].

2.5 – Red-Seaweed and Carrageenan

The classification of algae is difficult and has changed over time as the understanding of these organisms has improved. Algae refers to a broad class of aquatic photosynthetic organisms, ranging from microscopic single-celled creatures to larger multicellular forms [49]. In the past, algae were categorized according to their pigments, cell structures, and characteristics related to reproduction. Algae can be divided into several significant groups being the most acceptable and comprehensive, although the most exhaustive classification, developed by the Father of Phycology, F. E. Fritsch, who divided this classification into 11 groups. It can be consulted in his book “Structure and Reproduction of the Algae” or in the publication chapter of Pooja Baweja et al. [50].

In Asian countries, like China, Korea and Japan, edible algae are an important part of the human diet due to their nutritional and functional properties for a long time [51]. In addition to being an essential ingredient in Asian cuisine, seaweed is also used to produce cosmetics, stabilizers, thickening agents, and gellants. This is accomplished by extracting agar and carrageenan from red seaweed and alginate from brown seaweed [52]. In this study, the focus is on the *Rhodophyta* phyla.

The oldest eukaryotic algae (macroalgae) known to exist in freshwater and marine habitats are thought to be red seaweeds. They belong to the most diverse taxonomic category, with around 6500 species presently known [53]. The pigments phycoerythrin, phycocyanin, and chlorophyll a give red seaweed their vibrant red color [50]. Due to the abundance of vitamins, minerals, calcium, magnesium, and many other nutrients, they provide numerous advantages for people, such as serving as a food source. In addition, they support bone health, strengthens the immune system, and nourishes the skin [54]. The concentrations of total polysaccharides in the species of seaweed of interest range from 4 to 76% of dry weight. Agar, alginate, and carrageenan are the three seaweed polysaccharides used for commercial purposes [52]. The primary red seaweed species *Kappaphycus*, *Gigartina*, *Eucheuma*, *Chondrus*, and *Hypnea* are used to extract carrageenan. The two main species frequently used in carrageenan production are *Kappaphycus alvarezii* and *Eucheuma denticulatum* (Figure 6) [53].

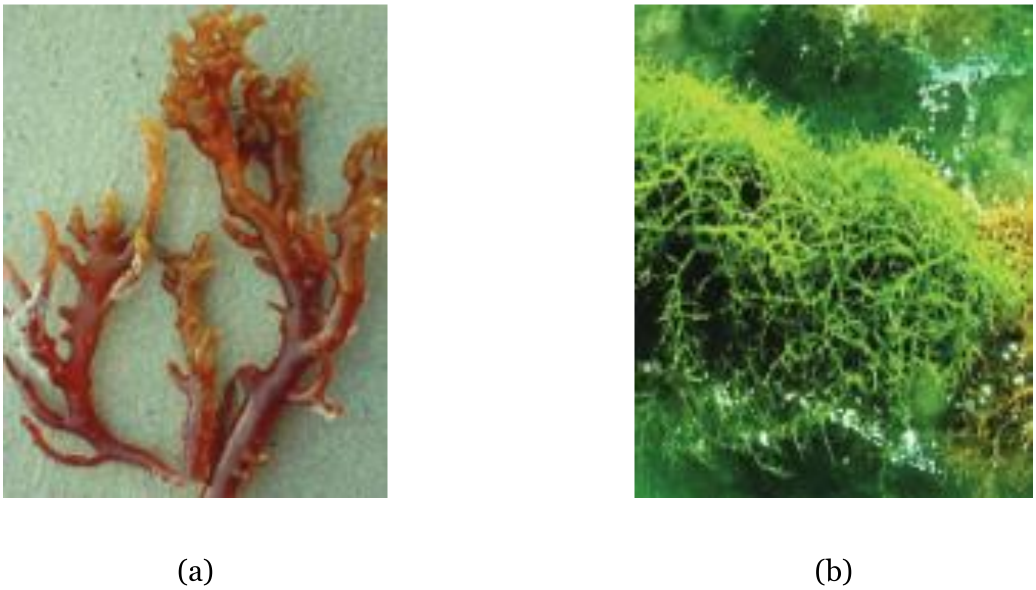


Figure 6. Images of different types of seaweed used as a source of carrageenans. (a) *Euchema denticulatum*, (b) *Kappaphycus sp.*[55].

Historically, carrageenan was derived from wild seaweeds; however, the supply of carrageenan obtained from wild seaweed, need to be improved and in certain areas, such as the United Republic of Tanzania, wild seaweed became depleted. As a direct result, the effective growth of carrageenan-containing seaweeds in tropical seas began in the 1970s [56]. The production of seaweed grown worldwide, in 2019 was 34.7 million tons, or almost $14.7 billion. 52.6% of this total, or more than USD 7.1 billion, comes from red seaweed (*Rhodophyta*), the most important cultivated species being *Kappaphycus/Eucheuma*, *Gracilaria* (warm-water genera), and *Porphyra* (cold-water genus) [53]. Given its rapid growth rates, short harvest periods (100-120 days), high polysaccharide yields, and relatively stable carrageenan composition (which is unaffected by the alga's life history stage), the *Kappaphycus* genus has become one of the essential sources of carrageenan [57]. Seaweed was used to extract carrageenans for the first time in 1837, and the *Rhodophyceae* family has been the principal source of carrageenan extraction. *Irish moss*, or *C. crispus*, is thought to be the original source of carrageenans and mostly consists of a mixture of kappa- and lambda-carrageenans [55].

After receiving the dried and sliced weeds at the processing facility and testing the samples to determine the quality of the extractive, the extraction procedure may begin [55]. The unrefined carrageenan must initially be isolated from the source. First, the raw materials will be cleaned to remove any traces of sand, salt, or other contaminants. Alkaline treatment, ranging from 95 to 110°C, is often used to extract carrageenan; however, the success of this process is highly dependent on the quality of the alkaline solution used. Once a solution like sodium hydroxide (NaOH), potassium hydroxide (KOH), or calcium hydroxide ($CaOH_2$) is added to the extract of seaweed for a long time, an alkaline environment can be made [57]. Filtration or centrifugation is used to refine the extracted carrageenan. Following the extraction, the solution is filtered through activated charcoal or porous silica to provide a carrageenan concentration of 1%-2%. A further significant step is to concentrate the carrageenan to 2%-3%. This typically occurs with multistage vacuum evaporators [55]. The carrageenan can then be separated from the solution in one of two ways: by adding alcohol (mainly isopropyl alcohol) or by making a gel press in a potassium chloride solution. All carrageenans can be purified using the alcohol-precipitation technique, but k-carrageenan is the only one that can be extracted using the gel approach. Once the gel was formed, any residual water may be squeezed out in a press [57]. To produce a clear solution of pure carrageenan, the precipitated carrageenans must be dried, crushed, and redissolved [57].

Carrageenans are high-molecular-weight polysaccharides (between 100 and 1000 kDa [58] and density of 1.002 g/cm^{-3} [59]) composed of repeated *D*-galactose units and 3,6-anhydro-*D*-galactose [52]. Glycosidic connections of the types α-1-3 and β-1-4 alternate between joining the units, as shown in Figure 7 [60].

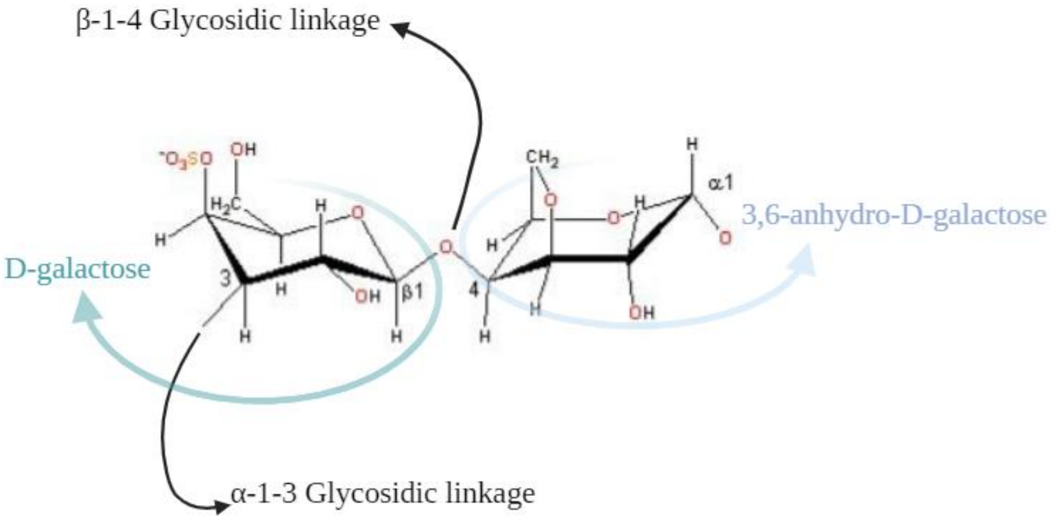


Figure 7. Chemical structure of k-carrageenan (adapted from [60]).

Kappa (k), Iota (ι), Mu (μ), Nu (ν), Theta (θ), and Lambda (λ) carrageenan are the six primary types. Both their chemical classification and commercial manufacturing can benefit from this nomenclature [49, 58, 61]. Carrageenan can dissolve in water, producing extremely viscous solutions, and maintain stability over a wide pH range (6-10) according to their unique characteristics, hence, they are used in several industrial operations having the EU additive E-number E407 or E407a when used in food products [58, 60].

The research into producing carrageenan-based films for food packaging is a relatively recently established and growing field. Although there are some studies where they are applied in packaging for fresh and frozen fish, fruit coatings, and meats, among others [62–64], there is still, a limited number of published papers that provide a comprehensive assessment of the prospects for these films, as well as the current approaches to improve their properties [63].

Carrageenan polymers have a compact film structure, and their high gelling capacity confers excellent film-forming properties [62].

Therefore, acquiring knowledge on enhancing the characteristics of these films, distinguished by their biodegradability, biocompatibility, and cost-effectiveness, becomes imperative [63, 65].

2.6– Essential Oils

The history of EOs may be traced back to the East as well as the concept of distillation, which forms the technological foundation of the EO business. They were originally thought and put into practice in the Orient, particularly in Egypt, India, and Persia [66]. The terminology "essential oil" was originally used in the 16th century by Paracelsus von Hohenheim, who called the active ingredient in a remedy "quinta essentia" [66].

The synthesis of volatile compounds takes place in specialized cell types that may be found in almost every section of plants, like trees (sandal), flowers (jasmine, rose), grasses, roots (ginger), and fruits (anise) [67, 68]. These glandular trichome cells (Figure 8) also include adduct cavities and osmophores, the sites where the chemicals are collected after being biosynthesized [67].

Figure 8. Microscopic sideview of a glandular trichome and branched protective trichomes in the background, magnified 400×; from *Lavandula angustifolia* [69].

Derived from aromatic and medicinal plants through expression, or steam distillation, EOs are a complex mixture of volatile plant compounds, manly terpenes and terpenoids [70]. However, it should be noted that the chemical composition of an EO can change from plant to plant, and even within the same species. Numerous elements, including abiotic and biotic factors, post-harvest treatment, extraction techniques, and storage conditions, are linked to these variations in the chemical composition [71].

EOs are one of the finest alternatives to synthetic food additives, due to their potent biological activities, and are employed in various applications in the food and beverage sector. These applications include providing a natural taste and scent and serving for preservation [72]. EO ingredients are so effective at preventing food from spoiling that they are included in the packing films development and production [73]. Not only they are used in the food industry, but also in cosmetics with numerous skin regenerating properties and in pharmacology as active ingredients [67, 74].

The most applied techniques for chemical analysis of EOs are, for their widespread use for chemical profiling, gas chromatography (GC), gas chromatography combined with mass spectrometry (GC-MS), for its ease of use, environmental friendliness, and long-term cost-effectiveness [71].

GC is based on separating a single molecule from a complex matrix between two phases according to the phases' affinities, shapes, and masses. The mobile phase is the one that travels by capillarity or pressure moving substances toward the stationary phase more quickly or more slowly. In GC, the stationary phase is solid at room temperature and the mobile phase is a gas. However, the stationary phase will partially melt at high temperatures but will still be stuck on the column. Typically, the column is coupled to a flame ionization detector (FID) used for EO analysis [71].

2.6.1 – *Cymbopogon winterianus*, Jowitt

There are two different *Cymbopogon* species, *C. winterianus*, Jowitt (Java citronella) (Figure 9) and *C. nardus* L. Rendle (Ceylon). They have similar volatile oil scents and therapeutic applications and present differing citronellal contents. Geraniol (36.0%) and citronellal (42.7%) are abundant in Java citronella [75].

Figure 9. *Cymbopogon winterianus* [76].

There are 140 species in the genus *Cymbopogon* (*Poaceae* family), more than 52 of which have been documented to occur in Africa, 45 in India, 6 in Australia and 6 in South

America, 4 in Europe, 2 in North America, and the remainder spread across South Asia [77].

Resilient and aromatic, Java citronella, henceforth referred as citronella, is a kind of grass that may reach a height of 2.5 m from a rootstock. The leaves are long and thin, measuring between 0.5 and 1.6 cm in width and growing to a length of 1 m. They have a greenish-white tip, a white midrib, and an upper cauline wider than 9 mm but narrowing toward the base [78].

They are very stress-tolerant plants that readily adapt to a variety of edaphoclimatic conditions [79]. Although genetically controlled, the ambient, harvest, and post-harvest conditions significantly impact on the production of secondary metabolites [75].

Cymbopogon species thrive in the warm, humid conditions of the tropics and subtropics. They often like hot, humid climates with plenty of sunshine during all the year [80].

Citronella grows in soil with a pH range of 5.8 to 8.0. Water logging should be avoided to produce the best yields because the crop is particularly sensitive. High atmospheric humidity and evenly distributed rainfall positively impact plant development, production, and EO quality. Lower yields are reportedly the result of citronella's growth being constrained at greater altitudes above 400 m [76].

The preferred multiplication method for citronella is often vegetative since seed propagation is time-consuming and poses the potential for hybridization between *C. winterianus* and *C. nardus* [81]. The optimal time for planting is in subtropical areas with reliable irrigation, just before it starts to rain. However, it can also be done in the months of January through February, February through March, and June through July [82, 83]. September and October saw the highest yields and citronellal concentrations. Geraniol and citronellal content decreased during a drought whereas citronellol content increased [75].

Overall, EO is present in every section of the plant, but it is most abundant in the leaves. Also, the quality of the EO from other regions is poor. Thus, it should only have its leaves harvested [83]. Following the process of harvesting, the citronella leaves are first left in the field to undergo the natural progression of drying or can be dried at room temperature (28°C) until brittle [81]. To boost the amount of EO, the leaves are cut before extraction, so they contain the greatest surface area [83].

Many elements, including soil, climate, age of the crop, and distillation efficiency method, impact the EO content of the leaves. EOs can be extracted using traditional distillation techniques including steam distillation and hydro-distillation. The produced

EO is a natural source of key compounds used in fragrance, such as citronellol, geraniol, among others, widely used in the global soap, perfumery, cosmetic, and flavoring industries [83].

Although citronella EO has several applications in aromatherapy, it is most well-known for its inherent ability to repel insects. Multiple investigations have shown that citronella EO is mainly composed of citronellal, with citronellol and geraniol being the subsequent major compounds (Figure 10) [83–86]. Moreover, it has been shown that the repellent capacity is mostly attributed to the presence of citronellol and citronellal, hence substantiating their potential as repellents [84]. Among other EO, citronella EO proved to be the most effective at repelling insects for two hours [87].

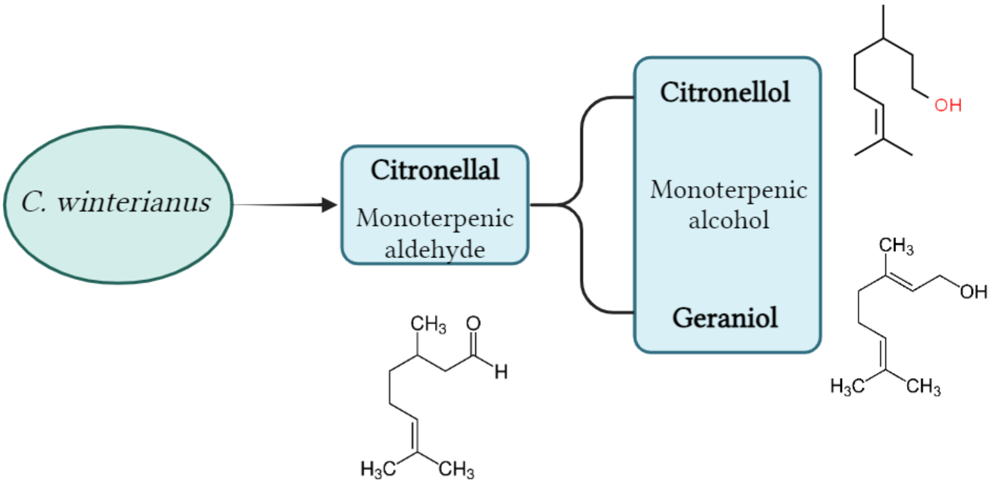


Figure 10. Main constituents of *C. winterianus* [88–90].

The principal chemical compounds found in citronella are monoterpenes and are classified as a vast and heterogeneous group of naturally occurring compounds. Monoterpenes, also known as monoterpenoids, are composed of two interconnected isoprene units, forming their fundamental structure. They can suffer cyclization and oxidation allowing EOs to have low molecular weight. Monoterpenes have several pharmacological activities, including antibacterial, anti-inflammatory, antioxidant, antipruritic, hypotensive, and analgesic effects [91].

In terms of therapeutic applications, most citronella EOs are restricted to their usage as a mosquito repellent, as said before, but they can also be used as antiparasitic, nematocidal, antifungal, and antibacterial agents [83, 92]. Simic's et al. research demonstrated the potential of citronella to possess antifungal and antibacterial effects, notably against Gram-positive bacteria [85]. The study conducted by Oliveira et al. provides more support for the antifungal properties of citronella EO, specifically concerning its effectiveness against *Candida albicans* and *Trichophyton* species [93].

It can also be applied as a massage oil for sore muscles and joints. With respect to traditional treatments, fever, intestinal parasites, digestive issues, and menstruation problems are among the applications [83, 94].

2.6.2 – *Lavandula viridis* L.Her.

One of the most well-liked medicinal plants with significant commercial importance is *Lavandula* (*Lamiaceae*). About 39 different species constitute the genus *Lavandula*, but only *L. angustifolia* (lavender), *L. hybrids* (lavandin), and *L. latifolia* (spike lavender) are harvested for their EOs [69, 95]. Species belonging to the genus *Lavandula* are native to the Mediterranean area. However, they have been successfully grown in other parts of the globe, including Europe, Southwest Asia, India, the Arabian Peninsula, and North and South America [96]. *Lavandula viridis* L.Her. is a xerophytic aromatic shrub that may grow to be as tall as 40 cm and has ovoid-cylindrical spikes composed of white flowers, which can range around 6 and 8 mm in diameter (Figure 11) [96].

Figure 11. *Lavandula viridis* plant [96, 97].

Several studies reported the antibacterial, antifungal, and anti-cholinesterase properties of many EOs from this genus. The neuroprotective, anti-inflammatory, and antioxidant activities of *Lavandula* extracts were also shown [98, 99].

Due to their use in the food and fragrance sectors and more recently in aromatherapy, *Lavandula* EOs are very interesting. The flavoring of beverages, ice cream, candy, and chewing gum has been achieved in the food industry by using volatile chemicals. The EOs have been used in cosmetics such skin lotions, soaps, colognes, perfumes, and other skin care products in the fragrance industry [99]. The physiological effects of the inhaled volatile chemicals lead to the use of *Lavandula* species in aromatherapy. In addition to their effects on the central nervous system, animal investigations have demonstrated that *Lavandula* species have antispasmodic effects on the smooth muscles of the uterus and the ileum. Additionally, acaricidal and antimicrobial activity against several bacterial and fungal species have been documented for lavender EOs [100].

Considering that is a perennial plant, lavender may grow in the same location from 8 to 15 years up to 20 or 30 years. It is possible to begin planting the crop from spring through October; however, farmers discovered that planting in the fall results in more blossoms the following year [69].

Lavender thrives best in a climate that has warm summers and chilly winters. Additionally, the soil in which it grows should be porous, somewhat rich, and have a pH that ranges from neutral (6.4) to slightly alkaline (8.2). For both the germination process and the transition into the vegetation stage, a reasonably high temperature is required: 10–15ºC [69].

Propagation of lavender plants may be accomplished by cultivating seeds, cuttings, division of existing plants or layering [69]. If the plant grows from seed, the quality of the EO, the color of the flowers, or the growth habit of the plant would not be known precisely [101]. Most of the lavender may be grown from cuttings at any moment of the year, but the end of summer or the beginning of fall is often suggested. The most successful cuttings are often taken late in the summer from robust plants because these plants have thick stems that root easily [101]. When taking tip cuttings from mature plants, the length of the cutting should be between 3 and 8 cm. After the cuttings have been placed in a propagation mix that drains well, they should be shielded from sunlight and frost, and they should be misted many times each day. Within three to four weeks, it is possible to get plants that have roots. [101].

Lavandula viridis, has the potential to reach a height of 70 cm and a diameter of 80 cm. It blooms in the spring or summer with white flowers. Although it can survive at temperatures as low as 5ºC, it grows best in bright sunlight or partially shaded but well-lit locations [69, 101].

The southern region of Portugal, Algarve, is extremely abundant in aromatic plants, particularly lavender species. There are several species of *Lavandula*, of which the *L. viridis* (“rosmaninho-menor”, “rosmaninho-verde” or “rosmaninho-branco”) is part. The Southwest Iberian Peninsula, Madeira, and the Azores Islands are all home for *L. viridis* [105].

The blossoming of the lavender plants, which occurs between June and July, marks the beginning of the harvesting process in the second year of the crop. The flowering tops and inflorescence of the plant are what is taken to extract the EO. These plant parts are picked after the plant is fully blossoming. The volatile EO content of fresh lavender flowers ranges from roughly 0.7–1.4%. Some of the volatile EO in the plant material is lost during the drying process [69]. Nowadays, most herbal EOs are distilled via steam distillation. When exposed to steam, the EO glands burst, resulting in oily splotches. The steam condenses on the outside of the plant, releasing latent heat and increasing the temperature of the top layers. After the condensing steam has risen the temperature to about 98 ºC, the vapor pressures of the water and EO combine to bring the liquids to a boil due to their additive qualities. Saturated vapors of EO ascend, and when cooled in a condenser, they return to a liquid state. The liquid is directed into a separator. The EO is separated off the surface of the water because it is less dense than water therefore it floats [101].

According to the research conducted by Vairinhos *et al.*, the major compound of *L. viridis* EO is 1,8-cineole, a monoterpenic ether [96]. According to Machado's et al. investigation, camphor, a monoterpenic ketone, was identified as the second principal compound (Figure 12) [102].

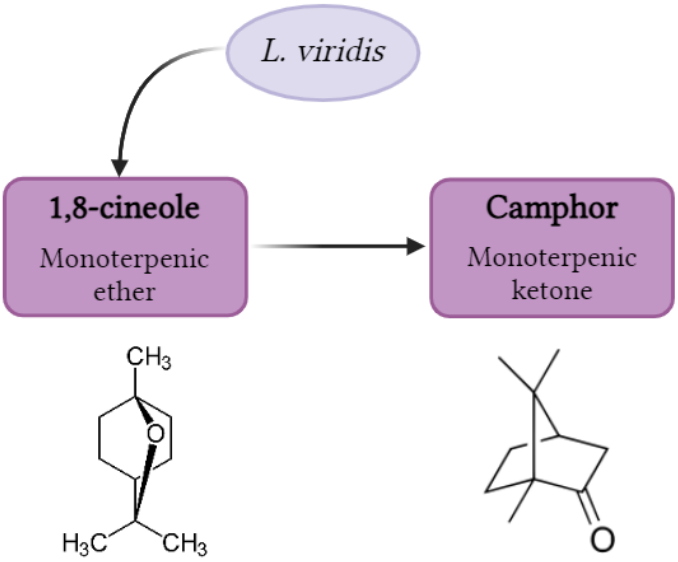


Figure 12. Main constituents of *L. viridis* [103, 104].

Lavender EO is widely sold because of its versatility in many different contexts, including fragrance, medicine (for treating wounds), aromatherapy (for relieving migraines), and even as a body oil [69, 101].

2.7 –Preparation of Films

The use of biopolymers producing edible films has received significant attention over the past two decades as an effective substitute of petroleum-based packaging materials [40]. Nevertheless, it has been reported that the structure of films made from these biopolymers is brittle due to substantial intermolecular forces, which can be reduced by adding a plasticizer [40].

Plasticizers are a group of nonvolatile compounds with a low molecular weight extensively used as additives in the polymer industry. The primary function of these compounds is to enhance the flexibility and ability to process polymers by reducing the glass transition temperature (Tg) [105]. Glycerol is broadly incorporated into most hydrocolloid films, since it is an extremely hygroscopic molecule that allows to avoid film fragility [106]. Despite the capacity of glycerol to enhance elasticity, it ultimately presents a drawback. Elasticity enhancement reduces the strength of internal dipole-dipole interactions among the polymeric chains, increasing intermolecular space. This increased space facilitates the penetration of oxygen and moisture into the films [107].

When producing an active packaging with active compounds incorporated in the polymer matrix, the active agent is intimately mixed with the polymer by dissolving both substances in an appropriate solvent (casting procedure) or by melting the polymer and incorporating the antioxidants into the melt (extrusion procedure). Another technique involves the immobilization of antioxidants on a polymer surface that has been treated with physical or chemical agents to promote the adhesion of the active compound (coating procedure) [108].

The casting method (Figure 13) includes dissolving the polymer that will serve as the film matrix in an appropriate solvent while simultaneously incorporating the intended active agent. After pouring the polymer/active agent solution into a surface and allowing the solvent to evaporate, a plastic-like film is produced. This method typically produces films containing natural bioactive agents (EOs and natural extracts) [108].

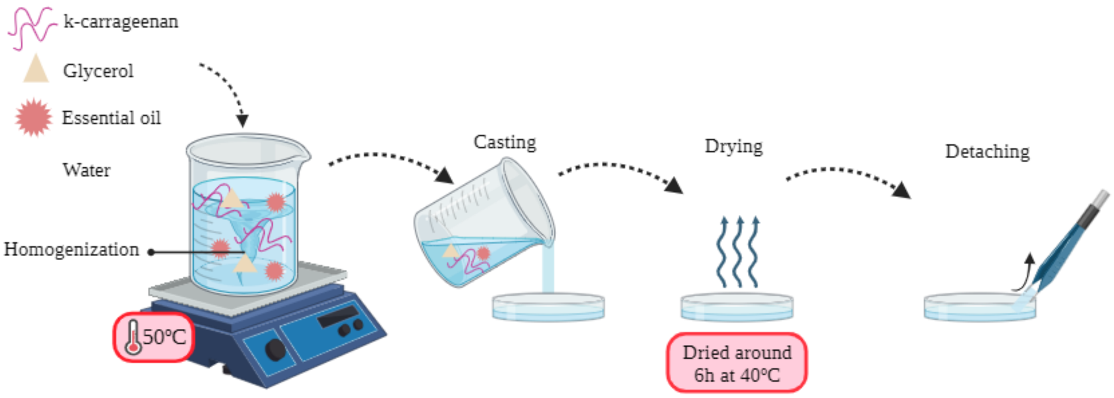


Figure 13. Casting method with the materials used in this work [109].

In the extrusion method, the antioxidant is integrated into the polymer matrix, which melts because of the extruder's high shear forces and temperatures [108].

The coating process involves using and immobilizing antioxidant compounds on the film's surface. This method has specific benefits over alternative methods. The active ingredient is spread on the package's interior layer, and no aggressive thermal or mechanical processes are employed, enabling the incorporation of active compounds with minimal activity loss [108].

After the obtention of the films, they must be characterized for their physical properties, which influence aspects such as the films' flexibility and hydrophobicity, barrier and optical properties (which are crucial for the consumer to judge the quality of the packaged product), and bioactive properties (Figure 14).

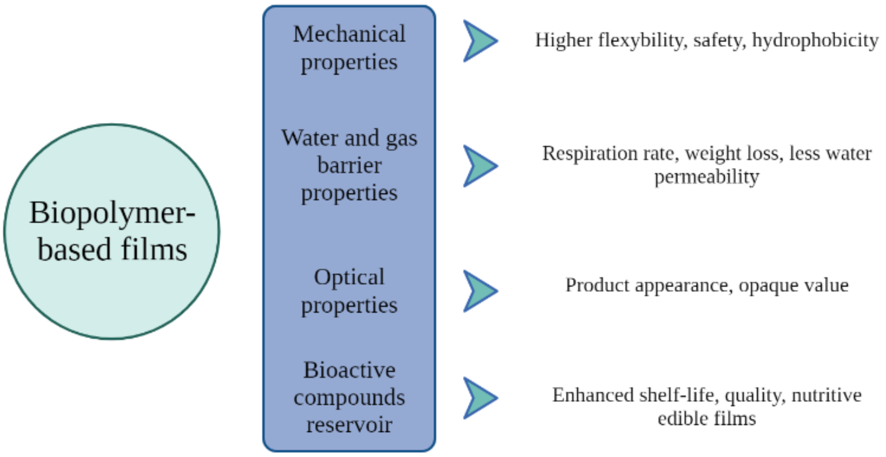


Figure 14. Biopolymers impact special functionalities in the film matrix, such as mechanical, water and gas barrier, and optical qualities, as well as a reservoir for bioactive substances (adapted from [40]).

2.8 – FTIR and DSC Analysis

The main technique of infrared spectroscopy is the Fourier-Transform Infrared Spectroscopy (FTIR) [110]. Infrared spectroscopy, which relies on the study of absorption peaks at certain wave numbers (cm^{-1}), is one of the most helpful methods for detecting polysaccharide structures [111]. In this method, infrared radiation is sent through the sample, with some of it being absorbed and the rest being transmitted. The spectrum acquired is the consequence of molecule absorption and emission, which forms a fingerprint zone of the sample, allowing identification, assessing their quality, and the number of components in the samples [110].

Differential Scanning Calorimetry (DSC) analysis is a thermodynamic technique that permits direct evidence to support the heat sustained by a sample in a controlled environment where the temperature is either increasing or decreasing. Calorimetry is the only direct method for determining the enthalpy associated with the process of interest, and it is a major technique for measuring the thermal characteristics of materials to establish a link between temperature and certain physical properties of substances, by performing a mathematical integration on the curve that represents the variation in heat flow over time [112]. This enables the determination of phase transition modification such as melting, crystallization, and others [113, 114].

2.9 - Evaluation of Physical Properties of the Films

Packaging is a crucial part of today's economy since it ensures that food items retain their original quality throughout transport. It's also vital since it keeps foods fresh and prevents contamination from harmful environmental factors [35].

Films used for food packaging should have favorable mechanical characteristics, such as Tensile Strength (TS) and Peak Elongation (PE). Materials with the right combination of mechanical properties may protect food from damage throughout handling, storage, distribution, and consumption [40]. Materials' tendency to break under stress constitutes their tensile characteristics [115].

The tensile test is used to measure several of the films' mechanical properties, and the results (TS, PE Tensile Index (TI), and Elastic Modulus (EM)) are reported here. A material's TS (N/m) is the maximum stress that may be applied before it breaks. The TI is calculated dividing the tensile strength (N/m) by the grammage (g/m^2) [116]. EM (MPa) assesses how well a material resists elastic deformation. When stretched, materials with a low EM can stretch a lot. The reverse occurs with high EM materials [117]. The PE is the percentage of the new length as compared to the original length after the specimen has been broken. It indicates how much a material can withstand deformation without developing cracks [118].

It is possible to calculate the surface free energy of a packaging material using the approach developed by Owens and Wendt (OW) by measuring the contact angle of the material's surface with three pure liquids (typically, water, ethyleneglycol and diiodomethane). It's a thermodynamic metric that describes the equilibrium of atoms in a material's surface layer. Each material has its own signature amount. The phase border between the two media, reveals the unique imbalance in intermolecular interactions. Methods of calculation based on contact angle measurements are grounded on the Young equation. It was developed from the equilibrium of forces representing surface tensions at the interface between a solid, a liquid, and a gas. It is also possible to obtain the Young equation given the triple point energy balance:

$$\gamma_S = \gamma_{SL} + \gamma_L \, cos\theta \quad (1)$$

Where γ_S is the surface free energy of solids in vacuum, γ_{SL} is the surface tension on solid-liquid phase separation, γ_L is the surface free energy of the liquid and θ is the contact angle measured on the studied surface [119].

Based on the Bethelot theory, the OW method states that the interactions between molecules of the two substances present in their surface layer are equivalent to the geometric mean of intermolecular interactions inside each substance, involves identifying components of dispersion and polar surface free energy. When taken together, polar, hydrogen, inductive, and acid-base interactions make up what is known as the polar factor. To calculate surface free energy the following formula can be used:

$$\gamma_S = \gamma_S^d + \gamma_S^p \quad (2)$$

Where γ_S is the surface free energy, γ_S^d is the dispersive component of the surface free energy and γ_S^p is the polar component of the surface free energy [119].

Customers react differently depending on the kind of packaging, the product's brand, and the colors and shapes employed [120]. The main desired color factor is that the films do not present color or opacity, so that the packaged food can be fully visible to the consumer. By maintaining a steady correlation between color values and how they are perceived, the CIE L*a*b* color system enables numerical representation of colors. The color coordinates a* and b* range from +a* (red) to -a* (green) and +b* (yellow) to -b* (blue), whereas L* corresponds to brightness and ranges from 0 (black) to 100 (white) [121].

In optics, transparency refers to an object's physical attribute of allowing light to flow through it freely. Transparent materials allow the greatest amount of incoming light to flow through them; translucent materials allow some light to pass through but at a reduced intensity; and opaque materials prevent any light from passing through at all [122].

Chemical groups present in the polymer (polar or not), Tg, and crystallinity play a role in the polymer's barrier properties, which are in turn strictly correlated to the polymer's intrinsic structure [123]. The mechanical characteristics of the materials have a direct impact on the barrier qualities, which are often assessed for water vapor, oil, and oxygen [124].

2.10 - Evaluation of Antioxidant and Antimicrobial Properties

Numerous health benefits have promoted antioxidants to the status of a crucial nutraceuticals [125]. Substances that have the potential to scavenge free radicals are often characterized by their antioxidant capacity. In biological systems, this ability is linked to the availability of chemicals that can scavenge free radicals [126]. The well-known antioxidant tests rely on the scavenging capacity of 2,2-difenil-1-picrilhidrazil (DPPH) radicals and β-carotene bleaching assay [127].

The widely used DPPH• antioxidant test is based on the scavenging of DPPH radicals [125]. When a DPPH radical solution is combined with an antioxidant/reducing compound, its color changes from purple to the corresponding hydrazine's yellow. By tracking a reduction in absorbance at 517 nm as the forming hydrazine $DPPH_2$ produces a yellow solution, or by electron spin resonance, the reducing capacity of antioxidants regarding DPPH• can be determined [126].

In the β-carotene bleaching assay the major component of the reaction medium is a β-carotene/linoleic acid emulsion in water with produced peroxyl radicals (LOO•). During the experiment, the concentration of β-carotene is measured in both a control sample and a sample containing the investigated antioxidant. Immediately after the depletion of the studied antioxidant, β-carotene interacts with peroxyl radicals in the sample. The spectrophotometric measurement of β-carotene loss occurs at 470 nm. Antioxidant activity is often represented as a percentage compared to an untreated control in this approach [128].

The most common and fundamental techniques to evaluate antibacterial properties are disk diffusion and broth or agar dilution. A standard inoculum of the microorganism under investigation is used to inoculate agar plates. After that, the necessary concentration of the test substance is spread throughout the surface of the agar in cellulose paper discs with 6 mm in diameter. The Petri dishes are kept warm and humid in an incubator. The diameters of inhibition zones where the microorganism was unable to growth are typically assessed after an antimicrobial agent has diffused into the agar [129].

Chapter 3
Objectives

3.1 – Main and Specific Objectives

The main purpose of this work was to develop and characterize two innovative bioactive food packaging materials using k-carrageenan films incorporated with two EOs, namely *C. winterianus* and *L. viridis*. As a result, the following specific goals were established:

- Chemical characterization using GC and FTIR of *C. winterianus* and *L. viridis* EOs;
- Evaluation of the antioxidant, antibacterial and anti-quorum sensing properties of both EOs;
- Production of k-carrageenan films incorporating different volumes of *C. winterianus L. viridis* EOs;
- Characterization of bioactive films using DSC and FTIR;
- Physical characterization of the produced films, determining the following properties: structural (thickness and grammage), mechanical (TS, TI, PE, EM) and optical (color coordinates and transparency);
- Determination of the contact angles of the films, as well as their surface free energies;
- Evaluation of the barrier properties (water and oil) of the bioactive films;
- Evaluation of the antioxidant, antibacterial and anti-biofilm properties of the films;
- Simulation of the application of films on a food model, by wrapping and sealing cheese slices.

Chapter 4

Materials and Methods

4.1. – Reagents

The polysaccharide k-carrageenan (CAS number: 11114-20-8) was purchased from Tokyo Chemical Industry CO.LTD (Tokyo, Japan). The *C. winterianus* EO was achieved from "Herdade Vale Côvo", extracted by hydrodistillation, using the leaves. *L. viridis* EO was purchased in "D'Alenguadiana". Plant's aerial parts were obtained to extract the EO by hydrodistillation. Glycerol (anhydrous) (CAS Number: 56-81-5) was purchased from Merck (Darmstadt, Germany).

4.2. – EO Chemical Analysis: GC-MS

The EOs were stored at room temperature (±25ºC) and protected from light until used. The chemical composition of the *C. winterianus* and *L. viridis* EOs was analyzed by the company CHROMESSENCE through GC-MS, by using the standard protocol ISO 7609:1985. The program ran at a temperature of 50°C for 6 minutes before increasing by 2°C per minute to 190°C and then by 4°C per minute to 220°C. After remaining at 220°C for 10 minutes, the program switched to its final two modes, which were 4°C per minute up to 240°C and remained at that temperature for 10 minutes. Helium served as the carrier gas, with injection volumes of 0.1 µL (FDI) and 0.1 µL (MSD) and head pressures of 33 Psi (FID) and 25.5 Psi (MSD), respectively [130, 131].

4.3 – Evaluation of the Antioxidant Activity

The antioxidant activity of the two EOs was evaluated using the DPPH free radical scavenging method and the β-carotene bleaching test.

4.3.1 – DPPH Free Radical Scavenging Method

Briefly, 3.9 mL of three different DPPH• methanolic solutions (0.08, 0.1242 and 0.2 mM) were combined with 100 µL of various concentrations of both EOs (5%; 3.75%; 2.5%; 1.25%; 0.5% and 0.25%). The absorbances were measured at 517 nm after 90 min of incubation at room temperature in the dark using a spectrophotometer. Equation (3) was used to determine the radical scavenging activity.

$$\%Inhibition = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (3)$$

Where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance in the presence of the EO.

By graphing the concentrations of the EOs against the corresponding %Inhibition, a calibration curve was built to estimate the IC_{50} and the results were presented as Antioxidant Activity Index (AAI), which was calculated, using equation (4):

$$AAI = \frac{final\ concentration\ of\ DPPH\ in\ the\ negative\ control}{IC_{50}} \quad (4)$$

According to the work of Scherer and Godoy, AAI values could be categorized as poor (AAI ≤ 0.5), moderate (0.5 < AAI ≤ 1.0), strong (1.0 < AAI < 2.0), or very strong (AAI ≥ 2.0). The DPPH• solutions were made each day, and the assays were performed in duplicate [132, 133].

4.3.2 - β-Carotene Bleaching Test

For the β-carotene bleaching test, 40 µL of linoleic acid, 400 µL of Tween 40, and 1 mL of chloroform were combined with 500 µL of a β-carotene solution (50 mg/mL in chloroform). Following the vacuum-assisted evaporation of the chloroform, 100 mL of distilled water previously saturated with oxygen were added to the mixture to produce an emulsion. After that, 300 µL of EOs methanolic solutions were added to test tubes along with 5 mL of the emulsion. 5 mL of the emulsion and 300 µL of methanol served as the negative control. The tubes were then agitated and submerged for 1 h at 50°C in a water bath. Using a spectrophotometer and a blank emulsion without β-carotene, the absorbance of the samples was measured at 470 nm. At the beginning of the incubation period (t=0 h) and the end of the incubation period (t=1 h), measurements were made in duplicate. The antioxidant activity of the EOs was determined as % Inhibition of β-carotene [134].

$$\%Inhibition = \frac{A_{sample}^{t=1h} - A_{control}^{t=1h}}{A_{control}^{t=0h} - A_{control}^{t=1h}} \times 100 \quad (5)$$

where $A^{t=1h}$ is the absorbance of the sample or negative control at the final time of incubation, and $A^{t=0h}$ is the absorbance of the negative control at the initial time of incubation [134].

4.4 - Antibacterial Properties Assessment

4.4.1 – Solid Diffusion Assay

The antibacterial activity of both EOs was determined by the disc diffusion method. Six bacterial species: three Gram-positive (*S. aureus* ATCC 25923, *L. monocytogenes* LMG 16779 and *E. faecalis* ATCC 29212), and three Gram-negative (*E. coli* ATCC 25922, *Salmonella* Typhimurium ATCC 13311 and *P. aeruginosa* ATCC 27853) were used. The bacterial strains stock cultures were maintained in 20% (v/v) glycerol at -80°C. Before the antibacterial assays, all the strains were subcultured in Brain-Heart Infusion agar (BHI) for 24 h [134].

For the solid diffusion assay, the bacteria were suspended in a sterile saline solution (NaCl, 0.85% *w/v*) to a cell suspension of 0.5 McFarland to obtain a bacterial density of $1\text{-}2\times10^8$ colony-forming units/mL (CFU/mL). Bacterial suspensions were used to inoculate Müeller-Hinton agar (MHA) plates. Sterile blank cellulose discs (6 mm) were soaked with 15 µL of the EOs that were then applied on the top of the inoculated culture medium. The plates were incubated at 37°C for 24 hours and the diameters of inhibition zones were visually examined using a digital pachymeter [134]. Additionally, the plates were observed by optical microscopy, using a Nikon Labophot-2 microscope (Nikon, Tokyo, Japan) equipped with a Leica MC190 HD camera and (Leica, Wetzlar, Germany) controlled by the LAS v4.13 software [135], to verify the microbial growth inhibition after the incubation period. The results were achieved by three independent assays.

4.4.2 – Resazurin Microtiter Assay

The resazurin microtiter assay was used to determine the EOs' Minimum Inhibitory Concentrations (MIC). Serial two-fold dilutions of both EOs (from 32 to 0.25 µL/mL) were made in a 96-well plate (50 µL/well) using MHB. Following 30 µL of fresh MHB, the resazurin indicator solution (10 µL, 0.1% *w/v*) was added to each well. The wells were then filled with the bacterial suspension (10 µL, 0.5 McFarland). The plates were made in triplicate and incubated for 24 h at 37°C. Following a visual evaluation, the color transition from purple to pink or colorless was noted as favorable. The MIC value was determined to be the lowest concentration at which the color shift was noticed [134].

4.4.3 – Quorum-sensing Inhibition

The anti-quorum sensing properties of both EOs were evaluated using the biomonitor bacteria *Chromobacterium violaceum* ATCC 12472. The *C. violaceum* ATCC 12472 bacterial suspension was produced by aerobic overnight growth (30°C, 250 rpm) in Luria-Bertani (LB) medium. To seed *C. violaceum* ATCC 12472 suspension on LB agar plates, its turbidity was adjusted to an OD_{620nm} of 1. The plates were covered with sterile blank filter discs soaked with 15 µL of the EOs and incubated for 24 h at 30°C. After the incubation period, a digital pachymeter was used to assess the anti-quorum sensing activity, which was determined by the inhibition of the violacein pigment formation around the disc (a ring of colorless but living cells) [134, 136].

4.5 – Production of k-Carrageenan/EO Films

The solution casting technique was used to produce the bioactive films. Initially, 1 g of k-carrageenan was dissolved in 50 mL of distilled water, and the mixture was agitated for 10 minutes using a magnetic stirrer at 50°C. To further investigate the effect of incorporating both the EOs, three different volumes (62.5 µL, 125 µL, and 250 µL) were added and then stirred again for 7 minutes, at 50°C. Then, 250 µL of glycerol were added, and the mixture was agitated for 7 minutes at 830 rpm at 50°C. Control films without the addition of the EOs were also prepared. The films were made by depositing the filmogenic solution, ≈15 g, in polystyrene Petri dishes, which were then placed in an oven with forced ventilation for around 6 h at 40°C. Finally, the dried films were peeled off from the Petri dishes and stored in a laboratory with controlled temperature (23±2°C) and relative humidity (RH) (50±5%) [134, 137].

4.6 – Characterization of the Films

4.6.1 – FTIR

FTIR of the films was performed using a Nicolet iS10 smart iTRBasic Fourier-Transform Spectrometer model (Thermo Fisher, Whaltham, MA, USA). The spectra were obtained between 4000 and 600 cm^{-1} with 64 scans per spectrum and a resolution of 4 cm^{-1}.

4.6.2 – DSC

The samples were previously dried at 105 °C for 24 h. Initially, baselines were determined for each sample. An inert atmosphere was present during the test, which took place at temperatures ranging from room temperature to 400 °C at a heating rate of 10 °C/min [138].

4.6.3 – Physical Properties

The structural properties, such as grammage and thickness, were determined according to ISO 536:1995, the grammage of the films was determined using the mass-to-area ratio (g/m^2). A digital micrometer was used to measure the thickness (µm) of the films in accordance with ISO 534:2011 taking several random readings [139].

Mechanical properties such as TS (N/m), TI (N.m/g), PE (%) and EM (MPa) of the films were measured using a tensile tester, with the initial gap set at 50 mm and the constant rate of elongation applied is 20 mm/min in accordance of ISO 1924/2 with some changes [139].

Using a Color Touch 2, model ISO spectrophotometer (Technidyne Corporation), the optical properties of the films, color, and transparency, were evaluated using the illuminant D65 and the observer angle 10°. The color of the films was evaluated through the lightness/brightness (L*), redness/greenness (a*), and yellowness/blueness (b*) color coordinates against a white standard background, these measurements were made on several randomly selected points of the films [139].

4.6.4 – Contact Angles and Surface Free Energy

The sessile drop method was used to determine the contact angles of the films. Measuring the contact angles using deionized water, ethyleneglycol and diiodomethane, as test prove liquids to determine the surface free energy of the films (including the total, dispersive, and polar components). The equipment's software provided the reference liquids' surface tension components. For each liquid and each sample, contact angle data were acquired from at least six measurements, and the surface free energies of the samples were determined using the Owens, Wendt, Rabel and Kaelble (OWRK) approach [134].

4.6.5 – Barrier Properties

The water vapor transmission rate (WVTR) of polymer films is frequently tested for quality assurance and to determine their suitability as packaging materials. In this work the gravimetric method, also known as steady-state measurement, was employed. WVTR is the water mass that penetrates a film of a given thickness and area per unit time at a given temperature and relative humidity (RH) gradient [140].

According to the established protocol ASTM E96-00, Water Vapor Permeability (WVP) (g/Pa.day.m) and WVTR (g/m^2.day) were measured. The films were adhered to the tops of cups containing a desiccant (15 g of anhydrous $CaCl_2$, dried at 105°C before use). After that, the test cups were kept in a cabinet at 23±2°C and 50±5% RH. Over the course of 48 h, the weight fluctuations were tracked every 2 h [141].

The slope of a linear regression of the weight gain *vs* time was used to calculate the gradient. The Equations (6) and (7) were used to determine WVTR and WVP:

$$WVTR = \frac{\frac{\Delta m}{\Delta t}}{A} \qquad (6)$$

where A is the test area (m^2), t is the test period (day), and Δm is the weight changes of the test cups (g).

$$WVP = \frac{WVTR}{\Delta p} = \frac{WVTR}{p \times (RH_1 - RH_2)} \times e \qquad (7)$$

where e is the film thickness (m), p is the water vapor pressure at 23 °C (Pa), RH_1 is the cabinet's relative humidity (50%) and RH_2 is the humidity within the cups (0%) [139].

To study the films Oil Permeability (OP), they were cut and placed at the top of the tubes containing 5 mL of edible vegetal oil from sunflower seeds. The tubes were placed upside-down over a filter paper that was dried at 105°C for 24 h before being weighed. The weight difference of the filter paper, the thickness of the films, the effective contact area, and the storage time (24 h) were used to calculate the OP (g.mm/ m^2day) [141].

$$OP = \frac{\Delta W \times e}{A \times T} \qquad (8)$$

A is the contact area (m^2), ΔW is the weight difference of the filter paper (g), e is the film's thickness (mm), and T is the storage time (days) [141].

4.6.6 – Antioxidant Activity

The antioxidant activity of the bioactive films was evaluated by both methods described above for EOs, with slight modifications. For the DPPH•, 3 discs of the films (6 mm) were added to 2.9 mL of a DPPH• methanolic solution (0.1 mM). Then, the absorbances were measured over a 5 h period at 517 nm every 30 min against a blank of methanol. The control sample consisted of 100 µL methanol plus 2.9 mL DPPH• solution [141]. The equation 9 was used to determine the films' antioxidant activity:

$$Inhibition = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (9)$$

Where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of the solutions in the presence of the films.

Using the β-carotene bleaching test, the volume of the sample was substituted by 3 discs of the films (6 mm) [141].

4.6.7 - Antibacterial and Anti-quorum Sensing Properties Assessment

Using the methods outlined above for EOs, the antibacterial and anti-quorum sensing properties of k-carrageenan films were assessed using the solid diffusion assay. Briefly, discs of the films (6 mm) were cut in an aseptic environment and applied on the surface of seeded agar plates [134]. Additionally, the plates were observed by optical microscopy, to verify the microbial growth inhibition and the integrity of the films after the incubation period [141]. The results were achieved by three independent assays.

4.6.8 – Anti-biofilm Activity

Scanning Electron Microscopy (SEM) was used to examine the anti-biofilm activity of the bioactive films against *L. monocytogenes* LMG 16779. Bacterial biofilms were produced directly on the discs of the films (about 1 cm^2) that were set up on 12-well plates. *L. monocytogenes* LMG 16779 was grown in Tryptic Soy Broth (TSB) overnight at 37°C and 250 rpm. The suspension's turbidity was adjusted to an OD_{610nm} of 0.7. Then, 700 µL of TSB were added to 300 µL of the bacterial suspension. The plates were incubated for 24 h at 37°C. After the incubation period, the biofilms were washed twice with sterile saline solution. Next, they were fixed with 2.5% (*v*/*v*) glutaraldehyde at 4°C for 4 h. After that, samples were given a single Phosphate Buffered Saline (PBS) solution wash before being dehydrated in ethanol for 20 min at each of the following concentrations: 30, 50, 70, 80, 90% (*v*/*v*) and absolute. The samples were then left to dry in a desiccator overnight. Then, using a metal evaporator, they were spray-coated with gold. VP SEM Hitachi S-3400N analysis of biofilms was completed with a voltage of 20.0 kV and a 120.0 A emission [132, 134].

4.7 – Application of Films in a Food Model

A food model was used to assess the films' effectiveness. Four cheese slices were wrapped in the produced bioactive films (4×4 cm) and in the control films (4×3 cm). The packages were kept in a refrigerator at 4°C for 15 days after first weighing and color measurement. The cheese slices were then weighed once more, and the cheese color was measured to determine any variations [142].

4.8 – Statistical Analysis

Generally, the results were presented as mean values ± standard deviation (SD). The Microsoft Excel® was used to analyze the raw values, employing the Student's *T*-test (assuming the normal distribution of the continuous variables). Differences among means were considered to be significant if the *p*-value is <0.05 (a confidence level of 95%).

Chapter 5

Results and Discussion

5.1 – Chemical Composition of *C. winterianus* EO

The GC-MS analysis of the *C. winterianus* EO allowed the identification 73 compounds, representing more than 98% of its chemical composition (Table 2). Citronellal, a monoterpenic aldehyde, was identified as the major compound of the EO representing 41.12%, followed by geraniol (19.97%), citronellol (11.94%) along with limonene, geranyl acetate, citronellyl acetate, elemol with relative percentages of 3.50%, 2.63%, 2.0%, 1.98%, respectively (Table 2). These results are in accordance with what was reported previously about EO-bearing grasses of the genus *Cymbopogon* focused on *C. nardus* [78, 83, 86, 132, 143, 144]. Citronellal is present in several plants, including in those of *Cymbopogon* genus. It has a considerable value in the perfume, cosmetic, and aromatherapy sectors, giving the EO a lemony scent [83].

Citronellal and citronellol are employed as intermediates in the synthesis of 1-menthol, α-tocopherol and irones, important products in the fragrance industry [145]. Citronellal has many therapeutic benefits, including antifungal, anthelmintic, anticancer and antioxidant effects [146, 147].

Monoterpenes and sesquiterpenes predominate among the identified compounds (Table 2). The most prevalent types of terpenes are called monoterpenes [148]. They are a vast and diversified class of naturally occurring compounds, many of them are present in EOs because of their low molecular weight. Several monoterpenes have medicinal benefits including antimicrobial, anti-inflammatory, antioxidant, antipruritic and analgesic effects [91]. Sesquiterpenes have a richer aroma, are more stereochemically diverse, are less volatile than terpenes and they have antibacterial and anti-inflammatory properties [149, 150].

Table 2. Chemical composition of *C. winterianus* EO.

Retention time (min)	Compounds	% Relative	Chemical family
6.09	Acetone	0.02	Aliphatic ketone
13.06	Tricyclene	0.01	Monoterpene
13.85	α-Pinene	0.22	Monoterpene
16.49	Camphene	0.04	Monoterpene
19.18	β-Pinene	0.01	Monoterpene
20.04	Sabinene	0.01	Monoterpene
21.99	Δ-3-Carene	0.02	Monoterpene
22.89	β-Myrcene	0.06	Monoterpene
23.13	α-Phellandrene	0.01	Monoterpene
25.58	Limonene	3.50	Monoterpene
26.29	β-Phellandrene	0.02	Monoterpene
26.60	1,8-Cineole	0.07	Monoterpenic ether
28.02	*cis*-β-Ocimene	0.11	Monoterpene

28.97	γ-Terpinene	0.01	Monoterpene
29.26	*trans*-β-Ocimene	0.06	Monoterpene
30.69	*p*-Cymene	0.02	Monoterpene
31.72	α-Terpinolene	0.05	Monoterpene
35.51	6-Methyl-5-Hepten-2-one	0.07	Aliphatic ketone
36.77	Melonal	0.08	Aliphatic aldehyde
36.99	*cis*-Rose oxide	0.02	Monoterpenic ether
38.13	*trans*-Rose oxide	0.01	Monoterpenic ether
44.62	α-Cubebene	0.01	Sesquiterpene
45.08	Menthone	0.04	Monoterpenic ketone
45.79	Citronellal	**41.12**	Monoterpenic aldehyde
48.98	β-Bourbonene	0.08	Sesquiterpene
50.00	Linalool	0.75	Monoterpenic alcohol
51.71	*neo*-Isopulegol	0.42	Monoterpenic alcohol
52.21	Isopulegol	1.25	Monoterpenic alcohol
53.11	*trans*-α-Bergamotene	0.07	Sesquiterpene
53.45	β-Elemene	1.17	Sesquiterpene
53.87	β-Copaene	0.04	Sesquiterpene
54.11	Terpinen-4-ol	0.02	Monoterpenic alcohol
54.24	*trans*-β-Caryophyllene	0.31	Sesquiterpene
54.91	Citronellyl formate	0.04	Monoterpenic ester
55.75	cis-β-Terpineol	0.05	Monoterpenic alcohol
56.43	*trans*-Muurola-3,5-diene	0.02	Sesquiterpene
57.63	Citronellyl acetate	2.00	Monoterpenic ester
58.09	*trans*-Cadina-1(6),4-diene	0.03	Sesquiterpene
58.89	α-Humulene	0.08	Sesquiterpene
58.98	Neral	0.63	Monoterpenic aldehyde
59.88	α-Amorphene	0.21	Sesquiterpene
60.17	Germacrene-*D*	1.09	Sesquiterpene
61.49	(*Z,E*)-α-Farnesene	0.12	Sesquiterpene
62.02	Geranial	1.28	Monoterpenic aldehyde
62.76	Bicyclogermacrene	0.01	Sesquiterpene
63.37	Geranyl acetate	2.63	Monoterpenic ester
63.83	Citronellol	**11.94**	Monoterpenic alcohol
63.93	Δ-Cadinene	1.21	Sesquiterpene
64.25	γ-Cadinene	0.51	Sesquiterpene
65.01	Lavandul	0.01	Monoterpenic alcohol
65.59	*trans*-Cadina-1,4-diene	0.04	Sesquiterpene
65.86	Nerol	0.19	Monoterpenic alcohol
66.14	α-Cadinene	0.12	Sesquiterpene
68.54	Geraniol	**19.97**	Monoterpenic alcohol
71.48	Geranyl butyrate	0.15	Monoterpenic alcohol
71.71	*cis*-Muurol-5-en-4-β-ol	0.11	Sesquiterpenic alcohol
74.59	*trans*-Muurol-5-en-4-β-ol	0.13	Sesquiterpenic alcohol
77.48	Methyl eugenol	0.06	Phenylpropanoid
80.15	Germacrene-*D*-4-ol	1.11	Sesquiterpenic alcohol
80.63	1,10-Di-Epi-Cubenol	0.07	Sesquiterpenic alcohol
81.03	1-Epi-Cubenol	0.02	Sesquiterpenic alcohol
81.24	Elemol	1.98	Sesquiterpenic alcohol
81.93	8-Hidroxy-neo-menthol	0.18	Alcohol
84.28	Eugenol	0.90	Phenylpropanoid
84.83	*trans*-Methyl-isoeugenol	0.16	Alcohol
85.79	*T*-Muurolol	0.25	Sesquiterpenic alcohol
86.23	α-Muurolol	0.07	Sesquiterpenic alcohol
86.70	Elemicine	0.05	Ether
87.30	Citronellic acid	0.26	Carboxylic acid
87.42	α-Eudesmol	0.16	Sesquiterpenic alcohol
87.75	α-Cadinol	0.55	Sesquiterpenic alcohol
87.87	β-Eudesmol	0.15	Sesquiterpenic alcohol
92.87	(*E,E)*-Farnesol	0.06	Sesquiterpenic alcohol

5.2 – Chemical Composition of *L. viridis* EO

Through the GC analysis results it was possible to identify 84 compounds in *L. viridis* EO, representing 95.47% of its chemical composition (Table 3).

The major compound of the EO is 1,8-cineole, a monoterpene ether (33.23%). Then, camphor at 22.72%, linalool at 3.58%, borneol at 3.12% and α-pinene at 2.06%, were also identified. These results reinforce the findings of Machado's et al. investigation, in which 1,8-cineole was similarly shown to be the principal compound of *L. viridis* [99].

One of the most common cyclic monoterpene ethers in nature is eucalyptol (1,8-cineole). It's most often associated with the *Eucalyptus globulus* EO, but it's also present in other plants including rosemary, sage, and mint. Because of its antibacterial, mucolytic, broncholytic, and anti-inflammatory properties, 1,8-cineole is used to treat illnesses of the respiratory system [96, 151].

Once again it can be seen that the most prevalent chemical families are the monoterpenes.

Table 3. Chemical composition of *L. viridis* EO.

Retention time (min)	Compounds	% Relative	Chemical family
9.29	3-Methyl-2-butenal	0.02	Aldehyde
14.91	*α*-Thujene	0.01	Monoterpene
15.00	Tricyclene	0.15	Monoterpene
15.29	*α*-Pinene	**2.06**	Monoterpene
16.02	Camphene	1.07	Monoterpene
16.25	Verbenene	1.02	Monoterpene
16.40	Methyl butenol	0.11	Alcohol
16.48	Benzaldehyde	0.03	Aromatic aldehyde
17.34	*β*-Pinene	0.54	Monoterpene
17.54	6-Methyl-5-Hepten-2-one	0.08	Aliphatic ketone
20.30	Lavander lactone	0.19	Aliphatic Lactone
21.59	*n*-Octanol	0.07	Aliphatic alcohol
21.71	Sabinene	0.10	Monoterpene
22.69	*trans*-Linalool oxide (Furanoid)	0.45	Monoterpenic epoxide
23.19	Linalool	**3.58**	Monoterpenic alcohol
23.41	Hotrienol	0.90	Monoterpenic alcohol
24.10	Fenchol	0.03	Monoterpenic alcohol
24.22	Octan-3-yl actetate	0.13	Aliphatic ester
24.62	Chrysanthenone	0.08	Monoterpenic ketone
24.86	4-Acetyl-1-methylcyclohexene	0.11	Aliphatic ketone
25.34	Nopinone	0.27	Aliphatic ketone

26.60	2,3-Dehydro-1,8-cineole	0.11	Monoterpenic ether
26.97	Limonene	0.27	Monoterpene
27.48	*p*-Cymen-8-ol	0.64	Monoterpenic alcohol
28.13	Myrtenol	0.35	Monoterpenic alcohol
28.30	1,8-Cineole	**33.23**	Monoterpenic ether
28.84	Verbenone	1.62	Monoterpenic ketone
29.12	*cis*-β-Ocimene	0.08	Monoterpene
30.14	*γ*-Terpinene	0.07	Monoterpene
30.37	Carvone	0.24	Monoterpenic ketone
30.63	Linalyl acetate	0.20	Monoterpenic ester
31.71	*p*-Cymene	0.55	Monoterpene
32.02	4-Hidroxy-3-methylacetophenone	0.13	Ketone
32.22	Lavandulyl acetate	0.30	Monoterpenic ester
32.33	Bornyl acetate	0.21	Monoterpenic ester
32.71	*α*-Terpinolene	0.04	Monoterpene
36.63	Octyl butyrate	0.06	Monoterpenic ester
37.04	*β*-Bourbonene	0.08	Sesquiterpene
38.76	*β*-Cedrene	0.06	Sesquiterpene
40.55	*β*-Acoradiene	0.08	Sesquiterpene
41.46	*β*-Selinene	0.75	Sesquiterpene
41.94	Geranyl isobutyrate	0.07	Monoterpenic ester
42.01	*β*-Bisabolene	0.09	Sesquiterpene
42.82	*α*-p-Dimethylstyrene	0.34	Monoterpene
42.84	*trans*-Calamenene	0.10	Sesquiterpene
42.86	10-Epizonarene	0.15	Sesquiterpene
43.43	Filifolone	0.02	Monoterpenic ketone
43.44	Unknown sesquiterpene	0.72	Sesquiterpene
43.72	*cis*-Linalool oxide (Furanoid)	1.07	Monoterpenic epoxide
44.38	Germacrene B	0.04	Sesquiterpene
45.03	Camphenilone	0.38	Aliphatic ketone
46.69	*α*-Campholenal	0.16	Monoterpenic aldehyde
47.27	Phenethyl hexanoate	0.03	Phenolic ester
48.96	Camphor	**22.72**	Monoterpenic ketone
52.25	Bornyl formate	0.26	Monoterpenic ester
52.66	Isobornyl acetate	0.22	Monoterpenic ester
52.89	Methyl heptadienone	0.17	Aliphatic ketone
53.76	Terpinen-4-ol	0.77	Monoterpenic alcohol
55.34	Octyl-2-methyl butyrate	0.22	Monoterpenic ester
55.54	Myrtenal	0.88	Monoterpenic aldehyde
55.87	Sabina ketone	0.08	Aliphatic ketone
56.82	*trans*-Pinocarveol	0.73	Monoterpenic alcohol
57.08	*p*-Mentha-1,3-dien-8-ol	0.27	Monoterpenic alcohol
57.80	*δ*-Terpineol	0.92	Monoterpenic alcohol
58.23	*trans*-Verbenol	0.53	Monoterpenic alcohol
59.24	*α*- Terpineol	1.42	Monoterpenic alcohol
59.57	Borneol	**3.12**	Monoterpenic alcohol
60.19	*α*-Bulnesene	0.22	Sesquiterpene
60.66	*p*-Mentha-1,5-dien-8-ol	0.49	Monoterpenic alcohol

61.07	*cis*-α-Bisabolene	1.33	Sesquiterpene
61.25	*α*-Selinene	0.42	Sesquiterpene
62.37	Geranyl acetate	0.90	Monoterpenic ester
62.61	*trans*-Linalool oxide (Pyranoid)	0.15	Monoterpene epoxide
63.30	*γ*-Cadinene	0.16	Sesquiterpene
63.48	*p*-Lethyl acetophenone	0.13	Aromatic hydrocarbon
63.98	Cuminaldehyde	0.06	Monoterpenic aldehyde
64.58	Euderma-3,7(11)-diene	1.61	Sesquiterpene
65.09	*α*-Cadinene	0.08	Sesquiterpene
66.67	*cis*-Carveol	0.21	Monoterpenic alcohol
68.53	Benzyl isovalerate	0.08	Phenolic ester
74.19	Phenethyl-2-methyl butyrate	0.42	Phenolic ester
84.72	(*E*)-1-(6,10-Dimethylundec-5-en-2-yl)-4-methylbenzene	0.46	Diterpene
85.07	Cadalene	0.07	Sesquiterpene
85.57	*α*-Cadinol	0.53	Sesquiterpenic alcohol
87.82	(*E*)-1-(6,10-Dimethylundec-5,9-dien-2-yl)-4-methylbenzene	0.54	Diterpene

5.3 – Antioxidant Activity of the EOs

All foods suffer degradation throughout the storage period because of their perishable nature, although at different rates [17]. The occurrence of off-odors, off-flavors, alterations in texture, and changes in color resulting from lipid oxidation processes may significantly impact consumer acceptability and preference [108]. In light of this knowledge, evaluating the potential impact of any active substance on lipid oxidation is essential. This may be accomplished by the use of specific assays, such as DPPH• and β-carotene bleaching test. The DPPH free radical scavenging test, based on reduction reactions, and the β-carotene bleaching test, linked to lipid peroxidation, stand out among the known *in vitro* techniques for assessing antioxidant capacities of natural products thus, the antioxidant properties of *C. winterianus* and *L. viridis* EOs were evaluated by these both methods (Table 4) [143, 152].

Regarding the results obtained by the DPPH• method, the *C. winterianus* EO showed powerful antioxidant activity, results that are consistent with bibliographical analyses of other authors [144, 153]. Regarding *L. viridis* EO, it is evident that it possesses very strong antioxidant capacity. These results are also consistent with other published works [154, 155].

One of the determining elements for use in food packaging is the capacity to decrease lipid oxidation and it is clear from the data and the IC_{50} that minimal amounts of *C. winterianus* EO are required to be able to inhibit lipid oxidation by 50%. Therefore, evaluating previous reports and the results obtained, it can be concluded that this EO has the capacity to inhibit lipid oxidation. *L. viridis* EO results indicate that a higher EO quantity is required to exert an effect on lipid oxidation.

In an effort to sustainably contribute to the food chain, research has expanded in the area of combining EOs into packaging produced from materials derived from renewable and biodegradable sources, and having this promising result, *C. winterianus* EO is a promising substance for food packaging whose organoleptic properties are highly dependent on the preservation of the lipid content [156].

Table 4. Antioxidant properties of *C. winterianus* and *L. viridis* EOs.

	Parameters	***CW EO***	***LV EO***
DPPH• method	**IC_{50} (%, v/v)**	0.06 ± 0.01	0.35 ± 0.06
	AAI	85.60 ± 13.42	15.26 ± 4.03
	Antioxidant activity	Very strong	Very Strong

β-carotene bleaching assay	**IC$_{50}$(%, v/v)**	3.16 ± 0.48	5.01 ± 0.15

Results presented as mean ± SD; AAI: Antioxidant Activity Index; IC$_{50}$: average inhibitory concentration; *CW*: *C. winterianus; LV: L. viridis.*

5.4 – Antibacterial and Anti-quorum Sensing Properties of the EOs

Concerning the antibacterial activity of *C. winterianus* EO, it is clear that Gram-positive bacteria, particularly *L. monocytogenes* LMG 16779, were more susceptible than Gram-negative bacteria (Table 5). This result is consistent both in terms of antibacterial activity and Gram-positive prevalence, considering other studies [85, 153, 157].

The results of the tests for *L. viridis* EO antibacterial activity indicate that this EO may be effective against bacteria in general, with more significant inhibitory zones for *S. aureus* ATCC 25923. When compared to the research of Ait Said et al., it was found that they are consistent with the results now obtained [100].

By determining its MIC values (Table 5), the antibacterial activity of the EOs was further assessed. For *C. winterianus* EO, the lowest MIC values were found for Gram-positive bacteria, particularly for *L. monocytogenes* LMG 16779 and *E. faecalis* ATCC 29212, supporting the findings from the solid diffusion assay. The conclusion that may be inferred with the MIC test is that *C. winterianus* EO has significant inhibitory effects on Gram-positive bacteria, which is consistent with other publications [85].

The MIC values obtained for *L. viridis* EO demonstrate that the minimal value was achieved for the Gram-negative bacterium *E. coli* ATCC 25922. The observed outcome may be reasonably anticipated due to the antibacterial properties of α-pinene, one of the main components of *L. viridis* EO, particularly against *E. coli*. Previous research has shed light on the antibacterial potential of this monoterpene, primarily in relation to its effects against *E. coli* [158].

The opportunistic pathogen *L. monocytogenes* in animals and humans leads to life-threatening infections. This facultative intracellular bacterium is common in nature and contaminates various surfaces through cross-contamination with the development of biofilms in industrial machinery, plumbing, and other surfaces even reaching the final product for consumption [28]. Eating foods that have been contaminated can result in illnesses [159]. *Staphylococcus aureus* can produce an extensive range of toxins, some of which are known as staphylococcal enterotoxins. Foodborne diseases caused by staphylococcal enterotoxins are a significant concern, often resulting from the consumption of various foods, particularly processed meats, and dairy products. Improper handling and subsequent storage of such foods at elevated temperatures can lead to contamination with *S. aureus*, thereby increasing the risk of food poisoning [160]. The primary cause of food contamination is attributed to food handlers who carry enterotoxin-producing *S. aureus* either in their airways or in their hands. This can occur through direct manual interaction or via respiratory secretions [161]. In the context of food packaging, the effects that *C. winterianus* and *L. viridis* EOs have against *L. monocytogenes* LMG 16779 and *S. aureus* ATCC 25923 are pertinent because it would inhibit their growth.

In a sort of microbial communication called quorum sensing, bacteria communicate and work together through various chemical signals called auto-inducers to keep a certain process in balance and have an impact on populations. Additionally, it affects how genes are expressed in response to cell density and triggers the activation of several transcription factors in bacteria [162]. *C. winterianus* EO was able to inhibit the violacein production by *C. violaceum* ATCC 12472 (Table 5), making it possible to confirm its anti-quorum sensing capacity. Similarly, the *L. viridis* EO also demonstrated the ability to inhibit the violacein production.

Table 5. Antibacterial and anti-quorum sensing properties of *C. winterianus* and *L. viridis* EOs.

	***C. winterianus* EO**		***L. viridis* EO**	
Strains	**Diameter of inhibition zones (mm)** [1]	**MIC values (µL/mL)** [2]	**Diameter of inhibition zones (mm)** [1]	**MIC values (µL/mL)** [2]
S. aureus ATCC 25923	15.53 ± 2.12	32	15.63 ± 1.87	>32
L. monocytogenes LMG 16779	31.67 ± 5.16	8	13.58 ± 0.53	>32
E. faecalis ATCC 29212	12.03 ± 0.18	16	9.06 ± 0.04	>32
E. coli ATCC 25922	9.98 ± 0.66	>32	12.37 ± 0.15	32
***S.* Typhimurium** ATCC 13311	6.00 ± 0.00	32	10.18 ± 0.21	>32
P. aeruginosa ATCC 27853	7.77 ± 0.00	>32	7.64 ± 0.63	>32
C. violaceum ATCC 12472	10.93 ± 0.81	-	18.88 ± 0.70	>32

[1]Results are presented as mean±SD; [2]Results are presented as modal values.

5.5 – Characterization of Films

5.5.1 – FTIR Spectra of the Films

The FTIR spectra for k-carrageenan, glycerol, *C. winterianus* EO and *L. viridis* EO were firstly obtained.

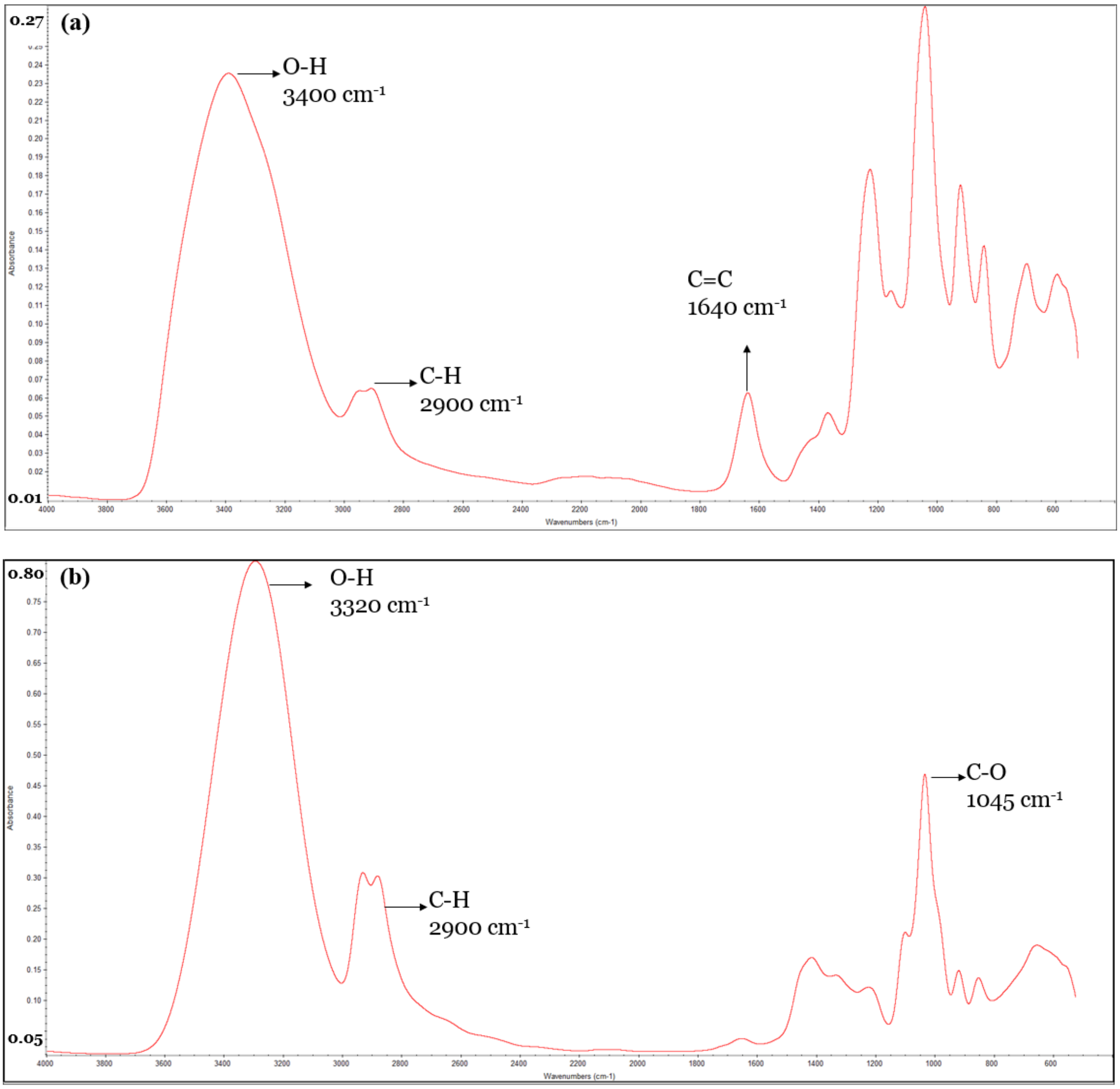


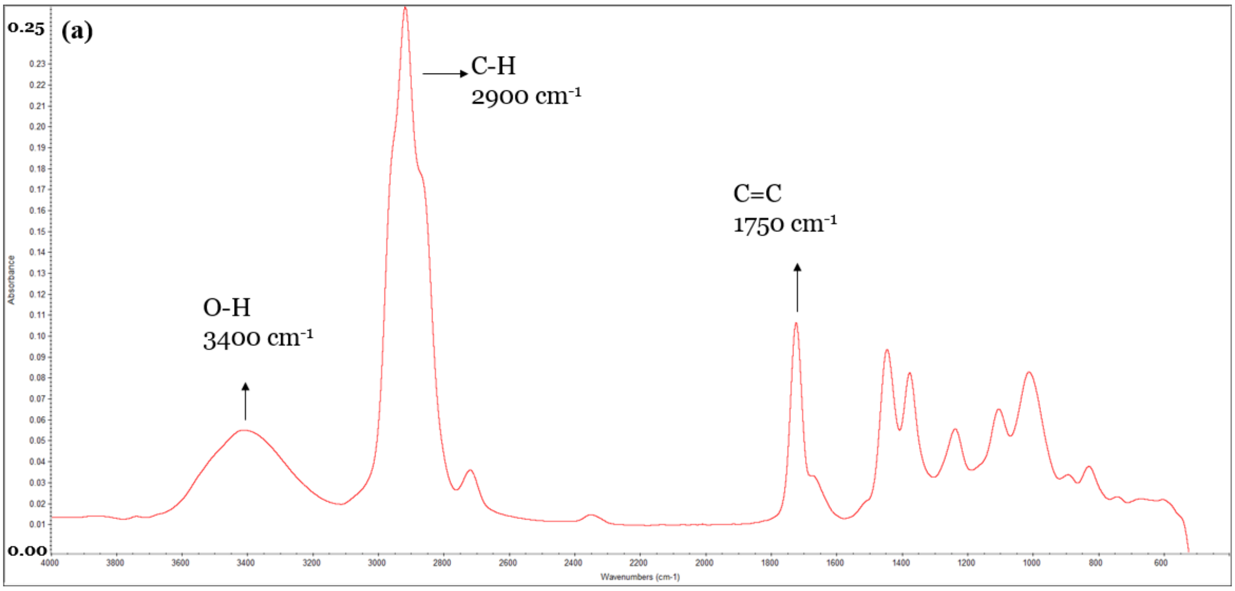
Figure 15. FTIR spectra of the individual components: (a) k-Carrageenan, (b) Glycerol.

The FTIR analysis of k-Carrageenan and glycerol (Figure 15) reveals the presence of peaks with comparable wavenumber values. One notable observation is the considerable disparity in absorbance between the stretching of the O-H bond in glycerol and k-carrageenan [163].

The FTIR spectrum of k-carrageenan (Figure 15(a)) showed several representative bands: at 3000-3500 cm^{-1} corresponding to O-H stretching. Although the FTIR used is not the most sensitive, based on published work it is expected a peak at 1220 cm^{-1} that corresponds to the asymmetric stretching of ester sulfate groups (O=S=O); 1030 cm^{-1} is attributed to C-O and C-OH stretching and 930 cm^{-1} corresponds to C-O-C stretching in 3,6-anhydro-D-galactose. The band at 845 cm^{-1} is related to C-O-SO_3 stretching in (1-3)-D-galactose. The intense bands at 1592 cm^{-1} and 1382 cm^{-1} are probably related to the structural water deformation band [164, 165].

The spectral region that includes the fingerprint region in FTIR is typically delineated as the range covering from 400 cm^{-1} to 1500 cm^{-1}. The compound in question exhibits a multitude of peaks, thereby posing a challenge in discerning and isolating individual bonds [166, 167].

In contrast with glycerol, there is a broad spectral peak seen at 3320 cm^{-1}, which may be attributed to the stretching of O-H bonds [163].



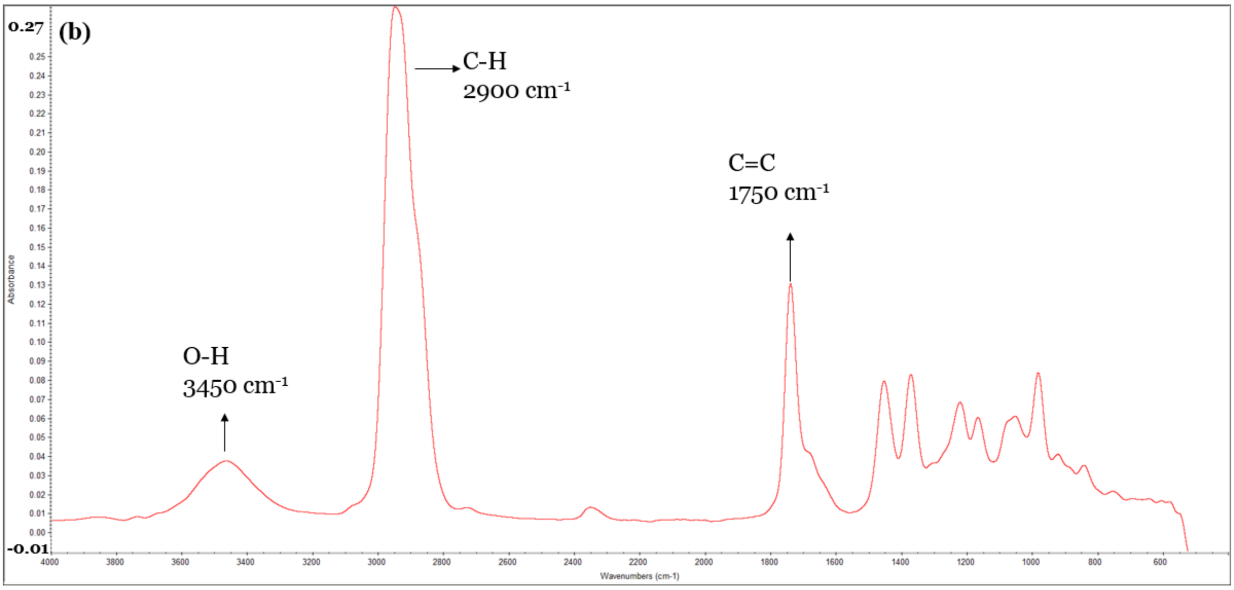
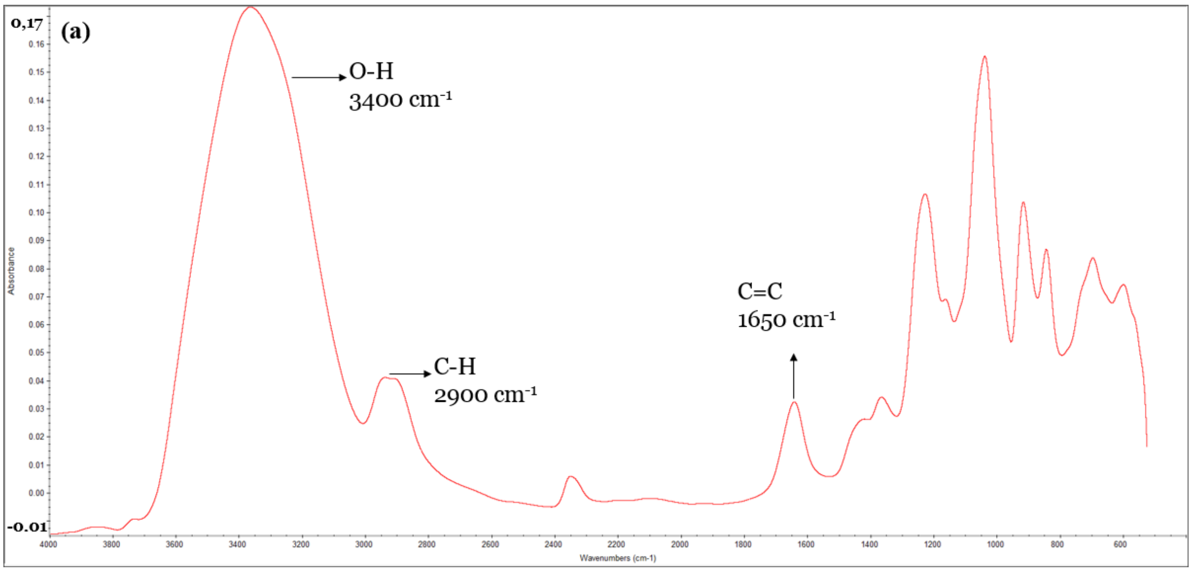


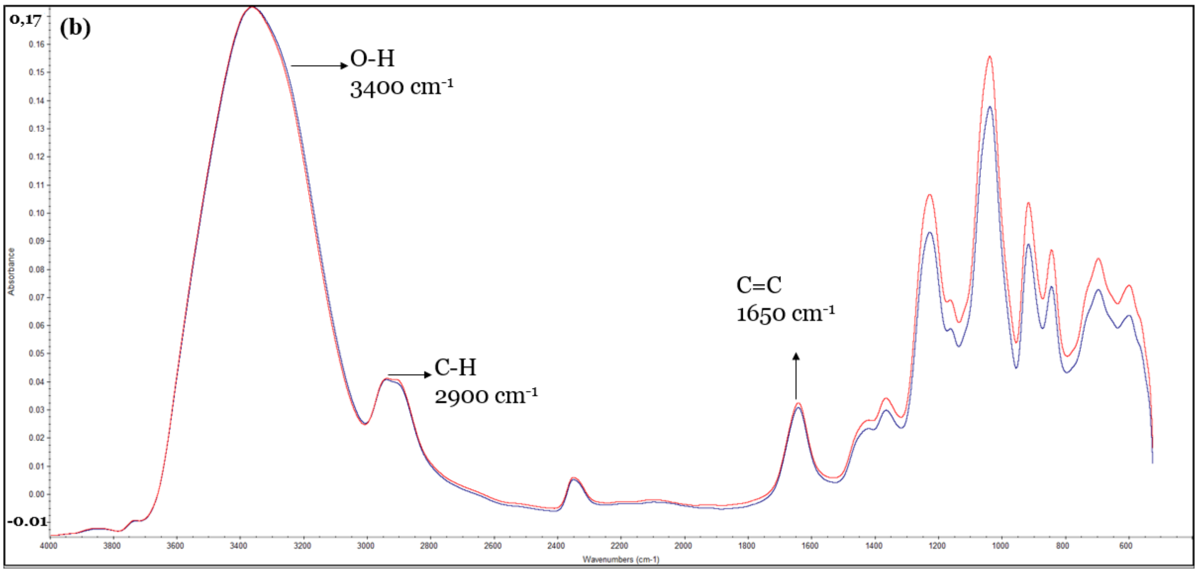
Figure 16. FTIR spectra of the EOs: (a) *C. winterianus* and (b) *L. viridis*.

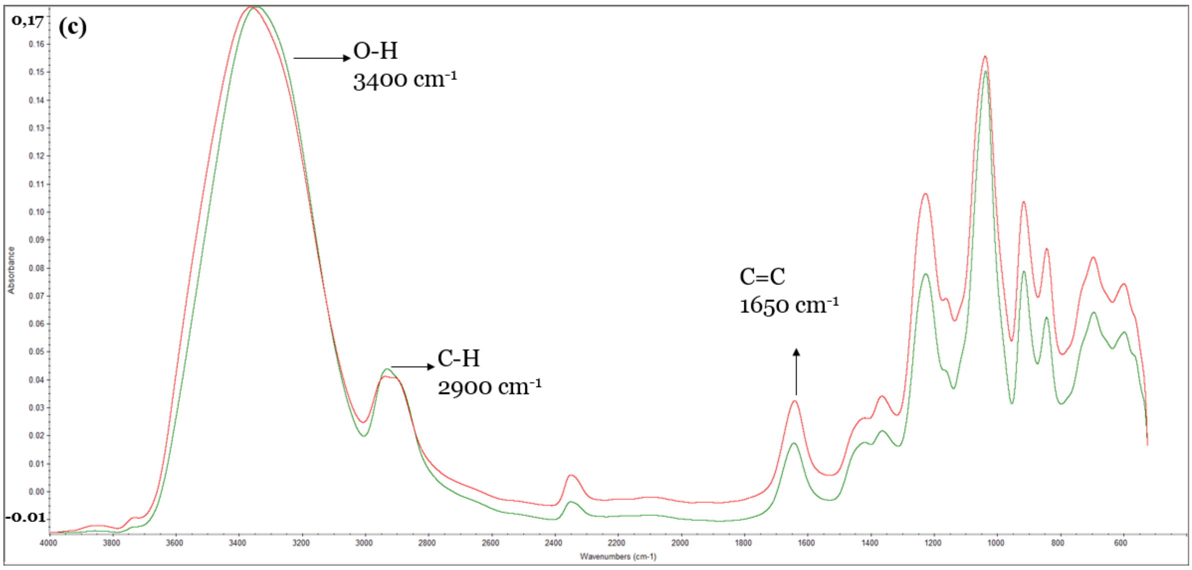
The FTIR spectra of *C. winterianus* and *L. viridis* EOs (Figures 16(a), 16(b)) are identical, with the hydrocarbon group (2900 cm^{-1}), hydroxyl group (3450 cm^{-1}), and unsaturated group as the three main peaks (1750 cm^{-1}) [167].

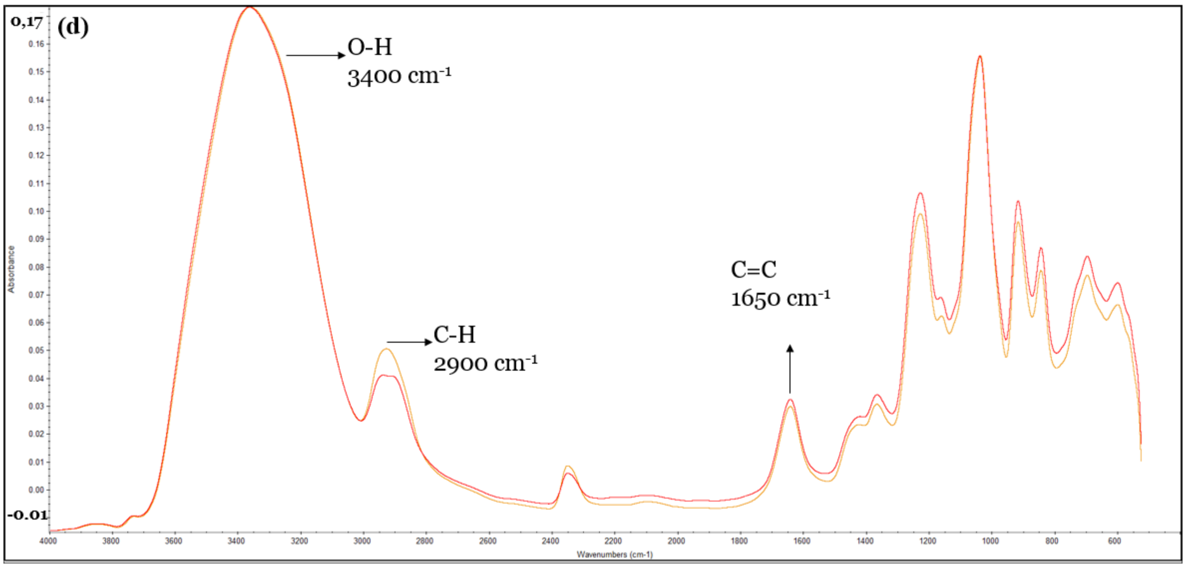
Based on Songkro's et al. study, it is expected to see for *C. winterianus* absorption peaks principally for citronellal, citronellol and geraniol being identified at 3413 cm^{-1} (O–H stretch) which is approximate with what was obtained; 2721 cm^{-1} (aldehydic C–H stretch), which is also on the margin of the peak obtained; 1727 cm^{-1} (C=O stretch), 1645 cm^{-1} (O–H bend), 1378 cm^{-1} (deformation of C–O–H group), and 1016 cm^{-1} (C–O stretch) [168].

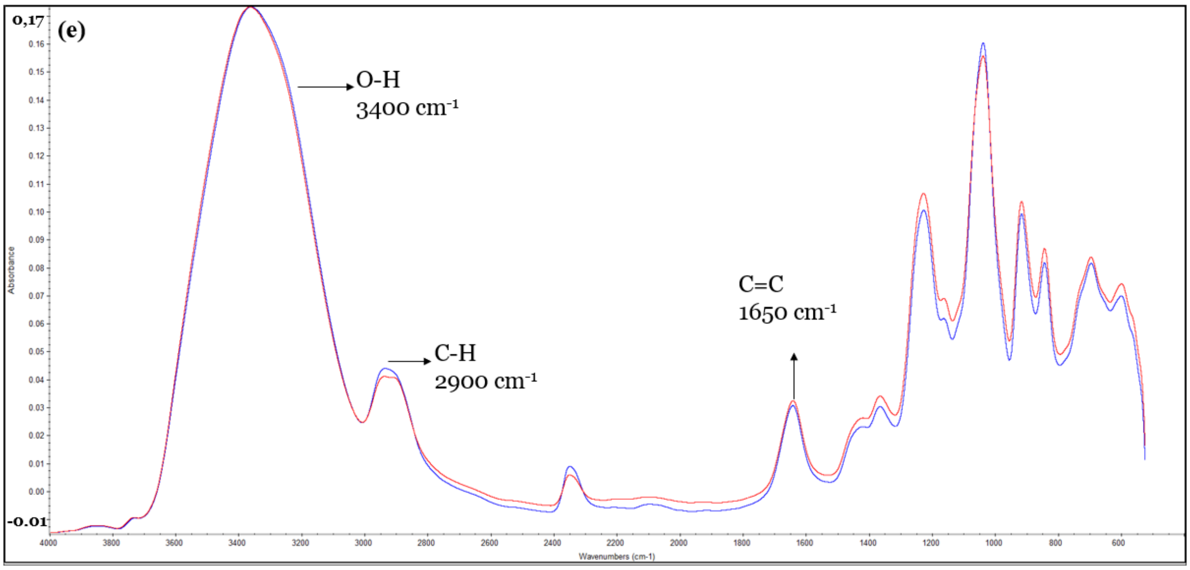
Given the absence of any research examining FTIR in *L. viridis*, the best approach would be to compare the results that focused on the principal constituent 1,8-cineole, in this case being in *Eucalyptus* EO. Based on Garcia's et al. study, 1,8-cineol presents bands at 1079 cm^{-1} and 1214 cm^{-1} attributed to symmetric and asymmetric stretches of C-O-C group and a CH_3 deformation at 1374 cm^{-1} [169]. The remaining peaks were subjected to analysis using an infrared table in order to interpret the observed peaks [167].

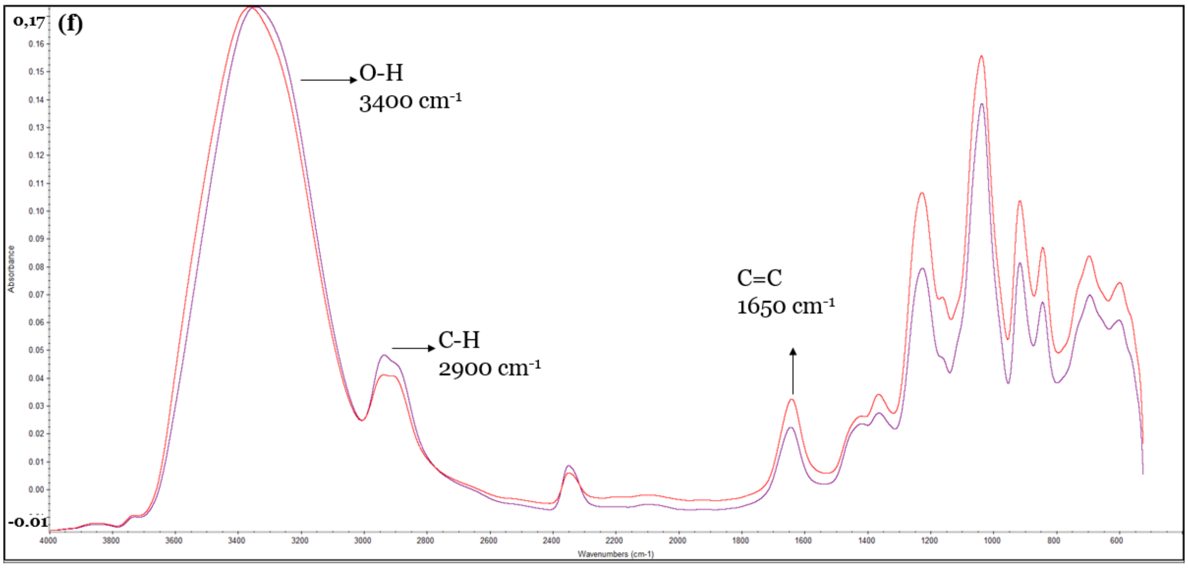

0,17
(a)
0.16
0.15
0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
-0.01
Absorbance
O-H
3400 cm^{-1}
C-H
2900 cm^{-1}
C=C
1650 cm^{-1}
4000
3800
3600
3400
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
Wavenumbers (cm-1)


0,17
(b)
0.16
0.15
0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
-0.01
Absorbance
O-H
3400 cm^{-1}
C-H
2900 cm^{-1}
C=C
1650 cm^{-1}
4000
3800
3600
3400
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
Wavenumbers (cm-1)


0,17
(c)
0.16
0.15
0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
-0.01
Absorbance
O-H
3400 cm^{-1}
C-H
2900 cm^{-1}
C=C
1650 cm^{-1}
4000
3800
3600
3400
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
Wavenumbers (cm-1)


(d)
O-H
3400 cm-1
C-H
2900 cm-1
C=C
1650 cm-1
0,17
0.16
0.15
0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
-0.01
Absorbance
4000
3800
3600
3400
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
Wavenumbers (cm-1)


(e)
O-H
3400 cm-1
C-H
2900 cm-1
C=C
1650 cm-1
0,17
0.16
0.15
0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
-0.01
Absorbance
4000
3800
3600
3400
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
Wavenumbers (cm-1)


(f)
O-H
3400 cm-1
C-H
2900 cm-1
C=C
1650 cm-1
0,17
0.16
0.15
0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
-0.01
Absorbance
4000
3800
3600
3400
3200
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
Wavenumbers (cm-1)

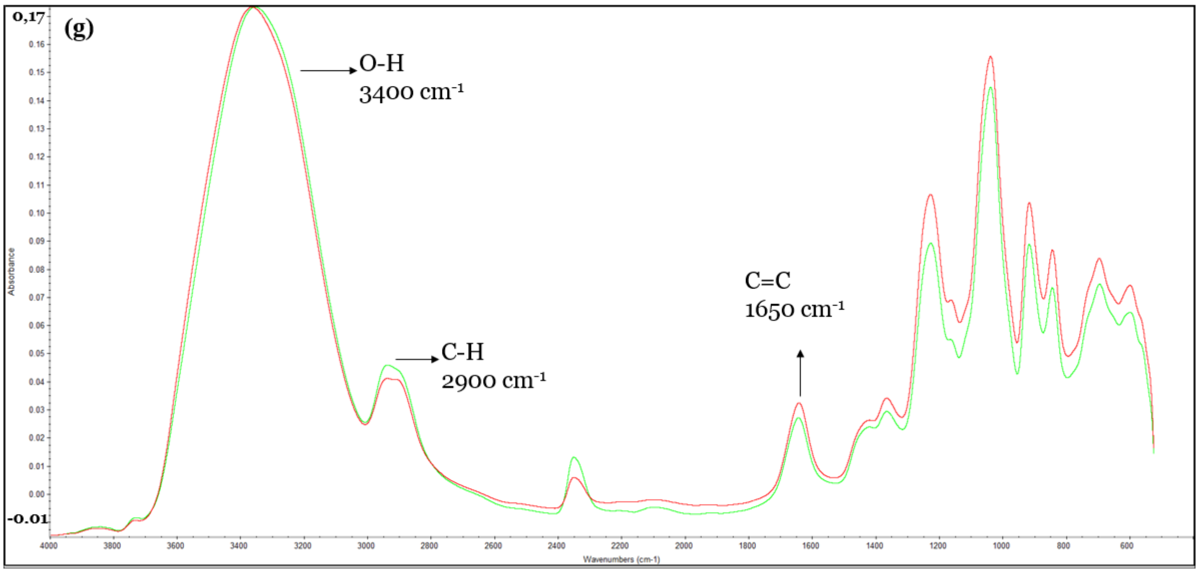


Figure 17. FTIR spectra of the films: (a) Control, (b) with 62.5 µL of *CW* EO, (c) with 125 µL of *CW* EO, (d) with 250 µL of *CW* EO. (e) with 62.5 µL of *LV* EO, (f) with 125 µL of *LV* EO and (g) with 250 µL of *LV* EO. *CW: C. winterianus; LV: L. viridis*

As a baseline, it was possible to observe on Figure 17 (a) an O-H (3400 cm^{-1}) bond peak in the control film, followed by a smaller peak for the C-H bond (2900 cm^{-1}) [164]. The C=C connection (1650 cm^{-1}) may be seen close to the digital print zone [167]. The digital print zone typically has many peaks, making it challenging to distinguish between them and pick out individual bonds [166, 167]. These peaks are in agreement with other FTIR analyzes as is the case with the Paşcalau's et al. study with respect to k-carrageenan [170]. The FTIR spectra of the k-carrageenan films incorporating the *C. winterianus* EO (Figures 17 (b), 17 (c) and 17 (d)) showed that the existing peaks were taller, despite no new peaks have appeared, indicating also that the EO compounds are present as its concentration increased. Regarding k-carrageenan films incorporated with *L. viridis* (Figure 17 (e), 17 (f) and 17 (g)), the same can be concluded.

As the peaks are found in the same wavenumbers, it was once thought that it would be hard to tell between the presence and absence of the EOs. Nevertheless, it can now be said that there is generally a difference in height, this means that as the volume of the EO used increased, the FTIR was able to capture more of it, which shows that the EOs are present in both films [167].

5.5.2 – Thermal Analysis (DSC)

To comprehend this assay, the materials used to produce the films must be taken into account. The control film was composed of k-carrageenan and glycerol. In films incorporating EOs, the previous base reagents were used together with the EOs. In this way, changes that involve these elements or the connections that link them are searched. The thermograms of control film, 250 µL *CW* EO, and 250 µL *LV* EO films were obtained (Figure 18). By performing the test for these 3 films, it can be better understood the behavior of k-carrageenan alone and with the incorporation of both EOs. In general, the characteristics of the control and 250 µL *LV* EO films are comparable, whereas the 250 µL *CW* EO film exhibits distinct state transitions. One crucial consideration in interpreting these findings is the absence of studies assessing k-carrageenan using the DSC technique. The previously obtained results solely rely on thermogravimetric analysis (TGA), which is a method that shares certain similarities with DSC, specifically in the examination of thermal properties and characteristics of materials. Consequently, the test mentioned previously was used as a point of reference in order to establish a correlation with the results that were obtained.

Using Velásquez's et al. and Liu 's et al. published works in which present a similar profile for k-carrageenan [47, 137], at temperatures between 170 and 199°C (exothermic), occurs the first peak, when glycerol will probably be lost due to its decomposition. The second peak occurs at temperatures between 200 and 230°C (endothermic), where the maximal decomposition of k-carrageenan occurs [137]. The third peak ranging from 290 to 400°C (exothermic) with a maximal decomposition rate culminating at 305°C, may be associated with residual pyrolytic decomposition. During this procedure, the crosslinked products formed during sucrose dehydration and its reaction with other film components decompose gradually [47].

As mentioned before, the profile of the 250 µL *LV* EO film is quite similar to the profile of the control film, but the temperature range is different. In the first peak, the exothermic curve, is the loss of glycerol in an interval 170 to 200°C, in the second peak, the maximal decomposition of k-carrageenan in a temperature interval of 200 to 240°C and in the last peak, temperatures of 290 to 400°C (endothermic), there is the residual pyrolytic decomposition. No studies have been conducted on any of the EOs; however, using Dębczak's et al. study on *Lavandula angustifolia* as a reference, it can be estimated the behavior of this EO [171]. In this study [171], a small endothermic peak was observed between 50 and 80°C. If this interval is examined attentively, it can be seen that there is an endothermic peak around 5 °C, which may correspond to *L. viridis* EO, and as an EO, it is expected that it volatilizes at low temperatures.

Finally, the 250 µL *CW* EO film thermogram differs from the others in that it displays two significant peaks, the loss of glycerol (170 to 200ºC, exothermic) and the residual pyrolytic decomposition (280 to 350ºC, endothermic). In the absence of studies with this EO, it can be observed that its endothermic peak is comparable to that of lemongrass, however, this study does not show a peak immediately after the exothermic one like the one we obtained. [172].

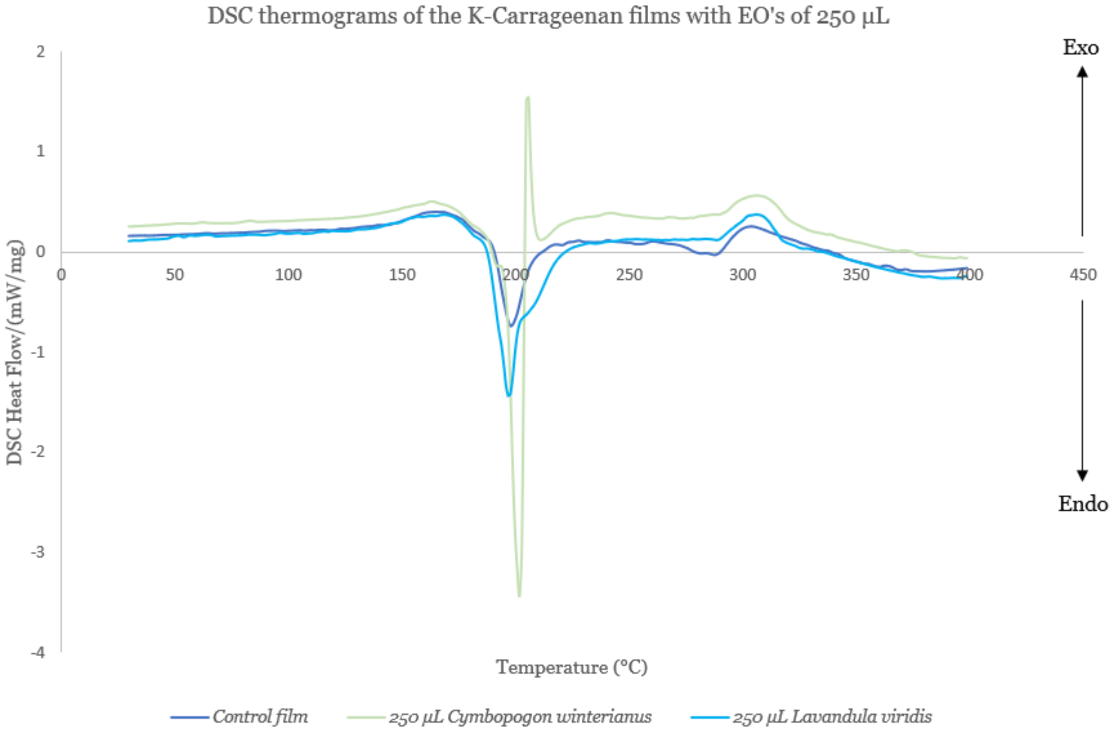


Figure 18. DSC thermograms of the k-Carrageenan films with 250 µL of the EOs.

5.5.3 -Physical Properties of the Films

The impact of incorporating the EOs on the structural properties of films is presented in Table 6.

Table 6. Structural properties of k-Carrageenan films.

	Property	**Control^{a}**	**62.5 µL *CW* EO^{b}**	**125 µL *CW* EO^{c}**	**250 µL *CW* EO^{d}**	**62.5 µL *LV* EO^{e}**	**125 µL *LV* EO^{f}**	**250 µL *LV* EO^{g}**	***p*-values**
Structural	Grammage (g/m^{2})	65.18 ± 2.91	65.36 ± 5.26	66.43 ± 5.87	69.12 ± 4.09	70. 77 ± 7.15	74.23 ± 12.93	60.74 ± 0.44	0.926^{ab} 0.557^{ac} 0.030^{ad*} 0.3067 ^{ae} 0.3489 ^{af} 0.0064 ^{ag*}
	Thickness (µm)	38.51 ± 4.01	41.45 ± 4.98	44.17 ± 5.51	64.93 ±7.89	42.6 ± 4.94	44.57 ± 6.29	40.53 ± 4.50	0.002^{ab*} <0.001^{ac} * <0.001^{ad*} 0.0090 ^{ae} * 0.0037 ^{af} * 0.1396 ^{ag}

Results are presented as mean±SD; * Indicates a significant result (*p*-value<0.05); Superscript letters (a–g) indicate the samples under statistical comparison. *CW: C. winterianus; LV: L. viridis.*

The k-carrageenan films thickness varied from 38.51 mm to 64.93 mm with incorporation of *C. winterianus* EO significantly affecting (*p*-value<0.05) the resulting films thickness, as well as the grammage, due to the difference on total solid contents in the films [139]. Visually, it was feasible to confirm that as the amount of *C. winterianus* EO increased, the film's roughness increased. The surface roughness may increase as a result of the EO integration into the polymer matrix, which affects their thickness [73].

The thickness of films containing *L. viridis* EO increased from 38.51 mm to 44.57 mm, having statistically significant variation (*p*-value<0.05), while the 250 µL film did not exhibit the greatest value, it exhibited an increase when compared to the control film. In terms of grammage, the findings were consistent, increasing for the first two volumes of EO while decreasing for the final amount (250 µL), with statistically significant deviations (*p*-value<0.05).

The effects of incorporating the EOs on the mechanical properties of the films are shown in Table 7.

Table 7. Mechanical properties of k-Carrageenan films.

	Property	**Control^{a}**	**62.5 µL *CW* EO^{b}**	**125 µL *CW* EO^{c}**	**250 µL *CW* EO^{d}**	**62.5 µL *LV* EO^{e}**	**125 µL *LV* EO^{f}**	**250 µL *LV* EO^{g}**	***p*-values**
Mechanical	Tensile strength (N/m)	3006.99 ± 80.56	2783.54 ± 236.82	2821.52 ± 201.98	1432.29 ± 14.38	3239.61 ± 183.43	1915.48 ± 244.14	3068.54 ± 79.84	0.242^{ab} 0.256^{ac} 0.019^{ad*} 0.153^{ae} 0.009^{af*} 0.523^{ag}
	Tensile index (N.m/g)	46.15 ± 1.20	42.60 ± 3.63	42.46 ± 3.02	20.75 ± 0.21	45.76 ± 2.608	25.8 ± 3.26	50.5 ± 1.27	0.230^{ab} 0.163^{ac} 0.018^{ad*} 0.839^{ae} 0.004^{af*} 0.073^{ag}
	Peak elongation (%)	1.91 ± 0.01	2.38 ± 0.09	3.73 ± 0.47	1.46 ± 0.12	5.5 ± 1.18	2.63 ± 0.15	4.89 ± 0.48	0.009^{ab*} 0.021^{ac*} 0.114^{ad} 0.034^{ae*} 0.014^{af*} 0.072^{ag}
	Elastic modulus (MPa)	7978.67 ± 312.77	6946.07 ± 545.55	5558.45 ± 337.19	3017.49 ± 2.64	5820.72 ± 128.88	4678.19 ± 476.43	5728.24 ± 355.37	0.075^{ab} 0.008^{ac*} 0.028^{ad*} 0.043^{ae*} 0.003^{af*} 0.022^{ag*}

Results are presented as mean±SD; * Indicates a significant result (*p*-value<0.05); Superscript letters (a–g) indicate the samples under statistical comparison. *CW: C. winterianus; LV: L. viridis.*

TS, TI, PE and EM are the mechanical properties of the films, which are dependent on the kind of polymer matrix, the sort of additives, and the interactions between them [173]. The ability to endure a tensile force is measured by assessing the breakpoint of a specimen under tensile stress [174]. Increasing the content of *C. winterianus* EO in the films causes a reduction in TS and TI, which significantly changed (*p*-value<0.05) when the volume was 250 mL but did not significantly change (*p*-value>0.05) for the other two volumes. In the film network, weaker polymer-EO connections have partially replaced stronger polymer-polymer interactions, being a possible reason for the reduction [175, 176].

PE is the increase in the specimen's length from its starting length to the breakpoint length, which is related to a polymeric material's elasticity. In this study, PE increased as volume of *C. winterianus* EO increased to 62.5 μL and 125 μL, and there is statistical variation in these (*p*-value<0.05). The value of the 250 μL is lower than that of the control, it likely already contains too much EO, and there are less interconnections between the structures, which limits its flexibility. The EM decreases with the increase in EO volume incorporation, being significant for the two highest volumes (*p*-value<0.05), resulting in less rigid films. According to Lima Barizão et al. rigid structures should promote low water vapor permeability (WVP) [177]. Adding the EO to the polymer may not result in a homogenous combination, giving the films a roughness that creates minuscule weak areas and makes them more prone to breaking [73, 173]. The TI, being the relation between the TS and the grammage, it is natural that these values follow the behavior of these two factors, having these decreased, with no statistical variations (*p*-value<0.05).

Except for the 125 μL film, which saw statistical variances, the films that included *L. viridis* EO (Table 7) showed only very modest changes in terms of TS and generally stayed fairly comparable to the control film. The outcomes in terms of TI did not demonstrate a consistent shift. For 62.5 μL, the value stayed almost the same as the control film, for 125 μL, there is a large drop that is also subject to statistical variances (*p*-value<0.05), and lastly, for 250 μL, the value increased just slightly above the control film. In terms of the PE generally, this value grew as the EO volume increased, with statistical deviations between 62.5 μL and 125 μL. Finally, values decreased for EM, resulting in less rigid films, and indicating statistical differences between all of them (*p*-value<0.05).

When combining polysaccharides and EOs, the loss of properties is frequently observed, according to analysis of findings from other authors [174, 175, 178].
If compared with plastic films used in food industry, low density polyethylene (LDPE), for example, it presents TS values equal to or greater than 10 MPa and PE values equal to or greater than 200% [179]. Therefore, compared to the existing plastics in the industry, the films now developed have lower TS.

The impact of incorporating the EOs on the optical properties of the films are shown in Table 8.

Table 8. Optical properties of k-Carrageenan films.

	Property	**Control^{a}**	**62.5 µL *CW* EO^{b}**	**125 µL *CW* EO^{c}**	**250 µL *CW* EO^{d}**	**62.5 µL *LV* EO^{e}**	**125 µL *LV* EO^{f}**	**250 µL *LV* EO^{g}**	***p*-values**
Optical	L* (Lightness)	93.01 ± 0.23	93.15 ± 0.17	93.26 ± 0.15	94.07 ± 0.51	93.11 ± 0.13	93.04 ± 0.23	93.33 ± 0.18	0.448^{ab} 0.201^{ac} 0.052^{ad} 0.559 ^{ae} 0.881 ^{af} 0.135 ^{ag}
	a* (Redness)	1.58 ± 0.04	1.60 ± 0.07	1.51 ± 0.02	1.33 ± 0.08	1.58 ± 0.01	1.53 ± 0.055	1.45 ± 0.038	0.676^{ab} 0.069^{ac} 0.018^{ad}• 1 ^{ae} 0.275 ^{af} 0.014 ^{ag} •
	b* (Yellowness)	-5.94 ± 0.11	-5.93 ± 0.40	-5.56 ± 0.11	-4.38 ± 0.42	-5.92 ± 0.077	-5.73 ± 0.27	-5.23 ± 0.186	0.970^{ab} 0.014^{ac}• 0.018^{ad}• 0.813 ^{ae} 0 ^{af} • 0.009 ^{ag} •
	Transparency (%)	95.72 ± 0.454	94.54 ± 0.384	93.83 ± 0.348	91.65 ± 1.463	95.43 ± 0.49	95 ± 0.482	94.03 ± 0.291	0.028^{ab}• 0.006^{ac}• 0.031^{ad}• 0.494 ^{ae} 0.133 ^{af} 0.009 ^{ag} •

Results are presented as mean±SD; • Indicates a significant result (p-value<0.05); Superscript letters (a–g) indicate the samples under statistical comparison. *CW: C. winterianus; LV: L. viridis.*

Since color and transparency directly affect the consumers acceptance, the optical properties of the films were evaluated (Table 8). The addition of *C. winterianus* and *L. viridis* EOs did not significantly change (p-value>0.05) the L*, a* and b* coordinates. The incorporation of *C. winterianus* EO decreased the transparency values, nonetheless, the films show high transparency values, from 96% to 92%, which might be related to the amorphous arrangement in films composed with k-carrageenan and connected to the light scattering brought on by the distribution of the EO droplets inside the biopolymer matrix [180]. This conclusion is consistent with findings from other studies since adding more EO often makes the films less transparent, and depending on the kind of EO, adding more may also affect the color of the films [178, 181]. No significant changes in transparency were seen for the films that included *L. viridis* EO, maintaining within the control film values of 95%.

5.5.4 – Contact Angle and Surface Free Energies

The most popular technique for determining a surface's hydrophobicity or hydrophilicity is to measure the water contact angle. A hydrophobic surface has a water contact angle higher than 90°, whereas a hydrophilic surface has a water contact angle smaller than 90° [182]. The values of the contact angles obtained on both sides of the films are listed in Table 9. k-Carrageenan is composed of around 25% to 30% organosulfates, exhibiting a notably high level of solubility [183]. By incorporating EOs, it is expected an increase in hydrophobicity. It was crucial to carefully examine both sides of the films because they displayed different behaviors. This can be an important consideration when deciding which side to pack food on; if it is desired to pack food with more water content, it must turn the hydrophobic side inward in order to prevent water loss.

Generally, there were no changes in the hydrophobicity of the films with *C. winterianus* EO (Table 9), relative to the upper face (p-value<0.05). Therefore, the upper face is hydrophobic, contrary to the lower face, which has a contact angle inferior to 90° and this tends to decrease with increasing EO volumes. It is curious to note that there is a certain affinity of the materials with the surface for the different faces of the films, since the values obtained in the control films are similar for both sides, whereas this is not the case with the addition of the EOs. In the films incorporating the EOs it can be said that a decrease in hydrophobicity on the bottom faces is observed, while on the upper faces, except for 125 µL having p-value<0.05, the hydrophobicity tends to increase.

Films containing *L. viridis* EO (Table 9) exhibit hydrophobicity on the top side with water contact angles ranging from 101.43 to 104.81, except for the film with 62.5 µL, which has a water contact angle of 89.74. The hydrophobicity of the bottom face is only seen with the incorporation of 125 µL of the EO. In these tests, no statistically significant differences were observed (p-value<0.05). In the bottom face of the films with *L. viridis* EO, it tended to increase the water contact angle, which implies that hydrophobicity increased. This contrasts with the films incorporating *C. winterianus* EO, in which the hydrophilicity increased on the bottom face.

EOs are hydrophobic due to their non-polar chemical structure, and then, when integrating them into hydrophilic polymer matrices increases their barrier properties [176]. In addition to the EOs' natural hydrophobicity, another explanation for the rise in this property relates to the roughness produced in the films, which may also increase their hydrophobicity since the surface of structures is crucial in determining a surface's hydrophobicity [175].

There are numerous applications for surface free energy. It establishes how the solid will perform in the presence of liquids. High surface free energy materials are often easily wettable by any liquid [184]. Measurements of contact angles were frequently used to determine the material's surface free energy for some particular liquids [185]. It will be possible to determine the dispersion and polar components of the free energy of the surface of the films using the contact angles through the OW approach. This model allows to describe both the polarity and the surface free energy [185].

The total surface free energy (Table 9) of the films with *C. winterianus* EO ranged between 13.95 ± 2.59 and 38.60 ± 1.49 mN/m and, analyzing the evolution with the increase of the EO volume, it increased relatively to the control film; however, the results with statistical significance were confirmed in all values (*p*-value<0.05).

Regarding the polar component, it decreased with the addition of the EO and there were no verified statistical differences (*p*-value>0.05). The dispersive component also tended to increase with the addition of the EO; however, statistically significant values were observed for this parameter (*p*-value<0.05).

The incorporation of the *L. viridis* EO in the films lead to results similar to those obtained for films with *C. winterianus* EO. Regarding the dispersive component, the values increased in comparison with the control film and each of them exhibited statistical variance (*p*-value<0.05). When compared to the control film, the polar component decreased, and statistical analysis revealed no significant differences in this regard. Finally, the total component increased relatively to the control film, and there were statistical deviations (*p*-value<0.05) in all the films.

The values for total surface free energy obtained from the films are comparable to some petroleum-derived products, like that of polypropylene [186].

Table 9. Contact angles (◦) of the films.

Films			**Water contact angle (◦)**	**Diiodomethane contact angle (◦)**	**Ethyleneglycol contact angle (◦)**	**Dispersive component, γ^D (mN/m)**	**Polar component, γ^P (mN/m)**	**Total surface free energy, γ^T (mN/m)**
	Control	Top face^{a}	93.22 ± 5.02	93.16 ± 5.58	93.22 ± 5.02	9.84 ± 2.08	4.35 ± 1.88	14.19 ± 2.80
		Bottom face^{b}	93.09 ± 5.60	93.90 ± 4.92	93.22 ± 5.02	9.97 ± 1.87	3.98 ± 1.79	13.95 ± 2.59
C. winterianus	62.5 µL EO	Top face^{c}	97.95 ± 6.98	47.27 ± 0.96	62.81 ± 2.48	35.79 ± 0.52	0.38 ± 0.25	36.17 ± 0.58
		Bottom face^{d}	93.39 ± 4.79	47.04 ± 1.70	60.00 ± 1.95	35.90 ± 0.92	0.74 ± 0.30	36.64 ± 0.97
	125 µL EO	Top face^{e}	86.81 ± 8.44	41.67 ± 2.86	66.82 ± 0.84	38.53 ± 1.46	0.00 ± 0.01	38.53 ± 1.46
		Bottom face^{f}	69.00 ± 8.44	41.45 ± 2.83	73.77 ± 5.08	38.40 ± 1.44	0.20 ± 0.37	38.60 ± 1.49
	250 µL EO	Top face^{g}	95.10 ± 4.76	44.26 ± 4.74	65.50 ± 5.43	37.11 ± 2.46	0.23 ± 0.36	37.35 ± 2.49
		Bottom face^{h}	84.43 ± 5.47	50.47 ± 1.38	79.09 ± 0.75	33.72 ± 0.77	0.33 ± 0.12	34.05 ± 0.78
L. viridis	62.5 µL EO	Top face^{i}	89.74 ± 4.10	47.77 ± 0.54	63.92 ± 1.00	35.49 ± 0.30	0.36 ± 0.11	35.85 ± 0.31
		Bottom face^{j}	82.12 ± 7.11	42.01 ± 0.56	70.27 ± 9.72	38.58 ± 0.29	1.07 ± 1.07	39.65 ± 1.11
	125 µL EO	Top face^{k}	101.43 ± 8.92	47.58 ± 0.81	64.41 ± 2.06	35.62 ± 0.44	0.25 ± 0.17	35.86 ± 0.48
		Bottom face^{l}	94.39 ± 4.74	45.50 ± 2.78	66.57 ± 3.41	36.54 ± 1.48	0.16 ± 0.22	36.70 ± 1.49
	250 µL EO	Top face^{m}	104.81 ± 7.39	45.78 ± 0.83	62.32 ± 0.92	36.61 ± 0.45	0.33 ± 0.10	36.94 ± 0.46
		Bottom face^{n}	90.21 ± 3.89	40.27 ± 1.92	69.66 ± 0.55	39.04 ± 0.96	0.08 ± 0.06	39.11 ± 0.96
***p*-values**			0.210^{ac} 0.933^{bd} 0.147^{ae} 0.004^{bf*} 0.495^{ag} 0.031^{bh*}	$<0.001^{ac*}$ $<0.001^{bd*}$ $<0.001^{ae*}$ $<0.001^{bf*}$ $<0.001^{ag*}$ $<0.001^{bh*}$	$<0.001^{ac*}$ $<0.001^{bd*}$ $<0.001^{ae*}$ $<0.001^{bf*}$ $<0.001^{ag*}$ $<0.001^{bh*}$	$<0.001^{ac*}$ $<0.001^{bd*}$ $<0.001^{ae*}$ $<0.001^{bf*}$ $<0.001^{ag*}$ $<0.001^{bh*}$	0.065^{ac} 0.085^{bd} 0.057^{ae} 0.062^{bf*} 0.058^{ag} 0.071^{bh*}	$<0.001^{ac*}$ $<0.001^{bd*}$ $<0.001^{ae*}$ $<0.001^{bf*}$ $<0.001^{ag*}$ 0.003^{bh*}
			0.407^{ai} 0.109^{bj} 0.255^{ak} 0.763^{bl} 0.097^{am} 0.519^{bn}	$0.004^{ai\,*}$ $0.004^{bj\,*}$ $0.003^{ak\,*}$ $0.001^{bl\,*}$ $0.003^{am\,*}$ $0.002^{bn\,*}$	$0.008^{ai\,*}$ $0.035^{bj\,*}$ $0.004^{ak\,*}$ $0.004^{bl\,*}$ $0.007^{am\,*}$ $0.018^{bn\,*}$	$0.0018^{ai\,*}$ $0.0011^{bj\,*}$ $0.0015^{ak\,*}$ $0.0001^{bl\,*}$ $0.0013^{am\,*}$ $0.0002^{bn\,*}$	0.0662^{ai} 0.0874^{bj} 0.0624^{ak} 0.0640^{bl} 0.0654^{am} 0.0634^{bn}	$0.0050^{ai\,*}$ $0.0001^{bj\,*}$ $0.0045^{ak\,*}$ $0.0007^{bl\,*}$ $0.0042^{am\,*}$ $0.0013^{bn\,*}$

Results are presented as mean±SD; * Indicates a significant result (p-value < 0.05); Superscript letters (from a to n) indicate the samples under statistical comparison.

5.5.5 – Barrier Properties

Food products should be transported, handled, and commercialized in a protective environment created by packaging materials. By serving as an effective barrier to moisture and gases (CO_2, O_2), they should help lengthen the shelf-life of perishable items [187].

The barrier properties of the films developed in this work were evaluated in terms of barrier to water vapor and to oil (Table 10). Regarding the WVP of the films, it was observed that adding *C. winterianus* EO slightly changed WVTR and WVP values. Although only 62.5 μL and 250 μL values increased generally for the WVTR, no statistical variations were found for the values (*p*-value<0.05). While EO values increased, the WVP values showed a pattern of stagnation; however, the 250 μL exhibits significant statistical variation (*p*-value<0.05).

The WVTR values for the films containing *L. viridis* EO increased overall, except for the WVTR with 62.5 μL, which is lower than the control film; nonetheless, both studies exhibit statistical variances (*p*-value<0.05).

A. Farhan et al. reported in their work that OP of k-carrageenan/glycerol films ranged between 0.37 and 0.97 g.mm/m^2.day [9]. These values are very low when compared with the obtained results [188]. Carrageenan films are permeable to water vapor due to the abundance of hydroxyl and sulfate groups present in their molecular structures, resulting in a highly hydrophilic nature of carrageenan film; however, it has been previously established that the retention of water in polysaccharide-based films is connected to potential interactions between polysaccharide molecules and glycerol hydroxyl groups, resulting in a more compact polymeric network [9, 63].

Considering the OP results, they lacked a clear pattern and were somewhat dispersed; however, it did not show statistically significant results (*p*-value>0.05).

Table 10. Barrier properties of films.

Films		**Water Vapor**		**Oil**
		WVTR (g/m^2.day)	**WVP (g/Pa.day.m)**	**(g.mm/m^2.day)**
	Control ^{a}	532.58 ± 1.76	$1.55\times10^{-5} \pm 5.14\times10^{-08}$	4.79 ± 0.39
C. winterianus	62.5 µL EO ^{b}	534.14 ± 33.95	$1.68\times10^{-5} \pm 1.06\times10^{-6}$	13.32 ± 2.65
	125 µL EO ^{c}	503.27 ± 12.35	$1.68\times10^{-5} \pm 4.13\times10^{-7}$	6.93 ± 1.06
	250 µL EO ^{d}	539.75 ± 8.38	$2.65\times10^{-5} \pm 4.12\times10^{-7}$	9.64 ± 2.08
L. viridis	62.5 µL EO ^{e}	517.30 ± 11.02	1.62E-5 ± 3.46E-07	4.69 ± 0.074
	125 µL EO ^{f}	542.56 ± 83.78	1.81E-5 ± 2.80E-06	12.154 ± 1.382
	250 µL EO ^{g}	529.77 ± 85.11	2.60E-5 ± 4.18E-06	6.874 ± 0.702
	p-values	0.959 ^{ab} 0.178 ^{ac} 0.434 ^{ad} 0.040 $^{ae\,*}$ 0.040 $^{af\,*}$ 0.040 $^{ag\,*}$	0.348 ^{ab} 0.136 ^{ac} 0.015 ^{ad*} 0.040 $^{ae\,*}$ 0.040 $^{af\,*}$ 0.040 $^{ag\,*}$	0.401 ^{ab} 0.127 ^{ac} 0.509 ^{ad} 0.083 ^{ae} 0.230 ^{af} 0.125 ^{ag}

Results are presented as mean±SD; * Indicates a significant result (p-value < 0.05); Superscript letters (a–g) indicate the samples under statistical comparison.

5.5.6 – Antioxidant Activity of the Films

The results of the antioxidant activity of the films determined by the DPPH• method are shown in Figures 19 and 20. The control film did not show antioxidant activity, which is not verified with the films incorporating the EOs. An antioxidant activity pattern was observed for films containing *C. winterianus* EO, after the initial phase they remain steady, but after 2 h an increase in the antioxidant activity was verified. After 2.5 h of reaction, the films with 250 µL of the EO showed 6% and at 4 h 5% of DPPH free radicals inhibition. The films containing 62.5 and 125 µL of the EO showed only one major peak, being almost 1.5% and 2.8% inhibition of DPPH free radicals, respectively.

Antioxidant activity was observed in the films with *L. viridis* EO. The initial spike is seen in the first hour, with the greatest percentage being achieved by 62.5 µL, around 5.7%. The peak is more modest for the other two volumes, at 2.9% and 1.5% inhibition of DPPH free radicals for the 250 µL and 125 µL volumes, respectively. After some time (3h), there is still a second peak for the volume of 125 µL, and this one has a percentage of roughly 1.3% inhibition of DPPH free radicals.

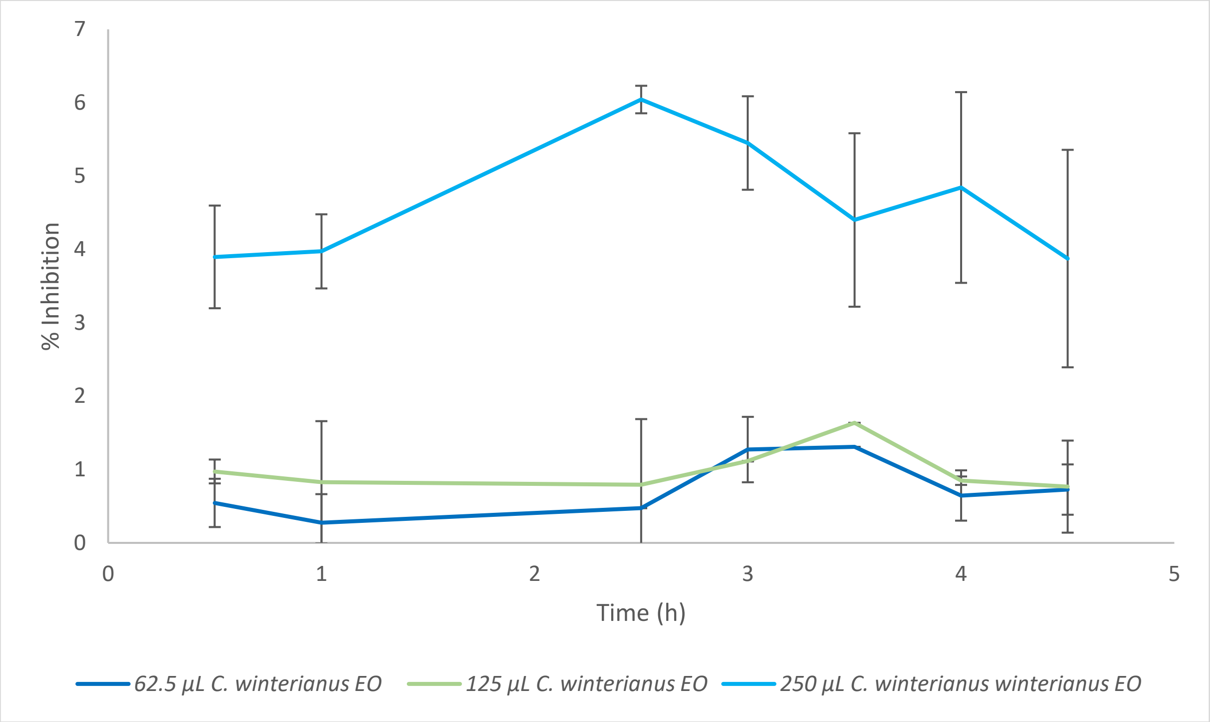


Figure 19 Antioxidant activity of k-carrageenan films incorporating *C. winterianus* EO evaluated by DPPH radical. Results presented as mean±SD.

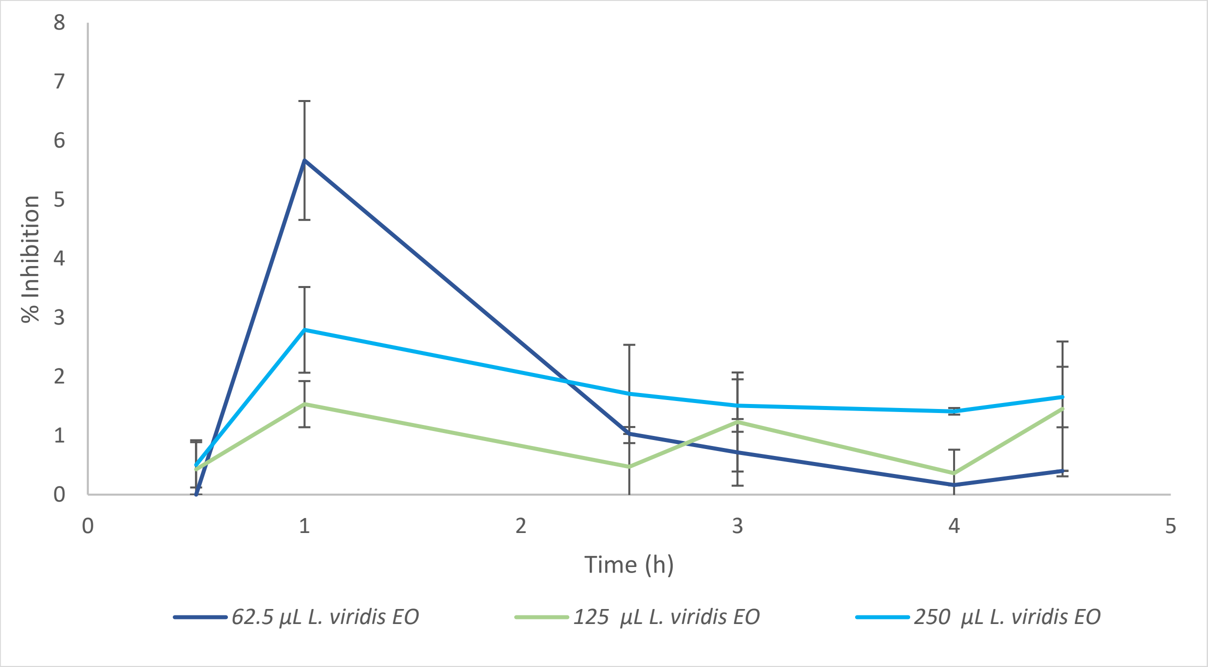


Figure 20 Antioxidant activity of k-carrageenan films incorporating *L. viridis* EO evaluated by DPPH radical. Results presented as mean±SD.

The β-carotene bleaching test results, where the ability of the films to inhibit the lipid peroxidation was evaluated, are presented in Table 11.

Table 11. Antioxidant properties of the films determined by β-carotene bleaching test.

Films	**% Inhibition**	***p*-values**
Control^{a}	79.35 ± 5.64	-
62.5 µL *C. winterianus EO* ^{b}	90.14 ± 2.68	0.375^{ab}
125 µL *C. winterianus EO* ^{c}	100.00 ± 3.29	0.068^{ac}
250 µL *C. winterianus EO* ^{d}	100.00 ± 4.64	0.355^{ad}
62.5 µL *L. viridis EO* ^{e}	14.65 ± 3.05	0.011 $^{ae\,*}$
125 µL *L. viridis EO* ^{f}	21.11 ± 4.62	0.009 $^{af\,*}$
250 µL *L. viridis EO* ^{g}	22.66± 2.53	0.008 $^{ag\,*}$

Results presented as mean ± SD; Upper letters (a-g) indicate the samples under statistical comparison.
* Indicates a significant result (*p*-value<0.05).

Given the positive outcome shown by the β-carotene bleaching assay on the *C. winterianus* EO, it is reasonable to anticipate similar results in the films with this EO. The findings demonstrated that films containing *C. winterianus* EO exhibit a high level of lipid peroxidation inhibition and for that reason they have potential to be used as substitutes for the traditional food packaging materials, particularly for foods with a high fat content.

In contrast to this EO, *L. viridis* EO demonstrated much lower values, comparing with *C. winterianus* EO films and control films. The higher the efficiency of an antioxidant, the slower will be the color depletion, hence, the duration of *L. viridis* EO antioxidant activity is less prolonged in comparison to *C. winterianus* EO.

5.5.7 – Antibacterial and Anti-quorum Sensing Properties of the Films

The solid diffusion method was used to assess the film's antibacterial and anti-quorum sensing properties, being the inhibition zones presented (Table 12). It was possible to verify that in some cases there was contact inhibition of bacterial growth (6 mm) as for example in 125 µL and 250 µL of *C. winterianus* EO in *L. monocytogenes* LMG 16779 and in the control film with the bacterium *S*. Typhimurium ATCC 13311.

Regarding the films with *L. viridis* EO, there are also certain cases that seem to have contact inhibition, such as in the case of *L. monocytogenes* LMG 16779 and *P. aeruginosa* ATCC 27853.

By using optical microscopy, the antibacterial activity results were also verified, the colonies and the edge of the film were marked in blue and red, respectively. Comparing the findings of the values of Table 12 with the pictures in Figures 21-26, it can be concluded that, for the strains that did not exhibited inhibition, bacterial growth takes place below the film. Concerning the anti-quorum properties of the films, it was noticed that the EOs capacity to inhibit the bacterial intercellular communication is not maintained when they were incorporated in k-carrageenan films.

Table 12.Diameters of inhibition zones (mm). *CW: C. winterianus; LV: L. viridis.*

	Control Film	**62.5 µL *CW* Film**	**125 µL *CW* Film**	**250 µL *CW* Film**	**62.5 µL *LV* Film**	**125 µL *LV* Film**	**250 µL *LV* Film**
E. coli ATCC 25922	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
E. faecalis ATCC 29212	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
L. monocytogenes **LMG** 16779	0.00 ± 0.00	0.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
P. aeruginosa ATCC 27853	6.85 ± 1.20	7.93 ± 0.67	8.17 ± 0.37	7.62 ± 0.16	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00
S. aureus ATCC 25923	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
***S.* Typhimurium** ATCC 13311	6.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C. violaceum ATCC 12472	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00	6.00 ± 0.00

Results presented as mean ± SD.

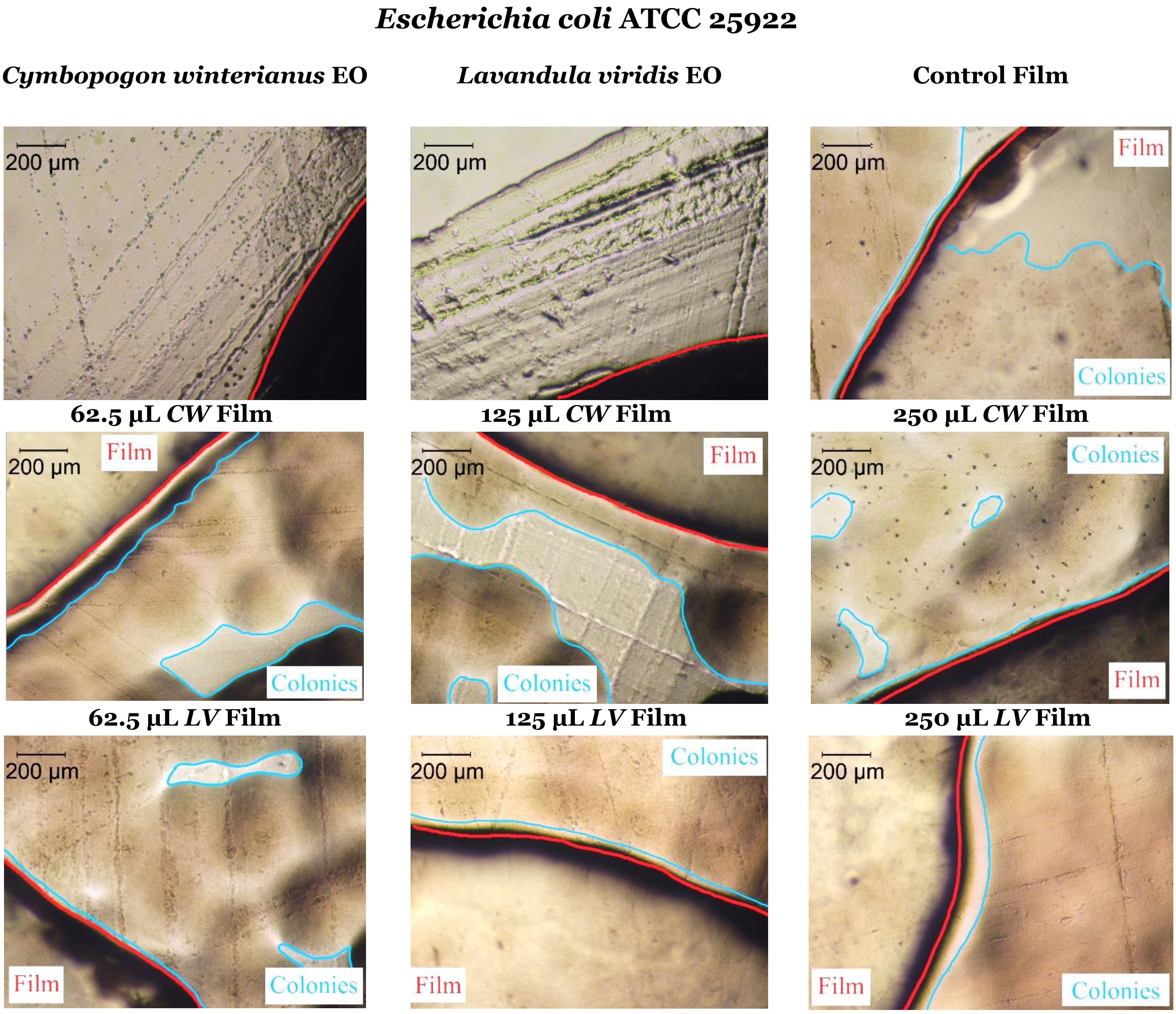
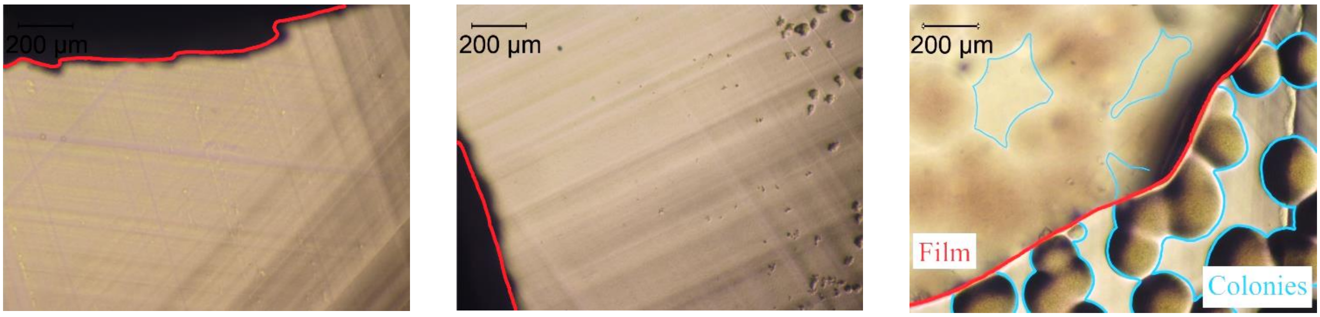


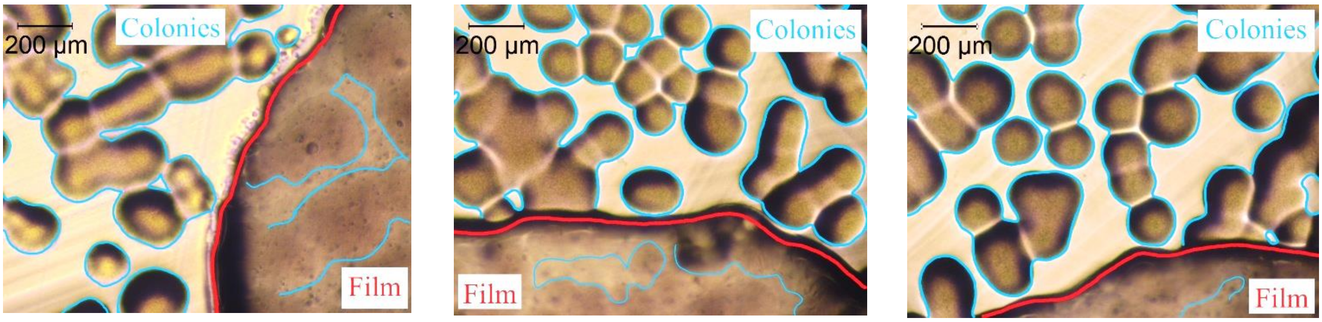
Figure 21. Optical microscopy images of the antibacterial activity of the films against *Escherichia coli* ATCC 25922. *CW: C. winterianus; LV: L. viridis.*

Enterococcus faecalis ATCC 29212

***Cymbopogon winterianus* EO** **|** ***Lavandula viridis* EO** **|** **Control Film**



62.5 µL *CW* Film **125 µL *CW* Film** **250 µL *CW* Film**



62.5 µL *LV* Film **125 µL *LV* Film** **250 µL *LV* Film**

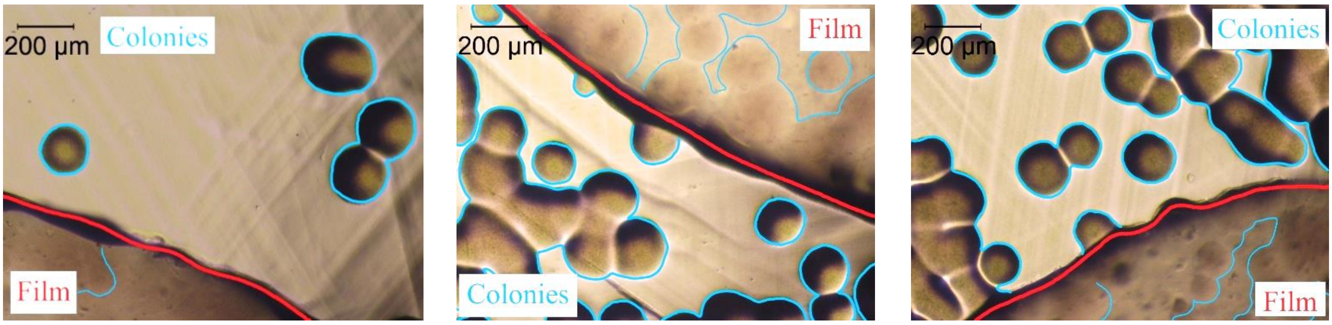
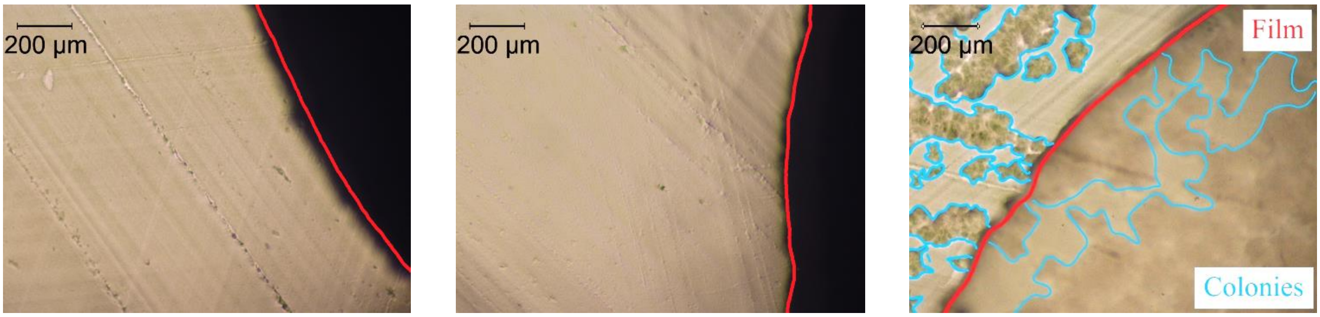


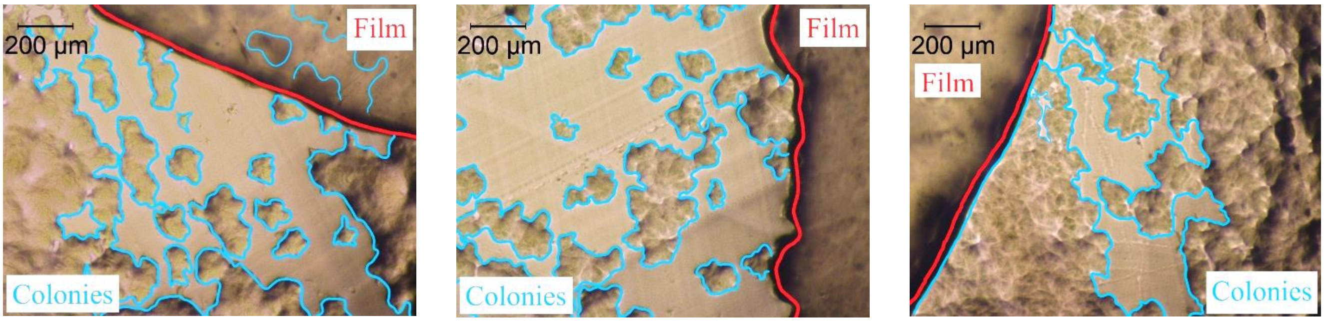
Figure 22 Optical microscopy images of the antibacterial activity of the films against *Enterococcus faecalis* ATCC 29212. *CW: C. winterianus; LV: L. viridis.*

***Listeria monocytogenes* LMG 16779**

***Cymbopogon winterianus* EO** | ***Lavandula viridis* EO** | **Control Film**



62.5 µL *CW* Film | **125 µL *CW* Film** | **250 µL *CW* Film**



62.5 µL *LV* Film | **125 µL *LV* Film** | **250 µL *LV* Film**

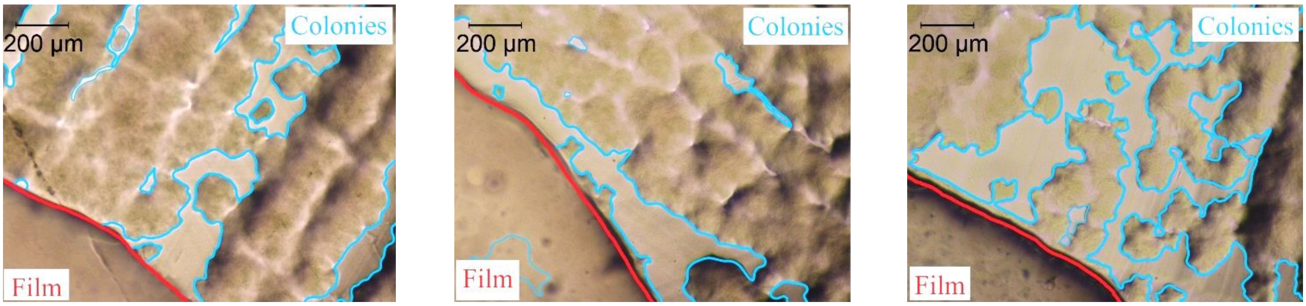
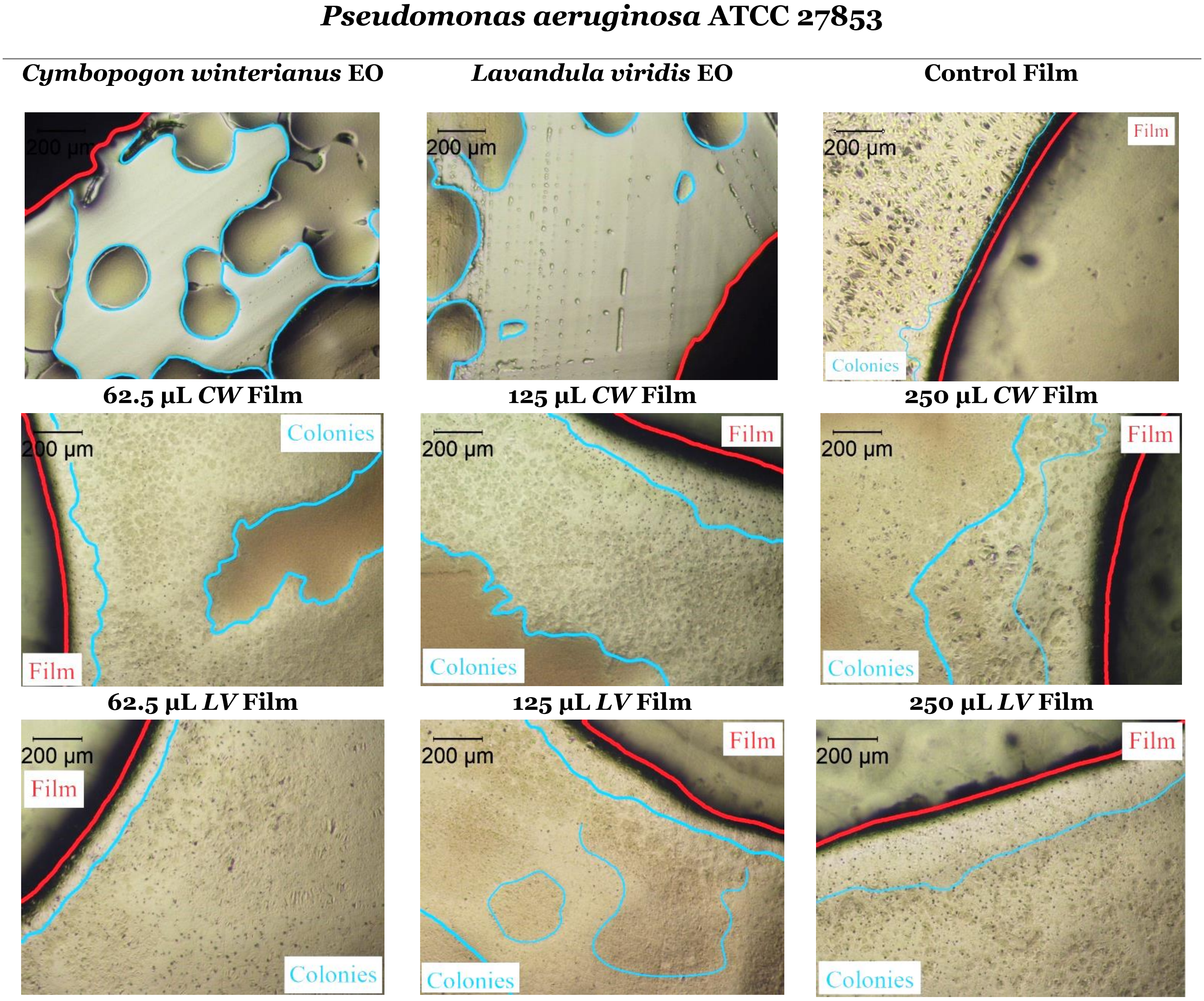


Figure 23 Optical microscopy images of the antibacterial activity of the films against *Listeria monocytogenes* LMG 16779. *CW: C. winterianus; LV: L. viridis.*



***Pseudomonas aeruginosa* ATCC 27853**

***Cymbopogon winterianus* EO** | ***Lavandula viridis* EO** | **Control Film**

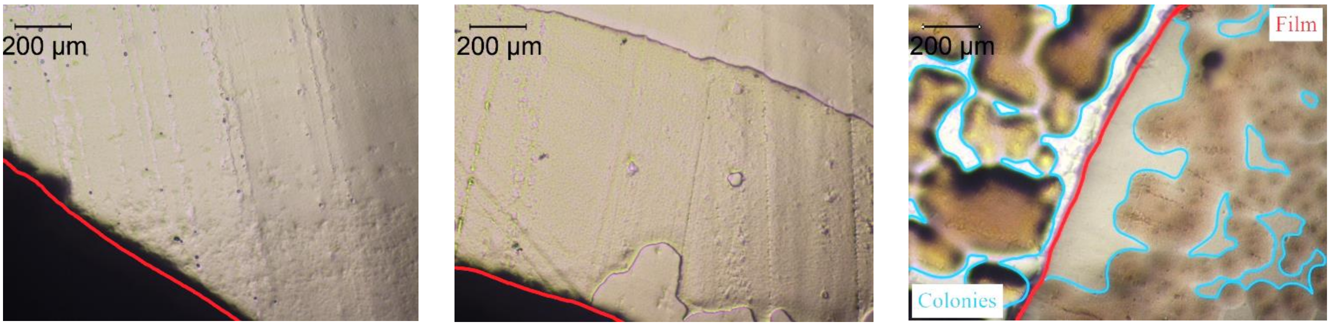
62.5 µL *CW* Film | **125 µL *CW* Film** | **250 µL *CW* Film**

62.5 µL *LV* Film | **125 µL *LV* Film** | **250 µL *LV* Film**

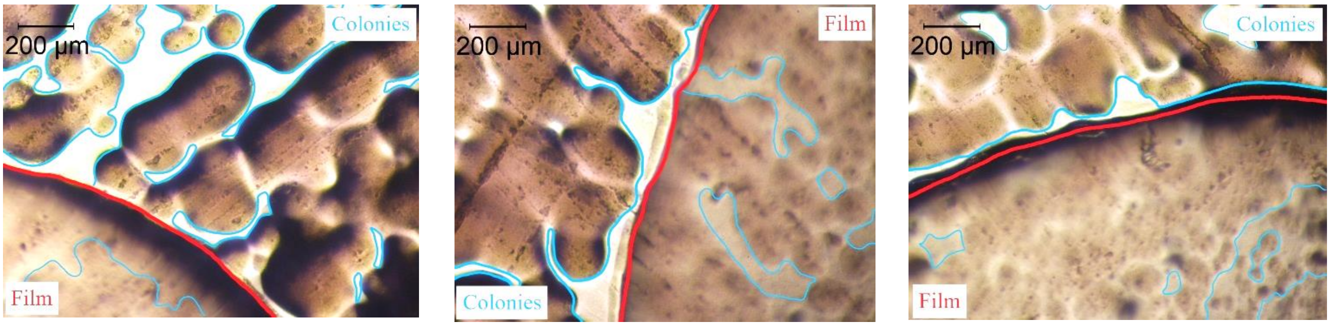
Figure 24. Optical microscopy images of the antibacterial activity of the films against *Pseudomonas aeruginosa* ATCC 27853. *CW: C. winterianus; LV: L. viridis.*

***Staphylococcus aureus* ATCC 25923**

***Cymbopogon winterianus* EO** **_Lavandula viridis_ EO** **Control Film**



62.5 µL *CW* Film **125 µL *CW* Film** **250 µL *CW* Film**



62.5 µL *LV* Film **125 µL *LV* Film** **250 µL *LV* Film**

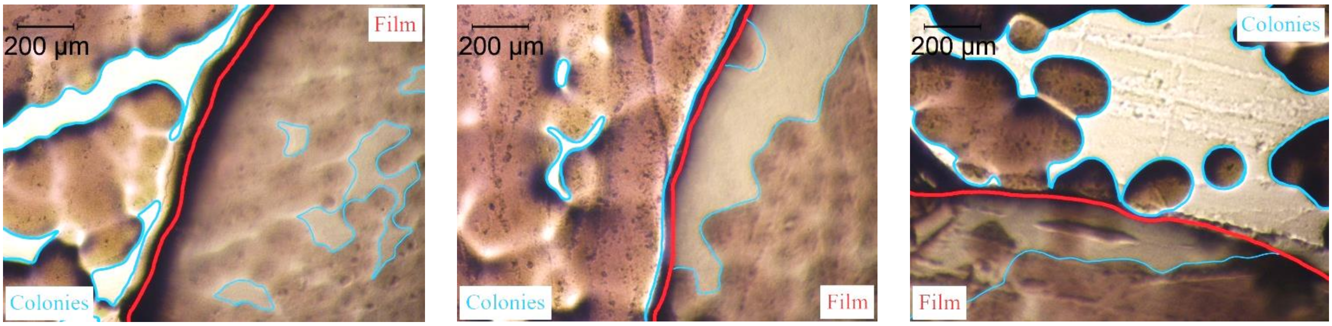
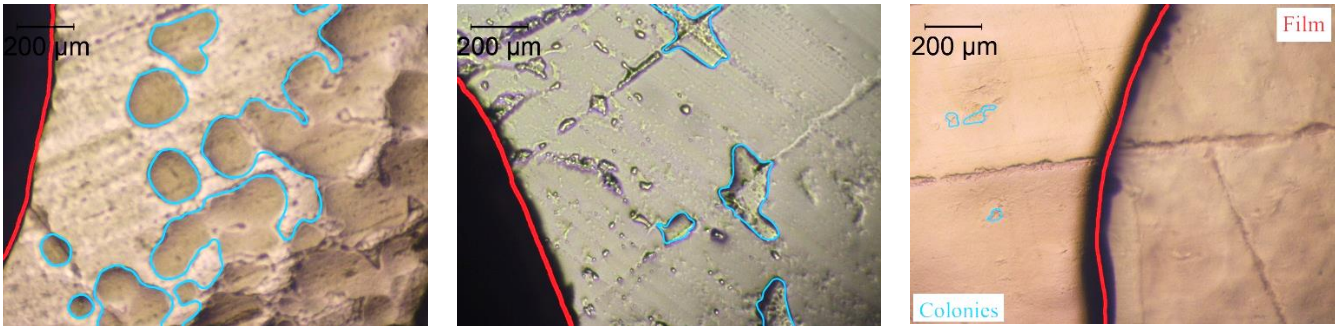


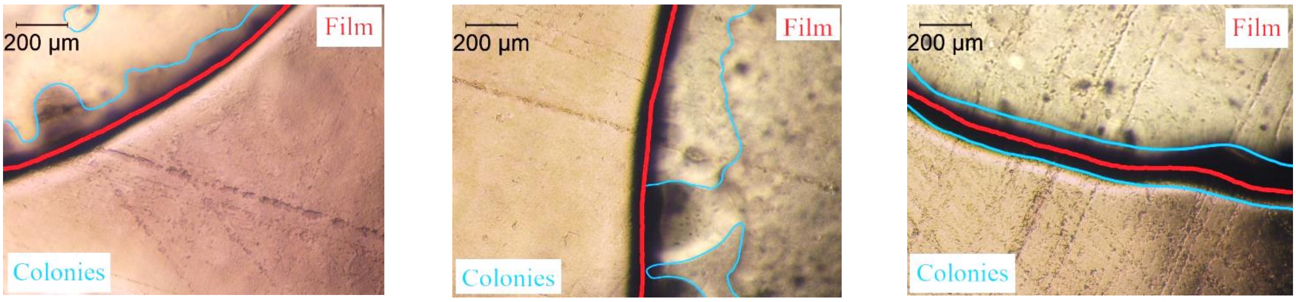
Figure 25 . Optical microscopy images of the antibacterial activity of the films against *Staphylococcus aureus* ATCC 25923. *CW: C. winterianus; LV: L. viridis.*

Salmonella Typhimurium ATCC 13311

***Cymbopogon winterianus* EO** | ***Lavandula viridis* EO** | **Control Film**



62.5 µL *CW* Film | **125 µL *CW* Film** | **250 µL *CW* Film**



62.5 µL *LV* Film | **125 µL *LV* Film** | **250 µL *LV* Film**

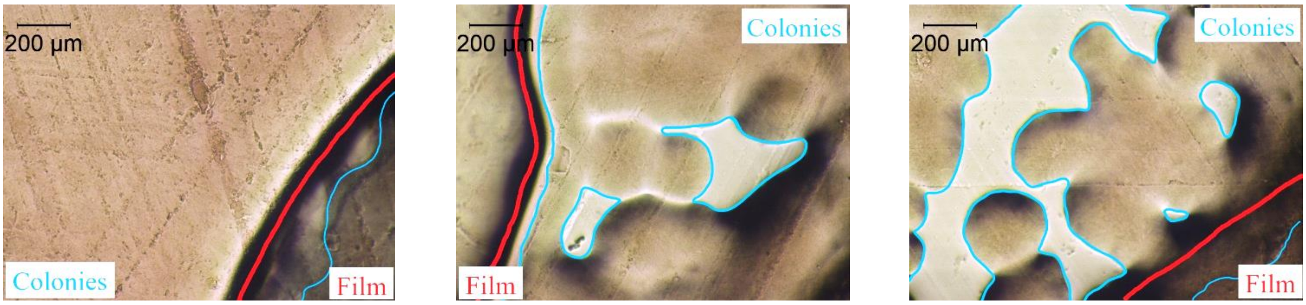


Figure 26 Optical microscopy images of the antibacterial activity of the films against *Salmonella* Typhimurium ATCC 13311. *CW: C. winterianus; LV: L. viridis.*

5.5.8 – Anti-biofilm Activity of the Films Against *L. monocytogenes*

Since the *C. winterianus* and *L. viridis* EOs primarily affected the growth of *L. monocytogenes* LMG 16779, and since this bacterium is a well-known foodborne pathogen, the antibiofilm activity of the films incorporating 250 µL of the EOs was evaluated by SEM (Figure 27). These films were chosen since increasing the volume of the EOs incorporated in the films, it would give a better antibiofilm effect because the EOs have antibacterial activity for this bacterium [189].

Figure 27-A shows the *L. monocytogenes* biofilm formed in the control film, showing several layers of bacteria. As shown in Figures 27-C1, biofilms formed in the surface of k-carrageenan films containing 250 µL of *C. winterianus* EO are sparse and consist of just one layer of cells. In addition, it was also visible that the cell integrity was lost (examples marked in red). Figure 27-C2 shows that the number of bacteria still decreased when the biofilms were formed on the bioactive k-carrageenan films. Some bacteria have compromised cell membrane integrity, but there were also some bacteria with good integrity for comparison (examples marked in blue).

The films containing 250 µL of *L. viridis* EO also showed promising results (Figures C1 and C2), since there is a noticeable reduction in the total number of bacteria when compared to the control film. Therefore, it is possible to conclude that these films have an anti-biofilm capability, which is a property that might be extremely advantageous when used for packaging food.

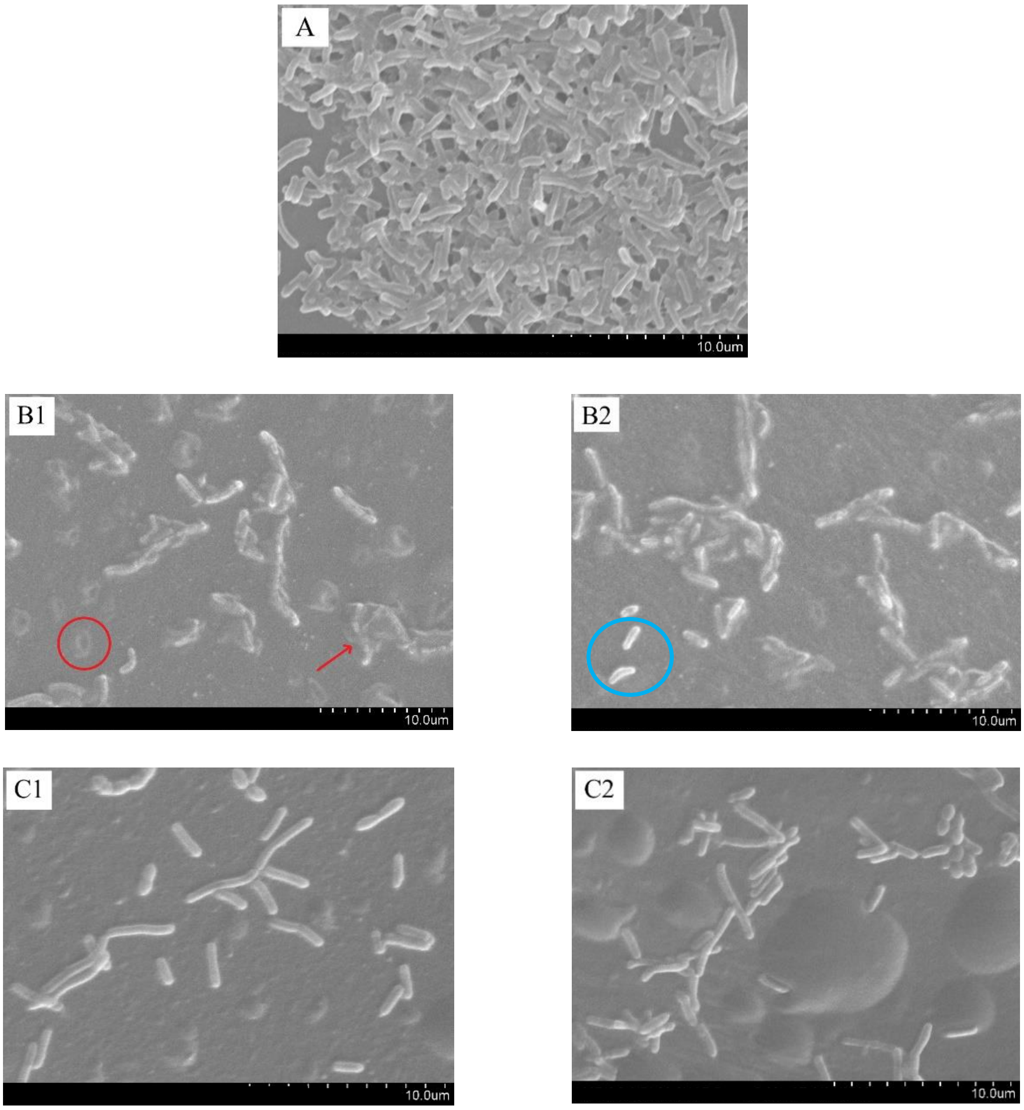


Figure 27. SEM images of *Listeria monocytogenes* LGM 16779 biofilms formed directly on the surface of the control film (A); on the film containing 250 µL of *C. winterianus* EO (B1, B2); and on the film containing 250 µL of *L. viridis* EO (C1, C2)

5.5.9 – Application of Films in a Food Model

As previously mentioned, a food model was used to evaluate the film's effectiveness. To do that, cheese slices were wrapped with control film and with films containing 250 µL of *C. winterianus* and *L. viridis* EOs. After initial weighing and evaluation of color parameters of the cheese slices, the small packages were maintained in a refrigerator at 4°C for 15 days (Figure 28). Then, the color of the cheese slices was measured again to identify any differences, and the cheese slices were also weighed [142].

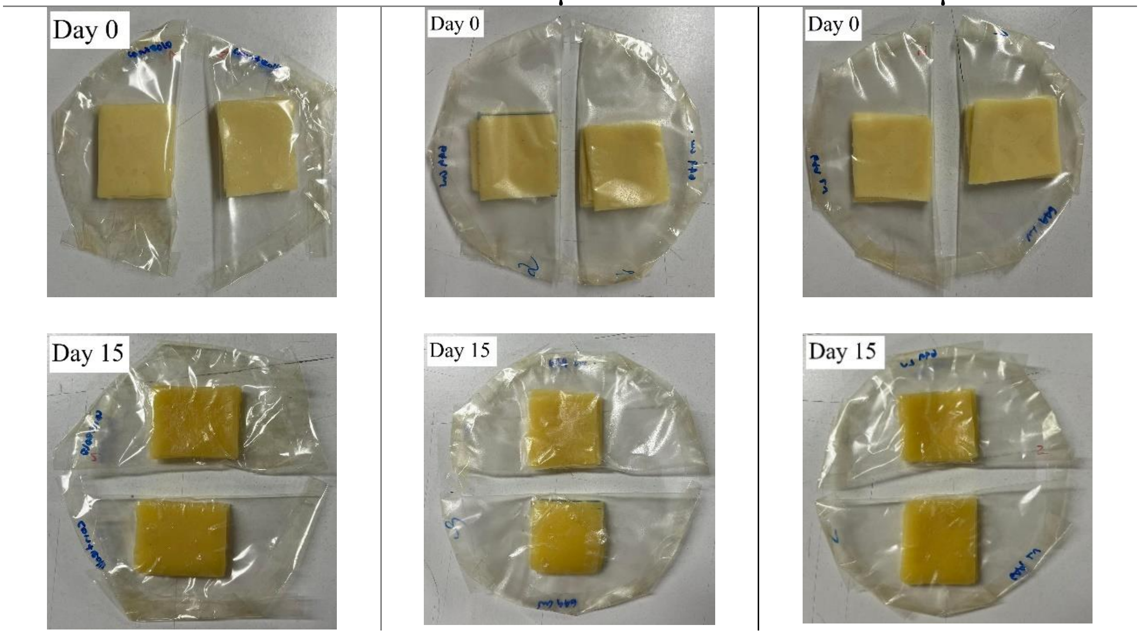

Cheese wrapped in control film | **Cheese wrapped in film with 250 µL *CW* EO** | **Cheese wrapped in film with 250 µL *LV* EO**

Figure 28. Cheese slices wrapped in three types of films. *CW: C. winterianus; LV: L. viridis*

Table 13. Water loss in cheese during 15 days.

Film	Water loss	*p*-value
Control ^{a}	2.98 ± 0.04	-
250 µL *CW* EO ^{b}	3.57 ± 4.45	0.88 ^{ab}
250 µL *LV EO* ^{c}	4.19 ± 0.52	0.19 ^{ac}

Results are presented as mean±SD; Superscript letters (a-c) indicate the samples under statistical comparison. *CW: C. winterianus; LV: L. viridis.*

The water loss was approximately comparable when the cheese slices were wrapped with the three different films, with no statistically significant variances (*p*-value>0.05) (Table 13). As expected, there were small changes in the yellow color (Table 14). Additionally, it should be mentioned that no visible signs of cheese rot or mold were observed.

Table 14. Color of the cheese slices wrapped with the k-carrageenan films.

Color			
			***p*-Value**
Cheese (Initial)	L* ^{a}	81.05 ± 0.902	-
	a* ^{b}	-0.21 ± 0.165	
	b* ^{c}	17.99 ± 0.71	
Control film (15 days)	L* ^{d}	65.8 ± 2.057	0.0020 ^{ad*}
	a* ^{e}	-0.41 ± 0.298	0.3814 ^{be}
	b* ^{f}	22.55 ± 0.782	0.0018 ^{cf*}
	L* ^{g}	65.33 ± 2.001	0.0016 ^{ag*}
	a* ^{h}	-0.52 ± 0.351	0.2649 ^{bh}
	b* ^{i}	22.35 ± 0.572	0.0014 ^{ci*}
250 µL CW EO film **(15 days)**	L* ^{j}	65.21 ± 1.926	0.0014 ^{aj*}
	a* ^{k}	-0.75 ± 0.314	0.0772 ^{bk}
	b* ^{l}	22.18 ± 0.26	0.0047 ^{cl*}
	L* ^{m}	63.03 ± 2.764	0.0042 ^{am*}
	a* ^{n}	-1.61 ± 0.711	0.0694 ^{bn}
	b* ^{o}	17.58 ± 3.366	0.8542 ^{co}
250 µL LV EO **(15 days)**	L* ^{p}	68.69 ± 3.51	0.0204 ^{ap*}
	a* ^{q}	-0.42 ± 0.412	0.4802 ^{bq}
	b* ^{r}	22.2 ± 0.694	0.0018 ^{cr*}
	L* ^{s}	65.97 ± 3.195	0.0102 ^{as*}
	a* ^{t}	-0.67 ± 0.453	0.2139 ^{bt}
	b* ^{u}	21.75 ± 0.582	0.0024 ^{cu*}

Results are presented as mean±SD; * Indicates a significant result (p-value<0.05); Superscript letters(a–u) indicate the samples under statistical comparison. *CW: C. winterianus; LV: L. viridis.*

Chapter 6

Conclusions and Future Perspectives

6 - Conclusions and Future Perspectives

This work demonstrated the viability to produce k-carrageenan bioactive and eco-friendly films incorporating *C. winterianus* and *L. viridis* EOs that can be applied as innovative food packaging materials, avoiding using conventional plastics. The produced films were transparent (>90%), hydrophobic (water contact angle>90°), and presented antioxidant activity related to free radicals scavenging and lipid peroxidation inhibition. Moreover, the films inhibited the growth of planktonic and biofilm cells of *L. monocytogenes,* an important foodborne pathogen.

These films have many attractive properties for use in the food packaging industry, but they still present some challenges that should be investigated in future work, namely the mechanical and barrier properties. One potential approach to enhance the mechanical properties of films is the production of a biocomposite, for example, combining k-carrageenan with nanosilica. The integration of nanosilica into a biocomposite has attracted considerable interest due to its ability to enhance mechanical and thermal characteristics.

In order to enhance the barrier properties, more investigation in using hydrophobic substances like lipids, and resins may be pursued. For the case of EOs, an alternative strategy might include the emulsification to mitigate variations in the face hydrophobicity, as it was observed by contact angle measurements.

A few questions that may emerge in this investigation relate to the biodegradability of the films and the cytotoxicity of EOs. While the existing literature indicates that k-carrageenan is biodegradable, it is essential to conduct studies to verify these claims. A further significant consideration is the potential toxicity of the EOs, how they might interact with the human body, and the existence of any established dose limitations. As an example, *C. citratus*, which shares geraniol as its primary constituents with *C. winterianus*, exhibits cytotoxicity against human blood cells.

Since these films cannot be sealed using heat, it should be investigated biodegradable compounds with adhesion properties and, afterwards, consider the potential of a scale-up. The introduction of these films to the market may then be studied, which would boost the circular economy.

Attachments

foods

MDPI

Article

Production and Characterization of k-Carrageenan Films Incorporating *Cymbopogon winterianus* Essential Oil as New Food Packaging Materials

Catarina Santos [1,2], **Ana Ramos** [2,3], **Ângelo Luís** [1,3,*] and **Maria E. Amaral** [2,3]

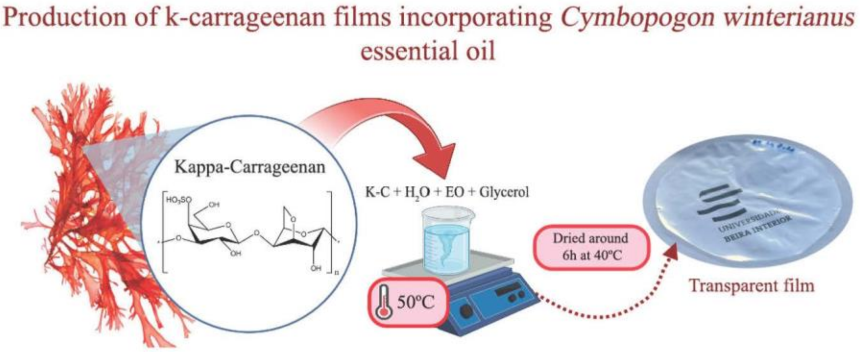
[1] CICS-UBI, Health Sciences Research Centre, University of Beira Interior, Av. Infante D. Henrique, 6200-506 Covilhã, Portugal; catarina.filipa.santos@ubi.pt
[2] FibEnTech-UBI, Fiber Materials and Environmental Technologies Research Unit, University of Beira Interior, Rua Marquês d'Ávila e Bolama, 6201-001 Covilhã, Portugal; ammr@ubi.pt (A.R.); mecca@ubi.pt (M.E.A.)
[3] Chemistry Department, Faculty of Sciences, University of Beira Interior, Rua Marquês d'Ávila e Bolama, 6201-001 Covilhã, Portugal
* Correspondence: angelo.luis@ubi.pt

Abstract: The global production of synthetic plastics from petroleum-based raw ingredients exceeds 150 million metric tons. The environment is threatened by tons of plastic waste, thus endangering wildlife and the public's health. These consequences increased the interest in biodegradable polymers as potential substitutes for traditional packaging materials. This study aimed to produce and characterize k-carrageenan films incorporating *Cymbopogon winterianus* essential oil, in which citronellal was determined to be the major compound (41.12%). This essential oil presented remarkable antioxidant activity, as measured through DPPH (IC_{50} = 0.06 ± 0.01%, v/v; AAI = 85.60 ± 13.42) and β-carotene bleaching (IC_{50} = 3.16 ± 0.48%, v/v) methods. The essential oil also showed antibacterial properties against *Listeria monocytogenes* LMG 16779 (diameter of inhibition zone = 31.67 ± 5.16 mm and MIC = 8 μL/mL), which were also observed when incorporated in the k-carrageenan films. Moreover, scanning electron microscopy showed the reduction of the biofilms of this bacterium, and even its inactivation, due to visible destruction and loss of integrity when the biofilms were created directly on the developed k-carrageenan films. This study also revealed the quorum sensing inhibition potential of *Cymbopogon winterianus* essential oil (diameter of violacein production inhibition = 10.93 ± 0.81 mm), where it could impede intercellular communication and, hence, lower violacein synthesis. The produced k-carrageenan films were transparent (>90%) and slightly hydrophobic (water contact angle > 90°). This work demonstrated the viability of using *Cymbopogon winterianus* essential oil to produce k-carrageenan bioactive films as new food packaging materials. Future work should focus on the scale-up production of these films.

Keywords: k-carrageenan; *Cymbopogon winterianus*; essential oil; films; food packaging; antibacterial activity

Citation: Santos, C.; Ramos, A.; Luís, Â.; Amaral, M.E. Production and Characterization of k-Carrageenan Films Incorporating *Cymbopogon winterianus* Essential Oil as New Food Packaging Materials. *Foods* **2023**, *12*, 2169. https://doi.org/10.3390/foods12112169

Academic Editor: Amalia Conte


Production of k-carrageenan films incorporating *Cymbopogon winterianus* essential oil

Bibliographic References

[1] Borrelle SB, Ringma J, Law KL, Monnahan CC, Lebreton L, Mcgivern A, Murphy E, Jambeck J, Leonard GH, Hilleary MA, Eriksen M, Possingham HP, De Frond H, Gerber LR, Polidoro B, Tahir A, Bernard M, Mallos N, Barnes M, Rochman CM *Predicted growth in plastic waste exceeds efforts to mitigate plastic pollution.*
https://doi.org/10.1126/science.aba3656

[2] Borg K, Lennox A, Kaufman S, Tull F, Prime R, Rogers L, Dunstan E (2022) *Curbing plastic consumption: A review of single-use plastic behavior change interventions*. J Clean Prod.
https://doi.org/10.1016/j.jclepro.2022.131077

[3] Brandon JA, Jones W, Ohman MD (2019) *Multidecadal increase in plastic particles in coastal ocean sediments*. Sci Adv 5:1–7. https://doi.org/10.1126/sciadv.aax0587

[4] Lim XZ (2021) *Microplastics are everywhere - but are they harmful?* Nature 593:22–25.
https://doi.org/10.1038/d41586-021-01143-3

[5] O'Farrell K (2018) *2016-17 Australian Plastics Recycling Survey*.

[6] Villarrubia-Gómez P, Cornell SE, Fabres J (2018) *Marine plastic pollution as a planetary boundary threat – The drifting piece in the sustainability* puzzle. Mar Policy 96:213–220.
https://doi.org/10.1016/j.marpol.2017.11.035

[7] Phelan A, Meissner K, Humphrey J, Ross H (2022) *Plastic pollution and packaging: Corporate commitments and actions from the food and beverage sector*. J Clean Prod 331.
https://doi.org/10.1016/j.jclepro.2021.129827

[8] Zarina S, Ahmad I (2015) *Biodegradable composite films based on κ-carrageenan reinforced by cellulose nanocrystal from kenaf fibers*. Bioresources 10:256–271.
https://doi.org/10.15376/biores.10.1.256-271

[9] Farhan A, Hani NM (2017) *Characterization of edible packaging films based on semi-refined kappa-carrageenan plasticized with glycerol and sorbitol*. Food Hydrocoll 64:48–58.
https://doi.org/10.1016/j.foodhyd.2016.10.034

[10] Alves VD, Castelló R, Ferreira AR, Costa N, Fonseca IM, Coelhoso IM (2011) *Barrier properties of carrageenan/pectin biodegradable composite films*.

Procedia Food Sci 1:240–245.
https://doi.org/10.1016/j.profoo.2011.09.038

[11] Robertson GL, Environment FP (2019) *History of Food Packaging*. Elsevier. https://doi.org/10.1016/B978-0-08-100596-5.22535-3

[12] Krochta JM (2007) *Food Packaging*, 2nd Edition.

[13] Risch SJ (2009) *Food Packaging History and Innovations*. J Agric Food Chem 57:8089–8092. https://doi.org/10.1021/jf900040r

[14] Wilbey RA (2005) *Food Packaging Technology*. Int J Dairy Technol 58:125–125. https://doi.org/10.1111/j.1471-0307.2005.00157.x

[15] Napper IE, Thompson RC (2020) *Plastic Debris in the Marine Environment: History and Future Challenges*. Global Challenges. https://doi.org/10.1002/gch2.201900081

[16] Han JH (2005) *New technologies in food packaging: Overview*. Innovations in Food Packaging 3–11. https://doi.org/10.1016/B978-012311632-1/50033-4

[17] Man CMD (2016) *Food storage trials*. Elsevier Ltd. https://doi.org/ 10.1016/B978-0-08-100435-7.00006-X

[18] Young NWG, O'Sullivan GR (2011) *The influence of ingredients on product stability and shelf life*. Woodhead Publishing Limited. https://doi.org/10.1533/9780857092540.1.132

[19] Torrieri E (2015) *Storage Stability: Shelf-Life Testing*, 1st ed. Elsevier Ltd. https://doi.org/10.1016/B978-0-12-384947-2.00666-8

[20] Thammavongs B, Corroler D, Panoff JM, Auffray Y, Boutibonnes P (1996) *Physiological response of Enterococcus faecalis JH2-2 to cold shock: Growth at low temperatures and freezing/thawing*. Lett Appl Microbiol 23:398–402. https://doi.org/10.1111/j.1472-765X.1996.tb01345.x

[21] Meng L, Zhang Y, Liu H, Zhao S, Wang J, Zheng N (2017) *Characterization of Pseudomonas spp. and associated proteolytic properties in raw milk stored at low temperatures*. Front Microbiol 8:1–7. https://doi.org/10.3389/fmicb.2017.02158

[22] Manzocco L, Calligaris S, Nicoli MC (2010) *Methods for food shelf-life determination and prediction*. Woodhead Publishing Limited. https://doi.org/10.1533/9780857090447.1.196

[23] Ragnarsson JO, Labuza TP (1977) *Accelerated shelf-life testing for oxidative rancidity in foods-A review*. Food Chem 2:291–308. https://doi.org/10.1016/0308-8146(77)90047-4

[24] Corrales M, Fernández A, Han JH (2013) *Antimicrobial Packaging Systems*. https://doi.org/10.1016/B978-0-12-394601-0.00007-2

[25] Hoelzer K, Pouillot R, Gallagher D, Silverman MB, Kause J, Dennis S (2012) *Estimation of Listeria monocytogenes transfer coefficients and efficacy of bacterial removal through cleaning and sanitation*. Int J Food Microbiol 157:267–277. https://doi.org/10.1016/j.ijfoodmicro.2012.05.019

[26] Coimbra A, Carvalho F, Duarte AP, Ferreira S (2022) *Antimicrobial activity of Thymus zygis essential oil against Listeria monocytogenes and its application as food preservative*. Innovative Food Science and Emerging Technologies 80. https://doi.org/10.1016/j.ifset.2022.103077

[27] Carvalho F, Coimbra AT, Silva L, Duarte AP, Ferreira S (2023) *Melissa officinalis essential oil as an antimicrobial agent against Listeria monocytogenes in watermelon juice*. Food Microbiol 109. https://doi.org/10.1016/j.fm.2022.104105

[28] Camargo AC, Woodward JJ, Call DR, Nero LA (2017) *Listeria monocytogenes in Food-Processing Facilities, Food Contamination, and Human Listeriosis: The Brazilian Scenario*. Foodborne Pathog Dis 14:623–636. https://doi.org/10.1089/fpd.2016.2274

[29] (2009) *MAPA* - Ministério da Agricultura, Pecuária e Abastecimento.

[30] Gasanov U, Hughes D, Hansbro PM (2005) *Methods for the isolation and identification of Listeria spp. and Listeria monocytogenes: A review*. FEMS Microbiol Rev 29:851–875 https://doi.org/10.1016/j.femsre.2004.12.002

[31] Rooney ML (1995) *1 Overview of active food packaging*. 1–37.

[32] Bhowmik S, Agyei D, Ali A (2022) *Bioactive chitosan and essential oils in sustainable active food packaging: Recent trends, mechanisms, and applications*. Food Packag Shelf Life. https://doi.org/10.1016/j.fpsl.2022.100962

[33] López-Rubio A (2011) *Bioactive food packaging strategies*. Multifunctional and Nanoreinforced Polymers for Food Packaging 460–482. https://doi.org/10.1533/9780857092786.3.460

[34] Song H geon, Choi I, Lee JS, Chang Y, Yoon CS, Han J (2022) *Whey protein isolate coating material for high oxygen barrier properties: A scale-up study from laboratory to industrial scale and its application to food packaging*. Food Packag Shelf Life 31:100765. https://doi.org/10.1016/j.fpsl.2021.100765

[35] Wyrwa J, Barska A (2017) *Innovations in the food packaging market: active packaging*. European Food Research and Technology 243:1681–1692. https://doi.org/10.1007/s00217-017-2878-2

[36] Mohammed Wasim Siddiqui *Fresh-cut fruits and vegetables*. Academic Press.

[37] Bilohan M (2021) *Desenvolvimento de trilaminados de pululana/celulose/zeína para embalagem alimentar*.

[38] Nathalie Lavoine *Conception, mise en oeuvre et caractérisation de nouveaux bio-nano-matériaux fonctionnels*. Autre. Université de Grenoble, 2013. Français. <NNT : 2013GRENI042>. <tel-01124263>

[39] Han JH (2005) *Innovations in food packaging*. Elsevier Academic https://doi.org/10.1016/B978-012311632-1/50044-9

[40] Abdullah, Cai J, Hafeez MA, Wang Q, Farooq S, Huang Q, Tian W, Xiao J (2022) *Biopolymer-based functional films for packaging applications: A review*. Front Nutr.
https://doi.org/10.3389/fnut.2022.1000116

[41] Palazzo M, Vollero A, Siano A (2023) *Intelligent packaging in the transition from linear to circular economy: Driving research in practice*. J Clean Prod 388:135984.
https://doi.org/10.1016/j.jclepro.2023.135984

[42] Huff K, Student G *Active and Intelligent Packaging: Innovations for the Future*

[43] Imran M, Revol-Junelles AM, Martyn A, Tehrany EA, Jacquot M, Linder M, Desobry S (2010) *Active food packaging evolution: Transformation from micro- to nanotechnology*. Crit Rev Food Sci Nutr 50:799–821. https://doi.org/10.1080/10408398.2010.503694

[44] Kaushik K, Sharma RB, Agarwal S (2016) *Natural polymers and their applications*. Int J Pharm Sci Rev Res 37:30–36

[45] Stick R V., Williams SJ (2009) *Formation of the Glycosidic Linkage*. Carbohydrates: The Essential Molecules of Life 133–202. https://doi.org/10.1016/b978-0-240-52118-3.00004-1

[46] Blackwood AD, Chaplin MF (2000) *Disaccharide, Oligosaccharide and Polysaccharide Analysis*. Encyclopedia of Analytical Chemistry 1–24. https://doi.org/10.1002/9780470027318.a0303

[47] Velásquez P, Montenegro G, Valenzuela LM, Giordano A, Cabrera-Barjas G, Martin-Belloso O (2022) *k-carrageenan edible films for beef: Honey and bee pollen phenolic compounds improve their antioxidant capacity*. Food Hydrocoll 124.
https://doi.org/10.1016/j.foodhyd.2021.107250

[48] Manivasagan P, Oh J (2016) *Marine polysaccharide-based nanomaterials as a novel source of nanobiotechnological applications*. Int J Biol Macromol 82:315–327.
https://doi.org/10.1016/j.ijbiomac.2015.10.081

[49] Zia KM, Tabasum S, Nasif M, Sultan N, Aslam N, Noreen A, Zuber M (2017) *A review on synthesis, properties and applications of natural polymer-based carrageenan blends and composites*. Int J Biol Macromol 96:282–301. https://doi.org/10.1016/j.ijbiomac.2016.11.095

[50] Baweja P, Sahoo D (2015) *Classification of Algae*. pp 31–55
https://doi.org/10.1007/978-94-017-7321-8_2

[51] Alves C, Silva J, Freitas R, Pinteus S, Reboleira J, Pedrosa R, Bernardino S (2018) *Red algae*. Elsevier Inc.
https://doi.org/10.1016/B978-0-12-812491-8.00051-5

[52] Tasende MG, Manríquez-Hernández JA (2016) *Carrageenan properties and applications: A review*. Carrageenans: Sources and Extraction Methods, Molecular Structure, Bioactive Properties and Health Effects 17–49

[53] Rawiwan P, Peng Y, Paramayuda IGPB, Quek SY (2022) *Red seaweed: A promising alternative protein source for global food sustainability*. Trends Food Sci Technol 123:37–56.
https://doi.org/10.1016/j.tifs.2022.03.003

[54] Shafie MH, Kamal ML, Zulkiflee FF, Hasan S, Uyup NH, Abdullah S, Mohamed Hussin NA, Tan YC, Zafarina Z (2022) *Application of Carrageenan extract from red seaweed (Rhodophyta) in cosmetic products: A review*. Journal of the Indian Chemical Society 99:100613.
https://doi.org/10.1016/j.jics.2022.100613

[55] Ali A, Ahmed S (2019) *Carrageenans: Structure, Properties and Applications*. Marine Polysaccharides 0:29–52.
https://doi.org/10.1201/9780429058929-3

[56] Valderrama D, Cai J, Hishamunda N, Ridler N (2013) *Social and economic dimensions of carrageenan seaweed farming: a global synthesis*

[57] Rupert R, Rodrigues KF, Thien VY, Yong WTL (2022) *Carrageenan From Kappaphycus alvarezii (Rhodophyta, Solieriaceae): Metabolism, Structure, Production, and Application*. Front Plant Sci 13.
https://doi.org/10.3389/fpls.2022.859635

[58] Necas J, Bartosikova L (2013) *Carrageenan: A review*. Vet Med (Praha) 58:187–205.
https://doi.org/10.17221/6758-VETMED

[59] Fenton T, Kanyuck K, Mills T, Pelan E (2021) *Formulation and characterisation of kappa-carrageenan gels with non-ionic surfactant for melting-triggered controlled release*. Carbohydrate Polymer Technologies and Applications 2. https://doi.org/10.1016/j.carpta.2021.100060

[60] Kulkarni VS, Shaw C (2016) *Use of Polymers and Thickeners in Semisolid and Liquid Formulations. In: Essential Chemistry for Formulators of Semisolid and Liquid Dosages*. Elsevier, pp 43–69. https://doi.org/10.1016/b978-0-12-801024-2.00005-4

[61] Campo VL, Kawano DF, Silva DB da, Carvalho I (2009) *Carrageenans: Biological properties, chemical modifications and structural analysis - A review*. Carbohydr Polym 77:167–180. https://doi.org/10.1016/j.carbpol.2009.01.020

[62] Liyanapathiranage A, Dassanayake RS, Gamage A, Karri RR, Manamperi A, Evon P, Jayakodi Y, Madhujith T, Merah O (2023) *Recent Developments in Edible Films and Coatings for Fruits and Vegetables*. Coatings. https://doi.org/10.3390/coatings13071177

[63] Sedayu BB, Cran MJ, Bigger SW (2019) *A Review of Property Enhancement Techniques for Carrageenan-based Films and Coatings*. Carbohydr Polym 216:287–302. https://doi.org/10.1016/j.carbpol.2019.04.021

[64] Hambleton A, Fabra MJ, Debeaufort F, Dury-Brun C, Voilley A (2009) *Interface and aroma barrier properties of iota-carrageenan emulsion-based films used for encapsulation of active food compounds*. J Food Eng 93:80–88. https://doi.org/10.1016/j.jfoodeng.2009.01.001

[65] Kanmani P, Rhim JW (2014) *Development and characterization of carrageenan/grapefruit seed extract composite films for active packaging*. Int J Biol Macromol 68:258–266. https://doi.org/10.1016/j.ijbiomac.2014.05.011

[66] D. VAN NOSTRAND COMPANY INC *The Essential Oils*.

[67] Baptiste Hzounda Fokou J, Michel Jazet Dongmo P, Fekam Boyom F (2020) *Essential Oil's Chemical Composition and Pharmacological Properties*. In: Essential Oils - Oils of Nature. IntechOpen. https://doi.org/10.5772/intechopen.86573

[68] Asbahani A El, Miladi K, Badri W, Sala M, Addi EHA, Casabianca H, Mousadik A El, Hartmann D, Jilale A, Renaud FNR, Elaissari A (2015) *Essential oils: From extraction to encapsulation*. Int J Pharm 483:220–243. https://doi.org/10.1016/j.ijpharm.2014.12.069

[69] Crișan I, Ona A, Vârban D, Muntean L, Vârban R, Stoie A, Mihăiescu T, Morea A (2023) *Current Trends for Lavender (Lavandula angustifolia Mill.) Crops and Products with Emphasis on Essential Oil Quality*. Plants. https://doi.org/10.3390/plants12020357

[70] Kontominas *BIOACTIVE FOOD PACKAGING Strategies, Quality, Safety*.

[71] Baptiste Hzounda Fokou J, Michel Jazet Dongmo P, Fekam Boyom F (2020) *Essential Oil's Chemical Composition and Pharmacological Properties*. In: Essential Oils - Oils of Nature. IntechOpen. https://doi.org/10.5772/intechopen.86573

[72] Gui H, Zhao M, Zhang S, Yin R, Hu C, Fan M, Li L (2022) *Active Antioxidant Packaging from Essential Oils Incorporated Polylactic Acid/Poly (butylene adipate-co-terephthalate)/Thermoplastic Starch for Preserving Straw Mushroom*. Foods. https://doi.org/10.3390/foods11152252

[73] Ojagh SM, Rezaei M, Razavi SH, Hosseini SMH (2010) *Development and evaluation of a novel biodegradable film made from chitosan and cinnamon essential oil with low affinity toward water*. Food Chem 122:161–166. https://doi.org/10.1016/j.foodchem.2010.02.033

[74] Worwood VA (2020) *The Complete Book of Essential Oils and Aromatherapy*

[75] Blank AF, AGC, Arrigoni-Blank M de F, H. SC, Cavalcanti, Alves PB, Renato Innecco PADE, ISousa nagá F de (2007) *Influence of season, harvest time and drying on Java citronella (Cymbopogon winterianus Jowitt) volatile oil*. 17:557–564. https://doi.org/10.1590/S0102-695X2007000400014

[76] Katiyar R, Gupta S, Yadav KR (2011) *Cymbopogon winterianus: An Important Species for Essential Java Citronella Oil and Medicinal Value*. National Conference on Forest Biodiversity: Earth's Living Treasure 115–118

[77] Fatima S, Abad Farooqi AH, Sharma S (2002) *Physiological and metabolic responses of different genotypes of Cymbopogon martinii and C. winterianus to water stress*. Plant Growth Regul 37:143–149. https://doi.org/10.1023/A:1020531609126

[78] Kaur H, Bhardwaj U, Kaur R (2021) *Cymbopogon nardus essential oil: a comprehensive review on its chemistry and bioactivity*. Journal of Essential Oil Research 33:205–220. https://doi.org/10.1080/10412905.2021.1871976

[79] SINGH-SANGWAN N, ABAD FAROOQI AH, SINGH SANGWAN R (1994) *Effect of drought stress on growth and essential oil metabolism in lemongrasses*. New Phytologist 128:173–179. https://doi.org/10.1111/j.1469-8137.1994.tb04000.x

[80] Singh K, Kothari SK, Singh D V, Singh VP, Singh PP (2000) *Agronomic studies in cymbopogons - a review*. J of Spices and Arom Crops 9:13–22.

[81] C.C. de Guzman & R.A. Reglos *Cymbopogon winterianus*. https://uses.plantnet-project.org/e/index.php?title=Cymbopogon_winterianus_(PROSEA)&mobileaction=toggle_view_desktop. Accessed 29 Aug 2023.

[82] Gawali AS, Meshram NA (2019) *Scientifically cultivation of lemon grass - a potential aromatic crop*. Plant Arch 19:2860–2864

[83] Aakanksha Wany, Shivesh Jha VKN and DMP (2013) *CHEMICAL ANALYSIS AND THERAPEUTIC USES OF CITRONELLA OIL FROM CYMBOPOGON WINTERIANUS: A SHORT REVIEW*. Int J Adv Res (Indore) 1:1–6

[84] Songkro S, Hayook N, Jaisawang J, Maneenuan D, Chuchome T, Kaewnopparat N (2012) *Investigation of inclusion complexes of citronella oil, citronellal and citronellol with b-cyclodextrin for mosquito repellent*. J Incl Phenom Macrocycl Chem 72:339–355. https://doi.org/10.1007/s10847-011-9985-7

[85] Simic A, Rančic A, Sokovic MD, Ristic M, Grujic-Jovanovic S, Vukojevic J, Marin PD (2008) *Essential oil composition of Cymbopogon winterianus and Carum carvi and their antimicrobial activities*. Pharm Biol 46:437–441. https://doi.org/10.1080/13880200802055917

[86] Luthra R, Singh N, Sharma S (1991) *Changes in monoterpene content accompanying development of Cymbopogon winterianus Jowitt leaves*. J of Essential Oil Research 3:349–354. https://doi.org/10.1080/10412905.1991.9697955

[87] Curtis CF, Lines JD, Ijumba J, Callaghan A, Hill N (1987) *The relative efficacy of repellents against mosquito vectors of disease*. Med and Vet Entom 109–119.

[88] Pubchem - *Citronellol*. https://pubchem.ncbi.nlm.nih.gov/compound/8842#section=Structures. Accessed 30 Aug 2023.

[89] Pubchem - *Citronellal*. https://pubchem.ncbi.nlm.nih.gov/compound/7794#section=Structures. Accessed 30 Aug 2023.

[90] Pubchem - *Geraniol.* https://pubchem.ncbi.nlm.nih.gov/compound/637566#section=2D-Structure. Accessed 30 Aug 2023.

[91] Zielińska-Błajet M, Feder-Kubis J (2020) *Monoterpenes and their derivatives—recent development in biological and medical applications*. Int J Mol Sci 21:1–38.
https://doi.org/10.3390/ijms21197078

[92] Thiel AM, Quinlan MB (2022) *Cymbopogon winterianus, Neurolaena lobata, and Ruta chalepensis-Recurring Herbal Remedies in Guatemalan Maya Q'eqchi' Homegardens*. Letters 13:41–48.
https://doi.org/10.2307/48718109

[93] Araújo De Oliveira W, De Oliveira Pereira F, Carol G, Gomes De Luna D, Lima IO, Alves Wanderley P, Baltazar De Lima R, De Oliveira Lima E (2011) *ANTIFUNGAL ACTIVITY OF CYMBOPOGON WINTERIANUS JOWITT EX BOR AGAINST CANDIDA ALBICANS*. Brazilian Journal of Microbiology 42:433–441.
https://doi.org/10.1590/S1517-83822011000200004

[94] Abdulazeez MA, Abdullahi AS, James BD (2015) *Lemongrass (Cymbopogon spp.) Oils*. Elsevier Inc.
https://doi.org/10.1016/B978-0-12-416641-7.00058-4

[95] Boelens MH (1995) *Chemical and sensory evaluation of {Lavandula} oils*. Perfumer & Flavorist 20:23–51

[96] Vairinhos J, Miguel MG (2020) *Essential oils of spontaneous species of the genus Lavandula from Portugal: A brief review*. Section C Journal of Biosciences 75:233–245.
https://doi.org/10.1515/znc-2020-0044

[97] *Lavandula viridis, Lamiaceae* - Jekka's. https://www.jekkas.com/products/green-lavender. Accessed 18 Sep 2023.

[98] Costa P, Gonçalves S, Andrade PB, Valentão P, Romano A (2011) *Inhibitory effect of Lavandula viridis on Fe2+-induced lipid peroxidation, antioxidant and anti-cholinesterase properties*. Food Chem 126:1779–1786. https://doi.org/10.1016/j.foodchem.2010.12.085

[99] Machado M, Martins N, Salgueiro L, Cavaleiro C, Sousa MC (2019) *Lavandula luisieri and Lavandula viridis essential oils as upcoming anti-protozoal agents: A key focus on leishmaniasis*. Applied Sciences (Switzerland). https://doi.org/10.3390/app9153056

[100] Ait Said L, Zahlane K, Ghalbane I, El Messoussi S, Romane A, Cavaleiro C, Salgueiro L (2015) *Chemical composition and antibacterial activity of Lavandula coronopifolia essential oil against antibiotic-resistant bacteria*. Nat Prod Res 29:582–585. https://doi.org/10.1080/14786419.2014.954246

[101] John Mason *Growing and knowing Lavander*.

[102] Machado M, Martins N, Salgueiro L, Cavaleiro C, Sousa MC (2019) *Lavandula luisieri and Lavandula viridis essential oils as upcoming anti-protozoal agents: A key focus on leishmaniasis*. Applied Sciences (Switzerland). https://doi.org/10.3390/app9153056

[103] Cai ZM, Peng JQ, Chen Y, Tao L, Zhang YY, Fu LY, Long Q De, Shen XC (2021) *1,8-Cineole: a review of source, biological activities, and application*. J Asian Nat Prod Res 23:938–954. https://doi.org/10.1080/10286020.2020.1839432

[104] Pubchem - *Camphor*. https://pubchem.ncbi.nlm.nih.gov/compound/Camphor. Accessed 30 Aug 2023.

[105] Vieira MGA, Da Silva MA, Dos Santos LO, Beppu MM (2011) *Natural-based plasticizers and biopolymer films: A review*. Eur Polym J 47:254–263. https://doi.org/10.1016/j.eurpolymj.2010.12.011

[106] Karbowiak T, Hervet H, Léger L, Champion D, Debeaufort F, Voilley A (2006) *Effect of plasticizers (water and glycerol) on the diffusion of a small molecule in iota-carrageenan biopolymer films for edible coating application*. Biomacromolecules 7:2011–2019. https://doi.org/10.1021/bm060179r

[107] Karbowiak T, Ferret E, Debeaufort F, Voilley A, Cayot P (2011) *Investigation of water transfer across thin layer biopolymer films by infrared spectroscopy*. J Memb Sci 370:82–90. https://doi.org/10.1016/j.memsci.2010.12.037

[108] Domínguez R, Barba FJ, Gómez B, Putnik P, Bursać Kovačević D, Pateiro M, Santos EM, Lorenzo JM (2018) *Active packaging films with natural antioxidants to be used in meat industry: A review*. Food Research International 113:93–101. https://doi.org/10.1016/j.foodres.2018.06.073

[109] Prakoso FAH, Indiarto R, Utama GL (2023) *Edible Film Casting Techniques and Materials and Their Utilization for Meat-Based Product Packaging*. Polymers (Basel) 15:2800. https://doi.org/10.3390/polym15132800

[110] Thermo Nicolet (2001*) Introduction to Fourier Transform Infrared Spectrometry.*

[111] Gómez-Ordóñez E, Rupérez P (2011) *FTIR-ATR spectroscopy as a tool for polysaccharide identification in edible brown and red seaweeds*. Food Hydrocoll 25:1514–1520.
https://doi.org/10.1016/j.foodhyd.2011.02.009

[112] Gill P, Moghadam TT, Ranjbar B (2010) *Differential Scanning Calorimetry Techniques: Applications in Biology and Nanoscience.*

[113] Pooria Gill, Tahereh Tohidi Moghadam, and Bijan Ranjbar1, D (2017) *Differential Scanning Calorimetry Techniques: Applications in Biology and Nanoscience.* Diabetic Medicine 34:99–107.
https://doi.org/10.1111/dme.13173

[114] *An insight into Differential Scanning Calorimetry*. https://setaramsolutions.com/techniques/differential-scanning-calorimetry-dsc. Accessed 11 Sep 2023.

[115] Rahman R, Putra SZFS (2018) *Tensile properties of natural and synthetic fiber-reinforced polymer composites. In: Mechanical and Physical Testing of Biocomposites, Fibre-Reinforced Composites and Hybrid Composites*. Elsevier, pp 81–102.
https://doi.org/10.1016/B978-0-08-102292-4.00005-9

[116] Joshi A, Bhanot V (2005) *Effect of accelerated aging on the tensile index of a synthetic insulation paper*. NDT and E International 38:394–396. https://doi.org/10.1016/j.ndteint.2004.11.001

[117] Jones DRH, Ashby MF (2019) *Elastic Moduli. In: Engineering Materials 1.* Elsevier, pp 31–47
https://doi.org/10.1016/b978-0-08-102051-7.00003-8

[118] Djafari Petroudy SR (2017) *Physical and mechanical properties of natural fibers. In: Advanced High Strength Natural Fibre Composites in Construction*. Elsevier Inc., pp 59–83.
https://doi.org/10.1016/B978-0-08-100411-1.00003-0

[119] Rudawska A, Jacniacka E (2009) *Analysis for determining surface free energy uncertainty by the Owen-Wendt method.* Int J Adhes 29:451–457. https://doi.org/10.1016/j.ijadhadh.2008.09.008

[120] Ozcan A, Arman Kandirmaz E, Kandirmaz A *The effect of packaging color on product sales.*

[121] Kashiri M, Cerisuelo JP, Domínguez I, López-Carballo G, Muriel-Gallet V, Gavara R, Hernández-Muñoz P (2017) *Zein films and coatings as carriers and release*

systems of Zataria multiflora Boiss. essential oil for antimicrobial food packaging. Food Hydrocoll 70:260–268. https://doi.org/10.1016/j.foodhyd.2017.02.021

[122] Guzman-Puyol S, Benítez JJ, Heredia-Guerrero JA (2022) *Transparency of polymeric food packaging materials*. Food Research International. https://doi.org/10.1016/j.foodres.2022.111792

[123] Siracusa V (2012) *Food packaging permeability behaviour: A report*. Int J Polym Sci 2012. https://doi.org/10.1155/2012/302029

[124] Mueller K, Schoenweitz C, Langowski HC (2012) *Thin laminate films for barrier packaging application - Influence of down gauging and substrate surface properties on the permeation properties*. Packaging Technology. and Science 25:137–148. https://doi.org/10.1002/pts.966

[125] Sharma OP, Bhat TK (2009) *DPPH antioxidant assay revisited*. Food Chem 113:1202–1205. https://doi.org/10.1016/j.foodchem.2008.08.008

[126] Pyrzynska K, Pękal A (2013) *Application of free radical diphenylpicrylhydrazyl (DPPH) to estimate the antioxidant capacity of food samples*. Analytical Methods 5:4288–4295. https://doi.org/10.1039/c3ay40367j

[127] Prieto MA, Rodríguez-Amado I, Vázquez JA, Murado MA (2012) *β-Carotene assay revisited. Application to characterize and quantify antioxidant and prooxidant activities in a microplate*. In: Journal of Agricultural and Food Chemistry. pp 8983–8993. https://doi.org/10.1021/jf302218g

[128] Dawidowicz AL, Olszowy M (2010) *Influence of some experimental variables and matrix components in the determination of antioxidant properties by β-carotene bleaching assay: Experiments with BHT used as standard antioxidant*. European Food Research and Technology 231:835–840. https://doi.org/10.1007/s00217-010-1333-4

[129] Balouiri M, Sadiki M, Ibnsouda SK (2016) *Methods for in vitro evaluating antimicrobial activity: A review*. J Pharm Anal 6:71–79. https://doi.org/10.1016/j.jpha.2015.11.005

[130] Sedikelo GK, Lenetha GG, Malebo NJ (2022) *Chromatography-mass spectrometry and chemical characteristics of Thymus zygis and Cymbopogon*

winterianus essential oils: Possible insect repellents. Sci Afr. https://doi.org/10.1016/j.sciaf.2022.e01095

[131] Verma RS, Verma SK, Tandon S, Padalia RC, Darokar MP, Verma RS, Verma SK, Tandon S, Padalia RC (2020) *Chemical composition and antimicrobial activity of Java citronella (Cymbopogon winterianus Jowitt ex Bor) essential oil extracted by different methods*. Journal of Essential Oil Research 00:1–7. https://doi.org/10.1080/10412905.2020.1787885

[132] Luís Â, Pereira L, Domingues F, Ramos A (2019) *Development of a carboxymethyl xylan film containing licorice essential oil with antioxidant properties to inhibit the growth of foodborne pathogens*. Lwt 111:218–225. https://doi.org/10.1016/j.lwt.2019.05.040

[133] Scherer R, Godoy HT (2009) *Antioxidant activity index (AAI) by the 2,2-diphenyl-1-picrylhydrazyl method*. Food Chem 112:654–658. https://doi.org/10.1016/j.foodchem.2008.06.026

[134] Luís Â, Ramos A, Domingues F (2020) *Pullulan films containing rockrose essential oil for potential food packaging applications*. Antibiotics 9:1–20. https://doi.org/10.3390/antibiotics9100681

[135] *Leica LAS v4.13 Software*. https://imillermicroscopes.com/pages/software-download. Accessed 31 Jan 2021.

[136] Kamath A, Shukla A, Patel D (2023) *Quorum Sensing and Quorum Quenching: Two sides of the same coin*. Physiol Mol Plant Pathol. https://doi.org/10.1016/j.pmpp.2022.101927

[137] Liu Y, Qin Y, Bai R, Zhang X, Yuan L, Liu J (2019) *Preparation of pH-sensitive and antioxidant packaging films based on κ-carrageenan and mulberry polyphenolic extract*. Int J Biol Macromol 134:993–1001. https://doi.org/10.1016/j.ijbiomac.2019.05.175

[138] de Lima Barizão C, Crepaldi MI, Junior O de OS, de Oliveira AC, Martins AF, Garcia PS, Bonafé EG (2020) *Biodegradable films based on commercial κ-carrageenan and cassava starch to achieve low production costs*. Int J Biol Macromol 165:582–590.
https://doi.org/10.1016/j.ijbiomac.2020.09.150

[139] Luís Â, Gallardo E, Ramos A, Domingues F (2020) *Design and characterization of bioactive bilayer films: Release kinetics of isopropyl palmitate*. Antibiotics 9:1–18.
https://doi.org/10.3390/antibiotics9080443

[140] Sängerlaub S, Schmid M, Müller K (2018) *Comparison of water vapour transmission rates of monolayer films determined by water vapour sorption*

and permeation experiments. Food Packag Shelf Life 17:80–84. https://doi.org/10.1016/j.fpsl.2018.06.004

[141] Luís Â, Ramos A, Domingues F (2021) *Pullulan–apple fiber biocomposite films: Optical, mechanical, barrier, antioxidant and antibacterial properties*. Polymers (Basel) 13:1–15. https://doi.org/10.3390/polym13060870

[142] *Bilohan M, Ramos A, Domingues F, Luís Â (2022) Production and characterization of pullulan/paper/zein laminates as active food packaging materials*. J Food Process Preserv 1–12. https://doi.org/10.1111/jfpp.17083

[143] Antonio K, Rodrigues F, Dias CN, Maria F, Amaral M, Moraes DFC, Filho VEM, Andrade EHA, Maia JGS (2013) *Molluscicidal and larvicidal activities and essential oil composition of Cymbopogon winterianus*. Pharm Biol 1–5. https://doi.org/10.3109/13880209.2013.789536

[144] Leite BLS, Bonfim RR, Antoniolli AR, Thomazzi SM, Araújo AAS, Blank AF, Estevam CS, Cambui EVF, Bonjardim LR, Albuquerque Júnior RLC, Quintans-Júnior LJ (2010) *Assessment of antinociceptive, anti-inflammatory and antioxidant properties of Cymbopogon winterianus leaf essential oil*. Pharm Biol 48:1164–1169. https://doi.org/10.3109/13880200903280000

[145] Victoria FN, Radatz CS, Sachini M, Jacob RG, Alves D, Savegnago L, Perin G, Motta AS, Silva WP, Lenardão EJ (2012) *Further analysis of the antimicrobial activity of α-phenylseleno citronellal and α-phenylseleno citronellol*. Food Control 23:95–99. https://doi.org/10.1016/j.foodcont.2011.06.017

[146] Fatima K, Luqman S (2021) *Citronellal suppress the activity of ornithine decarboxylase in hypopharyngeal carcinoma cells*. South African Journal of Botany 143:443–448. https://doi.org/10.1016/j.sajb.2021.07.043

[147] Kazuhiko NAKAHARA NSA, YOSHIHASHI T, NGUYEN HTT, TRAKOONTIVAKORN and G *Chemical Composition and Antifungal Activity of Essential Oil from Cymbopogon nardus (Citronella Grass)*. https://doi.org/10.6090/jarq.37.249

[148] Xie S, Wu G, Ren R, Xie R, Yin H, Chen H, Yang B, Zhang Z, Ge M (2023) *Transcriptomic and metabolic analyses reveal differences in monoterpene profiles and the underlying molecular mechanisms in six grape varieties with different flavors*. LWT.

https://doi.org/10.1016/j.lwt.2023.114442

[149] Waterman PG (1993) *Volatile oil crops : their biology, biochemistry, and production*. Longman Scientific & Technical.

[150] Ishnava KB, Chauhan JB, Barad MB (2013) *Anticariogenic and phytochemical evaluation of Eucalyptus globules Labill.* Saudi J Biol Sci 20:69–74. https://doi.org/10.1016/j.sjbs.2012.11.003

[151] Aprotosoaie AC, Luca VS, Trifan A, Miron A (2018) *Antigenotoxic Potential of Some Dietary Non-phenolic Phytochemicals. In*: Studies in Natural Products Chemistry. Elsevier B.V., pp 223–297. https://doi.org/10.1016/B978-0-444-64181-6.00007-3

[152] Sacchetti G, Maietti S, Muzzoli M, Scaglianti M, Manfredini S, Radice M, Bruni R (2005) *Comparative evaluation of 11 essential oils of different origin as functional antioxidants, antiradicals and antimicrobials in foods*. Food Chem 91:621–632. https://doi.org/10.1016/j.foodchem.2004.06.031

[153] Shrestha D, Sharma P, Pandey A, Dhakal K, Baral RP, Adhikari A (2022) *Chemical Characterization, Antioxidant and Antibacterial Activity of Essential Oil of Cymbopogon winterianus Jowitt (Citronella) from Western Nepal*. Curr Biotechnol 11:86–91. https://doi.org/10.2174/2211550111666220405133558

[154] Costa P, Grosso C, Gonçalves S, Andrade PB, Valentão P, Gabriela Bernardo-Gil M, Romano A (2012) *Supercritical fluid extraction and hydrodistillation for the recovery of bioactive compounds from Lavandula viridis L'Hér*. Food Chem 135:112–121. https://doi.org/10.1016/j.foodchem.2012.04.108

[155] Hussain AI, Anwar F, Iqbal T, Bhatti IA (2011) *Antioxidant attributes of four Lamiaceae essential oils*. Pak J Bot 43:1315–1321

[156] Ribeiro-Santos R, Andrade M, Melo NR de, Sanches-Silva A (2017) *Use of essential oils in active food packaging: Recent advances and future trends*. Trends Food Sci Technol 61:132–140. https://doi.org/10.1016/j.tifs.2016.11.021

[157] Naik MI, Fomda BA, Jaykumar E, Bhat JA (2010) *Antibacterial activity of lemongrass (Cymbopogon citratus) oil against some selected pathogenic bacterias*. Asian Pac J Trop Med 3:535–538. https://doi.org/10.1016/S1995-7645(10)60129-0

[158] Allenspach M, Steuer C (2021) *α-Pinene: A never-ending story*. Phytochemistry.

[159] Ragon M, Wirth T, Hollandt F, Lavenir R, Lecuit M, Monnier A Le, Brisse S (2008) *A new perspective on Listeria monocytogenes evolution*. PLoS Pathog. https://doi.org/10.1371/journal.ppat.1000146

[160] Argudín MÁ, Mendoza MC, Rodicio MR (2010) *Food Poisoning and Staphylococcus aureus Enterotoxins*. Toxins (Basel) 2:1751–1773. https://doi.org/10.3390/toxins2071751

[161] Hennekinne JA, De Buyser ML, Dragacci S (2012) *Staphylococcus aureus and its food poisoning toxins: Characterization and outbreak investigation*. FEMS Microbiol Rev 36:815–836. https://doi.org/10.1111/j.1574-6976.2011.00311.x

[162] Babalola OO (2010) *Beneficial bacteria of agricultural importance*. Biotechnol Lett 32:1559–1570. https://doi.org/10.1007/s10529-010-0347-0

[163] Guimarães JL, Cursino ACT, Saul CK, Sierrakowski MR, Ramos LP, Satyanarayana KG (2016) *Evaluation of castor oil cake starch and recovered glycerol and development of "green" composites based on those with plant fibers*. Materials. https://doi.org/10.3390/ma9020076

[164] Gulzar S, Balange AK, Nagarajarao RC, Zhao Q, Benjakul S (2022) *Microcapsules of Shrimp Oil Using Kidney Bean Protein Isolate and κ-Carrageenan as Wall Materials with the Aid of Ultrasonication or High-Pressure Microfluidization: Characteristics and Oxidative Stability*. Foods. https://doi.org/10.3390/foods11101431

[165] Daniel-Da-Silva AL, Lopes AB, Gil AM, Correia RN (2007) *Synthesis and characterization of porous κ-carrageenan/calcium phosphate nanocomposite scaffolds*. J Mater Sci 42:8581–8591. https://doi.org/10.1007/s10853-007-1851-z

[166] Ramírez-Hernández A, Aguilar-Flores C, Aparicio-Saguilán A (2019) *Fingerprint analysis of ftir spectra of polymers containing vinyl acetate*. DYNA (Colombia) 86:198–205. https://doi.org/10.15446/dyna.v86n209.77513

[167] sigmaaldrich *IR Spectrum Table & Chart*. https://www.sigmaaldrich.com/PT/en/technical-documents/technical-article/analytical-chemistry/photometry-and-reflectometry/ir-spectrum-table. Accessed 31 Aug 2023.

[168] Songkro S, Hayook N, Jaisawang J, Maneenuan D, Chuchome T, Kaewnopparat N (2012) *Investigation of inclusion complexes of citronella oil, citronellal and*

citronellol with b-cyclodextrin for mosquito repellent. J Incl Phenom Macrocycl Chem 72:339–355. https://doi.org/10.1007/s10847-011-9985-7

[169] García C, Montero G, Coronado MA, Valdez B, Stoytcheva M, Rosas N, Torres R, Sagaste CA (2017) *Valorization of Eucalyptus Leaves by Essential Oil Extraction as an Added Value Product in Mexico*. Waste Biomass Valorization 8:1187–1197. https://doi.org/10.1007/s12649-016-9695-x

[170] Paşcalau V, Popescu V, Popescu GL, Dudescu MC, Borodi G, Dinescu A, Perhaiţa I, Paul M (2012) *The alginate/k-carrageenan ratio's influence on the properties of the cross-linked composite films*. In: Journal of Alloys and Compounds. https://doi.org/10.1016/j.jallcom.2011.12.026

[171] Dębczak A, Tyśkiewicz K, Fekner Z, Kamiński P, Florkowski G, Konkol M, Rój E, Grzegorczyk A, Malm A (2022) *Molecular Distillation of Lavender Supercritical Extracts: Physicochemical and Antimicrobial Characterization of Feedstocks and Assessment of Distillates Enriched with Oxygenated Fragrance Components*. Molecules. https://doi.org/10.3390/molecules27051470

[172] Martins P, Sbaite P, Benites C, Maciel M (2011) *Thermal characterization of orange, lemongrass, and basil essential oils*. Chem Eng Trans 24:463–468. https://doi.org/10.3303/CET1124078

[173] Zhang W, Shu C, Chen Q, Cao J, Jiang W (2019) *The multi-layer film system improved the release and retention properties of cinnamon essential oil and its application as coating in inhibition to penicillium expansion of apple fruit*. Food Chem. https://doi.org/10.1016/j.foodchem.2019.125109

[174] Liang J, Yan H, Zhang J, Dai W, Gao X, Zhou Y, Wan X, Puligundla P (2017) *Preparation and characterization of antioxidant edible chitosan films incorporated with epigallocatechin gallate nanocapsules*. Carbohydr Polym 171:300–306. https://doi.org/10.1016/j.carbpol.2017.04.081

[175] Sharma S, Barkauskaite S, Jaiswal AK, Jaiswal S (2021) *Essential oils as additives in active food packaging*. Food Chem. https://doi.org/10.1016/j.foodchem.2020.128403

[176] Sánchez-González L, Vargas M, González-Martínez C, Chiralt A, Cháfer M (2009) *Characterization of edible films based on hydroxypropylmethylcellulose and tea tree essential oil*. Food Hydrocoll 23:2102–2109. https://doi.org/10.1016/j.foodhyd.2009.05.006

[177] de Lima Barizão C, Crepaldi MI, Junior O de OS, de Oliveira AC, Martins AF, Garcia PS, Bonafé EG (2020) *Biodegradable films based on commercial κ-carrageenan and cassava starch to achieve low production costs*. Int J Biol Macromol 165:582–590. https://doi.org/10.1016/j.ijbiomac.2020.09.150

[178] Atarés L, Chiralt A (2016) *Essential oils as additives in biodegradable films and coatings for active food packaging*. Trends Food Sci Technol 48:51–62. https://doi.org/10.1016/j.tifs.2015.12.001

[179] Szlachetka O, Witkowska-Dobrev J, Baryła A, Dohojda M (2021) *Low-density polyethylene (LDPE) building films – Tensile properties and surface morphology*. Journal of Building Engineering. https://doi.org/10.1016/j.jobe.2021.103386

[180] Sánchez-González L, Cháfer M, Chiralt A, González-Martínez C (2010) *Physical properties of edible chitosan films containing bergamot essential oil and their inhibitory action on Penicillium italicum*. Carbohydr Polym 82:277–283. https://doi.org/10.1016/j.carbpol.2010.04.047

[181] de Lima Barizão C, Crepaldi MI, Junior O de OS, de Oliveira AC, Martins AF, Garcia PS, Bonafé EG (2020) *Biodegradable films based on commercial κ-carrageenan and cassava starch to achieve low production costs*. Int J Biol Macromol 165:582–590. https://doi.org/10.1016/j.ijbiomac.2020.09.150

[182] Niu B, Shao P, Chen H, Sun P (2019) *Structural and physiochemical characterization of novel hydrophobic packaging films based on pullulan derivatives for fruits preservation*. Carbohydr Polym 208:276–284. https://doi.org/10.1016/j.carbpol.2018.12.070

[183] Dawam Abdullah AH, Firdiana B, Nissa RC, Satoto R, Karina M, Fransiska D, Nurhayati, Agusman, Irianto HE, Priambudi P, Marliah S, Ismadi (2021) *EFFECT OF κ-CARRAGEENAN ON MECHANICAL, THERMAL AND BIODEGRADABLE PROPERTIES OF STARCH–CARBOXYMETHYL CELLULOSE (CMC) BIOPLASTIC*. Cellulose Chemistry and Technology 55:1109–1117. https://doi.org/10.35812/CelluloseChemTechnol.2021.55.95

[184] Wang G, Guo Z, Liu W (2014) *Interfacial effects of superhydrophobic plant surfaces: A Review*. J Bionic Eng 11:325–345. https://doi.org/10.1016/S1672-6529(14)60047-0

[185] (2023) *"What is surface free energy?"*. Biolin Scientific. Available online: https://www.biolinscientific.com/blog/what-is-surface-free-energy Accessed on 02/05/2023.

[186] Rbihi S, Aboulouard A, Laallam L, Jouaiti A (2020) *Contact Angle Measurements of Cellulose based Thin Film composites: wettability, surface free energy and surface hardness*. Surfaces and Interfaces 21:100708. https://doi.org/10.1016/j.surfin.2020.100708

[187] Chibowski E, Terpilowski K (2009) *Surface free energy of polypropylene and polycarbonate solidifying at different solid surfaces*. Appl Surf Sci 256:1573–1581. https://doi.org/10.1016/j.apsusc.2009.09.024

[188] Sousa AMM, Gonçalves MP (2015) *Strategies to improve the mechanical strength and water resistance of agar films for food packaging applications*. Carbohydr Polym 132:196–204. https://doi.org/10.1016/j.carbpol.2015.06.022

[189] Tonyali B, Cikrikci S, Oztop MH (2018) *Physicochemical and microstructural characterization of gum tragacanth added whey protein-based films*. Food Research International 105:1–9. https://doi.org/10.1016/j.foodres.2017.10.071