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Emulsified Pullulan Films Incorporating Geraniol: Production and Characterization

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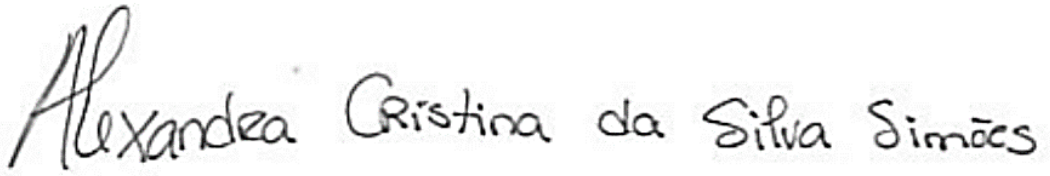

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Resumo

Os plásticos derivados do petróleo têm sido amplamente utilizados como materiais de embalagem, devido à sua disponibilidade a baixo custo e às suas boas propriedades mecânicas. No entanto, o uso de plásticos tornou-se restrito, uma vez que estes são altamente resistentes à biodegradação, causando problemas ambientais. Para utilização como potenciais substitutos dos plásticos convencionais, este trabalho pretende produzir e caracterizar filmes emulsionados de pululana incorporando geraniol para aplicação como material de embalagem alimentar.

O geraniol apresentou atividade antimicrobiana contra *Staphylococcus aureus* ATCC 25923 (diâmetro da zona de inibição = 15.44 ± 1.72 mm); *Enterococcus faecalis* ATCC 29212 (diâmetro da zona de inibição = 11.37 ± 0.75 mm); *Salmonella* Typhimurium ATCC 13311 (diâmetro da zona de inibição = 8.61 ± 0.51 mm); *Pseudomonas aeruginosa* ATCC 27853 (diâmetro da zona de inibição = 7.31 ± 0.61 mm); *Escherichia coli* ATCC 25922 (diâmetro da zona de inibição = 11.48 ± 0.18 mm) e *Listeria monocytogenes* LMG 16779 (diâmetro da zona de inibição = 25.62 ± 0.36 mm). Quando o geraniol foi incorporado nos filmes, estes apresentaram atividade antimicrobiana contra *Enterococcus faecalis* ATCC 29212 (diâmetro da zona de inibição = 15.19 ± 0.66 mm) e *Pseudomonas aeruginosa* ATCC 27853 (diâmetro da zona de inibição = 10.99 ± 1.82 mm). Para além disso, a microscopia eletrónica de varrimento mostrou a redução dos biofilmes de *Enterococcus faecalis* ATCC 29212 quando os biofilmes foram formados diretamente nos filmes emulsionados de pululana incorporando geraniol. Este estudo também revelou o potencial anti-quorum sensing do geraniol (diâmetro da zona de inibição = 9.12 ± 0.22 mm), o que indica que este inibe a comunicação entre as células microbianas.

Os filmes produzidos demonstraram ainda uma elevada transparência (>90%) e superfícies hidrofílicas em ambos os lados (ângulo de contacto com a água <90º).

Esta investigação provou que os filmes bioativos de pululana incorporando o geraniol podem ser utilizados como materiais inovadores de embalagem alimentar. A expansão da produção destes filmes deve ser o foco de trabalhos futuros.

Palavras-chave

pululana, geraniol, filmes emulsionados, embalagem alimentar, atividade antioxidante, atividade antibacteriana.

Abstract

Petroleum-based plastics have been widely used as packaging materials because of their low-cost availability and good mechanical properties. However, the use of plastics has become restricted as they are highly resistant to biodegradation, causing environmental problems. For use as potential substitutes for conventional plastics, this work aims to produce and characterize emulsified pullulan films incorporating geraniol for application as food packaging material.

Geraniol showed antimicrobial activity against *Staphylococcus aureus* ATCC 25923 (inhibition zone diameter = 15.44 ± 1.72 mm); *Enterococcus faecalis* ATCC 29212 (inhibition zone diameter = 11.37 ± 0.75 mm); *Salmonella Typhimurium* ATCC 13311 (inhibition zone diameter = 8. 61 ± 0.51 mm); *Pseudomonas aeruginosa* ATCC 27853 (inhibition zone diameter = 7.31 ± 0.61 mm); *Escherichia coli* ATCC 25922 (inhibition zone diameter = 11.48 ± 0.18 mm) and *Listeria monocytogenes* LMG 16779 (inhibition zone diameter = 25.62 ± 0.36 mm). When geraniol was incorporated into the films, they showed antimicrobial activity against *Enterococcus faecalis* ATCC 29212 (inhibition zone diameter = 15.19 ± 0.66 mm) and *Pseudomonas aeruginosa* ATCC 27853 (inhibition zone diameter = 10.99 ± 1.82 mm). Furthermore, scanning electron microscopy showed the reduction of *Enterococcus faecalis* ATCC 29212 biofilms when they were directly formed on the emulsified pullulan films incorporating geraniol. This study also revealed the anti-quorum sensing potential of geraniol (inhibition zone diameter = 9.12 ± 0.22 mm), where it could prevent communication between microbial cells.

The produced films also demonstrated high transparency (>90%) and hydrophilic surfaces on both sides (water contact angle <90°).

This research proved that bioactive pullulan films incorporating geraniol can be used as innovative food packaging materials. Expanding the production of these films should be the focus of future work.

Keywords

pullulan, geraniol, emulsified films, food packaging, antioxidant activity, antibacterial activity.

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List of Acronyms

AAI	Antioxidant Activity Index
BHI	Brain-Heart Infusion
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSC	Differential Scanning Calorimetry
EC	*Escherichia coli*
EF	*Enterococcus faecalis*
FTIR	Fourier-Transform Infrared Spectroscopy
GRAS	Generally Recognized As Safe
LB	Luria-Bertani
LM	*Listeria monocytogenes*
MHA	Müeller-Hinton Agar
MHB	Müeller-Hinton Broth
MIC	Minimum Inhibitory Concentrations
OP	Oil Permeability
OWRK	Owens, Wendt, Rabel and Kaelble
PA	*Pseudomonas aeruginosa*
PBS	Phosphate-Buffered Saline
PCL	Polycaprolactone
PGA	Polyglycolic Acid
PLA	Polylactic Acid
Pu	Pullulan
Pu40	Pullulan, Tween 40
PuGer	Pullulan, Geraniol
PuGer40	Pullulan, Geraniol, Tween 40
PVA	Polyvinyl Alcohol
QS	Quorum Sensing
QSI	Quorum Sensing Inhibition
RH	Relative Humidity
ROS	Oxygen-containing Reactive Species
SA	*Staphylococcus aureus*
SEM	Scanning Electron Microscopy
ST	*Salmonella Typhimurium*
Tg	Glass transition temperature
WVP	Water Vapor Permeability
WVTR	Water Vapor Transmission Rate

1. Introduction

1.1. Polymers and Plastics

Although they do not have the same meaning, the words "plastics" and "polymers," particularly in the packaging industry, are frequently used together. Indeed, most plastic packaging materials are made of polymers (70–99%), which are always mixed with different amounts of additives like plasticizers, antioxidants, dyes, antistatic, fillers and many other substances. Since those substances are necessary to deliver the desired functionality, the finished products are not unquestionably polymers (Piergiovanni & Limbo, 2016).

The idea of polymers as long-chain molecules formed of repeated units joined by primarily covalent connections has been established throughout the past century. A significant progress has been achieved in creating various forms of commodity and specialized polymers, allowing the production of plastic things in a range of sizes, shapes and uses (Millican & Agarwal, 2021).

This leads us to reflect on the life cycle of plastics, presented in Figure 1, from their production until they accumulate and start generating pollution.

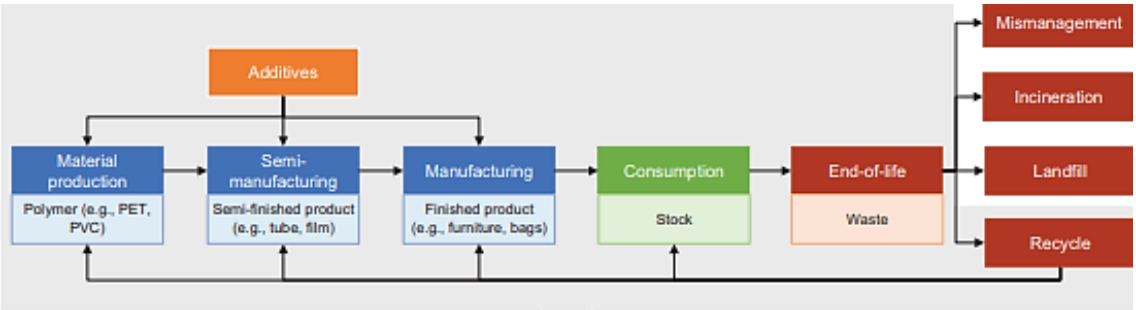


Figure 1: Plastics' life cycle. Adapted from Wang et al. (2021).

1.2. Plastic Life Cycle

Petrochemistry and fossil feedstocks play an important role in the manufacture of polymers. 99% of the raw materials used to make plastic are derived from fossil fuels, which make up 8–9% of the world's total gas and oil usage (Andrady, 2015).

As petrochemical corporations make substantial investments in new production facilities and infrastructures, there is a predicted rise in global demand and production capacity for conventional plastic (Nielsen et al., 2019).

Despite the universal features of plastic manufacture, each type of plastic (polyethylene, polypropylene, polyethylene terephthalate, polystyrene, polyvinyl chloride and polyamide) used in the industry sector requires unique features and characteristics in its production process.

The lifespans (or service lives) of various plastics goods vary, affecting how long plastics are used as stocks and when those stocks turn into plastic waste (Wang et al., 2021).

Research on consumption frequently concentrate on how people behave in relation to specific plastic items, like plastic bags, plastic bottles, throwaway cups, and food packaging (Nielsen et al.,

2019). Microplastics, little plastic fragments between 1 μm and 5 mm in size, are found everywhere, including inside people and animals. While some microplastics are purposefully created, such as for use in cosmetics, the majority are the byproducts of the decomposition of bigger plastics. Microplastics can contain potentially harmful compounds and can bind heavy metals and poisons (Rist et al., 2018). Single-use plastics are heavily focused on plastic food packaging. Yet, it is advocated that plastic packaging is essential to maintain food quality, sanitation and lifespan (Verghese et al., 2015). The usefulness and necessity of plastics are being called into doubt in different contexts. Contrary to popular belief, convenience drove the adoption of plastics in the medical field rather than an effort to improve hygiene (Nielsen et al., 2019).

Plastics are useful in everyday life in various communities, but if their post-use is not properly managed, they can change various aspects of the planet (Evode et al., 2021). Several waste management techniques, including recycling, incineration, bioremediation, and landfills have a scientific foundation. These procedures are designed to promote environmental cleanliness and effective plastic trash disposal (Awoyera & Adesina, 2020).

Recycling is a waste management practice that involves gathering waste materials and turning them into raw materials that may be used again to create other useful products (Evode et al., 2021). One benefit of this technique is the protection of human life by reducing the amount of carbon dioxide and other dangerous gases in the atmosphere that can result from burning or incinerating garbage. Recycling uses less energy, decreases pollutants across the ecosystem and promotes the preservation of the environment. It reduces the demand for fossil fuel usage and frees up space in landfills that are quickly running out. Although there are disadvantages too, during the recycling process, some chemicals are released into the environment. These gases can result in acid rain, the greenhouse effect and global warming, all of which have different negative environmental effects (Shen & Worrell, 2014).

Plastic pollution poses a hazard to ecosystems everywhere as well as an elevated risk to human health on a global scale (Herrmann et al., 2022).

1.3. Alternatives and Solutions

Plastics are widely employed in a variety of industrial fields because they are lightweight, manageable, and affordable. As no solution to reduce this issue is 100% effective, it is vital to develop solutions that act at the production level with bio-alternatives, such as bioplastics or films produced with biopolymers.

1.3.1 Food and Active Packaging

Food packaging nowadays serves more than just a passive role in the protection and promotion of an edible product. The requirements of the consumer must currently be met by an alimentary package. A practical diet that is ready to eat, secure, in the right proportion, and beneficial to health are requirements of modern life. An innovative food container can delay oxidation, does not allow food respiration processes, prevent microbiological contamination or humidity entry,

and release antioxidants while being stored. Additional features include CO_2 absorbers and emitters, aroma emitters, temperature and time sensors, maturation indicators, and biosensors. Despite the benefits mentioned, these packaging nevertheless face numerous obstacles, whether at the consumer or industrial investment levels (Petkoska et al., 2021).

There are two distinct categories of innovative packaging: intelligent packaging that focuses on communication and active packaging that seeks to protect food. Typically, combinations of intelligent systems and active systems are used to increase food safety and shelf life and to communicate about the quality of the food along the distribution and ultimate consumer chain (Kuswandi, 2020).

An active package is considered as one that contains parts that can interact with the internal environment and the food to extend its shelf life and increase its margin of safety. Active packages include those that can absorb or release substances from the environment around them or from the interior or exterior of the foods they contain (Rangappa et al., 2020). It is possible to distinguish the active packaging based on the type of material that has been incorporated inside. This material may have a chemical origin, such as potassium permanganate to extend the shelf life, or a biological one, such as essential oils with antimicrobial activity (Sharma et al., 2021).

1.3.2. Biopolymers or Bioplastics

Biopolymers are polymers of natural origin made up of monomeric units that are covalently bound to one another to produce molecules that resemble chains (Othman, 2014). Packaging described as "bioplastic" is typically made from renewable resources (mainly plant material) and offers several sustainability advantages over conventional plastic, such as a lower production level of greenhouse gases. Yet, despite their unique manufacturing process, bioplastics are not necessarily biodegradable (Herrmann et al., 2022). In addition, bioplastics face different economic difficulties than traditional plastic, such as greater production costs (Neves et al., 2020).

1.3.2.1. Types of Biopolymers

Natural and synthetic biopolymers are the two categories that make it easy to categorize biopolymers based on their origin, as presented in Figure 2. Synthetic biopolymers are created via microbial production and fermentation, or chemical synthesis with biomass, whereas natural biopolymers are made from polysaccharides or proteins (Calori et al., 2020).

The development of synthetic biopolymers, such as polylactic acid (PLA), polycaprolactone (PCL), polyglycolic acid (PGA), polyvinyl alcohol (PVA) and polybutylene succinate (PBS), was facilitated by modern technology. Synthetic biopolymers have several benefits, including the potential to develop a sustainable business and improvements in several qualities, including toughness, flexibility, high gloss, transparency, and mechanical strength. PLA is the most popular synthetic biopolymer that has been researched to date (Othman, 2014). Lactic acid is converted into PLA in an industrial setting, and it is not only biocompatible but also biodegradable. It is produced by fermenting sugar or polysaccharides using non-fossil renewable natural resources. Due to the

high cost, limited availability and low molecular weight of the polymer, the primary uses of PLA were first restricted to medical applications such as implant devices, tissue scaffolds and internal sutures. In recent years, new technologies have significantly increased its use, and PLA has drawn a lot of interest as a substitute for synthetic polymers based on petrochemicals in the packaging and/or textile industries. (Murariu & Dubois, 2016).

Large biological molecules known as proteins are composed of one or more long chain amino acid residues (Calori et al., 2020). Polysaccharides are bonded with glycosidic bonds, which are composed of monosaccharides. Typically hydrophilic in nature, polysaccharides generate hydrophilic films with excellent mechanical properties and pronounced gas barrier qualities. Hydrophobic polymers, on the other hand, offer a strong barrier to water vapor (Dhumal & Sarkar, 2018). Natural polymers had several issues, including instability, replication issues, aesthetic changes during storage, and unpredictable formulation issues. However, the advantages that these polymers also inherit include their natural abundance, relative simplicity of isolation and potential for chemical modification to fulfill technological requirements. Moreover, these polymers degrade enzymatically and hydrolytically in a biological environment, producing body-friendly byproducts (Bhatia & Bhatia, 2016). Additionally, natural biopolymers present reduced packing volume, weight and waste; edibleness; they were able to enhance the organoleptic qualities of food, such as appearance, odor and flavor; they can incorporate antibacterial and antioxidant compounds, being capable to control over the intercomponent migration of moisture, gases, lipids and solutes; and prolong shelf life and enhance quality of typically unpackaged products (Rhim & Ng, 2007).

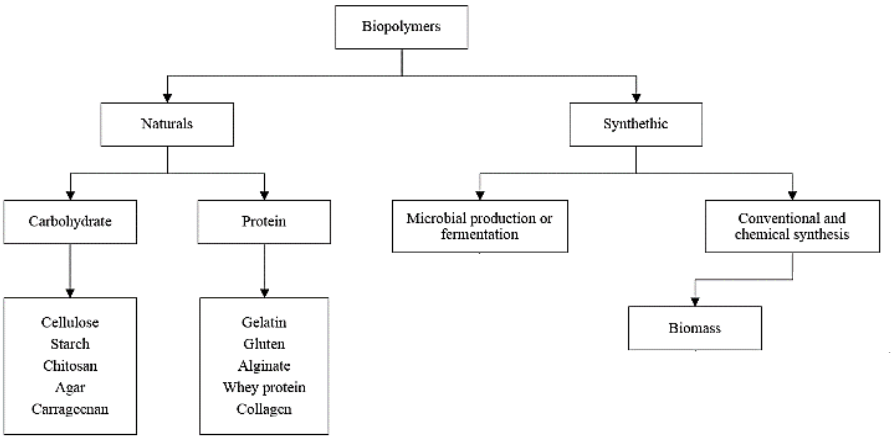


Figure 2: Categories of biopolymers. Adapted from Othman (2014).

1.3.2.2. Properties of Biopolymer Films

Food-grade proteins and polysaccharides can be used to create biopolymer films, which can also incorporate additional ingredients including water, lipids, minerals, and sugars. Many variables will determine which proteins and polysaccharides are used to produce biopolymer films (Jones & McClements, 2010).

The characteristics of packaging materials made from biopolymers rely on a wide range of diverse variables. Due to the origin and processing variables, biopolymer films often exhibit higher water vapor permeability values than synthetic films. Moreover, the thickness of the film, which also influences other physical properties, determines the water resistance (Galus et al., 2020).

The two most significant optical characteristics of edible films are their color and transparency, which are both primarily influenced by selective light absorption and light scattering, respectively (McClements, 2002).

Regarding its use in various types of foods, biopolymer effects on optical characteristics have significant practical consequences because some goods should be clear while others should be opaque (Jones & McClements, 2010).

1.3.3. Biopolymer Composites

Either a stable emulsion or a bilayer can be used to create a composite film. Lipid forms a second layer on the top of the polysaccharide or protein layer in bilayer composite films. The lipid is distributed and trapped in the protein or polysaccharide matrix in emulsion composite films (Pérez-Gago & Krochta, 2005).

By combining two or more constituent materials with notably different properties, a film forming dispersion is obtained. After casting and drying processes, composites are heterogeneous structural material mixes (Christian, 2020), like presented in Figure 3.

Figure 3: Schematic illustration of the composite film formation strategy. Adapted from Abdullah et al. (2022).

To increase the stiffness and tensile strength of the produced composite, a reinforcement agent in discontinuous phase can be added to the biopolymer matrix in continuous phase. By using natural biopolymers, this type of composite is intended to produce a product with good durability performance (Aaliya et al., 2021).

Biopolymer composites have several benefits over conventional composites. They are compatible with any hydrophobic polymer and increase the durability and prolonged release of vitamins and medications included inside them (Aaliya et al., 2021).

Beside advantages, the biopolymer composites are created for commercial manufacturing using fiber treatment, biopolymer blending, plasticizer, coating, and other processing techniques. Natural fibers and biopolymers are less expensive than their synthetic counterparts, although some processing techniques require significant capital expenditure and energy consumption (Christian, 2020).

1.3.3.1. Properties of Biopolymer Composites

The structure and design of composite, interfacial adhesion of the matrix, presence of voids and incorporation of additives like plasticizers, compatibilizers, nanofiller and binding agent all have an impact on the properties of biopolymer composites (Kabir et al., 2012).

Biopolymer composites show notable mechanical characteristics required for many applications. The chemical composition and physical characteristics of biopolymer, composite processing techniques (temperature and force applied), processing environment and plasticization are factors that affect the mechanical properties of biopolymer composites (Aaliya et al., 2021). Waxy substances, which affect the composites' features of adhesion and wettability, have an impact on the mechanical properties as well (Periyasamy et al., 2018) .

The strength and ductility of a biopolymer composite affect the toughness and hardness qualities (Periyasamy et al., 2018).

A biopolymer composite under stress may be regarded as brittle or ductile depending on whether it breaks with or without obvious deformation. If the biopolymer composites are brittle, they break under stress, whereas ductile biopolymer composites tend to deform before failing entirely. Before breaking, brittle biopolymer composites gain considerably more energy than ductile ones (Aaliya et al., 2021).

Tensile tests are one of the most often used methods for figuring out the mechanical characteristics and understanding the structural layout of biopolymer composites (Azammi et al., 2020). Biopolymers are reinforced to the biopolymer matrix in order to increase the tensile properties of the composite because fibers are stronger and more rigid than biopolymers (Periyasamy et al., 2018).

The features and qualities of biopolymer composites are adversely impacted by natural weathering factors such as humidity, temperature, rain, and UV radiation. Determining biopolymer composites' longevity is therefore crucial to realize the use and functionality of the materials over the long term. Many parameters, including moisture absorption, thermal stability, flame retardancy, UV resistance and the biodegradability of natural fiber, biopolymer, and its composites, influence the longevity of biopolymer composites (Aaliya et al., 2021).

1.3.3.2. Bilayer Films

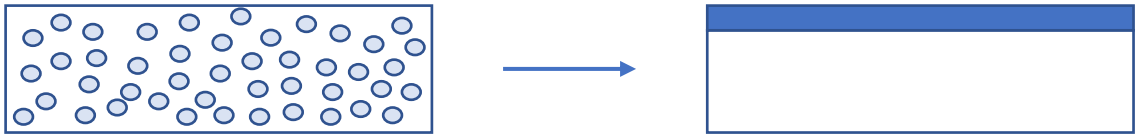
Bilayer films are defined as two materials with various properties linked into a single layered structure (Gartner et al., 2015).

Multi-layered films, an emerging technique, combine the distinctive qualities of many polymers to provide packaging with superior performance in terms of safety and tensile strength. By

enhancing the mechanical and barrier qualities of edible films, the multi-layer film can enhance the properties of food packaging. These benefits of multi-layer films have sparked an increase in interest for uses in food packaging (Alias et al., 2022).

An inner barrier layer film typically comprises of polymers with better oxygen barrier qualities, while polymers with higher water vapor barrier and stronger mechanical capabilities serve as an outside layer to improve the functional properties of polymers (Fabra et al., 2014). Either the "coating technique" or the "emulsion technique" can be used to create bilayer film systems. The coating technique entails casting a lipid layer, either molten or from solvent, onto a polysaccharide or protein film that has already been created. When the continuous phase is unable to stabilize the emulsion and phase separation takes place during drying, the "emulsion technique"—a one-step coating technique—involves distributing the lipid into the film-forming solution before film casting (Figure 4) (Pérez-Gago & Krochta, 2005).

Coating Technique: Two - step technique.



Emulsion Technique: One - step technique.

Figure 4: Bilayer Film: Lipid on hydrophilic film. Adapted from Pérez-Gago and Krochta (2005).

Because bilayer films have a continuous hydrophobic phase, they are often better barriers against water vapor than emulsified films. The coating technique's main drawbacks, however, are that it involves numerous processes, the use of solvents, or the handling of molten waxes (Pérez-Gago & Krochta, 2005).

Concerning the physical properties of bilayer films, the polymer used determines the transparency characteristics. Because they are sensitive to moisture and have low melting and dissolving temperatures, protein-based films are typically exceedingly unstable. The application of multi-layer films made from synthetic or microbial materials, however, have the potential to enhance the thermal properties of protein-based films (Alias et al., 2022).

The hydrophilic behavior of biomass polymers like polysaccharides and proteins is well recognized to cause poor water permeability in biodegradable films made from such materials. In order to boost the hydrophobicity of the final structure and improve the water vapor barrier of the multi-layer films, another polymer with a high hydrophobic layer is laminated onto the biomass films (Rhim et al., 2013).

Referring to the mechanical and gas barrier properties of the bilayer films, they are more mechanically robust than lipid and polysaccharide-based films. The barrier qualities of single-layer films can be improved by applying multi-layer films (Alias et al., 2022).

1.3.3.3. Emulsified Films

Emulsified film systems can only be generated via the emulsion technique, which requires the dispersion of the lipid into either the polysaccharide or the protein film-formation solution to make a stable emulsion. In general, proteins are suitable for this approach because to their emulsifying properties. However, polysaccharides are not as efficient as emulsifiers, and in order to increase emulsion stability, emulsifiers are incorporated in the formulation (Figure 5) (Pérez-Gago & Krochta, 2005).

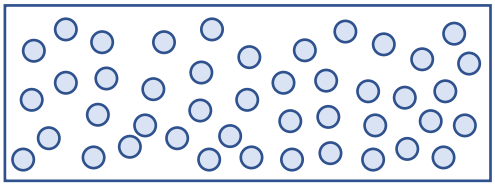


Figure 5: Emulsified Film: The hydrophilic phase contained lipid droplets. Adapted from Pérez-Gago and Krochta (2005).

The properties of emulsion formulations depend on the type of interactions that exist between proteins and lipids or between polysaccharides and lipids. Proteins are a key component in stabilizing protein-lipid emulsions. Proteins are amphiphilic, which causes them to align at the protein-lipid interface so that the polar groups point toward the aqueous phase and the non-polar groups toward the oil. As a result, the stabilization of the emulsions is the result of a delicate balance between several forces, mainly electrostatic and hydrophobic forces. Polysaccharides stabilize emulsions through stearic effects, as opposed to protein-lipid emulsions, which are primarily stabilized by electrostatic forces. To generate a polymeric layer or network with an appreciable thickness, polysaccharides must be tightly bonded to the surface of the lipid and significantly protrude into the continuous phase (Callegarin et al., 1997). Nevertheless, polysaccharides frequently have a limited amphiphilic character, therefore adding emulsifiers, like Polysorbate, is necessary to increase emulsion stability.

Protein and polysaccharide-based emulsified films' barrier properties are related with lipid type, position, volume fraction, and drying conditions in emulsified composite films (Pérez-Gago & Krochta, 2005).

Because of their hydrophilic nature, proteins and polysaccharides typically produce films with strong mechanical qualities but poor moisture barriers (Hambleton et al., 2012).

The addition of lipid to films causes an increase in oxygen permeability. Yet, the chemical nature of lipids has a significant impact on the carbon dioxide permeability of emulsified films (Galus & Kadzińska, 2015). Due to lipids' hydrophobic nature, waxes are among the best moisture barriers, and generally, most research show a decrease in water vapor permeability (Bourtoom, 2009).

Emulsified films have strong mechanical strength but a less effective water barrier than bilayer films because a homogenous distribution of lipids is not obtained (Galus & Kadzińska, 2015). The optical properties of dried films are significantly influenced by the lipid fraction and droplet particle size distribution in film-forming emulsions. Emulsified films often exhibit high lightness values with a little trend to decline, which is related with an increase in lipid concentration (Monedero et al., 2010). The increased homogeneity in the film structure is correlated with the high transparency of emulsified films. By obstructing light transmission through the film, the lipid droplets spreaded in the matrix of the film have an impact on transparency, because the droplets have the capacity to diffuse the light. Hence, the addition of lipids is what causes the significant increase in opacity values that is generally observed (Galus & Kadzińska, 2015).

Due to the difficulty of homogenizing an emulsion, that is, mixing the lipid in the water-based filmogenic solution, some problems leading to heterogeneity of the mixture can occur.

Drainage and gravity-driven separations, such as creaming and sedimentation, flocculation, and coalescence, are the primary mechanisms influencing the aging of emulsions. While the difference in densities between the two liquid phases primarily controls creaming and sedimentation, the other phenomena are more or less directly related to the characteristics of the liquid-liquid interface. The interaction of the adsorbed layers at the surface of the emulsion droplets causes flocculation, which results in the development of droplet aggregates. A droplet coalesces into another one when a liquid film between them thins and ruptures. This process is known as coalescence, and it is greatly controlled by the interfacial characteristics of the adsorbate layers. In emulsions, coalescence can happen when moving droplets come into touch with one another or because of their flocculation (Ravera et al., 2021). All these mechanisms are illustrated in Figure 6.

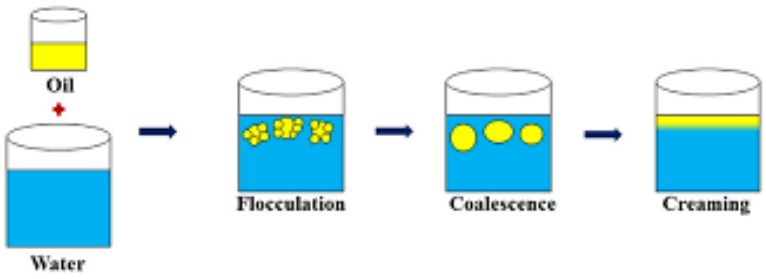


Figure 6: Mechanisms of oil instability at water–oil interactions. Adapted from Santhosh et al. (2021).

Emulsified films can have their water vapor barrier capabilities increased by modifying their hydrophilic/hydrophobic nature. The majority of food packaging applications require a barrier against water vapor, particularly for dried foods (to prevent texture changes, the agglomeration of powdered items, and even microbial growth). An additional benefit of an oil phase on films is that it serves as a vehicle to transport active compounds, many of which are hydrophobic, resulting in the formation of active films and coatings, like films with essential oils, which may present antioxidant and antibacterial activities concurrently. Different essential oils can be

incorporated in films with emulsions while retaining their antibacterial activity against Gram-positive and Gram-negative bacteria (Niro et al., 2021). For these reasons they will be developed in this work.

In the next four sections, the components (pullulan as a polysaccharide, glycerol as a plasticizer, Tween 40 as emulsifier and geraniol as active compound) used in the emulsified films preparation will be described.

1.3.3.4. Pullulan

The extracellular polysaccharide known as pullulan, which is produced by the polymorphic fungus *Aureobasidium pullulans*, is a linear α-*D*-glucan that has a regularly repeating trisaccharide residue (Catley et al., 1986) (Figure 7). It is produced aerobically in cultures of starch and sugar (Tiwari et al., 2019). A lot of agro-based industries produce waste that is extremely rich in the organic and inorganic compounds that *A. pullulans* needs to grow. These wastes can be used as an alternative substrate for submerged or solid-state fermentation to produce pullulan (Singh et al., 2019).

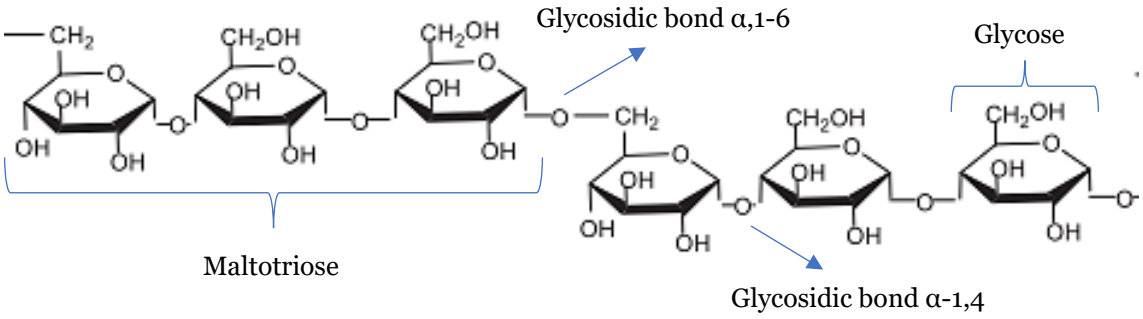


Figure 7: Pullulan molecular structure. A glycosidic bond of α-1,4 connects three glucose units, while a glycosidic bond of α-1,6 links two maltotriose units together. Adapted from Tiwari et al. (2019).

Pullulan has good fiber and film forming capabilities, is water soluble, biodegradable and exhibits great adhesion. Pullulan films are advantageous for food coating, packaging, medication delivery and biomedical applications because they are colorless, nontoxic, heat-sealable, edible, and anti-static. Additionally, pullulan is 'generally recognized as safe' (GRAS). Pullulan films may be dissolved, although this restricts its use in food preservation (Niu et al., 2019).

Pullulan has numerous pharmaceutical uses, which are encouraged by regulatory acceptance. It is a commercially accessible substitute for natural gums made from plants and sea algae. Even at high concentrations, it dissolves in aqueous media without producing a gel, unlike other polysaccharides. The solution demonstrates superior film-forming abilities. The production of active food packaging, capsule shells and mucosal film compositions are some uses for transparent, odorless pullulan films (Tiwari et al., 2019).

Hydrophobicity is introduced (physically, not chemically) to edible coating formulations with the intention of regulating the water vapor permeability. Pullulan may have a hydrophobic moiety

added to it (chemical bonding), which could reduce the permeability of the films to water vapor and increase the range of coating applications for food preservation (Wang et al., 2018).
Pullulan is a stabilizing, thickening and gelling agent used in food products. The pullulan films have high transparency, strong mechanical properties and low oxygen permeability that extend the shelf life of food products. The coating enhances foods sensory qualities while acting as a selective barrier against moisture, gases, and vapors. Additionally, it allows the minimization of petrochemical-based packages usage (Tiwari et al., 2019).

1.3.3.5. Plasticizer

Low molecular weight substances called plasticizers increase the flexibility of films. Polysorbates and glycerol (Figure 8) are the most widely used plasticizers. Because plasticizers can lessen the intermolecular forces in a polymer, they are often added to thin films to improve the transmission of water vapors and water permeability. While choosing plasticizers, compatibility, effectiveness, permanence and economics are taken into account (Chhikara & Kumar, 2021).

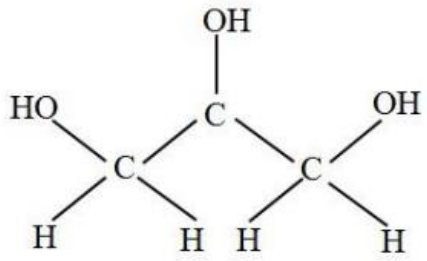


Figure 8: Chemical structure of glycerol. Adapted from (Bangi et al., 2020).

In addition to various physical qualities, other characteristics are also impacted, including the degree of crystallinity, optical clarity, electric conductivity, fire behavior and resistance to biological deterioration (Białecka-Florjańczyk & Florjańczyk, 2007).
The need for plasticizers rises in tandem with the expansion of the plastics sector. The current market offers a wide range of plasticizer options with a variety of characteristics that can be chosen for certain applications to satisfy important material requirements (Vieira et al., 2011). It makes sense to assume that biopolymer plasticizers should ideally be also biodegradable (Choi & Park, 2004).
The compatibility of the parts, the amount of plasticization required, processing characteristics, the desired thermal, electrical, and mechanical properties of the finished product, permanence, resistance to water, chemicals, and solar radiation, toxicity and cost are typically considered when choosing a system. Effective plasticization depends critically on the compatibility between the plasticizer and the polymer, which can be determined by several characteristics, such as polarity, hydrogen bonding, dielectric constant, and solubility parameters. Solvation is another crucial element because plasticizers with solubility characteristics like the polymers require less energy to fuse or solvate the polymer. The plasticizers solvation strength and molecule size are related to the temperature at which fusion or gelation occurs. Volatility and resistance to evaporation,

migration and oil, solvent and water extraction are connected to permanence. The plasticizer should therefore have a low vapor pressure and low rate of diffusion in the polymer (Vieira et al., 2011).

Yet, several studies have also documented detrimental impacts of plasticizers on the properties of edible films. A decrease in cohesion primarily affects mechanical properties and the majority of these indicate an increase in gas, solute and water vapor permeability. Hence, the properties of biopolymer-based films depend on striking a balance between the degree of matrix cross-linking (sometimes required to lower solubility in water but causes brittleness) and the addition of plasticizers for improved workability (da Silva et al., 2009). Phase separation with plasticizer exclusion is typically observed when the plasticizer exceeds the biopolymers compatibility limit above a critical concentration (Vieira et al., 2011).

Glycerol that acts as a plasticizer, reduces intermolecular forces, increases the mobility of biopolymer chains, reduces the H-bonding interactions, can change how water expands, and how much surface energy an aqueous solution has. These effects are the main reasons for the interest in using glycerol (Lavorgna et al., 2010).

1.3.3.6. Emulsifier

Tween 40, or Polysorbate 40, is a detergent/surfactant used as an emulsifier. Emulsifiers could make primary emulsion formation easier and boost their stability after formation by reducing interfacial tension through adsorption at the boundary between the oil and aqueous phases (Figure 9).

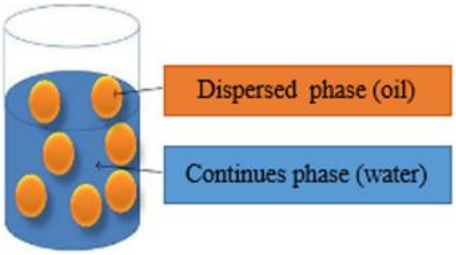


Figure 9: Oil in water emulsion representation. Adapted from Marhamati et al. (2021).

In products made with emulsions, emulsifiers are crucial for long-term shelf life and resilience to environmental challenges. To demonstrate its functional qualities, an emulsifier needs to be suitably distributed in a solution. The place of solubilization, the rate of solubilization and the maximum amount of the dissolved compounds per unit mass of the emulsifier are some of the crucial factors that determine functional characteristics (Marhamati et al., 2021). Emulsifier should lower the interfacial tension, fast adsorb at the surface of the droplets and have the ability to shield the droplets from one another and repel other particles by steric and electrostatic attraction (Yuji et al., 2007). They must also be chemically stable in an emulsion, cost-effective, odorless and nontoxic (Lauridsen, 1976).

Based on their chemical nature, emulsifiers are divided into four categories: artificial, natural, finely dispersed solids, and auxiliary agents.

Synthetic food-grade emulsifiers are made of fats, oils, glycerol, organic acids, sugars, and polyols. Anionic, cationic, non-ionic, and amphoteric classifications are used to describe synthetic emulsifiers. A negative charge is present on the hydrophilic head of anionic emulsifiers. Cationic emulsifiers have exceptional preservative, antiseptic and antibacterial capabilities in addition to having a positive charge on the hydrophilic head of the molecule. Amphoteric emulsifiers can become cationic, anionic, or non-ionic in a solution depending on the environment's acidity or pH. They have both positive and negative charges on a molecule. Only lipophilic (carrying saturated/unsaturated fatty acids or fatty alcohols) and hydrophilic (containing polyoxyethylene, polyoxypropylene or polyols) units are present in nonionic emulsifiers, which lack an electric charge (Marhamati et al., 2021).

The most often used natural emulsifiers are proteins, phospholipids, polysaccharides, lipopolysaccharides, bio-emulsifiers and bio-emulsifiers produced through fermentation with bacteria, yeasts, fungi or separated from plant materials (e.g., glycolipids, lipoproteins and lipopeptides) (Yuji et al., 2007).

In terms of finely dispersed solids, by producing swelling and the creation of a particulate layer around the dispersed phase particles, bentonite and magnesium hydroxide enhance the viscosity of the dispersed phase and decrease the contact between the dispersed particles (Lauridsen, 1976).

Several fatty acids, fatty alcohols and fatty esters are examples of auxiliary agents. Since these chemicals have no great emulsifying abilities, co-emulsifiers should be used in conjunction with them (Lauridsen, 1976).

Membranes go through various stages of disintegration with an increase in detergent, starting with lysis or rupture. Selective extraction of membrane proteins is possible at greater detergent to membrane ratios (about 0.1–1 mg detergent per milligram membrane lipid), but the membrane bilayer essentially stays intact. The membrane becomes soluble at even greater ratios, forming soluble lipid protein-detergent, protein-detergent and mixed lipid-detergent micelles (approximately 2 mg detergent per milligram lipid). Use 10 mg of detergent or more per milligram of lipid to get the greatest possible exchange of lipid for detergent around the proteins (delipidation) (Helenius et al., 1979).

The durability and utility of emulsions are largely determined by the physicochemical characteristics of the interfacial layer generated when emulsifier molecules adsorb to an oil-water interface. Depending on the molecular size, packing and interactions of the molecules of the adsorbed emulsifier, the interface may change in thickness, density, and rheology. The physical stability of emulsions is strongly influenced by these variations in interfacial characteristics. For instance, the intensity and range of the steric contacts between emulsion droplets are significantly influenced by the thickness of the interface, but the strength of the electrostatic interactions is significantly influenced by electrical properties. Whereas globular proteins and other thin-interface emulsifiers require both electrostatic and steric repulsion to stabilize droplets, thick-interface emulsifiers (such as polysaccharides) can frequently stabilize emulsions exclusively by steric repulsion. The interactions between the droplets that constitute an emulsion have a significant impact on its rheological characteristics, and these interactions can be changed by

adjusting the interfacial qualities including thickness, charge and hydrophobicity (McClements & Jafari, 2018).

1.3.3.7. Geraniol

One of the most widely used fragrance components in consumer goods on the European market is geraniol, which is the major compound of several essential oils like the one obtained from *Cymbopogon martinii* (palmarosa). It possesses a quality that is well-liked by customers and is described as being sweet, floral, rose-like, with a tinge of citrus fruit (Hagvall et al., 2012). With the molecular formula $C_{10}H_{18}O$ (Figure 10), geraniol is an acyclic monoterpene alcohol that can be extracted from palmarosa essential oil. Geraniol appears as a transparent to pale yellow oil that is soluble in most organic solvents but insoluble in water (Chen & Viljoen, 2010).

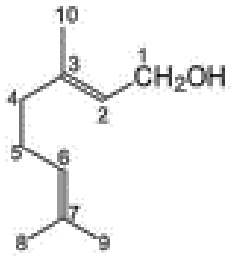


Figure 10: Chemical structure of geraniol. Adapted from Chen and Viljoen (2010).

Due to their insecticidal, repellant, and/or antifeedant qualities, essential oils and their major compounds are emerging as viable pest management agents. Their development is aided by their low mammalian toxicity and biodegradability. Geraniol had showed in many studies significantly more repellent activity than other essential oils (Chen & Viljoen, 2010).

A review of geraniol's antimicrobial properties reveals that this compound's antimicrobial activity may be primarily due to its non-polar nature, which may allow it to interact with the components of the microorganism's cell membrane and disrupt its lipid structure, making it more permeable to other substances, such as antibiotics. Geraniol has the ability to enter the cells and bind to intracellular sites crucial for the survival of the bacteria, hence limiting its growth (Maczka et al., 2020). Geraniol shown antibacterial efficacy against 78 bacteria and fungi of various species. The organisms most thoroughly investigated for geraniol antibacterial activity were strains of the genus *Candida*, followed by *Staphylococcus*. Because both genera contain opportunistic species, such as *C. albicans* and *S. aureus*, there may be interest in developing new antimicrobial agents (such as geraniol) specifically against these two genera. Using MIC evaluation test, *E. aerogenes*, *S. aureus*, *E. coli*, *L.monocytogenes*, *S. epidermidis*, *B. cereus*, *S. Typhimurium*, *T. rubrum*, *T. mentagrophytes*, *M. canis*, *M. gypseum*, *C. albicans*, *C. krusei*, *C.glabrata*, *C. tropicalis*, *C. parapsilosis*, *T. asahii* and *C. neoformans* were evaluated as having an optimal sensitivity (≤600 µg/mL of geraniol) (Lira et al., 2020).

As a result of biomolecules being oxidized by free radicals, aging, arteriosclerosis, cancer, Alzheimer's disease, diabetes, and asthma are all molecular conditions that are caused by these changes (Edris, 2007). Essential oils have recently received attention from researchers as antioxidants or free radical scavengers. Significant reactive oxygens species (ROS) protection was demonstrated by geraniol. These findings showed geraniol's pharmacological potential in inflammatory conditions where oxidative stress is a crucial control factor (Chen & Viljoen, 2010). *In vitro* and *in vivo* tests using several human cancer models have shown that geraniol possesses anticancer properties (Burke et al., 1997). Geraniol's capacity to lower DNA synthesis and so stop the cell cycle was related to its antiproliferative actions on human colon cancer cells (Chen & Viljoen, 2010).

2. Objectives

There are serious environmental concerns as a result of the increasing use of synthetic materials in food packaging and their subsequent improper disposal, particularly due to the materials' resistance to degradation. By extending the shelf life of foods and supporting the idea of circular economy given the repurposing of agricultural waste, the development of biopolymer films with the addition of active compounds emerges as a viable substitute for plastic packaging. This also helps to increase food security. In this context, in the current work, emulsified films incorporating geraniol were developed to create an active food packaging. Thus, the following specific goals were established:

- Production of emulsified pullulan films with geraniol incorporation;
- Analysis of the films using Fourier Transform Infrared Spectroscopy (FTIR);
- Analysis of the thermal behavior of films using Differential Scanning Calorimetry (DSC);
- Characterization of films in terms of their grammage, thickness, mechanical, barrier, and optical properties;
- Determination of the film's contact angles and surface energies;
- Evaluation of the films' antioxidant properties;
- Evaluation of the antimicrobial activity of the films against several foodborne pathogens;
- Study of the films' biodegradability in soil and ocean water.

3. Materials and Methods

3.1. Reagents

Pullulan (CAS: 9057-02-7), molar mass 574.57 g/mol was acquired from TCI Europe NV. Glycerol (anhydrous, extra pure) (CAS: 56- 81-5) was purchased at Merck. Geraniol (CAS: 106-24-1), molar mass 154.24 g/mol was bought in TCI Europe NV. Tween 40 (CAS: 9005-66-7), molar mass 1283.63 g/mol was acquired from Alfa Aesar.

3.2. Evaluation of the Antioxidant Activity of Geraniol

Two previously established methods were used to assess the geraniol's antioxidant activity: β-carotene bleaching test and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay (Luís, Pereira, et al., 2019).

3.2.1. DPPH Free Radical Scavenging Assay

By using the DPPH free radical scavenging test, the antioxidant activity of the geraniol was assessed**.**

In summary, three DPPH methanolic solutions were prepared by dissolving 19.7, 12.25, and 7.9 mg of DPPH (Sigma-Aldrich, St. Louis, MS, USA) in 250 mL of methanol (Fluka, Milwaukee, WI, USA) (0.2, 0.1242, and 0.8 mM solutions, respectively). Then, 3.9 mL of the previous solutions were mixed with 0.1 mL of geraniol dilutions (100, 75, 50, 25, 15, 10% v/v). 0.1 mL of methanol and 3.9 mL of each DPPH solution made up the negative control. Using a spectrophotometer (Helios-Omega, Thermo Scientific, Waltham, MA, USA), the absorbances were measured at 517 nm following the incubation time (90 min) at room temperature in the dark (Luis et al., 2017). The radical scavenging activity was calculated using the following equation:

$$\%\ Inhibition\ = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad , \qquad \textbf{(1)}$$

where $A_{control}$ was the absorbance of the control sample and A_{sample} was the absorbance in the presence of geraniol. The IC_{50} (concentration providing 50% of inhibition), was determined using a calibration curve in the linear range of the graphic by plotting the geraniol concentration *versus* the corresponding scavenging effect (% Inhibition). The antioxidant activity was expressed as the antioxidant activity index (AAI), calculated as follows:

$$AAI = \frac{final\ concenteration\ of\ DPPH\ in\ the\ control\ sample}{IC_{50}} \quad . \qquad \textbf{(2)}$$

Accordingly, the AAI was calculated considering the mass of the DPPH and the mass of geraniol in the reaction, yielding a constant that was independent of the concentration of DPPH or geraniol used. The AAI allowed the following classification of the antioxidant activity: poor (AAI ≤ 0.5), moderate (0.5 < AAI ≤ 1.0), strong (1.0 < AAI < 2.0), or very strong (AAI ≥ 2.0) (Luís et al., 2016). The assays were conducted in duplicate, and all DPPH solutions were prepared daily.

3.2.2. β-Carotene Bleaching Test

At first, 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 (20 mg/mL in chloroform). Following the vacuum-assisted evaporation of the chloroform, 100 mL of distilled water saturated with oxygen were added to the mixture to create an emulsion. Then, test tubes containing 300 µL of geraniol dilutions (100, 75, 50, 25, 15, 10% v/v) were filled with 5 mL of this emulsion. The tubes were then vortexed and heated to 50°C in a water bath for 1 h.

The samples' absorbances were measured at 470 nm in comparison to a blank containing an emulsion without the β-carotene solution. 300 µL of methanol and 5 mL of the emulsion were used as control samples. The antioxidant activity of the geraniol was determined as the percentage of inhibition of β-carotene oxidation by the following equation (Luis, Gallardo, et al., 2020):

$$\%\ Inhibition\ = \frac{A^{\,t\,=\,1h}{}_{sample} - A^{\,t\,=\,1h}{}_{control}}{A^{\,t\,=\,0h}{}_{control} - A^{\,t\,=\,1h}{}_{control}} \quad , \qquad \textbf{(3)}$$

where $A^{t=1h}$ is the absorbance of the sample (geraniol) or control at the final time of incubation, and $A^{t=0h}$ is the absorbance of the control at the initial time of incubation.

3.3. Evaluation of the Antibacterial and Anti-Quorum Sensing Activities of Geraniol

Three Gram-positive bacterial strains (*Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* LMG 16779, and *Staphylococcus aureus* ATCC 25923) and three Gram-negative bacterial strains (*Salmonella* Typhimurium ATCC 13311, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922) were employed for the antibacterial experiments. The anti-quorum sensing activity of the geraniol was evaluated using the biosensor strain *Chromobacterium violaceum* ATCC 12472. The American Type Culture Collection (ATCC, Manassas, VT, USA) and the BCCM/LMG Bacteria Collection (Belgian Co-Ordinated Collections of Micro-organisms, Gent, Belgium) provided the reference strains. The bacterial strains' stock cultures were stored at -80°C in 20% (v/v) glycerol (Himedia, Mumbai, India). 24 h prior to the assays, all the strains for the antibacterial activity were sub-cultured in brain-heart infusion agar (BHI) and *Chromobacterium violaceum* was sub-cultured in Luria-Bertani broth (LB).

3.3.1. Solid Diffusion Assay

Bacteria were suspended in sterile saline solution (NaCl 0.85% (w/v)) to a cell suspension of 0.5 McFarland (1-2 × 10^8 colony-forming units/mL (CFU/mL) to create the inoculums for this test. 6 mm diameter sterile blank filter discs were saturated with 15 µL of geraniol. Then, the previously prepared discs were placed on top of the BHI or Müeller-Hinton Agar (MHA) plates that had already been inoculated with bacteria. The plates were then incubated for 18 h at 37°C. Following incubation, the diameters of the inhibition zones were measured with a digital pachymeter on each plate (Lopez et al., 2005). Additionally, the plates were observed by optical microscopy to verify the microbial growth inhibition after the incubation period (Luis et al., 2021). This assay was performed three independent times.

3.3.2. Determination of MIC Values: Resazurin Microtiter Method

The resazurin microtiter assay was used to determine the minimum inhibitory concentrations (MIC) of geraniol. To improve its solubility, dimethyl sulfoxide (DMSO) (maximum 2% (v/v)) was used. Müeller-Hinton broth (MHB) (Liofilchem, Italy) was employed as culture medium for *Salmonella* Typhimurium ATCC 13311, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. BHI was used for *Enterococcus faecalis* ATCC 29212 and *Listeria monocytogenes* LMG 16779. Serial two-fold dilutions of geraniol (from 32 to 0.25 µL/mL) were made in a 96-well plate (50 µL/well). Then, 30 µL of fresh MHB or BHI was added to each well, followed by 10 µL of resazurin (TCI Europe N.V., Belgium) indicator solution (0.1% w/v diluted in MHB or BHI). The wells were then filled with a bacterial suspension (10 µL, 0.5 McFarland units; total volume: 100 µL/well). 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 geraniol concentration at which the color change occurred (Luís, Sousa, et al., 2019).

3.3.3. Anti-Quorum Sensing Activity

The *C. violaceum* ATCC 12472 suspension was cultured aerobically in LB broth at 30°C overnight (Alvarez et al., 2014).

C. violaceum ATCC 12472 suspension was seeded into LB agar (Pronadisa, Madrid, Spain) plates after being adjusted to an OD_{620nm} of 1. The plates were topped with sterile discs (6 mm in diameter) saturated with 15 L of geraniol and incubated at 30°C for 24 h. Following the incubation time, the amount of pigment production inhibition around the disc (a ring of live, colorless cells) was assessed. Antimicrobial action is indicated by the absence of microbial growth. The diameter of bacterial growth inhibition was then subtracted from the overall diameter to determine the quorum sensing inhibition (QSI), which was assessed by the violacein pigment inhibition. These experiments were performed in three independent assays (Luis et al., 2017).

3.4. Preparation of Films

The initial preparation of the pullulan aqueous solution (3%, w/v) involved magnetic stirring (300 rpm) at room temperature for 5 min of 3 g of pullulan with 100 mL of distilled water. Then, 0,45g of glycerol were added as plasticizer (15%, w/w relative to pullulan), and the solution was stirred for 30 min at 50°C. 150 mg of geraniol (5%, w/w relative to pullulan) and 0,5 mL of Tween 40 were added to the solution and agitated for 10 min under the same conditions. Before the distribution of the solution to the polystyrene Petri dishes (16 mL/plate), it was subjected to homogenization at 21500 rpm for 5 min using an IKA T25 Digital Ultra-Turrax rotor-stator homogenizer. This process leads to large accumulation of foam in the solution so, a vacuum cycle with a Rotavapor RE 111 regulated with a Vacuum Controller V-850 up to 130 mbar was required. After that, the Petri dishes were placed for 3 h in a ventilated oven at 60°C. For the preparation of the control films, the method used was similar, except the addition of Tween 40 (in the PuGer films), the addition of Ger (in the Pu40 films) and the addition of Tween 40 and Ger (in the Pu films). After being dried, the films were removed from the plates and stored at a temperature of 23 ± 2°C and a relative humidity (RH) of 50 ± 5% (Chu et al., 2019).

3.5. Characterization of Films

3.5.1. Fourier-Transform Infrared Spectroscopy (FTIR)

Using a Nicolet iS10 smart iTRBasic (Thermo Fisher Scientific, Waltham, MA, USA) model with 64 scans and a 4 cm^{-1} resolution, FTIR spectra of the films were acquired between 4000 and 600 cm^{-1} (Nunes et al., 2018).

3.5.2. Differential Scanning Calorimetry (DSC)

With a calorimeter Netzsch DSC 204 operating in an inert atmosphere and heating the films at a rate of 10°C/min from 25 to 400°C, DSC thermograms of the films were acquired. Samples of the films were held at 105°C for 24 h prior to the thermal examination in order to totally evaporate the water (Bilohan et al., 2022).

3.5.3. Grammage, Thickness and Mechanical Properties

According to ISO 536:1995, the grammage of the films was estimated using the mass-to-area ratio (g/m^2) (Luis, Gallardo, et al., 2020).

The thickness was measured using an Adamel Lhomargy type MI 20 micrometer in accordance with ISO 534:2011. Each film sample was subjected to five measurements at various positions, and the mean values were used to determine the mechanical properties (Silva et al., 2016).

Elongation (%), tensile strength (N/m), tensile index (N.m/g), and elastic modulus (MPa) of the films were measured in accordance with ISO 1924/2 using a tensile tester (Thwing-Albert

Instrument Co., West Berlin, NJ, USA), with the initial grip set at 50 mm and the crosshead speed set at 10 mm/min (Luis, Ramos, et al., 2020).

3.5.4. Contact Angle and Surface Free Energies

The sessile drop contact angle method was used to ascertain the contact angles of the films, and the model OCAH 200 (DataPhysics Instruments, Filderstadt, Germany) that allowed image acquisition and data analysis was used. Deionized water, ethylene-glycol, and diiodomethane were used to measure the contact angles to calculate the surface free energy (total, dispersive, and polar components) of the films. The software of the equipment provided the surface tension components of the reference liquids (Luis, Gallardo, et al., 2020). 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) method (Silva et al., 2016).

3.5.5. Optical Properties

A Technidyne Color Touch 2 spectrophotometer was used to evaluate the optical properties of the films, such as color coordinates and transparency. Several random measurements were carried out on each sample using the D65 illuminant (daytime light with the ultraviolet component) and the observation angle of 10º. Color coordinates L* (lightness), a* (redness; ± red-green) and b* (yellowness, ± yellow-blue) were obtained. The ISO 22891 equation **(4)** was used to calculate the transparency (T) of the samples (Luís, Pereira, et al., 2019):

$$T = \sqrt{(R_w - R_0)\left[\frac{10000}{R_{(w)}} - R_0\right]} \quad \textbf{(4)}$$

where, R_W is the reflectance of the sample in % when positioned against a white background, R_o is the reflectance of the sample against a black background and $R_{(w)}$ = 90.41 is the reflectance of the standard white background used in the test.

3.5.6. Antioxidant Activity

The two previously implemented methods were used to assess the antioxidant activity of the films: β-carotene bleaching test and DPPH free radical scavenging assay (Luís, Pereira, et al., 2019).

3.5.6.1. DPPH Free Radical Scavenging Assay

Twelve disks of each film (6 mm in diameter) were mixed with 2.9 mL of a DPPH methanolic solution (0.1 mM). The absorbances were then measured over one hour period at 517 nm every 30 min against a methanol blank (Luis et al., 2019).

3.5.6.2. β-Carotene Bleaching Test

To perform this test, the methodology described in Section 3.2.2 was used, however, instead of 300 µL of geraniol, 12 disks (6 mm of diameter) were inserted in each tube (Luis, Gallardo, et al., 2020).

3.5.7. Evaluation of the Antibacterial and Anti-Quorum Sensing Activities of the Films

The procedure outlined in Section 3.3 was used to conduct these tests.

3.5.7.1. Solid Diffusion Assay

Although this test was conducted using the methods outlined in Section 3.3.1., some changes were made. Instead of paper disks with geraniol, films' disks (6 mm in diameter) were used (Lopez et al., 2005).

3.5.7.2. Anti-Quorum Sensing Activity

Although this test was conducted using the methods outlined in Section 3.3.3., some changes were made. Instead of the sterile discs, the plates were topped with discs from each film and incubated at 30°C for 24 h (Luis et al., 2017).

3.5.7.3. Anti-Biofilm Activity

Scanning electron microscopy (SEM) was used to study the antibiofilm activity of the films for one Gram-positive bacterial specie (*E. faecalis* ATCC 29212) using the Pu and PuGer40 biofilms. Bacterial biofilms were formed for that purpose directly on the discs of the films (about 1 cm^2) that were set up on 12-well plates. The suspensions' turbidity was adjusted to have an $OD_{610\ nm}$ of 0.7. The BHI medium was then added in a 700 µL volume to the bacterial suspensions in a volume of 300 µL. The plates were incubated for 24 h at 37°C. The biofilms were then fixed with 2.5% (v/v) glutaraldehyde (Sigma-Aldrich, USA) diluted with Phosphate-buffered saline solution (PBS) and incubated at 4°C for 4 h after being washed twice with sterile saline solution. After that, samples were given a single PBS wash before being dehydrated in ethanol for 20 min at each of the following concentrations: 30, 50, 70, 80, and 90% (v/v) and absolute. The samples were then left to dry in a desiccator overnight. Then they were mounted on the proper stubs and coated with gold using a metal evaporator (Quorum Q150R ES, East Sussex, UK). VP SEM Hitachi S-3400N

was used to observe the biofilms, with a voltage of 20.0 kV and a 120.0 A emission (Luis, Ramos, et al., 2020).

3.5.8. Barrier Properties

3.5.8.1. Oil Permeability

First, test tubes were filled with 5 mL of edible vegetal oil from sunflower seeds, which was then covered with each film. The tubes were placed upside-down on a filter paper surface that had been dried at 105°C for 24 h. The weight difference of the filter paper (before and after oil exposition), the thickness of the films, the effective contact area, and the storage time (24 h) were used to calculate the oil permeability (OP) (g.mm/m².day) as follows:

$$OP = \frac{\Delta W \times e}{A \times \mathrm{t}} \quad , \tag{5}$$

where ΔW is the weight difference of the filter paper (g), *e* corresponds to the thickness of the film (mm), A is the contact area (m²), and t is the storage period (days) (Luis et al., 2021).

3.5.8.2. Water Vapor Permeability (WVP)

According to the standard procedure ASTM E96-00, water vapor permeability (WVP) (g/Pa.day.m) and water vapor transmission rate (WVTR) (g/m².day) were measured. The films were adhered to the top of adjusted cups that contained a desiccant (13 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 variations were tracked every 2 h. The slope of a linear regression of the weight gain *vs* time was used to calculate the gradient. Equations **(6)** and **(7)** were used to determine respectively the WVTR and WVP (Luis, Ramos, et al., 2020):

$$WVTR = \frac{\frac{\Delta m}{\Delta t}}{A} \quad , \tag{6}$$

where Δm is the weight changes of test cups (g), A is the test area (m²), and t is the test time (day).

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

where *p* is the vapor pressure of water at 23◦C (Pa), RH1 is the RH of the cabinet (50%), RH2 is the RH inside the cups (0%), and e is the thickness (m) of the films.

3.5.9. Biodegradability

The biodegradability assay was conducted in two different environments: sea water from *Peniche de Cima* beach and in an enriched sample of soil. In both environments, strips of each film were cut (dimensions 2 × 7 cm), weighed and placed in the sea water (100 mL) and soil samples (depth of 10 cm) for 24 h. The % of biodegradability was established with the difference between the initial and final film strips mass.

3.6. Statistical Analysis

Generally, the results were presented as mean ± standard deviation (SD). The analytic tool Microsoft Excel was used to evaluate the data. Student's T-test was used to examine significant variations in mean (assuming the normal distribution of the continuous variables). *P*-values lower than 0.05 were considered as statistically significant.

4. Results and Discussion

4.1. Evaluation of the Antioxidant Activity of Geraniol

Since geraniol is extracted from palmarosa and is the major compound of its essential oil, it is expected that it has potential biological activities like antioxidant activity. The geraniol's antioxidant activity was assessed by DPPH free radical scavenging assay and β-Carotene bleaching test.

4.1.1. DPPH Free Radical Scavenging Assay

Due to their numerous health advantages, antioxidants are regarded as vital nutraceuticals. The necessity of a standard test is crucial for comparing the findings of various laboratories and validating the conclusions. A stable free radical called DPPH possesses an unpaired valence electron at the bridge of one nitrogen atom (Sharma & Bhat, 2009).

Using the DPPH assay to obtain the absorbances of geraniol in three DPPH concentrations, it was possible to assess the antioxidant activity index results (Table 1). Supported by a calibration curve, we were capable of design equations to stablish the IC_{50} (%, v/v) (Table 1) of geraniol's needed to scavenge in 50% the DPPH radicals.

Table 1: Geraniol's DPPH half maximal inhibitory concentration and Antioxidant Activity Index (AAI). (Results presented as mean ± standard deviation).

IC_{50} (%, v/v)	AAI	Antioxidant activity
24.51 ± 6.85	0.21 ± 0.03	Poor

Comparing the IC_{50} results (Table 1) with the ones obtained by Widiyarti et al. (2019), it is possible to conclude that in the present study a bigger concentration of geraniol was necessary to inhibit the DPPH free radicals. Accordingly with this result, the geraniol's AAI allowed the classification of its antioxidant activity as poor (AAI ≤ 0.5) (Luís et al., 2016). In Andrade et al. (2014), a study with Wistar rats was conducted with geraniol inhalation and this exposition caused oxidative stress. Only the combined action of geraniol and other *Cymbopogon martinii* compounds presented significant results of antioxidant activity.

4.1.2. β-Carotene Bleaching Test

The β-carotene bleaching test is used to quantify the concentration of geraniol necessary to inhibit 50% of the oxidation of β-carotene, which, thanks to the oxidizing effect, is discolored. Usually, the absorbance measurements are conducted at 470nm (Danet, 2021).

The IC_{50} result is presented on the Table 2.

Table 2: Geraniol's β-carotene half maximal inhibitory concentration. (Results presented as mean ± standard deviation).

IC_{50} (%, v/v)
70.81 ± 11.23

According to the results and description of Kulisic et al. (2004), it is possible to affirm that an increase in the concentration of oregano essential oil, and in the present case geraniol, causes an increase in the percentage of inhibition of β-carotene oxidation. In the same study, the IC_{50} of oregano essential oil is 2 g/L, approximately, however, the concentration of geraniol to produce a similar effect is quite bigger (70.81 (%, v/v)). So, to conclude, the antioxidant capacity of geraniol is lower than that of oregano essential oil. As far as we know, no studies with this assay have been realized with geraniol.

Overall, it is possible to conclude that in the present study, geraniol did not demonstrate effective antioxidant activity.

4.2. Evaluation of the Antibacterial and Anti-Quorum Sensing Activities of Geraniol

The antibacterial properties of Geraniol were evaluated against six foodborne pathogens (*Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* LMG 16779, *Staphylococcus aureus* ATCC 25923, *Salmonella* Typhimurium ATCC 13311, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922). The anti-quorum sensing (QS) impact of geraniol was evaluated using the biosensor strain *Chromobacterium violaceum* ATCC 12472.

4.2.1. Solid Diffusion Assay

The gold standard for determining if bacteria are susceptible is the disk diffusion method (Khan et al., 2019). This test is used to verify if geraniol has antimicrobial activity against the bacterial strains used by measuring the microbial growth inhibition zone that is created around a geraniol-impregnated disk.
In Table 3 it is possible to see the diameters obtained.

Table 3: Diameters of the inhibition zones obtained with a pachymeter. (Results presented as mean ± standard deviation).

Strain	Diameter of inhibition zones (mm)
Staphylococcus aureus ATCC 25923	15.44 ± 1.72
Enterococcus faecalis ATCC 29212	11.37 ± 0.75
Salmonella Typhimurium ATCC 13311	8.61 ± 0.51
Pseudomonas aeruginosa ATCC 27853	7.31 ± 0.61
Escherichia coli ATCC 25922	11.48 ± 0.18
Listeria monocytogenes LMG 16779	25.62 ± 0.36

The results of the adapted solid diffusion assay were analyzed in the optical microscope to a better interpretation than the naked eye analysis of the Petri dishes. The images obtained are presented in Figure 11.

In Figure 11a is represented the paper disc delineated with a red line and around it there is no bacterial colonies or single bacteria, so geraniol has an antibacterial activity against *Staphylococcus aureus*. In Figure 11b with the geraniol against *Enterococcus faecalis* it is possible to observe a total bacterial inhibition. In Figure 11c, the paper disc at left and at right a group of *Salmonella* Typhimurium colonies, however between the paper and the colonies there is a ring of total inhibition without bacteria.
In Figure 11d, the paper disc marked with red and in its neighborhood, there is some dispersal colonies of *Pseudomonas aeruginosa*. Comparing these dispersal colonies with the ones marked with a blue line, it is possible to admit that there was a partial growth inhibition of this bacterial strain. In Figure 11e and 11f the scenario is similar with a complete inhibition of the bacterial growth (*Escherichia coli* and *Listeria monocytogenes*, respectively) around the geraniol impregnated disc.

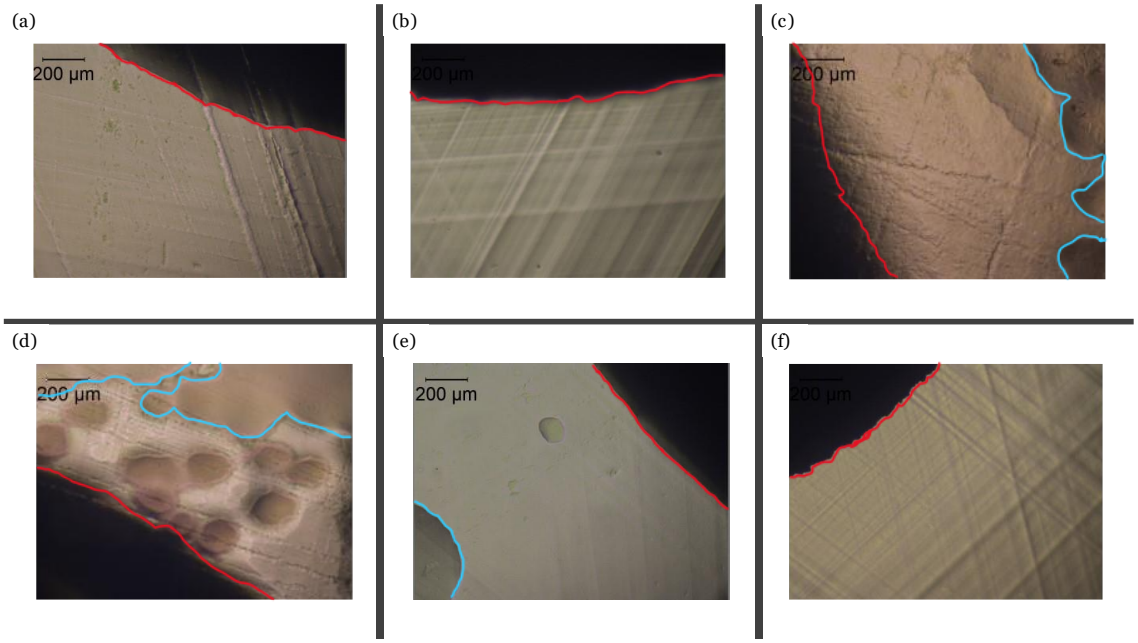


Figure 11: Optical microscopy images of geraniol's antibacterial activity (Amplification: 100×). (a) *Staphylococcus aureus* ATCC 25923; (b) *Enterococcus faecalis* ATCC 29212; (c) *Salmonella* Typhimurium ATCC 13311; (d) *Pseudomonas aeruginosa* ATCC 27853; (e) *Escherichia coli* ATCC 25922; (f) *Listeria monocytogenes* LMG 16779. The red lines delineate the contour of the sterile blank filter discs and the blue lines delineate the bacterial colonies. The space between blue and red lines is the inhibition zone.

If compared, the values of Table 3 agree with the microscopy images. The diameters of inhibition zones obtained for *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Listeria monocytogenes* are the most significant and if compared with the images are the ones without bacterial colonies because the inhibition ring size did not allow the entire capture. In Figure 11c and 11d, *Salmonella* Typhimurium and *Pseudomonas aeruginosa* respectively, showed total and partial inhibition rings, but with smaller sizes allowing the capture of the bigger bacteria colonies. Comparing these results with a previous study using palmarosa essential oil against *Staphylococcus aureus*, *Salmonella* Typhimurium, *Pseudomonas aeruginosa*, *Escherichia coli* and *Listeria monocytogenes*, they were quite different. In that study, palmarosa essential oil only have antibacterial activity against *Listeria monocytogenes* and *Staphylococcus aureus*. Although, the diameter of the inhibition zone in *Staphylococcus aureus* was very similar to the one obtained in the present study (Ghabraie et al., 2016).

4.2.2. Determination of MIC Values: Resazurin Microtiter Method

The lowest concentration of an antimicrobial at which bacterial growth is totally inhibited is known as the MIC value. Resazurin, a weakly fluorescent blue dye that is converted by active bacteria to fluorescent resorufin (pink), can be used to speed up reading in the broth microdilution method (Elshikh et al., 2016). It is necessary to repeat tests where bacteria do not

grow at lower antimicrobial concentrations but do proliferate visibly at higher concentrations. This could be the result of several factors, such as technical mistakes related to improper antimicrobial dilution, for example. The identification of the resistance mechanism is not required to evaluate antimicrobial resistance based on the MIC value (Kowalska-Krochmal & Dudek-Wicher, 2021).

The example of a result of a 96-well plate is shown in Figure 12.

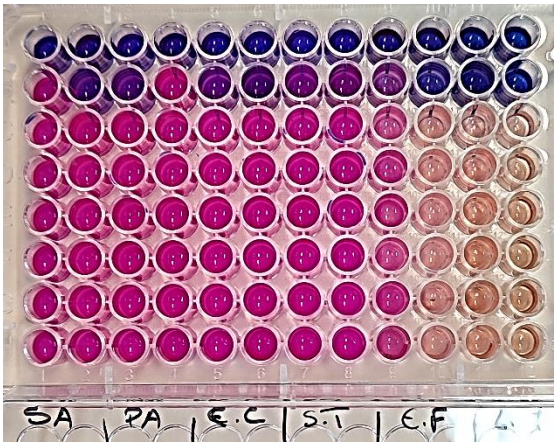


Figure 12: Example of a trial of MIC's assay. From left to the right: *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27583, *Escherichia coli* ATCC 25922, *Salmonella* Typhimurium ATCC 27853, *Enterococcus faecalis* ATCC 29212 and *Listeria monocytogenes* LMG 16779. From top to bottom: 32 µL/mL; 16 µL/mL; 8 µL/mL; 4 µL/mL; 2 µL/mL; 1 µL/mL; 0.5 µL/mL; 0.25 µL/mL.

After the analysis of all the replicates it was possible to conclude the geraniol's MIC value for each strain of bacteria (Table 4).

For a comparison exercise, Lira et al. (2020) presents a collection of MIC's of geraniol against several bacterial and fungal from other studies. In that collection, the MIC values presented were: for *Staphylococcus aureus* 0.25 mg/mL; for *Pseudomonas aeruginosa* 2773.6 µg/mL; for *Escherichia coli* 1386.8 µg/mL; for *Salmonella* Typhimurium 0.03 mg/L; for *Enterococcus faecalis* 1386.8 µg/mL and finally for *Listeria monocytogenes* 2773.6 µg/mL. Doing the adequate conversation with the density of geraniol, it is possible to conclude that the present values are better than in Lira et al. (2020), indicating that we use less quantity of antimicrobial to achieve the same result.

Table 4: Geraniol's MIC values to all the bacterial strains.

Strain	MIC value (µL/mL)
Staphylococcus aureus ATCC 25923	16
Enterococcus faecalis ATCC 29212	32
Salmonella Typhimurium ATCC 13311	16
Pseudomonas aeruginosa ATCC 27853	16
Escherichia coli ATCC 25922	16
Listeria monocytogenes LMG 16779	16

4.2.3. Anti-Quorum Sensing Activity

Quorum sensing is the use of signal molecules to control gene expression in response to changes in cell population density. It has been revealed that QS controls bacterial motility, biofilm formation, and extracellular enzyme synthesis as virulence factors (Yu et al., 2022).

In the Figure 13 it is possible to observe an example of the results obtained:

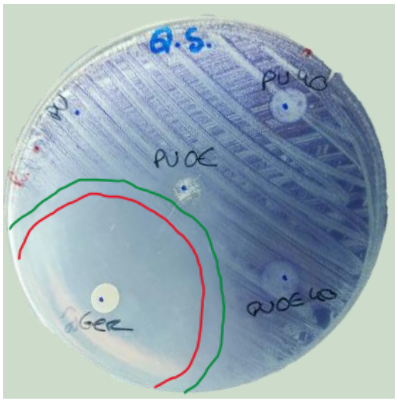


Figure 13: Geraniol's Anti-Quorum Sensing activity. The red lines delineate the bacterial growth inhibition, and the green lines delineate the zones with bacterial growth but without pigment.

Table 5: Diameters of the inhibition zones obtained with a pachymeter. (Results presented as mean ± standard deviation).

Sample	Diameter of inhibition zones (mm)
Geraniol	9.12 ± 0.22

As previously mentioned, anti-microbial action is indicated by the absence of microbial growth. The diameter of bacterial growth inhibition was then subtracted from the overall diameter to determine the QSI, which was assessed by the violacein pigment inhibition (Luis et al., 2017). Analyzing the results, it was possible to conclude that geraniol has anti-QS activity. The use of a pachymeter enable the measurement of the green and red rings' diameters to conclude that the mean diameter in millimeters is 9.12 ± 0.22. Comparing this study with a previous one using geraniol against *Pseudomonas fluorescens*, the outcomes are hugely similar. One more time, geraniol demonstrates its anti-QS activity (Yu et al., 2022).

4.3. Preparation of Films

The preparation of the films was a process that led us through a strategy of trial and error until we arrived at the final protocol for each type of film according to the characteristics we wanted. For example, initially we used a large amount of geraniol and the films became extremely greasy. We also tried a smaller amount of glycerol, but the texture of the films was too brittle.
The final appearance of the four types of films produced (Pu, PuGer, PuGer40, Pu40) is shown in the Figure 14.

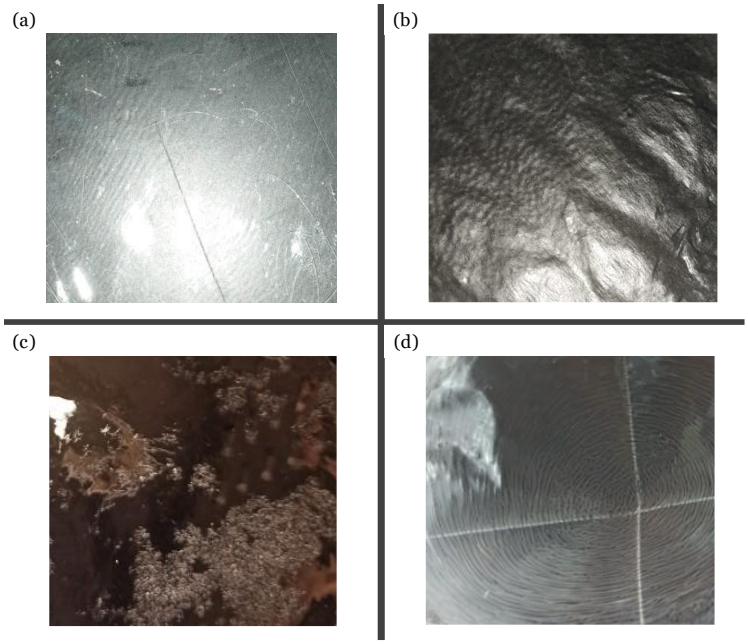


Figure 14: All types of films produced: a) Pu; b) Pu40; c) PuGer; d) PuGer40.

In Figure 14 (a), the Pu film only have pullulan and the final result was a film very transparent and clear, but only with the pullulan addition the film has no antioxidant nor antibacterial properties. In Figure 14 (b), the Pu40 film only have pullulan and Tween 40. The obtained film appears with too much oiliness and some roughness because of the bubbles due to the homogenization process. In Figure 14 (c), the PuGer film have in its constitution pullulan and geraniol. In the picture it's possible to see that the film presents some stains, because in the drying process, the geraniol without the emulsifier Tween 40 have tendency to evaporate. This film is not homogenous. In Figure 14 (d), thePuGer40 film has all the compounds: pullulan, geraniol, and Tween 40. This film is very clear and transparent, without geraniol stains, no brittle aspect, less greasy than the others obtained, homogenous aspect and no roughness despite the design of the film with those marks' characteristic of the mixture.

4.4. Characterization of Films

Four types of films were prepared. PuGer40 with all compounds (pullulan, geraniol, glycerol and Tween 40), PuGer without Tween 40, Pu40 without geraniol and Pu without geraniol and Tween 40.

4.4.1. Fourier-Transform Infrared Spectroscopy (FTIR)

FTIR was performed for all the components used in the production of the films and for all the films obtained. This test allowed us to verify the constitution of the films obtained by identifying the main functional groups commonly present in the compounds used and in the films obtained. The FTIR spectra obtained by a Nicolet iS10 smart iTRBasic are showed below:

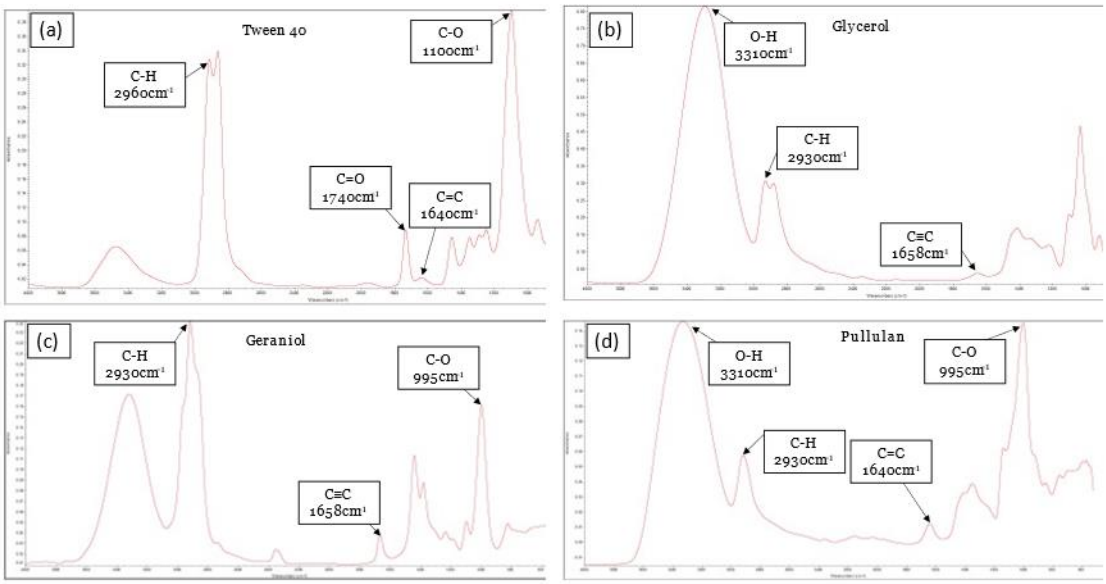


Figure 15: FTIR spectra of film's components: (a) Tween 40; (b) Glycerol; (c) Geraniol; (d) Pullulan.

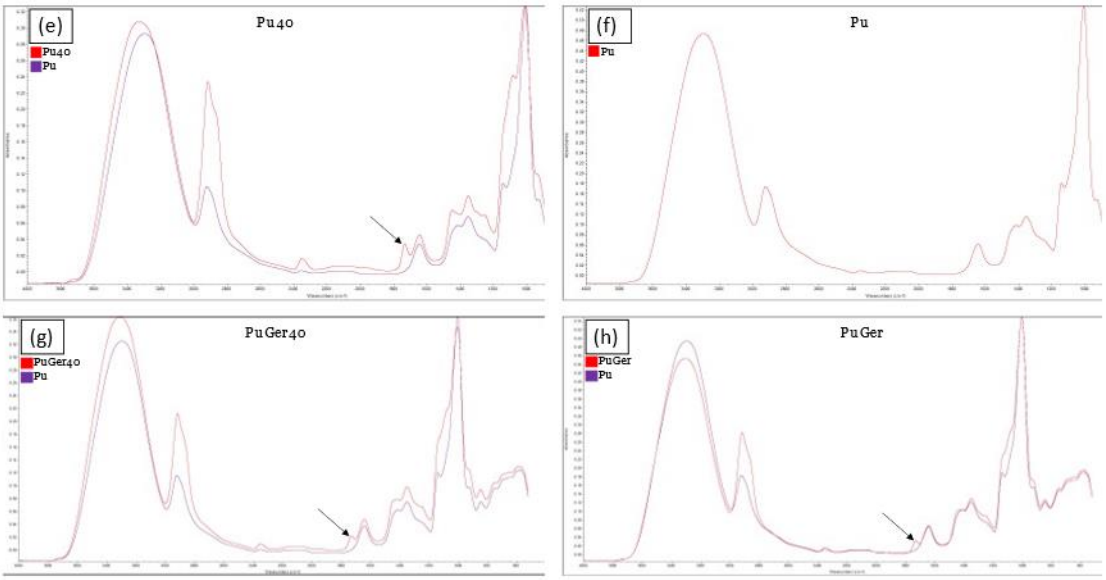


Figure 16: FTIR spectra of each type of film: (e) Pu40; (f) Pu; (g) PuGer40; (h) PuGer.

In Figure 15 it is possible to observe some of the principal functional groups of all the components of the films in the FTIR spectra. In Figure 15a, the spectrum correspondent to Tween 40 shows that it owns a methyl group, an ester group, an alkene group, and an ether group. Chu et al. (2019) have done the same FTIR analysis with Tween 80 and both analysis shows a band near 1735 cm^{-1} associated to the stretching vibration of carbonyl group. In Figure 15b, the spectrum correspondent to glycerol shows that this compound owns an alcohol group that indicates the presence of water, a methylene group and an alkyne group and it is almost fully corroborated by AlOmar et al. (2016). In Figure 15c, the spectrum correspondent to geraniol shows that this compound owns a methylene group, an alkyne group, and an ether group. Accordingly with Wany et al. (2013) the geraniol's spectrum have similarities. In Figure 15d, the spectrum correspondent to pullulan shows that it owns a bending oscillation of hydroxyl groups (observed frequently in the structure of polysaccharides), a methyl group, an alkene group, and an ether group. In Haghighatpanah et al. (2020) it is possible to see a similar pullulan FTIR analysis.
Comparing the FTIR spectra of the films' components with the spectra of each film is possible to conclude that pullulan spectra and Pu spectra are the same, Pu40 spectra just has a different peak comparing to Pu spectra (marked with an arrow in Figure 16e) that could be associated with Tween 40 spectra. This situation could be seen in Figure 16g with the same peak associated to Tween 40. In Figure 16h there is a different peak too comparing with Pu spectra, but this peak isn't associated with geraniol, because geraniol's FTIR spectra do not present that peak.

4.4.2. Differential Scanning Calorimetry (DSC)

It is possible to detect changes in the physical and chemical properties of materials as a function of temperature using the differential scanning calorimetry. To put it another way, below the glass transition temperature (Tg), a material is rigid and fragile, whereas above it, it becomes more

flexible and brittle. The Tg is the temperature at which a material undergoes a structural transition from a solid, amorf state to one that is more viscous and elastic (Luís et al., 2020).
The thermal profiles of pullulan films with (PuGer40) or without (Pu) geraniol incorporated were evaluated by DSC (Figure 17).

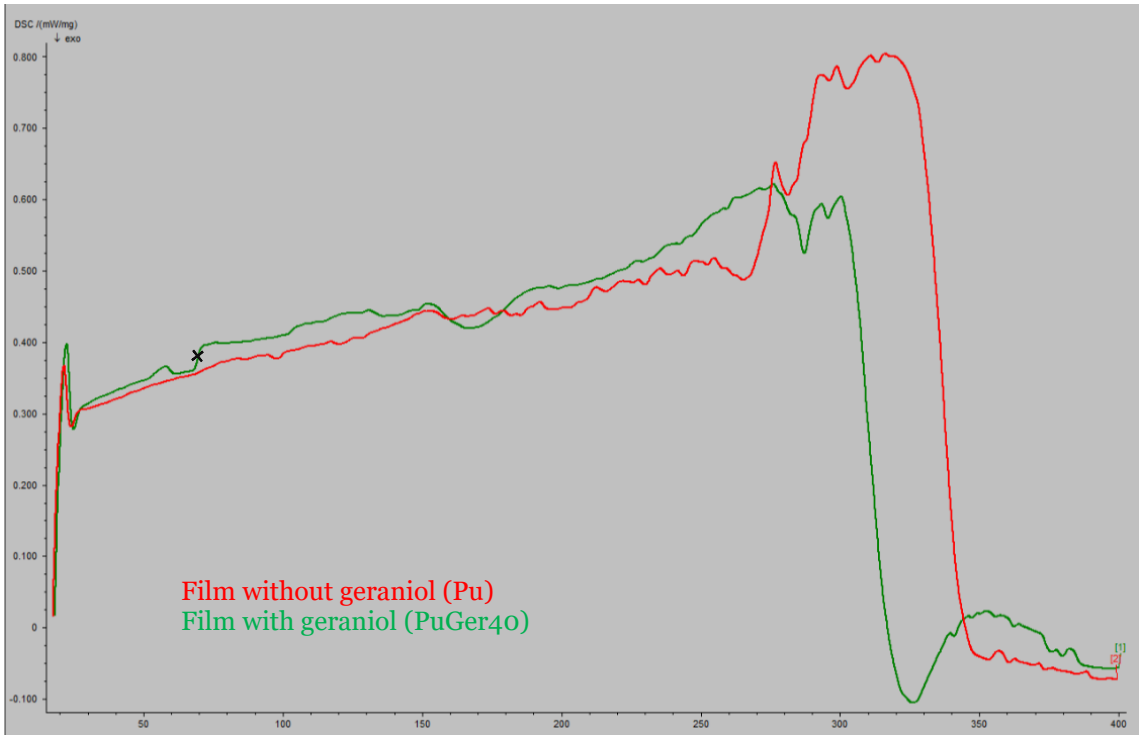


Figure 17: DSC thermograms of the pullulan-based films.

The stablished pullulan glass transition temperature is 154.5 ºC. The DSC thermogram shows that the glass transition temperature is 71 ºC (signed with a cross). This transition are ends in a baseline. The peaks that appear bellow this baseline represent exothermic reactions and the peaks above the baseline represent endothermic reactions.
The decrease of the pullulan glass transition temperature from 154.5 ºC to 71 ºC means that geraniol present a plasticizing effect on the produced films.
In accordance with the results presently obtained, the plasticizing effect of the essential oil was observed in Luís et al. (2020) because its incorporation caused a decrease in Tg in the produced pullulan films.

4.4.3. Grammage, Thickness and Mechanical Properties

Young's modulus, elongation and other mechanical properties are influenced by the construction of the film as well as its constituent parts (M. Vieira et al., 2011). Because packaging films are designed to endure external forces while preserving their integrity, these characteristics are crucial. By measuring these properties, it is possible to forecast how the material would react

under various food processing circumstances and to compare it with commercial polymers (Cazón et al., 2017).

The results obtained for grammage, thickness and mechanical properties assays executed to all produced films are presented on the Table 6:

Table 6: Grammage, thickness and mechanical properties of all produced films. (Results presented as mean ± standard deviation; *indicates a significant result).

	PuGer40[a]	**Pu[b]**	**Pu40[c]**	**PuGer[d]**	***P*-values**
Grammage (g/m²)	93.27 ± 3.81*	83.56 ± 3.39	97.24 ± 5.56*	85.93 ± 6.63	<0.001[ab*] <0.001[cb*] 0.360[db]
Thickness (µm)	79.84 ± 18.67*	61.43 ± 14.48	84.24 ± 17.35*	59.26 ± 13.42	0.001[ab*] <0.001[cb*] 0.610[db]
Tensile Strength (N/m)	1022.09 ± 69.15*	2315.61 ± 60.46	1043.13 ± 85.65*	1763.41 ± 277.49*	<0.001[ab*] <0.001[cb*] 0.020[db*]
Elongation (%)	0.80 ± 0.08*	1.28 ± 0.07	0.92 ± 0.13*	1.23 ± 0.10	<0.001[ab*] <0.001[cb*] 0.440[db]
Elastic Modulus (MPa)	2375.66 ± 364.87*	3943.34 ± 132.00	2241.93 ± 113.26*	3126.90 ± 353.66*	<0.001[ab*] <0.001[cb*] 0.010[db*]

The grammage and thickness are higher in the films containing Tween 40, which is probably due to the foam production in the homogenization process. Although the vacuum cycle, some films keep bubbles in their composition.

Films are stiff in terms of their mechanical properties, hence plasticizers are required to increase their flexibility (Sun et al., 2018). The tensile strength of all films with Tween 40 decreased compared to Pu and PuGer films, so this means, as explained before, that the responsible is the foam formation. The same situation was verified in elongation and in the elastic modulus parameters. These results could be compared with a previous work with pullulan plasticized films where in some conditions, the tensile strength, elongation and elastic modulus also decreased with the nanocrystals addition (Kristo & Biliaderis, 2007).

4.4.4. Contact Angle and Surface Free Energies

Through the measurement of the contact angle, the interaction between a surface and a certain liquid can be examined. This is defined as the angle between a plan parallel to a liquid drop and a plan containing the surface where the liquid is deposited (Rabockai, 1979), schematized in Figure 18. Smaller water contact angle values (<90º) indicate a material's hydrophilic nature, which is characterized by a strong affinity of water molecules for the substrate. Materials with active polar functional groups facilitate the adsorption of water molecules in these materials. The hydrophobic nature of the surface is shown by the greater water contact angle (>90º) (Hebbar et al., 2017).

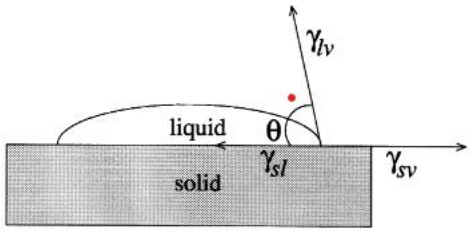


Figure 18: Schematic of a sessile drop contact angle system, where θ is the contact angle, γ_{SV} and γ_{LV} are the surface energy of the solid and the liquid's surface tension when it is in equilibrium with the vapor, respectively; γ_{SL} is the solid-liquid interface energy (Kwok & Neumann, 1999).

Using a liquid, as an example, can make the idea of surface energy easier to understand. Atoms and liquid molecules are free to move to occupy a position with lower potential energy. A location where they and the forces—both attractive and repellent—acting in all directions are in balance. On the other hand, the forces that the particles on the material's surface experience are just those that are directed toward the liquid (Figure 19). This results in surfaces always being higher energy regions. And precisely this difference between species' energies in the material's surface and inside is what gives rise to the terms "energy of surface" or "interfacial tension" (Rabockai, 1979).

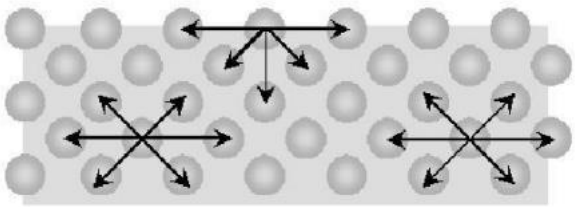


Figure 19: Forces acting on atoms or molecules, inside and outside, of a material (Burkarter, 2010).

The results obtained in this assay using the method described in chapter 3.5.4. are showed above:

Table 7: Contact angle (°) measurements using three different liquids. (Results presented as mean ± standard deviation).

	Water	Diiodomethane	Ethylene glycol
Pu top	55.13 ± 6.05	36.78 ± 3.50	37.87 ± 3.24
Pu bottom	56.86 ± 0.84	34.15 ± 2.95	42.04 ± 2.33
PuGer top	64.34 ± 7.24	45.72 ± 1.62	42.67 ± 1.51
PuGer bottom	73.08 ± 5.41	34.44 ± 3.47	42.21 ± 2.67
Pu40 top	45.79 ± 5.90	20.28 ± 1.72	37.89 ± 1.50
Pu40 bottom	44.19 ± 6.23	26.61 ± 2.12	37.03 ± 2.18
PuGer40 top	41.15 ± 5.01	21.68 ± 2.29	37.27 ± 1.55
PuGer40 bottom	38.42 ± 3.11	21.82 ± 3.40	37.72 ± 1.35

The experiments conducted with a water droplet, presented in Table 7, shows that both faces of each film have a hydrophilic behavior because all values are smaller than 90°. Measurements were performed in both surfaces because the ideal package have different characteristics in top and bottom faces to absorb or release substances from the inside to the outside, or the opposite. In this study, bottom and top faces have similar behaviors for water, diiodomethane and ethylene glycol. The addition of geraniol to the Pu films, increased the water contact angle value. It can be explained because of the hydrophobic nature of geraniol that keeps the droplets less dispersed. However, when this addition is made with Tween 40, the water contact angle decreases because geraniol is in emulsion, losing its hydrophobic characteristic. In other hand, with an apolar liquid like diiodomethane, the contact angle decreases with the addition of Tween 40 in the Pu40 films and with the addition of geraniol and Tween 40 in the PuGer40 films compared to Pu films. With only the addition of geraniol in PuGer films compared to Pu films, the contact angle remains quite similar.

The sum of the polar and dispersive components can be used to represent the total surface free energy of the solid and liquid phases (Ruckenstein & Lee, 1987). In this work, we applied the OWRK method, which calculates a solid surface's total energy as the sum of all interactions at the

solid/liquid interface, divided into two contributions: polar and dispersive resulting from London interactions (Encinas et al., 2010).

Table 8: Surface free energies. (Results presented as mean ± standard deviation).

	Dispersive component (mN/m)	Polar component (mN/m)	Total surface free energy (mN/m)
Pu top	40.25 ± 1.64	5.23 ± 1.05	45.48 ± 1.95
Pu bottom	38.87 ± 1.23	8.45 ± 0.08	47.32 ± 1.47
PuGer top	36.50 ± 0.87	4.51 ± 0.56	41.01 ± 1.03
PuGer bottom	41.95 ± 1.57	2.80 ± 0.72	44.74 ± 1.72
Pu40 top	47.52 ± 0.51	2.15 ± 0.29	49.66 ± 0.58
Pu40 bottom	45.15 ± 0.79	3.38 ± 0.51	48.53 ± 0.94
PuGer40 top	46.69 ± 0.72	2.76 ± 0.36	49.44 ± 0.80
PuGer40 bottom	44.57 ± 1.03	3.83 ± 0.46	48.40 ± 1.13

This study indicated that the dispersive component's value was higher than the polar component, however, there was no apparent difference in the ratio of the two components amongst the various kinds and faces. Strong correlations suggest that the surfaces have a lot of chemical similarities. In a study with pullulan films containing rockrose essential oil, the pullulan-based films' total surface free energy and corresponding polar component were significantly reduced (P-value 0.05), but the dispersive component increased (P-value 0.05). These outcomes are also connected to the rockrose essential oil hydrophobicity (Luis, Ramos, et al., 2020). In other hand, in the present study, the addition of geraniol in the PuGer40 film compared with the Pu film conduct to an increase of the dispersive component and of the total surface free energy. However, the polar component has decreased.

4.4.5. Optical Properties

The optical properties are one of the most important characteristic of the films. As a consumer, the first thing that we evaluate and take opinions of is the appearance of the packaging so in the film's production it is privileged the transparency, brightness, homogeneity, and the facility to see and assess the products inside of the package.

The results of the color coordinates and transparency can be seen in Table 9 and remembering the meaning of the parameters L* (lightness), a* (redness; ± red-green) and b* (yellowness, ± yellow-blue), it is possible to verify that the addition of Tween40 have not negatively affected the

color coordinates in comparison to the Pu values, an interesting conclusion as long as Tween40 is a detergent with greasy appearance. The addition of geraniol to the Pu films have not affected the color coordinates, similar results were found by Agarwal et al. (2020); however, when adding geraniol together with Tween40, the values turn into more light than in Pu or PuGer. It can be explained because Tween40 is an emulsifier and it can make the PuGer40 films more homogenous than PuGer, increasing the Lightness.

Table 9: Color coordinates and transparency of each type of film. (Results presented as mean ± standard deviation; *indicates a significant result).

	PuGer40[a]	**Pu[b]**	**Pu40[c]**	**PuGer[d]**	***P*-values**
L*	94.56 ± 0.19*	93.12 ± 0.23	94.64 ± 0.31*	92.94 ± 0.42	<0.001[ab*]
					<0.001[cb*]
					0.560[db]
a*	1.66 ± 0.01*	1.84 ± 0.02	1.64 ± 0.05*	1.84 ± 0.06	<0.001[ab*]
					<0.001[cb*]
					1.000[db]
b*	-6.68 ± 0.03*	-7.30 ± 0.06	-6.68 ± 0.15*	-7.28 ± 0.28	<0.001[ab*]
					<0.001[cb*]
					0.920[db]
Transparency (%)	90.41 ± 0.50*	95.25 ± 0.53	89.36 ± 0.65*	95.59 ± 0.76	<0.001[ab*]
					<0.001[cb*]
					0.760[db]

In Table 9, we verify that the addition of geraniol is the one that did not affect the transparency values, because without the addition of Tween40 (emulsifier), geraniol may evaporate during the drying time. According with Trinetta et al. (2011), that added glycerin with decreasing of transparency, in this study the addition of Tween40 also decreases the transparency values, with or without geraniol incorporation.

In summary, L* and Transparency values are >85% and a* and b* were not high or they were negative so we can conclude that all types of films have great optical properties. The negative b values suggest a tendency to the blue color, this values can be probably explained because of the bubble's formation.

4.4.6. Antioxidant Activity

In this section are presented the results from films' antioxidant activity measured by DPPH and β-Carotene assays.

4.4.6.1. DPPH Free Radical Scavenging Assay

As previously mentioned, it is believed that antioxidants are essential nutraceuticals. To compare the results of different laboratories and to verify the conclusions, a standard test is essential. One nitrogen atom's bridge is home to an unpaired valence electron, creating the stable free radical DPPH (Sharma & Bhat, 2009).

Table 10: % of inhibition of the films' antioxidant activity in DPPH assay. (Results presented as mean ± standard deviation).

	1/2 hour	1 hour
Pu	0.00	0.00
Pu40	0.00	0.00
PuGer	0.02 ± 0.001	0.11 ± 0.01
PuGer40	0.13 ± 0.02	0.16 ± 0.03

The antioxidant % were considered null in films Pu and Pu40 because they have not antioxidant compounds in their composition. It can be said that the addition of Tween 40 to the PuGer induced the increase of the antioxidant activity, because the absence of the emulsifier may cause the geraniol evaporation during films drying. With the emulsifier, all the geraniol properties are kept in the film. The time exposition also influences the antioxidant activity, exposing films to DPPH radicals for one hour allowed better antioxidant activity.

By incorporating essential oils, pure k-carrageenan film's antioxidant activity was significantly increased. The antioxidant properties of the films were improved by adding more essential oils content (Shojaee-Aliabadi et al., 2014).

4.4.6.2. β-Carotene Bleaching Test

The principle of this assay is equal to the one developed to the β-carotene assay to evaluate the antioxidant activity of geraniol.

Table 11: % of inhibition of β-carotene bleaching test in the different types of films. (Results presented as mean ± standard deviation).

	Pu	Pu40	PuGer	PuGer40
% Inhibition	0.00	0.00	37.01 ± 7.26	35.25 ± 2.67

In Table 11 are presented the results of the percentage of inhibition for each type of film. In films Pu and Pu40 the percentage of inhibition is null because these films do not have antioxidant compounds in its composition.

In the other two types of films, it was possible to observe that the addition of Tween 40 has a diminishing effect between PuGer and PuGer40. In terms of explanation, the emulsify power of

Tween 40 could prevent the release of geraniol from the films to the involving solution, decreasing its antioxidant activity.

In a previous study with rosemary essential oil using the β-carotene bleaching assay to compare two types of this essential oil with butylated hydroxytoluene (BHT), a synthetic antioxidant, showed that rosemary has a significant decrease in the percentage of inhibition (Yeddes et al., 2019). The results obtained in the present study compared with the one using rosemary show a % of inhibition almost three times bigger in some cases.

4.4.7. Evaluation of the Antibacterial and Anti-Quorum Sensing Activities of the Films

The antibacterial agents have the ability to stop or slow down the microbial growth that causes fast food spoilage and disease transmission (Majhi et al., 2019). Given that geraniol has antimicrobial activity, the antimicrobial activity of the films was also evaluated. The antibacterial properties of the films were evaluated against six foodborne pathogens (*Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* LMG 16779, *Staphylococcus aureus* ATCC 25923, *Salmonella* Typhimurium ATCC 13311, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922) with the solid diffusion assay. The anti-QS activity of the films was evaluated using the biosensor strain *Chromobacterium violaceum* ATCC 12472.

4.4.7.1. Solid Diffusion Assay

The results of this assay are present in Table 12 and 13:

Table 12: Measurements of the inhibition rings in millimeters. EF: *Enterococcus faecalis* ATCC 29212; LM: *Listeria monocytogenes* LMG 16779; EC: *Escherichia coli* ATCC 25922; ST: *Salmonella* Typhimurium ATCC 13311; PA: *Pseudomonas aeruginosa* ATCC 27853; SA: *Staphylococcus aureus* ATCC 25923. (Results presented as mean ± standard deviation).

	EF	LM	EC	ST	PA	SA
Pu	0.00	0.00	0.00	0.00	8.58 ± 0.66	0.00
Pu40	17.73 ± 2.33	0.00	0.00	0.00	13.33 ± 2.33	6.00
PuGer	0.00	0.00	0.00	0.00	6.56 ± 0.49	0.00
PuGer40	15.19 ± 0.66	0.00	0.00	0.00	10.99 ± 1.82	0.00

Initially, the measurements made in the Petri dishes will be explained and analyzed and after that the results will be compared with the pictures obtained in the optical microscope.

In the *Enterococcus faecalis* column, only Pu40 and PuGer40 had an inhibition zone. Comparing with the pictures, the results are different. In a naked eye measure, only the ones that inhibit colonies turning them into single bacteria (Pu40 and PuGer40) had a significant and measurable inhibition zone. The size of the zone is quite similar.

The obtained results for *Listeria monocytogenes* showed that no inhibition zones were observed for this strain, however the microscopy amplification has enabled the view of a reduced activity ring.

An equal situation occurred in *Escherichia coli*, in a naked view to measure the inhibition zones it was not possible. Whereas, when observed in microscopy with a 200× amplification, this zone becomes visible.

In *Salmonella* Typhimurium case, there were no inhibition zones and the microscope images showed colonies in all types of films. So, both results are correspondent.

In *Pseudomonas aeruginosa* case, only one microscopical image do not present an inhibition zone, although all film discs have a measure of the inhibition zone.

Comparing the diameters of inhibition zones of *Staphylococcus aureus* it was possible to conclude that they are in concordance with the microscopy images. The pictures showed microbial colonies in all types of films with no inhibition zones.

With their primary constituents being linalool, thymol, and carvacrol, respectively, natural antimicrobial agents including basil, thyme, and oregano essential oils are well adapted to be used as food preservatives and as prospective substitutes for synthetic food additives. The potential for applications in antimicrobial packaging systems that could lower the risk of food-borne illness related to microbial contamination in food products is demonstrated by packaging materials using antibacterial agents (Kuorwel et al., 2011). As far as we know, no studies with this assay have been realized with geraniol.

Table 13: Optical microscopy images of the antibacterial activity (Amplification: 100×). The blue lines and arrows delimitate the bacterial zones and the red lines contour the films disks.

Pseudomonas aeruginosa ATCC 27853

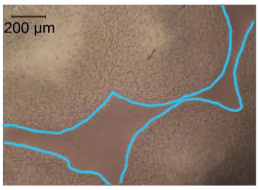
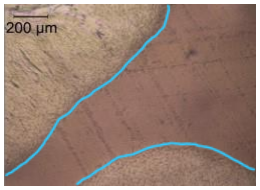
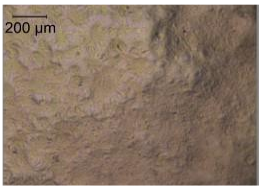
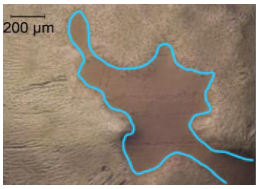
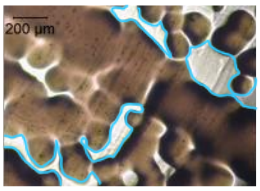
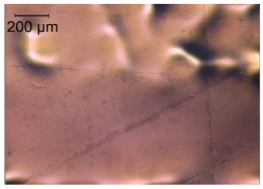
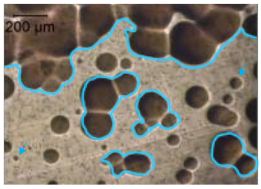
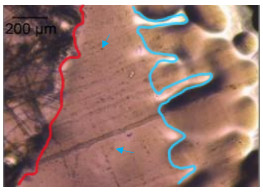
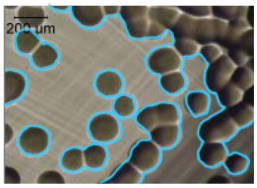
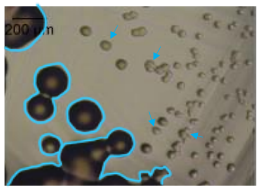
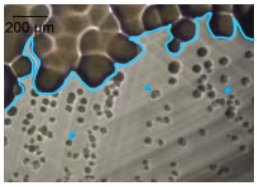
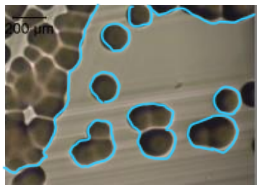
Pu	PuGer40	Pu40	PuGer
			

Table 13: Continuation.

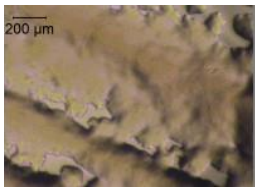
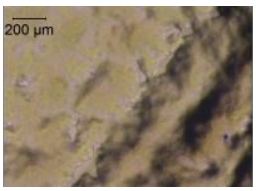
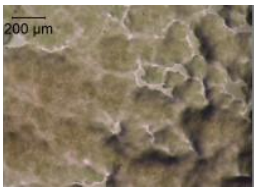
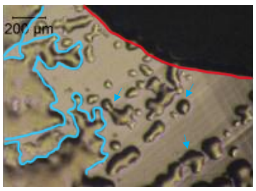
Staphylococcus aureus ATCC 25923

Pu	PuGer40	Pu40	PuGer
			

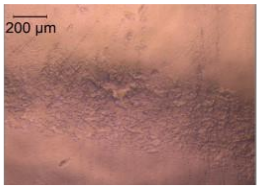
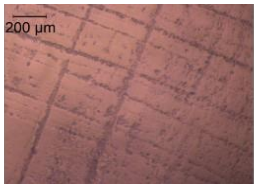
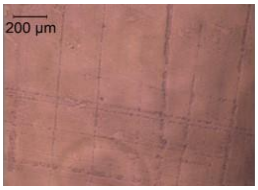
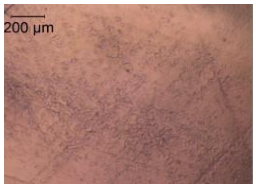
Enterococcus faecalis ATCC 29212

Pu	PuGer40	Pu40	PuGer
			

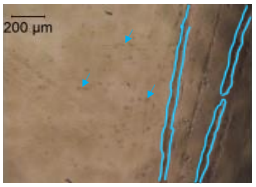
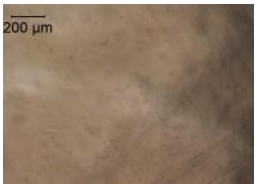
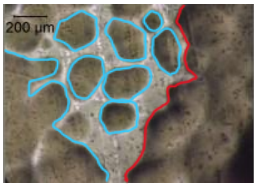
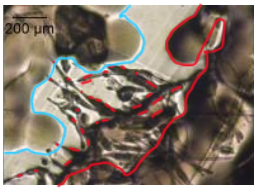
Listeria monocytogenes LMG 16779

Pu	PuGer40	Pu40	PuGer
			

Salmonella Typhimurium ATCC 13311

Pu	PuGer40	Pu40	PuGer
			

Escherichia coli ATCC 25922

Pu	PuGer40	Pu40	PuGer
			

The pictures correspondent to *Pseudomonas aeruginosa* appear with inhibition zones (marked with blue) in all types of films except in Pu40, where it is possible to see a homogenous layer of bacteria. There are not red lines marking the films because they get dissolved during the incubation.

In *Staphylococcus aureus* there is an inhibitory effect in all films. In PuGer40 film the presented inhibitory zone shows total bacterial inhibition. The Pu film presents some little and disperse inhibitory zones, probably because of a contact inhibition. About Pu40, there is a group of colonies far from the film area and when we get closer to the film we start having some dispersal colonies and single bacteria (marked with blue arrows). The film is only recognized in PuGer (marked with a red line), it did not get dissolved and similarly to Pu40, near the film there is only single bacteria and forward and homogenous number of colonies.

In *Enterococcus faecalis*, there is an antibacterial activity in all types of films and it was not possible to see the films in all pictures. Here, the activity is characterized by a change of growing by the film's neighborhood to the peripheral ring area. Near the films we are just capable to find single bacteria or very dispersal single and little colonies, as we get further away from the action area, the bacteria turn into colonies and the single colonies get bigger and in an increased number. Otherwise, in *Listeria monocytogenes* only show some inhibition activity in PuGer films (Table 13). In the other situations, we have a layer of this strain with almost total homogeneity and in PuGer it was possible to see the film and the antibacterial activity around even if little.

The film disks dissolved in all *Salmonella* Typhimurium tests and there was no inhibitory action, all pictures show microbial colonies.

At last, *Escherichia coli* with a middle situation. If on the one hand we have Pu and PuGer40 without activity and with the incapacity to detect the film disk, on the other hand Pu40 and PuGer films totally dissolved and show minimal inhibition zones, maybe another contact inhibition situation.

Comparing with the results obtained in geraniol's solid diffusion assay, it is possible to conclude that against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus faecalis*, geraniol has antibacterial activity in oil form and in the PuGer40 film structure. In *Salmonella* Typhimurium, *Escherichia coli, and Listeria monocytogenes* the results do not agree with the ones obtained in the assay performed with geraniol. Probably when in emulsion, geraniol has the difficulty of release, decreasing the antibacterial activity in this strains.

4.4.7.2. Anti-Quorum Sensing Activity

As geraniol showed anti-QS activity, we tested the anti-QS potential of the films and the results are presented as follows:

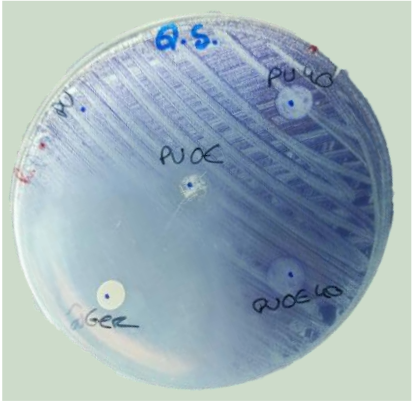


Figure 20: Quorum Sensing assay for each film type. Correction: PuOE-PuGer; PuOE40-PuGer40.

The Figure 20 reveals an absence of anti-QS activity of the different four types of films, with or without geraniol. In Akyuz et al. (2018) the control films had an % of violacein pigment inhibition under 20% that was increased because of the rise of the capsaicin (the active compound of peppers). The inhibition zones increased from 4 mm in the control to 14, 20 and 22 mm with the rise of capsaicin.

4.4.7.3. Anti-Biofilm Activity

Initially, biofilms were described as organized bacterial cell populations that adhered to inert or alive surfaces inside of self-produced polymeric matrix (Maifreni et al., 2015). Evidence suggests that anti-biofilm bacterial agents change biotic and abiotic surfaces as well as the physical characteristics of bacterial surfaces involved in processes of cell-to-cell adhesion and aggregation (Miquel et al., 2016).

In this study, the anti-biofilm activity of the films was evaluated against *Enterococcus faecalis* ATCC 29212, because in the antibacterial assays PuGer40 film showed great activity against this strain.

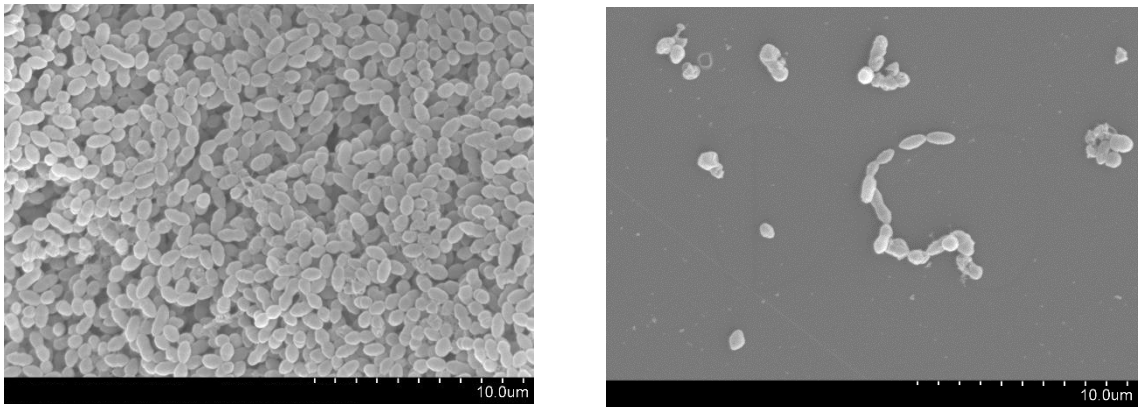


Figure 21: SEM images of the anti-biofilm activity.

In Figure 21 it is possible to see at left the SEM image of the Pu film and at right the SEM image of PuGer40 film. Looking to both pictures we are capable of conclude that the addition of geraniol,

as active agent, and Tween40, as emulsifier, increase the anti-biofilm activity of pullulan films. With the Pu film, the *Enterococcus faecalis* biofilm grown with a regular structure and in a considerable quantity. In the PuGer40 film, the bacteria number decreased substantially and the biofilm conformation and structure appear compromised. Similarly to Khan et al. (2020), where they studied the anti-biofilm activity of chitosan, it was proved that charged interactions alter the permeability of the cell membrane and cause cytoplasmic material to flow out, which ultimately results in cell death.

4.4.8. Barrier Properties

In order to maintain the integrity of the food throughout the entire handling process until it reaches the ultimate consumer, one of the barrier properties of the food materials is its ability to create a protective atmosphere at the food's exit (Luís et al., 2020).

In this section, the results of oil permeability and water vapor permeability assays will be analyzed. Strong barrier qualities are a reliable predictor of eco-friendly packaging materials over time (Luis et al., 2021).

4.4.8.1. Oil Permeability

For oil permeability (g.mm/m².day) the results obtained are presented in the Table 14, determined with the weight difference of the filter paper (before and after vegetal oil exposition), the thickness of the films, the effective contact area, and the storage time (24 h) (Luis et al., 2021).

Table 14: Oil permeability results for each film.

	PuGer40[a]	**Pu[b]**	**Pu40[c]**	**PuGer[d]**	***P*-values**
OP (g.mm/m².day)	87.82 ± 8.40*	9.38 ± 0.99	41.42 ± 5.13	9.64 ± 1.42	0.040[ab*] 0.060[cb] 0.850[db]

In a first sigh it is clear that OP of Pu films and PuGer films are almost the same, explained by the absence of Tween 40. Without emulsifier, geraniol may evaporate during casting having very similar characteristics with Pu films. Although, the addition of Tween 40 and geraniol increase the OP of the films, these two components enlarged the free volume between the pullulan molecules, which made oil permeate easily, in concordance with (Luis et al., 2021).

4.4.8.2. Water Vapor Permeability (WVP)

When assessing the practical uses of functional films, water permeability is a crucial factor. A polymeric film's barrier qualities are essential for calculating or projecting the product's shelf life. Plastics typically have a low barrier to tiny molecules like gases, water vapor, organic vapors, or

liquids. These substances might penetrate the polymer package wall from the interior or exterior environment, causing a constant change in the quality of the product and shortening its shelf life (Siracusaa, 2008).
The results of this assay are shown in Table 15.

Due to the hydrophobic nature of geraniol, it was expected that the films with geraniol presented lower water permeability. However, the results showed that the addition of geraniol increases the WVP, but not significantly.

Table 15: Water vapor permeability.

	PuGer40[a]	**Pu[b]**	**Pu40[c]**	**PuGer[d]**	***P*-value**
WVTR (g/(m².day))	71.94 ± 0.56	80.29 ± 7.87	67.17 ± 2.81	97.78 ± 10.12	0.370[ab]
					0.230[cb]
					0.200[db]
WVP (g/(Pa.day.m)) (×10⁻⁶)	4.35 ± 0.03	3.73 ± 0.37	4.28 ± 0.18	4.38 ± 0.45	0.250[ab]
					0.240[cb]
					0.260[db]

These WVP results could be interpretated by forming a parallelism with the thickness results. PuGer film is thinner than Pu film so it could explain the rise of WVP. Although in Pu40 films and PuGer40 films, the thickness values are higher than Pu films results and the WVP keeps higher too. It demonstrates that the films' water barrier qualities were weakened by the foam. In a previous study with pullulan films addicted with rice wax (a hydrophobic substance like geraniol) the increase of wax, declined the WVP in contrast with the present study (Shih et al., 2011).

4.4.9. Biodegradability

One of the primary requirements for new packaging materials that seek to replace those made of synthetic polymers is their ability to biodegrade (Qamar et al., 2020). Pullulan films due to pullulan characteristics are biodegradable with multiple blends and composites (Tabasum et al., 2018).
In this study, strips of each type of film were buried in a sample of soil and immersed in sea water for 24 h with a previous measure of their mass. The amount of films biodegradability was calculated by the difference between initial and final films mass, however, after the 24 h of contact of the films with both environments, the % of biodegradability was 100%. The films' strips completely degraded in soil and sea water.

5. Conclusions

Different plastic products have different service lifetimes, which affects how long plastics are used as stocks and when those stocks become plastic garbage. It is commonly known that plastic pollution places a high risk on human health globally as well as a threat to ecosystems all over the world.

For this reason, it is crucial to develop solutions that function at the production level with biobased-alternatives, such as bioplastics or films made with biopolymers, as no solution to address this issue is 100% effective.

In the present work, a film made using pullulan, a biopolymer, was developed with the incorporation of geraniol as an antioxidant and antibacterial agent. The studies performed revealed that the incorporation of geraniol into the pullulan films enrich their properties like described below.

The mechanical properties of the films were influenced by the geraniol addition. The grammage and thickness increased, and the tensile strength, elongation and the elastic modulus decreased.

About the optical properties, the films presented great transparency, that is a major factor in the consumers acceptance.

The PuGer40 film appears with weak barrier properties, the oil permeability increased because of the enlarge of the free volume between pullulan molecules. The geraniol addition increased the WVP but not significantly compared with Pu film.

Both faces of the produced films are hydrophilic with a contact angle smaller than 90º.

The films have promising antibacterial activity proved in the several experiments performed.

At last, the biodegradability was evaluated and the films get completely degraded in soil and sea water after 24 h.

Although the promising results, more tests should be performed. We suggest applying the developed films in a food model to verify the films' characteristics in a real situation, as a future perspective. Another crucial aspect would be to investigate the geraniol's release into the food using food simulants. It would be interesting to research the process of scaling up the production of these new materials for industrial application in the future introduction to the market.

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