# Food Coating 2.0: Optimization of Sustainable and Natural Food Coatings

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*This thesis does not contain the suggestions proposed by the jury.*

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**Acknowledgments**

I thank God for having given man the faculty of reasoning and to exploit the varieties of the universe.

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I dedicate this work to my family, especially to the dearest people in the world, my father and my mother, who are the light of my eyes, shadow of my steps and the happiness of my life. Who have supported me during all my years of study, for these sacrifices and support, and who give me tenderness, confidence, courage, and security.

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**List of abbreviations**

**AMP** Ampicillin

**STR** Short tandem repeats

**CDD** Central composite design

**BB** Box-Behnken design

**DMSO** Dimethyl sulfoxide

**DPPH** 2,2-diphenyl-1-picrylhydrazyl

**E392** Food additive code (extracts of rosemary)

**$EC_{50}$** Effective concentration 50%

**EOs** Essential oils

**INT** Iodonitrotetrazolium chloride

**KET** Ketoconazole.

**MBC** Minimum bactericidal concentration

**MEB** Malt extract broth

**MET** Methicillin

**MFC** Minimum fungicidal concentration

**MIC** Minimal inhibition concentration

**PBS** Phosphate-buffered saline

**PCS** Phosphorylated chitosan

**ROOH** Organic hydroperoxide

**ROS.CON** Rosemary extract

**TOC** α-Tocopherol

**CA** Citric acid

**RSM** Response surface methodology

**AP** Ascorbyl palmitate

**TBARS** Thiobarbituric acid reactive substances

**TSB** Standardized tryptone soy broth

**TTP** Tripolyphosphate

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**Abstract**

Synthetic film manufacturing used in food packaging has increased dramatically in recent decades, raising major environmental issues due to the resistance of synthetic plastics to degradation. Biopolymeric polymers as raw material for food packaging and preservation have been the subject of research. As a result, the scientific community has been working to develop novel materials for edible and biodegradable films, based primarily on renewable and abundant natural resources. The objective of this study was to enhance the antioxidant and antimicrobial properties of a food coating solution using a Box-Behnken experimental design to optimize the bioactive properties. The three ingredients investigated were rosemary extract, α-tocopherol and ascorbic acid. The findings revealed that the coating solutions did not display much antimicrobial activity against Gram-negative bacteria at the highest tested concentrations. However, for Gram-positive bacteria, the coating solutions containing only rosemary extract exhibited the most effective antimicrobial potential, specifically against *Bacillus cereus* and *Staphylococcus aureus*. Notably, higher concentrations of ascorbic acid demonstrated superior results against *Listeria monocytogenes*. The minimum inhibitory concentrations for different bacteria revealed distinct behaviors, with a linear model describing the response of *B. cereus* and quadratic models being more appropriate for *L. monocytogenes* and *S. aureus*. The influence of individual ingredients varied, with ascorbic acid significantly affecting *B. cereus*, while α-tocopherol and ascorbic acid played a more substantial role in inhibiting *L. monocytogenes*. Regarding the antioxidant activity, the experimental design successfully optimized the combination of rosemary extract, α-tocopherol, and ascorbic acid, yielding a well-fitting quadratic model with strong performance. The optimal blend for maximal antioxidant activity consisted of 0.14 g/100 mL rosemary extract, 1.81 g/100 mL α-tocopherol, and 1.66 g/100 mL ascorbic acid. This study emphasized the significance of each ingredient in both the antimicrobial and antioxidant properties of the coating solution and provided valuable insights into their individual and combined effects.

Keywords: Biofilms, antioxidant polymers, food packaging, α-tocopherol, *Lysteria monocytogenes*, response surface methodology.

**Resumo**

O fabrico e o uso de filmes sintéticos em embalagens alimentares aumentaram drasticamente nas últimas décadas, levantando importantes questões ambientais devido à resistência dos plásticos sintéticos à degradação. Assim, os biopolimeros como matéria-prima para embalagens e conservação de alimentos têm sido alvo de investigação. A comunidade científica tem reunido esforços para desenvolver novos materiais para filmes comestíveis e biodegradáveis, baseados em recursos naturais, renováveis e abundantes. O objetivo deste estudo foi o incremento das propriedades antioxidantes e antimicrobianas de uma solução de revestimento alimentar com recurso a um planeamento experimental Box-Behnken, utilizando extrato de alecrim, o α-tocoferol e ainda o ácido ascórbico. Os resultados revelaram que as soluções de revestimento não apresentaram uma boa atividade antimicrobiana contra bactérias Gram-negativo e fungos na maiores concentrações testadas. Para bactérias Gram-positivo, as soluções contendo apenas extrato de alecrim exibiram maior potencial antimicrobiano, especificamente contra *Bacillus cereus* e *Staphylococcus aureus*. Notavelmente, concentrações mais altas de ácido ascórbico demonstraram resultados superiores contra *Listeria monocytogenes*. As concentrações inibitórias mínimas para diferentes bactérias revelaram comportamentos distintos, em que o modelo linear descreveu a atividade de *B. cereus* e os modelos quadráticos revelaram-se apropriados para o comportamento de *L. monocytogenes* e *S. aureus*. A influência de ingredientes individuais foi variável; o ácido ascórbico inibiu significativamente *B. cereus*, enquanto o α-tocoferol e ácido ascórbico inibiram *L. monocytogenes*. Em relação à atividade antioxidante, o desenho experimental otimizou com sucesso a combinação de extrato de alecrim, α-tocoferol e ácido ascórbico, produzindo um modelo quadrático bem ajustado com forte desempenho. A mistura ideal para atividade antioxidante máxima consistiu em 0,14 g/100 mL de extrato de alecrim, 1,81 g/100 mL de α-tocoferol e 1,66 g/100 mL de ácido ascórbico. Este estudo enfatizou a importância de cada ingrediente nas propriedades antimicrobianas e antioxidantes da solução de revestimento e forneceu informações valiosas sobre seus efeitos individuais e combinados.

**Palavras-chave:** Biofilmes, Polímeros antioxidantes, Embalagens alimentares, α-tocoferol, *Lysteria monocytogenes*, Metodologia de Superfície de Resposta.

## Objectives

The primary aim of this proposal was to enhance the performance of a natural food coating solution by varying the combinations among the constituents and polymers by using the response surface methodology, thereby improving the antioxidant and antimicrobial activities. To accomplish this objective, numerous approaches were conducted, including antioxidant and antimicrobial analyses, by varying the contents of antioxidants in an optimized coating solution previously developed within SusTEC-IPB, called “SpraySafe”. Therefore, this proposal endeavors to delve deeply into the constituents and polymers of a natural food coating, employing response surface methodology, which will enable the optimization of the coating's various components, ultimately leading to elevated levels of both antioxidant and antimicrobial activities.

## 1. Introduction

The packaging and food industries have recently teamed up to reduce the amount of packaging materials used for food. The potential to prolong the shelf life of numerous agricultural products has been demonstrated via edible coatings. A food covering film that can be consumed along with the food is called an edible food coating. While wax films (beeswax, carnauba wax, etc.) and lipid or lipid derivative films have improved water vapor barrier properties, films based on polysaccharides (cellulose, starch, dextrin, chitosan and other gums, etc.) and proteins (gelatin, gluten, casein, etc.) based films have suitable mechanical and organoleptic properties. It gives particular qualities of food while preserving the quality of the coated food product by reducing the major sources of alteration through a variety of processes, such as preventing moisture losses, lowering the rates of unfavorable chemical reactions, and acting as barriers to gas exchange (Cristofoli *et al.*, 2023).

In the food sector, edible food and coatings have gained popularity because they produce less waste, are affordable, and provide protection once the container has been opened. The quality of food products depends on organoleptic, nutritional, and hygienic characteristics, but these evolve during storage and commercialization (Cha & Chinnan, 2004). These changes are primarily caused by changes between foods and surrounding media or migrations between the different components in a composite food, although the use of edible films in food products seems new, edible films and coatings were first used to cover food products many years ago (Debeaufort *et al.*, 1998). Therefore, if focus on edible films and coatings as packaging and food components, they must meet some requirements. They should have good sensory qualities, high barrier and mechanical efficiencies, enough biochemical, physicochemical, and microbial stability, be free of contaminants and safe for human health, simple technology, low cost of raw materials and processes, and be non-polluting (Yener, 2007).

This work aims to optimize the previous results of food coating 1.0 to a new enhanced and optimized food biofilms to improve antimicrobial activity and find the best balance in the mixture between antioxidant polymers and other components.

### 1.1. Polymers as food coatings

Synthetic film manufacturing and use in food packaging has increased dramatically in recent decades, raising major environmental issues due to the resistance of synthetic plastics to degradation (Muscat *et al*., 2012). Consumers currently want to lessen the ecological impact of food packaging by demanding biodegradable materials. Biopolymeric polymers as a raw material for food packaging and preservation have been the subject of research (Persin *et al*., 2011). Due to their ability to minimize moisture loss, fragrance loss, solute movement, water absorption in the food matrix, and oxygen penetration, edible and biodegradable films could be a viable alternative to synthetic packaging materials in a variety of applications (Aider, 2010; Dutta *et al.*, 2009).

As a result, food scientists and engineers are working to develop novel materials for edible and biodegradable films, based primarily on renewable and abundant natural resources. These materials are generally inexpensive, and several of them are considered waste or by-products (Kim *et al*., 2006). Disposable cutlery, drinking cups, lids, plates, wrap and lamination films, straws, stirrers, and containers for food distributed in gourmet food stores and fast-food places are currently examples of biodegradable polymer applications in the food sector (Siracusa *et al*., 2008).

A polymer is a big molecule (macromolecule) made up of structural units that repeat. Covalent chemical bonds are commonly used to join these subunits. Although both synthetic and natural polymers are accessible, natural polymers are preferred for food applications because they are cost-effective, easily available, and nontoxic. They can be chemically modified, are biodegradable, and, with a few exceptions, are biocompatible (Satturwar *et al*., 2003).

#### 1.1.1. Cellulose

Anselme Payen, a French scientist, discovered cellulose in 1838 after isolating it from plant matter and determining its chemical formula. Cellulose is an organic polysaccharide with formula $(C_6H_{10}O_5)n$ that is made up of a linear chain of hundreds to thousands of $\beta(1\rightarrow4)$ connected D-glucose units (Nishiyama *et al*., 2002). Plant cell wall

polysaccharides include mainly cellulose, hemicelluloses, and pectin (Scheller *et al.*, 2007).

Cellulose is the most prevalent organic polymer on the planet and is a fundamental structural component of the cell walls of higher plants. Many parallel cellulose molecules combine to produce crystalline microfibrils that are mechanically and enzymatically resistant. These are aligned with each other to give the cell wall structure. Cellulose is insoluble in water and cannot be digested by humans (Aquilera & Stanley, 1999; Cosgrove, 2005). Figure 1 represents the different cellulose derivatives, and Figure 2 represents the chemical structure of cellulose.

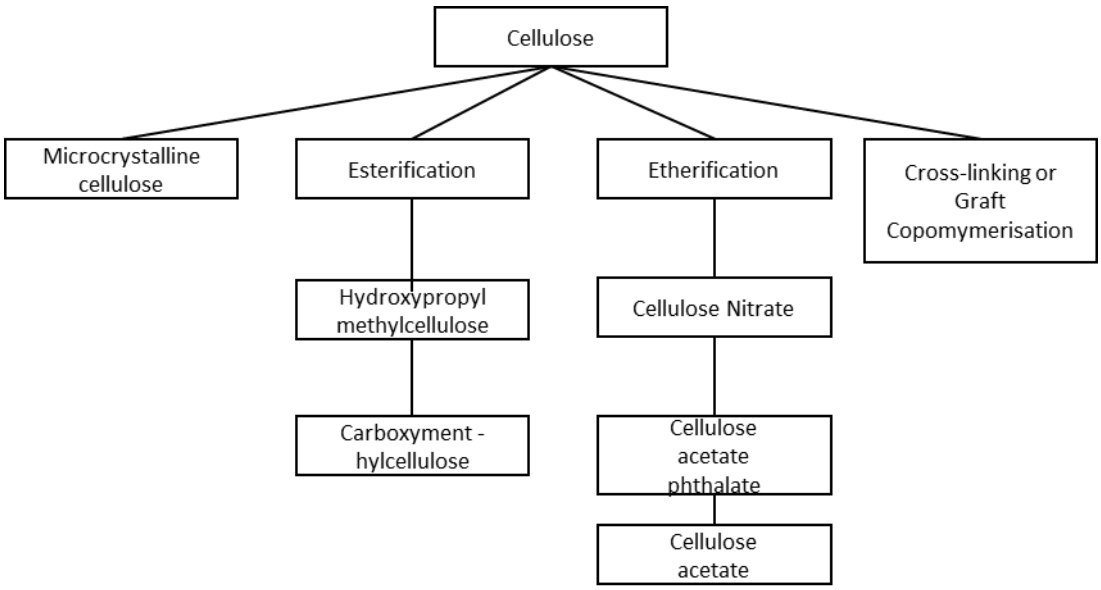



**Figure 1**. Cellulose derivatives (Vishakha & Kishor, 2012).

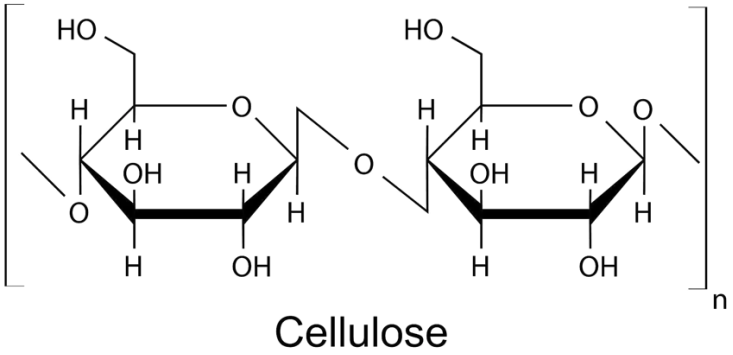



**Figure 2**. Structure of cellulose (Vishakha & Kishor, 2012).

The raw material for creating partially depolymerized cellulose is native cellulose. Microcrystalline cellulose and powdered cellulose are the end products. Additionally, the production of functional cellulose ethers such as methylcellulose, hydroxypropyl cellulose, cellulose gum, ethylcellulose, or hydroxypropylcellulose begins with highly purified cellulose in the form of pulps. Then, as technological additives, these partially or entirely water-soluble cellulose derivatives are added to a variety of foods. Rarely, if ever, is natural untreated cellulose used as a functional ingredient in food on its own; the only purpose it serves is as a filler. Cellulose serves as a processing aid in the filtering of beverages, which is an area of indirect application (Wuestenberg, 2014).

### 1.1.2. Starch

Starch, also known as amylum, is a carbohydrate made up of a large number of glucose units linked by glycosidic bonds. All green plants synthesize this polysaccharide to store energy. It is the most common type of carbohydrate reserve in green plants, with seeds and subterranean organs being particularly abundant. Granules of starch can be found (starch grains). Several starches have been approved for use in pharmaceuticals; these include maize (*Zea mays*), rice (*Oryza sativa*), wheat *(Triticum aestivum*), and potato (*Solanum tuberosum*) (Trease & Evans, 2002). Starch is made up of two polymers: amylose (a nonbranching helical polymer made up of 1,4 linked D-glucose monomers) and amylopectin (a highly branched polymer made up of both 1,4 and 1,6 linked D-glucose monomers) (Vishakha & Kishor, 2012). Figure 3 represents the chemical structure of amylose and amylopectin.

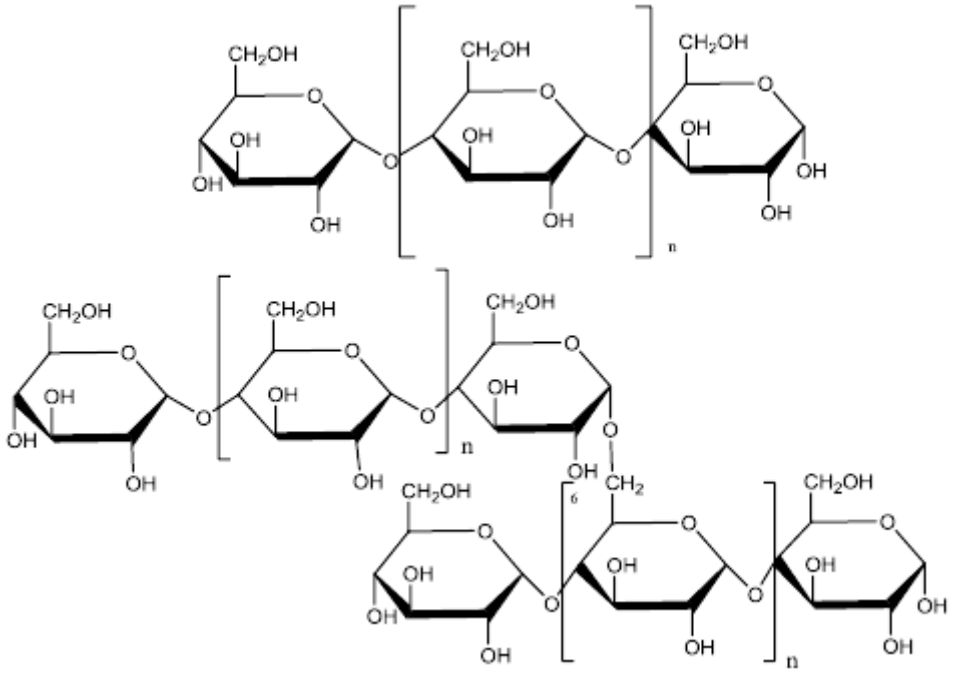


**Figure 3**. Structure of Amylose (above) Amylopectin (below)

Starch-based edible coatings improve the shelf life of coated fruits and vegetables by controlling the respiratory exchange ratio, preventing their natural senescence. As mentioned above, compared to ordinary synthetic materials, starch-based coatings show highly selective gas permeability ratios ($CO_2/O_2$), reduced gas exchange and gas transfer rates result from the changed atmosphere the coating creates, which causes physical capture of $CO_2$ inside the fruit or vegetable and partial closure of the pores (Versino *et al.*, 2016).

The importance of coating integrity depends on the flexibility, surface tension, and adherence of the film to the food product. Strong interactions between the polymer chains in matrices without plasticizer cause them to be hard and brittle, and they can also cause aggregates to develop. These structures might not work well with crooked surfaces, as those on some fruits. This issue can be resolved by adding plasticizers because they make the coating more flexible, High plasticizer concentrations impair barrier properties and may result in segregation from the matrix, hence the plasticizer/polymer ratio should be controlled (García *et al*., 1998).

### 1.1.3. Aloe Gel

Parenchyma tissue, which contains the mucilaginous gel, is found in the interior section of the leaves of Aloe Vera (L.) Baum. f. (*Aloe barbadensis*) (Ni *et al*., 2004). The acetone precipitate was crushed directly in matrix systems containing diclofenac sodium as a model medicine after extraction of the Aloe Vera gel from the leaves and a filtration step. The mucilage created direct compressible matrix tablets with good swelling and long-lasting drug release (Jani *et al*., 2007). Polysaccharides found in the gel of Aloe Vera leaves are believed to be responsible for many of the health benefits. Wound healing, antifungal activity, hypoglycemia or antidiabetic effects, anti-inflammatory, anticancer, immunomodulatory, and gastroprotective qualities are among these biological actions (Vishakha & Kishor, 2012).

These effects include the capacity of Aloe Vera whole leaf or inner fillet gel liquid solutions to improve intestine absorption and bioavailability of co-administered substances, as well as skin permeation. Important medicinal applications, such as the use of dried Aloe Vera gel powder as an excipient in long-acting pharmaceutical dosage forms, are also available (Josias & Hamman, 2008).

Although aloe vera gel has strong antioxidant and antimicrobial properties, it has not been used frequently in the formulation of edible films and coatings because it does not form films well enough (Pinzon *et al*., 2018). Therefore, the aloe vera gel film may have low barrier properties and some water permeability. To enhance the quality of the film, additional substances with desirable properties for film formation, such as starch, cellulose, gelatin, gellan gum, etc., have been added to the aloe vera solution (Alvarado-González *et al*., 2012). Figure 4 represents the structure of Aloin according to the National Center for Biotechnology Information.

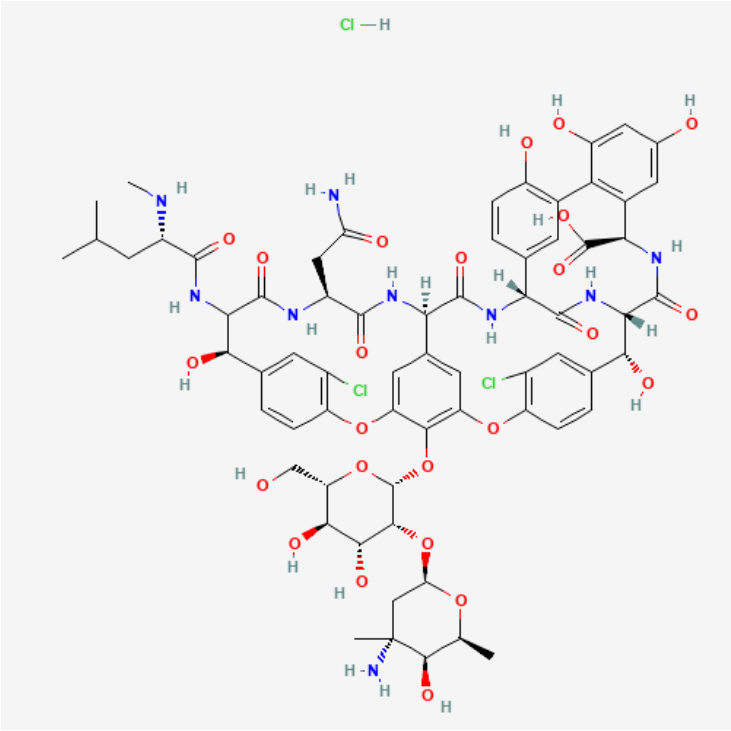


**Figure 4**. Structure of Aloin (National Center for Biotechnology Information ,2023)

### 1.1.4. Chitin

Chitin is a polysaccharide derivative with amino and acetyl groups that are the most prevalent organic constituent in invertebrates' skeletal structure. Mollusks, annelids, and arthropods all contain it, as well as mycelia and spores of various fungi (Kokate *et al.*, 2003). Phosphorylated chitosan (PCS)-based polyelectrolyte complex gel beads were produced for the regulation of the release of ibuprofen in oral administration. Using an ionotropic gelation with counter polyanion, tripolyphosphate (TPP), at pH 4.0, PCS gel beads were easily made from soluble phosphorylated chitosan. The PCS gel beads had a high concentration of ibuprofen, more than 90%. The percentages of ibuprofen released from PCS gel beads increased as the pH of the dissolution liquid increased (Phyu *et al.*, 2003). Figure 5 represents the structure of chitin.

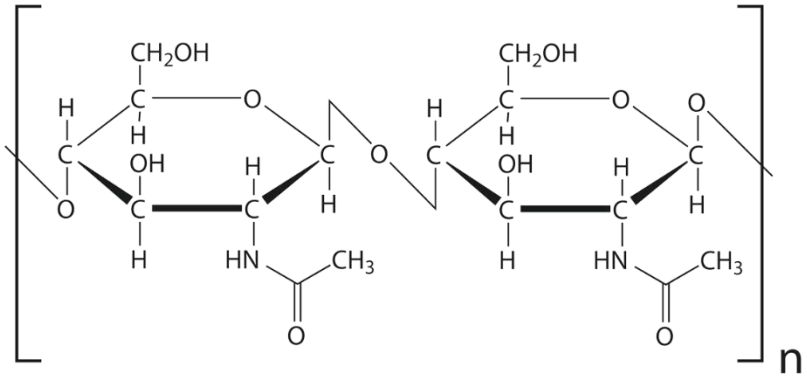


**Figure 5**. Structure of Chitin

The food sector can use chitin and its derivatives as food preservatives (Sethulekshmi, 2014; Barikani *et al*., 2014). They can protect food from microbial degradation because of their antibacterial activities. The positive charges in chitinous materials interact with the negative charges of bacterial cell walls to cause leakage of the internal molecules of microorganisms, which is thought to be the cause of their antibacterial effect (Khoushab & Yamabhai, 2010). Chitosan limits the growth of microbes in food, avoiding poor appearance, odorless flavors, and economic losses. According to El-Diasty *et al*. (2012), adding chitosan to cheese enhanced its mycological quality. Shelf life was increased and mold and yeast growth was prevented.

### 1.1.5. Alginates

Alginates, also known as alginic acids, are anionic polysaccharides found in brown seaweed and marine algae such as *Laminaria hyperborea*, *Ascophyllum nodosum* and *Macrocystis pyrifera*. Alginic acid can be transformed into its salts, the most common of which being sodium alginate. These polymers are made up of two distinct monomers in various quantities, namely β-D-mannuronic acid and -L-guluronic acid, which are linked in α- or β -1,4 glycosidic linkages as homopolymeric or heteropolymeric blocks of just β-D-mannuronic acid or α-L-guluronic acid (Aquilera & Stanley, 1999; Liew *et al*., 2006). Alginates have been studied and employed as emulsion stabilizers, suspending agents, tablet binders, and disintegrants (Sudhakar *et al*., 2006). Figure 6 represents the structure of alginates.

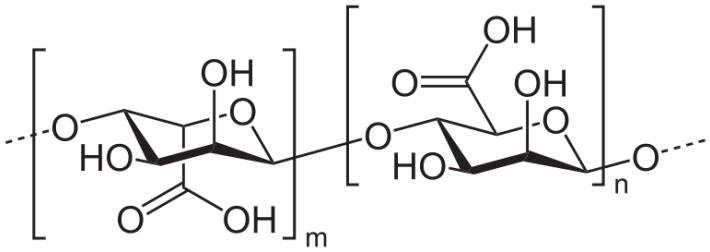



**Figure 6**. Structure of Alginates.

Alginates are just one type of numerous polysaccharides and proteins that have been used as edible coatings for various meals. Food coatings made of sodium alginate have been shown to have high tensile strength, elasticity, and resistance to rippage, as well as being impermeable to oils. However, because alginate gels are porous, these coatings frequently have high oxygen and water permeability (Wang *et al*., 2007). The use of alginate food coatings may be helpful in a variety of culinary applications because they can also be created ionotropically at ambient temperature, antimicrobial agents have been shown to be an effective barrier to microbial surface spoilage of vegetables, meat, and other foods when added to alginate gel (Oussalah *et al*., 2007). The alginate gel coating's cold formation minimizes harm to both the antimicrobial agents and the food itself. Furthermore, this characteristic has been shown to be advantageous for coating a variety of fresh fruit and vegetable products, including lettuce (Tay & Perera, 2004).

### 1.1.6. Psyllium

Psyllium mucilage is made by milling the outer layer of *Plantago ovata* seeds. Its tablet binding properties have been tested, but also to create hydrogels using radiation-induced crosslinking for controlled release of the model drug 5-fluorouracil (Kulkarni *et al*., 2002; Singh *et al*., 2008). To produce a novel sustained release, swelling and bio-adhesive gastroprotective drug delivery system for ofloxacin, psyllium husk was combined with various excipients such as hydroxypropyl methylcellulose (Chavanpatil *et al*., 2006). Figure 7 represents the structure of psyllium.

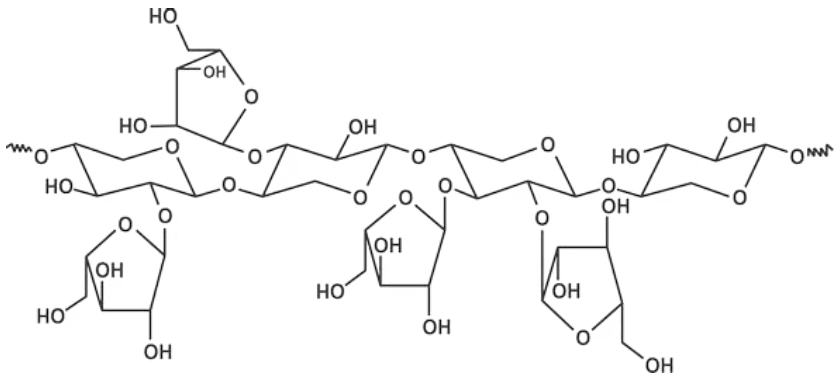


**Figure 7**. Structure of Psyllium.

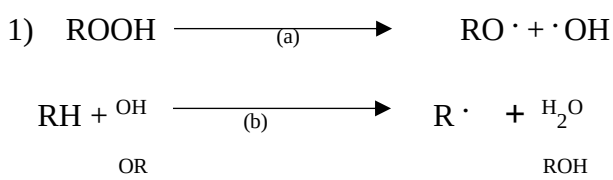
### 1.2. Antioxidants as food coatings

Most carbon-based materials degrade as a result of a chemical interaction with ambient oxygen. Recently, reactive species involved in autoxidation have been linked to several diseases in vivo. Over the past century, autoxidation has been shown to be a common factor in the deterioration of the lubricating properties of hydrocarbon oils, the rancidification of fats and oils, and the loss of physical properties of rubbers and plastics. (Caspary & Lorentzen, 1977; Melhorn & Cloe, 1985).

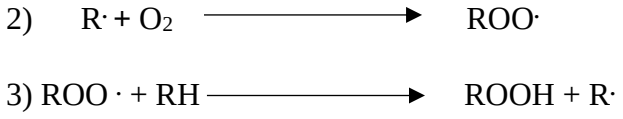
Early in the development of rubber as a technical material, the phenomenon of "ageing" of rubber and the impact of very minute amounts of additional chemicals, dubbed "antioxygens" or "antioxidants," in suppressing this process were noted. In the early 1900s, Ostwald discovered a connection between oxygen absorption and the ageing of rubber (Glumbic, 1946).

The creation of an intermediate peroxide is a crucial aspect of the procedure (reactions (1) through (6)). It is now understood that the concentration of this species in various organic substrates directly affects the rate of autoxidation and that once it is present in the autoxidizing system, no further initiator is needed (Reaction 1) (Glumbic., 1946).

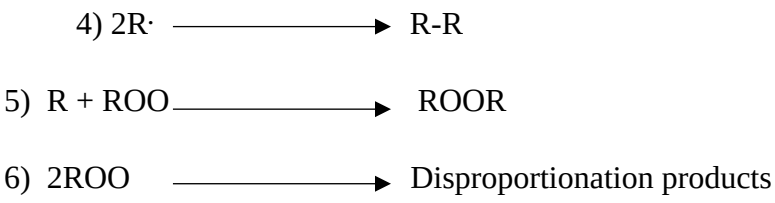
**Initiation**



**Propagation**




**Termination**




### 1.2.1. Natural antioxidants

Any agent that can delay, retard, or prevent the development of rancidity in food or other flavor deterioration due to oxidation is considered a food antioxidant. Due to potential hazards, the existence of synthetic antioxidants in food is disputed, and rigorous legislative regulations are necessary. The use of natural antioxidants, particularly tocopherol, plant extracts, and essential oils (EOs), or their constituents, is an alternate strategy that is receiving extensive research (Tovar *et al*., 2005; Wessling *et al*., 1999).

#### 1.2.1.1 Rosemary

Due to its pleasant flavor and perfume, rosemary (*Rosmarinus officinalis* L.), is highly well-liked throughout Europe. Between 1% and 3% of the mass of leaves and blossoms is made up of essential oils, which are the source of this perfume, in which 1,8-cineol, -pinene, and camphor make up the bulk of the oil. Due to the phenolic chemicals found primarily in flowers, rosemary has antioxidant activity. Carnosol, carnosic acid, and rosmarinic acid are phenolic chemicals that can be found in rosemary (Santos-Sanchez *et al*., 2017).

Since 2008, rosemary extract has been authorized for use in the European Union as a food additive. Its official name is "Extracts of Rosemary E392." Carnosol and carnosic acid are cited by the European Union Regulatory Commission as (Santos-Sanchez *et al*.,

2017). Figure 8 represents the chemical structure of two major diterpene phenolics carnosol and carnosic acid.

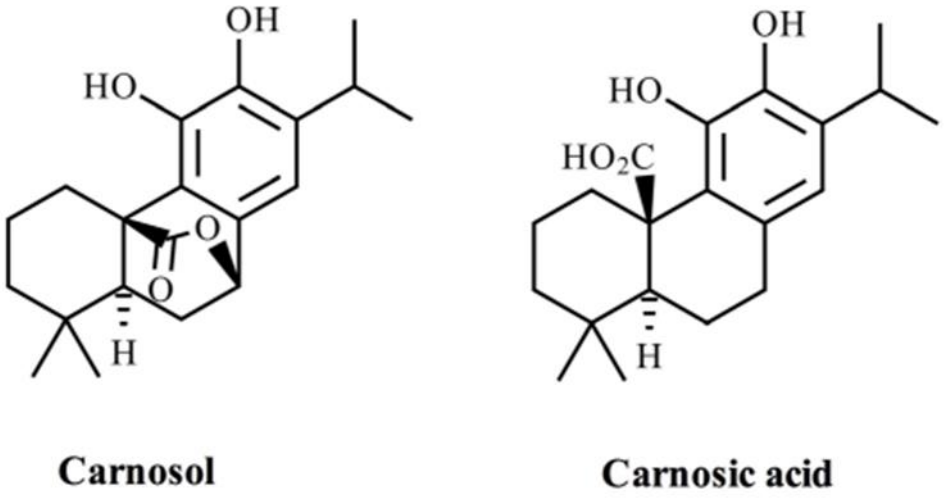



**Figure 8**. Chemical structure of two major diterpene phenolics (Santos-Sanchez *et al.*, 2017).

Antioxidants in rosemary extracts, and their combined amount should not be less than 90% of the extract's total phenolic diterpenes Flavonoids with luteolin and apigenin-like structures are also present in rosemary extracts. To preserve meat, fish, and oils, rosemary extracts are used (de Raadt *et al*., 2015).

In a separate trial, lambs in the fattening stage received rosemary extract supplements at concentrations of 200 and 400 mg/kg of carnoside acid and carnosol, respectively. The findings of this study demonstrated that lamb meat had a longer half-life (Ortuño *et al*., 2014).

### 1.2.1.2 α-Tocopherol

Traber (2007) explains that α-Tocopherol, a kind of vitamin E, is believed to be one of the most physiologically active forms of this nutrient. It is a fat-soluble substance that is present primarily in plant oils such as soybean, wheat germ, and sunflower oil. α-Tocopherol is a strong antioxidant that has been shown to neutralize free radicals and stop

lipid oxidation, which can cause cellular damage and oxidative stress (Brigelius-Flohé & Traber, 1999; Azzi & Stocker, 2000).

Supplementing with α-tocopherol may have preventive benefits against neurological disorders, cancer, and cardiovascular diseases, according to studies. To fully understand the possible health advantages of α-Tocopherol, further study is required because several studies have produced contradictory results (Lee *et al*., 2005; Meydani *et al*., 1997).

Radical-chain breaker tocopherol has been described as acting in a lipid environment because of its hydrophobic nature (Barclay, 1981). Thus, the antioxidant properties of α-Tocopherol are limited to their direct actions on membranes and lipoprotein domains. Other terms such as "secondary antioxidant," "antioxidant as inhibitor of "enzymes that create radicals" or "activator of "genes coding for antioxidant enzymes"" are therefore unclear and do not aid in understanding the molecular mechanism of tocopherol action in vivo" (Azzi, 2007).

Despite the debate over vitamin E supplements, α-Tocopherol is still a crucial nutrient with potential health benefits. A lack of vitamin E can cause several health problems, since the body needs it to function correctly (Traber, 2007). Because of this, it is crucial to have a balanced diet that contains enough vitamin E from foods such as nuts, seeds, and leafy green vegetables (National Institutes of Health, 2021).

#### 1.2.1.3 Ascorbic acid

The primary antioxidant in plasma and cells is ascorbic acid, or vitamin C, but it can also interact with the plasma membrane by giving the α-tocopheroxyl radical an electron and activating a trans-plasma membrane oxidoreductase. Thus, the plasma membrane receives and transmits the reducing capacity produced by ascorbate. The recycling of a-tocopherol by ascorbate aids in preventing peroxidation of membrane lipids (May, 1999).

Vitamin C, also known as ascorbic acid, has the ability to protect the cytosol and membrane components of cells from oxidative damage. Ascorbate functions as a major antioxidant in the cytosol to scavenge free radical species produced as by-products of cellular metabolism. The reduction of tocopheroxyl radicals to a-tocopherol may have an indirect antioxidant effect on cellular membranes. In liposomes and cellular organelles,

ascorbate has been shown to recycle α-tocopherol (Mehlhorn *et al*.,1989; Scarpa *et al*., 1984). Figure 9 represents the redox metabolism of ascorbic acid.

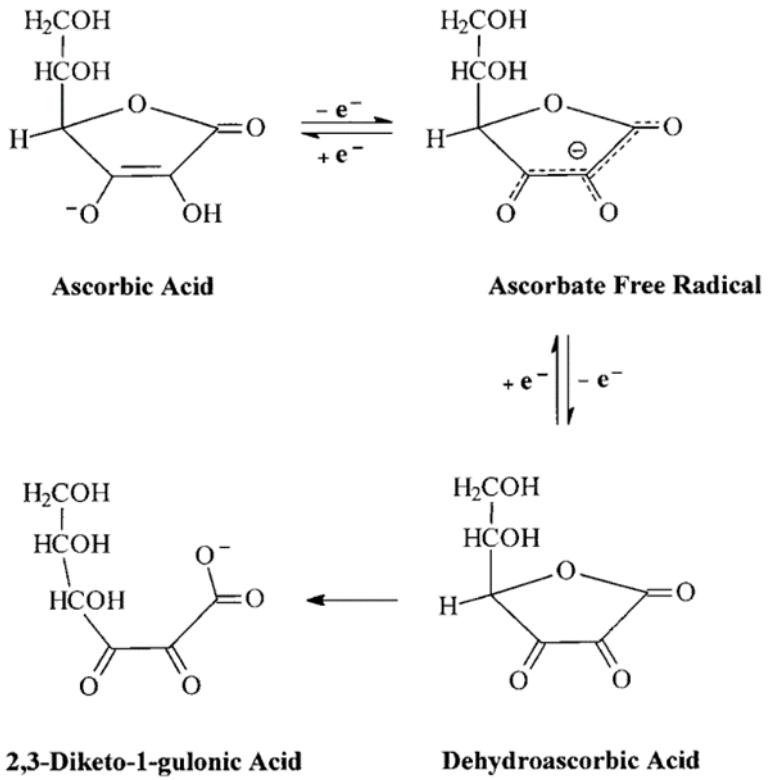



**Figure 9**. Redox metabolism of ascorbic acid (May, 1999).

### 1.2.2. Application of Antioxidant Compounds in Films and Coatings

Primary antioxidants are free radical acceptors that slow or prevent the initiation of autoxidation or stop it in its tracks. Through a variety of ways, secondary antioxidants reduce the rate of oxidation, but they are unable to turn free radicals into more stable molecules (Reische *et al*., 2002). Numerous studies have been conducted on the effects of antioxidant inclusion on the functional characteristics of various biopolymer films and coatings. Antioxidants derived from plants or other natural sources, such as essential oils (Bonilla *et al.,* 2013; Ruiz-Navajas *et al*., 2013; Perdones *et al*., 2014) and other substances have antioxidant properties, such as tocopherol (Akthar *et al*.,2012; Zeng *et al*., 2013; Li *et al*., 2014).

After peeling or cutting, dipping the sample in antioxidant solutions is one technique to prevent the fruit from browning. This method depends on modified environment packaging and low-temperature storage to lengthen the shelf life of the product (Baldwin

*et al*., 1995). The shelf life of fresh cut fruit can also be extended by films and edible coatings. Additional antioxidants can enhance the preservation activity of films and coatings, decrease browning, and lessen the negative consequences of nutrient oxidation (Pastor *et al*., 2013; Bonilla *et al*., 2013).

#### 1.2.2.1. Pure compounds

By adding active substances, new trends in edible films and coatings hope to increase their functionality. Adding an antioxidant as a pure component, such as ascorbic acid, citric acid, resveratrol, or tocopherol, is an intriguing approach that might endow these materials with functional capabilities. These are typically the preferred chemicals, as they serve as models for antioxidants, complement diets, and preserve the nutritional and sensory qualities of the food itself (León & Rojas, 2007).

There is little information in the literature on how adding substances such as resveratrol, ascorbic acid, α-tocopherol, butylated hydroxytoluene and butylated hydroxyanisole alters the properties of films. However, physicochemical techniques have been extensively used to study their antioxidant activity and, in some circumstances, their antibacterial characteristics. For example, ascorbic acid prevents the enzymatic browning of fruits by lowering the o-quinones produced by polyphenoloxidase enzymes. Unfortunately, quinones can reaccumulate and go through browning after ascorbic acid completely oxidizes to dehydroascorbic acid (Rojas-Graü *et al*., 2008).

The findings of many studies have indicated that functional edible films with additional pure antioxidants may be useful in extending the shelf life of foods that are susceptible to oxidative processes. Studies have concentrated on the enhancement of various coatings to make them carriers of pure chemicals. These coatings have shown to effectively maintain the qualitative characteristics of many meals; however, most of the antioxidants used can still degrade quickly as a result of oxidative processes (Pierucci *et al*., 2004).

Alginate, hydroxypropylmethylcellulose, pectin, and gellan are intriguing alternatives among the biopolymers utilized to create coatings because they are odorless, tasteless, and biodegradable (Krochta & De Mulder-Johnson, 1997). The edible coverings in fruits and vegetables contain antibrowning chemicals (Soliva-Fortuny & Martin-Belloso, 2003; Chiumarelli *et al*., 2010).

#### 1.2.2.2. Essential oils

Fewer chemicals must be used in minimally processed fruits and vegetables, according to consumer demands. Therefore, it is crucial to look for natural compounds that can serve as substitute antioxidants. Antioxidants can increase lipid stability and stop loss of sensory and nutritional quality, extending the shelf life of food products (Ponce *et al*., 2008).

Essential oils are naturally occurring aromatic antioxidant and antibacterial compounds that are physically extracted from vegetables. They are made up of a complex blend of natural substances; most of them contain a combination of terpenes, terpenoids, phenolic acids, and other aromatic and aliphatic substances, however, their make-up may differ depending on where they come from. The use of essential oils in food products may increase shelf life because they can reduce lipid oxidation (Tongnuanchan *et al*., 2013; Perdones *et al*., 2014).

Because they exhibit the hydrophobic nature characteristic of lipids, essential oils can also increase the water barrier qualities of the film in addition to their strong antioxidant capacity (Atarés *et al*., 2010). According to numerous authors, the amount of essential oils applied to a biodegradable film affects how powerful an antioxidant it is; in other words, the antioxidant activity increases as the concentration of essential oils in the film increases (Gómez-Estaca *et al*., 2009; Moradi *et al*., 2012; Shojaee-Aliabadi *et al*., 2013; Tongnuanchan *et al*., 2013; Jouki *et al*., 2014).

#### 1.2.2.3. Natural Extracts

Researchers have focused on films that contain antioxidant compounds from natural sources, such as natural extracts, because synthetic antioxidants have aroused some safety concerns, and regulatory agencies have prohibited their usage as food additives (Murcia & Martínez-Tomé, 2001; De'nobili *et al*., 2013). These extracts should improve the nutritional value and overall quality of the food product without compromising its integrity (Guilbert *et al*., 1996).

Numerous authors have studied the various functions of antioxidant extracts. Fruit and vegetable extracts have recently been taken into consideration for use as natural bioactive additives due to their coloring potential, pharmacological activities and bioactivity in

areas of cleanliness, nutrition, and environmental awareness (Akhtar *et al*., 2012). Several studies on antioxidant and antiradical extracts that confer color to films have been published (Gómez-Estaca *et al*., 2009a, b; Norajit *et al*., 2010; Akhtar *et al*., 2012; Bitencourt, 2013; LI *et al*., 2014).

Because the extract and the film matrix interact well, the physical characteristics of the film, such as moisture content and water solubility, remain unchanged after the addition of extracts (Kaliana Sitonior *et al*., 2014). The use of edible coatings on fruits and vegetables could improve food quality and shelf life, similar to how natural compounds can be successfully combined into biodegradable films. However, during storage, light may cause the active ingredient to degrade and affect optical qualities such as brightness. Unlike samples without coating, several studies have indicated that this method can better regulate weight loss and respiration rates, allowing extended storage times (Pastor *et al*., 2013; Supapvanich *et al*., 2012; Das *et al*., 2013).

### 1.2.3. Synergistic, antagonistic, and additive antioxidant effects

#### 1.2.3.1. Synergism

Antioxidants may engage in a variety of interactions. The antioxidant activity of natural mixtures can be influenced favourably or negatively by interactions between antioxidants (Olszowy-Tomczyk, 2020).

Synergism is one of the antioxidant effects observed in a complex mixture. The Greek term "synergos" implies "working together." To put it another way, synergism is the coordinated or correlated activity of two or more structures, agents, or physiological processes so that the combined effect is higher than the sum of the individual effects of each work alone (Thoo *et al*. 2013; Sonam & Guleria 2017; Tavadyan & Minasyan 2019). The protective action of one antioxidant through its sacrificial oxidation can also result in a synergistic antioxidant effect (Choe & Min, 2009).

The synergism can be explained by (Choe & Min, 2009):

- Regeneration of the stronger antioxidant by the weaker antioxidant (with a lower reduction potential) (with a higher reduction potential)
- Antioxidants generate persistent intermolecular complexes that have more antioxidant activity than their parent substances.

- creation of new phenolic products having a stronger antioxidant capacity than the mixture of the parents' compounds, including dimers and adducts.
- Differential antioxidant phase distributions and variations in their solubility (near and at the interface)
- Unexpected interactions between the substances under study.

#### 1.2.3.2. Antagonism

In chemistry, antagonism is a phenomenon in which the combined effect of two or more agents is smaller than the total effect of each agent acting alone. When the combined effects are less than the mathematical total that would be projected from the separate components, antagonism results (Wang *et al*. 2011).

Several hypotheses have been proposed to explain the mechanism of the antagonistic antioxidant effect (Wang *et al*. 2011):

- The more potent the antioxidant replenishes, the less potent.
- Possible development of complexes and adducts between antioxidants.
- Antioxidants could potentially polymerize, which would reduce their antioxidant capabilities.
- The final decomposition of free antioxidant radicals is the result of their irreversible reactions; as a result, they do not react with the neutralized radical.
- Antioxidants have unknown interactions with each other.

In a study conducted by Hras *et al*. (2000), the antioxidant activity was compared in mixtures of different antioxidants (rosemary, acetic acid α-tocopherol ascorbylpalmitate) by applying it in vegetable oil at 60°C, where the measurement of oxidation activity was by the presence of the peroxide value and the anisidine value (Wang *et al*. 2011). The peroxide values of sunflower oil at 60 ° C with single antioxidants added are shown in Figure 10. Rosemary extract (ROS.CON) retarded the hydroperoxide formation, ascorbyl palmitate (AP) also showed a considerable but not significant stabilization effect, sunflower oil's oxidative stability was slightly improved by citric acid's antioxidative properties, α-Tocopherol (TOC) exhibited a prooxidative effect. The peroxide value of the sample with added a-tocopherol started to fall after 9 days of storage at 60 ° C.

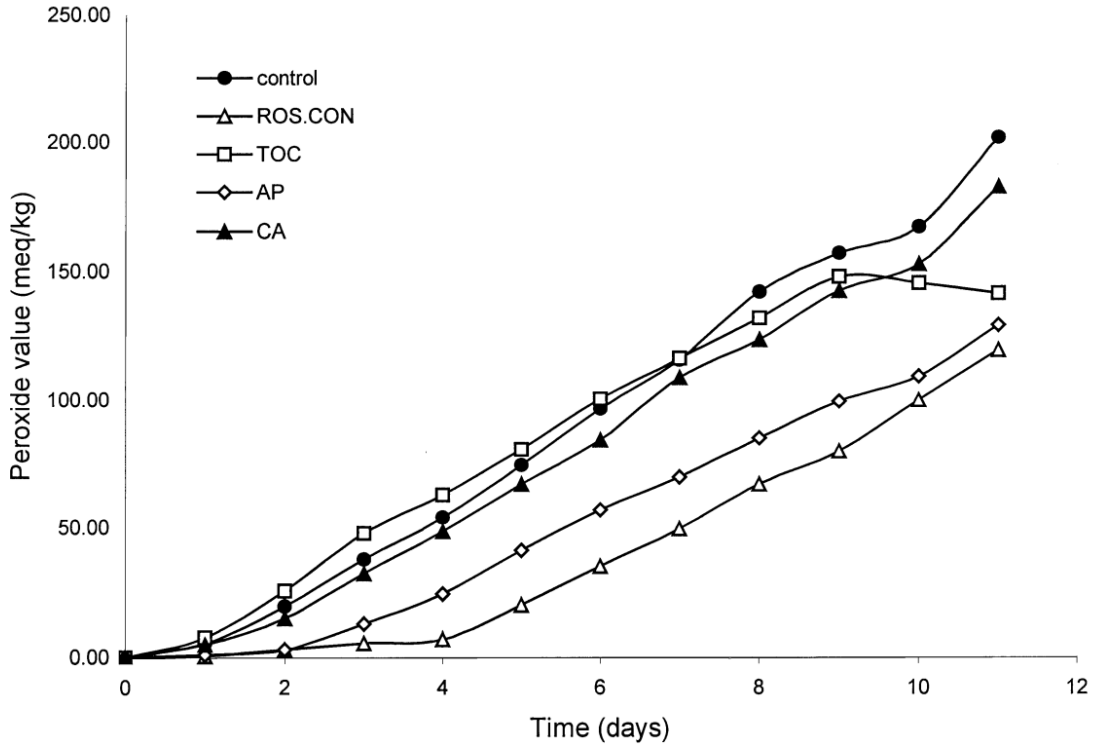



**Figure 10**. Peroxide values of sunflower oil without antioxidants and with added rosemary extract (ROS.CON), α -Tocopherol (TOC), ascorbyl palmitate (AP) and citric acid (CA) during storage at 60 °C (HrasÏ et al., 2000).

The effect of ROS.CON+ AP was significantly better compared to the effect of ROS.CON, and the combination of citric acid and rosemary extract showed a small synergistic effect. Hydroperoxide formation was significantly reduced in ROS.CON, ROS.CON + AP and ROS.CON+CA samples compared to the control sample, as shown in Figure 11. The antioxidant effect of rosemary extract was reduced by a-tocopherol, but rosemary extract improved the stability of α-Tocopherol this is consistent with the research of Hopia *et al*. (1996), who discovered that tocopherol reduced the two important elements of the oxidative stability of rosemary carnosol and carnosic acid (HrasÏ *et al*., 2000).

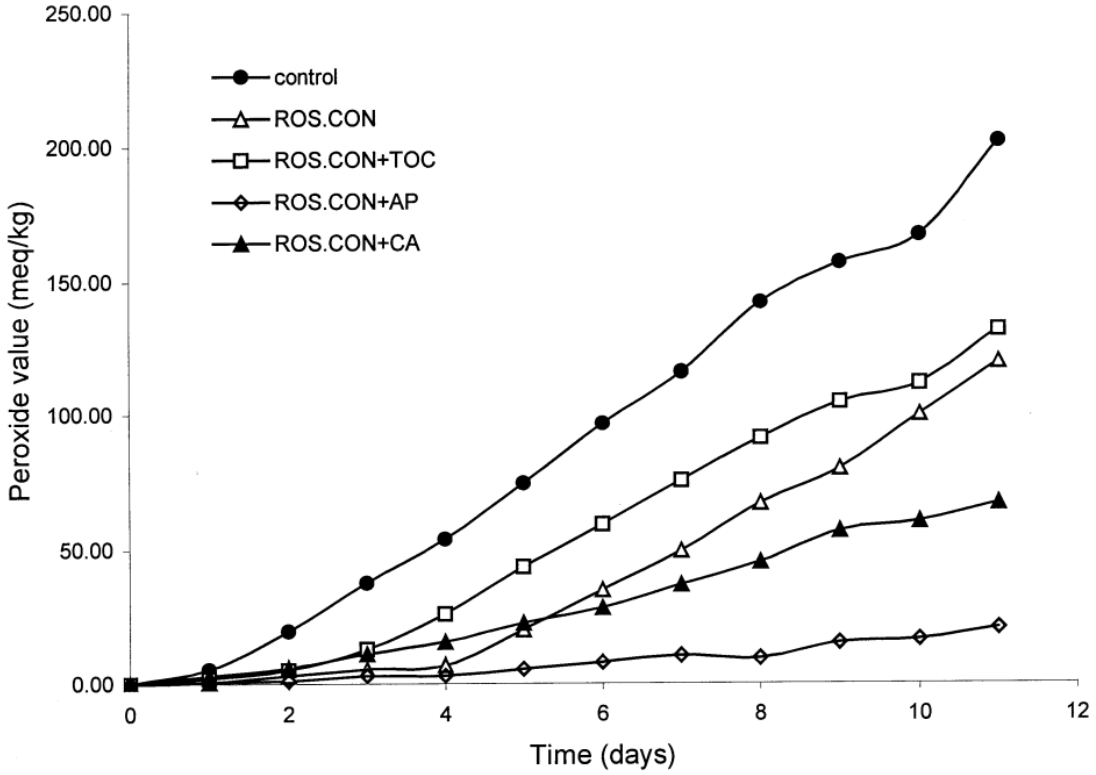



**Figure 11**. Peroxide values of sunflower oil without antioxidants and with added antioxidant mixtures of rosemary extract+a-tocopherol (ROS.CON+TOC), rosemary extract+ascorbyl palmitate (ROS.CON +AP) and rosemary extract+citric acid (ROS.CON+CA) during storage at 60 ° C (A.R. HrasÏ *et al*., 2000).

### 1.3. Response Surface Methodology (RSM)

Box and coworkers created the response surface methodology in the 1950s (Gilmour, 2006; Bruns, 2006). This phrase was coined from the graphical perspective created following the fitness of the mathematical model, and it has since been commonly used in chemometrics texts. RSM is a collection of mathematical and statistical methods based on fitting empirical models to experimental data collected in accordance with experimental design. To achieve this goal, the system under study is described using linear or square polynomial functions, which are then used to investigate (by modelling and displacing) the experimental conditions until their optimal (Teofilo, 2006).

The following are some phases in the use of RSM as an optimization technique:

1. Delineation of the experimental area according to the study's goal and the researcher's experience, as well as selection of independent variables with significant impacts on the system through screening investigations.
2. Choosing an experimental design and conducting experiments using the chosen experimental matrix.
3. The mathematical and statistical analysis of the gathered experimental data using a polynomial function fit.
4. The fitness of the model being evaluated.
5. Determining whether it is necessary and feasible to move in the direction of the ideal region.
6. Obtaining the optimum values for each variable studied.

### 1.3.1. Advantages

Using RSM and the Box-Behnken design to extract essential oils more effectively from *Eucalyptus globulus* leaves (Gullón *et al*., 2017), as well as Response Surface Methodology as a tool for bioprocess design and optimization by Kalil *et al*. (2000), the studies also shed light on the benefits of RSM:

- Establishes a link between responses (activity, yield, cell viability, oxygen level, etc.) and the factors under control (temperature, pressure, initial concentration, power input, agitation rate, etc.)
- For a particular range of control variables, it forecasts the response values.
- It enables the evaluation of the relevance of control variables using statistical testing.
- Determines the optimal values of the control variable that will produce the highest activity or yield across the region evaluated in a particular experimental design.

### 1.3.2. Optimization using Central Composite Design (CCD)

The CCD statistical technique, which is based on a multivariate nonlinear model, has been widely utilized to optimize adsorption process factors and to extract the regression model equations and operating conditions from the relevant experiments (Kalavathy *et al*., 2009). It is also useful for studying the interactions of the various parameters that affect the process (Sugashini & Begum, 2012).

One of the strategies used in the response surface methodology for designing the experimental protocols is Central Composite Design (CCD). CCD optimization can screen a variety of parameters and determine the contribution of each factor (Şahin *et al*., 2011). The single variable or the combined influence of the variables on the response can likewise be evaluated via CCD. Even though this ability is shared with other experimental design types such as the complete factorial technique and partial factorial method, it differs in that the number of experimental runs is condensed. For instance, the full factorial technique will recommend at least 81 experimental trials plus replication when there are only four independent variables (Box & Wilson, 1951).

Otherwise, only 31 experimental points (16 factorial, 8 axial, and 7 center) are required when utilizing the CCD technique (Sun *et al*., 2010). In order to optimize complicated processes and systems, Central Composite Design (CCD), is frequently employed in several disciplines, including chemical engineering, food science, and biotechnology. The capacity of CCD to model and optimize non-linear and quadratic interactions between the input factors and response variables is one of its key benefits. This capability can result in appreciable increases in process effectiveness and product quality. Numerous studies have shown how effective CCD is at streamlining various procedures, including the extraction of pigment from microalgae (González-Vega *et al*., 2021). Treatment of wastewater by electrocoagulation (Shah *et al*., 2017), as well as the production of bioethanol from rice straw (Takano & Hoshino, 2018). However, the selection of relevant input variables, the precision of the experimental design, and the veracity of the model assumptions are all critical considerations that affect how well CCD performs (Anderson-Cook *et al*., 2009).

Despite being a popular optimization technique, Central Composite Design (CCD) has some restrictions and disadvantages. The need for several tests, particularly when researching complicated systems with various components, is one of the fundamental drawbacks of CCD. This may make it more difficult, expensive, and time-consuming to conduct experiments. Furthermore, CCD makes predictions using statistical analysis and mathematical models, which may not always accurately reflect actual circumstances. Additionally, CCD might not be appropriate for responses that are discontinuous or nonlinear; in a study by Bezerra *et al*. (2008), the scientists pointed out that overfitting, which happens when a mathematical model is overly complicated and unable to generalize to new data, might result from CCD. As a result, there may be a lot of noise or

unpredictability, making it challenging to evaluate the findings. In another study by Das *et al*. (2022), scientists noted that outliers might distort the data and provide false conclusions because CCD is prone to them.

### 1.3.3. Optimization using Box Behnken (BB) Design

Another approach in the Response Surface Methodology is called Box-Behnken (BB), and its goal is to identify the factors that will result in the best possible response or output. According to Maran *et al*. (2013), a design known as a Box-Behnken design is one that lacks an embedded factorial or fractional factorial point that could be used to identify the variable condition at both the midpoint and the center of the variables space (Maran *et al*., 2013).

This optimization method known as Box-Behnken design (BB) is popular and has been employed in a variety of research domains. (BB) has been employed in the pharmaceutical sector to optimize medication delivery systems. For example, (BB) was used to optimise hydrogel-based nasal drug delivery systems in the work by Nathiely *et al*. (2018), (BB) has also been used in the preparation of food, as seen in the Wang *et al*. (2007) study, for reference, where apple pectin was produced, the processing conditions were optimised using (BB).

(BB) has been applied to environmental engineering to improve wastewater treatment procedures. Optimization using Box Behnken (BB) has been applied to environmental engineering to improve wastewater treatment procedures, where (BB) was employed to maximize the adsorption of heavy metals from wastewater using a biosorbent (Choińska-Pulit *et al*., 2018). In another study by Thirugnanasambandham & Shine (2018), (BB) was used to optimize the electrocoagulation method used to remove chromium from wastewater.

Compared to other optimization techniques, (BB) has the benefit of being simpler and easier to use. In a research conducted by Abla *et al*. (2023), the formulation of orally disintegrating tablets of a medication with poor water solubility was optimized using (BB). According to the authors, BB was able to speed up and lower the cost of the optimization process by identifying the best formulation with fewer trial runs.

The fact that (BB) requires a quadratic connection between the input variables and the result, however, is one of its drawbacks since when the relationship is non-linear, (BB) might not be able to correctly forecast the ideal circumstances. For example, the synthesis of biodiesel from leftover cooking oil was optimized using (BB) (Hamze *et al*., 2015). Although (BB) offered a fair fit to the response surface, the authors claimed that it was unable to fully capture the non-linear relationship between the input variables and the response.

## 2. Methodology

### 2.1. Sample and Extract Preparation

Samples of dried rosemary leaves were obtained from "Cantinho das Aromáticas" from Vila Nova de Gaia (Portugal) (Figure 12), a company that commercializes aromatic plants. The dried leaves were grounded to obtain a fine powder (approximately 20 mesh) and stored until further analysis. The extract of rosemary leaves was prepared by infusion, in which 1L of boiled distilled water was added to 25 g of grounded rosemary and left for 5 min. Afterwards, the mixture was left to cool for another 5 min. After this process, the mixture was filtered to remove any solid particles. Finally, the liquid phase was transferred to sterilized glass containers, frozen and lyophilized to obtain a fine powder of rosemary extract, as shown in Figure 13.

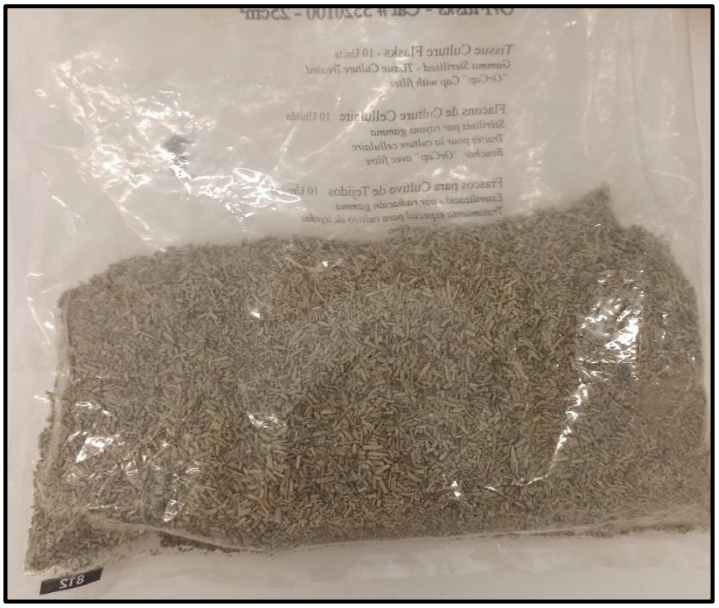


**Figure 12**. Rosemary leaves.

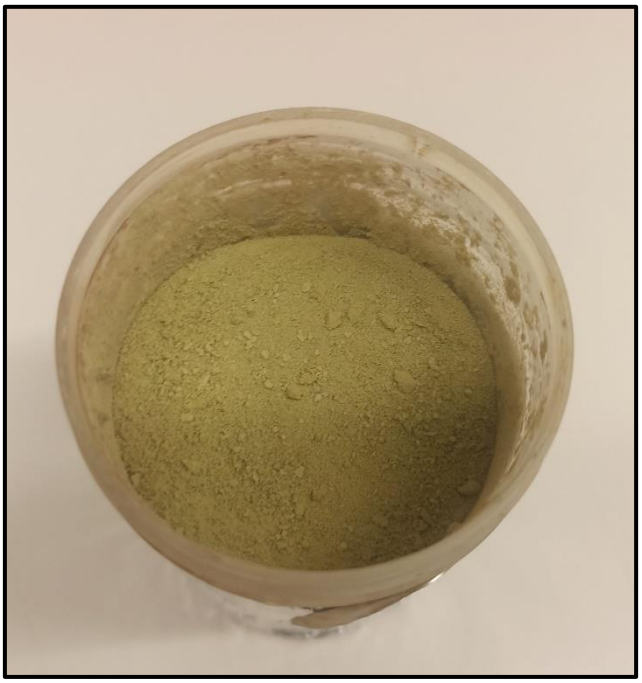


**Figure 13**. lyophilized rosemary extract.

## 2.2. **Spraysafe Formulation**

The SpraySafe solution was prepared varying the contents of its components: 17 samples (runs) with 100 mL of each Spraysafe solution with different concentrations of α-tocopherol, ascorbic acid and rosemary were obtained as shown in Table 1, to study the antioxidant activity, as well as synergistic and antagonistic effects. The carrousel extractor was used to mix the compounds of the mixture as shown in Figure 14.

**Table 1**. Different concentrations of α-tocopherol, ascorbic acid, and rosemary.

| Run | Rosemary extract (g/L) | α-tocopherol (g/L) | Ascorbic acid (g/L) |
|---|---|---|---|
| 1 | 0.7 | 0.2 | 0 |
| 2 | 0.35 | 0.2 | 0.2 |
| 3 | 0.35 | 0.2 | 0.2 |
| 4 | 0.7 | 0 | 0.2 |
| 5 | 0.35 | 0.2 | 0.2 |
| 6 | 0 | 0.2 | 0 |
| 7 | 0.35 | 0.4 | 0.4 |
| 8 | 0 | 0.2 | 0.4 |
| 9 | 0.35 | 0.2 | 0.2 |
| 10 | 0 | 0 | 0.2 |
| 11 | 0.7 | 0.4 | 0.2 |
| 12 | 0.7 | 0.2 | 0.4 |
| 13 | 0.35 | 0 | 0 |
| 14 | 0.35 | 0.4 | 0 |
| 15 | 0 | 0.4 | 0.2 |
| 16 | 0.35 | 0 | 0.4 |
| 17 | 0.35 | 0.2 | 0.2 |

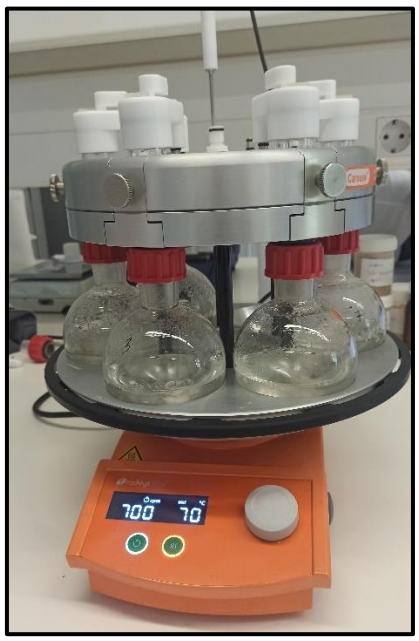



**Figure 14**. Carrousel extractor used to prepare the Spraysafe solutions.

After mixing the solutions in the carrousel equipment, 17 individual samples were obtained. These samples were prepared with different concentrations of α tocopherol, ascorbic acid and rosemary extract according to Table 1 and Figure 15.

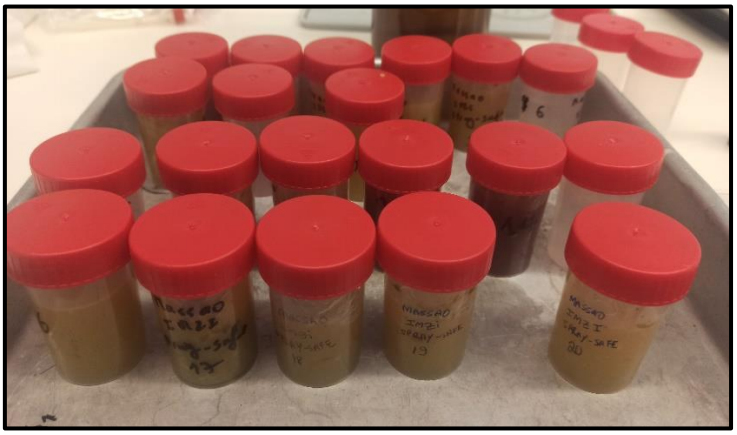


**Figure 15**. Spraysafe samples prepared for the antioxidant activity test.

## 2.3. Bioactivity

### 2.3.1. Antioxidant Activity (DPPH Assay)

The determination of antioxidant activity using the DPPH radical scavenging method was carried out according to Aibarro-Ortega *et al*. (2020). The mixture of each of the 96-wells (Figure 16) consisted of 30 µL of sample solution and 270 µL of DPPH methanolic solution (6 x $10^{-5}$ M), prepared and added to different dilutions of the sample's extracts. The mixtures were incubated in the dark for 1 hour at room temperature. After the incubation period, the reading was undertaken at 515 nm (Figure 17) using a SPECTROstar Nano spectrophotometer (BMG LABTECH, Ortenberg, Germany). Trolox was used as a positive control. The results were expressed as $EC_{50}$ values (mg/mL).

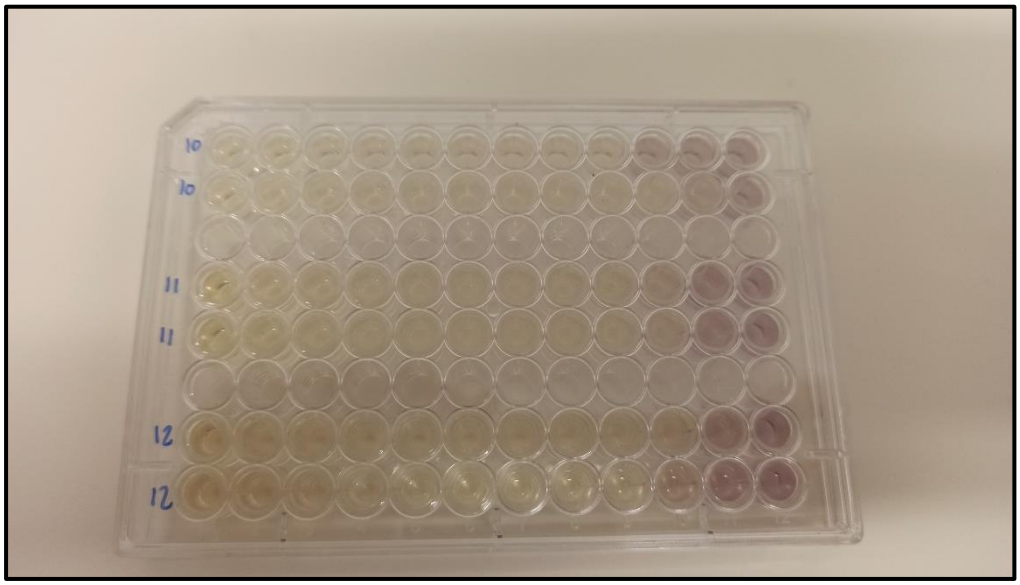



**Figure 16**. Microplate of 96-wells used in the DPPH assay .


**Figure 17.** spectrometer used for the DPPH readings.

### 2.3.2. Antimicrobial activity

The antimicrobial potential of the extracts was evaluated following the methodology applied by Pires *et al*. (2018) for the following set of microorganisms acquired from the Frilabo company in Porto, Portugal. Gram-negative Bacteria: *Enterobacter cloacae*

(ATCC 49741), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica subsp* (ATCC 13076), *Yersinia enterocolitica* (ATCC 8610); Gram-positive Bacteria: *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 19111), *Staphylococcus aureus* (ATCC 204305); and Fungi: *Aspergillus fumigatus* (ATCC 204305), *Aspergillus brasiliensis* (ATCC 16404). The microorganisms were previously incubated under different conditions to obtain them in exponential growth phase for use in the assays. The bacteria *E. coli*, *S. enterica*, *P. aeruginosa*, and *Y. enterocolitica* were incubated at 37 ± 0.5 ° C in MacConkey agar culture medium for 24 hours. The other bacteria were incubated under the same conditions, but on blood agar. The fungi were incubated in Malt Extract Broth (MEB) at 25 ± 0.5 °C for 72 hours. The bacteria suspensions were prepared on TSB standardized at $1.5x10^6$ CFU/mL and quantified using a densitometer. Suspensions of the fungi were prepared in PBS, and TWEEN (0.1%) standardized at $1.0x10^6$ CFU/mL, quantified by counting in a Neubauer chamber. A stock solution of 20 mg/mL extract was prepared in DMSO (5%; *v/v*) and Tryptone Soy Broth (TSB) culture medium. In a 96-well microplate, 90 µL of the extract solution was added to 100 µL of TSB and a serial dilution was performed. Subsequently, 10 µL of inoculum was added in each of the wells, obtaining effectively tested extract concentrations, in duplicates, between 10 – 0.075 mg/mL. Negative controls of the extract and TSB culture medium were prepared. Ketoconazole and streptomycin, methicillin and ampicillin were used as positive controls for the antifungal and antibacterial activities, respectively. All work was performed with sterile materials handled in laminar flow. The plates with bacteria were covered and incubated at 37 ± 0.5 °C for 24 hours. After this period, 40 µL of a 0.2 mg/mL solution of the colorimetric indicator *p*-iodonitrotetrazolium chloride (INT) prepared in sterile water was added, and the plate was incubated at 37 ± 0.5 °C for 30 minutes. The minimum inhibitory concentration (MIC) was defined as the lowest concentration that inhibits visible bacterial growth determined by changing coloration from yellow to pink if the microorganisms are viable. To determine the minimum bactericidal concentration (MBC), defined as the lowest concentration required to kill the bacteria, 50 µL of liquid from each well that showed no color change was seeded onto a solid medium and incubated at 37 ± 0.5 °C for 24 hours. The lowest concentration that produced no growth determined the MBC. The plate with fungi was incubated at 25 ± 0.5 °C for 72 hours. After this period, the MIC was determined directly from the comparison with the positive control to identify the lowest concentration in which no visible fungal growth was determined by visualizing spores.

To determine the minimum fungicidal concentration (MFC), the plate was incubated for another 72 hours at 25 ± 0.5 ° C and a new observation was made to check for visible fungal growth.

### 2.4. Optimization Procedure and Statistical Analysis

For the antimicrobial activity, the optimization of the proportions of each component was carried out in terms of the minimum inhibitory concentration for the most sensitive microorganisms of each evaluated group, namely *B. cereus*, *L. monocytogenes* and *S. aureus*. The proportions used in the antimicrobial capacity test were converted to coded factors of the Box-Behnken design (Montgomery, 2017), ranging between -1 and 1. From this conversion, multivariate quadratic mathematical models (Equation 1) were constructed to represent the potential antimicrobial agent of the mixture, in the form:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + ... + \beta_k X_k + \beta_{11} X_1^2 + \beta_{22} X_2^2 + ... + \beta_{kk} X_k^2 + \beta_{12} X_1 X_2 + ... + \beta_{ij} X_i X_j \text{ (Eq. 1)}$$

Where Y represents the response variable; $X_1$, $X_2$, … $X_k$ are the proportions of each component in harcoded values; and $\beta_0$, $\beta_1$, $\beta_2$, ..., $\beta_k$, $\beta_{11}$, $\beta_{22}$, ..., $\beta_{kk}$, $\beta_{12}$, ..., $\beta_{ij}$ are the regression coefficients representing the intercept, linear effects, quadratic effects, and interaction effects between factors, respectively.

The choice of the polynomial order of the mathematical model was defined based on the analysis of variance (ANOVA), in which the values of the mean square error, Fisher and *p*-values, and the determination coefficients ($R^2$ and adjusted $R^2$) were determined. The confidence intervals and statistical significance for the model β coefficients were calculated and the nonstatistically significant β coefficients for the model performance were discarded. Finally, for antioxidant activity, the same methodology was also applied (Montgomery, 2017), but the response in terms of $EC_{50}$ values (μg/mL). All data analysis was carried out using Stat-Ease 360 Software.

## 3. Results and Discussion

### 3.1. DPPH Results

The optimization of the antioxidant activity through the DPPH assay of the three different ingredients of the solution followed an experimental design using the Box-Behnken

model, which only considers points within the given range of the different factors. This experimental design allowed to understand the optimal concentration of the three antioxidants used for the solution and to analyse possible synergistic or antagonistic effects. Thus, the three varying ingredients were rosemary extract, α-tocopherol and ascorbic acid. Thus, ingredient A, rosemary extract varied between 0 and 7 g/100 mL, while ingredient B and C, α-tocopherol and ascorbic acid both varied between 0 and 0.4 g/100 mL. In Table 2, the 17 different experimental runs are shown with the response (R1), which represents the antioxidant activity, measured using the DPPH assay. The highlighted rows represent the centre points, which are used to measure the variance between repetitions.

**Table 2**. Experimental design runs and response.

| Run | A: Rosemary Extract (g/100 mL) | B: α-tocopherol (g/100 mL) | C: Ascorbic Acid (g/100 mL) | R1: DPPH ($EC_{50}$ - µg/mL) |
|---|---|---|---|---|
| 1 | 0.7 | 0.2 | 0 | 136.63 |
| 2 | 0.35 | 0.2 | 0.2 | 46.77 |
| 3 | 0.35 | 0.2 | 0.2 | 46.83 |
| 4 | 0.7 | 0 | 0.2 | 74.98 |
| 5 | 0.35 | 0.2 | .02 | 47.52 |
| 6 | 0 | 0.2 | 0 | 45.34 |
| 7 | 0.35 | 0.4 | 0.4 | ~~34.15~~ |
| 8 | 0 | 0.2 | 0.4 | 27.3 |
| 9 | 0.35 | 0.2 | 0.2 | 66.9 |
| 10 | 0 | 0 | 0.2 | 72.4 |
| 11 | 0.7 | 0.4 | 0.2 | 65.2 |
| 12 | 0.7 | 0.2 | 0.4 | 95.3 |
| 13 | 0.35 | 0 | 0 | 689.13 |
| 14 | 0.35 | 0.4 | 0 | 65.76 |
| 15 | 0 | 0.4 | 0.2 | 32.65 |
| 16 | 0.35 | 0 | 0.4 | 120.55 |
| 17 | 0.35 | 0.2 | 0.2 | 72.85 |

In terms of the optimization, as seen in Table 3, the obtained quadratic model showed a significant fit, and a non-significant lack of fit. In terms of the proportion of variance, symbolized by $R^2$, it showed a very good fit of the model, while the adjusted $R^2$ is just slightly lower, but still excellent. Overall, to achieve these results, one run was ignored as the result represented an outlier, namely run 7. The responses used a square root transformation to better fit the model.

**Table 3.** Fit statistics of the quadratic model obtained for R1.

| Parameter | Value | Info |
|---|---|---|
| Model | *p*-value | 0.0007 - Significant |
| Lack of Fit | *p*-value | 0.0637 - Not significant |
| $R^2$ | 0.9692 | - |
| Adjusted $R^2$ | 0.9231 | - |

Coded equation -

$$Ec_{50}=7.45+1.50A-1.21B-0.4949C+0.5532AB-0.1052AC-4.21A^2+4.49B^2+5.10C^2$$

The antioxidant activity measured using the DPPH assay was expressed in $EC_{50}$ values, which means the concentration of the solution that can quench 50% of the DPPH radicals. Thus, the minimize function was used to obtain the lowest response provided by the combination of the ingredient concentration. In this way, by showing the lowest value, the model provides the lowest $EC_{50}$, which means the combination of ingredients that has the highest antioxidant activity. In Figure 18, the optimal points are shown for each ingredient. To obtain an $EC_{50}$ of 7.23 μg/mL (under the lowest $EC_{50}$ of all individual runs, the lowest was 27.3) only 0.14 g/100 mL of rosemary extracts are needed, while tocopherol and ascorbic acid showed very similar results of 1.81 and 1.66 g/100 mL, respectively. In all figures, for clarity, the results are displayed in g/L, although the discussion is made in g/100 mL as it is the volume that the package contains the solution.

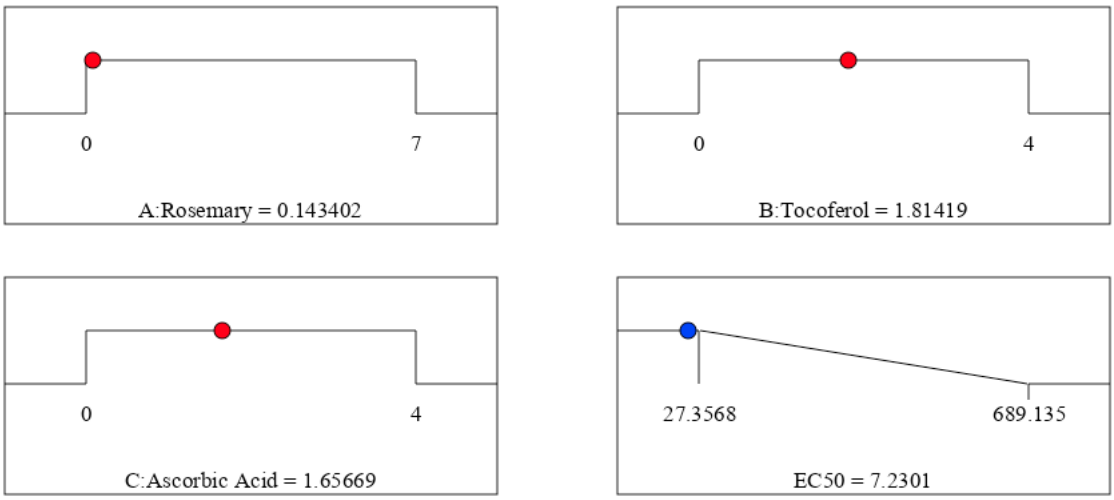



**Figure 18**. Graphical representation of the optimal points for the antioxidant activity of the solution.

In Figure 19, each ingredient is plotted showing its antioxidant activity over the concentrations tested, showing its contribution to the overall antioxidant activity. It should be noted that lower values, closer to 0 µg/mL show better antioxidant activity ($EC_{50}$ values). Therefore, ingredient A (rosemary extract) shows a decrease in antioxidant activity from 0 to 4 g/100 ml, which then increases to 7 g/100 ml. B, α-tocopherol inversely shows a rising antioxidant activity until 2 g/100 mL, maintaining it constant until 3.5. and lowering from there to 4 g/100 mL. This behavior is very similar for ascorbic acid (C).

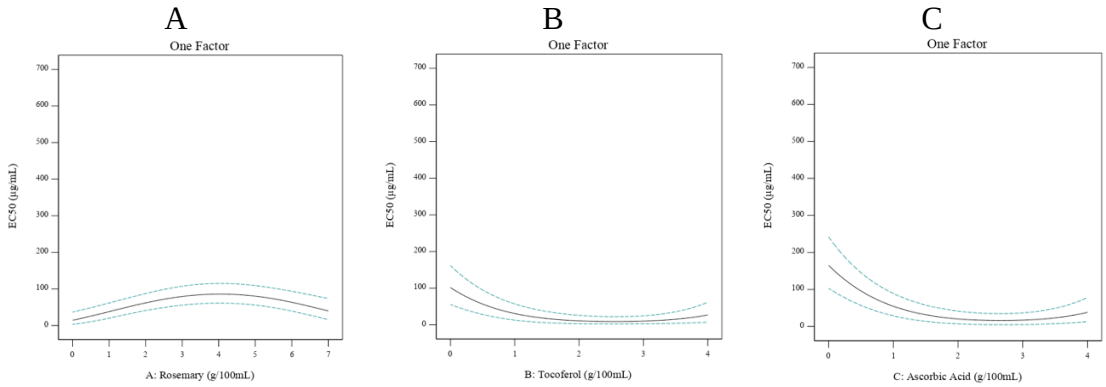



**Figure 19**. Individual contribution of each factor (ingredient) to overall antioxidant activity.

In Figure 20, the 3D surface plots of each factor (ingredient) combined in pairs are shown, allowing for a better understanding of how each combined factor behaves in the concentrations tested. Plot A, that combines rosemary extract with α-tocopherol, shows a higher antioxidant activity at the lowest and highest concentrations of rosemary extract, while showing a better antioxidant activity at about 2 g/100 mL of tocopherol. This phenomenon recorded for rosemary is also shown in plot B, in which the best antioxidant activity is found in the extremities of the concentration of rosemary extract. This behavior is probably due to antagonistic effects of intermediate concentrations of the rosemary extract with the other antioxidants, while promoting synergisms with lower and maximum values. In plot B, ascorbic acid at concentrations of 1 to 3 g/100 mL showed the best antioxidant activity when plotted against the rosemary extract, while in plot C the highest antioxidant activity was sough at concentrations superior to 2 g/100 mL of either extracts.

Overall, in terms of contributions to the antioxidant activity of the solution, ascorbic acid and tocopherols showed the highest importance, most probably working in tandem and showing synergistic effects. This is also apparent when taking into account the coded equation shown in Table 3, where the individual factors (A, B, and C) are shown with constants. The constants for α-tocopherol and ascorbic acid, B and C, respectively, show negative values, which shows greater importance compared to the rosemary extract that has a positive constant (1.5).

It should be noted that the DPPH assay represents a chemical assay that takes into account only one antioxidant mechanism (electron transfer), and thus is not completely representative of a total antioxidant solution. Therefore, these same concentrations of ingredients, chosen based on previous studies of the solution, should be used in other antioxidant assays to better understand the important contribution of rosemary extracts to overall antioxidant mechanisms. Assays such as thiobarbituric acid reactive substances (TBARS), reducing power, cellular antioxidant activity, and others should be performed in future studies to complement this preliminary study.

| A | B | C |
|---|---|---|

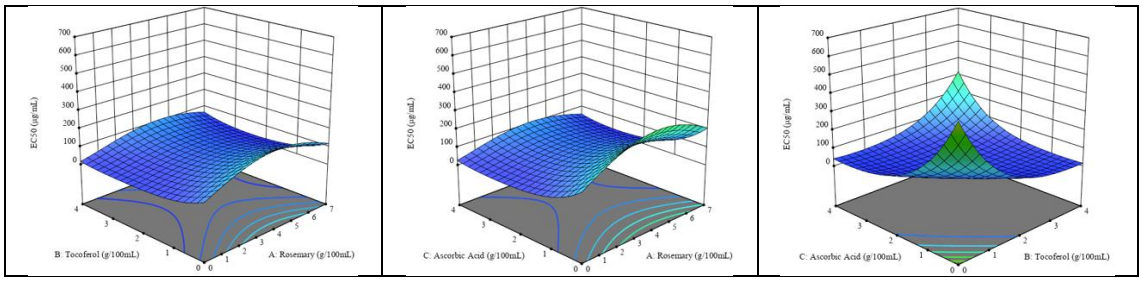



**Figure 20.** 3D surface plots of the factors combined in pairs.

### 3.2. Antimicrobial activity

Tables 4, 5 and 6 presents the antimicrobial activity of the coating solutions against several Gram-negative and Gram-positive bacteria, as well as against fungi, considered food contaminants. The maximum concentration tested (mg/mL) was different for each sample, considering the sum of all components in each coating solution (Table 1).

For Gram-negative bacteria (Table 4), all coating solutions did not present antibacterial or antifungal activity at the maximum concentration tested, with the exception of *Y. enterocolitica* and *E. cloacae* (sample 4) in which the concentration of the rosemary extract was higher in comparison with the concentration of ascorbic acid.

The main distinction between Gram-positive and Gram-negative bacteria is found in the chemical composition and organization of their cell walls. Compared to Gram-positive bacteria, which have an outer membrane made of lipopolysaccharides (LPS) and a thicker coating of peptidoglycan, gram-negative bacteria have a thin layer of peptidoglycan around them. Their sensitivity to preservative chemicals is significantly influenced by this difference in cell wall structure (Silhavy et al., 2010).

**Table 4.** Gram-negative antibacterial activity (mg/mL) of the coating solutions.

| Run | Gram-negative bacteria | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ***Enterobacter cloacae*** | | ***Escherichia coli*** | | ***Pseudomonas aeruginosa*** | | ***Salmonella enterica*** | | ***Yersinia enterocolitica*** | |
| | **MIC** | **MBC** | **MIC** | **MBC** | **MIC** | **MBC** | **MIC** | **MBC** | **MIC** | **MBC** |
| 1 | >9 | >9 | >9 | >9 | >9 | >9 | >9 | >9 | 9 | >9 |
| 2 | >7.5 | >7.5 | >7.5 | >7.5 | >7.5 | >7.5 | 7.5 | >7.5 | 7.5 | >7.5 |
| 3 | >7.5 | >7.5 | >7.5 | >7.5 | >7.5 | >7.5 | 7.5 | >7.5 | 7.5 | >7.5 |
| 4 | 9 | >9 | >9 | >9 | >9 | >9 | 9 | >9 | 9 | >9 |
| 5 | >7.5 | >7.5 | >7.5 | >7.5 | >7.5 | >7.5 | >7.5 | >7.5 | 7.5 | >7.5 |
| 6 | >2 | >2 | >2 | >2 | >2 | >2 | >2 | >2 | 2 | >2 |
| 7 | >11.5 | >11.5 | >11.5 | >11.5 | >11.5 | >11.5 | 11.5 | >11.5 | 11.5 | >11.5 |
| 8 | >6 | >6 | >6 | >6 | >6 | >6 | 6 | >6 | 6 | >6 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 9 | >7.5 | >7.5 | >7.5 | >7.5 | >7.5 | >7.5 | 7.5 | >7.5 | 7.5 | >7.5 |
| 10 | >2 | >2 | >2 | >2 | >2 | >2 | >2 | >2 | 2 | >2 |
| 11 | >13 | >13 | >13 | >13 | >13 | >13 | 13 | >13 | 13 | >13 |
| 12 | >13 | >13 | 13 | >13 | >13 | >13 | 13 | >13 | 13 | >13 |
| 13 | >3.5 | >3.5 | >3.5 | >3.5 | >3.5 | >3.5 | >3.5 | >3.5 | 3.5 | >3.5 |
| 14 | >7.5 | >7.5 | >7.5 | >7.5 | >7.5 | >7.5 | >7.5 | >7.5 | 7.5 | >7.5 |
| 15 | >6 | >6 | >6 | >6 | >6 | >6 | >6 | >6 | 6 | >6 |
| 16 | >7.5 | >7.5 | >7.5 | >7.5 | >7.5 | >7.5 | 7.5 | >7.5 | 7.5 | >7.5 |
| 17 | >7.5 | >7.5 | 3.75 | >7.5 | >7.5 | >7.5 | >7.5 | >7.5 | 7.5 | >7.5 |
| STR | 0.007 | 0.007 | 0.01 | 0.01 | 0.06 | 0.06 | 0.007 | 0.007 | 0.007 | 0.007 |
| MET | n.t. | n.t. | n.t. | n.t. | n.t. | n.t. | n.t. | n.t. | n.t. | n.t. |
| AMP | 0.15 | 0.15 | 0.15 | 0.15 | 0.63 | 0.63 | 0.15 | 0.15 | 0.15 | 0.15 |

MIC – minimal inhibitory concentration; MBC – minimal bactericidal concentration; STR – Streptomicin 1 mg/mL; MET – methicillin 1 mg/mL; AMP – ampicillin 10 mg/mL; n.t. – not tested.

On the other hand, for Gram-positive bacteria (**Table 5**), the increase on the concentration of the ingredients other than rosemary may reduce antimicrobial activity (samples 1, 4, 11 and 12). Solutions containing only rosemary (sample 13) showed the best antimicrobial activities, even at lower concentrations, but only for *B. cereus* and *S. aureus*. For *S. aureus* and *B. cereus*, coating solutions containing rosemary extracts showed the best antimicrobial activities against these two bacteria. Therefore, the addition of other ingredients created a possible antagonistic effect on antimicrobial activity. Finally, the best results against *L. monocytogenes* were obtained with samples 8 and 10, in which sample 10 contained only ascorbic acid in its formulation and sample 8 the major component was also ascorbic acid.

**Table 5.** Gram-positive antibacterial activity (mg/mL) of the coating solutions.

| Run | Gram-positive bacteria | | | | | |
|---|---|---|---|---|---|---|
| | ***Bacillus cereus*** | | ***Listeria monocytogenes*** | | ***Staphylococcus aureus*** | |
| | **MIC** | **MBC** | **MIC** | **MBC** | **MIC** | **MBC** |
| 1 | 4.5 | 9 | 4.5 | >9 | 1.125 | >9 |
| 2 | 3.75 | >7.5 | 7.5 | >7.5 | 7,5 | >7.5 |
| 3 | 7.5 | >7.5 | 7.5 | >7.5 | 7.5 | >7.5 |
| 4 | 2.25 | >9 | 4.5 | >9 | 2.25 | >9 |
| 5 | 7.5 | >7.5 | 7.5 | >7.5 | 7.5 | >7.5 |
| 6 | >2 | >2 | >2 | >2 | >2 | >2 |
| 7 | 5.75 | >11.5 | 11.5 | >11.5 | 6.75 | 11.5 |
| 8 | 6 | >6 | 1.5 | >6 | >6 | >6 |
| 9 | 3.75 | >7.5 | 7.5 | >7.5 | 7.5 | >7.5 |
| 10 | >2 | >2 | 1 | >2 | >2 | >2 |
| 11 | 6.5 | >13 | 13 | >13 | 3.25 | >13 |
| 12 | 13 | >13 | 6.5 | >13 | 3.25 | >13 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 13 | 1.75 | >3.5 | >3.5 | >3.5 | 0.875 | 3.5 |
| 14 | 3.75 | 7.5 | >7.5 | >7.5 | 3.75 | 7.5 |
| 15 | >6 | >6 | >6 | >6 | >6 | >6 |
| 16 | 7.5 | >7.5 | >7.5 | >7.5 | 3.75 | >7.5 |
| 17 | >7.5 | >7.5 | 3.75 | >7.5 | 3.75 | >7.5 |
| STR | 0.007 | 0.007 | 0.007 | 0.007 | 0.007 | 0.007 |
| MET | n.t. | n.t. | n.t. | n.t. | 0.007 | 0.007 |
| AMP | n.t. | n.t. | 0.15 | 0.15 | 0.15 | 0.15 |

MIC – minimal inhibitory concentration; MBC – minimal bactericidal concentration; STR – Streptomicin 1 mg/mL; MET – methicillin 1 mg/mL; AMP – ampicillin 10 mg/mL; n.t. – not tested.

Regarding the antifungal activity (Table 6), for both *A. brasiliensis* and *A. fumigatos,* samples 6 and 10 were the most effective in inhibiting/killing these fungi as it can be seen by the lower MIC/MBS values. These samples have the presence of only α-tocopherol and ascorbic acid, respectively. In this case, the presence of the rosemary extract decreased the antifungal potential of the samples.

**Table 6.** Antifungal activity (mg/mL) of the coating solutions.

| Run | *Apergillus brasiliensis* | | *Aspergillus fumigatus* | |
|---|---|---|---|---|
| | **MIC** | **MFC** | **MIC** | **MFC** |
| 1 | 9 | >9 | 9 | >9 |
| 2 | >7.5 | >7.5 | 7.5 | >7.5 |
| 3 | >7.5 | >7.5 | 7.5 | >7.5 |
| 4 | 9 | >9 | 9 | >9 |
| 5 | 7.5 | >7.5 | 7.5 | >7.5 |
| 6 | 2 | 2 | 2 | 2 |
| 7 | 11.5 | >11.5 | 11.5 | >11.5 |
| 8 | 6 | >6 | 6 | >6 |
| 9 | 7.5 | >7.5 | 7.5 | >7.5 |
| 10 | 2 | >2 | 2 | >2 |
| 11 | 13 | >13 | 13 | >13 |
| 12 | 13 | >13 | 13 | >13 |
| 13 | 3.5 | >3.5 | 3.5 | >3.5 |
| 14 | 7.5 | >7.5 | >7.5 | >7.5 |
| 15 | 6 | >6 | 6 | >6 |
| 16 | >7.5 | >7.5 | 7.5 | >7.5 |
| 17 | >7.5 | >7.5 | >7.5 | >7.5 |
| KET | 0.06 | 0.125 | 0.5 | 1 |

MIC: minimal inhibitory concentration; MFC - minimal fungicidal concentration; KET – ketoconazole 1 mg/mL.

Although several studies have noted beneficial interactions between rosemary extract, tocopherol, and ascorbic acid, little is known about their possible detrimental interactions with antimicrobial activity. However, according to Del Campo *et al.* (2000), the antimicrobial activity of rosemary extract (*R. officinalis*) against various foodborne pathogens, including *L. monocytogenes*, the rosemary extract had strong antimicrobial activity against *L. monocytogenes*, with a minimum inhibitory concentration (MIC) of 0.5% and a minimum bactericidal concentration (MBC) of 1%. The study also investigated the mechanism of action of rosemary extract against *L. monocytogenes*. The results showed that rosemary extract disrupted the cell membrane of *L. monocytogenes*, leading to leakage of intracellular contents and cell death (Del Campo *et al*., 2000).

Przekwas *et al*. (2020) showed that vitamin C (ascorbic acid) has a notable inhibitory effect on the growth of *L. monocytogenes* in biofilms. The sensitivity to vitamin C is strain dependent, for *L. monocytogenes*, and the inhibition of bacterial growth was observed at concentrations ranging from 0.25 to 25.0 mg/mL. Statistically significant differences in bacterial growth inhibition after vitamin C treatment compared to the positive control were observed for the concentrations of 25.0 mg/mL, 2.50 mg/mL, 0.25 mg/mL, and 25.0 µg/mL. The only concentration that did not show a difference compared to the positive control was 2.50 µg/mL. There were no statistically significant differences between the strong and weak biofilm producer groups of *L. monocytogenes* (Przekwas *et al*., 2020).

The results presented in Table 7 represent the fit statistics of the models obtained for the MIC values of antimicrobial activity for the bacteria *B. cereus*, *L. monocytogenes* and *S. aureus*, respectively. The minimum bactericidal concentration was not evaluated in this methodology, since it exceeds the limits of the maximum concentration tested and, therefore, is outside the optimization domain. Only Gram-positive bacteria were evaluated since they were the only ones that showed antimicrobial activity below the maximum concentration tested. *P*-values less than 0.05 indicate that the model terms are significant.

**Table 7.** Fit statistics of the models obtained for MIC values of Gram-positive bacteria.

| | Parameter | Value | Info |
|---|---|---|---|
| *B. cereus* | Model | p-value | 0.0033 - Significant |
| | Lack of Fit | p-value | 0.83 Not significant |

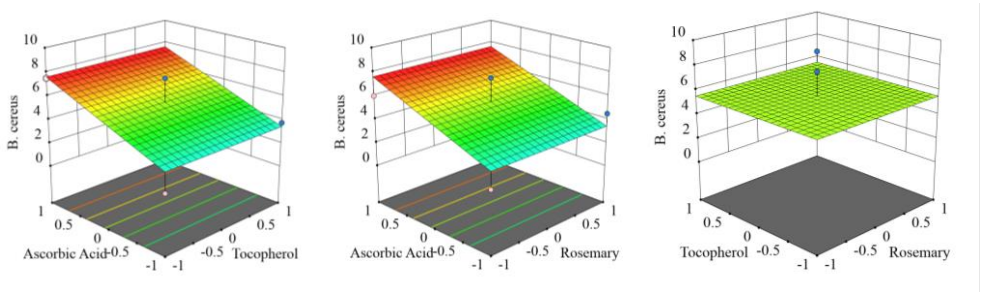
| | | | |
|---|---|---|---|
| | $R^2$ | 0.4714 | - |
| | Adjusted $R^2$ | 0.4186 | - |
| | $MIC_{B.\ cereus} = 5.51 + 2.09\ C$ | | |
| *L. monocytogenes* | Model | p-value | 0.0137 - Significant |
| | Lack of Fit | p-value | 0.0019 Not significant |
| | $R^2$ | 0.9245 | - |
| | Adjusted $R^2$ | 0.9115 | - |
| | $MIC_{L.\ monocytogenes} = 0.84 - 2.10\ A + 1.52\ B - 1.20\ C + 4.54\ AB + 2.15\ B^2 + 5.22\ C^2$ | | |
| *S. aureus* | Model | p-value | < 0.0001 - Significant |
| | Lack of Fit | p-value | - |
| | $R^2$ | 0.9929 | - |
| | Adjusted $R^2$ | 0.9544 | - |
| | $MIC_{S.\ aureus} = 7.50 - 0.77\ A + 1.36\ B + 1.5\ C - 0.7AB - 0.47\ AC - 2.41\ A^2 - 1.72\ B^2 - 2.00\ C^2$ | | |

MIC: minimum inhibitory concentration; A: concentration of rosemary; B: concentration of tocopherol; C: concentration of ascorbic acid (g/100 mL)

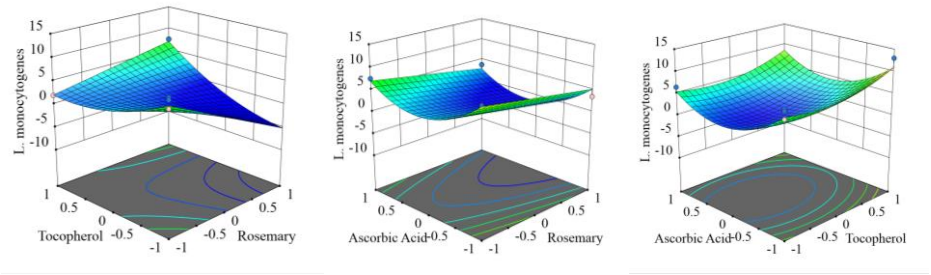
For *B. cereus*, a linear model best described the behavior of the bacteria against the coating solution, while for *L. monocytogenes* and *S. aureus*, a quadratic model showed the best results. Although the 3 models were statistically significant (*p*-value < 0.05) and did not present lack of fit (*p-value* > 0.05), only *L. monocytogenes* and *S. aureus* showed a good adjusted $R^2$ result (above 0.9), while the statistical model of *B. cereus* presented an adjusted $R^2$ of 0.4186, not considered adequate.

Figure 21 shows the 3D surface plots of the factors (rosemary, alpha-tocopherol, and ascorbic acid concentration) combined in pairs for *B. cereus*, *L. monocytogenes* and *S. aureus*, respectively. The evaluation of the plots is based on the lowest possible results (blue coloration), since the lower the MIC values, the better the antimicrobial activity of the coating solution.

(a)




(b)




(c)

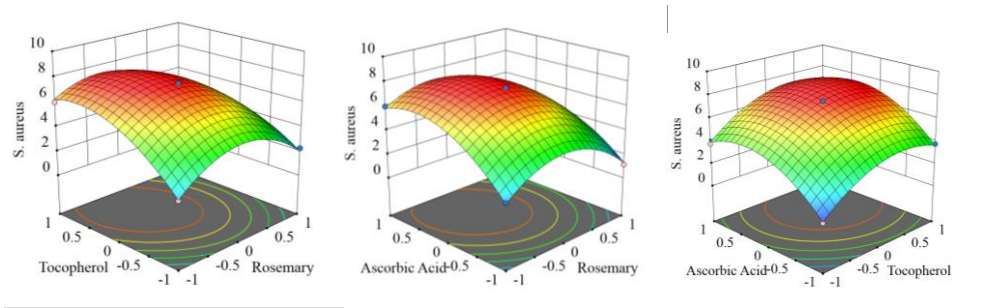



**Figure 21.** 3D surface plots of the factor combined in pairs for (a) *B. cereus*, (b) *L. monocytogenes*, and (c) *S. aureus.*

From the 3D surface plots (Figure 21) and the optimal points (Table 8), it is possible to see the influence of each component on the antimicrobial activity for each Gram-positive bacteria tested.

**Table 8.** Optimal points for each Gram-positive bacteria

| Concentration (g/100 mL) | | | MIC values (mg/mL) | | |
|---|---|---|---|---|---|
| Rosemary | Tocopherol | Ascorbic Acid | *B. cereus* | *L. monocytogenes* | *S. aureus* |
| 0.00 | 0.20 | 0.20 | 3.59 | - | - |
| 0.03 | 0.20 | 0.20 | - | 1.154 | - |
| 0.68 | 0.20 | 0.20 | - | - | 0.864 |

MIC: minimum inhibitory concentration

For *B. cereus*, ascorbic acid seems to have a greater influence on the antimicrobial activity compared to the other ingredients (rosemary and α-tocopherol). This result is also observed in the third plot (Figure 21a), in which no variations in antimicrobial activity were observed when the ascorbic acid concentration was fixed. Despite the obtained results, it should be noted that for *B. cereus,* the model may not be adequate to evaluate the behavior of the coating solution due to the low adjusted $R^2$ value (Table 7).

For *L. monocytogenes,* the concentration of α-tocopherol and ascorbic acid has a greater influence on the antimicrobial activity, while for *S. aureus,* the concentration of rosemary extract is able to contribute to a better activity compared to the other ingredients (Table 8). Therefore, the results reinforce the importance of each ingredient in the coating solution as a whole, as each component is capable of assigning a preservative function to different bacteria.

## 4. Conclusions

In conclusion, the study on the balance of antioxidants in the SpraySafe formulation composed of rosemary extract, ascorbic acid, and tocopherol highlights the importance of biological and sustainable food packaging solutions. The development of innovative and sustainable packaging materials is crucial in addressing the environmental issues associated with synthetic plastics and their resistance to degradation.

By focusing on edible and biodegradable films based on renewable and abundant natural resources, such as the antioxidants investigated in the present work, it was possible to harness cost-effective and non-toxic materials for food packaging and preservation purposes. The combination of rosemary extract, ascorbic acid, and tocopherol offers a promising avenue for enhancing both antioxidant and antimicrobial activities in food biofilms.The presence of natural antioxidants in the formulation not only contributes to the preservation and shelf life of food products, but also provides health benefits by reducing oxidative damage and the growth of harmful microorganisms. By optimizing the ratios and concentrations of these antioxidants, the study aimed to identify the ideal combination that maximized the overall antioxidant and antimicrobial properties of food biofilms. The results showed that the coating solutions did not exhibit antimicrobial activity against Gram-negative bacteria at the maximum tested concentration. However,

for Gram-positive bacteria, coating solutions containing only rosemary extract showed the best antimicrobial activity, particularly against *B. cereus* and *S. aureus*. Higher concentrations of ascorbic acid demonstrated the best results against *L. monocytogenes*, maybe due to the disruption of the cell membrane. The results obtained from the analysis of minimum inhibitory concentration (MIC) values revealed different patterns for different bacteria. *B. cereus* showed a linear behavior, whereas quadratic models were found to be more appropriate for describing the behavior of *L. monocytogenes* and *S. aureus*. The impact of individual ingredients varied, with ascorbic acid demonstrating significant efficacy against *B. cereus*, while α-tocopherol and ascorbic acid exhibited greater influence against *L. monocytogenes*. Regarding antioxidant activity, the experimental design successfully optimized the combination of rosemary extract, α-tocopherol, and ascorbic acid. A quadratic model provided a good fit and indicated a strong relationship. The ideal composition for achieving the highest antioxidant activity was determined to be 0.14 g/100 mL of rosemary extract, 1.81 g/100 mL of α-tocopherol, and 1.66 g/100 mL of ascorbic acid. This study emphasized the significance of each ingredient in both antimicrobial and antioxidant properties of the coating solution. It also provided valuable insights into the individual and combined effects of these ingredients.

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