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Investigation and development of innovative protocols and technologies to enhance the safety of food and to reduce the food loss

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Abstract

Food technologies have played a crucial role since the beginning of human civilization. The evolution of food processing and packaging have led to an increase of food quality and safety, improving the quality of human life. Recently, academic research and industries have gained awareness about the economic and environmental impact of conventional technologies. This consciousness oriented the efforts towards more sustainable techniques and materials, paving the way to a new "green era" of food technology. This PhD project is an example of multidisciplinary approach in which food microbiology, biomaterial science, extraction techniques, and statistic tools are synergistically applied to develop and test sustainable protocols and active packaging materials with promising applications in food sector. An outline of the thesis chapters is provided below.

Chapter 1 introduces sustainable non-thermal technologies as promising substitutes of conventional thermal treatments to ensure food safety and quality. A focus is dedicated to biodegradable polymers from renewable sources (e.g., agri-food by-products) and their application to produce active packaging films with antimicrobial and antioxidant properties. Moreover, this chapter gives to the readers an overview about the concept of "hurdle technology".

Chapter 2 aims to evaluate cold storage coupled with gaseous ozone as a prospective strategy to inhibit pathogenic and spoilage bacteria growth in food storage cold chambers. The research investigated the impact of gaseous ozone (0.05 ppm, different exposures) on the bacterial contamination of internal surfaces and air in a cold chamber (3°C). The effectiveness of this combination of technologies was also tested in vitro against *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enterica* Typhimurium, *Campylobacter jejuni*, and *Pseudomonas fluorescens*.

Chapter 3 focuses on the complex interaction between the properties of biodegradable films and the manufacturing parameters, leading the need for statistical models to describe and predict this interdependence. This study analysed the impact of 8 compositional and drying factors on microstructural and functional properties of films based on chitosan and pectin through a multivariate approach. 32 formulations were developed and the results were analysed through principal component analysis (PCA). An in-depth discussion of the results was provided, highlighting the suitability of multivariate data analysis to predict the technical behaviour of films related to the manufacture process.

Chapter 4 explores the use of plant leaf extracts to produce active biodegradable films with antioxidant properties. This research aimed to set up an optimised protocol to extract polyphenols from sage and nettle leaves and to incorporate them into films based on chitosan/hydroxypropyl methylcellulose (CS/HPMC) blend, hence characterising their structural, technical, and antioxidant performances. The results showed that the obtained natural antioxidant films could be employed as valuable alternative to synthetic plastics to prolong the shelf-life of food products.

In **Chapter 5**, cellulose nanocrystals (CNCs) were isolated from orange peel discarded by orange juice industry using an alkaline/H_2O_2 bleaching followed by sulfuric acid hydrolysis. Extracted CNCs were added as reinforcing agent into CS/HPMC films enriched with lauroyl arginate ethyl (LAE) to produce nanocomposite antimicrobial films. The biocidal activity of the films against *E. coli*, *S. enterica*, *L. monocytogenes*, and *P. fluorescens* was tested. Overall, nanocomposite films enriched with LAE showed potentiality as a suitable strategy to replace antimicrobial petroleum-derived materials and to valorise discarded orange peels, using food waste to reduce food loss.

Riassunto

Le tecnologie alimentari giocano un ruolo fondamentale dall'inizio della civiltà umana. L'evoluzione dei processi e del packaging hanno determinato un aumento della sicurezza e della qualità alimentare, migliorando la vita delle persone. Attualmente, il mondo accademico e l'industria sono divenuti consapevoli dell'impatto ecologico ed economico delle tecnologie convenzionali, spingendoli ad orientarsi verso tecniche e materiali sostenibili e inaugurando l'era "green" delle tecnologie alimentari. Questo progetto di PhD ha avuto lo scopo di sviluppare e testare protocolli e biomateriali attivi sostenibili per applicazioni alimentari attraverso un approccio multidisciplinare comprendente microbiologia alimentare, scienza dei materiali, tecniche estrattive e strumenti statistici. Si riporta una descrizione dei capitoli della tesi.

Il **Capitolo 1** introduce le tecnologie non termiche come alternativa ai trattamenti convenzionali per garantire la sicurezza degli alimenti. Un particolare focus è dedicato ai polimeri biodegradabili da fonti rinnovabili (es. sottoprodotti alimentari), impiegati per la produzione di packaging attivo con funzione antimicrobica e antiossidante. Il capitolo introduce inoltre il concetto di "teoria degli ostacoli".

Il **Capitolo 2** valuta l'impiego sinergico della refrigerazione e dell'ozono gassoso come strumento per inibire la crescita di batteri ambientali e patogeni all'interno di celle frigo per lo stoccaggio di alimenti. La ricerca ha valutato l'impatto dell'ozono a bassa concentrazione (0.05 ppm, diversi periodi) sulla popolazione batterica presente sulle pareti e nell'aria interna a una cella appositamente costruita. L'efficacia del trattamento è stata testata sulla crescita in vitro di *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* Typhimurium, *Campylobacter jejuni*, and *Pseudomonas fluorescens*.

Il **Capitolo 3** mette in luce la complessa interazione tra proprietà dei film biodegradabili e parametri di produzione, con conseguente necessità di strumenti statistici che descrivano e predicano in modo efficace tale interdipendenza. La ricerca ha analizzato l'influenza di 8 fattori compositivi e di essiccazione sulle proprietà strutturali e tecniche di film in chitosano e pectina attraverso un approccio statistico multivariato. Sono state sviluppate 32 formulazioni di packaging in base alla variazione dei suddetti parametri e i risultati sono stati analizzati mediante PCA. La discussione dei risultati ha confermato l'idoneità degli strumenti di analisi multivariata per lo studio del comportamento tecnico dei film biodegradabili sulla base del processo produttivo.

Il **Capitolo 4** esplora il mondo degli estratti vegetali applicati alla produzione di biofilm attivi con funzione antiossidante. E' stato messo a punto un protocollo ottimizzato per l'estrazione di polifenoli

da foglie di salvia e ortica, i quali sono stati incorporati all'interno di film in chitosano e idrossipropil metilcellulosa (CS/HPMC). Le proprietà tecniche e antiossidanti dei film sono state discusse, mettendo in luce la possibilità di impiegare questi film in sostituzione a packaging sintetico per il confezionamento di alimenti sensibili all'ossidazione.

Nel **Capitolo 5**, sono stati isolati nano-cristalli di cellulosa da bucce di arancia di scarto mediante un processo di bleaching alcalino seguito da idrolisi acida. I nano-cristalli sono stati impiegati per la produzione di biofilm nano-rinforzati in CS/HPMC, ai quali è stato aggiunto lauroil etil arginanto (LAE) con funzione antimicrobica. Le proprietà tecniche e battericide dei film sono state misurate. I film prodotti si sono dimostrati promettenti per la sostituzione di film antimicrobici sintetici. Inoltre, l'utilizzo di bucce di arancia di scarto ha dimostrato come i sottoprodotti alimentari, opportunamente valorizzati, possano diventare uno strumento atto a ridurre lo spreco alimentare.

Chapter 1

Non-thermal techniques and sustainable active packaging: the new era of food technologies

Abstract

Food technologies have played a crucial role since the beginning of human civilization. The evolution of food processing and packaging have led to an increase of food quality and safety, improving the quality of human life. Recently, academic research and industries have gained awareness about the economic and environmental impact of conventional technologies. This consciousness oriented the efforts towards more sustainable techniques and materials, paving the way to a new "green era" of food technology.

My third year of PhD was partially focused on providing a schematic overview about emerging sustainable technologies and materials as promising substitutes of conventional thermal treatments and packaging systems to ensure food safety and quality.

In the first section of this "review project", seven novel non-thermal technologies were highlighted, describing their theoretical principles, mechanism of action, effect on microorganisms, advantages, and limitations. The described technologies are: ozone, non-thermal plasma (NTP), high pressure processing (HPP), pulsed electric field (PEF), ultrasounds (US), ionizing irradiation, and ultraviolet irradiation (UV). Besides, the concept of hurdle technology to overcome the criticisms related to single processing techniques, was explored.

In the second section, a focus was dedicated to biodegradable polymers from renewable sources (e.g., agri-food by-products) and their application to produce active packaging films with antimicrobial and antioxidant properties. The concept of active packaging was introduced. Then, the incorporation of plant extracts and food waste derivatives as emerging green strategy to produce biodegradable active films with enhanced technical performances and to valorise agri-food by-products was investigated.

1. Introduction

Food technologies have played a crucial role since the beginning of human civilization. Throughout the centuries, the evolution of food processing and packaging has led to an increase of food quality and safety, improving the quality of human life (Kaavya et al., 2021).

Recently, academic research, industries, and consumers have gained awareness about the impact of agri-food practices on economy, environment, and human health. These concerns have oriented the food sector towards the development and adoption of novel and sustainable technologies, paving the way to a "green era" of food technology.

Among the main pillars of this complex and multifaceted process (Dugmore, Clark, Bustamante, Houghton, & Matharu, 2017), it is worth citing three lines of research, that have deeply contributed to re-define the concept of "*food technology*" (Batiha et al., 2021; Lòpez-Pedrouso et al., 2019):

(I) Substitution of thermal techniques such as sterilization and pasteurization, and chemical sanitisation with green alternatives, in order to reduce energy (i.e., combustion of fossil fuels) and chemicals consumptions, and the impact on the nutritional and sensorial properties of food;

(II) Development of biodegradable and active packaging based on renewable biopolymers, aiming to (i) reduce the use of petroleum-derived plastics in the food packaging sector, and (ii) prolong the shelf-life of the packed products, preventing the generation of food waste;

(III) Extraction of added-value biological compounds from renewable resources (e.g., food waste and by-products; herbs, spices, etc.) through a biorefinery approach, and their application as alternatives to conventional preservatives and additives.

In this context, this work gives a synthetic overview about the trend strategies which are leading the food industry towards green technology and sustainability criteria, reducing the energy consumption, waste generation, and footprints on the environment. The first section explores the world of "non-thermal technologies" for food sanitisation, and the concept of "hurdle technology" as a strategy to overcome the criticisms related to single processing techniques. The second section is dedicated to biodegradable polymers from renewable sources (e.g., agri-food by-products) and their application to produce active packaging items with antimicrobial, antioxidant and nano-reinforced properties as prospective substitute of conventional plastic materials.

2. Emerging non-thermal technologies to enhance food safety

Food safety has always constituted a matter of public interest in both developing and developed countries. In fact, foodborne diseases are amongst the major concerns affecting public health (Luksiene, 2021). Foodborne diseases are mainly caused by the growth, spread, and metabolic activity of microorganisms such as bacteria, yeasts, moulds, and protozoa in food. Besides, the spread of spoilage microorganisms can compromise the quality and thus the shelf life of the product, generating a relevant economical damage. Most food products are highly exposed to the risk of cross-contamination during the several processing steps from harvest to consumption. Therefore, the control of unintentional microbial contamination is compulsory in the food processing industries to guarantee the safety and quality of the products (Sakudo, 2017).

In ancient times, food preservation techniques mainly consisted in the control of parameters such as water activity (aw), pH, and environmental conditions. Later, plant sanitisation with chemical disinfectants, high-temperature treatments, and chemical preservatives gained wide recognition due to their significant efficiency in food decontamination (Kaavya et al., 2021). These technologies, and particularly thermal treatments, represent a fundamental pillar of the food processing industry. Over years and years, these methods have been extensively applied to ensure a proper decontamination of foods, so much that today they are regarded as the "conventional" strategies to guarantee the food safety (Aaliya et al., 2021).

Despite the obvious benefits, the use of conventional decontamination technologies based on thermal treatment hides not negligible criticisms. Firstly, high temperature can hinder food quality by lowering its nutritional values and freshness, affecting the organoleptic properties, and generating chemical derivatives harmful for consumer health (Rifna, Singh, Chakraborty, & Dwivedi, 2019). Besides, these technologies are highly energy consuming. This energy mainly derives from the combustion of fossil fuels, and this generates a remarkable ecologic and economic impact on human society and environment (Chakka, Sriraksha, & Ravishankar, 2021).

The drawbacks related to the conventional decontamination processes prompted the development and adoption of novel non-thermal technologies, having the potential to improve the product quality and safety with reduced water and energy consumptions (Fengying, Min, Kai, & Arun, 2020). These physical processing techniques are effective for microbial decontamination, non-toxic, environment-friendly, and residual-free. However, they could present setbacks related to investments in equipment, maintenance costs, and required skills, which can hinder their spread in food industry (Priyadarshini, Rajauria, O'Donnell, & Tiwari, 2019). Moreover, the diffusion of some of these techniques is limited

by the low consumer acceptability and the resistance of regulatory authorities (Khan, Tango, Miskeen, Lee, & Oh, 2017).

The current section aims to highlight seven novel non-thermal technologies that are currently emerging as promising substitutes of conventional decontaminating treatments, describing their theoretical principles, mechanism of action, effect on microorganisms, advantages, and limitations. The described technologies are: ozonation, non-thermal plasma (NTP), high pressure processing (HPP), pulsed electric field (PEF), ultrasounds (US), ionizing irradiation, and ultraviolet irradiation (UV). Besides, the concept of hurdle technology, namely the combination of two or more preservation technologies at mild levels to overcome the criticisms related to single processing techniques, is explored.

2.1. Ozonation

Ozone (O_3) is a triatomic allotrope of oxygen. Discovered by Schoembein in 1840 and regarded as one of the most effective and sustainable biocidal agents, ozone treatment is commonly employed as sanitising technology in a broad range of processes to ensure and enhance the safety and quality of many food products (Niveditha et al., 2021). Ozone, as a powerful oxidant agent, is highly effective against several types of microorganisms including viruses, algae, bacteria, and fungi, and ably counteracts the presence of mycotoxin contaminants within foods (Deng et al., 2020). As ozone can spontaneously decompose into oxygen, leaving no hazardous residues on foods, its usage as a decontaminating agent has been approved as safe by the US Food and Drug Administration (FDA) since 2001 (Rice & Graham, 2001). Progressively, ozone application either in aqueous or gaseous forms has been promisingly extended to the stages of post-harvest, processing, and storage of foods, including fruits, vegetables, meat and poultry at levels set by central agencies (Pandiselvam et al., 2020; Prabha, Barma, Singh, & Madan, 2015).

The production of ozone requires a generator, an oxygen source, dust filters, a contact unit, and a destruct unit (Pandiselvam et al., 2019). Ozone production involves two subsequent processes, namely the splitting, also known as photodissociation or photolysis, and further re-combination of oxygen molecules. When a certain amount of energy is provided to diatomic oxygen, which splits into free radicals, the resultant oxygen atoms undergo numerous collisions with each other, and with stable O_2. These collisions release a combination of O_2 and O with an excess of energy, whose dissipation leaves intact and stable ozone molecules (Niveditha et al., 2021). This reaction is called a "three-body collision" and can be triggered by means of two key approaches: UV-light radiation and

corona discharge. In the corona discharge method, the most commonly applied for ozone generation, pure oxygen or a gas mixture containing oxygen (e.g., air) passes through the gap between two electrodes supplied with a high-energy electrical field, which enables diatomic oxygen to split into free radicals. The two electrodes are separated by a dielectric material like glass. Another technique widely applied to produce ozone is the dielectric barrier discharge (DBD) method, which gained popularity due to the reduced power consumption and higher conversion efficiency. This technology employs high-voltage alternating current between two electrodes separated by an insulating dielectric barrier. Moreover, ozone can also be generated directly in water by applying electrolytic ozone generators. In these conditions, an electrical discharge is used to split the water molecules into H_2 and O_2, which is furtherly converted into O and then O_3 (Kaavya et al., 2021).

Due to its strong oxidizing power, ozone possesses a broad range of activities, which result in a countless number of prospective applications such as pathogens disinfection (Yeoh, Ali, & Forney, 2014), reduction of mycotoxins (Rifna et al., 2019), starch modification (Pandiselvam et al., 2019), storage of fresh products (Feliziani, Romanazzi, & Smilanick, 2014), sanitation of processing equipment and packaging films (Almeida & Gibson, 2016), and pesticide removal (Pandiselvam et al., 2020).

Concerning the antimicrobial activity of ozone, this gas aids in microbial destruction by interacting with the cellular components, which are gradually led to oxidation. Firstly, ozone interacts with the polyunsaturated fatty acids (PUFAs) of the cell envelope, converting them into acid peroxides (Khan et al., 2017). This process causes the progressive disintegration of the cell membrane, resulting in the leakage of cell contents, and eventually cellular lysis. Secondly, ozone oxidizes amino acids, peptides, proteins, and enzymes, mainly attacking the sulfhydryl groups in these molecules. Accordingly, as the gas enters the microbial cells, all essential components, proteins, RNA, DNA, and enzymes undergo a complete oxidation and result in the destruction of cells (Brodowska, Nowak, & Smigielski, 2018). As well, ozone has been discovered to actively degrade mycotoxins. In this case, the chemical pathway of degradation relates to the chemical structure of mycotoxin (Temba et al., 2016).

The key factors that affect the ozone efficacy for all its specific applications are the treatment mode (i.e., aqueous or gaseous), inlet gas composition, exposure time, ozone concentration, and the intrinsic microbial sensitivity (Bahrami, Baboli, Schimmel, Jafari, & Williams, 2020; Rifna et al., 2019). For example, many studies highlighted that the spores are far more resilient to ozone than vegetative cells (Ramaswamy, Ahn, Balasubramaniam, Saona, & Yousef, 2019). As well, bacteria are more vulnerable than yeasts and fungi, while the greater or lower resistance of gram-positive and gram-negative bacteria is still debated (Deng et al., 2019). Another crucial condition that determines the

effectiveness of ozone treatment is the environment of application. The presence of organic material such as proteins and fat in the medium drastically decreases the effectiveness of ozone. Also, the stability of ozone increases with a lower pH of the medium (Pandiselvam, Sunoj, Manikantan, Kothakota, & Hebbar, 2017).

Disinfection of food products by aqueous and gaseous ozone treatment has been extensively studied and reviewed. Many studies tested the efficacy of this treatment for the decontamination of vegetables such as onions, lettuce, and fruits, including tomatoes, strawberries, and peaches (Chakka et al., 2021; Xu & Wu, 2014). This studies mainly focused on the capacity of ozone to inactivate food-borne pathogens such as *Salmonella enterica* (Kaavya et al., 2021) and *Escherichia coli* (Niveditha et al., 2021), but a comprehensive overview about the impact of ozone on the intrinsic features of food was also provided. The application of this technique was extended also to the meat and poultry sector (Kalchayanand, Worlie, & Wheeler, 2019), and cheese storage (Cavalcante, Leite Júnior, Tribst, & Cristianini, 2013) with promising results

Overall, the oxidising performances and the global sustainability of ozone make it a valuable potential alternative to conventional agents such as chlorine for food-related operations (e.g., cleaning and sanitisation) (Chakka et al., 2021). With about 50% higher oxidizing potential than chlorine, ozone efficiently inactivates fungi, viruses, bacteria, plus reducing the impact of mycotoxins and contaminants such as pesticides. It requires shorter contact time for disinfection when compared to other techniques. An excess amount of ozone rapidly auto-decomposes to O_2, leaving no chemical residues in food (Dilmaçünal & Kuleaşan, 2018). The treatment requires no thermal energy, and so it is suitable for heat-sensitive products and reduces the need for input energy. Not requiring storage or transportation, the treatment has relatively low running costs. Thus, it comprehensively constitutes an environmentally sustainable and commercially feasible technology.

Despite the relevant benefits, ozone at low concentrations or short exposures could insufficient to remarkably affect the spread of microorganisms and mycotoxins. On the other hand, higher ozone concentration can become corrosive, leading to modification in sensorial and nutritional attributes of food products, with notable differences related to the food category. Furthermore, several degradation products and/or by-products can be formed during ozone treatment, which may have toxicological properties (Temba et al., 2016). Globally, ozone represents a toxic gas with high oxidation capacity, and thus the safety of operators and the rate of surfaces corrosion of equipment should be carefully considered.

2.2. Atmospheric Cold Plasma (ACP)

Plasma treatment is an innovative biocidal technology which is becoming more and more popular in the food processing sector for decontaminating foods, plants, and packaging. Plasma represents the fourth state of matter after solid, liquid, and gas (Sakudo, 2017). It is mainly constituted by partially or fully ionized gases, involving the coexistence of positively and negatively charged ions, free radicals, excited molecules, photons, and other reactive species. It can be distinguished into quasi-equilibrium (thermal) plasma, produced at high pressure and temperature, and not in equilibrium (non-thermal or atmospheric cold) plasma based on its thermodynamic equilibrium (Shashi, Zifan, & Keener, 2018; Scholtz Pazlarova, Souskova, Khun, & Julak, 2015). Atmospheric cold plasma (ACP) is generated through different electric discharge methods such as dielectric barrier discharge (DBD), corona discharge (CD), radio frequency low discharge (RFLD), micro hollow cathode discharge (MHCD), plasma needle (PN) and atmospheric pressure plasma jet (Deng et al., 2020). The gases currently subjected to ionization are air, nitrogen (N_2), oxygen (O_2) or specific mixtures of noble gases such as argon (Ar), helium (He) or neon (Ne). The ACP initiation leads to the formation of active species like OH•, O_3, H_2O_2, NO, NO_2, N_2O and other reactive species (RS), widely known as reactive oxygen species (ROS) and nitrogen active species (RNS) (Deng et al., 2019).

ACP possesses an enhanced diffusion coefficient and a wide range of activity against pathogens and spoilage microorganisms, biofilms, and spores (Bahrami et al., 2020). ACP mechanisms for triggering microbial injury or death include: ROS and RNS accumulation on the cell surface, which provokes the oxidation of amino acids and lipids and the disruption of the cell membrane; UV photons intrinsic photo-desorption; direct gene damage (Misra & Jo, 2017). The efficacy of ACP depends on the ionizing source, voltage, frequency, gas composition, environmental parameters, mode of exposure, and microbial characteristics (Aaliya et al., 2021).

ACP represents a versatile technology, whose application has been investigated in several sectors of the food industry, including the decontamination of foods, production plants, and packaging.

Amongst food products, ACP was tested to ensure the safety and extend the shelf life of vegetables such as romaine lettuce (Min et al., 2016) and fresh-cut carrots (Kumar Mahnot, Siyu, Wan, Keener, & Misra, 2020) as a green alternative to chlorine treatment for its disinfectant properties against *E. coli* O157:H7, *Salmonella*, and *Listeria monocytogenes*. The application of ACP was extended to animal products such as avian eggs (Gavahian, Peng, & Chu, 2019) and smoked salmon (Oh, Roh, & Min, 2016), as well as fresh refrigerated mushrooms (Gavahian, Sheu, Tsai, & Chu, 2020b). ACP technology was also applied on dairy and cereal products to reduce the impact of mycotoxin-producing fungi such as *Aspergillus*, *Alternaria*, and *Penicillium* (Gavahian & Cullen, 2020a).

The effect of ACP treatment on edible packaging materials was investigated by different authors, with considerable differences related to the packaging composition and the treatment parameters. Despite the sanitizing purpose of ACP, this treatment was found to enhance or worsen some specific features of the packaging material such as gas and water vapour barrier, microstructure, mechanical strength and stretchability (Beikzadeh et al., 2020). The variety of effects is due to the specific pathway of interactions between the ionized gases and the constituents of packaging like carbohydrates, lipids, proteins, phenolic compounds, and water.

ACP is an eco-friendly and flexible decontamination technology with low energy requirements, no utilisation of harmful solvents, and reduced physicochemical damage of the treated product (Qiu, Zhang, Tang, Adhikari, & Cao, 2019). In addition, the ionized gases are unstable and tend to recombine and react to form the original gas mixture. Thus, ACP was demonstrated to release non-toxic substances and can be regarded as a "clean label" (Misra & Roopesh, 2019).

Despite the benefits, ACP presents some drawbacks. High-fat products were demonstrated to be not suitable for the treatment due to the oxidation and derived rancidity of the lipids (Liao et al., 2020). As well, ACP was found to increase the acidity, reduce the firmness, and induce discolouration of some fruits and vegetables (Ekezie, Sun, & Cheng, 2017). Moreover, the presence of roughness and irregularities on the food surface can hinder the biocidal efficacy of ACP. Clearly, the interaction of the several radical species with multicomponent systems like food constitutes a complex phenomenon, far from being widely understood (Denoya, Colletti, Vaudagna, & Polenta, 2021).

2.3. High-Pressure Processing (HPP)

High-Pressure Processing (HPP), also known as pascalization, is a non-thermal decontamination technology in which the food matrix, placed in an aqueous medium, is subjected to 100-1000 MPa pressure at ambient temperature (do Rosàrio, Rodrigues, Bernardes, & Conte-Junior, 2020). This technology has been defined as "cold pasteurisation" by FDA since it has the potential to replace the conventional pasteurisation process due to its high biocidal efficacy (Guyon, Meynier, & de Lamballerie, 2016).

HPP application obeys and takes advantage of three physical principles. The first principle, known as isostatic principle, relies on the instant and uniform transmittance of pressure within a medium. In other words, a product subjected to HPP is uniformly compressed independently from its geometry and size, since the transmission of pressure to the core is not mass and/or time dependent and thus requires a minimum processing time (Neeto & Chen, 2018). The second principle, known as Le

Chatelier's principle, defines that "when the pressure of a system in equilibrium varies, the system tries to re-establish its stability by adjusting the volume" (Rifna et al., 2019). The third principle, known as microscopic ordering theory, states that a pressure increase at constant temperature enhances the degree of molecular ordering of the sample subjected to the treatment (Chatterjee & Abraham, 2018).

HPP exerts its biocidal effect against a wide range of bacterial, yeast, and molds vegetative cells by hindering the DNA synthesis, denaturing proteins and enzymes, and disrupting the cytoplasmatic membrane (Deng et al., 2020). The antimicrobial efficacy of HPP depends on several parameters such as pressurization level, treatment time and temperature, composition and a_w of the matrix, and intrinsic sensitivity of the microbial strains (Rifna et al., 2019).

HPP has been tested as sanitising treatment for both solid and liquid food. This process was shown to be particularly efficient when applied to foods stored at low temperature and/or with high acid content. For instance, researchers focused their efforts on testing this technology on milk (Stratakos et al., 2019), citrus juice (Tappi, Tylewicz, & Dalla Rosa, 2020), coconut water (Raghubeer et al., 2020), cheese, and smoothies (Ramaswamy et al., 2019) as a prospective substitute to thermal treatment to inhibit the exploitation of foodborne pathogens and to extend their shelf life. HPP showed different advantages including enhanced nutrient retention and/or reduced loss of nutrients, minimal heat impact, shorter processing time, and unaltered colour and organoleptic properties of foods with respect to other sanitising techniques (Qiu et al., 2019). Nevertheless, HPP resulted ineffective on low acid, shelf stable products since the microbial spores can survive even at a pressure of 1200 MPa when maintained at environmental temperature. This drawback can be overcome by combining this technology with other hurdles such as mild heat treatment, refrigeration or ultrasonication (Aaliya et al., 2021). Besides, the application of the principle of Le Chatelier's results in a reduction of the volume, which may affect the structure of larger molecules like proteins within matrix. This process can either produce positive effects, leading the inactivation of microorganisms and enzymes, or negative effects, causing undesirable changes in sensory and physicochemical characteristics of food (do Rosàrio et al., 2020). Moreover, the initial and maintenance costs of this system represent another significant drawback of this technology (Tappi et al., 2020).

2.4. Pulsed electric field (PEF)

Pulsed electric field (PEF) represents a relatively novel and green technology, whose promising application in the food sector for various purposes has been extensively investigated in the last few years. During PEF treatment, the food product is placed between two electrodes and subjected to high-voltage electrical pulses (20-80 kV/cm) for short and repeated periods of time (1-100 μs) (Ziuzina, Los, & Bourke, 2018). Currently, PEF process is broadly employed to inhibit the spread of microorganisms within foods, to enhance the extraction efficacy of bioactive compounds from natural sources, and to enhance the performances of drying and dehydration processes (Kaavya et al., 2021). In particular, the enhanced lethality of this technology against a broad range of microbes such as molds, yeasts, and bacterial vegetative cells makes it a prospective substitute to thermal pasteurization for several high-moisture food matrices including milk, fruit juices, yogurts, and liquid egg (Bermùdez-Aguirre, 2018).

PEF is able to induce cellular loss of integrity and death. The chief effects of this process are known as electroporation (or electropermeabilisation) and dielectric breakdown (Bahrami et al., 2020). When a strong electrical pulse is applied to the cell cytoplasmic membrane, the transmembrane potential is altered, inducing the formation of small hydrophilic pores. Repeated electrical shocks progressively increase the size and number of the pores, and the electroporation changes from reversible to irreversible. This phenomenon causes the leakage of intracellular compounds towards the external environment, and then the cellular rupture (Slavov et al., 2019).

The biocidal efficacy of PEF relies on multiple factors. Firstly, the performances of the process are strictly related to the design of the plant, which comprises treatment chamber, pulse generator, monitoring system, and fluid handling system (Khan et al., 2017). Besides, the physical and dielectric properties of the microbial cells, along with the chemical composition and conductivity (pH, ionic strength) of the food product, play another important role. Based on these parameters, treatment conditions such as electric field intensity, time-per-pulse, and number of repetitions should be carefully selected to maximise the expected effect and reduce the impact on the food quality (Ramaswamy et al., 2019).

PEF processing allows to obtain high rates of decontamination with minimum increase of the product temperature. Thus, a relevant advantage of PEF consists in the ability to retain the utmost nutritional and organoleptic qualities of the food material with respect to conventional pasteurisation (Indriani, Amalia, Sumarlan, & Barunawati, 2019). Moreover, this technique has been reported to decrease the environmental impact of pasteurization, supporting food and nutrition safety with minimal usage of energy and water, and without evident toxicity on human health (Arshad et al. 2021; Pal, 2017a).

Despite the relevant benefits of this green technology, its industrial implementation in the different sectors of food industry is still difficult. One of the main obstructions is related to the high costs of set-up and maintenance of the processing plants, still not comprehensively justified through up-to-date literature enabling an efficient cost analysis of the process. Besides, the biocidal efficacy of PEF strongly depends on the electrical conductivity of the product, which mainly relies on its moisture content. Thus, solid and semi-solid foods are less suited than liquid foods for PEF treatment (Rifna et al., 2019).

2.5. Ultrasounds (US)

Ultrasound (US) is defined as a sound wave that exceeds the threshold of human hearing (20 kHz) (Yu et al., 2020). Basically, US represents a type of vibrational energy produced by specific transducers able to convert electrical energy into acoustic energy (Chen, Zhang, Yang, & Hui, 2020). According to the frequency range, US can be classified as: power (20-100 kHz), high-frequency (100 kHz-1 MHz), and diagnostic (1-500 MHz). Currently, the high-frequency and diagnostic US are extensively utilized in the biomedical sector for soft tissue surgery, diagnostic imaging, and stimulation of tissue regeneration (Wischhusen & Padilla, 2019). Conversely, power US have experienced an increasing interest in the food processing sector as a green, non-thermal, and reliable technology, with a variety of prospective applications including sterilization, decontamination, hydrolysis, crystallization, homogenization, drying, and extraction (Bahrami et al., 2020; Deng et al., 2020).

The mechanism of US action, and thus its usefulness for several applications, is mainly related to a specific phenomenon known as cavitation effect. As the US waves propagate within a liquid medium, they generate a periodic alternance of positive and negative pressure, which makes the liquid molecules expand and stretch. This process induces the formation of micro-sized bubbles that grow, oscillate and burst, realising a large amount of energy that increases the local temperature up to 5000 K and the pressure to 50 MPa. This process is accompanied by strong micro-jetting, shock waves, shear forces, and free radical formation (Chen et al., 2020).

Many researchers investigated the opportunity to employ the disruptive effects of US to kill microorganisms and to inactivate enzymes in the food products (Slavov et al., 2019). The acoustic cavitation was found to affect the cellular vitality both directly, by damaging the cell wall and membrane through physical processes, and indirectly, by generating free radicals with strong oxidative properties that break or modify the DNA structure (Liao et al., 2018; Wu et al. 2015). US

biocidal efficacy strictly depends on many parameters such as power intensity, frequency, exposure time, temperature, geometry of the reactor, characteristics of food, and inner resistance of the microbial strain (Bahrami et al., 2020). Nevertheless, the correct evaluation of these factors allowed to maximise the performances of this technology, obtaining valuable results against hazardous food-borne pathogens like *Salmonella enteritidis*, *E. coli*, *Staphylococcus aureus*, and *Listeria innocua* (Aalyia et al., 2021).

Besides the ability of US treatment to reduce the microbial load of food, many researchers have investigated the effects of US on the nutritional and sensorial quality of food (Yuan et al., 2021). It was demonstrated that US may exert positive or negative effects on food quality depending on the applied processing conditions. For example, ultrasound was found to eventually improve or worsen the textural properties of meat, depending on the severity of treatment (Gallo, Ferrara, & Naviglio, 2018; Shi et al., 2020). Excessive exposures to US were shown to cause non enzymatic browning of food (Khanal, Anand, Muthukumarappan, & Huegli, 2014), and degradation of pigments (Bi, Hemar, Balaban, & Liao, 2015). However, a recent study highlighted that a proper treatment can significantly enhance the colour of blueberry wine, which may be due to the protection of anthocyanins by ultrasound (Li, Zhang et al., 2020). As well, an appropriate US treatment can reduce the loss of volatile components in food, and increase the availability of bioactive substances by affecting the cell membranes and making intracellular substances exude (De Souza Carvalho et al., 2020).

Overall, US treatment constitutes a promising green technology with transversal applications for the food industry, supported by impressive academic results provided by several studies. Nevertheless, some challenges need to be solved before its commercial application. The first and most relevant challenge is the development of ultrasound equipment able to satisfy the needs of food industry for large-scale and continuous set-ups, which should be encountered by the willing of manufacturers to customise new designs according to the requirements (Azam et al., 2020). Secondly, further research is needed to explore more in depth the physical and chemical phenomena occurring on the food product during US treatment, which may cause adverse effects on food properties. These studies are fundamental to minimize the impact of this emerging technology on the product quality while maintaining its decontamination efficacy (Abesinghe et al., 2020).

2.6. Ionizing irradiation

Ionizing irradiation is a physical process during which a matrix is exposed to the energy emitted by a source of ionizing radiation for a defined period of time. Irradiation treatment has been tested in many sectors, including the food industry, in which this technology is currently applied on different kind of matrices as a valuable alternative to conventional pasteurization for the microbial decontamination of foods (Deng et al., 2020). In view of that, in 1990 the FDA approved ionizing irradiation as an effective and safe biocidal technique for the treatment of fish, meat, eggs, poultry, vegetables, fruits, cereals, spices, and sprouts (Ramaswamy et al., 2019).

The process of irradiation can be performed by applying three alternative types of radiation: gamma ray (γ-ray), electron beam (e-beam), or Roentgen ray (X-ray) (Bahrami et al., 2020). γ-rays are emitted during the decay of radioisotopes such as cobalt-60 (^{60}Co) or cesium-137 (^{137}Cs). The e-beams are generated by means of particle accelerators, and aimed at specific targets. X-rays are emitted within linear accelerators or Rhodotron-style accelerators when high energy electrons strike a metal plate (Aaliya et al., 2021).

According to the applied dose of radiation, the industrial application of gamma irradiation can be distinguished in radurization (0.1-2.5 kGy), radicidation (3.0-10.0 kGy), and radappertization (> 10 kGy). In particular, radurization and radicization were shown to effectively inactivate pathogenic and spoilage microorganisms without causing alterations and damages of the food properties (do Rosàrio et al., 2020).

The main mechanism by which ionizing radiations induce inactivation and/or destruction of vegetative cells and spores is known as radiolysis. The exposed microorganisms undergo a direct damage of the DNA, compromising their ability to replicate. Additionally, radiolysis generates reactive molecules (hydroxyl radicals, hydrogen peroxide, atomic hydrogen) which corrupt the cellular metabolic pathways, leading to cellular oxidation and lysis (Ziuzina et al. 2018). This degradation process may involve a wide range of organic molecules, which makes this technology able to inhibit the germination of spores, disinfest insects and parasites, delay of ripening, and reduce the incidence of mycotoxins and mycotoxigenic fungi, besides the destruction of non-spore-forming bacteria (Bahrami et al., 2020).

The biocidal efficacy of ionizing radiation relies on many factors, including radiation source and dosage, intrinsic resistance and physiological state of the microbial strain, the food composition, and the environmental conditions. In-depth investigations were conducted to describe the specific sensitivity of the main microbial classes to ionization, comparing them with each other. These studies

highlighted that the prokaryotes are more resistant than eukaryotes, as well as gram-positive bacteria resulted less susceptible than gram-negatives (Deng et al., 2019). Song et al. (2014) demonstrated that *S.* Typhimurium resulted more resilient to ionization than *E. coli*, but their intrinsic sensitivity was significantly affected by the type of food matrix. The authors also showed how the efficacy of the process depends on the moisture content of treated foods.

Despite the evident advantages of ionizing radiations as non-thermal decontamination process, they include certain limitations. The first constraints are strictly technical, since the free radicals produced during irradiation may cause the oxidation of nutrients such as lipids and vitamins, colour changes of the product, and formation of off-flavours. In addition, irradiation may induce the mutagenesis and selection of resistant pathogenic strains, while sub-inhibitory doses could stimulate the sporulation, growth and secondary metabolism of fungi. Other drawbacks are related to the large capital costs required and the lack of acceptance of consumers related to health and safety (Denoya et al., 2021).

2.7. Ultraviolet irradiation (UV)

Ultraviolet (UV) light comprises the region of electromagnetic spectrum ranging between 100 and 400 nm. According to the wavelengths range, UV light can be classified as UV-V (100-200 nm), UV-C (200-280 nm), UV-B (280-315 nm), and UV-A (315-400 nm) (Sanchez-Maldonado, Lee, & Farber, 2018). UV light represents a non-ionizing electromagnetic radiation, widely employed as an effective alternative for thermal and chemical decontamination processes (Ochoa-Velasco et al., 2018).

The germicidal efficiency of UV light against prokaryotic and eukaryotic microorganisms is due to the ability of UV photons to induce a complex of chain reactions within the cell structure leading to DNA damage (Kaavya et al., 2021). In particular, UV absorption promotes the formation of bonds between adjacent thymine bases, forming dimers. The thymine dimers block DNA replication and transcription, affecting the metabolic functions of the microorganism and causing its death. The maximum microbial inactivation rate is reached using light in the UV-C region, since the absorbance of DNA (around 254 nm) aligns with the emitted wavelength (Green et al., 2018).

Along with its own antimicrobial effect, UV light can be employed to trigger a non-thermal process known as photodynamic inactivation (PDI) mechanism (Corrêa et al., 2020). PDI relies on a series of simultaneous photochemical and photophysical interactions which involve oxygen, light at specific wavelengths, and a non-toxic photosensitizer (Hamblin, 2016). Photosensitizers are light-sensitive molecules, which can be either naturally present (e.g., intercellular porphyrin) or supplied externally. When the cells are exposed to light at specific wavelengths, the photosensitizers absorb it and move

to a higher energy state. While returning to their ground energy state, photosensitizers collide with the cytoplasmic molecules and release their energy. This process, conducted in presence of O_2, results in the formation of ROS such as peroxides, superoxide ion, and singlet oxygen. These compounds accomplish a cytotoxic effect by reacting with proteins, lipids, and nucleic acids (Du, Prasad, Ganzle, & Roopesh, 2020).

UV irradiation has also been considered as a potential effective tool to degrade the mycotoxins and inhibit their production by molds into low moisture foods like cereals and nuts. In particular, aflatoxin B_1 (AFB_1) was shown to absorb UV light in the range 220-370 nm, with a maximum of absorption at 362 nm. It was demonstrated that UV light affects the structure of the terminal furan ring and interacts with the active binding sites of AFB_1, hindering its toxicity (Hojnik, Cvelbar, Tavčar-Kalcher, Walsh, & Križaj, 2017).

The disinfection efficacy of UV light depends on multiple parameters, including wavelength and intensity of emission, type of emitting source, O_2 concentration, properties of the product (e.g., composition, thickness, geometry, surface structure), exposure time, and microbial strains and load (Lopez et al., 2018). The conventional sources for emitting continuous UV light on laboratory and industrial scales are low-pressure mercury (LPM) lamps, medium-pressure mercury (MPM) lamps, low-pressure amalgam (LPA) lamps, and excimer lamps (do Rosàrio et al., 2020). Among these sources, LPM and MPM lamps, the most widespread ones for industrial applications, have different criticisms. In particular, these lamps are usually bulky and fragile, have a low life span, and require specific disposal due to their toxicity (Murashita, Kawamura, & Koseki, 2017). Hence, novel UV emitting sources based on light emitting diode (LED) technology have recently gained attention for decontaminating processes (Josewin, Kim, & Yuk, 2018). LEDs are semiconductive diodes which produce light through electroluminescence principle. These diodes emit radiations with wavelengths at a narrow bandwidth, related to the nature of the employed semiconductive material (Prasad, Ganzle, & Roopesh, 2019). Recent researches were conducted in order to develop LEDs with radiating spectrum in the range 100-380 nm, defined as UV-LEDs (Hinds, O'Donnell, Akhter, & Tiwari, 2019). These LEDs can be used to produce either continuous or pulsed light, giving the opportunity to replace both conventional and xenon lamps for commercial applications.

The application of continuous UV irradiation in the food sector represents a promising approach due to its broad spectrum of lethality, low cost, energy efficiency, easy setup and maintenance, harmlessness, no residues generation, and minimal loss of sensory and nutritional quality of the food products (Delorme et al., 2020). Besides, the enhanced versatility of this technology prompted researchers and industries to extend its applications from water and water-like transparent fluids to

liquid foods like tea, milk, coffee, vegetal juices, and sugar syrups (Koutchma, 2016). Lately, UV irradiation efficacy was tested on different kind of solid food products including fresh and minimally processed fruits and vegetables (Bermùdez-Aguirre, 2018), aromatic herbs (Dogu-Baykut & Gunes, 2018), and meat (Corrêa et al., 2020).

Despite the advantages, UV treatment has specific hindering factors, mainly related to its limited penetration ability and the complex surface structure of some products, which creates the so-called "shade effect". Pathogens and spoilage microorganisms can reside in wounds, crevices, and pores, being protected from UV light. Besides, light-sensitive nutritional compounds like unsaturated vitamins, fatty acid, and pigments can be altered by UV exposure, along with the organoleptic characters of food (Deng et al., 2020).

2.8. Hurdle technologies

In recent years, many novel non-thermal technologies have gradually emerged across the food sector as promising substitutes of conventional treatments such as pasteurization or sterilization. These technologies are usually effective, non-harmful, environment friendly, and residual-free. Nevertheless, the "one-technology-per-treatment" strategy could face specific criticisms. On the one hand, some decontamination techniques could be not enough to guarantee the safety of a product when applied alone. On the other hand, single processing technologies with high dosage or exposure time could negatively affect the organoleptic and nutritional attributes of food, reducing the consumer acceptability (Khan et al., 2017). These evident drawbacks prompted the adoption of a novel strategy, consisting in the successive or simultaneous application of different techniques at mild levels to improve the safety, nutritional and sensory qualities of the products, using lower individual treatment intensities (Liao et al., 2020). This approach is commonly known as "hurdle technology".

The hurdle concept can be defined as "the combination of existing and novel preservation techniques to establish a series of preservative factors (hurdles) that any microorganisms present should not be able to overcome" (Oh, Khan, & Tango, 2019). The primary scope of hurdle technology is to obstruct the growth and stability of targeted microorganisms by subjecting them to alternate or concurrent physical, chemical, and environmental stresses. This task is achieved by three main mechanisms: hindrance of homeostasis condition; metabolic exhaustion; deprivation of the stress reaction mechanism (Pal et al., 2017b). These mechanisms are strongly related to each other. Firstly, homeostasis represents the natural tendency of the organisms to maintain an internal status of uniformity and stability (Tsironi, Houhoula, & Taoukis, 2020). The most feasible way to disturb the

homeostasis of microorganisms is by applying continuous and multiple hurdles. When this happens, microbes try to restore their homeostasis, and the loss of energy aids to metabolic exhaustion. This process induces the so-called "auto-sterilization" phenomenon (Pal et al., 2017b). Moreover, the concurrence of different stressors impedes the expression of genes related to the synthesis of stress shock proteins, commonly produced by some microorganisms to face stress conditions and starvation.

Hurdle technology is currently employed in various food processing sectors with the chief purposes to ensure the microbial safety and to prolong the shelf life of food matrices. In the last years, this strategy has been extended to several products including meat and derivatives, fruits and vegetables, milk and dairies, and starch products. Multiple combinations of technologies have been tested, either showing synergistic, additive, or competing effects (Aalyia et al., 2021). Some promising examples of hurdle technology involving the application of the most relevant non-thermal techniques are depicted in the following sections.

2.8.1. Ozonation

Ozone sanitization constitutes a well-established technology, with a broad range of consolidated applications in the food sector. Lately, the opportunity to combine ozone with other techniques to enhance the efficiency and to overcome the criticisms has gained increasing attention (Kaavya et al., 2021).

Many researches highlighted the synergistic effect of UV-C light irradiation with ozone in aqueous and gaseous forms. This combination was tested for the disinfection of foodborne pathogens in flowing water (Hernàndez-Arias et al., 2019), lemons (Hasani et al., 2019), fresh beef (Yang, Kalchayanand, Belk, & Wheeler, 2019), and flour slurries (Jewsuwan & Thipayarat, 2015). Authors concluded that the direct impairment of nucleic acids and enzymes induced by UV-C photons was boosted by the oxidation of cell components caused by ROS release from ozone breakdown.

The biocidal efficacy of aqueous ozone was also found to surge when combined with physical treatments like high frequency ultrasounds or chemical additives such as lactic acid at low concentrations for the sanitization of fresh vegetables, without any change in the quality attributes of the treated matrices (Mustapha et al., 2020; Wang, Wang et al., 2019). Moreover, Khan et al. (2017) described that the sequential application of mild heat and ozone could represent a suitable substitute to conventional and more severe thermal treatments.

2.8.2 Atmospheric Cold Plasma

ACP constitutes a sustainable and effective biocidal technique. However, the individual application of ACP might be not sufficient to fully inactivate foodborne microorganisms. For example, when subjected to ACP process, some microorganisms could enter into a sub-lethal state known as the viable but non-culturable (VBNC) state (Chakka et al., 2021). Thus, the combination of this technology with other hurdles (hybrid technology) becomes essential to enhance the efficacy of each single treatment, eliminating also the microorganisms in VBNC state (Chaplot, Yadav, Jeon, & Roopesh, 2019).

Since ACP represents a highly versatile technique, other hurdles could be introduced before, after, or during the plasma treatment, allowing the use of plasma at lower intensity and protecting the quality of the treated products.

The first form of hurdle technology based on ACP is known as chemical augmentation of plasma. This method consists in the introduction of specific compounds such as water or essential oils in the plasma generating chamber, which modify the plasma composition and improve its biocidal performances. In particular, water-augmented plasma was shown to possess a higher density of active molecular species such as H or H_2O_2 with respect to normal plasma (Kaavya et al., 2021). As well, the presence of essential oils was proven to confer to the plasma an additive antimicrobial effect due to the high variety of compounds with intrinsic antimicrobial activity (Niemira, 2012).

ACP was applied in combination with H_2O_2, either in liquid or aerosolized forms. Govaert, Smet, Verheyen, Walsh, & Van Impe (2019) tested this synergy in vitro, against the production of biofilms by some major foodborne pathogens. Song & Fan (2020) extended this combination to the disinfection of fresh fruits and vegetables (in vivo). A similar effect was observed with the addition of peracetic acid (PAA) for the disinfection of *S.* Typhimurium in raw poultry meat (Chaplot et al., 2019), and vitamin C, which was observed to relevantly reduce also the VBNC microbial population (Liao et al., 2020).

Plasma processing was employed to improve the antibacterial properties of active packaging material based on silk fibroin nanofibers and incorporated with nano-capsules of thyme essential oil. The release of thyme oil by the nanofibers was relevantly boosted by ACP, with higher performances for the disinfection of *S.* Typhimurium in poultry meat wrapped using these treated nanofibers (Lin, Liao, & Cui, 2019).

Xiang et al. (2019) employed plasma-activated water along with mild heat treatment to reduce the population of *E. coli* O157:H7. The experiment revealed that the combined treatments affected the

intracellular components and outer structure of bacteria by synergistic effect. As well, Lis et al. (2018) combined ACP with refrigeration temperature (8 °C) to inactivate *S.* Typhimurium in ham samples stored into N_2 flushed sealed packages.

Liao et al. (2018) successfully demonstrated the synergistic effect of ultrasounds and ACP against *S. aureus* in saline solution. During the process, the plasma generated abundant ROS in the medium. These active species (hydroxide (OH·), nitric oxide (NO), hydrogen peroxide (H_2O_2)) effectively passed through the microbial cell membrane thanks to the ultrasonic microjet effect, triggering oxidative damages in the intracellular components.

2.8.3. High-Pressure Processing

The optimization of HPP biocidal efficacy by applying a hurdle approach has been extensively investigated, and different combination of technologies were tested aiming for synergistic effects.

HPP was successfully combined either with low-temperature, mild heat and/or ultrasound treatments to inactivate bacterial pathogens and spores in processed products based on fruit and vegetables (Evelyn & Silva, 2016; Mukhopadhyay et al., 2016).

Some studies showed the enhanced potential of HPP for the elimination of pathogens such as *Vibrio cholerae*, *Shigella flexneri*, and *L. monocytogenes* when paired with virulent bacteriophages to treat milk, ground beef, and seafood (Ahmadi, Anany, Walkling-Ribeiro, & Griffiths, 2015; Komora et al., 2020).

2.8.4. Pulsed electric field

Recently, PEF was applied in combination with one or more decontamination methodologies at low intensity to overcome the drawbacks related to this technology, depending on product composition and the specific microorganisms to be inactivated. These combinations allowed to achieve the required lethality with lower electrical input and a less intense electrical field.

Different researches showed the benefits related to the application of PEF under mild heated conditions to decontaminate liquid foods such as fruit juice, egg, and milk (Alvarèz & Heinz, 2007). The lethality of this treatment was furtherly enhanced by the addition of essential oils including citral, carvacrol, and limonene (Espina, Monfort, Alvarèz, García-Gonzalo, & Pagàn, 2014).

Similar effects were observed by treating processed vegetal products with HPP followed by moderate-intensity PEF, reducing the microbial population below the detection limit (García-Parra et al., 2018).

As well, ultrasound-PEF combination was tested for the decontamination of whole liquid eggs, with promising results comparable to conventional pasteurization treatments (Kaavya et al., 2021).

2.8.5. Ultrasounds

The improved antimicrobial efficiency of US combined with other preservation techniques is well documented (Aalyia et al., 2021).

The combination of US with physical treatments including heat, pressure, and UV-C irradiation was extensively investigated (Pinela & Ferreira, 2017).

Thermosonication, meaning the combination of US with mild heat, was shown to induce macromolecule depolymerization on the structure of heat-shocked microbial cells, developing either a cumulative or synergistic effect (Guerrero, Ferrario, Schenk, & Carrillo, 2017). Thermosonication can be categorised as sublethal (<45-50 °C) or lethal (>45–50 °C) according to the temperature range. Mano-thermosonication treatment, which combines pressure, heat and US, was able to inactivate most of the thermal-resistant enzymes, such as peroxidases and lipoxygenase, and have a broad antimicrobial spectrum. Moreover, it requires lower processing times than conventional US processing (Chatterjee & Abraham, 2018). Besides, photo-sonication, combining UV irradiation with sonication at high intensity, was successfully applied to decontaminate and prolong the shelf life of large leaf vegetables (Khandpur & Gogate, 2016).

US was also tested in association with synthetic or natural compounds with antimicrobial activity, showing promising results against pathogenic and spoilage microorganisms (Afari, Hung, King, & Hu, 2016). do Rosàrio et al. (2017) highlighted that the bactericidal impact of peracetic acid on pathogens like *S. enterica* was positively affected by US treatment, maintaining the sensory characteristics of strawberries. US, slightly acidic electrolyzed water (SAEW), and temperature (60 °C) were applied to guarantee the microbiological safety of bell pepper (Luo & Oh, 2016). Besides, the inactivation of *S. enterica* in green peppers was achieved by associating US with the addition of 1% lactic acid. Regarding to animal-based products, US treatment was paired with the addition of cinnamon essential oil to sanitise milk, and a strong biocidal effect was observed against both gram-positive (*L. monocytogenes*) and gram-negative (*S.* Typhimurium) bacteria (Mortazavi & Aliakbarlu, 2019).

2.8.6. Ionizing irradiation

When an excessive dose of irradiation, necessary to attain an adequate microbial inactivation rate, is provided to a food matrix, its nutritional and organoleptic properties could face a more or less severe degradation (Deng et al., 2020). Low-dose irradiation along with other preservation treatments including heat, refrigeration, freezing, and modified atmosphere packaging (MAP) aided to achieve satisfying results, comparable to those obtained by applying severe irradiation treatments alone (Khan et al., 2017). These synergistic combinations were mainly applied to treat fresh and processed meat, poultry and seafood. For example, Mahto, Ghosh, Das, & Das (2015) demonstrated the synergistic effect of γ-irradiation and freezing on the microbial, nutritional and organoleptic quality of prawns. Nevertheless, this approach seems to have promising perspectives also for the disinfection of large leaf vegetables (Olanya, Niemira, Cassidy, Boyd, & Uknalis, 2020).

2.8.7 Ultraviolet irradiation

In order to overcome the issues related to the single UV-C treatment (e.g., low microbial inactivation rates; adverse impact on product quality), different researchers suggested the opportunity to apply UV in combination with other technologies like mild heat treatment, cold plasma, and modified atmospheric packaging, causing a hurdle effect (Bang, Kim, Lee, & Min, 2020).

UV-C irradiation and mild heat treatment exhibited a synergistic effect for the disinfection of pathogenic and spoilage bacteria including *S.* Typhimurium, *E. coli*, *L. innocua*, and *Bacillus* spp., with minimum quality changes (Cheon, Shin, Park, Chung, & Kang; 2015; Fan, Huang, & Chen, 2017).

UV-C irradiation was also tested in association with refrigeration and/or modified atmospheric packaging (MAP), showing a relevant increase of the product shelf life (e.g., fruits and fruit-derived products) and the effective reduction of bacterial population (Choi, Park, Choi, Kim, & Chun; 2015; Gòmez, Welti-Chanes, & Alzamora, 2011).

Some researches were performed washing the products (mainly fruits or vegetables) with water or diluted antimicrobial substances followed by UV-C irradiation. Studies conducted on berries (Cao, Huang, & Chen, 2017) and tomatoes (Guo, Huang, & Chen, 2017) demonstrated that water-assisted UV-processing, which combined washing in turbulent water with UV light, had better biocidal performances than dry UV treatment, regardless of the inoculation method and the storage conditions. Moreover, the addition of antimicrobial substances including chlorine (Huang & Chen, 2018),

peracetic acid (Collazo et al., 2019), or blended sanitizers (Leng et al., 2020) during washing furtherly improved the efficacy of this hurdle approach.

2.8.8. Challenges

Hurdle technology represents a fascinating trend area of research. Novel combinations of preservation technologies are constantly tested on laboratory or pilot scale to maximise their technological performances, ensuring the safety of food products without compromising their quality aspects. Despite that, perfecting the hurdle approach still involves notable challenges and requires efforts from the academic research and industries in view of their massive application on industrial scale (Aalyia et al., 2021).

- In-depth investigations highlighting the microbial mechanisms of stress reaction, adaptation and "cross-tolerance" (both generic and specific of the single strains) is strongly required to overcome the actual limitations of this approach;
- The combination of hurdles might have an additive, synergistic or an antagonistic effect compared to a single hurdle. Thus, it is extremely important to assess the uncertainty related to combining different preservation techniques;
- Monitoring and validation of microbial inactivation mechanisms and rates by each hurdle approach represents a central area of interest, to maximise the efficacy of the treatment and avoid the generation of undesired chemical reactions;
- Implementation of tools such as predictive microbiology and data integration from genomics, metabolic pattern recognition, and protein expression could be used to predict the microbial activity in food and clarify the response of microbial cells, helping to set-up a suitable combination of treatments for each specific case study;
- Nowadays, most of the hurdle approaches are not economical and time-consuming because of its high set-up cost and the need for trained personnel. Cost-effectiveness of hurdle technologies should be taken into account in future studies;
- The lack of regulations regarding hurdle technology is a major limitation for establishing the real potentialities of this approach. However, due to the promising results obtained on research and pilot scale, official standards regulating the application of this novel approach are keenly expected in the next future.

3. Sustainable active packaging: general considerations and new trends

Food packaging is a coordinated system aiming to preserve the safety and quality of the food products from their production to their end-use (Moeini, Germann, Malinconico, & Santagata, 2021). It plays a crucial role in human society as a fundamental component of the food supply chain (Petkoska, Danilosk, D'Cunha, Naumovski, & Broach et al., 2021).

Over the world, it is estimated that one-third of the produced food is disposed every year due to various factors including wrong harvesting procedures, mechanical damages, and inadequate storage conditions, which result in microbial decay, oxidation, degradation of nutrients, and loss of consumer acceptability (Hoseinnejad, Jafari, & Katouzian, 2018; Youssef & El-Sayed, 2018). Therefore, the selection of adequate packaging solutions able to protect each targeted product from potential hazards and to maintain its quality is crucial to extend the food shelf life, thus preventing waste generation (Aguirre-Joya et al., 2018).

Conventional packaging is commonly constituted by a one-time use item, immediately discarded after reaching the intermediate or final user of the packed content (Petkoska et al., 2021). Over a broad variety of materials, fossil-based plastics have dominated the food packaging industry since their first appearance during the Second World War (Risch, 2009), thanks to their enhanced barrier and mechanical properties, chemical resistance, durability, lightweight, availability, and cost-effectiveness (Park, Koo, Cho, & Lyu, 2017).

Currently, global production of plastics is about 320 million tons/year, with an increasing demand due to their broad-spectrum of applications in various fields (Paletta, Leal Filho, Balogun, Foschi, & Bonoli, 2019). Data reveal that one-third of all plastic is dedicated to packaging materials (Schwarzbock, Van Eygen, Rechberger, & Fellner, 2016). Hence, the food packaging industry is closely involved in the production of massive amounts of plastics, generating severe economic burdens and ecological impact.

One the main concerns of fossil-based plastics is related to their non-sustainable nature, since their source (petroleum) is not renewable. Besides, single-use plastics are generally considered as not "environmentally friendly" due to their non-compostable nature and low recycling rate (Geyer, Jambeck, & Law, 2017). This ends up in the accumulation of tremendous masses of waste in landfills and oceans, raising wildlife mortality from ingestion and entanglement (Hale, Seeley, La Guardia, Mai, & Zeng, 2020). Another negative implication related to plastics is the generation of CO_2 and

other harmful greenhouse gases during their disposal through incineration (Aguirre-Joya et al., 2018; Jeevahan & Chandrasekaran, 2019).

In the last few years, human awareness about the environmental impact of plastic have grown both at personal and at community level. On the one hand, consumers are increasingly demanding for natural, high-quality, safe, and convenient foods, and for food packaging that does not create pollution. On the other hand, governments are pushing towards the reduction of human impact on environment. For example, the European Parliament focused its Sustainable Development Goals on the partial replacement of oil-based polymers for packaging purposes with biodegradable polymers coming from renewable resources by 2030 (European Commission, 2015).

All these reasons prompted researchers, institutions, and packaging companies to shift their efforts and investments on the exploration and exploitation of novel renewable resources, and the development of sustainable and biodegradable solutions, including films, coatings, and other items (Jeevahan & Chandrasekaran, 2019).

Specifically, films are thin layers of material prepared through different technologies such as solution casting or extrusion as stand-alone structures. The prepared films are used to wrap the foods or to be placed between the layers of food products. Coatings are thin layers of material which are directly applied on the food surface, and act as a barrier between the external environment and the product during transportation, processing, and storage. Coatings are applied either by dipping the product in the coating solution or directly spraying them over the product surface.

These novel packaging systems are designed to perform multiple functions. Along with the "classic" packaging activity, namely the interposition of a physical barrier between food and environment, they may operate as carriers of bioactive compounds (e.g., plant extracts) with antioxidant, antimicrobial, or nutritional properties. These "active ingredients" aim to prolong the shelf life or increase the nutritional value of the packaged product (Amin et al, 2021). Moreover, the addition of bioactives can result in modified physicochemical, mechanical and barrier properties since they chemically interact with the biopolymer structure. Hence, their wise application may allow to improve or even adapt the functional features of the packaging solution for a broad variety of applications (Mir, Dar, Wani, & Shah, 2018). Overall, biodegradable packaging systems are constantly subjected to engineering process finalised to achieve technical, manufacturing, and economic characteristics and performances comparable to conventional synthetic materials, while maintaining their sustainable nature (Jeevahan et al., 2020).

The aim of this section is to provide a schematic overview on biodegradable materials from renewable resources as promising substitutes of conventional plastics for food packaging applications. The

concept of active packaging is introduced. Then, the incorporation of natural extracts, synthetic biodegradable compounds, and nano-fillers as emerging green strategies to produce biodegradable active packaging with enhanced structural, technical, and functional performances is discussed.

3.1. Compostable, biodegradable or renewable?

Research and industries are pushing towards the usage of biodegradable polymers for food packaging purposes, in order to reduce the pollution derived by the over-use of conventional materials. As well, the extensive exploitation of renewable resources has the potential to reduce the use of oil and other fossil fuels. However, plastics produced by renewable resources are not necessarily compostable or biodegradable, and vice versa (Lambert & Wagner, 2017). For example, cellulose, starch and gelatin maintain their biodegradability also when obtained synthetically. Equally, when castor oil monomers are polymerized to produce Nylon 9, they lose their biodegradability due to chemical modification (Asgher, Qamar, Bilal, & Iqbal, 2020). In fact, biodegradation is positively correlated to the chemical structure of the compound rather that its origin. In this context, it is important to clearly state the definitions of biodegradation and compostability, which allows to further introduce the concept of biopolymer.

Biodegradation broadly defines an event in which a biomass is decomposed over 90% within 6 months via the action of enzymes and/or chemical degeneration associated to living organisms such as moulds, yeasts, and bacteria (UNI EN 13432:2002). This process can be conducted both in aerobic and anaerobic conditions (Al-Tayyar, Youssef, & Al-hindi, 2020). Other processes such as photodegradation, hydrolysis, and oxidation may also have an impact on the structure of biomass prior to or during biodegradation (Zhong, Godwin, Jin, & Xiao, 2020). Compostability involves a series of processes (mainly conducted in industrial conditions) that exploit biodegradation to convert organic matter into the so-called “compost”, which must completely degrade in soil within 3 months by producing water, carbon dioxide, and other inorganic compounds (Al Hosni, Pittman, & Robson, 2019).

In light of these statements, it is worth noting that the synthesis of compostable bioplastic using 100% renewable resources has not yet been realized at large scale. Until now, bioplastic usually comprises more than 50% (w/w) of renewable sources (Asgher et al., 2020). Several bioplastics include mixtures of synthetic compounds (e.g., polymers or additives) to boost up the technical properties of the final product, extending its potential applications (Saravanan, Leena, & Selvamurugan, 2016). Despite that, the current tendency is to replace synthetic additives with natural compounds with comparable

functional properties, and to enhance the use of biopolymers over fossil-derived materials to produce approximately 100% renewable and biodegradable plastics (Reinders, Onwezen, & Meeusen, 2017).

3.2. Biopolymers

According to the European Bioplastics association, biopolymers are defined as biodegradable, compostable, and biocompatible polymers derived from renewable resources (Santagata et al., 2017). They are broadly regarded as the most promising sustainable alternative to petrol-based synthetic polymers for food packaging applications, due to their compostable nature and film-forming ability (Zhong et al., 2020).

Thanks to their technical variability, biopolymers are adaptable to various packaging technologies, offering a range of package products, including cups, covers, separation layers, wraps, and food containers. In particular, they can be used to prepare composite films and multilayered coatings to guarantee food safety and to prolong the shelf-life of food products. Moreover, biopolymers are compatible with functional ingredients including nutraceuticals, antioxidants, antimicrobials, probiotics, texture enhancers, pigments, and flavors (Rangaraj, Rambabu, Banat, & Mittal, 2021).

Biopolymers have been classified into three categories according to their sources and synthesis methods: (I) polymers directly extracted from renewable biomasses, including polysaccharides, polypeptides, and lipids; (II) polymers synthetized from chemical polymerization of bio-monomers (e.g., polylactic acid and polyvinyl alcohol); (III) polymers derived from microbial fermentation (e.g., polyhydroxy alkanoates) (Al-Tayyar et al., 2020). Besides, biopolymers can be furtherly distinguished according to their hydroplastic or thermoplastic behavior (Moeini et al., 2021).

Most of the biopolymers possess remarkable features as technical materials for packaging applications due to their chemical complexity. A brief description of the most commonly explored biopolymers is detailed in the following sub-sections.

3.2.1. Polysaccharides

Polysaccharides represent the first class of biopolymers extensively investigated for packaging purposes. Several studies reported their suitability for producing packaging products with tailored behavior (Gao, Pollet, & Avèrous, 2017).

Polysaccharides are complex macromolecules consisting of repeated mono or disaccharide units linked via glycosidic bonds (Ferreira, Alves, & Coelhoso, 2016). They are natural, easily accessible, non-toxic, and renewable.

Due to their complex structure, polysaccharides exhibit adequate mechanical resistance and high barrier to oxygen (O_2) and carbon dioxide (CO_2). The presence of hydroxyl groups leads to the formation of hydrogen bonds, responsible for inter-intra macromolecular association and thus film-forming ability. However, their hydrophilic nature entails poor moisture resistance and reduced capacity to hinder water vapor transmission (Rangaraj et al., 2021). To overcome these drawbacks, polysaccharides are modified through chemical pathways to obtain derivatives with enhanced performances or by blending them with hydrophobic materials and nanofillers.

3.2.1.1. Chitosan

Chitosan, or β-(l-4)-2-amino-2-deoxy-D-glucopyranose, is a cationic linear polysaccharide consisting of N-acetyl-glucosamine and N-glucosamine units. It derives from alkaline N-deacetylation of chitin, the second abundant natural polysaccharide after the cellulose. The primary sources of chitin are shellfish waste, insect cocoons, crabs peritrophic membranes, and fungi (Nešić, Moeini, & Santagata, 2020).

Chitosan is biodegradable, non-toxic, bio compatible, and broadly available. Due to its chemical and biological properties, it is widely used for many applications in biomedical, cosmetic, agricultural, and food sectors (Malinconico, Cerruti, Santagata, & Immirzi, 2014; Moeini et al., 2018; Straccia, Romano, Oliva, Santagata, & Laurienzo, 2014).

Chitosan is insoluble in water but soluble in a slightly acid aqueous solutions due to the protonation of the NH_2 groups (Haghighi, Licciardello, Fava, Siesler, & Pulvirenti, 2020b). It exhibits good antimicrobial activity against a wide range of microorganisms including gram-positive and gram-negative bacteria, filamentous fungi, and yeasts (Cazòn, Velazquez, Ramírez, & Vàzquez, 2017).

Chitosan shows an excellent film-forming ability. However, extrusion technology is inadequate to produce chitosan-based films, due to the low degradation temperature of this polymer and its non-

thermoplastic behaviour. As a result, the production of films is mainly conducted through solution casting method (Pelissari, Yamashita, & Grossmann, 2011).

These films perform good mechanical properties and effectively obstruct O_2 and CO_2 transmission (Machado et al., 2020). Meanwhile, they are highly sensitive to moisture transmission, which compromise their use to preserve fresh or fatty food products. To overcome this criticism, authors investigated different strategies including chemical crosslinking and grafting with secondary components (Higueras, Lòpez-Carballo, Gavara, & Hernàndez-Muñoz, 2015). These methods provide a more interpenetrating structural network to the resulting films, improving their hydrophobicity. Another suitable technique is blending chitosan with compatible polymers such as polysaccharides (e.g., cellulose derivatives) or proteins (e.g., gelatin) to induce a strong inter-intramolecular hydrogen bonding, which results in improved barrier and mechanical performances of the blend films (Ahmed & Ikram, 2016; Kumar, Mukherjee, & Dutta, 2020).

3.2.1.2. Cellulose and derivatives

Cellulose, or (1→4)-β-D-glucopyranosyl, is a linear chain polysaccharide in which anhydrous glucose rings (($C_6H_{10}O_5$) n) are bound through β1-4 glycosidic bonds, and the number of repeat units depends on the source material (Wang, Lu & Zhang, 2016). It constitutes the most abundant biopolymer in nature, giving structure and rigidity to the plant cell walls.

Native cellulose is water-insoluble due to its structural complexity, high crystallinity, and tightly packed hydrogen bonds, and thus unable to form stable gels (Lindman, Karlström, & Stigsson, 2010). This limitation is overcome by applying an alkali treatment followed by acidification using hydrophilic agents such as chloroacetic acid, methyl chloride, or propylene oxide to produce cellulose hydroplastic and thermoplastic derivatives. Cellulose derivatives are commonly isolated from wood, hemp, cotton, and other plant components (Xu, Chen, Rosswurm, Yao, & Janaswamy, 2016). These derivatives have been extensively investigated to develop biodegradable composites and films due to their high abundance, non-toxicity, low cost, biodegradability, and chemical stability.

Hydroplastic polymers obtained from cellulose are highly hydrophilic, and possess excellent gelling capacity. They include carboxy methylcellulose (CMC), methylcellulose (MC), hydroxypropyl methylcellulose (HPMC), hydroxypropyl cellulose (HPC), and others (Han, Yu & Wang, 2018). Films and coatings based on these polymers are transparent, odorless, resistant to oxidation, and show enhanced mechanical and gas barrier properties (Al-Tayyar et al., 2020; Hasheminya, Mokarram, Ghanbarzadeh, Hamishekar, & Kafil, 2018). However, these derivatives are highly sensitive to water

vapor transmission due to their hydrophilic nature, which limits their application to dried and low-fat foods (Hernandez-Izquierdo & Krochta, 2008). In this context, several strategies have been investigated to confer hydrophobicity to cellulose-based films, thus reducing their WVP value. Shahbazi, Ahmadi, Seif, & Rajabzadeh (2016) applied surface modification of CMC based films via reaction with sodium benzoate and glutaraldehyde vapor, followed by photo-crosslinking or chemical-crosslinking with gelatin, respectively. Authors observed that photo-crosslinking improved hydrophobicity and water barrier property more than the chemical crosslinking. Another study tested cellulose-based films obtained via chemical crosslinking of CMC with hydroxy ethylcellulose (HEC) using citric acid (Singh et al., 2019).

Cellulose acetate is the most researched and widely consumed thermoplastic polymer derived from native cellulose. This derivative is obtained treating technical-grade cellulose with a methylene chloride-acetic acid solution to substitute hydroxyl groups with acetyl groups (Tyagi, Salem, Hubbe, & Pal, 2021). FDA tagged cellulose acetate as GRAS, which prompted the food packaging industry to develop and test novel applications of this polymer (Shaghaleh, Xu, & Wang, 2018). Cellulose acetate is commonly used to wrap fresh products and baked goods. Cellulose acetate films and coatings are tough and resistant to puncture, which makes them suitable for many applications. Conversely, they possess relatively poor moisture barrier properties, high rigidity, and lower thermal resistance compared to conventional thermoplastics (Paunonen, 2013). These criticisms can be partially solved by adding plasticizers, which impart good gloss, clarity, tailored rigidity and dimensional stability. Moreover, when employed for prolonged applications, cellulose acetate may undergo partial hydrolysis to produce acetic acid (Puls, Wilson, & Hölter, 2011).

3.2.1.3. Starch

Starch represents the primary energy reserve biosynthesized in the plant kingdom and one of the most plentiful renewable feedstocks known by humans. Native starch consists of two types of glucose polymers: amylose, a linear polysaccharide with (1 → 4)-α-D-glucopyranosyl units, and amylopectin, branched amylose with (1 → 6)-α- D-glucopyranosyl side units. Starch has been extensively studied as a low-cost biodegradable plastic and food hydrocolloid component, thanks to its renewability, biodegradability, easy availability, and excellent film-forming capacity. Starch-based films and coatings exhibit remarkable mechanical strength, elasticity, transparency, and low oxygen permeability (Amin et al., 2021). The major challenges related to films based on native starch are brittleness and high hydrophilicity, which results in poor water vapor barrier properties (Hassan, Chatha, Hussain, Zia, & Akhtar, 2018). These drawbacks preclude the application of starch-based

films and coatings to package foods sensitive to moisture and oxidation (Zhong et al., 2020). To enhance the flexibility and water resistance, food-grade plasticizers (e.g., glycerol, glycol, sorbitol) and hydrophobic substances such as lipids can be incorporated into the film forming solution (Cazòn et al., 2017).

3.2.1.4 Pectin

Pectin is an anionic, hydro soluble and high-molecular weight heteropolysaccharide. It is one of the main components of the plant cell wall, contributing to tissue rigidity and integrity.

Pectin is chemically composed by poly α-(1–4)-D-galacturonic acid chains (Abid et al., 2017), commonly known as homogalacturonan (Cazòn et al., 2017). Its linear structure is usually interrupted by rhamnose residues, on which secondary chains containing galactose, xylose and arabinose are grafted. Consequently, pectin is composed of three different polysaccharide domains. The first domain is the homogalacturonan, which is the smooth component of the molecule. The second domain is named rhamnogalacturonan I and it is constituted by a chain of α-(1,2)-linked L-rhamnopyranose residues. The third one, rhamnogalacturonan II, is characterised by a complex and heterogeneous structure. The second and the third domains form the hairy components of pectin by clumping together in specific regions of the polymer (Espitia, Du, Avena-Bustillos, Soares, & McHugh, 2014).

The carboxyl groups of galacturonic acid are partially esterified with methanol to form methoxylated groups, and can be converted to amide groups via reaction with ammonia (Nešić et al., 2017). According to the esterification degree (DE), pectin can be classified as low methoxyl (<50%) and high methoxyl (>50%) pectin. DE strongly influence the gelling properties of pectin. High methoxyl pectin requires high concentrations of solids such as sugars and low pH to form gel, while low methoxyl pectin gels only in presence of divalent ions as Ca^{2+} (De Cindio et al., 2016).

The main industrial sources of pectin are orange pulp and apple pomace (Cazón et al., 2017; Zannini, Dal Poggetto, Malinconico, Santagata, & Immirzi, 2021).

Pectin is widely applied in food industry as gelling, thickening, and stabilizing agent for jam, drinks, and ice cream. It is recognized as safe (GRAS) by the FDA (2013).

It is well known for its biocompatibility, non-toxicity, biodegradability, and its good gelling ability. Thus, the ability of pectin to form biodegradable and edible films and coatings has been largely investigated (Espitia et al., 2014). Some researchers suggested the scarce potential of pectin as film-forming polymer, due to the limited physicochemical and mechanical performances (Šešlija et al.,

2018). Despite that, several investigations have been conducted to improve pectin-based filming and coating properties. To enhance the mechanical stability of the film and the surface adhesion on the food substrate, pectin has been blended with food-grade plasticizers (e.g., glycerol, polyethylene glycol, and sucrose) and polymers (e.g., polyvinyl alcohol and cellulose derivatives). As well, pectin has been combined with hydrophobic compounds like lipids to enhance its resistance to moisture and water vapor transmission.

3.2.2. Proteins

Proteins are complex macromolecules characterized by variable molecular structures exerting different functional properties (Calva-Estrada, Jimènez-Fernàndez, & Lugo-Cervantes, 2019). Protein derivatives are commonly isolated from natural resources and represent promising biopolymers to produce biodegradable packaging with interesting properties, including excellent physicochemical, optical, mechanical, and gas barrier performances (Ribeiro-Santos et al., 2018). In particular, the enhanced capacity of protein-based packaging to control gas transmission allows to hinder the loss of flavors and restrict the migration of active components, thus improving food shelf-life (Murrieta-Martínez et al., 2018). Besides, protein-based packaging can be easily degraded in the environment, and acts as a good biofertilizer due to its high nitrogen content (Chen et al., 2019).

The film-forming ability of protein derivatives strongly depend on their structure, molecular weight, solubility, and charge (Calva-Estrada et al., 2019). The mechanical properties of protein films and coatings vary based on several factors, including the sequence of amino acids of the protein structure, chain flexibility, and amount of intra-protein interactions (Koshy, Mary, Thomas, & Pothan, 2015). Besides, proteins can be effectively combined with other biopolymers, resulting in active composite films with improved features (Umaraw & Verma, 2017).

3.2.2.1. Gelatin

Gelatin is a water-soluble protein obtained through the partial hydrolysis of native collagen, a primary component of bones, tendons, skin, and connective tissues of animals. This protein consists of a triple helix structure with repeated glycine-proline-hydroxyproline units. It is composed by a mixture of α-chains (one polymer/single chain), β-chains (two crosslinked α-chains), and γ-chains (three crosslinked α-chains), with relevant variability depending on the source (Chen et al., 2019). According to the synthesis method, gelatin is broadly classified as (I) Type A, derived from acid-

treated collagen, and (II) Type B, obtained from alkali-treated collagen (Guerrero, Stefani, Ruseckaite, & de la Caba, 2011).

Among biopolymers, gelatin has the peculiar capacity to form thermo-reversible gels with a melting point close to 40°C. This attribute, along with the low price and abundance, prompted its widespread use in food, nutraceutical, and pharmaceutical industries as stabilizing agent and for the production of biodegradable active films and coatings (Łupina et al., 2019).

Gelatin-based films exhibits low O_2 permeability and acceptable mechanical properties, mainly dependent on the amino acid sequence, molecular weight, and structure of the polymer (Ramos, Valdès, Beltràn, & Garrigòs, 2016). Also, gelatin can act as a carrier for natural antioxidants and antimicrobial agents. However, these films are highly sensitive to moisture and permeable to water vapor due to their hygroscopic behavior.

Numerous studies have been conducted evaluating the incorporation of crosslinkers, strengthening nanofillers, plasticizers, vegetable oils (e.g., corn oil, sun flower oil, essential oils), and natural polyphenolic antioxidants as promising methods to improve the technical performances of gelatin-based films and to support their bioactivity (Zhao et al., 2016). In particular, the cross-linking reaction was found to affect the intermolecular forces (ionic and hydrogen bonds) within the triple helix structure, resulting in an interpenetrated network structure of the film matrix (IPN) (Lin, Pan et al., 2019). Moreover, gelatin has been blended with other biopolymers including chitosan (Haghighi et al., 2019a), and zein protein (Xia, Wang, Wang, Liu, & Xiao, 2019) to produce a series of unique hybrid active films with improved barrier and mechanical characters.

3.2.2.2. Corn zein

Zein is a prolamin protein mainly isolated from corn seeds. It is an alcohol-soluble and biodegradable protein, whose hydrophobic nature relies on the high density of non-polar amino acids (Sahraee, Milani, Regenstein, & Kafil, 2019). Moreover, it exerts a thermoplastic behavior and outstanding film-forming properties (Moeini et al., 2021). These characteristics makes zein a good candidate for developing natural biodegradable films and coatings with enhanced technical features.

Zein-based films are smooth, thermally stable, and possess lower WVP values than other protein-based films (Zhang et al., 2019). These attributes are mainly related to the presence of hydrogen and disulfide bonds which are formed between zein chains during solvent evaporation and confer the films a hydrophobic behavior. For this reason, zein-based films can be tailored to act as selective barriers to oxygen, carbon dioxide, and oils. Despite that, these films generally exhibit poor

mechanical properties and fragility, which can compromise their wide application as food packaging system. Thus, many strategies have been explored to improve their structural properties, including the addition of plasticizers and combination with other polymers to produce bilayer and composite films (Amin et al., 2021).

3.2.3. Polylactic acid (PLA)

Polylactic acid (PLA) is a compostable (under industrial conditions), biocompatible and thermoplastic aliphatic polyester. It is obtained either through direct polycondensation of L- and/or D-lactic acid monomers or from the ring-opening polymerization of lactide monomers. The first pathway is generally followed to produce low-molecular weight PLA, while the second method is usually applied to produce high-molecular weight PLA (Zhong et al., 2020).

PLA is mainly synthetized by microbial fermentation from agricultural renewable sources such as corn, cassava, sugar beet pulp and sugarcane, and from their transformation residuals. Although 90% of total LA is obtained by bacterial fermentation, the remaining 10% is synthetically produced by hydrolysis of lactonitrile (Marra, Silvestre, Duraccio, & Cimmino, 2016). Currently, the annual production of PLA is estimated to be 140,000 tons, with an increasing trend due to its potential as substitute of petroleum-based materials for packaging production (Siakeng et al., 2019).

PLA properties include tensile strength, thermal stability and gas permeability, and are comparable to those of synthetic polymers from fossil fuels, such as polypropylene (PP), polyethylene (PE), polyethylene terephthalate (PET), and polystyrene (PS) (Mahmoodi, Ghodrati, & Khorasani, 2019). Moreover, PLA exhibits a better thermal processability compared to other thermoplastic biopolymers, and thus can be processed through conventional equipment and techniques, including blow filming, injection molding, fiber spinning, thermoforming, and cast filming (Rasal, Janorkar, & Hirt, 2010).

PLA has been accepted as GRAS by the FDA (Moeini, van Reenen et al., 2020). As a result, this polymer has been increasingly employed in the food packaging industry to produce disposable cutlery, plates, lids, cups, overwrap, plates, containers, and lamination films. Despite that, the high cost and the drawbacks, such as brittleness, low extensibility (<10% elongation at break), low resistance to oxygen permeation, and low degradation rate still deter the mass use of this polymer (Moeini et al., 2021).

Considerable efforts have been made to improve PLA performances. Different blends of PLA with other natural biopolymers were tested. For example, blending with thermoplastic starch (TPS) was proved to enhance the mechanical properties and the biodegradability rate of the biopolymer, and to

reduce the production cost (Turco et al., 2019). On the other hand, PLA/PHB blend obtained by melt blending showed improved oxygen barrier, water resistance and mechanical properties compared to pure PLA.

The addition of plasticizers represents another suitable strategy to improve the PLA mechanical performances. Indeed, they should be compatible with the polymer, biodegradable, and non-toxic. Thus, the demand for new "green" plasticizers based on natural and renewable resources such as vegetable oils is rapidly increasing (Moeini et al., 2020).

3.2.4. Polyhydroxy butyrate (PHB)

Polyhydroxy butyrate (PHB) belongs to the family of the polyhdroxy alkanoates (PHAs), a series of biodegradable, crystalline and thermoplastic polyesters synthesized from microbial fermentation of organic biomass. It is produced by the gram-positive bacterium *Bacillus megaterium* (Hoseinabadi, Rasooli, & Taran, 2015).

PHB exhibits remarkable technical performances, comparable to those of polyethylene and polypropylene. Moreover, owing to its lamellar structure, it performs superior aroma and water vapor barrier properties, with a lower carbon footprint and avoidance of "white pollution" with respect to conventional plastics. In fact, it is easily biodegraded by the action of PHA hydrolases depolymerases, which form (R)- and (S)-hydroxybutyrates and other non-toxic compounds under aerobic or anaerobic conditions (Markl, Grünbichler, & Lackner, 2018).

These attributes make PHB a sustainable replacing candidate to fossil commodity polymers for short-term food packaging applications. Despite that, some criticisms, i.e., high brittleness, low thermal stability and reduced processability, along with insufficient barrier properties, still limit its widespread use (Panaitescu et al., 2018). Thus, many attempts have been made to overcome the limitations related to the use of PHB for food packaging. Arrieta, Fortunati, Dominici, Lòpez, & Kenny (2015) blended PHB with PLA thanks to their comparable melting point temperatures, showing improved flexibility with respect to pure PHB. Also, extensibility can be enhanced by incorporating plasticizer or by fabricating composites through the addition of nanofillers (Panaitescu et al., 2018).

3.3. Bio-active packaging

In the last decades, food packaging has evolved beyond its use as simple containers and barriers against external factors. The impact of the traditional food preservation techniques (e.g., thermal treatments) on the nutritional and organoleptic characteristics of food, along with the consumer demand for healthy and safe products, prompted scientists and industries to develop packaging materials able to actively ensure food safety and extend the shelf-life, thus maintaining its quality and taste (Battisti et al., 2017). This new packaging approach is known as "active packaging" (Valerio et al., 2017).

Active packaging items are designed as "*materials and articles that are intended to extend the shelf-life or to maintain or improve the condition of packaged food*; *they are designed to deliberately incorporate components that would release or absorb substances into or from the packaged food or the environment surrounding the food*" (European regulation [EC] No. 450/2009).

Active food packaging aims to improve and expand the features of traditional packaging including containment, protection, preservation, and communication, shifting from a passive defensive role towards an active role. It acts as a medium of interaction among product, environment and packaging itself, altering the native environment of the packed product and thus enhancing its safety, shelf life and quality (Yildirim et al., 2018).

Depending on its functioning mode, active packaging can be classified under two major categories: scavenging and emitting systems. Scavengers are materials employed to absorb undesirable substances from the internal packaging environment, including moisture, oxygen, carbon dioxide, ethylene, and odours/flavours. Conversely, emitter systems are designed to discharge specific substances with desirable properties to produce a positive impact in the packaging headspace (Chawla, Sivakumar, & Kaur, 2021). These active compounds can be either part of the packaging material or enclosed inside the package, separated from the packed food. The advantages related to the first solution with respect to the second one are: (I) no possible manipulation by the consumer, decreasing the chance of contamination; (II) packaging is produced with conventional equipment, decreasing the complexity of the production process.

Some examples of substances commonly added to the packaging system with different purposes are antioxidant and antimicrobial agents (e.g., polyphenols; essential oils), enzymes, aromatic compounds, nutraceuticals and pre- or pro-biotics. Among these, antimicrobial and antioxidant active compounds (either synthetic or natural-based) have been recognized as the most attractive ones to be

incorporated into packaging systems, since microbial spoilage and lipid oxidation can be considered as the two major causes of food deterioration (Jideani & Vogt, 2016).

3.3.1. Antimicrobial packaging

Antimicrobial packaging has received increasing attention from food and packaging industries as a valuable alternative over thermal treatments and other preservation technologies to control the growth and avoid the spread of targeted pathogenic and spoilage microorganisms in the food products (Zhong et al., 2020).

Concisely, antimicrobial packaging is obtained by incorporating an antimicrobial agent in the packaging material (Asgher et al., 2020). This represents a potential alternative to the direct addition of bioactive agents into or on the surface of food, which could lead to the immediate depletion of the antimicrobial functionality, thus failing the restriction of microbial growth for a medium/long-term period (Landi, Paciello, de Alteriis, Brambilla, & Parascandola, 2015). In this sense, antimicrobial packaging can exert a controlled release of the antimicrobial compounds, whose migration kinetics depend upon different factors such as the molecular structures of the polymer and antimicrobial compounds, the physicochemical characteristics of the packaging item (e.g., the thickness of the film), and the environmental conditions, both internal and external (Asgher et al., 2020). In this context, the design of an antimicrobial packaging system is complex, since it requires a thorough knowledge of five major factors and their interactions: the food product; the internal package atmosphere; the targeted microorganisms; the packaging material; the antimicrobial agent (Gonçalves & Rocha, 2017).

Different approaches have been explored for the development of biobased antimicrobial packaging solutions. According to their structure and production process, antimicrobial packaging systems can be categorized in five classes (Chawla et al., 2021). The first class consists in antimicrobial sachets which are included in the package, and from which the active compound is gradually released during the storage period. In the second class, the active molecules are directly blended in the polymer matrix to produce antimicrobial items, and therefore should be carefully evaluated for their thermal and chemical stability. The third class of antimicrobial packages are obtained by adsorbing a specific matrix, serving as a carrier of the antimicrobial additive, onto the packaging surface. This production method overcomes the disadvantages related to the second class, since the active compounds are not exposed to high temperatures and shearing forces related to the production of the packaging material. In the fourth class, the antimicrobial agent is immobilized on the polymer matrix through ionic or covalent bonds between their functional groups. In this case, polymers and additives should share

compatible functional groups, and the release of the active agent from the matrix largely depends on the type of bonding. The fifth class of antimicrobial packaging involves the application of polymers with intrinsic antimicrobial properties (e.g., chitosan). This approach requires the direct contact between packaging material and the food product for effective inhibition, which could be considered a limiting factor for two reasons: inhibition process is restricted to superficial contact layers; the polymer must be approved as a food additive (Sofi et al., 2018).

The compounds which have been explored and implemented for the production of active packaging due to their antimicrobial features belongs to several categories of molecules, either synthetic or extracted from plant, animal and microbial biomasses (Qamar, Asgher, & Bilal, 2020). The widespread tendency to reduce the application of conventional synthetic additives has led to the broad diffusion of bio-based (in some cases, natural-based) antimicrobial compounds which include organic acids, essential oils, polyphenols, polysaccharides (e.g., chitosan), and polypeptides (e.g., lysozyme, lactoferrin, bacteriocins) (Mendonca, Jackson-Davis, & Motiq, 2018). All these classes of molecules have been successfully integrated into bio-based packaging, with promising results against pathogenic and spoilage bacteria and fungi (Moeini et al., 2021).

A brief overview of the main classes of compounds which were investigated for the development of bio-based antimicrobial packaging is provided in the next sections.

3.3.1.1. Essential oils (EOs)

Essential oils (EOs) are aromatic secondary metabolites, present in various plants. They consist in complex oily blends of 20-60 components (at different concentrations), extracted from different plant parts including roots, leaves, flower and bark. They are extracted through various techniques, such as solvent extraction, distillation, cold pression, and non-conventional technologies (e.g., microwaves; ultrasounds; supercritical fluids) (Mendonca et al., 2018). The composition of EOs includes monoterpenes and sesquiterpenes as the predominant quantitative components, followed by phenolic acids, aldehydes, ketones, and terpenoids. Due to the presence of various active molecules with different functional groups, EOs have been reported to exert a broad number of biological activities, including antibacterial, antifungal, and antioxidant (Moeini et al., 2021). These features were extensively tested both in food and packaging research fields, in view of their promising application as novel substitute to synthetic antimicrobial compounds.

Due to the variety of compounds constituting their composition, the biocidal action of EOs is exerted through different pathways. However, it is common agreement that the main target of EOs is the

cytoplasmic membrane of the microbial cell, whose permeability is influenced by the composition and the hydrophilic/hydrophobic behavior of the compounds interacting with it (Nazzaro, Fratianni, De Martino, Coppola, & De Feo, 2013). Since EOs are hydrophobic, their presence induces a change in the structure and fluidity of the cell membrane. This process triggers a cascade of chain reactions, resulting in internal pH disorder, electrical potential alteration, and impairment of the sodium-potassium pump, ultimately culminating in cell death (Batiha et al., 2021).

Several studies investigated the ability of EOs either free or incorporated in biodegradable packaging to impede the growth of gram-positive bacteria (e.g., *S. aureus; L. monocytogenes*), gram-negative bacteria (e.g., *Aeromonas hydrophila; E. coli, S. enterica, Campylobacter jejuni, Pseudomonas aeruginosa*) and fungi (*Fusarium spp.; Aspergillus spp.; Penicillium spp.*) (Guo et al., 2021; Haghighi et al., 2019a; Kumar et al., 2020; Wang et al., 2018). These researches highlighted that the antimicrobial effectiveness of EOs depend on their specific composition and source, as well as the defensive strategies fielded by the tested microorganisms (Asgher et al., 2020).

The most common EOs which were applied as active agents in food packaging include cinnamon (cinnamaldehyde) (Mohammadi, Mirabzadeh, Shahvalizadeh, & Hamishehkar, 2020), rosemary (Choulitoudi et al., 2017), ginger (Bonilla, Poloni, Lourenço, & Sobral., 2018), oregano (Lee, Garcia, Shin, & Kim, 2019), tea tree (Souza et al., 2017), thyme (thymol and carvacrol) (Sharma, Ahuja, Izrayeel, Samyn, & Rastogi, 2021), and citrus (Li, Tang, & He, 2021).

All these researches demonstrated that, along with the antimicrobial activity, EOs presence can remarkably affect the structure of the packaging materials such as films and coatings, either improving or worsening their technical performances by interacting with the polymer matrix and the plasticizers (Haghighi et al., 2019a). Besides, their antimicrobial effect can be compromised by the fast release of volatile active compounds before the end of the shelf-life period. Furthermore, EOs may also influence the sensory and organoleptic attributes of foods, which could be disadvantageous for their widespread application (Tongnuanchan & Benjakul, 2014). A strategy to solve these issues is represented by the micro or nanoencapsulation of EOs and subsequent addition to the polymer matrix. This process allows to perform a controlled delivery of the bioactive compounds, protecting them from the high temperatures reached during the polymer-extrusion step, and avoiding an exceeding impact on the sensorial profile of the food product (Lee et al., 2019).

3.3.1.2. Animal-derived polypeptides

Polypeptides are the most common animal-derived antimicrobial compounds. They are mainly secreted as a defence mechanism against bacterial spread and infections. Among these, lysozyme, lactoperoxidase and lactoferrin have been extensively investigated for active packaging applications due their enhanced antibacterial properties (Pisoschi et al., 2018).

Lysozyme is an animal-derived enzyme which was recognized as safe (GRAS) for direct inclusion in food matrices (FDA, 1998). It is stable over broad ranges of temperature (4-95°C) and pH (2-10). The biocidal activity of lysozyme has been tested against a wide range of pathogens and spoilage bacteria, founding its main effectiveness on gram-positive bacteria such as *Clostridium tyrobutyricum*, *Micrococcus spp.*, and *L. monocytogenes* (Davidson, Taylor, & Smith., 2013).

Lysozyme expresses its antibacterial activity by disrupting the peptidoglycan layer of bacterial cell walls, which is achieved through the hydrolysis of the bond between N-acetyl-d-glucosamine and N-acetyl-muramic acid (Aloui & Khwaldia, 2016). This specific mechanism of action makes lysozyme extremely effective against gram-positive bacteria, while the lipo-polysaccharidic layer of gram-negative bacteria inhibits its access to the site of action. Many researches suggested the possibility to enhance the lysozyme activity against gram-negative bacteria by modifying its molecular structure through different pathways including covalent attachment of saturated fatty acids to lysine residues, thermal denaturation, glycosylation, reduction of disulfide linkages, and application of chelating molecules (Lèsnierowski & Yang, 2021). Nowadays, lysozyme is mainly used on large-scale industry to challenge undesired butyric fermentation and late blowing caused by *C. tyrobutyricum* in semi-hard cheeses such as “Grana Padano” (Soggiu et al., 2016).

Lactoperoxidase is another animal-derived enzyme, secreted in the epithelial cells of the mammary gland and largely present in cow’s milk (20 times higher compared to human milk) (Saeed, Afzaal, Tufail, & Ahmad, 2019). It is extremely effective against enteric bacteria including *Salmonella spp.*, *Shigella spp.* and *E. coli.* It catalyses the oxidation of thiocyanate groups by hydrogen peroxide to yield thiocyanogen, which is then hydrolysed to hypothiocyanite. These unstable molecules react with the sulfhydryl groups of the bacterial cell membrane proteins, causing microbial death. This enzyme can be applied at ambient temperature, and thus is recommended as an alternative to chilling for the preservation of raw milk (Buys & Sefu, 2022).

Lactoferrin is a globular glycoprotein exerting antioxidant, anti-carcinogenic, anti-obesity and antibiotic properties. It is found in secretions of reproductive, respiratory and digestive systems of humans and other mammalians. Besides, it is present in large quantity in colostrum milk (Niaz et al., 2019).

The antimicrobial activity of lactoferrin is due to its ability to chelate iron, which results in the disruption of the external membrane of gram-negative bacteria. Along with the biocidal activity, lactoferrin was demonstrated to exert a bacteriostatic action, decreasing the microorganisms' proximity to nutrients. Thus, it resulted highly effective against many food-borne pathogenic bacteria such as *E. coli, Klebsiella spp.*, and *L. monocytogenes* (Gyawali & Ibrahim, 2014).

Due to their enhanced antimicrobial activity and naturality, lysozyme, lactoperoxidase and lactoferrin have been applied as additive to produce biodegradable packaging able to inhibit the growth and exploitation of pathogenic microorganisms contaminating the surface of ready-to-eat foods.

3.3.1.3. Antagonistic microorganisms and bacteriocins

Some microorganisms and their metabolites are able to prevent the growth of others. This ability has attracted the attention of researchers and industries, eager to investigate the possibility to apply them as a "natural shield" to the growth of spoilage and pathogenic microorganisms in food. Nowadays, the application of "antagonistic microorganisms" and their derivatives for preserving food has become widespread, and it is commonly referred to as "bio-preservation" (Quinto et al., 2019).

The prominent class of antagonistic microorganisms that has found wide employment in food systems are the Lactic Acid Bacteria (LAB). LAB have been defined as GRAS by the FDA, and have obtained the Qualified Presumption of Safety (QPS) by the European Food Safety Authority (EFSA) (Agriopoulou, Stamatelopoulou, Sachadyn-Kròl, & Varzakas, 2020).

The use of LAB to compete against pathogenic and spoilage microorganisms in food matrices have been investigated, along with their ability to produce nutrients and metabolites with antimicrobial properties. Successful results were achieved by applying them to fruit and vegetables (Ramos, Brandão, Teixeira, & Silva, 2020), fresh dairy products (Cheong et al., 2014) and cooked meat (Gao, Li & Liu, 2015). In this studies, different species of *Lactobacillus* showed their capacity to thrive in competition with bacterial (e.g., *L. monocytogenes*) and fungal (e.g., *Penicillium spp.*) populations.

Bacteriocins are proteinaceous metabolites mainly produced by LAB as a defence mechanism against other microbial strains. Their promising application has been assessed on a wide range of food products, including minimally processed fruits and vegetables, dairy products, meat and fish. In particular, their maximal potency is expressed when combined with other technologies through a hurdle approach (Batiha et al., 2021). Moreover, their use should follow already used good manufacturing practices, efficient processing conditions, and perfect storage and distribution practices.

Among the large variety of bacteriocins produced by LAB, nisin and pediocin are the major and most prominent bacteriocins that have received attention as promising food bio-preservatives (Aloui & Khwaldia, 2016).

Nisin is a heat-stable protein produced by specific *Lactococcus lactis* strains. It possesses a strong antibacterial activity against gram-positive bacteria such as *Staphylococcus*, *Bacillus cereus*, *Clostridium botulinum*, *Clostridium sporogenes*, *L. monocytogenes*, *Micrococcus* and others. However, it exhibits a lower inhibiting activity against gram-negative bacteria and fungi (Tiwari et al., 2009). In fact, nisin hinders the growth of gram-positive cells by binding to specific functional groups of the cell wall, which results in the poration of the cell membrane and the loss of intracellular constituents (Bauer & Dicks, 2005). Thus, nisin found one of its most promising applications in controlling the populations of *L. monocytogenes* and *Clostridium spp.* in dairy products, both singularly and in combination with other technologies (Ibarra-Sánchez, El-Haddad, N., Mahmoud, Miller, & Karam, 2020).

Pediocin is produced by different species of *Pediococcus*, a group of gram-positive, homofermentative bacteria belonging to the family of *Lactobacillaceae* (Haakensen, Dobson, Hill, & Ziola 2009). Pediocin acts by generating holes in the cytoplasmic membrane of the target cells, reducing the intrinsic pH and inhibiting the proteins responsible for energy production (Gabrielsen, Brede, Nes, & Diep, 2014). The addition of concentrated pediocin has been tested for the preservation of food commodities susceptible to spoilage, including vegetables, dairy products (Verma, Sood, Saini, & Saini, 2017), and processed meat (Castellano, Pèrez Ibarreche, Blanco Massani, Fontana, & Vignolo, 2017). The activity of pediocin in food is mainly influenced by pH, osmotic equilibrium, enzymes activity, and temperature (Niamah, 2018).

Bacteriocins have been applied as antimicrobial additives incorporated in active packaging, to ensure the safety and extend the shelf life of the products in direct contact with the package. For example, nisin has been successfully employed in antimicrobial films (both petroleum-derived and bio-based), used to wrap raw and processed meat, and tested against *Listeria spp.* (Blanco Massani et al., 2014). Moreover, its impact on the technical properties of biodegradable films was evaluated in a recent study (Sun et al., 2019).

3.3.2. Antioxidant packaging

Antioxidant packaging represents another trend category of active packaging. In this case, packaging is enriched with active compounds able to delay the oxidation rate of the packed products (Mir et al., 2018). Thus, the use of antioxidants to produce active packaging items has the potential to reduce the generation of food waste and its disposal costs.

With respect to the food sector, the activity of an antioxidant agent is mainly addressed to suppress the ignition of lipid oxidation chain reactions, which naturally occurs within biological matrices. This process causes the gradual alteration and decay of colour (i.e., enzymatic oxidation), odour and flavour (oxidative rancidity), structure (i.e., softening), and nutrients (Arnon-Rips & Poverenov, 2016). Antioxidants strongly differ from each other for their reaction pathways. Some molecules act as "direct" antioxidants, reacting with intermediate peroxyl radicals (produced in the early stages of oxidation), and blocking the subsequent reactions (e.g., glutathione, ascorbic acid, polyphenols). Other molecules act as "preventative" antioxidants, binding cationic metals such as Fe (II) and Cu (II) (e.g., albumin) (Petkoska et al., 2021). According to their molecular nature and reactive mechanism, antioxidant agents can be employed to produce release-type packaging, which transfer the active substances to the food surface at a sustainable rate, or scavenging-type packaging, which sequester target radicals and ions without affecting the food composition (Rangaraj et al., 2021).

The development of an antioxidant packaging system starts with the selection of the bioactive agent, which must comply with two main requirements: (I) suitability for the target product to-be-preserved, and (II) compatibility with the polymer matrix, to achieve a homogeneous distribution of the substance in the packaging item (Gòmez-Estaca, Lòpez de Dicastillo, Hernàndez Muñoz, Català, & Gavara, 2014). Focusing on bio-based and edible packaging, antioxidant films and coatings are mainly obtained through direct incorporation of the active molecule in the biopolymer matrix, followed by solution casting. Other techniques involve the functionalization of the packaging material via physical (e.g., encapsulation) or chemical (e.g., crosslinking, plasticizer addition) processes, which affect the adhesion of the active compounds to the polymer matrix. These processes allow to tailorize the rate of release and/or scavenging mechanism of the active molecule, adapting the materials for a broad range of applications (Castro-López, López de Dicastillo, López Vilariño, & González Rodríguez, 2013).

A broad variety of antioxidants have been evaluated for the development of active packaging, including synthetic molecules like butylated hydroxy toluene (BHT) and butylated hydroxy anisole (BHA). Despite their strong antioxidant efficacy, the use of these molecules has been questioned for food safety reasons, since they are toxic and harmful for human health (Barbosa-Pereira et al., 2013).

As a result, the current trend is focused on replacing synthetic additives with natural and harmless alternatives.

3.3.2.1. Natural antioxidants

Natural antioxidant molecules can be mainly categorised into three sub-groups: (I) vitamins (e.g., ascorbic acid; α-tocopherol), (II) carotenoids (e.g., carotens; xantophylls), and (III) phenolic compounds.

Polyphenols constitute the most popular and important group of naturally-occurring antioxidant compounds employed for the production of active packaging due to their strong free-radicals scavenging effect (Papuc, Goran, Predescu, Nicorescu, & Stefan, 2017).

The antioxidant activity of polyphenols is commonly ascribed to single-electron transfer and hydrogen transfer mechanisms, which allow the active molecule to react with active radical species of the matrix, producing stable and harmless oxidized molecules.

Related to their composition, polyphenols can be classified into (ii) non-flavonoids and (ii) flavonoids. Among them, flavonoids are the most largely studied for packaging applications due to their strong biological and antioxidant activity. Flavonoids are present in the form of flavonols, flavones, isoflavones, anthocyanins, and others sub-groups. Most of them are polar, which makes them compatible with most of the hydroplastic polymers, and extracted through protic solvents (e.g., water, ethanol, methanol, isopropanol) from non-edible portions of fruit and vegetable by-products, such as peels, stones, and seeds (Goulas & Manganaris, 2012; Nguyen, Pham, Bowyer, van Altena, & Scarlett, 2016).

Generally, polyphenols are not employed in active packaging singularly, but mostly exist as complex mixtures which include aqueous and alcoholic plant extracts, essential oils from spices and herbs, and a broad variety of phenolic concentrates obtained from various waste bio-sources (Tuberoso, Boban, Bifulco, Budimir, & Pirisi, 2013). For this reason, the overall antioxidant activity of these products not only refers to their polyphenolic content, but it strongly depends on their source, chemical composition and extraction process (Oroian & Escriche, 2015).

A brief description of the main sources of natural antioxidants is provided in the next sections.

- *Plant extracts*

The inclusion of plant extracts, as complex systems containing numerous molecular components at variable concentrations, has an interesting potential to functionalize biobased packaging materials with antioxidant bioactivity. These mixtures are isolated from several botanical sources through solvent-extraction technology. The extraction efficiency, and thus the phenolic content of the extracts, can be varied by changing the operational parameters, such as time, temperature, solvent type, solvent concentration, and pH (Nguyen et al., 2016). Moreover, physical processes such as microwave, ultra-sonication, and milling allow to further enhance the extraction rate of these antioxidants, increasing the polar components in the extracts (Garcia-Vaquero, Ummat, Tiwari, & Rajauria, 2020).

The main vegetal sources of polyphenolic extracts used in food packaging comprise medical plants (leaves, roots, and stems), and various edible and non-edible parts of fruits and vegetables. Among medical plants, extracts from thyme (Talón et al., 2017a), black tea (Peng; Wu & Li; 2013), green tea (Siripatrawan & Harte, 2010), mint (Raghav & Saini, 2018), rosemary (Bonilla & Sobral, 2016), and sage (Oudjedi, Manso, Nerin, Hassissen, & Zaidi, 2019) have been added to film forming solutions based on single polymers (e.g., chitosan, gelatin, starch etc.) and their combination to produce antioxidant films for packaging purposes. About edible fruits, grape seed (Xiong, Chen, Warner, & Fang, 2020), pomegranate peel (Kumar, Ojiha & Singh, 2019), thinned apple (Sun et al., 2017) and other fruit-derived matrices have been evaluated as sources of polyphenolic extracts. All these researches highlighted the ability of the extracts to enhance the radical scavenging capacity of the polymer, mainly due to their high phenolic component.

The polyphenolic prolife of an extract strongly changes in relation with its source. According to their composition, different extracts diversely interact with the polymer matrix, creating variable hydrogen-bonding patterns (Siripatrawan & Harte, 2010). This fact not only influences the final antioxidant property of the film, but can alternatively affect the mechanical and barrier properties of the packaging item. For example, in some cases the large number of viable hydroxyl groups induce an increase of the free volumes in the blend matrix, leading to highly flexible films (Bonilla & Sobral, 2016). In contrast, the rigid aromatic and heterocyclic rings of flavonoids can act as physical crosslinkers of the polymer chains, improving the tensile strength and elastic modulus of the film (Lòpez de Dicastillo, Bustos, Guarda, & Galotto, 2016).

- *Essential oils*

The incorporation of essential oils extracted from plants in the packaging material not only exerts an antimicrobial function, but can also improve oxidation stability, due to their high polyphenolic content (Pandey, Kumar, Singh, Tripathi, & Bajpai, 2017).

In particular, oregano (Jouki, Yazdi, Mortazavi & Koochevi, 2014; Lee et al. ,2019), thyme (Ruiz-Navajas, Viuda-Martos, Perez-Alvarez, & Fernandez-Lopez, 2013), clove bud (Nisar et al., 2018), and rosemary (Abdollahi, Rezaei & Farzi, 2012) EOs showed promising results as antioxidant additive to produce active edible films. Their bioactivity was mainly ascribed to the large presence of polyphenols such as thymol and carvacrol, responsible for both their antioxidant and antimicrobial characters.

3.3.3. Challenges

Although a large number of natural antioxidant and antimicrobial agents have already been investigated, and many efforts have been devoted on developing biobased active packaging solutions, there is still a deep gap between laboratory-scale research and real-time applications and commercialization (Moeini et al., 2021).

The first root of this gap is technological. For example, laboratory-scale researches highlighted that EOs possess a strong biocidal efficacy on a broad range of microorganisms, which virtually makes them suitable alternatives to conventional preservatives. However, each of them also possesses a peculiar aromatic profile, which could negatively affect the smell and taste of the food product, and thus drastically limits its wide usage (Malhotra, Keshwani, & Kharkwal, 2015).

The second root is economical. As an example, several authors suggested to reduce the sensory impact of EOs by entrapping them in nano-emulsions or sachets. However, these approaches find economic restrictions since they are not cost-effective (Ozogul et al., 2017). Besides, talking about natural-derived metabolites, their production relies on the availability of their resource, on the extraction procedure (i.e., time, solvents, energy), the purification steps etc. All these aspects contribute to increase the final price of the product.

The third root of this gap is related to the impact of these compounds on human health and environment. In this sense, the composition of each active agent, its specific migration rate from the packaging material, and the interactions with the food product should be fully characterized to avoid any possible hazard for human health and to ensure the quality of the whole package.

Last, but critically worthy, regulatory issues strongly limit the commercialization of bioactive packaging systems, which relies on severe legal regulations to ensure protection of consumer interests and to enforce the prevention and repression of food fraud (Moeini et al., 2021).

These concerns certainly set a significant drawback for the pilot and industrial exploitation of natural compounds as additives in novel, upgraded, bioactive food packaging materials.

3.4. Nanotechnology in biodegradable packaging

Biobased packaging possesses specific drawbacks, such as brittleness, moisture sensitivity, low heat distortion temperature, and limited industrial applicability (Farhoodi, 2016). Among various possible approaches, nano-technology represents one of the major research topics of the packaging sector due to the huge number of prospective applications and advantages (Abdollhai, Rezaei, & Farzi, 2012).

The use of nano-materials traditionally covers many aspects of the food sector, including food safety, nano-sensors, nutrients delivery, and pathogen detection (Petkoska et al., 2021). Lately, nano-technology have been utilised to improve the technical performances of conventional bio-based materials, and to give them additional features. Besides, this novel approach is laying the basis for the development of a new generation of smart and intelligent food packaging systems, able to localize, sense, and remote control the food items (Nile et al., 2020).

The use of nano-structures (i.e., nano-fillers, bio-nanocomposites, and nano-capsules) is expected to broadly enhance the potentialities of biobased packaging, and extend the number of smart packaging solutions in the next few years. Thus, it is worthy to provide a brief description of these concepts, which follows in the last sections of this work.

3.4.1. Bio-nanocomposite materials

Nanoparticles are characterized by nanoscale dimensions, usually <100 nm. When nanoparticles are incorporated into a biopolymer material with specific technological purposes, they take the name of "nanofillers", and the resulting item is called "bio-nanocomposite" (Chaturvedi & Dave, 2020; Sharma, Jafari, & Sharma, 2020). Bio-nanocomposite materials may be defined as a multiphase material in which a continuous phase (i.e., a biopolymer) is embedded with a non-continuous nano-dimensional phase (i.e., a nanofiller), either inorganic or organic (Zubair & Ullah, 2020).

Due to their small size, high aspect ratio, and large interfacial areas, nanofillers have been firstly explored as structural reinforcing agents, with the function to improve the technological properties of

packaging materials. When uniformly distributed in the polymer matrix, nanofillers are able to interact with the polymer chains, creating a tangled network of hydrogen bonds that fill the free spaces within the matrix and restricts its molecular mobility (Chawla et al., 2021). In this way, nanoparticles provide an overall enhancement of mechanical, gas barrier, water barrier, and thermal properties of the packaging material with respect to traditional non-composite systems (Honarvar, Hadian, & Mashayekh, 2016; Maftoonazad & Ramaswamy, 2018). In particular, it was demonstrated that low concentrations of fillers (< 5%) are able to significantly improve the biopolymer properties, which is economically advantageous in view of their large-scale application (Rhim, Park, & Ha, 2013a).

Along with the structural function, the incorporation of nanofillers also represents a suitable strategy to confer additional functions to the packaging material. On the one hand, nanofillers can serve as bioactive additives, since some of them exhibit inner antimicrobial, antioxidant, and scavenging properties (Nile et al., 2020; Shah et al., 2015). In this sense, they can exert a direct function, assuring food safety from microbial spoilage and stability against oxidation. On the other hand, nanofillers incorporation allows to tailorize the retainment and release kinetic of bioactives from the polymer matrix, and to adapt the barrier performances of the packaging item (Gòmez-Estaca et al., 2014). As a result, the correct selection of a nanofiller (nature, quantity) and suitable process parameters allow to customize bio-nanocomposite materials for countless potential applications (Vasile, 2018).

3.4.1.1. Nano-clays

Clays have gained remarkable interest as reinforcing fillers to improve the mechanical, thermal, and barrier properties of biopolymers (Vengatesan, Singh, Pillai, & Mittal, 2016). These siliceous compounds mainly exist in the form of laminated one-dimensional (1D) or two-dimensional (2D) fibrous structures that can be easily dispersed into a polymer through two possible mechanisms, namely intercalation or exfoliation (de Azeredo, 2009). The latter mode represents the best strategy to incorporate these compounds into a polymer matrix, since it allows the complete delamination of the particles, and thus their homogeneous diffusion at nanoscale level (Farhoodi, 2016).

Some widespread nano-clays applied to develop bio-nanocomposite materials are montmorillonite, bentonite, palygorskite, and sepiolite. Among these, montmorillonite have been largely tested due to its excellent technical behavior, abundance, low cost, and compatibility with a wide range of biopolymers (Alboofetileh, Rezaei, Hosseini, & Abdollahi, 2013). It consists of a hydrated aluminum silicate layered structure, with a modest negative charge which varies from layer to layer (de Azeredo, 2009). It possesses a high surface ratio and interfacial area, which contributes to its uniform distribution into biopolymer matrices.

The features of a clay-reinforced film strongly depend on the polymer matrix, chemical and physical nature of the clay, the clay-polymer interactions, and processing conditions (Sothornvit, Hong, An, & Rhim, 2010). Besides, surface-modification methods have been tested on clays to enhance their capacity of interfacial interaction, including the use of alkylammonium cation surfactants. However, these surfactants are not appropriate for modifying clay surfaces in bio-applications due to their toxicity (Kirkmeyer, Puetter, Yahil, & Winey, 2003). As a result, most clay-composites are prepared using unmodified clay materials.

3.4.1.2. Metal nanoparticles

Metal nanoparticles such as copper (Cu), silver (Ag), gold (Au), and their alloys have been widely applied to produce nanocomposite active films and coatings due to their strong antimicrobial activity (Bajpai et al., 2018).

Different mechanisms have been postulated to explain the antimicrobial action of metal nanoparticles. In particular, Tamayo, Azòcar, Kogan, Riveros, & Pàez (2016) suggested a 3-step mechanism to explain the antimicrobial activity of Cu-nanoparticles on the bacterial cell in Cu/polymer nanocomposites: (I) the biopolymer gradually releases Cu^{2+} ions, which permeate the cell wall and interact with the membrane proteins and lipopolysaccharides; (II) the cell wall collapses due to the weakening of the membrane, which leads to the loss of cell organelles; (III) ions interact with the bacterial DNA, causing its rupture and producing reactive oxygen species (ROS), which lead to oxidative damage and bacterial death. A similar mechanism was also proposed to describe the activity of Ag-doped edible packaging (Bajpai et al., 2018).

Despite their antimicrobial activity, metal nanoparticles possess a certain antioxidant activity, exerted *via* the radical scavenging mechanism (Bhakya, Muthukrishnan, Sukumaran, & Muthukumar, 2016). Moreover, they are compatible with various natural antioxidant extracts and EOs, and thus can be used in synergy with them to produce films with enhanced antimicrobial antioxidant performances (dos Santos, Ingle, & Rai, 2020). Additionally, the incorporation of metal nanoparticles can alter the barrier properties of the material, by filling the voids in the porous matrix and interacting with the polymer chains (de Moura, Mattoso, & Zucolotto, 2012).

3.4.1.3. Metal oxides

Nanocrystalline metal oxides have been extensively studied for food packaging applications due to their strong antimicrobial properties, which makes them promising alternative to organic agents. They

include titania (TiO_2), silica (SiO_2), magnesium oxide (MgO), zinc oxide (ZnO), iron oxide (Fe_3O_4), and others. Among these, TiO_2 and ZnO were the most widely tested in the food packaging sector due to their specific physico-chemical characteristics (e.g., high interfacial area), chemical stability, low price, and biocompatibility (Oun, Shankar, & Rhim, 2020). These nanoparticles have been tested both as a reinforcing agent to improve the technical properties of edible films, and as antimicrobial additives. Specifically, they possess a remarkable photocatalytic activity in the near UV region: under UV illumination, they generate reactive oxygen species (ROS) that can directly damage the cell walls, leading to microbial death (Rajakumar et al., 2018).

As an example, Siripatrawan & Kaewklin (2018) developed TiO_2-enriched chitosan films. Authors showed that increasing concentrations of TiO_2 enhanced the photodegradation rate of ethylene. Besides, the film exhibited a broad antimicrobial activity against gram-negative and gram-positive bacteria.

3.4.1.4. Bio-nanofillers

Bio-nanofillers are ultrathin structures produced by different methods (e.g., electrospinning; acid hydrolysis etc.) from organic materials such as carbohydrates, proteins, lipids. They are biodegradable renewable, easily available, and possess a high surface-to-volume ratio and low density. These particles have been extensively tested in the food packaging sector as reinforcing agents, and to modulate the delivery of bioactive compounds from the packaging material to the food surface (Johansson et al., 2012).

Nano-sized cellulose derivatives are the most widespread bio-nanofillers to fabricate biodegradable composites (Vilarinho, Sanches Silva, Vaz, & Farinha, 2018). Cellulose nanoparticles can be classified into three types, related to their structure and dimensions: (I) cellulose nanocrystals (CNCs), which are rod-like microcrystals with 5–70 nm width and 100–250 nm length; (II) cellulose nanofiber (CNFs), that possess a fibrous structure with a width of 5–60 nm and length of several nanometers; (III) bacterial cellulose, which consists in ribbon-shaped fibrils with 70–80 nm width (Bharimalla, Patil, Mukherjee, Yadav, & Prasad, 2019).

Cellulosic nanofillers exhibit a strong and characteristic self-association property, deriving from the inter- and intramolecular hydrogen bonding involving their surface hydroxyl groups (Dufresne, 2013). This promotes the strong adhesion of these materials on and within the polymer matrix, enhancing the mechanical characteristics of the composite material by creating tortuosity, crystal nucleation, and chain immobilization (Yang et al., 2016). In addition, the highly tortuous structure

induced by crystalline fibers can hamper the water vapor diffusion, resulting in low WVP values. Due to their surface reactivity, they can also serve as bio-scaffolds.

Cellulosic nanoparticles possess an enormous amount of active surface hydroxyl groups that can be modified by chemical reactions such as cationization, silylation, carboxylation, polymer grafting, and hybridation with metals and metal oxides (Rangaraj et al., 2021). In particular, surface-modified nanofillers possess higher interfacial compatibility with a wide range of biopolymer matrices with respect to un-modified ones. Surface-modification can also influence the overall polarity and hydrophilic behavior of the material, enhancing its ability to hinder vapor diffusion throughout the packaging system (Almasi, Ghanbarzadeh, Dehghannya, Entezami, & Asl, 2015).

3.4.2. Nano-encapsulation and nano-emulsions

Encapsulation is a technology which consists in packing a target substance into a solid envelope, with the double purpose to protect it from external interactions and provide a controlled release under specific environmental conditions (e.g., moisture, heat, pressure) (Pabast, Shariatifar, Beikzadeh, & Jahed, 2018). Commonly, hydrophilic materials (i.e., polysaccharides, proteins) are used to encapsulate hydrophobic substances, while lipids or other substances with hydrophobic character are used to pack hydrophilic compounds (Petkoska et al., 2021).

According to their size, capsules can be categorized as macro-, micro-, and nano- (Donsì, Annunziata, Sessa, & Ferrari, 2011). Specifically, nano-capsules have been widely applied as carriers of nutraceuticals (macronutrients, enzymes, prebiotic, probiotic, vitamins, omega-3-fatty acids) and technological additives (antioxidant, antibacterial, and antifungal chemicals; colourants; flavors; aromas) to produce functional food with enhanced safety and stability (Donsì, 2018). In addition, they were used to dope biodegradable films and coatings to fabricate nanocomposite packaging solutions with variable bioactivities (Hosseini, Rezaei, Zandi, & Farahmandghavi, 2016). As an example, Liu et al. (2017) developed films based on gelatin and enriched with different concentrations of tea polyphenols/chitosan nanoparticles. The incorporation of nanoparticles decreased the WVP of the resultant films. Moreover, the release kinetic of tea polyphenols from the film surface was evaluated by means of two food simulants (i.e., 50% ethanol at 4°C; 95% ethanol at 25°C). The study highlighted a slow releasing rate of the polyphenols for both the simulants, which was probably due to the film's tortuosity and increased diffusion pathways induced by the dispersed nanoparticles. Similar results were obtained by Cui, Surendhiran, Li, & Lin (2020) for zein films doped with pomegranate polyphenols/chitosan nanoparticles.

Nano-emulsification represents another technique which allows to increase the bioavailability and stability of bioactive compounds, and to guarantee their proper delivery in the surrounding environment. In this way, it helps providing food products with better physiological properties, microbiological stability, and enhanced nutritional and organoleptic properties (Robledo et al., 2018).

A nano-emulsion is a system composed by two immiscible liquids in which one is homogenously dispersed in the other, forming nano-sized globules (50–500 nm). Due to their high ratio of droplet surface/mass unit, nano-emulsions possess a high delivery/encapsulation ability. Thus, the bioactivity and bioavailability of active agents are commonly enhanced through nano-emulsification (Hasan, Ferrentino, & Scampicchio, 2020).

The most widespread application of nano-emulsions in food industry consists of the retainment and controlled delivery of active agents to solid foods. Bioactive molecules like EOs can be directly incorporated into a food system (i.e., functional food) or entrapped in polymer matrices to produce active packaging (Kumar et al., 2020). In particular, the incorporation of nano-emulsified EOs into biodegradable materials has the double advantage to minimize the concentration of active agent required to perform a valuable antimicrobial activity (by increasing its accessibility), and to reduce its sensory impact.

3.4.3. Challenges

Despite the advantages related to the use of nano-technologies in food packaging, the impact of nanoparticles (e.g., metal particles) on human health and on the environment represents a major concern (Rangaraj et al., 2021). The risks related to nanomaterials are mainly due to the lack of knowledge about their mechanisms of migration from the packaging to the food product and the environment. In this sense, food regulatory bodies such as FDA and EFSA have expressed their reservation about the extensive application of these materials, and established strict regulations on the transfer threshold of these compounds to food products. For example, EFSA established that the upper limit for silver migration in food packaging is 0.05 mg/ L in water and 0.05 mg/kg in food (EFSA, 2021). In this sense, further and in-depth research about the migration pathways of these particles is strictly required to sustain their regulatory approval, in view of the massive application of these novel materials on industrial scale (Sharma et al., 2020).

3.5. Biodegradable packaging from agri-food waste

To date, about 30-50% of food is wasted from post-harvesting to processing, storage, and consumer usage. Typical examples of food by-products are vegetable peels, fruit pomace, seeds, husks, barks, oil cakes, and low-quality whole fruits and vegetables (Girotto, Alibardi & Cossu, 2015). The large part of these matrices is still discarded in landfills, while a small portion is valorised as feedstock or as raw materials for value-added bioprocessing (Xiong et al., 2019).

A feasible strategy to valorise food waste and by-products consists of their use for the production of bio-based packaging materials. This approach involves two remarkable benefits. On the one side, food by-products constitute a cheap, renewable and under-utilised source of polysaccharides, lipids (e.g., wax), proteins, organic acids, essential oils, and many other valuable components (Dilucia, Lacivita, Conte, & Del Nobile, 2020). These components can be either employed as the major constituent of the packaging material or as minor additives, which results in the reduction of the overall costs of production and added-value generation. On the other side, one of the greatest challenges faced when developing biodegradable packaging materials is their insufficient engineering properties (namely, poor water resistance, mechanical strength, thermal stability, and processability) compared to conventional plastics (Coelho et al., 2020). The inclusion of food by-products components has been demonstrated to improve the engineering properties of the packaging material, thus conferring it additional features such as antioxidant and antimicrobial capacities (Rodsamran & Sothornvit, 2019b).

A brief overview on this topic is provided in the following paragraphs.

3.5.1. Pre-treatments of by-products and application for packaging production

The most common way to prepare bio-based films and coatings containing food by-products involves to directly blend the whole by-product or one/more of its components (i.e., fillers) with biopolymers and additional additives to form composite film solutions (Moghadam, Salami, Mohammadian, Khodadadi & Emam Djomeh, 2020). In this sense, a necessary step to apply by-products for packaging production is represented by their pre-treatment.

The first step of pre-treatment usually involves drying and milling processes. Drying process allows to stop the microbiological decay (related to the high moisture content) and to enhance the handling of the product. Milling process involves the mechanical breaking down of the product into fine and uniform-sized particles, which furtherly improves the processability, uniformity and dispersibility for blending (Coelho et al., 2020; Gouw, Jung, Simonsen & Zhao, 2017).

A further step of treatment can involve the isolation of specific purified components through the application of conventional or non-conventional (e.g., microwave or ultrasound-assisted) extraction techniques (Zhang et al., 2019). This process allows to isolate and purify specific fractions of the raw material, which is subsequently added to the film-forming solution for specific purposes such as technical properties enhancement (e.g., polysaccharides to improve mechanical properties; lipids to improve water-barrier properties etc.) and to provide additional features to the packaging material (e.g., polyphenols to confer antioxidant capacity; essential oils for antimicrobial activity etc.).

In recent years, some novel approaches have shown their potentialities as valuable ways to valorise food by-products for packaging development. Among several promising strategies, it is worthy to cite: isolation of nano-sized cellulose and their employment as bio-fillers to improve the mechanical and water-related properties of packaging material (this topic was already discussed in the previous paragraphs) (Yang, Bai & Wang, 2018); chemical modifications of the raw material by different methods (e.g., grafting) (Gowman et al., 2018); fermentation of fruit juice pulp to obtain thermoplastic biodegradable polymers like poly-hydroxy alkanoates (Melendez-Rodriguez et al., 2018).

3.5.2. Impact on the engineering properties of packaging

Finding suitable strategies to improve the engineering properties of biodegradable packaging, particularly for mechanical properties or water barrier properties, represents one of the main topics on which researchers mostly focused their efforts. In recent years, particular attention was dedicated to by-products (both whole and fractionated) as sustainable and green bio-fillers to produce materials with enhanced technical characteristics.

Taking into account the mechanical properties, Nair, Chen, Peng, Huang & Yan (2018) showed that inclusion of 5-15% of wood-based CNCs leaded to a significant increase in tensile strength of PLA films, mainly ascribed to the densified volume fraction of fibrils. Yang et al. (2018). observed that, according to the treatments performed on nano-sized cellulose (e.g., presence or absence of solid-state shear milling), their addition to the polymer matrix can either decrease or increase the tensile strength of the final film. This effect mainly depends on the interfacial contact area achieved between the nano-sized fibers and the polymer chains. Overall, it is interesting to note that many bio-based materials enriched with nano-sized cellulose have tensile strength comparable to commonly used low-density polyethylene (7.0-25.0 MPa), while the elongation percentage of most films are significantly lower than those of commercial products. This indicates that the addition of fillers reduces the

molecular mobility of polymer chains, resulting in unsatisfactory flexibility, and highlights the need for further and more-in-depth research on this topic (Gowman et al., 2018). Besides cellulose-based bio-fillers, other compounds derived from by-products can help improving the mechanical properties of packaging materials. As an example, pomegranate peel extract was found to enhance the elongation percentage of protein-based (from 81% to 173%) and PVA films (from 48% to 182%), based on the polyphenol interaction with the material matrices, which chemically strengthened the composite (Moghadaman et al., 2020).

The addition of by-products can reduce the water vapor permeability of a packaging material by altering its overall hydrophilicity (reducing the available hydrogen groups) and the structure of the biopolymer (increasing the tortuosity for the passage of water molecules).

Grape seed extract (Munir, Hu, Liu & Xiong, 2019), lime peel extract (Rodsamran & Sothornvit, 2019b), and other extracts were found to improve the water barrier properties of the tested materials when applied at specific concentration (excessive or not sufficient concentrations can either have no significant effect or worsen the mentioned properties).

Overall, most of the biodegradable packaging films still do not provide a sufficient water barrier for moisture-sensitive foods, and so their feasible applications are mainly restricted to dry products or as disposable food wrappers for fast foods that do not require improved water barrier properties (Yang et al., 2018). Further efforts are needed to pre-treat or modify food by-products or their components to improve their capacity to interact with the polymer matrices and modulate their final engineering properties.

Aside from the above-mentioned properties, some researchers highlighted the changes in oxygen barriers, optical properties, thermal properties, and the morphology of bio-based materials induced by food by-products addition. For example, the introduction of discarded balsamic vinegar or tea leaves waste extract remarkably decreased the oxygen permeability of PVA films (Quilez-Molina et al., 2020). As well, thermal stability could be enhanced by strengthening the chemical bonding pathways within the biopolymer matrix (Munir et al., 2019), or by including high thermal-stable components such as lignin (Nair et al., 2018).

3.5.3. Impact on antioxidant and antimicrobial capacities of packaging

Food waste and by-products usually contain a large number of bioactive compounds (i.e., polyphenols, organic acids, EOs) with variable properties. Recently, the application of these compounds has caught the interest of many researchers as an appealing strategy to confer targeted capacities (e.g., antioxidant capacity, antimicrobial capacity) to packaging systems, thus valorising the raw by-product.

Regarding the antioxidant activity, the addition of pomegranate peel extracts (Moghadam et al., 2020) apple skin powder (Choi et al., 2017), and black plum peel extract (Zhang et al., 2019) resulted in a significant increase of the antioxidant capacity of the final films compared to the films without the extracts. Some researchers obtained successful results by applying various by-product extracts to prevent the oxidation of lipid-rich foods. As an example, chitosan films enriched with olive pomace resulted in significantly lower peroxide values in walnuts compared to control (without extract) and polyethylene plastic films after 31 days of storage (de Moraes Crizel et al., 2018).

Along with antioxidant capacity, various by-products can also confer antimicrobial properties to the packaging material, especially in the form of extracts. Two examples are pine needle extract (Yu et sl., 2019) and black plum peel extract (Zhang et al., 2019).

The variable antimicrobial activities of extracts from by-products mainly result from the mixed active compounds that characterise their specific composition. Moreover, the antimicrobial efficacy strongly depends on the applied concentration and the interaction with other components, which can be either synergistic or contrasting.

To date, most of the studies focused on antimicrobial and antioxidant properties of biodegradable packaging enriched with food by-products extract are still performed on *in-vitro* level. For the future, it would be worthy to take a step further, testing the currently achieved findings for larger-scale applications (e.g., application *in-vivo* on different foods) in order to provide the food industry with more specific data about the impact of these extracts on food safety, quality, and shelf life.

Chapter 2

Impact of low-dose gaseous ozone treatment to reduce the growth of in vitro broth cultures of foodborne pathogenic/spoilage bacteria in a food storage cold chamber

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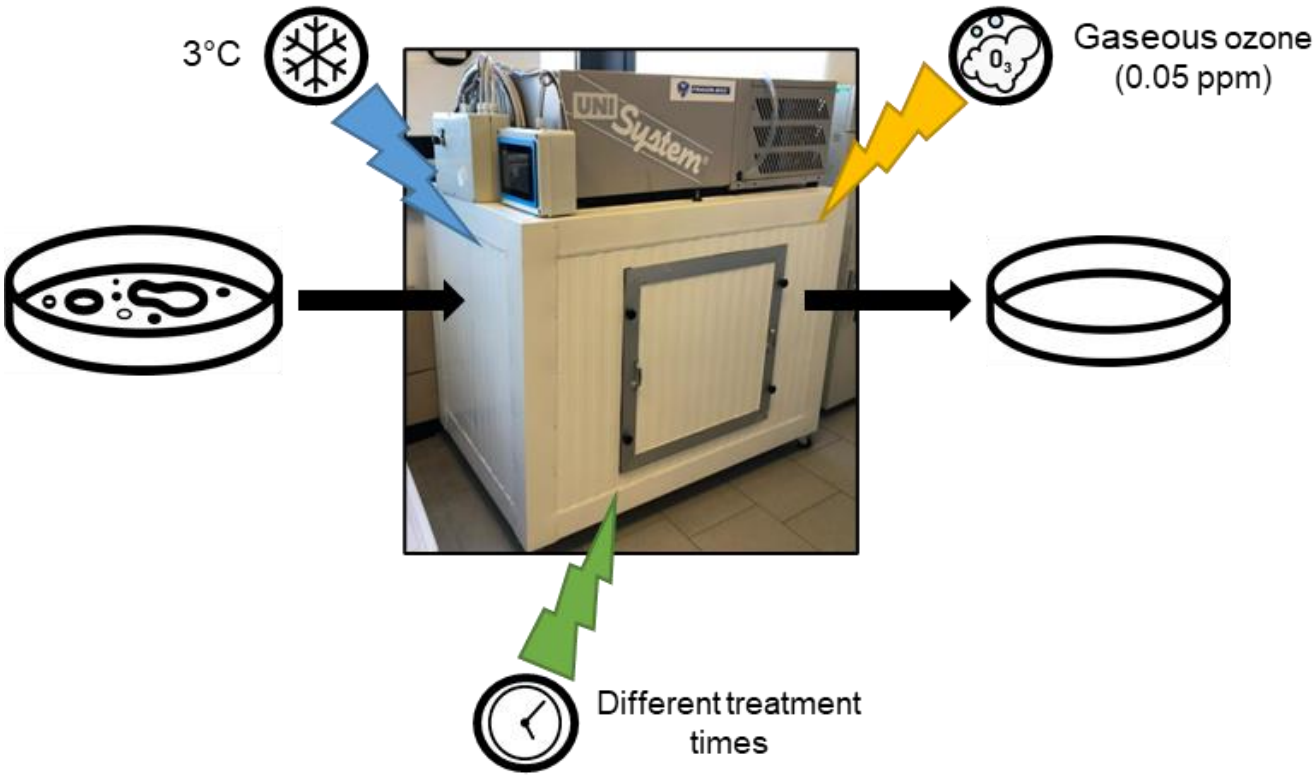
Graphical abstract



Keywords

Gaseous ozone; Food storage cold chamber; Surface sanitization; Air-borne spoilage; Food pathogenic bacteria.

Abstract

Cold storage coupled with gaseous ozone represents a potential strategy to reduce or inhibit the presence of pathogenic and spoilage bacteria in a food storage cold chamber. This study aims to evaluate the impact of gaseous ozone treatment (0.05 ppm at exposure times of 30 and 60 min) on the bacterial contamination of internal surface and air in a cold chamber (3°C) intended for food storage. The bacterial load of internal surfaces was reduced by 0.99 ± 0.24 and 1.35 ± 0.27 log after 30 and 60 min ozone treatment, respectively. Airborne bacterial load was reduced by 0.93 ± 0.24 log after 30 min ozone treatment and became non-detectable after 60 min. Gaseous ozone treatments (0.05 ppm at exposure times of 1, 2, 6, 24, 30, and 48 h) of the cold chamber were investigated to evaluate the effectiveness of this technology against *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enterica* Typhimurium, *Campylobacter jejuni*, and *Pseudomonas fluorescens* cultured in broth cultures. Ozone treatment was effective against *C. jejuni* since the population at the highest concentration of inoculum (3.34 log CFU/plate) was reduced by 2.23 log after 1 h, and it was completely undetectable after 2 h. *S. enterica* and *E. coli* showed the highest resistance to short ozone treatment since 6 h treatment did not show antibacterial activity. Whereas, after 24 h treatment 2 log reduction was observed for both pathogens. Short ozone treatment did not affect *L. monocytogenes* viability. *P. fluorescens* showed high sensitivity to short treatments, with 0.75–1.32 log reductions after 1 h, and further 0.22–0.53 log reductions after 6 h.

1. Introduction

Nowadays, consumers are increasingly conscious of the nutritional benefits of minimally processed food products. Minimally processed foods are considered highly perishable since they are not subjected to preservative processes that delay undesirable biological and biochemical changes (Chisari, Barbagallo, Spagna, & Artes, 2011; Timmons, Pai, Jacob, Zhang, & Ma, 2018). In particular, the incidence of foodborne pathogens outbreaks related to minimally processed food products has risen with their consumption because they are directly served without further processing or cooking (Cui, Ma, Li, & Lin, 2016; Gao, Daliri et al., 2019; Guo, Huang, & Wang, 2015; Liao et al., 2017). Their contamination by pathogenic bacteria, such as *Escherichia coli*, *Salmonella* spp., *Campylobacter jejuni*, and *Listeria monocytogenes*, as well as spoilage bacteria such as *Pseudomonas fluorescens* represents a critical challenge for the food industry (EFSA, 2017; Min et al., 2016; Ziuzina, Han, Cullen, & Bourke, 2015).

Cold storage represents the most common preservation method to maintain the quality of raw and minimally processed products, and to extend their shelf life (Savini et al., 2017). However, refrigeration does not allow to completely inactivate foodborne microbial growth, since it only slows down their growth during storage (Choi et al., 2015; Daş, Gürakan, & Bayindirli, 2006). On the other side, good sanitization practices of surfaces, equipment, tools, and indoor air are fundamental pre-requisites to guarantee the microbiological quality of food products (Masotti, Cattaneo, Stuknytė, & De Noni, 2019a). Thus, an approach that combines cold storage with antimicrobial technology to reduce the microbial growth during the storage period and preserving the food quality could be an effective strategy (Choi et al., 2015; La Storia et al., 2012).

Ozone (O_3) treatment represents one of the most promising techniques to be combined with cold storage, due to its effectiveness against food and airborne microorganisms. Besides, ozone rapidly decomposes to oxygen without leaving harmful residuals (Cullen & Norton, 2012; Ketteringham, Gausseres, James, & James, 2006; Pérez-Calvo, 2019). The use of ozone has been proved to be effective as an antimicrobial approach in different contexts, including medical, agricultural, marine, and food (Guo & Wang, 2017). It was approved as GRAS (generally recognized as safe) by the Food and Drug Administration (FDA) (Rice & Graham, 2001). Ozone is commonly applied in aqueous and gaseous forms (Sheng et al., 2018). The antimicrobial activity of aqueous and gaseous ozone has been reported against a wide range of microorganisms, including gram-positive bacteria such as *L. monocytogenes* (Concha-Meyer, Eifert, Williams, Marcy, & Welbaum, 2014) and *L. innocua* (Fan, Sokorai, Engemann, Gurtler, & Liu, 2012), gram-negative bacteria such as *E. coli* O157:H7 and

Salmonella spp. (Bialka & Demirci, 2007b; Daş et al., 2006; Fan et al., 2012; Singh, Singh, Bhunia, & Stroshine, 2002), and fungi such as *Botrytis cinerea* (Aguayo, Escalona, & Artés, 2006; Feliziani, et al., 2014; Minas, Karaoglanidis, Manganaris, & Vasilakakis, 2010). Gaseous ozone showed better antimicrobial performances than aqueous ozone to treat fresh products because of its penetration capacity, which enables to reach microbial spoilers and pathogens in less accessible sites (Shynkaryk, Pyatkovskyy, Mohamed, Yousef, & Sastry, 2015). However, gaseous ozone usually requires higher concentrations and exposure time to achieve an antimicrobial efficacy comparable with aqueous ozone (Bialka & Demirci, 2007a). The efficacy of gaseous ozone against foodborne pathogens has been tested in vitro (Alwi & Ali, 2014; Mahfoudh et al., 2010), highlighting the potential of this technique to inhibit bacterial growth. The effectiveness of gaseous ozone against pathogenic and spoilage bacteria was also tested in fresh products such as tomato (Bermúdez-Aguirre & Barbosa-Cánovas, 2013), fresh papaya (Yeoh et al., 2014), fresh-cut cantaloupe (Selma, Ibáñez, Cantwell, & Suslow, 2008), kiwifruit (Minas et al., 2010), and table grapes (Feliziani et al., 2014). Besides, gaseous ozone has been investigated as an alternative method to inactivate the microbial spoilage on surfaces (Cavalcante, Leite Júnior, Tribst, & Cristianini, 2014), as well as airborne microorganisms (Cullen & Norton, 2012; Masotti, Vallone, et al., 2019b) in food storage chambers. In view of the obtained results, several organizations and countries approved the application of gaseous ozone as an antimicrobial agent for the decontamination of storage facilities and/or food, including vegetables, meat, fish, and dairy products (Brodowska et al., 2018; Segat et al., 2014). Italian Ministry of Health (2010) approved the use of gaseous ozone for disinfecting empty cheese ripening and storage facilities, while the application on food is still not permitted. Despite the advantages related to the application of gaseous ozone, its application at high concentrations during the storage of food commodities could compromise the oxidative stability of high-fat foods such as dairy products (Sert, Mercan, & Kara, 2020; Torlak, Sert, & Ulca, 2013). Besides, ozone can affect the tissues of the respiratory tract causing lung injury. For this reason, the U.S. Occupational Safety and Health Administration recommended that the ozone exposure should not exceed 0.1 ppm by volume (equivalent to 0.2 mg/m^3 of air) under normal working conditions (8 h daily) (Brodowska et al., 2018). The same guidelines were adopted both by the Italian Ministry of Health (2010) and by the European Commission through the EU Biocidal Products Regulation No. 528.

To the best of our knowledge, there are only few reports focused on food storage cold chambers equipped with a gaseous ozone generator. In particular, the biocidal performances of gaseous ozone at low concentrations that are safe for human beings (0.05 ppm) were barely investigated. Furthermore, the application of gaseous ozone at low concentrations could potentially avoid the oxidative processes affecting the food commodities stored in the cold chamber. Therefore, the aims

of this study were (a) to investigate the effect of gaseous ozone (0.05 ppm) on the bacterial contamination of air and internal surfaces of a food storage cold chamber and its potential application as a sanitizing tool of food storage cold chambers, and (b) to evaluate the effectiveness of gaseous ozone treatment coupled to low temperature against in vitro broth cultures of *E. coli*, *L. monocytogenes*, *S. enterica* Typhimurium, *C. jejuni*, and *P. fluorescens* up to 48 h (1, 2, 6, 24, 30, and 48 h). This work constitutes exploratory research that was conducted in absence of specific kinds of food commodities. The achievement of positive findings will allow us to evaluate and eventually optimize the application of the process to actual industrial conditions, thus identifying which food commodities are most suitable in view of their oxidative stability and the market requirements.

2. Materials and Methods

2.1 Equipment

A novel prototype of a cold chamber for food storage (120 × 85 × 80 cm^3) was provided by a local company (Frigor Box International s.r.l., Reggio Emilia, Italy). The internal walls of the chamber were made of pre-varnished galvanized sheets, resistant to oxidation and corrosion, following the standard UNI EN 10142:2002. The cold chamber was coupled with a barrier dielectric discharge generator system, equipped with cylindrical electrodes. The operational voltage was set at 3 kV. A coaxial fan was placed at the interface between the generator and the chamber to direct the air resulting from the ionization process inside the storage chamber at a constant rate. The prototype was equipped with a sensor device Aeroqual SM 50 OZU GSS (Aeroqual Limited, Auckland 1026, New Zealand), with an accuracy of calibration < ± 0.005 ppm. The sensor was interfaced to the power supply of the ozone generator to maintain an average concentration of ozone of 0.05 ± 0.01 ppm.

2.2 Bacterial strains and culture preparation

Five bacterial strains were used in this study, including *L. monocytogenes* (ATCC 19115), *E. coli* (ATCC 43888), *Salmonella enterica* Typhimurium (ATCC 14028), *C. jejuni* (ATCC 33250), and *P. fluorescens* (ATCC 13525). Bacterial cultures were prepared according to Timmons et al. (2018) with slight modifications. Each strain from frozen stock was streaked onto separate plate count agar plates (PCA, Biolife, Milan, Italy) and incubated at 37°C for 24 h. A single colony of each culture was inoculated in tryptic soy broth (TSB, Biolife) and incubated at 37°C overnight. The bacterial concentration of each liquid culture was determined by serially diluting the culture in a sterile saline

solution (0.9% NaCl) (VWR Chemicals, Milan, Italy) and plated on PCA, followed by incubation at 37°C for 24–48 h.

2.3 Ozone treatment and microbiological analysis

2.3.1 Microbial sampling of surfaces of the storage chamber

The ozone generating system was activated for 30 and 60 min to evaluate the sanitizing effect of low-dose gaseous ozone treatment on internal surfaces. The temperature of the storage chamber was set at 3°C, whereas the relative humidity (RH%) was fixed at 90%. These conditions of temperature and RH% were maintained for all the experiments. The recontamination of the chamber after each treatment was induced by fluxing external air inside the chamber through a coaxial fan (EBM PAMPST mod. 5606S, Mozzate, Italy) for 24 h, to create similar starting conditions. The mesophilic aerobic charge on the treated surfaces of the storage chamber was counted. Surfaces not treated with ozone were analysed as a reference. Each test was performed separately. The microbiological analysis of the internal surfaces was performed according to the standard UNI EN ISO 18593:2018 with slight modifications. After each ozone treatment (30 and 60 min), a sterile plastic frame with a 100 cm^2 inner area (VWR International) was aseptically placed on one of the internal surfaces of the storage chamber. A sterile swab (Cultiplast, LP Italiana SPA, Milan, Italy) was moistened in a sterile saline solution (0.9% NaCl) and streaked on the sampling area. The swab was then dipped in 10 ml sterile saline solution (0.9% NaCl), vortexed and 10-fold serially diluted. The samples were finally plated on PCA, followed by incubation at 30°C for 24 h. All experiments were performed in triplicate. For each replicate, three surfaces were randomly selected between the bottom, top, and sidewalls. The results were expressed as $\log_{10}$ CFU/m^2 in Table 1, and the microbial inactivation effect was calculated through the Eq. (1), as described by Alwi & Ali (2014):

$$\text{Microbial log reduction (log CFU/m}^2\text{)} = \log (N_0/N) \quad \text{Eq. (1)}$$

where N_0 is the microbial load of control, N is the microbial load of samples after treatment. The results expressed as log reduction in the main text.

2.3.2 Air microbial sampling of the storage chamber

The same experimental condition described in Section 2.3.1 was applied to explore the impact of low-dose gaseous ozone treatment on the microbiological quality of the air inside the storage chamber. In this study, the total mesophilic bacterial count was investigated. The microbiological analysis of air was performed using a microbiological air sampler (SAS, Surface Air System, SAS mod. Super ISO 100 Petri, VWR International). The analysis was conducted according to the standard UNI EN ISO 14698-1:2004 (Masotti, Vallone, et al., 2019b) with slight modifications. A Petri dish (90 mm diameter) containing PCA was aseptically clipped in place on the sampler and covered by an aluminium lid with a precision pattern of holes (n = 256). The SAS sampler was then placed inside the storage chamber. Each treatment was performed according to the experimental conditions. After the treatment, the SAS sampler was remotely activated without opening the chamber door to maintain the microbiological quality of air induced by ozonation. The volume of air sampling was set at 250 L. The defined volume of air was fluxed onto the Petri dish at a constant rate of 100 L/min. At the end of the suction step, the SAS sampler lid was removed, the agar plate was recovered and incubated at 30°C for 72 h. Each ozone treatment was performed in triplicate. The results were expressed as $\log_{10}$ CFU/m^3 in Table 1. The antimicrobial effect on internal air was calculated through Eq. (1) and the derived results were reported as log reduction in the main text.

2.4 Preparation of inocula and in vitro assay

The preparation of inocula was assessed according to Timmons et al. (2018) with slight modifications. One millilitre of each bacterial culture was harvested by centrifugation at 5000 rpm for 10 min and the resulting pellets were washed twice with sterile saline solution (0.9% NaCl). The cells were finally resuspended in 10 ml sterile saline solution. Appropriate dilutions of each fresh culture were separately streaked onto PCA to obtain inocula concentrations of 1.3–1.4, 2.3–2.4, and 3.3–3.4 log CFU/plate, and incubated for 1 hr at room temperature. The inoculated plates were placed on the internal floor of the storage chamber and treated with ozone for exposure times of 0 (control), 1, 2, 6, 24, 30, and 48 h. After ozone exposure, the plates were incubated at 37°C for 24 h for *E. coli*, *S. enterica* Typhimurium and *C. jejuni*, and 48 h for *L. monocytogenes*. The plates containing *P. fluorescens* were incubated at 25°C for 30 h. The results were expressed in log CFU/plate in Table 2 and the antibacterial activity of gaseous ozone treatment against each bacterial strain was calculated following the Eq. (1) and reported as log reduction in the main text.

2.5 Statistical analysis

The statistical analysis of the data was performed through one-way analysis of the variance using SPSS statistical program (SPSS 20 for Windows, SPSS, Inc., IBM, New York). The differences between means were evaluated by Tukey's multiple range test ($p < .05$). All tests were performed in triplicate. The data were expressed as the mean ± SD.

3. Results and Discussion

3.1. Effect of gaseous ozone on the bacterial load of internal surfaces

The bacterial viability detected on surfaces treated with low-dose gaseous ozone (30 and 60 min) was compared with untreated ones as a control. The results of microbiological analyses performed on the internal walls of the cold storage chamber are reported in Table 1. The average bacterial load of untreated surfaces was 3.6 log CFU/m^2. Treatment of the internal walls with gaseous ozone for 30 min reduced the bacterial population to 2.61 log CFU/m^2 ($p < 0.05$). The increase of exposure time to 60 min slightly decreased the cell viability to 2.28 log CFU/m^2 ($p > 0.05$). These results demonstrated the effectiveness of low-dose gaseous ozone for the reduction of surface bacterial load into a cold storage chamber in a short period (30 min).

3.2 Effect of gaseous ozone on airborne bacterial contamination

The bacterial load of air treated with gaseous ozone for 30 and 60 min was compared with untreated air as a control (Table 1). The bacterial population of untreated air accounted 1.81 log CFU/m^3. Ozone treatment for 30 min caused 0.93 ± 0.24 log reductions on air bacterial population ($p < 0.05$). After 60 min exposure, the bacterial population of air became undetectable.

Table 1.

Bacterial loads of internal surfaces (log_{10} CFU m^{-2}) and air (log_{10} CFU m^{-3}) of the food storage cold chamber before (control) and after air ozonation for 30 and 60 min.

Bacterial loads	Control	Ozone treatment (30 min)	Ozone treatment (60 min)
Internal surfaces (log_{10} CFU m^{-2})	3.60 ± 3.18^{b}	2.61 ± 2.23^{a}	2.28 ± 2.02^{a}
Air (log_{10} CFU m^{-3})	1.81 ± 1.27^{b}	0.90 ± 0.60^{a}	$0_{\dagger}$

Values are given as mean ± SD (n = 3).
Different lowercase letters in the same row indicate significant differences ($p < 0.05$).
† 0: no growth (0 CFU) revealed in the plate.

3.3 In vitro assay

The effect of low-dose ozone treatment on the viability of *C. jejuni*, *S. enterica* Typhimurium, *E. coli*, *L. monocytogenes*, and *P. fluorescens* is reported in Table 2. Petri dishes were inoculated with an average bacterial load of 1.3–1.4, 2.3–2.4, and 3.3–3.4 log CFU/plate for each culture. Petri dishes were exposed to gaseous ozone for 1, 2, 6, 24, 30, and 48 h. Increasing treatment time enhanced the antibacterial effect of ozone against all the selected bacterial strains ($p < 0.05$). Nevertheless, cellular behaviour in relation to ozone treatment and then its antimicrobial effectiveness drastically changed depending on the bacterial strain and the inoculum concentration. Ozone was mostly effective against *C. jejuni*. A noticeable decrease in cellular viability was observed after 1 h, regardless of the concentration of inoculum. Gaseous ozone treatment for 1 h was enough to reduce the highest concentration of inoculum (3.34 log CFU/plate) by 2.23 ± 0.16 log reductions, while no bacterial growth was observed at the lowest concentrations.

S. enterica Typhimurium and *E. coli* were the most resistant pathogens among the tested ones to short-term gaseous ozone treatment (1–6 h). Petri dishes with higher concentration loads were uncountable (≥3.00 log CFU/plate) even after 6 h. Thus, the first assessment was performed on Petri dishes with lower concentrations (2.3–2.39 and 1.3–1.39 log CFU/plate). For both *S. enterica* Typhimurium and *E. coli*, antibacterial activity was not achieved during the first 6 h of treatment ($p > 0.05$). An increase of exposure time to 24 h drastically affected the higher microbial inocula (3.30–3.40 log CFU/plate), inducing 1.62 ± 0.12 log reductions for *S. enterica* and 2.07 ± 0.04 for *E. coli* ($p < .05$). In particular, the behaviour of the two pathogens was quite similar up to 24 h of treatment. Further increase of gaseous ozone exposure to 48 h showed slightly greater resistance of *S. enterica* Typhimurium to long-term treatments (6–48 h) with respect to *E. coli*.

L. monocytogenes showed resistance to short-term gaseous ozone treatments at all the concentrations of inoculum. No significant antimicrobial effect of ozone treatment on *L. monocytogenes* viability was detected during the first 6 h, even against the lowest concentration of inoculum ($p > 0.05$). Treatment for 24 h induced a remarkable reduction on *L. monocytogenes* population. For the highest inoculum concentration, *L. monocytogenes* population was reduced by 1.95 log ($p < 0.05$), while the inoculum at 1.3–1.4 log CFU/plate became undetectable at the same exposure period. Further increase of treatment time to 48 h did not affect the bacterial load ($p > 0.05$).

P. fluorescens showed high sensitivity to short-term ozone treatment. The antibacterial activity of ozone rapidly increased during the first 6 hr, inducing 0.75–1.32 log reductions after 1 h with respect to untreated plates, and further 0.22–0.53 log reductions after 6 h. The population of *P. fluorescens*

at 1.30–1.4 log CFU was undetectable after 2 h. Further increasing of time from 6 to 48 h showed a negligible effect against *P. fluorescens* ($p>0.05$), which showed high resistance of surviving cells against a long-term ozone treatment.

Table 2.
Effect of low dose gaseous ozone for different treatment times (1, 2, 6, 24, 30, 48 hours) on *Campylobacter jejuni*, *Salmonella enterica* sv. Typhimurium, *Escherichia coli*, *Listeria monocytogenes* and *Pseudomonas fluorescens* (log_{10} CFU/plate) at different concentrations of inoculum (1.30-1.40, 2.30-2.40, 3.30-3.40 log_{10} CFU/plate).

Bacterial strains log_{10} CFU/plate	**Control**	**Ozone Treatment**					
		1h	**2h**	**6h**	**24h**	**30h**	**48h**
C. jejuni	3.34 ± 1.86^{b}	1.11 ± 0.63^{a}	$0_{‡}$	$0_{‡}$	--	--	--
	2.35 ± 1.69	$0_{‡}$	$0_{‡}$	$0_{‡}$	--	--	--
	1.34 ± 0.45	$0_{‡}$	$0_{‡}$	$0_{‡}$	--	--	--
S. enterica	3.30 ± 1.89^{c}	N.C.†	N.C.†	N.C.†	1.69 ± 1.19^{b}	1.06 ± 0.55^{b}	0.15 ± 0.00^{a}
	2.37 ± 1.10^{b}	2.34 ± 1.79^{b}	2.34 ± 1.85^{b}	2.35 ± 1.92^{b}	1.28 ± 0.15^{a}	0.60 ± 0.15^{a}	$0_{‡}$
	1.39 ± 0.33^{b}	1.30 ± 0.45^{b}	1.41 ± 1.25^{b}	1.42 ± 0.69^{b}	0.30 ± 0.15^{a}	0.18 ± 0.00^{a}	$0_{‡}$
E. coli	3.38 ± 1.72^{b}	N.C.†	N.C.†	N.C.†	1.31 ± 0.33^{a}	1.24 ± 0.33^{a}	1.16 ± 0.33^{a}
	2.37 ± 1.03^{e}	2.32 ± 0.45^{d}	2.10 ± 0.55^{c}	2.01 ± 0.15^{b}	0.60 ± 0.15^{a}	0.48 ± 0.15^{a}	$0_{‡}$
	1.38 ± 0.63^{b}	1.00 ± 0.15^{a}	1.04 ± 0.15^{a}	0.90 ± 0.15^{a}	$0_{‡}$	$0_{‡}$	$0_{‡}$
L. monoc.	3.34 ± 1.63^{b}	N.C.†	N.C.†	N.C.†	1.39 ± 0.33^{a}	1.20 ± 0.15^{a}	1.04 ± 0.45^{a}
	2.32 ± 1.19^{c}	2.29 ± 1.23^{bc}	2.21 ± 1.28^{b}	2.18 ± 1.56^{ab}	0.60 ± 0.15^{a}	0.18 ± 0.33^{a}	$0_{‡}$
	1.38 ± 0.63^{a}	1.26 ± 1.10^{a}	1.27 ± 1.03^{a}	1.00 ± 0.45^{a}	$0_{‡}$	$0_{‡}$	$0_{‡}$
P.fluoresc.	3.32 ± 1.55^{c}	2.12 ± 1.08^{b}	1.70 ± 0.93^{a}	1.63 ± 0.45^{a}	1.61 ± 0.55^{a}	1.54 ± 0.96^{a}	1.45 ± 0.96^{a}
	2.34 ± 1.00^{d}	1.22 ± 0.33^{c}	1.06 ± 0.33^{b}	0.90 ± 0.15^{ab}	0.60 ± 0.45^{ab}	0.60 ± 0.15^{ab}	0.48 ± 0.15^{a}
	1.28 ± 0.45^{b}	0.30 ± 0.15^{a}	$0_{‡}$	$0_{‡}$	$0_{‡}$	$0_{‡}$	$0_{‡}$

Values are given as mean ± SD (n = 3).
Different lowercase letters in the same row indicate significant differences ($p < 0.05$).
† N.C.: not countable.
‡ 0: no growth (0 CFU) revealed in the plate.

3.4. Discussion

In this study, the effect of ozone treatment on the microbiological quality of air and internal surfaces of a food storage cold chamber and its potential application as an effective sanitizing tool was investigated. Microbial air contamination represents a critical issue for food manufacturers, since it could occur also in well designed, constructed, and maintained factories. In fact, bacteria and molds are common and ubiquitous airborne contaminants in the food production environment (Masotti, Vallone, et al., 2019b). The results obtained in this study were in accordance with those obtained by Masotti, Vallone, et al. (2019b) in dairy production environments, showing that the periodic (3 h/day) generation of ozone allowed to reduce the growth of bacteria up to 100%, with an initial average

contamination of 2.21 log CFU/m^3. The tests performed on the internal surfaces of the storage chamber with or without ozone treatment confirmed the effectiveness of gaseous ozone to enhance the hygiene of the surfaces. Low dose gaseous ozone treatment allowed to obtain an average one log reduction of the total bacterial population on surfaces after 30 min treatment.

The antibacterial activity of gaseous ozone is due to its oxidizing power (Pérez-Calvo, 2019), which affects the growth of airborne and surface bacteria both directly and indirectly. Ozone can induce bacterial cell inactivation by introducing oxidative stress due to the presence of reactive species of oxygen (ROS), which derive from ozone decomposition (Alwi & Ali, 2014). The accumulation of ROS inside the cell membrane can lead to several damaging effects on the cell structure, such as lipid peroxidation and membrane disruption, oxidation of amino acids of proteins, and mutation of DNA (Brodowska et al., 2018; Greene, Güzel-Seydim, & Seydim, 2012). These damages compromise the cellular metabolism, leading to the death of microorganisms (Torlak et al., 2013). Besides the direct impact on cellular structure, ozone modifies the composition of environmental air, providing an atmosphere with no reducing compounds and obstructing the survival of bacteria inside aerosols. Therefore, gaseous ozone treatment can be considered as an outstanding method to improve the sanitization and disinfection of food storage cold chamber (Cullen & Norton, 2012).

Low-dose gaseous ozone treatment significantly reduced the growth of *C. jejuni*, *E. coli*, *S. enterica* Typhimurium, *L. monocytogenes*, and *P. fluorescens* populations. The in vitro assay confirmed that the effectiveness of gaseous ozone increased with exposure time for all the microorganisms. Nevertheless, the strains reacted differently to the contact with ozone. The degree of bacterial inactivation by gaseous ozone is affected by different factors, including their gram-positive or gram-negative nature, the protective mechanisms of the cells to oxidation stress, the physiological state of cells, as well as their resistance to temperature. Besides, the susceptibility of microorganisms to ozone vary according to the environmental factors (e.g., pH of the medium, composition of the medium, environmental temperature, and humidity), along with the amount of organic matter surrounding the cells. Thus, it is barely feasible to compare the findings of this study with other previous research conducted under different experimental conditions (Pirani, 2010). However, selected studies are reported as a reference to explain the main mechanisms describing the different sensitivity observed among the strains.

C. jejuni represents the most common bacterial source of zoonosis in the EU (EFSA, 2017), representing a critical issue for the food industry. Nevertheless, few comparative data highlighting the effect of low-dose gaseous ozone on *C. jejuni* were found in the literature. The effect of aqueous ozone in combination with peracetic acid on *C. jejuni* has been studied by Dittoe et al. (2019). Authors

highlighted a similar behaviour of *C. jejuni*, *E. coli*, and *S. Typhimurium* against ozone treatment. In our case, the inactivating performance of gaseous ozone against the three pathogens was significantly different. *C. jejuni* was shown as the most sensitive one to the gaseous ozone treatment in this study. The higher sensitivity to ozone of *C. jejuni* with respect to the other enteric pathogens could be due to its strict microaerophilic nature, requiring <10% ambient oxygen to grow (Crushell, Harty, Sharif, & Bourke, 2004).

S. enterica Typhimurium, *E. coli*, and *L. monocytogenes* are considered as three key foodborne pathogens in the EU (De Leo et al., 2018; EFSA, 2017). *S. enterica* Typhimurium outbreaks are related to the consumption of minimally processed products such as fresh vegetables (Choi et al., 2015). *E. coli* can cause hemolytic uremic syndrome and hemorrhagic colitis in human (Kim, Lee, Puligundla, & Mok, 2020). *L. monocytogenes* is responsible for listeriosis in humans, primarily the elderly, pregnant women, and immunocompromised individuals (Ferreira, Wiedmann, Teixeira, & Stasiewicz, 2014). *S. Typhimurium*, *E. coli*, and *L. monocytogenes* showed high resistance to short-term ozone treatments (1–6 h), with no significant differences in bacterial population at all the concentrations. The analysis of long-term ozone treated plates showed that *L. monocytogenes* was more resistant than *E. coli* and *S.* Typhimurium to long-term ozone treatment (24–48 h).

The good resistance of *S.* Typhimurium to ozone could be attributed to the high density of its cell membrane, related to the great content of phospholipid components such as phosphatidylethanolamine and phosphatidylglycerol. These components enhance the rigidity of the cell wall, providing extra protection for the cell membrane against ozone (Alwi & Ali, 2014; Torlak et al., 2013).

The resistance of *L. monocytogenes* compared to *S. enterica* Typhimurium and *E. coli* might be attributed to its gram-positive nature. The thicker membrane of gram-positive bacteria due to the greater presence of peptidoglycan in the cell wall may represent a barrier to the diffusion of reactive gaseous species such as ozone through the bacterial cell wall, thus impacting the antimicrobial efficacy (Akata, Torlak, & Erci, 2015; Yeoh et al., 2014; Ziuzina, Patil, Cullen, Keener, & Bourke, 2014). Rey, Sellés, Baluja, & Otero (1995) demonstrated that the resistance of *Listeria* to aqueous ozone was significantly enhanced by the presence of N-acetyl glucosamine, a constituent of the peptidoglycan of the bacterial cell wall. Besides, the resiliency of *L. monocytogenes* to the tested conditions could be promoted by its psychrophilic behaviour, as described by previous researches (Concha-Meyer et al., 2014; Sheng et al., 2018).

The presence of *Pseudomonas* spp. in food is risky because they can produce hydrolytic enzymes, pigments, and slimes, mostly in refrigerated foods (Rajmohan, Dodd, & Waites, 2002). The results

obtained for *P. fluorescens* demonstrated the higher sensitivity of this pathogen to short-term treatments (1–6 h) compared to *L. monocytogenes*, in accordance with the results obtained by Marino, Maifreni, Baggio, & Innocente (2018). This could be attributed to the lack of protective mechanisms to oxidative stress, causing an overall loss of cell viability. However, our research showed that a long-term ozone treatment (24–48 h) was not able to inactivate the surviving cells, inducing negligible changes in the residual population. This effect could be due to the high density of *P. fluorescens* colonies (Marino et al., 2018). The presence of biological material derived from dead cells might have represented the first target of the ozone activity, acting as a protective shield for the viable cells against oxidative damages. Furthermore, the great resistance of *P. fluorescens* to long-term treatment in our experimental conditions could be partially related to its high cold tolerance. Similar results were reported by previous research (Ripamonti, Bersani, Pirani, & Stella, 2009; Rusch & Kraemer, 1989), which showed the higher resilience of *Pseudomonas* strains to ozone treatment under refrigerated conditions with respect to Enterobacteriaceae such as *E. coli* and *S. enterica*, and to *L. monocytogenes.*

4. Conclusion

This study demonstrated the effectiveness of low-dose gaseous ozone treatment on inactivating bacterial pathogens and to promote the optimal sanitization of surfaces and air of food storage cold chambers. However, the translation of these findings to an in-use food storage cold chamber (real industrial conditions) is still difficult to predict, since the penetration capacity and thus the biocidal activity of gaseous ozone could be countered by many factors such as residual debris on the walls, as well as the complexity of food products, including their geometry, dimension, and composition. Furthermore, the scratches and crevices on the floor, derived from the prolonged use, could represent hiding places for microbes and pathogens. For this reason, further investigations aiming to confirm the biocidal performances of gaseous ozone at 0.05 ppm in real industrial and/or commercial conditions will be strongly required. Nevertheless, the obtained results strongly encourage the design of innovative food storage chambers with ozone generators as valuable sanitizing tools. In fact, this technique could be used as a supplementary disinfection step, integrating the periodic sanitization procedures, and thus contributing to the fulfilment of the microbial reduction due to its effectiveness against a wide microbial spectrum.

Chapter 3

Multivariate exploratory data analysis by PCA of the combined effect of film-forming composition, drying conditions, and UV-C irradiation on the functional properties of films based on chitosan and pectin

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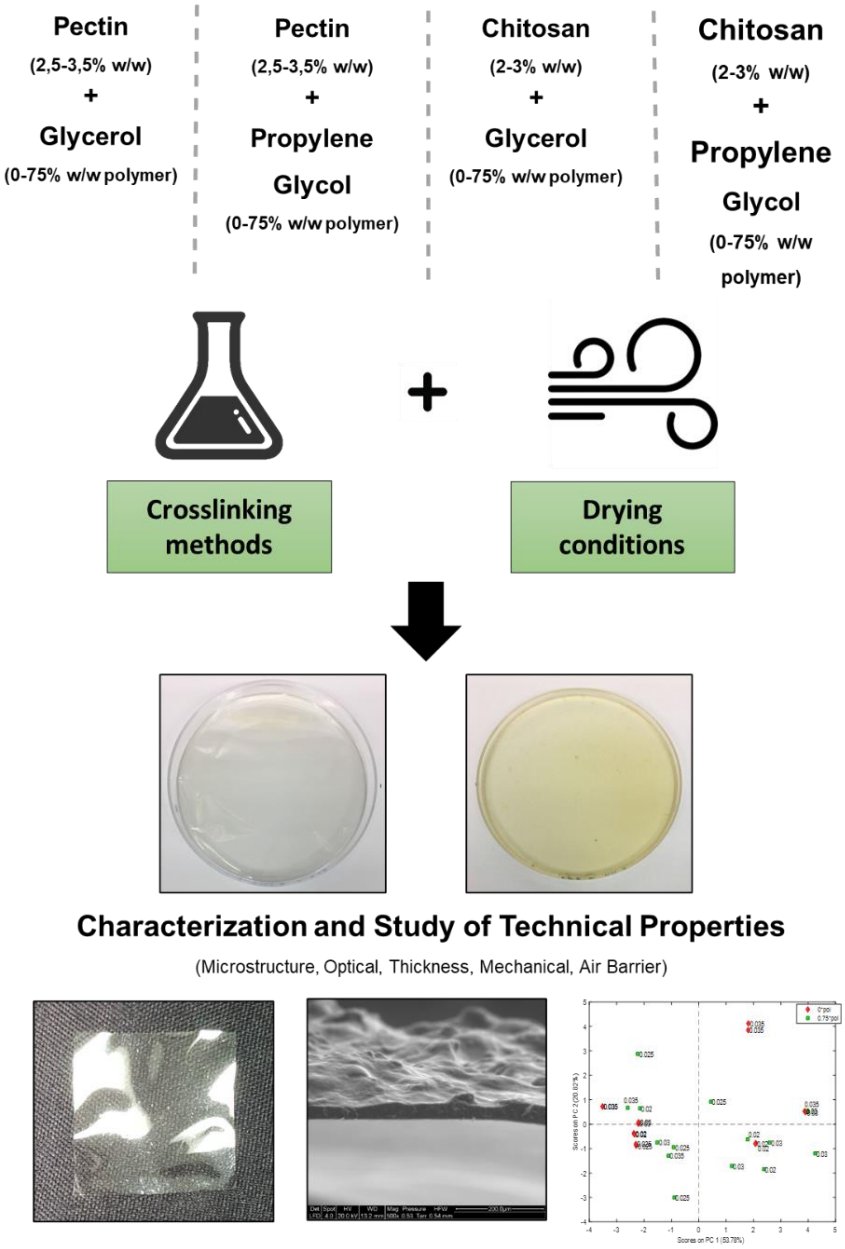
Graphical abstract



Keywords

Biodegradable films; Air permeability; Microstructure; Sodium trimethaphosphate; Drying process; Principal component analysis.

Abstract

The technical features of biodegradable films depend on several manufacturing parameters. Exploiting these films on an industrial basis requires statistical models for the fast and effective evaluation of these parameters. This study analyses the impact of compositional and drying factors on the microstructural and functional properties of films based on chitosan and pectin through a multivariate approach. A total of 32 formulations were developed and the results were analyzed through principal component analysis (PCA). Four formulations did not form films due to the photodegradation caused by prolonged UV-C irradiation. Films containing glycerol had a higher elasticity (1.5–39%) than those with propylene glycol or that were not plasticized (1.2–12%). Glycerol increased the thickness of pectin films (56–77 μm). Sodium trimethaphosphate (STMP) enhanced the UV mean absorbance of both pectin and chitosan films (0.7–1.7). In addition, STMP led to an increase of thickness (72–129 μm) and air permeability (38.6 × 103 to 9206.1 × 103 cm^3/m^2 × day × kPa) of the chitosan films, while $CaCl_2$ led to a decrease of thickness (31–59 μm) and air permeability (23.9 × 103 to 46.8 × 103 cm^3/m^2 × day ×kPa) of the pectin films.

1. Introduction

The production of petroleum-derived plastics for food packaging has grown exponentially, thanks to their versatility, easy processing, and low price. However, they are unsustainable in environmental terms because of their resistance to degradation. Biopolymers such as polysaccharides, proteins, lipids, and their derivatives are promising green alternatives to non-biodegradable plastics (Cazòn et al., 2017).

Chitosan has great potentials as a biopolymer for packaging due to its biodegradability, stability, commercial availability, and antioxidant/antimicrobial properties. It is a linear polysaccharide obtainable through the N-deacetylation of chitin, which is the second most abundant polysaccharide in nature after cellulose (Haghighi et al., 2020b). It is soluble in low-pH solutions due to the protonation of amino groups in the polymer backbone. Due to its cationic behaviour, it could prolong the shelf life of food products by inhibiting the growth of a wide range of microorganisms such as yeasts, molds, as well as gram-positive and gram-negative bacteria (Khwaldia, Basta, Aloui, & El-Saied, 2014).

Pectin is an anionic heteropolysaccharide extracted from citrus peel and apple pomace (De Leo et al., 2018). It is one of the main components in the plant's primary cell wall and is chemically constituted by poly α-(1–4)-D-galacturonic acids (Abid et al., 2017), whose carboxyl groups are partially esterified with methanol. The degree of methylation is used to classify pectin as high (> 50%) and low (< 50%) methoxy pectin (Thakur, Singh, Handa, & Rao, 1997). The low methoxy amidated (LMA) pectin used in this study is generated from the conversion of some of the ester groups into amide groups using ammonia. This pectin derivative is more stable in natural or slightly alkaline conditions (May, 1990).

The functional properties of chitosan and pectin films and their potential for food packaging depend on several technological parameters, related to the composition of the film-forming solution (FFS) and the drying conditions. For example, the addition of a plasticizer leads to a decrease in intermolecular forces along with the polymer matrix. This enhances the film flexibility, decreases brittleness, and prevents shrinking during handling and storage (da Silva, Bierhalz, & Kieckbusch, 2009). In this study, among the many possible plasticizers, glycerol and propylene glycol were used to improve elasticity and increase the mobility of the biopolymeric chains (Jantrawut, Chaiwarit, Jantanasa-Kulwong, Brachais, & Chambin, 2017).

Crosslinking is another key technique for improving the physico-chemical properties of polysaccharide-based films by adding intra- and intermolecular bonds randomly distributed in the

biopolymer backbone. In this study, sodium trimetaphosphate (STMP) and $CaCl_2$ were applied as chemical cross-linkers to reduce the solubility of the films by decreasing the number of free hydroxyl groups that could react with water (Prezotti, Meneguin, Evangelista, & Ferreira Cury, 2012). UV-C irradiation was applied as a physical cross-linker. This technique has no negative effects on human health and can also be used to kill or inactivate a wide range of microorganisms. However, it was reported that UV-C irradiation negatively affects the chitosan film matrix, since photodegradation processes occur on the film surface with the formation of carboxyl and hydroxyl groups (Sionkowska, Kaczmarek, Gnatowska, & Kowalonek, 2015).

Besides plasticizers and crosslinkers, drying conditions significantly influence the optical, mechanical, and barrier performance of the film by affecting the degree of re-organization or crystallinity of the polymers (Srinivasa, Ramesh, Kumar, & Tharanathan, 2004).

The impact of the above experimental factors on the functional properties of the films is generally investigated through a one-variable-at-a-time approach. This method is time-consuming and does not consider the interactions between the experimental parameters (Chaichi, Badii, Mohammadi, & Hashemi, 2019). To the best of our knowledge, a statistical model that explores the effects of many independent variables on a set of final properties of films based on chitosan and pectin has not yet been described. In this study, due to the high number of factors that were considered, a Plackett-Burman screening design (Plackett & Burman, 1946) was used to define the experimental conditions. Then, given the high number of film properties that were measured for each experimental condition, principal component analysis (PCA) was used as a multivariate exploratory data analysis tool to easily estimate the technical behaviour of biodegradable films depending on the simultaneous variations of several experimental conditions. In fact, following a generally adopted approach consisting of the calculation of a generalized linear model for every single property would have led to a too high number of models, making their interpretation difficult. Furthermore, the analysis of the single models would not have allowed the overall evaluation of the correlations between the final properties measured for the different polymers. The main aim of this study was thus to use a holistic approach to explore the effect of seven different experimental factors (type and concentration of the biopolymer, plasticizer, crosslinking agent, drying temperature, presence or absence of convective ventilation, and UV-C irradiation) on the whole set of twelve parameters reflecting microstructural, mechanical, UV- and air-barrier properties of chitosan and pectin films for food packaging applications.

2. Materials and Methods

2.1. Materials and reagents

Low methoxy amidated pectin from orange peels (degree of esterification 30–36%, degree of amidation 14–20%) was purchased from Herbstreith & Fox (Neuenbürg/Württ, Germany). Chitosan with a molecular weight of 100–300 kDa was obtained from Acros Organics™ (Beijing, China). Glycerol (≥99.5%), propylene glycol (≥99.5%), STMP, $CaCl_2$, and acetic acid (≥99.5%) were purchased by Sigma-Aldrich (St. Louis, USA).

2.2. Infrared-ultraviolet (IR-UV) oven prototype

An innovative IR-UV oven was designed based on the evolution of a conventional oven by ZTM Mechatronic (Correggio, Reggio Emilia, Italy) to create the specific drying conditions defined by the experimental design. A heating chamber (40 × 40 × 40 cm^3) was equipped with infrared-emitting quartz lamps (1200 W), a coaxial fan to produce convective ventilation inside the chamber, two UV-C mercury lamps (18 W), and flat glass support.

2.3. Preparation of film-forming solutions (FFSs) and films

The preparation of pectin and chitosan FFSs without STMP was adapted from the procedure of Galus, Turska, & Lenart (2012) and Fernàndez-Pan, Ziani, Pedroza-Islas, & Matè (2010), respectively. The pectin FFSs were prepared by dissolving 2.5 and 3.5 g pectin in 100 mL distilled water. The chitosan FFSs were prepared by dissolving 2 and 3 g chitosan in acetic acid solution (1 g acetic acid in 100 mL distilled water). Depending on the experimental conditions, glycerol or propylene glycol (0.75 mL/g of biopolymer) were then added as plasticizers and stirred for 10 min at 60°C. Pectin FFSs containing STMP were prepared according to Carbinatto, de Castro, Evangelista, & Cury (2014). Chitosan FFSs containing STMP were prepared by adapting the methods described by Zhang & Xia (2014) and Lack, Dulong, Picton, Le Cerf & Condamine (2007) which included the dissolution of chitosan into an alkali environment. The concentration of STMP in the FFS was set at 0.3 g on 1 g of polymer. Pectin FFSs were acidified by adding 1 M HCl solution (pH ≤ 4.4). The FFSs were degassed under a vacuum pump (Vacuumbrand GMBH + CO KG - Wertheim, Germany) at 80 kPa for 15 min to remove bubbles. Films were obtained by casting 20 mL of the FFS into Petri dishes (140 mm in diameter). The resulting films were sprayed with $CaCl_2$ (5 g in 100 mL water) when required by the

experimental design. The films were dried under conditions defined by the experimental design (25 or 75°C; presence/absence of convective ventilation; presence/absence of UV-C treatment), manually peeled off from the plates, and stored at 25 ± 2°C overnight in a chemical hood at 45% relative humidity.

2.4. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) images from the surface and cross-section of the films were obtained with the use of a scanning electron microscope (FEI, Quanta 200, Hillsboro, Oregon, USA). Film samples were fixed on stainless-steel supports with a double-sided conductive adhesive. The analysis was conducted in a low vacuum (80 Pa) at an acceleration voltage of 20 kV.

2.5. Thickness and mechanical properties

Film thickness was measured with a digital micrometer (IP65, SAMA Tools, Viareggio, Lucca, Italy) at five different randomly chosen positions. The means of these five measurements were recorded. Tensile strength (TS), elongation at break percentage (E%), and Young's modulus (YM) were determined according to the ASTM D882 (ASTM, 2001a). Films with known thickness were cut into rectangular strips (9 × 1.5 cm^2), the initial grip separation and cross-head speed were set at 70 mm and 50 mm/min, respectively. The measurements were repeated 10 times using a dynamometer (Zwick/Roell, Ulm, Germany). The results were elaborated using the software TestXpert®II (V3.31).

2.6. Air permeability

Air permeability (AP) was determined in triplicate, according to the ASTM D3985-05 (ASTM, 2010). AP was measured using a LISSY GPM500 analyser (Lausanne, Switzerland) at 23°C and 0% relative humidity, coupled with gas chromatography (GC320, GL Science®, Tokyo, Japan) for the quantitative evaluation of the gas transmission. Film samples were masked with aluminium foil leaving a circular test area of 25 cm^2 on the surface. Films were clamped between two chambers. The lower chamber was swept by a helium flux while the test gas (a mixture of nitrogen and oxygen with the same percentages of atmospheric air) was introduced to the upper chamber. AP was reported as (cm^3 / m^2 × day × KPa) and calculated according to Eq. (2):

$$AP = \text{Air transmission rate} \times \text{Average film thickness}/\Delta P \quad (2)$$

where the air transmission rate is in cm^3 / m^2 × day, and ΔP is the difference in partial pressure between both sides of the film.

2.7. UV barrier property

Film samples (3 × 3 cm^2) were fixed on a paper frame used as a support. The transmittance percentage was measured in triplicate in the 200–380 nm wavelength range using a UV–Vis spectrophotometer (Jasco V-550 UV/VIS; Orlando, U.S.A). The values of transmittance were then converted into absorbance units. The absorbance values at fixed wavelengths (200, 260, 320, and 380 nm) were evaluated. The average absorbance (A_{MEAN}) and unitary absorbance (UA) of each film calculated following Eqs. (3) and (4):

$$A_{MEAN} = (A200 + A260 + A320 + A380)/4 \quad (3)$$

$$UA = A_{MEAN}/\text{film thickness} \quad (4)$$

where A_{MEAN} is the mean absorbance at 200, 260, 320, and 380 nm, and the film thickness is in μm. The UV barrier property of the films was determined in triplicate.

2.8. Principal component analysis (PCA)

PCA is a well-established multivariate statistical technique that enables the information contained in the dataset to be summarized and visualised (Lever, Krzywinski, & Altman, 2017). Essentially, PCA splits data variability into two separate parts: the first contains the systematic variation of the data (data structure) which provides useful information, while the second part (residuals) contains random variability due to experimental errors. The data structure is described by a set of orthogonal variables, the principal components (PCs), which are linear combinations of the original variables (i.e., of the properties measured on the film samples). The first PC (PC1) is defined as the direction of maximum variance, PC2 is the second direction of maximum variance orthogonal to the previous one(s), and so on. Each PC is defined by two vectors: the score vector, reporting the position of the samples along with that PC, and the loading vector, representing the contribution of each original variable to the PC. If e.g. two PCs are sufficient to describe the data structure, the score plot of PC1 vs. PC2 identifies clusters of similar samples and outliers. The corresponding PC1 vs. PC2 loading plot highlights correlations among the original variables, based on their direction with respect to the axes origin, and estimates the significance of the variables, based on their distance from the axes origin. By comparing

the score plot with the corresponding loading plot, it is possible to interpret the relationships between samples and variables. For example, if a cluster of samples is found in the same direction with respect to the axes origin as a given set of variables, the samples of this cluster will have high values of these variables. Further details on PCA theory and application can be found in Bro & Smilde (2014).

2.9. Experimental designs and statistical analysis

Four biopolymer/plasticizer combinations were investigated using a Plackett-Burman design (Plackett & Burman, 1946) including pectin/glycerol, pectin/propylene glycol, chitosan/glycerol, and chitosan/propylene glycol (Table 3).

The study explored the impact of the experimental factors on the whole set of response variables. All the properties were collected into a dataset with a size equal to {32 experiments, 12 response variables} and explored using PCA. Five PCA models were calculated using the response variables pretreated by autoscaling, which consists of transforming each variable by subtracting its average value and then dividing it by its standard deviation. The first PCA model was calculated for the whole dataset and the remaining ones for each separate biopolymer/plasticizer combination. All the PCA models were calculated using the PLS Toolbox chemometric software (ver. 8.5, Eigenvector Research Inc., Manson, WA, USA).

The statistical analysis of the data was performed through analysis of variance (ANOVA) using SPSS statistical program (SPSS 20 for Windows, SPSS INC., IBM, New York, USA). Each experimental condition described in Table 3 was performed in three replicates and the number of repeated measurements of each parameter varied depending on the measured parameter and is reported in each subsection. The differences between means were evaluated by Tukey's multiple range test ($p < 0.05$). The data were expressed as the mean ± SD (standard deviation).

Table 3.
Experimental design based on Plackett Burman model including 7 experimental factors; X1: biopolymer (pol.) concentration into the film-forming solution (FFS); X2: plasticizer concentration with respect to polymer mass; X3: presence/absence of $CaCl_2$; X4: presence/absence of sodium trimetaphosphate (STMP); X5: drying temperature; X6 presence/absence of ventilation; X7: presence/absence of ultraviolet-C radiation(UV-C) for the production of biodegradable films based on low methoxy amidated pectin (1-16) and chitosan (17-32).

FFS	Biopolymer	Plasticizer	Biopolymer Concentration (g/100 mL FFS)	Plasticizer Concentration (mL/g pol.)	$CaCl_2$ (g/g pol.)	STMP (g/g pol.)	Temp (°C)	Ventilation	UV-C
1	Pectin	Glycerol	3.5	0.75	0.125	0	75	-	-
2	Pectin	Glycerol	2.5	0.75	0.125	0.3	25	+	-
3	Pectin	Glycerol	2.5	0	0.125	0.3	75	-	+
4	Pectin	Glycerol	3.5	0	0	0.3	75	+	-
5	Pectin	Glycerol	2.5	0.75	0	0	75	+	+
6	Pectin	Glycerol	3.5	0	0.125	0	25	+	+
7	Pectin	Glycerol	3.5	0.75	0	0.3	25	-	+
8	Pectin	Glycerol	2.5	0	0	0	25	-	-
9	Pectin	Propylene glycol	3.5	0.75	0.125	0	75	-	-
10	Pectin	Propylene glycol	2.5	0.75	0.125	0.3	25	+	-
11	Pectin	Propylene glycol	2.5	0	0.125	0.3	75	-	+
12	Pectin	Propylene glycol	3.5	0	0	0.3	75	+	-
13	Pectin	Propylene glycol	2.5	0.75	0	0	75	+	+
14	Pectin	Propylene glycol	3.5	0	0.125	0	25	+	+
15	Pectin	Propylene glycol	3.5	0.75	0	0.3	25	-	+
16	Pectin	Propylene glycol	2.5	0	0	0	25	-	-
17	Chitosan	Glycerol	3	0.75	0	0.3	25	+	-
18	Chitosan	Glycerol	2	0.75	0	0.3	75	-	+
19	Chitosan	Glycerol	2	0	0	0.3	75	+	-
20	Chitosan	Glycerol	3	0	0	0	75	+	+
21	Chitosan	Glycerol	2	0.75	0	0	25	+	+
22	Chitosan	Glycerol	3	0	0	0.3	25	-	+
23	Chitosan	Glycerol	3	0.75	0	0	75	-	-
24	Chitosan	Glycerol	2	0	0	0	25	-	-
25	Chitosan	Propylene glycol	3	0.75	0	0.3	25	+	-
26	Chitosan	Propylene glycol	2	0.75	0	0.3	75	-	+
27	Chitosan	Propylene glycol	2	0	0	0.3	75	+	-
28	Chitosan	Propylene glycol	3	0	0	0	75	+	+
29	Chitosan	Propylene glycol	2	0.75	0	0	25	+	+
30	Chitosan	Propylene glycol	3	0	0	0.3	25	-	+
31	Chitosan	Propylene glycol	3	0.75	0	0	75	-	-
32	Chitosan	Propylene glycol	2	0	0	0	25	-	-

"+" presence and "-" absence.

3. Results and Discussion

Plackett-Burman design was used to develop 32 different formulations in three replicates (Table 3). Two formulations (3 and 11) did not result in film formation, mainly due to the photodegradation caused by prolonged UV-C irradiation. A white powder was observed in the Petri dishes. These formulations were characterized by the presence of STMP and $CaCl_2$ as cross-linkers, and treated with UV-C radiation. Kaczmarek, Bayer, Galka & Kotnowska (2007) showed that pectin is highly sensitive to photodegradation induced by UV-C radiation, which occurs through mechanisms such as dehydrogenation, dehydroxylation, de-esterification, decarboxylation, chain scission, and ring rupture. Souto-Maior, Reis, Pedreiro & Cavalcanti (2010) investigated the chemical mechanism of crosslinking induced by STMP in high methoxy pectin (degree of esterification 72%). The authors highlighted a general decrease in the inter- and intramolecular hydrogen bonds to the carboxylic groups due to the cross-linking of the hydroxyl groups and the formation of phosphate linkages. A similar polymer re-organization was observed for low methoxy pectin crosslinked with STMP at pH 6 by Carbinatto, De Castro, Cury, Magalhães, & Evangelista (2012) and Chen et al. (2015). In our study, after the crosslinking process with STMP (occurring in alkali environment), the acidification of FFS by HCl (pH: 4.4) resulted in better gelling properties of crosslinked LMA pectin than at neutral pH. This result was in accordance with Löfgren, Guillotin, and Hermansson (2006), who reported that the gel formation of LMA pectin was promoted by a reduction of pH presumably by hydrogen bonds to the amide groups.

Calcium ions from $CaCl_2$ were reported to react with both unreacted STMP ($Na_3P_3O_9$) and phosphate groups in an aqueous solution, producing a variety of calcium phosphates such as $CaNaP_3O_9$ and $Ca_3(PO_4)_2$, thus compromising the crosslinking mechanism (Averbuch - Pouchot & Durif, 1996). This process, together with the enhanced UV-C absorption induced by the high reactivity of STMP to UV-C wavelengths, promoted the photo-disruption of the polymer (Halmann & Platzner, 1965).

Formulations 15 and 21 formed films with inadequate mechanical resistance. Both the formulations were dried at 25°C and treated with UV-C radiation. Long UV-C treatments induced photodegradation both in pectin and chitosan films (Kowalonek, 2017).

The formulations able to form coherent films were subjected to further analyses to characterise their structural, functional, optical, mechanical, and barrier properties. The numerical results are summarised in Tables 4-7 and applied as raw data to compute PCA models whose objective was to investigate the mutual relation between the film properties (dependent variables) and their

dependence on the manufacturing parameters of the films (independent variables), explained in the next paragraphs.

Table 4.
Thickness, tensile strength (TS), elongation at break (E%), Young modulus (YM), air permeability (AP), and UV mean absorbance (A_{MEAN}) of the films based on pectin/glycerol as reported by the experimental design (**1-8**).

Film sample	Thickness (µm)	TS (MPa)	E (%)	YM (MPa)	AP × 10^3 (cm^3/m^2×day× kPa)	A_{MEAN}
1	59 ± 4^{b}	14 ± 1^{bc}	16 ± 1^{c}	386 ± 32^{a}	46.8 ± 0.1^{b}	0.6 ± 0.01^{b}
2	56 ± 5^{b}	2.8 ± 0.1^{a}	1.5 ± 0.2^{a}	258 ± 22^{a}	46.3 ± 0.2^{b}	0.7 ± 0.05^{b}
3	---	---	---	---	---	---
4	33 ± 3^{a}	7.5 ± 1^{ab}	0.2 ± 0.04^{a}	2316 ± 467^{c}	22145.6 ± 2.1^{e}	1.4 ± 0.07^{c}
5	77 ± 7^{c}	3.0 ± 1^{a}	39 ± 3^{d}	5.5 ± 0.5^{a}	25.9 ± 0.1^{a}	0.5 ± 0.06^{ab}
6	32 ± 3^{a}	109 ± 9^{d}	2.5 ± 0.3^{a}	5783 ± 379^{d}	25.9 ± 1.6^{a}	0.4 ± 0.05^{a}
7	62 ± 3^{b}	1.1 ± 0.01^{a}	6.5 ± 0.1^{b}	11 ± 1^{a}	578.9 ± 0.1^{d}	1.7 ± 0.15^{d}
8	30 ± 2^{a}	21 ± 2^{c}	3.5 ± 0.2^{ab}	1152 ± 71^{b}	74.6 ± 0.4^{c}	0.5 ± 0.01^{ab}

Values are given as mean ± SD (n = 3).
Different lowercase letters in the same column indicate significant differences ($p < 0.05$).

Table 5.
Thickness, tensile strength (TS), elongation at break (E%), Young modulus (YM), air permeability (AP), and UV mean absorbance (A_{MEAN}) of the films based on pectin/propylene glycol as reported by the experimental design (**9-16**).

Film sample	Thickness (µm)	TS (MPa)	E (%)	YM (MPa)	AP × 10^3 (cm^3/m^2×day× kPa)	A_{MEAN}
9	31 ± 3^{b}	66 ± 4^{c}	1.6 ± 0.3^{b}	4513 ± 356^{c}	39.9 ± 0.02^{b}	0.6 ± 0.05^{bc}
10	32 ± 3^{b}	17 ± 1^{ab}	1.2 ± 0.1^{b}	1576 ± 66^{a}	43 ± 0.03^{c}	1 ± 0.09^{d}
11	---	---	---	---	---	---
12	32 ± 3^{b}	6.5 ± 1^{a}	0.2 ± 0.04^{a}	2313 ± 619^{b}	22144.3 ± 0.4^{f}	1.4 ± 0.07^{e}
13	23 ± 2^{a}	79 ± 6^{c}	1.7 ± 0.2^{bc}	6065 ± 146^{d}	13117.6 ± 0.1^{e}	0.7 ± 0.01^{c}
14	31 ± 3^{b}	104 ± 10^{d}	2.2 ± 0.1^{c}	6229 ± 394^{d}	23.9 ± 0.1^{a}	0.4 ± 0.05^{a}
15	---	---	---	---	---	---
16	29 ± 3^{b}	22 ± 2^{b}	3.4 ± 0.3^{d}	1095 ± 61^{a}	74.6 ± 0.02^{d}	0.4 ± 0.08^{ab}

Values are given as mean ± SD (n = 3).
Different lowercase letters in the same column indicate significant differences ($p < 0.05$).

Table 6.
Thickness, tensile strength (TS), elongation at break (E%), Young modulus (YM), air permeability (AP), and UV mean absorbance (A_{MEAN}) of the films based on chitosan/glycerol as reported by the experimental design (**17-24**).

Film sample	Thickness (µm)	TS (MPa)	E (%)	YM (MPa)	AP × 10^3 (cm^3/m^2×day× kPa)	A_{MEAN}
17	122 ± 3^{e}	2.9 ± 0.2^{ab}	22 ± 2^{b}	14 ± 1^{a}	1250.8 ± 0.7^{e}	1.3 ± 0.14^{d}
18	113 ± 3^{d}	1.4 ± 0.2^{a}	26 ± 1^{c}	5.1 ± 0.3^{a}	135.5 ± 0.2^{d}	1.3 ± 0.16^{d}
19	110 ± 4^{d}	5.4 ± 0.5^{b}	2.7 ± 0.1^{a}	427 ± 31^{b}	1464.9 ± 0.8^{f}	1 ± 0.12^{cd}
20	32 ± 3^{a}	39 ± 3^{d}	2.3 ± 0.5^{a}	3045 ± 298^{d}	11 ± 0.04^{b}	0.3 ± 0.13^{ab}
21	---	---	---	---	---	---
22	114 ± 3^{d}	10 ± 1^{c}	1.1 ± 0.05^{a}	1013 ± 87^{c}	9206.1 ± 0.2^{g}	1.4 ± 0.25^{d}
23	71 ± 3^{c}	4.3 ± 0.3^{ab}	32 ± 0.1^{d}	17 ± 1^{a}	18.1 ± 0.1^{c}	0.8 ± 0.35^{bc}
24	43 ± 3^{b}	47 ± 2^{e}	1.8 ± 0.1^{a}	3503 ± 102^{e}	7 ± 0.01^{a}	0.2 ± 0.08^{a}

Values are given as mean ± SD (n = 3).
Different lowercase letters in the same column indicate significant differences ($p < 0.05$).

Table 7.
Thickness, tensile strength (TS), elongation at break (E%), Young modulus (YM), and air permeability (AP), and UV mean absorbance (A_{MEAN}) of the films based on chitosan/ propylene glycol as reported by the experimental design (**25-32**).

Film sample	Thickness (μm)	TS (MPa)	E (%)	YM (MPa)	AP × 10^3 (cm^3/m^2×day× kPa)	A_{MEAN}
25	129 ± 4^{f}	13 ± 1^{b}	2.7 ± 0.1^{c}	858 ± 71^{b}	1935.2 ± 0.08^{g}	1.3 ± 0.05^{c}
26	72 ± 3^{d}	7.9 ± 0.3^{ab}	12 ± 1^{e}	350 ± 23^{a}	38.6 ± 0.03^{d}	1.2 ± 0.10^{c}
27	111 ± 4^{e}	4.7 ± 0.4^{a}	2.6 ± 0.2^{bc}	401 ± 36^{a}	1463.5 ± 0.38^{f}	1.3 ± 0.005^{c}
28	27 ± 3^{a}	39 ± 4^{d}	2.1 ± 0.2^{bc}	3166 ± 238^{d}	10.8 ± 0.31^{c}	0.4 ± 0.04^{a}
29	26 ± 3^{a}	54 ± 5^{e}	2.8 ± 0.3^{c}	3781 ± 320^{e}	120.6 ± 0.020^{e}	0.6 ± 0.06^{b}
30	114 ± 4^{e}	10 ± 1^{ab}	1.1 ± 0.1^{a}	1193 ± 43^{b}	9205.4 ± 0.13^{h}	1.6 ± 0.02^{d}
31	58 ± 3^{c}	26 ± 2^{c}	6.1 ± 1.2^{d}	2081 ± 99^{c}	9.9 ± 0.01^{b}	0.6 ± 0.02^{b}
32	35 ± 3^{b}	38 ± 4^{d}	1.9 ± 0.1^{ab}	3393 ± 203^{de}	7 ± 0.07^{a}	0.4 ± 0.06^{a}

Values are given as mean ± SD (n= 3).
Different lowercase letters in the same column indicate significant differences ($p < 0.05$).

3.1. Surface and cross-section morphology

The surface and cross-section images of pectin and chitosan films are reported in Fig. 1 and Fig. 2. Film microstructure depends on the compatibility and miscibility between the film components (Haghighi et al., 2020a). SEM images of pectin films (Fig. 1) showed a smooth surface and a compact cross-section, with some exceptions. Formulations 1 and 9 had large bubbles on the surface. This was probably due to the presence of $CaCl_2$ inducing the formation of Ca^{2+}-mediated networks in the molecular structure of LMA pectin, as reported by Cui et al. (2017) and Löfgren et al. (2006). This promoted the retention of small amounts of air inside the FFS. The two plasticizers had different effects on the films. This difference was probably due to the different surface tension of the FFSs due to their composition. These effects were more evident in formulations 2 and 10, which were prepared with the lowest concentration of pectin, the presence of STMP and $CaCl_2$, and by drying at 25°C with convective ventilation. The films developed a huge number of small bubbles due to prolonged ventilation. Similar results have been reported by Galus et al. (2012).

The surface and cross-section morphologies of chitosan films are reported in Fig. 2. The films without a cross-linker developed a smooth and uniform surface, without a discontinuous or porous structure. The addition of STMP induced significant changes in the microstructure of the biopolymers due to the formation of aggregates on the film surface. This could be related to the decrease of the polymer solubility in water caused by the crosslinking, with the consequent aggregation of the polymer chains due to intra- and intermolecular bonds. The influence of drying temperature on the microstructure of films was not significant, contrary to the result obtained by Fernàndez-Pan et al. (2010), and in accordance with Srinivasa et al. (2004). In addition, the UV-C radiation did not lead to a significant modification of the topography of the chitosan films, as observed by Sionkowska et al. (2006). The

cross-section analysis of the films suggested that the thickness of the films was greatly affected by the concentration of the polymer and by the presence of the plasticizer (Galus et al., 2012).

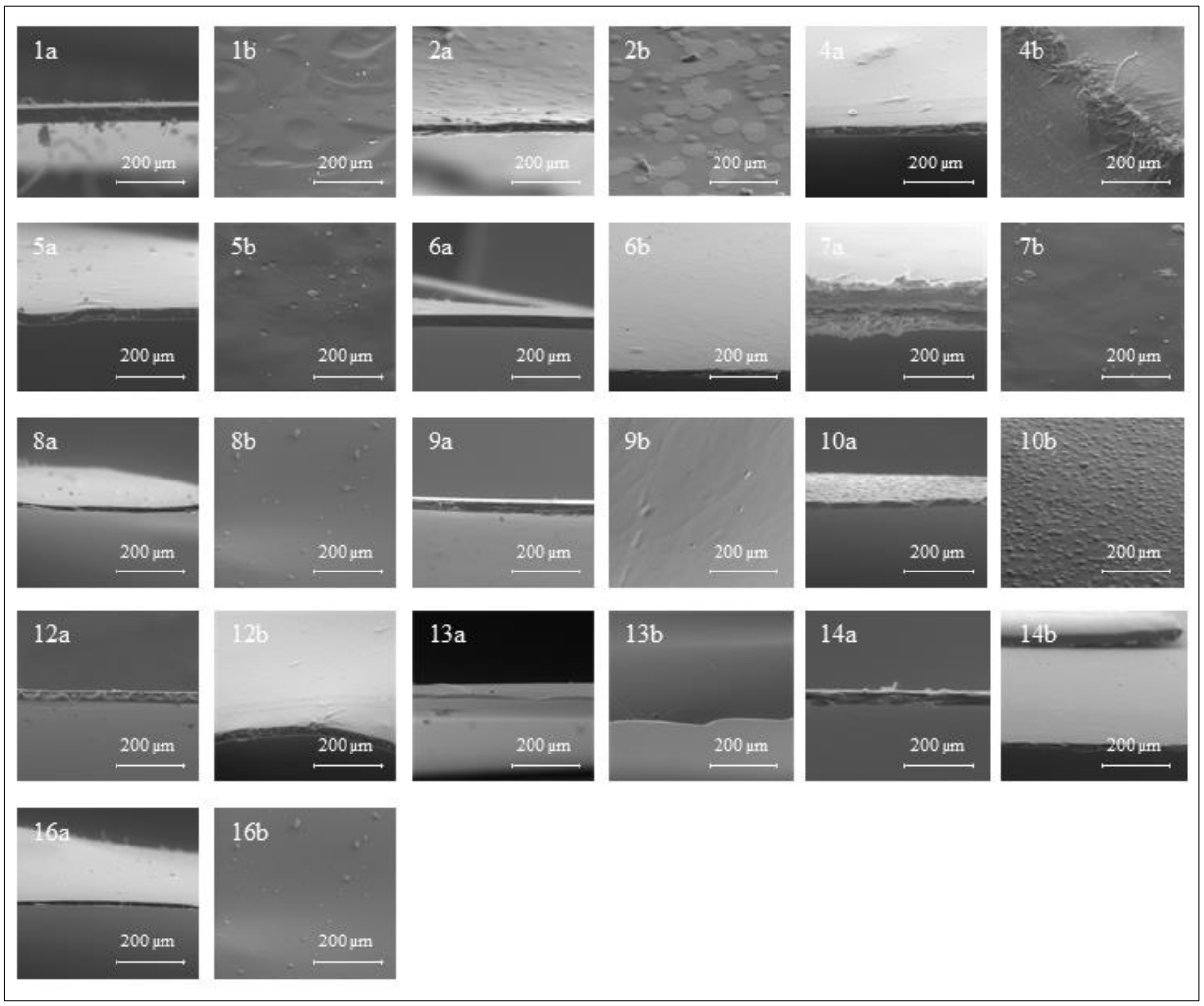


Fig. 1. SEM images on (a) cross-section and (b) surface of low methoxy amidated pectin films based on experimental design (**Table 3**).

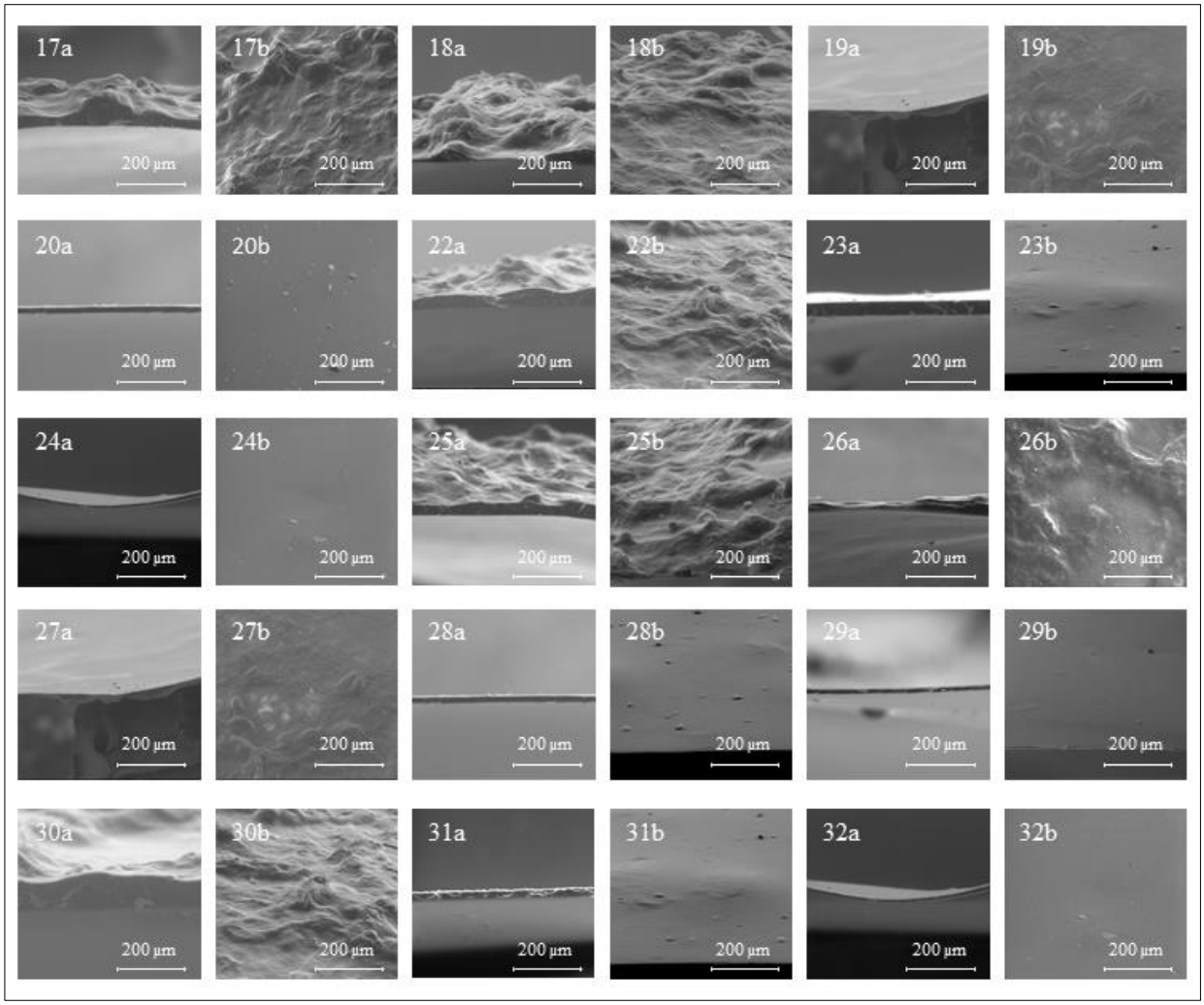


Fig. 2. SEM images on (a) cross-section and (b) surface of chitosan films based on experimental design (**Table 3**).

3.2. Principal component analysis (PCA)

3.2.1. PCA model I: whole dataset

Fig. 3 reports the score and loading plots of PC1 vs. PC2 of the model computed on the whole dataset. These two PCs accounted for about 75% of the total data variance.

The PC1 vs. PC2 loading plot (Fig. 3a) highlighted that PC1 (53.78% of total data variance) was mainly influenced by the UV–vis absorbance values. The grouping of all the absorbance values indicated their mutual positive correlation. PC1 was also positively correlated with the film thickness. Young modulus (YM) and tensile strength (TS) were found on the opposite side of the thickness with respect to the axes origin, indicating that the two latter variables were positively correlated with each other and negatively correlated with the thickness.

PC2 (20.82% of total data variance) was influenced by four main factors: unitary absorbance (UA) and air permeability (AP) at positive values, elongation at break percentage (E%), and thickness at negative values. In particular, E% was negatively correlated with TS and YM, while AP and UA were highly correlated with each other.

Fig. 3b reports the PC1 vs. PC2 score plot. The symbols on the plot refer to the different formulations (polymer and plasticizer), while the numbers indicate the corresponding experimental conditions according to Table 3. Most of the pectin-based films were at negative values of PC1, corresponding to low thickness (ranging from 23 to 77 μm, Tables 4 and 5). Conversely, the chitosan films were split into two groups: one at positive values and one at negative values of PC1.

The effect of the plasticizer was observed throughout PC2. The analysis of the labels (Fig. 3b) demonstrated that most of the films containing glycerol (formulations 1, 5, 17, 18, and 23) were placed at the bottom part of the score plot. This position corresponded to a high E % (ranging from 16 to 39%, Tables 4 and 6) and low TS (ranging from 1.4 to 14 MPa, Tables 4 and 6). Samples containing propylene glycol or without plasticizer were placed on the upper side of the score plot, showing a lower E% (ranging from 0.2 to 12%, Tables 4–7). These results confirmed the high performance of glycerol as a plasticizer for the production of biodegradable films with respect to propylene glycol, as already shown by Jantrawut et al. (2017). Depending on whether glycerol was present or not, pectin samples were divided along the diagonal line from the upper left side to the bottom right side of the PC1 vs PC2 score plot. This distribution indicated that pectin films plasticized with glycerol were significantly thicker (ranging from 56 to 77 μm, Table 4) than films containing propylene glycol or without plasticizer (ranging from 23 to 33 μm, Tables 4 and 5). This effect was probably due to the high hygroscopicity of glycerol (Galus, Uchański, & Lenart, 2013). Conversely, chitosan films were not clearly separated in the model according to the type and concentration of the plasticizer.

PC1 vs PC2 score plot (Fig. 3b) shows that there are superimposed samples in the model. They corresponded to samples 4–12, 6–14, and 8–16 for pectin films, and 19–27, 20–28, 22–30, and 24–32 for chitosan films. Each data couple can be considered as a double replication of a single experimental condition, according to Table 3. Their superimposition on the PC1 vs PC2 score plot indicated the high reproducibility of the film preparation and response variable measurement. Notably, samples 22–30 of chitosan films were also superimposed on formulation 7. This similarity was mainly due to the high absorbance values of these three films (Tables 4, 5 and 7). A strong correlation between the absorbance of the films and the crosslinking methods can be assumed, irrespectively of the biopolymer/plasticizer combination.

In order to investigate the effect of the experimental parameters on the film properties, the objects in the PC1 vs PC2 score plot were also analysed using different symbols corresponding to the levels of each factor. Referring to the whole dataset model, this analysis highlighted that the only factor leading to a noticeable separation of the objects in the PC1 vs. PC2 score plot was STMP, as reported in Fig. 3c. Films treated with STMP were located on the right of the plot while films without STMP were on the left. A comparison with the loading plot (Fig. 3a) showed that the films with STMP were less transparent to UV light than films without STMP (Tables 4–7). This was probably due to the compact surface formed by intra- and intermolecular interactions within the polysaccharide chains. The same effect was observed by Wang, Liao et al. (2019) for chitosan-methylcellulose composite films.

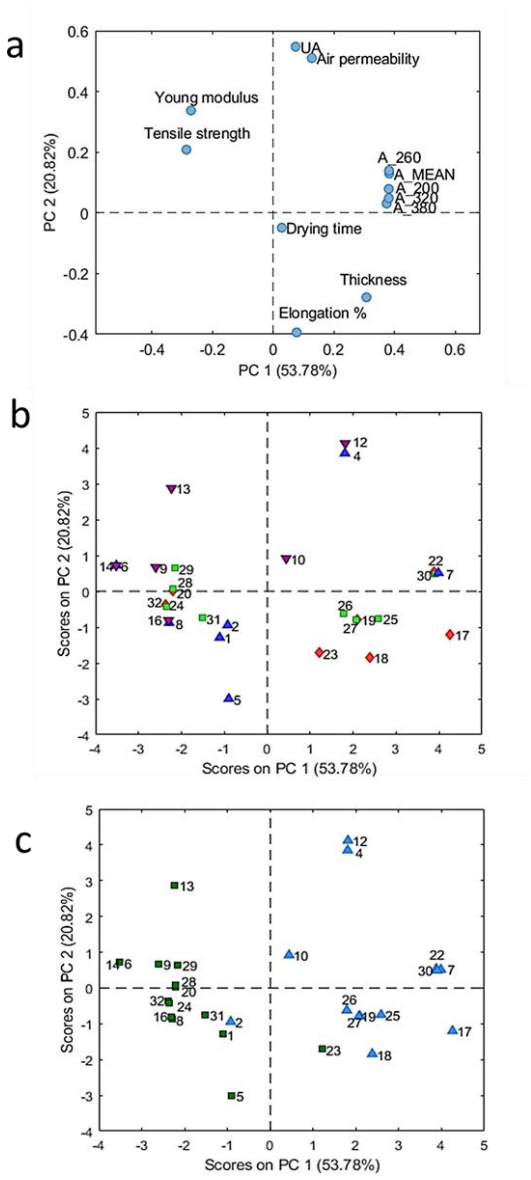


Fig. 3: PCA model I on the whole dataset. a) PC1 vs. PC2 loading plot and b) PC1 vs. PC2 score plot and c) PC1 vs. PC2 score plots according to presence/absence of sodium trimetaphosphate (STMP). ▲ pectin + glycerol; ▼ pectin + propylene glycol; ♦ chitosan + glycerol; ■ chitosan + propylene glycol; ■ absence of STMP; ▲ presence of STMP

3.2.2. PCA model II: pectin/glycerol

Fig. 4 reports the score and loading plots of PC1 vs. PC2 of the model computed for the pectin/glycerol combination. The loading plot of PC1 vs. PC2 (Fig. 4a) confirmed the high influence of the UV absorbance variables on PC1, which accounted for 52.61% of total data variance. PC2 (25.16% of total data variance) was mainly influenced by the thickness and E% at positive values, and by TS and YM at negative values. The drying time was not significant in the space of the first two PCs.

The PC1 vs. PC2 score plot (Fig. 4b) of this model was used to analyse the impact of each factor, by plotting the objects with different symbols corresponding to the factor levels. The most noticeable effect was related to the presence/absence of the plasticizer. Samples plasticized with glycerol (formulations 1, 2, 5, and 7) were located at positive values of PC2, and were thicker, more extensible, and with lower YM than those that were not plasticized (formulations 4, 6, and 8) ($p < 0.05$). These results are in line with those shown in Table 4. This effect might be due to the reliable plasticizing effect (Galus et al., 2013) and to the high hygroscopicity of glycerol, which retains a large amount of water inside the polymer matrix, thus forming a large hydrodynamic plasticizer-water complex (Jantrawut et al., 2017).

The two formulations (4 and 7) at positive values of PC1 (Fig. 4b) were those containing STMP without $CaCl_2$. These samples showed high absorbance values, which could be related to the increase in UV light absorption induced by STMP. Conversely, all the samples containing 2.5 g polymer/100 mL FFS (formulations 2, 5, and 8) and $CaCl_2$ as crosslinker (formulations 1, 2, and 6) were located on the left of the plot. These samples showed lower UV absorbance values than formulations 4 and 7 ($p < 0.05$) (Table 2). This effect was probably due to the presence of $CaCl_2$, which induced the molecular re-organization of the polymer matrix (Cui et al., 2017).

The loading plot of PC3 vs PC4 (Fig. 5a) highlighted the great influence of the drying time at positive values of PC3 and negative values of PC4. The score plot showed that the samples were divided along PC3 according to the drying temperature (Fig. 5b). This confirmed that the high temperature (75°C), synergistically with the convective ventilation, significantly reduced the drying time ($p < 0.05$) (Table 4), as already described by Fernàndez-Pan et al. (2010) for chitosan films.

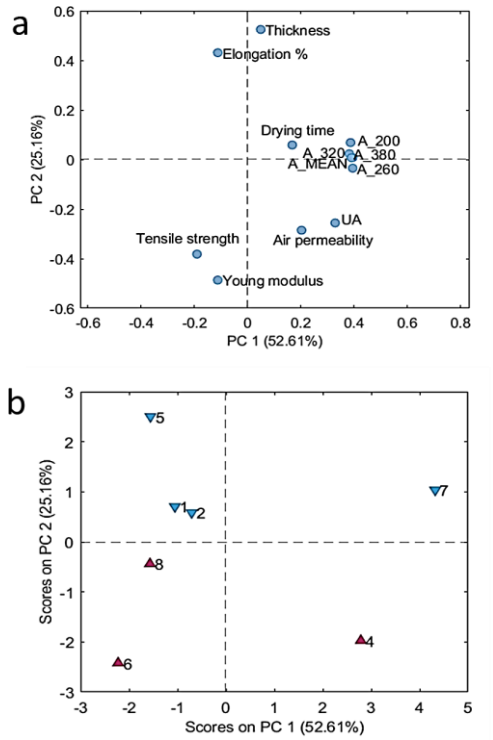


Fig. 4. PCA models II on low methoxy amidated pectin/glycerol combination. a) PC1 vs. PC2 loading plot and b) PC1 vs. PC2 score plot according to the presence/absence of glycerol. ▲ absence of glycerol; ▼ presence of glycerol

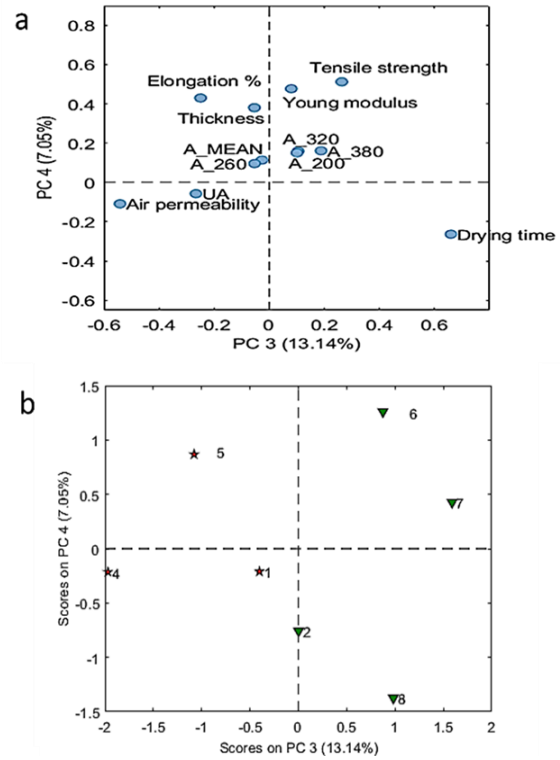


Fig. 5. PCA model II on low methoxyl amidated pectin /glycerol combination. a) PC3 vs. PC4 loading plot, b) PC3 vs. PC4 score plot according to drying temperature. ▼ drying temperature 25 ºC; ★ drying temperature 75 ºC.

3.2.3. PCA model III: pectin/propylene glycol

The loading plot of PC1 vs. PC2 of the pectin/propylene glycol FFSs (Fig. 6a) confirmed the influence of UV absorbance on PC1, which accounted for about 86% of total data variance. UA was located close to the other absorbance-related variables, due to the slight thickness variations in all the pectin/propylene glycol formulations (Table 5). For the same reason, the thickness was very close to the origin of the axes. Both TS and YM were located at the positive values of PC2. Unlike pectin/glycerol formulations, the influence of the drying time at negative values of PC2 was significant.

The analysis of the corresponding score plot showed a clear separation of the samples along PC1, depending on the presence/absence of STMP (Fig. 6b), as described for model II (pectin/glycerol formulations). In contrast to the pectin/glycerol formulations, the presence of propylene glycol did not lead to significant modifications in the final film properties, and especially the mechanical properties (Table 3). This analysis demonstrated the low quality of propylene glycol as a plasticizer for the production of the pectin films, with respect to glycerol (Jantrawut et al., 2017). The analysis of the PC1 vs PC3 loading plot (Fig. 7a) showed that PC3 was mainly influenced by thickness at positive values, and by AP at negative ones. The corresponding score plot showed a perfect division of the data throughout PC3 based on the presence/absence of $CaCl_2$ (Fig. 7b), in line with the experimental results reported in Table 5. The films crosslinked with $CaCl_2$ developed a slightly thicker structure, while the AP of the films was drastically reduced by the presence of $CaCl_2$ ($p<0.05$).

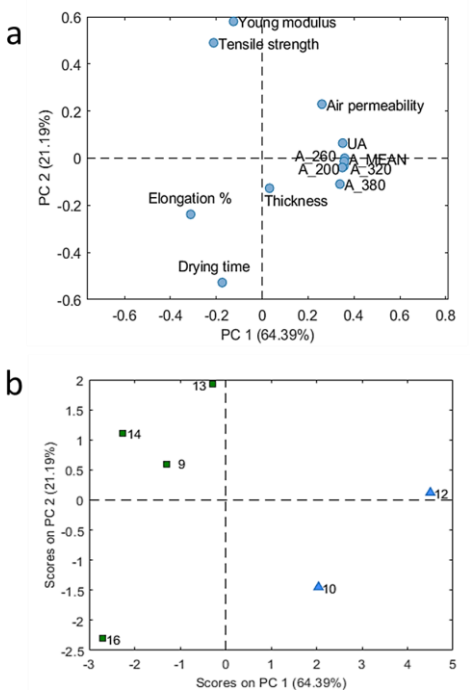


Fig. 6. PCA models III on low methoxy amidated pectin/propylene glycol combinations. a) PC1 vs. PC2 loading plot and b) PC1-PC2 score plot according to presence/absence of sodium trimetaphosphate (STMP). ■ absence of STMP; ▲ presence of STMP.

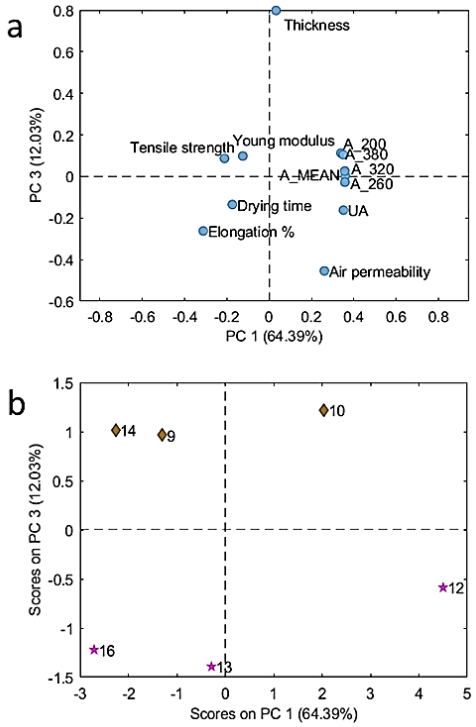


Fig. 7. PCA model III on low methoxyl amidated pectin /propylene glycol combination. a) PC1 vs. PC3 loading plot, b) PC1 vs. PC3 score plots according to presence/absence of $CaCl_2$. ★ absence of $CaCl_2$; ♦ presence of $CaCl_2$.

3.2.4. PCA model IV: chitosan/glycerol

The loading plot of PC1 vs PC2 for chitosan/glycerol (Fig. 8a) confirmed that the direction of PC1 depended on the absorbance values. PC2 was mainly influenced by drying time and by AP at positive values, and by E% at negative ones.

The score plot of PC1 vs. PC2 (Fig. 8b) showed that the samples with STMP had positive values of PC1, due to their high absorbance and thickness (Table 6). The addition of STMP to the FFS induced the formation of three-dimensional aggregates on the film surface, probably due to the creation of inter- and intra-molecular bonds inside the polymer structure. In contrast, Wang, Liao et al. (2019) demonstrated that the chitosan-methyl cellulose composite films crosslinked with STMP developed more uniform and compact microstructures than the untreated ones. Formulation 23 showed positive values of PC1, due to its high UV absorbance (Table 6), high thickness, and enhanced E%: in fact, sample 23 was the only formulation without STMP that contained glycerol. The presence of glycerol (formulations 17, 18, and 23 in the bottom right corner of Fig. 8b) led to a higher E%, confirming the dependence of E% on the content of plasticizer (Thakhiew, Devahastin, & Soponronnarit, 2010).

The samples in Fig. 8b were also divided along the diagonal from the bottom left to the upper right corner, meaning that the films treated with STMP (formulations 17, 18, 19, and 22) were also more

permeable to air than the untreated ones (Table 6). This effect is probably related to the structural discrepancies observed through the microstructural analysis of the films crosslinked with STMP. Concerning the effect of drying temperature, the samples heated to 25°C tended to show positive (or less negative) PC2 values due to their long drying time.

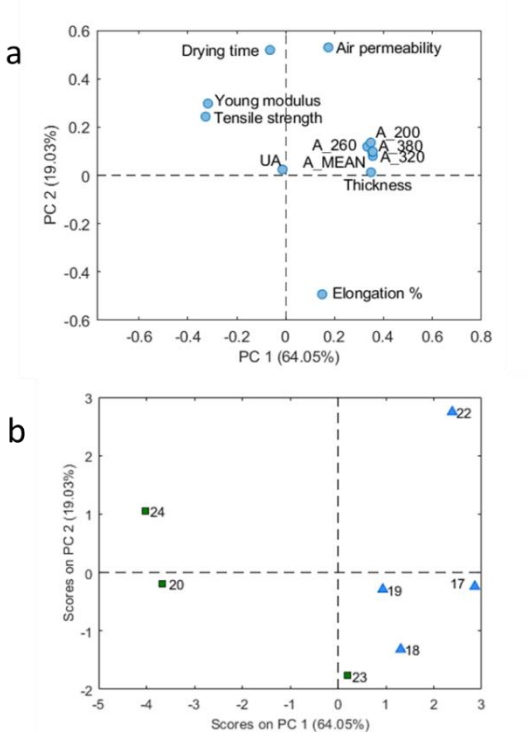


Fig. 8. PCA model IV on chitosan/glycerol. a) PC1 vs. PC2 loading plot and b) PC1 vs. PC2 score plot according to presence/absence of sodium trimetaphosphate (STMP). ■ absence of STMP; ▲ presence of STMP.

3.2.5. PCA model V: chitosan/propylene glycol

The PC1 vs. PC2 loading plot of the chitosan/propylene glycol dataset (Fig. 9a) was similar to the one for chitosan/glycerol. The model suggested that the presence of propylene glycol did not significantly affect the functional properties of the films, including the mechanical ones. This result was confirmed by comparing the trends observed in the PCA model with the numerical data reported in Table 7.

The samples containing STMP (formulations 25, 26, 27, and 30) were separated from the others at positive values of PC1 (Fig. 9b), according to the high thickness and UV absorbance (Table 7). In addition, the drying temperature (Fig. 9c) divided the samples along PC2, confirming the mutual relations between the experimental factors and the final properties of the films. The PC1 vs. PC2 score plot based on the other experimental factors did not highlight any further trends.

The PC1-PC3 loading plot (Fig. 10a) showed that UA mainly contributed to define the PC3 direction. The samples were separated on the corresponding score plot (Fig. 10b) according to the UV-C radiating treatment; in fact, the treated films absorbed UV light more than the non-treated ones (Table 7). A long-term UV-C treatment (formulation 6), combined with the STMP crosslinking process, significantly increased the AP of the films. These effects were due to the degradation induced on the polymer structure by a long radiating process (Sionkowska et al., 2006). The UV-C treatment helped to reduce the drying time, in synergy with the drying temperature and the convective ventilation (Table 7).

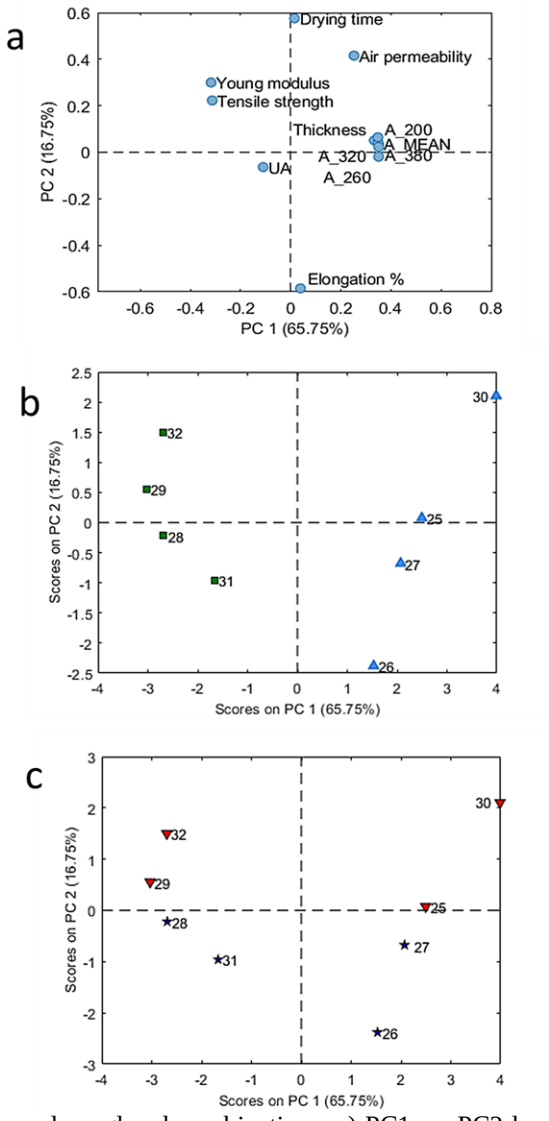


Fig. 9. PCA model V on chitosan/propylene glycol combinations. a) PC1 vs. PC2 loading plot, b) PC1 vs. PC2 scores plot according to presence/absence of sodium trimetaphosphate (STMP) and c) PC1 vs. PC2 score plot according to drying temperature. ■ absence of STMP; ▲ presence of STMP. ▼ drying temperature 25 ºC; ★ drying temperature 75 ºC.

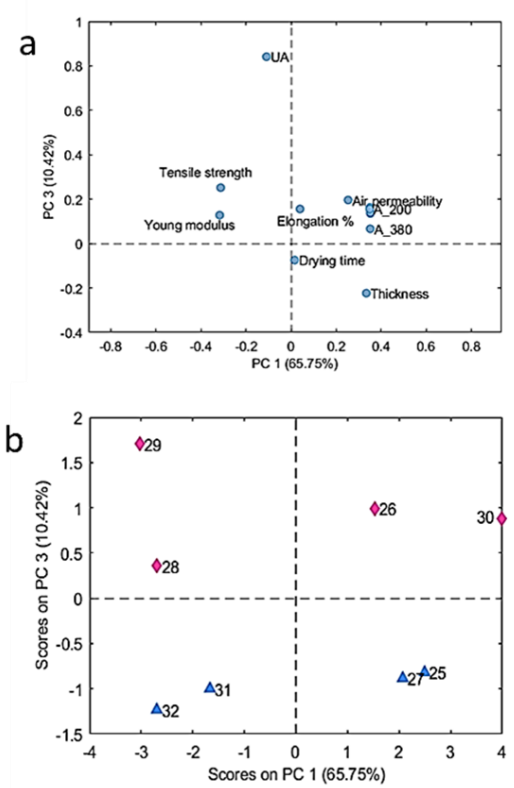


Fig. 10. PCA model V on chitosan/propylene glycol combination. a) PC1 vs. PC3 loading plot, b) PC1 vs. PC3 score plot according to presence/absence of UV-C irradiation. ▲ absence of UV-C irradiation; ♦ presence of UV-C irradiation.

4. Conclusions

The functional properties of chitosan and pectin films depend on several experimental factors including compositional features, crosslinking process, and drying conditions. The complex interaction between these parameters can also be important. In this study, PCA facilitated exploring the dependence of the final properties of chitosan- and low methoxy amidated pectin-based biodegradable films on the experimental parameters considered in a Plackett-Burman experimental design. The trends observed in the PCA models were compared with the raw numerical dataset (Tables 4–7). PCA easily highlighted the main sources of data variance and the main interactions between the experimental factors, e.g., the presence of STMP, and the functional properties of the films. In conclusion, this work lays the groundwork for the creation of more refined experimental designs and statistical models which could help predicting the trend of film performances related to the wide range of experimental conditions. This approach represents a useful tool for the future industrial production and design quality of these materials.

Chapter 4

Characterization of chitosan-hydroxypropyl methylcellulose blend films enriched with nettle or sage leaf extract for active food packaging applications

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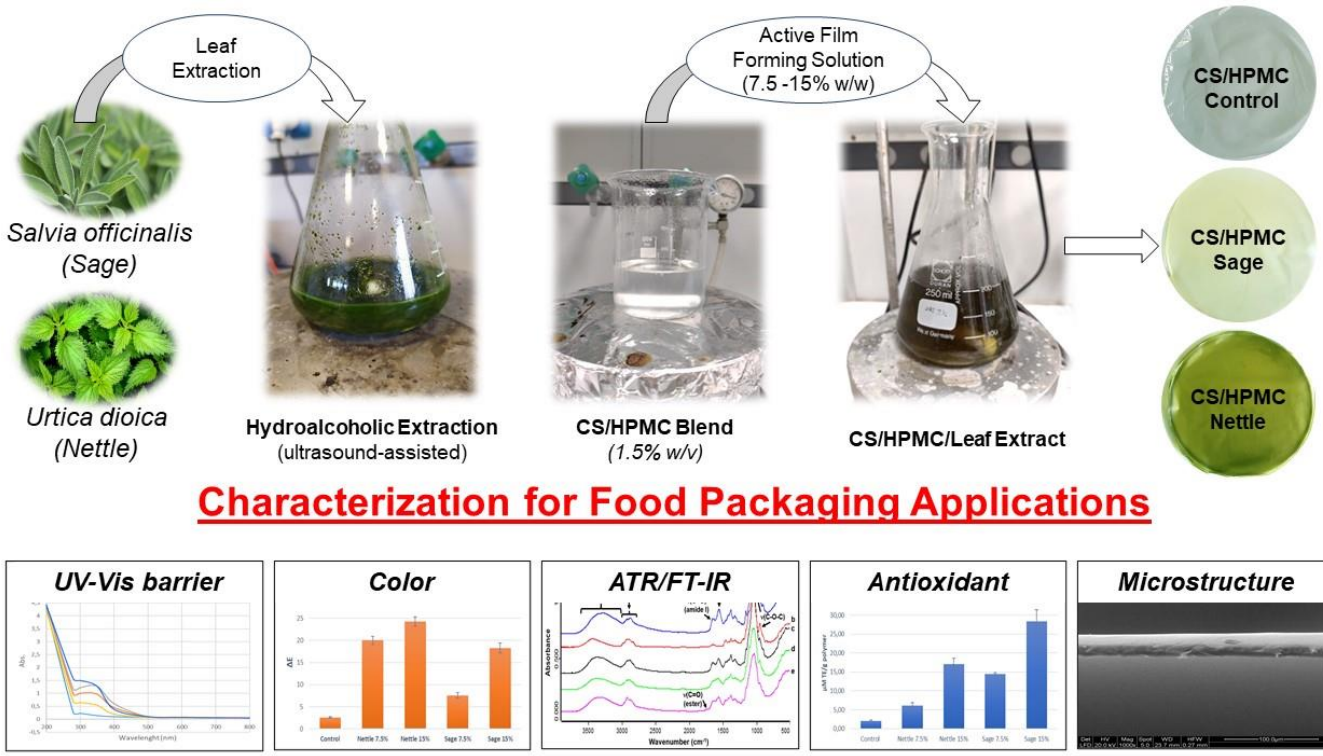
Graphical abstract



Keywords

Active film; Chitosan/hydroxypropyl methylcellulose blend; Leaf extracts; Antioxidant activity; Herb extracts; Polyphenols.

Abstract

The incorporation of plant leaf extracts into biodegradable food packaging materials is a promising green approach to develop active films with antioxidant and antimicrobial activities. In this context, this study aimed to develop active films based on chitosan/hydroxypropyl methylcellulose (CS/HPMC) blend enriched with sage (SLE) and nettle (NLE) leaf extracts (7.5–15% w/w of biopolymer) to characterize their surface and cross-section morphology, optical, mechanical, water barrier, and antioxidant properties for food packaging applications. Scanning electron microscopy confirmed microstructural integrity and compatibility between CS and HPMC and incorporated leaf extracts. The successful incorporation of plant extracts was confirmed by Fourier transform infrared spectroscopy. Active films showed improvement in UV–Vis light barrier properties ($p < 0.05$) with opacity value lower than five. Addition of leaf extracts induced a slightly darker colour by inducing a green and yellow shade. Water vapor transmission rate increased at lower concentration of leaf extracts (7.5% w/w) while further increase in leaf extracts (15% w/w) caused a reduction of these parameters ($p < 0.05$). Addition of NLE increased the water solubility and water vapor permeability compared to the control film ($p < 0.05$). Total phenolic content and antioxidant activity were increased upon addition of leaf extracts. Overall, CS/HPMC films incorporated with SLE and NLE could be employed as a green alternative for partial substitution of synthetic plastics with antioxidant activity and to prolong the shelf-life of food products.

1. Introduction

Biodegradable food packaging materials based on biopolymers have recently gained widespread interest as a green alternative to the ecological impacts caused by the massive application of synthetic, fossil-based plastics (Espitia et al., 2014; Hosseini & Gòmez-Guillèn, 2018). Among biopolymers, hydroxypropyl methylcellulose (HPMC) and chitosan (CS) represent good candidates for food packaging since they are abundant, renewable, non-toxic, biocompatible, and biodegradable (da Silva et al., 2019; Wang, Dong, Men, Tong, & Zhou, 2013). HPMC is a semi-synthetic, non-ionic, low cost, and transparent cellulose-derived ether which is approved as a food additive for direct addition to the food for human consumption by the US Food and Drug Administration (21 CFR 172.874) and the European Parliament and Council (Directive No. 95 No 95/2/EC) (Akhtar et al., 2013). This biopolymer is water-soluble and is widely utilized in the food industry as an emulsifier, stabilizer, thickener, suspending agent, and film-forming material (Möller, Grelier, Pardon, & Coma, 2004). HPMC-based films possess good transparency, flexibility, low aroma permeability, resistance to fat and oils with moderate strength, and efficient permeability to oxygen transmission (Sánchez-González, Vargas, González-Martínez, Chiralt, & Cháfer, 2009). However, they are highly susceptible to moisture because of their inherent hydrophilic nature. This represents a drawback for their industrial applications for packaging foods with high water activities (Lee et al., 2019; Rotta et al., 2009). CS is a polysaccharide obtained from the deacetylation of chitin. CS is the second most abundant polysaccharide in nature after cellulose and is abundant in the exoskeleton of crustaceans and insects (Muxika, Etxabide, Uranga, Guerrero, & de la Caba, 2017). CS is soluble in low-pH aqueous solutions due to the protonation of the NH_2 groups (Haghighi et al., 2020b). It inhibits the growth of a broad range of microorganisms due to its cationic behaviour. Moreover, this polysaccharide shows excellent film-forming properties, and an acceptable barrier to gases such as CO_2 and O_2 (Machado et al., 2020). However, these positive properties are affected by drawbacks such as low mechanical properties and high production costs. Since CS and HPMC are hydrophilic and highly compatible biopolymers, their combination (CS/HPMC) may improve the physical and mechanical properties of the individual biopolymer and reduce the cost as well (Liang, Zhao, Xiao, Cheng, & Zhao, 2020; Möller et al., 2004; Song, Feng et al., 2020). This synergy might be related to the formation of strong intermolecular hydrogen bonds between the NH_2 groups of CS and the OH functionalities of HPMC (Rotta et al., 2009).

Antioxidant and antibacterial packaging represent the main categories of active packaging addressed to ensure a high level of food safety and to maintain food quality (Zimoch-Korzycka, Bobak, & Jarmoluk, 2016). The use of natural antioxidants and/or antimicrobial compounds of plant origin (e.g.,

essential oils, polyphenols, etc.) allow developing active packaging based on a sustainable approach (Akhtar et al., 2013; Bajić et al., 2019; Bonilla & Sobral, 2016; Mir et al., 2018; Quilez-Molina et al., 2020; Sirisha Nallan Chakravartula et al., 2020). In this context, leaves of stinging nettle (*Urtica dioica* L.) and sage (*Salvia officinalis* L.) are promising sources of bioactive compounds for incorporation into biopolymeric matrices. Stinging nettle is a perennial plant that belongs to the family of Urticaceae and has been spread in many places throughout the world (Haghju, Beigzadeh, Almasi, & Hamishehkar, 2016). Its aqueous and alcoholic extracts were found to exert a wide range of antioxidant and antimicrobial activities. Various studies confirmed the presence of active compounds such as vitamins (e.g., B1, B2, C, and K), caffeic acid derivatives, terpenoids, ceramides, carotenoids, essential oils, fatty acids, minerals (e.g., Fe, Ca, Zn, Cu, Mn, and Ni), phytosterols, glycosides, and many other polyphenolic compounds (Di Virgilio et al., 2015; Orcic et al., 2014; Upton, 2013). On the other hand, sage is a member of the Lamiaceae family, native to the Mediterranean region. This plant has antioxidant, antibacterial, and fungistatic activities related to the high polyphenolic contents (Eidi & Eidi, 2009). Previous studies confirmed the presence of phenolic compounds, terpenoids, and flavonoids such as rosmarenic acid, carnosic acid, carnosol in sage leaves (Oudjedi et al., 2019).

To the best of our knowledge, the development of CS/HPMC films incorporated with NLE or SLE has not been reported in the literature. Therefore, the present study aimed to investigate surface and cross-section morphology, optical, mechanical, water barrier, and antioxidant properties of CS/HPMC films enriched with NLE or SLE at different concentrations for active food packaging applications.

2. Materials and Methods

2.1. Materials and Reagents

Chitosan (CS) with medium molecular weight (100–300 kDa) was supplied from Acros Organics™ (China). Hydroxypropyl methylcellulose (hydroxypropyl 5–8%, methoxy 28–30%) was purchased from ACEF SPA (Piacenza, Italy). Ethanol, acetic acid, glycerol, 6-hydroxy 2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′ azinobis (3 ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Folin-Ciocalteu's reagent, gallic acid monohydrate (GA), barium chloride, calcium chloride, and potassium persulfate with analytical grade were provided by Sigma-Aldrich (St. Louis, USA).

2.2. Preparation of plant leaf extracts

Stinging nettle (Urtica dioica L.) and sage (Salvia officinalis L.) leaves were collected from a local farm (Reggio Emilia, Italy) between May–June 2020. The leaves were dried in a ventilated oven (ZTM Mechatronic, Reggio Emilia, Italy) at 40°C for 4 h. A total of 5 g of dried and ground leaves were mixed with 150 mL of EtOH–H_2O solution (50:50 v/v) and stirred for 5 min. Then, the mixture was sonicated (ARGO Lab. Mod. DU-45, Modena, Italy) for 50 min (180 W; 40 kHz; 40°C). After filtration (Whatman filter paper No. 93, Little Chalfont, Buckinghamshire, UK), the extracts were dried under vacuum at 40°C using a rotary evaporator (VV2000, Heidolph Instruments GmbH & CO., Schwabach, Germany) and stored at −10°C (Zeković, Cvetanović, et al., 2017a).

2.3. Preparation of film-forming solutions (FFSs) and films

Pure CS FFS was prepared by dissolving 1.5 g CS in 100 mL of acetic acid solution (1% v/v) with stirring at 55 °C for 15 min (Leceta, Guerrero, Ibarburu, Dueñas, & de la Caba, 2013). Pure HPMC FFS was prepared by dissolving 1.5 g HPMC in 100 mL hot water (80°C) for 60 min (Lee et al., 2019) followed by cooling down at room temperature. Glycerol (30% w/w of CS or HPMC) was then added into both FFS with additional stirring for 30 min. CS/HPMC blend was prepared by mixing CS and HPMC FFSs at a 1:1 ratio. CS/HPMC blend film without leaf extract was used as a control. NLE and SLE at two different concentrations (7.5 and 15% w/w of biopolymer) were added to CS/HPMC blend FFS under stirring at room temperature for 30 min (Talón, Trifkovic, Vargas, Chiralt, & González Martínez, 2017b). A total of 20 mL FFS were cast onto Petri dishes (14.4 cm

diameter) and dried at an ambient condition (25°C and 45% relative humidity) for 24 h in a chemical hood.

2.4. Scanning electron microscopy (SEM)

The surface and cross-section morphology of the films were examined using a field emission scanning electron microscope (NovaNano SEM 450, FEI, USA) in a low vacuum mode (80 Pa) with an acceleration voltage of 10 kV. Film samples were cut (2 × 2 mm^2) to mount on stainless steel stubs with double-sided tape.

2.5. Attenuated total reflection (ATR)/Fourier-transform infrared (FT-IR) spectroscopy

The FTIR spectra of the films were obtained using an ATR/FT-IR spectrometer (Alpha, Bruker Optik GmbH, Ettingen, Germany). Each spectrum was recorded from the top and bottom of the same sample in the infrared region between 4000 and 400 cm^{-1} at a spectral resolution of 4 cm^{-1} by accumulating 64 scans. Each measurement was performed in duplicate.

2.6. Thickness and mechanical properties

The average thickness of each film at five different random positions was calculated by a digital micrometer (IP65, SAMA Tools, Viareggio, Italy). Mechanical properties including tensile strength (TS), elasticity (E%), and Young's modulus (YM) were determined using a dynamometer (Z1.0, ZwickRoell, Ulm, Germany) equipped with a 1 kN loading cell according to ASTM standard method D882-12 (ASTM, 2001a). The initial grip separation was 70 mm with 10 mm/min crosshead speed. The measurements were repeated 15 times. TestXpert® II 161 (V3.31) (ZwickRoell, Ulm, Germany) was used to calculate TS (MPa), E (%), and YM (MPa).

2.7. UV-Vis light transmittance, opacity, and colour

The UV–Vis light transmittance of films (2 × 2 cm^2) was measured at 200–800 nm wavelengths using a spectrophotometer (VWR®Double Beam UV × VIS 6300 PC spectrophotometer, China). The opacity of the films was calculated by Eq. (5):

$$\text{Opacity value} = -\log T600/d \quad (5)$$

where T600 is the fractional transmittance at 600 nm and d is the film thickness (mm). The average of four replicate measurements was calculated.

The CIELAB coordinates L* (lightness), a* (redness/greenness), and b* (yellowness/blueness) were obtained at room temperature through a CR-400 Minolta colourimeter (Minolta Camera, Co., Ltd., Osaka, Japan) with D65 illuminant and 10° observer angle. The instrument was calibrated by a white standard (L* = 99.36, a* = −0.12, b* = −0.06). The total colour variation (ΔE*) was calculated using Eq. (6):

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (6)$$

where ΔL*, Δa*, and Δb* represent the differences between the corresponding colour parameter of the samples and that of a white standard used as the film background (De Leo et al., 2018). An average of 10 measurements was recorded for each film.

2.8. Water solubility (WS)

The WS was determined by measuring the initial dry weight of each film (2 × 2 cm^2) after drying to constant weight in an oven at 105 ± 2 °C (W_i) and subsequently each film was immersed in 50 mL distilled water at 25°C for 24 h. Undissolved parts were dried in an oven at 105 ± 2°C (W_f). The WS was then calculated according to Eq. (7):

$$WS\ (\%) = ((W_i - W_f)/W_i) \times 100 \quad (7)$$

where W_i and W_f are the initial and final dry weights (g) of the films, respectively. An average of four measurements was recorded for WS analysis.

2.9. Water vapor transmission rate (WVTR) and water vapor permeability (WVP)

WVTR was determined in triplicate according to the ASTM E96 method (ASTM, 2001b) with slight modifications. Films were sealed on top of 25 mL glass bottles containing 2 g anhydrous $CaCl_2$ (0% RH). Test bottles were placed into a desiccator containing $BaCl_2$ (90% RH) at 45°C. WVTR was determined by plotting the daily weight gain of bottles as a function of time. WVTR (g/day m^2) and WVP (g mm/kPa day m^2) were calculated according to the following Eqs. (8) and (9):

$$WVTR = \Delta W/\Delta t \times A \quad (8)$$

$$WVP = WVTR \times L/\Delta P \quad (9)$$

where $\Delta W/\Delta t$ is the weight gain of the bottles as a function of time (g/day), A is the area of the exposed film surface (m^2), L is the mean film thickness (mm) and ΔP is the difference of vapor pressure across the film (kPa).

2.10. Antioxidant properties of the films

2.10.1. Trolox-equivalent-antioxidant-capacity (TEAC) assay

The antioxidant capacity was determined by ABTS [2,2′-azino-bis (3-ethylbenzothiazo-line6-sulfonic acid)] assay with slight modifications (Gavril et al., 2019; Martini, Conte, & Tagliazucchi, 2018). Trolox (6-hydroxy.2,5,6,7-tetramethylchroman-2-carboxyl acid) was used as standard. The ABTS radical cation (ABTS+•) was generated by mixing a total of 7 mM aqueous solution of ABTS with 2.45 mM potassium persulfate allowing the mixture to react in the dark for 16 h. The ABTS+• solution was diluted in methanol to obtain an absorbance value (A_0) of 0.705 ± 0.005 at 734 nm. A total of 5 mg film sample was mixed with 2800 µL of ABTS+• solution. The samples were shaken in darkness at 20°C for 15 min and the absorbance at 734 nm was recorded for each sample. The final absorbance at 734 nm (A_f) of each sample was recorded. The percentage of scavenging (S%) was calculated for each sample according to Eq. (10):

$$S\% = ((A_0 - A_f)/A_0) \times 100 \quad (10)$$

where A_0 is the absorbance of the control and A_f the absorbance of the sample. The TEAC of films was determined by comparing the corresponding scavenging percentage to a calibration curve obtained with Trolox 50–500 µmol/L. The results were expressed as µmol Trolox Equivalent/g dried film (µmol TE/g). All tests were performed in triplicates.

2.10.2. Total phenolic compounds (TPC) assay

TPC of NLE and SLE were determined using the Folin-Ciocalteu assay with slight modifications (Singleton, Orthofer, & Lamuela Raventós, 1999). The samples were prepared by mixing separately 50 µL of each sample with 3950 µL of distilled water and 250 µL of Folin-Ciocalteau reagent. The mixture was stirred and left in the dark for 1 min. A total of 750 µL of 20% (w/w) Na_2CO_3 solution was then added to the sample. The mixture was incubated in the dark for 2 h at room temperature.

The absorbance at 765 nm of the mixtures was measured. Gallic acid was used as a phenolic standard to create a calibration curve (concentration range of 0–500 mg/L). The TPC of the herbal extract was expressed as mg of gallic acid equivalents/g extract (mg GAE/g).

A modification of the protocol described above was used to directly determine TPC in the control and active films. Briefly, 20 mg of the dried film was mixed with 4000 μL of distilled water and shaken for 5 min. A total of 250 μL of Folin - Ciocalteau reagent was added, and the mixture was left in the dark for 1 min after stirring. A total of 750 μL of 20% (w/w) Na_2CO_3 solution was then added, followed by incubation for 2 h. The liquid mixture was separated from the film sample through centrifugation and the absorbance at 765 nm was measured. Results were expressed as mg GAE/g dried film. The experimental results obtained from the films (TPC_E) were compared with the predicted values (TPC_P), derived from the TPC of the leaf extracts to estimate the release of phenolic compounds from films in the water medium. All tests were performed in triplicate. This method aimed to avoid a further extraction of the phenolic compounds from the dried polymer matrix through soaking and to avoid overestimating the actual phenolics release from the films (Licciardello, Wittenauer, Saengerlaub, Reinelt, & Stramm, 2015).

2.11. Statistical analysis

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's multiple range test ($p < 0.05$) using SPSS statistical program (SPSS 20 for Windows, SPSS INC., IBM, New York). The results were expressed as mean ± standard deviation (SD). A correlation test was applied between TPC and TEAC results and the derived regression model was then computed.

3. Results and Discussion

3.1. Surface and cross-section morphology

The morphological structure of the film depends on the interactions between the film components, which affect its final physical, optical, mechanical, and barrier properties (Bigi, Haghighi, De Leo, Ulrici, & Pulvirenti, 2021a).

The surface and cross-section morphology of control films based on CS/HPMC blend and those enriched with SLE and NLE (7.5 and 15% w/w of biopolymer) are presented in Fig. 11 and Fig. 12. The surface of the control film was smooth and did not show pores, bubbles, and cracks (Fig. 11a) indicating the formation of an ordered matrix and the high compatibility between CS and HPMC to form blend (Song, Feng et al., 2020). Addition of SLE (7.5 and 15% w/w) did not affect the surface morphology of the films (Fig. 11b and c). This confirmed that SLE was well-distributed into the polymer matrix (Bajić et al., 2019; Gao, He, Sun, He, & Zeng, 2019; Sun et al., 2017). A similar result was observed in a film containing 7.5% (w/w) NLE (Fig. 11d). A less compact surface with small particles was observed for the films containing 15% (w/w) NLE (Fig. 11e). This might be related to the partial release of low molecular weight compounds such as phenolic acids on the film surface. A similar result was reported in CS film incorporated with different amounts of grape seed extract (Rubilar et al., 2013). The differences observed between films enriched with SLE and NLE may be attributed to the different compositional profiles which characterize the two plant extracts (Zeković, Cvetanović, et al., 2017a; Zeković, Pintać, et al., 2017b). Nevertheless, the combined analysis of surface and cross-section allowed us to confirm that this process only affected the surface of the polymers and did not cause holes and structural disruptions of the biopolymer.

The cross-section of control film (Fig. 12a) showed an homogenous microstructure without irregularities and phase separation indicating excellent structural integrity and compatibility between CS and HPMC. The cross-section of all active films showed also continuous and compact cross-section morphology suggesting a good level of miscibility between the polymer matrix and the plant extracts (Fig. 12b–e). The increased number of biopolymer-polyphenol interactions might contribute to the formation of compact structures in active films containing leaf extracts (Wu et al., 2013).

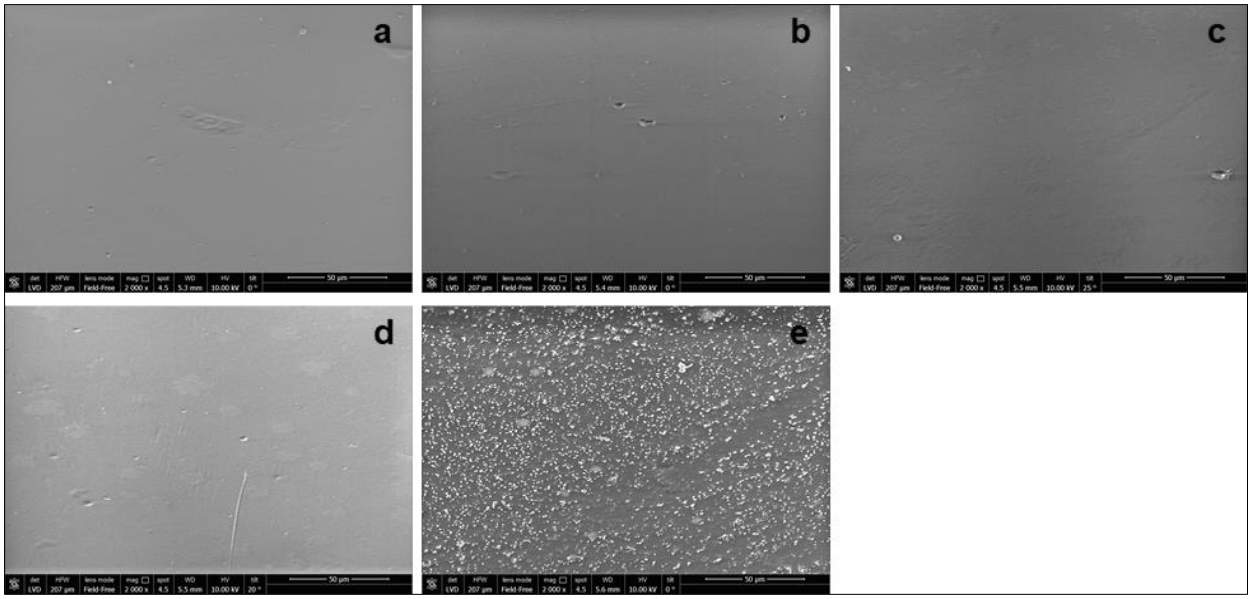


Fig. 11. SEM images (surface) of films based on: a) chitosan/hydroxypropyl methylcellulose blend (CS/HPMC) as control, b) CS/HPMC/SLE7.5% (w/w), c) CS/HPMC/SLE15% (w/w), d) CS/HPMC/NLE7.5% (w/w), e) CS/HPMC/NLE15% (w/w).

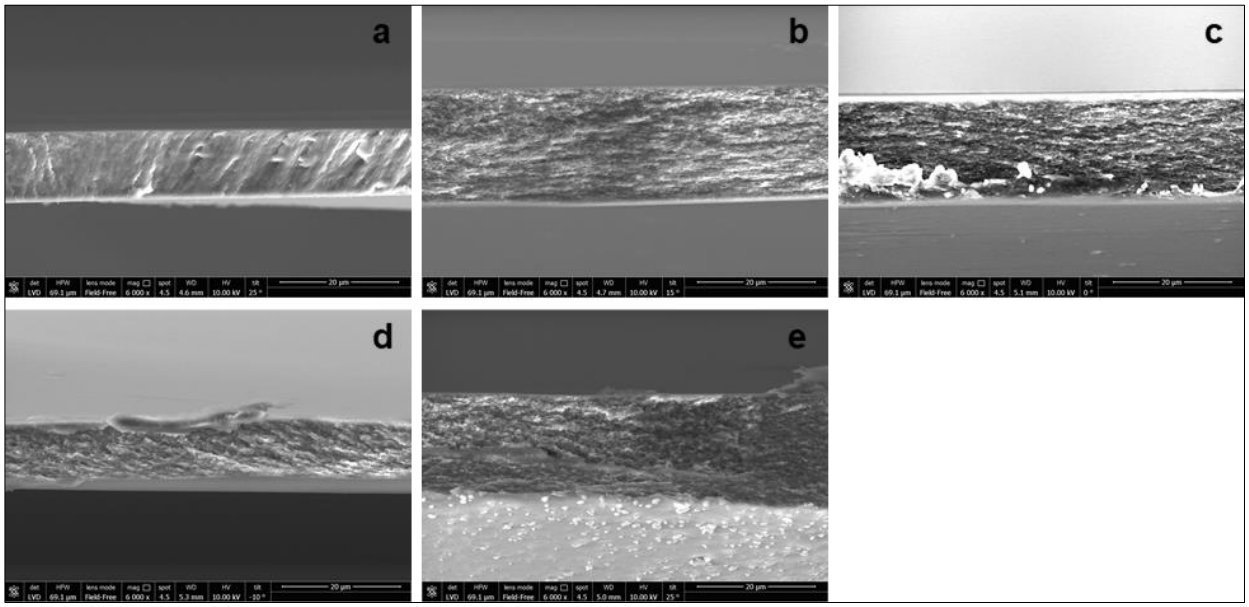


Fig. 12. SEM images (cross-section) of films based on: a) chitosan/hydroxypropyl methylcellulose blend (CS/HPMC) as control, b) CS/HPMC/SLE7.5% (w/w), c) CS/HPMC/SLE15% (w/w), d) CS/HPMC/NLE7.5% (w/w), e) CS/HPMC/NLE15% (w/w).

3.2. ATR/FT-IR spectroscopy

ATR/FT-IR spectroscopy was used as a powerful, non-destructive, and rapid technique with minimum sample preparation, because it provides information on the different chemical functionalities of CS, HPMC, and the incorporated SLE and NLE. Thus, valuable details on the structural changes and intermolecular interactions of the polymers and additives can be derived from the interpretation of the spectroscopic changes (Haghighi et al., 2019b). The IR spectrum of pure CS film (Fig. 13a) showed characteristic peaks at 1641 and 1555 cm^{-1} that can be assigned to the amide-I and amide-II bands, respectively. The peak at 1380 cm^{-1} can be assigned to the ν(C–O) stretching vibration of primary alcoholic groups (-CH_2OH) and to the symmetric bending vibration of residual CH_3 groups. Peaks from 896 to 1151 cm^{-1} can be assigned to saccharide structures (Silva-Weiss, Bifani, Ihl, Sobral, & Gómez-Guillén, 2013) which indicated that the glycoside bond of CS had a β-configuration (Guo et al., 2019). The band doublet at 2920/2873 cm^{-1} can be primarily assigned to overlapping antisymmetric/symmetric $\nu_{as}(CH_2)/\nu_s(CH_2)$ and ν(CH) stretching vibrations of the corresponding CS chain functionalities. The broad absorption band between 3600 and 3000 cm^{-1} is composed of ν(OH), ν(NH), and $\nu_{as}(NH_2)/\nu_s(NH_2)$ vibrational contributions (Ma, Qiao, Wang, Yao, & Xu, 2019).

The IR spectrum of pure HPMC film (Fig. 13b) showed a very strong absorption band at 1049 cm^{-1} associated with the ν_{as}(C–O–C) of pyranose ring skeletal vibrations (Akhtar et al., 2012). The peak at 945 cm^{-1} also represents vibrations from ether linkages and appears as a weaker signal superimposed on the low-wavenumber wing of the 1049 cm^{-1} band. These two peaks verify the presence of ether groups in the HPMC backbone (Akinosho, Hawkins, & Wicker, 2013). The peak at 3441 cm^{-1} corresponds to the ν(OH) stretching vibration of hydrogen bonded HPMC chains (da Silva et al., 2019). The band complex between 3000 and 2800 cm^{-1} can be assigned to $\nu(CH_3)/\nu(CH_2)/\nu$(CH) stretching vibrations of the methyl, propyl and CH groups of HPMC. The band at 1638 cm^{-1} can be assigned to bound water (Hay et al., 2018) and the deformation vibration of OH-functionalities. The asymmetric bending vibration of the methyl group in CH_3O appears at 1454 cm^{-1} alongside the $\delta(CH_2)$ deformation vibration.

The IR spectrum of the CS/HPMC blend film differed from the spectra of the individual CS and HPMC films by slight band shifts and intensity changes. Comparison of the spectra of the CS/HPMC blend film (Fig. 13c) and the pure CS film (Fig. 13a) showed that the amide-I and amide-II peaks of pure CS shifted from 1641 to 1647 cm^{-1} and from 1555 to 1559 cm^{-1}, respectively, associated with hydrogen-bonding changes of the C=O and NH-functionalities. Similarly, slight shifts of the $\nu(OH)/\nu(NH)/\nu(NH_2)$ stretching vibrations of CS support changes of intermolecular hydrogen bonds

between CS and HPMC. In conclusion, the spectroscopic data showed that CS and HPMC were miscible and compatible (Liang et al., 2020).

Active films containing SLE (15% w/w) also showed some slight shifts of the amide-I/amide-II bands. The other major bands of the control CS/HPMC blend film have not been influenced by the incorporation of SLE (Fig. 13d). Similar results were reported by Sun et al. (2017) in CS films enriched with thinned young apple polyphenols.

Active films containing NLE showed an extra characteristic band at 1734 cm^{-1} (Fig. 13e) that can be assigned to an ester structure of the NLE (Wu et al., 2013). Slight band shifts have been observed in the ν(OH), ν(NH), as well as amide-I/amide II band areas. The different spectroscopic behaviour of CS/HPMC blend films enriched with SLE and NLE, respectively, could be related to the specific compositions of the two extracts. NLE was partially released on the film surface as described by microstructural analysis and this could explain the new band at 1734 cm^{-1} in the IR profile of CS/HPMC film containing NLE compared to SLE. Overall, the FT-IR results confirmed, that changes in hydrogen bond formation between the hydroxyl and amino groups of the CS/HPMC film matrix and the hydroxyl groups of the polyphenolic compounds of SLE and NLE take place.

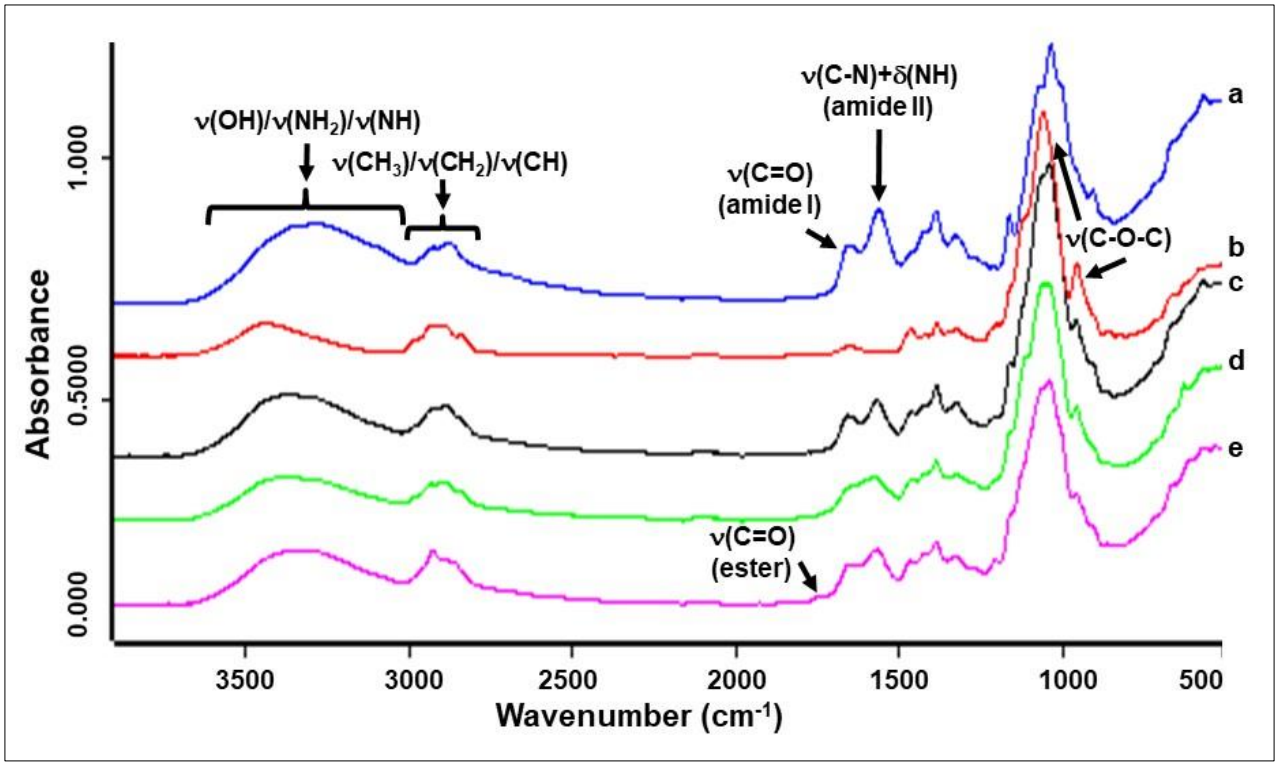


Fig. 13. ATR/FT-IR spectra of films based on: a) chitosan (CS), b) hydroxypropyl methylcellulose (HPMC), c) chitosan/hydroxypropyl methylcellulose (CS/HPMC), d) CS/HPMC/SLE15% (w/w), e) CS/HPMC/NLE15% (w/w).

3.3. Thickness and mechanical properties

The thickness and mechanical properties of control and active films are presented in Table 8. In this study, thickness ranged from 22.5 to 30 µm. The thickness of the control film was 26.1 µm. Addition of SLE (7.5 and 15% w/w) reduced the thickness to 22.5 and 22.8 µm, respectively ($p < 0.05$). A similar result was reported for CS films enriched with sage essential oil and thyme ethanolic extract (Souza et al., 2017; Talón et al., 2017b). Both sage and thyme belong to the family of the *Lamiaceae* (*Labiatae*). The aerial parts of these species are known to contain a range of secondary metabolites including flavonoids and terpenoids (Roby, Sarhan, Selim & Khalel, 2013). Rosmarinic acid, carnosic acid, ferulic acid, apigenin, methyl carnosate, rosmadial, and rosmanol were highlighted as the predominant phenolic and diterpenoid compounds (Cuvelier, Berset, & Richard, 1994; Zeković, Pintać, et al., 2017b). It is possible to assume that the presence of specific classes of phenolic and terpenoid compounds in the polymer matrix with lipophilic nature and low solubility in water could lead to a decrease of water retained in the polymer matrix, inducing the development of a dense network and hence reduction in thickness. These interactions may also be partially due to the opposite charge of CS and polyphenols in acidic media (Gibis, Ruedt, & Weiss, 2016).

In contrast, addition of NLE (7.5 and 15% w/w) increased the thickness to 28.1 and 30 µm, respectively ($p < 0.05$). These results are in agreement with the film surface morphology observed by SEM analysis. Several authors also reported an increase in thickness upon addition of polyphenolic extracts or essential oils into the FFS mainly due to the restructuring of the intermolecular polymer network (Gao, He et al., 2019; Khezrian & Shahbazi, 2018; Lee et al., 2019). The different behaviour characterizing the films enriched with NLE compared to those with SLE could be due to the different chemical compositions of the two extracts. The phenolic profile of NLE is mainly dominated by two classes of polyphenols: phenolic acids including chlorogenic acid, 2-O-caffeoylmalic acid, p-hydroxybenzoic acid, and sinapic acid which can account for 75% of total phenolic content; flavonoids, such as rutin, quercetin, isorhamnetin, and kaempferol (Pinelli et al., 2008; Zeković, Cvetanović, et al., 2017a). Many of these molecules were also detected by Sun et al. (2017) in thinned young apple polyphenolic extract. Authors concluded that the presence of apple polyphenols induced a remarkable increase of film thickness.

Adequate mechanical strength and extensibility are strongly required to withstand external stress that occurs during processing, shipping, handling, and storage of the packaged materials and to maintain the integrity and properties of the packaged foods (Ahmed, Mulla, Arfat, & Thai T, 2017; Jridi et al., 2014). Tensile strength (TS), elongation at break (E%), and Young's modulus (YM) represent the main parameters allowing to define the strength and flexibility of films (Souza et al., 2017). The TS

measures the film's strength. The control film showed the highest TS value (22.8 MPa). The films incorporated with NLE at 7.5 and 15% (w/w) showed a slight decrease of TS (20.1 and 20.4 MPa, respectively) ($p<0.05$), as previously described by Haghju et al. (2016). The incorporation of SLE at the same concentrations led to a sharper decline in the mechanical resistance of the films (16.4 and 16.8 MPa). A similar result was reported by Talón et al. (2017a) for chitosan-starch films enriched with thyme polyphenols at different concentrations. Specifically, the presence of thyme polyphenols decreased the TS from 9.5 to 8.2 MPa. This decline could be mainly due to the disruption of the crystalline order of the polymer matrix induced by the polyphenols, thus weakening the intermolecular hydrogen bonding, hindering the polymer-polymer interactions, and providing flexible domains within the films (Sun et al., 2017). Nevertheless, the TS of packaging films should be higher than 3.5 MPa, according to the conventional standards (Hosseini, Rezaei, Zandi, & Farahmandghavi, 2015). In our study, the TS values of CS/HPMC films with or without NLE or SLE ranged from 16.4 to 22.8 MPa, being comparable with those of high-density or low-density polyethylene films (Rhim, Wang, & Hong, 2013b). E% represents the flexibility and stretchability of the films by calculating the maximum change in length of a film before its breakage compared to the initial length (Jantrawut et al., 2017). The E% values of the control and active films ranged from 9.4 to 21.5%. The addition of SLE at different concentrations significantly reduced the E% of the films ($p < 0.05$) while films incorporated with NLE showed significantly higher E% values than control films ($p < 0.05$). The addition of natural polyphenols to the FFS could decrease the film matrix density, weakening the intermolecular forces between adjacent macromolecules, increasing the free volume, and facilitating movements of polymer chains under stress, thus enhancing their flexibility (Akhtar et al., 2013; Bonilla & Sobral, 2016). In our case, the different behaviour described for films enriched with SLE and NLE could be due to their different chemical composition, such as the presence of fatty acids and terpenoids, which could diversely affect the plasticizing effect of the extracts inside the polymer matrix (Oudjedi et al., 2019). In contrast with our results, Souza et al. (2017) reported that the incorporation of natural hydroalcoholic extracts to CS-based FFS did not significantly affect the mechanical properties and particularly the E% of the resulting films ($p > 0.05$). The YM defines the resistance of the film to elastic deformation. In other words, this parameter measures the intrinsic stiffness of the film. A low YM generally is an indication of a more flexible material (Jantrawut et al., 2017). The addition of NLE at 7.5% w/w polymer did not significantly affect the YM of the CS/HPMC film ($p > 0.05$), while an increase in concentration up to 15% w/w induced a consistent reduction in the stiffness of the films (from 738.8 to 667.4 MPa), indicating that films became more flexible and less rigid. On the contrary, the incorporation of SLE seemed to enhance the rigidity of films, showing the highest YM values in this research (783.7 and 828.9 MPa).

These results confirmed the trends observed in the E% values of the films. Overall, it seems that NLE acted as an effective plasticizer, reducing the film strength and enhancing its extensibility. On the contrary, the incorporation of SLE into the FFS resulted in a reduction of the mechanical performances of the films.

Table 8.
Thickness, tensile strength (TS), elasticity (E%), and Young's modulus (YM) of films based on chitosan/hydroxypropyl methylcellulose blend (CS/HPMC) as control and active films enriched with sage leaf extract (SLE) or nettle leaf extract (NLE), 7.5% and 15% w/w of biopolymer.

Film Sample	Thickness (μm)	TS (MPa)	E (%)	YM (MPa)
CS/HPMC	26.1 ± 2.2^{b}	22.8 ± 2.0^{c}	16.5 ± 1.6^{b}	738.8 ± 77.6^{b}
CS/HPMC/SLE7.5% (w/w)	22.5 ± 1.7^{a}	16.4 ± 1.5^{a}	10.2 ± 1.1^{a}	783.7 ± 74.2^{bc}
CS/HPMC/SLE 15% (w/w)	22.8 ± 2.1^{a}	16.8 ± 1.7^{a}	9.4 ± 1.5^{a}	828.9 ± 67.1^{c}
CS/HPMC/NLE7.5% (w/w)	28.1 ± 2.0^{c}	20.1 ± 2.0^{b}	20.1 ± 1.8^{c}	739.1 ± 99.2^{b}
CS/HPMC/NLE15% (w/w)	30.0 ± 2.8^{c}	20.4 ± 2.0^{b}	21.5 ± 2.9^{c}	667.4 ± 64.4^{a}

Values are given as mean ± SD (n = 3).
Different letters in the same column indicate significant differences ($p<0.05$).

3.4. UV barrier, light transmittance, and opacity value

The UV–Vis light barrier represents an important parameter for the development of food packaging materials since it can prevent photo-oxidation, degradation of vitamins and other pigments, nutrient loss, discolouration, and also minimize the various off-flavours and undesirable compounds from lipids oxidation, thereby extending food shelf-life (Di Filippo et al., 2021). UV–Vis light transmittance at specific wavelengths (200–800 nm) and opacity values of control and active films based are presented in Table 9. All films showed excellent barrier property to UV-C at 200 nm (transmittance < 0.1). Active films also showed lower transmittance at UV-B and UV-A wavelengths (280 and 350 nm, respectively). In particular, active films containing SLE and NLE (15% w/w of biopolymer) behaved as effective UV barriers, since the transmittance value was below 10% at UV-B and UV-A (280 and 350 nm, respectively). This result may be explained by the presence of phenolic compounds in active films which are rich in unsaturated bonds responsible for the absorption of UV radiation (Bitencourt, Fávaro-Trindade, Sobral, & Carvalho, 2014; Kanmani & Rhim, 2014).

The transmittance of visible wavelengths (400–800 nm) was higher than 80% in control film indicating that CS/HPMC films were very clear and transparent. The incorporation of leaf extracts into the film matrix slightly reduced the transparency of the film. Therefore, the transparency of the active films did not change even though the UV barrier property significantly increased. Similar results were reported by Wang et al. (2013) and Kanmani & Rhim (2014). The opacity value ranged from 1.4 to 4.6. The control film showed the lowest value suggesting that this film was very

transparent. The opacity value was increased with the addition of SLE and NLE and their concentration ($p<0.05$). Despite the increasing opacity value by addition of plant leaf extract, all films showed opacity values lower or strictly close to the values measured for LDPE films, a commercial plastic intended for packaging application (Hosseini, Ghaderi, & Gòmez-Guillèn, 2021). Therefore, the active films developed in this study could provide a clear view of the food content and its condition which is an important parameter for consumer acceptance. Similar results have been reported for CS films incorporated with different amount of tea extracts (Peng et al., 2013; Wang et al., 2013).

Table 9.
UV-Vis light transmittance (%) and opacity of the films based on chitosan/hydroxypropyl methylcellulose blend (CS/HPMC) as control and active films enriched with sage leaf extract (SLE) or nettle leaf extract (NLE), 7.5% and 15% w/w of biopolymer.

Film sample	Light transmittance (%) at different wavelengths (nm)								Opacity (600 nm)
	200	280	350	400	500	600	700	800	
CS/HPMC	1.1	56.6	72.4	80.3	86.4	89.0	90.3	90.9	1.7 ± 0.2^{a}
CS/HPMC/SLE7.5%	<0.1	29.9	37.7	68.8	85.8	88.5	89.7	90.3	3.7 ± 0.3^{c}
CS/HPMC/SLE15%	<0.1	4.3	7.7	45.9	80.7	85.5	87.9	89.1	4.5 ± 0.5^{d}
CS/HPMC/NLE7.5%	<0.1	11.8	11.8	37.1	79.2	84.3	86.9	88.6	2.6 ± 0.2^{b}
CS/HPMC/NLE15%	<0.1	6.7	7.0	25.5	79.3	84.9	87.6	89.4	3.6 ± 0.3^{c}

Values are given as mean ± SD (n = 3).
Different letters in the same column indicate significant differences ($p<0.05$).

3.5. Colour

The colour parameters (L*, a*, and b*) and total colour variation (ΔE*) of control and active films are reported in Table 10. The L* value, measuring the lightness of the films, varied from 98.6 to 93.4 indicating high brightness levels for all the films, even if the incorporation of both leaf extract significantly reduced the L* values ($p < 0.05$) (Haghju et al., 2016). Films containing SLE were lighter than films containing NLE. Increasing concentration of leaf extract from 7.5 to 15% (w/w) did not influence the L* value ($p > 0.05$). Similar findings were reported by Moradi et al. (2012) and Siripatrawan & Harte (2010).

The a* parameter, expressing the greenness - redness component of the films, was negative for all films indicating that the leaf extracts caused a green shade which increased with increasing leaf extracts concentration ($p < 0.05$). The b* parameter, which measures the blueness - yellowness component, increased by the addition of plant extracts ($p < 0.05$). Positive b* values indicate a stronger yellow character of active films with respect to control (da Silva et al., 2019). Increasing leaf extract concentration led to increasing b* value ($p < 0.05$) (Bonilla & Sobral, 2016; Souza et al., 2017). Addition of leaf extract significantly increased the total colour variation (ΔE*), especially at

the highest leaf extract concentration ($p < 0.05$). Higher ΔE* values suggest that colour changes in active films could be easily detected by the naked eye (Wang et al., 2013). Overall, addition of leaf extracts caused films to become slightly darker and gain more green and yellow colour with respect to control film (Fig. 14). This effect could be attributed to the original colours of NLE and SLE (Peng et al., 2013; Sun et al., 2017).

Table 10.
Colour parameters (L*, a*, and b*) and total colour variation (ΔE*) of films based on chitosan/hydroxypropyl methylcellulose blend (CS/HPMC) as control and active films enriched with sage leaf extract (SLE) or nettle leaf extract (NLE) 7.5% and 15% w/w of biopolymer.

Film sample	Colour parameters			
	L*	**a***	**b***	**ΔE***
CS/HPMC	98.6 ± 0.1^{c}	-0.6 ± 0.03^{d}	2.4 ± 0.1^{a}	2.6 ± 0.1^{a}
CS/HPMC/SLE7.5%	96.5 ± 0.2^{b}	-1.9 ± 0.1^{c}	7.0 ± 0.6^{b}	7.6 ± 0.6^{b}
CS/HPMC/SLE15%	96.9 ± 0.3^{b}	-4.0 ± 0.2^{b}	17.2 ± 1.1^{c}	18.3 ± 1.1^{c}
CS/HPMC/NLE7.5%	93.9 ± 0.4^{a}	-4.6 ± 0.2^{b}	18.6 ± 0.8^{c}	20.0 ± 0.9^{c}
CS/HPMC/NLE15%	93.4 ± 0.4^{a}	-5.6 ± 0.4^{a}	22.7 ± 1.0^{d}	22.2 ± 1.0^{d}

Values are given as mean ± SD (n = 3).
Different letters in the same column indicate significant differences ($p<0.05$).

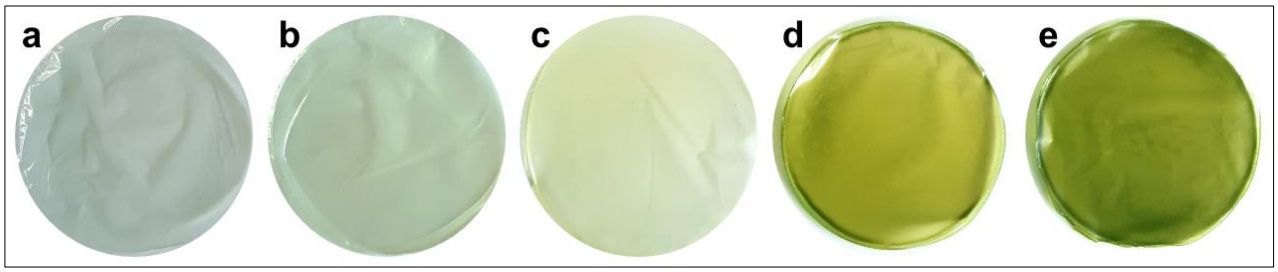


Fig. 14. Visual appearance of films based on: a) chitosan/hydroxypropyl methylcellulose blend (CS/HPMC) as control, b) CS/HPMC/SLE7.5% (w/w), c) CS/HPMC/SLE15% (w/w), d) CS/HPMC/NLE7.5% (w/w), e) CS/HPMC/NLE15% (w/w).

3.6. Water solubility and water vapor permeability

The water solubility (WS) and water vapor permeability (WVP) of control and active films are reported in Table 11. The WS represents an important property of bio-based films for food packaging applications since it reflects the water resistance of the film (Nisar et al., 2018). High WS may represent a limitation for the application to food products with high water content when integrity and high water resistance are required (Ghasemlou et al., 2013; Souza et al., 2017). Moreover, the WS is linked with the biodegradability of the film. Furthermore, the modulation of this property allows to effectively adjust the release of antimicrobial or antioxidant substances from the film to the food surface (Abdollahi et al., 2012). The WS of the control film was 60.4%. This result was in accordance with those observed in previous researches for CS/HPMC films (Liang et al., 2020; Möller et al.,

2004; Rotta et al., 2009). Authors concluded that the enhanced WS of the films depended on the high hydrophilicity of HPMC mainly due to its hydroxyl groups. Thus, the solubility of CS/HPMC films can be actively controlled by changing the proportion of each polymer used in their preparation enabling a wide range of industrial applications (Rotta et al., 2009). The addition of SLE (7.5 and 15% w/w) did not affect the WS of the films ($p > 0.05$), while addition of NLE (7.5 and 15% w/w) significantly increased the WS ($p < 0.05$). Similar results were observed by Wang et al. (2013) for CS films enriched with tea polyphenols. This might be due to the different contents in both hydrophilic molecules such as polyphenols and hydrophobic molecules such as diterpenoids, which are characteristic of these leaf extracts. Indeed, the hydrophilic groups of polyphenols which are free from binding with the polymer matrix can easily interact with water molecules, thereby improving its solubility (Sun et al., 2017).

The shelf life of food products depends, among other factors, on the transfer of water between the product and the surrounding environment. Therefore, the determination of water barrier property is of key importance in food packaging (Kanatt, Rao, Chawla, & Sharma, 2012). Generally, the capacity to reduce the water transfer represents a desirable property for a food packaging material, since it allows preserving food from chemical and microbiological degradation induced by moisture (Hosseini et al., 2016). The WVP value of control film was 6.3 (g mm/kPa day m^2) (Wang et al., 2013). The presence of SLE at different concentrations did not significantly affect the water permeability of the films ($p > 0.05$). A similar result was reported for chitosan/gelatin blend films enriched with boldo, cinnamon, guarana, and rosemary mixed plant extracts (Bonilla & Sobral, 2016). The authors highlighted that the presence of extracts and the proportion of CS into the FFS neither improved nor impaired the WVP of the films. The same behaviour was also observed in gelatin-based films enriched with curcuma ethanolic extract (Bitencourt et al., 2014). Contrary to SLE, addition of NLE increased the WVP ($p < 0.05$). However, there were no significant differences between different amounts of leaf extracts (7.5 and 15% w/w). In contrast to this result, the WVP of films based on chitosan-gelatin blend incorporated with Chinese hawthorn fruit extract first decreased and then increased by increasing extract concentration (Kan et al., 2019). Authors concluded that the extract could disperse well in the film matrix at low levels, thereby inhibiting water transfer while at higher extract concentration, the microstructure of films was disrupted. Several factors could affect the high permeability of films including molecular weight and deacetylation degree of chitosan, microstructure, the plasticizer concentration, hydrophilic nature of biopolymers, film thickness, intermolecular interaction between film components, and incorporated leaf extracts (Wang et al., 2013). Overall, the WVP values obtained in this study were high compared to those of high barrier synthetic polymers such as polyvinyl chloride (0.11 g mm/kPa day m^2), low-density polyethylene

(0.08 g mm/kPa day m^2), and high-density polyethylene (0.02 g mm/kPa day m^2) (Ghasemlou et al., 2013).

Table 11.
Water solubility (WS) and water vapor permeability (WVP) of the films based on chitosan/hydroxypropyl methylcellulose blend (CS/HPMC) as control and active films enriched with sage leaf extract (SLE) or nettle leaf extract (NLE), 7.5% and 15% w/w of biopolymer.

Film sample	WS (%)	WVP 90:0% RH (g mm/kPa day m^2)
CS/HPMC	60.4 ± 0.9[a]	6.5 ± 0.8[a]
CS/HPMC/SLE7.5%	61.0 ± 0.7[a]	6.4 ± 0.7[a]
CS/HPMC/SLE15%	60.7 ± 0.7[a]	6.8 ± 0.7[a]
CS/HPMC/NLE7.5%	63.4 ± 0.5[b]	9.8 ± 0.7[b]
CS/HPMC/NLE15%	63.7 ± 0.7[b]	9.9 ± 0.9[b]

Values are given as mean ± SD (n = 3).
Different letters in the same column indicate significant differences ($p<0.05$).

3.7. Antioxidant activity

The antioxidant activity of control and active films was determined by the Trolox Equivalent Antioxidant Capacity (TEAC) assay (Table 12). The control film exhibited a slight antioxidant activity (2.03 μmol TE/g film), probably due to the ability of CS to form stable macromolecular radicals through the reaction between residual amino groups ($-NH_2$) with free radicals (Siripatrawan & Harte, 2010). The incorporation of SLE and NLE led to a significant increase of film antioxidant activity ($p < 0.05$), more noticeable for SLE loaded films. The highest TEAC value was observed for CS/HPMC/SLE15% (w/w) with 28.35 μmol TE/ g film, followed by CS/HPMC/NLE15% (w/w) with 17.08 μmol TE/g film. Films enriched with SLE showed higher antioxidant capacity than films containing NLE at the same concentrations ($p < 0.05$). This may be related to the higher content in polyphenols in the SLE with respect to NLE (as it can be inferred from the predicted total polyphenol content presented in Table 12). Furthermore, a strong correlation between the extract concentration into the FFS and the antioxidant activity of the dried films was observed for both NLE and SLE (Fig. 15). A similar relation was reported for pectin/chitosan films enriched with tea polyphenols (Gao, He al., 2019; Giménez, López de Lacey, Pérez-Santín, López-Caballero, & Montero, 2013).

The bioactive properties of SLE and NLE can be attributed to the high content of phenolic compounds such as phenolic acids and flavonoids. Presence of carnosic acid, carnosol, rosmarinic acid, and lutèolèine-7-O-α-glucopyranoside, and many other phenolic compounds such as diterpenes, triterpenes, and flavonoids have been reported in SLE (Oudjedi et al., 2019). Similarly, presence of quercetin and ursolic acid has been reported in NLE (Bourgeois et al., 2016). The antioxidant function

of polyphenols is closely related to their chemical structure and their redox properties which can be explained by various possible mechanisms such as free-radical scavenging activity, hydrogen donor property, transition-metal-chelating activity, and/or singlet oxygen-quenching capacity. These mechanisms depend on the high reactivity of hydroxyl group substituents, concentration of the active compound, temperature, light, and presence of micro-components acting as pro-oxidants or synergists (Ruiz-Navajas et al., 2013).

Table 12.
Trolox-equivalent-antioxidant capacity (TEAC), experimental (measured) total phenolic content (TPC_E), and predicted total phenolic content (TPC_P) of films based on chitosan/hydroxypropyl methylcellulose blend (CS/HPMC) as control and active films enriched with sage leaf extract (SLE) or nettle leaf extract (NLE), 7.5% and 15% w/w of biopolymer.

Film sample	TEAC (μmol TE/g film)	TPC_E (mg GAE/ g film)	TPC_P (mg GAE/ g film)
CS/HPMC	2.03 ± 0.04^{a}	0.15 ± 0.01^{a}	--
CS/HPMC/SLE7.5%	14.48 ± 0.32^{c}	4.85 ± 0.23^{c}	5.09^{b}
CS/HPMC/SLE15%	28.35 ± 3.05^{e}	7.35 ± 0.48^{e}	9.91^{d}
CS/HPMC/NLE7.5%	6.63 ± 0.76^{b}	3.45 ± 0.08^{b}	3.69^{a}
CS/HPMC/NLE15%	17.08 ± 1.46^{d}	6.22 ± 0.55^{d}	7.13^{c}

Values are given as mean ± SD (n = 3).
Different lowercase letters in the same column indicate significant differences ($p<0.05$).

3.8. Total phenolic content (TPC)

The total phenolic content (TPC_E) of control and active films are reported in Table 12. Control film showed a TPC of 0.15 mg GAE/g film. This finding might be attributed to the formation of chromogens due to the reaction of the Folin - Ciocalteu reagent with non-phenolic reducing groups in the biopolymer backbone that can be detected by spectrophotometry (Genskowsky et al., 2015; Moradi et al., 2012). The incorporation of leaf extracts increased the TPC_E ($p < 0.01$) ranging from 4.85 to 7.35 mg GAE/g for films containing SLE and 3.45–6.22 mg GAE/g film for films containing NLE ($p < 0.05$). A direct relation between the concentration of the extract into the FFS and the TPC_E of the films was also detected as already observed for antioxidant activity (Ruiz-Navajas et al., 2013). The films enriched with SLE showed higher TPC_E values than the ones with NLE at similar concentrations ($p<0.05$).

The experimental results obtained from the films (TPCE) slightly differed from the predicted values (TPC_P). This discrepancy may depend on the fact that the applied method can detect only a portion of the actual phenolic compounds, which are strictly retained into the polymer matrix and not able to react with the chromogenic reagents. The minor differences observed between TPC_E and TPC_P confirmed that a small part of the bioactive compounds has been lost through oxidation during the

preparation of the films. However, the high repeatability and reproducibility of the results support the scientific reliability of this novel approach.

As already described, a close correlation between the content of polyphenols and the antioxidant activity of the films should be expected (Wang et al., 2013). Thus, a regression model was computed to infer the actual contribution of the SLE and NLE on the antioxidant features of the films (Fig. 15). A positive and significant correlation was observed between the TPC (mg GAE/g film) and the TEAC (µmol TE/g film) values, whose relation was described by an exponential regression model ($y = 2.0241e^{0.3597x}$; $R^2 = 0.9785$). In contrast to this result, a linear correlation between TPC and TEAC was reported by (Jouki et al., 2014; Ruiz-Navajas et al., 2013). Nevertheless, the high R^2 value suggests a high accuracy of the present regression model.

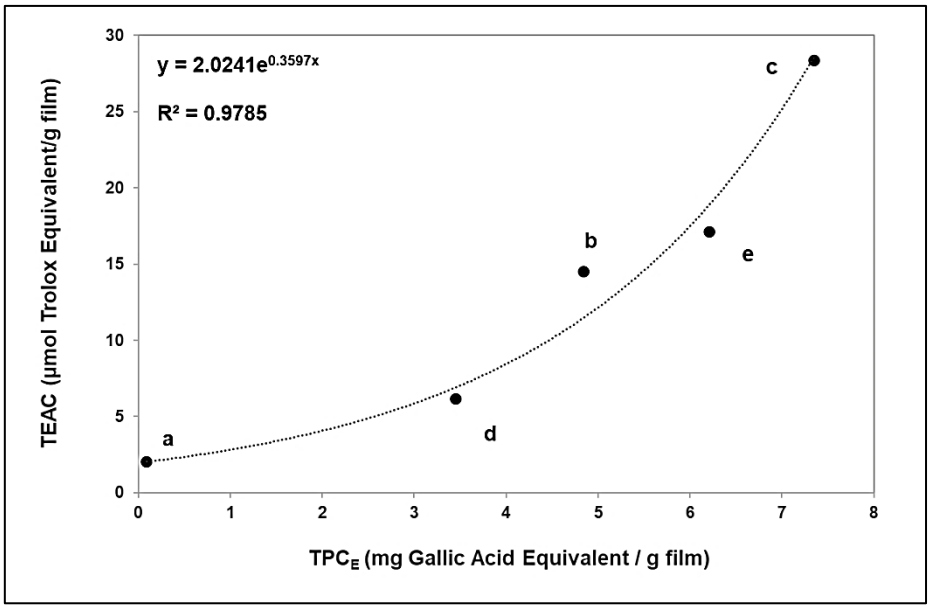


Fig. 15. Correlation analysis between experimental total phenolic content (TPC_E) and Trolox-equivalent-antioxidant capacity (TEAC) and relative exponential regression model of films based on: a) chitosan/hydroxypropyl methylcellulose (CS/HPMC) as control, b) CS/HPMC/SLE7.5% (w/w), c) CS/HPMC/SLE15% (w/w), d) CS/HPMC/NLE7.5% (w/w), e) CS/HPMC/NLE15% (w/w).

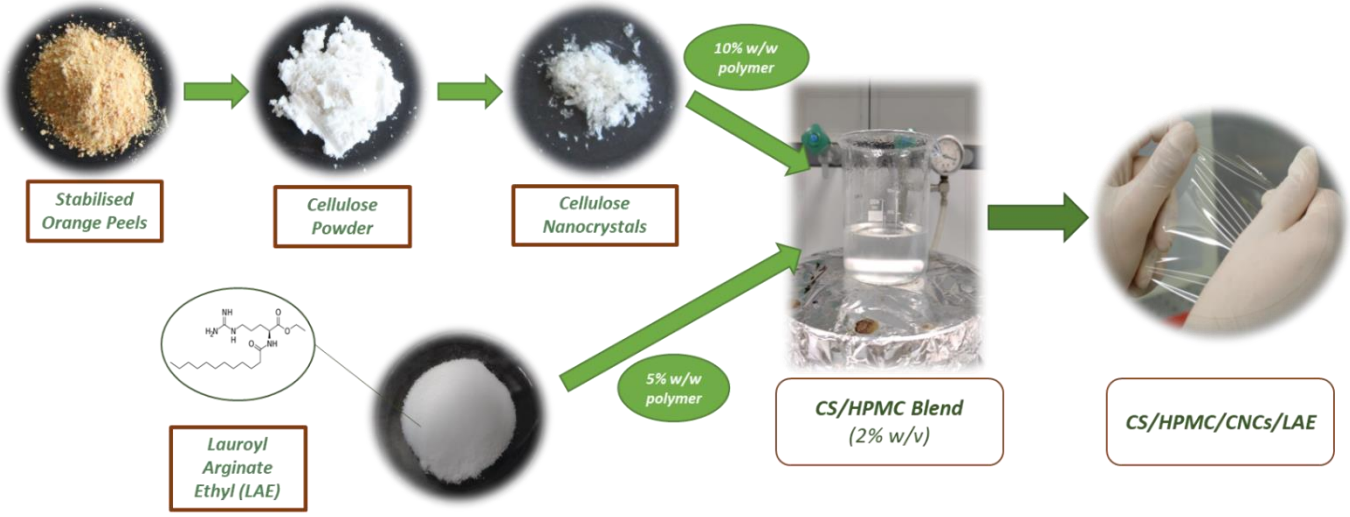
4. Conclusions

This study aimed to develop bio-based CS/HPMC blend films enriched with NLE and SLE at different concentrations and to investigate their functional properties for active food packaging applications. SEM analysis confirmed the high compatibility between CS and HPMC and incorporated leaf extracts. The ATR/FT-IR spectra confirmed intermolecular interactions between the hydroxyl and amino groups of the biopolymer network and the functional groups of the leaf extracts, related to their polyphenolic content. Incorporation of SLE and NLE improved the UV/Vis light barrier properties with opacity values lower than five. The antioxidant activity of the films was significantly enhanced by the addition of leaf extracts due to the high quantity of bioactive polyphenols. Addition of leaf extracts caused films to become slightly darker and to gain more green and yellow colour with reference to the control film. Addition of NLE caused an increase in water solubility and water vapor permeability while the addition of SLE had a negligible effect on these parameters. The obtained results suggest the possibility of employing these films as a green and environmentally friendly approach for partial substitution of synthetic plastics for packaging foods that are sensitive to oxidation and to increase their shelf life. The high WS and WVP restrict their potential application to low moisture products such as bakery products and spices. Nevertheless, this specific behaviour perfectly fits some specific industrial applications, such as the single-dose primary packaging of spices, herbal infusions, and nutraceutical powder supplements, which are extremely sensitive to oxidation. In these scenarios, mechanical resistance, UV barrier properties, antioxidant activity, and immediate water solubility are indispensable to satisfy the needs of industries and consumers.

Chapter 5

Nanocomposite active films based on chitosan/hydroxypropyl methylcellulose blend enriched with orange peel cellulose nanocrystals and lauroyl arginate ethyl as a novel food packaging solution

Graphical abstract



Keywords

Active food packaging; Cellulose nanocrystals; Chitosan/hydroxypropyl methylcellulose blend; Nano-reinforced materials; Orange peel waste; Circular economy.

Abstract

Cellulose nanocrystals (CNCs) were extracted from orange peels obtained as the main by-product of the orange juice industry using an alkaline/H_2O_2 bleaching pre-treatment followed by sulfuric acid hydrolysis. Extracted CNCs were added as reinforcing agents into chitosan/hydroxypropyl methylcellulose (CS/HPMC) blend films enriched with lauroyl arginate ethyl (LAE) to produce bio nanocomposite active films. The size and morphology of CNCs were characterized by transmission electron microscopy. In addition, the effect of CNCs (10% w/w of biopolymer) and LAE (5% w/w of biopolymer) on microstructural, optical, physical, mechanical, water barrier, and antimicrobial features of the CS/HPMC films were evaluated. CNCs displayed needle-like shapes with an average length of 500 nm and an average width of 40 nm, corresponding to an aspect ratio of 12.5. Scanning electron microscopy illustrated high compatibility between CS/HPMC with CNCs and/or LAE. Hydrogen bonds formation between the hydroxyl and amino groups of the CS/HPMC and the functional groups of CNCs and/or LAE was confirmed by Fourier-transform infrared spectroscopy. These nanocomposite active films exhibited improved UV-Vis light barrier performances ($p<0.05$). The reinforcement of CS/HPMC blend with CNCs induced a reduction in water solubility and water vapor permeability, and improvement in tensile strength. LAE incorporation improved the elasticity of the films and also enhanced the biocidal activity against four major foodborne bacterial pathogens including *E. coli*, *S. enterica*, *L. monocytogenes,* and *P. fluorescens.* Overall, this study showed that nanocomposite films based on CS/HPMC blend and enriched with LAE could represent a suitable green solution for a partial replacement of conventional materials to protect food from microbial exploitation and quality decay.

1. Introduction

Nowadays, the social awareness about the ecological impact of petroleum-based plastics is rapidly growing. Consumers are demanding safe, fresh, and healthy food products free of artificial additives and preservatives (Costa et al., 2021). In this context, researchers and industries are focusing their efforts on the development of sustainable active food packages, employing novel and greener preservation technologies (Mittal et al., 2021; Yildirim et al., 2018). This makes the food packaging sector an emergent trend segment with remarkable business potential (Panseri et al., 2018).

Lately, antimicrobial bio-based films have emerged as valuable solutions able to reduce the environmental impact of plastic disposal, and to prolong the food shelf life by inhibiting the growth of microorganisms (Singh, Gaikwad & Lee, 2018; Zhong et al., 2020). Among many biopolymers, hydroxypropyl methylcellulose (HPMC) and chitosan (CS) have received much attention, mainly due to their commercial availability, non-toxicity, biodegradability, and excellent film-forming features (Bigi, Haghighi, Siesler, Licciardello, & Pulvirenti, 2021b; Liang et al., 2020). HPMC represents a renewable, cheap, and semi-synthetic cellulose-derived ether, approved as human food additive by the US Food and Drug Administration (FDA, 21 CFR 172.874) and the European Parliament and Council (Directive No. 95 No 95/2/EC) (Di Filippo et al., 2021). This biopolymer has been applied for the preparation of packaging materials since it forms transparent, flexible, odorless, and oil-resistant films (Sánchez-González et al., 2009). Even so, HPMC films show remarkable sensitivity to moisture, limiting their industrial application in the food sector (Rotta et al., 2009). CS is a linear polysaccharide obtained through the *N*-deacetylation of chitin, the main structural constituent of insects and crustaceans exoskeleton. CS is soluble in low-pH solutions due to the protonation of -NH_2 functional groups in the polymer backbone (Bigi et al., 2021a). This biopolymer exhibits excellent film-forming properties, with an acceptable barrier to gases such as CO_2 and O_2 (Machado et al., 2020). However, CS films have high production costs and low mechanical properties.

HPMC and CS are highly compatible biopolymers (Rotta et al., 2009). Blending CS and HPMC could represent a feasible approach to improve the functional properties of the single components. This is mainly due to the formation of intermolecular hydrogen bonds between amino functional groups of CS and hydroxyl functional groups of HPMC (Liang et al., 2020; Song et al., 2020).

Enrichment of packaging materials with natural and/or synthetic active compounds represents a novel strategy aiming to guarantee the safety and to prolong the shelf-life of food (Pezo, Navascuès, Salafranca, & Nerin, 2012). This approach is commonly known as “active packaging”, and the current trend is to develop biobased films enriched with biodegradable compounds with a strong bioactivity,

in order to replace the wide use of traditional preservatives with more sustainable solutions. Among many food additives which were tested for this purpose, lauroyl arginate ethyl (LAE) has gained the attention of many researchers due to its wide range of activity against food pathogens and spoilage microorganisms (i.e., gram-positive and gram-negative bacteria, yeast, and molds), sustainability, and non-toxicity (Becerril, Manso, Nerin, & Gómez-Lus, 2013). LAE is a synthetic molecule composed of an ethyl-arginine head esterified with a lauroyl tail. (Rubilar, Candia, Cobos, Díaz, & Pedreschi, 2016), This molecule acts as a cationic surfactant, disrupting the cytoplasmic membrane of microorganisms and affecting their metabolic processes (Muriel-Galet, Lopez-Carballo, Hernández-Muñoz, & Gavara, 2016). LAE was approved as a food preservative by the European Food Safety Authority (EFSA, 2007) and by the U.S. FDA (FDA, 2005). LAE is easily degraded by the human body into L-arginine, lauric acid, and ethanol through chemical and metabolic pathways (Asker, Weiss, & Mc Clements, 2009). Several studies reported that LAE significantly improves the capacity of bio-based packaging to maintain the safety and quality of packed foods (De Leo et al., 2018; Haghighi et al., 2020; Moreno, Cárdenas, Atarés, & Chiralt, 2017a).

Along with the addition of active compounds, the polymer matrix can also be reinforced with nanomaterials to overcome its technical criticisms (Doh, Dunno, & Whiteside, 2020). The uniform dispersion of nanomaterials can contribute organizing the polymer matrix in a strong and dense nanoscale intermolecular network through hydrogen bonding linkages, resulting in an enhancement of the physical, optical, mechanical, and barrier performances of the packaging system (Coelho et al., 2020; El Achabi, Kassab, Aboulkas, Gaillard, & Bakard, 2018; Li, Shi, He, Fei, & Peng, 2020). Among nanomaterials, cellulose-derived nanostructures such as cellulose nanocrystals (CNCs) from natural resources have received considerable interest due to their renewability, biocompatibility, low cost, and improved mechanical, barrier, and optical properties (Dai, Ou, Huang, & Huang, 2018; Vilarinho et al., 2018). CNCs are highly crystalline particles with needle-shaped morphology. The structural characteristics of CNCs vary with the plant source and extraction method (Melikoğlu, Bilek & Cesur, 2019). CNCs are conventionally obtained by partially dissolving cellulose fibrils through sulfuric acid hydrolysis. In this method, sulfuric acid esterifies the surface hydroxyl groups of cellulose, whose amorphous regions are easily hydrolysed and removed. Meanwhile, the crystalline regions which are more resistant remain intact (Feng et al., 2015).

Shifting from a linear to a circular economy model in the agro-food sector is leading to evaluate agricultural by-products and waste as promising and sustainable cellulosic feedstocks to extract CNCs. The application of these by-products allows to improve the rural economies without jeopardizing food supplies. In this context, CNCs were extracted from different agricultural by-

products such as pineapple peel (Dai et al., 2018), grape pomace (Coehlo et al., 2018), tomato peel (Jiang & Hsieh, 2015), sugarcane bagasse (Kumar, Negi, Choudhari, & Bhardwaj, 2014), oil palm biomass (Haafiz et al., 2013), banana peel (Tibolla, Pellissari, & Menagalli, 2014), and orange peel (Naz et al., 2016). The processing of orange fruit to produce juice generates huge amounts of peels and pulp mainly discarded as waste (Rodsamran & Sothornvit, 2019a). Due to its high cellulose content, the valorization of this waste as a valuable source of CNCs deserves particular attention, representing a remarkable business opportunity (Naz et al., 2016).

To the best of our knowledge, literature considering CS/HPMC film reinforced with CNCs from orange peel is unavailable. Therefore, the first part of this study aimed to design an optimized protocol for the extraction of cellulose from orange peel, and its conversion to CNCs. The extracted CNCs were applied as reinforcing fillers to CS/HPMC films, which were enriched with LAE as an antimicrobial compound. Then, the effect of CNCs (10% w/w of biopolymer) and LAE (5% w/w of biopolymer) on microstructural, optical, physical, mechanical, water barrier, and antimicrobial properties of the CS/HPMC film were evaluated.

2. Materials and Methods

2.1. Materials and reagents

The orange peels were kindly provided by Macè s.r.l. (Terre del Reno, Ferrara, Italy). CS (molecular weight 100-300 kDa) was acquired from Acros Organics™ (China). HPMC (hydroxypropyl 5-8%, methoxy 28-30%) was supplied by ACEF SPA (Piacenza, Italy). Glycerol (≥ 99.5%) was obtained from Merck (Darmstadt, Germany). Ethanol, acetic acid, sulfuric acid, sodium hydroxide, hydrogen peroxide, sodium dodecyl sulfate, EDTA, and cetyltrimethylammonium bromide were provided by Sigma-Aldrich (St. Louis, USA). LAE (Minerat NSF®) was obtained from Vedeqsa (Terrassa, Barcelona, Spain). Brain heart infusion agar (BHIA), and brain heart infusion broth (BHIB) were purchased by Biolife (Milan, Italy).

2.2. Pre-treatment and chemical composition analysis of orange peels

The fresh orange peels were ground in a grinder (Philips HR2100/00, Amsterdam, Holland) and dried at 50ºC in an infrared oven (ZTM Mechatronic, Reggio Emilia, Italy) until their moisture content reached 10 ± 0.5%. The dried peels were then milled to obtain orange peel powder and stored at -

18°C. Moisture, protein, fat, and ash contents of orange peel powder were measured according to the international standard AOAC (2012). Van Soest (1963) protocols with slight modifications were applied to determine cellulose, hemicelluloses, lignin, and non-fiber soluble components. The analyses were performed with a crude fiber extractor FIWE6 (VELP Scientifica, Velate, MB, Italy). Each analysis was performed in triplicate.

2.3. Cellulose isolation

Cellulose was obtained from dried orange peel following a five-steps procedure, as depicted by Coelho et al. (2020). The ratio between orange peel and solvent was maintained constant at 1:20 (w/v) for each step. Briefly, a total of 50 g of dried orange peel was mixed with 50% ethanol solution (60°C for 5 h) under stirring to remove sugars, vitamins, pigments, and polyphenols. The recovered solid material was dried and treated with 2% HCl solution (70°C for 5 h) to separate acid-soluble polysaccharides and polyphenolic compounds. The material was washed with double distilled water to neutral pH and treated with 5% NaOH solution (90°C for 6 h) to dissolve hemicelluloses, lignin, and the remaining polysaccharides. The alkaline-treated material was washed to neutral pH and bleached twice by 5% H_2O_2 (pH 11.5, 60°C for 6 h) to remove residual lignin and phenolic monomers. The final product was washed to neutral pH and freeze-dried (1.35 Pa, -50°C for 24 h) by a lyophilizer (Labconco Corporation, Kansas City, Missouri, USA). The yield of extraction (%) was calculated by dividing the final dried mass of extracted cellulose by the initial dry mass of orange peel.

2.4. Production of cellulose nanocrystals (CNCs) and yield calculation

The acid hydrolysis method was applied to produce CNCs according to Coelho et al. (2018) with modifications. A total of 3 g of orange cellulose were mixed with 64 wt% sulfuric acid solution (1:30 ratio, w/v) at 45°C under stirring for 60 min (500 rpm). The hydrolysis process was stopped by diluting the mixture with cold distilled water (1:15 ratio, v/v). CNCs were precipitated under centrifugation (6000 rpm, 45 min, 20°C) and washed with deionized water through four repeated centrifuge cycles (6000 rpm, 15 min, 20°C). The washing cycles were followed with ultrasonication (28 kHz, 180 w, 3 min) by an ultrasound (Argo Lab, DU45, Italy) to disrupt large CNCs aggregates. The obtained suspension was dialyzed using a cellulose dialysis membrane with 10-12 kDa molecular weight cut off (Sigma-Aldrich, Milan, Italy) against double distilled water to neutral pH, and freeze-dried (-50°C, 1.35 Pa) by a lyophilizer (Labconco Corporation, Kansas City, Missouri, USA) to obtain

CNCs powder. The production yield (%) was calculated by dividing the initial dry mass of orange cellulose and the final mass obtained after freeze-drying.

2.5. Preparation of film-forming solutions and nanocomposite films

The CS FFS was produced by dissolving 2 g CS in 100 mL of acetic acid solution (1% v/v) at 55 ºC for 15 min (Leceta et al., 2013). 2 g HPMC were dissolved in 100 mL hot water (80 °C) for 60 min to prepare the HPMC FFS (Lee et al., 2019), which was then cooled down at room temperature. Glycerol (30% w/w of CS or HPMC) was added into both FFSs under additional stirring for 30 min. CS and HPMC FFSs were mixed at a 1:1 ratio to prepare CS/HPMC blend, and used as a control. CNCs (10% w/w biopolymer) and/or LAE (5% w/w biopolymer) were separately added to the CS/HPMC FFSs These concentrations were selected based on preliminary tests, which highlighted that higher concentrations of CNCs and LAE could compromise the structural integrity of the films (e.g., formation of aggregates; surface discrepancies) without inducing any functional benefit. Meanwhile, lower concentrations of CNCs and LAE did not exert relevant effects on the technical performances of the films. After additives addition, ultrasonication (28 kHz, 180 w, 30 min) and additional stirring (500 rpm) for 30 min were applied to promote a homogeneous distribution of these compounds into the polymer matrix. The obtained FFSs were degasified with a vacuum pump (Vacuumbrand GMBH + CO KG, Wertheim, Germany) at 70 kPa for 15 min to remove bubbles. Films were obtained by casting 20 mL FFS onto Petri dishes (14.4 cm diameter) and dried overnight at 25°C (45% relative humidity).

2.6. Transmission electron microscopy (TEM)

The size and morphology of CNCs were characterized by TEM. The investigations were performed using a Talos F200S G2 microscope (Thermo Scientific, Brno, Czech Republic) operating at a 200 kV acceleration voltage. A total amount of 0.2 g of freeze-dried CNCs was dispersed in 5 mL of double distilled water with ultrasonication (28 kHz, 180 w, 20 min). A drop of CNCs suspension was deposited on a formvar/carbon-coated copper TEM grid (200 mesh). TEM micrographs were analysed using ImageJ software (v. 1.53a, National Institute of Health, USA). The average width and length of 100 randomly chosen CNCs were determined.

2.7. Scanning electron microscopy (SEM)

The surface and cross-section morphology of the films were obtained using a field emission scanning electron microscope (Nova NanoSEM 450, FEI, Hillsboro, Oregon, USA). Film samples were cut (2 x 2 mm^2) and mounted on a stainless-steel stub with double-sided tape. The analysis was performed in a low vacuum mode (80 Pa) with an acceleration voltage of 10 kV.

2.8. Attenuated total reflection (ATR)/Fourier-transform infrared spectroscopy (FT-IR)

The infrared spectra were measured with an ATR/FT-IR spectrometer (Alpha, Bruker Optik GmbH, Ettlingen, Germany). The spectra were recorded in the wavenumber region 4000 - 400 cm^{-1} at a spectral resolution of 4 cm^{-1} by accumulating 64 scans. Each determination was performed in triplicate.

2.9. Thickness and mechanical properties

The thickness of each film was measured at different random positions with a digital micrometer (IP65, SAMA Tools, Viareggio, Italy). A dynamometer (Z1.0, ZwickRoell, Italy) equipped with a 1KN loading cell was used to measure the tensile strength (TS), elongation (E%), and Young's modulus (YM) of the films according to ASTM D882-12 (ASTM, 2001a). The initial grip separation was 70 mm with a 10 mm/min cross-head speed. The recording of the tensile strength curve and the calculation of TS (MPa), E% (%), and YM (MPa) were performed through the TestXpert® II software (V3.31) (ZwickRoell, Ulm, Germany). 10 measurements were recorded for each film.

2.10. UV-Vis light transmittance, opacity, and colour

The barrier properties of films against UV and visible light were determined at the UV (200, 280, and 350 nm) and visible (400, 500, 600, 700, and 800 nm) wavelengths. The optical parameters were determined by a spectrophotometer (VWR®Double Beam UV × VIS 6300 PC 152 spectrophotometer, China) using film samples (2 × 2 cm^2) as described by Haghighi et al. (2019b). The opacity of the films was calculated by Eq. (5):

$$\text{Opacity value} = -\text{Log}T_{600} / d \quad (5)$$

where: T_{600} is the transmittance at 600 nm and d is the film thickness (mm). The average of four repeats was calculated.

The coordinates L* (lightness), a* (redness/greenness), and b* (yellowness/blueness) were obtained using a CR-400 Minolta colourimeter (Minolta Camera, Co., Ltd., Osaka, Japan) with D65 illuminant and 10° observer angle. The calibration of the instrument was executed by a white standard (L* = 99.36, a* = -0.12, and b* = -0.06). The total colour variation (ΔE*) was calculated using Eq. (6):

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (6)$$

where: ΔL*, Δa*, and Δb* are defined as the differences between the corresponding colour parameter of the samples and that of a white standard used as the film background (De Leo et al., 2018). 10 measurements were recorded for each film.

2.11. Water content (WC) and water solubility (WS)

The WC of the films (2 x 2 cm^2) was measured based on the mass loss upon drying in an oven at 105 ± 2°C for 24 h, according to the Eq. (11):

$$WC\ (\%) = ((W_0 - W_1) / W_0) \times 100 \quad (11)$$

where: W_0 and W_1 are film weights (g) before and after drying.

The WS was determined by measuring the initial dry weight of each film (2 × 2 cm^2) dried to constant weight in an oven at 105 ± 2°C (W_i). Each film was then immersed in 50 mL distilled water at 25°C for 24 h and the undissolved parts were dried to constant weight in an oven at 105 ± 2°C (W_f). The WS was calculated according to Eq. (7):

$$WS\ (\%) = ((W_i - W_f) / W_i) \times 100 \quad (7)$$

where: W_i and W_f represent initial and final dry weight (g) of the films, respectively. The experiments were performed in triplicate.

2.12. Water vapor permeability (WVP)

Water vapor permeability (WVP) of the films was determined according to the ASTM E96 method (ASTM, 2001b) with slight modifications. The film samples were sealed on top of glass test cups with an internal diameter of 10 mm and a depth of 55 mm. The test cups had been previously filled

with 2 g anhydrous $CaCl_2$ (0% RH). The cups were placed in desiccators containing $BaCl_2$ (90% RH), which were maintained into an incubator at 45°C. The cups were weighed every day for a week to guarantee steady-state permeation. The slope of the mass gain versus time was obtained by linear regression ($r^2 \geq 0.99$). WVTR (g/day m^2) and WVP (g/kPa day m^2) were calculated according to the following Eqs. (8) and (9):

$$WVTR = \Delta W/\Delta t \times A \quad (8)$$

$$WVP = WVTR \times L/\Delta P \quad (9)$$

where $\Delta W/\Delta t$ is the weight gain as a function of time (g/day), A is the surface area of the exposed film (m^2), L is the mean thickness of the film (mm) and ΔP is the vapor pressure gap across the film (kPa). The analyses for WVTR and WVP were performed in triplicate.

2.13. In vitro antimicrobial activity

Biocidal activity of the films was tested against four major food bacterial pathogens including *S. enterica* sv. Typhimurium (ATCC 14028), *L. monocytogenes* (ATCC 19115), *E. coli* (ATCC 43888), and *P. fluorescens* (ATCC 13525) through the disk diffusion assay, as described by Haghighi et al. (2020a) with slight modifications.

A loop of each pure strain was transferred into 10 mL of sterile BHIB and incubated at 30°C for 24 h. A total of 100 µL of inoculum containing 10^6 CFU/mL of each tested bacteria were separately streaked on the surface of sterile BHIA plates. Films were cut into disks (18 mm diameter) and sterilized under UV light and then placed on the surface of the inoculated plates. The plates were incubated at 30°C for 24 h. The diameter of the inhibition zone was measured. Each analysis was performed in triplicate.

2.14. Statistical analysis

A one-way analysis of variance (ANOVA) was applied to evaluate the statistical significance of the recorded data. Difference among means were determined by Tukey's multiple range test ($p < 0.05$) using SPSS statistical program (SPSS 20 for Windows, SPSS INC., IBM, New York). The results were expressed as mean ± standard deviation.

3. Results and Discussion

3.1. Chemical analysis of orange peel, CNCs yield, and visual appearance

Identifying the chemical composition of a raw material is fundamental to estimate its potential yield in CNCs and to set the chemical purification stages aiming to remove the impurities (Melikoğlu, Bilek, & Cesur, 2019). The chemical analysis showed that dried orange peel (Fig. 16a) used in this study contained 9.8% moisture, 2.0% crude fat, 5.9% protein, and 2.75% ash. A total of 64.2% non-fibrous carbohydrates, 0.2% hemicelluloses, 1.0% lignin, and 14.2% cellulose were determined.

The orange peel powder was subjected to a combined treatment including ethanolic extraction, acid, and alkali washing, and two repeated bleaching steps (H_2O_2) to isolate cellulose by removing non-cellulosic components (Coehlo et al., 2020). A total of 5.8 g of pre-treated material was obtained from 50 g of dried orange peel, consisting of a fine white powder (Fig. 16b) mainly composed of cellulose (86.4%). The extraction yield (~11.5%) was similar to the value reported by Naz et al. (2016) for citrus (Kinnow) peel. Overall, the ethanolic extraction followed by the alkaline NaOH/H_2O_2 route allowed to effectively extract cellulose from untreated orange peel powder. In addition, this step was reported to reduce the amorphous character of cellulose, improving its reactivity to the subsequent acid treatment for the production of nanocrystals (Coehlo et al., 2018).

Orange peel cellulose was hydrolysed with 64 wt% sulfuric acid for 60 min to generate CNCs with 27% yield. The aqueous CNC suspension was transparent and stable, probably due to the negatively charged sulphate groups introduced by sulfuric acid hydrolysis (Jiang & Hsieh, 2015). Freeze-dried CNCs appeared as light and well-distributed crystals (Fig. 16c), and were subjected to further analyses for morphological and size determination.

Fig 16. The visual appearance of a) raw orange peel powder after drying, b) bleached orange peel cellulose, and c) cellulose nanocrystals (CNCs).

3.2. Transmission electron microscopy (TEM)

The morphology and size of orange CNCs were characterized by TEM (Fig. 17). CNCs exhibited a rod and needle-shaped morphology. The average length and width of the nanoparticles were 500 nm and 40 nm, corresponding to an aspect ratio of 12.5. These results agreed with previous studies highlighting that plant CNCs have a length of 100-500 and a width of 5-70 nm. The authors concluded that the plant source and acid hydrolysis parameters significantly influenced these parameters (Abdul-Khalil et al., 2014; Oun & Rhim, 2016).

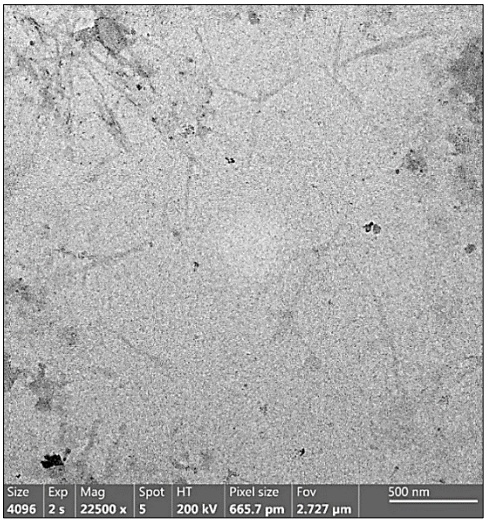


Fig 17. TEM image of cellulose nanocrystals (CNCs) from orange peel.

3.3. Characterization of CS/HPMC nanocomposite films

3.3.1. Surface and cross-section morphology

The microstructure of a film depends on the miscibility and compatibility between the film constituents, and directly affect the optical, physical, mechanical, and barrier properties (Bigi et al., 2021a). The surface and cross-section of films based on CS/HPMC as control and those reinforced with CNCs and/or enriched with LAE are presented in Fig. 18 and Fig. 19.

The surface of the control film was homogeneous, smooth, and compact (Fig. 18a). Phase separation was not observed in CS/HPMC blend, indicating that CS and HPMC were highly compatible due to associative interactions (Song, Feng et al., 2020). Active films enriched with LAE (5% w/w biopolymer) (Fig. 18b) revealed a less compact surface with small pores and discontinuities, as reported by Haghighi et al. (2020b) for CS/PVA blend films enriched with LAE up to 7.5% (w/w). This effect might be caused by the partial release of this compound on the surface of the film. A rough surface with small particles and aggregates was observed for CS/HPMC/CNCs (Fig. 18c) and CS/HPMC/CNCs/LAE (Fig. 17d). This effect was probably due to the strong interactions between

the hydroxyl groups of the CNCs which promoted the partial agglomeration of the CNCs on the film surface (Costa et al., 2021; El Achaby, Kassab, Aboulkas, Gaillard, & Barakat, 2018).

The cross-section of the control film (Fig. 19a) exhibited a compact and homogenous microstructure, indicating excellent structural integrity and compatibility between CS and HPMC. The film containing LAE (Fig. 19b) also showed a continuous and compact microstructure suggesting the high level of miscibility between the polymer matrix and this active compound. Films reinforced with CNCs showed a slightly irregular and sponge-shaped structure (Fig. 19c and 19d). This confirmed the partial agglomeration of the CNCs in the polymer matrix mainly due to the predominance of nanocrystal-nanocrystal associations over nanocrystal-polymer interactions.

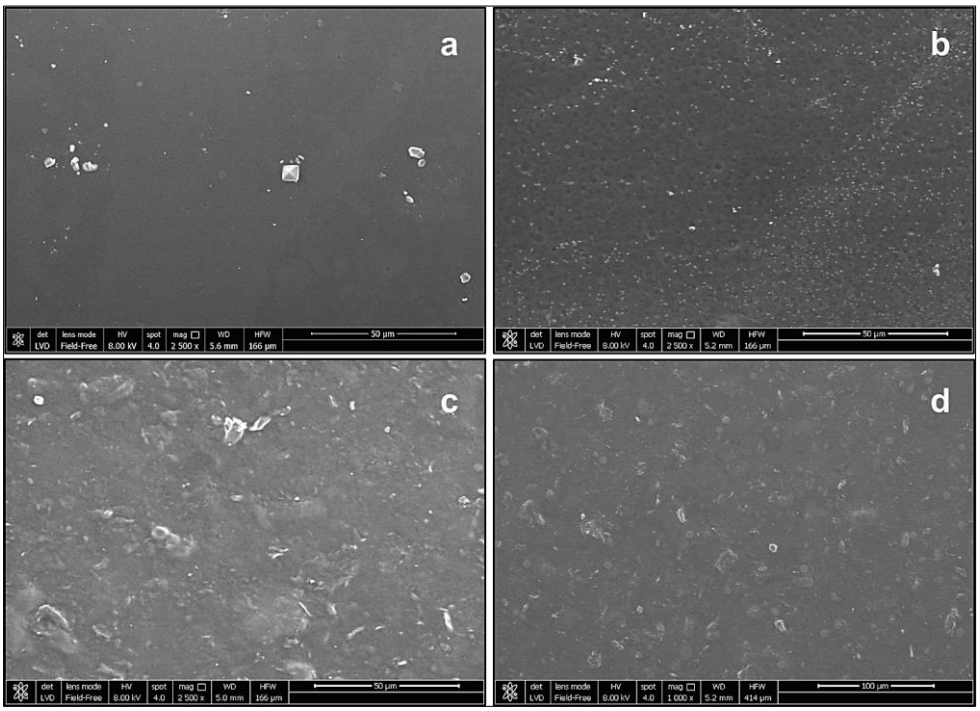


Fig. 18. SEM images (surface) of films based on a) chitosan/hydroxypropyl methylcellulose blend (CS/HPMC), b) CS/HPMC/LAE, c) CS/HPMC/CNCs, and d) CS/HPMC/CNCs/LAE.

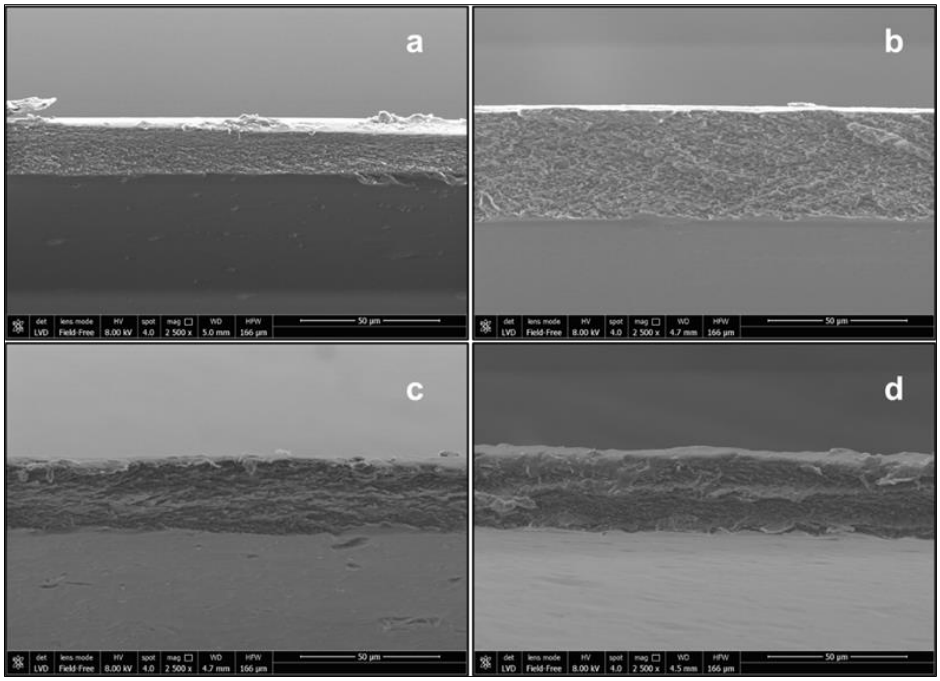


Fig. 19. SEM images (cross-section) of films based on a) chitosan/hydroxypropyl methylcellulose blend (CS/HPMC), b) CS/HPMC/LAE, c) CS/HPMC/CNCs, and d) CS/HPMC/CNCs/LAE.

3.3.2. ATR/FT-IR spectroscopy

ATR/FT-IR spectroscopy was performed on orange peel cellulose and CNCs to highlight whether sulfuric acid hydrolysis induced any significant change in the chemical backbone of the cellulose biopolymer (Fig. 20). The spectra of orange peel cellulose and CNCs exhibited the typical peaks of cellulose. The bands located in the region of 3000-3600 cm^{-1} and the peak at about 1647 cm^{-1} correspond to the stretching and bending vibrations of OH functionalities of cellulose and small amounts of absorbed water, respectively (Dechant, 1972; Dai et al., 2018). The peaks in the 2800-3000 cm^{-1} region can be assigned to stretching vibrations of CH/CH_2 groups and the peaks in the 1350-1500 cm^{-1} region contain primarily bands of CH/CH_2 and OH bending vibrations (Dechant, 1972; Li et al., 2020). Typical "finger-prints" of cellulose were also found at 1160 cm^{-1}, 900 cm^{-1}, and 560 cm^{-1}, indicating β-glycosidic linkages in cellulose structure and OH out-of-plane bending vibrations (Kale & Gorade; 2019). The peak at 1315 cm^{-1} arose from the bending vibrations of the CH and C-O groups of the rings in polysaccharides, while the very intense bands in the 1030-1160 cm^{-1} range, correspond to C-O-C stretching and C-H rocking of the pyranose ring (Costa et al., 2021; Dechant, 1972). No significant differences were identified between the two spectra, suggesting that the chemical structure was retained in the CNCs after acid hydrolysis. Both spectra were characterized by the complete absence of peaks around 1735 cm^{-1}, which are commonly ascribed to the C=O stretching of the acetyl and uronic ester groups of hemicelluloses, and the ester linkages of carboxyl groups in ferulic and p-coumaric acids of lignin and hemicelluloses. Overall, these results indicated

that the applied purification treatment and subsequent acid hydrolysis effectively removed the non-cellulosic impurities from the raw matrix and reduced the amorphous character of cellulose without affecting its chemical structure.

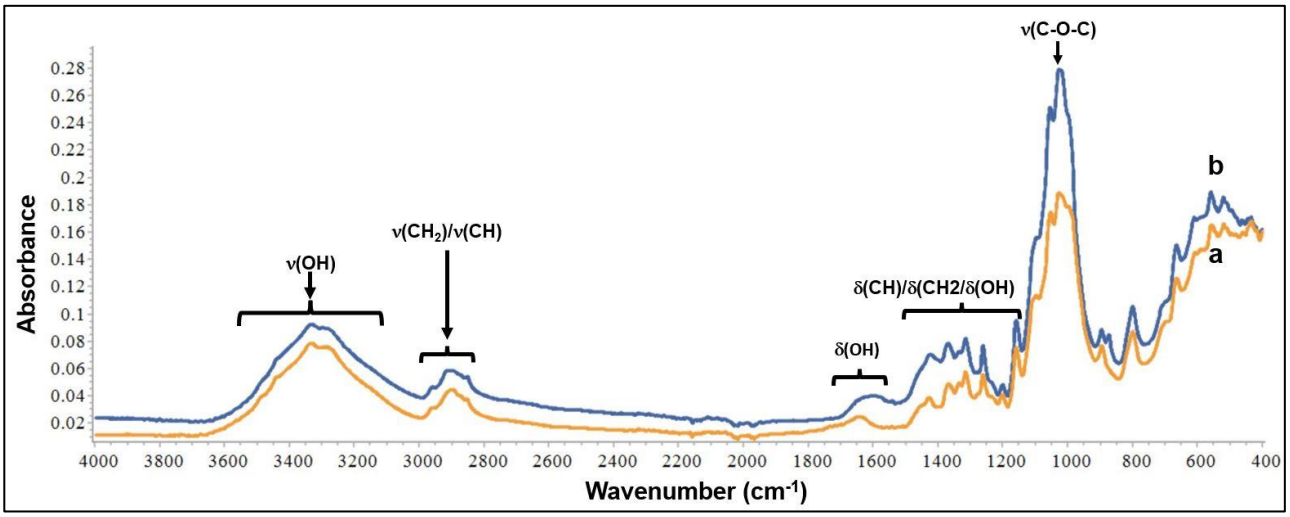


Fig. 20. ATR/FT-IR spectra of a) bleached orange peel cellulose and b) cellulose nanocrystals (CNCs) with the assignment of the most prominent absorption bands.

ATR/FT-IR spectroscopy was also performed to identify the spectroscopic changes induced by the addition of CNCs (10% w/w of biopolymer) and/or LAE (5 % w/w of biopolymer) into a CS/HPMC film matrix (Fig. 21).

The IR spectrum of pure CS/HPMC control film (Fig. 21a) showed the characteristic peaks at 1647 and 1557 cm^{-1}, which can be associated with the amide-I and amide-II bands, respectively. The broad absorption bands in the range 3600-3000 cm^{-1} are related to the stretching vibrations of OH, NH, and NH_2 functionalities. The band complex in the range 2800-3000 cm^{-1} can be assigned to the stretching vibrations of the CH_3, CH_2, and -CH groups of HPMC. The bending vibrations of the CH_3/CH_2 groups were located in the 1300 – 1500 cm^{-1}region. The most intense bands between 930 and 1150 cm^{-1} can be assigned to the saccharide structure, indicating the ν(C-O-C) stretching vibrations of the ß-glycosidic linkages within the polymer structure. Overall, the spectroscopic data confirmed that CS and HPMC are highly compatible to produce biodegradable films, as described in previous works (Bigi et al., 2021b).

Active films containing LAE (5% w/w of biopolymer) (Fig. 21b and 21d) revealed new absorption bands in the 2800-3000 cm^{-1} region, and at 1739 cm^{-1}, corresponding to the contributions of anti-symmetric ν(CH_2) and ν(C=O) stretching vibrations, respectively (Gamarra, Missagia, Urpì, Moratò, & Munoz-Guerra, 2018; Moreno et al., 2017a). The shift of the amide II band at 1557 cm^{-1} to 1565 cm^{-1} (Fig. 21b) and 1574 cm^{-1} (Fig. 21d) confirmed that the molecular functionalities of LAE such as

carbonyl, amino, and imino groups contributed to create competitive intermolecular interactions with the hydroxyl, amino, and ether groups of the CS/HPMC blend. Furthermore, the increased intensity of the peak at 1645 cm^{-1} suggested that this additive contributes to create amide C=O groups to the polymer matrix (Haghighi et al., 2020a).

The addition of CNCs (10% w/w of biopolymer) slightly affected the shape and intensity of the main bands of CS/HPMC blend films (Fig. 21c and 21d). A shift of the peak at 3346 cm^{-1} to 3335 cm^{-1} was observed, suggesting that hydrogen bonding took place between hydroxyl groups of the CNCs and OH, NH, NH_2, and C=O groups of the CS/HPMC matrix. The peak at 1557 cm^{-1} shifted to 1571 cm^{-1} and 1574 cm^{-1}, confirming the molecular interactions occurring between the nanofillers and the main polymer backbone (Haghighi et al., 2021).

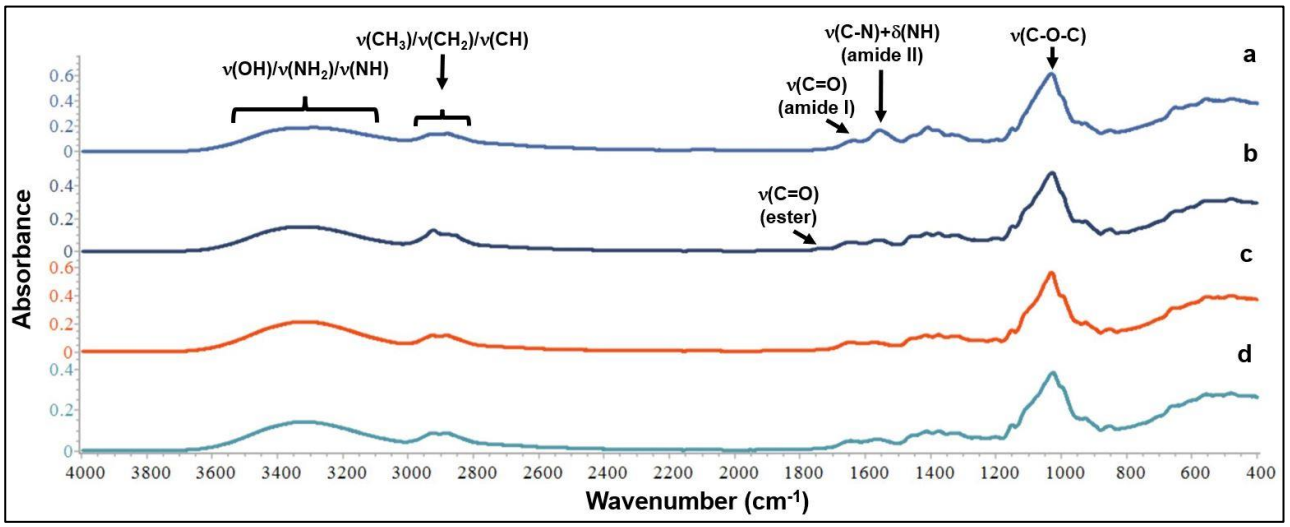


Fig. 21. ATR/FT-IR spectra of films based on a) chitosan/hydroxypropyl methylcellulose blend (CS/HPMC), b) CS/HPMC/LAE, c) CS/HPMC/CNCs, and d) CS/HPMC/CNCs/LAE.

3.3.3. Thickness and mechanical properties

The thickness, tensile strength (TS), elongation (E%), and Young's modulus (YM) of CS/HPMC blend films as control and those reinforced with CNCs and/or enriched with LAE are presented in Table 13. Thickness ranged from 32.7 to 38.1 µm. The control film showed the lowest thickness value. The reinforcement with CNCs (10% w/w of biopolymer) increased the thickness due to the higher solid content ($p < 0.05$) (Costa et al., 2021). The incorporation of LAE (5% w/w of biopolymer) slightly increased the thickness, probably due to its emulsifying behaviour, which induced major retention of water molecules in the film matrix. The co-presence of CNCs and LAE induced a further increase in thickness which might be due to the synergic effect of the two additives.

TS measures the highest stress that films can withstand before breakage. The TS of CS/HPMC control film was 17.5 MPa. The presence of CNCs significantly increased the TS of the films ($p < 0.05$). The improvement of TS of films induced by CNCs incorporation has been reported by previous studies (Li, Shi et al., 2020; Perumal, Sellamuthu, Nambiar, & Sadiku, 2018; Sukyai et al., 2018). This behaviour can be attributed to the large number of hydroxyl functionalities on the CNCs surface, which strongly interact with the CS/HPMC blend to form hydrogen bonds (Wang, Shankar, & Rhim, 2017), and to fill the free spaces between the polymer chains (Xu, Willis, Jordan, & Sismour, 2018). The addition of LAE to the polymer matrix slightly decreased the TS compared to the control film, but the difference was statistically negligible ($p > 0.05$).

E% reflects the flexibility and stretchability of the films. It compares the highest level of elongation of a film with its initial length (Jantrawut et al., 2017). The E% of the control film (18.9%) was not influenced by the incorporation of CNCs ($p > 0.05$). Similar results were reported by Gonzàles, Gastelù, Barrera, Ribotta, & Igarzabal (2019) for soy protein films reinforced with cellulose nanofibers (0-10% w/w of biopolymer). The authors concluded that addition of cellulose nanofibers mainly had a crosslinking effect on the polymer matrix, causing an increase in TS and a decrease in the elasticity value (Coehlo et al., 2020; Xu et al., 2018). LAE incorporation significantly influenced the film flexibility. Films containing LAE became less rigid and more extensible compared to the control film. This effect could be related to the plasticizing effect of LAE, acting as an emulsifier and reducing the adhesion forces within the film matrix (Moreno, Dìaz, Atarès, & Chiralt, 2016).

YM quantifies the intrinsic stiffness of a film. Low YM values usually indicate a flexible material (Jantrawut et al., 2017). An increase in YM was observed with the incorporation of CNCs. The CS/HPMC control film showed an YM of 644.7 MPa, which increased to 705.8 MPa in the presence of CNCs ($p < 0.05$). This effect may be ascribed to the high crystallinity of CNCs and the inter- and

intra-molecular interactions induced by the distribution of the nanoparticles within the polymer matrix (Costa et al., 2021). Overall, these findings demonstrated that CNCs acted as an excellent reinforcing agent for CS/HPMC films. Conversely, the addition of LAE notably reduced the YM of CS/HPMC films (407.0 MPa) ($p < 0.05$). This effect could be attributed to the competitive interaction between the molecular functionalities of LAE and the CS/HPMC blend ones, which restrains the cohesion forces between the polymers and reduces the degree of physical crosslinking by weakening the intermolecular hydrogen bonding (Haghighi et al., 2020a). This effect was sightly mitigated by the presence of both LAE and cellulose nanocrystals in CS/HPMC/CNCs/LAE films (529.1 MPa).

Table 13.
Thickness, tensile strength (TS), elongation (E%), and Young's modulus (YM) of films based on chitosan/hydroxypropyl methylcellulose blend (CS/HPMC) as control and those reinforced with cellulose nanocrystals (CNCs 10% w/w of biopolymer) and/or enriched with lauroyl arginate ethyl (LAE 5% w/w of biopolymer).

Film sample	Thickness (µm)	TS (MPa)	E (%)	YM (MPa)
CS/HPMC	32.7±2.0^{a}	17.5±1.0^{a}	18.9±0.9^{a}	644.7±25.2^{c}
CS/HPMC/CNCs	35.3±1.8^{b}	25.4±2.7^{b}	19.9±1.6^{a}	705.8±57.2^{d}
CS/HPMC/LAE	34.1±2.6^{ab}	15.1±0.9^{a}	23.9±1.0^{b}	407.0±28.9^{a}
CS/HPMC/CNCs/LAE	38.1±1.8^{c}	26.4±1.4^{b}	27.8±1.9^{c}	529.1±48.8^{b}

Values are presented as mean ± standard deviation (n = 3).
Different letters in the same column indicate significant differences ($p<0.05$).

3.3.4. Colour

The colour values (L*, a*, and b*) and the total colour difference (ΔE*) of films based on CS/HPMC blend as control and those reinforced with CNCs and/or enriched with LAE are presented in Table 14.

L*, a*, and b* parameters allow to evaluate the appearance of the films. L* (quantifying the film lightness) ranged from 97.5 to 95.6, indicating that all the films were bright. The incorporation of CNCs slightly decreased the L* value ($p < 0.05$) (Coehlo et al., 2020), while LAE did not significantly affect this colour parameter ($p > 0.05$) (Haghighi et al., 2019b). The a* value, which expresses the greenness-redness colour component, was negative for all the films. Both CNCs and LAE induced a slight increase of this value ($p < 0.05$). The b* value, measuring the blueness–yellowness, increased upon the addition of both CNCs and LAE ($p < 0.05$). ΔE* of the films varied from 7.5 to 10.8. The addition of CNCs and LAE to CS/HPMC film matrix increased the ΔE* ($p < 0.05$). This increase could be related to the change of b*, and to the variation in film thickness induced by the CNCs. A similar observation was reported by Li, Shi et al. (2020) for polysaccharide-based films enriched with plant-based nanocellulose.

Table 14.
Colour parameters (L*, a*, and b*) and total colour variation (ΔE*) of films based on chitosan/hydroxypropyl methylcellulose blend (CS/HPMC) as control and those reinforced with cellulose nanocrystals (CNCs 10% w/w of biopolymer) and/or enriched with lauroyl arginate ethyl (LAE 5 % w/w of polymer).

Film sample	Colour parameters			
	L*	**a***	**b***	**ΔE***
CS/HPMC	97.2 ± 0.3^{c}	-1.1 ± 0.1^{a}	7.2 ± 0.6^{a}	7.5 ± 0.6^{a}
CS/HPMC/CNCs	95.6 ± 0.2^{a}	-0.9 ± 0.06^{b}	10.2 ± 0.4^{c}	10.8 ± 0.4^{b}
CS/HPMC/LAE	97.5 ± 0.3^{c}	-0.8 ± 0.06^{c}	7.5 ± 0.6^{a}	7.8 ± 0.7^{a}
CS/HPMC/CNCs/LAE	96.3 ± 0.2^{b}	-0.9 ± 0.03^{b}	8.9 ± 0.8^{b}	10.0 ± 1.0^{b}

Values are presented as mean ± standard deviation (n = 3).
Different letters in the same column indicate significant differences ($p<0.05$).

3.3.5. UV barrier, light transmittance, and opacity value

UV-Vis light barrier property represents a key attribute of active films, which allows to protect the packed products from photo-oxidation and to avoid the loss of nutritive components (Cazòn et al., 2017).

The UV-Vis light transmittance values at wavelengths from 200 nm to 800 nm and opacity values of films based on CS/HPMC blend as control and those reinforced with CNCs and/or enriched with LAE are presented in Table 15.

All the films performed excellent barrier features to UV-C at 200 nm, with a light transmittance lower than 0.1%. The incorporation of LAE did not significantly affect the percentage of light transmittance in the wavelength range between 280 and 350 nm (UV-B and UV-A light), while CNCs drastically improved the performance of the films against UV light. This result may be attributed to the hindrance of light passage induced by CNCs dispersion in the polymer matrix (Li, Shi et al., 2020).

The transmission of wavelengths in the visible range (400-800 nm) was higher than 80% for the control films and those containing LAE, indicating the high transparency and brightness of CS/HPMC films. The presence of LAE alone did not exert a remarkable effect on the transmission of visible light. Conversely, the reinforcement of films by CNCs caused a slight decrease in film transparency. Similar findings were reported by Costa et al. (2021) for CS film reinforced with CNCs. The authors concluded that the addition of CNCs promoted a slight decrease in film's transparency due to their accumulation in the polymeric network, causing light diffusion.

The opacity values ranged from 1.5 to 3.1. The control film exhibited the lowest value, suggesting the high transparency of this film. The opacity was enhanced by the addition of CNCs ($p < 0.05$). The addition of LAE to CS/HPMC/CNCs film seemed to mitigate the effect of CNCs, reducing the

opacity. This novel result confirms that this compound acted as an emulsifier, promoting a more homogeneous distribution of CNCs within the matrix and avoiding their agglomeration.

Overall, all films had opacity values strictly close to the values measured for low-density polyethylene films (Hosseini et al., 2021). Thus, the active films developed in this study could allow to clearly see the food content and its condition, an important parameter for consumer acceptance.

Table 15.
UV-Vis light transmittance (%) and opacity of the films of films based on chitosan/hydroxypropyl methylcellulose blend (CS/HPMC, 2% w/v) as control and reinforced with cellulose nanocrystal (CNCs 10% of w/w biopolymer) and/or enriched with lauroyl arginate ethyl (LAE 5% w/w of biopolymer).

Film sample	Light transmission (%) at different wavelengths (nm)								Opacity (600 nm)
	200	280	350	400	500	600	700	800	
CS/HPMC	<0.1	25.5	45.7	69.2	83.2	87.2	89.4	90.2	1.5 ± 0.1[a]
CS/HPMC/CNCs	<0.1	12.4	31.8	54.4	67.2	73.1	76.2	78.0	3.1 ± 0.2[d]
CS/HPMC/LAE	<0.1	26.8	51.2	71.7	83.7	87.4	88.7	89.4	1.9 ± 0.06[b]
CS/HPMC/CNCs/LAE	<0.1	22.3	44.6	64.4	76.8	81.2	83.8	85.1	2.4 ± 0.2[c]

Values are presented as mean ± standard deviation (n = 3).
Different letters in the same column indicate significant differences ($p<0.05$).

3.3.6. Water content, water solubility, and water vapor permeability

The sensitivity to water represents the main criticism related to biodegradable active films. In fact, biopolymers tend to absorb water when surrounded by a moist environment. Thus, the measurement of water-related properties represents a key step to tailor a packaging system for targeted applications (Aguirre-Loredo, Rodrìguez-Hernandez, Morales-Sànchez, Gòmez-Aldapa, & Velazquez, 2016).

The water content (WC), water solubility (WS), and water vapor permeability (WVP) of films based on CS/HPMC blend as control and those reinforced with CNCs and/or enriched with LAE are presented in Table 16.

The WC values ranged from 19.7 to 23.9%. LAE addition did not significantly affect WC ($p > 0.05$). Similar results were reported by Moreno, Gil, Atarès, & Chiralt, (2017b) for starch-gelatin films. Conversely, the incorporation of CNCs slightly decreased this value, probably due to their strong interaction with the CS/HPMC network, which led to a reduction of the film hydrophilicity (Dufresne, Dupeyre, & Vignon, 2000; Gonzàles et al., 2019). The WS of the control film was 52.5%. The addition of CNCs decreased this value to 35.6% ($p < 0.05$). This finding may be associated with the formation of hydrogen bonds between the hydroxyl groups of CS/HPMC blend and the CNCs, restricting the movement of hydrophilic compounds toward water (Noshirvani, Hong, Ghanbarzadeh, Fasihi, & Montazami, 2018). CS/HPMC films containing LAE showed the highest values of WS

(62.5%). This effect was probably due to the low oil-water equilibrium partition coefficient (K_{ow} < 0.1) of LAE, indicating the high affinity of this compound with water (Rubilar et al., 2016).

The ability of a film to hinder the transmission of moisture from the environment to the packaged foodstuffs and vice-versa is strongly important to maintain the quality of food during handling, storage, and selling. Both fresh foods (e.g., fruits and meat) and dry foods (e.g., bakery products and spices) take advantage of this feature since it avoids dehydration for the formers and moisture uptake for the latters' (Cazòn et al., 2017). The WVP of packaging depends on several factors including the composition and hydrophilic-hydrophobic nature of the film, the co-presence of crystalline and amorphous zones, and the mobility of the polymer chains. The WVP of the control film was 7.2 (g mm/day kPa m^2). The addition of CNCs reduced the WVP value to 5.8 (g mm/day kPa m^2) corresponding to a 19.5% reduction. This result confirmed that the addition of CNCs represents an effective strategy to counteract the film sensitivity to moisture. In fact, their homogeneous dispersion into the FFS reduces the availability of free hydroxyl groups within the polymer matrix, and creates a three-dimensional frame that slows down the diffusion of water molecules (Singh et al., 2018). Conversely, LAE addition significantly increased the WVP of CS/HPMC film to 9.5 (g mm/day kPa m^2). The WVP value of CS/HPMC/CNCs/LAE film was 6.8 (g mm/day kPa m^2). This evidence may be attributed to the partial disruption and re-construction of the hydrogen bonds induced by LAE, promoting the creation of amorphous patterns within the polymer chains (Ma, Zhang, & Zhong, 2016b).

Table 16.
Water content (WC), water solubility (WS), and water vapor permeability (WVP) of films based on chitosan/hydroxypropyl methylcellulose blend (CS/HPMC, 2% w/v) as control and those reinforced with cellulose nanocrystals (10% w/w of biopolymer) and/or lauroyl arginate ethyl (LAE 5 % w/w of biopolymer).

Film sample	WC (%)	WS (%)	WVP 90:0% RH (g mm/kPa day m^2)
CS/HPMC	22.6 ± 2.6^{b}	52.5 ± 1.8^{b}	7.2 ± 0.5^{b}
CS/HPMC/CNCs	19.7 ± 0.8^{a}	35.6 ± 0.8^{a}	5.8 ± 0.3^{a}
CS/HPMC/LAE	23.9 ± 1.1^{b}	62.5 ± 1.2^{d}	9.5 ± 0.7^{c}
CS/HPMC/CNCs/LAE	22.8 ± 0.7^{b}	56.9 ± 1.0^{c}	6.8 ± 0.5^{ab}

Values are presented as mean ± standard deviation (n = 3).
Different letters in the same column indicate significant differences ($p<0.05$).

3.3.7. In vitro antimicrobial activity

Food products are highly susceptible to microbial spoilage which can occur during production, processing, and storage. In particular, bacteria are amongst the main responsible for food deterioration, and can induce harmful effects on human health (Costa et al., 2021). Thus, the application of bio-based active films with antimicrobial properties represents an effective strategy to avoid the growth and exploitation of spoilage and pathogenic bacteria.

The antimicrobial activity of control film and those reinforced with CNCs and/or enriched with LAE against *S. enterica* subsp. Typhimurium, *E. coli*, *L. monocytogenes*, and *P. fluorescens* were investigated through the disk diffusion assay (Table 17). The films without LAE did not affect the growth of the tested microorganisms. The absence of antimicrobial character may be explained by the low diffusivity of CS in the agar medium (Leceta et al, 2013) and the incapability of cellulose derivatives to inhibit the bacterial growth (Di Filippo et al., 2021). Active films enriched with LAE inhibited the growth of all tested bacteria. A similar result was reported by Muriel-Galet, Lopez-Carballo, Gavara, & Hernandez-Munoz (2015). The antimicrobial activity of LAE has been widely associated with its action as a cationic surfactant on the cytoplasmic membrane of gram-positive and gram-negative bacteria. In particular, this compound affects the membrane potential and the cytoplasm permeability resulting in cell growth inhibition and loss of viability (Moreno et al., 2017b). Furthermore, LAE has been reported to induce changes in the DNA structure, causing aggregations through ionic bridging (Ma, Davidson, Critzer, & Zhong, 2016a).

In this study, LAE showed slightly higher activity against *L. monocytogenes* than *E. coli* and *S.* Typhimurium, as already described by Muriel-Galet et al. (2012). Authors concluded that gram-negative bacteria result less susceptible to the action of antibacterial compounds due to the presence of an outer membrane which surrounds the cell wall and restricts the diffusion of hydrophobic compounds through its lipopolysaccharide covering. The good resistance of *S.* Typhimurium with respect to the other pathogens could be attributed to the high density of its cell membrane, related to the great content of phospholipid components such as phosphatidylethanolamine and phosphatidylglycerol. These components enhance the rigidity of the cell wall, providing extra protection for the cell membrane (Alwi & Ali, 2014). An exception was observed for *P. fluorescens*, which resulted the most sensitive among tested bacteria. This could be attributed to the lack of protective mechanisms to oxidative stress, causing an overall loss of cell viability. Moreover, the higher antibacterial efficacy observed for CS/HPMC/LAE film with respect to CS/HPMC/CNCs/LAE one may be attributed to the dense nano-scale network induced by the presence of CNCs, which could effectively retain LAE, thus hindering its migration in the external

medium. Overall, these results proved that the addition of LAE to CS/HPMC film could represent an effective strategy to protect packed foods against major food bacterial pathogens.

Table 17.
Inhibition zone diameters (expressed in mm) of the film disks (18 mm diameter) based on chitosan-hydroxypropyl methylcellulose blend (CS/HPMC) as control and those reinforced with cellulose nanocrystals (CNCs 10% w/w of biopolymer), and/or enriched with lauroyl arginate ethyl (LAE 5% w/w of biopolymer).

Film sample	*S. enterica*	*E. coli*	*L. monocytogenes*	*P. fluorescens*
CS/HPMC	N.D.	N.D.	N.D.	N.D.
CS/HPMC/CNCs	N.D.	N.D.	N.D.	N.D.
CS/HPMC/LAE	0.9 ± 0.09^{bA}	4.4 ± 0.4^{bB}	6.7 ± 0.6^{aC}	8.5 ± 0.7^{bD}
CS/HPMC/CNCs/LAE	0.5 ± 0.07^{aA}	3.4 ± 0.4^{aB}	6.5 ± 0.6^{aD}	5.7 ± 0.4^{aC}

Values are presented as mean ± standard deviation (n = 3). N.D. means not detected.
Different lowercase letters in the same column indicate significant differences ($p<0.05$).
Different capital letters in the same row indicate significant differences ($p<0.05$).

4. Conclusions

The aim of this study was to provide an extraction protocol based on alkaline/H_2O_2 bleaching treatment followed by sulfuric acid hydrolysis to convert orange peel into CNCs. The extracted CNCs were used as a reinforcing agent to produce nano-composite biodegradable films based on CS/HPMC blend, which were enriched with LAE to confer antimicrobial activity. The microstructural, optical, physical, mechanical, water barrier, and antimicrobial properties of the nanocomposite active films were evaluated. SEM showed that both CNCs and LAE were evenly distributed in the polymer matrix to form homogeneous films, indicating the high compatibility between the polymers and the additives. The ATR/FT-IR spectra confirmed the interaction between the hydroxyl and amino groups of the biopolymer network and the molecular functionalities of CNCs and LAE to form hydrogen bonds. CNCs significantly improved the UV and light barrier properties of the films which may be useful to protect food from photo-oxidation and UV degradation. Meanwhile, the opacity values remained lower than 5 for all the films. On the one hand, the addition of CNCs significantly improved the tensile strength and stiffness of the films, while it decreased the water solubility and water vapor permeability. On the other hand, LAE addition improved the elasticity of the films due to its emulsifying behaviour. Additionally, films containing LAE successfully inhibited the growth of all the tested food-borne bacterial pathogens. Overall, these findings suggest the possibility to employ these films as a green substitute to conventional plastic materials for packaging foods sensitive to microbiological decay and photo-oxidation. Furthermore, CNCs extraction from agri-food waste and their subsequent application as the nano-reinforcing agent could be considered as an effective strategy to reduce the economic and environmental impact of waste disposal, thus creating a new opportunity of business with high-growth potential.

Concluding remarks

The aim of this PhD project was to investigate the potential of non-thermal techniques, bio-based active packaging and multivariate statistical analysis as powerful tools to reduce the economic and environmental impact of agri-food technologies. During these 3 years, our efforts were focused on the development of novel and sustainable protocols and packaging solutions, by applying a multidisciplinary approach which put in contact biopolymer sciences, extraction techniques, food microbiology, and statistical tools. In this work, different fields of research were addressed and combined with each other. This allowed us to set a transversal cultural background, useful to deal with the future challenges related to this "new green era" of food technologies.

The research items of this PhD thesis may be summarised as follows:

- Evaluation of gaseous ozone (0.05 ppm) in combination with low temperature as a prospective strategy to guarantee the microbial quality of cold chambers for food storage, and to inhibit the exploitation of pathogenic bacteria in food transformation environments (**Chapter 2**);
- Testing of Principal Components Analysis (PCA) as a tool to interpret (and, prospectively, predict) the interdependence between the technical behaviour of chitosan and pectin-based films and the complex of manufacturing parameters (**Chapter 3**);
- Development of antioxidant and antimicrobial bioactive films based on renewable biopolymers (i.e., chitosan/hydroxypropyl methylcellulose blend) and enriched with herbal extracts (i.e., nettle and sage) or with biodegradable, non-toxic synthetic molecules (e.g., LAE) to overcome the drawbacks related to the massive use of petroleum-derived plastics and conventional preservatives (**Chapter 4** and **Chapter 5**, respectively).

The promising achievements of this project confirmed the suitability of these strategies to reduce the impact of the agri-food sector in the next future. Nevertheless, as discussed in **Chapter 1**, further studies are needed to overcome the current drawbacks related to these technologies. Referring to the world of non-thermal technologies, there is still a big gap between laboratory research scale and the industrial application of these techniques, mainly due to economic (e.g., high set-up and maintenance costs), and regulatory issues. This also applies to biopolymer-based active packaging. Moreover, these new packaging solutions still suffer technical criticisms, mainly related to their poor barrier properties (e.g., gases, water vapour etc.), thermal instability and (in some cases) non-thermoplastic behaviour, which implies their scarce applicability to well-stated technologies such as extrusion and thermoforming.

Hence, more-in-depth investigations and engineering processes are necessary to further expand our knowledge and, subsequently, to shift the application of these technologies toward a real industrial scale.

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Bigi, F., Maurizzi, E., Siesler, H.W., Pulvirenti, A., & Haghighi, H. (2021). Characterization of active chitosan/hydroxypropyl methylcellulose/orange cellulose nanocrystals films enriched with LAE for food packaging applications. Published by *MDPI* in: *The 2nd International Electronic Conference on Foods - "Future Foods and Food Technologies for a Sustainable World* (POSTER). https://doi.org/10.3390/Foods2021-10933

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Congress contributions

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