Development of edible coating functionalized with hydroxyapatite, complexed with bioactive compounds for the shelf-life extension of food products

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Malvano, F.; Montone, A.M.I.; Capuano, F.; Colletti, C.; Roveri, N.; Albanese, D.; Capparelli, R. Effects of active alginate edible coating enriched with hydroxyapatite-quercetin complexes during the cold storage of fresh chicken fillets. Food Packag. Shelf Life2022, 32, doi:10.1016/j.fpsl.2022.100847

Montone, A.M.I., Malvano, F., Pham, P.L., Cinquanta, L., Capparelli, R., Capuano, F. and Albanese, D. (2022), Alginate-based coatings charged with hydroxyapatite and quercetin for fresh-cut papaya shelf life. Int J Food Sci Technol, 57: 5307 5318. https://doi.org/10.1111/ijfs.15860

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Summary

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Abstract

The main goal of food packaging is to protect the food from physical, chemical and biological contaminations. The environmental impact of conventional food packaging directed research towards new packaging strategies based on environmentally friendly and biodegradablenatural polymers. The nature of biopolymers influences the physicochemical and mechanical properties of films and coatingssuch as mechanical stability, transparency, moisture and gas barrier. Generally, polysaccharides are employed to avoid gas permeability, lipids limit water vapour transmission, and proteins improve the mechanical stability of the structure.

Anovel way to preserve the safety of food products and prolong their shelf life is represented by the incorporationinto edible coatingsof active compounds, such as nutrients, antioxidants, antimicrobials, colorants, and flavorings, which are mainly used to improve the functional properties of coatings. However, the poor stability of these bioactive compoundsunder processing conditions and during storage makes it necessary to use a carrier system for the release of the compound from the coating to the product surface.

Among severalcarriersystems adopted to protect active compounds, hydroxyapatite crystals seem to be attractive candidates for this application.

Hydroxyapatiteis a calcium phosphate similar to that present in the human hard tissues as regard morphology and composition. It is used especially for the fabrication of inorganic scaffolds for bone replacement and tissue engineering.Thanks to its structure and composition, this mineral is able to chemically interact with different organic molecules such as proteins and antimicrobial peptides, representing a potential carrier for the delivery of bioactive compounds in the development of active systems.

On the basis of the above considerations, my research project has been focused on the development and optimization of an alginate-based edible coating enriched with hydroxyapatite crystals complexed with active compounds, for the shelf life extension of food products.

Among active compounds, the flavonoid quercetinhas been already used in different active systems mainly for its antioxidant capacity; However, my interest was also focused on its potential antimicrobial activity.The results on antimicrobial activity of quercetin glycoside compounds against *Pseudomonas fluorescens*, one of the most abundant Gram negative bacteria responsible for meat and meat products spoilageon obtained, showed a total bacterial reduction at 1000 mg/L and 500 mg/L of quercetin,Similar results were obtained when quercetin was loaded into hydroxyapatite structure, at the same quercetin amount.

Hydroxyapatite/quercetin complexes were preliminarily analysed by SEM, FTIR and zeta potential analysis, confirming the adsorption of quercetin into hydroxyapatite crystals that showed a micro-size dimension. Then, alginate-based coatings, developeddunder optimized conditions, were characterized for their ability to release quercetin in an aqueous medium, which represents the base condition during the development of active edible food packaging. The results showed a very fast release of the active compound, highlighting the ability of the developed coating to be really used in food applications, where the release of the active compounds should occur in the first hours of contact, to avoid or slow down the starting of spoilage and extend the shelf life.

Thus, the effectiveness of alginate-based edible coating enriched with hydroxyapatite/quercetin complexes was evaluated on fresh chicken fillets and fresh-cut papaya.

The microbiological analysis performed during the cold storage of coated chicken fillets highlighted the capability of alginate coating with hydroxyapatite/quercetin complexes to significantly inhibit the growth of spoilage bacteria, as well as the total volatile basic nitrogen.

Moreover, the comparison among the coated samples pointed out a positive effect of hydroxyapatite to slow down the changes in hardness during the storage time of 11 days. Finally, the outcomes of colour, odour and taste evaluation both in raw and cooked fillets demonstrated that the coating with hydroxyapatite/quercetin complexes was able to preserve the sensory attributes of fresh poultry meat until the 11th day of storage.

Also in the case of fresh-cut papaya, the microbial analysis carried out during the storage period pointed out the positive effect of hydroxyapatite charged with quercetin to inhibit the growth of spoilage bacteria, as well as to slow down the respiration rate of fresh-cut papaya, preserving the antioxidant compounds naturally present in papaya, including carotenoid compounds.

With the aim to increase the antimicrobial activity of quercetin glycoside compounds, lactoferrin was added to hydroxyapatite/quercetin complexes considering its well-known antimicrobial activityversus*Pseudomonas fluorescence*.Thus, the synergistic affectof lactoferrin and quercetin loaded into hydroxyapatite structure was evaluated. The results highlighted that the antimicrobial activity depends both on the lactoferrin and quercetin concentrationandonwhich molecule is adsorbed firstinto HA structure.In particular, the highest inhibition versus *Pseudomonas fluorescence* was obtained when the HA was first incubated with lactoferrin and then with quercetin, at concentration of 100 ppm (w/v) for both compounds.

The kinetic release of quercetin and lactoferrin from alginate-based coating in an aqueous medium pointed out a faster release of lactoferrin than quercetin due to the different solubility of the active compounds in the aqueous medium desides the different interactions of the active compounds

could show with hydroxyapatite structure.However, the equilibrium was reached in 70 h and 30 h for quercetin and lactoferrin respectively. Finally,the effectiveness of alginate-based coating loaded with hydroxyapatite/lactoferrin/quercetin complexes on fresh-pork meat was evaluated.

The activated alginate-based coating with showed a high capability to slow down the growth of spoilage bacteria during the storage of 15 days at 4°C, as well as the production of the total volatile basic nitrogen. The activated coating showed also high efficacy in preserving the colour and texture parameters of pork meat. Finally, according to sensory results, fresh pork meat samples coated with alginate-based coating loaded with hydroxyapatite/ lactoferrin/ quercetin complexes still showed, at the end of the storage period, suitable sensory attributes for commercial purposes.

Introduction

1.1 Food Packaging: traditional role and new tendencies

In the food industry supply chain, there is a great interest in the quality and process of finding, preparing, and analyzing food products. The food industry seems to show little interest in food processing practices, which allow food preservation over time and until the end of its commercial life. Food packaging is part of the complex food industry chain, as a fundamental element requiring specific professional figures with adequate knowledge of the product characteristics and food packaging technologies.

Food packaging is an essential point of the food supply chain. It plays a critical role in protecting food products from sources of degradation and preserving the quality and hygiene of products. Food preservation is one of the most important steps of the food chain. Most of the food, both animal and plant-derived, tend to undergo chemical-physical changes, leading to progressive deterioration.

Biochemical modifications are the main cause responsible for the commercial loss of food products. They result from the action of endogenous enzymes, such as the enzyme autolysis of post-mortem meat, or exogenous enzymes generated by microorganisms, generally bacteria or mold (Daniloski*et al*., 2019;Jeevahan e Chandrasekaran *et al*., 2019; Umaraw*et al*., 2020).

Many of these microorganisms, in particular pathogens, could cause severe health problems to consumers, especially if food is handled and distributed under inappropriate conditions. Therefore, processed food requires protection from spoilage during preparation, storage, and distribution to extend the shelf-life and preserve food quality.

Traditional preservation techniques, such as heat treatment, salting, or acidification, have been applied for a long time by the food industry to prevent the growth of spoilage and pathogenic microorganisms in food. However, they often result in unacceptable losses in food nutritional values. New strategies related to food storage have been developed. They consist in novel preservation techniques, which ensure food protection, without hampering organoleptic and nutritional properties.

A good packaging system also reduces food waste, at both industrial and domestic levels. Data from the United Nations Food and Agriculture Organization (FAO Food and Agriculture Organization of the United Nations) indicated that about one-third of the entire world's agricultural

products destined for human consumption are wasted every year. Therefore,the " Global food loss and waste " program was instituted. Food waste, as well as being unacceptable from an ethical point of view, has serious and significant social, economic and environmental consequences. It is responsible for 10% of greenhouse gas emissions and large consumption of water resources.

For this reason, the UN has included the Sustainable Development Goals of the 2030 Agenda, which aims to "halve the global per capita food waste at retail and consumption by 2030, as well as reduce the losses food along the production and supply chain ". Even the European Commission in presenting the "A 'Farm to Fork' Strategy for a fair, healthy and environmentally friendly food system" to the EU Parliament contemplated the promotion of more sustainable food consumption and the reduction of food losses and waste. Therefore, developing suitable food packaging can help to reduce and prevent the generation of food waste (Pooja Saklani*et al*., 2019).

Conventional food packaging is characterized by synthetic, non-renewable, and non-biodegradable plastic materials, responsible for serious environmental drawbacks. They have been considered a major source of environmental pollution by consumers. Globally, the food packaging sector produces large volumes of plastic, which represent a danger for terrestrial and aquatic organisms. All of this is due to the changing habits of society, a common choice is in fact to prefer single-use packs, to eat meals out (Ncube LK *et al*., 2020).

The traditional role of food packaging continues to evolve in response to changing market needs. The increasing demand for safer, healthier, and higher quality foods, ideally with a long shelf life; for convenient and transparent packaging and the preference for more sustainable packaging materials have led to the development of new packaging technologies.

1.2 Biobased food packaging

The increased interest in sustainable development have attracted great attention in biopolymers as a potential replacement for conventional materials. Biopolymers are polymeric biodegradable materials, which can have a composition of biobased (Torres-Giner*et al*., 2018). Biopolymers include plant-derived materials (starch, cellulose, other polysaccharides, protein), animal products (proteins, polysaccharides), microbial products (polyhydroxy butyrate), and polymers synthesized chemically from monomers of natural origin (polylactic acid) (Fig 1.1).

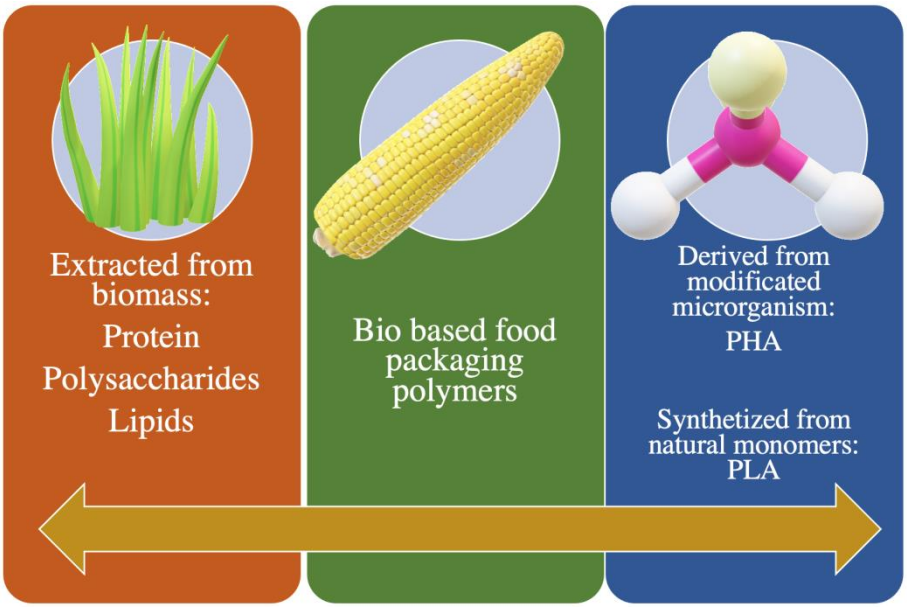

Figure 1.1:*Schematic representation of Biobased food packaging polymers. (PHA=PolyHydroxyAlkanoate; PLA=PolyLactic Acid).*

The increased interest in sustainable development has attracted great attention to biopolymers as a potential replacement for conventional materials. Biopolymers are polymeric, biodegradable materials, which can have a composition of biobased (Torres-Giner*et al*., 2018). Biopolymers include plant-derived materials (starch, cellulose, other polysaccharides, protein), animal products (proteins, polysaccharides), microbial products (polyhydroxy butyrate), and polymers synthesized chemically from monomers of natural origin (polylactic acid) (Fig 1.1).
Often, the term biopolymer or bioplastic are considered synonyms. The term biopolymer refers to a polymer produced by living organisms, therefore entirely bio-based, and biodegradable. The term bioplastic, on the other hand, has a broader meaning, and bioplastics do not necessarily mean biodegradable polymers derived from renewable sources. According to European
Bioplastics, a material is called bioplastic if at least one of the two characteristics following is verified:

• It derives, even partially, from renewable resources,
• It is biodegradable.

The term bioplastic, therefore, basically is used to address three families of polymers:

• Polymers derived from renewable and biodegradable sources (biopolymers in the strict sense),
• Polymers derived from renewable sources but not biodegradable,
• Polymers derived from non-renewable sources (petroleum), but biodegradable.

Biopolymers are the basis of a series of alternative packaging systems, safe for the consumer and with minimal environmental waste such as biodegradable packaging, active packaging, and edible packaging.
Therefore, the new packaging systems are not only intended to provide barrier and protective functions but interact with the food actively, exerting an oxygen scavenging function, antimicrobial activity, humidity, and others. As defined in the European regulation (EC) No 450/2009, active packaging systems are designed to "deliberately incorporate components that would release or absorb substances into or from the packaged food or the environment surrounding the food." Currently known active packaging systems are: oxygen scavengers, carbon dioxide emitters/absorbers, moisture absorbers, ethylene absorbers, emitters of ethanol, flavor and odor release/absorption systems, antioxidants, and antimicrobials (Ozdemir&Floros *et al.*, 2004).
An interesting innovation in active packaging is antimicrobial packaging, which serves to improve the safety and shelf life of food products. It constitutes a new system development that incorporates the antimicrobial agent in the packaging material or the formulation of the film, in order to inactivate the vital functions of microorganisms that contaminate food, reduce the rate of growth and decrease rapidly their number, thus maintaining microbiological security. The addition of active substances, such as antimicrobials and antioxidants, using active packaging instead of direct addition to the food, may decrease the number of such substances required. In fact, for the freshest and most processed food, food degradation or microbial growth occurs at the surface of the food. Therefore, the addition of active substances via active packaging may be more effective than their addition to the bulk of the food. In this context, packaging and food become "the same", as elements that can interact. In recent years, the research sector has developed packaging systems that always play an active role but become an integral part of food: active edible packaging

1.3 Active edible packaging

Active edible packaging is a real innovation in the field of food packaging. It offers a physical barrier to external factors and acts as a carrier of active substances to maintain organoleptic properties and ensure food safety, reducing food waste.

In Europe, consumers are increasingly looking for sustainable and environmentally friendly products, especially for food packaging. According to a 2021 study by the FMCG Packaging Observatory, (FMCGgurus, 2021) " 14% of Italians have stopped buying products due to a packaging that did not present elements of sustainability and more than half of the respondents said that it could do so in the near future" in the case of food packaging, the new frontier is represented by edible packaging. The term edible packaging indicates packaging that not only protects food, prolonging its shelf-life, but that can be consumed with it. There are, however, several problems, which limit the growth of edible packaging, such as problems of contamination of raw materials during storage, design considerations, investments, and replacement costs of competing technologies. To overcome these uncertainties, long-term laboratory- and pilot-scale experiments are needed to assess the performance and reliability of these preservation systems. In this regard, the consumer demand for naturally preserving food has led to extending the use of edible biopolymers obtained from industrial by-products. However, items that are edible or are in contact with food should be generally recognized by qualified experts as being safe under the conditions of their intended use, with the amount applied following good manufacturing practices. As consequence, these food-safe materials must typically have the approval of the Food and Drug Administration (FDA) (Pavlath*et al*., 2009).

A central role in edible packaging is played by edible film and edible coating.

The use of biopolymers from natural polymer substrates (polysaccharides, lipids, and proteins) (Fig 1.2) finds wide application in the case of edible films and coatings that have attracted the interest of researchers and the food industry since they can improve not only the shelf-life of but also the safety, quality, and functionality of food products (Gallego *et al*., 2020). The physical and chemical characteristics of biopolymers greatly influence the properties of the resulting films and coatings. In particular, the proteins, that are often used as film-forming materials, have the feature to be easily modified in the tertiary and quaternary structures, obtaining desirable properties of the film.

Protein film-forming materials were obtained from various animal and plant sources, including animal tissues, milk, eggs, cereals, and oilseeds (Andrade *et al*., 2012).

Film-forming materials can also be obtained from polysaccharides. The conformations of polysaccharides are more complicated and unpredictable than proteins, forming larger molecules with higher molecular weight. Most carbohydrates are neutral, some are negatively charged with very exceptional cases of a positive charge. Due to a large number of hydroxyl groups and other hydrophilic fractions in the structure of neutral

carbohydrates, hydrogen bonds play the most significant role in the formation and characteristics of the film (Bertuzzi&Slavutsky *et al*., 2016).

Some negatively charged substituents, such as alginate, pectin, and carboxymethylcellulose, exhibit significantly different rheological properties in acid than in neutral or alkaline conditions, as well as in the presence of multivalent cations (TugceSenturkParreidt*et al*., 2018). Lipids are also used as film-forming materials. It is improper to define them as biopolymers; however, they produce biodegradable and edible materials (Bertuzzi&Slavutsky *et al*., 2016). Most edible lipids and resins are soft solids. They can be manufactured into any shape by casting and molding systems after heat treatment, causing reversible phase transitions between fluid, soft-solid, and crystalline-solid states. Due to the hydrophobic nature, films or coatings made with lipid film-forming have a high-water resistance and low surface energy. Lipids can be combined with other film-forming materials, such as proteins or polysaccharides, such as emulsion particles or multilayer coatings to increase water penetration (Alves da Silva Rios *et al*., 2022).

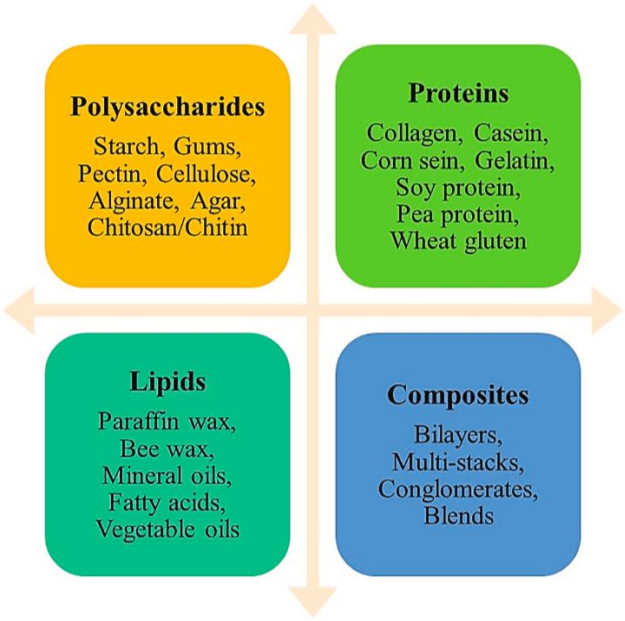

Figure 1. 2:*Edible materials (Petkoska et al.2021)*

1.3.1 Alginate based coatings

Alginates belong to the categories of adjuvants, gelling agents, and thickeners. Their function is to interact with free water, engaging it in stable structures present in food.

In molecular terms, alginates are a family of binary copolymers unbranched units of β - D - mannuronic acid (a) and α - L - guluronic acid (b) joined by α (1-4) and β (1-4) bonds, having different composition and sequential structure (Fig 1.3).

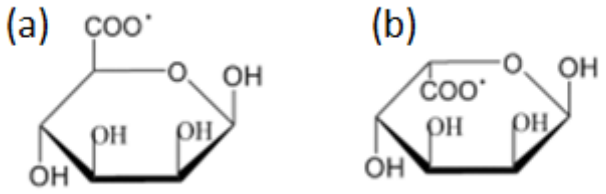

Figure 1.3:*Cyclic structure of the two acids*

Commercial alginates are mainly obtained from about ten species of algae, such as *Laminaria hyperborea*, *L. digitata* and *Ascophillum nodosum* (widespread in Europe, England, Norway, France), *Macrocystis pyrifera* (U.S.A.), *Ascophillum nodosum* (Canada) and to a lesser extent by *Laminaria japonica*, *Ecklonia maxima*, *Lessonia negrescens*, and *Sargassum spp*. (Senturk Parreidt *et al*.,2018). The differences in the structure and monomeric composition of algal alginates make the products available on the market extremely variable in terms of chemical-physical characteristics Alginates with very different compositions can be isolated from bacteria: e.g., *Azotobacter vinelandi* and also from different species of *Pseudomonas spp*. Sodium alginate is the most preferred product given a large number of industrial applications; alginic acid and calcium, potassium, ammonium, and propylene glycol alginates are also available.

The chemical-physical factors that influence the thickening properties of aqueous solutions of alginates are: molecular weight, temperature, pH, concentration, and presence of other solvents, chelating agents, monovalent salts, polyvalent cations and ions ammonium (Qin *et al*., 2006). As the temperature increases, the viscosity of the alginate solutions decreases by approximately 12% for each increase of 15.5 ° C. As the temperature decreases, viscosity increases without gel formation. The viscosity of alginate solutions is not affected by freezing and subsequent thawing. The alcohols and glycols cause an increase in viscosity in the alginate solutions and its eventual precipitation. For example, 1% sodium alginate solutions tolerate up to 20% ethanol and 70% glycerol before precipitation occurs. Sodium alginate is sufficiently stable for pH between 4 and 11, while it loses stability and precipitates at pH below 4 (Sellimi*et al*., 2015).

Traditional models present the gel as formed by simple one's ionic bonds between two carboxyl groups of two adjacent polymer chains and a calcium

cation. The ability to form these chemical bonds was mainly found for L-guluronic acid polymers with a degree of polymerization greater than 20 monometric units (Qin *et al*., 2006). The pairs of polygonal sequences tend to correlate along the axes, giving rise to a series of cavities that act as active sites for calcium ions. Long sequences of these sites lead to the formation of cross-links with similar sequences present in other alginate molecules, creating a very compact structure called "egg box" (Fig 1.4) (Daohao*et al*., 2015).

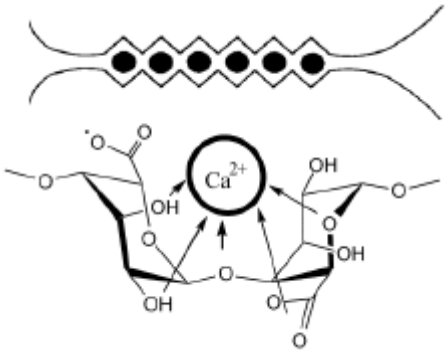

Figure 1.4:*Egg box model for the gelation of alginates(Alistair e Stephen, 1995).*

Calcium ions are not only attracted to carboxyl groups, but also other electronegative atoms, such as oxygen and hydroxyl groups. In any case, there is a so-called "primary" aggregation with the carboxylic ions and a "secondary" one thanks to the polar interactions (Daohao*et al*., 2015). In general, it can be noted that the capture of calcium ions depends on the pH of the solution as a result of the balance between hydrogen ions and calcium ions: at pH 3 the excess of H+ ions does not allow the binding of Ca2+ ions to the polymer.

Stoichiometrically, to obtain complete saturation of the alginate of sodium, calcium must be added in an amount equal to 7.2% of the weight of the alginate itself. By adding 3%, you have a good gel, while with 1% the aggregation is very weak (Lii*et al*., 2003). The property of alginate and its ability in forming gel by reaction with a certain number of divalent cations is very useful and exploited in the food sector.

1.3.2Edible film and coating

Depending on the production method, it is possible to differentiate between edible film and edible coating.

An edible coating is a thin layer of edible material formed directly as a coating on the food product, while an edible film is a thin film of edible material that can only be applied to the food after it has been performed. The main difference between these two systems is that edible coating is applied in liquid form on the food, usually by dipping the product in a solution formed by a structural matrix (carbohydrates, proteins, lipids, or a multicomponent mixture), while the edible film is first modeled as solid sheets which are then applied in the form of a film on the food product.

1.3.3Methods of edible film formation

The edible films can be obtained from edible materials through two different methods: wet and dry processes; they are also called solvent casting and extrusion processes, respectively. The solvent casting method involves three steps to prepare a film from biopolymers:

solubilization of biopolymer in a suitable solvent, the casting of the solution in the mold, and drying of casted solution (La Fuente*et al*., 2022).

The air-drying procedure for casting edible film is a very important step for polymerization. Quick drying can have negative effects on physical and structural properties; the high temperature used for drying the film can modify the nature of biopolymers and inhibit the action of antimicrobial substances. Extrusion is another method used for producing polymeric films and is one of the major polymer processing techniques currently in use at a commercial scale, but the main disadvantages of extrusion film procedures are mainly focused on the limitation of processing only temperature tolerant and low moisture raw material blends, which restricts the use of certain polymers. Also, a higher initial cost of specialized equipment and a higher maintenance cost affect the usage of this process.

Electrospinning, on the other hand, is a system that allows nanotechnology to be applied to the edible film sector. It was possible thanks to the introduction of nanohydrogels, nanoemulsions, and nanoparticles, the control of gaseous exchanges, and the vehicle of substances such as enzymes, antimicrobials, and antioxidants, able to provide greater effectiveness in prolonging the shelf life of food (TrajkovskaPetkoska*et al*., 2021).

1.3.4Methods of edible coating applications

Edible coatings are applied directly onto the surface of the food and can be consumed together with the product.

Fruits, vegetables, and meat are foods that can be coated, but the effectiveness of applying edible coatings may have interfered with the type

of technique used (Bhuiyan *et al*., 2020). Among the main methods of application of the edible coating, immersion, spraying, fluidized bed, and panning are the most frequent (Suhag *et al*., 2020).

There are several methods of applying edible coatings. The choice of an appropriate method depends on the characteristics of the food, coating materials, the desired effect, and cost (Andrade *et al*., 2012).

Edible coatings can be applied by dipping the products into the solution and then letting the excess coating drain as it dries and solidifies. This method is advantageous when the products require a total coating, it allows to obtain good uniformity around a complex and rough surface. This method is commonly used for coating fruit, vegetables, and meat products (Andrade*et al*., 2012).

The brushing method of applying the coating is the most economical. It consists in applying the coating directly on the surface of the product, with the use of special brushes. Edible coatings generally are applied to highly perishable fruits and vegetables such as beans, strawberries, and berries (Raghav *et al*., 2016).

Fluid bed coating is a technique that can be used to apply a very thick layer thin on dry particles of very low density or small size. It was originally developed as a pharmaceutical coating technique, but its use in the food industry is gradually increasing. It can be used to enhance the effect of ingredients and additives such as processing aids, preservatives, flavorings, and other additives that improve the taste and color of food.

Panning is usually used for coating candy, nuts, and some fruits

which are characterized by a smooth and regular surface. The product to be coated is deposited in a large rotating bowl called a "pan". The coating solution is then applied through a pump that sprays it inside the rotating pan, distributing it evenly on the surface of the food product. Forced air, is applied to dry the coating (Andrade *et al*., 2012).

When a thin and uniform coating is required for certain surfaces, spraying is the most used. This is the most popular method for coating whole fruit and vegetables, with the development of high-pressure spray applicators and atomization systems of the air.

Coating application methods on food products have demonstrated numerous merits/demerits. The most important factor is that these methods are dependent on the properties of food products and physical characterizations such as surface tension, density, and viscosity of coating materials. The physical parameters of food products such as shape and size, and desired coating thickness, influence the selection of the coating system and device, the advantages are reduced costs and the absence of solvents that evaporate at high temperatures.

1.3.5 Layer-by-layer coating deposition technique

Layer-by-Layer (LbL) is a versatile approach introduced by Decher and coworkers, which allows the fabrication of finely tuned layered materials.

LBL self-assembly is a technique that uses the intermolecular forces (electrostatic forces, covalent bonds, hydrogen bonds, host-host interactions, etc.) of two or more assembled materials (polyelectrolytes, biomolecules, inorganic nanomaterials, small organic molecules, etc.) (Chenghui*et al*., 2022) to deposit them alternately on the surface of the model. The most common chemical forces used to prepare LbL structures are electrostatic interactions. The first LbL systems were based on the sequential deposition, on a charged surface, of polyelectrolytes, polycations, and polyanions (Arnon-Rips *et al*., 2018).

The multilayer coating or the LbL technique is based on the immersion of the food product in different coating solutions containing polyelectrolytes of opposite charge to obtain a physical and chemical link between them (Arnon-Rips *et al*., 2018).

Currently, the chemical principles behind the formation of LbL are based on the charge overcompensation mechanism, which is based on intrinsic and extrinsic charge compensations and a combination with competitive ions. Extrinsic compensation is based on ion pairing (on the surface) with charged counter-ions (which are present within the bulk of the multilayer). During the LbL development, this extrinsic charge compensation is converted into intrinsic charge compensation, leading to the release of counter-ions and the release of solvating water molecules, with an entropic driving force for the LbL assembly process.

The LbL process will follow a linear growth mode when there is a strong attraction between polyelectrolytes, while when an attraction between two polyelectrolytes is relatively weak, the LbL method will follow an exponential growth mode, where the amount of the adsorbed polyelectrolytes will exponentially increase with the number of bilayers (Joseph *et al*., 2014).

Poverenov (2014) developed an LbL edible coating with an inner negatively charged alginate layer with good adhesive properties.

Alginate can form strong gels or insoluble polymers through cross-linking with Ca++ by post-treatment of $CaCl_2$ solution.

The LbL technique is optimal for edible coating applications, as the appropriate coating components. According to the application, it is possible to choose the appropriate number of layers, their concentrations and the order of application. Some studies compared multilayer coatings with single coatings, showing better performance of the first, on the shelf life of products, (Arnon*etal*., 2015; Poverenov*et al*., 2014; Velickova*et al*., 2013). Other studies showed that a single alginate-based coating layer, reinforced with hydroxyapatite nanocrystals, is also able to improve and extend the

shelf life of fresh-cut fruit (Montone*et al*., 2022) and chicken fillets (Malvano*et al*., 2022), up to 15 and 11 days respectively.

LbL is applied to fruit, vegetables and meat products, with satisfactory results in terms of the formation of edible coatings and improvement of food quality. The LbL method can also be employed in the field of edible coatings with its ability to apply various biopolymers in a controlled manner to fine-tune the coating properties. The electrostatic interaction between negatively charged carboxylic groups of well-adhesive polyelectrolyte alginate and positively charged Ca^{2+}group has been exploited to obtain the coating on meat samples (Malvano*et al*., 2022).

The application of edible coatings, on hydrophobic surfaces such as fruit and vegetables or hydrophilic surfaces such as fresh-cut fruit, presents adhesion difficulties, which can be solved by modulating the first layer (Olivas *et al*., 2005; Rojas-Graü*et al*., 2009).The degree of adhesion depends on the chemical and electrostatic affinity of the coating material with the surface of the fruit. Higher adhesion could be obtained by adding functional ingredients such as emulsifiers (soy lecithin, stearic acid, and Tweens) and surfactants (Tweens) to improve coating adhesion (Miteluț *et al*.,2021).

The alternation of lipid and hydrocolloid layers showed better control over the water vapor and gas permeability properties of the coatings, compared to the same components mixed in film (Velickova*et al*., 2013).

LbL edible coatings have also been extensively studied, as an encapsulation matrix for active compounds. (Quirós*et al*., 2014).

The incorporation of active agents in LbL edible coatings is still at an early stage and there are only a few examples of such studies (Brasil*et al*., 2012; Sipahi*et al*., 2013; Martiñon*et al*., 2014; Moreira *et al*., 2014).

In these studies, nanoemulsions, nanoparticles, and other transport systems can be combined with the LbL method. These systems have a great advantage, by introducing active compounds into a layer and controlling its release, thus protecting the active substance from degradation, thanks to the formation of an overlapping layer. The LbL coatings can also protect the active compounds from undesired reactions with the environment.

From a practical point of view, the application of edible LbL coatings does not require special equipment or tools. The waxing process, for example, which is carried out on citrus fruits, apples, and other fruits and

vegetables, includes numerous drippers that spread the coating material on the product surface.

These systems can be adapted, at an industrial level, to different food products. No substantial changes would be required for the different production lines. Fresh and processed foods products may require a dipping process instead of the spraying method.

1.4Incorporation of functional ingredients

The growing interest in the use of edible packaging can also be associated with the coating that can be considered an excellent carrier for incorporating an extensive variety of compounds such as crosslinkers, strengthening agents, plasticizers, nutrients, antioxidants, antimicrobials, colorants, and flavorings, which are mainly used to improve the functional properties of coatings and to prolong the shelf life of food products (Cantú-Valdéz*et al*., 2020).

One of the characteristics of edible coatings is the ability to incorporate different ingredients in the matrix to improve its functionality. The plasticizers are mixed in the edible coating solution to increase the property mechanics, low molecular weight plasticizers bonded to the coating material proteins are used, to modify and improve the coating structural capacity. Plasticizers are generally hygroscopic and attract water molecules (Lin *et al*., 2007). The most used plasticizers are glycerol, fatty acids, sorbitol, polyethylene glycol, sucrose and monoglycerides. water can function as a plasticizer; however, it is easily lost because of dehydration in an environment with low relative humidity (Raghav *et al*., 2016).

Emulsifiers are surfactant agents capable of reducing the surface tension of the interface water-lipids or water air. Emulsifiers are essential for the formation of protein coatings or polysaccharides containing lipid emulsion particles. In addition, they also modify the energy surface to check the adhesion and wet ability of the coating surfaces (Lin *et al*., 2007).

The incorporation of antimicrobial compounds into edible coatings provides a way to improve food safety and the shelf life of foods ready for consumption. Common antimicrobial agents used in the food system, such as acid benzoic, sodium benzoate, sorbic acid, potassium sorbate and protonic acid, can be incorporated into edible films and coatings (Appendini*et al*., 2002).

Antioxidants can be added to the matrix coating to protect certain foods from oxidative rancidity, degradation e discoloration. Examples of these substances are butyl hydroxyanisole (BHA), butyl hydroxytoluene (BHT) and citric acid. The edible coating is an excellent vehicle for nutritional enhancement through the introduction of nutrients, flavors, and dyes. For example, in fruit and vegetable applications, they provide basic nutrients and nutraceuticals that are lacking or only present in small quantities (Lin *et al*., 2007)

1.4.1 Antimicrobial and antioxidant compounds

Consumption of contaminated food or beverages represents a serious threat to human health. According to the World Health Organization (WHO) estimates, approximately 10% of the human population worldwide experiences foodborne diseases every year (Heeyoung*et al*., 2021). Although

self-limiting gastroenteritis is the main clinical manifestation of foodborne illnesses, more severe signs could occur, especially in immunocompromised patients (Schirone*et al*., 2021). Consequently, the need for intensive care and hospitalization contributes to an increase in the economic burden. In this context, there is a continuous search for alternative approaches, aiming to prevent the occurrence of foodborne diseases and the resulting socioeconomic impact.

Products manufactured from food-producing animals are the major source of foodborne diseases (Heredia *et al*., 2018). Food-producing animals are a significant reservoir of microbial pathogens. Salmonella, Campylobacter, Staphilococcus aureus, Escherichia coli and Lysteria monocytogenes are the major pathogens responsible for foodborne diseases.

Microbial contamination could derive from different factors. Soil, air, and water, together with the processing environment are important sources of bacterial contaminants. Therefore, monitoring programs and/or adequate hygienic rules could represent an effective strategy to control food contamination. Contamination may also occur at different stages of the food chain, from production to consumption.

The last step of the food chain (transition from the producer to the consumer) is the most critical step in increasing the risk of food contamination. To ensure food quality and safety, active antimicrobial food packaging has emerged (Malhotra *et al*., 2015).

As an emerging alternative to the traditional techniques used for food preservation (salting, acidification, heat treatment, or preservative addition) (Mellinas*et al*., 2015), the active antimicrobial edible coating has attracted the interest of researchers and the food industry. The reasons of this growing interest are: 1) a longer antimicrobial activity due to the slow release of the antimicrobial agent; 2) maintenance of both nutritional and organoleptic properties; 3) antimicrobial agent stability (Dhumal *et al*., 2018).
Antimicrobial agents can be distinguished into two categories: organic or inorganic materials, which display high activity against both Gram-positive and Gram-negative bacteria (Sharma *et al*., 2020). Compared to inorganic substances, which are more resistant, organic compounds are inactivated by high temperature and pressure (Huang *et al*., 2019; Joerger*et al*., 2007).

Therefore, the functionalization of these sensitive compounds with biopolymers, to produce active coatings, is a valid strategy to improve their stability and ensure their function.

Organic acids are the most common antimicrobial agents used in active coatings. However, a great variety of antimicrobial agents have been reported to be used, including natural molecules. For the selection of the appropriate antimicrobial agent, different aspects must be considered. First, the good interaction with the polymer matrix; second, the presence of other additives; third, the microbial target (Valdes *et al*., 2017). By decreasing

bacterial growth, they also improve the chemical-physical parameters of food products.

In meat and fish products, spoilage psychotropic bacteria are responsible for the transformation of NPN compounds into ammonia, amines, and other basic nitrogenous compounds, which gives information about the content of ammonia and organic amines which affect the sensory acceptability besides being toxic for human health (Bekhit*et al*., 2021; Takma*et al*., 2019).

Along with antimicrobial compounds, novel active food packaging incorporates antioxidant compounds. Active food packaging is defined as a material enriched in active compounds (antimicrobials and antioxidants), to release or absorb substances into or from the environment or the packaged food (Ozdemir*et al*., 2004) and ensure quality, integrity, stability, and shelf-life extension of food products.

According to their mechanism of action, antioxidant substances can be classified into free radical scavengers, metal chelators, UV absorbers, oxygen quenchers, and oxygen scavengers. Free radical scavengers aim to convert free radicals into stable substances, while the remaining aim to reduce or prevent the occurrence of oxidation reactions. As a consequence, antioxidant packaging can: release antioxidant compounds into the food or adsorb and scavenge from the food substances responsible for the lipid oxidation (e.g. oxygen, radical oxidative species, or metal ions from the surface of the food product). The main advantage of active packaging containing antioxidant agents is that the release of the compound is controlled, and the incorporation of biopolymers is a good option to protect the antioxidant agent. Table 1 provides some studies on antimicrobial and antioxidant substances with alginate-based coating for food packaging applications.

Plant foods are one of the major sources of antioxidant compounds. They contain many phenolic compounds, which are synthesized - via the shikimate pathway - following stress conditions (Macheroux*et al*., 1999). Due to the hydroxyl group on the phenol ring, phenolic compounds exert an important antioxidant activity, by interacting with a great variety of free radicals. Phenolic compounds can be classified into three chemical classes: phenolic acids, flavonoids, and nonflavonoid polyphenols (Manach*et al*., 2004).Flavonoids are the most abundant polyphenolic compounds. 8000 different flavonoids have been identified in plants and, specifically, in plant cells or cell surfaces (Mutha*et al*., 2021). Quercetin is a flavonoid present in several fruits, vegetables, and plants, with onion as the major source. A wide range of beneficial properties has been attributed to quercetin. It possesses significant antimicrobial and antioxidant properties. Moreover, as GRAS (Generally Recognized as Safe) compound, quercetin is a good candidate usable as a food additive in polymeric matrixes (Nguyen *et al*., 2022). More specifically, it could represent a valid active compound in alginate-based coatings. Several studies have documented the ability of alginate to interact

with phenolic compounds. In detail, the hydroxyl group of the phenol ring forms hydrogen bonds with both the hydroxyl and carboxyl groups of alginates.

Among bioactive compounds, peptides and proteins also play key roles. Biopeptides showing antioxidant properties are protein fragments characterized by 3-16 amino acids, including histidine, tyrosine, methionine, cysteine, lysine, and tryptophan (Sánchez *et al*., 2017). They display antioxidant activities by controlling both enzymatic and non-enzymatic oxidation processes or inactivating free radicals (Tkaczewska*et al*., 2020). Many biopeptides also show antimicrobial properties. The most important sources of bioactive peptides are food-derived plants and animals or marine species, including algae (Tadesse *et al*., 2020).

The application of bioactive peptides obtained from the enzymatic hydrolyses of proteins, in the field of active packaging, is emerging (de Oliveira Filhoa*et al*., 2019). In addition, recent studies have demonstrated that alginate-based coating improves the protein hydrolysates properties (de Oliveira Filhoa*et al*., 2019), thus representing an interesting alternative to traditional coatings.

Table 1: Studies on antimicrobial and antioxidant substances with alginate-based coating for food packaging applications.

Matrix/Antimicrobial	**Principalresult**	**Reference**
Freshcutmelon/Chitosan	The coating was associated with microbial decrease, enhanced fruit texture up to 21 days	Poverenov et al. 2014
chicken fillets/essential oil of Ziziphora persica	Edible coating is able to reduce Pseudomonas spp., and Enterobacteriaceae, during 12 days of storage	Hussein et al.2021
ground beef/lactic acid,EDTA, nisin, potassium sorbate	The incorporetedsubstamnces inhibiting the growth of E. coli O157:H7 in ground beef at 10°C	Tony Jfang et al. 2003

seabass/tea polyphenols	The coating is able to reduce total bacteria expecially when was incorporeted the tea polyphenols	Nie, et al.2018
chilled chicken fillets/lactoperoxidase system	Results indicated that the coating had a substantial inhibitory effect on Enterobacteriaceae, and Pseudomonas	Molayi et al.2018
	Active compounds with potassium sorbate decreased the growth rate of Pseudomonas spp. and Enterobacteriaceae.	Lucera, A et al.2014
fresh-cut papaya/quercetin	the total bacteria count in samples covered, was significantly lower respect to uncoated	Montone et al 2022
chicken fillets/quercetin	Alginate coatings significantly inhibit the growth of spoilage bacteria during the cold storage period	Malvano et al.2022

Matrix/Antioxidants	**Principalresult**	**Reference**
porkchops/rosemaryoleoresin	Lipidoxidationwasinhibited	Wu et al.2016
slicedcarrots/citric acid	Coating process, when applied together with a modified atmosphere, enhanced the shelf-life extension effect.	Amanatidou, A. et al.2000
rainbowtroutfillet/resveratrol	Edible coating significantly decreased the lipid oxidation	Jalali, N. et al.2016

fresh-cut papaya/quercetin	Alginate coatings promoted firmness, lightness and totalphenoliccontent	Montone et al 2022

1.4.2 Nanosystem in edible coating

When functional ingredients are incorporated into a polymer, they have the disadvantage that the migration and release of the molecules cannot be easily predicted and controlled. This often involves an initial rapid release of the active constituent in the food, thus limiting its availability over time. The morphology of the polymer matrix plays an important role in the release rate of the active molecule. Biopolymers as packaging materials are relatively poor materials because of their mechanical and barrier properties which currently limit their industrial use. A particular challenge is the development of moisture barrier properties because of the hydrophilic nature of biopolymers (Zhang *et al.*, 2020). The principal problems are lower mechanical, thermal, and barrier properties, processability, the degree of degradation under certain conditions, changes in the mechanical properties during the disposal, the potential growth of microorganisms, and the release of harmful substances.

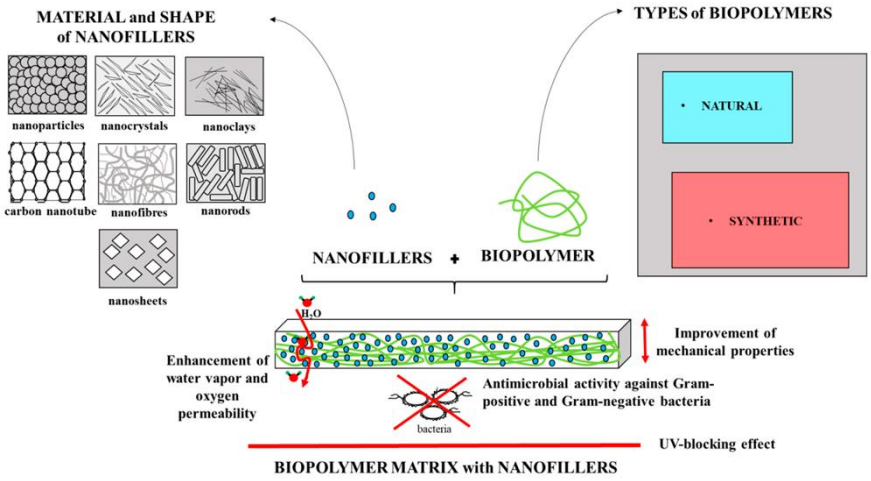

Figure 1.5:*Schematic representation of nanosystems with biopolymer matrix (Jamróz et., al 2019)*

However, the application of nanotechnology, by incorporation of nanoparticles or preparing polymer nanocomposites can help to overcome these problems (Sharma *et al*., 2017).Nanotechnology in the food segment

aims to develop new packaging from several types of nanocomposites, including nanotubes, nanoparticles, nanolaminates, and nanodispersions.
Nanoparticles have a high specific surface, and the advantage of ultra-large contact area per unit of volume between the polymer matrix and nanoelements, greater functionality per unit of weight, and the lack of high surface energy results in agglomeration of the particles. To exploit specific functional properties of polymer nanocomposites, a good dispersion of nanoparticles were required. The final properties of polymer nanocomposites depend on the uniform dispersion of nanoparticles (no agglomeration) (Vasile*et al*., 2018), which depends on the type of nanoparticles (size, specific surface area), the concentration of nanoparticles and polymer matrix type, the interaction between the nanoparticles and polymer matrix, and the manner of production (in solvent evaporation, in situ polymerization, blending in the melt). The challenge in obtaining new materials is the structure of polymer nanocomposites with desired properties.
In food packaging, the potential of nanotechnology is being actively explored, including active packaging. Emphasis is given to inorganic nanoparticles which can be complex with functional compounds. Thanks to their small size, nanomaterials can interact at different levels of the bacterial cell, causing damage and/or destruction (Jamróz*et al*., 2019). In general, they can penetrate the cell envelope, produce harmful secondary metabolites, and oxidize cellular components. TiO2 nanoparticles inserted in packaging systems have shown bactericidal effects against*Salmonella strain*, andStaphylococus *aureus*. similarly, films coated with nano-ZnO showed antimicrobial effects against*ListeriaMonocytones*and*S. Enteritis*. However, it has been shown that the disadvantages of packaging materials based on biopolymers can be reduced by the application of nanocomposite technology, which also represents a good strategy for bacteria control.

1.4.2.1 Hydroxyapatite

Hydroxyapatite is the most widely used biomaterial in the biomedical field for the preparation of bioceramics. the chemical similarity to the inorganic components s of bones and teeth gives this material good biocompatibility with living tissues. For this reason, this material has aroused considerable interest over the past few decades and has become the most important material used for the construction of artificial bones or their regeneration (Ptáček*et al*., 2016).
The inorganic mineral phase that makes up calcified tissues, such as bones and teeth, is calcium phosphate, which can be traced back to the structure of synthetic hydroxyapatite, Ca10 (PO4) 6 (OH) 2 (Fig 1.6).

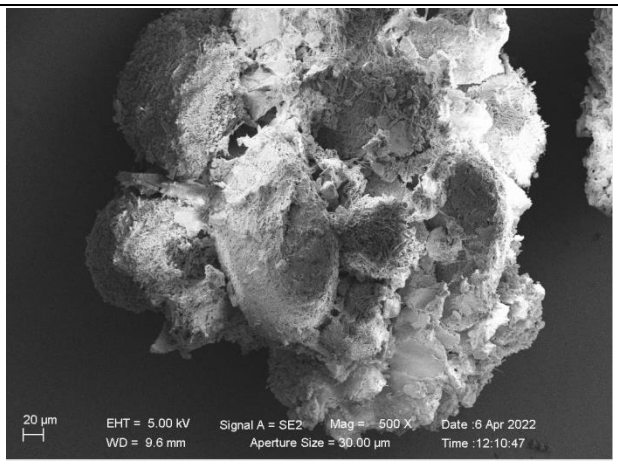

Figure 1.6: *SEM image of hydroxyapatite nonocristals*

Biological apatites differ from the synthetic form for several aspects: in fact, they are less crystalline and less stoichiometric, going to incorporate foreign ions such as Na +, K +, etc. (Bigi*et al*., 2004).
Besides its utilization to replace bones, HA is also used as a catalyst, in products of the pharmaceutical or fertilizer industry, in water treatment processes, and also as a controlled drug release.
Various methods have been used in the literature for the synthesis of calcium phosphates, such as solid-state reactions, hydrothermal processes, dry processes, and wet processes (Fig1.7).
The most commonly used method is precipitation, in which the chemical reaction between calcium ions and phosphate ions takes place while keeping both the temperature and the pH of the solution under control. This is a simple technique that uses inexpensive raw materials, low temperatures of reaction (not higher than 100 ° C) with low operating costs.

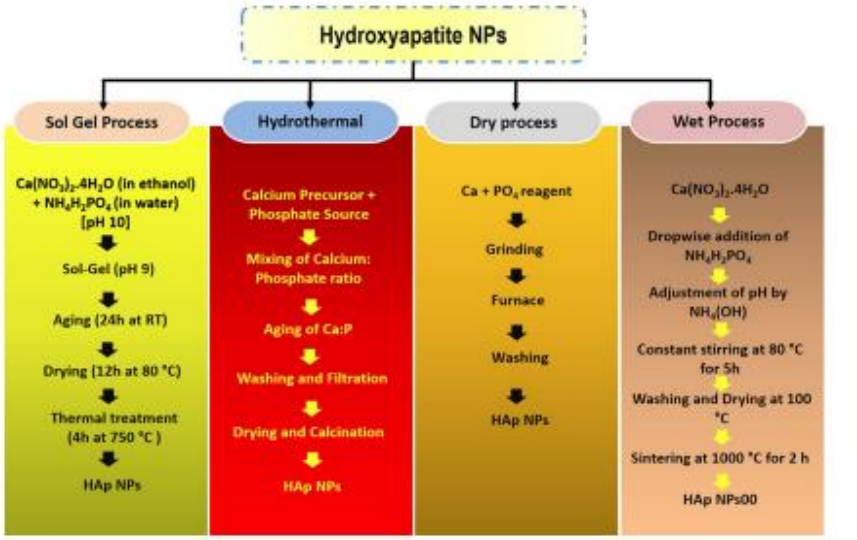

Figure 1.7: *Schematic representation of physicochemical synthesis of hydroxyapatite (Selvam Sathiyavimala et., al 2020)*

However, according to the method and conditions of synthesis, chemical, structural and morphological properties of synthetic hydroxyapatites can be modulated aspects (Palazzo *et al*., 2007). the properties of the synthesized material can also be changed and improved by functionalizing the surface with different molecules, metal ions, proteins, or drugs. *(*Sathiyavimala*et al*., 2020*)* Hydroxyapatite is known for its ability to bind a wide variety of molecules and therapeutic agents that can act both as scaffolds for the formation of new tissues and for the release of biomolecules over time previously functionalized (Koutsopoulos*et al*., 2002).

Some investigations have focused on using hydroxyapatite as filler to reinforce some natural polymers like alginate, gelatin, chitosan, and collagen and produce high-quality composites (Wang *et al*., 2016; Raz *et al*., 2018). Most biopolymers contain numerous groups of hydroxyls, amines, and carboxyls. These functional groups are not only beneficial for enhancing the interfacial bonding between HA and polymer but are also effective when combined with antibiotics. Besides, biopolymers also have the advantages of plasticity and biodegradability. To enhance the interaction between the inorganic apatite and the organic polymers, in-situ and ex-situ processes have been used. Ex situ refers to introducing hydroxyapatite briefly synthesized and then simply mixed into the biopolymers, while in the case of in situ procedures, apatite is formed by a process of alternately soaking in CaCl2 solution and a MK2HPO4 solution (Tachaboonyakiat*et al*., 2001). Among the studies of medical applications and dental care collagen-HA

composite materials have attracted considerable attention and have good prospects as they have excellent biocompatibility and bioactivity, as well as improved mechanical properties (Iafisco*et al*., 2009). Few studies have focused attention on the application of hydroxyapatite in the food packaging sector, a scientific work that exploited the electrodeposition of hydroxyapatite on plastic food films for the preservation of sliced apples (Nocerino*et al*., 2014) and a study that attempted to prepare apatite in situ in a cowhide collagen film for food packaging applications (Wang *et al*., 2016). The use of hydroxyapatite in active edible coating based on natural biopolymers was investigated only in these two studies (Malvano*et al*., 2022;Montone*et al*., 2022), where alginate-based coatings functionalized with hydroxyapatite have been developed for the preservation of meat products and fresh-cut fruit. Both works will be detailed in chapters three and four of this thesis.

The use of hydroxyapatite in food is authorized by Reg. (UE) n. 231/2012 laying down specifications for food additives established by Regulation (EC) No 1333/2008. The use of hydroxyapatite as a carrier in active packaging could protect the active compound from environmental factors, processing, and storage conditions allowing to maintain of the compound's antimicrobial, and antioxidant properties to extend the shelf life of foods.

1.5 Release of bio active compounds

One of the main objectives of bioactive edible coatings is the release of active compounds with controlled kinetics, to extend the shelf life of food products. The control of the release process, with well-determined release rates and migration amounts, is a critical aspect, especially in the biotechnology field.

Zactiti and Kieckbusch (2009) determined the permeability and release of potassium sorbate from alginate films and evaluated the effect of three levels of alginate cross-linking (with different concentrations of calcium chloride). It has been shown that with the increase of the cross-linking agent (calcium ions), the mobility of the active substance has been prevented.

The kinetics of the release of the active ingredient contained in the functionalized nanostructures and embedded in a matrix usually formed by edible polymers are mainly influenced by the choice of the polymeric matrix based on the edible coating and different barriers that the bioactive substance must cross to interact with the food surface.

Therefore, the study of the release kinetics of functionalized bioactive materials in nanostructures is of great importance since it allows for an increase in the shelf life of the food (Dan *et al*., 2016).

It is possible to control the release of active compounds from edible coatings, considering parameters such as humidity, temperature, pH, and antimicrobial and nutraceutical effect on the matrix, at different times during the storage or ingestion of food (Quirós-Sauceda*et al*., 2014).

There are different mechanisms to release of an active compound through the components of edible coatings, such as melting, degradation, swelling, or rupture of nanostructures and diffusion release (González-Reza *et al*., 2018). Different release models can be used to explain this. According to both time and how the matrix is modified, different kinds of release kinetics can be identified. The non-time-dependent zero-order release kinetic

$$Mt=k0t+M0 \quad (1)$$

favors a constant release of the active compound (k0), maintaining its concentration and preserving food quality (Peppas et al 2014). However, it has the disadvantage that active compounds can be removed quickly from the coating surface, affecting food-conservation properties.

The kinetic release time-dependent considers variations in the concentration of the bioactive compound favoring an increasing release during storage. Results were expressed as a percentage ratio of $Mt/M\infty$ (Mt is the concentration of bioactive compounds diffused at time t, and $M\infty$ represents the concentration of bioactive compounds diffused at equilibrium) (Malvano*et al*., 2021).

The release of a bioactive substance with low molecular weight can produce the diffusion of water into the polymeric matrix, followed by its relaxation with the subsequent diffusion of the active compound on the food surface (Mastromatteo*et al*., 2009). Modifications of the edible polymer coating by the nanostructures employed in the formulation of the edible coating can provide other release kinetics, compared to the kinetics that is observed when the polymer matrix is not functionalized by nanostructures (Tamjidi*et al*., 2013; Acevedo-Fani*et al*., 2017).

Some studies have analyzed the release of the active ingredient only "in vitro", and only a few have analyzed its applications in food (Magnuson *et al*., 2013; Jain *et al*., 2022). Bustos, Alberti, and Matiacevich(2016) studied the release of microencapsulated citronella oil from the alginate matrix. The efficacy of the release was tested by evaluating the antimicrobial activity against*E. coli*. Moreover, the release of the active compound incorporated in nanosystems or microcapsules was compared, with the release of the same unbound active compound, released directly from the coating matrix.

1.6 Effects of edible coating on shelf life of foods

1.6.1 Microbial spoilage

Bacteria are the main cause of food spoilage. They colonize the food, using it as a growth medium. The first layers of bacteria develop the conditions for the favorable growth of other species that are more pathogenic and dangerous for human health. The mechanical protection of edible coatings allows for to reduce of microbial attacks. Additionally, edible coatings often

include antimicrobial components as part of their composition which provides them with the ability to reduce harmful microorganisms.

Damaged foods (showing cuts or cracks) are the most susceptible to microbial attack.

For example, when fresh fruits are cut or minimally processed (peeling, cutting, or slicing), they are susceptible to chemical, physical, sensory, and microbiological changes.

Fresh-cut fruits are prone to the invasion and colonization of microorganisms such as mesophiles and psychrophiles, molds, and yeasts. Deteriorative microorganisms cause damage to fresh-cut fruits and make them sensorily unacceptable. For example, this type of microorganism causes the production of lactic acid, acetic acid, hydrogen gases, and carbon dioxide, which results in sour odors and the puffing up of packages. As these products are consumed without any thermal treatment, it is essential to keep the microbial loads of fresh-cut fruits as low as possible to avoid food-borne diseases (Iturralde-García *et al*., 2022).

Meat products are also highly susceptible to microbiological contamination. Microbiological growth in meat products is influenced by the intrinsic and extrinsic parameters of the food. The major intrinsic factors that govern microbial spoilage in meat are pH, moisture content, water activity (aw), oxidation/reduction potential, nutritional composition, anti-microbial constituents, and biological structures. It has been well established that most microorganisms grow best at pH values around 7.0 (in the range 6.6–7.5) (Jay J. M*et al*., 2005).

The pH of fresh meat is neutral, and therefore appropriate for microorganism proliferation. Similarly, the growth of microorganisms (particularly pathogenic bacteria that do not grow below aw = 0.91) is also favored by aw, which in meat is higher than 0.9. In the case of oxidation/reduction potential, this property refers to the oxidizing or reducing conditions prevailing in meat that influences the growth of microorganisms and the evolution of oxidative reactions (Aymerich*et al*., 2008).

Another relevant aspect that can influence the quality of meat is the effect of extrinsic factors, which are independent of substrate characteristics and thus plays an important role in developing preservation strategies towards meat and derived products. For instance, the temperature of storage, relative humidity of the environment, presence and concentration of gases, and presence/absence of other microorganisms can be cited as relevant extrinsic factors (Gómez *et al*., 2012).

The temperature has a major role in governing spoilage and deterioration of meat. Higher storage temperature and long storage time favor the loss of quality, including an increase in the purge, color, and odor deterioration, and microbial spoilage, of fresh meat products (Antoniewski*et al*., 2010).

All of these factors, either alone or in combination, can result in detrimental changes in the color, odor, texture, and flavor of the meat.

Microbial contamination has an economic impact on food loss because it reduces the product's shelf life through deterioration and risks the public's health by causing foodborne illnesses (Yousuf *et al*., 2020).

1.6.2 Weight loss, water vapor permeability (WVP), water holding capacity (WHC)

Weight loss caused by moisture escape leads to economic losses. Employing water-insoluble or poorly soluble components in edible coating formulation reduces their water vapor permeability (WVP) and inhibits weight loss. In fresh-cut fruits, fluidity-dependent membranes can solidify at low temperatures, causing membrane integrity/loss problems, solute diffusion, tissue water loss, and membrane-bound protein agglomeration. These phenomena can accelerate senescence, caused by the accumulation of reactive oxygen species (Iturralde-García *et al*., 2022).

The WHC is one of the primary determinants of the visual appeal of meat products. It is defined as the ability of fresh meat to retain its water during storage and strongly depends on the structure of muscle proteins and their spatial arrangement. The water released can be described as drip loss or exudate loss. Water, which acts as a plasticizer of muscle proteins, is lost from the myofibrillar lattice structure because of protein denaturation and consequent reductions in the muscle fiber volume, reducing the quality of raw and cooked meat (Kim *et al*., 2014).

1.6.3 Texture

The texture of the product is influenced by various factors, such as moisture loss, water loss, and microbiological action. Edible coatings can improve the texture of products thanks to the different functional compounds incorporated. Water-insoluble or poorly soluble compounds can be used in the formulation of the coating, as well as Ca^{2+} ions that increase the degree of crosslinking of the coating, to reduce water loss. Another aspect that can improve the texture of food products is reducing the structural damage caused by microorganisms by incorporating antimicrobial components.

The texture of food is one of the main quality indicators for consumers and is a parameter that is always evaluated during the shelf life of fruit and meat products. The consistency of fresh-cut fruits is correlated to the components of the cell wall (pectin, hemicellulose, and cellulose), in fact, there is a decrease in the intermolecular bond between the polymers of the cell wall. Consequently, an increase in solubility is observed, generating softening (Iturralde-García *et al*., 2022).

In meat products, however, the phenomenon is due to the oxidation of proteins, causing fragmentation of the proteins. Consequently, the functionality of proteins is affected by determining different effects on

nutritional quality, water retention capacity, and tenderness (Jongberg*et al*., 2017).

1.6.4 Permeability to gases and organic volatiles

Gas permeability is a parameter concerning fresh fruit and vegetables (Dhall*et al*., 2013). Edible coatings must slow down the gas exchange, to avoid the phenomena of accelerated senescence and the formation of alcoholic compounds produced by microorganisms in anaerobic conditions. (Mishra*et al*., 2010). Correct gas exchange can be regulated through edible coatings due to an adequate spatial arrangement of the coating components and the thickness which is regulated by the concentration of the components.

1.6.5 Total volatile basic nitrogen (TVB-N)

The TVB-N value is an important reference index to evaluate the freshness of meat. The TVB-N is a measure of the volatile, basic nitrogen-containing substances, for example, ammonia, primary amines, and secondary amines, produced by the decomposition of protein under the action of enzymes and bacteria (Ruiz-Capillas*et al*., 2001; Zhang *et al*., 2016). The application of the edible coating and the use of appropriate antimicrobial substances can reduce bacteria contamination and consequently the amine compounds production (Shuguo Sun *et al*., 2020)

1.6.6 Appearance

Appearance refers to color, glossiness, and the absence of visual defects on food products. During their storage, food products are susceptible to oxidation and enzymatic browning, which cause undesired color changes and moisture loss leading to size shrinkage and gloss reduction. Skin blemishes may be caused by exposure to pests and microbial and mechanical damage.

Polyphenol oxidase (PPO) and peroxidase (POD) are the main enzymes causing color degradation and deprivation of sensory properties, such as taste and flavor, in fresh-cut fruits.

The production of aldehydes, ketones, alkenes and alcohols, due to Lipid oxidation, causes off-flavors and odors in meat, which affect negatively the acceptability and overall quality of meat and meat products (Kumar *et al*., 2015).

All these factors, either alone or in combination, can result in detrimental changes in the color, odor, texture and flavor of fresh fruit and meat.

Application of edible coatings prior the storage can inhibit these deleterious effects. Moreover, appropriate coating components can enhance the gloss and visual attractiveness of the food product (Falguera*et al*., 2011).

1.7 Edible coatings on meat product: state of art

The high content of proteins and fats, the acidity of the meat, and the specific structure of the meat tissue, together with hygiene and temperature conditions make the meat perishable, thus limiting its shelf life. Special technical operations are used during meat processing and, inadequacy at any stage results in a negative impact on the product and/or process in the next stage. in addition, meat is one of the most expensive foods. A huge quantity of fresh meat is withdrawn from the market every day, because of inadequate storage associated with the expiry of the shelf life. This could be avoided bybetter preservation methods, which would lead to significant savings in the entire supply chain.

Although edible coatings and films are not widely applied for commercial purposes for meat and meat products, the scientific community is trying hardto find new solutions and opportunities for their wider use.

Different types of coatings and films have been tested to maintain the quality of fresh meat, pork, poultry and frozen meat products (Bojorges*et al*., 2020; Dinika*et al*., 2020; Yordshahi*et al*., 2020).

Fat oxidation is the main cause of deterioration in the quality of meat and meat products, as it leads to discoloration, unpleasant taste and nutrient loss. Kim (2013) showed that plant extracts prevent or minimize fat oxidation in meat products by improving their nutritional quality. Essential oils are interesting because they have antioxidant action and antimicrobial protection, prolonging the stability of the packaged product. Even if the added quantities vary from 0.5 to 4% (v / v), the aromatic profile is extremely intense, if not correctly dosed it can cause variations in the organoleptic characteristics (smell, aroma) of the final product.

Considering that different compounds can be used as polymers (polysaccharides: chitosan, alginate, pectin, carboxymethylcellulose, etc., proteins: collagen, soy proteins, casein, whey proteins, etc., waxes: carnauba, etc., fats and combinations, with different additives and nanocomposites, numerous essential oils, and isolated active components), it is not surprising that the resulting combinations are numerous.

In addition, it is possible combining it with new processing and storage methods (cold plasma, high pressure, ultrasound, pulsating electric field, etc.). In the literature, polysaccharides such as alginate and carrageenan have been widely used in the development of edible coatings, and the introduction of various types of phenolic compounds, essential oils, nanomaterials and other potential substances have enhanced their functionality. Examples of scientific research conducted on fresh beef show that beef wrapped in edible tapioca film (Utami*et al*., 2019) or alginate (Zhang *et al*., 2021) with cinnamon and essential oils can keep up to 15 days a 4 ° C. Alginate in combination with turmeric has a promising effect on the physical-chemical and structural properties of fresh chicken, beef and pork, contributing to an important antioxidant effect with the consequent reduction in the values of

the reactive substance thiobarbituric acids (TBARS). In the case of the meat, it was demonstrated that the bovine lactoferrin and the quercetin glycoside compounds act efficiently against *Pseudomonas fluorescens* (Montone*et al*., 2021).

Alginate coating crosslinked with $CaCl_2$ and applied on lamb meat, led to a decrease in the total volatile nitrogen for refrigerated meat, with respect to frozen meat. Although alginate coating achieved psychrophilic bacterial inhibition during the frozen storage (Koushki*et al*., 2015). Alginate coating with tea polyphenols, applied on Japanese sea bass, provided the greatest effect on quality (TVB-N, lipid oxidation, protein decomposition) and sensory results compared to their effects alone (Nie*et al*., 2018). Another study, about chicken breast and chicken thigh meat, showed that the lactoperoxidase addition into the alginate-based coating system led to higher bacterial and sensorial quality values of chicken meat (Yousefi*et al*., 2018-Molayi*et al*., 2018).

However, the coating based on alginate helped stabilize the color of the meat, reduce shrinkage, and achieve sensory scores more than uncoated meat samples (Parreidt*et al*., 2018). Edible coatings have also been used to coat pre-cooked pork meatballs to address flavor and texture problems (Yu *et al*., 2008). The coating reduced oxidative rancidity, cooking losses and increased tenderness of the meat, indicating that the increased structural integrity given by the coating was favored by consumers (Yu *et al*., 2008). The use of gelatinized alginate in freezing fishery products was patented in Norway, providing greater protection against thawing and water loss (Helgerud*et al*., 1956). Their application not only improves the integrity and healthiness of fresh products but can replace some of the less efficient plastic packaging used today. Additionally, edible coatings can be a valid alternative to the use of preservatives and synthetic flavorings.

1.8 Application of edible coating on fruit: state of art

Often fruit and vegetables are already equipped with a natural wax-based coating (cuticle). However, additives have always been used to control permeability and exchanges of oxygen and carbon dioxide with the outside, and to limit damage from fruit handling during the various phases preceding storage. The properties that characterize an edible coating are transparency, water vapor and solutes permeability, and the capability to exchange gas (Jongsri*et al*., 2016); another property not to be overlooked is that the edible coating must be odorless and tasteless, to maintain the original sensory profile (Iturralde-García *et al*., 2022). The edible coating causes a slowdown in cellular respiration and, consequently, of senescence, as well as slowing the development and proliferation of fungi and bacteria and improving the external appearance of the fruit (Dhall*et al*., 2013; Mahajan*et al*., 2014). Some adjuvants such as antimicrobial, acidifying and antioxidant solutions are commonly used (Valdés *et al*., 2017) to delay oxidative processes, but in

a world where sustainability plays a very central role, it becomes essential to find valid alternatives to chemicals while maintaining the quality of theproduct. Since the 1950s, waxes, colloidal suspensions or emulsions have been used to coat fresh fruit. The most successful coatings were lipid films, hydrocolloids (polysaccharides and proteins) and waxes (beeswax, carnauba and candelilla wax, paraffin and bran of rice). Hydrocolloids represent the group of biopolymers most used in the production of edible materials. They can be obtained directly from plants, animals, or microorganisms. Among these, cellulose derivatives, starch, alginate, chitosan, and carrageenans are the most used polysaccharides in the production of edible films and coatings, while soy proteins, gluten, zein, gelatin, casein and keratin are among the most used proteins (Falguera*et al*., 2011). However, being these materials of hydrophilic nature, the need to incorporate vegetable oils or fats in the hydrocolloid matrix arises, to improve the barrier effect of the coating against the external environment (Galus*et al*., 2015). The edible coating is applied by dipping (dipping technique) the fruit in the solution o byspraying (spraying technique) the solution on the surface of the fruit. Whereas the immersion technique determines a greater consumption of the product and its greater absorption by the fruit -often causing a visible stratification of the edible coating on its surface - the spraying technique involves the delivery of a thin layer of edible coating, which can be applied several times to create a different layering of the coating (LBL - layer by layer technique). This enables the generation of a different barrier effect according to the needs of the fruit. However, as reported in the literature (carried out both on IV range and on the whole product), the immersion technique is preferred. Among the positive effects detected by the physicochemical analyses on whole fruits treated with an edible coating, there are: the maintenance of consistency and compactness since the presence of edible coating keeps cell wall pectins bound; the increase in titratable acidity and vitamins, thanks to the presence of antioxidant substances and/or antimicrobials. While on fresh-cut fruit they significantly delay water loss, they don't alter the content of soluble solids and keep the color of the products unaltered.

Therefore, the positive effects of edible coatings, have stimulated the interest of researchers, who wanted to deepen the studies concerning the correlation between the matrices of the coating and the physiological characteristics of fruits to coat. Studies have shown that the application of an edible coating can reduce the enzymatic activity of polyphenol oxidase (PPO) and peroxidase (POD) and slow down the browning and discoloration of the fruit. Some edible coatings, such as those based on Aloe vera, appear to induce a reduction of PPO activity (Chauhan *et al*., 2011) leading to an increase in the phenol content during storage. In addition, citric acid plays the role of a chelating agent, resulting in the inactivation of PPO, reduction of pH and chelation of copper on the active site of the enzyme (Iturralde-García *et al*., 2022). In conclusion, the edible coating improves shelf life,

and reduces water losses and gas exchange, favoring both the breath and oxidative processes of cells. Furthermore, it is harmless for the environment, in fact, it can be considered a valid green alternative to synthetic coatings and other post-harvest chemical treatments.

1.9 Aim of the work

This work aimed to develop and characterize a novel edible coating for food applications, containing both antimicrobial and antioxidant compounds. Alginate was selected as a biodegradable polymer, used to vehicle two bioactive compounds: quercetin and lactoferrin as antimicrobial and antioxidant compounds. The obtained system was supplemented with hydroxyapatite nanoparticles to improve the bioaccessibility of active compounds.
The development of the research project took place in three main phases:

The first phase focused on the selection of the most effective antimicrobial molecules in controlling food contamination with the greatest impact on consumer health and food shelf-life, such as those caused by: *Staphylococcus aureus, Lysteria monocytogenes, Salmonella spp, Escherichia coli, Pseudomonas spp*. In this step, we also identified food matrices (meat and meat preparations; fresh fruit and fresh cut fruit) to use for the experiments.

The second phase was focused on the study of the interaction of hydroxyapatite with the molecules selected in the first phase (quercetin glycosides compounds and lactoferrin). We investigated the kinetic release of compounds complexed with hydroxyapatite, and immobilized in alginate-based coatings, including CaCl2 as a crosslinker. Morphological analysis of neo-synthesized crystals with active molecules and the stability of the complexes were evaluated with FT-IR and zeta potential analysis.

In the third phase, the activity of the developed coatings was explored, using specific food products, (chicken fillets, fresh-cut papaya, loin pork fillets). temperature-controlled shelf-life tests were performed, to monitor chemical-physical, microbiological and sensory parameters responsible for the initial quality of the food.

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This part is an edited version of:

Development of a Novel Active Edible Coating Containing Hydroxyapatite for Food Shelf-life Extension

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Development of a novel active edible coating containing hydroxyapatite for food shlf -life extension

2.1 Abstract

In this work, active alginate-based coatings were developed using hydroxyapatite nanoparticles as potential carriers for quercetin glycoside compounds. The coatings were produced through the layer-by-layer method and loaded with different concentrations of quercetin and hydroxyapatite/quercetin complexes. In-vitro release studies of the quercetin through the coatings were performed in an aqueous medium: even if the hydroxyapatite nanocrystals slow down the diffusion process, quercetin released reached the equilibrium in one day for all coatings. Lastly, antimicrobial tests show that all active coatings display antibacterial activity against *Pseudomonas fluorescens.* This study highlights the real possibility of applying active edible coating loaded with hydroxyapatite/quercetin complexes to the food shelf life extension.

2.2. Introduction

The principal roles of food packaging are to protect food products from physical, chemical and biological influences by delaying food deterioration, retaining and prolonging the beneficial effect of processing and maintaining the quality and the safety of the food, therefore, extending its shelf life. Non-renewable e nonbiodegradable packaging materials have serious environmental drawbacks: they have been considered a major source of environmental pollution by consumers. Recently, there has been increased interest in new packaging strategies with environmentally friendly, biodegradable and edible packaging materials made from renewable natural polymers, that can be used as edible coatings to improve food safety and the shelf life of food products (Seixas et al., 2013). However, the commercial applications of the above-mentioned materials as edible coatings are still currently limited because different materials present different problems: some of them lack good adhesion properties, some do not provide sufficient protection, while others hamper normal gas exchange in fresh products (Vargas et al., 2008). Rationally, designed multi-component coatings and films could help to satisfy the diverse practical requirements that cannot be met by a single material. One of the most straight forward approaches for preparing multicomponent edible coating requires blending different filmforming components. The layer-by-layer electrostatic deposition technique is an advanced approach that originated in materials science and is used for the preparation of multi-component coatings. It is based on the alternate deposition of oppositely charged polyelectrolytes, to produce thin layers on a surface. Edible coatings for food products based on a combination of oppositely charged polyelectrolyte natural polymers have been largely reported in literature (Arnon-Rips et al., 2018), highlighting the possibility to prepare advanced edible coatings resulting in notable

enhancement of the coated food quality. Studies on edible coatings with antimicrobial properties are currently increasing. In particular, in the last years, several active systems have been developed using the flavonoid quercetin, exploiting principally its antioxidant capacity to prevent oxidation phenomena in food products (Farrag et al., 2018; Silva-Weiss et al., 2018). Besides its established antioxidant activity, some authors report the antimicrobial activity of this flavonoid against Gram-positive (*Staphylococcus aureus, Listeria spp*) and Gram-negative bacteria (*E.coli, SalmonellaEnteritidis, Salmonella Typhimurium)* (Hirai et al. 2010). It must be pointed out that the release time of the preservative from a film or coating has a strong impact on its effectiveness. In some applications, a quick release is required to prevent microbial growth in the food; on the contrary, in other food systems, a slow release is necessary to assure a certain level of the preservative at the surface to control the external contamination.

Hydroxyapatite (HA) is a calcium phosphate similar to that present in the human hard tissues in morphology and composition. It possesses several properties such as biocompatibility, biomimetic dimensions and degradability, and thus it is an attractive candidate as a biomaterial with several potential applications. The composition and structure of HA nanoparticles, reported by Fulgione et al. (2019) highlighted the capability of this mineral to chemically interact with different organic molecules and thus could represent a potential carrier for the delivery of bioactive compounds in the development of edible coating systems.

In literature, the application of emerging technologies to the development of edible coatings for food preservation have included various nanosystems, including polymeric nanoparticles, nanoemulsions and nanocomposites, in effort to enhance solubility, improve bioavailability, facilitate controlled release, and protect bioactive components during manufacture and storage. Liu et al. (2013) reported elaborating curcumin nanospheres using chitosan as the biopolymer, while Lv et al. (2014) described the incorporation of jasmine essential oil nanocapsules composed of two biocompatible polymers (gelatin and arabic gum) by complex coacervation. In another approach, a chitosan-based nanoemulsion containing lemongrass oil droplet was developed by Oh et al. (2017).

To the best of our knowledge, no previous studies have been published on the application of HA as a component of edible coating for the delivery of bioactive compounds. Based on above, the aim of this study has been focused on the development of alginate-based edible coatings, charged with hydroxyapatite/quercetin complexes. The potential of the developed coatings in the food shelf life extension has been evaluated in vitro by the evaluation of the kinetic release of quercetin glycoside compounds and the antimicrobial properties versus *Pseudomonas fluorescens.*

2.3 Experimental Section

2.3.1 Materials and preparation of the alginate based active coatings

Quercetin glycoside compound (QUE) (98.6% food grade) was purchased from Oxford® Vitality Company (Bicester, UK). Sodium alginate, calcium chloride, glycerol, calcium acetate, phosphoric acid, ammonium hydroxide solution and 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyl tetrazolium bromide (MTT) were all purchased from Sigma-Aldrich (Milano, Italy). HA [Ca5(PO4)3(OH)] nanocrystals were provided by Laboratory of Environmental and Biological Structural Chemistry (University of Bologna-Italy) and synthesized according to Palazzo et al. (2009). Details on the dimension and morphology of HA are reported elsewhere (Fulgione et al., 2019). Hydroxyapatite/quercetin (HA-QUE) complexes were prepared by mixing hydroxyapatite solution opportunely diluted (1:100) with different QUE concentrations 200, 300, 400 and 500 mg/L. The solutions were gently mixed at 37°C for 24 hours. At the end of the adsorption process of QUE onto HA crystals, the effective amount of QUE entrapped into the structure was evaluated. HA/QUE solutions were centrifuged at 8500 rpm for 5 min; the amount of QUE in the collected supernatant was determined using UV–vis spectrophotometer (Perkin Elmer Lambda 25) at 369 nm based on a quercetin-3 glucoside standard curve.

Alginate-based coatings were prepared according to *layer –by –layer* (LBL) technique: glass slides were dipped, for 2 minutes, at first in the sodium alginate solutions (2%,1.5%, w/v) then in calcium chloride ones (2%, 1% and 0.75% w/v) for the crosslinking and the formation of the gel. Finally, the coatings were air-dried at room temperature. Alginate solutions were prepared by mixing sodium alginate in a glycerol solution (2% w/v) and stirred at 70°C for 2 h. Active based alginate coatings were produced dissolving quercetin glycoside compounds and Hydroxyapatite/Quercetin complexes in sodium alginate solution. Preliminary tests on the coatings, developed with sodium alginate (2% w/v) and calcium chloride (2% and 1% w/v), highlighted the formation of biopolymeric matrices very thick and extremely resistant to the diffusion of active compounds (data not reported). Therefore, in this work, sodium alginate 1.5% w/v and calcium chloride 0.75%w/v were selected as the best condition for the development of suitable alginate-based coatings for food applications.

2.3.2 Characterization of the alginate-based active coatings

Coatings thickness was determined using a digital micrometer with a precision of 0.001 mm. The thickness was analyzed in three randomly selected points on each film and then an average value was calculated. Thecolour of the coatings was measured using a CR-300 colorimeter

(Konica Minolta, Inc., Osaka, Japan) to determine the values of L*, a*, and b* (CIE 1976 L*a*b* colour space). The colour measurements were performed by placing the film specimens over the withe standard tile and by measuring at least four points of each sample selected randomly to analyze the colour parameters of the coating. The total colour differences:

$$(\Delta E = \sqrt{(L* - L)2 + (a* - a)2 + (b* - b)2}) \quad (1)$$

and whiteness

$$(WI = 100 - \sqrt{(100 - L*)2 + a*2 + b*2}) \quad (2)$$

were also determined, according to Albanese et al. (2014), where L*, a* and b* are the colour values of the coatings and L, a and b are the colour parameters of the white standard tile. The release kinetic of active alginate-based coatings was studied according to Farrag et al. (2018). Pieces of coatings of dimension 2x2 cm^2(5 g) were cut and immersed in 20 mL water solution under a slight agitation at room temperature. The amount of the QUE in the release medium was determined periodically using UV–vis spectrophotometer at a wavelength of 369 nm based on a Quercetin-3-glucoside standard curve. Results were expressed as percent ratio of M_t/M_∞ (M_t is the concentration of QUE (mg/mL) diffused at time t, and M_∞ represents the concentration of QUE diffused at equilibrium).

2.3.4 Antimicrobial activity

Pseudomonas fluorescens strain ATCC 13525 was provided from the Laboratory of Microbiological Food Control—Department of Food Microbiology of the IstitutoZooprofilatticoSperimentale del Mezzogiorno in Portici (Naples, Italy). *P. fluorescens* was grown overnight at 37°C in the liquid culture medium Buffered Peptone Water. To identify bacterial growth phase, the turbidity of the medium was determined by optical density measurement at 600 nm on a UV/Vis spectrophotometer. Minimal Inhibitory Concentration (MIC) of QUE was determined by a colorimetric method, using 3-4,5-dimethylthiazol 2,5-diphenyltetrazolium bromide solution (MTT). The colorimetric assay was performed in accordance with the Clinical & Laboratory Standards Institute guidelines (CLSI, 2015). Briefly, bacterial suspensions were prepared to contain 10_5-10_6 cfu/mL (OD: 0.08 - 0.13 nm) and transferred into 96-well plates with different dilutions of QUE aqueous solutions (from 1000 to 5 mg/L). The plate was then incubated at 37 °C for 24 h. After bacterial cells attachment, 10 µL of MTT (5 mg/mL) was added to each well and the plate incubated for 2 hours at room temperature. Finally, the content of each well was removed and 100 µL of DMSO was added. Bacterial MTT reductase activity was determined by measuring the absorbance of DMSO extracts at 570 nm. Bacterial cell growth inhibition was calculated and reported as percentage, while the MIC value was

recorded as the lowest concentration of molecule able to inhibit bacterial growth.

2.4 Statistical analysis

Experiments were performed in triplicate. Data reported were the mean and standard deviation calculated from three replicates. The analysis of variance (ANOVA) was applied to the data. The least significant differences were obtained using an LSD test ($P < 0.05$). Statistical analysis was performed using Analysis Lab software.

2.5. Results and Discussions

2.5.1 Antimicrobial activity of QUE

Besides their established antioxidant activity, many phenolic compounds may exhibit significant antibacterial activity. Contamination by *Pseudomonas spp*. plays an important role in meat products spoilage because *Pseudomonas spp*. produce many lipolytic and proteolytic enzymes, which reduce both the quality and shelf life of fresh and processed meat products. In order to verify the antibacterial activity of quercetin glycoside compounds MIC tests at different concentrations of QUE against P. *fluorescens* were performed. The antibacterial activity results (Figure 2.1)
showed a MIC value of 500 mg/L while the bacterial inhibition was observed at lower concentrations of QUE. This latter exhibits a value close to 98% for QUE amounts of 300 and 150 mg/L and then gradually decreases up to 28% for concentrations equal to 5 mg/L. In literature, the recent works on the antibacterial activity of quercetin and its glucoside derivatives pointed out higher values than those obtained in this study. Adamczak et al. (2020) reported the MIC value of 500 mg/L for quercetin-3-O-rutinoside against *Pseudomonas aeruginosa,* while no significant effect at 1000 mg/L for quercetin. Bouarab-Chibane et al. (2019) registered a bacterial inhibition of 16% against *Pseudomonas aeruginosa* when quercetin 3-β-D-glucoside was used at 1000mg/L. The higher antibacterial activity observed in this study could be explained by the different quercetin glycosides, with higher activity, present in the QUE standard used for the experiments

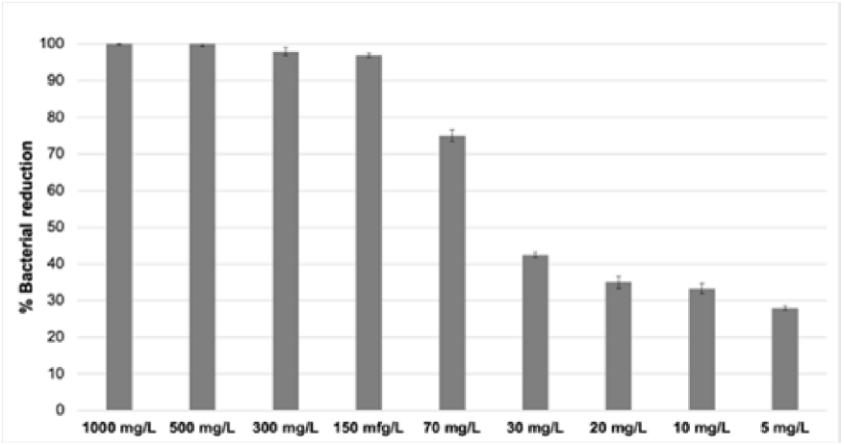

Figure 2.1: *Antimicrobial activity at different concentrations of QUE against Pseudomonas fluorescens (10^6cfu/mL)*

2.5.2 Evaluation of HA-QUE complex antimicrobial activity

HA nanoparticles were loaded with different amounts of QUE (Table 2.1). The results of adsorption evaluation showed, in the tested experimental conditions, that not all QUE is adsorbed in the crystalline structure of hydroxyapatite and the higher the QUE amount in contact with a fixed HA amount, the lower is the yield of adsorption of QUE

Table 2.1:*Yield of the adsorption QUE onto hydroxyapatite nanocrystal*

HA %w/v	Theoretical QUE amount [mg/L]	Adsorbed QUE [mg/L]	Yield
4	500	411.29	82.26
4	400	362.45	90.61
4	300	290.83	96.94
4	200	195.06	97.53

Based on antimicrobial and adsorption results the HA-QUE complexes with a QUE amount of 400 and 300 mg/L were selected to attest the preserved antibacterial activity of the loaded QUE against Pseudomonas fluorescens (Figure 2). The results pointed out a bacterial inhibition of HA-QUE at 400 mg/L equal to 98% close to that observed for free QUE at 500 mg/L while for the HA-QUE at 300 mg/L only a bacterial inhibition of 70% was registered.

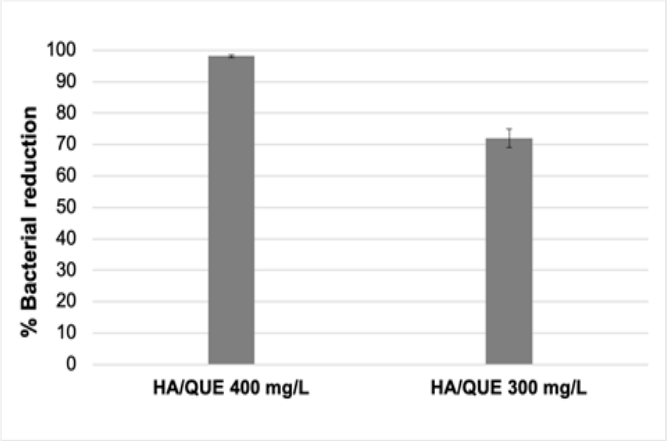

Figure 2.2:*Antimicrobial activity at different concentrations (400–300 mg/L) of HA-QUE against Pseudomonas fluorescens (10^6cfu/mL)*

2.5.3 Coatings characterization

The effects of adding QUE and HA-QUE complex at different concentrations on colour coatings are shown in Table 2. Adding QUE and HA to alginate coatings slightly affected L* (lightness/darkness), a*(redness/greenness) and b* (yellowness/blueness) values of the coating surface. The obtained results indicated that QUE and HA-QUE coatings had higher b* values (and lowest WI) than the control, indicating a tendency to yellowness which becomes more significant at the increasing of QUE amount. No significant differences ($p < 0.05$) in b* values were found among QUE and HA/QUE coatings at different concentrations. Control coating showed the highest WI that significantly decreased with the increasing of QUE amount. Finally, no significant differences ($p > 0.05$) were reported in thickness values for all coatings.

Table 2.2:*Colour parameters and thickness of alginate-based coatings*

	L*	a*	b*	ΔE	WI	Thickness [µm]
Control	92.05±1.28a	-0.36±0.15a	-4,24±0.38a	8.05±0.73a	90.98±1.04a	860.00±50.00a
HA-QUE300mg/L	87.05±1.87b	-0.41±0.16a	9,35±0.17b	12.65±1.58b	84.02±1.59b	850.00±50.00a
QUE 300mg/L	83.05±2.37b	-0.67±0.14a	9,52±0.27b	16,13±1.94c	80.55±1.92b	810.00±40.00a
HA-QUE400mg/L	80.51±0.91b	-0.36±0.05a	11.43±0.20b	19.27±0.76d	77.41±0.75c	800.00±30.00a
QUE 400mg/L	76.26±0.91c	-0.48±0.05a	12.20±0.45b	23.39±2.09d	73.30±2.08d	810.00±10.00a

Mean values in the same column with different letters (a, b, c...) are significantly different ($p < 0.05$)

The kinetic release of QUE from the active alginate-based coatings is shown in figure 2.3. As expected, the amount of QUE released in the outer medium increased until equilibrium was reached for all the tested coating samples occurs after 24 h. Moreover, the kinetic release of QUE was noted to be similar in all coating samples, due to the homogeneous distribution of active compound; however, the coatings loaded with HA- QUE complexes are characterized by a slightly slower release, respect to the coating loaded with free QUE, at the same QUE amount. In general, the release of an active agent from a coating exerts a great influence on its effectiveness. The molecular release from a biopolymeric network occurs in two stages: in the primary step, water penetrates and diffuses into film from the outer solution. Thus, meshes of the polymeric network become increasingly wider, allowing active agent diffusion through film into outer solution until a thermodynamic equilibrium between the two phases is reached (Buonocore et al., 2003).

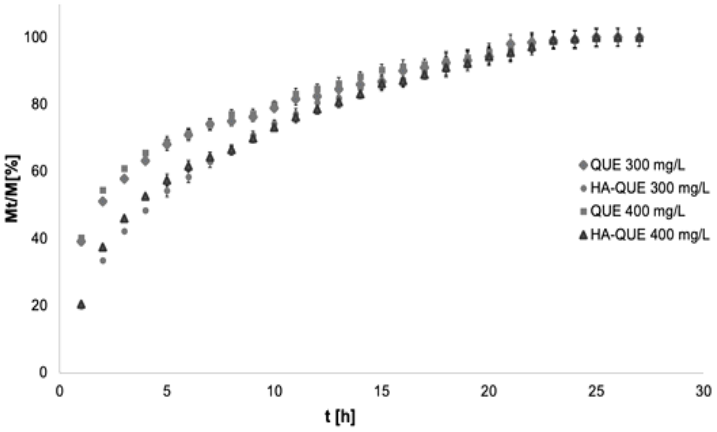

Figure 2.3: *kinetic profile of quercetin glycoside compounds from alginate-based coatings.*

As shown in figure 2.3, in the first 12 hours of release process, the greatest fraction/amount of QUE was released from coatings: in particular, in this time, for coatings with free QUE 80% release has already been achieved, while for the other coatings the hydroxyapatite slows down the diffusion process releasing a slightly smaller amount (70%). Therefore, it has been noted a harder diffusion of HA-QUE complexes than free QUE from alginate coatings. A possible explanation was linked to coating structure stability: hydroxyapatite nanocrystals, with their calcium phosphate-based composition, interact with $CaCl_2$ molecules causing a change in the coating morphology which is reflected in a slower release of the active compound. However, the release time obtained with developed coatings are very fast compared to the results obtained in literature: starch-based films of different origins and loaded with QUE microparticles release the active compound in 4 days in the case of starch deriving from cereals and in more than a week in the case of starch of legum origin (Farrag et al., 2018). A carboxymethyl cellulose active edible film loaded with QUE was able to release in a simulant medium the 50% of active compound amount in 21 days, making it hardly usable for food applications (Silva-Weiss et al., 2018). It is necessary to highlight, therefore, the real possibility to use the developed active edible coatings for food applications, where the release of the active compounds must necessarily occur in the first hours of contact, in order to preserve the safety of the product and prolong its shelf life.

2.5.4 Evaluation of coatings antimicrobial activity

The antibacterial activity against *Pseudomonas fluorescens*of alginate-based coatings charged with QUE and HA-QUE was also evaluated. The results showed for both coatings (HA-QUE at 400mg/L and HA-QUE at 300 mg/L) that the % bacterial reduction decreased by almost 50% compared to free HA-QUE complexes. This behaviour could be due to release kinetic of the active compound that is lower than bacterial growth rate and thus the amount of the hydroxyapatite complexed with QUE which is released in the broth culture is not sufficient to effectively inhibit the bacteria growth.

2.6 Conclusions

An active edible coating loaded with hydroxyapatite/quercetin complexes was developed and optimized. The antibacterial activity tests of quercetin glycoside compounds (free and complexed with HA) have shown satisfactory results for the inhibition of *Pseudomonas fluorescens*. Specifically, in-vitro studies showed a fast and homogeneous release of the quercetin glycoside compound through the coatings, reaching the equilibrium in 24 hours. These results make the coatings potential carriers for the controlled release of the quercetin hydroxyapatite complexes, paving the way for the development of coatings designed for the food shelf-life extension, i.e fresh meat and fish products. Additional in-vivo studies are needed to further characterize the effects of quercetin glycoside coatings.

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Effects of active alginate edible coating enriched with hydroxyapatite-quercetin complexes during the cold storage of fresh chicken fillets

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3.1 Abstract

Alginate edible coatings enriched with quercetin glycosides and hydroxyapatite/quercetin complexes were evaluated as effective active coatings able to slow down the spoilage of fresh chicken fillets during the cold storage. Hydroxyapatite/quercetin complexes were preliminarily analysed by SEM, FTIR and zeta potential. Then alginate coatings were characterized for their ability to release quercetin in an aqueous medium which represents the base condition during the development of active edible food packaging. The microbiological parameters (total viable count, psychotropics bacteria count, *Pseudomonas spp*. and *Enterobacteriaceae*), measured during the cold storage period at 6 °C, highlighted the capability of alginate coatings with hydroxyapatite/quercetin glu- cosides complexes to significantly inhibit the growth of spoilage bacteria, as well as the total volatile basic ni- trogen during the cold storage period of 11 days. Likewise, the texture, colour and sensory parameters pointed out a positive effect of quercetin when complexed with hydroxyapatite to maintain the quality of fresh chicken fillet during the storage.

3.2 Introduction

Chicken meat is one of the most consumed meat sources for many people all over the world. Chicken meat in fact is widely available, relatively inexpensive and from a nutritional content point of view shows a high protein, a lower fat content and a desirable fatty acid profile (Marangoni et al., 2015).

However fresh chicken meat is highly perishable due to the rapid microbial growth of the *Pseudomonas* species that represent the most common spoilage bacteria recorded in poultry meat (Rouger, Tresse, &Zagorec, 2017).

Food packaging such as vacuum packaging (VP), modified atmo- sphere packaging (MAP) and active packaging have been previously reported for the shelf life extension of fresh chicken meat (Baltic et al., 2019; Meredith et al., 2014).

The new packaging strategy to preserve quality and extend the shelf life of food products is represented by edible films and coatings obtained by natural biopolymers. The nature and the number of biopolymers influence the film and coating properties such as mechanical stability, transparency, moisture and gas barrier. Generally, polysaccharides are employed to avoid gas permeability, lipids limit water vapor trans- mission, while proteins improve mechanical stability to the structure (Pavlath& Orts, 2009). Additionally, edible coatings and films can be enriched with natural bioactive substances such as antimicrobials or antioxidants (Sahraee, Milani, Regenstein, &Kafil, 2019; Umaraw et al., 2020). Edible coatings are developed directly on the

food surface unlike edible films that are before produced and then used as wrapping ma- terials. Due to the easy application method for edible coatings, a wide number of scientific papers on food quality, food safety and shelf life extension have been published (SenturkParreidt, Muller & Schmid, 2018; Zambrano-Zaragoza et al., 2018). As regards the extension of cold storage period of fresh chicken meat, different polysaccharide-based active coatings have been widely proposed in the literature (Abdou, Galhoum, & Mohamed, 2018; Hamedi, Kargozari, shotorbani, Mogadam&Fahimdanesh, 2017; Yousefi, Farshidi, &Ehsani, 2018; Molayi, Ehsani, &Yousefi, 2018; Wagle et al., 2019; Mehdizadeh&Langroodi, 2019; Zhou, et al., 2021). Most of the works just mentioned incorporated in the edible coating natural extract or essential oils as active substances to inhibit the microbial growth and oxidation phenomena. Quercetin and quercetin glucoside compounds have been widely studied and applied as active compounds for their antioxidant (Silva-Weiss et al., 2018) and antimicrobial properties against some Gram-positive and Gram-negative bacteria (Hirai et al., 2010). Quercetin and quercetin glucoside compounds have been little studied as active compounds in edible food packaging to date. Previous studies on the antibacterial activity of a large number of flavonoids against Staphylococcus aureus have pointed out that quercetin has a considerable inhibition against the selected pathogenic bacteria (Hirai et al., 2010; Manner, Skogman, Goeres, Vyorela&Fallarero, 2013; Yanishlievaa, Marinovaa, & Pokorny, 2006). Despite the well-known quercetin biocidal properties, only a few studies concerning the incorporation of quercetin into edible films are present in literature (Arcan&Yemeniciog˘lu, 2011; Basu et al., 2017; Benbettaieb, Karbowiak, Brachais, &Debeaufort, 2015; Dutta, Tripathi, & Dutta, 2012; Silva-Weiss et al., 2018; Yadav, Mahrotra, Bhartiya, Singh, & Dutta, 2020, Olewnik-Kruszkowska et al., 2021). In this context, the poor stability of quercetin under processing conditions and during storage, as well as its low water solubility and bioavailability limited its application in food purposes (Silva-Weiss et al., 2018). However, different systems for encapsulation and controlled release could overcome these limitations. Among several techniques adopted to protect the bioactive com- pounds, such as polymeric nanoparticles, nanoemulsions, and nano- systems (Zambrano-Zaragoza et al., 2018), hydroxyapatite crystals seem to be attractive candidates for this application. Hydroxyapatite (HA) is a biomaterial present as a major component of bone, tooth and cartilag- inous tissues. Thanks to its low toxicity and remarkable stability, as well as biocompatibility, biomimetic dimensions, osteoconductivity, and degradability, HA is suitable for several applications, such as building bone scaffolds and implant coating materials and drug delivery in the pharmaceutical industry (Palazzo et al., 2009). The use of HA in food is allowed by Reg. (UE) n. 231/2012 laying down

specifications for food additives established by Regulation (EC) No 1333/2008. The use of HA as a carrier in food active packaging could protect the active compound from environmental factors, processing and storage conditions allowing to maintain the compound's antimicrobial, antioxidant properties to extend the shelf life of foods. To the best of our knowledge, the use of hydroxyapatite alone or combined with quercetin for fresh food pres- ervation has never been studied. Based on the above, the aim of this work is to study the effect of an alginate edible coating enriched with biomimetic HA crystals complexed with quercetin on the microbial, physical, and sensory properties of fresh chicken fillets during cold storage.

3.3 Materials and methods

3.3.1 Materials

Fresh chicken breast fillets were bought in a local butcher's shop (Salerno, Italy) and after soon cold transported in cold conditions to the laboratory of food technology (University of Salerno) for processing. Biomimetic Hydroxyapatite (HA) were provided by Research and development department (Chemical Center Srl, Italy) and synthesized following the method used by Palazzo et al. (2009).

Quercetin glucoside compounds (QUE) 98.6% food grade) were ob- tained from Oxford® Vitality Company (Biecester, United Kingdom).

3.3.2 Preparation and characterization of functionalized biomimetic Hydroxyapatite with QUE

Based on previously minimal inhibitory concertation (MIC) results observed for quercetin glucoside compounds against Pseudomonas fluo- rescence (Malvano et., al 2021), we decided to prepare HA/QUE com- plexes with a final concentration in QUE of 500 mg/L as follows.

HA solution (4% w/v) with QUE water solution (1000 mg/L) were gently mixed, by a ratio1:1 (v:v) The mixtures were then incubated at 37 ◦ C for 24 h. Then HA/QUE solutions were centrifuged at 8500 rpm for 5 min. The precipitate was collected, then re-suspend with distilled water. The effective amount of QUE into HA was evaluated, according to Malvano, Montone, Capparelli, Capuano, and Albanese (2021).

3.3.3. Scanning electron microscopy (SEM)

To make the surface of the samples electrically conductive, a very thin film of gold was deposited on them, using a vacuum coating unit; then they were fixed on electron microprobe stubs.

Afterward, the surface morphology of biomimetic HA crystals and HA/QUE complexes was examined using a Scanning Electron Micro- scope (Zeiss,

EVOMA10) with an accelerating voltage equal to 20 kV. Scanning electron microscope (SEM) is equipped with a tungsten filament and secondary electron and backscattered electron detectors that operate under vacuum pressure.

3.3.4. Zeta potential analysis

Zeta potential values were determined using a Coulter DELSA 440sx. The suspensions of pure HA and HA/QUE complexes were prepared by mixing 50 mg/L of HA in 10^{-2} M KNO3.

3.3.5. FT-IR analysis

FT-IR analysis of HA, QUE, and HA/QUE complexes were carried out with a Thermo Nicolet 380 Fourier transform infrared (FT-IR) spec- trometer equipped with an attenuated total reflectance (ATR) element according to Nocerino et al. (2014).

3.4 Coating solutions

Alginate-based edible coatings were carried out by layer-by-layer technique based on the sequentially dipping of food product into oppositely charged polyelectrolytes solutions (alginate - CaCl2). Three different Sodium Alginate solutions (SAs) were prepared: control SAs, SAs with QUE and SAs with HA/QUE. SAs was prepared by dissolving, under magnetic stirring, sodium alginate (1.5% w/v, Sigma-Aldrich, Milano, Italy) and then glycerol (2% w/v, Sigma-Aldrich, Milano, Italy) for 2 h at 70 ◦C.
QUE and HA/QUE solutions were added to the control SAs to produce active alginate solutions with a concentration in QUE of 500 mg/L of SAs. Calcium chloride solution (0.75% w/v) was used as a cross-linker for the alginate-based biopolymer.

3.4.1. Release profile of quercetin loaded alginate-based coating

Alginate-based coatings (2 ×2 cm2; 5 g) with QUE and HA/QUE respectively were cut and immersed in distilled water (20 mL) under agitation. Periodically, the concentration of QUE in water solution was detected according to Malvano et al. (2021).

3.5 Breast Chicken fillets coating

Breast chicken fillets were cut into portions with a weight of about 100–120 g and then separated into four groups. The coating process was carried out by dipping sequentially the chicken fillets into sodium alginate solutions, above described, and calcium chloride solution. The dipping time for both solutions was 2 min. Next, samples were air-dried by a fan at room

temperature for 10 min. Finally, breast chicken slices were put away into polyethylene terephthalate boxes provided with a lid.
The shelf-life tests were performed by compared four different chicken samples prepared as below:
•Chicken fillets without coating (C).
•Chicken fillets covered with control SAs coating (SAs).
•Chicken fillets covered with QUE-SAs coating (QUE-SAs).
•Chicken fillets covered with HA/QUE-SAs coating - (HA/QUE-SAs).
A total of 12 boxes for each treatment were prepared and stored at 6°C for 11 days. At regular interval times (0, 2, 4, 7 and 11 days) the overall quality of chicken fillets was evaluated by physicochemical, microbiological, texture analysis in addition to sensory evaluation. At each cold storage time, the chicken fillets from three replicate boxes were used.

3.6 Chicken physicochemical analysis

3.6.1. Colour and pH measurement

Chicken surface colour was determined with a CR-300 colorimeter (Konica Minolta, Inc., Osaka, Japan) for the detection of L* , a* , b* (CIE 1976 L*a*b* colour space) values. Five measurements at randomly selected points were recorded per sample. The total colour differences (Δ*E*) was calculated as given by Eq. (1):

$$\Delta E = (Lt - L)2 + (at - a)2 + (bt - b)2 \quad \text{(a)}$$

Where Lt, at and bt are the colour values of the chicken samples at different storage time, while L, a and b are the colour values at time zero. The pH was measured using a digital pH-meter (Model 2001, Crison, Barcelona, Spain) equipped with a penetration pH-electrode.

3.6.2. Water Holding Capacity

Water holding capacity (WHC) was determined as the drip loss percentage that reveals the amount of free water that exudes during the storage. The chicken samples were removed from their coatings at 2, 4, 7 and 11 days of storage and their weights were recorded. For each sample, WHC values were calculated as a percentage of weight loss relative to its initial weight, with the following equation:

$$WHC = \frac{\textit{Initial Weight } (day0) - \textit{Weight } (\textit{analysis day})}{\textit{Initial Weight } (day0)} * 100 \quad \text{(b)}$$

3.6.3. Texture Profile Analysis (TPA)

The samples were submitted to a double compression up to 50% of the original height by a cylindrical probe of 1 cm diameter with a speed of 1 mm/s using a texturometer (LRX Plus, Lloyd Instruments, Chicago). The force–deformation curve, obtained by specific software (Nexy- gen batch 4.1), allowed to determine the Hardness (expressed in N), Cohesiveness (Dimensionless), Springiness (mm) Gumminess (N) and Chewiness (N * mm) parameters. Data are reported as the average of 7 measurements per sample.

3.6.4. Microbiological analysis

Chicken meat (10–20g) was homogenized for 3min with an appropriate amount of 0.1% (w/v) peptone water (ratio 1:10) in a Stomacher Lab-Blender 400. Decimal serial dilutions were prepared and plated onto appropriate culture media as reported below. Total viable count (TVC), and psychotropic bacteria count (PBC) were plated in plate count agar (Oxoid, ThermoScientificTM, Italy) and incubated at 30 °C for 3 days and 4 °C for 10 days, respectively.

Pseudomonas counts were obtained after 48 h of incubation at 25 °C on cetrimide-fucidin-cephaloridine agar (Oxoid, ThermoScientificTM, Italy) The green-blue, brown or fluorescent colonies were identified as presumptive *Pseudomonas spp*. but no further identification was carried out. For *Enterobacteriaceae*, violet-red bile glucose agar (Oxoid, ThermoScientificTM, Italy) was used and incubated at 37 °C for 48 h. The results were reported as log CFU/g meat.

3.6.5. Determination of Total Volatile Basic Nitrogen

Total Volatile Basic Nitrogen (TVB-N) was measured according to Albanese, Cinquanta, Lanorte, and Di Matteo (2005) based on Kjeldahl method with a vapor distillation apparatus. TVB-N values were expressed in mg N_2/100 g of chicken fillet.

3.7 Overall acceptability, colour, odour and taste evaluation

Raw chicken fillet samples at each storage time were evaluated by colour and odour, and after cooking, for taste, odour and overall acceptability. Cooking was performed by broiling, turning the fillets during the heating. In order to guarantee to the samples the same cooking process the inner temperature was verified by thermocouple type K until to reach 72 °C (McMinn et al., 2018).

All sensory attributes of chicken fillets were evaluated by 5 members (3 women and 2 men, aged between 25 and 50) engaged from the Department of Industrial Engineering, University of Salerno. The members were selected for their experience in the freshness evaluation of chicken fillets on the basis of the frequency in consumption of poultry meat.
A hedonic scale with five points from 5 (very good) to 1 (very poor) was used to score the samples during the storage period.

3.8 Statistical analysis

Data were reported as mean and standard deviation and subjected to analysis of variance (ANOVA). The significance of differences ($p < 0.05$) among samples was determined by Student's t-test using Analysis Lab software.

3.9 Results and discussion

3.9.1 Hydroxyapatite and hydroxyapatite/quercetin complexes characterization

FT-IR spectra of HA, QUE and HA-QUE complexes were shown in Fig. 3.1a. The range of HA spectra revealed the adsorption bands of phosphates groups at 1034 cm^{-1} and 1100 cm^{-1} and other bands at 880 cm^{-1}, 1466 cm^{-1}, 1422 cm^{-1} and 1545 cm^{-1} that were according to carbonate type A (hydroxyl site)-substituted and type B (phosphate site)-substituted HA nanocrystals (Nocerino et al., 2014).
FT-IR spectrum of HA-QUE confirmed the presence of QUE showing the characteristic peaks of QUE at 3248 cm^{-1} (O-H stretching), 1670 cm^{-1} (C– O stretching) and 1500 cm^{-1} (C– C stretching).
Moreover, the absorption bands in the region between 650 and 1000 cm^{-1} highlight the presence of the aromatic compounds (Milanezi et al., 2019).
SEM images of HA and HA-QUE complexes showed in both cases μm particle size (Fig. 3.1b-c). As reported previously by Fulgione et al. (2016) hydroxyapatite nanocrystal aggregated spontaneously in micrometric clusters probably justified by their zeta-potential values close to − 0.0505 mV.
Zeta-potential values between – 20 and + 20 mV are related to poor stability and a greater tendency to particle aggregation due to the absence of electrostatic repulsions of individual particles that hinder the aggregation process (Bhattacharjee, 2016). The Zeta-potential was measured also for QUE showing a value of 0.0441 mV which justify the interaction with HA during the absorption process.
According to previous studies (Fulgione et al., 2019; Nocerino et al., 2014), the positive electrostatic surface potential of QUE produces a strong surface interaction with the HA nanocrystals having a negative surface charge

related to their amorphous surfaces. This electrostatic interaction leads to the formation of HA-QUE complexes.

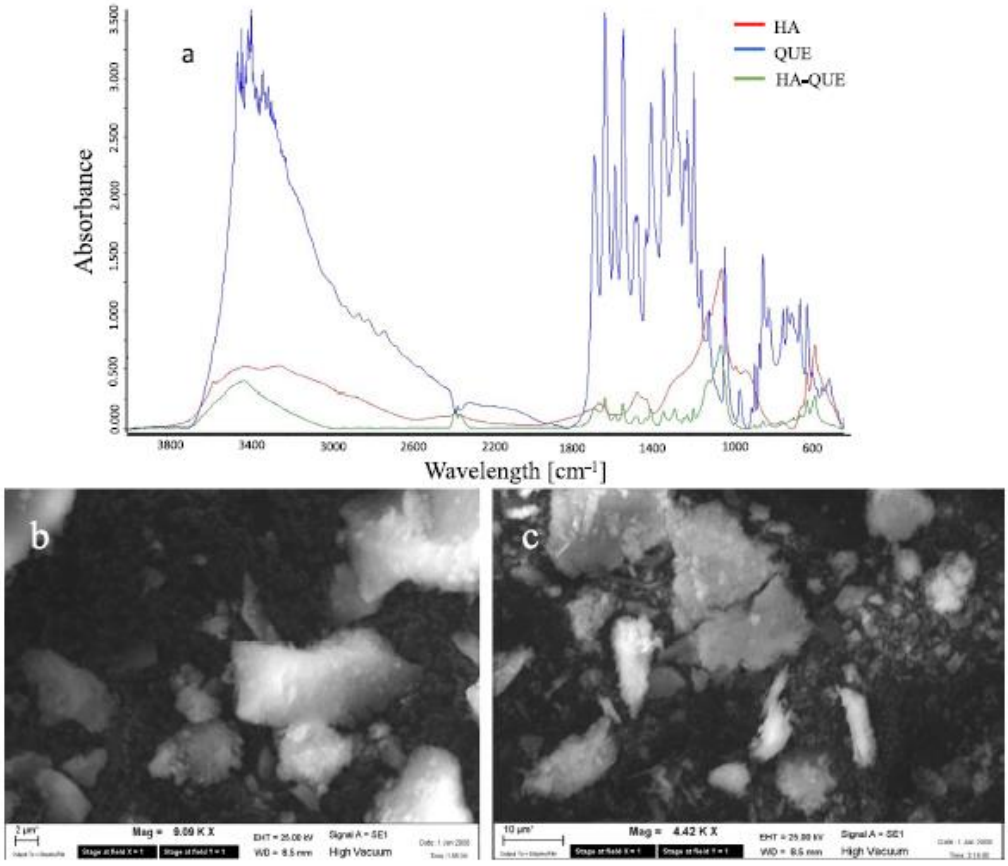

Fig. 3.1. *Fourier transform infrared spectrum of HA crystals, QUE and HA-QUE complexes (a), SEM image of crystals clusters of HA (b); SEM image of crystals clusters of HA-QUE (c).*

3.9.2 Release study

The kinetic release of free QUE from QUE-SAs and HA/QUE-SAs coatings were reported in Fig. 3.2. The release of the active compounds, from a biopolymer network, into aqueous medium occurs in two steps: first, from the external solution, the water molecules penetrate and diffuse into the film; therefore, the meshes of the polymeric network widen allowing the diffusion of the compound from the film to the aqueous medium until a thermodynamic equilibrium is reached (Imran, Klouj, Revol-Junelles&Desorby, 2014). The release behaviour is the same for both coatings in 12 h, the higher amount of quercetin was already released for QUE-SAs and HA/ QUE-SAs coatings which released 84.71% and 78.63% respectively, compared to the concentration reached the equilibrium state.

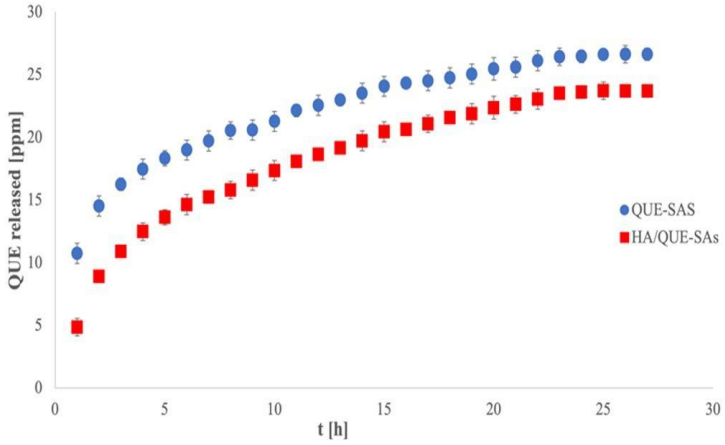

Fig. 3.2. *Release profile of quercetin glucoside compounds from QUE-SAs and HA/QUE-SAs coatings.*

These results were similar to our previously published studies (Malvano et al., 2021) and indicated a relatively higher ability of quercetin retention from HA crystalline structure that could be due to the interactions be- tween HA nanocrystals, based on a phosphate composition, with CaCl2 molecules present into the coating structure.

QUE-SAs and HA/QUE-SAs coatings showed a very faster release time of active compound compared to some recent active packaging systems reported in the literature. The complete release of quercetin from a starch-based coating and from carboxymethyl cellulose–based film (Farrag et al., 2018; Silva-Weiss et al., 2018) into a fatty food simulant was estimated to be 4 and 21 days respectively. The release of ferulic acid and caffeic acid from chitosan-fish gelatin edible films immersed in ethanol

required up to 25 days (Benbettaieb, Tanner, Cayot, Karbowiak, &Debeaufort, 2018). Essential oils, such as thyme and cinnamon, incorporated in chitosan (Hassan, Ali, Mostafa, &Maz- rou, 2021) and fish skin gelatin (Wu, Sun, Guo, Ge, & Zhang, 2017) films respectively take 2 and 7 days to release into an ethanol-based medium.

Our results highlighted the ability of the developed coatings to be really used in food applications, where the release of the active com- pounds should occur in the first hours of contact, to avoid or slow down the starting of spoilage and extend the shelf life.

3.10 Effect of active edible coating on shelf-life of chicken meat

3.10.1. Microbiological analysis

The effect of active edible coatings on total viable count (TVC), psychrotrophic bacteria count (PBC), *Pseudomonas spp*. and *Enterobacteriaceae*during the storage period of fresh chicken breast fillets was shown in Fig. 3.

The initial microbial count (day 0) for TVC and *Pseudomonas spp*. (Fig.3.3a, c) on chicken samples were close to 5.11 log cfu/g, which is a relatively high bacterial load, in comparison with previous reports on shelf-life studies on poultry fillets (Majdinasab, Niakousari, Shang-haghian&Dehghani, 2020; Meredith et al., 2014; Mehdizadeh& Lan- groodi, 2019) probably attributed to the poor hygienic condition of the food retailer during the manual processing of the whole chicken breast.

Pseudomonas spp. contribute significantly to spoilage of poultry meat, due to their psychrotrophic, proteolytic and lipolytic activity contribute to the development of off odours and off flavours in chicken meat under refrigeration conditions (Pooni& Mead, 1984; Gallo, Schmitt, & Schmidt-Lorenz, 1988; Wickramasinghe, Ravensdale, Coorey, Vhandry& Dykes, 2019). As expected, the growth of all microbial parameters was observed as the storage time increased. For each storage day, signifi- cantly lower values were observed for QUE-SAs and HA/QUE-SAs in comparison with control and SAs samples. In view of the value of 7–8 log CFU/g, which is the recognized and accepted limit of TVC value for fresh poultry meat (Senter, Arnold, & Chew, 2000), in HA/QUE-SAs samples this limit was reached at 11 days that is 3 days later compared to the control, SAs and QUE-SAs samples.

These results indicated that coating containing QUE delayed the growth of TVC, PBC, *Pseudomonas spp*and*Enterobacteriaceae*. Different authors proved antimicrobial properties of quercetin glucoside com- pounds against meat spoilage microorganisms such as *Pseudomonas spp*. (Jaisinghani, 2017; Adamczak, Zarowski, & Karpinski, 2020; Malvano et al., 2021) The antibacterial activity of QUE is probably due to its interaction with the

bacterial cell membrane, resulting in an increase in membrane permeability. Moreover, it has been proposed that phenolic components suppress the calcium and potassium transport by parti- tioning in the lipid phase of the membrane and subsequently altering the local environment of calcium channels (Altiok, Altiok, &Tihminlioglu, 2010).

The comparison between QUE-SAs and HA/QUE-SAs samples highlighted a higher capability to delay bacterial growth (TVC, PBC, *Pseudomonas spp*. and *Enterobacteriaceae*) when quercetin glucoside compounds were adsorbed in HA crystals (Fig. 3.3). This effect is more evident at 7 and 11 days where it is observed a higher capability for HA/QUE-SAs to slow down the microbial growth compared to QUE-SAs. This result could be due to the biomimetic property of HA crystals.

Different authors report the use of HA, which mimic extracellular structures, as delivery carriers of antibacterial agents. Nocerino et al. (2014) and Fulgione et al. (2016) observed significantly enhanced lac- toferrin antibacterial, antioxidant and anti-inflammatory activities when adsorbed into HA nanocrystals surface. Calasans-Maia et al. (2019) showed a higher in vitro antibacterial activity and a sustained release profile for antibiotic minocycline when adsorbed on

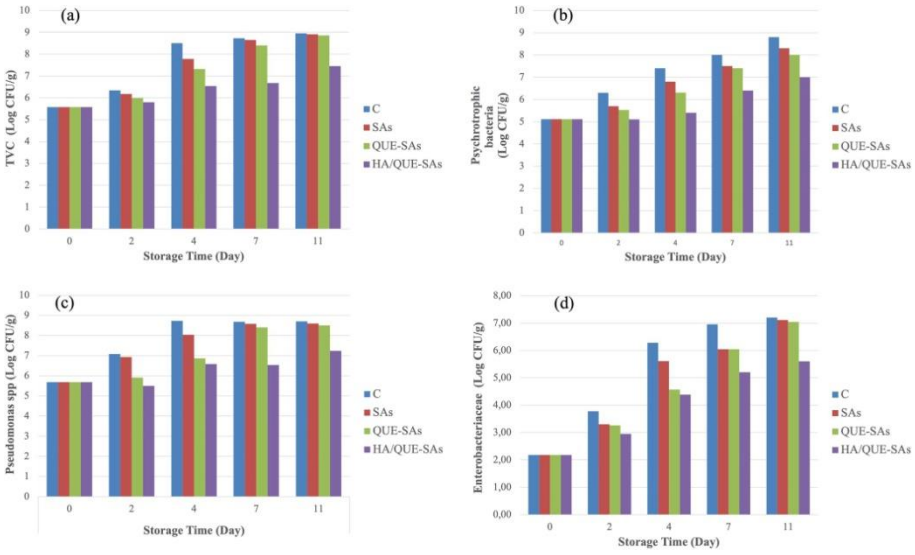

Fig. 3.3. *Changes in total viable bacterial count (TVC) (a), Psychrotrophic bacterial (b) Pseudomonas spp (c) and Enterobacteriaceae (d) of chicken fillet samples during the storage period.*

nanocrystalline carbonated HA powder and encapsulated into alginate microspheres.

3.11 pH, total volatile basic nitrogen and colour

The effect of alginate active coating application on the pH of chicken fillets are shown in Table 3.1. pH values showed significant differences ($p < 0.05$) due to storage time and conditions. The initial pH value of chicken fillets was 6.15, similar to that one reported by other authors in fresh chicken meat (Ruiz-Cruz et al., 2018; Takma&Korel, 2019) and this value increased as the storage time progressed in C, SAs and QUE-SAs samples: however, the pH values were always lower in the coated samples than in control one. In particular, the coating containing hydroxyapatite-quercetin complexes grants almost no variation of the pH during the storage period. The increase of pH during the storage could be explained by the accumulation of nitrogen basic compounds which are due to the transformation of NPN compounds into ammonia, amines and other basic nitrogenous compounds as a result of spoilage psychotropic bacteria (Albanese et al., 2005, Cao et al., 2013).

TVB-N is a common parameter for shelf-life evaluation of meat and fish products which gives information about the content of ammonia and organic amines which affect the sensory acceptability besides being toxic for human health (Bekhit, Holman, Giteru, & Hopkins, 2021; Zhou et al., 2021). Variations in the TVB-N values of control and coated chicken fillets are shown in Table 1. TVB-N value at time 0 was 8.44 mg N/100 g and significantly increase during the cold storage period in all treatments. The amount of TVB-N detected in this study was in agreement with previous shelf-life studies of chicken fillets in active edible coatings (Majdinasab, Niakousari, Shaghaghian, &Dehghani, 2020). The comparison of the TVB-N among the uncoated and coated fillet chicken samples pointed out significant ($p < 0.05$) lower values, for each storage day, in QUE-SAs and HA/QUE-SAs compared to control and SAs samples. According to the microbial results, discussed above, the lowest amount of TVB-N, at any time of storage, was measured for coated fillets containing HA/QUE complexes. Considering that the acceptability limit of TVB-N for initiation ofmicrobial spoilage and deterioration of meat and meat products is 25mgN/100g (Bekhit et al., 2021), control, SAs, QUE-SAs and HA/QUE-SAs exceeded this limit on 7th storage days while HA/QUE-SAs reached the value of 26.48 at the end storage period (11 days).

The colour, mainly associated with the packaging, type and ageing of the meat, influences significantly the decision of consumers regarding the purchase of fresh meat (Suman, Hunt, Nair, &Rentfrow, 2014).

Effects of active alginate edible coating enriched with hydroxyapatite-quercetin complexes during the cold storage of fresh chicken fillets

Changes in surface CIE L* (Lightness), a* (redness), b* (yellowness), and total colour differences (ΔE) of chicken fresh meat samples throughout storage time and treatments are shown in Table 3.1.

The L* values of coated chicken fillets remain constant until day 7 and slightly decrease the last day of storage. On the contrary, the control showed a constant decrease of the lightness from day 0 to the end of storage. Because the decrease in lightness suggests that the chicken meat became darker during storage, alginate-based coatings were able to partially preserve meat colour.

The initial a* values ranged from 0.03 to 0.44. At the beginning of cold storage, a* values of active coated samples were maintained stable and slightly increase from day 2 to the end of the storage period. On the contrary, control samples showed a continuous significant ($p < 0.05$) a* value increase during all the storage period.

By exploring CIE b* values, it was noted in the QUE-SAs and HA/ QUE-SAs coated chicken fillets a higher yellowness value than in con- trol and coated SAs samples. However, this parameter increased during the storage for all coated samples and for the control.

No significant ($p < 0.05$) changes were observed in total colour during the storage period for coated samples, while in the control samples the storage period influenced ΔE value for which it was recorded a significant increase ($p < 0.05$).

Table 3.1:*Effect of different packaging treatments on pH and colour of fresh chicken fillets during the storage time.*

Parameter	Storage time (days)					
	Sample	0	2	4	7	11
pH	C	6.15±0.02 aA	7.31±0.10 cB	8.51±0.07 dC	8.93±0.04 aD	9.28±0.06 dE
	SAs	6.15±0.02 aA	7.10±0.08 aB	7.69±0.11 aC	8.92±0.02 aD	9.12±0.08 aE
	QUE-SAs	6.15±0.02 aA	6.32±0.01 bB	7.28±0.06 bC	7.92±0.05 bD	8.11±0.05 bE
	HA/QUE-SAs	6.15±0.02 aA	6.17±0.10 bA	6.95±0.04 cB	6.98±0.03 cB	7.04±0.06 cB
TVB-N	C	8.44±1.02 aA	15±1.05a B	24.78±2.56aC	33.52±2.0 aD	40.30±3.67aE
	SAs	8.44±1.02 aA	15.3±1.02 aB	23.67±2.01aC	32.94±2.45aD	38.59±2.78aE
	QUE-SAs	8.44±1.02 aA	13.2±1.45 bB	18.44±1.98bC	26.87±2.1 bD	35.21±2.19bE

	HA/QUE-SAs	8.44±1.02 aA	12.5±1.05 bB	16.88±1.09 bC	20.51±2.06 cD	26.48±2.0 cE
L*	C	59.91±0.82 bA	53.66±0.10 bB	51.81±0.05 bC	49.14±0.94 bD	48.69±0.78 bD
	SAs	66.69±0.78 aA	65.57±0.90 aA	65.14±0.08 aA	64.32±0.53 aA	63.00±0.53 aB
	QUE-SAs	59.35±0.90 bA	58.09±3.10 bA	56.91±2.34 bA	53.79±3.34 bA	51.24±1.08 bB
	HA/QUE-SAs	59.14±1.10 bA	57.02±3.64 bA	55.40±2.38 bA	53.92±1.57 bA	51.31±2.98 bB
a*	C	0.06±0.03 aA	0.60±0.03 dB	1.11±0.08 bC	2.99±0.05 aD	3.40±0.05 cE
	SAs	0.03±0.01 aA	0.09±0.02 aA	1.74±0.39 aB	2.27±0.92 aB	2.24±0.26 aB
	QUE-SAs	0.32±0.02 bA	0.32±0.10 bA	1.08±0.06 bB	2.91±0.02 aC	2.90±0.07 bC
	HA/QUE-SAs	0.44±0.05 cA	0.45±0.03 cA	1.82±0.17 aB	1.96±0.02 aB	2.73±0.03 bC
b*	C	6.83±0.07 cA	8.66±0.06 aB	9.81±0.09 aC	10.78±0.06 aD	11.42±0.06 dE
	SAs	8.01±0.68 aA	8.02±1.37 aA	10.04±0.63 aA	11.28±0.89 aB	12.29±0.77 aB
	QUE-SAs	12.68±0.05 bA	14.50±0.03 bB	15.33±0.04 bC	16.10±0.03 bD	16.77±0.05 bE
	HA/QUE-SAs	12.40±0.06 bA	13.88±0.05 bB	15.30±0.08 bC	15.61±0.02 bD	15.06±0.05 cE
ΔΔE	C	-	6.57±0.80 bA	8.86±0.59 bB	11.91±0.01 bC	12.63±0.01 bD
	SAs	-	2.87±0.05 aA	3.49±0.09 aA	4.71±0.99 aA	6.19±1.16 aB
	QUE-SAs	-	3.70±1.25 aA	4.39±1.01 aA	7.12±2.36 aB	9.44±0.82 bB
	HA/QUE-SAs	-	3.99±1.82 aA	5.29±1.38 aA	6.48±1.32 aA	8.83±2.79 bA
WHC	C	-	4.92±0.05 aA	8.54±0.5a B	10.25±0.78 aC	12.25±0.89 aD
	SAs	-	3.14±0.02 bA	4.26±0.34 bB	6.17±0.14 bC	8.39±0.73 bD
	QUE-SAs	-	3.61±0.0b A	4.25±0.23 bB	6.76±0.16 bC	8.50±0.23 bD
	HA/QUE-SAs	-	1.11±0.01 cA	2.77±0.12 cB	4.57±0.18 cC	6.56±0.12 cD

Mean values in the same row with different capital case letters (A, B, C, D, E) are significantly different ($p < 0.05$) during the storage time, and mean values in the same column with different lower letters (a, b, c, d, e) are significantly different ($p < 0.05$) among the samples.

Table 3.2 *TPA parameters of fresh chicken fillets during the storage time.*

		Storage time (days)				
	Sample	0	2	4	7	11
Hardness [N]	C	17.84±0.02aA	17.84±0.02aA	18.77±0.03aB	30.26±0.03aC	35.84±0.02aD
	SAs	18.66±0.02bA	18.66±0.03bA	19.78±0.05bB	23.94±0.19bC	25.18±0.07bD
	QUE-SAs	19.95±0.01cA	19.95±0.05cA	20.97±0.04cB	22.03±0.04cC	23.08±0.01cD
	HA/QUE-SAs	20.31±0.02cA	20.31±0.01cA	21.22±0.03cA	22.89±0.03cB	23.02±0.04cC
Cohesiveness	C	0.01±0.00aA	0.01±0.00aA	0.01±0.00aA	0.01±0.00aA	0.01±0.00aA
	SAs	0.02±0.00bA	0.02±0.00bA	0.01±0.00aB	0.01±0.00aB	0.01±0.00aB
	QUE-SAs	0.02±0.00bA	0.02±0.00bA	0.02±0.00bA	0.01±0.00aB	0.01±0.00aB
	HA/QUE-SAs	0.02±0.00bA	0.02±0.00bA	0.02±0.00bA	0.01±0.00aB	0.01±0.00aB
Springiness [mm]	C	0.22±0.00aA	0.22±0.00aA	0.14±0.00aB	0.14±0.00aB	0.11±0.00aC
	SAs	0.24±0.00bA	0.24±0.00bA	0.15±0.00aB	0.14±0.00aB	0.14±0.00bC
	QUE-SAs	0.24±0.00bA	0.24±0.00bA	0.20±0.00bB	0.18±0.00bC	0.18±0.00cC
	HA/QUE-SAs	0.24±0.00bA	0.24±0.00bA	0.17±0.00cB	0.17±0.00bB	0.17±0.00cB
Gumminess [N]	C	0.32±0.00aA	0.32±0.00aA	0.22±0.00aB	0.11±0.00aC	0.11±0.00aC
	SAs	0.26±0.00bA	0.26±0.00bA	0.26±0.00bA	0.23±0.02bB	0.21±0.00bC
	QUE-SAs	0.26±0.00bA	0.26±0.00bA	0.23±0.00aB	0.17±0.00cC	0.18±0.00cC
	HA/QUE-SAs	0.41±0.00cA	0.41±0.00cA	0.38±0.00cB	0.24±0.00bC	0.31±0.00dD

Chewiness [N*mm]	C	0.04±0.00 aA	0.04±0.00 aA	0.03±0.00 aA	0.04±0.00 aA	0.04±0.00 aA
	SAs	0.09±0.00 bA	0.09±0.00 bA	0.03±0.00 aB	0.03±0.00 bB	0.04±0.00 aB
	QUE-SAs	0.10±0.00 bA	0.10±0.00 bA	0.08±0.00 bB	0.04±0.00 bC	0.04±0.00 aC
	HA/QUE-SAs	0.10±0.00 cA	0.10±0.00 bA	0.07±0.00 bB	0.04±0.00 bC	0.04±0.00 aC

Mean values in the same row with different capital case letters (A, B, C, D, E) are significantly different ($p < 0.05$) during the storage time, and mean values in the same column with different lower letters (a, b, c, d, e) are significantly different ($p < 0.05$) among the samples**.**

3.11.1. Water holding capacity and texture profile analysis

The WHC is one of the primary determinants of visual appeal of meat products. It is defined as the ability of fresh meat to retain its own water during storage and strongly depends on the structure of muscle proteins and their spatial arrangement. The water released can be described as drip loss or exudate loss. Water, that acts as a plasticiser of muscle proteins, is lost from the myofibrillar lattice structure because of protein denaturation and consequent reductions in the muscle fibre volume, reducing the quality of raw and cooked meat (Hughes, Oiseth, Purslow, & Warner, 2014). Exudative loss progressively increased for all samples during the eleven days of cold storage ranging from 3.14% to 8.39%, 3.61–8.50%, 1.11–6.56%, 4.92–12.25% for SAs, QUE-SAs, HA/QUE-SAs and C samples respectively. However, the water losses in control were significantly ($p < 0.05$) higher than in all chicken fillet samples with coating. This beneficial effect of the alginate-based coating may be due to its water barrier properties, which prevent exudation and avoiding meat dehydration (Song, Liu, Shen, You, & Luo, 2011). Furthermore, the obtained results suggested that the presence of the HA nanostructured network led to a strong water holding capacity. The use of active edible coatings could thus make trays appear more attractive to consumers due to the decrease in meat exudative losses, one of the main undesirable quality changes during the storage. Texture properties of meat products is an important parameter for consumers acceptability strongly affected by storage time. Texture parameters of the control sample and coated chicken fillets during the cold storage time were shown in Table 3.2. All texture parameters in coated chicken samples were significantly different ($p < 0.05$) from control probably due to the effect of the alginate layer that modifies the texture of fresh chicken fillets. A significant increase in hardness was registered in all chicken samples starting from 4 storage day. As elsewhere reported, the hardness increasing in meat

products could be related to water losses of the meat that justify the highest hardness value observed for control samples at the end of the storage period (Gallego, Arnal, Talens, Toidra, & Mora, 2020). The comparison among the coated samples pointed out a positive effect of HA/QUE-SAs to slow down the changes in hardness during the storage time compared to time 0. In contrast, no significant differences ($p < 0.05$) were observed as regard cohesiveness, probably due to the strength of the internal bonds of the sample. From a sensory point of view, they are parameters related to the effect of the first bite on the internal structure of the product. As hardness increase during the storage period for all chicken samples, a decrease of springiness values was observed, which represent the ability of the sample to recover its original form after the removal of the force. The control samples, that showed the highest hardness values at the end of the storage period, registered the lowest values of springiness ($p < 0.05$) due to the low elasticity of cellular structure resulting from a high water loss. In contrast, chewiness, which can be associated with the energy required to masticate a solid food product, decrease in coated samples compared to the control, in which it remains almost constant. However, these results indicate that the alginate-based coating would not modify the chewiness of the chicken which, at the end of the storage period, would be the same among all samples.

3.11.2. Overall acceptability, colour, odour and taste evaluation

Overall acceptability, colour, odour and taste evaluation was carried out before and after cooking of chicken fillets with the aim to evaluate the impact of alginate coating during the cooking and thus on the taste and acceptability of the chicken fillets. The results of the sensory attributes evaluated during the storage period were shown in Table 3.3. The scores of all the treatments were decreased with storage time, which probably originated from microbial growth and TVB-N production. However, the extent of this decrease in HA/QUE-SAs fillets was lower than in other samples. The outcomes of sensory attributes both in raw and cooked fillets demonstrated that the HA/QUE-SAs treatment was able to preserve the fresh sensory attribute of fresh poultry meat reaching a satisfactory score until the 11th day of storage.

Conversely, control, SAs and QUE-SAs fillets reached unacceptable sensory at 7 and 11 storage days respectively. As regards the impact of alginate coating on the taste and overall acceptability of the cooked fillets, the obtained scores at time 0 pointed out no significant differences ($p < 0.05$) between control and coated fillets probably due to the high temperatures reached during the cooking on the plate, that caused the thermal degradation

of sodium alginate layer. Moreover, alginate-based edible films are characterized by neutral taste (SenturkParreidt, Mu¨ller, & Schmid, 2018).

Table 3.3 :*Sensory evaluation of raw and cooked chicken fillets during the storage time.*

		Sample	Storage Time (days)				
			0	2	4	7	11
Fresh Chicken Meat	Odour	C	5.00±0.00aA	4.80±0.45aB	3.80±0.45aB	2.80±0.45aC	1.20±0.45aD
		SAs	4.80±0.45aA	4.80±0.45aA	4.80±0.45aA	3.80±0.45bB	2.00±0.00bC
		QUE-SAs	4.80±0.45aA	4.80±0.45aA	4.80±0.45aA	3.80±0.45bB	2.80±0.45cC
		HA/QUE-SAs	4.80±0.45aA	4.80±0.45aA	4.80±0.45aA	4.80±0.45cA	3.80±0.45dB
	Colour	C	5.00±0.00aA	4.80±0.45aA	3.80±0.45aB	2.80±0.45aC	2.80±0.45aC
		SAs	4.40±0.55bA	4.40±0.55aA	4.20±0.45bA	3.80±0.45bB	3.80±0.45bB
		QUE-SAs	4.40±0.55bA	4.40±0.55aA	4.20±0.45bA	3.80±0.45bB	3.60±0.45bB
		HA/QUE-SAs	4.40±0.55bA	4.40±0.55aA	4.40±0.55bA	3.80±0.45bB	3.80±0.45bB
Cooked Chicken Meat	Taste	C	5.00±0.00aA	4.80±0.45aA	3.40±0.55aB	2.60±0.55aC	1.20±0.45aD
		SAs	4.80±0.45aA	4.60±0.55aA	3.80±0.45aB	2.80±0.45aC	2.20±0.45bC
		QUE-SAs	4.80±0.45aA	4.80±0.45aA	3.80±0.45aB	3.30±0.55bB	2.80±0.45bC
		HA/QUE-SAs	5.00±0.00aA	4.80±0.45aA	3.80±0.45aB	3.60±0.55bB	3.80±0.45cB
	Odour	C	4.80±0.45aA	4.80±0.45aA	3.40±0.55aB	1.80±0.45aC	1.20±0.45aD
		SAs	4.80±0.45aA	4.60±0.55aA	3.80±0.45aB	2.00±0.45aC	1.20±0.45aD
		QUE-SAs	5.00±0.00aA	4.80±0.45aA	3.80±0.45aB	3.00±0.55aB	1.80±0.45aC
		HA/QUE-SAa	4.80±0.45aA	4.80±0.45aA	4.60±0.55bA	3.80±0.45bB	3.60±0.45cB

Overall Acceptability	C	4.80±0.45aA	4.80±0.45aA	3.00±0.00aB	2.40±0.55aC	1.20±0.45aD
	SAs	4.80±0.45aA	4.60±0.55aA	3.80±0.45bB	2.80±0.45aC	1.20±0.45aD
	QUE-SAs	4.80±0.45aA	4.80±0.45aA	3.80±0.45bB	3.60±0.45bB	2.80±0.45bC
	HA/QUE-SAs	4.80±0.45aA	4.80±0.45aA	3.80±0.45bB	3.80±0.55bB	3.60±0.45cB

Mean values in the same row with different capital case letters (A, B, C, D, E) are significantly different ($p < 0.05$) during the storage time, and mean values in the same column with different lower letters (a, b, c, d, e) are significantly different ($p < 0.05$) among the samples

3.12 Conclusions

Alginate edible coatings loaded with quercetin glycosides and hydroxyapatite/quercetin complexes were developed and used to extend the shelf-life of fresh chicken fillets. Preliminary in-vitro release studies exhibited a fast release of the bioactive compounds from the coatings also when the quercetin glycoside compounds were complexed with hydroxyapatite. The microbiological analysis performed during the cold storage period of coated chicken fillets highlighted the capability of alginate coating with hydroxyapatite/quercetin complexes to significantly inhibit the growth of spoilage bacteria, as well as the total volatile basic nitrogen. Moreover, the comparison among the coated samples pointed out a positive effect of hydroxyapatite to slow down the changes in hardness during the storage time of 11 days. Finally, the outcomes of colour, odour and taste evaluation both in raw and cooked fillets demonstrated that the coating with hydroxyapatite/quercetin complexes was able to preserve the sensory attributes of fresh poultry meat until the 11th day of storage. Therefore, the development of alginate coatings loaded with hydroxyapatite/quercetin complexes could be an effective method to slow down spoilage and increase the cold storage period of fresh meat such as chicken fillets.

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This part is an edited version of:

Alginate-based coatings charged with hydroxyapatite and quercetin for fresh-cut papaya shelf life

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4.1 Summary

In this study, the effect of alginate-based coatings charged with quercetin glycoside compounds and hydroxyapatite/quercetin glycoside compounds (HA/QUE) on the microbiological quality, and on bioactive compounds of fresh-cut papaya, was evaluated for 14 days at 6 °C. Alginate coatings with hydroxyapatite/quercetin showed a high capability to slow down the growth of all microbiological parameters investigated. At the end of cold storage, the total bacteria count in papaya samples covered with HA/QUE alginate coating was 4.8 log CFU g_{-1} which is significantly lower ($P < 0.05$) than 8.3 log CFU g_{-1} for uncoated samples. Total carotenoids' percentage decrease, at the end of storage, was about 20% in papaya with active coatings, with respect to the losses of 39 and 35%, registered in uncoated and alginate-coated samples respectively. Vitamin C content and the antioxidant activity measured in papaya coated with HA/QUE alginate showed significantly higher values ($P < 0.05$) for each storage day than those detected for control- and alginate-coated samples. Based on the sensory evaluation, active-coatedfresh-cut papaya reached, at the end of the storage period, suitable values for commercial purposes.

4.2 Introduction

Papaya (Carica papaya L.) is a fruit native to Central America and widespread in all tropical and subtropical regions (Brazil, Florida, India, Indonesia and Sri Lanka). The demand for ready to eat papaya is increasing, due to the interest of consumers for convenient fruits with high nutritional and healthy properties. However, in minimally processed fruit, the qualitydecay occurs more rapidly than the whole ones due to the influence of different processing steps during the preparation. The quality losses can be reduced by the application of several preservation techniques such as modified atmospheres packaging and edible coating.

Edible coatings (EC) could be an effective method to extend the shelf-life of fresh sliced fruit: They form a thin layer on the surface of the food that can be consumed with it or after their removal; they provide partial moisture, oxygen and carbon dioxide barrier, reduce water loss and slow down fruit ripening (Pinzon et al., 2019). Proteins, lipids, polysaccharides and composite materials are the principal substances employed for the production of edible coatings. Sometimes, other compounds showing antimicrobial and antioxidant activity are added in the formulations (Mohamed et al., 2020). Different studies investigated the capability of active edible coatings to preserve the quality of fresh-cut fruits during the storage (Hasan et al., 2020; Marringgal et al., 2020). Among active compounds, quercetin is a phenolic compound showing a strong antioxidant activity due to its capability to reduce the formation of free radicals and

proinflammatory substances (Xu et al., 2019). In addition, it showed antimicrobial activity against spoilage bacteria such as *Pseudomonas fluorescens* (Malvano et al., 2021). Since quercetin is a compound sensitiveto pH, temperature, light and oxygen, the use of carriers for its controlled release could be useful to overcome the problem of its limited use in foods as ingredient (Silva-Weiss et al., 2018). Hydroxyapatite (HA) is a calcium phosphate with numerous characteristics such as biocompatibility, biomimetic dimensions and biodegradability (Fulgione et al., 2019). Severaltechniques were studied to protect the bioactive compounds, such as polymeric nanoparticles, nanoemulsions and nanosystems (Zambrano-Zaragoza et al., 2018). Since HA is able to interact with organic compounds (Malvano et al., 2021), it could be a novel and promising carrier for the release of active substances in the development of edible coatings to applyfor the shelf-life extension of minimally processed fruits. Thus, activated edible coatings could be innovative strategies to preserve papayas by controlling fungal rot and post-harvest quality parameters. To the best of our knowledge, for the first time, we were here evaluated the effects of an alginate-based edible coating loaded with quercetin/hydroxyapatite complexes on the shelf-life of fresh-cut fruit (papaya). Physicochemicaland microbiological parameters, as well as sensory attributes, have been evaluated during a 14-day storage period at 6 °C.

4.3 Material and methods

4.3.1Materials

Biomimetic hydroxyapatite (HA), in form of colloidal dispersion, was provided by Research and development department (Chemical Center S.r.l., Bologna,Italy). Quercetin glucoside compounds (QUE, 98.6% food grade) were obtained from Oxford® Vitality Company (Biecester, Oxford, UK). Papaya fruits (C.papaya L, cv. Formosa) were bought from a farm in Palermo (Italy).

4.3.2Alginate-based coatings

Edible coatings were produced whit the layer-by-layer technique, using sodium alginate (1.5% w/v; Sigma Aldrich, Milano, Italy) and CaCl2 (1% w/v; Sigma Aldrich) as negatively and positively charged coating solutions respectively. Coating solutions were prepared in a water solution of sodium alginate and glycerol (2% w/v; Sigma Aldrich) by mixing for 2 h at 70 °C. According to Malvano et al. (2022), three different sodium alginate solutions were prepared: control sodium alginate solution (SA), sodium alginate with 500 ppm of quercetin glycosides (QUE) and sodium alginate with hydroxyapatite/quercetin glucosides complexes (HA/QUE) with 500 ppm of quercetin glycosides.

HA-QUE complexes were produced by adsorption method, as reported by Montone et al. (2021).

4.3.4Coated fresh-cut papaya

Papayas were selected for ripeness level based on external colour, then washed and hand peeled. The peeled fruits were cut into cubes of approximate 3 cm, then washed in NaClO solution (5% v/v) for 1 min and finally dried with paper. The coating process was carried out according to the layer-by-layer technique by dipping papaya cubes into sodium alginate solutions and calcium chloride solution. The dipping time for both solutions was 2 min. After that, the coated samples were air-dried for 5 min and finally packed into food polyethylene terephthalate boxes (13.5 × 12.5 × 5 cm) provided with a lid. The shelf-life tests were performed by comparing four different fresh-cut papaya samples prepared as below:

- fresh-cut papaya without coating (Control);
- fresh-cut papaya covered by alginate coating (SA);
- fresh-cut papaya covered in alginate coating enriched with QUE (QUE);
- fresh-cut papaya covered in alginate coating enriched with HA/QUE (HA/QUE).

A total of 12 boxes, for each treatment, were prepared and kept at 6 °C for 14 days (Albanese et al., 2007). Measurements of the microbiological and quality parameters were performed on three replicates at regular interval times (0, 3, 7, 10 and 14 days).

4.3.5Thickness and water vapour permeability

The thickness of different coatings was measured with P6 stylus profilometer (KLA-Tencor, Milpitas, CA,USA). Coatings' water vapour permeability (WVP) was determined gravimetrically according to Vieira et al. (2020). The weight of each capsule was monitored regularly for 7 days. WVP was calculated as follows:

$$WVP = \frac{\Delta g}{\Delta t} * \left(\frac{X}{A * \Delta P} \right) \quad \text{(a)}$$

where Δg is the rate of weight change (g h^{-1}), x is the Δt thickness (mm), A is the area (0.00025434 m^2), and ΔP is the partial pressure difference (3.169 kPa at 25 °C).

4.3.6 Headspace gas composition and pH

O2 and CO2 in the headspace of the packages were determined by an O2/CO2 gas analyser (PBI Dansensor,
Checkmate3, Ringsted-Denmark) according to Liguori et al. (2021). The pH was measured using a digital pH-meter (Model 2001; Crison, Barcellona, Spain) with a penetration pH-electrode**.**

4.3.7 Analysis of microbiological parameters

Ten gram of papaya sample was homogenised in 90 mL of peptone-saline solution (PSS) by a homogenising mixer for 3 min at room temperature, and after, decimal serial dilutions were prepared. Total viable count (TVC) was plated in plate count agar (Oxoid; Thermo Fisher Scientific™, Milano, Italy) and incubated at 30 °C for 3 days. LAB was analysed by spread plate method onto de Man, Rogosa and Sharpe (MRS) agar and kept in aerobiosis, at 30 °C for 72 h. The count of moulds and yeasts was carried out using the spread plate method in Rose Bengal Agar and then kept at 25 °C for 5–7 days. The plates with 10 to 150 colonies were selected, and all the different colony morphologies (fungal, yeast-like, etc.) were selectively counted.

4.3.8 ABTS and DPPH radical-scavenging assays

Methanolic extracts of papaya samples were prepared by homogenisation of 4 g samples in 20 mL of methanol: water solution (1:1 v/v). The mixture was stirred at dark for 20 min and then centrifuged at 2516 g at 4 °C for 8 min. The antioxidant activity (AA) of extracts was evaluated through both DPPH and ABTS, performed according to Liguori et al. (2019) and Gayosso-Garcı´a Sancho et al., 2010 respectively. For both radical-scavenging assays, a Trolox standard calibration curve was carried out and the antioxidant activity results were expressed as Trolox equivalent (TE) mg/gdw.

4.3.9 Total carotenoids content

Total carotenoid (TC) extraction was carried out according to Gayosso-Garcı´a Sancho et al. (2011). TC content was measured by a Perkin Elmer Lambda 25 spectrophotometer at the wavelength of 450 nm. β- carotene standard was employed for the construction of a calibration curve to calculate TC.

4.3.10 Vitamin C content

Extracts of uncoated and coated papaya for vitamin C content detection were prepared after homogenization of 5 g of pulp with 50 mL of bidistilled water using an Ultraturrax Homogenizer (T25; IKA, Staufen, Germany).

The homogenised extracts were then centrifuged (4000 rpm, 8 min, 4 °C) and filtered with 0.45-μm filter. Ionic exchange chromatography was used for the analysis of Vit C content according to the method previously reported (Liguori et al., 2017).

4.3.11 Texture profile analysis

Texture profile analysis (TPA) of fresh-cut papaya samples was performed at room temperature by a texturometer (LRX Plus; Lloyd Instruments, Chicago). The samples underwent a double compression up to 50% of the original height by a cylindrical probe (diameter 1 cm) at 1 mm s−1. The parameters evaluated were hardness, cohesiveness, springiness and chewiness. The data are reported as the average of five measurements per sample.

4.3.12 Assessment of sensory attributes

Sensory attributes of fresh-cut papaya samples were evaluated by five members from the University of Salerno who were selected based on their experience in the sensory analysis. All panellists were specifically trained using different samples of fresh papaya, allowing them to recognize the quality characteristics to be assessed and to familiarize themselves with the scales and procedures adopted. Fresh-cut papaya samples of about 10 g were presented to each taster in random order and evaluated, at each storage day, by odour, colour, taste, firmness, off flavours and overall acceptability using a 5-points hedonic scale (5 = very good - 1 = very poor). Scores equal to or lower than 3 were considered unacceptable for the marketing.

4.4Statistical analysis

Thickness and WVP of the three edible coatings as well as the microbiological parameters, antioxidant activity, vitamin C, total carotenoids and TPA of the fresh-cut papaya samples were reported as mean and standard deviation and subjected to analysis of variance (ANOVA).
The significance of difference ($P < 0.05$) among different samples (C, SA, HA and HA/QUE) and for each sample during the storage time (0, 3, 7, 10 and 14 days) were determined by LSD test by the Analysis Lab software.

4.5 Results and discussions

4.5.1Physical properties of edible coatings

The main physical properties (thickness and WVP) of alginate-based coatings developed for fresh-cut papaya are reported in Table 4.1.

The thickness of alginate-based coatings varied from 6.12 μm (QUE) to 7.17 μm (HA/QUE). A significant increase ($P < 0.05$) in thickness was observed when HA crystals were added to the coatings. No significant differences were found between SA and QUE coatings (Table 1). This result suggests that the presence of HA crystals influenced the density of film probably due to the increase in solid content (Vieira et al., 2020). Similar data were reported by Vieira et al. (2020) and Arfat et al. (2017) during the characterisation of edible films containing gold and silver nano/microparticles. One of the main actions of edible coatings is control water transfer between the food and headspace of the

Table 4.1*Thickness and water vapour permeability (WVP) of developed alginate-based coatings*

	Thickness (μm)		WVP (g·mm/h·m2·kPa)	
SA	6.21	0.44°	0.11	0.0020a
QUE	6.12	0.080	0.091	0.0010
HA/QUE	7.17	0.48	0.11	0.0010

Mean values in the same column with different letters (a, b, c) are significantly different ($P < 0.05$) among the coatings.

packages. The WVP of coatings changed significantly from 0.09 to 0.11 g_mm/h_m2_kPa in the different edible coatings (Table 4.1). The hydrophilic nature of sodium alginate leads to a water-permeable coating, as well as the addition of plasticiser glycerol increases the WVP values due to the reduction in intermolecular bonds between polymer chains (Parreidt et al., 2018). SA coating showed the highest value of WVP compared with QUE and HA/QUE coatings probably due to the presence of HA crystals and quercetin glycoside compounds which reduce the diffusion of water molecules.

4.5.2 Headspace gas evaluation

Fruits keep breathing even after harvesting and cutting (Tabassum & Khan, 2020). The effect of alginatebased coatings on the O2 and CO2 levels in the headspace of the packed fresh-cut papaya is shown in Fig. 4.1 CO2 production and O2 consumption were recorded in all samples, without significant differences ($P < 0.05$) among samples, until day 7. A higher O2

decrease for uncoated fruits was observed at the 10th storage day, whereas no significant differences ($P < 0.05$) were found between coated samples during the storage, that reached after 14 days a O2 value close to 10%. As regards the CO2 increase, uncoated and QUE samples showed the highest CO2 production during the last days of storage. No differences ($P < 0.05$) in CO2 production were observed between SA and HA/QUE samples, that reached CO2 final values close to 10%. These results pointed out the capability of alginate-based coatings to slow down the respiration rate of fresh-cut fruits, according to Azarakhsh et al. (2012) who studied the effect of alginate coatings for fresh-cut pineapples. The high level of respiration rate recorded for the QUE samples may be due to a degradation of the alginate polymer caused by the oxidation of quercetin glycoside compounds. Although quercetin is a strong antioxidant compound, it is also chemically unstable and can be subjected to chemical and enzymatic oxidation by peroxidase and polyphenol oxidase enzymes, which are contained in fresh papaya naturally (Cano et al., 1995). The oxidation products, mainly phenolic acids (Zenkevich et al., 2007), may have induced the degradation of alginate-based coatings, which are easily unstable in acidic conditions (Tonnesen& Karlsen, 2002).

4.5.3 Microbiological analysis of papaya and pH evaluation

The cut surface of fresh-cut fruit is exposed to environment, inducing the degradation and causing an increase in microbial count as consequence (Fig. 4.2). The absence of a protective physical barrier in control samples makes the fruit more prone to spoilage by microorganisms. As shown in Fig. 4.2a, the initial microbial charge (day 0) was characterised by the presence of a total bacterial count (TBC) ranging from 2.7 log CFU g−1 in HA/QUE to 4.0 log CFU g−1 in control samples, acceptable charge if we consider the commonvalues related to the microbial population of fruits and vegetables ranging between 5 and 7 log CFU g−1 (Di Cagno et al., 2013). The lowest values(4.5 log CFU g−1) observed for activated coated

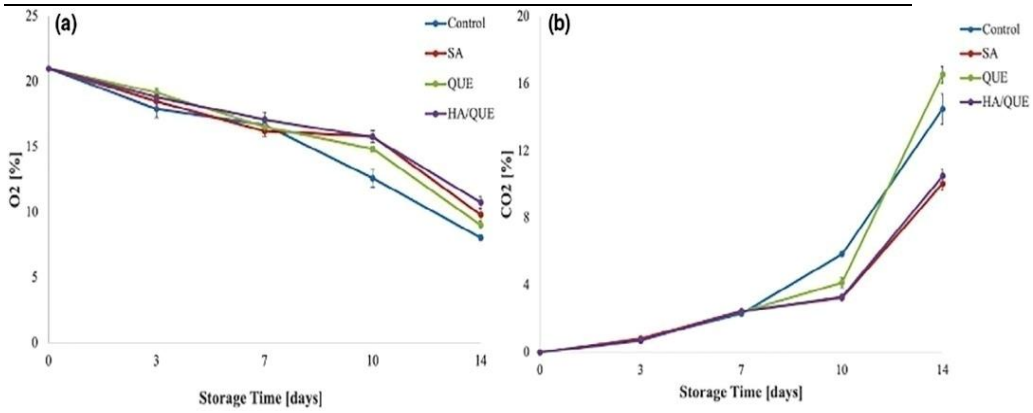

Figure 4.1 *Changes in O2 (%) (a) and CO2 (%) (b) in fresh-cut papaya packages during the storage time at 6 °C.*

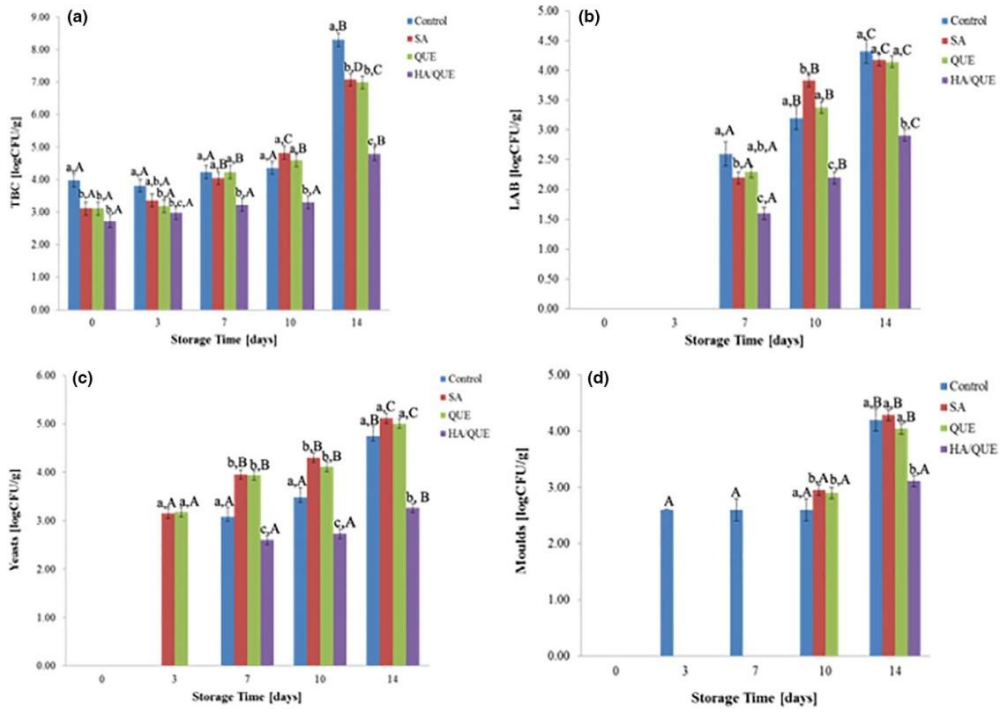

Figure 4.2 *Changes in total bacterial count (TBC) (a), lactic acid bacteria (b), yeasts (c) and moulds (d) in fresh-cut papaya during the storage time at 6 °C. Different letters (a, b, c, d) reveal significant differences (P < 0.05) among the samples for each storage time, and different letters (A, B, C.) reveal significant differences (P < 0.05) among treatments during the storage time.*

samples showed the antimicrobial effect of quercetin glycosides as reported in our previous study (Malvano et al., 2021). These results are in accordance with Tabassum & Khan (2020) who evaluated the effect of alginate-based coating enriched with 2% of thyme or oregano essential oil on the TBC growth in fresh-cut papaya stored for 12 at 4 °C. Microbial growth in fruits and vegetables is a complex phenomenon that involves microbial ecology, microbial competition, substrate composition and Ph values. Many microorganisms, in particular LAB and fungi (yeasts and moulds), are capable of using fruit as substrate and cause spoilage (Tournas et al., 2006). Microbial competition and the initial acidic pH of papaya samples (Fig. 4 3) could justify the no detectability of LAB, yeasts and moulds at time 0 measured in all investigated samples (Jin& Kirk, 2018). As expected and according to previous study on the subject (Tabassum & Khan, 2020; Cortez-Vega et al., 2014; Brasil et al., 2012), the growth of all microbiological parameters was observed as the storage time increased. As regards TBC, significantly lower values were observed for HA/QUE in comparison with control, SA and QUE samples, during all storage periods.Given the value ranging from 6 to 7 log CFU g−1, which are recognized as accepted limits of TBC value for minimally processed fruit (Corbo et al., 2006; Lavelli et al., 2006), HA/QUE showed TBC values lower than acceptable limits for all storage periods, unlike control, SA and QUE, whichreached these limit values after 14 days. LAB was registered in all papaya samples with the highest value of 2.50 log CFU g−1 in control sample and the lowest in HA/QUE (1.50 log CFU g−1) only after the 7th day, probably due to microbial competition and to the lowering of pH that reached in all samples values close to 5.4 or lower. As observed for total bacteria counts significant ($P < 0.05$), lower LAB charges were measured for HA/QUE during the following storage days (Fig. 2b). These results highlighted that, according to literature (Raybaudi-Massilia et al., 2008; Tabassum & Khan, 2020), the coating with only alginate did not show effective antimicrobial activity.

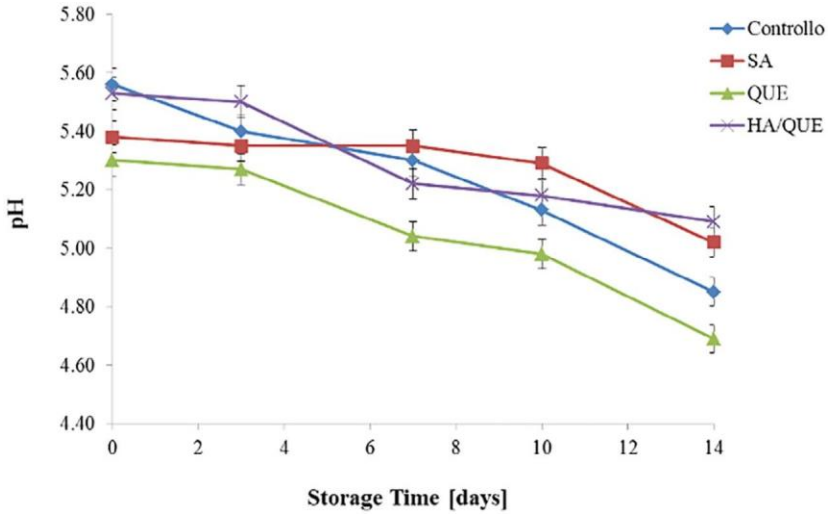

Figure 4.3 *pH of fresh-cut papaya samples during the storage time at 6 °C.*

Moreover, the comparison between QUE and HA/QUE samples pointed out that coating with QUE alone did not effectively inhibit the microbiological growth, showing a similar charge as in the SA sample. The effectiveness ofquercetin glycoside compounds in slowing down microbial growth is shown only when it is adsorbed into HA crystals. Thanks to their biomimetic property, different authors have underlined the capability of HA crystalsto be used as a delivery carrier of antimicrobial and antioxidant agents (Nocerino et al., 2014; Fulgione et al., 2016; Calasans-Maia et al., 2019; Fulgione et al., 2019). Our previous studies have shown a very fast release of quercetin glycoside compounds from alginate-based coatings when the bioactive compounds were complexed with HA crystals, pointing out how anedible coating enriched with hydroxyapatite/quercetin complexes could be an interesting alternative for the shelf-life extension of fresh chicken fillets (Malvanoet al., 2022). A similar trend to the TBC was observed for yeast and mould count where the control samples had values much above the consumable limit, while among the coated samples, HA/QUE sample exhibited the lowest mould count with 3.30 log CFU g−1. The detected data showed an antimicrobial effect of quercetin glycoside compound versus moulds that appears in QUE and QUE/HA samples after 10 and 14 days

respectively. Conversely, the yeasts seemed to benefit from the presence of quercetin compounds in the alginate coatings, probably due to the significant decrease in pH values.

4.5.4 Total carotenoids content and antioxidant activity evaluation

Fresh-cut papaya shows a high nutritional interest as it is an excellent source of carotenoids, nutritional compounds that act as natural antioxidants, protecting cellular components from oxidative damage. The alginate-based coating showed a positive effect in maintaining the total carotenoids content of the freshcut papaya, as shown in Fig. 4.4 Carotenoid compounds, susceptible to enzymatic and non-enzymatic oxidation continue to be synthesised during ripening. In fact, until storage day 3, the amount of total carotenoids increased in both coated and uncoated fruits, reaching the maximum values ranging from 0.16 to 0.19 mg gdw −1, respectively, close to the total carotenoids content related to the maximum level of ripeness of the papaya fruit (Rajyalakshmi et al., 2003; Gayosso-Garcı´a Sancho et al., 2011). The reduction in total carotenoid content started from day 7 for all papaya samples. QUE and HA/QUE samples showed the highest carotenoids content for all days of the storage period: No significant changes ($P < 0.05$) from day 7 to day 10 for both samples were recorded. At the end of storage, the carotenoids percentage decrease compared with time 0 in QUE and HA/QUE was about 20%, while in control and SA, carotenoids showed losses of 39 and 35%, respectively. Brasil et al. (2012) observed a total carotenoids reduction of 50% in uncoated fresh-cut papaya after 15 days at 4 °C in contrast a more limited losses were registered when papaya samples were covered by betacyclodextrin based coatings. The losses of carotenoids could be due to the oxygen exposure to the product since b-carotene is oxidised when exposed to light and oxygen (Rivera-Lopez et al., 2005). The lowest losses in total carotenoids observed for QUE and HA/QUE, recorded in all storage days, could be explained by the presence of quercetin glycoside compounds which, acting as a reducing agent, protect carotenoids from oxidative degradation and thus preserve their content in the fruits (Wang et al., 2016). According to the total carotenoids results, during the first 3 days of the storage, while the ripening went on, the antioxidant activity of coated and uncoated fresh-cut papaya increased, reaching their maximum values.

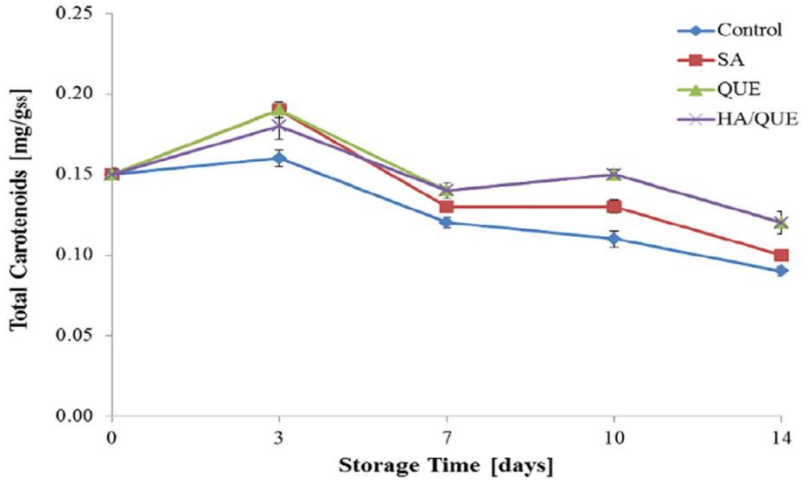

Figure 4.4 *Changes in total carotenoid content in coated and uncoated fresh-cut papaya during the storage time at 6 °C.*

Fig. 4.5 shows the percentage change in antioxidant activity of all samples, evaluated through both DPPH and ABTS radical-scavenging assays were reported. From the 3rd day until the end of the storage time, the oxidation reactions became prominent and, along with the degradation of carotenoid compounds, the antioxidant activity, measured by DPPH, was subjected to a reduction in all samples (Fig. 4.5a). However, smaller reductions in the antioxidant activities were found in QUE and HA/QUE samples during storage period. The reduction in antioxidant activity in control and SA samples derived from the degradation of antioxidant compounds (carotenoids and ascorbic acid) during the storage period, while in the activated coatings, the presence of quercetin glycoside allowed preserving the antioxidant activity.

ABTS results (Fig. 4.5b) showed the same trend of DPPH ones: A lower degradation of antioxidant compounds in coated fruits with quercetin was reached in all days of the storage period, with an antioxidant activity significantly ($P < 0.05$) higher for HA/QUE samples than the other ones after 14 days of storage. These results confirmed the effectiveness of the alginate-based edible coating enriched with HA/QUE complexes to preserve antioxidant compounds of fresh-cut papaya during the entire storage period.

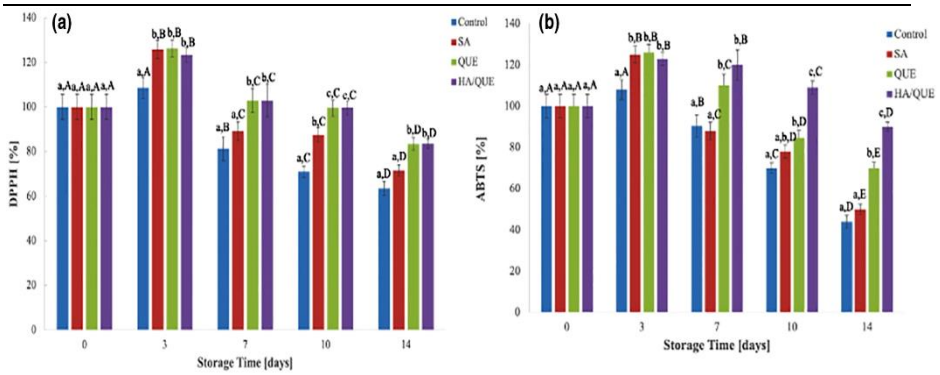

Figure 4.5 *Percentage change in antioxidant activity of coated and uncoated samples, evaluated through DPPH (a) and ABTS (b) radicalscavenging assays. Different letters (a, b, c, d) reveal significant differences (P < 0.05) among the samples for each storage time, and different letters (A, B, C. . .) reveal significant differences (P < 0.05) among treatments during the storage time.*

4.5.5 Vitamin C evaluation

The measurements of vitamin C content were carried out at the beginning, at the middle time and the end of the storage period. At time 0, fresh-cut papaya showed about 48 mg/100 gfw of vitamin C according to data previously reported for Formosa fresh papaya (Oliveira et al., 2010).As shown in Fig. 4.6, the slight but significant (P < 0.05) increase in vitamin C content occurred in all samples from the beginning until the 7th storage day due to the ripening of papaya, while a remarkable reduction was recorded at the end of storage period. In particular, the drastic reduction in vitamin C content in control samples, which is in accordance with previous study on active edible coating on minimally processed papaya (Brasil et al., 2012), is due to the direct exposure of fresh cut fruit to air, which caused the oxidation of the compound. A lower reduction in vitamin C content was observed for fresh-cut papaya covered with active coatings compared with SA samples: This behaviour is explained by the presence of quercetin glycoside compounds that acted as reducing agents, by partially protecting vitamin C from oxidative degradation. The higher reduction in vitamin C in the QUE compared with HA/QUE could be due to the quercetin glycoside compounds oxidation, with the production of acidic compounds, which may have induced a partial degradation of the coating and thus a higher air exposition of the fruits, which caused the losses of vitamin C.

4.6 Texture profile analysis

Texture parameters of coated and uncoated fresh-cut papaya during the storage time are shown in Table 4.2. It can be seen that the hardness values of all papaya samples decreased during the storage period. The hardness decrease in papaya is due to enzymatic degradation of the cell wall and decomposition of intracellular materials (dos Passos Braga et al., 2020). The comparison among the samples pointed out the positive effect of HA/QUE coating to slow down in the hardness decrease during the cold storage. The reduced loss of firmness in HA/QUE-coated papaya could be due to a reduction in enzymatic activity in the fruit as a consequence of decreased endogenous ethylene production and respiratory rate (Monz´on-Ortega et al., 2018). At the end of storage time, HA/QUE sample registered a 44.24% loss of hardness in contrast to 82.59%, 75.89% and 72.54% shown in Control, SA and QUE samples respectively. Cortez-Vega et al. (2014) observed a 17.64% loss of firmness after 12 days of storage at 4 °C in minimally processed papaya coated with edible coatings from protein isolate with organoclay montmorillonite. The difference with the data obtained in this study could be due to the different cold temperature used for the storage as well as to the ripening state of fresh papaya employed. A decrease in cohesiveness values was shown for all samples during the storage time. The lowest cohesiveness in control samples at the end of the storage period indicated a prominent decay of cell tissues. The HA/QUE samples appeared to have the highest cohesiveness, which implied the samples still retained their texture also after 14 days of storage.

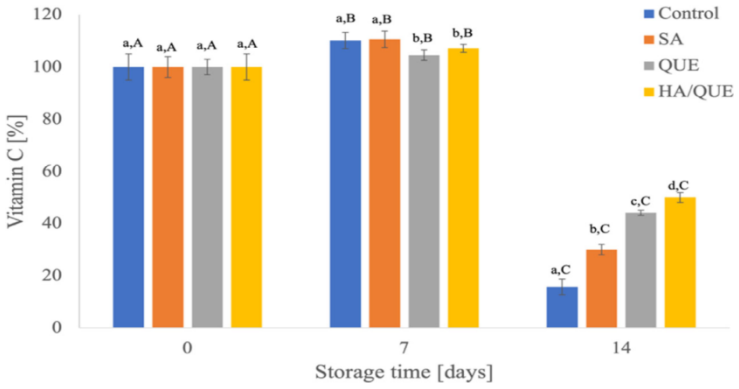

Figure 4.6 *Percentage change in vitamin C content of coated and uncoated samples. Different letters (a, b, c, d) reveal significant differences (P < 0.05) among the samples for each storage time, and different letters (A, B, C. . .)*

reveal significant differences (P < 0.05) among treatments during the storage time.

Table 4.2 *Texture profile analysis parameters of fresh-cut papaya during the storage time at 6 °C*

		Storagetime(days)				
	Sample	0	3	7	10	14
Hardness [N]	Control	12.06±0.40a,A	7.10±0.72a,B	5.47±0.18a,B	3.15±0.06a,C	2.10±0.07a,D
	SA	13.77±1.97a,A	7.62±0.38a,B	4.99±0.06a,C	4.46±0.07b,D	3.32±0.19b,E
	QUE	12.93±0.80a,A	7.74±0.60a,B	5.02±0.09a,C	4.53±0.10b,D	3.55±0.10b,E
	HA/QUE	12.93±0.67a,A	11.37±0.39bB	9.46±0.12b,D	8.19±0.20c,E	7.21±0.20c,F
Cohesivenes	Control	12.06±0.40a,A	7.10±0.72aB	5.47±0.18a,B	3.15±0.06a,C	2.10±0.07a,D
	SA	13.77±1.97a,A	7.62±0.38a,B	4.99±0.06a,C	4.46±0.07b,D	3.32±0.19bE
	QUE	0.41±0.02c,A	0.36±0.01a,B	0.32±0.01aC	0.26±0.02c,D	0.20±0.02b,E
	HA/QUE	0.40±0.01c,A	0.40±0.02bA	0.30±0.01aB	0.30±0.01dB	0.30±0.01cB
Springiness [mm]	Control	0.41±0.03a,A	0.41±0.02aA	0.40±0.01aA	0.38±0.01a,AB	0.37±0.01a,B
	SA	0.59±0.07b,A	0.54±0.01bA	0.53±0.02bA	0.51±0.01bAB	0.51±0.01b,B
	QUE	0.56±0.03b,A	0.55±0.03bA	0.53±0.01bA	0.51±0.01b,B	0.51±0.01b,B
	HA/QUE	0.64±0.02c,A	0.62±0.03cA	0.62±0.02,A	0.61±0.01c,A	0.60±0.01c.A
Chewiness [N*mm]	Control	0.35±0.05a,A	0.31±0.01aA	0.29±0.01aA	0.21±0.01a,B	0.20±0.01aB
	SA	0.40±0.01abA	0.40±0.02bA	0.39 ±0.01bA	0.37±0.01bA	0.34±0.02,bB
	QUE	0.41±0.01abA	0.41±0.01bA	0.39±0.02bA	0.37±0.01bB	0.35±0.02bB
	HA/QUE	0.42±0.01abA	0.42±0.02bA	0.40±0.01bA	0.38±0.02bAB	0.36±0.01bB

Different letters (a, b, c, d) reveal significant differences (P < 0.05) among the samples for each storage time, and different letters (A, B, C, .. .) reveal significant differences (P < 0.05) among treatments during the storage time.

On the contrary, no significant differences (P < 0.05) during the storage time were registered in the springiness values in all coated and uncoated papaya. The higher springiness values recorded in all coated samples with respect to uncoated ones could be due to the elasticity of the alginate-based coatings. Finally, the chewiness, defined as the energy required to chew a solid food

until its swallowing, was significant ($P < 0.05$) lower in control samples with respect to coated ones, on each day of the storage period. As reported by Dhall (2013), edible coating works to modify the internal gas composition of individual fresh-cut fruit, with a high influence on the lowering respiration rate. By limiting the respiration of papaya, the coatings have delayed the maturation phenomena, thus preventing the softening of
fresh-cut papaya. The effectiveness of polysaccharidesbased coating in slowing down the tissue breakdown of fresh-cut fruits was also reported elsewhere (Brasil et al., 2012).
Texture profile analysis results highlighted the high potential of coatings loaded with HA/QUE in retaining the texture of fresh-cut papaya.

4.7 Assessment of sensory attributes

The evaluation of coated and uncoated sample at time 0 highlighted that the alginate-based coatings did not affect the sensory attributes of fresh-cut papaya, but a slight decrease in odour for the coated samples was evaluated (Fig. 4.7). During the first 7 storage days, a slight decrease in sensory attributes was recorded in all coated papaya samples in comparison with time 0. A strong loss in the scores was observed on the 10th day in all samples except for HA/QUE samples that preserved the attributes of colour, taste and firmness. This difference could be explained by the higher microbial load, responsible for spoilage with consequent production of unpleasant compounds. On the 14th day, the sensory evaluation was carried out only for the HA/QUE samples, due to the presence of visible mould
colonies on the surface of other samples. Finally, the evolution of sensory attributes during the storage highlighted the positive effects of alginate-based coatings charged with HA/QUE complexes which were still assessed acceptable after 14 storage days.

4.8 Conclusion

The capability of alginate-based coatings, incorporated with bioactive quercetin glycoside compounds to preserve the quality parameters of fresh-cut papaya stored for 14 days at 6 °C, was evaluated. The physical characterisation of alginate-based coatings with and without active compounds showed how the presence of HA crystals allowed an increase in the film thickness as well as a decrease in water vapour transmission.
The microbial analysis carried out during the storage period pointed out the positive effect of HA charged with QUE to inhibit the growth of spoilage bacteria, as well as to slow down the respiration rate of freshcut papaya. Moreover, the activated coatings have shown high efficacy in preserving the antioxidant compounds naturally present in papaya, including carotenoid

compounds. Therefore, the use of hydroxyapatite/quercetin complexes loaded into an alginatebased coating could be an effective method of preserving the quality of fresh-cut papaya, by extending its shelf-life.

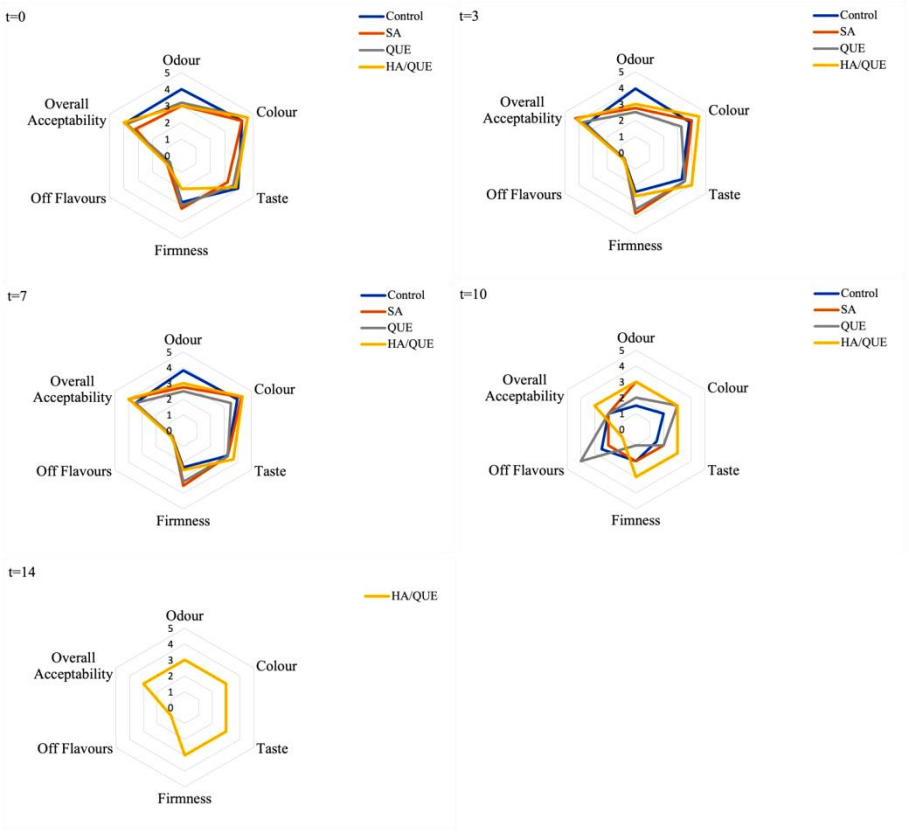

Figure 4.7 *Spider plots of sensory attributes (odour, colour, taste, firmness, off flavours and overall acceptability) of fresh-cut papaya during the storage time at 6 °C.*

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Lactoferrin, Quercetin, and Hydroxyapatite Act SynergisticallyagainstPseudomonasfluorescens

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5.1 Abstract

Pseudomonas fluorescens is an opportunistic, psychotropic pathogen that can live in different environments, such as plant, soil, or water surfaces, and it is associated with food spoilage. Bioactive compounds can be used as antimicrobials and can be added into packaging systems. Quercetin and lactoferrin are the best candidates for the development of a complex of the two molecules absorbed on bio combability structure as hydroxyapatite. The minimum inhibiting concentration (MIC) of single components and of the complex dropped down the single MIC value against *Pseudomonas fluorescens*. Characterization analysis of the complex was performed by means SEM and zeta-potential analysis. Then, the synergistic activity (Csyn) of single components and the complex was calculated. Finally, the synergistic activity was confirmed, testing in vitro its anti-inflammatory activity on U937 macrophage-like human cell line. In conclusion, the peculiarity of our study consists of optimizing the specific propriety of each component: the affinity of lactoferrin for LPS; that of quercetin for the bacterial membrane. These proprieties make the complex a good candidate in food industry as antimicrobial compounds, and as functional food.

5.2 Introduction

Pseudomonas fluorescens is a Gram-negative, aerophile, and psychotropic bacterium. The psychotropic property of this bacterium favors its growth—even in cold rooms— and reduces the shelf life of food products. Thus, *Pseudomonas fluorescens* contributes particularly to the deterioration of fish, milk, and meat stored at low temperatures.

Pseudomonas spp.—including *Pseudomonas fluorescens*—account for up to 97% of bacteria isolated in cattle meat stored in cold rooms [1–4]. These species secrete several enzymes (protease, lipase, and thermostable lecithinase) that cause deterioration of food products [5,6], a source of heavy economic losses to the food industry [7]. So far, numerous antimicrobial additives have been exploited in order to control microbial contamination during the food supply chain [8–10]. Generally, consumers consider food additives as very unhealthy. Since consumers see chemical additives as dangerous, the food industry has decided to use natural molecules to control bacterial contamination [11–13]. Natural molecules often are active only at high concentrations and against a limited number of bacterial species. This limit can partially be reduced using lower concentration of several components. The food industry generally prefers adding additives to the packaging, rather than directly to the product [14]. However, this solution

needs using a carrier to control the additive release and a scaffold to preserve the activity of the additives throughout the shelf life of the product.

Among the potential components to use against *Pseudomonas fluorescens*, we exploited quercetin, lactoferrin, and hydroxyapatite. Quercetin (que) is a polyphenolic molecule common in plants and known as an efficient antioxidant. In plants, quercetin is present in the forms of quercetin-3-O-rutinoside (rutin), quercetin-3-O-glycoside, and quercetin-3-O- (6"-O-acetil)-glycoside. Quercetin exerts its antioxidant activity principally by eliminating free radicals [15], chelating metallic ions [16], and inhibiting lipid peroxidation [17]; in addition, it is active against bacteria and yeast (*Pseudomonas aeruginosa, Salmonella enteritidis, Staphylococcus aureus, Escherichia coli, Proteus spp.*, and *Aspergillus flavus*) [18].

Quercetin exerts its antibacterial activity by degrading the bacterial cell wall, altering cell permeability, protein synthesis, enzymatic activity, and inhibition of nucleic acid synthesis [18–22].

Lactoferrin (lacto) is a glycoprotein with a molecular weight of 78 Kd. Its peculiar property is of binding iron—abundant in animal and human milk—and in exocrine secretions.

Originally, the antimicrobial activity of lactoferrin was attributed to iron chelation; at present, we know that it directly damages the external membrane of Gram-negative bacteria binding to the lipid A of LPS (Lipopolysaccharide) and promotes its removal from the bacterial surface [23]. Further, lactoferrin and quercetin are both anti-inflammatory molecules [24]. In particular, quercetin controls the activation of NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) [25] and the release of pro-inflammatory cytokine (TNF-a, IL-1b, IL-6, IL-8) [26]. Lactoferrin modulates the expression of proinflammatory cytokine [27,28], chemiotactic factor production, and the expression of adhesion molecules [29]. This property as suggested their use against COVID-19 [30].

Hydroxyapatite (HA) [Ca5(PO4)3(OH)]—an inorganic mineral and basic component of the bones—has also been shown efficient as a carrier-molecule for its property of interacting with antimicrobial peptides, and organisms such as bacteriophages [31,32]. Moreover, it has already been successfully tested as a stabilizer of quercetin [33] and lactoferrin [34] against Pseudomonas spp. and Salmonella spp., respectively.

The purpose of the present study was to test the potential utility of a complex that included quercetin and lactoferrin as antimicrobials, and hydroxyapatite as a carrier for the food industry.

First, we tested in vitro the antimicrobial activity of quercetin and lactoferrin against *Pseudomonas fluorescens*, individually and in combination as a complex with hydroxyapatite.

We established the minimum inhibiting concentration (MIC) of single components and of the complex. Then, we calculated the synergistic activity (Csyn) of single components and of the complex. Finally, we confirmed the synergistic activity of the complex, testing in vitro its anti-inflammatory activity on U937 macrophage-like human cell line.

5.3 Results

5.3.1 Antimicrobial Activity of Que and Lacto Alone and Complexes with HA

First, we tested separately Que and Lacto against *Pseudomonas fluorescens*. Que displayed a slightly higher antimicrobial activity (Figure 1a). Next, we tested Que and lacto, each in combination with HA. Que and Lacto displayed the same MIC values (500 ppm) (Figure 5.1b), which is much higher, compared to the concentration of 250 ppm reported as the maximum concentration approved [29,35], suggesting that HA does not show a significant improvement of Que and Lacto antimicrobial activity.

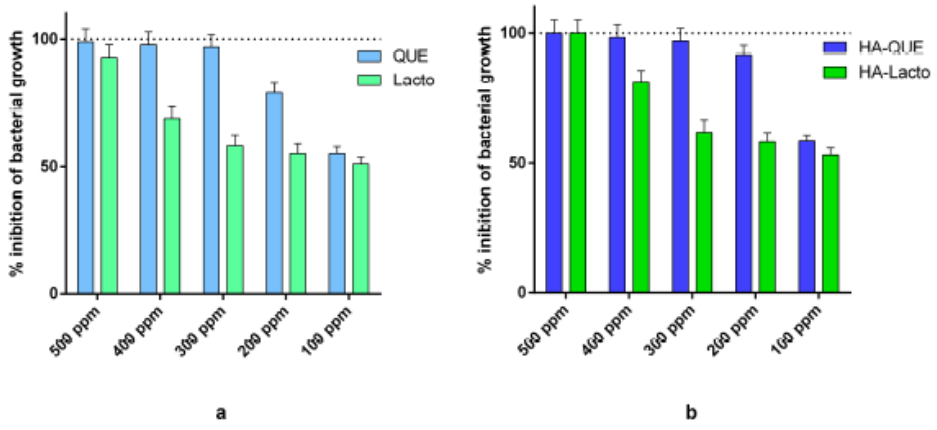

Figure 5.1. *(a) Antimicrobial activity at different concentrations of Que and Lacto against Pseudomonas fluorescens (10^6 CFU/mL). (b) Antimicrobial activity at different concentrations of HA–Que and HA–Lacto against Pseudomonas fluorescens (10^6 CFU/mL).*

In order to determine their optimal concentrations, Que and Lacto were tested together against *Pseudomonas fluorescens*. The MIC of the two molecules together was 200 ppm (w/v) (Figure 5.2). This MIC value (200 ppm) was combined with HA and tested at different concentrations and different combinations. Specifically, interaction of Que and Lacto with HA

displayed different antibacterial activity, depending upon which molecule was added first to HA. The highest inhibition was obtained when the HA was first incubated with Lacto and then with Que (smaller compared to lacto) at a concentration of 100 ppm (w/v) (Figure 5.3b). Instead, when the order was inverted (HA–Que–Lacto) at the same concentration (100 ppm), the inhibition was only 40% (Figure 5.3a).

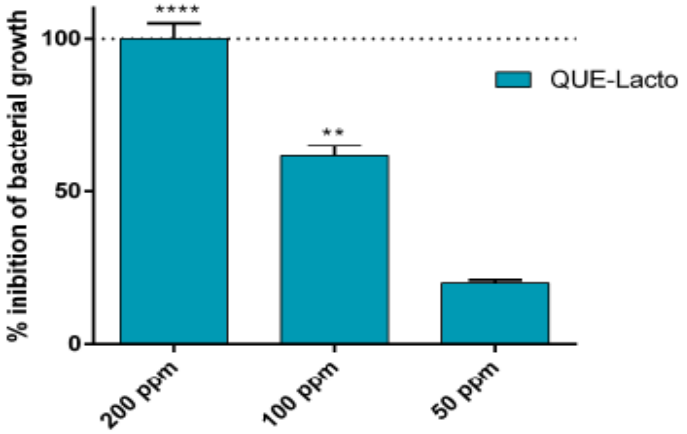

Figure 5.2. *Antimicrobial activity of Que and Lacto together against Pseudomonas fluorescens (10^6 CFU/mL). Statistical analysis was performed and considered statistically significant when* $p < 0.05$ *(**$p < 0.01$, ****$p < 0.0001$) according to two-way ANOVA multiple comparisons*

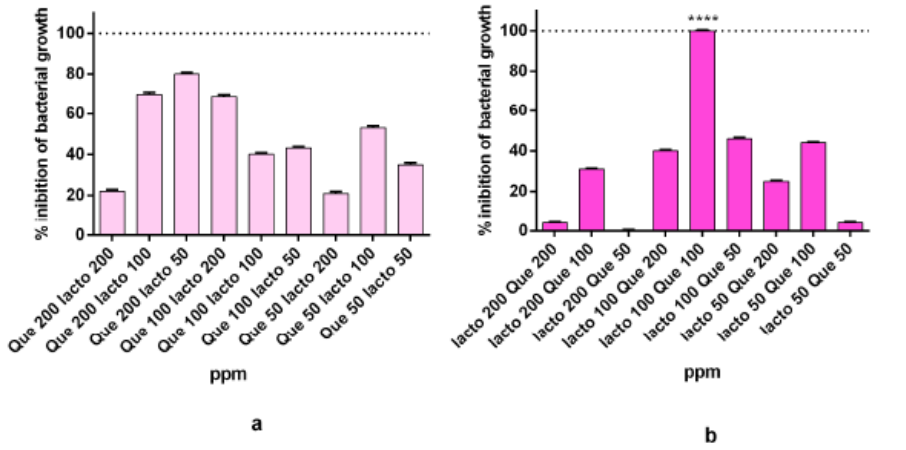

Figure 5.3. *Antimicrobial activity of the Que and Lacto complex against Pseudomonas fluorescens (10^6 CFU/mL). Different concentrations of Que and Lacto were absorbed on HA. The absorption order was Que and then Lacto (a) Lacto and then Que (b). Statistical analysis was performed and considered statistically significant when* $p < 0.05$ *(****$p < 0.0001$*) according to two-way ANOVA multiple comparisons*

5.3.2. Evaluation of HA Absorption Capacity

Results of the absorption showed that Que was fully absorbed on HA structure (1:100 w/v) for all quantities of Que tested (50–200 ppm) (Figure 5.4a). Additionally, for Lacto the absorption on HA is total, even between 50 and 100 ppm the absorbance results in negative values (Figure 4b). The orange points in both graphs correspond to supernatant's absorption after reaction with HA.

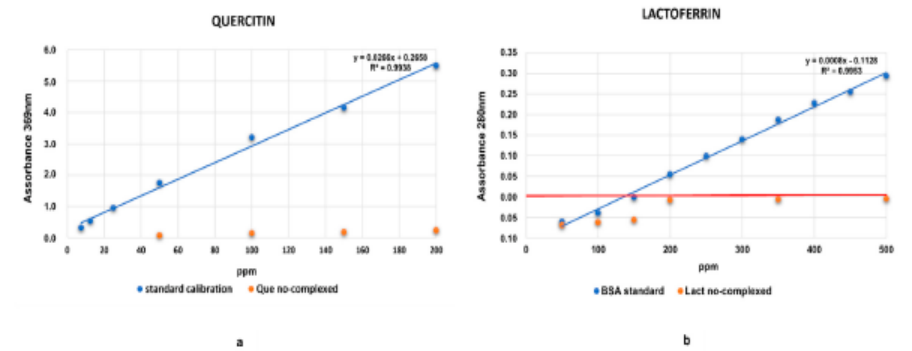

Figure 5.4. (**a**) The effective amount of Que (50–200 ppm) at the end of the adsorption process on HA. (**b**) The effective amount of Lacto (50–200 ppm) at the end of the adsorption process on HA.

5.3.3. Synergistic Activity of the Complex

The complex HA–Lacto–Que, at the concentration of 100 ppm (w/v)—alone—can inhibit *Pseudomonas fluorescens* (Figure 5.3b). Next, we looked for a synergistic activity of the complex. The results shown in Table 5.1 clearly demonstrate that the highest activity occurs when the complex HA–Lacto is used with Que at 100 ppm (Csyn= 6.75 ± 2.67). However, the synergistic effect is also observed when the Que was used at 200 or 50 ppm (suboptimal concentrations). The fractional inhibitory concentration index (FIC index) was calculated to confirm the synergistic effect as reported by Bidaud et al. [36]. The complex HA–Lacto–Que, at the concentration of 100 ppm (w/v) has a synergistic effect with a value of 0.4 according to the interpretation that a FIC index of ≤0.5 suggests the synergistic interaction

Table 5.1. *The values (*C_{syn}*) are the result of the mathematical analysis used to determine the combination and concentrations of Que and lacto, with the best synergistic antimicrobial activity. The combination of HA–Lacto–Que (100 ppm* w/v*) showing the best synergistic* C_{syn} *values.*

	Lacto 200	**Lacto 100**	**Lacto 50**
Que 100	0.83 ± 0.18	1.27 ± 0.39	0.89 ± 0.21
Que 100 + HA	1.77 ± 0.50	0.88 ± 0.35	1.51 ± 0.52
	Que 200	**Que 100**	**Que 50**
Lacto 100	1.67 ± 0.33	0.85 ± 0.30	2.19 ± 0.44
Lacto 100 + HA	2.91 ± 0.77	6.75 ± 2.67	2.09 ± 0.66

5.3.4. Characterization of the HA–Lacto Complex in Presence of Que

To examine the interaction of HA with Lacto and Que at molecular level, we used the scanning electron microscope (SEM). HA show porous spherical aggregates of elongated crystallites. The average dimension of the particles was above the expected size of nanometer. This finding may result from the self-aggregation of the HA from a few hundred nanometers to a few microns (Figure 5.5a) this hypothesis is in line with the absence of net charge of zeta potential [37]. The functionalization of HA with Lacto alone (Figure 5.5b) and with Lacto–Que (Figure 5.5c) reduced the porosity of HA as effect of adsorption of the two compounds inside the crystalline structure.

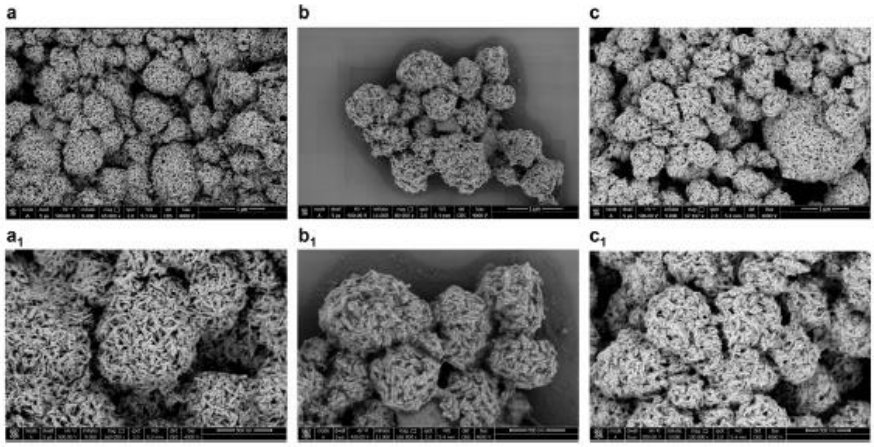

Figure 5.5. *SEM image of: (a,a1) HA 65,000×, scale bar 1 µm, and 160,000×, scale bar 500 nm respectively; (b,b1) HA–Lacto 80,000×, scale bar 1 µm, and 160,000×, scale bar 500 nm respectively; (c,c1) HA–Lacto–Que 68,000×, scale bar 1 µm, and 130,000×, scale bar 500 nm respectively.*

This result is confirmed by independent z-potential data. In Figure 5.6the HA–Lacto, with a z potential of −30 mV compared to HA alone, with values of −15 mV, show a strong aggregation of HA nanocrystals. The HA–Lacto–Que complex shows a positive z potential (11 mV), which demonstrates that the positive electrostatic surface potential of Que produces a strong surface interaction with the HA nanocrystals and stabilizes the previous HA–Lacto bond, which suggests less repulsion between the components of the complex [14].

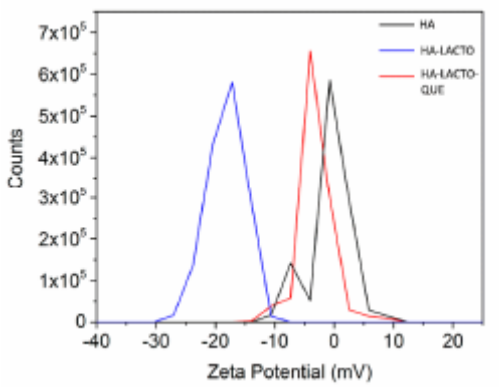

Figure 5.6. *Zeta potential analysis of HA, HA–Lacto, and HA–Lacto–Que.*

5.3.5. Anti-Inflammatory Activity of the Complex

The human macrophage cell line U93 upon incubation after 6 h with *Pseudomonas fluorescens* displayed activation of the pro-inflammatory cytokines TNF-α, IL6, and IL8 (Figure 5.7). Instead, when the same cell line was incubated with the complexes or the single components, we observed a significant reduction of cytokine production. The experiment confirms, once more, the synergistic activity of the complex (HA–Lacto–Que). IL8 is the only cytokine that shows a marked difference in the presence of Lacto (4.08 Fc) or Que (11.74 Fc). In other words, IL8 is down regulated when Que is more exposed on a complex surface.

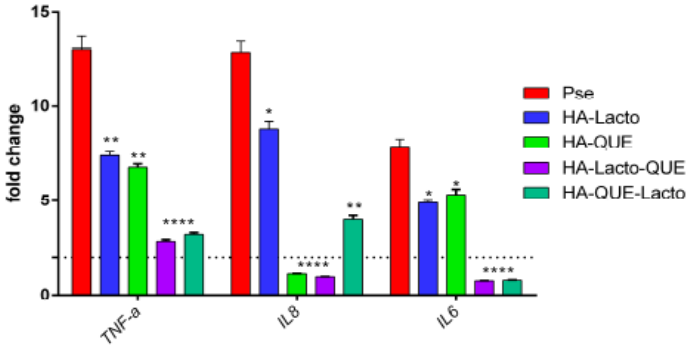

Figure 5.7. *Real-time PCR cytokine expression profile of the different genes involved in the pro-inflammatory response of U937 cells in the presence of Pseudomonas fluorescens and the complexes for 6 h. Statistical analysis was performed and considered statistically significant when* $p < 0.05$ *(** $p < 0.05$*, *** $p < 0.01$*, ***** $p < 0.0001$*) according to two-way ANOVA multiple comparisons.*

5.4 Discussion

Pseudomonas fluorescens is an opportunistic, psychotropic pathogen that can live in different environments, such as plant, soil, or water surfaces. *Pseudomonas fluorescens* has enzymes acting on proteins, lecithin, and lipids conferring to the aliment an undesirable taste [38]. Further, Pseudomonas fluorescens damages several plants causing chlorotic and necrotic lesions on leaves and fruits, with heavy economic losses to the agriculture and the food industry. The chemical additives, at present used to preserve the food against the pathogens, are perceived unhealthy by the consumer.

This study provides clear evidence that the bovine lactoferrin and the quercetin glycoside act against *Pseudomonas fluorescens* efficiently. Provided that data reported in literature are generally difficult to compare, our results (antimicrobial activities of lactoferrin and quercetin equal or up to 300 ppm) (Figure 5.1a) agree with the literature [39,40]. However, when the two molecules were combined, the MIC dropped to 200 ppm (Figure 5.2).

In the next step, the two molecules (Lacto and que) were combined with HA. The MIC dropped further down to 100 ppm (Figure 5.3a). The best results were obtained when Lacto was added first to HA (Figure 5.3b). One more positive feature emerging from our data is that Que and Lacto are completely adsorbed to HA (Figure 5.4a,b) and the complex is stable, with a z-potential value of 11 mV (Figure 6). The z-potential measures the repulsive forces between particles: the higher is the repulsive force, the lower is the probability of aggregate formation. Moreover, to assess if the effect induced by the simultaneous treatment with the HA–Lacto–Que and HA–Que–Lacto were additive or synergistic were analyzed two parameters: normalization as a function of control absorbance (ρ); and synergistic coefficient (Csyn). Since high Csyn values indicate a clear synergistic effect, the best result was obtained when the antimicrobial treatment was performed using HA–Lacto–Que complex (Table 5.1).

Pseudomonas fluorescens is generally considered as non-pathogenic for humans. However, this pathogen has been detected in human clinical samples and shown to be highly hemolytic and able to induce cytotoxic and pro-inflammatory response [41]. An antigen from the same pathogen has been isolated from the serum of patients with Crohn's disease [42].

Back to our study, in order to evaluate in vitro the anti-inflammatory activity of the complex, we infect the human macrophage cell line U937 with *Pseudomonas fluorescens*. We noticed a statistically significant reduction of several pro-inflammatory cytokines (Figure 5.7). The high level of IL8 may be assigned to the presence of intracellular bacteria not killed by the Lacto (known to have a moderate antimicrobial activity) [43]. In the presence of que, IL8 is down regulated. This result is in line with the known activation of IL8 through the TLR4-independent pathway [44]. From this result, we can also infer that Que is not induced by LPS, a TLR4-dependent pathway. Consequently, the down regulation of IL8 reported above can appropriately be attributed to the presence of Que exposed on the complex (HA–Lacto–Que).

In conclusion, the peculiarity of our study consists of optimizing the specific propriety of each component: the affinity of Lacto for LPS; that of Que for the bacterial membrane, a property that we amplified by adding Que to the complex last and, therefore, more exposed (Figure 5.8). These properties make the complex as good candidate for antimicrobial use in the food industry, and as functional food.

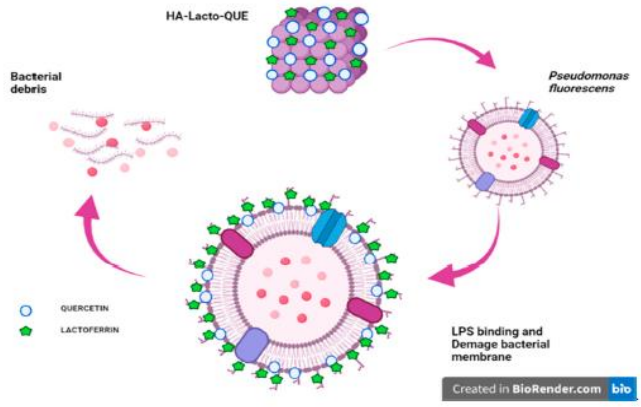

Figure 5.8. *Schematic representation of the activity of HA–Lacto–Que complex against the cell wall of* Pseudomonas fluorescens. *The synergistic activity is due to the specific aptitude of each component: the affinity of Lacto for LPS and of Que for the bacterial membrane. Created with Biorender (https://biorender.com/, accessed on 4 August 2021).*

5.5 Materials and Methods

5.5.1. Bacterial Strains and Culture Conditions

The bacterial strains of *Pseudomonas fluorescens* strain ATCC 13525 were provided from the "IstitutoZooprofilatticoSperimentale del Mezzogiorno" in Portici (Naples, Italy). Pseudomonas fluorescens was grown overnight at 37 °C in the liquid culture medium (Buffered peptone water, BPW). To identify bacterial growth phase, turbidity of medium was measured by optical density measurement at 600 nm on a UV/Vis spectrophotometer.

5.5.2. Antimicrobial Compounds

Quercetin glycoside compound (98.6% food grade) was purchased from Oxford® Vitality Company (Bicester, UK) and lactoferrin (95% food grade) from Fagron the UK. The stock solution of each compound was dissolved in water, to obtained a final concentration of 1 mg/mL.

5.5.3. Antimicrobial Activity

The minimal inhibitory concentration (MIC) of each compound was determined by a colorimetric method, using 3-4,5-dimethylthiazol 2,5-diphenyltetrazolium bromide solution (MTT), using the standard broth microdilution method according to [33]. All antimicrobial tests performed in this work were carried out using the same method.

5.5.4. Biomimetic HA Nanocrystal Synthesis and Characterization

Biomimetic hydroxyapatite nanocrystals were produced as described by [45]. HA was precipitated from a solution of (CH3COO)2Ca (75 mM) by the slow addition of an aqueous solution of H3PO4 (50 mM) and maintaining pH at 10 (addition of NH4OH). The synthesis was carried out at room temperature (RT). Finally, the suspension of HA was washed with distilled water to remove ammonium ions and favor the interaction between nanocrystals.

5.5.5. Evaluation of HA Absorption Capacity and Complex Preparation

HA–Que and HA–Lacto complexes were prepared adding HA solution diluted (1:100) (4% w/v) with known concentrations (500, 400, 300, and 200 ppm) of Que or Lacto, respectively. The complexes were gently mixed at room temperature for 24 h. After the incubation, the solutions were centrifuge and the supernatants and the effective amount of Que and Lacto entrapped into the structure were measured. Specifically, the amount of Que

was evaluated using UV-vis spectrophotometer (Perkin Elmer Lambda 25) at a wavelength of 369 nm; the standard curve consisted of 10–200 ppm Quercetin-3 glucoside. The amount of Lacto was evaluated using spectrophotometer Nanodrop ONEC at a wavelength of 280 nm and the standard curve consisted of 10–500 ppm BSA (Bovine Serum Albumin). Finally, the complexes HA–Que and HA–Lacto were vacuum evaporated for 2 h, then the second molecule—Que or Lacto (2000–100–50 ppm)—was added; finally, the complexes were mixed for 24 h and their antimicrobial activity was evaluated.

5.6 Statistical Analysis for the Complex Synergy

The additive or synergistic interaction of each complex was calculated using the parameters ρ (normalization as a function of control absorbance) and Csyn (synergistic coefficient) statistical approach:

Once defined, the ρ parameter was:

$$\rho a = 1 - (abs_{ctrl} - abs_a)/abs_{ctrl} \quad (1)$$

It was possible to estimate the synergistic coefficient Csyn as:

$$Csyn = \rho_a * \rho_b/\rho_{ab} \quad (2)$$

Error analysis was performed according to [46].

Moreover, the FIC index can be used to confirm the effect of a tested combination. The FIC is designed by division of the MIC of the complex and the MIC of the molecules alone according to the following formula:

FIC index = FIC A + FIC B = (MIC complex1/MIC 1 alone) + (MIC complex2/MIC 2 alone) (3)

5.7 Zeta Potential

The dimensions of HA, HA–Lacto, and HA–Lacto–Que were measured with the zeta potential using a Zetasizer Nano ZS (Malvern Instruments, DTS1070, Malvern, UK). Each sample was tested in triplicate using 1 mL of sample at 25 °C.

5.8 SEM Image

Water suspensions of HA, HA–Que, HA–Lacto, and HA–Lacto–Que samples were centrifuged at 13,000 rpm for 15 min and then deposited on 5 × 5 mm silicon chips; the solvent was evaporated under vacuum at 30 °C; the silicon supports were mounted as described in [46]. SEM microscopy was recorded with a NovaNanoSem 450 field emission gun scanning electron microscope (FEGSEM) (FEI/Thermofisher, Hillsboro, OR, USA), under high-vacuum conditions.

5.9 In Vitro Infection Studies

To further confirmed the synergistic activity of the complexes, the effect of HA–Lacto, HA–Que, HA–Lacto, and HA–Que-Lacto on human macrophage-like (U937) cell line, after 6 h of *Pseudomonas fluorescens* infection was carried out. The cells were maintained in RPMI supplemented with 10% FBS, 1% of Pen/strep, and cultured in 5% CO2 atmosphere. For infection, U937 cells were seeded onto 12-well plates at a density of 0.5 × 106 cells/well, without antibiotics. Subsequently, the cells were infected with Pseudomonas fluorescens strains at a multiplicity of infection (MOI) of 100 with or without different treatment for 6 h. After the incubation period, the cells centrifuged for 5 min at 1500 rpm. The supernatant was discarded and the RNA was extracted using Trizol protocol [47]. A NanoDrop One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to assess total RNA quantity. The retro-transcriptase was performed in order to synthetize first-strand cDNA (SuperScript® III Reverse Transcriptase, Invitrogen). Real-time PCR reactions were carried out in triplicate; expression values were calculated according to the $2^{-\Delta\Delta Ct}$ method [48], and all samples were normalized to GAPDH as a housekeeping gene. A negative sample (cell untreated) was used as calibrator.

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Antimicrobial activity of hydroxyapatite complexes against food pathogenic bacteria

6.1. Introduction

To evaluate the efficacy of active edible coating in inhibiting the growth of bacteria responsible for food poisoning, complexes characterized by the combination of hydroxyapatite (HA), quercetin glycosides compounds (QUE), and lactoferrin (LACTO) were tested against three bacteria - commonly present in fresh and processed food - such as *Salmonella typhimurium, Staphylococcus aureus*, and *Listeria monocytogenes*.
Salmonella is a group of Gram-negative bacteria that can cause gastrointestinal diseases, generally called salmonellosis. Salmonellosis occurs when people eat raw or undercooked foods. Salmonellosis outbreaks have been associated with meat products, poultry products, raw or undercooked eggs and doughs, dairy products, fruits, green leafy vegetables, raw sprouts, fresh vegetables, nut butter and spreads, and foods for pets. Poor hygiene practice is the major cause of bacteria spread.
Listeria monocytogenes (Gram-positive bacteria) is a species of ubiquitous pathogenic (disease-causing) bacteria. It can survive and even grow under refrigeration and other food storage measures. When people eat food contaminated with *Listeria monocytogenes*, they can develop a disease called listeriosis.
Listeria monocytogenes is generally transmitted when foods are collected, processed, prepared, packaged, transported or stored in environments contaminated with the bacterium.
Outbreaks of Listeriosis have been linked to unpasteurized raw milk and cheeses, ice cream, raw or processed vegetables, raw or processed fruit, raw or undercooked poultry, sausages, hot dogs, cured meats and raw or smoked fish and other seafood. These days, a toxic infection of *Listeria monocytogenes* has been reported in Nord-Italy, due to the consumption of wurstel eaten raw, where three deaths and 66 isolations of the bacterium from contaminated human samples were observed. Gram-positive *Staphylococcus aureus* is an important pathogen causing food poisoning

because of its high presence on human skin and nasal passages, which can be easily transferred to food. Foodborne *Staphylococcus aureus* infections occur through a toxigenic mechanism caused by the production of heat-stable staphylococcal enterotoxins in foods, most commonly in dairy products (e.g. milk, cheese, and cream), as well as in meat and fish. Contamination with these bacteria can occur during different steps of food preparation, transport, and consumption. Given this scenario, the role of active edible coating, containing antimicrobial compounds was explored in several scientific papers. (Chengcheng*et al.*,2019, Bin *et al.*,2021). In the previous chapters, the in vitro antimicrobial capacity of QUE loaded with HA against *Pseudomonas fluorescens* was evaluated. We observed that the inhibition occurred with a MIC of 500 ppm (Chapter 2). Subsequently, the synergistic power that the natural compounds can play during the formation of antimicrobial complexes was exploited. By adding LACTO, the MIC obtained against *Pseudomonas fluorescens* decreases (100 ppm), suggesting that 1) QUE and LACTO, at the same concentration, operate different mechanisms of bacterial killing, and 2) the formation of the complex with HA, by introducing the first LACTO and then QUE or reverse, influences the antimicrobial response in a different way (Chapter 5). The hydroxyapatite co-delivery system, first loading the LACTO and then the QUE, inhibited the growth of *Pseudomonas fluorescens*. Based on these results, we investigated whether this complex also exerts the same antimicrobial activity against *Salmonella typhimurium*, *Staphylococcus aureus*and *Listeria monocytogenes.*

6.2. Antimicrobial activity of quercetin and lactoferrin against food pathogenic bacteria: state of art

In the literature, the antimicrobial effect of quercetin was assessed by using both Gram-positive and Gram-negative bacteria, and selective antimicrobial activity was revealed against *Staphylococcus epidermidis*, *Staphylococcus. aureus*, and methicillin-resistant *Staphylococcus aureus* (Ramadan *et al.*,2009; Hirai *et al.*,2010).

Vaquero (2011) demonstrated that the anti-Listeria activity of the flavonoid group including quercetin and rutin was more effective than that of other flavonoid groups and non-flavonoid groups.

Several studies have been published about the antimicrobial properties of quercetin (Ramadan *et al.*,2009; Hirai *et al.*,2010). In these works, the poor water solubility, absorption, permeability, instability, and light-induced decomposition of quercetin emerged. Moreover, they revealed increased antiradical and chelating properties of quercetin when combined with nanoparticles (Pool *et al.*,2012). The antibacterial mechanism of quercetin could be attributed to the inhibition of nucleic acid synthesis, cytoplasmic

membrane function, bacterial cell wall synthesis, or energy metabolism (Cushnie*et al.*,2005; Hirai *et al.*,2010).

Hirai (2010) showed that quercetin and quercetin-loaded NPs present antimicrobial properties only against gram-positive bacteria. The complex cell wall structure of gram-negative bacteria acts as a barrier against quercetin and quercetin-loaded nanoparticles (Ramadan *et al.*,2009; Espitia*et al.*,2012; Arasoglu*et al.*,2015; Li *et al.*,2015; Suleiman *et al.*,2015).

Although the mechanism of the antimicrobial activity of lactoferrin is not fully understood, there is extensive research related to the synergistic effects of lactoferrin in combination with antibodies (Bullen *et al.*,1981), antibiotics (Chimura*et al.*,1993), and antifungal agents (Simpson *et al.*,1997).

Payne (1994) also reported that the combination of apolactoferrin and lysozyme retarded the growth of *Listeria. monocytogenes*. However, there are considerable reports on the antimicrobial activity of lactoferricin against emerging pathogens such as *Salmonella Enteritidis, Salmonella Typhimurium, Salmonella Montevideo, E. coli, Campylobacter jejuni, Staphylococcus aureus, Clostridium perfringens, Listeria monocytogenes,* and *Pseudomonas aeruginosa*.

The activity of lactoferrin as an antibacterial is partly due to its ability to sequester iron (Fe3+) away from bacteria. Bacteria use iron in DNA and RNA synthesis, tricarboxylic acid cycle, cytochrome and toxin production and as a source of energy (Farnaud*et al.*,2003; Min *et al.*,2005).

Reduction of environmental iron levels could eventually lead to less energy for pathogenic bacteria. Against gram-negative bacteria, lactoferrin targets the lipid A portion of the LPS layer, causing its release from the membrane, thus reducing bacterial survival (Wang *et al.*,2019).

The antimicrobial mechanism against gram-positive bacteria is not well known but is thought to target teichoic acid in the bacterial cell wall; moreover, evidence indicates that the effect of lactoferrin may be even greater against some gram-positive bacteria.

Brown (2007) looked at the effect of lactoferrin in chitosan edible films against Escherichia coli and *Listeria monocytogenes* and found that up to 2 mg/mL, lactoferrin alone did not significantly inhibit the growth of either bacterium until the protein was paired with nisin or EDTA. This study by (Biernbaum*et al.*,2021) provides relevant information on bovine lactoferrin as an antimicrobial against 2 high-profile gram-negative pathogens, although it may not be applicable if used alone.The general mechanisms of antibacterial action of quercitin and lactoferrin are reported in the literature, but studies reporting their combined antimicrobial effects against food pathogens are not described, much less when transported by nanosystems. The combination of the two substances together, quercitin with a greater

active on Gram-positive and lactoferrin on Gram negative, could be a valid antimicrobial strategy.

6.2.1 MIC's results of HA-LACTO-QUE complexes against food pathogenic bacteria

MIC is defined as the minimum concentration required to inhibit microbial growth, and it is often used to test the bacteriostatic activity of potential antimicrobial compounds. The MIC was determined, using the standard broth microdilution method according to (Malvano*et al.*,2021). Figure 6.1 shows the inhibition percentage of *Salmonella typhimurium*, *Staphylococcus aureus*, *Listeria monocytogenes* growth. Complete inhibition of all bacteria growth was observed at concentrations of 100 ppm (w / v) of both LACTO and QUE complexed with HA.

As for *Pseudomonas fluorescens*, the antimicrobial activity of QUE and LACTO combined with HA was dependent on the order of insertion during the complex production. The highest inhibition was obtained when HA was first incubated with LACTO and then with QUE at a concentration of 100 ppm (w/v). Instead, when the order was inverted (HA–QUE–LACTO) at the same concentration (100 ppm), the inhibition was 15% for *Salmonella typhimurium*, 30% *Staphylococcus aureus* and 86% *Listeria monocytogenes*. All the complexes with different mass ratios showed good inhibition against Lysteria monocytogenes, except when quercetin or lactoferrin was 50 ppm. Relevant inhibition of *Salmonella* and *Staphylococcus* growth was observed when the mass ratios of QUE and LACTO were 100/200 ppm (w / v).

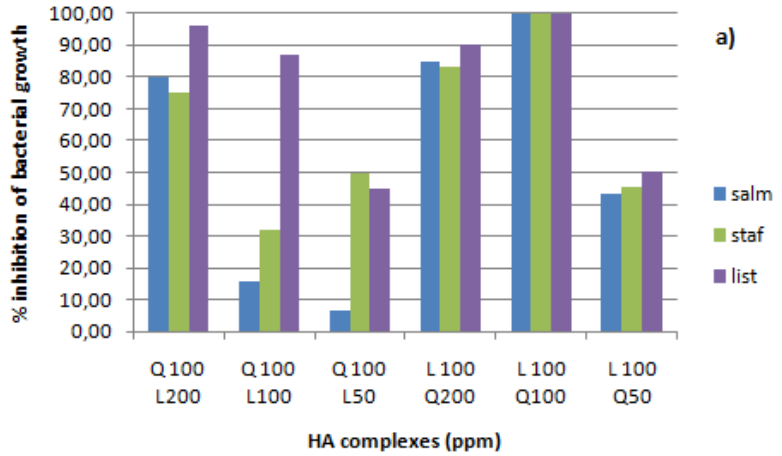

Figure 6.1:*Atimicrobial activity of hydroxiapatite charged with different concentrations of QUE and LACTO, in different combinations againstSalmonella typhimurium, Staphylococcus aureus, Listeria monocytogenes* (10^6CFU/mL).

The antimicrobial activity results between bacteria strains showed significant differences. The first reason for this is the variation between the cell walls.
Listeria monocytogenes presented a lower resistance and highest susceptibility to HA's complexes, probably justified by its surface charges.
According to the zeta potential results present in literature, *Listeria monocytogenes* and *Staphylococcus aureus* (–2.05 mV and –7.04 mV) have more positive surface charges than *Salmonella typhimurium* (–13.1 mV).
Synthesized HA's NPs with QUE and Lacto have negative surface charges (zeta potential: –15 mV), so it was thought that their interaction with microorganisms having a positive surface charge is stronger (Arasoğlu*et al.*,2016;Montone*et al.*,2021).
The pore size of cell membranes is also important because it has been stated in the literature that due to the smaller pore structure (Sun *et al.*,2018), penetration of active substances released by NPs in cells it is limited.
Was been demonstrated that QUE loaded with HA nanoparticles at 500 ppm inhibits the growth of *Pseudomonas fluorescens* that is a Gram-negative bacteria (Malvano*et al.*,2021), and that when the LACTO was added to the HA- QUE complex, the MIC value was 100 ppm. (Montone*et al.*,2021)
It has also been shown that thanks to the contribution of LACTO, in quercetin complex with hydroxyapatite, the concentration of 100 ppm was capable of inhibiting also *Salmonella typhimurium*, *Staphylococcus aureus*, *Listeria monocytogenes.*

6.3 Zinc could increase the antimicrobial activity of hydroxyapatite: state of art of its activity

Hydroxyapatite nanocrystals loaded with LACTO and QUE shows antimicrobial activity against a large group of microorganisms, including foodborne pathogens.
The development of nanosystems has also extended to the transport and manufacture of nanoparticles loaded with antimicrobial agents, such as titanium oxide, silver, zinc, copper, gold, selenium, etc., which are considered appropriate agents as they have a high efficacy at very low levels and can be harmful because they have a high degree of penetration into cell membranes. These nanoparticles are promising new candidates for application in food packaging applications due to their high antimicrobial capacity, particularly with the current growing interest in findingalternative

approaches to produce novel and cost-effective antimicrobial agents for food storage.

Typical antimicrobial agents include titania, silver, zinc, selenium, copper, magnesia and gold. By implementing these nanoparticles as antimicrobial materials coated on surfaces, various industries can take advantage of these elements at the nanoscale, including food processing and packaging, water purification, synthetic fabrics, medical equipment, etc.

In addition, the substitution of some ions present in the structure of HA with other ones makes possible modifications of the HA properties (Thian*et al.*, 2006; Porter *et al.*,2004; Patel *et al.*,2002). For example, the substitution of part of calcium ions with zinc increases the antimicrobial activity of the new synthesized structure (Chen *et al.*,2010; Rameshbabu*et al.*,2007).

Furthermore, it has been shown that variation in the concentration of the reagents, temperature, time and pH of the reaction, makes it possible to obtain HA molecules with specific structural characteristics (size, porosity, morphology) and binding properties, according to the exact application (Walsh *et al.*,2007; Koutsopoulos*et al.*,2002; Palazzo *et al.*,2007).

ZnO is a sub-group of zinc derivatives, which can be used individually or together with other compounds like gelatin, to preserve foods from spoilage (Umamaheswari*et al.*,2015).

Among metal oxides, ZnO has shown great inhibitory effects on the growth of microorganisms, such as *Escherichia coli, Salmonella spp, Staphylococcusaureus, Campylobacter jejuni*, and *Listeria monocytogenes* (Zarrindokht*et al.*,2011).There are several possible mechanisms for the antibacterial action of zinc ions. It has been suggested that zinc binds to the membranes of microorganisms, similarly to mammalian cells; prolongs the lag phase of the microorganism growth cycle and increases the generation time of the organisms so that it takes each organism more time to complete cell division (Sirelkhatim*et al.*,2015). In a study by Södeberg (1990), it was found that gram-positive bacteria are the most susceptible to zinc ions, unlike Gram-negative aerobic bacteria, which are extremely resistant to zinc, except at high concentrations (1024 µl/ml). it was also reported that a combination of zinc oxide and rosin or resin acids shows synergistic effects on antibacterial activity against gram-positive bacteria but not against gram-negative bacteria (Hoseinnejad*et al.*,2017).

As a promising candidate, a new zinc substitute hydroxyapatite (HAZn) has been developed and functionalized with LACTO and QUE. The antimicrobial activity of the new complex was tested against two Gram-negative and two Gram-positive bacteria, among these, were included pathogenic bacteria.

Antimicrobial activity of hydroxyapatite complexes against food pathogenic bacteria

6.3.1 MIC's results of HAZn-LACTO-QUE against Gram-negative and Gram-positive bacteria

Figure 6.2 shows the inhibition percentage of bacterial growth, for *Salmonella typhimurium, Pseudomonas fluorescens, Stafilococco aureus* and *Listeria monocytogenes.* No MIC values were observed, with the mass ratios of QUE and LACTO complexed on HAZn used, for all bacteria tested. The antimicrobial activity of these complexes with HAZn presents more difference among bacteria tested and respects the hydroxyapatite's complexes without zinc.
Again, this is probably due to the characteristic differences of the different bacterial species tested and the interaction between the complex and the bacterial surface.

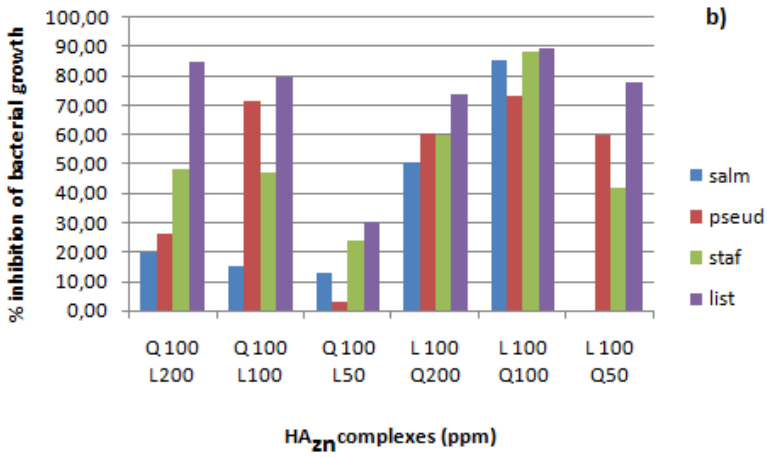

Figure 6.2: Atimicrobial activity of HA_{Zn} with QUE and LACTO complexes against *Salmonella typhimurium, Stafilococco aureus, Listeria monocytogenes* (10^6CFU/mL).

From Figure 6.2 it was shown that the balanced ratio of lactoferrin and quercetin 100/100, which occupy the same ratio as the binding sites of hydroxyapatite and the loading order of lactoferrin before quercetin, showed a better bacterial inhibition than other combinations.
Despite the good note antimicrobial properties of zinc, the data reported in figure 6.2 was similar to the data reported by (Uskoković-Marković*et al.*,2020), which highlighted, that do not increase the antimicrobial activity when it was added to the flavonoid.

If the effects of zinc complexes and the flavonoids themselves are compared, the MIC values are the same against many of the tested strains. The result present in figure 6.2, was similar to the result present in other work, where was reported that Zn–quercetin exhibited slightly better effectiveness against Gram-positive, but less against the Gram-negative *K.pneumoniae, Salmonella abony,* and *Pseudomonas aeruginosa*.

In conclusion, we have determined that the metal ions can chelate with quercetin and form a metal complex, which significantly changes the chemical properties of quercetin. In general, the antimicrobial activity of a compound depended on the dose. However, this can be true in the case of unique and free compounds. In the case of multiple compounds transported by nanosystems, this can be explained by the controlled and sustained release system operated by these systems. The study of antimicrobial activity highlighted that the presence of zinc in the hydroxyapatite structure influences the nature of active compounds.

Therefore, considering the best antimicrobial performances of the HA / LACTO-QUE complex, this was chosen as an additive for the development of an edible coating based on alginate. Its ability to extend the shelf life was evaluated on a meat product.

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Alginate coating charged by Hydroxyapatite complexes with lactoferrin and quercetin enhances the pork meat shelf life

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7.1 Abstract

In this work, the effect of an alginate-based coating loaded with hydroxyapatite/lactofer- rin/quercetin (HA/LACTO-QUE) complexes during the storage of pork meat was evaluated. FT-IR spectra of HA/LACTO-QUE complexes confirmed the adsorption of QUE and LACTO into HA crys- tals showing the characteristic peaks of both active compounds. The kinetic releases of QUE and LACTO from coatings in an aqueous medium pointed out a faster release of LACTO than QUE. The activated alginate-based coating showed a high capability to slow down the growth of total viable bacterial count, psychotropic bacteria count, Pseudomonas spp. and Enterobacteriaceae during 15 days at 4 °C, as well as the production of the total volatile basic nitrogen. Positive effects were found for maintaining the hardness and water-holding capacity of pork meat samples coated with the acti- vated edible coatings. Sensory evaluation results demonstrated that the active alginate-based coat- ing was effective to preserve the colour and odour of fresh pork meat with overall acceptability up to the end of storage time.

7.2 Introduction

Meat and meat products are important sources of food nutrients such as proteins and B-complex vitamins [1]. However, their composition makes them highly perishable prod- ucts with a short shelf life. The main phenomenon related to the spoilage of meat is the microbial growth that occurs during storage, causing off-odours and flavours that make the product unsuitable for human consumption [2]. Recently, several approaches have been proposed to preserve the safety and quality of meat and meat products, such as edible films and coatings from biodegradable biopol- ymers [3]. Among several polysaccharide-based biopolymers commonly used for edible films and coating for food preservation, chitosan and sodium alginate are the most exploited for meat and meat products [4,5]. However, unlike chitosan, which has shown to be an effective natural antimicrobial agent against Gram-positive and Gram-negative bacteria [6], alginate-based layers act only on the water and gas exchanges [5] with no effect on Pseudomonas spp. and Enterobacteriaceae growth during the cold storage of chicken fillets [7]. Similar results were reported by other authors [8,9], highlighting the same total viable count increment rate during the storage of uncoated and alginate-based coated fresh chicken breast meat. However, active edible films and coatings loaded with antimicrobial and antioxidant compounds seem to have great potential for preserving the quality and prolonging the shelf life of meat products [10]. Recently, different studies evaluated the ability of active edible coatings enriched with essential oils [11–13],

phenolic compounds [14], organic ac- ids [15] and natural extracts [16,17] to preserve the quality of fresh meat and meat prod- ucts during storage. Positive effects of coatings enriched with essential oils [11–13], phe- nolic compounds [14], organic acids [15] and natural extracts [16,17] were obtained in ex- tending the shelf life of meat, inhibiting microbial growth as well as lipid oxidation and weight loss. As regards the application of active alginate-based coatings in fresh meat and different types of meat products, we report in Table 7.1 some of the published reports in the last 5 years.

Table 7.1 *Recent applications of active alginate-based coatings in fresh meat and different types of meat products*

Food	Active Substance	Results	Reference
Chicken meat.	Quercetin Glycoside compounds	Coating significantly inhibited the growth of spoilage bacte- ria, as well as the total volatile basic nitrogen and slowed down the changes in hardness during the storage time of 11 days	[7]
Chicken meat.	Citrus and Lemon	Coating resulted in less growth of microorganisms in the samples	[18]
Chicken meat	Black cumin	High antimicrobial activity versus Escherichia coli, less vari- ation in pH and lower colour changes, over 5 days of stor- age at 4 °C.	[19]
Chicken meat	Lactoperoxidase	Lactoperoxidase addition into the alginate-based coating system led to higher bacterial and sensorial quality values of chicken meat. The effect was even increased with the in-	[20]

		creasing concentration of lactoperoxidase.	
Beef	Ginger essential oils	Active coating increased the shelf life of chilled beef slices by 9 days, delaying lipid oxidation and microbial spoilage.	[21]
Beef	Basil	The active coating increased antioxidant activity and re- duced meat lipid oxidation and weight loss.	[22]
Pork Meat	epigallocatechin gallate	The results showed that fresh pork coated with the active coating had a significant inhibitory effect on its microbial growth.	[14]
Lamb meat	Essential oils of thyme and garlic	The active coating had an effect on lamb meat quality that helps maintain its characteristics during its shelf life after thawing. Thyme led to lower lipid oxidation and better col- our maintenance.	[13]

Among active compounds, the flavonoids′ quercetin and quercetin glycosides with antioxidant [23] and antimicrobial activity [24] are present in various fruit and vegetables besides being used in numerous consumer applications such as dietary supplements. Lac- toferrin is a glycoprotein with well-known antimicrobial activity [25,26]. The use of lac- toferrin as a nutritional supplement is GRAS by the US Food and Drug Administration and a novel food ingredient after the positive scientific opinion provided in 2012 by Eu- ropean Food Safety Authority.

Due to the high sensitivity of these compounds to pH, temperature and light, a carrier for their prompt release is necessary to overcome the problem of their use as active compounds in food packaging. Moreover, the efficacy of

an antimicrobial packaging system also depends on the ki- netic release of the compound from the coating to the food surface. The latter depends on both the solubility of the compounds in an aqueous medium and the type and strength of the polymer network used to produce the coating. Moreover, to protect the bioactive com- pounds from degradation that could occur in the edible coating during the storage period, carrier systems such as polymeric nanoparticles [27–29], nanoemulsion [30–32] and nano- composites [33] have been proposed in the literature of alginate-based edible film and coatings. Within the delivery systems of active compounds, hydroxyapatite (HA), due to its chemical physical properties including biocompatibility, stability, and degradability, could be an interesting candidate for carriers in food packaging applications. It represents the major component of cartilaginous tissues, such as bone and tooth; due to the biomi- metic crystal structure and properties, HA crystals are widely employed in different med- ical practices [34]. Furthermore, the use of HA in food is allowed in Europe by Regulation (EC) No 1333/2008 on food additives classifying the HA by the code E341. To the best of our knowledge, no previous studies exist in the literature on the appli- cation of hydroxyapatite as a component of edible coatings for food shelf-life extension, except those published by our research group [7,35,36]. Our research group recently de- veloped alginate-based coatings loaded with hydroxyapatite/quercetin complexes for the shelf-life extension of fresh chicken fillets [7]. To increase the antimicrobial activity versus Pseudomonas spp. we also evaluated the synergistic effect of lactoferrin and quercetin loaded in hydroxyapatite crystals at different active compound concentrations [25]. Based on these considerations, this work aimed to evaluate the effectiveness of HA lactoferrin and quercetin complexes loaded in an alginate-based coating during the cold storage of fresh pork fillets. For this purpose, the effects of alginate coating charged with free lactoferrin and quercetin and HA lactoferrin and quercetin complexes were carried out by the evaluation of microbiological, physical and sensory properties of pork fillets stored at 4 °C for 15 days. Moreover, the physical characterisation of Hydroxyapatite com- plexes was performed besides studying the kinetic release of lactoferrin and quercetin from activated alginate-based coatings.

7.3 Materials and Methods

7.3.1. Materials

Fresh pork meat was purchased at a food retailer meat counter (Salerno Italy) and cold (4 ± 1 °C) was transported to the laboratories of the University of Salerno. Sodium alginate, calcium chloride and glycerol were all obtained from Sigma-Al- drich (Milano, Italy). Quercetin glycoside compounds (QUE, 98.6% food grade) were pur- chased from Oxford®

Vitality Company (Bicester, UK) and Lactoferrin (LACTO, 95% food grade) from Fargon (Newcastle, UK). Biomimetic Hydroxyapatite (HA) was obtained by Chemical Center Srl (Research and Development Department, Italy) and synthesised ac- cording to the procedure of Palazzo et al. [34].

7.3.2. Preparation and characterization of Hydroxyapatite loaded with quercetin glycoside compounds and lactoferrin

Based on previous antimicrobial activity results of the LACTO and QUE complexes against Pseudomonas fluorescent [20], HA/LACTO-QUE complexes were prepared with 100 mg/L in LACTO and QUE. LACTO and QUE were adsorbed in HA crystals according to the procedure reported by Montone et al. [25]. The morphology of HA/LACTO-QUE complexes was evaluated by a Scanning Elec- tron 33 Microscope (Leo, model EVO 50). Before the analysis, samples were placed on a conductive graphite surface and then coated with a thin layer of silver in a sputter coater (Edwards, S150B) for 3 min through a flux of argon ions. After this time, the argon flow was stopped, and the samples were left under vacuum for 24 h. Fourier Transformed Infrared analysis of HA, QUE, LACTO and HA/LACTO-QUE complexes was performed according to Nocerino et al. [37], using a Thermo Nicolet 380 FT-IR spectrometer.

7.3.3 Preparation of coating solutions

Alginate solution was prepared by dissolving sodium alginate in distilled water according to the procedure described by Malvano et al. [7]. Successively, two types of so- dium alginate solutions were prepared: the first one contained 100 ppm of LACTO and QUE, and the second one HA/LACTO/QUE complexes with 100 ppm of LACTO and QUE.

Quercetin and Lactoferrin Release from Coatings

The release study of quercetin and lactoferrin from alginate-based coatings charged with HA/LACTO, HA/QUE and HA/LACTO-QUE complexes was performed following the procedure developed in our previous studies [24,25].

Alginate coating charged by Hydroxyapatite complexes with lactoferrin and quercetin enhances the pork meat shelf life

7.4 Preparation of Coatings and Study of Pork Meat Fillets Storage

7.4.1. Pork Meat Fillets Coatings and Storage Study

Fresh pork meat fillets (60–80 g) were obtained from whole pork meat piece using an automatic slicer. The edible coating process was carried out by exploiting the layer-by- layer method. Pork fillet samples were dipped before into sodium alginate solutions for 1 min and then in calcium chloride solution (1.5% w/v) for 1 min, according to Malvano et al. [7]. After, the samples were got dried at room temperature for 15 min and then put into PET boxes provided with a lid. Storage study was performed by comparing three different pork meat samples prepared as below:

- Uncoated Pork meat fillets (C)
- Pork meat fillets covered by alginate solution charged with free LACTO and QUE (LACTO-QUE)
- Pork meat fillets covered by alginate solution charged with HA/LACTO-QUE complexes (HA/LACTO-QUE)

Fifteen boxes for each treatment were maintained at 4 °C for 15 days. At 0, 2, 4, 7, 11 and 15 days physicochemical, microbiological, texture and sensory analyses were carried out. For each storage time, three replicate pork fillet boxes were employed.

7.4.2. Microbiological analysis

The microbiological parameters investigated during the storage of uncoated and coated pork fillets samples were Total viable bacterial count (TVC), Psychrotrophic bacteria count (PBC), *Pseudomonas spp.* and *Enterobacteriaceae*. The analysis of the above micro- biological parameters was performed according to Malvano et al. [7].

7.4.3. Total Volatile Basic Nitrogen (TVB-N)

Total Volatile Basic Nitrogen (TVB-N) was evaluated by a Kjeldahl distillation unit (UDK139 VelpScientifica) according to Albanese et al. [19].

7.4.4. Water Holding Capacity

Water-holding capacity (WHC) was calculated as a percentage of weight loss con- cerning its initial weight, according to the Equation (1):

$$\mathrm{WHC} = \frac{InitialWeight(day\ 0) - Weight\ (analysisday)}{InitialWeight(day\ 0)} \quad (1)$$

Coatings were manually removed from pork samples and the weight of the samples was recorded.

7.4.5 pH and colour evaluation

The pH values, as well as total colour differences (ΔE), were determined according to Malvano et al. [7].

7.4.6. Texture profileanalysis

A Texture Analyzer (LRX Plus, Lloyd Instruments, Chicago) provided with a 100 N load cell was employed to evaluate Hardness (N), Gumminess (N), Cohesiveness (dimen- sionless), Chewiness (N*mm) and Springiness (mm) parameters. Two consecutive com- pressions with a cylindrical probe of 1 cm diameter at 1 mm/min were performed on the pork fillet samples. All measurements were performed in triplicate for each fillet sample.

7.4.7. Sensory evaluation

Coated (LACTO-QUE; HA/LACTO-QUE) and control (C) pork samples were as- sessed by colour and odour before the cooking, and after the cooking, performed by broil- ing, for taste, odour and overall acceptability. The active alginate-based coatings were not removed from the samples to evaluate their impact on the colour, odour and taste before and after the cooking of pork fillets. Ten members (4 male and 6 female) of the Department of Industrial Engineering at the University of Salerno (Italy) were engaged, based on their frequency of consumption of pork meat. Before the sensory trials, all Panel Participants released signed Informed Consent according to American Meat Science Association [39]. The sensory attributes were rated on a 5-point scale from “none” (1) to “high” (5). Scores equal to or lower than 3 were considered not suitable for marketing.

7.5 Statistical analysis

All the analyses were performed in triplicates. Experimental data were reported as mean and standard deviation and subjected to analysis of variance (ANOVA). The signif- icance of differences ($p < 0.05$) among samples was determined by Student’s *t*-test with SPSS software version 13.0 for Windows (SPSS, Inc., Chicago, IL, USA).

7.6. Results and Discussion

7.6.1. HA Complex Characterisation

FT-IR spectra of HA, LACTO, QUE and HA/LACTO-QUE complexes are reported in Figure 7.1

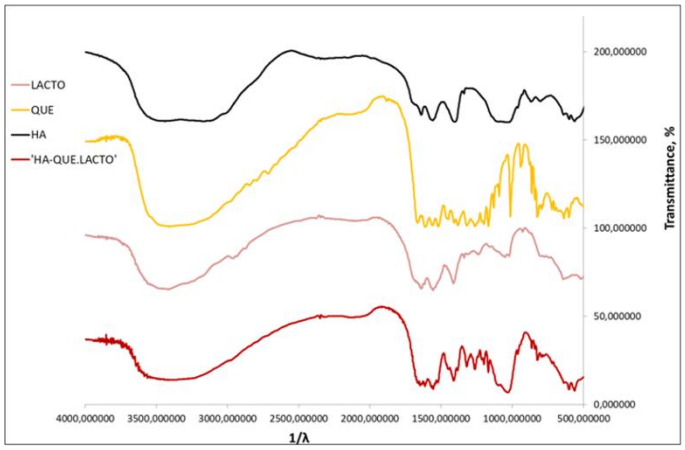

Figure 7.1. FT-IR spectra of HA, LACTO, QUE and HA/LACTO-QUE complexes

According to previous studies [7,40] the FT-IR spectra of HA show the characteristic adsorption bands at 1000–1100 cm−1 related to the asymmetric stretching mode of vibration for PO4 group and other bands at, 880 cm−1, 1466 cm−1, 1545 cm−1 and 3497 cm−1 due to the carbonate type A (hydroxyl site)-substituted and type B (phosphate site)-substitute HA crystals. Moreover, the FT-IR analysis of HA/LACTO-QUE complexes revealed both active compounds. In particular, QUE showed its characteristics peaks at 1500 cm−1 (correspond- ing to C=C stretching), 1670 cm−1 (corresponding to C=O stretching), 3248 cm−1 (corre- sponding to O-H stretching) and other peaks in the range of 650 and 1000 cm−1 pointed out the presence of the aromatic compounds [7]. LACTO showed its characteristic peaks at 1655 cm−1 (C=O), 1532 cm−1 (C-H) and 1392 cm−1 (C-N) [41]. SEM images of HA, LACTO, QUE, HA/LACTO and HA/LACTO-QUE complexes were reported in Figure 7.2

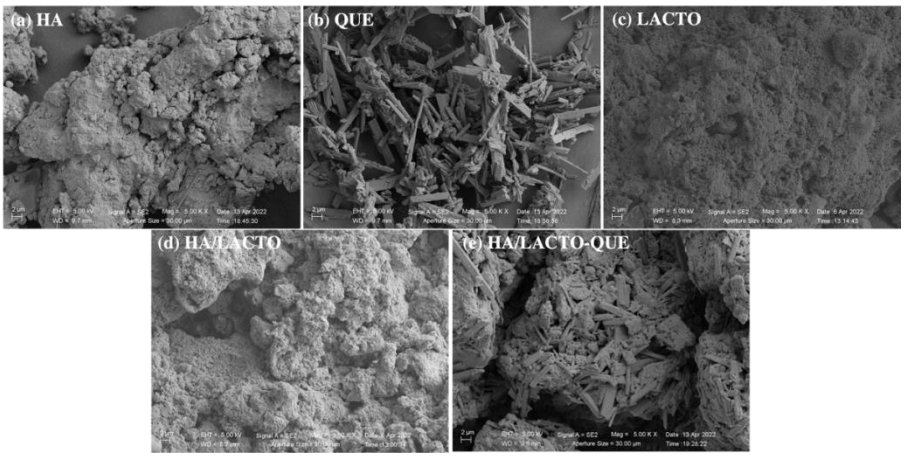

Figure 7.2. SEM images of HA (a), QUE (b), LACTO (c) HA/LACTO complexes (d) and HA/LACTO-QUE (e) complexes.

According to our previous study [7], SEM images of HA showed μm particle size (Figure 7.2a). HA nanoparticles tend to agglomerate in micrometric clusters probably due to Ostwald ripening [42] and their Z-potential values near 0 mV [7]. The characteristic strip-like structures of quercetin glycoside compounds [43] and the spherical structures of lactoferrin [44] are clearly shown in Figure 7.2b and Figure 7.2c respec- tively. SEM images of HA complexes highlighted the first doping with LACTO (Figure 7.2d) and after with QUE (Figure 7.2e) showing the characteristic structures of QUE and LACTO, respectively, in the crystal structure.

7.7 Release study

The release of a bioactive compound from a biopolymer matrix into an aqueous me- dium happens thanks to physical phenomena occurring in sequence and is ruled by physicochemical interactions between polymer, solvent and the bioactive compound. At first, the water penetrates and diffuses into the structure causing the polymeric network widens which allows the diffusion of the active compound until the equilibrium is reached [45]. The kinetic releases of QUE and LACTO from alginate-based coating loaded with HA/QUE, HA/LACTO and HA/LACTO-QUE complexes are shown in Figure 7.3. As can be observed for the coating loaded with HA/LACTO-QUE complexes, the release of LACTO, as well as the achievement of equilibrium, occurs in a shortertime than QUE. In particular, the release of LACTO started after 9 h compared with the QUE release that occurred after 31 h. Moreover, QUE reached the equilibrium 42 h later than LACTO.

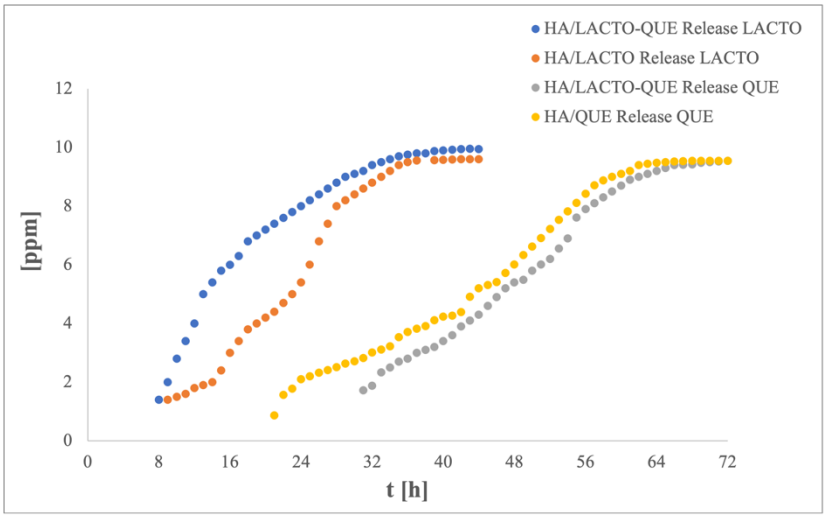

Figure 7.3. Release profile of QUE and LACTO from HA complexes

As regards the influence of simultaneous loading of the active compounds into HA crystals, the presence of QUE seems to influence the release of LACTO, which is faster in the case of HA/LACTO-QUE complexes than in HA/LACTO ones. In contrast, the coating loaded with HA/QUE complexes starts releasing quercetin in a shorter time before than the coating loaded with HA/LACTO-QUE ones, even if no kinetic release differences were observed. The observed different release behaviour in active alginate-based coatings could be due to the difference in the solubility of the active compounds in the aqueous medium (10 g/L for LACTO and 1.61 g/L for QUE) besides the different interactions of the active com- pounds could show with HA structure

7.8 Changes in Microbial, Chemical-Physical and Sensory Properties of Pork Meat

7.8.1. Microbiological changes

Microbial changes in the coated and uncoated fresh pork meat are reported in Figure 7.4. For all microbial parameters, an increase was measured at the increase of the storage time.

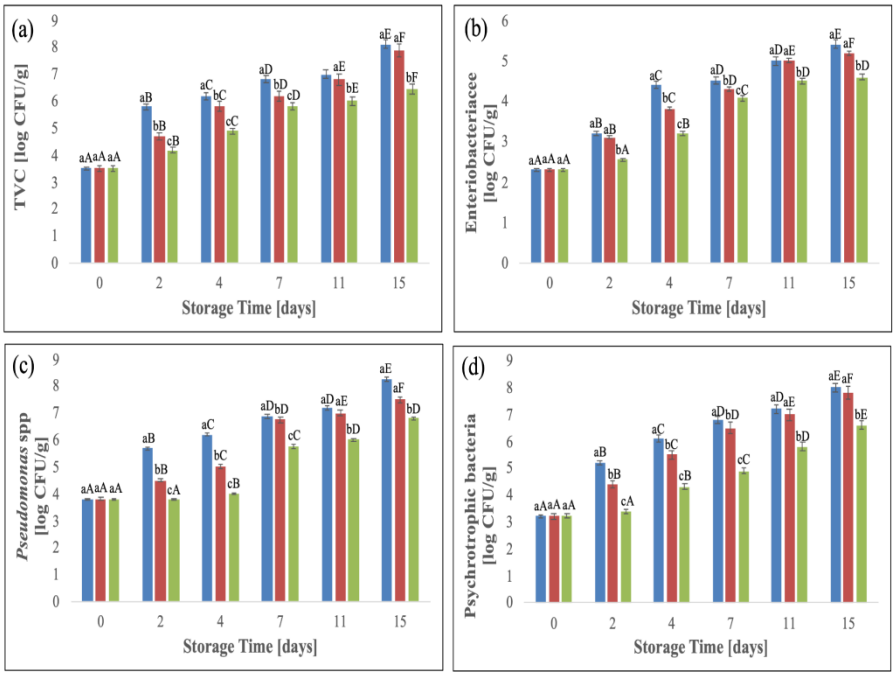

Figure 7.4. Changes in total viable bacterial count (a), Enterobacteriaceae (b), Pseudomonas spp. (c) and psychrotrophic bacteria count (d) of pork meat samples during the storage time at 4 °C. Different letters (a, b, c, ...) reveal significant differences ($p<0.05$) among the samples for each storage time, and different letters (A, B, C, ...) reveals significant differences ($p<0.05$) for each treatment during the storage time

The initial TVC of pork meat samples (Figure 7.4a) was 3.50 log CFU/g, according to previous studies on the shelf-life evaluation of pork meat [14,17]. TVC values increased with the increase of storage days for coated and uncoated sam- ples. On day 7, uncoated samples reached TVC values close to 7 log CFU/g, considered the maximum acceptable limit for fresh meat [46]. LACTO-QUE coated samples reached the TVC threshold at 11 days while this value was never reached by HA/LACTO-QUE samples until the end of the storage period. This indicates that, from a microbiological point of view, a shelf-life extension, of at least 7 and 4 days was obtained for HA/LACTO-QUE samples compared with the control and HA/LACTO, respectively. Coating loaded with HA/LACTO-QUE complexes inhibited the growth of the *Enterobacteriaceae* (Figure 7.4b) and the final charge (4.6 log CFU/g), at 15 storage days, was lower (1 log cycle) significantly ($p < 0.05$) compared to the control sample. Meat spoilage is mainly caused by the metabolic activity of psychrotrophic bacteria, in particular *Pseudomonas*

spp., the prevailing spoilage flora of food from the animal origin [21] causing off-odours and off-flavours during storage in cold conditions. Figure 7.4c,d show that PBC and *Pseudomonas spp*. for uncoated and coated pork meat samples showed a similar trend to the TVC, increasing with the increase of storage time. In particular, during the storage period, LACTO-QUE and HA/LACTO-QUE sam- ples showed significantly ($p < 0.05$) lower *Pseudomonas spp* and PTC, as compared to the control. The results of LACTO-QUE and HA/LACTO-QUE coated samples highlighted the higher capability of the coating to slow down bacterial growth when the active com- pounds were adsorbed into HA crystals. The antimicrobial activity of both quercetin glycoside compounds and lactoferrin against meat spoilage microorganisms such as *Pseudomonas spp*. has been tested by our group and other authors [24,37,47–49]. In addition, our group also verified the enhanced antibacterial activity of lactoferrin and quercetin when adsorbed into HA crystals [25]. The capability of alginate-based coating charged with HA/LACTO-QUE to slow down the microbial charge coated pork fillets during the stor- age agrees with our previous study focused on synergistic inhibition effect versus Pseudo- monasfluorescens of LACTO and QUE loaded in the structure of hydroxyapatite [25]. When HA was incubated firstly with LACTO and then with QUE, the highest inhibition was reached. The complex HA/LACTO-QUE (100 pm) exhibited a synergistic effect on the growth of *Pseudomonas fluorescens*, showing a fractional inhibitory concentration (FIC) in- dex equal to 0.4, according to the interpretation that a FIC index of ≤0.5 suggests the synergistic interaction [50].

7.8.2. pH, Total volatile Basic Nitrogen and colour evaluation

Meat pH is related to quality and freshness characteristics, such as colour, tenderness, and microbial growth [33]. The effects of alginate-based coatings on pork meat pH during the storage period are shown in Figure 7.5a.

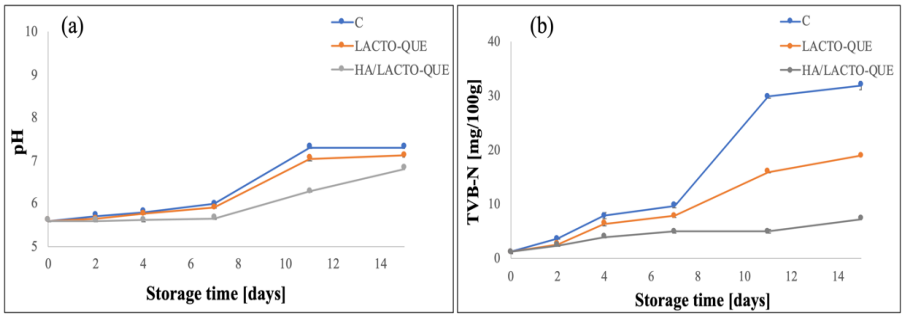

Figure 7.5. Effects of alginate-based coatings on pH (a) and TVB-N (b) of pork meat samples during the storage time

The pH value of the fresh pork meat sample was 5.59, close to the values reported by other authors [14,16,17], and it increased with the increase of the storage days in all sam- ples. In particular, until day 4 no pH change was observed in HA/LACTO-QUE samples. On day 11, a significant increase in pH was observed for all meat samples: however, the pH values were always lower in the coated samples than in the control ones. At the end of the storage, the coating containing HA/LACTO-QUE complexes granted the lowest pH value, with an increase of 21.82% concerning day 0. An increase of 30.59% and 27.19% was reached for the control and LACTO-QUE samples, respectively, at the end of the storage period. The rise of pH in pork samples may be explained as related to the degradation of proteins that occur at the last stage of storage that allows the production of volatile alka- line nitrogen molecules, including ammonia and amines, through microbial activity and meat endogenous proteases [14,38]. pH results agreed with the amount of total volatile basic nitrogen (TVB-N), which represents the main product of protein decomposition by spoilage bacteria in pork meat [52] affecting the sensory acceptability of the meat besides being toxic for human health [53]. Changes in TVB-N values for all fillet pork samples are reported in Figure 4b. The initial value of fresh meat was 1.15 mg/100 g, and this value increased with the storage time. In fact, according to microbiological results, microbial growth causes protein degra- dation and damage to the muscle cell structure. As result, endogenous enzymes were re- leased that accelerated protein degradation and the release of amino acids [54]. On each day of storage, the TVB-N values of uncoated samples were significantly ($p < 0.05$) higher than coated ones. The minimumTVB-N value was guaranteed, at any time of storage, by the coating containing hydroxyapatite–lactoferrin–quercetin complexes. Considering that the TVB-N acceptable threshold for pork meat is ≤15 mg/100 g [55], control and LACTO-QUE samples exceeded the limit on 11th cold storage, in contrast to the HA/LACTO-QUE samples, which did not reach this limit for the whole storage period. Changes in L*, a*, b* and total colour differences ΔE of pork meat samples during the storage time are shown in Table 7.2 At the beginning of storage (day 0), coated samples showed similar lightness, lower than the control sample. This difference could be due to the colour of the film-forming solution, which is the result of the pale-yellow colour of sodium alginate adding to the yellow and pink colour of quercetin and lactoferrin, respec- tively.

Table 7.2. Effects of alginate-based coatings on colour of pork meat samples during the storage time

Parametr	Sample	Storage Time (days)					
		0	2	4	7	11	15
L*	C	68.84 ±0.69 aA	68.84 ±0.65 aA	67.95 ±0.68 aA	65.95 ±0.66 aB	52.33 ±0.52 aC	50.16 ±0.50 aD
	QUE-LACTO	63.88 ±0.64 bA	62.88 ±0.63 bA	62.82 ±0.62 bA	60.89 ±0.61 bB	59.78 ±0.60 bB	58.04 ±0.58 bC
	HA/QUE-LACTO	64.84 ±0.65 bA	64.84 ±0.65 cA	63.95 ±0.64 bA	62.45 ±0.62 cB	61.23 ±0.61 cC	59.92 ±0.60 cD
a*	C	4.40± 0.04a A	4.40± 0.03a A	5.68± 0.06a B	7.27± 0.07a C	11.76 ±0.12 aD	13.50 ±0.13 aE
	QUE-LACTO	7.12± 0.07b A	7.12± 0.07b A	7.30± 0.07b B	8.78± 0.09b C	9.87± 0.09b D	12.15 ±0.12 bE
	HA/QUE-LACTO	7.20± 0.07c A	7.20± 0.06b A	7.60± 0.08c B	8.78± 0.09b C	10.20 ±0.10 cD	11.56 ±0.12 cE
b*	C	3.25± 0.03a A	3.25± 0.02a A	4.08± 0.04a B	5.54± 0.05a C	7.01± 0.07a D	7.26± 0.07a E
	QUE-LACTO	3.79± 0.04b A	3.79± 0.03b A	3.84± 0.04b A	4.20± 0.04b B	4.59± 0.05b C	5.36± 0.05b D
	HA/QUE-LACTO	3.09± 0.03c A	3.09± 0.03c A	3.20± 0.03c B	3.60± 0.02c C	4.09± 0.04c D	4.78± 0.05c E
Δ_E	C	-	0.00± 0.00a A	1.77± 0.01a B	4.80± 0.02a C	18.46 ±1.23 aD	21.16 ±1.78 aE
	QUE-LACTO	-	0.00± 0.00a A	1.57± 0.02b B	2.62± 0.03b C	4.22± 0.51b D	7.10± 0.34b E
	HA/QUE-LACTO	-	0.00± 0.00a A	0.98± 0.01c B	2.91± 0.02c C	4.80± 0.23c D	6.78± 0.48c E

Different letters (a, b, c, ...) reveal significant differences ($p<0.05$) among the samples for each storage time, and different letters (A, B, C, ...) reveals significant differences ($p<0.05$) for each treatment during the storage time

L* values of coated and uncoated pork meat samples remained constant until day 7, after that a rapid decrease in lightness was registered for uncoated samples. The coated samples showed a slight decrease in lightness from day 7 until the end of storage. This means that the alginate-based coating was able to protect meat oxygen responsible for browning phenomena, while active compounds as antioxidants reduced oxidation. Similar results were reported also by Ruan et al. [9]. The initial a* value, which indicates the freshness of pork meat, ranged from 4.40 to 7.20 and these values didn't change until day 2. After that, the coated samples showed a slight increase during the entire storage period, while a marked increase was registered for uncoated ones. As regards b*, our results showed that the values increased during the storage period (Table 1). This behaviour could be due to the formation of hydrogen sulfide (H2S), which is produced by microorganisms and enzymes that degraded proteins, thus binding to haemoglobin to form yellow complexes [37]. In contrast to uncoated samples that increase quickly from day 2 to the end of the storage period, a slight b* increase was observed for coated samples, thanks to the beneficial effect of coating against microbial spoilage. Moreover, the addition of hydroxyapatite in alginate-based edible coating had a better colour protection effect on fresh pork meat. Finally, a constant increase in total colour was observed during the storage period for coated samples, while a significantly ($p<0.05$) higher ΔE value was obtained for uncoated ones.

7.8.3. WHA and TPA

Water-holding capacity (WHC) is an important attribute related to fresh meat quality,influencing the freshness, cooking yield and sensory palatability, as well as nutritionalprofile [56].As reported in Table 7.3, water losses gradually increased for all pork samples duringstorage time, ranging from 1.41% to 12.03% for uncoated samples and from 0.93% to 9.43%and from 0.41% to 8.06% for LACTO-QUE and HA/LACTO-QUE samples, respectively.

This behaviour could be due to the water-barrier properties of alginate-based coatings that prevent exudation and meat dehydration [5]. The comparison between coated porksamples pointed out a significantly ($p < 0.05$) higher WHC in HA/LACTO-QUE than coatingsloaded with free quercetin and lactoferrin. These results agree with our previousstudies [7,35], highlighting the capability of HA structure to lead to a strong water-holdingcapacity.

Texture parameters measured of coated and uncoated pork samples are reported in Table7.3. As can be observed, the alginate layer on the surface of pork fillets influences thetexture parameters of pork fillets showing significant differences in comparison with uncoatedsamples. The hardness increased significantly at 15 storage days only in controland LACTO-QUE samples. As previously stated, the increased hardness in meat productsmay be correlated to water leakage because of the evaporation of water from the meatsurface and the reduced capability of meat proteins to hold water [13]. The decrease inspringiness values was registered at the end of the storage period for all pork filletsamples. The lowering in springiness could be explained by a lower-elasticity meat structure arising from water leaks. Values of cohesiveness, gumminess and chewiness did notshow significant ($p < 0.05$) differences among samples during the cold storage.

Table 7.3. Effects of alginate – based coatings on WHC and textural parameters of pork meat samples during the storage time

		Storage Time (days)					
Parameter	Sample	0	2	4	7	11	15
WHC	C	-	1.41±0.02aA	4.73±0.03aB	6.69±0.08aC	11.12±1.12aD	12.04±0.98aE
	LACTO-QUE	-	0.93±0.01bA	3.61±0.02bB	5.83±0.04bC	7.44±0.05bD	9.43±0.78bE
	HA/LACTO-QUE	-	0.41±0.01cA	1.36±0.02cB	3.59±0.03cC	5.97±0.06cD	8.06±0.56cE
Hardness [N]	C	10.25±0.30aA	13.02±0.50aB	12.77±0.50aB	12.31±0.70aB	13.35±0.10aC	15.66±0.10aD
	LACTO-QUE	13.03±0.40bA	13.83±0.60aA	12.54±0.60aB	12.70±0.50aB	13.28±0.60aB	14.83±0.10bC
	HA/LACTO-QUE	13.27±0.28bA	13.27±0.30aA	12.45±0.60aA	12.98±0.20aA	12.62±0.60aA	13.09±0.50cA
Cohesiveness	C	0.03±0.01aA	0.03±0.01aA	0.01±0.01aA	0.02±0.00aA	0.02±0.01aA	0.02±0.01aA
	LACTO-QUE	0.03±0.00aA	0.03±0.00aA	0.03±0.01aA	0.02±0.01aA	0.03±0.00aA	0.02±0.01aA
	HA/LACTO-QUE	0.03±0.00aA	0.03±0.00aA	0.02±0.01aA	0.02±0.01aA	0.02±0.01aA	0.03±0.00aA
Springiness [mm]	C	0.35±0.01aA	0.40±0.06aA	0.43±0.08aA	0.44±0.06aA	0.42±0.05aA	0.25±0.01aB

	LACTO-QUE	0.38±0.01bA	0.39±0.00aA	0.42±0.07aA	0.44±0.06aA	0.36±0.07aA	0.34±0.01bB
	HA/LACTO-QUE	0.38±0.00bA	0.36±0.03aA	0.35±0.07aA	0.39±0.07aA	0.38±0.04aA	0.36±0.02bA
Gumminess [N]	C	0.37±0.00aA	0.37±0.10aA	0.22±0.10aA	0.30±0.10aA	0.40±0.10aA	0.36±0.10aA
	LACTO-QUE	0.38±0.00bA	0.38±0.10aA	0.37±0.10A	0.25±0.10aA	0.33±0.05aA	0.27±0.00aA
	HA/LACTO-QUE	0.39±0.00bA	0.39±0.10aA	0.37±0.10A	0.23±0.10aA	0.20±0.10aA	0.33±0.10aA
Chewiness [N*mm]	C	0.11±0.00aA	0.13±0.00aB	0.09±0.00aC	0.13±0.00aD	0.12±0.00aE	0.08±0.00aF
	LACTO-QUE	0.14±0.00bA	0.13±0.00aB	0.15±0.10aB	0.06±0.02bB	0.12±0.10aB	0.09±0.00bC
	HA/LACTO-QUE	0.14±0.00bA	0.14±0.00bA	0.09±0.00aB	0.08±0.00bC	0.07±0.10aC	0.12±0.00cD

Different letters (a, b, c, ...) reveal significant differences ($p<0.05$) among the samples for each storage time, and different letters (A, B, C, ...) reveals significant differences ($p<0.05$) for each treatment during the storage time

7.8.4. Sensory evaluation

Changes in sensory properties (colour, odour, taste and overall acceptability) ofcoated (LACTO-QUE; HA/LACTO-QUE) and control (C) pork meat samples before andafter cooking are shown in Table 7.4. The scores of samples showed a similar downwardtrend that, as expected, decreased with the increase in storage time.

On the fourth day, thecolour of uncoated samples was significantly ($p < 0.05$) lower than coated pork samples,reaching unacceptable scores on the seventh day. Between coated pork samples, LACTOQUEreached a score of 3 on the 15th day, while HA/LACTO-QUE samples remained acceptableduring the whole storage period.

Until day 4 there were no significant ($p < 0.05$) differences between coated and controlpork fillet samples, for both raw and cooked pork fillets. However, from the seventh day,the odour score of coated samples was significantly ($p < 0.05$) higher than those of uncoatedones.

At the end of the storage period, HA/LACTO-QUE samples showed valueshigher than the acceptability threshold. The overall acceptability of uncoated cooked porksamples was insufficient on the seventh day of storage.

The coating with alginate coating charged with LACTO and QUE allowed pork filletsto reach overall acceptability scores of up to 11 storage days,

while the addition ofHA/LACTO-QUE made pork samples acceptable for the whole investigated storage period.

These results were consistent with the values of TVB-N and TVC reported in thesections discussed above.

As regards the taste evaluated at time 0 for the cooked porkfillets no significant differences ($p < 0.05$) were observed among the control and coatedsamples. In particular, the cooking, due to the high temperature, caused the thermalbreakdown of the alginate coating, though characterised by a neutral taste [7].

Table 7.4 Effects of alginate-based coatings on sensory parameters of pork meat samples during thecold storage.

	Parameter	Sample	Storage Time (days)					
			0	2	4	7	11	15
Fresh porkmeat	Colour	C	5.00± 0.00a A	4.80± 0.20a A	4.00± 0.10a B	2.40± 0.40a C	1.10± 0.10a D	1.00± 0.00a D
		LACTO-QUE	5.00± 0.00a A	4.60± 0.40a A	4.40± 0.20b A	3.80± 0.20b B	3.20± 0.18b C	3.00± 0.20b C
		HA/LACTO-QUE	5.00± 0.00a A	4.80± 0.20a A	4.60± 0.20b A	4.00± 0.60b A	3.60± 0.20c B	3.40± 0.10c B
	Odour	C	5.00± 0.00a A	4.80± 0.40a A	4.40± 0.40a A	3.40± 0.10a B	1.40± 0.20a C	1.00± 0.00a D
		LACTO-QUE	5.00± 0.00a A	5.00± 0.00a A	4.60± 0.20a B	4.40± 0.10b B	3.80± 0.10b C	3.00± 0.10b D
		HA/LACTO-QUE	5.00± 0.00a A	5.00± 0.00a A	4.60± 0.20a B	4.40± 0.20b B	3.80± 0.20b C	3.60± 0.20c C
Cookedpork meat	Taste	C	5.00± 0.00a A	4.60± 0.20a B	3.40± 0.10a C	2.00± 0.00a D	1.40± 0.20a E	1.00± 0.00a F
		LACTO-QUE	5.00± 0.00a A	4.80± 0.40a B	4.40± 0.40b B	3.80± 0.10b C	3.60± 0.10b C	3.00± 0.20b D

Alginate coating charged by Hydroxyapatite complexes with lactoferrin and quercetin enhances the pork meat shelf life

	HA/LACTO-QUE	5.00± 0.00a A	5.00± 0.00a A	4.60± 0.20b B	4.40± 0.20c B	4.00± 0.00c C	3.80± 0.20c C
Odour	C	5.00± 0.00a A	4.80± 0.20a A	4.60± 0.20a A	3.40± 0.10a B	1.80± 0.40a C	1.20± 0.10a D
	LACTO-QUE	5.00± 0.00a A	5.00± 0.00a A	4.60± 0.20a B	4.40± 0.10b B	3.00± 0.20b C	1.80± 0.20b D
	HA/LACTO-QUE	5.00± 0.00a A	5.00± 0.00a A	4.60± 0.20a B	4.60± 0.20b B	3.80± 0.20c C	3.60± 0.20c C
Overall acceptability	C	5.00± 0.00a A	4.80± 0.20a A	4.20± 0.30a B	3.00± 0.20a C	1.20± 0.20a D	1.00± 0.10a D
	LACTO-QUE	5.00± 0.00a A	4.80± 0.20a A	4.60± 0.30a A	4.20± 0.10b B	3.80± 0.10b C	3.00± 0.10b D
	HA/LACTO-QUE	5.00± 0.00a A	4.80± 0.20a A	4.60± 0.60a A	4.40± 0.10b A	3.80± 0.10b B	3.80± 0.10c B

Different letters (a, b, c, ...) reveal significant differences ($p<0.05$) among the samples for each storage time, and different letters (A, B, C, ...) reveals significant differences ($p<0.05$) among treatments during the storage time

7.9 Conclusions

An alginate- based coating activated with hydroxyapatite/lactoferrin/quercetin complexes was developed and applied to fresh pork meat to extend its shelf life.

The morphological analysis confirmed the adsorption of both bioactive compounds into the hydroxyapatite network, while in-vitro studies showed a homogeneous releaseof quercetin glycoside compounds and lactoferrin through the coating, reaching equilibriumin 70 h and 30 h, respectively.

The activated alginate-based coating showed a high capability to slow down the growth of the main microorganism responsible for the spoilage of fresh meat products,during storage for 15 days at 4 °C, reducing the production of the volatile basic nitrogencompounds.

Moreover, the comparison among samples pointed out a positive effect of the coating charged with hydroxyapatite/lactoferrin/quercetin complexes to slow down the changesin hardness during the storage time as well as the sensory attributes for both uncookedand cooked pork fillets. Finally, the results of the sensory evaluation showed that the presenceof edible coating

did not affect the visual and taste attributes in raw and cookedfillets, making the proposed active edible coating a potential application for the shelf-life extension of fresh meat products.

7.10 References

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Conclusions

This work has been focused on the development and optimization of edible coatings, based on alginate, incorporated with hydroxyapatite as alternative carrier for the delivery of bioactive compounds.
At first, quercitin was selected - for its antimicrobial and antioxidant properties - and the formation of the complex, its effectiveness of bacterial inhibition, as well as its homogeneous release from the coating were evaluated. The hydroxyapatite/quercitin complex at 500 ppm, loaded in a coating based on 1.5% alginate and 0.75% calcium chloride, are the best conditions obtained for the developed edible coatings, that has been targeted for the shelf-life extension of fresh food products under cold storage.
The effect of the coatings on the microbial, physical and sensory properties of the products during the cold storage was evaluated.
In particular, my interest has been turned to two different types of food products: meat and fruit. Both of these products are highly perishable and susceptible to environmental and microbiological degradations phenomena which consequently influence the quality, safety and shelf life of fresh meat and fruit. The application of developed coatings on fresh-cut fruit and meat products have shown satisfactory results for the inhibition of microbial growth as well as for the preservation of safety and quality parameters.
The microbiological analysis performed during the cold storage period of coated chicken fillets highlighted the capability of alginate coating with hydroxyapatite/quercetin complexes to significantly inhibit the growth of spoilage bacteria, as well as the total volatile basic nitrogen. Moreover, the comparison among the coated samples pointed out a positive effect of hydroxyapatite to slow down the changes in hardness during the storage time of 11 days. The coating with hydroxyapatite/quercetin complexes was able to preserve the sensory attributes of fresh poultry meat until the 11th day of storage and the edible coating doesn't affect the properties of the product also of a sensory point of view. The alginate-based coating loaded with hydroxyapatite/quercetin complexes was applied on fresh-cut papaya. Changes in microbial, physicochemical and sensory properties of fresh-cut papaya were evaluated for 14 days at 6°C.
The results highlighted the ability of the coating loaded with hydroxyapatite/quercetin to slow down the respiration rate of fresh-cut fruit, as well as to preserve its antioxidant compounds during the entire storage period. The microbial analysis carried out during the storage period pointed out the positive effect of HA charged with QUE to inhibit the growth of spoilage bacteria. Finally, the evolution of sensory attributes during the storage highlighted the positive effects of alginate-based coatings charged

with hydroxyapatite/quercetin complexes which were still assessed acceptable after 14 storage days.

After that, in order to obtain the same results, but with a lower concentration of quercetin, another bioactive compound was selected- the synergistic effect of quercitin and lactoferrin was evaluated against different bacteria strains (spoilage and pathogenic bacteria). Different amounts of quercetin and lactoferrin were adsorbed into HA structure and the results highlighted that the antimicrobial activity also depend upon which molecule is added firstly to HA.

The highest inhibition was obtained when the HA was first incubated with lactoferrin and then with quercetin, at concentration of 100 ppm. Then was evaluated the influence of simultaneous loading of two bioactive compounds in HA - the release study performed on Ha simultaneous charged with Lacto and QUE highlight that the presence of QUE speed up the release of Lacto, while the simultaneous loading of lacto and que not affect the realese of que.

The observed different release behaviour in active alginate-based coatings could be due to the difference in the solubility of the active compounds in the aqueous medium, besides the different interactions of the active compounds could show with HA structure. The performance of edibile coating with the ha /lacto/que complex was evaluated another time on fresh meat product. In particular was evaluated the shelf life extension of fresh pork loin fillets, covered with the edibile coating charged with ha lacto que, compered to the cating without ha e un coated samples as control. The parameters monitoring was the same seen for the chicken fillet study,but stored for 15 days a 4 C.

The comparison between LACTO-QUE and HA/LACTO-QUE coated samples highlighted a higher capability of the coating to delay bacterial growth when the active compounds were adsorbed into HA crystals, highlight a shelf life equal of 15 days. The activated alginate-based coating with ha /lacto/que showed a high capability to slow down the production of the total volatile basic nitrogen. The coating with alginate coating charged with LACTO and QUE allowed pork fillets to reach overall acceptability scores of up to 11 storage days, while the addition of HA/LACTO-QUE made pork samples acceptable for the whole investigated storage period.

In conclusion, the application of alginate-based coatings loaded with hydroxyapatite/quercetin and with hydroxyapatite/lactoferrin/quercetin complexes on fresh-cut fruit and fresh meat products have obtained satisfactory results as regards the inhibition of microbial growth, as well as for the preservation of quality parameters. These results make the hydroxyapatite potential structure able to preserve the properties of quercetin and lactoferrin, paving the way for the development of activated coatings designed for food shelf- life extension.