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Development of a novel Active Edible Coating containing Hydroxyapatite for food shelf-life extension

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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 free quercetin and hydroxyapatite/quercetin complexes. In-vitro release studies of the quercetin glycoside compound 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, preliminary 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.

1. 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 (Pinto et al., 2019). Non-renewable e non-biodegradable 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 straightforward approaches for preparing multicomponent edible coating requires blending different film-forming 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 (Harnon-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*, *Salmonella Enteritidis, 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 (Zambrano-Zaragoza et al., 2018). Liu et al. (2013) reported elaborating curcumin nanospheres using chitosan as the biopolymer, while Lv et al. (2014) described the incorporation of jasmin essential oil nanocapsules composed of two biocompatible polymers (gelatin and arabic gum) by complex coacervation. In another study, Coradini et al. (2014) reported the co-encapsulation of resveratrol and curcumin in PCL/grape seed oil nanocapsules. In yet another approach, a chitosan-based nanoemulsion containing lemongrass oil droplet was developed by Oh et al. (2017). A similar system, based on the use of nanoemulsions-based edible coating containing oregano essential oil, was used by Artiga-Artigas et al. (2017) onto low-fat cut cheese.
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. Experimental Section

2.1 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 [$Ca_5(PO_4)_3(OH)$] 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 hydroxyapatite crystals, the effective amount of QUE entrapped into the structure was evaluated. Hydroxyapatite/quercetin 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 a wavelength of 369 nm based on a quercetin-3 glucoside standard curve. The characterization of HA-QUE complexes is under study.
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.

2.2 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. The colour 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 (ΔE) and whiteness index (WI) were also determined as given by equations (1) and (2) (Albanese et al., 2014)

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

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

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) with some modifications. 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 $M_t/M^{\bullet}$ (M_t is the concentration of QUE (mg/mL) diffused at time t, and $M^{\bullet}$ represents the concentration of QUE diffused at equilibrium.

2.3 Antimicrobial activity

Pseudomonas fluorescens strain ATCC 13525 was provided from the Laboratory of Microbiological Food Control—Department of Food Microbiology of the Istituto Zooprofilattico Sperimentale del Mezzogiorno in Portici (Naples, Italy). *P. fluorescens* was grown overnight at 37°C in the liquid culture medium Buffered Peptone Water, BPW). 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 by using the standard broth microdilution method on polystyrene 96-well plates (Nunc) 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.

3. Results and Discussions

3.1 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 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-^{2} -D-glucoside was used at 1000mg/L. In plants, quercetin occurs mainly in a sugars-bounded form (Quercetin O-glycosides) in vast quantities and in highly diversified forms (Fossen et al., 1998). 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.

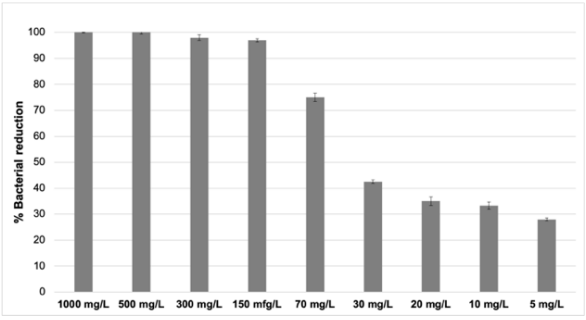

Figure 1: Antimicrobial activity at different concentrations of QUE against Pseudomonas fluorescens (10^6 cfu/mL)

3.2 Evaluation of HA-QUE complex antimicrobial activity

HA nanoparticles were loaded with different amounts of QUE (Table1). 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 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 close to 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.

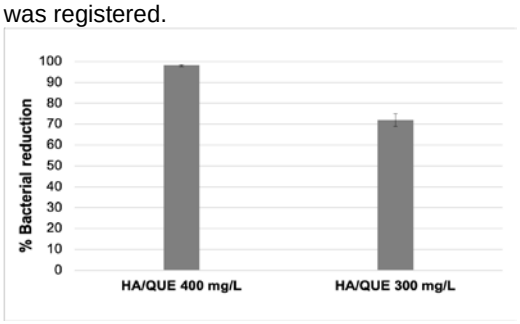

Figure 2: Antimicrobial activity at different concentrations (400–300 mg/L) of HA-QUE against Pseudomonas fluorescens (10^6 cfu/mL)

3.3 Coatings characterization

The effects of adding QUE and HA-QUE complex at different concentrations on colour coatings are shown in Table 2.

Table 2: Colour parameters and thickness of alginate-based coatings

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

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

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.
The kinetic release of QUE from the active alginate-based coatings is shown in figure 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).

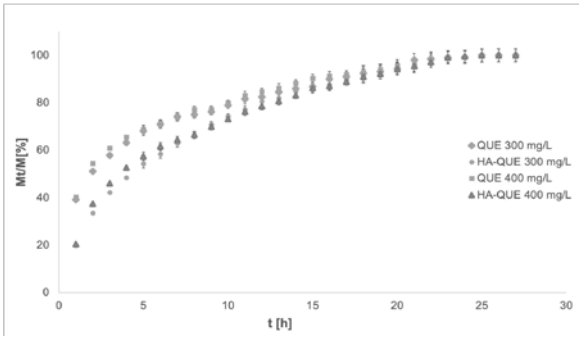

Figure 3: kinetic profile of quercetin glycoside compounds from alginate-based coatings.

As shown in figure 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.

3.4 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 obtained data 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.

4. 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 preliminary 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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