Biopolymer hybrid materials: development, characterization, and food packaging applications

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Abstract

Current trends in food packaging systems are oriented to biodegradable materials, especially with enhanced features. The latter are often provided by the combination of natural biopolymers, clays and bioactive molecules. This review summarises recent developments in the production of biopolymers-clay hybrid materials for food packaging. The main production methods of bio-composites and the improvement of their physicochemical properties due to the clay addition are discussed. The improvement of the film properties is explained with a focus on the molecular interactions between biopolymer matrix, clay, and bioactive compounds. Recent research on their food application as active and intelligent packaging are summarised. This review shows that the application of biodegradable bio-composites as packaging material is a promising green option to ensure food quality and safety. The research on the chemical properties of tailored and tunable biopolymers could lead to new functional films, suitable for the industrial scale-up.

Keywords: biopolymer, hydrocolloids, clay, film properties, analytical characterization, food packaging

Abbreviation list: AMOS, aqueous miscible organic solvents; AMP, antimicrobial peptides; CAGR, compound annual growth rate; CBHs, clay-bioactive compounds hybrids; CEO, clove essential oil; CMC, carboxymethyl cellulose; DSC, differential scanning calorimetry; DPPH, 2,2-diphenyl-1-picrylhy-drazyl; EOs, essential oils; EOCs, essential oil compounds; EU, European Union; FDA, Food and Drug Administration; HAL, halloysite; HNTs, halloysite nanotubes; JFE, Jamaica flower extract; LDH, layered double hydroxide; MMT, montmorillonite; OMMT, organo-modified montmorillonite; PA, polyamide; PEC, polyelectrolyte complex; PHAs, poly(hydroxyalkanoate)s; PHBs poly(hydroxybutyrate)s; PHC, palygorskite; PLA, poly(lactic acid); PVA, poly(vinylalcohol); RH, relative humidity; SAS, synthetic amorphous silica; TGA, thermogravimetric analysis; TPS, thermoplastic starch; TTI, time temperature indicator; TVB-N, total volatile base nitrogen; WPI, whey protein isolate.

1. Introduction

Active packaging is defined as "packaging in which subsidiary constituents have been deliberately included in or on either the packaging material or the package headspace to enhance the performance of the package system" (Robertson, 2006). As recently reviewed by Janjarasskul and Suppakul (2018), the most important active packaging systems are the O_2 scavengers, the CO_2 scavengers and emitters, the moisture and ethylene gas regulators, releasing/adsorption systems of flavours and odours as well as those with the ability to release antimicrobial agents or antioxidants.

Intelligent packaging refers to a packaging system that can detect environmental changes, since it possesses logic capabilities and intelligent functions (e.g., detecting, sensing, recording, tracing, communicating, and applying algorithms to extend the shelf-life), in turn providing information, and warning about potential issues (Janjarasskul & Suppakul, 2018).

More than 30% of the worldwide plastic production is used as packaging materials. The 47% of this percentage is represent by bioplastics. Interestingly, the food sector represents over the 75% of the bioplastic applications as packaging material (European bioplastics, 2020; Coherent Market Insights, 2018; Zhao, Cornish, & Vodovodtz, 2020). Recently, there is an increased demand for biodegradable packaging materials, and, according to recent records, the biodegradable plastics market is predicted to grow at an 8.4% compound annual growth rate (CAGR) in the period 2016-2022, up to 16.8 million dollars by 2022 (MRFR, 2020). However, biopolymer-based packaging materials are not widely used for food packaging, mainly because of their limited mechanical, barrier, and processing properties as well as the high production cost. To overcome such limitations, the use of biopolymer/clay composites has attracted attention as an alternative to conventional polymers, due to the improvement in the mechanical and physical film properties such as rigidity, stiffness or flexibility, durability, temperature and moisture stability, and barrier effect against water, oxygen, and other gases (Fatyeyeva, Chappey, & Marais, 2017). Moreover, the inclusion of clay bioactive compound hybrids (CBHs) supplies additional properties (i.e., antimicrobial or antioxidant) to these films (Cirillo, Kozlowski, & Spizzirri, 2018).

In Figure 1 the biopolymers, the clays, and the organic antimicrobials/antioxidants used for the manufacture of biopolymers-clay hybrid composite materials are classified.

Hydrocolloids can be found as hydrosol or hydrogel, depending on the water content. Novel biopolymers-clay hybrid composite materials for food packaging could be developed using hydrogels, as reported in this review. Hydrogels are defined as colloidal gels, as they disperse in water and present viscoelastic and structural properties. In the swollen state and when exposed to certain pressures, their three-dimensional structure allows the absorption of target fluids without structural changes (Batista et al., 2019). They could be sensitive to different pH, temperature, pressure, or photo irradiation conditions. Moreover, hydrogels can be used as humidity adsorbents, antimicrobial materials, or to modulate the release of antioxidant compounds (Batista et al., 2019).

The aim of this review is the description and discussion of innovative, economically sustainable, and time-saving methods for the manufacture of CBHs, and bio-composite films, as well as the improvement of their physicochemical properties, determined by molecular interactions among biopolymer matrix, clays, and bioactive compounds. Finally, selected applications as active or intelligent packaging of these bio-composites for the food preservation and the food safety are reported. Since the European Plastic Strategy promotes the environmental-friendly use of different biopolymers, polymer blends, and additives (Elliot, Gillie, & Thomson, 2020), the manufacturing of novel bio-composite films and their approval for food contact purposes is expected. Therefore, in this review, the regulations related to food application of the nanocomposite materials are reported.

2. Biopolymers, clays, and bioactive compounds

The bio-composite films described in this review are composed of the biopolymer matrix, the clays, and the bioactive compounds loaded into the clay hybrids. Their description for the development of bio-composite films is reported in the following sections.

2.1 Biopolymers

A plastic material is defined as a bioplastic if it is either biobased, biodegradable, or features both properties. Biopolymers described in this review are a sub-class of biobased and biodegradable bioplastics (European bioplastics, 2020). According to the method of production, biopolymers can be polymers directly extracted from biomass of vegetable or animal origin, such as polysaccharides, proteins, lipids, but also polymers produced by classical chemical synthesis starting from renewable bio-based monomers such as poly (lactic acid) (PLA), or polymers produced by wild or genetically modified microorganisms, such as poly(hydroxyalkanoate)s (PHAs), poly(hydroxybutyrate)s (PHBs), bacterial cellulose, xanthan, gellan, pullulan. However, depending on their source, biopolymers can be polysaccharides, proteins, lipids, or aliphatic polyesters (for a review see Sivakanthan, Rajendran, Gamage, Madhujith, & Mani, 2020). Polysaccharides include starch, cellulose, chitosan, alginate and carrageenan, pectin and gums or their derivatives.

Proteins and lipids have limited use for the biopolymers manufacturing. Protein films can be classified according to the animal (gelatin, casein, whey, and collagen) or vegetable (zein, soy, gluten) origin (Fig. 1). Beeswax and carnauba wax are the most used lipids for the manufacture of emulsion coatings including clays, to be used in the food sector (Klangmuang & Sothornvit, 2016; Motamedi et al., 2018).

Aliphatic polyesters used for biopolymer manufacture are PLA, PHA, PHB, and some classes of polyamides (PA 6.6 and PA 4.6) (Radzik, Leszczyńska, & Pielichowski, 2020). Aliphatic polyesters are not hydrocolloids, but in some cases, they can be used in multilayer composites based on these compounds. In particular, their good gas barrier properties, heat resistance, and the possibility to be processed into different products including films, trays and coatings on other biobased materials offer opportunities to be applied as food packaging materials (Ragaert et al., 2019).

Among all biopolymers, hydrogels show promising features for the production of bio-polymeric clay-reinforced materials. For instance, a Pickering emulsion hydrogel system using chitosan as Pickering emulsifier, and Ca-alginate hydrogel beads as carrier was developed (Lim et al., 2020). The chitosan particles remained adsorbed on the oil droplets upon release, showing potential application as food delivery system (Lim et al., 2020). Recently, the possibility to stabilize the Pickering oil-in-water emulsion through the clay addition was reported, as in the case of alginate/MMT composite which thickened the continuous phase, created a gel-like environment around the droplets, and enhanced their electrostatic force (Wang, Deng, Sung, & Yang, 2020). Based on these results, bioactive antioxidant compounds could be loaded into hydrogel-clay matrix to be released during food storage in the future.

Composite emulsion hydrogels could be prepared using a continuous phase of oil-in-water concentrated emulsion templates, as demonstrated for gelatin (water phase)/cinnamon oil (oil phase) hydrogel with antimicrobial activity (Wang et al., 2018), and whey protein concentrates/high methoxy pectin hydrogel particles with improved oxidative stability (Cao et al., 2020). These findings suggest the potential of the composite emulsion hydrogels to be used as delivery systems of bioactive compounds. In the future, the application of hydrogels-clay hybrids in the production of innovative bio-composites could become a trending research topic. Their application in the food packaging could meet the demand of functional and tailored biomaterials.

2.2 Clays

Film properties like melt strength, impact strength, thermal stability, and gas permeability of biopolymers do not meet the demand for some food applications, especially for fresh products.

Therefore, the incorporation of nanosized reinforcement agents, like commercially available clays, is a good strategy to improve these properties. To this aim, the layered silicates of natural or synthetic origin mostly used are the montmorillonite, the halloysite, the silica, the Laponite®, and the hydrotalcite. In addition, as recently reviewed by Pires, de Paula, Souza, Fernando, & Coelhoso (2021) the inclusion of clays in biopolymeric films has several advantages due to their low cost, availability, and good surface area. However, possible concerns related to potential toxicity and migration from packaging material should be taken into account.

Montmorillonite (MMT) is the principal compound (*ca.* 90%) of the 2:1 clay bentonite. This hydrated alumina-silicate-layered clay consists of two tetrahedral sheets fused to an edge-shared octahedral sheet of aluminium hydroxide. MMT has a high slightly negative surface area, high elastic modulus (178 GPa) as compared to other clays, is plate-shaped, and typically 1 nm in thickness (Giannakas & Leontiou, 2018). The negative charge is balanced with exchangeable cations, as Na^{+} and Ca^{2+} (natural sodium montmorillonite, Na-MMT, and natural calcium montmorillonite, Ca-MMT). However, it is difficult to disperse homogeneously MMT in an organic polymer phase, due to the hydrophilic nature of its surface. Therefore, the hydrophobicity of MMT needs to be improved by means of the surface modification. This modification is often carried out by cation exchange, leading to organically modified-MMT (OMMT) with higher distance between the silicate layers. The most used modifiers are the quaternary alkyl ammonium cations (Fatyeyeva et al., 2017).

Halloysite nanotubes (HNTs) are aluminosilicates with an outer surface of a silica sheet, and the inner surface of an alumina sheet, rolled up resulting in a hollow tube. From a structural point of view, HNTs have non-polar external surface and a polar inner part loading charged molecules. The non-polar surface allows HNTs to be easily dispersed into many polymer systems, increasing the stiffness of the biopolymers thank to their rigid nature. The main properties of the HNTs are the porous structure, good mechanical properties and biocompatibility, the thermal and acid/basic stability. The intercalation of organic compounds is favoured in the hydrated form.

The cycloaddition of azides and alkynes (click reaction) in the HNT cavity is one of the modification procedures to produce bio-nanocomposite with HNT fillers (Darie –Niţă & Vasile, 2018).

Silica, Laponite®, and hydrotalcite are less used for food bio-nanocomposite manufacturing compared to MMT and HNTs. Silicon dioxide, or silica, has been used in food applications in the form called synthetic amorphous silica (SAS). The material is marked as the food additive E551 and consists of primary particles, aggregates, and agglomerates. Only part of the particle size distribution of SAS is less than 100 nm (Fruijtier-Pölloth, 2016). Laponite® is synthetic clay with a disk-like shape, small size (1 nm in thickness), great ability to form transparent colloidal dispersions, and high surface area (Fatyeyeva et al., 2017). Hydrotalcite (formerly layered double hydroxide, LDH) is a lamellar inorganic material with large surface area, great thermal and mechanical properties, and tendency to interchange their interlayer anions with other anions such as larger organic and inorganic ones. The structure of LDH composes of brucite-like sheets and the thickness of each sheet is about 0.5 nm (Mallakpour & Khodadadzadeh, 2020).

Layered silicates do not have antimicrobial activity but they interact with microbial cell membranes due to their high electrostatic activity and, for this reason, they may be useful in combination with antimicrobials (Azeredo et al., 2019).

2.3 Bioactive compounds

The incorporation of bioactive compounds into the packaging represents an interesting strategy to avoid the use of preservatives or other additives directly on foods. In this manner, the active packaging material could extend the shelf-life of foods, maintain their freshness and quality, and improve the food safety. Among different natural bioactive compounds, essential oils (EOs), extracts composed by different volatile compounds (e.g., rosemary, clove, thyme essential oil), or essential oil compounds (EOCs) (e.g., eugenol, thymol, cinnamaldehyde) are the mostly used antimicrobials to prepare biopolymer-CBHs films. EOs and EOCs are well known for their antifungal (Pinto et al., 2020, 2021) and antibacterial (Bevilacqua, Speranza, Perricone, Sinigaglia, & Corbo, 2017; Perricone, Arace, Corbo, Sinigaglia, & Bevilacqua, 2015) activity. Clays can load different EOs or EOCs that can be released over time. They also act as a carrier to disperse hydrophobic molecules into hydrophilic matrices as observed with marjoram essential oil and clove essential oil in alginate and chitosan, respectively (Azeredo et al., 2019). In addition, EOs or EOCs could display both antimicrobial and antioxidant properties (Garrido-Miranda, Rivas, Pérez-Rivera, Sanfuentes, & Peña-Farfal, 2018).

The antimicrobial peptides (AMPs) of animal (Baruzzi et al., 2015) and microbial origin (Meira, Zehetmeyer, Wemer, & Brandelli, 2017), and the organic acids are other natural compounds used for the inclusion into bio-based packaging materials, and to prepare CBHs.

In addition to antimicrobials, antioxidants such as plant polyphenols can be successfully loaded into clays. As recently found by Muráth, Szerlauth, Sebők, and Szilágyi (2020), the antioxidant-clay hybrid could overcome the problem related to the low solubility of certain antioxidants in water. Therefore, these hybrids can be included in hydrophilic biopolymers for food contact purposes. Other natural antioxidants suitable for the inclusion into biopolymers are the hydro-alcoholic extract from vegetable tissues. They can interact with the biopolymer matrix, such as chitosan, trough hydroxyl groups of polyphenols and the carbonyl groups of phenolic acids (Souza et al., 2017).

3. Development of clay bioactive compound hybrids and composite biopolymers

The development of the bio-composite material usually starts with the production of CBHs, here defined as clay material loaded with organic molecules (antimicrobials, antioxidants), and to be included within the biopolymer matrix. Sometimes, the addition of the bioactive molecules follows, or is concurrent with, the production of the biopolymer-clay composite material (Giannakas, Stathopoulou, Tsiamis, & Salmas, 2020). In Table 1, the main production methods of the CBHs and the composite biopolymers are reported.

3.1 Development of biopolymer-clay composites

The interaction of clay particles with the biopolymer matrix has a pivotal role to obtain homogeneous films. As reported by Ramos et al. (2018), layered clays can be phase separated or immiscible (microcomposite), intercalated, exfoliated, and disordered intercalated (partially exfoliated) in the polymer matrix. Exfoliated structure allows the maximum reinforcement because of the proper clay distribution, resulting in a large surface area (Fatyeyeva et al., 2017). Moreover, the exfoliated structures show good barrier to water vapor, being suitable for food packaging applications (Pires et al., 2021).

In Fig. 2, the train-loop-tail conformation of the polymer matrix (xanthan gum) at the clay interface (kaolinite) is depicted. This conformation affects the water absorption capacity of the biopolymer. Indeed, at low xanthan gum concentration, more loops cross-link between clay particles, exposing a significant length of xanthan gum chains to the water molecules. This exposure allows the gum to adsorb large amounts of water. On the contrary, at high xanthan gum concentration, polymer chains saturate clay particles with the train conformation. The limited exposed chain loops reduce the amount of water that the xanthan gum can adsorb (Sherif, Dilip, & Dilip, 2019). Therefore, the amount of biopolymer added to the clay suspension drives the water absorption capacity of the composite material.

According to Alcântara and Darder (2018), two strategies can be followed for the composite production. In the blocks assembly approach, the biopolymers and the inorganic units are assembled together to obtain the nanostructured bio-composite, whereas in the molecular assembly approach the clay hybrids can be synthesized in the presence of the biopolymer.

The mostly used methods for the biopolymer-clay production follow the block assembly approach. In this respect, Alcântara, Darder, Aranda, and Ruiz-Hitzky (2020) have compared the ion-exchange, the co-precipitation, and the calcination-rehydration reaction method for the production of zein-LDH hybrids. In the latter, the dehydrated layered double oxide is obtained through calcination. Then, the oxide is mixed with the solution of zein at pH above 11, as in the case of ion-exchange and co-precipitation methods. Results show that the co-precipitation method and the calcination-rehydration reaction determine partial, or complete, intercalation of zein into LDH, through electrostatic interactions with the clay (Alcântara et al., 2020). An innovative hybrid preparation method was developed by Alcântara, Darder, Aranda, and Ruiz-Hitzky (2016). The authors prepared a zein/MMT hybrid through the adsorption of the zein phases, previously segregated in absolute ethanol, to which a water/ethanol suspension of the clay is added (Alcantara et al., 2016).

The production methods can be classified in dry and wet processes. Wet processes include the solvent casting and the solution intercalation methods. Dry processes include the melt extrusion, the injection molding, the compression molding, and *in situ* polymerization (layer-by-layer, electrospinning, simple coating) (Ramos et al., 2018; Zubair & Ullah, 2020). The solvent casting method (wet process) is the most used to produce biopolymer/clay/bioactive composites, such as gelatin/MMT/ginger essential oil (Alexandre, Lourenço, Bittante, Moraes, & do Amaral Sobral, 2016), karaya gum/cloisite/cinnamaldehyde (Cao & Song, 2019), starch/HNT/AMPs (Meira et al., 2017), k-carragenan/MMT/*Zataria multiflora* extract (Nouri et al., 2020), and chitosan/PVA/MMT/lactate on a polyethilene support (Zhang et al., 2017). This wet process needs three steps to form the films, including solubilisation, casting, and drying. The clay is usually dispersed in water or water/ethanol solutions to open clay stacks. The biopolymer is added to the clay solution, in presence of a plasticizers such as glycerol (Cao & Song, 2019) or triethyl citrate (Rodriguez et al., 2019), at 70-80 °C under magnetic stirring. The film forming solution is then sonicated, degassed, and casted onto a glass or plastic plate to form a thin film (Kashiri, Maghsoudlo, & Khomeiri, 2017), and dried at 20-25 °C and relative humidity (RH) of 50% to reach the steady state (Cao & Song, 2019). Ortiz, de Moraes, Vicente, Laurindo, & Mauri (2017) produced a large-scale soy protein film by the tape casting method, another wet process in which the film-forming solution is deposited on a support with a doctor blade device, controlling the suspension thickness.

In the solution intercalation method, clay and biopolymer are dispersed in the solvent separately. Then, the solutions are mixed, also with the help of the ultrasonication. The full exfoliation of the clay and better biopolymer/clay dispersion can be achieved compared to other methods (Li et al., 2013; Zubair & Ullah, 2020). Once the solvent is removed, by evaporation or freeze-drying, the material is then shaped by compression molding or extrusion.

As regards the dry processes, Campos-Requena et al. (2017) produced thermoplastic starch (TPS) added with MMT and carvacrol or thymol by the melt extrusion process. The starch/plasticizer blend was mixed with MMT/EOCs slurry. The mixture was then processed in a twin-screw extruder, hot pressed, and cooled (Campos-Requena et al., 2017). Poly (butylene succinate)/LDH/l-ascorbate composite was produced by the compression molding method (Marek et al., 2020). In this process, the mixture is thermally compressed between pre-heated stainless-steel platens covered with aluminium foil. In protein-based polymers, these conditions lead to the protein denaturation. The cooling of the mixture controls its formation by the development of new interactions such as covalent, ionic, hydrogen bonding and hydrophilic interactions (Zubair & Ullah, 2020). The films are then peeled from the aluminium foil layers after cooling at room temperature.

As far as the *in-situ* polymerization methods are concerned, the simple coating and the electrospinning are the most used approaches. A coating composed by carnauba wax/MMT/orange peel essential oil can be directly applied on the surface of oranges (Nasirifar, Maghsoudlou, & Oliyaei, 2018). Bugatti, Vertuccio, Viscuso, and Gorrasi (2018) developed a bio-based polyamide-11/HNT/lysozyme composite by electrospinning. In this procedure, the solution containing polyamide-11, HNT-lysozyme filler, and hexafluoroisopropanol is injected in a syringe connected to an electrode. Fibrous mats are collected on a ground aluminium collector. In our opinion, this procedure could be extended in the future to other biopolymers such as polyamide 6.6 and 4.6 (PA 6.6 and PA 4.6), produced by fermentation and microbial transformation.

Recently, Wang et al. (2021) successfully produced a chitosan/HNT/tea polyphenols film by means of 3D printing. Although authors concluded that material characteristics, process parameters, and post-processing methods need further investigation, this technology provides a new strategy for the film processing.

3.2 Clay-bioactive compound hybrids (CBHs)

Several protocols have been described for the preparation of CBHs. By a molecular point of view, the organic guest molecule can be intercalated or adsorbed onto the clay surface (Lobo-Sánchez et al., 2018). Complexation interactions are often hydrogen bonding, electrostatic, hydrophobic and π-π interactions between the molecule and the clay surface. These interactions are driven by the pH, and the cation exchange capacity of the clay surface. In addition to the acid-base chemistry, the conformation of the molecule across the nanopores (parallel, tilted, or vertical conformation), the exfoliation phenomenon, the surface area, and the hydrophilic character play a key role to determine the sorption rate (Nakhli et al., 2017).

The conformation of the polymer matrix also affects the adsorption of bioactive molecules by the composite material. Generally, as the polycation charge density of the biopolymer decreases, its conformation at the clay surface shifts towards ‘loops and tails’, and the composite surface is more hydrophobic (less charged monomers). Hence, the adsorption of non-ionic molecules by such composites increases due to both higher hydrophobicity and a more dangling conformation. Conversely, anionic molecules are preferentially adsorbed onto composites with high charge density through electrostatic interactions (Kohay, Bilkis, & Mishael, 2019). The inclusion of the guest molecules (EOs, EOCs, AMPs) into the clay-hybrid during shear mixing is the most used preparation method (Meira et al., 2017; Pires, Souza, & Fernando, 2018; Tenci et al., 2017). In this procedure, the clay and a solution of the organic compound are mixed in a fixed ratio. Then, the dispersion of the clay in the suspension is promoted by means of the ultrasonication. The following centrifugation and evaporation allow the removal of the non-adsorbed compound (Meira et al., 2017; Tenci et al., 2017). For EOCs, Tenci et al. (2017) compared the preparation of clay-carvacrol hybrids by the adsorption in saturation conditions or by the shear mixing. The first consists in the exposure of clay surface in a sealed thermostatic environment saturated with the EOC vapours for 2 to 5 days (Tenci et al., 2017). The shear mixing method determined higher loading capacity of carvacrol than adsorption in saturation conditions, with the highest values observed for the fibrous PHC, followed by the tubular HAL and finally by the lamellar MMT (Tenci et al., 2017).

Although the preparation of LDH-EO hybrids is still limited explored, the co-precipitation method is currently used (Bugatti et al., 2019; Lobo-Sánchez et al., 2018). Zn and Al nitrate solutions are dropped with EO solution in ethanol at pH 10. The resulting slurry is sonicated in an ultrasonic vessel to crystallize LDH. The solid is then precipitated, washed, and dried (Lobo-Sánchez et al., 2018).

The reduction of the length and the increase of the inner diameter of the clay particles can increase the loading capacity of the bioactive compound. Indeed, Makaremi et al. (2017) revealed higher loading efficiency of salicylic acid into shorth-tubular HNT with high inner diameter values, than long-tubular HNT with low inner diameter.

4. Physicochemical properties of the clay-reinforced bio-composites

The inclusion of clay into the biopolymer matrix can improve some physicochemical properties such as barrier, mechanical, and thermal properties, as summarized in Table 2. The following section describes the physicochemical and structural characterization of the bio-composites, highlighting the interactions between the polymer matrix and the clays. In addition, biodegradability, compostability, and release properties of these films are also reported.

4.1 Barrier properties

The barrier properties of the polymers are associated with their inherent ability to modulate the permeation of substances with low molecular weight, such as gases. Clay nanoparticles are naturally impermeable and improve the polymer barrier properties by creating the tortuous path that delays the diffusion of vapour or water molecules through the polymer matrix (Fatyeyeva et al., 2017).

The incorporation of clove essential oil (CEO) and HNT into the chitosan polymer significantly reduced the water vapour permeability, the moisture content, and the water adsorption of the film, as a function of the clay concentration (Lee, Kim, & Park, 2018). Dairi, Ferfera-Harrar, Ramos, and Garrigós (2019) reported that the bio-composite cellulose acetate/Ag-MMT/thymol showed higher oxygen transmission rate than cellulose film. This phenomenon was ascribed to the presence of predominantly intercalated layered silicate than exfoliated ones (Dairi et al., 2019). However, the addition of the clay could overcome the increase in oxygen permeability, due to the plasticizing effect of the thymol addition (Dairi et al., 2019). Sun et al. (2019) produced a chitosan/bentonite/poplar extract film with reduced water vapour permeability, oxygen permeability, and water solubility compared with chitosan/poplar extract films. Authors postulated that these properties might be attributed to the hydrogen bonding and covalent bonding between the chitosan network and Poplar extract (composed by hemicellulose-derived sugars and lignin) (Sun et al., 2019). Similarly, chitosan and HNTs showed a synergistic effect in reducing the moisture uptake, water solubility, and water swelling degree of agar films. These effects are probably caused by the H-bonds formation between the polymer matrix and the HNTs, and the electrostatic interactions between sulphates groups of agar chain and the protonated amino group of chitosan (Huang et al., 2020). Overall, the addition of clays into the biopolymer matrix reduces the water vapor and oxygen permeability of the bio-composite films, thanks to their conformation and molecular interaction with the hydrocolloids.

4.2 Mechanical properties

Mechanical properties are of great importance for food packaging application, and mechanical durability is expected from bio-composite films. The maximum tensile stress, i.e., the maximum stress a material can stand before it breaks, increased in methylcellulose/MMT/carvacrol film as function of the MMT added in the polymer matrix. At the same time, the maximum tensile strain, the maximum ratio of extension to original length, was reduced with increasing MMT concentration. The MMT addition also increased the film Young's modulus, namely the parameter expressing the stiffness of the material. This reinforcing effect is due to the high aspect ratio and high specific surface area of MMT (Tunc, Duman, & Polat, 2016). Wang, Kang, Zhang, Zhang, and Li (2017) developed a soy protein/tannic acid-coated MMT film with higher tensile strength and Young's modulus than those of the pristine film. The authors, following the ATR-FTIR characterization of the film, explained the improvement of the mechanical properties through the strong interactions between the amino or thiol groups of soy protein polymer and tannic acid-MMT hybrid (Wang et al., 2017). Similarly, an improvement of the Young's modulus and tensile strength was found for a multilayer bio-composite film based on chitosan/PVA/thiabendazoluim-MMT in comparison to pristine film (El Bourakadi et al., 2019). The addition of clays into a hydrogel matrix can improve the rheological, mechanical, and thermal properties of the bio-composite. A protein-clay hydrogel was prepared using an aqueous suspension of Laponite® nanosheets added with sodium polyacrylate and elastomeric proteins rich in arginine. The resulting hydrogel showed enhanced mechanical properties thanks to the interaction of arginine-rich proteins with clays, and cross-linking of the nanosheets into hydrogels (Lv, Duan, & Li, 2019). The addition of MMT into xanthan gum-based hydrogel was useful to tune up the rheological properties of the hydrogel. In particular, the addition of 2% w/w of clay produced a consolidated matrix and MMT aggregation (Garcia-Hernandez, Lobato-Calleros, Vernon-Carter, Sosa-Hernandez, & Alvarez-Ramirez, 2017). In conclusion, the inclusion of clays into the biopolymeric films improves their mechanical properties, increasing stiffness and tensile strength.

4.3 Optical properties

Optical properties of the bio-composite film have a direct impact on the appearance of the packed food product, as well as affect the possibility to incorporate non-destructive sensor systems that allow the direct detection of the microbiological status or oxygen leakage during the shelf-life of the products (Kelly, Santovito, Cruz-Romero, Kerry, & Papkovsky, 2020). Therefore, optical parameters such as opacity, UV-light transmission, and colorimetric parameters (a* and b* values) are evaluated after clay-bioactive hybrid addition into the biopolymer matrix. The addition of the essential oils such as rosemary essential oil into the chitosan matrix produced more saturated colour (higher chroma), opaque and yellowish films (Souza et al., 2018). ~~The EO droplets interrupt the continuous polymer matrix, and their interaction with water molecules modifies the refractive index of the chitosan, increasing its opacity.~~ Moreover, the rosemary essential oil addition turned the material to a yellow colour, and reduced UV-light transmittance, protecting the packed food towards light-mediated oxidation processes. However, the clay addition did not affect optical properties of the film, suggesting that the optical properties were modified by the EO incorporation (Souza et al., 2018).
This finding was also confirmed in k-carragenan/MMT/*Zataria multiflora* extract (Nouri et al., 2020) nanocomposite film in which the reduction of the UV-light transmittance and the increase of the opacity were correlated only with the essential oil concentration included in the polymer matrix. Recently, the addition of nanoclay and catechin-lysozime into the rice flour/gelatin matrix decreased the light transmission in the UV and the visible range, but significantly increased a* (redness) and b* (yellowness) values of the film (Pattarasiriroj, Kaewprachu, & Rawdkuen, 2020). Generally, the reduction of UV-light transmission, the increase of opacity,

and changes in colorimetric parameters of the bio-composite film, seem more related to the bioactive compound inclusion than the clay addition.

4.4 Thermal properties

The addition of CBHs into the biopolymer can improve the thermal stability of the nanocomposite film. The addition of MMT and citric acid into a whey protein isolate (WPI) film was found to reduce the mobility of the polymer chains and, consequently, the thermal stability as demonstrated by high initial degradation temperature values of the composite film (Azevedo, Silva, Pereira, da Costa, & Borges, 2015). The reduction of the glass transition temperature upon incorporation of MMT nanoparticles was attributed to the plasticization effect of the intercalated compound dispersed in the WPI matrix (Azevedo et al., 2015). TGA analysis revealed that WPI/MMT/citric acid showed higher decomposition temperature and lower mass loss variation than WPI film, therefore more stable to the thermal decomposition (Azevedo et al., 2015). Similar results were obtained by Makaremi et al. (2017) with pectin/long tubular HNT film which showed higher residual matter at 700 °C, and higher temperature at maximum weight loss rate than pectin film.

The addition of sepiolite to carboxymethyl cellulose (CMC) hydrogel increased the thermal stability of the biopolymer, as revealed by the increase of the glass transition temperature and the melting temperature (Palem et al., 2021). The results described in this section show higher stability to the thermal decomposition of biopolymer-CBHs films than pristine films.

4.5 Release properties

The ability of a bio-composite material to release functional molecules, such as antimicrobials or antioxidants, into food they are in contact with, is commonly referred as release properties. A release study using chitosan/HNT/clove essential oil film and ethanol solutions as simulants (95, 50, and 10% v/v), showed that the highest amount of essential oil was released in 50% ethanol solution, probably due to the swelling of the film by the water molecules as well as the high EO solubility in ethanol. The clay-reinforced film showed higher amounts of released EO in comparison to chitosan/EO film, except for the first stages of contact (Lee et al., 2018). The main environmental parameter affecting the release of bioactive compounds from biopolymer-clay composite films is the storage temperature. Indeed, as demonstrated by Biddeci et al. (2016), the release of menthone, the main EOC of peppermint essential oil, from pectin/HNT/cucurbit-6-uril/peppermint essential oil film into 50% v/v ethanol solution was lower at 4 °C than 25 °C. The release profile followed a first order exponential kinetic but, at both temperatures, a not complete release was detected (Biddeci et al., 2016).

Release properties of bioactive compound-clay hybrid are also characterized. Recently, Cheng et al. (2019) studied the release behaviour of p-hydroxybenzoic acid from LDH clay into 10% ethanol as simulant. The release of intercalated p-hydroxybenzoic acid from LDH was incomplete and occurred in a slower rate, compared to pure acid, as a consequence of the LDH intercalation and the protonation of anions. In addition, the release was fitted with a zero-order model, which indicates that the release mechanism is a combination of diffusion and LDH dissolution (Cheng et al., 2019). Conversely, two first order desorption processes fitted the experimental data related to the release of salicylic acid from LDH (Ghezzi et al., 2018). In conclusion, the controlled release of the bioactive compound included in the clay-reinforced biopolymer is not always achieved, being affected by the storage conditions and the interaction with the clays.

4.6 Biodegradability and compostability

Bio-based clay hybrid composite materials are characterized by different level of biodegradability and compostability, depending on the clay concentration, additives used for the manufacture, and the biopolymer origin. For instance, the addition of clays into composite films can improve the biodegradation rate, thanks to the formation of imperfect crystals, whereas the polymer additives such as oleic acid or glycerol monooleate seem to have limited effect on the biodegradation time (Cesur, Köroğlu, & Yalçın, 2018). An increase in the biodegradation degree is observed in PLA/cloisite films added with thymol or cinnamaldehyde in comparison to PLA/cloisite film, due to the plasticizer effect of these EOCs. Indeed, these small molecules increase polymer chain mobility, speeding up the hydrolytic degradation process (Villegas et al., 2019). As far as the compostability of the material is concerned, biodegradable materials could be not compostable due to toxic effects of clay hybrids added in the composite formulation (Gutiérrez, Toro-Márquez, Merino, & Mendieta, 2019). Indeed, compost enriched with increasing concentration of TPS/organomodified-MMT/Jamaica flower extract powder negatively affected the length of lettuce primary root. However, the use of natural MMT, or other clay modifications did not impair the percentage of the primary root elongation (Gutiérrez et al., 2019). Given these findings, biodegradability of the bio-composite film is not affected after the clay addition, whereas a limited compostability rating could be obtained using biopolymer-CBHs materials.

5. Bioactivity of bio-composite films

The following section describes the antimicrobial and antioxidant activities, and the food packaging applications of bio-based CBHs composite materials (Table 3). In addition, the development of novel bio-composites as intelligent packaging will be proposed.

5.1 Antimicrobial activity of bio-composites

The clay hybrids used as fillers of biopolymers can result in materials endowed with antimicrobial activities thanks to the inclusion of antibacterial or antifungal compounds from different sources. Essential oils and their compounds are the most used natural antimicrobials to be included in clay hybrids. Alboofetileh, Rezaei, Hosseini and Abdollahi (2018) reported that the film forming solution containing sodium alginate, montmorillonite and marjoram essential oil partially inhibited *Escherichia coli*, *Listeria monocytogenes*, *Bacillus cereus*, and *Staphylococcus aureus*. However, the films prepared from the abovementioned solutions showed a reduced antimicrobial activity, probably caused by the loss of the concentration of the active compounds during the film drying. Conversely, cassava starch/bentonite/cinnamon essential oil films showed promising antibacterial activity against *E. coli*, *Salmonella typhimurium*, and *S. aureus*. At 2.5% cinnamon oil, the zone of inhibition ranged from 10 mm (*S. aureus*) to 15 mm (*E. coli*) (Iamareerat, Singh, Sadiq, & Anal, 2018). Rodriguez et al. (2019) compared the antibacterial activity against *E. coli* of cellulose acetate/MMT/cinnamaldehyde films produced by casting solution and melt-compounding extrusion techniques. The antibacterial test performed according to the ASTM E2149–13 standard (ASTM, 2013), highlighted the higher antibacterial activity of melt-compounding films compared to those prepared by casting solution, up to day 7 (Rodriguez et al., 2019). Recent findings showed the possibility to develop hydrogel-based antibacterial composites. Indeed, the composite hydrogel PVA/sodium alginate/modified LDH/thyme essential oil showed limited antibacterial activity against *S. aureus* and *P. aeruginosa* (Boccalon et al., 2020), as well as the emulsion hydrogel composed by alginate and CEO displayed antibacterial activity against *E. coli* and *S. aureus* (Wang et al., 2018).

As regards the sensitivity of bacteria to biopolymer/clay/essential oil films contact, it is possible to define a sensitivity scale. Some authors report higher resistance of Gram-positive bacteria than Gram-negative bacteria (Kashiri et al., 2017), whereas other groups found higher sensitivity of Gram-positive bacteria than Gram-negative ones (Alboofetileh et al., 2018; Iamareerat et al., 2018). These controversial results could be due to the different chemical composition of the essential oil included into the biopolymer matrix as well as the different releasing behaviour of the antimicrobial compounds.

As far as the antifungal activity of these bio-composite films is concerned, Pola et al. (2016) found that cellulose acetate/MMT/oregano essential oils films inhibited the growth of *Alternaria alternata*, *Rhizopus stolonifer*, and *Geotrichum candidum* by both direct and vapour contact.

Similarly, PHB/TPS/OMMT/eugenol films inhibited the mycelium growth of *Botrytis cinerea* by direct contact (Garrido-Miranda et al. 2018). Other antimicrobial compounds used to prepare CBHs are the cationic peptides and the organic acids. Carboxymethyl cellulose-based nanocomposites reinforced with MMT and ε-poly-L-lysine showed appreciable antimicrobial activity against *E. coli*, *S. aureus*, *B. cinerea* and *R. oligosporus*, reaching 90% of growth inhibition at 12.5% ε-poly-L-lysine (He et al., 2020). Recently, a gelatin-based composite including LDH and p-hydroxy benzoic acid showed inhibitory activity (inhibition zone of 15-16 mm) against *S. aureus* and *Candida albicans* (Cheng et al., 2019).

Other plant extracts can also be included into biopolymer-reinforced matrix to produce antimicrobial packaging. For instance, the inclusion of *Nigella arvensis* extract into chitosan/OMMT nanocomposite determined antibacterial properties, close to that of the gentamicin, against Gram-negative and Gram-positive bacteria (İlk, Şener, Vural, & Serçe, 2018).

The biopolymer used to prepare the bio-composite can be antimicrobial itself as in the case of chitosan. The widely accepted mode of action is the interaction with negatively charged surface components of microorganisms, causing extensive alterations to the cell surface, and leading to leakage of intracellular substances that results in cell death (Irastorza, Zarandona, Andonegi, Guerrero, & de la Caba, 2021). The composite polycaprolactone/MMT/chitosan using glycerol monooleate as additive showed antibacterial activity against *E. coli*, *P. aeruginosa* and *C. albicans*. Pristine chitosan films inhibited only the growth of *E. coli* (Cesur et al., 2018).

The antibacterial and antifungal activity of the CBHs or the bio-composite films can be modelled following different approaches. For instance, heat flow curves of the metabolic activity of *P. fluorescens* IMA 19/5, exposed to HAL/salicylic acid (20 mM) hybrid, showed a single exponential growth phase followed by a single exponential decay (Ghezzi et al., 2018). The radial growth of fungi in contact with starch/HAL/oregano essential oil films can be modelled through a modified Gompertz model. The comparison of Gompertz parameters allowed defining

Fusarium spp. strains as the most resistant, followed by *Rizophus* spp., and finally *Aspergillus* spp. strains (Aguilar-Sánchez et al., 2019).

In some cases, the antimicrobial activity of the bio-composite with the CBHs is comparable to that of the pristine film (Iamareerat et al., 2018; Rodriguez et al., 2019). However, de Souza, dos Santos, da Silva Torin, and dos Santos Rosa (2020) demonstrated higher antibacterial activity of the biopolymer with the inclusion of the CBHs than that displayed by the antimicrobial compound into the polymer, as revealed for the TPS/MMT/carvacrol film against *E. coli*.

As far as the anti-biofilm activity of these bio-composites is concerned, limited results are available for their application in the food sector. Ambrogi et al. (2017) developed a chitosan/MMT/chlorhexidine composite to prevent microbial colonization in wounds. The film showed good anti-biofilm activity against *S. aureus* and *P. aeruginosa* strains, independently by the concentration of chlorhexidine loaded into the film, suggesting a possible anti-biofilm activity of the chitosan/MMT film. This activity could be the result of the interference with the cell-cell communication mechanisms (quorum sensing), as reported for the cellulose/OMMT composite material (Demircan, Ilk, & Zhang, 2017). The use of bio-composites able to reduce the biofilm development on food packaging materials is expected to gain attention in the future. Therefore, further research on this topic will be appreciated by the scientific community.

5.2 Antioxidant activity of bio-composites

The antioxidant activity of bio-composite materials including clay hybrids is mainly due to the scavenging activity displayed by specific natural compounds, such as EOCs or plant polyphenols. As reported by Alexandre et al. (2016), the composite gelatin/MMT/ginger essential oil showed a 2,2-diphenyl-1-picrylhy-drazyl (DPPH) scavenging activity of 0.35 ± 0.12 μmol Trolox equiv/g of dried film.

High antioxidant activity was found for the PHB/TPS/MMT/eugenol film, which showed 92% of radical scavenging activity in DPPH assay, close to 95% displayed by 3 mM ascorbic acid as positive control (Garrido-Miranda et al., 2018). Conversely, a lower radical scavenging activity (41 %) using DPPH assay was detected for the pectin/HNT/cucurbituril/peppermint essential oil (Biddeci et al., 2016).

A bio-hybrid material containing porous starch, HNT and the antioxidant fucoxanthin was developed by Oliyaei, Moosavi-Nasab, Tamaddon and Fazaeli (2020). The heat and light sensitive fucoxanthin showed a gradual release during time. Its inclusion in HNT and porous starch showed high retention rate following exposure to light or 50 °C during 4 weeks of storage (Oliyaei et al., 2020). These results could lead to the development of bio-composite films as antioxidant releasing systems. Alginate/sepiolite/myrtle berries extract films showed higher antioxidant activity, as revealed by ABTS assay, than pristine films and with long stability over one year of storage (Cheikha, Martín-Sampedro, Majdouba, & Darder, 2020). The chitosan/HNT/tea polyphenol film showed appreciable antioxidant activity (DPPH radical scavenging activity of 75%) but, with the increase of HNT content the antioxidant activity decreased. The authors postulated that this phenomenon was determined by the aggregation of HNT particles which, in turns, produced cracks on the surface of the film and the loss of tea polyphenols into the chitosan matrix (Wang et al., 2020).

Recently, Muràth et al. (2020) characterized the antioxidant activity of LDH/ellagic acid hybrids treated with different aqueous miscible organic solvents (AMOS). Among different AMOS, ethanol treatment was found to enhance the antioxidant activity of LDH/ellagic acid, as revealed by DPPH and cupric reducing antioxidant capacity (CuPRAC) assays. On the basis of these results, authors postulated that this hybrid could be used in bio-composite in contact with foods sensitive to lipid oxidation.

5.3 Bio-composites for food preservation and food safety

Several applications of bio-composite materials, reinforced with clays, and including antimicrobial and antioxidant compounds have been explored for foods of both vegetable and animal origin.

Poultry meat wrapped in chitosan/MMT-Na/rosemary essential oil film showed a reduction of 3 log cfu/g in total mesophilic aerobic bacteria load, and of 2.3 log MPN/g in coliforms compared to unwrapped meat (Pires et al., 2018). As regards the lipid oxidation, starting from day 7 and until day 15 of cold storage, the malonaldehyde content of the meat samples wrapped in active film was significantly ($P \leq 0.05$) lower than that detected in unwrapped samples. These positive effects were attributed to the Fe-chelating activity of chitosan, the oxygen barrier effect of the film, and the antioxidant activity of the essential oil (Pires et al., 2018). Later, the same research group found that this composite delayed the meat discolouration of poultry meat, and the increase of pH values (Souza et al., 2019). Pork meatballs packed in cassava starch/bentonite/cinnamon essential oil film showed total bacterial count at 25 °C below the acceptable level recommended by the FDA (10^6 cfu/g) until 96 h, whereas this limit was exceeded in meat samples packed in plastic bags or starch film (Iamareerat et al., 2018).

Bio-composite films were also evaluated to extend the shelf-life of fish products (Alboofetileh, Rezaei, Hosseini, & Abdollahi, 2016; Echeverría, López-Caballero, Gómez-Guillén, Mauri, & Montero, 2018; Dias et al., 2019). In particular, rainbow trout samples wrapped in alginate/MMT/majoran essential oil film and stored for 15 days at 4 °C, showed a reduction of 1 log cfu/g in inoculated *L. monocytogenes* load, and a total volatile base nitrogen (TVB-N) content never exceeding the limit of acceptability of 25 mg N/100 g flesh (Alboofetileh et al., 2016). Tuna fillets covered with the bio-composite soy protein/MMT/clove essential oil showed a significant ($P \leq 0.05$) reduction of *Pseudomonas* spp. load, reduced malonaldehyde content, and low triglycerides degradation and ketones accumulation in comparison to fillets covered with the polyethylene film (Echeverría et al., 2018). The protection towards lipid oxidation was also found by Dias et al. (2018) in salmon wrapped in chitosan/MMT/α-tocopherol film, showing low levels of malonaldehyde (acceptable level of 2 mg/Kg) until the day 6 of cold storage (Dias et al., 2018).

Bio-composite films are also used to extend the shelf-life of fruits and vegetables. As regards strawberries, TPS/MMT/EOCs film caused 50% reduction of *B. cinerea* mycelial growth, with a IC_{50} value of 5.9 g kg^{-1} for the thymol-carvacrol mixture (Campos-Requena et al.,2017), as well as carboxymethyl cellulose/MMT/ε-poly-L(lysine) coating material inhibited mould development up to 7 days at room temperature (He et al., 2019)

Blood oranges coated with carnauba wax/MMT, with or without the addition of orange peel essential oil, showed higher juice antioxidant activity and higher content of vitamin C after 100 days of storage, than fruit coated with wax without MMT addition (Nasirifar et al., 2017). The bio-composite films can be used to preserve quality parameters of fresh-cut fruits. As reported by Azevedo et al. (2018), fresh-cut apples packed in whey protein isolate/MMT/citric acid showed strong reduction of the enzymatic browning and maintained colour characteristics during cold storage. This effect was induced by the anti-browning effect of citric acid and the improvement of gas barrier properties after MMT addition (Azevedo et al., 2018). Recently, the application of Konjac glucomannan/carrageenan/nano-silica films during cold storage of white mushrooms reduced the browning index, and delayed the weight loss and softening (Zhang, Wang, Wang, & Cheng, 2019). Authors proposed a possible explanation for these results, considering the silicon–oxygen bond in the composite film that could affect the absorption, dissolution, diffusion, and release of carbon dioxide and oxygen, inhibiting the respiration and ethanol evolution (Zhang et al., 2019).

5.4 Bio-composites as intelligent packaging systems

To date, few reports related to the development of intelligent biopolymer-clay composites have been published. For this reason, the research related to the development of intelligent bio-composites using clay as reinforcing agents deserves further investigation.

In a recent work, Asdagh and Pirsa (2020) developed a pectin/nanoclay composite film, loaded with *Carum copticum* essential oils and β-carotene, to protect butter from oxidation. In this composite film, β-carotene changed the film color from orange to yellow in response to oxidizing agents, providing a visual indicator of butter quality (Asdagh & Pirsa, 2020). A pectin/nanoclay/methylene blue film was also developed for the measurement of vitamin C (accuracy over 90%) in kiwi, orange and tangerine juice. The bio-composite film colour changes showed high sensitivity and selectivity to ascorbic acid concentration (Pirsa, 2020).

The development of bio-composite films including clays and natural pH-sensing vegetable extracts attracted great attention during recent years. The colour changes of the packaging, associated with pH value variations, could be associated with frauds, noncompliance of the cold chain, or freshness of the food and can be used to develop time-temperature indicator (TTI) systems (Pereira, de Arruda, & Stefani, 2015). An early work of Gutiérrez, Ponce and Alvarez (2017) showed the production of pH-sensitive nanoclay hybrids. In particular, natural and modified MMTs were added with blueberry extract, rich in pH-sensitive anthocyanins, to produce a hybrid packaging with antioxidant activity and intelligent behaviour (Gutiérrez et al., 2017). Unfortunately, the inclusion of these hybrids into TPS matrix exposed the blueberry anthocyanins to high-temperature extrusion conditions, losing the pH-sensitive properties in the composite films (Gutiérrez & Alvarez, 2018). The same research group developed a TPS/MMT/Jamaica flower extract film (JFE), using natural or modified MMT/JFE pH-sensitive clays. The addition of the fillers produced stronger H-bonding interactions between JFE and the TPS matrix, improving the hydrophilic properties of the surfaces (Gutiérrez et al., 2019).

Another work described the production of a chitosan/PVA/bentonite/black carrot anthocyanins film, in which, the addition of clay hybrids improved the thermal stability of the pH-sensing material (Koosha & Hamedi, 2019). These results highlight that novel processing conditions should be developed to preserve the pH response of clay hybrids, or to confer other intelligent properties to these films.

Other intelligent systems are the optical oxygen sensors (OOSs), a class of oxygen sensors based on luminescence quenching (Cruz-Romero, Santovito, Kerry, & Papkovsky, 2019). The most used luminescent probes are metal porphyrin dyes that can be adsorbed onto clay minerals in order to overcome porphyrin leaching problems, increase thermal stability, and prevent porphyrin aggregation. In particular, Čeklovský and Takagi (2013) successfully loaded palladium porphyrins onto the synthetic clay mineral Sumecton SA, showing good sensitivity of the hybrid material under aerobic conditions. A porphyrin-LDH/poly (butylene succinate) composite film with photoactive properties was also developed (Káfuňková et al., 2010). Overall, these results could support the development of biopolymer-based porphyrin-clay composite films with optical oxygen-sensing properties. These intelligent films could be used for the packaging of foods sensitive to oxidation processes (e.g., fat foods, fresh meat).
In addition to biopolymers, the hydrogel matrix could be also used as sensing material for food safety purposes. Indeed, Feng et al. (2020) developed a thermosensitive hydrogel combining starch, alginate, poly-(N-isopropylacrylamide), and kaolin. The thermosensitive hydrogel was able to release non-toxigenic *Aspergillus flavus* spores to be used as biocontrol agent against peanut aflatoxin contamination. This innovative hydrogel could be applied in the future during grain storage to reduce mycotoxins contamination.

6. Regulation and safety issues

This section aims to describe the current regulation on bio-composite materials, and emerging food safety issues related to the use of clay hybrids and/or biopolymers. The bio-composites herein described consist of a biopolymer including natural or synthetic clay, eventually loaded with a bioactive molecule. Since several clays belong to the nanomaterials class, safety issues might arise with their use. In this context, the use of bio-composite materials for food packaging is covered by regulations on nanomaterials. However, different definitions of nanomaterials are available based on different regulatory frameworks. Moreover, the European Union (EU) and

USA follow different approaches for the approval of nanomaterials in the food packaging sector. The EU definition of nanomaterial has been established by the Commission recommendation of 18 October 2011 (European Commission, 2011b). In addition, engineered nanomaterials are considered novel foods and, in this case, they are covered by the Regulation (EU) 2015/2283 (European Commission, 2015). Nanomaterials, defined as material that has one or more dimensions of the order of 100 nm or less, used in food contact materials must be explicitly authorized on the basis of the "Regulation on plastic materials and articles intended to come into contact with food" (European Commission, 2011a). In the Annex I of this Regulation, the additives (e.g., clays) or polymers admitted for the production of plastic materials in contact with food are reported. For instance, bentonite, hydrotalcite, eugenol, tannic acid, sorbic acid, pectin, Arabic gum, beeswax, cellulose, starch, alginate, gelatin, and casein are included in this list. Specific provisions for nanomaterials are also required under the Regulation (EU) No 528/2012 related to biocidal products (European Commission, 2012).

The specific definitions differ in the size, the material structure, the material source and characteristic properties. Several recommendations for the nanomaterial regulation update were proposed: the nanomaterial definitions should be clarified by avoiding ill-defined terms and by including clear thresholds, the 50% by number threshold should be replaced by a threshold of 1% by weight to make the definitions workable with current particle analysis methods. Furthermore, nano-specific regulations require adaptation and harmonization, and the product manufacturers should be responsible for the nanomaterials' origin (Miernicki, Hofmann, Eisenberger, von der Kammer, & Praetorius, 2019). According to the EU regulations, nanomaterials in contact with food must also be indicated in the food label (Rauscher, Rasmussen, & Sokull-Klüttgen, 2017). On the basis of novel food regulation and biocidal products regulation reported above, a specific risk assessment of nanomaterials is required.

On the contrary, the FDA approach does not consider the nanosize of the material, but only its chemical and biological nature. Therefore, if a substance has already been approved for the use as food packaging in its bulk form, it can also be used in its nanoform (Fatyeyeva et al., 2017). Food safety issues related to nanomaterial packaging can be the release of nanoparticles, metals, or the toxicity of the material. Bott and Franz (2019) exposed nanocomposites materials to thermal, chemical and mechanical stress followed by mechanical abrasion of their surface. They observed that even under dynamic stress conditions, Laponite®, with high potential to be released from a polymer matrix, is not release in its nano-form. As recently reviewed by Bandyopadhyay and Ray (2019), the migration of LDH or MMT from PLA-based films ranges from 0.1 to 32 mg dm^{-2}, depending on the material and the simulant used in the migration test.

The soy protein isolate/MMT/clove essential oil film subjected to solubility test in water showed a great release of Si, dependent by the MMT concentration, without an increase of Si content in tuna fillets packed with this film (Echeverría, López-Caballero, Gómez-Guillén, Mauri, & Montero, 2016). The inclusion of clove essential oil hindered the release of Si from the films, suggesting the interaction between MMT, protein, and the EO (Echeverría et al., 2018).

On the contrary, salmon fillets packed with a chitosan/MMT/α-tocopherol film showed Mg content (up to 1.6 mg/100 g salmon) higher than that detected in salmon samples not exposed to MMT (Dias et al., 2018).

As regards the toxicological evaluation of nanomaterials reinforced with clays, the *in vitro* toxicity of clay minerals is evaluated through basal cytotoxicity assays using human or rodents cell lines. Common toxicity responses are the oxidative stress generation, the genotoxicity, the inflammation, or the cell death. Several clays (LDH and MMT) showed *in vitro* cytotoxic effects, but not mutagenic activities. However, PLA/clay composites were found to be safe for use in food packaging (Bandyopadhyay & Ray, 2019). *In vivo* studies are limited in comparison to *in vitro* studies but, generally, they showed no toxic effects of the nanocomposite material. In addition, clays such as MMT showed low systemic toxicity in human and animals, being suitable for inclusion in food packaging materials (Pires et al., 2021). However, a case-by-case toxicological evaluation of nanomaterials in contact with food is suggested (for a review see Maisanaba et al., 2015).

Recently, Saha et al. (2018) reported that cellulose acetate butyrate/MMT film is compatible with human blood up to the concentration of 20 mg/mL, not affecting plasma lactate dehydrogenase concentration, an important marker of cytotoxicity, and plasma free haemoglobin. Overall, current regulation on nanomaterials and data on food safety risks highlight the need for a global harmonization of rules and definitions as well as the development of specific risk assessment plans on the basis of the packaging material, food type, and storage conditions.

7. Conclusions

In conclusion, the development of bio-composite films using hydrocolloids as biopolymer, clays as reinforcement agents and natural antimicrobials and antioxidants, offers the possibility to obtain active biodegradable and, sometimes, compostable packaging. These bio-composite films may be able to exhibit improved barrier, mechanical, thermal, optical, and release properties. These characteristics are the result of the molecular interactions among biopolymer matrix, clay surface and target molecule loaded into clay hybrids. Furthermore, these clays can be suitably modified with specific compounds endowed of antimicrobial or antioxidant activity. In this scenario, the replacement of chemical preservatives with natural additives is perceived to be safer from the consumer. Therefore, the use of bio-based active and/or intelligent packaging, also including specific biosensors, represents a sustainable approach to extend the shelf-life of food products and to improve the food safety. The research on the production of hydrogel-based bio-composites for the food sector is expected to continue towards films with multiple and novel functionalities (e.g., pH, oxidative damage and spoilage-sensitive packaging systems or able to release bioactive compounds). Further research on risk assessment issues should be promoted in order to sustain harmonic regulation on bio-composite materials.

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Figure captions

Figure 1. Biopolymers, clays, and organic compounds used for bio-composite films preparation.

Figure 2. Train-loop-tail conformation of polymer adsorbed onto the clay surface.