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Active bionanocomposite films

Antibacterial activity against *L. monocytogenes*

<table>
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<th>Elemental atomic composition of clays (%)</th>
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Antibacterial activity of gelatin / copper (II)-exchanged montmorillonite films

Josefa F. Martucci & Roxana A. Ruseckaite*

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ABSTRACT

Cu (II) - exchanged montmorillonite (Cu\textsuperscript{2+}MMt) was prepared, characterized and introduced into a bovine gelatin (Ge) matrix via a dissolution-intercalation method to get antibacterial nanocomposite films. The maximum amount of exchanged cation did not exceed the cation exchange capacity of the pristine montmorillonite (Na\textsuperscript{+}MMt), as assessed by energy dispersive X-ray (EDX) spectroscopy. Cu\textsuperscript{2+}MMt showed antibacterial activity in vitro against *Escherichia coli O157:H7* (Gram-negative) and *Listeria monocytogenes* (Gram-positive) as revealed by the agar disc-diffusion assay.

The dispersion of clays in Ge films was monitored by X-ray diffraction (XRD) and Scanning Electron Microscopy (SEM). Blending gelatin with 5 % w/w of clay increased the tensile strength of the nanocomposite films in around 280 % while the elongation at break and the water vapor permeability decreased in about 42 and 30 %, respectively, regardless of the cation in clay. The Ge/Cu\textsuperscript{2+}MMt film exhibited antibacterial effectiveness against both pathogens tested under the same conditions, demonstrating a stronger effect on *L. monocytogenes* than on *E. coli O157:H7*, since the cell wall of the latter differs significantly and such difference could influence their vulnerability and response to the active films. Therefore, the incorporation of low clay levels as a vehicle for copper ions into gelatin matrix has demonstrated to be a good method for developing functional materials that can be potentially applied to the design of food contact items.

*Keywords*: Bovine gelatin; Cupric ions; Montmorillonite; Nanocomposite; Active film; Antimicrobial activity.
1. Introduction

The inclusion of long-lasting biocide agents in natural polymer matrices is acting as a driving force for the development of new environmentally sound packaging concepts that extend shelf-life, while maintaining the food safety and quality of packed food (Rhim et al. 2013).

Certain naturally occurring metal ions such as copper, silver, zinc, palladium, and titanium, which, in some cases, are essential minerals, are active antimicrobials against a very broad spectrum of bacteria, yeasts and fungi with no adverse effects on eukaryotic cells (Llorens et al. 2012). CuSO$_4$ and Cu(OH)$_2$ have been widely applied to animal production as traditional inorganic antibacterial materials (Hu et al. 2005). However, the direct inclusion of Cu$^{2+}$ in polymer formulations has been limited by uncontrolled leaching. One way to prevent early burst is by immobilizing Cu$^{2+}$ ions onto inorganic carriers, including zeolites (Drellich et al. 2011) and clay minerals (Mosser et al. 1997; He et al. 2001; Zhou et al. 2004; Tong et al. 2005; Hu et al. 2005; Hu & Xia 2006). Montmorillonite (MMt) is a hydrophilic and highly water dispersible 2:1 layered aluminium phyllosilicate with good adsorption ability, high cation-exchange capacity, and drug-carrying capability (Xia et al. 2010) combined with other favorable features such as high surface area and chemical inertness (Drellich et al. 2011). The negatively charged interlayer regions of MMt are mainly filled with exchangeable positively charged ions, such as Na$^+$, K$^+$, Ca$^{2+}$, etc., thus, active Cu$^{2+}$ ions can be accommodated in the interlayer space, providing materials with a long-lasting action period (He et al. 2001; Hu et al. 2005; Hu & Xia 2006). The immobilization of Cu$^{2+}$ onto MMt, together with its antimicrobial action, has been extensively documented (Mosser et al. 1997; He et al. 2001; Zhou et al. 2004; Tong et al. 2005; Kloprogge et al. 2006; Hu et al. 2005; Hu and Xia 2006; Malachovà et al. 2009, 2011; Pereira et al. 2013). Even so, scant
literature explores the antimicrobial activity of Cu\textsuperscript{2+}MMt-polymer nanocomposites. Bruna and others (2012) developed low density polyethylene (LDPE)/Cu\textsuperscript{2+}MMt films and reported a reduction of 94\% of *Escherichia coli* O157:H7 colonies at 4\% w/w nano-clay loading. Similarly, cellulose acetate (CA)/Cu\textsuperscript{2+}MMt (3\% w/w clay) films yielded high levels (>98\%) of inhibitory action against *Escherichia coli* ATCC 25922 (Bruna et al. 2014), whereas poly(lactic acid) (PLA)/Cu\textsuperscript{2+}MMt films (Bruna et al. 2015) were effective at reducing up to 99\% of *Escherichia coli* ATCC 25922 and *Listeria innocua* ATCC 33090, when 5\% w/w Cu\textsuperscript{2+}MMt was added to each matrix. To the best of the author’s knowledge, there are no studies dealing with the potential of protein/Cu\textsuperscript{2+}MMt nanocomposites used as active food contact materials. Amongst proteins, gelatin (Ge) is a water-soluble animal protein, obtained from the hydrolysis of bone-collagen or connective tissues. It can be found as abundant waste/by product in slaughter houses, and poultry and fish industries at reasonable cost (Hernandez-Muñoz et al. 2004). Gelatin can be taken as a biogenic alternative to active films for being classified as a “Generally Recognized as Safe” (GRAS) substance in the food additive list by the U.S. Food and Drug Administration (FDA); also due to its biodegradability, excellent film-forming ability, high oxygen barrier and satisfactory mechanical properties at low or intermediate relative humidity (Hernandez-Muñoz et al. 2004; Martucci et al. 2012). Nonetheless, the limited water resistance and mechanical strength of gelatin films in moist environments still pose a problem to their wide application. In earlier studies, the authors successfully demonstrated that blending gelatin with sodium montmorillonite (Na\textsuperscript{+}MMt) could enhance barrier, mechanical and moisture resistance properties of films (Martucci et al. 2007; Martucci & Ruseckaite 2010), while preserving their eco-friendliness (Martucci & Ruseckaite 2009). The best results were obtained from films amended with 5\% w/w of Na\textsuperscript{+}MMt, presenting the highest tensile
strength and Young's modulus, and the lowest WVP and hydrophilic surface (Martucci & Ruseckaite 2010). This work presents the synthesis and characterization of Cu<sup>2+</sup>MMt by acid-activated MMt through ion-exchange procedure, and the effect of incorporating 5 % w/w of modified clay on the physical properties and antimicrobial potential of nanocomposite gelatin films against *E. coli* and *L. monocytogenes*, as a model of the pathogens commonly found in foodstuffs.

2. EXPERIMENTAL SECTION

2.1. Chemicals and source of bacteria

Bovine hide gelatin (Ge) type B (Bloom strength 150, isoionic point (Ip) 5.3) was kindly supplied by Rousselot (Buenos Aires, Argentina) and used with no further treatment. Sodium montmorillonite (named as Na<sup>+</sup>MMt) was obtained from Southern Clay Products Inc. (Texas, USA) under the trade name Cloisite Na<sup>+</sup>. The cation-exchange capacity (CEC) was 92.6 meq/100 g of clay and the interlayer distance was 1.17 nm (as it was determined by X-ray diffraction on dry powder). Glycerol (Gly, 98 % reagent grade) and cupric sulphate pentahydrated (CuSO<sub>4</sub>.5H<sub>2</sub>O, 99.99 % purity) were purchased from DEM (Mar del Plata, Argentina) and Anedra (Buenos Aires, Argentina), respectively. All the other chemicals used were of analytical grade and brought from Aldrich (St. Louis, MO, USA). Food-borne pathogens were selected to assess the antibacterial properties: *Escherichia coli* O157:H7 ATCC 32158 (ATCC, American Type Culture Collection) and *Listeria monocytogenes* ATCC 25923. Both strains were plated onto eosin-methylene blue agar (EMB) and Baird Parker agar, respectively (Martucci et al. 2015). The vegetative cells of each microorganism were streaked on Mueller Hinton agar and incubated at 37 ± 0.5 °C for 24 h. Microbial broth was then suspended in double distilled sterile water. The density of bacteria suspension
was adjusted until the visible turbidity was equal to 0.5 McFarland standards before testing.

2.2. Preparation of modified MMt

Cu$^{2+}$MMt was obtained from acid-activated MMt (H$^+$MMt) by ion exchange according to the procedure described by Hu & Xia (2006) with minor modifications. H$^+$MMt was produced by suspending 10 g of Na$^+$MMt in 75 mL of 0.05 M HCl solution. This dispersion was kept for 24 h under constant stirring (400 rpm) at room temperature in a hot plate (Cole Palmer, USA) and then centrifuged (Sartorius type 4-15, Germany) at 5000 rpm for 5 min. The recovered sediment was washed with bi-distilled water until no acid in the supernatant was detected, and then dried at 80 °C overnight in an air-circulating oven (Memmert UFE550, Germany). The dry product was pulverized to an average size of less than 300 mesh sieve. Cu$^{2+}$MMt was produced by dispersing 5 g of dry H$^+$MMt in 100 mL of a 0.05 M CuSO$_4$·5H$_2$O solution under gentle stirring (400 rpm) at 60 °C for 6 h. Afterwards the sample was submitted to the same purification protocol than its acid-activated counterpart.

2.3. Film forming process

Gelatin films added with clay (i.e., Na$^+$MMt, H$^+$MMt and Cu$^{2+}$MMt; 5 % w/w dry gelatin basis) and plasticized with glycerol (30 % w/w dry gelatin basis) were prepared by the solution–intercalation method based on early works by the group (Martucci et al. 2007; Martucci & Ruseckaite 2009, 2010). Plasticizer and clay contents were fixed on the basis of previous studies (Martucci & Ruseckaite 2008, 2010). Formulations with glycerol content lower than 30 % w/w resulted in films behaving similarly to their unplasticized counterparts, while the incorporation of a glycerol level higher than 30 % w/w induced plasticizer segregation and migration (Martucci & Ruseckaite 2008). In the case of clay, gelatin nanocomposites containing 5 % w/w of Na$^+$MMt displayed the best
A set of thermal, mechanical, barrier and optical properties (Martucci & Ruseckaite 2008, 2010), so this percentage was selected for synthesis of the antibacterial nanocomposite films. All films were preconditioned at 25 ± 2 ºC and 65 ± 2 % RH for 48 h in an environmental chamber before further experimental analysis. Films were designated as Ge/Na\textsuperscript{+}MMt, Ge/H\textsuperscript{+}MMt and Ge/Cu\textsuperscript{2+}MMt, respectively, depending on the clay used.

2.4. Characterization

2.4.1. X-ray diffraction (XRD). XRD patterns were recorded at room temperature on a PANalytical X’Pert Pro diffractometer (Almelo, The Netherlands) equipped with a Cu K\textsubscript{α} radiation source (λ = 0.1546 nm) at a generator voltage of 45 kV and 30 mA as the applied current. The incidence angle ranged from 5º to 50º at a scanning rate of 1 º/min. The interlayer spaces were calculated by the Bragg equation.

2.4.2. Fourier Transform Infrared Spectroscopy (FTIR). FTIR analyses were performed on a Mattson Genesis II spectrophotometer in transmission mode. The measurements were recorded between 4000–400 cm\(^{-1}\) at 32 scans. Pulverized specimens were pressed into pellets with KBr. The background noise was corrected with pure KBr data.

2.4.3. Energy dispersive X-ray spectroscopy (EDX). EDX was used to assess the presence of copper in MMt samples by using an spectrometer EMAX (Horiba Co. Ltd., Wycombe, U.K.) operated at Vacc ¼ 15 kV.

2.4.4. Thickness. Film thickness was measured by a hand-held micrometer (Dial Thickness gauge 7301, Mitutoyo Corporation, Kanagawa, Japan) with an accuracy of 0.01 mm. Measurements were taken at ten random locations from three films of each formulation, and the mean thickness values were used to calculate the physical properties.
2.4.5. Visible light–barrier properties. The light absorption of nanocomposite films was measured in a wavelength ranging from 400 to 800 nm, using a UV-visible spectrophotometer Shimadzu 1601 PC (Tokyo, Japan) according to a method described elsewhere (Irissin-Mangata et al. 2001). Each film specimen was cut in rectangular strips and placed directly in the spectrophotometer test cell. Air was used as reference. Film opacity was expressed as the area under the absorption curve (arbitrary units/nm) per thickness unit (mm). Reported values are the average of five measurements.

2.4.6. Equilibrium moisture content (MC). The squared-shape strips of each film sample (dimensions 4 cm$^2$) were weighed in an analytical balance (±0.0001 g; Ohaus, USA) to determine the initial mass. Then samples were dried in an air circulating oven (Memmert, Germany) at 105 °C for 24 h according to the procedure reported in the ASTM D644-94, 1994. The equilibrium moisture content (MC) was expressed as the percentage of initial film weight lost during drying. Reported values are the average of three replicates.

2.4.7. Water vapor permeability (WVP). Water vapor permeability (WVP) was performed gravimetrically at 25 °C, following the ASTM E96-95 desiccant method. All specimens were equilibrated at 65 ± 2 % RH at 25 ± 2 °C for 48 h. Afterwards, test films were fixed onto opening cells containing silica gel (0 % RH), and the cells were placed in a controlled humidity chamber at 65 ± 2 % RH and 25 ± 2 °C. The air gap inside the cell was ~1.2 cm and the film area exposed for water vapor transmission was 13.8 cm$^2$. The cells were weighed on an hourly basis over a 10 h period. WVP was calculated from the following equation:

$$WVP(Kg \cdot m \cdot s^{-1} \cdot Pa^{-1} \cdot m^{-2}) = \frac{W}{A \Delta P} e$$  \hspace{1cm} (1)
where \( w \) is the weight gain of the cup (Kg) at time \( t \) (s); \( e \) is the film thickness (m); \( A \) is the film exposed area (m\(^2\)); \( \Delta P \) is the vapor pressure difference across the film (Pa). All measurements were taken in quadruplicate.

### 2.4.8. Tensile properties

The tensile strength (TS) and percentage of elongation at break (\( \varepsilon\% \)) were measured according to the ASTM 638 94 D standard using an Instron 4467 Universal Testing Machine (Buckinghamshire, England) with a 5 kN load cell at a crosshead speed of 10 mm/min. Reported results were obtained from at least 10 samples for each type of film.

### 2.4.9. Testing of antimicrobial activity

The \textit{in vitro} antibacterial activity of films and clays was assessed following our previous work (Martucci et al. 2015) using agar disc-diffusion assay. Test bacteria (100 \( \mu \)L of inoculums containing approximately \( 10^5 - 10^6 \) CFU/mL of each bacterium) were plated onto Mueller Hinton (Merck, Darmstadt, Germany) agar medium. Discs (10 mm in diameter) were cut from the films with a circular knife and placed onto the inoculated plates. The antimicrobial activity of clay specimens was assessed in a similar way. Each clay (3 mg), was dispersed in 1 mL of bi-distilled water and submitted to an ultrasonic bath (Testlab, 160 W, 40 KHz) for 20 min. Then 30\( \mu \)L of the obtained suspension was poured into agar wells (5 mm diameter). All plates were incubated at 37 \(^\circ\)C for 24 h. The diameter of the inhibition zone surrounding the film discs or wells (in the case of clays) was measured with a manual caliper (Mitutoyo, Japan) from the center of the film. The antimicrobial activity of clay specimens was assessed in a similar way. The result was determined as the mean of three separate experimental runs.

### 2.4.10. \( \text{Cu}^{2+} \) ion desorption studies

\( \text{Cu}^{2+}\text{MMt} \) (0.1 g) was extensively washed with bi-distilled water under stirring for 24 h. The resulting dispersion was centrifuged at 8000 rpm (Sartorius type4-15, Germany) for 10 min. The concentration of the cupric cation
left in the washed Cu\textsuperscript{2+}MMt was determined by EDX. The \textit{in vitro} antibacterial activity of the washed clay was qualitatively measured with the agar disc-diffusion method, as previously described for clays.

2.5. Statistical analysis

Experimental data were statistically analyzed by the one-way analysis of variance (ANOVA) using the Origin Pro 8 software and Turkey’s test for comparison of means at a 5% significance level. All the results are expressed as the mean ± standard deviation.

3. RESULTS AND DISCUSSION

3.1. Modified clay characterization

X-ray diffraction patterns of pristine and modified clay were used to determine the variations in the basal \(d_{001}\)-spacing due to cation switching (Figure 1). Na\textsuperscript{+}MMt exhibited a diffraction peak at \(2\theta = 7.3^\circ\) (1.21 nm, according to Bragg eq.) corresponding to the basal interlayer \(d_{001}\)-spacing (Mosser et al. 1997). Upon acid activation, this reflection slightly shifted to lower angles corresponding to an interlayer distance of 1.25 nm (Figure 1), in line with the exchange of Na\textsuperscript{+} for H\textsuperscript{+} with larger ionic radius (ca. H\textsuperscript{+} hydrated: 0.900 nm vs. Na\textsuperscript{+} hydrated: 0.450 nm). The slight difference in the basal reflection of montmorillonite caused by switching Na\textsuperscript{+} for H\textsuperscript{+} (Figure 1), suggests that acid activation had a minor effect on the layered structure (Zhao et al. 2013). After treating H\textsuperscript{+}MMt with CuSO\textsubscript{4}, the \(d_{001}\)-spacing increased up to 1.30 nm (corresponding to \(2\theta = 6.8^\circ\)) confirming the intercalation of Cu\textsuperscript{2+}. The small increment can be ascribed to the disparity between the ionic radius of the hydrated forms of Cu\textsuperscript{2+} (i.e., hexaaqua) and Na\textsuperscript{+} cations (He et al. 2001; Tanaka et al. 2007). A small new reflection also appeared at around \(2\theta = 13^\circ\) in the diffraction patterns of Cu\textsuperscript{2+}MMt, probably attributed to an amorphous cupric hydroxide such as Cu(OH)\textsubscript{2}·H\textsubscript{2}O, as already
accounted for by others (Zhou et al. 2004). The increment in d-spacing of MMt due to
Cu$^{2+}$ ion exchange was previously observed, but values might vary due to differences in
the composition of the raw clay and the treatment performed on it (Zhou et al. 2004; Hu

EDX data further supported the presence of Cu$^{2+}$ ions in the clay (Figure 2 c). Na$^+$MMT
was distinguished by the presence of a peak at 0.05 eV/K in EDX spectrum assigned to
Na$^+$ which was absent in H$^+$MMt and Cu$^{2+}$MMt spectra (Figures 2 a, b and c,
respectively). The occurrence of a new peak at 8 eV/K in Cu$^{2+}$MMt spectrum (Figure 2 c)
is a strong experimental evidence of copper exchange (Bagchi et al. 2013; Das et al.
2013). Since the intensity of such peak is proportional to the element concentration, the
loading of Cu$^{2+}$ cation onto MMt was estimated in about 3 % (on element basis) (Figure
2 c).

The effect of the cation exchange on the clay structure was analyzed by FTIR (Figure
3). All the spectra exhibited relevant absorption bands at 3631 cm$^{-1}$ (stretching vibration
of structural OH group (Al-OH)), 3432 and 1631 cm$^{-1}$ (stretching and bending
vibrations of interlayer H$_2$O, respectively), 1045 cm$^{-1}$ (stretching vibration of Si-O), and
915-18 cm$^{-1}$ (Al-Al-OH bending vibration) characteristic of clay structure (Zhou et al.
2004; Zhao et al. 2013; Pereira et al. 2013). The absorption feature of Na$^+$MMT
remained unchanged after acid activation, thereby suggesting that the aluminum cations
of montmorillonite seemed not to be leached by the acid treatment, as postulated by
others (Tong et al. 2005). The peak situated at 3631 cm$^{-1}$ was slightly shifted
downwards upon Cu$^{2+}$ exchange, which is explained by the presence of interactions
between cupric ions and clay, primarily in the inter-lamellar space, through
complexation of copper ions as previously indicated by other authors (Bagchi et al.
2013; Pereira et al. 2013). The intensity of the hydration band barely changed due to
differences in the water coordination capacity of Na$^+$ and Cu$^{2+}$ ions. Finally other characteristic vibrations of the tetrahedral sheets, namely Si-O-Si stretching (993 cm$^{-1}$) and Si-O-Al bending (521 cm$^{-1}$), reached higher wave length values and decreased intensity indicating a somewhat disordered MMt structure (Xia et al. 2010).

The inhibitory activity of pristine and modified clay against Gram-negative and Gram-positive bacteria was investigated with the agar disc diffusion method, and results are summarized in Table 1. Both pathogens revealed sensitivity to all clay suspensions, indicating certain Na$^+$MMt antibacterial activity. The ability of Na$^+$MMt, Ca$^{2+}$MMt and H$^+$MMt to reduce the bacterial plate counts of *E. coli* was previously observed by Hu & Xia (2006), though no reports on the inhibitory effect on Gram-positive bacteria have come to light. Cu$^{2+}$MMt, on the other hand, exerted a powerful antibacterial action against both bacteria tested (p<0.05), related to the higher adsorption capacity of this clay (Guo et al. 2011) and the intrinsic antibacterial activity of Cu$^{2+}$ (He et al. 2001; Hu and Xia 2006; Malachová et al. 2011). The presence of Cu$^{2+}$ cations leads to a surplus of positive charge onto the mineral surface. They serve as potential attachment sites for negatively charged cell surface (Stotzky 1980), and result in the appearance of defects in the bacterial outer membrane responsible for the cell permeability, so that cell contents are lost. The higher susceptibility of *L. monocytogenes* to Cu$^{2+}$MMt (Table 1) could be related to differences in the composition and thickness of the outer membrane of Gram-positive and Gram-negative bacteria (Hu et al. 2005; Malachovà et al. 2009).

The cell wall of Gram-positive bacteria is thicker than that of Gram-negative bacteria due to the presence of a thick peptidoglycan layer (20-80 nm) containing phosphate and carboxylic groups. This layer provides a negatively charged site onto the cell wall of Gram-positive bacteria where cations bind. Gram-negative bacteria have a thinner monolayer of peptidoglycan, lipopolysaccharide and phospholipids, phospholipids being
the only main binding site for cations (Vaara 1992). Such differences in the cell wall structures of Gram-positive and Gram-negative bacteria turned *L. monocytogenes* more vulnerable to Cu$^{2+}$MMt.

Cu$^{2+}$MMt slightly reduced its antibacterial activity (about 10 % of the original, Table 1) after extensive washing for 24 h, evidencing that copper is retained onto the clay surface (Hu et al. 2005; Hu & Xia 2006). EDX results also confirmed the high retention rate of copper after washing, copper desorption being below 8 % (data not shown).

3.2. Characterization and comparison of the functional properties of control and Ge/Cu$^{2+}$MMt films

3.2.1. Structural analysis

The diffractogram of the unfilled gelatin film (control) displayed a broad and low intensity peak at 2θ = 6.2-9.5 ° representing the typical amorphous state of gelatin films produced at a temperature higher than the helix-coil transition ($T_{helix-coil} \sim 35$ °C) (Figure 4) (Martucci et al. 2007). The XRD of the nanocomposite films was characterized by the presence of shoulders at 2θ < 7° denoting a certain degree of matrix component intercalation, i.e., gelatin and/or glycerol, into the clay galleries causing widening of the d-space relative to that of the pristine MMt (Figure 4). (Martucci et al. 2007; Rao et al. 2007; Martucci & Ruseckaitė 2010; Farahnaky et al. 2014; Nagarajan et al. 2014).

The FTIR spectra of un-filled Na$^+$MMt and Cu$^{2+}$MMt- incorporated gelatin films (Figure 5) presented characteristic peaks in the amide region at 1631 cm$^{-1}$ (amide I, C=O stretching), 1551 cm$^{-1}$ (amide II, N-H bending), and 1237 cm$^{-1}$ (amide III, C-N and N-H) (Sionkowska et al. 2004; Martucci & Ruseckaite 2010). The addition of clay shifted amide-I, amide-II and amide-III to higher frequency c.a. 1646, 1553, and 1245 cm$^{-1}$, respectively, confirming the occurrence of hydrogen bonding interactions between gelatin and acceptor atoms such as oxygen from free-OH and Si–O–Si groups in MMt,
as documented for other protein-MMt composites (Kumar et al. 2010; Martucci & Ruseckaite 2010).

### 3.2.2. Optical properties

The neat gelatin film was transparent without any color tint, while nanocomposites were less transparent and colored as Ge/Cu$^{2+}$MMt. The light transmission capacity of gelatin films was reduced by clay addition (Table 2, p<0.05), indicating a strong light scattering effect due to clay particles with sizes higher than the wave-length of the visible light (Martucci & Ruseckaite 2010; Shotornivit et al. 2010; Rhim 2013). The sharp decrease in transparency experienced by the Ge/Cu$^{2+}$MMt film should be attributed not only to the scattering explained by some clay structures but also to coloration increase due to the transformation of some cupric ions to cupric oxides during the drying stage of the film manufacturing process, as previously described by Bruna and co-workers (Bruna et al. 2012). The significant differences noticed in the parameter analyzed were not detected when the films’ visual appearance was qualitatively observed, since all specimens remained transparent.

### 3.2.3. Moisture content, water vapor permeability and tensile properties

The average moisture content (MC) of all films remained around 13.8 ± 1.6 g of water/100 g of film (Table 2). The constancy of MC at any cation in MMt suggests that the hydration capacity of cations did not affect the moisture uptake capacity of the nanocomposite films.

The water vapor barrier property of gelatin films was substantially improved (p<0.05, Table 2) by adding 5 % w/w MMt regardless of the cation intercalated, suggesting that switching the cation marginally alters the hydrophilic/hydrophobic balance of the filler. The strong interactions between gelatin and nano-clays (Martucci & Ruseckaite 2010) consume some hydrophilic groups, reducing the water uptake by capillarity at the
interface. The presence of water vapor impermeable silicate platelets or other structures with large aspect ratios dispersed in the polymer matrix also contribute to obstructing and delaying the transmission of water vapor through the matrix, as postulated for other protein nanocomposite films (Farahnaky et al. 2014; Kanmani & Rhim 2014; Nagarajan et al. 2014).

The incorporation of 5 % w/w MMt noticeably improved (p<0.05) TS values as compared to control. The great affinity of biopolymer and nano-clay limiting the molecular mobility of protein chains, together with the uniform dispersion of the nano-reinforcements might lead to an increase in TS (Martucci & Ruseckaite 2010; Farahnaky et al. 2014; Nagarajan et al. 2014). The differences in tensile strength between our nanocomposites and other reported in the literature could be attributed to differences in clay type, matrix source, processing technologies, or a combination thereof. The extensibility decreased significantly (p<0.05) in about 42 % when adding 5 % w/w clay, and no major effects (p>0.05) were detected with cation exchange in MMt (Table 2). The reduction in ε% was previously reported in gelatin-based nanocomposites (Rao et al. 2007; Martucci & Ruseckaite 2010; Kanmani & Rhim 2014; Farahnaky et al. 2014), and has been attributed to the restricted motion of gelatin molecules due to interfacial interactions between gelatin and nano-clay.

3.2.4. Antimicrobial activity

Antibacterial assays (Table 3) indicate that free-MMt gelatin films have shown little antimicrobial activity against both Gram-negative and Gram-positive pathogenic bacteria, probably due to the presence of short chain polypeptides (Minervini et al 2003; Di Bernardini et al. 2010). Clay addition enlarged the diameter of the clearing zone (Table 3) and the activity visibly varied with the cation in MMt and the pathogen tested. The Ge/Cu$^{2+}$MMt film exhibited the highest inhibitory activity, in agreement with the
antimicrobial performance of clays (Table 1). Overall, Ge/Cu$^{2+}$MMt film greatly
inhibited the growth of Gram-positive (*L. monocytogenes*) as compared to Gram-
negative (*E. coli*) pathogens (Table 3). The inhibitory action of Ge/Cu$^{2+}$MMt against
both microorganisms was assumed to be similar to that described above for Cu$^{2+}$MMt:
attachment to clay surface, followed by cell wall damage, loss of cell content and,
eventually microbial death (Hu et al. 2005; Guo et al. 2011). These results are consistent
with the reduction of 98 % of *E. coli* colonies exposed to cellulose acetate films
incorporated with 5 % w/w of Cu$^{2+}$MMt (Bruna et al. 2014) and 99 % of *Escherichia
coli* ATCC 25922 and *Listeria innocua* ATCC 33090 colonies in contact with PLA/5 %
8/w Cu$^{2+}$MMt (Bruna et al. 2015).

4. CONCLUSION

This manuscript shows the feasibility of preparing antibacterial Ge/Cu$^{2+}$MMt films with
extended time of action by immobilizing Cu$^{2+}$ through complexation with hydroxyl
groups in montmorillonite. Cu$^{2+}$MMt demonstrated low leaching level in the tested
conditions and retained about 90 % of its inhibitory activity against *E. coli* and *L.
monocytogenes*. Yet its sensitivity varied with the ability of the tested bacteria to attach
to the positively charged clay surface. The addition of Cu$^{2+}$MMt as inorganic
antibacterial into the gelatin matrix enhanced several key properties for packaging
applications (tensile strength increased 280 %, and water vapor permeability declined
43 %) and sufficed to inhibit Gram negative and Gram positive bacteria at relatively low
loading growth (c.a. 5 % w/w Cu$^{2+}$MMt). Studies on the evaluation of copper ions
release from nanocomposite films exposed to food simulants are being conducted in
order gain insight into the potential risk assessment of their use as food contact
materials.
5. REFERENCES


Table 1. Antimicrobial activity against *E. coli* and *L. monocytogenes* of clays measured as inhibition zone.

<table>
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<th>Inhibition zones (mm)</th>
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<td></td>
</tr>
<tr>
<td><em>d₀</em>: 5mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na⁺MMt</td>
<td>12.0±2.8 a</td>
<td>12.5±2.1 a</td>
<td>15.5±0.7 b</td>
<td>14.0±1.0 b</td>
</tr>
<tr>
<td>H⁺MMt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu²⁺MMt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu²⁺MMt (after release)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Any two means in the same row followed by the same letter are not significantly (P>0.05) different according to Turkey test.
**Table 2.** Thickness, opacity, Water vapor permeability (WVP), moisture content (MC), tensile strength (TS) and elongation at break (ε%) of obtained gelatin films.

<table>
<thead>
<tr>
<th></th>
<th>Thickness (mm)</th>
<th>Opacity (uA*nm)</th>
<th>WVP*10^{13} (Kg/Pa.s.m) RH 65:0</th>
<th>MC (%)</th>
<th>TS (MPa)</th>
<th>ε% (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ge</td>
<td>0.21±0.04 a</td>
<td>33.0±2.0 a</td>
<td>2.50±0.13 a</td>
<td>14.00±1.25 a</td>
<td>3.9±1.0 a</td>
<td>96.9±11.9 a</td>
</tr>
<tr>
<td>Ge/Na^{+}MMt</td>
<td>0.15±0.02 a</td>
<td>49.0±3.0 b</td>
<td>1.62±0.14 b</td>
<td>13.74±0.62 a</td>
<td>11.9±1.6 b</td>
<td>56.4±6.8 b</td>
</tr>
<tr>
<td>Ge/H^{+}MMt</td>
<td>0.18±0.06 a</td>
<td>51.0±9.0 b</td>
<td>2.04±0.30 b</td>
<td>13.78±0.58 a</td>
<td>9.9±1.6 b</td>
<td>49.8±6.9 b</td>
</tr>
<tr>
<td>Ge/Cu^{2+}MMt</td>
<td>0.16±0.03 a</td>
<td>89.8±13.0 c</td>
<td>1.43±0.40 b</td>
<td>13.58±1.03 a</td>
<td>10.9±1.4 b</td>
<td>48.4±7.8 b</td>
</tr>
</tbody>
</table>

Any two means in the same column followed by the same letter are not significantly (P>0.05) different according to Turkey test.
Table 3. Antimicrobial activity against *E. coli* and *L. monocytogenes* measured as inhibition zone expressed as millimeter (mm) of Ge films with and without clays.

<table>
<thead>
<tr>
<th>Ge</th>
<th>Ge/Na^+^MMt</th>
<th>Ge/H^+^MMt</th>
<th>Ge/Cu^{2+}MMt</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td>15.0±0.0 a</td>
<td>19.5±0.7 b</td>
<td>24.0±1.4 c</td>
</tr>
<tr>
<td><strong>L. monocytogenes</strong></td>
<td>16.0±1.0 a</td>
<td>30.5±0.7 b</td>
<td>33.5±0.7 c</td>
</tr>
</tbody>
</table>

Any two means in the same row followed by the same letter are not significantly (P>0.05) different according to Turkey test.
FIGURE CAPTIONS

Figure 1. XRD pattern of Na⁺MMt (___), H⁺MMt (…) and Cu²⁺MMt (----).

Figure 2. Chemical composition of a) Na⁺MMt, b) H⁺MMt and c) Cu²⁺MMt determined by EDX.

Figure 3. FTIR spectra between 4000–400 cm⁻¹ for Na⁺MMt(____), H⁺MMt (…. ) and Cu²⁺MMt (----).

Figure 4. XRD pattern of Ge (.-.-.-), Ge/Na⁺MMt (___), Ge/H⁺MMt (…..) and Ge/Cu²⁺MMt (----) films.

Figure 5. FTIR spectra of Ge (.-.-.-), Ge/Na⁺MMt (___), Ge/H⁺MMt (…..) and Ge/Cu²⁺MMt (----) films.
<table>
<thead>
<tr>
<th>Elemental atomic composition of clays (%)</th>
<th>C</th>
<th>O</th>
<th>Al</th>
<th>Si</th>
<th>Fe</th>
<th>Na</th>
<th>Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na&amp;Mn</td>
<td>2.69</td>
<td>60.30</td>
<td>9.148</td>
<td>19.92</td>
<td>1.75</td>
<td>6.17</td>
<td>0</td>
</tr>
</tbody>
</table>

![Graph showing elemental composition](image-url)
<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>O</th>
<th>Al</th>
<th>Si</th>
<th>Fe</th>
<th>Na</th>
<th>Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMMt</td>
<td>1.56</td>
<td>73.10</td>
<td>7.82</td>
<td>16.65</td>
<td>0.85</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

![Elemental atomic composition of clays (%)](image)
<table>
<thead>
<tr>
<th>Element</th>
<th>C</th>
<th>O</th>
<th>Al</th>
<th>Si</th>
<th>Fe</th>
<th>Na</th>
<th>Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu^2+MMt</td>
<td>1.04</td>
<td>63.38</td>
<td>9.77</td>
<td>21.26</td>
<td>1.11</td>
<td>0</td>
<td>3.42</td>
</tr>
</tbody>
</table>
Highlights

1. Cu\textsuperscript{2+}MMt was obtained by ion exchange from acid activated Na\textsuperscript{+}MMt

2. Cu\textsuperscript{2+}MMt showed strong activity against *E. coli* and *L. monocytogenes*

3. Inclusion of 5 % w/w clay enhanced tensile strength and water vapor barrier of gelatin films

4. Significant antibacterial property was observed for Ge/Cu\textsuperscript{2+}MMt films