Accepted Manuscript

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Please cite this article as: Martucci, J.F., Ruseckaite, R.A., Antibacterial activity of gelatin/copper (II)-exchanged montmorillonite films, *Food Hydrocolloids* (2016), doi: 10.1016/j.foodhyd.2016.10.030.
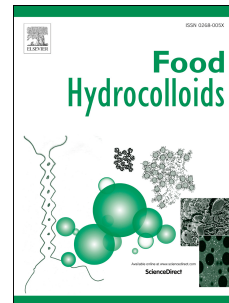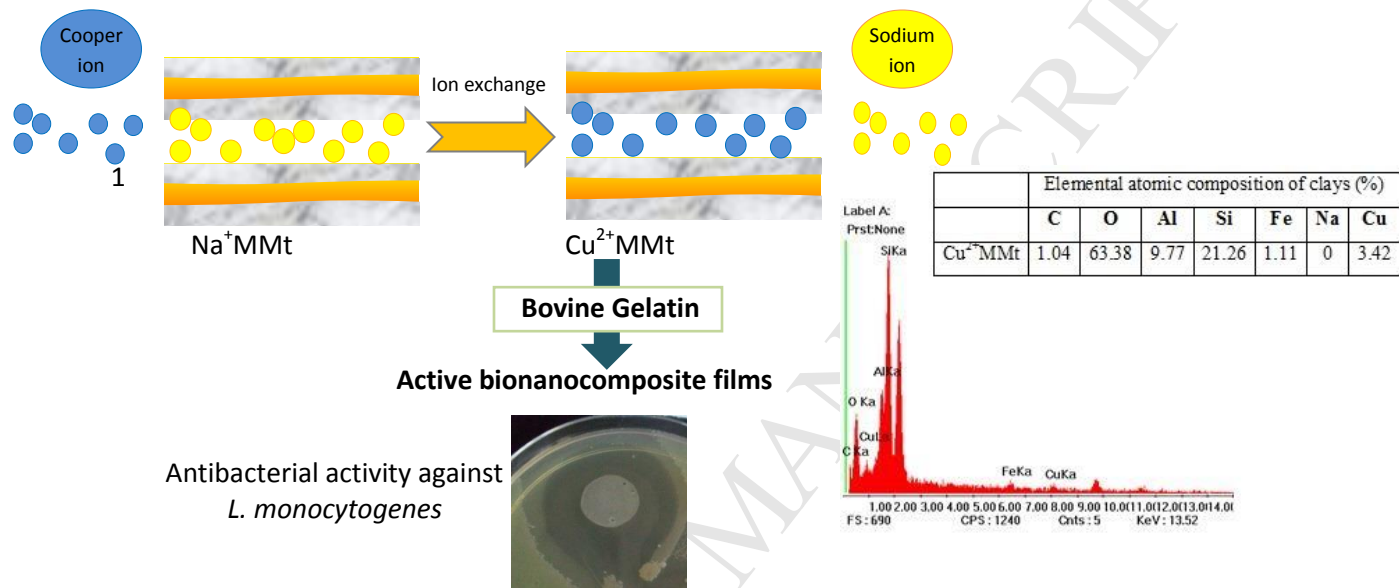
1   **Antibacterial activity of gelatin / copper (II)-exchanged montmorillonite films**

2

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ABSTRACT

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18   Cu (II) - exchanged montmorillonite (Cu^{2+}MMt) was prepared, characterized and

19   introduced into a bovine gelatin (Ge) matrix via a dissolution-intercalation method to

20   get antibacterial nanocomposite films. The maximum amount of exchanged cation did

21   not exceed the cation exchange capacity of the pristine montmorillonite (Na^+MMt), as

22   assessed by energy dispersive X-ray (EDX) spectroscopy. Cu^{2+}MMt showed

23   antibacterial activity *in vitro* against *Escherichia coli O157:H7* (Gram-negative) and

24   *Listeria monocytogenes* (Gram-positive) as revealed by the agar disc-diffusion assay.

25   The dispersion of clays in Ge films was monitored by X-ray diffraction (XRD) and

26   Scanning Electron Microscopy (SEM). Blending gelatin with 5 % w/w of clay increased

27   the tensile strength of the nanocomposite films in around 280 % while the elongation at

28   break and the water vapor permeability decreased in about 42 and 30 %, respectively,

29   regardless of the cation in clay. The Ge/Cu^{2+}MMt film exhibited antibacterial

30   effectiveness against both pathogens tested under the same conditions, demonstrating a

31   stronger effect on *L. monocytogenes* than on *E. coli O157:H7,* since the cell wall of the

32   latter differs significantly and such difference could influence their vulnerability and

33   response to the active films. Therefore, the incorporation of low clay levels as a vehicle

34   for copper ions into gelatin matrix has demonstrated to be a good method for

35   developing functional materials that can be potentially applied to the design of food

36   contact items.

37

38   *Keywords: Bovine gelatin; Cupric ions; Montmorillonite; Nanocomposite; Active film;*

39   *Antimicrobial activity.*

40

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 (Drelich 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 (Drelich 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

66 literature explores the antimicrobial activity of Cu^{2+}MMt-polymer nanocomposites.

67 Bruna and others (2012) developed low density polyethylene (LDPE)/Cu^{2+}MMt films

68 and reported a reduction of 94 % of *Escherichia coli O157:H7* colonies at 4 % w/w

69 nano-clay loading. Similarly, cellulose acetate (CA)/Cu^{2+}MMt (3 % w/w clay) films

70 yielded high levels (>98 %) of inhibitory action against *Escherichia coli* ATCC 25922

71 (Bruna et al. 2014), whereas poly(lactic acid) (PLA)/Cu^{2+}MMt films (Bruna et al. 2015)

72 were effective at reducing up to 99 % of *Escherichia coli* ATCC 25922 and *Listeria*

73 *innocua* ATCC 33090, when 5 % w/w Cu^{2+}MMt was added to each matrix.

74 To the best of the author´s knowledge, there are no studies dealing with the potential of

75 protein/Cu^{2+}MMt nanocomposites used as active food contact materials. Amongst

76 proteins, gelatin (Ge) is a water-soluble animal protein, obtained from the hydrolysis of

77 bone-collagen or connective tissues. It can be found as abundant waste/by product in

78 slaughter houses, and poultry and fish industries at reasonable cost (Hernandez-Muñoz

79 et al. 2004). Gelatin can be taken as a biogenic alternative to active films for being

80 classified as a "Generally Recognized as Safe" (GRAS) substance in the food additive

81 list by the U.S. Food and Drug Administration (FDA); also due to its biodegradability,

82 excellent film-forming ability, high oxygen barrier and satisfactory mechanical

83 properties at low or intermediate relative humidity (Hernandez-Muñoz et al. 2004;

84 Martucci et al. 2012). Nonetheless, the limited water resistance and mechanical strength

85 of gelatin films in moist environments still pose a problem to their wide application. In

86 earlier studies, the authors successfully demonstrated that blending gelatin with sodium

87 montmorillonite (Na^+MMt) could enhance barrier, mechanical and moisture resistance

88 properties of films (Martucci et al. 2007; Martucci & Ruseckaite 2010), while

89 preserving their eco-friendliness (Martucci & Ruseckaite 2009). The best results were

90 obtained from films amended with 5 % w/w of Na^+MMt, presenting the highest tensile

91 strength and Young's modulus, and the lowest WVP and hydrophilic surface (Martucci

92 & Ruseckaite 2010). This work presents the synthesis and characterization of $Cu^{2+}MMt$

93 by acid-activated MMt through ion-exchange procedure, and the effect of incorporating

94 5 % w/w of modified clay on the physical properties and antimicrobial potential of

95 nanocomposite gelatin films against *E. coli* and *L. monocytogenes,* as a model of the

96 pathogens commonly found in foodstuffs.

97

98 2. **EXPERIMENTAL SECTION**

99 **2.1. Chemicals and source of bacteria**

100 Bovine hide gelatin (Ge) type B (Bloom strength 150, isoionic point (Ip) 5.3) was

101 kindly supplied by Rousselot (Buenos Aires, Argentina) and used with no further

102 treatment. Sodium montmorillonite (named as Na^+MMt) was obtained from Southern

103 Clay Products Inc. (Texas, USA) under the trade name Cloisite Na^+. The cation-

104 exchange capacity (CEC) was 92.6 meq/100 g of clay and the interlayer distance was

105 1.17 nm (as it was determined by X-ray diffraction on dry powder). Glycerol (Gly, 98

106 % reagent grade) and cupric sulphate pentahydrated ($CuSO_4.5H_2O$, 99.99 % purity)

107 were purchased from DEM (Mar del Plata, Argentina) and Anedra (Buenos Aires,

108 Argentina), respectively. All the other chemicals used were of analytical grade and

109 brought from Aldrich (St. Louis, MO, USA). Food-borne pathogens were selected to

110 assess the antibacterial properties: *Escherichia coli O157:H7* ATCC 32158 (ATCC,

111 American Type Culture Collection) and *Listeria monocytogenes* ATCC 25923. Both

112 strains were plated onto eosin-methylene blue agar (EMB) and Baird Parker agar,

113 respectively (Martucci et al. 2015). The vegetative cells of each microorganism were

114 streaked on Mueller Hinton agar and incubated at 37 ± 0.5 °C for 24 h. Microbial broth

115 was then suspended in double distilled sterile water. The density of bacteria suspension

116   was adjusted until the visible turbidity was equal to 0.5 Mc Farland standards before

117   testing.

2.2. Preparation of modified MMt

119    $Cu^{2+}MMt$ was obtained from acid-activated MMt (H^+MMt) by ion exchange according

120   to the procedure described by Hu & Xia (2006) with minor modifications. H^+MMt was

121   produced by suspending 10 g of Na^+MMt in 75 mL of 0.05 M HCl solution. This

122   dispersion was kept for 24 h under constant stirring (400 rpm) at room temperature in a

123   hot plate (Cole Palmer, USA) and then centrifuged (Sartorius type4-15, Germany) at

124   5000 rpm for 5 min. The recovered sediment was washed with bi-distilled water until no

125   acid in the supernatant was detected, and then dried at 80 °C overnight in an air-

126   circulating oven (Memmert UFE550, Germany). The dry product was pulverized to an

127   average size of less than 300 mesh sieve. $Cu^{2+}MMt$ was produced by dispersing 5 g of

128   dry H^+MMt in 100 mL of a 0.05 M $CuSO_4.5H_2O$ solution under gentle stirring (400

129   rpm) at 60 °C for 6 h. Afterwards the sample was submitted to the same purification

130   protocol than its acid-activated counterpart.

2.3. Film forming process

132   Gelatin films added with clay (i.e., Na^+MMt, H^+MMt and $Cu^{2+}MMt$; 5 % w/w dry

133   gelatin basis) and plasticized with glycerol (30 % w/w dry gelatin basis) were prepared

134   by the solution–intercalation method based on early works by the group (Martucci et al.

135   2007; Martucci & Ruseckaite 2009, 2010). Plasticizer and clay contents were fixed on

136   the basis of previous studies (Martucci & Ruseckaite 2008, 2010). Formulations with

137   glycerol content lower than 30 % w/w resulted in films behaving similarly to their un-

138   plasticized counterparts, while the incorporation of a glycerol level higher than 30 %

139   w/w induced plasticizer segregation and migration (Martucci & Ruseckaite 2008). In the

140   case of clay, gelatin nanocomposites containing 5 % w/w of Na^+MMt displayed the best

141 set of thermal, mechanical, barrier and optical properties (Martucci & Ruseckaite 2008,

142 2010), so this percentage was selected for synthesis of the antibacterial nanocomposite

143 films. All films were preconditioned at 25 ± 2 ºC and 65 ± 2 % RH for 48 h in an

144 environmental chamber before further experimental analysis. Films were designated as

145 Ge/Na$^+$MMt, Ge/H$^+$MMt and Ge/Cu^{2+}MMt, respectively, depending on the clay used.

146 **2.4.   Characterization**

147 **2.4.1. X-ray diffraction (XRD).** XRD patterns were recorded at room temperature on a

148 PANalytical X'Pert Pro diffractometer (Almelo, The Netherlands) equipped with a Cu

149 Kα radiation source (λ= 0.1546 nm) at a generator voltage of 45 kV and 30 mA as the

150 applied current. The incidence angle ranged from 5º to 50º at a scanning rate of 1 º/min.

151 The interlayer spaces were calculated by the Bragg equation.

152 **2.4.2. Fourier Transform Infrared Spectroscopy (FTIR).** FTIR analyses were

153 performed on a Mattson Genesis II spectrophotometer in transmission mode. The

154 measurements were recorded between 4000–400 cm^{-1} at 32 scans. Pulverized specimens

155 were pressed into pellets with KBr. The background noise was corrected with pure KBr

156 data.

157 **2.4.3. Energy dispersive X-ray spectroscopy (EDX).** EDX was used to assess the

158 presence of copper in MMt samples by using an spectrometer EMAX (Horiba Co. Ltd.,

159 Wycombe, U.K.) operated at Vacc ¼ 15 kV.

160 **2.4.4. Thickness.** Film thickness was measured by a hand-held micrometer (Dial

161 Thickness gauge 7301, Mitutoyo Corporation, Kanagawa, Japan) with an accuracy of

162 0.01 mm. Measurements were taken at ten random locations from three films of each

163 formulation, and the mean thickness values were used to calculate the physical

164 properties.

165 **2.4.5. Visible light–barrier properties**. The light absorption of nanocomposite films

166 was measured in a wavelength ranging from 400 to 800 nm, using a UV-visible

167 spectrophotometer Shimadzu 1601 PC (Tokyo, Japan) according to a method described

168 elsewhere (Irissin-Mangata et al. 2001). Each film specimen was cut in rectangular

169 strips and placed directly in the spectrophotometer test cell. Air was used as reference.

170 Film opacity was expressed as the area under the absorption curve (arbitrary units/nm)

171 per thickness unit (mm). Reported values are the average of five measurements.

172 **2.4.6. Equilibrium moisture content (MC**). The squared-shape strips of each film

173 sample (dimensions 4 cm^2) were weighed in an analytical balance (±0.0001 g; Ohaus,

174 USA) to determine the initial mass. Then samples were dried in an air circulating oven

175 (Memmert, Germany) at 105 ℃ for 24 h according to the procedure reported in the

176 ASTM D644-94, 1994. The equilibrium moisture content (MC) was expressed as the

177 percentage of initial film weight lost during drying. Reported values are the average of

178 three replicates.

179 **2.4.7. Water vapor permeability (WVP**). Water vapor permeability (WVP) was

180 performed gravimetrically at 25 ℃, following the ASTM E96-95 desiccant method. All

181 specimens were equilibrated at 65 ± 2 % RH at 25 ± 2 ℃ for 48 h. Afterwards, test

182 films were fixed onto opening cells containing silica gel (0 % RH), and the cells were

183 placed in a controlled humidity chamber at 65 ± 2 % RH and 25 ± 2 ℃. The air gap

184 inside the cell was ∼1.2 cm and the film area exposed for water vapor transmission was

185 13.8 cm^2. The cells were weighed on an hourly basis over a 10 h period. WVP was

186 calculated from the following equation:

187 $$WVP(Kg \cdot m \cdot s^{-1} \cdot Pa^{-1} \cdot m^{-2}) = \frac{w}{At\Delta P}e \qquad\qquad (1)$$

188   where w is the weight gain of the cup (Kg) at time t (s); e is the film thickness (m); A is

189   the film exposed area (m^2); ΔP is the vapor pressure difference across the film (Pa). All

190   measurements were taken in quadruplicate.

191   **2.4.8. Tensile properties**. The tensile strength (TS) and percentage of elongation at

192   break ($\varepsilon\%$) were measured according to the ASTM 638 94 D standard using an Instron

193   4467 Universal Testing Machine (Buckinghamshire, England) with a 5 kN load cell at a

194   crosshead speed of 10 mm/min. Reported results were obtained from at least 10 samples

195   for each type of film

196   **2.4.9. Testing of antimicrobial activity.** The *in vitro* antibacterial activity of films and

197   clays was assessed following our previous work (Martucci et al. 2015) using agar disc-

198   diffusion assay. Test bacteria (100 µL of inoculums containing approximately 10^5 - 10^6

199   CFU/mL of each bacterium) were plated onto Mueller Hinton (Merck, Darmstadt,

200   Germany) agar medium. Discs (10 mm in diameter) were cut from the films with a

201   circular knife and placed onto the inoculated plates. The antimicrobial activity of clay

202   specimens was assessed in a similar way. Each clay (3 mg), was dispersed in 1 mL of

203   bi-distilled water and submitted to an ultrasonic bath (Testlab, 160 W, 40 KHz) for 20

204   min. Then 30µL of the obtained suspension was poured into agar wells (5 mm

205   diameter). All plates were incubated at 37 ºC for 24 h. The diameter of the inhibition

206   zone surrounding the film discs or wells (in the case of clays) was measured with a

207   manual caliper (Mitutoyo, Japan) from the center of the film. The antimicrobial activity

208   of clay specimens was assessed in a similar way. The result was determined as the mean

209   of three separate experimental runs.

210   **2.4.10. Cu^{2+} ion desorption studies**. Cu^{2+}MMt (0.1 g) was extensively washed with bi-

211   distilled water under stirring for 24 h. The resulting dispersion was centrifuged at 8000

212   rpm (Sartorius type4-15, Germany) for 10 min. The concentration of the cupric cation

213   left in the washed Cu^{2+}MMt was determined by EDX. The *in vitro* antibacterial activity

214   of the washed clay was qualitatively measured with the agar disc-diffusion method, as

215   previously described for clays.

216   **2.5. Statistical analysis**

217   Experimental data were statistically analyzed by the one-way analysis of variance

218   *(*ANOVA) using the Origin Pro 8 software and Turkey's test for comparison of means at

219   a 5 % significance level**.** All the results are expressed as the mean ± standard deviation.

220

221   **3.   RESULTS AND DISCUSSION**

222   **3.1. Modified clay characterization**

223   X-ray diffraction patterns of pristine and modified clay were used to determine the

224   variations in the basal d_{001}-spacing due to cation switching (Figure 1). Na^+MMt

225   exhibited a diffraction peak at $2\theta = 7.3°$ (1.21 nm, according to Bragg eq.)

226   corresponding to the basal interlayer d_{001}-spacing (Mosser et al. 1997). Upon acid

227   activation, this reflection slightly shifted to lower angles corresponding to an interlayer

228   distance of 1.25 nm (Figure 1), in line with the exchange of Na^+ for H^+ with larger ionic

229   radius (ca. H^+ hydrated: 0.900 nm vs. Na^+ hydrated: 0.450 nm). The slight difference in

230   the basal reflection of montmorillonite caused by switching Na^+ for H^+ (Figure 1),

231   suggests that acid activation had a minor effect on the layered structure (Zhao et al.

232   2013). After treating H^+MMt with $CuSO_4$, the d_{001}-spacing increased up to 1.30 nm

233   (corresponding to $2\theta = 6.8°$) confirming the intercalation of Cu^{2+}. The small increment

234   can be ascribed to the disparity between the ionic radius of the hydrated forms of Cu^{2+}

235   (i.e., hexaaqua) and Na^+ cations (He et al. 2001; Tanaka et al. 2007). A small new

236   reflection also appeared at around $2\theta = 13°$ in the diffraction patterns of Cu^{2+}MMt,

237   probably attributed to an amorphous cupric hydroxide such as $Cu(OH)_2 \cdot H_2O$, as already

238   accounted for by others (Zhou et al. 2004). The increment in *d*-spacing of MMt due to

239   Cu^{2+} ion exchange was previously observed, but values might vary due to differences in

240   the composition of the raw clay and the treatment preformed on it (Zhou et al. 2004; Hu

241   & Xia 2006; Tanaka et al. 2007; Bruna et al. 2012, 2014, 2015).

242   EDX data further supported the presence of Cu^{2+} ions in the clay (Figure 2 c). Na^+MMT

243   was distinguished by the presence of a peak at 0.05 evK in EDX spectrum assigned to

244   Na^+ which was absent in H^+MMt and Cu^{2+}MMt spectra (Figures 2 a, b and c,

245   respectively). The occurrence of a new peak at 8 evK in Cu^{2+}MMt spectrum (Figure 2 c)

246   is a strong experimental evidence of copper exchange (Bagchi et al. 2013; Das et al.

247   2013). Since the intensity of such peak is proportional to the element concentration, the

248   loading of Cu^{2+} cation onto MMt was estimated in about 3 % (on element basis) (Figure

249   2 c).

250   The effect of the cation exchange on the clay structure was analyzed by FTIR (Figure

251   3). All the spectra exhibited relevant absorption bands at 3631 cm^{-1} (stretching vibration

252   of structural OH group (Al-OH)), 3432 and 1631 cm^{-1} (stretching and bending

253   vibrations of interlayer H_2O, respectively), 1045 cm^{-1} (stretching vibration of Si-O), and

254   915-18 cm^{-1} (Al-Al-OH bending vibration) characteristic of clay structure (Zhou et al.

255   2004; Zhao et al. 2013; Pereira et al. 2013). The absorption feature of Na^+MMt

256   remained unchanged after acid activation, thereby suggesting that the aluminum cations

257   of montmorillonite seemed not to be leached by the acid treatment, as postulated by

258   others (Tong et al. 2005). The peak situated at 3631 cm^{-1} was slightly shifted

259   downwards upon Cu^{2+} exchange, which is explained by the presence of interactions

260   between cupric ions and clay, primarily in the inter-lamellar space, through

261   complexation of copper ions as previously indicated by other authors (Bagchi et al.

262   2013; Pereira et al. 2013). The intensity of the hydration band barely changed due to

263   differences in the water coordination capacity of Na^+ and Cu^{2+} ions. Finally other

264   characteristic vibrations of the tetrahedral sheets, namely Si-O-Si stretching (993 cm^{-1})

265   and Si-O-Al bending (521 cm^{-1}), reached higher wave length values and decreased

266   intensity indicating a somewhat disordered MMt structure (Xia et al. 2010).

267   The inhibitory activity of pristine and modified clay against Gram-negative and Gram-

268   positive bacteria was investigated with the agar disc diffusion method, and results are

269   summarized in Table 1. Both pathogens revealed sensitivity to all clay suspensions,

270   indicating certain Na^+MMt antibacterial activity. The ability of Na^+MMt, Ca^{2+}MMt and

271   H^+MMt to reduce the bacterial plate counts of *E. coli* was previously observed by Hu &

272   Xia (2006), though no reports on the inhibitory effect on Gram-positive bacteria have

273   come to light. Cu^{2+}MMt, on the other hand, exerted a powerful antibacterial action

274   against both bacteria tested (p<0.05), related to the higher adsorption capacity of this

275   clay (Guo et al. 2011) and the intrinsic antibacterial activity of Cu^{+2} (He et al. 2001; Hu

276   and Xia 2006; Malachová et al. 2011). The presence of Cu^{2+} cations leads to a surplus

277   of positive charge onto the mineral surface. They serve as potential attachment sites for

278   negatively charged cell surface (Stotzky 1980), and result in the appearance of defects

279   in the bacterial outer membrane responsible for the cell permeability, so that cell

280   contents are lost. The higher susceptibility of *L. monocytogenes* to Cu^{2+}MMt (Table 1)

281   could be related to differences in the composition and thickness of the outer membrane

282   of Gram-positive and Gram-negative bacteria (Hu et al. 2005; Malachovà et al. 2009).

283   The cell wall of Gram-positive bacteria is thicker than that of Gram-negative bacteria

284   due to the presence of a thick peptidoglycan layer (20-80 nm) containing phosphate and

285   carboxylic groups. This layer provides a negatively charged site onto the cell wall of

286   Gram-positive bacteria where cations bind. Gram-negative bacteria have a thinner

287   monolayer of peptidoglycan, lipopolysccharide and phospholipids, phospholipids being

288   the only main binding site for cations (Vaara 1992). Such differences in the cell wall

289   structures of Gram-positive and Gram-negative bacteria turned *L. monocytogenes* more

290   vulnerable to $Cu^{2+}MMt$.

291   $Cu^{2+}MMt$ slightly reduced its antibacterial activity (about 10 % of the original, Table 1)

292   after extensive washing for 24 h, evidencing that copper is retained onto the clay surface

293   (Hu et al. 2005; Hu & Xia 2006). EDX results also confirmed the high retention rate of

294   copper after washing, copper desorption being below 8 % (data not shown).

295   **3.2.   Characterization and comparison of the functional properties of control**

296   **and Ge/$Cu^{2+}MMt$ films**

297   **3.2.1. Structural analysis**

298   The diffractogram of the unfilled gelatin film (control) displayed a broad and low

299   intensity peak at $2\theta = 6.2\text{-}9.5$ ° representing the typical amorphous state of gelatin films

300   produced at a temperature higher than the helix-coil transition ($T_{helix\text{-}coil} \sim 35$ °C) (Figure

301   4) (Martucci et al. 2007). The XRD of the nancomposite films was characterized by the

302   presence of shoulders at $2\theta < 7°$ denoting a certain degree of matrix component

303   intercalation, i.e., gelatin and/or glycerol, into the clay galleries causing widening of the

304   *d*-space relative to that of the pristine MMt (Figure 4). (Martucci et al. 2007; Rao et al.

305   2007; Martucci & Ruseckaite 2010; Farahnaky et al. 2014; Nagarajan et al. 2014).

306   The FTIR spectra of un-filled Na^+MMt and $Cu^{2+}MMt$- incorporated gelatin films

307   (Figure 5) presented characteristic peaks in the amide region at 1631 cm^{-1} (amide I,

308   C=O stretching), 1551 cm^{-1} (amide II, N-H bending,), and 1237 cm^{-1}1 (amide III, C-N

309   and N-H) (Sionkowska et al. 2004; Martucci & Ruseckaite 2010). The addition of clay

310   shifted amide-I, amide-II and amide-III to higher frequency c.a. 1646, 1553, and 1245

311   cm^{-1}, respectively, confirming the occurrence of hydrogen bonding interactions between

312   gelatin and acceptor atoms such as oxygen from free-OH and Si–O–Si groups in MMt,

313 as documented for other protein-MMt composites (Kumar et al. 2010; Martucci &

314 Ruseckaite 2010).

315 **3.2.2. Optical properties**

316 The neat gelatin film was transparent without any color tint, while nanocomposites were

317 less transparent and colored as $Ge/Cu^{2+}MMt$. The light transmission capacity of gelatin

318 films was reduced by clay addition (Table 2, $p<0.05$), indicating a strong light scattering

319 effect due to clay particles with sizes higher than the wave-length of the visible light

320 (Martucci & Ruseckaite 2010; Shotornivit et al. 2010; Rhim 2013). The sharp decrease

321 in transparency experienced by the $Ge/Cu^{2+}MMt$ film should be attributed not only to

322 the scattering explained by some clay structures but also to coloration increase due to

323 the transformation of some cupric ions to cupric oxides during the drying stage of the

324 film manufacturing process, as previously described by Bruna and co-workers (Bruna et

325 al. 2012). The significant differences noticed in the parameter analyzed were not

326 detected when the films´ visual appearance was qualitatively observed, since all

327 specimens remained transparent.

328 **3.2.3. Moisture content, water vapor permeability and tensile properties**

329 The average moisture content (MC) of all films remained around 13.8 ± 1.6 g of

330 water/100 g of film (Table 2). The constancy of MC at any cation in MMt suggests that

331 the hydration capacity of cations did not affect the moisture uptake capacity of the

332 nanocomposite films.

333 The water vapor barrier property of gelatin films was substantially improved ($p<0.05$,

334 Table 2) by adding 5 % w/w MMt regardless of the cation intercalated, suggesting that

335 switching the cation marginally alters the hydrophilic/hydrophobic balance of the filler.

336 The strong interactions between gelatin and nano-clays (Martucci & Ruseckaite 2010)

337 consume some hydrophilic groups, reducing the water uptake by capillarity at the

338   interface. The presence of water vapor impermeable silicate platelets or other structures

339   with large aspect ratios dispersed in the polymer matrix also contribute to obstructing

340   and delaying the transmission of water vapor through the matrix, as postulated for other

341   protein nanocomposite films (Farahnaky et al. 2014; Kanmani & Rhim 2014; Nagarajan

342   et al. 2014).

343   The incorporation of 5 % w/w MMt noticeably improved ($p<0.05$) TS values as

344   compared to control. The great affinity of biopolymer and nano-clay limiting the

345   molecular mobility of protein chains, together with the uniform dispersion of the nano-

346   reinforcements might lead to an increase in TS (Martucci & Ruseckaite 2010;

347   Farahnaky et al. 2014; Nagarajan et al. 2014). The differences in tensile strength

348   between our nanocomposites and other reported in the literature could be attributed to

349   differences in clay type, matrix source, processing technologies, or a combination

350   thereof. The extensibility decreased significantly ($p<0.05$) in about 42 % when adding 5

351   % w/w clay, and no major effects ($p>0.05$) were detected with cation exchange in MMt

352   (Table 2). The reduction in $\varepsilon\%$ was previously reported in gelatin-based

353   nanocomposites (Rao et al. 2007; Martucci & Ruseckaite 2010; Kanmani & Rhim 2014;

354   Farahnaky et al. 2014), and has been attributed to the restricted motion of gelatin

355   molecules due to interfacial interactions between gelatin and nano-clay.

356   **3.2.4. Antimicrobial activity**

357   Antibacterial assays (Table 3) indicate that free-MMt gelatin films have shown little

358   antimicrobial activity against both Gram-negative and Gram-positive pathogenic

359   bacteria, probably due to the presence of short chain polypeptides (Minervini et al 2003;

360   Di Bernardini et al. 2010). Clay addition enlarged the diameter of the clearing zone

361   (Table 3) and the activity visibly varied with the cation in MMt and the pathogen tested.

362   The Ge/Cu^{2+}MMt film exhibited the highest inhibitory activity, in agreement with the

363 antimicrobial performance of clays (Table 1). Overall, Ge/Cu^{2+}MMt film greatly

364 inhibited the growth of Gram-positive (*L. monocytogenes*) as compared to Gram-

365 negative (*E. coli*) pathogens (Table 3). The inhibitory action of Ge/Cu^{2+}MMt against

366 both microorganisms was assumed to be similar to that described above for Cu^{2+}MMt:

367 attachment to clay surface, followed by cell wall damage, loss of cell content and,

368 eventually microbial death (Hu et al. 2005; Guo et al. 2011). These results are consistent

369 with the reduction of 98 % of *E. coli* colonies exposed to cellulose acetate films

370 incorporated with 5 % w/w of Cu^{2+}MMt (Bruna et al. 2014) and 99 % of *Escherichia*

371 *coli* ATCC 25922 and *Listeria innocua* ATCC 33090 colonies in contact with PLA/5 %

372 w/w Cu^{2+}MMt (Bruna et al. 2015).

373

374 **4. CONCLUSION**

375 This manuscript shows the feasibility of preparing antibacterial Ge/Cu^{2+}MMt films with

376 extended time of action by immobilizing Cu^{2+} through complexation with hydroxyl

377 groups in montmorillonite. Cu^{2+}MMt demonstrated low leaching level in the tested

378 conditions and retained about 90 % of its inhibitory activity against *E. coli* and *L.*

379 *monocytogenes.* Yet its sensitivity varied with the ability of the tested bacteria to attach

380 to the positively charged clay surface. The addition of Cu^{2+}MMt as inorganic

381 antibacterial into the gelatin matrix enhanced several key properties for packaging

382 applications (tensile strength increased 280 %, and water vapor permeability declined

383 43 %) and sufficed to inhibit Gram negative and Gram positive bacteria at relatively low

384 loading growth (c.a. 5 % w/w Cu^{2+}MMt). Studies on the evaluation of copper ions

385 release from nanocomposite films exposed to food simulants are being conducted in

386 order gain insight into the potential risk assessment of their use as food contact

387 materials.

388

5. **REFERENCES**

Bagchi, B., Kar, S., Dey, S., Bhandary, S., Roy, D., Mukhopadhyay, T., Das, S., & Nandy, P. (2013). In situ synthesis and antibacterial activity of copper nanoparticle loaded natural montmorillonite clay based on contact inhibition and ion release. Colloids and Surfaces B: Biointerfaces 108, 358–365

Bruna, J. E., Galotto, M. J., Guarda, A., & Rodriguez, F. (2014). A novel polymer based on MtCu2+/cellulose acetate with antimicrobial activity. Carbohydrate Polymers 102, 317-323

Bruna, J. E., Peñaloza, A., Guarda, A., Rodríguez, F., & Galotto, M. J. (2012). Development of MtCu2+/LDPE nanocomposites with antimicrobial activity for potential use in food packaging. Applied Clay Science 58, 79–87

Bruna, J. E., Quilodrán, H., Guarda, A., Rodríguez, F., Galotto, M. J., & Figueroa, P. (2015). Development of antibacterial MtCu/PLA nanocomposites by casting method for potential use in food packaging Journal of the Chilean Chemical Society 60, 2868-2873

Das, D., Nath, B. C., Phukon, P., & Dolui, S. K. (2013). Synthesis and evaluation of antioxidant and antibacterial behavior of CuO nanoparticles. Colloids and Surfaces B: Biointerfaces 101, 430-433

Di Bernardini, R., Harnedy, P., Bolton, D., Kerry, J., O'Neill, E., Mullen, A. M. (2011) Antioxidant and antimicrobial peptidic hydrolysates from muscle protein sources and by-products. Food Chemistry 124, 1296-1307

Drelich, J., Li, B., Bowen, P., Hwang, J-Y, Mills, O., & Hoffman, D. (2011). Vermiculite decorated with copper nanoparticles: novel antibacterial hybrid material. Applied Surface Science 257, 9435–9443

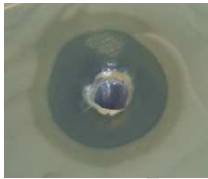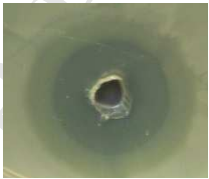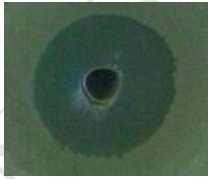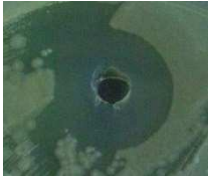412 Farahnaky, M., Dadfar, S. M. M., & Shahbazi, M. (2014). Physical and mechanical

413 properties of gelatin–clay nanocomposite. Journal of Food Engineering 122, 78–83

414 Guo, Z., Li, Y., Zhang, S., Niu, H., Chen, Z., & Xu, J. (2011). Enhanced sorption of

415 radiocobalt from water by Bi (III) modified montmorillonite: a novel adsorbent. Journal

416 of Hazardous Materials 192, 168–175

417 He, H. P., Guo, J. G., Xie, X. D., & Peng, J. L. (2001). Location and migration of

418 cations in Cu2+−adsorbed montmorillonite. Environment International 26 (5–6), 347–

419 352

420 Hernandez-Muñoz, P., Villalobos, R., & Chiralt, A. (2004). Effect of cross-linking

421 using aldehydes on properties of glutenin rich films. Food Hydrocolloid 18, 403-411

422 Hu, C-H., & Xia, M-S. (2006). Adsorption and antibacterial effect of copper-exchanged

423 montmorillonite on Escherichia coli K88. Applied Clay Science 31 (2006) 180–184

424 Hu, C-H., Xu, Z-R., & Xia, M-S. (2005). Antibacterial effect of Cu2+-exchanged

425 montmorillonite on Aeromonas hydrophila and discussion on its mechanism Veterinary

426 Microbiology 109, 83–88

427 Irissin-Mangata, J., Bauduin, G., Boutevin, B., & Gontard, N. (2001). New plasticizers

428 for wheat gluten films. European Polymer Journal 37, 1533-1541

429 Kanmani, P., & Rhim, J-W. (2014). Physical Mechanical and Antimicrobial Properties

430 of Gelatin Based Active Nanocomposite Films Containing AgNPs and Nanoclay. Food

431 Hydrocolloids 35, 644–652

432 Kloprogge, J. T., Mahmutagic, E., & Frost, R. L. (2006). Mid Infrared and Infrared

433 Emission Spectroscopy of Cu-Exchanged Montmorillonite. Journal of Colloid and

434 Interface Science 296(2), 640-646

435 Kumar, P., Sandeep, K. P., Alavi, S., Truong, V. D., & Gorga, R. E. (2010). Preparation

436 and characterization of bio-nanocomposite films based on soy protein isolate and

437 montmorillonite using melt extrusion. Journal of Food Engineering 100(3), 480-489

438 Llorens, A., Lloret, E., Picouet, P. A., Trbojevich, R., & Fernandez, A. (2012). Metallic-

439 based micro and nanocomposites in food contact materials and active food packaging

440 Trends in Food Science & Technology 24, 19-29

441 Malachová, K., Praus, P., Pavlíčková, Z., & Turicová, M. (2009). Activity of

442 antibacterial compounds immobilised on montmorillonite. Applied Clay Science 43,

443 364–368

444 Malachová, K., Praus, P., Rybková, Z., & Kozák, O. (2011). Antibacterial and

445 antifungal activities of silver, copper and zinc montmorillonites. Applied Clay Science

446 53, 642–645

447 Martucci, J. F., & Ruseckaite, R. A. (2010). Biodegradable Bovine Gelatin/Na+-

448 Montmorillonite Nanocomposite Films. Structure, Barrier and Dynamic Mechanical

449 Properties. Polymer-Plastics Technology and Engineering 49(6), 581-588

450 Martucci, J. F., & Ruseckaite, R. A. (2009). Biodegradation of three-layer laminate

451 films based on gelatin under indoor soil conditions. Polymer Degradation and Stability

452 94, 1307–1313

453 Martucci, J.F., & Ruseckaite, R.A. (2008). Structure and properties of

454 gelatine/montomorillonite nanocomposite films, in Recent Advances in Research on

455 Biodegradable Polymers and Sustainable Polymers. Volume 1, A. Jimenez, G.E. Zaikov

456 (Eds), Nova Publishers, Huntington, NY, pp. 27-36. ISBN 978-1-60692-095-4

457 Martucci, J.F., Accareddu, A.M.E., & Ruseckaite, R.A. (2012). Preparation and

458 Characterization of Plasticized Gelatin Films Cross-Linked With Low Concentrations of

459 Glutaraldehyde. Journal of Material Science 47(7), 3282-3292

460 Martucci, J. F., Gende, L. B., Neira, L. M., & Ruseckaite, R. A. (2015). Oregano and

461 lavender essential oils as antioxidant and antimicrobialadditives of biogenic gelatin

462 films. Industrial Crops and Products 71, 205–213

463 Martucci, J. F., Vázquez, A., & Ruseckaite, R. A. (2007). Nanocomposites based on

464 gelatin and montmorillonite. Morphological and thermal studies. Journal of Thermal

465 Analysis and Calorimetry 89(1), 117-122

466 Minervini, F., Algaron, F., Rizzello, C. G., Fox, P. F., Monnet, V., & Gobbetti, M.

467 (2003). Angiotensin I-converting-enzyme-inhibitory and antibacterial peptides from

468 Lactobacillus helveticus PR4 proteinase-hydrolysec casein of milk from six species.

469 Applied and Environmental Microbiology 69, 5297-5305

470 Mosser, C., Michot, L. J., Villierns, F., & Romeo, M. (1997). Migration of cations in

471 Cu(II)-exchanged montmorillonite and laponite upon heating. Clays and Clay Minerals

472 45, 789–802

473 Nagarajan, M., Benjakul, S., Prodpran, T., & Songtipya, P. (2014). Characteristics of

474 bio-nanocomposite films from tilapia skin gelatin incorporated with hydrophilic and

475 hydrophobic nanoclays Journal of Food Engineering 143, 195-204

476 Pereira, F. A. R., Sousa, K. S., Cavalcanti, G. R. S., Fonseca, M. G., de Souza, A. G., &

477 Alves, A. P. M. (2013). Chitosan-montmorillonite biocomposite as an adsorbent for

478 copper (II) cations from aqueous solutions. International Journal of Biological

479 Macromolecules 61, 471–478

480 Rao, Y. (2007). Gelatin-clay nanocomposites of improved properties. Polymer 58(18),

481 5369-5375

482 Rhim, J-W., Park, H-M., & Ha, C-S. (2013). Bio-nanocomposites for food packaging

483 applications. Progress in Polymer Science 38(10-11), 1629–1652

484   Sionkowska, A., Wisniewski, M., Skopinska, J., Kennedy, C. J., & Wess, T. J. (2004).

485   Molecular interactions in collagen and chitosan blends. Biomaterials 25, 795–801

486   Stotzky, G. (1980) Surface interactions between clay minerals and microbes, viruses,

487   and soluble organics, and the probable importance of these interactions to the ecology

488   ofmicrobes in soil. In Microbial Adhesion to Surfaces (eds. R. C. BERKELEY et al.),

489   pp. 233-247. Soc. Chem. Industry, London.

490   Tanaka, M., Itadani, A., Abe, T., Taguchi, H., & Nagao, M. (2007). Observation of

491   characteristic IR band assignable to dimerized copper ions in montmorillonite. Journal

492   of Colloid and Interface Science 308, 285–288

493   Tong, G., Yulongo, M., Peng, G., & Zirong, X. (2005). Antibacterial effects of the

494   Cu(II)-exchanged montmorillonite on Escherichia coli K88 and Salmonella

495   choleraesuis. Veterinary Microbiology 105(2), 113–122

496   Vaara M. (1992). Agents that increase the permeability of the outer membrane.

497   Microbiology and Molecular Biology Reviews 56, 3395-3411.

498   Xia, M., Jiang, Y., Zhao, L., Li, F., Xue, B., Sun, M., Liu, D., & Zhang, X. (2010). Wet

499   grinding of montmorillonite and its effect on the properties of mesoporous

500   montmorillonite Colloids and Surfaces A: Physicochemical and Engineering Aspects

501   356, 1–9

502   Zhao, H., Zhou, C. H., Wu, L. M., Lou, J. Y., Li, N., Yang, H. M., Tong, D. S., & Yu,

503   W. H. (2013). Catalytic dehydration of glycerol to acrolein over sulfuric acid-activated

504   montmorillonite catalysts. Applied Clay Science 74, 154–162

505   Zhou, Y. H., Xia, M. S., Ye, Y., & Hu, C. H. (2004). Antimicrobial ability of Cu^{2+}-

506   montmorillonite. Applied Clay Science 27 (3–4), 215–218.

507

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

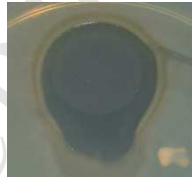| d$_0$: 5mm	Inhibition zones (mm)			
	Na$^+$MMt	H$^+$MMt	Cu^{2+}MMt	Cu^{2+}MMt (after release)
E. coli	12.0±2.8 a	12.5±2.1 a	15.5±0.7 b	14.0±1.0 b
L. monocytogenes	11.0±1.4 a	15.5±0.8 bc	16.5±0.7 b	14.5±0.5 c
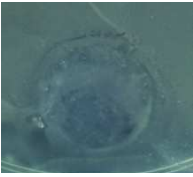
Any two means in the same row followed by the same letter are not significantly (P>0.05) different according to Turkey test.

Table 2. Thickness, opacity, Water vapor permeability (WVP), moisture content (MC), tensile strength (TS) and elongation at break (ε%) of obtained gelatin films.

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

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.

d_0:15mm	Inhibition zone (mm)			
	Ge	Ge/Na$^+$MMt	Ge/H$^+$MMt	Ge/Cu^{2+}MMt
E. coli	15.0±0.0 a	19.5±0.7 b	24.0±1.4 c	23.5±0.7 c
L. monocytogenes	16.0±1.0 a	30.5±0.7 b	33.5±0.7 c	37.0±2.8 c

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

1   **FIGURE CAPTIONS**

2   **Figure 1.** XRD pattern of Na$^+$MMt (___), H$^+$MMt (….) and Cu^{2+}MMt (----).
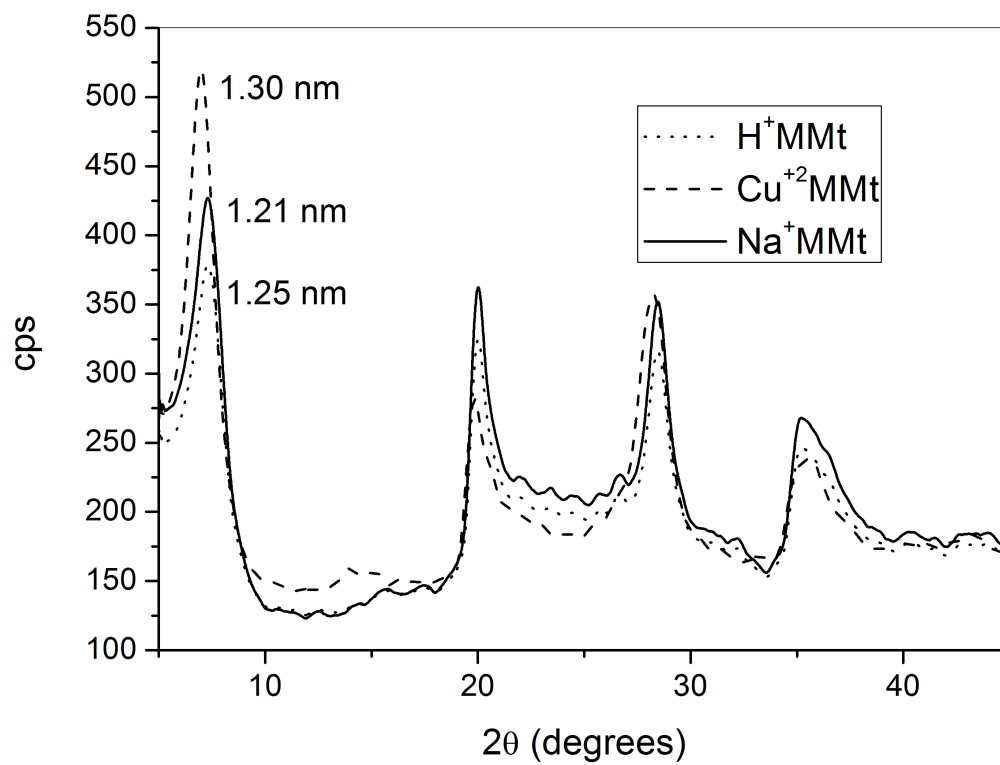
3   **Figure 2.** Chemical composition of a) Na$^+$MMt, b) H$^+$MMt and c) Cu^{2+}MMt

4   determined by EDX.

5   **Figure 3.** FTIR spectra between 4000–400 cm^{-1} for Na$^+$MMt(___), H$^+$MMt (….) and
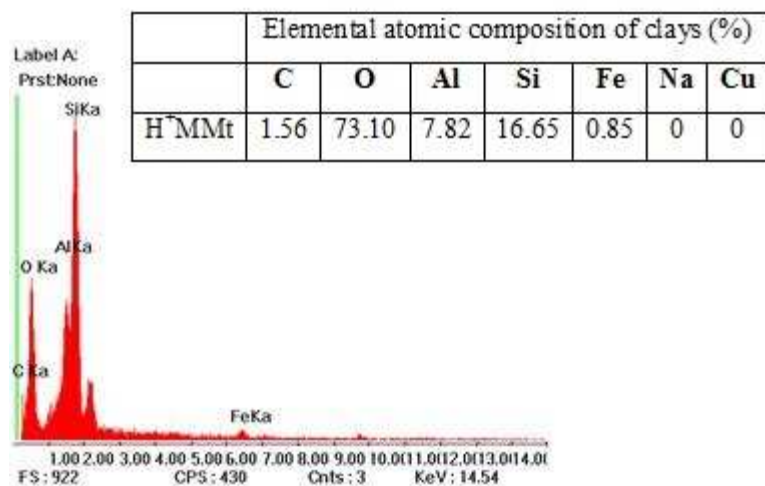
6   Cu^{2+}MMt (----).

7   **Figure 4.** XRD pattern of Ge (-.-.-), Ge/Na$^+$MMt (___), Ge/H$^+$MMt (….) and
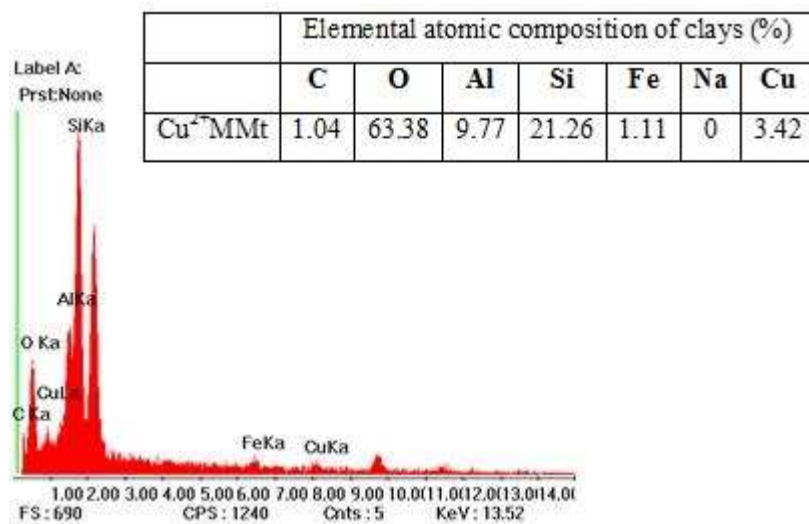
8   Ge/Cu^{2+}MMt (----) films.

9   **Figure 5.** FTIR spectra of Ge (-.-.-), Ge/Na$^+$MMt (___), Ge/H$^+$MMt (….) and

10   Ge/Cu^{2+}MMt (----) films.

11

	Elemental atomic composition of clays (%)						
	C	O	Al	Si	Fe	Na	Cu
Na$^+$MMt	2.69	60.30	9.148	19.92	1.75	6.17	0

	Elemental atomic composition of clays (%)						
	C	O	Al	Si	Fe	Na	Cu
H⁺MMt	1.56	73.10	7.82	16.65	0.85	0	0

Label A:
Prst:None

SiKa

AlKa
O Ka

C Ka

FeKa

1.00 2.00 3.00 4.00 5.00 6.00 7.00 8.00 9.00 10.00 11.00 12.00 13.00 14.00
FS : 922   CPS : 430   Cnts : 3   KeV : 14.54

	Elemental atomic composition of clays (%)						
	C	O	Al	Si	Fe	Na	Cu
$Cu^{2+}MMt$	1.04	63.38	9.77	21.26	1.11	0	3.42

Label A:
Prst:None

SiKa

AlKa

O Ka

CuL

C Ka

FeKa   CuKa

1.00 2.00 3.00 4.00 5.00 6.00 7.00 8.00 9.00 10.0(11.0(12.0(13.0(14.0(
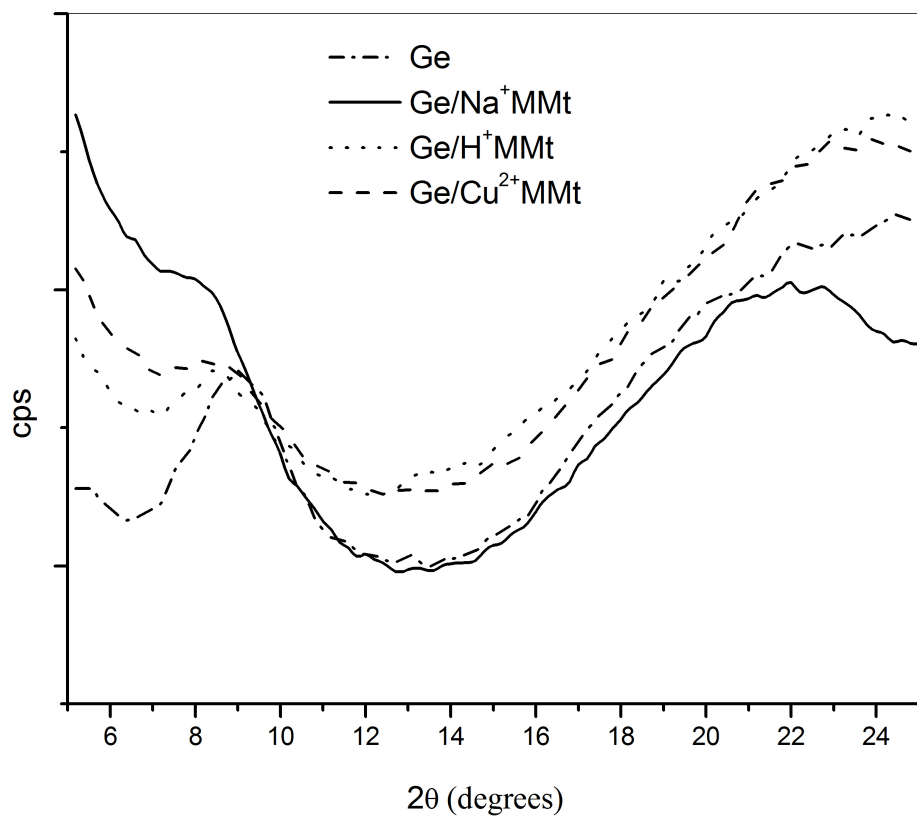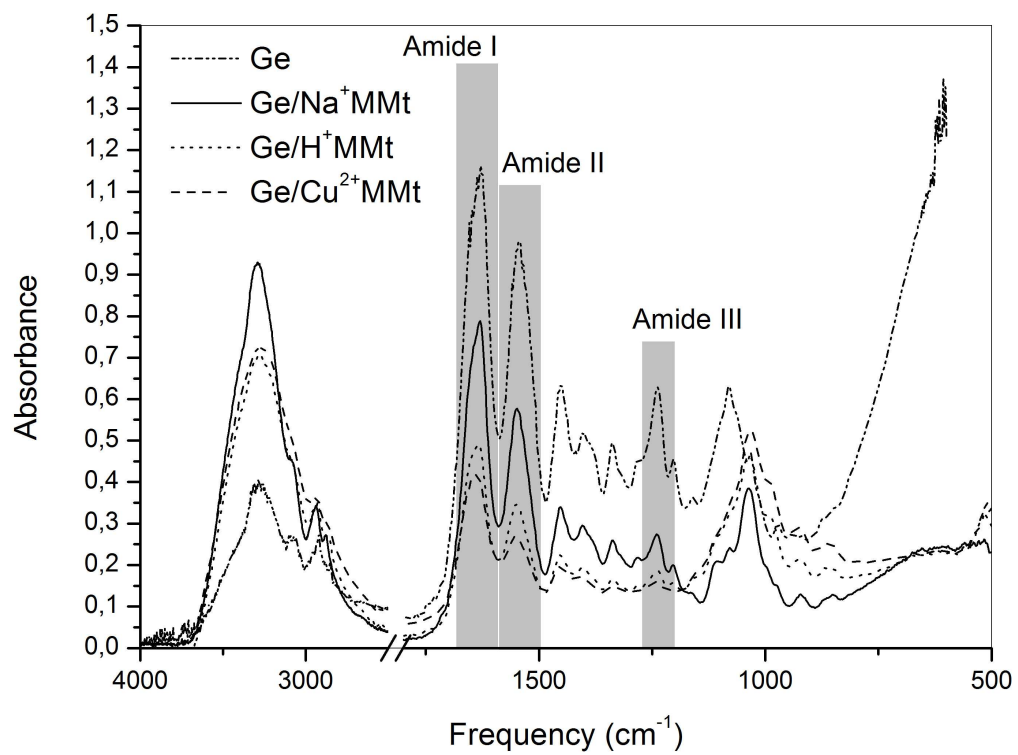FS : 690   CPS : 1240   Cnts : 5   KeV : 13.52

cps

2θ (degrees)

Highlights

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

2. Cu^{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^{2+}MMt films