Improving ham shelf life with a polyhydroxybutyrate/polycaprolactone biodegradable film activated with nisin

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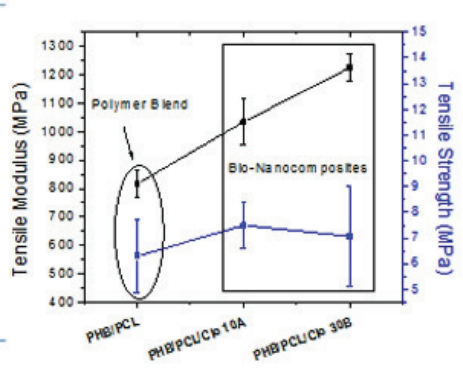
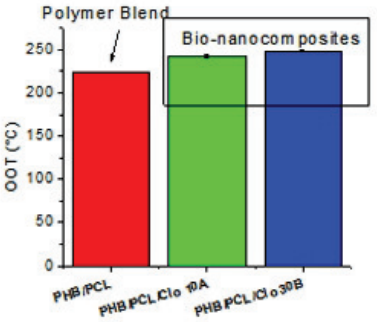
Biodegradable PHB/PCL polymer blend

Organo-modified clays addition

Bio-nanocomposites with mechanical, barrier properties and degradation temperature improvements





Nisin activation

Biodegradable and antimicrobial polymeric films

Nisin activation parameters
- ➢40 °C
- ➢10 min
- ➢4000 IU cm^{-3}

Active against *L. plantarum* CRL691 inoculated in sliced ham

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Abstract

Environmental polution and food shelf life extension are issues of global concern. In this work, biodegradable Polyhydroxybutyrate/Polycaprolactone (PHB/PCL) films and organo-clays (Cloisite® 30B and 10A) based nanocomposites were prepared. Tensile and thermal properties, water vapor barrier and activation with nisin were studied. Organo-clays addition promoted a reinforcement effect of the polymer blend, increasing barrier properties and degradation temperature. The optimal parameters for nisin adsorption to PHB/PCL film were 4000 IU cm^{-3}, 40°C and 10 min. Organo-clays exerted antimicrobial activity against *Lactobacillus plantarum* CRL691; nevertheless, their inclusion into the polymer blend did not lead to antimicrobial films. Nisin adsorption to PHB/PCL film was not afected by clays presence. PHB/PCL nisin activated film was effective against *L. plantarum* CRL691 (used as processed meat spoilage bacterium model) inoculated on sliced ham, thus extending its shelf life. PHB/PCL blend and its nanocomposites activated with nisin showed potential for their application in processed meat packaging.

Keywords: Biodegradable nanocomposite films; active packaging; nisin; ham; shelf life extension.

1. Introduction

Spoilage bacteria lead to economic losses in meat processing industry (Giaouris et al., 2014). To overcome this problem, and according to an increased negative perception towards chemical agents, natural antimicrobial agents have been extensively screened and tested for their effectiveness in meat and processed meat foods (Woraprayote et al., 2016). Active packaging containing bacteriocins showed the ability to inhibit unwanted microorganisms in various meat products (Blanco Massani et al., 2014; Marcos, Aymerich, Garriga, & Arnau, 2013; Woraprayote et al., 2013). In contrast to plant extracts and other natural antimicrobial preservatives, bacteriocins present colorless, odorless, and tasteless characteristics. These characteristics make bacteriocins good candidates for their application in active packaging, since their use does not interfere with sensory quality of food products (Elsser-Gravesen & Elsser-Gravesen, 2013; Woraprayote et al., 2016).

Regulatory requirements for active packaging technologies in the United States are not very different from the requirements for conventional antimicrobial additives. The material exerting antimicrobial effect on food through migration or controlled release would constitute a ‘‘direct additive” and would be subject to FDA regulatory requirements (Restuccia et al 2010). For the European Union, regulation 450/2009/EC set legal basis for the correct use, safety and marketing of active packaging. The use of the active substance must accomplish a technological need and active substances migrating from the packaging shall comply with the conventional rules laid down in the EU Directives (Commission Regulation 1333/2008/EC, Reg 1129/2011 and Reg 1130/2011) (Restuccia et al 2010).

Among hundreds of bacteriocins, nisin is the only commercial bacteriocin approved for food applications (Elsser-Gravesen & Elsser-Gravesen, 2013; Reg 1129/2011). Its

mechanism of action involves initial interaction with the membrane and further lipid II binding. In this way, a stabilized poration complex is formed in the target site and at the same time, sequestration of lipid II causes cell wall biosynthesis inhibition. This dual mode of action, in which inhibition of peptidoglycan synthesis and pore formation are most efficiently combined, makes nisin a very potent antimicrobial agent and impedes the emergence of resistant strains (Islam, Nagao, Zendo, & Sonomoto, 2012).

Bacteriocins adsorption on polymer surfaces offers a way for setting up antibacterial systems. Other strategy is adding antimicrobial agents into polymers. In this regard, commercially montmorillonites modified with quaternary alkyl ammonium salts Cloisite® 10A and 30B showed potent antimicrobial activity; thus, active packaging obtained with these and other modified clays was reported (Nigmatullin, Gao, & Konovalova, 2008; Rhim, Hong, & Ha, 2009; Tornuk, Hancer, Sagdic, & Yetim, 2015).

Polyhydroxybutyrate (PHB) is a renewable thermoplastic material exhibiting intermediate oxygen and water permeability compared to petroleum based polymers. Also, PHB is more attractive than other biopolyesters like polylactic acid (PLA) in terms of barrier properties (Corre, Bruzaud, Audic, & Grohens, 2012). Nevertheless, PHB has limited food packaging applications due to its cost, narrow processability window, stiffness and brittleness properties. Blending PHB with other polymers offers a way to obtain materials with enhanced properties. Polycaprolactone (PCL) is a good candidate for this purpose due to its biodegradable nature, its flexibility, toughness and thermal stability (Lovera et al., 2007). Aditionally, in order to improve PHB mechanical, gas barrier, and thermal properties, nanofillers such as organo-clays could be incorporated at low filler content (less than 5% by weight) and nanoscale distribution (Bordes, Pollet, Bourbigot, & Avérous,

77 2008; Botana, Mollo, Eisenberg, & Torres Sanchez, 2010). However, the incorporation of
78 nanofillers has raised concerns among several researchers, regarding the effects of particles
79 migration from the nanocomposites to the packaged food. The European regulation on
80 plastic materials and articles intended to come into contact with food (Commision
81 Regulation 10/2011) is rather specific with regard to nanoparticles and states that risk
82 assessment of materials in nanoform has to be performed on a case-by-case basis, until
83 more information is known in relation to this new technology. According to Souza &
84 Fernando (2016), nanoparticles could have potential to migrate to the packaged foodstuff,
85 but migration assays and risk assessment are still not conclusive. This scarce information
86 regarding migration is related to the lack of suitable and validated test methods for the
87 identification, characterization and detection of nanoparticles in complex matrices as food
88 (EFSA, 2011). Even when in vitro toxicological research on neat clays is of high interest
89 nowadays, studies dealing with nanocomposites containing clays are still scarcer.
90 Maisanaba et al. (2015) reported that different clays have their own cytotoxic profile with
91 dependence on the experimental conditions (type of clay, modifier, cell line,
92 concentrations used, etc). Despite neat clay Cloisite 30B showed high toxicity effects
93 (Maisanaba et al., 2015), particularly for a Organoclay/Poly(butylene adipate-co-
94 terephthalate) nanocomposite, no citotoxicity was observed with 10% of clay incorporation
95 (Fukushima, Wu, Bocchini, Rasyida & Yang, 2012). Similar results have been revealed for
96 in vitro and in vivo toxicity of organo-clays/PLA nanocomposites (Maisanaba et al. 2014a;
97 Maisanaba et al. 2014b). These authors also concluding that organo-clays/polymer based
98 nanocomposites need to be studied in greater detail, regarding safety issues, in order to
99 support their conclusions.

In this work, a PHB/PCL blend, with a high (50%) content of renewable polymer and organo-clays (Cloisite® 30B and 10A) PHB/PCL based nanocomposites were activated with nisin in order to develop antimicrobial films with enhanced material properties. The effects of the type of organo-clay (Cloisite® 30B and 10A) on the intercalation of polymeric chains into clay galleries, and on the final mechanical properties of the biodegradable nanocomposites were studied. Nisin adsorption at different temperatures was investigated for the PHB/PCL matrix looking for the best conditions to obtain an antimicrobial biodegradable film. Nisin adsorption on PHB/PCL matrix with and without clays was studied and compared to find out possible synergistic antimicrobial effects. Finally, the ability of nisin activated PHB/PCL films to inhibit *Lactobacillus plantarum* CRL691 (used as processed meat spoilage bacterium model) was explored for cooked ham during 4-week period.

2. Materials and methods

2.1 Materials

Polyhydroxybutyrate (PHB) homopolymer commercial grade "Biocycle 1000" was obtained from PHB Industrial S.A., Brazil, in the form of powder with a weight average molecular weight of approximately 600,000 g/mol. Polycaprolactone (PCL) commercial grade "FB-100" was obtained from Perstorp, United Kingdom, in the form of pellets with a weight average molecular weight of 100,000 g/mol. Organo-clays Cloisite® 30B and 10A (Clo 30B and Clo 10A) were obtained from Southern Clay Products, USA.

2.2 *Bacterial strains and growth conditions*

121 *Lactobacillus plantarum* CRL691 was grown in (Man, Rogosa and Sharpe) MRS broth
122 (Britania, Argentina) at 30ºC. The strain, kindly transferred by CERELA-CONICET
123 (Argentina), was maintained and stored at -20 ºC in 0.15 g cm^{-3} of glycerol until use.
124 *2.3. Nanocomposite films preparation*
125 Materials were dried during 8 h under vacuum at 80 °C (PHB and organo-clays) and 40 °C
126 (PCL). For this study, PHB/PCL in a 50/50% (w/w) proportion was used as the blend with
127 the highest content of renewable polymer without detrimental consequences on mechanical
128 properties and processabilty, due to the stiffness and high flowability of PHB. The 50/50
129 PHB/PCL blend and organo-clay based nanocomposites with a Clo10A and Clo30B fixed
130 content at 5% (w/w) were prepared by melt intercalation in an internal mixing chamber
131 Brabender Plasti-corder (30 cm^3) at 165 °C and 50 rpm rotor speed for 5 min.
132 Nanocomposites were labeled as PHB/PCL/Clo10A and PHB/PCL/Clo30B. Films were
133 obtained by compression molding at 175 °C using an 8 min molding cycle.
134 *2.4. Polymer and nanocomposites characterization*
135 *2.4.1. Oxidation onset temperature (OOT)*
136 OOT was performed by dynamic scanning in a differential scanning calorimeter (DSC)
137 Mettler 822e/500/1473 using the method described in ASTM E 2009 (American Society
138 for Testing and Materials, 2014a). Measurements were carried out under an oxygen
139 atmosphere and analyzed from 30 °C to 350 °C at a heating rate of 10°C/min. Results of
140 two determinations were averaged and informed.
141 *2.4.2. X-Ray diffraction (XRD)*
142 Organo-clays structure and their PHB/PCL composites were evaluated with XRD
143 measurements. XRD patterns were taken with a Phillps PW 1730/10 X-ray diffractometer,

144 operated at 40 kV and 30 mA, equipped with Cu Ka radiation at a wavelength of 0.1546
145 nm. Diffraction data was collected over a 2θ range of 1°–10°, with a step width of 0.02°
146 and a counting time of 2.0 s/step. Silicate layer basal spacing (d001) was calculated using
147 the Bragg's equation (1)

148 $\lambda=2d \sin \theta$ (1)

149 Where λ is the wavelength of the X-ray radiation used (0.1546 nm), d is the spacing
150 between diffractional lattice planes and θ is the measured diffraction angle.

151 *2.4.3. Tensile properties*

152 Tensile testing was performed with an INSTRON universal testing machine model 5569
153 according to ASTM D 882-12 method (American Society for Testing and Materials, 2012).
154 The tests were carried out at 23 °C, with a constant rate of 5 mm/min, an initial grip
155 separation of 50 mm and ribbon-shape samples (10 mm width) according to ASTM D 882-
156 12 method.

157 *2.4.4. Water Vapor Permeability (WVP)*

158 Films WVP was measured using the cups method described in ASTM E 96/E96M -14
159 (American Society for Testing and Materials, 2014b). For this test, a cylindrical vessel
160 filled with desiccant (10 g of silica) was sealed with the investigated film and stored in a
161 chamber with controlled temperature and relative humidity (23°C and 50%, respectively).
162 Water mass uptake was monitored as a function of time and the Water Vapor Transmission
163 Rate (WVTR) was calculated from the slope of the mass uptake profile versus time at the
164 steady state. Then, WVP ($g \cdot s^{-1} \cdot m^{-1} \cdot Pa^{-1}$) was calculated using equation (2)

165 $WVP = WVTR \cdot d/\Delta P$ (2)

166 Where WVTR is the water vapor transmission rate ($g \cdot s^{-1} \cdot m^{-2}$), d is the film thickness (m)
167 and ΔP is the water vapor partial pressure difference (Pa). Experiment was run in four
168 replicates.

169 *2.5. Activation with nisin*

170 *2.5.1. Antimicrobial solutions*

171 A nisin stock solution (8000 IU cm^{-3}) was prepared by dissolving 800 mg of commercial
172 nisin powder (Maxinis®, AMG, Buenos Aires, Argentina) in 100 cm^3 of distilled water.
173 Thereafter different nisin concentration solutions (4000, 2000, 500, 200, 100 and 50 IU
174 cm^{-3}) were prepared from the stock solution.

175 *2.5.2. Adsorption kinetic and equilibrium.*

176 500 IU·cm^{-3} of nisin was previously definded as the minimum inhibitory concentration
177 necessary to obtain a PHB/PCL film active against *L. plantarum* CRL691 (data not
178 shown). To optimize nisin adsorption times and temperatures the PHB/PCL film (0.95
179 cm^2) was contacted with 0.260 cm^3 of 500 IU cm^{-3} nisin solution during different times (1,
180 5, 10, 15, 30, 60 and 120 min) at 20, 30 and 40 °C.

181 Nisin adsorption isotherms were obtained by contacting the PHB/PCL film with different
182 peptide concentration solutions (4000, 2000, 500, 200, 100 and 50 IU cm^{-3} of nisin) during
183 30, 15 and 10 min, respectively at 20, 30 and 40 °C.

184 Bacteriocin adsorption capacity on PHB/PCL; PHB/PCL/Clo10A and PHB/PCL/Clo30B
185 was studied by contacting nisin (4000 IU cm^{-3}) during 10 min at 40 °C.

186 After activation treatments, activity on the films and in nisin solutions were evaluated in
187 semisolid agar as described in section 2.5.3. In all cases, triplicate samples were run in two
188 independent experiments.

189 *2.5.3. Activity quantification*

190 Nisin activity in solution (titer) was determined by the agar well diffusion assay
191 (Pongtharangkul & Demirci, 2004). Serial two-fold dilutions of the bacteriocin solutions
192 (15 µl) were added to 5 mm diameter wells cut in semisolid MRS agar plates seeded with
193 *L. plantarum* CRL691 (10^7 CFU·cm^{-3}). After incubation (18 h at 30 ºC) nisin titer,
194 expressed in arbitrary units (AU cm^{-3}), was defined as the reciprocal of the highest dilution
195 yielding a visible zone of inhibition on the sensitive strain. All determinations were
196 performed in triplicate.

197 To quantify nisin on film surfaces the zone of inhibition assay was used. This method is
198 based on the fact that the area of each inhibition zone directly corresponds to the quantity
199 of bacteriocin retained by samples (Massani et al., 2013; Bower et al., 2002; Blanco
200 Massani et al., 2012; Green, Fulghum, & Nordhaus, 2011). For this evaluation, nisin
201 treated film circles (0.95 cm^2) were placed face down on semisolid agar plates seeded with
202 *L. plantarum* CRL691 (10^7 CFU·cm^{-3}) and analized for inhibition zones. Clo30B, Clo10A
203 and their PHB/PCL nanocomposites inhibition zones were also studied. ImageJ 1.47t
204 (Wayne Rasband, National Institutes of Health, USA) was used to measure antimicrobial
205 activity as relative inhibition areas (RIA=inhibition zone area/film area).

206 *2.6. Sliced cooked ham active packaging*

207 *2.6.1. Cooked ham elaboration*

208 Cooked ham was manufactured in a meat processing pilot plant according to Argentinean
209 regulations ("C.A.A.," 1893). A raw pork leg was trimmed off and minced (Themis 32
210 mincer). Extension of 60% was obtained by mixing the pulp with brine (water,
211 carrageenan, sucrose, maltodextrin, sodium chloride, erythorbate, phosphate, nitrate and

212 nitrite). Massage during 40 min was performed (KitchenAid® Stand Mixer) to allow brine
213 homogeneous distribution. Pulp was cooked by a cook-in process in a 80 °C water bath to
214 a core temperature of 72 °C. After cooling (3 °C), the ham was sliced, vacuum-packed
215 (90%-Erlich Best Vacuum), post pasteurized (10 min, 80 °C) and stored (5-6 °C).

216 *2.6.2. Ham inoculation and active packaging*

217 *L. plantarum* CRL691 was resuspended in steryle saline solution (NaCl 8.5 mg cm^{-3}) to
218 reach 10^5 CFU·cm^{-3} (0.15 Abs_{530}). Ham slices (250 g) were inoculated by immersion (30 s)
219 in 25 cm^3 of the *L. plantarum* CRL691 suspension, reaching a final concentration of 10^3
220 CFU per gram of ham. After drying, three PHB/PCL nisin activated films (36 cm^2 each)
221 were used to separate five inoculated slices (25 g) which were included inside a packaging
222 made with untreated Cryovac films (96 cm^2). Following the same procedure PHB/PCL
223 control (without nisin) films were used to obtain control ham packages. Packages were
224 thermo-sealed under vacuum (90%-Erlich Best Vacuum) and stored (5 °C, 4 weeks). This
225 experiment was run in two independet experiments by duplicates.

226 *2.6.3. Microbiological determinations*

227 After inoculation and at 7, 14, 21 and 28 days of storage (5 °C) microbiological
228 evaluations were performed in 10 g obtained by transversely cutting the sliced hams. Each
229 sample was minced with 90 cm^3 of sterile saline solution for 1 min. Homogenate dilutions
230 were obtained with sterile saline solution and duplicate counts of *L. plantarum* CRL691
231 were obtained after incubation (anaerobic conditions, 35 °C, 48h) in MRS. Results were
232 expressed as log CFU g^{-1}. Lag time λ was estimated using the MicroFit v 1.0 software
233 (Institute of Food Research, United Kingdom). pH was measured in all packages.

234 *2.7. Statistical analysis*

Experimental data was subjected to analysis of variance (ANOVA). Tukey test was applied at 0.05 of significance level. All statistical analyses were performed using Minitab Statistic Program, release 12 (Pennsylvania, USA).

3. Results and discussion

3.1. Polymer and nanocomposites characterization

Table 1 presents OOT values, mechanical and barrier properties of PHB/PCL blend and PHB/PCL-based nanocomposites. The addition of organo-clays improved nanocomposites degradation temperature compared to the blend without clays (18 °C for Clo10A and 24 °C for Clo30B). It is known that layered silicates improve thermal stability of the polymer matrix, acting as a heat barrier, raising the overall thermal stability of the system, and enhancing oxygen and oxidation products barrier during thermal decomposition (Alexandre & Dubois, 2000). From an industrial point of view, this is an interesting result since the improvement of the OOT values for the nanocomposites indicates an increase in the processing temperature upper limit for these materials without a significant level of degradation, which implies a wider processing window.

Clo30B and Clo10A X-ray diffraction (XRD) patterns (Fig. 1) revealed diffraction peaks at $2\theta = 4.94$ and $2\theta = 4.53$ respectively, corresponding to 17.9 Å (Clo30B) and 19.5 Å (Clo10A) layer distance. Clay´s peaks were shifted to lower angle for nanocomposites, corresponding to higher interlayer distances for PHB/PCL/Clo30B (37.1 Å) and PHB/PCL/Clo10A (36.9 Å). The higher basal spacings of clays in the nanocomposites as compared to the pristine organo-clays are due to the intercalation of polymer chains inside the clay layers (Alexandre & Dubois, 2000). The extent of this intercalation calculated as an increase in d-spacing (Δd_{001}) was higher for PHB/PCL/Clo30B than for

258 PHB/PCL/Clo10A (19.2 and 17.5 Å, respectively). This result could be related to the
259 strong hydrogen bonding and interactions between the carboxyl groups of PHB and PCL in
260 the blend and the hydroxyl group in the gallery of Clo30B. Similar results were reported
261 for the preparation of other biodegradable polyester/organo-clay nanocomposites (Bordes
262 et al., 2008; Botana et al., 2010; Ray & Bousmina, 2005). The peaks around 2θ= 4.54
263 (PHB/PCL/Clo30B) and 4.38 (PHB/PCL/Clo10A) may be originated by agglomerated
264 organo-clay particles caused by collapse of interlayer space of clays due to the thermal
265 degradation of the alkyl ammonium ions during nanocomposites processing and/or the d_{002}
266 reflection (Botana et al., 2010; Najafi, Heuzey, & Carreau, 2012).
267 According to Alexandre & Dubois, (2000) and Carli, Crespo, & Mauler, (2011), clays
268 intercalation process increases tensile modulus. Results of mechanical properties (Table 1)
269 showed that clays addition promoted a reinforcement effect given by a significant increase
270 in the tensile modulus (around 27% for Clo10A and 50% for Clo30B), while tensile
271 strength was not affected in comparison with PHB/PCL neat blend. However this
272 enhancement in tensile properties was at the expense of the deformation at break with both
273 types of clays. This behavior is in agreement with published results on
274 PHA/montmorillonite nanocomposites, and can be attributed to either degradation of the
275 clay organomodifier that may alter physical and mechanical properties of the polymer
276 matrix, or embrittlement due to the presence of some non intercalated clay stacks (Bordes
277 et al., 2008; Carli et al., 2011).
278 WVP is an important property because it indicates the amount of water vapour that
279 permeates per unit of area and time through the packaging material. WVP values of the
280 PHB/PCL and PHB/PCL-based nanocomposites (Table 1) showed that organically

281 modified clays significantly increased barrier properties, since a decrease in
282 nanocomposites WVP when comparing to that of neat PHB/PCL 50/50 blend was
283 observed (59% for films compounded with Clo10A and 52% for films with Clo30B).
284 Reduction of the WVP values observed in the nanocomposites can be attributed to long
285 and tortuous paths created by clay platelets distributed in the polymer matrix, slowing
286 down the progress of water molecules through the matrix (Alexandre & Dubois, 2000).
287 This result is important since lower WVP values indicate better moisture protection to the
288 packaged food and, consequently, a potential reduction of food deterioration due to
289 excessive gain of water during storage (Siracusa, 2012). Also, WVP values of PHB/PCL-
290 based nanocomposites found in our work (Table 1) are similar to the informed for neat
291 PLA commercial extruded films (1.8×10^{-11} $g \cdot s^{-1} \cdot m^{-1} \cdot Pa^{-1}$) (Corre et al., 2012).
292 The nanocomposites obtained in this study presented improved thermal stability,
293 mechanical, and barrier properties, compared to the neat PHB/PCL blend. The feasibility
294 of improving these properties after organo-clays addition to the blend reveals true potential
295 to expand the range of biodegradable materials currently used for various packaging
296 applications.

297 *3.2. Films activation*

298 *3.2.1. Adsorption kinetic and equilibrium*

299 For the development of active polymers for potential food applications, the study of
300 bacteriocins adsorption on a determined surface yields to the knowledge of activation
301 parameters (optimal time/temperature, shape of adsorption curves and saturation point),
302 which can be obtained from the analyses of kinetics an equilibrium of adsorption (Massani,
303 Vignolo, Eisenberg, & Morando, 2013). When assaying relative inhibition areas, the size

304 of the inhibition zone is related to the diffusion constant for the antimicrobial agent
305 evaluated in the media as well as to the total amount of agent that is available to diffuse
306 (Green, Fulghum, & Nordhaus, 2011). That is to say, for the same experimental conditions
307 (adsorbed antimicrobial tested, culture media, sensitive strain and inoculum size,
308 incubation temperature for culture growth, etc.) and for bacteriocins that completely elute
309 from the surface, the area of each inhibition zone directly corresponds to the quantity of
310 bacteriocin adsorbed by samples (Bower et al., 2002; Green et al., 2011; Massani, Vignolo,
311 Eisenberg, & Morando, 2013). In order to find the kinetic of adsorption at different
312 temperatures, a 500 IU cm^{-3} nisin solution was contacted with the PHB/PCL film during
313 different times (1 to 120 min) and the correspondings RIA were analyzed. Adsorption
314 plateau was defined as the time, at a fixed temperature, from which RIA were not
315 significantly different ($p \geq 0.05$) (Fig. 2). It was observed that activation temperature
316 affected both, the maximum of adsorption (plateau) and the time needed to reach the
317 plateau. Thus, at lower activation temperature higher times were required to reach the
318 adsorption plateau, being respectively 30, 15 and 10 minutes, the necessary times for
319 adsorption equilibrium attainment at 20, 30 and 40 °C (Fig. 2). In contrast, Guerra et al.,
320 (2005a) studying nisin adsorption in rubber, PET and stainless steel at 8, 25, 40 and 60 °C
321 reported that temperature had no influence on adsorption time (12 h). In our study
322 adsorption acceleration by temperature was observed, obtaining the lowest time for
323 adsorption equilibrium (10 min) at 40 °C this probably indicating that temperature increase
324 favoured dehydration of nisin in aqueous solution, promoting bacteriocin adsorption to the
325 surface as already explained by Norde (1996) and previously found for other bacteriocins
326 (Massani et al., 2013). Nevertheless, the highest RIA at the plateu attainment was observed

327 at 30°C, then films nisin activity following the order 20 °C>40 °C (Fig. 2). Temperature
328 rise could be leading to a decrease in bacteriocin active concentration available to be
329 adsorbed on the film, as earlier reported (Massani et al., 2013). The results on adsorption
330 kinetics in our system (nisin-PHB/PCL film) led to adsorption times (10 min at 40°C; 15
331 min at 30°C and 30 min at 20°C) lower than those reported for activation with nisin of
332 other surfaces such as cellophane, PET, and stainless steel (12 h at 8°, 25°, 40° and 60°C)
333 (Guerra et al., 2005a; Guerra, Macias, Agrasar, & Castro, 2005b).
334 For the purpose of modeling nisin adsorption to the PHB/PCL film at different
335 temperatures and constructing adsorption isotherms, the bacteriocin was contacted with the
336 film in different concentrations at 20, 30 and 40 °C. Results of nisin RIA as a function of
337 the bacteriocin titers after adsorption were fitted as shown in Figure 3. For all the assayed
338 temperatures, it was observed that nisin RIA on the activated film increased with the
339 concentration of nisin contact solution up to a point (nisin contact solution 4000 IU cm^{-3},
340 34133 AU·cm^{-3} after adsorption) from which RIA were not significantly different from
341 each other ($p \geq 0.05$). Thus, a steep initial slope of bacteriocin adsorption on PHB/PCL
342 followed by an adsorption plateau at higher concentrations was observed (Fig. 3). This
343 behavior could be subjected to an empirical treatment according to Langmuir equation
344 which implies monolayer adsorption (Guerra et al., 2005a). From this, a PHB/PCL film
345 saturation point begining with 4000 IU cm^{-3} nisin contact solution was defined. From
346 equilibrium determinations (Fig 3) the same adsorption plateau at different temperatures
347 was obtained, suggesting that 40 °C could be the best activation temperature, due to the
348 reduction of contact time in comparison to 20 and 30 °C. As a consequence, 40 °C, 10 min

349 and a 4000 IU cm^{-3} nisin contact solution were defined as the optimum parameters for
350 PHB/PCL matrix activation.
351 *3.2.2. Materials antimicrobial activity and activation comparison*
352 Clo10A, Clo30B, PHB/PCL and their nanocomposites were assayed against *L. plantarum*
353 CRL691 to study their antimicrobial activity. It was observed that even when Clo10A and
354 30B were active against the microorganism, antimicrobial activity was not observed for
355 either film without nisin treatment (Table 2). Clay/polymer nanocomposites biocidal
356 activity is a function of antimicrobial interchanged cation, and the composites
357 nanostructure. It appears that at least two mechanisms are responsible for the antimicrobial
358 activity of the organo-clays, i.e., activity of surfactants released from the clay and the
359 action of the solid surface (Nigmatullin et al., 2008). Partial degradation of alkyl
360 ammonium ions during nanocomposites processing as previously discussed (section 3.1;
361 Botana et al., 2010; Carli et al., 2011) could be a possible explanation for antimicrobial
362 activity lack in PHB/PCL nanocomposites. On the other hand, intimate contact between a
363 bacterial cell and organo-clay antimicrobial surface may be crucial to affect the cell
364 (Nigmatullin et al., 2008). In order to describe interlayer organo-clays structure, it is
365 believed that the cationic head group of the alkyl ammonium molecule preferentially
366 resides at the layer surface, leaving the organic tail radiating away from the surface
367 (Alexandre & Dubois, 2000). Botana et al., (2010) reported that when PHB crystallizes in
368 presence of clay mineral particles, crystals could grow on the particle surfaces. Moreover,
369 PHB/PCL nanocomposites thermal studies (DSC) suggested that clays may be acting as
370 crystallization agents (Correa et al., unpublished results). Then, interchanged cations from
371 clays filled into PHB/PCL matrix might be entrapped into the PHB crystals, being not

372 available to migrate in a concentration above the minimum inhibitory concentration needed
373 to inhibit the sensitive microorganism. Tornuk et al. (2015) reported antimicrobial activity
374 of LLDPE nanocomposites (based on MMT and halloysite) grafted with bioactive
375 compounds when using in processed meat packaging applications. In our work, since
376 organoclays addition did not impart antimicrobial activity to the PHB/PCL based film,
377 nisin adsorption was suggested in order to reach an active material capable of inhibiting
378 lactic acid bacteria (LAB) during cooked ham storage.
379 PHB/PCL organo-clay films were treated with nisin using the optimized parameters (40
380 °C, 10 min, 4000 IU·cm^{-3} nisin contact solution), assayed against *L. plantarum* CRL691
381 and compared to nisin activated PHB/PCL (Table 2). All surfaces exerted uniform
382 inhibition areas and RIA presented no significant differences ($p \geq 0.05$) (Table 2). As
383 aboved mentioned, the relative inhibition area displayed by a bacteriocin treated surface
384 has already been associated to the amount of bacteriocin adsorbed; i.e., the smallest
385 inhibition zone corresponded to the smallest bacteriocin adsorbed mass (Massani, Vignolo,
386 Eisenberg, & Morando, 2013; Bower et al., 2002). Based on this assumption, the lack of
387 significant differences between the RIA found after activating PHB/PCL and organo-clay
388 films with nisin (Table 2) could be indicating that nisin activation was not influenced by
389 organo-clays addition to the PHB/PCL film. Karam et al. (2013) performing XPS and
390 time-of-flight secondary ion mass spectrometry measurements on nisin adsorbed to LDPE
391 and the polymer grafted with acrylic acid confirmed that the peptide formed a multilayer
392 coverage, and films antimicrobial activity was related to nisin distribution on the surfaces.
393 In our work, nisin antimicrobial activity on a neat PHB/PCL and PHB/PCL nanocomposite

394 films was uniform (Table 2) and peptide adsorption seemed to occur by monolayer
395 coverage (Fig. 3).
396 As discussed in section 3.1, nanocomposites presented better properties when compared
397 with neat PHB/PCL blend. However, either the barrier or the mechanical properties
398 observed in our work were not sufficient to satisfy all the requirements imposed on the
399 materials used for meat products packaging (Sukhareva, Yakovlev, Legonkova, & Zaikov,
400 2008; Kerry, Kerry & Ledwar, 2002). For this reason, to asses the efectiveness in a
401 processed meat product, the materials obtained in this work were proposed as ham
402 separating films. Also, considering that either PHB/PCL film or organo-clays added
403 PHB/PCL films showed the same performance as nisin carriers, and until having more
404 insights into the safety issues concerning the use of nanocomposites in direct contact with
405 foods (European Commission, 2011; EFSA, 2011; Maisanaba et al, 2015; Souza &
406 Fernando, 2016), neat PHB/PCL films were used as nisin support for experiments
407 performed to asses effectiveness in food.
408 *3.3. PHB/PCL film as active material for ham storage*
409 During ham production, the aim of thermal processing is to reduce the number of bacteria
410 present to a reliable level which ensures the safety and stability of the product.
411 Nevertheless, cross-contamination occurs throughout subsequent product handling, slicing
412 and packaging (Dušková, Kameník, Lačanin, Šedo, & Zdráhal, 2016). LAB were informed
413 as a microbial population capable of reaching numbers corresponding to the spoilage
414 threshold (around 6 to 7 log CFU g^{-1}) in cooked hams (Dušková et al., 2016; Pothakos,
415 Samapundo, & Devlieghere, 2012). Figure 4a shows the growth of *L. plantarum* CRL691
416 obtained for inoculated active (PHB/PCL nisin activated separating films) and control

417 (PHB/PCL separating films) ham packages. In control packages, typical LAB grown was
418 observed with an initial count of 3.48±0.13 log CFU g^{-1} units, raising to 6.60±0.20 log
419 CFU g^{-1} at the end of the experiment (28th day) (Fig 4a). Meanwhile for active packages,
420 inhibition of the LAB was observed up to the 21st day (3.16±0.56 log CFU g^{-1}), then
421 reaching 3.99±0.20 log CFU g^{-1} (28th day) (Fig 4a). These results are in agreement with
422 Kalschne et al., (2014) who examined the possibility of controlling LAB development on
423 vacuum-packed sliced cooked ham by the addition of nisin. The mentioned authors found a
424 decrease of LAB growth by 2 or 3 log cycles upon antimicrobial addition. Data shown in
425 Fig 4a clearly indicates a bacteriostatic inhibition effect over *L. plantarum* CRL691
426 inoculated on ham, with 3.4 log cycles reduction at the 21st day, and 2.6 log cycles
427 reduction at the end of the experiment (28th day). Lag phase extention is essential in order
428 to avoid unwanted microorganisms growth. Lag phase, calculated with MicroFit software,
429 was extended from 7.03 to 22.39 days due to nisin effect in the active packages, avoiding
430 LAB counts to reach more than 6 log units (Fig 4a), thus prolonging ham shelf life up to 28
431 days (Dušková et al., 2016; Kalschne, Geitenes, Veit, Sarmento, & Colla, 2014; Pothakos
432 et al., 2012).
433 pH changes in active and control packages during storage time are shown in Figure 4b. A
434 slight decrease in control packages at the end of the storage was observed (6.36 to 6.10),
435 while pH levels remained unaffected for active packages (Fig 4b). pH decrease in control
436 packages could be explained by the inoculated LAB growth. Our results of pH invariability
437 around 6.3 due to LAB inhibition in packages containing nisin are in coincidence with data
438 reported by Kalschne et al. (2014).
439 **4. Conclusions**

440 PHB/PCL biodegradable polymer blend and organo-clay based nanocomposites were
441 studied and characterized as nisin carriers for their application in food packaging.
442 Cloisite® 30B showed better intercalation and interaction with the PHB/PCL matrix than
443 Cloisite® 10A. The nanocomposites obtained in this study presented better thermal
444 stability, mechanical and barrier properties compared to the neat PHB/PCL blend. The
445 feasibility of improving these properties offers the possibility to expand the range of
446 biodegradable materials that could be used for different packaging applications. The
447 optimal parameters for PHB/PCL film activation with nisin were 4000 $IU \cdot cm^{-3}$ contact
448 solution, 40 °C and 10 min. Organo-clay fillers did not exert synergistic antimicrobial
449 effects when combined with nisin upon films activation. PHB/PCL nisin activated film was
450 effective in inhibiting *L. plantarum* CRL691 inoculated on ham. This work opens the
451 possibility to consider nisin activated PHB/PCL polymer blend and/or PHB/PCL/organo-
452 clay based nanocomposites as active materials for potential food packaging applications.
453 The feasibility of obtaining an entirely biodegradable antimicrobial packaging with nisin,
454 PHB/PCL and nanocomposites involving organoclays is further to be studied. So far, we
455 demonstrated the workability of these blends upon nisin activation, and nisin-PHB/PCL
456 effectiveness as shelf life extender for vacuum-packed sliced cooked ham.

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632 **Figure captions**

633 **Figure 1.** XRD patterns of pristine Cloisite 10A (Clo 10A), Cloisite 30B (Clo 30B)
634 organo-clays and their Polyhydroxybutirate/Polycaprolactone/Clay based nanocomposites
635 (PHB/PCL/Clo 10A and PHB/PCL/Clo 30B). d(001) basal spacings were included in Å.

636 **Figure 2.** Nisin adsorption on PHB/PCL at 20°C (▼), 30°C (●) and 40°C (■) during
637 different contact times. Continuous lines mark tendencies. Error bars indicate standard
638 deviations from two independent experiments by triplicates.

639 **Figure 3.** Nisin adsorption isotherms on PHB/PCL at 20°C (▼), 30°C (●) and 40°C (■).
640 The curves drawn through the data follow Langmuir equation. Error bars indicate standard
641 deviations from two independent experiments by triplicates.

642 **Figure 4.** *L. plantarum* CRL961 growth (a) and pH changes (b) during 28 days at 5 ºC in
643 active (▲) and control (●) ham packages. Lines between points mark tendencies. Error
644 bars indicate standard deviations from two independent experiments by duplicates.
645

Abbreviations and acronyms

PHB, Polyhydroxybutyrate/

PCL, Polycaprolactone

IU, International units

AU, Arbitrary units

Clo 30B, Cloisite® 30B

Clo 10A, Cloisite®10A

OOT, Oxidation onset temperature

DSC, Differential scanning calorimeter

XRD, X-Ray diffraction

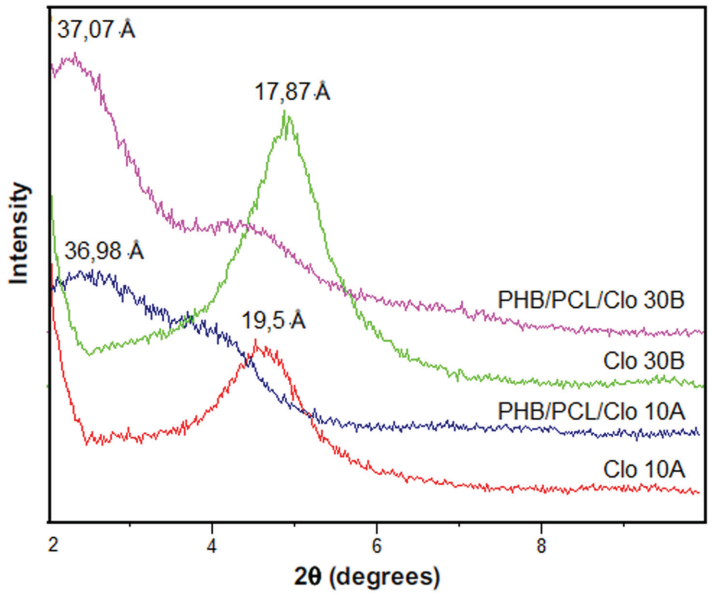
WVP, Water Vapor Permeability

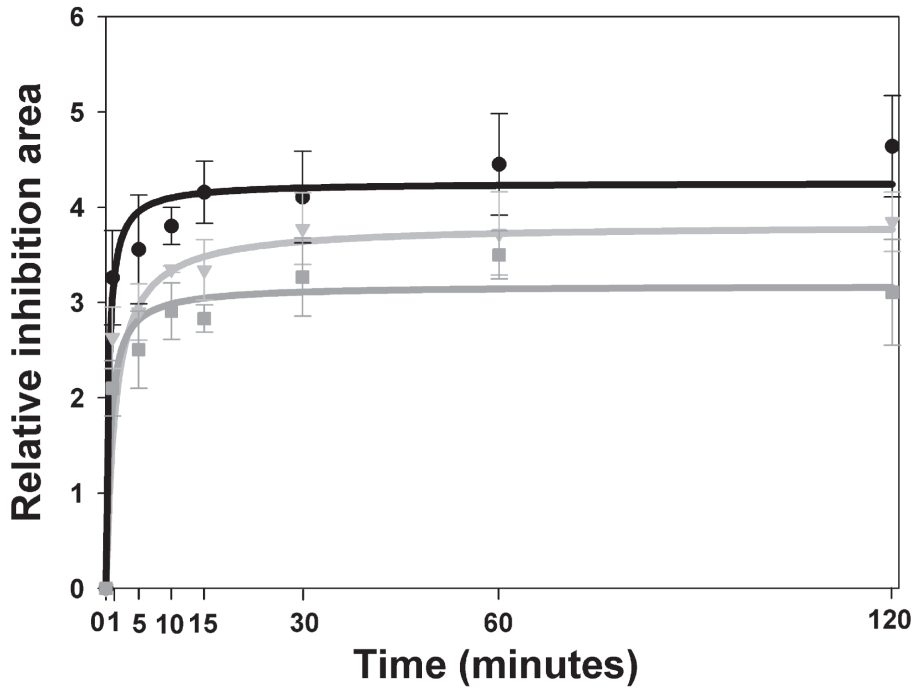
RIA, relative inhibition areas

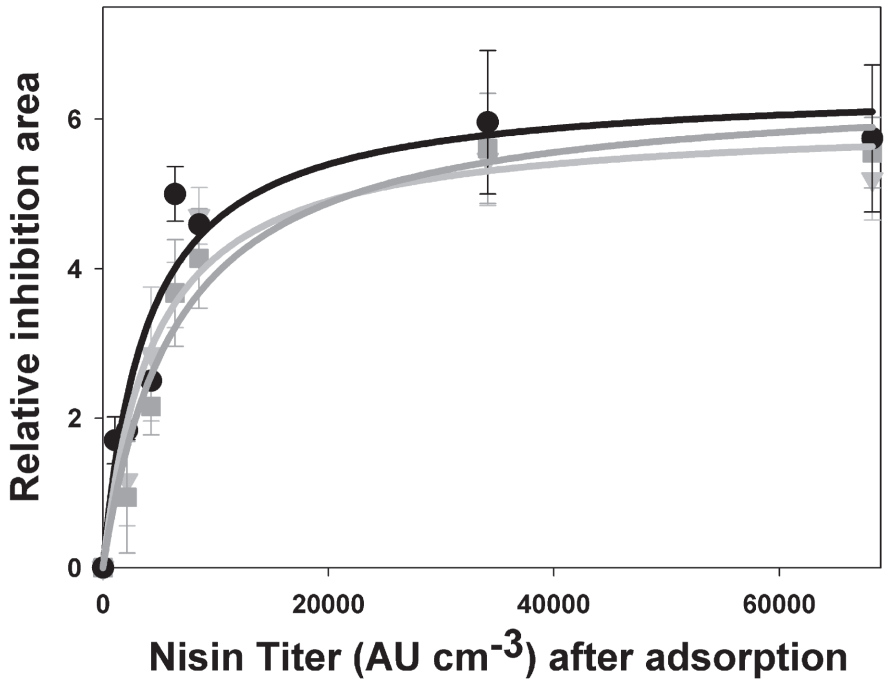
PLA, Polylactic acid

MMT, Montmorillonite

LAB, lactic acid bacteria


37,07 Å
17,87 Å
36,98 Å
19,5 Å
PHB/PCL/Clo 30B
Clo 30B
PHB/PCL/Clo 10A
Clo 10A
Intensity
2
4
6
8
2θ (degrees)


Relative inhibition area
0
1
2
3
4
5
6
0 1 5 10 15 30 60 120
Time (minutes)


Relative inhibition area
0
2
4
6
0
20000
40000
60000
Nisin Titer (AU cm-3) after adsorption

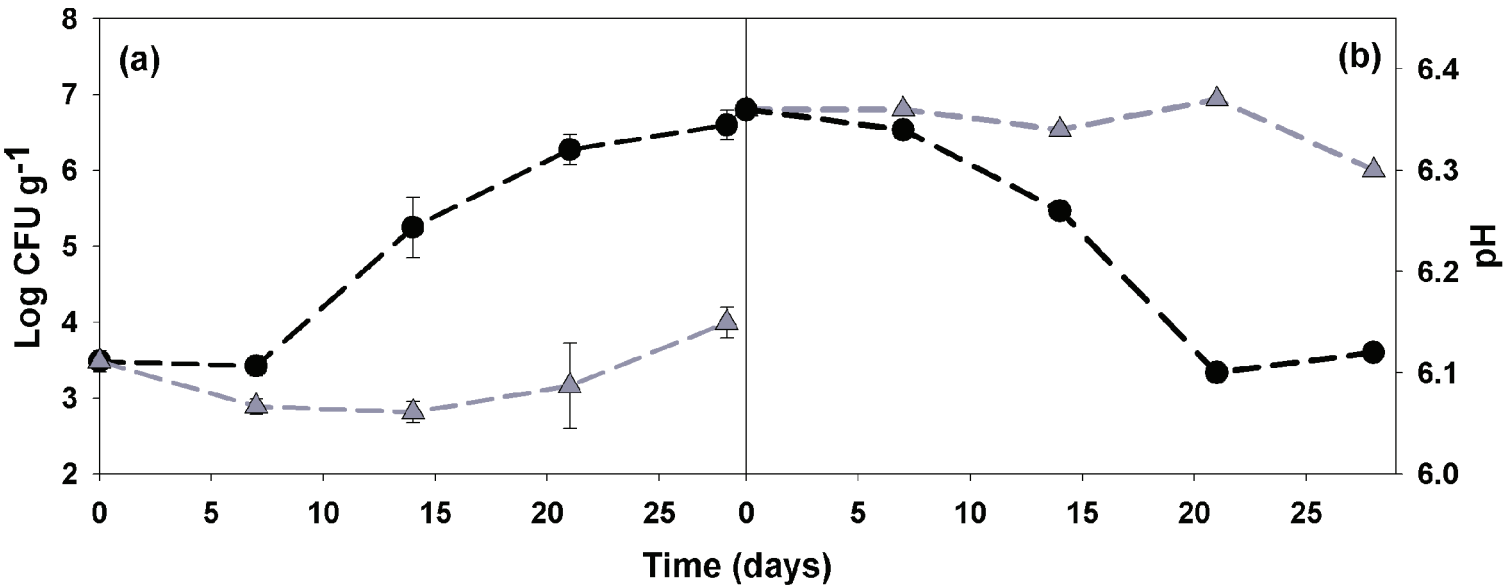

(a)
(b)
Log CFU g-1
pH
Time (days)
8
7
6
5
4
3
2
6.4
6.3
6.2
6.1
6.0
0
5
10
15
20
25

Table 1. Indicated material properties average for neat PHB/PCL 50/50 blend and PHB/PCL/clay nanocomposite films.

Property	PHB/PCL	PHB/PCL/ Clo 10A	PHB/PCL/ Clo 30B
OOT (°C)†	224.1±0.2 [a]	242.4±2.1 [b]	248.2±0.9 [c]
Tensile Modulus (MPa)‡	817 ± 45 [a]	1035 ± 81 [b]	1226 ± 46 [c]
Tensile Strength (MPa) ‡	6.29± 1.42 [a]	7.49± 0.89 [a]	7.06 ± 1.96 [a]
Elongation at break (%) ‡	3.03± 1.71 [a]	0.91 ± 0.16 [b]	0.72 ± 0.19 [b]
WVP (x10^{-11} g·s^{-1}·m^{-1}·Pa^{-1})†	2.62± 0.15 [a]	1.05± 0.06 [b]	1.26± 0.07 [c]

[a-c] Different letters in the same line indicate significative differences ($p < 0.05$).

† Mean of four determinations ± standard deviation.

‡ Mean of five replications ± standard deviation.

PHB/PCL, Polyhydroxybutyrate/ Polycaprolactone

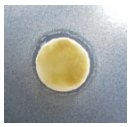
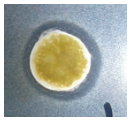
Clo10A, Cloisite® 10A

Clo30B, Cloisite® 30B

OOT, Oxidation onset temperature

WVP, Water Vapor Permeability

Table 2. Antimicrobial activity of organically modified montmorillonites, nisin treated and non-treated films.

Sample	Relative inhibition area against *L. plantarum* CRL691	Inhibition behavior on semisolid agar
Clo 10A	1.5±0.3 [a]	
Clo 30B	2.2±0.2 [b]	
PHB/PCL	ND [†]	
PHB/PCL/Clo 10A	ND [†]	
PHB/PCL/Clo 30B	ND [†]	
PHB/PCL + nisin	5.5±0.5 [c]	
PHB/PCL/Clo 10A + nisin	5.9±0.7 [c]	
PHB/PCL/Clo 30B + nisin	5.9±0.9 [c]	

[a-c] Different letters within the table indicate significative differences ($p<0,05$) from two independent experiments by triplicates.

[†] ND, not detected

Clo10A, Cloisite® 10A

Clo30B, Cloisite® 30B

PHB/PCL, Polyhydroxybutyrate/ Polycaprolactone

Highlights

Polyhydroxybutyrate/polycaprolactone (PHB/PCL) films were reinforced with organoclays

Organoclays addition increased barrier properties and degradation temperatures

PHB/PCL and its nanocomposites were used as nisin support

PHB/PCL nisin activated film was effective against *Lactobacillus* inoculated in ham

Biodegradable active film allowed extending ham shelf life from 7 to 28 days