

Green pesticides based on cinnamate anion incorporated in layered double hydroxides and dispersed in pectin matrix

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*Original*

Green pesticides based on cinnamate anion incorporated in layered double hydroxides and dispersed in pectin matrix / Bugatti, V., Vertuccio, L., Zara, S., Fancello, F., Scanu, B., Gorrasi, G.. - In: CARBOHYDRATE POLYMERS. - ISSN 0144-8617. - 209:(2019), pp. 356-362. [10.1016/j.carbpol.2019.01.033]

*Availability:*

This version is available at: 11388/220074 since: 2022-05-23T10:40:25Z

*Publisher:*

*Published*

DOI:10.1016/j.carbpol.2019.01.033

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note finali coverpage

(Article begins on next page)

Manuscript Number: CARBPOL-D-18-04372R1

Title: Green pesticides based on cinnamate anion incorporated in layered double hydroxides and dispersed in pectin matrix

Article Type: Research Paper

Keywords: pectin, LDH, cinnamate, green pesticide, antimicrobial activity

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Abstract: This paper reports the preparation of green pesticides based on nano-hybrids composed of a Layered Double Hydroxide (LDH) with cinnamate anion. The dispersion into a pectin matrix was obtained using high energy ball milling in wet conditions. Structure and physical properties of the fillers and the composites films were evaluated. Controlled release of cinnamate was followed using UV spectrophotometry and the release kinetics were found to be dependent on the filler loading. The experimental results were analyzed by the Gallagher-Corrigan model. Antimicrobial activity was evaluated on different bacterial strains, as well as plant pathogens belonging to the genus *Phytophthora* using modified agar diffusion, broth microdilution and dual culture methods, respectively. Experimental results suggested the possibility to use the analyzed composites as green protective coatings for crops' protection.

Dear Professor Kennedy,

I send you the original paper “*Green pesticides based on cinnamate anion incorporated in layered double hydroxides and dispersed in pectin matrix*” by Valeria Bugatti, Luigi Vertuccio, Severino Zara, Francesco Fancello, Bruno Scanu and myself to be considered for publication in *Carbohydrate Polymers*.

In this study, the preparation of a green pesticide based on nano-hybrids composed of a Layered Double Hydroxide (LDH) with cinnamate anion is reported. The dispersion into a pectin matrix was obtained using high energy ball milling in wet conditions. Structure and physical properties of the fillers and the composites films were evaluated. Controlled release of cinnamate was followed using UV spectrophotometry and the release kinetics were found to be dependent on the filler loading. The experimental results were analyzed by the Gallagher-Corrigan model. Antimicrobial activity was evaluated on different bacterial strains, as well as plant pathogens belonging to the genus *Phytophthora*, using modified agar diffusion, broth microdilution and dual culture methods, respectively. Experimental results suggested the possibility to use the analyzed composites as green protective coatings for crops’ protection.

I do believe that this paper could be of great interest for *Carbohydrate Polymers*’ Readers, and I hope that you will positively take it into account.

I declare:

- any conflict of interest
- that the manuscript is original, not submitted or under consideration in any other journal
- that all the co-authors have agreed for submission to *Carbohydrate Polymers*
- that all figures and tables are original

I thank you for your time and concern and I send you my best regards,

Giuliana Gorrasi

*prof. Giuliana Gorrasi*  
*Department of Industrial Engineering-University of Salerno-*  
*via Giovanni Paolo II 132, 84084 Fisciano (SA)-Italy*  
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*tel: +39089964146-4019; fax: +39089964057*

Dear Editor,

I send you the revised version of the original paper “*Green pesticides based on cinnamate anion incorporated in layered double hydroxides and dispersed in pectin matrix*” (Ms. Ref. No.: CARBPOL-D-18-04372) by Valeria Bugatti, Luigi Vertuccio, Severino Zara, Francesco Fancello, Bruno Scanu and myself to be considered for publication in *Carbohydrate Polymers*.

We thank you for your Editorial Report and the Reviewers for their appreciation at work and very useful comments and suggestions that greatly helped to improve the manuscript quality.

Our modifications are highlighted in yellow in the text.

Following our point by point answer to the Reviewers. In *Italic* font our answers.

We hope now the paper can be accepted for publication in *Carbohydrate Polymers*.

I thank you for your time and concern and I send you my best regards,

Giuliana Gorrasi

*prof. Giuliana Gorrasi*  
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### **Reviewer #1: Minor Revisions for acceptance**

The paper written by Bugatti et al reported the chemical modification of LDH by cinnamate counter anions in order to produce pectin nanocomposites for antimicrobial activities. In addition, this paper highlighted the final properties of the resulting nanocomposites by studying the water barrier properties and the mechanical performances. This paper is well-structured and described. This work can be accepted in *Carbohydrate Polymers* after minor revisions.

*We thank very much the Reviewer for her/his appreciation at work*

-Change the Figure 3 by one table summarizing the mechanical data and their standard deviations.

*We removed Figure 3 and summarized the mechanical parameters in Table 2*

-Don't use the term galleries but rather layers and use basal spacing or interlayer distances for XRD diffraction

*We changed the text accordingly to Reviewer's suggestions*

-As FTIR is a technique of surface, it is not representative of the intercalation of cinnamate into LDH layers. Please, remove this part because TGA and XRD are sufficient to justify the intercalation of LDH.

*We removed the FTIR analysis and deleted this part in the text*

-The conclusion should highlight the good results obtained, not in the form of a list but in the form of a discussion. Please re-write (rearrange) the conclusion.

*We re-wrote the conclusions*

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The manuscript numbered CARBPOL-D-18-04372 and entitled: Green pesticides based on cinnamate anion incorporated in layered double hydroxides and dispersed in pectin matrix by Bugatti et al. describes the fabrication of composite based on varying quantities of pectin and layered double hydroxides intercalated with cinnamate which serves as the active molecule with a reported loading of 36%. A full investigation of the film properties is conducted as well as the controlled release of cinnamate over time. Furthermore, the antimicrobial activity of various bacteria and phytophthora was investigated. The largest percentage growth inhibition was 53.3% for *P. cinnamomic*.

The manuscript is novel, valuable to the scientific community and fits well with the readership of Carbohydrate Polymers. I recommend the manuscript for publication pending the address of a few minor questions and comments.

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- 1- In my experience pectin is degradable in water, how will the dispersion of LDH layer in pectin prevent its re-aggregation? To be clear is it just the retardation of this effect that is accomplished with pectin?

*Water was used to dissolve the pectin (and filler) for obtain films from casting. This is the only way to obtain pectins' films (manufactures to be analysed) because this material does not melt with temperature. A possible effect of water degradation on the material is excluded because the evaporation of the solvent is fast (24 h under fume cupboard for our thin samples). In addition, we further dried the films in a vacuum oven at room temperature for 3 days, as reported in the experimental part.*

- 2-In line 70 the sentence should read antimicrobials to reduce... or antimicrobials for the reduction...

*The sentence was corrected as suggested from the Reviewer*

- 3 In line 112 the authors mention all the films has a thickness of approximately 300um could the author include how they measured the thickness.

*We reported the method for the thickness measurement (see section 2.3)*

- 4 Could author clarify the sentence starting in line 263: "The absence of any diffraction peak relative to the filler, the spectra of the composited, suggests the exfoliation of the LDH- cinnamate in the used processing conditions." For less familiar readers could the authors specifically stated what is the filler and where they would expect the filler peak and what is the consequence of the LDH layer delamination.

*We better clarified this concept (see section 3.2 -XRD analysis-)*

5 In figure 4 there are fits conducted but not comment of the error of the fit and parameters obtained.

*We thank the Reviewer for this useful comment. In order to better evidence the errors for any experimental data, we preferred to put them in Table 3. Being the diffusion data on logarithmic scale, it would have been difficult to see them on the graph.*

6-Could authors comment on the choice of pectin and 10% LDH-cinnamate composite for bacterial testing.

*Such sample was chosen as model sample because contains the maximum active specie. It was used to investigate the effect of the active molecule bonded to the LDH and dispersed into the pectin. Work is in progress in order to investigate the same antibacterial properties on composites with different active molecule loading.*

7- The results with errors should be presented to the same decimal places as the error. Example in Table 4 all results should be presented to 2 decimal places.

*We thank the Reviewer for this very useful comment. The Table 4 (now table 5) was follows her/his indications.*

8- Fungicides are known to mask pathogens in the plants and the plants go on to develop the disease at a later stage (Jung. et al. 2018) could authors comment on how their composite would outperform the fungicides on this front.

*In this work, we tested the effects of our composite on the growth of two Phytophthora species through an in vitro experiment. The strong inhibition rate detected on P. cinnamomi represents itself a very promising result, comparable to some fungicides. However, in order to determine whether this compound could outperform synthetic fungicides in controlling disease development, further studies are needed to investigate its effect on the survival structures of the pathogen (chlamydospores and oospores) in planta (see comments in the conclusions)*

Nano-hybrid composites pesticide were prepared incorponating cinnamate into LDH

The dispersion into a pectin matrix was obtained using high energy ball milling

Antimicrobial activity was evaluated on plant pathogens

The composites show promising application as green coatings for crops' protection.

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# Green pesticides based on cinnamate anion incorporated in layered double hydroxides and dispersed in pectin matrix

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## Abstract

This paper reports the preparation of green pesticides based on nano-hybrids composed of a Layered Double Hydroxide (LDH) with cinnamate anion. The dispersion into a pectin matrix was obtained using high energy ball milling in wet conditions. Structure and physical properties of the fillers and the composites films were evaluated. Controlled release of cinnamate was followed using UV spectrophotometry and the release kinetics were found to be dependent on the filler loading. The experimental results were analyzed by the Gallagher-Corrigan model. Antimicrobial activity was evaluated on different bacterial strains, as well as plant pathogens belonging to the genus *Phytophthora* using modified agar diffusion, broth microdilution and dual culture methods, respectively. Experimental results suggested the possibility to use the analyzed composites as green protective coatings for crops' protection.

*Keywords: pectin, LDH, cinnamate, green pesticide, antimicrobial activity*

## 1. Introduction

The protection of crops against pest involves an heavy application of highly toxic synthetic pesticides that can cause serious environmental problems (Hiller, Cernanský, Krascenits, & Milicka, 2009; Miglioranza, de Moreno, & Moreno, 2004; Newton, Cole, & Tinsley, 2008; Tilman et al., 2001; Tilman, Cassman, Matson, Naylor, & Polasky, 2002). Besides the growing use of synthetic pesticides for crops protection, different control strategy based on the use of chemical

34 antimicrobials have been developed to reduce the post-harvest contamination of plant and human  
35 pathogens, such as *Botrytis cinerea*, *Colletotichum gloeosporioides*, *Rhizopus stolonifera*,  
36 *Alternaria alternate*, *Erwinia* spp., *Salmonella*, *Listeria monocytogenes*, *Staphylococcus* spp.  
37 (Bautista-Baños et al., 2006; Lobo-Sánchez, M., 2018).

38 Synthetic pesticides are molecules that contaminate soil, water, air, and their accumulation causes  
39 irreversible damage on all kinds of bio-systems. At the same time the indiscriminate use of these  
40 synthetic antimicrobial compounds poses serious issues for the spread of antimicrobial resistance in  
41 bacteria and fungi. In this context one of the main goal is represented by the possibility to protect  
42 crops without harmful effects on nature. Next to the methodologies of genetic engineering and  
43 natural enemies (Mao, Lewis, Lumsden, & Hebbar, 1998; Navon, 2000; Stevens & Lee, 1979), that  
44 have to be further validate for real applications, it is possible to use the tools of nanotechnology to  
45 assess alternative nature-compatible approaches. Layered Double Hydroxides (LDHs) are a class of  
46 inorganic lamellar solids that possess the characteristic to be soil-compatible. Their general formula  
47 is  $[M(II)_{1-x}M(III)_x(OH)_2](A_{x/n}) \cdot mH_2O$ , where M(II) is a divalent cation such as Mg, Ni, Zn, Cu, Co  
48 and M(III) is a trivalent cation such as Al, Cr, Fe or Ga with  $A^{n-}$  an exchangeable anion of charge n.  
49 The x value generally ranges between 0.2 to 0.4 and determines the positive layer charge density  
50 and the anion exchange capacity (Cavani, Trifiro, & Vaccari, 1991; Costantino, Ambrogi, Perioli, &  
51 Nocchetti, 2008; Herrero, Labajos, & Rives, 2009; Leroux & Taviot-Guého, 2005). The interlayer  
52 anions can be exchanged by other inorganic, organic or metallo-organic compounds in anionic form  
53 and the obtained structures can be used as active nano-hybrid fillers for polymers for targeted  
54 applications (Chen & Qu, 2003; Costantino et al., 2009; Muksing, Magaraphan, Coiai, & Passaglia,  
55 2011; Qiu, Chen, & Qu, 2005; Romano, Naddeo, Guadagno, & Vertuccio, 2014; Zammarano et al.,  
56 2006). LDHs are also cheap materials that can be produced with high level of purity. This makes  
57 LDHs ideal matrices to carry active molecules in soils and control their sustained release into the  
58 desired medium. Furthermore, the intercalated molecule between the inorganic layers could be  
59 safely protected against chemical and biological degradations in soils. The dispersion of the active  
60 nano-hybrid into the soil is a crucial point because the simple dispersion in water causes, after water  
61 evaporation, a re-aggregation of the LDH layers and subsequent loss of adhesion on the plant and  
62 soil to be protected. An interesting alternative could be the dispersion of the nano-hybrid into a bio-  
63 based matrix soluble in water. Pectins are a class of complex water-soluble polysaccharides widely  
64 used to form coatings. They are carbohydrate products obtained by aqueous extraction of some  
65 edible plant material, usually citrus fruits or apples, available in high volume mainly in agricultural  
66 wastes. Pectin coatings have been also studied for their ability to retard lipid migration and moisture  
67 loss, and to improve appearance and handling of foods. This paper reports the preparation of a

68 nano-hybrid composed by LDH and cinnamate, and its possible use as green pesticide against an  
69 important group of plant pathogens, such as *Phytophthora* spp. (Jung et al., 2018), and  
70 antimicrobials for reduce the pathogens post-harvest contamination, at different active molecule  
71 loading. The dispersion into a pectin matrix was conducted through high energy ball milling in  
72 presence of water. Cast films were obtained and analyzed. Structural, thermal, mechanical, barrier  
73 properties were evaluated and correlated to the filler loading. The controlled release analysis of  
74 cinnamate was followed as function of time. Antimicrobial activity of the nano-hybrid composed by  
75 LDH and cinnamate was also assayed. Particularly, several strains of bacteria and *Phytophthora*  
76 belonging to different pathogen species were analyzed.

77

## 78 2. Experimental

79

### 80 2.1 Materials

81

82  $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ , NaOH and trans-cinnamic acid were purchased from Sigma-  
83 Aldrich (Italy). Pectins from apples were purchased from Sigma Aldrich in powder form. The  
84 molecular weight is 30,000-100,000 and the degree of esterification about 70-75%, on a dry basis,  
85 total impurities  $\leq 10\%$  water (CAS Number: 9000-69-5).

86

87

### 88 2.2 Preparation of ZnAl-o-BzOH by coprecipitation method

89 30 mL of an aqueous solution of  $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$  (12.9 g, 43.4 mmol) and  $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$  (8.14 g,  
90 21.7 mmol) were added to 30 ml of a trans-cinnamic sodium salt solution (6.3 g, 36.9 mmol) under  
91 stirring and under nitrogen flow. The pH slowly reached the value of 7.5 by adding 1M NaOH. At  
92 the end, the precipitate was washed with distilled water and left in oven at 50° C for 24h, under  
93 vacuum (Frunza, Lisa, Popa, Miron, & Nistor, 2008). The chemical formula obtained from the  
94 elemental analysis was the following:  $[\text{Zn}_{0.65}\text{Al}_{0.35}(\text{OH})_2] (\text{C}_9\text{O}_2\text{H}_7)_{0.35} \cdot 0.7 \text{H}_2\text{O}$  with value of the  
95 molar fraction  $x = \frac{\text{M}^{\text{III}}}{\text{M}^{\text{III}} + \text{M}^{\text{II}}}$  of 0.35 and molecular weight of 149.99 g/mol; the amount of trans-  
96 cinnamic anion intercalated in ZnAl-o-BzOH is 34.3 wt % of the total weight. Therefore almost all  
97 the alluminium is co-precipitated with the zinc ions to obtain a solid with the stoichiometry of two  
98 Zn(II) atoms for each Al(III) atom. This corresponds to an ideal arrangement of the brucite-like  
99 sheet with each aluminium atom surrounded by six zinc atoms (Oswald & Asper, 1977).

100

### 2.3 Composites Pectin/LDH-cinnamate: preparation and characterization

Composites based on pectin plasticized with glycerol and 2.5 wt%, 5 wt% and 10 wt% of nano-hybrid were prepared by dissolving the powder of pectin and LDH-cinnamate, in weight ratio (pectin: LDH) 97.5:2.5, 95:5 and 90:10, in 30 ml of water-glycerol solution at 4 vol % of glycerol, and left stirring at 80 °C for 60 min. Nano-hybrid LDH-cinnamate, the pectin powders, and water-glycerol were then milled at room temperature in a Retsch (Germany) planetarium ball mill (model PM 100), using a cylindrical steel jar of 50 cm<sup>3</sup> with 5 steel balls of 10 mm of diameter. The rotation speed used was 580 rpm and the milling time was 1 h. The mixtures obtained were slowly evaporated in Petri dishes. Films of pure pectin and pectin/LDH-cinnamate/glycerol for each percentage of nano-hybrid were obtained in the same described experimental conditions. All films, having the same thickness ~300 µm, were dried in a vacuum oven at room temperature for 3 days.

### 2.4 Methods of investigation

*X-ray diffraction (XRD)* patterns were taken, in reflection, with an automatic Bruker diffractometer equipped with a continuous scan attachment and a proportional counter, using nickel-filtered Cu K $\alpha$  radiation (K $\alpha$  = 1.54050 Å) and operating at 40 kV and 40 mA, step scan 0.05° of 2 $\theta$  and 3 s of counting time.

*Thermogravimetric analyses (TGA)* were carried out in air atmosphere with a Mettler TC-10 thermobalance from 30°C to 800 °C at a heating rate of 10 °C/min.

*Fourier transform infrared (FT-IR)* absorption spectra were recorded by a Bruker spectrometer, model Vertex 70 (average of 32 scans, at a resolution of 4 cm<sup>-1</sup>).

*Mechanical properties* of the samples were evaluated, in tensile mode, at room temperature and ambient humidity (about 50%) using a dynamometric apparatus INSTRON 4301. Experiments were conducted at room temperature on pectin and composites' films with the deformation rate of 2 mm/min. The specimens were 10 mm wide and  $\cong$  250 µm thick. The initial length of the samples was 10 mm. Elastic modulus was derived from the linear part of the stress-strain curves, giving to the samples a deformation of 0.1%. Data were averaged on five samples.

*Barrier properties* of water vapor were evaluated using conventional Mc Bain spring balance system, which consists of a glass water-jacketed chamber serviced by a high vacuum line for sample degassing and vapor removal. Inside the chamber, samples were suspended to a helical quartz spring supplied by Ruska Industries (Houston, TX) having a spring constant of 1.52 cm/mg. The temperature was controlled to 30  $\pm$  0.1 °C by a constant temperature water bath. Samples were

134 exposed to the water vapor at fixed pressures, P, giving different water activities  $a = P/P_0$ , where  $P_0$   
 135 is the saturation water pressure at the experimental temperature. The spring position was recorded  
 136 as a function of time using a cathetometer. The spring position data were converted to mass uptake  
 137 data using the spring constant, and the process was followed to a constant value of sorption for at  
 138 least 24 h. Data averaged on three samples. Measuring the increase of weight with time, for the  
 139 samples exposed to the vapor at a given partial pressure, it is possible to obtain the equilibrium  
 140 value of sorbed vapor,  $C_{eq}(\text{g}_{\text{solvent}}/100 \text{ g}_{\text{polymer}})$ . Moreover, in the case of Fickian behavior, that is a  
 141 linear dependence of sorption on square root of time, it is possible to derive the mean diffusion  
 142 coefficient from the linear part of the reduced sorption curve, reported as  $C_t/C_{eq}$  versus square root  
 143 of time, by Equation (1): (Koros, Burgess, & Chen, 2015)

$$144 \quad \frac{C_t}{C_{eq}} = \frac{4}{d} \left( \frac{Dt}{\pi} \right)^{1/2} \quad (1)$$

145 where  $C_t$  is the penetrant concentration at the time t,  $C_{eq}$  the equilibrium value, d (cm) the thickness  
 146 of the sample and D ( $\text{cm}^2/\text{s}$ ) the average diffusion coefficient. The sorption parameter (S), is  
 147 obtained from the equilibrium concentration ( $C_{eq}$ ) of the permeant vapor as a function of the partial  
 148 pressure:

$$149 \quad S = \frac{d(C_{eq})}{dp} \quad (2)$$

150 All the samples showed a Fickian behavior during the sorption of water vapor at different activities.  
 151 Using Equation (1) it was possible to derive the diffusion coefficient, D, at every fixed vapor  
 152 activity ( $a = p/p_0$ ), and the equilibrium concentration of solvent into the sample,  $C_{eq}(\text{g}_{\text{solvent}}/100$   
 153  $\text{g}_{\text{polymer}})$ . For polymer-solvent systems, the diffusion parameter is usually not constant, but depends  
 154 on the vapor concentration, according to the empirical Equation (3):

$$155 \quad D = D_0 \exp(\gamma C_{eq}) \quad (3)$$

156 where  $D_0$  ( $\text{cm}^2/\text{s}$ ) is the zero concentration diffusion coefficient (related to the fractional free  
 157 volume and to the microstructure of the polymer);  $\gamma$  is a coefficient, which depends on the fractional  
 158 free volume and on the effectiveness of the penetrant to plasticize the matrix (Koros, Burgess, &  
 159 Chen, 2015). The permeability (P) coefficient is described as the product of a thermodynamic  
 160 parameter which is the sorption coefficient (S) and a kinetic parameter which is the zero diffusivity  
 161 or diffusion coefficient ( $D_0$ ):

$$162 \quad P = S \times D_0 \quad (4)$$

163 *The release kinetics* of cinnamate were performed by ultraviolet spectrometric measurement using a  
 164 Spectrometer UV-2401 PC Shimadzu (Japan). The tests were performed using rectangular  
 165 specimens of  $2 \text{ cm}^2$  and same thickness ( $\cong 200 \mu\text{m}$ ), placed into 25 mL of ethanol with 0.9 wt% of  
 166 tetrabutylammonium chloride and stirred at 100 rpm in an orbital shaker (VDRL MOD. 711+ Asal

167 S.r.l.). The release medium was withdrawn at fixed time intervals and replenished with fresh  
168 medium. The considered band was at 268 nm.

169  
170 *2.5 Microbial strains*

171  
172 The microorganisms used in in this work for the antimicrobial tests are listed in Table 1. Bacteria  
173 were cultured in BHI broth or BHI agar (Microbiol, Cagliari, IT) and incubated at 37°C for 24 h,  
174 while *Phytophthora* spp. were cultured on carrot agar (CA) (Scanu et al., 2014), and incubated at 20  
175 °C for 24-48 h.

176  
177 *Table 1: microorganisms used in the present work and sources*

Tested microorganisms	Sources
<i>Bacteria</i>	
<i>Staphylococcus aureus</i> DSMZ 20231	DSMZ
<i>Listeria monocytogenes</i> DSMZ 20600	DSMZ
<i>Escherichia coli</i> DSMZ 30083	DSMZ
<i>Salmonella bongori</i> DSMZ 13772	DSMZ
<i>Phytophthora</i>	
<i>Phytophthora cinnamomi</i> PH105	UNISS
<i>Phytophthora palmivora</i> PH090	UNISS

178  
179 *DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, German Collection of Microorganism*  
180 *of Cell Cultures; UNISS, Collection of Dipartimento di Agraria – University of Sassari, Italy*

181  
182 *2.5.1 Broth microdilution test*

183  
184 The minimal inhibitory concentration (MIC) of the cinnamic acid of the bacterial species was tested  
185 by the microdilution broth method, according to Fancello et al. (2016). Briefly, cinnamic acid stock  
186 solution was first prepared with a concentration of 25 mg/mL in a 75% ethanol aqueous solution.  
187 Stock solutions were then diluted in sterile distilled water, to give a series of concentrations ranging  
188 from 25 mg/mL to 0.097 mg/mL. Overnight cultures were then used to prepare microbial  
189 inoculation used for the test. Aliquots of 100 µL of diluted inoculation at desired cells concentration  
190 were added to each well in the 96-well micro-dilution plate already containing 100 µL of desired  
191 cinnamic acid dilutions. The plates were then incubated at 37 °C for 24 h. After incubation, MICs  
192 (mg/mL) values were determined as the lowest concentration that inhibited visible growth of the  
193 tested microorganism, which was indicated by absence of turbidity. Each test was performed in  
194 quadruplicate and the experiments were repeated twice.

## 196 2.5.2 Modified Agar diffusion and dual culture tests

197

198 The growth of bacteria was monitored after exposure of Pectin/LDH-cinnamate 10wt% as the  
199 following procedure. Bacteria were grown overnight on the specific media as mentioned before.  
200 The day after,  $5 \times 10^6$ /mL cells were stricken on BHI agar medium (Microbiol, Cagliari, IT). Disks  
201 of 10 mm of Pectin/LDH-cinnamate 10 wt% and Pectin/cinnamic acid 3.6 wt% were seeded on  
202 plates. To confirm this data, the same quantity of cinnamic acid contained in the Pectin/LDH-  
203 cinnamate was spotted (5  $\mu$ L /spot) onto Whatman 3 MM Chromatographic paper disks (0.34 mm  
204 paper thickness, 460 $\times$ 570 mm) and seeded on plates. For both tests the inhibition halos were  
205 measured after 24 h of incubation at 37 °C. Each assay was replicated 3 times. The diameter of the  
206 clear zone around the disc was measured and expressed in millimeters (disk diameter included). The  
207 rate of inhibition was determined according to Sagdic et al. (2003), a diameter of 10 to 15 mm was  
208 considered as slight antibacterial activity; a diameter of 16 to 20 mm as moderate antibacterial  
209 activity and a diameter of 20 mm as strong antibacterial activity. The antifungal properties of the  
210 biofilm against *Phytophthora* spp. was also tested using the dual culture method. A mycelial plug (5  
211 mm diameter) were cut from the margin of actively growing 5-day-old colony, using a flamed cork  
212 borer, and placed on one side of a Petri dish containing 20 ml of CA (Scanu et al., 2014).  
213 Meanwhile a 10 mm disk of Pectin/LDH-cinnamate 10 wt% was placed on the opposite side of the  
214 plate, with a 30 mm of distance between the two plugs. Plates containing the *Phytophthora* species  
215 without the biofilm were used as negative control. The plates were incubated at 20°C in the dark.  
216 There were six replicates for each pathogen-biofilm combination and the test was repeated twice.  
217 The radial growth of the two *Phytophthora* species tested was recorded when the control treatments  
218 covered the plate surface. The percent growth inhibition was calculated according to the formula:  
219  $PGI = 100 (DC-DT)/DC$  where PGI = the percentage of inhibition of mycelia growth; DC = the  
220 radial growth of *Phytophthora* spp. in control plate; DT = the radial growth of *Phytophthora* spp.  
221 towards the biofilm.

222

## 223 3. Results and discussion

224

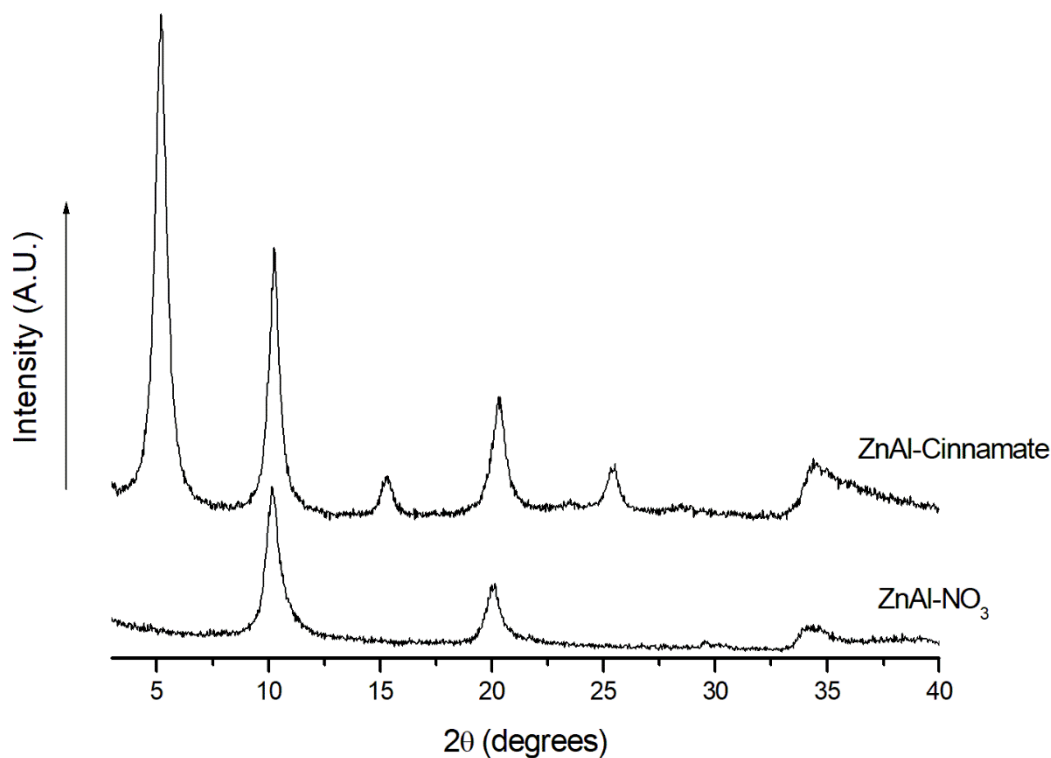
### 225 3.1 Characterization of filler

226

227 Figure 1 reports the XRD spectra of pristine LDH-NO<sub>3</sub> and the LDH modified with cinnamate  
228 anion. It is evident that the nitrate form of LDH presents the main peaks at about 10° and 20° of 2 $\theta$ ,  
229 relative to the basal spacing (003) and (006), respectively. The intercalation of cinnamate molecule

230 is evident from the modification of the basal spacing of the LDH with the shifting of the diffraction  
231 peaks at lower angle (Weiling, Qinglin, & Yong, 2007).

232



233

234 **Figure 1: XRD spectra of pristine LDH-NO<sub>3</sub> and the LDH modified with cinnamate molecule**

235

236 TGA analysis was carried out on LDH-NO<sub>3</sub> (A), cinnamic acid (B) and LDH-cinnamate (C). The  
237 TGA curve of LDH-NO<sub>3</sub>, reported in the supporting information (SI 1), shows three steps of  
238 decomposition: i) the first at around 150°C, corresponding to the loss of absorbed water between  
239 LDH layers, ii) a second, occurring around at 250°C, is due to the thermal decomposition of nitrate  
240 anions, iii) a third, at about 400°C, due to the dehydroxylation of the LDH sheets (Park et al., 2010).  
241 Experimental results demonstrate the stabilization of cinnamate molecule within the interlayer  
242 space of LDH. In fact, free cinnamic acid (B) exhibits its degradation in one step, above 150°C. The  
243 intercalation into the inorganic matrix results in a significant improvement in thermal stability: the  
244 main thermal decomposition of the hybrid takes place at around 374°C. The hydroxide framework  
245 transforms finally into its corresponding oxide by dehydroxylation above 500°C. Such behavior,  
246 already found for several molecules incorporated into LDH layers (Gorrasi & Bugatti, 2016),  
247 suggests a protecting effect of the LDH respect to the cinnamate and a stable interaction LDH-

248 organic molecule due to electrostatic forces.  
249 FTIR spectra of LDH-NO<sub>3</sub> (A), cinnamic acid (B) and LDH-cinnamate (C) in the range 1000-2000  
250 cm<sup>-1</sup> are reported in the supporting information (SI 2). The cinnamic acid shows characteristic  
251 vibrations at 1682 cm<sup>-1</sup> attributed to C=O stretching, at 1626 cm<sup>-1</sup> due to C=C stretching, at 1313  
252 cm<sup>-1</sup> for C-O stretching, and at 1418 cm<sup>-1</sup> for OH in-plane bending, respectively. The spectrum of  
253 the nano-hybrid LDH-cinnamate shows most of the vibrations assigned to both cinnamate and  
254 LDH, although several vibrations are overlapped. In particular, the strong vibrations at 1638 cm<sup>-1</sup> is  
255 due to COO<sup>-</sup> stretching of the intercalated cinnamate. This result suggests that cinnamate anion is  
256 stably intercalated between the LDH galleries, and its anion form electrostatically interact with  
257 positively charged LDH layers.

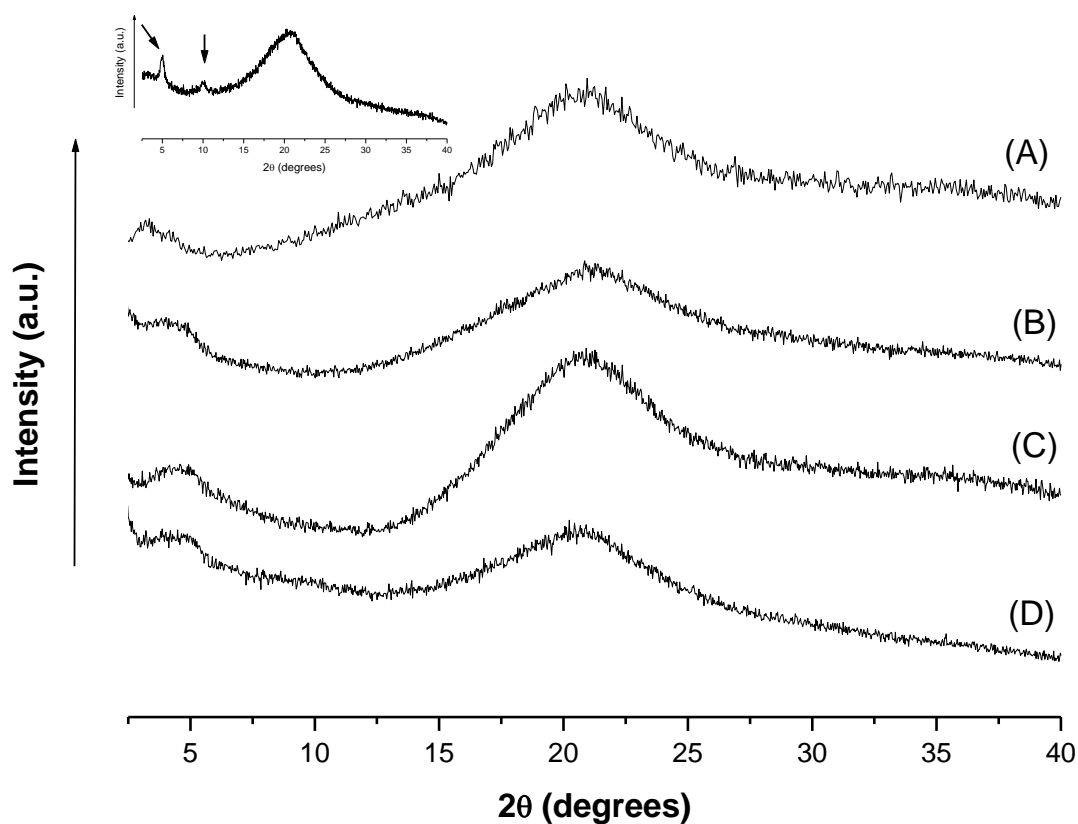
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### 259 *3.2 Characterization of composites*

260

261 Figure 2 reports the XRD analysis on pectin and composites. Pectin spectrum shows the typical  
262 form of plasticized material, with a broad halo centered at about 21° of 2θ. Such amorphous  
263 organization is retained in all composites, at all filler loading (Masuelli & Renard, 2017). The  
264 absence of any diffraction peak relative to the filler, in the spectra of the composites, suggests the  
265 exfoliation of the LDH-cinnamate in the used processing conditions. The mechanical action, in  
266 presence of water, allows to hypothesize that the LDH layers are completely delaminated at any  
267 filler composition. In order to better support this hypothesis we prepared a mechanical mixture of  
268 pectins powder with 2.5% of LDH-cinnamate (inset of Figure 2). It is evident that the simple  
269 grinding of the filler with the polymer did not induced any structural modification in both  
270 components. In particular, the basal X-ray reflections of the inorganic filler remained intense and  
271 sharp, with the XRD pattern being just a superposition of the two components' spectra.

272



273

274 **Figure 2: XRD on films (A) pectin, (B) pectin/2.5% LDH-cinnamate, (C) pectin/5% LDH-cinnamate, (D)**  
 275 **pectin/10% LDH-cinnamate. The inset reports XRD on a mechanical mixture composed of pectin and 2.5%**  
 276 **LDH-cinnamate**

277

278 Thermal behavior was evaluated on the composites through thermogravimetric analysis (TGA and  
 279 DTG). Results are reported in the supporting information (SI 3). It is also shown the  
 280 thermogravimetric curve of the pure pectin, for comparison. The thermo-oxidative degradation of  
 281 pectins is a series of complex events that involves three steps of degradation: i) the first one,  
 282 centered at about 90°C, due to loss of water; ii) the second one, between 150°C and 280°C, due to  
 283 pyrolytic decomposition consisting of a primary and secondary decarboxylation involving the acid  
 284 side group and a carbon in the ring (Gorrasi, 2015; Shim, Hajaligol & Baliga, 2004; Waymack,  
 285 Belobe, Baliga, & Hajaligol, 2004;); iii) the third step between about 650°C and 720°C,  
 286 corresponding to the oxidation region. The second step of degradation occurs at the same  
 287 temperatures either for pectin or for the composites independently of the filler loading; whereas the  
 288 third degradation step is dependent on the filler amount. Its temperature decreases on increasing the

289 filler loading, as evidenced by the DTG analysis (part B of the figure). It has been reported that the  
290 glycerol percentage has a significant effect on the degradation of pure pectin (Yang & Yang, 2016),  
291 but in this case the glycerol amount is the same in all composites. It can be hypothesized that oxides  
292 of Zn and Al, that are formed for the decomposition of LDH at high temperatures, can catalyze the  
293 oxidation of pectin matrix.

294 Mechanical properties were estimated on all samples (Figure 3). From the stress-strain curves, not  
295 reported, they were evaluated elastic moduli (MPa), stress at break point (MPa) and elongation at  
296 break (%). The elastic modulus, E (MPa), of the unfilled pectin is lower than the one evaluated on  
297 pectin film treated in the same conditions, but with no glycerol (Gorrasi, Bugatti, & Vittoria, 2012).  
298 This is due to the plasticizing effect of the glycerol that lowers the mechanical resistance of the  
299 material (Yang & Yang, 2016). The elastic modulus (A) increases on increasing the filler content  
300 and the stress at break point (B) does not change up to 5 wt% of filler and increases significantly for  
301 10 wt % of LDH-cinnamate. This could be due to the reinforcing effect of the nano-hybrid into the  
302 polymeric matrix. The inorganic lamellae, well dispersed into the organic phase (see XRD results)  
303 directly enhances the stiffness of the nanocomposites, because the exfoliated LDHs nanolayers are  
304 thoroughly dispersed into the pectin matrix, and each nanolayer could contribute to the  
305 reinforcement of the nanocomposites. This is particularly evidenced in the improvement of the  
306 elastic modulus. As expected, the strain at break (C) decreases with filler content for the different  
307 chemical nature of both composites' components. The dispersed phase, at high elongation and  
308 loading, behaves as "defects" into the polymer matrix.

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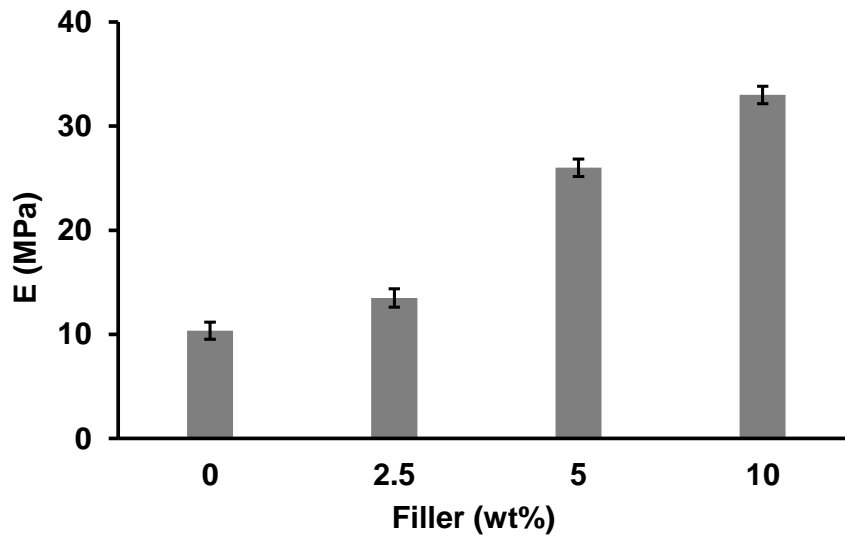
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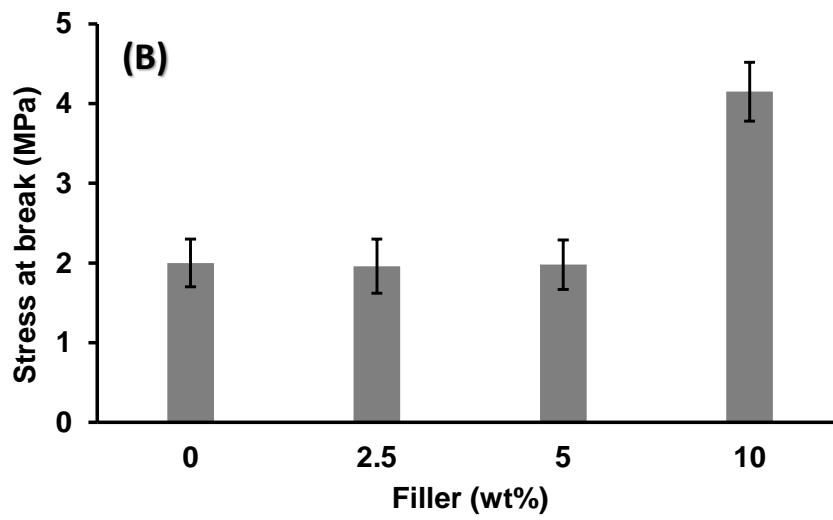
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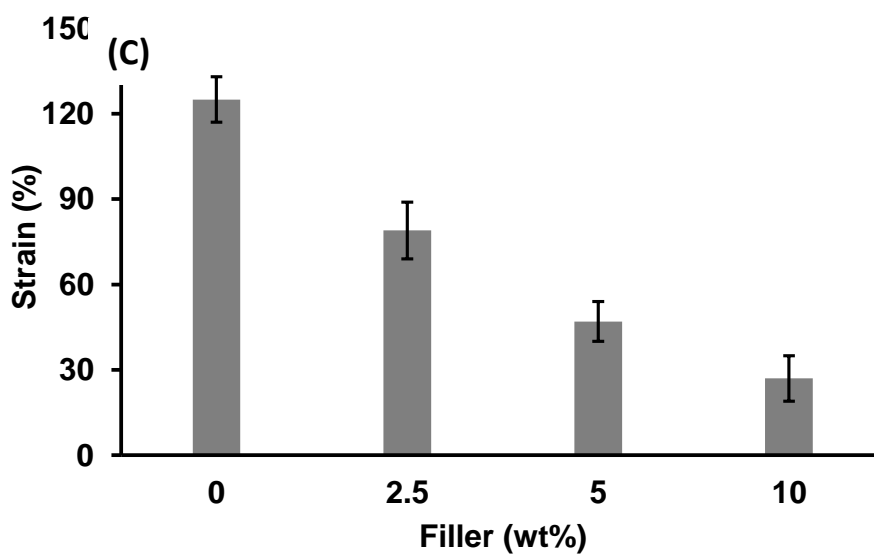
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Figure 3: Mechanical properties evaluated on pectin and composites

321 Figure 4 shows the barrier properties, sorption (A) and diffusion (B), evaluated on all the  
322 composites. Data relative to unfilled pectin are also reported, for comparison. The sorption isotherm  
323 of pectin plasticized with glycerol follows a typical Langmuir adsorption (Koros, Burgess, & Chen,  
324 2015) where the solvent molecules are absorbed on specific sites at low vapour pressure; when all  
325 the sites are occupied a constant value of concentration is shown on increasing the vapor activity.  
326 Equation (2) allowed to evaluate the sorption coefficients for all the samples. It is evident a  
327 significant reduction of water sorption in the composites at 5 and 10 wt% of filler loading. From  
328 XRD results it was evidenced that the structure of the pectin does not change for the filler addition,  
329 in terms of degree of crystallinity, thus the variation in the sorption must be attributed to other  
330 factors and not to a reduction of the amount of amorphous permeable phase. Being the water a polar  
331 solvent it is assumed that the adsorption occurs on polar groups of the pectin matrix. The less  
332 availability of the polar sites causes, then, a decrease of sorption. The preferential interaction of the  
333 pectin matrix with the polar groups of the LDH-cinnamate could be a possible explanation of the  
334 sorption reduction with filler loading.

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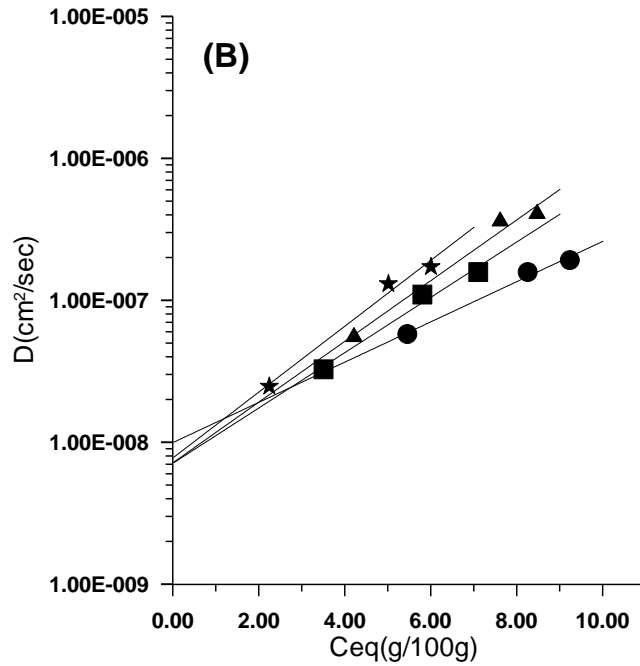
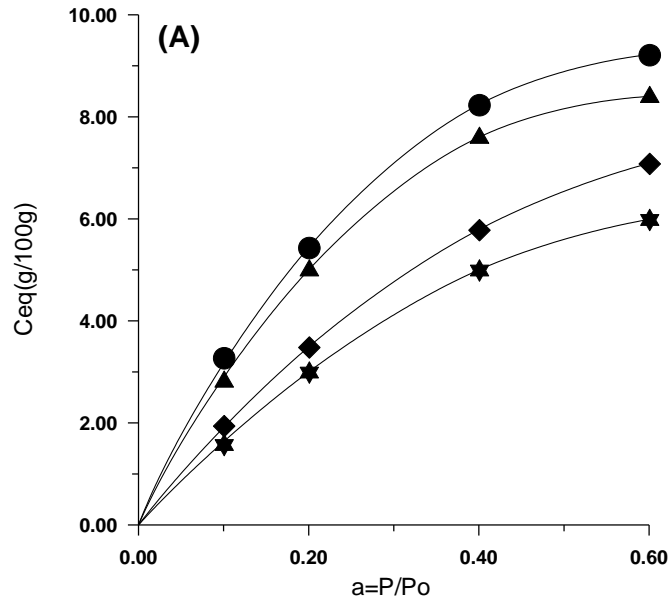
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350 **Figure 4: (A) Sorption isotherm for samples: pectin (●), Pectin/2.5% LDH-cinnamate (Δ), Pectin/5% LDH-**  
 351 **cinnamate (◊), Pectin/10% LDH-cinnamate (\*); (B) Diffusion for samples: pectin (●), Pectin/2.5% LDH-**  
 352 **cinnamate (Δ), Pectin/5% LDH-cinnamate (◊), Pectin/10% LDH-cinnamate (\*)**  
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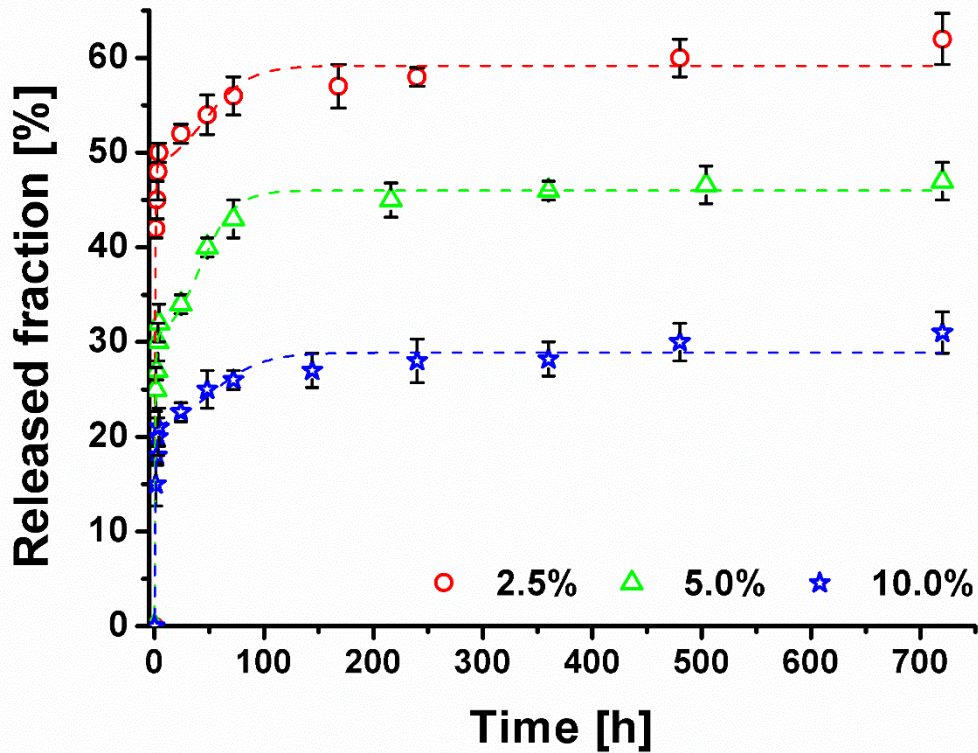
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*Table 2: barrier parameters, sorption diffusion and permeability, of pectin and composites*

Sample	Sorption (g/100g/mmHg)	Diffusion (cm <sup>2</sup> /s)	Permeability (g/100g/mmHg)*(cm <sup>2</sup> /s)
Pectin	28.32	1.01*10 <sup>-8</sup>	2.86*10 <sup>-7</sup>
Pectin/LDH-cinn 2.5%	25.64	7.27*10 <sup>-9</sup>	1.86*10 <sup>-7</sup>
Pectin/LDH-cinn 5%	17.86	7.34*10 <sup>-9</sup>	1.31*10 <sup>-7</sup>
Pectin/LDH-cinn 10%	15.16	7.87*10 <sup>-9</sup>	1.19*10 <sup>-7</sup>

355

356 Figure 5 reports the release of cinnamate anion (%) from the composites at different nano-hybrid  
357 loading, as function of time (h).



358

359 **Figure 5: Release of cinnamate molecule, as function of contact time (h), for composites at 2.5, 5 and 10% of**  
360 **nano-hybrid loading. Dotted lines are the fitting with the model expressed from Equation (5)**

361

362 The release can be visualized in two steps: the initial one is related to the burst of the molecules  
363 located on the external surfaces of the films, and a second step that can be attributed to the diffusion  
364 of the cinnamate molecules from the bulk. The second step is followed by a plateau. It is worth to  
365 note that the amount of released molecule decreases with increasing the filler loading. In order to  
366 give a phenomenological interpretation to the experimental data, we used the Gallagher and  
367 Corrigan model (Gallagher & Corrigan, 2000). The model assumes that the drug release at any time  
368 is the sum of two processes: an initial diffusion controlled phase and a subsequent polymer  
369 degradation controlled phase. In particular it describes a two-stage drug release kinetics: the first  
370 part of the equation reflects the diffusion controlled dissolution of drug to the medium, which is  
371 characterized by the first order kinetics; the second part describes that the drug release rate depends  
372 on the polymer relaxation (Dunne, Ramtoola, & Corrigan, 2009; Gallagher & Corrigan, 2000;  
373 Milallos, Alexander, & Riga, 2008; Zhong & Mi, 2005). Therefore  $f_t$ , the total fraction of drug  
374 released at a given time  $t$  is given by:

375

$$f_t = f_b * (1 - e^{-k_1 t}) + (f_{t_{max}} - f_b) \left( \frac{e^{k_2(t-t_{2max})}}{1 + e^{k_2(t-t_{2max})}} \right) \quad (5)$$

where  $f_t$  is the accumulative drug release percentage at time  $t$ ,  $k_1$  is the first order release constant (Stage 1),  $k_2$  is the second stage release constant due to the polymer relaxation,  $f_b$  is the accumulative drug release percentage during the Stage 1,  $f_{t_{max}}$  is the maximum drug release percentage during the whole process,  $t_{2max}$  is the time at which drug release rate reaches the maximum. The correlation coefficient ( $R^2$ ) is an indicator of the best fitting for the considered model.

Table 3 reports the kinetic parameters derived using Equation 5. It can be noted that the burst ( $f_b$ ) parameter and the first order release constant,  $k_1$ , decrease with filler loading, while the time at which the drug release rate reaches the maximum,  $t_{2max}$ , increases. The  $k_2$  constant remains almost unvaried at any filler composition. It is hypothesized that such behavior could be related either to the hindrance effect created by the LDH platelets, that delay the counter-diffusion of the active molecule (Bugatti, Vertuccio, Viscusi, & Gorrasi, 2018), or preferential to hydrogen bonds between the cinnamate and the system pectin-glycerol (see sorption data).

Table 3: kinetic parameters derived from Equation (5)

Sample	$f_b$ (%)	$t_{2max}$ (h)	$k_1$ ( $h^{-1}$ )	$k_2$ ( $h^{-1}$ )	$R^2$
Pectin/LDH-cinn 2.5%	47	45	1.84	$4.89 \times 10^{-2}$	0.991
Pectin/LDH-cinn 5%	29	41	1.50	$6.40 \times 10^{-2}$	0.994
Pectin/LDH-cinn 10%	20	51	1.08	$4.27 \times 10^{-2}$	0.984

### 3.3 Antimicrobial activity

The antimicrobial activity was evaluated firstly performing an in vitro test to determine the MIC of the cinnamic acid against the microbial species considered in this work and reported in table 1 (*Staphylococcus aureus* DSMZ 20231, *L. monocytogenes* DSMZ 20600, *E. coli* DSMZ 30083 and *S. bongori* DSMZ 13772 and two strains of *Phytophthora* namely *P. cinnamomi* (isolate PH105) and *P. palmivora* (isolate PH090) ), by the microdilution broth test. The different strains tested showed the same sensitivity against the cinnamic acid with a MIC value of 1.56 mg/mL (10.52 mM), in accordance with the values found by other authors (Guzman, 2014). The MIC value obtained was used to set up the concentration of cinnamic acid in subsequent experiments.

The in vitro antimicrobial activity was, then, evaluated on a composite based on pectin and 10% of LDH-cinnamate (3.6% of active molecule), taken as model sample to investigate the effect of the

412 active molecule bonded to the LDH and dispersed into the pectin. For the bacteria, results on  
 413 modified agar diffusion test (disks of pectin/LDH-cinnamate directly seeded on the agar plates (see  
 414 Table 4) indicated that the cinnamate bonded to the LDH and dispersed into the pectin exhibited  
 415 slight antimicrobial activity against *S. aureus* DSMZ 20231, with a diameter halo of about  
 416  $11.5\pm 0.07$  mm and a moderate activity against *E. coli* DSMZ 30083 with a diameter halo of about  
 417  $16.5\pm 0.07$  mm while exerted an activity against *L. monocytogenes* DSMZ 20600 and *S. bongori*  
 418 DSMZ 13772 with a diameter  $< 10$  mm. The agar diffusion test used as control with cinnamic acid  
 419 alone imbibed in Whatman paper discs, showed an antimicrobial activity against the four pathogen  
 420 strains used, with halos that varied from *S. aureus* DSMZ 20231 with about  $14 \pm 0.0$  mm, *S. bongori*  
 421 DSMZ 13772 with about  $13.5\pm 0.07$  mm, *E. coli* DSMZ 30083 with about  $12.8\pm 0.04$  mm and  
 422 finally *L. monocytogenes* DSMZ 20600 with about  $12\pm 0.02$  mm (Table 4). The mechanism under  
 423 this phenomenon is quite complex. A possible explanation of the different antimicrobial ability of  
 424 LDH-cinnamate into pectin could be found in the different cell surface charge of the different  
 425 pathogens used and/or different hydrophobicity of cell surface that can influence the reaction of the  
 426 bacterial strains (Dickson & Koohmaraie, 1989).

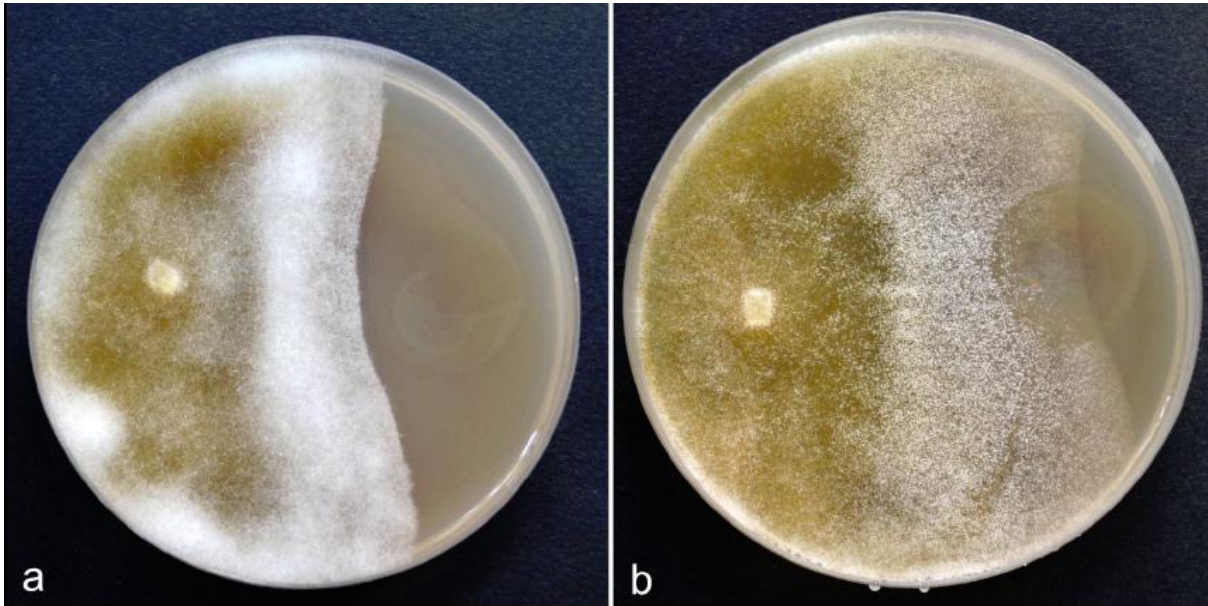
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428 *Table 4. Antimicrobial activity by modified agar diffusion test (according to Sagdic et al., (2003))*

Bacterial strains	Pectin/10% LDH-cinn (Ø mm)	Cinnamic acid (Ø mm)
<i>S. aureus</i> DSMZ 20231	$11.5\pm 0.07$	$14 \pm 0.0$
<i>E. coli</i> DSMZ 30083	$16.5\pm 0.07$	$12.8\pm 0.04$
<i>L. monocytogenes</i> DSMZ 20600	$<10$	$12\pm 0.02$
<i>S. bongori</i> DSMZ 13772	$<10$	$13.5\pm 0.07$

429

430 For the *Phytophthora* spp, the dual culture assay generated significant inhibitory effects on the  
 431 radial growth of the tested pathogens. This inhibition was clearly discerned by a limited growth and  
 432 a complete absence of pathogen mycelium around the biofilm disk (Figure 6).



433

434

435 **Figure 6: In-vitro evaluation of Pectin/10% LDH-cinnamate in dual culture assay with *Phytophthora* spp.: colony**  
 436 **of *P. cinnamomi* (a) and *P. palmivora* (b) after 5 days at 20°C**

437

438 There was a significant reduction in mycelial growth of both pathogens. The highest percent of  
 439 inhibition of mycelial growth was observed in the case of *P. cinnamomi*, with a percent growth  
 440 inhibition averaging 53.3%. The mycelial growth of *P. cinnamomi* was entirely limited when in  
 441 contact with the biofilm disk (Figure 6a). The growth rate of *P. palmivora* was also influenced by  
 442 the presence of the film Pectin/LDH-cinn, however the inhibition was lower and around 36.7%, and  
 443 the pathogen was able to grow above the biofilm (Figure 6b). The strong inhibition rate against  
 444 *Phytophthora* spp. suggests that this compound could be a valid alternative to the use of synthetic  
 445 fungicides, which are limited by the development of antimicrobial resistance and the harmful effects  
 446 to human health (Parra & Ristaino, 2001). Additionally, many *Phytophthora* spp. (including *P.*  
 447 *cinnamomi* and *P. palmivora*) are emerging pathogens in natural and forest ecosystems, where due  
 448 to the lack of legal authorisations and for environmental reasons, the use of fungicides is not a  
 449 realistic option for the control of *Phytophthora* diseases in most countries (Jung et al., 2018). The  
 450 film Pectin/LDH-cinn was able to reduce significantly the growth of both *Phytophthora* spp. tested;  
 451 however, it is interesting to note that it was less effective at inhibiting mycelial growth in *P.*  
 452 *palmivora* as compared to *P. cinnamomi*. Further investigations are needed in order to explore the  
 453 LDH-cinnamate effect on the different life cycle stages of *Phytophthora* species as well as its  
 454 efficacy in *in planta* inoculation trials.

455

456

457 **Conclusions**

458

459 This paper reported the preparation of green composites based on pectins and layered double  
460 hydroxides (LDH) intercalating cinnamate anion, as active molecule. The cinnamate loading into  
461 the LDH was 36%. Composites at 2.5, 5 and 10 wt% were prepared using ball milling technology in  
462 presence of water. Films were obtained and tested, respect to structural and functional properties.

- 463 • XRD analysis showed the successful intercalation of cinnamate molecule, evidenced from  
464 the modification of the basal spacing of the LDH, and a delamination of the nano-hybrid  
465 into the pectin matrix at any filler composition.
- 466 • TG-DTG analysis allowed to hypothesize that the organic molecule is protected by the LDH  
467 layers, and the degradation of the pectin matrix was not greatly influenced from the nano-  
468 hybrid filler, except for the oxidation stage at high temperatures, that resulted anticipated.
- 469 • Mechanical properties showed an improvement of the elastic modulus and the stress at break  
470 point, especially at 10 wt% of filler loading. Such reinforcing effect is mainly due to the  
471 well dispersed inorganic lamellae that enhance the stiffness of the composites. The strain at  
472 break point decreases with the filler content, because to the incompatibility of the inorganic  
473 nature of the filler and organic nature of the matrix.
- 474 • Barrier properties to water vapour revealed a decrease of sorption with the increasing the  
475 filler loading, while the diffusion was unvaried. Interaction between polar groups of pectin  
476 and filler were hypothesized, resulting in a lower sorption of the polar water molecules
- 477 • The release kinetics of composites' membranes were found to be dependent on the nano-  
478 hybrid loading and were well fitted the Gallagher-Corrigan model. It was demonstrated that  
479 varying the filler loading it is possible to tune the cinnamate release for desired applications.
- 480 • The antimicrobial activity of the membrane filled with 10 wt % of LDH-cinnamate revealed  
481 an antimicrobial activity particularly against *E. coli*, *S. aureus*, *P. cinnamomic* and *P.*  
482 *palmivora*,

483

484 **Acknowledgements**

485 This work was supported by the project “High Performing Advanced Material Platform For Active  
486 and Intelligent Food Packaging: Cronogard™” (H2020-SMEINST-2-2016-2017). Grant agreement  
487 n. 783696

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# Green pesticides based on cinnamate anion incorporated in layered double hydroxides and dispersed in pectin matrix

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## Abstract

This paper reports the preparation of green pesticides based on nano-hybrids composed of a Layered Double Hydroxide (LDH) with cinnamate anion. The dispersion into a pectin matrix was obtained using high energy ball milling in wet conditions. Structure and physical properties of the fillers and the composites films were evaluated. Controlled release of cinnamate was followed using UV spectrophotometry and the release kinetics were found to be dependent on the filler loading. The experimental results were analyzed by the Gallagher-Corrigan model. Antimicrobial activity was evaluated on different bacterial strains, as well as plant pathogens belonging to the genus *Phytophthora* using modified agar diffusion, broth microdilution and dual culture methods, respectively. Experimental results suggested the possibility to use the analyzed composites as green protective coatings for crops' protection.

*Keywords: pectin, LDH, cinnamate, green pesticide, antimicrobial activity*

## 1. Introduction

The protection of crops against pest involves an heavy application of highly toxic synthetic pesticides that can cause serious environmental problems (Hiller, Cernanský, Krascenits, & Milicka, 2009; Miglioranza, de Moreno, & Moreno, 2004; Newton, Cole, & Tinsley, 2008; Tilman et al., 2001; Tilman, Cassman, Matson, Naylor, & Polasky, 2002). Besides the growing use of synthetic pesticides for crops protection, different control strategy based on the use of chemical

34 antimicrobials have been developed to reduce the post-harvest contamination of plant and human  
35 pathogens, such as *Botrytis cinerea*, *Colletotichum gloeosporioides*, *Rhizopus stolonifera*,  
36 *Alternaria alternate*, *Erwinia* spp., *Salmonella*, *Listeria monocytogenes*, *Staphylococcus* spp.  
37 (Bautista-Baños et al., 2006; Lobo-Sánchez, M., 2018).

38 Synthetic pesticides are molecules that contaminate soil, water, air, and their accumulation causes  
39 irreversible damage on all kinds of bio-systems. At the same time the indiscriminate use of these  
40 synthetic antimicrobial compounds poses serious issues for the spread of antimicrobial resistance in  
41 bacteria and fungi. In this context one of the main goal is represented by the possibility to protect  
42 crops without harmful effects on nature. Next to the methodologies of genetic engineering and  
43 natural enemies (Mao, Lewis, Lumsden, & Hebbar, 1998; Navon, 2000; Stevens & Lee, 1979), that  
44 have to be further validate for real applications, it is possible to use the tools of nanotechnology to  
45 assess alternative nature-compatible approaches. Layered Double Hydroxides (LDHs) are a class of  
46 inorganic lamellar solids that possess the characteristic to be soil-compatible. Their general formula  
47 is  $[M(II)_{1-x}M(III)_x(OH)_2](A_{x/n}) \cdot mH_2O$ , where M(II) is a divalent cation such as Mg, Ni, Zn, Cu, Co  
48 and M(III) is a trivalent cation such as Al, Cr, Fe or Ga with  $A^{n-}$  an exchangeable anion of charge n.  
49 The x value generally ranges between 0.2 to 0.4 and determines the positive layer charge density  
50 and the anion exchange capacity (Cavani, Trifiro, & Vaccari, 1991; Costantino, Ambrogi, Perioli, &  
51 Nocchetti, 2008; Herrero, Labajos, & Rives, 2009; Leroux & Taviot-Guého, 2005). The interlayer  
52 anions can be exchanged by other inorganic, organic or metallo-organic compounds in anionic form  
53 and the obtained structures can be used as active nano-hybrid fillers for polymers for targeted  
54 applications (Chen & Qu, 2003; Costantino et al., 2009; Muksing, Magaraphan, Coiai, & Passaglia,  
55 2011; Qiu, Chen, & Qu, 2005; Romano, Naddeo, Guadagno, & Vertuccio, 2014; Zammarano et al.,  
56 2006). LDHs are also cheap materials that can be produced with high level of purity. This makes  
57 LDHs ideal matrices to carry active molecules in soils and control their sustained release into the  
58 desired medium. Furthermore, the intercalated molecule between the inorganic layers could be  
59 safely protected against chemical and biological degradations in soils. The dispersion of the active  
60 nano-hybrid into the soil is a crucial point because the simple dispersion in water causes, after water  
61 evaporation, a re-aggregation of the LDH layers and subsequent loss of adhesion on the plant and  
62 soil to be protected. An interesting alternative could be the dispersion of the nano-hybrid into a bio-  
63 based matrix soluble in water. Pectins are a class of complex water-soluble polysaccharides widely  
64 used to form coatings. They are carbohydrate products obtained by aqueous extraction of some  
65 edible plant material, usually citrus fruits or apples, available in high volume mainly in agricultural  
66 wastes. Pectin coatings have been also studied for their ability to retard lipid migration and moisture  
67 loss, and to improve appearance and handling of foods. This paper reports the preparation of a

68 nano-hybrid composed by LDH and cinnamate, and its possible use as green pesticide against an  
69 important group of plant pathogens, such as *Phytophthora* spp. (Jung et al., 2018), and  
70 antimicrobials for the reduction the pathogens post-harvest contamination, at different active  
71 molecule loading. The dispersion into a pectin matrix was conducted through high energy ball  
72 milling in presence of water. Cast films were obtained and analyzed. Structural, thermal,  
73 mechanical, barrier properties were evaluated and correlated to the filler loading. The controlled  
74 release analysis of cinnamate was followed as function of time. Antimicrobial activity of the nano-  
75 hybrid composed by LDH and cinnamate was also assayed. Particularly, several strains of bacteria  
76 and *Phytophthora* belonging to different pathogen species were analyzed.

77

## 78 2. Experimental

79

### 80 2.1 Materials

81

82  $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ , NaOH and trans-cinnamic acid were purchased from Sigma-  
83 Aldrich (Italy). Pectins from apples were purchased from Sigma Aldrich in powder form. The  
84 molecular weight is 30,000-100,000 and the degree of esterification about 70-75%, on a dry basis,  
85 total impurities  $\leq 10\%$  water (CAS Number: 9000-69-5).

86

### 87 2.2 Preparation of ZnAl-o-BzOH by coprecipitation method

88 30 mL of an aqueous solution of  $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$  (12.9 g, 43.4 mmol) and  $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$  (8.14 g,  
89 21.7 mmol) were added to 30 ml of a trans-cinnamic sodium salt solution (6.3 g, 36.9 mmol) under  
90 stirring and under nitrogen flow. The pH slowly reached the value of 7.5 by adding 1M NaOH. At  
91 the end, the precipitate was washed with distilled water and left in oven at 50° C for 24h, under  
92 vacuum (Frunza, Lisa, Popa, Miron, & Nistor, 2008). The chemical formula obtained from the  
93 elemental analysis was the following:  $[\text{Zn}_{0.65}\text{Al}_{0.35}(\text{OH})_2] (\text{C}_9\text{O}_2\text{H}_7)_{0.35} \cdot 0.7 \text{H}_2\text{O}$  with value of the  
94 molar fraction  $x = \frac{M^{\text{III}}}{M^{\text{III}} + M^{\text{II}}}$  of 0.35 and molecular weight of 149.99 g/mol; the amount of trans-  
95 cinnamic anion intercalated in ZnAl-o-BzOH is 34.3 wt % of the total weight. Therefore almost all  
96 the aluminium is co-precipitated with the zinc ions to obtain a solid with the stoichiometry of two  
97 Zn(II) atoms for each Al(III) atom. This corresponds to an ideal arrangement of the brucite-like  
98 sheet with each aluminium atom surrounded by six zinc atoms (Oswald & Asper, 1977).

99

100

### 2.3 Composites Pectin/LDH-cinnamate: preparation and characterization

Composites based on pectin plasticized with glycerol and 2.5 wt%, 5 wt% and 10 wt% of nano-hybrid were prepared by dissolving the powder of pectin and LDH-cinnamate, in weight ratio (pectin: LDH) 97.5:2.5, 95:5 and 90:10, in 30 ml of water-glycerol solution at 4 vol % of glycerol, and left stirring at 80 °C for 60 min. Nano-hybrid LDH-cinnamate, the pectin powders, and water-glycerol were then milled at room temperature in a Retsch (Germany) planetarium ball mill (model PM 100), using a cylindrical steel jar of 50 cm<sup>3</sup> with 5 steel balls of 10 mm of diameter. The rotation speed used was 580 rpm and the milling time was 1 h. The mixtures obtained were slowly evaporated in Petri dishes. Films of pure pectin and pectin/LDH-cinnamate/glycerol for each percentage of nano-hybrid were obtained in the same described experimental conditions. All films, having the same thickness ~300 µm, were dried in a vacuum oven at room temperature for 3 days.

The average film thickness was evaluated by a "Hacloser" digital micrometer (Accuracy: 0.01mm/0.0005" - 0.001mm /0.00005")

### 2.4 Methods of investigation

*X-ray diffraction (XRD)* patterns were taken, in reflection, with an automatic Bruker diffractometer equipped with a continuous scan attachment and a proportional counter, using nickel-filtered Cu K $\alpha$  radiation (K $\alpha$  = 1.54050 Å) and operating at 40 kV and 40 mA, step scan 0.05° of 2 $\theta$  and 3 s of counting time.

*Thermogravimetric analyses (TGA)* were carried out in air atmosphere with a Mettler TC-10 thermobalance from 30°C to 800 °C at a heating rate of 10 °C/min.

*Mechanical properties* of the samples were evaluated, in tensile mode, at room temperature and ambient humidity (about 50%) using a dynamometric apparatus INSTRON 4301. Experiments were conducted at room temperature on pectin and composites' films with the deformation rate of 2 mm/min. The specimens were 10 mm wide and  $\cong$  250 µm thick. The initial length of the samples was 10 mm. Elastic modulus was derived from the linear part of the stress-strain curves, giving to the samples a deformation of 0.1%. Data were averaged on five samples.

*Barrier properties* of water vapor were evaluated using conventional Mc Bain spring balance system, which consists of a glass water-jacketed chamber serviced by a high vacuum line for sample degassing and vapor removal. Inside the chamber, samples were suspended to a helical quartz spring supplied by Ruska Industries (Houston, TX) having a spring constant of 1.52 cm/mg. The temperature was controlled to 30  $\pm$  0.1 °C by a constant temperature water bath. Samples were exposed to the water vapor at fixed pressures, P, giving different water activities  $a = P/P_0$ , where P<sub>0</sub>

134 is the saturation water pressure at the experimental temperature. The spring position was recorded  
 135 as a function of time using a cathetometer. The spring position data were converted to mass uptake  
 136 data using the spring constant, and the process was followed to a constant value of sorption for at  
 137 least 24 h. Data averaged on three samples. Measuring the increase of weight with time, for the  
 138 samples exposed to the vapor at a given partial pressure, it is possible to obtain the equilibrium  
 139 value of sorbed vapor,  $C_{eq}(g_{solvent}/100 g_{polymer})$ . Moreover, in the case of Fickian behavior, that is a  
 140 linear dependence of sorption on square root of time, it is possible to derive the mean diffusion  
 141 coefficient from the linear part of the reduced sorption curve, reported as  $C_t/C_{eq}$  versus square root  
 142 of time, by Equation (1): (Koros, Burgess, & Chen, 2015)

$$143 \quad \frac{C_t}{C_{eq}} = \frac{4}{d} \left( \frac{Dt}{\pi} \right)^{1/2} \quad (1)$$

144 where  $C_t$  is the penetrant concentration at the time  $t$ ,  $C_{eq}$  the equilibrium value,  $d$  (cm) the thickness  
 145 of the sample and  $D$  ( $cm^2/s$ ) the average diffusion coefficient. The sorption parameter ( $S$ ), is  
 146 obtained from the equilibrium concentration ( $C_{eq}$ ) of the permeant vapor as a function of the partial  
 147 pressure:

$$148 \quad S = \frac{d(C_{eq})}{dp} \quad (2)$$

149 All the samples showed a Fickian behavior during the sorption of water vapor at different activities.  
 150 Using Equation (1) it was possible to derive the diffusion coefficient,  $D$ , at every fixed vapor  
 151 activity ( $a = p/p_0$ ), and the equilibrium concentration of solvent into the sample,  $C_{eq}(g_{solvent}/100$   
 152  $g_{polymer})$ . For polymer-solvent systems, the diffusion parameter is usually not constant, but depends  
 153 on the vapor concentration, according to the empirical Equation (3):

$$154 \quad D = D_0 \exp(\gamma C_{eq}) \quad (3)$$

155 where  $D_0$  ( $cm^2/s$ ) is the zero concentration diffusion coefficient (related to the fractional free  
 156 volume and to the microstructure of the polymer);  $\gamma$  is a coefficient, which depends on the fractional  
 157 free volume and on the effectiveness of the penetrant to plasticize the matrix (Koros, Burgess, &  
 158 Chen, 2015). The permeability ( $P$ ) coefficient is described as the product of a thermodynamic  
 159 parameter which is the sorption coefficient ( $S$ ) and a kinetic parameter which is the zero diffusivity  
 160 or diffusion coefficient ( $D_0$ ):

$$161 \quad P = S \times D_0 \quad (4)$$

162 *The release kinetics* of cinnamate were performed by ultraviolet spectrometric measurement using a  
 163 Spectrometer UV-2401 PC Shimadzu (Japan). The tests were performed using rectangular  
 164 specimens of  $2 \text{ cm}^2$  and same thickness ( $\cong 200 \mu\text{m}$ ), placed into 25 mL of ethanol with 0.9 wt% of  
 165 tetrabutylammonium chloride and stirred at 100 rpm in an orbital shaker (VDRL MOD. 711+ Asal

166 S.r.l.). The release medium was withdrawn at fixed time intervals and replenished with fresh  
167 medium. The considered band was at 268 nm.

## 168 2.5 Microbial strains

169  
170 The microorganisms used in in this work for the antimicrobial tests are listed in Table 1. Bacteria  
171 were cultured in BHI broth or BHI agar (Microbiol, Cagliari, IT) and incubated at 37°C for 24 h,  
172 while *Phytophthora* spp. were cultured on carrot agar (CA) (Scanu et al., 2014), and incubated at 20  
173 °C for 24-48 h.

174

175 *Table 1: microorganisms used in the present work and sources*

Tested microorganisms	Sources
<i>Bacteria</i>	
<i>Staphylococcus aureus</i> DSMZ 20231	DSMZ
<i>Listeria monocytogenes</i> DSMZ 20600	DSMZ
<i>Escherichia coli</i> DSMZ 30083	DSMZ
<i>Salmonella bongori</i> DSMZ 13772	DSMZ
<i>Phytophthora</i>	
<i>Phytophthora cinnamomi</i> PH105	UNISS
<i>Phytophthora palmivora</i> PH090	UNISS

176

177 DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, German Collection of Microorganism  
178 of Cell Cultures; UNISS, Collection of Dipartimento di Agraria – University of Sassari, Italy

179

### 180 2.5.1 Broth microdilution test

181

182 The minimal inhibitory concentration (MIC) of the cinnamic acid of the bacterial species was tested  
183 by the microdilution broth method, according to Fancello et al. (2016). Briefly, cinnamic acid stock  
184 solution was first prepared with a concentration of 25 mg/mL in a 75% ethanol aqueous solution.  
185 Stock solutions were then diluted in sterile distilled water, to give a series of concentrations ranging  
186 from 25 mg/mL to 0.097 mg/mL. Overnight cultures were then used to prepare microbial  
187 inoculation used for the test. Aliquots of 100 µL of diluted inoculation at desired cells concentration  
188 were added to each well in the 96-well micro-dilution plate already containing 100 µL of desired  
189 cinnamic acid dilutions. The plates were then incubated at 37 °C for 24 h. After incubation, MICs  
190 (mg/mL) values were determined as the lowest concentration that inhibited visible growth of the  
191 tested microorganism, which was indicated by absence of turbidity. Each test was performed in  
192 quadruplicate and the experiments were repeated twice.

193

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195

196

## 197 2.5.2 Modified Agar diffusion and dual culture tests

198

199 The growth of bacteria was monitored after exposure of Pectin/LDH-cinnamate 10wt% as the  
200 following procedure. Bacteria were grown overnight on the specific media as mentioned before.  
201 The day after,  $5 \times 10^6$ /mL cells were stricken on BHI agar medium (Microbiol, Cagliari, IT). Disks  
202 of 10 mm of Pectin/LDH-cinnamate 10 wt% and Pectin/cinnamic acid 3.6 wt% were seeded on  
203 plates. To confirm this data, the same quantity of cinnamic acid contained in the Pectin/LDH-  
204 cinnamate was spotted (5  $\mu$ L /spot) onto Whatman 3 MM Chromatographic paper disks (0.34 mm  
205 paper thickness, 460 $\times$ 570 mm) and seeded on plates. For both tests the inhibition halos were  
206 measured after 24 h of incubation at 37 °C. Each assay was replicated 3 times. The diameter of the  
207 clear zone around the disc was measured and expressed in millimeters (disk diameter included). The  
208 rate of inhibition was determined according to Sagdic et al. (2003), a diameter of 10 to 15 mm was  
209 considered as slight antibacterial activity; a diameter of 16 to 20 mm as moderate antibacterial  
210 activity and a diameter of 20 mm as strong antibacterial activity. The antifungal properties of the  
211 biofilm against *Phytophthora* spp. was also tested using the dual culture method. A mycelial plug (5  
212 mm diameter) were cut from the margin of actively growing 5-day-old colony, using a flamed cork  
213 borer, and placed on one side of a Petri dish containing 20 ml of CA (Scanu et al., 2014).  
214 Meanwhile a 10 mm disk of Pectin/LDH-cinnamate 10 wt% was placed on the opposite side of the  
215 plate, with a 30 mm of distance between the two plugs. Plates containing the *Phytophthora* species  
216 without the biofilm were used as negative control. The plates were incubated at 20°C in the dark.  
217 There were six replicates for each pathogen-biofilm combination and the test was repeated twice.  
218 The radial growth of the two *Phytophthora* species tested was recorded when the control treatments  
219 covered the plate surface. The percent growth inhibition was calculated according to the formula:  
220  $PGI = 100 (DC-DT)/DC$  where PGI = the percentage of inhibition of mycelia growth; DC = the  
221 radial growth of *Phytophthora* spp. in control plate; DT = the radial growth of *Phytophthora* spp.  
222 towards the biofilm.

223

## 224 3. Results and discussion

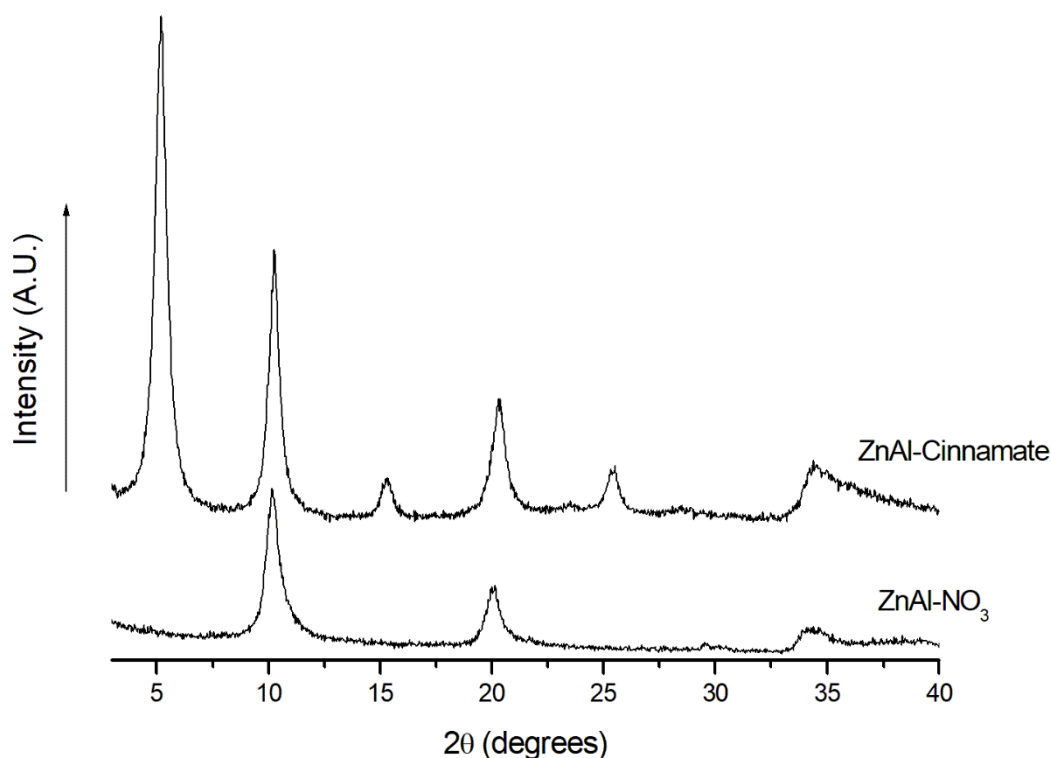
225

### 226 3.1 Characterization of filler

227

228 Figure 1 reports the XRD spectra of pristine LDH-NO<sub>3</sub> and the LDH modified with cinnamate

229 anion. It is evident that the nitrate form of LDH presents the main peaks at about  $10^\circ$  and  $20^\circ$  of  $2\theta$ ,  
230 relative to the basal spacing (003) and (006), respectively. The intercalation of cinnamate molecule  
231 is evident from the modification of the basal spacing of the LDH with the shifting of the diffraction  
232 peaks at lower angle (Weiling, Qinglin, & Yong, 2007).  
233



234  
235 **Figure 1: XRD spectra of pristine LDH-NO<sub>3</sub> and the LDH modified with cinnamate molecule**  
236

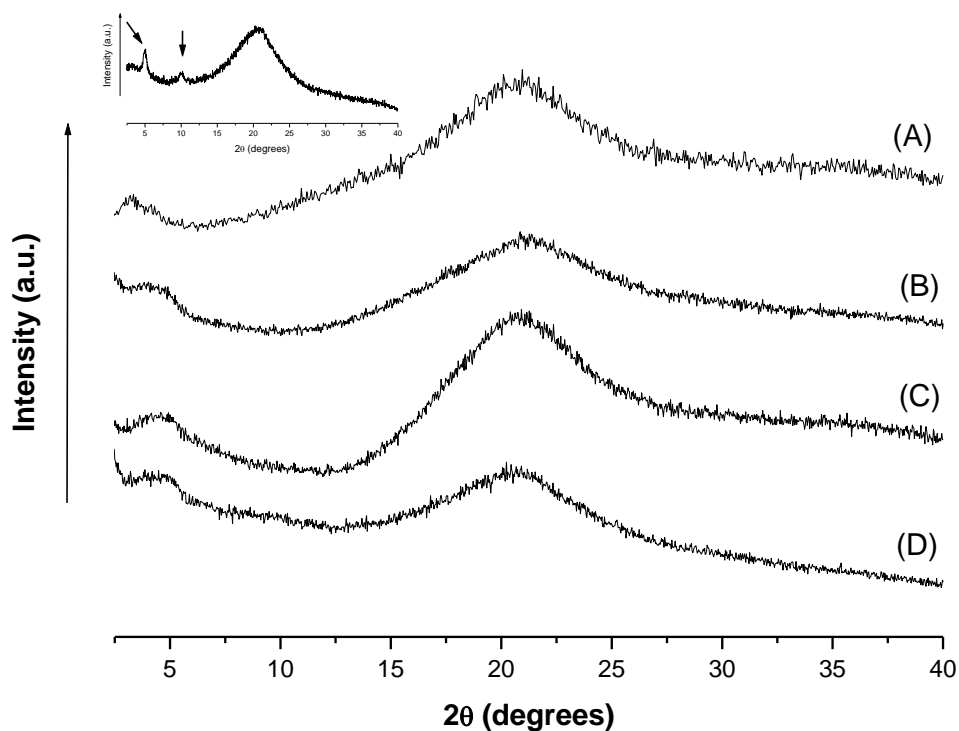
237 TGA analysis was carried out on LDH-NO<sub>3</sub> (A), cinnamic acid (B) and LDH-cinnamate (C). The  
238 TGA curve of LDH-NO<sub>3</sub>, reported in the supporting information (SI 1), shows three steps of  
239 decomposition: i) the first at around  $150^\circ\text{C}$ , corresponding to the loss of absorbed water between  
240 LDH layers, ii) a second, occurring around at  $250^\circ\text{C}$ , is due to the thermal decomposition of nitrate  
241 anions, iii) a third, at about  $400^\circ\text{C}$ , due to the dehydroxylation of the LDH sheets (Park et al., 2010).  
242 Experimental results demonstrate the stabilization of cinnamate molecule within the interlayer  
243 space of LDH. In fact, free cinnamic acid (B) exhibits its degradation in one step, above  $150^\circ\text{C}$ . The  
244 intercalation into the inorganic matrix results in a significant improvement in thermal stability: the  
245 main thermal decomposition of the hybrid takes place at around  $374^\circ\text{C}$ . The hydroxide framework  
246 transforms finally into its corresponding oxide by dehydroxylation above  $500^\circ\text{C}$ . Such behavior,

247 already found for several molecules incorporated into LDH layers (Gorrasi & Bugatti, 2016),  
248 suggests a protecting effect of the LDH respect to the cinnamate and a stable interaction LDH-  
249 organic molecule due to electrostatic forces.

### 250 *3.2 Characterization of composites*

251

252 Figure 2 reports the XRD analysis on pectin and composites. Pectin spectrum shows the typical  
253 form of plasticized material, with a broad halo centered at about  $21^\circ$  of  $2\theta$ . Such amorphous  
254 organization is retained in all composites, at all filler loading (Masuelli & Renard, 2017). The  
255 absence of any diffraction peak relative to the filler, in the spectra of the composites, suggests the  
256 exfoliation of the LDH-cinnamate in the used processing conditions. **The mechanical action, in**  
257 **presence of water, favors a completely delamination of the LDH interlayers at any filler**  
258 **composition. No re-aggregation of the LDH is observed in the composites' films after the water**  
259 **evaporation.** In order to better support this hypothesis we prepared a mechanical mixture of pectins  
260 powder with 2.5% of LDH-cinnamate (inset of Figure 2). It is evident that the simple grinding of  
261 the filler with the polymer did not induced any structural modification in both components. In  
262 particular, the **basal spacing** from X-ray reflections of the inorganic filler remained intense and  
263 sharp, with the XRD pattern being just a superposition of the two components' spectra.



264

265 **Figure 2: XRD on films (A) pectin, (B) pectin/LDH-cinnamate 2.5%, (C) pectin/ LDH-cinnamate 5%, (D)**  
 266 **pectin/LDH-cinnamate 10%. The inset reports XRD on a mechanical mixture composed of pectin and 2.5% of**  
 267 **LDH-cinnamate**

268 Thermal behavior was evaluated on the composites through thermogravimetric analysis (TGA and  
 269 DTG). Results are reported in the supporting information (SI 2). It is also shown the  
 270 thermogravimetric curve of the pure pectin, for comparison. The thermo-oxidative degradation of  
 271 pectins is a series of complex events that involves three steps of degradation: i) the first one,  
 272 centered at about 90°C, due to loss of water; ii) the second one, between 150°C and 280°C, due to  
 273 pyrolytic decomposition consisting of a primary and secondary decarboxylation involving the acid  
 274 side group and a carbon in the ring (Gorrasi, 2015; Shim, Hajaligol & Baliga, 2004; Waymack,  
 275 Belobe, Baliga, & Hajaligol, 2004;); iii) the third step between about 650°C and 720°C,  
 276 corresponding to the oxidation region. The second step of degradation occurs at the same  
 277 temperatures either for pectin or for the composites independently of the filler loading; whereas the  
 278 third degradation step is dependent on the filler amount. Its temperature decreases on increasing the  
 279 filler loading, as evidenced by the DTG analysis (part B of the figure). It has been reported that the  
 280 glycerol percentage has a significant effect on the degradation of pure pectin (Yang & Yang, 2016),  
 281 but in this case the glycerol amount is the same in all composites. It can be hypothesized that oxides

282 of Zn and Al, that are formed for the decomposition of LDH at high temperatures, can catalyze the  
283 oxidation of pectin matrix.

284 Mechanical properties were estimated on all samples. From the stress-strain curves, not reported,  
285 they were evaluated elastic moduli (MPa), stress at break point,  $\sigma_b$  (MPa), and elongation at break,  
286  $\epsilon_b$  (%). Table 2 reports the experimental data. The elastic modulus, E (MPa), of the unfilled pectin  
287 is lower than the one evaluated on pectin film treated in the same conditions, but with no glycerol  
288 (Gorrasi, Bugatti, & Vittoria, 2012). This is due to the plasticizing effect of the glycerol that lowers  
289 the mechanical resistance of the material (Yang & Yang, 2016). The elastic modulus increases on  
290 increasing the filler content and the stress at break point does not change up to 5 wt% of filler and  
291 increases significantly for 10 wt % of LDH-cinnamate. This could be due to the reinforcing effect  
292 of the nano-hybrid into the polymeric matrix. The inorganic lamellae, well dispersed into the  
293 organic phase (see XRD results) directly enhances the stiffness of the nanocomposites, because the  
294 exfoliated LDHs nanolayers are thoroughly dispersed into the pectin matrix, and each nanolayer  
295 could contribute to the reinforcement of the nanocomposites. This is particularly evidenced in the  
296 improvement of the elastic modulus. As expected, the strain at break decreases with filler content  
297 for the different chemical nature of both composites' components. The dispersed phase, at high  
298 elongation and loading, behaves as "defects" into the polymer matrix.

299

300

301

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303

**Table 2 : Mechanical parameters evaluated from stress-strain curves**

<b>Sample</b>	<b>E (MPa)</b>	<b><math>\sigma_b</math> (MPa)</b>	<b><math>\epsilon_b</math> (%)</b>
<b>Pectin</b>	<b>11 ± 0.81</b>	<b>3.96 ± 0.33</b>	<b>1.25 ± 0.13</b>
<b>Pectin/LDH-cinnamate 2.5%</b>	<b>14 ± 0.88</b>	<b>1.96 ± 0.34</b>	<b>0.79 ± 0.12</b>
<b>Pectin/LDH-cinnamate 5%</b>	<b>26 ± 0.83</b>	<b>1.98 ± 0.31</b>	<b>0.47 ± 0.11</b>
<b>Pectin/LDH-cinnamete 10%</b>	<b>33 ± 0.84</b>	<b>4.15 ± 0.37</b>	<b>0.27 ± 0.10</b>

304

305 Figure 3 shows the barrier properties, sorption (A) and diffusion (B), evaluated on all the  
306 composites. Data relative to unfilled pectin are also reported, for comparison. The sorption isotherm  
307 of pectin plasticized with glycerol follows a typical Langmuir adsorption (Koros, Burgess, & Chen,  
308 2015) where the solvent molecules are absorbed on specific sites at low vapour pressure; when all  
309 the sites are occupied a constant value of concentration is shown on increasing the vapor activity.  
310 Equation (2) allowed to evaluate the sorption coefficients for all the samples. It is evident a  
311 significant reduction of water sorption in the composites at 5 and 10 wt% of filler loading (Table 3).

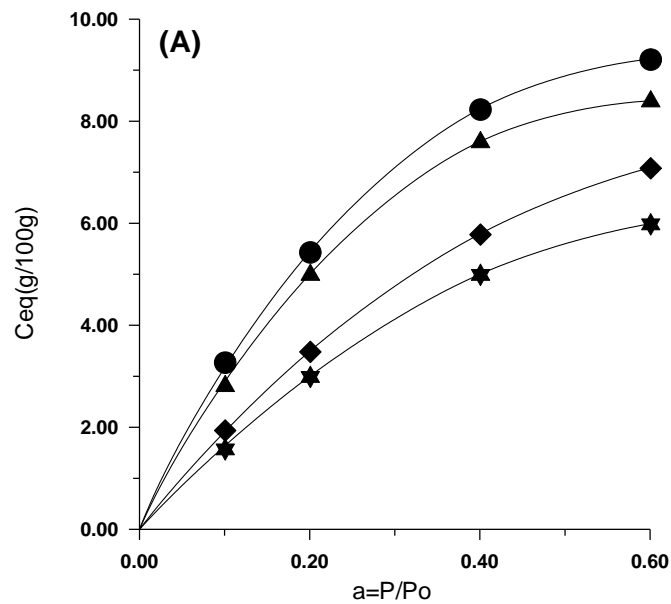
312 From XRD results it was evidenced that the structure of the pectin does not change for the filler  
313 addition, in terms of degree of crystallinity, thus the variation in the sorption must be attributed to  
314 other factors and not to a reduction of the amount of amorphous permeable phase. Being the water a  
315 polar solvent it is assumed that the adsorption occurs on polar groups of the pectin matrix. The less  
316 availability of the polar sites causes, then, a decrease of sorption. The preferential interaction of the  
317 pectin matrix with the polar groups of the LDH-cinnamate could be a possible explanation of the  
318 sorption reduction with filler loading.

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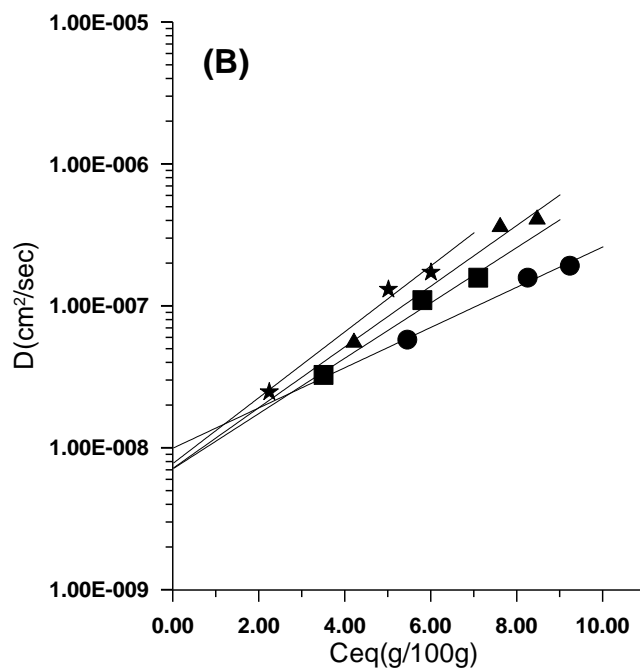
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325

326 Figure 3: (A) Sorption isotherm for samples: pectin (●), Pectin/LDH-cinnamate 2.5% (Δ), Pectin/LDH-  
 327 cinnamate 5% (◇), Pectin/LDH-cinnamate 10% (\*); (B) Diffusion for samples: pectin (●), Pectin/LDH-  
 328 cinnamate 2.5% (Δ), Pectin/LDH-cinnamate 5% (◇), Pectin/LDH-cinnamate 10% (\*)

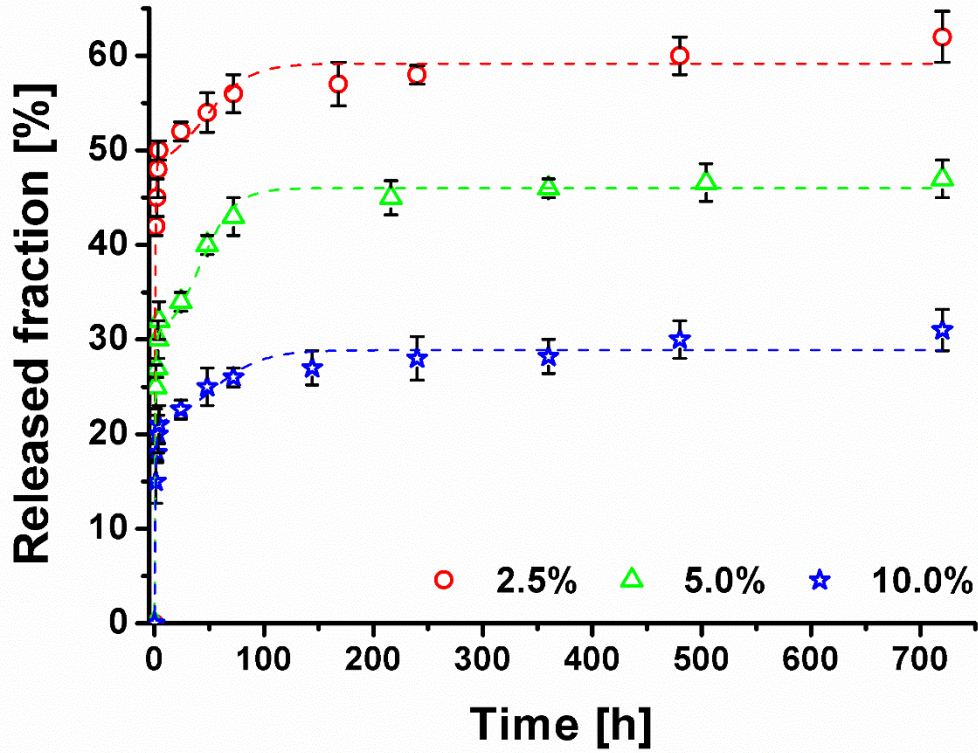
329

330 Table 3: barrier parameters, sorption diffusion and permeability, of pectin and composites

Sample	Sorption (g/100g/mmHg)	Diffusion (cm <sup>2</sup> /s)*10 <sup>9</sup>	Permeability (g/100g/mmHg)*(cm <sup>2</sup> /s)*10 <sup>9</sup>
Pectin	28.32±0.02	10.10±0.08	286.03±2.46
Pectin/LDH-cinnamate 2.5%	25.64±0.07	7.27±0.05	186.40±1.79
Pectin/LDH-cinnamate 5%	17.86±0.05	7.34±0.03	131.09±0.90
Pectin/LDH-cinnamate 10%	15.16±0.02	7.87±0.06	119.31±1.07

331

332 Figure 4 reports the release of cinnamate anion (%) from the composites at different nano-hybrid  
 333 loading, as function of time (h).



334

335 **Figure 4: Release of cinnamate molecule, as function of contact time (h), for composites at 2.5, 5 and 10% of**  
 336 **nano-hybrid loading. Dotted lines are the fitting with the model expressed from Equation (5)**

337

338 The release can be visualized in two steps: the initial one is related to the burst of the molecules  
 339 located on the external surfaces of the films, and a second step that can be attributed to the diffusion  
 340 of the cinnamate molecules from the bulk. The second step is followed by a plateau. It is worth to  
 341 note that the amount of released molecule decreases with increasing the filler loading. In order to  
 342 give a phenomenological interpretation to the experimental data, we used the Gallagher and  
 343 Corrigan model (Gallagher & Corrigan, 2000). The model assumes that the drug release at any time  
 344 is the sum of two processes: an initial diffusion controlled phase and a subsequent polymer  
 345 degradation controlled phase. In particular it describes a two-stage drug release kinetics: the first  
 346 part of the equation reflects the diffusion controlled dissolution of drug to the medium, which is  
 347 characterized by the first order kinetics; the second part describes that the drug release rate depends  
 348 on the polymer relaxation (Dunne, Ramtoola, & Corrigan, 2009; Gallagher & Corrigan, 2000;  
 349 Milallos, Alexander, & Riga, 2008; Zhong & Mi, 2005). Therefore  $f_t$ , the total fraction of drug  
 350 released at a given time  $t$  is given by:

351

352

$$f_t = f_b * (1 - e^{-k_1 t}) + (f_{tmax} - f_b) \left( \frac{e^{k_2(t-t_{2max})}}{1 + e^{k_2(t-t_{2max})}} \right) \quad (5)$$

353 where  $f_t$  is the accumulative drug release percentage at time  $t$ ,  $k_1$  is the first order release constant  
 354 (Stage 1),  $k_2$  is the second stage release constant due to the polymer relaxation,  $f_b$  is the  
 355 accumulative drug release percentage during the Stage 1,  $f_{t_{max}}$  is the maximum drug release  
 356 percentage during the whole process,  $t_{2max}$  is the time at which drug release rate reaches the  
 357 maximum. The correlation coefficient ( $R^2$ ) is an indicator of the best fitting for the considered  
 358 model.  
 359

360 Table 4 reports the kinetic parameters derived using Equation 5. It can be noted that the burst ( $f_b$ )  
 361 parameter and the first order release constant,  $k_1$ , decrease with filler loading, while the time at  
 362 which the drug release rate reaches the maximum,  $t_{2max}$ , increases. The  $k_2$  constant remains almost  
 363 unvaried at any filler composition. It is hypothesized that such behavior could be related either to  
 364 the hindrance effect created by the LDH platelets, that delay the counter-diffusion of the active  
 365 molecule (Bugatti, Vertuccio, Viscusi, & Gorrasi, 2018), or preferential to hydrogen bonds between  
 366 the cinnamate and the system pectin-glycerol (see sorption data).  
 367

368 *Table 4: kinetic parameters derived from Equation (5)*

Sample	$f_b$ (%)	$t_{2max}$ (h)	$k_1$ ( $h^{-1}$ )	$k_2$ ( $h^{-1}$ )	$R^2$
Pectin/LDH-cinnamate 2.5%	47	45	1.84	$4.89 \times 10^{-2}$	0.991
Pectin/LDH-cinnamate 5%	29	41	1.50	$6.40 \times 10^{-2}$	0.994
Pectin/LDH-cinnamate 10%	20	51	1.08	$4.27 \times 10^{-2}$	0.984

### 376 3.3 Antimicrobial activity

377  
 378 The antimicrobial activity was evaluated firstly performing an in vitro test to determine the MIC of  
 379 the cinnamic acid against the microbial species considered in this work and reported in table 1  
 380 (*Staphylococcus aureus* DSMZ 20231, *L. monocytogenes* DSMZ 20600, *E. coli* DSMZ 30083 and  
 381 *S. bongori* DSMZ 13772 and two strains of *Phytophthora* namely *P. cinnamomi* (isolate PH105)  
 382 and *P. palmivora* (isolate PH090) ), by the microdilution broth test. The different strains tested  
 383 showed the same sensitivity against the cinnamic acid with a MIC value of 1.56 mg/mL (10.52  
 384 mM), in accordance with the values found by other authors (Guzman, 2014). The MIC value  
 385 obtained was used to set up the concentration of cinnamic acid in subsequent experiments.

386 The in vitro antimicrobial activity was, then, evaluated on a composite based on pectin and 10% of  
 387 LDH-cinnamate (3.6% of active molecule). Such sample was chosen as model sample because  
 388 contains the maximum active specie. It was used to investigate the effect of the active molecule

389 bonded to the LDH and dispersed into the pectin. For the bacteria, results on modified agar  
 390 diffusion test (disks of pectin/LDH-cinnamate directly seeded on the agar plates (see Table 5)  
 391 indicated that the cinnamate bonded to the LDH and dispersed into the pectin exhibited slight  
 392 antimicrobial activity against *S. aureus* DSMZ 20231, with a diameter halo of about  $11.5\pm 0.07$  mm  
 393 and a moderate activity against *E. coli* DSMZ 30083 with a diameter halo of about  $16.5\pm 0.07$  mm  
 394 while exerted an activity against *L. monocytogenes* DSMZ 20600 and *S. bongori* DSMZ 13772 with  
 395 a diameter < 10 mm. The agar diffusion test used as control with cinnamic acid alone imbibed in  
 396 Whatman paper discs, showed an antimicrobial activity against the four pathogen strains used, with  
 397 halos that varied from *S. aureus* DSMZ 20231 with about  $14 \pm 0.0$  mm, *S. bongori* DSMZ 13772  
 398 with about  $13.5\pm 0.07$  mm, *E. coli* DSMZ 30083 with about  $12.8\pm 0.04$  mm and finally *L.*  
 399 *monocytogenes* DSMZ 20600 with about  $12\pm 0.02$  mm (Table 5). The mechanism under this  
 400 phenomenon is quite complex. A possible explanation of the different antimicrobial ability of LDH-  
 401 cinnamate into pectin could be found in the different cell surface charge of the different pathogens  
 402 used and/or different hydrophobicity of cell surface that can influence the reaction of the bacterial  
 403 strains (Dickson & Koohmaraie, 1989).

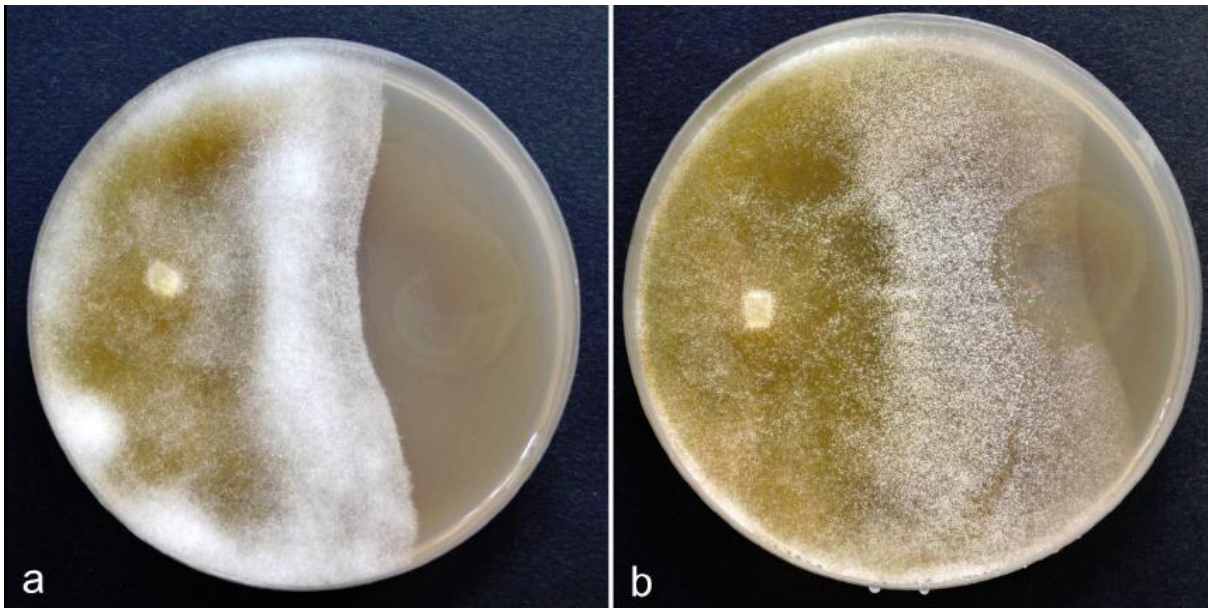
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405 Table 5. Antimicrobial activity by modified agar diffusion test (according to Sagdic et al., (2003))

Bacterial strains	Pectin/LDH-cinnamate 10% (Ø mm)	Cinnamic acid (Ø mm)
<i>S. aureus</i> DSMZ 20231	$11.50\pm 0.07$	$14.00\pm 0.01$
<i>E. coli</i> DSMZ 30083	$16.50\pm 0.07$	$12.75\pm 0.04$
<i>L. monocytogenes</i> DSMZ 20600	<10.00	$12.00\pm 0.02$
<i>S. bongori</i> DSMZ 13772	<10.00	$13.50\pm 0.07$

406

407 For the *Phytophthora* spp, the dual culture assay generated significant inhibitory effects on the  
 408 radial growth of the tested pathogens. This inhibition was clearly discerned by a limited growth and  
 409 a complete absence of pathogen mycelium around the biofilm disk (Figure 5).



410

411

412 **Figure 5: In-vitro evaluation of Pectin/10% LDH-cinnamate in dual culture assay with *Phytophthora* spp.: colony**  
413 **of *P. cinnamomi* (a) and *P. palmivora* (b) after 5 days at 20°C**

414

415 There was a significant reduction in mycelial growth of both pathogens. The highest percent of  
416 inhibition of mycelial growth was observed in the case of *P. cinnamomi*, with a percent growth  
417 inhibition averaging 53.3%. The mycelial growth of *P. cinnamomi* was entirely limited when in  
418 contact with the biofilm disk (Figure 5a). The growth rate of *P. palmivora* was also influenced by  
419 the presence of the film Pectin/LDH-cinn, however the inhibition was lower and around 36.7%, and  
420 the pathogen was able to grow above the biofilm (Figure 5b). The strong inhibition rate against  
421 *Phytophthora* spp. suggests that this compound could be a valid alternative to the use of synthetic  
422 fungicides, which are limited by the development of antimicrobial resistance and the harmful effects  
423 to human health (Parra & Ristaino, 2001). Additionally, many *Phytophthora* spp. (including *P.*  
424 *cinnamomi* and *P. palmivora*) are emerging pathogens in natural and forest ecosystems, where due  
425 to the lack of legal authorisations and for environmental reasons, the use of fungicides is not a  
426 realistic option for the control of *Phytophthora* diseases in most countries (Jung et al., 2018). The  
427 film Pectin/LDH-cinn was able to reduce significantly the growth of both *Phytophthora* spp. tested;  
428 however, it is interesting to note that it was less effective at inhibiting mycelial growth in *P.*  
429 *palmivora* as compared to *P. cinnamomi*. Further investigations are needed in order to explore the  
430 LDH-cinnamate effect on the different life cycle stages of *Phytophthora* species as well as its  
431 efficacy in *in planta* inoculation trials and considering also lower active molecule's concentration.

432

433

434 **Conclusions**

435

436 This paper reported the preparation of green composites based on pectins and layered double  
437 hydroxides (LDH) intercalating cinnamate anion, as active molecule. The cinnamate loading into  
438 the LDH was 36%. Composites at 2.5, 5 and 10 wt% were prepared using ball milling technology in  
439 presence of water. Films were obtained and tested, respect to structural and functional properties.

440 The successful intercalation of cinnamate molecule, evidenced from the modification of the basal  
441 spacing of the LDH, was observed from XRD analysis. Data demonstrated a delamination of the  
442 nano-hybrid into the pectin matrix at any filler composition. The thermal analysis, conducted using  
443 TGA, demonstrated that the cinnamate molecule is thermally protected by the LDH layer. In  
444 addition,

445 the degradation of the pectin matrix was not greatly influenced from the nano-hybrid filler, except  
446 for the oxidation stage at high temperatures, that resulted anticipated. The nano-hybrid filler also  
447 improved the mechanical properties of the pectin matrix. Such an improvement is greatly evident at  
448 10 wt% of filler loading. Such reinforcing effect is mainly due to the well dispersed inorganic  
449 lamellae that enhance the stiffness of the composites. The strain at break point decreases with the  
450 filler content, because to the incompatibility of the inorganic nature of the filler and organic nature  
451 of the matrix.

452 The analysis of barrier properties, sorption and diffusion, to water vapour demonstrated a decrease  
453 of sorption with the increasing the filler loading, and no effect on the diffusion. Interaction between  
454 polar groups of pectin and filler were then hypothesized, resulting in a lower sorption of the polar  
455 water molecules. It was analyzed in vitro the release of the cinnamate molecule from all  
456 composites. It was correlated the filler loading to the release kinetics. The release kinetics of  
457 composites' membranes were found to be dependent on the nano-hybrid loading and were well  
458 fitted the Gallagher-Corrigan model. It was demonstrated that varying the filler loading it is  
459 possible to tune the cinnamate release for desired applications. The effects of the composite on the  
460 growth of two *Phytophthora* species through an in vitro experiment was tested. The strong  
461 inhibition rate detected on *P. cinnamomi* represents itself a very promising result, comparable to  
462 some fungicides. However, in order to determine whether this compound could outperform  
463 synthetic fungicides in controlling disease development, further studies are needed to investigate its  
464 effect on the survival structures of the pathogen (chlamydospores and oospores) in planta.

465

466 **Acknowledgements**

467 This work was supported by the project “High Performing Advanced Material Platform For Active  
468 and Intelligent Food Packaging: Cronogard™” (H2020-SMEINST-2-2016-2017). Grant agreement  
469 n. 783696

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