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

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Keywords: pectin, LDH, cinnamate, green pecticide, 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 pubblication 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
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via Giovanni Paolo II 132, 84084 Fisciano (SA)-Italy
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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 accondingly 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.

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-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.

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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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   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

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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.

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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.

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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)

***Highlights (for review)**

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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Giuliana Gorrasi[1]*

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[2]Nice Filler s.r.l., via Loggia dei Pisani, 25; 80133 Napoli -Italy-

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

Synthetic pesticides are molecules that contaminate soil, water, air, and their accumulation causes irreversible damage on all kinds of bio-systems. At the same time the indiscriminate use of these synthetic antimicrobial compounds poses serious issues for the spread of antimicrobial resistance in bacteria and fungi. In this context one of the main goal is represented by the possibility to protect crops without harmful effects on nature. Next to the methodologies of genetic engineering and natural enemies (Mao, Lewis, Lumsden, & Hebbar, 1998; Navon, 2000; Stevens & Lee, 1979), that have to be further validate for real applications, it is possible to use the tools of nanotechnology to assess alternative nature-compatible approaches. Layered Double Hydroxides (LDHs) are a class of inorganic lamellar solids that possess the characteristic to be soil-compatible. Their general formula 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 and M(III) is a trivalent cation such as Al, Cr, Fe or Ga with A^{n-} an exchangeable anion of charge n. The x value generally ranges between 0.2 to 0.4 and determines the positive layer charge density and the anion exchange capacity (Cavani, Trifiro, & Vaccari, 1991; Costantino, Ambrogi, Perioli, & Nocchetti, 2008; Herrero, Labajos, & Rives, 2009; Leroux & Taviot-Guého, 2005). The interlayer anions can be exchanged by other inorganic, organic or metallo-organic compounds in anionic form and the obtained structures can be used as active nano-hybrid fillers for polymers for targeted applications (Chen & Qu, 2003; Costantino et al., 2009; Muksing, Magaraphan, Coiai, & Passaglia, 2011; Qiu, Chen, & Qu, 2005; Romano, Naddeo, Guadagno, & Vertuccio, 2014; Zammarano et al., 2006). LDHs are also cheap materials that can be produced with high level of purity. This makes LDHs ideal matrices to carry active molecules in soils and control their sustained release into the desired medium. Furthermore, the intercalated molecule between the inorganic layers could be safely protected against chemical and biological degradations in soils. The dispersion of the active nano-hybrid into the soil is a crucial point because the simple dispersion in water causes, after water evaporation, a re-aggregation of the LDH layers and subsequent loss of adhesion on the plant and soil to be protected. An interesting alternative could be the dispersion of the nano-hybrid into a bio-based matrix soluble in water. Pectins are a class of complex water-soluble polysaccharides widely used to form coatings. They are carbohydrate products obtained by aqueous extraction of some edible plant material, usually citrus fruits or apples, available in high volume mainly in agricultural wastes. Pectin coatings have been also studied for their ability to retard lipid migration and moisture loss, and to improve appearance and handling of foods. This paper reports the preparation of a nano-hybrid composed by LDH and cinnamate, and its possible use as green pesticide against an important group of plant pathogens, such as *Phytophthora* spp. (Jung et al., 2018), and antimicrobials for reduce the pathogens post-harvest contamination, at different active molecule loading. The dispersion into a pectin matrix was conducted through high energy ball milling in presence of water. Cast films were obtained and analyzed. Structural, thermal, mechanical, barrier properties were evaluated and correlated to the filler loading. The controlled release analysis of cinnamate was followed as function of time. Antimicrobial activity of the nano-hybrid composed by LDH and cinnamate was also assayed. Particularly, several strains of bacteria and *Phytophthora* belonging to different pathogen species were analyzed.

2. Experimental

2.1 Materials

$Zn(NO_3)_2$*$6H_2O$, $Al(NO_3)_3$*$9H_2O$, NaOH and trans-cinnamic acid were purchased from Sigma-Aldrich (Italy). Pectins from apples were purchased from Sigma Aldrich in powder form. The molecular weight is 30,000-100,000 and the degree of esterification about 70-75%, on a dry basis, total impurities ≤10% water (CAS Number: 9000-69-5).

2.2 Preparation of ZnAl-o-BzOH by coprecipitation method

30 mL of an aqueous solution of $Zn(NO_3)_2$*$6H_2O$ (12.9 g, 43.4 mmol) and $Al(NO_3)_3$*$9H_2O$ (8.14 g, 21.7 mmol) were added to 30 ml of a trans-cinnamic sodium salt solution (6.3 g, 36.9 mmol) under stirring and under nitrogen flow. The pH slowly reached the value of 7.5 by adding 1M NaOH. At the end, the precipitate was washed with distilled water and left in oven at 50° C for 24h, under vacuum (Frunza, Lisa, Popa, Miron, & Nistor, 2008). The chemical formula obtained from the elemental analysis was the following: $[Zn_{0.65}Al_{0.35}(OH)_2]$ $(C_9O_2H_7)_{0.35}$ *0.7 H_2O with value of the molar fraction $x=M^{III}/M^{III}+M^{II}$ of 0.35 and molecular weight of 149.99 g/mol; the amount of trans-cinnamic anion intercalated in ZnAl-o-BzOH is 34.3 wt % of the total weight. Therefore almost all the alluminium is co-precipitated with the zinc ions to obtain a solid with the stoichiometry of two Zn(II) atoms for each Al(III) atom. This corresponds to an ideal arrangement of the brucite-like sheet with each aluminium atom surrounded by six zinc atoms (Oswald & Asper, 1977).

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^3 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 Kα radiation (Kα = 1.54050 Å) and operating at 40 kV and 40 mA, step scan 0.05° of 2ϑ 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^{-1}).

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 ≅ 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 ± 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_0 is the saturation water pressure at the experimental temperature. The spring position was recorded as a function of time using a cathetometer. The spring position data were converted to mass uptake data using the spring constant, and the process was followed to a constant value of sorption for at least 24 h. Data averaged on three samples. Measuring the increase of weight with time, for the samples exposed to the vapor at a given partial pressure, it is possible to obtain the equilibrium value of sorbed vapor, $C_{eq}(g_{solvent}/100\ g_{polymer})$. Moreover, in the case of Fickian behavior, that is a linear dependence of sorption on square root of time, it is possible to derive the mean diffusion coefficient from the linear part of the reduced sorption curve, reported as C_t/C_{eq} versus square root of time, by Equation (1): (Koros, Burgess, & Chen, 2015)

$$\frac{Ct}{Ceq} = \frac{4}{d}\left(\frac{Dt}{\pi}\right)^{1/2} \qquad (1)$$

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

$$S = \frac{d(Ceq)}{dp} \qquad (2)$$

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

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

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

$$P = S \times D_0 \qquad (4)$$

The release kinetics of cinnamate were performed by ultraviolet spectrometric measurement using a Spectrometer UV-2401 PC Shimadzu (Japan). The tests were performed using rectangular specimens of 2 cm^2 and same thickness (≅ 200 μm), placed into 25 mL of ethanol with 0.9 wt% of tetrabutylammonium chloride and stirred at 100 rpm in an orbital shaker (VDRL MOD. 711+ Asal

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

2.5 Microbial strains

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

Table 1: microorganisms used in the present work and sources

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

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

2.5.1 Broth microdilution test

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

2.5.2 Modified Agar diffusion and dual culture tests

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

3. Results and discussion

3.1 Characterization of filler

Figure 1 reports the XRD spectra of pristine LDH-NO_3 and the LDH modified with cinnamate anion. It is evident that the nitrate form of LDH presents the main peaks at about 10° and 20° of 2ϑ, relative to the basal spacing (003) and (006), respectively. The intercalation of cinnamate molecule is evident from the modification of the basal spacing of the LDH with the shifting of the diffraction peaks at lower angle (Weiling, Qinglin, & Yong, 2007).

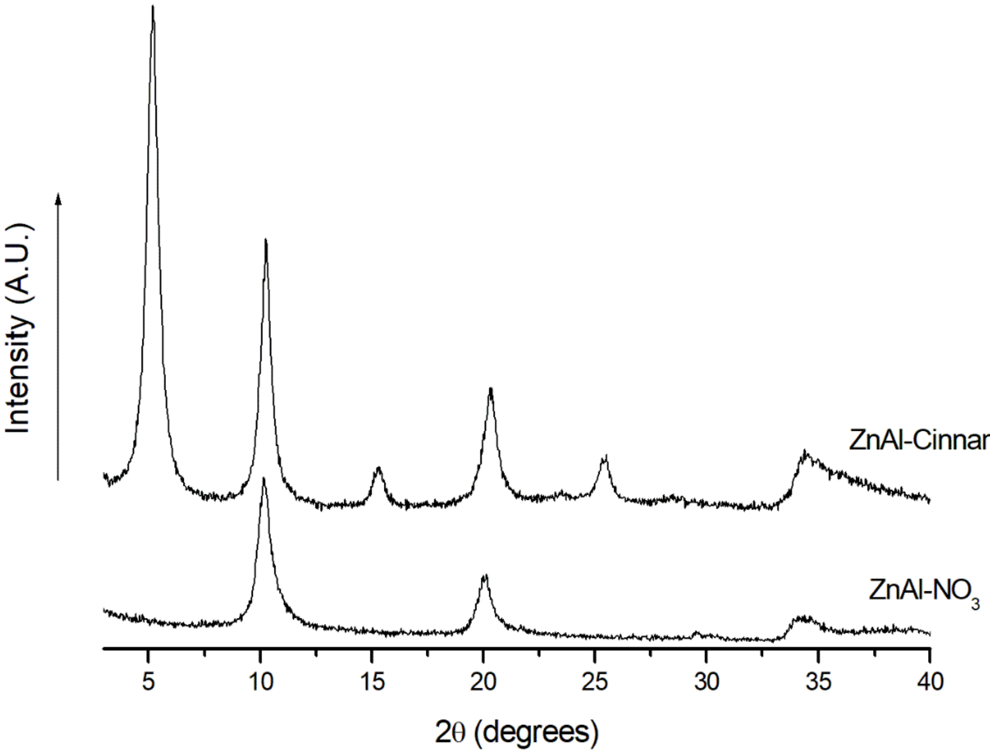

Figure 1: XRD spectra of pristine LDH-NO$_3$ and the LDH modified with cinnamate molecule

TGA analysis was carried out on LDH-NO$_3$ (A), cinnamic acid (B) and LDH-cinnamate (C). The TGA curve of LDH-NO$_3$, reported in the supporting information (SI 1), shows three steps of decomposition: i) the first at around 150°C, corresponding to the loss of absorbed water between LDH layers, ii) a second, occurring around at 250°C, is due to the thermal decomposition of nitrate anions, iii) a third, at about 400°C, due to the dehydroxylation of the LDH sheets (Park et al., 2010). Experimental results demonstrate the stabilization of cinnamate molecule within the interlayer space of LDH. In fact, free cinnamic acid (B) exhibits its degradation in one step, above 150°C. The intercalation into the inorganic matrix results in a significant improvement in thermal stability: the main thermal decomposition of the hybrid takes place at around 374°C. The hydroxide framework transforms finally into its corresponding oxide by dehydroxylation above 500°C. Such behavior, already found for several molecules incorporated into LDH layers (Gorrasi & Bugatti, 2016), suggests a protecting effect of the LDH respect to the cinnamate and a stable interaction LDH-organic molecule due to electrostatic forces.

FTIR spectra of LDH-NO_3 (A), cinnamic acid (B) and LDH-cinnamate (C) in the range 1000-2000 cm^{-1} are reported in the supporting information (SI 2). The cinnamic acid shows characteristic vibrations at 1682 cm^{-1} attributed to C=O stretching, at 1626 cm^{-1} due to C=C stretching, at 1313 cm^{-1} for C-O stretching, and at 1418 cm^{-1} for OH in-plane bending, respectively. The spectrum of the nano-hybrid LDH-cinnamate shows most of the vibrations assigned to both cinnamate and LDH, although several vibrations are overlapped. In particular, the strong vibrations at 1638 cm^{-1} is due to COO^- stretching of the intercalated cinnamate. This result suggests that cinnamate anion is stabily intercalated between the LDH galleries, and its anion form electrostatically interact with positively charged LDH layers.

3.2 Characterization of composites

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

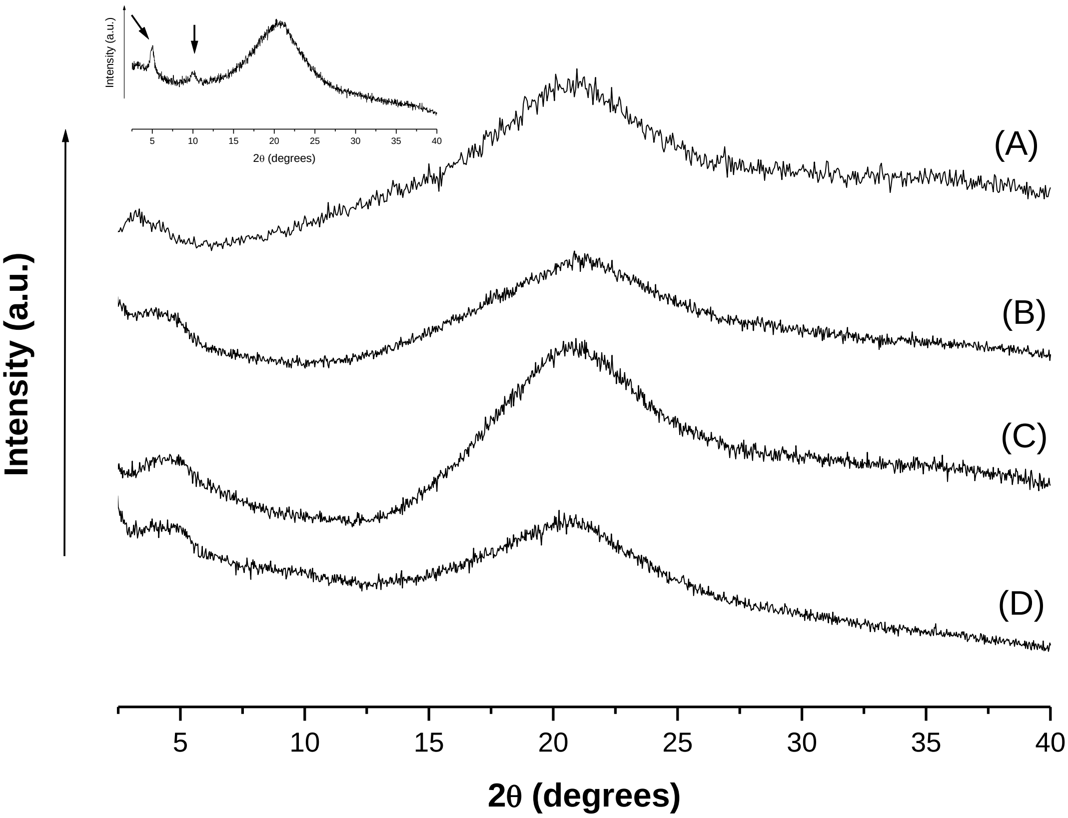

Figure 2: XRD on films (A) pectin, (B) pectin/2.5% LDH-cinnamate, (C) pectin/5% LDH-cinnamate, (D) pectin/10% LDH-cinnamate. The inset reports XRD on a mechanical mixture composed of pectin and 2.5% LDH-cinnamate

Thermal behavior was evaluated on the composites through thermogravimetric analysis (TGA and DTG). Results are reported in the supporting information (SI 3). It is also shown the thermogravimetric curve of the pure pectin, for comparison. The thermo-oxidative degradation of pectins is a series of complex events that involves three steps of degradation: i) the first one, centered at about 90°C, due to loss of water; ii) the second one, between 150°C and 280°C, due to pyrolytic decomposition consisting of a primary and secondary decarboxylation involving the acid side group and a carbon in the ring (Gorrasi, 2015; Shim, Hajaligol & Baliga, 2004; Waymack, Belobe, Baliga, & Hajaligol, 2004;); iii) the third step between about 650°C and 720°C, corresponding to the oxidation region. The second step of degradation occurs at the same temperatures either for pectin or for the composites independently of the filler loading; whereas the third degradation step is dependent on the filler amount. Its temperature decreases on increasing the filler loading, as evidenced by the DTG analysis (part B of the figure). It has been reported that the glycerol percentage has a significant effect on the degradation of pure pectin (Yang & Yang, 2016), but in this case the glycerol amount is the same in all composites. It can be hypothesized that oxides of Zn and Al, that are formed for the decomposition of LDH at high temperatures, can catalyze the oxidation of pectin matrix.

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

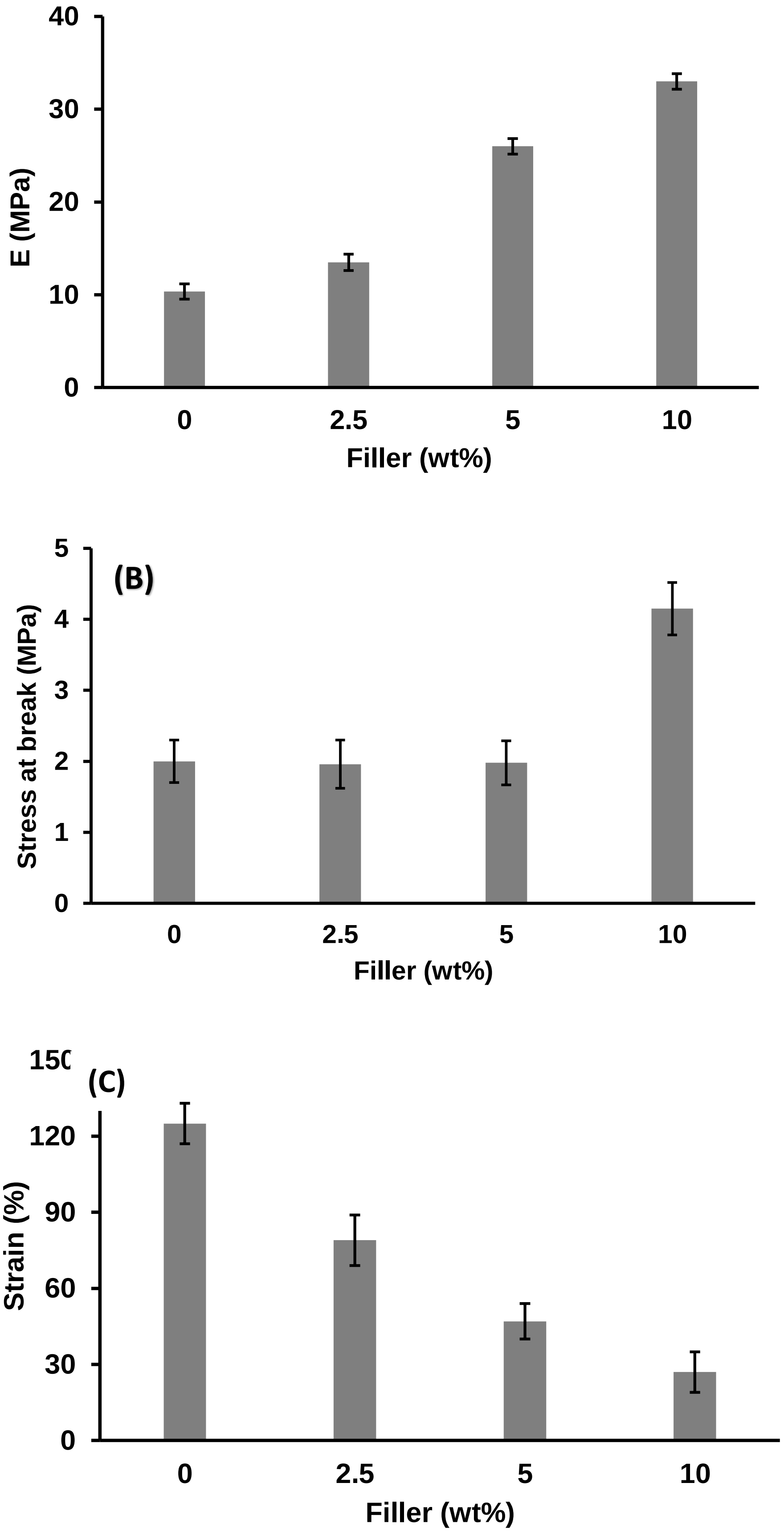

Figure 3: Mechanical properties evaluated on pectin and composites

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

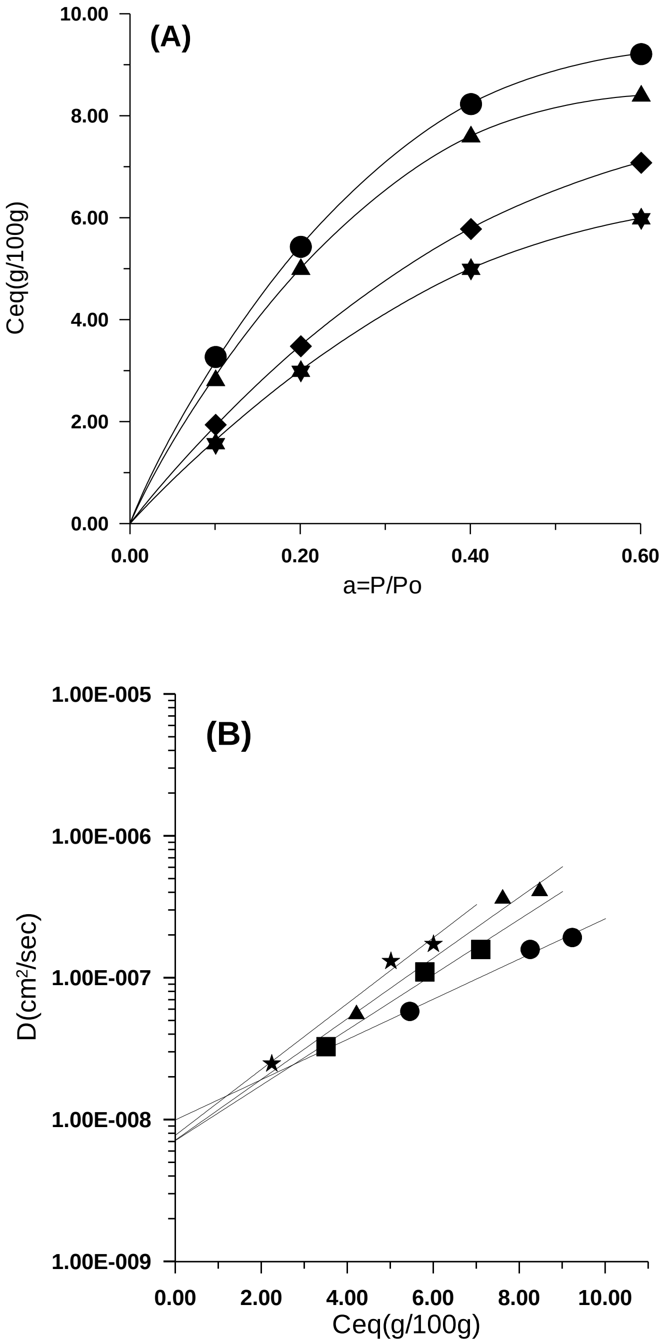

Figure 4: (A) Sorption isotherm for samples: pectin (•), Pectin/2.5% LDH-cinnamate (Δ), Pectin/5% LDH-cinnamate (◊), Pectin/10% LDH-cinnamate (⁎); (B) Diffusion for samples: pectin (•), Pectin/2.5% LDH-cinnamate (Δ), Pectin/5% LDH-cinnamate (◊), Pectin/10% LDH-cinnamate (⁎)

Table 2: barrier parameters, sorption diffusion and permeability, of pectin and composites

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

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

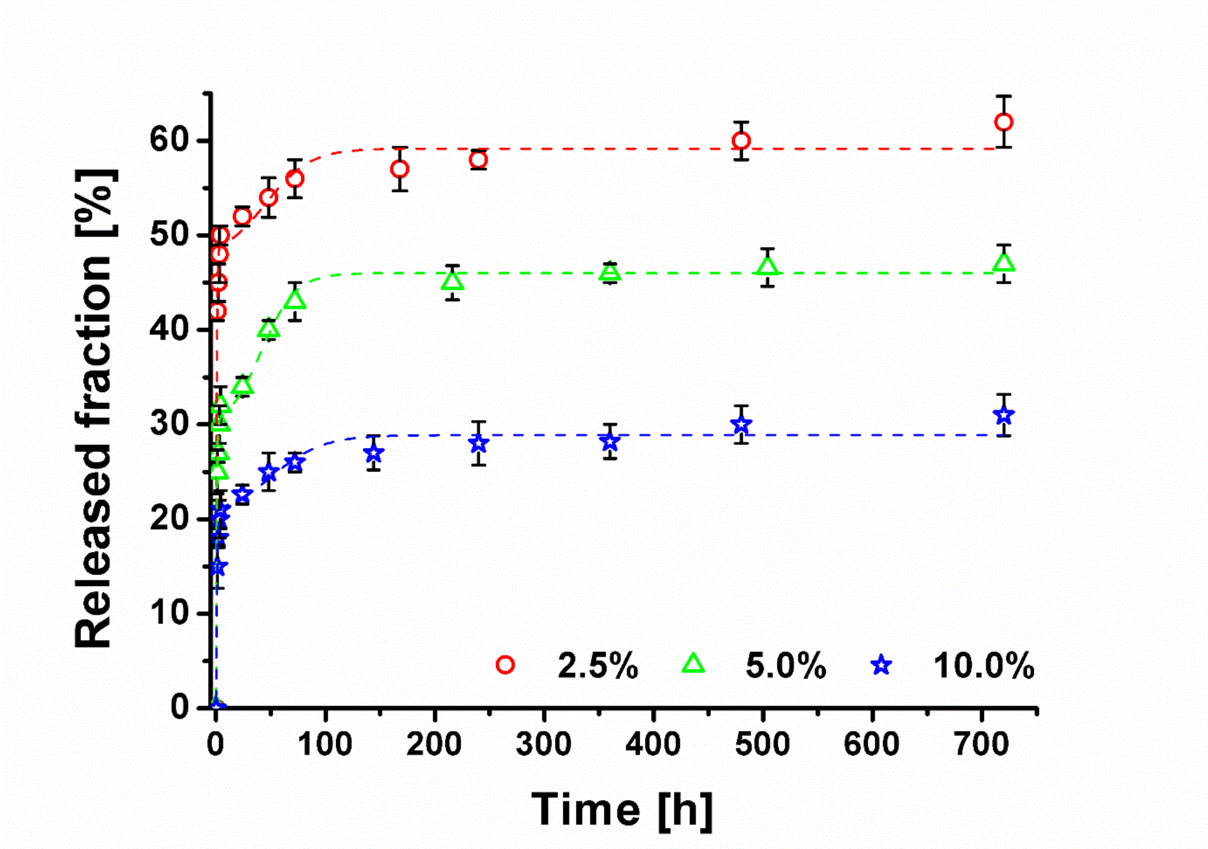

Figure 5: Release of cinnamate molecule, as function of contact time (h), for composites at 2.5, 5 and 10% of nano-hybrid loading. Dotted lines are the fitting with the model expressed from Equation (5)

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

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

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_{tmax} 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	4.89×10^{-2}	0.991
Pectin/LDH-cinn 5%	29	41	1.50	6.40×10^{-2}	0.994
Pectin/LDH-cinn 10%	20	51	1.08	4.27×10^{-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 active molecule bonded to the LDH and dispersed into the pectin. For the bacteria, results on modified agar diffusion test (disks of pectin/LDH-cinnamate directly seeded on the agar plates (see Table 4) indicated that the cinnamate bonded to the LDH and dispersed into the pectin exhibited slight antimicrobial activity against *S. aureus* DSMZ 20231, with a diameter halo of about 11.5±0.07 mm and a moderate activity against *E. coli* DSMZ 30083 with a diameter halo of about 16.5±0.07 mm while exerted an activity against *L. monocytogenes* DSMZ 20600 and *S. bongori* DSMZ 13772 with a diameter < 10 mm. The agar diffusion test used as control with cinnamic acid alone imbibed in Whatman paper discs, showed an antimicrobial activity against the four pathogen strains used, with halos that varied from *S. aureus* DSMZ 20231 with about 14 ±0.0 mm, *S. bongori* DSMZ 13772 with about 13.5±0.07 mm, *E. coli* DSMZ 30083 with about 12.8±0.04 mm and finally *L. monocytogenes* DSMZ 20600 with about 12±0.02 mm (Table 4). The mechanism under this phenomenon is quite complex. A possible explanation of the different antimicrobial ability of LDH-cinnamate into pectin could be found in the different cell surface charge of the different pathogens used and/or different hydrophobicity of cell surface that can influence the reaction of the bacterial strains (Dickson & Koohmaraie, 1989).

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

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

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

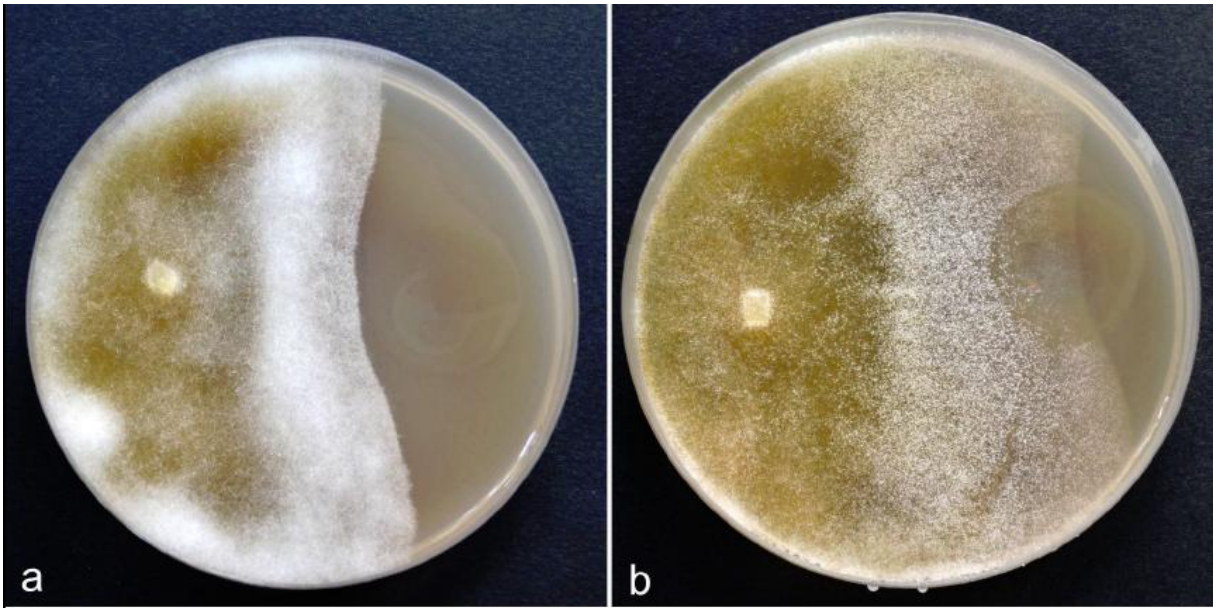

Figure 6: In-vitro evaluation of Pectin/10% LDH-cinnamate in dual culture assay with *Phytophthora* spp.: colony of *P. cinnamomi* (a) and *P. palmivora* (b) after 5 days at 20°C

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

Conclusions

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

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

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

Synthetic pesticides are molecules that contaminate soil, water, air, and their accumulation causes irreversible damage on all kinds of bio-systems. At the same time the indiscriminate use of these synthetic antimicrobial compounds poses serious issues for the spread of antimicrobial resistance in bacteria and fungi. In this context one of the main goal is represented by the possibility to protect crops without harmful effects on nature. Next to the methodologies of genetic engineering and natural enemies (Mao, Lewis, Lumsden, & Hebbar, 1998; Navon, 2000; Stevens & Lee, 1979), that have to be further validate for real applications, it is possible to use the tools of nanotechnology to assess alternative nature-compatible approaches. Layered Double Hydroxides (LDHs) are a class of inorganic lamellar solids that possess the characteristic to be soil-compatible. Their general formula 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 and M(III) is a trivalent cation such as Al, Cr, Fe or Ga with A^{n-} an exchangeable anion of charge n. The x value generally ranges between 0.2 to 0.4 and determines the positive layer charge density and the anion exchange capacity (Cavani, Trifiro, & Vaccari, 1991; Costantino, Ambrogi, Perioli, & Nocchetti, 2008; Herrero, Labajos, & Rives, 2009; Leroux & Taviot-Guého, 2005). The interlayer anions can be exchanged by other inorganic, organic or metallo-organic compounds in anionic form and the obtained structures can be used as active nano-hybrid fillers for polymers for targeted applications (Chen & Qu, 2003; Costantino et al., 2009; Muksing, Magaraphan, Coiai, & Passaglia, 2011; Qiu, Chen, & Qu, 2005; Romano, Naddeo, Guadagno, & Vertuccio, 2014; Zammarano et al., 2006). LDHs are also cheap materials that can be produced with high level of purity. This makes LDHs ideal matrices to carry active molecules in soils and control their sustained release into the desired medium. Furthermore, the intercalated molecule between the inorganic layers could be safely protected against chemical and biological degradations in soils. The dispersion of the active nano-hybrid into the soil is a crucial point because the simple dispersion in water causes, after water evaporation, a re-aggregation of the LDH layers and subsequent loss of adhesion on the plant and soil to be protected. An interesting alternative could be the dispersion of the nano-hybrid into a bio-based matrix soluble in water. Pectins are a class of complex water-soluble polysaccharides widely used to form coatings. They are carbohydrate products obtained by aqueous extraction of some edible plant material, usually citrus fruits or apples, available in high volume mainly in agricultural wastes. Pectin coatings have been also studied for their ability to retard lipid migration and moisture loss, and to improve appearance and handling of foods. This paper reports the preparation of a nano-hybrid composed by LDH and cinnamate, and its possible use as green pesticide against an important group of plant pathogens, such as *Phytophthora* spp. (Jung et al., 2018), and antimicrobials for the reduction the pathogens post-harvest contamination, at different active molecule loading. The dispersion into a pectin matrix was conducted through high energy ball milling in presence of water. Cast films were obtained and analyzed. Structural, thermal, mechanical, barrier properties were evaluated and correlated to the filler loading. The controlled release analysis of cinnamate was followed as function of time. Antimicrobial activity of the nano-hybrid composed by LDH and cinnamate was also assayed. Particularly, several strains of bacteria and *Phytophthora* belonging to different pathogen species were analyzed.

2. Experimental

2.1 Materials

$Zn(NO_3)_2*6H_2O$, $Al(NO_3)_3*9H_2O$, NaOH and trans-cinnamic acid were purchased from Sigma-Aldrich (Italy). Pectins from apples were purchased from Sigma Aldrich in powder form. The molecular weight is 30,000-100,000 and the degree of esterification about 70-75%, on a dry basis, total impurities ≤10% water (CAS Number: 9000-69-5).

2.2 Preparation of ZnAl-o-BzOH by coprecipitation method

30 mL of an aqueous solution of $Zn(NO_3)_2*6H_2O$ (12.9 g, 43.4 mmol) and $Al(NO_3)_3*9H_2O$ (8.14 g, 21.7 mmol) were added to 30 ml of a trans-cinnamic sodium salt solution (6.3 g, 36.9 mmol) under stirring and under nitrogen flow. The pH slowly reached the value of 7.5 by adding 1M NaOH. At the end, the precipitate was washed with distilled water and left in oven at 50° C for 24h, under vacuum (Frunza, Lisa, Popa, Miron, & Nistor, 2008). The chemical formula obtained from the elemental analysis was the following: $[Zn_{0.65}Al_{0.35}(OH)_2]\ (C_9O_2H_7)_{0.35}\ *0.7\ H_2O$ with value of the molar fraction $x=M^{III}/M^{III}+M^{II}$ of 0.35 and molecular weight of 149.99 g/mol; the amount of trans-cinnamic anion intercalated in ZnAl-o-BzOH is 34.3 wt % of the total weight. Therefore almost all the alluminium is co-precipitated with the zinc ions to obtain a solid with the stoichiometry of two Zn(II) atoms for each Al(III) atom. This corresponds to an ideal arrangement of the brucite-like sheet with each aluminium atom surrounded by six zinc atoms (Oswald & Asper, 1977).

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^3 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 Kα radiation (Kα = 1.54050 Å) and operating at 40 kV and 40 mA, step scan 0.05° of 2ϑ 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 ≅ 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 ± 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_0

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

$$\frac{Ct}{Ceq} = \frac{4}{d}\left(\frac{Dt}{\pi}\right)^{1/2} \qquad (1)$$

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

$$S = \frac{d(Ceq)}{dp} \qquad (2)$$

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

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

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

$$P = S \times D_0 \qquad (4)$$

The release kinetics of cinnamate were performed by ultraviolet spectrometric measurement using a Spectrometer UV-2401 PC Shimadzu (Japan). The tests were performed using rectangular specimens of 2 cm^2 and same thickness (≅ 200 μm), placed into 25 mL of ethanol with 0.9 wt% of tetrabutylammonium chloride and stirred at 100 rpm in an orbital shaker (VDRL MOD. 711+ Asal

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

2.5 Microbial strains

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

Table 1: microorganisms used in the present work and sources

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

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

2.5.1 Broth microdilution test

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

2.5.2 Modified Agar diffusion and dual culture tests

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

3. Results and discussion

3.1 Characterization of filler

Figure 1 reports the XRD spectra of pristine LDH-NO_3 and the LDH modified with cinnamate anion. It is evident that the nitrate form of LDH presents the main peaks at about 10° and 20° of 2ϑ, relative to the basal spacing (003) and (006), respectively. The intercalation of cinnamate molecule is evident from the modification of the basal spacing of the LDH with the shifting of the diffraction peaks at lower angle (Weiling, Qinglin, & Yong, 2007).

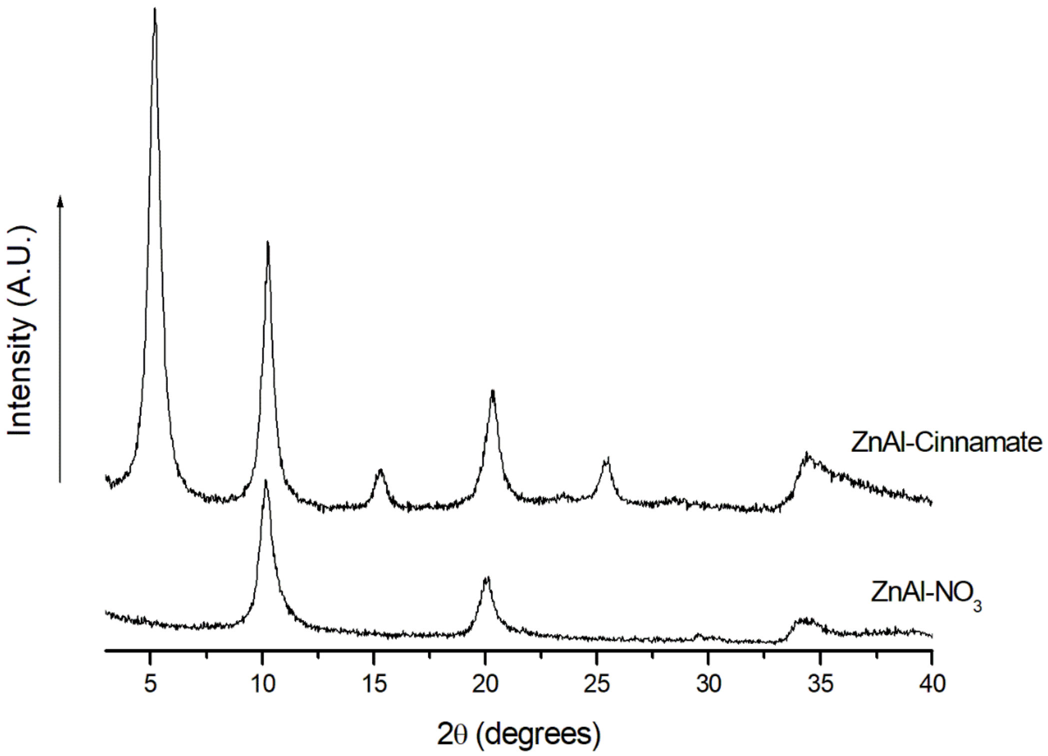


Figure 1: XRD spectra of pristine LDH-NO_3 and the LDH modified with cinnamate molecule

TGA analysis was carried out on LDH-NO_3 (A), cinnamic acid (B) and LDH-cinnamate (C). The TGA curve of LDH-NO_3, reported in the supporting information (SI 1), shows three steps of decomposition: i) the first at around 150°C, corresponding to the loss of absorbed water between LDH layers, ii) a second, occurring around at 250°C, is due to the thermal decomposition of nitrate anions, iii) a third, at about 400°C, due to the dehydroxylation of the LDH sheets (Park et al., 2010). Experimental results demonstrate the stabilization of cinnamate molecule within the interlayer space of LDH. In fact, free cinnamic acid (B) exhibits its degradation in one step, above 150°C. The intercalation into the inorganic matrix results in a significant improvement in thermal stability: the main thermal decomposition of the hybrid takes place at around 374°C. The hydroxide framework transforms finally into its corresponding oxide by dehydroxylation above 500°C. Such behavior, already found for several molecules incorporated into LDH layers (Gorrasi & Bugatti, 2016), suggests a protecting effect of the LDH respect to the cinnamate and a stable interaction LDH-organic molecule due to electrostatic forces.

3.2 Characterization of composites

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

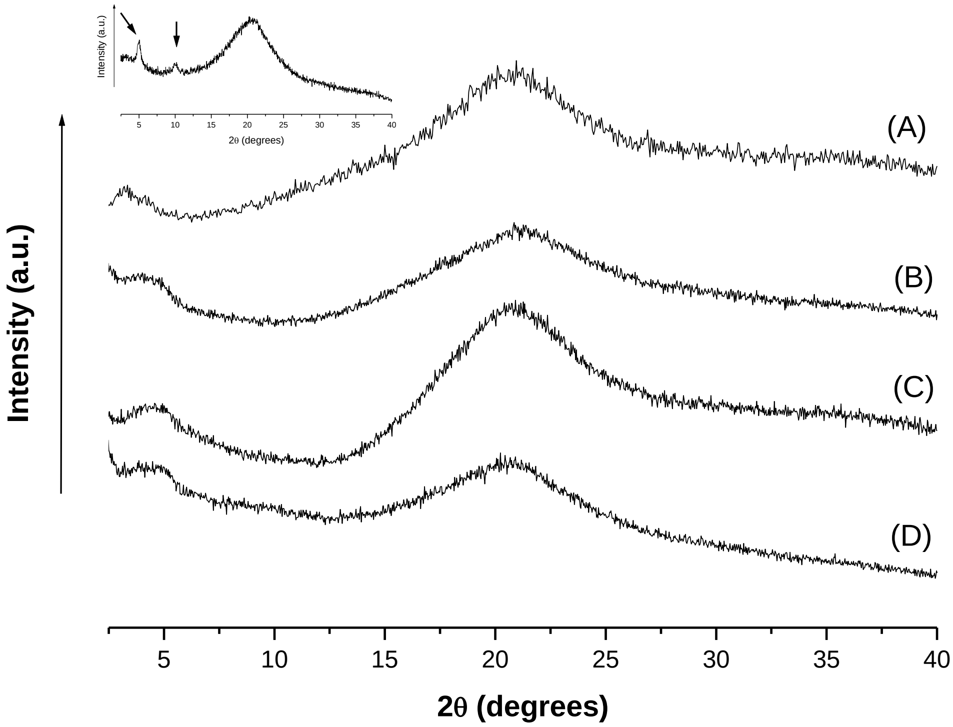


Figure 2: XRD on films (A) pectin, (B) pectin/LDH-cinnamate 2.5%, (C) pectin/ LDH-cinnamate 5%, (D) pectin/LDH-cinnamate 10%. The inset reports XRD on a mechanical mixture composed of pectin and 2.5% of LDH-cinnamate

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

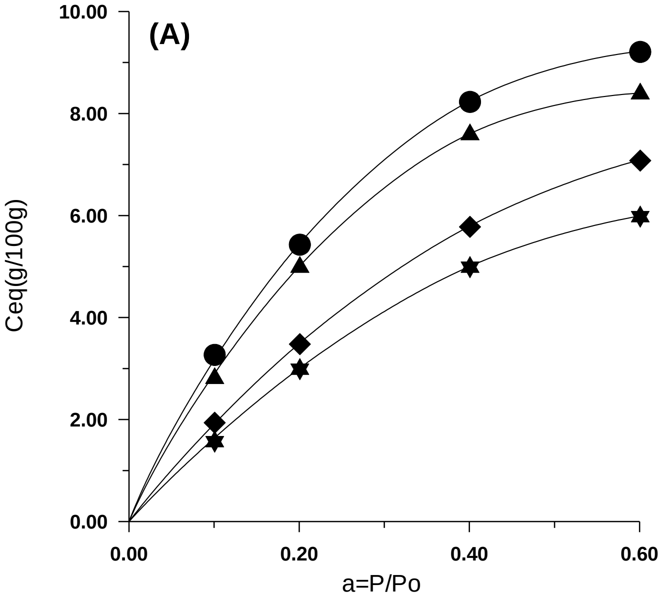
Mechanical properties were estimated on all samples. From the stress-strain curves, not reported, they were evaluated elastic moduli (MPa), stress at break point, σ_b (MPa), and elongation at break, ε_b (%). Table 2 reports the experimental data. The elastic modulus, E (MPa), of the unfilled pectin is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

Table 2 : Mechanical parameters evaluated from stress-strain curves

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

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

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



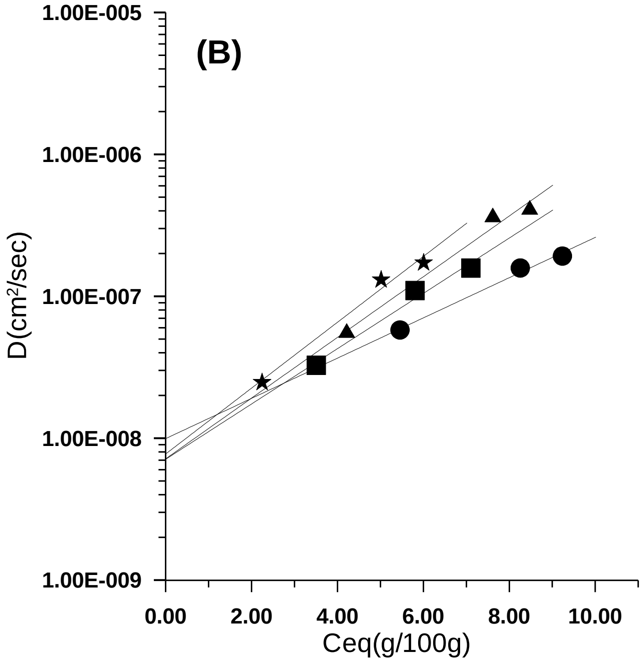


Figure 3: (A) Sorption isotherm for samples: pectin (●), Pectin/LDH-cinnamate 2.5% (Δ), Pectin/LDH-cinnamate 5% (◊), Pectin/LDH-cinnamate 10% (*); (B) Diffusion for samples: pectin (●), Pectin/LDH-cinnamate 2.5% (Δ), Pectin/LDH-cinnamate 5% (◊), Pectin/LDH-cinnamate 10% (*)

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

Sample	**Sorption** (g/100g/mmHg)	**Diffusion** $(cm^2/s)*10^9$	**Permeability** $(g/100g/mmHg)*(cm^2/s)*10^9$
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

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

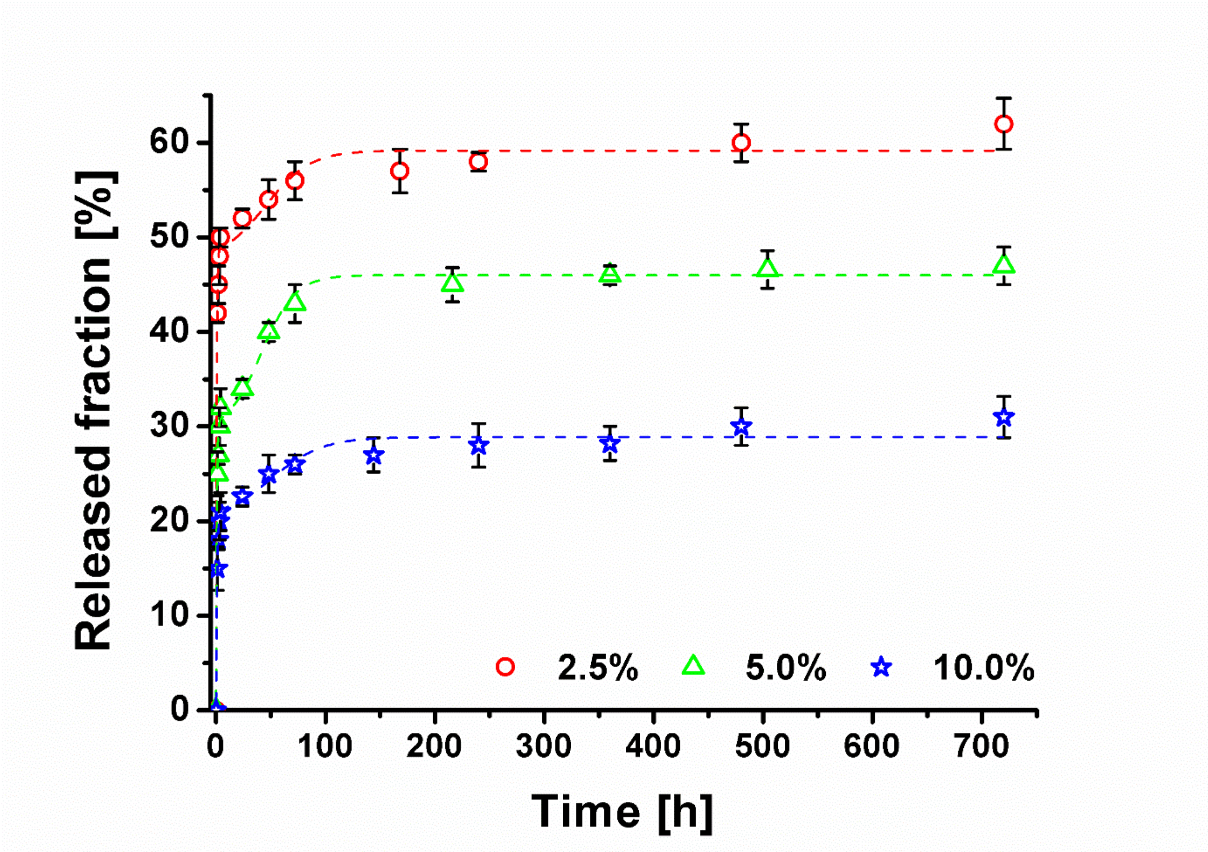


Figure 4: Release of cinnamate molecule, as function of contact time (h), for composites at 2.5, 5 and 10% of nano-hybrid loading. Dotted lines are the fitting with the model expressed from Equation (5)

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

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

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_{tmax} 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 4 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 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	4.89×10^{-2}	0.991
Pectin/LDH-cinnamate 5%	29	41	1.50	6.40×10^{-2}	0.994
Pectin/LDH-cinnamate 10%	20	51	1.08	4.27×10^{-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). 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. For the bacteria, results on modified agar diffusion test (disks of pectin/LDH-cinnamate directly seeded on the agar plates (see Table 5) indicated that the cinnamate bonded to the LDH and dispersed into the pectin exhibited slight antimicrobial activity against *S. aureus* DSMZ 20231, with a diameter halo of about 11.5±0.07 mm and a moderate activity against *E. coli* DSMZ 30083 with a diameter halo of about 16.5±0.07 mm while exerted an activity against *L. monocytogenes* DSMZ 20600 and *S. bongori* DSMZ 13772 with a diameter < 10 mm. The agar diffusion test used as control with cinnamic acid alone imbibed in Whatman paper discs, showed an antimicrobial activity against the four pathogen strains used, with halos that varied from *S. aureus* DSMZ 20231 with about 14 ±0.0 mm, *S. bongori* DSMZ 13772 with about 13.5±0.07 mm, *E. coli* DSMZ 30083 with about 12.8±0.04 mm and finally *L. monocytogenes* DSMZ 20600 with about 12±0.02 mm (Table 5). The mechanism under this phenomenon is quite complex. A possible explanation of the different antimicrobial ability of LDH-cinnamate into pectin could be found in the different cell surface charge of the different pathogens used and/or different hydrophobicity of cell surface that can influence the reaction of the bacterial strains (Dickson & Koohmaraie, 1989).

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

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

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

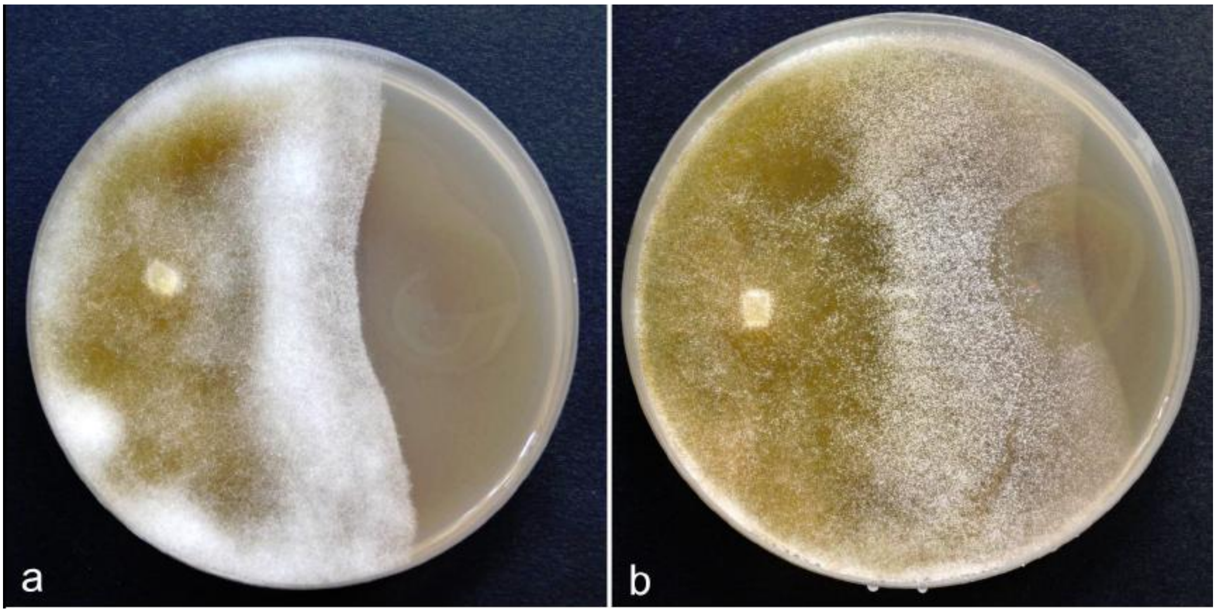


Figure 5: In-vitro evaluation of Pectin/10% LDH-cinnamate in dual culture assay with *Phytophthora* spp.: colony of *P. cinnamomi* (a) and *P. palmivora* (b) after 5 days at 20°C

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

Conclusions

This paper reported the preparation of green composites based on pectins and layered double hydroxides (LDH) intercalating cinnamate anion, as active molecule. The cinnamate loading into the LDH was 36%. Composites at 2.5, 5 and 10 wt% were prepared using ball milling technology in presence of water. Films were obtained and tested, respect to structural and functional properties. The successful intercalation of cinnamate molecule, evidenced from the modification of the basal spacing of the LDH, was observed from XRD analysis. Data demonstrated a delamination of the nano-hybrid into the pectin matrix at any filler composition. The thermal analysis, conducted using TGA, demonstrated that the cinnamate molecule is thermally protected by the LDH layer. In addition,

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

The analysis of barrier properties, sorption and diffusion, to water vapour demonstrated a decrease of sorption with the increasing the filler loading, and no effect on the diffusion. Interaction between polar groups of pectin and filler were then hypothesized, resulting in a lower sorption of the polar water molecules. It was analyzed in vitro the release of the cinnamate molecule from all composites. It was correlated the filler loading to the release kinetics. The release kinetics of composites' membranes were found to be dependent on the nano-hybrid loading and were well fitted the Gallagher-Corrigan model. It was demonstrated that varying the filler loading it is possible to tune the cinnamate release for desired applications. The effects of the composite on the growth of two *Phytophthora* species through an in vitro experiment was tested. 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.

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