International Journal of Biological Macromolecules 89 (2016) 360–368

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Effect of cellulose and lignin on disintegration, antimicrobial and antioxidant properties of PLA active films

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ARTICLE INFO

Article history:
Received 9 March 2016
Received in revised form 22 April 2016
Accepted 22 April 2016
Available online 25 April 2016

Keywords:
Lignin nanoparticles
Cellulose nanocrystals
Poly (lactic acid) nanocomposites
Bacterial plant pathogens
Active packaging

ABSTRACT

This study reports the effects on antimicrobial, antioxidant, migration and disintegrability activities of ternary nanocomposite films based on poly(lactic acid) incorporating two biobased nanofillers, (cellulose nanocrystals (CNC) and lignin nanoparticles (LNP)), in two different amounts (1 and 3% wt.). Results from antimicrobial tests revealed a capacity to inhibit the Gram negative bacterial growth of *Xanthomonas axonopodis* pv. *vesicatoria* and *Xanthomonas arboricola* pv. *pruni* along the time, offering innovative opportunities against dangerous bacterial plant pathogens. LNP proved to be highly efficient in antioxidation activity, based on the disappearance of the absorption band at 517 nm of the free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH) upon reduction by an antiradical compound; moreover the combination of LNP and CNC generates a synergistic positive effect in the antioxidation response of PLA ternary films. Furthermore, all the studied formulations showed a disintegrability value up to 90% after 15 days of incubation in composting conditions. Migration results showed that the films can be considered suitable for application in food packaging field.

1. Introduction

The increasing public awareness regarding synthetic antioxidant application not only for food but also for stabilization of polymeric materials moved the interest towards antioxidants of natural origin, in particular polyphenols that, due to their biodegradability and significantly lower toxicity, can represent a good alternative to the synthetic ones [1]. However, most of the naturally originated antioxidants are not efficient enough for the protection of plastics and composites, due to their lower molecular mass and lesser thermal stability in comparison with synthetic systems [2]. On the contrary, biorenewable polymeric polyphenol lignin is a suitable natural antioxidant for use in polymers, due to its lower sensitivity to high temperatures and higher molecular mass in comparison with the low molecular weight natural antioxidants, which are commonly proposed for the stabilization of polymeric materials, e.g. α-tocopherol [3]. Lignin, as the second most abundant renewable and biodegradable natural resource next to cellulose, is a heteropolymeric aromatic compound that has a complex chemical structure, such as carboxyl, carbonyl, phenolic or aliphatic hydroxyls that can be found in different proportions according to lignin origin. Due to the presence of these functional groups, oxidation propagation reaction can be terminated through hydrogen donation. The antioxidant capacity of lignin has been widely studied and its application in fields as medical, pharmaceutical (anti carcinogenic agent) or polymeric applications (thermal behaviour enhancement) has been proved [4]. However, this property depends on the used lignocellulosic material, the extraction methods, and the secondary applied treatments. As a result, studies were carried out with regard to the incorporation of lignin into materials for manufacturing value added antioxidant products in the various fields, aiming to tune the rate of autooxidation [5,6]. For higher antioxidative efficiency, lignin should have higher quantity of phenolic hydroxyl and methoxy groups, lower quantity of aliphatic hydroxyl groups, narrow polydispersity and lower molecular weight. Furthermore, the effects of chemical characterisation, structure of side chain, π-conjugated systems of lignin on its antioxidant activity have been studied [7,8]. It was determined that the main factor that contributes to lignin antioxidant activity was the high content of phenolic moieties, which underwent proton coupled electron transfer mechanism. The remaining factors that could contribute

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http://dx.doi.org/10.1016/j.ijbiomac.2016.04.068
0141-8130/

to antioxidant activity included relative content of phenylpropane units, phenylpropane units with CH_2 groups in the α-position of side chains, phenylpropane units with oxygen-containing groups in the side chains and size of the π-conjugated systems. This effect has been studied, for example, by Azadfar et al. [9], who revealed that the antioxidant activity of alkali lignin was comparable to the commercial antioxidants (such as butylated hydroxytoluene), as the 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition percentages of lignin and butylated hydroxytoluene were 86.9% ± 0.34% and 103.3% ± 1%, respectively. However, despite the well proved antioxidant properties, the absence of rationalized conceptions about lignin structural features and physical–chemical properties (including size dimensions) influencing its antioxidant activity hinders the realization of lignin potential in the area of stabilization of materials and food/feed products against oxidative damage. Domenek et al. [10] investigated the antioxidant activity of microscaled lignin as a natural substitute of synthetic antioxidants for the protection of the food, paving the way to the fabrication of active packaging materials compounded with natural antioxidant substances. After that, Yearla et al. investigated the radical scavenging activity of nanosized lignin and demonstrated that its antioxidant and UV protection properties could be also further exploited in food, pharmaceutical and cosmetic industries [11]. They studied how structure-activity relationship is crucial to identify the most optimal antioxidants and to determine the most promising directions for upgrading of technical lignins, in order to produce effective antioxidants for various application systems. However, no previous studies have been reported on how lignin at the nanoscale dimension could work as antioxidant agent when incorporated in polymeric systems.

Concerning the antibacterial properties of lignin, it seems to be also a promising green principle useful against dangerous microorganisms. Its biocidal activity allows to reduce the environmental problems related to the silver nanoparticles use and could improve, as an example, the release of active principles in agriculture [12,13]. Some studies have shown that lignin has an antimicrobial effect [14], even when it was incorporated into polyethylene film and applied in the finishing processes of textiles [15,16]. However, none of them investigated the mechanism of the antibacterial activity of lignin when included in polymeric matrices. A recent study [17] revealed that the main determining factor of the antimicrobial effect of lignin lies on the phenolic components, specifically the side chain structure and the nature of the functional groups. Typically, the presence of a double bond in α, β positions of the side chain and a methyl group in the γ position grants the phenolic fragments with the most potency against microorganisms.

In addition to lignin based nanoparticles, cellulose nanocrystals (CNC), that can be easily extracted from cellulose based sources, have been proved to be effective in enhancing the barrier to gases when introduced in polymeric systems, due to the synergistic tortuosity, crystal nucleation and chain immobilization effects [18,19]. In the meanwhile, they present the advantage of being renewable when compared to other inorganic fillers, supporting their use in biodegradable materials, such as PLA [5,20,18,1,21,22,23,24,25]. Some previous research works have also highlighted the significant advantages of incorporating CNC in PLA, including important water vapour, oxygen barrier effects, migration level below packaging normative requirements [26] and antimicrobial properties when combined with silver nanoparticles [27] or essential oil [18,28], demonstrating the antimicrobial potential of PLA/CNC films as a promising bioactive packaging to preserve fresh food products against food pathogens.

Composting is nowadays a general treatment method for municipal solid waste. In the European Union, composting is regarded as one recycling method for packages and this will probably favour compostable packages, like films, papers and boards. Tuomela et al. [29] found that the elevated temperatures during the thermophilic phase are essential for rapid degradation of lignocelluloses. Complex organic compounds like lignin are mainly degraded by thermophilic micro fungi and actinomycetes, and then form humus. The optimum temperature for thermophilic fungi is 40 ± 50 °C which is also the optimum temperature for lignin degradation in compost. We have demonstrated [19] that lignin, even at the nanodimension, strongly influences the degradation process in compost of PLA films, and the degradation rate can be modified changing both lignin nanoparticles content and processing methods (solvent casting or extrusion). In that study, PLA filled with 0, 1 and 3 wt.% of lignin nanoparticles (LNPs) was investigated. Disintegrability in composting conditions has been tested and visual observation, chemical, thermal and morphological investigations proved that the incorporation of 1 wt.% of LNP seems to hinder the disintegration of PLA matrix, due to the hydrophobic nature of the filler; when the LNP content rises up to 3 wt.%, some LNP aggregations and rougher film surface structure form, inducing higher degradation rate. For PLA filled with CNC, Bitinis et al. and Fortunati et al. [5,26] studied that the addition of the unmodified CNC did not affect the disintegration rate of the material. Although the addition of hydrophilic filler is expected to accelerate the degradation rate of the PLA/natural rubber material, CNC could also inhibit water diffusion, explaining the obtained results. Actually, the two effects appear to counterbalance each other.

With the aim of evaluating their possible synergic effects, binary and ternary PLA based nanocomposites, prepared by combining two processing procedure (masterbatch and reactive melt grafting) and containing both CNC and LNP without any further modification, were produced. Antibacterial activity, oxidative behaviour, migration and disintegrability activity of all produced films were tested and here reported, taking into account a possible final application of the produced formulation as food packaging materials.

2. Experimental

2.1. Materials

Poly (lactic acid) (PLA 3251D—specific gravity of 1.24 g cm^{-3} and melt flow index of 35 g $(10\,min)^{-1}$ (190 °C, 2.16 kg) was supplied by NatureWorks LLC, USA. Pristine lignin was supplied by CRB (Centro Ricerca Biomasse, University of Perugia) and lignin nanoparticles were synthesized as previously reported [19]. Microcrystalline cellulose (MCC, dimensions of 10–15 μm), used as cellulose nanocrystals precursor during the hydrolysis procedure, was supplied by Sigma-Aldrich®. All the chemical reagents were supplied by Sigma-Aldrich® and used as received.

2.2. Preparation of masterbatches (MBs)

PLA grafting with glycidyl methacrylate was performed in a twin-screw microextruder (DSM Explorer 5&15CC Micro Compounder) in presence of dicumyl peroxide as initiator. Before being used for grafting, GMA was stored in the refrigerator at 5° C. DCP content was set as 1 wt.% of the PLA weight, while GMA was fixed at 10 wt.% of the PLA weight. DCP and GMA (DCP/GMA ratio = 0.1) were mixed and the obtained solution was sprayed onto dried PLA matrix. Screw speed of 100 rpm, mixing time of 8 min and a temperature profile of 165–175–180 °C were selected to realize the grafting reaction. The content of PLA grafted with GMA (g-PLA), to be used as a compatibilizer in the resulting nanocomposites, was fixed at 15 wt.%. The compositions of different masterbatches are MB1 (PLA+1.17 wt.% CNC), MB2 (PLA+3.53 wt.% CNC), MB3 (grafted PLA+6.67 wt.% LNP) and MB4 (grafted PLA+20 wt.% LNP).

Table 1
Nanocomposite formulations.

Formulations	PLA (wt.%)	g-PLA (wt.%)	LNP (wt.%)	CNC (wt.%)
PLA	100	–	–	–
PLA-1LNP/1CNC	83	15	1	1
PLA/3CNC	82	15		3
PLA-1LNP/3CNC	81	15	1	3
PLA/3LNP	82	15	3	–
PLA-3LNP/1CNC	81	15	3	1
PLA-3LNP/3CNC	79	15	3	3

2.3. PLA nanocomposite processing

Binary and ternary nanocomposite films were manufactured by using a twin-screw microextruder. Conditions of 100 rpm screw speed, 2 min of dwell time and a kneading temperature of 180–195–210 °C were employed to optimize material final properties, after which, a film forming process with a head force of 180 N and a die temperature of 200 °C was used, in order to obtain PLA and PLA nanocomposite films with a thickness ranging from 20 up to 80 μm. The materials, designed as PLA/3CNC and PLA-3LNP with direct incorporation of 3 wt.% . of CNC and LNP (mixing time 2 min) after 8 min of PLA heating, were produced as control, while the ternary PLA nanocomposite films having the code PLA-1LNP/1CNC consisted of 85% wt. MB1 and 15% wt. MB3, respectively. The system containing 85 wt.% of MB2 and 15 wt.% of MB3 was denoted as PLA-1LNP/3CNC, while PLA-3LNP/1CNC represented the material composed of 85 wt.% of MB1 and 15 wt.% of MB4. Finally, PLA-3LNP/3CNC was indicated as the material obtained through the mixture of 85 wt.% MB2 and 15 wt.% MB4. The final compositions of the formulations, in terms of LNP and CNC content, are reported in Table 1.

The microorganisms used for antimicrobial assay were *Pseudomonas syringae pv. tomato*, *Xanthomonas arboricola pv. pruni* and *Xanthomonas axonopodis* pv. vesicatoria. The bacteria were provided by French Collection of Plant Pathogenic Bacteria (CFBP) and are labelled CFBP 1323, CFBP 3894 and CFBP 3274, respectively.

2.4. Methods

2.4.1. Antibacterial activity

Biocidal activity of neat PLA film and PLA nanocomposites containing lignin nanoparticles and/or cellulose nanocrystals was evaluated against different plant pathogenic bacteria, such as *Pseudomonas syringae* pv. *tomato* (*Pst*), the causal agent of tomato (*Lycopersicon esculentum* Mill.) bacterial speck, able to determine serious damages on all aerial tomato organs (leaves, stems, petioles, flowers, fruits), *X. axonopodis* pv. *vesicatoria* (*Xav*), the causal agent of bacterial spot disease in pepper and tomato plants, which causes symptoms throughout the above-ground portion of the plant including leaf spots, fruit spots and stem cankers [30], and *X. arboricola* pv. *pruni* (*Xap*), the causal agent of relevant damages (bud necrosis, leaf spot, leaf defoliation, cankers on twigs, fruit deformation) on *Prunus* spp. (almond, peach, cherry, plum, apricot). These bacteria affect worldwide some fruits and vegetables grown/production, causing serious damages on all organs (including fruits that resulted unmarketable) and economically important yield losses [31]. In particular, the antibacterial activity of the binary and ternary nanocomposites produced with the higher content of LNP nanoparticles (PLA/3LNP and PLA-3LNP/1CNC) was evaluated and tested with respect to known (CFBP) *Pst*, *Xav* or *Xap* strains, utilised at a concentration of 1×10^6 CFU mL^{-1}. PLA neat film was also tested and used as control thesis. *Pst*, *Xav* and *Xap* subcultures were obtained by growing bacteria for 48–72 h at 25 ± 2 °C using Nutrient Agar (NA), supplemented by Sucrose (S) 5% (NAS). The experiments were carried out using Nutrient Broth (NB) 4% by the broth medium test [32]. The thesis (PLA as control, PLA/3LNP and PLA-3LNP/1CNC) were constituted by 3 replicates each; 5 sterile glass tubes per thesis, each containing 5 mL of liquid broth (NB) with 1×10^6 CFU/mL of *Pst*, *Xav* or *Xap* and 1 sample (3.24 cm^2) per nanocomposite films, were arranged. All tubes were placed on a reciprocal shaker for 48 h at 25 ± 2 °C at 150 rpm. Samplings from all tubes/thesis were carried out at 0, 3, 12, 24 h after bacterial inoculation and serial dilution were carried out and plated on NAS. After 48 h at 25 ± 2 °C the number of *Pst, Xav* or *Xap* bacterial colonies was counted. All data were statistically elaborated using GraphPad Prism 4 software by analysis of variance (ANOVA) and significance of treatments was determined using Tukey's HSD test ($p \leq 0.05$) [33].

2.4.2. Antiradical activity of LNP solution and PLA nanocomposite films

The antiradical activity of LNP solution was tested by using a spectroscopic method, based on the disappearance of the absorption band at 517 nm of the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich), upon reduction by an antiradical compound [34]. The test consisted in adding certain amount of the LNP aqueous solution into 2 mL of a DPPH solution in methanol (25 mg L^{-1}), then different concentrations of LNP solution (0, 10, 25, 50 mg L^{-1}) were prepared and the intensity of the 517 nm absorption band was measured overtime by using an ultraviolet-visible (UV–vis) spectrophotometer (Varian (Cary 4000, USA)). In the case of film samples, we adopted the method reported in [10]. The antioxidant capacity was expressed as the ability to scavenge the stable radical DPPH, which was calculated as radical scavenging activity (RSA) using the following equation (Eq.(1)):

$$(RSA, \%) = \left[\frac{A_{control} - A_{sample}}{A_{control}}\right] * 100 \quad (1)$$

where $A_{Control}$ and A_{Sample} are the absorbance of the control (methanol) at t = 0 min and tested sample at different incubation times, respectively.

2.4.3. Antiradical activity of migrating substances

DPPH radical scavenging activity for PLA films containing lignin nanoparticles was determined according to the method proposed by Byun et al. [35] and Domenek et al. [10], with a slight modification. Film (0.1 g) was cut into small pieces and immersed in 2 mL of methanol for 24 h at room temperature. The obtained supernatant was analyzed for evaluation of DPPH radical scavenging activity: an aliquot of methanol extract (1 mL) was mixed with 1 mL of DPPH in methanol (50 mg L^{-1}). The mixture was maintained at room temperature in the dark for 60 min. The absorbance was measured at 517 nm using a UV–vis spectrometer (Varian (Cary 4000, USA). The mixture solution of methanol extracted from neat PLA and DPPH methanol was used as control. DPPH radical scavenging activity was calculated by using Eq. (1), where A_{sample} was the absorbance of sample and $A_{control}$ was the absorbance of the control.

2.4.4. Overall migration activity of PLA nanocomposite films

Taking into account the possible application of the produced PLA nanocomposites for fresh food packaging, overall migration tests were run in triplicate in simulant A (10% (v/v) ethanol/water solution) and alternative simulant D2 (isooctane). Rectangular strips of 10 cm^2 in 10 mL of food simulants were used. Samples were kept in the ethanol solution in a controlled atmosphere at 40 °C for 10 days, according to European Commission Regulation EU 10/2011, while samples in isooctane were kept at 20 °C for 2 days, according to European Standard EN 1186-1/2002. At the end of the experiment, films were removed and the simulants evaporated. The residues were weighed with an analytical balance with ±0.01 mg precision

and the migration value in mg kg^{-1} of each simulant was determined.

2.4.5. Disintegrability activity in composting conditions

Disintegrability of PLA and PLA nanocomposites films was evaluated by means of a disintegration test in composting conditions according to the ISO-20200 standard. A specific quantity of compost, supplied by Gesenu S.p.a. (Italy), was mixed together with the synthetic biowaste, prepared with certain amount of sawdust, rabbit food, starch, sugar, oil and urea. The water content of the substrate was around 50 wt.% and the aerobic conditions were guaranteed by mixing it softly. The samples were buried at 4–6 cm depth in perforated boxes, containing the prepared mix, and incubated at 58 °C. The (15 mm × 15 mm) films were recovered at different disintegration steps, washed with distilled water, dried in oven at 37 °C for 24 h, and weighed. The disintegrability value was obtained normalizing the sample weight, at different stages of incubation, to the initial ones.

3. Results

3.1. Antibacterial activity

The antibacterial response induced by PLA based formulation containing LNP and/or a combination of LNP and CNC against *Pst*, *Xav*, and *Xap* bacteria (due to their remarkable negative impact on important crops (tomato, pepper and several stone fruit)), was investigated. The idea was to elucidate the mechanisms of antibacterial activity of PLA based films containing the two different lignocellulosic nanoparticles.

Control thesis (PLA) film revealed its inability respect to the bacterial cell multiplication over the time, indeed it supported *Xav* bacteria growth. PLA showed *Xav* values ranging from 5.6×10^5 to 1×10^8 CFU mL^{-1} and, in particular, they were of 5.6×10^5 CFU mL^{-1}, 6.1×10^5 CFU mL^{-1}, 6.0×10^6 CFU mL^{-1}, 1.2×10^8 CFU mL^{-1}, and 1.9×10^7 CFU mL^{-1} at 0, 3, 12, 24, and 48 h, respectively (Fig. 1a).

At the same time of samplings, in the case of PLA film containing 1 wt.% of CNC and 3 wt.% of LNP, *Xav* values recorded were as 6.3×10^5 CFU mL^{-1}, 3.4×10^5 CFU mL^{-1}, 6.4×10^6 CFU mL^{-1}, 1.3×10^7 CFU mL^{-1} and 2.9×10^6 CFU mL^{-1} at 0, 3, 12, 24, 48 h, respectively. In the PLA-3LNP system, the values were registered as 6.4×10^5 CFU mL^{-1}, 3.1×10^5 CFU mL^{-1}, 4.4×10^6 CFU mL^{-1}, 9.8×10^7 CFU mL^{-1}, 5.9×10^5 CFU mL^{-1} at 0, 3, 12, 24, 48 h, respectively. The better antibacterial activity resulted from PLA films loaded with both LNP and CNC. In the case of *Xap* bacterial test, PLA showed *Xap* values 1×10^6 CFU mL^{-1}, 2×10^6 CFU mL^{-1}, 8.0×10^7 CFU mL^{-1}, 9.0×10^7 CFU mL^{-1} and 1.0×10^7 CFU mL^{-1} at 0, 3, 12, 24, 48 h, respectively (Fig. 1b).

In the PLA-3LNP system, the values were registered as 1.0×10^6 CFU mL^{-1}, 2.0×10^6 CFU mL^{-1}, 4.0×10^7 CFU mL^{-1}, 7.0×10^7 CFU mL^{-1}, 3.0×10^6 CFU mL^{-1} at 0, 3, 12, 24, 48 h, respectively (Fig. 1b). Meanwhile, in the case of PLA-3LNP/1CNC, *Xap* values recorded were as 1.0×10^6 CFU mL^{-1}, 3.0×10^6 CFU mL^{-1}, 7.0×10^6 CFU mL^{-1}, 6.0×10^7 CFU mL^{-1} and 7.0×10^6 CFU mL^{-1} at 0, 3, 12, 24, 48 h, respectively (Fig. 1b). At the beginning of the tests (after 3 h), no antibacterial activity was detected in the PLA and PLA-3LNP systems, while an evident antibacterial activity was observed after 12 h of *Xap* inoculation, in the PLA-3LNP/1CNC system (Fig. 1b). In the case of *Pst*, lignin nanoparticles and cellulose nanocrystals induced an antibacterial activity of PLA matrix.

By using PLA-3LNP/1CNC based nanocomposite, the antibacterial activity resulted much more evident with respect to PLA control few hours (0–3 h) after *Pst* inoculation, while, at the end of the experiments (48 h after *Pst* inoculation), the antibacterial activity of the different systems tends to become quite uniform (Fig. 1c).

The antibacterial activity of lignin is usually in relation to its origin, and specifically due to the presence of phenolic compounds and different functional groups containing oxygen (–OH, –CO, –COOH) in its structure. Furthermore, it is known that its biocidal activity is expressed on bacterial cell membrane causing severe damages and lysis of bacterial cells with consequent release of cell content. Dong et al. [36] extracted lignin from residue of corn stover, which exhibited antimicrobial activities against Gram positive bacteria (*Listeria monocytogenes* and *Staphylococcus aureus*) and yeast (*Candida lipolytica*), but not Gram negative bacteria (*Escherichia coli* O157:H7 and *Salmonella Enteritidis*) or bacteriophage MS2. However, in our cases, lignin nanoparticles also exhibited antimicrobial activities against Gram negative bacteria. It is worth to mention that LNP and CNC even show a synergistic effect on the *Xav* and *Xap* in some time.

Among the nanoparticles here utilised, our results point out original and relevant biocidal properties of PLA ternary system containing both cellulose nanocrystals (1 wt.%) and lignin nanoparticles (3 wt.%) (PLA-3LNP/1CNC). Their capacity to reduce the bacterial survival/multiplication over the time seems to be related to a specific negative influence on a biological property of the plant pathogenic bacteria (*X. a.* pv. *vesicatoria*, *X. a.* pv. *pruni*) tested. During initial host-pathogen interaction, for these bacteria it is fundamental to adhere on host surface. This phenomenon it is possible when they are able to determine the production of the biofilm; this organic matrix is mainly constituted by lipopolysaccharides (LPSs) and exopolysaccharides (EPSs) and results fundamental for bacterial growth [37,38]. In this sense, the combination of lignocellulosic particles, even at a low concentration, resulted effective against the LPSs/EPSs development. In fact, within other physicochemical factors, such as solution chemistry and presence of multivalent ion species, they induce the *Xav* and *Xap* bacterial aggregation, inhibiting the subsequent bacterial initial surface adhesion and the biofilm formation [39]. Different results were obtained with respect to the causal agent of bacterial spot (*Pst*). In this case, its capacity to be an epiphytic bacteria with a resident phase on the host [40] explains its specific property to produce a biofilm and to maintain almost unchanged its fitness capacity (growth, colonization) along the time [41].

3.2. Antiradical activity of LNP solution and polymeric films

Radicals originating from oxygen exist naturally in the atmosphere or can be created by thermal processing or irradiation of packaging and food [42]. Those radicals act as initiators of the chain oxidation of lipids. It is therefore interesting to eliminate theses radicals from the headspace and the bulk of the food, as an alternative route to eliminate oxygen from the package. The radical scavenging efficiency of an antiradical substance depends on the rate of hydrogen atom abstraction from the phenyl group and also on the stability of the resulting radical. Strategies employed in active packaging generally include (1) the design of active compound releasing systems and (2) undesired compound scavenging systems [43]. In the first strategy, the generation of low molecular weight substances inside the packaging film and promotion of their migration into the foodstuff is interesting. In the second strategy, one of the advantages of radical scavengers is their efficiency upon contact without need for release of active compounds. This has been shown for hydroxyl radicals in the gas phase scavenged by essential oils supported on active packaging films containing essential oils [44]. The principle has been applied, as an example, in the use of catechins in poly (ethylene terephthtalate) [45,46]. Although different methods exist to measure the radical scavenging activity of plastic films, the antiradical activity of LNP solutions and lignin

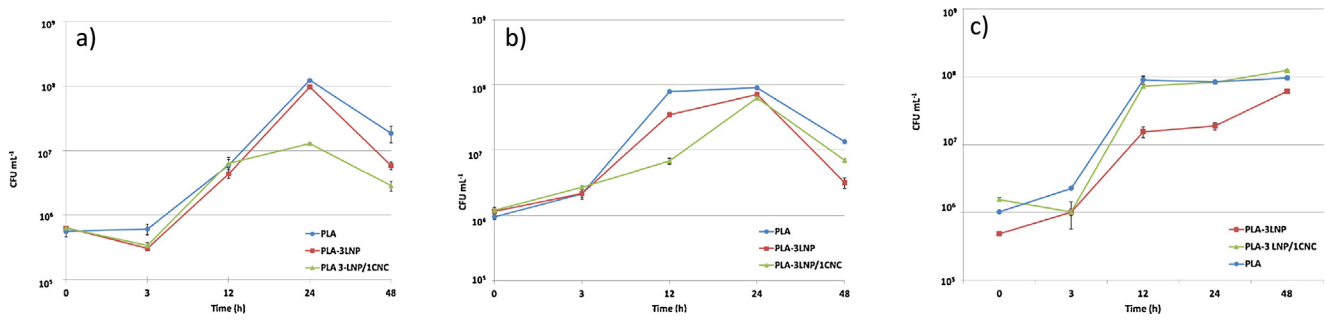

Fig. 1. (a) Antibacterial activity of different films based on neat PLA, PLA film with 3 wt.% of lignin nanoparticles (PLA-3LNP), PLA ternary system containing both cellulose nanocrystals (1 wt.%) and lignin nanoparticles (3 wt.%) (PLA-3LNP/1CNC), on the multiplication of plant pathogenic bacteria *Xanthomonas axonopodis* pv. *vesicatoria* (Xav) (CFBP 3274) 1×10^6 CFU/mL; (b) Antibacterial activity of different films based on neat PLA, PLA film with 3 wt.% of lignin nanoparticles (PLA-3LNP), PLA ternary system containing both cellulose nanocrystals (1 wt.% .) and lignin nanoparticles (3 wt.%) (PLA-3LNP/1CNC), on the multiplication of plant pathogenic bacteria Xanthomonas arboricola pv. pruni (Xap) (CFBP 3894) 1×10^6 CFU/mL; (c) Antibacterial activity of different films based on neat PLA, PLA film with 3% wt. of lignin nanoparticles (PLA-3LNP), PLA ternary system containing both cellulose nanocrystals (1 wt.%) and lignin nanoparticles (3 wt.%) (PLA-3LNP/1CNC), on the multiplication of plant pathogenic bacteria Pseudomonas syringae pv. tomato (Pst) (CFBP 1323) 1×10^6 CFU/mL. Data submitted to the analysis of variance (ANOVA) resulted significant ($p \leq 0.05$).

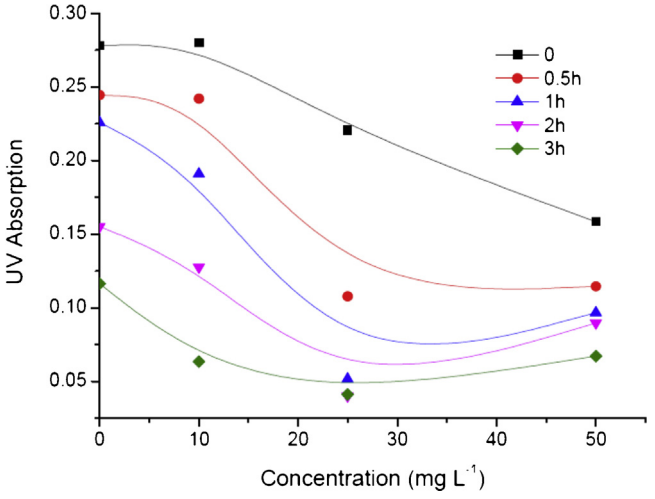

Fig. 2. Absorption of the band at 517 nm for the free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH) upon reduction by different concentrations (from 0 to 50 mg/L) of LNP at different times.

Table 2
Radical scavenging activity (RSA) values for LNP solutions at different concentration and after different times.

Concentration (mg L^{-1})	*DPPH scavenging activity, RSA (%)*				
Time (hs)	0	0.5	1	2	3
0	0	12.12	18.81	44.23	58.18
10	−0.72	13.02	31.32	54.12	77.24
25	20.68	61.31	81.45	85.62	85.26
50	43.04	58.83	65.30	67.78	75.91

based PLA film was tested by the DPPH test, that is readily available, largely used and already employed for lignin based materials [7,10,34,47].

Fig. 2 and Table 2 show both the scavenging activity of free radical DPPH over the time for different concentrations (from 0 to 50 mg L^{-1}) of LNP in the methanol/DPPH solution. The results show that DPPH scavenging activity of the lignin nanoparticles was strong and concentration/time dependent. Increasing the incubation time and LNP concentrations, the absorbance band at 517 nm decreased, while DPPH scavenging activity increased, up to 81.5% after 1 h of incubation for 25 mg L^{-1} of LNP in methanol solution.

This activity was maintained stable until 3 h, indicating how a sustained protective action for food due to slow release kinetics might be possible. However, when the concentration rose up to 50 mg L^{-1}, the absorbance band increased and the DPPH scavenging activity declined: this result may be attributed to the presence of both water soluble and organic solvent insoluble fractions of lignin [48], since at that concentration, lignin nanoparticles precipitated and aggregated during the incubation over time in DPPH methanol solution, inducing higher absorbance band and lower scavenging activity than those registered at 25 mg L^{-1} [49].

Fig. 3a and b shows the antioxidant activities of migrating substances for different PLA nanocomposite films immersed directly in the methanol solution for 24 h. The neat PLA and PLA/3CNC samples did not show any DPPH radical scavenging activity as expected, and high plateau values of absorbance (0.9462 and 0.9306 for PLA and PLA/3CNC, respectively) were detected. When incorporating 1 wt.% of LNP, the RSA value of PLA-1LNP/3CNC film increased from 1.65 to 7.85%, highlighting a noticeable antioxidation response. When the dose of LNP raised up to 3 wt.% (PLA-3LNP film), a more remarkable DPPH scavenging activity could be obtained (19.85%). Interestingly, the combination of LNP and CNC provokes a synergistic positive effect in the antioxidation response of PLA ternary formulations, since higher RSA values (25.17 and 28.45%) were registered for PLA-3LNP/1CNC and PLA-3LNP/3CNC sample, respectively. On the basis of this study, the LNP seems to be reasonably attractive from the safety and marketing point of view, having lower cost and higher antioxidant ability than α-tocopherol (vitamin E), a common commercial antioxidant used in food preservation [35], moreover PLA film incorporating a combination of lignocellulosic nanoparticles can be used as a competitive antioxidant film in food or medical packaging industry.

3.3. *Overall migration activity of PLA nanocomposite films*

Overall migration tests with food simulants were carried out and the results are shown in Fig. 4. The idea was to demonstrate the possible practical application of the produced nanocomposite films in the food packaging field. After 10 days of incubation at 40 °C in ethanol 10% (v/v), the maximum migration level was 62.5 mg kg^{-1} of simulant measured for the PLA-3LNP/1CNC formulation. This value is acceptable as migration limits for food contact materials, 60 mg kg^{-1} (or 10 mg dm^2) simulant, established by the current European legislation. All the other PLA nanocomposite films are all below this limit. The different migration values detected for various formulations can be ascribed to the morphology of these systems, that showed a porous structure, detailed discussed in [12], and are also related to the plasticizing effect induced by the presence of the glycidyl methacrylate that facilitated the diffusion processes [50]. In the meanwhile, the difference in thickness of the films could be also a critical factor for the different overall migration behaviour.

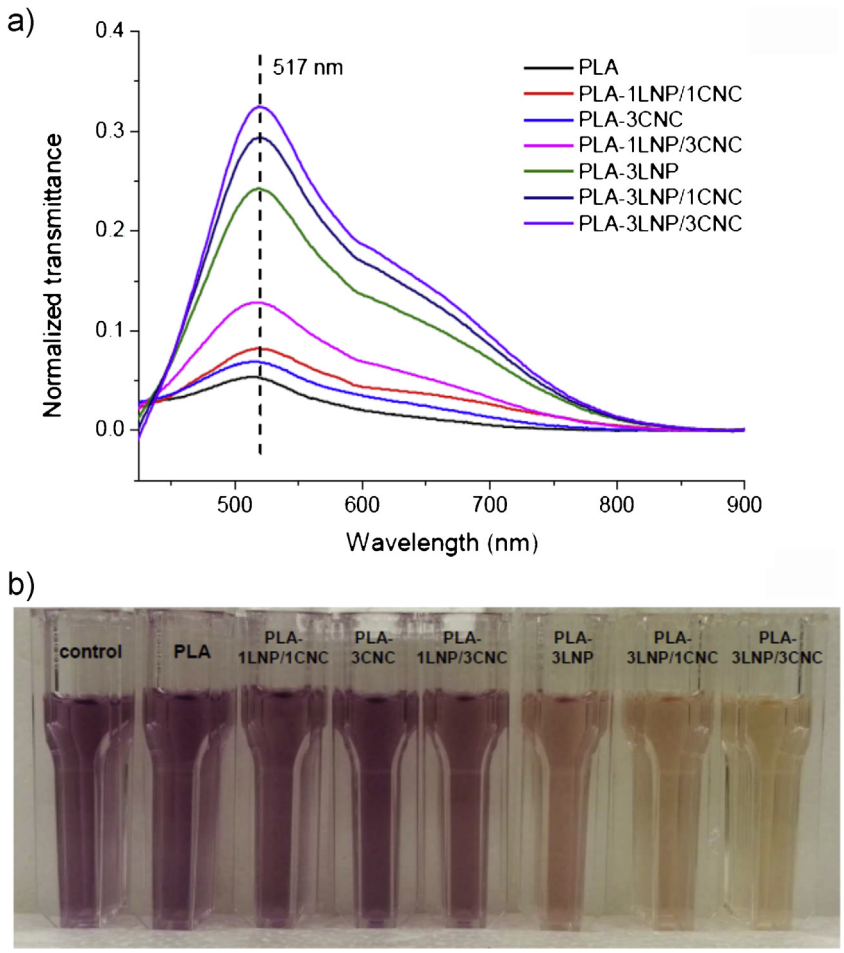

Fig. 3. Antioxidant activities of migrating substances for different PLA nanocomposite films immersed directly in the methanol solution for 24 hs: monitoring of the absorbance for band at 517 nm (a) and colour variation of the DPPH methanol solution (b).

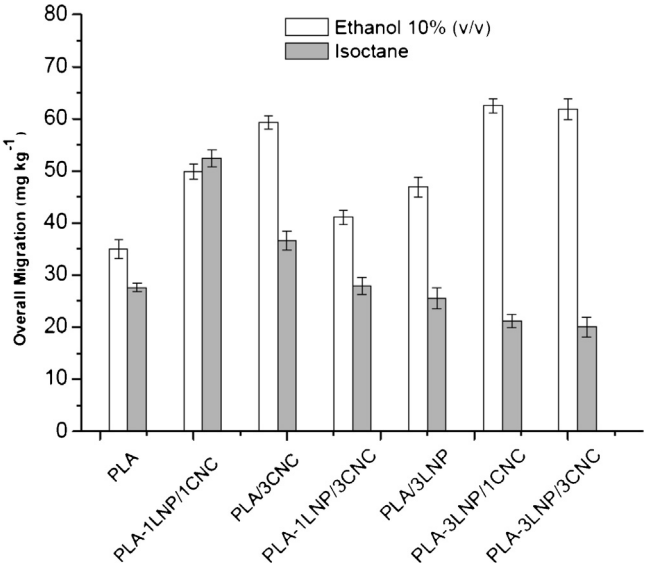

Fig. 4. Overall migration in 10% (v/v) ethanol and isooctane for PLA and PLA nanocomposites.

For the migration values in isooctane for 2 days in 20 °C, the results showed the opposite tendency respect to ethanol 10% (v/v) simulant. The highest overall migration value detected was 52.4 mg kg^{-1} in the case of PLA-1LNP/1CNC. It should be pointed out that all the migration values for the studied systems are well below the overall migration legislative limits, suggesting the possibility to use the proposed formulations for food packaging. Actually, based on the migration results, the studied films are more suitable in packaging for fatty food, as lower overall migrating values were detected when incubating in isooctane simulant in comparison with ethanol 10% (v/v) simulant.

3.4. Disintegrability activity in composting conditions

The disintegration in composting conditions of PLA is one of the most attractive properties for packaging applications [51]. Its degradation usually starts with the hydrolysis of the PLA chains induced by the diffusion of water into the materials. The influence over the degradation process strongly depends on the hydrophilicity/hydrophobicity and dispersion of the nanoparticles. It is well known that a delayed biodegradability due to the improved barrier properties of the nanomaterials was previously observed, which could hinder the water diffusion through the bulk [52]. Fig. 5 shows photographs of all the samples at different composting times up to 15 days. Whitening and deformation of the film surfaces were detected just after the first day of incubation for all materials and these effects were enhanced after 3 days in composting conditions. These results are indicative of the beginning of the hydrolytic degradation, when taking into account the thickness difference between them. The loss of transparency observed for all samples could be attributed to changes in the refractive index due to the water absorption and the formation of low molecular

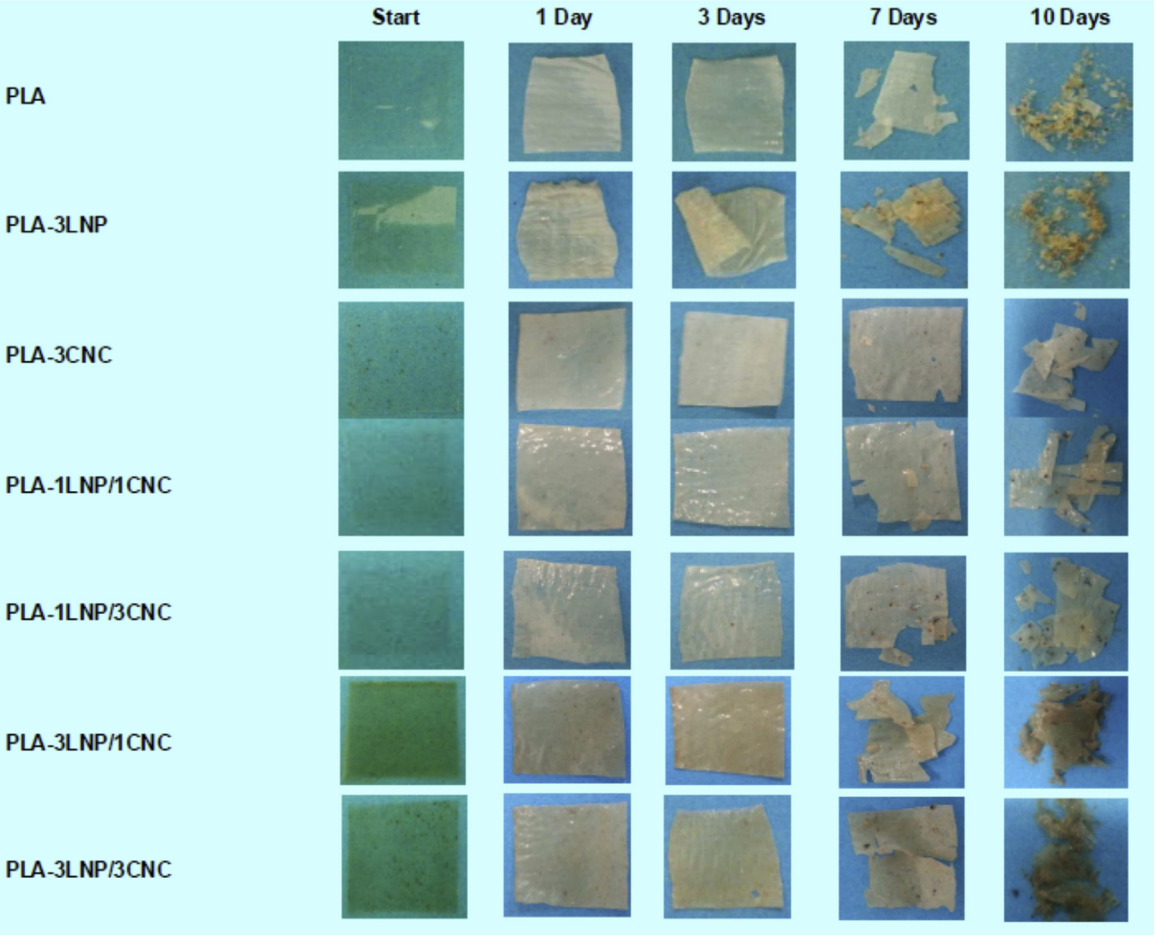

Fig. 5. Visual observation of PLA and PLA nanocomposites before (0 day) and after different stages of disintegration in composting at 58 °C.

weight compounds after the hydrolytic degradation [53,50]. The colour changes are also linked with sample degradation process and embrittlement, leading to their breakage. It has been indicated that the PLA hydrolysis begins in the amorphous region of the polymer structure and all these effects would also result in changes in the polymer cristallinity and consequently in loss of transparency [54]. Moreover, it should be taken into account that the degradation experiments took place at 58 °C, which is the region of glass transition of PLA. This could increase the chain mobility, inducing the crystallization of the PLA matrix. The disintegrability was also evaluated in terms of mass loss at different incubation times.

The disintegration process takes place between 7 and 10 days of incubation, as reported in Fig. 6, in accordance with our previous study [19]. The disintegrability value remains constant for all systems until 3 days of incubation, while reaches 30–50% at 10 days for all PLA nanocomposite films. The highest disintegration values (around 50%) were detected for PLA and PLA formulations loaded with 3 wt.% of LNP (binary and ternary films), while lower values of disintegration (around 20–30%) were detected, after 10 days of incubation, for PLA/3CNC binary film and PLA-1LNP/1CNC or PLA-1LNP/3CNC ternary formulations. Finally, all the studied formulations showed a disintegrability value up to 90% after 15 days of incubation in composting conditions.

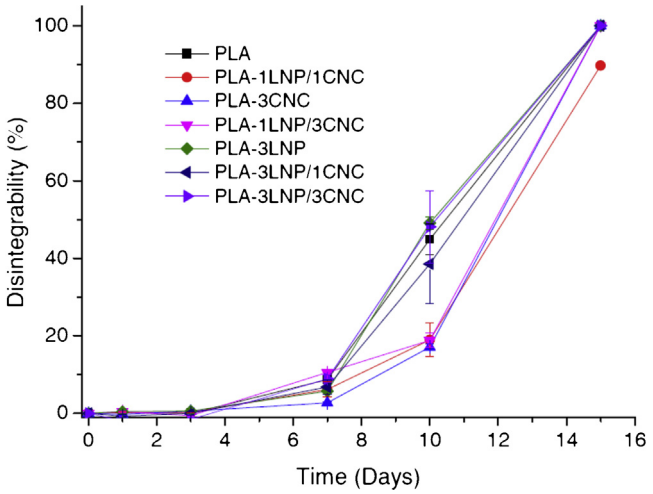

Fig. 6. Disintegrability values of PLA and PLA binary and ternary nanocomposites at different stages of incubation in compost.

4. Conclusions

PLA systems containing cellulose nanocrystals and lignin nanoparticles revealed an innovative capacity to inhibit the bacterial growth along the time. Their biodegradability and the environmental properties offer innovative opportunities against dangerous bacterial plant pathogens like *X. axonopodis* pv. *vesicatoria* and *X. arboricola* pv. *pruni*. Risk of remarkable economic losses caused by several microorganism, like these phytobacteria, during post-harvest phases until to consumers, are a concrete problem to be solved and these nanoparticles reveal a green potentiality in this sense.

Radical scavenging activity of free radical DPPH over the time for different concentrations of LNP in the methanol/DPPH solution reveals high antioxidation activity even at 25 mg L^{-1} due to the hydrogen atom abstraction from the phenyl group in LNP, which will eliminate oxygen from the package. In the meanwhile, migrating substances for the films containing LNP also show high antioxidation activity. Based on these results, the LNP can be employed in both active packaging strategies, generally including (1) the design of active compound releasing systems and (2) undesired compound scavenging systems, which seems to be reasonably attractive from the safety and marketing point of view, having lower cost and higher antioxidant ability. Migration values for the studied systems are well below the overall migration legislative limits, suggesting the possibility to use the proposed formulations for food-packaging. Actually, the studied films are more suitable in packaging for fatty food based on the lower overall migrating values detected when incubating in isooctane simulant when comparing with ethanol 10% (v/v) simulant. Results of disintegrability tests in composting conditions proved that PLA and PLA formulations loaded with 3 wt.% of LNP (binary and ternary films) have higher values of disintegration after 10 days of incubation in comparison to PLA/3CNC binary film and PLA-1LNP/1CNC or PLA-1LNP/3CNC ternary formulations, which may be ascribed to the lower crystallization ability for LNP.

Acknowledgement

Weijun Yang appreciates the funding support from China Scholarship Council (CSC).

References

[1] M. Jamshidian, E.A. Tehrany, S. Desobry, Release of synthetic phenolic antioxidants from extruded poly lactic acid (PLA) film, Food Control 28 (2012) 445–455.
[2] I. Volf, I. Ignat, M. Neamtu, V.I. Popa, Thermal stability, antioxidant activity, and photo-oxidation of natural polyphenols, Chem. Pap. 68 (2014) 121–129.
[3] F. Manzanarez-López, H. Soto-Valdez, R. Auras, E. Peralta, Release of α-tocopherol from poly (lactic acid) films, and its effect on the oxidative stability of soybean oil, J. Food Eng. 104 (2011) 508–517.
[4] H. Faustino, N. Gil, C. Baptista, A.P. Duarte, Antioxidant activity of lignin phenolic compounds extracted from kraft and sulphite black liquors, Molecules 15 (2010) 9308–9322.
[5] N. Bitinis, E. Fortunati, R. Verdejo, J. Bras, J.M. Kenny, L. Torre, M.A. López-Manchado, Poly(lactic acid)/natural rubber/cellulose nanocrystal bionanocomposites: part II: properties evaluation, Carbohydr. Polym. 96 (2013) 621–627.
[6] D. Kai, M.J. Tan, P.L. Chee, Y.K. Chua, Y.L. Yap, X.J. Loh, Towards lignin-based functional materials in a sustainable world, Green Chem. (2016).
[7] T. Dizhbite, G. Telysheva, V. Jurkjane, U. Viesturs, Characterization of the radical scavenging activity of lignins-natural antioxidants, Bioresour. Technol. 95 (2004) 309–317.
[8] V. Ugartondo, M. Mitjans, M.P. Vinardell, Comparative antioxidant and cytotoxic effects of lignins from different sources, Bioresour. Technol. 99 (2008) 6683–6687.
[9] M. Azadfar, A.H. Gao, S. Chen, Structural characterization of lignin: a potential source of antioxidants guaiacol and 4-vinylguaiacol, Int. J. Biol. Macromol. 75 (2015) 58–66.
[10] S. Domenek, A. Louaifi, A. Guinault, S. Baumberger, Potential of lignins as antioxidant additive in active biodegradable packaging materials, J. Polym. Environ. 21 (2013) 692–701.
[11] S.R. Yearla, K. Padmasree, Preparation and characterisation of lignin nanoparticles: evaluation of their potential as antioxidants and UV protectants, J. Exp. Nanosci. 11 (2016) 289–302.
[12] M. Norgren, H. Edlund, Lignin: recent advances and emerging applications, Curr. Opin. Colloid Interface Sci. 19 (2014) 409–416.
[13] A.P. Richter, J.S. Brown, B. Bharti, A. Wang, S. Gangwal, K. Houck, E.A.C. Hubal, V.N. Paunov, S.D. Stoyanov, O.D. Velev, An environmentally benign antimicrobial nanoparticle based on a silver-infused lignin core, Nat. Nanotechnol. 10 (2015) 817–823.
[14] V.K. Thakur, M.K. Thakur, Recent advances in green hydrogels from lignin: a review, Int. J. Biol. Macromol. 72 (2015) 834–847.
[15] A. Gregorová, S. Redik, V. Sedlařík, F. Stelzer, Lignin-containing polyethylene films with antibacterial activity, NANOCON 2011-Conference Proceedings, 3rd International Conference. TANGER Ltd. (2011).
[16] M. Zimniewska, R. Kozłowski, J. Batog, Nanolignin modified linen fabric as a multifunctional product, Mol. Cryst. Liq. Cryst. 484 (2008) 43/[409]–450/[416].
[17] S. Dumitriu, V.I. Popa, Polymeric Biomaterials, CRC Press, 2013.
[18] E. Fortunati, S. Rinaldi, M. Peltzer, N. Bloise, L. Visai, I. Armentano, A. Jiménez, L. Latterini, J.M. Kenny, Nano-biocomposite films with modified cellulose nanocrystals and synthesized silver nanoparticles, Carbohydr. Polym. 101 (2014) 1122–1133.
[19] W. Yang, E. Fortunati, F. Dominici, J.M. Kenny, D. Puglia, Effect of processing conditions and lignin content on thermal, mechanical and degradative behavior of lignin nanoparticles/polylactic (acid) bionanocomposites prepared by melt extrusion and solvent casting, Eur. Polym. J. 71 (2015) 126–139.
[20] N. Bitinis, E. Fortunati, R. Verdejo, J. Bras, J.M. Kenny, L. Torre, M.A. López-Manchado, Poly (lactic acid)/natural rubber/cellulose nanocrystal bionanocomposites: part II: properties evaluation, Carbohydr. Polym. (2013) 96, 96 621 -627.
[21] M. Jonoobi, J. Harun, A.P. Mathew, K. Oksman, Mechanical properties of cellulose nanofiber (CNF) reinforced polylactic acid (PLA) prepared by twin screw extrusion, Compos. Sci. Technol. 70 (2010) 1742–1747.
[22] M.R. Kamal, V. Khoshkava, Effect of cellulose nanocrystals (CNC) on rheological and mechanical properties and crystallization behavior of PLA/CNC nanocomposites, Carbohydr. Polym. 123 (2015) 105–114.
[23] N. Lin, J. Huang, P.R. Chang, J. Feng, J. Yu, Surface acetylation of cellulose nanocrystal and its reinforcing function in poly(lactic acid), Carbohydr. Polym. 83 (2011) 1834–1842.
[24] Q. Shi, C. Zhou, Y. Yue, W. Guo, Y. Wu, Q. Wu, Mechanical properties and in vitro degradation of electrospun bio-nanocomposite mats from PLA and cellulose nanocrystals, Carbohydr. Polym. 90 (2012) 301–308.
[25] W. Yang, F. Dominici, E. Fortunati, J.M. Kenny, D. Puglia, Melt free radical grafting of glycidyl methacrylate (GMA) onto fully biodegradable poly (lactic) acid films: effect of cellulose nanocrystals and a masterbatch process, RSC Adv. 5 (2015) 32350–32357.
[26] E. Fortunati, M. Peltzer, I. Armentano, L. Torre, A. Jiménez, J. Kenny, Effects of modified cellulose nanocrystals on the barrier and migration properties of PLA nano-biocomposites, Carbohydr. Polym. 90 (2012) 948–956.
[27] E. Fortunati, I. Armentano, Q. Zhou, A. Iannoni, E. Saino, L. Visai, L.A. Berglund, J. Kenny, Multifunctional bionanocomposite films of poly (lactic acid), cellulose nanocrystals and silver nanoparticles, Carbohydr. Polym. 87 (2012) 1596–1605.
[28] S. Salmieri, F. Islam, R.A. Khan, F.M. Hossain, H.M. Ibrahim, C. Miao, W.Y. Hamad, M. Lacroix, Antimicrobial nanocomposite films made of poly (lactic acid)-cellulose nanocrystals (PLA-CNC) in food applications—part B: effect of oregano essential oil release on the inactivation of Listeria monocytogenes in mixed vegetables, Cellulose 21 (2014) 4271–4285.
[29] M. Tuomela, M. Vikman, A. Hatakka, M. Itävaara, Biodegradation of lignin in a compost environment: a review, Bioresour. Technol. 72 (2000) 169–183.
[30] F. Thieme, R. Koebnik, T. Bekel, C. Berger, J. Boch, D. Büttner, C. Caldana, L. Gaigalat, A. Goesmann, S. Kay, Insights into genome plasticity and pathogenicity of the plant pathogenic bacterium *Xanthomonas campestris* pv. vesicatoria revealed by the complete genome sequence, J. Bacteriol. 187 (2005) 7254–7266.
[31] A. Quattrucci, E. Ovidi, A. Tiezzi, V. Vinciguerra, G. Balestra, Biological control of tomato bacterial speck using Punica granatum fruit peel extract, Crop Prot. 46 (2013) 18–22.
[32] W. Chun, N.W. Schaad, J.B. Jones, Laboratory Guide for Identification of Plant Pathogenic Bacteria, American phytopathological society, 2001.
[33] R.G. Steel, J.H. Dickey, Principles and Procedures of Statistics a Biometrical Approach, WCB/McGraw-Hill, 1997.
[34] Q. Lu, M. Zhu, Y. Zu, W. Liu, L. Yang, Y. Zhang, X. Zhao, X. Zhang, X. Zhang, W. Li, Comparative antioxidant activity of nanoscale lignin prepared by a supercritical antisolvent (SAS) process with non-nanoscale lignin, Food Chem. 135 (2012) 63–67.
[35] Y. Byun, Y.T. Kim, S. Whiteside, Characterization of an antioxidant polylactic acid (PLA) film prepared with α-tocopherol, BHT and polyethylene glycol using film cast extruder, J. Food Eng. 100 (2010) 239–244.
[36] X. Dong, M. Dong, Y. Lu, A. Turley, T. Jin, C. Wu, Antimicrobial and antioxidant activities of lignin from residue of corn stover to ethanol production, Ind. Crops Prod. 34 (2011) 1629–1634.
[37] P.C. Bogino, Mde.L. Oliva, F.G. Sorroche, W. Giordano, The role of bacterial biofilms and surface components in plant-bacterial associations, Int. J. Mol. Sci. 14 (2013) 15838–15859.
[38] T. Danhorn, C. Fuqua, Biofilm formation by plant-associated bacteria, Annu. Rev. Microbiol. 61 (2007) 401–422.
[39] X. Sun, Q. Lu, Y. Boluk, Y. Liu, The impact of cellulose nanocrystals on the aggregation and initial adhesion of Pseudomonas fluorescens bacteria, Soft Matter 10 (2014) 8923–8931.
[40] C. Leben, How plant-pathogenic bacteria survive, Plant Dis. 65 (1981) 633–637.
[41] J. Yu, A. Peñaloza-Vázquez, A.M. Chakrabarty, C.L. Bender, Involvement of the exopolysaccharide alginate in the virulence and epiphytic fitness of *Pseudomonas syringae* pv. syringae, Mol. Microbiol. 33 (1999) 712–720.
[42] C. Loípez de Dicastillo, C. Nerin, P. Alfaro, R. Catalá, R. Gavara, P. Hernaíndez-Mun˜oz, Development of new antioxidant active packaging films based on ethylene vinyl alcohol copolymer (EVOH) and green tea extract, J. Agric. Food Chem. 59 (2011) 7832–7840.

[43] C. Nerin, L. Tovar, J. Salafranca, Behaviour of a new antioxidant active film versus oxidizable model compounds, J. Food Eng. 84 (2008) 313–320.
[44] D. Pezo, J. Salafranca, C. Nerín, Determination of the antioxidant capacity of active food packagings by in situ gas-phase hydroxyl radical generation and high-performance liquid chromatography-fluorescence detection, J. Chromatogr. A 1178 (2008) 126–133.
[45] M. Colon, C. Nerin, Role of catechins in the antioxidant capacity of an active film containing green tea, green coffee, and grapefruit extracts, J. Agric. Food Chem. 60 (2012) 9842–9849.
[46] C. López-de-Dicastillo, D. Pezo, C. Nerín, G. López-Carballo, R. Catalá, R. Gavara, P. Hernández-Muñoz, Reducing oxidation of foods through antioxidant active packaging based on ethyl vinyl alcohol and natural flavonoids, Packag. Technol. Sci. 25 (2012) 457–466.
[47] M.P. Vinardell, V. Ugartondo, M. Mitjans, Potential applications of antioxidant lignins from different sources, Ind. Crops Prod. 27 (2008) 220–223.
[48] Z. Yuan, S. Cheng, M. Leitch, C.C. Xu, Hydrolytic degradation of alkaline lignin in hot-compressed water and ethanol, Bioresour. Technol. 101 (2010) 9308–9313.
[49] W. Yang, J.M. Kenny, D. Puglia, Structure and properties of biodegradable wheat gluten bionanocomposites containing lignin nanoparticles, Ind. Crops Prod. 74 (2015) 348–356.
[50] H. Kargarzadeh, R.M. Sheltami, I. Ahmad, I. Abdullah, A. Dufresne, Cellulose nanocrystal: a promising toughening agent for unsaturated polyester nanocomposite, Polymer 56 (2015) 346–357.
[51] G. Kale, R. Auras, S.P. Singh, R. Narayan, Biodegradability of polylactide bottles in real and simulated composting conditions, Polym. Test. 26 (2007) 1049–1061.
[52] W.-K. Lee, R.W. Nowak, J.A. Gardella, Hydrolytic degradation of polyester blend monolayers at the air/water interface: effects of a slowly degrading component, Langmuir 18 (2002) 2309–2312.
[53] K. Fukushima, D. Tabuani, C. Abbate, M. Arena, L. Ferreri, Effect of sepiolite on the biodegradation of poly(lactic acid) and polycaprolactone, Polym. Degrad. Stab. 95 (2010) 2049–2056.
[54] L. Zaidi, M. Kaci, S. Bruzaud, A. Bourmaud, Y. Grohens, Effect of natural weather on the structure and properties of polylactide/Cloisite 30B nanocomposites, Polym. Degrad. Stab. 95 (2010) 1751–1758.