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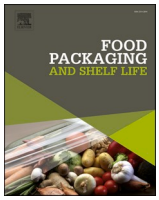

Development of a sustainable and antibacterial food packaging material based in a biopolymeric multilayer system composed by polylactic acid, chitosan, cellulose nanocrystals and ethyl lauroyl arginate

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ABSTRACT

Biodegradable packaging materials with antimicrobial properties are main key for developing sustainable active packages able to protect different foodstuff. Thus, the aim of this research was developing a biopolymeric and antibacterial packaging material with a trilayer structure through the combination of extrusion, electrospinning and coating techniques. This trilayer system was composed by a support extruded layer of polylactic acid (PLA), an intermediate PLA electrospun layer loaded with ethyl lauroyl arginate (LAE) and cellulose nanocrystals (CNC), and a third inner chitosan coating. Morphological, wettability, antibacterial and disintegrability properties of this material were evaluated. The morphological surface analysis evidenced the heterogeneity of the chitosan coating over the electrospun PLA layer, and this effect affected negatively the evaluation of wettability of this trilayer material. Antibacterial assays evidenced a fast and strong bactericidal effect against Gram(+) and (−) bacteria that maintained this activity for 15 days. Finally, the biodegradable properties of different layers favored the disintegration of developed trilayer material at 21 days under composting conditions.

1. Introduction

The widespread use of polyolefins for the development of food packaging materials and the lack of rigorous regulations about the post-use management of these materials involving adequate recycling or final disposal have negatively impacted the environment (Guillard et al., 2018). In this context, the use of compostable and biodegradable biopolymers has arisen as a highly attractive alternative to the design of eco-friendly food packaging materials (Popović et al., 2018). Among biopolymers, polylactic acid (PLA) appears as one the most interesting alternative polymer to replace the conventional ones due to its good performance. The biodegradability of PLA is promising for several applications and can compete with polyethylene terephthalate (PET) for many food packaging functions because of its good mechanical properties, high transparency, easy processing and market availability (Ulloa et al., 2019). This fact in turn have also encouraged the use of PLA in the development of active packaging in order to extend the food shelf-life, reduce food losses and increase the profitability of food industry. Previous works have already compiled the description of active PLA film developments through different processing techniques, conditions and by adding several types of active agents, including essential oils, natural extracts and metallic nanoparticles (Velásquez et al., 2021). In the last years, the incorporation of strong antimicrobial substances as ethyl lauroyl arginate (LAE) has allowed the development of highly functional packaging materials able to protect food products sensitive to microbial growth for longer periods (Haghighi et al., 2020; Patiño Vidal et al., 2022). LAE is a cationic surfactant obtained from L-arginine, lauric acid and ethanol and is widely used by the food industry due to its low-doses application (Ma et al., 2020). Nevertheless, the incorporation of several antimicrobial substances into PLA-based materials has generally

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produced undesirable changes in their physical properties, decreasing their inherent barrier properties to gases and water vapor (Rojas et al., 2021). In this context, the design of multilayer films through the combination of conventional methods, such as thermo-compression and coating, has emerged as an alternative for obtaining functional materials from PLA with good barrier properties (Andrade et al., 2022; Nilsuwan et al., 2020; Trinh et al., 2021). Recently, the conventional electrospinning has been proposed as a non-thermal technique to design active bilayer structures able to improve the release of active substances (Gulzar et al., 2022). However, the burst release kinetics of active compounds from these structures would impact negatively the maintenance of their active effect along time, as it has been reported in some studies (Joy et al., 2022; López de Dicastillo et al., 2018). Thus, coaxial electrospinning has been recently employed to slow down the release kinetic of active substances due to the generation of concentrical core/shell fibrillar structures. This structure allows both the protection of sensitive active substances through their encapsulation into the core and the slowdown of its release due to the presence of the shell layer (Han et al., 2021). Specifically, LAE has already been encapsulated into coaxial electrospun fibers by selecting polyvinyl alcohol (PVOH) and PLA in the core and shell structures, respectively (Patiño Vidal et al., 2021). Research evidenced the protection of this antimicrobial substance and released LAE concentrations above minimum inhibitory concentration (MIC) values for Gram(+) and (−) bacteria. Furthemore, the incorporation of cellulose nanocrystals (CNC) in the shell of the coaxial fiber has been also proposed as a feasible method to improve barrier properties and to decelerate in more extent the release kinetic of active substances due to the creation of a tortuous path through the shell layer (López de Dicastillo, Garrido, et al., 2021; Rojas et al., 2020). Patiño Vidal et al. (2022) have recently confirmed the decrease of the diffusion coefficients of LAE from coaxial electrospun fibers including CNC to both aqueous and fatty food simulants.

On the other hand, chitosan, a biopolymer resulting from the chitin deacetylation has been widely used for developing multilayer active materials. This fact has been due to its intrinsic antimicrobial activity produced by the presence of its glucosamine and N-acetylglucosamine units (Fiore et al., 2021; Oladzadabbasabadi et al., 2022).

In this framework, the aim of this study was developing a novel antibacterial food packaging material with a trilayer structure consisting of an outer PLA extruded layer, an antibacterial intermediate electrospun layer composed by PLA-LAE/PLA-CNC core/shell fibers, and an inner thin film layer of chitosan. Thus, the main goal of designing the trilayer structure including coaxial electrospun fibers was slowing down the release of LAE and achieving a extended antibacterial activity. The antibacterial activity of this trilayer system was assessed against Gram (−) and Gram (+) bacteria. Furthermore, a disintegration test of the trilayer material under composting conditions was carried out in order to obtain information related to its compostability as a suitable waste management mechanism.

2. Material and methods

2.1. Polymers, solvents, microorganisms and solid synthetic waste

Polylactic acid (2003D grade) with a specific gravity of 1.24 was purchased from Natureworks® (Minnetonka, USA). High molecular weight chitosan (310,000–375,000 Da) was obtained from Sigma-Aldrich (Santiago, Chile). Cellulose nanocrystals with dimensions of 5–20 nm width and 150–200 nm length were purchased from University of Maine (Maine, USA). Ethyl lauroyl arginate was supplied by PRINAL (Santiago, Chile). Chloroform (CLF), dimethylformamide (DMF), trifluoroacetic acid (TFA), ethanol (EtOH), acetic acid and isopropyl alcohol obtained of Sigma-Aldrich (Santiago, Chile) were used as solvents.

Salmonella enterica (CECT 378, ATCC 13525) as Gram negative bacteria, and *Listeria innocua* (CECT 910, ATCC 33090) as Gram positive bacteria were obtained from the Packaging Laboratory of IATA-CSIC (Valencia, Spain).

Solid synthetic waste used for disintegration test under composting conditions was composed of sawdust (40 wt%), rabbit food (30 wt%), mature compost (10 wt%), corn starch (10 wt%), sugar (5 wt%), corn oil (4 wt%) and urea (1 wt%). Mature compost was supplied by Gesenu S.p.a (Perugia, Italy). Furthermore, a ratio 50:50 of waste:water was maintained in accordance with the standard ISO 20200. This ratio was considered and maintained in order to maintain constant the relative humidity during the analysis and guarantee the growing of microorganisms at 58 °C.

2.2. Development of antibacterial packaging material with a multilayer structure

Antibacterial food packaging material with a trilayer structure was obtained through the combination of extrusion, coaxial electrospinning and coating techniques. The development of the layers was as follows:

- *Outer layer* consisted of an extruded PLA film obtained through a co-rotating twin-screw extruder Scientific Lab Tech LTE20 (Samutprakarn, Thailand) connected to a chill roll attachment Labtech LBCR-150 (Samutprakarn, Thailand), using a temperature profile from 195 to 210 °C and a screw speed fixed at 20 rpm. This layer was named as "PLA*ext*". Furthermore, a thickness of approx. 85 μm was determined for this layer through a digital micrometer Mitutoyo ID-C112 (San Sebastian, Spain).
- *Intermediate layer* corresponded to an electrospun mat composed by antibacterial polymeric fibers with a core/shell structure obtained through a coaxial horizontal electrospinning system that included a high-voltage power supply Acopian (Easton, USA), two syringe pumps KD Scientific (Holliston, USA), and a collector plate. 10 % (w/v) PLA solutions were prepared using a solvent mixture of CLF:DMF at ratio 7:3. LAE at 15 wt% and CNC at 1 wt% respect to PLA mass were incorporated in the core and shell structures, respectively. Both solutions were electrospun using a coaxial device composed of two concentric stainless-steel needles with diameters of 0.7 and 2.1 mm. Electrospinning parameters were set as follows: external and internal flow rates, respectively at 1.8 and 0.75 mL h^{-1}, distance between needle tip and collector plate of 14 cm, a voltage between 18 and 20 kV and a collecting time of 40 min. This resulting layer was made of core/shell PLA-LAE/PLA-CNC fibers and was named as PLA*ele*. Coaxial electrospun PLA/PLA-CNC fibers (without LAE in the core structure) were also produced to study the effect of incorporating LAE on the overall behaviour of electrospun mat and multilayer material. Electrospinning parameters were equal to the parameters used for obtaining the antibacterial coaxial PLA*ele* layer. This layer maintained a thickness of approx. 404 μm.
- *Inner layer* based on a thin film of high molecular weight chitosan (Chi) with a thickness of 26 μm was obtained from a 2 % (w/v) polymeric solution prepared in an acetic acid solution at 1 % (v/v). Isopropyl alcohol at 1 % (v/v) was also incorporated to the chitosan solution for improving its wettability.

In order to obtain the resulting trilayer antibacterial structure, the assembly was done as follows: a square sample of PLA*ext* material (11 cm × 12 cm) was put on the collector plate of electrospinning equipment. Polymeric solutions for obtaining the core/shell antibacterial electrospun mat were electrospun and collected over the PLA*ext* film for 40 min. Subsequently, this bilayer system was subjected to a corona treatment process for 6 min by using a high frequency corona surface treater BD-20 AC (Chicago, USA) in order to improve the adherence with the third inner layer. Chitosan solution was then coated over the electrospun layer through an automatic coating applicator Elcometer 4340 (Mataró, Spain). The material was dried at room temperature for 24 h, and the resulting trilayer structure was named "PLA*ext*/PLA*ele*/Chi".

Furthermore, a thickness of approx. 515 μm was determined for this material.

Furthermore, a bilayer material named as PLA*ext*/Chi with a thickness of approx. 110 μm was prepared by coating a chitosan solution over the PLA*ext* material. The PLA*ext* material was previously subjected to a corona treatment before to be coated with the chitosan solution. PLA*ext* and PLA*ext*/Chi materials were used as controls in some analysis.

2.3. Characterization of morphological and surface properties of the trilayer packaging system

The surface of materials was observed through field emission scanning electron microscope (FESEM) Supra 25-Zeiss (Dresden, Germany). Samples were coated with a thin gold foil and FESEM micrographs were obtained with a voltage acceleration at 5 kV.

The surface wettability of materials was determined by water contact angle measurements (WCA) at room temperature through sessile-drop method. A drop of distilled water (20 μL) was deposited over the surface of material, and WCA was analyzed using a First Ten Angstroms Inc., FTA 1000 analyzer (Portsmouth, United Kingdom) equipped with a camera and a drop shape analyzer SW21, FTA32 2.0 software. WCA values of eight drops randomly deposited over the materials surface were analyzed with the DROP image Software, and the average value was reported.

2.4. Analysis of antibacterial activities

The antibacterial activity of the trilayer PLA*ext*/PLA*ele*/Chi material was evaluated through two methods: i) in liquid medium through the full contact between the material and the bacterial solution; and ii) in solid medium through the measurement of bacterial inhibition zone (halo).

Both analyses were carried out against *S. enterica* and *L. innocua* as surrogates strain for *Listeria monocytogenes*. These bacteria were chosen as Gram (−) and Gram (+) models, respectively, because of their relevance in foodborne illnesses. The following samples were used as controls: i) CNT: bacterial solutions and solid growing medium without material; and ii) bacterial solutions and solid growing medium in contact with PLA*ext* and PLA*ext*/Chi materials.

Liquid medium: this assay was carried out following the methodology of López de Dicastillo, Settier-Ramírez, et al. (2021) with some modifications. 10 mL of Mueller hinton broth (MHB) into assay tubes were inoculated with a bacterial overnight culture concentration $\approx 10^5$ CFU mL^{-1}, and put in contact with 6 cm^2 of trilayer material. The tubes were stored at 4 °C for 15 days, and periodically, serial dilutions in peptone water were done from the samples and plated in petri dishes containing selective medium brilliant green agar and palcam agar for *S. enterica* and *L. innocua*, respectively. The petri dishes were incubated at 37 °C for 48 h, and the resulting colony forming units were counted. The results were expressed as the logarithm of bacterial concentrations ($\log_{10}$ CFU mL^{-1}).

Solid medium: 200 μL of a bacterial concentration $\approx 10^6$ CFU mL^{-1} were growth in petri dishes containing tryptone soya agar (TSA). Subsequently, disks of material with a diameter of 2.5 cm were put in contact with the agar, by placing the inner layer of trilayer material in contact with the medium. The petri dishes were incubated at 37 °C for 24 h, and the inhibition halo was measured (Liu et al., 2020). The disks were posteriorly removed and the petri dishes were incubated again for 24 h. The inhibition halo was measured in order to determine the type of bacterial effect: bacteriostatic or bactericidal.

2.5. Disintegration test of multilayer food packaging material under composting conditions

The disintegration under composting conditions of the trilayer PLA*ext*/PLA*ele*/Chi material was evaluated following the UNE-EN ISO 20200:2016 Normative. The disintegration of non-antibacterial coaxial PLA/PLA-CNC fibers, as well as PLA*ext*, PLA*ele* and PLA*ext*/Chi materials was also carried out in order to analyze the effect of incorporating LAE and the different layers in the trilayer material. The synthetic solid waste was prepared by the mixture of sawdust, rabbit food, mature compost, corn starch, sugar, corn oil and urea, maintaining the water content around 50 %. The synthetic solid waste was transferred to perforated boxes in order to ensure the gas exchange between inner atmosphere and outside environment. Every day, the solid waste was softly mixing for guarantee the aerobic conditions during the test. On the hand, squares of material (1.5 × 1.5 cm^2) were dried at 40 °C for 24 h, and posteriorly weighed. The samples were put into meshed plastic bags for ensuring the free contact with the solid waste and avoiding the loss of small fragments during the test. The bags were buried into the synthetic solid waste at 4–6 cm depth and incubated at 58 °C and 50 % relative humidity. The bags in contact with the synthetic solid waste were recovered after 1, 3, 7, 10, 14, 17 and 21 days. The samples were washed and dried at 40 °C for 24 h, and posteriorly weighed. The degree of disintegration (*Dis*) of each material was calculated by Eq. (1):

$$\mathrm{Dis} = \frac{m_i - m_r}{m_i} \mathrm{x}100 \quad (1)$$

where m_i and m_r are the initial and residual dry weights of the materials, respectively.

The physical changes of the samples along the test were analyzed through visual comparison by photographs. The changes on the surface morphologies of the initial and disintegrated materials after 10 days were observed with a field emission scanning electron microscope (FESEM) Supra 25-Zeiss (Dresden, Germany). The samples were previously coated with a thin gold foil and the micrographs were obtained with an accelerating voltage of 5 kV.

2.6. Statistical analysis

Data resulted from surface, antibacterial and disintegration studies of multilayer packaging material were statistically analyzed by variance analysis (ANOVA) and Tukey's Test, using the InfoStat program (2008) in order to detect differences between the samples with a confidence level of 95 % ($p < 0.05$).

3. Results and discussions

3.1. Morphological analysis of developed materials

Fig. 1 shows the morphology of materials surface observed through FESEM analysis. PLA/PLA-CNC coaxial fibers were uniform and exhibited a diameter of approx. 457 ± 51 nm. As Fig. 1 shows, these non active coaxial fibers presented some beads in their surface. PLA*ele* material composed by these coaxial fibers containing LAE in the core structure also showed morphology and diameter (425 ± 47 nm) similar to those of PLA/PLA-CNC fibers. However, the incorporation of LAE produced fibers free of beads. This fact could be related to the surfactant effect of LAE that reduced the viscosity of the polymeric solution and favored the electrospinning process (Patiño Vidal et al., 2022; Shao et al., 2019). Fig. 1 also shows smooth surfaces without imperfections in chitosan film and PLA*ext* material. Likewise, the surface of PLA*ext*/Chi material exhibited a morphology similar to that of the two above mentioned materials.

On the other hand, the corona treatment of PLA*ele* layer collected over PLA*ext* material and its subsequent coating with chitosan produced a non-smooth and non-homogeneous surface in PLA*ext*/PLA*ele*/Chi material. As Fig. 1 shows, the electrospun layer of the trilayer material exhibited some sites satisfactorily coated with the chitosan (light grey color). This result could be produced by intrinsic factors of electrospinning process and the antibacterial electrospun layer. The type of electrospinning collector plays an important role in the obtaining

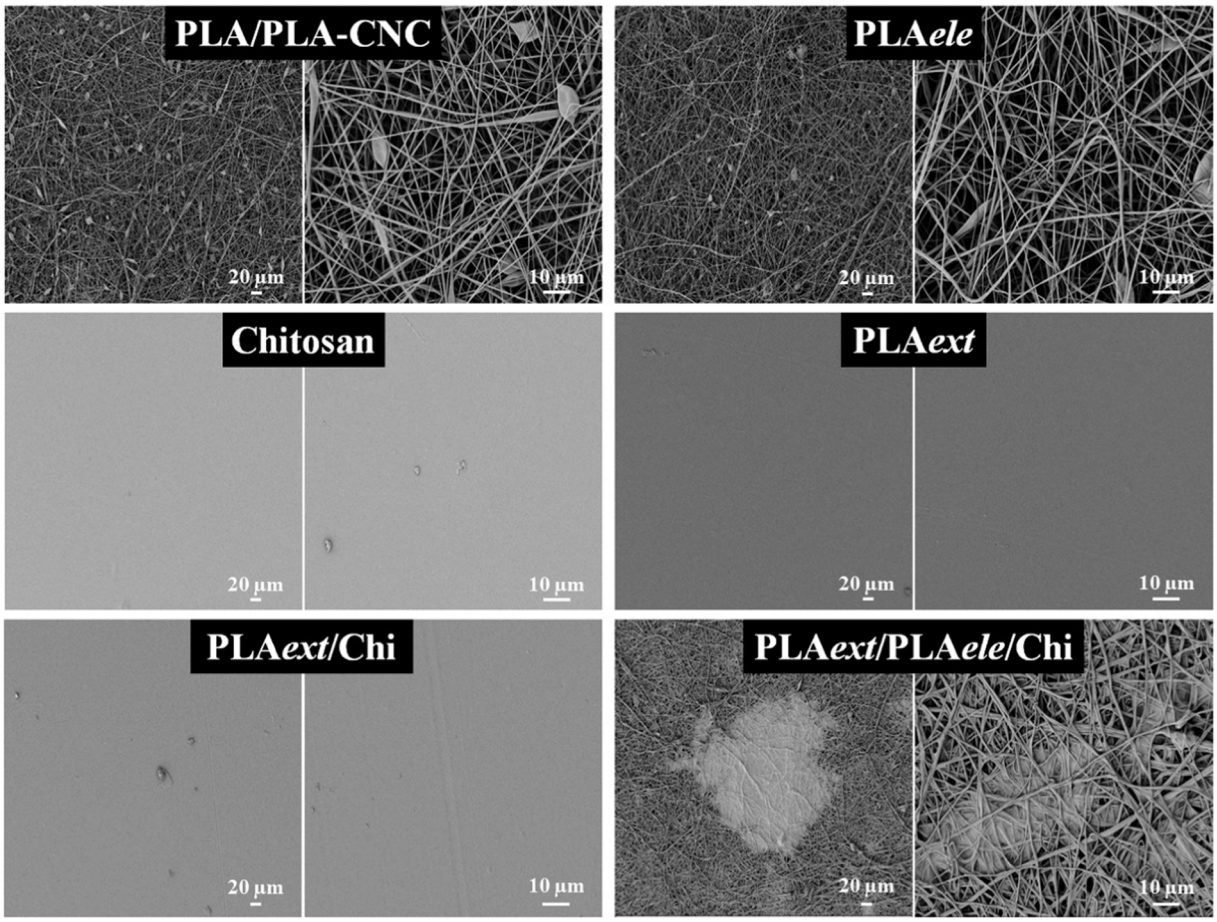

Fig. 1. FESEM micrographs of material surfaces obtained with at 500 × and 2500 × magnification.

process of multilayer materials including their thickness, alignment of the fibers and the compactability of material. A rotatory collector produces electrospun mats with a highly homogeneous thickness and a compacted structure due to the better alignment of the fibers (Angel et al., 2022). On the contrary, the collector plate favors the obtaining process of heterogeneous thicknesses in the materials since fibers are largely deposited in the middle of the collection zone by effect of Taylor's Cone. This phenomenon occurs because the high electric field applied to the polymeric solution exposed in the tip of the capillary generates its stretching. This fact in turn favors the formation of a jet that adopts the shape of a conical structure named "Taylor's cone" as it is stretched, where the solvent is evaporated and finally the fibers are deposited in the collector (Velásquez et al., 2021). Furthermore, this type of collector produces a random deposition of the fibers (Wang et al., 2022). In this context, the use of the plate collector for coating the PLA*ext* material with the fibers produced different deposition grades, where the zones with lower amount of collected electrospun fibers favored their coating with chitosan. Therefore, the spaces generated between the fibers due to the random collection were filled by chitosan during the coating process, avoiding the formation of a smooth and homogeneous surface.

3.2. Water contact angle measurements

The evaluation of wettability of a plastic surface, usually evaluated through contact angle measurements (Ruzi et al., 2022), is considered a valuable tool for food packaging materials in order to establish the response of a polymeric film to highly wet products or through the exposure to high humidities along the food chain (Iglesias-Montes, Luzi, et al., 2021). Because the interactions between the packaging material and foodstuff can have a major effect on product quality, the performance of materials with liquid over time under certain environmental conditions has to be monitored to control their hydrophobic/hydrophilic behavior. Therefore, the wettability of PLA*ext*/-PLA*ele*/Chi trilayer material and their different layers was evaluated by water contact angle measurements (WCA), and their values are shown in Fig. 2.

All surfaces of materials exhibited a hydrophobic behaviour since WCA values were higher than 74° (Ezati & Rhim, 2020). Electrospun PLA/PLA-CNC and PLA*ele* materials exhibited the highest WCA values of nearly 120° (Figs. 2A and 2B). These values evidenced that CNC were mainly embedded within the shell of coaxial fibers since the high hydrophilic character of nanoparticles did not modify the intrinsic WCA value of electrospun PLA (approx. 120°) (Shen et al., 2021). A similar phenomen was also reported by Zhang et al. (2015) when PLA electrospun fibers were loaded with CNC at 1 wt% and 5 wt%. Furthermore, because LAE is a cationic surfactant, the reduction of the diameter of the fibers and an increase of their roughness could be favored, contributing also to the increase of the hydrophobicity in the surface of PLA*ele* material (Li et al., 2021; Szewczyk et al., 2018).

As Fig. 2C shows, chitosan film presented a WCA value of 75° in accordance with a recent study (Infurna et al., 2022). Furthermore, this value was statistically similar to WCA value of PLA*ext* material (73°), as is shown in Fig. 2D. The hydrophobic nature of PLA films obtained through extrusion or casting have been also evidenced in previous studies (Roy & Rhim, 2020; Villegas et al., 2019). On the other hand, the chitosan coating over PLA*ext* material produced an increase of water contact angle (Fig. 2E). The WCA value of 94° from PLA*ext*/Chi material indicated that the surface of chitosan exhibited a higher hydrophobic character compared with chitosan film (Fig. 2C). This fact was probably

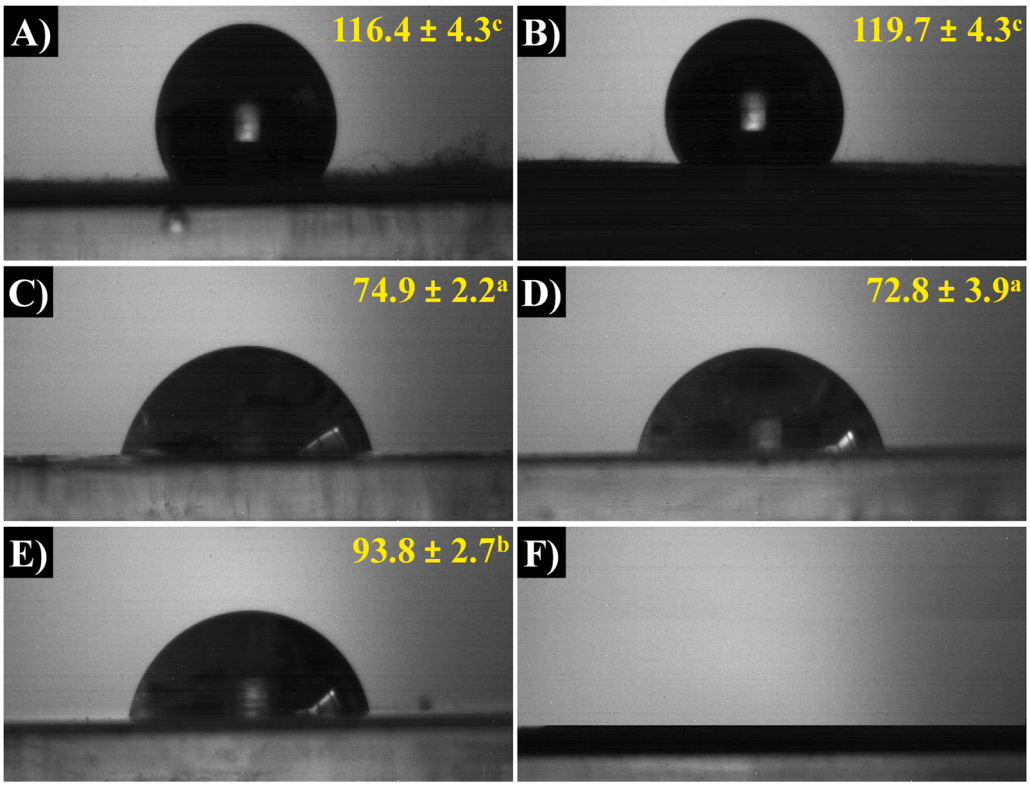

Fig. 2. Photographs of WCA analysis of: A) coaxial PLA/PLA-CNC fibers, B) antibacterial PLA*ele* layer, C) chitosan film; D) PLA*ext*, E) PLA*ext*/Chi, and F) PLA*ext*/PLA*ele*/Chi. Lower case letters a–c indicate significant differences among materials ($p < 0,05$) according to ANOVA analysis and Tukey's test.

associated to the heterogeneity of the bilayer surface and the swollen superficial deformation of chitosan layer since the water drop could have produced a swelling effect of this layer. The swollen chitosan layer was able to increase the drop volume and result on high WCA value (Kurek et al., 2014). This phenomenon has been also evidenced in previous studies when different materials were coated with polymers of hydrophilic nature. Recently, the coating of a PLA film with an active solution composed by gelatine, sodium alginate and inclusion complexes produced a WCA value from 72° (uncoated PLA film) to 86°. This fact was associated to strong interaction forces existing between the polymers (Chen et al., 2021). Similarly, the coating of a polyethylene film with chitosan also produced a high WCA value (Al-Naamani et al., 2016).

As Fig. 2F shows, the WCA analysis of *PLAext*/PLA*ele*/Chi material was not measurable since the drop was rapidly absorbed on the surface. In this case, the heterogeneous coating of chitosan over PLA*ele* material (shown in Fig. 1) possibly affected the analysis. Thus, the sites generated between the electrospun fibers and not occupied by the chitosan layer could have favored a "soak" of water drop between PLA*ele* and chitosan layers.

3.3. Antibacterial activity of trilayer material

3.3.1. Liquid medium

The antibacterial activity of PLA*ext*/PLA*ele*/Chi trilayer material was evaluated in liquid medium against *S. enterica* and *L. innocua* as Gram(−) and Gram(+) bacteria, respectively. The trilayer material was put in contact with bacterial solutions for 15 days at 4 °C in order to simulate its antibacterial protection towards foodstuck with high relative humidities and stored under refrigeration conditions.

Fig. 3A shows a similar behaviour in the growth curves of *L. innocua* with CNT and PLA*ext* samples. Latency phase of initial inoculum was maintained for 24 h, and posteriorly, microorganism growth exponentially until day 10. Finally, the microbial growth was slower reaching the stationary phase around 15 days.

On the other hand, chitosan in PLA*ext*/Chi material showed an inhibitory effect against *Listeria* microbial growth that was extended during the analysis period (15 days). Fig. 3A manifests an initial bacterial reduction of 1 log after 72 h by the bilayer material, reaching the maximum effectivity after 10 days with 3 log reduction. This antibacterial effect shown by chitosan occurred because this biopolymer is able to bind to the bacterial membrane through electrostatic attraction. This fact in turn modificates the properties of bacterial membrane, inhibiting the metabolism of cell and producing its death (Esmaeili et al., 2021; Xavier et al., 2021).

PLA*ext*/PLA*ele*/Chi material mainly exhibited a strong antibacterial activity inhibiting 3 log after 2 h followed by bactericide effect after 6 h, as can be seen in Fig. A1 in Supplementary material, Appendix A. This result was associated to the amount of released LAE from the coaxial electrospun fibers to the liquid medium, as well as the presence of chitosan (Patiño Vidal et al., 2022). Therefore, the synergic effect of antibacterial property of LAE and chitosan favored a fast bactericidal effect against this microorganism. Likewise the chitosan, LAE is able to interact electrostatically with the bacterial cell due to the cationic surfactant behavior. Therefore, the antimicrobial compound is able to generate disruption and instability in the cell envolve, causing finally the death of microorganism (Haghighi et al., 2019; Ma et al., 2020).

On the other hand, Fig. 3B shows that the latency phase of initial inoculum of S. *enterica* with CNT, PLA*ext* and PLA*ext*/Chi was maintained along the test. PLA*ext*/Chi material also showed antibacterial activity against *S. enterica*, but this effect was lower than against *L. innocua*. This pathogen is a mesophilic bacterium so the microbial growth was inhibited at refrigeration temperatures retarding the latency phase, although *Listeria* is a psychrotrophic microorganism able to growth a 4 °C (Settier-Ramírez et al., 2021). PLA*ext*/Chi material inhibited 1 log after 15 days of refrigeration storage. A higher antibacterial effect of chitosan against *L. innocua* was associated to a higher susceptibility of this type of bacteria. This fact was explained because

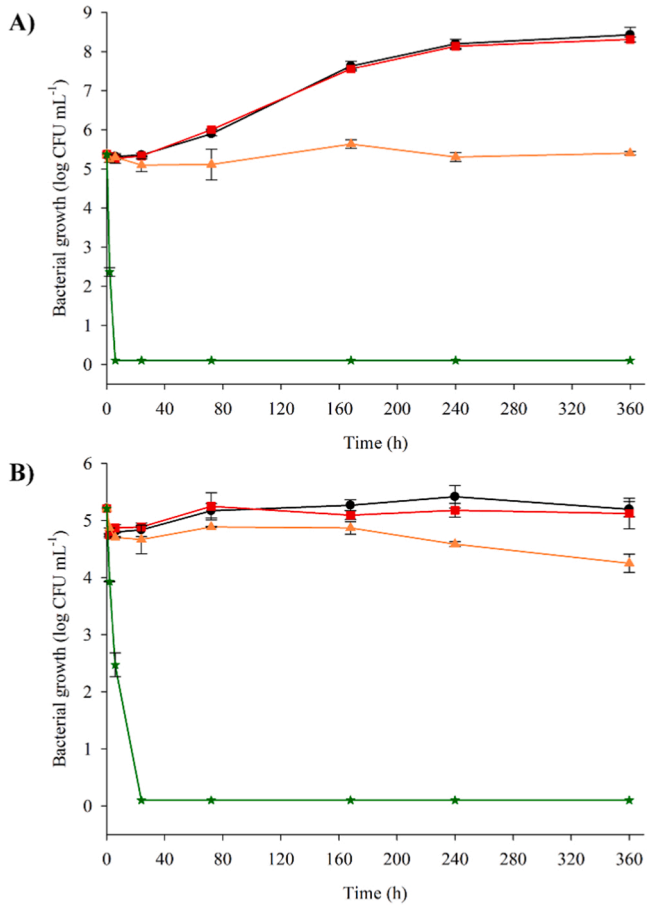

Fig. 3. Growth curves of *L. innocua* (A) and *S. enterica* (B) in liquid medium for 15 days at 4 °C with samples: CNT: black circes, PLA*ext*: red squares, PLA*ext*/Chi: orange triangles and PLA*ext*/PLA*ele*/Chi: green stars.

the cell wall of Gram(+) bacteria is composed by a thick peptidoglycan layer and teicoic acid. Therefore, in the presence of chitosan, the chain of teicoic acid of bacteria was negatively charged and interacted through electrostatic attraction with positively charged chitosan chains. Thus, the properties of bacterial membrane were modified and the microorganisms died (Li & Zhuang, 2020). Besides, microorganisms are more resistant in latency phase than in exponential phase, and this fact justified the higher susceptibility of *L. innocua* in the exponential phase than that of *S. enterica* (Li & Zhuang, 2020).

Analogously, the antibacterial effect of PLA*ext*/PLA*ele*/Chi material against *S. enterica* was lower than towards *L. innocua*. The growth curve of *S. enterica* evidenced a reduction of 1 log after 2 h, followed by a total inhibition of bacterial growth after 24 h (see Fig. A2 in Supplementary material, Appendix A). As it was above mentioned, this difference between the type of bacteria are probably due to their different cellular structures and microbial growth at 4 °C. Furthemore, the bactericide effect of the trilayer material against *L. innocua* due to the synergic effect of released LAE and chitosan was also exhibited against *S. enterica* after 24 h.

Several studies about biodegradable films and coatings containing LAE have also demonstrated the strong antimicrobial activity of this compound. For instance, Apicella et al. (2019) prepared bilayer materials composed by a poly(ethylene terephthalate) film substrate coated with PLA loaded at 5, 10 and 20 wt% LAE were tested against *Escherichia coli*. The films were put in contact with the bacterial solution overnight, and a total bacterial inhibition growth occurred with the highest LAE concentration. Likewise, Silva et al. (2019) developed cellulose nanofibrils films that contained LAE at 0.5, 1, 5 and 10 wt% and were put in contact with *E. coli*, *Listeria monocytogenes* and *Salmonella Typhymurium* for 24 h. The results evidenced the strong antibacterial activity of LAE since a total inhibition of bacterial growth was obtained when the antimicrobial compound was added at 1 wt%. Recently, Gracia-Vallés et al. (2022) incorporated a complex based on keggin-type polyoxometalate and different concentrations of LAE (0.1, 0.5, 1, 5 and 10 wt%) into carboxymethyl cellulose films afforded high antibacterial activities. A bacterial reduction around 7 log for *E. coli* and *L. monocytogenes* was obtained with the active films contained the highest concentrations of LAE. Also, Muriel-Galet et al. (2015) developed active ethylene vinyl alcohol (EVOH) films loaded with LAE at 5 and 10 wt% and tested these against microbiota from chicken and surimi sticks. The direct contact between the active films and the microbiota during 10 days at 4 °C promoted a reduction of 3–7 log for aerobic bacteria, 3 log for *Pseudomonas*, 3 log for lactic acid bacteria and 6 log for psychrotrophic bacteria.

3.3.2. Solid medium

The bacteriostatic or bactericidal effect afforded by PLA*ext*/PLA*ele*/Chi material was evaluated in solid medium against the same bacteria used in liquid medium. The inhibition halo obtained after the contact between bacteria and materials for 24 h at 37 °C is shown in Fig. 4. The bacteriostatic or bactericidal effect of trilayer material was determined through the measurement of inhibition halo produced after the materials were removed and incubated at 37 °C for 24 h.

PLA*ext* and PLA*ext*/Chi materials did not show antibacterial effect against both bacteria because high molecular weight chitosan acted only through direct contact with the bacteria cells, and therefore, the nearby cells were not affected, as it was observed for trilayer material. Therefore, the inhibition halo was not observed.

On the other hand, the antibacterial activity exhibited by PLA*ext*/PLA*ele*/Chi material against both bacteria was directly related to the LAE released from the material and diffused in agar medium. Likewise, a higher antibacterial effect was observed against *L. innocua* compared to *S. enterica*. As it was mentioned in the liquid medium test, the different cellular structure of both bacteria favored this result (Li & Zhuang, 2020). Furthemore, Fig. 4 shows the bactericidal effect of LAE released from trilayer material against both bacteria. This fact was evidenced since there was not growth of microorganisms in the sites of agar where LAE diffused. Therefore, the inhibition halo determined to *L. innocua* (3 mm) and *S. enterica* (2.7 mm) were maintained after the second incubation of microorganisms showing bactericide effect.

Previous works about packaging materials loaded with LAE have also evidenced the antibacterial effectivity of this compound against bacterial concentrations around 10^6 CFU mL^{-1} (Gracia-Vallés et al., 2022; Kim & Park, 2016; Patiño Vidal et al., 2021). For example, chitosan/polyvinyl (alcohol) films loaded with LAE were able to inhibit the growth of *L. monocytogenes*, *E. coli*, *S. typhymurium* and *Campylobacter jejuni*. Inhibition halos between 0.5 and 5.2 mm were obtained after the direct contact between the active films and the bacteria (Haghighi et al., 2020). Similarly, inhibition halos between 16 and 24.3 mm for *L. monocytogenes*, *E. coli*, *S. typhymurium* and *C. jejuni* were reached through the contact of active films of chitosan/gelatin containing LAE with these microorganisms (Haghighi et al., 2019). Chitosan/polyethylene oxide nanofibers loaded with LAE also exhibited a strong antibacterial activity against *Staphylococcus aureus* and *E. coli*, reaching inhibition halos between 18.3 and 29.2 mm (Deng et al., 2018).

3.4. Disintegration under composting conditions of trilayer material

The disintegration under composting conditions is a decomposition process of organic matter carried out by microorganisms to carbon dioxide, water and heat. Therefore, the result of this process is a soil enriched with nutrients favorable for the growing of plants (Iglesias-Montes, Soccio, et al., 2021). In the case of plastic materials based on PLA and chitosan, they are converted to small fragments during this

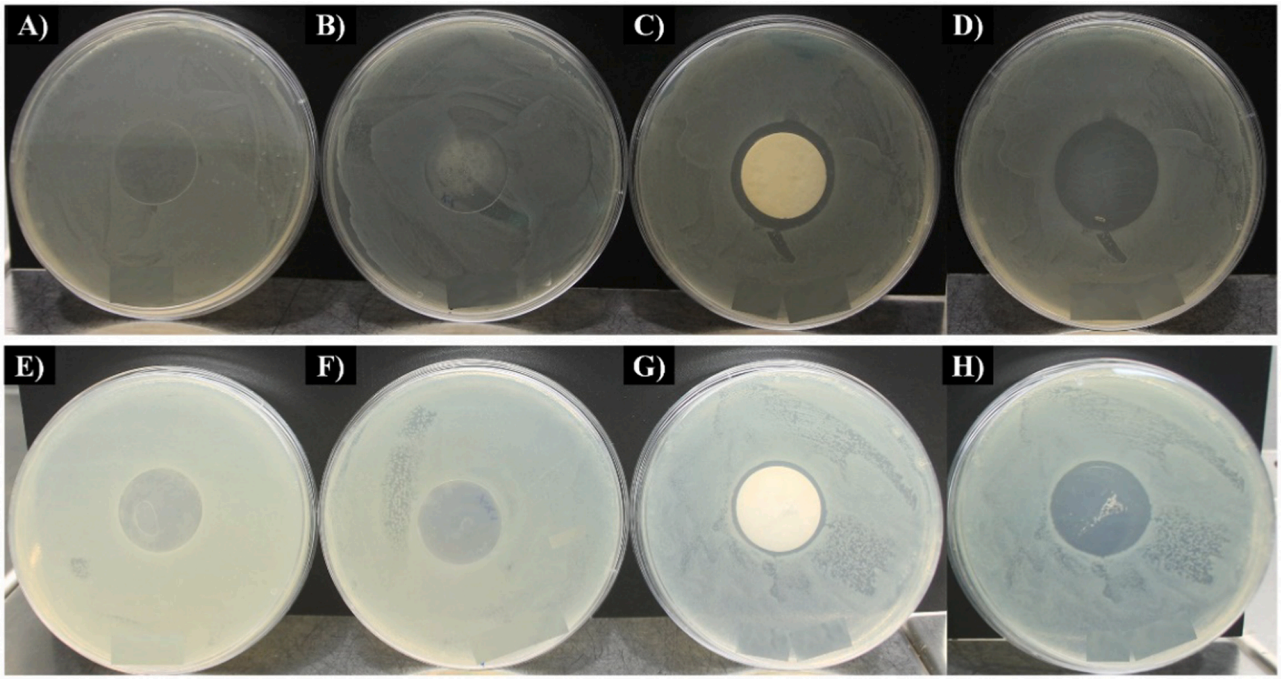

Fig. 4. Photographs of petri dishes containing *L. innocua* (up) and *S. enterica* (down) in contact with: PLA*ext* (A and E), PLA*ext*/Chi (B and F) and PLA*ext*/PLA*ele*/Chi (C and G) materials and incubated in solid medium. D) and H) correspond to photographs of petri dishes containing *L. innocua* and *S. enterica* after PLA*ext*/PLA*ele*/Chi material was removed and dishes were incubated.

natural process (Weligama Thuppahige & Karim, 2022).

The biodegradation of PLA starts with the water diffusion through the polymeric matrix during the first two weeks. This phenomen produces a non-enzymatic hydrolysis of the polymer that reduces its molecular weight as result of random chain scissions through their ester groups (Iglesias-Montes, Soccio, et al., 2021; Shah et al., 2008). Subsequently, oligomers and lactic acid obtained from the fragmentation of the polymer are assimilated by microorganisms to be finally converted to carbon dioxide and water (Kalita et al., 2019). On the contrary, the biodegradation of chitosan is mainly produced through enzymatic hydrolysis by action of lysozyme and some chitinases produced by microorganisms (Dash et al., 2011; Kean & Thanou, 2010). This process begins with the random breaking of β-1,4 glycosidic bonds (depolymerization) followed by the hydrolysis of N-acetyl bonds (deacetylation). This fact produces a reduction of the molecular weight of chitosan and the increase of its deacetylation grade. Simultaneously to this stage, the scission of amine, amide, carbonile and hydroxyl groups can also occur. Finally, the structure of polymer is modified, and thus, its physico-chemical properties are lost (Matica et al., 2017).

On the other hand, the biodegradation process of polymeric materials can be affected by: i) the characteristics of the polymer (crystallinity, composition, molecular weight), ii) the environment conditions (temperature, humidity), and iii) the presence of additives. Therefore, the effect of the presence of LAE and the incorporation of different layers on the disintegration of PLA*ext*/PLA*ele*/Chi trilayer material under composting conditions was evaluated through macroscopic changes (color, size and texture), disintegration level or loss weight and morphological properties.

3.4.1. Macroscopic changes and disintegration grade of developed materials

The physical changes occurred during composting of all materials can be observed in Fig. 5A. Photographs indicated in all materials evidenced homogeneous structures without visible breaks at initial test time (day 0). Furthermore, an obvious transparency was also observed in the samples, except for PLA/PLA-CNC fibers, PLA*ele* and PLA*ext*/PLA*ele*/Chi materials that exhibited a white color due to the electrospun mat.

Chitosan film was the sample that presented the fastest disintegration process, evidencing a total fragmentation and brown coloration at day 1 followed by the total disintegration at day 7. The hydrophilic nature of this polymer and the high humidity of the synthetic solid waste produced the swelling of this material and its change to a gelly structure, favoring its fast disintegration. Two recent studies also observed such physical changes on chitosan-based materials during the first three days of test (Bonilla & Sobral, 2020; Oberlintner et al., 2021). As Fig. 5A shows, electrospun PLA/PLA-CNC and PLA*ele* fibers exhibited several physical changes, such as brown coloration, reduction of size, increase of roughness and swelling since the first day of composting. At day 7, PLA/PLA-CNC fibers showed a high fragility and the presence of minimum surface cracks, obtaining a notorious fragmentation at day 10 followed by total degradation after 14 days of test. On the contrary, the presence of LAE into PLA*ele* material delayed its degradation. This fact was evidenced because physical changes obtained in PLA/PLA-CNC were also shown by PLA*ele* after 10 days. Furthermore, the total degradation of this antibacterial layer was reached after 21 days of incubation. This result could have occurred because LAE was released from electrospun material to the synthetic solid waste favored by the high amount of available water in the medium and the test temperature nearly to PLA glass transition (approx. 55–65 °C) (López de Dicastillo, Garrido, et al., 2021; Velásquez et al., 2019). Thus, the strong antimicrobial activity of LAE at low concentrations probably slowed down the action of the microorganisms from the solid waste (Patiño Vidal et al., 2021). The incorporation of antimicrobial compounds as umberifellone or limonene into PLA-based materials have also delayed the polymer degradation (Arrieta et al., 2014; Iglesias-Montes, Luzi, et al., 2021).

PLA*ext* and PLA*ext*/Chi materials suffered similar physical changes during the test. Whitening of extruded layer of both materials was exhibited at first day. This fact was associated to the water intake principally at the amorphous sites of polymeric matrix, favoring thus a increase of the opacity (Iglesias-Montes, Soccio, et al., 2021). At the third day, the bilayer PLA*ext*/Chi material exhibited a brown coloration associated with the start of chitosan degradation. After 7 days of

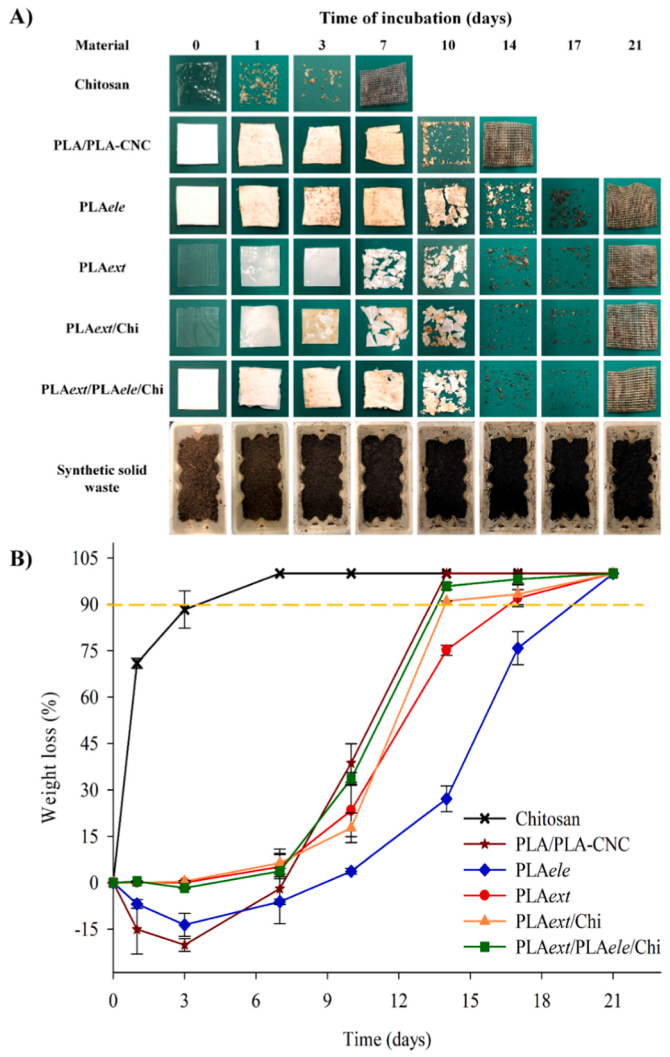

Fig. 5. A) Photographs of physical changes obtained during the disintegration process of materials under composting conditions, and B) Curves of weight loss of chitosan: black crosses; coaxial fibers PLA/PLA-CNC: brown stars; antibacterial layer PLA*ele*: blue diamonds; and materials PLA*ext*: red circles; PLA*ext*/Chi: orange triangles, and F) PLA*ext*/PLA*ele*/Chi: green squares.

composting, both materials exhibited a reduced thickness, increased fragility and fragmentation followed by the total degradation after 21 days.

The trilayer PLA*ext*/PLA*ele*/Chi material presented physical changes similar to PLA*ext* and PLA*ext*/Chi materials at the same time of incubation. This fact evidenced that LAE did not delay the degradation of PLA and chitosan in the trilayer material. Two recent studies also confirmed the total disintegration of PLA films after 21 days of composting (Beltrán et al., 2021; Cerro et al., 2021). On the other hand, Fig. 5A also shows a color change of synthetic solid waste along the test. The progressive darkening from the first day concurred with the degradation process of materials. The disintegration in turn favored the presence of some compounds and chemical elements responsible for the color change (Wei et al., 2017).

Fig. 5B shows the weight loss of materials and the objective of the disintegration test represented by the dotted yellow line. The highest weight loss of approx. 71% was evidenced by the chitosan film at the first day. This fact demonstrated its fast disintegration process that was reached at the third day of test. On the contrary, electrospun materials PLA/PLA-CNC and PLA*ele* exhibited two different behaviors. As Fig. 5B shows, an increase of weight of both materials associated to the capability of sorption of water and other components from the synthetic solid waste by these polymeric matrixes was observed until the day 7. After 10 days, PLA/PLA-CNC fibers lost approx. a 39 % of its weight, and it was totally disintegrated after 14 days. The effect of delay in the disintegration of PLA*ele* material by the presence of LAE observed in the above analysis was confirmed with a minimum weight loss of 3 % at day 10, reaching the objective of 90 % disintegration after 21 days.

Monolayer PLA*ext*, bilayer PLA*ext*/Chi and trilayer PLA*ext*/PLA*ele*/Chi materials began their disintegration processes at day 7. Monolayer and bilayer materials showed a similar weight loss (approx. 6 %), however, LAE contained in the trilayer system reduced this value to 4 %. All materials exhibited different behaviours after 10 days of incubation. PLA*ext* material lost 23 % of weight and this value was reduced to 18 % by the presence of chitosan film in the bilayer PLA*ext*/Chi. This result could be associated to the intrinsic antimicrobial activity of chitosan that favored the delay of the disintegration of the bilayer material.

Two recent studies have also evidenced a similar behavior when water-insoluble and antimicrobial nanoparticles have been incorporated into PLA films. In both works, lignin nanoparticles delayed the disintegration of this nanocomposite and researchers linked this phenomen to intrinsic characteristic of nanoparticles (Cerro et al., 2021; Iglesias-Montes, Luzi, et al., 2021). On the contrary, the presence of PLA*ele* layer and chitosan coating in the trilayer PLA*ext*/PLA*ele*/Chi favored an increase of weight loss of 31 % after 10 days. In this case, the result was associated to the heterogenous coating of chitosan over the electrospun layer that was already previously confirmed through FESEM analysis (Fig. 1). Thus, the non-coated zones in the material surface allowed an easy entrance of water molecules, accelerating the hydrolysis process.

After 14 days of incubation, PLA*ext*/Chi and PLA*ext*/PLA*ele*/Chi materials reached approx. 91 % weight loss, while PLA*ext* material only lost 75 % weight. The disintegration of monolayer material was extended until day 17. These results confirmed the above mentioned about the biodegradation process of PLA, that normally begins with the non-enzymatic hydrolysis of polymer during the first two weeks of test.

3.4.2. *Morphological analysis (FESEM)*

A comparison of morphologies between non-disintegrated and disintegrated materials after 10 days of disintegration test is shown in Fig. 6. The chitosan film was not analyzed due to its fast and total degradation reached at day 7.

PLA/PLA-CNC and PLA*ele* electrospun materials showed uniform fibers at day 0. After 10 days of disintegration test, PLA/PLA-CNC fibers presented a high degree of self-agglomeration probably as a result of loss of their morphology during the beginning of PLA hydrolysis (Arrieta et al., 2016). Furthermore, these structures lost uniformity and the presence of cracks and holes in their surfaces was evidenced. On the contrary, antibacterial coaxial fibers of PLA*ele* material were minimally agglomerated and their morphology was maintained.

Likewise, the presence of little holes in their surface was also observed. These observations even more confirmed the delay of disintegration of PLA by the effect of LAE already previously mentioned in Section 3.4.1.

PLA*ext* and PLA*ext*/Chi materials displayed smooth and homogeneous surfaces before the disintegration test. However, big cracks and deep fractures in both materials appeared after 10 days of test. Furthermore, as Fig. 6 shows, residual bacteria and ovoid holes were also detected in the surface of PLA*ext* and PLA*ext*/Chi materials, respectively. These observations were associated to the hydrolytic degradation of materials that favored the break of the chains in the inner structure of the polymeric matrix. In addition, the presence of superficial cracks was caused by the water diffusion in the amorphous zones of the polymer and the migration of low molecular weight compounds to the solid waste (Iglesias-Montes, Soccio, et al., 2021; Luo et al., 2012). Some studies have also observed that PLA-based materials experienced these changes after they were subjected to disintegration process under

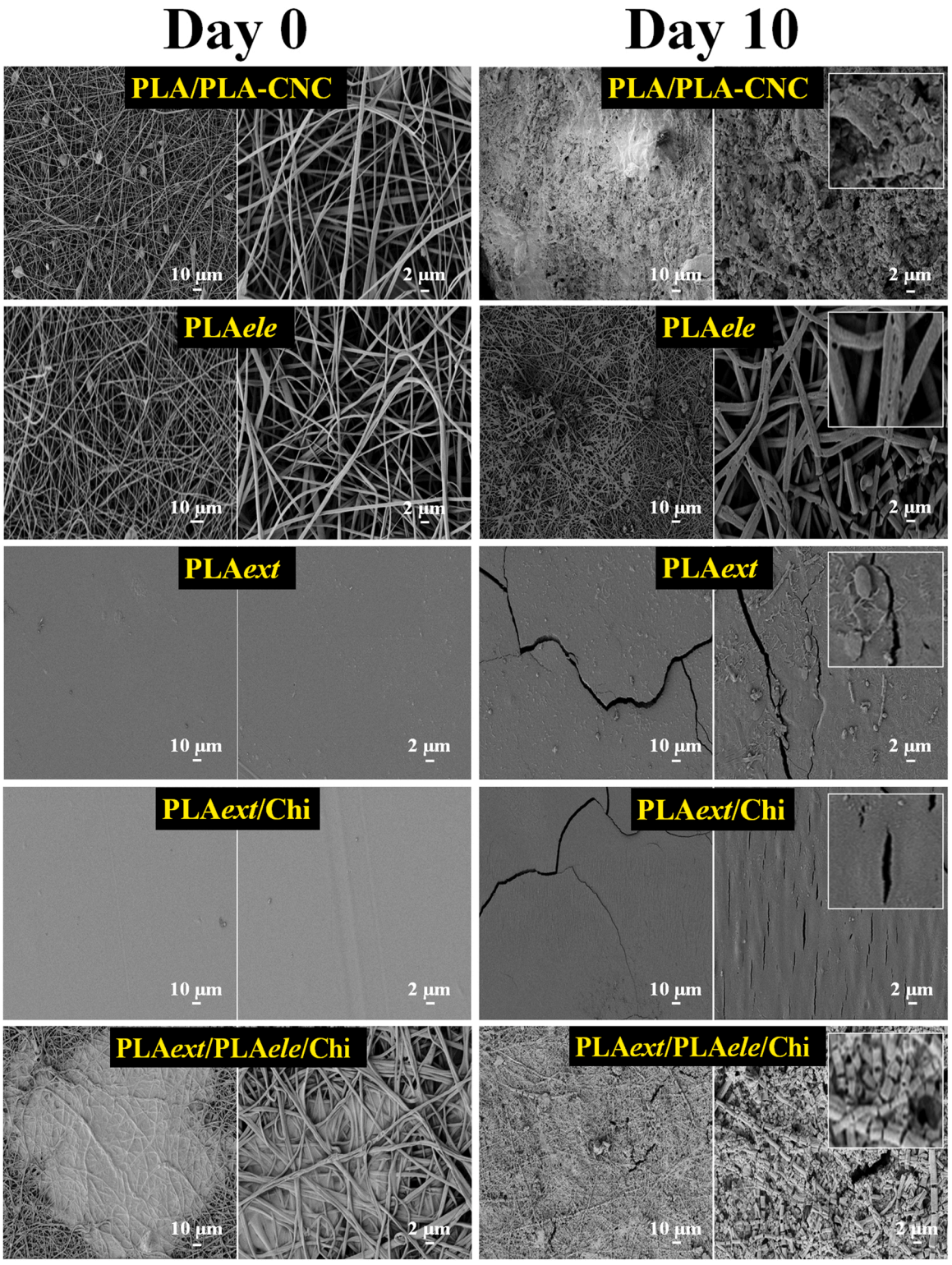

Fig. 6. FESEM micrographs of the surface of materials before and after of disintegration process under composting conditions.

composting conditions (Fortunati et al., 2014; Yang et al., 2015).

Specifically, the trilayer PLA*ext*/PLA*ele*/Chi material manifested a non-homogeneous surface at day 0. After 10 days, small cracks in the surface of this material were also evidenced. Additionally, agglomerated, non-uniform and sliced fibers were also detected during the analysis. These facts resulted from the presence of chitosan and its non-homogenous coating over the PLA*ele* layer that possibly favored a high and fast plasticizing effect of electrospun layer. Therefore, the water could be easily diffused into electrospun polymeric matrix and accelerated the disintegration process. This effect was not noticed by PLA*ele* material since the integrity of the fibers was practically fully maintained after 10 days of composting conditions.

4. Conclusions

A biodegradable trilayer material with a strong antibacterial

property was satisfactorily developed by combining the extrusion, electrospinning and coating techniques. Trilayer material exhibited a non-smooth and non-homogeneous surface, and this fact negatively affected its surface properties since the wettability could be not determined. Trilayer material exhibited a strong and fast bactericidal effect against Gram(+) and (−) bacteria that was maintained for 15 days. Furthermore, the presence of LAE into electrospun layer did not delay the degradation of the trilayer material if it is compared with the control sample PLA*ele*. However, the presence of the chitosan layer in the bilayer material PLA*ext*/Chi favors a delay in the disintegration of PLA. Thus, this polymeric material could be used for the development of a sustainable antibacterial package oriented to protect dairy and meat products.

CRediT authorship contribution statement

Cristian Patiño Vidal: Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Francesca Luzi:** Methodology, Validation, Formal analysis. **Debora Puglia:** Conceptualization, Writing – review & editing, Supervision. **Gracia López-Carballo:** Methodology, Formal analysis, Writing – original draft. **Adrián Rojas:** Writing – original draft. **María José Galotto:** Resources. **Carol López de Dicastillo:** Conceptualization, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Conflicts of interest

The authors declare no conflict of interest.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fpsl.2023.101050.

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