UNIVERSITAT
POLITÈCNICA
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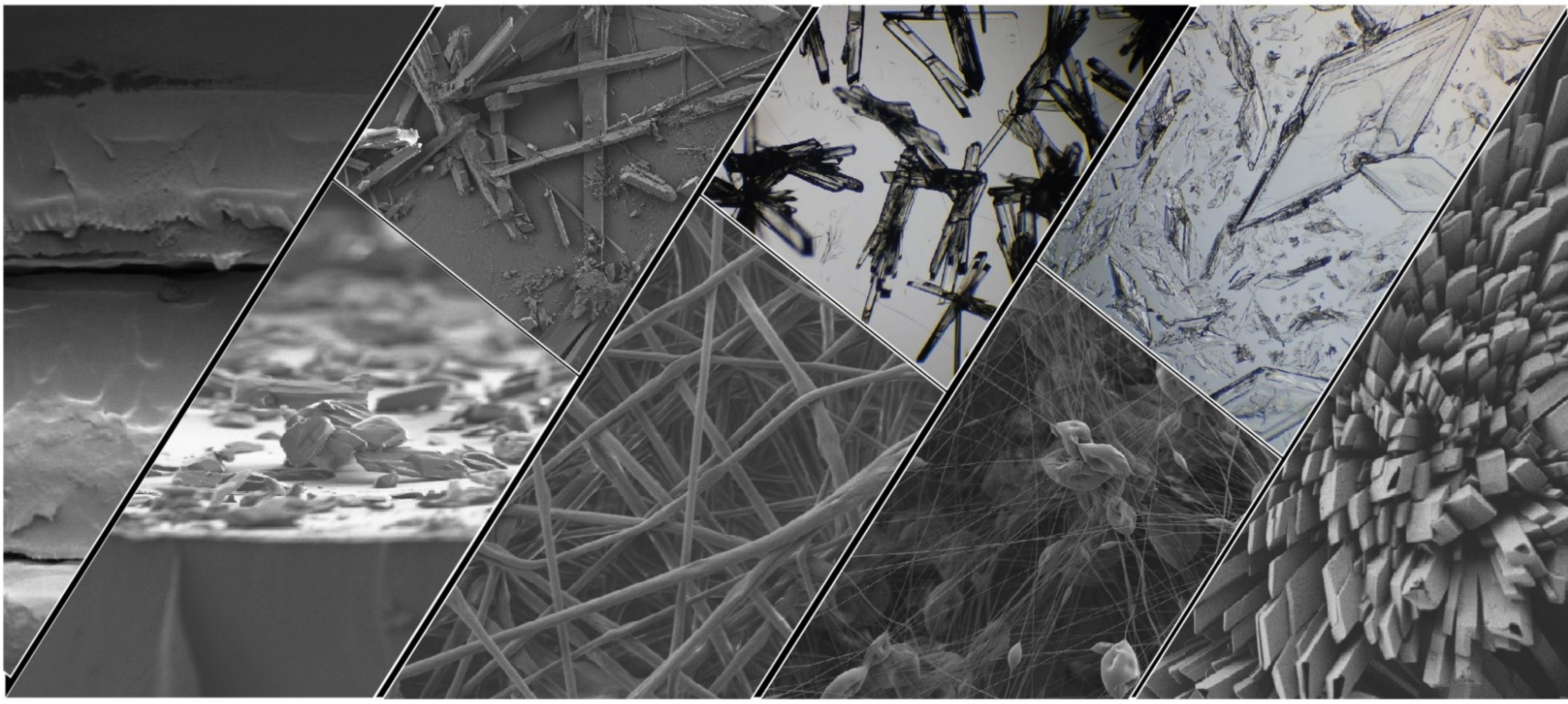
DOCTORAL THESIS

USE OF FERULIC AND CINNAMIC ACIDS TO OBTAIN ACTIVE FILMS BASED ON STARCH AND PLA FOR FOOD PACKAGING APPLICATIONS



Presented by:
Ramón Ordoñez Lagos

Supervisors:
Amparo Chiralt Boix
Lorena Atarés Huerta

Valencia, June 2022

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INSTITUTO UNIVERSITARIO DE INGENIERÍA DE ALIMENTOS PARA EL DESARROLLO

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Hacen constar que:

La memoria titulada: **"USE OF FERULIC AND CINNAMIC ACIDS TO OBTAIN ACTIVE FILMS BASED ON STARCH AND PLA FOR FOOD PACKAGING APPLICATIONS"** que presenta Dⁿ **Ramón Alberto Ordoñez Lagos** para optar al grado de Doctor por la Universitat Politècnica de València, ha sido realizada en el Instituto Universitario de Ingeniería de Alimentos para el Desarrollo (IIAD) bajo su dirección y que reúne las condiciones para ser defendido por su autor.

Valencia, marzo de 2022

Fdo. Amparo Chiralt Boix

Fdo. Lorena Atarés Huerta

A Vilma & Héctor,

"Las buenas cosas cuestan…"

Agradecimientos

Hace mucho tiempo me propuse la meta de obtener un doctorado, nunca imagine el camino que recorrería y ni los obstáculos que enfrentaría, hoy ese sueño se cristaliza con este trabajo. Viendo en retrospectiva solo puedo pensar, que hermosa vista desde las cimas de las montañas. Aunque este logro requirió mucho esfuerzo de mi parte, he tenido la dicha de encontrar muchas personas especiales en cada etapa de mi vida. Ustedes me han hecho quien soy, me han enseñado, apoyado y marcado de muchas maneras, hoy quiero agradecerles por ayudarme a alcanzar mi meta y sobre todo por formar parte de este mi viaje.

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Este ha sido un viaje largo y a veces difícil, sin embargo, estoy seguro de que lo volvería a hacer mil veces. El doctorado ha sido una experiencia única para desarrollarme profesionalmente, aprender y fascinarme con la ciencia como nunca, pero aún más que eso ha sido una oportunidad increíble para crecer como persona. No soy capaz de nombrar a tantas personas que me han apoyado y marcado en este camino, pero puedo decirles a todos, me debo a ustedes, les deseo lo mejor.

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Abstract

Food packaging waste is a major contributor to the current environmental crisis caused by conventional plastic. Therefore, the food industry needs more sustainable environmentally friendly packaging solutions, such as using biodegradable and active materials to extend the food shelf-life. In this sense, the present doctoral thesis aims to obtain active films based on starch and PLA by incorporating ferulic or cinnamic acids as active compounds, in order to develop multilayer assemblies useful to meet food packaging requirements, with antibacterial capacity to extend the product shelf life. First, monolayer films of starch or PLA with active compounds were studied and finally multilayer films combining starch and PLA sheets were designed.

The incorporation of ferulic and cinnamic acids into cassava starch matrices through melt-blending process, at 1 and 2% w/w, promoted the film plasticization, probably due to the partial hydrolysis of polymer during the melt-blending process. This enhanced the film plasticity and stretchability, decreasing its stiffness and resistance to break. Acid incorporation did not improve the barrier properties of the films, but significantly reduced their water solubility. The analyses of the antibacterial activity of the films against *E. coli* and *Listeria innocua* strains, in culture medium, revealed that films with cinnamic acid were more effective at inhibiting the bacterial growth, and that *L. innocua* was more sensitive to both acids than *E. coli.*

When ferulic and cinnamic acids were incorporated into PLA matrices at 1 and 2% w/w by melt blending, the functional properties of the films were not greatly affected. However, the in vitro antimicrobial tests with *L. innocua* and *E. coli*, did not show antibacterial action, which could be attribute to the lack of effective release of the compounds from the PLA matrix. Release studies showed very limited release of the ferulic and cinnamic acids from the PLA matrix due to the glassy state of polymer matrix and its hydrophobic nature that limits the polymer swelling and relaxation in contact with aqueous media, such as many food matrices. Therefore, in order to promote the release of active compound from the PLA matrix, different strategies were analysed: 1) plasticizing the thermoprocessed films with PEG 1000, 2) increasing the concentration of the compounds in the film, 3) processing the films by casting and 4) anchoring the active compounds to the film surface. The superficial incorporation was carried out by adsorption of the actives from the hydro-ethanolic solutions of the active compounds, by spraying active ethanolic solutions on the film surface, or coating the films by electrospinning of polymer-active solutions, using compatible solvent mixtures.

Only the surface incorporation of ferulic or cinnamic on PLA films gave rise to significant bacterial growth inhibition. Pulverization of PLA films with ferulic or cinnamic acid ethanol solutions allows the formation of an overconcentrated layer of active compounds on the films with crystalline formations that led to an adequate release of actives to inhibit the bacterial growth. PLA-acid solutions, using blends of ethyl acetate

(E) with dimethyl sulfoxide (DMSO) or glacial acetic acid (A), were electrospun on the film surface. Structural differences of the obtained mats, associated to the solvent system, determined the antimicrobial power of the material. DMSO systems produced fibre-structured mats with high specific surface that significantly inhibited bacterial growth, whereas acetic acid systems produce bead-based mats that not had antimicrobial activity.

The surface incorporation of the active compounds was considered for obtaining three-layered films PLA/starch/PLA (PSP) with improved functional properties and antimicrobial capacity. Superficially loaded films by both electrospinning or pulverisation, showed effective growth inhibition of *E. coli* and *L. innocua*, electrospun films being more effective than pulverised, suggesting greater ability to release the active compounds.

Therefore, laminates PLA/starch/PLA, with surface incorporation of ferulic or cinnamic acids, represent a good alternative to obtain active food packaging materials with the capacity to preserve food quality and extend shelf life. Nonetheless, further studies on the material stability and specific applications in real foods are necessary to determine the possible industrial application and economic viability of these materials.

Resumen

Los residuos de envases alimentarios contribuyen en gran medida a la actual crisis medioambiental provocada por los plásticos. Por ello, la industria alimentaria necesita soluciones de envasado más sostenibles y respetuosas con el medio ambiente, utilizando materiales biodegradables y activos para alargar la vida útil de los alimentos. En este sentido, la presente tesis doctoral tiene como objetivo la obtención de películas activas a base de almidón y PLA mediante la incorporación de ácidos ferúlico o cinámico como compuestos activos, con el fin de desarrollar laminados multicapa que cumplan con los requisitos de envasado de alimentos, con capacidad antibacteriana para alargar la vida útil del producto. En primer lugar, se estudiaron películas monocapa de almidón o PLA con compuestos activos y, finalmente, se diseñaron películas multicapa combinando láminas de almidón y PLA.

La incorporación de los ácidos ferúlico y cinámico a las matrices de almidón de yuca mediante el proceso de mezclado en fundido, al 1 y 2% p/p, promovió la plastificación de la película, probablemente debido a la hidrólisis parcial del polímero durante el proceso de mezclado. Esto aumentó la plasticidad y la extensibilidad de las películas, disminuyendo su rigidez y su resistencia a la rotura. La incorporación de ácidos no mejoró las propiedades de barrera de las películas, pero redujo significativamente su solubilidad en agua. Los análisis de la actividad antibacteriana de las películas frente a *Escherichia coli* y *Listeria innocua*, en medio de cultivo, revelaron que las películas con ácido cinámico eran más eficaces para inhibir el crecimiento bacteriano, y que Listeria innocua era más sensible a ambos ácidos que *E. coli*.

Cuando los ácidos ferúlico y cinámico se incorporaron a las matrices de PLA al 1 y al 2% mediante mezclado en fundido, las propiedades funcionales de las películas no se vieron muy afectadas. Sin embargo, los ensayos antimicrobianos in vitro con *L. innocua* y *E. coli*, no mostraron acción antibacteriana, lo que podía atribuirse a la falta de liberación efectiva de los compuestos desde la matriz de PLA. Los estudios de liberación mostraron una liberación muy limitada de los ácidos ferúlico y cinámico desde la matriz de PLA debido al estado vítreo de la matriz polimérica y a su naturaleza hidrofóbica, que limitan el hinchamiento y la relajación del polímero en contacto con medios acuosos, como muchas matrices alimentarias. Por lo tanto, para promover la liberación del compuesto activo desde la matriz de PLA, se analizaron diferentes estrategias: 1) plastificar las películas termoprocesadas con PEG 1000, 2) aumentar la concentración de los compuestos en la película, 3) procesar las películas por casting y 4) anclar los compuestos activos a la superficie de la película. La incorporación superficial se realizó por adsorción de los activos a partir de las soluciones hidro-etanólicas de los compuestos activos, por pulverización de las soluciones etanólicas de los compuestos sobre la superficie de la película, o por recubrimiento de las películas por electrospinning de las soluciones polímero-activo, utilizando mezclas de disolventes compatibles.

Sólo la incorporación superficial de ferúlico o cinámico en las películas de PLA dio lugar a una inhibición significativa del crecimiento bacteriano. La pulverización de las películas de PLA con soluciones etanólicas de ácido ferúlico o cinámico permitió la formación superficial de una capa sobre concentrada de compuestos activos en las películas, con formaciones cristalinas que dio lugar a una adecuada liberación de activos para inhibir el crecimiento bacteriano. Disoluciones de PLA-ácido, utilizando mezclas de acetato de etilo (E) con dimetilsulfóxido (DMSO) o ácido acético glacial (A), fueron electrodepositadas en la superficie de las películas. Las diferencias estructurales de las capas obtenidas, asociadas al sistema solvente, determinaron el poder antimicrobiano del material. Los sistemas con DMSO produjeron fibras con una elevada superficie específica que inhibieron significativamente el crecimiento bacteriano, mientras que los sistemas con ácido acético produjeron estructuras particuladas sin actividad antimicrobiana.

Se consideró la incorporación superficial de los compuestos activos para obtener películas de tres capas PLA/almidón/PLA (PSP) con mejores propiedades funcionales y capacidad antimicrobiana. Las películas cargadas superficialmente, tanto por electrospinning como por pulverización, mostraron una eficaz inhibición del crecimiento de *E. coli* y *L. innocua*, siendo las películas recubiertas por electrospinning más eficaces que las pulverizadas, lo que sugiere una mayor capacidad de liberación de los compuestos activos.

Por lo tanto, los laminados PLA/almidón/PLA, con incorporación superficial de ácidos ferúlico o cinámico, representan una buena alternativa para obtener materiales de envasado de alimentos activos, con capacidad para preservar la calidad de los alimentos y prolongar su vida útil. No obstante, son necesarios más estudios sobre la estabilidad del material y aplicaciones específicas en alimentos reales para determinar la posible aplicación industrial y la viabilidad económica de estos materiales.

Resum

Els residus d' envasos alimentaris contribueixen en gran mesura a l' actual crisi mediambiental provocada pels plàstics. Per això, la indústria alimentària necessita solucions d' envasament més sostenibles i respectuoses amb el medi ambient, utilitzant materials biodegradables i actius per allargar la vida útil dels aliments. En aquest sentit, aquesta tesi doctoral té com a objectiu l' obtenció de pel·lícules actives a base de midó i PLA mitjançant la incorporació d' àcids ferúlic o cinàmic com a compostos actius, per tal de desenvolupar laminats multicapa que compleixin amb els requisits d' envasament d' aliments, amb capacitat antibacteriana per allargar la vida útil del producte. En primer lloc, es van estudiar pel·lícules monocapa de midó o PLA amb compostos actius i, finalment, es van dissenyar pel·lícules multicapa combinant làmines de midó i PLA.

La incorporació dels àcids ferúlic i cinàmic a les matrius de midó de iuca mitjançant el procés de mesclament en fos, a l'1 i 2% p/p, va promoure la plastificació de la pel·lícula, probablement a causa de la hidròlisi parcial del polímer durant el procés de barret. Això va augmentar la plasticitat i l'extensibilitat de les pel·lícules, disminuint la seva rigidesa i la seva resistència al trencament. La incorporació d'àcids no va millorar les propietats de barrera de les pel·lícules, però va reduir significativament la seva solubilitat en aigua. Les anàlisis de l'activitat antibacteriana de les pel·lícules enfront d'*Escherichia coli* i *Listeria innocua*, en medi de cultiu, van revelar que les pel·lícules amb àcid cinàmic eren més eficaces per inhibir el creixement bacterià, i que Listeria innocua era més sensible a tots dos àcids que *E. coli*.

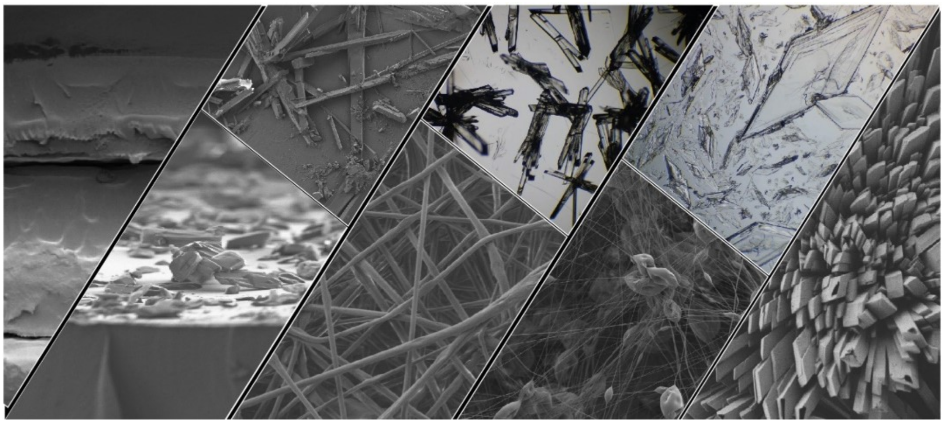
Quan els àcids ferúlic i cinàmic es van incorporar a les matrius de PLA a l'1 i al 2% mitjançant mesclament en fos, les propietats funcionals de les pel·lícules no es van veure gaire afectades. No obstant això, els assajos antimicrobians in vitro amb L. innocua i *E. coli*, no van mostrar acció antibacteriana, la qual cosa podia atribuir-se a la falta d'alliberament efectiu dels compostos des de la matriu de PLA. Els estudis d'alliberament van mostrar un alliberament molt limitat dels àcids ferúlic i cinàmic des de la matriu de PLA a causa de l'estat vitri de la matriu polimèrica i a la seva naturalesa hidrofòbica, que limiten l'hinxament i la relaxació del polímer en contacte amb mitjans aquosos, com moltes matrius alimentàries. Per tant, per promoure l' alliberament del compost actiu des de la matriu de PLA, es van analitzar diferents estratègies: 1) plastificar les pel·lícules termoprocessades amb PEG 1000, 2) augmentar la concentració dels compostos a la pel·lícula, 3) processar les pel·lícules per casting i 4) ancorar els compostos actius a la superfície de la pel·lícula. La incorporació superficial es va realitzar per adsorció dels actius a partir de les solucions hidro-etanòliques dels compostos actius, per polvorització de les solucions etanòliques dels compostos sobre la superfície de la pel·lícula, o per recobriment de les pel·lícules per electrospinning de les solucions polímer-actiu, utilitzant mescles de dissolvents compatibles.

Només la incorporació superficial de ferúlic o cinàmic a les pel·lícules de PLA va donar lloc a una inhibició significativa del creixement bacterià. La polvorització de les pel·lícules de PLA amb solucions etanòliques d'àcid ferúlic o cinàmic va permetre la formació superficial d'una capa sobreconcentrada de compostos actius a les pel·lícules, amb formacions cristal·lines, que va donar lloc a un adequat alliberament d'actius per inhibir el creixement bacterià. Dissolucions de PLA-àcid, utilitzant mescles d'acetat d'etil (E) amb dimetilsulfòxid (DMSO) o àcid acètic glacial (A), van ser electrodepositades a la superfície de les pel·lícules. Les diferències estructurals de les capes obtingudes, associades al sistema solvent, van determinar el poder antimicrobià del material. Els sistemes amb DMSO van produir fibres amb una elevada superfície específica que van inhibir significativament el creixement bacterià, mentre que els sistemes amb àcid acètic van produir estructures particulades sense activitat antimicrobiana.

Es va considerar la incorporació superficial dels compostos actius per obtenir pel·lícules de tres capes PLA/midó/PLA (PSP) amb millors propietats funcionals i capacitat antimicrobiana. Les pel·lícules carregades superficialment, tant per electrospinning com per polvorització, van mostrar una eficaç inhibició del creixement d'*E. coli* i *L. innocua*, sent les pel·lícules recobertes per electrospinning més eficaços que les polvoritzades, la qual cosa suggereix una major capacitat d'alliberament dels compostos actius.

Per tant, els laminats PLA/midó/PLA, amb incorporació superficial d'àcids ferúlic o cinàmic, representen una bona alternativa per obtenir materials d'envasat d'aliments actius, amb capacitat per preservar la qualitat dels aliments i prolongar la seva vida útil. No obstant això, calen més estudis sobre l' estabilitat del material i aplicacions específiques en aliments reals per determinar la possible aplicació industrial i la viabilitat econòmica d' aquests materials.

Preface



Dissertation outline

This doctoral thesis has been structured in five sections:

I. Introduction
II. Objectives
III. Chapters
IV. General discussion
V. Conclusions

The **Introduction** stablishes the state of the art of the research involved in the development of active biodegradable materials for food packaging applications, specifically in the potential of phenolic acids as active compounds with antioxidant and antimicrobial capacities. This section also dwells on the effects of phenolic acids, or plant extracts rich in these compounds, when incorporated into biodegradable polymeric matrices, also discussing the factors affecting the release of active compounds from these matrixes into food systems. This bibliographic research and discussion are presented in the format of a review article entitled “Biodegradable Active Materials Containing Phenolic Acids for Food Packaging Applications”, This work was submitted to the journal “Comprehensive Reviews in Food Science and Safety”.

Objectives section defines the general objective of the thesis and the specific objectives stablished to reach this main goal.

The **Chapters** display the different obtained results. This section is divided in six chapters corresponding to the specific objectives detailed in the **Objectives** section. All **chapters** are subdivided as follow:

- Introduction
- Materials and Methods
- Results and Discussion
- Conclusions

In the **General Discussion** section, the most relevant results are exposed as an overview and analysed from the global perspective of the whole study.

Lastly, the final **Conclusions** of this thesis are presented in the corresponding section.

Dissemination of results

As part of the doctoral program most of the research results and findings had been published or divulged in several formats as described below.

Publications in International JRC Journals

Edible coatings controlling mass loss and *Penicillium roqueforti* growth during cheese ripening | Journal of Food Engineering.
https://doi.org/10.1016/j.jfoodeng.2020.110174
Research article | Status: Published

Physicochemical and antimicrobial properties of cassava starch films with ferulic or cinnamic acid | LWT.
https://doi.org/10.1016/j.lwt.2021.111242
Research article | Status: Published

Biodegradable active materials containing phenolic acids for food packaging applications | Comprehensive Reviews in Food Science and Food Safety
Review article | Status: Under review

Effect of ferulic and cinnamic acids on the functional and antimicrobial properties in thermo-processed PLA films | Food Packaging and Shelf Life.
Preprint: https://doi.org/10.2139/SSRN.3978978
Research article | Status: Under review

Antibacterial properties of cinnamic and ferulic acids incorporated to starch and PLA monolayer and multilayer films | Food Control.
https://doi.org/10.1016/j.foodcont.2022.108878
Research article | Status: Published

Properties of PLA films with cinnamic acid: effect of the processing method | Food and Bioproducts Processing
https://doi.org/10.1016/j.fbp.2022.02.002
Research article | Status: Published

Antilisterial action of PLA films with ferulic acid as affected by the method of incorporation | Food Bioscience.
Research article | Status: Under review

Multilayer antimicrobial films based on starch and PLA with superficially incorporated ferulic or cinnamic acids for food packaging purposes | Food Packaging and Shelf Life.
Preprint: http://dx.doi.org/10.2139/ssrn.4042077
Research article | Status: Under review

International congress participations

Poster presentation:
Aplicación de recubrimientos en quesos para el control del crecimiento fúngico superficial | XII Iberoamerican Congress of Food Engineering (CIBIA 2019)

Oral communication:
Antimicrobial electrospun materials based on polylactic and ferulic acids for active food packaging developing | 6th International ISEKI Food Conference (ISEKI FOOD 2021)

Communications in Scientific Events

University representative in the national competition #HiloTesis
http://twitter.com/monchyff/status/1384569256642326528?s=20

Materiales activos para envase alimentario a base de PLA, almidón y ácidos fenólicos. | "Jornada de Investigación en Ciencia y Tecnología de Alimentos 2021". Valencia, Spain, July, 2021.

Bachelor thesis co-direction

Efecto de la incorporación de ácidos fenólicos antimicrobianos en las propiedades de films de almidón de yuca | **Student:** Maria del Mar Ortiz Gimeno opting for Food Science & Tehcnology degree (UPV) | **Directors**: Amparo Chiralt Boix, Lorena Atarés Huerta; **Experimental director:** Ramón Ordoñez Lagos.

Influencia de ácidos fenólicos antimicrobianos en las propiedades de films de ácido poliláctico (PLA) | **Student:** Francisco DaSilva Guardia opting for Food Science & Tehcnology degree (UPV) | **Directors**: Amparo Chiralt Boix, Lorena Atarés Huerta; **Experimental director:** Ramón Ordoñez Lagos.

Propiedades fisicoquímicas y antibacterianas de películas de poliácido láctico y ácido cinámico | **Student:** Jaroslaw Szmaj Aguilar opting for the Food Science & Tehcnology degree (UPV) | **Directors**: Amparo Chiralt Boix, Lorena Atarés Huerta; **Experimental director:** Ramón Ordoñez Lagos.

Obtención de nanomateriales de poliácido láctico y ácido ferúlico con acción antimicrobiana | Student: Enrique Roig Tormo opting for the Food Science & Tehcnology degree (UPV) | **Directors**: Amparo Chiralt Boix, Lorena Atarés Huerta; **Experimental director:** Ramón Ordoñez Lagos.

Estrategias de incorporación de ácido ferúlico en films de PLA para facilitar la acción antimicrobiana | Student: Silvia Sabina Fajardo opting for the Biotechnology degree (UPV) | **Directors**: Amparo Chiralt Boix, Lorena Atarés Huerta; **Experimental director:** Ramón Ordoñez Lagos.

Master thesis co-direction

Efecto antibacteriano de films de almidón con ácidos fenólicos: aplicación en matrices alimentarias | Student: Rosa Elena González Guevara opting for the Master in Food Science & Engineering degree (UPV) | **Directors**: Amparo Chiralt Boix, Lorena Atarés Huerta; **Experimental director:** Ramón Ordoñez Lagos.

Ácidos fenólicos como agentes antibacterianos en el desarrollo de materiales activos para el envasado de alimentos | Student: Andrés Soriano Altamiñana opting for the Master in Food Science & Engineering degree (UPV) | **Directors**: Amparo Chiralt Boix, Lorena Atarés Huerta; **Experimental director:** Ramón Ordoñez Lagos.

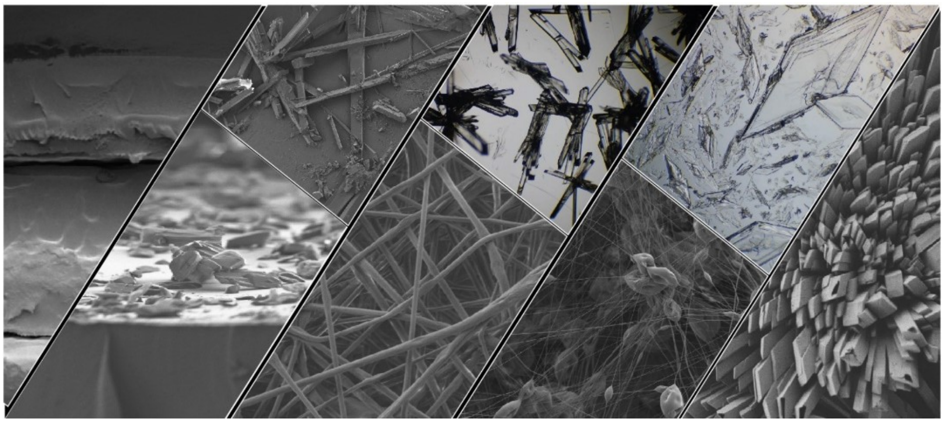
Table of Contents

Nomenclature

A	Acetic acid
ASTM	American Society for Testing Materials
BP	Biodegradable polymer
C	Cinnamic Acid
C_{ab}^*	Chroma
CECT	Spanish Type Culture Collection
CFU	Colony Forming Unit
D	Diffusion coefficient
Da	Dalton
DMSO	Dimethyl Sulfoxide
DSC	Differential Scanning Calorimetry
DTGA	Derivative thermal gravimetric analysis
E	Elongation at break
E. coli	*Escherichia coli* bacterial strain
EM	Elastic Modulus
EtAc	Ethyl Acetate
ES	Electrospinning
F	Ferulic acid
FESEM	Field Emission Scanning Electron Microscopy
FFS	Film Forming Solution
h_{ab}^*	Hue
HRFESEM	High Resolution Field Emission Scanning Electron Microscopy
k	Rate constant for Korsmeyer-Peppas' model
k_1	Rate constant for Peleg's model
k_2	Equilibrium constant for Peleg's model
L^*	Luminosity
L. innocua	*Listeria innocua* bacterial strain
M_∞	Equilibrium value of release active
MIC	Minimum Inhibitory Concentration
MTT	Thiazolyl Blue Tetrazolium Bromide
n	Difussional exponent for Korsmeyer-Peppas' model
OP	Oxygen Permeability
OTR	Oxygen Transmission Rate
PA	Phenolic acid
PBAT	Polybutyrate Adipate Terephthalate
PBS	Phospate-Buffered Saline
PCL	Poly- ε-caprolactone
PEG	Polyethylene Glycol
PHAs	Polyhydroxyalkanoates
PHBV	Poly(3-hydroxybutyrate-co-3-hydroxivalerate)
PLA	Polylactic Acid
PVA	Poly(Vynil Alcohol)
RH	Relative Humidity
S	Starch

t	Time
T	Temperature
T_g	Glass Transition Temperature
TGA	Thermogravimetric Analysis
T_i	Internal Transmittance
T_m	Melting Temperature
TP	Thermal processing
TPP	Thermal processing and plasticized
T_{peak}	Peak Degradation Temperature
TPS	Thermoplastic Starch
T_{onset}	Onset thermal degradation temperature
TS	Tensile Strength at Break
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UV	Ultraviolet
Vis	Visible
WTR	Water Transmission Rate
WVP	Water Vapor Permeability

I. Introduction



I. Introduction

Biodegradable Active Materials Containing Phenolic Acids for Food Packaging Applications

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(Submited)

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Abstract

The development of new materials for food packaging applications is necessary to reduce the excessive use of disposable plastics and their environmental impact. Biodegradable polymers represent an alternative means of mitigating the problem. To add value to biodegradable materials, and to enhance food preservation, the incorporation of active compounds into the polymer matrix is an affordable strategy. Phenolic acids are plant metabolites that can be found in multiple plant extracts, which exhibit antioxidant and antimicrobial properties. As compared with other natural active compounds, such as essential oils, phenolic acids do not present a high sensorial impact while exhibiting similar minimal inhibitory concentrations against different bacteria. This work summarizes and discusses recent studies about the potential of both phenolic acids/plant extracts and biodegradable polymers as active food packaging materials, their properties, interactions, and the factors that could affect their antimicrobial efficiency. The molecular structure of the phenolic acids greatly affects their potential antioxidant and antimicrobial capacity, as well as their specific interactions with the polymer matrices and the food substrate. These interactions, in turn, can lead to plasticizing or cross-linking effects. In the present paper, the antioxidant and antimicrobial properties of different biodegradable films with phenolic acids have been described, as well as the main factors affecting the active properties of these films as useful materials for active packaging development. More studies applying these active materials in real foods are required.

1. Introduction

In the search for environmentally responsible operations, bioplastics-based materials have shown potential for replacing traditional plastics in the food packaging industry, thanks to their renewable origin and/or biodegradability (Choi et al., 2018; Zhu, 2021). On the other hand, active packaging is a novel approach aimed at developing packaging materials that also carry active agents to improve food quality and safety (Woranuch et al., 2015). These agents' functionalities could be nutrient supplementation, antimicrobial or antioxidant activity, and more (Cheng et al., 2015; Fabra et al., 2011). Active materials could perform either by interacting with the food or with the headspace in the container (Sung et al., 2013). These materials have been widely studied as a means of extending food shelf life while reducing the use of synthetic chemical preservatives, as demanded by consumers who prefer additive-free, natural food products. Some studies have reported accumulations of these compounds along the food chain, which could become harmful for consumer health or lead to the development of microbial resistance (Guil-Guerrero et al., 2016; Jaiswal & Jaiswal, 2015). Naturally occurring compounds, such as essential oils (EO), phenolic acids (PA) and other plant metabolites, are considered to be highly promising alternatives to synthetic preservatives (Guil-Guerrero et al., 2016).

There are numerous previous studies into active packaging materials, using essential oils or their main compounds as natural preservatives, on the basis of their antimicrobial and antioxidant properties. Essential oils and their main compounds have been reported not only as good antimicrobial agents themselves but also as effective additives in bioplastic films (Tampau et al., 2018). Requena et al. (2019b) studied the minimum inhibitory concentration (MIC) of several of the main compounds of EO in both Gram-positive and negative bacteria, reporting values of between 0.5 and 1.75 mg/ml, cinnamaldehyde being the most active (0.5 mg/ml for both types of bacteria). Carvacrol and thymol also presented MIC values below 1 mg/ml and a promising synergistic effect of carvacrol and cinnamaldehyde was also reported. The effect of EO compounds on the organoleptic properties of food products is the main drawback that greatly limits their application in food products (Atarés & Chiralt, 2016). Sapper et al. (2020) reported a great persistence of the flavour and aroma of carvacrol in apples coated with polyvinylalcohol-starch-carvacrol based coatings, which negatively affected the sensorial acceptance.

Phenolic acids are naturally occurring compounds mostly derived from benzoic or cinnamic acids. They play a major role in the natural defences of plants against viruses and fungal and bacterial species (Heleno et al., 2015). Being plant metabolites, they can be found in multiple natural extracts linked to consumer health benefits (Rashmi & Negi, 2020). The antioxidant and antimicrobial properties of phenolic acids have been widely studied (Kumar & Goel, 2019; Wang et al., 2019), and some of them have shown great potential as food preservatives. Because of these properties, as well as the reported potential health benefits (Rashmi & Negi, 2020), phenolic acids could be effective active agents when looking to obtain active food packaging. Nevertheless, their sensory impact when applied as food additives or active compounds in packaging materials needs to be evaluated. In contrast to the highly negative sensory impact produced by essential oils, organoleptic modifications occur to a much lower extent with phenolic acids (Meira et al., 2017; Miyague et al., 2015).

Active packaging materials that incorporate substances that are intended to be released into food should comply with the requirements of the authorisation under the specific legislation on food, such as legislation on food additives (European Union, 2009). The incorporation of phenolic acids as food additives is not generally contemplated, although these are usual components of many food products and with recognized health properties (Rashmi & Negi, 2020). In the US, the Food and Drug Administration (FDA) classifies as GRAS (generally recognized as safe) numerous phenol-rich plant extracts such as grape pomace, cranberry or coffee. Tannic and cinnamic acids are listed as flavouring and adjuvant agents. In Europe, natural plant extracts fall under Directive 2002/46/EC (Martillanes et al., 2017). Some specific compounds have already been approved for different uses. Carminic acid (E-120) is a widely used colorant, and benzoic acid (E-210) is used as a preservative. Recent research on phenolic compounds demonstrates their potential as natural preservatives to be applied to highly perishable products. Given that these compounds represent a promising alternative to synthetic additives, more work is needed in order to determine their maximum daily intake for a safe application in food products.

The objective of this work is to analyze the reported studies on phenolic acids, or plant extracts rich in these compounds, in terms of their antimicrobial or antioxidant capacity when incorporated into biodegradable polymeric materials and, therefore, their potential for the food packaging industry. The different biodegradable polymers available for food packaging are summarized as well as the main studies on the antimicrobial and antioxidant properties of these phenolic compounds when incorporated to obtain active films and their impact on the properties of polymeric matrices. Finally, the main factors affecting the effective action of active films in food substrates are also analysed.

2. Biodegradable polymers for food packaging applications

The development of biobased polymers as a means of substituting petroleum-based synthetic plastics has been widely studied given the non-degradable and non-renewable nature of traditional plastics (DeGruson, 2016). The so-called bioplastics are polymeric materials from renewable sources, and/or are biodegradable or compostable (Zhong et al., 2020). Biodegradation is a natural decomposition process involving enzymatic processes triggered by microorganisms (Vert et al., 2012). Physical factors, such as sunlight, temperature or moisture, can also intervene to turn certain polymers into viable substrates for microorganisms (Hernández-García et al., 2021a). Composting is a degradation process implying the hydrolysis of organic fractions (Wei et al., 2017) and involving thermophilic bacteria. In the initial stages of the degradation, a temperature rise takes place due to the production of small molecules, such as sugars or amino acids, by mesophilic bacteria. Then, thermophilic bacteria reduce larger compounds (fats, cellulose, lignin, etc.) to small molecules that can be assimilated and metabolized by microorganisms. As the temperature drops back, the mesophilic organisms recolonize and degrade the metabolic residues producing water and CO_2 (Rastogi et al., 2020). PLA is an example of a polymer that degrades effectively in composting conditions.

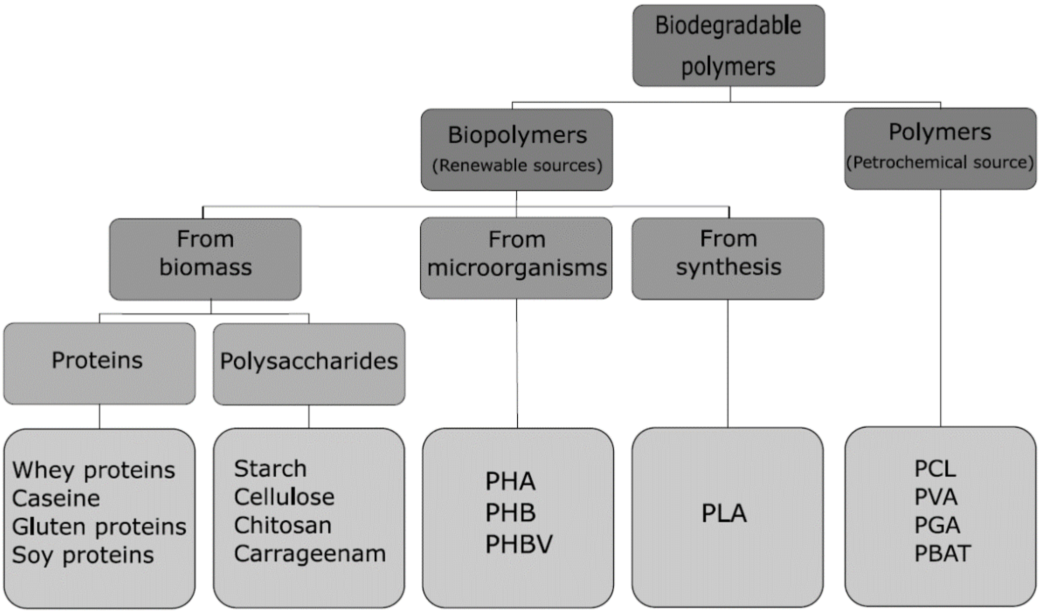


Figure 1. Classification of biodegradable polymers based on the source from which they are obtained. Adapted from Zhong et al. (2020).

Biopolymer is the term used for polymers originating from living organisms (Vert et al., 2012). Although most biopolymers are biodegradable, this is not the case for naturally sourced PET. Also, not all biodegradable polymers are bioderived (DeGruson, 2016), and some (such as polycaprolactone, PCL or polyvinylalcohol, PVA) come from petrochemical sources. **Figure 1** shows that biodegradable polymers can be classified according to their source, primarily either renewable or petrochemical and secondly according to the renewable source used. The most common natural source for bioplastic materials is biomass that is rich in polysaccharides (such as starch, cellulose, pectin, or chitosan) and proteins (gelatin, casein, whey protein, or soy protein) (Cheng et al., 2015). Bioplastics can also be synthesized from bioderived sources, which is the case of polylactic acid (PLA) (Vinod et al., 2020), or biosynthesized by bacteria (such as poly-hydroxy-alkanoates). These bioplastics still present some problems that hinder their practical application, such as unsatisfactory mechanical performance (brittleness, insufficient elongation, etc.) or deficient barrier properties in comparison to petroleum-based plastics. Polysaccharides tend to have low oxygen permeability, but given their hydrophilic nature, they offer poor resistance to water vapor. In contrast, polyesters such as PLA are very hydrophobic, with a higher water vapor barrier capacity, while being more permeable to oxygen and other gases (Requena et al., 2018). **Table 1** summarises recently reported data on the functional properties of some biopolymers frequently studied for food packaging. Both proteins and polysaccharides present good barrier properties to oxygen, but given their hydrophilic nature, they provide the films with a poor barrier to water vapor.

Table 1. Properties of some biodegradable polymers for food packaging applications: oxygen permeability (OP), water vapor permeability (WVP), elastic modulus (EM), tensile strength at break (TS) and percentual elongation (E).

	Polymer	Raw source	OP*10^{14} (cm^3/msPa)	WVP*10^{12} (g/msPa)	EM (MPa)	TS (Mpa)	E (%)	Reference
Polysaccharides	Starch	Cassava	6.37	2120	55	4.7	26	Menzel et al. (2019)
	MC	Vegetal biomass	147	870	1604	66	15	Pastor et al. (2013)
	HPMC	Vegetal biomass	1.06	1600	1327	71	14	Atarés et al. (2011)
	Alginate	Brown seaweed	1.16	250	3200	80	5	Cofelice et al. (2019)
	Chitosan	Crustacea	16.7	1200	2739	94	14	Pastor et al. (2013)
Proteins	Sodium caseinate	Dairy	63.7	169	380	6	19	Fabra et al. (2011)
	Gluten	Wheat	-	2.66	-	1.1	204	Hager et al. (2012)
	Gelatin	Animal biomass	-	840	1970	61	11	Riahi et al. (2021)
Polyesters	PLA	Lactic acid	1500	24	1599	49	3.5	Muller et al. (2017)
	PHBV	Several bacteria	-	5.7	1688	37	3.4	Requena et al. (2018)
	PCL	Petroleum based	-.	32	304	18	1100	Ortega-Toro et al. (2015)
	PBAT	Petroleum based	-	10	47	16	440	Cardoso et al. (2017)
Othe	PVA	Petroleum based	0.11	686	80	153	135	Andrade et al. (2020)

Different promising strategies have been proposed to overcome these issues. The combination of two or more bioplastics with complementary barrier properties has been tested using two approaches: polymer blending and multilayer assemblies. Blending is usually done by melt blending in order to obtain a mixed polymeric matrix. This technique has been used to add hydrophobic compounds or polymers to hydrophilic matrices, such as starch (Acosta et al., 2015; Ortega-Toro et al., 2015). However, the different polymer polarities and lack of chemical affinity could lead to phase separation and heterogeneous matrices, as observed in polycaprolactone and starch blends (Ortega-Toro et al., 2015). On the other hand, multilayer materials can be obtained with fewer compatibility problems than polymer blending (Muller et al., 2017). These materials can be assembled by thermocompression (Requena et al., 2018), coextrusion (Zhu et al., 2016), casting (Uz & Altınkaya, 2011), or by electrospinning layers on already formed monolayers (Tampau et al., 2018).

In the following subsections, some of the most promising biodegradable polymers for food packaging applications are described

2.1. Polysaccharides

Starch has been widely studied, and it is one of the most naturally abundant polymers. It comes from multiple plant sources (cassava, corn, potato…) and is the least expensive biopolymer on the market (DeGruson, 2016). Its plastic properties can be improved by adding a plasticizer and other strategies. The blending and compatibilization of starch with other biodegradable polymers have been proven to enhance this low-cost material. For example, when starch is blended with other polysaccharides, such as xanthan or gellan gum, can improve some mechanical limitations (Hernández-García et al., 2021b). Blending starch with polyesters could also help overcome the main drawbacks of starch (poor water vapor permeability and low resilience), yielding more homogeneous materials with the help of compatibilizers (Zhong et al., 2020).

Cellulose is the most abundant organic compound on earth. Its derivates, methylcellulose (MC), hydroxypropyl methylcellulose (HPMC) and carboxymethyl cellulose (CMC) have shown excellent film-forming capacity, giving rise to films with excellent barrier capacities to hydrophobic compounds and good mechanical performance (Rubilar et al., 2015). In fact, biodegradable polymers consisting of starch and/or cellulose fibres or derivates have growing possibilities of application (Zhong et al., 2020). Alginate can be obtained from different species of brown seaweeds at a low cost, and it has shown desirable film-forming properties (Cofelice et al, 2019). Chitin, which can be found in the exoskeleton of crustaceans and other biological materials, is the second most common naturally-occurring biopolymer. Chitosan results from chitin deacetylation, and it has been proven to show good film-forming capacity, as well as antimicrobial activity (Mohamed et al., 2020; Priyadarshi & Rhim, 2020; Talón et al., 2017).

2.2. Proteins

Proteins, such as caseinate, whey protein, gluten or gelatin, have been studied as potential food packaging materials. In general, due to their unique structure, protein films have better mechanical properties than polysaccharide films (Mohamed et al., 2020). However, they present poor mechanical strength, and their main drawback is their high water vapor permeability.

2.3. Synthetic and microorganism-produced biopolymers

Polyhydroxyalkanoates (PHAs) are a family of biopolyesters produced by many bacteria as intracellular storage carbon and an energy source against starvation (Chen et al., 2011). Although they present a high degree of crystallinity, they can be improved by blending with other polymers (Adeleye et al., 2020). Keskin et al. (2017) proposed several applications for PHAs, such as coated cardboard for milk or drink packaging. PLA is also a polyester that can originate from starch or other biomass polymers that are hydrolysed to dextrose, which is then transformed via fermentation into lactic acid, which finally is polymerized by different chemical routes (Nampoothiri et al., 2010). PLA is a very versatile material exhibiting excellent barrier properties against water vapor. It has received great interest as evidenced by the number of international patents it holds. In fact, it is considered to be the most promising compostable polymer at present (Elvers et al., 2016; RameshKumar et al., 2020). Biodegradable polymers from petrochemical sources, such as PCL or polybutylene succinate (PBS), present ideal mechanical and thermal properties. PCL modifications had been demonstrated to overcome hydrophobicity and fast degradation problems, thanks to the fact that it is highly permeable to several agents, PCL has been widely studied for use in drug delivery applications (Dash & Konkimalla, 2012).

3. Active properties of phenolic acids

Phenolic acids are a subclass of phenolic compounds mainly encompassing hydroxybenzoic acids, hydroxyphenyl acetic acids and hydroxycinnamic acids, which exhibit antioxidant and antimicrobial properties. They can be found in various plant sources such as fruits, vegetables, spices and grains. Rashmi & Negi (2020) reviewed the composition in phenolic acids of a great variety of plants. Both conventional and novel techniques have been used for phenolic acid extraction as reported by Al Jitan et al. (2018). These include Soxhlet extraction, liquid-liquid extraction, maceration, extraction assisted by microwave or ultrasound, accelerated solvent extraction and supercritical fluid extraction. The selected method will be determined by the final application and the cost.

PAs profile and content greatly affect the organoleptic quality of some food products, such as fruits and vegetables (Kumar & Goel, 2019) and have been correlated with the sensory acceptance of different wheat flours (Challacombe et al.,2012). In fact, these compounds can provide the food matrix with a variety of flavors, ranging from sweet to bitter and astringent (Soto-Vaca et al., 2012) and colors (mainly their oxidized forms). Therefore, the use of these compounds as food additives or active packaging formulations should require specific studies about the sensory impact that these have on the final product.

3.1. Antioxidant properties of phenolic acids

Not only phenolic acids but also some of the products of phenolic acid metabolism retain strong antioxidant properties (Piazzon et al., 2012). The antioxidant activity of phenolic acids is strongly affected by the acidic group (carboxyl, acetyl or ethylenil) and the number and position of hydroxyl groups in the molecule (Rice-Evans et al., 1996). The electron-withdrawing properties of the carboxylate group in benzoic acids has a negative influence on the H-donating capacity of the hydroxy benzoates, whereas hydroxylated cinnamates or phenylacetates are more effective than benzoates. The insertion of an additional ethylene or methylene group between the phenyl ring and the carboxyl group in the hydroxycinnamic or hydroxyphenyl acetic acids mitigates the negative impact of the carboxylate group and promotes the antioxidant capacity of the molecules. This effect is more favourable on the reducing properties of the OH group in the hydroxycinnamic structures.

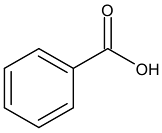
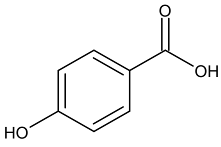
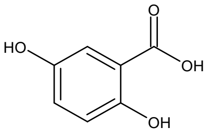
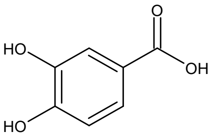
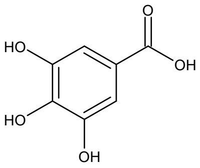
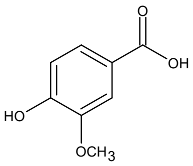
Table 2 gives the Trolox Equivalent Antioxidant Activity (TEAC, mM), as reported by Rice-Evans et al. (1996) for the different phenolic acids, allowing their different antioxidant activity to be ranked depending on the hydroxyl position in the aromatic ring relative to the carboxyl group. The monohydroxy benzoic acids in the ortho and para positions show no hydrogen-donating capacity, whereas in the meta position they show a TEAC antioxidant activity of 0.84 mM. The ethylenic group in the hydroxycinnamic acids promotes the antioxidant properties of the OH group in any position of the aromatic ring, compared with the equivalent OH position in phenolic acid. The equivalent m- and o-hydroxycinnamic acids have TEAC values closer to unity. The dihydroxybenzoic acid derivatives also show an antioxidant response dependent on the relative positions of the hydroxyl groups in the ring; 2,3-dihydroxy benzoic acid gives a TEAC value of 1.46 mM which is slightly elevated compared to the 3,4 positions (TEAC 1.2 mM) and 2,5 positions (TEAC 1.1 mM). Thus, the proximity of the carboxyl group to the hydroxyl substituents affects their hydrogen donating capacity, the m-position being the most effective. Therefore, the chemical structure of polyphenols, defining the availability of the phenolic hydrogens as radical scavengers, determines their antioxidant activity. However, the antioxidant activity of phenolic compounds also depends on the type of prooxidant and the antioxidant to prooxidant ratio, under certain conditions certain antioxidants including PAs can behave as prooxidants (Carocho & Ferreira, 2013). Both factors are important for the efficiency of phenolics in the inhibition of catalysed oxidations. Therefore, when a particular phenolic compound is selected for an application, the type and content of different prooxidative agents should be known, since different reactions are involved in interactions between pro and antioxidants.

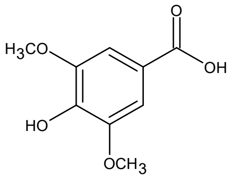
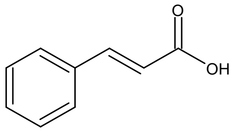
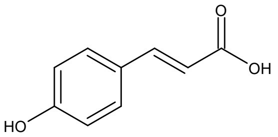
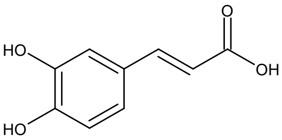
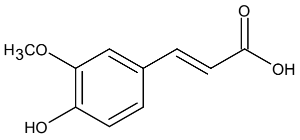
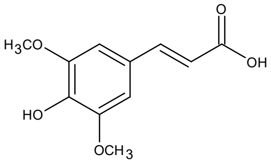
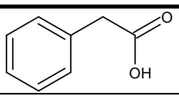
3.2. Antimicrobial properties of phenolic acids

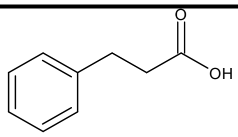
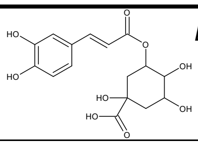
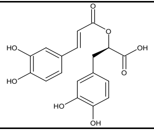
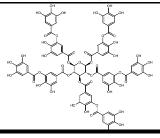
The capacity of PAs to inhibit bacterial growth is due to their prooxidative properties and their ability to alter the cell’s superficial charge and hydrophobicity, causing fissures and cytoplasmic deposition (Maddox et al., 2010). Previous studies (Cueva et al., 2010) demonstrated that Gram-positive bacteria are more susceptible to the action of phenolic acids than Gram-negative. This activity can be influenced by the molecular structure of the PA, specifically the type and position of the substituents in the aromatic ring and the length of the lateral chain. This structural effect was widely studied by Sánchez-Maldonado et al. (2011) working on lactic acid bacteria with different conclusions for hydroxycinnamic and hydroxybenzoic acids. The antibacterial activity of hydroxybenzoic acids decreased as the number of hydroxyl groups rose, which promoted the hydrophilic nature of the compounds and highly increased as the hydroxyl groups were substituted by methoxy groups, thus boosting the lipophilic character of the molecule. However, the activity of hydroxycinnamic acids was not significantly affected by the aromatic ring substitutes since it was heavily dependent on the conjugated double bond on the side chain. This insight could facilitate the selection of PAs for food antimicrobial applications.

Table 2 shows the values of the Minimum Inhibitory Concentration (MIC) against different bacteria of different PAs, as reported by several authors. *C*oumaric, ferulic and syringic acids exhibited inhibition capacity against *L. monocytogenes* (Miyague et al., 2015), this activity being higher at lower pH levels when PAs are not ionized and can easily cross the plasma membrane; once into the cytoplasm they can disrupt many metabolic pathways. These authors also tested the efficacy of combinations of some EO compounds and PAs against *L. monocytogenes*; finding several synergistic effects at pH 5, which points to the potential use of these compound combinations in acidic foods to control bacterial growth. Malheiro et al. (2018) reported a significant growth inhibition of planktonic and sessile bacteria for cinnamic acid, cinnamaldehyde and their derivates. Merkl et al. (2010) studied the antimicrobial and antioxidant capacities of several alkyl esters of phenolic acids and reported enhanced antioxidant and antimicrobial activity of the esters of protocatechuic and caffeic acids as compared to the respective acids, while the antimicrobial power of these derivates increased as the alkyl chain became longer.

Table 2. Minimal inhibitory concentration (MIC) of different phenolic acids for several microorganisms. Trolox Equivalent Antioxidant Activity (TEAC, mM) of phenolic acids was also included, as reported by Rice-Evans et al. (1996).

	Acid	Structure	Microorganism	MIC (mg/g)	Reference
Hydroxybenzoic acids	Benzoic		*Escherichia coli*	1.00	Cueva et al. (2010)
			Lactobacillus plantarum	1.45	Sánchez-Maldonado et al. (2011)
			Lactobacillus hammesii	0.97	Sánchez-Maldonado et al. (2011)
			Bacillus subtilis	0.04	Sánchez-Maldonado et al. (2011)
	p-hydroxybenzoic		*Escherichia coli*	0.12	Sánchez-Maldonado et al. (2011)
	TEAC (mM): 0.08		*Escherichia coli*	0.34	Merkl et al. (2010)
			Listeria monocytogenes	0.69	Miyague et al. (2015)*
			Listeria monocytogenes	1.00	Cueva et al. (2010)
			Listeria monocytogenes	0.69	Merkl et al. (2010)
			Lactobacillus plantarum	1.57	Sánchez-Maldonado et al. (2011)
			Lactobacillus hammesii	1.12	Sánchez-Maldonado et al. (2011)
			Bacillus subtilis	0.13	Sánchez-Maldonado et al. (2011)
			Bacillus cereus	0.69	Merkl et al. (2010)
	Gentistic		*Escherichia coli*	0.77	Merkl et al. (2010)
	TEAC (mM): 1.04		*Listeria monocytogenes*	0.77	Merkl et al. (2010)
			Bacillus cereus	0.77	Merkl et al. (2010)
	Protocatechuic		*Escherichia coli*	0.31	Sánchez-Maldonado et al. (2011)
	TEAC (mM): 1.19		*Escherichia coli*	1.54	Merkl et al. (2010)
			Listeria monocytogenes	1.54	Miyague et al. (2015) *
			Listeria monocytogenes	0.77	Merkl et al. (2010)
			Lactobacillus plantarum	1.57	Sánchez-Maldonado et al. (2011)
			Lactobacillus hammesii	1.12	Sánchez-Maldonado et al. (2011)
			Bacillus subtilis	0.13	Sánchez-Maldonado et al. (2011)
			Bacillus cereus	0.77	Merkl et al. (2010)
	Gallic		*Escherichia coli*	0.49	Sánchez-Maldonado et al. (2011)
	TEAC (mM): 3.01		*Listeria monocytogenes*	1.70	Miyague et al. (2015)*
			Lactobacillus plantarum	3.74	Sánchez-Maldonado et al. (2011)
			Lactobacillus hammesii	4.56	Sánchez-Maldonado et al. (2011)
			Bacillus subtilis	0.64	Sánchez-Maldonado et al. (2011)
	Vanillic		*Escherichia coli*	1.00	Cueva et al. (2010)
	TEAC (mM): 1.43		*Escherichia coli*	1.64	Merkl et al. (2010)
			Listeria monocytogenes	0.84	Miyague et al. (2015)*
			Listeria monocytogenes	0.82	Merkl et al. (2010)
			Bacillus cereus	0.82	Merkl et al. (2010)

	Syringic ***TEAC (mM): 1.36***		*Escherichia coli*	0.39	Sánchez-Maldonado et al. (2011)
			Listeria monocytogenes	0.99	Miyague et al. (2015)*
			Lactobacillus plantarum	1.75	Sánchez-Maldonado et al. (2011)
			Lactobacillus hammesii	1.15	Sánchez-Maldonado et al. (2011)
			Bacillus subtilis	0.26	Sánchez-Maldonado et al. (2011)
Hydroxycinnamic acids	Cinnamic		*Escherichia coli*	0.11	Sánchez-Maldonado et al. (2011)
			Listeria monocytogenes	1.48	Miyague et al. (2015)*
			Lactobacillus plantarum	0.79	Sánchez-Maldonado et al. (2011)
			Lactobacillus hammesii	0.86	Sánchez-Maldonado et al. (2011)
			Bacillus subtilis	0.07	Sánchez-Maldonado et al. (2011)
	p-Coumaric ***TEAC (mM): 2.22***		*Escherichia coli*	0.12	Sánchez-Maldonado et al. (2011)
			Listeria monocytogenes	0.82	Miyague et al. (2015)*
			Lactobacillus plantarum	1.21	Sánchez-Maldonado et al. (2011)
			Lactobacillus hammesii	1.04	Sánchez-Maldonado et al. (2011)
			Bacillus subtilis	0.25	Sánchez-Maldonado et al. (2011)
	Caffeic ***TEAC (mM): 1.26***		*Escherichia coli*	0.23	Sánchez-Maldonado et al. (2011)
			Escherichia coli	0.25	Matejczyk et al. (2018)
			Escherichia coli	1.80	Merkl et al. (2010)
			Listeria monocytogenes	1.80	Merkl et al. (2010)
			Lactobacillus plantarum	1.52	Sánchez-Maldonado et al. (2011)
			Lactobacillus hammesii	0.63	Sánchez-Maldonado et al. (2011)
			Bacillus subtilis	0.30	Sánchez-Maldonado et al. (2011)
			Bacillus cereus	1.80	Merkl et al. (2010)
	Ferulic ***TEAC (mM): 1.90***		*Escherichia coli*	0.16	Sánchez-Maldonado et al. (2011)
			Escherichia coli	1.94	Merkl et al. (2010)
			Listeria monocytogenes	0.97	Miyague et al. (2015)*
			Listeria monocytogenes	1.94	Merkl et al. (2010)
			Lactobacillus plantarum	1.68	Sánchez-Maldonado et al. (2011)
			Lactobacillus hammesii	0.89	Sánchez-Maldonado et al. (2011)
			Bacillus subtilis	0.38	Sánchez-Maldonado et al. (2011)
			Bacillus cereus	1.94	Merkl et al. (2010)
	Sinapinic		*Listeria monocytogenes*	1.12	Miyague et al. (2015)*
Phenylacetic	Phenylacetic		*Escherichia coli*	0.50	Cueva et al. (2010)
	3-hydroxyphenylacetic	***TEAC (mM): 0.90***	*Escherichia coli*	1.00	Cueva et al. (2010)
	3,4-dihydroxyphenylacetic	***TEAC (mM): 2.19***	*Escherichia coli*	1.00	Cueva et al. (2010)

Phenylpropionic	Phenylpropionic 	*Escherichia coli*	0.50	Cueva et al. (2010)
	3-hydroxyphenylpropionic	*Escherichia coli*	1.00	Cueva et al. (2010)
	3,4-dihydrophenylpropionic	*Escherichia coli*	1.00	Cueva et al. (2010)
Other phenolic acids	Chlorogenic 	*Escherichia coli*	1.00	Cetin-Karaca & Newman (2015)
	Rosmarinic 	*Escherichia coli*	0.25	Matejczyk et al. (2018)
	Tannic 	*Escherichia coli* *Listeria monocytogenes*	0.025 0.025	de Almeida Roger et al. (2018) de Almeida Roger et al. (2018)

*Value at pH = 6.

Based on the aforementioned studies, phenolic acids appear to be promising compounds for the purposes of obtaining active materials for food packaging application with a double-action, antioxidant and antimicrobial capacity, which confers great potential for food preservation and shelf-life extension. Nevertheless, their incorporation into the packaging films may provoke changes in the functional properties in the materials and their release from this would be required to ensure an effective role in food preservation. The following sections discuss different studies where phenolic acids, or plant extracts potentially rich in these compounds, have been incorporated into different polymer matrices, analysing their effect on the barrier and tensile properties and antimicrobial or antioxidant properties of the materials.

4. Functional and active properties of biodegradable films containing phenolic acids/ plant extracts

As commented on above, hydrophilic biopolymer-based films and coatings exhibit water sensitivity, along with poor mechanical and barrier properties, which limits their practical applications. Phenolic compounds, and particularly phenolic acids, have been tested as crosslinkers to improve the properties of some biopolymer films, such as those that are protein-based, involving the formation of both covalent and non-covalent bonding (Zhang et al., 2010). Phenolic acids are present in many natural foods and due to their antioxidant capacity among other properties, these compounds provide food with health benefits and oxidative stability (Soto-Vaca et al., 2012). The following sections discuss the effect of phenolic acid incorporation on the functional or active properties of biodegradable materials intended for food packaging applications.

4.1. Functional properties

Table 3 summarizes recent studies in which phenolic acids or plant extracts have been incorporated into biodegradable films based on different materials, analyzing their mechanical and barrier properties.

Table 3. Studies into the effect of the incorporation of phenolic acids /plant extracts on the functional properties (Elastic Modulus: EM, Tensile Strength at break: TS, elongation at break: E, water vapor permeability: WVP or oxygen permeability: OP) of several biodegradable polymeric matrices.

Material	Active compound	Highlights	Reference
Alginate	Grape seed and green tea extracts	WVP improved with the highest concentrations of extracts.	Fabra et al. (2018)
Alginate	Tannic acid	Plastic behaviour was improved, reduced surface hydrophilic character. OP and WVP were increased.	Kaczmarek (2020)
Casein	Tannic acid	TS increased while E decreased in relation to the acid concentration, WVP decreased greatly at low concentrations.	Picchio et al., (2018)
Cassava starch	Rosemary extract	TB decreased in every sample, WVP greatly increased at high extract concentrations.	Piñeros-Hernandez et al. (2017)
Chitosan	Propolis extract	E, TS and OP increased while WVP decreased.	Siripatrawan & Vitchayakitti (2016)
Chitosan	Gallic, ferulic, p-coumaric, salicilyc, and vanillic acids	All acids reduced E, TS and water solubility. Ferulic and gallic acids gave rise to the best properties.	Liu et al. (2021)
Chitosan and chitosan-corn starch	Murta leaf extract	Extract created discontinuity in films and reduced E.	Silva-Weiss et al. (2013)
Chitosan, gelatin and MC	Tannic acid	E increased in chitosan and MC but decreased in gelatin. OP decreased in chitosan and gelatin. WVP decreased in all formulations.	Halim et al. (2018)
Chitosan-alginate	Ferulic acid	E decreased in the mixed material, WVP also decreased in every sample.	Li et al. (2019)
Chitosan-PVA	Mint and pomegranate peel extracts	Extracts increased TS but did not alter WVP or OP.	Kanatt et al. (2012)
Fish myofibrilar protein	Caffeic, ferulic and tannic acids	EM and TS increased while E decreased in every acid concentration.	Prodpran et al. (2012)
Bovine gelatin	Rosmarinic acid	Reduced TS, EM and WVP while E increased.	Ge et al. (2018)
Gellan gum	Coffee parchment waste	E and TS decreased.	Mirón-Mérida et al. (2019)
Gluten	Gallic and tannic acids	Gallic acid increased E and WVP, while decreasing TS. The opposite was observed for tannic acid.	Hager et al. (2012)
PLA-starch-PBAT blend	Ferulic acid or ferulic acid-coupled chitosan	WVP increased but OP decreased, E increased for the mixed material but decreased for chitosan.	Woranuch et al. (2015)
PLA-PBAT blend	Ferulic acid	TS and thermal stability increased and internal transmittance decreased by the acid incorporation.	Sharma et al. (2020)

PVA	Cinnamic and ferulic acids	Films containing ferulic acid were mechanically stiffer and had enhanced barrier properties	Andrade et al. (2021)
Potato starch	Sunflower hull extracts	EM increased while E, WVP and OP decreased	Menzel et al. (2019)
Soy protein	Ferulic, caffeic and gallic acids	All the acids increased E and TS while reducing WVP.	Insaward et al. (2015)
Turmeric and gelatin	Tannic and caffeic acids	No significant differences with unoxidized forms. With oxidized acids, WVP decreased, and E increased.	Choi et al. (2018)

4.1.1. Mechanical properties

The mechanical properties of film-forming materials play an important role in preserving the physical integrity of packaged foods and some values are required to ensure food integrity, avoiding discontinuities (Azeredo & Waldron, 2016).

Cross-linking is frequently used to improve the performance and resistance of biopolymeric films (Choi et al., 2018). In this reaction, bonds between a cross-linking agent and the polymeric chains are created (Hager et al., 2012). Chemical cross-linking is attained if covalent bonds are predominant, while physical cross-linking refers to hydrogen, ionic, and other non-covalent interactions. Given their high number of functional groups, these techniques have been commonly utilized in protein films (Azeredo & Waldron, 2016). Cross-linking results in a reduction of molecular mobility and free volume in the system, which, in turn, gives rise to improved barrier properties, an increased glass transition temperature, improved resistance to light and heat, and a general solubility reduction, while making the structure stiffer (Azeredo & Waldron, 2016). Cross-linking may also retard the biodegradation process in polysaccharides and proteins. The main drawback of cross-linking for food packaging purposes is that the most commonly used agents, such as lineal short-chain aldehydes, have been reported as toxic (Makishi et al., 2013). Genipin and transglutaminase have been recognized as safe crosslinkers, but they are too expensive for industrial applications. Therefore, there is a demand for new low-cost, safe crosslinkers (Picchio et al., 2018).

Phenolic compounds have been reported to act as cross-linking agents in different polymeric matrices, which can benefit the properties of the material (Azeredo & Waldron, 2016). The oxidation of phenolic compounds yields quinones, which can react with the side chains of proteins, forming new covalent bonds (C-N or C-S) and leading to hydrogen bonding (Makishi et al., 2013). Liu et al. (2017) suggested similar mechanisms for this cross-linking effect on chitosan grafted with phenolic acids. Strong intermolecular connections between chains reduce the free space in the polymeric matrix, resulting in a denser, stiffer, and more tightly packed structure, with reduced molecular mobility (Makishi et al., 2013). Nevertheless, some studies describe phenolic acids as film plasticizers rather than cross-linking agents, by forming hydrogen bonds with the polymer matrix, replacing polymer-polymer interactions and increasing the free volume of the matrix, hence reducing tensile strength (Azeredo & Waldron, 2016). Hager et al. (2012) reported both effects of different phenolic acids on the gluten matrix: gallic acid affected the mechanical properties acting as a plasticizer, while tannic acid exhibited cross-linking behavior. Choi et al. (2018) found that, for phenolic oxidized substances, the cross-linking effect predominated at low concentrations whereas the plasticizing effect did at high concentrations. Arcan and Yemenicioğlu (2011) studied the mechanical properties of zein films with several incorporated phenolic acids and found that these had a plasticizing effect, eliminating the brittleness of the films and increasing their elongation capacity. This effect was explained by the binding of phenolic compounds to the zein protein surface, leading to an increased free volume in the matrix. Sharma et al. (2020) also found a crosslinking effect in PLA/PBAT (poly (butylene adipate co-terephthalate)) blend films, obtained by casting, when ferulic acid was incorporated into the polyester matrix at 10% w/w, increasing the film tensile strength and thermal stability. Nevertheless, this effect was not so clearly observed at lower concentrations of ferulic acid.

4.1.2. Barrier properties

The barrier capacity of films is mainly affected by the film-forming material and the additives, as well as by the film preparation process (Cheng et al., 2015). Phenolic acid incorporation tends to reduce the water vapor permeability (WVP) of some biodegradable films, this trend being predominant at low concentrations, where the hydrophobic nature of phenolic compounds hinders water diffusion through the matrix (Cheng et al., 2015). Moreover, the possible cross-linking that could rearrange the matrix occupying the empty molecular space could also difficult the migration of both water and oxygen molecules (Choi et al., 2018). At high concentrations, phenolic acids could promote a heterogeneous arrangement, creating non-bound phenolic fractions and oligomers (Zhang et al., 2010), which could end up permitting the easy passage of water molecules, as observed by Piñeros-Hernandez et al. (2017). Apart from the concentration effect, Choi et al. (2018) reported the specific effect of each phenolic acid by comparing caffeic and tannic acids at the same concentration. Tannic acid reduced WVP to a greater extent, which was attributed to its high number of hydroxyl groups. In most studies, oxygen migration is clearly reduced by phenolic acid incorporation. As mentioned above, this could stem from the cross-linking rearrangement together with the antioxidant effect of phenolic compounds that could promote an oxygen scavenging effect (Bonilla et al., 2012).

4.1.3. Optical properties

Optical properties that might influence the characteristics of a film’s appearance, such as color or transparency, are also important for practical applications. Cheng et al. (2015) reported a significant reduction in lightness (L*) brought about by the addition of phenolic acids to zein and chitosan films, as well as hue or chrome changes in the material. Azeredo and Waldron (2016) also concluded that a general disadvantage of the incorporation of phenolic compounds into films is the promoted color changes. The darkening effect, which was dependent on the phenolic acid added, was partly attributed to the oxidation of phenolic compounds yielding quinones.

4.2 Active properties

The effectiveness of an active packaging material depends on its capacity to preserve food quality and safety. **Table 4** summarizes recent studies into the antimicrobial and antioxidant properties of biodegradable films, mainly based on proteins or polysaccharides, with incorporated phenolic acids or plant extracts rich in phenolic acids. The effectiveness of the film at controlling bacterial growth has predominantly been studied using *in vitro* tests in inoculated culture media, whereas few studies analyse the antimicrobial effects in real foods. Likewise, several methods have been used to test the antioxidant activity of the materials, such as DPPH radical inhibition (Menzel et al., 2019), ABTS radical inhibition (Fabra et al., 2018) or ferric antioxidant power assay (FRAP) (Salgado et al., 2012). In most cases, films loaded with phenolic acids exhibited the expected antimicrobial or antioxidant capacities with differing intensities, depending on the polymer matrix or compound concentration. Azeredo and Waldron (2016) discussed the mechanism of the antioxidant activity caused by phenolic acids. The antioxidant power of PAs, and their potential to reduce the oxygen permeability of the material due to cross-linking, may act together to reduce oxidation reactions in food systems.

In most studies into the active properties of materials with phenolics, biopolymers from biomass (proteins and polysaccharides) prepared by solvent casting were used. However, thermal treatments for film production, which are normally used on an industrial scale, do not seem to significantly reduce their antioxidant activity. Menzel et al. (2019) incorporated sun-flower hull extract into thermoplastic starch and found antioxidant activity in films obtained by melt-blending and compression moulding. Woranuch et al. (2015) used the extrusion method to incorporate pure ferulic acid and ferulic acid coupled to chitosan by means of its bonding to the amino group, into a biodegradable material composed of PLA, thermoplastic starch and PBAT. The radical scavenging activity of materials with incorporated ferulic acid coupled to chitosan was almost 3 times higher than those with pure ferulic acid. Quiles-Carrillo et al. (2019) incorporated gallic acid into PLA by using electrospinning. The antioxidant nanofibers obtained were able to release the active compound directly or through a thin layer of thermally processed PLA.

Table 4 Studies into the incorporation of phenolic acids/plant extracts into biodegradable polymeric matrices and their effect on the film's active properties (antioxidant or antimicrobial).

Material	Active compound	Highlights	Reference
Alginate	Grapeseed or green tea extracts	Both extracts impaired the antiviral (against murine norovirus and hepatitis A virus) and antioxidant (TEAC) activities of the films.	Fabra et al. (2018)
Alginate	Tannic acid	Films exhibited antioxidant activity as a function of the concentration of tannic acid.	Kaczmarek (2020)
Bovine gelatin	Rosmarinic acid	Long-term antibacterial activity was observed against *E. coli* and *S. aureus.* The ABTS method showed effective antioxidant activity.	Ge et al. (2018)
Cassava starch	Rosemary extract	DPPH scavenging activity increased as did the polyphenol content of the films.	Piñeros-Hernandez et al. (2017)
Chitosan	Propolis extract	Enhanced antimicrobial activity against *E. coli, S. aureus, Pseudomonas aeruginosa and Salmonella enteriditi*s. Antioxidant capacity (DPPH) greatly increased.	Siripatrawan & Vitchayakitti (2016)
Chitosan	Gallic, ferulic, p-coumaric, salicilyc, and vanillic acids	DPPH scavenging activity was significantly increased in all formulations except with salicilyc acid. Antibacterial activity was enhanced by all acids. Ferulic and p-courmaric formulations extended Shrimp shelf life.	Liu et al. (2021)
Chitosan, gelatin or MC	Tannic acid	Growth inhibition of *E. coli* and *S. aureus* was reported for all the formulations containing tannic acid.	Halim et al. (2018)
Chitosan-PVA	Mint or pomegranate peel extracts	Films exhibited antibacterial activity against *S. aureus* and *B. cereus* but did not inhibit the growth of Gram-negative bacteria, such as *E. coli or P. fluorescens.* Extracts conferred antioxidant properties on the films as determined by the DPPH method.	Kanatt et al. (2012)
Corn starch	Tannic acid	Films inhibited the growth of *E. coli* and *L. monocytogenes*. Tannic acid solutions exhibited antioxidant activity, protecting vegetable oils from air oxidation (Rancimat method). Tannic acid was reported to gain activity after thermal processing.	Pyla et al. (2010)
Gellan gum	Coffee parchment waste extract	Gallic, chlorogenic, p-coumaric and synaptic acids were identified in the extract. Films exhibited antifungal activity against *Fusarium verticillioides*, *Fusarium sp.*, and *Colletotrichum gloeosporioide*.	Mirón-Mérida et al. (2019)
PLA (electrospun)	Gallic acid	DPPH assay showed the high antioxidant capacity of electrospun mats. The release of the acid through a multilayer assembly was demonstrated in the saline medium.	Quiles-Carrillo et al. (2019)
PLA-starch-PBAT blend.	Ferulic acid (FA) or ferulic acid-coupled chitosan	DPPH and reducing power assay showed that FA-coupled chitosan was a significantly stronger antioxidant agent than FA. The peroxide value of potato chips stored in the films containing FA-coupled chitosan was reduced by around 32%.	Woranuch et al. (2015)
PLA-PBAT blend	Ferulic acid	Films effectively inhibited *E. coli* and *L. monocytogenes* growth in liquid media, as a function of ferulic acid concentration.	Sharma et al. (2020)
Potato starch	Sunflower hull extracts	Chlorogenic acid was identified by the DPPH method as the main active compound with demonstrated antiradical activity.	Menzel et al. (2019)
Sodium alginate	Tannic acid	The antioxidant activity increased with the increasing content of tannic acid	Kaczmarek (2020)

Sunflower protein concentrate	Naturally present phenolic compounds	Films exhibited considerable antioxidant capacity (ABTS, FRAP and PCL assays) but no antimicrobial activity against 26 microbial strains.	Salgado et al. (2012)
Tuna or bovine gelatin	Oregano or rosemary extracts	Rosmarinic acid was the most abundant phenolic compound of the extracts. Both extracts promoted the antioxidant activity (FRAP and ABTS) of the gelatin matrices. In the same extract concentration, bovine gelatin was more active than tuna gelatin.	Gómez-Estaca et al. (2009)
Turmeric-gelatin	Oxidized green tea extract, tannic, or caffeic acids	Turmeric-gelatin films exhibited DPPH inhibition capacity, which was not improved by the incorporation of phenolics. When applied to ground pork meat, all the films were effective at reducing TBA values.	Choi et al. (2018)
Zein	Gallic acid	Films exhibited antioxidant capacity (ABTS) and inhibited the growth of *Listeria monocytogenes* and *Campylobacter jenuni.*	Arcan & Yemenicioğlu (2011)
Zein	Gallic, vanillic or cinnamic acids	Films containing PA inhibited plant pathogens, such as *Erwinia amylova, Erwinia cartavora, Xatnhomas vesicatoria* and *Pseudomonas syringae*. Gallic acid exhibited the highest activity.	Alkan & Yemenicioğlu (2016)
Zein-chitosan	Ferulic or gallic acids	Both acids in the composite films enhanced the growth inhibition of *E. coli* and *S. aureus,* while also conferring antioxidant activity (DPPH and ABTS).	Cheng et al. (2015)

4.2.1. Antioxidant properties

Arcan and Yemenicioğlu (2011) studied the incorporation of gallic acid into zein films and obtained films with a high ABTS radical inhibition, which release 88% of the incorporated acid. This high release rate, associated with the high antioxidant capacity, was attributed to the relaxation of the hydrophilic matrix in contact with water. Piñeros-Hernandez et al. (2017) studied the antioxidant activity of starch incorporated with rosemary extract which exhibited high release rates of active compounds in water, but only a very small quantity of phenolics was delivered when using ethanol as a fatty simulant. Phenolic acids can also exert their antioxidant capacity through their oxidized form, as reported by Choi et al. (2018) in the case of turmeric-gelatin films loaded with oxidized extracts. While the DPPH radical scavenging activity of turmeric-gelatin films was not significantly affected by any component, the films with oxidized extracts notably limited the oxidation of stored pork meat. Nevertheless, a good antioxidant capacity is not necessarily correlated with a good antimicrobial activity, Salgado et al. (2012) developed films with sunflower protein concentrate, which presented good antioxidant capacity that was attributed to the main PAs found in the concentrate (chlorogenic and caffeic acids). However antimicrobial assays showed no growth inhibition against any of the 21 different strains tested, which was attributed to the high pH value (around 11) in the film-forming dispersions that promoted the dissociation of acids, thus reducing their antimicrobial activity.

4.2.2. Antimicrobial activity

Antimicrobial activity of potentially active films has been estimated by different methods; qualitative methods offer an initial approach to discover the potential activity while quantitative methods should be carried out for final conclusions. Pyla et al. (2010) used disc diffusion in agar medium and viable cell counts to test the antimicrobial activity against *E. coli* and *L. monocytogenes* of starch-based films with tannic acid submitted or not to thermal treatment. The zone of inhibition successfully increased along with tannic acid concentration for both treatments. However, cell counts for untreated tannic acid did not significantly inhibit *L. monocytogenes* in TSB media. Likewise, Fabra et al. (2018) proved the antiviral properties of alginate films carrying grape seed or green tea extracts against murine norovirus and hepatitis A virus. Although some antiviral activity was imparted by the films with extracts, there was significantly less than in the pure extracts, which suggests that a part of the active compounds was retained in the films by bonding with the alginate matrix. Cheng et al. (2015) incorporated gallic and ferulic acids into zein-chitosan composite films and observed growth inhibition of *E. coli* and *S. aureus* for films with both acids. The greatest antibacterial effect was obtained in the case of gallic acid, which contrasts with previous studies which report lower MIC values for ferulic acid than for gallic acid against *E. coli* (Sánchez-Maldonado et al., 2011). This was explained by the interactions of chitosan and ferulic in the film matrix that limit its release and effective action against bacteria. Ge et al. (2018) observed antibacterial activity against *E. coli and S. aureus* for gelatin films with rosmarinic acid (with very low MIC values). They described morphological changes associated with the destruction of the bacterial cell membrane after being in contact with the films, using SEM observations.

Few studies analysed the sensory impact of PAs incorporated into the packaging materials on the food matrices in contact. Choi et al. (2018) studied the organoleptic acceptance of pork meat packaged with turmeric-gelatin films loaded with oxidized tannic and caffeic acid and no negative effect on the product sensory acceptance was observed while lipid oxidation was prevented. Liu et al. (2021) observed an improved sensory score in shrimps (*Penaeus vannamei*) stored in chitosan films with ferulic and p-coumaric acids, in comparison with those in contact with films of sole chitosan. While limited, these studies showed promising results for active food packaging with phenolic acids to enhance food shelf life without altering organoleptic properties.

Ferulic, gallic and tannic acids are among the most widely-studied phenolic acids as regards the development of active packaging applications. These and other promising compounds, such as cinnamic or rosmarinic acids, are yet to be further explored for antimicrobial packaging applications in food matrices. However, the antimicrobial effectiveness of potentially active films is not only dependent on the effectiveness of the incorporated active compound, but also on other factors affecting the release kinetics of the active agent from the food-packaging material and its final partition between packaging and food. The following section analyses these factors in order to understand the different activities found for determined compounds, depending on the polymeric matrix and food substrate.

5. Factors affecting the antimicrobial efficiency of biodegradable active materials in contact with food products

Target microorganism(s) must be identified prior to adequate selection of the active ingredient for the purposes of designing effective antimicrobial films for food packaging. Moreover, polymers must be selected according to their functional properties and the specific requirements of the food product. The incorporation of an antimicrobial agent into the polymeric material might modify its functional properties; therefore, these must be reanalysed to assess the true effectivity of the food packaging system. As discussed above, some antimicrobial additives, such as several phenolic acids, can act as plasticizers or crosslinkers, modifying the tensile properties of the films. However, in general, the film strength has been observed to decrease while the concentration of such active substances increases (Choi et al., 2018). Interactions of the active compounds and the polymer through hydrogen bonds, hydrophobic or electrostatic forces can influence the physical properties of the material linked to its functionality (Zhu, 2021). Likewise, the presence of antimicrobial compounds in the material may affect its biodegradation behaviour since the release of these compounds could alter the microbial population responsible for this phenomenon; while these compounds modify the sensitivity of the polymer matrix to the physical degradation processes through changes in the matrix cohesion forces or wetting capacity (Hernández-García et al., 2021a).

5.1. Factors determining the release and efficiency of the antimicrobial agent

As concerns the antimicrobial effectiveness of the material containing antimicrobial agents for food preservation purposes, **Figure 2** shows different factors that can affect the release of the active compounds from the polymeric matrix and their migration to the food product surface, which will in turn, affect their antimicrobial activity. The compositions of both the polymeric matrix and the foodstuff, as well as the interactions between components, determine the migration mechanisms between both phases (packaging material and food) in contact, resulting from the differences in the chemical potential of each component in the respective phase. Thus, both the release kinetics and the amount of antimicrobial at equilibrium released into the food system determine its effective action. Moreover, the food composition also affects the effectiveness of the antimicrobial agent. The foodstuff composition impacts on the microbial growth and the food components can exert different/opposite functions, thus affecting antimicrobial action. In fact, some components are adequate nutrients for the microbial population, enhancing its vitality and resistance, while some of these can act as sequestrants on the active compound due to specific interactions. Requena et al. (2019a) found that the microbial growth inhibition of essential oil compounds, such as carvacrol and eugenol incorporated into poly(3-hydroxybutyrate-co-3-

hydroxyvalerate) (PHBV) matrices, was different in several food matrices, such as cheese, chicken breast, and fresh-cut melon or pumpkin. Food components can modify the interactions of antimicrobial compounds and pathogens; certain lipids and proteins are known to exert a protective action on bacterial targets (Juneja et al., 2012). It has also been suggested that, given the lipophilic nature of many antimicrobial compounds, they tend to dissolve in the lipid phase or bind to other components of the food and become less available to bacteria in the aqueous phase, hence requiring higher concentrations to exert their antimicrobial activity (Prakash et al., 2018). Likewise, phenolic compounds can react with free radicals stemming from fat oxidation, which could also reduce their effectiveness (Pyla et al., 2010). Additionally, some other characteristics of the food substrate, such as water activity and pH, can alter the MIC for a given microorganism – antimicrobial combination (Juneja et al., 2012; Miyague et al., 2015).

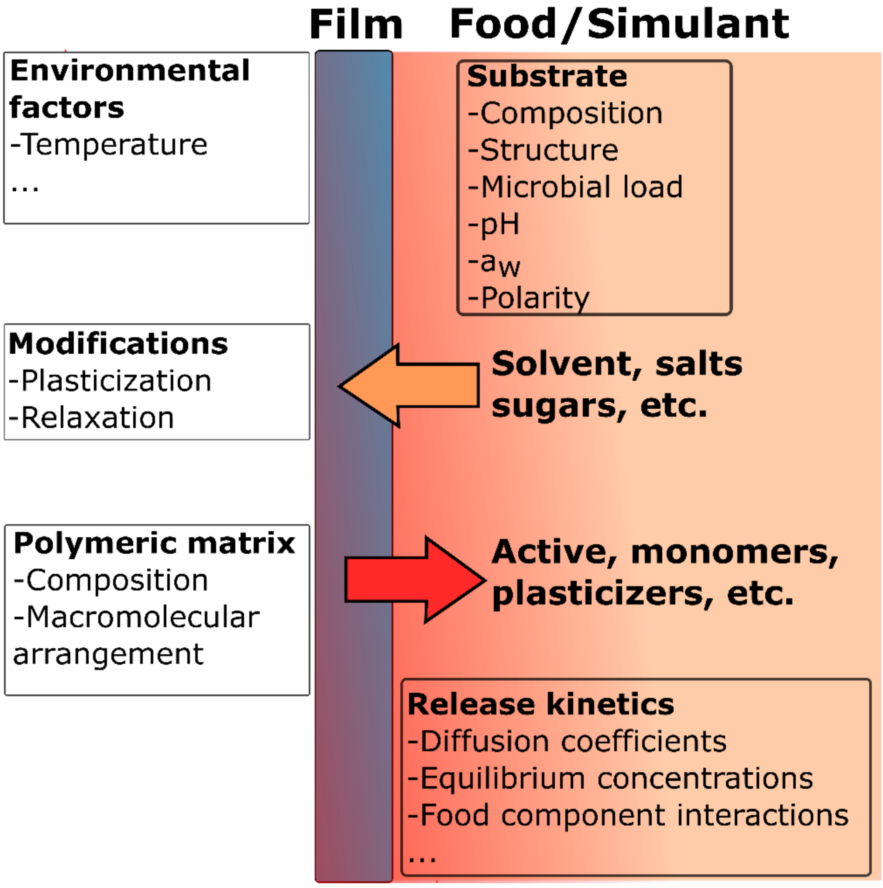


Figure 2 Mass transfer phenomena between film packaging and food systems, showing the factors influencing the antimicrobial effectiveness of active films.

The active compound concentration released during the storage time is an essential factor when it comes to ensuring antimicrobial efficiency and food safety (Requena et al., 2019a). Therefore, the antimicrobial efficiency of films carrying active compounds not only depends on the nature of the additive, but also on the capacity of the film to release it into the food substrate at an adequate rate, finally maintaining an appropriate concentration at equilibrium. This, in turn, depends on the active compound interactions with the polymer matrix and its solubility in the food system (Requena et al., 2017). The chemical affinity of the active substance with both phases (packaging material and food system) will greatly affect the equilibrium concentrations reached in both phases (partition coefficient). In some cases, the entire antimicrobial compound is rapidly released into the food matrix and its effect ceases once it is depleted. The minimal inhibitory concentration of the compound for a target microorganism must be locally reached in the contaminated point of the food system.

Some chemical species also migrate from the food substrate towards the polymeric matrix. The release kinetics of the active compound is, hence, affected by three mechanisms: (1) the diffusion of food components, such as water or fat, towards the polymeric matrix, (2) the relaxation of the macromolecular structure due to diffusion/plasticization, and (3) the diffusion of the active compound through the relaxed polymeric matrix until reaching thermodynamic equilibrium between film and food (Requena et al., 2017). Matrix plasticization improves molecular mobility, and so the rate of all the diffusional processes can markedly increase. In consequence, the ability of the polymeric matrix to be plasticized by migrated food components (such as water or oil) can greatly accelerate the diffusion process.

The MIC value of target microorganisms must be reached in the food matrix in contact with the active film in order to effectively prevent the antimicrobial growth. This state must be reached at the right time to avoid the initial proliferation that could augment the resistance of the target microorganism. Nevertheless, the food matrix composition, pH, water activity and other factors can change the MIC value for a specific microorganism and active compound. pH plays a particularly important role in the effectivity of ionizable compounds in aqueous media, such as phenolic acids, since it influences the proportion of dissociated and protonated forms with different activities. Miyague et al. (2015) demonstrated that the antimicrobial activity of phenolic acids is higher at a pH below their pKa, when the protonated form predominates.

Environmental factors, such as temperature, can affect the release rate and the antimicrobial effectiveness of active compounds. Provided the antimicrobial additive is not thermo-sensitive, a high temperature can potentially increase the release rate and improve its antimicrobial effect to a greater extent than microbial growth. However, this balance could ultimately lead to a counterproductive result. The temperature has a great impact on the molecular mobility of the polymeric matrix, especially if it is close to the glass transition temperature of the material. Increased molecular mobility would result in improved antimicrobial properties due to the enhanced release of the additive.

5.2. Modelling release kinetics

For a given active compound, analysing the release kinetics from a polymeric matrix into food simulants of varying polarities may help to predict the antimicrobial activity in a similarly polar food product. Several types of food simulants can be used. As some examples, 10% ethanol (Simulant A) and 3% acetic acid (Simulant B) aqueous solutions are used to simulate aqueous food matrices with pH above and below 4.5, respectively. 50% ethanol aqueous solution (Simulant D1) is used to simulate less polar matrices, such as oil-water emulsions or highly alcoholic products, and isooctane is used to simulate lipid matrices (Commission Regulation (EU) No 10/2011 of 14 January 2011, 2011). Several authors (Muller et al., 2017; Requena et al., 2017, 2018; Tampau et al., 2018) studied the release kinetics of essential oil compounds incorporated into biodegradable films using these four simulants. To this end, the amount of active compound released into the simulant as a function of contact time was modelled using a Fickian approach, in terms of the mass of active compound released related to the total mass of the active in the film (M_t/M_o). Thus, the diffusion coefficient (D) and the mass of the active compound released at equilibrium: (M_∞/M_o) were determined. **Table 5** summarizes the obtained diffusion coefficient (D) and the released percentage at equilibrium of different active compounds incorporated into different polymeric matrices in contact with the abovementioned food simulants. Other mathematical models have been applied by different authors, such as first order kinetics (Tawakkal et al., 2016), Peppas and Weibull models (Bustos et al., 2016) or the Fick model (Tawakkal et al., 2016), to determine the release rate of active compounds and the maximum release at equilibrium. The models permit the concentration of the active substance reached in a food matrix with similar polarity at a given time to be estimated from a determined concentration of active substance incorporated into the packaging and a food/packaging mass ratio (Requena et al., 2017).

Table 5. Diffusion coefficient (D) and mass percentage of active released at equilibrium with respect to the total mass in the film (M_∞/Mo) for several polymeric matrices with active compounds in contact with food simulants.

Polymeric matrix	Active compound	Simulant A		Simulant B		Simulant D1		Simulant D2		Reference
		$Dx10^{13}$ (m^2/s)	M_∞/Mo (%)	$Dx10^{13}$ (m^2/s)	M_∞/Mo (%)	$Dx10^{13}$ (m^2/s)	M_∞/Mo (%)	$Dx10^{13}$ (m^2/s)	M_∞/Mo (%)	
PHBV	Carvacrol	3.2	22	2.8	23	7.2	96	0.15	65	Requena et al. (2017)
PHBV	Eugenol	0.5	47	0.5	52	5.5	92	0.023	71	Requena et al. (2017)
PLA-PHBV (Blend)	Carvacrol	30	19	2	25	280	76	6	86	Requena et al. (2018)
PLA-PHBV/ Starch	Carvacrol	5	22	15	11	5	100	2	70	Requena et al. (2018)
PLA	Cinnamaldehyde	0.024	48	0.019	45	0.63	55	0.0031	5.5	Muller et al. (2017)
PLA/Starch	Cinnamaldehyde	0.53	35	0.15	29	13.8	27	0.018	8	Muller et al. (2017)
Starch/PCL /Starch	Carvacrol	3.9	75	5.3	57	46	100	100	96	Tampau et al. (2018)

As can be observed in **Table 5,** both the release rate (diffusion coefficient, D) and release percentage of an active compound were greatly affected by both the polarity of the food simulant and the polymeric matrix. This is related to the different molecular interactions and chemical affinity between the active compound, the polymeric matrix and the simulant. The diffusion coefficient of a determined compound from a polymer matrix was significantly affected by the polarity of the simulant, as a consequence of the potential simulant migration into the film matrix and the resulting plasticization effect. Thus, the release of carvacrol and eugenol from the PHBV matrix was greatly promoted when the polarity of the simulant decreased (D1) due to both the higher degree of polymer relaxation in contact with the simulant and the greater affinity of active additives with the simulant, which contribute to a partition of the compounds that is more favourable to the simulant. In this sense, it is remarkable that there were differences between the release behaviour of carvacrol and eugenol for the same polymer matrix and simulant; carvacrol was delivered faster but to a lower extent from PHBV than eugenol in every aqueous simulant, in line with their different chemical affinity with polymer and simulants. In general, given the less polar nature of the active compounds, more compound is delivered when the simulant polarity decreases; however, considering the different polar and non-polar phases present in the food systems, the release behaviour can differ substantially in real foods, depending on the food structure. Film structure also has a great impact on the release kinetics and the concentrations at equilibrium. Therefore, particular molecular interactions inside the polymeric matrix and food system play an important role in the release kinetics of active compounds and, in complex systems, such as foods, it is difficult to easily predict the release behaviour and so the antimicrobial effects. Therefore, specific release and antimicrobial studies are required to ensure the effectiveness of a determined potentially active material applied to a determined food system. These interactions become more complicated for multilayer films consisting of different polymeric layers, where the process is affected by the interactions and migration phenomena between interlayers and simulant/food. In fact, Requena et al. (2018) observed different carvacrol diffusion and equilibrium release in PHBV/PLA blend-starch bilayer films to that observed in PHBV monolayers (Table 5). Quiles-Carrillo et al. (2019) studied the release of gallic acid from electrospun fibres of PLA with and without a 10-µm PLA coating. The PLA coating acted as a barrier that affected the release kinetics; a sustained release was observed when the thin PLA barrier was used, whereas it was fast and abrupt otherwise. Similar results were reported by Neo et al. (2013) while electrospinning zein and gallic acid solutions, obtained fibers presented fast release with a fickian behaviour.

6. Conclusions

Biodegradable polymeric films coming from natural sources offer a promising alternative for the food packaging sector. Through the incorporation of different compounds into these matrices, active packaging materials can be obtained for specific applications, adding value to these materials with high production costs. In this sense, phenolic acids represent an alternative to synthetic additives and essential oils with a high sensory impact. Different polysaccharides and proteins have proven to have good properties as carriers of these active compounds, although their application is, in practice, limited by their highly hydrophilic nature. Studies into hydrophobic matrices, such as PLA, PHB or PCL, with incorporated phenolic acids are still scarce, but they point at the viability of these materials for application in high water activity systems. Of the phenolic acids, hydroxycinnamic acids exhibit antimicrobial activity regardless of the substituents in the benzoic ring, while their antioxidant capacity is also enhanced when compared to their equivalent hydroxybenzoic acid. However, the specific molecular structure would also affect the release kinetics from the film into the food system due to the particular interactions between the active compound, the polymer matrix and the food substrate. Therefore, although the prospects for future applications of phenolic acids in active packaging seems promising, more research is required into the release kinetics and efficiency of different phenolic acid – polymer combinations when applied to specific real foods.

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Author contributions

Ramón Ordoñez: Conceptualization, Investigation, Writing-original draft. **Lorena Atarés**: Conceptualization, Data curation, Supervision, Writing-reviewing and editing. **Amparo Chiralt**: Conceptualization, Supervision, Writing-reviewing and editing, Project administration, Funding acquisition.

Conflicts of Interest

Authors declare no conflicts of interest.

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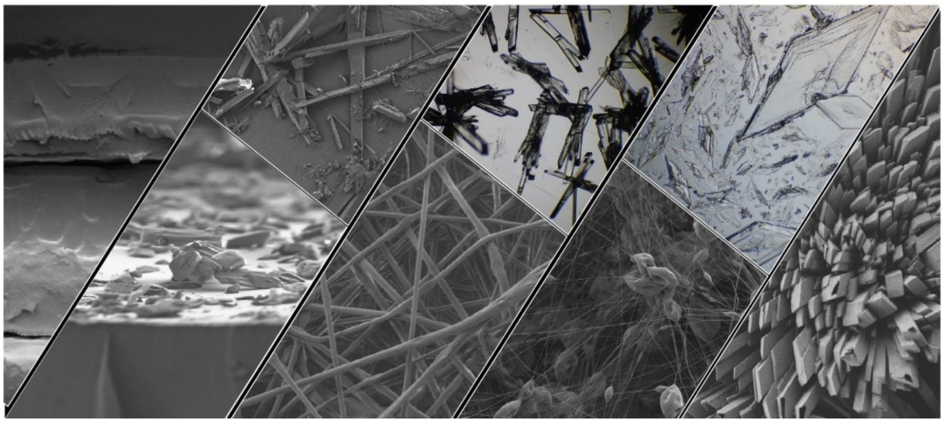
I. Introduction

II. Objectives



II. Objectives

Objectives

The **general objective** of the thesis was to obtain active films based on starch and PLA by incorporating ferulic or cinnamic acids as active compounds, in order to develop active multilayer assemblies useful to meet food packaging requirements in terms of barrier and mechanical properties as antibacterial capacity to extend the product shelf life.

To reach the main objective, the following **specific objectives** and working plan were proposed:

1) Analyse the effect of incorporation of ferulic or cinnamic acids on functional properties of cassava starch films, as well as their antibacterial action

For that purpose, starch films with 1 and 2 % ferulic or cinnamic acid were obtained by melt blending and compression moulding and their physical and antibacterial action were evaluated, using inoculated culture media, chicken breast and fresh-cut melon with *Escherichia coli* and *Listeria innocua*.

2) Analyse the effect of incorporation of ferulic or cinnamic acids on functional properties of thermoprocessed PLA films and the active release and antimicrobial action.

The same concentration (1 and 2 %) of ferulic or cinnamic acids were incorporated into thermoprocessed PLA films, analysing their effect on the physical properties of the films, the active release in food simulants and the antibacterial action.

3) Analyse the factors affecting the antibacterial properties of cinnamic and ferulic acids incorporated into starch and PLA monolayer and multilayer films.

Ferulic (F) and cinnamic (C) acid at higher concentration (2 or 3%) were incorporated into starch and PLA films, using several strategies to promote their release from PLA, in order to obtain effective antimicrobial activity: film thermoprocessing, thermo-processing with the addition of plasticiser (TPP), solvent casting, and three-layer PLA assemblies with an internal starch film loaded with F or C, using TP-PLA, TPP-PLA, or cast-PLA. The antibacterial activity against *Escherichia coli* and *Listeria innocua* were analysed in the different films.

4) Analyse the effect of processing method on the properties of PLA films with different concentration of cinnamic acid.

PLA films with cinnamic acid up to 10 % were processed by melt blending and compression moulding and by solvent casting. Their physical properties and antimicrobial activity were evaluated. The influence of the film processing method was analysed on both physical and antibacterial properties.

5) Analyse the effectiveness of the superficial incorporation of ferulic acid, using different methods (adsorption, surface pulverization and electrospinning) on the antimicrobial action of PLA films.

Thermoprocessed PLA films were submitted to adsorption process from ferulic acid solutions, spraying with ferulic acid solutions and coating by electrospinning with PLA-ferulic acid solutions, using different solvent systems. The surface film microstructure and antibacterial activity were analysed.

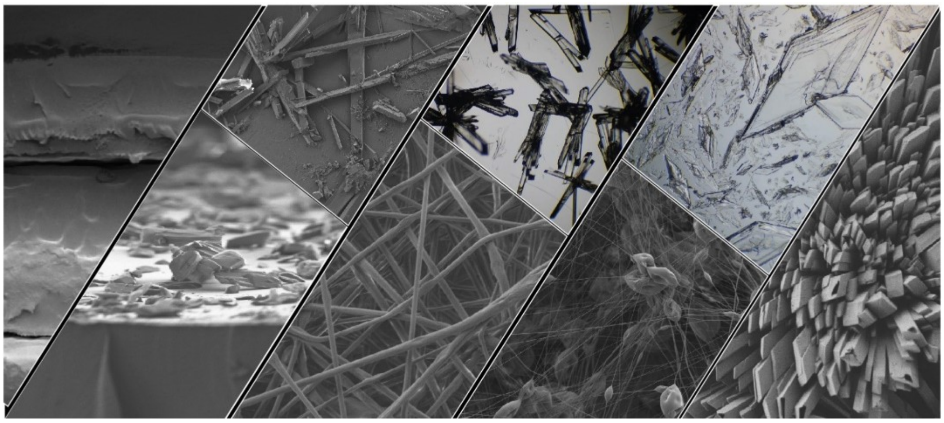
6) Design and characterization of multilayer films based on cassava starch and PLA layers with ferulic and cinnamic acids with effective release of the active compounds and antimicrobial activity.

Three-layer films with internal starch layer and external PLA layers were obtained and coated with ferulic or cinnamic acids by pulverization with ethanol solutions of the active compounds or electrospinning with fibre-forming PLA-acid solutions. The physical and antimicrobial properties of the three-layers were evaluated.

The results of the thesis have been structured in different **chapters** that correspond to scientific publications that cover the different specific objectives.

II. Objectives

III. Chapters



Chapter list

Chapter 1:

Physicochemical and antimicrobial properties of cassava starch films with ferulic or cinnamic acid.

Chapter 2:

Effect of ferulic and cinnamic acids on the functional and antimicrobial properties in thermo-processed PLA films.

Chapter 3:

Antibacterial properties of cinnamic and ferulic acids incorporated to starch and PLA monolayer and multilayer films.

Chapter 4:

Properties of PLA films with cinnamic acid: effect of the processing method.

Chapter 5:

Antilisterial action of PLA films with ferulic acid as affected by the method of incorporation.

Chapter 6:

Multilayer antimicrobial films based on starch and PLA with superficially incorporated ferulic or cinnamic acids for active food packaging purposes.

Chapter 1:

Physicochemical and antimicrobial properties of cassava starch films with ferulic or cinnamic acid.

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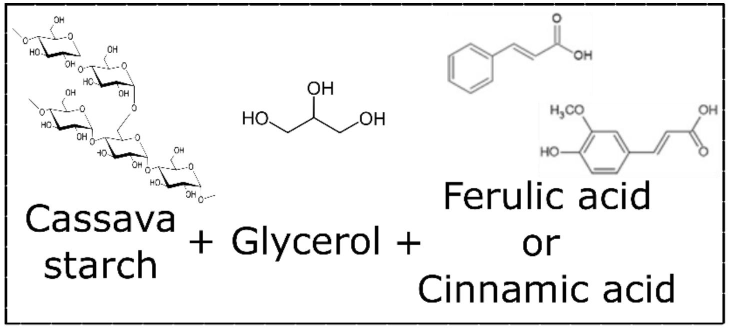
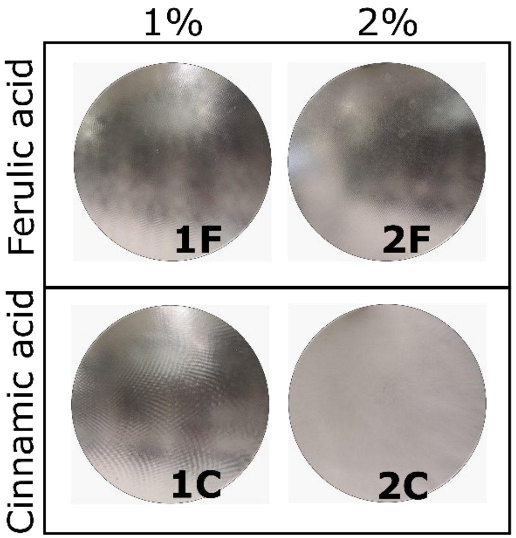


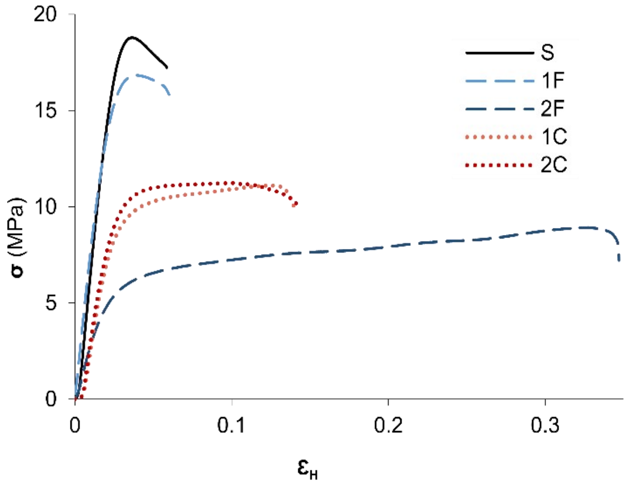
Cassava starch + Glycerol + Ferulic acid or Cinnamic acid

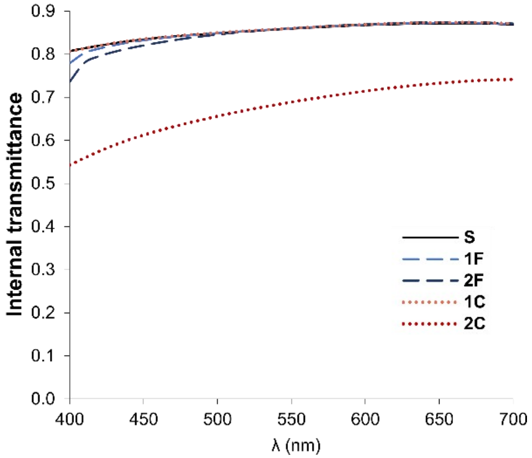
Melt blending & Compression moulding

Antimicrobial starch films



Functional characterization

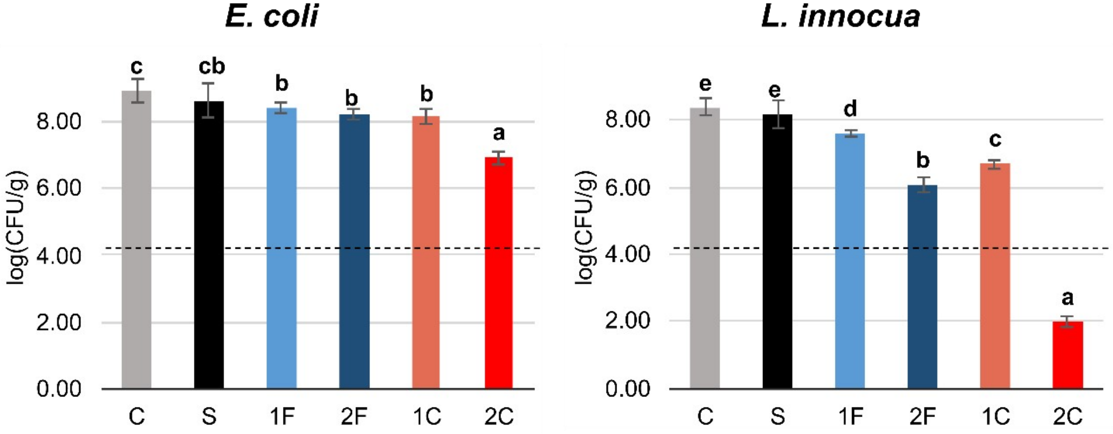




Phenolic acids decreased water solubility and did not cause significant changes on barrier properties.

Antimicrobial tests

Bacterial counts at 6 days of incubation (*in vitro* tests)


E. coli

L. innocua

Films were also effective on chicken breast and fresh cut melon.

Abstract

Plasticized cassava starch films with 1-2% ferulic or cinnamic acid were obtained by melt blending and compression moulding. Partial losses (about 40%) of the more thermo-sensitive ferulic acid occurred during processing. Incorporation of phenolic acids did not provoke remarkable changes in the oxygen and water vapour permeability of starch films, but these became less water soluble (13-25% reduction), more extensible and less resistant to break, especially with the highest concentration of ferulic acid. Films with 2% of either acid effectively inhibited the growth of *E. coli* and *L. innocua* in tests carried out in culture medium; being cinnamic acid more effective than ferulic and *L. innocua* more sensitive than *E. coli*; a Log CFU reduction, with respect to the control, higher than 6 was obtained for *L. innocua* with 2% of cinnamic acid in the films. The growth of both strains was also inhibited in chicken breast (log CFU reduction>2) with 2 % of either acid. In fresh-cut melon, the *L. innocua* growth was significantly inhibited by films with 2% cinnamic acid, but lower growth inhibition of *E. coli* in fresh-cut melon was observed.

1. Introduction

The food industry currently relies on mostly single-use, petroleum-derived non-biodegradable plastic materials to satisfy the food packaging requirements. Specifically in Europe, only 30% of the plastic produced is recycled (Gong, Putnam, You, & Zhao, 2020; Moreno, Cárdenas, Atarés, & Chiralt, 2017). The environmental impact of plastic use and mishandling is reaching a critical point (Lestari & Trihadiningrum, 2019; Qi, Jones, Li, Liu, & Yan, 2020; Rodrigues et al., 2019). Furthermore, consumers demand more sustainable and less processed products with fewer or no synthetic additives (Carocho, Barreiro, Morales, & Ferreira, 2014; Kushwah, Dhir, & Sagar, 2019). However, food safety needs to be ensured and both packaging and additives are needed for this. The development of active packaging materials using biodegradable polymers and naturally-occurring antimicrobial/antioxidant compounds is necessary to improve food preservation and extend shelf-life in a more sustainable way (Atarés & Chiralt, 2016).

Starch is a naturally occurring polymer, constituted by amylose and amylopectin, which can be obtained from a variety of renewable sources, such as cassava, potato or maize (Domene-López, Delgado-Marín, Martin-Gullon, García-Quesada, & Montalbán, 2019). Starch has proven to be a good alternative for traditional food packaging, since it can be thermo-processed with plasticisers to obtain adequate materials (Menzel, González-Martínez, Chiralt, & Vilaplana, 2019; Ochoa-Yepes, Di Giogio, Goyanes, Mauri, & Famá, 2019). It is cheap and provides transparent films with excellent oxygen barrier capacity (Ortega-Toro, Muñoz, Talens, & Chiralt, 2016; Tampau, González-Martínez, & Chiralt, 2018). However, given its hydrophilic nature, starch films are very sensitive and permeable to water vapour, making applications on high moisture foods very difficult (Acosta, Jiménez, Cháfer, González-Martínez, & Chiralt, 2015; Ochoa-Yepes et al., 2019). Several strategies to overcome this drawback have been studied, such as promoting crosslinking with different chemicals or combining starch with other polymers with complementary properties, either by blending them or using multilayer film assemblies (Fang, Fu, Tao, Liu, & Cui, 2020; Muller, González-Martínez, & Chiralt, 2017; Ochoa-Yepes et al., 2019; Requena, Vargas, & Chiralt, 2018).

Phenolic acids are characterised by the presence of a benzene ring, a carboxylic group and at least one hydroxyl or methoxyl group (Lima et al., 2016). The antioxidant and antimicrobial activities of these naturally-occurring compounds have been studied in the last few years (Guil-Guerrero et al., 2016; Lima et al., 2019). Specifically, ferulic and cinnamic acids have shown effective growth inhibition of both gram positive and negative bacterial strains at concentrations below 1 mg/g (Meira, Holley, Bordin, Macedo, & Luciano, 2017; Miyague, Macedo, Meca, Holley, & Luciano, 2015; Olasupo, Fitzgerald, Gasson, & Narbad, 2003). The incorporation of highly active phenolic acids, such as ferulic and cinnamic, into a biodegradable polymeric matrix, such as starch, could result in a filmogenic material with antimicrobial properties for food packaging. Although previous studies (Benbettaïeb, Karbowiak, Brachais, & Debeaufort, 2015; Fabra, Hambleton, Talens, Debeaufort, & Chiralt, 2011) analysed the effect of ferulic acid on the properties of sodium caseinate and chitosan films, antibacterial capacity of these films was not reported.

The aim of this study was to analyse the effect of the incorporation of different ratios of cinnamic and ferulic acids into thermoplastic cassava starch films, by analysing the changes induced in the films' functional properties as packaging material. The antimicrobial action of the phenolic acid-loaded films in both the culture media and different food substrates was also analysed.

2. Materials and methods

2.1. Materials

Cassava starch produced by Asia Co., LDT (Kalasin, Thailand) and purchased from Quimidroga S.A. (Barcelona, Spain), as well as glycerol as plasticiser (Panreac Química, Barcelona, Spain) were used to obtain films. Cinnamic and ferulic acids (Sigma-Aldrich, Saint Louis, USA) were used as antimicrobial compounds. Magnesium chloride and phosphorus pentoxide, used to control the sample relative humidity (RH) were supplied by Panreac Química (Barcelona, Spain). Tryptone soy broth, tryptone soy agar, phosphate-buffered saline, and peptone water for microbial test were purchased from Scharlab (Barcelona, Spain). Strains of *Listeria innocua* (CECT 910) and *Escherichia coli* (CECT 101) were from the Spanish Type Collection (CECT, University of Valencia, Spain).

2.2. Film preparation

Glycerol (G) was added to cassava starch in a ratio of 0.30 g/g starch. Cinnamic (C) or ferulic (F) acids were added at 0, 1 or 2 g acid / 100 g dry film. Melt blending of components was carried out in an internal mixer (Haake PolyLab QC, Thermo Fisher Scientific, Germany) at 130 ºC and 50 rpm for 10 min. The obtained melts were cold ground and conditioned at 33% RH and 25 ºC in desiccators containing an oversaturated solution of $MgCl_2$. Films of about 20 cm in diameter were obtained with 4 g of conditioned samples, using a hydraulic heating press (LP20, Labtech engineering, Thailand). Samples were preheated at 160 ºC for 1 min and compressed at 5 MPa for 2 min, plus 10 MPa for 6 min. Finally, a 3 min cooling step to 70 ºC was applied. The films obtained were conditioned at 33% RH and 25 ºC for at least 7 days before characterization. The different samples were coded as S (starch films without actives), 1F, 2F (starch with ferulic acid at 1 or 2 % w/w); and 1C, 2C (starch with cinnamic acid at 1 or 2 % w/w).

2.3. Characterization of the films

2.3.1. Thermal analysis

The thermal properties of the films were measured using a differential scanning calorimeter (DSC, 1 StareSystem, Mettler-Toledo, Switzerland) and a thermogravimetrical analyser (TGA/SDTA 851e, Mettler-Toledo, Switzerland). Prior to the analyses, all the samples were conditioned at 0% RH for 1 week in a desiccator with P_2O_5. DSC samples (about 10 mg) were placed into aluminium pans and sealed. Samples were heated from room temperature to 160 ºC at 10 K/min, maintaining 160 ºC for 5 min, cooling at 50 K/min to -10 ºC, maintaining at -10 ºC for 5 min and applying a second heating to 160 ºC at 10 K/min. An empty aluminium pan was used as reference. For TGA analyses, samples of about 10 mg were placed into alumina crucibles and heated from 25 to 600 ºC at 10 K/min, under a nitrogen flow (50 mL/min). Both analyses were performed in duplicate for each sample.

2.3.2. Retention of active compounds, moisture content and water solubility of the films

The final content of ferulic and cinnamic acids in the films was determined through methanol extraction and spectrophotometric quantification. Strip samples (about 100 mg) preconditioned at 0% RH with P_2O_5, were immersed in 10 ml methanol while being gently stirred for 48 hours. Methanol extracts were filtered and properly diluted for the absorbance measurements at the wavelength of maximum absorbance (320 nm for F and 270 nm for C), by using a UV-visible spectrophotometer (Thermoscientific Evolution 201, USA). The respective methanol extract of the film without active compounds was used as blank. The absorbance measurement was transformed into a concentration using the previously determined calibration curve for each active compound. All measurements were taken in triplicate.

The moisture content of films conditioned at a specific RH was gravimetrically determined by completely drying the conditioned films in a vacuum oven at 60ºC for 48 hours and their subsequent conditioning at 0% RH (using P_2O_5) at room temperature until constant weight (Cano, Cháfer, Chiralt, & González-Martínez, 2015). The results were expressed as g water/100 g of film.

In order to quantify the film's water solubility, the samples conditioned at 0% RH were immersed in vials with 10 ml distilled water and gently stirred for 24 hours. The content of the vials was filtered and the liquid phase was dried in a vacuum chamber at 60 ºC until reaching constant weight. Solubility was expressed as the total solid content of the aqueous phase (solubilised film) with respect to the mass of the initial sample.

2.3.3. Mechanical, barrier and optical properties

A texture analyser TA-XT plus (Stable Micro Systems, Haslemere, England) was used to determine the tensile properties of each film formulation, following the ASTM D882 method (American Society for Testing Materials, 2002). Film strips (25 mm x 100 mm) were mounted on tensile grips (50 mm separation) and stretched at 50 mm/min. Tensile strength at break (TS), elastic modulus (EM) and elongation at break (%E) were obtained from the stress-strain curves. A hand-held electronic digital micrometer (Comecta S.A., Barcelona, Spain) was used to measure film thickness to the closest 0.001 mm, at six random points in each strip. Eight replicates per sample were performed.

Water vapour permeability (WVP) was measured gravimetrically following the ASTM E96-95 method (American Society for Testing Materials, 1995), using Payne permeability cups (Elcometer SPRL, Hermelle/s Argenteau, Belgium) of 3.5 cm in diameter. Cups were filled with 5 ml of distilled water and mounted with circular film samples. The cups were placed into desiccators containing $MgCl_2$ oversaturated solution and kept at 25 ºC. Cups were weighed periodically (1.5 h-24 h) using an analytical balance (±0.00001 g). The rate of weight loss over time was used to calculate WVP (American Society for Testing Materials, 1995) when the stationary state was reached. The measurements were taken in triplicate for each sample.

The oxygen permeability (OP) was determined by following the ASTM D3985-05 method (American Society for Testing Materials, 2010) using Ox-Tran 1/50 equipment (Mocon, Minneapolis, USA). The film test area was 50 cm^2 and measurements were taken at 25 ºC and 53% RH. OP was calculated by dividing the oxygen transmission rate by the difference between the oxygen partial pressure on both sides of the film and multiplying by the average film thickness. Two replicates per formulation were obtained.

The measurement of colour coordinates and internal transmittance was taken with a CM-5 spectrocolorimeter (Konica Minolta, Inc., Japan). Reflection spectra (400-700 nm) of the films were obtained for three film replicates and at three points per replicate, backed on both black and white plates. Internal transmittance (T_i) was calculated by applying the Kubelka-Munk theory of multiple dispersion (Hutchings, 1999). CIEL*a*b* colour coordinates were obtained from the determined reflectance of an infinitely thick layer of material, considering the D65 illuminant and 10º observer (Cano et al., 2014). Finally, the lightness (L*), chroma (C_{ab}*) and hue (h_{ab}*) psychometric coordinates were calculated (Mahy, Van Eycken, & Oosterlinck, 1994).

2.4. Antibacterial effectiveness of the films

2.4.1. Minimal inhibitory concentration of phenolic acids

Minimal inhibitory concentrations (MIC) of both ferulic and cinnamic acids against *E. coli* and *L. innocua* were determined using the thiazyl blue tetrazolium bromide (MTT) colorimetric assay, using a 96-well disposable sterile microtiter plate(Requena, Vargas, & Chiralt, 2019b). Stock solutions of each compound were obtained using DMSO. Diluted solutions ranging between 0.5 to 2.0 mg/mL were prepared using TBS broth medium, and 100 µL aliquots of each dilution were placed in the corresponding wells. Plates were inoculated with 100 µL of bacterial suspension (10^5 CFU/mL) and covered with a sterile sealer mat to act as vapour barrier. Sterile and growth controls were prepared for each plate, and the outer wells were left empty in order to prevent border effect. After 24 hours of incubation at 37 ºC, 10 µL of MTT reconstituted in PBS at 5 mg/mL were added to all the wells, and the plate was left to incubate for 4 h before MIC was assessed by observation of the plate. MIC was considered as the lowest concentration at which no purple colour was observed in the well. Experiments were carried out in duplicate inside a laminar flow sterile cabinet.

2.4.2. Antimicrobial activity of films tested with culture medium

Tryptic Soy Agar (TSA, Scharlab, Barcelona, Spain) was used for culture medium tests, following the methodology described by previous studies (Requena, Vargas, & Chiralt, 2019a; Tampau et al., 2018). 10 mL samples of TSA were poured into 55 mm petri dishes and inoculated with 100 µL of *E. coli* or *L. innocua* bacterial suspension (10^6 CFU/mL) on the plate surface using a L-form rod to uniformly spread the inoculum. Then, samples were covered with 55 mm round film samples. A non-covered inoculated control was also included. Dishes were closed with their lids, sealed with Parafilm^{tm} and incubated for 6 days at 10 ºC in a culture chamber. After incubation, TSA and films were homogenised for 3 min in buffer peptone water (Scharlab, Barcelona, Spain) by means of a Masticator paddle blender (IUL Instruments, Barcelona, Spain). Serial dilutions were plated and covered with selective media: violet red vile agar (VRBA) for *E. coli* and palcam agar base (PAB) enriched with palcam selective supplement for *Listeria* (Scharlab, Barcelona, Spain), as described by

Requena et al., (2019a). After 48 h incubation at 37 ºC, the colonies were counted. Each film formulation and control was tested in duplicate. Microbial counts were also performed in duplicate for each sample.

2.4.3. Antimicrobial activity of films tested with food matrices

The antibacterial activity of the films was also tested on two different food matrices, namely chicken breast and melon (var. *Cucumis melo*). Chicken breast and melon were selected as respective models of protein-rich and carbohydrate-rich matrices. These products were purchased from the local market and handled in sterile conditions inside a laminar flow cabinet. The chicken breast was cut into thin fillets, while the fresh melon was peeled and cut into thin slices. Samples (about 10 g) were shaped as cylinders (55 mm in diameter and about 6 mm thickness) and placed on petri dishes of 55 mm in diameter. The samples were inoculated with 100 µL of gram-negative (*E. coli)* or gram-positive (*L. innocua)* bacterial suspension (10^6 CFU/mL), by uniformly spreading the inoculum on the sample surface using a L-form rod. Then, samples were covered with the films, closed with their lids, sealed with Parafilm^{tm} and incubated for 6 days at 10 ºC in a culture chamber. Non-covered growth controls were included. After incubation, samples were processed as described in section 2.4.2 to determine bacterial counts.

2.5. Statistical analysis

A statistical analysis of data was performed through analysis of variance (ANOVA) and regression analyses using Statgraphics Centurion XVII software. Fisher's least significant difference was used at 95% confidence level.

3. Results and discussion

3.1. Physical properties of films

A DSC analysis of the obtained films revealed the glass transition of the plasticised starch that was affected by the presence of phenolic acids. **Table 1** shows the midpoint T_g values of the different samples**.** The T_g values found for glycerol plasticised starch films (S) were similar to those previously reported by other authors for the same type of starch (Menzel et al., 2019; Muller et al., 2017). Acid incorporation led to a decrease in T_g values, coherently with the proportion of acid. This could be due to a partial reduction of the starch chain molecular weight associated with a hydrolytic effect provoked by the acids during melt blending, when high temperatures and shear forces were applied to the material (Menzel et al., 2019). The T_g reduction was higher for the ferulic blends than for the cinnamic, thus indicating a greater hydrolytic potential of the former as its concentration increases.

Table 1. Glass transition temperature (second heating) and onset and peak temperature of the polymer thermal degradation of the different starch films without and with 1 or 2 % of ferulic (F) or cinnamic (C) acids.

	DSC	TGA	
Formulation	**Tg (ºC)**	**T_{onset} (°C)**	**T_{max} (°C)**
S	98 ± 11^{b}	247 ± 1^{a}	282± 1^{ab}
1F	75 ± 2^{ab}	258 ± 2^{c}	286 ± 1^{b}
2F	56 ± 13^{a}	252 ± 1^{ab}	285 ± 2^{ab}
1C	97 ± 3^{b}	253 ± 3^{b}	281± 4^{a}
2C	80 ± 2^{ab}	255 ± 1^{bc}	285 ±2^{ab}

Superscript letters in each column indicate significative differences between groups ($p < 0.05$).

The TGA analyses of the films showed the mass loss curves of each film formulation as a function of the temperature, which agreed with those described by other authors (Mathew and Abraham, 2008) for starch films. **Figure 1** shows the mass loss curves absolute and derivative for each sample. A first, progressive mass loss took place below 240 ºC, at which point bonded water was evaporated, glycerol was evaporated/degraded (Muller et al., 2017) and phenolic acids could also be partially degraded. The TGA curves of pure phenolic acids revealed that ferulic decomposition started at 167 ºC, with the peak at 212 ºC, while cinnamic started degradation at 180 ºC reaching a maximum at 215 ºC. In fact, samples with ferulic acid exhibited greater mass loss in this first step, while samples with cinnamic acid presented a shoulder in the main weight loss step starting at about 250 ºC, which could be attributed to a slightly retarded cinnamic acid degradation in the presence of a polymer. The second and more intense mass loss step occurred between 250 ºC and 300 ºC, when the degradation of the main polymeric chains occurred. The onset and peak temperatures of the polymer degradation are shown in **Table 1**.

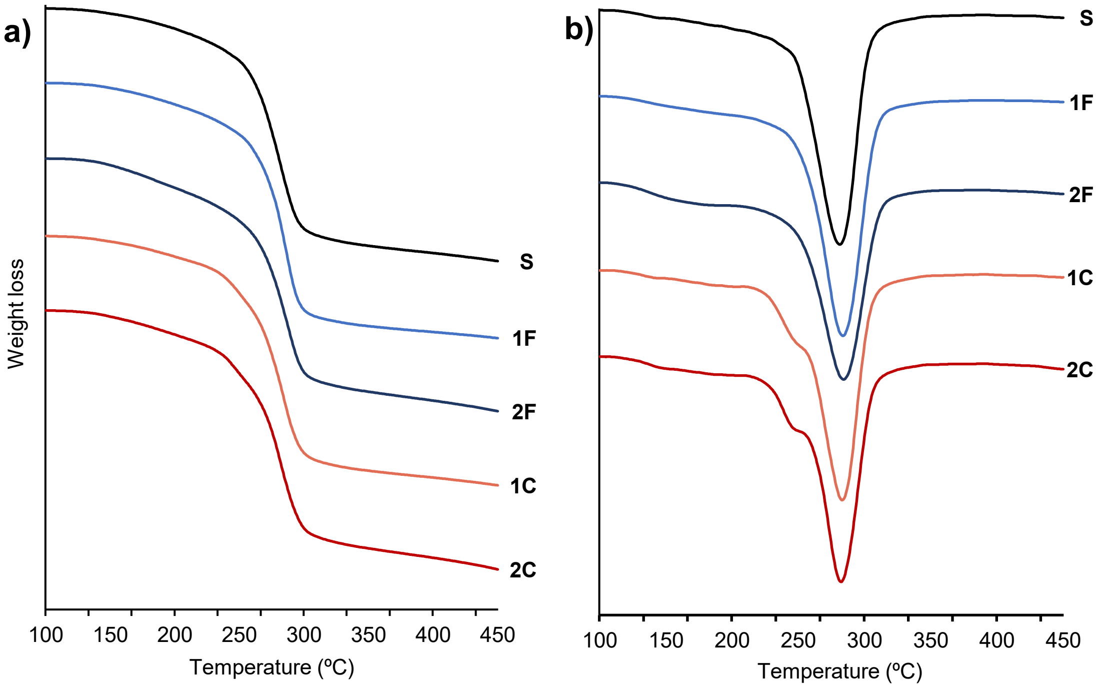


Figure 1. TGA curves of starch films with and without phenolic acids, a) weight *vs* temperature curve and b) first derivative of weight loss *vs* temperature. Starch without active compounds (S); starch with ferulic acid at 1 and 2% w/w (1F, 2F); starch with cinnamic acid at 1 and 2% w/w (1C, 2C).

The incorporation of phenolic acids into the starch matrices slightly increased the onset and peak temperatures of starch degradation, the ferulic acid exerting the greatest effect. These changes could be attributed to differences in the mean molecular weight of the starch polymers associated with the hydrolytic effect of phenolic acids during melt blending and compression moulding (Menzel et al., 2019).

3.2. Active compound retention, moisture content and water solubility

Table 2 shows the active compound retention (AC) expressed as g of ferulic or cinnamic acid per 100 g of the compound initially incorporated into the film formulation. Both films containing ferulic acid showed acid retention values of around 60%, which could be due to its partial degradation during the melt blending and compression moulding steps, as a result of the thermal and shear stress. Although TGA on pure ferulic showed an initial degradation at nearly 170 ºC, previous studies (Yilmaz et al., 2011) reported the degradation of phenolic compounds at temperatures as low as 50 ºC when exposed to high pressures. Additionally, chemical interactions between ferulic acid, through hydrogen bonds between phenolic group and the starch hydroxyls, could be limiting the release of this acid from the film matrix into the extraction solvent. In contrast, cinnamic formulations presented higher compound retention values (around 90%), hence reduced acid loss, coherently with the greater thermal stability of cinnamic acid.

Table 2. Properties (Mean values ± standard deviations) of starch films with phenolic acids conditioned at 33% RH; active compound retention (AC, %), moisture (M), water solubility (WS), thickness (t), tensile strength (TS), elongation at break (E), elastic modulus (EM), water vapor permeability (WVP), oxygen permeability (OP), lightness (L*), hue (h_{ab}*) and chrome (C_{ab}*).

	S	**1F**	**2F**	**1C**	**2C**
AC (%)	-	61 ± 3^{a}	59 ± 2^{a}	90 ± 3^{b}	97 ± 4^{c}
M (%)	7.6 ± 0.3^{ab}	7.9 ± 0.2^{b}	8.2 ± 0.3^{b}	7.2 ± 0.3^{a}	7.9 ± 0.2^{b}
WS (%)	86 ± 2^{d}	73 ± 3^{c}	71 ± 5^{bc}	61 ± 2^{a}	67 ± 2^{b}
t (µm)	142 ±10^{a}	137 ±14^{a}	143 ±13^{a}	135 ±12^{a}	143 ±13^{a}
TS (MPa)	17 ± 4^{c}	17 ± 3^{c}	8.4 ± 0.9^{a}	11 ± 2^{b}	9.6± 1.2^{ab}
EM (MPa)	830 ± 110^{e}	720 ± 90^{d}	280 ± 40^{a}	550 ± 100^{c}	440 ± 80^{b}
E (%)	4 ± 2^{a}	6 ± 2^{a}	30 ± 5^{c}	12 ± 5^{b}	14 ± 3^{b}
WVP (g·mm/kPa·h·m^2)	10 ± 2^{ab}	9 ± 2^{a}	10 ± 1^{ab}	14 ± 2^{c}	13 ± 2^{bc}
OP (x10^{14}·cm^3/m·s·Pa)	4.2 ± 0.4^{a}	5.2 ± 0.4^{bc}	5.8 ± 0.5^{c}	4.7 ± 0.1^{ab}	4.6 ± 0.1^{ab}
L*	76.4 ± 0.7^{a}	77.5 ± 0.7^{b}	76.9 ± 1.3^{ab}	77.0 ± 0.7^{ab}	79.9 ± 0.8^{c}
h_{ab}*	76.8 ± 1.2^{c}	76.7 ± 0.5^{c}	79.4 ± 0.3^{d}	74.7 ± 0.2^{b}	72.0 ± 0.2^{a}
C_{ab}*	11.5 ± 0.8^{b}	12.3 ± 0.5^{c}	16.4 ± 0.7^{d}	12.2 ± 0.6^{bc}	10.2 ± 0.3^{a}

S: starch without active compound, 1F: with 1% ferulic acid, 2F: with 2% ferulic acid, 1C: with 1% cinnamic acid, 2C: with 2% cinnamic acid. Superscript letters in each column indicate significant differences between groups ($p < 0.05$).

The incorporation of the phenolic acids did not remarkably affect the equilibrium moisture content (M) of the films (**Table 2**) , and nor was any clear pattern observed as a result of the proportion of acid in the film. The obtained values were coherent with those previously reported for cassava starch/glycerol films (Cano et al., 2014; Menzel et al., 2019). In contrast, the film's water solubility (WS) was clearly reduced by the addition of phenolic acids. Given its hydrophilic nature, films of pure starch exhibit high ws (Cano et al., 2014). The concentration of ferulic acid did not affect the WS reduction and the films g with cinnamic acid (especially 1C) presented the most marked reduction in WS. This suggests that interactions between the carboxyl groups of acids and hydroxyls of starch chains reduced the water affinity of the matrix, inhibiting the formation of hydrogen bonds with water molecules, thus reducing the water solubility. The milder effect of ferulic acid could be attributed to the phenolic group in the aromatic ring that also favours the water interactions with the chain bonded phenolic compound.

3.3. Mechanical, barrier and optical properties

Table 2 also shows the mean values of film thickness (t) and the tensile parameters of conditioned films: tensile strength (TS), Young's modulus (EM) and percentage of elongation at break (%E). No significant differences in film thickness were observed ($p>0.05$), while the values were similar to those previously reported for cassava starch/glycerol monolayers processed under the same conditions (Muller et al., 2017).

As concerns tensile behaviour, **Figure 2** shows the stress-strain curves of every film formulation in which a significant effect of phenolic acid incorporation can be observed. Formulations with 1% ferulic acid did not notably differ from pure starch films and exhibited similar tensile parameters. In contrast, 2 % of ferulic acid provoked a strong plasticising effect, as revealed by the sharp increase in the film's extensibility (elongation at break, 6 times higher) and the reduction in the elastic modulus (EM), by more than 500 MPa, and resistance to break (TS). This mechanical plasticisation was consistent with the notable Tg decrease observed in DSC analyses for the highest concentration of ferulic acid. Coherently, it is remarkable that these films with 2 % F became very sticky when conditioned at a RH higher than 33%.

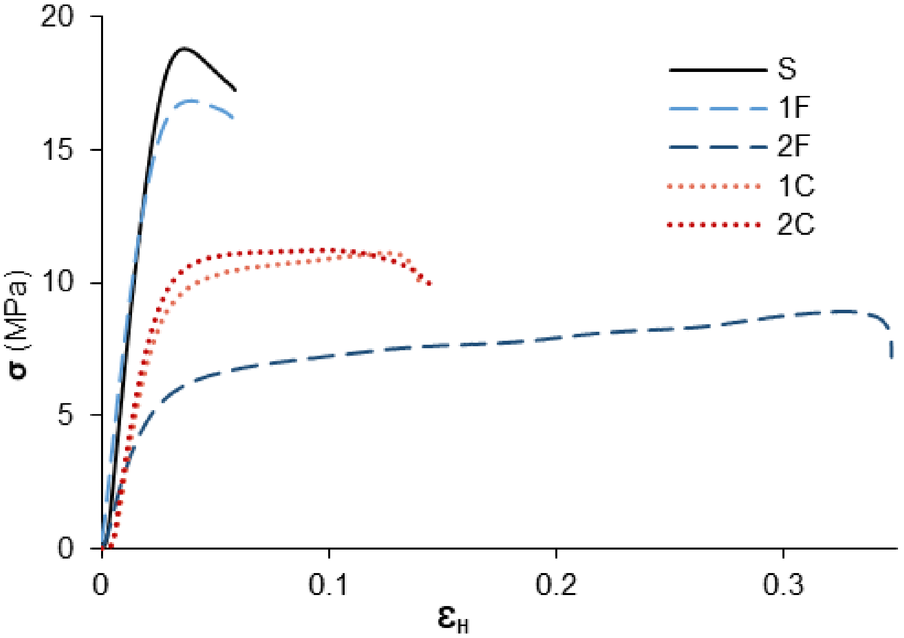


Figure 2. Stress-strain curves of the films. Starch without active compound (S); starch with ferulic acid at 1 and 2% w/w (1F, 2F); starch with cinnamic acid at 1 and 2% w/w (1C, 2C).

Films containing cinnamic acid (1 and 2 %) exhibited very similar tensile behaviour, while being more extensible (3 times more) than starch films and having a reduced elastic modulus and lower resistance to break. This was coherent with the similar, small change in the Tg values of the starch matrix observed in DSC analyses for films with 1 and 2 % of cinnamic acid. These results indicated that the starch matrix was highly sensitive to the hydrolytic action of ferulic acid as a function of its concentration, whereas the increase in the cinnamic acid content in the matrix did not provoke such important changes in the properties of the starch matrix.

The barrier properties (OP and WVP values) of the film formulations are also shown in Table 2. Both OP and WVP values were in the range reported in previous studies (Menzel et al., 2019; Muller et al., 2017) for cassava starch films and were hardly affected by the presence of the phenolic acids. Ferulic acid did not significantly affect the water vapour barrier capacity of the films at 1 or 2 %, despite the observed differences in the matrix plasticisation, although it did slightly promote OP. However, Mathew and Abraham (2008) reported an improvement in both the water vapour and oxygen barrier capacity after the addition of a low ratio of ferulic acid in starch/chitosan films.

Although all the films were transparent and homogenous, with 2% C they exhibited a certain whiteness. This is reflected in **Figure 3**, where the internal transmittance (Ti) spectra are shown with lower values for the 2C sample, while similar spectra were observed for the rest of the films. Colour parameters, lightness, hue and chroma (**Table 2)** revealed the greater lightness of the less transparent 2C films and the slightly yellowish and more saturated colour of films with 2% ferulic acid**.**

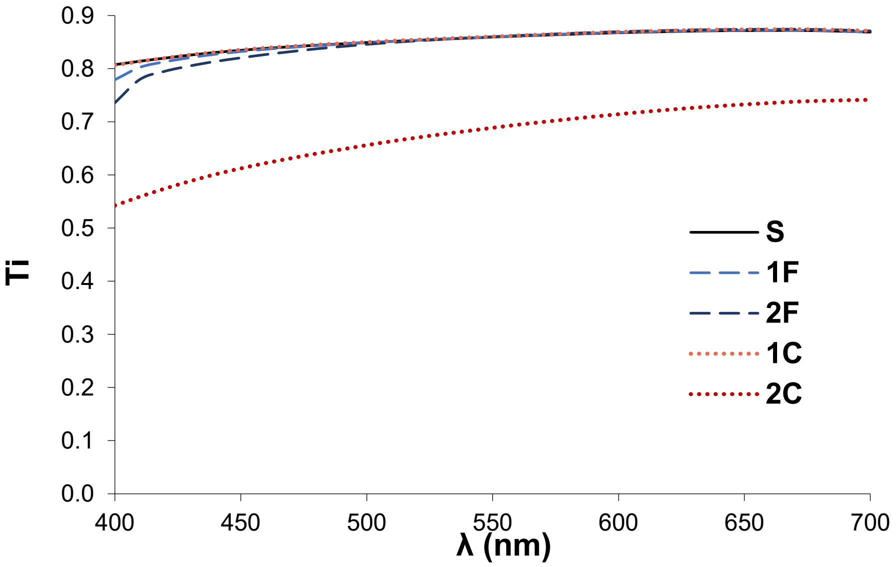


Figure 3. Internal transmittance (T_i) spectra (400-700nm) of the films. Starch without active compound (S); starch with ferulic acid at 1 and 2% w/w (1F, 2F); starch with cinnamic acid at 1 and 2% w/w (1C, 2C).

3.4 Antibacterial effectiveness of the films

The MIC values of phenolic acids for *E coli* and *L. Innocua* were determined to evaluate their antibacterial effectiveness against these specific strains. For *E. coli*, this value was 0.80 mg/mL for ferulic acid*,* slightly lower than that reported by Meira et al. (2017). However, the MIC value found for *L. innocua* with ferulic acid was 0.70 mg/mL, in the range of that reported by Miyague et al. (2015) who observed a marked effect of the pH on the MIC values of *Listeria*. In the case of cinnamic acid, the MIC values were 0.70 and 0.65 mg/mL, respectively, for *E. coli* and *L. innocua.* These were also in the range of those previously reported by other authors (Miyague et al., 2015; Olasupo et al., 2003). These values indicate that cinnamic acid was more effective than ferulic acid against both bacteria and that both compounds exhibited a greater antibacterial effect against *L. innocua* than against *E. coli*. These MIC values were used to determine the dose of active compounds required in the films in order to obtain active films against the tested bacteria, assuming a complete active release in the culture medium or food substrate.

Figure 4a shows the microbial counts obtained in the antibacterial test in the culture medium and **Table 3** shows the reduction obtained in the log CFU, with respect to the uncovered control plates. The counts in TSA samples covered with phenolic-free starch films were similar to those obtained in the uncovered control plates; hence, the antimicrobial effectiveness (count reduction) of the rest of the films could be attributed to the action of the incorporated phenolic acids. In fact, the antimicrobial effectiveness of the films was highly dependent on the phenolic acid concentration and those containing the lowest proportion (1F and 1C) did not exhibit a significant inhibitory effect against the tested bacteria, according to the small amount potentially released of the active, compared to the bacteria MIC. In contrast, a reduction of more than 2 log in the bacterial counts of *L. Innocua* was obtained for films with 2 wt.% of both acids; cinnamic acid being much more effective. However, these active compounds at 2 wt.% were less effective against *E coli*, where lower growth inhibition was observed. In general, a more remarkable growth inhibition was observed for films containing cinnamic acid than for those with ferulic acid, while the films were more effective against *L. innocua* than against *E. coli*, coherently with the respective MIC values. It is remarkable that films with 2 wt.% cinnamic acid provoked a reduction of more than 6 log CFU in the listeria counts in the culture medium after 6 incubation days.

Table 3. Log CFU reduction for *E. coli* and *L. innocua* in TSA and food matrices obtained with the different films: active-free starch (S), with 1 or 2 wt.% ferulic acid (1F, 2F,) and with 1 or 2 wt.% cinnamic acid (1C, 2C). Mean values and standard deviations.

		TSA	**Chicken**	**Melon**
S	*E. coli*	0.3 ± 0.5^{a1}	0.2 ± 0.1^{a1}	0.1 ± 0.1^{a1}
	L. innocua	0.2 ± 0.4^{a1}	0.4 ± 0.2^{a1}	0.1 ± 0.1^{a1}
1F	*E. coli*	0.5 ± 0.2^{a1}	1.8 ± 0.2^{a2}	0.4 ± 0.2^{a1}
	L. innocua	0.8 ± 0.1^{a2}	1.3 ± 0.1^{a2}	0.4 ± 0.1^{a1}
2F	*E. coli*	0.7 ± 0.2^{a1}	3.1 ± 0.2^{b2}	0.8 ± 0.1^{a1}
	L. innocua	2.3 ± 0.2^{b2}	2.2 ± 0.2^{b1}	0.7 ± 0.6^{a1}
1C	*E. coli*	0.8 ± 0.2^{1}	1.9 ± 0.2^{a2}	0.8 ± 0.2^{a1}
	L. innocua	1.7 ± 0.1^{2}	1.8 ± 0.1^{a2}	1.3 ± 0.1^{b1}
2C	*E. coli*	2.0 ± 0.2^{a1}	4.0 ± 0.1^{b2}	1.9 ± 0.1^{b1}
	L. innocua	6.4 ± 0.2^{b2}	2.9 ± 0.0^{a1}	3.3 ± 0.5^{c2}

Superscript letters (a-c) in each column and numbers (1-2) in lines indicate significant differences between values ($p < 0.05$).

Figures 4b and **4c** show the results of the antibacterial tests performed on chicken breast and fresh-cut melon with the films containing 1 and 2 wt. % of phenolic acids. As shown in **Figure 4b**, the starch films with 2 wt. % phenolic acids effectively protected chicken breast from bacterial growth (more than 2 Log reduction), both for *E. coli* and *L. innocua.* In this food matrix, cinnamic acid was also more active than ferulic acid against both bacteria and films with 2 wt. % of cinnamic acid exhibited an almost bacteriostatic effect in both cases.

As compared to TSA and chicken breast, the effectiveness of the films against *E.coli* in fresh-cut melon was less remarkable. The growth of both bacteria was only significantly with inhibited (more than 2 log reduction) by cinnamic acid at 2 %, while ferulic acid slightly reduced the CFU counts of both bacteria at the same concentration. Nevertheless, a notable antilisterial effect was observed in this food matrix for films containing cinnamic acid, with more than 3 log CFU reduction. Requena et al. (2019a) also found a very slight growth inhibition of these bacteria in fresh-cut melon coated with active films containing carvacrol and eugenol. This was explained both by the fact that melon tissue provides a source of good nutrients for the bacteria and by the potential interactions of the active compounds with the melon components that could limit their antibacterial action. However, the antilisterial effect observed for cinnamic acid from starch films in this food matrix opens the possibility of their use for controlling the growth of this foodborne pathogen, resistant at low temperatures, such as those used for the preservation of fresh-cut fruits.

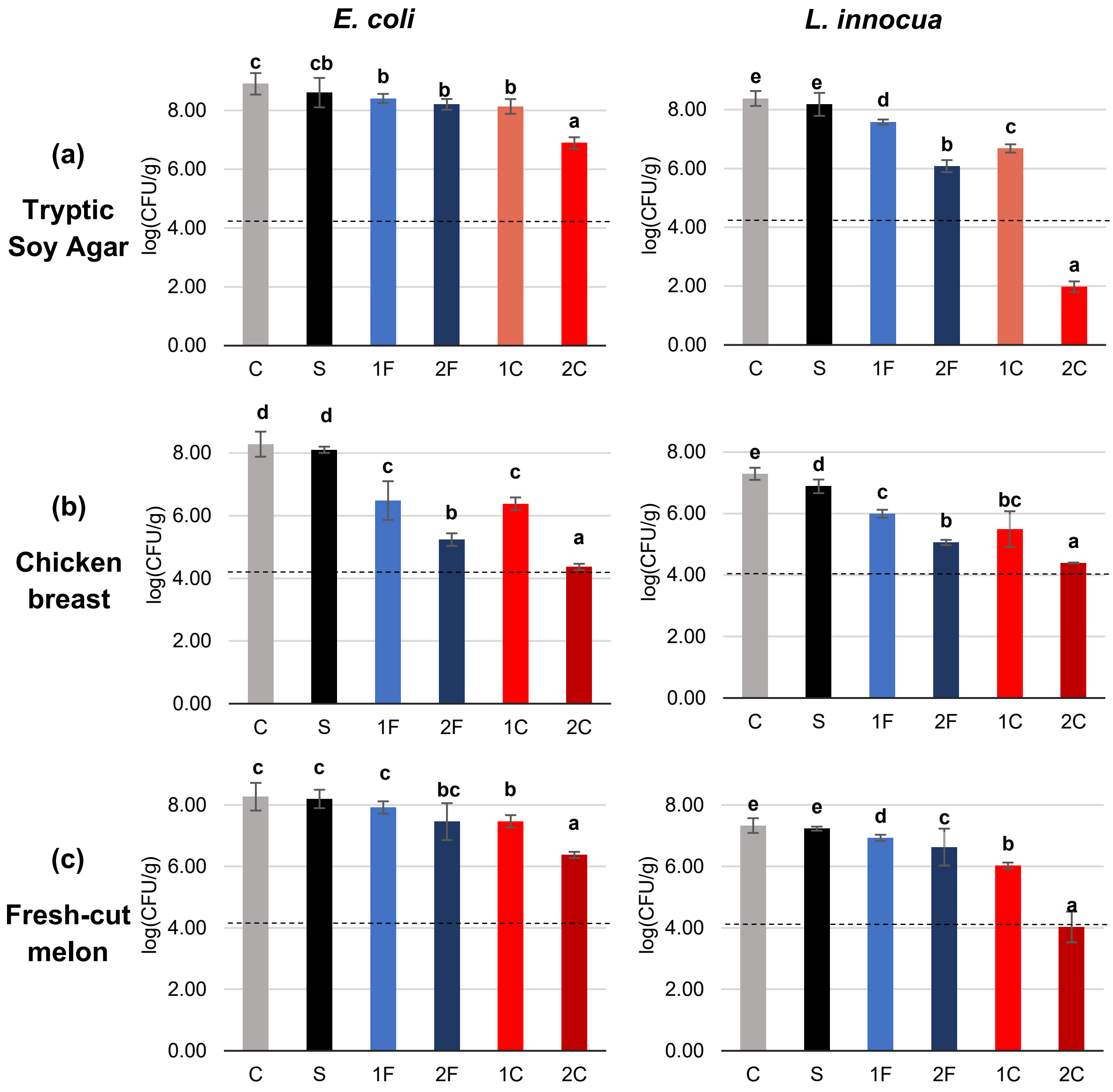


Figure 4. Microbial counts for *E. coli* and *L. innocua,* obtained after 6 days of incubation at 10 ºC in (a) TSA culture media, chicken breast (b) and fresh-cut melon (c). Uncovered control sample (C) and samples covered with films of active-free starch (S), with 1 or 2 wt.% ferulic acid (1F, 2F) and with 1 or 2 wt. % cinnamic acid (1C, 2C). Dotted lines show the initial microbial counts. Different letters above the bars show homogeneous sample groups ($p < 0.05$).

A comparison of bacterial growth inhibition in TSA and food matrices reveals the significant role that the interactions between the food components, bacterial cells and released active compounds play in the antimicrobial effectiveness of the films. Food nutrients promote bacterial growth to a different extent, and these can also interact with active compounds limiting or enhancing their ability to act against the cells. The amount of active released from the films, as well as the release rate, also plays an important role in the active film's effectiveness. An amount that is sufficient to reach the MIC value of bacteria is required, and its release should occur when the bacterial growth is still in the exponential phase when bacteria are more sensitive to the antimicrobial action. In highly water sensitive starch films, a fast and almost complete release of acids could be expected due to the relaxation of the polymer matrix in contact with the moist food. Polymer relaxation associated with the film hydration favours the diffusion of active compound into the food in contact with the film. In this sense, phenolic acid-starch coatings could be used as active layers for food contact in multilayer assemblies with other hydrophobic polymer sheets to obtain active laminate materials with adequate functional properties for food packaging.

4. Conclusions

Active starch films with ferulic or cinnamic acid could be obtained by thermo-processing with small modifications of the films' functional properties. Incorporation did not notably affect the barrier properties of the starch films, although these became less water soluble, more extensible, and less resistant to break. Both cinnamic and ferulic acids conferred antibacterial activity to the starch films against *E. coli* and *L. innocua,* which was probed in culture medium and food systems (chicken breast and fresh cut melon). Films with cinnamic acid were more effective than with ferulic acid and both were more active against *L. Innocua* than against *E. coli*. The antibacterial activity of the films was affected by the kind of acid and strain and the food substrate. The highest effectiveness was obtained for films with cinnamic acid, against *L. innocua,* in chicken. breast and fresh-cut melon.

Therefore, although highly hydrophilic starch films had limitations for wet food packaging, starch layers with 2 % ferulic or cinnamic acid could be used as active layers for food contact in multilayer assemblies, combined with other hydrophobic polymer sheets of complementary properties, to obtain active laminates that could provide remarkable antimicrobial capacity and, in particular, antilisterial activity.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ramón Ordoñez: Investigation, Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Lorena Atarés**: Conceptualization, Methodology, Data curation, Writing – original draft, Writing – review & editing, Supervision. **Amparo Chiralt**: Conceptualization, Methodology, Data curation, Writing – review & editing, Supervision, Project administration.

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Chapter 2:

Effect of ferulic and cinnamic acids on the functional and antimicrobial properties in thermo-processed PLA films.

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Submitted to Food Packaging and Shelf Life.

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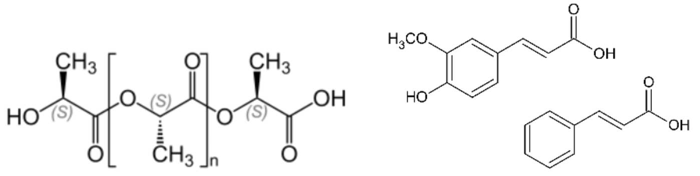
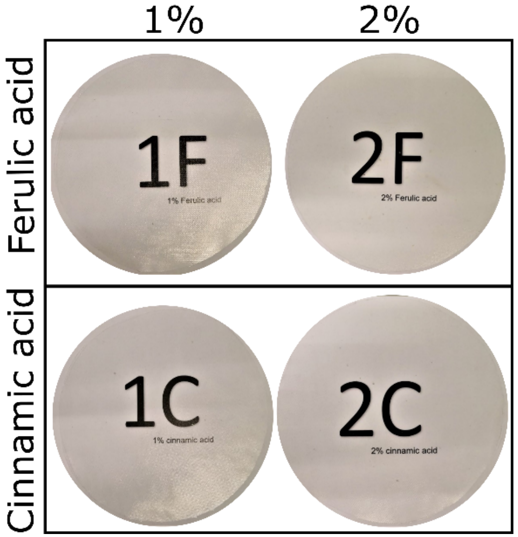


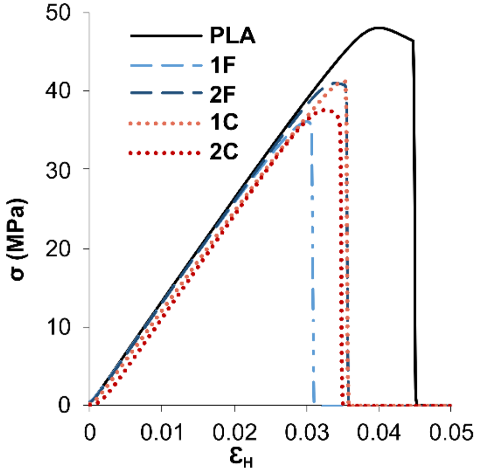
Polylactic acid + Ferulic acid or Cinnamic acid

Melt blending & Compression moulding

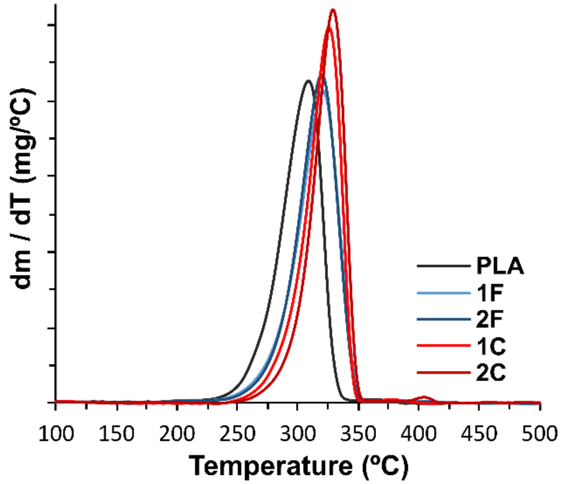
PLA active films



Functional characterization

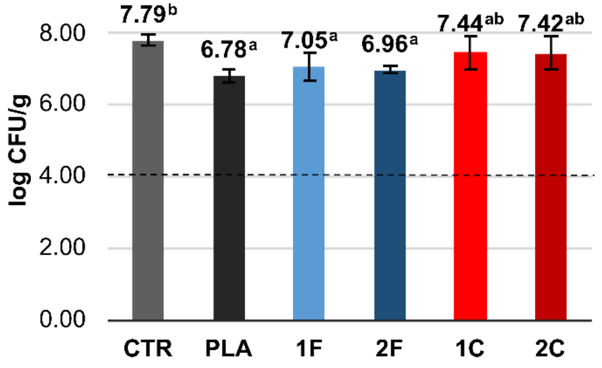


Tensile behaviour of PLA films.



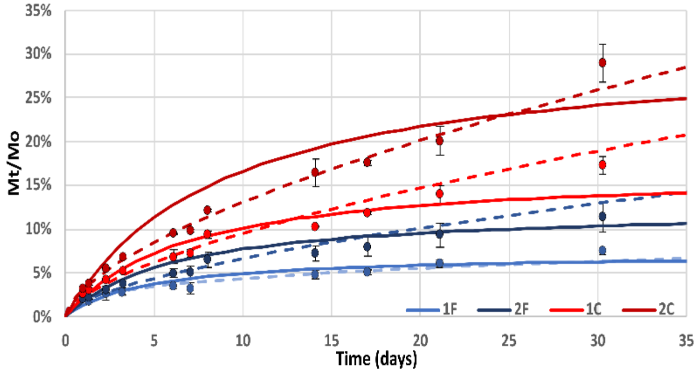
Thermogravimetric derivative curves.

Antimicrobial tests



E. coli growth test in contact with obtained films.

Release kinetics



Active compounds release profiles in D1 simulant.

Abstract

The incorporation of ferulic or cinnamic acids as antimicrobial agents into PLA matrices to produce films with active properties for food packaging applications is evaluated in this study. The acids were incorporated by thermal processing at 1 and 2 wt.% presenting good stability with 84-98% retentions. The addition of acids provoked about a 5% reduction of elastic modulus and up to 20% reduction in the resistance to break in the PLA films, slightly decreasing their extensibility. Oxygen and water vapor barrier capacities were enhanced by about 6 and 45% respectively for the highest acid concentrations. The acids did not change the glass transition of the polymer but increased the thermal resistance. No significant antimicrobial activity was detected, despite the proven activity of the acids, due to their limited release into the aqueous culture media. Release kinetic studies revealed that significant release was only achieved in intermediate polarity simulant (50% aqueous ethanol), where the final percentages of the active released increased along with the total amount in the films (15 and 31 %, respectively, for 2 % of ferulic and cinnamic acids). Further studies are necessary to improve the compound release into food matrices and antimicrobial effectiveness.

1. Introduction

Despite the widely known environmental issue it represents and the fact that recycling rates for plastics are very low as compared to other packaging materials, such as metal, glass or paper, plastic production continues to increase and represents around 40% of the total production of the packaging industry (European Commision, 2018). In this context, biodegradable bioplastics represent an alternative means of developing sustainable food packaging (Keränen et al., 2021). Polylactic acid (PLA) possesses good properties for food packaging purposes (Muller et al., 2016) and is considered to be the most promising bioplastic, based on its production and the growing tendency on patents filings (Elvers et al., 2016). PLA is a biodegradable polyester obtained by synthesis from lactic acid that can be obtained by the fermentation of biomass, and its production is considered to be sustainable (Ahmad et al., 2020). The beneficial properties of PLA include good mechanical strength and thermoforming ability, biocompatibility, composting ability and monomer renewability, but its inherent brittleness and low thermal resistance are disadvantages that limit its use in packaging applications (Jamshidian et al., 2010). In this sense, its functional properties can be improved by incorporating active compounds into the polymer matrix, which can contribute to the enhancement of its ability for food preservation and shelf-life extension, thus adding value to the material and increasing its competitiveness in the packaging market. Active packaging is considered a new alternative means of preserving food products while keeping them free from traditional synthetic additives (Sharma et al., 2021).

The potential of PLA for use in antimicrobial packaging applications has been studied by different authors and several substances, such as organic acids, bacteriocins, plant extracts, essential oils, enzymes, chelating agents or metals, have been incorporated into the PLA matrices to provide the films with antimicrobial activity (Jamshidian et al., 2010; Scaffaro et al., 2018; Tawakkal et al., 2014). The use of natural phenolic compounds or phenolic-rich extracts incorporated into biodegradable polymeric matrices has been widely studied, with promising results for active food packaging applications (Sapper et al., 2020). Contrary to the essential oils widely studied as natural active components, phenolic acids do not impact severely on the food's organoleptic properties (Miyague et al., 2015), while presenting good antioxidant and antimicrobial properties (Merkl et al., 2010). Cinnamic acid (C) is a major constituent of the oriental Ayurvedic plant Cinnamomum cassia, being the precursor of hydroxycinnamic acids, the prevalent family of phenolic acids. It can be extracted from a wide range of vegetable sources (Rashmi & Negi, 2020), and exhibits antimicrobial properties, as demonstrated in different studies (Miyague et al., 2015; Sánchez-Maldonado et al., 2011). It is especially active against *Listeria*, which constitutes a dangerous food pathogen due to its persistency at cold temperatures (Tasara & Stephan, 2006). This compound and its derivatives have been reported to possess various biological properties of which its antioxidant activity is a prime one (Patra et al., 2012), showing specific interactions at reducing the oxidative stress in different vegetables (Sun et al., 2012), interfering the mechanisms involved in the protection against the intracellular reactive oxygen species (ROS) that produce oxidative cellular damage.

Ferulic Acid (F) is a hydroxycinnamic acid, ubiquitously present in the plant cell wall (Sharma et al., 2020), with proven antimicrobial (Pernin et al., 2019; Shi et al., 2016) and antioxidant properties (Itagaki et al., 2009; Zduńska et al., 2018). It has low toxicity and possesses many physiological functions (anti-inflammatory, antioxidant, antimicrobial activity, anticancer, and antidiabetic effect), is widely used in the pharmaceutical, food, and cosmetics industry. Ferulic acid is a free radical scavenger, but also an inhibitor of enzymes that catalyse free radical generation and an enhancer of scavenger enzyme activity (Zduńska et al., 2018).

In previous studies, cinnamic and ferulic acids have both been incorporated into starch matrices, where they exhibited a marked growth inhibition capacity with both Gram-positive and Gram-negative bacteria (Ordoñez et al., 2021). In fact, the minimum inhibitory concentration (MIC) values of these compounds against *E. coli* and *L. innocua* are in the range of 0.7 mg/mL (Ordoñez et al., 2021) as the most active compounds from essential oils, such as cinnamaldehyde or carvacrol (Requena et al., 2019b). Despite the great potential of this application in polysaccharide matrices, such as starch, these are not water-resistant and can easily disintegrate in contact with high moisture foodstuffs (Zhong et al., 2020). In contrast, PLA is hydrophobic, does not exhibit water sensitivity and has a good barrier capacity to water vapor (Muller et al., 2016). In this sense, incorporating antimicrobial ferulic and cinnamic acids into a PLA matrix could lead to materials that are suitable for active food packaging in a wide range of applications. Ferulic acid has been incorporated into cast PLA/PBAT (98:2) blend films, which exhibited antibacterial activity (Sharma et al., 2020). Andrade et al. (2022) also incorporated ferulic acid into PLA/PVA/PLA three-layer films, which were effective at controlling microbial growth in cold storage beef meat. Nevertheless, no previous studies analysed the incorporation of ferulic or cinnamic acid to produce thermo-processed PLA antimicrobial films.

However, potentially active compounds can affect the functional properties of the packaging material when incorporated into the polymeric matrix, which can compromise their use. Likewise, an efficient release (adequate rate and ratio) of the active compound into the food substrate should occur for the development of the active capacity, such as antimicrobial or antioxidant action (Requena et al., 2017). The release kinetics of active compounds from the polymer matrix into the food substrate and the partition coefficient at equilibrium is greatly affected by the compound-polymer interactions, the potential polymer relaxation in contact with the food substrate and the respective chemical affinity of the compound with the polymer and food substrate or the simulant (Requena et al., 2017). These factors define the concentration of active in the food substrate as a function of time. Then, the compound release varies as a function of the polymer structure, the compound molecular characteristics (molecular weight, polarity and functional groups) and the environmental conditions; for example, the release rate is slow when it is governed by diffusion in the polymer matrix, and fast when governed by swelling or dissolution (Jamshidian et al., 2012).Therefore, the development of active PLA films with ferulic or cinnamic acid requires the verification of the influence of actives on the functional properties of polymer as well as the compound’s effective release to exert the antimicrobial effect on a determined system.

The objective of this study was to obtain and characterize thermo-processed PLA films with ferulic or cinnamic acids at different concentrations, for food packaging applications. The effect of acid incorporation on the mechanical, barrier and optical properties of PLA films, as well as the release of active compounds into food simulants and the antibacterial properties against *L. innocua* (Gram-positive) and *E. coli* (Gram-negative) of the films were analysed.

2. Materials and methods

2.1. Materials

Amorphous PLA 4060D with 106 kDa average molecular weight (Nature Works, MN, USA), cinnamic and ferulic acids (Sigma-Aldrich, Saint Louis, USA) were used for film production. Magnesium nitrate and phosphorus pentoxide, supplied by Panreac Química (Barcelona, Spain), were used to equilibrate the sample relative humidity (RH) at 53% and 0%, respectively. Tryptone soy broth, tryptone soy agar, phosphate-buffered saline and peptone water for microbial tests were purchased from Scharlab

(Barcelona, Spain). Strains of *Listeria innocua* (CECT 910) and *Escherichia coli* (CECT 101) were supplied by the Spanish Type Collection (CECT, University of Valencia, Spain). Scharlab (Barcelona, Spain) supplied the tryptic Soy Agar, buffer peptone water and selective media: violet-red bile agar (VRBA) for *E. coli* and palcam agar base (PAB) enriched with palcam selective supplement for *Listeria*. Spectrophotometric grade solvents: methanol, ethanol, acetic acid, and isooctane were all purchased from Panreac Química (Barcelona, Spain).

2.2. Film preparation

PLA pellets were first dried at 60 °C overnight to prevent hydrolysis during thermal processing. The components were melt blended in an internal mixer (Haake PolyLab QC, Thermo Fisher Scientific, Germany) at 150 ºC and 50 rpm for 10 min. Cinnamic (C) or ferulic (F) acid was added at 1 or 2 g acid / 100 g dry film. This concentration was established with the aim of using the minimal amount as possible, but ensuring an antibacterial effect, according to previous studies (Ordóñez et al., 2021). The melts obtained were cold ground using an M20 IKA mill (Staufen, Germany). Films of 20 cm in diameter were obtained by compressing 4 g of powder, using a hydraulic heating press (LP20, Labtech engineering, Thailand). Samples were preheated at 200 ºC (Muller et al., 2017) for 4 min and compressed at 10 MPa for 4 min. Finally, a 3 min cooling step to 70 ºC was applied. The films obtained were conditioned at 53% RH and 25 ºC before characterization. The different samples were coded as PLA (PLA films without acids), 1F, 2F (PLA with ferulic acid at 1 or 2 % w/w); and 1C, 2C (PLA with cinnamic acid at 1 or 2 % w/w).

2.3. Characterization of the films

2.3.1. Microstructure observation

A High-resolution field emission scanning electronic microscope (HR-FESEM) (GeminiSEM 500, Zeiss, Germany) was used to observe the cross-section microstructure of obtained films. Samples were cryofracture using liquid nitrogen (only for cross-section observations), mounted in supports with carbon tape and platinum-coated before observation.

2.3.2. Thermal analyses

The thermal properties of the films were studied using a differential scanning calorimeter (DSC, 1 StareSystem, Mettler-Toledo, Switzerland) and a thermogravimetric analyser (TGA/SDTA 851e, Mettler-Toledo, Switzerland). Before the analyses, samples were conditioned at 0% RH. For the purposes of the DSC analyses, samples (about 10 mg) were placed into aluminium pans and sealed. The analyses consisted of the following steps: heating from room temperature to 200 ºC at 10 K/min, maintaining 200 ºC for 5 min, cooling at 50 K/min to -10 ºC, maintaining -10 ºC for 5 min, and heating to 200 ºC at 10 K/min. An empty aluminium pan was used as reference. For TGA analyses, samples of about 10 mg were placed into alumina crucibles and heated from 25 to 600 ºC at 10 K/min, under a nitrogen flow (50 mL/min). Both analyses were performed in duplicate.

2.3.3. Content of active compounds

The final content of ferulic and cinnamic acids in the films was determined through methanol extraction and spectrophotometric quantification. Film samples (about 100 mg) preconditioned at 0% RH were immersed in 10 ml methanol and gently stirred for 48 hours at room temperature. Methanol extracts were filtered and properly diluted, and the absorbance measurements were taken using a UV-visible spectrophotometer (Thermoscientific Evolution 201, USA) at the maximum absorbance wavelengths, i.e. 320 nm for F and 270 nm for C. The respective methanol extract of the PLA samples was used as blank. The absorbance measurements were transformed into acid concentration using the previously determined calibration curves. The results were presented as the mass percentage of acid released referred to the amount initially incorporated into the film. All the measurements were taken in triplicate.

2.3.4 Mechanical, barrier and optical properties

A texture analyser TA-XT plus (Stable Micro Systems, Haslemere, England) was used to determine the tensile properties of each film formulation, following the ASTM D882 method (American Society for Testing Materials, 2002). Film strips (25 mm x 100 mm, eight replicates per formulation) were cut, and a hand-held electronic digital micrometer (Comecta S.A., Barcelona, Spain) was used to measure film thickness to the closest 0.001 mm, at six random points per strip. The samples were mounted on tensile grips (50 mm separation) and stretched at 50 mm/min. Tensile strength at break (TS), elastic modulus (EM) and elongation at break (%E) were obtained from the stress-strain curves.

Water vapour permeability (WVP) was measured following the ASTM E96-95 method (American Society for Testing Materials, 1995). Payne permeability cups (Elcometer SPRL, Hermelle/s Argenteau, Belgium) of 3.5 cm in diameter were filled with 5 ml of distilled water and mounted with circular film samples whose thickness had been previously measured at six random positions. The cups were placed into desiccators containing $MgNO_3$ oversaturated solution and kept at 25 ºC. Cups were weighed periodically (1.5 h-24 h) using an analytical balance (±0.00001 g). The rate of weight loss over time was used to calculate WVP once the stationary state had been reached according to E96-95 method. The measurements were taken in triplicate for each film formulation.

The oxygen permeability (OP) was determined by following the ASTM D3985-05 method (American Society for Testing Materials, 2010) using Ox-Tran 1/50 equipment (Mocon, Minneapolis, USA). The film test area was 50 cm^2 and measurements were taken at 25 ºC and 53% RH. OP was calculated by dividing the oxygen transmission rate by the oxygen partial pressure gradient on both sides of the film and multiplying by the average film thickness, which had been previously measured at six different positions on the film sample. Two replicates per formulation were obtained.

The optical properties of the films were analysed with a CM-5 spectrocolorimeter (Konica Minolta, Inc., Japan). Reflection spectra (400-700 nm) were obtained for three film replicates and at three points per replicate, backed on both black and white plates. Internal transmittance (T_i) was calculated by applying the Kubelka-Munk theory of multiple dispersion (Hutchings, 1999). CIEL*a*b* colour coordinates were obtained from the determined reflectance of an infinitely thick layer of material, considering the D65 illuminant and 10º observer (Cano et al., 2014). Finally, the lightness (L*), chroma (C_{ab}^*) and hue (h_{ab}^*) psychometric coordinates were calculated (Mahy et al., 1994).

2.4 Antibacterial effectiveness of the films

Tryptic Soy Agar (TSA) was used as culture medium for antimicrobial tests, following the methodology described in previous studies (Requena et al., 2019a; Tampau et al., 2018). Round film samples (55 mm diameter) were sterilised on both faces using UV-light in a laminar flow cabinet (Bio II advance, Telstar Spain). 10 mL of TSA were poured into 55 mm petri dishes and inoculated with 100 µL of *E. coli* or *L. innocua* bacterial suspension (10^6 CFU/mL) on the plate surface using a L-form rod to uniformly spread the inoculum. Then, the plates were covered with the film samples, and non-covered inoculated plates were included (control). The Petri dishes were sealed with Parafilm^{tm} and incubated for 6 days at 10 ºC. After incubation, samples were homogenised for 3 min in buffer peptone water, employing a Masticator paddle blender (IUL Instruments, Barcelona, Spain). Serial dilutions were plated and covered with selective media: violet red vile agar (VRBA) for *E. coli* and palcam agar base (PAB) enriched with palcam selective supplement for *Listeria* (Requena et al., 2019a; Tampau et al., 2018). After 48 h incubation at 37 ºC, the colonies were counted. Each film formulation and control were tested in duplicate. Microbial counts were also performed in duplicate for each sample.

2.5 Release kinetics of ferulic and cinnamic acids from the polymeric matrix

Release kinetics of ferulic and cinnamic acids from PLA film samples were evaluated as described by Requena et al. (2017). Four simulants were used to represent different food systems: 10%v/v ethanol solution (simulant A) imitates aqueous food systems; acetic acid 3%w/v (simulant B) simulates aqueous food systems with pH values lower than 4.5; ethanol 50%v/v (simulant D1) represents alcoholic food and oil-in-water emulsions, and isooctane (simulant D2) simulates fatty continuous phase food systems (Commission Regulation (EU) No 10/2011). Film samples (500 mg each, three per formulation) were immersed in 100 ml of the food simulant and kept at 20°C for 30 days under magnetic stirring. F or C concentration released in the simulants was periodically evaluated. To this end, an aliquot was taken from the simulant at different times and analysed as to its acid content using UV-visible spectroscopy as described in section 2.3.2.

Peleg's equation (**Equation 1)** was used to model the release kinetics. The concentration at equilibrium (M_∞), partition coefficient (M_∞/M_o) and release rate were calculated (Peleg, 1988).

$$\frac{t}{M_t} = k_1 + k_2 t \tag{1}$$

Where:
M_t: mass of active compound released at time t

k_1 and **k_2** are model constants, k_1 is inversely related to the initial release rate ($1/k_1$) and k_2 with the asymptotic release value at equilibrium (**M_∞**$= 1/k_2$).

The Korsmeyer-Peppas model (**Equation 2**) was also used to fit the experimental data up to 60% of the total acid release. The corresponding parameters were calculated to evaluate the release mechanism involved (Siepmann & Peppas, 2011).

$$\frac{M_t}{M_\infty} = kt^n \tag{2}$$

Where:

M_t/M_∞ is the released fraction with respect to the equilibrium concentration at time t
k is the release rate constant incorporating several factors involved in the diffusion process
n is a constant indicating the release mechanism; thus, for a thin film sample, an n value of 0.5 indicates a Fickian diffusion, n values below 0.5 are considered a quasi-Fickian diffusion and n values higher than 0.5 are known as anomalous transport, involving the coupling of polymer relaxation and compound diffusion.

2.6 Statistical analysis

Statistical data analysis was performed through an analysis of variance (ANOVA) and regression analyses using Statgraphics Centurion XVII software. Fisher's least significant difference was used at a 95% confidence level.

3. Results and discussion

Homogeneous, transparent and malleable films were obtained for all the formulations where no phase separation was observed in the microscopic analyses. **Figure 1a** shows the FESEM micrographs of PLA films with 2 % ferulic and cinnamic acids where a good integration of the acids in the polymer matrix during the melt blending process was obtained. No separated acid particles were detected in any case as revealed in the FSEM micrographs.

3.1. Thermal behaviour of the films.

Figure 1a shows the first and second heating thermograms obtained for the different samples. In every case, the lack of crystalline regions in the amorphous material was confirmed by the absence of the typical melting endotherms of semi-crystalline PLA at about 175ºC (Muller et al., 2017). The glass transition and subsequent relaxation endotherm were the only thermal events observed. **Table 1** shows the glass transition temperatures for both the first and second heating steps in the DSC analyses (Tg_1 and Tg_2). These Tg values were in the range of those reported in previous studies for thermally processed PLA (Badia et al., 2012; Collazo-Bigliardi et al., 2019; Gómez-Contreras et al., 2021). This temperature was only slightly reduced by the incorporation of the acids, as reported in previous studies for PLA (Dintcheva et al., 2017; Jin et al., 2002; Quynh et al., 2007; S. Yang et al., 2008), but no significant plasticization effect was observed in the second heating step for the incorporated amounts of acids. The thermograms (**Figure 1**a) also showed the typical relaxation endotherms just after the glass transition in the first heating scan, as previously observed by other authors (Cai et al., 1996; Muller et al., 2016). The enthalpy of relaxation is quantitatively related with the physical aging of the material and the slight differences between samples, as well as between the glass transition temperatures from the two heating scans, could be attributed to the thermal history of the material.

Table 1 Glass transition temperatures obtained from the first (Tg_1) and second heating (Tg_2) steps and onset and peak degradation temperatures from TGA. PLA: film without acids; 1F and 2F: films with 1 or 2 % ferulic acid; 1C and 2C: films with 1 or 2% cinnamic acid.

	DSC		TGA	
Formulation	**Tg_1 (°C)**	**Tg_2 (°C)**	**T_{onset} (°C)**	**T_{max} (°C)**
PLA	54.1 ± 0.9^b	52.3 ± 0.1^a	265 ± 3^a	309 ± 1.3^a
1F	52.3 ± 0.2^a	52.6 ± 0.6^a	280 ± 8^b	320 ± 5^b
2F	52.7 ± 0.2^a	50.0 ± 2.0^a	281 ± 7^b	319 ± 4^b
1C	52.1 ± 0.1^a	51.6 ± 1.8^a	292 ± 0^{bc}	326 ± 0^{bc}
2C	51.7 ± 0.0^a	51.6 ± 0.2^a	299 ± 2^c	329 ± 0^c

Superscript letters in each column indicate significant differences between groups ($p < 0.05$).

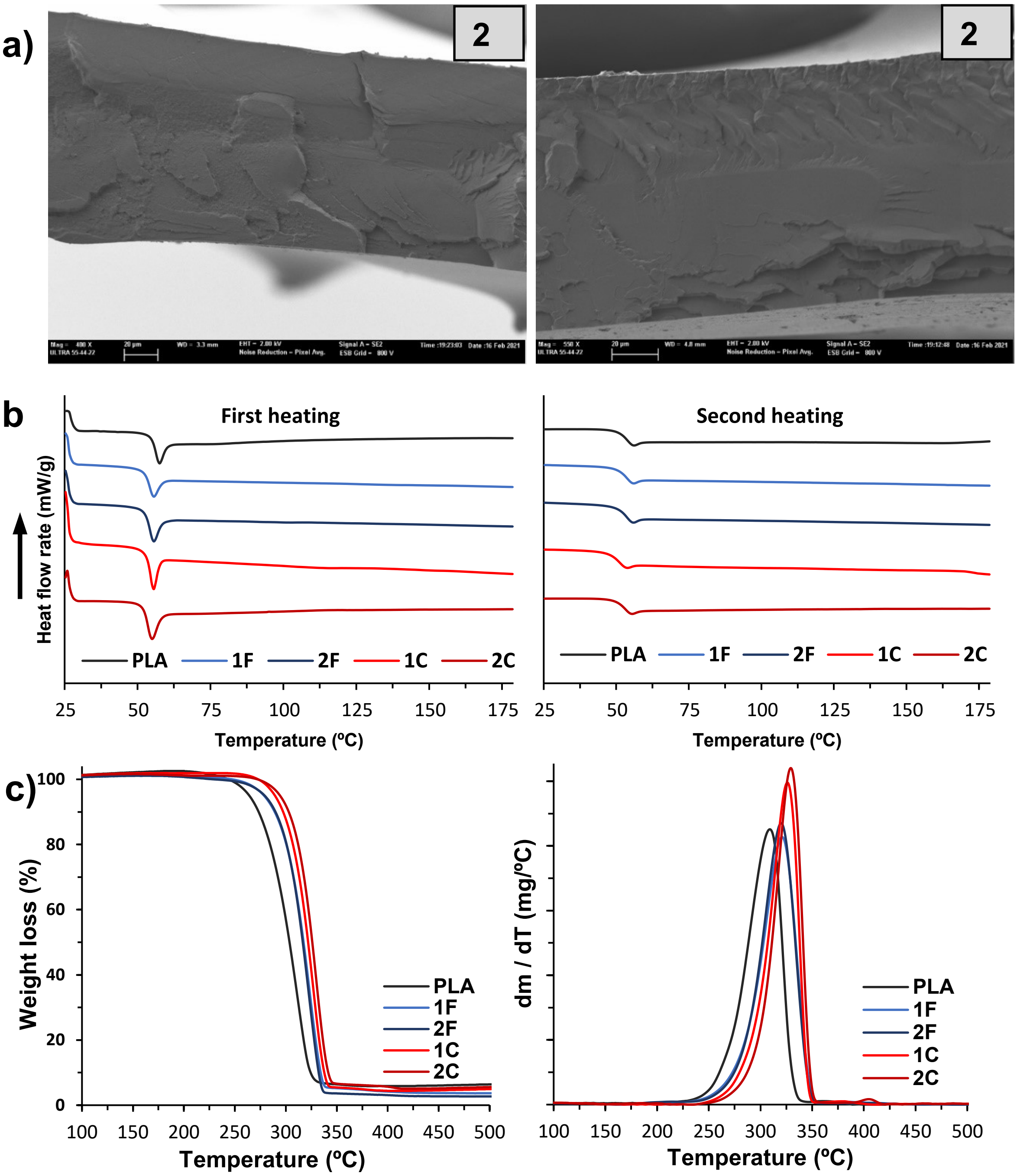


Figure 1 a) FESEM micrographs of PLA films with 2 % of ferulic (left) or cinnamic (right) acids. **b)** DSC thermograms for the first and second heating steps of PLA films. **c)** TGA curves weight vs temperature and weight loss vs temperature. PLA: film without acids; 1F and 2F: films with 1 or 2 % ferulic acid; 1C and 2C: films with 1 or 2% cinnamic acid.

As concerns the thermal degradation behaviour, the TGA curves of the different films are shown in **Figure 1b**. Likewise, the peak and onset temperatures of thermal degradation are also shown in **Table 1**. The thermal degradation of PLA is based on a hydroxyl end initiated ester exchange process and chain homolysis (Zou et al., 2009). Reactive end groups and impurities have been reported to enhance the thermal degradation of PLA (Signori et al., 2008). In the TGA curves of the films (**Figure 1b**), the protective effect of both ferulic and cinnamic acids on the thermal degradation of PLA can be observed. The onset and peak degradation temperatures (**Table 1**) significantly increased when acid was added. The thermal degradation curve of PLA was similar to that reported by Zou et al. (2009) at the same heating rate (10 ºC/min) and N_2 flow, whereas F addition caused an increase in thermal degradation of about 10ºC and the incorporation of C resulted in an increase in T_{max} of as much as 20ºC in the case of the 2C formulation. Degradation temperatures are affected by the structural properties of the matrix, such as molecular weight or crystallinity. However, the DSC analysis did not reveal notable structural changes reflected in the phase transitions of the materials. Therefore, the thermal protective effect of ferulic and cinnamic acids could be related to their antioxidant activity. During the thermal degradation process of PLA, radical reactions take place above 250ºC (Nicolae et al., 2008). The radical scavenging capacity of phenolics could partially inhibit degradative reactions at high temperatures, thus delaying the polymer degradation. At high temperatures, the thermal degradation of the acids initially implies their decarboxylation (Cheng et al., 2014), but their decarboxylated forms also exhibit antioxidant capacity (Belvedere & Tursi, 1981; Cheng et al., 2014; Tung et al., 2012). The decarboxylation of cinnamic acid produces styrene that has been used to produce films, (styrene-butadiene-styrene (SBS) block copolymer) of potential use as oxygen-scavenging polymers for barrier applications (Tung et al., 2012).

On the other hand, Tran et al. (2015) successfully obtained novel PLAs with the conjugation of both chain terminals with cinnamic acid and its derivates and proved the significant improvement in their thermal properties. In this sense, partial esterification of end chain -OH of PLA with ferulic or cinnamic acids, during thermal processing, could also induce thermal protection in the PLA chains. In fact, no thermo-protective effect was observed for PLA films with ferulic or cinnamic acid obtained by casting (Ordóñez et al. 2022). Therefore, although the true mechanism is unclear, the enhancing of thermal stability of thermo-processed PLA by these acids is an interesting finding since the heat resistance of PLAs is relatively low, which limits their applications.

3.2. Physical properties of the films and final content of active compounds

3.2.1. Active compound retention

Table 2 shows the different physical properties of the PLA films with and without ferulic and cinnamic acids at 1 or 2 wt. %, and their final retention (in percentage) after film processing, referred to the amount of compound initially incorporated (1 or 2 %). High ratios (84-98 % of the incorporated amount) of both compounds were retained in the films during processing, despite the thermal stress that the material undergoes (**Table 2**). Retention seemed to be favoured at low acid concentrations, more particularly in cinnamic acid formulations. The lowest recovery ratios found for sample 1F, as compared to sample 1C, could be attributed to the higher thermolability of ferulic acid, with a higher antioxidant capacity associated with its molecular structure (o-hydroxycinnamic acid with a o-methoxy group with respect to the hydroxyl) that promotes the hydrogen donation capacity (Li et al., 2020). A reduction of 40% in the initial content of ferulic acid and under 10 % of cinnamic acid was also observed during the thermo-processing at 130 °C of starch films containing these compounds (Ordoñez et al., 2021). Likewise, both ferulic and cinnamic acids with pKa of 4.58 and 4.37, respectively, could be partially bonded by ester formation with the end chain OH groups of PLA during thermal processing. This covalent bond would completely limit compound release during the extraction process, thus also contributing to the less than total recovery of the compounds during extraction. This potential bonding was also inferred by the promoted thermal stability of the PLA commented on above.

Table 2. Functional properties of PLA films: active compound retention (AC) with respect to the initially incorporated amount, film thickness (t), tensile strength (TS), elongation at break (E), elastic modulus (EM), water vapour permeability (WVP), oxygen permeability (OP), lightness (L*), hue (h_{ab}*) and chroma (C_{ab}*). PLA: film without acids; 1F and 2F: films with 1 or 2 % ferulic acid; 1C and 2C: films with 1 or 2% cinnamic acid.

	PLA	1F	2F	1C	2C
AC (wt.%)	-	92.0 ± 3.2^{b}	88.7 ± 3.4^{ab}	97.6 ± 2.3^{c}	84.6 ± 2.4^{a}
t (µm)	207 ± 7^{ab}	217 ± 10^{c}	209 ± 6^{bc}	201 ± 5^{ab}	198 ± 5^{a}
TS (MPa)	44 ± 5^{a}	35 ± 8^{b}	40 ± 4^{a}	42 ± 2^{a}	38 ± 4^{ab}
EM (MPa)	1310 ± 80^{a}	1260 ± 40^{b}	1240 ± 60^{b}	1250 ± 40^{b}	1270 ± 14^{b}
E (%)	4.3 ± 0.5^{a}	2.8 ± 0.7^{b}	3.5 ± 0.2^{ab}	3.6 ± 0.2^{ab}	3.2 ± 0.4^{ab}
WVP (g·mm/kPa·h·m^{2})	0.26 ± 0.01^{b}	0.24 ± 0.08^{b}	0.14 ± 0.01^{a}	0.18 ± 0.03^{ab}	0.19 ± 0.03^{ab}
OP (x10^{14}·cm^{3}/m·s·Pa)	187 ± 4^{ab}	191 ± 5^{b}	179 ± 0^{ab}	184 ± 6^{ab}	176 ± 8^{a}
L*	82.8 ± 1.1^{a}	86.5 ± 1.5^{bc}	82.2 ± 1.3^{a}	87.5 ± 1.4^{c}	85.3 ± 1.0^{b}
h_{ab}*	102.3 ± 0.8^{c}	96.5 ± 2.0^{b}	90.4 ± 1.4^{a}	108.0 ± 2.0^{d}	98.0 ± 0.6^{b}
C_{ab}*	5.1 ± 0.4^{a}	9.3 ± 0.7^{d}	9.7 ± 0.6^{d}	6.7 ± 0.4^{b}	8.3 ± 0.5^{c}

Different superscript letters indicate significant differences between film formulations ($p < 0.05$).

3.2.2. Mechanical properties

Figure 2 shows the typical stress-strain curves of the films tested, and the mechanical parameters are shown in **Table 2**. The mechanical behaviour of PLA films without acids was similar to that reported in previous studies for the same kind of PLA (Muller et al., 2017). The incorporation of the acids into the proportions tested did not lead to an improvement in the mechanical behaviour of the films, since these become less resistant to break and less extensible, with a small reduction in the elastic modulus. This also indicates the lack of notable cross-linking or plasticising effects deduced from the DSC analyses. The slight reduction in EM caused by acid incorporation could be attributed to the thermal and mechanical stress caused by the melt-blending process in the presence of acids, which could favour the partial degradation of PLA chains giving rise to a small fraction of oligomers and the subsequent loss of cohesion forces in the matrix, as reported by other authors (Signori et al., 2008). Although no clear effect of this potential hydrolysis was reflected on the Tg values, the mechanical response could be more sensitive to the presence of oligomers since these contribute to the weakening of the matrix cohesion forces, reducing the elastic modulus and resistance to break. In fact, higher concentration of cinnamic acid (5 and 10 %) in PLA films notably reduced the Tg value by 5-15 ºC (Ordoñez et al., 2022).

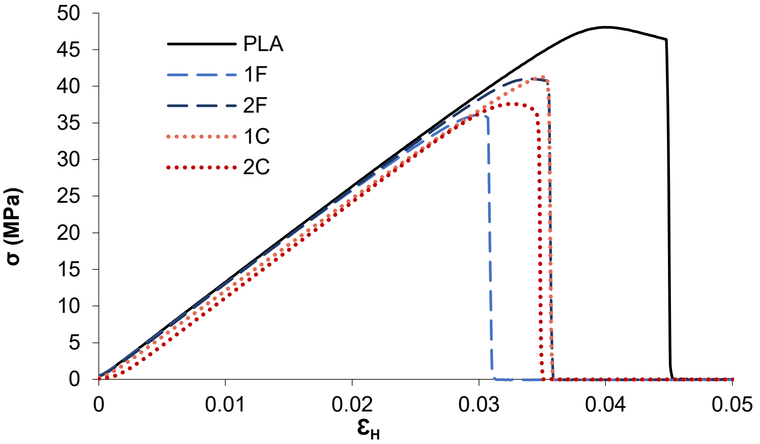


Figure 2 Stress-strain curves of films conditioned at 53% RH. PLA: film without acids; 1F and 2F: films with 1 or 2 % ferulic acid; 1C and 2C: films with 1 or 2% cinnamic acid.

3.2.3. Barrier properties

Table 2 shows the values of the WVP and OP of the different films. Little variations in the barrier properties were caused by the incorporation of the acids. Statistically significant reductions in WVP were found for films 2F, 1C and 2C, with formulation 2F exhibiting the lowest value of WVP. This effect could be attributed to the previously mentioned bonding of -OH groups at the end of PLA chains with the acid, thus reducing the water affinity of the matrix even more. This behaviour was also observed in other more hydrophilic polymer matrices, such as sodium caseinate, where the WVP was reduced in relation to the amount of ferulic acid incorporated (Fabra et al., 2011). On the other hand, acid incorporation did not have a clear effect on the oxygen permeability, whose values were only slightly reduced for the highest ratio of the acids. This reduction could be related to the oxygen scavenging capacity of the added acids (Bonilla et al., 2013).

3.2.4. Optical properties

Figure 3a shows the internal transmittance spectra of the films, and **Figure 3b** shows the locus of the different films in the a*b*chromatic plane. Similar T_i spectra were observed for amorphous PLA films without acids to those obtained by Collazo-Bigliardi et al. (2019), whereas a slight increase in internal transmittance was observed for all the film formulations containing the acids. The higher the Ti the greater the film transparency. Thus, the transparency of the films was slightly enhanced by ferulic and cinnamic acids, which may be attributed to the slight loss in cohesion forces of the polymer matrix, as deduced by the mechanical analysis, and the subsequent decrease in the refractive index. In contrast, the colour coordinates (**Table 2**) were slightly modified by acid incorporation. Film lightness (L*) ranged between 82 and 87, and no clear pattern of variation was observed as a result of acid concentration. All the films showed some yellowish hue, the values of h_{ab}* ranging between 90 and 108. Ferulic acid promoted yellowness to a greater extent than cinnamic acid. Colour saturation (C_{ab}*) increased when acid was incorporated, the increase being most remarkable in the case of ferulic acid. This could be, in part, attributed to the natural colour of the acids (more yellowish in ferulic acid) or their partial oxidation during the film's thermal processing, leading to the development of browning in the film. This effect was more remarkable in films containing ferulic acid that is more coloured and whose thermal degradation temperature is lower (167 ºC) than that of cinnamic (180 ºC).

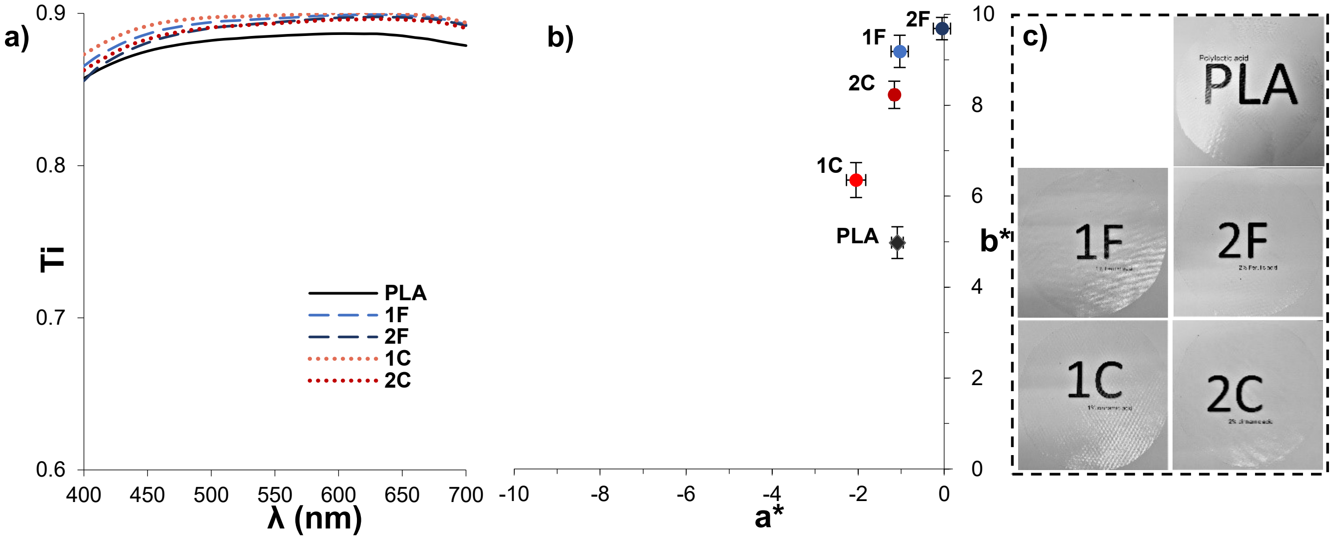


Figure 3 Optical properties of films a) Internal transmittance spectra, b) a*b*chromatic diagram. PLA: film without acids; 1F and 2F: films with 1 or 2 % ferulic acid; 1C and 2C: films with 1 or 2% cinnamic acid. c) Film images displayed to show film transparency.

3.3. Antibacterial effectiveness of the films

Figure 4 shows the results of the *in vitro* assays carried out to determine antibacterial effectiveness against *E. coli* and *L. innocua* of the films. No antibacterial activity against *L. innocua* was observed for any films since no significant differences in the viable counts were found for film formulations or the uncovered control plates. In contrast, a mild inhibitory effect was found against E*. coli* for some films. Nevertheless, none of the film formulations caused a 2 log CFU reduction, which is needed if they are to be considered as effective antibacterial material (Requena et al., 2019a). Lower reductions are in the range of natural variability of this kind of test. However, the amount of acids incorporated in the films was enough to reach the MIC values of both compounds (around 0.7 mg/mL) in the plates for the bacteria tested, considering the amount of active in the film per plate and the volume of the culture medium, if a complete release was achieved. This indicates that the active compounds were not completely released from the film matrix and the released amount was not enough to cause any antimicrobial effect. In a previous study, remarkable growth inhibition of both bacteria, mainly *Listeria*, was observed when using thermoplastic starch as the carrier matrix of these acids in the same ratio (2% wt,%), (Ordoñez et al., 2021). However, the hydrophobic nature of the PLA matrix and the lack of polymer relaxation when in contact with the culture media, greatly limits the effective release of active compounds, which is necessary to reach MIC values of the bacteria in the culture plate. These findings were corroborated through the study of the release kinetics of compounds into food simulants of differing polarities, as discussed in the next section.

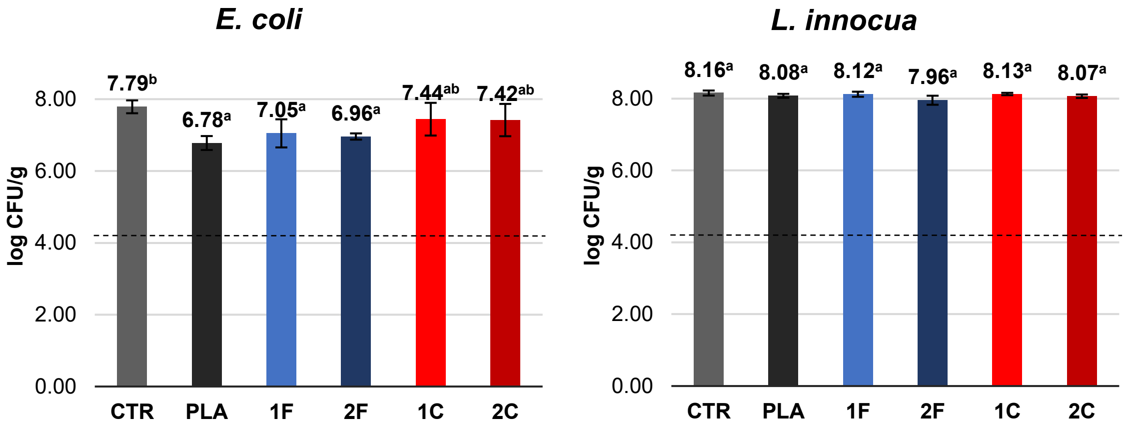


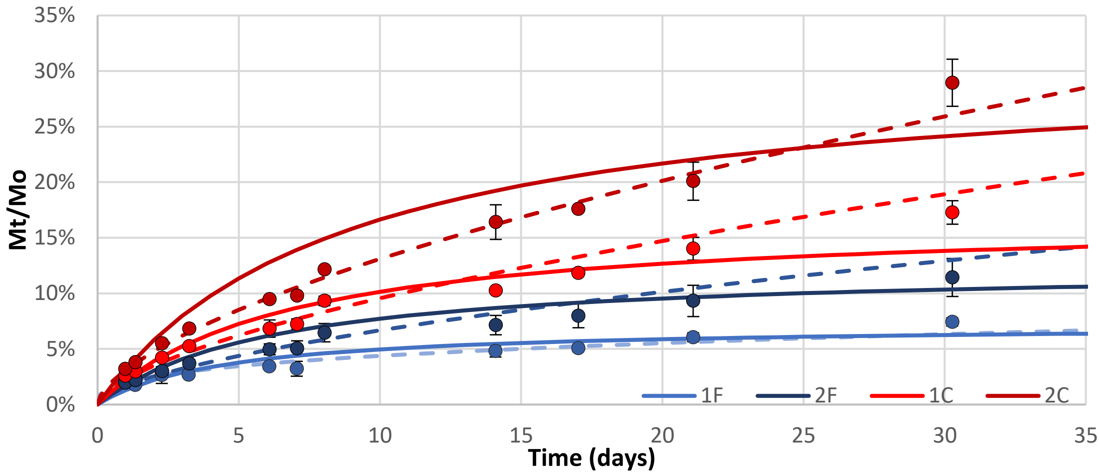
Figure 4 Microbial counts for *E. coli* and *L. innocua* obtained after 6 days of incubation at 10°C in TSA medium. Uncovered control sample (CTR), and covered samples with PLA: film without acids; 1F and 2F: films with 1 or 2 % ferulic acid; 1C and 2C: films with 1 or 2% cinnamic acid.

3.4. Release kinetics of cinnamic and ferulic acids from the polymeric matrix

The concentration of cinnamic and ferulic acids in the simulant solutions in which the film samples were immersed was monitored for 30 days. Whereas no detectable acid release occurred in either the polar food simulants (A and B) or the non-polar simulant

(D2), a significant release was detected into the D1 simulant, which indicates that the acids could only be released from the polymer matrix into less polar food substrates, such as alcoholic foods or oil-in-water emulsions, as simulated by the D1 solvent. In the D1 simulant with 50% ethanol, a relaxation of the PLA matrix brought about by ethanol penetration into the polymer matrix would favour the compound release to some extent. Other authors also found an increase in the compound release from PLA matrices when the simulant had a higher ratio of ethanol (Jamshidian et al., 2012). The ethanol migration into the PLA matrix provokes the polymer matrix relaxation which enhances molecular mobility and compound diffusion, thus promoting its release in the simulant. They also report a partial degradation of the polymer matrix in contact with methanol, which also favoured the compound release. Yang et al. (2019)Yang et al. (2019) also observed a higher release of active compounds from PLA:PBSA (9:1) films when the ethanol ratio rose in the food simulant. In the absence of matrix relaxation, the glassy state of the polymer at the release temperature (Tg>20 ºC) greatly limited molecular mobility and diffusion-controlled mass transfer process. Therefore, film plasticisation could be a possible strategy to promote the release of active compounds, although adequate plasticizers should be used to fit the film functional properties (mechanical and barrier properties) to the food packaging requirements.

The experimental values of M_t/M_o (amount of compound released with respect to the initial amount in the films), as well as the fitted Peleg model (curves) are shown in **Figure 5**. The kinetic parameters obtained from the fitting of the Peleg and Korsmeyer-Pepas models are also shown. The initial release rate ($1/k_1$) and final release ratio M_∞/M_o) increased when the acid concentration rose in the film for both acids, in agreement with the higher mass transfer driving force. However, significant differences were observed in the delivery behaviour of both acids. The release of cinnamic acid from the PLA matrix into the food simulant took place more extensively and at a higher rate than that of ferulic acid. This is probably linked to the structural differences of both molecules. Whereas the molecule of ferulic acid has one hydroxyl group in para position with respect to the carboxyl, cinnamic acid does not have hydroxyls in the phenolic ring. The *p*-OH in ferulic acid could favour the establishment of interchain hydrogen bonds through the oxygens of ester groups, thus favouring the bonding of molecules in the matrix and limiting their diffusion in contact with the food simulant. Therefore, the most remarkable release level was found for films with 2 % cinnamic acid, whose asymptotic value (after 30 days' contact) reached 31% of the initial content of the film. In contrast, only 15% of the initial ferulic acid content (nearly 2%) was released at equilibrium. This behaviour indicates that the compound release occurs very slowly and to a limited extent, which compromises the active role of the compounds in different food substrates of intermediate polarities.



Formulation	Peleg				Korsmeyer-Peppas		
	$1/k_1$ (μg/day)	M_∞ (g/ 100g film)	M_∞/M_0 (%)	R^2	n	k ($days^{-n}$)	R^2
1F	53±11^{a}	0.080±0.003^{a}	8.8±0.2^{a}	>0.85	0.37±0.08^{a}	0.23±0.02^{c}	>0.94
2F	131±3^{c}	0.261±0.032^{b}	14.9±2.5^{ab}	>0.95	0.57±0.06^{b}	0.15±0.04^{b}	>0.96
1C	93±6^{b}	0.200±0.018^{b}	20.4±1.2^{b}	>0.94	0.56±0.01^{b}	0.14±0.02^{b}	>0.94
2C	263±16^{d}	0.451±0.065^{c}	31.2±3.8^{c}	>0.93	0.62±0.03^{c}	0.09±0.03^{a}	>0.97

Figure 5 Experimental values (dots) and concentration predicted by Peleg's model (solid lines) and by Korsmeyer-Peppas model (dashed lines) at different contact times for release into the D1 food simulant of ferulic (F) or cinnamic (C) acid incorporated (1 or 2%) into PLA films. Parameters of release; Peleg model: release rate ($1/k_1$), active compound release at equilibrium (M_∞) and release ratio at equilibrium (M_∞/M_0). The Korsmeyer-Peppas model: diffusional exponent (n) and rate constant (k). 1F and 2F: films with 1 or 2 % ferulic acid; 1C and 2C: films with 1 or 2% cinnamic acid.

The Korsmeyer-Pepas model was used to deduce the mechanisms involved in the acid release from the film matrix through the values of the n parameter, as reported by the authors (Siepmann & Peppas, 2011). As shown in **Figure 5**, all the n values were close to 0.5, corresponding to the Fickian or diffusional process. Nevertheless, except for the sample with 1 %f ferulic acid, the values slightly exceed this, which is associated with an anomalous non-Fickian mechanism, where polymer relaxation is coupled with the compound diffusion. Thus, the progressive penetration of ethanol molecules into the PLA matrix, induced by the solvent-polymer chemical affinity, provoked the structural relaxation of the film matrix, increasing molecular mobility and promoting the diffusion of the acid compounds. Therefore, the release of active compounds from the PLA matrix requires its plasticisation, which does not occur when in contact with aqueous systems, such as the culture medium used in antimicrobial analyses. Only when the PLA is in contact with food systems from which compound migration can plasticise the polymeric matrix, can the antimicrobial effect of the acids be expected. It is necessary to carry out antimicrobial tests on different real foods to prove these effects.

4. Conclusions

The total recovery of ferulic and cinnamic acids in thermo-processed PLA films was relatively high (84 - 98%), which suggests that these compounds can be incorporated by using the usual thermoplastic process of the plastic industry. These acids provoked a weakening effect in the polymer slightly reducing the stiffness and resistance to break, mainly with 2 % ferulic acid, although this was not notably reflected in the PLA glass transition. However, a 2 % content of both acids improved the water vapour and oxygen barrier capacity of the films. Likewise, both acids notably improved the PLA thermal stability.

The films with ferulic and cinnamic acid at 1 or 2 % did not show a significant antimicrobial action against *E. coli* and *L. innocua*, which was attributed to the scarce release of these compounds in the culture medium during the *in vitro* test. In fact, no quantitative release was detected into aqueous or non-polar media, but only into the D1 simulant (50% ethanol in water), where a low release rate and limited release ratio were also observed, mainly in the case of ferulic acid.

Further research is needed in order to favour the release of ferulic and cinnamic acids so as to improve their antimicrobial activity. Given the great potential of PLA - ferulic and cinnamic acid combinations for packaging purposes, further antimicrobial and antioxidant studies into food matrices are required to test the capacity of these films in real systems from where compound migration (e.g., fat) could favour the relaxation of the polymer matrix, facilitating the delivery of active compounds from the film.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ramón Ordoñez: Investigation, Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Lorena Atarés**: Conceptualization, Methodology, Data curation, Writing – original draft, Writing – review & editing, Supervision. **Amparo Chiralt**: Conceptualization, Methodology, Data curation, Writing – review & editing, Supervision, Project administration.

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Chapter 3:

Antibacterial properties of cinnamic and ferulic acids incorporated to starch and PLA monolayer and multilayer films.

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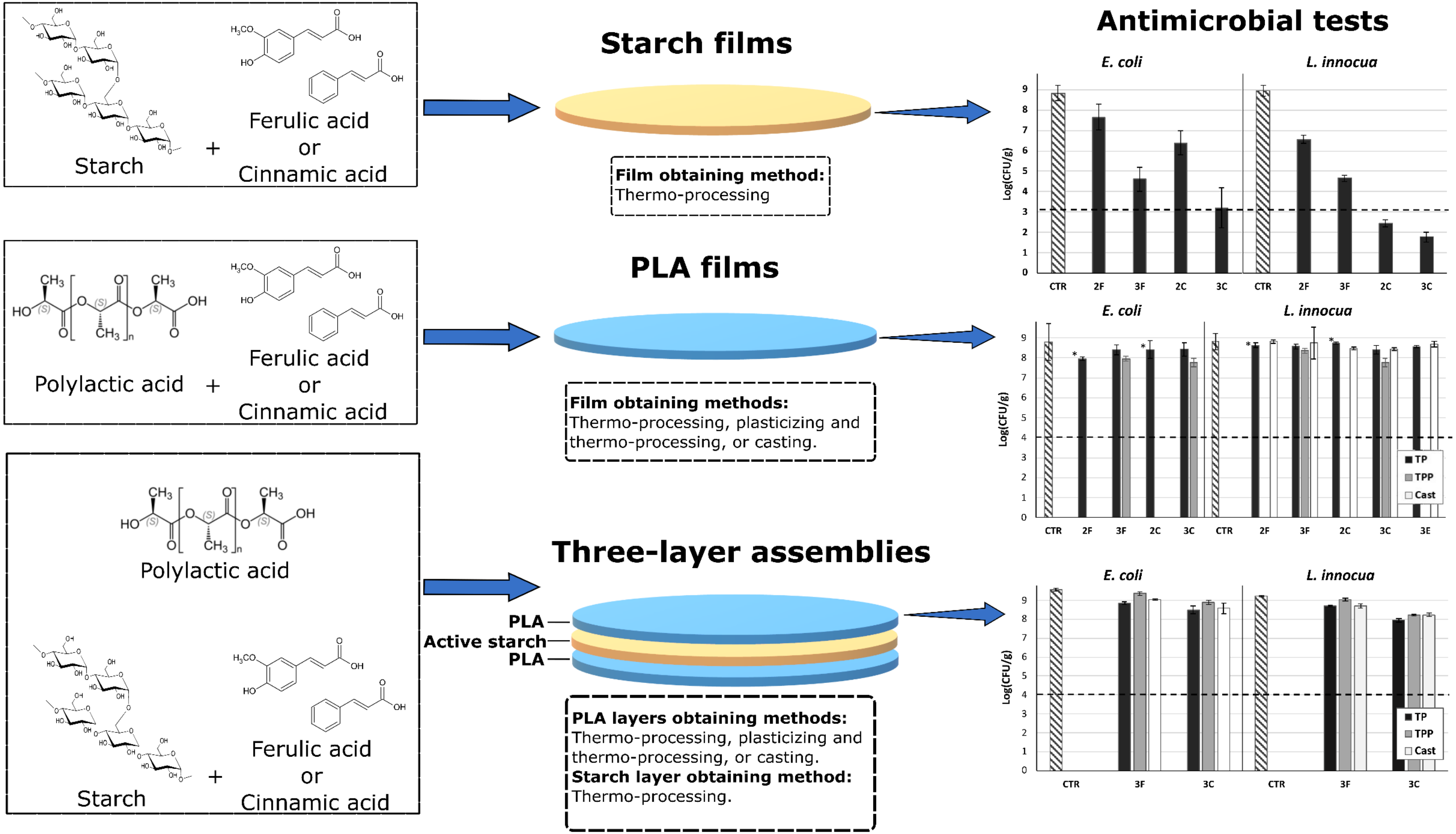

Starch + Ferulic acid or Cinnamic acid

Starch films

Film obtaining method: Thermo-processing

Polylactic acid + Ferulic acid or Cinnamic acid

PLA films

Film obtaining methods: Thermo-processing, plasticizing and thermo-processing, or casting.

Polylactic acid

Starch + Ferulic acid or Cinnamic acid

Three-layer assemblies

PLA

Active starch

PLA

PLA layers obtaining methods: Thermo-processing, plasticizing and thermo-processing, or casting.
Starch layer obtaining method: Thermo-processing.

Antimicrobial tests

Abstract

Ferulic (F) and cinnamic (C) acids have shown great potential as antimicrobial additives in the development of active food packaging based on biodegradable polymers. However, previous studies on thermo-processed (TP) PLA matrices with 1 or 2%w/w of these compounds found no microbial growth inhibition due to their limited release. In this study, 2 or 3% of F and C were incorporated into starch and PLA films, using several strategies to promote their release from PLA, in order to obtain effective antimicrobial activity against *E. coli* or *L. innocua*. TP starch films with F or C presented antimicrobial capacity, C being more effective than F for both bacteria and *L. innocua* being more sensitive to both acids. However, PLA films obtained by three different techniques: TP, thermo-processing with the addition of plasticiser (TPP), and solvent casting did not exhibit antibacterial action. The incorporation of ferulic acid methyl ester into PLA films did not improve the antimicrobial effect, which suggested that the release limitation may not be related to chemical interactions with the end chain hydroxyl of PLA. Three-layer PLA assemblies, with an internal starch film loaded with F or C, were tested using TP-PLA, TPP-PLA, or cast-PLA films as contact layer. Diffusion of F and C through the PLA layer was highly hindered, regardless of the film preparation method. Therefore, no significant bacterial growth inhibition was observed for the three-layered films. The reduced molecular mobility in the PLA matrix was the limiting factor for the compound release and subsequent antimicrobial effect.

1. Introduction

Active food packaging based on renewable sources, such as biodegradable polymers, could be an alternative to tackle the environmental crisis generated by traditional plastic packaging (Atarés & Chiralt, 2016). While extending the food shelf life, antimicrobial and antioxidant active packaging also reduces the additives directly incorporated into foodstuffs (Sharma et al., 2021). PLA and starch are two widely studied biobased/biodegradable polymers thanks to their economically viable production and the great variety of biomass from which both can be obtained (Nevoralová et al., 2019; Vinod et al., 2020). Phenolic acids are common, naturally-occurring compounds with known antimicrobial and antioxidant properties (Merkl et al., 2010) similar to those exhibited by essential oil compounds (Requena et al., 2019b) but with the advantage of exerting a milder sensory impact in food applications (Miyague et al., 2015).

The incorporation of small concentrations of ferulic or cinnamic acids into starch matrices obtained by melt-blending showed promising bacterial growth inhibition (Ordoñez et al., 2021). However, starch materials are highly sensitive to moisture, which limits their application as food contact materials in high moisture foods (Vinod et al., 2020). PLA is an aliphatic polyester with good water vapour barrier capacity, which makes it an ideal material for high moisture systems (Madhavan Nampoothiri et al., 2010). However, a previous study (Ordoñez et al., 2022) did not show antibacterial activity for PLA films containing 1 and 2 % of ferulic or cinnamic acids, which was attributed to the limited diffusion of the active compounds through the PLA matrix into the culture medium. This raises the need to increase the molecular mobility of PLA films in order to enhance the release of the active compounds, thus favouring their antimicrobial action. The release kinetics of active compounds is greatly affected by the active-polymer interactions and the relaxation of the polymer in contact with the packaged material (Jamshidian et al., 2012) and can also be highly affected by the method used for the incorporation of active compounds into the polymeric matrix and the film formation process. Film formation by solvent casting tends to create matrices with higher molecular mobility (more plasticised), mainly due to the retained solvent that acts as plasticiser (Suhag et al., 2020), which could facilitate the compound release. Likewise, the incorporation of plasticisers is widely used on an industrial scale to improve molecular mobility and to modulate the film properties (Suhag et al., 2020). On the other hand, given their complementary barrier properties, PLA and starch can be assembled in multilayer systems with enhanced oxygen and water vapour barrier capacities (Muller et al., 2017). These multilayers can contain active layers loaded with antimicrobial/antioxidant compounds in order to obtain active food packaging materials that can also serve as a means to control the release of the active compounds (Quiles-Carrillo et al., 2019).

This study aimed to analyse the release behaviour of cinnamic and ferulic acids incorporated into non-polar PLA matrices prepared by different techniques and compare it to their release from polar (starch) matrices, through the evaluation of the antimicrobial activity in agar culture media inoculated with Gram + (*Listeria innocua*) and Gram – (*Escherichia coli*) bacterial strains. Both kinds of polymer matrices with differing water sensitivities will undergo different degrees of swelling and relaxation when in contact with the aqueous culture media, which should also contribute to the release of active compounds. The casting and thermo-processing of PLA, as well as its plasticisation with PEG 1000, were used to increase molecular mobility in the matrix. Ferulic acid methyl ester was also used instead of ferulic acid to discard the possible chemical bonding of the acid to the end chain hydroxyl group of PLA during the film processing as the main cause of the limited compound diffusion through the polymer matrix. Likewise, the active loaded starch-PLA multilayer assemblies were also tested in order to analyse the potential compound release from the starch sheet through the PLA matrix.

2. Materials and methods

2.1. Materials

Amorphous PLA pellets 4060D produced by Nature Works (MN, USA), cassava starch by Asia Co., LDT (Kalasin Thailand), PEG 1000 and glycerol (Panreac Química, Barcelona, Spain) were used to produce film monolayers. Cinnamic acid, ferulic acid

(Sigma-Aldrich, Saint Louis, USA) and ferulic acid methyl ester (Alfa Aesar, Lancashire, UK) were used as antimicrobial agents incorporated into the films. Ethyl acetate (Indukern S.A., Barcelona, Spain) was used as the food contact solvent for PLA. Magnesium chloride and magnesium nitrate (Panreac Química, Barcelona, Spain) were used for the relative humidity control and conditioning of the films. Tryptone soy broth, tryptone soy agar (TSA) and buffered peptone water (BPW) for microbial testing purposes were purchased from Scharlab (Barcelona, Spain). The strains of *Listeria innocua* (CECT 910) and *Escherichia coli* (CECT 101) were obtained from the Spanish Type Collection (CECT, University of Valencia, Spain). Selective media for plate counts were supplied by Scharlab (Barcelona, Spain): violet-red bile agar (VRBA) for *E. coli* and palcam agar base (PAB) enriched with palcam selective supplement for *Listeria*.

2.2. Film preparation

All the starch films were prepared using glycerol as a plasticiser in a ratio of 0.30 g/g starch. For plasticising PLA, PEG 1000 at 0.15 g/g PLA ratio was used. Active compounds (ferulic or cinnamic acids) were added at 0, 2, or 3 g/g film. Likewise, an additional experimental series with ferulic acid methyl ester at 3 % w/w were carried out using PLA. To obtain thermally processed (TP) monolayer films, all of the components were incorporated into a Haake PolyLab QC internal mixer (Thermo Fisher Scientific, Germany) where the 10-minute melt-blending process takes place at 50 RPM and 130 ºC for starch blends and 160 ºC for PLA films. The obtained melts were cold grounded and conditioned at 53% in a desiccator with a $MgNO_3$ oversaturated solution for one week. The pellets obtained were heated and compression-moulded using a hydraulic heating press (LP20, Labtech engineering, Thailand), 4 g samples were placed in a circular mould to obtain films of approximately 15 cm in diameter. In the case of the starch films, the pellets were preheated at 160 ºC with no pressure applied for 1 min, followed by an initial compression at 5 MPa for 2 min and a second compression at 10 MPa for 6 min; finally, there was a 3-min cooling step until 70 ºC. The PLA pellets were preheated for 4 min at 200 ºC before a single compression step was applied at 10 MPa for 4 min, followed by the 3-min cooling step until 70 ºC.

Casting was used as described by Muller et al. (2017). To obtain film-forming solutions (FFS), PLA pellets were dissolved in ethyl acetate with or without the actives at 10 g PLA/g solution and left under magnetic stirring overnight at room temperature. To obtain cast monolayers, 40 g of FFS were poured into 15 cm diameter Teflon plates in order to obtain 4 g solid films, the solvent was left to evaporate overnight, and the films were peeled from the plates.

Three-layer assemblies (PLA/starch/PLA) were obtained by thermo-compressing the monolayers together. To this end, films were preheated for 2 min at 110 ºC before a single step compression at 25 MPa took place at the same temperature; afterwards, the samples were cooled to 70 ºC in 3 min. These conditions were suitable for good layer adhesion. Three-layer films were composed of a central layer of starch with the 3 % of active compounds and two thinner PLA external layers obtained through different methods: thermo-processing (TP, 100 µm), thermo-processing with a plasticiser (TPP, 100 µm), and casting of the PLA in ethyl-acetate solutions (C, 60 µm). The possible migration of phenolic acids from the starch internal layer through the PLA layer was analysed through the antimicrobial activity in the inoculated culture media in contact with the external PLA sheet.

2.3. Antimicrobial activity tests

The *in vitro* antimicrobial activity was assessed following the methodology described in previous studies (Requena et al., 2019a; Tampau et al., 2018). 10 mL samples of TSA were poured into 55 mm Petri dishes and inoculated with 100 µL of *E. coli* or *L. innocua* bacterial suspension (10^6 CFU/mL) on the plate surface using an L-form rod to achieve a uniform spread, obtaining a final initial inoculum of 10^4 CFU/mL. The samples were covered with 55 mm round film samples. A non-covered inoculated control was also included. The dishes were closed with their lids, sealed with Parafilm^{tm}, and incubated for 6 days at 10 ºC in a culture chamber. After incubation, the TSA and films were homogenised for 3 min in 100 mL of BPW employing a Masticator paddle blender (IUL Instruments, Barcelona, Spain). The serial dilutions were plated with selective media corresponding to each bacterial strain. After 48 h incubation at 37 ºC, the colonies were counted. Each film formulation and control were tested in duplicate. The microbial counts were also performed in duplicate for each sample.

Antimicrobial tests were carried out with films with 2 and 3 % of ferulic and cinnamic acids for starch films, but only with 3 % for thermo-processed PLA or three-layer films in order to have increased driving force for the active compound release, given the release limitations previously observed from the PLA matrix with 2% of the active compounds (Ordoñez et al., 2022). Some data with 2% acid into PLA films obtained in the previous study were included for comparison purposes. Likewise, *E. coli* was not tested in some films, given the lower sensitivity of this bacterium to the studied acids, *L. innocua* being more sensitive as an indicator of the effective release of the active compounds.

2.4. Statistical analysis

A statistical analysis of data was performed through analysis of variance (ANOVA) and regression analyses using Statgraphics Centurion XVII software. Fisher’s least significant difference was used at a 95% confidence level.

3. Results and discussion

3.1. Starch monolayers

Starch monolayers containing 1 and 2 %w/w of F and C were previously studied (Ordoñez et al, 2021) and microbial growth inhibition was found for films containing 2% acid. **Figure 1** shows the bacterial counts obtained for the *in vitro* microbial tests on TP starch films with 2 and 3% acid, as compared to the bacterial growth of the non-covered control samples. The films with cinnamic acid were more effective than those with ferulic acid at inhibiting the growth of *E. coli* and, as expected, the effectiveness of both compounds increased when their concentration rose in the films. In the case of *L. innocua*, the growth inhibition brought about by all the films was higher than 2 logs in every case, while cinnamic acid in the films showed greater antibacterial potential than ferulic acid. The films with 3% cinnamic acid (3C) exhibited remarkable growth inhibition for both bacterial strains, higher than 5 and 7 Log(CFU/mL) for *E. coli* and *L. innocua*, respectively. **Table 1** summarises the growth inhibition (expressed as the difference in Log(CFU/mL) between the sample and the respective control sample) obtained for the different films. In general, the films with cinnamic acid were more effective than films with ferulic acid and *L. innocua* was more sensitive to these active compounds than *E. coli*, this can also be deduced from the respective values of the minimally inhibitory concentration (MIC) of these acids against both bacteria, reported in a previous study (Ordoñez et al., 2021). Therefore, when active compounds were incorporated into starch films, these were effectively released into the culture media, as deduced from their antibacterial effect that was dependent on the amount incorporated into the film. The water-sensitive starch films likely swelled in contact with the aqueous culture medium, thus promoting the compound diffusion from the film to the plate surface, which promoted the antibacterial action of the compounds.

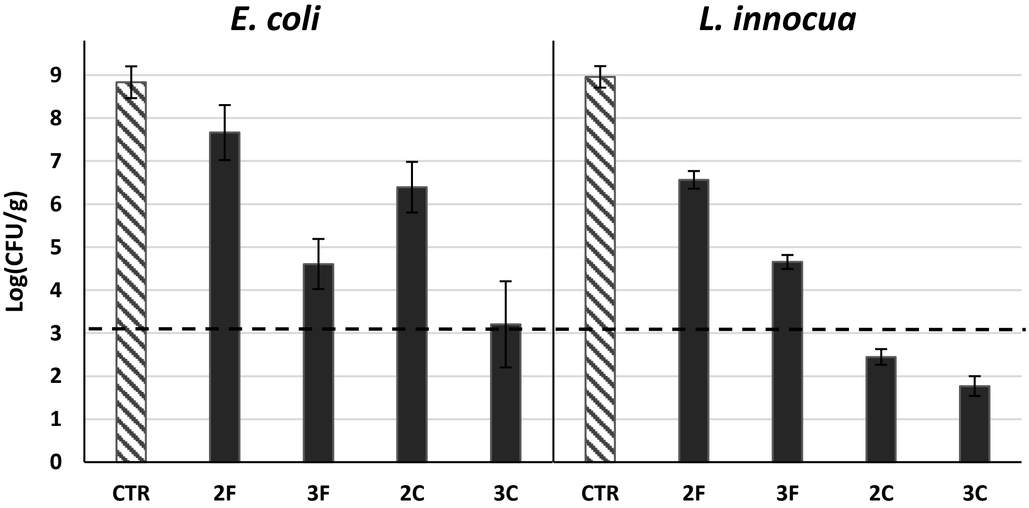

Figure 1 *E. coli* and *L. innocua* growth after 6 days of incubation at 10 ºC for samples in contact with thermally processed starch monolayers with 2 or 3% ferulic (F) acid or cinnamic (C) acid and uncoated control growth sample (CTR, striped bar). The dotted line represents initial inoculation.

Table 1 Growth inhibition of *E coli* and *L. innocua* found in the *in vitro* tests with the different biodegradable films with ferulic (F) acid, cinnamic acid (C), or ferulic acid methyl-ester (E).

Experimental series	PLA film obtaining method(1)	Active compound	Active concentration (% w/w)	Growth inhibition (Log(CFU/mL)	
				E. coli	***L. innocua***
Active starch monolayers	TP	F	2	1.17±0.64^{fg}	2.39±0.21^{h}
	TP	F	3	4.23±0.58^{i}	4.30±0.16^{i}
	TP	C	2	2.44±0.59^{h}	6.51±0.18^{j}
	TP	C	3	5.63±1.00^{ij}	7.19±0.23^{k}
PLA thermo-processed monolayers	TP(2)	F	2	0.83±0.2^{efg}	0.2±0.12^{ab}
	TP(2)	C	2	0.37±0.3^{ab}	0.09±0.05^{a}
	TP	F	3	0.39±0.08^{a}	0.25±0.08^{e}
	TP	C	3	0.36±0.3^{ab}	0.42±0.05^{d}
PLA thermo-processed & plasticised monolayers	TPP	F	3	0.83±0.13^{efg}	0.47±0.21^{ef}
	TPP	C	3	1.01±0.21^{g}	1.04±0.21^{hg}
Cast PLA monolayers	Casting	F	2	N.D.	0.01±0.08^{a}
	Casting	F	3	N.D.	0.08±0.31^{ab}
	Casting	C	2	N.D.	0.34±0.07^{cd}
	Casting	C	3	N.D.	0.38±0.08^{d}
PLA TP and cast monolayers with Ferulic acid methyl ester	TP	E	3	N.D.	0.26±0.06^{c}
	Casting	E	3	N.D.	0.14±0.12^{ab}
Multilayer: Active starch with TP PLA	TP	F	3	0.71±0.08^{def}	0.53±0.03^{f}
	TP	C	3	1.07±0.19^{g}	1.27±0.09^{h}
Multilayer: Active starch with TPP PLA	TPP	F	3	0.20±0.09^{a}	0.20±0.08^{bc}
	TPP	C	3	0.67±0.12^{de}	1.01±0.03^{g}
Multilayer: Active starch with cast PLA	Casting	F	3	0.52±0.03^{bc}	0.12±0.12^{ab}
	Casting	C	3	0.98±0.28^{fg}	0.99±0.08^{g}

(1) TP: Thermo-processing, TPP: Plasticised & thermo-processing,

(2): data from a previous study (Ordoñez et al. 2022)

N.D.: Not determined.

3.2. PLA monolayers

TP PLA films loaded with cinnamic or ferulic acid at 1 or 2% were obtained in previous studies, where no significant microbial inhibition was detected, which was attributed to the insufficient active release from the film in the culture media (Ordoñez et al., 2022). In order to improve the molecular mobility and compound diffusion in the film, thereby making the release of the acids from the PLA matrix easier, different strategies were tested, namely incorporating a plasticiser into the PLA matrix (Suhag et al., 2020) and obtaining the films by casting. Likewise, the greatest concentration of acids (3% w/w) was only used in thermo-processed PLA films, with and without plasticiser, in order to increase the acid diffusion driving force through the films into the culture medium.

Figure 2 shows the microbial counts of plates in contact with PLA thermo-processed (TP), with plasticiser (TPP), and cast films, compared to the non-covered control samples. **Table 1** summarises the numerical values of growth inhibition in the different samples compared to the respective growth control, expressed as Log(CFU/mL) difference. None of the PLA monolayers showed a significant antimicrobial action since growth inhibition values were lower than 2 Log(CFU/mL) (Requena et al. 2019a) while the observed differences among samples are in the variability range typically observed in microbial counts. *E. coli* was not tested for cast films since *L. innocua* is more sensitive than *E. coli* to the active compounds and so, no significant activity against *E. coli* was expected. The lack of antibacterial activity of PLA films must be attributed to the fact that too small a quantity of compound was released to reach the MIC of each bacterium. Probably, only the acid molecules near the film surface were delivered, which constitutes a very limited amount and is not enough to reach the MIC values. This could be due either to the strong bonding of acids to the PLA chains (e.g. by the formation of esters with the end chain hydroxyl groups of PLA) that could occur during film processing or to the greatly reduced compound diffusion in the films associated with the glassy state of the matrix at the incubating temperature (10ºC) and to the hydrophobic nature of PLA that limits both the polymer swelling and the relaxation of the matrix in contact with the aqueous culture medium. Reduced molecular mobility in the polymer matrix would seriously affect the release of the internal acid molecules from the film and their potential antimicrobial effect. In contrast, Sharma et al. (2020) reported the antimicrobial effect of PLA/PBAT cast films with ferulic acid on both *E. coli* and *L. monocytogenes*, which may be attributable to the higher incubation temperature (37ºC) and the PLA blending with PBAT, likely affecting the interactions between the acid molecules and the polymer matrix. To rule out the hypothesis of the binding of active acids to PLA chains during film processing, two strategies were followed: a) the incorporation of ferulic acid methyl ester (with similar antibacterial activity to ferulic acid) as an active compound (Merkl et al., 2010) at the same concentration and b) the analysis of the antibacterial activity of three-layer films, with starch loaded with actives in the core and with PLA in the outer layers.

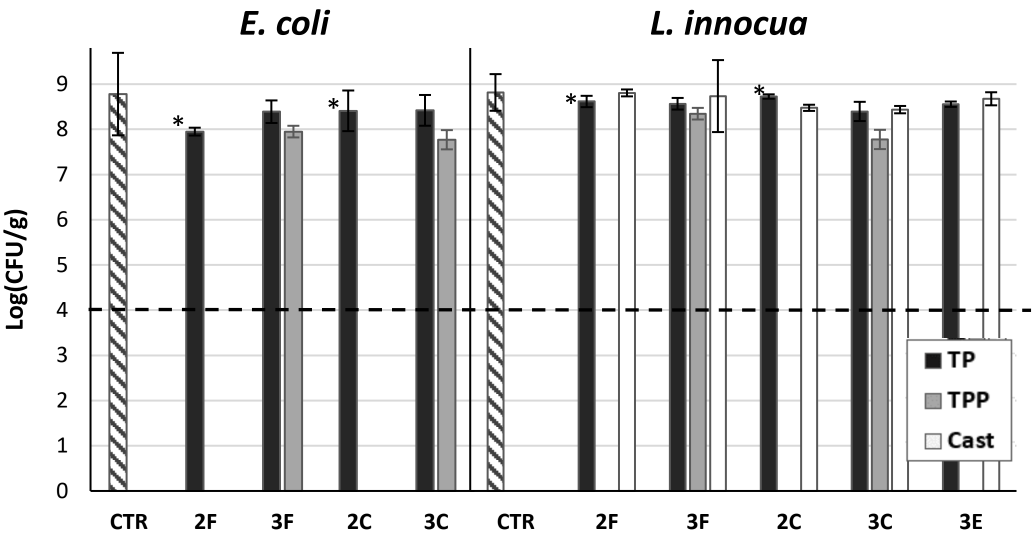

Figure 2 *E. coli* and *L. innocua* counts after 6 days of incubation at 10 ºC for samples in contact with PLA monolayers with 2 or 3% ferulic (F) acid cinnamic (C) acid or ferulic acid methyl €er (E); obtained by thermal processing (TP, black bars), plasticising and thermal processing (TPP, grey bars) or casting (white bars). Mean counts of uncoated control growth sample (CTR, striped bar). The dotted line represents initial inoculation. *Data from Ordoñez et al., 2022.

Figure 2 also shows the counts of *L. Innocua* for PLA films containing ferulic acid methyl ester at 3 wt.% € obtained by both thermo-processing and casting. In no case was the growth inhibition significant, which suggests that the bonding of active acids to the PLA chains was not the cause of the lack of effective release of these compounds into the culture media, since the ferulic acid methyl ester does not diffuse either. Therefore, the reduced molecular mobility in the PLA matrix must be the limiting factor as regards the antimicrobial action of the films. This was also corroborated by the three-layer films discussed in the next section.

3.3 Three-layer assemblies of active loaded starch and PLA

A three-layer assembly, composed of active loaded starch (with 3% ferulic or cinnamic acid) as an internal layer and external PLA layers, was also tested in order to analyse the potential diffusion of active compounds from the starch layer into the culture media through the PLA external layers obtained by the different treatments (TP, TPP, and casting). **Figure 3** shows the corresponding microbial counts, including the control plates. None of the three-layer assemblies provided significant growth inhibition of the bacteria since all inhibition values were lower than 2 Log (CFU/mL). The three-layered films with 3% cinnamic acid reached 1 Log(CFU/mL) growth inhibition of *L. innocua*, as also observed for TPP PLA monolayers, which suggests some release of the active compound. The MIC value of cinnamic acid with *L. innocua* was the lowest (Ordoñez et al., 2021) and this could explain the greater growth inhibition observed, although this was not enough to be considered effective.

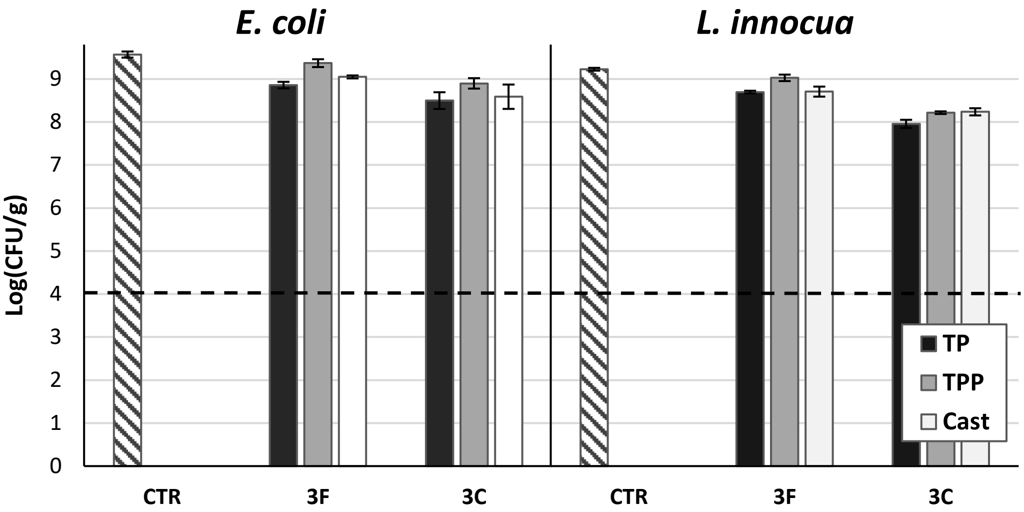

Figure 3 *E. coli* and *L. innocua* counts after 6 days of incubation at 10 ºC for samples in contact with the different three-layer films (internal starch layers loaded with 2 or 3% ferulic (F) acid or cinnamic (C) acid). The different PLA contact layers were thermo-processed (TP, black bars), plasticised and thermo-processed (TPP, grey bars), or obtained by casting (white bars). Uncoated control growth sample (CTR, striped bar). The dotted line represents initial inoculation.

Therefore, the presence of a plasticiser and the production method of the PLA layers were not decisive for promoting the active compound release from the PLA matrix. The expected improvement in the molecular mobility in TPP or cast films was not enough to favour the diffusion of ferulic or cinnamic acids through the PLA matrix into aqueous systems, such as a culture medium, and the obtained PLA films were not effective as antimicrobial packaging materials. Superficial incorporation of the actives on the PLA films could be considered to obtain PLA active films with these acids, thus avoiding the diffusion problems through the matrix. In this sense, Quiles-Carrillo et al. (2019) found a slow but consistent release of gallic acid into a saline medium from electrospun coated PLA films with PLA fibres containing gallic acid. This release is probably favoured by the high surface to volume ratio exhibited by the electrospun fibres. Therefore, the surface load of PLA films, using electrospinning or other techniques, could be used to obtain active PLA films with ferulic or cinnamic acids. Further studies are necessary to probe the effectiveness of these methods.

4. Conclusions

Starch monolayers loaded with cinnamic and ferulic acids showed notable growth inhibition capacity against *E. coli* and *L. innocua,* in line with the effective release of active compounds from the starch matrix. In contrast, PLA monolayers containing the highest proportion of these acids, or ferulic acid methyl ester, did not exhibit antimicrobial capacity regardless of the film processing conditions (casting or thermo-processing with and without plasticiser). Multilayer systems, where a layer of active starch was sandwiched between two PLA layers obtained by different methods, also exerted very little inhibition. The reduced molecular mobility in the polyester matrix is the limiting cause affecting the release of acids and their potential antimicrobial activity. Further research on strategies to improve the release of antimicrobial acids from PLA into the food systems could provide a viable and sustainable active material suitable for food packaging applications.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ramón Ordoñez: Investigation, Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Lorena Atarés**: Conceptualization, Methodology, Data curation, Writing – original draft, Writing – review & editing, Supervision. **Amparo Chiralt**: Conceptualization, Methodology, Data curation, Writing – review & editing, Supervision, Project administration.

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Chapter 4:

Properties of PLA films with cinnamic acid: effect of the processing method.

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Food and Bioproducts Processing (2022), 133, 25-33

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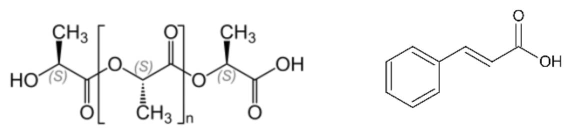
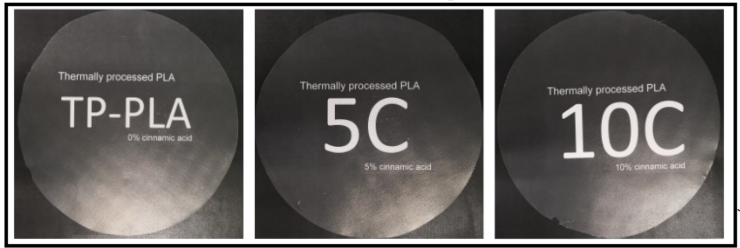
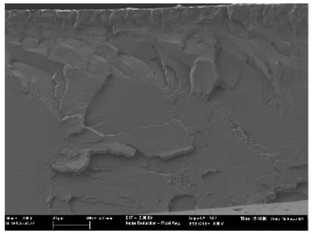


Polylactic acid (PLA) + Cinnamic acid (C)

Film obtaining method: Thermo-processing (TP)
C concentrations (w/w): 0, 5 & 10%

TP films

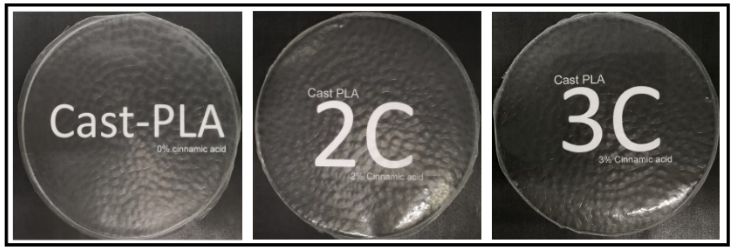




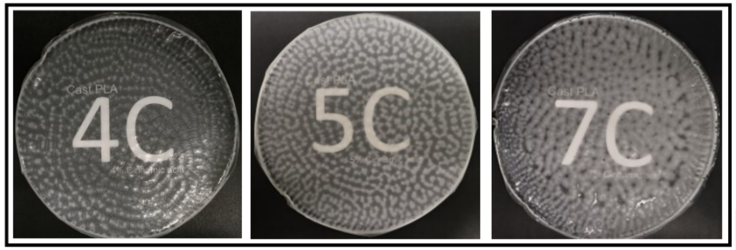
FESEM: No crystalline formations detected.

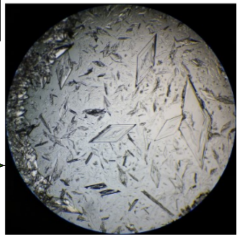
Film obtaining method: Casting
C concentrations (w/w): 0, 2, 3, 4, 5, 7 & 10%

Cast films



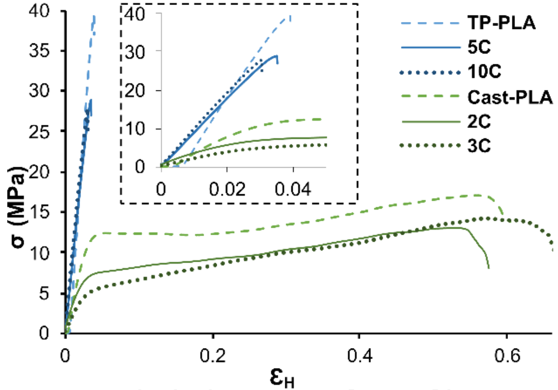
Homogeneous films, no crystals detected.



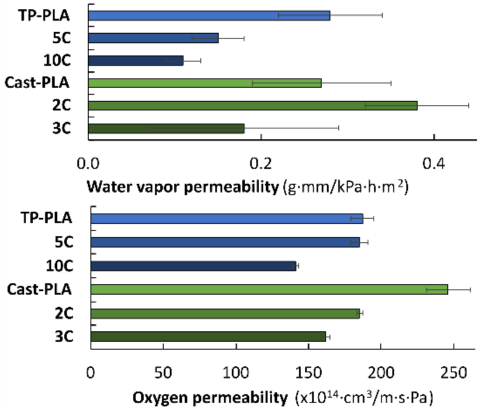


Optical microscopy: Crystalline cinnamic acid formations detected.

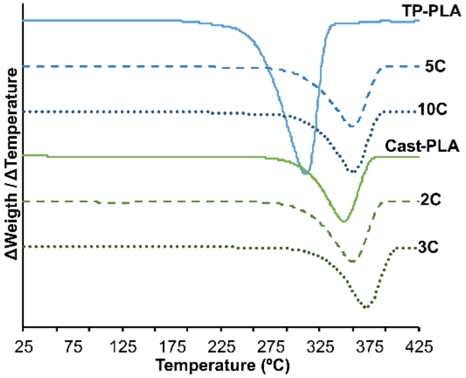
Functional characterization



Tensile behaviour of PLA films.

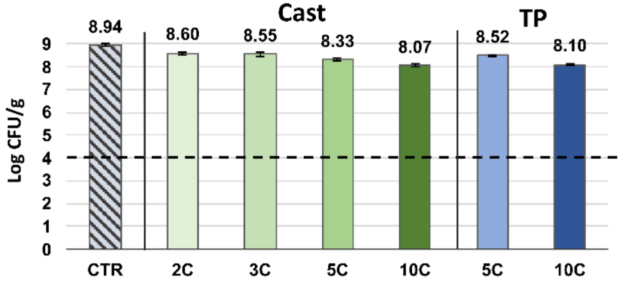


Barrier properties of obtained films.



Thermogravimetric derivative curves.

Antimicrobial tests



Listeria innocua growth test in contact with obtained films.

Abstract

Polylactic acid (PLA) is a sustainable material for food packaging applications. The incorporation of cinnamic acid (C) into PLA films was studied to obtain antimicrobial films, using two different techniques for film production, namely casting and thermal processing (TP). Whereas C could be successfully incorporated at 5 and 10% w/w into TP films, only 3% w/w could be used in cast films without observing any crystallization of C. The acid reduced the glass transition temperature in TP films, while also improving their thermal resistance. Cast films retained a little proportion of the casting solvent which contributed to their plasticisation. The processing method greatly affected the mechanical properties of the films, with the cast films being significantly more plastic and stretchable. C reduced the film's stiffness and resistance to break and improved their water vapour and oxygen barrier capacity. *A*ntibacterial assays with *Listeria innocua* did not show significant growth inhibition for any film, but the observed inhibition could be related to the acid concentration. This suggested that only a small amount of acid near the film surface could be released. Further research to promote the superficial concentration of cinnamic acid into PLA films is required to obtain active materials.

1. Introduction

As the environmental global crisis escalates, the consumption of single-use plastic increases. Traditional plastics still figure as the most commonly used material for food packaging (Harnkarnsujarit et al., 2021). It is estimated that less than 30% of the plastic produced in Europe is recycled (Gong et al., 2020). Alternatives, such as biodegradable polymers, with suitable properties for foodstuff preservation purposes have been widely studied and are beginning to have a small presence in the market (Harnkarnsujarit et al., 2021). Creating more applications for these materials could help increase their demand and economic viability (Mangaraj et al., 2018). In this sense, active packaging represents an alternative means of providing biodegradable polymers with improved capacity to preserve the safety and quality of food products.

Active compounds from renewable sources, with known antioxidant or antimicrobial capacities, have been studied in the few past years as possible substitutes for synthetic food preservatives (Guil-Guerrero et al., 2016). Several phenolic compounds are a promising alternative since they are abundant in nature, have low minimal inhibitory concentrations (MIC) for several pathogens, and do not possess marked organoleptic properties, unlike widely studied essential oil compounds (Choi et al., 2018; Miyague et al., 2015). Cinnamic acid (CA) is the precursor of the prevalent family of phenolic acids, known as hydroxycinnamic acids, and can be found in a wide range of vegetable sources (Rashmi and Negi, 2020). Cinnamic acid exhibits antimicrobial properties, as different studies have demonstrated (Miyague et al., 2015; Sánchez-Maldonado et al., 2011), being especially active against Listeria, which constitutes a dangerous food pathogen due to its persistency at cold temperatures (Tasara and Stephan, 2006).

Cinnamic acid has been previously incorporated into thermoplastic starch matrices to obtain active biodegradable films, which exhibited growth inhibition of both Gram-positive and negative bacterial strains (Ordoñez et al., 2021). Nevertheless, starch and other hydrophilic polymers present limited applications given their high degree of sensitivity to water (Zhong et al., 2020). Hydrophobic biodegradable or compostable polyesters, such as polylactic acid (PLA), could be the base for water-resistant materials suitable for high moisture environments. PLA has been widely studied as a potential carrier for active compounds in food packaging applications as recently reported by Rojas et al., (2021). Different essential oils and their compounds, as well as other phenolic compounds, have been incorporated into the PLA matrices, provoking different changes in the film functional properties while providing them with antioxidant or antimicrobial capacity, depending on the film processing conditions and the nature (volatility, solubility or thermo-sensitivity) of the active agent (Velásquez et al., 2021).

A previous study (Ordoñez et al., 2022) showed no effective antimicrobial activity when cinnamic acid was incorporated at low concentrations (up to 2% w/w) into amorphous PLA matrices obtained by thermal processing (TP). This was attributed to the limited molecular mobility of the compound inside the glassy matrix, limiting its diffusion and release into the culture medium. Nevertheless, the release of active compounds from PLA films can highly be affected by the film processing method (Rojas et al., 2021; Velásquez et al. 2021). Considering the great potential of PLA with C incorporated as possible active packaging material, several alternative means of enhancing the rates and ratio of the release of C into the food system should be studied, such as the incorporation of a higher dose of active compounds in the films or plasticising the films to favour the mass transfer phenomena.

Polymeric films studied for food packaging applications have mainly been obtained by two types of methods: thermal processing (TP) techniques (such as melt-blending, compression-moulding, or extrusion) or solvent casting (Suhag et al., 2020). Casting consists of preparing polymeric solutions in suitable solvents and casting a certain amount of the solutions on levelled plates, letting the solvent evaporate in order to obtain thin film layers. While casting has been widely used in research, its use on an industrial scale is not realistic, given the energy requirements and process disadvantages for solvent evaporation. Therefore, TP methods are the standard industrial method, whereas casting could be used to coat thermo-processed materials with a thin layer of film-forming solution carrying active compounds (Tampau et al., 2020b). Casting does not apply high temperatures that can alter thermosensitive active compounds, while cast films tend to present more plastic behaviour (Muller et al., 2017a; Rhim et al., 2006), which could potentially favour the migration of active compounds, such as C, to the food substrate. Requena et al. (2018) obtained cast PLA-PHBV blend films with carvacrol with a great capacity to inhibit bacterial growth on inoculated aqueous culture media. Despite the proven antimicrobial properties of cinnamic acid, no previous studies have analysed the incorporation of cinnamic acid into PLA films by casting or thermal processing in a wide range of the compound concentration, in order to obtain active films with an effective compound release and antimicrobial action.

This study focused on incorporating cinnamic acid into PLA matrices at concentrations up to 10%, analysing the effect of the processing method (thermal processing or casting) on the antimicrobial activity of the films against *Listeria innocua*, as well as on the thermal behaviour and functional (mechanical, barrier and optical) properties of these materials.

2. Materials and methods

2.1. Materials

Amorphous PLA 4060D (Nature Works, MN, USA; 106 kDa average MW) was used to obtain films with and without cinnamic acid (Sigma-Aldrich, Saint Louis, USA) at different concentrations. Magnesium nitrate and phosphorus pentoxide (Panreac Química, Barcelona, Spain) were used to balance the sample relative humidity (RH) at 53% and 0% respectively. Ethyl acetate (EtAc) from Indukern (Barcelona, Spain) was used as food-grade solvent. *Listeria innocua* strain (CECT 910) supplied by Spanish Type Collection (Valencia, Spain) was used in antimicrobial *in vitro* assays. Tryptic soy broth (TSB), tryptic soy agar (TSA), buffer peptone water, palcam agar base (PAB), and palcam selective supplement for *Listeria* were all supplied by Scharlab (Barcelona, Spain).

2.2. Film preparation

Different C concentrations, up to 10% w/w, were tested both for cast and thermally processed (TP) PLA films. The cast films were produced as described by Muller et al. (2017a), using ethyl acetate (EtAc) as solvent. Film-forming solutions (FFS) with PLA at 10 wt.% were obtained, by stirring overnight at room temperature. To obtain 4 g films, 40 g of FFS were poured into 15 cm diameter Teflon plates and left to dry overnight. A formulation without C was produced (Cast-PLA), and C concentrations in the cast films ranged from 2% to 10% w/w in solids (2C to 10C).

To obtain thermo-processed films, PLA pellets were first dried at 60 °C overnight to prevent hydrolysis during the process. TP films were obtained by adding the components in an internal mixer (Haake Polylab QC, Thermo Fisher Scientific, Germany) where a melt blending process was taking place at 50 rpm and 160 ºC for 5 min. The obtained pellets were cold ground employing an M20 mill (IKA, Staufen, Germany). To produce the films, 4 g pellet samples were placed in circular Teflon moulds, and 15 cm diameter films were obtained through compression moulding with a thermal-hydraulic press (LP20, Lactech Engineering, Thailand). The samples were preheated at 200 ºC for 4 min before the compression step at 10 MPa for 4 minutes. Finally, the films were cooled down to 70 ºC in 3 min. The concentrations of C in TP films were 5 and 10 % (w/w), giving rise to formulations TP-5C and TP-10C. A control formulation without C was also obtained (TP-PLA).

2.3. Film characterisation

2.3.1. Thermal analysis

The thermal behaviour of the film formulations was analysed using a differential scanning calorimeter (DSC, 1 StareSystem, Mettler-Toledo, Switzerland) while thermal degradation was studied with a thermogravimetric analyser (TGA/SDTA 851e, Mettler-Toledo, Switzerland). Both tests were duplicated for each formulation. Before the analyses, the samples were conditioned at 0% RH. For the DSC, the samples (about 10 mg) were sealed inside aluminium pans and placed into the calorimeter. The temperature was initially lowered from 25 ºC to -10 ºC and then raised to 200 ºC at 10 ºC/min. Following a 5 min isothermal step at 200 ºC, a fast-cooling step took place at 50 ºC /min until -10 ºC was reached. This temperature was maintained for 5 min and, finally, a second heating to 200 ºC took place at 10 ºC /min. For the TGA analyses, the samples (about 10 mg) were placed into aluminium crucibles in the TGA system and maintained under a nitrogen flow of 50 mL/min while heating took place from 25 to 600 ºC at 10 ºC /min. The initial degradation temperature (T_{onset}), (mass loss of 5%) and the maximum degradation rate temperature (T_{peak}) were registered.

2.3.2. Retention of cinnamic acid

The retention of C in the films was quantified in three samples per formulation via methanolic extraction and spectrophotometric quantification. Dry film samples of around 100 mg were immersed in vials with 10 mL of methanol and magnetically stirred for 48 hours at room temperature. The methanolic extracts were filtered and properly diluted to measure their absorbance at 270 nm in a UV-visible spectrophotometer (Thermoscientific Evolution 201, USA). The concentration of C was calculated with a previously obtained calibration curve and the results were expressed as g C per 100 g of C initially incorporated into the film sample.

2.3.3. Mechanical, barrier, and optical properties

The tensile properties of the films were determined using a texture analyser TA-XT plus (Stable Micro Systems, Haslemere, England), following the ASTM D882 method (American Society for Testing Materials, 2002). Eight film strip samples (25 x 100 mm) per formulation were tested. An electronic micrometer (Comecta S.A., Barcelona, Spain) was used to determine the thickness of each strip at six random points. Strips were placed on tensile grips with 50 mm of separation and were stretched at 50 mm/min to obtain the stress-Henky strain curves, from which tensile strength at break (TS), elastic modulus (EM) and percent elongation at break € were obtained.

The ASTM E96-95 (American Society for Testing Materials, 1995) method was followed to measure the water vapour permeability (WVP), using 3.5 cm diameter Payne permeability cups (Elcometer SPRL, Hermelle/s Argeteau, Belgium). Three circular film samples per formulation were cut to match the diameter of the cups, and the thickness was measured at six different random points. The cups were filled with 5 ml of distilled water, the films were mounted and sealed before placing the cups into desiccators with $MgNO_3$ saturated solution and kept at 25 ºC. The cup mass was monitored using an analytical balance (±0.00001 g), and the mass-loss rate was used to calculate the WVP once the stationary state had been reached, according to Andrade et al. (2020).

The oxygen permeability (OP) was determined following ASTM D3985-05 (American Society for Testing Materials, 2010), using Ox-Tran 1/50 equipment (Mocon, Minneapolis, USA). The film samples (two per formulation, the thicknesses of which were measured at six random points) were cut to fit the equipment setup, with 50 cm^2 of exposed area. The tests were performed at 25 ºC and 53% RH to obtain the oxygen transmission rate (OTR). The OP was calculated by dividing the OTR by the gradient of oxygen partial pressure on both sides of the film and multiplying it by the film thickness.

A CM-5 Spectrocolorimeter (Konica Minolta Inc., Japan) was used to evaluate the optical properties of the films. Reflection spectra (400-700 nm) were obtained by measuring three films per formulation at three different points, backed by black and white backgrounds. The CIEL*a*b* coordinates were calculated from the reflectance of an infinitely thick layer of material, considering D65 illuminant and 10º observer (Cano et al., 2014). The psychometric coordinates of lightness (L*), chroma (C_{ab}*), and hue (h_{ab}*) were calculated (Mahy et al., 1994). Kubelka-Munk's theory of multiple dispersion was used to determine the internal transmittance (T_i) of the film samples (Hutchings, 1999).

2.4. Antibacterial activity

In vitro bacterial growth inhibition tests were carried out against *Listeria innocua* according to the methodology used by Ordoñez et al. (2021). Circular film samples were cut to match the inner diameter of a 55 mm Petri dish and sterilised on both faces with UV light in a laminar flow cabinet (Bio II advance, Telstar, Spain). 10 ml of TSA were poured into Petri dishes and left to solidify before being inoculated with 100 µl of 10^6 UFC/mL *L. innocua* using an L-rod to evenly distribute the inoculum on the medium's surface. Afterwards, the inoculated medium was covered with the different films. The dishes were closed and sealed with Parafilm^{tm} and left to incubate for 6 days at 10 ºC. After the incubation, the medium and films were homogenised in 100 ml of peptone water employing a masticator paddle blender (IUL Instruments, Barcelona, Spain). Serial dilutions were plated and covered with PAB selective media and left to incubate for 48 hours at 37 ºC before counting the colonies. Two film samples were included per formulation and all of the dilutions were plated in duplicate. An uncoated growth control sample was included as a growth reference.

2.5. Microscopic observations

Optical microscopy (B-353PL, Optika, Italy) was used to observe the C crystalline structures in cast films. Field emission scanning microscopy (FESEM) was used to analyse the potential presence of crystals in TP films. To this end, the samples were cryofractured and platinum-coated. The cross-sectional observations were carried out under a 2 kV acceleration voltage in an Ultra 55 Zeiss, UK microscope.

2.6. Statistical analysis

Statistical data analysis was performed through analysis of variance (ANOVA) using Statgraphics Centurion XVII software. Fisher’s least significant difference was used at a 95% confidence level.

3. Results and discussion

3.1. Film structure and appearance

The films obtained by TP (C ratio up to 10% w/w) were homogeneous and transparent to the naked eye (**Figure 1**), and FESEM cross-sectional images (**Figure 2**) revealed that no crystalline structures were formed in any TP film despite the high concentration of cinnamic acid. The films obtained by casting (**Figure 1**) with C up to 3% w/w were homogeneous and translucent as well. However, cast films with C concentrations above 3% presented visible crystalline formations whose number and size increased accordingly with the proportion of C. Optical microscopy observations of the formed crystals (Figure 2) revealed the characteristic monoclinic structure of cinnamic acid crystals, as reported by Hsieh et al. (2005). The crystallisation of cinnamic acid during the casting process suggests that compound oversaturation occurred during solvent evaporation and film formation. The crystallisation was not inhibited by the viscous effects of the PLA solution or any polymer-compound interactions that could limit the nucleation and growth of cinnamic acid. In contrast, the high viscosity of the PLA-acid melt blend inhibited the crystallisation of cinnamic acid, which remains well dispersed in the film matrix. Likewise, more intense interactions between the polymer chains and acid molecules could be promoted at the high temperature used in TP conditions. In this sense, in the absence of a solvent, the acid could be bonded to the hydroxyl end chain groups of PLA at high temperature, and form hydrogen bonds with carbonyl groups of the chains. Therefore, the processing procedure would determine not only the proportion of C that can be incorporated into the film but also all of its final properties derived from the different polymer-acid interactions. Given the heterogeneous structure of the cast films with more than 3% CA, which hinders their real application, the physicochemical characterisation of cast films was limited to 2C and 3C formulations.

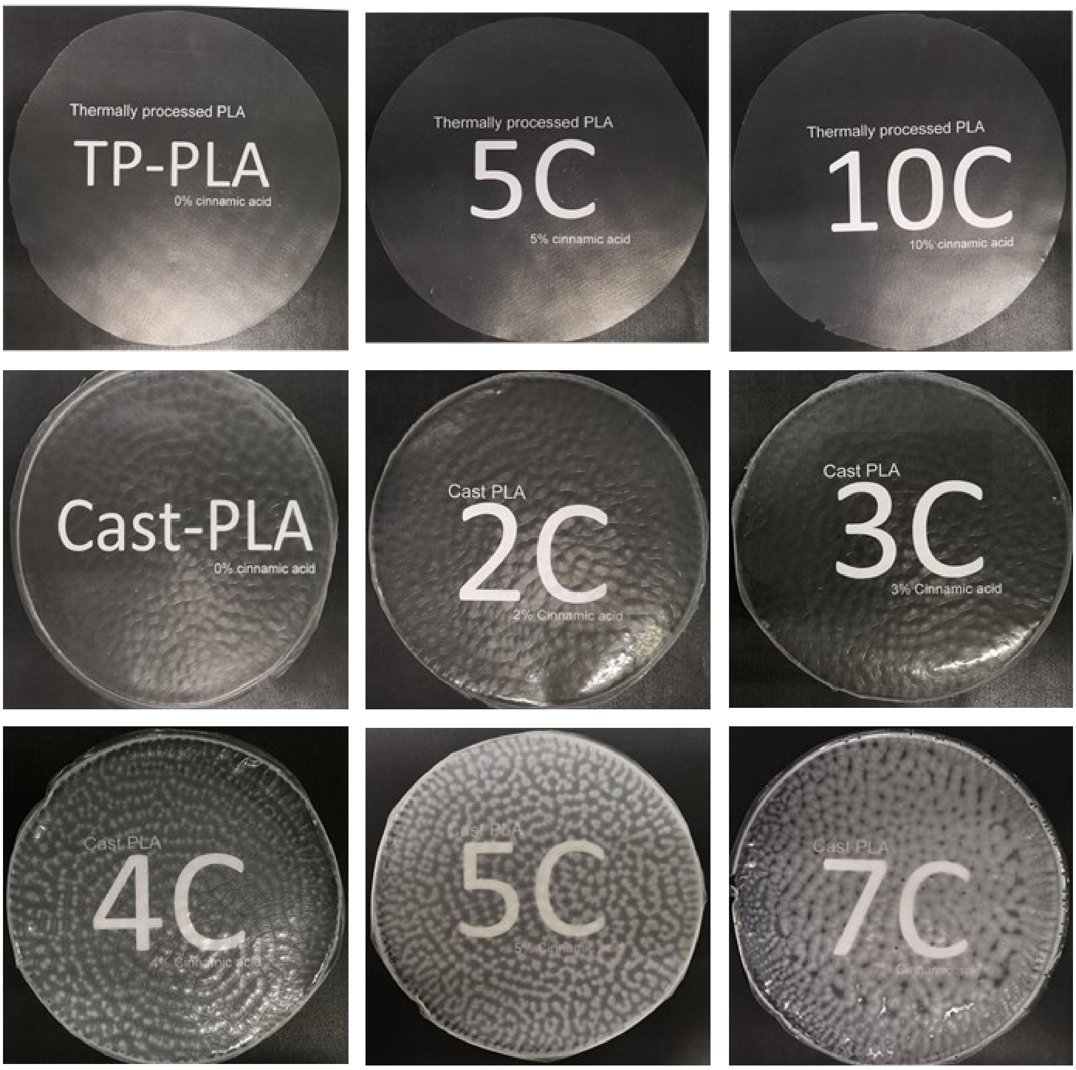


Figure 1 Thermally processed (first row) and cast (second and third rows) PLA films with different cinnamic acid concentrations.

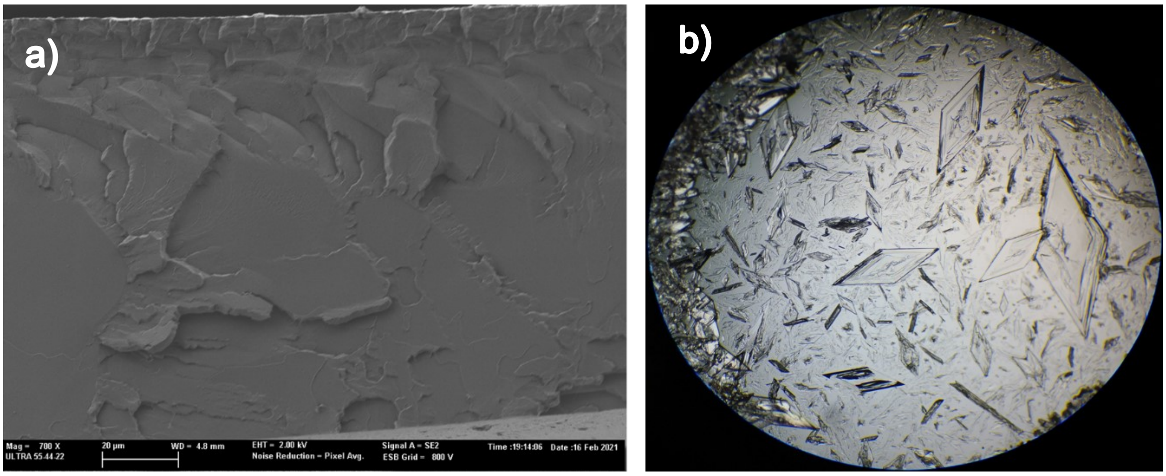


Figure 2. a) FESEM micrograph of TP-10C film cross-section at 700X. **b)** Crystals of cinnamic acid observed by optical microscopy (x10) in cast PLA films with 5%w/w CA.

3.2. Thermal behaviour and degradation

Figure 3 shows the first and second heating thermograms obtained by DSC analyses for the different films obtained by casting and thermoprocessing with differing concentrations of cinnamic acid. Coherently with the amorphous nature of PLA, no melting peak was detected but only the glass transition, in some cases followed by the subsequent relaxation endotherms (only in the first heating step) associated with the effects brought about by matrix ageing. In cast films, small endotherms can be observed at about 85 ºC that may be attributed to the total or partial evaporation of the solvent retained (EtAc) in the film during the film formation step, which was released during the thermal scan. The integration of the peaks permits the estimation of the amount of solvent evaporated by comparing it with the evaporation enthalpy of the pure solvent (404 J/g). This amount was estimated as about 2% w/w of the film samples, with a certain variability between samples.

Table 1 shows Tg values (mid-point) obtained for the different samples from the first and second heating scans of DSC analyses. For the TP films, the incorporation of 5 and 10 % C promoted a significant plasticisation of the PLA matrix: the higher the C concentration, the lower the Tg values. This could be attributed to a hydrolytic effect of the acid under thermal conditions, favouring the formation of oligomers that contribute to plasticising the PLA matrix. The second heating step did not reveal the significant changes in the Tg values obtained in the first scan, but as expected, no relaxation endotherms were observed after glass transition since the first heating erased the sample's thermal history. This plasticising effect was also observed (Ordoñez et al., 2022), but to a lesser extent, in thermo-processed PLA films with 1 and 2 % cinnamic acid, which indicates that the effect was proportional to the compound concentration.

The cast films with cinnamic acid exhibited lower Tg values than TP PLA (28 vs. 54 ºC), which could be attributed to the solvent retained in the matrix, which plasticises the polymer. The incorporation of the acid at 2 and 3 % also reduced the Tg values to the same extent (a reduction of approximately 7 ºC), which points to additional effects of cinnamic acid on polymer plasticisation. The solvent evaporation (at least in part) during the first heating scan led to higher Tg_2 values, as compared to Tg_1, while the Tg_2 values of cast films were still lower than those of TP films. Muller et al. (2017a) reported slightly higher values of Tg for the cast films of amorphous PLA also obtained from EtAc solutions, as well as a plasticisation effect of cinnamaldehyde. Tampau et al. (2020a) also report that the EtAc absorption in cast PLA matrices took place to a different extent, seriously affecting the plasticisation level of the polymer.

Table 1 Thermal properties of thermo-processed (TP) and cast PLA films with different cinnamic acid ratios. Glass transition temperatures in the first and second DSC heating scans (T_{g1} and T_{g2}, respectively). Onset and peak degradation temperatures from TGA.

	DSC		TGA	
	T_{g1} (ºC)	T_{g2} (ºC)	T_{onset} (ºC)	T_{peak} (ºC)
TP-PLA	54.1 ± 0.9^{f}	54.1 ± 0.9^{d}	265 ± 2^{a}	310 ± 2^{a}
5C	49.1 ± 0.1^{d}	41.5 ± 0.1^{c}	312 ± 2^{b}	363 ± 7^{b}
10C	38.9 ± 0.2^{c}	35.3 ± 0.2^{b}	310 ± 9^{b}	355 ± 5^{b}
Cast-PLA	28.6 ± 0.3^{b}	37.4 ± 0.2^{b}	333 ± 17^{b}	368 ± 14^{b}
2C	22.0 ± 0.5^{a}	31.0 ± 2.0^{a}	311 ± 2^{b}	354 ± 7^{b}
3C	22.6 ± 0.4^{a}	35.1 ± 1.8^{b}	323 ± 13^{b}	365 ± 9^{b}

Different super indices in the same row indicate significant differences between film formulations ($p<0.05$). 2-10 C stands for the mass percentage of C in the dry film.

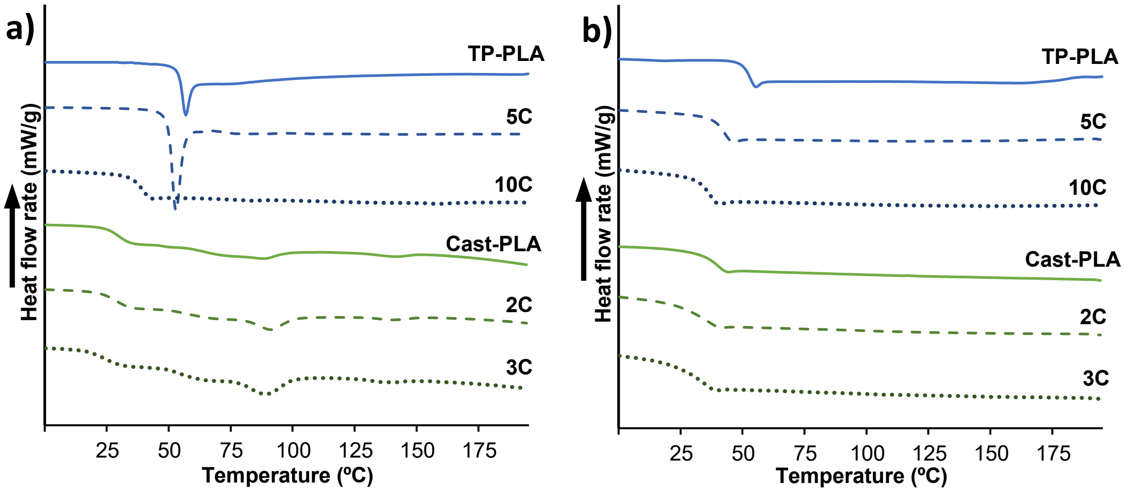


Figure 3 DSC heating thermograms of PLA films obtained by thermal processing (TP) or casting. Effect of cinnamic acid (CA). a) First heating b) Second heating. 2-10 C stands for the mass percentage of C in the dry film.

Figure 4 shows the typical derivative curves of TGA analyses for the different film formulations and Table 1 gives the onset and peak temperatures in each sample. In TP films, only the thermal degradation of the polymer was observed, whereas in cast films small weight loss events were observed at about 115 ºC, which can be attributed to the solvent evaporation (1-3 % wt.), as observed in DSC analyses. The thermal degradation of PLA is due to a random main-chain scission reaction, as well as depolymerisation, oxidative degradation and transesterification reactions. Reactive end groups and impurities have been reported to enhance thermal degradation (Signori et al., 2008). As observed in **Figure 4**, the differing film-forming methods and proportions of C affected the thermal degradation behaviour of PLA. The thermal resistance of TP PLA films was significantly lower than that of cast PLA films. This can be explained by the different packing of PLA chains in each treatment, with differing thermal resistance, or by the partial degradation that occurred in TP films during thermo-processing. Thermo-processing could give rise to the formation of oligomers which can reduce the onset and peak temperatures of the polymer matrix degradation. The incorporation of C at 5 and 10 % exerted a thermal protective effect on TP films, thus delaying degradation by more than 40ºC. The protective effect of C on TP PLA films was previously observed at lower C concentrations (Ordoñez et al., 2022). Tran et al. (2015) successfully obtained novel PLAs with the conjugation of both chain terminals with C and its derivates and proved the significant improvement in their thermal properties. In contrast, cinnamic acid did not exert a thermo-protective action in cast films which suggests no effective interactions of this compound with PLA chains. This was coherent with the observed crystallisation of C in cast films, which suggested poor interactions between C and PLA chains during the solvent casting process, which led to phase separation and compound crystallisation, as previously commented on. Therefore, the bonding of cinnamic acid to PLA chains during thermo-processing could be deduced from the thermal analyses.

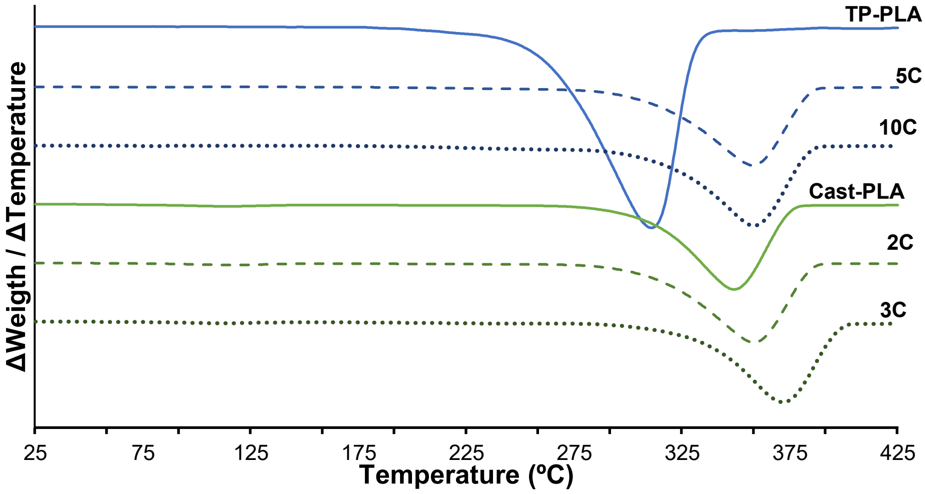


Figure 4 DTGA curves of PLA films obtained by thermal processing (TP) or casting with different ratios of cinnamic acid (CA). 2-10 C stands for the mass percentage of C in the dry film.

3.3. Cinnamic acid retention

The results of C retention in the films are shown in **Table 2**. The casting method preserved C in the films to a remarkable extent, and percentages close to 100% were reached for both 2C and 3C formulations. The complete recovery of C is probably due to the non-thermal nature of this method, where the films are formed at room temperature by the evaporation of the solvent. Under these conditions, C is not oxidized or degraded by thermal stress. In contrast, C extraction from TP films showed lower C recovery values (89, 75 %). This points to the partial losses of the compound caused by the thermal stress undergone by the matrix in both melt blending and compression moulding steps. In fact, previous studies reported the initial degradation of C at 180ºC under a nitrogen atmosphere (Ordoñez et al., 2021), whereas in thermal processing occurred at lower temperatures.

Table 2 Functional properties of PLA films obtained by thermal processing (TP) and casting with different ratios of cinnamic acid (C). C retention (%), film thickness (t), tensile strength at break (TS), elongation at break (E), elastic modulus (EM), water vapour permeability (WVP), oxygen permeability (OP), lightness (L*), hue (h_{ab}*), chroma (C_{ab}*) and internal transmittance (T_i) at 400 and 700nm.

	TP-PLA	**5C**	**10C**	**Cast-PLA**	**2C**	**3C**
C retention (%)	-	89±7^{b}	75±5^{a}	-	98± 3^{c}	98± 3^{c}
t (µm)	203±9^{a}	213±16^{a}	204±31^{a}	247±13^{b}	246±22^{b}	251±11^{b}
TS (MPa)	33±5^{d}	30±3^{cd}	28±2^{c}	21±7^{b}	13±3^{a}	15±2^{a}
EM (MPa)	1254±71^{e}	912±113^{d}	905±97^{d}	466±104^{c}	296±53^{b}	208±62^{a}
E (%)	3.0±0.5^{a}	3.6±0.3^{a}	3.3±0.3^{a}	67±25^{c}	52±12^{b}	65±9^{c}
WVP (g·mm/kPa·h·m^{2})	0.28±0.06^{c}	0.15±0.03^{b}	0.11±0.02^{a}	0.27±0.08^{cd}	0.38±0.06^{d}	0.18±0.11^{bc}
OP (x10^{14}·cm^{3}/m·s·Pa)	187±8^{c}	185±6^{c}	141±2^{a}	246±15^{d}	185±2^{c}	162±3^{b}
L*	83.1±0.6^{bc}	83.5±0.6^{bc}	82.6±0.4^{ab}	80.6±3.0^{b}	84.7±2.8^{c}	83.5±0.3^{bc}
h_{ab}*	97.3±1.0^{d}	74.7±2.0^{a}	73.3±0.9^{a}	83.6±2.8^{bc}	84.3±9.0^{c}	79.4±1.0^{b}
C_{ab}*	8.2±0.4^{c}	7.1±0.2^{bc}	7.8±0.2^{b}	5.9±1.3^{a}	6.4±1.9^{ab}	6.58±0.3^{ab}
T_i 400 nm (x1000)	882±3^{b}	883±3^{b}	882±2^{b}	858±9^{a}	864±2^{a}	863±1^{a}
T_i 700 nm (x1000)	907±1^{b}	906±1^{b}	906±1^{b}	891±8^{a}	898±1^{a}	897±1^{a}

Different super indices in the same row indicate significant differences between film formulations ($p<0.05$). 2-10 C stands for the mass percentage of C in the dry film.

3.4. Mechanical, barrier and optical properties

Table 2 shows the obtained values for thickness (t), tensile strength at break (TS), elastic modulus (EM), and percent elongation at break (%E) for all of the formulations and **Figure 5** shows the typical stress-strain curves of the different films. Despite having the same mass of solids (PLA+C), the TP films were significantly thinner than the cast films ($p<0.05$), which suggests a different structural arrangement of the polymer chains, depending on the production method. A more compact film structure would, hence, be obtained by TP, and the chain arrangement caused by thermal treatment may favour the cohesiveness of the matrix (Rhim et al., 2006). The previously commented on solvent retention in the cast films would also contribute to the swelling and greater thickness of the film. In no case was the film thickness significantly affected by the incorporation of cinnamic acid.

The film tensile behaviour was also barely affected by the production method. Compared to TP films, the cast films were less stiff and resistant to break with barely enhanced plastic deformability, (**Figure 5**). This agrees with that reported in a previous study (Rhim et al., 2006). The high degree of cohesiveness of the TP films led to their reduced stretchability and increased stiffness. Additionally, the solvent retained in the cast films would promote their plastic behaviour, facilitating the molecular mobility of the polymeric chains, as previously observed by Muller et al. (2017a). The incorporation of cinnamic acid-modified the mechanical performance of both the TP and cast films, reducing the TS and EM while having no significant effect on the %E.

This could be attributed to the plasticising effect of C on the amorphous PLA matrices, as described for organic acids in previous studies (Azeredo & Waldron, 2016). The mechanism for this effect was attributed to the interruption of interchain polymer interactions by acid molecules, hence promoting a decrease in the matrix cohesion forces. In the TP films, the weakening effect provoked by C could also be related to the potential hydrolytic effect of the acid during the melt-blending step, causing some degradation of PLA to yield oligomers, leading to a reduction in the material cohesiveness (Mangeon et al., 2017; Signori et al., 2008).

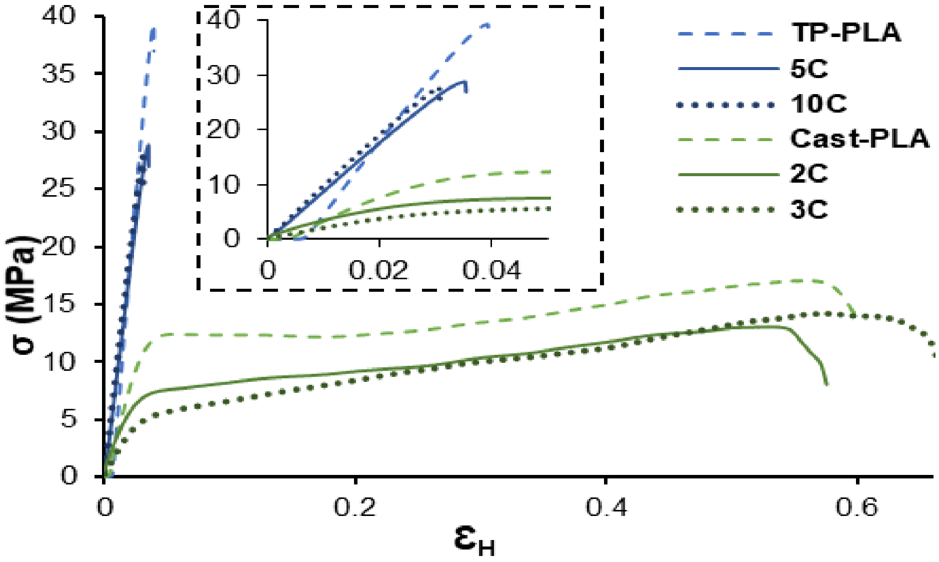


Figure 5 Typical stress-strain curves of the PLA films obtained by thermal processing (TP) and casting methods. 2-10 C stands for the mass percentage of cinnamic acid in the dry film.

The obtained water vapour permeability (WVP) values are shown in **Table 2**. Additionally, specific arrangements in the polymer matrix could further improve this capacity. Both control PLA films (TP-PLA and Cast-PLA) exhibited similar water barrier capacity, as has been reported in previous studies (Muller et al., 2017a). The incorporation of cinnamic acid greatly reduced the WVP of the films, mainly in TP films; the greater the C concentration the higher the WVP reduction. This could be related to the bonding of acid molecules to PLA chains through the end-chain OH groups and to the carbonyl groups inside the chains, forming hydrogen bonds (Collazo-Bigliardi et al., 2019; Madhavan Nampoothiri et al., 2010). This would contribute to a reduction in the little water affinity of the polymer matrix, thus reducing the solubility and permeability of water molecules through the polymer. The improvement in the water barrier properties brought about by the incorporation of C was also observed in other more hydrophilic polymer matrices, such as sodium caseinate; in this case, the observed reduction in WVP was even significant when compared with what was promoted by the incorporation of ferulic acid (Fabra et al., 2011). The milder effect of

C on the WVP reduction in the cast films agrees with the inhibition of C-PLA interaction, in the presence of the solvent, as commented on above.

A high degree of oxygen permeability is a problem for the application of PLA films in food packaging (Muller et al., 2017b). The processing method had a significant effect on the oxygen barrier capacity of the control films, and the TP-PLA presented a lower OP than the Cast-PLA films (**Table 2**). This could be related to the more compact structure attained by TP films, in part due to the solvent retention in the cast films, as deduced from the tensile behaviour. For both the TP and cast films, the oxygen barrier capacity was improved by the incorporation of C; the greater the proportion of C, the bigger the improvement in the oxygen barrier capacity. Despite the lower proportion of C, the cast films experienced the most remarkable OP reduction since the C-loaded cast films exhibited similar OP values to those of the TP films, despite the higher OP of the cast PLA. This suggests that cinnamic acid could act as an oxygen scavenger in the films, as observed for other phenolic compounds (Bonilla et al., 2013).

The internal transmittance (T_i) spectra between 400 and 700 nm are shown in **Figure 6**. The TP films exhibited higher T_i values than the cast films over the whole wavelength range, similar to those obtained by Collazo-Bigliardi et al. (2019) for amorphous TP PLA films; this indicates that the TP films were more transparent than the cast films. This could be attributed to the more compact and homogenous structure of the TP films. The solvent retention in the cast films and their greater thickness can help to make them less transparent. The incorporation of cinnamic acid did not significantly affect the transparency of either the TP or cast films.

The colour parameters of the films are shown in **Table 2.** As a result of the different light interactions with the films, the cast PLA samples tended to show lower hue values, with lower colour saturation than the TP films. In the cast films, the addition of C set a trend towards greater saturation, which matches the values reported by Muller et al. (2017a), for cast PLA films with cinnamaldehyde. In the case of the TP films, the incorporation of C caused a considerable reduction in hue along with a small decrease in chroma, indicating that films became slightly brownish. This effect can be attributed to the partial oxidation of C during the melt-blending process, as discussed above. However, none of these changes were appreciable to the naked eye.

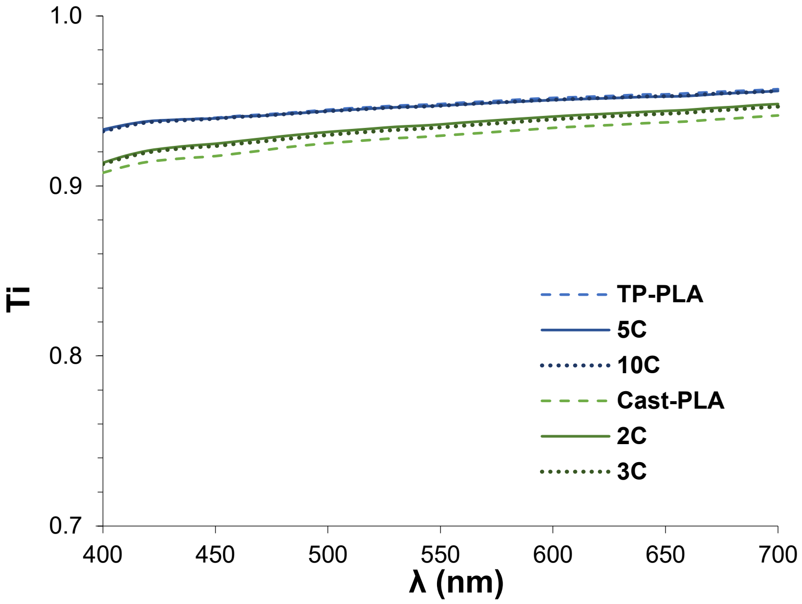


Figure 6: Internal transmittance (T_i) spectra of thermoprocessed (TP) films (TP-PLA, 5C and 10C), and cast films (Cast-PLA, 2C and 3C).

3.6. Antibacterial activity of the films

Figure 7 shows the growth of *L. innocua* in the TSA medium in contact with the different PLA films, in comparison to the uncoated TSA control growth sample. Despite the crystalline formations found for 5C and 10C cast films, these were also submitted to the antimicrobial test in order to elucidate a possible relationship between the concentration of C and *L innocua* growth inhibition. All of the samples exhibited growth of over 4 logs(CFU/g) compared to the initial inoculum (10^4 CFU/g). Despite the high proportion of C, the films proved to have a very little antimicrobial effect. A slight tendency linking growth inhibition and the ratio of C in the film could be observed. In a previous study, Ordoñez et al. (2021) determined the minimal inhibitory concentration (MIC) of C for *L. innocua*, which was 0.65 mg/ml. This value would have already exceeded in the culture medium for 2C films if a total release of the C incorporated into the film has occurred. However, not even 10C formulations reached a growth inhibition of 1log. This indicates that C was retained tightly in the polymer matrix in all cases, thus being unable to exert its antimicrobial capacity. Despite the high proportion of active presence in the films to enhance the mass transfer driving force, the lack of polymer swelling and relaxation when in contact with the aqueous culture medium greatly limited the release of cinnamic acid from the film to exert its antimicrobial action against *L. innocua.* The obtained results suggest that only the compound near the film surface was delivered to the medium, but in too small a quantity to be able to significantly inhibit the bacterial growth. Therefore, other strategies, such as the surface application of the active compound, compound solution spraying or electrospinning, could yield more efficient active materials that may inhibit the bacterial growth in aqueous media such as those present in many foods.

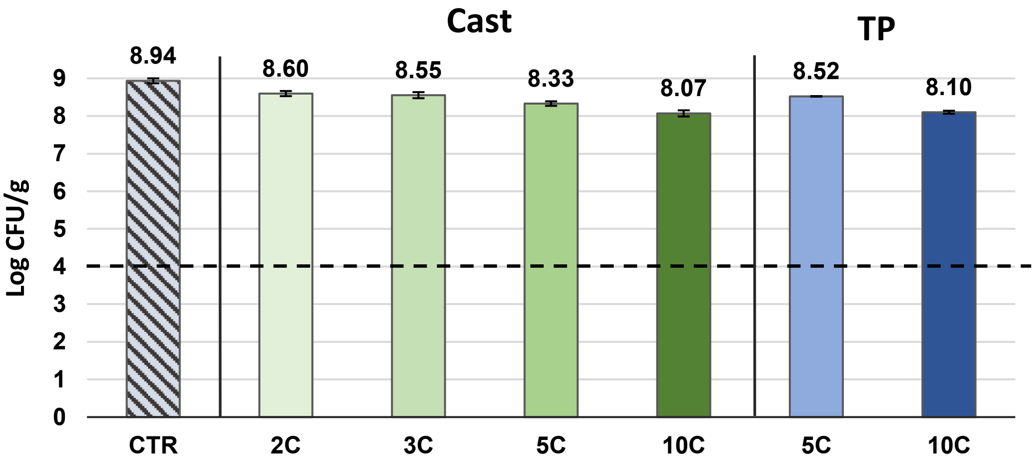


Figure 7 Microbial growth of *Listeria innocua* after 6 days of incubation at 10 ºC in TSA in contact with cast (green bars) and thermally processed (TP, blue bars) PLA films with different cinnamic acid (CA) wt %. CTR: non-covered growth control. The dotted line represents the initial inoculum (10^4 CFU/g). 2C-10 C stands for the mass percentage of C in the dry film.

4. Conclusions

Over 3% w/w cinnamic acid could not be homogeneously incorporated in cast PLA films, whereas 10% acid was successfully integrated by melt blending with PLA. Thermo-processed PLA films were significantly stiffer and had greater resistance to break than the cast films, whereas the latter exhibited a high degree of plastic deformability, in part due to the solvent retention in the cast films. The incorporation of phenolic acids reduced the film's stiffness and resistance to break in both the TP and cast films while it improved the water vapour and oxygen barrier capacity of the films. The TP films were more transparent than the cast films and turned slightly brownish after the addition of cinnamic acid.

Despite the high concentration of the active compound in the films, no significant antibacterial activity was observed against *Listeria innocua* in the *in vitro* assays, although a positive effect was observed when the acid concentration increased. This suggests that only the acid molecules near the film surface were released into the medium to exert their antibacterial action. Given the limited release of the acid from both the cast and thermo-processed PLA matrices, future research should focus on promoting it through the surface application of the active compound, such as compound solution spraying or electrospinning, which could yield active materials that are more efficient at inhibiting the bacterial growth in aqueous media, such as those present in many foods, thus helping to extend the shelf life of sensitive foodstuffs.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ramón Ordoñez: Investigation, Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Lorena Atarés**: Conceptualization, Methodology, Data curation, Writing – original draft, Writing – review & editing, Supervision. **Amparo Chiralt**: Conceptualization, Methodology, Data curation, Writing – review & editing, Supervision, Project administration.

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Chapter 5:

Antilisterial action of PLA films with ferulic acid as affected by the method of incorporation.

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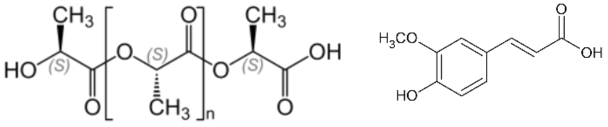
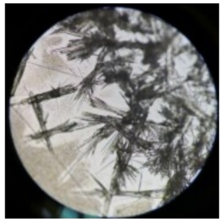


Polylactic acid (PLA) + Ferulic acid (F)

Solvent casting films:
F concentrations (w/w):
0, 2, 3, 4, 5, 7 & 10%

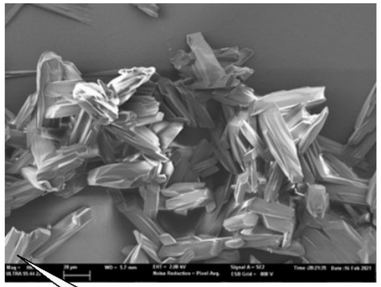
Cast films



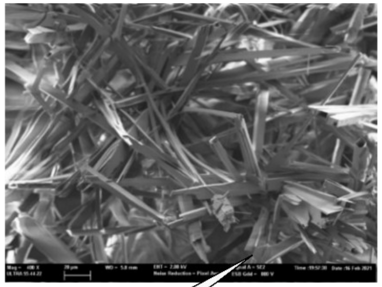
Crystalline F formations.

F superficial incorporation on thermoprocessed films:
Adsorption & pulverisation

Adsorption films



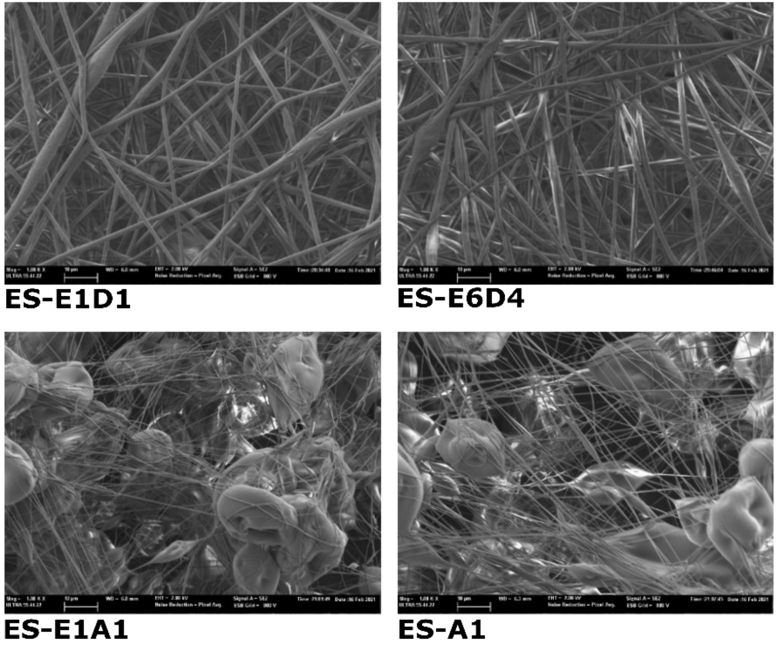
Pulverised films



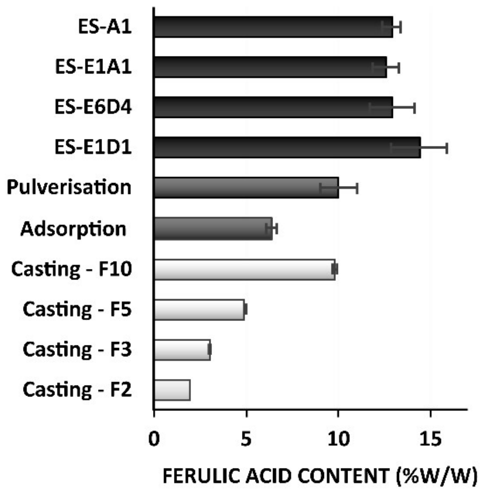
Crystalline F formations.

Electrospinning(ES):
Solvent systems with ethyl acetate (E), DMSO (D) & acetic acid (A).

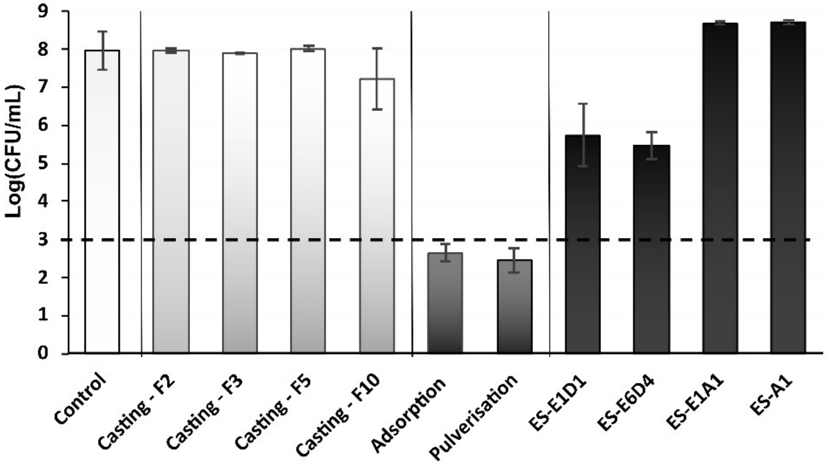
Electrospun mats



Ferulic acid content



Antimicrobial tests



Listeria innocua count in *In vitro* tests.

Abstract

The combination of Polylactic acid (PLA) and ferulic acid (F) is a viable approach to develop active materials with antimicrobial properties that could extend the shelf life of food products. Previous research incorporating F into PLA matrices revealed limitations to the release of F, preventing its antimicrobial activity. In this study, alternative incorporation strategies were studied and while evaluating the antibacterial activity. PLA films up to 10% w/w F were obtained by solvent casting, presenting crystalline formations at all concentrations, and no antibacterial activity. F crystals were successfully anchored on the surface of PLA films by adsorption from an ethanolic solution or by direct pulverisation with this solution. A bacteriostatic effect was observed for both cases. Electrospinning of PLA and F solutions using different solvent systems was also studied. DMSO systems produced fibre mats that significantly inhibited bacterial growth, whereas acetic acid systems produced bead mats with no antimicrobial activity.

1. Introduction

As the global environmental crisis escalates due to the uncontrolled use of petroleum-based plastics, the food packaging industry needs alternative solutions capable of ensuring food quality and safety while driving the operations to more sustainable practices. These practices also include the reduction of chemical additives used for food preservation; hence, there is growing interest in the use of naturally occurring compounds with antimicrobial and antioxidant properties (Atarés & Chiralt, 2016). Polylactic acid (PLA) is a widely studied material, it is the principal biodegradable polymer used in food packaging applications (Velásquez et al., 2021). This polymer is compostable, economically viable and can be obtained from a wide array of biomass sources (Armentano et al., 2013). Of the naturally occurring bioactive compounds, ferulic acid (F) is a common metabolite in a great variety of plant species. It is a hydroxycinnamic phenolic acid, with known antimicrobial and antioxidant activities (Miyague et al., 2015; Rice-Evans et al., 1996) and health benefits (Paiva et al., 2013).

Combining active compounds, such as F, and biodegradable polymers, such as PLA, could result in active packaging materials which have the capacity to extend food shelf life (Muller, Casado Quesada, et al., 2017), and a better environmental performance than conventionally used plastics (Lorite et al., 2017). Previous studies have dealt with the incorporation of F into PLA matrices by standard methods, such as melt-blending and subsequent compression moulding, to obtain films (Ordoñez et al., 2022). The resulting materials showed little to no antimicrobial activity under food storage conditions which was attributed to the limited molecular mobility in the polymeric matrix, hence severely limiting the release of F into the food system (Chung & Kwak, 2019). In this sense, studying alternative methods with which to incorporate F into the PLA matrix so as to favour its antimicrobial action is of great interest given the marked potential of PLA active films for food preservation purposes. These methods should favour the internal diffusion of the compound through the polymer matrix (e.g. by plasticising) or the superficial anchoring of the compound on the film to avoid internal mass transfer through the film.

Solvent casting is a -used method at laboratory scale to obtain polymer-based films. It consists of formulating polymeric solutions where additives (such as plasticisers, active components…) can be incorporated, and later allowing the solvent to evaporate in order to form the film (Suhag et al., 2020). Given the use of solvents and the energy demanded evaporation, casting is not viable at industrial scale, where thermoplastic processing is usually employed (Ochoa-Yepes et al., 2019). However, the resulting film microstructure, with residual solvent retention, promotes the formation of more plasticised matrices (Muller et al., 2017), leading to higher molecular mobility that could enhance the active release, compared to those polymer matrices obtained by thermal processing (Muller et al., 2017). The coating of thermo-processed PLA films with a thin layer of F-loaded PLA solution could be used to obtain active films, facilitating the release of the active in contact with the food substrate.

As concerns the load of active compound on the film surface, the compound adsorption from a concentrated solution or its simple spraying on the film surface could be a suitable strategy to obtain F-PLA active films. Previous studies reported the separation of ferulic acid via its surface adsorption on Amberlite XAD16 from organic waste effluents, with promising results (Dávila-Guzman et al., 2012). The chemical potential increase in line with the substrate concentration is the main driving force of the adsorption phenomena. Adsorption mechanisms and the yield of the process can be affected by a wide number of factors, such as the chemical affinity between the substrate and the surface, the solvent used, temperature and pH (Dávila-Guzman et al., 2012; Pholosi et al., 2020). The superficial anchoring of the ferulic acid on the PLA film surface could be achieved by the film's immersion in a concentrated solution of the acid. Likewise, the pulverisation of an acid solution using a volatile solvent, which would lead to a fast-drying process (Main et al., 1978), could also be used to produce ferulic surface-loaded PLA films.

Electrospinning also represents an alternative strategy with which to incorporate ferulic acid into PLA nanostructures, with high specific areas, which may be deposited on the film surface. This technique is used to obtain nanoscale materials, such as fibres and beads. It consists of propelling a stream of a polymeric solution when submitted to an electrical field that accelerates and spins the stream while evaporating most of the solvent. As a result, the polymeric material is deposited on a collector plate (Wang & Ryan, 2011). **Figure 1** shows a basic electrospinning setup.

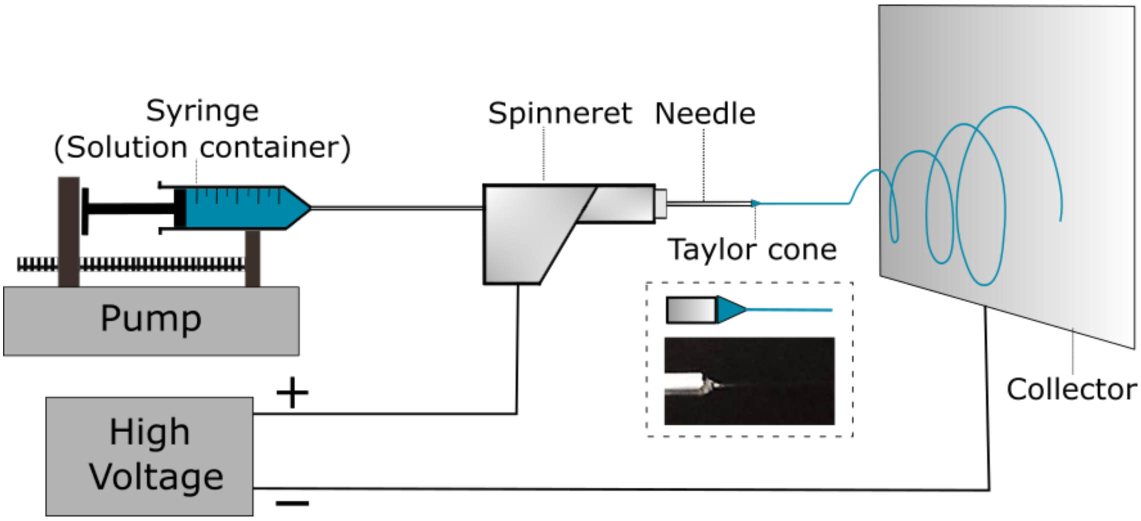


Figure 1 Basic electrospinning setup with main components and Taylor cone illustration.

In previous studies, electrospinning has been used to incorporate active compounds into biopolymer matrices in order to develop active materials for food packaging (Tampau et al., 2020). The electrospun mats present a high surface area that enhances the release of encapsulated active compounds into the food matrices or simulants (Alonso-González et al., 2020). The properties of the polymeric solutions, such as surface tension, electrical conductivity or viscosity, determine the stability of the operation and the viability of the electrospinning process. A stable cone formation of the stream at the tip of the needle, known as the Taylor cone, is considered as an indicator of steady-state process (Bhattacharjee & Rutledge, 2017). In order to reach operational stability, a number of variables must be controlled, both related to the solution properties (solvent volatility, conductivity, viscosity or surface tension) and the process conditions, such as flow rate, electric potential applied and needle-collector distance (Castro Coelho et al., 2021). Tampau et al. (2020) studied the combination of several solvents to obtain electrospun fibres of PLA, and found that volatile solvents, such as ethyl acetate (E), needed to be combined with other less volatile solvents, such as DMSO (D) or acetic acid (A), to achieve a stable electrospinning operation.

In this study, four different strategies were used to obtain PLA - ferulic acid bioactive materials for food packaging applications. These were: a) cast films from ethyl acetate polymeric solutions, b) the surface adsorption of ferulic acid on thermoprocessed PLA films by immersion in concentrated solutions of the compound, c) the pulverisation of a concentrated solution of ferulic acid on the surface of thermoprocessed PLA films and d) the electrospinning of PLA-F solutions with different solvent systems to obtain electrospun mats. After producing the materials, their structural and antimicrobial properties against *L. innocua* were studied.

2. Materials and methods

2.1. Materials

Amorphous PLA 4060D (106 kDa MW, Nature Works, MN, USA) and ferulic acid (Sigma-Aldrich, St. Louis, USA) were used to formulate active films. Ethyl acetate (Indukern, Barcelona, Spain), dimethyl sulfoxide (DMSO), glacial acetic acid, methanol and ethanol 96% (obtained from Panreac Química, Barcelona, Spain) were used as solvents. Tryptone soy broth (TSB), bacteriological agar, peptone water and Palcam agar base (PAB) enriched with palcam selective supplement for Listeria were obtained from Scharlab (Barcelona, Spain) and used for antibacterial tests. The bacterial strain, *Listeria innocua* (CECT 910), was supplied by the Spanish Type Collection (CECT, University of Valencia, Spain).

2.2. Cast PLA films with ferulic acid

PLA films with incorporated F (0-10% w/w in the film) were obtained by casting (Muller, González-Martínez, et al., 2017). Film-forming solutions were formulated by dissolving PLA and F in ethyl acetate, at 10 g of solids per 100 g of solution. 40 g of solution were poured onto 15 cm diameter Teflon plates and left to dry overnight before peeling the films from the plate.

2.3. Production of compression-moulded PLA films

Pure PLA films were also obtained by compression moulding using a heat plates hydraulic press (LP20, Labtech Engineering, Thailand). Ground and dried PLA samples (3.5 g) were placed into 15 cm circular Teflon moulds in the press. An initial heating step took place for 4 min at 200 ºC followed by a compression step at 10 MPa and the same temperature for a further 4 min; finally, a cooling step lowered the temperature to 70 ºC in 3 min.

2.4. Adsorption of ferulic acid on the PLA films

F was adsorbed on the surface of PLA films by immersion in three different F-saturated solution systems, namely distilled water (0.1 % w/w F), ethanol 10 % v/v (0.1 % w/w F) and ethanol 96% v/v (11.2 % w/w F). Film strips (1 x 4.5 cm and 1± 0.1 g) were immersed in the solutions and kept under magnetic stirring at 150 rpm and 25 ºC. The maximum immersion time was 96 h for water and 10% ethanol, and only 4 h for 96% ethanol, due to film deformation. The samples were removed from the F solutions at different times, drained for 1 min and vacuum (0.1 atm) dried at 60 ºC for 24-36 h until reaching constant weight. The F content was determined spectrophotometrically (UV-visible spectrophotometer Thermoscientific Evolution 201, USA) after the methanolic extraction of dry films. To this end, the samples were immersed in 10 ml methanol and kept under magnetic stirring at room temperature for 72 h. The absorbance measurements at 320nm were carried out after filtration and proper dilution. A previously determined F- methanol calibration curve was used to quantify the F adsorbed on the film surface, and the results were expressed as mg F/ g treated film. To determine the real superficial anchoring of F, some samples were washed in their corresponding solvent before F was quantified and the resulting amounts were compared.

To study the F adsorption kinetics, the experimental results were fitted to the Peleg model (Peleg, 1988) (Eq. 1).

$$M\,(t)\;=\;M_0+\frac{t}{k_1+k_2{\cdot}t} \qquad \text{(Eq. 1)}$$

Where: M(t) is the mass of ferulic acid incorporated per mass unit of film at a given time (mg/g); M_o is the initial mass of F per mass unit of film (0 mg/g); t is the adsorption time (h); k_1 and k_2 are model constants: k_1 is inversely related to the release rate (1 / k_1), while k_2 is inversely related to the asymptotic adsorption value ($M_{\square}$= 1/k_2).

A second batch of samples was prepared to evaluate the antimicrobial effect of PLA films with adsorbed F. These were 5.5 cm diameter circular samples to cover the Petri dishes. They were immersed in the 11.2% F solution (solvent ethanol 96% v/v) for 30 min to reach a determined load of F.

2.5. Pulverisation of ferulic acid solutions on PLA films

Ethanol was selected as the solvent for pulverisation due to its volatility and the high solubility of F. A saturated solution of F in 96% (v/v) ethanol (11.2 g of F per 100 g of solution) was filtered and loaded in an airbrush (E4182, Elite pro) container equipped with a 0.8 mm nozzle. The solution was sprayed on circular 5.5 cm diameter PLA films for 5 seconds, at 7.83 ml/s airbrush flow. The pulverised film samples were vacuum dried at 60 ºC and 0.1 atm for 24-36 hours until reaching constant weight. The incorporation of F to the film surface was quantified gravimetrically.

2.6. Production of electrospun PLA-F materials

PLA materials encapsulating F were also obtained by electrospinning. The polymeric solutions had constant concentrations of PLA (15 g/ 100 g solution) and F (15 g/ 100 g solids). Four different food contact solvent systems whose electrospinning operations were of proven stability were used, based on previous studies (Tampau et al., 2020). Ethyl acetate (E) is a good solvent for amorphous PLA, but its high degree of volatility (boiling point: 77 ºC) prevents a steady electrospinning stream. E was mixed with DMSO (D, boiling point: 189 ºC) in two volumetric ratios 1:1 and 6:4 (E:D). Acetic acid (A, boiling point: 118 ºC) was used both pure and in a 1:1 volume mixture with E. Hence, the polymeric solutions of PLA and F were referred to according to the solvent system used: ED1:1, ED6:4, EA1:1, and A1.

The relevant properties of the PLA-F solutions in the different solvents were characterised. The density (20 ºC) was determined in duplicate with an immersion densimeter (Alla France). The conductivity (20 ºC) was measured twice per formulation using a Mettler Toledo (Switzerland) SevenEasy conductometer. The surface tension (25 ºC) was assessed by the pendant drop method with an OCA 20 instrument (Dataphysics, Germany) using software SCA 20, performing 9 repetitions per formulation. The rheological behaviour (25 ºC) was measured in triplicate per formulation, using a rheometer (HAAKE Rheostress 1, Thermo Electric Corporation, Germany) with a coaxial cylinder system (Z34DIN Ti), between 0 and 600 s^{-1}.

The electrospinning operation was carried out in Fluidnatek LE-10 (Bioinicia SA, Valencia, Spain) equipment, using single flow monoaxial mode at 25±1 ºC and 45% RH. 5 mL disposable syringes (BD Plastik) were used to load the solutions into a PTFE tube connected to the spinneret with a 0.6 mm stainless-steel needle. The stream was deposited on a stainless-steel collector covered with previously weighed aluminium foil. Both needle and collector were connected to a high voltage source (0-30 kV).

Relevant operation parameters, such as flow rate, voltage and injector-collector distance, were adjusted empiricallMiy aiming for the formation of a stable Taylor cone in the tip of the spinneret (Tampau et al., 2020). The content of ferulic acid in the mats was determined by methanolic extraction and UV-visible spectrophotometry, as described above. The materials obtained were stored in desiccators with silica gel at room temperature until their use. The operation time in samples used for antimicrobial analyses was adjusted for each formulation in order to obtain a mass of mats containing the amount of ferulic acid necessary to reach the minimal inhibitory concentration of the bacteria in the culture plate when coated with the obtained mat.

2.7. Microstructural observation

The materials obtained were observed using a field emission scanning electronic microscope (FESEM, Ultra 55, Zeiss, UK) at an acceleration voltage of 2 kV. The surface observations were carried out for all materials while images of the film cross-section were taken for materials obtained by adsorption and pulverisation Before observation, samples were cryofractured (only for cross-section observations), mounted on sample holders with carbon tape and platinum coated. ImageJ software (v.153c, National Institute of Health, Bethesda, USA) was used to measure the diameter of beads and fibres in the electrospun mats.

2.8. Antibacterial activity assessment

In vitro antibacterial activity with *Listeria innocua* was performed according to previous studies (Ordoñez et al., 2021; Tampau et al., 2018). All materials, equipment and previously prepared media were sterilised with UV-light inside a laminar flow cabinet (Bio II advance, Telstar, Spain). 10 mL of TSA were poured into 55 mm Petri dishes and inoculated with 100 µL of *L. innocua* bacterial suspension (10^5 CFU / mL) on the plate surface using a L-form rod, aiming for an initial concentration of 10^3 CFU / mL. The TSA was covered with films from the obtained materials; the Petri dishes were sealed with Parafilm^{TM} and left to incubate at 10 ºC for 6 days. After this time, the TSA and samples were homogenised in 100 mL of buffered peptone water for 2 min, employing a masticator paddle blender (IUL Instruments, Barcelona, Spain). The serial dilutions were plated and covered with Palcam Agar base previously enriched with *Listeria* selective supplement. After 48 hours at 37 ºC incubation, the colonies were counted. Each material was tested in triplicate and all the dilutions were plated and counted in duplicate.

2.9. Statistical analysis

Data statistics were evaluated through analysis of variance (ANOVA) using Statgraphics Centurion XVIII software. Fisher's least significant difference was used at a 95% confidence level.

3. Results and discussion

3.1. Cast PLA films with ferulic acid

Figure 2 shows images of the PLA and PLA-F films (2-10% F in the film) obtained by casting. All the formulations with F presented crystalline formations, which were visible to the naked eye. **Figure 2** also shows an optical microscope image of the F crystals in the cast films. A ferulic acid trans isomer tends to crystallise forming prolonged white crystalline aggregates, as previously observed by other authors (Paiva et al., 2013; Chen et al. 2020). In a previous study (Ordoñez et al., 2022), F was homogeneously incorporated into thermally processed PLA films at 2 g of F per 100 g of film without evident crystallisation. However, due to the slower rate of film formation brought about by progressive solvent evaporation, the crystallisation of F at low concentrations occurred in cast films. During this process, the compound would reach saturation level in the solvent and the lack of compatibility with the polymer in the solution would lead to phase separation and the subsequent crystallisation of ferulic acid inside the polymer matrix. Despite the inadequacy of these materials for packaging purposes, they were proven to exert antibacterial activity, since they contain growing amountsof active compound that could be released into the culture media to differing extents, giving rise to varying levels of bacterial growth inhibition.

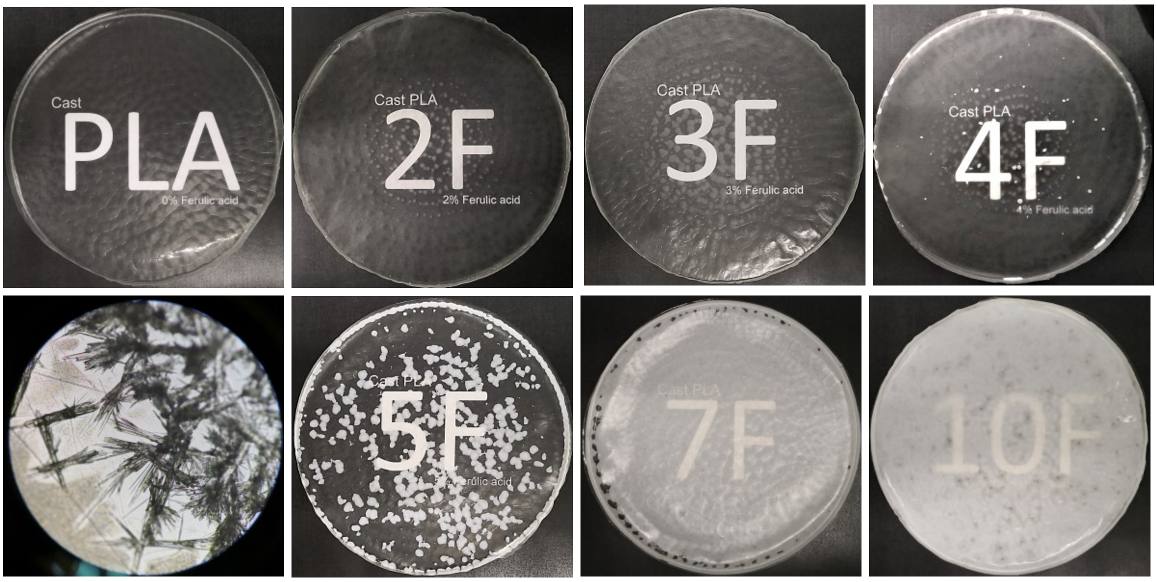


Figure 2: Cast PLA and PLA-F films (2-10 % w/w F in the film). The bottom left picture shows crystalline formations of ferulic acid in the films as observed through an optical microscope (10X).

3.2. Adsorption of ferulic acid on PLA preformed films

Ferulic acid surface adsorption was successfully achieved, and F crystals were formed on the surface of the film samples immersed in all three F solutions, both with and without washing the films with their corresponding solvent. **Figure 3** shows the experimental values of F adsorbed on the PLA surface (mg F/g film) as a function of the immersion time. The low solubility of F in water (Mota et al., 2008), and the subsequent low concentration of F in the saturated solution, limited the adsorption of the acid molecules on the PLA films from the aqueous systems. In the 10% ethanol solution, only 0.27 mg F/ g treated film were adsorbed after 24 h immersion; hence, this system was excluded from further study. In pure water, a small but steady increase in the F adsorbed on the films was achieved, this being 0.76 mg/g after 96 hours. On the other hand, the samples immersed in 96% ethanol with 11.2% F presented remarkably higher values of adsorbed F, reaching 63 mg/g after an immersion of only 15 min. The highest concentration of F in the solution implied this compound had a high gradient of chemical potential between the solution and the film surface, hence representing a great driving force for the adsorption process. However, after 4 hours of immersion, the film strips started to deform, which was attributed to the ethanol diffusion into the matrix, promoting polymer relaxation and swelling and even partial degradation, as reported by other authors in the case of the PLA films in contact with ethanol rich aqueous solvents (Jamshidian et al., 2012).

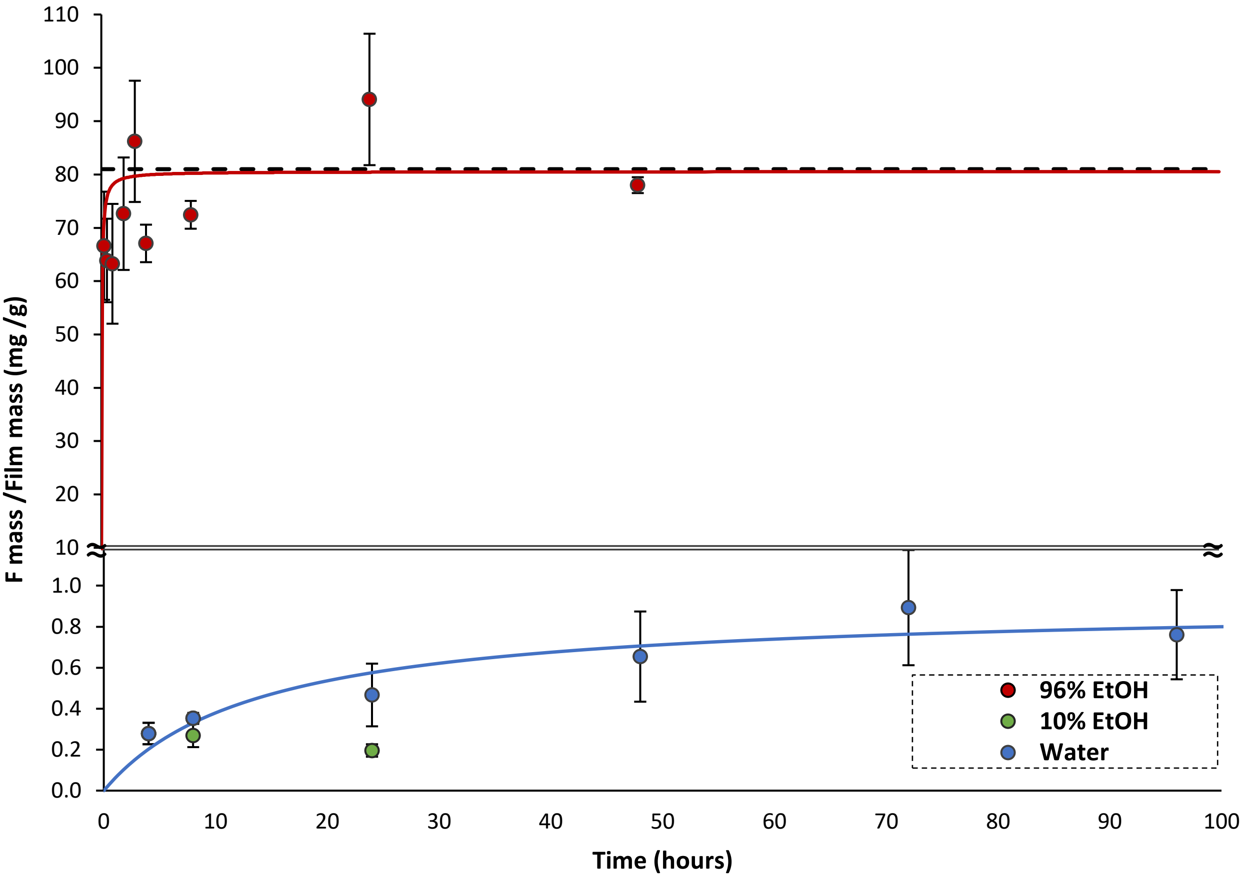


Figure 3 Experimental values of ferulic acid adsorption per PLA film mass (dots) as a function of time, in different solution systems (distilled water with 0.1 % F, 10% v/v ethanol with 0.1 % F and 96% v/v ethanol with 11.2% F). Continuous lines show the Peleg model's prediction, and the dotted lines represent the asymptotic limits.

To assess the real anchoring of ferulic acid on the surface of the films, a set of samples was washed with the corresponding solvent after the adsorption process. While the samples immersed in water lost about 84% of F by washing, those immersed in 96% ethanol only lost 55% of the initially retained acid. Apart from confirming the differences between the adsorption media used, this result suggests that most of the F molecules are attached to the film's surface by weak interactions, thus allowing an effortless release; F diffusion into the PLA matrix, however, was limited.

The Peleg equation was used to model the adsorption kinetics of F from both water and 96% ethanol solutions and to determine the immersion time needed to prepare samples for the antimicrobial tests with a determined F load. The resulting parameters are shown in **Table 1** and the corresponding predictions are represented in **Figure 3.**

Table 1 The Peleg model's kinetic parameters for ferulic acid adsorption on PLA films during immersion in F saturated solutions in water and 96% ethanol.

Parameter	Water	96% Ethanol
k_1 (h · g · mg^{-1})	15.4	$4 \cdot 10^{-4}$
k_2 (g/mg)	1.09	0.012
M_∞(mg/g)	0.912	80.6
R^2	0.956	0.992

Despite the highly variable nature of the experimental data, which could be attributed to a heterogenous film drainage, the high values of R^2 demonstrate the good fit of the Peleg model. Given the high adsorption rate of F in the 96% ethanolic system, k_1 (inversely related to the process rate) was a great deal lower for this series. The resulting asymptotic values of F adsorption (M_∞) are inversely related to k_2. Coherently with the experimental data, M_∞ was two orders of magnitude higher for the 96% ethanolic system than for the water system. Reaching 90% of M_∞ took 72 h for the samples immersed in the water, while only 8 h were necessary in the 96 % ethanol. Due to the small amount of F adsorbed from the aqueous solution, these samples were discarded for both antimicrobial testing and further characterisation. For the samples immersed in the 96% ethanol, an immersion time of 30 min (when 66 % of M_∞ had been reached) was fixed to prepare film samples destined for antibacterial tests, reaching a compromise between the adsorption of F on the film surface and the lower degree of film deformation.

3.3. Pulverisation of ferulic acid solutions on PLA films

Spraying PLA films with ethanolic ferulic acid solution (11.2% w/w) led to the formation of a uniform layer of firmly adhered F crystals when dried. This method was more effective than adsorption at loading PLA films, since the incorporation of F reached 10 % w/w F in the film, while consuming less solvent and time. FESEM observations were carried out to compare the microstructure of the ferulic-loaded film surface obtained by ethanolic adsorption and pulverisation , with F crystalline formations (**Figure 4).** Notable differences in the morphology of the crystals could be observed. Crystalline formations in the adsorption films were broader and shorter than those obtained by pulverisation, which could be related to the different crystallisation kinetics and internal diffusion of F molecules that occurred in each case, with different process times. A very limited internal diffusion of F could be expected in the pulverisation process due to the fast evaporation of ethanol. This needle-like morphology of the crystals is similar to that reported by (Chen et al., 2020) for ferulic acid agglomerates. Despite the fact that solvent absorption and the internal diffusion of F into the film could take place during the processes, no F penetration front or crystal formation at the film's inner surface could be detected in the cross-section of the films from the FESEM images (**Figure 4**). This could indicate that most of the F anchoring occurred on the film surface as crystalline formations. This structural arrangement suggests that F would be easily and almost totally released when the films are put in contact with any food or culture media, thus promoting the antimicrobial activity.

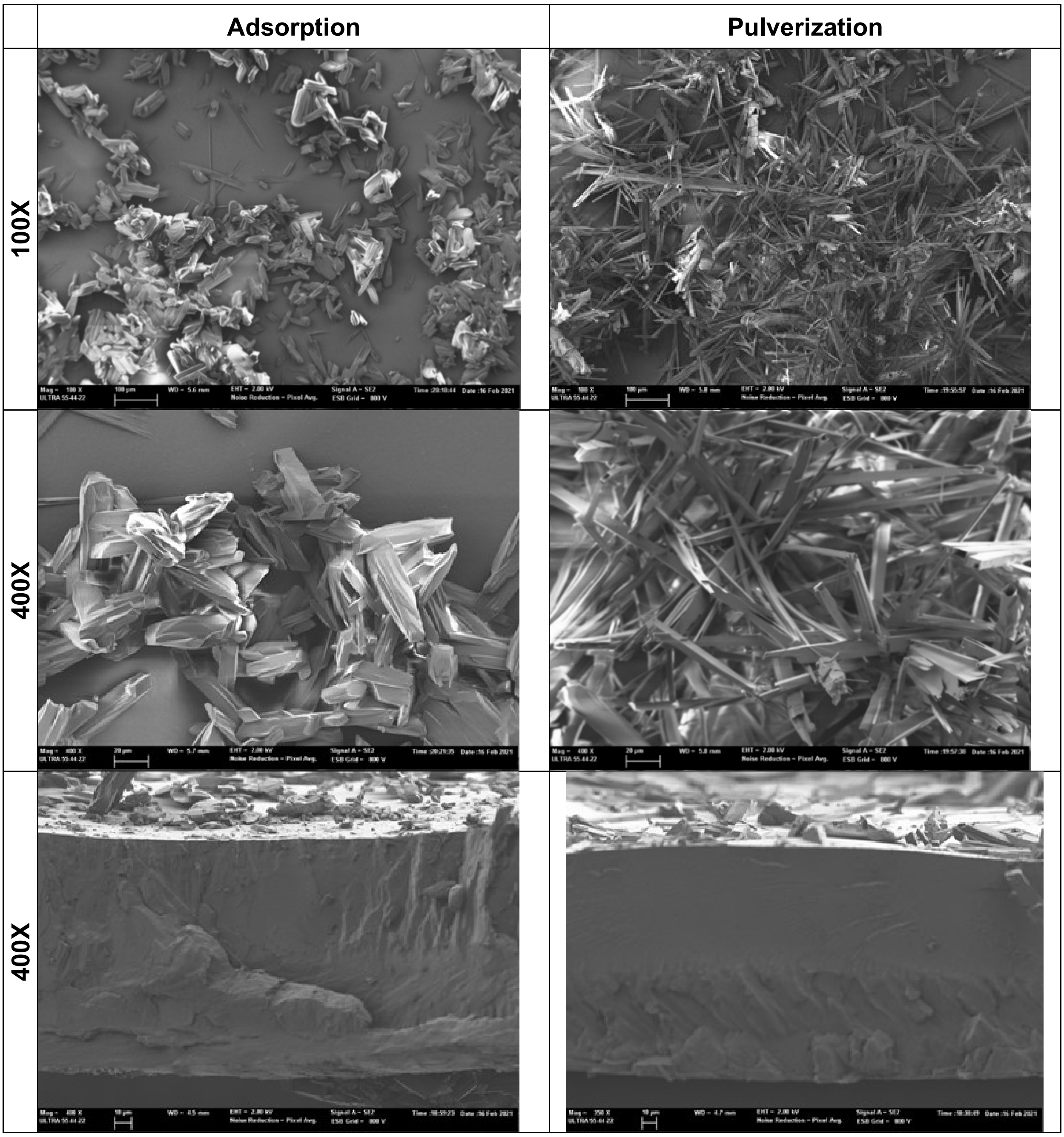


Figure 4 FESEM images for ferulic acid incorporated on the surface of PLA films using 96% ethanol solutions through adsorption (left) or pulverisation (right).

3.4. Production of electrospun PLA-F materials

The most relevant properties of the PLA-F solutions (density, conductivity, viscosity and surface tension) are shown in **Table 2,** along with the different solvent systems used in the electrospinning process. The density of all the solutions was close to 1 g/cm^3, and Newtonian behaviour was found in every case with relatively small differences in viscosity. A1 was the densest and most viscous solution. Density, viscosity and surface tension were reduced when the proportion of ethyl acetate rose in the solvent systems, in coherence with the properties of the pure solvents (Smallwood, 1996). The solvent mixtures containing DMSO exhibited significantly higher values of conductivity due to the higher value of the dielectric constant of DMSO (46), as compared to those of ethyl acetate and acetic acid (about 6) (Smallwood, 1996). Conductivity can have a great impact on the electrospinning process, since low conductivity can limit the effect of the electric field on the solution stream (Agrahari et al., 2017).

Table 2. Properties of the PLA-F solutions (15 g PLA / 100 g solution, 15 g F / 100 g solids) for electrospinning processes with different solvents: ethyl acetate (E), DMSO (D) and acetic acid (A) at different volumetric proportions.

Solution	Density (g/cm^3)	Conductivity (µS/cm)	Surface tension (mN/m)	Viscosity (Pa·s)
ED1:1	1.046±0.001^c	3.08±0.08^c	31.0±1.4^c	0.982±0.004^c
ED6:4	1.027±0.001^b	2.60±0.09^b	28.2±0.8^b	0.840±0.030^b
EA1:1	1.020±0.002^a	0.07±0.01^a	25.2±0.3^a	0.723±0.019^a
A1	1.085±0.002^d	0.08±0.01^a	25.0±0.4^a	1.263±0.016^d

Different superscripts ^{abc} indicate significant differences ($p<0.05$).

Successful operating conditions were obtained for the four solvent combinations (**Table 3)**. The initial values of these operating conditions were based on previous studies into PLA using similar solvent systems (Tampau et al., 2020), and were empirically adjusted until a stable Taylor cone was observed. The solvents containing acetic acid (A) required a slightly lower voltage, and a stable Taylor cone could only be achieved at shorter distances to the collector. For the EA 1:1 solvent, the volumetric flow had to be increased. The process time was adjusted to prepare the samples destined for microbiological testing: the purpose was to obtain mats which were thick enough to reach the same target concentration of F in the sample (about 0.45 mg/cm^2), which would imply about 1.1 mg/mL when put in contact with the culture medium in the antimicrobial assays, assuming a total release.

Table 3. The electrospinning operation parameters and morphological dimensions of the nanomaterials obtained using different PLA and ferulic acid polymeric solutions (15 g PLA / 100 g solution, 15 g F / 100 g solids) with different solvents: ethyl acetate (E), DMSO (D) and acetic acid (A) in different volumetric proportions.

Formulation	Electrospinning operation parameters				Nanomaterial dimensions	
	Flow rate (µL/h)	Collector distance (cm)	Processing time (min)	Voltage (KV)	Fibre diameter (µm)	Bead diameter (µm)
ED1:1	1250	20	150	12-14	2,00±0,90^{c}	-
ED6:4	1250	20	150	12-14	1,50±0,30^{b}	-
EA1:1	2500	15	75	10-13	0,60±0,16^{a}	25±6^{b}
A1	1250	15	150	10-13	0,44±0,15^{a}	19±5^{a}

Different superscripts ^{abc} indicate significant differences ($p<0.05$).

Significant structural differences of the electrospun materials could be appreciated at a macroscopic scale. DMSO solvent systems gave rise to fibrous mats, while those with acetic acid yielded an electro-sprayed powdered layer, which was corroborated by FESEM analyses (**Figure 5**). **Table 3** shows the results of image analysis in order to quantify the characteristic diameters of fibres and beds. As can be observed, DMSO based systems created continuous nano-fibre structures, whose diameter was reduced when the DMSO ratio in the solvent was lower. On the other hand, the structure of acetic acid systems was based on beads connected with thinner fibres, where most of the material is concentrated in the beads. When pure acetic acid was used, the diameters of both beads and fibres were smaller than those obtained in the EA1:1 solvent. These structural differences could be mainly attributed to the differences between the conductivity of the formulations. The greater conductivity of the systems with DMSO would give rise to a higher acceleration of the stream and the maintenance of a continuous flow before reaching the collector plate (Agrahari et al., 2017), thus promoting fibre formation.

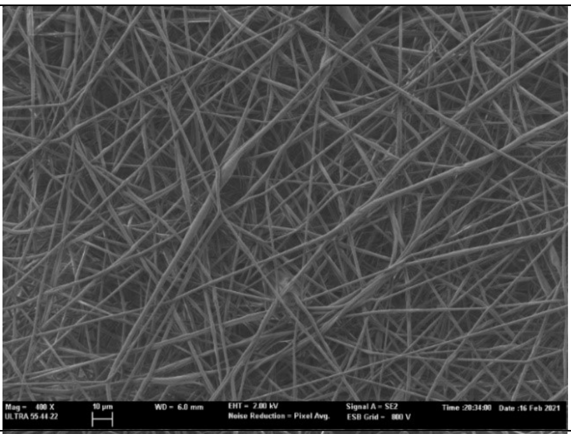
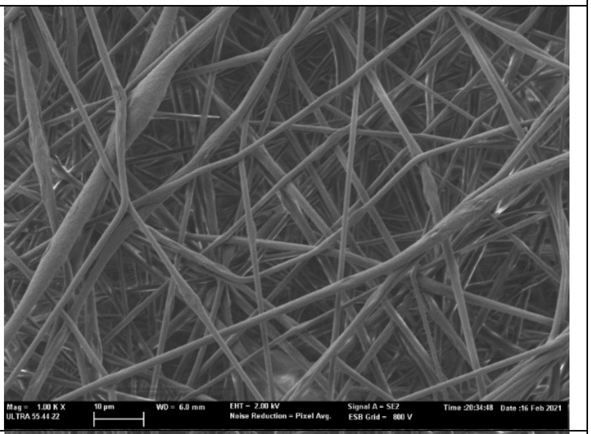
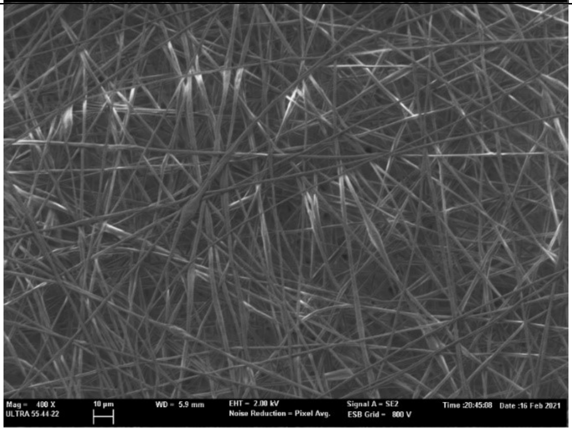
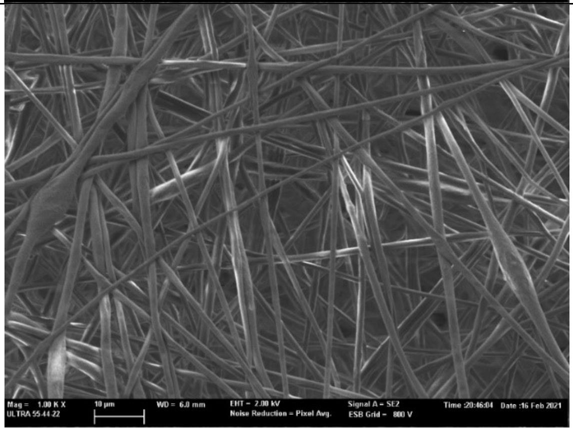
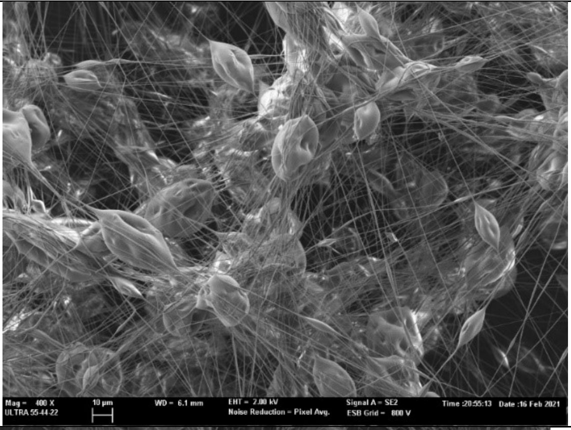
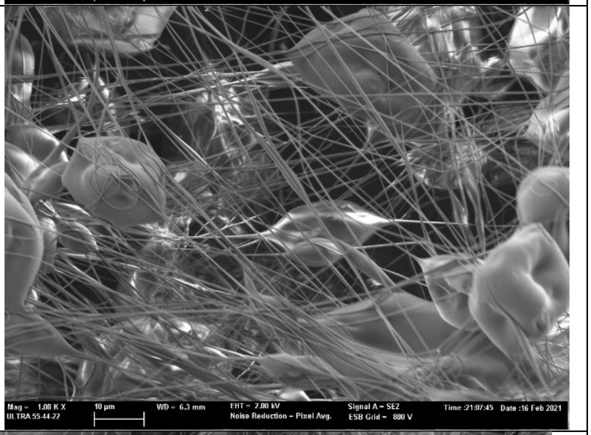
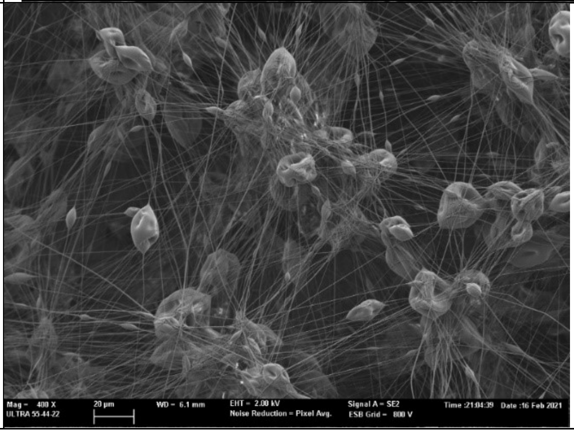
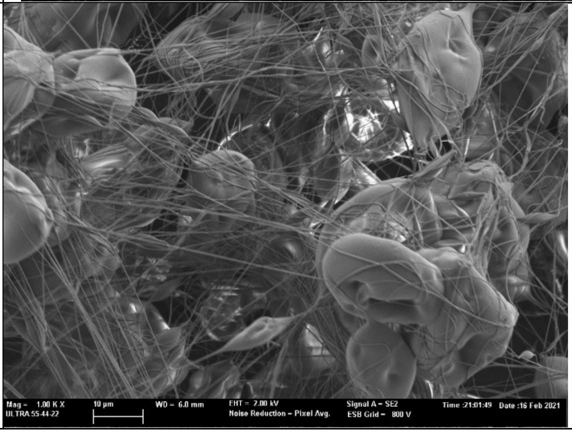
	400X	1000X
E1D1		
E6D4		
E1A1		
A1		

Figure 5 FESEM images of electrospun PLA nanomaterials containing ferulic acid at different magnifications.

3.5. Ferulic acid content in the materials obtained

The F content of the different materials determined by methanolic extraction and spectrophotometry is shown in **Table 4**, expressed as g of F per 100 g of material, and the encapsulation efficiency (g of F in the material per 100 g of nominally incorporated F). All the cast films exhibited high encapsulation efficiency (97-100 %) while the lowest value was obtained for some electrospun materials (84-86%), which indicates that a small proportion of the initially incorporated F could be oxidised during the electrospinning process. Nevertheless. electrospun materials had the highest F content per gram of material, close to the amount incorporated into the solution (15% of total solids). In the case of the superficial incorporation of F, the pulverisation procedure proved to be more efficient than adsorption. This was due not only to the fact that more F was incorporated with lower solvent and time were requirements, but also to the lack of film deformation that occurred during immersion in the adsorption process. Nevertheless, the different distribution of F in the different samples (inside the PLA matrix in cast films or electrospun mats and on the film surface in samples prepared by adsorption or pulverisation) could imply a variation in the antimicrobial activity of the films, depending on the amount of active released into the inoculated culture medium. Table 5 shows the estimated theoretical concentration reached by F in the culture medium, assuming a complete release from the films. A wide range of theoretical concentrations can be seen, the electrospun samples providing the media with the lowest concentration due to their lower thickness. Nevertheless, the real amount of active released in each case would be related with the antibacterial effect observed in the *in vitro* test.

Table 4 Ferulic acid content in the different PLA materials, encapsulation efficiency (% ratio with respect to that nominally incorporated), theoretical F concentration (TFC) in the culture medium, assuming a complete release from the different PLA materials, and growth inhibition of *L innocua*, with respect to the uncovered control samples, obtained in the *in vitro* tests with the different PLA materials.

Formulation	F content (% w/w)	Encapsulation efficiency (%)	TFC (mg/mL)	*Growth inhibition (Log CFU/mL)*
Casting - F2	1.96±0.04^{a}	97.0±2.0^{a}	1.05	0.01±0.02^{a}
Casting - F3	3.00±0.02^{b}	99.9±1.0^{a}	1.58	0.07±0.05^{a}
Casting - F5	4.90±0.11^{c}	98.2±2.2^{a}	2.63	0.01±0.07^{a}
Casting - F10	9.80±0.30^{e}	97.9±2.8^{a}	5.26	0.25±0.79^{a}
Adsorption	6.4±1.0^{d}	-	2.49	4.30±0.31^{c}
Pulverisation	10,0±1.5^{e}	-	3.87	4.11±0.42^{c}
ES-E1D1	14.4±1.2^{g}	96±7^{a}	1.02	2.99±0.81^{b}
ES-E6D4	12.9±0.7^{f}	86±5^{b}	0.92	3.26±0.33^{b}
ES-E1A1	12.6±0.5^{f}	84±3^{b}	0.90	0.05±0.04^{a}
ES-A1	12.9±0.3^{f}	86±2^{b}	0.92	0.03±0.06^{a}

Different superscripts ^{abc} indicate significant differences ($p<0.05$)

3.6. Antimicrobial activity of the materials obtained

Figure 6 shows the bacterial counts for *L. innocua* obtained in the *in vitro* tests with the different F loaded PLA materials, while the different growth inhibition values for the different PLA samples, with respect to the uncoated plate, can be observed in **Table 4**. According to a previous study, the Minimum Inhibitory Concentration (MIC) of ferulic acid for *L. innocua* is 0.7 mg/ml (Ordoñez et al., 2021), which would be surpassed in all cases provided the release of the acid from the materials into the culture media is complete. However, only in four cases did the materials reach 2 Log CFU/mL growth inhibition and, hence, an efficient antibacterial activity (Requena et al., 2019): these were those obtained by F adsorption or pulverisation and electrospun materials obtained with DMSO solvent systems (E1D1 and E6D4) that were fibre structured. Despite the high concentration of F that could be theoretically reached in the culture media with a total release of F from cast films (up to 5.26 mg/mL in the cast film with 10% F), no significant growth inhibition occurred for these materials. The ineffectiveness of these films against *L. innocua* revealed no release of ferulic acid from the films regardless of their concentration. This was also observed in a previous study into thermoprocessed PLA films incorporating ferulic and cinnamic acids (Ordoñez et al., 2022). The glassy state of the polymer and the lack of swelling and relaxation when in contact with the aqueous culture media limited the molecular mobility and diffusion of ferulic molecules through the film matrix and only the most superficial amount of the compound was released to exert its antimicrobial power.

The active films obtained by the adsorption and pulverisation of F solutions, where the active compound was mainly incorporated onto the film surface, could easily release the active compound in contact with the culture medium, thus being the most effective at inhibiting the growth of *L. innocua.* These films exhibited similar antimicrobial power given the excess amount of F present on the film surface, thus easily releasing the acid and provoking a bacteriostatic effect.

Electrospun materials contained about 14 % ferulic acid. However, the plate-covering materials were very thin, with just enough electrospun material to reach the MIC of ferulic acid on the plate, assuming a complete release. The effectiveness of electrospun materials was dependent on their morphology. DMSO based materials, having a fibre structure, exhibited a significant bacterial growth inhibition whereas the mainly bed-structured mats showed no antibacterial effect. This could be explained by the higher specific surface area of the fibres (Alonso-González et al., 2020) that can release the active compound near the surface more effectively, reaching F concentration values closer to the MIC of bacteria in the culture medium. In contrast, acetic acid-based materials with bead structures did not have enough ferulic acid near the surface to reach the MIC values and to inhibit the bacterial growth.

The results obtained reveal that the release of ferulic acid is greatly hindered when encapsulated in PLA matrices, the only possibility of enhancing the compound delivery being its surface retention. Therefore, it is the location of the active compound in the material, and not its global concentration, that limits the obtaining of antimicrobial materials with ferulic acid and PLA.

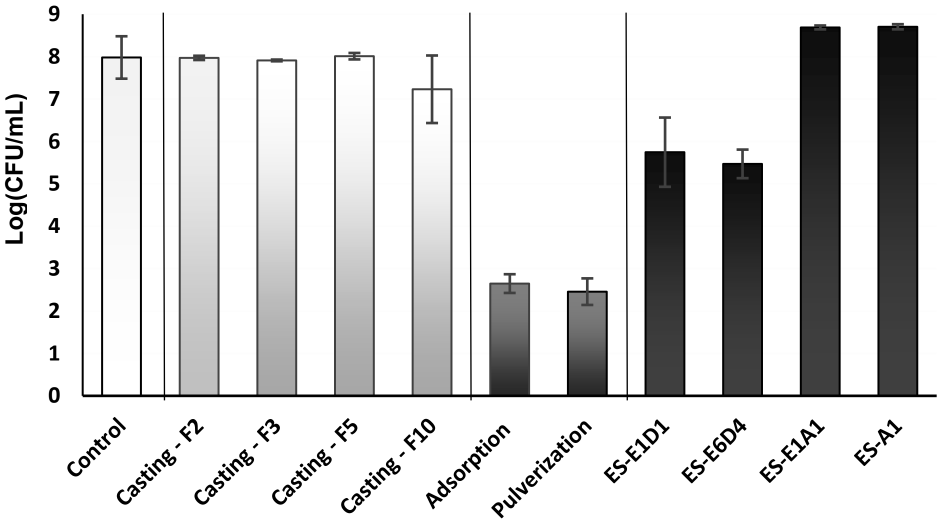


Figure 6 *Listeria innocua* counts after 6 days of incubation at 10 ºC for samples in contact with PLA-based materials loaded with ferulic acid. The control bar shows mean growth for uncoated samples and the dotted line represents the initial inoculation.

4. Conclusions

Four different strategies were tested for the purposes of developing antimicrobial materials based on polylactic acid with ferulic acid. The films obtained by casting with different amounts of the active compound presented crystalline formations of ferulic acid, whose release from the polymeric matrix was greatly limited, as deduced from the lack of any antibacterial effect, despite the fact that these contained a high enough amount of active to exceed the MIC of the bacteria. Casting is, hence, not considered as a viable alternative to obtain active PLA packaging materials with ferulic acid. A good yield of ferulic acid adsorption from an ethanolic oversaturated solution on the surface of PLA films was achieved after a relatively short immersion time, whereas the pulverisation of the same solution was a more effective method with a higher amount of ferulic acid deposited on the PLA surface. The films with ferulic acid incorporated via adsorption and pulverisation inhibited the growth of *L. innocua*, exerting a bacteriostatic effect in the concentrations applied. The structure of the electrospun materials, which, in turn, was determined by the solvent system, affected their antimicrobial efficacy. When DMSO was used, fibres were obtained, which favoured the antimicrobial power, due to their higher specific surface area. In contrast, electrospun materials obtained from acetic acid solvent systems exhibited a bead-based structure, which led to no significant antibacterial activity. The lower specific surface area of the beads could imply a more limited availability of ferulic acid near the surface for an efficient release to achieve the MIC of bacteria in the culture media. On the basis of these results, the surface application of ferulic acid by adsorption or the pulverisation of F solutions, as well as electrospun depositions of PLA-F mats using DMSO solvent systems, represent viable strategies to obtain PLA food packaging materials with strong antibacterial activity. More research is required in order to implement these processes in complex food packaging materials, such as multilayer assemblies, and study their antimicrobial impact in both model systems and specific food matrices.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ramón Ordoñez: Investigation, Conceptualisation, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Lorena Atarés**: Conceptualisation, Methodology, Data curation, Writing – original draft, Writing – review & editing, Supervision. **Amparo Chiralt**: Conceptualisation, Methodology, Data curation, Writing – review & editing, Supervision, Project administration.

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Chapter 6:

Multilayer antimicrobial films based on starch and PLA with superficially incorporated ferulic or cinnamic acids for active food packaging purposes.

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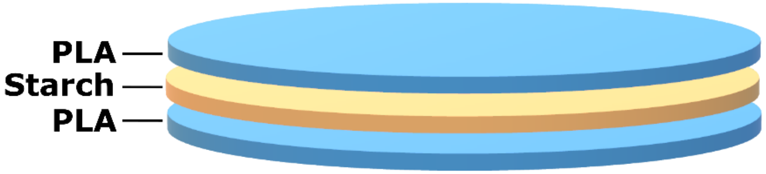
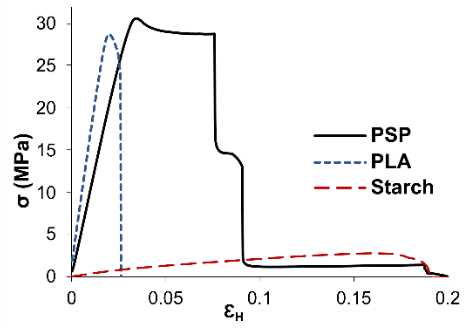
PSP: three-layered films

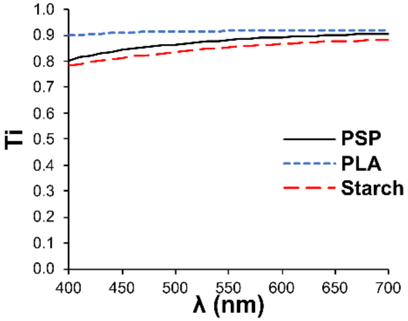


Functional characterization:

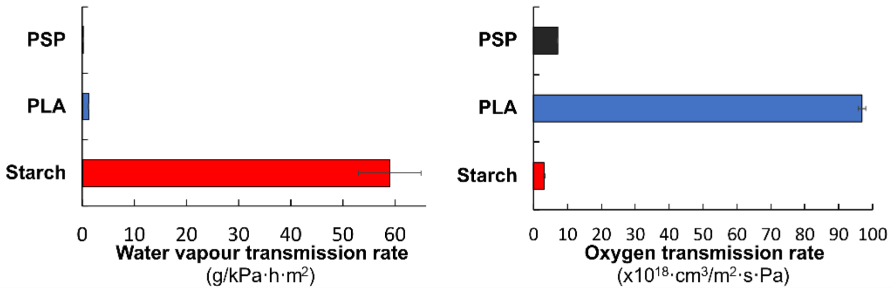
Mechanical behaviour



Internal transmitance

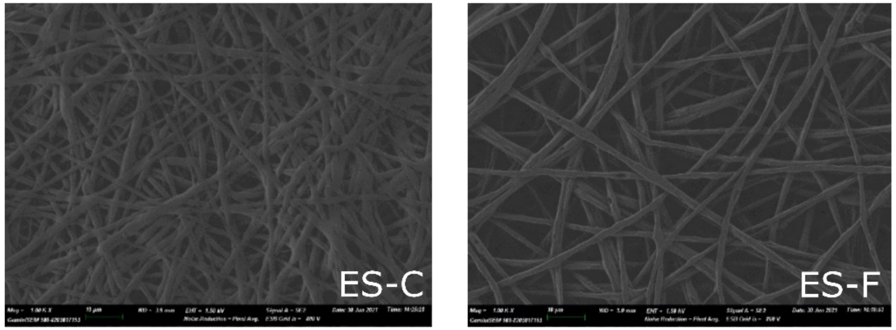


Barrier properties

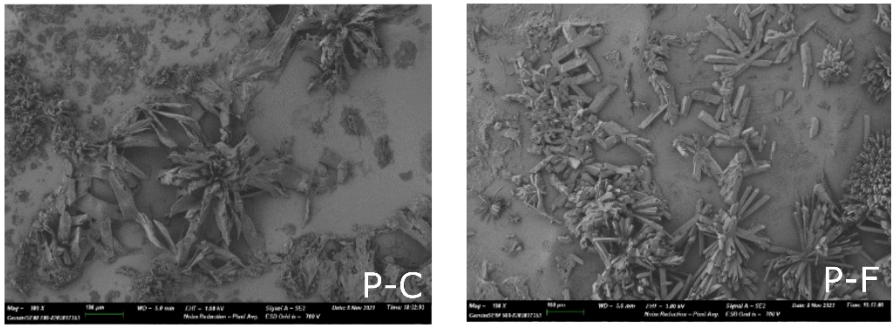


Superficial incorporation of cinnamic (C) or ferulic (F) acids:

Electrospinning (ES):

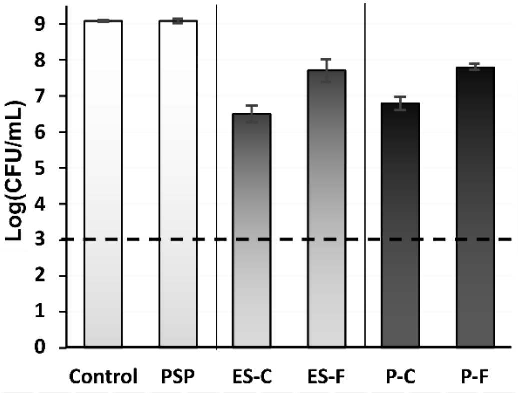


Pulverization (P):

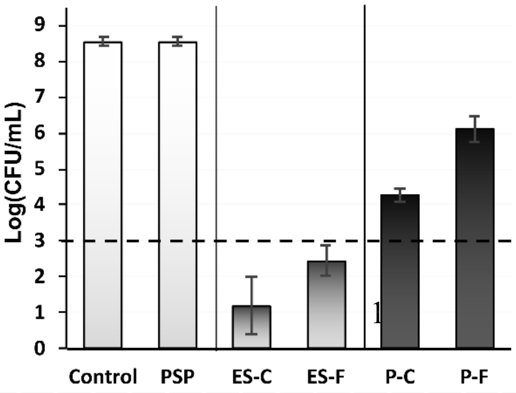


Antimicrobial tests

E. coli



L. innocua



Abstract

Active packaging based on biodegradable polymers with naturally occurring active compounds, such as ferulic (F) and cinnamic (C) acids, have been studied to reduce the environmental impact of food packaging while prolonging food shelf-life. Three-layered films PLA/starch/PLA (PSP) were designed based on the complementary barrier properties of the polymers. The superficial incorporation of F or C through different methods was studied to promote compound release. Electrospinning of PLA-acid solutions was effective at producing fibre mats encapsulating the acids. Pulverisation of concentrated ethanolic solutions of F or C onto the PSP surface produced compound crystalline formations firmly attached to the film surface. Superficially loaded films by both electrospinning or pulverisation, showed effective growth inhibition of *E. coli* and *L. innocua*. Listeria was more sensitive to both active compounds while C showed greater antibacterial activity. Electrospun films were more effective than pulverised, suggesting greater ability to release the active compounds.

1. Introduction

Although a great effort to regulate the use of single-use petrochemical plastics has been made, the food packaging industry still relies on these materials given their great functional properties and low cost (Porta et al., 2020). To solve this crisis, biodegradable and renewably- sourced polymers, such as cassava starch or polylactic acid (PLA), have been exhaustively studied as economically viable alternatives to conventional plastics (Elvers et al., 2016; Mangaraj et al., 2018). Likewise, there is greater and greater consumer demand for a reduction in the synthetic additives in food products (Carocho et al., 2014), while food safety needs to be ensured. To this end, antimicrobial and antioxidant agents are one of the main tools used in the food industry. Active packaging is an innovative approach to extending food shelf-life by deliberately incorporating components into the packaging material that would interact with the food product (Yildirim et al., 2018). Incorporating naturally-occurring active agents, with antimicrobial or antioxidant properties, into biodegradable polymers could bring renewable food packaging materials, meeting consumer demand and ensuring food safety (Atarés & Chiralt, 2016; Mangaraj et al., 2018; Yildirim et al., 2018).

Starch is one of the most common means of storing energy in plants. This can be obtained from multiple sources, such as corn, potato or cassava (Carvalho, 2008). Native starch can be gelatinised by heating with plasticisers to obtain thermo-plastic starch that can be used to obtain plastic materials, such as films or other thermoformed pieces (Carvalho, 2008; Menzel et al., 2019; Ordoñez et al., 2021). Starch films exhibit extensible and plastic behaviour and good oxygen barrier capacity, but poor water vapour barrier capacity and a high degree of water sensitivity and solubility, all of which represents a drawback to the wrapping of high moisture foods, since their functional properties are greatly affected by moisture content (La Fuente Arias et al., 2021; Ordoñez et al., 2021; Zhong et al., 2020). In contrast, PLA is a hydrophobic renewable polyester, with relatively poor oxygen barrier capacity but high barrier capacity for water vapour and mechanical strength. It is obtained through the lactic fermentation of carbohydrates, such as starch, and subsequent lactic acid polymerisation (Jamshidian et al., 2010). The properties of PLA films make them suitable for food packaging applications, and its production is becoming more economically viable (Elvers et al., 2016; Muller et al., 2017). Starch and PLA present complementary barrier properties, and so multilayer assemblies of these two materials can offer great barrier properties both to water vapour and oxygen (Muller et al., 2017). Moreover, PLA external layers could potentially protect the inner layer of starch from moisture intake from the environment or the packaged food products.

Phenolic acids and their precursors are naturally-sourced antimicrobial and antioxidant agents that can be found in a wide range of plant species (Rashmi & Negi, 2020). Contrary to essential oil compounds, phenolic acids are not volatile and have lower organoleptic impact in food matrices (Choi et al., 2018; Liu et al., 2021). Cinnamic and ferulic acids have been previously incorporated into starch matrices, exhibiting a great antimicrobial activity against Gram + and Gram – bacteria (Ordoñez et al., 2021). However, given the limited applicability of hydrophilic starch films on high moisture food products (Muller et al., 2017a), the incorporation of these active compounds into more hydrophobic matrices, such as PLA, could potentially result in highly applicable active food packaging materials. Nevertheless, previous studies (Ordoñez et al., 2022a) using thermoprocessed PLA films found no significant release of ferulic and cinnamic acids from the films into aqueous media, such as sensitive foods to microbial spoilage, which greatly limited their antimicrobial action. In contrast, subsequent studies (Ordoñez et al., 2022b) incorporating ferulic acid onto the PLA film surface or into fibre-structured electrospun mats demonstrated how effective this was at controlling the growth of *Escherichia coli* and *Listeria innocua*. The distribution of active compound near the film surface was effective at promoting its antimicrobial action, thus avoiding the diffusion limitations of the compound through the PLA matrix, where the mass transfer process was greatly hindered due to the glassy state of the polymer and the lack of matrix relaxation when in contact with aqueous systems.

Electrospinning is a novel approach to obtaining nanofibers of polymeric materials. These structures offer a high specific surface area that facilitates the active compound release (Huang & Thomas, 2018). Electrospinning polymeric solutions with biodegradable polymers and naturally-occurring active compounds have been used as an efficient strategy to obtain fast active release rates(Quiles-Carrillo et al., 2019; Tampau et al., 2018). Quiles-Carrillo et al. (2019) studied the incorporation of gallic acid into electrospun PLA nanofibers, using chloroform as solvent, reporting good release rates of the compound in saline media. Tampau et al. (2020) also incorporated carvacrol into PLA fibres, using food contact solvents such as ethyl acetate and DMSO. In this sense, the incorporation of ferulic or cinnamic acid into PLA nanofibers using ethyl acetate and DMSO blend solvents could give rise to antimicrobial mats for the purposes of coating films with adequate properties to meet food packaging requirements.

The surface application of cinnamic and ferulic acids onto packaging films by means of solution spraying also represents an easy method to prepare active packaging materials. These compounds exhibit a high degree of solubility in ethanol (Noubigh & Akremi, 2019; Shakeel et al., 2017), which is a cheap, safe solvent of great volatility that favours its fast evaporation. Hence, the pulverisation of ethanolic solutions with F or C could lead to a quick surface oversaturation during solvent evaporation, triggering the surface crystallisation of the compounds (Batista et al., 2019; Chen et al., 2020). These crystalline structures could be initially attached to a PLA surface thanks to the tendency of PLA towards electric polarisation and static electricity (Urbaniak-Domagala, 2013): once a small crystal is attached, it could grow if continuously exposed through the oversaturated stream.

The aim of this study was to develop biodegradable materials for food packaging purposes, offering barrier capacity to oxygen and water vapour, with the ability to extend food shelf life. To this end, PLA and starch were laminated as three-layer assemblies, with two PLA outer layers to protect the interior of the starch from moisture, incorporating ferulic or cinnamic acid onto the PLA surface through electrospinning or the pulverisation of their solutions. The functional, structural, and antibacterial properties of these materials were evaluated.

2. Materials and methods

2.1. Materials

Amorphous PLA 4060D (106 kDa MW, Nature Works, MN, USA), cassava starch (Asia Co., LDT, Kalasin, Thailand) and glycerol (Panreac Química, Barcelona, Spain) were used to formulate film monolayers. Magnesium nitrate and phosphorus pentoxide supplied from Panreac Química were used to control relative humidity (RH) for film conditioning purposes. Cinnamic and ferulic acid were used as active components and were purchased from Sigma-Aldrich (Saint Louis, USA). Ethyl acetate, from Indukern (Barcelona, Spain), and dimethyl sulfoxide (DMSO), from Panreac Química, were used as solvents in the electrospinning solutions. Ethanol (96%), from Panreac Química, was used as the solvent for pulverisation solutions. Tryptone soy broth, bacteriological agar, and peptone water obtained from Scharlab (Barcelona, Spain) were used for antibacterial tests. Bacterial strains, *Listeria innocua* (CECT 910) and *Escherichia coli* (CECT 101), were supplied by the Spanish Type Collection (CECT, University of Valencia, Spain). Selective media: violet-red bile agar (VRBA) for *E. coli* and palcam agar base (PAB) enriched with palcam selective supplement for *Listeria* were obtained from Scharlab.

2.2. Film preparation

Starch and PLA monolayers were obtained separately and assembled in three layer systems with two outer PLA layers enclosing an inner starch sheet; ferulic or cinnamic acid were incorporated by electrospinning or the surface pulverisation of one of the PLA sheets.

2.2.1. Starch and PLA monolayers

The starch monolayers were prepared according to previous studies (Ordoñez et al., 2021). Cassava starch was melt-blended with glycerol at 0.3 g/ g starch ratio in an internal mixer (Haake PolyLab QC, Thermo Fisher Scientific, Germany) at 130 ºC and 50 rpm for 10 min. The obtained melts were ground with an M20 IKA mill (Staufen, Germany) and conditioned for 1 week inside a desiccator at 53% RH, using $MgNO_3$ oversaturated solution, at 25 ºC. The samples (4 g) were placed onto 20 cm diameter Teflon moulds to obtain films of the same diameter by compression-moulding, using a hot-plate hydraulic press (LP20, Labtech engineering, Thailand). The samples were preheated at 160 ºC for 1 min and subsequently compressed at 5 MPa for 2 minutes, followed by a second compression at 10 MPa. Finally, a cooling step to 70 ºC in 3 min was applied. The films were stored at 53% RH and 25 ºC until their use.

The PLA monolayers were obtained using the previously described conditions (Muller et al., 2017). PLA pellets were cold ground and 2 g samples were placed onto 20 cm diameter Teflon moulds to be compression moulded. An initial preheating step took place at 200 ºC for 4 min before a 10 MPa compression at 200 ºC for 4 min, ending with a cooling step to 70 ºC for 3 min.

2.2.2. Multilayer assembly

The multilayer films were obtained by the thermo-compression of two external PLA layers and one inner starch layer (PSP). All three monolayers, in the abovementioned arrangement inside the heating hydraulic press, were preheated for 2 min at 110 ºC and then compressed at 2.5 MPa and 110 ºC for 1 min before being cooled to 70 ºC for 3 min. No irregularities and good layer adhesion were observed in these conditions.

Both monolayers and PSP assemblies were characterised as to their functional properties. Likewise, PSP films were surface coated with ferulic or cinnamic acid by spraying or electrospinning, as described below, and tested as to their antimicrobial activity.

2.3. Characterisation of the film properties

The thermal properties of the PSP assemblies and the monolayers were studied using a differential scanning calorimeter (DSC, 1 StareSystem, Mettler-Toledo, Switzerland) and a thermogravimetric analyser (TGA/SDTA 851e, Mettler-Toledo, Switzerland). The samples were conditioned at 0% RH in desiccators with P_2O_5 before the tests. The DSC analysis consisted of the following steps: cooling from room temperature to -10 ºC at 10 K/min, heating to 160 ºC at the same heating rate, maintaining 160 ºC for 5 min, cooling at 50 K/min to -10 ºC, maintaining -10 ºC for 5 min, and heating back to 160 ºC at 10 K/min. An empty aluminium pan was used as a reference. For TGA analyses, 10 mg samples in alumina crucibles were heated from 25 to 600 ºC at 10 K/min, under a nitrogen flow (50 mL/min). Both tests were performed twice per formulation.

A TA-XT plus (Stable Micro Systems, Haslemere, England) texture analyser was used to measure the tensile properties of each film formulation, following the ASTM D882 method (American Society for Testing Materials, 2002). Film samples of 25 mm x 100 mm were cut (eight replicates per formulation) and their thickness was determined at six random points using an electronic digital micrometer (Comecta S.A., Barcelona, Spain). The samples were mounted on tensile grips (50 mm separation) and stretched at 50 mm/min. Tensile strength at break (TS), elastic modulus (EM) and elongation at break (%E) were calculated from the stress-Henky strain curves.

The water vapour permeability (WVP) was determined following the ASTM E96-95 method (American Society for Testing Materials, 1995). Payne permeability cups (3.5 cm diameter, Elcometer SPRL, Hermelle/s Argenteau, Belgium) were filled with 5 ml of distilled water and covered with circular film samples whose thickness had been previously measured at six random points. The cups were placed into desiccators containing $MgNO_3$ oversaturated solution inside a temperature controlled chamber at 25 ºC. Cups were weighed periodically at one-hour intervals, starting after 16 h in the chamber, using an analytical balance (±0.00001 g). The weight loss rate *vs.*: time data, in the stationary state, were used to calculate WVP, following the E96-95 method, as described by (Andrade et al., 2020). Three replicates were obtained per formulation.

Oxygen permeability (OP) was measured following the ASTM D3985-05 method (American Society for Testing Materials, 2010) employing an Ox-Tran 1/50 equipment (Mocon, Minneapolis, USA). The film test area was 50 cm^2 and measurements were taken at 25 ºC and 53% RH. The OP was calculated by dividing the oxygen transmission rate by the oxygen partial pressure gradient on both sides of the film and multiplying by the average film thickness, which was previously measured at six different positions of the film sample. Two replicates per formulation were obtained.

The optical properties of the films were analysed with a CM-5 spectrocolorimeter (Konica Minolta, Inc., Japan). The reflection spectra (400-700 nm) were obtained for three film replicates and at three points per replicate, backed on both black and white plates. The internal transmittance (T_i) was calculated by applying the Kubelka-Munk theory of multiple dispersion (Hutchings, 1999). The CIEL*a*b* colour coordinates were obtained from the determined reflectance of an infinitely thick layer of material, considering the D65 illuminant and 10º observer (Cano et al., 2014). Finally, the lightness (L*), chroma (C_{ab}*) and hue (h_{ab}*) psychometric coordinates were calculated (Mahy et al., 1994).

2.4. Coating of three-layered PSP with active compounds

To obtain active PSP laminates, they were coated with ferulic or cinnamic acid using two alternative procedures: via the electrospinning of PLA-active solutions on one of the PLA external layers or by the direct spraying of ethanolic solutions of the actives.

2.4.1. Electrospinning of active PLA solutions

Based on previous studies (Ordoñez et al., 2022b; Tampau et al., 2020), polymeric solutions for the electrospinning process were formulated using an ethyl acetate-DMSO blend (Ratio 6:4) as a food-grade solvent. The PLA concentration was 15 g/g solution and ferulic or cinnamic acid was added at 15 g/g solids. The solutions were electrospun in a Fluidnatek LE-10 (Bioinicia SA, Valencia, Spain) setup, with 5 mL syringes (BD Plastik) as solution container and a 0.6 mm stainless steel needle at the spinneret. The operation conditions were adjusted empirically based on the formation of a stable Taylor-cone in the solution stream. The collector-syringe distance was fixed at 20 cm, the flow rate at 2500 µL/h and the voltage ranged from 12-14 KV. The PSP film samples were placed on the collector and a continuous stream was electrospun for 75 min in order to achieve a theoretical active concentration of 0.45 mg/cm^2 on the film surface. After the electrospinning process, annealing was subsequently applied to the coated films to promote the compaction of the fibre mats and to ensure a good adhesion to the PSP films. To this end, the films were placed inside the hydraulic hot-plate press, between steel plates, and heated at 50 ºC for 1 min, without additional pressure.

2.4.2. Superficial pulverisation of active solutions

Cinnamic or ferulic acid were pulverised on the surface of PSP films by means of an airbrush (E4182, Elite pro) loaded with 5% w/v active solutions in 96% ethanol. Circular film samples of 5.5 cm were placed at a vertical distance of 14.7 cm below the airbrush nozzle and were sprayed at 8 µL/s flow for 5 seconds. After pulverisation, the samples were vacuum dried at 60 ºC and 0.1 atm overnight.

2.5. Active compound quantification

The ferulic and cinnamic acid contents of three-layered PSP were obtained by methanolic extraction and UV-visible spectrophotometry. The dry samples (100 mg) were immersed in 10 ml of methanol and kept under magnetic stirring at room temperature for 72 hours. The absorbance measurements for F and C were taken at 320 nm and 270 nm, respectively, after filtration and proper dilution. A similarly extracted sample without actives was used as blank. A previously determined methanol calibration curve for each active was used to quantify the concentration of ferulic or cinnamic acid in the films. Results were expressed in mg of active per film unit area.

2.6. Microstructural analyses

A high-resolution field emission scanning electronic microscope (HR-FESEM) (GeminiSEM 500, Zeiss, Germany) was used to observe the superficial and cross-section microstructures of the obtained materials. The samples were cryofractured using liquid nitrogen (only for cross-section observations), mounted in supports with carbon tape and platinum coated before observation. ImageJ software (v.153c, National Institute of Health, Bethesda, USA) was used for image analysis.

2.7. Antibacterial activity assessment

The antibacterial capacity of the active coated films was assessed though the growth inhibition of *L. innocua* and *E. coli,* according to previous studies (Ordoñez et al., 2021; Tampau et al., 2018). The sterilised media, materials and film samples were placed inside a laminar flow cabinet (Bio II advance, Telstar, Spain) and exposed to UV-light for 15 min. The Petri dishes (55 mm diameter) were filled with 10 mL of TSA media and left to solidify before being inoculated with 100 µL of a bacterial suspension at 10^5 CFU/mL in order to obtain an initial concentration of 10^3 CFU/mL in the plate. The TSA was covered with the film active surface, and the plates were sealed. All the films were tested in duplicate. After 6 days of incubation at 10 ºC, the samples were homogenised in 100 mL of peptone water using a paddle masticator (IUL Instruments, Barcelona, Spain) and serial dilutions were plated (in duplicate) in selective growth media: Palcam agar base enriched with *Listeria* selector for *L. innocua,* and red vile agar for *E. coli*. After 48 hours of incubation at 37 ºC, the colonies were counted and compared with the uncoated growth control samples.

2.8. Statistical analysis

Data statistics were evaluated through an analysis of variance (ANOVA) using Statgraphics Centurion XVIII software. Fisher's least significant difference was used at a 95% confidence level.

3. Results and discussion

3.1. Properties of the PSP laminates

Multilayer films were successfully assembled, exhibiting good adhesion between layers, without irregularities. These were characterised as to their main functional properties as packaging material, in comparison with their constitutive monolayers, as shown in **Table 1**. The three-layered PSP was not as thick as the sum of the individual thicknesses of the mono layers (a reduction of approximately 25 %), which indicates that additional radial flow occurred in the different sheets during the adhesive thermocompression step, reducing the final thickness of the three-layered PSP. The individual reduction of each polymer sheet was estimated from the cross-sectional observations by FESEM (**Figure 1**). Comparing the initial monolayer thickness with that measured in the three-layered assemblies (about 90 and 170 μm for PLA and starch layers, respectively), a greater thickness reduction was observed for PLA than for starch monolayers, coherently with the higher degree of flowability of PLA. Similar results were observed by other authors for starch-polyester bilayers during the thermocompression step (Hernández-García et al., 2021).

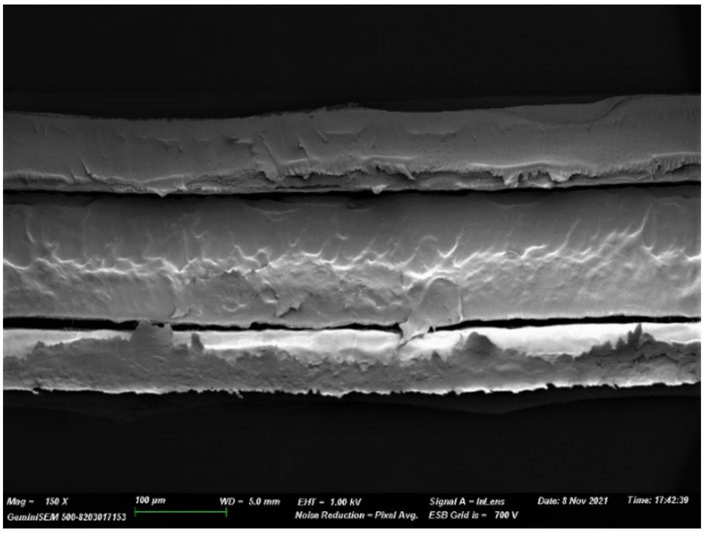


Figure 1 Cross-sectional HR-FESEM observation of PSP three-layered assemblies.

Table 1 Properties of PLA and starch monolayers, and PSP multilayer assemblies. Film thickness (t), tensile strength (TS) and elongation at break (E%), elastic modulus (EM); glass transition temperature (Tg) from the second heating scan, thermal degradation temperature (onset (T_{onset}) and peak (T_{peak})), water vapour transmission rate (WVTR), oxygen transmission rate (OTR), colour parameters (lightness (L*), hue (hab*), chroma (Cab*)) and internal transmittance (Ti) at 400 and 700 nm.

	PLA	**Starch**	**PSP**
t (µm)	129±6^{a}	211±8^{b}	352±20^{c}
TS (MPa)	29.2±0.8^{b}	2.8±0.4^{a}	29.0±2.0^{b}
EM (MPa)	1510±40^{c}	40±8^{a}	1030±70^{b}
E (%)	2.9±0.4^{a}	19.2±0.2^{c}	8.0±2.0^{b}
Tg$_{PLA}$ (ºC)	54±2^{a}	-	50±3 ^{a}
Tg$_{Starch}$ (ºC)	-	100±9^{a}	100±5^{a}
T_{onset} (ºC)	265±3^{b}	246±2^{b}	247±2^{b}
T_{peak} (ºC)	309±2^{b}	280±2^{a}	308±2^{b}
WVTR (g/kPa·h·m^{2})	1.28±0.05^{b}	59±6^{c}	0.12±0.01^{a}
OTR x 10^{-18} (cm^{3}/m^{2}·s·Pa)	96.9±1.1^{c}	3.1±0.2^{a}	7.12±0.12^{b}
L*	82.4±0.7^{c}	61.5±3.1^{b}	59.2±1.1^{a}
h$_{ab}$*	99.0±3.0^{c}	74.0±0.1^{a}	76.7±0.5^{b}
C$_{ab}$*	7.4±1.1^{a}	11.4±0.6^{b}	13.9±0.3^{c}
Ti (400 nm)	0.89±0.02^{b}	0.78±0.02^{a}	0.80±0.01^{a}
Ti (700 nm)	0.92±0.02^{a}	0.88±0.01^{a}	0.90±0.01^{a}

Different superscript letters indicate significant differences between film formulations ($p < 0.05$).

The tensile behaviour of three-layered films, compared with the monolayers, is shown in **Figure 2a**. The lower strength and more plastic behaviour of the starch layer can be observed, as compared with the stiffer and more brittle PLA films, as previously reported (Collazo-Bigliardi et al., 2019; Muller et al., 2017). As expected, three-layered assemblies exhibited similar strength to PLA monolayers, but with enhanced plastic deformation. In general, the rupture of the PSP films during stretching occurs when the PLA layers break while the internal starch sheet is still becoming longer, as can be observed in **Figure 2a**. The tensile parameters, elastic modulus, tensile strength and deformation at break are shown in **Table 1** for PSP assemblies and monolayers, in which it can be observed that PSP presented the same resistance to break (TS) as the PLA monolayers. The PLA layers offered the three-layered structure great resistance and stiffness, whereas the stretchability was better in the three-layered structure than in the PLA films. This effect suggests that the PLA in contact with starch could be partially hydrolysed, gaining plasticity and slightly reducing its stiffness.

Therefore, combining PLA and starch layers in multilayer assemblies implied a significant improvement in the mechanical performance of the material, better meeting the food packaging requirements than the individual films. The partial migration of glycerol and water molecules from the starch layer to the PLA matrix could modify the PLA's tensile behaviour in the multilayer. The high degree of sensitivity of PLA to hydrolysis in the presence of the migrated water (Rocca-Smith et al., 2017) would largely explain the observed behaviour. The hydrolysed oligomers could plasticise the PLA sheets making them more stretchable.

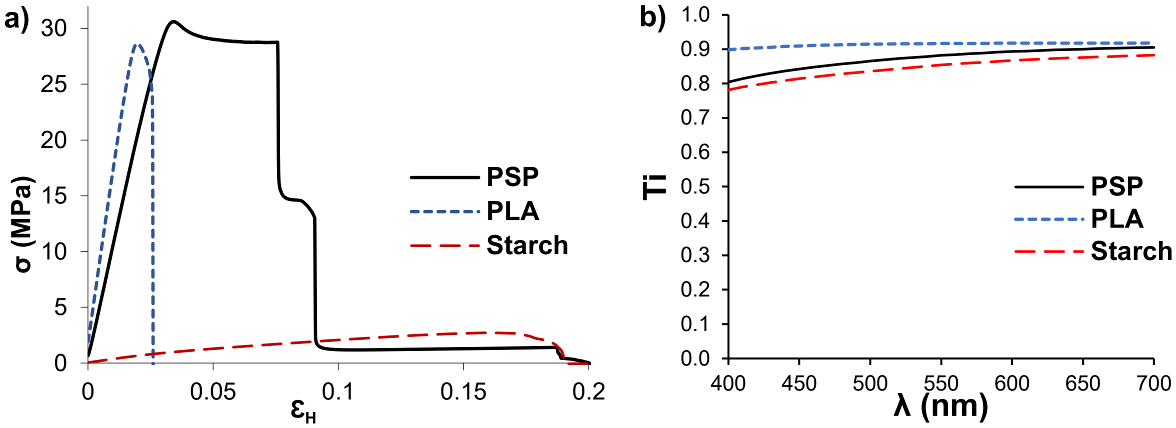


Figure 2 a) Strain-stress curves and **b)** Internal transmittance (Ti) spectra of PLA and starch monolayers and PSP multilayer assemblies.

The DSC analyses of monolayers in the second heating scan showed the typical glass transition of amorphous PLA and thermoplastic cassava starch, with midpoint Tg values at 54 ºC and 100 ºC, respectively, which agrees with that previously reported for these polymers (Collazo-Bigliardi et al., 2019; Muller et al., 2017). For the three-layered assembly, thermograms also exhibited both glass transitions, with no significant changes in the Tg values. For the PLA, a decreasing trend in the Tg value was observed in the three-layered structure which could be explained by the partial migration of the plasticiser or the water hydrolysis effect, as previously commented on. Likewise, the thermodegradation pattern of the three-layered structure was similar to the monolayers, exhibiting overlapped thermal degradation of both polymers due to the similar temperature at which this occurs in both cases. PLA is slightly more thermostable than starch and the obtained To and Tp values were in the range of those previously reported by other authors for these polymers (Collazo-Bigliardi et al., 2019; Muller et al., 2017). The degradation temperatures of both materials overlapped in a single degradation curve starting at the starch onset temperature and exhibiting the peak near to the PLA peak temperature, as observed by other authors (Collazo-Bigliardi et al., 2019; Gómez-Contreras et al., 2021) for PLA/Starch blends.

As concerns the barrier properties, the water vapour transmission rate (WVTR) and oxygen transmission rate (OTR) are shown in **Table** 1. The starch and PLA monolayers exhibited similar values to those previously reported for PLA and starch films (Collazo-Bigliardi et al., 2019; Ordoñez et al., 2021); the starch layer exhibited a good oxygen barrier capacity whereas PLA exhibited good barrier capacity to water vapour. The PSP assemblies exhibited a highly reduced water vapour transmission rate with respect to the starch films, as well as a reduced oxygen transmission rate with respect to the PLA films. Thus, this material presented an improved overall barrier capacity for water vapour and oxygen. This is coherent with the additive effect of the assembled parallel resistances to mass transfer offered by the layer combination. Applying the mass transfer model for successive resistances assembled perpendicularly to the mass flow (**Eq. 1**), and considering the thickness of the different layers in the three-layered assembly, the values of the WVTR and the OTR were 2.5 g/kPa.h.m^2 and 6.2x10^{18} cm^3/Pa.s.m^2, respectively, which indicates that the experimental OTR values practically meet the model predictions, whereas the experimental WVTR was 20 times lower than the predicted value. This additional improvement in the water vapour barrier capacity of PSP films could be attributed to the interfacial interactions of the PLA-S layers that could confer more barrier capacity onto the multilayer assembly. Likewise, the gradient of water vapour established in the pure starch films during the WVTR measurements will be different than that established in the internal starch layer when the measurement was taken in the PSP films, thus affecting the obtained value. This also demonstrates the protective effect of the PLA sheet against the moisturising of the internal starch. In contrast, with PLA being more permeable to oxygen than starch, the external PLA layer did not significantly limit the oxygen flow or the establishment of the corresponding oxygen gradient in the starch sheet.

$$\frac{t_{ML}}{TR_{ML}} = \sum_i \frac{t_i}{TR_i} \qquad \textbf{(Eq. 1)}$$

Where *t* is the thickness of the multilayer (ML) or each layer (i) and *TR* is the transmission rate of the multilayer (ML) or each layer (i).

The colour coordinate values for the PSP assemblies and monolayers are shown in **Table 1**. The monolayers exhibited similar colour parameter values to those previously reported for starch or PLA films (references), the starch layer being slightly darker and redder; the multilayer assembly, however, presented similar colour parameter values to the starch films, as expected from the parallel arrangement of the layers. This behaviour was corroborated by the internal transmittance spectra (**Figure 2b**), where the PSP assembly exhibited very similar internal transmittance spectra to the starch films but with slightly higher values due to the reduction in the thickness of the starch sheet during the adhesive thermocompression process. Therefore, the three-layered assembly exhibited slightly greater transparency than starch monolayers with colour parameters similar to those of the starch monolayers.

3.2. Superficial incorporation of ferulic and cinnamic acids on PSP laminates

The active compounds were successfully incorporated onto the surface of the PSP assembly by electrospinning or solution pulverisation. The microstructure of the electrospun mats over the films was observed by HR-FESEM, and the images are shown in **Figure 3a**. In both cases, fibre mats were obtained, as observed in a previous study (Ordoñez et al., 2022b) for ferulic acid-PLA solutions using DMSO:EtAc (6:4) solvent. Electrospun mats with FA were highly fluffy and were submitted to the annealing process to favour their compaction and good adhesion to the PLA surface, as described in the methods section. This was not required in PLA-cinnamic acid mats, which exhibited a denser structure well adhered to the PLA film surface. In **Figure 3a**, the final structure of the electrospun mats encapsulating ferulic and cinnamic acids can be observed, where the more open nanofiber structure can be seen for samples with ferulic acid, despite the annealing treatment. This structural difference could be due either to the effect of CA on PLA fibres, inhibiting their repulsive interactions, or to the higher degree of solvent retention, leading to the depositing of not completely dry PLA fibres on the mat, as observed by other authors for PLA-carvacrol electrospun materials from DMSO solutions (Tampau et al., 2020). Solvent retention in the electrospun material could favour both fibre adhesion and flattening when deposited on the collector. In both cases, the high specific surface area of the fibre structure could enhance the release rate of the active compounds (Huang & Thomas, 2018). The image analysis in the micrographs revealed a similar fibre diameter for both ferulic and cinnamic acids, encapsulating fibres with 1.5±0.1 μm and 1.5 ±0.3 μm, respectively. The cross-sectional HR-FESEM observations of the PSP films coated with the acid-loaded fibres are shown in **Figure 3a**, where the good adhesion of the electrospun mats to the film surface can be observed.

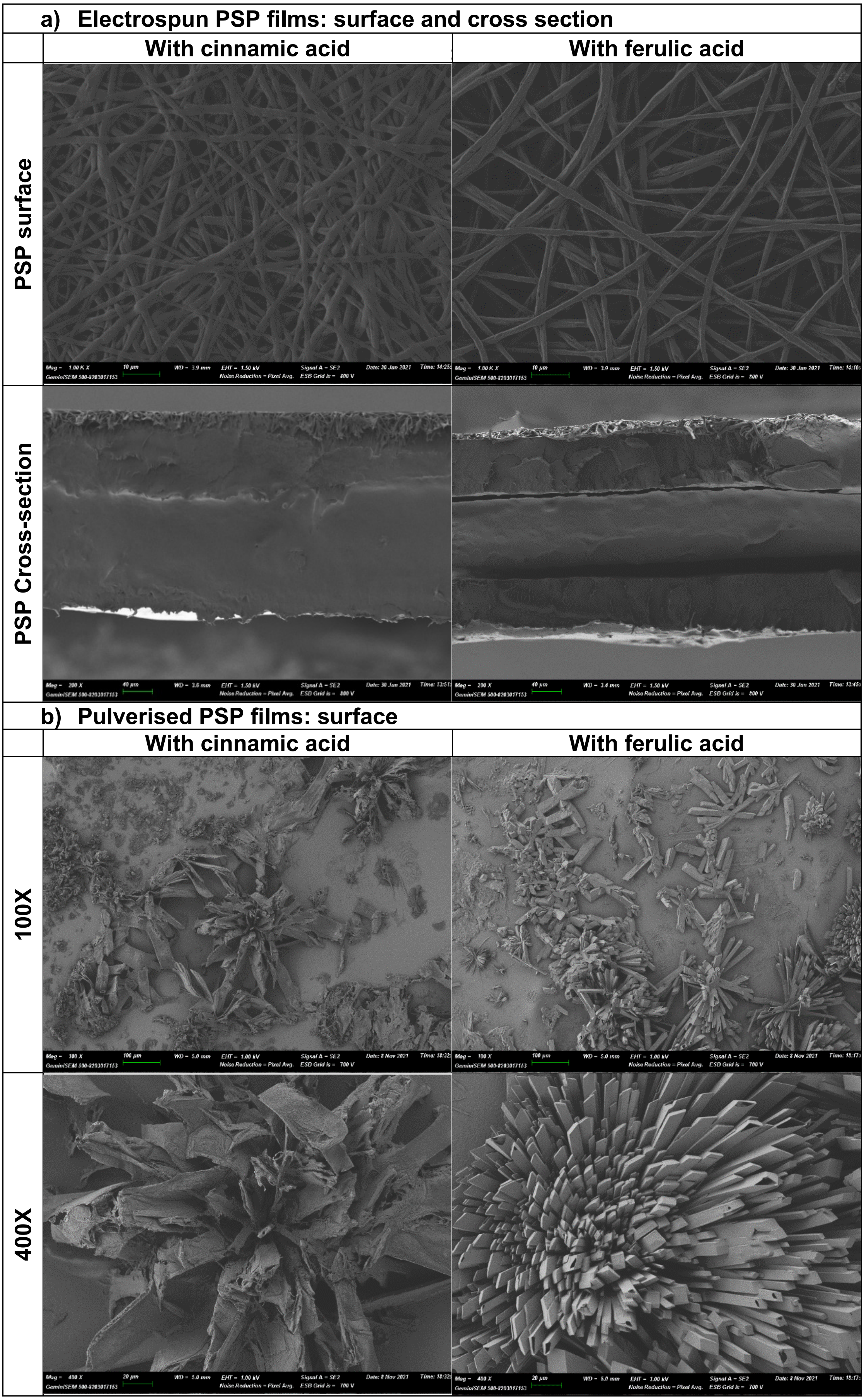


Figure 3 HR-FESEM images of **a)** electrospun PLA fibres with ferulic or cinnamic acid on the surface of the PSP multilayer (x1000) and cross-sections of the coated PSP films (x200) and **b)** Crystalline structures of cinnamic or ferulic acid on the surface of superficially pulverised PSP multilayer films.

Pulverised solutions of ferulic or cinnamic acid on the PSP surface gave rise to the formation of crystalline structures of the respective acid, which were well attached to the PLA surface after the solvent evaporation, as can be observed in the HR-FESEM micrographs of the film surface (**Figure 3b**). FA crystalline agglomeration structures were similar to those observed by Chen et al. (2020). These authors reported that FA tends to agglomerate in needle-like structures when precipitated in oversaturated solutions. Thus, the fast solvent evaporation in pulverised films promoted the crystallisation of F on the film surface, forming well-adhered crystalline structures. Batista et al. (2019) also studied the crystallisation of CA, observing similar crystalline structures but smaller in size. Due to the crystallisation, no homogeneous distribution of cinnamic and cinnamic could be observed on the film surface, but agglomerations of crystalline forms of differing sizes in highly intricate structures that could affect the release rate of active compounds when the coated film is put into contact with an aqueous medium, such as an inoculated culture or a real food. In fact, the solubilisation rate of the active compound in the aqueous media may be affected by the crystalline structure and crystal sizes (Batista et al., 2019). An even distribution and a smaller, homogeneous size of crystals could be achieved by changing the solvent or spraying conditions, such as nozzle type or flow.

The contents of active compounds incorporated onto the PSP films, determined by methanolic extraction and spectrophotometric quantification, are shown in **Table 2** for both electrospun and sprayed PSP films. Although the surface concentration (mg/cm^2) reached similar values for both treatments, it was slightly higher for the pulverised samples. By taking these contents, the film area and the volume of culture medium in the plates into consideration, the theoretical concentration of both active compounds in the culture medium was estimated, assuming their complete release. These values were very close to 1 mg/mL, according to the target concentration established as necessary to reach the minimal inhibitory concentration (MIC) of the bacteria in the antimicrobial test. According to a previous study (Ordoñez et al. 2021), the MIC values of *L. innocua* were 0.70 and 0.65 mg/mL for FA and CA respectively, while for *E. coli* these were 0.80 and 0.70 mg/mL, for FA and CA respectively. Therefore, the MIC of both active compounds for the bacteria would be exceeded in all cases if their complete release is achieved in the culture medium.

Table 2 Superficial concentration of ferulic and cinnamic acids in the PSP films, theoretical concentration in the culture plate (TCCP), assuming a complete release from the different films, and growth inhibition of *E. coli* and *L. innocua* with respect to the uncovered control samples, obtained in the *in vitro* tests with the different PSP materials.

Formulation	**Surface concentration (mg/cm^2)**	**TCCP (mg/mL)**	***E. coli* growth inhibition (Log (CFU/mL))**	***L. innocua* growth inhibition *(Log (CFU/mL))***
ES-C	0.44 ± 0.02^a	1.04	2.6 ± 0.2^b	7.3 ± 0.8^c
ES-F	0.40 ± 0.03^a	0.95	1.4 ± 0.3^a	6.1 ± 0.4^c
P-C	0.52 ± 0.06^b	1.23	2.3 ± 0.2^b	4.3 ± 0.2^b
P-F	0.48 ± 0.08^{ab}	1.14	1.3 ± 0.1^a	2.5 ± 0.4^a

Different superscript letters indicate significant differences between film formulations ($p < 0.05$).

3.3. Antibacterial activity of PSP films surface loaded with ferulic or cinnamic acid

Figure 4 shows the bacterial counts for *E. Coli* and *L. innocua* after 6 days of incubation at 10 ºC for the different active PSP materials coating the culture plates and compared with the control uncoated plates and those coated with PSP films without active compounds. In Table 2, the growth inhibition values with respect to the control samples are shown. All the films coated with active compounds, by spraying or electrospinning, inhibited the growth of both *E. coli* and *L. innocua* to a different extent, depending on the active compound, the bacterial strain and the active incorporation method. This revealed that the extent of the effective release of the incorporated compounds into the culture medium was enough to reach the corresponding MIC values. Both F and C were more effective at controlling the growth of *L. innocua* than *E. coli*, coherently with the lower MIC values of *L. innocua* with both compounds. In the same way, cinnamic acid was more effective than ferulic acid, also coherently with the lower MIC values of this compound for both strains. These results agree with those obtained in a previous study (Ordoñez et al. 2021) where both compounds were incorporated into thermoprocessed starch films and tested as to their antibacterial capacity with the same strains. As concerns the incorporation method, different behaviour was observed for *E. coli* and *L. Innocua*. No significant differences in the inhibition capacity of pulverised or electrospun films were observed for *E. coli*, whereas electrospun films were more effective than pulverised at inhibiting the growth of L. innocua, despite the fact that they contained a slightly lower number of active compounds. This indicates that releasing the incorporated compounds from the electrospun mats coating the PSP films was more effective than doing so from the crystalline coating obtained by pulverisation. The different release rate had a significant effect on the more sensitive bacteria (*L. innocua*) but did not notably affect the growth inhibition of the less sensitive bacteria (*E. coli*). Electrospun PSP films containing cinnamic acid exhibited a bactericidal effect on

L. innocua, reducing the initial inoculum after 6 incubation days, while films with electrospun ferulic acid exhibited a bacteriostatic effect due to it being less active against this bacteria. Quiles-Carrillo et al. (2019) also observed an adequate release of gallic acid from PLA electrospun fibres, reporting the main role of the active accumulated on the fibre surface that is it is easily releasable in contact with aqueous systems.

Therefore, electrospun PLA fibres loaded with active compounds, such as ferulic or cinnamic acid, were very effective at promoting the release of actives from their high specific surface area, providing the food packaging with active properties to extend the food shelf life. The pulverisation of concentrated solutions of these active compounds on the packaging material is a method that is both more easily available and cheap; however, the surface crystallisation of the compounds could limit their solubility in the microbially sensitive aqueous systems, making them less effective in terms of their antimicrobial action. Nevertheless, other solvents and pulverisation conditions should be studied to optimise the release of actives in order to favour the antibacterial activity.

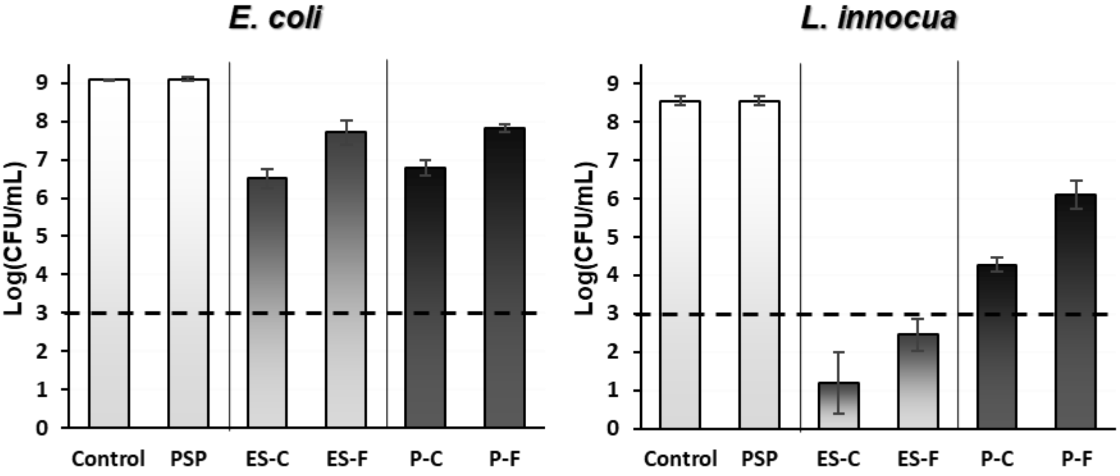


Figure 4 Bacterial growth obtained from *in vitro* tests after 6 days of incubation at 10 ºC for plates coated with PSP multilayers with and without cinnamic (C) or ferulic (F) acid superficially incorporated by electrospinning (ES) or pulverisation (P). The control bar shows the mean growth for uncoated plates and the dotted line represents initial inoculation.

4. Conclusions

Three-layered PSP assemblies exhibited an improved mechanical and barrier capacity with respect to the PLA and starch monolayer films with adequate functional properties to meet food packaging requirements. PLA provides the laminates with mechanical resistance and water vapour barrier capacity while the internal starch layer was protected from the moisture sensitivity by the external

PLA sheets, providing the laminates with oxygen barrier capacity. The optical properties of the multilayer were similar to those of the starch monolayers. The incorporation of ferulic and cinnamic acids onto the PSP surface by the electrospinning of PLA-acid solutions in EtAc-DMSO (6:4) were effective at producing fibre mats encapsulating the acids with high specific surface area and the ability to release the active compounds so as to exert antimicrobial action. The pulverisation of concentrated ethanolic solutions of ferulic or cinnamic acid onto the PSP surface produced crystalline structures of the compounds that were firmly attached to the film surface with potential antimicrobial activity. The antimicrobial tests of surface-loaded PSP laminates with F or C, by either electrospinning or pulverisation, exhibited effective growth inhibition of *E. coli* and *L. innocua*, the latter being more sensitive to both active compounds and cinnamic acid showing greater antibacterial activity. For the more sensitive bacteria (*L. innocua*), significant differences in the growth inhibition were observed for the electrospun and pulverised PSP laminates. The electrospun films were more effective than the pulverised, thus indicating their greater ability to release the active compounds into the culture medium. However, pulverisation conditions could be optimised in order to produce more soluble crystalline formations with smaller particle size and easy release. Therefore, laminates of PLA/starch/PLA with the surface incorporation of ferulic or cinnamic acid represent a good alternative means of obtaining active food packaging materials with the capacity to preserve and enhance food quality and shelf life. Nonetheless, further studies into their stability and specific applications in real foods of these materials are necessary in order to determine their possible industrial application and economic viability.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ramón Ordoñez: Investigation, Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Lorena Atarés**: Conceptualization, Methodology, Data curation, Writing – original draft, Writing – review & editing, Supervision. **Amparo Chiralt**: Conceptualization, Methodology, Data curation, Writing – review & editing, Supervision, Project administration.

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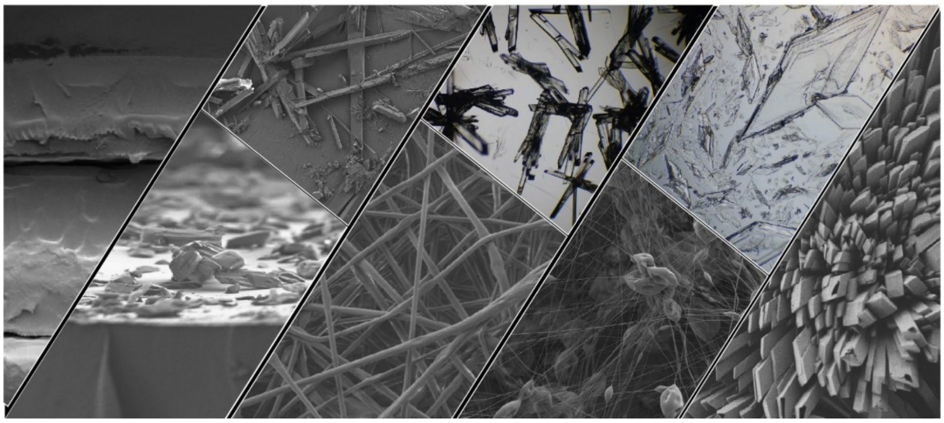
IV. General Discussion



IV. General Discussion

General Discussion

Food packaging waste is a major contributor to the current environmental crisis caused by conventional plastic. For this reason, the food industry needs more sustainable environmentally friendly packaging solutions, which has driven the research focus to the development of novel materials suitable for food packaging applications. Likewise, the growing demand of the consumers for less chemical additives has motivated the search for improved food safety and quality through active packaging materials with bioactive (antimicrobial or antioxidant) properties, which could be based on biodegradable polymers and naturally occurring active compounds.

Research on active packaging incorporating natural additives has been mainly focused on the use of essential oils or their main compounds. However, high volatility and the organoleptic impact of these components represent an important drawback for food application, since they may alter the flavour of food products. In contrast, phenolic acids and their precursors, such as ferulic or cinnamic acids, have low volatility and less sensory effect while also have proven antimicrobial/antioxidant activity and health benefits. These have not been extensively studied for food packaging uses and their incorporation to packaging materials represent an opportunity to develop tailor-made active materials which could inhibit the bacterial growth over storage without compromising the quality characteristics of the food product.

Both polylactic acid (PLA) and starch have been widely studied in the recent years as potential bio-based plastics for food packaging applications. Both can be biodegraded; while starch is easily degraded in humid environments, PLA can be degraded under composting conditions. Both polymers have been studied in the present Doctoral Thesis as carriers of ferulic and cinnamic acids as naturally occurring antimicrobial compounds, in order to obtain active food packaging materials. PLA and starch exhibit complementary barrier properties due to their different polarities; hence, PLA films shows high water vapour barrier capacity while starch films are good oxygen barriers. Likewise, PLA films present higher mechanical resistance and stiffness than starch films. Therefore, their combination in multilayer assemblies could provide the laminate with improved barrier capacity and mechanical performance, which is highly desirable for food packaging materials.

The present doctoral thesis studied the effect of incorporation of ferulic (F) and cinnamic (C) acids into PLA and starch matrices through different techniques, aiming to obtain antimicrobial materials to extend food shelf life while preserving food quality. The functional and antimicrobial properties of the films obtained were evaluated and discussed. First, monolayer films of starch or PLA with active compounds were studied and finally multilayer films combining starch and PLA sheets were designed in order to obtain packaging material with improved properties to better meet the food packaging requirements. The incorporation method of the active compounds into the films has been deeply analysed in order to obtain an effective release of the active compounds for ensuring their antimicrobial action.

Chapter 1 deals with the incorporation of ferulic and cinnamic acids into cassava starch matrices through melt-blending process, at 1 and 2% w/w. The incorporation of both acids promoted the film plasticization, with a notable decrease in the Tg values of starch, probably due to the partial polymer hydrolysis taking place during the melt-blending process. This hydrolysis promoted the plasticity and the stretchability of the films while decreased their stiffness and resistance to break, mainly at the highest ferulic acid concentration. In formulations with C, TGA curves presented a shoulder before the main degradation peak, which may be related to newly formed oligomeric fractions degrading before the full-lengthened polymeric chains. Acid incorporation did not improve the barrier properties of the films but significantly reduced their water solubility, which may represent a significant improvement given that the hydrophilic nature of starch is a main reason limiting its food packaging applications.

The antibacterial activity of the films was tested against *E. coli* and *Listeria innocua* strains. Cinnamic acid formulations were found to be more efficient at inhibiting the bacterial growth, and *Listeria innocua* was more sensitive to both acids than *E. coli*. The antimicrobial effectiveness of the films was also tested in real food matrices, using inoculated chicken breast and fresh-cut melon. Whereas both acids were effective against both bacteria on the chicken breast, their action was more limited on melon. These results corroborated the great potential of phenolic acids and their precursors in antimicrobial food packaging applications.

In **Chapter 2**, ferulic and cinnamic acids were also incorporated into PLA matrices at 1 and 2% w/w by melt blending, and films were obtained by compression moulding. The hydrophobic character of this polymer represents an important advantage for food packaging applications, where water intake from the moist food may compromise the integrity of the material. The functional properties of the films were not greatly affected by the incorporation of the acids, whereas these improved the thermal stability of the polymer. However, the *in vitro* antimicrobial tests with *L. innocua* and *E. coli*, did not show antibacterial action for these films with the same concentration of the active compounds as the starch films, which suggested the lack of effective release of the compounds from the PLA matrix.

Release kinetics of F and C from PLA films was analysed in four food simulants with different polarity: A (10% ethanol in water, simulating aqueous foods), B (3%

acetic acid in water, simulating acid foods), D1 (50% ethanol in water, simulating emulsions or alcoholic foods) and D2 (isooctane, simulating fatty foods). Only in D1 simulant did the film samples release a quantifiable concentration of the acids, with slow release rate and low release ratio (only up to 15 or 30 % of the incorporated amount for ferulic or cinnamic). Therefore, the release of the F and C acids from the PLA matrix was very limited, due to the glassy state of polymer matrix and its hydrophobic nature that limit the polymer swelling and relaxation in contact with aqueous media, such many food matrices. In contrast this relaxation occurred to a great extent in hydrophilic starch films, thus promoting the molecular mobility and diffusion of active compounds through the polymer into aqueous contact media.

On the basis of the inefficient antibacterial effect of PLA films with 1 and 2 % of F or C acids, **Chapter 3** explored the antibacterial effect of PLA films, with 2 and 3 % w/w of these compounds, prepared by different techniques, in comparison with those obtained for starch films with the same concentration of the active compounds, in order to identify possible mechanisms to enhance the antibacterial effect. Casting and thermoprocessing of PLA, as well as its plasticisation with PEG 1000, were used to obtain a different molecular mobility in the polymer matrix. Likewise, three-layer assemblies PLA/active loaded starch/PLA were also tested in order to analyse the potential compound diffusion from the starch sheet through the PLA matrix. The PLA layers were produced by the three different methods previously described. The two PLA outer layers would protect the starch active film from hydration when the three-layer is in contact with aqueous systems. The microbial test with L. *innocua* and *E. coli*, using the different PLA processing methods did not showed significant antibacterial action of the films, despite the presence of the plasticizer and the more plasticized cast films by the residual solvent retention. The three-layer films also showed no antibacterial activity, thus indicating no diffusion of the active compounds from the starch layer through the external PLA sheets.

Considering the possibility that the end chain OH groups of the polymeric chains could be bonded to the reactive carboxylic group of the acids during thermoprocessing, ferulic acid methyl ester, with the same antimicrobial effectiveness as ferulic acid, was incorporated into PLA films at 3% w/w, using casting and thermoprocessing methods, and the films were tested as to their potential antibacterial effect. Nevertheless, no significant antimicrobial activity was detected in any case. Hence, it was concluded that chemical bonding of acids was not the limiting factor that reduced their molecular mobility and their subsequent antimicrobial inefficacy.

In contrast, thermoprocessed starch films with the same concentration of active compounds (2 and 3% w/w) were highly effective. While the 2% formulations inhibited the growth of both strains (up to 6.5 Log UCF for C with *L. innocua*) as compared to the control plate, the 3% formulations exerted a bacteriostatic effect for both bacteria during the incubation period. Therefore, molecular mobility in the starch matrix, which was enhanced by its swelling in contact with the aqueous culture media, promoted the release of active compounds and their antibacterial action, coherently with the compound concentration in the film. Nevertheless, when the active compounds must diffuse through the PLA matrix into the culture medium, no effective antibacterial action was observed, regardless the incorporation method tested. This study indicated that the active compounds have very limited molecular mobility into the PLA matrix and these can not be released to exert their antimicrobial action at the incorporated amount in the films. This reduced molecular mobility was not caused by chemical bonding of the acid molecules to the end-chain hydroxyl group, but this must be attributed to the glassy state of the polymer and the lack of swelling and matrix relaxation in contact with the aqueous microbial sensitive media. Therefore, other strategies should be proved to enhance antimicrobial action of PLA films with ferulic or cinnamic acids.

At this point of the study, increasing the concentration of the acids in the film formulations was considered in order to increase the driving force for mass transfer process, thus promoting the release of acids from the PLA. This was explored in **Chapter 4** with cinnamic acid, which was incorporated into PLA films by both thermoprocessing and casting methods, up to 10% wt. Cast films presented crystallization of cinnamic acid at concentrations higher than 3% w/w, which suggests the incompatibility of cinnamic acid and PLA in the casting ethyl acetate solutions, provoking phase separation and acid crystallization when this become saturated during solvent evaporation and film formation. At concentrations lower than 3 %, crystallisation was probably inhibited by the viscous effects of the PLA solution, or prevalent polymer-compound interactions, that could limit the nucleation and growth of cinnamic acid crystals. Therefore, only 2 and 3% cast films were characterized. In contrast, homogeneous thermoprocessed films were successfully produced with 5 and 10% w/w of C acid and no crystalline structures of cinnamic acid were detected by FESEM observations. Probably, the high viscosity of the PLA-acid melt blend inhibited the crystallisation of the acid, which remained homogenously dispersed in the film matrix. Likewise, more intense interactions between the polymer chains and acid molecules could be promoted at the high temperature of the process.

The effect of the processing method on the functional properties of the PLA films were also evaluated. While thermally processed films were stiff and rigid, cast films presented a remarkable plastic behaviour. This was attributed to a small fraction of casting solvent remaining in the structure and plasticizing the polymer matrix. The elongation of cast films was close to 60%, while TP films failed at around 3%. Cinnamic acid affected positively the barrier capacity of the films both to water vapour and oxygen. Given that the high oxygen permeability of PLA is a drawback for its application in the food packaging industry, this reduction represents an interesting improvement.

Antibacterial *in vitro* tests for these films were carried out with *L. innocua*. Nevertheless, despite the high concentration of cinnamic acid in these formulations, no significant antimicrobial activity was observed once again (Log CFU reduction <2). However, the small growth inhibition values obtained were dependent on the total concentration in the film. This suggests that the corresponding amount of acid near the film surface could be released to culture media, contributing to the bacterial growth inhibition. Thus, superficial incorporation of active compounds on the PLA films could be an appropriate alternative to obtain active films with PLA and phenolic acids, thus avoiding the problem of the hindered migration though the PLA matrix.

On the basis of these results, **Chapter 5** evaluated different strategies to incorporate ferulic acid on the surface of PLA films while comparing their antimicrobial and structural properties to cast films with high concentration of this acid. Cast films were formulated with ferulic acid concentrations, ranging from 2 to 10% w/w, and F crystalline formations into the film matrix could also be observed at every acid concentration. Superficial adsorption of ferulic acid by film immersion in different oversaturated solutions of the acid was studied. The low solubility of the acid in aqueous media limited the adsorption from pure water solutions and 10% v/v ethanol aqueous solutions. In order to increase the concentration of ferulic acid in the solution, 96% v/v ethanol was chosen as solvent. In this medium the acid adsorption was greatly promoted, both in terms of the asymptotic concentration value and the adsorption rate, as deduced from the applied Peleg's model parameters. However, the immersion time in this ethanolic solution had to be reduced due to the relaxation of the polymer matrix and film deformation in this solvent. Spraying the 96% v/v ethanolic solution of ferulic acid on the PLA films surface was also tested as a means to achieve the surface incorporation of the acid while limiting film deformation. With this method, the target superficial concentration of ferulic acid could be reached with lower requirements of solvent and time, hence being potentially more cost efficient than the film immersion to promote ferulic adsorption. In both adsorption and pulverised films, well adhered crystalline formations of ferulic acid were formed on the film surface after the solvent evaporation, whose solubilization rate could compromise their antibacterial action in contact with the microbial sensitive aqueous media.

Electrospinning of PLA-acid solutions was also studied as an alternative method to superficial coating of PLA films. PLA solutions with ferulic acid in different solvent systems based on blends of ethyl acetate (E) with dimethyl sulfoxide (DMSO) or glacial acetic acid (A) were electrospun. The properties of these solutions were studied, and little differences were found in density, viscosity and surface tension, whereas the conductivity of solutions with DMSO was significantly higher than those with acetic acid. This high conductivity positively affected the electrospinning process. FESEM observations of the electrospun mats revealed that the solutions with acetic acid gave rise to powder-like materials with a beaded nanostructure, whereas DMSO based solutions produced thin fibre structured materials, with higher specific surface area.

The antibacterial activity of the all the obtained materials was tested against *L. innocua*. Despite their high acid concentration, the cast films did not exert significant growth inhibition due to the limited release of the acid into the culture medium, as already observed in the previous study with cinnamic acid. In contrast, film samples with superficially incorporated ferulic acid by adsorption and spraying not only inhibited the bacterial growth but showed a bacteriostatic effect. Likewise, a great difference in the antibacterial capacity was observed for electrospun materials depending on their nanostructure. Whereas beaded materials did not inhibit bacterial growth, fibrous mats reduced the bacterial counts up to 3 Log (CFU/mL). This different effect was attributed to the different specific surface area of the electrospun mats. The fibre structure, with higher superficial area, could release a higher amount of the active compound into the culture medium, due to the higher ratio of compound loaded on the fibre surface, whereas the bead structured mats did not possess enough surface loaded ferulic acid to reach the MIC of the bacterium. The results of this chapter proved that superficial incorporation of ferulic acid could overcome the release limitations caused by the low molecular mobility in PLA matrices, hence yielding materials with antimicrobial properties for food packaging applications.

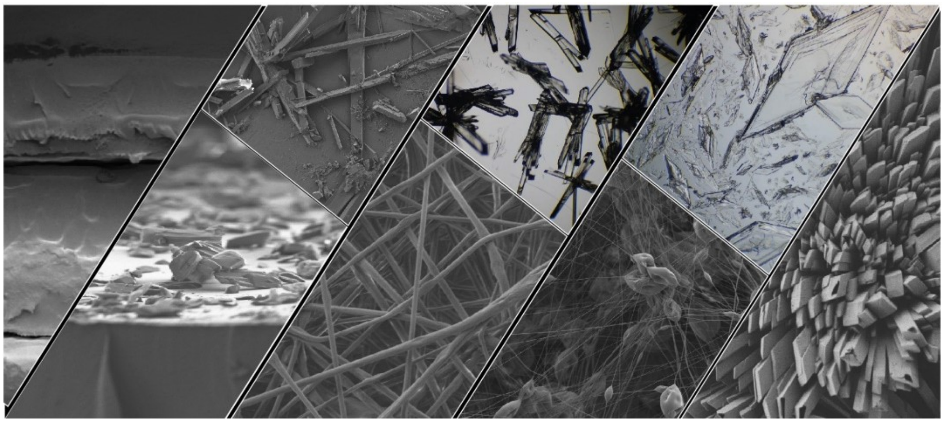
Given the effectiveness of the surface incorporation of the active compounds, this strategy was considered for obtaining food packaging materials with improved functional properties and antimicrobial capacity to extend the food shelf life. To this aim, **Chapter 6** deals with the assembly and characterization of three-layered films consisting of two external PLA layers with an inner starch layer (PSP), with surface incorporation of ferulic and cinnamic acids both by spraying and electrospinning, as stablished in the previous chapter. The thermo-adhesion of the three-layer could be optimised to reach well adhered laminates with homogenous structure, where the starch sheet is protected by the outer PLA layers from the moisture uptake when in contact with moist foods. Given the complementary barrier properties of PLA and starch, PSP films showed improved barrier capacity to both water vapour and oxygen with respect to individual films of starch or PLA, respectively, while exhibited more plastic behaviour than net PLA films. Interlayer migration of water from starch sheet could provoke a partial hydrolyses of PLA layers, and the formed oligomers enhanced the film plasticity and stretchability. Therefore, the obtained laminates could better meet the food packaging requirements than the individual films. These were incorporated with ferulic and cinnamic acid by surface coating using pulverisation or electrospinning under the process conditions optimised in chapter 5.

Electrospun fibres containing cinnamic acid were well adhered directly to the surface of PSP films by electrospinning, whereas an annealing step was required for those with ferulic acid. This consisted of heating the films with deposited fibres in the thermal-hydraulic press with no additional pressure. FESEM observations revealed that the materials maintained their fibre structure after annealing and that fibres loaded with cinnamic acid were more compact despite no annealing process was carried out. FESEM also revealed crystalline formations of ferulic or cinnamic acid, with the characteristic crystalline morphology, which well adhered on the film surface after spraying and solvent evaporation. The surface concentration of the acids in the coated PSP films ranged between 0.40 to 0.50 mg/cm^2, which would allow to reach the MIC of the bacteria if a total released occurs into the culture media.

Antibacterial tests with *E. coli* and *L. innocua* showed that both pulverised and electrospun coated PSP films inhibited the bacterial growth of both strains. As previously observed, cinnamic acid was more effective than ferulic acid against both bacterial, *L. Innocua* being more sensitive to both active compounds. For *E. coli,* the incorporation method did not have a significant effect on the bacterial growth inhibition. However, the most sensitive L. *innocua* exhibited higher growth inhibition for the films coated by electrospinning, which exerted a bacteriostatic effect, whereas the sprayed samples inhibited 4.3 and 2.5 Log (CFU/mL) for cinnamic and ferulic acid, respectively. These results suggested that electrospun PSP films were more effective at releasing the active compounds than those pulverised, which could be attribute to the limited solubility of formed crystals on the film surface. This could be optimised by modifying the spraying conditions in order to promote lower size crystal and faster solubility.

Therefore, laminates PLA/starch/PLA obtained in **Chapter 6**, with surface incorporation of ferulic or cinnamic acids, represent a good alternative to obtain active food packaging materials with the capacity to preserve food quality and extend shelf life. These exhibited improved barrier and functional properties for food packaging applications while the surface incorporation of cinnamic or ferulic acids proved to be an effective strategy to inhibit bacterial growth Nonetheless, further studies on the material stability and specific applications in real foods are necessary to determine the possible industrial application and economic viability of these materials.

V. Conclusions



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Chapter 1

Active starch films with ferulic or cinnamic acid could be obtained by thermo-processing with small modifications of the films' functional properties. Incorporation did not notably affect the barrier properties of the starch films, although these became less water soluble, more extensible, and less resistant to break. Both cinnamic and ferulic acids conferred antibacterial activity to the starch films against *E. coli* and *L. innocua,* which was probed in culture medium and food systems (chicken breast and fresh cut melon). Films with cinnamic acid were more effective than with ferulic acid and both were more active against *L. Innocua* than against *E. coli*. The antibacterial activity of the films was affected by the kind of acid and strain and the food substrate. The highest effectiveness was obtained for films with cinnamic acid, against *L. innocua,* in chicken breast and fresh-cut melon.

Therefore, although highly hydrophilic starch films had limitations for wet food packaging, starch layers with 2 % ferulic or cinnamic acid could be used as active layers for food contact in multilayer assemblies, combined with other hydrophobic polymer sheets of complementary properties, to obtain active laminates that could provide remarkable antimicrobial capacity and, in particular, antilisterial activity.

Chapter 2

The total recovery of ferulic and cinnamic acids in thermo-processed PLA films was relatively high (84 - 98%), which suggests that these compounds can be incorporated by using the usual thermoplastic process of the plastic industry. These acids provoked a weakening effect in the polymer slightly reducing the stiffness and resistance to break, mainly with 2 % ferulic acid, although this was not notably reflected in the PLA glass transition. However, a 2 % content of both acids improved the water vapour and oxygen barrier capacity of the films. Likewise, both acids notably improved the PLA thermal stability.

The films with ferulic and cinnamic acid at 1 or 2 % did not show a significant antimicrobial action against *E. coli* and *L. innocua*, which was attributed to the scarce release of these compounds in the culture medium during the *in vitro* test. In fact, no quantitative release was detected into aqueous or non-polar media, but only into the D1 simulant (50% ethanol in water), where a low release rate and limited release ratio were also observed, mainly in the case of ferulic acid.

Further research is needed in order to favour the release of ferulic and cinnamic acids so as to improve their antimicrobial activity. Given the great potential of PLA-ferulic and cinnamic acid combinations for packaging purposes, further antimicrobial and antioxidant studies into food matrices are required to test the capacity of these films in real systems from where compound migration (e.g., fat) could favour the relaxation of the polymer matrix, facilitating the delivery of active compounds from the film.

Chapter 3

Starch monolayers loaded with cinnamic and ferulic acids showed notable growth inhibition capacity against *E. coli* and *L. innocua,* in line with the effective release of active compounds from the starch matrix. In contrast, PLA monolayers containing the highest proportion of these acids, or ferulic acid methyl ester, did not exhibit antimicrobial capacity regardless of the film processing conditions (casting or thermo-processing with and without plasticiser). Multilayer systems, where a layer of active starch was sandwiched between two PLA layers obtained by different methods, also exerted very little inhibition. The reduced molecular mobility in the polyester matrix is the limiting cause affecting the release of acids and their potential antimicrobial activity. Further research on strategies to improve the release of antimicrobial acids from PLA into the food systems could provide a viable and sustainable active material suitable for food packaging applications.

Chapter 4

Over 3% w/w cinnamic acid could not be homogeneously incorporated in cast PLA films, whereas 10% acid was successfully integrated by melt blending with PLA. Thermo-processed PLA films were significantly stiffer and had greater resistance to break than the cast films, whereas the latter exhibited a high degree of plastic deformability, in part due to the solvent retention in the cast films. The incorporation of phenolic acids reduced the film’s stiffness and resistance to break in both the TP and cast films while it improved the water vapour and oxygen barrier capacity of the films. The TP films were more transparent than the cast films and turned slightly brownish after the addition of cinnamic acid.

Despite the high concentration of the active compound in the films, no significant antibacterial activity was observed against *Listeria innocua* in the *in vitro* assays, although a positive effect was observed when the acid concentration increased. This suggests that only the acid molecules near the film surface were released into the medium to exert their antibacterial action. Given the limited release of the acid from both the cast and thermo-processed PLA matrices, future research should focus on promoting it through the surface application of the active compound, such as compound solution spraying or electrospinning, which could yield active materials that are more efficient at inhibiting the bacterial growth in aqueous media, such as those present in many foods, thus helping to extend the shelf life of sensitive foodstuffs.

Chapter 5

Four different strategies were tested for the purposes of developing antimicrobial materials based on polylactic acid with ferulic acid. The films obtained by casting with different amounts of the active compound presented crystalline formations of ferulic acid, whose release from the polymeric matrix was greatly limited, as deduced from the lack of any antibacterial effect, despite the fact that these contained a high enough amount of active to exceed the MIC of the bacteria. Casting is, hence, not considered as a viable alternative to obtain active PLA packaging materials with ferulic acid. A good yield of ferulic acid adsorption from an ethanolic oversaturated solution on the surface of PLA films was achieved after a relatively short immersion time, whereas the pulverisation of the same solution was a more effective method with a higher amount of ferulic acid deposited on the PLA surface. The films with ferulic acid incorporated via adsorption and pulverisation inhibited the growth of *L. innocua*, exerting a bacteriostatic effect in the concentrations applied. The structure of the electrospun materials, which, in turn, was determined by the solvent system, affected their antimicrobial efficacy. When DMSO was used, fibres were obtained, which favoured the antimicrobial power, due to their higher specific surface area. In contrast, electrospun materials obtained from acetic acid solvent systems exhibited a bead-based structure, which led to no significant antibacterial activity. The lower specific surface area of the beads could imply a more limited availability of ferulic acid near the surface for an efficient release to achieve the MIC of bacteria in the culture media. On the basis of these results, the surface application of ferulic acid by adsorption or the pulverisation of F solutions, as well as electrospun depositions of PLA-F mats using DMSO solvent systems, represent viable strategies to obtain PLA food packaging materials with strong antibacterial activity. More research is required in order to implement these processes in complex food packaging materials, such as multilayer assemblies, and study their antimicrobial impact in both model systems and specific food matrices.

Chapter 6

Three-layered PSP assemblies exhibited an improved mechanical and barrier capacity with respect to the PLA and starch monolayer films with adequate functional properties to meet food packaging requirements. PLA provides the laminates with mechanical resistance and water vapour barrier capacity while the internal starch layer was protected from the moisture sensitivity by the external PLA sheets, providing the laminates with oxygen barrier capacity. The optical properties of the multilayer were similar to those of the starch monolayers. The incorporation of ferulic and cinnamic acids onto the PSP surface by the electrospinning of PLA-acid solutions in EtAc-DMSO (6:4) were effective at producing fibre mats encapsulating the acids with high specific surface area and the ability to release the active compounds so as to exert antimicrobial action. The pulverisation of concentrated ethanolic solutions of ferulic or cinnamic acid onto the PSP surface produced crystalline structures of the compounds that were firmly attached to the film surface with potential antimicrobial activity. The antimicrobial tests of surface-loaded PSP laminates with F or C, by either electrospinning or pulverisation, exhibited effective growth inhibition of *E. coli* and *L. innocua*, the latter being more sensitive to both active compounds and cinnamic acid showing greater antibacterial activity. For the more sensitive bacteria (*L. innocua*), significant differences in the growth inhibition were observed for the electrospun and pulverised PSP laminates. The electrospun films were more effective than the pulverised, thus indicating their greater ability to release the active compounds into the culture medium. However, pulverisation conditions could be optimised in order to produce more soluble crystalline formations with smaller particle size and easy release. Therefore, laminates of PLA/starch/PLA with the surface incorporation of ferulic or cinnamic acid represent a good alternative means of obtaining active food packaging materials with the capacity to preserve and enhance food quality and shelf life. Nonetheless, further studies into their stability and specific applications in real foods of these materials are necessary in order to determine their possible industrial application and economic viability.

General conclusion

Thermoprocessed starch films were good carriers for ferulic or cinnamic acids, exhibiting remarkable antimicrobial activity at low concentrations. This was not the case of PLA, where the reduced molecular mobility and its hydrophobic nature prevented the release of these active compounds from the polymer matrix. An efficient release of the actives from the PLA matrix was only obtained by superficial incorporation of the active compounds by different techniques.

Laminates PLA/starch/PLA with superficially incorporated ferulic or cinnamic acid, by solution pulverisation or electrospinning, exhibited good mechanical and barrier properties for food packaging applications and notable growth inhibition capacity of both Gram-positive and Gram-negative bacteria. Further studies on the capacity of these films to extend shelf-life of specific food products are required in order to assess their true potential as active food packaging materials.

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