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

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A B S T R A C T

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

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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-min melt-blending process takes place at 50 RPM and 130 °C for starch blends and 160 °C 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 °C 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 °C. The PLA pellets were preheated for 4 min at 200 °C before a single compression step was applied at 10 MPa for 4 min, followed by the 3-min cooling step until 70 °C.

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 °C before a single step compression at 25 MPa took place at the same temperature; afterwards, the samples were cooled to 70 °C 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

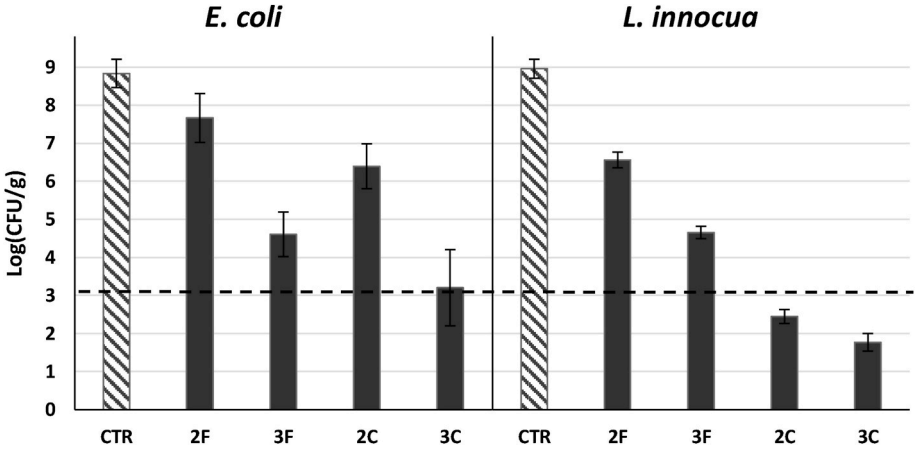

Fig. 1. *E. coli* and *L. innocua* growth after 6 days of incubation at 10 °C for samples in contact with thermally processed starch monolayers with 2 or 3% ferulic (F) acid or cinnamic (C) acid and uncoated control growth sample (CTR, striped bar). The dotted line represents initial inoculation.

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[a]	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[b]	F	2	0.83 ± 0.2^{efg}	0.2 ± 0.12^{ab}
	TP[b]	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}

N.D.: Not determined.
[a] TP: Thermo-processing, TPP: Plasticised & thermo-processing.
[b] Data from a previous study (Ordoñez et al., 2022).

(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 °C 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 °C, 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. Fig. 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.

3.2. PLA monolayers

TP PLA films loaded with cinnamic or ferulic acid at 1 or 2% were

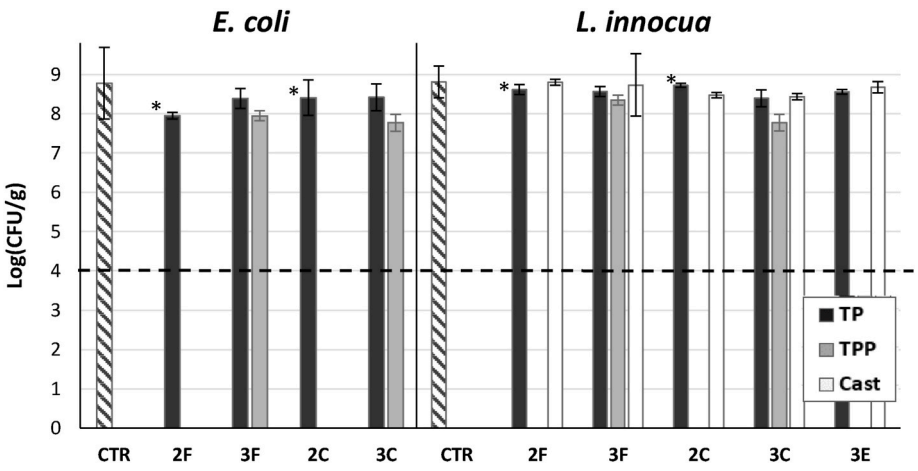

Fig. 2. *E. coli* and *L. innocua* counts after 6 days of incubation at 10 °C for samples in contact with PLA monolayers with 2 or 3% ferulic (F) acid cinnamic (C) acid or ferulic acid methyl €er (E); obtained by thermal processing (TP, black bars), plasticising and thermal processing (TPP, grey bars) 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.

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.

Fig. 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 (10 °C) 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 (37 °C) 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.

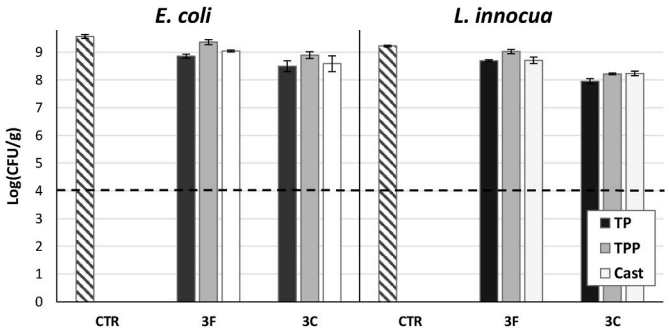

Fig. 3. *E. coli* and *L. innocua* counts after 6 days of incubation at 10 °C for samples in contact with the different three-layer films (internal starch layers loaded with 2 or 3% ferulic (F) acid or cinnamic (C) acid). The different PLA contact layers were thermo-processed (TP, black bars), plasticised 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.

Fig. 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). Fig. 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.

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 thermoprocessing 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.

Conflict of interest and authorship confirmation

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 (Release problems of phenolic acids from PLA films to obtain active food packaging materials.) submitted to the journal Food Control.

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.

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.

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