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Additional Information

1 Effect of ferulic and cinnamic acids on the functional and antimicrobial properties in thermo- 2 processed PLA films

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7 Abstract

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

21 **Keywords:** Polylactic acid, ferulic acid, cinnamic acid, release kinetics, antimicrobial packaging, active
22 food packaging.

23 1. Introduction

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

41 The potential of PLA for use in antimicrobial packaging applications has been studied by different authors
42 and several substances, such as organic acids, bacteriocins, plant extracts, essential oils, enzymes,
43 chelating agents or metals, have been incorporated into the PLA matrices to provide the films with
44 antimicrobial activity (Jamshidian et al., 2010; Scaffaro et al., 2018; Tawakkal et al., 2014). The use of
45 natural phenolic compounds or phenolic-rich extracts incorporated into biodegradable polymeric matrices
46 has been widely studied, with promising results for active food packaging applications (Andrade et al. 2021
47 and 2022; Hernández-García et al. 2022; Velásquez et al., 2021). Contrary to the essential oils widely
48 studied as natural active components, phenolic acids do not impact severely the food's organoleptic
49 properties (Miyague et al., 2015), while presenting good antioxidant and antimicrobial properties (Merkel
50 et al., 2010). Cinnamic acid (C) is a major constituent of the oriental Ayurvedic plant *Cinnamomum cassia*,
51 being the precursor of hydroxycinnamic acids, the prevalent family of phenolic acids. It can be extracted
52 from a wide range of vegetable sources (Rashmi & Negi, 2020), and exhibits antimicrobial properties, as
53 demonstrated in different studies (Miyague et al., 2015; Sánchez-Maldonado et al., 2011). It is especially
54 active against *Listeria*, which constitutes a dangerous food pathogen due to its persistence at cold
55 temperatures (Tasara & Stephan, 2006). This compound and its derivatives have been reported to
56 possess various biological properties of which its antioxidant activity is a prime one (Patra et al., 2012),
57 showing specific interactions in reducing the oxidative stress in different vegetables (Sun et al., 2012),

58 interfering the mechanisms involved in the protection against the intracellular reactive oxygen species
59 (ROS) that produce oxidative cellular damage.

60 Ferulic Acid (F) is a hydroxycinnamic acid, ubiquitously present in the plant cell wall (Sharma et al., 2020),
61 with proven antimicrobial (Pernin et al., 2019; Shi et al., 2016) and antioxidant properties (Itagaki et al.,
62 2009; Zduńska et al., 2018). It has low toxicity and possesses many physiological functions (anti-
63 inflammatory, antioxidant, antimicrobial activity, anticancer, and antidiabetic effect), is widely used in the
64 pharmaceutical, food, and cosmetics industries. Ferulic acid is a free radical scavenger, but also an
65 inhibitor of enzymes that catalyse free radical generation and an enhancer of scavenger enzyme activity
66 (Zduńska et al., 2018).

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

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

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

102 2. Materials and methods

103 2.1 Materials

104 Amorphous PLA 4060D with 106 kDa average molecular weight (Nature Works, MN, USA), cinnamic and
105 ferulic acids (Sigma-Aldrich, Saint Louis, USA) were used for film production. Magnesium nitrate and
106 phosphorus pentoxide, supplied by Panreac Química (Barcelona, Spain), were used to equilibrate the
107 sample relative humidity (RH) at 53% and 0%, respectively. Tryptone soy broth, tryptone soy agar,
108 phosphate-buffered saline and peptone water for microbial tests were purchased from Scharlab
109 (Barcelona, Spain). Strains of *Listeria innocua* (CECT 910) and *Escherichia coli* (CECT 101) were supplied
110 by the Spanish Type Collection (CECT, University of Valencia, Spain). Scharlab (Barcelona, Spain)
111 supplied the tryptic Soy Agar, buffer peptone water and selective media: violet-red bile agar (VRBA) for *E.*
112 *coli* and palcam agar base (PAB) enriched with palcam selective supplement for *Listeria*.

113 Spectrophotometric grade solvents: methanol, ethanol, acetic acid, and isooctane were all purchased from
114 Panreac Química (Barcelona, Spain).

115

116 2.2 Film preparation

117 PLA pellets were first dried at 60 °C overnight to prevent hydrolysis during thermal processing. The
118 components were melt blended in an internal mixer (Haake PolyLab QC, Thermo Fisher Scientific,
119 Germany) at 150 °C and 50 rpm for 10 min. Cinnamic (C) or ferulic (F) acid was added at 1 or 2 g acid /
120 100 g dry film. **This concentration was established with the aim of using the minimal amount as possible,
121 but ensuring an antibacterial effect, according to previous studies (Ordóñez et al., 2021).** The melts
122 obtained were cold ground using an M20 IKA mill (Staufen, Germany). Films of 20 cm in diameter were
123 obtained by compressing 4 g of powder, using a hydraulic heating press (LP20, Labtech engineering,
124 Thailand). Samples were preheated at 200 °C (Muller et al., 2017) for 4 min and compressed at 10 MPa
125 for 4 min. Finally, a 3 min cooling step to 70 °C was applied. The films obtained were conditioned at 53%
126 RH and 25 °C before characterization. The different samples were coded as PLA (PLA films without acids),
127 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).

128 2.3 Characterization of the films

129 2.3.1 Microstructure observation

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

134

135 2.3.2 Thermal analyses

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

145 2.3.3 Content of active compounds

146 The final content of ferulic and cinnamic acids in the films was determined through methanol extraction
147 and spectrophotometric quantification. Film samples (about 100 mg) preconditioned at 0% RH were
148 immersed in 10 ml methanol and gently stirred for 48 hours at room temperature. Methanol extracts were
149 filtered and properly diluted, and the absorbance measurements were taken using a UV-visible
150 spectrophotometer (Thermoscientific Evolution 201, USA) at the maximum absorbance wavelengths, i.e.
151 320 nm for F and 270 nm for C. The respective methanol extract of the PLA samples was used as blank.
152 The absorbance measurements were transformed into acid concentration using the previously determined
153 calibration curves. The results were presented as the mass percentage of acid released referred to the
154 amount initially incorporated into the film. All the measurements were taken in triplicate.

155 2.3.4 Mechanical, barrier and optical properties

156 A texture analyser TA-XT plus (Stable Micro Systems, Haslemere, England) was used to determine the
157 tensile properties of each film formulation, following the ASTM D882 method (American Society for Testing
158 Materials, 2002). Film strips (25 mm x 100 mm, eight replicates per formulation) were cut, and a hand-held
159 electronic digital micrometer (Comecta S.A., Barcelona, Spain) was used to measure film thickness to the

160 closest 0.001 mm, at six random points per strip. The samples were mounted on tensile grips (50 mm
161 separation) and stretched at 50 mm/min. Tensile strength at break (TS), elastic modulus (EM) and
162 elongation at break (%E) were obtained from the stress-strain curves.

163 Water vapour permeability (WVP) was measured following the ASTM E96-95 method (American Society
164 for Testing Materials, 1995). Payne permeability cups (Elcometer SPRL, Hermelle/s Argenteau, Belgium)
165 of 3.5 cm in diameter were filled with 5 ml of distilled water and mounted with circular film samples whose
166 thickness had been previously measured at six random positions. The cups were placed into desiccators
167 containing MgNO₃ oversaturated solution and kept at 25 °C. Cups were weighed periodically (1.5 h-24 h)
168 using an analytical balance (±0.00001 g). The rate of weight loss over time was used to calculate WVP
169 once the stationary state had been reached according to E96-95 method. The measurements were taken
170 in triplicate for each film formulation.

171 The oxygen permeability (OP) was determined by following the ASTM D3985-05 method (American
172 Society for Testing Materials, 2010) using Ox-Tran 1/50 equipment (Mocon, Minneapolis, USA). The film
173 test area was 50 cm² and measurements were taken at 25 °C and 53% RH. OP was calculated by dividing
174 the oxygen transmission rate by the oxygen partial pressure gradient on both sides of the film and
175 multiplying by the average film thickness, which had been previously measured at six different positions on
176 the film sample. Two replicates per formulation were obtained.

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

184 **2.4 Antibacterial effectiveness of the films**

185 Tryptic Soy Agar (TSA) was used as culture medium for antimicrobial tests, following the methodology
186 described in previous studies (Requena et al., 2019a; Tampau et al., 2018). Round film samples (55 mm
187 diameter) were sterilised on both faces using UV-light in a laminar flow cabinet (Bio II advance, Telstar
188 Spain). 10 mL of TSA were poured into 55 mm petri dishes and inoculated with 100 µL of *E. coli* or *L.*
189 *innocua* bacterial suspension (10⁶ CFU/mL) on the plate surface using a L-form rod to uniformly spread the
190 inoculum. Then, the plates were covered with the film samples, and non-covered inoculated plates were
191 included (control). The Petri dishes were sealed with Parafilm[™] and incubated for 6 days at 10 °C. After
192 incubation, samples were homogenised for 3 min in buffer peptone water, employing a Masticator paddle
193 blender (IUL Instruments, Barcelona, Spain). Serial dilutions were plated and covered with selective
194 media: violet red vile agar (VRBA) for *E. coli* and palcam agar base (PAB) enriched with palcam selective
195 supplement for *Listeria* (Requena et al., 2019a; Tampau et al., 2018). After 48 h incubation at 37 °C, the
196 colonies were counted. Each film formulation and control were tested in duplicate. Microbial counts were
197 also performed in duplicate for each sample.

198 **2.5 Release kinetics of ferulic and cinnamic acids from the polymeric matrix**

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

209 Peleg's equation (**Equation 1**) was used to model the release kinetics. The concentration at equilibrium
210 (M_∞), partition coefficient (M_∞/M₀) and release rate were calculated (Peleg, 1988).

$$211 \quad \frac{t}{M_t} = k_1 + k_2 t \quad (1)$$

212 Where:
213 M_t : mass of active compound released at time t
214 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
215 release value at equilibrium ($M_\infty = 1/k_2$).

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

$$219 \frac{M_t}{M_\infty} = kt^n \quad (2)$$

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

226 2.6 Statistical analysis

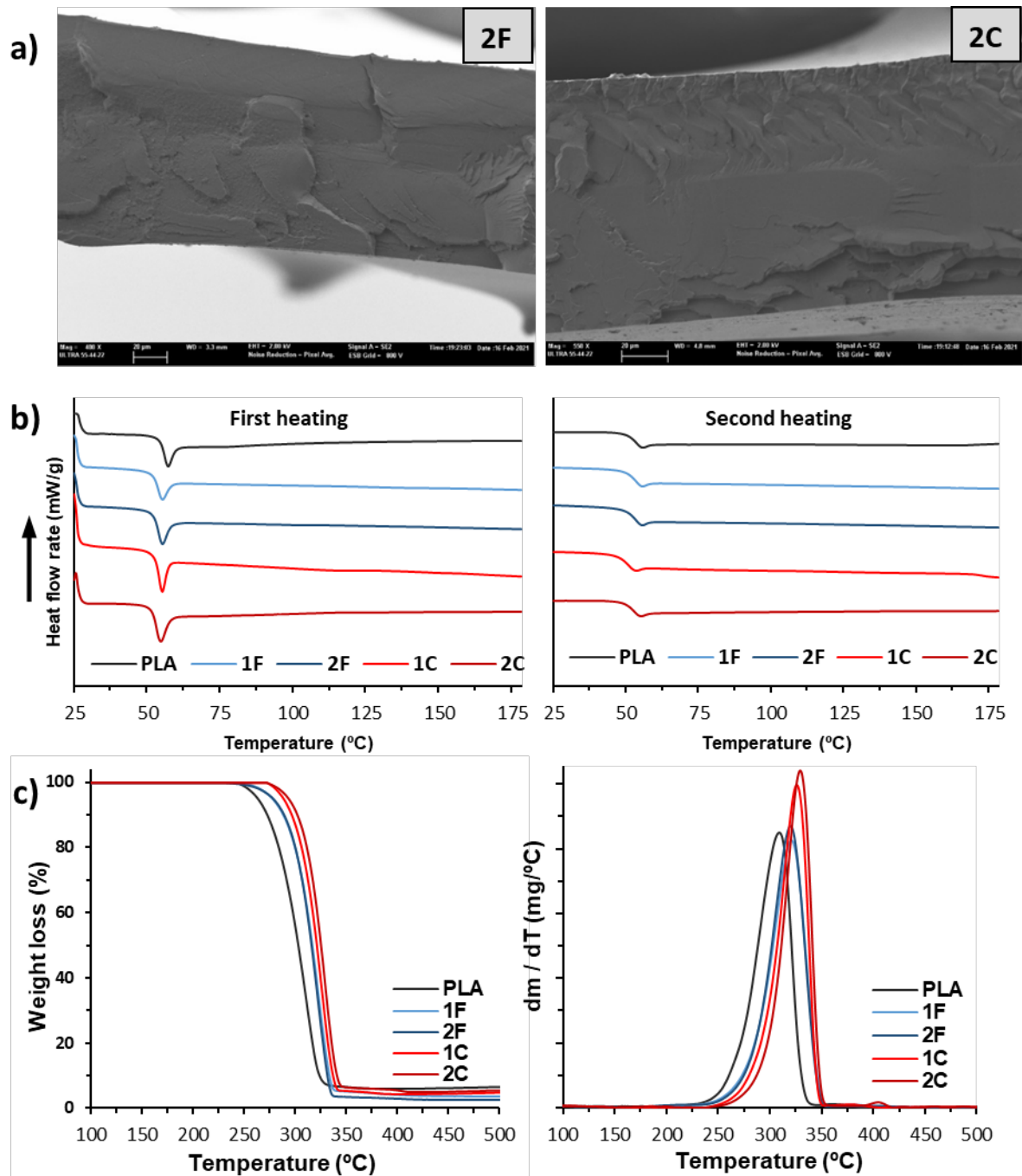
227 Statistical data analysis was performed through an analysis of variance (ANOVA) and regression analyses
228 using Statgraphics Centurion XVII software. Fisher's least significant difference was used at a 95%
229 confidence level.

230 3. Results and discussion

231 Homogeneous, transparent and malleable films were obtained for all the formulations where no phase
232 separation was observed in the microscopic analyses. No segregated acid particles were detected in films
233 with ferulic or cinnamic acid, as revealed by the FESEM micrographs and homogenous microstructure was
234 observed in every case. **Figure 1a** shows the FESEM micrographs of PLA films with 2 % ferulic and
235 cinnamic acids where the good integration of the acids in the polymer matrix during the melt blending
236 process can be observed, even at the highest acid concentration used.

237 3.1 Thermal behaviour of the films.

238 **Figure 1b** shows the first and second heating thermograms obtained for the different samples. In every
239 case, the lack of crystalline regions in the amorphous material was confirmed by the absence of the typical
240 melting endotherms of semi-crystalline PLA at about 175°C (Muller et al., 2017). The glass transition and
241 subsequent relaxation endotherm were the only thermal events observed. **Table 1** shows the glass
242 transition temperatures for both the first and second heating steps in the DSC analyses (T_{g1} and T_{g2}).
243 These T_g values were in the range of those reported in previous studies for thermally processed PLA
244 (Badia et al., 2012; Collazo-Bigliardi et al., 2019; Gómez-Contreras et al., 2021). This temperature was
245 only slightly reduced by the incorporation of the acids, as reported in previous studies for PLA (Dintcheva
246 et al., 2017; Jin et al., 2002; Quynh et al., 2007; S. Yang et al., 2008), but no significant plasticization effect
247 was observed in the second heating step for the incorporated amounts of acids. The thermograms (**Figure**
248 **1a**) also showed the typical relaxation endotherms just after the glass transition in the first heating scan, as
249 previously observed by other authors (Cai et al., 1996; Muller et al., 2016). The enthalpy of relaxation is
250 quantitatively related with the physical aging of the material and the slight differences between samples, as
251 well as between the glass transition temperatures from the two heating scans, could be attributed to the
252 thermal history of the material.



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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 (left) and DTGA (right) curves. PLA: film without acids; 1F and 2F: films with 1 or 2 % ferulic acid; 1C and 2C: films with 1 or 2% cinnamic acid.

258
259
260

Table 1 Glass transition temperatures obtained from the first (T_{g1}) and second heating (T_{g2}) 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.

Formulation	T_{g1} (°C)	T_{g2} (°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

261

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

262 As concerns the thermal degradation behaviour, the TGA curves of the different films are shown in **Figure**
263 **1c**. Likewise, the peak and onset temperatures of thermal degradation are also shown in **Table 1**. The
264 thermal degradation of PLA is based on a hydroxyl end initiated ester exchange process and chain
265 homolysis (Zou et al., 2009). Reactive end groups and impurities have been reported to enhance the
266 thermal degradation of PLA (Signori et al., 2008). In the TGA curves of the films (**Figure 1c**), the protective
267 effect of both ferulic and cinnamic acids on the thermal degradation of PLA can be observed. The onset
268 and peak degradation temperatures (**Table 1**) significantly increased when acid was added. The thermal
269 degradation curve of PLA was similar to that reported by Zou et al. (2009) at the same heating rate (10
270 °C/min) and N₂ flow, whereas F addition caused an increase in thermal degradation of about 10°C and the
271 incorporation of C resulted in an increase in T_{max} of as much as 20°C in the case of the 2C formulation.
272 Degradation temperatures are affected by the structural properties of the matrix, such as molecular weight
273 or crystallinity. However, the DSC analysis did not reveal notable structural changes reflected in the phase
274 transitions of the materials. Therefore, the thermal protective effect of ferulic and cinnamic acids could be
275 related to their antioxidant activity. During the thermal degradation process of PLA, radical reactions take
276 place above 250°C (Nicolae et al., 2008). The radical scavenging capacity of phenolics could partially
277 inhibit degradative reactions at high temperatures, thus delaying polymer degradation. At high
278 temperatures, the thermal degradation of the acids initially implies their decarboxylation (Cheng et al.,
279 2014), but their decarboxylated forms also exhibit antioxidant capacity (Belvedere & Tursi, 1981; Cheng et
280 al., 2014; Tung et al., 2012). The decarboxylation of cinnamic acid produces styrene that has been used to
281 produce films, (styrene-butadiene-styrene (SBS) block copolymer) of potential use as oxygen-scavenging
282 polymers for barrier applications (Tung et al., 2012). **On the other hand, Tran**

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

290

291 **3.2 Physical properties of the films and final content of active compounds**

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

308

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

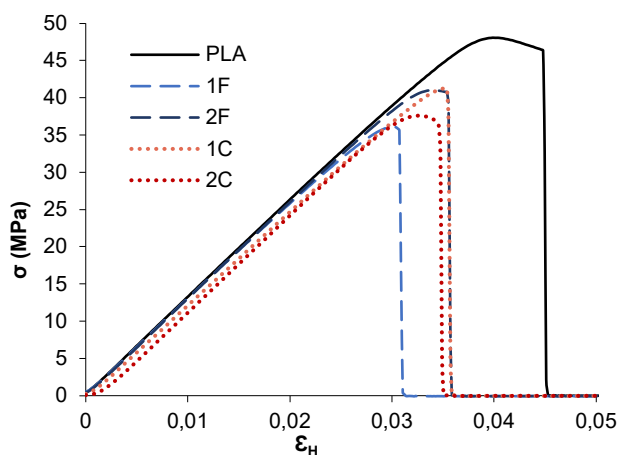
	PLA	1F	2F	1C	2C
AC (wt.%)	-	92.0±3.2 ^b	88.7±3.4 ^{ab}	97.6±2.3 ^c	84.6±2.4 ^a
t (µm)	207±7 ^{ab}	217±10 ^c	209±6 ^{bc}	201±5 ^{ab}	198±5 ^a
TS (MPa)	44±5 ^a	35±8 ^b	40±4 ^a	42±2 ^a	38±4 ^{ab}
EM (MPa)	1310±80 ^a	1260±40 ^b	1240±60 ^b	1250±40 ^b	1270±14 ^b
E (%)	4.3±0.5 ^a	2.8±0.7 ^b	3.5±0.2 ^{ab}	3.6±0.2 ^{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

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

316 3.3 Mechanical, barrier and optical properties

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

331



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

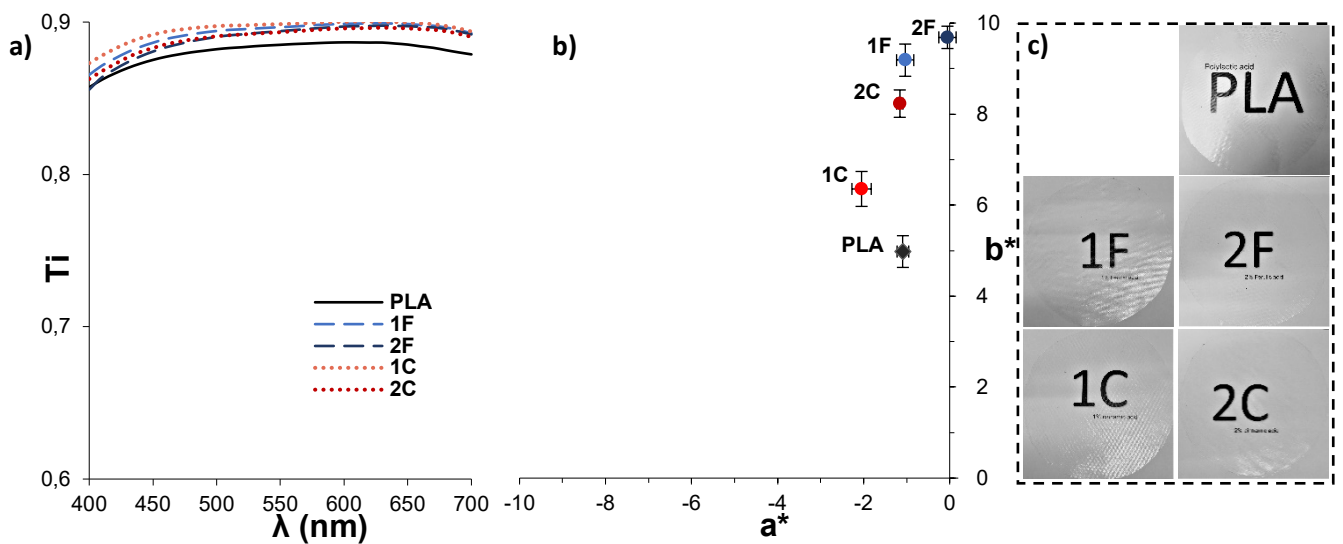
334

335 **Table 2** shows the values of the WVP and OP of the different films. Little variations in the barrier
 336 properties were caused by the incorporation of the acids. Statistically significant reductions in WVP were
 337 found for films 2F, 1C and 2C, with formulation 2F exhibiting the lowest value of WVP. This effect could be

338 attributed to the previously mentioned bonding of -OH groups at the end of PLA chains with the acid, thus
 339 reducing the water affinity of the matrix even more. This behaviour was also observed in other more
 340 hydrophilic polymer matrices, such as sodium caseinate, where the WVP was reduced in relation to the
 341 amount of ferulic acid incorporated (Fabra et al., 2011). On the other hand, acid incorporation did not have
 342 a clear effect on the oxygen permeability, whose values were only slightly reduced for the highest ratio of
 343 the acids. This reduction could be related to the oxygen scavenging capacity of the added acids (Bonilla et
 344 al., 2013).

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

361



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

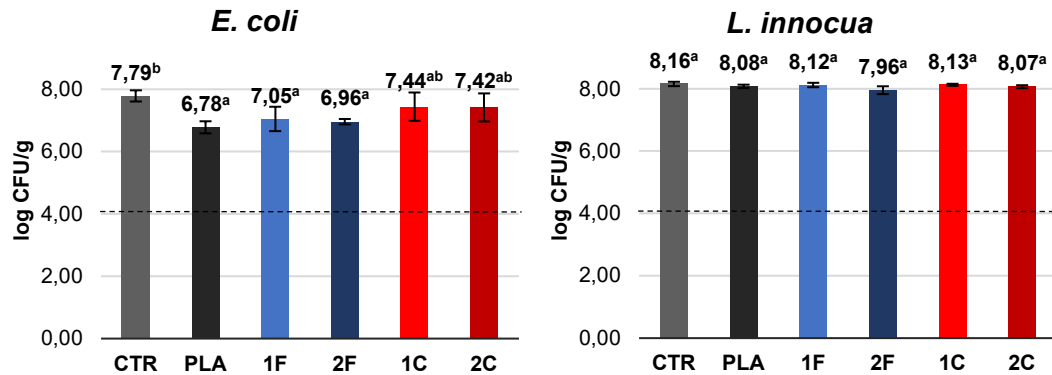
365

366 3.4 Antibacterial effectiveness of the films

367 **Figure 4** shows the results of the *in vitro* assays carried out to determine antibacterial effectiveness
 368 against *E. coli* and *L. innocua* of the films. No antibacterial activity against *L. innocua* was observed for any
 369 films since no significant differences in the viable counts were found for film formulations or the uncovered
 370 control plates. A mild inhibitory effect was found against *E. coli* for some films. Nevertheless, none of the
 371 film formulations caused a 2 log CFU reduction, which is the usual reduction to be considered an effective
 372 antibacterial material (Requena et al., 2019a). Lower reductions are in the range of the natural variability in
 373 these kinds of tests. However, the amount of acid incorporated in the films was enough to reach the MIC
 374 values of both compounds (around 0.7 mg/mL) in the plates for the bacteria tested, if a complete release
 375 of the active occurs in the volume of the culture medium of the plate. This indicates that the active
 376 compounds were not completely released from the film matrix and the released amount was not enough to

377 cause any antimicrobial effect. In a previous study, remarkable growth inhibition of both bacteria, mainly
 378 *Listeria*, was observed when using thermoplastic starch as the carrier matrix of these acids in the same
 379 ratio (2% wt.%), (Ordoñez et al., 2021). However, the hydrophobic nature of the PLA matrix and the lack of
 380 polymer relaxation when in contact with the culture media, greatly limits the effective release of active
 381 compounds, which is necessary to reach MIC values of the bacteria in the culture plate. These findings
 382 were corroborated through the study of the release kinetics of compounds into food simulants of differing
 383 polarities, as discussed in the next section.

384

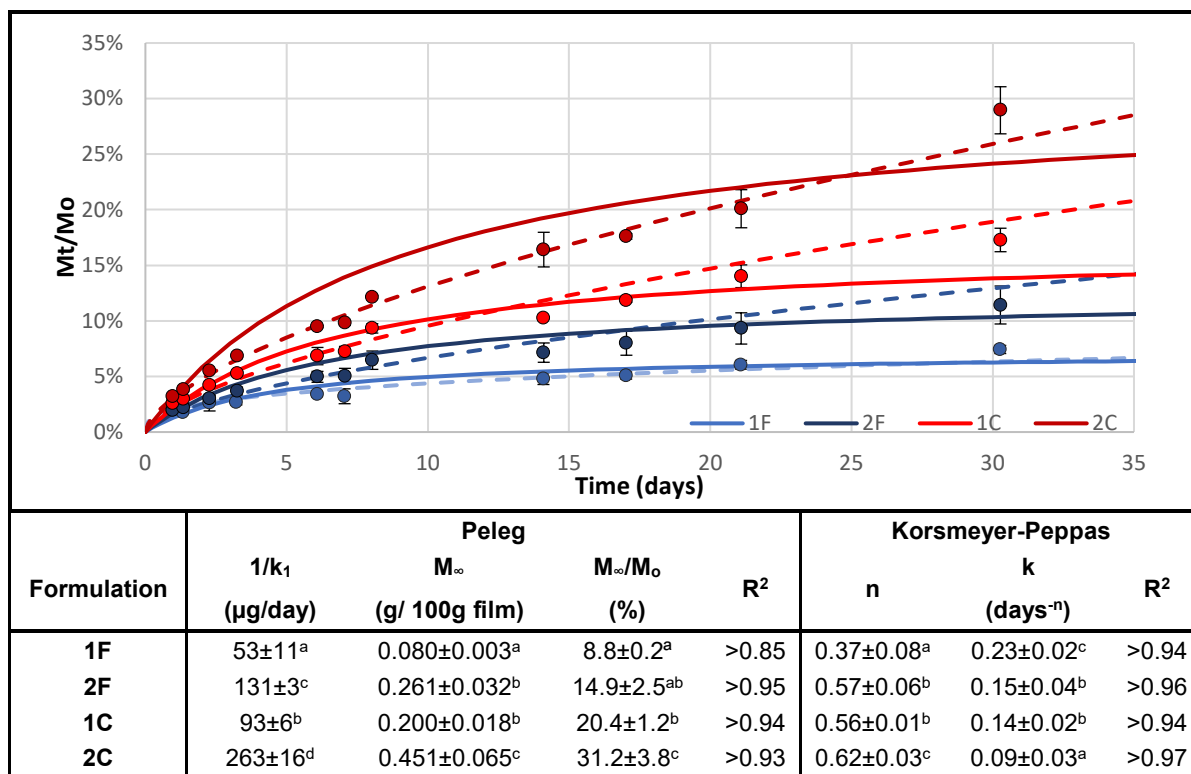


385 **Figure 4** Microbial counts for *E. coli* and *L. innocua* obtained after 6 days of incubation at 10°C in TSA
 386 medium. Uncovered control sample (CTR), and covered samples with PLA: film without acids; 1F and 2F:
 387 films with 1 or 2 % ferulic acid; 1C and 2C: films with 1 or 2% cinnamic acid.

388 3.5 Release kinetics of cinnamic and ferulic acids from the polymeric matrix

389 The concentration of **cinnamic and ferulic** acids in the simulant solutions in which the film samples were
 390 immersed was monitored for 30 days. Whereas no detectable acid release occurred in either the polar
 391 food simulants (A and B) or the non-polar simulant (D2), a significant release was detected in the D1
 392 simulant, which indicates that the acids could only be released from the polymer matrix into less polar food
 393 substrates, such as alcoholic foods or oil-in-water emulsions, as simulated by the D1 solvent. In the D1
 394 simulant with 50% ethanol, a relaxation of the PLA matrix brought about by ethanol penetration into the
 395 polymer matrix would favour the compound release to some extent. Other authors also found an increase
 396 in the compound release from PLA matrices when the simulant had a higher ratio of ethanol (Jamshidian
 397 et al., 2012). **The ethanol migration into the PLA matrix provokes the polymer matrix relaxation which
 398 enhances molecular mobility and compound diffusion, thus promoting its release in the simulant. They also
 399 report a partial degradation of the polymer matrix in contact with methanol, which also favoured the
 400 compound release. Yang et al. (2019) Yang et al. (2019) also observed a higher release of active
 401 compounds from PLA:PBSA (9:1) films when the ethanol ratio rose in the food simulant. In the absence of
 402 matrix relaxation, the glassy state of the polymer at the release temperature ($T_g > 20^\circ\text{C}$) greatly limited
 403 molecular mobility and diffusion-controlled mass transfer process. Therefore, film plasticisation could be a
 404 possible strategy to promote the release of active compounds, although adequate plasticizers should be
 405 used to fit the film functional properties (mechanical and barrier properties) to the food packaging
 406 requirements.**

407 The experimental values of M_t/M_0 (amount of compound released with respect to the initial amount in the
 408 films), as well as the fitted Peleg model (curves) are shown in **Figure 5**. The kinetic parameters obtained
 409 from the fitting of the Peleg and Korsmeyer-Pepas models are also shown. The initial release rate ($1/k_1$)
 410 and final release ratio M_∞/M_0 increased when the acid concentration rose in the film for both acids, in
 411 agreement with the higher mass transfer driving force. However, significant differences were observed in
 412 the delivery behaviour of both acids. The release of cinnamic acid from the PLA matrix into the food
 413 simulant took place more extensively and at a higher rate than that of ferulic acid. This is probably linked to
 414 the structural differences of both molecules. Whereas the molecule of ferulic acid has one hydroxyl group
 415 in para position with respect to the carboxyl, cinnamic acid does not have hydroxyls in the phenolic ring.
 416 The *p*-OH in ferulic acid could favour the establishment of interchain hydrogen bonds through the oxygens
 417 of ester groups, thus favouring the bonding of molecules in the matrix and limiting their diffusion in contact
 418 with the food simulant. Therefore, the most remarkable release level was found for films with 2 % cinnamic
 419 acid, whose asymptotic value (after 30 days' contact) reached 31% of the initial content of the film. In
 420 contrast, only 15% of the initial ferulic acid content (nearly 2%) was released at equilibrium. This behaviour
 421 indicates that the compound release occurs very slowly and to a limited extent, which compromises the
 422 active role of the compounds in different food substrates of intermediate polarities.



424 **Figure 5.** Experimental values (dots) and concentration predicted by Peleg's model (solid lines) and by
 425 Korsmeyer-Peppas model (dashed lines) at different contact times into the D1 food simulant of
 426 ferulic (F) or cinnamic (C) acid incorporated (1 or 2%) into PLA films. Parameters of release; Peleg model:
 427 release rate ($1/k_1$), active compound release at equilibrium (M_∞) and release ratio at equilibrium (M_∞/M_0).
 428 The Korsmeyer-Peppas model: diffusional exponent (n) and rate constant (k). 1F and 2F: films with 1 or 2
 429 % ferulic acid; 1C and 2C: films with 1 or 2% cinnamic acid.

430

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

444

445 4. Conclusions

446 The total recovery of ferulic and cinnamic acids in thermo-processed PLA films was relatively high (84 -
 447 98%), which suggests that these compounds can be incorporated by using the usual thermoplastic
 448 process of the plastic industry. These acids provoked a weakening effect in the polymer slightly reducing
 449 the stiffness and resistance to break, mainly with 2 % ferulic acid, although this was not notably reflected in
 450 the PLA glass transition. However, a 2 % content of both acids improved the water vapour and oxygen
 451 barrier capacity of the films. Likewise, both acids notably improved the PLA thermal stability. The films with
 452 ferulic and cinnamic acid at 1 or 2 % did not show a significant antimicrobial action against *E. coli* and *L.*
 453 *innocua*, which was attributed to the scarce release of these compounds in the culture medium during the
 454 *in vitro* test. In fact, no quantitative release was detected into aqueous or non-polar media, but only into the

455 D1 simulant (50% ethanol in water), where a slow and very limited release was observed for both acids.
456 Further research is needed in order to favour the release of ferulic and cinnamic acids so as to improve
457 their antimicrobial activity. Given the great potential of PLA - ferulic and cinnamic acid combinations for
458 packaging purposes, further antimicrobial and antioxidant studies into food matrices are required to test
459 the capacity of these films in real systems from where compound migration (e.g., fat) could favour the
460 relaxation of the polymer matrix, facilitating the delivery of active compounds from the film.

461

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465 Declaration of competing interest

466 The authors declare that they have no known competing financial interests or personal relationships that
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468 CRediT authorship contribution statement

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

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