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Physical crosslinking of pea protein-based bioplastics: Effect of heat and UV treatments

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ABSTRACT

Climate change and the enhancement of ecology have generated the need to create packaging that is biodegradable and, at the same time, allows food to be preserved efficiently in order to avoid the accumulation of plastic and minimize food waste. In this sense, protein-based bioplastics are a promising alternative, but due to their limited properties they need additional crosslinking in order to compete with conventional plastics. Among them, physical crosslinking is of special interest in the food industry, as it does not generate toxicity problems. In this way, the overall objective of this work was to develop pea protein-based bioplastics by injection moulding, using two different physical crosslinking methods: heat treatment (50ºC-24 h, 120 ºC-4 h and 120 ºC-24 h) and ultraviolet (UV) treatment (50, 120 and 500 mJ/cm^2). Thus, different bioplastics were compared based on their mechanical, functional and antimicrobial properties. The relevance of this study is based on the improvement of certain aspects of the mechanical and functional properties of bioplastics by the addition of an extra physical crosslinking stage to the fabrication process. In fact, UV treatment improves the antimicrobial activity of bioplastics, which gives it a significant improvement to compete with conventional plastics in the food sector.

1. Introduction

Climate change and sustainable development are two of the greatest challenges that society is currently facing. For this reason, it is increasingly important for companies to reduce the environmental impact of their products and services throughout their entire life cycle (Cai & Li, 2018). In this sense, one of the key points for companies to minimize the environmental impact is related to packaging (Han, Ruiz-Garcia, Qian & Yang, 2018). This means that packaging must be designed using the minimum amount of resources for its purpose, so that once its functions are fulfilled, its value is maximized. Desirable products are those whose manufacturing is effective and, besides, help to recycle conventionally used polymers (Wikström et al., 2019). For this reason, packaging materials that compensate both lines have not been found yet, and an average of 121 kg of food per person is wasted every year at the consumer level (Forbes, Quested, & O'Connor, 2021). Therefore, there is a growing interest in the use of polymer-based materials in packaging (Naveena & Sharma, 2020; Tyuftin & Kerry, 2020). There are currently three types of bio-based polymers on the market: those derived from starch (Shafqat, Tahir, Mahmood, Tabinda, Yasar & Pugazhendhi, 2020) and polylactic acid (PLA) (Barletta, Aversa, & Puopolo, 2020), and those derived from cellulose (Brodin, Vallejos, Opedal, Area & Chinga-Carrasco, 2017). However, many of these bioplastics are mixed or combined with synthetic components to improve their functional characteristics and to expand the range of uses, which does not benefit their environmental character (Luzi, Torre, Kenny & Puglia, 2019).

A promising alternative to these materials is protein-based bioplastics (Thiruchelvi, Das, & Sikdar, 2020). These bioplastics are made up of proteins mainly from agri-food waste, which makes them relatively cheap and ecological. In addition, their processing is simple (similar to that of conventional plastics), easily modifying their functional properties (Chan, Wong, Hassan & Zakaria, 2021). In this sense, the production of composites (Sagnelli et al., 2017) or the formation of new bonds to modify the properties of protein-based materials is of great significance to compete with conventional plastics in applications such

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as food packaging (Reshmy et al., 2021). The formation of new bonds to induce changes in protein-based bioplastics is achieved by including an additional crosslinking stage. This crosslinking stage may produce structural changes that not only influence the mechanical and microstructural properties of the material, but which could also make modifications in the functional and application properties (Garavand, Rouhi, Razavi, Cacciotti & Mohammadi, 2017).

There are three different crosslinking methods: physical crosslinking with the use of physical agents (Garavand et al., 2017), chemical crosslinking that uses chemical agents (Perez-Puyana, Jiménez-Rosado, Romero & Guerrero, 2019) or enzymatic crosslinking that implies the use of enzymes (Giosafatto, Fusco, Al-Asmar & Mariniello, 2020). Physical crosslinking consists in the modification of the structure of a material through non-covalent secondary interactions such as hydrogen bonds, electrostatic interactions and hydrophobic forces (Garavand et al., 2017; Yu, Lian, Kong, Lopez Hernandez, Qin & Appel, 2021). There are several advantages to the use of physical crosslinking. First, the ease of carrying out the process without unnecessary extra reactions. In fact, the use of a physical crosslinking method avoids the controversy of the use of chemicals in a natural product. In this sense, it does not require the addition of secondary reagents that could produce undesirable secondary reactions or even increase the toxicity of the material or possible-side products obtained, especially relevant for food industry applications. Some examples of physical crosslinking techniques are heat treatment, UV treatment and ultrasound treatment (Jiménez-Rosado, Bouroudian, Perez-Puyana, Guerrero & Romero, 2020). Several authors have studied the possibility of including a physical crosslinking treatment to improve the properties of biopolymer-based materials. Most of these are related to biomedical applications, since toxicity is a potential drawback when using chemical reagents. Weadock et al. (1983) evaluated the influence of different crosslinking techniques on the properties of biopolymers, highlighting a heat treatment for the specific case of biopolymers for biomedical purposes (Weadock, Olson, & Silver, 1983). In the field of bioplastics, Jiménez-Rosado et al. (2020) studied whether the addition of a heat treatment may improve the production of protein-based bioplastics.

However, most of the previous studies related to the modification of bioplastics properties via physical crosslinking are only based on the incorporation of a heat treatment. Nevertheless, only a few considered UV radiation is seen as a promising candidate to improve the properties of protein-based materials (Gonçalves de Moura, Vasconcelos de Sá, Lemos Machado Abreu & Alves Machado, 2017). Its use is highly recommended since a double function can be performed. This technique can not only induce intramolecular crosslinking, producing improved properties, but also sterilize the materials. In this sense, the main novelty of this study is the production and characterization of protein-based bioplastics with mechanical and antimicrobial properties enhanced with the addition of two different physical crosslinking methods (UV and heat treatments).

Thus, the main objective of this work was to assess the influence of different physical crosslinking methods on the properties of pea protein-based bioplastics. In this sense, the mechanical, functional and antimicrobial properties of bioplastics crosslinked through UV and heat treatments were analyzed.

2. Materials and methods

2.1. Materials

Pea protein isolate with a protein content higher than 90 wt% was used as raw material. It was provided by Roquette (Lestrem, France). The rest of the composition was already studied in Perez, Felix, Romero, and Guerrero (2016). On the other hand, the plasticiser used, glycerol, was purchased from Panreac Química, S.A. (Spain).

2.2. Bioplastics production

2.2.1. Sample preparation

Pea protein-based bioplastics were produced via injection moulding (Fig. 1). This process consisted of two stages: Firstly, in order to obtain a homogeneous blend, pea protein isolate and glycerol were mixed in a Polylab QC two-blade counter-rotating batch mixer (ThermoHaake, Karlsruhe, Germany). The mixing conditions were selected according to previous studies (Carvajal-Piñero, Ramos, Jiménez-Rosado, Perez-Puyana & Romero, 2019; Perez et al., 2016): a 60/40 pea protein/glycerol ratio (for a total 60 g blend correspond to 36 g pea protein and 24 g of glycerol) was homogenized for 10 min at 50 rpm. Secondly, the dough-like blend was processed using a MiniJet Piston Injection Moulding System (ThermoHaake) to obtain bioplastics. Once again, the conditions for the injection stage were optimized in previous studies (Perez-Puyana, Felix, Romero & Guerrero, 2016): a cylinder and mould temperatures of 50 and 130 °C, respectively; an injection pressure of 500 bar for 20 s and a post-injection pressure of 200 bar for 200 s. Two different moulds were used to obtain specimens with different geometries: 1. 60 × 10 × 1 mm^3 rectangular-shaped specimen for dynamic mechanical temperature analysis (DMA) experiments. 2. Dumbbell-shaped specimen (type V) according to ISO 527–1:2012 for tensile strength measurements of plastics.

2.2.2. Crosslinking methods

In this study, two different physical crosslinking methods were assessed to evaluate their influence on the properties of the pea protein-based bioplastics developed.

2.2.2.1. Heat treatment. One of the crosslinking methods evaluated consisted of a heat treatment. This heat treatment consisted of a thermal stage in a conventional oven (Memmert, Germany). According to Álvarez-Castillo, Del Toro, Aguilar, Guerrero, and Bengoechea (2018), this method could favour the formation of covalent bonds in the structure of protein-based bioplastic. As shown in previous studies, the exposure of collagen to a denaturing agent (heat in this case) led to partially unfold the collagen fibrils and, therefore, increase the reactivity of amino groups enhancing their susceptibility of different protein chains to interact (Cheung, Tong, Perelman, Ertl & Nimni, 1990).

This heat treatment was conducted at different temperatures (50 and 120 ºC) and different times (4 and 24 h) according to previous studies conducted with other protein-based bioplastics (M. Jiménez-Rosado, Bouroudian, et al., 2020).

2.2.2.2. UV treatment. The second crosslinking method was a UV post-treatment. UV irradiation forms bonds from less important aromatic tyrosine and phenylalanine residues (Davidenko et al., 2016). The application of UV allows the rearrangement of the structure and improves the properties of the bioplastics. UV-light is a zero-length crosslinking agent that predominantly or exclusively crosslinks proteins at their contact points (Moss, Dimitrov, & Houde, 1997). In other words, it facilitates protein-protein covalent interactions at aminoacid level (Chodosh, 1996; Stützer et al., 2020). In this sense, the interaction of UV light with proteins at molecular level involved the formation of photoadducts in aminoacids such as arginine, as previously described by other authors (Ahsan, 2018).

This crosslinking stage was carried out by placing bioplastics on a Microprocessor-Controlled UV Crosslinkers XLE-1000 (Select™, USA). The intensity was modified to determine whether all the bioplastic properties change uniformly with the application of the treatment. In this sense, different intensities were studied (50, 120 and 500 mJ/cm^2) at 30 min and 254 nm.

A summary of the different crosslinking treatments has been included in Table 1.

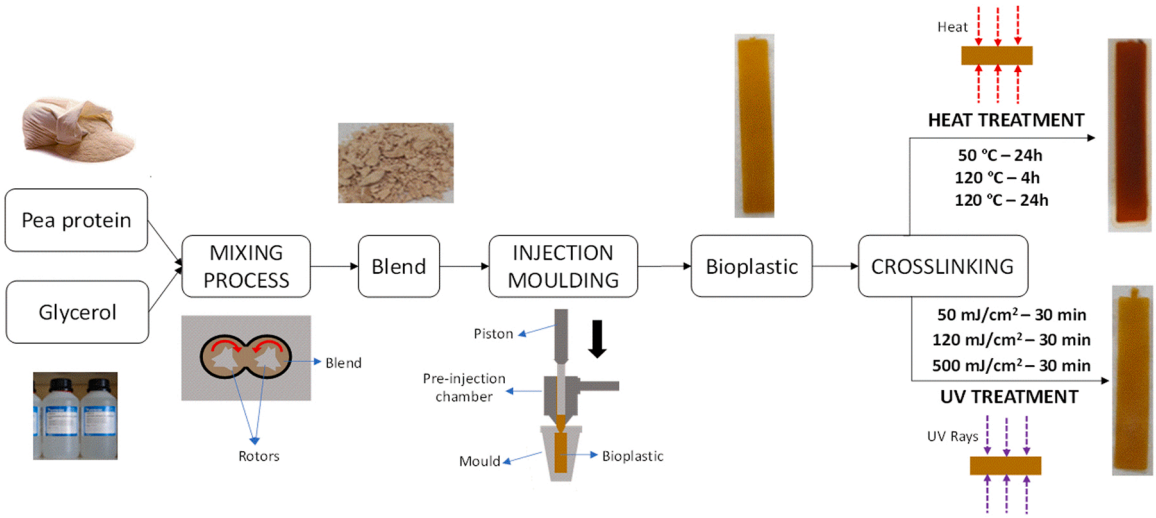

Fig. 1. Schematic overview of the fabrication process of pea protein-based bioplastics with physical crosslinking (heat treatment or UV radiation).

Table 1
Summary of the different crosslinking stages carried out to pea protein-based bioplastics.

Systems	Temperature / UV Intensity	Time
Heat treatment	50 °C	24 h
	120 °C	4 h
	120 °C	24 h
UV Treatment	50 mJ/cm^2	30 min
	120 mJ/cm^2	30 min
	500 mJ/cm^2	30 min

2.3. Characterization of bioplastics

2.3.1. Dynamic Mechanical Analysis (DMA)

DMA tests were carried out with a dynamic-mechanical rheometer RSA3 (TA Instruments, New Castle, DE, USA) on rectangular probes using a dual cantilever geometry in flexural mode. Firstly, strain sweep tests (from 0.002% to 1% of strain at 1 Hz and room temperature) were carried out in order to obtain the linear viscoelastic range and the critical strain. Secondly, a frequency sweep test was carried out within the linear viscoelastic range at room temperature (from 0.02 to 20 Hz).

2.3.2. Tensile strength measurements

Tensile tests were performed by using the Insight 10 kN Electromechanical Testing System (MTS, Eden Prairie, MN, USA). Measurements were carried out with an extensional rate of 10 mm/min at room temperature, following the ISO 527–2:2012 for Tensile Properties of Plastics (ISO 527–2:2012, 2012). The obtained parameters were: maximum tensile strength (σ_{max}), Young's modulus (E) and strain at break (ε_{max}).

2.3.3. Water uptake capacity and soluble matter loss

Absorption in the bioplastics was evaluated by water uptake capacity, measured following the ASTM D570 (ASTM, 2005) standard. Rectangular 60 × 10 × 1 mm specimens were previously subjected to drying (conditioning) in an oven at 50 ± 2 °C for one hour to determine the initial dry weight, followed by the weighing of the sample after immersion in distilled water for 24 h. Finally, they were exposed to drying again (reconditioning) and weighed to determine the soluble matter loss. Water uptake capacity and soluble matter loss were determined by Eqs. (1) and (2):

$$\%\ Water\ uptake\ capacity = \frac{Wet\ \ Weight - Initial\ \ Dry\ \ Weight}{Initial\ \ Dry\ \ Weight}\ x\ 100 \quad (1)$$

$$\%\ Soluble\ \ matter\ \ loss = \frac{Initial\ \ Dry\ \ Weight - Final\ \ Dry\ \ Weight}{Initial\ \ Dry\ \ Weight}\ x\ 100 \quad (2)$$

2.3.4. Transparency and colour measurements

Transparency measurements were performed on a Genesys-20 (Thermo Scientific, USA) spectrophotometer. The transmittance (%) of 1-mm-thick rectangular specimens at a selected wavelength of 600 nm was measured. The bioplastic without any crosslinking stage was used as reference. In order to compare the transparency of different bioplastics, a transmittance index (*TI*) was used:

$$TI = \frac{\%\ \ Transmittance}{\%\ \ Transmittance\ \ of\ \ reference\ \ bioplastic}\ x\ 100 \quad (3)$$

Colour measurements were carried out using a KONICA MINOLTA CM-700D spectrocolorimeter in the CIELAB colour space. The average of 5 scans was used to determine the value of L^* as the perceptual lightness, and a and b as the four unique colours of human vision (red, green, blue, and yellow) of each system. Δa, Δb and ΔL were obtained as the difference between each system and the reference system without any crosslinking method.

2.3.5. Fourier transform infrared spectroscopy (FTIR)

FTIR profiles of the different bioplastics were obtained with a Hyperion 100 spectrometer (Bruker, USA), using an ATR diamond sensor. The measurements were performed as a mean of 200 scans between 3600 and 750 cm^{-1} with an opening of 4 cm^{-1}.

2.3.6. Crosslinking degree

The crosslinking degree of the different bioplastics was measured following the same procedure previously described by Zárate-Ramírez et al. (2014). Briefly, a portion of bioplastic was immersed in a denaturing solution to denaturalize the uncrosslinked protein. Then, the amount of protein contained in the denatured solution was determined using a modification of the Lowry's method (Markwell, Haas, Bieber & Tolbert, 1978). This method consists in the formation of a complex between the soluble protein chains (specifically, tyrosine, tryptophan, and cysteine) and the Folin-Ciocalteau reagent in an alkaline medium. This reaction generates a blue-green colour in the solutions that could be measured at a wavelength of 750 nm. The amount of soluble protein is obtained by using a calibration curve.

The crosslinking degree was calculated between a reference system (protein bioplastic without any crosslinking stage, 0% crosslinking) and a denaturing solution without bioplastics (100% crosslinking).

2.3.7. Antimicrobial assay

The bactericidal activity of the bioplastics was tested against Staphylococcus epidermidis (*S. epidermidis,* Gram+) and Escherichia coli (*E. coli*, Gram-). Pea protein-based bioplastics with a circular shape (dia: 10 mm) were placed on agar petri plates, previously cultured with either *S. epidermidis* or *E. coli* with 10^8 colony-forming units. Then, agar petri plates were incubated at 37 ºC for 24 h before measuring the inhibition zone. The resulting inhibition potential was calculated by measuring the diameter of the inhibition zone of the samples. The ratio between the inhibition observed for each system respect the reference one has been calculated to evaluate the improvement in the antimicrobial activity.

2.4. Statistical analysis

At least three replicates of each measurement were carried out. The statistical analyses were performed using t-test and one-way analysis of variance (ANOVA, $p < 0.05$) using the statistical package Excel 2013 (Microsoft, Redmond, WA, USA). Standard deviations from some selected parameters were calculated.

3. Results and discussion

3.1. Effect of heat treatment

3.1.1. Crosslinking degree

Table 2 shows the crosslinking degree of the systems modified with an additional heat treatment. The crosslinking degrees included are referred to the reference system without any additional treatment. According to the results obtained, all the systems presented a degree of crosslinking between 14% and 25% higher than the reference system, being more significant when the heat treatment is conducted at a higher temperature and time (24.3 ± 0.3% for the system at 120 ºC and 24 h). As described by Domenek, Morel, Bonicel, and Guilbert (2002), heat treatment favours the formation of sulfhydryl groups interchain bonds with the cysteine residues from different protein chains. On the other hand, further studies revealed that protein unfolding takes place upon heating, leading to the branching of the biopolymer towards a more crosslinked structure (Domenek, Morel, Redl & Guilbert, 2003).

The amino acid composition of the pea protein is shown in Table 3. The amino acid profile showed glutamic and aspartic acids as the major amino acid components, as shown by Leterme et al. (1990). Moreover, it also presented a high content in Lysine, Leucine and Arginine. Similar results were found by Gorissen et al. (2018). Taking into account the amino acid profile obtained for pea protein (Table 3), the heat treatment may alter the structure in terms of branching, according to the studies of Domenek et al. (2003).

Table 2
Amino acid profile of the pea protein isolate.

AMINO ACID COMPOSITION	
Amino acid	**g/100 g protein**
Alanine	4.11
Arginine	8.58
Aspartic Acid	11.91
Cystine/Cysteine	1.78
Glutamic Acid	17.41
Glycine	3.79
Histidine*	1.94
Isoleucine*	3.30
Leucine*	7.91
Lysine*	8.18
Methionine*	0.70
Phenylalanine*	5.35
Proline	3.59
Serine	5.45
Threonine*	3.43
Tyrosine	3.36
Valine*	3.57

Table 3
Crosslinking degree of bioplastics crosslinked by heat treatment (50 ºC – 24 h, 120 ºC – 4 h, 120 ºC – 24 h) and ultraviolet treatment (50, 120 and 500 mJ/cm^2). Pea protein-based bioplastic without any crosslinking was used as reference, being all the crosslinking additional to this system. Different letters were included as superscripts to denote significant differences between the values.

Systems		Crosslinking degree (%)
Heat treatment	**50 ºC – 24h**	16.8 ± 2.9^{A}
	120 ºC – 4h	14.4 ± 6.8^{A}
	120 ºC – 24h	24.3 ± 0.3^{B}
UV Treatment	**50 mJ/cm^2**	3.6 ± 1.7^{C}
	120 mJ/cm^2	14.1 ± 2.6^{A}
	500 mJ/cm^2	18.1 ± 2.2^{A}

3.1.2. FTIR measurements

Fig. 2 A shows the FTIR profile of the reference bioplastic and those crosslinked by heat treatment. According to the results obtained, all the systems presented a similar profile to the reference system. A band between 3500 and 3000 cm^{-1} (maximum peak at 3278 cm-1) show the stretching of OH bonds, stretching of NH bonds present in amide A and B and harmonic vibration of Amide II (Türker-Kaya & Huck, 2017). The 2925 and 2871 cm^{-1} bands correspond to CH and CH_2 stretching, while the 1738 cm^{-1} band is attributed to C═O ester, all of them present in the protein chains (Türker-Kaya & Huck, 2017). Nevertheless, the most important bands of the proteins are those present at 1625, 1544 and 1227 cm^{-1}, which correspond to amide I, II and III, respectively (Wang et al., 2011). Interestingly, these amide bands present differences when a heat treatment is applied. Thus, the system obtained with the most drastic conditions (120 ºC during 24 h) showed a severe decrease in the intensity of these peaks as a consequence of the chemical modification of the protein network. This reduction was assigned to an increase in protein aggregation in previous works (Jiménez-Rosado, Maigret, Lourdin, Guerrero & Romero, 2022), which is consistent with the degree of crosslinking obtained (higher in these systems). Other bands are those present between 1460 and 1380 cm^{-1} (OH, CH_2 and COO^-) and at 1037 cm^{-1} (C-O-C) (Baker et al., 2014).

3.1.3. Mechanical properties

Fig. 3 shows the evolution of the elastic modulus along frequency for pea protein-based bioplastics with and without heat treatment. All the profiles show a similar profile, with a slight increase in the E' values with increasing time and temperature at the frequency range studied. Comparing the different systems, the application of the heat treatment induced a pronounced increase in the elastic character of the bioplastics, as shown in Table 4 by the marked increase in the E'_1 values, from 5.5 ± 0.18 MPa for the reference system to the range 2600–3300 MPa for the crosslinked systems. Together with the increase in the E' values, there is a decrease in tan $(\delta)_1$ values. These two effects are correlated. Thus, the higher the E' values, the smaller the tan $(\delta)_1$ values; in other words, a more solid character. Interestingly, the effect was greater when a higher temperature (120 ºC) and time (24 h) were used. Moreover, a slight decrease of the slope is also observed with heat treatment, so a lower dependency of E' values with frequency and giving rise to more elastic systems.

Concerning the critical strain values (Table 4), 50 ºC as heat treatment did not show any significant difference with the reference system, although the application of 120 ºC reduced such values by 33% and 45% when the heat treatment was applied for 4 and 24 h, respectively. This reduction highlights the solid character of these bioplastics, which are stronger but also more rigid.

On the other hand, concerning the stress-strain curves (Fig. 4), two different profiles are observed. The bioplastics submitted to the heat treatment at 120 ºC showed a marked elastic region followed by a small plastic zone until their break. However, the reference system and the one

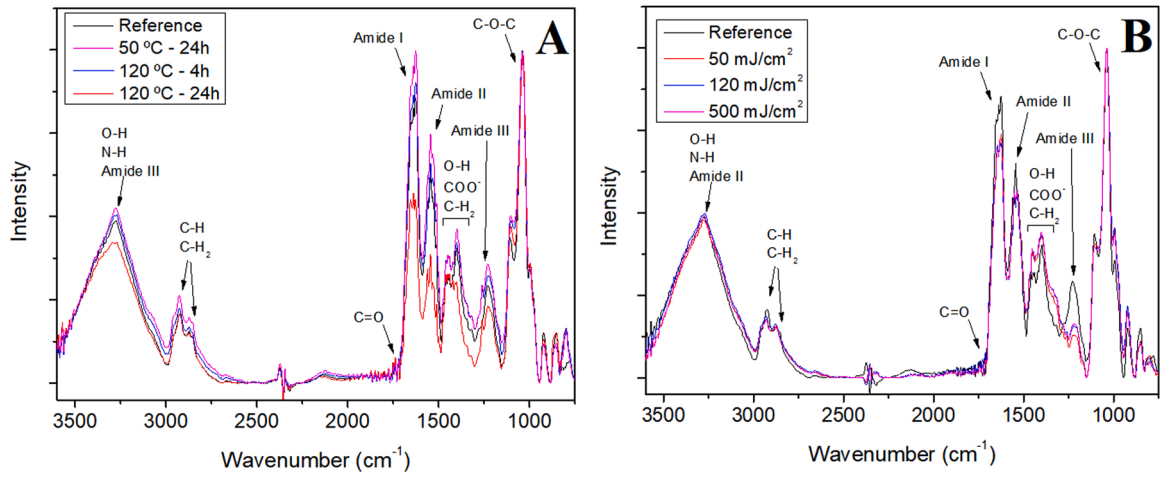

Fig. 2. FTIR profile of bioplastics crosslinked by (A) heat treatment (50 °C – 24 h, 120 °C – 4 h, 120 °C – 24 h) and (B) ultraviolet treatment (50, 120 and 500 mJ/cm^2). Pea protein-based bioplastic without any crosslinking method was also included as reference.

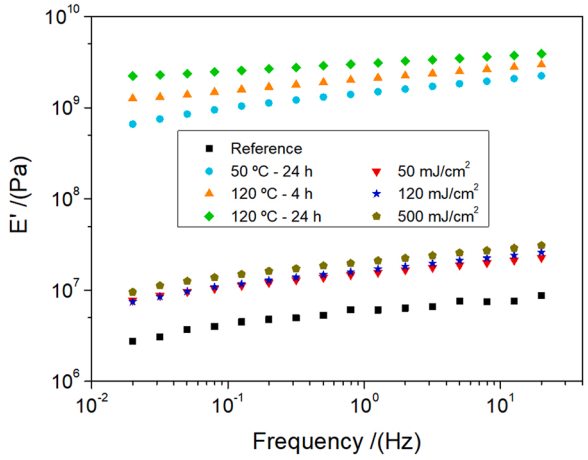

Fig. 3. Flexural frequency tests of bioplastics crosslinked by heat treatment (50 °C – 24 h, 120 °C – 4 h, 120 °C – 24 h) and ultraviolet treatment (50, 120 and 500 mJ/cm^2). Pea protein-based bioplastic without any crosslinking method was also included as reference.

crosslinked at 50 °C showed a longer plastic region defined by the higher deformability of these systems. Table 4 shows a summary of the parameters obtained from the strain-stress profiles. The system at 50 °C did not present significant differences with respect to the reference system, whereas the systems crosslinked at 120 °C exhibited a significant increase in both Young's Modulus and Maximum stress, although with a marked reduction of the strain at break. Therefore, the heat treatment at 120 °C produced more rigid systems with a more brittle character, as shown in the dynamic mechanical tests described above.

To sum up, a heat treatment at 120 °C (at both 4 and 24 h) produced more rigid and less deformable bioplastics, whereas applying a heat treatment at 50 °C did not influence the tensile properties of the bioplastics, although it increased their elastic modulus with respect to the reference system without any crosslinking method.

3.1.4. *Water uptake capacity and soluble matter loss*

Fig. 5 A shows the evolution of the water uptake capacity and soluble matter loss of pea protein-based bioplastics subjected to a heat treatment together with the reference system. In a similar way as the mechanical properties, the results of the heat treatment at 50 °C and 24 h were similar to those of the reference system, with an uptake capacity of over 100%. Furthermore, the heat treatment at 120 °C worsened the water absorption of the specimens, decreasing by 22% and 50% when the treatment lasted 4 and 24 h. This decrease is related to the increase in the mechanical properties of the systems. In other words, the heat treatment at 120 °C produced systems with enhanced mechanical properties but, subsequently, with lower water uptake capacity. This behaviour could be due to the fact that the more brittle bioplastics have a less facility to swell and increase their size, making the amount of water that they can retain is less. Similar results were found in the bibliography (Espigulé, Puigvert, Vilaseca, Mendez, Mutjé & Girones,

Table 4
Dynamic flexural parameters (Elastic Modulus at 1 Hz: E'$_1$; Loss Tangent at 1 Hz: tan (δ)$_1$; and Critical Strain) and Tensile parameters of bioplastics crosslinked by heat treatment (50 °C – 24 h, 120 °C – 4 h, 120 °C – 24 h) and ultraviolet treatment (50, 120 and 500 mJ/cm^2). Pea protein-based bioplastic without any crosslinking method was also included as reference. Different symbols were included as superscripts to denote significant differences between the values for each column.

SYSTEMS			Critical Strain (%)	E'$_1$ (Pa)·10^{-7}	tan (δ)$_1$	Young's Modulus (MPa)	Maximum Stress (MPa)	Strain at break (mm/mm)
Reference			0.158 ± 0.050^{a}	0.55 ± 0.18 ^{A}	0.220 ± 0.010$^{\alpha}$	59.6 ± 8.9^{I}	2.83 ± 0.32$^{\#}$	0.72 ± 0.02 *
Heat Treatment	**50 °C**	**24 h**	0.159 ± 0.051^{a}	260.7 ± 7.4^{B}	0.214 ± 0.011$^{\alpha}$	58.7 ± 10.4^{I}	2.60 ± 0.17$^{\#}$	0.66 ± 0.11 *
	120 °C	**4 h**	0.105 ± 0.074^{a}	262.1 ± 15.5^{B}	0.187 ± 0.004$^{\beta}$	216.9 ± 33.5^{II}	5.71 ± 0.89$^{\#\#}$	0.18 ± 0.04 * *
		24 h	0.088 ± 0.048^{a}	328.3 ± 9.3 ^{C}	0.149 ± 0.006$^{\gamma}$	238.3 ± 9.1^{II}	7.92 ± 0.28$^{\#\#\#}$	0.22 ± 0.12 * *
UV Treatment	**50 mJ/cm^2**		0.398 ± 0.127^{a}	1.82 ± 0.11^{D}	0.204 ± 0.001$^{\delta}$	67.1 ± 4.8$^{I\text{-}III}$	2.80 ± 0.10$^{\#}$	0.74 ± 0.04 *
	120 mJ/cm^2		0.251 ± 0.080^{ab}	1.81 ± 0.27^{D}	0.201 ± 0.008$^{\alpha\delta}$	78.5 ± 8.3^{III}	3.00 ± 0.22$^{\#}$	0.60 ± 0.10 *
	500 mJ/cm^2		0.626 ± 0.102^{c}	1.99 ± 0.06^{D}	0.203 ± 0.006$^{\alpha\delta}$	63.5 ± 5.5^{I}	2.90 ± 0.20$^{\#}$	0.88 ± 0.09 * **

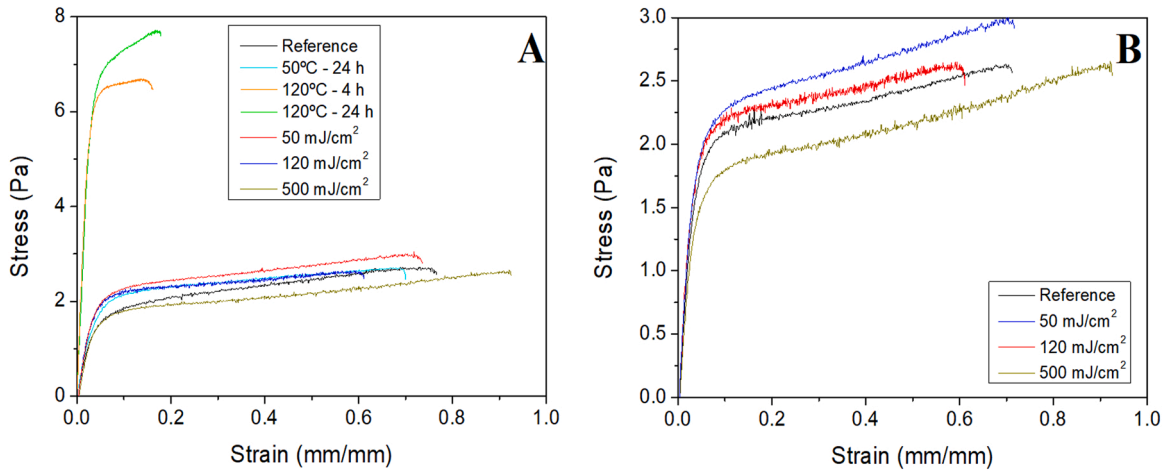

Fig. 4. (A) Tensile tests of bioplastics crosslinked by heat treatment (50 ºC – 24 h, 120 ºC – 4 h, 120 ºC – 24 h) and ultraviolet treatment (50, 120 and 500 mJ/cm^2). (B) Magnification of tensile tests of bioplastics crosslinked by ultraviolet treatment. Pea protein-based bioplastic without any crosslinking method was also included as reference.

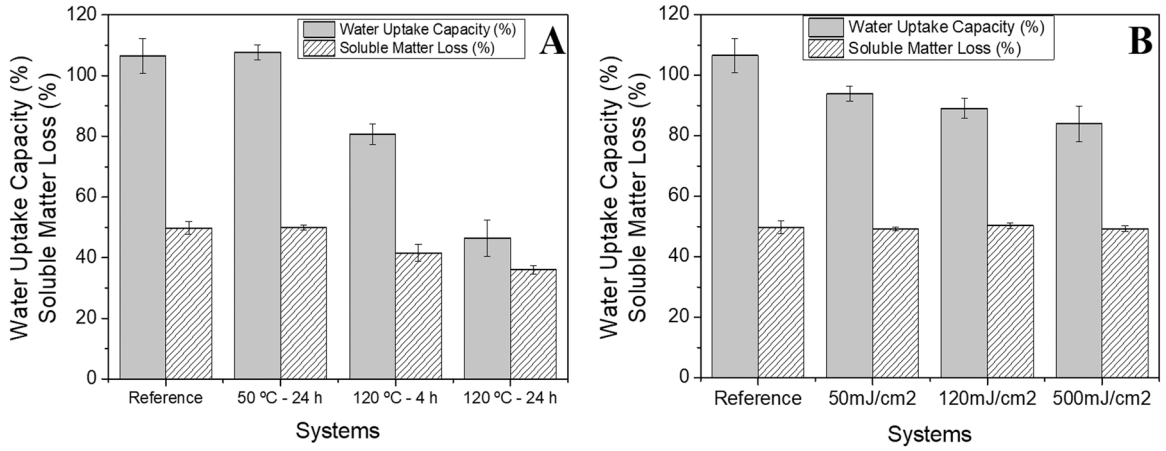

Fig. 5. Water uptake capacity and soluble matter loss of bioplastics crosslinked by (A) heat treatment: 50 ºC – 24 h, 120 ºC – 4 h, 120 ºC – 24 h and (B) ultraviolet treatment: 50, 120 and 500 mJ/cm^2. Pea protein-based bioplastic without any crosslinking method was also included as reference.

2014; Jiménez-Rosado, Rubio-Valle, Perez-Puyana, Guerrero & Romero, 2021).

The crosslinking stage produced more structured systems with a higher interconnected network. So, there is a higher structure conforming with the additional heat treatment and, as a result, the soluble matter loss decreased, indicating the strengthening of the system (Jiménez-Rosado et al., 2021). Similar results were found by Alvarez-Castillo et al. who observed a reduction in water uptake capacity with the exposure time and temperature of heat treatment in similar bioplastics obtained with soy protein isolate (Álvarez-Castillo et al., 2018).

3.1.5. *Transparency and colour measurements*

The influence of the heat treatment on the morphological properties of the bioplastics was studied with the evaluation of both the transparency and the colour of the samples. The application of a heat treatment induces a decrease in the transparency index of the bioplastics. The higher the temperature and exposure time, the lower the *TI* (Table 5). As a consequence, more crystalline products are obtained (Perez et al., 2016). However, this is not the only effect, since the heat treatment also induces the formation of secondary bonds promoted by the Maillard reaction, deriving to more brownish bioplastics (Gerrard & Brown, 2002; Zárate-Ramírez, Romero, Martínez, Bengoechea, Partal & Guerrero, 2014). This change in the colour of the bioplastics was also

Table 5
Transparency index (*TI*) and colour parameters of bioplastics crosslinked by heat treatment (50 ºC – 24 h, 120 ºC – 4 h, 120 ºC – 24 h) and ultraviolet treatment (50, 120 and 500 mJ/cm^2). Pea protein-based bioplastic without any crosslinking method was also included as reference.

SYSTEMS			*a*	*Δa*	*b*	*Δb*	*L** **	*ΔL*	*TI*
Reference			3.71 ± 0.20	–	13.13 ± 0.76	–	31.47 ± 0.6	–	1
Heat Treatment	**50 ºC**	**24 h**	4.53 ± 0.35	0.82	14.83 ± 1.23	1.70	33.83 ± 0.75	2.36	0.81 ± 0.03
	120 ºC	**4 h**	5.73 ± 0.15	2.02	11.20 ± 0.40	-1.93	31.67 ± 0.31	0.20	0.78 ± 0.04
		24 h	4.98 ± 0.15	1.27	8.21 ± 0.08	- 4.92	27.49 ± 0.26	-3.98	0.78 ± 0.01
UV Treatment	**50 mJ/cm^2**		4.78 ± 0.08	1.07	15.93 ± 0.81	2.80	34.02 ± 1.01	2.55	0.48 ± 0.03
	120 mJ/cm^2		3.45 ± 0.49	-0.26	13.81 ± 0.98	0.68	33.66 ± 0.18	2.19	0.53 ± 0.05
	500 mJ/cm^2		3.37 ± 0.23	-0.34	12.52 ± 0.69	-0.61	33.75 ± 0.28	2.28	0.19 ± 0.02

analyzed and the results were summarized in Table 5. The system crosslinked at 50 ºC is brighter ($\Delta L>0$) and more yellow ($\Delta b>0$) than the reference system. Moreover, the systems crosslinked at 120 ºC present a more brownish colour ($\Delta a>0$ and $\Delta b<0$), which is also darker ($\Delta L<0$) at longer times (24 h).

3.2. Effect of UV treatment

3.2.1. Crosslinking degree

The crosslinking degree of the systems treated under UV radiation was also measured (Table 2). All the systems presented a degree of crosslinking between 3% and 18%. The evolution of the crosslinking degree presented a polynomial increase in respected UV intensity, from 3.6 ± 1.7% to 18.1 ± 2.2% for the systems at 50 mJ/cm^2 and 500 mJ/cm^2, respectively.

Among the different amino acids, phenylalanine and tyrosine get excited with UV light improving the crosslinking effect (Fernández-d'Arlas, 2019), as shown by the increase in the crosslinking degree of the UV systems related to the reference system. In this sense, UV radiation may improve the properties of pea protein-based bioplastics, considering the relatively high content of these amino acids in pea protein (Table 3). For this reason, the beneficial effect of this treatment is twofold: On the one hand, the improvement of the properties of the bioplastics by applying a UV treatment, while, on the other hand, it allows the sterilization of the samples (Riley, Bavastrello, Covani, Barone & Nicolini, 2005). The latter effect is quite useful for several potential applications such as packaging, wound healing, etc.

3.2.2. FTIR measurements

Fig. 2B shows the FTIR profile of the reference bioplastic and those obtained by UV crosslinking. These profiles present a similar structure. Nevertheless, as previously shown by Aldayel and El Semary (2020), UV radiation can induce photochemical changes that may alter the protein chains of the bioplastics. These changes are corroborated by the FTIR profiles obtained, since the UV radiated systems showed a similar profile to the reference system but with a lower intensity of the amide bands (1625, 1544 and 1227 cm^{-1}), being more pronounced in amide III. Furthermore, the systems obtained with additional UV radiation exhibited a higher intensity in the bands between 1460 and 1380 cm^{-1}.

3.2.3. Mechanical properties

The influence of UV treatment on the mechanical properties is also shown in Figs. 3 and 4. The elastic moduli of the samples are slightly higher than that of the reference system (as shown in Table 4), regardless of the intensity used. However, considering the critical strain, the most striking change occurs when the intensity used is 500 mJ/cm^2. In this sense, the application of UV irradiation enhanced the deformability of the bioplastics, probably due to the different interactions induced by UV rays.

Concerning their tensile properties, the profile of the UV crosslinked systems was similar to that of the reference system without any crosslinking method (Fig. 4). With respect to the parameters obtained from the strain-stress measurements (Table 4), a maximum in Young's Modulus and Maximum stress was obtained at 120 mJ/cm^2. However, the strain at break reached its maximum at 500 mJ/cm^2; thus the bioplastics subjected to the UV treatment at 500 mJ/cm^2 are more deformable, as corroborated by the increase in both the critical strain and the strain at break. This higher deformability may be due to the structural reorganization produced by the new bonds formed between the tyrosine and phenylalanine residues of the protein. Thus, there are long chains with higher mobility (Davidenko et al., 2016).

3.2.4. Water uptake capacity (WUC) and soluble matter loss

WUC measurements (Fig. 5B) showed a progressive decrease from the reference system up to the crosslinked bioplastic produced at the highest intensity (500 mJ/cm^2). Comparing the effect of the UV treatment with the heat treatment, the effect produced is not as pronounced as on the systems crosslinked at 120 ºC. Thus, the improvement in the mechanical properties produces a decrease in the water uptake capacity, as mentioned above, although in a lesser extent in UV crosslinked bioplastics because these systems have higher deformability (higher values of critical strain and strain at break). On the other hand, the soluble matter loss of the studied systems showed no significant differences with respect to the reference bioplastic. So, the soluble protein fractions of the bioplastics are likewise exposed after UV treatment.

3.2.5. Transparency and colour measurements

The effect of the UV treatment on the colour properties of the pea protein-based bioplastics was also measured by means of transparency measurements and colour analyses. Table 5 shows the evolution of the transparency index as a function of the intensity of the UV treatment. Although it is true that the samples became opaquer with the UV crosslinking, there are no significant differences based on the intensity used. In other words, the new bonds generated improved the crystallinity of the bioplastics. However, the changes are not as dramatic as the heat treatment described in the previous section (Section 3.1).

On the other hand, colour values showed that UV treatment produced brighter ($\Delta L > 0$) and slightly more yellowish ($\Delta b > 0$) systems. However, as it can be observed from the bioplastics' images included in Fig. 6, this effect is not as significant as the resulting bioplastics when applying the heat treatment.

3.3. Antimicrobial assessment

Finally, the antimicrobial potential was assessed against Gram+ and Gram- bacteria. The bioplastics crosslinked with UV irradiation were compared to the bioplastics processed without any further treatment. They were also compared with the system, which exhibited further changes after performing the heat treatment compared to the reference system (120 ºC for 24 h). According to the results shown in Table 6, the UV treatment improved the antimicrobial behaviour of the pea protein-based bioplastics, which already presented a certain activity against Gram- bacteria (*E. coli*). UV radiation is mostly non-ionizing, although it causes photochemical changes. In this sense, the improvement of the antimicrobial activity of pea bioplastics by applying UV radiation on them may be due to two possible derived effects: On the one hand, the application of this radiation may have generated metabolites that interfere with bacterial growth. On the other hand, it is also possible that a high optical energy of UV irradiation may have a significant impact on the electron transitions in the molecules of the bioplastic's proteins, rendering some of them more effective in their antibacterial action, as shown previously by Aldayel and El Semary (2020).

On the other hand, the heat treatment worsened the antimicrobial activity of the bioplastics (7% lower with respect to the reference system and 14–16% lower than the UV crosslinked bioplastics), probably due to the induced degradation of the protein system. However, neither of the

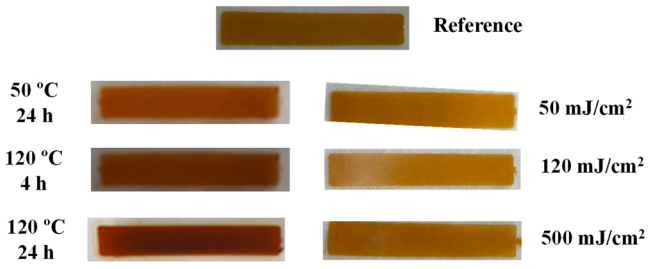

Fig. 6. Images of bioplastics crosslinked by heat treatment (50 ºC – 24 h, 120 ºC – 4 h, 120 ºC – 24 h) and ultraviolet treatment (50, 120 and 500 mJ/cm^2). Pea protein-based bioplastic without any crosslinking method was also included as reference.

Table 6
Antimicrobial ratios of bioplastics crosslinked by heat treatment (120 ºC – 24 h) and ultraviolet treatment (50 and 500 mJ/cm^2). Pea protein-based bioplastic without any crosslinking method was also included as reference.

System	*E. Coli*		*S. Epidermidis*	
	Diameter (cm)	Ratio	Diameter (cm)	Ratio
Reference	2.85	–	–	–
50 mJ/cm^2	3.10	+ 9%	–	–
500 mJ/cm^2	3.05	+ 7%	–	–
120 ºC – 24 h	2.65	-7%	–	–

enhanced bioplastics showed antimicrobial activity against Gram + bacteria, as indicated by the null activity against *S. epidermidis*.

Gram + bacteria have a thick polypeptide layer (wall size ca. 55 nm) and have no outer lipid membrane, whereas Gram - bacteria have a thin polypeptide layer (ca. 2 nm) and have an outer lipid membrane (Salton, 1953). Thus, to attack Gram + bacteria, the synthesis of peptidoglycans, essential elements for the constitution of the polypeptide wall, has to be affected. However, for Gram – bacteria, active agents are needed that disrupt the lipid portion of the bacterial membrane, causing bacterial lysis (Mai-Prochnow, Clauson, Hong & Murphy, 2016). Therefore, since our system is effective against Gram- bacteria but not against Gram + bacteria, it should act on the lipid layer, causing defects in the bacterial membrane and, as a consequence, causing its lysis. Nevertheless, it does not act so strongly on the polypeptide layer as to induce lysis of Gram + bacteria.

Similar results were observed by other authors concerning different types of materials, such as the studies of Goy et al. (2009) with colloidal silver or Vila Domínguez et al. (2020) with chitosan.

4. Conclusions

Pea protein-based bioplastics with enhanced mechanical and antimicrobial properties were obtained by carrying out an additional physical crosslinking stage to the fabrication process. Specifically, heat treatment produced systems with improved mechanical properties, although lowering their critical strain and water uptake capacity. In fact, heat treatment at 50 ºC produced brighter bioplastics, whereas at 120 ºC it led to brownish samples. UV irradiation improved the critical strain and strain at break of the samples. Furthermore, it also produced an increase in the crosslinking of the bioplastics together with a slight decrease in the water uptake capacity. Comparing both treatments, the heat treatment produced more rigid and brittle systems, whereas the UV radiation improved the deformability together with the possible sterilization of the samples.

Finally, pea protein-based bioplastics exhibited good antimicrobial properties against Gram+ bacteria. In fact, the antimicrobial activity was improved by 7–9% when applying an UV treatment to the bioplastics. However, the heat treatment modified the protein structure, worsening the antimicrobial character of the derived bioplastics.

CRediT authorship contribution statement

Victor Perez-Puyana: Conceptualization, Investigation, Methodology, Writing – original draft. **Pablo Cuartero**: Investigation, Data curation, Software. **Mercedes Jiménez-Rosado**: Conceptualization, Data curation, Validation, Visualization. **Inmaculada Martínez**: Formal analysis, Supervision, Project administration, Writing – review & editing. **Alberto Romero**: Conceptualization, Supervision, Writing – review & editing, Funding acquisition.

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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fpsl.2022.100836.

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