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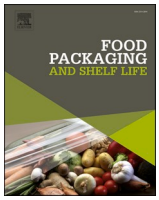

Metal-free organic polymer for the preparation of a reusable antimicrobial material with real-life application as an absorbent food pad

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ABSTRACT

There is a strong need to reduce food waste while maintaining the quality of packaged food. Thus, we have prepared a new fully organic and metal-free antimicrobial polymer, with the aim of increasing both the shelf life and safety of packaged meat. This antimicrobial polymer is based on widely available commercial acrylic monomers with covalently linked vanillin motifs, which are naturally occurring essential oils with antimicrobial characteristics. The film-shaped antibacterial polymeric material shows antibacterial activity for *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes* with an R parameter of up to 3.18, 3.37 and 2.00 and inhibition % of up to 99.95%, 99.96%, and 99.02%, respectively. To show the potential of these materials, we conducted a proof of concept experiment in which the antimicrobial polymer film was used as an absorbent food pad. The results show that the use of the antimicrobial polymer film can increase the shelf life of a packaged meat product by 50%. Since the antimicrobial activity is based on a covalently anchored group, there is no antimicrobial agent diffusion, and the antimicrobial activity persists beyond the first use because it is easily washable and reusable for at least 10 cycles.

1. Introduction

A biocide is defined in European legislation as a chemical substance or microorganism intended to destroy, deter, render harmless or exert a control effect on any harmful organism. In Europe, biocides are divided into different types of products, depending on their intended use, as stated in the Biocidal Products Regulation (BPR) of the EU No. 528/2012 (European Chemicals Agency, 2012): "Biocidal products are necessary for the control of organisms that are harmful to human or animal health and for the control of organisms that cause damage to natural or manufactured materials". However, it is necessary to consider some of the disadvantages of the actual antimicrobial and biocidal products. This need is stressed today due to the current pandemic, where the continuous cleaning of surfaces to eliminate SARS-CoV-2 has become mandatory (Mallakpour et al., 2021).

Antimicrobial products can pose risks to humans, animals, and the environment as a result of their intrinsic properties and associated use patterns (Sharma et al., 2021). Our first objective in this work was to prepare inherently antimicrobial materials that do not cause the migration of antimicrobial agents towards living beings, eliminating the dangers referred to in the aforementioned regulations.

Although there are some reviews concerning antimicrobial polymers as advanced materials, only a few are relevant in the field (Borjihan & Dong, 2020; Krishnamoorthy et al., 2014; Olmos et al., 2021). In fact, these reviews are mostly related to the antimicrobial activity exhibited by hybrid structures containing metals (Braun et al., 2020; Otoni et al., 2016; Shao et al., 2015; Tang et al., 2013; Wang et al., 2014), which makes these studies interesting from a scientific viewpoint but irrelevant to real-life applications due to both their high production costs and the health and environmental concerns related to the use of metal cations (Carraher & Roner, 2014; Ding et al., 2019; Fernández et al., 2009; Luo et al., 2021; Tamayo et al., 2016; Zhang et al., 2014). The advantages of the antimicrobial activity of polymers over conventional antimicrobial agents against microorganisms (such as bacteria, fungi, and protozoans) include their nonvolatility, chemical stability, nontoxicity (difficulty in permeating through the skin of animals), ability to prolong product shelf life, increased efficiency, and selectivity while minimizing their environmental impact (Norrrahim et al., 2021; Sharma et al., 2021).

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Although the preparation of materials with antibacterial properties is a topic that is booming (Cheng et al., 2021; Liu et al., 2020; Su et al., 2020), there is an urgent need to develop a scalable synthetic strategy involving different kinds of polymers whose potency against specific microorganisms is accompanied by less hazardous effects (Otoni et al., 2016). The second objective of our work involves more than just these advantages, proposing fully organic antimicrobial polymers (i.e., metal-free) that have a biocompatible main chain and pendant chemically anchored vanillin derivative motifs. At this point, the use of vanillin is significant because it is a natural product that is also a widely used and accepted flavouring food additive (U.S. Food & Drug Administration, 2021).

The preparation of new polymers with antimicrobial activities requires the synthesis, purification, and characterization of new compounds to be used as monomers, comonomers, or simple reagents for dispersion into the polymer matrix. This process is complex and time-consuming, and the resulting materials are not economically viable, so our third objective was to prepare these materials in a simple and easily scalable way for future applications. For this reason, we propose an antimicrobial polymer based on the antimicrobial activity of phenols (Park et al., 2001), specifically essential oils containing phenol structures (Fiore et al., 2021; Gañán & Brignole, 2011; He et al., 2021; Javed et al., 2011; Reyes et al., 2021; Syafiq et al., 2020), and even more specifically vanillin (Cerrutti et al., 1997; Fitzgerald et al., 2004). Furthermore, we designed a very easily prepared film-shaped polymer that is 100% based on inexpensive and widely available commercial monomers (such as *N*-vinyl-2-pyrrolidone, methyl methacrylate, and 4-aminostyrene) and can be easily modified by simply dipping the dense membrane in two solutions. 4-Aminostyrene acts as a functional motif in the polymer's main chain to anchor the vanillin motifs (phenol derivative) through an azo-coupling reaction (Bustamante et al., 2019). The proportion of this anchoring monomer is very low (mol 1%), so the polymers can be easily scaled up for industrial production (i.e., there is no need for costly modification of the current production processes for prepared goods that contain polymers), and, at the same time, are safe for the environment and living beings.

Additionally, in this work, we have carried out a proof of concept experiment for the designed polymers as absorbents for packaged meat products (Han et al., 2018) to demonstrate the possible applications of these new polymers in one of the expected real-life scenarios. The current common absorbents are single-use products composed of silica gel or cellulose coated in plastic (Han et al., 2018) and generally do not have antimicrobial functionality or their antimicrobial activity ceases when all antimicrobial agents are delivered (Oral et al., 2009). These pads are not accepted for recycling or composting in the United States at this time, so they pose an environmental problem (Gaspar et al., 2019). Furthermore, their reusability is economically and environmentally impossible due to their porous nature. Microorganisms, such as bacteria, highly contaminate the absorbent pad, which necessitates the use of advanced washing processes in such a way that it is not profitable.

In short, the novelty of our proposal lies in the combination of the following characteristics: (1) the dense nature of the material allows it to be washable and reusable; (2) the antimicrobial agent is covalently anchored to the polymer chains, so the polymers do not lose antimicrobial activity after use and do not deliver any substance; (3) metal-free composition; (4) easy preparation procedure; (5) materials contain a high amount of a hydrophilic monomer (*N*-vinyl-2-pyrrolidone, mol 49.5%), which ensures the absorbent properties of the material, and a hydrophobic monomer (methylmethacrylate, mol 49.5%), which ensures that the material can withstand washing processes.

2. Experimental

2.1. Materials

All materials and solvents used were commercially available and used as received unless otherwise indicated. The following materials and solvents were used: methylmethacrylate (**MMA**, 99%, Merck, Darmstadt, Germany), *N*-vinyl-2-pyrrolidone (**VP,** 99%, Acros Organics, Geel, Belgium), 4-aminostyrene (**SNH2**, 98%, TCI, Zwijndrecht, Belgium), ethylene glycol dimethacrylate (97.5%, Merck, Darmstadt, Germany), hydrochloric acid (37%, VWR International, Leuven, Belgium), sodium hydroxide (99%, VWR International, Leuven, Belgium), peracetic acid (35%, Acros Organics, Geel, Belgium), sodium nitrite (99%, Applichem Panreac, Darmstadt, Germany), pork meat (Carrefour, Burgos, Spain), streptomycin thallous acetate agar (OXOID LTD, Basingstoke, England), STA selective supplement (OXOID LTD, Basingstoke, England), MRS agar (OXOID LTD, Basingstoke, England), *Pseudomonas* agar base (OXOID LTD, Basingstoke, England), ringer solution (OXOID LTD, Basingstoke, England), plate count agar (PCA, Condalab, Torrejón de Ardoz, Spain), *Listeria* agar base (Scharlau, Barcelona, Spain), glycerol (VWR International, Leuven, Belgium), tryptone bile x-glucuronide agar (TBX, Scharlau, Barcelona, Spain), Baird Parker agar base (Condalab, Torrejón de Ardoz, Spain), violet red bile lactose agar (VRBL, VWR International, Leuven, Belgium), brain heart infusion (BHI, OXOID LTD, Basingstoke, England), *Listeria* chromogenic agar base Acc. Ottaviani & Agosti (ALOA, OXOID LTD, Basingstoke, England), tellurite egg yolk emulsion (Condalab, Torrejón de Ardoz, Spain), *Staphyloccocus aureus* ATCC 29923 (American Type Culture Collection, Manassas, USA), *Escherichia coli* CECT 50 (Colección Española de Cultivos Tipo, Valencia, Spain), *Listeria monocytogenes* ILSI 9 (University of Burgos, Burgos, Spain), propidium iodide (1 mg/mL solution in water, Invitrogen, Waltham, USA), and stock solution of phosphate buffered saline (PBS 10X, Merck, Darmstadt, Germany). Azo-bis-isobutyronitrile (AIBN, 98%, Merck, Darmstadt, Germany) was recrystallized twice from methanol.

2.2. Instrumentation

Infrared spectra (FTIR) were recorded with an infrared spectrometer (FT/IR-4200, Jasco, Tokyo, Japan) equipped with an ATR-PRO410-S single reflection accessory. High-resolution electron-impact mass spectrometry (EI-HRMS) was carried out using a spectrometer (Micromass AutoSpec Waters mass, Micromass Holdings Ltd., Cary, USA) with an ionization energy of 70 eV and a mass resolving power $> 10{,}000$. ^{1}H and $^{13}C\{^{1}H\}$ NMR spectra (Avance III HD spectrometer, Bruker Corporation, Billerica, USA) were recorded at 300 MHz for ^{1}H and 75 MHz for ^{13}C using deuterated solvents such as dimethyl sulfoxide (DMSO-d_6) or deuterated chloroform ($CDCl_3$) at 25 °C.

The thermal and mechanical properties of the material were measured using thermogravimetric analysis, with 10–15 mg of the sample exposed to synthetic air and a nitrogen atmosphere at a heating rate of 10 °C/min (Q50 TGA analyser, TA Instruments, New Castle, USA); differential scanning calorimetry, with 10–15 mg of the sample exposed to a nitrogen atmosphere at a heating rate of 10 °C/min (Q200 DSC analyser, TA Instruments, New Castle, USA); and the tensile properties were analysed with 5 × 30 × 0.103 mm samples tested at 5 mm/min, dried at 60 °C for 1 h, with an inter-jaw distance of 9.44 mm (EZ Test Compact Table-Top Universal Tester, Shimadzu, Kyoto, Japan).

The weight percentage of the water taken up by the films upon soaking in pure water at 20 °C until equilibrium (water-swelling percentage, WSP) was obtained from the weight of a dry sample film (ωd) and its water-swelled weight (ωs) using the following expression: $WSP = 100 \times [(\omega s \times \omega d)/\omega d]$.

AFM-RAMAN spectra were recorded with an Alpha300R – Alpha300A AFM (WITec, Ulm, Germany) using laser radiation at wavelengths of 532 nm and 785 nm at 100 × magnification. RAMAN spectra were obtained at room temperature at 5 μm intervals on the Z-axis (thickness) to check the homogeneity of the material. Scanning electron microscopy (SEM) experiments were carried out for the material's surface and the cross-section using an electron microscope (FEI Quanta 600, FELMI-ZFE, Graz, Austria). Films were dried in air,

fractured and gold sputtered in vacuum to assure the electrical conductivity of the films.

Images of bacterial cells stained with propidium iodide (PI) were acquired using a microscope (CTR6000, Leica Microsystems, Wetzlar, Germany) equipped with an N21 (red) filter cube using the following settings: excitation wavelengths of 515–560 nm and an emission wavelength of 590 nm. Meat packaging was carried out with a modified atmosphere packaging (MAP) machine (Efabind, Murcia, Spain) coupled with an OxyBaby 6.0 gas analyser (Witt-Gasetechnik GmbH & Co KG, Witten, Germany).

The powder X-ray diffraction (PXRD) patterns were obtained using a diffractometer (D8 Discover Davinci design, Bruker Corporation, Billerica, Massachusetts, USA) operating at 40 kV with a Cu(Kα) radiation source and a scan step time of 2 s. Each spectrum was acquired from 5° to 70° using a step size of 0.05° (2θ). The wavelength of the X-ray radiation was 1.54060 nm with an intensity of 30 mA.

2.3. Antimicrobial polymeric film preparation

The starting material was prepared by the bulk radical polymerization of three commercial monomers (**VP**, **MMA**, and **SNH_2**) in different molar ratios with 0.1% mol of a crosslinker (ethylene glycol dimethacrylate) and 1% by weight of AIBN as a radical thermal initiator (**see** Scheme 1). The polymerization was carried out at 60 °C overnight in a mould (width, length, and thickness of 90 ×120 ×0.1 mm, respectively) comprised of two silanized glasses in an oxygen-free atmosphere. The films were removed from the mould and washed once with methanol and twice with water. The films were then dipped in a sodium nitrite acid solution (125 mL water, 12 mL HCl (37%), 0.5 g $NaNO_2$) for 90 min into which a benzene diazonium salt was formed. Finally, the films were dipped into a basic vanillin solution (60 mL NaOH 1 M, 40 mL methanol, 1 g vanillin) to facilitate an azo-coupling reaction. The obtained antimicrobial polymeric films were washed exhaustively with basic water and water, dried at 60 °C, and then sterilized with a UV lamp (365 nm) for 24 h.

Given the low proportion of aniline side groups in the starting materials (0.2, 1 and 2 mol%), as well as the low ratio of groups derived from vanillin in the antimicrobial polymeric films, we prepared an additional polymer with a higher molar ratio of vanillin motifs (mol 10%) to enable characterization by FT-IR spectroscopy.

2.4. Bacterial strains and inoculum preparation

In this study, Staphylococcus aureus ATCC 29923, Escherichia coli CECT, and different strains of Listeria monocytogenes were used: Listeria monocytogenes ILSI 29 (International Life Sciences Institute North America), Listeria monocytogenes ILSI 9, and Listeria monocytogenes C6 1449 isolated from a mincing machine in a poultry processing plant (Department of Biotechnology and Food Science Collection, Burgos, Spain). Staphylococcus aureus ATCC 29923 was grown onto plate count agar (Oxoid), Escherichia coli on tryptone bile X-glucuronide agar (Scharlau), and Listeria strains on agar listeria ALOA (Oxoid). One colony of each strain was transferred into BHI broth (Oxoid) and grown at 37 °C for 24 h to achieve a viable cell population of 8 log CFU/mL. Decimal dilutions were performed in Ringer solution (Oxoid) to achieve the proper inoculum concentration (5 log CFU/mL).

2.5. Experimental procedure used for the evaluation of antimicrobial capacity and efficacy

The study aimed to evaluate the antimicrobial capacity of the prepared material against different bacteria, such as *E. coli, S. aureus, and L. monocytogenes*, following established procedures (*ISO Standard 22196:2011* and *JIS Standard Z 2801:2010 +A:2012*).

The tested materials were cut into square shapes (4 ×4 cm). After sterilization with ethanol (70%), 300 μL of the bacterial inoculum (*E. coli, S. aureus, or L. monocytogenes*) was streaked onto the square pieces, and the inoculum was covered with another square piece (4 ×4 cm) of sterile polyethylene. The samples were incubated for 24–48 h at 35 °C ± 1 °C, and the bacteria were collected by sterile homogenization with 10 mL of culture broth (Ringer solution). One hundred microlitres of the homogenate was taken, inoculated in the corresponding culture

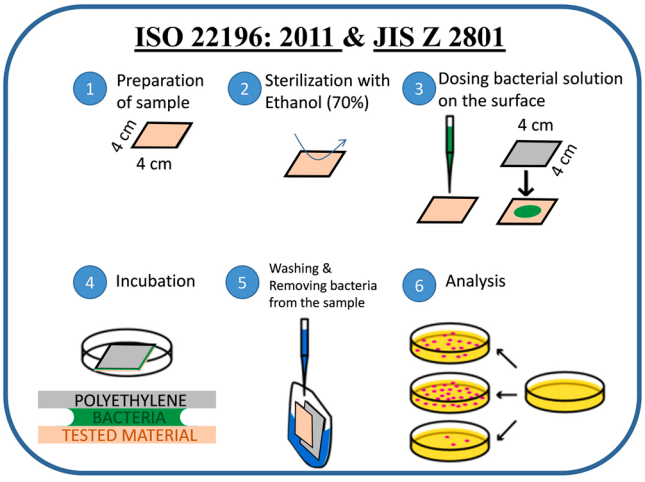
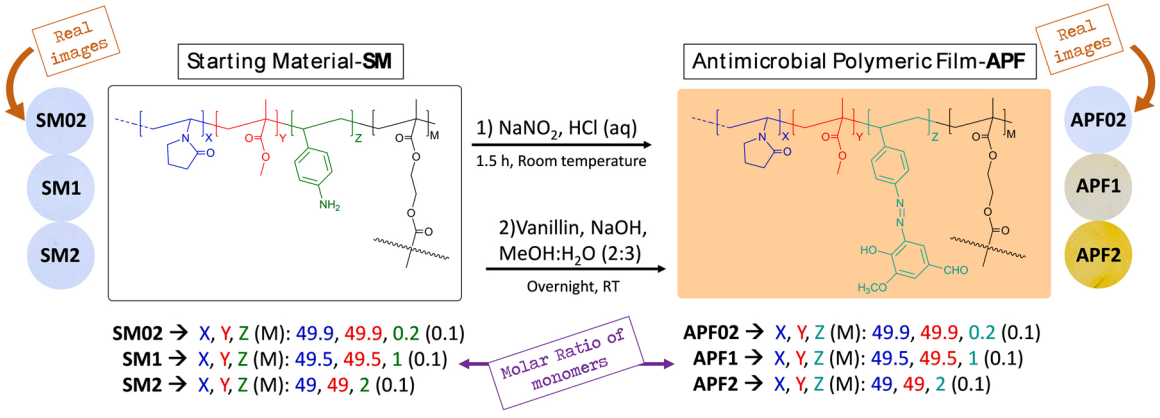


Fig. 1. Graphical abstract of the experimental procedure according to the ISO 22196: 2011 & JIS Z 2801 standards.



Scheme 1. Preparation of the antimicrobial polymer films from the starting materials. The scheme shows the molar ratios used for each polymer, and real images (adapted as circular shapes) of the films taken with a camera to illustrate the colours of the materials.

medium, and allowed to incubate for the necessary time for each bacterium (Fig. 1). The count of viable bacteria was performed by counting the colonies on the incubated plates and comparing this with the initial inoculum concentration. The antibacterial activities of the antimicrobial polymeric films were compared to the starting materials and calculated using the final counts of viable bacteria that were incubated with the antimicrobial polymeric films. Both the inhibition % and R parameter were calculated using the following formulae:

- Inhibition (%) = 100-((Final count (CFU/cm2)* 100)/(Initial count (CFU/cm2))
- R (antibacterial activity) = Ut – At
- Ut = average of the logarithmic number of viable bacteria after inoculation on starting materials after 24 h.

At = average of the logarithmic number of viable bacteria after inoculation on antimicrobial polymeric films after 24 h.

2.6. Migration study

The migration study was *carried out both qualitatively (Kirby-Bauer method) and quantitatively* (European Commission, 2011*)*. For the former, an 8 mm diameter disc of starting material **SM1** was first immersed in a vanillin solution (3 mL NaOH 1 M, 2 mL MeOH and 50 mg vanillin) to obtain a material with dispersed vanillin, i.e., non-covalent binding). The material was incubated at 37 °C for 24 h in petri dishes containing TBX culture medium with *E. coli.* The same procedure was followed for the antimicrobial polymer film **APF1**. For the latter, migration was studied as described in EU No. 10/2011 (European Commission, 2011*)* and amendments, which relates to plastic materials and articles oriented towards food contact applications. The standard test methods were carried out at high temperatures (100 °C or reflux temperature) with aqueous solutions of acetic acid (3%) and ethanol (10%), and olive oil (*UNE-EN 1186–1, 2002*, *and UNE-EN 1186–3, 2002)*.

2.7. Determination of the bacterial cell membrane integrity

The bacterial cell membrane integrity was studied by using propidium iodide (PI) and further optical microscopy analysis. Bacteria (*E. coli* and *S. aureus*) were collected from the surface of the materials used in Section 3.5 after 24 h of contact with starting material **SM1** and antimicrobial polymeric film **APF1** using 1X phosphate-buffered saline (PBS). Cells were diluted 1/100 with 1X PBS and incubated for 5 min at room temperature with 5 μg/mL PI. As a negative control, bacterial cells were grown at 37 °C for 24 h, and, as a positive control, bacteria were treated with heat at 80 °C for 4 min

2.8. Preparation of meat packages for the proof of concept

All packages were prepared the same day and with the same piece of meat to minimize experimental errors. First, 200–220 g of fresh pork loin meat was placed in hermetically sealed packages with a modified atmosphere (75% N_2 - 25% CO_2). In total, 21 meat packages were prepared, 7 **CONTROLs**, 7 with starting material **SM1**, and 7 with antimicrobial polymeric film **APF1**, and these packages were stored at 4 °C.

Samples were removed vertically with a scalpel so that the sample could be visualized as a sandwich that contained the outer Part 1 (in contact with the atmosphere), the internal part of the meat, and the outer Part 2 (in contact with **APF1**). The microbiota for these samples were evaluated at different times (0, 2, 4, 7, 9, 11, and 15 days) and for each type of packaging. This evaluation was carried out by using conventional experimental procedures in microbiology (Pascual Anderson & Calderón y Pascual, 2000; *ISO Standard 7218:2007*; *ISO Standard 6887–1:2017*).

2.9. APF1 washing process

We carried out different antimicrobial polymeric film **APF1** washing procedures to ensure that this process is cheap as possible for future applications and to achieve an environmentally sustainable washing process. Specifically, we carried out four different washing processes (WP) for the antimicrobial polymeric film **APF1** used in the proof of concept described in Section 3.7 after 15 days.

- WP-A: Antimicrobial polymeric film **APF1** was dipped in water, which was renewed four times every 30 min, and finally dried with a sterile gauze.
- WP-B: After WPA, antimicrobial polymeric film **APF1** was dipped in ethanol, which was renewed four times every 30 min. The material was finally rinsed with water.
- WP-C: After WPB, antimicrobial polymeric film **APF1** was exposed to 365 nm UV radiation for 15 min.
- WP-D: After WPA, antimicrobial polymeric film **APF1** was dipped in an aqueous peracetic acid solution (5%) for 30 min at 55 °C. The material was finally rinsed with water.

Washed materials were incubated in peptone water at 25 °C (since it is a high nutrient culture broth) overnight with orbital shaking in such a way that any residual bacteria on the material surface could grow. Then, 100 μL of the culture broth was taken and distributed on PCA culture media and incubated in aerobic and anaerobic conditions at 30 °C before the final bacterial count. These culture media are general and nonspecific, and they give an idea of the general microbial population.

2.10. Statistical analyses

Statistical analyses were performed using GraphPad Prism v8. First, the normality and homoscedasticity of the data were analysed. When the data fulfilled both assumptions, one-way or two-way ANOVA followed by Tukey's multiple comparisons test ($p < 0.05$) was run. When the data fulfilled the assumption of normality but not homoscedasticity, Welch's ANOVA followed by Unpaired t with Welch's correction ($p < 0.05$) was performed. When the data did not fulfil the assumption of normality, a parametric Kruskal–Wallis test ($p < 0.05$) was performed followed by the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli to correct for multiple comparisons by controlling the False Discovery Rate.

3. Results and discussion

3.1. Chemical characterization of the materials

As a crosslinked material, the characterization of the newly formed motifs is impossible due to the requirement of polymer solubilisation, so we studied the polymer with the higher molar ratio of vanillin motifs (mol 10%) by FT-IR spectroscopy. Thus, this film is valid as a model for understanding the changes in the material's chemical structure measured using FT-IR (Fig. S1, **ESI-Section 1**). Bands at 2270 cm^{-1} and 1577 cm^{-1} confirm the formation of both the benzene diazonium salt and azo compound in the polymer (Bustamante et al., 2019).

Our material is a 100 μm thick dense membrane for which we have made chemical changes, so it is necessary to show that the material is homogeneous after the reaction, i.e., that the reaction does not occur only in the outer part but also in the inner part of the material. RAMAN spectra confirmed the homogeneity of the material since no difference in the data was observed at different depths, as shown in Fig. S1 (**ESI-Section 1**).

3.2. Physical characterization of the materials

Scanning electron microscopy (SEM) tests were performed for the

starting materials and antimicrobial polymeric films. The results all showed the same dense structure, which is crucial to prevent the bacteria from entering the material and to demonstrate not only the antimicrobial effect of the antimicrobial polymeric film surface but also that such films are easily cleanable, washable and reusable.

Additionally, PXRD measurements for the starting materials and antimicrobial polymeric films were carried out (Fig. S1, **ESI-Section 1**). These measurements show typical amorphous haloes, which give the mean distance between the polymer chains and allow for the elucidation of the effect of the azo-coupling reaction on the mean distance (<R> = 5/8(λ/sin θ_{max}). In other words, the higher the θ_{max}, the lower the distance between the chains. This angle is increased from starting material **SM02** to **SM2**, meaning that <R> is decreased with increasing content of amino groups, indicating an increase in the interchain interactions promoted by this polar group; whereas <R> is increased from antimicrobial polymeric film **APF02** to **APF2**, showing the influence of the bulky lateral groups in increasing the distance between polymer chains.

3.3. Thermal and mechanical characterization

We consider a material to be manageable when it has suitable properties and can be easily manually manipulated without special care. In our case, given that one of the possible applications of the prepared material is as an absorbent food pad, this manageability must also be adequate when the material exists in a swollen state. Therefore, we optimized the monomer and crosslinker molar ratios to obtain materials with not only an appropriate WSP (see Table 1) but also good manageability.

The mechanical properties of the prepared materials were more objectively studied, and the resulting Young's modulus was determined to be 730 MPa. These data confirm the manageability that was visually observed upon film handling. The dimensions of the samples were chosen based on our experience with generic polyacrylic materials (Guembe-García et al., 2020, 2021).

Additionally, we carried out TGA and DSC experiments with all starting materials and antimicrobial polymeric films. Table 1 shows T_5 and T_{10} (temperatures at which 5% and 10% weight loss is observed, respectively) obtained from the thermogravimetric analysis and the glass transition temperature (Tg) obtained from DSC measurement. Both the TGA and DSC patterns are shown in Fig. S1 (**ESI-Section 1**).

3.4. Migration tests

First, as one of the potential applications of these materials is related to food packaging, we checked that no migration of any substance occurs through a study of the antimicrobial polymeric film **APF1** and starting material **SM1** by the Kirby-Bauer method (Dunkelberg, 1981). This qualitative and simple test allows for the examination of the anchoring of the antimicrobial substances that we have covalently anchored to the material, i.e., to determine if these substances are anchored, or, on the contrary, simply remain dispersed in the material. Over time, the vanillin contained in the material migrates to the solution, and a halo is observed around the disc, as shown in Fig. 2. However, when the same assay was performed with the antimicrobial polymeric film **APF1**, no halo was observed, confirming the lack of migration of the antimicrobial substances towards the culture media.

Table 1
Water swelling percentage (WSP), glass transition temperature (Tg), T_5 and T_{10} for different starting materials and antimicrobial polymer films.

	SM02	SM1	SM2	APF02	APF1	APF2
T_5 (°C)	335	332	334	327	329	330
T_{10} (°C)	349	347	350	343	345	346
Tg (°C)	140	143	145	139	144	143
WSP	35	50	35	40	65	45

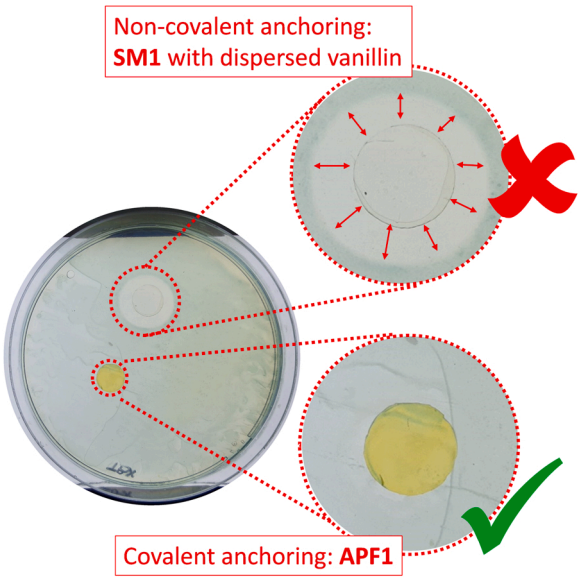


Fig. 2. Migration test for both covalently and noncovalently anchored vanillin based on the Kirby-Bauer method. The presence of a halo in the test for the starting material **SM1** with dispersed vanillin indicates the migration of vanillin towards the media.

Additionally, a migration study following the EU No. 10/2011 regulations (European Commission, 2011) and amendments shows that the antimicrobial polymeric film **APF1** complies with the restriction for the overall migration limit (<10 mg dm^{-2}), as defined in the aforementioned European regulation. Specifically, the migration results obtained for antimicrobial polymeric film **APF1** in 3% acetic acid, 10% ethanol and olive oil were 0.7, 5.9, and 4.8 mg dm^{-2}, respectively.

The chemical formulation of the antimicrobial polymeric film **APF1** mainly comprises N-vinyl-2-pyrrolidone and methylmethacrylate. Regarding the former, both the monomer (*N*-vinyl-2-pyrrolidone) and polymer (polyvinylpyrrolidone) are authorized food contact materials (FCMs), with FCM numbers of 376 and 552, respectively, according to EU No. 10/2011. Regarding the latter, crosslinked methyl methacrylate copolymers are authorized food contact materials (FCM number: 664). Moreover, methyl methacrylate copolymers with maximum level percentages for other polymers are also authorized FCMs with FCM numbers of 865–869. However, due to the material's smart antimicrobial behaviour, the EU No. 450/2009 regulation (European Commission, 2009) for active and intelligent materials and articles that are intended to come into contact with food is applied, so the material should be further optimized prior to commercialization.

3.5. Antimicrobial capacity and efficacy

The assays were performed with *E. coli* (gram-negative) and *S. aureus* (gram-positive) for the different antimicrobial polymeric films. Table 2 shows the results obtained for the initial/final count of bacteria, inhibition percentage, and antibacterial efficiency of the material expressed as "R", which should be higher than 2, as expressed in the standards.

As explained in the standards, antimicrobial polymeric film **APF02** cannot be considered an antimicrobial material since its R parameter is lower than 2, and it is worth noting that antimicrobial polymeric film **APF2** contains twice the amount of 4-aminostyrene, which is the most expensive reagent used in the polymer synthesis. Thus, we selected antimicrobial polymeric film **APF1** as the polymer with the best antimicrobial activity/cost ratio, and we chose it as an absorbent food pad for the proof of concept experiment discussed below. Due to this application, we also analysed this material's antimicrobial capacity with *Listeria monocytogenes*, since these bacteria can cause very severe

Table 2
Evaluation of the antimicrobial capacity and efficacy of APFs (ISO 22196:2011; JIS Z 2801:2010 +A: 2012).

		Antimicrobial polymeric film		
		APF02	APF1	APF2
E. coli *CECT 50**	Initial count (log CFU/cm^2)	5.46 ± 0.00 b	6.41 ± 0.00 a	5.46 ± 0.00 b
	Final count (log CFU/cm^2)	4.85 ± 0.02c	3.58 ± 0.02 d	2.19 ± 0.11 e
	Inhibition (%)	75.86 ± 0.69 a	99.85 ± 0.01 b	99.95 ± 0.02 b
	Antibacterial activity (R)	0.55 ± 0.03 a	2.77 ± 0.03 b	3.18 ± 0.08c
S. aureus *ATCC 29923**	Initial count (log CFU/cm^2)	5.41 ± 0.00 b	6.56 ± 0.00 a	5.41 ± 0.00 b
	Final count (log CFU/cm^2)	4.77 ± 0.02 c	3.44 ± 0.04 d	1.97 ± 0.03 e
	Inhibition (%)	77.05 ± 0.80 a	99.92 ± 0.01 b	99.96 ± 0.01 b
	Antibacterial activity (R)	0.58 ± 0.03 a	3.06 ± 0.03 b	3.37 ± 0.02 c

* CECT (Colección Española de Cultivos Tipo); ATCC (American Type Culture Collection), ILSI (International Life Sciences Institute North America). Data are the means of the ± SD for three replicates. Different letters indicate significant differences within the bacterial counts ($p < 0.05$, Two-way ANOVA followed by Tukey's multiple comparisons test), inhibition and antibacterial activity ($p < 0.05$, One-way ANOVA followed by Tukey's multiple comparisons test) data.

problems in the food industry (European Commission, 2005; *EFSA and ECDC, 2019*). For this assay, the antimicrobial polymeric film **APF1** showed 99.05 ± 0.06% inhibition and an R parameter of 2.00 ± 0.01.

3.6. Study of the antimicrobial effect on bacterial cells

The objective of this study carried out with propidium iodide (PI) was to characterize the effect of antimicrobial polymeric films on bacterial cells. PI is a nonmembrane permeable intercalating agent that binds DNA. Therefore, PI cannot penetrate healthy cells, resulting in low fluorescence, but can penetrate cells with damaged membranes and bind DNA, leading to a 20- to 30-fold increase in the fluorescence intensity of the cells.

As shown in Fig. 3, the fluorescence enhancement observed in the bacterial cells in contact with antimicrobial polymeric film **APF1** demonstrates that the cell membrane integrity is compromised. These results agree with previous studies in which the antimicrobial effect of vanillin was produced by destabilization of cell membrane integrity, resulting in a loss of pH homeostasis and ionic balance (Fitzgerald et al., 2004; Saibabu et al., 2020). Therefore, the covalent immobilization of vanillin does not alter the mode of action of this essential oil.

3.7. Proof of concept: antimicrobial polymeric films as absorbent food pads with biocidal activity

In this work, we wanted to demonstrate the potential of these new materials with a proof of concept experiment that is pertinent to real life applications. Undoubtedly, these materials will be tested soon as surface coatings, sanitary material coatings, fibre coatings, etc. However, the proof of concept experiment herein involves the use of the prepared material as an absorbent food pad. For this, we chose packaged pork loin steaks as an example and prepared 3 types of packaging: without an absorbent food pad (**CONTROL**), with starting material **SM1**, and with the antimicrobial polymeric film **APF1**.

All data can be found in **ESI-Section 2**, but as a summary, as shown in Fig. 4, we show the meat packages with starting material **SM1** and antimicrobial polymeric film **APF1** on Day 15 and the inhibition % for both materials at different times.

The analysed samples contain the outer Part 1 (in contact with the 75% N_2 - 25% CO_2 modified atmosphere), the internal part of the meat, and the outer Part 2 (in contact with **APF1**). Considering that bacterial proliferation occurs mainly in the external parts of meat (outer Parts 1 and 2) to colonize the internal part, our interpretation is that **APF1** exerts great bacterial inhibition in outer Part 2 but never in outer Part 1 because no antimicrobial substance is released. However, as shown by

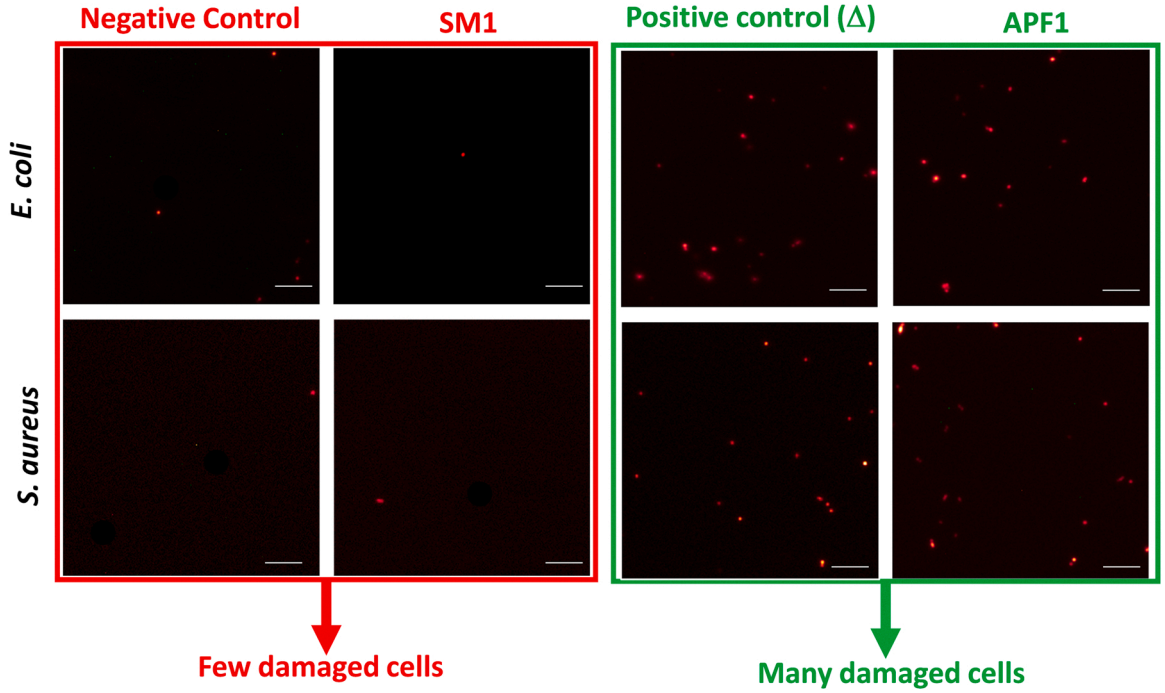


Fig. 3. Fluorescence images of *E. coli* and *S. aureus* cells after treatment with propidium iodide. Microscope images of the control and starting material **SM1** show a very low number of damaged cells (red points), while many damaged cells (red points) can be observed both in the antimicrobial polymeric film **APF1** and heated samples. Scale bars in the images correspond to 25 μm.

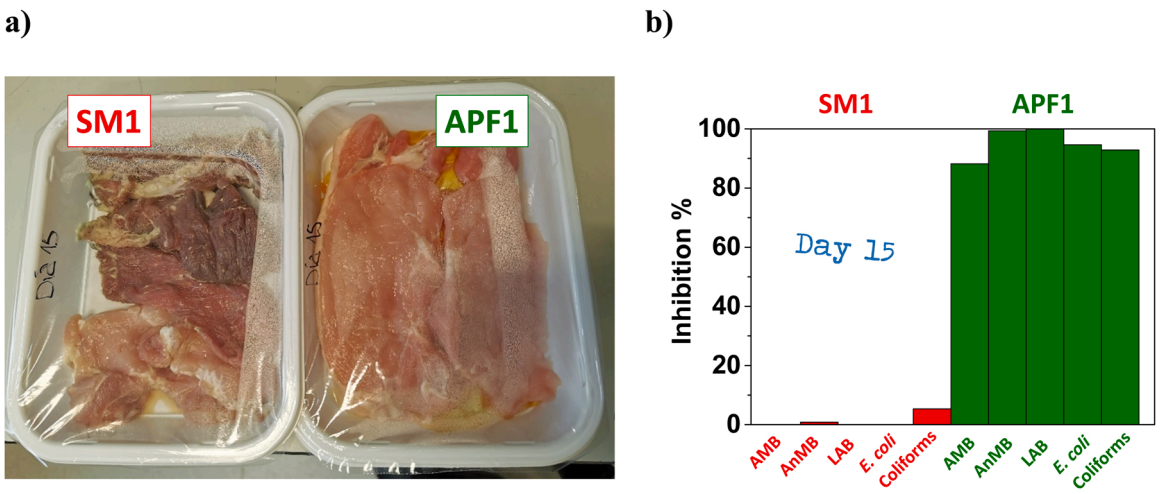


Fig. 4. (**a**) Image of packaged meat after 15 days with starting material **SM1** and antimicrobial polymeric film **APF1**. (**b**) Inhibition % for both the starting material **SM1** and antimicrobial polymeric film **APF1** at Day 15 for the studied bacteria: aerobic mesophilic bacteria (AMB), anaerobic mesophilic bacteria (AnMB), lactic acid bacteria (LAB), *E. coli*, and coliforms.

the experimental results, the antibacterial effect exerted on outer Part 2 greatly impacts the bacterial proliferation for the entire sample, probably due to the combination of the antimicrobial effect with the gel behaviour of **APF1**, which absorbs meat exudates, further delaying the formation of bacterial colonies.

As shown in Fig. 4a, after 15 days, meat's appearance remarkably changes, which correlates with the inhibition % results obtained for the studied bacteria. However, the most important result for these assays is the estimation of the shelf life for the packaged pork meat. Thus, the shelf life of the packaged meat both without packaging food pads (**CONTROL**) and using starting material **SM1** is estimated to be 10 days (considering that meat is not suitable for consumption when the count of total aerobic mesophilic bacteria is greater than 7 log CFU/g, as indicated in the literature (Chouliara et al., 2007; ICMSF, 2005; Kanatt et al., 2013; Mouafo et al., 2020). Nevertheless, by using our antimicrobial polymeric film **APF1** as the packaging food pad, the shelf life is increased to 15 days. In other words, the shelf life of the same product is increased by 50%.

A few months later, the proof of concept was repeated, following exactly the same steps. Despite different bacterial counts on Day 0, the same results for increased shelf-life were obtained. The data for this second proof of concept are shown in **ESI-Section S2**.

The obtained results have enormous impact, since the extension of the expiration date reduces food waste, generates social and environmental benefits, and increases competitiveness, profit, and employment for the companies that market this packaging.

3.8. *Reusing antimicrobial polymeric film APF1*

First, we studied the antimicrobial polymeric film **APF1** used in Section 3.7 by SEM after 15 days in contact with pork meat. Fig. 5 shows SEM images of the surface and cross-section of the material. This preliminary characterization is crucial to understanding the antimicrobial mechanism for antimicrobial polymeric films, since the dense structure of the material makes it impossible for bacteria to enter the material's interior. Therefore, antimicrobial activity occurs only via contact with the material's surface. Additionally, this dense structure makes the washing (reusability) of the material much easier, totally effective, and more pertinent to real-life applications since bacteria only contaminate the exterior of the antimicrobial polymeric films; thus, we different washing processes were developed.

Second, the antimicrobial polymeric film **APF1** was "used & washed" up to 10 times. The subsequent antimicrobial activity was studied with *E. coli* as described in Section 3.5, and the washing process was carried out by the simplest method described in Section 2.8 (washing process WP-A) since the final bacterial count for all the WPs was 0 CFU/cm^2. This means that all the washing processes were effective, even for the simple process using only water. The inhibition % and the "R" parameter for the original material were determined to be 99.85 and 2.77, respectively. After 8 "use & wash" cycles, no statistically significant difference was observed in these data, as shown in Table S5 (**ESI-Section S3**). In addition, for the case of the material after 10 "use & wash" cycles, the inhibition % and the "R" parameter were determined to be 99.76 and 2.52, respectively, so the material maintained high antimicrobial

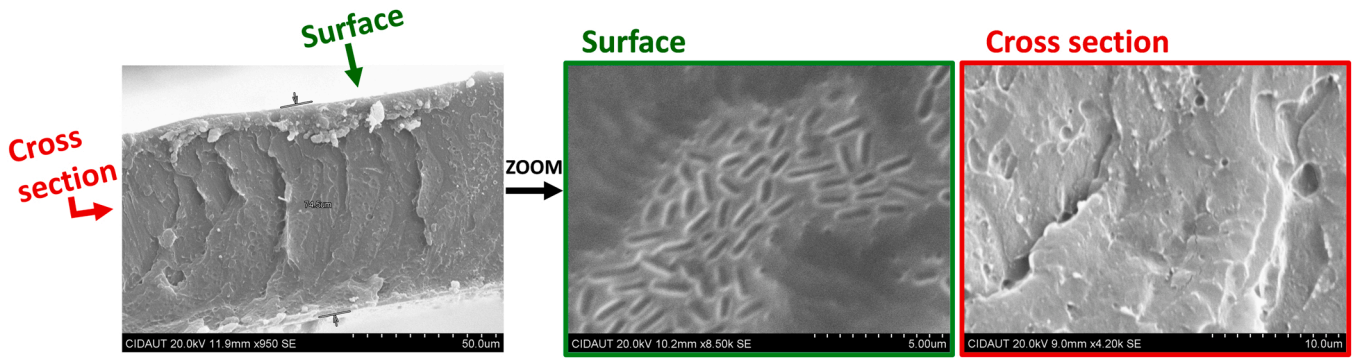


Fig. 5. SEM images of antimicrobial polymeric film **APF1**: material's surface containing bacteria (green); cross-section after fracture showing no bacteria contamination in the material's interior (red).

Table 3
Comparison of the data for different antimicrobial materials.

Material	Antimicrobial agent	Antimicrobial activity (Inhibition % or CFU/g reduction)	Reusable (Y/N)	Ref.
Poly(ethylene oxide)	Metal-free. Tea tree oil and beta-cyclodextrin	- 99.99% inhibition against *E. coli.*	–	(Cui et al., 2018; Limjaroen et al., 2016)
Polyethylene	Metal-free. Carvacrol-HNT	- 85% inhibition against *Aeromonas hydrophila.* 48% against aerobic count.	–	(Alkan Tas et al., 2019)
- Poly(ethylene-co-vinyl acetate) - Linear Low Density Polyethylene - Polypropylene	Metal-free. Isobutyl-4-hydroxybenzoate	- 6 log CFU/g reduction (LLDPE) - 4 CFU/g reduction (EVA) - 1 CFU/g reduction (PP) against *S. aureus*	–	(Cottaz et al., 2019)
Polylactide films	Metallic. Nanostructured aluminium-doped zinc oxide (AZO)	- [60–99%] against viable *E. coli*	–	(Valerini et al., 2018)
Poly(lactic acid)	Metal-free. Lauroyl arginate	- 3.43 log CFU/g reduction against *E. coli*	–	(Li et al., 2021)
Polylactide films	Metallic. Bimetallic Ag–Cu Nanoparticles and Essential Oils	- 3 log CFU/mL reduction against *S. typhimurium* counts and 1 for *C. jejuni*.	–	(Ahmed et al., 2018)
Chitosan	Metal-free. Graphitic carbon nitride	- 99.8% inhibition against *E. coli* - 99.9% inhibition against *S. aureus*.	–	(Ni et al., 2021)
Nanocellulose films	Metallic. Dextran coated silver nanoparticles	- 99.9% inhibition against *E. coli* viable bacteria and 83.3–97.8 against *S. aureus* viable bacteria.	–	(Lazić et al., 2020)
Polylactic acid	Metal-free. Nisin	- 4 log CFU/mL reduction against *E. coli* - 2 log CFU/mL reduction against *S. enteriditis*.	–	(Jin & Zhang, 2008)
Polyethylene terephthalate	Metallic. Silver nanoparticles	- Ag concentrations of 0.05 mg/L reduced *E. coli* by 10^5 CFU/mL - Ag concentrations of 0.015 mg/L reduced *E. coli* by 102CFU/mL	–	(Braun et al., 2021)
N-vinyl-2-pyrrolidone & methylmethacrylate copolymer	Metal-free. Vanillin derivative	- 99.95% inhibition against *E. coli* - 99.96% inhibition against *S. aureus*.	At least 10 times	This work

activity. This fact is a direct consequence of the covalent anchoring of vanillin motifs to the polymer. According to these results, the antimicrobial polymeric film **APF1** is easily washable, and, therefore, reusable (full data for all "use & wash" cycles can be found in **ESI-Section 3**). Table 3 depicts a selection of polymeric materials used in food packaging as antimicrobial materials, with which we compare our development in terms of antimicrobial agent, antimicrobial activity, and especially in terms of material reusability, since we have not found any publications reporting this advantage.

4. Conclusions

There is a strong need to increase the safety and shelf life of packaged food to maintain food quality and reduce food waste. Within this frame, we designed and prepared an innocuous polymeric material with antimicrobial characteristics and exploited it to increase the shelf life of packaged meat products by 50%. The antimicrobial polymer is prepared with commercially available monomers having pendant vanillin motifs, with this phenol-based essential oil acting as an antimicrobial agent. The polymer can be prepared as an absorbent food pad for packaged meat and has the following characteristics: a) it is safe, with no migration into the meat; b) antimicrobial activity occurs without the need for microorganisms to assimilate the antimicrobial agent; and c) the environmental impact of the developed film and its products is very low, since they can be reused at least 10 times by simply washing with water. The methodology used can be extended to the study of other types of phenol derivative-based essential oils, such as eugenol, thymol, and carvacrol. Additionally, the polymer nature of the antimicrobial material and its persistent antimicrobial characteristic envisage its use in other fields, such as antimicrobial coatings, fibres, nanofibres, and composites.

CRediT authorship contribution statement

Lara González-Ceballos: Methodology, Investigation, Writing – original draft. **José Carlos Guirado-Moreno:** Methodology, Investigation. **Marta Guembe-García:** Methodology, Investigation. **Jordi Rovira:** Resources, Conceptualization, Methodology, **Beatriz Melero:** Conceptualization, Writing – review & editing, Visualization. **Ana Arnaiz:** Methodology, Investigation. **Ana María Díez:** Conceptualization, Methodology, Writing – review & editing. **Jose M. García:** Resources, Conceptualization, Methodology, Writing – review & editing, Funding acquisition. **Saúl Vallejos:** Investigation, Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Visualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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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.2022.100910.

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