



Preventing the harmful biofilm increase on the polylactic-acid/Mg surface by the addition of quercetin

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ABSTRACT

Biodegradable composites with antimicrobials properties designed to reduce biofilm formation can sometimes produce the opposite effect to that expected. This is what happened with the antibacterial response of the biomaterial polylactic acid (PLA) doped with Mg: previous studies have shown that Mg is excellent for reducing early biofilm but enhances biofilm after 24 h. For this reason, a natural dopant, quercetin (Qr) is proposed in this work to prepare PLA/Mg/Qr matrices which not only prevent the increase in biofilm formation but also reduce it effectively. PLA crystallinity affects both the mechanical properties and the distribution of dopants inside the matrix and influences the degradation and release rate of the embedded compounds: a semicrystalline state places the dopants closer to the surface, it hardens less with time than the amorphous one, and it results in a higher release of the active compounds and a greater reduction of biofilm. The antibacterial character of Qr comes from its degradation products since, due to its high chemical instability, these derivatives are the ones that appear in the physiological-like environment after releasement. Exposure of the PLA/Mg/Qr matrix to physiological environments also alters its surface physicochemical properties, thus disfavoring bacterial colonization on the material. Therefore, the new sustainable antimicrobial biocomposite PLA/Mg/Qr is able to combine mechanical enhancement with antimicrobial performance.

1. Introduction

The antibacterial properties of polylactic acid (PLA) matrices enriched with magnesium particles during the initial stages of bacterial colonization have been previously reported [1]. Nonetheless, after 24 h, these matrices exhibit a concerning increase in biofilm formation compared to their undoped counterparts. This phenomenon has been attributed primarily to the rapid and explosive release of Mg within short periods, coupled with the absence of sustained antibacterial activity over time. The present study aims to mitigate this undesirable outcome by incorporating the natural compound quercetin, thereby

reducing long-term bacterial colonization on PLA surfaces mechanically reinforced with Mg particles.

1.1. Quercetin: antimicrobial action

Quercetin (Qr) is a natural flavonoid commonly found in leaves, fruits and flowers, which has gained considerable attention in recent years due to its diverse bioactive effects. These include antidiabetic, anti-inflammatory, antioxidant, anti-Alzheimer, antiarthritic, cardiovascular, wound-healing, and antimicrobial properties. The environment has also benefited from the antioxidant effects of QR:

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supplementing plant food with quercetin promotes growth and protects the plant from oxidative stress [2]. Different studies have demonstrated the efficacy of Qr in significantly reducing bacterial colonization on various surface materials, involving both Gram-positive and Gram-negative strains. The beneficial effects of Qr are primarily attributed to its free radical scavenging capacity, which underpins its antioxidant properties and promotes tissue healing [3–9]. Although recent works point out that the reasons for its anti-biofilm [3,7] action against *Staphylococci* are not exactly known, there are very interesting results which suggest that Qr acts by inhibiting the synthesis of nucleic acids and proteins and suppressing certain bacteria-bacteria signals associated with quorum sensing pathways in the bacterial membrane [8,10,11]. In addition, Qr can modify surface physical properties such as cell surface hydrophobicity, membrane permeability [7] or bilayer thickness, and can also inhibit the efflux pump through hydrogen bonds with the hydroxyl group of Qr, downregulating the intercellular adhesion (ica) locus or reducing polysaccharide intercellular adhesion (PIA) production [4,5,12,13], both of which are mechanisms of bacterial resistance. Qr has also been described to be able to suppress virulence enzyme and modulate DNA and protein synthesis. Specifically, inhibition of topoisomerase IV activity, disruption of ATP synthesis and disruption of membrane potential have been demonstrated [10,14,15]. It has also been shown in gram-negative strains such as *Escherichia coli* to reduce oxidative stress and DNA fragmentation, with increased ROS, leading to apoptosis-like bacterial death [16].

In addition to the direct action of quercetin, various studies suggest that Qr has synergistic effects when combined with other antimicrobials, allowing for reduced concentrations and overcoming resistance barriers. For example, Siri Wong et al. have demonstrated that quercetin has a synergistic effect with ampicillin against strains of *Staphylococcus epidermidis* resistant to amoxicillin, via different modes of actions: inhibition of peptidoglycan synthesis and β -lactamases activity, increase of the cytoplasmic membrane permeability and protein amide I and II, and decrease of the fatty acid content in bacterial cells [17]. A recent study has also shown the effectiveness of Qr in enhancing the bactericidal effect of tetracycline antibiotics against *S. aureus* [18]. Qr has also been used in the fabrication of silver nanoparticles to increase their potential as a potent antibacterial formulation [19].

Although Qr shows promise for preventing and treating biofilm-associated infections, three key limitations may reduce its therapeutic potential. The most significant concern is the high Qr concentrations required for efficacy, which some authors deem clinically irrelevant. Reported values range from 250 to 500 $\mu\text{g mL}^{-1}$ for amoxicillin-resistant *S. epidermidis* [20] and up to 1000 $\mu\text{g mL}^{-1}$ for *S. aureus* [21] and *E. coli* [22]. Despite the fact that lower concentrations (e.g. 2 $\mu\text{g mL}^{-1}$) can reduce bacterial viability by 60 %, they rarely achieve complete inhibition [6,12]. Combining Qr with antibiotics may enhance antibacterial effects, as Qr can destabilise bacterial membranes, improving antibiotic action and potentially overcoming resistance. The second and third limitations of quercetin (Qr) involve its poor solubility and high chemical instability in aqueous media. Various delivery systems have been proposed to address these issues, including cationic nanolipoidal carriers, electrospun hybrid fibres, cellulose nanofibers, and bioinspired nanoparticles [23]. Additionally, the reactivity of Qr has been exploited to enhance its activity by forming complexes with metal cations, facilitated by its -OH groups, which efficiently chelate ions such as zinc, copper, iron, or magnesium.

Nevertheless, Qr is more effective against planktonic bacteria than biofilm-embedded bacteria, which can resist antibiotics at concentrations of up to a thousand times higher due to their low metabolic activity and limited antimicrobial penetration [8]. To enhance Qr efficacy, it can be incorporated into biomaterial surfaces or bulk to prevent bacterial colonization and promote bacterial death through controlled release. In this context, it is worth mentioning research in biomaterial science and the food/packaging industry. Wiggers et al. proved the efficacy of Qr-loaded chitosan films against the colonization of *S. aureus* and *E. coli*

in a biomedical context [24]. Li et al. used Qr to fabricate antimicrobial composite microgels based on poly(vinyl alcohol) for potential application in wound dressings [25]. Last year Rani et al. incorporated Qr to soy protein isolate films, for the food industry, observing that they exhibit significant antibacterial activity against *Listeria monocytogenes* and *E. coli* [26]. Research on the same lines includes He et al. who prepared smart food packaging material with Qr which was able to both indicate food quality and extend shelf life [27], or the work of Olewnik-Kruszkowska et al. who integrated Qr into a PLA-PEG copolymer to achieve biocidal properties for food packaging [28].

1.2. Polylactic acid: enhancement functionality after doping

Since its commercialization in 1992, PLA has become a versatile biopolymer widely used as a sustainable alternative to petroleum-based plastics [29]. Its non-toxic degradation products make it suitable for medical applications such as sutures, tissue engineering, and regenerative medicine [30,31]. PLA's adaptability arises from its controllable bulk and surface properties, which can be adjusted by modifying its crystallinity or adding dopants [32–37]. Incorporation of Mg particles enhances its mechanical strength, resulting in composites with a Young's modulus comparable to bone, making them ideal for osseous repairs [1].

There are different strategies to confer antibiofilm properties to PLA, including doping with natural compounds, peptides, enzymes, metals, chelating agents, antibiotics [28,38], or by modifying the PLA surface chemistry through surface functionalization with polyamines by aminolysis reaction [39]. Bacterial colonization is not only influenced by the active substances released from the polymer matrix but also by the physical particularities of the surface, such as hydrophobicity, surface tension, surface mechanical properties and surface electrical charge [9,28,37,40].

An essential factor to consider in biodegradable biomaterials is their response to aging. PLA degradation initiates at its surface and gradually progresses into the polymer matrix, affecting both surface and bulk properties, including mechanical, optical, and electrical characteristics [41,42]. This degradation process depends not only on environmental conditions but also on intrinsic factors such as crystallinity, molecular weight, sample morphology, and the presence of dopants [31,42–44]. Crystallinity plays a key role in PLA degradation, as crystalline domains are less permeable to water, slowing down hydrolysis [31]. Consequently, in semicrystalline polymers, degradation begins in amorphous regions before progressing to crystalline zones.

Dopants, such as Mg, can accelerate PLA hydrolysis by increasing the matrix's hydrophilicity, enhancing its interaction with aqueous media. Mg particles also corrode in physiological environments, raising the local pH and neutralising the acidic conditions caused by PLA hydrolysis. By adjusting the size, shape, distribution, and concentration of Mg its influence on the PLA degradation rate can be controlled [45–49]. Furthermore, Mg promotes bioactivity and osseointegration by forming a protective magnesium acetate layer in alkaline conditions [50,51]. Incorporation of quercetin into PLA/Mg composites helps to counteract some drawbacks of Mg within the PLA matrix while offering its biomedical benefits. Recent findings from our group indicate that the antioxidant properties of Qr, despite its rapid degradation in physiological environments, effectively protect PLA against photo-degradation, maintaining the matrix's mechanical stability [9].

Within this framework, the objective of this study is to prevent the harmful biofilm increase on the PLA/Mg surface by reinforcing the PLA matrix with the natural compound Qr. A biofilm-forming strain will be tested on the modified PLA surfaces, while material properties such as crystallinity, surface hydrophobicity, surface free energy, Young's modulus, and chemical composition will be analysed. These physico-chemical properties will be assessed before and after degradation in an *in vitro* physiological buffer. Bacterial colonization results will be interpreted based on the combined antibacterial effects of the surface

and the compounds released from the matrix.

Based on this approach, the PLA/Qr/Mg biocomposite will be proposed as a good candidate for the manufacture of implants with dual functionality: mechanical reinforcement and antimicrobial action.

2. Materials and methods

Fig. 1 shows a diagram of the experimental design workflow for this research, which includes information from sample preparation to the methodology used to physically characterize the surface of the films and quantify biofilm formation.

2.1. Sample preparation

2.1.1. Sample materials and chemical reagents

PLA with two different degrees of crystallinity was used to manufacture the matrices: amorphous PLA (Mw = 22,100 g/mol, [52], PURASORB®, PDL 04, Corbion, Amsterdam, The Netherlands) and semicrystalline PLA (Mw = 230,000 g/mol, [53], PLA2003D, NatureWorks L.L.C., Blair, NE, USA), both used without any further purification.

The PLA solutions were deposited on acid-cleaned glass disks (25 mm diameter and 2 mm thickness). Chloroform (Sigma-Aldrich, MO, USA, purity >99.5 %) was used to dissolve the PLA particles and ethanol absolute pure (PanReac AppliChem, Barcelona, Spain, purity >99.5 %) and DMSO (Panreac Química SA, Barcelona, Spain) were used to dissolve the quercetin powder (Sigma-Aldrich, China, purity >95 %). For matrices containing magnesium, magnesium particles ($\leq 50 \mu\text{m}$ NitroParis, Castellón, Spain), were selected. Also, *m*-SBF solution was employed to degrade the matrices and was prepared according to Oyane et al. [54].

2.1.2. Preparation of PLA matrices

Fig. 2 represents a scheme of the PLA preparation. PLA matrices were fabricated by the solvent-casting method, previously described by Luque-Agudo et al. [9,40,55]. Briefly, amorphous and semicrystalline PLA granules were dissolved in chloroform using a magnetic rotator at room temperature. Then, the PLA solutions were divided in four and those corresponding to Qr, Mg and Qr/Mg were mixed with the specific dopants using a rotator stirrer (JP Selecta, Barcelona, Spain) for 30 min. Amorphous (PLAa) and semicrystalline PLA (PLAc) matrices were doped

with the same quantity of active fillers in order to compare the results. Magnesium and quercetin amounts were calculated based on the percentage by weight relative to semicrystalline PLA.

In the case of PLA/quercetin matrices, the suitable volume of a quercetin solution in ethanol of concentration 25 mg mL^{-1} was added to the initial PLA solution, to obtain a quercetin to PLA ratio of 27 % v/v. For PLA/Mg matrices, 10 % w/w of magnesium particles was added to the PLA solution. Finally, for PLA/Qr/Mg matrices, first the solution containing quercetin was prepared and then the magnesium particles were added in the proportions indicated above [9].

Once the solutions were prepared, 0.51 mL of each one was deposited onto glass disks and left to dry first at room temperature for 4 h and later in an oven at 65°C for 48 h to completely remove any remaining solvent.

It is worth noting that the solution of Qr, prepared in absolute ethanol, used to prepare the working solutions of PLA/Qr and PLA/Mg/Qr is stable when stored at 4°C , in a refrigerator. It was verified by recording the UV-Vis spectra of this solution over the storage period and comparing them with the UV-Vis spectrum of a freshly prepared solution of Qr in ethanol.

2.2. Characterization of PLA matrices

The characterization of matrices was carried out on pristine matrices (without degradation) and after 24 h [49] of degradation in *m*-SBF media, at 37°C . This degradation condition is related to the time and temperature involved in the biofilm formation process.

2.2.1. FT-IR analysis of PLA matrices

Fourier Transform Infrared Spectroscopy (FT-IR) analysis was carried out by a VERTEX 70 spectrophotometer (Bruker, Billerica, Massachusetts, USA) with a Praying Mantis cell (Harrick Scientific, Pleasantville, NY, USA). The working mode was Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTS), where irradiation does not cross the whole sample but is diffusely reflected by the outermost surface layers of only a few microns in depth and then recorded by a Deuterated Lanthanum α Alanine doped TriGlycine Sulphate (DLATGS) detector (Bruker, Billerica, Massachusetts, USA). The spectral resolution was 0.4 cm^{-1} in the $4000\text{--}400 \text{ cm}^{-1}$ spectral range studied. The visual comparisons between spectra were made with the raw data, without baseline correction, to avoid any loss of important information

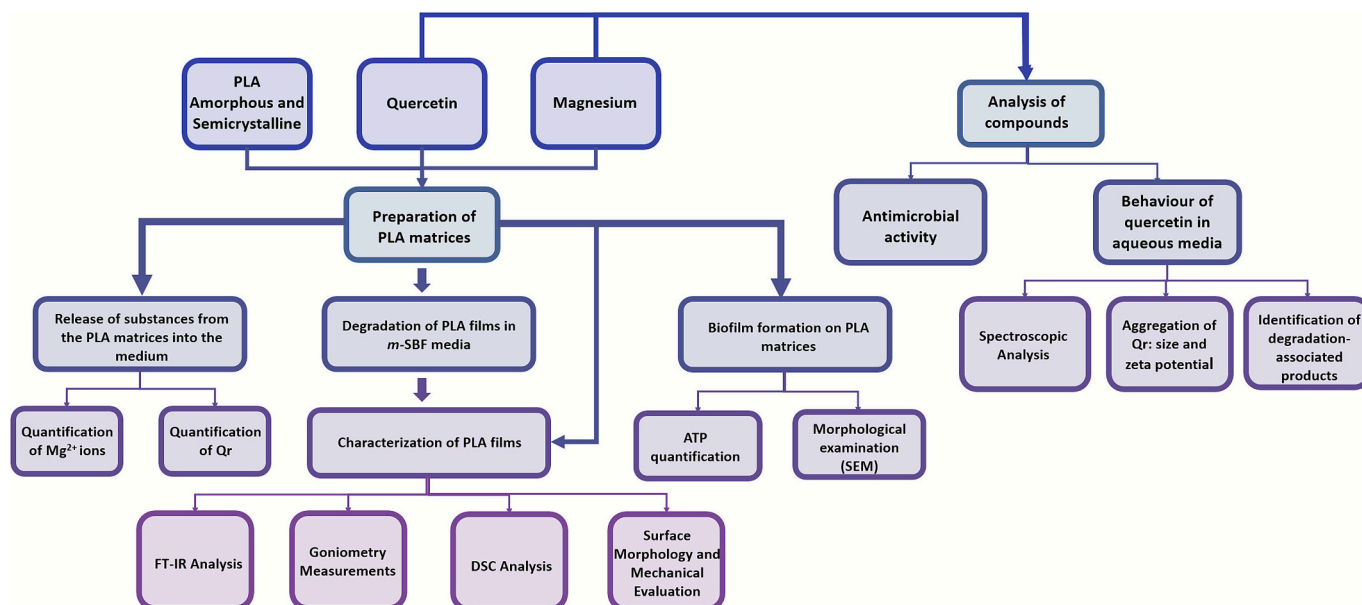


Fig. 1. Workflow of the experimental design of this research.

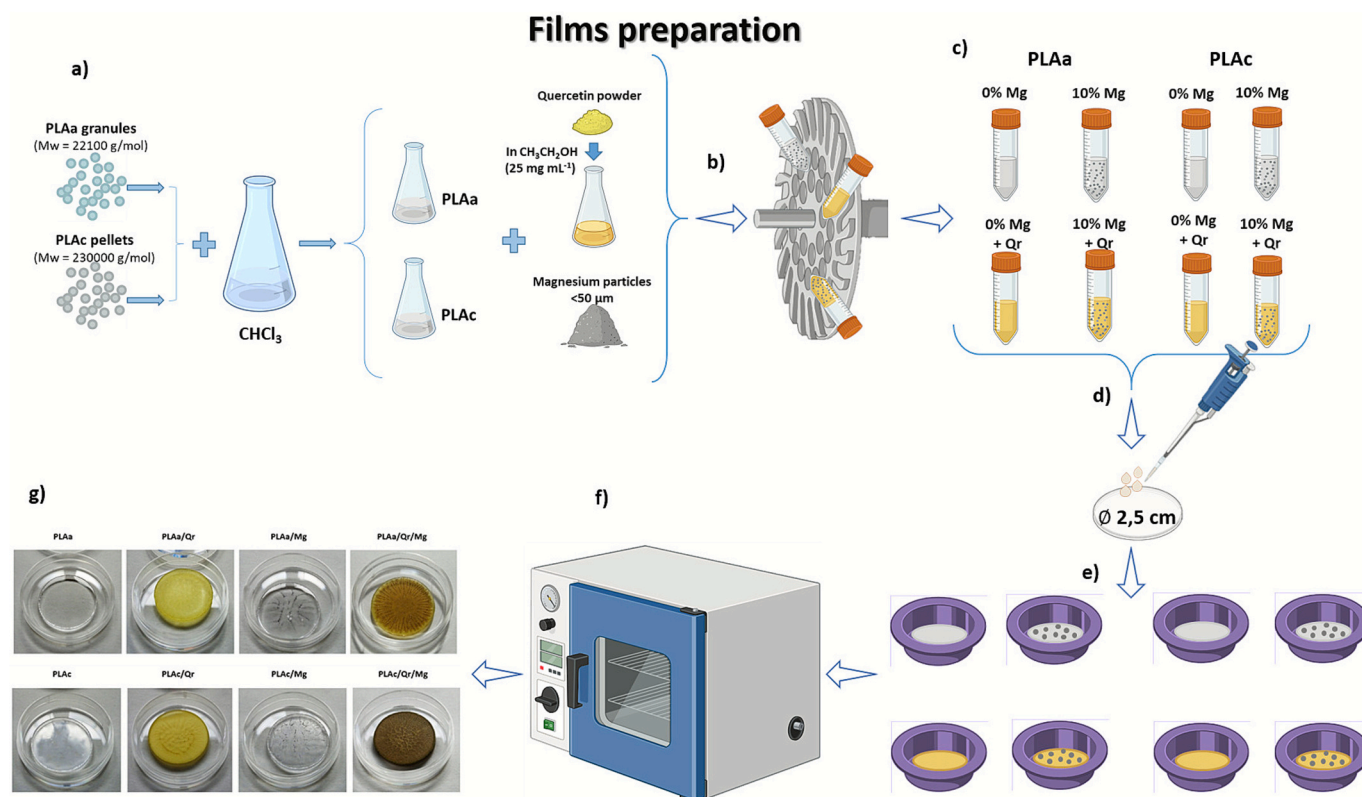


Fig. 2. Scheme of different steps of the PLA film preparation: the solution in chloroform of the amorphous and semicrystalline PLA with the different dopants (a); mixing of the dopants with the PLA solutions in the rotator stirrer (b); different solutions obtained after mixing the dopants with the PLA solutions (c); deposition of 0.51 mL of each solution on the glass disks (d); drying process at room temperature for 4 h (e); drying process in an oven at 65 °C for 48 h (f); actual images of the prepared films (g). (The actual images of the films are better observed in Figs. 1S and 2S).

regarding the possible appearance of specific bands after degradation.

The stability of Qr in PLA films containing this compound was checked by comparing the FTIR spectra of such films with the spectra of pure quercetin (the pure quercetin spectra can be found in the supplementary information of reference [9]).

2.2.2. DSC analysis

Differential Scanning Calorimetry analysis was performed with a 214 POLYMA DSC (NETZSCH GmbH, Selb, Germany). The measurements were carried out with a resolution of 0.1 μW and an enthalpy precision of ± 0.05 to ± 0.2 %. At least 3 mg from each film was obtained to make measurements between the aluminium platelets. The total flux was 100 mL min^{-1} , and the heating was performed from 0 to 200 °C or from -20 to 200 °C with a ramp of 10 °C min^{-1} . The measurements were taken in closed aluminium pans under a nitrogen atmosphere. The equipment calibration was made with control samples from NETZSCH: 2100401 (In), 22032501 (Sn), 130720 (Bi), MKCQ7668 (Zn), 20033101 (CsCl). Glass transition temperature (T_g), melting temperature (T_m) and % of crystallinity (X_c) were obtained through DSC curves by *Proteus*® software (NETZSCH, Germany). Crystallinity calculation was done by linear integration considering a melting enthalpy, ΔH_m^0 , of 93 J g^{-1} for 100 % crystalline PLA [50,56]. T_m was taken as the peak of the melting endotherm. T_g was taken as the inflection point of the specific heat decrement at the glass transition region.

2.2.3. Goniometry measurements

Contact angle measurements were performed using the sessile drop method with a Krüss goniometer (Krüss, Hamburg, Germany) at room temperature, and drop analysis was carried out with the Drop Shape Analyzer software (Version 1.12.2.06901, Krüss GmbH, Hamburg, Germany). Three liquids with different polarities were used to obtain the

surface tension of the samples: deionised water (Milli-Q Integral 5 System, Merck, Darmstadt, Germany), formamide (Fluka, Thermo Fisher Scientific, Waltham, MA, USA) and diiodomethane (Fluka, Thermo Fisher Scientific, Waltham, MA, USA), whose surface tension components are well known [57]. Five drops of 5 μL for water, 6 μL for formamide and 2 μL for diiodomethane were deposited on each of the three samples analysed per condition. Thus, the surface tension components of the matrices were calculated according to the model proposed by van Oss [57–60]. The contact angle and surface tension values will be expressed as mean $\pm$ standard deviation.

2.2.4. Surface morphology and mechanical evaluation

Morphology of the surface of the PLA matrices was evaluated by using an atomic force microscope (AFM) (Agilent AFM 5500, Agilent Technologies, California, CA, USA) operating at room temperature in contact mode. Rectangular cantilevers (FMV-PT, Bruker, Billerica, MA, USA) were used with a natural frequency of 75 kHz and a spring constant of 2.8 N m^{-1} . Four-sided pyramidal tip radius had a nominal curvature radius of 25 nm and a half-opening angle of 22.5°. Images were taken for two scanning lengths (10 $\times$ 10 μm^2 and 2 $\times$ 2 μm^2) and in each scanning area, topographical, deflection and friction images were acquired. Experiments were performed in triplicate; three different matrices were studied for each condition and three different areas for each matrix. The same cantilevers used for topography measurements were employed for the acquisition of Young's modulus values through force-distance (F-d) curves. Regarding previous technical works from AFM tip manufacturer (Bruker, Billerica, MA, USA), the spring constant value used in this work is suitable for samples with Young's modulus of MPa-GPa order [61]. The calibration and procedure methodology to obtain Young's modulus has been already published [9].

Statistical design was performed by the definition of factors

representing the different variables involved in each of the experiments. Statistical analysis was carried out with R software. Specifically, when some interaction between dopant presence, PLA crystallinity and *m*-SBF degradation was sought, a full-factorial ANOVA test was performed. All analyses were considered at a significance level of $p < 0.05$ and the main results will be presented as mean $\pm$ standard deviation. All the calculations and implementations needed to validate the ANOVA analysis and to obtain the main statistical parameters have been added to the Supplementary material (Tables 2S and 3S).

2.3. Release of substances from the PLA matrices into the medium

Release of the active substances loaded on the matrices (Mg and Qr) was performed with the help of silicone chambers that allowed control of the liquid volume and the exposed area. First, 2.3 mL of water was added, and then it was kept in an oven at 37 °C for 24 h. After this time, the supernatant was removed and the amount of Mg and Qr was quantified.

2.3.1. Quantification of Mg^{2+} ions

Inductively coupled plasma-mass spectroscopy (ICP-MS, 7900 Agilent Technologies, California, CA, USA) was used to quantify the amount of Mg^{2+} released to the medium. At least three samples per condition were used and the analysis was made with a pool of the samples.

2.3.2. Quantification of Qr

Quercetin quantification was made from calibration curves where the absorbance relative to the characteristic peak of Qr (380 nm) was plotted against different concentrations ranging from 0.001 to 0.1 mg mL⁻¹ (milli-Q water as solvent). Continuous absorbance spectra were recorded for all samples and the temporal stability of these spectra after 24 h at 37 °C was checked. The equation of the calibration curve was: $A = 11.0 [Qr] + 0.08$ ($R^2 = 0.9795$), A and $[Qr]$ being the absorbance and Qr concentration (in mg mL⁻¹), respectively. The coefficient values with their uncertainties were: 11.0 ± 0.6 (mg mL⁻¹)⁻¹ and 0.08 ± 0.03 .

2.4. Analysis of the behaviour of quercetin (and its degradation products) in aqueous media

Due to the low stability of the Qr molecule in aqueous-like media, its behaviour was followed in milli-Q water and TSB, both in the presence and absence of magnesium. Different solutions were prepared as follows: 100 µL of the stock solution of Qr in DMSO (100 mg mL⁻¹) was taken and 4.9 mL of aqueous medium (milli-Q water or TSB) was added. For Mg-containing media, 25 mg of Mg particles were also added. Each of these solutions was left in an oven at 37 °C for 3 days to ensure Mg saturation based on the work of Rodríguez-Sánchez et al. [62]. After this time, aliquots were taken for the different tests.

2.4.1. Spectroscopic analysis

A first assessment of whether there were chemical changes in quercetin after incubation in aqueous media was carried out by UV-visible spectroscopy (UV-1800 UV-VIS spectrophotometer, Shimadzu Corporation, Kyoto, Japan). For this purpose, the aliquots were scanned in the wavelength range between 190 and 900 nm.

2.4.2. Identification of degradation-associated products

Mass spectra were performed by infusion of the samples in the Agilent 6520 Accurate Mass QTOF LC/MS (Agilent Technologies, California, CA, USA) without passing through a column, with ESI-MS as ionisation source, with 4 infusions per sample (2500 V, 3000 V, 3500 V and 4000 V) in the capillary, and throughout each infusion the fragmentor voltage varied between 10 V, 40 V, 60 V and 100 V. The mass spectra of the different media allowed the identification of possible compounds present in them.

2.4.3. Aggregation of Qr: size and zeta potential

In order to discuss the possible interaction of Qr with bacteria, an analysis of the size and the interaction electrical potential (zeta potential) of Qr, when suspended in aqueous and non-aqueous media, was carried out with the help of a Zetasizer Nano (Malvern Panalytical, United Kingdom), and the zeta potential (ζ) obtained by means of the Helmholtz-Smoluchowsky equation.

Dynamic Light Scattering (DLS) was employed for size quantification using the polydispersity index as a control parameter for the sample to be characterized by DLS.

The Qr suspensions were prepared using ethanol, DMSO, and water. Mixed proportions of DMSO/water and ethanol/water were also tested to check the possible aggregation of Qr. In this sense, 50/50, 10/90, 2/98 and 0.2/99.8 v/v solutions were prepared, and the Qr concentrations of 2 mg mL⁻¹ and 0.2 mg mL⁻¹ were maintained in each one. The higher one corresponded to the higher Qr concentration in antimicrobial assays and the lower one is a 1/10 diluted solution, suitable for a good quality report in zeta potential measurements. At least three independent measurements were performed for each sample.

2.5. Antimicrobial activity of compounds

2.5.1. Preparation of compounds

The compounds loaded to the PLA matrices were tested separately and also mixed. The stock solution of quercetin was prepared by dissolving quercetin powder in dimethyl sulfoxide (DMSO). From Qr solution stock of 100 mg mL⁻¹ in DMSO, a first dilution was performed in Trypticase Soy Broth (TSB, BBL™, BD, Becton, Dickinson and Company, Sparks, NV, USA) to obtain a concentration of 2 mg mL⁻¹. Subsequent serial dilutions on microtiter plates were prepared (from approximately 1 mg mL⁻¹ to 8 µg mL⁻¹).

In relation to Mg particles, the antimicrobial activity of Mg^{2+} ions was checked since this ionic form of Mg is the one that is likely to be found when Mg particles dissolve from the PLA matrix. Mg^{2+} ions were obtained from Mg particles and MgCl₂ salt dissolved in water and TSB (culture medium of bacteria). To prepare the saturated aqueous Mg^{2+} solution, 25 mg of Mg particles was added to 5 mL of milli-Q water in a topaz-coloured flask and left, with gentle agitation, for 3 days at 37 °C. After this time, the supernatant was taken and kept for assays. A third solution, called "reaction medium" (RM) was checked by combining Qr and Mg particles. This RM was prepared as follows: 100 mg of quercetin was dissolved in 20 mL of ethanol. To this solution, a suspension of 40 mg of Mg particles in 20 mL of milli-Q water was added. The reaction was magnetically stirred and heated in a silicone bath at 35–40 °C inside the flask for three days. After this time, the reaction medium was extracted with dichloromethane (3 × 20 mL) to remove the ethanol, leaving the pure aqueous phase, which was filtered with a 0.45 µm syringe filter (Millipore, Merck, Darmstadt, Germany) and used without further purification.

2.5.2. Microorganisms and culture conditions

S. epidermidis ATCC 35983 (American Type Culture Collection, Manassas, VA, USA), a moderate biofilm producing strain [1] was inoculated on oxoid blood agar plates (OXOID, Ltd., Basingstoke, Hampshire, UK), and independent colonies were cultivated in tubes with 5 mL of TSB for the different experiments. After orbital shaking for 12–18 h at 37 °C incubation, the inoculum was adjusted to 82 % of transmittance at 492 nm in a vertical spectrophotometer (Helios epsilon Model, Thermospectronic, Waltham, MA, USA) to obtain the desired bacterial suspension, approximately 10⁶ colony forming units per milliliter (cfu·mL⁻¹).

2.5.3. Antibacterial activity

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were obtained in order to determine the *in vitro* activity of the Qr, Mg and reaction medium. The microdilution method

in 96-well flat-bottomed polystyrene microtiter plates (Greiner bio-one, Frickenhausen, Germany) was employed according to the Clinical and Laboratory Standards Institute guidelines [63].

Each well contained a total volume of 200 μL , 100 μL of antimicrobial compounds and another 100 μL of bacterial suspension adjusted as indicated in the previous section. Positive controls (inoculum in TSB only) and negative controls (antimicrobial in TSB only) were also performed. The effect of the solvents (DMSO and ethanol) was also examined.

The plates were incubated in aerobiosis and with orbital movement (20 rpm) (Heidolph, Rotamax 120, Schwabach, Germany) at 37 °C for 24 h in a Mermett oven (Model 855, Schwabach, Germany).

MIC was determined visually and by spectrometry method at a wavelength (λ) of 492 nm with an automatic microplate reader (ELx800; Bio-Tek Instruments, Inc. Winooski, VT, USA) and also visually. MBC, which indicates the amount of antimicrobial necessary to kill 99.9 % of a sample after a standard incubation period, usually 18–24 h [64], was also determined. A volume of 20 μL from the wells without visible growth was seeded on agar plates. The plates were incubated for 24–48 h at 37 °C and colony-forming units per milliliter ($\text{cfu}\cdot\text{mL}^{-1}$) were counted after the incubation period.

2.6. Biofilm formation on PLA matrices

Biofilm quantification was performed using the BacTiter-Glo™ Microbial Cell Viability Assay (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. This technique measures the amount of intracellular ATP directly correlated with the viability of

attached bacteria and allowing the detection of metabolically active cells attached to the surface. The method offers greater sensitivity and specificity with respect to traditional colorimetric methods such as crystal violet.

Fig. 3 displays a scheme of the biofilm formation process on the different PLA matrices. In particular, the bacterial suspension concentration was firstly adjusted as indicated in the previous section. The matrices, previously sterilized under ultraviolet light for 15 min, were carefully placed in sterile conditions into a tissue culture dish of 40 mm (TPP, Techno Plastic Products AG, Trasadingen, Switzerland). A silicone chamber (flexiPERM, Greiner bio-one, Germany) was fixed upon its surface in order to hold 1 mL of the bacterial suspension which was cultured on the matrices' surfaces for 24 h at 37 °C under orbital shaking (20 rpm). The selection of this biofilm formation time was based on previous work [1] and its interest lies in the fact that biofilm formation on biomaterials can occur within 24 h of insertion [65]. Postoperative prophylactic treatments beyond the first 24 h of implant insertion did not provide additional benefits in preventing infections [66].

Firstly, bacterial suspension was carefully removed after incubation and non-adherent bacteria were removed with fresh sterile PBS (twice). After that, 1 mL of the BacTiter-Glo™ Reagent per matrix was added and allowed to act for 15 min in the dark under orbital motion. The supernatant was measured by a luminometer (MicroplateFluorescent Reader FLX 800, Bio-Tek Instruments, USA) in polystyrene opaque/white sterile microplates (Greiner Bio-One) and the light emission reaction (luciferin-luciferase). Each assay was performed in duplicate and repeated at least three times with independent cultures to confirm reproducibility.

Biofilm viability data will be presented as mean and standard

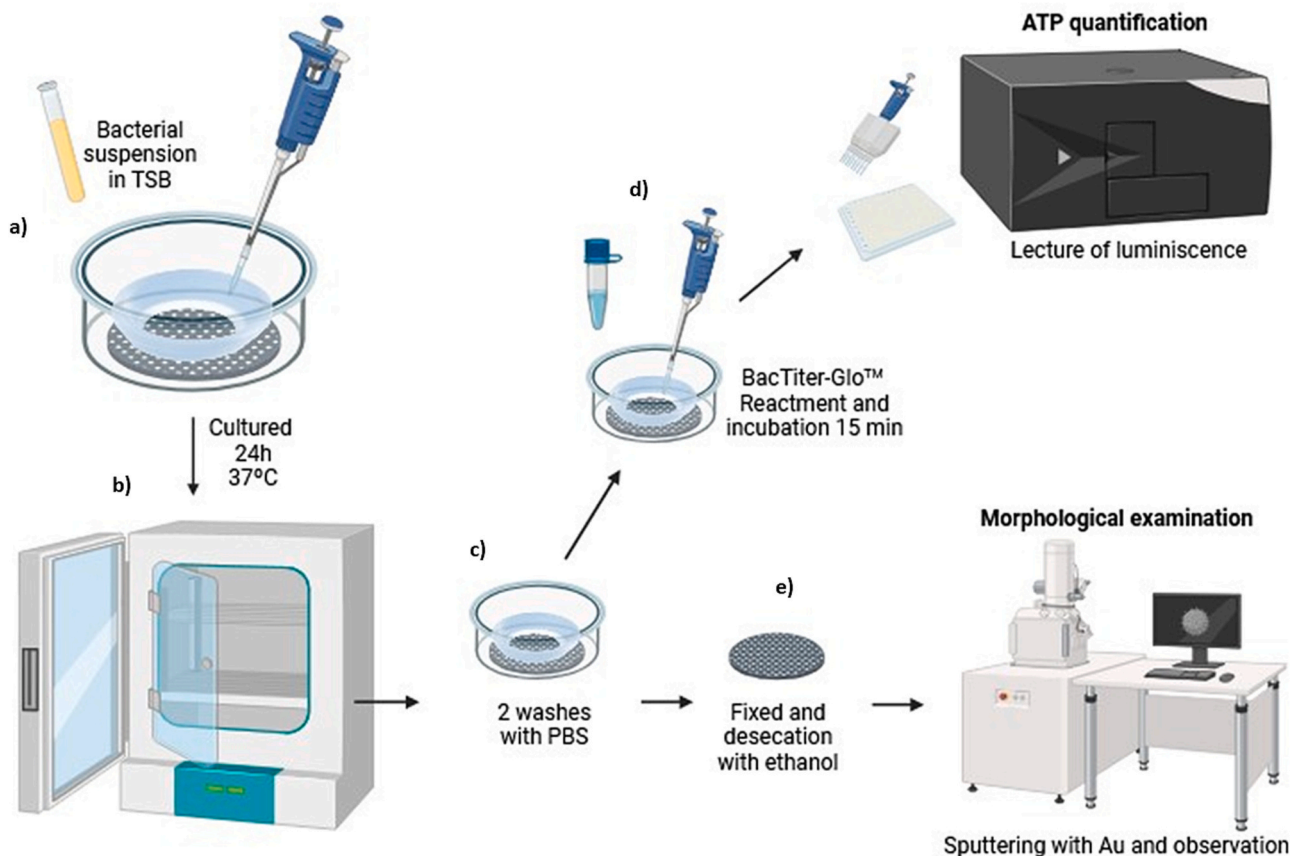


Fig. 3. Scheme of the procedure followed to study biofilm formation on the PLA matrices: PLA matrices were sterilized under UV light and placed in sterile culture dishes with Silicone chambers (a); bacterial suspension on surface incubated at 37 °C for 24 h (b); after incubation, the medium was removed and matrices were washed to eliminate non-adherent bacteria (c); adherent biofilm was quantified using ATP bioluminescence (BacTiter-Glo™) and read on a luminometer (d); in parallel, matrices were fixed with glutaraldehyde, dehydrated, gold-coated, and imaged by SEM (e). The luminescence results allowed quantification of viable biomass, while SEM provided a morphological examination of the biofilm structure.

deviation per experiment. Statistical tests were performed using R (R Core Team, version 4.5.0). The Shapiro-Wilk test was used to assess normality distribution. The Levene test was applied to confirm the homogeneity of variance, and a one-way analysis of variance (ANOVA) with the *post hoc* Dunnett test was used to compare biofilm viability of each doped PLA matrix with the undoped PLA control. The significance threshold (*p* value) was set at 0.05, which means that the differences were considered to be statistically significant at *p* values <0.05. Values within the range 0.051–0.059 suggest a trend toward significance (marginal significance) [67,68]. The results obtained have been included in Supplementary material.

A Scanning Electron Microscope (SEM) was used to visualize biofilms. For this purpose, the bacteria and biofilms were prepared as described previously [1]. After washing, the growth biofilms were fixed at room temperature with 3 % v/v glutaraldehyde (PanreacQuímica SAU, Barcelona, Spain) for approximately 15 h and dehydrated in a series of ethanol solutions (30, 50, 70, 90 and 100 % v/v) for 1 h each. The samples were then dried in a vacuum chamber, coated with a thin layer of gold (≤ 5 nm) using an EMITECH K575K (Quorum Technologies Ltd., West Sussex, UK) sputter coater, and finally, images were captured with a Scanning Electron Microscope (Quanta 3D FEG, FEI Company, Hillsboro, OR, USA).

3. Results

3.1. Qualitative surface characterization

3.1.1. Amorphous films

A visual inspection of amorphous films is presented in Fig. 1S (Supplementary material). Before *m*-SBF degradation, PLA control matrices were practically transparent with a glossy finish. When quercetin was added, the film acquired a yellowish colour and, after magnesium incorporation, the inorganic particles could be appreciated within the transparent polymer matrix following a random diffusion pattern. Likewise, when the dopant mixture was included, the matrix took on a dark-brownish yellow colour.

After degradation, the aspect of the PLAa matrices was slightly modified with a certain loss of the initial gloss. This fact is better appreciated in the PLAa and PLAa/Mg samples. When quercetin was present, there was a small variation in the yellowish tone (or dark-brownish yellow for the PLAa/Qr/Mg matrices). In essence, there was an increase of the dullness in amorphous surfaces.

3.1.2. Semicrystalline films

In the case of semicrystalline PLA matrices (Fig. 2S), control films acquired a more translucent and wavy aspect than the amorphous ones. When magnesium was incorporated into the crystalline PLA matrix, random diffusion paths could be also recognized. Once quercetin was integrated, the resulting surface acquired a uniform yellowish matt colour when added alone and turned to brown when added with Mg. After *m*-SBF degradation, there was also an increase of the surface dullness but less appreciated due to the crystalline surface texture. Surface form and waviness also increased after samples were immersed in *m*-SBF.

3.2. FT-IR evaluation

Comparing spectra of PLA films containing Qr with the spectra of pure Qr, there is no alteration in the structure of the quercetin molecule, which means that, during the fabrication process of the films, there is no degradation of the molecule (pure quercetin spectra in supplementary information of reference [9]).

To determine how the surface of PLA samples doped with quercetin, magnesium particles or both changed chemically after immersion for 24 h in *m*-SBF, FT-IR (DRIFTS) spectra were recorded before and after immersion (Fig. 4). Assignment of the most important bands of the polymer

and of each of the dopants in the control samples had been done previously [9]. Briefly, absorbance bands located at 2999, and 2945 cm^{-1} correspond to asymmetrical and symmetrical stretching modes of the -CH group. At 1787 cm^{-1} the most characteristic PLA band of the carbonyl (-C=O) group is observed. At 1469 and 1396 cm^{-1} the symmetrical and asymmetrical bending vibrations of -CH₃ are located, as well as the deformation mode of -CH at 1288 cm^{-1} . Some characteristic peaks of symmetrical and asymmetrical -C-O-C stretching, -CO stretching and -OH bending are found at 1236, 1144, 1100 and 1051 cm^{-1} , respectively. From 950 to 551 cm^{-1} , absorbance bands from -CH₃ rocking modes, -C-COO and -C-C stretching, -C=O bending and amorphous phase can be detected. Finally, in PLAc/Qr spectra, broad absorbance bands associated to hydroxyl groups in quercetin structure are located at 3431 and 3216 cm^{-1} , while absorbance bands in 1720, 1654 and 1618 cm^{-1} correspond to -C=O and -C=C (aromatic rings in quercetin) groups.

From the visual inspection of the spectra, it is noteworthy that the dopant bands are more intense in semicrystalline PLA matrices compared to amorphous PLA, which indicates that in the crystalline matrix dopants are closer to the surface than in the amorphous one. Moreover, after degradation in *m*-SBF for 24 h, an increase in the intensity of the bands is observed in the case of samples made with crystalline PLA, while there is scarcely any variation in those made with amorphous PLA. This difference in polymer behaviour as a function of crystallinity was already observed by Casares-López et al. [9] when analysing the influence of UV-C radiation on these same samples. Mainly, an increase in intensity is noticed in the band at 3400 cm^{-1} in the Qr-containing samples, which is a further indication that this compound is undergoing some transformation under these conditions.

3.3. DSC analysis

The thermal response of different PLA matrices doped with quercetin and/or magnesium degraded in *m*-SBF is summarised in Fig. 5. DSC parameters T_g , T_m , and X_c , have been evaluated and depicted in Table 1S (Supplementary material).

3.3.1. Amorphous films

Amorphous and semicrystalline PLA as provided by the manufacturer have also been included for comparisons. In the case of amorphous films, the most important information is that there was no melting zone, and it was only possible to discern a glass transition region. Despite that, visible changes were appreciated regarding dopant presence and *m*-SBF treatment. Before degradation, there was no significant change in the DSC curve after doping with quercetin and/or magnesium compared to the manufacturer curve. However, after degradation, PLAa and PLAa/Mg DSC curves underwent the most noticeable changes, the glass transition temperature decreasing considerably in the case of PLAa/Mg + SBF. Meanwhile, those DSC curves corresponding to amorphous PLA films with quercetin remained quite similar to the non-degraded conditions. There was a clear decrease in their DSC peaks, but T_g did not largely change.

3.3.2. Semicrystalline films

Analogous DSC curves were obtained with semicrystalline PLA films. Compared with the amorphous films, all the semicrystalline films showed a melting endotherm, proving the existence of a crystalline phase in these films. Before degradation, the semicrystalline films did not show important changes, only a decrease in T_m peak, compared to the manufacturer. After degradation, the undoped semicrystalline films and those containing only magnesium suffered few modifications. In PLAc + SBF and PLAc/Mg + SBF, some exothermic peaks appeared, corresponding to cold crystallization temperature values, as can be seen in Table 1S, which shows all the DSC parameters obtained from the thermal analysis and has been added to the Supplementary material document.

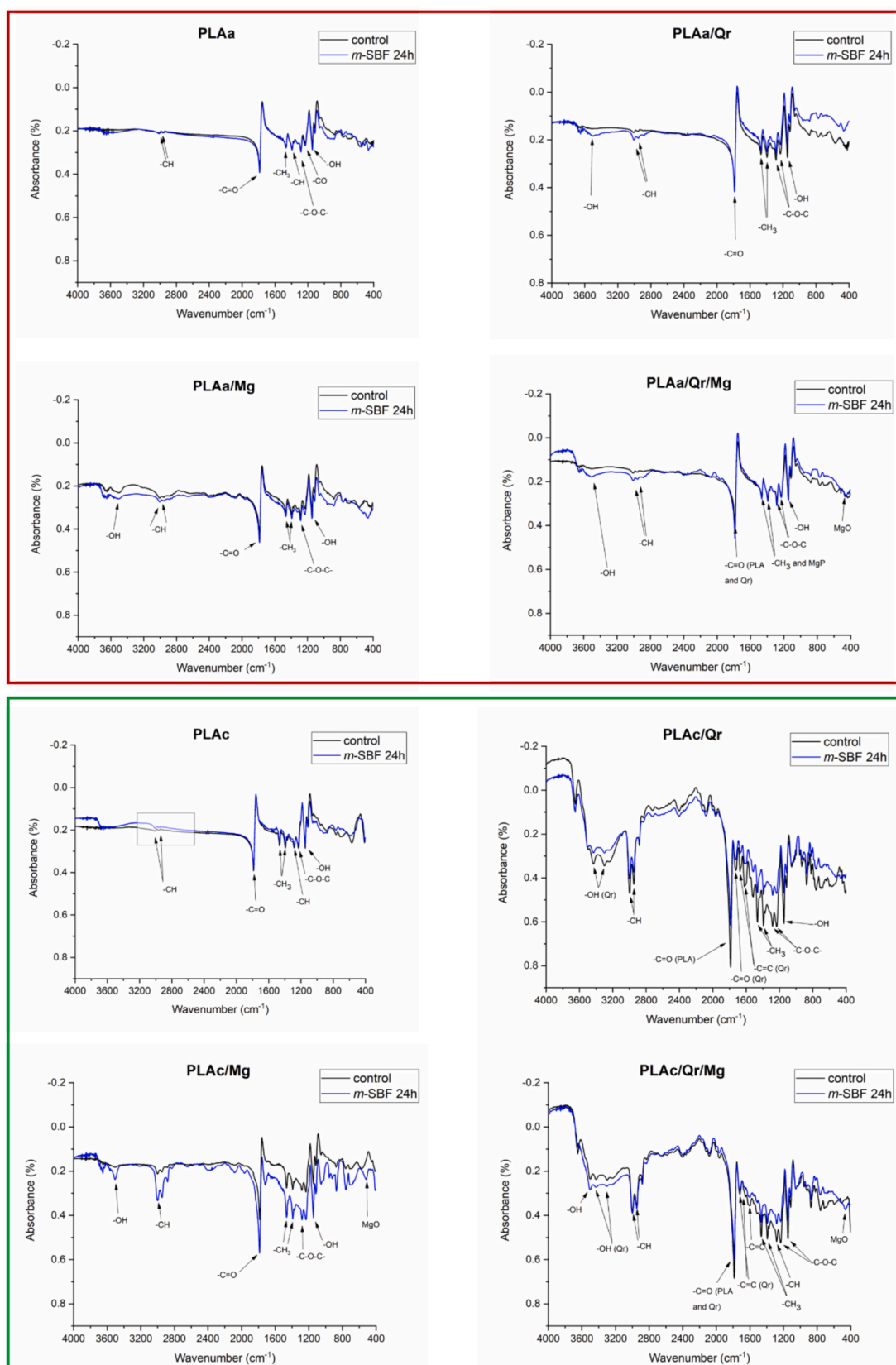


Fig. 4. FTIR spectra for amorphous PLA (in the red square) and semicrystalline PLA (in the green square) matrices. Without additives: PLAa and PLAc, after the addition of quercetin: PLAa/Qr and PLAc/Qr, after the addition of magnesium: PLAa/Mg and PLAc/Mg and after the addition of both quercetin and magnesium: PLAa/Qr/Mg and PLAc/Qr/Mg. Before degradation (black profiles) and after degradation (blue profiles).

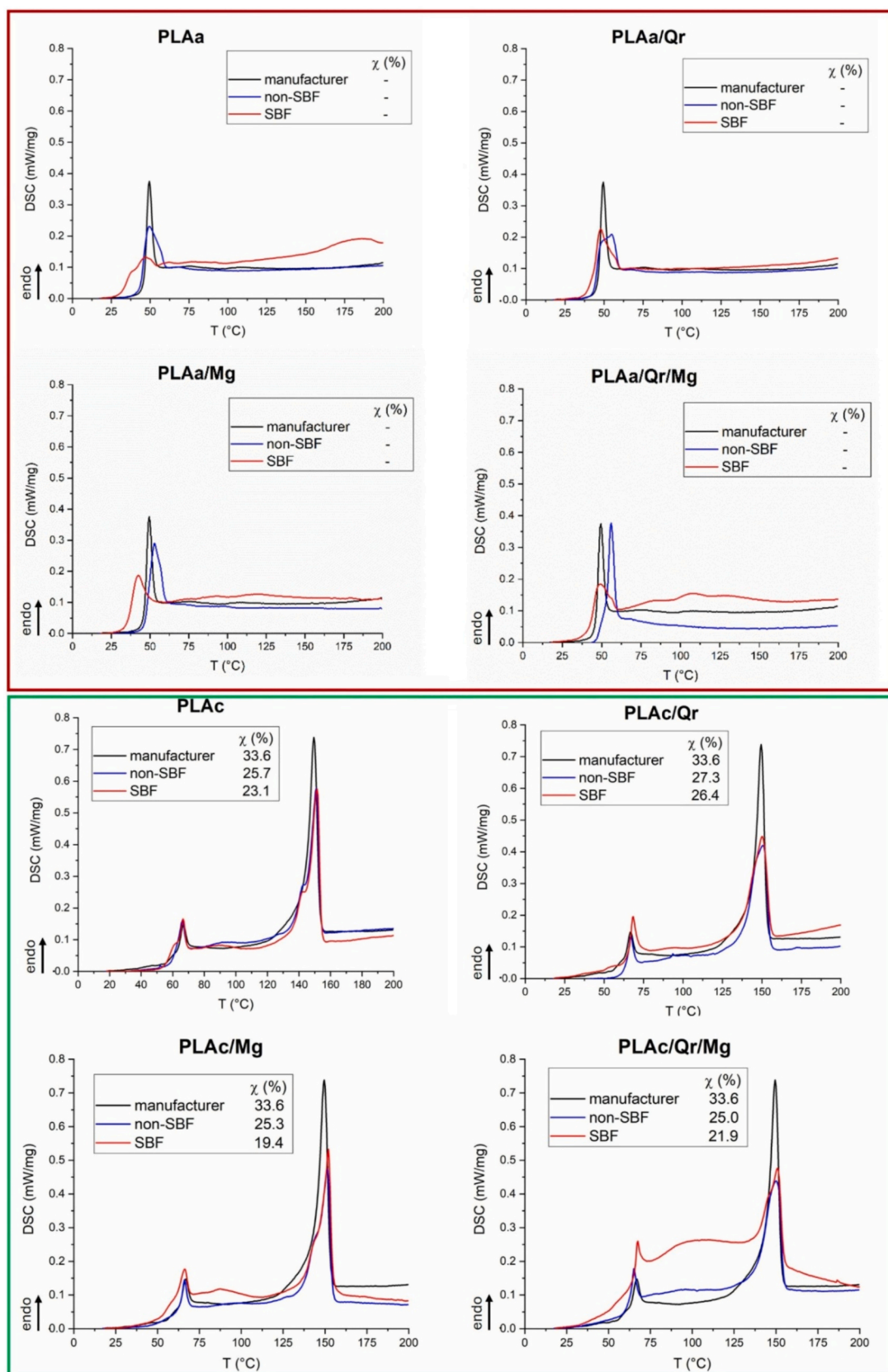


Fig. 5. DSC curves for amorphous PLA (in the red square) and semicrystalline PLA (in the green square) matrices. Without additives: PLAa, PLAc; after the addition of quercetin: PLAa/Qr, PLAc/Qr; after the addition of magnesium: PLAa/Mg, PLAc/Mg and after the addition of both quercetin and magnesium: PLAa/Qr/Mg, PLAc/Qr/Mg. Before degradation (blue profiles), after degradation (red profiles) and PLA as provided by the manufacturer (black profile).

Regarding crystallinity, the χ_c value decreases in all the samples manufactured with PLAc after immersion in *m*-SBF, the effect being higher in those containing Mg (PLAc/Mg and PLAc/Qr/Mg).

Similarly, other representations of DSC curves have been made and included in Supplementary material (Figs. 3S and 4S). These figures contain more analogous information than Fig. 4 but from a different perspective. They compare DSC curves regarding dopant incorporation in the same graphic, before and after *m*-SBF degradation, for both amorphous and semicrystalline cases.

3.4. Contact angles and surface free energy of PLA matrices

Table 1 shows the values of the water (θ_W), formamide (θ_F) and diiodomethane (θ_D) contact angles on semicrystalline and amorphous PLA matrices without and with Qr and/or Mg. There are no statistical changes in hydrophobicity (θ_W) between samples, regardless of crystallinity, the addition of active compounds or degradation state. Formamide is able to detect differences between degraded and non-degraded samples, θ_F decreasing in most cases. Conversely, with the apolar liquid diiodomethane, there is a slight increase of θ_D in most cases. It is interesting to note the increase in the standard deviations of most of the degraded samples, compared to the non-degraded ones, with the highest deviations in matrices with both active compounds: Qr and Mg.

Table 1 also provides the surface free energy of the matrices and their components (γ^{LW} and γ^{AB}) and the parameters electron-donor (γ^+) and electron-acceptor (γ^-). It is difficult to describe any difference between the matrices due to the high dispersions of the average values, especially in those with Qr and Mg, but one thing that can be noted is the increase in the mean value of the polar component γ^{AB} in the degraded matrices, mainly due to a non-zero parameter γ^+ in these cases, responsible for the mean polarity increase.

3.5. Morphology and mechanical properties of the PLA matrices' surfaces

3.5.1. Morphology examination

Fig. 6 shows the 2D-deflection error images of the amorphous and semicrystalline matrices before and after 24 h of degradation in *m*-SBF.

From a topographic point of view, the amorphous control matrices differ from the semicrystalline ones in terms of surface texture. Before *m*-SBF degradation, amorphous PLA had a homogeneous and flat surface, a few nanometers in height. When doped with quercetin and/or magnesium, the amorphous PLA roughness slightly increased in surface height distribution and in Sa values. In the case of semicrystalline matrices, the

characteristic surface texture of crystalline PLA, a smooth surface with wrinkled crystalline structures, seen in previous works, was reproduced.

These wrinkled structures seemed to remain on the PLAc/Mg surface, while doping with Qr the surface texture became softer and flatter. Mean roughness slightly increased in PLAc when Qr was added and when Mg was the only dopant, Sa changed from 33.0 ± 0.5 nm to 50.0 ± 0.4 nm.

After *m*-SBF degradation, amorphous matrices increased their Sa values and peak heights, especially in the PLAc/Qr/Mg samples, due to the appearance of some spot-like structures. Semicrystalline matrices increased their Sa values only in the case without additives, maintaining their wrinkled structures. A 3D-deflection topography images of the amorphous and semicrystalline matrices before and after 24 h of degradation in *m*-SBF has been added to the Supplementary material document (Fig. 5S) to provide complementary information to the surface characterization analysis.

3.5.2. Young's modulus measurements

The Young's modulus values are indicated in Fig. 6. A representation of this parameter for each condition has also been added to the Supplementary material (Fig. 6S), with its corresponding statistical result.

For non-degraded samples there are no significant differences in Young's modulus between semicrystalline and amorphous control matrices without additives. However, when dopants are incorporated, the conformation of the crystalline/amorphous polymeric structure determines the intensity of the observed changes: in an amorphous state there are no significant changes after introducing Qr and/or Mg, while in a crystalline state the presence of Qr or Mg decreases Young's modulus, but if both additives are added together this decrease disappears.

After degradation all amorphous matrices significantly increase their Young's modulus, reaching maximum values. In the case of semicrystalline matrices, only PLAc/Qr and PLAc/Mg increase their Young's modulus after *m*-SBF immersion, remaining unchanged in PLAc and PLAc/Qr/Mg.

3.6. Antibacterial activity of compounds: Qr, Mg and RM

In relation to the antibacterial activity of the active compounds added to the matrices, this has been tested in different ways as previously stated. In the case of Qr, it was found that MIC was in the range of $500\text{--}1000 \mu\text{g mL}^{-1}$, but Qr lacked bactericidal activity at the concentrations tested ($\text{MBC} > 2000 \mu\text{g mL}^{-1}$).

In addition, the absence of antibacterial activity of Mg particles in

Table 1

Contact angle values for amorphous and semicrystalline PLA matrices in control, quercetin, magnesium and quercetin/magnesium conditions, for non-degraded and degraded samples. Surface free energy values for amorphous and crystalline PLA matrices in control, quercetin, magnesium and quercetin/magnesium conditions, for non-degraded and degraded samples. Different components of the surface free energy (Lifshitz-van der Waals, γ^{LW} , acid-base, γ^{A-B} , electron acceptor, γ^+ , electron donor, γ^-) and surface tension of solid, γ_s , were obtained by means of the van Oss approximation.

	Samples	Contact angles			Surface free energy components				
		$\theta_W [^\circ]$	$\theta_F [^\circ]$	$\theta_D [^\circ]$	$\gamma^{LW} [\text{mJ m}^{-2}]$	$\gamma^{A-B} [\text{mJ m}^{-2}]$	$\gamma^+ [\text{mJ m}^{-2}]$	$\gamma^- [\text{mJ m}^{-2}]$	$\gamma_s [\text{mJ m}^{-2}]$
Non-degraded	PLAc	73 ± 1	62 ± 1	43 ± 1	32.6 ± 0.5	1.4 ± 0.8	0.03 ± 0.03	14.9 ± 1.4	34.0 ± 1.3
	PLAc/Qr	73 ± 1	61 ± 1	43 ± 1	32.7 ± 0.5	2.0 ± 0.8	0.07 ± 0.05	14.2 ± 1.4	34.7 ± 1.3
	PLAc/Mg	73 ± 1	62 ± 2	43 ± 1	32.6 ± 0.6	1.4 ± 1.4	0.03 ± 0.06	14.9 ± 1.9	34.0 ± 2.0
	PLAc+Qr/Mg	71 ± 2	59 ± 2	43 ± 2	32.6 ± 1.1	3.0 ± 1.7	0.15 ± 0.14	15.2 ± 2.8	35.6 ± 2.8
	PLAa	72 ± 1	62 ± 3	44 ± 1	31.9 ± 0.6	1.4 ± 2.1	0.03 ± 0.09	16.3 ± 2.6	33.3 ± 2.8
	PLAa/Qr	72 ± 1	62 ± 2	44 ± 2	31.9 ± 1.0	1.6 ± 1.6	0.04 ± 0.08	16.1 ± 1.9	33.5 ± 2.6
	PLAa/Mg	73 ± 4	64 ± 3	41 ± 4	33.3 ± 2.1	0.6 ± 2.4	0.005 ± 0.044	16.4 ± 5.6	32.7 ± 4.5
	PLAa/Qr/Mg	72 ± 2	58 ± 4	43 ± 1	32.9 ± 0.8	3.5 ± 2.8	0.23 ± 0.31	13.3 ± 3.4	36.4 ± 3.6
	PLAc + SBF	67 ± 7	49 ± 5	46 ± 4	31.4 ± 2.5	8.7 ± 5.7	1.4 ± 1.0	13.3 ± 8.2	40.1 ± 8.2
	PLAc/Qr + SBF	69 ± 3	50 ± 7	46 ± 1	31.7 ± 1.1	7.9 ± 5.1	1.3 ± 1.2	11.7 ± 5.0	39.7 ± 6.2
Degraded	PLAc/Mg + SBF	69 ± 2	54 ± 2	49 ± 2	29.8 ± 1.1	7.5 ± 2.1	1.0 ± 0.4	14.0 ± 2.6	37.3 ± 3.2
	PLAc/Qr/Mg + SBF	67 ± 9	55 ± 9	52 ± 3	27.7 ± 2.5	8.5 ± 9.2	1.1 ± 1.5	17.0 ± 12.9	36.3 ± 11.7
	PLAa + SBF	73 ± 2	49 ± 3	46 ± 2	32.7 ± 1.1	7.1 ± 2.3	1.7 ± 0.6	7.4 ± 2.1	39.8 ± 3.4
	PLAa/Qr + SBF	69 ± 3	48 ± 3	47 ± 2	31.4 ± 1.2	8.8 ± 2.9	1.8 ± 0.6	10.6 ± 3.4	40.2 ± 4.1
	PLAa/Mg + SBF	73 ± 1	52 ± 1	50 ± 2	30.3 ± 1.1	7.5 ± 1.7	1.6 ± 0.3	8.7 ± 1.0	37.8 ± 2.2
	PLAa/Qr/Mg + SBF	59 ± 11	46 ± 6	53 ± 5	26.7 ± 3.2	14.5 ± 9.7	2.5 ± 1.6	20.7 ± 14.7	41.2 ± 13.0

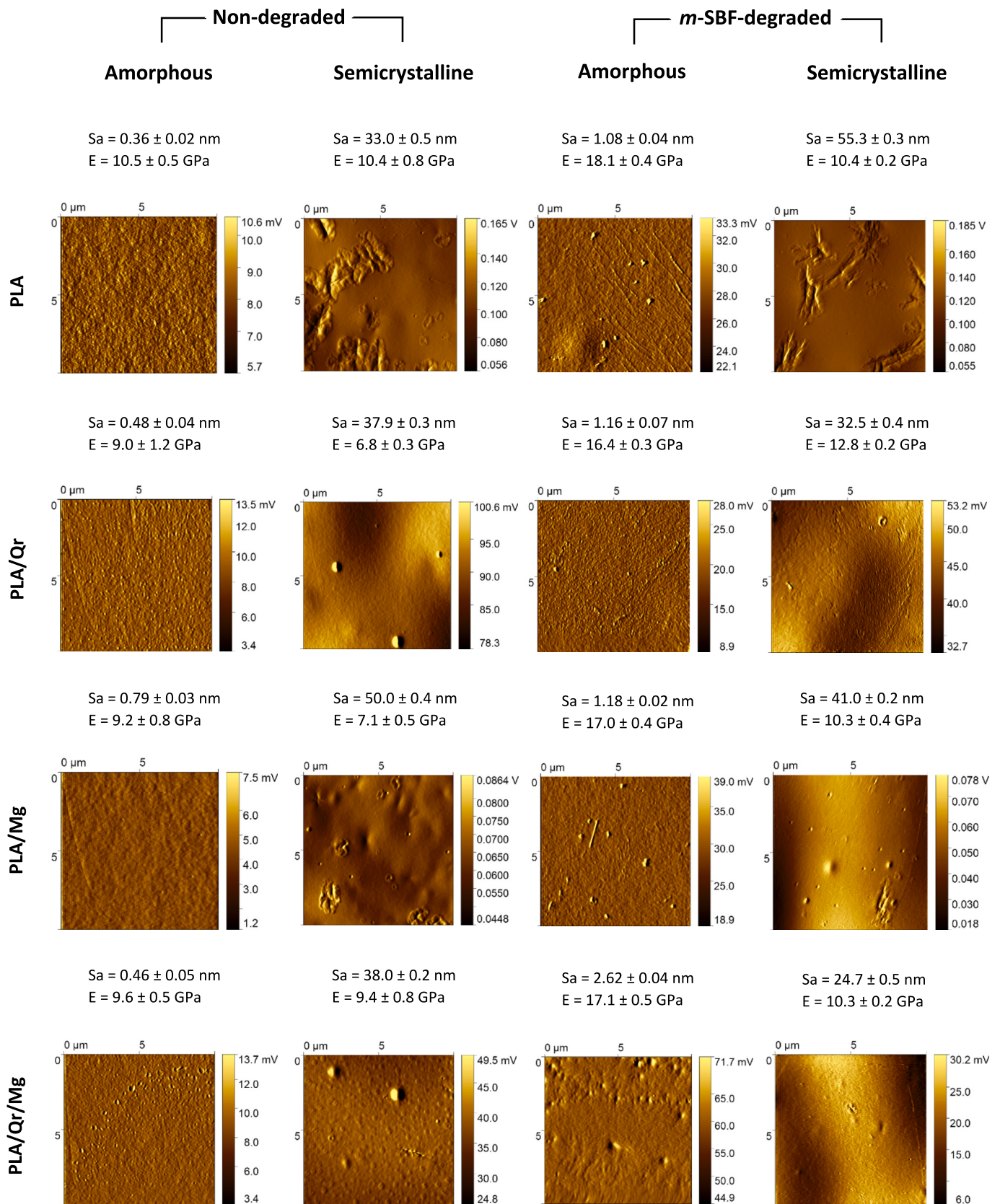


Fig. 6. 2D-deflection images of the amorphous PLA (amorphous) and semicrystalline PLA (semicrystalline) matrices in control (PLA), quercetin (PLA/Qr), magnesium (PLA/Mg) and quercetin/magnesium conditions (PLA/Qr/Mg), from top to bottom, before (non-degraded) and after *m*-SBF degradation for 24 h (*m*-SBF-degraded). All images have been processed only with a flattening filter in order to maintain a higher degree of rawness in the data. Mean surface roughness (S_a) and Young Modulus (E) are provided for each condition.

the concentrations of the matrices' was corroborated in different ways: with saturated solutions of magnesium particles in water or TSB, and from magnesium cations obtained from dissolution of MgCl_2 . In none of these cases was a MIC detected.

Nevertheless, the combination of Qr and Mg, known as RM, did present antibacterial activity in solution. In this sense, different preparation protocols were tested including milliQ-water and TSB. For combinations of Qr and Mg, concentrations of Qr $500 \mu\text{g mL}^{-1}$ + Mg dissolution 25 %, exhibited antibacterial activity (MIC value) and at the concentration of 1000 + 50 %, the MBC was obtained. For RM, $\frac{1}{4}$ of the stock concentration produced bactericidal activity.

3.7. Biofilm formation on the PLA matrices

Fig. 7.A summarises the biofilm formation on the different PLA surfaces, PLAA on the left, PLAc on the right. Viability, measured as the amount of ATP by bioluminescence, of the bacteria in the biofilm is represented.

The increase of biofilm after Mg incorporation into PLAc, already observed in the previous work of Fernández-Calderón et al., [1] is confirmed. Likewise, biofilm formation on PLAA/Mg samples is statistically higher than the control (95 % CI: 23.94 to 114.04, $p = 0.0020$). The incorporation of Qr as dopant in both PLA films decreases biofilm formation on their surface. The highest antibiofilm activity of the Qr is observed in semicrystalline PLA samples. The combined treatment with quercetin and magnesium PLAc/Qr/Mg exhibited a statistically significant reduction in the response variable compared to the control (95 % CI: -83.83 to -13.34, $p = 0.0054$). When Qr is introduced in combination with Mg, it is observed that Qr dampens the increase in biofilm formation that Mg alone produces.

The SEM images of biofilms on different surfaces are presented in Fig. 7.B. SEM images of the surfaces of the amorphous samples, as an example, show the bacterial reduction on the PLAA/Qr surface compared to the control, as well as how the addition of Mg to PLA increases the number of bacteria attached to the surface, and finally how on the surface with Mg and Qr the biofilm formation has been considerably reduced. Also, it can be appreciated that doping with Mg even enhances the activity that PLA/Qr samples alone displayed.

3.8. Quantification of the compounds released from the matrices into the medium

3.8.1. Mg^{2+} determination

Table 2 shows the amount of magnesium released to the medium from the matrices degraded during 24 h in water. Water is used as the degradation medium because it is the one in which Qr shows the highest stability over time (compared to *m*-SBF and TSB), allowing comparisons between samples.

The amount of Mg^{2+} released from semicrystalline matrices is much greater than that released from amorphous matrices, this amount being, in any case, higher in matrices containing Qr. The presence of Qr approximately doubles the amount of Mg^{2+} released from crystalline matrices and increases tenfold the amount released from amorphous matrices.

3.8.2. Qr determination

As mentioned, Qr is not chemically stable under certain conditions (slightly alkaline aqueous medium and in the presence of oxygen), so its quantification in solution under the conditions of antibacterial activity tests is a complex challenge, as it is transformed into other species. This was verified by recording the UV-Vis spectra of Qr solutions in *m*-SBF stored at 37°C for 24 h. It was observed that the absorption maximum of Qr shifted to lower wavelengths and the mixture turned to a red-like colour, changing from its initial yellow-like aspect.

For this reason, in order to make comparisons, the concentration of quercetin released to the medium by the different polymeric matrices

was determined by using water as a fundamental-model release environment. Quantification was inferred from the previous calibration curve described in Section 2.3.2.

The amount of Qr released after 24 h of degradation at 37°C was higher in semicrystalline matrices than in amorphous ones, obtaining concentrations of $0.016 \pm 0.002 \text{ mg mL}^{-1}$ and $0.009 \pm 0.003 \text{ mg mL}^{-1}$, respectively, indicating that PLAc could degrade faster than PLAA.

3.8.3. Qr-based species inside the solution

The chemical changes of Qr-slightly basic solutions when heated can be intensified if Mg^{2+} ions are present in the medium. In this sense, Fig. 8 presents the UV-Vis spectra of Qr and of different media in which Qr is brought into contact with Mg, at 37°C , using water, ethanol and DMSO. The RM used to test the antimicrobial activity is included.

Unequivocally, Qr is degraded and transformed into other molecule (s) depending on the reaction conditions, since, in some cases, in the UV-Vis absorption spectra the absorption maximum located at $\lambda = 380 \text{ nm}$ disappears and another one appears, located at a shorter wavelength.

One of the explanations found in the literature for the displacement of the absorption maximum of Qr after heating a solution containing this compound and the cations Ca^{2+} and Mg^{2+} is the possible formation of a Qr-Mg (or Ca) complex, with a stoichiometry of 2:1 [69,70]. Attempts to synthesize this complex, starting from the published experimental procedure [69–71] and with several subsequent modifications, covered different variables, such as solvent (water, water/ethanol, DMSO), Mg/Qr molar ratio, Mg source and heating form and time. In none of the cases has the complex been described in the literature obtained.

What is observed in Fig. 8 is that Qr degradation occurs to a greater extent in ethanol and water (so-called RM) than in DMSO (Fig. 8, dark red and dark blue, respectively), indicating that the solvent is involved in the process, and, in addition, degradation is slower when magnesium particles are not added to the medium (Fig. 8, light red and light blue, respectively). In any case, the degradation of Qr leads to the loss of its B-ring, as absorption maxima are only observed associated with band II (benzoyl), located at $\lambda = 256 \text{ nm}$ (Fig. 8, inset).

When the solvent is a mixture of water and ethanol and the molar ratio Mg/Qr is 5, the absorption maximum is at $\lambda = 292 \text{ nm}$ (Fig. 8, dark red), which matches with that of 3,4-dihydroxybenzoic acid (Fig. 8, green), and which is a molecule that could be obtained by fragmentation of the Qr. However, its peak ($m/z = 154.03$) is not observed in the mass spectrum (Fig. 8S), nor is that of one of its most common fragmentations, located at $m/z = 137$. A peak does appear in the spectrum, at $m/z = 225.01$, which could be attributed to the inset structure [72,73].

Moreover, in order to obtain more information on the system Mg/Qr, ICP-MS was used to quantify the different Mg^{2+} concentrations in different incubation media. $\text{H}_2\text{O} + \text{Mg}$: $3.5 \pm 0.4 \text{ ppm}$, TSB + Mg: $213 \pm 130 \text{ ppm}$, $\text{H}_2\text{O} + \text{Qr} + \text{Mg}$: $307 \pm 40 \text{ ppm}$ and TSB + Qr + Mg: $725 \pm 60 \text{ ppm}$. Interestingly, a higher concentration of this cation was found in the media containing Qr.

3.9. Qr aggregation in aqueous media

In all Qr solutions in DMSO and ethanol tested, at both concentrations (2 mg mL^{-1} and 0.2 mg mL^{-1}) Qr was completely dissolved, with an optical absorbance close to 0 in both cases (0.001–0.003 units). However, when Qr was in contact with water, the solutions turned opaque, with absorbance values as high as 1.7 units. Table 3 shows, as an example, some DMSO/water systems at 0.2 mg mL^{-1} and combines visual and quantitative information.

Water promotes the aggregation of Qr, increasingly so as the presence of water is greater. This change in the conformation of Qr molecules is also associated to changes in the ζ of Qr. In pure solvent DMSO is practically zero and as water is added to prepare the solution, ζ becomes progressively more negative.

A similar situation occurs in ethanol where ζ of Qr in the pure solvent

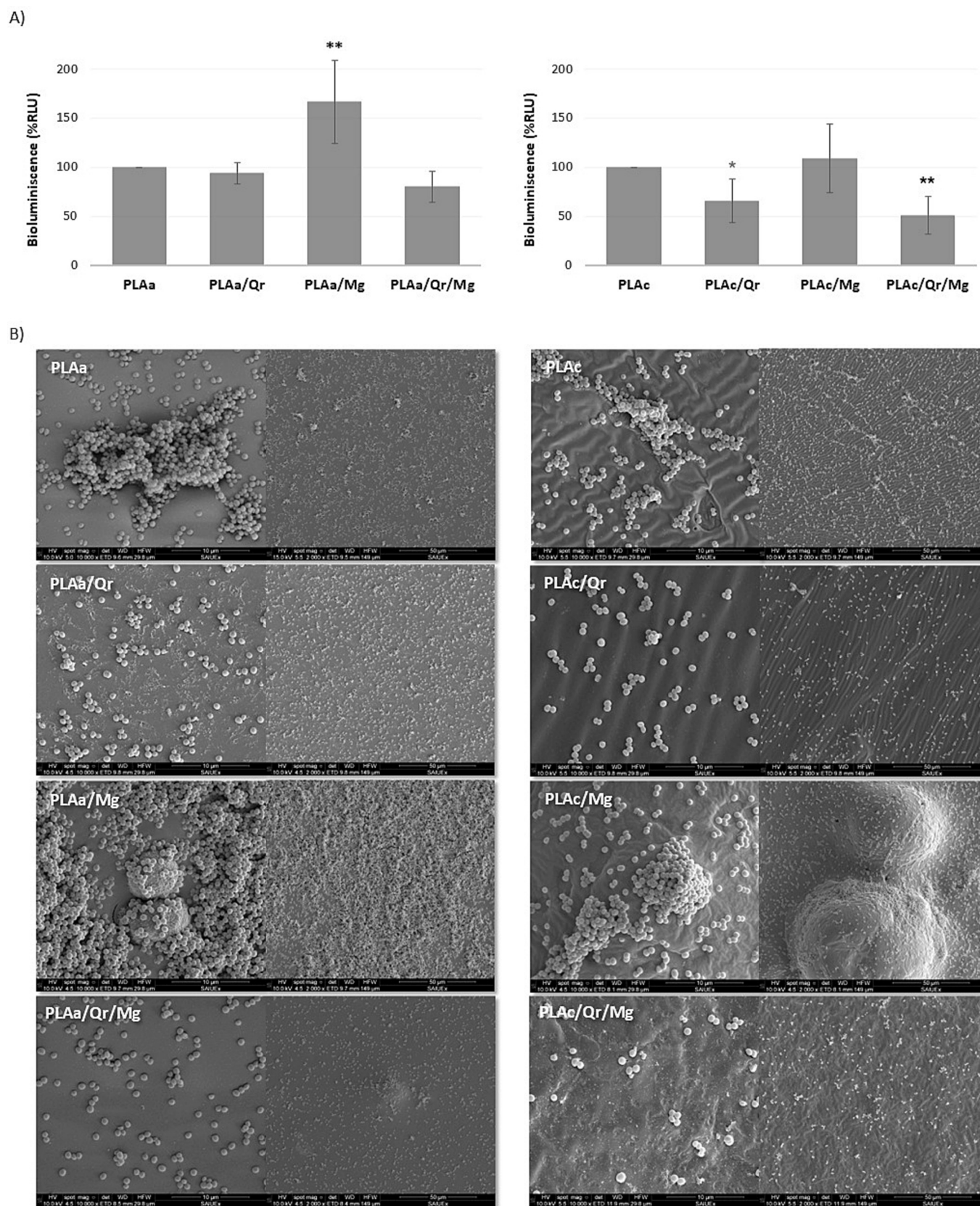


Fig. 7. Evaluation of *S. epidermidis* biofilm formation on PLA matrices after 24 h incubation (A). Quantification of biofilm viability through ATP bioluminescence assay on amorphous PLA (PLAa, left), and semicrystalline PLA (PLAc, right) under different doping conditions (control, Mg, quercetin, and Mg/quercetin). Values are expressed as mean $\pm$ standard deviation. Statistically differences compared to the undoped PLA control (**): $p = 0.0020$ for PLAa/Mg and $p = 0.0054$ for PLAc/Qr/Mg; difference close to statistically difference (*) $p = 0.0577$ for PLAc/Qr (B). Representative SEM images (2000 $\times$ and 10,000 $\times$) of biofilms developed on the surface of amorphous PLA (PLAa, left) and semicrystalline PLA (PLAc, right), illustrating the disposition of the attached bacteria.

Table 2
Determination of Mg^{2+} by ICP-MS in different incubation media.

PLA	Additive	$[\text{Mg}^{2+}]$ ($\pm 0.01 \text{ mg L}^{-1}$)
Semicrystalline	Mg	31.3
	Qr + Mg	67.8
Amorphous	Mg	1.8
	Qr + Mg	11

is $-0.3 \pm 0.3 \text{ mV}$ and when measured in water it reaches the value of $-27.1 \pm 0.9 \text{ mV}$, at which the formation of aggregates is also observed. Regarding the measurement of particle size, it was not possible to obtain a reproducible size in the suspensions containing water, the quality control of the sample before measurement was not satisfactory and the polydispersion indices were very high, sometimes reaching 0.9, which indicates a large dispersion of sizes in the samples.

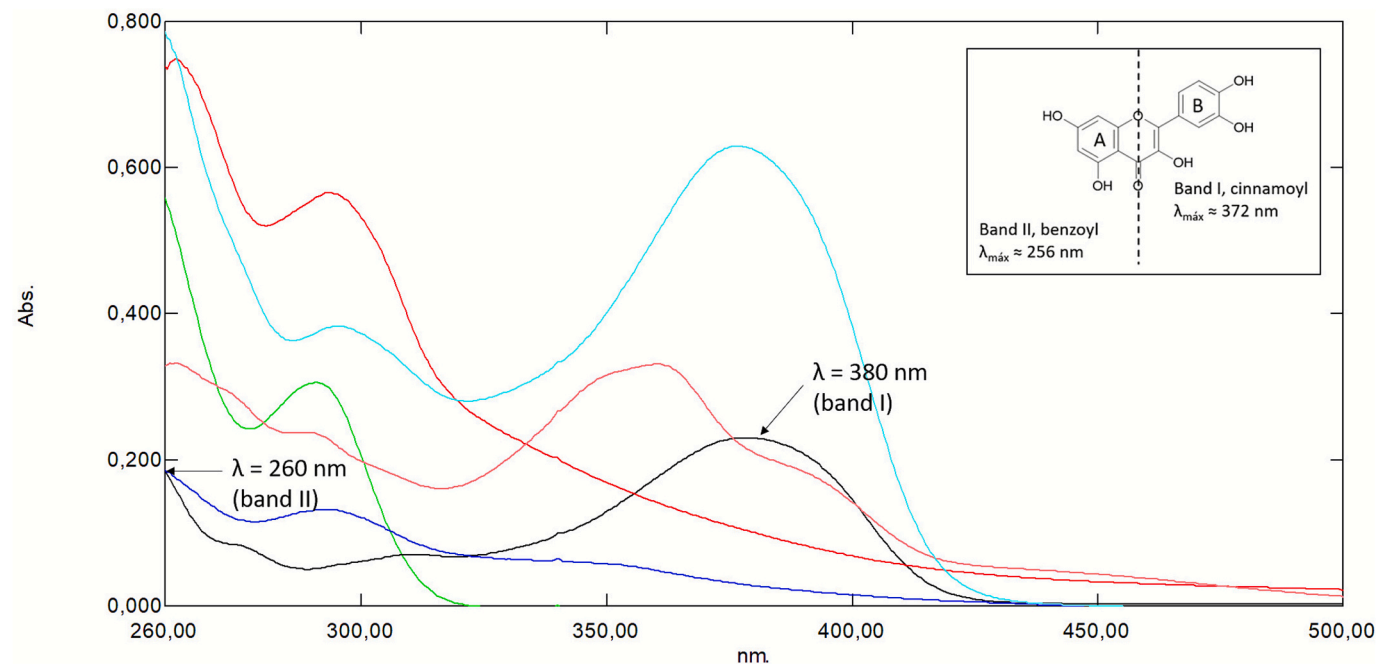
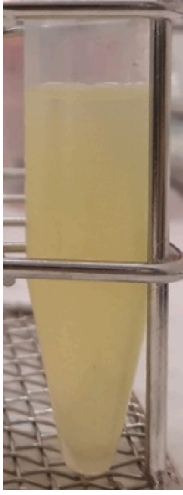


Fig. 8. UV-Vis spectra of Qr (black), of the reaction media after 3 days in EtOH+H₂O (RM) (dark red) and in DMSO (dark blue), of the reaction media after 3 days without Mg in EtOH (light red) and in DMSO (light blue) and of 3,4-dihydroxybenzoic acid (green). Inset corresponds to quercetin structure.

Table 3
Visual comparison between the different quercetin suspensions at 0.2 mg mL^{-1} changing the ratio DMSO/Water (specified in the image). Optical density (OD) measured at 492 nm and zeta potential (ζ) determination.

DMSO/water proportions			
100/0	50/50	10/90	0.2/99.8
			
OD:0.003 ζ (mV) -0.7 ± 0.4	OD:0.038 ζ (mV) -4.7 ± 1.9	OD:1.733 ζ (mV) -19.0 ± 2.0	OD: 1.338 ζ (mV) -26.3 ± 1.5

4. Discussion

Quercetin inclusion in PLA matrices not only reduces the formation of biofilms but also effectively suppress the biofilm increase observed in PLA/Mg matrices after 24 h (Fig. 7). In this sense, PLA/Mg/Qr matrices exhibit a significant reduction in bacterial colonization compared to PLA control matrices without dopants. This outcome is highly promising as it highlights the significant advances of combining Mg and Qr in the new composite material: Mg improves the mechanical properties of the polymer bulk [5,21,74] while Qr ensures antibacterial activity at 24 h of bacterial colonization. Table 4 compares the more significant results of the research and now we will explore the possible mechanisms that may be behind this fact, analysing not only the activity of the components released from the matrix but also the changes that the surface of the films may undergo during the time of bacterial colonization. While evaluating the impact of each analysed variables is challenging, their combined interaction is responsible for the observed benefits in PLA/Mg/Qr.

4.1. Antimicrobial action of released compounds

Regarding the components released from the matrix, the antibacterial activity of Qr has been analysed through the standard MIC and MBC method. Due to the low solubility of this molecule in aqueous media and its high chemical instability, its ability to aggregate in water and its possible binding to the Mg^{2+} cation to form the complexes described in the literature and which, supposedly, are responsible for the highest antimicrobial activity, have been also evaluated.

The MIC of Qr found for planktonic bacteria in this study ranged from 500 to 1000 $\mu g mL^{-1}$; although bactericidal activity (MBC) was not observed even at the highest tested concentration of 2000 $\mu g mL^{-1}$. These values are high when compared to conventional antibiotics against sensitive strains. For example, the MIC of linezolid and vancomycin is between 1 and 4 $\mu g/mL$ against *S. aureus* [75]. Clinical isolates of *S. epidermidis* also present MIC values in the order of a few $\mu g/mL$ [76]. These high MIC values may explain why complete biofilm inhibition was not achieved in any of the cases studied, aligning with previous research suggesting that Qr primarily exhibits bacteriostatic properties [5,74]. However, when incorporated into PLA matrices, Qr could still provide benefits, particularly in matrices with high crystallinity. Specifically, PLAc/Qr reduced biofilm formation by 34 % compared to PLAc, whereas amorphous matrices showed only a 6 % reduction. This effect may be linked to the greater release of Qr in crystalline matrices compared to amorphous ones, which, as indicated by FTIR results, could be attributed to a higher surface presence of Qr in semicrystalline matrices (Fig. 4).

The literature shows that antibacterial activity of Qr is quite variable, even under equal conditions and for the same bacterial strain [5,17,20,21,74,77,78]. This variability may be attributed to Qr aggregation in aqueous media, which would explain those cases where the

MIC values are notably high. It has been shown that, working with the same concentration of Qr, complete dispersion is achieved using typical solvents such as DMSO or ethanol, whereas if the solvent is mixed with water the aggregation becomes evident not only visually but quantitatively with a very significant increase in OD (Table 3). This aggregation also affects the physical properties of the Qr, which are decisive in their interaction with the bacterial wall: when Qr is fully dispersed in the solvent DMSO, its zeta potential is nearly zero, i.e. the net surface charge of the Qr under these conditions is close to zero (Table 3). This neutral charge would facilitate the approach and adhesion of Qr to the surface of the negatively charged bacteria. However, as the water content in the medium increases, the zeta potential of Qr becomes progressively more negative, due to the rearrangement of the electrical double layer surrounding Qr in this polar medium, reaching -26.3 ± 1.5 mV in nearly pure water (Table 3). The higher affinity of the hydroxyl ions of water compared to hydrons likely leads to the inner layer of the electric double layer surrounding the Qr molecule-particles being predominantly composed of negative ions. As a result, this negative electrical charge makes it more challenging for Qr to approach the bacterial surface, which is also negatively charged [79].

Aggregation of Qr may significantly hinder its ability to interact with bacterial membranes, limiting its access to molecular targets. In line with these results, a recent work in 2023 has shown that the physical properties of bacteria are altered in the presence of quercetin. Veiko et al. [14] observed an increase in both diameter and zeta potential of *S. aureus* after contact with Qr, which agrees with the aggregation of Qr in water and its negative zeta potential of Qr in polar media, as found in this research. These authors also detected a reduction in biofilm formation after contact with Qr.

Degradation of Qr in the presence of Mg has also been analysed. UV-Vis absorption spectra have shown that absorption maxima of Qr disappear in the presence of Mg (Fig. 8). Initially it was hypothesised that antibacterial activity in PLA/Mg/Qr films might be due to the formation of Qr-Mg complexes as suggested in previous studies [69,70]. However, our results show no evidence of such complex formation. Instead 3,4-dihydroxybenzoic acid was identified in RM, while the remaining degradation products have yet to be characterized, thus highlighting the complexity of the chemical degradation of Qr. Notably, this identified compound did not show activity against bacteria when acting alone, suggesting that the antibacterial activity of the PLA/Mg/Qr samples arises from the interplay of the different chemical species formed and the elevated pH values (pH 9.89) resulting from corrosion of the Mg particles. Interestingly, ICP-MS analysis revealed that Qr increases the concentration of Mg^{2+} ions in suspension across all the tested media, compared to media containing only Mg (Table 2). Based on the fact that Qr-Mg complex is not formed, it could be proposed that the higher concentration of Mg^{2+} found in media containing Qr is because the degradation of this molecule somehow dampens the rise in pH associated with the oxidation of the Mg particles and that the new molecules formed compete with the OH^- ions in the medium to form the

Table 4
Comparison table of the most relevant results and novelty.

Material	Release of components	Mechanical properties	Effect on biofilm	Effect on bacterial viability	Novelty/limitation
Pure PLA	No release of active agents	Only hardening of amorphous matrices after degradation.	No inhibition	No effect	Negative control
PLA/Mg	Mg^{2+} release: more in crystalline than in amorphous matrices	After degradation promotes the hardening of crystalline matrices. More hardening in amorphous state.	Biofilm increase	Limited effect	Not antimicrobial effect
PLA/Qr	Qr release: more in crystalline than in amorphous matrices	Similar to PLA/Mg: more hardening in amorphous than in crystalline matrices after degradation.	Some inhibition in crystalline matrix.	Reduction in viability	Some antimicrobial effect in crystalline matrices but weaker mechanics
PLA/Mg/Qr	Sustained Mg^{2+} & Qr release: more in crystalline than in amorphous matrices	Prevents the hardening of crystalline matrices.	Strong inhibition in crystalline matrix.	Strong reduction in viability	Novelty: reduction in biofilm formation in crystalline matrices with complete mechanical stability.

corresponding salts. These salts would be more soluble at this pH than $\text{Mg}(\text{OH})_2$ and MgO and therefore Mg^{2+} ions would remain in solution, except for what happens when the medium does not have Qr: in these cases, there is a greater amount of $\text{Mg}(\text{OH})_2$ and MgO precipitates. The higher concentration of positive Mg^{2+} ions in suspension, mediated by the presence of Qr, must be beneficial in favouring the electrostatic approximation and interaction of Qr-bacteria, and so promotes the antibacterial action of Qr (or degraded species), as seen in the PLAa/Mg/Qr and PLAc/Mg/Qr materials. Additionally, Qr appears to counteract the SOS bacterial stress response that PLA/Mg films show at 24 h of biofilm formation [1]: the interaction between Qr and bacteria may increase bacterial membrane permeability to Mg^{2+} , which can actively contribute to destabilizing vital functions of the bacteria both inside and on their negatively charged surface. Furthermore, Qr may chemically interfere with the polysaccharide production by inhibiting the transcriptional regulator SarA [78]. The result is a smaller and/or less active biofilm, as evidenced by the activity measurement associated with ATP quantification [80]. Fig. 9 illustrates a model of the possible mechanism of Qr on the surface of the bacterium to reduce biofilm formation.

4.2. Antimicrobial action of the films surface

In parallel to the biological and chemical activity of the species released from the films, surface changes of the material must also play a role in the accommodation of bacteria on the surface and, consequently, in the formation of the biofilm.

Following degradation, all the samples increase their surface polarity (γ^{AB}) primarily due to a significant rise in electron-accepting capacity (γ^+) (Table 1). This change is likely associated with the deposition of salts and/or ionized species on their surface, which is in agreement with

the high deviations observed in the contact angle measurements for degraded samples. Visual inspection further supports this, as a decrease in surface brightness in PLA matrices, especially in amorphous ones, after *m*-SBF degradation suggests the formation of a layer of degradation salts (Figs. 1S and 2S). A similar phenomenon was reported by Ben Abdeljawad et al. [47] who observed increased surface opacity in PLA after immersion in *m*-SBF. They reported that this modification in opacity after hydrolysis in buffered medium could be explained by various phenomena such as light scattering due to the water absorbed through the material, the formation of holes and the cracks on the surface as well as in the bulk of the samples, degradation products formed during hydrolysis, and changes in PLA crystallinity.

Crystallinity is also relevant in the surface hardening of the polymer after degradation, as shown in Young's modulus (Figs. 6 and 6S). Semicrystalline matrices are more mechanically stable after degradation than amorphous matrices, with complete mechanical preservation observed in crystalline samples containing both Qr and Mg. However, Mg alone accelerates polymer degradation by increasing structural disorder, or amorphous character of the material, as indicated by χ_c values (Fig. 5 and Table 1S). In amorphous samples, post-degradation hardening may result from greater liquid diffusion, leading to polymer compaction after drying. These findings align with previous studies on PLA degradation at similar immersion times in physiological media [43,44,51,81], which linked increases in Young's modulus after *m*-SBF immersion to surface salt deposition [51]. This hardening effect may be related to a lower capacity to release the active compounds from the matrix, explaining why biofilm formation is more significantly reduced in crystalline matrices compared to amorphous ones. This hypothesis is further supported by the higher release of Qr and Mg^{2+} ions in amorphous matrices than in semicrystalline matrices.

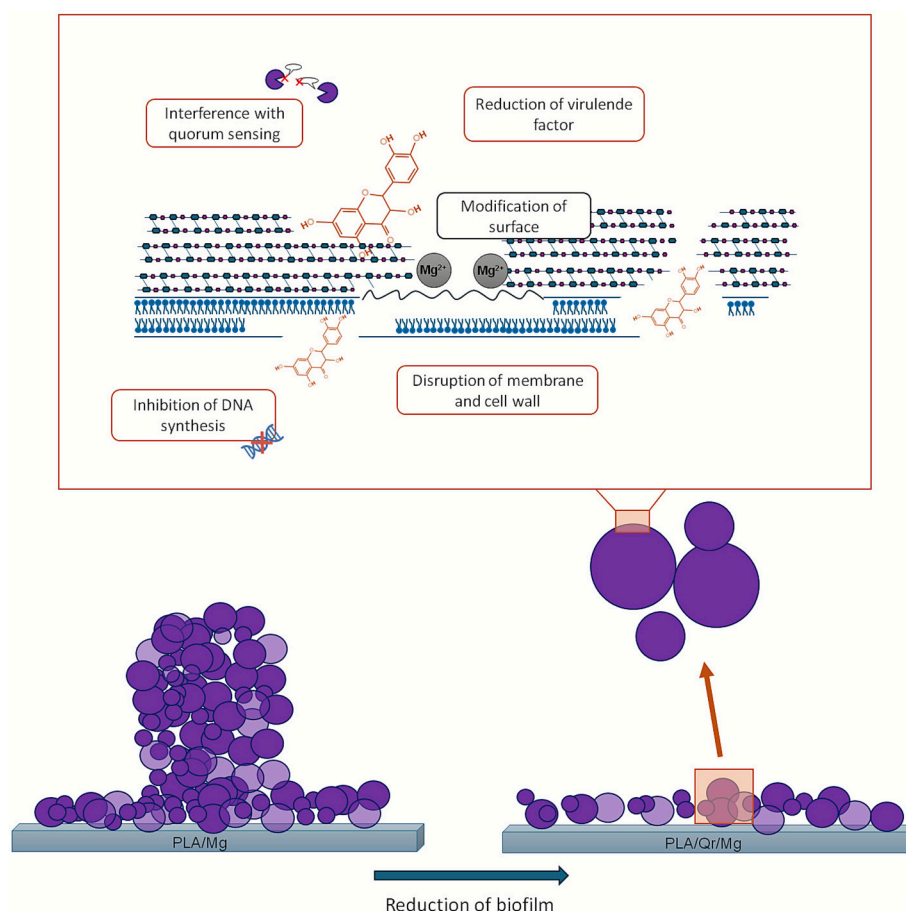


Fig. 9. Illustration of the possible mechanism of quercetin's biofilm inhibition.

Regarding thermal properties, the T_g values of semicrystalline matrices increased slightly after degradation, with the lowest values observed for PLAc and PLAc/Mg (Table 1S and Fig. 5). This is consistent with findings by Araque-Monrós et al. [43], who studied PLA in PBS solution at 37 °C for 12 months, and Ali et al. [49], who immersed PLA in c-SBF at 37 °C for 180 days. In particular, Ali et al. [49] also recorded a major decrease when magnesium was present in the PLA matrix. In contrast, amorphous matrices exhibited a clear decrease in T_g likely due to a further PLA chain scission. However, in the case of T_m values (Table 1S and Fig. 5), there is no variation of the melting point after degradation for semicrystalline matrices, in the same line as other authors [43,44]. In particular, Moetazedian et al. [44] studied the hydrolytic degradation of natural PLA filaments in PBS at various incubation temperatures (37, 50 and 65 °C) and found that DSC parameters remained almost unchanged after degradation at physiological temperatures, with T_m remaining constant beyond 250 days. They suggested that in the early stage of degradation, crystallinity changes have a greater impact on mechanical properties than molecular weight reduction due to chain scissions. The crystallinity degree results confirm those obtained from the IR spectra for semicrystalline matrices after *m*-SBF degradation: the loss of crystallinity of PLAc matrices containing Mg (PLAc/Mg and PLAc/Qr/Mg) may be caused by the catalytic action of these particles on the degradation of the polymeric bulk. In contrast, for samples containing only Qr, degradation appears to be more focused on Qr than PLA, resulting in a less pronounced decrease in crystallinity.

The lower mechanical hardening of semicrystalline matrices than amorphous ones, after degradation (as indicated by Young's modulus), and the higher thermal instability of Mg-containing matrices after degradation (as seen in DSC), are both beneficial for biofilm reduction. First, improved release of active substances from crystalline films containing Mg enhances antimicrobial activity. Second, increased surface deterioration of these films hinders biofilm formation by creating a less favourable attachment surface for bacteria.

Overall, the design of biodegradable materials for implants based on the PLA/Qr/Mg combination can be highly beneficial in the field of biomedicine thanks to its dual approach, which addresses both the mechanical reinforcement of the biopolymer and the antibacterial properties of the new material.

Finally, it is worth considering how the antimicrobial factors in this study (released Qr/Mg + degraded film surface) may be altered in an *in vivo* scenario. The presence of other contributions such as proteins, polymicrobial communities or fluid shear stress may increase the complexity of Qr degradation products and generate precursor layers, in the case of proteins, on the surface of the films, with different physical-chemical properties. It would therefore be necessary to continue with *in vivo* validation, analysing cytotoxicity testing, multi-strain biofilm modes and longer degradation times. Despite this, the good response shown *in vitro* by the PLAc/Qr/Mg biocomposite leads us to believe that the changes observed *in vitro* can be transferred to *in vivo* trials.

5. Conclusions

The results presented in this study are aligned with the idea of developing new biomaterials for implants combining sustainable antimicrobial performance.

The presence of Qr in Mg-doped polylactic acid (PLA) films effectively reduces the excessive biofilm formation at 24 h observed in films containing only Mg.

When released into an aqueous medium, Qr is chemically and structurally unstable, leading to the formation of large aggregates with a negative surface charge and the disappearance of its chemical fingerprint (UV-Vis spectra), particularly in the presence of Mg. Despite this instability, the degraded Qr species and Mg^{2+} ions interact at the membrane level, preventing the SOS response that bacteria exhibit in the presence of Mg^{2+} alone.

The crystallinity of PLA plays a crucial role in determining the extent

of these effects. In semicrystalline matrices, dopants remain closer to the surface, and the material hardens less over time during colonization. This leads to a more effective release of active components and a greater reduction in biofilm formation in PLA/Qr/Mg films.

These findings provide evidence for considering Qr as a promising additive for the design of PLA/Mg-based biomedical devices, given the effectiveness of the PLA/Qr/Mg biocomposite surface in combating early infections, especially if the polymer presents a certain degree of crystallinity.

It is always important to acknowledge that complex *in vivo* environments may influence the intensity of effects observed *in vitro*. Nonetheless, the significant differences observed *in vitro* between the different materials are likely to translate into *in vivo* applications.

CRedit authorship contribution statement

María Luisa Navarro-Pérez: Writing – original draft, Investigation, Formal analysis. **Juan Manuel Casares-López:** Writing – original draft, Investigation, Formal analysis. **María Coronada Fernández-Calderón:** Writing – original draft, Supervision, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Margarita Hierro-Oliva:** Writing – original draft, Investigation, Formal analysis. **Amparo María Gallardo-Moreno:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Verónica Luque-Agudo:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Amparo M. Gallardo Moreno reports financial support was provided by Spain Ministry of Science and Innovation. If there are other authors, they 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. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2025.149241>.

Data availability

Raw datasets are in SI

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