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A novel biodegradable active packaging based on PHB/PCL for controlling *Listeria monocytogenes* in fresh salmon

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ABSTRACT

Our study was focused on developing a novel active packaging material with antibacterial properties to ensure food safety, especially against *Listeria monocytogenes*. For this purpose, blends formed by a mixture of biodegradable polymers, polyhydroxybutyrate (PHB) and polycaprolactone (PCL), and different ε-poly-L-lysine (EPL) concentrations, were produced by extrusion. Then, active laminates were prepared by thermoforming and characterized by TGA, DSC and FT-IR. Besides, an EPL release assay into food simulants was performed and a sustained release was observed. Laminates activity was evaluated against *Listeria monocytogenes* and *Salmonella enterica*, both in liquid and solid media, where they exhibited high antibacterial activities, especially in the case of *Listeria*. To further study whether the developed materials maintained their active properties when in contact with contaminated food, a challenge test was performed on *Listeria monocytogenes*-inoculated fresh salmon. For that purpose, tray packaging prototypes containing 3 and 5 % EPL were produced by injection moulding and used to store salmon for 12 days. Microbial counts (*Listeria monocytogenes,* Lactic Acid Bacteria, *Pseudomonas* spp. and Total Viable Counts), and total volatile basic nitrogen (TVB-N) were determined over time. Results showed that active tray prototypes did not affect the quality of the stored salmon in terms of TVB-N. Moreover, they yielded significant reductions on microbial counts for all bacterial groups when compared to control samples. Thus, biodegradable antimicrobial developed trays effectively controlled *Listeria monocytogenes* and other spoilage bacteria present in fresh salmon.

1. Introduction

Ensuring safety in the food chain is a crucial issue as food contamination with pathogens can occur in all stages from primary production, processing, distribution, or preparation (CDC, 2024; EFSA, 2024a). All food types are susceptible to contamination although it is more usual in those of animal origin such as eggs, fish, meat or cheese (Heredia & García, 2018), and some common bacterial pathogens include *Salmonella* spp., *Campylobacter* spp., or *Listeria monocytogenes* (Bintsis, 2017; EFSA, 2024a). The European Food Safety Authority (EFSA) in its last report on zoonosis and associated foodborne outbreaks (EFSA, 2024b) determined that the relative number of cases, hospitalizations and deaths due to foodborne outbreaks in the European Union (EU) had increased with respect to the previous year. In that regard, taking action to reverse this tendency and ensure food safety is urgently needed.

Among all foodborne pathogens, *Listeria monocytogenes* and its infection called listeriosis, is of special relevance due to its virulence (Bintsis, 2017; Disson et al., 2021). In infections related to foodborne breaks in the EU in 2023, *Listeria monocytogenes* was the most severe, with 63.8 % of listeriosis cases ending up in hospitalizations and 11.3 % ending up in death (EFSA, 2024b). Furthermore, *Listeria monocytogenes* is known to affect, in a preferred manner, to elderly people, pregnant women, newborns and immunocompromised patients (Disson et al., 2021). This bacterial species is often found in cheeses -especially in those prepared from raw milk (Gérard et al., 2018), fish (like salmon or cod) (Zakzrewski et al., 2024), or meat products such as ham (Pérez-Baltar et al., 2021).

To control food contamination with *Listeria monocytogenes* and other pathogens in food systems, different strategies have been used, such as thermal treatments, high-pressure processing (HPP) (Cava et al., 2021),

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ultraviolet radiation (Gervilla-Cantero et al., 2024), biocontrol methods (Gutiérrez et al., 2017), or the use of chemical agents (Luu et al., 2021). Nevertheless, controlling *Listeria* spp. effectively typically requires a combination of methods, rather than a single approach, as some cells and biofilms can survive to some of these treatments (Araújo et al., 2023; Gupta & Adhikari, 2022).

Active food packaging is an example of an advantageous technology that could be used in combination with other food processing methods to reduce food contamination. For instance, antimicrobial food packaging materials incorporate different active agents that inhibit microbial growth, while maintaining food quality (Upadhyay et al., 2024). Due to their numerous advantages such as the reduction on the amount of preservatives needed or their effect on food shelf-life extension, antimicrobial packaging has recently been used in controlling different foodborne bacteria, such as *Salmonella enterica* (Rupérez et al., 2025; Wrona et al., 2023) or *Listeria monocytogenes* (Khalil et al., 2024; Ramos et al., 2024) in food products. A wide range of antimicrobial agents have been explored and used in the last decades, such as metal nanoparticles, citric acid or essential oils (Yang et al., 2022; Wu et al., 2017; Clemente et al., 2016). Nowadays, antimicrobial peptides have gained attention due to their biological origin and broad range of antimicrobial action (Liu et al., 2021). An example of an antimicrobial peptide which is currently at the cutting edge in food packaging applications is ε-poly--L-lysine (EPL) (Cai et al., 2024; Zhang et al., 2023), a peptide produced by several microorganisms, mainly in those from the genre *Streptomyces* (Wang et al., 2022), and Generally Recognised As Safe by the U.S. Food and Drug Administration (U.S. FDA, 2011). EPL has shown excellent antimicrobial properties against a wide range of microorganisms, including bacteria, yeasts and moulds (De Sousa et al., 2024; Jiao et al., 2020; Padilla-Garfias et al., 2022). In the case of bacteria, it is particularly effective against those Gram-positive (Ranjbar et al., 2023), such as *Listeria* spp., although some Gram-negative species are also sensible to EPL (Geornaras & Sofos, 2005; Liu et al., 2020; Rodrigues et al., 2020).

In the food industry, polymers are usually the main components of packaging. Due to the environmental and safety concerns derived from plastic pollution, switching to biodegradable polymers instead of conventional persistent plastics is becoming more and more a sound alternative to develop ecofriendly packaging (Sani et al., 2021). Poly-(3-hydroxybutyrate) (PHB) and polycaprolactone (PCL) are examples of biodegradable polymers that have recently gained attention from the industry. PHB is a rigid thermoplastic produced in nature and industry by different microorganisms such as bacteria and algae (Popa et al., 2022). In the case of PCL, it is a synthetic polyester with a biodegradability on a time basis of weeks or months (Krasowska et al., 2016). Due to its low fusion temperature around 60 °C, PCL is mainly used in combination with other polymers (García-García et al., 2017; Shi et al., 2022). For instance, it has been used in combination with PHB for enhancing thermal and mechanical properties such as flexibility (Garcia-Garcia et al., 2016) or water barrier properties for flexible applications (Ramos et al., 2023). In active food packaging applications, Correa et al. (2017) studied the use of PHB/PCL with a combination of organoclays and nisin in controlling the meat-spoilage bacteria *Lactobacillus plantarum*. However, to the extent of our knowledge, a blend of PHB/PCL with EPL, a potent natural antibacterial agent, has not been previously reported. Our hypothesis is that, by including this antimicrobial peptide in the biodegradable blend, it could inhibit the growth of *Listeria monocytogenes* and other foodborne pathogens and thus serve to control food contamination along the food chain distribution and retail levels.

Thus, the purpose of this research was to develop an antimicrobial packaging made of a mixture of biodegradable polymers (PHB and PCL) containing EPL as the active agent and evaluate its effectiveness against *Listeria monocytogenes* in artificially contaminated fresh salmon. Furthermore, as many studies on developing active packaging with biodegradable polymers use laboratory scale procedures or techniques that cannot be scaled-up such as solvent-casting or spin coating (Briassoulis et al., 2022; Diken et al., 2022; Shi et al., 2022), hindering their transition to industry, our antimicrobial packaging design is based on techniques already used in the food packaging industry such as extrusion and injection moulding.

2. Materials and methods

2.1. Materials

ε-poly-L-lysine hydrochloride (EPL; CAS: 28211-04-3) (3500–4500 Da) was provided by BioSynth Ltd (United Kingdom). For the material preparation, two polymers in pellet form were used: polycaprolactone and poly(3-hydroxybutyrate). Polycaprolactone CAPA 6250 (PCL) was supplied by Perstorb (Malmö, Sweden). After confirmation by NMR-H analysis, the commercial product BIO-FED GP1012, acquired in M·VERA® (Cologne, Germany) was used as poly(3-hydroxybutyrate) (PHB).

2.2. Methods

2.2.1. Masterbatch preparation

A masterbatch containing a mixture of PCL/PHB and EPL was prepared by extrusion using a 16 mm co-rotating twin-screw extruder (LabTech Engineering Company Ltd, Thailand) with a two-step extrusion. Firstly, a PCL/PHB (50:50 w/w) blend was prepared by extruding together a mixture of PCL and PHB previously dried (overnight at 50 and 70 °C, respectively) to reduce moisture. Information on extrusion conditions can be found in Supplementary Information (Table S1). The PHB/PCL filament obtained (2 mm diameter) was pelletized in 1 mm-length pellets. After drying the pellets at 50 °C for 6 h, the masterbatch was prepared by adding EPL (powder) at 15 % (w/w) to the PHB/PCL blend, followed by homogenization, extrusion and pelletizing in the same way as described above.

2.2.2. Dilution and laminate preparation

Dried pellets (masterbatch and PCL/PHB blends) were used to prepare dilutions at different concentrations of EPL in the blend (1, 2, 3, 5 and 10 % (w/w)). Table 1 shows the nomenclature used for each material. The extrusion process for each diluted sample was conducted in the same way as with the masterbatch. Once the pellets of each diluted sample were dried, different laminates were prepared with them by using a hydraulic thermopressing machine (Darragon). For each laminate (lam), 10 g of pellets were disposed inside a 10 cm × 10 cm aluminium square-frame mold (1 mm thickness) and they were placed in between two steel plates, which were set in the thermopressing machine. The thermopressing cycle consisted of 3 min at 175 °C with no pressure, followed by 2.5 min of pressure (200 bar) and then cooling at room temperature without pressure. After that, laminates (Fig. 1a) were stored.

Besides the preparation of blend laminates which contained none to different EPL concentrations, pure PHB and PCL laminates were also prepared for TGA analysis. In the case of PHB, laminates were produced after extrusion process followed by thermopressing, in the same conditions as blends. In the case of PCL, extrusion process could not be performed due to the low melting temperature and laminates were

Table 1
Nomenclature used in the study for different developed blends.

Material	Abbreviation
PHB/PCL	0EPL
PHB/PCL +1 % EPL	1EPL
PHB/PCL +2 % EPL	2EPL
PHB/PCL +3 % EPL	3EPL
PHB/PCL +5 % EPL	5EPL
PHB/PCL +10 % EPL	10EPL

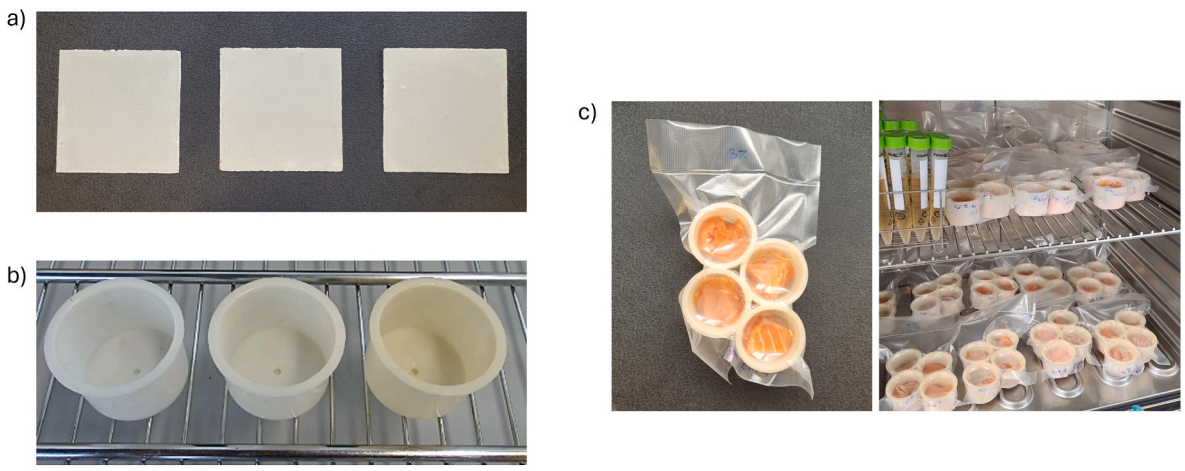

Fig. 1. Examples of a) laminates developed by thermopressing of extruded pellets b) trays (packaging prototypes) developed by injection moulding and c) packaged salmon in vacuum bags during challenge test.

prepared directly from commercial product by thermopressing after drying at 50 °C overnight.

2.2.3. Fabrication of packaging prototypes (trays)

Small trays (tr) with the shape of a cup (44.9 × 32.7 × 1.3 mm) (Fig. 1b) were produced as packaging prototypes by injection moulding of extruded pellets (Babyplast 10/12; Rambaldi Group, Italy) for the *in vivo* tests with salmon. Injection moulding conditions are available at Table S2 of Supplementary Information. The developed trays were named $0EPL_{tr}$, $3EPL_{tr}$ and $5EPL_{tr}$.

2.2.4. Laminate characterization

Thermogravimetric analysis (TGA) was performed directly on laminate samples (10 mg) without any further preparation with a TGA 400 instrument (Perkin, USA). The thermal analysis consisted of a heating ramp (10 °C/min) from 30 to 650 °C on air.

Regarding laminates mechanical properties, tensile strength and elongation at break were determined with a texture analyser TA.XT2i (Stable Micro Systems, U.K.) coupled with an A/TG probe. For the tests, laminates (80 × 20 mm) were placed in the centre of the traction clamps (35 × 35 mm) of the probe, at an initial separation of 50 mm, with a traction speed of 1 mm/s. Prior to the tests, samples width was determined with a digital micrometre (Mitutoyo, Germany), and at least 7 replicates of each material were studied.

Scanning electron microscopy (SEM; Inspect F50, 15 kV) was used to evaluate cross-sectional morphology of laminates. Prior to visualization, samples were subjected to cryogenic fracture under nitrogen and coated with palladium.

Other characterization techniques such as differential scanning calorimetry (DSC) and Fourier-Transformed Infrared Spectroscopy (FT-IR) were performed directly on laminates. Information on the methodology and results obtained can be found in Supplementary information.

2.2.5. EPL release assays

Given their intended use as food contact materials, an EPL release study was carried out in two food simulants: simulant A (10 % ethanol (v/v)) and simulant D2 (95 % ethanol (v/v)). These simulants were chosen according to the recommendations of Regulation EC 10/2011 on the food simulants for fresh fish.

For the release experiments, only the active laminates of $3EPL_{lam}$ and $5EPL_{lam}$ were used, besides the control samples ($0EPL_{lam}$). In the assay, samples of 2 × 0.5 cm of the laminates were dried at 30 °C overnight to remove moisture from the surface. After, they were cool down to room temperature in a desiccator, weighted and immersed in 1 mL of each simulant. Samples were incubated at 7 °C for 12 days, with sampling at 1 h, 2 h, 4 h, 6h, 8h, 24h (1 d), 48 h (2 d), 120 h (5 d), 168 h (7 d), 216 h (9 d) and 288 h (12 d). At each measuring point, three independent replicates of each sample were performed. Furthermore, a blank containing only the simulant was included in each sampling point.

The determination of EPL released to the simulants was performed by absorbance measurements using the Pierce™ bicinchoninic acid assay (BCA) Protein Assay Kit (ThermoFisher Scientific, USA) following manufacturer instructions for the microplate procedure. Absorbance measurements were performed on a Multiskan FC microplate reader (Thermo Scientific) at 562 nm. Prior to quantification, an EPL calibration curve in the range of 20–100 μg/ml was carried out. Samples were properly diluted in miliQ-H_2O to ensure that EPL concentration determined was within the range of the calibration curve. In the case of simulant D2, at least a 1:10 dilution was made to ensure that ethanol percentage during quantification was below 10 % not to interfere in the BCA assay.

2.2.6. Antibacterial activity tests

2.2.6.1. Bacterial strains and growth conditions. For the antibacterial activity tests, reference strains *Listeria monocytogenes* CECT 911 (*L. monocytogenes*) and *Salmonella enterica* subsp. *enterica* CECT 556 (*S. enterica*) were provided by the Spanish Culture Type Collection (CECT; Valencia, Spain). Bacterial strains were stored at – 80 °C in the form of a concentrated suspension in adequate culture medium supplemented with 20 % (v/v) glycerol. Prior to experiments, they were defrosted and subcultured twice in cation-adjusted Muller Hinton agar (MHA; Oxoid, UK) for *S. enterica* and Brain Heart Infusion agar (BHIA, Scharlau, Spain) for *L. monocytogenes* at 37 °C. Then, overnight grown colonies were used to prepare bacterial suspensions in 0.9 % NaCl. For inoculum preparation, these suspensions were diluted until obtaining an absorbance at 600 nm (OD_{600}) of 0.1 (approximately 10^8 CFU/mL). Then, a 1:100 dilution of the inoculum was prepared in MH broth (MHB) for *S. enterica* and BHI broth (BHIB) for *L. monocytogenes* to obtain a final inoculum concentration of 1 × 10^6 CFU/mL.

2.2.6.2. In vitro antibacterial evaluation of active laminates. The antibacterial effect of active laminates was evaluated in both liquid and solid media against each bacterial strain. Prior to each experiment, laminates were sterilised under UV light for 30 min each side. For liquid media assay, laminate samples of 2 × 0.5 cm were placed at the bottom of glass tubes with the help of sterile tweezers and immersed in 1 mL of the inoculum (1x10^6 CFU/mL) of either *L. monocytogenes* and *S. enterica*. Glass tubes containing the samples were kept at 37 °C for 24 h in aerobic conditions. After that, the MIC, defined as minimum concentration of

EPL in laminate samples that prevented visible growth (CLSI, 2012), as well as the MBC, defined as the minimum concentration of EPL in the sample that was lethal to 99.9 % bacterial colonies (CLSI, 1999) were determined. Three replicates of each laminate sample were used in each experiment, and at least three independent experiments were performed.

For the solid medium assay, MHA or BHIA plates were inoculated with 100 μL of the inoculum and spread evenly on the surface of the agar and allowed to dry. Afterwards, 2 × 2 cm laminate samples were placed in the centre of the plate, in direct contact with the inoculated agar. Petri dishes were incubated at 37 °C for 24 h under aerobic conditions. After incubation, inhibition zones, zones without visible bacterial colonies, were measured using a digital gauge (Comecta S.A., Spain). Three replicates of each laminate were used in each experiment, and at least three different independent experiments were performed for each strain and laminate. An example of how inhibition zones were measured is available in Supplementary Information (Fig. S1). For both antibacterial tests, $0EPL_{lam}$ (control) as well as growth controls for each strain were also included. To evaluate how the bacterial cells appeared after being in contact with the developed materials ($0EPL_{lam}$ and $5EPL_{lam}$), scanning electron microscopy images (SEM; Inspect F50) were acquired from *L. monocytogenes* agar plugs (0.7 cm diameter) close to the inhibition zone previously fixated and dehydrated. Inoculated agar plugs without being exposed to the active materials were also included. Fixation was performed by immersing the plugs in sodium cacodylate buffer containing glutaraldehyde (2.5 % w/v) and saccharose (0.3 % w/v) for 2 h at 37 °C. After that, plugs were transferred to different ethanol solutions (30, 50, 70, 90 and 100 % v/v, each of them was repeated twice), where they stayed for 20 min each to ensure dehydration. Before SEM visualization, dehydrated samples were coated with palladium.

2.2.7. Challenge test with inoculated raw salmon

The evaluation of the active prototypes against *L. monocytogenes* contamination in fresh salmon was performed using $0EPL_{tr}$ as control, and the active $3EPL_{tr}$ and $5EPL_{tr}$ as antimicrobial materials. To sterilize the trays, they were placed under UV light in a safety cabinet (MSC-Advantage, Thermo Fisher Scientific, Inc., MA, USA) for 30 min prior to the test, and the lamp from the laminar hood was used as the UV light source (36 W, 254 nm). The procedure for the challenge test (food artificially contaminated) was adapted from Eicher et al. (2020) with some modifications using a reference bacterial strain. Prior to the beginning of the test, and following the guidelines provided by EURL Lm (2021), cold adaptation of the bacterial strain was performed to mimic the physiological state of *L. monocytogenes* most likely to contaminate the salmon. To achieve this, the strain was defrosted and subcultured in solid medium (BHIA), and a colony forming unit was transferred to BHIB and maintained at 8 °C for several days until an OD_{600} of 0.5 was reached. Inoculum preparation for the challenge test was performed from this cold-adapted culture.

Fresh raw Norwegian salmon (*Salmo salar*) (without bones and skin) was acquired in a supermarket the day before the beginning of the *in vivo* experiment, carried to the laboratory on ice and kept inside the fridge at 4 °C overnight. All the following procedures were also performed on ice to maintain the cold chain.

On day zero of the assay, salmon was cut into 20 g cubic pieces and each of them was inoculated by immersion with a cold-adapted *L. monocytogenes* inoculum to reach a microbial *L. monocytogenes* load of approximately 10^3 CFU/g. After immersion, salmon pieces were placed inside the trays (20 g per tray). Four trays of each material were placed inside vacuum bags and vacuum sealed (Fig. 1c). Vacuum-sealed bags containing the trays were kept at 7 °C for 12 days until sampling. This temperature was chosen based on EURL (2021) guidance on cold storage conditions at retail level. Sampling was performed at days 0, 2, 5, 7, 9 and 12 of the challenge test. Each sampling day, three bags, one for $0EPL_{tr}$, $3EPL_{tr}$ and $5EPL_{tr}$ containing 4 trays each (Fig. 1c) were open, and the salmon pieces were analysed in terms of microbiological counts (*L. monocytogenes*, Total viable counts (TVC), Lactic Acid Bacteria (LAB) and *Pseudomonas* spp.) and total volatile basic nitrogen (TVB-N).

2.2.7.1. Total volatile basic nitrogen determination.

Total volatile basic nitrogen (TVB-N) was determined using the Conway microdiffusion method as explained in Ouahioune et al. (2022), with some modifications. Briefly, 4 g of each salmon sample were weighted, transferred to an individual Stomacher bag and diluted by adding 15 mL sterile distilled water. Samples were homogenised using a Stomacher-400 Circulator (Seward, UK) for 2 min at 265 rpm. To eliminate protein content, 10 mL of 10 % (w/v) trichloroacetic acid (Sigma-Aldrich, USA) were added to each bag and homogenised again for 4 min at 265 rpm. After that, the obtained slurry was transferred to microtubes, centrifuged and the supernatant was collected for further analysis. The determination was performed using a Conway microdiffusion chamber previously prepared with 1 mL of saturated potassium carbonate in the outermost area of the chamber and 1 mL of sulfuric acid (0.01N) in the central area of the chamber. 1 mL of the sample supernatant was placed in the outermost area, where the potassium carbonate was placed. Finally, the chamber was closed, Parafilm® sealed and shaken carefully, and it was incubated for 1 h at 35 °C. After that, a titration was performed using sodium hydroxide (0.01N) and Tashiro indicator (methyl red/methylene blue). TVB-N content was calculated as follows (Equation (1)) and expressed in mg N per 100 g of salmon.

$$TVB-N\,(mgN/100\,g\,sample) = [((V_{ac} - V_{ba}) \times 0.14 \times 25) / (V_s \times M_s)] \times 100 \quad (1)$$

Where V_{ac} is the volume of sulfuric acid, V_{ba} is the volume of sodium hydroxide used in the titration, V_s is the volume of supernatant placed in the chamber and M_s is the weight of salmon sample.

2.2.7.2. Microbiological counts.

Microbiological counts were performed following the procedure described by Silva et al. (2018), with slight alterations. 4 g of each salmon piece were placed aseptically into individual Stomacher bags, diluted 1:10 in peptone water (Scharlau) and homogenised in the stomacher for 2 min at 265 rpm. After that, serial dilutions were prepared and 100 μL of each sample were spread-plated on different agar plates, according to the microbial analysis to be performed. For L. *monocytogenes* enumeration, PALCAM selective agar (PALCAM, Scharlau) was used and incubated 48 h at 37 °C. TVC were spread on Plate Count Agar (PCA, Scharlau) plates and incubated for 48 h at 30 °C. Pseudomonads were plated on Cetrimide selective agar (Cetrimide, Scharlau) plates at 25 °C for 48 h. Finally, LAB were determined using Man Rogosa Sharpe agar (MRSA) incubated at 30 °C for 72 h under microaerophilic conditions.

2.3. Statistical analysis

Statistical analysis of the results was performed using GraphPad Prism 8.02. Differences in mean scorings were analysed by a two-way ANOVA and separated using post-hoc Tukey's significant difference test ($p < 0.05$).

3. Results and discussion

3.1. Materials characterization

3.1.1. Thermal analysis

In DSC analysis, results on blends (Supplementary Information, Fig. S3) showed a low miscibility of both polymers. These results correlate with those stated in literature (Garcia-Garcia et al., 2017; Lovera et al., 2007) and with TGA results (Fig. 2), with very differentiated degradation steps, slightly different with respect to characteristic degradation temperatures of each polymer separately. Thus, our material would be composed of two different phases, with some domains rich

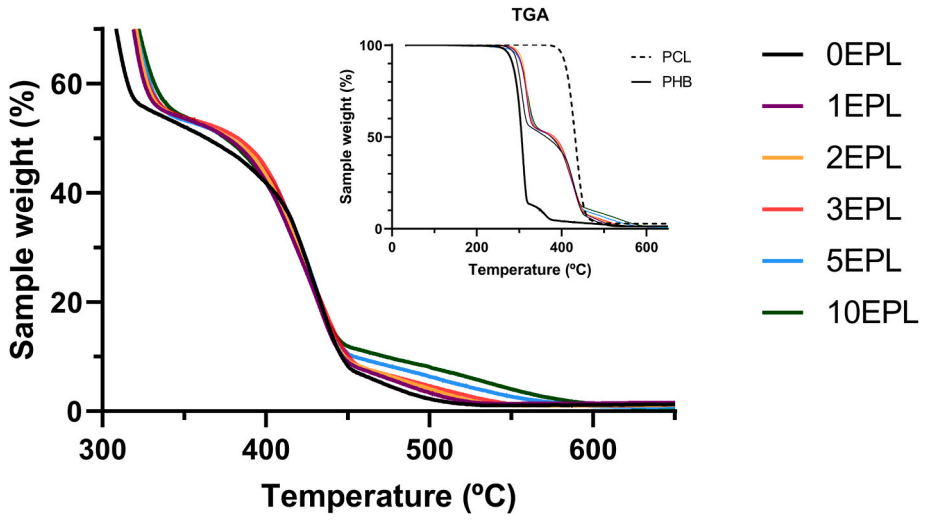

Fig. 2. TGA curves of prepared laminates. In top graph: TGA curves from 30 to 650 °C of all samples, including pure PCL and PHB curves. In bottom graph: TGA blend laminates profiles from 300 to 650 °C, depicting the influence of increasing EPL concentration.

in PHB while others rich in PCL.

TGA curves showed that in blend control laminates ($0EPL_{lam}$), weight loss occurred in three steps. The first step of weight loss occurred between 260 and 340 °C in which almost 43–44 % of the sample was degraded for all samples. During the second weight loss step (340-450 °C), another 43–48 % of the samples was degraded. These first two steps of degradation may correspond to each polymer degradation, PHB and PCL, respectively, as can be seen when compared to those of PHB and PCL alone (Fig. 2, top graph). Furthermore, the third step of weight loss in blend laminates is shown between 450 and 600 °C. In this step, 6–12 % of sample weight was lost. This degradation corresponds not only to the EPL content in the sample (weight loss is higher as the EPL content rises in this range of temperatures), but also to PHB, as shown in the PHB curve. We hypothesized that this finding could be related to any additional compound such as plasticizers, fillers or other additives present in this commercially available PHB (Garcia-Garcia et al., 2016; Prakalathan et al., 2014).

3.1.2. Morphology and mechanical properties

Regarding mechanical properties, tensile strength of 0EPL, 3EPL and 5EPL laminates was determined at 16.2 ± 0.8, 16.3 ± 0.6, 17.5 ± 1.3 MPa, while their elongation at break percentages were 10.0 ± 0.2, 10.3 ± 0.2, 10.6 ± 0.2 %, respectively, showing no differences in the mechanical behaviour when EPL is included in the blend. Furthermore, these values account for a relatively moderate strong and ductile material, sometimes found in biomaterials (Bezerra et al., 2019; Culenova et al., 2021). In 2016, Garcia-Garcia et al. determined the tensile strength and elongation at break of PHB/PCL (50:50) blends at 19.8 ± 0.2 MPa and 17.6 ± 1.2 %, respectively. Mechanical properties of blends are often affected by composition of the blend, processing conditions, temperatures, storage time and the lack of miscibility in the blends, which can weaken the interfacial bonding and reduce tensile strength and elongation at break values (Fernández-Tena et al., 2023; Garcia--Garcia et al., 2016; Srubar et al., 2012).

SEM images of cross-sectional areas of laminates are shown in Fig. 3. As observed, the morphology of the cross-sectional area was heterogeneous, and different disordered structures, such as spherical particles, could be identified. These structures are typically formed when mixing two immiscible polymers, such as PHB and PCL, due to phase separation during processing, and they are consistent with DSC and TGA results (section 3.1.1). When EPL was present, a slight increase in homogeneity is seen, with less and smaller spherical particles formed, and with greater areas of continuous phases, indicating that EPL could be enhancing the miscibility of the blend.

3.2. Release of EPL from active materials

EPL release in food simulants is shown in Fig. 4. Although EPL presence was not very well determined in DSC and FT-IR results (Supplementary Information), when the protein content of laminates was determined with the specific bicinchoninic acid assay (BCA), it became clear that EPL was effectively included in the active laminates. For the release studies, food simulants were chosen instead of other media such as water or culture broth to better understand how the laminates would

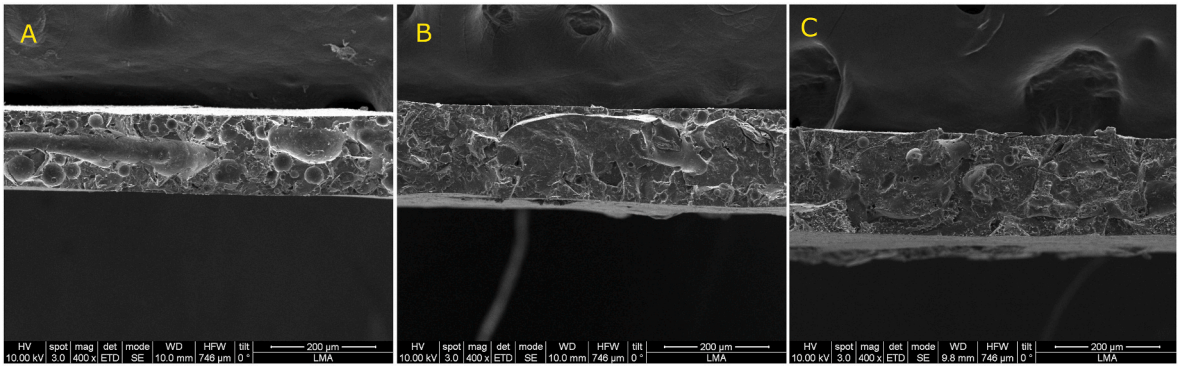

Fig. 3. Scanning electron micrographs (6000x) of cross-sectional area of developed materials a) $0EPL_{lam}$, b) $3EPL_{lam}$ and c) $5EPL_{lam}$.

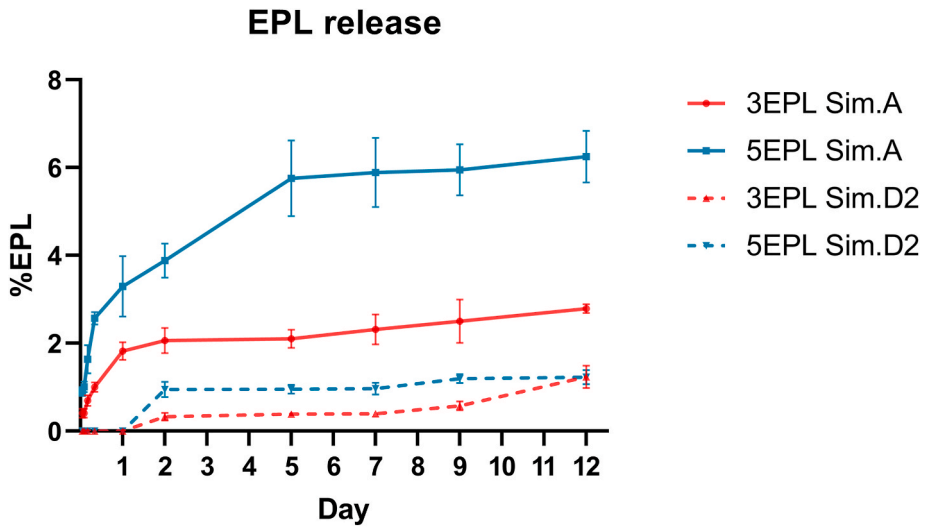

Fig. 4. EPL Release. Percentage of EPL (referred to sample weight) released to food simulants A (line) and D2 (dash) during time (measurements at 1 h–2 h – 4 h–6 h – 8 h–1 d – 2 d–5 d – 7 d–9 d – 12 d). 3EPL$_{lam}$ material appears in red while 5EPL$_{lam}$ is shown in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

behave when in contact with food.

Differences between simulants were observed, being simulant A (10 % ethanol) the one with higher EPL release. In this simulant, an initial burst in EPL release was found in both laminates during the first 24 h, probably due to the hydrophilicity of the peptide, which prefers the outer hydrophilic medium than the inner hydrophobic polymer matrix. Although this initial burst occurred for both laminates; it was more pronounced in the case of 5EPL$_{lam}$ samples. Additionally, other differences in the behaviour of EPL released among active laminates were seen. In the most concentrated sample (5EPL$_{lam}$), the release of EPL continued to rise strongly up to day 5 followed by a slower release until the last day. For 3EPL$_{lam}$, however, after the initial burst, the release did not rise and was maintained for some days up to day 7, followed by a slight increased release again until day 12, attaining a final concentration of 2.8 % EPL per weight of sample at day 12.

In simulant D2 (95 % ethanol), the amount of EPL released from samples was lower, only reaching a 1 % of EPL at day 12 for both 3EPL$_{lam}$ and 5EPL$_{lam}$. Furthermore, EPL was not determined in the simulant until the second day for both laminates, proving that not only the amount of EPL but also the release rate was lower in this simulant. From day 2 to day 7, no significant amount of EPL was released. In the last days of the experiment, significant amounts of EPL were released from the laminates, which were higher in the case of 3EPL$_{lam}$. In any case, the final amount of EPL released in this simulant for any of the laminates barely reached a 1.2 ± 0.2 % EPL per weight of sample in both active materials.

To our knowledge, the release of EPL from PHB/PCL extruded blends into food simulants has not been studied to date. Ding et al. (2018) developed PHB/PCL membranes by electrospinning containing levofloxacin and studied its release as a drug model. After an initial burst, the controlled drug release lasted 72 h. In 2023, Erci & Sariipek developed a nanofibrous mat of PHB/PCL containing an inclusion complex based on β-cyclodextrin and curcumin as antimicrobial agent, and found a controlled release for 360 min (Erci and Sariipek, 2023). Furthermore, Gorrasi et al. (2020) studied the incorporation of curcumin into PHB and PCL alone or in combined blends. In this case, authors found that the initial burst in the antimicrobial release in the blend was reduced when compared to PHB or PCL alone. In our case, we have reached a longer sustained release up to several days. Differences with respect to those found in literature could be attributed to the material processing technique used, polymer's concentration in the blend and interactions between the antimicrobial used and the polymer blend matrix. We hypothesized that the initial release burst in our samples could be due to the release of the EPL found in the surface of laminates or inside porous in the blends. When the material, composed of a mixture of hydrophobic polymers, is in contact with hydrophilic medium, the EPL, either in the surface or in pores inside the blend matrix, is released because of its hydrophilic nature and affinity to the food simulant. Apart from this initial burst, however, by mixing polymers by extrusion process, we have reached a sustained release of EPL in time, which is especially relevant for active materials intended to be used in contact with food.

Special attention may be drawn to the differences found between EPL content in samples and theoretical EPL content (based on the amount of EPL added during the extrusion process). For instance, 5EPL$_{lam}$ showed an EPL content of 6.25 ± 0.59 % while for 3EPL$_{lam}$ samples, an EPL content of 2.8 ± 0.10 was found. These differences may be due to the differences in particle size of EPL and PHB/PCL pellets when mixed in the twin screw-extruder, which could have ended up on a masterbatch with different content on EPL than expected.

3.3. Antibacterial activity of laminates

3.3.1. In liquid media

Active laminates showed antibacterial activity in liquid medium against both tested strains. In the case of *L. monocytogenes*, MIC values could not be determined (≤1EPL$_{lam}$), as even the laminate with the minimum EPL concentration caused a complete growth inhibition. In the case of *S. enterica*, MIC was determined when the bacterial suspension was in contact with the 3EPL$_{lam}$, whereas MBC was found only with 5EPL$_{lam}$ samples. The fact that EPL was more effective against *Listeria* than *Salmonella* is explained due to the differences between bacterial cell walls. *S. enterica*, as a Gram-negative bacteria, presents a cell wall surrounded by an outer membrane rich in lipopolysaccharides, which gives an extra protection to the cell. By contrast, *L. monocytogenes*, as a Gram-positive bacteria, lacks an outer membrane, and its cell wall is more exposed to the action of some antimicrobials. These findings concerning EPL being more active against Gram-positive bacteria are in agreement with previous works (Fang et al., 2022; Ye et al., 2013).

3.3.2. In solid media

Results on the antibacterial effect (inhibitory zones) of active laminates in direct contact with inoculated plates are shown on Fig. 5a. For both strains, inhibitory effect was higher as the EPL concentration rise (10EPL$_{lam}$ > 5EPL$_{lam}$ > 3EPL$_{lam}$ > 2EPL$_{lam}$ > 1EPL$_{lam}$), and no

a)

Material	*Listeria monocytogenes*			*Salmonella enterica*		
	Mean (mm)	sd (mm)	CV (%)	Mean (mm)	sd (mm)	CV (%)
$1EPL_{lam}$	25.82	1.55	6.00	22.80	0.42	1.85
$2EPL_{lam}$	27.09	1.08	4.00	23.77	0.54	2.27
$3EPL_{lam}$	28.81	2.10	7.29	25.01	0.70	2.81
$5EPL_{lam}$	30.44	1.39	4.55	26.70	0.82	3.07
$10EPL_{lam}$	32.68	0.45	1.36	28.80	0.60	2.09

b)

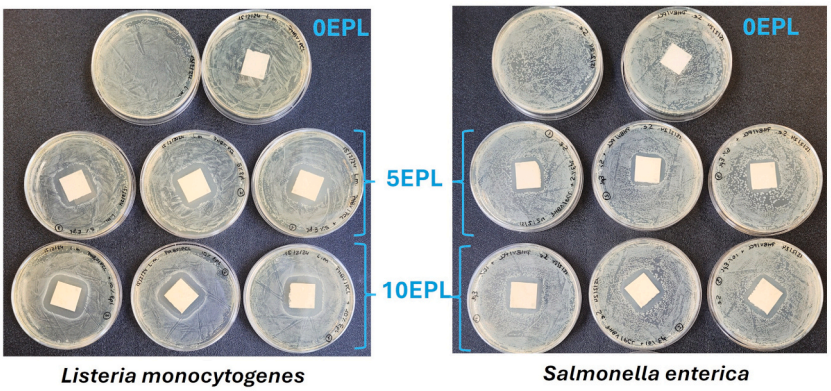

Fig. 5. Inhibition zones of active materials in solid media. a) Zones of inhibition (mm; mean, standard deviation (sd) and coefficient of variation (CV)) of active laminates for *L. monocytogenes* and *S. enterica*. b) Examples of representative agar plates (one experiment) for $5EPL_{lam}$ and $10EPL_{lam}$ for *L. monocytogenes* (left) and *S. enterica* (right). One replicate of the bacterial growth in agar and another for $0EPL_{lam}$ growth is shown for each strain.

inhibitory effect was found in the case of $0EPL_{lam}$. Furthermore, for *L. monocytogenes,* the inhibitory effect was higher than for *S. enterica*, which is in accordance with the susceptibility profiles obtained in liquid media. Furthermore, a visible marked barrier was found on the outermost part of the inhibition halo where the EPL concentration was lower due to diffusion (Fig. 5b).

Antibacterial effects in both liquid and solid medium cannot be compared due to different methodologies used and possibly different EPL diffusion profiles in both cases. However, it could be seen that in general these results are in accordance with the ones showed in liquid medium, being active samples more effective against *L. monocytogenes*, and showing increasing antibacterial effect as the content in EPL rises. Taking into account these results, $3EPL_{lam}$ and $5EPL_{lam}$ were chosen as effective materials with the lowest EPL content enough to yield high antibacterial activity in any case. These concentrations (3 and 5 % EPL) were used to prepare active packaging prototypes (trays) to be used in the challenge test with fresh salmon, and 10EPL was not included in further studies.

3.3.3. Morphological changes

SEM images (6000x) of *L. monocytogenes* cells without any treatment and after being exposed to control ($0EPL_{lam}$) and active material ($5EPL_{lam}$) are shown in Fig. 6. As seen in SEM micrographs, no morphological differences were found between untreated cells and cells exposed to $0EPL_{lam}$ material, and bacterial surfaces appeared perfectly smooth, rod-shaped with rounded ends. However, when exposed to the active material ($5EPL_{lam}$), *L. monocytogenes* cells lost their smoothness and appeared with an irregular rough surface. This effect would be the result of the stress induced by EPL and its mode of action, mainly focused on the adhesion to the cells surface due to electrostatic interactions, which triggers membrane destabilization (Hyldgaard et al., 2014). Although SEM analysis provided valuable morphological insights, it is insufficient on its own to elucidate the mechanism of action of EPL within the active material. Further comprehensive studies, including assessments of its effects on bacterial cell membrane integrity would be required to fully characterize its antimicrobial function and potential applications. Even so, since only the active films containing EPL have shown antimicrobial activity and morphological changes, it is expected that this antimicrobial mode of action is solely due to EPL release. So,

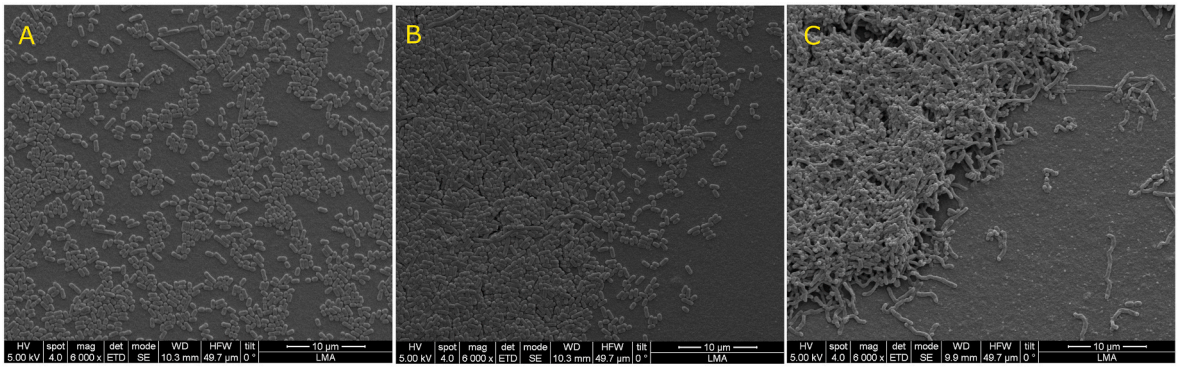

Fig. 6. Scanning electron micrographs of a) agar-grown *L. monocytogenes* cells (no treatment) and after being exposed to b) $0EPL_{lam}$ and c) $5EPL_{lam}$ (2 × 2 cm sample; 24 h at 37 °C in aerobic conditions; direct contact assay in solid (agar) media).

the mechanism of action expected would be the one already described in detailed by Hyldgaard et al. (2014) for EPL in Gram-positive bacteria (*Listeria innocua*).

3.4. Challenge test

3.4.1. Results on TVB-N of salmon

Total volatile base-nitrogen (TVB-N) results are shown in Fig. 7. During the experiment, TVB-N content in samples increased over time. This finding is expected as TVB-N is an indicator of freshness in fish products. Typically, a content of 30–35 mg of nitrogen per 100 g of fish is considered as a marker of spoilage, although this value is species-dependant (Martin et al., 2023). For *Salmo salar* species, the TVB-N threshold is set at 35 mg of nitrogen per 100 g of fish in the EU (Regulation EC 1022/2008). This content is only reached for control samples at the last day (day 12) of storage at 7 °C. No significant differences were found in terms of TVB-N content in salmon samples stored in active trays compared to those stored in $0EPL_{tr}$. This finding evidenced that active trays did not affect the quality of the fish with regard to TVB-N. Increase of TVB-N value in fish may occur by different ways such as enzymatic hydrolysis or the metabolism of some spoilage bacteria (Aguilera-Barraza et al., 2015; Fidalgo et al., 2019) and can be affected by storage conditions like temperature (Dondero et al., 2004). Although sometimes an increase of TVB-N values has been related to the microbial content in terms of TVC, it has also been stated that some specific spoilage bacterial species such as *Photobacterium* spp. or hydrogen sulphide producing bacteria take a more specific role in production of TVB-N in salmon (Fogarty et al., 2018), and microbial content is not always correlated with TVB-N (Fidalgo et al., 2019; Holman et al., 2021). This could explain the fact that even when our active trays affected the microbial growth (section 3.4.2), they did not diminish TVB-N content, which may have been produced by specific non-inhibited bacterial species or by other means such as enzymatic degradation. Moreover, our results agree with the fact that artificial inoculation with the non-spoilage bacteria *L. monocytogenes* does not affect TVB-N values, as reported by Benabbou et al. (2018), who observed similar values of TVB-N for *L. monocytogenes*-inoculated salmon and untreated control samples.

3.4.2. Results on microbiological counts

Results on microbiological counts for *L. monocytogenes* and spoilage *bacteria* (LAB, *Pseudomonas* spp. and TVC) over time are shown in Fig. 8. In general, for both active trays, $3EPL_{tr}$ and $5EPL_{tr}$, significant differences were found in all bacterial groups when compared to control trays, more pronounced for those with higher EPL concentration ($5EPL_{tr}$).

In the case of *L. monocytogenes*, $5EPL_{tr}$ significantly reduced its growth since day 5 up to the end of the experiment. Compared to $0EPL_{tr}$, $5EPL_{tr}$ reduced *L. monocytogenes* growth in more than 1 logarithm for several days (days 5–9). On the last day of the experiment, a slight increase in the number of colonies was found, but it remained significantly lower than in $0EPL_{tr}$ samples. For $3EPL_{tr}$, a reduction on the number of colonies was also observed since day 5, although it was not as noticeable as the one yielded by $5EPL_{tr}$. On day 12 of storage, *L. monocytogenes* growth on salmon stored on $3EPL_{tr}$ did not differ significantly from the one determined in $0EPL_{tr}$, proving that the content of EPL released from $3EPL_{tr}$was not enough to completely inhibit *L. monocytogenes* growth at the end of the experiment, suggesting that at least 5 % of EPL should be incorporated into the blend to maintain an anti-listerial effect over time. In literature, authors studied the addition of EPL directly into *L. monocytogenes* inoculated cold-smoked salmon and found negligible effect (Kang et al., 2014). Recently, Ramos et al. (2024) developed an active packaging based on polyethylene, a conventional plastic, containing silver nanoparticles as antimicrobial agents with similar action than EPL (direct contact with bacteria). When evaluating its effectiveness in *L. monocytogenes* inoculated Canastra cheese, authors found that their active packaging did not reduce bacterial counts in cheese stored at 5 and 10 °C. In our case, we found that the developed trays exhibited a better performance than those examples present in literature. On the one hand, the addition of the protein to the packaging instead of directly to the food enhanced its antibacterial effect and allowed a reduction of *L. monocytogenes* over time. On the other hand, our tray prototypes enabled a better and long-lasting sustained release, which has not been stated in other active designs such as the one developed by Ramos et al. (2024).

On day 0 of the experiment, TVC were present at a 4 log CFU/g. As expected, TVC continued to growth up to 9 log CFU/g in salmon placed in $0EPL_{tr}$ at day 9, when it stabilized. In salmon stored in active trays, a significant reduction in TVC was found from day 7 onwards when compared to control trays due to the sustained release of EPL. No

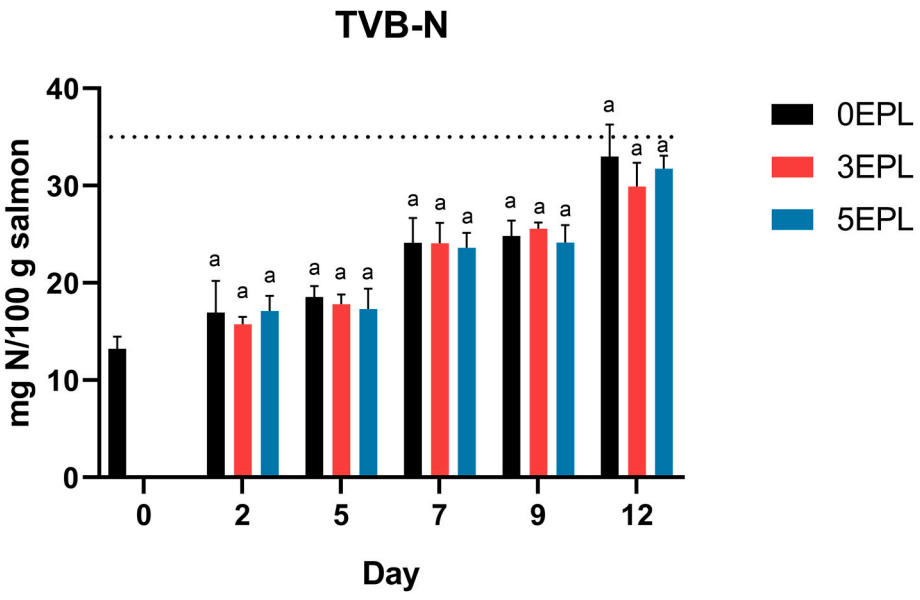

Fig. 7. TVB-N results of salmon stored in developed trays ($0EPL_{tr}$, $3EPL_{tr}$ and $5EPL_{tr}$). Values of TVB-N expressed as mg nitrogen (N) per 100 g of fish. Dot lines represent threshold limit in *Salmo salar* by EC 1022/2008. Different letters for each day indicate a significant difference ($p < 0.05$) between treatments according to post-hoc Tukey test.

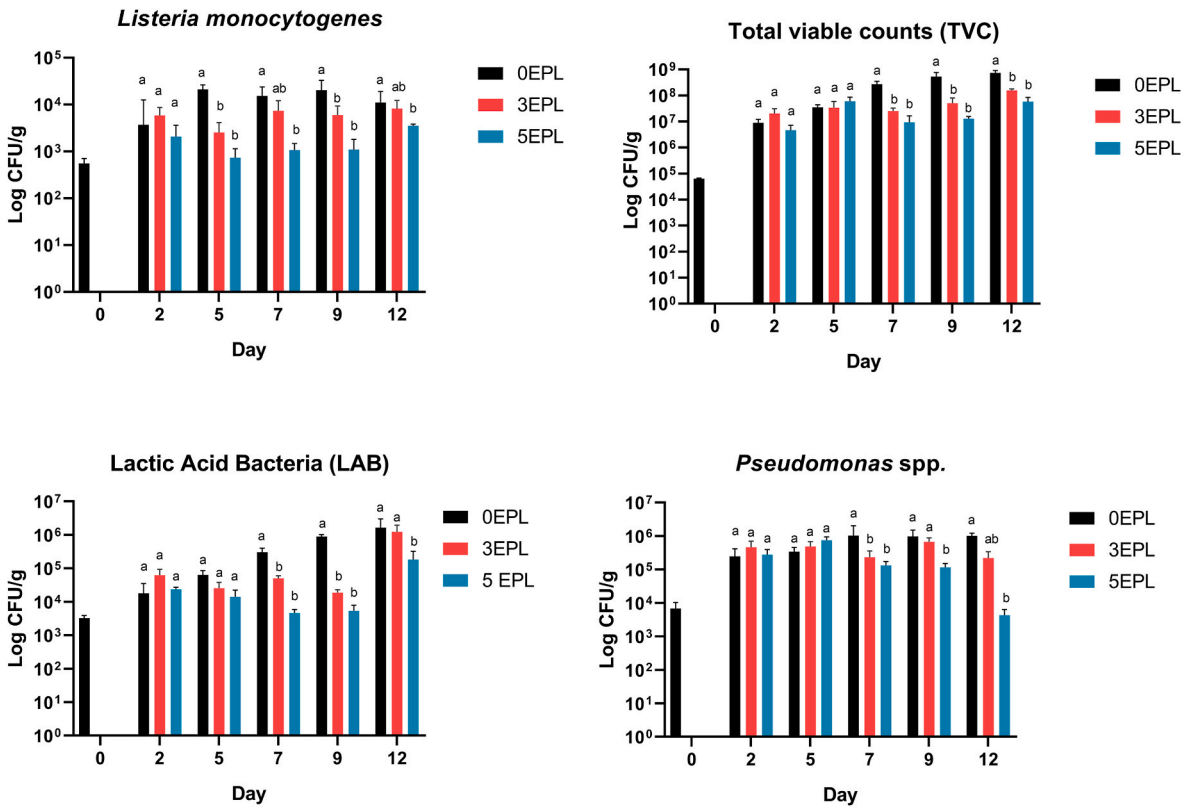

Fig. 8. Results on microbiological counts of *L. monocytogenes*, Total Viable Counts (TVC), Lactic Acid Bacteria (LAB) and *Pseudomonas* spp. of salmon stored in developed trays ($0EPL_{tr}$, $3EPL_{tr}$ and $5EPL_{tr}$) at days 0, 2, 5, 7, 9 and 12, expressed in logarithmic scale per gram of salmon. Different letters for each day indicate a significant difference ($p < 0.05$) between treatments according to post-hoc Tukey test.

significant differences were found between the effect of $3EPL_{tr}$ and $5EPL_{tr}$ in terms of TVC, being both active enough to reduce this bacteria spoilage group in salmon.

Pseudomonas spp. and some LAB such as *Carnobacterium maltaromaticum* or *Lactococcus piscilum* are natural spoilage bacteria found in salmon (Macé et al., 2012). For LAB, active trays were effective since day 7, when they reached a 2-log reduction when compared to salmon stored in control samples, and it was maintained at day 9. However, at the end of the experiment, this reduction only remained significant for the $5EPL_{tr}$, due to the higher EPL concentration.

It was not until day 7 that significant reduction in *Pseudomonas* spp. growth was observed and kept constant until day 12 for $5EPL_{tr}$. Conversely, in the case of $3EPL_{tr}$, the reduction on the growth only occurred at day 7, as on day 9 and 12 of the experiment there was no significant difference between $3EPL_{tr}$ and control trays. This could be explained by the fact that *Pseudomonas* is Gram-negative genre and a higher content of EPL may be needed to inhibit its growth, as was already seen in the *in vitro* assays for *S. enterica*.

By and large, these results can be related to the EPL release profiles obtained for food simulant A (90 % water) and the fact that salmon, as a fish, is composed of large amount of water (between 65 and 70 %) (Aursand et al., 2009). In this simulant, after an initial burst, EPL was sustainedly released, and that could be the reason why the effect of EPL is mainly seen when the mid-days of the challenge assay (5-7-9) are reached. Even so, from all these data we could say that the active materials developed, specially $5EPL_{tr}$, were effective controlling *L. monocytogenes* and other bacteria naturally present in fresh salmon over time, even at refrigerated storage. Lastly, when using antimicrobial peptides as preservatives, it is worth considering the sensorial impact they may have on the food, as it is known that peptides and proteins can yield a bitter food taste at high concentrations (Sarker, 2022). In the case of EPL, Takahashi et al. (2011) found that the maximum concentration of EPL in minced tuna and salmon roe which did not affect food sensorial quality was 1000 ppm. In Japan, EPL is commonly used in many foods, including sliced fish and fish sushi, at concentrations ranging from 10 to 5000 ppm (Chheda & Vernekar, 2015). Given the results available, one may expect that the antimicrobial package developed herein does not yield strong organoleptic changes, as the concentrations used in our study fall within these ranges.

4. Conclusion

By mixing EPL, PHB and PCL we have developed a novel biodegradable active material with proved antimicrobial efficacy against pathogenic bacteria such as *L. monocytogenes* and *S. enterica* at low EPL contents up to 5 % *in vitro*. Besides, a sustained release of the protein in food simulants was achieved, which is especially relevant for food contact materials. Apart from being active against both Gram-negative and Gram-positive bacteria *in vitro*, our novel packaging prototypes effectively controlled *L. monocytogenes* and other psychrotrophic bacteria in fresh salmon, demonstrating their potential as an alternative hurdle technology to enhance food safety. Furthermore, we strongly believe that the industrial development of our active packaging is feasible due to the use of rapid and scalable methods such as extrusion and injection moulding. Moreover, the food packaging industry needs to provide greener alternatives to conventional plastics. Because of their added value as antimicrobial materials, our prototypes are ideal candidates to be used soon in the market as biodegradable tray containers for fresh fish products or other products with high water activity, for instance. However, comprehensive studies addressing its antibacterial mode of action are necessary to fully define its application potential.

CRediT authorship contribution statement

Laura Aguerri: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Frédéric Leonardi:** Writing – review & editing, Supervision, Resources. **Filomena Silva:** Writing – review & editing, Supervision, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2025.111475.

Data availability

Data will be made available on request.

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