

# Effect of novel bottle inner surface coatings made with polylactic acid and plant extracts on the physiological status of *Pseudomonas aeruginosa* in real liquid foods using flow cytometry

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## Funding information

Ministry of Higher Education and Scientific Research; Principality of Asturias, Grant/Award Number: GRUPIN IDI/2018/000127

## Abstract

Poly(lactic acid) (PLA) was used, together with a novel optimized mixture (OM) of *Thymus fontanesii* essential oil (EO<sub>d</sub>) and the ethyl acetate (EA) fraction from *Scirpus holoschoenus* extract, to develop antimicrobial coatings for the inner surface of bottles for the preservation of food liquids against the pathogenic bacterium *Pseudomonas aeruginosa*. The effect of these coatings on the physiological status of *P. aeruginosa* was tested by flow cytometry (FC), filling coated bottles with previously contaminated fish soup or egg whites. As was expected, the release of phenolic compounds (PCs) from the inner coatings of the bottles to the liquid was temperature dependent. Although their antimicrobial activity in contaminated egg whites was negligible, probably due to the neutralization of bioactive substances by ovalbumin, the PLA-OM-coated bottles had a high antimicrobial effect when they were filled with fish soup. In this latter case, the percentage of viable cells greatly decreased, and the percentage of dead cells, killed by membrane damage, increased.

## KEYWORDS

antibacterial activity, bottle inner surface coatings, flow cytometry, *P. aeruginosa*, PLA, *T. fontanesii*

## 1 | INTRODUCTION

The agri-food industry is currently looking for substances with a high preservative capacity in order to develop new natural and non-toxic products that better meet the consumer's requirements. The antimicrobial activity of secondary metabolites from plants is well demonstrated in synthetic media and in food models. However, applied at high doses, these bioactive substances could be toxic.<sup>1</sup> For example,

the food tolerance threshold for thymol has been set by the World Health Organization at less than 50 mg/kg,<sup>2</sup> so the search for compounds with high bioactivity at low concentrations is being actively encouraged.

According to some researchers, essential oils have a pronounced antimicrobial effect, probably due to a synergistic effect between their constituents.<sup>3</sup> Antimicrobial synergism has also been found between different essential oils<sup>4</sup> and between their components and other

antibiotic agents.<sup>5</sup> This synergistic effect is even produced when mixing essential oils from different sources, which could be used to advantage in order to decrease their concentration while retaining their antimicrobial properties.

A previous study tested the individual and combined effects of diluted *Thymus fontanesii* essential oil (EO<sub>d</sub>) and the ethyl acetate (EA) fraction of *Scirpus holoschoenus* extract.<sup>6</sup> Its findings showed that an optimized mixture (OM) solution of EO<sub>d</sub> and EA was highly effective against *Pseudomonas aeruginosa* at 7°C. As is well known, bacteria of the genus *Pseudomonas* are important spoilage bacteria that can be found profusely in fish<sup>7</sup> and eggs.<sup>8</sup> In addition, among the members of the *Pseudomonas* genus, *P. aeruginosa* is the most dangerous species,<sup>7</sup> since it is a food-borne pathogen responsible for nosocomial infections,<sup>9</sup> able to grow in a biofilm mass that can produce a large number of virulence factors.<sup>10</sup>

Turning now to the question of how to evaluate the antimicrobial activity of these compounds, fluorescence methods can provide information on the functional and structural properties of cells. In addition to its rapidity in use, flow cytometry (FC) detects microorganisms even at a relatively low concentration<sup>11</sup> and allows the assessment of the cell membrane integrity, enzymatic activities and the membrane potential value.<sup>12</sup> For these reasons, FC methods have been used for microbial inhibition experiments by several researchers. In this field, FC was used to evaluate ultrasound-induced bacterial damage,<sup>13</sup> the antibacterial effect of metals,<sup>14</sup> nisin,<sup>15</sup> essential oils and other phenolic compounds (PCs).<sup>16</sup> Furthermore, FC was reported as a fast and effective method to analyse bacterial populations in several food matrices, such as milk,<sup>17</sup> juice<sup>18</sup> and yoghurt.<sup>19</sup>

Finally, and bearing in mind the potential of these bioactive compounds for the food industry, the current trend is to incorporate herb-derived compounds into biopolymer films, which are known to be good vehicles for these active agents. A range of biopolymers can be used, but some studies have focused on the preservative effect of polylactic acid (PLA) films containing bioactive compounds.<sup>20,21</sup> PLA originates from bio-derived monomers<sup>22</sup> and is recognized as GRAS. While the biodegradability of PLA is low under physiological and acidic conditions, its elimination is rapid in an alkaline environment. Due to its low immunogenicity and good mechanical performance, PLA is preferable for preparing packaging films.<sup>23</sup> Additionally, PLA manufacturing processes such as drying and crystallization can be controlled as needed. Owing to its water resistance capacity, it is already being used to manufacture several commercial packaging-related products for liquid foods, such as the Biota™ and Noble™ PLA-based bottles for water and juice respectively and Dannon yogurts.<sup>24</sup> Applied alone, PLA did not contribute any antimicrobial activity, but it constitutes an interesting antimicrobial packaging when it is supplemented with active substances. In this sense, Jin et al.<sup>24</sup> prepared composite films with PLA and nisin to protect liquid egg white or orange juice from *Listeria monocytogenes*, coated bottles with PLA-nisin to protect egg white or skimmed milk from *L. monocytogenes*,<sup>25</sup> and coated bottles with PLA-nisin and allyl isothiocyanate to protect liquid egg white from *Salmonella enterica* and *S. enteritidis*.<sup>21</sup>

Hence, by taking advantage of the enhanced antimicrobial properties of plant extracts such as *T. fontanesii* and *S. holoschoenus*, this study proposes to develop PLA-OM and PLA-EO<sub>d</sub> inner coatings for bottles. For that purpose, inner coated bottles were filled with fish soup or egg whites previously contaminated with *P. aeruginosa*, a pathogenic bacterium usually found in fish and eggs. The antimicrobial effects of these inner coatings on the growth and physiology of *P. aeruginosa* were then tested by FC.

## 2 | MATERIAL AND METHODS

### 2.1 | Chemicals

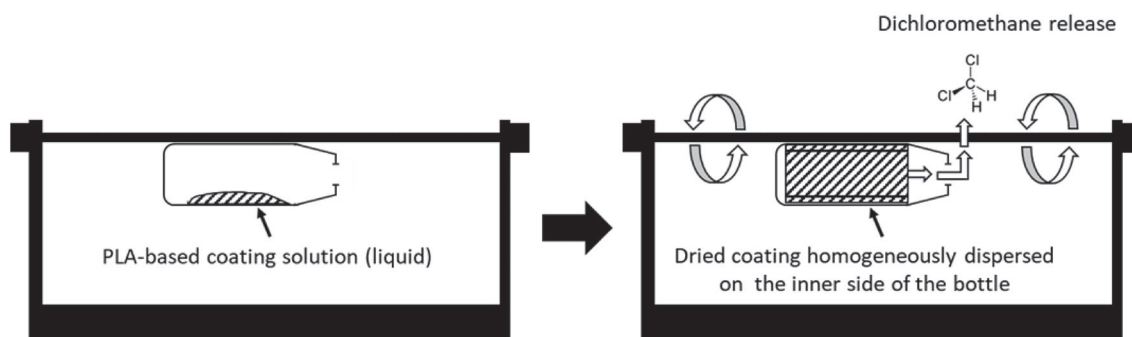
Dimethylsulfoxide (DMSO) was purchased from Biochem-Chemopharma (Loire, France). Ethylenediaminetetraacetic acid (EDTA) and dichloromethane were obtained from Sigma (St. Louis, United States). The PLA (180 kDa) was produced by NatureWorks (4032D, Minnesota, United States). SYBR Green and propidium iodide were purchased from Molecular Probes (Eugene, United States), sodium bicarbonate (Na<sub>2</sub>CO<sub>3</sub>) from Panreac (Barcelona, Spain), and Folin-Ciocalteu phenolic reagent was provided by Prolabo (Loire, France). Fish soup and pasteurized liquid egg whites were purchased in a local market (Oviedo, Spain).

### 2.2 | Bacterial strain and culture conditions

The bacterial strain used in this work, *P. aeruginosa* ATCC 27853, a psychrophilic strain recognized for its opportunistic and pathogenic power, was supplied by the American Type Culture Collection. The strain, which was previously stored at -20°C in BHIB, was supplemented with glycerol (30% v/v), revived in BHIB at 37°C, seeded on BHI Agar medium and finally incubated at 37°C for 24 h before being used.

### 2.3 | Preparation of coatings

Essential oil (EO<sub>d</sub>) was obtained by hydrodistillation from *T. fontanesii*, while the phenolic extract consists of the EA fraction of *S. holoschoenus* extract. The extraction procedures were described in a previous study.<sup>6</sup> The coating-forming solution was prepared at room temperature by solubilizing PLA in dichloromethane (3%, w/v), and afterwards, this solution was mixed with the OM consisting of 75% essential oil (EO<sub>d</sub>) (12.5%, v/v) and 25% EA (70 mg/ml) at five different concentrations (C1 = 5%, C2 = 4%, C3 = 3%, C4 = 2% and C5 = 1% [v/v]) or with EO<sub>d</sub> alone at 5% (v/v). Coatings containing EA alone were not prepared due to the lower capacity of this EA fraction to inhibit or kill *P. aeruginosa* in comparison to EO<sub>d</sub> alone or the EO<sub>d</sub>-EA mixture.<sup>6</sup> One millilitre of the coating solution was introduced into 15-ml glass bottles attached horizontally to a rotary laboratory mixer and the solvent then evaporated as the bottle revolved around



**FIGURE 1** Diagram of the bottle attached to the rotary mixer to produce the coating of the inner surface of the bottle

its central axis (Figure 1) at room temperature. The operation was repeated four times for the same bottle, and the wall and base of the bottle were covered with coating. According to the previous tests performed, a more homogenous inner coating thickness was obtained if the application of the coating solution was divided in aliquots of 1 ml. After four times, the thickness of the inner coating was sufficient to contain an appropriate amount of essential oil for its antimicrobial properties to be detected. In order to determine the thickness of the films, they were carefully detached from the inner surface of the bottles and measured with a digital micrometer (Mitutoyo Co., Japan) with a precision of  $\pm 1.0 \mu\text{m}$ . The thickness of the inner coating was always lower than 0.25 mm. The control was prepared with PLA without any active substance. After the preparation of the coatings, bottles were put under UV for 2 min.

## 2.4 | Release profile of PCs from the bottle coating to the liquid medium

To ensure that there was no interaction between PC and the components of the synthetic medium or food products, distilled water was chosen to study the release of PC from the coating. The active coatings were prepared as previously described, and the bottles were filled with 15 ml of distilled water and stored at 37°C, 7°C or 4°C, respectively. The temperature of 37°C was assessed because it is the optimal growth temperature of the bacteria, and the effect of the coatings on the physiological status of *P. aeruginosa* at its most favourable temperature will be tested in the following section. In addition, EFSA recommends refrigeration temperatures below 5°C for the storage of food products,<sup>26</sup> so the temperature of 4°C was also selected. Furthermore, the most usual temperature in Southern European domestic refrigerators is  $7.0 \pm 2.7^\circ\text{C}$ ,<sup>27</sup> so the temperature of 7°C was also assessed.

The Folin–Ciocalteu colorimetric method, described by Georgé et al.,<sup>28</sup> was used for the determination of the total phenolic content (TPC) released at different temperatures and time intervals. For that purpose, 100  $\mu\text{l}$  of the medium was added to 1 ml of Folin–Ciocalteu reagent, which had been diluted 10 times with distilled water beforehand. After 5 min, 1 ml of a 7.5% aqueous solution of sodium

carbonate ( $\text{Na}_2\text{CO}_3$ ) was added as a pH regulator, and then the mixture was put in a water bath at 50°C for 15 min. The calibration curve was prepared with gallic acid as standard and the absorbance was measured at 760 nm using a spectrophotometer (Shimadzu, UV 1203 model, Kyoto, Japan). The TPC was expressed in  $\mu\text{g}$  gallic acid equivalent/ml. Each test was repeated three times.

To calculate the diffusion coefficient of the PC ( $D$ ), the analytical solution of Crank<sup>29</sup> (Equation 1) to study the diffusion phenomena in a plane sheet was used. To apply this equation, it was assumed there was no reaction between PC and the film matrix, a homogeneous concentration distribution of PC, that the amount of solvent could be considered infinite, a negligible edge effect, and therefore, unidirectional diffusion of the active agent from the surface of the film into the liquid.

$$\frac{M_t}{M_\infty} = 1 - \sum_{n=0}^{\infty} \frac{8}{(2n+1)^2 \pi^2} e^{\{-D(2n+1)^2 \pi^2 t / 4L^2\}}, \quad (1)$$

where  $M_t$  is the amount of PC released at each time,  $M_\infty$  is the amount of PC released after infinite time, and  $L$  is the half-thickness of the films tested. If instead of considering all the data collected during this experiment, only the values of  $M_t/M_\infty > 0.4$  are taken; Equation 2 can be rewritten as the following linear regression model<sup>30</sup>:

$$\ln\left(1 - \frac{M_t}{M_\infty}\right) = \ln \frac{8}{\pi^2} - \frac{D\pi^2 t}{4L^2}. \quad (2)$$

When the first term of this equation is plotted vs. time, the  $D$  parameter can be calculated from the slope.

To further characterize the PC release mechanism, the data obtained were modelled using a first-order equation 3 and the Korsmeyer–Peppas equation 4.<sup>31</sup>

$$\frac{M_t}{M_\infty} = 1 - e^{-kt}, \quad (3)$$

$$\frac{M_t}{M_\infty} = k \times t^n, \quad (4)$$

where  $k$  is the release constant of PC and  $n$  is the diffusion exponent that characterizes the release mechanism of the PC.

## 2.5 | Effect of the inner coating on bacterial growth

In order to verify the antimicrobial properties of the inner coatings loaded with several concentrations of OM (C1–C5) and EO<sub>d</sub> (5%), the bottles were filled with a suspension of *P. aeruginosa*, and the bacterial growth curves were obtained. For that purpose, the inner surface of the bottles was coated as described above and then filled with the bacterial suspension prepared at a concentration of 10<sup>5</sup> UFC/ml in nutrient broth. Finally, the bottles were incubated at 37°C for 16 h, since previous tests indicated that after 16 h the bacterial culture entered the stationary phase. Samples were taken after 2, 4, 6, 8, 10, 13 and 16 h. The bacterial growth was expressed as optical density at 600 nm (OD<sub>600</sub>), which was measured with a spectrophotometer. A control was prepared with PLA without extract. A blank was prepared for each sample, and its absorbance was measured over the incubation time period.

## 2.6 | Effect of free OM and EO<sub>d</sub> on the physiological status of *P. aeruginosa* in liquid synthetic medium

A bacterial suspension of 10<sup>5</sup> CFU/ml was prepared from a fresh culture. Five microliter of this suspension was added to 95 µl of BHIB. Then, 100 µL of BHIB containing either EO<sub>d</sub> (2.5 µl/ml) or OM (2.5 µl/ml) was added to this diluted suspension.

The concentrations of the EO<sub>d</sub> and OM correspond in each case to the minimal bactericidal concentration (MBC) as determined in previous work.<sup>6</sup> A control was prepared by replacing the extracts with the culture medium. After incubation for 24 h at 37°C, the inoculums were centrifuged at 13,000×g for 10 min, then the cells were washed twice with phosphate-buffered saline (PBS, pH 7.4, sterile and filtered at 0.22 µm) and resuspended in 1 ml of the same buffer and treated with PI and SYBR Green. PI dye stock diluted in sterile distilled water (1 mg/ml) was added to 200 of cell suspension at a final concentration of 5.25 µg/ml. In addition, 2 µl of SYBR green prepared by diluting 10 µl of the stock solution in 990 µl of EDTA was also added to the same cell suspension.<sup>32</sup>

FC studies were performed using a Cytomics FC 500 flow cytometer (Beckman Coulter, Brea, United States) with a 488- and 633-nm excitation light source. FC measurements of fluorescence corresponding to SYBR-stained cells were collected on the FL1 channel (520 nm), while PI fluorescence was measured on the FL4 channel (675 nm). Data acquisition was analysed with Kaluza Analysis software. Cell percentages were expressed as a percentage of the total stained cells in the upper left, upper right and the lower right quadrants. Those of the lower right quadrant represent dead cells with damaged membrane, while the cells in the upper right quadrant correspond to sublethal cells with compromised membranes.<sup>33</sup>

## 2.7 | Effect of bottle inner coatings on the physiological status of *P. aeruginosa* in fish soup and egg whites

*P. aeruginosa* was inoculated into fish soup and egg whites (10<sup>5</sup> UFC/ml), and bottles with their inner surface coated with PLA-OM and PLA-EO<sub>d</sub> were filled with these contaminated liquid foods. The bottles were incubated for 35 days at 7°C and 4°C. At specific times, 200 µl was taken from each of these incubations and treated as described in the previous section. The bottles were quickly returned to the refrigerated temperature as soon as the sample was taken. FC with a dual staining approach (SYBR green, PI) was used.

## 2.8 | Statistical analysis

Experiments were performed in triplicate and are shown as the mean value ± standard deviation of three independent experiments ( $n = 3$ ). Least significant differences (LSDs) were calculated by Fisher's test to determine significant differences between the tested samples. These analyses were performed using the statistical software Statgraphics® V.15.2.06.

# 3 | RESULTS AND DISCUSSION

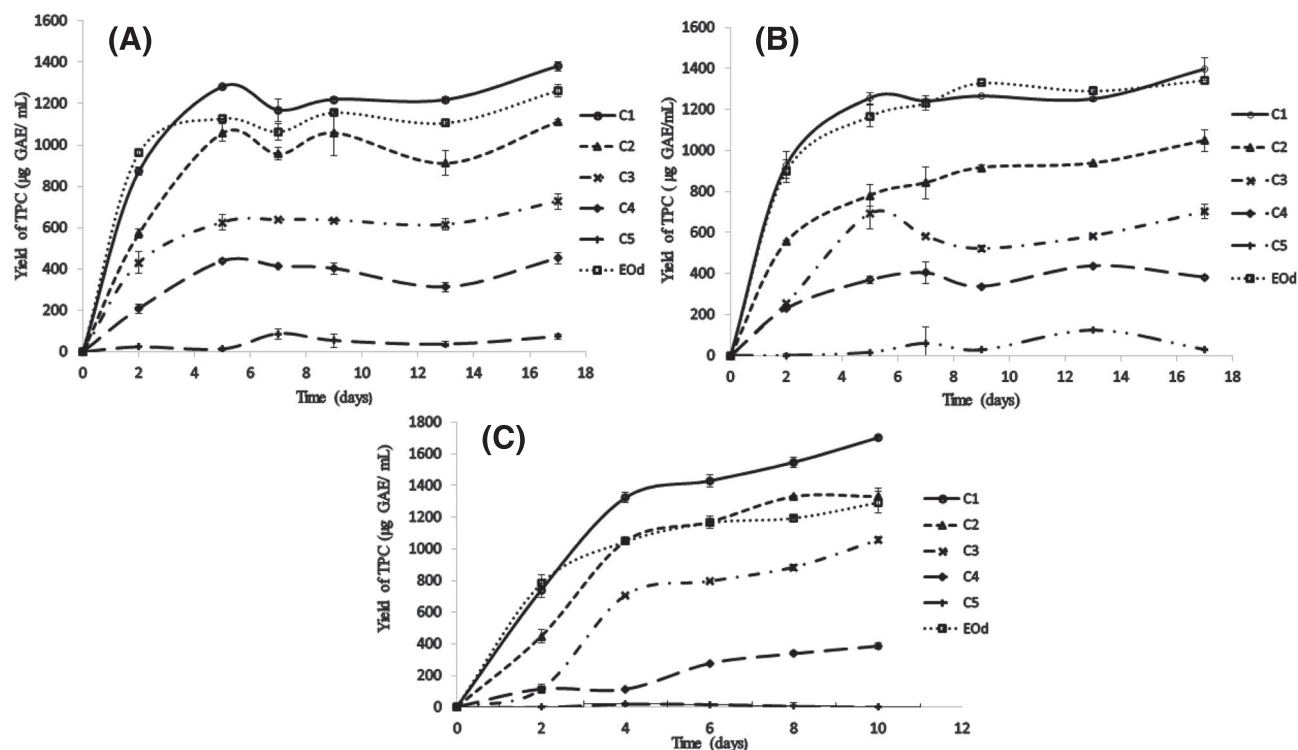
## 3.1 | Release of PCs from the coatings to the liquid medium

The purpose of this experiment was to determine the kinetics of the release of PC from the inner coatings of the bottles into the liquid medium.

The shape of the PC release curve for every inner coating tested shows a high release of plant extract during the first 2 days at low temperatures (4°C and 7°C) and during the first 4 h at 37°C (Figure 2). This profile was reported by several researchers who demonstrated that the diffusion of active compounds from polymer films occurs in two stages: First, water penetrates into the film, causing an increase in diffusivity, and second, equilibrium is reached when the polymer is saturated with water.<sup>34</sup>

In some cases, individual PCs do not behave in the same way during their release from a coating. Indeed, Benbettaieb et al.<sup>35</sup> demonstrated that the different release characteristics of these compounds were due to retention induced by charge-charge interactions with the polymer.

The diffusion coefficients for Samples C1, C2, C3 and EO<sub>d</sub> are shown in Table 1. The samples with the lowest essential oil concentrations, C4 and C5, were not considered because at such low concentrations of PC, the release curves, in particular at 37°C, did not fit adequately to Equation 2. As is shown in Table 1, at each temperature, the diffusion coefficients were found to be very similar at every essential oil concentration tested, which suggests that the release kinetics were not dose dependent. In this respect, del Nobile et al.<sup>36</sup>



**FIGURE 2** Release of phenolic compounds from inner coatings loaded with OM at five different concentrations (C1 = 5%, C2 = 4%, C3 = 3%, C4 = 2% and C5 = 1% [v/v]) or EOd (5%) into water at 4°C (A), 7°C (B) and 37°C (C)

**TABLE 1** Diffusion coefficients of PC released from bottle inner coatings into water

|                 | Diffusion coefficients (cm <sup>2</sup> /s)      |                                                  |                                                  |
|-----------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|
|                 | 4°C                                              | 7°C                                              | 37°C                                             |
| C1              | $9.25 \times 10^{-11} \pm 0.30 \times 10^{-11a}$ | $9.82 \times 10^{-11} \pm 0.10 \times 10^{-11a}$ | $2.45 \times 10^{-10} \pm 0.25 \times 10^{-10a}$ |
| C2              | $9.82 \times 10^{-11} \pm 0.20 \times 10^{-11a}$ | $5.20 \times 10^{-11} \pm 2.30 \times 10^{-11b}$ | $3.43 \times 10^{-10} \pm 0.10 \times 10^{-10b}$ |
| C3              | $7.90 \times 10^{-11} \pm 2.05 \times 10^{-11a}$ | $8.90 \times 10^{-11} \pm 1.00 \times 10^{-11a}$ | $2.94 \times 10^{-10} \pm 0.33 \times 10^{-10a}$ |
| EO <sub>d</sub> | $9.00 \times 10^{-11} \pm 0.01 \times 10^{-11a}$ | $4.48 \times 10^{-11} \pm 3.00 \times 10^{-11b}$ | $1.96 \times 10^{-10} \pm 0.40 \times 10^{-10c}$ |

Note: Values are expressed as mean  $\pm$  standard deviation ( $n = 3$ ). Different letters in the same column indicate significant differences ( $P < 0.05$ ).

studied the release kinetics of thymol from zein films at several thymol concentrations in a water solution, finding in a similar way that the diffusion coefficients showed non-dose-dependent behaviour.

When the effect of temperature was observed, it was found that the release rate of PC was faster at 37°C than at low temperatures in any bottle inner coating tested. Thus, the highest amount of PC released from C1 and EO<sub>d</sub> coatings was  $1701.03 \pm 10.93$  and  $1295.1 \pm 71.07$  µg GAE/ml respectively, at 37°C after 10 h; while the PC release fell to only  $900 \pm 65.6$  and  $927 \pm 54.67$  µg GAE/ml respectively at 7°C after 48 h, and  $871 \pm 19.13$  and  $960 \pm 13.66$  µg GAE/ml respectively at 4°C after 24 h. Additionally, the diffusion coefficient values at 4°C and 7°C were very similar, as was expected, but the value of this parameter noticeably increased when the temperature increased from the 4–7°C range to 37°C. These results are in accordance with those reported by several researchers who demonstrated that temperature is one of the main parameters that impels the

movement of the PC from the biopolymer matrix to the liquid medium.<sup>37,38</sup> In fact, Marcet et al.<sup>39</sup> assessed the thymol release profile from PLA nanoparticles and recovered 93% and 60% at 35°C and 5°C respectively in 6 h.

In order to characterize the mechanism of release of the PC from the bottle inner coating, the release data of the C1 sample were fitted to Equations 3 and 4 at 4°C, 7°C and 37°C, obtaining the release rate constant ( $k$ ) and the diffusion exponent indicative of the mechanism of PC release ( $n$ ) (Table 2). In this case, only Sample C1 was tested, since, as was shown above, the release kinetics were not dose dependent. The data fits these equations with a high correlation coefficient value at every temperature tested ( $R^2 > 0.98$ ). In the case of the first-order kinetic equation, the good fit of the experimental data to this equation suggests that the driving force of the movement of bioactive agents from the inside to the outside of the PLA film is the difference in concentration. Moreover, parameter  $k$  showed a similar value at

**TABLE 2** Release kinetic parameters of PC from bottle inner coatings into water

|      | First order              |                | Korsmeyer–Peppas         |                |
|------|--------------------------|----------------|--------------------------|----------------|
|      | k                        | R <sup>2</sup> | n                        | R <sup>2</sup> |
| 4°C  | 0.24 ± 0.04 <sup>a</sup> | 0.98           | 0.43 ± 0.05 <sup>a</sup> | 0.99           |
| 7°C  | 0.28 ± 0.05 <sup>a</sup> | 0.98           | 0.37 ± 0.11 <sup>a</sup> | 0.99           |
| 37°C | 0.46 ± 0.01 <sup>b</sup> | 0.99           | 0.83 ± 0.01 <sup>b</sup> | 0.98           |

Note: Values are expressed as mean ± standard deviation ( $n = 3$ ). Different letters in the same column indicate significant differences ( $P < 0.05$ ).

4°C and 7°C, as was expected, but the value of this parameter increased from 0.24 at 4°C to 0.46 at 37°C, corroborating that the release of PCs at the highest temperature tested is faster than at the lowest. Regarding the parameter  $n$ , the values obtained at the lowest temperature tested suggest a release mechanism by diffusion following Fick's law. Therefore, in this case, the release of the PCs is produced due to the transportation of these molecules from higher concentration areas to those of lower concentration, as was also suggested by the first-order equation kinetic results. However, when the same parameter was obtained at 37°C, the main driving force of PC release is an anomalous transport, being a mixture of Fickian diffusion and the movement of the PCs due to the swelling of the polymeric matrix.<sup>40</sup> In this case, the release of the PCs is facilitated to some extent by the interaction of the water with the PLA matrix, increasing its size and producing the disentanglement of the PLA biopolymers.

### 3.2 | Effect of the inner coatings of the bottles on bacterial growth

Bottles were coated with PLA-OM at five OM concentrations or with PLA-EO<sub>d</sub> at 5% (v/v) EO<sub>d</sub>, then filled with *P. aeruginosa* in nutrient broth, and finally the bacterial growth was followed for 16 h at 37°C (Figure 3). This temperature was selected because it is the optimal growth temperature for *P. aeruginosa*,<sup>41</sup> and in this manner, any synergetic effect between the temperature and the antimicrobial effect of the plant extracts is avoided.

In this case, an increase in optical density was observed after 2–3 h for every sample tested. However, when the coatings were loaded with OM, it was found that the higher the concentration of bioactive agent in the films was, the more the microbial growth was hampered. Moreover, at the highest concentrations of plant extract tested (PLA-EO<sub>d</sub> and C1 coatings), the microbial growth was almost completely inhibited.

Furthermore, when correlation analysis was performed on the relation between the OD<sub>600</sub> and the concentration of OM in the PLA coatings at 16 h of incubation, it indicated a negative correlation with a coefficient of determination  $R^2 = 0.91$ , which suggests that the microbial inhibition was positively correlated with antimicrobial concentrations over the range of concentrations tested.

Studies demonstrating the antibacterial activity of species belonging to the *Thymus* genus are numerous.<sup>42</sup> Some researchers attribute the antibacterial potential of thyme to its hydrophobic phenolic constituents such as carvacrol and thymol, which can be incorporated into the lipid layer of the cell membrane, disrupting it.<sup>43</sup> The development of various films incorporating this oil has been investigated in many studies. In this context, Patel et al.<sup>44</sup> reported that chitosan films incorporating thyme oil exhibited an antibacterial effect against *Pseudomonas*. However, only a few studies have reported on the inhibitory effect of *S. holoschoenus* essential oil against *Bacillus subtilis* and *Staphylococcus aureus*<sup>45</sup> and *P. aeruginosa*,<sup>6</sup> so it is difficult to compare this study with those in the literature. The activity of the plant could be attributed to its PCs, such as resveratrol, which is present in *S. holoschoenus* in relatively large quantities.

### 3.3 | Effect of free OM and EO<sub>d</sub> on the physiological status of *P. aeruginosa* in synthetic medium

The importance of double staining is to be able to distinguish dead cells from living ones, but it can also detect damaged cells, which may recover their activity in the future. In this case,  $51.35 \pm 1.65\%$  of the total cell population was found to stain with PI/SYBR green in the control sample, which represented stressed cells.

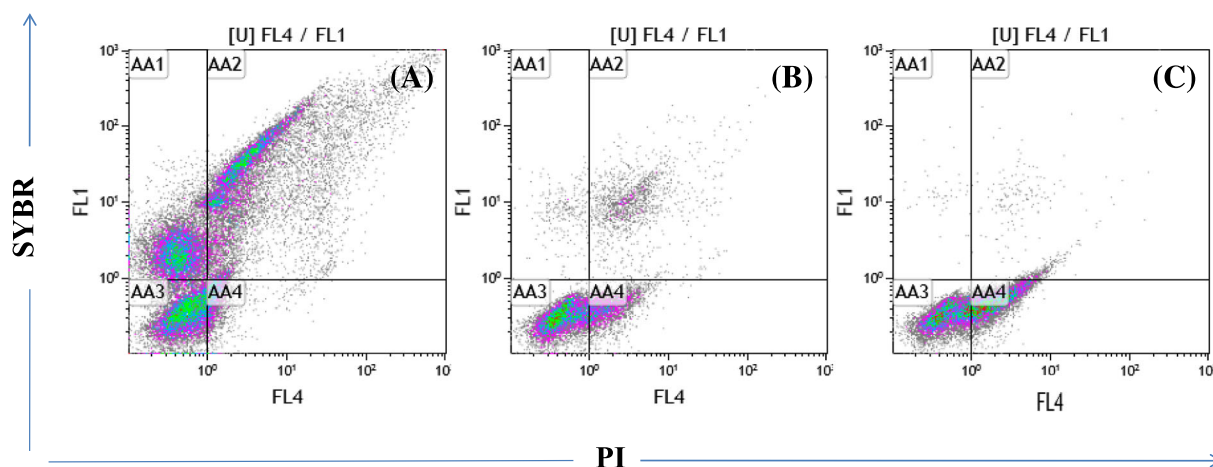
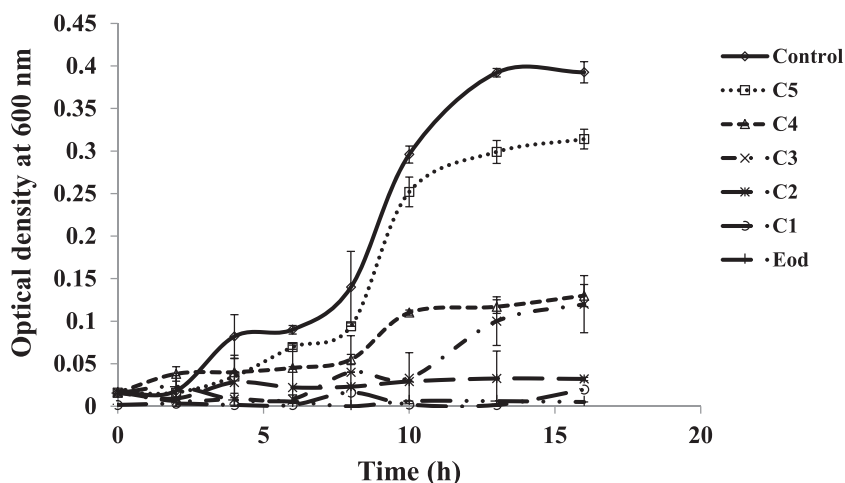
Furthermore, the treatment of the cells with the antibacterial compounds rendered cells more permeable to the entry and uptake of PI (Figure 4 and Table 3). The degree of uptake of PI gives an estimate of the integrity of the membrane; if the membrane is damaged, the PI penetrates the cell and binds to the nucleic acids. The results obtained with *P. aeruginosa* growing in BHIB at 37°C revealed that the cells treated with OM at the concentration corresponding to the MBC showed higher PI uptake ( $91.60 \pm 1.6\%$ ) compared to that of EO<sub>d</sub>, while the control sample, without treatment, was relatively poorly PI stained ( $11.87 \pm 1.80\%$ ). Thus, the tested extracts were found to affect mainly the integrity of the bacterial membrane.

### 3.4 | Effect of coatings on the physiological status of *P. aeruginosa* in fish soup and egg whites

As was shown in Figure 2, the release of the essential oils at fridge temperatures was slower than the release at 37°C. The bioactive compounds were almost completely released in 5 days and remained in the liquid medium for a long period of time, probably exerting their antimicrobial properties. In order to test these antimicrobial properties in a real liquid food over a long period of refrigerated storage, the effect of OM concentration and temperature of incubation on the physiology of *P. aeruginosa* in fish soup was assessed (Table 4) at 7°C and 4°C.

In the control sample at 7°C, after 35 days of incubation,  $45.67 \pm 1.78\%$  of the bacterial population was viable (SYBR-stained). However, at the highest OM concentration tested (C1), the

**FIGURE 3** Bacterial growth of *Pseudomonas aeruginosa* at 37°C in nutrient broth-filled bottles coated with PLA-OM at five different OM concentrations (C1 = 5%, C2 = 4%, C3 = 3%, C4 = 2% and C5 = 1% [v/v]) or EO<sub>d</sub> (5%)



**FIGURE 4** Dot plots representing SYBR green fluorescence versus PI fluorescence of *Pseudomonas aeruginosa* cells in BHIB and grown in liquid medium (A), in liquid medium with free EO<sub>d</sub> at its MBC (B), in liquid medium with free OM at its MBC (C)

**TABLE 3** Cell subpopulations in samples treated with the MBC of free EO<sub>d</sub> and OM, after 24 h at 37°C

| Cell subpopulations   | Control                    | EO <sub>d</sub>            | OM                         |
|-----------------------|----------------------------|----------------------------|----------------------------|
| SYBR green I stain    | 37.92 ± 1.01 <sup>a1</sup> | 2.71 ± 2.42 <sup>a2</sup>  | 0.36 ± 0.27 <sup>a2</sup>  |
| PI stain              | 11.87 ± 1.80 <sup>b1</sup> | 71.73 ± 6.91 <sup>b2</sup> | 91.60 ± 1.61 <sup>b3</sup> |
| SYBR green I/PI stain | 51.35 ± 1.65 <sup>c1</sup> | 25.55 ± 4.49 <sup>c2</sup> | 8.03 ± 1.89 <sup>c3</sup>  |

Note: Values are expressed as mean ± standard deviation (n = 3). Different letters in the same column and different numbers in the same row indicate significant differences (P < 0.05).

Abbreviations: EO<sub>d</sub>, diluted essential oil; OM, optimized mixture.

percentage of viable bacteria after 35 days of assay decreased to 0.96% of the total cell population. Furthermore, the percentage of viable bacteria after 35 days increased as the concentration of OM decreased, as was to be expected. In fact, at the minimum OM concentration tested (C5), the percentage of viable bacteria was similar to the control value (49.49 ± 4.94%). Regarding the percentage of non-viable bacteria with membrane damage at 7°C, the highest percentage for the PI-stained cell subpopulation was seen at the maximum concentration of OM tested (C1, 85.78 ± 0.86%), which, as

explained previously, was due to the membrane-damaging effect of the essential oil compounds. In addition, the percentage of this cell subpopulation decreases as the concentration of OM decreases from Samples C1–C5. In relation to the percentage of double-stained cells (SYBR/PI), which represent cells whose survival is compromised, the highest populations found with this physiology were found in the control and at the lowest OM concentrations tested (C4 and C5). In fact, C4 and C5 samples showed an overall staining behaviour similar to that found for the control sample, which could be because the

TABLE 4 Cell subpopulations (%) in untreated soup and soup treated with OM at different concentrations

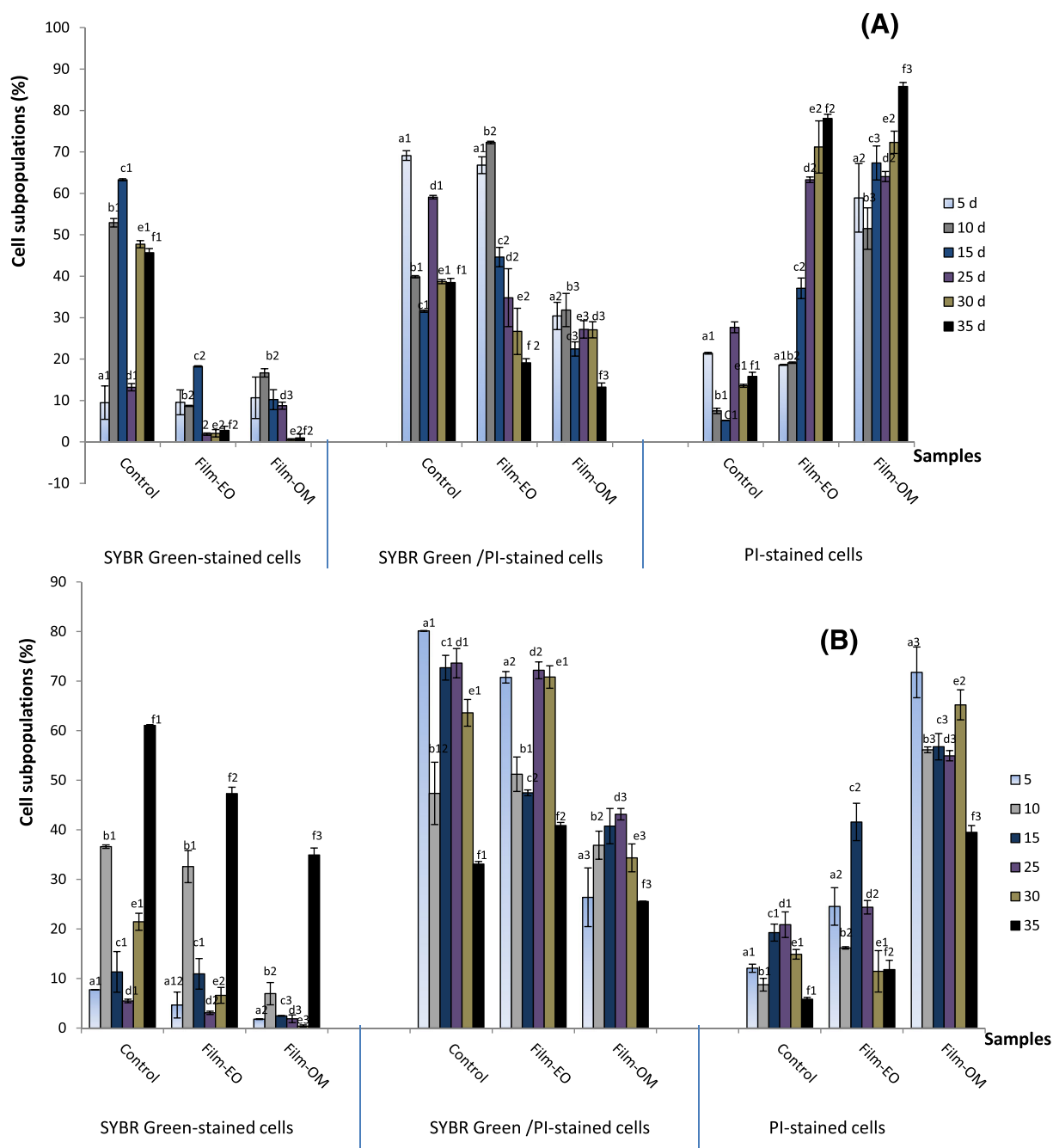
| Temperature   |  | 7°C                         |                             |                             |                             |                            | 4°C                         |                             |                            |                             |                             |
|---------------|--|-----------------------------|-----------------------------|-----------------------------|-----------------------------|----------------------------|-----------------------------|-----------------------------|----------------------------|-----------------------------|-----------------------------|
| Days          |  | 5                           | 10                          | 25                          | 30                          | 35                         | 5                           | 10                          | 25                         | 30                          | 35                          |
| Samples       |  |                             |                             |                             |                             |                            |                             |                             |                            |                             |                             |
| SYBR stain    |  |                             |                             |                             |                             |                            |                             |                             |                            |                             |                             |
| C1            |  | 10.69 ± 5.04 <sup>a1</sup>  | 16.65 ± 1.00 <sup>b1</sup>  | 08.75 ± 0.87 <sup>c1</sup>  | 00.60 ± 0.20 <sup>d1</sup>  | 00.96 ± 0.21 <sup>e1</sup> | 1.82 ± 0.079 <sup>a1</sup>  | 06.95 ± 2.25 <sup>b1</sup>  | 01.89 ± 0.78 <sup>c1</sup> | 00.42 ± 0.21 <sup>d1</sup>  | 34.92 ± 1.41 <sup>e1</sup>  |
| C2            |  | 14.02 ± 1.6 <sup>a1</sup>   | 27.65 ± 7.97 <sup>b2</sup>  | 24.01 ± 1.88 <sup>c2</sup>  | 02.56 ± 0.23 <sup>d1</sup>  | 01.84 ± 0.05 <sup>e1</sup> | 1.54 ± 0.078 <sup>a1</sup>  | 13.62 ± 1.37 <sup>b1</sup>  | 02.99 ± 0.46 <sup>c1</sup> | 00.44 ± 0.18 <sup>d1</sup>  | 36.07 ± 2.03 <sup>e1</sup>  |
| C3            |  | 10.77 ± 0.1 <sup>a1</sup>   | 42.39 ± 1.59 <sup>b3</sup>  | 23.72 ± 6.71 <sup>c2</sup>  | 11.14 ± 0.60 <sup>d2</sup>  | 09.27 ± 0.75 <sup>e2</sup> | 2.78 ± 0.08 <sup>a1</sup>   | 12.62 ± 2.16 <sup>b2</sup>  | 04.73 ± 0.36 <sup>c1</sup> | 02.62 ± 0.22 <sup>d2</sup>  | 38.49 ± 3.57 <sup>e1</sup>  |
| C4            |  | 18.89 ± 4.4 <sup>a12</sup>  | 51.47 ± 0.24 <sup>b3</sup>  | 10.69 ± 0.01 <sup>c1</sup>  | 31.52 ± 0.65 <sup>d3</sup>  | 35.34 ± 1.88 <sup>e3</sup> | 4.12 ± 0.42 <sup>a1</sup>   | 27.47 ± 7.12 <sup>b2</sup>  | 02.08 ± 0.06 <sup>c1</sup> | 04.77 ± 0.44 <sup>d3</sup>  | 37.86 ± 4.22 <sup>e1</sup>  |
| C5            |  | 25.53 ± 1.5 <sup>a2</sup>   | —                           | 13.61 ± 6.48 <sup>c12</sup> | 32.59 ± 0.309 <sup>d3</sup> | 49.49 ± 4.94 <sup>e4</sup> | 16.88 ± 10.18 <sup>a2</sup> | 24.92 ± 4.13 <sup>b2</sup>  | 08.75 ± 4.32 <sup>c1</sup> | 10.06 ± 0.95 <sup>d4</sup>  | 36.23 ± 1.88 <sup>e1</sup>  |
| PI stain      |  |                             |                             |                             |                             |                            |                             |                             |                            |                             |                             |
| C1            |  | 58.90 ± 8.28 <sup>f1</sup>  | 51.51 ± 5.01 <sup>g1</sup>  | 64.07 ± 1.21 <sup>h1</sup>  | 72.31 ± 2.17 <sup>i1</sup>  | 85.78 ± 0.86 <sup>j1</sup> | 71.78 ± 5.11 <sup>f1</sup>  | 56.13 ± 0.59 <sup>g1</sup>  | 54.94 ± 1.93 <sup>h1</sup> | 65.22 ± 3.04 <sup>i1</sup>  | 39.99 ± 1.37 <sup>j1</sup>  |
| C2            |  | 54.57 ± 3.95 <sup>f1</sup>  | 33.36 ± 1.94 <sup>g2</sup>  | 45.60 ± 3.08 <sup>h12</sup> | 66.95 ± 2.29 <sup>i2</sup>  | 77.66 ± 0.62 <sup>j2</sup> | 80.38 ± 18.14 <sup>f1</sup> | 44.0 ± 2.22 <sup>g2</sup>   | 50.63 ± 2.06 <sup>h1</sup> | 57.03 ± 0.89 <sup>i2</sup>  | 33.38 ± 0.55 <sup>j1</sup>  |
| C3            |  | 48.32 ± 5.36 <sup>f12</sup> | 21.85 ± 0.21 <sup>g3</sup>  | 43.69 ± 7.10 <sup>h12</sup> | 48.88 ± 0.57 <sup>i3</sup>  | 47.88 ± 1.20 <sup>j3</sup> | 65.75 ± 5.14 <sup>f12</sup> | 40.22 ± 5.16 <sup>g2</sup>  | 54.18 ± 0.44 <sup>h2</sup> | 43.54 ± 0.74 <sup>i3</sup>  | 29.21 ± 2.08 <sup>j1</sup>  |
| C4            |  | 36.87 ± 2.99 <sup>f2</sup>  | 13.92 ± 2.19 <sup>g4</sup>  | 49.30 ± 3.59 <sup>h1</sup>  | 29.89 ± 0.37 <sup>i4</sup>  | 29.02 ± 0.10 <sup>j4</sup> | 51.00 ± 2.9 <sup>f2</sup>   | 24.90 ± 5.21 <sup>g3</sup>  | 38.72 ± 0.41 <sup>h2</sup> | 19.55 ± 2.81 <sup>i4</sup>  | 24.12 ± 0.31 <sup>j1</sup>  |
| C5            |  | 20.06 ± 0.11 <sup>f3</sup>  | —                           | 25.81 ± 16.83 <sup>h2</sup> | 25.86 ± 1.97 <sup>i4</sup>  | 15.20 ± 1.63 <sup>j5</sup> | 22.27 ± 2.38 <sup>f3</sup>  | 20.89 ± 3.99 <sup>g3</sup>  | 30.58 ± 0.74 <sup>h2</sup> | 12.22 ± 1.03 <sup>i5</sup>  | 16.71 ± 0.41 <sup>j1</sup>  |
| SYBR/PI stain |  |                             |                             |                             |                             |                            |                             |                             |                            |                             |                             |
| C1            |  | 30.42 ± 3.28 <sup>k1</sup>  | 31.83 ± 04.01 <sup>l1</sup> | 27.17 ± 2.09 <sup>m1</sup>  | 27.07 ± 1.96 <sup>n1</sup>  | 13.25 ± 0.65 <sup>o1</sup> | 26.39 ± 5.91 <sup>k1</sup>  | 36.90 ± 2.84 <sup>l1</sup>  | 43.15 ± 1.15 <sup>m1</sup> | 34.35 ± 2.82 <sup>n12</sup> | 25.57 ± 0.36 <sup>o1</sup>  |
| C2            |  | 31.40 ± 2.30 <sup>k1</sup>  | 38.98 ± 06.03 <sup>l1</sup> | 30.38 ± 1.19 <sup>m1</sup>  | 30.47 ± 2.05 <sup>n1</sup>  | 20.49 ± 0.67 <sup>o2</sup> | 38.7 ± 2.62 <sup>k12</sup>  | 42.34 ± 0.84 <sup>l12</sup> | 46.36 ± 1.60 <sup>m2</sup> | 42.55 ± 0.70 <sup>n1</sup>  | 30.53 ± 153 <sup>o12</sup>  |
| C3            |  | 40.89 ± 5.47 <sup>k1</sup>  | 35.74 ± 1.38 <sup>l1</sup>  | 32.58 ± 0.39 <sup>m1</sup>  | 39.97 ± 0.006 <sup>n2</sup> | 42.83 ± 0.45 <sup>o3</sup> | 31.46 ± 5.23 <sup>k12</sup> | 47.40 ± 2.99 <sup>l2</sup>  | 41.07 ± 0.81 <sup>m3</sup> | 53.82 ± 0.97 <sup>n3</sup>  | 32.28 ± 1.48 <sup>o23</sup> |
| C4            |  | 44.23 ± 1.43 <sup>k1</sup>  | 34.60 ± 2.19 <sup>l1</sup>  | 40.00 ± 3.60 <sup>m1</sup>  | 38.58 ± 01.38 <sup>n2</sup> | 35.62 ± 1.99 <sup>o4</sup> | 44.86 ± 2.48 <sup>k2</sup>  | 47.61 ± 1.91 <sup>l2</sup>  | 59.18 ± 0.40 <sup>m4</sup> | 28.56 ± 4.14 <sup>n2</sup>  | 38.00 ± 3.9 <sup>o3</sup>   |
| C5            |  | 54.45 ± 1.41 <sup>k1</sup>  | —                           | 60.56 ± 10.34 <sup>m2</sup> | 41.47 ± 1.05 <sup>n2</sup>  | 35.29 ± 3.30 <sup>o4</sup> | 60.83 ± 7.8 <sup>k3</sup>   | 54.18 ± 0.13 <sup>l3</sup>  | 65.50 ± 1.48 <sup>m5</sup> | 37.21 ± 4.47 <sup>n1</sup>  | 47.05 ± 2.30 <sup>o4</sup>  |

Note: C1–C5, concentration of OM; (—), undetermined. Values are expressed as mean ± standard deviation ( $n = 3$ ). Means with different letters were significantly different at the level of  $P < 0.05$ . a–r are notations for differentiation between concentrations, and 1–3 are notations for the level of statistical differences.

essential oil concentration was too low to have any effect on bacterial growth. This good performance of essential oils against food borne pathogens under refrigerated temperatures has been studied previously by many researchers. In this sense, to cite just a few examples, Heydari-Majid et al.<sup>46</sup> prepared PLA-based films loaded with ZnO and essential oils from *Mentha piperita* L. and *Zataria multiflora* Boiss to wrap fish fillets, extending the shelf life of the fillets from 7 to 16 days. In addition, Porter et al.<sup>47</sup> studied the effect of mustard essential oil and carvacrol on ground chicken previously inoculated

with *Salmonella* under refrigerated temperatures at 4°C and 10°C, resulting in a slight decrease in the population of *Salmonella* at 4°C and a bacteriostatic effect at 10°C.

Furthermore, temperature was found to be an important variable to consider, since at 4°C, a decrease in the percentage of viable bacteria was detected compared with the same percentage at 7°C in the earlier and intermediate stages of bacterial growth. For example, after 5 days and in every sample tested, the percentage of viable cells (SYBR stained) was lower at 4°C than at 7°C. A similar outcome was



**FIGURE 5** Time-course profiles of cell subpopulations in soup model during storage for up to 35 days and at 7°C (A) and 4°C (B). Values are expressed as mean  $\pm$  standard deviation ( $n = 3$ ). Means with different letters were significantly different at the level of  $P < 0.05$ ; a–r are notations for the different number of days in storage, and 1–3 are notations for the level of statistical differences

**TABLE 5** Cell subpopulations in untreated and treated liquid eggs

|             |                 | SYBR strain                 |                            | SYBR/PI strain             |                            | PI strain                   |                           |
|-------------|-----------------|-----------------------------|----------------------------|----------------------------|----------------------------|-----------------------------|---------------------------|
| Time (days) |                 | 5                           | 35                         | 5                          | 35                         | 5                           | 35                        |
| 4°C         | Control         | 38.03 ± 3.45 <sup>a1</sup>  | 77.74 ± 1.38 <sup>b1</sup> | 60.02 ± 3.63 <sup>c1</sup> | 19.35 ± 0.34 <sup>d1</sup> | 1.94 ± 0.17 <sup>e1</sup>   | 1.15 ± 0.02 <sup>f1</sup> |
|             | EO <sub>d</sub> | 31.90 ± 6.47 <sup>a12</sup> | 66.42 ± 0.39 <sup>b2</sup> | 64.20 ± 7.00 <sup>c1</sup> | 32.28 ± 0.27 <sup>d2</sup> | 3.88 ± 0.58 <sup>e2</sup>   | 0.75 ± 0.90 <sup>f2</sup> |
|             | OM              | 26.64 ± 2.57 <sup>a2</sup>  | 53.62 ± 0.51 <sup>b3</sup> | 70.40 ± 2.90 <sup>c1</sup> | 45.31 ± 0.42 <sup>d3</sup> | 3.30 ± 0.33 <sup>e2</sup>   | 1.60 ± 0.11 <sup>f1</sup> |
| 7°C         | Control         | 41.46 ± 1.36 <sup>a1</sup>  | 51.70 ± 5.16 <sup>b1</sup> | 57.52 ± 0.98 <sup>c1</sup> | 43.86 ± 3.22 <sup>d1</sup> | 1.01 ± 0.37 <sup>e1</sup>   | 4.42 ± 1.94 <sup>f1</sup> |
|             | EO <sub>d</sub> | 38.39 ± 0.01 <sup>a1</sup>  | 50.86 ± 10.5 <sup>b1</sup> | 58.52 ± 1.37 <sup>c1</sup> | 28.66 ± 1.03 <sup>d2</sup> | 3.08 ± 0.12 <sup>e1,2</sup> | 2.45 ± 0.59 <sup>f1</sup> |
|             | OM              | 31.55 ± 7.48 <sup>a1</sup>  | 44.83 ± 1.69 <sup>b1</sup> | 65.34 ± 7.61 <sup>c1</sup> | 51.99 ± 6.92 <sup>d1</sup> | 3.09 ± 0.07 <sup>e2</sup>   | 3.16 ± 0.66 <sup>f1</sup> |

Note: Values are expressed as mean ± standard deviation (n = 3). Means with different letters were significantly different at the level of *P* < 0.05; a–f are notations for the difference in n° of days in storage, and 1–3 are notations for the level of statistical difference.  
Abbreviations: EA, ethyl acetate fraction; EO<sub>d</sub>, diluted essential oil; OM, optimized mixture.

obtained after 30 days, but in contrast, after 35 days of bacterial growth, the percentage of SYBR-stained bacteria (viable cells) was higher at 4°C than at 7°C. In addition, the percentage of dead bacteria (PI stained) was higher at earlier stages at 4°C than at 7°C, but after 30 days of bacterial growth, the percentage of PI-stained bacteria was higher at 7°C than at 4°C. This was offset, however, by the percentage of the sublethal bacteria population, which was higher at 4°C than at 7°C after 30 and 35 days. These differences detected in the behaviour of OM at 4°C and 7°C in the early stages of this experiment may be explained by the higher efficacy of essential oils at lower temperature, which is also tied to a decrease in bacterial growth at lower temperatures. Moreover, in the later stages of the experiment (35 days), the SYBR-stained bacterial subpopulation was higher at 7°C than at 4°C, which may be explained by faster essential oil depletion at the lowest temperature tested, maybe enhanced by temperature-dependent interactions between the food matrix and the essential oil compounds.

Additionally, to detect any difference in the antimicrobial performance of OM compared to that of EO<sub>d</sub>, bottles coated with PLA containing EO<sub>d</sub> (5%) were filled with *P. aeruginosa*-contaminated soup and their antimicrobial properties were tested at 7°C and 4°C. The percentage value of each *P. aeruginosa* subpopulation was compared to that found in control and PLA-OM C1 samples (Figure 5). In this case, after 35 days, the effect of EO<sub>d</sub> on the physiology of the bacteria at both 7°C and 4°C led to a slightly higher percentage of viable cells and a lower percentage of bacteria killed by membrane damage with respect to those percentages for the C1 sample. This higher antimicrobial activity shown by the C1 sample may be due to the existence of interactions between the constituents of EO<sub>d</sub> and those in the phenolic extract EA contained in OM.

Finally, to test the performance of the PLA coatings loaded with OM and EO<sub>d</sub> in another food matrix, the same experiment was conducted, but in this case, filling the bottles with egg white contaminated with *P. aeruginosa*. In Table 5 are shown the bacterial subpopulation percentages after 5 and 35 days of assay and only for EO<sub>d</sub> and the highest OM concentration tested (C1), since lower OM concentrations (from C2 to C5) did not exert any antimicrobial effect in this

food matrix. As is shown in this table, after 35 days of incubation at both 7°C and 4°C, the percentage of viable cells rose noticeably for both essential oils tested. Moreover, the percentage representing the PI-stained bacterial subpopulation was lower than 5% for every time, temperature and essential oil tested. Therefore, and contrary to the clear antimicrobial effect shown by the PLA bottle coatings with contaminated fish soup, with egg whites, these plant extracts have exhibited scarce membrane-damaging action and a poor antimicrobial effect, which may be due to the presence of ovalbumin in this liquid food. In this respect, it is interesting to refer to the findings of Veldhuizen et al.,<sup>48</sup> who attribute a decrease in the antimicrobial activity of carvacrol, the major component of thyme and oregano essential oils, to binding interactions between this bioactive compound and bovine serum albumin, an animal protein similar to ovalbumin.

## 4 | CONCLUSIONS

The release profile of the PC contained in the coatings on the inner side of bottles into the liquid medium was assessed, the highest release of almost all the active antimicrobial compounds being obtained in few hours at 37°C. Therefore, taking into consideration this result, it would be recommendable to fill these inner coated bottles at the lowest temperatures tested in order not to produce a massive release of the bioactive agent within such a short time span. Furthermore, FC was found to be a valuable technique for following the physiological changes undergone by *P. aeruginosa* in these experiments, providing more detailed knowledge on the effect of the plant extracts on this bacterium. When the bottles with inner coatings were filled with fish soup contaminated with *P. aeruginosa*, the novel OM active agent was seen to be more highly effective at killing this pathogenic bacterium at 7°C and 4°C than the *T. fontanesii* essential oil (EO<sub>d</sub>) alone. This is likely to have been due to the synergy between the phenols from different sources present in the OM composition. Therefore, this innovative bottle inner surface coating was found to be suitable for killing *P. aeruginosa* at refrigerated temperatures, but

the food matrix must be considered, so changes in the composition of the liquid food may lead to an improvement or reduction of its effectiveness.

## ACKNOWLEDGEMENTS

This work was financially supported by the Principality of Asturias, within the project GRUPIN IDI/2018/000127 and by the grant of the Ministry of Higher Education and Scientific Research of Algeria to S. Oussaid.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**How to cite this article:** Oussaid S, Madani K, Houali K, et al. Effect of novel bottle inner surface coatings made with polylactic acid and plant extracts on the physiological status of *Pseudomonas aeruginosa* in real liquid foods using flow cytometry. *Packag Technol Sci.* 2021;1-12. doi:10.1002/pts.2620