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PHBV active bilayer films obtained by compression-molding applying essential oils at the interface

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Abstract

Four active components, oregano essential oil (OR) and its respective main compound, carvacrol (CA); clove essential oil (CLO) and its respective main compound, eugenol (EU), were used separately to obtain poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) bilayer films with antimicrobial activity, by compression molding. The active compounds were sprayed (15% w/w, polymer: active compound ratio) at the interface between two layers of PHBV, which were joined by thermo-compression. Tensile, barrier and optical properties, as well as thermal behaviour of the films, were characterized after 1 week at 25° C and 53 % relative humidity. Likewise, the antimicrobial activity of the films was evaluated against *Escherichia coli* and *Listeria innocua*. Although the tensile properties of the films were not improved with respect to pure PHBV films by the addition of the active compounds, more transparent materials with better water vapour barrier capacity were obtained. Thermogravimetric analyses showed that CA and EU slightly decreased the polymer thermal stability, while OR and CLO led to more thermo-resistant material. Miscibility of actives with the polymer was assessed through the promoted decrease in its melting temperature and crystallinity degree. PHBV films allowed the release of active compounds in adequate amounts and rate into culture media to control the microbial growth of the two tested bacteria. The

films were significantly more effective against *E. coli* than against *L. innocua*. Both bacteria were more sensitive to OR and to its main compound, CA, due to the higher antimicrobial effectiveness of these components.

Keywords: poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV), carvacrol, eugenol, oregano, clove, *Listeria innocua*, *Escherichia coli*, antimicrobial.

1. Introduction

The packaging industry is the main consumer of plastic materials, most of them oil-based. In this context, the food sector is the main producer of packaging waste, since packaging plays an essential role in the transport and preservation of food. Nevertheless, the increasing concern about the environmental impact generated by packaging waste has promoted the search for biodegradable alternatives.[1,2] One of the promising options in biomaterial development for packaging are polyhydroxyalkanoates (PHAs), which are completely biodegradable linear polyesters that are produced by bacteria.[3] These biopolymers can be synthetized from renewable resources such as sucrose, starch, cellulose, etc.[1] Within this large family of PHAs, poly-3-hydroxybutyrate (PHB) is the most studied, since it shows similar properties to some synthetic thermoplastic polymers such as polypropylene.[4] However, the high crystallinity of its structure makes it yield rigid and brittle materials, thus limiting its application range.[5,6] In order to solve these drawbacks, copolymers such as polyhydroxybutyrate-*co*-hydroxyvalerate (PHBV) have been developed.[7,8]

In order to compensate for the drawbacks of biodegradable materials in comparison to conventional synthetic packaging systems an increased functionality of the former is required. Nevertheless, biodegradable films with antioxidant and antimicrobial properties have the advantage of reducing or inhibiting the microbial growth and the

oxidative processes in foods.[9] Natural compounds used in the formulation of biodegradable films such as essential oils (EOs) have shown antioxidant and antimicrobial activity and have been recognised as safe by the Food and Drug Administration (FDA).[10,11] EOs are oily, aromatic and volatile liquids of complex composition with 2 or 3 major components, which can represent up to 85 % of the total. There are other minor components present in trace amounts.[12-14] Oregano essential oil and clove essential oil are among the most effective EOs in controlling microbial growth.[15] The antimicrobial activity of these oils is mainly attributed to their major components, carvacrol and eugenol, respectively.[13] Nevertheless, some studies have concluded that the whole EO has greater antibacterial activity than the mixture of its major components[16,17], which suggests that the minor components are critical for the activity and may have a synergistic or potentiating effect .[13]

The incorporation of EOs as active compounds in biodegradable films involves high losses of volatiles when the films are made, both in extension and drying of the film-forming dispersions (casting), as in the thermo-processing (extrusion or melt blending). Nevertheless, the incorporation of EOs by spraying them on one side of the film and the subsequent thermo-compression of two films, obtaining a bilayer film with the active compounds at the interface, could be an appropriate strategy to improve the process of obtaining such films. In this way, the release of the active compounds would occur progressively from the package to the food surface or to the headspace of the packaging. This approach would avoid the direct application of the active compounds on the food, which has been previously found to have serious drawbacks.[18]

The aim of the present work was to develop bilayer PHBV films incorporating oregano or clove essential oils and their major components, carvacrol (CA) or eugenol (EU). The resulting films were evaluated in their antimicrobial and functional properties.

2. Materials and methods

2.1. Materials

Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) 8% (PHBV) was provided in pellet form by NaturePlast (Caen, France). Oregano (OR) and clove (CLO) essential oils were obtained from Herbes del Molí (Alicante, Spain). Polyethylenglycol 1000 (PEG100), used to plasticize the polymer, carvacrol (CA), eugenol (EU), and UV methanol were supplied by Sigma-Aldrich (Sigma–Aldrich Chemie, Steinheim, Germany).

2.2. Preparation of films

2.2.1. Preparation of PHBV films

PHBV films were obtained by compression-molding. To this end, PHBV was mixed with 10 % w/w of PEG1000 on a two-roll mill (Model LRM-M-100, Labtech Engineering, Thailand) at 180 ºC and 15 rpm for 10 min. Afterwards, the pellets were compression-moulded using a hydraulic press (Model LP20, Labtech Engineering, Thailand). Then, 3.5 grams of pellets were put onto steel sheets and pre-heated on the heating unit for 5 min. Next, compression was performed at 160 °C for 4 min at 10 MPa followed by a cooling cycle of 3 min.

2.2.2. Incorporation of active compounds into bilayer structures

The obtained PHBV monolayers were sprayed with a constant amount of active compound (OR, CLO, CA, EU) of 15% w/w with respect to the polymer matrix (polymer plus plasticiser) and were covered with another monolayer. Finally, in order to obtain bilayer films the ensemble monolayers were compressed at 160 ºC for 2 min at a pressure of 7 MPa followed by a cooling cycle of 3 min. Thus, five kinds of films were

obtained: pure polymer bilayer films without active compounds (PHBV), as a control, and films with active compounds (PHBV-CA, PHBV-EU, PHBV-OR, PHBV-CLO).

2.3. Film characterization

2.3.1. Retention of active compounds

Quantification of the active compound retention in the films was carried out by two different methods. The first was to weigh the film before and after the pressing process. Thus, the retention percentage can be estimated by the difference in weight. The second was to extract the active compound with UV methanol, followed by spectrophotometric quantification using a UV-visible spectrophotometer (Evolution 201, Thermo Scientific). This was performed to determine the retention percentage in PHBV bilayer films with pure CA or EU. To this end, film samples of 100 mg were cut in strips, as thin as possible to promote the total release of the active compounds from the polymer matrix. The strips were placed in flasks with 10 mL of UV methanol, which were kept stirring at 20 ºC for 24 hours. After that, samples were filtered and appropriately diluted to obtain absorbance values between 0.2 and 0.8. Then, CA and EU were quantified through the absorbance measurement at 275 and 282 nm, respectively. In order to ensure total extraction of active compounds, the solvent was replaced by new solvent after 24 hours, and samples were kept stirring at 20 ºC for 72 hours more. Then, samples were analysed spectrophotometrically in the same way. PHBV bilayer films without active compounds were also submitted to the same extraction procedure in order to use their methanol extract as blank solution. Measurements were taken in quintuplicate per formulation and all absorbance measurements were taken in triplicate. Standard calibration curves for CA and EU were obtained to determine their

concentration from the absorbance values by using an initial solution with 500 µg/mL and the subsequent dilutions.

2.3.2. Scanning electron microscopy

Microstructure of the cross-sections of the films was observed using a Scanning Electron Microscope (JEOL JSM-5410, Japan). Film samples were cryofractured by immersion in liquid nitrogen, fixed on copper stubs and gold coated. Then the images were captured using an accelerating voltage of 10 kV.

2.3.3. Optical properties

The surface reflectance spectra of the films were determined from 400 to 700 nm using a spectro-colorimeter CM-3600d (Minolta Co., Tokyo, Japan). The measurements were taken in duplicate in three films of each formulation. Transparency of the films was evaluated applying the Kubelka–Munk theory for multiple scattering to the reflection spectra (Eqs 1-3)[19], where R_0 is the reflectance of the film on an ideal black background and R is the reflectance of the sample layer backed by a known reflectance (R_g).

$$a = \frac{1}{2} \cdot \left(R + \frac{R_0 - R + R_g}{R_0 \cdot R_g} \right) \quad (1)$$

$$b = (a^2 - 1)^{\frac{1}{2}} \quad (2)$$

$$T_i = \sqrt{(a - R_0)^2} - b^2 \quad (3)$$

The reflectance of an infinitely thick layer of the material (R_∞) was calculated by means of Eq. 4 in order to obtain the colour coordinates: Lightness (L^*), Chroma (C_{ab}^*) (Eq. 5)

and hue (h_{ab}^{*}) (Eq. 6), using illuminant D65 and 10º observer. Moreover, to evaluate the colour differences between the different films and the control film (PHBV) Eq. 7 was used.

$$R_{\infty} = a - b \tag{4}$$

$$C_{ab}^{*} = \sqrt{\left(a^{*2} + b^{*2}\right)} \tag{5}$$

$$h_{ab}^{*} = arctg\left(\frac{b^{*}}{a^{*}}\right) \tag{6}$$

$$\Delta E = \sqrt{\left(\Delta L^{*}\right)^{2} + \left(\Delta a^{*}\right)^{2} + \left(\Delta b^{*}\right)^{2}} \tag{7}$$

2.3.4. Tensile properties

Tensile properties of the films were evaluated using a universal testing Machine (TA.XTplus model, Stable Micro Systems, Haslemere, England) according to ASTM D882 standard method.[20] The typical mechanical parameters used in this kind of analysis, tensile strength (TS), elastic modulus (EM) and elongation at break (E), were obtained from the stress-strain curves of the different samples. To this end, the film strips (2.5 x 10 cm) were placed in film-extension grips and stretched until breaking at 50 mm min^{-1}. Measurements were taken in eight replicates per formulation in films conditioned at 25 ° C and 53% RH for one week

2.3.5. Water vapour permeability

The water vapour permeability (WVP) was determined in quadruplicate in films conditioned at 25 ° C and 53% RH for one week, according to gravimetric method ASTM E-96-95.[21] For this purpose, the film samples were placed on Payne permeability cups (3.5 cm in diameter, Elcometer SPRL, Hermelle/s Argenteau, Belgium) with distilled water inside to get 100% RH on one side of the film. Each cup was placed in a

desiccator at 25 °C with an oversaturated solution of magnesium nitrate (53% RH). In order to reduce the resistance to transport of water vapour, a fan was placed above each cup. The cups were weighed periodically over four days and the WVP was calculated from the slopes of the curves of weight loss versus time [22]

2.3.6. Thermal properties

A thermogravimetric analyser (Star^{e}System, Mettler-Toledo, Inc., Switzerland) was used to evaluate the thermal stability of the different types of films. Measurements of the thermal weight loss of each type of film were performed in duplicate in a temperature range between 25 ºC and 600 ºC at a heating speed of 10 ºC/min under nitrogen stream of 20 mL/min.

Differential scanning calorimetry (DSC) analyses were performed on a differential scanning calorimeter (Stare System, Mettler-Toledo, Inc., Switzerland). Film samples (~10 mg) were weighted, placed into aluminium pans and analysed by a multiple scan. Firstly, a scan from 25ºC to -60 ºC at a rate of 10 ºC/min. Then, samples were heated to 200 ºC and cooled down to -60 ºC at the same rate. Lastly, a second heating scan wasperformed (10 ºC/min). All measurements were taken in duplicate under nitrogen stream of 20 mL/min. The sample crystallinity was calculated from the enthalpy of melting of 100% crystalline PHB (ΔH^{0}_{PHB} = -132 J/g polymer)[23,24] and the measured melting enthalpy of different samples (ΔH), using Eq. 8.

$$X(\%) = \frac{\Delta H}{\Delta H^{0}_{PHB}} \cdot 100 \qquad (8)$$

2.3.7. Antimicrobial activity

The methodology followed for the determination of the antimicrobial activity of the films was adapted from Jiménez et al.[25]. *Listeria innocua* (CECT 910) and *Escherichia coli* (CECT 101) were supplied by the Spanish Type Culture Collection (CECT, Burjassot, Spain). These bacterial cultures were regenerated (from a culture stored at -

194 25 ºC) by transferring a loopful into 10 mL of Tryptone Soy Broth (TSB, Scharlab, Barcelona, Spain) and incubating at 37 ºC for 24 hours. From this culture, a 10 µL
196 aliquot was again transferred into 10 mL of TSB and grown at 37 ºC for 24 hours more in order to obtain a culture in exponential phase of growth. Afterwards, this bacterial
198 culture was appropriately diluted in TSB tubes to get a target inoculum of 10^5 CFU/mL. Circular samples of 55 mm in diameter, obtained from the different types of film
200 formulations, were placed in inoculated tubes and incubated for 13 days at 10 ºC. Immediately after the inoculation and after 2, 6, 9 and 13 days, the microbial counts on
202 Tryptone Soy Agar (TSA, Scharlab, Barcelona, Spain) plates were examined. To this end, serial dilutions were made and poured onto TSA dishes which were incubated for
204 24 hours at 37 ºC. All tests were performed in duplicate.

The amounts of CA and EU that migrated from the films to the culture media at the
206 different times were estimated from the release kinetics of these compounds in food simulants (data not shown). To this end, film samples of 500 mg were placed in flasks
208 with 100 mL of solvent and stirred at 20 ºC. The released compound was analysed at different contact times. Simulant A (10% ethanol) was selected for the release studies
210 after considering its similar composition to that of the culture media.

2.4. Statistical analysis

212 Analysis of variance (ANOVA) with Fisher′s least significant difference (LSD) at 95% confidence level was performed to analyse the data statistically To this end,
214 Statgraphics Centurion XVI (Manugistics Corp., Rockville, MD, USA) was used.

216 3. Results

3.1. Active compound retention in the films

The weight losses of the films after the thermo-compression were quantified in order to determine the loss of the active compounds from a simple approach. The weight loss of the films with active compounds ranged between 3 and 6 % with respect of the initial mass, showing great variability, which does not allow us to obtain significant differences between formulations. This loss must be mainly attributed to volatilization of actives, since moisture content of PHBV films was 0.9% and no significant water loss can be assumed. Based on the weight loss, 20-44 % of the incorporated amount of actives could be lost by volatilization during thermo-compression, although the weight measurements could imply notable errors.

On the other hand, CA and EU contents in PHBV-CA and PHBV-EU films, determined by methanol extraction and spectrophotometric quantification, were 11.5±1.3 and 8.1±1.4 g/ 100 g polymer matrix, respectively. Comparison of these values with the incorporated amount (15 g/100 g polymer matrix), leads to retention percentages of 80 % ± 6 %, for CA, and 58 % ± 6 % for EU. Significant differences in the retention level of both compounds and the similarity in their boiling points (237.7ºC and 253.2ºC, respectively) suggest that a part of eugenol could be more strongly bonded to the polymer matrix and that no total extraction in methanol of this compound from the films could be obtained. In this sense, the extraction procedure is limited by the bonding of actives to the polymer matrix, and greater amounts of these (not extractable) could be present in the film.

3.2. Film microstructure

Figure 1 shows the SEM micrographs of the cross-section of PHBV bilayer films without and with different active compounds. No layer separation was observed in control films (PHBV), which indicates a good join of the layers after the thermo-pressing process. In the same way, the micrographs of the bilayer films with active

compounds show no separation between the layers, which proves the good miscibility of the active compounds in the polymer matrix. The micrographs reveal that active compounds diffuse from de interface to the matrix sinus, where they are retained in a homogenous way. Thermo-compression led to a good incorporation of active compounds in the bilayer films, as deduced from the homogeneity of the film and the complete adhesion of the two polymer layers containing actives in between.

3.3. Optical properties

Table 1 shows the optical properties evaluated for each formulation. The high values of T_i are coherent with the great film homogeneity and transparency. On the contrary, lower values of Ti are typical of more opaque films. Incorporation of the studied active compounds at the interface of PHBV bilayer films gave rise to films with significantly higher T_i values at 550 nm (where the spectra show the biggest difference), meaning that the PHBV films with active compounds were slightly more transparent than the control film (pure PHBV). According to the SEM micrographs, these results reflected again the good miscibility of the active compounds in the polymer layers, where they diffuse from the interface. This diffusion could debilitate the polymer interchain forces, decreasing the matrix compactness, which would give rise to more transparent films. This effect would impact on the film mechanical behaviour, giving rise to lower TS and EM values, as commented below.

Regarding the film colour, the addition of the different active compounds did not provoke significant differences in film lightness, as has been reported by Martucci et al.[28]. When CA or EU were incorporated, the film chrome significantly decreased, whereas the EOs addition did not modify this parameter as reported by Muriel-Galet et al. [29] and Teixeira et al.[30] for ethylene-vinyl alcohol copolymer (EVOH) and fish protein films added with OR. Film hue was significantly affected by the incorporation of both

268 OR and its main component, CA. Unlike these, CLO and its main component, EU, had no significantly effect on this parameter.

270 Films with CA showed the highest colour differences with respect to control films. Nevertheless, these differences are not relevant in practical terms since colour

272 differences below 2.4 cannot be perceived by the human eye.[31]

3.4. Tensile properties

274 Table 2 shows the tensile parameters, elastic modulus (EM), tensile strength (TS) and elongation at break (%E) for each film formulation. The incorporation of the studied

276 active compounds in PHBV bilayer films significantly decreased the EM, TS and %E values. Nevertheless, although the addition of all compounds reduced the material

278 stretchability, carvacrol gave rise to films that were more extensible than those containing EU, OR or CLO. Films containing EU, OR or CLO did not show significant

280 differences among them in terms of mechanical behaviour. Previous studies have shown similar results due to the addition of CLO, OR or EU in fish protein and thermoplastic

282 flour films.[30,32] On the contrary, other studies performed with CA and OR incorporated in triticale protein, polypropylene and alginate films led to films that were more

284 extensible than the pure polymer films.[33-36] In the same way, the decrease in the EM and TS values due to the incorporation of these active compounds, has also been described

286 for EU, OR and CA by Woranuch and Yoksan[32], Aguirre et al.[33] and Ramos et al.[35], respectively. Given the poor stretchability of the obtained PHBV films, their application

288 would be limited to packaging uses where ductility is not required, such as preformed trays or lids.

290 The disparity in the effect of actives on the film tensile behaviour can be due both to the different quantity of active compound incorporated in the polymer matrix, as well as to

292 the different interactions between components. When active compounds are

incorporated at the interface between two layers, these are not directly included in the sinus of the polymer matrix. As a result, these compounds could provoke a discontinuity between both layers that could affect the mechanical behaviour of the layer assembly. Nevertheless, this discontinuity was not observed in the microstructural images and thus, it is possible to assume that the active compounds diffuse fast into the polymer matrix. Therefore, the balance of molecular interactions between the active compounds and the polymer chains near the interface will affect the overall cohesion forces of the polymer network. Given the good adhesion between PHBV layers, interactions between added compounds and polymer chains will govern the resulting mechanical properties of the films. In this sense, no relevant differences in tensile behaviour were observed for active bilayers. The only remarkable fact is the lower reduction in the film stretchability when CA was used, which could be attributed to its effect at the interfacial level, increasing the layer adhesion forces. The molecules that diffused into the PHBV matrix could provoke a weakening effect of the chain forces of polymer, making the chain slippage easier during the tensile test. Other phenols of the tested actives could promote some cross-linking effect in the matrix, thus lowering the ductility of the material, as deduced from the films' thermal behaviour commented on below.

3.5. Water vapour permeability

The water vapour permeability (WVP) values of PHBV bilayer films are shown in Table 2. The incorporation of active compounds in PHBV bilayer films gave rise to significantly lower values of WVP, except for the CLO that showed no differences with respect to the control film. OR and its main component, CA, were the most effective at decreasing WVP values. Analogously, Woranuch and Yoksan[32] and Benavides et al[34] reported a higher water barrier capacity in thermoplastic flour and alginate films after the incorporation of EU and OR, respectively. However, other studies reported the

318 opposite effect (Aguirre et al.[33], Teixeira et al.[30] and Rojas-Graü et al.[36])- As previously mentioned, these differences can be attributed to both the different quantity of active
320 compound incorporated in the polymer matrix, as well as to the different interactions between components that define the matrix strength.

322 The differences in chemical composition and molecular polarity of the matrix components can explain the different WVP values of the bilayer films with active
324 compounds, since the water vapour transfer trough the film is greatly affected by the hydrophilic-lipophilic balance of the film components.[30] In this sense, all active
326 compounds significantly increased the water vapour barrier capacity of the films since they are all hydrophobic substances. Among them, the most effective to promote the
328 water barrier capacity was CA, followed by OR, which main component is CA..

3.6. Thermal behaviour

330 Figure 2 shows the degradation pattern of pure PHBV films and of those containing different active compounds. Pure films showed a degradation pattern with two main
332 thermal events. The first one corresponds to PHBV degradation, while the second one corresponds to the plasticizer degradation as can be observed in Figure 2A, where the
334 behaviour of the polymer without plasticizer is included. Likewise, the effect of the second compression step on bilayer films can also be observed. A decrease in the
336 thermo-resistance of the polymer is promoted by this second compression, which indicates that the thermal treatment induced structural changes in the polymer network.
338 When the active compounds were added, a weight loss started at about 150ºC due to the progressive volatilization of actives from the film (boiling points of CA and EU,
340 237.7ºC and 253.2ºC, respectively).[37] In fact, thelosses of the active compoundes overlapped with the degradation step of PHBV, which did not permit the estimation of
342 their release from the film by TGA.

Table 3 gives the onset ant peak temperatures for the main degradation step of the polymer in the different films. The incorporation of pure CA or EU enhanced the thermo-sensitivity of PHBV, decreasing the onset and peak of degradation temperature, whereas the whole essential oils (OR and CLO) slightly promote the thermal stability of the polymer. Incorporation of some additives to polymer matrices usually results in a reduction of the thermo-resistance of the network due to the weakening effect of the chain bonds.[38] Nevertheless, some authors[39,40] reported an increase in the polymer thermo-resistance when some essential oils were added. Among all formulations, the film with CLO was the most thermo-resistant, which could be associated with stronger interactions between PHBV and some components of the EO, thus reinforcing the polymer network. This is coherent with the lower extraction capacity for the EU from the films using methanol, which suggests the strong bonding of a part of this compound to the polymer network.

Figure 3 shows the DSC thermograms for the first heating scan (A) and the cooling scan (B) of control PHBV films and those containing active compounds. Likewise, monolayer (pressed once) PHBV films with and without plasticizers were also analysed to evaluate the effect of processing. Table 4 shows the peak temperature values for melting (first and second heating scan) and crystallization (cooling scan) of PHBV for the different samples, as well as the corresponding enthalpy values of these transitions. Glass transition temperature (T_g) of the amorphous phase of polymer was also determined in the first heating scan. Plasticizer addition slightly reduced both temperature and enthalpy associated to either melting or crystallization process of PHVB, whereas plasticizers did not significantly affect T_g. This fact indicate that PEG interacts with the polymer lattice, affecting crystallization behaviour and reducing the crystallization degree of PHVB from 99 to 60 %. However, the application of a second

368 thermo-compression step in the bilayer formation promoted crystallization of the polymer (Table 4) in line with the increase in the molecular mobility during the heating
370 step. As expected, this effect disappears in the second heating scan where the thermal history of the samples has been deleted. The addition of active compounds modified
372 thermal behaviour of PHBV in agreement with their miscibility with the polymer. Both, crystallization and melting peaks were shifted to lower temperature values when any of
374 the active components was present in the film. Likewise, all of them provoked a reduction in the crystallinity degree of polymer in the film (Table 4). This effect was
376 more pronounced for the whole EOs, and especially for clove essential oil. This indicates that other EOs' components interact with polymer chains to a greater extent,
378 thus affecting thermal behaviour. This effect of the EOs on the crystallinity of polymers was also reported for polypropylene films with CA and thymol by Ramos et al.[35]. The
380 film thermal properties obtained from the first and the second heating scans were very similar, which indicates no sensitivity of the polymer to the different thermal history.
382 On the other hand, the differences between T_c and T_m were about 50 ºC in all cases, which indicates that the same supercooling effect occurred in all cases and so any of the
384 added compounds showed nucleating action, but only a depressing effect on the melting point according to the miscibility effects. All samples showed a small glass transition at
386 T_g about 7 ºC[41] without significant differences between them. The thermal response of the films agreed with that observed in TGA analysis where the whole EOs seemed to
388 have greater impact on the polymer structure than pure CA or EU.

3.7. Antimicrobial properties.

390 Figure 3 shows the microbial counts at different incubation times of different films in contact with the culture media with *L. innocua* (Fig. 3A) and *E. coli* (Fig. 3B). The
392 respective amounts (g/mL) of CA and EU released from the film to the culture medium,

assuming the same kinetic release as in simulant A, were also given for films containing
394 CA or EU. Films without active compounds had no effect on growth of both bacteria, which indicates that the antimicrobial effect cannot be attributed to the polymer. The
396 incorporation of active compounds in PHBV bilayer films, regardless of their nature, significantly decreased the growth of *L. innocua* and of *E. coli. L.innocua* was more
398 sensitive to OR and its main component (CA), than to CLO and its main component (EU). Similar results were reported by Teixera et al.[30]. Although EU was more retained
400 within the polymer matrix when methanol extraction was performed, the available ratio of EU was released faster into simulant A, reaching an equilibrium value higher than
402 that obtained for CA. In this way, at every time, the EU concentration in the media was significantly higher than the CA level. Therefore, the greater antimicrobial activity of
404 CA in the films must be attributed to its higher effectiveness as reported by Burt[13]. The main inhibition mechanism of this type of actives, as reported by other authors[42-44], is
406 the disturbance of the cytoplasmic membrane of the cells, disrupting the proton motive force, electron flow, active transport and coagulation of cell contents. Nevertheless, a
408 different intensity of the effects must be expected as a function of the molecular structure of the active.

410 Films containing CA or OR showed similar antimicrobial effect at 2 and 13 days. However, they had a different antimicrobial effect within this period. While CA led to a
412 gradual decrease in the *L. innocua* population, OR showed higher antimicrobial activity from day 2. A faster kinetic release of OR from the films, or a synergic effect of CA
414 with the other compounds present in the oil, which lend it higher activity, could explain this behaviour. Films containing EU were more effective against *L. innocua* than those
416 with CLO throughout the first 9 days. Nevertheless, the effect was reversed after 13 days. This behaviour suggests the delayed release of active components of CLO.

In general, *E. coli* was more sensitive than *L. innocua* to all tested compounds, since a total inhibition was rapidly observed when *E.coli* was cultured in presence of films containing CA, EU or OR. Previous studies in films with OR and CA have reported similar antimicrobial activities against *E. coli*.[33, 45, 46, 28, 35]. For both bacteria, PHBV-CLO films were the least effective, showing higher activity against *E. coli*. This behaviour agrees with the possible crosslinking effect of oil compounds into the polymer matrix, as deduced from the thermogravimetric analyses, which could inhibit their effective release to the culture media, thus decreasing their antimicrobial action.

At all contact times, the estimated CA concentrations in the media were significantly higher than the minimum inhibitory concentrations (MICs) reported for *L. innocua* ($3.7 \cdot 10^{-4}$ g/mL)[13] and *E. coli*. ($3.1 \cdot 10^{-4}$ g/mL)[47], as deduced from the found antimicrobial activity. In the same way, the EU concentrations were also higher than the respective MICs reported for the same bacteria ($1.5 \cdot 10^{-3}$ g/mL)[48] and ($1.2 \cdot 10^{-3}$ g/mL)[47]. The obtained results reveal that PHBV is an adequate matrix as a carrier of EU or CA to obtain antimicrobial films against Gram-positive or Gram-negative bacteria such as *E. coli* or *L. innocua*. The studied active agents were more effective against Gram-negative bacteria, such as *E. coli*, than against Gram-positive bacteria, such as *L. innocua.* On the contrary, other studies performed with antimicrobial films containing the same active compounds showed higher antimicrobial activity against Gram-positive cells, without outer membrane.[33, 38, 34, 13, 49, 35, 30] However, Aldana et al.[50] have also observed a higher antimicrobial activity of films with lime EO against Gram-negative bacteria. This fact indicates that the nature and structural characteristics of the matrix from which the essential oil is released, as well as the film formation method, could greatly affect the films' antimicrobial properties.[46]

4. Conclusion

PHBV bilayer films with active compounds incorporated at the interface between the layers were obtained by compression molding, with a good layer adhesion and homogeneous structure. This method produced antimicrobial films with appropriate tensile, optical and water vapour barrier properties, and with good thermal stability, even if the incorporation of active compounds significantly affected the physical properties of the films. Although, the studied actives did not improve the tensile properties of the films, more transparent materials with better water vapour barrier capacity were obtained. As regards thermal stability, while CA and EU gave rise to a slight decrease in the thermal stability of the polymer matrix, OR and CLO led to more thermo-resistant materials. The compound miscibility into the polymer matrix was confirmed by DSC analyses, where a notable decrease in the polymer melting temperature and crystallinity was observed when active compounds, especially whole EOs, were incorporated. The release of the active ingredients from the films was adequate to control the growth of *E. coli* and *L. innocua* in the culture media. Active films were significantly more effective against *E. coli* than against *L. innocua*, and both bacteria were more sensitive to OR and its main compound, CA. The greater antimicrobial activity of films containing CA was attributed to the higher effectiveness of CA, since the amount of EU released from de film was higher than that of CA. Therefore, incorporation of natural antimicrobials such as those studied, especially CA and OR, into bilayer films of PHBV could be a promising option for the development of active biodegradable films.

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580 **Figures**

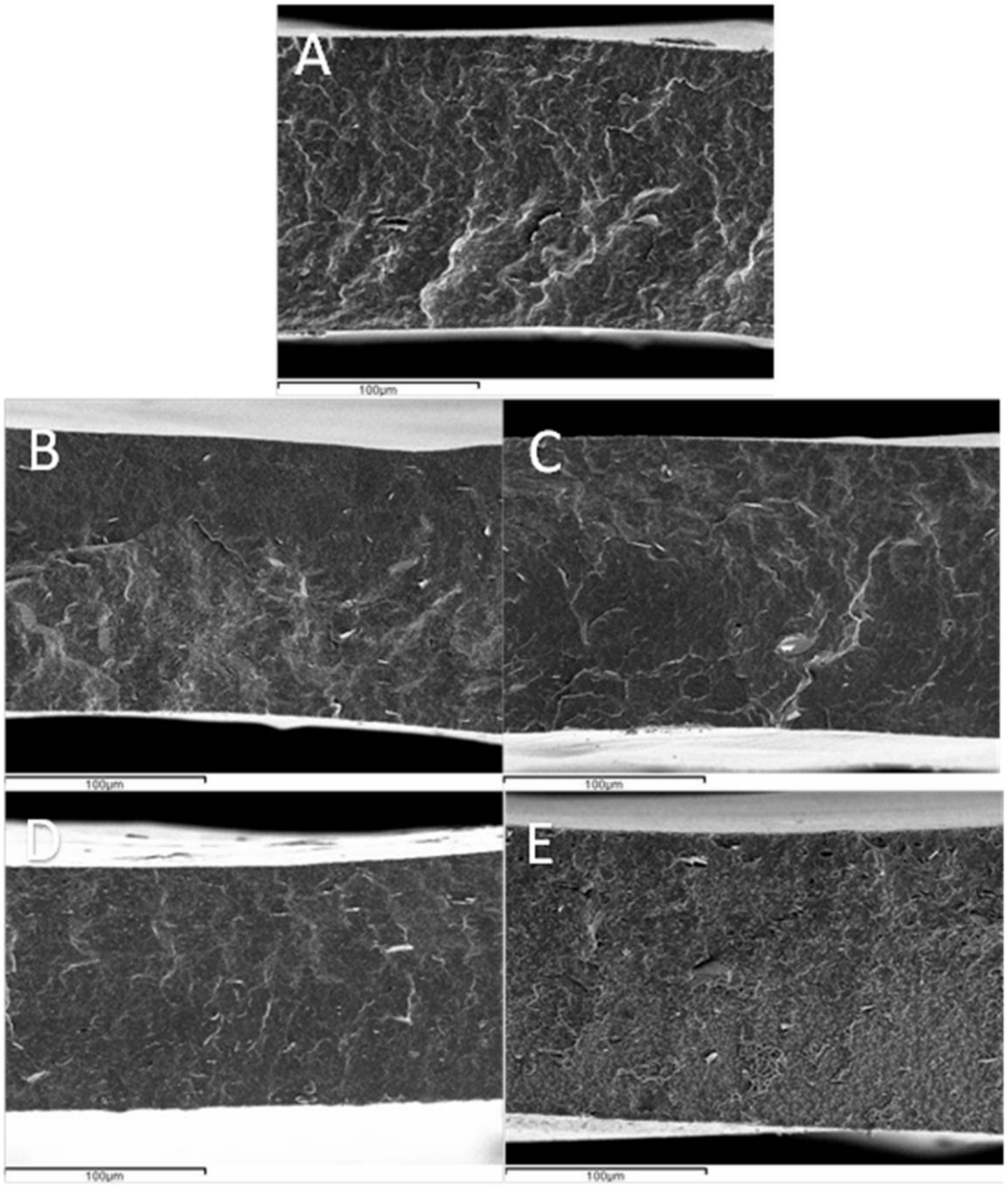

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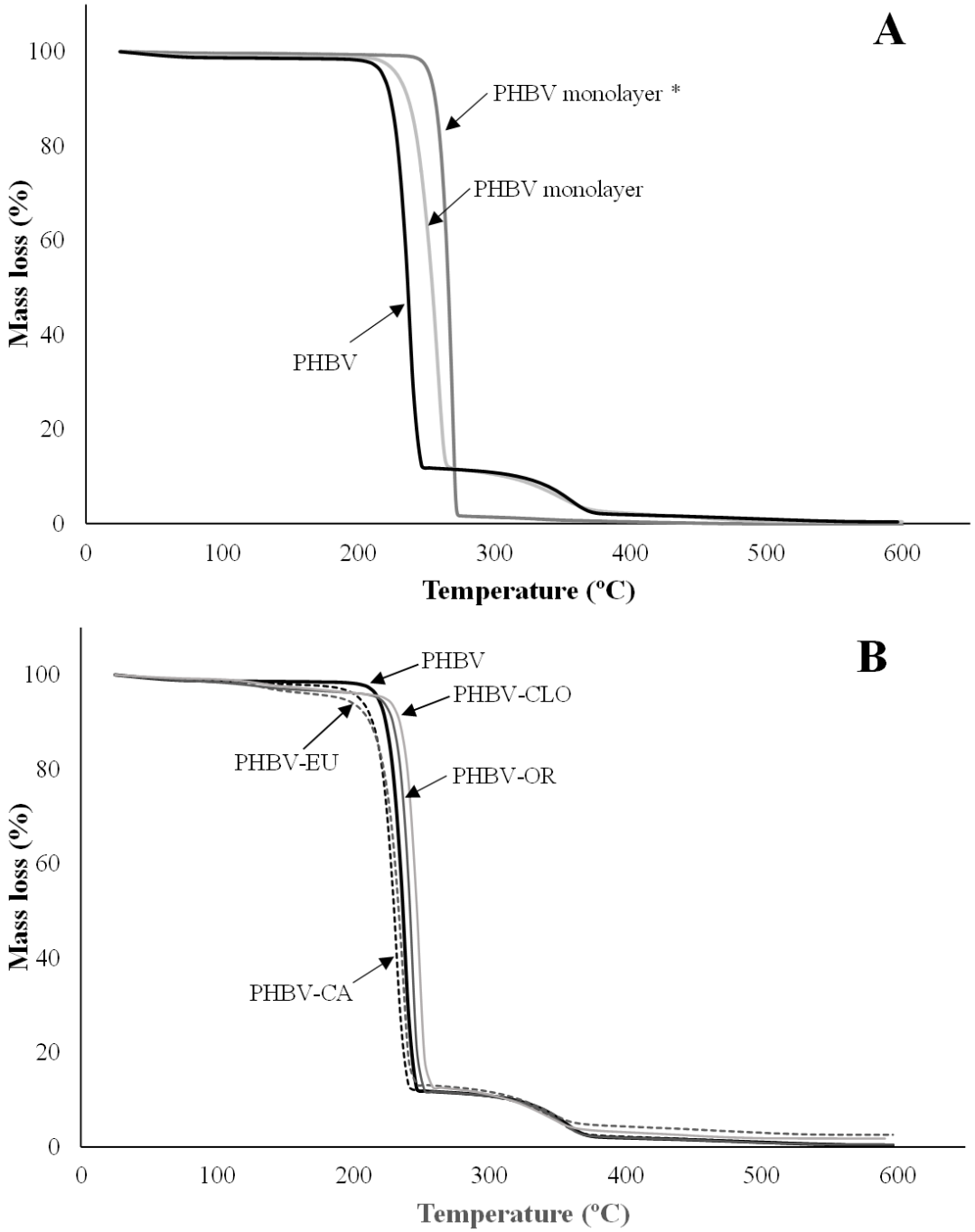
Figure 1. SEM micrographs of the cross section of PHBV bilayer films without (A) and

584 with active compounds: carvacrol (B), eugenol (C), oregano essential oil (D), clove essential oil (D).

586



588

Figure 2. Thermogravimetric curves of PHBV films. A: PHBV monolayer and bilayer
590 films containing or not (*) plasticizer; B: PHBV bilayer films with active compounds (CA: carvacrol, OR: oregano essential oil, CLO: clove essential oil; EU: eugenol).

592

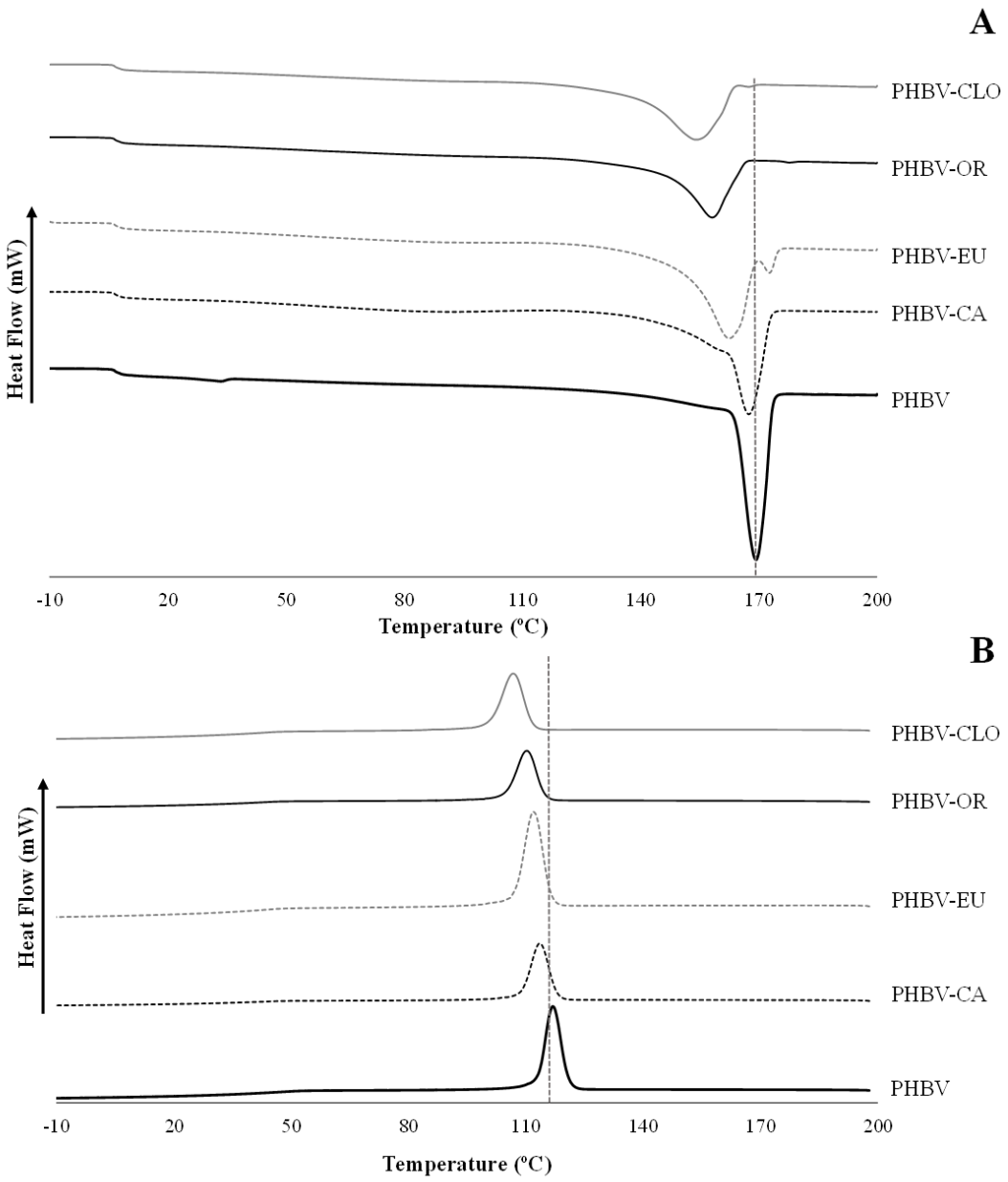


Figure 3. DSC thermograms for the first heating scan (A) and the cooling scan (B) of control PHBV film and with active compounds (CA: carvacrol, OR: oregano essential oil, CLO: clove essential oil; EU: eugenol).

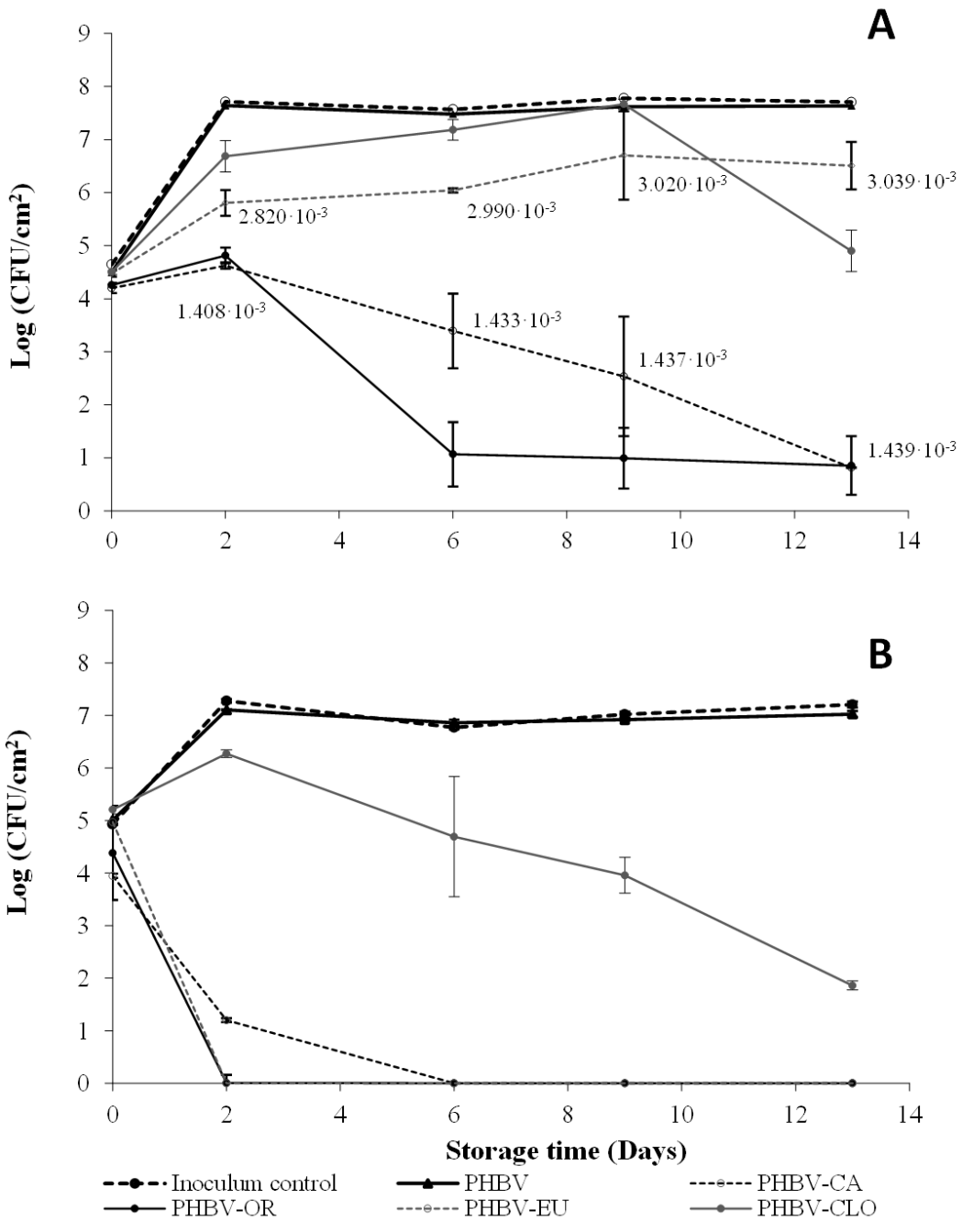


Figure 4. Effect of different PHBV bilayer films on the growth and survival of *L. innocua* (A) and *E. coli* (B) at 10 ºC. Mean values and standard deviation. The released amounts (g/mL of media, assuming the same release kinetics as in A simulant) of CA and EU at the different contact times were indicated.

Tables

Table 1. Internal transmittance (Ti) at 550 nm and colour parameters of PHBV bilayer films with different active compounds (CA: carvacrol; EU: eugenol; OR: oregano essential oil (OR); CLO (clove essential oil) and without them (PHBV). Mean values ± standard deviation.

Formulation	Ti (550 nm)	L*	C_{ab}^*	h_{ab}^*	ΔE
PHBV	$50.2 \pm 0{,}8^{a}$	75.7 ± 0.2^{a}	20.79 ± 0.16^{a}	80.32 ± 0.13^{a}	-
PHBV-CA	60 ± 2^{b}	76.1 ± 0.3^{a}	19.3 ± 0.4^{b}	81.8 ± 0.2^{b}	1.64
PHBV-EU	$58.8 \pm 0{,}4^{b}$	75.8 ± 0.3^{a}	20.2 ± 0.2^{c}	80.28 ± 0.10^{a}	0.56
PHBV-OR	58 ± 2^{b}	75.8 ± 0.6^{a}	20.0 ± 0.4^{ac}	81.2 ± 0.5^{b}	0.47
PHBV-CLO	58 ± 2^{b}	75.58 ± 0.12^{a}	20.3 ± 0.2^{ac}	80.73 ± 0.13^{a}	0.50

a-c: Different superscripts within the same column indicate significant differences among formulations ($p < 0.05$).

Table 2. Tensile properties (elastic modulus (EM), tensile strength (TS) and elongation at break (E)) and water vapour permeability (WVP) of the bilayer films with different active compounds and without them (PHBV). Mean values ± standard deviation.

Formulation	**EM (MPa)**	**TS (MPa)**	**E (%)**	**WVPx10^{12} (g/m·s·Pa)**	**Thickness (µm)**
PHBV	1141 ± 12^{a}	27.6 ± 0.6^{a}	5.3 ± 0.4^{a}	21 ± 4^{a}	188 ± 7^{a}
PHBV-CA	780 ± 85^{b}	17.6 ± 1.1^{b}	4.4 ± 0.6^{b}	9.2 ± 1.2^{b}	169 ± 15^{bc}
PHBV-EU	773 ± 118^{b}	17 ± 2^{b}	3.8 ± 0.4^{c}	15 ± 2^{c}	183 ± 13^{a}
PHBV-OR	768 ± 71^{b}	17.3 ± 0.6^{b}	3.8 ± 0.4^{c}	12 ± 2^{bd}	163 ± 6^{c}
PHBV-CLO	808 ± 91^{b}	18.2 ± 1.2^{b}	3.9 ± 0.4^{c}	21 ± 6^{a}	178 ± 11^{ab}

a-d: Different superscripts within the same column indicate significant differences among formulations ($p < 0.05$).

Table 3. Initial degradation temperature (T_{onset}) and maximum rate temperature (T_{peak}) for the main degradation step of PHBV monolayer films, containing or not (*) plasticizer, and PHBV bilayer films with active compounds (CA: carvacrol; OR: oregano essential oil; CLO: clove essential oil; EU: eugenol). Mean values ± standard deviation.

Formulation	**T_{onset} (ºC)**	**T_{peak} (ºC)**
PHBV monolayer*	261.42 ± 0.04^{a}	269.7 ± 0.2^{a}
PHBV monolayer	255 ± 2^{b}	265 ± 1^{b}
PHBV	227.9 ± 0.6^{c}	238.42 ± 1.06^{c}
PHBV-CA	222.38 ± 1.51^{d}	233.17 ± 1.18^{d}
PHBV-EU	223.3 ± 1.7^{d}	236 ± 2^{cd}
PHBV-OR	234.2 ± 0.8^{e}	244.0 ± 0.2^{e}
PHBV-CLO	238.4 ± 1.6^{f}	248.4 ± 0.6^{f}

a-f: Different superscripts within the same column indicate significant differences among formulations ($p < 0.05$).

Table 4. Thermal properties: melting temperature (T_m), crystallization temperature (T_c), glass transition temperature (T_g), melting enthalpy (ΔH_m), crystallization enthalpy (ΔH_c) and degree of crystallinity (X_c) for PHBV monolayer films, containing or not (*) plasticizer, and PHBV bilayer films with active compounds (CA: carvacrol; OR: oregano essential oil; CLO: clove essential oil; EU: eugenol). Mean values ± standard deviation.

Film	**1st heating scan**				**Cooling**		**2nd heating scan**	
	T_g (º C)	**T_m (ºC)**	**ΔH_m (J/g)**	**X_c (%)**	**T_c (ºC)**	**ΔH_c (J/g)**	**T_m (ºC)**	**ΔH_m (J/g)**
PHBV-monolayer*	7.0 ± 0.2[a]	170.6 ±0.2[a]	99.1 ±0.2[a]	99.06 ± 0.24[a]	121.2 ± 0.2[a]	91.6 ± 0.7[a]	168.7 ± 0.2[a]	106.4 ± 0.3[a]
PHBV-monolayer	6.98 ± 0.02[a]	167.20 ±0.06[b]	79.8 ±0.6[b]	60.5 ± 0.4[b]	118.8 ± 0.7[b]	70.5 ± 0.3[b]c	164.90 ± 0.07[b]	84.8 ± 0.6[b]
PHBV	7.030 ± 0.113[a]	169.0 ± 0.8[c]	88 ± 2[c]	66.5 ± 1.2[c]	117.9 ± 0.4[a]	71 ± 3[b]	164.8 ± 0.5[b]	83.907 ± 1.006[b]
PHBV-CA	6.9 ± 0.3[a]	166.97 ± 0.05[b]	74 ± 3[d]	56 ± 2[d]	113.7 ± 0.5[c]	65.0 ± 0.8[d]	161.3 ± 0.5[c]	74.5 ± 1.2[c]
PHBV-EU	6.91 ±0.07[a]	162.2 ± 0.3[d]	71.6 ± 0.9[d]	54.2 ± 0.7[d]	113.4 ± 0.6[c]	67.8 ± 0.8[cd]	161.0 ± 0.7[c]	76.93 ± 0.12[d]
PHBV-OR	7.02 ± 0.04[a]	158.2 ± 0.5[e]	66.9 ± 0.6[e]	50.7 ± 0.5[e]	110.4 ± 0.4[d]	66.48 ± 0.12[d]	158.5 ± 0.3[d]	76.21 ± 0.09[d]
PHBV-CLO	7.00 ± 0.02[a]	153.9 ± 0.2[f]	63.15 ± 0.12[f]	47.84 ± 0.09[f]	106.9 ± 0.5[e]	59.5 ± 0.7[e]	154.6 ± 0.7[e]	69.6 ± 0.5[e]

a-f: Different superscripts within the same column indicate significant differences among formulations ($p < 0.05$).