Food Chemistry 277 (2019) 38–45

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Eugenol and carvacrol migration from PHBV films and antibacterial action in different food matrices

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ARTICLE INFO

Keywords:
PHBV films
Essential oils
Carvacrol
Eugenol
Migration
Antibacterial activity
Food applications

ABSTRACT

The antibacterial effect of PHBV films with oregano or clove essential oil, or their main compounds, carvacrol (CA) and eugenol (EU), respectively, was analysed in food matrices (cheese, chicken breast and pumpkin and melon) and *in vitro* test for Escherichia coli and Listeria innocua. The migration of CA and EU in the different food matrices was determined to analyse the food matrix effect on the film's antimicrobial effectiveness. The antimicrobial activity in foods was less remarkable than in *in vitro* test. Despite the antilisterial effect in the *in vitro* test, this was not noticed in any food matrix. The most significant antibacterial effects against E. coli were observed in cheese and pumpkin, whereas the highest migration of both CA and UE took place in melon. This lack of correlation reflected that many compositional factors affect the active compound's availability to exert its antibacterial action in a specific food.

1. Introduction

Nowadays, the microbial contamination of perishable products is the main reason for food spoilage and foodborne diseases, since they are transported and stored until consumption, which requires an extended shelf-life (Pavlath & Orts, 2009). In this context, active packaging has appeared as a novel strategy for the control of microbial growth, thereby increasing food quality and safety of the packaged foodstuffs (Wen et al., 2016). In turn, consumer demand is moving towards more natural foods containing lower amounts of synthetic preservatives. In this sense, healthy compounds, such as essential oils (EOs) and their main compounds, which are considered as flavoring substances by the European Regulation 1999/217/CE and Generally Recognized As Safe (GRAS) substances by the Food and Drug Administration (FDA), have been widely studied as to their antimicrobial properties against spoilage and pathogenic microorganisms and constitute an effective alternative to synthetic preservatives (Jaiswal & Jaiswal, 2014).

EOs are complex mixtures of volatile compounds where two or three major components can constitute up to 85% of the oil, the phenolic compounds being mainly responsible for their antimicrobial properties. However, minor compounds are reported to have an important effect on the EO antimicrobial activity, possibly due to synergistic effects (Burt, 2004; Gutierrez, Barry-Ryan, & Bourke, 2008). Of the EOs, oregano (OR) and clove essential oil (CLO) are two of the most effective at inhibiting microbial growth of food-borne pathogens and spoilage microorganisms (Burt, 2004) and much research has focused on the development of antimicrobial films containing these actives. Whey protein films containing OR have inhibited the microbial growth of *Listeria innocua*, *Salmonella enteritidis* and *Staphylococcus aureus* (Royo, Fernández-Pan, & Maté, 2010), whereas starch-chitosan films with OR exhibited antimicrobial properties against *Bacillus cereus*, *Escherichia coli*, *S. enteritidis* and *S. aureus* (Pelissari, Grossmann, Yamashita, & Pineda, 2009). Likewise, Muppalla, Kanatt, Chawla, and Sharma (2014) have reported significant antimicrobial effects against *S. aureus* and *B. cereus* by incorporating CLO into carboxymethyl cellulose-polyvinyl alcohol films. Similar results were obtained with pectin films containing CLO for *S. aureus*, *E. coli* and *Listeria monocytogenes* (Nisar et al., 2018). The antimicrobial properties of both OR and CLO have mainly been attributed to their major compounds, carvacrol (CA) and eugenol (EU), respectively (Burt, 2004). Many *in vitro* studies with active biodegradable matrices containing CA (Requena, Jiménez, Vargas & Chiralt, 2016; Rojas-Graü et al., 2007) or EU (Narayanan and Ramana, 2013; Requena et al., 2016) demonstrated their effectiveness as antimicrobial agents against a broad spectrum of microorganisms. However, significantly higher EO amounts are required to achieve similar antimicrobial effects when applied to food matrices, probably due to the interactions between the active compounds and different food components, which could limit their effectiveness as antimicrobials (Burt, 2004; Gutierrez et al., 2008). In this sense, some authors reported that

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https://doi.org/10.1016/j.foodchem.2018.10.093
Received 22 June 2018; Received in revised form 10 September 2018; Accepted 19 October 2018
Available online 21 October 2018

high fat and protein contents in the food matrix can inhibit the antimicrobial activity of the EOs, which was attributed to the protective action of these food components for the bacteria (Canillac & Mourey, 2004; Gutierrez et al., 2008; Higueras, López-Carballo, Hernández-Muñoz, Catalá, & Gavara, 2014; Kim, Ruengwilysup, & Fung, 2004; Shelef, Jyothi, & Bulgarellii, 1984; Veldhuizen, Creutzberg, Burt, & Haagsman, 2007). Thus, no significant antimicrobial activity has been reported against several foodborne pathogens after applying chitosan films with CA on chicken samples. The scavenging effect of chicken protein on the CA gave rise to a very low available active concentration (Higueras et al., 2014). Likewise, EOs were less effective in full-fat products than in their corresponding low-fat alternative, such as was observed in cheese (Smith-Palmer, Stewart, & Fyfe, 2001) or hotdogs (Singh, Singh, Bhunia, & Singh, 2003). One of the main hypotheses for the higher microbial resistance to EOs in high protein and fat foods, compared to *in vitro* tests, is the greater nutrient availability in the food, which allows bacteria to repair their damage faster than in the culture medium (Gill, Delaquis, Russo, & Holley, 2002; Veldhuizen et al., 2007). Some authors also suggested that, due to their lipophilic nature, EOs generally dissolve in the fat-lipid phase of food, thus being less available to interact with the bacteria present in the aqueous phase (Mejlholm & Dalgaard, 2002; Veldhuizen et al., 2007). Moreover, phenolic compounds, the main antimicrobial agents in EOs can react with fatty free radicals, resulting from autoxidation in fatty products, thus obtaining reaction products less effective than the original phenolic compounds (Kim et al., 2004). On the contrary, high salt and water levels in the food are reported to increase the bacterial sensitivity to EOs (Shelef et al., 1984). As regards the carbohydrate content of foods, Gutierrez et al. (2008) reported that EO antimicrobial activity was reduced at high starch concentrations, in contrast to that observed by Shelef et al. (1984). Therefore, it is clear that the development of active films for food packaging applications require antimicrobial *in vivo* tests performed with the specific microorganisms inoculated into the food matrix where the films containing EOs should be applied, in order to assess whether food safety requirements are met.

As concerns film composition, the increasing environmental awareness advises the replacement of conventional plastic materials for more environmentally-friendly, biodegradable ones obtained from natural sources. In this context, biopolymers obtained from renewable resources through bacterial action, such as the polyhydroxyalkanoates (PHA), are a promising option for food packaging applications, since they can be produced by 300 species of Gram-positive and Gram-negative bacteria as well as a wide range of archaea and are completely biodegradable (Laycock, Halley, Pratt, Werker, & Lant, 2013). Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) is one of the most common PHA, since this biopolymer has physical properties comparable to some synthetic polymers, such as polypropylene and polyethylene, although PHBV leads to more brittle materials with lower elongation at break (Laycock et al., 2013). PHA films have been previously used as carriers of the different EO compounds to obtain biodegradable active materials, whose antimicrobial activity has been proved in *in vitro* tests (Narayanan and Ramana, 2013; Requena et al., 2016; Xavier, Babusha, George, & Ramana, 2015).

The aim of this study was to assess the antibacterial effect of PHBV films containing OR or CLO, as well as their main compounds, CA and EU respectively, in several food matrices of distinct composition, in order to determine the food matrix effect on the film antimicrobial properties.

2. Materials and methods

2.1. Materials and reagents

PHBV (8% of hydroxyvalerate) was provided in pellet form by NaturePlast (Caen, France). The polyethyleneglycol 950–1050 Da (PEG1000) used as plasticizer, as well as carvacrol (CA) and eugenol (EU), and UV-grade methanol were supplied by Sigma-Aldrich (Steinheim, Germany). Oregano (OR) and clove essential oils (CLO) were obtained from Herbes del Molí (Alicante, Spain). Gas chromatography standard 2-pentanol was purchased from Sigma-Aldrich Corp. (St. Louis, MO).

The different food submitted to the antimicrobial test (fresh cheese, chicken breast, pumpkin and melon) were purchased from a local market.

2.2. Film preparation

PHBV monolayer films were prepared by melt blending and compression-moulding as described by Requena et al. (2016). Briefly, PHBV in pellet form was blended with PEG1000 (10% w/w, in the film) in a two-roll mill (Model LRM-M-100, Labtech Engineering, Thailand) at 180 °C and thermo-compressed by using a hot plate hydraulic press (Model LP20, Labtech Engineering, Thailand) at 10 MPa and 180 °C for 4 min. Then, PHBV active films with different active compounds were obtained by spraying a constant amount (13% w/w, in the film) of each active compound (OR, CLO, CA or EU) as reported by Requena et al. (2016). To sum up, PHBV monolayers were sprayed with the corresponding active compound, covered with another PHBV sheet and compressed using the hydraulic press. Thus, five kinds of films were obtained: control films without active compounds (PHBV), and films with the corresponding active compounds (PHBV-OR, PHBV-CLO, PHBV-CA, PHBV-EU).

2.3. Physicochemical analysis of raw materials

Physicochemical properties of foods directly related with the microbial growth sensitivity, such as pH and water activity (a_w), were analysed in each of the food matrices by using a pH puncture electrode (Seven Easy™ pH, Mettler Toledo, Switzerland) and an a_w meter (Aqualab 4TE, METER FOOD, USA), respectively. The °Brix were also determined in fruit samples (pumpkin and melon), as ripeness indicator, by using a refractometer (ATAGO™ NAR-3T Abbe, Japan). For a_w measurements, all samples were peeled and cut into small pieces, whereas for the determination of the total soluble solids in the fruit, the sample juice was obtained by liquefying. All measurements were taken in triplicate.

2.4. Antibacterial effectiveness of active films: in vitro and in vivo tests

2.4.1. Processing of food matrices

The antibacterial activity of the different active films was tested in food matrices with high protein or fat content (fresh cheese and chicken breast) and high carbohydrate content (fresh-cut pumpkin and melon), in order to study the food composition effect on the film antimicrobial activity. All the food was purchased from a local market, transported to the laboratory immediately and handled in a laminar flow cabinet in sterile conditions. The chicken breasts were cut into thin fillets and the fresh cheese was prepared in slices. Both vegetable matrices were superficially disinfected by dipping them in sodium hypochlorite 1% (v/v) solution for 1 min, peeled and also cut into thin slices. 10 g samples of 55 mm in diameter were obtained from each food matrix and placed in petri dishes.

2.4.2. Antibacterial activity assessment

Listeria innocua (CECT 910) and *Escherichia coli* (CETC 101) lyophilized strains were supplied by the Spanish Type Culture Collection (CECT, Universitat de València, Spain), and stored at −25 °C with 30% glycerol. Bacterial cultures on exponential growth phase were prepared by inoculating the microbial stock suspensions into TSB, followed by their incubation at 37 °C for 24 h. The inoculums were properly diluted to obtain bacterial suspensions of 10^6 CFU/mL.

Tryptic Soy Agar (TSA, Scharlab, Barcelona, Spain) culture medium,

used for *in vitro* tests, and the different food samples placed in petri dishes (55 mm in diameter) were inoculated with 100 µl of *L. innocua* or *E. coli* suspension and covered with the different active film samples of the same diameter. Both samples covered with active-free PHBV films and those non-covered were also tested as film control and inoculum control, respectively. Petri dishes were closed with their lids, sealed with Parafilm™ and incubated for 6 days. Incubation was carried out at 10 °C in all cases. TSA culture media and fresh cheese samples were also previously tested at 4 °C. Microbial counts in each sample were performed in duplicate after inoculation and after 6 incubation days. To this end, each sample was homogenized in buffer peptone water (Scharlab, Barcelona, Spain) for 3 min, by using a Stomacher Lab-blender (Masticator, IUL Instruments, Barcelona, Spain) and properly diluted. The plate counts of *L. innocua* were performed on Palcam agar base (Scharlab, Barcelona, Spain) containing Palcam selective supplement for *Listeria* (Scharlab, Barcelona, Spain), after incubation at 37 °C for 48 h, whereas the plate counts of *E. coli* were performed on Fecal Coliforms agar (Scharlab, Barcelona, Spain) dyed with 1% Rosolic acid solution (Scharlab, Barcelona, Spain) after pre-incubation at 37 °C for 2 h and transferred to 44 °C for 22 h. Counts were expressed as log CFU/g food matrix or TSA.

2.5. CA and EU migration to the food matrices

2.5.1. Quantification in the films by methanol extraction

The active amount that migrated from the films to the different matrices during incubation time was estimated indirectly by determining the active compound remaining in the films after the contact time, through methanol extraction and subsequent spectrophotometric quantification. Thus, after 6 days at 10 °C and prior to the extraction, PHBV-CA and PHBV-EU films were detached from the different matrices and kept in phosphorus pentoxide ($a_w = 0$) for 24 h to remove adsorbed water. Then, methanol extraction of the active compounds was carried out using 10 mg film in 1 mL solvent in contact for 72 h. The resulting extracts were filtered and properly diluted to obtain absorbance values between 0.2 and 0.8. In this way, CA and EU amounts in the extracts were quantified through absorbance measurements at 275 and 282 nm, respectively, by using an UV–visible spectrophotometer (Evolution 201, Thermo Scientific). PHBV bilayer films without active compounds in contact with samples for 6 days and submitted to the same extraction procedure were used to obtain the background solutions. All the determinations were run in triplicate. CA and EU standard calibration curves were previously obtained to convert absorbance values to active content. The active amount that migrated from the active films to the different matrices was estimated by subtracting the active mass remaining in the films after the incubation from the initial active amount in the films, which was assessed using the same extraction procedure.

2.5.2. Analyses in the food matrices by gas chromatography–mass spectrometry (GC–MS)

The active compounds that migrated from the active films to the different food matrices were extracted by purge and trap thermal desorption (Perdones, Escriche, Chiralt, & Vargas, 2016). 100 µl of the internal standard 2-pentanol (10 mg/l) and 10 g of food purée, properly diluted with water, were placed into a purging flask and kept in a water bath under the extraction conditions shown in Table 1, previously optimized for each food matrix. Throughout the extraction time, purified nitrogen (100 mL/min) flowed through the glass frit at the bottom of the flask. Thus, volatile compounds were dragged by the nitrogen stream, which passed through the sample and were adsorbed in a 100 mg porous polymer (Tenax® TA, 20–35 mesh) packed into a glass tube placed at the end of the system.

The adsorbed volatile extract was thermally desorbed by a direct thermal desorber (TurboMatrix TD, Perkin-Elmer TM, CT-USA). Desorption was performed under a 10 mL/min helium flow at 220 °C for 10 min, and the volatiles were cryofocused in a cold trap at 30 °C. After 1 min, the cold trap was heated up to 250 °C (at a rate of 99 °C/min) and volatiles were directly transferred onto the head of the capillary column. The GC–MS analysis was performed using a Finnigan TRACE™ MS ThermoQuest, Austin, USA). Volatile compounds were separated using a DB-WAX capillary column (1.0 µm × 0.32 mm × 60 m, SGE, Australia). Helium was used as carrier gas at a constant flow rate of 1 mL/min. The oven was kept at an initial temperature of 40 °C for 2 min. Then, the temperature was increased to 190 °C at a rate of 4 °C/min, maintained for 5 min and finally increased to 230 °C at 10 °C/min. The MS interface and source temperatures were 250 and 200 °C, respectively. Electron impact mass spectra were recorded in impact ionisation mode at 70 eV and with a mass range of m/z 33–433. At least five extracts were obtained for each food sample. The identification of CA and EU was performed by comparing their mass spectra with spectral data from the National Institute of Standards and Technology 2002 library as well as the published retention indices. The CA and EU quantification was carried out after calibration following the standard addition method, in order to avoid the food matrix composition effect. 10 g of food purée, homogenised with 100 µl of internal standard 2-pentanol (10 mL/L) and 5 different concentrations (0–5 mg/g) of CA or EU were tested in quadruplicate, following the procedure already described.

Table 1
Optimum extraction conditions established for each food matrix for the purge and trap method.

Matrix	Sample:Water	Time (min)	Temperature (°C)
Cheese	1:1	25	70
Chicken	1:1	15	40
Pumpkin	1:2	25	50
Melon	1:0	25	50

2.6. Statistical analyses

Experimental data were analysed by analysis of variance (ANOVA) using Fisher's Least Significant Difference (LSD) test at 95% confidence level. To this end, Statgraphics Centurion XVIs for Windows 5.1 (Manugistics Corp., Rockville, MD, USA) was used.

3. Results

3.1. Characterization of raw materials

Moisture content is not directly related with the microbial spoilage of foods, since foodstuffs with the same moisture content can deteriorate to a different extent, whereas the water activity (a_w), or free water available for the microbial growth, has been widely used as an indicator of microbial spoilage sensitivity. All foodstuffs are suitable substrates for bacterial development in terms of a_w, since all matrices show a_w values (Table 2) above the required threshold for bacterial growth (0.9) (Beuchat, 1981). The pH can also be used as a predictor of the bacterial growth, since it generally occurs optimally at pH values in the range 6–7 and falls as the pH moves away from this region (Adams &

Table 2
Water activity (a_w) and pH values, and Brix level of the different food matrices. Mean value ± standard deviation.

Matrix	pH	a_w	Brix level (°)
Cheese	6.61 ± 0.02	0.985 ± 0.002	–
Chicken	5.98 ± 0.02	0.991 ± 0.001	–
Pumpkin	5.79 ± 0.02	0.991 ± 0.001	11.7 ± 0.2
Melon	6.17 ± 0.04	0.988 ± 0.001	12.7 ± 0.2

Table 3
Microbial counts of *Listeria innocua* and *Escherichia coli* on inoculated TSA agar and fresh cheese (log CFU/g sample) after contact with films containing carvacrol (PHBV-CA), eugenol (PHBV-EU), oregano essential oil (PHBV-OR), clove essential oil (PHBV-CLO) and without active compounds (PHBV), for 6 days at 4 °C. Inoculated, non-covered samples were also considered before (C_0) and after the incubation time (C_6).

Matrix	C_0	C_6	PHBV	PHBV-CA	PHBV-EU	PHBV-OR	PHBV-CLO
Listeria innocua							
Agar	3.2 ± 0.1^{b}	3.4 ± 0.1^{a}	3.3 ± 0.1^{ab}	2.8 ± 0.1^{c}	3.5 ± 0.3^{a}	2.6 ± 0.2^{c}	3.5 ± 0.1^{a}
Cheese	3.6 ± 0.1^{d}	5.7 ± 0.2^{a}	5.3 ± 0.2^{c}	5.4 ± 0.4^{bc}	5.4 ± 0.1^{abc}	5.6 ± 0.1^{abc}	5.6 ± 0.2^{ab}
Escherichia coli							
Agar	3.6 ± 0.1^{a}	2.8 ± 0.2^{c}	2.8 ± 0.2^{c}	nd	nd	Nd	3.0 ± 0.2^{b}
Cheese	4.7 ± 0.1^{a}	4.9 ± 0.5^{a}	4.1 ± 0.2^{b}	3.8 ± 0.7^{b}	4.6 ± 0.1^{a}	4.7 ± 0.1^{a}	4.6 ± 0.1^{a}

a-d: Different letters in the same line show significant differences between film formulations ($p < 0.05$). nd: non-detected microbial growth.

Nicolaides, 1997). Similarly to that reported for a_w values, all the food products showed pH values in the optimal range for bacterial growth (Table 2). Therefore, the physicochemical properties of the foodstuffs did not hinder the bacterial growth in any case. As regards the soluble solid content of the plant foodstuffs, the melon samples showed higher values, which could mean more nutritious media for the bacterial development.

3.2. Antibacterial effect of active films at 4 °C.

Microbial counts of *L. innocua* and *E. coli* in TSA culture media performed at 4 °C are shown in Table 3. At this temperature, the lack of bacterial growth in the culture medium after incubation was remarkable for both bacteria, especially for *E. coli* where a decrease in the initial counts was observed. Likewise, no significant differences in the microbial counts of either bacteria were observed after 6 days of incubation for any sample covered with PHBV films without actives, in comparison with the non-covered samples (C_6). Therefore, the antibacterial activity of the films with active compounds was attributed to the incorporation of active compounds. Both PHBV-CA and PHBV-OR films exerted a significant bactericidal effect against *L. innocua*, as reported by Requena et al. (2016) in TSB liquid media. However, no antilisterial activity of the PHBV-EU or PHBV-CLO films was observed, which agrees with the higher minimum inhibitory concentration (MIC) of EU for *L. innocua* (1.05 mg/mL) compared to the corresponding MIC value of CA (0.75 mg/mL) (Requena, Vargas, & Chiralt, 2018). However, the total inhibition of *E. coli* was observed with all the active films, excluding PHBV-CLO, despite the higher EU MIC value against *E. coli* (1.35 mg/mL) (Requena, Vargas, & Chiralt, 2018) compared to the corresponding value against *L. innocua*. This could be attributed to the different combined effect of the low temperature (4 °C) and actives on both bacteria: bacteriostatic for *Listeria* and bactericidal for *E. coli*. Although EU is the main compound in CLO, the EU content in the PHBV-CLO films could be below its MIC against *E. coli* at 4 °C (Burt, 2004).

In contrast to the results of the *in vitro* test, significant microbial growth of *L. innocua* was observed in fresh cheese samples after 6 days of incubation at 4 °C (Table 3). This may be due to the composition of the dairy product, which makes it very sensitive to the growth of *Listeria* (Gutierrez et al., 2008). In contrast with the *Listeria*'s capacity to grow at temperatures as low as 4 °C (Al-Nabulsi et al., 2015), the mesophilic status of *E. coli* did not allow its growth (Francis & O'Beirne, 2001). PHBV films containing active compounds did not show any remarkable effects on the growth of *L. innocua* and *E. coli* inoculated into fresh cheese samples, unlike the effects observed in *in vitro* tests; this is likely due to the combined bacteriostatic or bactericidal effect of the low temperature, which could mask the antibacterial action of the active PHBV films. Thus, additional tests were conducted at 10 °C in order better to reflect the role of the active compounds.

3.3. Antibacterial effect of active films at 10 °C

Due to the potential combined antimicrobial effect of the low temperature, the antimicrobial tests were carried out at 10 °C, within the limits of cold preservation. The *in vivo* test was performed to obtain the microbial counts of *L. innocua* and *E. coli* in two kinds of high-protein foods (fresh cheese and chicken breast) and in two kinds of vegetable matrices (fresh-cut pumpkin and melon), previously inoculated and stored at 10 °C for 6 days. No bacteriostatic effects were observed for *L. innocua* and *E. coli* at this storage temperature, whose population increased more than 4 log after 6 days in the TSA culture medium (Fig. 1). The microbial growth of both bacteria was quite similar, with small differences depending on the foodstuff that are associated with the different food composition.

In line with the *in vitro* results at 4 °C, PHBV films with CA or OR significantly reduced the microbial counts of *L. innocua* and *E. coli*, thus showing an antibacterial effect against both bacteria, although those counts were always higher than the initial inoculum (grey dotted lines). Both bacteria were more affected by PHBV films containing CA than by those containing OR, as reported by Rojas-Grau et al., 2007 in *in vitro* studies for alginate-apple puree films and *E. coli*, since the OR antimicrobial activity has been mainly attributed to CA, which only represents 46% of this EO (Perdones, Tur, Chiralt, & Vargas, 2016). In contrast with that obtained in the *in vitro* test at 4 °C, PHBV-EU and PHBV-CLO films significantly reduced both bacterial growths, with no significant differences between formulations. Similar reductions were reported at 10 °C for *L. monocytogenes* and *E. coli* by Alboofetileh, Rezaei, Hosseini, and Abdollahi (2014) with alginate films containing CLO.

3.3.1. Antibacterial effect of active films in high-protein food

The microbial counts of *L. innocua* and *E. coli* obtained in fresh cheese and chicken breast samples coated with the different films are shown in Fig. 1. It is remarkable that no significant growth reduction of *L. innocua* was observed in fresh cheese samples with any film formulation, in contrast with that obtained in the *in vitro* test (Fig. 1). This lack of antilisterial activity could be due to the protective effect of the fats and proteins present in the cheese exert on bacteria, which inhibited any potential antimicrobial effect of the EO compounds, as reported by several authors (Canillac & Mourey, 2004; Gutierrez et al., 2008; Higueras et al., 2014; Kim et al., 2004; Shelef et al., 1984; Veldhuizen et al., 2007). Smith-Palmer et al. (2001) and Singh et al. (2003) reported significantly greater antilisterial effect of the EO on low-fat cheese and hotdogs than in the corresponding full-fat products. However, all the active film formulations exhibited significant antimicrobial activity against *E. coli*, in line with the greater sensitivity of *E. coli* to these active compounds (Raybaudi-Massilia, Mosqueda-Melgar, & Martin-Belloso, 2006; Requena et al., 2018; Teixeira et al., 2013). However, despite the higher sensitivity of *E. coli* to CA compared to the other studied actives (Burt, 2004; Pei, Zhou, Ji, & Xu, 2009), the PHBV films containing CA gave rise to the lowest microbial reduction (1.5 log), followed by PHBV-OR (2.5 log). As reported by several authors, the whole EOs are often more effective than their pure main compounds; this is because there are some other minor compounds that could be critical in the antimicrobial activity (Gill et al., 2002; Mourey

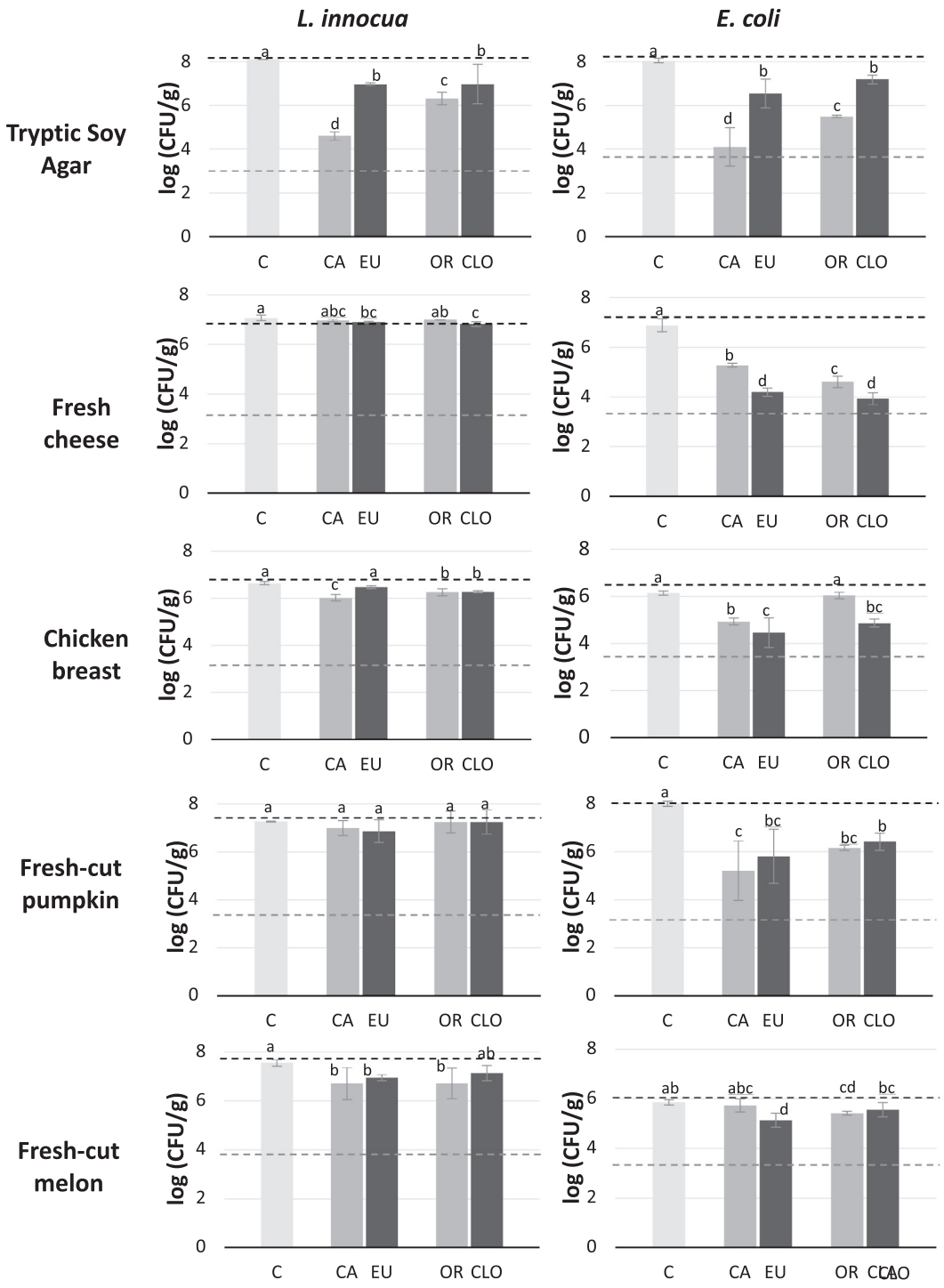

Fig. 1. Microbial counts for *L. innocua* and *E. coli*, obtained after 6 days of incubation at 10 °C in TSA culture media (*in vitro* test) and different food matrices covered with PHBV films containing carvacrol (CA), eugenol (EU), oregano (OR) or clove essential oil (CLO) and without actives (C). Dotted lines show the microbial count of the inoculum before (grey) and after 6 days of incubation (black). Different letters above the error bars of the histogram show homogeneous sample groups ($p < 0.05$).

& Canillac, 2002), which, to a different extent, can also be affected by the presence of the cheese components. Notwithstanding the lower antimicrobial effect of EU against *E. coli* when compared to CA and OR (Burt, 2004; Pei et al., 2009), PHBV films containing EU or CLO inhibited the microbial growth by 3 log, with no significant differences between either. The different behaviour observed for active films in cheese with respect to that exhibited in the *in vitro* test suggests that the antimicrobial activity of OR and CA could be inhibited by the interaction with the fats or proteins present in the cheese, whereas EU and CLO could be more available in the fatty-protein matrix to act against bacteria. Therefore, as reported by Glass and Johnson (2004), higher amounts of antimicrobials are often required when they are applied to real systems, since some compounds present in the foodstuffs can interfere with both the microorganism's viability and the potential antimicrobial activity of the active compounds.

Significant differences were observed in the *L. innocua* counts for

chicken breast samples incubated with and without active PHBV films, although without any remarkable reductions in practical terms (> 1 log). Nevertheless, the PHBV-EU films resulted in significant growth inhibitions of *E. coli* (2 log) in chicken samples. Likewise, PHBV films containing CLO or CA also led to significant bacterial growth reductions, but to a lesser extent, whereas no antimicrobial activity was observed by applying PHBV-OR films. In the same way, Shekarforoush, Nazer, Firouzi, and Rostami (2007) reported a significant antimicrobial effect of OR against *E. coli* in *in vitro* studies, but no effect was observed when this EO was applied to roast chicken. Differences between the *in vitro* and *in vivo* tests in chicken samples could again be explained by the scavenging effect of the protein matrix on the active, probably due to their high chemical compatibility (Higueras et al., 2014).

3.3.2. Antibacterial effect of active films in plant food

Antimicrobial studies were also performed on two matrices with high carbohydrate content and low fat and protein content in order to compare the antimicrobial activity of the active films depending on the food matrix composition. Fig. 1 shows the microbial counts of *L. innocua* and *E. coli* in inoculated fresh-cut pumpkin and melon, incubated for 6 days at 10 °C, in contact with the different films. The initial population of *L. innocua* increased 4 log in both products, whereas the microbial growth of *E. coli* was food matrix-dependent; counts increased 5 log in pumpkin samples and less than 3 log in melon samples, highlighting the importance of the food matrix in the bacterial growth.

As regards the antimicrobial activity of the active films, no film formulation led to important growth inhibitions (> 1 log) of *L. innocua* in either of the two vegetable matrices. Similarly, no antilisterial activity was observed in fresh broccoli packaged in plastic bags containing an active trilayer film based on a mixture of organic acids, extract of rosmarinic acid and Italian or Asian EO (Takala et al., 2013). However, all the active films had a significant antimicrobial effect against *E. coli* in fresh-cut pumpkin samples, but not in fresh-cut melon samples, in all likelihood because of the active interaction with some of the melon components.

The growth inhibition effects of the studied active films on the different food matrices, in terms of the reduction in log CFU, with respect to the corresponding inoculum control, are shown in Table 4. In *in vitro* studies with TSA culture medium, a significant growth inhibition of both bacteria was observed (between 1 and 4 log depending on the film formulation), whereas in *in vivo* tests, the antimicrobial effects were less remarkable in every case with the exception of the antimicrobial effect against *E. coli* of PHBV-EU and PHBV-CLO films, which were more effective in cheese, chicken meat and pumpkin samples. The greater antimicrobial effect of these actives in the food matrices suggests that the presence of some matrix compounds strengthen the mechanism of antibacterial action. The most significant antimicrobial effects against *E. coli* were obtained in fresh cheese and fresh-cut pumpkin. PHBV-EU and PHBV-CLO films were more effective in cheese, while PHBV-CA and PHBV-OR films exerted a greater effect in pumpkin samples. Despite the antilisterial effects observed in the *in vitro* tests, particularly in the case of CA, these were not noticed in any of the food matrices studied (< 1log). The presence of nutrients that have a protective, nutritious effect on bacteria, as well as the interactions of the actives with the food compounds, significantly decreased their potential antilisterial effect. In this sense, noteworthy results were obtained for the plant foods, where, despite the low content of fat and protein in the matrix, a marked reduction in the antilisterial activity was observed for all the films. In this sense, Gutierrez et al. (2008) also reported that starch concentrations of 5% and 10% had a negative effect on the OR efficacy against *L. monocytogenes*. In general, the active compound was more effective against *E. coli* than *L. innocua* when applied to the food matrices, as well as in the culture medium, which may also be attributed to the higher MIC values of the active compounds for *Listeria*, which, in turn, will be affected by the substrate of growth (Pei et al., 2009; Raybaudi-Massilia et al., 2006; Requena et al., 2018; Teixeira et al., 2013). The lack of any significant antibacterial effect of active films on melon is remarkable, where very low values of growth inhibition were observed for both *E. coli* and *L. innocua*. Likewise, no inhibition of the *L. innocua* growth was observed in cheese samples.

3.4. Quantification of CA and EU migration to the food matrices

The total migration of actives from the films to the food systems was analysed to better understand the differences in their antibacterial effect. This was carried out for CA and EU in the case of PHBV-CA and PHBV-EU films. In this sense, the remaining CA and EU content in the active films was analysed after 6 days in contact with each matrix, under the same conditions given in the antimicrobial assays, as well as the active content present in the respective food matrix, as described in Section 2.6. Table 5 shows the contents of CA and EU in the film after 6 contact days, and the estimated amount delivered (% respect to the initial content in the film: 107 ± 7 mg CA/g film or 61 ± 6 mg EU/g film) to the food system, as well as the content determined in the food matrix, using both the data obtained from the remaining content in the films (1 subscript) and that quantified in the food matrix (2 subscript). Although the amount of actives released into the food matrices by both methods did not coincide completely, the tendencies observed were quite coherent; this is except for the case of EU in chicken meat samples, where no significant release was detected through the analyses of the remaining content in the films. Nevertheless, a direct analysis in the food matrix would offer more reliable values since film manipulation throughout the extraction process could imply uncontrolled losses of the actives.

Melon was the food system in which the highest migration of both CA and UE occurred, whereas the pumpkin samples and the culture medium exhibited the lowest content of actives. Likewise, except for the pumpkin samples, the percentages of released CA and EU led to active contents in the food matrices higher than the MIC (CA: 0.75 mg/mL for *L. innocua* and 0.70 mg/mL for *E. coli*; EU: 1.05 mg/mL for *L. innocua*

Table 4
Count reduction of *Listeria innocua* and *Escherichia coli* with films containing carvacrol (PHBV-CA), eugenol (PHBV-EU), oregano essential oil (PHBV-OR) and clove essential oil (PHBV-CLO), as compared to the inoculum, (Log CFU difference) in the culture medium and in the different food matrices after 6 days of incubation at 10 °C.

Matrix	PHBV-CA		PHBV-EU		PHBV-OR		PHBV-CLO	
	L. innocua	*E. coli*	*L. innocua*	*E. coli*	*L. innocua*	*E. coli*	*L. innocua*	*E. coli*
Agar	3.6 ± 0.2^{b1}	4.2 ± 0.9^{d1}	1.2 ± 0.1^{b1}	1.7 ± 0.7^{ab1}	1.9 ± 0.3^{c1}	2.7 ± 0.1^{c2}	1.2 ± 0.9^{b1}	1.1 ± 0.2^{b1}
Cheese	nd	2.1 ± 0.1^{bc}	nd	3.2 ± 0.2^{c}	nd	2.8 ± 0.2^{c}	Nd	3.5 ± 0.3^{d}
Chicken	0.8 ± 0.2^{a1}	1.7 ± 0.2^{b2}	0.4 ± 0.1^{a1}	2.2 ± 0.6^{b2}	0.6 ± 0.2^{ab1}	0.6 ± 0.1^{a1}	0.6 ± 0.1^{ab1}	1.7 ± 0.2^{c2}
Pumpkin	0.5 ± 0.3^{a1}	2.8 ± 1.2^{c2}	0.6 ± 0.5^{a1}	2.3 ± 1.1^{bc2}	0.2 ± 0.5^{a1}	1.9 ± 0.1^{b2}	0.2 ± 0.5^{a1}	1.7 ± 0.4^{c2}
Melon	0.9 ± 0.7^{a1}	0.4 ± 0.2^{a1}	0.6 ± 0.1^{a1}	1.0 ± 0.3^{a1}	0.9 ± 0.6^{b1}	0.7 ± 0.1^{a1}	0.5 ± 0.3^{ab1}	0.6 ± 0.3^{a1}

a-c: Different letters in the same column show significant differences between samples ($p < 0.05$), whereas different numbers for the same matrix-film formulation show significant differences between bacteria. nd: non-detected inhibition.

Table 5
Carvacrol (CA) and eugenol (EU) contents remaining in the films after the food contact (mg active/g film), active release percentages into the food matrix respect to the initial content in the films and active amounts migrated to the food matrices (mg active/g food matrix) Subscript 1 corresponds to the values obtained from the film analyses. Subscript 2 corresponds to the values obtained from the food matrix analyses.

Matrix	Active remaining in the film		Active released from film (% with respect to initial content in film)				Active migrated to the matrix			
	CA	EU	CA_1	CA_2	EU_1	EU_2	CA_1	CA_2	EU_1	EU_2
Agar	93 ± 5^{b}	75 ± 20^{a}	13 ± 5^{c}	14 ± 5^{c}	ND	11 ± 3^{d}	0.8 ± 0.5^{d}	0.9 ± 0.3^{b}	ND	0.4 ± 0.1^{c}
Cheese	61 ± 5^{c}	40 ± 12^{b}	43 ± 5^{b}	17 ± 8^{c}	35 ± 11^{a}	29 ± 18^{bc}	2.8 ± 0.5^{b}	1.1 ± 0.6^{b}	1.3 ± 0.6^{a}	1.1 ± 0.5^{b}
Chicken	87 ± 8^{b}	70 ± 12^{a}	19 ± 7^{c}	33 ± 8^{b}	ND	55 ± 10^{ab}	1.3 ± 0.5^{c}	2.2 ± 0.6^{a}	ND	2.1 ± 0.4^{a}
Pumpkin	104 ± 5^{a}	54 ± 11^{b}	3 ± 5^{d}	ND	11 ± 2^{b}	16 ± 11^{cd}	0.2 ± 0.3^{d}	ND	0.4 ± 0.2^{b}	0.6 ± 0.4^{bc}
Melon	0^{d}	30 ± 20^{b}	100^{a}	48 ± 10^{a}	50 ± 34^{a}	74 ± 31^{a}	6.6^{a}	3.2 ± 0.6^{a}	1.9 ± 1.3^{a}	2.8 ± 1.1^{a}

Different letters in the same column show significant differences between samples ($p < 0.05$). ND: non-detected active compound.

and 1.35 mg/mL for *E. coli*; Requena et al., 2018). However, no remarkable antimicrobial effects were observed on the melon samples coated with PHBV-CA or PHBV-EU films, which could be attributed to a scavenging effect of the melon components on the actives or to their fast diffusion into the internal tissue, provoking a dilution effect on the sample surface where the bacteria grow. In contrast, despite the scarce CA and EU migration into the pumpkin samples, it was sufficient to reduce the microbial growth of *E. coli* by 2.8 or 2.3 log, respectively. Likewise, very low CA or EU concentrations were estimated in the agar medium, when compared to food matrices, while the most significant antibacterial effect was obtained in this culture medium. This suggests that both CA and EU were more concentrated at the sample surface, with minor internal diffusion, which allowed for a more effective antibacterial action. On the other hand, although no significant differences between the migration of CA and EU into the chicken or cheese samples were observed, the migration values were higher in the chicken breast. However, a more marked growth inhibition of *E. coli* was observed in the cheese samples for both active compounds.

The lack of coherence between the active migration from the films into the food matrices and the antibacterial action observed in the different cases make a specific antimicrobial analysis necessary for each food to prove the effectiveness of a determined antimicrobial material. Many factors affect the availability of the potentially active compounds to exert their action. The interactions of actives with the food components, which can provoke a scavenging effect of the compound (Higueras et al., 2014), their specific diffusion into the internal part of the food matrix and the subsequent dilution effects on the contaminated surface or the reinforced vitality of bacteria induced by food components, compromise the antimicrobial effectiveness of the potentially active compounds included in a determined packaging material. On the other hand, a study of the release kinetics of the active compounds in food simulants did not give the same values of compound migration than those obtained in real foods or culture medium. Previous studies (Requena, Vargas, & Chiralt, 2017) with the same films containing CA or EU in food simulants A (10% ethanol aqueous solution) and D1 (50% ethanol aqueous solution), which could emulate less fatty foods (chicken breast, melon and pumpkin samples or agar culture medium) and more fatty systems (cheese), respectively, would permit the estimation of the migration values of these components in the less and more fatty systems. The estimated values were 1.8 and 7.7 mg CA/g matrix and 3.8 and 7.4 mg EU/g matrix, respectively, for less and more fatty systems. These values differ noticeably from those obtained in the real foods, where specific components play an important role in both the active compound migration and availability and the bacterial vitality. Then, studies into the real foods are required to ensure that the potential antibacterial material can exert adequate protection against bacterial proliferation.

4. Conclusion

PHBV films with active essential oil compounds were highly effective against *L. innocua* and *E. coli* in *in vitro* tests, but they were much less effective in the real foods tested, with the exception of the effect against *E. coli* in cheese samples coated with PHBV-EU or PHBV-CLO films. No antilisterial effect was observed in any food matrices. The most significant antimicrobial effects against *E. coli* were observed in fresh cheese, for PHBV-EU and PHBV-CLO films, and fresh-cut pumpkin, for PHBV-CA and PHBV-OR films. In general, although the percentages of CA and EU migration led to active contents in the food matrices that were higher than their MICs, they were not always effective. The highest migration of both CA and UE took place in melon, whereas the lowest migration was quantified in pumpkin samples and in the culture medium. In contrast, no significant antimicrobial activity of the films was observed in melon, while they were very effective against *E. coli* in the culture medium and the pumpkin samples. The lack of correlation between the amount of active that migrated to the food and the antibacterial effect observed in the different matrices reflected the fact that many compositional factors affect the active compound's availability to exert its antibacterial action on a specific food composition, which, in turn, has a different nutritious/protective effect on the bacteria. Therefore, antimicrobial analyses are required that are specific to the food in question to ensure the effectiveness of a particular antimicrobial packaging material.

Acknowledgements

The authors thank the Ministerio de Economía y Competitividad (Spain) for the financial support provided through Project AGL2016-76699-R. Author Raquel Requena thanks the Ministry of Education, Culture and Sport (Spain) for the FPU (FPU13/03444) Grant.

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