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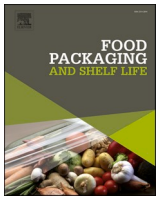

Tailored antimicrobial PHBV-based packaging for extended shelf life of processed cheese

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ABSTRACT

A coupled experimental and modelling approach was used to develop an adapted PHBV active film with Lauroyl Arginate Ethyl (LAE®) as an antimicrobial compound incorporated in the bulk or as a coating to prolong the shelf life of cheese. The minimum inhibitory concentration (MIC) of LAE® was evaluated in the range of 25–100 ppm against spoilage and pathogenic microorganisms, such as *Micrococcus luteus*, *Penicillium roqueforti*, *Salmonella enteritidis,* and *Pseudomonas putida*. Film production induced a loss of 50 % of LAE® when introduced into the bulk, whereas only 4 % was lost in the case of incorporation by coating. When in contact with food simulant D1 (50 % ethanol), a release of 43 % and 34 % of LAE® was achieved in 10 days at 20 °C for LAE® incorporated as a coating and in the bulk, respectively, while a lower release level of 34 % and 14 % respectively was observed in food simulant C (20 % ethanol), confirming the interest to use active coating for fatty foods. The developed packaging was well adapted to reach the MIC while remaining below the admissible daily intake. The results also showed that the PHBV film activated with LAE® incorporated as a coating was as effective as modified atmosphere packaging (MAP) to preserve cheese against microorganism growth.

1. Introduction

The main function of conventional packaging is to protect food from external agents and consequently slow down the deterioration of food during storage (Coffigniez et al., 2021). To reinforce food protection, active packaging could be developed based on the integration of active molecules in the packaging material, such as antioxidants or antimicrobial compounds, thus increasing food shelf life and avoiding food loss and waste (Rzayeva et al., 2023). During the last two decades, a huge quantity of antimicrobial packaging has been developed, using various active molecules for their capacity to inhibit microorganism growth. For example, the efficacy of organic acids (benzoic acid, propionic acid etc.), enzymes (lysozyme), essential oils (carvacrol, thymol etc.), metals (silver) and many other molecules (nisin, pediocin, lauroyl ethyl arginate, etc.) was already been proven (Appendini & Hotchkiss, 2002; Malhotra et al., 2015). In this context, Lauroyl Ethyl Arginate (LAE), synthesized through esterification of L-arginine with ethanol and lauric acid, is a molecule with a high potential owing to its broad-spectrum antimicrobial activity (Becerril et al., 2013). Indeed, the Minimum Inhibitory Concentration (MIC) reported in the literature ranges between 12 and 100 mg/kg or between 4 and 100 µg/mL against mold, yeast, Gram-negative and Gram-positive bacteria (Nerin et al., 2016; Becerril et al., 2013; Ma et al., 2023). These antimicrobial properties are due to the cationic surfactant action on the outer or cell membrane of bacteria (Luchansky et al., 2005). This leads to an alteration of membrane permeability and, consequently, to a modification of intracellular potassium and proton balance, inducing a reduction in cell growth or even cell death (Rodriguez et al., 2004; Pattanayaiying et al., 2014). Therefore, LAE seems to be a good candidate to protect food, as cheese from microorganisms and mold growth occurring during storage (Ricciardi et al., 2015; Laslo & György, 2018; Casalta et al., 2009; Ledenbach and Marshall, 2009).

Generally, the active molecules in active packaging systems are either contained in sachets or pads, or directly integrated into the packaging material, for instance, in the bulk of the polymer, coated on its surface, or immobilized through covalent links (Appendini & Hotchkiss, 2002; Suppakul et al., 2003; Rzayeva et al., 2023). The choice of one of these incorporation strategies is based on to the characteristics of the antimicrobial molecules, and more specifically, their volatility, thermal resistance, and interaction with the polymer matrix (Han, 2005; Nerin et al., 2016). In the case of LAE, the molecule is non-volatile and rather thermostable, which enables its incorporation into the bulk of the

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polymer or on its surface (Manso et al., 2021). Indeed, several authors have successfully integrated the molecule in the bulk or on the surface of different polymers: low-density polyethylene (LDPE), polystyrene (PS), polyethylene terephthalate (PET), polyvinyl alcohol (PVOH), polylactic acid (PLA), chitosan, gelatin, starch, and zein. (Manso et al., 2021; Li et al., 2021; Vidal et al., 2021; Haghighi et al., 2020; Moreno et al., 2017a,b; Kashiri et al., 2016; Higueras et al., 2013; Aznar et al., 2013). The integration of LAE in polyhydroxyalcanoates (PHA), such as polyhydroxy(butyrate-co-valerate) (PHBV), has never been attempted, although this polymer was identified as one of the most sustainable ones thanks to its production from renewable resources through bioprocesses and its biodegradability, coupled with good diffusional and thermo-mechanical properties (Rzayeva et al., 2023).

The effectiveness of LAE-based active packaging is possible only if the molecule diffuses from the polymer into food. This diffusion needs to be controlled to (1) have an antimicrobial effect on microorganisms present in the food matrix and (2) avoid any ingestion by consumers at levels above the recommended limits, being identified as admissible daily intake (ADI) (as established by EFSA)(Rzayeva et al., 2023). If the LAE migration in food simulants has been widely studied in the literature (Vidal et al., 2021; Aznar et al., 2013), the dimensioning of LAE-based active packaging allowing the inhibition of microorganisms without overpassing an uptake threshold value has never been done. Indeed, even if the antimicrobial impact of LAE active film used in close-to-real conditions, i.e. in contact with food and not simulants or agar, on different food products as meat, fruits, or vegetables was already studied (Higueras et al. 2013, Ma et al., 2023) none of them confirmed the feasibility regarding the EFSA recommended limitation of LAE® ingestion.

In this context, the aim of the present study was to design an active packaging based on the integration of LAE® in the bulk or as a coating of PHBV film, allowing the inhibition of microorganism growth into food, while conforming to EFSA rules (no overpass the admissible daily intake) to ensure consumer safety. A proof of concept of LAE® dimensioning was done on spreadable paste cheese. The study also assessed whether LAE®/PHBV antimicrobial packaging could represent an alternative to the modified atmosphere packaging.

2. Materials and methods

2.1. Materials

Ethyl Lauroyl Arginate (LAE®) was used in this study as an antimicrobial compound through a product called MIRENAT®-D (white powder), provided by VEDEQSA (Barcelona, España), and composed of 55 % LAE® and 45 % vegetable fibers. Pure Ethyl Lauroyl Arginate used for the calibration curve was purchased from USP (Twinbrook Pkwy, Rockville, MD, United States). The 3-hydroxybutyrate-co-3hydroxyvalerate with 3 wt% of 3HV (P(3HB)-co3HV)) used to produce films with LAE® in bulk (called bulk-active-PHBV film) was a commercial Tianan grade (China), used in the powder form without additives (PHBV), and was purchased from Naturplast (France) (PHI003 grade). The 3-hydroxybutyrate-co-3hydroxyvalerate with 3 wt% of 3HV (P(3HB)-co3HV)) used to produce the support of LAE® containing coating (coat-active-PHBV film) was a commercial Tianan grade (China), used in pellet form with additives, and was purchased from Naturplast (France) (PHI002 grade). The medium used for microbial tests was the trypto-casein soy agar (TSA) purchased from Biokar diagnostics (Beauvais, France; REF. BK047HA), whose composition (g/L) is: tryptone 15, papaic digest of soybean meal 5, sodium chloride 5, bacteriological agar 15. Ultrapure water was obtained from a Millipore Milli-Q system (Millipore, Bedford, MA, USA). Ethanol 96 %, methanol 99 %, formic acid 96 % and acetonitrile 99.9 % were purchased from Sigma Aldrich, France.

The microbial strains used in experiments were the following ones: mould *Penicillium roqueforti* (ATCC 1129), Gram negative bacteria *Pseudomonas fluorescens* (NCTC 10038), Gram positive bacteria *Micrococcus luteus* (ATCC 15307) and gram negative bacteria *Salmonella enteriditis* (ATCC 25928).

2.2. Determination of LAE® properties

2.2.1. LAE® stability

Thermal gravimetric analysis (TGA) was carried out in triplicate on LAE® using a Mettler apparatus equipped with an XP5U balance (precision of 0.0001 mg). For this purpose, 8 mg of Mirenat D (LAE®) was heated at 50 °C/min from 25 °C to 180 °C, fixed at 180 °C for 20 min, then heated at 50 °C/min from 180 °C to 900 °C. Experiments were performed under a nitrogen flow of 50 mL/min. The mass loss was recorded as a function of temperature, and the maximum degradation temperature (Tdeg) was identified.

2.3. Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) was evaluated for different microorganisms and yeasts: *Penicillium roqueforti*, *Pseudomonas fluorescens, Micrococcus luteus,* and *Salmonella enteriditis.* Microorganisms were prepared by incubation in Water (BPW: peptone 10 g/L; sodium chloride 5 g/L; disodium phosphate anhydrous 3.57 g/L; potassium dihydrogen phosphate 1.5 g/L; pH of the ready to use medium at 25 °C: 7.0 ± 0.2) for 5 days at 25 °C for *Penicillium*, 48 h at 30 °C for *Pseudomonas*, 48 h at 37 °C for *Micrococcus* and *Salmonella*. Then, 1 mL of a suspension of each microorganism or yeast at concentrations between 10^3 and 10^4 CFU/mL (spectrophotometric measurement at 625 nm) was deposited on Petri dishes containing agar medium and LAE® at concentrations ranging from 25 to 600 mg/kg. Inoculated Petri dishes were then incubated 24 h at 37 °C for *Micrococcus*, *Salmonella* and *Pseudomonas* and 5 days at 25 °C for *Penicillium* (NF V08–059) (Normes francaises et européennes, 2002), time after which counts were performed using the standard method NF EN ISO 7218 (Normes francaises et européennes, 2007).

2.4. Preparation of the active films

2.4.1. Active PHBV incorporating LAE® in the bulk

PHBV powder (PHI003) was mixed with MIRENAT® D (6.5 % w/w Mirenat® D, 3.6 % w/w LAE) and boron nitride (0.5 % w/w) and dried at 60 °C for 48 h. The mixture was melt-blended using a corotating twin-screw microextruder (model “process 11″ Thermofisher). The barrel temperature profile was set at 160 °C (from top to bottom), pressure of 3 bar, screw speed of 200 rpm, and residence time of 1.5 min. After cooling the blend to room temperature, it was pelletized (Pelletizer, Thermofisher, Germany). The pellets were dried for 24 h at 60 °C under vacuum and then transformed into films using a hydraulic thermopress (CFM 20 T, Pinette Emidecau Industries, Chalon sur Saone cedex, France) at 180 °C for a 1 min at 5 bar and then 1 min at 150 bar. The film was rapidly cooled down thanks to the use of a cold water bath, put on the surface of the metal form used to produce the films. The films were stored at room temperature for a maximum of two months before evaluation of migration, microscopic analysis, and measurements of the evolution of microorganisms in spreadable cheese. The average thickness of the films was approximately 381.3 ± 21.5 µm (see supplementary figure 1).

2.4.2. Active PHBV incorporating LAE® as a coating

The PHBV support of coating was prepared from PHBV pellets (PHI002), which were dried for 48 h at 60 °C and transformed into films using a hydraulic thermopress (CFM 20 T, Pinette Emidecau Industries, Chalon sur Saone cedex, France) at 178 °C for a cycle of 5 min at 5 bar, 30 s at 50 bar, 30 s at 100 bar, and 1 min at 150 bar. After cooling, two layers of a chloroform solution containing PHBV (PHI002, dissolved in chloroform after stirring overnight at 60 °C) (4.7 % w/w) and MIRENAT® D (4.7 % w/w Mirenat D, so 2.6 % w/w LAE®) were deposited

on the PHBV support using a bar coater (Erichsen, GmbH, Germany) at a speed of 2 mm/s. Chloroform was dried at room temperature for a few minutes. The final film contained 1.8 % LAE® (0.12 g of LAE® for 6.6 g of film). The average thickness of the final coated film was approximately 418.3 ± 22.2 μm (see supplementary figure 1). The films were stored at room temperature for a maximum of two months before evaluation of migration, microscopic analysis, and measurements of the evolution of microorganisms in spreadable cheese.

2.5. Determination of the film's properties

2.5.1. Evaluation of LAE® recovery and distribution in the films

From a macroscopic point of view, the PHBV/LAE® (in bulk or coating) sheets were divided into four equal parts. From each part, LAE® was extracted twice in methanol for 24 h at 20 °C. The amount of LAE® was determined by UV quantification in an Aquity UPLC system (Waters, Milford, MA). LAE® was isolated on Waters 100 mm × 2.1 mm, HSS T3 column (1.8 μm particles size), coupled with a photodiode array detector (DAD) set at 208 nm. The injection volume was 5 μL. The mobile phase consisted of a gradient of A: 0.1 % formic acid in water (v/v) and B: 0.1 % formic acid in acetonitrile (v/v). The gradient conditions were as follows: from 0 to 4 min, 0–60 % B; from 4 min to 5 min, 60–70 % B; from 5 min to 6 min, 70–80 % B; from 6 min to 8 mi;, 80–99 % B; from 8 min to 9 min, 99 % B; and from 9 min to 10 min, 99–0 % B. The flow rate was 0.55 mL/min and lauroyl ethyl arginate was detected at 4.7 min retention time. The LAE® was quantified after external calibration with LAE® for standards dissolved in methanol.

From a microscopic point of view, PHBV/LAE® films of 0.5 cm width were deposited on a carbon adhesive tape and covered with a thin layer (4 nm) of Au/Pd sputter coating. The films were then observed with a benchtop Phenom Pro X scanning electron microscope (Phenom World, Eindhoven, The Netherlands) with a backscattered electron detector and an acceleration voltage of 10 kV.

2.5.2. LAE® migration in food simulants

LAE® migration was determined after total immersion of samples in the food simulants based on standardized testing conditions set out for long-term storage of refrigerated foods according to the European Standard EN 10/2011 regulation (European Commission, 2011). The food simulants used for this analysis were simulant D1 (50 % ethanol v/v) and simulant C (20 % ethanol v/v). Six discs of 10 cm^2 cut from the films (so 60 cm^2), were incubated in 100 mL of food simulant (area--to-volume ratio of 6 dm^2/L) at 20 °C for 10 days in a climatic chamber (Memmert, Germany). The analysis was conducted in triplicate, and a blank was carried out (PHBV film without LAE®) for each food simulant. 2 mL of migration solvent were collected each day and LAE® was quantified by UPLC using the procedure described in section 2.4.1.1. As described by Di Giuseppe et al., (2022), the corresponding percentage of LAE® diffused in the food simulant was determined using the following equation:

$$\%\text{of LAE diffused in food simulant} = \frac{C_x \times V_{FS}}{m_f \times \%LAE} \quad (1)$$

with, C_x the mass concentration of LAE® in the simulant (mg/L), V_{FS} the volume of food simulant (L), m_f the mass of film (mg) and % *LAE* the percentage w/w of LAE® included in the PHBV film (3.6 % and 1.8 % for LAE® in bulk and coating, respectively).

The apparent diffusion of LAE® in the food simulant was modeled using Fick's second law (Eq. (2)), assuming that (i) the film is a one dimensional infinite plane sheet (ii) the thickness of the film is homogenous and unchanged during the experiments (no swelling), and (iii) LAE® is homogeneously distributed in the film and in the food simulant (Di Giuseppe et al., 2022).

$$\frac{M_t}{M_\infty} = 1 - \sum_{n=0}^{\infty} \frac{2\alpha(1+\alpha)}{1+\alpha+\alpha^2 q_n^2} \exp\left\{-\frac{Dq_n^2 t}{L^2}\right\} \quad (2)$$

And

$$\alpha = \frac{1}{K_{P,F}} \quad \frac{V_F}{V_P} \quad (3)$$

M_t is the total amount of LAE® in food simulant at time t and M_∞ is the total amount of additives in food simulant at the steady state or maximal point, D is the apparent diffusion coefficient of LAE from film into food simulant (m^2/s), L is the thickness of the film where LAE was incorporated (m), V_P is the polymer volume and V_F the food simulant volume, (q_n), the positive roots of the equation $tanq = -\alpha q$ and $K_{P,F}$, the partition coefficient of the additive in the polymer/food simulant system.

The apparent diffusivity was identified through the Matlab optimization routine, and more specifically, the lsqnonlin function. The model fitting is assured by minimizing the percent Root Mean Square Error (RMSE):

$$RMSE = \frac{1}{M_0}\sqrt{\frac{1}{N}\sum_{i=1}^{N}((M_t)experimental - (M_t)predicted)^2} \times 100 \quad (4)$$

where M_0 is the initial mass of LAE® in the film and M_t is the mass of LAE® into a food simulant at time *t*.

2.5.3. Antimicrobial effectiveness of the active films on inoculated cheese

Spreadable processed cheese (290 g, Carrefour, France) was artificially inoculated with 5.5 mL of diluted *Micrococcus* solution (prepared as described in Section 2.2) to obtain an initial concentration of approximately 10^4 CFU/g. To obtain homogenous inoculation, the contaminated cheese was mixed in a stomacher (Bagmixer 400, interscience, France) for 1 min. The contaminated cheese was placed in polypropylene trays (volume of 750 cm^3) (Futurplast, France) with oxygen and carbon dioxide permeabilities of 1.03×10^{-17} mol•m^{-1}•s^{-1}•Pa^{-1} and 4×10^{-17} mol•m^{-1}•s^{-1}•Pa^{-1}, respectively, sealed with a PP/PA top film (Politherm 65 peel, Boulanger, France) with oxygen and carbon dioxide permeabilities of 9×10^{-18} mol•m^{-1}•s^{-1}•Pa^{-1} and 3.6×10^{-17} mol•m^{-1}•s^{-1}•Pa^{-1}, respectively. In the package was added, either a PHBV pad containing 3.6 % of LAE® in bulk (11.8 ×5.9 cm, 3.4 g), or a PHBV pad containing 1.8 % of LAE® in surface coating (11.8 ×5.9 cm, 2.9 g), or a neat PHBV pad (11.8 ×5.9 cm, 2.8 g) (negative control), or an injection of 100 % CO_2 (positive control) (see supplementary figure 2). The top films were sealed on trays using a Reepack chamber machine (Reetray 20 VGT, Reepack, Italy). Contaminated packed samples were incubated at 20 °C for 10 days in a climatic chamber (Memmert, Germany). Ten grams of non-incubated cheese (0 days) and 10 days-incubated cheese in one of the 4 modalities tested were each mixed in a stomacher (bagmixer 400, interscience, France) with 100 mL of peptone water, and 1 mL was pour plated with growth medium in Petri dishes. After incubation at 37 °C for 24 h, counts were performed using the standard NF EN ISO 7218 method (Normes francaises et européennes, 2007).

2.5.4. Statistical analyses

The LAE extraction from bulk-active-PHBV and coat-active-PHBV film in methanol, the LAE migration in food simulant C and D1 after 10 days from bulk-active-PHBV film and coat-active-PHBV film, as well as the *Micrococcus* contamination in cheese were all statistically analyzed using an ANOVA and Tukey test with a significance level of 5 % ($p < 0.05$).

3. Results and discussion

3.1. Determination of the minimum inhibitory concentration (MIC) of LAE®

The experimental results showed the maximum growth of all test

microorganisms on the control Petri dishes (without LAE®), while total growth inhibition was observed at an LAE® concentration of 25 ppm for *Micrococcus luteus* and *Penicillium roqueforti*, and 100 ppm for *Salmonella enteritidis* and *Pseudomonas putida* (Table 1 and supplementary figure 3). Therefore, these results showed a strong effectiveness of LAE® against all types of tested microorganisms: molds, Gram-positive and Gram-negative bacteria, either spoilage or pathogenic microorganisms. These results are similar to those found in the literature: indeed, various authors identified an MIC range for LAE between 4 and 100 ppm against a wide variety of Gram-positive and Gram-negative bacteria (*Pseudomonas*, *Salmonella*, *Listeria*…), yeasts, and molds (*Aspergillus*) (Nerin et al., 2016; Ma et al., 2023).

3.2. Impact of production process on LAE® stability and distribution in the active films

PHBV/LAE® sheets were divided into four identical parts, and the LAE® contained in each part was extracted and quantified as described in Section 2.5.1. The results showed a similar amount of LAE® in each part of the film, with a result of 1.81 ± 0.23 g/100 g for bulk-active-PHBV film and 1.72 ± 0.07 g/100 g for coat-active-PHBV film (p = 0501). However, the total amount of LAE® extracted from the film was only 50 ± 6 % of the initial amount of LAE® from the bulk samples, against 96 ± 4 % recovered from the coated samples. Therefore, it can be concluded that the extrusion and thermopressing processes used to produce the film (LAE® in bulk) caused a 50 % loss in LAE®. This loss could not be attributed to thermal degradation, as TGA showed only 4–5 % LAE® degradation during a thermal process of 20 min at 180 °C and a Tdeg (maximum degradation temperature) of 287 °C. However, high temperatures could lead to bond formation between LAE® and PHBV (the amine group from LAE® can react with the ketone group from PHBV to form an imine), making LAE® extraction impossible when it is introduced in the bulk of PHBV and when both undergo the thermal process (contrary to the LAE® coating). This hypothesis was confirmed in the literature, where other authors demonstrated the formation of a bond between LAE and polymer during film production (Haghighi et al., 2020; Moreno et al., 2017a,b). For example, Haghighi et al., (2020) showed the formation of ester, amide and imine bonds between LAE and chitosan-polyvinyl alcohol when the concentration of LAE in the film was higher than 2.5 %.

From a microscopic point of view, some differences could be observed between the bulk-active-PHBV and coat-active-PHBV films (Fig. 1). Indeed, the surface of the bulk-active-PHBV film looked similar to the PHBV one: smooth and homogeneous, while the cross-sectional view showed some non-homogeneous and porous filaments. However, the surface of the coat-active-PHBV film displayed non-homogeneous porous crystals or filaments, whereas the cross-sectional view showed a smooth part (corresponding to the PHBV self-supporting film) and a filamentous one (corresponding to the LAE® coated part). Therefore, the incorporation of LAE® modified the structure of the PHBV film, as observed by Haghighi et al., (2019) and Li et al., (2021) on PLA, chitosan, and gelatin films. Moreover, from microscopic point of view, the thickness of the coated layer appeared non-homogeneous (Fig. 1) with a value ranging from 20.2 μm to 53.7 μm (31.5 ± 8.7 μm), probably due to the furrows of the bar coater.

Table 1
Minimal inhibitory concentration (MIC) of LAE®.

Microorganisms	MIC (ppm)
Micrococcus luteus	25
Salmonella enteritidis	100
Pseudomonas putida	100
Penicillium roqueforti	25

3.3. Migration of LAE® in food simulant

The concentration (mg/L of food simulant) and percentage (Eq.1) of LAE® diffused from the active films in food simulants D1 and C are shown in Fig. 2.

For both food simulants, the results showed an increase in LAE® migration with time until reaching a plateau after a few days (between 4 and 5 days) for coat-active-PHBV films, whereas the plateau was not reached in the considered time frame for bulk-active-PHBV films. Corroborating this observation, migration was significatively higher (p = 0.037) from the coated film (112 mg/kg and 86 mg/kg after 10 days in simulants D1 and C, respectively, corresponding to 43 % and 33 % of the initial LAE® amount), than from the PHBV bulk (93 mg/kg and 37 mg/kg after 10 days in simulants D1 and C, respectively, corresponding to 34 % and 14 % of the initial LAE® amount). This faster release from the coating was due to the lower thickness of the layer containing the active molecule (32 μm against 381 μm for the bulk-active-PHBV film), which consequently decreased the distance between the active molecule and the food simulant. These observations were also reported by other authors (Vasile & Baican, 2021; Buonocore et al., 2005; Jipa et al., 2012). For example, Buonocore et al., (2005) showed that a two-fold higher quantity of lysozyme migrated in aqueous media when the molecule was initially present in a monolayer film of PVOH compared with migration from the inner layer of a three-layer film.

LAE® migration was significatively higher (p = 0.007) in food simulant D1 (112 mg/kg and 93 mg/kg after 10 days for the coat-active-PHBV and bulk-active-PHBV films, respectively) than in food simulant C (86 mg/kg and 37 mg/kg after 10 days for the coat-active-PHBV and bulk-active-PHBV films, respectively) due to the higher solubility of LAE® in ethanol than in water. These results are similar to those reported by Vidal et al. (2021) and Aznar et al. (2013), who showed a higher quantity of LAE diffusing from active PVOH/PLA and PET films into a fatty simulant (substitute for D2 simulant=95 % ethanol) than into an aqueous one (simulant A= 10 % ethanol). Consequently, a higher LAE® release is expected in fatty products, such as cheese or alcoholic beverages, than in more polar products.

The apparent LAE® diffusivities showed values in the same order of magnitude between the two food simulants (Table 2), with a value of 2.1–2.2 × 10^{-14} m^2 s^{-1} for LAE® diffused from the bulk-active-PHBV film. The apparent diffusivity of LAE® was more than twenty fold lower when the molecule was initially present in the coated film for both food simulants (7.9 ×10^{-16} $m^2.s^{-1}$ for food simulant D1 and 1.0 ×10^{-15} $m^2.s^{-1}$ for food simulant C). This is probably due to local accumulation of the molecule at the interface with the food simulant, which is determined, in turn, by a lack of agitation of the food simulant, generating a boundary layer between the film and food simulant, which hampers the identification of the diffusivity. It must also be noted that the structure and crystallinity of the PHBV coating layer obtained via a solvent process is probably different than the thermo-mechanically processed film (Pal et al., 2020). Therefore, the diffusivity of LAE® in the PHBV coating layer may be lower in the solvent-based process material than in the thermo-mechanically processed material. It should also be noticed that from mechanistically point of view, the apparent diffusivity of LAE® integrated its diffusion through the polymer and its external transfer from polymer to food simulant. The apparent diffusivity values were in the same order of magnitude as those reported by other authors (Higueras et al., 2013; Kashiri et al., 2016). For example, Kashiri et al., (2016) showed an apparent diffusivity of LAE from zein film into food simulant A in the range of 1.0 × 10^{-16} $m^2.s^{-1}$ to 8.0 × 10^{-14} $m^2.s^{-1}$ for temperatures from 4 °C to 37 °C.

3.4. Application of antimicrobial LAE®-PHBV for cheese packaging

3.4.1. Film dimensioning for cheese tray

Two major parameters should be considered to develop safe and

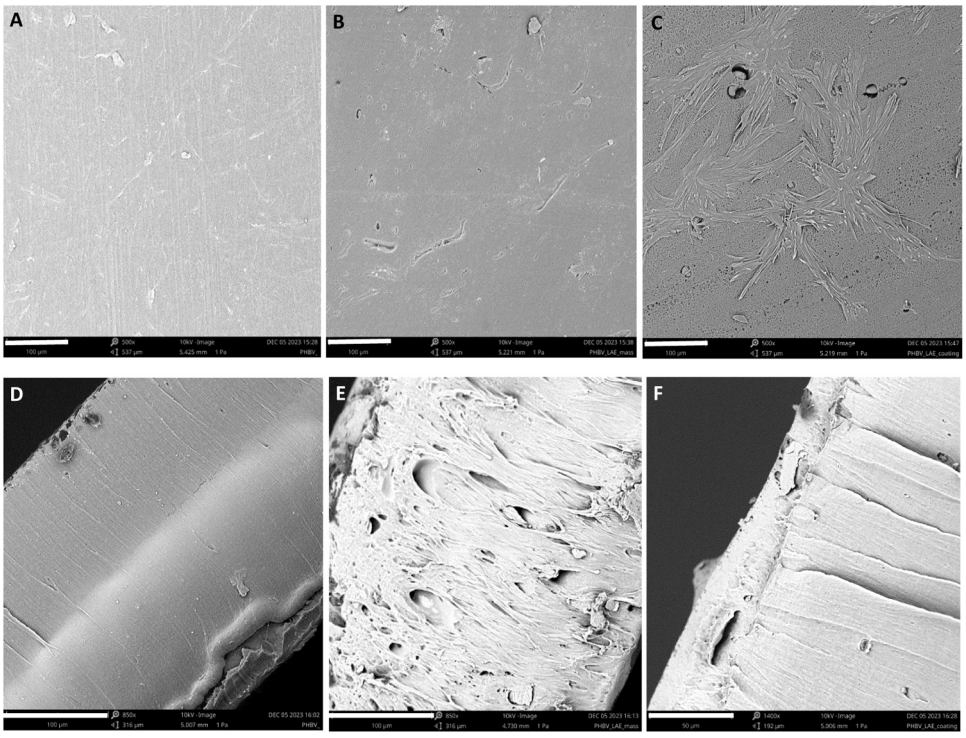

Fig. 1. Scanning electron microscopy images of PHBV film, PHBV film with 3.6 % of LAE® in bulk and PHBV film with 1.8 % of LAE® as coating on surface view ((A), (B) and (C) respectively) and cross sectional view ((C), (D), and (E) respectively). The scale bar represented 100 μm (×500 magnification for (A), (B), and (C), × 850 magnification for (D) and (E) and × 1400 magnification for (F)).

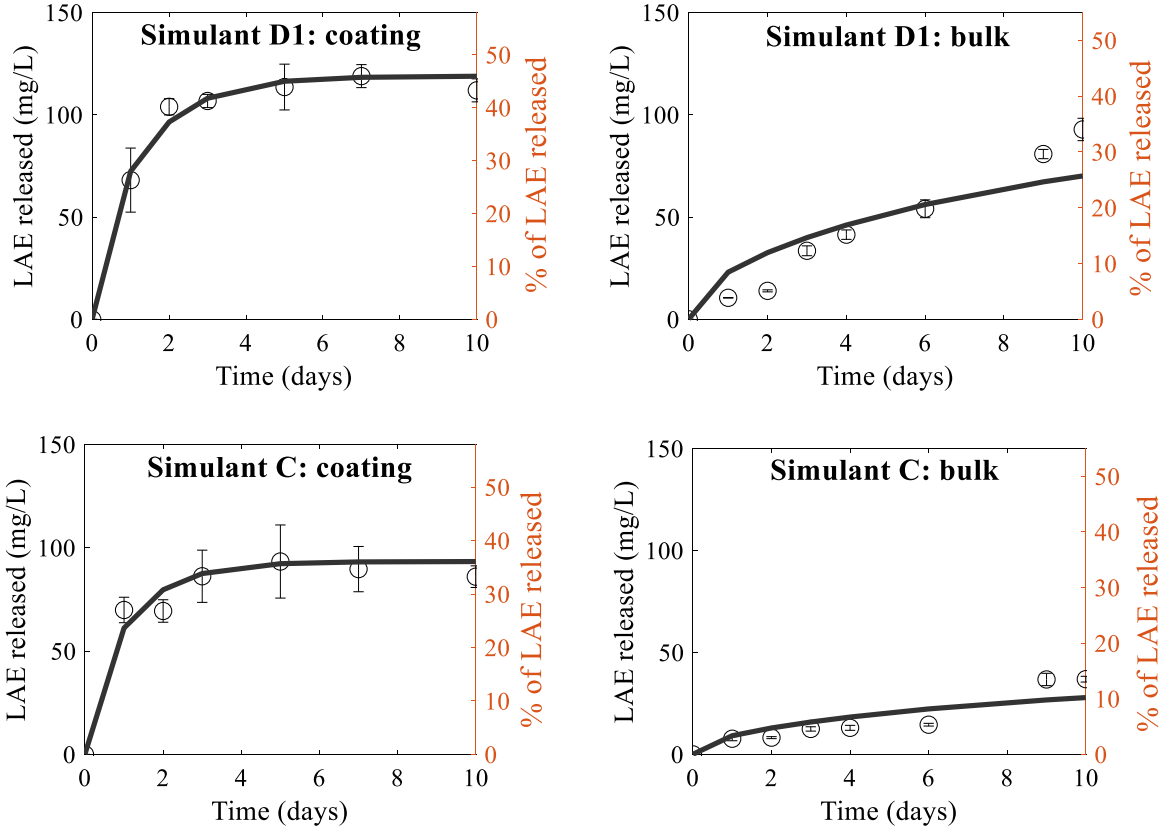

Fig. 2. Migration of LAE® (mg/mL or %) from coat-active-PHBV and bulk-active-PHBV films into simulants D1 and C at 20 °C. Dots represented experimental data, while lines represented simulated data. The error bars represented the standard deviation (n = 3).

Table 2
Estimated apparent diffusivity ($m^2.s^{-1}$) of LAE® migration from PHBV film. RMSE: Root mean square error between experimental and predicted concentrations (as %).

Type of food simulant	Diffusivity ($m^2.s^{-1}$)	RMSE (%)
LAE® in bulk (381.3 µm)		
Simulant D1	2.2×10^{-14}	13.5
Simulant C	2.1×10^{-14}	16.8
LAE® in coating (31.5 µm)		
Simulant D1	7.9×10^{-16}	3.9
Simulant C	1.0×10^{-15}	6.5

efficient packaging: the MIC (minimum inhibitory concentration) and ADI (acceptable daily intake) (Fig. 3). First, the ADI is defined as the quantity of the active compound that can be ingested in one day without having a negative impact on health. The ingestion of this molecule results from both: (i) natural exposure and (ii) exposure due to the migration of active molecules from packaging into food. Consequently, the quantity of antimicrobial agents found in food after migration from active packaging should not exceed the difference between ADI and natural exposure. This should also be an efficient quantity value (Fig. 3). In the case of LAE, the ADI is set to 0.5 mg/kg body weight (EFSA, REG. (UE) N. 506/2014 (European Commission, 2014) and EN 10/2011 (European Commission, 2011)), which represents 30 mg of LAE for a person weighing 60 kg. In France, the natural exposure to LAE by the adult population represents 3.81 % of the ADI, which represents 1.1 mg of LAE for an adult weighing 60 kg (Guillard et al., 2019). Consequently, the quantity remaining available to develop active packaging is 28.9 mg (per day and body weight). Second, this efficient quantity value should be close to or higher than the consumed food quantity multiplied by the MIC, which is the minimal concentration of the active compound that can inhibit the visible growth of a microorganism (Andrews, 2002). In the present study (see Section 3.1), MIC was defined to be ranged between 25 and 100 ppm.

According to Regulation 10/2011 (European Commission, 2011), spreadable cheese could be assimilated to food simulant D1, and diffusion for 10 days at 20 °C is assumed to represent diffusion for 67 days at 4 °C (the shelf life of spreadable cheese). From previous results (see Sections 3.2 and 3.3), for the coat-active-PHBV film, we know that 96 % of LAE® remained in the film after the production process, and 43 % of the remaining LAE® diffused in food simulant D1. As the pad (2.9 g) obtained from coat-active-PHBV film contained 1.8 % w/w of LAE®, 21.5 mg (2900 ×0.018 ×0.96 ×0.43) of active principle should diffuse in the packed cheese (290 g), resulting in a concentration of 74 ppm (21.5 ×1000/290) in spreadable cheese. Similarly, for the LAE® contained in bulk-active-PHBV, 50 % remained in the film after the thermal process and 34 % of the remaining LAE® diffused in food simulant D1. As the pad (3.4 g) obtained from bulk-active-PHBV film contained 3.6 % w/w of active principle, 20.8 mg (3400 ×0.036 ×0.5 ×0.34) of LAE® should diffuse in the packed cheese (290 g), resulting in a concentration of 72 ppm (20.8 ×1000/290) in spreadable cheese. Although the bulk-active-PHBV film was initially more concentrated than the coated film, the bonding with the PHBV network during the thermal process and the limited diffusivity of LAE® in the bulk-active-PHBV film made this solution as effective as the one relying on incorporation by coating. Therefore, this dimensioning of active packaging for spreadable cheese allowed the fulfilment of the two constraints: (i) be below the ADI (minus the natural exposure) of 28.9 mg (per body weight and day), assuring consumer safety; (ii) reach or be close to the MIC of several microorganisms (25 ppm for *Micrococcus luteus* and *Penicillium roqueforti*, and 100 ppm for *Salmonella enteritidis* and *Pseudomonas putida,* see Section 3.1).

3.4.2. *Antimicrobial effectiveness of the dimensioned active packaging*

Table 3 and supplementary figure 4 show the results of the antimicrobial activity of the dimensioned packaging with pads containing in bulk or coated LAE® towards *Micrococus luteus* inoculated in fresh spreadable cheese, during a storage period of 10 days at 20 °C. When PHBV pads without LAE® were used as a negative control, a 2.8 log growth (statistically significant) of *Micrococcus luteus* was observed on spreadable cheese after 10 days of incubation (Table 3). Modified atmosphere packaging of 100 % CO_2 was used as a positive control and

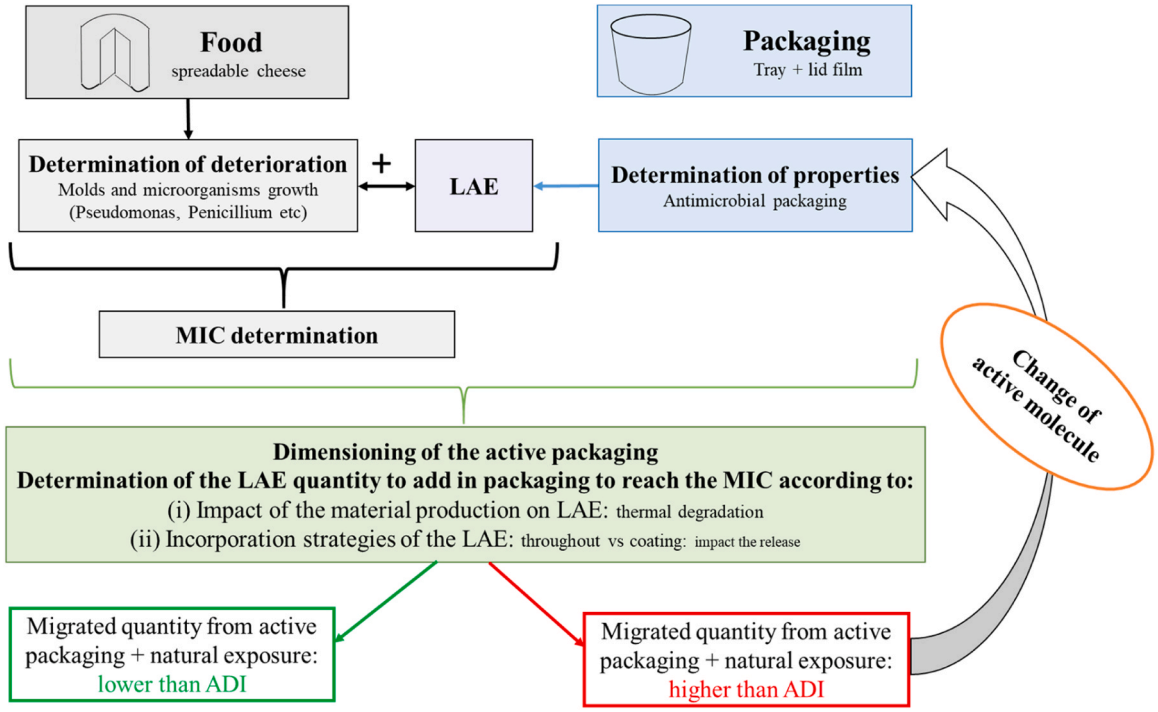

Fig. 3. Principle of the dimensioning of active packaging: example of an LAE active film for a fresh spreadable cheese (ADI=admissible daily intake and MIC=minimum inhibitory concentration).

Table 3
Evolution of *Micrococcus* contamination in fresh cheese depending on conditions of storage (with or without LAE® or MAP) after 10 days at 20 °C. Values with same letters were not significantly different ($p < 0.05$).

Conditions	Initial contamination ($\times 10^3$ CFU/g)	Contamination after storage ($\times 10^3$ CFU/g)	Log evolution during storage	Log evolution against negative control
Negative reference (PHBV pads)	$1.8^{c} \pm 0.3$	$1200^{a} \pm 64$	+ 2.8	
Positive reference (MAP)		$0.55^{c,d} \pm 0.44$	- 0.5	-3.3
bulk-active-PHBV pad		$45^{b} \pm 4.3$	+ 1.4	-1.4
coat-active-PHBV pad		$0.36^{d} \pm 0.28$	- 0.7	-3.5

allowed to obtain a reduction of 0.5 log (non statistically significant) against *Micrococcus luteus* after 10 days of incubation (Table 3). The presence of bulk-active-PHBV pads did not prevent *Micrococcus luteus* growth, as the microorganism counts showed a + 1.4 log growth evolution (increase statistically significant) (Table 3). In contrast, the presence of coat-active-PHBV pads in packaging prevented *Micrococcus luteus* growth, as the microorganism counts showed a - 0.7 log reduction (statistically significant). However, in all cases, a significant reduction in microbial contamination after 10 days of storage at 20 °C was observed for all active packaging compared to the negative control (1.4, 3.5 and 3.3 log for systems with bulk-active-PHBV, coat-active-PHBV, and MAP, respectively, Table 3), indicating that the active packaging was, in any case, a better solution than conventional packaging. Moreover, the system with LAE® as a coating showed an efficiency similar to the positive control (Table 3), indicating that this strategy could replace MAP or strengthen the effect of MAP, also guaranteeing protection in the case of MAP failure (due to seal failure) or after opening.

Although, dimensioning showed a similar effective impact on microorganisms (similar MIC) for bulk-active-PHBV and coat-active-PHBV pads (section 3.5.1), the experimental results showed a higher and significant impact on microorganisms when LAE® was coated. The pads were deposited below the cheese, while microorganism development usually occurs on the surface (Batiha et al., 2021). The migration of LAE® to the surface depends on the diffusivity of the molecule into the cheese, which generates a delay to reach the MIC on the food surface. In the case of coat-active-PHBV pads, the diffusion of LAE® from the film reached a plateau in 4 days (Section 3.3), letting time for LAE® to diffuse into the cheese before the end of the 10 days of the experiment. In the case of bulk-active-PHBV pads, the diffusion from the film was continuous during the 10 days (Section 3.3), with the delay due to the LAE® migration into the cheese; a lower concentration than expected was probably reached after 10 days of experiment.

The effectiveness of LAE® films against microorganism growth (*L. monocytogenes*, *E. coli*, *S enterica*, *C. jejuni*, *S. aureus*, *P. putida* etc) was also demonstrated by other authors in liquid or solid media (Muriel-Galet et al., 2012; Higueras et al., 2013; Kashiri et al., 2016; Haghighi et al., 2020), and in the food matrix as meat, fruits or vegetables and by (Higueras et al., 2013; Muriel-Galet et al., 2015; Moreno et al., 2017b; Kashiri et al., 2019; Hassan & Cutter, 2020; Li et al., 2021). For example, Higueras et al., (2013) showed a reduction of 2–3 log against different mesophiles, psychrophiles, and *Pseudomonas spp.* after incubation for 8 days at 4 °C in chicken breast filets in chitosan packaging containing 4 % LAE®, while Hassan & Cutter (2020) showed a reduction of 2.9 to 3.5 log against *E.coli*, *Salmonella spp*, *L. monocytogenes* and *S. aureus* after incubation for 28 days at 4 °C in raw beef in pullulan/gelatin/xanthane gum film containing 2.5 % LAE®.

Although active pads seems a good strategy of food conservation, to go beyond and use less packaging material, the processability of active material should be explored to use it as a self-support packaging. For that, it is necessary to explore and obtain good mechanical and barrier properties of the active packaging, that will probably go through a better understanding of the interaction between LAE® and PHBV. Moreover, the possibility to use a less thickness active film could be explored to use it as a lid film of packaging, the improvement of flexibility and mechanical properties of PHBV films being obtainable thanks to higher 3HV molar fraction (Doineau et al., 2023). In the case of active coated film, the chloroform is not usable for food contact and consequently, other strategy or solvent should be found to integrate LAE® in packaging.

4. Conclusion

In the present study, active films were developed based on a PHBV polymer, in which LAE® was added in bulk or embedded in a PHBV-based layer coated on the film surface. The dimensioning of active packaging was a crucial step and necessitated to fulfil two conditions: (1) LAE® must be transferred in food to a sufficient extent to reach the MIC of targeted microorganisms; (2) LAE® must be in small enough quantity to ensure a consumer's intake below the ADI. Both of these conditions were reached in the present study.

The results showed that unlike the coat-active-PHBV film, the available concentration of LAE® contained in the bulk-active-PHBV film is halved due to the formation of covalent bonds with PHBV. Moreover, 43 % and 34 % of the remaining LAE® from the coated and bulk films diffused in food simulant D1, respectively. From these outcomes, it can be concluded that the use of a 11.8 × 5.9 cm PHBV pad with 3.6 % of LAE® in bulk or 1.8 % of LAE® in the coating layer could lead to a concentration of LAE® in spreadable cheese (72–74 ppm) in the MIC value range measured against *Micrococcus luteus*, *Penicillium roqueforti*, *Salmonella Enteritidis* and *Pseudomonas putida* (25–100 ppm). Even in the worst case (migration of all LAE®® in food, and all food ingested by one consumer in one day) the quantity ingested by consumers would remain below the ADI, assuring consumer safety.

These calculations were confirmed by an experiment conducted on a spreadable cheese. However, although both LAE®/PHBV pads (in bulk or coating) induced a decrease in *Micrococcus* growth, the pads with LAE® in the coating seemed to be a better candidate to reinforce a CO_2 modified atmosphere. In the future, the use of LAE® packaging needs to be validated from an industrial processability and economic point of view.

CRediT authorship contribution statement

Valérie Guillard: Writing – review & editing, Investigation, Conceptualization. **Fabrice Gaubiac:** Writing – review & editing, Investigation. **Fanny Coffigniez:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis, Conceptualization. **Fabio Licciardello:** Writing – review & editing, Investigation, Conceptualization. **Jennifer Bruni:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization.

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

Acknowledgments

JB, FC, FL, and VG conceived and designed the experiments. JB performed the experiments. JB, FC, FL, VG and FG analysed the data and wrote the paper. FC supervised the work.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fpsl.2024.101319.

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