LWT - Food Science and Technology 218 (2025) 117486

Contents lists available at ScienceDirect

LWT

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

Development of solvent-cast antimicrobial PHBV films for the inhibition of spoilage microflora

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ARTICLE INFO

Keywords:
Active packaging
Food waste prevention
Shelf life
Nisin
LAE
poly(3-hydroxybutyrate-co-3-hydroxyvalerate)

ABSTRACT

Antimicrobial packaging based on poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) incorporating Ethyl Lauroyl Arginate (LAE®) was developed and demonstrated effectiveness in inhibiting spoilage bacteria commonly encountered during household food storage. An exploratory study first evaluated the antimicrobial efficacy of nisin and LAE® against spoilage microflora from household environments. Due to its effectiveness against spoilage mixed cultures and resistance to organic solvents, LAE® was selected for further assessment and incorporation into active films. The minimum bactericidal concentration (MBC) of LAE® was determined against *Listeria monocytogenes* ATCC 19115 and *Pseudomonas* sp. isolated from spoiled almond beverage. Active PHBV-LAE® films were assessed for antimicrobial efficacy in both broth medium and almond beverage, showing substantial microbial inhibition. This study supports the potential of biodegradable active packaging with LAE® to reduce food waste throughout the food supply chain, both before and after package opening.

1. Introduction

Food waste is a critical global issue, with about one-third of all food produced for human consumption lost or wasted globally, leading to significant resource wastage and environmental impact (FAO 2011, 2013). Reducing food loss and waste is a key target of the United Nations' Sustainable Development Goals (SDG 12) within the 2030 Agenda. According to the latest available report on the progress of the SDGs, 17% of the total food available to consumers is wasted at the consumer level (household, food service, and retail levels) (Jensen 2022), with 60% of this waste occurring in households (UNEP 2024). Despite food wastage occurring along the entire food supply chain, in medium- and high-income regions, the greatest amount is produced in the downstream phases of the food chain, including the consumption phase (FAO 2011; FAO 2013). This highlights the need to reduce food waste at the consumer level (Nicosia et al. 2023). Achieving this requires understanding the root causes of food waste by the end-user, which are primarily food durability and spoilage, linked to attention to expiration dates (Giordano et al. 2019; Herzberg et al. 2020; WRAP, 2013). Two promising strategies to reduce household waste include the scientific reassessment of labeled secondary shelf life (or used life) of foods, often shorter than the actual duration after opening (Nicosia et al., 2021, 2022, 2023), and the extension of the primary shelf life through improved packaging solutions.

Active packaging materials incorporating antimicrobial agents are a major focus in the food packaging industry due to their ability to inhibit or reduce microbial growth, maintaining food safety and increasing the product shelf life (Almasi et al. 2021; Dıblan and Kaya 2018). Among antimicrobials, growing interest has been directed toward Ethyl Lauroyl Arginate (LAE) and nisin, due to the wide range of antimicrobial activity and natural origin, respectively. LAE is a cationic surfactant, synthesized from L-arginine, lauric acid, and ethanol, active against Gram-positive and Gram-negative bacteria, yeasts, and molds. It was approved as food ingredient by the Food and Drug Administration (FDA) in 2005 (FDA, 2005) and by the European Food Safety Authority as food additive E243 (EFSA, 2007; Reg. (EC) No 1333/2008).

LAE has been incorporated into various active packaging systems, including both petroleum-based and biobased polymers. Most of these studies have shown promising results regarding the preservation of a variety of foods, such as raw meats (Hassan and Cutter 2020; Higueras et al. 2013; Moreno et al. 2018; Pattanayaiying et al. 2015), cured meats (Gracia-Vallés et al. 2022; Guo et al. 2014; Hassan and Cutter 2020; Pattanayaiying et al. 2015; Theinsathid et al. 2012), fish (Muriel-Galet et al. 2015), cheese products (Bruni et al. 2024), milk alternatives

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https://doi.org/10.1016/j.lwt.2025.117486
Received 8 August 2024; Received in revised form 30 December 2024; Accepted 2 February 2025
Available online 3 February 2025

(Nicosia and Licciardello 2025), and fresh fruit (Li et al. 2021). In a recent study we have elucidated the mechanism of LAE release from the active PHBV films developed in this study, proposing food products categories most suitable for this type of active packaging (Nicosia and Licciardello 2025).

Nisin is the most popular among bacteriocins, a group of antimicrobial peptides (AMPs) produced by bacteria, which have gained increasing interest as safe food preservatives (Popa et al. 2022; Santos et al. 2018). Nisin is a 34-amino acid residual peptide produced by strains of *Lactococcus lactis* subsp. *lactis* (Bahrami et al. 2019), that can inhibit many Gram-positive foodborne pathogens (Chai et al. 2015), while a few studies have demonstrated a slight effectiveness towards Gram-negative bacteria (Jin et al. 2009; Jin and Zhang 2008). It is generally recognized as safe (GRAS) and has been authorized as food additive E234 by the European Commission (Reg. (EC) No 1333/2008), the FDA, and the Food Standards Australia New Zealand (Popa et al. 2022). Several studies have demonstrated the incorporation of nisin into active packaging, showing antibacterial activity mainly against Gram-positive bacteria and reducing their growth in cheese products (Cao-Hoang et al. 2010; Cui et al. 2017; Divsalar et al. 2018; Freitas et al. 2020), meat products (Correa et al. 2017), and egg whites (Jin et al. 2009).

PHBV is a promising bioplastic from the polyhydroxyalkanoates (PHAs) family, produced via bacterial fermentation. It can be obtained from renewable carbon sources and food by-products, it is non-toxic and biodegradable in aerobic, anaerobic, and marine environments (Chea et al. 2016; Meereboer et al. 2020; Wang et al. 2006). PHBV is synthesized by incorporating 3-hydroxyvalerate (3-HV) units into polyhydroxybutyrate (PHB), which reduces crystallinity, melting temperature, and brittleness (Hernández-García et al. 2021). This modification provides a broader temperature/time processing window, making PHBV more processable than PHB (Soares da Silva et al. 2022). Additionally, it represents an attractive alternative to conventional plastics such as polypropylene (PP) and polyethylene terephthalate (PET), due to is physical properties comparable to some petroleum-based polymers, such as high viscosity in the liquid state, which facilitates extrusion processes, as well as good thermal and oxygen barrier properties (da Costa et al. 2020; Hernández-García et al. 2021; Ibrahim et al. 2021).

Unlike previous research that has exclusively focused on primary shelf life, our study shifts the focus to the often-overlooked but critical period following package opening, which is related with domestic food waste. The secondary shelf life (period after opening) for many food products is mainly linked with microbial alteration, its rate depending on the storage conditions and level of recontamination which, in turn, is affected by the domestic use conditions. Providing the package with additional features, such as antimicrobial capacity, is a promising approach to delay spoilage after opening and represents the only tool that can be implemented at the producer level, since most of the responsibility in the determination of life after opening lies with the consumer behaviour. Hence, in this study we propose a novel approach towards the reduction of household food waste by targeting the spoilage microflora that develop after package opening in domestic environments. To achieve this, the antimicrobial activity of nisin and LAE was assessed against spoilage microflora isolated from an almond beverage, which was naturally contaminated after package opening under realistic domestic conditions. Afterward, PHBV active films incorporating the most effective antimicrobial compound were developed, and their performance was investigated in broth medium and in an almond beverage for a real food application. This work demonstrates the potential of PHBV-LAE films in reducing food spoilage, offering innovative solutions and aligning with sustainability goals.

2. Materials and methods

2.1. Materials

PHBV (PHI 003; melt flow rate 15–30 g/10 min (190 °C, 2.16 Kg); density 1.24 g/cm^3) was kindly supplied by NaturePlast (Mondeville, France). LAE® was kindly provided by Vedeqsa (Barcelona, Spain) in its commercial formulation Mirenat®-GC (20% LAE®, 80% glycerol). Nisin from *Lactococcus lactis* was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Brain Heart Infusion (BHI) broth, Plate Count Agar (PCA), and Pseudomonas Selective Agar were purchased from Biolife (Milan, Italy). Qualitative analysis filter paper (FILTER-LAB® 1300/80) was purchased from Filtros Anoia S.A. (Barcelona, Spain). Formic acid (ACS reagent, ≥96%) and chloroform (ISO reagent, ≥99%) were supplied by Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

2.2. Obtaining target spoilage bacterial cultures

Ten cocktails of naturally occurring spoilage microorganisms and one culture of *Pseudomonas* sp. were isolated from spontaneously contaminated almond-based drinks, as described in Sections 2.2.1 and 2.2.2. *Lactiplantibacillus plantarum* UMCC 2996 was selected as a target for antimicrobial tests, as it represents a sensitive microorganism often used to evaluate the antimicrobial efficacy of nisin (Carl et al. 2004; Siroli et al. 2019). Additionally, the biocidal activity of LAE was assessed against *Listeria monocytogenes* ATCC 19115, a main foodborne pathogen widespread in the environment and associated with significant safety concerns (Bartula et al. 2023).

2.2.1. Environmental spoilage mixed cultures (SMC)

One of the objectives of this study was to evaluate the effectiveness of antimicrobials against spoilage microflora that develop after package opening. To this aim, we have proposed a novel approach to obtain spoilage mixed culture to be used for the assessment of antimicrobial activity.

The microflora that colonizes a particular food depends strongly on the product's characteristics, as well as how it is processed and stored (Raposo et al. 2017). However, recontamination that occurs after opening is closely linked to the environmental contamination of the surroundings where the package is opened (Shaltout 2024), leading to unacceptability usually within a few days after first opening. For this reason, the antimicrobial effectiveness of LAE and nisin was assessed against cocktails of spoilage microorganisms naturally developed in an almond beverage, subjected to end-use simulation in domestic environments.

An almond beverage was chosen as the target food because of its popularity as a plant-based milk alternative, which is increasingly demanded by consumers, and its susceptibility to rapid deterioration due to microbial growth (Sethi et al. 2016). A commercial UHT almond-based drink (4% almond, 2.6% fat, 3.4% carbohydrate, 0.8% protein) was purchased locally, stored at room temperature, and kept sealed until use, as indicated by the producers. A use simulation was carried out in ten domestic environments (labeled A to J) by ten consumers to obtain ten distinct spoilage microorganisms mixes. The procedure was adapted from previous research of our group, where we simulated worst-case scenarios of consumer's use and handling of the food products (Nicosia et al. 2021, 2022, 2023). In each environment, 100 mL of freshly opened beverage was poured into a non-sterile glass and left uncapped at room temperature for 20 min, stirring occasionally. The product was then transferred into a sterile 50 mL Falcon tube, capped, and left at room temperature for 1 h before being stored in the household refrigerator. This scenario simulates suboptimal use by consumers, when food is left at room temperature for instance during meal preparation and consumption. The following day, samples were transferred refrigerated to laboratory and stored for 7 days at 4 °C. Upon occurrence of alteration, 100 μL of each almond beverage sample was

spread onto PCA plates and incubated at 30 °C for 24–48 h to allow microorganisms from the ten households to grow on the medium. The environmental deteriorative microflora were then transferred and stored in liquid medium (BHI broth) for subsequent antimicrobial testing. In total, we obtained ten mixes of unidentified spoilage microorganisms, designated spoilage mixed cultures (SMC) A through J, each representing the household environment in which the product opening occurred. The efficacy of the antimicrobials nisin and LAE was tested *in vitro* against the obtained SMCs.

2.2.2. Isolation of *Pseudomonas* sp. from almond beverage

This study aimed to isolate a pure culture of *Pseudomonas* sp. from spoiled almond beverages, as *Pseudomonas* spp. are among the main spoilage bacteria in milk, dairy alternative drinks and derived products (more details in Section 3.1). The ten spoiled almond drinks (introduced in Section 2.2.1) were properly diluted, and 100 μL of each was spread on Pseudomonas Selective Agar plates, followed by incubation at 30 °C for 24–48 h. Nine isolated colonies showing the characteristic bright yellow-green coloration of *Pseudomonas* were found on the agar surface and were transferred by streaking onto sterile Pseudomonas Selective Agar plates. After five purification steps, basic phenotypic tests such as Gram staining and catalase activity were performed. Six out of nine isolates exhibited the characteristics of *Pseudomonas* (Gram-negative and catalase positive) and were selected for DNA sequencing.

Genomic DNA was extracted by Bio-Fab Research (Rome, Italy) using ZymoBIOMICS DNA Microprep kit (Catalog #D4301; Zymo Research Corporation, Irvine, CA). Amplification of the 16S rRNA gene was performed using primers 27f (5' - AGRGTTYGATYMTGGCTCAG - 3′) and 1492r (5' - TACGGYTACCTTGTTACGACTT - 3′). The PCR products were purified using MultiScreen plates (Millipore, Billerica, MA, USA). Sequencing was performed by Bio-Fab Research through capillary electrophoresis using an ABI PRISM 3730XL sequencer (Applied Biosystems, USA). Electropherograms were analyzed using Sequencing Analysis software version 5.2 (Applied Biosystems, USA). All sequences were processed with Mega X software (version 11.0.13) and aligned using the BLAST program in GeneBank.

2.3. Assessment of active compounds against spoilage mixed cultures

The agar disk diffusion method with slight modifications (Raeisi et al. 2016) was used to measure the inhibitory effect of the antimicrobial compounds towards the ten SMC, obtained as described in Section 2.2.1. Stock solutions of LAE (5 % w/v) and nisin (0.125 % w/v) were prepared in sterile distilled water and in 0.02 M HCl (Taylor et al. 2007), respectively, and stored at 4 °C until use for a maximum of seven days. The working solutions at defined concentrations were prepared daily. The antimicrobial compounds were tested in the following range of concentrations: nisin (0.01–0.125%), LAE (0.1–5%). The different concentration range used for the two compounds reflects their actual limit defined by Reg. (EC) No 1333/2008 on Food Additives, being 12.5 mg/kg of food for nisin and 160 mg/kg of food for LAE. Thus, nisin was tested in concentrations which were more than 10 times lower than LAE's concentration.

The agar disk diffusion method was adapted from Raeisi et al. (2016). Overnight cultures of the SMC (A to J) were diluted in sterile BHI broth to obtain inocula standardized at OD_{600} of 0.2 ± 0.02. Afterward, 100 μL of the standardized inoculum from each culture was spread on PCA plates. Sterilized filter paper disks (6 mm diameter) were placed on the surface of the seeded agar plates and were impregnated with 10 μL of each antimicrobial solution. Sterile distilled water was used as the control for LAE, while sterile 0.02 M HCl was used as the control for nisin solution. Inhibition zones were measured using ImageJ software after incubation for 24 h at 30 °C. All tests were performed in triplicate, and the diameter of the inhibition zones was adjusted by subtracting the disk diameter from each measure and recorded in millimeters.

2.4. Stability of active compounds following exposure to solvents

The effectiveness of LAE and nisin was assessed after contact with chloroform and formic acid to determine any potential variations in antimicrobial activity when incorporated into film forming solutions and during active film production via solvent casting. Stock solutions of LAE (2 %) and nisin (0.125 %) were prepared in chloroform and stirred for 1 h at 60 °C, and in formic acid for 30 min at 70 °C, replicating the conditions for film production. Afterward, the solvents were evaporated using a rotary evaporator, and the residual antimicrobials were resuspended in sterile distilled water (for LAE) and 0.02 M HCl (for nisin) to obtain the stock solutions after solvent contact. The antimicrobial activity of the treated compounds was evaluated by the disk-diffusion method, as described above, using *L. plantarum* as the target microorganism. This bacterium was chosen due to its sensitivity to both antimicrobials and its common use in antimicrobial assays with nisin, which has a narrower range of activity compared to LAE (Carl et al. 2004; Siroli et al. 2019). Using a microorganism sensitive to both antimicrobials was crucial to observe any reduction in antimicrobial efficacy following solvent treatment of the compounds.

2.5. Determination of the minimum bactericidal concentration (MBC) of LAE

The antimicrobial effectiveness of an active compound is commonly assessed by the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). MIC is defined as the lowest concentration of antimicrobial substance that inhibits visible growth of microorganisms, resulting in an optical density change at 600 nm (ΔOD_{600}) lower than 0.05 (Kowalczyk et al. 2020; Ma et al. 2013; Wiegand et al. 2008). MBC refers to the lowest concentration capable of inactivating at least 99.9% of the bacteria (a 3 log reduction of viable cells) after subculture onto an antimicrobial-free medium (Gracia-Vallés et al. 2022; Ma et al. 2013; NCCLS, 1999). While the MIC test typically relies on turbidity changes due to microbial growth, often measured via absorbance, determining the MIC was not possible in this study due to the turbidity caused by the addition of LAE to the broth medium. This turbidity interfered with the ability to assess changes in optical density resulting from microbial growth. In cases of turbidity issues, alternative methods that assess metabolic activity are typically used. However, for the scope of our work, which aimed to compare LAE's activity with existing literature, we employed the MBC test as suggested by Gherardi et al. (2016), who also performed only the MBC for LAE in line with our approach.

The MBC of LAE was assessed against *L. monocytogenes* ATCC 19115 and *Pseudomonas* sp. isolated from spoiled almond beverage using the tube dilution method (Kowalczyk et al. 2020; NCCLS, 1999) with a slight modification of the media used. Bacterial inocula were prepared from overnight cultures in sterile BHI broth to obtain OD_{600} of 0.2 ± 0.02. The antimicrobial stock solution was prepared at 2% (w/v) in sterile BHI broth. Proper volumes of stock solution were diluted in 9 mL of sterile BHI broth, to obtain concentrations of 50, 125, 250, 375, and 500 ppm of LAE in the final volume of 10 mL (after inoculum addition). A control test was conducted without the addition of antimicrobial. Subsequently, 1 mL of standardized *Pseudomonas* sp. and *L. monocytogenes* cultures was added to the tubes containing the different antimicrobial concentrations mentioned above, to achieve a final inoculum of 4.0×10^7 CFU/mL, as quantified by plating onto PCA agar plates. The tubes were incubated at 30 °C for 24 h, then serially diluted and subcultured on PCA plates. Microbial growth was quantified after plates incubation at 30 °C for 24–48 h. All tests were performed in triplicate.

2.6. Films formulation

PHBV film forming solutions were prepared according to Lo Faro et al. (2021), with slight modifications. Briefly, 5% (w/v) PHBV was

added to a pre-heated solvent, either chloroform (55 °C for 1 h) or formic acid (70 °C for 30 min), under continuous stirring to obtain a pure PHBV solution. For active films compounding, LAE was incorporated at 5% (w/w, based on polymer dry weight) into the film forming solution and further stirred for 5 min at room temperature. This antimicrobial concentration was chosen based on previous research by Bigi et al. (2023). Next, 11 g of the film forming solution were cast onto glass Petri dishes (140 mm diameter) under a fume hood (final film grammage: 1.70 g LAE/m^2). For the formic acid solution, the dishes were placed in a water bath, allowing the solvent to evaporate at 80 °C for approximately 10 min. The resulting neat PHBV and active PHBV-LAE films were used for further antimicrobial testing. The thickness of the films was measured using a digital micrometer with a sensitivity of 0.001 mm (S.A.M.A. Italia S.r.l.®, Viareggio, Italy).

2.7. Antimicrobial activity of active PHBV-LAE films

The antimicrobial effectiveness of the active PHBV-LAE films was studied in broth medium against *Pseudomonas* sp. isolated from almond beverage and *L. monocytogenes* ATCC 19115. The analysis was performed according to the liquid incubation method under continuous stirring, or dynamic shake flask test (ASTM E2149-01) with slight modifications. Briefly, the active PHBV-LAE and the control PHBV films (2 × 4 cm) were sterilized under UV light, placed into 100-mL Pyrex bottles containing 20 mL of sterile BHI broth, and stored at 30 °C under continuous stirring at 200 rpm. An initial period was allowed before inoculation to enable the active film to release the antimicrobial into the liquid medium. After 0, 1, 3, and 7 days of immersion, 10 μL of inoculum (standardized to an OD_{600} of 0.2 ± 0.02) were added into each bottle containing the active or control films, achieving a final concentration of 4.0×10^7 CFU/mL, as quantified by plating onto PCA agar plates. A film-free inoculated BHI broth and a BHI broth containing films but without inoculum were used as positive and negative controls, respectively. Samples were incubated at 30 °C and 200 rpm, simulating the worst-case scenario where food is left at room temperature rather than stored in the refrigerator (Jin et al. 2009). After 16 h of incubation, samples were serially diluted, 1 mL was poured onto PCA plates, and incubated at 30 °C for 24–48 h. The limit of detection was 1.0 $\log_{10}$ CFU/mL.

2.8. Antimicrobial tests in real food system

The antimicrobial effectiveness of LAE was also tested in a food matrix. UHT almond beverage was selected as the test food, and *Pseudomonas* sp., isolated from the same beverage, was used to assess the antimicrobial activity *in vivo*. The MBC test was performed as described in Section 2.5, using freshly opened UHT almond beverage as the medium. Additionally, the antimicrobial efficacy of active PHBV-LAE films was tested in almond beverage using the dynamic shake flask method, as described in Section 2.7. In this method, neat or active films were immersed in 20 mL of UHT almond beverage, stored at 30 °C, and inoculated after 0, 1, 3, and 7 days with 10 μL of *Pseudomonas* sp., achieving a final concentration of 4.0×10^7 CFU/mL.

2.9. Statistical analysis

Statistical analysis was performed using one-way and two-way analysis of variance (ANOVA), followed by Tukey's HSD post hoc test to evaluate significant differences among groups ($p < 0.05$). The relationships among variables were examined by applying Pearson correlation test. RStudio (version 2022.12.0 + 353; RStudio, Boston, MA) was used for statistical analysis. All experiments were performed in triplicate, and results are presented as means ± standard deviations (SD).

3. Results and discussion

3.1. Isolation of Pseudomonas sp. from spoiled almond beverage

Six colonies were isolated from naturally spoiled almond beverage after five purification steps on Pseudomonas Selective Agar, followed by morphological and phenotypic tests. The purification process yielded a pure culture of each isolate, which was then DNA sequenced for genus identification. The purpose was to isolate spoilage bacteria naturally occurring in spoiled almond beverages, with a specific target on *Pseudomonas,* as it is the most common spoilage microorganism found in dairy products (Ledenbach and Marshall 2009; Lopez et al. 2018; Quintieri et al. 2021), and other aerobically stored foods with high water content and neutral pH (Raposo et al. 2017), such as dairy alternative beverages (John et al. 2023; Mukuna et al. 2021). Additionally, Pseudomonads are psychrotrophs, allowing them to survive during refrigerated storage after package opening.

Among the sequenced strains, bacteria from the genera *Acinetobacter*, *Enterobacter*, *Klebsiella* and three strains of *Pseudomonas* were identified. The *Pseudomonas* sp. strain exhibiting the fastest growth on Pseudomonas Selective Agar was selected for subsequent antimicrobial testing.

3.2. Antimicrobial activity against the spoilage mixed cultures (SMC)

The antimicrobial activity of the two compounds was assessed against ten SMCs (A-J). LAE was effective towards all SMCs (Table 1a), while nisin inhibited only five (Table 1b). A positive correlation ($p < 0.001$) was found between antimicrobial concentrations and inhibition diameter in all samples with inhibition, with Pearson's correlation coefficients of 0.46 for LAE and 0.8 for nisin. LAE showed greater inhibition diameters than nisin. For instance, comparing the same concentration of the two antimicrobials (0.1%), LAE caused larger diameters of nearly 5 mm for SMC A, compared to nisin's maximum inhibition of 2.15 mm for SMC J.

While LAE showed broader inhibition overall, it is worth noting that the SMCs inhibited by nisin (A, C, D, H, J) also showed the greatest inhibition diameters with LAE, suggesting these five SMCs are the most sensitive, likely due to their microbial compositions. Particularly, SMCs A and J were among the most sensitive to both active compounds. The varied inhibition observed reflects both the compounds' spectra of activity and the heterogeneous compositions of the SMCs. It can be hypothesized that SMCs A, C, D, H, and J contain higher proportions of Gram-positive bacteria, which are more susceptible to LAE and nisin compared to Gram-negative bacteria, as explained hereafter. This susceptibility difference is attributed to the mechanisms of action of these compounds.

LAE is a cationic surfactant that binds to anionic microbial proteins on cell membranes or enzymatic systems, denaturing them and increasing membrane permeability (Dong et al. 2023; Muriel-Galet et al. 2015). It may also affect bacterial DNA conformation (Ma et al. 2020), altering metabolic processes and causing cell death (Haghighi et al. 2019). Although LAE has broad-spectrum activity against Gram-positive and Gram-negative bacteria, yeasts, and molds (Loeffler et al. 2020), Gram-negative bacteria exhibit greater resistance. This is due to their outer lipopolysaccharide layer, which prevents LAE from reaching the cell wall (Gracia-Vallés et al. 2022; Higueras et al. 2013; Wu et al. 2021).

Similarly, nisin binds to lipid II, a precursor for bacterial cell wall biosynthesis on the cytoplasmic membrane of Gram-positive bacteria, but it is inaccessible in Gram-negative bacteria (Bahrami et al. 2019). Additionally, nisin can infiltrate the bacterial membrane, creating pores, altering permeability, causing the loss of low-molecular-weight compounds and ions, and ultimately killing the bacteria (Chai et al. 2015). While this pattern of susceptibility is well-documented for LAE and nisin, it is important to note that antimicrobial susceptibility varies significantly among different microbial groups and compounds.

The varied inhibitions observed in this study are likely due to

Table 1
Inhibition diameters (mm) of LAE (a) and nisin (b) against ten spoilage mixed cultures (A to J). Data are presented as means and standard deviations.

a)	LAE (%)					
	0	0.1	0.5	1	2	5
A	0.10 ± 0.04 ^{bD}	4.99 ± 0.63 ^{aC}	7.34 ± 0.47 ^{aB}	8.54 ± 0.15 ^{aA}	8.76 ± 0.37 ^{aA}	9.42 ± 0.37 ^{aA}
B	0.10 ± 0.06 ^{bD}	1.16 ± 0.50 ^{deCD}	1.52 ± 0.43 ^{efBC}	1.92 ± 0.51 ^{efABC}	2.47 ± 0.13 ^{cdeAB}	3.05 ± 0.71 ^{cdeA}
C	0.15 ± 0.05 ^{abB}	1.47 ± 0.30 ^{cdB}	3.10 ± 0.73 ^{cdA}	3.34 ± 0.82 ^{cdA}	3.23 ± 0.54 ^{cdA}	3.83 ± 0.13 ^{cdA}
D	0.28 ± 0.12 ^{aC}	2.90 ± 0.63 ^{bB}	5.34 ± 0.44 ^{bA}	5.16 ± 0.76 ^{bA}	5.70 ± 0.93 ^{bA}	6.59 ± 0.34 ^{bA}
E	0.18 ± 0.03 ^{abC}	0.27 ± 0.10 ^{eC}	0.94 ± 0.55 ^{fC}	1.28 ± 0.19 ^{fBC}	2.38 ± 0.69 ^{deAB}	2.74 ± 0.43 ^{deA}
F	0.18 ± 0.05 ^{abD}	0.48 ± 0.28 ^{deCD}	1.01 ± 0.08 ^{fBC}	1.33 ± 0.17 ^{fB}	1.56 ± 0.19 ^{eB}	2.30 ± 0.30 ^{eA}
G	0.15 ± 0.04 ^{abD}	0.47 ± 0.24 ^{deD}	0.66 ± 0.11 ^{fCD}	1.36 ± 0.44 ^{fBC}	1.67 ± 0.28 ^{eAB}	2.13 ± 0.25 ^{eA}
H	0.12 ± 0.03 ^{abD}	1.28 ± 0.20 ^{cdeC}	2.47 ± 0.48 ^{deB}	2.79 ± 0.36 ^{deAB}	3.32 ± 0.59 ^{cAB}	3.91 ± 0.54 ^{cA}
I	0.12 ± 0.05 ^{abD}	0.51 ± 0.04 ^{deCD}	0.92 ± 0.12 ^{fC}	1.68 ± 0.13 ^{efB}	1.88 ± 0.24 ^{cdeB}	2.96 ± 0.25 ^{cdeA}
J	0.13 ± 0.02 ^{abD}	2.33 ± 0.55 ^{bcC}	4.00 ± 0.24 ^{cB}	4.35 ± 0.49 ^{bcB}	5.09 ± 0.63 ^{bAB}	5.90 ± 0.37 ^{bA}

b)	Nisin (%)						
	0	0.01	0.02	0.05	0.075	0.1	0.125
A	0.10 ± 0.06 ^{aC}	0.68 ± 0.35 ^{aBC}	0.73 ± 0.30 ^{aABC}	1.11 ± 0.37 ^{abAB}	1.05 ± 0.10 ^{abAB}	1.24 ± 0.22 ^{bAB}	1.50 ± 0.39 ^{abA}
C	0.15 ± 0.03 ^{aC}	0.49 ± 0.15 ^{aBC}	0.67 ± 0.28 ^{aABC}	0.71 ± 0.34 ^{bABC}	0.89 ± 0.09 ^{bAB}	0.98 ± 0.29 ^{bAB}	1.17 ± 0.24 ^{bA}
D	0.16 ± 0.05 ^{aC}	0.44 ± 0.28 ^{aBC}	0.48 ± 0.17 ^{aBC}	0.83 ± 0.22 ^{bABC}	0.97 ± 0.07 ^{bAB}	1.14 ± 0.16 ^{bAB}	1.29 ± 0.50 ^{bA}
H	0.13 ± 0.05 ^{aE}	0.39 ± 0.10 ^{aDE}	0.63 ± 0.37 ^{aCDE}	1.10 ± 0.09 ^{abCD}	1.32 ± 0.61 ^{abBC}	2.03 ± 0.07 ^{aAB}	2.35 ± 0.07 ^{aA}
J	0.08 ± 0.05 ^{aC}	0.45 ± 0.16 ^{aC}	0.56 ± 0.10 ^{aC}	1.54 ± 0.14 ^{aB}	1.79 ± 0.14 ^{aAB}	2.15 ± 0.36 ^{aAB}	2.40 ± 0.42 ^{aA}

Different lowercase letters within a column indicate significant differences among spoilage mixed cultures (SMCs) treated with the same antimicrobial concentration. Different uppercase letters within a row indicate significant differences in inhibition diameter for a specific SMC as the antimicrobial concentration increases.

different actions towards microbial groups and the heterogeneous microbial composition, resulting from environmental contamination from various households. The lack of inhibition by nisin and the reduced effectiveness of LAE against SMCs B, E, F, G, and I suggest a predominance of Gram-negative bacteria in these SMCs. Gram staining of colonies from some SMCs confirmed this, showing that SMC A was mainly (78 %) Gram-positive, while SMC I was predominantly (81%) composed of Gram-negative bacteria. Although the exact composition of the SMCs and the precise antimicrobial mechanisms were not studied, this test effectively identified the best-performing antimicrobial agent against the SMCs. These findings support the selection of LAE for developing active films targeting various spoilage microorganisms.

Due to its narrow action spectrum, nisin's activity is often enhanced by combining it with other antimicrobial agents or treatments (Hassan and Cutter 2020; Sun et al. 2019), including organic acids (Pintado et al. 2009), sodium lactate and benzoate (Neetoo and Mahomoodally 2014), potassium sorbate (Jin et al. 2010; Neetoo and Mahomoodally 2014), other bacteriocins (Scaffaro et al. 2011), the chelating agent EDTA (Bhatia and Bharti 2015; Jin et al. 2010), or in combination with high hydrostatic pressure (Jofré et al. 2008). The low antimicrobial efficacy of nisin may also be attributed to the low concentrations tested. The EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) has proposed the use of nisin up to a maximum of 25 mg/kg of food (or 25 ppm) (EFSA ANS Panel, 2017), which is double the amount currently approved under Reg. (EC) No 1333/2008. In contrast, the maximum permitted amount for LAE is 160 mg/kg of food (or 160 ppm), allowing for a higher antimicrobial effect.

3.3. Stability of active compounds following exposure to solvents

Besides the assessment of the effectiveness of LAE and nisin, the study also evaluated their activity after contact with solvents, considering their proposed application in active films produced by solvent casting. The results of the disk diffusion test of LAE and nisin solutions against *L. plantarum* are shown in Fig. 1.

A slight but significant reduction in antimicrobial activity was observed for both nisin and LAE after exposure to chloroform, a solvent widely used in solvent casting for active packaging development, as reported for LAE (Kayaci et al. 2013; Li et al. 2021) and nisin (Bastarrachea et al. 2010). However, exploring a new solvent, such as formic acid, became necessary due to production challenges with chloroform (explained in Section 3.5).

After contact with formic acid, LAE entirely maintained its effectiveness, whereas nisin completely lost its antimicrobial activity. Nisin has been solubilized in formic acid solutions at low acid concentrations (Ajingi et al. 2020; Prioult et al. 2000), while the solubilization in pure formic acid at high temperature (70 °C) as in our study might have led to nisin denaturation, causing a loss of antimicrobial capacity. Lower temperatures were found to be unsuitable due to insufficient PHBV solubilization.

Based on these findings, only LAE was selected for further testing and inclusion into active films via solvent casting.

3.4. Minimum bactericidal concentration (MBC) of LAE

The antimicrobial effectiveness of the LAE formulation was evaluated by determining its MBC against *Pseudomonas* sp. and *L. monocytogenes*, as the MIC assay could not be performed (see Section 2.5). The MBC is defined as the lowest antimicrobial concentration causing at least a 3-log reduction in viable cells. Fig. 2 illustrates the reduction in viable cells depending on LAE concentrations. In broth medium, the MBC of LAE was 125 ppm against *Pseudomonas* sp. and 50 ppm against *L. monocytogenes*.

The MBC for *L. monocytogenes* was slightly higher than the values reported in the literature, ranging from 12 to 32 ppm (Gracia-Vallés et al. 2022; Higueras et al. 2013; Muriel-Galet et al. 2012). These differences may arise from differences in LAE formulations used. This study employed Mirenat®-GC, containing 20% LAE and 80% glycerol, which could potentially provide additional nutrients to the bacteria. In

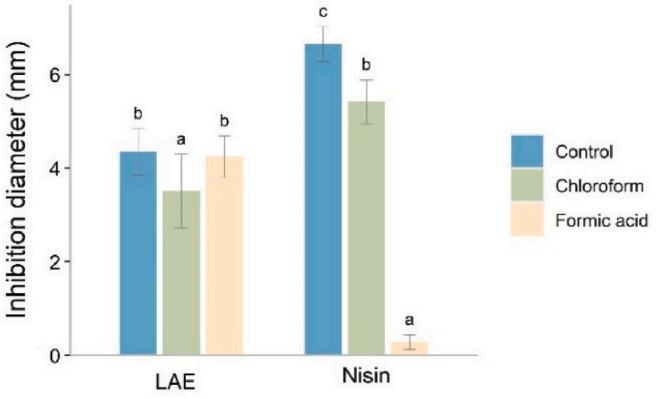

Fig. 1. Antimicrobial effectiveness of LAE and nisin against *L. plantarum* before (control) and after treatment with chloroform and formic acid.

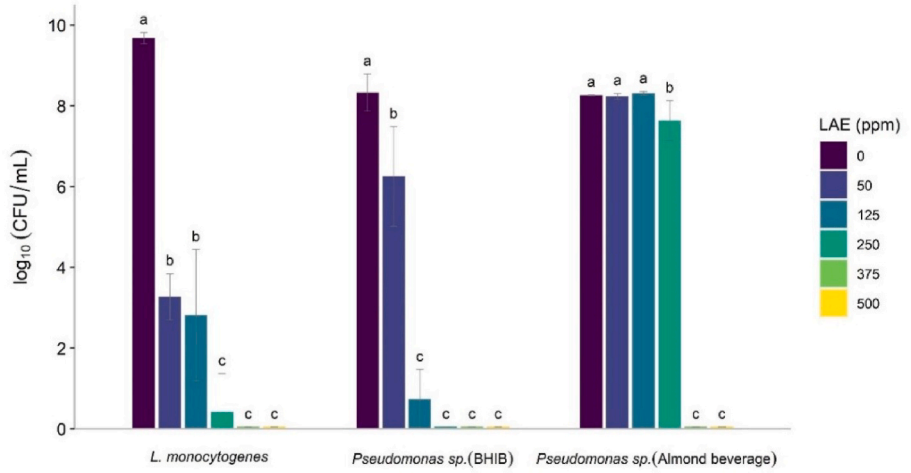

Fig. 2. Viable count of *L. monocytogenes* and *Pseudomonas* sp. in broth medium, and of *Pseudomonas* sp. in Almond beverage, with various concentrations of LAE.

contrast, prior studies used formulations such as 20% LAE dissolved in 80% ethanol (Gracia-Vallés et al. 2022) or a more concentrated LAE (69.3%) in 30.7% maltodextrins (Higueras et al. 2013). Other key factors influencing the MBC are the different methods and culture media used. Gracia-Vallés et al. (2022) used Mueller-Hinton-F (MH-F) broth, while Higueras et al. (2013) and Muriel-Galet et al. (2012) employed Tryptone Soy Broth (TSB), resulting in differences in nutrient composition and bacterial growth conditions.

The MBC of LAE for *Pseudomonas* sp. aligns with reports for *P. aeruginosa*, where an MBC of 100 ppm was observed, although the specific LAE formulation was not stated (Becerril et al. 2013). However, other researchers reported lower MBC and MIC values against *P. putida*, *P. fluorescens*, and *P. aeruginosa*, ranging from 15 to 32 ppm, using different LAE formulations, such as 14.5% LAE dissolved in 85.5% maltodextrins (Loeffler et al. 2020; Nübling et al. 2017), or 69.3% LAE in 30.7% maltodextrins (Higueras et al. 2013). The methods and culture media used also varied among studies, which employed media such as nutrient broth, MH, or TSB. The different bacterial growth can therefore affect the determination of MBC. These results underline the need for standardized methodologies in antimicrobial testing to ensure consistency and comparability among studies.

Overall, these results are consistent with previous findings, confirming that the MBC of LAE is higher against Gram-negative bacteria than against Gram-positive bacteria (Hassan and Cutter 2020; Higueras et al. 2013; Muriel-Galet et al. 2012).

3.5. Films formulation

Fig. 3 shows the PHBV films produced by solvent casting. The films solubilized in chloroform had a rougher and less uniform surface (Fig. 3a), likely due to the fast evaporation of chloroform occurring at room temperature which led to discontinuities and to an uneven surface. On the other hand, formic acid enabled the formation of a flat and smooth film, as shown in Fig. 3b. Unlike chloroform, formic acid is authorized for use as a polymer production aid in the Food Contact Material Regulation (Reg. (EU) No 10/2011). Therefore, formic acid represents a promising green alternative to chloroform, which is highly hazardous and toxic to human health (Prat et al. 2014), nonetheless it is widely used in published research on cast films. For these reasons, and based on the results from Section 3.3, this solvent was selected for active films production, as described in Section 2.6.

The measurement of samples thickness revealed no significant differences ($p = 0.064$) between the neat PHBV (Fig. 3c) and active PHBV-LAE films (Fig. 3d), with mean values of 35.58 ± 6.28 μm and 42.45 ± 7.36 μm, respectively.

3.6. Antimicrobial activity of PHBV-LAE films in liquid medium

The effectiveness of the PHBV-LAE films was evaluated in broth medium against *Pseudomonas* sp. and *L. monocytogenes*. The BHI broth with films and without inoculum (negative control) were poured on PCA plates and showed undetectable microbial growth (<1.0 $\log_{10}$ CFU/mL) after 20 h at 30 °C, demonstrating that neither the control nor the active film had accidentally contaminated the medium. Inoculation was performed immediately after immersion of the active films in the broth (day 0) and after 1, 3, and 7 days of immersion. The starting inoculum, quantified before incubation, was 4.3 ± 0.1 $\log_{10}$ CFU/mL in all samples. Fig. 4 shows the antimicrobial effectiveness of the active PHBV-LAE films against *L. monocytogenes* (Fig. 4a) and *Pseudomonas* sp. (Fig. 4b) in BHI broth.

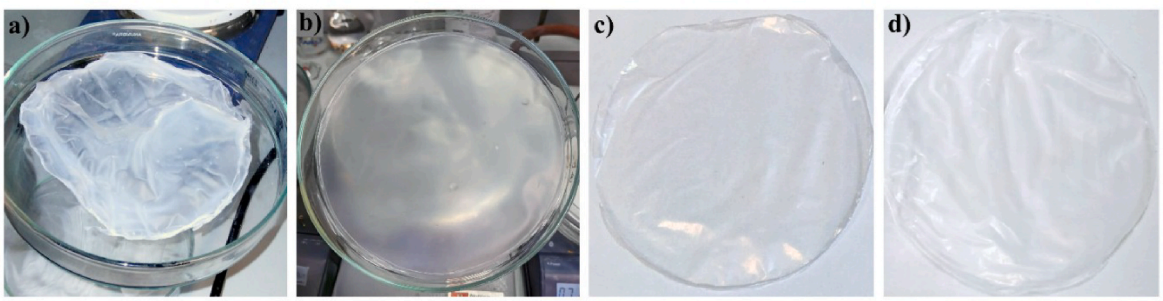

Fig. 3. Pictures of neat PHBV films produced by solvent casting, using chloroform (a) or formic acid (b). Once formic acid was selected as the best solvent, the neat PHBV (c) and PHBV-LAE with 5% LAE (d) were produced.

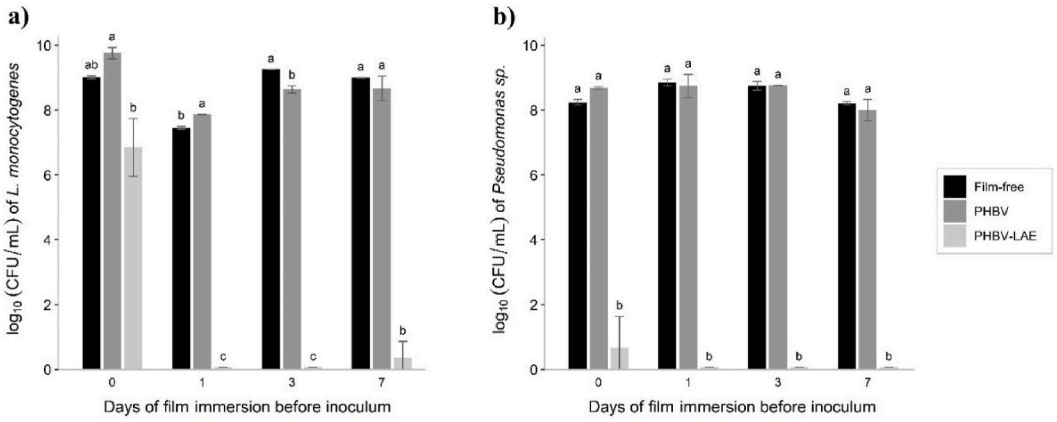

Fig. 4. Antimicrobial activity of PHBV-LAE films containing 5% LAE in BHI broth against *L. monocytogenes* (a) and *Pseudomonas* sp. (b) compared to the control PHBV films and the film-free inoculated BHI broth. Inoculum was performed after 0, 1, 3, and 7 days of immersion of the films into the medium.

In all samples, the film-free inoculated broth (positive control) and the control PHBV showed growth of both bacteria between 7.5 and 9.8 $\log_{10}$ CFU/mL after 16 h of incubation at 30 °C. Regarding L. *monocytogenes*, a reduction by 2.6 $\log_{10}$ CFU/mL was achieved when inoculation was performed at day 0. The antimicrobial effect of active films significantly increased when the films were immersed one day before inoculation, reaching total inhibition which continued in the following days. This behavior indicates that the antimicrobial compound released after one day was sufficient for complete inhibition, as it will be further explained at the end of this section. Similar inhibition of *L. monocytogenes* was previously reported by Kashiri et al. (2016), who achieved a 2.6 log reduction when zein films with 5% LAE where added to liquid medium. Other studies reported lower inhibition, approximately 0.5 log reduction of *L. monocytogenes* in liquid medium with chitosan films containing 20% LAE (w/w of polymer) (Ma et al. 2016). Slightly higher inhibition of *L. monocytogenes* were obtained by other authors, achieving approximately a 7-log reduction with PGGA-LAE active films (Gamarra-Montes et al. 2018), POM films with 5% LAE (Gracia-Vallés et al. 2022), and EVOH films with 5% LAE (Muriel-Galet et al. 2012).

The inhibitory effect of the active PHBV-LAE films against *Pseudomonas* sp. isolated from almond drink revealed a 7.6 log reduction at day 0 and total inhibition at day 1. Similar inhibition was reported in the literature against *P. putida* (Manso et al. 2021), where more than a 3-log reduction was obtained with polystyrene pads sprayed with LAE, and total inhibition was observed with pads prepared by total immersion in LAE solution. Other studies reported that chitosan films with 5% LAE in liquid medium exhibited a 2.6 log reduction of *P. putida* (Higueras et al. 2013).

Despite *Pseudomonas* being considered resistant to hydrophobic antimicrobials, the surfactant nature of LAE and the presence of hydrophilic groups may lead to a higher affinity for lipopolysaccharides in the Gram-negative's cell wall. This results in easier penetration of the cell wall and depolarization of the cytoplasmic membrane of *Pseudomonas*, making LAE a broad-spectrum active compound capable of inhibiting both foodborne spoilage and pathogen microorganisms (Zhuang et al. 2020). Additionally, previous research has shown that LAE, at concentrations as low as 10, 50 or 100 μM, can chelate iron, creating iron-limiting conditions that inhibit biofilm development in *P. aeruginosa*, which requires iron for growth (Kim et al. 2017).

To further explain the differences in inhibition observed after day 0 and day 1, it is useful to reference our study on the release kinetics of LAE from PHBV films, which studied the release behavior across various food simulants and at three different temperatures (Nicosia and Licciardello 2025). In compliance with European Commission Regulation (EU) No 10/2011, we employed food simulants, including simulant A (10% ethanol), which simulates hydrophilic foods with pH > 4.5 and has an intended use most closely aligned with the BHI broth used in this study. Since food simulants generally have higher extractive power than real foods or water, the release of LAE in BHI broth is expected to be slower than in simulant A. Therefore, simulant A provides an estimate for explaining the observed antimicrobial effects in this study. Release data for simulant A showed that the release rate of LAE is quite slow, even at high temperatures like 30 °C. For instance, only 30 ppm of LAE were released after 2 h, increasing to 40 ppm after 3 h. As discussed earlier in Section 3.4, these amounts are not sufficient to inhibit microbial growth, but they can help reduce it, particularly when initial contamination is very low, such as in the case of recontamination of UHT product after opening. In the initial hours, when only small amounts of antimicrobial are available, microorganisms are likely to follow their typical growth curve, often entering the exponential growth phase under favorable conditions (Greenwood 1985; Rudilla et al. 2018). Depending on the microorganism and antimicrobial agent, the lag phase may be slightly prolonged before the exponential phase begins (Frenkel et al. 2021; Sterniša et al. 2022). During this phase, the low antimicrobial concentration may minimally influence the growth kinetics, allowing microorganisms to grow with limited inhibition. On the other hand, the release study revealed that after 24 h, over 100 ppm of LAE had been released (Nicosia and Licciardello 2025), resulting in stronger inhibition of microorganisms' growth. This aligns with our MBC results, where 50 and 125 ppm of LAE were sufficient for complete bacterial inhibition (Fig. 2).

3.7. Antimicrobial activity of PHBV-LAE films in a real food system

3.7.1. Minimum bactericidal concentration (MBC) of LAE in almond beverage

The MBC test was performed against *Pseudomonas* sp., using almond beverage as the medium. The reduction of viable cells with the addition of LAE is shown in Fig. 2. Results highlighted an MBC of LAE against *Pseudomonas* sp. of 375 ppm, which corresponds to 3-fold the MBC observed in BHI broth (125 ppm), thus suggesting that the effectiveness of LAE is reduced in a real food system. Similar findings were reported in 2 % fat milk, likely due to the affinity of LAE for the lipid phase, leading to a reduction of LAE molecules in contact with the bacteria in the aqueous phase (Ma et al. 2013).

3.7.2. Antimicrobial activity of active PHBV-LAE films in almond beverage

In the almond beverage, active films caused significant microbial reductions of 2.4 and 2.6 log compared to control PHBV films at day 0 and day 1, respectively (Fig. 5). However, this effect diminished over time, with no antimicrobial efficacy observed after 3 and 7 days of film

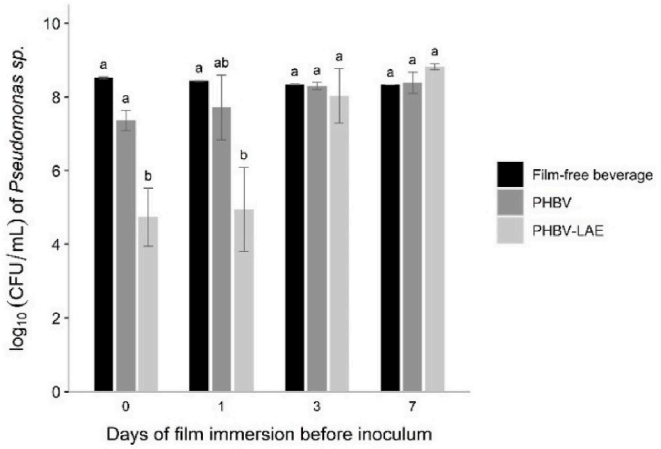

Fig. 5. Antimicrobial activity of PHBV-LAE films containing 5% LAE in almond beverage inoculated with *Pseudomonas* sp. compared to the control PHBV films and the film-free beverage. Inoculation was performed after 0, 1, 3, and 7 days of immersion of the films into the medium.

immersion, suggesting that LAE was inactivated by interacting with the matrix, allowing microorganisms to grow with no obstacles after inoculation. As expected, the antimicrobial effectiveness of active films observed after 0 and 1 day of film immersion was lower in almond beverage compared to BHI broth. This reduced microbial inhibition can be attributed to interactions between LAE and food components such as fats, polysaccharides, and proteins, which may hinder its activity (Ma et al. 2020). This is further supported by the significantly higher MBC we observed for LAE in almond beverage compared to broth medium, aligning with previous studies reporting reduced LAE efficacy in food systems compared to *in vitro* assays (Ma et al. 2013). Food matrices provide greater nutrient availability compared to broth media, which can enhance microorganisms' ability to repair cellular damage and maintain homeostasis (Ma et al. 2023). Additionally, in complex food matrices, LAE may form electrostatic interactions with charged molecules, such as polysaccharides and proteins, leading to the formation of inactive complexes (Loeffler et al. 2020). Non-charged components like starch, despite not interacting with LAE, can increase food viscosity, limiting LAE's access to microorganisms (Ma et al. 2013), while fats can reduce microbial sensitivity to antimicrobials, offering protection from LAE's action. The amphiphilic structure of LAE causes its adsorption at the water-fat interface or the formation of LAE micelles in the aqueous phase, reducing the availability of LAE to target microorganisms, which are typically in the water phase (Magrinyà et al. 2015). In our specific case, the almond beverage employed contains small amounts of carbohydrates (1.7%) and proteins (0.4%), along with emulsifiers (mono- and diglycerides of fatty acids) and stabilizers (gellan gum and xanthan gum). These components, while present in low concentrations, may facilitate micelle formation or slightly increase food viscosity, potentially limiting LAE's activity.

It is worth noting that under typical household conditions, contamination levels upon product opening are generally much lower, often just a few cells/mL. Additionally, the incubation temperature of 30 °C used in this study simulates a worst-case scenario, representing food exposed to high temperatures rather than being properly refrigerated. While higher temperatures, like 30 °C, can promote faster LAE release, which may accelerate the antimicrobial effect, refrigeration temperatures are expected to slow both microbial growth and LAE release. Our previous study demonstrated that in simulant A, there was a significant difference in release rates at 6 °C and 30 °C, however, no significant differences were observed in the amount of LAE released at equilibrium at these temperatures (Nicosia and Licciardello 2025). This study highlights performance under conditions that promote microbial growth and spoilage, such as improper handling or accidental storage at room temperature, while under typical household refrigeration conditions microbial contamination is usually lower, and the antimicrobial effect is sustained over time.

4. Conclusions

This study introduces a novel approach for evaluating and selecting antimicrobials to target environmental spoilage microflora commonly found in domestic environments, offering useful insights for the design of active films intended to reduce food waste.

Among the two compounds tested, LAE emerged as a suitable candidate for active packaging due to its broad-spectrum antimicrobial activity. Unlike prior studies that focused on individual microbial strains, this work extends the understanding of LAE's potential by evaluating its efficacy against spoilage mixed cultures. The active PHBV-LAE films demonstrated significant antimicrobial efficacy, reaching a 2.6-log reduction of *Listeria monocytogenes* and a 7.6-log reduction of *Pseudomonas* sp. when inoculated at day 0. For both bacteria, when the films were immersed one day before inoculation, they achieved total inhibition of microbial growth, which persisted in the following days. This suggests that after one day, the antimicrobial release was sufficient for complete inhibition, which is consistent with previous release kinetic studies.

This study represents the first application of a PHBV-LAE active packaging system for preserving a milk alternative, specifically almond beverage. Although the antimicrobial effect was reduced in almond beverage compared to the broth medium, the active films still achieved significant microbial growth reduction, with a 2.6-log reduction of *Pseudomonas* sp. after 24 h at 30 °C. These findings demonstrate the versatility of PHBV-LAE films to new food categories.

The developed PHBV-LAE active film demonstrates significant potential to reduce microbial spoilage in both broth medium and almond beverage as a model food system. Providing the packaging material with broad-spectrum, tunable antimicrobial activity, offers great potential for delaying spoilage of shelf-stable products (e.g. UHT beverages) after package opening in households, thus extending the secondary shelf life. Despite the use of solvent casting, which may not fully reflect industrial-scale production processes, these promising findings pave the way for future research, including testing actual packaging prototypes under real-world conditions, such as refrigeration, to validate their effectiveness in extending food shelf life and reducing domestic food waste.

CRediT authorship contribution statement

Carola Nicosia: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation. **Andrea Pulvirenti:** Supervision, Investigation, Funding acquisition. **Fabio Licciardello:** Writing – review & editing, Supervision, Resources, Investigation, Funding acquisition, Conceptualization.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of competing interest

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

Acknowledgement

The authors wish to thank Rebecca Secchi for her valuable contribution in laboratory experiments.

Data availability

Data will be made available on request.

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