International Journal of Food Microbiology 456 (2026) 111787

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Antimicrobial activity of a Tunisian ethanolic extract of propolis in ricotta cheese: *In vitro* assessment and *in situ* application

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ARTICLE INFO

Keywords:
Propolis
Listeria monocytogenes
Phenolic compounds
Dairy products
Shelf-life
Food safety

ABSTRACT

This study evaluated the antimicrobial potential of a Tunisian ethanolic extract of propolis in sweetened ricotta cheese, a perishable dairy product. The extract was chemically characterized by HPLC–PDA–MS and its antimicrobial activity was assessed through *in vitro* assays and *in situ* application. Chemical analysis revealed a high total phenolic content (7541.7 ± 117.2 mg/L) and 23 phenolic compounds, with isorhamnetin, pinobanksin-propionate, and 4-*O*-methyl-epigallocatechin as the most abundant. *In vitro* activity was evaluated against 15 bacterial strains using agar disc diffusion and broth microdilution assays to determine minimum inhibitory (MIC) and bactericidal (MBC) concentrations. The extract showed antibacterial activity against Gram-positive bacteria, while no inhibitory effects were observed against Gram-negative strains. MIC values ranged from 0.25 to 1.25 mg/mL and MBC values from 0.625 to 12.5 mg/mL, indicating predominantly bactericidal activity. *In situ* efficacy was assessed through a 14-day shelf-life study and a *Listeria monocytogenes* challenge test on industrial and artisanal sweetened ricotta supplemented with 0.1% and 1% extract. Shelf-life extension was limited, whereas a dose-dependent inhibition of *L. monocytogenes* was observed. Exploratory sensory evaluation revealed moderate changes. Overall, Tunisian propolis extract appears suitable for targeted control of L. *monocytogenes* rather than broad-spectrum preservation in ricotta cheese.

1. Introduction

The term propolis refers to the resinous material that honeybees collect from the buds, bark, and exudates of various plants and then mix with wax and salivary enzymes. This process yields a sticky, malleable substance that bees use to seal gaps in the comb, smooth and reinforce internal surfaces, and protect the hive entrance from intruders and environmental threats (Santos et al., 2020a, 2020b). Its composition varies according to botanical and geographical origin but generally consists of plant resins (45–50%), waxes (25–30%), essential oils (~10%), fatty acids, pollen, and a diverse fraction of organic compounds (~10%) (Irigoiti et al., 2021). This latter fraction includes a wide range of bioactive molecules such as polyphenols (flavonoids, phenolic acids, and esters), aromatic esters, and terpenoids, which underlie the numerous documented biological properties of propolis, including antibacterial, antioxidant, antiviral, anti-inflammatory, immunomodulatory, anticancer and antifungal activities (Pobiega et al., 2019a). These biological effects are increasingly exploited in various medical fields and natural remedies against bronchial asthma, gastric disorders, and wound healing (Bhatti et al., 2024; Siheri et al., 2025).

Propolis was also proposed as a natural preservative in different food productions according to the growing demand for natural ingredients increasingly appreciated by consumers as alternatives to chemical additives (El-Sakhawy et al., 2024; Segueni et al., 2023). Its antimicrobial activity against a wide range of pathogenic and spoilage microorganisms has been applied to ensure food safety and extend food shelf-life (Pobiega et al., 2019c; Nefzi et al., 2023). Propolis in food was used in different formulations with ethanolic extracts being among the most

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https://doi.org/10.1016/j.ijfoodmicro.2026.111787
Received 30 December 2025; Received in revised form 10 April 2026; Accepted 10 April 2026
Available online 13 April 2026

common due to ethanol's efficiency in extracting biologically active compounds (Pobiega et al., 2019b; Šuran et al., 2021). However, use of ethanolic extract of propolis (EEP) in foods presents notable technological challenges due to its low solubility in water and distinctive color, taste and odor which generally affect the sensorial characteristics of foods and may not be appreciated by consumers (Kara et al., 2022). Moreover, the presence of alcohol in EEP could limit its use for health and religious reasons.

Several studies have documented the potential use of EEP as an antimicrobial in foods of animal origin, such as fish, meat and honey, but few references are available concerning its application in dairy products (Chon et al., 2020; Gunes-Bayir et al., 2022; Pobiega et al., 2019a). Dairy products are particularly suitable for the incorporation of natural preservative substances, including spices and flavorings, in order to improve sensory qualities, extend shelf life, and prevent the growth of food-borne pathogens (Soutelino et al., 2024).

In this context, ricotta cheese, often flavored with spices such as cinnamon or pepper, or with aromatic herbs, represents an ideal candidate to evaluate the incorporation of propolis. This is particularly true for its sweetened variant, a traditional gastronomic product widely consumed in southern Italy, where the addition of aromatic ingredients is customary and contribute to its distinctive sensory profile (Middione, 2022).

The aim of the present study is therefore to evaluate the antimicrobial activity of a Tunisian EEP on sweetened ricotta cheese. Specifically, the antimicrobial efficacy was assessed *in vitro* and subsequently *in situ* using sweetened ricotta cheese as the food model to determine its shelf-life extension potential and activity against *Listeria monocytogenes*.

2. Materials and methods

2.1. Sampling of propolis and extract preparation

Samples of crude propolis from *Apis mellifera* hives were collected in September 2023 in the town of Tebourba, Tunisia (36°49′46″ N - 9°50′28″ E). Harvesting was performed by scraping the inner surfaces of the hives with the aid of a steel spatula.

Once collected, propolis was placed in sterile opaque plastic containers and transported under refrigeration to the Department of Veterinary Sciences, University of Messina (Messina, Italy).

The extraction protocol used was inspired by the one proposed by Mello and Hubinger (2012). Specifically, crude propolis was ground in a bench blender and an 80% (*v/v*) ethanol–water solution (Sigma Aldrich, St. Louis, MO, USA) was used for propolis extraction. The ethanolic extract was prepared in a ratio of 20% propolis and 80% solvent (*w/v*). The resulting mixture was sonicated in an ultrasonic bath (Sonorex RK100H, BANDELIN electronic GmbH & Co. KG, Berlin, Germany) at 40 °C for 30 min, then stored at room temperature in a sterile opaque plastic container for one week, stirring the container manually once a day. Subsequently, the mixture was centrifuged at 8800 rpm for 20 min, and the supernatant was filtered through Whatman No. 1 filter paper (Sigma Aldrich, St. Louis, MO, USA). The filtrate was kept under refrigeration for 3 h and filtered again for wax removal. The resulting EEP was concentrated by a rotary vacuum evaporator (Heidolph vv2000, Profcontrol GmbH, Schönwalde-Glien, Germany) at 45 °C and stored in the dark in a glass bottle at room temperature until the time of analysis. Prior to testing, the extract was re-dissolved in the 80% (*v/v*) ethanol–water solution to accurately obtain the concentrations desired in the assays.

2.2. Phenolic compounds characterization

Phenolic compounds identification and quantification were performed by means of high performance liquid chromatography (HPLC) coupled to a photodiode array detector (PDA) and a single quadrupole mass spectrometer (MS). HPLC-PDA-MS analyses were carried out on a Shimadzu Nexera X2 instrument serially coupled to a SPD-M30A detector and an LCMS-2020 mass spectrometer (Shimadzu, Duisburg, Germany). All the materials employed for chemical analyses were purchased from Merck KGaA (Darmstadt, Germany). The analytical conditions were the same previously employed by Russo et al. (2014). Briefly, the chromatographic separation was achieved on an Ascentis Express C18 column (150 × 4.6 mm, 2.7 μm) using water/ formic acid (99.9:0.1, *v/v*) (solvent A) and acetonitrile/formic acid (99.9:0.1, *v/v*) (solvent B) as mobile phases. The gradient elution program was as follows: 0 min, 5% B, 40 min, 25% B,60 min,100% B,70 min, 100% B, 73 min, 5%, 80 min 5% B. The flow rate and the injection volume were 0.7 mL/min and 2 μL, respectively.

PDA detection was performed in the range of 190–400 nm with a sampling frequency of 1.5625 Hz and a time constant of 0.64 s. Chromatograms were extracted at 280 and 325 nm.

MS acquisition was performed in the mass range 100–700 *m/z* using both an electrospray ionization source (ESI), in negative mode, and an atmospheric pressure chemical ionization source (APCI), in positive mode. ESI conditions were as follow: interval, 0.5 s; scan speed, 938 amu/s; N2 flow, 1.5 L/min; interface temperature, 350 °C; heat block, 300 °C; DL (desolvation line) temperature, 300 °C; DL voltage–34 V; probe voltage, +4.5 kV; Qarray voltage, 1.0 V and detection gain, 1.05 kV. APCI conditions were as follow: interval, 0.5 s; scan speed, 1500 amu/s; N2 flow, 4.0 L/min; interface temperature, 350 °C; heat block, 300 °C; DL temperature, 300 °C; DL voltage,–34 V; probe voltage, +4.5 kV; Qarray voltage, 1.0 V, detection gain, 1.05 kV.

Data acquisition and processing were conducted using the LabSolution ver. 5.95 software (Shimadzu, Duisburg, Germany).

Phenolic compounds were quantified by means of external calibration curves in PDA of the following standard compound: naringin, p-coumaric acid, rutin, hesperedin, and ferulic acid following a previously validated procedure (Russo et al., 2014; Russo et al., 2016).

2.3. In vitro antimicrobial activity

2.3.1. Preparation of bacterial strains

The antimicrobial activity of the EEP was tested against fifteen Gram-positive and Gram-negative bacterial strains (Table 1).

The strains were stored at −80 °C in cryovials containing a solution of brain heart infusion broth (Biolife, Milan, Italy) + 15% glycerol (Carlo Erba Reagents S.r.l., Milan, Italy).

After thawing at refrigeration, an aliquot of each bacterium was collected from the cryovial using a 10 μL sterile loop and plated in

Table 1
Bacterial strains used for the *in vitro* disc diffusion assay to evaluate the antibacterial activity of Tunisian ethanolic extract of propolis.

	Strains	
	ATCC	EURL
Gram-positive	*Listeria innocua* 33090	
	Listeria ivanovii 19119	
	Listeria monocytogenes 19112	*Listeria monocytogenes* 12MOB079LM
	Listeria monocytogenes 7644	*Listeria monocytogenes* 12MOB096LM
	Listeria monocytogenes 19111	*Listeria monocytogenes* 12MOB098LM
	Staphylococcus aureus 25923	
	Staphylococcus aureus 6533	
Gram-negative	*Escherichia coli* 25922	
	Escherichia coli 35218	
	Pseudomonas fluorescens 13525	–
	Salmonella Enteridis 13076	
	Salmonella Typhimurium 14028	

–. Not applicable.

Tryptone Soy Agar (TSA; Biolife, Milan, Italy) and incubated overnight at 37 °C for 24 h.

2.3.2. Agar-based disc diffusion assay

The antibacterial activity of EEP was preliminarily screened using the agar disc diffusion method, as described by Nefzi et al. (2023).

From the overnight TSA culture of each strain, morphologically similar colonies were picked with a sterile loop and suspended in a peptone water solution (PWS; Biolife, Milan, Italy) to achieve a final turbidity equivalent to 0.5 McFarland (~10^8 CFU/mL). A spectrophotometer (Biosigma, Cona, Italy), previously calibrated against a 0.5 McFarland turbidity standard, was used to adjust the density of the suspensions by adding PWS or more bacteria. Suspensions were used immediately after preparation and never beyond 15 min.

Müller–Hinton agar plates (MH; Biolife, Milan, Italy) were inoculated with 500 µL of each bacterial suspension. Sterile cellulose discs (6 mm diameter; Biolife, Milan, Italy) were impregnated with 10 µL of EEP at different concentrations (1000 mg/mL, 250 mg/mL and 125 mg/mL) and placed on the agar surface. Plates were incubated at 37 °C for 24 h (30 °C for *P. fluorescens* ATCC 13525), and the inhibition zones were measured using a digital caliper.

Each concentration was tested in three independent replicates using an 80% ethanol-water solution as the negative control to exclude any antimicrobial effect of the solvent.

2.3.3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC and MBC of the EEP were determined only against the bacterial strains found to be sensitive in the disc diffusion assay. The MIC was evaluated by the broth microdilution method according to Kowalska-Krochmal and Dudek-Wicher (2021).

A sterile 96-well microplate (Biosigma, Cona, Italy) was filled with serial twofold dilutions of EEP in Tryptic Soy Broth (TSB; Biolife, Milan, Italy), resulting in final concentrations of 25, 12.5, 6.25, 5, 2.5, 1.25, 0.25, 0.625, 0.125, 0.0625 and 0.0125 mg/mL in a final volume of 200 µL per well. A bacterial suspension from each overnight culture was added to achieve a final concentration of ~10^4 CFU/mL in each well. Then, the microplates were incubated at the appropriate temperature.

The positive control consisted of TSB inoculated with the bacterial suspension but without EEP, while the negative control consisted of uninoculated broth containing the same EEP concentrations. The MIC was defined as the lowest concentration of EEP showing no visible bacterial growth (absence of turbidity).

To determine the MBC, whole suspension from each well showing no visible growth was plated on TSA and incubated at the appropriate temperature. The MBC was defined as the lowest EEP concentration at which no bacterial colonies were observed.

The bactericidal or bacteriostatic nature of the EEP was interpreted according to the MBC/MIC ratio, as described by Pankey and Sabath (2004). The extract was considered bactericidal when the MBC/MIC ratio was ≤4, and bacteriostatic when the ratio was >4.

2.4. In situ antimicrobial activity

To further explore the potential use of propolis as a natural food preservative, both shelf-life and *L. monocytogenes* challenge tests were conducted on sweetened ricotta cheese samples supplemented with different concentrations of EEP. This ready-to-eat dairy product was chosen because its physicochemical properties, such as high water activity and near-neutral pH, make it highly perishable and particularly susceptible to the growth of spoilage and pathogenic microorganisms, including *L. monocytogenes*. Furthermore, sweetened ricotta, especially in certain traditional preparations that involve the use of aromatic ingredients, provides a clear rationale for the incorporation of EEP in this product, making it a suitable and sensitive model for evaluating the antimicrobial effectiveness of EEP under realistic storage conditions.

2.4.1. Preparation of ricotta cheese samples and experimental design

A total of ~2.5 kg of industrial ricotta cheese made from bovine's milk and 2.5 kg of artisanal ricotta cheese made from ovine's milk were purchased from large-scale retailers in the city of Messina (Italy) and transported under refrigerated conditions to the Food Microbiology Laboratory of the Department of Veterinary Sciences, University of Messina. Each type of ricotta was supplemented with 360 g of sugar per kilogram of product. Subsequently, each sample was divided, using a sterile steel spoon, into two equal portions. The first portion of each ricotta type was used to evaluate the effects of EEP on shelf life and sensory characteristics while the second portion was used to assess the antibacterial activity of the EEP against *L. monocytogenes* through a challenge test as described in the following paragraphs. Because the two experiments had different objectives, the concentrations of EEP applied differed between the shelf-life study and the challenge test. In the shelf-life study, a single concentration was used for each ricotta type to simulate realistic application conditions, whereas in the challenge test both concentrations were tested in each ricotta type in order to evaluate the dose-dependent inhibitory effect of EEP against *L. monocytogenes*. Each ricotta type was analyzed independently, and the effect of EEP was evaluated within each product by comparison with its corresponding control.

2.4.2. Shelf-life study and sensory evaluation in sweetened ricotta cheese

The portions of each type of ricotta were divided into two sub-aliquots: one supplemented with EEP at a concentration that varied according to the ricotta type (0.1% or 1%), and the other left untreated to serve as a control. Specifically, industrial ricotta was supplemented with 0.1% EEP (1 mg/g), whereas artisanal ricotta received a 1% EEP addition (10 mg/g).

Each sub-aliquot was then divided into five batches, corresponding to the five sampling days (0, 3, 6, 9, and 14). For each time point, three independent replicates were prepared and analyzed. Samples were sealed in sterile, heat-sealed plastic bags and stored at +7 °C, a temperature commonly used in shelf-life to represent potential domestic refrigeration abuse conditions (Giarratana et al., 2022).

At each sampling day, using a sterile wooden stick, 10 g of sample were collected, transferred into a sterile bag containing 90 mL of peptone water (Biolife, Milan, Italy), and homogenized for 60 s at 230 rpm using a Stomacher (400 Circulator, International PBI, Milan, Italy). The prepared samples were then analyzed for the following microbiological parameters: *i*) enumeration of total aerobic mesophilic bacteria (TAMC) on Plate Count Agar (Biolife, Milan, Italy) incubated at 30 ± 1 °C for 72 h (ISO 4833-1:, 2013); *ii*) enumeration of Enterobacteriaceae on Violet Red Bile Glucose Agar (Biolife, Milan, Italy) incubated at 37 ± 1 °C for 24 h (ISO 21528-2:2017); *iii*) enumeration of *Pseudomonas spp.* on Pseudomonas Agar Base (Biolife, Milan, Italy) incubated at 25 ± 1 °C for 48 h (ISO/TS 11059:2025); *iv*) enumeration of mesophilic lactic acid bacteria on M.R.S. agar (Biolife, Milan, Italy) incubated at 30 ± 1 °C for 72 h (ISO 15214:1998); and *v*) enumeration of yeasts and molds on DRBC agar (Biolife, Milan, Italy) incubated at 25 ± 1 °C for 4–5 days (ISO 21527-1:2008).

In parallel with microbiological analyses, exploratory sensory evaluations were performed according to ISO 6658:2017 and ISO 8589:2007 in order to investigate possible changes in the sensory characteristics of the product due to EEP addition. Five panelists from the Food Microbiology Laboratory of the Department of Veterinary Sciences, University of Messina, previously trained in the sensory evaluation of fresh sweetened ricotta according to ISO 5492:2008 and ISO 8586:2023, were selected for the assessment. Each panelist was asked to evaluate the flavor, color, and odor of the treated samples in comparison with the corresponding controls. Scores were assigned to indicate the degree of variation from the control sample on a 0–3 scale, where 0 = "typical", 1 = "slightly perceptible", 2 = "moderate", and 3 = "intense". It should be noted that this type of sensory evaluation would normally require a larger number of participants; therefore, the present assessment was

performed exclusively for exploratory purposes, as more extensive analyses are needed to fully characterize the sensory impact of EEP in ricotta cheese.

2.4.3. Challenge test for Listeria monocytogenes in sweetened Ricotta Cheese

The portions of each type of ricotta were divided into three sub-aliquots: two were supplemented with different concentrations of EEP (0.1% and 1%), which was directly added and mixed using sterile wooden sticks, while the third was left untreated and served as a control.

Three *L. monocytogenes* EURL strains isolated from cheese (12MOB079LM, 12MOB096LM and 12MOB098LM) were selected for experimental contamination, following an approach inspired by the EURL guideline for *Listeria monocytogenes* challenge test (EURL Lm, 2021).

From frozen stock cultures, each strain was inoculated into TSB and incubated at 37 °C for approximately 24 h. The broth cultures ($\sim 10^8$ CFU/mL) were then ten-fold diluted in fresh broth to obtain a concentration of 10^2 CFU/mL and subsequently incubated at 7 °C until they again reached $\sim 10^8$ CFU/mL, allowing strains to be pre-adapted to low-temperature growth. Finally, each culture was subjected to serial ten-fold dilutions to achieve a final concentration of $\sim 10^4$ CFU/mL for use in the contamination trials. Equal volumes of the three diluted cultures were mixed and used to inoculate each ricotta sub-aliquot (control, 0.1% EEP, and 1% EEP), achieving a final concentration of 100 CFU/g. The strains were used as a combined inoculum (strain mixture), as recommended by the EURL technical guidance for *Listeria monocytogenes* challenge tests, in order to account for strain variability. Consequently, the results reflect the overall behaviour of the strain mixture rather than that of individual strains. To rule out the presence of the pathogen prior to experimental inoculation, samples were analyzed for the detection of *L. monocytogenes* according to ISO 11290-1:2017.

Each sub-aliquot was then divided into five batches, corresponding to the five sampling days (0, 3, 6, 9 and 14). For each time point, three independent replicates were prepared and analyzed. Samples were sealed in sterile, heat-sealed plastic bags and stored at +7 °C, a temperature commonly used in challenge studies to represent potential domestic refrigeration abuse conditions (Giarratana et al., 2022).

Samples were processed as described above (see paragraph 2.3.2.) and analyzed for the enumeration of *L. monocytogenes* according to ISO 11290-2:2017, using Agar *Listeria* according to Ottaviani & Agosti (ALOA; Biolife, Milan, Italy) incubated at 37 ± 1 °C for 24–48 h. The enumeration results obtained were used to calculate the growth potential (Δ) of *L. monocytogenes*, defined as the difference between the maximum concentration ($\log_{10}$ CFU/g) observed during the test and the initial concentration at the time of inoculation (EURL Lm, 2021).

2.5. Statistical analysis

When applicable, results were expressed as mean ± standard deviation (SD) of independent replicates. Data normality was assessed using the D'Agostino-Pearson tests. When the assumptions of normality were not met or the sample size was limited, non-parametric approaches were adopted.

For the *in vitro* assays, inhibition zone diameters obtained at the three EEP concentrations were analyzed separately for each bacterial species. For each species, measurements corresponding to the different concentrations were treated as related samples and compared using the Friedman non-parametric test, followed by Dunn's multiple comparison test to identify significant pairwise differences among concentrations. MIC and MBC values were reported descriptively.

For the *in-situ* experiments, data from both the shelf-life evaluation and challenge test were analyzed by comparing the control and EEP-treated samples within each ricotta type. Comparisons were performed using the Mann-Whitney *U* test (two-tailed, unpaired).

The growth potential (Δ) of *L. monocytogenes* obtained from the challenge test was interpreted according to the criteria established by EURL Lm (2021). In particular, products showing a $\Delta \leq 0.5$ were classified as "unable to support the growth" of *L. monocytogenes*, whereas those with a $\Delta > 0.5$ were considered to "support growth".

Due to the exploratory design and limited panel size ($n = 5$), sensory data were analyzed descriptively by calculating mean ± SD for each attribute (odor, color and flavor) over time.

All statistical analyses were performed using GraphPad Prism version 9.1.1 (GraphPad Software, San Diego, CA, USA), with statistical significance set at $p < 0.05$.

3. Results

3.1. Phenolic compounds characterization

In order to identify and quantify bioactive molecules present in the propoli ethanolic extract, a HPLC-PDA/MS procedure was employed. The extract was analyzed without any pre-treatment and in triplicate. In Fig. 1 was reported an HPLC-PDA chromatogram of the propoli extract. As can be seen in Table 2, twenty-three bioactive compounds were identified. Total phenolic compounds concentration was 7541.7 ± 117.2 mg/L, with isorhamnetin, pinobanksin-proprionate, and 4-*O*-methyl-epigallocatechin as the most abundant bioactive compounds of the extract (1283.8 mg/L, 1489.1 mg/L, and 1185.0 mg/L respectively). From qualitative and quantitative data here presented, obtained by using an HPLC-PDA/MS methodology, it can certainly be stated that this propolis extract represents an excellent source of molecules with high biological value.

3.2. In vitro antibacterial activity

The antibacterial activity of the EEP was first evaluated *in vitro* by the agar disc diffusion assay. Significant differences in inhibition zones were observed among the three tested concentrations of the extract. In particular, the inhibition zones produced by EEP at 1000 mg/mL were significantly larger than those obtained at 250 mg/mL ($p = 0.0417$) and 150 mg/mL ($p = 0.0003$), whereas no significant differences were observed between 250 mg/mL and 150 mg/mL ($p = 0.4383$). Among all tested strains (Fig. 2), inhibition zone diameters varied across the different bacterial species, ranging from 10.5 ± 0.48 mm (*L. monocytogenes* EURL strain 12MOB079LM) to 18.0 ± 0.56 mm (*S. aureus* ATCC 6533). No inhibitory activity was observed at any of the tested concentrations against Gram-negative strains.

The antibacterial efficacy of EEP was further investigated by determining its MIC and MBC values against the strains that were sensitive in the diffusion assay (Table 3). MIC values ranged from 0.25 to 1.25 mg/mL, while MBC values varied between 0.625 and 12.5 mg/mL. The lowest MICs (0.25 mg/mL) were observed for *L. monocytogenes* ATCC 7644 and *L. monocytogenes* 19,111, whereas the highest MICs (1.25 mg/mL) were found for *S. aureus* 6533, *L. innocua* 33,090 and *L. monocytogenes* 19,112.

In most cases, the MBC values were equal to or slightly higher than the MICs, suggesting a predominantly bactericidal effect of the extract. Accordingly, the MBC/MIC ratios were ≤ 4 for the majority of the tested strains, confirming the bactericidal nature of EEP, with the exception of L. *innocua* 33,090 and *S. aureus* 6533, which exhibited an MBC/MIC ratio of 5 and 10, respectively.

3.3. In situ antimicrobial activity

3.3.1. Effect of ethanolic propolis extract on the shelf-life of ricotta cheese

Overall, the addition of EEP did not result in a meaningful extension of shelf life in either ricotta type. The two concentrations tested, 0.1% in the industrial ricotta and 1% in the artisanal ricotta, showed only minor differences compared with their respective controls and did not provide a substantial preservative effect under the evaluated storage conditions.

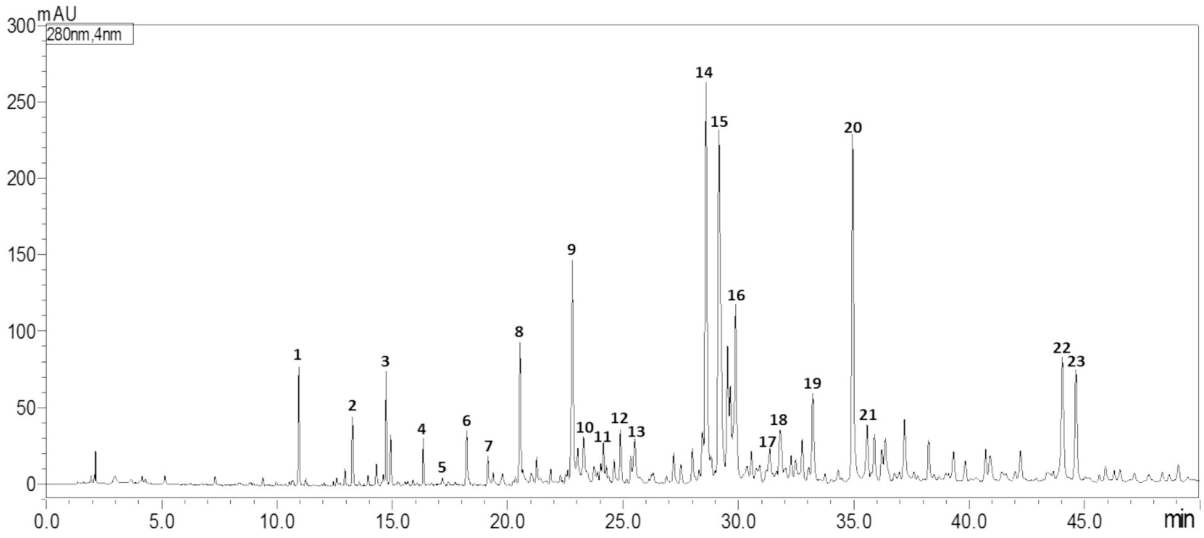

Fig. 1. HPLC/PDA chromatogram of phenolic compounds in propolis extracted at 280 nm. For peak identification see Table 2.

Table 2
Concentration (mg/L ± standard deviation) of phenolic compounds in propoli extract analyzed. The sample was analyzed in triplicate.

N°	Compound	Concentration
1	Dimethoxy-naringenin diglucoside[a]	10.3 ± 0.1
2	Hesperedin	182.4 ± 1.6
3	p-coumaric acid derivate[b]	39.1 ± 0.3
4	Pinobanksin-methyl-ether[c]	83.9 ± 0.9
5	Dihydroxy-dimethoxyflavone	0.5 ± 0.0
6	Pinobanksin[c]	151.4 ± 3.0
7	Hispidulin[d]	79.1 ± 1.5
8	Tricin[d]	389.1 ± 4.4
9	Myricetin derivate isomer[c]	658.8 ± 3.1
10	Myricetin derivate isomer[c]	159.8 ± 6.7
11	Chrysin[c]	63.4 ± 2.0
12	Pinocembrin[c]	138.4 ± 3.3
13	Pinobanksin-acetate[c]	112.5 ± 3.9
14	4-*O*-methyl-epigallocatechin[c]	1185.0 ± 20.1
15	Pinobanksin-propionate[c]	1489.1 ± 21.9
16	Pinobanksin-3-O-pentanoate[c]	478.4 ± 5.4
17	p-coumaric acid derivate[b]	16.9 ± 0.2
18	p-coumaric acid derivate[b]	42.6 ± 0.7
19	Ferulic acid dimer[e]	129.4 ± 1.2
20	Isorhamnetin[c]	1283.8 ± 14.1
21	Rhamnetin[c]	192.1 ± 6.7
22	Quercetin[c]	566.6 ± 9.2
23	p-coumaric acid derivate[b]	89.1 ± 6.6
	All	7541.7 ± 117.2

Phenolic compounds were quantified by means of calibration curves obtained with the corresponding standard compound: [a]naringin, [b]p-coumaric acid, [c]rutin, [d]hesperedin, [e]ferulic acid (Russo et al., 2014, 2016).

In the industrial samples, the TAMC increased in both treated and control samples, reaching approximately 5–6 log CFU/g after 14 days, with slightly lower mean values in the EEP treated ricotta (5.16 ± 0.29 log CFU/g) compared to the control (5.74 ± 0.46 log CFU/g; $p = 0.200$) (Supplementary Files-Fig. S1). All other microbial parameters, including lactic acid bacteria, *Pseudomonas spp.*, yeasts and molds, and Enterobacteriaceae, remained below the detection limit throughout storage.

In the artisanal samples, microbial growth was more pronounced overall, and only a minimal inhibitory effect was observed, limited to TAMC and lactic acid bacteria in the samples treated with 1% EEP compared with their controls. In detail, after 14 days, the TAMC reached 8.62 ± 0.25 log CFU/g in control samples and 7.37 ± 0.37 log CFU/g in treated ones ($p = 0.1000$). Similarly, lactic acid bacteria counts were lower in the treated samples (6.76 ± 0.04 log CFU/g) than in the control (7.37 ± 0.14 log CFU/g; $p = 0.1000$). Minor, non-significant reductions were also observed for *Pseudomonas spp.*, yeasts and molds, and Enterobacteriaceae at each time point (Supplementary Files-Fig. S2).

3.3.2. Sensory evaluation

Exploratory sensory evaluation revealed slight differences in odor, color, and flavor perception over storage time in both ricotta types treated with EEP, with the overall impact on sensory characteristics tending to decrease during storage (Supplementary Files-Fig. S3).

For the 1% EEP artisanal ricotta, the mean odor and flavor scores remained relatively stable from day 0 to day 14, ranging respectively from 3.00 ± 0.00 to 2.00 ± 0,00 for odor and 2.40 ± 0,5 to 1.6 ± 0.55 for flavor, indicating that changes were mostly perceived as moderate to slightly perceptible. Although color scores were slightly lower (ranging from 2.40 ± 0.55 to 1.40 ± 0.55), these differences were still rated as moderate to slightly perceptible after 14 days of storage.

In contrast, the 0.1% EEP industrial ricotta exhibited generally lower scores across all attributes, suggesting a milder sensory impact. Odor perception decreased gradually from 2.00 ± 0.00 at day 0 to 0.40 ± 0.55 on day 14, while color and flavor were rated between 1 and 0, corresponding to typical to slightly perceptible variations compared to the control.

Overall, EEP addition caused perceptible but moderate sensory changes, more evident at the higher concentration (1%). The treated ricotta samples maintained acceptable sensory quality during refrigerated storage, with no evidence of off-odor, flavors or color deterioration (data not shown).

3.3.3. Effect of ethanolic propolis extract on Listeria monocytogenes growth in ricotta cheese

The growth trend of *L. monocytogenes* in the different types of sweetened ricotta is shown graphically in Fig. 3.

In all analyzed samples, the initial bacterial concentration was approximately 1.90 log CFU/g.

In the industrial ricotta control, *L. monocytogenes* showed a rapid and sustained increase, reaching 8.03 ± 0.44 log CFU/g at day 14, corresponding to a growth potential of Δ = +6.13. The addition of 0.1% EEP slightly reduced bacterial proliferation, resulting in final counts of 7.87 ± 0.03 log CFU/g (Δ = +5.97), while 1% EEP considerably inhibited growth, limiting the bacterial counts to 5.74 ± 0.04 log CFU/g (Δ = +3.84).

In the artisanal ricotta, the overall growth of *L. monocytogenes* was less pronounced. The control reached 4.71 ± 0.24 log CFU/g on day 14 (Δ = +2.81). Treatment with 0.1% EEP further restrained growth to

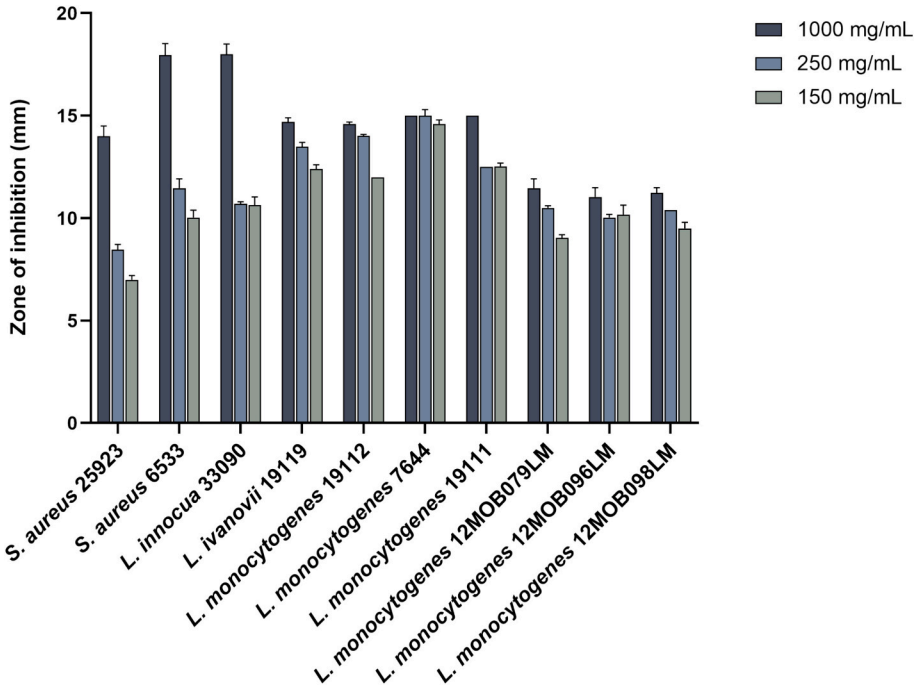

Fig. 2. Results of the agar disc diffusion assay evaluating the antimicrobial activity of different concentrations of Tunisian ethanolic extract of propolis. The diameters of the inhibition zones are presented as means ± standard deviations from three replicates, excluding the 6 mm paper disc.

Table 3
Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of the Tunisian ethanolic extract of propolis against different Gram-positive bacteria. Results of MIC and MBC are expressed in mg/mL.

	Strain	MIC	MBC	MBC/MIC
ATCC	*Listeria innocua* 33090	1.25	6.25	5
	Listeria ivanovii 19119	0.625	0.625	1
	Listeria monocytogenes 19112	1.25	1.25	1
	Listeria monocytogenes 7644	0.25	0.625	2.5
	Listeria monocytogenes 19111	0.25	0.625	2.5
	Staphylococcus aureus 25923	0.625	0.625	1
	Staphylococcus aureus 6533	1.25	12.5	10
EURL	*Listeria monocytogenes* 12MOB079LM	0.625	0.625	1
	Listeria monocytogenes 12MOB096LM	0.625	0.625	1
	Listeria monocytogenes 12MOB098LM	0.625	1.25	2

4.50 ± 0.10 log CFU/g (Δ = +2.60), and the 1% EEP treatment was the most effective, maintaining counts below 3.10 log CFU/g (Δ = +1.13).

In all tested conditions, Δ values exceeded 0.5 log CFU/g, indicating that *L. monocytogenes* was able to grow in both ricotta types.

4. Discussion

The present study provides new insights into the antimicrobial potential of Tunisian EEP in dairy products, specifically in sweetened ricotta cheese.

The antimicrobial potential of EEP has been widely documented, with numerous studies confirming its stronger efficacy against Gram-positive bacteria compared to Gram-negative ones (Przybyłek and Karpiński, 2019). This trend was also observed in the present study, where the EEP inhibited all tested Gram-positive strains, including *L. monocytogenes*, *L. innocua*, and *S. aureus*, while showing no effect against any of the Gram-negative strains tested. According to Vadillo-Rodríguez et al. (2021), the outer membrane of Gram-negative bacteria, rich in amphiphilic and highly charged lipopolysaccharides, acts as a selective permeability barrier that limits the diffusion of certain hydrophobic molecules. This structural feature may delay or even prevent the penetration of certain bioactive compounds into the cytoplasmic membrane, meaning that substantially higher extract concentrations are required to exert an antibacterial effect. However, when comparing our findings with those reported in other studies, a considerable variability in antimicrobial efficacy becomes evident. For instance, several authors have documented notable inhibitory effects of EEP against Gram-negative bacteria, including *Salmonella* Typhimurium and *Yersinia enterocolitica*, as well as *Escherichia coli* and *Pseudomonas aeruginosa* (Al-Ani et al., 2018; De Marco et al., 2017; Uzel et al., 2005). These discrepancies likely reflect the complex and still not fully elucidated mechanisms underlying propolis antibacterial activity. In our study, the limited efficacy observed against Gram-negative bacteria supports the hypothesis of a primarily structural mode of action, in which propolis compounds compromise the integrity of the cell envelope, leading to the leakage of intracellular material and irreversible lysis. Nonetheless, other authors have proposed that the antimicrobial effect may also arise from the biochemical activity of certain compounds, such as flavonoids and phenolic acids, that interfere with DNA, RNA, and protein synthesis, thereby inhibiting bacterial growth through functional rather than structural disruption (Vadillo-Rodríguez et al., 2021). In this regard, the predominance of flavonoid compounds identified in the present extract, such as pinobanksin derivatives and isorhamnetin, totally in accordance with previously pushed data on bioactive compounds in propoli samples (Ghallab et al., 2021; Nefzi et al., 2023; Vieira de Morais et al., 2021), may partly explain the antimicrobial pattern observed. Variability among studies is further compounded by the fact that even the same bacterial species can exhibit markedly different levels of susceptibility depending on the particular EEP tested. In this regard, the *in vitro* antibacterial efficacy observed in the present study appears to be generally lower than the values most commonly reported in literature. For instance, Rendueles et al. (2023) tested 31 Spanish EEP against clinical and wild isolates of *Listeria spp.*, reporting MIC values ranging from 78.1 to 625 μg/mL. Similarly, Grecka et al. (2019) observed MICs between 64 and 512 μg/mL for Polish EEPs against *S. aureus*, while Pedonese et al. (2019) found values from 250 to 1000 μg/mL against *L. monocytogenes* and *S. aureus*. Such discrepancies can likely be attributed to differences in the chemical composition of the propolis, strongly

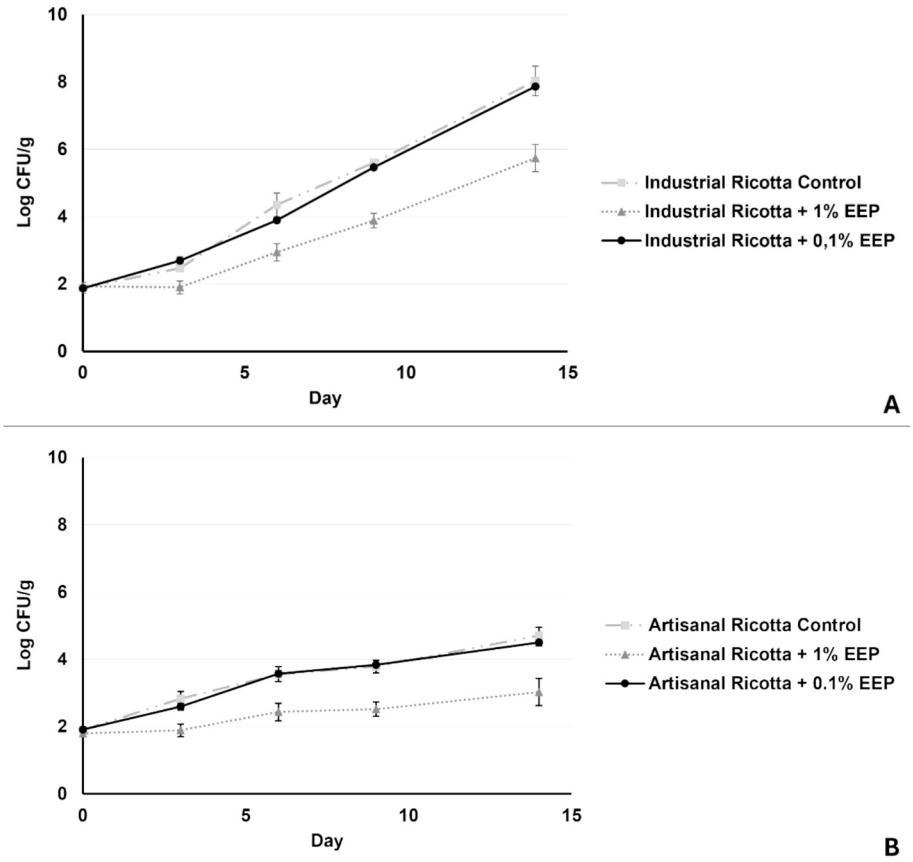

Fig. 3. Growth of *Listeria monocytogenes* in industrial ricotta (A) and artisanal ricotta (B) experimentally inoculated and treated with different concentrations of Tunisian ethanolic extract of propolis (EEP), compared with the untreated control (CTL). Bacterial counts were monitored during 14 days of refrigerated storage. Data are expressed as mean ± standard deviation of three replicates.

dependent on botanical and geographical origins, as well as strain-specific sensitivity to antimicrobial compounds.

In the present study, the addition of EEP to ricotta cheese showed limited effects on the spoilage microbiota, with only a modest inhibition at 1%, insufficient to meaningfully extend shelf life under the conditions tested. Such mild preservative activity aligns with previous findings in other dairy products, where EEP led to only slight reductions in background microbiota unless applied at higher concentrations (El-Deeb and Omar, 2017; Pedonese et al., 2019). In the artisanal ricotta treated with 1% EEP, slightly lower counts were observed compared with the corresponding controls for both TAMC and mesophilic lactic acid bacteria. The reduction in TAMC likely reflects, at least in part, the decreased levels of lactic acid bacteria, which constitute a substantial proportion of the background microbiota. In this type of product, lactic acid bacteria can act as spoilage microorganisms, contributing to unwanted acidification, textural changes, and gas production during storage (Scatassa et al., 2018). It is therefore possible that more evident effects on shelf life would have emerged if the monitoring period had been extended. The limited effect on the spoilage microbiota is also consistent with the compositional profile of the extract discussed above. Several common spoilage microorganisms in refrigerated dairy products, including *Pseudomonas spp.*, are Gram-negative and therefore less susceptible to propolis-derived compounds. In addition, the high water activity and protein-rich matrix of ricotta may further reduce antimicrobial efficacy through protein–polyphenol interactions that can limit the availability of bioactive phenolic compounds in the food system.

More encouraging outcomes regarding the potential application of EEP in foods emerged from the challenge test with *L. monocytogenes*. In this case, a dose-dependent inhibitory effect was observed, with the 1% EEP (10 mg/mL) treatment markedly reducing bacterial growth compared to the controls. Although the growth potential (Δ) values remained above the 0.5 threshold, indicating that *L. monocytogenes* was able to grow under the tested conditions. This apparent discrepancy between the strong bactericidal activity observed *in vitro* and the more limited inhibition in ricotta may be explained by the complexity of the food matrix. In dairy products, interactions between milk proteins and polyphenolic compounds can reduce the availability of bioactive molecules and limit their antimicrobial effectiveness. The inhibitory trend against *L. monocytogenes* in the present study was comparable to, or even stronger than, that reported in similar dairy products. For instance, Thamnopoulos et al. (2018) demonstrated that an ethanolic extract of Greek propolis added to pasteurized milk at 4 mg/mL completely inhibited *L. monocytogenes* growth during 30 days of storage at 4 °C, while lower concentrations significantly delayed proliferation. Similarly, Pedonese et al. (2019) tested higher concentrations (2% and 5%) of Italian EEP in cow's and goat's ricotta, observing significant inhibition only at 5%, whereas the 2% addition produced effects comparable to controls. In contrast, the present work achieved a substantial inhibitory effect with only 1% EEP, suggesting that the Tunisian extract may possess higher antimicrobial effect or that the specific *L. monocytogenes* strains used were more sensitive. A comparable antimicrobial trend was also observed in ice cream added with Egyptian EEP (150–600 mg/L), where increasing concentrations progressively reduced the survival of methicillin-resistant *S. aureus* during frozen storage, with complete inhibition achieved at the highest level (600 mg/L) (El-Bassiony et al., 2012). Together, these findings reinforce the hypothesis that the antimicrobial action of EEP is strongly dose-dependent and influenced by the composition and physical properties of the food matrix.

From a sensory perspective, the results of the present study indicate that the incorporation of EEP at both 0.1% and 1% did not compromise

the sensory acceptability of the product, as only moderate and non-unpleasant changes in odor, color, and flavor were perceived by panelists. These findings suggest that EEP can be incorporated into flavored dairy products such as sweetened ricotta without causing unacceptable sensory alterations, particularly at lower concentrations. This aspect is important for practical applications, since the sensory impact of propolis is often considered one of the main limitations to its use as a natural antimicrobial in foods. The sensory results obtained in this study are consistent with previous reports on the use of EEP in dairy products. For instance, Santos et al. (2020b) used a 0.05% (*w/v*) ethanolic extract of Brazilian red propolis in yogurt and reported that sensory acceptance remained high. Meanwhile, Thamnopoulos et al. (2018) applied an EEP deodorized using ion-exchange resins in milk at concentrations up to 0.2%, and observed no undesirable sensory attributes, although antimicrobial activity was somewhat reduced by deodorization. In contrast to these prior works, the present study extends the application to a higher concentration (1%) without requiring deodorization and demonstrates that the sensory impact further diminishes over storage while antimicrobial efficacy remains stable. This suggests that EEP can be utilized at functional levels in dairy products with minimal sensory compromise and ensuring preservative benefits.

Despite the encouraging antimicrobial activity demonstrated by the Tunisian EEP, several limitations of the present work should be acknowledged. The shelf-life evaluation did not show marked differences between treated and control samples for general spoilage microbiota, suggesting that EEP may be more effective against specific pathogens, such as *L. monocytogenes*, rather than as a broad-spectrum preservative. Additionally, the sensory assessment should be considered exploratory, as it involved a limited number of trained panelists. Consequently, the results should be interpreted with caution and cannot be regarded as fully representative of consumer acceptability, since the small panel size reduces the statistical robustness and generalizability of the sensory evaluation. Safety aspects also warrant attention. Although propolis is generally regarded as safe, allergic reactions, mainly due to caffeic acid esters, have been documented, with a prevalence of 1.2–6.6% in the general population and higher in individuals frequently exposed to raw propolis (Hausen, 2005; Oršolić, 2022; Walgrave et al., 2005). Strategies to reduce allergen content, such as enzymatic biotransformation, have been proposed and could improve its suitability for food applications (Gardana et al., 2012). From a technological perspective, formulation challenges remain. The intense flavor and aroma of propolis are known to limit its use, and although microencapsulation markedly reduces sensory impact and improves stability, its industrial application is still constrained by production costs and processing complexity (Pobiega et al., 2019a, 2019b, 2019c). Future research should therefore explore more feasible delivery systems, larger sensory panels, and the combination of propolis with other natural antimicrobials to enhance efficacy while maintaining product quality (Nefzi et al., 2023). Finally, regulatory aspects must also be considered. In the European Union, propolis is currently classified as a food supplement under Directive 2002/46/EC and its use as a technological additive is not yet harmonized. In addition, the European Food Safety Authority (EFSA) has not approved specific health claims for propolis-derived products, mainly due to the considerable variability in their chemical composition linked to botanical origin and extraction methods (EFSA, 2010). This highlights the need for more standardized characterization protocols, along with additional toxicological and clinical data, to support clearer regulatory guidance and facilitate its potential application in food systems.

5. Conclusion

This study provides new evidence on the antimicrobial potential of Tunisian EEP in sweetened ricotta cheese, confirming its pronounced activity against Gram-positive bacteria and its limited effectiveness toward Gram-negative species. While the addition of EEP had only a modest impact on the natural spoilage microbiota of ricotta, with small reductions observed only at the 1% level, these effects were not sufficient to meaningfully extend the product's shelf life under the conditions tested. In contrast, the challenge test suggested a dose-dependent inhibition of *L. monocytogenes*, indicating that the most promising application of EEP lies in targeting specific pathogens rather than broad-spectrum preservation. Sensory acceptability remained largely unaffected even at the highest concentration tested (10 mg/g), suggesting that EEP can be incorporated into dairy matrices without compromising product quality. However, some limitations should be acknowledged: the small sensory panel restricts the strength of conclusions on consumer acceptability, and external factors, such as the potential for allergic reactions to propolis constituents and current regulatory restrictions, may influence its practical use in foods. Overall, the findings support the potential of EEP as a functional antimicrobial ingredient in dairy products, while highlighting the need for further work to ensure safety, standardization, and regulatory compliance.

CRediT authorship contribution statement

Nermine Nefzi: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Marina Russo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Megdiche Ksouri Wided:** Validation, Conceptualization. **Giovanna Cafeo:** Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Paola Dugo:** Writing – review & editing, Validation, Supervision, Methodology, Data curation, Conceptualization. **Filippo Giarratana:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Luca Nalbone:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, 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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2026.111787.

Data availability

Data will be made available on request.

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