Original article

Combined antimicrobial use of essential oils and bacteriocin bacLP17 as seafood biopreservative to control *Listeria monocytogenes* both in planktonic and in sessile forms

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A B S T R A C T

The antilisterial activity of *Thymus vulgaris*, *Salvia officinalis* essential oils (EOs) and bacteriocin bacLP17 (previously isolated from seafood) was determined, using the compounds alone and in combination. The Disk Diffusion, Minimal Inhibitory Concentration (MIC) and Agar Well Diffusion assays were used to evaluate the effectiveness of the compounds against 12 *Listeria monocytogenes* in planktonic form, whereas the anti-*Listeria* biofilm activity was determined against the same strains in optical density (O.D.) at 570 nm, with crystal violet staining method. The lowest MIC values resulted for *T. vulgaris* EO and bacLp17 (0.5 μl/ml and 2 μl/ml, respectively). The combinations with the best results, expressed as FIC-Index, were *T. vulgaris*/*S. officinalis* EOs and EOs/bacLp17. The anti-biofilm activity of single EOs and bacLP17 was similar, whereas the combined use of the two kinds of EOs led to a synergistic activity. Lastly, the best anti-biofilm effect was observed with the combination bacLP17/*S. officinalis* and bacLP17/ *T. vulgaris*, compared to both control and the single use of the EOs. The present study suggests that the combination of natural compounds such as *T. vulgaris*, *S. officinalis* EOs and bacLp17 may be a useful approach to the control of planktonic and sessile cells of *L. monocytogenes* in seafood products.

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1. Introduction

Listeria monocytogenes is a psychotropic foodborne pathogen causing listeriosis, an illness mainly affecting immunocompromised population, pregnant women, young and elderly individuals, especially in the age group over 64 years old. This pathogen causes septicemia, meningitis or other infections of the central nervous system and, in pregnant women, the infection can determine spontaneous abortion, still birth or fetal death. Listeriosis has the second-highest fatality rate (20%) and the highest hospitalization rate (90%) [1], with 2536 European cases of listeriosis reported in 2016 [2]. *L. monocytogenes* can contaminate foods at pre- and post-harvest stages of production and its occurrence is highest in fish and fishery products (6%), followed by RTE salads (4.2%), RTE meat and meat products (1.8%), soft and semi-soft cheeses (0.9%), fruit and vegetables (0.6%) and hard cheeses (0.1%) [2]. Fish and seafood products, more commonly the ones consumed without further cooking and with extended shelf life at refrigeration temperatures (such as smoked fish), support the growth of the pathogen, that cannot only contaminate the raw seafood coming directly from the aquatic environment, but also it can be reintroduced as a post-processing contaminant [3]. Outbreaks of listeriosis associated with smoked mussels, smoked trout and raw oysters, and gravad and cold-smoked fish have been reported [4–7]. The persistence of *L. monocytogenes* in food processing plants is an important factor both in the transmission of this foodborne pathogen and in the contamination of foods and food associated environments and, in this context, a primary role is played by the biofilm [8,9]. Biofilm is a community of microbial cells enclosed in an extracellular polymeric structure and adherent to an inert or living surface at the interface with a liquid phase. Biofilm represents an evolutionary advantage for the microorganisms because it grants both nutrients for growth and protection from different adverse conditions [10–13]. Within the biofilm, microorganisms are less susceptible to the conventional treatments than their planktonic counterparts, so biofilm poses a challenge in food processing facilities, where new strategies to eradicate this microbial structure are needed [14,15].

In recent years, the consumer's demands of natural foods and the environmental concerns have highlighted the necessity to preserve a highly perishable product like seafood using natural additives. The refrigeration is the most common way to increase the shelf life of foods but it is unable to inhibit this psychrotrophic microorganism that survives and grows at 2−4 °C. The use of chemical preservatives has therefore become necessary but has often met the criticism and suspicion of consumers. To meet the consumers' requirements of high quality, minimally processed and additive free foods, new and natural technological approaches for food preservation must be found. Several natural preservatives from different sources have been widely studied, such as herbs and medical plants, microorganisms and animals, therefore gaining considerable attention [16,17]. For this reason, two types of natural compounds are proposed in this study: bacteriocins and essential oils (EOs). Bacteriocins are ribosomal synthesized peptide or proteins produced by bacteria and able to inhibit or kill other related or unrelated microorganisms. The antibacterial activity of bacteriocins is due to their interaction with the bacterial cell surface and cell membrane, with cell permeabilization and pore formation as major mechanisms of action [18]. Bacteriocins have attracted attention as potential antibiotic alternative to prevent bacterial infections and they are widely studied for food preservation against spoilage and pathogenic bacteria such as *L. monocytogenes* [19−25]. Essential oils, a mixture of volatile compounds extracted from aromatic plants, have already shown in previous investigations an important activity against human pathogens such as *L. monocytogenes* [26−29]. *Lamiaceae*, to which the two natural compounds used in the study belong, is one of the most important family, whose EOs are notoriously endowed with antimicrobial properties. Several lines of evidence support the bactericidal and bacteriostatic activity of *Salvia officinalis* against both Gram-positive and Gram-negative bacteria, even if Marino et al. (2011) [30] indicates a weak bacteriostatic effect only. This activity is mainly due to the bioactive components, such as terpenes, phenolic acids and flavonoids, responsible for a variety of effects, such as disturbing the cytoplasmic membrane integrity of bacteria, affecting the electron transport chain, changing the pH homeostasis, disrupting the proton motive force, and coagulation of cell contents. A lot of studies demonstrated that the *Thymus vulgaris* EO has both a potent bacteriostatic and bactericidal effects against many against Gram-positive and Gram-negative human pathogens, having carvacrol and thymol as its main active components. Regarding the mode of action, thyme EO has the potential to cause the rupture of the cell membrane, by penetrating the phospholipids layer of the bacterial cell wall and blocking the enzyme systems [31−34].

The first objective of this study was to confirm the anti-*Listeria* activity of bacLP17, produced by *Enterococcus mundtii* LP17, a strain previously isolated from red mullet and endowed with a strong activity toward the pathogen [25], and to evaluate the antibacterial activity of two EOs derived from *S. officinalis* and *T. vulgaris*, condiment plants commonly used in the Mediterranean area. These natural compounds, by themselves or in combination, have been studied against the pathogen. The second objective was to investigate the activity of these natural products against the mature biofilm formed by *L. monocytogenes*.

2. Materials and methods

2.1. Bacterial strains, media and culture conditions

The EOs, obtained by hydro-distillation of dried spices were bought from a local herbalist shop. To assess the antibacterial activity of *T. vulgaris* and *S. officinalis* EOs and bacLP17, twelve bacterial strains, 2 classified *L. monocytogenes* (NCTC and ATCC) and 10 *L. monocytogenes* wild type, were used (Table 1). Both *E. mundtii* Lp17 producer strain and all *L. monocytogenes* used as indicators were grown in Tryptic Soy Broth or Agar (TSB or TSA, Oxoid S.p.A, Milan, Italy). All strains were maintained in the same media containing 20% (w/v) glycerol at −80 °C until use.

2.2. Anti-Listeria activity determination

The preliminary determination of the antibacterial activity of *S. officinalis* and *T. vulgaris* against all *L. monocytogenes* strains, was carried out by using the agar disk diffusion assay, according to the standard procedure of the Clinical and Laboratory Standards Institute [35]. Sterile disks of 6 mm in diameter, containing 10 μL of each EO, were placed on Mueller Hinton Agar (MHA, Oxoid S.p.A, Milan, Italy) plates, previously seeded with 100 μl of 10^7-10^8 cfu/ml of bacterial suspensions. A sterile disk added with sterile distilled water was used as negative control. After incubation at 37 °C for 24 h, the antagonistic activity of the EOs was quantified by a clear zone of inhibition in the indicator lawn around the disks and the diameters in millimeters of these zones were measured [36].

The antibacterial activity of bacLP17 was determined by the agar well diffusion assay [37] against the same 11 strains of *Listeria* as indicators. To extract the bacteriocin released in liquid medium, the crude filtrate supernatant fluid (CFSF) of *E. mundtii* Lp17 was prepared from a culture in TSB broth grown at 37 °C for 24 h. Cultures were centrifuged at $12,000 \times g$ for 30 min at 4 °C and supernatant fluid was collected, dialysed against 30 mmol/l sodium acetate buffer (pH 5.3) and filter sterilized (0.45 mm-pore-size filter; Millipore Corp., Bedfort, Mass.). To eliminate the antimicrobial effect of organic acids, the pH of the supernatants was adjusted to 6.0 with sterile 1 M NaOH. The obtained crude bacteriocin bacLP17 was used at the concentration of 1280 arbitrary units per milliliter (AU/ml), previously determined as the reciprocal of the highest dilution of CFSF producing a distinct inhibition of the indicator lawn [25,38]. 50 μl of CFSF containing bacLP17 (1280 AU/ml) was brought to 100 μl volume with phosphate buffer (pH 6) and dispensed into 8 mm diameter wells previously performed in Tryptic Soy agar plates (TSA, Oxoid S.p.A, Milan, Italy). 100 μl of phosphate buffer was used as negative control. After diffusion of the solutions, plates were slowly seeded with 5 ml of warm TSA (0.7%) containing 10^7 cfu/ml from overnight cultures of the same indicators listed in Table 1 and incubated at 30 °C for 24 h. The presence of the antagonistic activity was determined by a clear zone of inhibition in the indicator lawn around the wells.

Table 1
Anti-*Listeria* activity detected with agar well diffusion assay deferred antagonism (BacLP17) and agar disk diffusion assays (*T. vulgaris* and *S. officinalis*).

Indicator strains	BacLP17	Thymus vulgaris	Salvia officinalis
L. monocytogenes NCTC 10888	+++	+++	++
L. monocytogenes ATCC 13932	+++	+++	++
L. monocytogenes 53 A	+++	+++	++
L. monocytogenes 25 C	+++	+++	++
L. monocytogenes 40 A	+++	+++	+
L. monocytogenes 33	+++	+++	+
L. monocytogenes 722	++	++	+
L. monocytogenes 30 C2	++	++	+
L. monocytogenes 37	+++	++	+
L. monocytogenes 66	+++	+++	+
L. monocytogenes 692	+++	++	+
L. monocytogenes 4 C	+++	+++	+

(−) = no inhibition zone; (+) = 1−3 mm inhibition zone; (++) = 3−5 mm inhibition zone; (+++) = >5 mm inhibition zone.

2.3. Minimal inhibitory concentration (MIC)

The MIC values of bacLP17, *S. officinalis* and *T. vulgaris* EOs were determined against *L. monocytogenes* by using the broth micro-dilution method in 96-well microplates, according to the Clinical Laboratory Standards Institute (CLSI) guidelines 2012 [35]. The test was performed in sterile 96-well microplates by dispensing into each well 95 μl of Tryptic Soy Broth (Oxoid S.p.A, Milan, Italy) and 5 μl of bacterial suspensions, to a final inoculum concentration of 10^6 CFU mL^{-1}. Then, 100 μl of bacLP17 and EOs serial dilutions were added to obtain concentrations ranging from 512 to 0.125 μl/ml [31,39]. Negative control wells consisted of bacteria in TSB without bacteriocin and EOs. The plates were incubated at 37 °C for 24 h, mixed on a plate shaker at 300 rpm for 20 s, and the MIC was defined as the lowest concentration of bacteriocin and EOs that inhibited visible growth of the tested microorganisms after the optical density (OD) measure at 570 nm using a microtiter-plate reader.

2.4. Combination of essential oils and bacteriocin

The combined effects of *T. vulgaris*/*S. officinalis* and EOs/bacLP17 were calculated in terms of fractional inhibitor concentration index (FIC-Index). The FIC-Index is calculated by comparing the value of the MIC of each agent alone with the combination-derived MIC. Antimicrobial combinations that result in a fold reduction in the MIC compared with the MICs of agents alone are synergistic (FIC ≤ 0.5), whereas FICs in the 0.5 to 1.0 range are non-synergistic or additive. FIC-Index values from 1 to 4 are defined as indifferent, while those with a value greater than 4 are antagonistic. For the determination of the FIC-Index we used the chessboard method with a 96-well microplate. The FIC-Index was calculated as follows: MIC of the combination of essential oils/MIC of the essential oil only. Essential oils have been combined with MIC + MIC; MIC + 1/2 MIC; MIC + 1/4 MIC; MIC + 1/8 MIC; 1/2 MIC + 1/2 MIC; 1/2 MIC + 1/4 MIC; 1/2 MIC + 1/8 MIC; 1/4 MIC + 1/4 MIC; 1/4 MIC + 1/8 MIC; and 1/8 MIC + 1/8 MIC. The same calculation was used for the evaluation of FIC-Index among essential oils and bacteriocins.

2.5. Anti-biofilm activity determination

The effects of both EOs, bacLP17 and the combination between bacLP17/EOs were tested on '3 days old' pre-formed biofilm, obtained using 96-well polystyrene microtiter plates, as previously described [40]. Polystyrene microtiter plates were inoculated with 200 μl of 18-h-old bacterial culture containing cell count of approximately 10^6 cfu/ml. The medium was refreshed every 24 h. After biofilm formation, the medium was gently aspirated and plates were washed three times with a sterile phosphate-buffered saline solution (PBS, pH 7.2) to remove planktonic bacteria and the natural compounds were added at MIC concentration. Following an additional incubation for 24 h at 37 °C, the biofilm biomass was determined by the crystal violet staining method [33,41]. Briefly, the supernatant was removed, and the wells were washed three times with PBS. For fixation of the biofilm's biomass, 150 μl of methanol for 15 min was added, and the supernatant was removed again. Then, 150 μl of crystal violet (CV) solution at 0.1% was added to each well and, 15 min later, the excess dye was removed by washing the plates three times with sterile PBS. The bound of crystal violet was released by adding 200 μl of 33% acetic acid followed by incubation for 10 min at room temperature. The optical density (O.D.) was measured at 570 nm using a microplate reader (Sunrise Tecan, Austria). Both 50% ethanol solution and TSB with bacterial culture only were used as negative and positive controls, respectively.

2.6. Statistical analysis

All the experiments were carried out in triplicate and the bacterial count was performed on three plates. The arithmetic means of the three determinations, expressed as log bacterial count, was plotted against the control. The results were analysed statistically with the Student's *t*-test and differences were considered significant when $p < 0.05$.

3. Results

3.1. Anti-Listeria activity determination

Both EOs were active against all *L. monocytogenes* strains, as demonstrated by using the agar disk diffusion assay, even if *T. vulgaris* resulted more effective than *S. officinalis* (Table 1). Difference in *T. vulgaris* and *S. officinalis* activity was observed, with the inhibition zone greater than 3 and 10 mm (8 out 11 indicators and 3 out 11 indicators, respectively). Fig. 1 shows an example of EOs anti-*Listeria* activity. The results obtained by the Agar Well Diffusion assay demonstrated the good anti-*Listeria* activity of bacLP17, with 9 out 11 showing inhibition zone greater than 5 mm, thus confirming the good anti-*Listeria* activity of the compound, already emerged in our previous investigation [25].

3.2. Minimal inhibitory concentration (MIC)

The MIC against all *L. monocytogenes* strains of EOs and bacLP17 confirmed the results of both the disk diffusion test and the deferred antagonism method. No strain showed resistance to both the bacLP17 and the EOs. A good antimicrobial activity was observed for bacLP17 and *T. vulgaris* EO (Table 2), with values ranging from 0.5 μl/ml to 4 μl/ml against all tested strains. *S. officinalis* EO, as already observed with the agar disk diffusion assay, resulted less active against all the indicators, with values ranging from 2 μl/ml to 16 μl/ml, and with the highest MIC value (16 μl/ml) observed for 4 out 11 indicator strains.

3.3. Combination of essential oils and bacteriocin

In general, the FIC-Index showed a good synergy between all the natural substances tested, EO/EO and EOs/bacLP17, with values equal to or less than 0.5 (Table 2). In particular, the combination EOs/bacLP17 revealed an excellent synergistic effect. The best
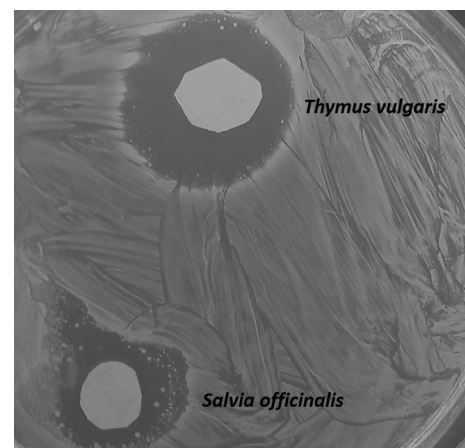

Fig. 1. Agar well diffusion assay: *Thymus vulgaris* and *Salvia officinalis* EOs against *Listeria monocytogenes* ATCC 10888.

Table 2
MICs of EOs and BacLP17 and synergistic interaction between EOs and for single EO in combinations with BacLP17 against *L. monocytogenes* strains.

Indicators strains	Essential oils MIC		FIC-Index of essential oils/SD	MIC of BacLP17	FIC-Index of BacLP17 and S. officinalis/SD	FIC-Index of BacLP17 and T. vulgaris/SD
	Salvia officinalis	*Thymus vulgaris*				
L. monocytogenes NCTC 10888	16	0.5	0.5 (S) ± 0.00	0.25	0.285 (S) ± 0.15	0.328 (S) ± 0.12
L. monocytogenes ATCC 13932	16	4	0.5 (S) ± 0.00	0.5	0.320 (S) ± 0.08	0.484 (S) ± 0.03
L. monocytogenes 4 C	2	2	0.312 (S) ± 0.12	2	0.328 (S) ± 0.1	0.289 (S) ± 0.1
L. monocytogenes 25 C	4	2	0.5 (S) ± 0.02	0.5	0.367 (S) ± 0.02	0.406 (S) ± 0.02
L. monocytogenes 30 C2	2	1	0.375 (S) ± 0.11	0.5	0.367 (S) ± 0.07	0.195 (S) ± 0.15
L. monocytogenes 33	4	2	0.5 (S) ± 0.03	1	0.367 (S) ± 0.00	0.464 (S) ± 0.03
L. monocytogenes 37	16	0.5	0.5 (S) ± 0.01	2	0.328 (S) ± 0.1	0.390 (S) ± 0.05
L. monocytogenes 40 A	8	0.5	0.375 (S) ± 0.08	2	0.484 (S) ± 0.01	0.445 (S) ± 0.05
L. monocytogenes 53 A	4	2	0.5 (S) ± 0.02	1	0.328 (S) ± 0.06	0.242 (S) ± 0.1
L. monocytogenes 66	16	2	0.36 (S) ± 0.13	4	0.375 (S) ± 0.02	0.5 (S) ± 0.00
L. monocytogenes 692	16	2	0.375 (S) ± 0.05	4	0.484(S) ± 0.00	0.406 (S) ± 0.04
L. monocytogenes 722	2	1	0.375 (S) ± 0.07	1	0.367 (S) ± 0.12	0.343 (S) ± 0.04

Minimal inhibitory concentration (MIC): data are expressed as µl/mL.
FIC-Index, fractional inhibitory concentration index: ≤0.5 represents synergy (S); >0.5 to ≤4.0 represents indifference (I); >4.0 represents antagonism (A).
SD, Standard deviation.

synergy (FIC-Index ≤ 0.5) was observed for the combination *T. vulgaris* EO/bacLP17, with values ranging from 0.195 to 0.484. A less evident synergy emerged when *S. officinalis* EO and bacLP17 were used together, with values ranging from 0.285 to 0.484. *T. vulgaris*/*S. officinalis* EOs also showed a synergistic effect (ranging from 0.312 to 0.5) against six strains out of twelve (50%) and with a FICIndex value of 0.5.

3.4. Anti-biofilm activity determination

All *L. monocytogenes* strains employed in the study proved to be good biofilm producers, nevertheless both the EOs and bacLP17

were effective against all of their mature biofilm's biomass (Fig. 2). The anti-biofilm activity of both single used EOs and bacLP17 was similar, with a significant difference to the controls (range of p-value from 0.0388 to 0.000014), whereas the association between EO/EO and EO/bacLP17 was synergic in reducing the mature biofilm's biomass. In particular, the synergic activity of combined EOs led to a significant reduction both with respect to the control (range of p-value from 0.018 to 0.00011) and when compared to the single EOs (range of p-value from 0.042 to 0.0028 for *S. officinalis* and from 0.046 to 0.00115 for *T. vulgaris*). The best anti-biofilm activity was observed with the combination bacLP17/*S. officinalis* and bacLP17/*T. vulgaris* both with respect to the control (p-value from 0.00089 to
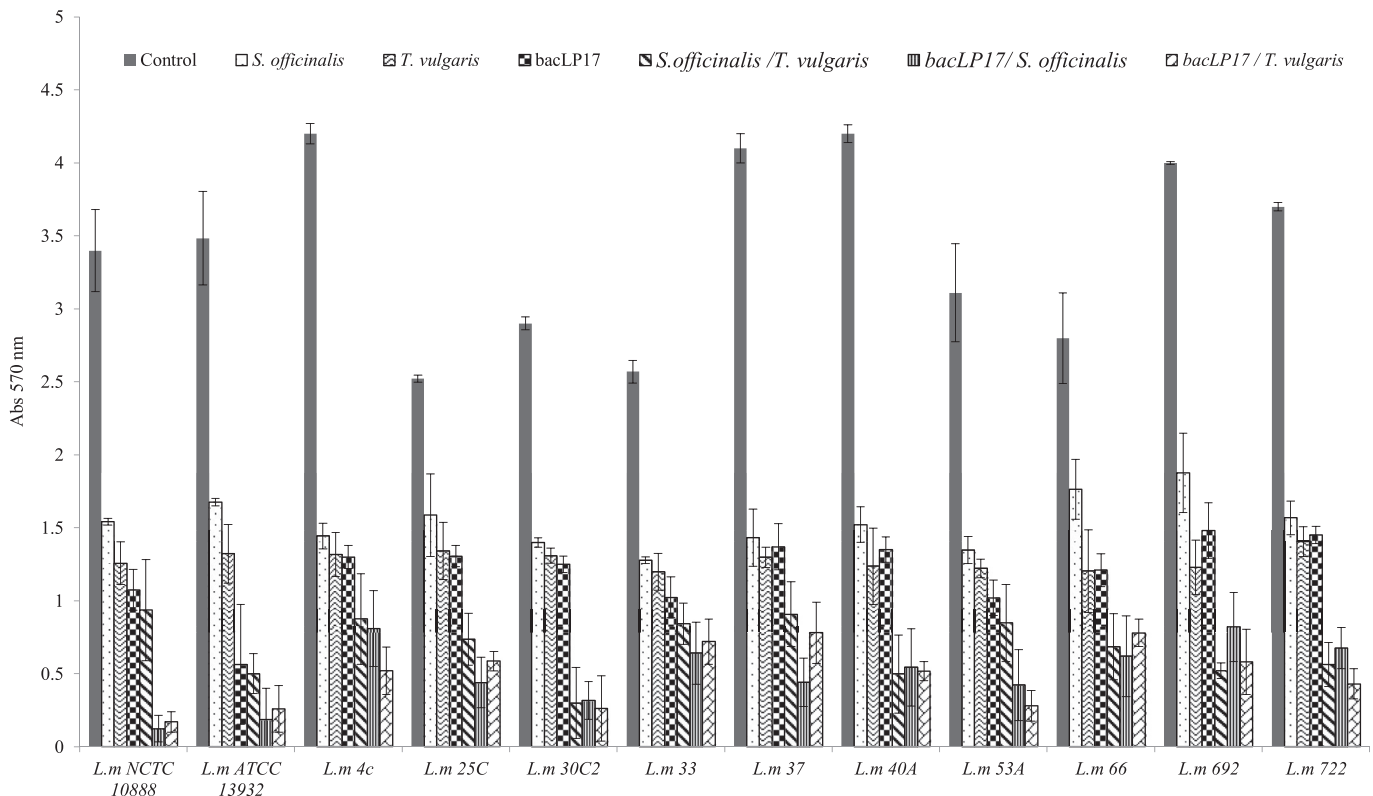

Fig. 2. Anti- *L. monocytogenes* ATCC 10888 Biofilm activity of *T. vulgaris*, *S. officinalis* EOs and bacLP17 used both alone and in combination. Error bars represent standard deviation.

0.0000009) and when compared to the single EOs (p-value from 0.062 to 0.0016 for *S. officinalis* and from 0.034 to 0.00023 for *T. vulgaris*).

4. Discussion

The consumption of minimally processed ready-to-eat (RTE) or raw foods has affected the incidence of diseases caused by psychotropic bacteria, such as *L. monocytogenes*. A major safety risk is associated to psychotropic pathogens in substrate where they can reach high viable counts during storage at refrigeration temperatures. In these products, and mainly in foods eaten without thermal treatments like ready to eat seafood, the employ of natural products as biopreservatives might be of great interest for producers and consumers [42]. Natural substances as bacteriocins and essential oils have already shown an important role in the control of pathogens, with feasible application in various foods. Their use in the food industry can help to reduce both the addition of chemical preservatives and the intensity of heat treatments, resulting in a more natural fresh food. Several bacteriocins already offer potential applications in food preservation, but to date, only few studies have described the anti-*Listeria* characteristic of a compound obtained from a bacteriocinogenic Lactic Acid Bacteria (LAB), a strain isolated from fish and therefore well adapted to growth in this organic matrix and therefore capable to compete with pathogens better than LAB from other sources. On the other hand, an increased focus on new preservation methods based on the use of natural substances like EOs has also been observed. Essential oils, which use in vegetable products have already been approved by the Food and Drug Administration (2001) [1] show their activity against both food-borne pathogens and spoilage bacteria [43–48]. It seems that the EOs are more effective in vegetables because they have a low-fat content [49]. *T. vulgaris* EO confirmed its good anti-*Listeria* effect observed in our previous investigation, where this essential oil led to a significant decrease in *L. monocytogenes* NCTC 10888 cell viability after 4 days of exposure and to a final reduction of 3 log cfu/g in artificially contaminated RTE vegetable, stored at refrigeration temperatures [29]. Another study [50] has shown a lot of pharmacological properties of *S. officinalis*, with its biological activity attributed to terpenes and terpenoids, compounds extensively found in the plant. The Authors refer a strong bactericidal effect on *L. monocytogenes*, whereas in the present investigation *S. officinalis* was less effective than *T. vulgaris* against all *L. monocytogenes* strains tested. Regarding the sensitivity of microorganisms to these natural antimicrobial substances, no resistance mechanisms developed by bacteria towards essential oils have been reported, most likely due to the complexity and to the variety of mechanisms of action of their active compounds. Moreover, the essential oil components may act synergistically with antibiotics, for their capability to affect multiple targets, to perform physicochemical interactions and to inhibit antibacterial-resistance mechanisms. Reports on bacteriocins resistance developed by some Gram-positive bacteria are referred for the most used bacteriocin nisin, compound already approved as food preservative. Many studies have however shown that the bacteriocin's resistance can be overcome by using the combination of different bacteriocins or by the association with other natural antimicrobial compounds like essential oils [51,52].

The combined use of the natural compounds against *L. monocytogenes* proposed in the present investigation has produced encouraging results, consistent with other studies. Turgis et al., 2012 [53] refer that the combination of nisin with *Origanum vulgare* EO induces a synergistic effect against *L. monocytogenes*. Addition of oregano or savory essential oil exhibited a synergistic effect with CAB (cell-adsorbed bacteriocin) to control *L. monocytogenes* in pork meat during storage at 4 °C, and anti-*Listeria* activity of AS-48 (30 μg/g) in ready-to-eat food was strongly enhanced by essential oils [54,55]. The natural substances employed in the present study are Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA) (2002) [56], but have some limits: bacteriocins do not have a broad host range, and the use of EOs is often limited for undesirable organoleptic impact. The synergistic effect observed in the present investigation consists of a more enhanced anti-*Listeria* activity of bacteriocin (used at its lower MIC) and a contextual significant reduction of the amount of EOs to use toward the pathogen. For this last reason, a low MIC permits the use of EOs as preservative without affecting the sensorial quality of foods. The combination EO/bacteriocin allows to overcome this limitation and could be an alternative to the traditional chemical preservatives. The combined use of EOs and bacteriocins could also help to overcome the problem of bacteriocin resistance in Gram-positive bacteria, as reported for nisin (0–200 IU/ml) and garlic extract (0–6 mg/ml) [57]. Lastly, the present study also showed a significant anti-*Listeria* biofilm activity of the single EOs and bacLP17. The synergistic association between EO/EO and EO/bacLP17 led to a significant reduction of the mature biofilm and the association between EOs and bacteriocin was the most active.

Our results demonstrate the antibacterial potential of *T. vulgaris*, *S. officinalis* and bacLP17, alone and in combination, both to control *L. monocytogenes*, and to impair the biofilm produced by the same. Although the natural antimicrobial compounds are becoming popular in the food industry for the control of foodborne pathogens, there are some difficulties with their effective use. Both natural antimicrobials used in the present investigation present limits like the reduced sensitivity of Gram-negative bacteria to LAB bacteriocins, and the strong smell of EOs. Our results show that the synergism emerged with the combined use of EOs and bacteriocin is a promising natural way to overcome both the narrow range of activity and the unpleasant sensory impact. The use of EOs and bacteriocins together opens new promising opportunities for the development of novel preservative agents effective in controlling *L. monocytogenes* growth in seafood and other minimally processed RTE foods.

Declaration of Competing Interest

The author reports no conflicts of interest in this work.

References

[1] FDA/USDA/CDC 2001 (US Food and Drug Administration, United States Department of Agriculture and Centers for Disease Control and Prevention). Draft assessment of the relative risk to public health from foodborne *Listeria monocytogenes* among selected categories of ready-to-eat foods.

[2] EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. EFSA Journal 2017;15(12):5077. https://doi.org/10.2903/j.efsa.2018.5500. 228.

[3] Guerra MM, Mclauchlin J, Bernardo FA. *Listeria* in ready-to-eat and unprocessed foods produced in Portugal. Food Microbiol 2001;18:423–9.

[4] Ericsson H, Ekløw A, Danielsson-Tham ML, Loncarevic S, Mentzing LO, Persson J, et al. An outbreak of listeriosis suspected to have been caused by rainbow trout. J Clin Microbiol 1997;35:2904–7.

[5] Brett MSY, Short P, McLauchlin J. A small outbreak of listeriosis associated with smoked mussels. Int J Food Microbiol 1998;43:223–9.

[6] Tham W, Ericsson H, Loncarevic S, Unnerstad H, Danielsson-Tham ML. Lessons from an outbreak of listeriosis related to vacuum-packed gravad and cold-smoked fish. Int J Food Microbiol 2000;62:173–5.

[7] ECDC (European Centre for Disease Prevention and Control) and EFSA (European Food Safety Authority). Multi-country outbreak of *Listeria monocytogenes* clonal complex 8 infections linked to consumption of cold-smoked fish products. EFSA Supporting Publication; 2019. p. 20. https://doi.org/10.2903/sp.EFSA. EN-1665.

[8] Ferreira V, Wiedmann M, Teixeira P, Stasiewicz MJ. *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. J Food Prot 2014;77:150—70.

[9] Overney A, Jacques-André-Coquin J, Ng P, Carpentier B, Guillier L, Firmesse O. Impact of environmental factors on the culturability and viability of *Listeria monocytogenes* under conditions encountered in food processing plants. Int J Food Microbiol 2017;244:74—81.

[10] Prakash B, Veeregowda BM, Krishnappa G. Biofilms: a survival strategy of bacteria. Curr Sci 2003;85:1299—307.

[11] Garrett TR, Bhakoo M, Zhang Z. Bacterial adhesion and biofilms on surfaces. Prog Nat Sci 2008;18:1049—56.

[12] Messi P. Biofilm formation, development and relevance. In Biofilm in bioengineering. Nova Science 2013;268:1—26.

[13] Mizan FR, Jahid IK, Ha SD. Microbial biofilms in seafood: a food-hygiene challenge. Food Microbiol 2015;49:41—55.

[14] Cortés ME, Bonilla JC, Sinisterra RD. Biofilm formation, control and novel strategies for eradication. In: Mendez-Vilas A, editor. Science against microbial pathogens: communicating current research and technological advances, vol. 2. Spain: Formatex Research Center; 2011. p. 896—905.

[15] Galié S, García-Gutiérrez C, Miguélez EM, Villar CJ, Lombó F. Biofilms in the food industry: health aspects and control methods. Front Microbiol 2018;9: 898. https://doi.org/10.3389/fmicb.2018.

[16] Gokoglu N. Novel natural food preservatives and applications in seafood preservation: a review. J Sci Food Agric 2019;99:2068—77.

[17] Baptista RC, Horita CN, Sant'Ana AS. Natural products with preservative properties for enhancing the microbiological safety and extending the shelf-life of seafood: a review. Food Res Int 2020;127:108762. https://doi.org/10.1016/j.foodres.2019.108762.

[18] Cintas Lm, Casaus MP, Herranz C, Nes IF, Hernandez PE. Review: Bacteriocins of lactic acid bacteria. Food Sci Tech Int 2001;7:281—305.

[19] Riley MA, Wertz JE. Bacteriocins: evolution, ecology, and application. Ann Rev Microbiol 2002;56:117—37.

[20] Anacarso I, de Niederhausern S, Iseppi R, Sabia C, Bondi M, Messi P. Anti-listerial activity of chitosan and Enterocin 416K1 in artificially contaminated RTE products. Food Control 2011;22:2076—80.

[21] Iseppi R, de Niederhausern S, Anacarso I, Messi P, Sabia C, Pilati F, et al. Anti-listerial activity of coatings entrapping living bacteria. Soft Matt 2011;7: 8542—8.

[22] Anacarso I, Messi P, Condò C, Iseppi R, Bondi M, Sabia C, et al. A bacteriocin-like substance produced from *Lactobacillus pentosus* 39 is a natural antagonist for the control of *Aeromonas hydrophila* and *Listeria monocytogenes* in fresh salmon fillets. LWT - Food Sci Technol 2014;55:604—11.

[23] Anacarso I, Gigli I, Bondi M, de Niederhäusern S, Stefani S, Condò C, et al. Isolation of two lactobacilli, producers of two new bacteriocin-like substances (BLS) for potential food-preservative use. Eur Food Res Technol 2017;243: 2127—34.

[24] Degli Esposti M, Toselli M, Sabia C, Messi P, de Niederhäusern S, Bondi M, et al. Effectiveness of polymeric coated films containing bacteriocin-producer living bacteria for *Listeria monocytogenes* control under simulated cold chain break. Food Microbiol 2018;76:173—9.

[25] Iseppi R, Stefani S, de Niederhäusern S, Bondi M, Sabia C, Messi P. Characterization of anti-*Listeria monocytogenes* properties of two bacteriocin-producing *Enterococcus mundtii* isolated from fresh fish and seafood. Curr Microbio 2019;76:1010—9.

[26] Barbosa LN, Mores Rall VL, Henrique Fernandes AA, Ikeda Ushimaru P, da Silva Probst I, Fernandes Jr R A. Essential oils against foodborne pathogens and spoilage bacteria in minced meat. Foodborne Path Dis 2009;6:725—8.

[27] Andrade BFMT, Barbosa LN, da Silva Probst I, Fernandes AJ. Antimicrobial activity of essential oils. J Essent Oil Res 2014;26:34—40.

[28] Gouveia AR, Alves M, de Almeida JMMM, Monteiro-Silva F, González-Aguilar G, Silva JA, et al. The antimicrobial effect of essential oils against *Listeria monocytogenes* in sous vide cook-chill beef during storage. J Food Proc Pres 2017;41:e13066. https://doi.org/10.1111/jfpp.13066.

[29] Iseppi R, Sabia C, de Niederhäusern S, Pellati F, Benvenuti S, Tardugno R, et al. Antibacterial activity of *Rosmarinus officinalis* L. and *Thymus vulgaris* L. essential oils and their combination against food-borne pathogens. Nat Prod Res 2019b;33:3568—72.

[30] Marino M, Bersani C, Comi G. Impedance measurements to study the antimicrobial activity of essential oils from *Lamiaceae* and *Compositae*. Int J Food Microbiol 2001;67(3):187—95. https://doi.org/10.1016/s0168-1605(01)00447-0.

[31] Afonso AF, Pereira OR, Fernandes Â, Calhelha RC, Silva AMS, Ferreira I, et al. Phytochemical composition and bioactive effects of *Salvia africana*, *Salvia officinalis 'Icterina'* and *Salvia mexicana* aqueous extracts. Molecules 2019;24(23):4327. https://doi.org/10.3390/molecules24234327. Published 2019 Nov 27.

[32] Sakkas H, Papadopoulou C. Antimicrobial activity of basil, oregano, and thyme essential oils. J Microbiol Biotechnol 2017;27(3):429—38. https://doi.org/10.4014/jmb.1608.08024.

[33] Wijesundara NM, Rupasinghe HPV. Bactericidal and anti-biofilm activity of ethanol extracts derived from selected medicinal plants against *Streptococcus pyogenes*. Molecules 2019;24(6):1165. https://doi.org/10.3390/molecules24061165. Published 2019 Mar 24.

[34] Wińska K, Mączka W, Łyczko J, Grabarczyk M, Czubaszek A, Szumny A. Essential oils as antimicrobial agents-myth or real alternative? Molecules 2019;24(11):2130. https://doi.org/10.3390/molecules24112130. Published 2019 Jun 5.

[35] Clinical and Laboratory Standard Institutes (CLSI). Performance standards for antimicrobial susceptibility testing: approved standard. Wayne, PA, USA: M100-S20; 2012.

[36] Klančnik A, Piskernik S, Jeršek B, Smole Možina S. Evaluation of diffusion and dilution methods to determine the antibacterial activity of plant extracts. J Microbiol Methods 2010;81:121—6.

[37] Rogers AM, Montville TJ. Improved agar diffusion assay for nisin quantification. Food Biotechnol 1991;5:161—8.

[38] Mayr-Harting A, Hedges AJ, Berkeley RCW. Methods for studying bacteriocins. In: Norris JR, Ribbons DW, editors. Methods in microbiology, vol. 7a. New York: Academic Press; 1972. p. 313—42.

[39] Sahin F, Güllüce M, Daferera D, Sökmen A, Sökmen M, Polissiou M, et al. Biological activities of the essential oils and methanol extract of *Origanum vulgare* ssp. vulgare in the Eastern Anatolia region of Turkey. Food Control 2004;15:549—57.

[40] Condò C, Anacarso I, Sabia C, Iseppi R, Anfelli I, Forti L, et al. Antimicrobial activity of spices essential oils and its effectiveness on mature biofilms of human pathogens. Nat Prod Res 2020;34:567—74.

[41] Stepanović S, Vuković D, Hola V, Di Bonaventura G, Djukić S, Irković IC, et al. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendation for assessment of biofilm production by staphylococci. APMIS 2007;115:891—9.

[42] Maté J, Periago PM, Palop A. Combined effect of a nanoemulsion of D-limonene and nisin on *Listeria monocytogenes* growth and viability in culture media and foods. Food Sci Technol Int 2015;22:146—52.

[43] Moreira MR, Ponce AG, del Valle CE, Rour SI. Inhibitory parameters of essential oils to reduce a foodborne pathogen. LWT - Food Sci Technol 2005;38: 565—70.

[44] Viuda-Martos M, Ruiz-Navajas Y, Fernandez-Lopez J, Perez-Alvarez JA. Antibacterial activity of different essential oils obtained from spices widely used in Mediterranean diet. Int J Food Sci Technol 2008;43:526—31.

[45] Samadi N, Sharifan A, Emam-Djomeh Z, Salehi Sormaghi MH. Biopreservation of hamburgers by essential oil of *Zataria multiflora*. Nat Prod Res 2011;26: 665—8.

[46] Ruiz-Navajas Y, Viuda-Martos M, Sendra E, Perez-Alvarez JA, Fernández-López J. Chemical characterization and antibacterial activity of *Thymus moroderi* and *Thymus piperella* essential oils, two *Thymus* endemic species from southeast of Spain. Food Control 2012;27:294—9.

[47] Mith H, Duré R, Delcenserie V, Zhiri A, Daube G, Clinquart A. Antimicrobial activities of commercial essential oils and their components against food-borne pathogens and food spoilage bacteria. Food Sci Nutr 2014;2:403—16.

[48] Marrelli M, Conforti F, Formisano C, Rigano D, Arnold NA, Menichini F, et al. Composition, antibacterial, antioxidant and antiproliferative activities of essential oils from three *Origanum* species growing wild in Lebanon and Greece. Nat Prod Res 2016;30:735—9.

[49] Singh N, Singh RK, Bhunia AK, Stroshine RL. Efficacy of chlorine di oxide, ozone and thyme essential oil or a sequential washing in killing *Escherichia coli* 0157:H7 on lettuce and baby carrots. LWT-Food Sci Technol 2002;35: 720—9.

[50] Ghorbani A, Esmaeilizadeh M. Pharmacological properties of *Salvia officinalis* and its components. J Trad Comp Med 2017;7:433—40.

[51] Langeveld WT, Veldhuizen EJ, Burt SA. Synergy between essential oil components and antibiotics: a review. Crit Rev Microbiol 2014;40(1):76—94. https://doi.org/10.3109/1040841X.2013.763219.

[52] Kumariya R, Garsa AK, Rajput YS, Sood SK, Akhtar N, Patel S. Bacteriocins: classification, synthesis, mechanism of action and resistance development in food spoilage causing bacteria. Microb Pathog 2019;128:171—7. https://doi.org/10.1016/j.micpath.2019.01.002.

[53] Turgis M, Vu KD, Dupont C, Lacroix M. Combined antimicrobial effect of essential oils and bacteriocins against foodborne pathogens and food spoilage bacteria. Food Res Int 2012;48:696—702.

[54] Ghalfi H, Benkerroum N, Doguiet DDK, Bensaid M, Thonart P. Effectiveness of cell-adsorbed bacteriocin produced by *Lactobacillus curvatus* CWBI-B28 and selected essential oils to control *Listeria monocytogenes* in pork meat during cold storage. Lett Appl Microbiol 2007;44:268—73.

[55] Cobo Molinos A, Abriouel H, López RL, Omar NB, Valdivia E, Gálvez A. Enhanced bactericidal activity of enterocin AS-48 in combination with essential oils, natural bioactive compounds and chemical preservatives against *Listeria monocytogenes* in ready-to-eat salad. Food Chem Toxicol 2009;47:2216—23.

[56] Food and Drug Administration. Secondary direct food additives permitted in food for human consumption. 2002.

[57] Singh B, Falahee MB, Adams MR. Synergistic inhibition of *L. monocytogenes* by nisin and garlic extract. Food Microbiol 2001;18:133—9.