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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**Abstract**

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 (OD) 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 combination 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.

**Keywords:** *Thymus vulgaris* 
*Salvia officinalis* 
Bacteriocin 
*Listeria monocytogenes* 
Biofilm 
Biopreservatives

**1. Introduction**

*Listeria monocytogenes* is a psychrotrophic 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].

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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 have attracted attention as potential antibiotic alternative to control Listeria monocytogenes both in planktonic and in sessile forms, Research in Microbiology, https://doi.org/10.1016/j.resmic.2020.07.002

2. Materials and methods

2.1. Bacterial strains, media and culture conditions

The EOs, obtained by hydro-distillation of dried spices were previously isolated from red mullet and endowed with a strong antibacterial 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.

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^−10^ 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 L. monocytogenes 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×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., Bedford, 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 [36,38]. 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 (TSB, 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

| 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 10^5 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 determined using 96-well polystyrene microtiter plates, as previously described [40]. Polystyrene microtiter plates were inoculated with bacterial 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; 1/4 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
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 FIC-Index 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

| Indicators strains | Essential oils MIC (μL/mL) | FIC-Index of essential oils/SD | MIC of BacLP17 (μL/mL) | FIC-Index of BacLP17 and S. officinalis/SD | FIC-Index of BacLP17 and T. vulgaris/SD |
|---------------------|-----------------------------|-------------------------------|-----------------------|------------------------------------------|----------------------------------------|
| 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.

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.

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0.00000009) 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 psychrotrophic bacteria, such as L. monocytogenes. A major safety risk is associated with psychrotrophic pathogens in substrates 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-L. monocytogenes 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 effective.

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.

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Declaration of Competing Interest

The author reports no conflicts of interest in this work.

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