

Comparison of the Effects of Essential Oils and Antibiotics on *Listeria monocytogenes* Isolates [†]

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Abstract: Food contamination with *Listeria monocytogenes* can cause health problems of increasing global concern. The resistance of *L. monocytogenes* to antibiotics requires finding alternative solutions to protect human health. This mini study was designed to evaluate the effects of antibiotics and some particular essential oils on *L. monocytogenes* isolates from food of animal origin and isolates from food-producing surfaces. This aim was to investigate the effects of seven antibiotics (amikacin, gentamicin, penicillin, cephalixin, ceftriaxone, tetracycline, and nalidixic acid) on *L. monocytogenes* isolated from food and surface samples. Also, the antibacterial activity of four essential oils (thyme, oregano, peppermint, and rosemary) against the growth of *L. monocytogenes* isolates was investigated. *Listeria monocytogenes* isolates were from food of an animal origin and food-producing surfaces. The isolation and determination of *L. monocytogenes* from food samples followed ISO 11290-1. Surface samplings were performed in compliance with ISO 18593, followed by the isolation and determination of *L. monocytogenes*. A disc diffusion method was used, and the tests were performed in triplicate. The results of this study confirmed the pronounced antibacterial activity of essential oils compared to antimicrobial drugs. The essential oil of *Thymus vulgaris* showed a bactericidal effect against all tested *L. monocytogenes* isolates.

Keywords: antibiotics; essential oils; *Listeria monocytogenes*



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1. Introduction

Food contamination with *Listeria monocytogenes* can cause health problems of increasing global concern. The disease caused by the presence of *Listeria monocytogenes* is mostly transmitted through contaminated food, inadequately pasteurised milk, soft cheeses, fermented sausages, and rarely directly from sick animals [1,2]. Besides animal-derived foods (ADFs), listeriosis often occurs as a result of consuming fresh cabbage and lettuce, green salads, celery, tomatoes, cucumbers, potatoes, radishes, and other vegetables [3,4].

The primary choice of antibiotic therapy for treating human listeriosis involves the use of β -lactams (penicillin and ampicillin) alone or in combination with an aminoglycoside, such as gentamicin or streptomycin. Additionally, tetracycline, vancomycin, rifampicin, chloramphenicol, and fluoroquinolones can also be used to treat listeriosis [5]. The increasing resistance of *L. monocytogenes* to antibacterial drugs worldwide is concerning [6]; therefore, the efforts to find alternative antibacterial treatments are intensifying [7,8]. Aouadhi et al. [9] tested eight essential oils for their antimicrobial activity against six species belonging to the genus of *Staphylococcus*, which were multi-resistant to antibiotics, and reported that the antimicrobial activity significantly varied depending on the tested essential

oils and bacterial species. Many studies have shown that essential oils from spice plants could reduce the number of *L. monocytogenes* in food [10,11]. Vidaković Knežević et al. [12] obtained results indicating the good activity of some selected essential oils on *L. monocytogenes*, with bacteriostatic, bactericidal, and antibiofilm effects.

This mini study is designed to evaluate the effects of antibiotics and some particularly essential oils purchased in pharmacies on *L. monocytogenes* isolates from food of animal origin and isolates from food-producing surfaces.

2. Material and Methods

2.1. Isolation of *L. monocytogenes*

Seven strains of *L. monocytogenes* isolated in the Laboratory for Food Microbiology, Feed and Water, PI Veterinary Institute RS “Dr. Vaso Butozan” Banja Luka, were used. The origin of isolates was as follows: isolate 1—fish, isolate 2—minced meat, isolate 3—minced meat, isolate 4—fresh meat, 5—minced meat, 6—surface sample from fish market, 7—surface sample from fish market.

Isolation and determination of *L. monocytogenes* from food samples were performed following ISO 11290-1 [13], including the API Listeria Test (bioMérieux, Craponne, France). Briefly, in the pre-enrichment step, 25 g of food was homogenised with 225 mL of half-Fraser broth (HFB) (HiMedia, Mumbai, India) and incubated at 30 °C for 24 h. After incubation, the primary broth was streaked on an ALOA agar (HiMedia, India) and Oxford agar (HiMedia, India) plates. Also, 0.1 mL from incubated HFB was transferred to 10 mL of Frazer broth (FB) (HiMedia, India). Inoculated FB was incubated at 37 °C for 48 h followed by a streak on an ALOA agar and Oxford agar plates. ALOA and Oxford agar plates were incubated at 37 °C for 24 h and prolonged for another 24 h. Blue–green colonies with opaque halo from ALOA agar and black colonies with black halo and a sunken centre from Oxford agar were streaked on tryptone soya extract yeast agar (TSYEA) (HiMedia, India) and incubated at 37 °C for 24 h. The single colonies from non-selective TSYEA were used for confirmation via the microscopic aspect, haemolysis test on blood agar plates (Torlak, Serbia), and the biochemical reaction by the API Listeria test (bioMérieux, France).

Surface samplings were performed in compliance with ISO 18593 [14], followed by isolation and determination of *L. monocytogenes*, as is mentioned above. Briefly, after wetting in 10 mL of Buffered Peptone Water (HiMedia, India), a sterile swab stick was used for sampling from 1000 cm² of the surface and returned to the tube with Buffered Peptone Water. In the laboratory, the received swab sample in 10 mL of Buffered Peptone water was homogenised, and 10 mL of Buffered Peptone Water was homogenised with 90 mL of HF in the pre-enrichment step of ISO11290-1 [13]. The enrichment, isolation, and identification of *Listeria monocytogenes* followed the steps described above in the food analysis.

Isolates from food samples and isolates from surface swabs were stored at a tryptone soya agar (TSA) (HiMedia, India) slant at 3 ± 2 °C, no more than three days before the antimicrobial test.

2.2. Antimicrobial Susceptibility Test

L. monocytogenes isolates were transferred from TSA to nutrient broth (Biolife, Milan, Italy) by loop and incubated for 18 h at 37 °C. The prepared bacterial suspension was adjusted to a 0.5 McFarland of turbidity. Petri dishes containing a Müller–Hinton agar (M-H) (Condalab, Spain) were inoculated with 100 µL of the *L. monocytogenes* suspension with an estimated 10⁵ CFU/mL concentration. The agar diffusion method [15] was used to determine the antibacterial activity of essential oils on the growth of *L. monocytogenes*. Paper discs with a diameter of 9 mm were placed on the surface of the previously inoculated M-H agar. Each disc was loaded with 100 µL of essential oil using a micropipette. The essential oils were purchased in pharmacies in Banja Luka, Bosnia, and Herzegovina. The following essential oils were used: *Thymus vulgaris* (Pharmamed, Travnik, Bosnia and Herzegovina), *Origanum vulgare* (Marnys, Spain), *Rosmarinus officinalis* (Biofarm, Croatia), and *Mentha*

piperita (Biofarm, Croatia). As a control, 100 µL of distilled water was dropped onto the disc. The test was performed in triplicate.

Furthermore, to test the antibacterial susceptibility of all seven *L. monocytogenes* isolates, the following antibiotic discs were used: amikacin (30 µg) (Liofilchem, Roseto degli Abruzzi, Italy), gentamicin (10 µg) (Liofilchem, Italy), penicillin (10IU) (Liofilchem, Italy), cephalixin (30 µg) (Liofilchem, Italy), ceftriaxone (30 µg) (Liofilchem, Italy), tetracycline (30 µg) (Liofilchem, Italy), and nalidixic acid (30 µg) (Liofilchem, Italy). Antibiotic discs were placed on the surface of the M-H agar. Subsequently, the M-H agar plates were left in the refrigerator for 30 min to allow the essential oils and antibiotics to diffuse into the medium followed by incubation at 37 °C for 24 h.

The parts of the agar with a visible inhibition zone were cut and transferred to the nutrient broth (HiMedia, India) to assess the mode of action of essential oils on the growth of *L. monocytogenes*. Incubation was carried out at 37 °C for 24 h. If the broth became cloudy after incubation, the action of the essential oil was considered bacteriostatic. If the broth remained clear, the action of the essential oil was considered bactericidal.

A two-way ANOVA with replications (Microsoft® Excel 2013) was used for statistical analysis of results.

3. Results and Discussion

The tested *L. monocytogenes* isolates are the results of routine analyses of the health safety of food of animal origin and of surfaces that come into contact with food. Das et al. [16] reported that *Listeria* spp. was detected in 32.3%, 27.1%, and 5% of fresh, frozen, and dry fish samples, respectively. In environmental samples from fisheries, *Listeria* spp. was detected in 29% of samples (20 out of 69) and *L. monocytogenes* in one sample (ice).

Some studies have shown that in the EU regions, most listeriosis outbreaks are associated with the contamination of crabs, shellfish, molluscs, meat and meat products, cheese, vegetables, and juices [17]. Following contamination of food with this pathogen, antibiotic resistance is another major public health concern and emphasises the need to pay particular attention to the control of the pathogen along the food chain and disease management in patients. Olaimat et al. [5] reported that strains of *L. monocytogenes* have shown resistance to cephalothin, nalidixic acid, cephalosporins, cefotaxime, cefepime, fosfomycin, oxacillin, and lincomycin.

Based on the results presented in Figure ??, amikacin showed an inhibitory effect on six *L. monocytogenes* isolates with inhibition zone diameters ranging from 17.00 mm to 28.33 mm, whereby one isolate (fresh meat) was not inhibited. The same isolate also showed the absence of an inhibition zone around the tetracycline disc. Penicillin showed inhibition zones from 15.7 mm to 28.3 mm, gentamicin 14.3 mm–25.0 mm, followed by weaker inhibition zones of cephalixin (10.3 mm–23.0 mm), and ceftriaxone (10.7 mm–19.3 mm). All isolates were resistant to nalidixic acid. Based on the ANOVA calculation, the sources of significant differences in the antibacterial effect ($p < 0.001$) are the antibiotics used, the isolates, and the interaction between antibiotics and isolates.

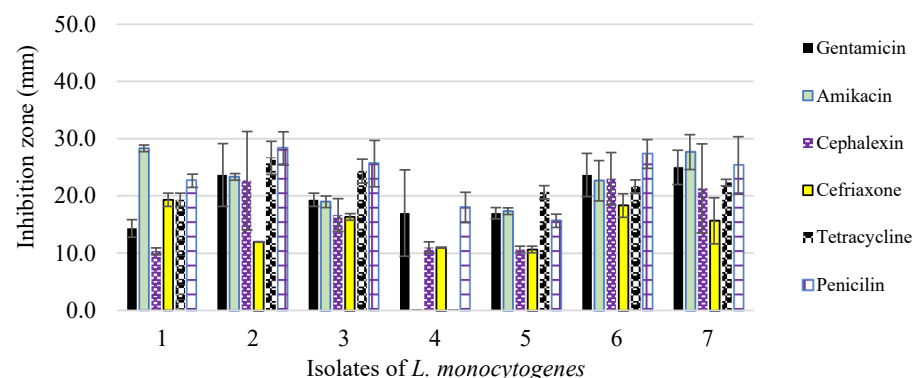


Figure 1. Antibacterial activity of antimicrobial drugs (except nalidixic acid) against *L. monocytogenes* isolates.

The *L. monocytogenes* isolates used in this study showed resistance profiles to multiple drugs, which is consistent with the results of other researchers [18,19]. Kumaraswamy et al. [20] found high levels of resistance to ampicillin, cefotaxime, and penicillin in *L. monocytogenes* isolates from raw and cooked meat and fish products. In addition, *L. monocytogenes* isolated from dairy cow farms showed resistance to ampicillin [21,22], penicillin [23], and tetracycline [24]. A higher resistance rate was found for penicillin, nalidixic acid, tetracycline, and chloramphenicol in samples of cakes, raw meat, ice cream, minced beef, fish, unpasteurised milk, and pizza [25]. Wieczorek et al. [26] found resistance of *L. monocytogenes* to ceftriaxone and gentamicin in a bovine-hide- and -carcass-processing plant. In a study conducted by Capita et al. [27], the sensitivity of 72 strains of *L. monocytogenes* to 26 antibiotics was investigated using the disc diffusion method. They found high resistance (51.4–93.1%) to cefoxitin, cefotaxime, cefepime, and nalidixic acid. In addition, lower resistance (3%) was found to ampicillin, gentamicin, rifampicin, chloramphenicol, enrofloxacin, vancomycin, trimethoprim–sulfamethoxazole, erythromycin, and tetracycline.

Furthermore, *L. monocytogenes* strains resistant to penicillin have been isolated from various sources: cabbage, water, the environment, food samples including dairy products and ready-to-eat (RTE) vegetables, and the environment of fish markets [23,28,29]. Resistance to tetracyclines has been detected in isolates from fish, fresh and frozen vegetables, ready-to-eat products, dairy products, fresh meat, production facilities, raw chicken meat, RTE, and in the processing environment [30–33].

The use of antimicrobial drugs is the main strategy for the treatment of infections and control of foodborne diseases caused by *L. monocytogenes*. However, indiscriminate or inappropriate use of antimicrobial drugs has serious consequences, including the emergence of resistant strains. Lyon et al. [34] reported that the percentage of resistance to antibiotic varied between 59% and 63%, depending on the sample from which listeria was isolated. In this study, gentamicin showed an inhibitory effect on all seven isolates, with inhibition zone diameters ranging from 10.00 mm to 25.00 mm. In the case of invasive listeriosis, gentamicin is an important drug for treatment. Although resistance to gentamicin in clinical strains of *L. monocytogenes* is still rare, several mechanisms and determinants of resistance have been discovered in recent decades [35]. Given the growing concern about antimicrobial resistance, it is important that antimicrobials are used judiciously and that resistance patterns are monitored. The discovery of new mechanisms and determinants of resistance emphasises the need for ongoing research and surveillance to effectively combat the emergence and spread of resistant *L. monocytogenes* strains.

The literature provides data on the effect of essential oils on the control and inhibition of *L. monocytogenes* in meat and poultry products. However, the interaction of essential oils with various food ingredients may reduce their antimicrobial properties [36,37]. Consequently, higher concentrations of the essential oil may be necessary to achieve an acceptable antibacterial effect. Essential oil constituents as food preservatives can cause a negative organoleptic effect when added in sufficient amounts to provide antimicrobial effect [38].

The antibacterial activity of the essential oils of thyme, oregano, rosemary, and peppermint tested on seven isolated strains of *L. monocytogenes* is shown in Figure 2. Figure 2 shows the antibacterial activity of essential oils of thyme, oregano, rosemary, and peppermint, tested on seven *L. monocytogenes* isolates. Based on the ANOVA calculation, the sources of significant differences in antibacterial activity ($p < 0.001$) are the essential oils used, the isolates, and the interaction between essential oils and isolates.

When comparing the effects of the essential oils and the antibiotics used in this study, it can be noted that some essential oils showed stronger antibacterial activity than antibiotics. Thyme essential oil showed a strong inhibitory effect on the growth of all seven *L. monocytogenes* isolates, with inhibition zone diameters ranging from 25.67 mm to 36.67 mm. Oregano essential oil showed inhibition zones ranging from 22.00 mm to 43.33 mm against the tested isolates. Rosemary essential oil inhibited the growth of six *L. monocytogenes* isolates, with inhibition zones between 10.00 and 40.67 mm, while it showed no inhibitory effect on one isolate. Peppermint essential oil showed an inhibitory

effect against all tested isolates, with inhibition zones ranging from 10.00 mm to 31.67 mm. The inhibition zones are much more pronounced by essential oils than antibiotics when the results presented in Section 3 and Figure 2 are compared.

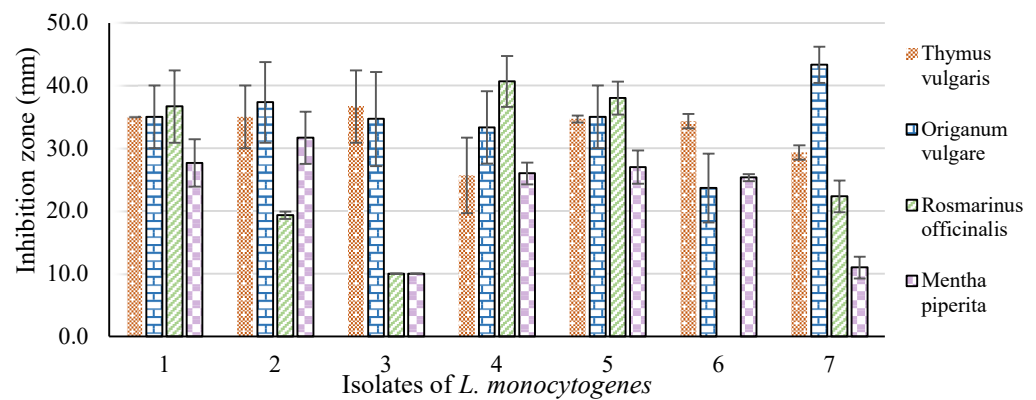


Figure 2. Antibacterial activity of essential oils against *L. monocytogenes* isolates.

The results obtained for the antibacterial activity of thyme, peppermint, oregano, and rosemary essential oils are consistent with other studies, confirming that essential oils have good antibacterial properties against *L. monocytogenes* isolates [39–41]. The results indicate the potential for preventive measures based on the application of essential oils against the hazards associated with this pathogen in food. Giarratana et al. [42] also found that a mixture of rosemary and thyme has a bacteriostatic effect on *L. monocytogenes*.

The results of the mode of action of the essential oils on *L. monocytogenes* are presented in Figure 3. Thyme essential oil showed a bactericidal effect on all *L. monocytogenes* isolates in all replicates. Oregano essential oils showed bactericidal activity against six isolates, and peppermint essential oil against five isolates. Mentha piperita essential oil showed bactericidal activity against five out of seven isolates of *L. monocytogenes*. Thyme essential oil showed no activity against one surface sample and bactericidal activity on five of six isolates.

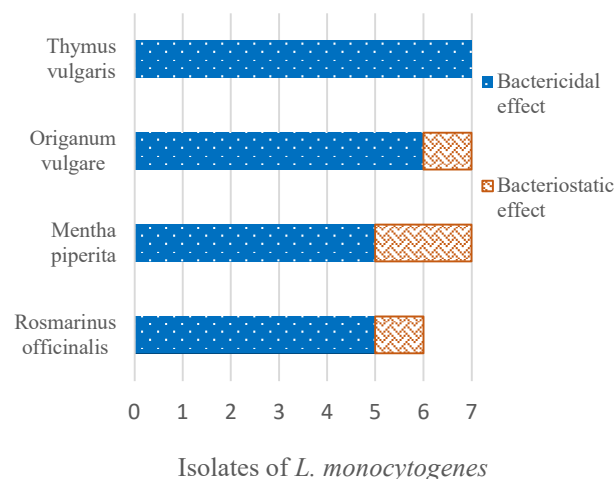


Figure 3. Mode of action of essential oils.

Bacteriostatic effects were observed in all three replicates for peppermint essential oil against two isolates. Gouveia et al. [41] concluded that essential rosemary oil can be an effective preservative. Many studies have shown that the antibacterial activity of essential oils depends on the concentration of phenolic compounds present in the oil. The antibacterial activity of oregano essential oil has been confirmed and used to control *L. monocytogenes* in meat and meat products [43,44]. The results showed a bacteriostatic effect of oregano against one isolate. The rosemary essential oil also showed a bacteriostatic

effect against one isolate. So, thanks to the antibacterial action of essential oils, the use of culinary herbs in food preparation, can lead to the prevention and hindered growth, as well as a reduction in the number, of *L. monocytogenes* in food.

4. Conclusions

Food contamination with *L. monocytogenes* can cause health problems of increasing global concern. Accordingly, it is desirable to know alternative ways to prevent its microbial growth. Results of antibiotic susceptibility testing provide valuable information about the resistance to antibiotics of *L. monocytogenes* bacteria isolated from food of animal origin and surfaces that come into contact with food. *L. monocytogenes* isolated from fresh meat showed the absence of an inhibition zone around tetracycline and amikacin discs. The essential oils, compared to antibiotics, showed more pronounced antibacterial activity against *L. monocytogenes*, based on the width of the inhibition zone. In particular, it should be emphasised that the essential oil of *Thymus vulgaris* showed a bactericidal effect against all tested *L. monocytogenes* isolates. However, rosemary essential oil showed no inhibitory effect on *L. monocytogenes* isolated from the surface sample, indicating the importance of the isolate origin and previous environmental conditions.

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Conflicts of Interest: Author Dragana Kalaba was employed by the company AU “Benu”. She participates in the paper: literature investigation, review and editing. AU “Benu” is not in any commercial or financial relationships with the paper.

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