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Antimicrobial Activity of Some Plant Essential Oils Against *Listeria monocytogenes*[1]

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ABSTRACT

The antimicrobial activity of 32 plant essential oils commonly used in food industry was examined against four strains of *Listeria monocytogenes* and one strain of *Listeria innocua*. Two different procedures were carried out to test the essential oils, a paper disc diffusion method and an inhibition curve. In the former procedure an absolute ethanolic solution (1:5 v/v) of each oil was tested on the plates inoculated with a bacterial concentration of 10^6 CFU/ml. Five of the 32 essential oils (cinnamon, clove, origanum, pimento, and thyme) showed antibacterial activity. Some of the five oils were also tested at lower concentration (1:50 v/v). The inhibition curve to study antilisteric efficacies of the five oils in a saline solution system was examined. Pimento oil showed marked and rapid activity (generally within 1 h of exposure), whereas clove, origanum, and thyme oils showed a more slow activity. The antilisteric activity of the tested oils seems to be strain dependent. A *L. monocytogenes* strain was also tested in a food matrix (minced pork meat) against thyme essential oil. Minced pork meat with thyme oil reduced the *L. monocytogenes* population by ca. 100-fold over the first week of storage.

Listeria monocytogenes is a gram-positive microorganism responsible for infections known as listeriosis in humans and many animal species. The disease mainly occurs in individuals whose immunosystem has been compromised as in pregnant women and their fetuses or neonates (*12*). The most common listeric infections fall into one of these categories: septicemia, meningitis, and a flu-like illness in pregnancy. Within the last 10 years, food-associated outbreaks of listeriosis in different countries have increased the concern for this microorganism, now fully recognized as a foodborne pathogen. *L. monocytogenes* is ubiquitous in nature and has been isolated from a variety of sources like soil, vegetation, feces of healthy humans, and food products including milk, cheeses, meat products, fish, and vegetables (*4,8-10,14,15*). As suggested by a World Health Organization report (*16*), transmission of foodborne listeriosis to humans is the result of environmental contamination involving both food supply and food processing plant.

Research is being conducted on the control or elimination of *L. monocytogenes* contamination in food, although only scattered results are available regarding the inhibitory activities of physical treatments and antimicrobial substances.

This concern prompted us to examine the possible antilisteric activity of some spices, commonly used in food industries. In the past few years, several studies have been carried out on the effect of spices for some antimicrobial and/or antifungal activity. Essential oils (EO) from spices have demonstrated inhibitory activity against foodborne pathogens such as *Aspergillus flavus, Staphylococcus aureus, Salmonella typhimurium*, and *Clostridium botulinum (5-7)*. The main purpose of this study was to determine the antilisteric effects of 32 essential oils.

MATERIALS AND METHODS

Bacterial strains

The *Listeria* strains used in this work were *L. monocytogenes* LL201 (human origin), and *L. innocua* PF59 (isolated from cheese) from Dr. J. Bille, Centre Hospitalier Universitaire Vaudois, Institut de Microbiologie, Lausanne, Switzerland; *L. monocytogenes* Scott A (human origin) from Dr. M. P., Doyle, University of Wisconsin, Madison; *L. monocytogenes* isolated from cheese (I.S.S. L12), from Dr. G. Terplan, Ludwig Maximilians Universitat, Munchen, Germany; *L. monocytogenes* (I. S. S. L28), from Dr. B. M. Hill, Dairy Research Institute, Palmerston North, New Zealand.

Essential oils (EO)

The EO used in this work are listed in Table 1. All EO were obtained from ABOCA (Arezzo) and CGI (Milano). The controlled title of EO was 99%, as indicated by the producers. All the EO were held at room temperature and stored in the dark when not in use.

Preparation of bacterial cultures for inhibitory tests

The strains were maintained in tryptic soy agar (TSA, Difco Laboratories, Detroit, MI) slants at 5°C. Stock cultures were grown in trypticase soy broth + 0.6% yeast extract at 32°C for 18-22 h.

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[2] *Deceased.*

TABLE 1. *Plant essential oils tested for antimicrobial properties.*

Basil	Neroly
Camomile-German-	Nutmeg
Camomile-Roman-	Onion
Celery	Orange
Cinnamon	Origanum
Clove	Parsley
Coriander	Pepper
Cumin	Peppermint
Estragon	Peppermint-Piedmontese-
Fennel-seed	Pettigrain
Garlic	Pimento-berry
Ginger	Rosemary
Laurel	Saffron
Lemon	Sage
Mandarin	Thyme
Marjoram	Vanilla*

* Oleoresin.

Determination of inhibitory activity of EO against Listeria *strains by paper disk diffusion method*

The agar diffusion method was used to detect the antimicrobial activity of EO. Filter paper disks (Oxoid, Basingstoke, England) (13 mm) were soaked with 50 µl of a solution 1:5 v/v of each EO in absolute ethanol. Each strain was tested against all EO. The experiment was performed with a bacterial inoculum of ca. 10^6 CFU/ml.

TSA was inoculated with each bacterial strain. The soaked disks were put in the middle of plates which were incubated at 32°C for 24 h in the inverted position

All experiments were conducted in duplicate and the results are expressed as average values of inhibition halo. For each strain, it had previously been determined that 40 µl of absolute ethanol did not give rise to any zone of inhibition; 1:50 dilution in EtOH of three EO (clove, thyme, and origanum) was also tested to compare the activity of two EO concentrations.

Inhibition curve

Five of the more effective EO in the Agar Diffusion Method were tested in a saline solution system. This test involves inoculation of *Listeria* strains in saline solution (0.9%), addition of EO followed by incubation and periodic sampling to determine survivals.

The bacterial suspension of each culture was diluted to 5-10 x 10^7 CFU/ml; 99 ml saline solution containing 500 µl of alcoholic solution of each EO (1:5 in absolute alcohol) was challenged with 1 ml of the testing suspension. Flasks were maintained in a shaking plate at room temperature and periodic sampling was performed at contact time of 0.5, 1, 2, 4, and 24 h.

At each sampling time, a dilution series was made in saline solution and 0.5 ml of each dilution was surface plated onto duplicate TSA plates. Plates were incubated at 32°C for 48 h and then counted for viable organisms.

All the experiments were performed in duplicate against a control with 500 µl of absolute ethanol to verify the lack of an antibacterial activity of the EO solvent. All the data are expressed as the average of the experimental results.

Inhibition activity in minced pork meat

Pork meat (ca. 500 g) was purchased at a retail market. The meat was examined to verify the absence of antibacterial residues, as described by Bogaerts and Wolf (*3*), and the absence of *Listeria* spp., following the McClain and Lee method (*11*). Total viable count (1.0-2.0 x 10^5 CFU/g) was estimated by a plate count method on TSA.

The pork meat was minced in our laboratory through a 0.49-cm grinder working for 5 min. The minced meat was divided into portions of 25 g that were aseptically put into sterile Stomacher bags.

Listeria monocytogenes L28 culture was decimally diluted in sterile saline solution in order to achieve a level of 1.0-2.0 x 10^7 CFU/g of the minced meat.

One hundred µl of alcoholic solution of thyme EO (1:5 in absolute alcohol) was added dropwise over the surface of inoculated minced meat, except the control meat samples. Both the inocula of *L. monocytogenes* L28 and the addition of EO were distributed in the minced pork meat by hand kneading for 5 min.

The Stomacher bags containing the inoculated meat were kept at 4°C (± 0.2) or 8°C (± 0.2) for 4 or 8 d. After this time, duplicate samples of inoculated meat were added with 225 ml of sterile saline solution. All samples were homogenized for 120 s using a Lab-Blender Stomacher (PBI 400).

Further serial dilutions of each sample were made using the same diluent. Duplicate spread plates were made at appropriate dilution for each sample on McBride's Listeria Agar. The plates were inverted and incubated 48 h at 32°C for the enumeration of viable *L. monocytogenes* L28.

Some of the colonies which were 0.5 to 1.5 mm in diameter, bluish or blue-grey, were confirmed by catalase reaction, gram analysis, haemolysis, nitrate reduction, carbohydrate reduction, tumbling motility, and obliquely transmitted light. The inoculated minced pork meat without thyme EO was used as control.

RESULTS AND DISCUSSION

Table 2 shows the effects of 32 EO on growth of *Listeria* strains by agar diffusion method. Twelve EO tested showed antibacterial activity, but only five (clove, cinnamon, thyme, origanum, pimento) were characterized by a significant and consistent inhibitory zone.

TABLE 2. *Inhibitory properties of undiluted plant essential oils towards the tested strains of* Listeria *spp., at a concentration of bacteria of approx. 10^6 CFU/ml.*

Plant oil	LI2	LL201	PF59	Scott A	L28
Basil	±	±	±	±	±
Camomile-German	-	±	-	-	-
Camomile-Roman	±	±	±	±	±
Celery	-	-	±	±	±
Cinnamon	+++	+++	+++	+++	++
Clove	++	++	++	+	++
Coriander	±	±	±	±	±
Cumin	±	±	±	±	±
Origanum	++++	++++	++++	++++	++++
Parsley	±	±	±	±	±
Peppermint-Piedmontese	±	±	±	±	±
Pimento-berry	+	+	+	+	++
Thyme	++++	++++	++++	++++	++++

LEGEND:
X = Arbitrarily defined ranges of inhibition zone diameter.
none: -
limited growth: ±
15 mm ≤ X < 25 mm: +
25 mm ≤ X < 35 mm: ++
35 mm ≤ X < 45 mm: +++
X ≥ 45 mm: ++++

NOTE: The following EO gave rise to no inhibition: estragon, fennel-seed, garlic, ginger, laurel, lemon, mandarin, marjoram, neroly, nutmeg, onion, orange, pepper, peppermint, pettigrain, rosemary, saffron, sage, vanilla.

Table 3 reports data on the activity of three EO (clove, thyme, and origanum) chosen among the more effective ones at two different concentrations. Results obtained showed a decreased activity for EO at lower concentration although an inhibitory zone was detected in all EO tested. Data also showed a possible interrelationship between lower concentrations of EO and strain characteristics. The five EO having a more effective antilisteric activity are among those recognized as the more effective in literature (*1,2,5,6*). In this regard, a further test of survival (an inhibition curve) was carried out to better evaluate this activity.

TABLE 3. *Comparison of inhibitory properties of three plant essential oils, diluted 1:5 and 1:50 towards the tested strain of* Listeria *spp. at a concentration of bacteria of approx. 10^6 CFU/ml - in brackets results with 1:5 dilution of EO.*

EO	LI2	LL201	PF59	Scott A	L28
Clove	+ (++)	+ (++)	+ (++)	+ (+)	+ (++)
Origanum	+ (++++)	+++ (++++)	+ (++++)	++ (++++)	+++ (++++)
Thyme	++ (++++)	+++ (++++)	++ (++++)	+++ (++++)	+++ (++++)

LEGEND:
X = Arbitrarily defined ranges of inhibition zone diameter.
none: - 25 mm ≤ X < 35 mm: ++
limited growth: ± 35 mm ≤ X < 45 mm: +++
15 mm ≤ X < 25 mm: + X ≥ 45 mm: ++++

Fig. 1 depicts the survival curve for *Listeria* strains in 0.1% preparations. We observed a marked decline in viability of *Listeria* strains suspended in saline solution to which EO were added.

The number of viable cells detected in the control for each strain tested remained quite stable. The time required for the reduction of microbial numbers below detection varied among the different EO and strains tested.

The antimicrobial activity of EO tested was similar against the five *Listeria* strains. A drastic decline in viable cells was noticed for each strain suspended with pimento EO within 1 h. In regard to thyme, clove, and origanum activity, the decline in viable cells was less evident in the first hour. Curve trend at the fourth hour was analogous for all the strains.

The cinnamon EO showed a weaker activity in inhibition of *Listeria* strains. In fact, the general trend indicates a lower antimicrobial activity within the first 2 h. At the fourth hour distinct patterns of behavior were observed. The decrease in viable cells of *L. monocytogenes* L12 and Scott A was attenuated in comparison with those of other strains. Moreover, an additional sampling time for the same five EO was performed at 24 h.

Ten subcultures of each test culture were incubated at 37°C for 48 h. No growth was detected (data not shown in Fig. 1). Our results indicate that *Listeria* is inhibited by the active EO tested either in a system allowing the bacterial growth (TSA medium) or in a saline solution. The latter result is significant because the *Listeria* is capable of surviving for long periods without multiplication.

As for the experiment conducted with the food matrix, the results obtained with the thyme EO had shown a reduction of the number of viable *L. monocytogenes* L28 cells in the meat where the EO was added (Table 4).

The transfer of antilisteric effectiveness of thyme EO from experimental procedures in agar and in liquid media to those conducted "in vivo" (the minced pork meat) had shown a decreased effectiveness of thyme EO. There also was a reduction of ca. 2 logs in the number of viable L28.

CONCLUSION

Spices and their derivatives are currently used with the primary purpose of flavoring foods and beverages although it has long been known some spices have an antimicrobial activity (*6*).

The most active constituents of spices having wide spectra of antimicrobial effectiveness are thymol (origanum, thyme), cinnamic aldehyde (cinnamon), eugenol (cinnamon, clove, and pimento), and carvacrol (origanum and thyme) (*1,2*).

Conner and Beuchat (*5*) have reported that clove, pimento, cinnamon, thyme, origanum, garlic, and onion were particularly inhibitory for some food-spoilage microorganisms. Other authors (*6,13*) have also shown the antimicrobial activity of several spices and EO on *S. aureus, Vibrio parahaemolyticus, S. typhimurium,* and *Escherichia coli.* No investigations were carried out on *Listeria* spp.

One of the major uses of spices as flavorings is in the corned meat industry. Moreover, the frequent isolation of *L. monocytogenes* strains in poultry, pork, beef, and ovine meat prompted us to verify the antilisteric activity of some EO. Our results suggest the potential use of some spices as antilisteric preservatives in food.

TABLE 4. *Activity of thyme EO on minced pork meat inoculated with* L. monocytogenes *L28.*

Time (h)	0 h		96 h		192 h	
Temperature °C						
Plate count	a	b	a	b	a	b
4	1-2x10^7 CFU/g	1-2x10^7 CFU/g	3x10^7 CFU/g	2x10^6 CFU/g	6x10^7 CFU/g	8x10^5 CFU/g
8	1-2x10^7 CFU/g	1-2x10^7 CFU/g	8x10^7 CFU/g	6x10^6 CFU/g	2x10^8 CFU/g	1x10^6 CFU/g

a: Minced pork meat without thyme EO, as control.
b: Minced pork meat with thyme EO.

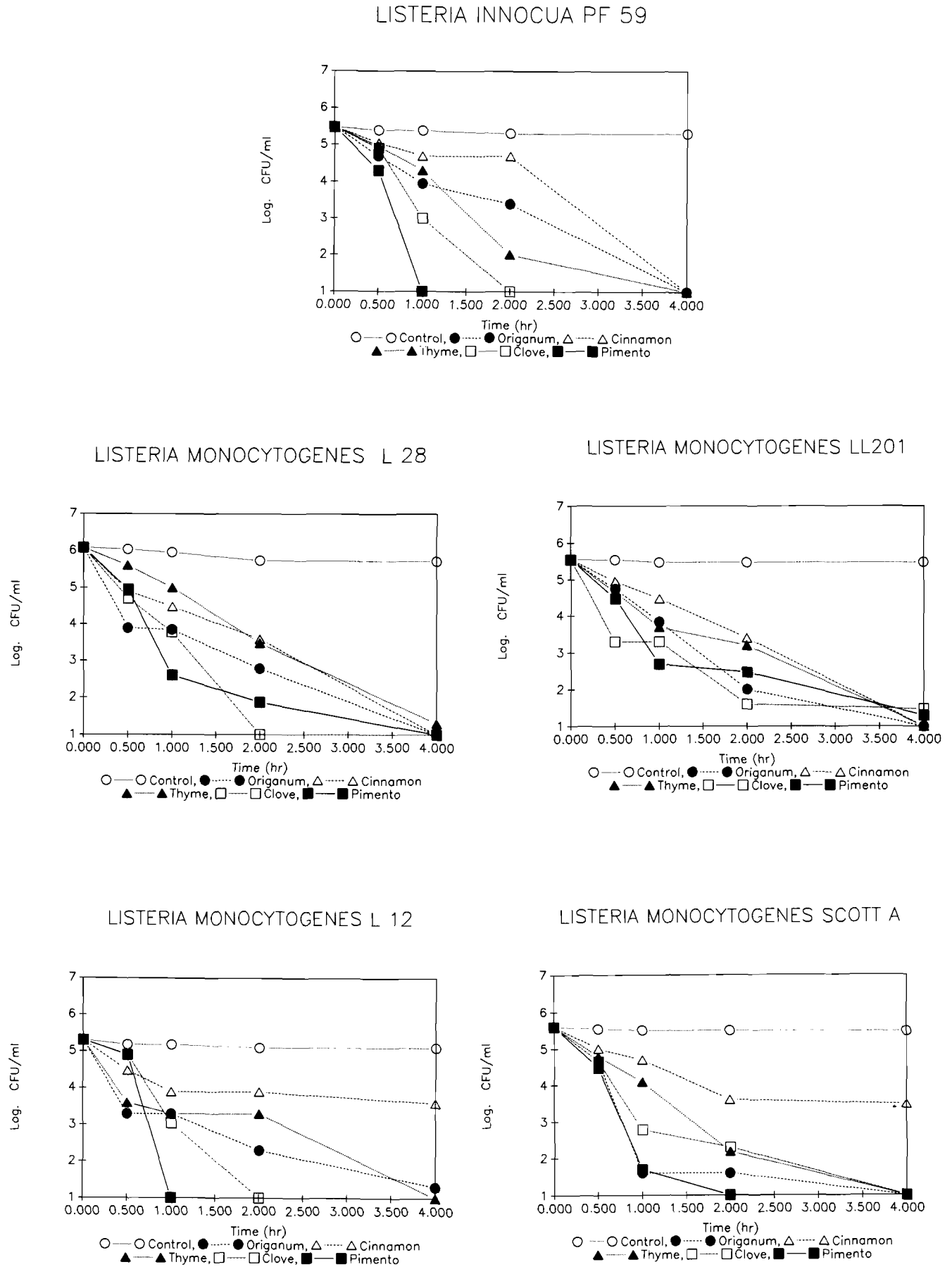

Figure. 1. *Survival curves for five* Listeria *strains against the tested EO in 0.9% saline solution added with 500 µl of alcoholic solution of each EO (1:5 in absolute alcohol).*

Additional research is required for a standardization of methods in order to thoroughly evaluate antilisteric activity of EO, considering also the interaction with the different food matrix and the palatableness. Further studies are needed for these purposes.

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