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Homologous and Heterologous Adaptation of *Listeria spp.* to Essential Oils of Condiment Plants

Jéssica M. P. Santos[1], Michelle C. Gonçalves[1], Heloisa A. Martins[1], Juliana J. Pinelli[1], Silas R. Isidoro[1], Roberta H. Piccoli[1,2*]

[1]Food Microbiology Laboratory, Universidade Federal de Lavras, Lavras, Brazil
[2]Food Science Department, Universidade Federal de Lavras, Lavras, Brazil
Email: *rhpiccoli@dca.ufla.br

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Abstract

The homologous and heterologous adaptation capacity of *L. monocytogenes* and *L. innocua* were determined for thyme, oregano and nutmeg essential oils, as well as their adaptation capacities to acidic stress. Minimum bactericidal concentrations (CMB) and minimum inhibitory and minimum growth pH, were established. The capacity for increased tolerance to essential oils and acidic stress, along with heterologous adaptation among the essential oils tested and to acidic pH was determined, and that between pH and essential oils. *L. monocytogenes* and *L. innocua* adapted to all essential oils and to the minimum inhibitory pH, after exposition to sub-lethal conditions. Both strains presented heterologous adaptation capacity. After previous exposition to sub-lethal essential oil concentrations, the regenerated cells were capable of growth under 3.5 pH values, and increased CMB values. Essential oil CMBs for previously cultivated cells under minimum growth pH environments also increased, attaining values 1.6 times superior to previous ones.

Keywords

Cross-Adaptation, Natural Antimicrobial, *L. monocytogenes*, Tolerance

1. Introduction

The *Listeria* genus is comprised by various species, of which *L. monocytogenes* is greatly relevant due to the number of diseases it can cause to humans and other animals. *L. innocua*, for other hand, is not pathogenic, but it presents ecological, biochemical and genetic similarity with *L. monocytogenes*, which serves as response when exposed to the same physiochemical treatments. They are capable of surviving under hostile environmental conditions, such as refrigeration temperatures, acidic pH environments and high concentrations of sodium chloride. It therefore has the ability to break through food security and preservation barriers, making it a relevant food pathogen [1].

Microbial cells' capacity to adapt to inhospif environmental conditions is well documented. Such physiological responses to stress are directly related to survival and growth strategies. Some of the most important adaptation responses are sensing mechanisms, regulatory protein expression (for instance, RpoS), homeostatic system and repair induction, synthesis of shock response proteins and modification of cell membranes, particularly that of their fatty acids and their physical properties [2]. Microbial cells also have the capacity to develop cross-adaptation. This form of adaptation can occur when different antimicrobial agents act on the same target in the cell, following a common route to their respective targets, or establish a common path to cell death [3].

The capacity of *L. monocytogenes* to adapt to stressful conditions, its persistence in processing environments and its resistance to antimicrobial agents has become worrying. When exposed to acidic stress, *L. monocytogenes* cells alter the fluidity of their cytoplasmic membrane, incorporating linear chain fatty acids and reducing ramified chain fatty acids [4] [5]. Stress tolerance in *L. monocytogenes* is partially explained by the induction of gene transcription that participates in homeostatic functions and provides protection against stress conditions [6].

Use of essential oils has been suggested as a way to reduce these adaptation and cross-adaptation mechanisms due to these oils' complexity, which assures that they act upon various targets within microbial cells because the essential oils can alter the permeability of microbial cells, damage cytoplasmic membranes, change membrane proteins, interfere with the generation system of energy (adenosine triphosphate (ATP)) and disrupts a cell homeostasis, resulting in cell death [7]. In addition to essential oils' high antimicrobial activity, it has been reported that important bacteria in the food industry do not present significant changes to their sensibility to other antimicrobial substances, physical processes or stress factors after having been exposed to sub-lethal concentrations of essential oils, or to their major components [8] [9].

Some accounts of adaptation and cross-adaptation capacities to essential oils and their major components can be found in the literature. Such accounts have scrutinized adaptation and/or cross-adaptation capacities of *L. monocytogenes* ATCC 19117 to carvacrol and eugenol [10], of *Salmonella* senftenberg to linalol and to basil oil [11], *S. enteritidis* ATCC 13076, *S. typhimurium* ATCC 13311, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 to *Origanum vulgare*, *Melaleuca alternifolia*, *Cinnamomum cassia* and *Thymus vulgaris* [12] and of *Staphylococcus aureus* (in two of the four strains tested) to carvacrol [13]. However, no studies have been carried out showing the ability or not of bacteria, especially *Listeria*, to develop adaptation between essential oils and acid stress. The goal of the present article is, therefore, to evaluate the capacity of *L. monocytogenes* and *L. innocua* to adapt to thyme, oregano and nutmeg essential oils, and to acidic stress, as well as their capacity for cross-adaptation among these essential oils and to acidic stress, when exposed to sub-lethal concentrations of essential oils and acidic stress.

2. Methods and Materials

2.1. Essential Oils

Nutmeg, oregano and thyme essential oils were acquired from FERQUIMA Indústria e Comércio Ltda (Brazil). Their composition is shown in Table 1.

2.2. Microorganisms and Cultivation Conditions

The strains employed were *L. monocytogenes* ATCC19117 sertype 4d and *Listeria innocua* ATCC 33090. Inoculum standardization (approximately 10^8 CFU/mL) was accomplished with a growth curve. Optic density (OD_{600nm}) was monitored along with plate counting, on trypticase soy agar (TSA) plus 0.5% (m/v) yeast extract.

2.3. Minimum Bactericidal Concentration of Essential Oils against *Listeria spp.*

The essential oils' minimum bactericidal concentration (CMB) was obtained with microdilution in adapted polystyrene microplates with 96 cavities [14]. Essential oil solutions were prepared in trypticase soy broth (TSB) plus yeast extract (TSB-YE) with a 0.5% Tween 80 addition. Analyses were done at concentrations of 4%; 2%; 1%; 0.5%; 0.2%; 0.1% and 0.05% (v/v). 150 μL aliquots of the solutions were added to the cavities and 10 μL of the standardized cultures were inoculated and incubated at 37˚C for 24 hours. After incubation, aliquots of the

Table 1. Essential oil composition.

Essential oils	Major components	Concentration (%)
Oregano*	Carvacrol	73.11
	E-Cariofileno	4.32
	γ-Terpineno	3.93
	Timol	2.97
Thyme*	Timol	50.89
	p-cinemo	24.97
Nutmeg**	Alpha-pineno	20
	Beta-pineno	14
	Sabineno	17
	Terpinen-4-ol	6

*According to Souza *et al.* (2016); **As provided by the company.

cultures were plated in TSA-YE and incubated at 37˚C for 24 hours.

A negative control was made with TSB-YE and 0.5% Tween 80 addition along with essential oils. The concentration of the essential oil solution in which growth was not observed was considered the CMB. The experiment was run in triplicate, with three repetitions.

2.4. Minimum Growth pH and Minimum Inhibitory pH of *Listeria spp.*

Minimum growth and minimum inhibitory pH of *L. innocua* and *L. monocytogenes* were determined with polystyrene microplates with 96 cavities. 150 µL of TSB-YE were dispensed into each cavity, with pH values adjusted at 6.0; 5.5; 5.0; 4.5; 4.0; 3.5; 3.0 and 2.5 with lactic acid (98%) along with 10 µl of standardized cultures and microplates were incubated at 37˚C for 24 hours and then plated in TSA-YE. Minimum inhibitory pH was defined through visual evaluation as the smallest value capable of completely inhibiting bacterial growth, while the minimum growth pH was defined as that immediately above the minimum inhibitory pH. The experiment was run in triplicate, with three repetitions.

2.5. *Listeria spp.* Adaptation to Sub-Lethal Conditions: Essential Oils and Acidic pH

L. monocytogenes and *L. innocua* were adapted through cultivation under sub-lethal concentrations. Sublethal concentrations were determined by CMB divided by 4 and by 8 (CMB/4 and CMB/8) of thyme, oregano and nutmeg essential oils in Falcon tubes containing 36 mL of TSB-YE with 0.5% Tween 80 added. 4 mL of the standardized culture were then added, and the tubes were subsequently placed under incubation at 37˚C for 6 hours. *L. innocua* and *L. monocytogenes* cultivation under acidic pH was done in Falcon tubes containing TSB-YE, pH 4.5, adjusted with lactic acid (98%), at 37˚C for 6 h.

After incubation, the cultures were centrifuged (5.000× g/5 min), the supernatant was discarded and the pellets were sanitized three times with saline solution (0.85% m/v). The pellets were again suspended in TSB-YE at around 10^8 CFU/mL. The experiment was run in triplicate, with three repetitions.

2.6. Homologous Adaptation of *Listeria spp.* to Essential Oils and to Acidic pH

The analysis of *Listeria spp.* adaptation to essential oils relied on suspensions of adapted standardized cells. 10 µL aliquots of the suspension were inoculated into 150 µL of essential oil solutions—the same oils to which cells had previously adapted—and incubated at 37˚C for 24 hours. Solutions of each essential oil were prepared at concentrations of 0.5 CMB; CMB; 1.2 CMB; 1.4 CMB; 1.6 CMB; 1.8 CMB and 2 CMB in TSB-YE with a 0.5% Tween 80 addition. After incubation, 10 µL aliquots of the cultures were plated in TSA-YE and incubated at 37˚C for 24 hours. *L. monocytogenes* and *L. innocua* cells were found to be capable of developing homologous adaptation if they continued plated growth after cultivation under concentrations equal or superior to CMB.

Tolerance to acidic stress was evaluated via inoculation of 10 µL aliquots of the standardized suspension of cells that had been adapted to the minimum growth pH (4.5) into 150 µL of TSB-YE under different pH values, adjusted with lactic acid (98%). pH values of 6.0; 5.5; 5.0; 4.5; 4.0; 3.5 and 2.5 were evaluated. Plates were incubated at 37˚C, for 24 hours. Subsequently, cultures were plated in TSA and incubated at 37˚C, for 24 hours. *L. innocua* and *L. monocytogenes* cells that grew in plating after cultivation under pH values inferior to minimum growth pH were considered capable of developing tolerance to acidic stress. Non-adapted cells were used as control.

2.7. Evaluation of Heterologous Adaptation among Essential Oils and among Essential Oils and Acidic pH

10 µL aliquots of the standardized *L. innocua* and *L. monocytogenes* cell solution that was adapted to 1/8 essential oil CMB for 6 hours were inoculated into 150 µL of the other essential oils. 0.5 CMB, CMB, 1.2 CMB, 1.4 CMB, 1.6 CMB, 1.8 CMB and 2 CMB concentrations were tested.

10 µL aliquots of the standardized cell suspension adapted to 1/8 essential oil CMB were inoculated into 150 µL TSB-YE with pH adjusted to 6.0; 5.5; 5.0; 4.5; 4.0; 3.5; 3.0 and 2.5 with lactic acid (98%), and incubated at 37˚C for 24 hours. Subsequently, the cultures were plated in TSA-YE. *L. innocua* and *L. monocytogenes* heterologous adaptation capacity was determined by analyzing plated growth after exposition to pH values inferior to minimum growth pH.

2.8. Evaluation of Heterologous Adaptation between Acidic Stress and Essential Oils

10 µL aliquots of the standardized cell suspension adapted to 4.5 pH were inoculated into 150 µL essential oil solutions under concentrations of 0.5 CMB; 1.2 CMB; 1.4 CMB; 1.6 CMB; 1.8 CMB and 2 CMB. The solutions were then incubated at 37˚C, for 24 hours. *L. innocua* and *L. monocytogenes* cultures that exhibited plated growth under essential oil concentrations equal or superior to CMB were considered capable of developing heterologous adaptation.

3. Results

Listeria innocua and *L. monocytogenes* presented the same behavior in all experiments. Essential oil CMB was 0.1% for thyme and oregano, and 0.2% for nutmeg. Minimum inhibitory pH and minimum growth pH were 4.0 and 4.5, respectively.

The two strains were capable of developing homologous adaptation to essential oils. After exposition for 6h to sublethal concentrations of 1/8 CMB of oregano (0.0125%), thyme (0.0125%) and nutmeg (0.025%) essential oils, they were able to grow under higher oil concentrations than the CMB. The new bactericidal concentration for cells adapted to oregano and thyme essential oils was 0.2% (2 CMB), while for those adapted to nutmeg oil the new concentration was impossible to obtain, as both *L. innocua* and *L. monocytogenes* were capable of growth under all concentrations tested (up to 2 CMB, or 0.4%).

Cells adapted to sub-lethal concentrations of 1/4 CMB (0.025% oregano and thyme; 0.05% nutmeg) were found to have a new CMB of 0.16% (1.6 CMB) for oregano and thyme essential oils, and 0.4% (2 CMB) for nutmeg oil.

Homologous adaptation to acidic stress was also observed. Minimum inhibitory pH, previously 4.0, decreased to 3.5 for both strains.

Both *L. innocua* and *L. monocytogenes* developed the some heterologous adaptations among essential oils (Table 2).

Both *L. innocua* and *L. monocytogenes* showed heterologous adaptation capacity to acidic stress. After being exposed to sub-lethal concentrations (1/8 CMB) of thyme and oregano essential oils (0.0125%) and nutmeg (0.025%), the strains were capable of growth in environments of pH 4.0, a lower value to that of the previously established minimum growth pH (4.5). The new minimum inhibitory pH was 3.5.

After adaptation to the minimum growth pH (pH 4.5), *L. innocua* and *L. monocytogenes* developed heterologous adaptation to thyme, oregano and nutmeg essential oils. The strains were capable of growth when plated with 1.4 times the CMB of each oil. The new CMB for both strains were 0.16% (1.6 CMB) for oregano and thyme essential oils, and 0.32% (1.6 CMB) for nutmeg oil.

4. Discussion

Nutmeg oil was determined to have had twice as high a CMB (0.2%) than its thyme and oregano counterparts (0.1%). Against *L. monocytogenes*, specifically, the literature has suggested that oregano essential oil CMB may vary between 0.005% and 0.6%, depending on strain and methodology [15]. The same observation may be made for thyme oil, which has been shown to wield various CMB values—0.1% [16], 0.2% [17], 0.39% [18] and 0.5% [19]. Minimum inhibitory concentration for nutmeg oil against *L. monocytogenes* was determined to be 0.2% [20], the same value found by this work.

Essential oils' antimicrobial activity is attributed mainly to their major compounds, although their trace components may contribute significantly to their

Table 2. Heterologous adaptation of *Listeria monocytogenes* and *Listeria innocua* to thyme, oregano and nutmeg essential oils.

Stress sublethal	Conc. Sublethal (%)	Stress lethal	Lethal Conc. (%)
Thyme	0.0125*	Oregano	0.18*
	0.0125	Nutmeg	0.36
Oregano	0.0125*	Thyme	0.18*
	0.0125	Nutmeg	0.36
Nutmeg	0.025*	Thyme	0.18*
	0.025	Oregano	0.18

**Listeria monocytogenes, Listeria innocua.*

effectiveness. Oregano, thyme and nutmeg essential oils are known antimicrobial compounds. Their antibacterial is associated with its phenolic major compounds, carvacrol, thymol, α-pinene and β-pinene [7]. Although these components present known antimicrobial activity, their biological activity is dependent on their chemical structure. Carvacrol, for instance, is twice as potent as α-pinene and β-pinene against gram positive bacteria [21]. The sum of α and β-pinene present in nutmeg oil amounted to 34% of its composition, while carvacrol and thimol in oregano, and thimol in thyme, 76.08% and 50.89% respectively. It may be said that the results of this work reflect the antimicrobial action of the major compounds of the essential oils studied.

Bacterial capacity for homologous and heterologous adaptation to transient conditions is well known, particularly with regards to oxidation, acidic conditions, thermal stress, high pressures, antibiotics and sanitizing agents. However, little is known about food-borne bacteria's capacity for adaptation to essential oils. In this work, both *Listeria* strains analyzed developed homologous as well as heterologous adaptation to these oils.

Adaptation mechanisms to essential oils and their major compounds are not yet well known, and there are few works published in the literature. *Salmonella* thompson exposition to sub-lethal doses of thymol has, however, been studied, leading to observations of protein synthesis associated with stress and damage to cellular metabolism [22]. *Bacillus cereus* cultivated under sub-lethal carvacrol concentrations has also been shown to present reductions to the fluidity of cells' cytoplasmic membrane, and alterations to their fatty acid make-up [23]. However, transcriptomic analyses show that *L. monocytogenes* response to sub-lethal stress caused by essential oils is complex [24].

Comparative transcriptomic analysis of *L. monocytogenes* serotype 4b adapted to sub-lethal concentrations of *Baccharis psiadioides* essential oil showed various changes to the regulation of genes associated to stress response and a decrease in gene expression associated to virulence factors [24]. Different *L. monocytogenes* isolates, after exposition to sub-lethal doses of lemongrass essential oil led to modifications to the expression of genes associated with fatty acid and peptideoglycan bio-synthesis, as well as to key genes associated with virulence factors [25].

Proteomic analyses also show that bacteria exposed to sub-lethal concentrations of essential oils or to their major compounds promote changes to the profiles of proteins associated to fatty acid bio-synthesis. This was shown for *E. coli*, *L. monocytogenes* and *S. enteritidis* that had their protein profiles altered, increasing unsaturated fatty acid concentrations after exposition to sub-lethal doses of thyme and oregano essential oils, as well as to carvacrol, thymol, trans-2-hexenal and citral [26]. This change to fatty acid composition may contribute to the understanding of stress response mechanisms employed by different pathogenic microorganisms responsible for food-borne disease, including with regards to exposition to sub-lethal concentrations of natural antimicrobial substances.

Weak acids affect cellular capacity to maintain pH homeostasis, interrupting substrate transportation and inhibiting metabolic pathways [27]. However, it is known that *L. monocytogenes* is capable of developing tolerance to acidic pH values, growing even in 3.5 pH environments [4], in line with the findings of the present work.

Heterologous adaptation was found, in this study, to be similar for both strains. This may have occurred due to the concurrent action of the essential oils and lactic acid over similar targets within the cell, namely, over common physiological mechanisms that lead to cell death, or due to common access pathways to their respective targets [3]. Juven *et al.* [28] state that the antibacterial properties of some essential oil components may be improved after exposition to low pH values. However, in this study, this behavior was not observed. Both strains exposed to 4.5 pH (minimum growth pH) presented higher tolerance to essential oils. Based on the concept of heterologous adaptation, cross-tolerance development was observed for both *L. innocua* and *L. monocytogenes* when previously exposed to sub-lethal pH and essential oil-rich environments.

The *Sigma* B alternative factor is a protein responsible for controlling important genes that regulate stress response, virulence, transcriptional regulation, carbohydrate metabolism and transportation. It affects mobility and chemotaxis in both *L. innocua* and *L. monocytogenes*. The regulation of both strains by alternative factor *Sigma* B show a common cluster of at least 49 genes [29] that may also be related to the similar behavior exhibited by these strains in this study.

Information regarding bacterial adaptational responses to sub-lethal conditions is important for food microbiology, seeing as many processes to which bacteria are submitted during processing are of non-lethal character, which represents risks to sanitation and consumer health.

5. Conclusion

Listeria innocua and *L. monocytogenes* both presented adaptational capacity when exposed to sub-lethal essential oil concentrations and to minimum growth pH. Both species developed heterologous adaptation among essential oils and to acidic stress, and between acidic stress and essential oil treatment. The ability of the strains to develop heterologous adaptation is extremely worrying, being a cause for alert for the use of essential oils in food in sublethal concentrations.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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