Acquired acid adaptation of *Listeria monocytogenes* during its planktonic growth enhances subsequent survival of its sessile population to disinfection with natural organic compounds

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INTRODUCTION

▸ The well-known ability of the bacterial foodborne pathogen *L. monocytogenes* to sense, adapt and respond to acid stress is crucial to its survival, given that this frequently encounters low-pH environments during its infectious life cycle (e.g. in acidic foods, during gastric transit) (1).

► Its striking capability to attach to surfaces is also believed to contribute to its persistence in food processing environments (2, 3).

► Given that attached bacteria seem to inherently present more resistance to disinfection, any supplementary phenotypic adaptation of these cells, such as acid adaptation (4), may further counteract the effectiveness of surface decontamination strategies.

► Organic acids and extracts of selected plants and herbs are examples of promising natural antimicrobial compounds, with some to be nowadays used both as food biopreservatives and for the disinfection of food surfaces (5, 6).

► In this study, the possible influence of acid adaptation of *L. monocytogenes* cells during their planktonic growth on their subsequent resistance against some such compounds (i.e. lactic acid, essential oil and hydrosol of Mediterranean spice *Satureja thymbra*) upon their attachment to stainless steel, was evaluated by simultaneously using the bead vortexing technique and a promising conductance method.

MATERIALS & METHODS

Bacterial strain & assay to induce acid tolerance response (ATR)

• *L. monocytogenes* strain Scott A (serotype 4b, epidemic strain, human isolate) was used.

➢ To prepare acid-adapted bacteria the experimental protocol of Buchanan and Edelson (7) was followed:

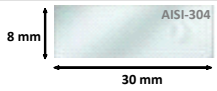
• After a preculture (at 30ºC for 24 h) in tryptone soy broth (TSB), bacteria were subcultured in either TSB containing 1% v/v glucose (TSB+G), or TSB without any glucose (TSB-G) (both at 30ºC for 24 h).

→ TSB-G does not result in the formation of an acidic environment, due to the lack of fermentable carbohydrate.

→ Conversely, TSB+G is acidogenic, supporting growth of the microorganism with an accompanying decrease of its pH.

Abiotic surface & attachment assay

• Rectangular stainless steel (SS) coupons were used as abiotic surfaces for bacterial attachment.



• Cleaned and sterilized SS coupons were individually placed in glass test tubes, each containing 3,5 ml of brain heart infusion (BHI) broth, in such a way that the upper part of each metallic surface (*ca.* 2 mm) was exposed to the air-liquid interface, given that this interface may favour bacterial attachment (8).

• Growth medium was inoculated with either nonadapted or acid-adapted *L. monocytogenes* to yield initial bacterial population of *ca.* 10^8 CFU/ml.

• Inoculated tubes were subsequently incubated at 5, 16 or 30ºC for 10 days (240 hours) under static conditions, without any nutrient refreshment.

Disinfection of attached cells with natural organic compounds

• On 10th day of incubation, SS coupons were carefully removed from glass test tubes, loosely attached cells were removed by strong rinsing and then coupons were individually introduced in new glass test tubes each containing 5 ml of disinfectant solution (see Table).

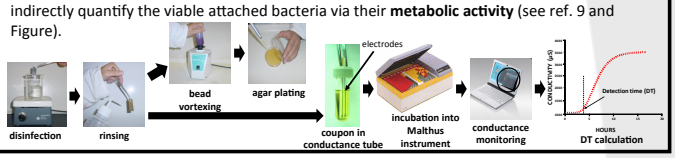
Treatment	Composition / parameters of treatment	Disinfection duration (min)
20% EtOH	20% (v/v) ethanol, 80% (v/v) distilled water (essential oil control)	6
LA (pH 3)	7 mmol/L lactic acid (pH 3)	60
1% e.o.	1% (v/v) essential oil of *S. thymbra*, 19% (v/v) ethanol, 80% (v/v) distilled water	6
hydrosol	100% (v/v) hydrosol of *S. thymbra*	6

Quantification of viable sessile populations

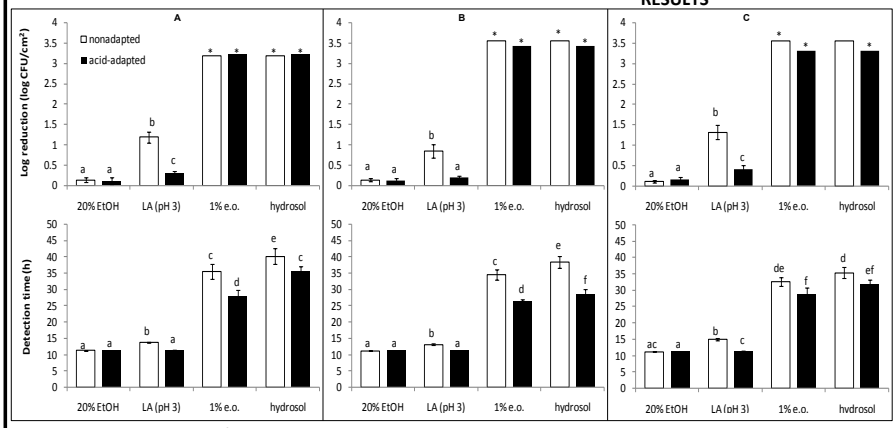
• Following each disinfection treatment, SS coupons were rinsed thoroughly and were then immersed (for 10 min at RT) into Dey-Engley Neutralizing broth (in order to stop disinfection action and help viable but possible injured cells to recover).

• Subsequently, coupons were either subjected to **strong vortexing with glass beads** in order to detach the strongly attached cells and enumerate them by agar plating (on TSA), and / or

• placed into a **conductance measuring tube** equipped with platinum electrodes in order to indirectly quantify the viable attached bacteria via their **metabolic activity** (see ref. 9 and Figure).



RESULTS



Log reductions (log CFU/cm²) of sessile *L. monocytogenes* populations following the 4 disinfection treatments and conductance DTs (h) corresponding to the remaining viable sessile populations.

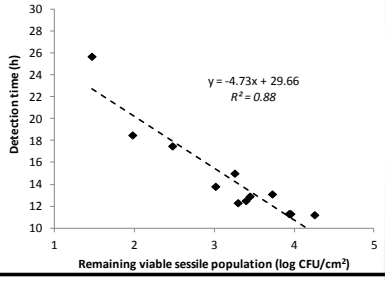
For bacterial attachment, SS coupons had initially been incubated for 10 days in BHI broth at 5 ºC (A); 16 ºC (B); or 30 ºC (C). This medium was inoculated with either nonadapted (□), or acid-adapted (■) bacteria. The bars represent the mean values ± standard deviations (n = 4). For each graph separately, mean values sharing at least one common letter shown above the bars are not significantly different at a P value of <0.05. *, count after treatment was below the detection limit (DL) of the agar plating method (1.03 log CFU/cm²).

• The biocide effect of each disinfection treatment was expressed as population log reduction (just before and after the treatment; log CFU/cm²), whereas when conductance measurements were used, this was expressed through the duration (h) of the detection time (DT) (see Figure).

➢ Both essential oil and hydrosol presented sufficient bactericidal activity against all formed sessile populations, always resulting in counts following disinfection below the plate counting detection limit.

➢ However, conductance method, able to detect metabolically active sessile bacteria unable to be recovered by the bead vortexing, revealed the positive influence of previously acquired acid adaptation on disinfection resistance of attached cells against these plant extracts.

➢ A similar effect of acid adaptation was also evident for lactic acid disinfection.



Linear regression plot between remaining viable sessile *L. monocytogenes* populations (log CFU/cm²) following the 60 min exposure to strong lactic acid challenge (pH 3) and corresponding conductance DTs (h).

CONCLUSIONS & PERSPECTIVES

✓ Use of some natural organic compounds, such as microbial derived ones (e.g. lactic acid) or extracts of selected plants and herbs (e.g. essential oil and hydrosol of *S. thymbra*) could provide alternative ways for the effective elimination of *L. monocytogenes* cells attached to food contact surfaces.

✓ Yet, acid adaptation of these sessile cells should be carefully considered when applying such ecofriendly interventions.

✓ Present findings, in combination with further research on the remaining gaps of knowledge on stress adaptive responses and disinfection efficiency (such as the effect of strain variability and of the possible simultaneously existence of different microbial species, together with the unraveling of the molecular mechanisms besides these phenomena) can be used as the basis for the development of effective interventions against the settlement of this important pathogenic bacterium onto food contact surfaces.

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The ability of *Listeria monocytogenes* to sense, adapt and respond to acid stress is crucial to its survival, given that this frequently encounters low-pH environments during its infectious life cycle. Its striking capability to attach to surfaces is also believed to contribute to its persistence in food processing environments. Organic acids and extracts of selected plants and herbs are examples of promising natural antimicrobial compounds, with some to be nowadays used both as food biopreservatives and for the disinfection of food surfaces. In this study, the possible influence of acid adaptation of *L. monocytogenes* cells during their planktonic growth on their subsequent resistance against some such compounds (i.e. lactic acid, essential oil or hydrosol of Mediterranean spice *Satureja thymbra*) upon their attachment to SS, was evaluated by simultaneously using the bead vortexing technique and a conductance method. Prior to disinfection, both nonadapted and acid-adapted stationary phase bacteria were left to attach to SS coupons statically incubated for 10 days under six different environmental conditions, simulating various food processing related stresses (with respect to temperature, acidity and salinity). Results revealed that both essential oil and hydrosol presented sufficient bactericidal activity against all formed sessile populations, always resulting in counts following disinfection below the plate counting detection limit. However, conductance method, able to detect metabolically active sessile bacteria unable to be recovered by the bead vortexing, revealed the positive influence of previously acquired acid adaptation on disinfection resistance of attached cells against these plant extracts. A similar effect of acid adaptation was also evident for lactic acid disinfection. To sum, use of some natural organic compounds, such as microbial derived ones or extracts of selected plants and herbs could provide alternative ways for the effective elimination of *L. monocytogenes* cells attached to food contact surfaces. Yet, acid adaptation of these sessile cells should be carefully considered when applying such ecofriendly interventions.

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