Food Control 59 (2016) 824–828

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

When nanoemulsified, D-limonene reduces *Listeria monocytogenes* heat resistance about one hundred times

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ARTICLE INFO

Article history:
Received 12 May 2015
Received in revised form
6 July 2015
Accepted 14 July 2015
Available online 15 July 2015

Keywords:
Nanoemulsions
D-limonene
Heat resistance
Listeria monocytogenes
Antimicrobial effect
Combined effect

ABSTRACT

The application of oily antimicrobials in form of nanoemulsion has been proved to solve the problem of their immiscibility in aqueous media, still preventing microbial growth and even improving the antimicrobial effect observed when applied directly. At present, only a few documented studies have evaluated the combined effect of nanoemulsions with other factors of stress for the microorganism. The present research shows very promising results on the combination of nanoemulsified D-limonene with thermal treatments on the inactivation of *Listeria monocytogenes*. The thermal resistance of *L. monocytogenes* was reduced two to five times when 0.5 mM D-limonene was added directly to the heating medium. However, when the same concentration of D-limonene was present in the heating medium in form of nanoemulsion, the heat resistance was reduced by one hundred times at all heating temperatures tested. The addition of nanoemulsified antimicrobials would allow to reduce greatly the intensity of the thermal treatments currently applied in the food processing industry.

1. Introduction

Listeria monocytogenes is a very ubiquitous microorganism, which is widely distributed in many environments, and can contaminate a wide variety of foods. Immunocompromised individuals, pregnant women and the elderly are those population groups most susceptible to this foodborne pathogen microorganism (Magalhães et al., 2014). The ubiquity of this organism and its ability to grow in the form of biofilms enables it to be present in food processing plants and foods, being ready-to-eat foods those most likely to be contaminated (Pilchová et al., 2014; Wang et al., 2005).

One of the objectives of the present food industry is to provide consumers with better sensorial quality foods, while keeping food safety. One way to achieve this goal is to combine conventional thermal treatments with the use of antimicrobials (Leistner & Gorris, 1995). For example, in 2012, Cava-Roda, Taboada, Palop, López-Gómez and Marín-Iniesta evaluated the thermal stability of *L. monocytogenes* in semi-skimmed milk supplemented with vanillin. In 2013, Juneja, Altuntaş, Ayhan, Hwang, Sheen and Friedman studied the thermal stability of this microorganism in minced meat with different concentrations of sodium chloride and polyphenols from apple. Espina, Condón, Pagán and García-Gonzalo (2014), Espina et al. (2012) and Luis-Villarroya et al. (2015) have proposed the use of citrus essential oils and a propolis-based dietary supplement, respectively, to reduce the intensity of the thermal treatment needed to inactivate *Escherichia coli* O157:H7 in acidic media such as fruit juices.

The effectiveness of some of these natural compounds as well as their degree of consumer acceptance, has led to an increase in the number of research publications related to natural antimicrobials in recent years. Among them, essential oils have shown better results against the growth of several microorganisms than other antimicrobials. Solomakos, Govarisa, Koidisb, and Botsoglou (2008) evaluated the antimicrobial effect of thyme essential oil and its combination with nisin in minced beef contaminated with *L. monocytogenes* and preserved under refrigeration, obtaining satisfactory results after several days of preservation. Delgado, Palop, Fernández, and Periago (2004) combined thymol and cymene to control de growth of *Bacillus cereus*, obtaining greater inhibition than when applying the antimicrobials separately. Also Esteban and

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http://dx.doi.org/10.1016/j.foodcont.2015.07.020

Palop (2011) and Periago and Moezelaar (2001) found interesting results for the combination of carvacrol and nisin on the growth of *L. monocytogenes* and *Bacillus cereus*, respectively. Govaris, Solomakos, Pexara, and Chatzopoulou (2010) also obtained satisfactory results assessing the antimicrobial effect of oregano essential oil and its combination with nisin against *Salmonella* Enteritidis in minced meat.

One of the major drawbacks of essential oils is their immiscibility in aqueous media. Therefore, an important part of the research in recent years has focused the attention on optimizing the application of these compounds in foods. Among the different techniques studied, the application of essential oils and their components in form of nanoemulsion is providing very promising results (Donsì, Sessa, & Ferrari, 2010; Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2014). It has been proved to solve the problem of the immiscibility of essential oils and their components in aqueous media, still preventing the growth of a wide variety of microorganisms even improving antimicrobial effect than when it is applied directly, not only in culture media, but also in foods. In 2012, Donsì, Annunziata, Vincensi and Ferrari evaluated the antimicrobial effect of a nanoemulsion of carvacrol, cinnamaldehyde and limonene against *E. coli*, *Lactobacillus delbrueckii* and *Saccharomyces cerevisiae* in culture medium with very interesting results. Later, in 2014, Zhang, Vriesekoop, Yuan and Liand studied the antimicrobial effect of a nanoemulsion of nisin and limonene against *Staphylococcus aureus*, *Bacillus subtilis*, *E. coli* and *S. cerevisiae* obtaining also satisfactory results in culture media. In 2014, Donsì, Cuomo, Marchese and Ferrari evaluated the antimicrobial effect of D-limonene emulsions and a mixture of monoterpenes of *Melaleuca alternifolia* and carvacrol emulsions on *L. delbrueckii*, *S. cerevisiae* and *E. coli* in juice and solid foods (sausages and zucchini) respectively, also obtaining satisfactory results. Recently, Maté, Periago, and Palop (2015) have also obtained satisfactory results when evaluating the antimicrobial effect of D-limonene nanoemulsions combined with nisin on *L. monocytogenes* in culture media and foods.

At present, only a few documented studies have evaluated the combined effect of nanoemulsions with other factors of stress for the microorganism. In 2014, Severino, Vu, Donsi, Salmieri, Ferrari and Lacroix evaluated the antimicrobial effect of some essential oils with different combined non-thermal treatments (ozonized water, ultra violet-C light and gamma irradiation) against *L. monocytogenes*. Severino et al. (2015) also studied the antimicrobial effect of an essential oil nanoemulsion combined with modified atmosphere packaging and gamma irradiation against *E. coli* and *Salmonella*. In both cases, interesting results were obtained. So far, there are no documented studies that have tested the antimicrobial effect of a combination of antimicrobial nanoemulsions with heat treatments. For this reason, the aim of this study was to evaluate the combined effect of a thermal treatment with a nanoemulsion of D-limonene on the inactivation of *L. monocytogenes* in tryptic soy broth (TSB).

2. Materials and methods

2.1. Bacterial strains

L. monocytogenes CECT 4032 was used in this study and it was provided by the Spanish Type Culture Collection (CECT, Valencia, Spain). This strain was stored at −80 °C (30% glycerol) until use. For growth and survival experiments, fresh cultures of *L. monocytogenes* were prepared by inoculating a loop of the cryopreserved culture in tryptic soy broth (TSB; Scharlau Chemie S.A., Barcelona, Spain) and incubating overnight at 37 °C until the stationary growth phase was reached.

2.2. Antimicrobials

D-limonene was obtained from Sigma Aldrich Chemie (Steinheim, Germany). For their direct addition to the culture media, they were dissolved in ethanol (Panreac, Barcelona, Spain) at 95% (v/v). The working solution was prepared to a final concentrations of 1 M and stored refrigerated until use.

2.3. Preparation of nanoemulsions

The nanoemulsions of D-limonene were prepared following the protocol described by Maté et al. (2015) and based on catastrophic phase inversion (CPI) method (Zhang et al., 2014). Briefly, aqueous phase was prepared by mixing sterile distilled water and propylene glycol (Panreac, Barcelona, Spain). To elaborate the oily phase, Tween 80 (Panreac) was mixed with D-limonene. Nanoemulsion was prepared by slowly adding aqueous phase into the oily phase with gentle agitation. The addition rate of aqueous phase was kept constant at approximately 1.0 mL/min with continuous stirring. A water-in-oil emulsion with a high oil-to-water ratio was formed, and then increasing amounts of water were added to the system, until a phase inversion occurred and an oil-in-water emulsion was formed, with continuous stirring for 6 h. Final concentration of D-limonene in the nanoemulsion was 1 M.

Nanoemulsions were aliquoted in pre-sterilized test tubes and stored in refrigeration until use. Droplet size was determinated at the beginning and at the end of the experiment. Size distribution of the oil droplets were determined by the laser light scattering method using Mastersizer 2000 (Malvern Instruments, Worcestershire, UK), as already described (Maté et al., 2015). No differences were found in size distribution along the time the present research was performed (data not shown).

2.4. Heat treatments

Thermal inactivation kinetics for *L. monocytogenes* in TSB supplemented with 0.5 mM D-limonene (direct addition or nanoemulsion) was determined at constant temperature in a thermoresistometer Mastia as described by Conesa, Andreu, Fernández, Esnoz, and Palop (2009). D-limonene was added to pre-sterilized TSB in sterile conditions. Then, the vessel of the thermoresistometer was filled with 400 mL of pre-sterilized TSB supplemented with D-limonene (direct addition or nanoemulsion). Heat treatments were conducted at 45.0, 50.0, 52.5 and 55.0 °C. Once the heating medium temperature had attained stability (±0.05 °C), it was inoculated with 0.2 mL of the cell culture (approx. 10^8 cells mL^{-1}). At preset intervals, 1 mL samples were collected into sterile test tubes, which were kept in ice until decimal dilutions were performed. Surviving cells were enumerated in tryptic soy agar (TSA, Scharlau Chemie). Plates were incubated for 24 h at 37 °C. Each treatment was assayed by triplicate in independent experiments performed in different days.

2.5. Data analysis

Decimal reduction times (D-values) were calculated as the inverse negative of the slope of the regression line of the survival curves, drawn plotting the logarithm of the survivors versus the corresponding heating times. Survival curves included all the counts obtained in the different repetitions.

z values were estimated from the decimal reduction time curves (DRTCs), drawn plotting the logarithm of the D values versus the corresponding temperatures, as the negative inverse of the slope.

Comparison of slopes of survival curves and DRTCs was performed by the slope homogeneity test as described by Steel and

Torrie (1960). Correlation coefficients (r_0) of the regression lines of survival curves and DRTCs and 95% confidence limits (CL) were calculated using Matlab softaware (Matlab, The Math Works, Natick, USA).

3. Results and discussion

Fig. 1 shows the effect of D-limonene added directly or nanoemulsified on the thermal resistance of *L. monocytogenes* in TSB at two different temperatures, 45.0 °C (Fig. 1A) and 55.0 °C (Fig. 1B). At 45.0 °C (Fig. 1A), the reduction in the survivors of the control without D-limonene hardly reached half log cycle in the 8 h (480 min) that the experiment lasted. A noticeable effect can be seen when the D-limonene is added directly to the heating medium, leading to a reduction in the survivors of one log cycle in 5 h (300 min). However, when D-limonene was present in form of nanoemulsion, the reduction in the survivors can be regarded as dramatic, decreasing more than four log cycles in less than 30 min. Similar results were observed at 55.0 °C (Fig. 1B): 1 log reduction in the survivors after 15 min for the control without D-limonene, 1 log reduction after 8 min when D-limonene was added directly to the heating medium and more than four log cycles in less than 1 min when D-limonene was nanoemulsified. Controls with all the ingredients used for the nanoemulsion (*i.e.* propylene glycol and Tween 80), but without D-limonene were also tested, showing no effect on the thermal resistance of this microorganism (data not shown).

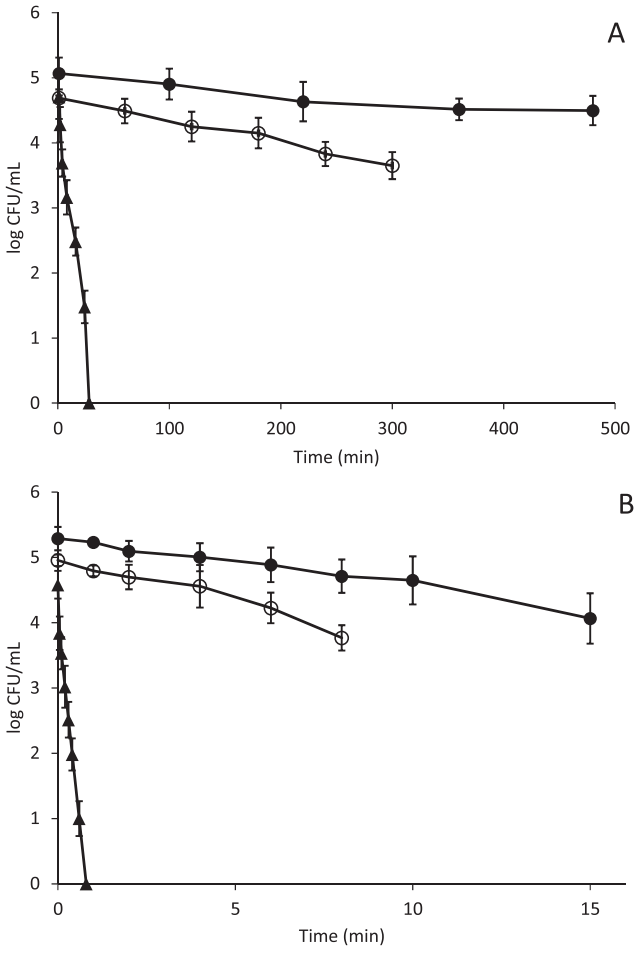

Fig. 1. Survival curves of *Listeria monocytogenes* in TSB broth at 45 °C (A) and 55 °C (B). Control: ●; 0.5 mM D-limonene added directly: ○; 0.5 mM D-limonene nanoemulsified: ▲.

Table 1 shows the heat resistance data obtained at all the temperatures tested. *L. monocytogenes* showed a $D_{55\ °C}$ value of 13.8 min in TSB when no antimicrobial was added. The $D_{55\ °C}$ was significantly reduced to about one half when D-limonene was added directly to the TSB. However, when D-limonene was added in form of nanoemulsion, the $D_{55\ °C}$ value was reduced down to 0.197 min, which means a 70 times lower D value than the control. Similar or even greater reductions were obtained at all other temperatures tested (up to 120 times at 50.0 °C; Table 1).

It is noteworthy that the $D_{45.0\ °C}$ value for the control was 784 min, *i.e.* more than 13 h to inactivate one log cycle, and a significantly lower $D_{45.0\ °C}$ value of 290 min (almost 5 h) was obtained when adding D-limonene directly. These values are far too high to get a proper inactivation of this microorganism at this temperature. When using D-limonene nanoemulsified, the $D_{45.0\ °C}$ value was reduced to 8.13 min (almost one hundred times regarding the control), which could be used for inactivation purposes. Moreover, this $D_{45.0\ °C}$ value with nanoemulsified D-limonene was lower than the control $D_{55.0\ °C}$ value (without D-limonene) and similar to the $D_{55.0\ °C}$ value with D-limonene added directly (Table 1). In fact, in a recent publication of our research group (Maté et al., 2015) some inactivation of *L. monocytogenes* was observed during the first 90 min of incubation at 37 °C when nanoemulsified D-limonene was added to different growth media, even when, eventually, the survivors were able to grow after a 2 day incubation period. These results mean that, even at growth temperatures, nanoemulsified D-limonene would cause some microbial inactivation, although, at these non-lethal temperatures, the survivors would eventually grow.

Fig. 2 shows DRTCs of *L. monocytogenes* in TSB without and with D-limonene added directly or as a nanoemulsion. DRTCs are parallel, which means that the effect of the D-limonene on the thermal resistance of *L. monocytogenes*, either if it is added directly or nanoemulsified, does not depend on the treatment temperature. Actually, no statistical significant differences were found between *z* values (Table 2).

Similar decreases of heat resistance were also observed in distilled water, peptone water and brain heart infusion broth fitted at different pH values (data not shown). Should this decrease in heat resistance be confirmed with other nanoemulsified antimicrobials and other microorganisms, this could mean a new way to improve thermal treatments applied to food or even to be used as a cleaning and disinfection method.

Combinations of antimicrobials with heat have been tested by several authors, showing interesting results. For example, Cava-Roda et al. (2012) added vanillin to milk, achieving an average reduction of 25% in the time needed for a 4 log reduction in the populations of *L. monocytogenes* when adding 1400 ppm. Also Juneja et al. (2013) used this microorganism to explore the combined effect of heat, sodium chloride and polyphenols from apple in minced meat, showing reductions of up to 68% in D-values. Char, Guerrero, and Alzamora (2009) also found a decrease in the thermal resistance of *Listeria innocua* to one third when adding 1100 ppm of vanillin to orange juice. These authors reached to similar results when combining mild heat with vanillin and citral (Char, Guerrero, & Alzamora, 2010). Cherrat et al. (2014) have also tested the combined effects of *Laurus nobilis* and *Myrtus communis* essential oils with several physical treatments, including heat, and found synergistic effects in the combination of these essential oils and heat, reducing in an average of 3 times the heat resistance of both *L. monocytogenes* and *E. coli* O157:H7. Similar reductions in the thermal resistance of *E. coli* O157:H7 in orange juice were found by Espina et al. (2014) when combining limonene or orange essential

Table 1
D values, 95% confidence limits and r_0 values of *Listeria monocytogenes* in TSB with 0.5 mM D-limonene added directly or nanoemulsified.

Medium	Temperature (°C)	D value (min)	95% − CL	95% + CL	r_0
TSB	45.0	784[i]	512	1670	0.961
	50.0	148[g]	119	194	0.979
	52.5	29.2[f]	24.7	35.8	0.987
	55.0	13.8[e]	12.4	15.6	0.992
TSB + 0.5 mM limonene (direct)	45.0	290[h]	253	339	0.995
	50.0	25.7[f]	22.1	30.9	0.983
	52.5	12.2[e]	9.70	16.2	0.970
	55.0	7.67[d]	6.38	9.63	0.985
TSB + 0.5 mM limonene (nanoemulsion)	45.0	8.13[d]	6.98	9.73	0.983
	50.0	1.21[c]	1.00	1.53	0.993
	52.5	0.420[b]	0.289	0.769	0.950
	55.0	0.197[a]	0.158	0.261	0.971

[a–i]: same letters indicate that there are no significant differences.

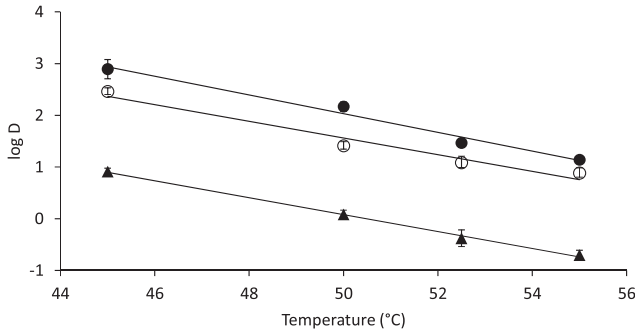

Fig. 2. Decimal Reduction Time Curves (DRTCs) of *Listeria monocytogenes* in TSB broth. Control: ●; 0.5 mM D-limonene added directly: ○; 0.5 mM D-limonene nanoemulsified: ▲.

Table 2
z values, 95% confidence limits and r_0 values of *Listeria monocytogenes* in TSB with 0.5 mM D-limonene added directly or nanoemulsified.

Medium	*z* value (°C)	95% − CL	95% + CL	r_0
TSB	5.52	3.90	9.48	0.990
TSB + 0.5 mM limonene (direct)	6.19	3.89	15.2	0.982
TSB + 0.5 mM limonene (nanoemulsified)	6.10	5.31	7.17	0.999

oil with heat. Among the highest reductions observed in thermal resistance when combining essential oils with heat, are those found by Espina et al. (2012) when treating *E. coli* O157:H7 in apple juice, where treatment times were down to 6 times lower when lemon essential oils were added.

All these researches show, in general, average decreases of three times in microbial heat resistance of different vegetative cells when adding different antimicrobials directly to the heating medium. These synergistic effects have been explained in terms of inactivation of heat-injured cells when the antimicrobials are present in the heating medium (Espina et al., 2012), *i.e.* heat causes injuries to different cell structures but is not able to inactivate these injured cells when applied alone. However, when combined, antimicrobials would help to inactivate these injured cells.

When considering bacterial spores, the picture is even worse, since they appear to be insensitive to the antimicrobials added directly to the heating medium and only when adding them to the recovery medium, some decreases in the apparent heat resistance can be observed (Esteban, Conesa, Huertas, & Palop, 2015; Huertas, Esteban, Antolinos, & Palop, 2014; Lekogo, Coroller, Mathot, Mafart, & Leguerinel, 2010).

The results here reported further confirm those results previously achieved with antimicrobials added directly, *i.e.* reductions of two to five times in the heat resistance (depending on the heating temperature; Table 1). However, the nanoemulsion of D-limonene implies a further decrease of the thermal resistance of *L. monocytogenes* of about one hundred times, or even more, depending on the treatment temperature. Only Luis-Villarroya et al. (2015) found comparable decreases in the thermal resistance of *E. coli* O157:H7 when adding a propolis-based dietary supplement to pH 4 buffer. In this case, the time needed to achieve 5 log reduction was more than 40 times lower. Interestingly, these authors also used propylene glycol as solvent. These authors explained the decrease in heat resistance found on the basis that heat might facilitate the diffusion of the antimicrobials into the liquid phase of the membrane, allowing them to penetrate the cell and reach more easily their target sites within the cytoplasm (Luis-Villarroya et al., 2015). In our case, the decrease in heat resistance could also be explained because of the use of the antimicrobial in form of nanoemulsion, since it has been shown that nanoemulsions improve the distribution and solubility of oily antimicrobials in aqueous media (Pan, Chen, Davidson, & Zhong, 2014), allowing them to reach more easily the target microorganisms.

In summary, these results show a very promising application of nanoemulsified oily antimicrobials, used in combined processes with heat, which would allow to reduce greatly (about one hundred times) the intensity of the thermal treatments currently applied in the food processing industry.

Acknowledgements

This research was financially supported by the Ministry of Science and Technology of the Spanish Government and European Regional Development Fund (ERDF) through Projects AGL-2010-19775 and AGL2013-48993-C2-1-R.

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