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Short communication

Fat and fibre interfere with the dramatic effect that nanoemulsified D-limonene has on the heat resistance of *Listeria monocytogenes*

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ABSTRACT

The application of D-limonene in form of nanoemulsion has been proved to reduce dramatically the thermal resistance of *Listeria monocytogenes* in culture media. The present research shows very promising results on the application in food products. The thermal resistance of *L. monocytogenes* was reduced 90 times when 0.5 mM nanoemulsified D-limonene was added to apple juice. This is the biggest reduction in the heat resistance of a microorganism caused by an antimicrobial described ever. However, no effect was found in carrot juice. A carrot juice system was prepared in an attempt to unravel which juice constituents were responsible for the lack of effect. When fat and fibre were not included in the carrot juice system formulation, the thermal resistance of *L. monocytogenes* was, again, dramatically reduced in presence of nanoemulsified D-limonene, so these components were shown to interfere with the effect. Once this interaction with food constituents becomes solved, 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

Fruit juices are healthy and nutritious drinks. Development of new packaging technologies has increased fruit juice consumption in the last decades. The trend of current society is to consume fresh-like products that keep their nutritional and quality properties next to the raw products. Hence, food industry needs to reduce the intensity of the thermal treatments and to seek strategies that permit to keep the same food safety levels without significantly increasing the costs of production (Devlieghere et al., 2004). However, mild heat treatments can result in the survival of pathogenic microorganisms.

Past occurrences of illnesses due to consumption of contaminated unpasteurized fruit juices have led the United States Food and Drug Administration (USFDA) to enact the federal Juice Hazard Analysis and Critical Control Point (HACCP) and ensure the safety of juice products (FDA, 2001). This program compels manufacturers to subject juice products to a processing step or combinations of processes capable of reducing the population of the target pathogen by 5 log10 cycles (Goodrich et al., 2005).

Bacterial pathogens pertinent to juice safety have been identified as *Escherichia coli* O157:H7, *Salmonella enterica* and *Listeria monocytogenes*. The identification of these pathogens was based on their historical association with juice products as well as the possibility of the pathogens to be involved in future outbreaks (FDA, 2001).

One of the strategies followed in recent years to reduce the thermal treatments has been the application of antimicrobials. When they have been applied combined with thermal treatments, a reduction of treatment temperatures and times can be achieved, improving the nutritional and sensory properties of the product while keeping its safety. Among natural antimicrobials, essential oils have been widely used. Essential oils contain a complex mixture of non-volatile and volatile compounds produced by aromatic plants as secondary metabolites (Bakkali et al., 2008). Essential oils have flavoring, antioxidant and antimicrobial properties (Tajkarimi et al., 2010). Antimicrobial action of essential oils

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has been attributed to their terpene or terpenoid nature and their interaction with microbial cell membranes. They are known to penetrate through the microbial membrane and cause the leakage of ions and cytoplasmic content, thus leading to cellular breakdown (Espina et al., 2013). The interest of incorporating essential oils in foods as preservatives is related to their recognition as safe natural compounds, being a potential alternative to produce foods free of synthetic additives. In recent years, only a few researches have evaluated the combined effect of heat treatments and essential oils applied directly in fruit juices. Somolinos et al. (2010) evaluated the combined effect of citral, a terpenoid that is present in some citric fruit essential oils, and mild heat treatments against *E. coli* in a culture medium. These authors found a reduction of the initial population in 5 $\log_{10}$ cycles when 200 μL of citral and a temperature of 53 °C were applied to the heating medium. Later, Espina et al. (2012), investigated the combination of mild heat treatments with citrus fruit essential oils against *E. coli* in apple juice finding that treatment times were about 6 times lower when lemon essential oil was added. Ait-Ouazzou et al. (2012) evaluated the bactericidal effect of carvacrol in combination with mild heat against *E. coli* O157:H7 in fruit juices, reaching a reduction of 75% of the time to reduce 5 $\log_{10}$ cycles when carvacrol was added to the heating medium. Espina et al. (2014) evaluated the combined effect of lemon essential oil and mild heat treatment in liquid whole egg against *Salmonella* Seftenberg and *L. monocytogenes*, inactivating up to 4 $\log_{10}$ of initial population in both cases.

The incorporation of antimicrobial essential oils to foods still presents several drawbacks due to their poor water solubility and their strong flavor (Burt, 2004). Hence, there is a need to develop technologies that allow to increase the solubility of the essential oils in water and to reduce the concentration of active ingredients. Nanoemulsions allow solving the non-solubility of the essential oils in water (Donsì et al., 2012). These properties of nanoemulsions are determined by their droplet size and size distribution. Nanometric range emulsion droplets fuse with the cell membrane of bacteria and destabilize it, resulting in leakage of intracellular constituents (Ghosh et al., 2014).

Recent researches have investigated the antimicrobial effect of essential oil nanoemulsions, but no thermal treatment was applied in combination. Ghosh et al. (2014) evaluated the antimicrobial effect of a nanoemulsion of eugenol in orange juice against *Staphylococcus aureus*. Donsì et al. (2014) evaluated the antimicrobial effect of a nanoemulsion of carvacrol in solid foods (zucchini and meat sausages) against *E. coli*. Severino et al. (2014) evaluated the antimicrobial effect of four nanoemulsions of essential oils, incorporated in chitosan based coatings over broccoli florets against *L. monocytogenes*. Maté et al. (2016a) also evaluated the antimicrobial effect of a nanoemulsion of D-limonene combined with nisin on different culture media and foods against *L. monocytogenes*. All these researches have provided satisfactory results regarding the application of essential oils in form of nanoemulsions.

Maté et al. (2016b) showed the synergistic effect of mild heat treatment and a D-limonene nanoemulsion on *L. monocytogenes* in culture medium. In this investigation, the thermal resistance of *L. monocytogenes* was reduced about one hundred times. The aim of this research was to explore the effect of this same combined treatment on *L. monocytogenes* in fruit juices and to unravel which components of fruit juices interact with the nanoemulsion interfering with its antimicrobial effect.

2. Materials and methods

2.1. Bacterial strains

Listeria monocytogenes CECT 4032 was used in this research and it was provided by the Spanish Type Culture Collection (CECT, Valencia, Spain). This strain was stored at −80 °C (30% glycerol) until use. Fresh cultures 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 and preparation of nanoemulsions

D-limonene was obtained from Sigma Aldrich Chemie (Steinheim, Germany). The nanoemulsions of D-limonene were prepared following the procedure described by Maté et al. (2016a) based on catastrophic phase inversion (CPI) method (Zhang et al., 2014). Briefly, the aqueous phase was prepared by mixing 55 mL of sterile distilled water and 27.5 mL of propylene glycol (Panreac, Barcelona, Spain). The oily phase was prepared mixing 6 mL of Tween 80 (Panreac) and 11.5 mL of D-limonene. Nanoemulsions were prepared by slowly adding aqueous phase into the oily phase with gentle magnetic 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. All the ingredients of the nanoemulsion (D-limonene, propylene glycol and Tween 80) are considered as GRAS substances and permitted as food additives in the European Union.

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., 2016a).

2.3. Fruit juices and juice systems

The natural carrot juice was prepared in the laboratory. Carrots obtained from a local market were washed with distilled water. Then carrots were homogenized with Thermomix® (Vorwerk, Germany) for 20 min at 10,000 rpm speed. The juice obtained was dispensed in bottles and autoclaved for 20 min at 120 °C.

A carrot juice system was also prepared by adding the majoritarian compounds described by the Agricultural Research Service of the USDA (2015) about nutritional composition of carrot juice. The composition per 100 g was the following: 0.9 g of protein hydrolysate from wheat gluten (Sigma Aldrich, Spain), 0.2 g of sunflower seed oil (Sigma Aldrich), 9.6 g of fructose (Sigma Aldrich), 2.8 g of cellulose fibre (Sigma Aldrich) and 86.5 g of distilled water. Then the carrot juice system was dispensed in bottles and sterilized in autoclave for 20 min at 120 °C. To explore the interaction of the food compounds with the nanoemulsion, some of these compounds were removed from the carrot juice system. When one (or more) compound(s) was (were) removed, its (their) corresponding weight was replaced by distilled water.

Pasteurized apple juice was acquired from a local market. The nutritional composition was 0.2 g of protein, 20.2 g of carbohydrates and 79.6 g of water. No fat neither fibre was present in the apple juice.

2.4. Heat treatments

Thermal inactivation kinetics were determined at constant temperature in a thermoresistometer Mastia as described by Conesa et al. (2009). Briefly, the vessel of the thermoresistometer

was filled with 400 mL of pre-sterilized fruit juice supplemented (or not) with 0.5 mM D-limonene nanoemulsified. This concentration was chosen as it did not change sensory properties of fruit juices (data not shown). Heat treatments were conducted at 52.5 °C. Once the heating medium temperature had attained stability (±0.05 °C), it was inoculated with 0.2 mL of the cell culture (approx. 10^9 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 pour plated in TSA (Scharlau Chemie). Plates were incubated for 24 h at 37 °C. Each treatment was assayed in triplicate in independent experiments performed in different days. Controls in sterile distilled water and foods with nanoemulsion components (tween 80 and 1, 2- propanediol. Panreac, Spain) without D-limonene were developed at the same temperature (52.5 °C) to evaluate the antimicrobial effect of these components and no antimicrobial effect was observed for both compounds (data not shown).

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 in front of the corresponding heating times. Survival curves included all the counts obtained in the different repetitions.

Correlation coefficients (r_0) of the regression lines of survival curves and 95% confidence limits (CL) were calculated by an appropriate statistical package.

3. Results and discussion

3.1. Droplet size distribution

Fig. 1 shows the droplet size distribution of the nanoemulsion of D-limonene, at the beginning of the experiment. The mean droplet size was of 0.399 μm, with a Sauter mean diameter (D (3,2)) of 0.262 μm, which falls into the consideration of nanoemulsion (Solans et al., 2005). No differences were found in size distribution along the time the present research was performed (data not shown). Phase separation was neither observed along this time. Data about size particles distribution showed similar results to these obtained previously (Maté et al., 2016a, 2016b).

3.2. Effect of nanoemulsified D-limonene on the heat resistance of Listeria monocytogenes in foods

Fig. 2 shows the survival curves of *L. monocytogenes* in apple juice (A) and carrot juice (B), as affected by the presence of 0.5 mM nanoemulsified D-limonene. The results were completely different in one and other juice. While the thermal resistance was reduced 90 times in apple juice with 0.5 mM nanoemulsified D-limonene added (from a $D_{52.5\ °C}$ = 1.49 min without D-limonene to a $D_{52.5\ °C}$ = 0.0166 min with 0.5 mM D-limonene; Fig. 2A and Table 1), this effect was completely absent in the carrot juice (Fig. 2B and Table 1). It is worth to note that the heat resistance of *L. monocytogenes* at 52.5 °C in apple juice with 0.5 mM nanoemulsified D-limonene was so low that it was necessary to use a fraction collector to take the samples, since the whole experiment lasted less than 3 s. Actually, more than 90% of the initial population was inactivated instantaneously, just before the first sample had been taken, *i.e.* in less than 0.5 s.

The dramatic decrease of the thermal resistance of *L. monocytogenes* found in apple juice when adding nanoemulsified D-limonene was of similar magnitude to the decrease previously reported for the same strain of this microorganism in TSB and other laboratory media (Maté et al., 2016b). All these decreases can be regarded as among the biggest reductions in thermal resistance caused by a combined process with heat and an antimicrobial. Only Luis-Villarroya et al. (2015) have found similar (although smaller, *i.e.* about 40 times) decreases of the thermal resistance of *E. coli* O157:H7 when adding propolis to pH 4 buffer. However, this effect was reduced to about 6.25 times when the thermal treatment was applied in apple juice (Luis-Villarroya et al., 2015). Opposite to this impressive decrease of the thermal resistance, the behavior of this same microorganism in carrot juice was completely different (Fig. 2B and Table 1). A $D_{52.5\ °C}$ = 22.6 min was obtained in natural carrot juice and no significant decrease of the thermal resistance was observed after the addition of nanoemulsified D-limonene (Table 1).

The reason for the lack of effect could be attributed to the food matrix, since it has been shown that the food matrix components

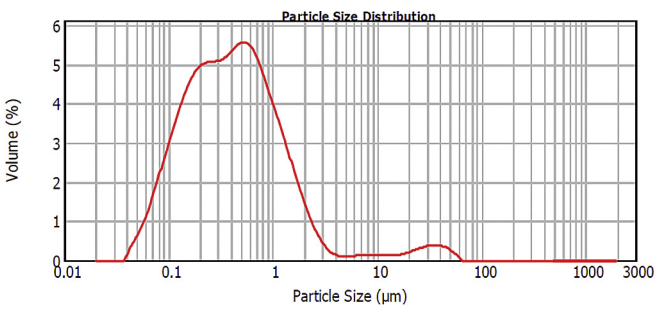

Fig. 1. Droplet size distribution of the nanoemulsion of D-limonene.

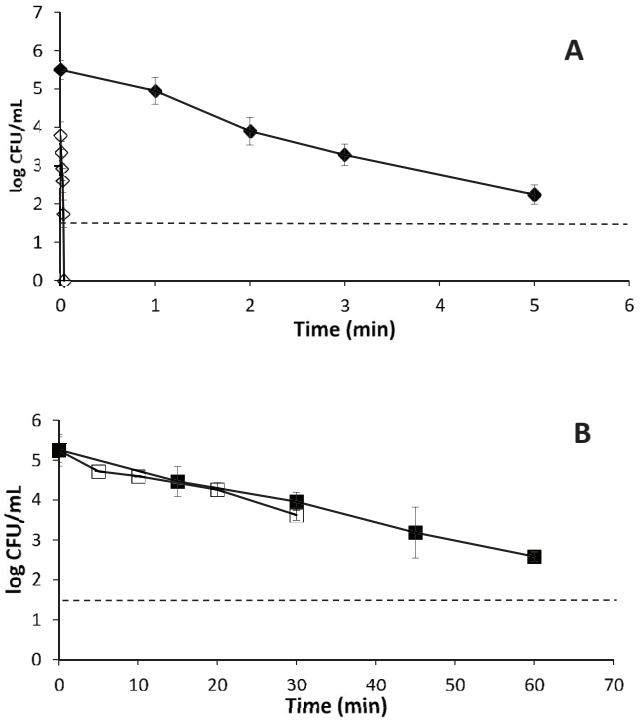

Fig. 2. Survival curves of *Listeria monocytogenes* at 52.5 °C in apple juice (A) and carrot juice (B). Apple juice: ◆; apple juice with 0.5 mM D-limonene nanoemulsified: ◊; carrot juice: ■; carrot juice with 0.5 mM D-limonene nanoemulsified: □. Dashed lines represent the detection level of survival counts. Error bars stand for the standard deviation.

Table 1
D values, 95% confidence limits and r_0 values of *Listeria monocytogenes* at 52.5 °C in different heating media without or with 0.5 mM D-limonene nanoemulsified.

Heating medium	D value (min)	95% − CL	95% + CL	r_0
Apple juice	1.49[c]	1.19	1.99	0.991
Apple juice with 0.5 mM D-limonene nanoemulsified	0.0166[a]	0.0124	0.0251	0.984
Natural carrot juice	22.59[g]	20.30	25.47	0.998
Natural carrot juice with 0.5 mM D-limonene nanoemulsified	20.45[fg]	14.90	32.60	0.981
Carrot juice system	24.76[fgh]	17.89	40.22	0.979
Carrot juice system with 0.5 mM D-limonene nanoemulsified	12.94[e]	11.72	14.44	0.998
Carrot juice system without fat	18.42[f]	16.37	19.11	0.982
Carrot juice system without fat with 0.5 mM D-limonene nanoemulsified	8.31[d]	7.17	10.31	0.987
Carrot juice system without fibre	68.49[i]	53.95	93.25	0.930
Carrot juice system without fibre with 0.5 mM D-limonene nanoemulsified	38.16[h]	32.77	45.53	0.950
Carrot juice system without fibre and fat	65.43[i]	50.37	93.33	0.968
Carrot juice system without fibre and fat with 0.5 mM D-limonene nanoemulsified	0.952[b]	0.805	1.165	0.995

[a–f]: Same letters indicate that there are no significant differences.

may interact with the essential oils, impairing their antimicrobial effects (Gutierrez et al., 2009; Hyldgaard et al., 2012; Weiss et al., 2014; Luis-Villarroya et al., 2015; Perricone et al., 2015; Rivera-Calo et al., 2015).

The food matrix also has an important effect on the thermal resistance of bacteria. Among food characteristics, the pH is one of the most influencing factors on the heat resistance of bacteria (Ocio et al., 1994; Palop et al., 1999; Esteban et al., 2013). Actually, in this research, the thermal resistance was greatly reduced from carrot juice, with a pH of 7.1 ($D_{52.5\,°C}$ = 22.6 min), to apple juice, with a pH of 3.5 ($D_{52.5\,°C}$ = 1.49 min), when no antimicrobials were added, so the pH seemed to have an effect on the thermal resistance of *L. monocytogenes*. Hence, in the same way, it could be expected that the pH would also be involved in the interaction with the essential oil nanoemulsion. However, the addition of nanoemulsified D-limonene led to a further decrease of 90 times in the $D_{52.5\,°C}$ in the acidic apple juice, but to no significant decrease in the *D* value in the neutral carrot juice. If any, neutral pH was inhibiting the effect of nanoemulsified D-limonene while acidic pH was favoring this effect. However, the dramatic decrease on the heat resistance of this same strain of *L. monocytogenes* was also shown in brain heart infusion broth at neutral pH when nanoemulsified D-limonene was present (Maté et al., 2016b), and no effect of the pH of the brain heart infusion broth was even observed. Therefore, the pH of the food did not seem to have a relevant role on the lack of effect of the nanoemulsion.

3.3. Effect of food constituents on the heat resistance of Listeria monocytogenes in presence of nanoemulsified D-limonene

An attempt was made to unravel the compounds present in carrot juice, which could be responsible for this drop of the antimicrobial effect. For this purpose, a carrot juice system, composed of its majoritarian components (USDA, 2015) was prepared. The thermal resistance in this carrot juice system was similar to that shown in the natural carrot juice (Figs. 2b and 3 and Table 1). No significant differences were found among the $D_{52.5\,°C}$ values obtained in both media, and only a slight, but significant, reduction of the thermal resistance, to about one half, was achieved when nanoemulsified D-limonene was present in the carrot juice system (Fig. 3 and Table 1). These results point out, first that the composition of a food can be mimicked with its majoritarian components, at least in which microbial thermal resistance regards and, second, that the lack of effect of nanoemulsified D-limonene was still present in the carrot juice system: although in this artificial juice some significant effect was shown, the effect was very low, at least in comparison to that found in apple juice (Fig. 2A).

Different constituents were removed from the carrot juice

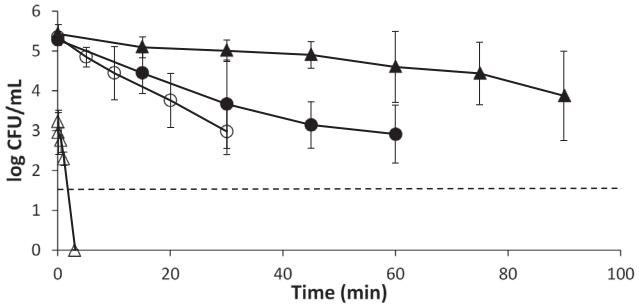

Fig. 3. Survival curves of *Listeria monocytogenes* at 52.5 °C in carrot juice system. Carrot juice system: ●; carrot juice system fat and fibre free: ▲; carrot juice system with 0.5 mM D-limonene nanoemulsified: ○; carrot juice system fat and fibre free with 0.5 mM D-limonene nanoemulsified: △. Dashed line represents the detection level of survival counts. Error bars stand for the standard deviation.

system. The effect of this removal on the heat resistance of *L. monocytogenes*, when the nanoemulsified D-limonene was not present in the heating medium, was as follows. The removal of proteins and fructose did not affect the thermal resistance of *L. monocytogenes* in the carrot juice system (data not shown). Also, the removal of fat led to a non-significant variation in the heat resistance (Table 1). However, it is noteworthy to point out the increase on the thermal resistance when fibre was removed from the formulation of the carrot juice system (from a $D_{52.5\,°C}$ = 24.76 min with fibre to a $D_{52.5\,°C}$ = 68.49 min without fibre; Table 1). This increase on the thermal resistance, of almost three times, would involve an important role of fibre on reducing the thermal resistance of bacteria. When both fibre and fat were removed, a similar increase was also observed (Table 1).

When nanoemulsified D-limonene was present in the heating medium, proteins and fructose neither have an effect, since the removal of these compounds from the carrot juice system led to no reduction of the thermal resistance of *L. monocytogenes* (data not shown). Fat had some effect, since the removal of fat from carrot juice system led to a reduction of the thermal resistance to about one half when adding nanoemulsified D-limonene (from a $D_{52.5\,°C}$ = 18.42 min without D-limonene to a $D_{52.5\,°C}$ = 8.31 min with nanoemulsified D-limonene; Table 1). Fat presence in foods has been linked to the lack of antimicrobial effect of essential oils, since they are supposed to move to the oily phase, turning absent from the watery phase, where the bacteria develop (Burt, 2004; Weiss et al., 2014; Perricone et al., 2015).

Also fibre had some effect, since the removal of fibre also led to a reduction of the thermal resistance of *L.* monocytogenes in carrot juice system to about one half when adding nanoemulsified D-

limonene (from a $D_{52.5\ ^\circ C}$ = 68.49 min without D-limonene to a $D_{52.5\ ^\circ C}$ = 38.16 min with nanoemulsified D-limonene; Table 1).

But the most impressive decrease of the thermal resistance of *L. monocytogenes* in presence of nanoemulsified D-limonene was evidenced when both fat and fibre were removed from the formulation of the carrot juice system. In this fat and fibre free carrot juice system a reduction of almost 70 times in the *D* value was observed (from $D_{52.5\ ^\circ C}$ = 65.43 min without nanoemulsified D-limonene to $D_{52.5\ ^\circ C}$ = 0.952 min with nanoemulsified D-limonene; Fig. 3 and Table 1). This decrease in the thermal resistance was comparable to that found in apple juice (90 times; Fig. 2A and Table 1) or laboratory media (Maté et al., 2016a), in which no fat neither fibre are present. As already observed in apple juice, or even in laboratory media (Maté et al., 2016a), an instantaneous inactivation of more than 90% of the initial population of *L. monocytogenes* was also shown in the fat and fibre free carrot juice system. It is hypothesised that this high percentage could correspond to an extremely sensitive portion of the population, and the less than 10% of the population remaining corresponding to a fraction more resistant to the combination of heat plus nanoemulsified D-limonene. To our knowledge, this is the first report of such a dramatic effect of a combined preservation process using an antimicrobial in a food or a food system. Food constituents are known to interact with antimicrobials (Burt, 2004; Hyldgaard et al., 2012; Weiss et al., 2014; Perricone et al., 2015) and so, when antimicrobials are applied in foods to be heat treated, much of their antimicrobial effect is lost (Espina et al., 2012; Luis-Villarroya et al., 2015). However, the application of the antimicrobials in form of nanoemulsion helps to maintain the effect.

It is noteworthy that fat and fibre separately interfered in a small amount with the effect of nanoemulsified D-limonene on the heat resistance of *L. monocytogenes*, so when only one of these compounds were removed from the formulation, the reduction of the thermal resistance was scarce, but when both compounds were absent, then the reduction of the thermal resistance caused by the presence of nanoemulsified D-limonene was very large. Then, some kind of interference with the effect of D-limonene has been shown, and this interference is only avoided when both compounds are removed from the food.

This research shows that nanoemulsions of essential oils can be applied in a wide variety of foods with very satisfactory results, largely solving the problems raised by the food industry for the application of these compounds. But, this research also shows that certain nutritional components of foods could influence the effect of nanoemulsions of essential oils. The mechanisms of interaction of the nanoemulsion with these food constituents should be elucidated, so improvements in their effect on the thermal resistance (and other properties of microorganisms) could be achieved. This fact would expand the variety of foods in which nanoemulsions of essential oils can be applied.

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