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Behavior of *Listeria monocytogenes* type1 355/98 (85) in meat emulsions as affected by temperature, pH, water activity, fat and microbial preservatives

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ABSTRACT

The aim of this work was to analyze the effect of temperature (10–30 °C), fat content (20–50%), sodium chloride (2.5–5.0%) and preservative concentrations: sodium nitrite (0–150 ppm), lactic acid (50–500 mM) and nisin (0–100 IU/g (international units per gram)) on the growth of *Listeria monocytogenes* in a meat emulsion system.

Individual and simultaneous effects of the parameters were tested and the results were mathematical modeled; inhibition indexes were calculated in each case. The addition of 7.5% NaCl inhibited the growth of *L. monocytogenes* at 20 and 30 °C, however, at 10 °C, microbial counts reached approximately 10^6 CFU/g. The addition of 50 mM of lactic acid to obtain a pH $\leq$ 5 inhibited the growth of *L. monocytogenes*. The combinations of lactic acid with sodium nitrite or with nisin showed an enhancement of the inhibitory effect. However, considering the low toxicity of nisin, the combination of lactic acid (50 mM) and nisin (20 IU/g) would be more acceptable in the prevention of the growth of *Listeria monocytogenes*.

1. Introduction

Listeria monocytogenes is a pathogen which causes miscarriages and mastitis in animals whereas in human beings, it can produce infections potentially fatal in susceptible individuals. This bacterium can cause miscarriages in pregnant women and meningitis in newborns, children and adults who are immunosuppressed (Donelly, 1994; Posfay-Barbe & Wald, 2004; Seeliger, 1961; Shih-Yu et al., 2007; Smith et al., 2009). *L. monocytogenes* and *Listeria innocua* are the most frequently isolated strains in food processing plants. However, most of the cases of listeriosis transmitted through food are due to the contamination of raw or cooked foods with *L. monocytogenes* (Altekruse, Cohen, & Swerdlow, 1997; Cox et al., 1989, WHO 1988). Listeriosis has become a serious public health problem considering that, although it presents low mortality rates depending on the susceptibility of the host, its mortality is high in inmunosupressed persons (20–30%) and has a long incubation period (Chhabra, Carter, Linton, & Cousin, 1999; Donelly, 1994). The minimum infective dose of *L. monocytogenes* has not been established yet (FDA Bad Bug Book, 2009) although it has been indicated that the intake of up to 100 cells does not affect the health of healthy consumers (Jay, 1994).

L. monocytogenes can develop at low temperatures in a wide pH range, with a high concentration of sodium chloride (NaCl) and reduced water activity. Hence, it can survive and multiply in a great variety of food products (Begot, Lebert, & Lebert, 1997; Bergey's, 1986; Cole, Jones, & Holyoak, 1990; Seman, Borger, Meyer, Hall, & Milkowski, 2002). *Listeria* has been involved in a large number of food-borne outbreaks transmitted through food all over the world. The most hazardous products are those "ready to eat" which are stored at room temperature for long periods of time. *Listeria* spp. has been found in a great variety of meats and meat products, even in sausages (Ingham, Beuge, Dropp, & Losinski, 2004; Jonson, Doyle, & Cassens, 1990).

Beef sausage has a limit in fat composition; that cannot exceed the 50% of the mass of the finished product. Lactic acid without any restrictions for use, and sodium nitrite are in the group of permitted products. According to the effective regulations the use of sodium nitrite, potassium nitrate or its combination must not exceed 200 ppm (0.2 mg/g) expressed as sodium nitrite in the final product (USDA-FSIS, 1999, pp. 72185–72186 (Chapter III)).

Fermented sausage is partially dehydrated to favor its preservation for a long period of time. This product reaches pH values ranging between 6.0 and 5.1 and water activities lower than 0.94

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due to the fermentation and drying processes (Frey, 1995) therefore it does not need to be preserved at refrigeration temperature (Girard, 1991). A large number of strains of Listeria have been isolated from dry sausages (Pellicer et al., 2002).

In fermented foods, preservation is achieved through the combined effects of lactic acid and sodium chloride. During fermentation, pH first falls from 5.8 to 5.4 or 5.3 and then it increases to 5.5–5.6. The lactic acid concentration in the final product varies according to the nature and quantity of sugars in the initial product, which in dry sausages can reach average values of 250 mM lactic acid at the beginning up to 600 mM lactic acid at the end of the fermentation (Girard, 1991). During fermentation and drying of sausage, *L. monocytogenes* tends to decrease substantially, however, this organism, which is ubiquitous, psychrotrophic and relatively resistant to curing ingredients, may survive (Ingham et al., 2004; Jonson et al., 1990; Juntilla, Him, Hill, & Nurmi, 1989).

Among the natural preservatives, lactic acid can be usually found in beef, specifically in the muscle as a consequence of the glycogen metabolism, in amounts which depend on its functional condition (0.01–0.02% in the muscle at rest, approximately 0.4% in the fatigued muscle and up to 1% after cadaveric rigidity appears) (Pellegrini, Silvestre, Ochoa La Puente, & Y Ochoa La Puente, 1986; Pränd, Fischer, Schmidhoger, & Y Hans-Jurgen, 1994).

It has been reported that the undissociated lactic acid inhibits the growth of microorganisms and is frequently used to extend the shelf life of foods (Barbuddhe, Malik, & Bhilegaonkar, 1999). Lactic acid is effective to inhibit the development of *L. monocytogenes* when it reaches pH values of 4.5 and 4.6 (Ariyapitipun, Mustapha, & Clarke, 2000; Sorrels, Engil, & Hatfield, 1989).

Curing salts, such as sodium nitrite, which is currently questioned due to the hazard of nitrosamine formation, inhibits *Clostridium botulinum* (Girard, 1991). Experimental data indicate that nitrites reduce the survival time of *L. monocytogenes* and also reduce its growth rate (Buchanan, Golden, Whiting, & Smith, 1994; Buchanan, Stahl, & Whiting, 1989; Whiting & Masana, 1994).

Bacteriocins are increasingly used to control the growth of *L. monocytogenes*; among them, nisin is an antimicrobial agent that can be used in meat products (Brandt et al., 2010; Breukink & Krujiff, 1999), in combination with pH reduction and the addition of NaCl (Bouttefroy, Mansour, Linder, & Millière, 2000).

Nisin is an antimicrobial peptide which acts against *L. monocytogenes* as well as other Gram-positive bacteria. Its use is permitted in dairy products (FAO Nutrition Meeting Report Series N° 45 A (1969); Directive 95/2/CE ratified by the Commission of European Communities FAO/WHO, 2007; FDA 2006). Although nisin still does not have approval for its application in meat products in South America, some countries such as Australia and New Zealand are trying to use it, proposing the inclusion of a limit for the use of nisin in meat products (Food Standards Agency of Australia and New Zealand; October 2007 (A565)). Its potential application is high, especially to avoid the deterioration caused by Gram-positive bacteria in processed meat such as sausage and meat paste in which the homogenization of the product allows the better distribution of nisin (Ariyapitipun et al., 2000; FDA 1988; Gonalves & Massaguer, 1999). The recommended concentration is up to 400 IU/g (international units per gram) of food, which represents 10 ppm.

The aims of this work were: (a) to analyze the effect of temperature (10–30 °C), fat content (20–50%), sodium chloride (2.5–5.0%) and preservative concentrations: sodium nitrite (0–150 ppm), lactic acid (50–500 mM) and nisin (0–100 IU/g) applied both individually and in combination, on the growth of *Listeria monocytogenes* in a meat emulsion representing a model system of sausage at different storage times and (b) to model mathematically the *L. monocytogenes* counts as a function of time evaluating the Inhibition Index under the different tested conditions.

2. Materials and methods

2.1. Sample preparation

Meat emulsions were prepared as sausage model systems, with fresh lean beef and bovine fat (20, 35 and 50%) with the addition of NaCl (2.5, 5.0 and 7.5%) (Anedra).

The emulsion was prepared by using a food processor (Rowenta Universo KA 900 8750/83); water activity was evaluated using Aqualab equipment. Different concentrations of preservatives were added according to each experiment: 75,150 ppm sodium nitrite (Anedra); 50, 100, 250 and 500 mM lactic acid (Anedra) which produced pH values ranging from 5.20 to 3.22 and 10, 20, 50 and 100 IU/g nisin (Nisaplin®, Danisco UK Ltd., Dorset, UK. given by the company AMG S.R.L Argentina).

Meat emulsion samples were packed in thermoresistant polyethylene bags (85 microns thickness) and were subjected to a thermal process (100 °C, 30 min) in order to reduce the microbial load originating from the different raw materials; this procedure enabled their inoculation with the selected strain of *L. monocytogenes*, assuring that the obtained counts, were due to the growth of the inoculated strain and not due to a microorganism from the different ingredients.

Samples were stored at different temperatures (10, 20, 30 °C).

2.2. Inoculum

Reference strain of *L. monocytogenes* type 1 355/98 (85) donated by Dr. N. Leardini of the INEI/ANLIS Institute Dr. Carlos G. Malbrán was grown in brain heart infusion broth (BHI) (Oxoid) for 18 h at 37 °C with agitation from which a microbial concentration of approximately 10^8 CFU/ml was obtained. Then 1 ml of that culture was diluted in 100 ml of BHI to achieve a count of 10^6 CFU/ml. From that dilution 1 ml of 10^6 CFU/ml was inoculated in 100 g of the meat emulsion in order to obtain an initial inoculum of 10^4 CFU/g in the preparation.

The inoculated emulsions were sub-divided under sterile conditions into portions of 10 g each and packed in sterile polyethylene films.

In all the cases the experiments were carried out in triplicates, with a maximum storage time of 16 days.

2.3. Experimental design

Experiments were carried out to analyze the effect of different factors on the growth and decline of *L. monocytogenes* in meat emulsions.

2.3.1. Effect of the addition of different fat percentages (experiment A)

Meat emulsions containing 20, 35 or 50% bovine fat, 2.5% NaCl, were inoculated with *L. monocytogenes* and stored at 20 °C (Total: 3 experiments in triplicates).

2.3.2. Effect of the addition of NaCl and the storage temperature (experiment B)

The effects of storage temperature and water activity (a_w) were analyzed using a *L. monocytogenes* inoculated meat emulsion with 20% fat, and applying a 3 × 3 factorial study, with three storage temperature levels (10, 20 and 30 °C) combined with 3 different NaCl concentrations 2.5, 5.0 and 7.5% resulting to a_w values of 0.985, 0.966 and 0.955 respectively (Total: 9 experiments in triplicates).

2.3.3. Effect of different sodium nitrite concentrations (experiment C)

Sodium nitrite (0, 75 or 150 ppm) was added to *L. monocytogenes* inoculated meat emulsions containing 20% fat, 2.5% NaCl and stored at 20 °C (Total: 3 experiments in triplicates).

2.3.4. Effect of nisin concentrations (experiment D)

Nisin concentrations of 0, 10, 20, 50 or 100 IU/g were added to *L. monocytogenes* inoculated meat emulsions containing 20% fat, 2.5% NaCl and stored at 20 °C.

Nisin stock solution was prepared from Nisaplin (with an activity of 1×10^6 IU/ml of nisin. Nisaplin (1g) was dissolved in nisin diluent (100 ml 0.02 N of HCl (Merck)) to obtain a final solution with 1×10^4 IU/ml of nisin, which was added to the emulsions (Total: 5 experiments in triplicates).

2.3.5. Effect of pH (experiment E)

To analyze the effect of pH on the growth of *L. monocytogenes* using lactic acid as a preservative, different concentrations of lactic acid (50, 100, 250 and 500 mM) were added resulting pH values of 5.20, 4.75, 3.90 and 3.22 in meat emulsions with 20% fat and 2.5% NaCl, stored at 20 °C (Total: 4 experiments in triplicates).

2.3.6. Simultaneous effect of lactic acid and sodium nitrite (experiment F)

The addition of lactic acid and different concentrations of sodium nitrite on the growth of *L. monocytogenes* was tested on meat emulsions with 20% fat and 2.5% NaCl, stored at 20 °C; 50 mM lactic acid was used in combination with 0, 75 or 150 ppm sodium nitrite (Total: 3 experiments in triplicates).

2.3.7. Simultaneous effect of lactic acid and nisin concentrations (experiment G)

This effect was analyzed on meat emulsions with 20% fat and 2.5% NaCl, stored at 20 °C; 50 mM lactic acid was combined with 0, 10 or 20 IU/g nisin (Total: 3 experiments in triplicates).

2.4. Sampling and counting of Listeria

The meat emulsion in each pack was microbiologically analyzed during a maximum storage period of 16 days. 10 g of each emulsion were homogenized with 90 ml of 0.1% peptone water (Biokar) in a Stomacher mixer for 1 min at medium intensity. Serial dilutions in 0.1% in peptone water were made and 1 ml was inoculated in Petri dishes using the pour plate procedure, with the addition of two culture media: (a) Selective medium prepared with Trypticase Soy Agar (Biokar) and the addition of 3.5% NaCl, LiCl (Anedra) 15 g/l and Acriflavine (Sigma) 4.6 mg/l and, (b) Selective medium PALCAM (Difco). All the determinations were carried out in triplicates.

2.5. Mathematical modeling

The microbial counts obtained were modeled using Gompertz equation (equation (1)) which is a double exponential function based on 4 parameters which describe an asymmetric sigmoid curve:

$$\log N = a + c\exp\{-\exp[-b(t-m)]\} \quad (1)$$

where log$N$ is the decimal logarithm of the microbial counts [log(CFU/g)] to time t, expressed in days, a is the logarithm of the asymptotic counts when time decreases indefinitely (roughly equivalent to the logarithm of the initial levels of bacteria [log(CFU/g)]; c is the logarithm of the asymptotic counts when the time is increased indefinitely (it is the number of log cycles of growth) [log(CFU/g)]; m is the required time to reach the maximum growth rate [day], b is the growth rate relative to time m [day]$^{-1}$.

The specific growth rate ($\mu = b \cdot c/e$ with $e = 2.7182$ [log (CFU/g)/day]), the duration of the lag phase (LPD $= m - 1/b$, [day]) and the maximum population density (MPD $= a + c$, [log(CFU/g)]) were derived from these parameters (Giannuzzi, Pinotti, & Zaritzky, 1998).

In the cases in which there was an inhibitory linear effect the following equation was applied (2):

$$\log N = \log N_0 + \mu t \quad (2)$$

where N is the microbial count at time t, N_0 is the microbial count at time 0, N/N_0 is the surviving fraction of microorganisms and μ is the slope of the line representing the specific microbial growth or decline. A negative value of μ corresponds to a bactericidal effect.

2.6. Inhibition index

In all the cases in which inhibition of the microbial growth was observed, the inhibition index (II) was calculated according to the following equation (3):

$$\text{II}: \frac{\left(\log\left(N/N_0\right)_{\text{control}} - \log\left(N/N_0\right)_{\text{treated}}\right)}{\log\left(N/N_0\right)_{\text{control}}} \quad (3)$$

where $\log(N/N_0)_{\text{treated}}$ and $\log(N/N_0)_{\text{control}}$ correspond to the sample treated with the different preservatives and the control sample respectively, being N = number of microorganisms at time t, N_0 = initial number of microorganisms. The control sample corresponds to a meat emulsion containing 20% fat and 2.5% NaCl.

Inhibition index values were obtained from experimental data. The $\log(N/N_0)_{\text{treated}}$ and $\log(N/N_0)_{\text{control}}$ were evaluated at the time the untreated control sample reached the stationary phase. If the inhibition index is equal to 0, it indicates a microbial growth similar to that of control samples ($\log(N/N_0)_{\text{treated}} = \log(N/N_0)_{\text{control}}$). The index takes values between 0 and 1 when there is growth inhibition; there is a bactericidal effect when the index is >1.

2.7. Statistical analysis

The ANOVA test (Analysis of Variance) provides the coefficients and their corresponding Standard Deviations. It has been recommended as one of the best methods to identify the relevant variables (Draper & Smith, 1981). The Analysis of Variance (ANOVA) was applied, with significance levels of 0.05 using a statistical package for computers (SYSTAT Inc. 1990, version 5.0, U.S.A) in the comparison of the counts obtained in PALCAM medium and Trypticase Soy Agar medium with the addition of inhibitors; in the same way the ANOVA was applied with significance levels of 0.05 using the same statistical package for computers in the comparison of the counts obtained from treated sample and its control for all the experiments.

3. Results and discussion

The counts of *L. monocytogenes* in both culture media (trypticase soya agar with inhibitors and PALCAM agar) did not show significant differences ($p > 0.05$). Therefore it was more convenient to use Trypticase Soy Agar medium with inhibitors because it was less expensive than PALCAM medium.

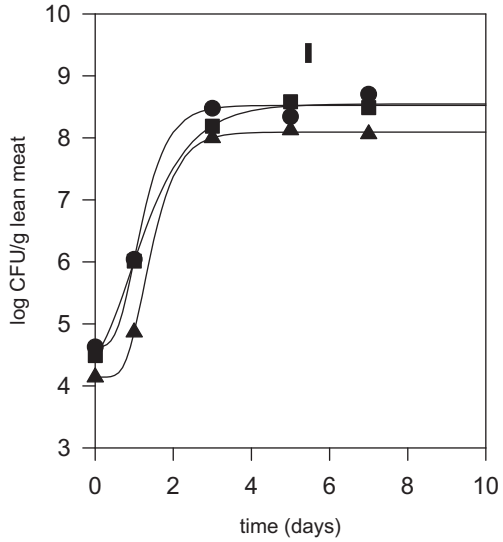

Fig. 1. *L. monocytogenes* growth in meat emulsions with bovine fat percentages contained 2.5% of NaCl and stored at 20 °C: ● 20% of fat, ■ 35% of fat, ▲ 50% of fat; expressed in log (CFU/g) of lean meat. Bar indicates average standard deviation.

3.1. Effect of the addition of different fat percentages on the growth of L. monocytogenes in meat emulsions

The counts expressed in log (CFU/g of emulsion) were higher in the emulsions with lower fat content. Considering that microorganisms grow in the aqueous phase of the emulsion, the microbial counts, which were originally expressed per gram of emulsion, were recalculated and expressed per gram of lean meat (Fig. 1). Once recalculated it was observed that the counts expressed in log (CFU/g of lean meat) did not show significant differences among the samples with different fat contents. Therefore "throughout the present work 20% fat emulsions were selected to analyze the effect of the different factors".

Fig. 1 shows the growth of *L. monocytogenes* in meat emulsions with different fat percentages (20, 30 and 50%), with 2.5% NaCl, stored at 20 °C, expressed per gram of lean meat (experiment A). In all cases LPD values were lower than 1 day and differences were not significant ($p > 0.05$).

3.2. Effect of the addition of NaCl and of the storage temperature on the development of L. monocytogenes in meat emulsions

Fig. 2a–c shows the counts of *L. monocytogenes* inoculated in meat emulsions with 20% fat, containing different concentrations of NaCl (2.5, 5.0 and 7.5%) and stored for 12 days at 30, 20 and 10 °C respectively (experiment B). Derived parameters from Gompertz equation (equation (1)) and Inhibition Index values (equation (3)) at different temperatures and NaCl concentrations are shown in Table 1.

As the temperature increased, the time necessary to reach *L. monocytogenes* counts between 10^7 and 10^9 CFU/g decreased. At 20 and 30 °C (Fig. 2a and b), with the addition of 2.5 and 5.0% NaCl the growth of Listeria increased, being more pronounced and faster with 2.5% NaCl.

At 30 °C μ values for 2.5 and 5.0% NaCl were higher than those obtained at 20 and 10 °C for the same concentrations of NaCl (Table 1). At 30 and 20 °C with the addition of 7.5% NaCl, negative μ values were obtained because of the inhibition of *L. monocytogenes*; however, it is interesting to note that at 10 °C and with 7.5% NaCl, growth was observed reaching final counts of 6.80 log (CFU/g) at 12 days of the storage. At 30 °C and 20 °C LPD values were lower than 1 day and differences were not significant ($p > 0.05$), however at 10 °C, LPD values ranged between 1 day for emulsions with 2.5% NaCl and 2 days for emulsions with 5.0 and 7.5% NaCl (Fig. 2). At 20 and 30 °C the inhibition of Listeria growth was significant with 5.0% NaCl, while with 7.5% NaCl a bactericidal effect was observed (Table 1).

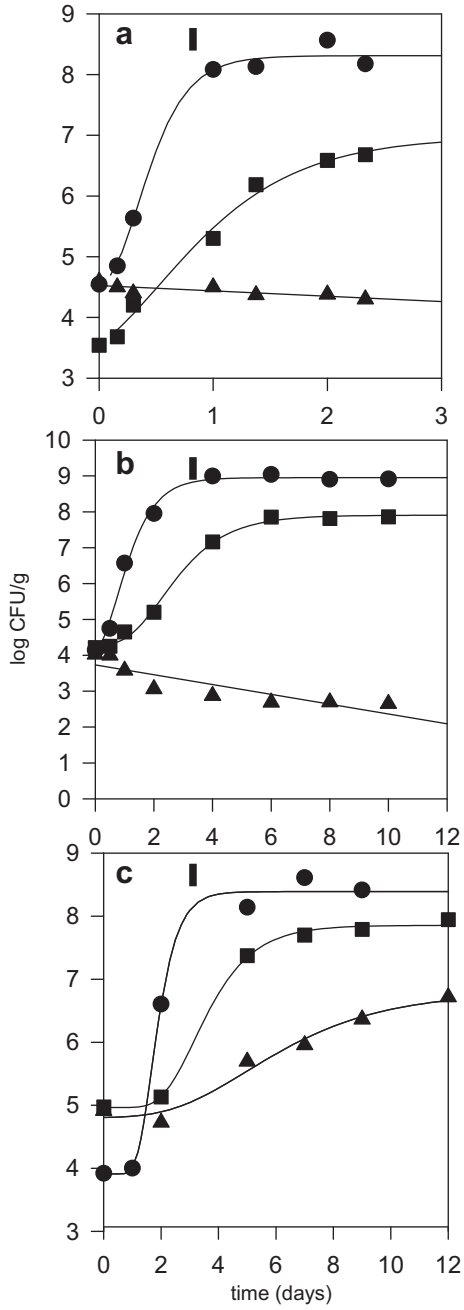

Fig. 2. *L. monocytogenes* growth in meat emulsions with 20% bovine fat and sodium chloride added: ● 2.5% of NaCl ($a_w = 0.985$); ■ 5.0% of NaCl ($a_w = 0.966$); ▲ 7.5% of NaCl ($a_w = 0.955$); stored at different temperatures (a) 30 °C, (b) 20 °C and (c) 10 °C. Bar indicates average standard deviation.

At 10 °C (Fig. 2c), for all NaCl tested concentrations, growth of *L. monocytogenes* was observed, reaching counts of 10^6 CFU/g after 12 days of storage. The inhibition index after 3 days of storage at 10 °C in meat emulsions containing 5.0 and 7.5% NaCl was twice as high than in the emulsion with 2.5% of NaCl. Then, *L. monocytogenes*

Table 1
Effect of temperature and NaCl concentration on the growth of *Listeria monocytogenes* in a meat emulsion containing 20% bovine fat: Gompertz derived parameters (specific growth rate; maximum population density) and Inhibition Index.

T (°C)	NaCl (%)	Gompertz derived parameters		Inhibition Index (equation (3))
		Specific growth rate	Maximum population density	
		μ log (CFU g^{-1} day^{-1})	MPD log (CFU g^{-1})	
30	2.5	2.87 ± 0.31	8.31 ± 0.39	0.08
	5	1.24 ± 0.63	6.97 ± 1.51	0.46
	7.5	−0.08 ± 0.02	–	1.04
20	2.5	2.23 ± 0.47	8.95 ± 0.61	0.00
	5	0.98 ± 0.70	7.89 ± 0.18	0.72
	7.5	−0.13 ± 0.03	–	1.28
10	2.5	2.17 ± 0.46	8.39 ± 0.35	0.35
	5	0.74 ± 0.05	7.85 ± 0.14	0.74
	7.5	0.16 ± 0.04	6.80 ± 0.49	0.85

μ: log CFU g^{-1} day^{-1}, MPD: log CFU g^{-1}.

could survive at high salt concentrations (10% NaCl) and at relative low temperature conditions (10 °C) in which metabolism is reduced, for longer periods than at 30 °C.

The results obtained in the present work agree with the findings of other authors (Cole et al., 1990). In addition, it has been shown that at 4°C *L. monocytogenes* can survive and growth in culture media with up to 10 and 12% NaCl (Galdeiro, Dİsanto and Aliberti 1997).

When *L. monocytogenes* is present in food, its adaptability to high salt concentrations and low temperatures is very important for its survival and possible growth. The ability of *L. monocytogenes* to survive at high salt concentrations is physiologically linked to the growth at low temperatures and this result can be attributed mainly to the accumulation of compatible solutes such as glycine-betaine or carnitine, or both under such conditions to compensate for the exclusion of water, which gives its osmotolerance and cryotolerance (Ko, Tombras Smith, & Smith, 1994; Smith, 1996; Wood et al., 2001). In several *L. monocytogenes* strains, the operon kdp locus has been identified as one of the possible responsible for development under conditions of high salt concentration (Kallipolitis & Ingmer, 2001). It has been indicated that the expression of the kdpE and OrfX genes of operon kdp is required for optimal growth when cells are shifted to higher concentrations of NaCl (Brøndstend, Kallipolitis, Ingmer, & Knöchel, 2003).

3.3. Effect of the different concentrations of sodium nitrite on the growth of L. monocytogenes in meat emulsions

Fig. 3 shows the effect of different concentrations of sodium nitrite (0, 75 and 150 ppm) in meat emulsions with 20% fat, 2.5% NaCl stored at 20 °C on the growth of *L. monocytogenes* (experiment C). Table 2 shows the derived parameters obtained from Gompertz equation (equation (1)) and the Inhibition Index (equation (3)) due to the addition of sodium nitrite to the meat emulsion. The changes of μ were not significant ($p > 0.05$) in emulsions with 75 and 150 ppm of sodium nitrite, in comparison with the emulsion without sodium nitrite. Counts close to 10^8 CFU/g were observed since the third day of storage. In all cases LPD values were lower than 1 day and differences were not significant ($p > 0.05$). Inhibition Index values were lower than 1 and differences were not significant ($p > 0.05$).

Concentrations higher than 100 ppm sodium nitrite in a bacteriological medium at 5 °C, pH 6, with 0.5–4.5% NaCl were reported as having inhibitory action against *L. monocytogenes* (Nerbrink, Borch, Blom, & Nesbakken, 1999).

Duffy, Vanderlinde, and Grau (1994) reported that the addition of 300 ppm sodium nitrite in combination with Na-ascorbate on meat slices, reduced the growth rate and increased twice the lag phase of *L. monocytogenes* with respect to control samples.

3.4. Effect of the addition of different concentrations of nisin on the growth of L. monocytogenes in meat emulsions

Fig. 4 shows the effect of different concentrations of nisin (0, 10, 20, 50 and 100 IU/g) in the inoculated meat emulsions containing 20% of fat, 2.5% NaCl and stored at 20 °C on the growth of *L. monocytogenes* (experiment D). Table 3 shows the derived parameters obtained from Gompertz equation (equation (1)) and the calculated Inhibition Index of Listeria (equation (3)) produced with the addition of nisin to the meat emulsion.

Concentrations of 10, and 20 IU/g nisin did not show inhibitory effect on *L. monocytogenes*, however they generated a reduction of the initial counts. For 50 and 100 IU/g nisin concentrations the changes in μ values were not significant ($p > 0.05$), in comparison with those of the emulsion without nisin (Table 3). In all cases LPD

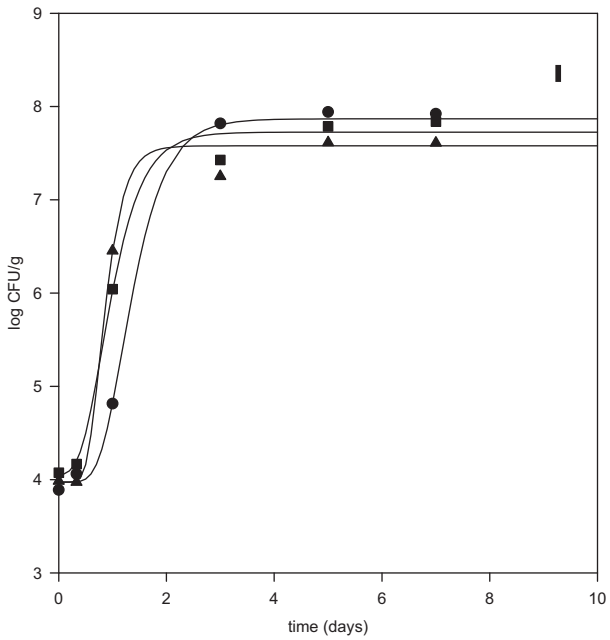

Fig. 3. *L. monocytogenes* growth in meat emulsions with 20% bovine fat, 2.5% of NaCl and stored at 20 °C, with different concentration of sodium nitrite (ppm): ● without sodium nitrite; ■ 75 ppm; ▲ 150 ppm of sodium nitrite. Bar indicates average standard deviation.

Table 2
Effect of sodium nitrite concentration on the growth of *L. monocytogenes* in a meat emulsion system with 20% bovine fat, 2.5% NaCl stored at 20 °C: Gompertz derived parameters (specific growth rate; maximum population density) and Inhibition Index.

Nitrite (ppm)	Gompertz derived parameters		Inhibition Index (equation (3))
	Specific growth rate	Maximum population density	
	μ log (CFU g^{-1} day^{-1})	MPD log (CFU g^{-1})	
0	2.41 ± 0.57	7.58 ± 0.84	0.00
75	2.13 ± 0.18	7.65 ± 0.39	0.03
150	1.94 ± 0.22	7.87 ± 0.13	0.13

μ: log CFU g^{-1} day^{-1}, MPD: log CFU g^{-1}.

values were lower than 1 day and differences were not significant ($p > 0.05$). In the case of the emulsions containing 50 and 100 IU/g nisin, the reduction of the initial counts was high and growth inhibition was significant ($p < 0.05$) (Table 3).

It was observed that as the concentration of nisin increased, a longer time was necessary to reach counts of 10^7 CFU/g. Besides, as the concentration of nisin increased, the initial counts decreased. These results agree with reports of Ariyapitipun et al. (2000), that worked with cubes of meat contaminated with Listeria, treated by immersion in a solution of 400 IU/ml nisin and vacuum packed, and with the results of Zhang and Mustapha (1999) that used a solution of 5000 IU/ml nisin.

The activity of 1 mg pure nisin is 40,000 IU; in food, the acceptable levels of nisin ranged from 100 to 400 international units (IU) per gram of food (or 2.5–10 ppm). Nisin is the only bacteriocin approved as food additive in Europe (Thomas, Clarkson, & Delves-Broughton, 2000) whereas in the U.S.A it has been granted the status of GRAS (generally recognized as safe) (FDA 1988; Jay, 2000, p. 679). In 1969 the FAO Expert Committee on Food Additives approved the use of nisin internationally, considering an acceptable daily consumption of up to 33,000 IU/kg body weight (<1 mg/kg body weight).

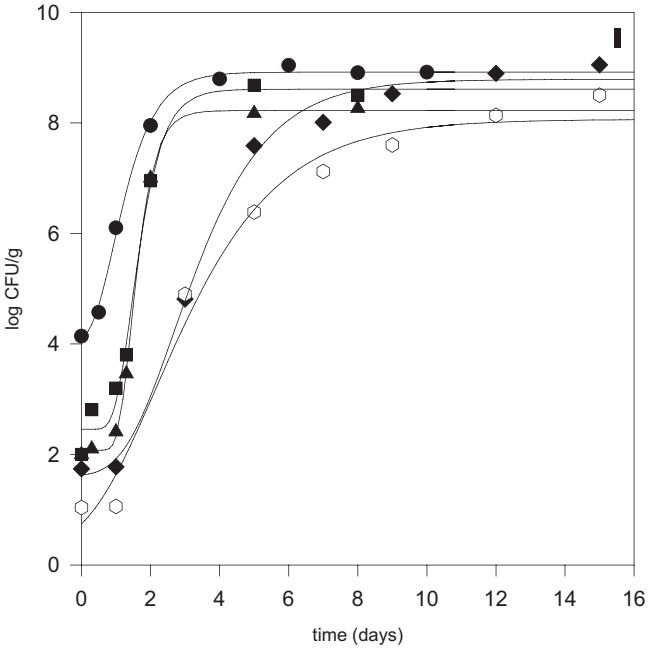

Fig. 4. *L. monocytogenes* growth in meat emulsions with 20% bovine fat, 2.5% of NaCl and stored at 20 °C, with different nisin concentrations (IU/g): ● without nisin; ■ 10 IU/g; ▲ 20 IU/g; ◆ 50 IU/g and ○ 100 IU/g of nisin. Bar indicates average standard deviations.

The effect observed in this work coincides with the significant reduction of the *L. monocytogenes* counts observed in dry Turkish sausage (sucuks) with the addition of nisin in concentrations of approximately 50 and 100 IU/g (Hampikyan & Musmmer, 2007).

The results of this work agree with Solomakos, Govaris, Koidis, and Botsoglou (2008), who observed a reduction of the initial counts in ground meat stored at 4 and 10 °C with the addition of 500 IU/g nisin which reduced the microbial counts of Listeria in 0.9 log (CFU/g).

3.5. Effect of the addition of different concentrations of lactic acid on the growth of *L. monocytogenes* in meat emulsions

Different concentrations of lactic acid (50, 100, 250 and 500 mM) were added to the inoculated meat emulsions with 20% fat, 2.5% NaCl stored for 10 days at 20 °C in order to observe the inhibitory effect produced by pH variations (5.20, 4.75, 3.90 and 3.22 respectively).

Fig. 5 shows the inhibitory effect of the pH on *L. monocytogenes* by the addition of different concentrations of lactic acid in meat emulsions (experiment E).

It is well known that the antimicrobial effect of organic acid is mainly caused by the action of the undissociated fraction of the acid rather than by hydrogen ions. It is due to the increase of the undissociated portion of weak acid which when present in the non-charged state, has greater activity to penetrate the cells than dissociated products (Giannuzzi & Zaritzky, 1996). The undissociated concentration of a weak acid such as lactic acid monoprotic can be calculated through equation (4).

$$[AH] = \frac{Ca[H^+]}{[H^+] + K_1} \quad (4)$$

where [AH] is the undissociated acid concentration (mM), and K_1 is the equilibrium constant of lactic ($K = 10^{-3.86}$), Ca is the total acid concentration (mM), and [H^+] proton concentration.

Table 4 shows the kinetic parameters and the Inhibition Index (equation (3)) of Listeria as affected by the addition of total lactic acid to the meat emulsion. In the same table are included undissociated lactic acid concentration calculated by equation (4). In all the cases a linear regression (equation (2)) was applied obtaining negative specific growth rate values which became more negative as the concentration of acid increased, proving its bactericidal effect.

Giannuzzi and Zaritzky (1996) and Sorrels et al. (1989) reported inhibitory effect of lactic acid on *L. monocytogenes* in culture media with pH values lower than 5.0. Besides Sorrels et al. (1989) reported similar effect on *L. monocytogenes* in cured fermented meats.

Table 3
Effect of nisin concentration on the growth of *L. monocytogenes* in a meat emulsion system with 20% bovine fat, 2.5% NaCl stored at 20 °C: Gompertz derived parameters (specific growth rate; maximum population density) and Inhibition Index.

Nisin (UI/g)	Gompertz derived parameters		Inhibition Index (equation (3))
	Specific growth rate	Maximum population density	
	μ log (CFU g^{-1} day^{-1})	MPD log (CFU g^{-1})	
0	2.20 ± 0.15	8.92 ± 0.22	0.00
50	1.45 ± 0.09	8.78 ± 0.44	0.80
100	1.29 ± 0.20	8.06 ± 1.50	0.82

μ: log CFU g^{-1} day^{-1}, MPD: log CFU g^{-1}.

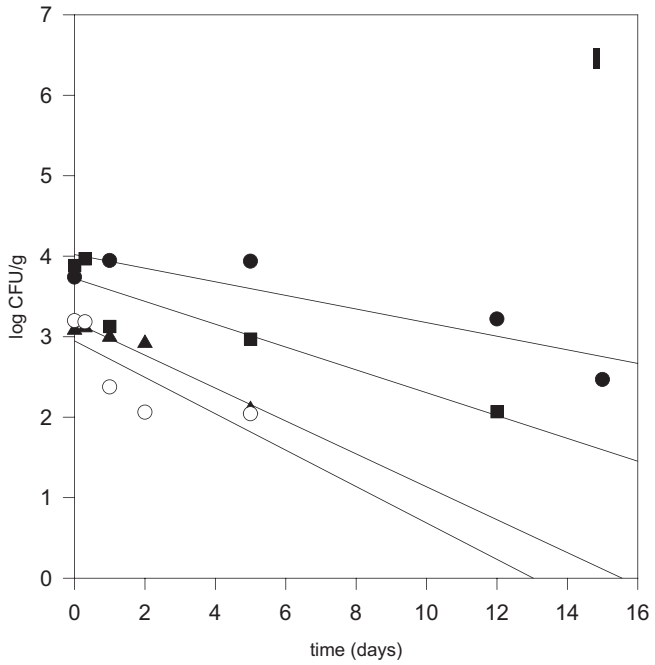

Fig. 5. Inhibition of *L. monocytogenes* in meat emulsion with 20% bovine fat, 2.5% of NaCl and stored at 20 °C with different lactic acid concentrations (mM): ● 50 mM; ■ 100 mM; ▲ 250 mM and, ○ 500 mM of lactic acid. Bar indicates average standard deviation.

Ahmad and Marth (1990) reported that *L. monocytogenes* was inhibited at several studied temperatures (7, 13, 21 and 35 °C) when the lactic acid concentrations in the culture media were of 0.3% or higher.

3.6. Simultaneous effect of lactic acid and sodium nitrite on the decline of L. monocytogenes in meat emulsions

Fig. 6a shows *L. monocytogenes* counts in meat emulsions with 2.5% NaCl, 20% fat and stored at 20 °C with the addition of 50 mM lactic acid and different concentrations of sodium nitrite (0, 75 and 150 ppm of sodium nitrite) (experiment F).

Table 5 shows the kinetic parameters of *L. monocytogenes* and the Inhibition Index (equation (3)) due to the addition of 50 mM lactic acid and different concentrations of sodium nitrite in the meat emulsion. In all the cases a linear model (equation (2)) was applied and the μ values were negative, becoming more negative as the concentration of sodium nitrite increased.

Sodium nitrite when applied individually did not present antimicrobial effect (Fig. 3). However, there was a decrease of 2 log cycles in the counts, when 50 mM lactic acid was combined with 75 ppm sodium nitrite. Besides a decrease of 2.75 log cycles was observed for the combination of 50 Mm lactic acid and 150 ppm of sodium nitrite after 10 days of storage. An enhancement of the bactericidal effect with the combination of lactic acid with 75 and 150 ppm sodium nitrite was observed in comparison with the emulsion containing lactic acid without sodium nitrite. The obtained results agree with those of McClure, Kelly, and Roberts (1991) who worked in culture media and reported that the inhibitory effect of the sodium nitrite is more marked at acid pH.

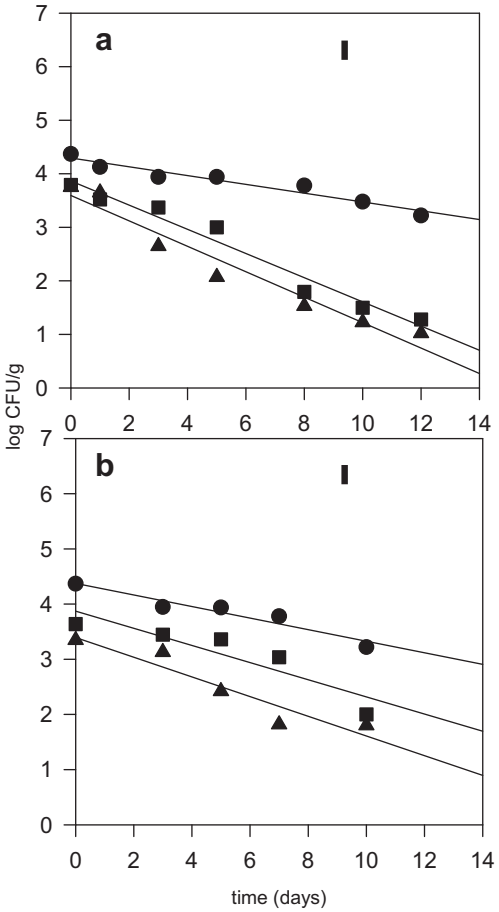

Fig. 6. Inhibition of *L. monocytogenes* in meat emulsion with 20% bovine fat, 2.5% of NaCl, and stored at 20 °C. (a) 50 mM of lactic acid with different concentrations of sodium nitrite: ● without sodium nitrite; ▲ 75 ppm and ■ 150 ppm of sodium nitrite. (b) 50 mM of lactic acid with different nisin concentrations: ● without nisin; ▲ 10 IU/g and ■ 20 IU/g of nisin. Bar indicates average standard deviation.

The combined effect of sodium nitrite and lactic acid showed a bactericidal effect in the presence of NaCl. These results are in agreement with findings of Buchanan et al. (1989), in culture media at pH < 6.0 and different concentrations of sodium nitrite containing also sodium chloride. Whiting and Masana (1994) reported that the bactericidal effect of sodium nitrite (up to 300 ppm in a model system of sausage) was enhanced lowering the pH between 4.0 and 5.0.

Table 4
Effect of total and undissociated lactic acid concentration producing different pH values (ranging between 3 and 7) on the inhibition of *L. monocytogenes* (Specific Decline Rate and Inhibition Index Values) in a meat emulsion system with 20% bovine fat, 2.5% NaCl stored at 20 °C.

Added preservative			Specific decline rate	Inhibition Index (equation (3))
Total lactic acid (mM)	pH	Undissociated lactic acid [AH] (mM)	log (CFU g^{-1} day^{-1})	
50	5.20	2.18	−0.08 ± 0.02	1.05
100	4.75	11.41	−0.14 ± 0.03	1.26
250	3.90	118.98	−0.20 ± 0.02	1.30
500	3.22	359.21	−0.23 ± 0.10	1.54

μ: log CFU g^{-1} day^{-1}.

Table 5
Simultaneous effect of lactic acid combined with sodium nitrite or nisin on the inhibition of *L. monocytogenes* in a meat emulsion system with 20% bovine fat, 2.5% NaCl stored at 20 °C: Specific Decline Rate and Inhibition Index values.

Added preservatives	Specific decline rate	Inhibition Index (equation (3))
Sodium nitrite (ppm)	log (CFU g^{-1} day^{-1})	
0 ppm Na nitrite + 50 mM lactic acid	−0.08 ± 0.01	1.05
75 ppm Na nitrite + 50 mM lactic acid	−0.22 ± 0.01	1.20
150 ppm Na nitrite + 50 mM lactic acid	−0.23 ± 0.02	1.39
Nisin (IU/g)	log (CFU g^{-1} d^{-1})	
0 IU nisin + 50 mM lactic acid	−0.10 ± 0.01	1.05
10 IU nisin + 50 mM lactic acid	−0.15 ± 0.04	1.18
20 IU nisin + 50 mM lactic acid	−0.18 ± 0.03	1.26

μ: log CFU g^{-1} day^{-1}.

3.7. Simultaneous effect of the addition of different concentrations of lactic acid and nisin on the decline of L. monocytogenes in meat emulsions

Fig. 6b shows the counts of *L. monocytogenes* in meat emulsions with 2.5% NaCl, 20% fat and stored at 20 °C with the addition of 50 mM lactic acid and different nisin concentrations (0, 10 and 20 IU of nisin per gram of emulsion) (experiment G).

Table 5 shows the kinetic parameters of the inhibition and the Inhibition Index (equation (3)) of Listeria as affected by the addition of 50 mM lactic acid and different concentrations of nisin in the meat emulsion. In all cases, the linear model (equation (2)) was applied and the μ values were negative, becoming more negative as the concentration of nisin increased.

With 50 mM lactic acid, a decrease of 1 log in the counts of *L. monocytogenes* in meat emulsions was observed. With the combination of 50 mM lactic acid and 10 IU/g nisin the decrease was of 1.6 log, and the decrease was of 2.5 log cycles with the combination of 50 mm of lactic acid and 20 IU/g of nisin after 10 days of storage. The values of the Inhibition Index increased as the lactic acid was combined with nisin.

Our results show an enhancement of the bactericidal effect of lactic acid with the addition of nisin. Similar results were reported by Bouttefroy et al. (2000) who observed in culture media an increase of the bactericidal effect of lactic acid on *L. monocytogenes* by the addition of 50 IU/g nisin and by Martinis, Crandall, Mazzotta, and Montville (1997) working with culture medium with 100 IU/ml nisin at pH 5.50.

Barbuddhe et al. (1999), reported an increase in the inhibition of *L. monocytogenes* in raw buffalo minced meat, with the addition of 2% lactic acid and nisin concentration (400–800 IU/g). Besides, an increase in the inhibition of *L. monocytogenes* was reported when the meat slices were immersed in solutions with nisin (5000 IU/ml) containing 3–5% lactic acid (Samelis et al., 2005).

The combination of lactic acid with sodium nitrite showed a higher inhibitory index than the combination of lactic acid with nisin. However considering that the maximum permitted concentration of sodium nitrite is 150 ppm and that its consumption could be hazardous for consumer's health and the low toxicity of nisin, the combination lactic acid (50 mM) and nisin (20 IU/g) would be more acceptable and safety to control *Listeria monocytogenes* growth in meat emulsions.

4. Conclusions

In the present work the individual and simultaneous effects of NaCl (2.5–7.5%), lactic acid (50–500 mM), sodium nitrite (0–150 ppm) and nisin (0–100 IU/g) on *L. monocytogenes* counts in a meat emulsion system were analyzed; the effects of fat content (20–50%) and temperature (10–30 °C) were also considered. Gompertz derived parameters and Inhibition Index values were determined in order to compare the different effects.

The fat content of the emulsions did not affect significantly ($p > 0.05$) the counts of *L. monocytogenes*, when the results were expressed as CFU/g of lean meat.

It was possible to confirm that *L. monocytogenes* has adaptation systems that give osmotolerance to this microorganism. In products with 7.5% NaCl, the storage at 10 °C did not inhibit the growth of *L. monocytogenes* in meat emulsions whereas at 20 and 30 °C the inhibition of the microorganism was observed.

The addition of sodium nitrite in the concentration range assayed (75–150 ppm) did not produce the inhibition of *L. monocytogenes*.

Nisin was added to the meat emulsions in concentrations ranging between 10 and 100 IU/g; as the concentration of nisin increased, the initial count of *L. monocytogenes* decreased and the time necessary to reach a final count higher than 10^7 CFU/g was extended from 1 day in the control samples to 8 days with 100 IU of nisin.

An inhibitory effect of lactic acid on Listeria was observed for all the tested acid concentrations (50–500 mM). In all cases negative values of μ were observed, ranging between −0.08 at −0.23 as lactic acid concentration increased from 50 at 500 mM.

The combination of lactic acid with sodium nitrite was more effective in the inhibition of *L. monocytogenes* than the combination of lactic acid with nisin. However, taking into account: a) the low limits established by the international regulations for the use of sodium nitrite (up to 150 ppm), b) the hazard for consumer's health that an excess of nitrite in food would imply and c) the safety that the use of nisin represents, it can be concluded that the use of the combination of lactic acid (50 mM) with nisin (20 IU/g) is safer for preventing the growth of *L. monocytogenes* in meat emulsions.

These results could be applied in the meat industry for the formulation of emulsified products.

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