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Combined effect of nisin and moderate heat on destruction of *Listeria monocytogenes* in milk

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Summary — The kinetics of destruction of two strains of *L monocytogenes* in skim milk heated at 60°C with and without addition of 25 or 50 IU/ml of nisin were studied. The survival curves displayed an initial lag phase followed by an accelerating killing phase. As both the length of the lag phase and the destruction rate depended on the temperature and the presence of nisin, a mathematical model was necessary to compare the survival curves and to determine the time to achieve a given reduction of the *Listeria* counts. Two models were compared, each allowing satisfactory goodness of fit. However, as they gave very diverging D-values, the time to achieve a given reduction (3 and 6 $\log_{10}$) of the numbers of *Listeria* in milk was calculated using one model. Addition of 25 or 50 IU/ml of nisin to milk heated between 54 and 65°C considerably reduced the heat resistance of one of the two strains of *Listeria* so that the time needed to achieve a 3 and 6 $\log_{10}$ reduction of populations of this bacterium was substantially diminished. For instance, 16 min at 54°C was sufficient to achieve a 10^3-fold reduction of the number of *Listeria* in milk containing 25 IU/ml nisin, compared to 77 min in absence of nisin. The combined effect of heat and nisin was somewhat enhanced if the bacteria were previously grown in milk at low temperatures. Factors that affect the heat resistance of *Listeria* are discussed as well as the possible mode of action of nisin and heat on destruction of bacterial cells.

***Listeria monocytogenes* / thermal inactivation / nisin / kinetics / mathematical model**

Résumé — Effet combiné de la nisine et d'un traitement thermique modéré sur la destruction de *Listeria monocytogenes* dans le lait. *Les cinétiques de destruction à 60°C de 2 souches de* Listeria monocytogenes *dans du lait écrémé additionné ou non de 25 ou 50 UI/ml de nisine ont été étudiées. Les courbes représentant le $\log_{10}$ de la population bactérienne survivante en fonction du temps de chauffage montrent une phase de latence suivie d'une phase linéaire de destruction. Le temps de latence et la vitesse de destruction dépendaient de la température de chauffage et de la concentration du lait en nisine. Un modèle mathématique était donc nécessaire pour comparer les cinétiques de destruction de* L monocytogenes. *Deux modèles ont été comparés. Ils permettaient l'un et l'autre un bon ajustement des données expérimentales. Cependant, les valeurs de D calculées à partir de ces modèles étaient très divergentes. On a donc préféré calculer le temps de chauffage nécessaire pour obtenir une destruction de 3 et 6 log du nombre de* Listeria *à partir des modèles. L'addition de 25 ou 50 UI/ml de nisine dans le*

lait diminue considérablement la résistance thermique de L monocytogenes *et le temps nécessaire pour avoir une réduction donnée du nombre de ces bactéries. Par exemple, à 54°C, 16 min sont suffisantes pour réduire d'un facteur 1000 le nombre de* Listeria *dans le lait contenant 25 UI/ml de nisine, alors que 77 min sont nécessaires en l'absence de nisine. L'effet combiné du traitement thermique et de la nisine augmente lorsque les bactéries sont précultivées dans le lait à basses températures. Les facteurs qui affectent la thermorésistance des* Listeria *sont discutés, ainsi que le mode d'action sur les cellules bactériennes de la nisine et de la chaleur.*

Listeria monocytogenes / *lait / cinétique de destruction / nisine / chauffage*

INTRODUCTION

The presence of *Listeria monocytogenes* in raw milk (Rodriguez *et al*, 1985; Beckers *et al*, 1987; Lovett *et al*, 1987; Fenlon and Wilson, 1989; Massa *et al*, 1990; Farber and Peterkin, 1991; Harvey and Gilmour, 1992), its ability to multiply at refrigeration temperatures (Wilkins *et al*, 1972; Rosenow and Marth, 1987; Junttila *et al*, 1988; Schaack and Marth, 1988; Papageorgiou and Marth, 1989; Walker *et al*, 1990; Siswanto and Richard, 1992), to survive during cheese manufacture, and to grow during the ripening of soft cheeses (Ryser *et al*, 1985; Ryser and Marth, 1987; Maisnier-Patin *et al*, 1992; Back *et al*, 1993; Sulzer and Busse, 1993) present a hazard for a group of consumers at risk, namely pregnant women and immunocompromised individuals.

The incidence of *L monocytogenes* in raw milk has prompted concern about the use of this material for making cheese and has resulted in numerous studies on the thermal inactivation of *L monocytogenes* in milk (Bradshaw *et al*, 1985; Donnelly and Briggs, 1986 ; Bradshaw *et al*, 1987; Donnelly *et al*, 1987; Doyle *et al*, 1987; Northolt *et al*, 1988; El-Shenawy *et al*, 1989; Farber, 1989; Fernandez, 1989; Lemaire *et al*, 1989; Mackey and Bratchell, 1989; Farber and Pagotto, 1992). Under pasteurization conditions, the holding times and temperatures required for destruction of *L monocytogenes* in milk are simply unsuitable for cheesemaking. For instance, Mackey and Bratchell (1989) calculated a $D_{54°C}$ of 20 min for *L monocytogenes*, meaning that 1 h of heating would be required to achieve a 3-$\log_{10}$ reduction. It is well known that the sensitivity of microorganisms to heat is influenced by many factors such as the nature and concentration of ingredients in the culture medium, the pH of the medium, the physiological state and age of the cells, and their temperature of growth (Hansen and Riemann, 1963; Allwood and Russell, 1970). Thus, it seems possible to find conditions enhancing the effect of heat, so that moderate heat treatment of milk would destroy the undesirable bacterial flora without significantly impairing the biological properties of milk proteins.

Nisin has been reported by many authors to be effective against *L monocytogenes* (Mohamed *et al*, 1984; Benkerroum and Sandine, 1988; Monticello and O'Connor, 1990; Harris *et al*, 1991; Bruno *et al*, 1992). It has also been demonstrated that a combination of heat and nisin was more effective against spores of bacilli and clostridia than nisin alone (Hurst, 1981; Scott and Taylor, 1981; Oscroft *et al*, 1990).

The purpose of the present study was: i) to explore a method of control of *Listeria* in cheese milk based on the interaction of nisin and moderate heat; ii) to draw the attention to factors that could affect the effectiveness of the process; and iii) to provide a model for analyzing the results, so that further developments would be facilitated. We report here the effect of nisin on thermal destruction of *L monocytogenes* in milk, and propose a rational approach of selecting time-

temperature combinations and nisin concentrations which could prevent or limit the milk protein denaturation in order to make safe cheeses with quality similar to those made from raw milk.

MATERIALS AND METHODS

Strains and culture

Two strains of *L monocytogenes*, V7 (serotype 1, milk isolate) and Scott A (serotype 4b, clinical isolate), both from the University of Wisconsin, Madison, USA, were selected for this study. The first one was selected for its relatively high resistance to nisin (Benkerroum and Sandine, 1988), the second because it was more thermoresistant than V7 (Bhadury *et al*, 1991). Both strains were stored at 4°C on slants of TSA (tryptone-soy agar, Difco) and transferred bimonthly. Three days before an experiment, the bacteria were transferred from the stock culture into TSB (tryptone-soy broth, Difco) and incubated for 18 h at 30°C. Reconstituted non-fat dry milk (11% solids, Nilac from NIZO, Ede, The Netherlands) in distilled water was autoclaved at 121°C for 10 min and inoculated with 1% of the TSB culture and incubated for 18 h at 30°C. A second culture prepared in the same manner was used for the milk heat treatments.

Since *L monocytogenes* can grow well in milk stored at low temperatures (Wilkins *et al*, 1972; Rosenow and Marth, 1987; Junttila *et al*, 1988; Schaack and Marth, 1988; Papageorgiou and Marth, 1989; Walker *et al*, 1990; Siswanto and Richard, 1992), cells grown at 4 and 7°C were also tested to assess the impact of growth temperature in milk on thermal resistance. In these experiments, the second culture in milk at 4 or 7°C was incubated 7 and 3 days, respectively, to reach the stationary phase of growth.

Nisin preparation

A 10^4 IU/ml stock solution was prepared by dissolving 277.8 mg of purified nisin (Aplin & Barrett Lod, Beaminster, UK, 3.6 x 10^6 IU/g) in 80 ml 0.02 N HCl. The solution was boiled for 5 min and after cooling at room temperature, the volume was raised to 100 ml in a volumetric flask and the solution was kept at –20°C. Before each experiment, the stock solution was added to milk to give nisin concentrations of 25 or 50 IU/ml.

Milk heat treatment

Capillary tubes (0.8–1.10 mm in diameter and 90 mm long, Wiretrol II, Drummond Scientific Company, USA) were used for heat inactivation experiments (El-Shenawy *et al*, 1989). The tubes were filled by capillary action with 50 µl of the culture previously diluted in milk to give an initial count of approximately 2 x 10^6 CFU/ml. Nisin was added to the inoculated milk just before filling. Both ends of the capillary tubes were sealed with cristoseal (Bioblock Scientific, France). The outside of the tubes was decontamined by soaking for 10 min in hypochlorite solution (500 ppm of available chlorine) at room temperature. The tubes were then stored in an ice-water mixture until their use. Before the heat treatment, they were rewarmed for 5–6 min at room temperature, then immersed in a water bath set at 54, 56, 58, 60, 62 or 65°C. After heating for specified times, the tubes were rapidly removed from the water bath and promptly cooled in an ice-water mixture. For enumeration, both ends of the tubes were broken and the contents were removed using a syringe to push the liquid out. The contents of two tubes (100 µl) were directly plated on the surface of TSAYE (tryptone-soy agar, Difco supplemented with 0.6% (w/v) yeast extract, Biomérieux, France). A preliminary study was carried out to determine the best conditions for plate incubation (2 *vs* 5 days at 30°C and anaerobic (Gaspak System) *vs* aerobic condition). Incubation for 5 days under aerobic conditions permitted the best recovery. For each holding-time, enumeration of the survivors was performed in triplicate and each experiment was repeated twice.

Calculations

The survivor curves were constructed by plotting the $\log_{10}$ of survivors (CFU/ml) against heating time. As the data did not fit the usual linear model of thermal destruction, two mathematical models claimed to more closely fit such kinds of data were used: i) the modified logistic equation of Kamau *et al* (1990) which is recommended when

survivor curves display an initial lag in death following by a one-phase killing:

$$\log_{10} N/No = \log_{10}[1 + e^{(-\beta t_{1/2})}] - \log_{10}[1 + e^{(\beta(t-t_{1/2}))}] \quad (1)$$

N and No (CFU/ml) are the initial and surviving numbers of bacteria at time t. β (min^{-1}) is the ratio of decrease, recorded at time t when the killing rate (dN/dt) is maximum. It is calculated as follows:

$$\beta = 4\ (dN/dt)_{max}/No$$

$t_{1/2}$, the time at which $N/N_o = 1/2$, is an estimate of the lag of killing. The D-values (min) are given by $D = 2/\beta$ when $(dN/dt)_{max}$ is reached.

ii) the second equation is proposed by Mackey and Derrick (1986) (see also Alderton and Snell, 1970; King *et al*, 1979) to fit non-linear curves survival data:

$$\log N/No = -(b \cdot t)1/a \quad (2)$$

where b (min^{-1}) is the death rate constant and a (no dimension unit) is a constant. The D-values (min) are given by D = 1/b.

To compare the two models, the differences between predicted (y_p) and observed (y_o) values were calculated, and the residual standard deviation determined as follows:

$$S_{y \cdot x} = [1/n \Sigma (y_o - y_p)^2]^{1/2}$$

with n, the number of observations.

RESULTS

Effect of nisin on the thermal resistance of L monocytogenes

Figure 1 illustrates the thermal destruction at 60°C of the two strains of *L monocytogenes* in milk. All survivor curves exhibited a similar pattern, regardless of the temperature and the nisin concentration of milk: a shoulder preceding an accelerating death rate. The presence of nisin dramatically reduced or eliminated the lag in killing and increased the death rate of *L monocytogenes*. This means that heating milk either: i) enhanced the bactericidal action of nisin; or ii) increased the susceptibility of the bacteria to nisin.

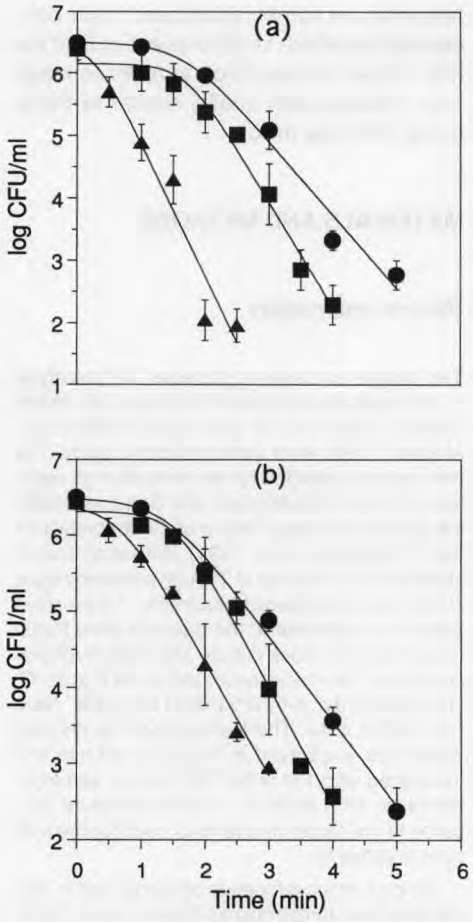

Fig 1. Kinetics of destruction of *Listeria monocytogenes* V7 **(a)** and Scott A **(b)** at 60°C in skim milk. ●, Control; addition of 25 (■) and 50 (▲) IU/ml of nisin.
Cinétiques de destruction de Listeria monocytogenes, *souches V7 **(a)** et Scott A **(b)** à 60°C dans du lait écrémé. (●) Témoin ; addition de 25 (■) et 50 (▲) UI/ml de nisine.*

Table I shows that both models fit more or less closely the survivor curves, depend-

Table I. Comparative statistical determination of D-values and variance regression (S^2yx) for *Listeria monocytogenes* (strain V7) using the mathematical model of Kamau *et al* (1990) (model 1) or the model of Mackey and Derrick (1986) (model 2).
Comparaison des valeurs de D et de la variance de régression (S^2yx) pour Listeria monocytogenes *(souche V7) déterminées d'après le modèle de Kamau* et al *(1990) (modèle 1) ou le modèle de Mackey and Derrick (1986) (modèle 2).*

Température (°C)	*Nisin (IU/ml)*	*Model 1*		*Model 2*	
		D(min)	*S^2yx*	*D(min)*	*S^2yx*
54	0	11.76	0.07	48.78	0.03
	25	3.77	0.25	6.14	0.32
	50	2.41	0.29	4.58	0.27
56	0	5.41	0.18	20.16	0.11
	25	2.90	0.12	3.39	0.11
	50	1.90	0.33	2.09	0.33
58	0	1.83	0.08	5.42	0.21
	25	0.85	0.28	1.69	0.28
	50	0.69	0.26	1.35	0.30
60	0	0.74	0.25	2.20	0.40
	25	0.52	0.31	1.97	0.21
	50	0.41	0.82	0.69	0.83
62	0	0.28	0.20	0.85	0.32
	25	0.09	0.49	0.63	0.80
	50	0.23	0.06	0.38	0.03
65	0	0.12	0.06	0.33	0.08
	25	0.07	0.06	0.27	0.15

ing on the heating temperature and the concentration of nisin in milk. Mean variance of model 1 (0.24) is slightly lower than that of model 2 (0.28) but the D-values calculated by model 2 were considerably higher than those derived from model 1. We decided to select and use one model to directly calculate the time needed to decrease the *Listeria* counts by a given factor (3 or 6 $\log_{10}$ reduction). The parameters of the two models were plotted against temperatures in order to select the most appropriate model for analysis. Figure 2 shows that a linear relationship can be found between the two parameters (β and $t_{1/2}$) of model 1 and the temperature. Thus, a kinetic study of *Listeria* destruction performed at two temperatures would be acceptable to determine by interpolation the parameters of equation 1 at all temperatures. The same representation of the parameters of model 2 did not result in a simple linear relationship. Therefore, model 1 was chosen for data analysis and calculation of heating time necessary to achieve a 10^3- or 10^6-fold decrease in the number of *Listeria*. *L monocytogenes* strain

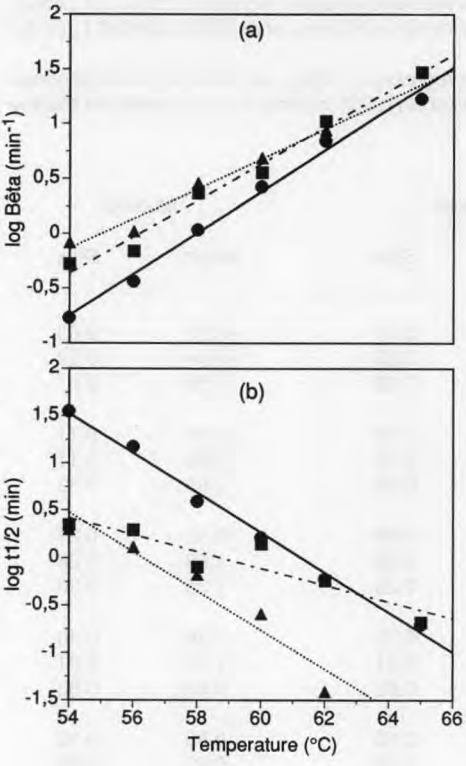

Fig 2. Heat temperature dependence of the 2 parameters, β and $t_{1/2}$, for model 1. ●, Control; addition of 25 (■) and 50 (▲) IU/ml of nisin in skim milk.
Relation entre les 2 paramètres, β et $t_{1/2}$, du modèle 1 et la température de chauffage. (●) Témoin ; addition de 25 (■) et 50 (▲) UI/ml de nisine dans le lait écrémé.

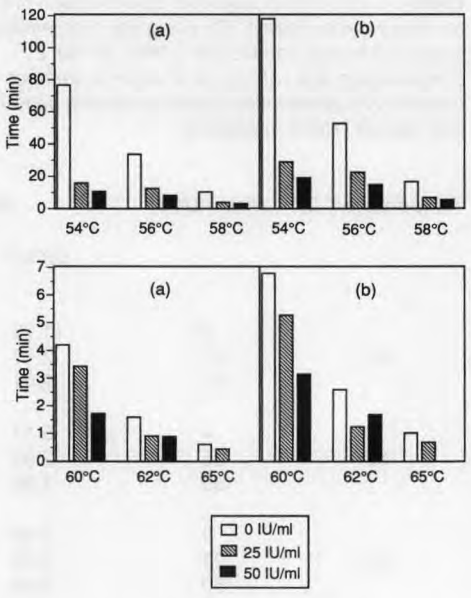

Fig 3. Calculated-times from model 1 for a 10^3- **(a)** and 10^6-fold **(b)** decrease in *Listeria monocytogenes* V7 number as a function of heating temperatures of skim milk.
Temps nécessaire (calculé à l'aide du modèle 1) pour réduire le nombre de Listeria monocytogenes *V7 par 10^3 **(a)** et 10^6 **(b)** en fonction de la température de chauffage du lait.*

V7 was also chosen because it was found to be more resistant to nisin than strain Scott A.

Compared to the control milk, 25 IU/ml of nisin reduced the heating time necessary to achieve a 3 $\log_{10}$ decrease in the number of *Listeria* by 80% at 54°C; 64% at 56°C; 63% at 58°C; 18% at 60°C; 43% at 62°C and 27% at 65°C (fig 3). The reduction of heating times was even higher with 50 IU/ml of nisin: 86% at 54°C; 76% at 56°C; 70% at 58°C; 59% at 60°C and 44% at 62°C.

Influence of growth temperature on thermal resistance of L monocytogenes *strain V7*

In control milk heated at 60°C, the time to achieve 10^3- or 10^6-fold decrease of *L monocytogenes* V7 grown at 7°C was lower than for cells grown at 30°C, whereas the culture at 4°C resulted in a marked increase in heat resistance (fig 4). However, this

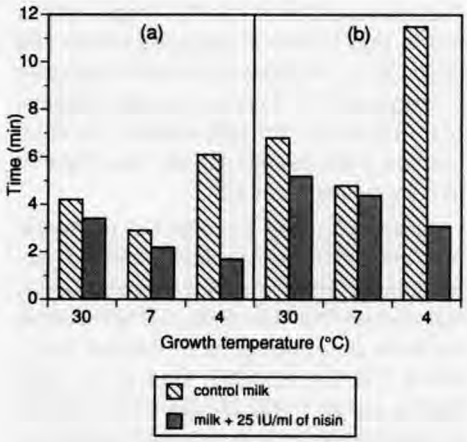

Fig 4. Calculated-times from model 1 for a 10^3- **(a)** and 10^6-fold **(b)** decrease of *Listeria monocytogenes* V7 heated at 60°C as a function of temperature of growth of this strain in skim milk.
Temps nécessaire (calculé à l'aide du modèle 1) pour réduire le nombre de Listeria monocytogenes *V7 par 10^3 (a) et 10^6 (b) à 60°C en fonction de la température de croissance de cette bactérie dans du lait écrémé.*

increased resistance at 4°C was not observed in the presence of 25 IU/ml of nisin.

DISCUSSION

There is extensive experimental evidence showing that the log survivor/time relationship for bacteria exposed to heat is not linear. This has been observed for different kinds of bacteria like *Enterococcus faecalis* (White, 1953), *Salmonella typhimurium* (Mackey and Derrick, 1986) as well as *Listeria monocytogenes* (Fedio and Jackson, 1989; Kamau *et al*, 1990; Bhaduri *et al*, 1991). However, concerning the last microorganism, linear thermal death curves were observed by Donnelly and Briggs (1986), El-Shenawy *et al* (1989), Linton *et al* (1992) and Huang *et al* (1992). Considering the great interest for application of these data, the phenomena underlying the observations on the survival curves of heated bacteria warrant discussion.

Thermal injury or death of bacterial cells is known to affect many cellular components such as membranes, ribosomes, nucleic acids and proteins (Hansen and Riemann, 1963; Allwood and Russel, 1970). But the sequence of damaging events accompanying heating, and the way in which these cause cellular death remain largely unknown. Thus, as stated by Hansen and Riemann (1963) there is no reason to believe that monomolecular reactions, resulting in linear log survivor/time relationship, occur in thermal inactivation of bacteria.

It has been assumed that curves with shoulders, corresponding to a lag in cell destruction, arise because different essential molecules have to be destroyed for the death of the cells to occur (Hansen and Riemann, 1963). Also, as pointed out by these authors, the heat damaged proteins in the cell can be brought back more or less to their original structure, provided the damage is not too severe, *eg* if only some of the S-S and hydrogen bonds have not been disrupted. Allwood and Russell (1970) observed that protein denaturation follows other changes within the cell which are responsible for death. According to these authors, the thermal destruction appears to be due to subtle changes in intracellular labile molecules (enzymes or RNA) and organized systems (cell membrane, ribosomes) which are difficult to reverse. However, they agreed with Hansen and Riemann (1963) that cells, not yet dead but damaged, may be able to repair, at least to some extent, the damage induced by heat. Reversible damages are particularly expected in the case of microorganisms exposed to moderate heating. Thus, the lag in cell destruction, as observed in the present study, could be attributed to the exis-

tence of efficient repair systems in *L monocytogenes*.

Conversely, curves showing a survivor tail (that are concave upward in the last part of the curves) are also often observed, and are attributed to non-uniform distribution of heat resistance among the individual cells: more resistant ones need more time for destruction (Hansen and Riemann, 1963; Allwood and Russell, 1970). This frequent phenomenon was not observed in the course of the present study probably because too low initial counts (5 x 10^6 CFU/ml) and small inocula (100 µl) were used.

It has been shown that time and temperature of incubation prior to heat treatment dramatically affect the heat resistance of both Gram-negative (Elliker and Frazier, 1938) and Gram-positive bacteria (White, 1953; Hansen and Riemann, 1963; Hurst *et al*, 1974). This phenomenon has recently been verified with *L monocytogenes* (Knabel *et al*, 1990; Smith *et al*, 1991; Farber and Pagotto, 1992; Linton *et al*, 1992). In particular, these workers have shown that microorganisms grown at higher temperatures survive heating better than when grown at low temperatures. Thus, our observation that strain V7 is more resistant to heating at 60°C in control milk when incubated at 4 than at 7°C (see fig 4) seems to contradict the above observations. However, it has also been reported (Hansen and Riemann, 1963) that increased resistance to heat occurs when the bacteria are incubated under their optimum temperature of growth. Under such growth conditions, more short carbon chain and unsaturated fatty acids are incorporated into the cell membrane. This results in the membrane becoming less viscous, and therefore more resistant to damage caused by moderate heat treatment. A possible difference in fatty acid composition of the membrane between cells grown at 7 and 4°C, to explain the difference in heat resistance observed in our study, is supported by the observation of Tadayon and Carroll (1971). These authors found that *L monocytogenes* strain 109 grown at 4°C exhibited a marked decrease of branched C17:0 and a dramatic increase of C18:0 and C18:1 fatty acids in the composition of the cell membrane, as compared to cells cultivated at 10°C.

Nisin enhanced the effect of moderate heat and had a greater effect when the temperature of growth in milk was lower. Taking into account that the mode of action of nisin involves pore formation in the cell membrane (Sahl *et al*, 1987; Gao *et al*, 1991; Garcera *et al*, 1993), the dramatic increase in thermal destruction of *L monocytogenes* in the presence of low levels of nisin can be attributed to a synergistic effect of heat and nisin on the membrane damage, leading to rapid efflux of cytoplasmic constituents (ATP, amino acids, potassium). However, it could be merely due to an increase in adsorption of nisin on the cell wall, as a result of modification of its surface properties brought about by heat (increased hydrophobicity by structural changes or loss of some cell wall components). In turn, this increased adsorption would result in better activity of nisin (Hurst, 1981). The combined effect of heat and nisin merits further investigation.

For a practical point of view, addition of minute amounts of nisin in conjunction with moderate milk heating (thermization) could considerably increase the margin of safety of raw milk cheeses with respect to milk-borne pathogens, without significantly increasing the cost of the process. Moreover, it can be expected that appropriate nisin concentrations and time-temperature combinations could be as efficient as regular pasteurization, without impairing the physico-chemical properties of the cheese milk proteins. In addition, as heated Gram-negative bacteria become sensitive to nisin (Kalchayanand *et al*, 1992), the additional effect of nisin and moderate heat could result in a considerable decrease of the counts of these bacteria in cheese milk.

Besides differences in heat resistance that naturally occur between strains of *Listeria* and as a result of growth environment discussed earlier, certain treatments prior to heating are known to enhance the heat resistance of *L monocytogenes* such as preheating (Fedio and Jackson, 1989) or acid shock (Farber and Pagotto, 1992). These factors must be considered when moderate heat treatment of milk is used.

Different mathematical models have been proposed to analyze the thermal death rate curves showing shoulders, and to derive D-values. The two models used in the present study fit the data, but the D-values were markedly diverging. To establish which model gave the true value would have required considerable work. Thus, it seemed simpler and more reliable to calculate the time needed to reduce the population by a fixed number of log units by using the most appropriate mathematical model. As both models used in our study gave comparable standard deviations, each of them could ensure the same degree of confidence of the results.

Future work should be directed at optimizing the process with the goal of eradicating *L monocytogenes* from cheese milk while preserving the biophysical properties of this raw material.

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