

## Inhibition of *Listeria monocytogenes* in Camembert cheese made with a nisin-producing starter

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(Received 21 November 1991; accepted 14 February 1992)

**Summary** — Camembert cheeses were made at 3 different times with milk containing initially  $10^1$ ,  $10^3$  or  $10^5$  *Listeria monocytogenes*/ml. A nisin-producing starter composed of a pair of isogenic protease positive and protease negative strains of *Lactococcus lactis* subsp *lactis* was used to inhibit *L. monocytogenes*. Nisin concentration in curd and in cheese paralleled the growth of lactococci. Maximum nisin concentration of ca 700 IU/g was obtained in curd at 9 h, then nisin concentrations decreased slowly during 9–24 h and dramatically during ripening. In the presence of nisin, the numbers of *L. monocytogenes* decreased rapidly from 6 h to 24 h. This inhibitory effect continued until the end of the second week of ripening in the core of Camembert cheeses, leading to a reduction of 3.3 log *Listeria*/g (average from 3 experiments) compared to the initial level in cheese milk. Thereafter, regrowth occurred in Camembert cheeses, sooner on the surface than in the interior. However, a difference of 2.4 log CFU/g between numbers of *Listeria* in cheese made with Nis<sup>+</sup> and Nis<sup>-</sup> starter cultures was maintained throughout ripening (6 weeks). Nisin was particularly effective when milk contained  $10^1$  or  $10^3$  *L. monocytogenes*/ml.

*Listeria monocytogenes* / starter / inhibition / nisin / Camembert cheese

**Résumé** — Inhibition de *Listeria monocytogenes* dans du Camembert fabriqué avec un levain producteur de nisine. Des Camemberts ont été fabriqués à 3 reprises avec du lait contenant  $10^1$ ,  $10^3$  ou  $10^5$  *Listeria monocytogenes*/ml. Deux souches isogéniques, protéase positive et protéase négative, productrices de nisine appartenant à l'espèce *Lactococcus lactis* subsp *lactis* ont été utilisées afin d'inhiber *L. monocytogenes*. L'évolution de la concentration en nisine, dans le caillé et les fromages était parallèle à l'évolution des lactocoques. La concentration maximale en nisine, environ 700 UI/g, était obtenue à 9 h, puis elle diminuait progressivement de 9 h à 24 h et ensuite rapidement lors de l'affinage. En présence de nisine, le nombre de *L. monocytogenes* diminuait rapidement de 6 à 24 h. L'effet inhibiteur se poursuivait à l'intérieur des fromages jusqu'à la deuxième semaine, conduisant à une réduction du nombre de *Listeria* de 3,3 log UFC/g (moyenne de 3 expériences) par rapport au nombre initial dans le lait. Puis, la croissance reprenait dans les fromages, plus précocement à la surface qu'à l'intérieur. Cependant, une différence de 2,4 log UFC/g entre les nombres de *L. monocytogenes* dans les fromages fabriqués avec des levains Nis<sup>+</sup> et Nis<sup>-</sup> était maintenue jusqu'à la fin de l'affinage (6 semaines). L'efficacité de la nisine était la plus importante quand le lait contenait  $10^1$  ou  $10^3$  *Listeria*/ml.

*Listeria monocytogenes* / levain lactique / inhibition / nisine / Camembert

## INTRODUCTION

It is well known that *Listeria monocytogenes*, which is potentially pathogenic, has the ability to withstand a wide variety of environmental conditions. In particular, *L. monocytogenes* has been shown to survive the processing and storage of fermented milk and cheeses. Studies with cheeses made from artificially inoculated milk indicated that the behaviour (growth, survival or inhibition) of *L. monocytogenes* in these products depends mainly upon pH and temperature conditions during manufacture, ripening, and storage. During ripening of Cheddar and storage of Colby cheeses, the numbers of *L. monocytogenes* decreased gradually from 3.5 to 1.5 log CFU/g, with a D-value of 58 days (Ryser and Marth, 1987a; Yousef and Marth, 1988), whereas in Blue cheese, after a slight increase during cheese making, the numbers dropped rapidly at the beginning of ripening and then stabilized (Papageorgiou and Marth, 1989). During Camembert manufacture, the numbers of *L. monocytogenes* increased 10-fold, then decreased during the first 18 days of ripening and finally reinitiated growth as a consequence of a rise in pH (Ryser and Marth, 1987b).

Apart from the use of effective fermentation, a potential means of controlling pathogens in cheese is through antimicrobial compounds produced by lactic acid bacteria (Tagg *et al*, 1976; Klaenhammer, 1988; Spelhaug and Harlander, 1989; Schillinger, 1990). Nisin, a polypeptide antibiotic of 34 amino acids, is one of these compounds which has been studied the most (Hurst, 1981). Nisin-producing strains of *Lactococcus lactis* subsp *lactis* were first employed by Hirsch *et al* (1951) to inhibit gas-blowing by anaerobic spore-formers in Swiss-type cheese. Later, for the same purpose, Lipinska (1977) used nisin-producing strains in combination with nisin-resistant ones possessing desirable

cheese making properties for the production of hard and semi-hard cheese. No further attention seems to have been paid to Nis<sup>+</sup> starters, probably because they did not have the required properties to make cheese (low rate of acidification, limited proteolytic activity, high sensitivity to phages, etc).

Recently, some authors (Mohamed *et al*, 1984; Benkerroum and Sandine, 1988; Asperger *et al*, 1989; Carminati *et al*, 1989; Harris *et al*, 1989) have revived interest in nisin by showing that this bacteriocin exerts a bactericidal effect towards *L. monocytogenes* in culture media as well as in some kinds of cheeses.

In the present study, a starter combining appropriate phenotypes, *ie* a lactose-positive, protease-positive (Prt<sup>+</sup>), nisin-producing (Nis<sup>+</sup>) strain of *Lactococcus lactis* subsp *lactis* along with a protease-negative (Prt<sup>-</sup>) variant was successfully used to make cheese. The purpose of this preliminary study was to evaluate the potential of this Nis<sup>+</sup> starter to inhibit *L. monocytogenes* during Camembert cheese manufacture and ripening, and identify problems that might be encountered in making Camembert cheese with such a starter.

## MATERIALS AND METHODS

### *Strains and media*

#### *L. monocytogenes*

One strain of *L. monocytogenes* V7 (milk isolate, serotype 1) was used. Stock cultures were maintained at 4 °C on Tryptic-Soy Agar (TSA, Difco) slants and transferred bimonthly. An intermediate active culture was prepared by transferring the stock culture into TSB (Tryptic-Soy Broth, Difco) and incubating for 18 h at 30 °C. A working culture was prepared by inoculating reconstituted skim milk (LJLN, Elle et Vire, France, 10% total solids) with the intermediate culture. After

18 h at 30 °C, the milk culture was in stationary growth phase (ca 5 x 10<sup>8</sup> CFU/ml). Appropriate dilutions of this culture were added to pasteurized milk in the cheese vats to obtain initial numbers of ca 10<sup>1</sup>, 10<sup>3</sup> or 10<sup>5</sup> *Listeria*/ml.

### Starter cultures

The strains of *Lactococcus lactis* subsp *lactis* were obtained from the collection of the Station de Recherches Laitières. *L. lactis* subsp *lactis* CNRZ 1076, a nisin-negative (Nis<sup>-</sup>), protease-positive (Prt<sup>+</sup>) strain and its protease-negative (Prt<sup>-</sup>) variant CNRZ 1075 were selected as control strains along with Nis<sup>+</sup>, Prt<sup>+</sup> and Nis<sup>+</sup>, Prt<sup>-</sup> strains of *L. lactis* subsp *lactis* (CNRZ 150). Active cultures of Prt<sup>+</sup> and Prt<sup>-</sup> strains in skim milk

were added to cheese milk to provide an inoculum of 2% (0.4% Prt<sup>+</sup> and 1.6% Prt<sup>-</sup>) giving a total of ca 10<sup>7</sup> CFU/ml. This association of isogenic Prt<sup>+</sup> and Prt<sup>-</sup> strains has been recommended to minimize bitterness in cheese (Vassal, personal communication) despite the fact that addition of Prt<sup>-</sup> strain to one Prt<sup>+</sup> decreases the rate of milk acidification (Juillard, 1991).

### Cheese manufacture

Camembert cheese was made in our pilot plant. Essential steps in the cheese making are outlined in table I. Pasteurized (72 °C/15 s) partially skimmed milk (28 g MG/l) warmed to 31–34 °C, was placed in 40-l vats and inoculated with *L*

**Table I.** Main characteristics of Camembert cheese-making.  
*Principales caractéristiques des fabrications de Camembert.*

Fabrication step		Trial 1		Trial 2		Trial 3	
		Nis <sup>-a</sup>	Nis <sup>+b</sup>	Nis <sup>-</sup>	Nis <sup>+</sup>	Nis <sup>-</sup>	Nis <sup>+</sup>
Milk inoculation (pH 6.6–6.7)	t <sup>c</sup>	0	0	0	0	0	0
	T <sup>d</sup>	30.8	30.8	33.8	33.9	33.0	33.0
Cutting curd (pH 6.0–6.1)	t	5.0	5.8	4.2	4.7	3.1	3.3
	T	28.4	27.4	29.9	29.6	31.1	31.0
Dipping (pH 5.6–5.8)	t	5.3	6.2	4.8	5.4	3.5	4.2
	T	28.0	27.0	28.3	27.8	29.0	30.0
1st turn (pH 4.9–5.0)	t	6.7 <sup>e</sup>	8.0	6.2	6.7	4.6	5.3
	T	26.8	25.7	27.6	27.2	29.1	29.1
2nd turn (pH 4.6–4.8)	t	8.8	9.1	8.7	8.5	9.0	9.0
	T	25.5	25.3	26.5	26.4	25.6	25.7
Salting (pH 4.4–4.6)	t	24.0	24.0	24.0	24.0	24.0	24.0
	T	19.0	19.0	19.0	19.0	22.5	22.2

<sup>a</sup> Non nisin-producing lactic acid starter (*Lactococcus lactis* subsp *lactis* CNRZ 1076 (strain Prt<sup>+</sup>) and 1075 (strain Prt<sup>-</sup>); <sup>b</sup> nisin-producing lactic acid starter (*Lactococcus lactis* subsp *lactis* CNRZ 150 (strain Prt<sup>+</sup> and Prt<sup>-</sup>)); <sup>c</sup> time (h); <sup>d</sup> temperature (°C); <sup>e</sup> pH was 5.2 in this vat.

<sup>a</sup> *Levain non producteur de nisine* (*Lactococcus lactis* subsp *lactis* CNRZ 1076 (souche Prt<sup>+</sup>) et 1075 (strain Prt<sup>-</sup>); <sup>b</sup> *levain producteur de nisine* (*Lactococcus lactis* subsp *lactis* CNRZ 150 (souches Prt<sup>+</sup> et Prt<sup>-</sup>)); <sup>c</sup> temps (h); <sup>d</sup> température (°C); <sup>e</sup> la valeur de pH dans cette bassine était 5.2.

*monocytogenes*, 2% lactic starter culture and spores of *Penicillium camemberti*. The clotting agent (rennet) was added at the same time. The coagulum was cut at pH 6.0 and 20–50 min later, it was dipped into 12-cm diameter hoops to allow the curd to drain. Hoops were turned twice, the first time when the pH was nearing 4.9–5.0, the second time when the pH was about 4.6–4.8. After draining overnight, cheese wheels were removed from hoops, dry-salted and stored at 11 °C with 85–95% relative humidity up to the end of the ripening period. This relatively high temperature was chosen to accelerate ripening and to provide favorable conditions of survival and growth for *Listeria*.

### Experimental design

Camembert cheeses were manufactured at 3 different dates. Each time, 2–5 vats were used. In the first experiment, 2 vats were used; 1 contained the Nis<sup>+</sup> and the other the Nis<sup>-</sup> starter. In both, milk was initially inoculated with 10<sup>5</sup> *Listeria*/ml. In the second experiment, the cheese milk of 1 vat was inoculated with the Nis<sup>-</sup> starter and 10<sup>1</sup> *Listeria*/ml, 3 others were inoculated with the Nis<sup>+</sup> starter and 10<sup>1</sup>, 10<sup>3</sup>, 10<sup>5</sup> *Listeria*/ml. The third experiment was the repetition of the second one, with 1 more vat which was inoculated with the Nis<sup>-</sup> starter and 10<sup>5</sup> *Listeria*/ml.

Samples were taken from each batch during cheese manufacture (at time of inoculation and at 3, 6, 9, 24 h) and weekly during ripening up to the 6th week. Milk and cheese were analyzed for numbers of viable *L. monocytogenes* and lactococci, pH, and nisin concentration.

### Sample preparation

Samples of milk (1 ml), curd (1 g) or cheese (3 portions of 3 g of core and crust taken separately from 3 wheels) were placed separately into sterile stomacher bags and diluted 1:10 with peptone water (0.1% Difco). Samples were homogenized with the stomacher for 2 min. Appropriate 10-fold dilutions were spread on the surface of enumeration media using a spiral plater or a hockey stick if less than 10<sup>3</sup> *Listeria*/ml were expected.

## Enumerations of bacteria

### *Listeria monocytogenes*

During the first experiment, 3 enumeration methods were compared: 1 relying on the use of selective agents, Oxford Agar incubated 48 h at 30 °C (Curtis *et al*, 1989), and the others relying only on incubation under selective conditions: TSA-YE (Tryptic-Soy Agar plus 0.6% Yeast Extract, Difco), the plates being incubated for 22 days at 4 °C under anaerobic conditions (Gas-pak System, H<sub>2</sub> + CO<sub>2</sub>), and BHIA (Brain Heart Infusion Agar, Difco) incubated for 28 days at 4 °C in Raibaud's tubes (long narrow tubes insuring anaerobic conditions). Incubations were performed at low temperature and under anaerobic conditions, to prevent growth of the lactococci and molds, respectively. As the 3 methods gave similar counts (see *Results and Discussion*), Oxford Agar was the only medium used for the enumeration of *L. monocytogenes* in the subsequent experiments.

### Lactococci

The enumeration of lactococci was performed on Fast Slow Differential Agar (FSDA, Huggins and Sandine, 1984). This medium gives the same lactococci numbers as M17 (Gratadoux, personal communication) and in addition allows differentiation of Prt<sup>+</sup> and Prt<sup>-</sup> strains.

### Detection of *Listeria*

When counts in cheese were expected to be less than 10<sup>1</sup> CFU/g, the procedure proposed by ISO (1989) for detection of *L. monocytogenes* in dairy products was used. A 25-g sample, resulting from blending of 1 wedge from each of 3 cheeses, was mixed with 225 ml of the Selective Enrichment Broth (SEB) using a Waring blender. This medium contained per l: 5 g of peptone from soy, 30 g of Tryptic Soy Broth, 6 g of yeast extract, 10 mg of acriflavin HCl, 40 mg of nalidixic acid and 50 mg of cycloheximide. *L. monocytogenes* were detected after 48 h of enrichment at 30 °C, by streaking 0.1 ml of SEB on Oxford Agar. The plates were incubated for 48 h at 37 °C.

### **Nisin determination**

The plate diffusion assay (Fowler *et al.*, 1975) was used to determine nisin concentrations in milk and cheese. To extract nisin from cheese, 15 g (5 g from each of 3 cheeses) of crust and core were placed separately in 60 ml of 0.02 N HCl and homogenized with a Waring blender for 2 min. After pH adjustment to 2.0, the suspension was heated for 5 min at 98 °C to release nisin and centrifuged (6 000 *g* for 10 min at 4 °C). The supernatants were used for the assay. *Micrococcus luteus* ATCC 10240 was used as the test-organism.

### **pH determination**

The pH of milk was determined using a pH-meter Knick (Portamess 654 Model) equipped with a combination glass electrode (Ingold). The pH at the surface and in the curd of cheeses was determined using a pH-meter Metrohm Herisau (Sofranie) equipped with a flat surface contact glass electrode.

## **RESULTS AND DISCUSSION**

### **Variations in cheese manufacture**

Variation between results of different experiments as well as within experiments occurred during the study (table I). In each experiment, milk fermentation was faster with the control starter than with the nisin-producing (Nis<sup>+</sup>) starter, regardless of the initial temperature of milk. Since pH was used to monitor the process, differences in rate of acidification resulted in delays at steps like cutting curd, hooping, turning, and so on, between control and experimental batches. Accordingly, temperatures at which these operations took place were not the same, those for control being higher than for experimental cheeses. Differences in time at cutting curd were observed until the first turn. Afterwards, they decreased rapidly, probably because pH

was nearing its final value in both control and experimental cheeses.

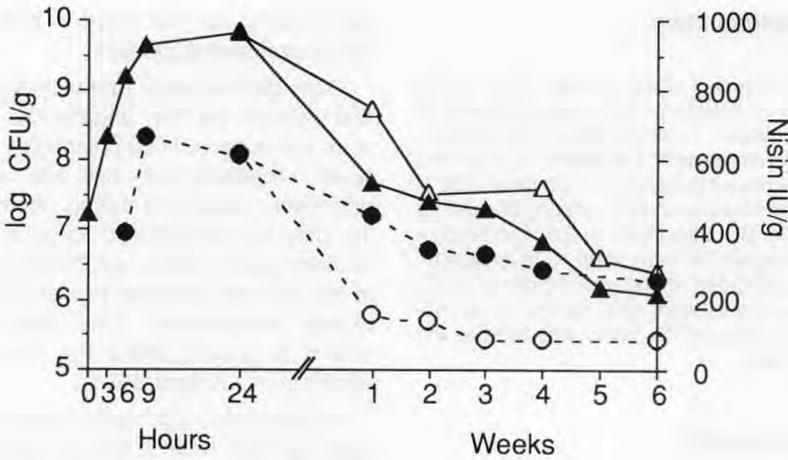
Such differences in rate of milk acidification between the Nis<sup>+</sup> and the Nis<sup>-</sup> starters were not observed in a preliminary study in which a reference skim milk was used. The differences observed during cheese making may be attributed to variations in the nutrient value of milk, resulting in changes in the balance between Prt<sup>+</sup> and Prt<sup>-</sup> cells during fermentation. This balance was shown to greatly affect the rate of milk acidification (Juillard, 1991).

As expected, with higher initial temperatures of milk (trial 2 and 3), acidification was faster, allowing earlier cutting and the following two steps. In trial 3, cheese temperature at salting was notably higher than in the other experiments (22.5–22.2 °C vs 19.0 °C).

### **Evolution of lactococci and nisin concentration during manufacture and ripening of cheese**

An illustration of the behaviour of the Nis<sup>+</sup> starter in milk and Camembert cheese is shown in figure 1. Increase in population during the first 6 h is mainly caused by concentration of cells entrapped in curd and a slight increase due to growth. After the first day of manufacture, the numbers of lactococci began to decrease in cheese. This lack of viability may be characteristic trait of the strain used or of any Nis<sup>+</sup> lactococci, since in other studies, counts of Nis<sup>-</sup> lactococci were found to be rather stable (Martley, 1975; Richard, 1984).

Nisin production paralleled the growth of *L. lactis*. There was an increase of nisin concentration in the first 9 h, nearing 700 IU/g. This increase was followed by a dramatic drop, between 24 h and 1 week of ageing. This observation is in agreement with that of Hurst (1966) who showed that



**Fig 1.** Behaviour of *Lactococcus lactis* subsp *lactis* CNRZ 150 ( $\blacktriangle, \triangle$ ) and concentration of nisin ( $\bullet, \circ$ ) during manufacture and ripening of Camembert cheeses (results of trial 2). Each point represents the mean of triplicate determinations on 3 different batches of cheese. Solid symbols indicate the determinations in the curd of the cheeses, open symbols determinations on the crust of cheeses.

*Évolution de Lactococcus lactis subsp lactis CNRZ 150 ( $\blacktriangle, \triangle$ ) et de la concentration en nisine ( $\bullet, \circ$ ) pendant la fabrication et l'affinage des Camemberts (résultats de l'essai 2). Chaque point représente la moyenne de 3 déterminations sur des fromages de trois bassines différentes. Les symboles pleins représentent l'intérieur des fromages, les symboles ouverts, la surface.*

in a culture medium, nisin was produced during the exponential phase of growth of the culture and that inactivation started just before the onset of the stationary phase of growth. According to this author and Ergorov *et al* (1982), this inactivation might be induced by an enzymatic system produced by the Nis<sup>+</sup> strains themselves. Thus, several phenomena may have occurred concurrently in the curd between 9 and 24 h: production of nisin and its concentration followed by a decrease due to enzymatic inactivation of the molecule and/or loss into whey. These facts probably explain why the nisin content of the curd was 2–3 times lower than that observed in milk by Jakubczyk *et al* (1973), Kalra and Dudani (1974), Oberman and Piatkiewicz (1978) and Ergorov (1980). Thereafter, the decrease was less rapid. It is worth mentioning that it was steeper in the crust than in the core of the

cheese. At least two reasons might explain the decrease of nisin concentration in cheese during ripening. Firstly, as nisin is a polypeptide it is susceptible to the action of proteases (Heinemann and Williams, 1966; Phillips *et al*, 1983). In the case of Camembert cheese, the proteases are those released by the lysis of the lactic acid bacteria and additionally, those produced by mold in the crust. As protein breakdown is more pronounced in the crust than in the interior of mold surface ripened cheeses, the decline of nisin concentration is consequently greater on the surface than in the core of the cheese. In addition, incomplete extraction of nisin due to binding to cheese components (fat and particular proteins) may also have occurred. This is suggested by the previous studies that showed poor recovery due to strong adsorption of nisin to meat proteins (Scott and Taylor, 1981; Bell

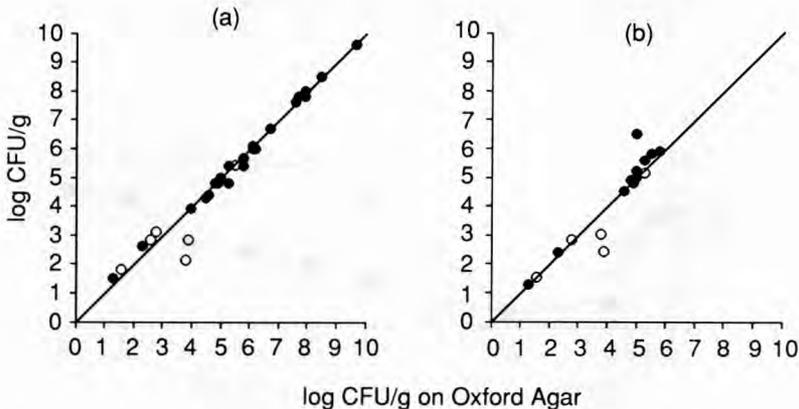
and De Lacy, 1986; Chung *et al.*, 1989) or binding to fat (Jones, 1974) or fat and proteins present in cheese spread (Somers and Taylor, 1987). Therefore, the nisin determinations in ripened cheese might represent only the unbound nisin and part of the bound molecules extracted by boiling cheese suspensions at pH 2.0, the remainder being inactivated by proteolysis or irreversibly adsorbed onto fat or proteins.

### Evolution of *L. monocytogenes* in milk and cheese

#### Comparison of media and cultures conditions

Counts of *L. monocytogenes* from control and experimental cheeses on Oxford agar

were close to those obtained on TSA-YE and BHIA (fig 2). The selective culture conditions used for the latter two media were effective in separating *L. monocytogenes* from the lactococci and mold: incubation at low temperature (4 °C) and anaerobiosis prevented growth of lactococci and mold, respectively. Nearly all the data points representing the numbers of *Listeria* were on the bisecting line, indicating that the two media gave similar results. However, two data points obviously underestimated *Listeria* counts on both TSA-YE and BHIA. They correspond to bacteria in 1 week-old cheese made with Nis<sup>-</sup> lactococci. This suggests that the selective conditions of culture (low temperature and/or anaerobiosis) could have had a greater inhibitory effect on cells injured by acidic conditions than the selective agents



**Fig 2.** Comparison of *Listeria monocytogenes* (strain V7) counts on Oxford and two non-selective media (results of trial 1, Nis<sup>-</sup> and Nis<sup>+</sup> cheeses): (a) TSA-YE (Tryptic-Soy Agar + Yeast Extract) incubated 22 days at 4 °C in anaerobic conditions (Gaspak System, H<sub>2</sub>+ CO<sub>2</sub>); (b) BHIA (Brain Heart Infusion Agar) incubated 28 days at 4 °C in long narrow tubes insuring anaerobic conditions (Raibaud's tubes). ●,○: bacteria in growth phase and in decline phase, respectively.

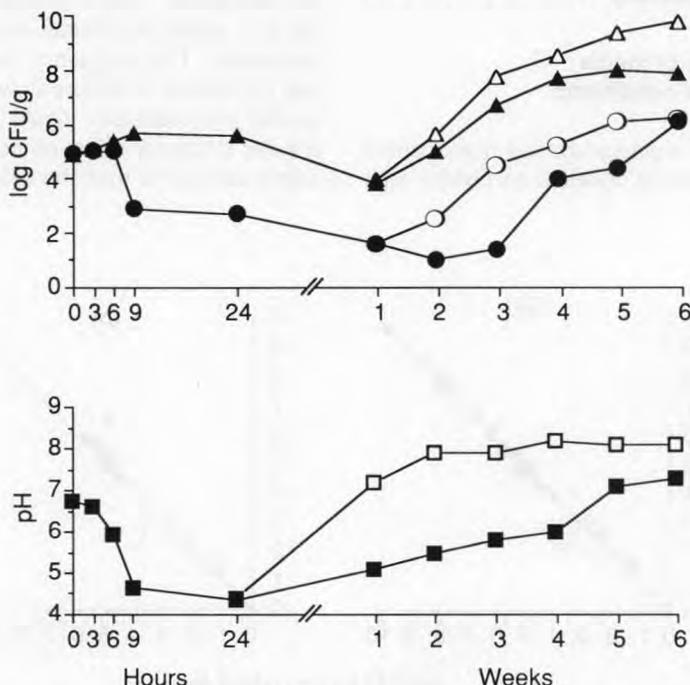
Comparaison du nombre de *Listeria monocytogenes* (souche V7) obtenu sur le milieu Oxford et sur 2 milieux ne contenant pas d'agents sélectifs (résultats de l'essai 1, fromages Nis<sup>-</sup> et Nis<sup>+</sup>): (a) TSA-YE («Tryptic-Soy Agar + Yeast Extract») incubé 22 j à 4 °C en conditions d'anaérobiose (Système Gaspak, H<sub>2</sub>+ CO<sub>2</sub>). (b) BHIA («Brain Heart Infusion Agar») incubé 28 j à 4 °C en condition d'anaérobiose (tubes de Raibaud). ●,○ : bactéries en phase de croissance et de déclin, respectivement.

used in Oxford medium. If this was true we should have observed the same in the experimental cheese. Oxford Agar medium was found equal or superior to other selective media, like Modified McBride Agar (Northolt, 1989; Tiwari and Aldenrath, 1990). Moreover, *Listeria* colonies on Oxford Agar are easily recognizable within 24 h incubation due to the aesculin-ferric ammonium citrate indicator system. These reasons led us to select this medium for enumerating *Listeria* in cheese throughout the study.

### Evolution of *L. monocytogenes* counts and pH during manufacture and ripening of cheese

#### *Cheeses made with high levels of Listeria*

No significant growth of *Listeria* was observed during the first 3 h of cheese making with either the Nis<sup>-</sup> or Nis<sup>+</sup> starter cultures and  $10^5$  *Listeria*/ml in milk (fig 3). This observation is consistent with results obtained by Ryser and Marth (1987b) who made Camembert cheese, using the same strain of *Listeria* (V7). The absence of



**Fig 3.** Behaviour of *Listeria monocytogenes* (strain V7) and pH in Camembert cheeses (results of trial 1, initial inoculum:  $10^5$  *Listeria*/ml).  $\blacktriangle, \triangle$ : cheese made with non nisin-producing starter;  $\bullet, \circ$ : cheese made with nisin-producing starter;  $\blacksquare, \square$ : mean value of pH determinations in cheese made with non nisin-producing and nisin-producing *Lactococcus lactis*. Solid symbols indicate the determinations in the core of the cheese. Open symbols indicate the determinations in the crust of cheese.

*Évolution de Listeria monocytogenes (souche V7) et du pH dans les Camemberts (résultats de l'essai 1, inoculum initial :  $10^5$  *Listeria*/ml)).  $\blacktriangle, \triangle$  : Camembert fabriqué avec des levains lactiques non producteurs de nisine;  $\bullet, \circ$  : Camembert fabriqué avec des levains lactiques producteurs de nisine;  $\blacksquare, \square$  : moyenne des pH mesurés à partir des fromages fabriqués avec des souches de *Lactococcus lactis* Nis<sup>-</sup> et Nis<sup>+</sup>. Les symboles pleins représentent l'intérieur du fromage, les symboles ouverts, la surface.*

growth may be explained by a particularly long lag phase for this strain. This is in agreement with Rosenow and Marth's results (1987) who recorded a 2 h lag phase when this strain was grown at 35 °C in autoclaved skim milk.

Between 3 and 9 h, the numbers of *Listeria* increased by 0.7 logCFU/g in the control curd. This increase could be due to cell entrapment in curd, as was previously noted by Ryser and Marth (1987b). During the same period of time, in curd fermented by Nis<sup>+</sup> lactococci, there was at first no increase in *Listeria* population (from 3–6 h) followed by a dramatic decrease (by ca 2.0 logCFU/g) between 6 and 9 h. Undoubtedly this is the result of accumulated nisin and its bactericidal activity. There was no further significant change in *Listeria* counts in both the control and experimental cheeses, between 9 h and salting time (24 h).

In both, control and Nis<sup>+</sup> cheeses, there was a decrease of 1–2 log CFU/g between 1 and 7 days of ripening at 11 °C. This decrease occurred both in the crust and in the core to a similar extent. Ryser and Marth (1987b) observed a similar decrease during 24 days of ripening with 3 other strains of *Listeria* (Scott A, California, and Ohio) but not with V7. This might indicate the possible differences among *Listeria* relative to their competitive responses against different lactic starters.

The surviving *Listeria* reinitiated growth in the crust and core of control cheese and only in the crust of the Nis<sup>+</sup> cheese between 1 and 2 weeks of ageing while in the core of Nis<sup>+</sup> cheese, there was a further decrease of *Listeria* (0.6 log CFU/g). This decrease of *Listeria* in the core of Nis<sup>+</sup> cheese occurred at a similar pH to that noted during the decrease of *Listeria* in curd, but at a lower temperature (11 °C vs 25 °C). The increase of *Listeria* in the crust of

Nis<sup>+</sup> cheese after 1 week of ageing, similar to that in the crust of control cheese, was unexpected because 400 IU/g of nisin were still present. In fact, 200 IU/ml was shown to cause a 2 log units decrease of *Listeria* in whole milk at pH 6.7 (Williams and Tatini, personal communication). A small percentage of the total assayable nisin (with acid extraction at pH 2.0 and boiling) could be the only part available in the aqueous portion of the cheese. This availability of nisin is further influenced by the changing pH (increasing) which influences the solubility of nisin: solubility decreased from 57 mg/ml at pH 2.0 to 1.5 mg/ml at pH 6.0 and 0.25 mg/ml at pH 8.5 (Liu and Hansen, 1990). An additional factor which might interfere with the activity of nisin is its adsorption to fat and proteins (Somers and Taylor, 1987) changing during ripening, in relation with the increase in pH. The activity of nisin is further influenced by breakdown of the molecule by the mold growth in the crust.

After 4 weeks of ripening at 11 °C, *L. monocytogenes* seemed to have reached maximum populations both on the surface and in the core of cheese made with Nis<sup>-</sup> lactococci (ca 9.10<sup>9</sup> and 1.10<sup>8</sup> CFU/g, respectively). However, in a simulation of a temperature abuse on 6-week-old cheeses (2 days of incubation at 20 °C), *Listeria* counts increased by 0.6 log CFU/g in the core (4-fold increase in counts) whereas no change in counts was observed on the surface. On the other hand, the same temperature abuse, when applied to cheese made with Nis<sup>+</sup> lactococci, gave counts increased by 0.4 log CFU/g in the core and 1.3 in the crust, i.e. more than in control cheese. Two factors may explain this apparent paradox: lower initial counts in experimental than in control cheese, allowing more possibility for growth, associated with more pronounced inactivation of nisin at higher temperature.

*Variation in behaviour of L monocytogenes during manufacture and ripening of cheeses*

The general behaviour of *Listeria monocytogenes* in Camembert cheese hitherto described for trial 1 was also observed in other experiments with some variations (table II). *Listeria* numbers at time of salting increased by 0.8 log CFU/g (average of 2 experiments) in control cheese. This increase is of the same order of magnitude as that observed by Ryser and Marth (1987b) for the same kind of cheese. Dur-

ing the same period of time, *Listeria* counts decreased by 1 to 2 log CFU/g in the experimental cheese, depending on the experiment. There is no clear relationship between the variations in cheese manufacture and the behaviour of *L monocytogenes* in curd. For instance, milk acidification was slower in trial 1 than in the other trials, but *Listeria* decrease was faster.

During the first week of cheese ripening, there was no difference in the level of decrease of *Listeria* between control and Nis<sup>+</sup>

**Table II.** Fate of *Listeria monocytogenes* (strain V7) in Camembert cheese made with milk containing non nisin-producing (Nis<sup>-</sup>) or nisin-producing (Nis<sup>+</sup>) strains of *Lactococcus lactis* subsp *lactis* and inoculated with 10<sup>5</sup> CFU/ml of *Listeria*. The variables in italic represent the average value of the variables printed above them.

*Comportement de Listeria monocytogenes (souche V7) dans du Camembert fabriqué avec du lait contenant des souches de Lactococcus lactis subsp lactis non-productrices de nisine (Nis<sup>-</sup>) ou productrices de nisine (Nis<sup>+</sup>) et 10<sup>5</sup> CFU/ml Listeria. Les variables en italique représentent la moyenne des variables situées au-dessus.*

Period of time	Changes in counts (log CFU/g)									
	Nis <sup>-</sup> starters				Nis <sup>+</sup> starters					
	Trial 1	Trial 3	Mean	SD	Trial 1	Trial 2	Trial 3	Mean	SD	
0-9 h	+0.8	+0.9	+0.85	0.07	-2.0	-0.9	-0.3	-1.07	0.86	
9-24 h	-0.1	0	-0.05	0.07	-0.2	-0.2	-1.1	-0.50	0.52	
0-24 h	+0.7	+0.9	+0.80	0.14	-2.2	-1.1	-1.4	-1.57	0.57	
24 h-1 week	core	-1.8	-1.5	-1.65	0.21	-1.1	-1.8	-2.0	-1.63	0.47
	crust	-1.7	-1.8	-1.75	0.07	-1.1	-1.9	-2.3	-1.76	0.61
0-1 week	<i>-1.05</i>	<i>-0.75</i>	<i>-0.90</i>	<i>0.21</i>	<i>-3.30</i>	<i>-2.95</i>	<i>-3.55</i>	<i>-3.27</i>	<i>0.30</i>	
1-2 weeks	core	+1.2	nd		-0.6	-1.0	nd	-0.80	0.28	
	crust	+1.7	nd		+0.9	-0.2	nd			
2-5 weeks	core	+2.9	nd		+4.7	+5.2	nd	+4.95	0.35	
	crust	+4.2	nd		+3.6	>+3.7	nd			
1-5 weeks	core	+4.1			+4.1	+4.2		+4.15	0.07	
	crust	+5.9			+4.5	>+3.5				

SD: Standard deviation; nd: not determined.

SD : écart type; nd: non déterminé.

cheeses or between core and crust. As pH of the interior of cheese was below 5, this confirms earlier studies showing that nisin was less effective or ineffective at a pH lower than 5.0 (Waites and Odgen, 1987; Monticello and O'Connor, 1990; Radler, 1990). However, some authors (using different systems) found that nisin was more effective at a low pH (Rayman *et al*, 1983; Mohamed *et al*, 1984; Sahl *et al*, 1987; Bekerroum and Sandine, 1988). For example, Mohamed *et al* (1984) found that the amount of nisin to completely inhibit *Listeria* was 16-fold lower at pH 5.5 than at pH 7.4. The decrease of *Listeria* in the crust of Nis<sup>-</sup> as well as Nis<sup>+</sup> cheeses, although the pH was rising, is more difficult to explain. It could be due to the combined effect of salt and low temperature on *Listeria* previously impaired by the acidic conditions. The absence of an extra inhibitory effect of nisin may be due to two factors: firstly, salt was shown to antagonize the action of nisin by interfering with nisin adsorption to bacteria cells (Bell and De Lacy, 1985); moreover, Mohamed *et al* (1984) showed that sensitivity of bacteria to nisin diminished when the temperature of incubation decreased. Nevertheless, an overall decrease of *Listeria* count of ca 1.0 log CFU/g in the control and 3.3 log CFU/g in the Nis<sup>+</sup> cheese was obtained from milk to cheese aged 1 week (table II).

During the second week, there was a further decrease in the core and only a small increase on the surface of Nis<sup>+</sup> cheese, whereas an increase of 1.2–1.7 log unit was observed in both the core and crust of control cheese. This continued decrease or delay in growth of *Listeria* in the core during the second week of ripening of Nis<sup>+</sup> cheeses could be due to the greater effectiveness of the remaining nisin as pH increased to values above 6.0–6.5, allowing greater nisin adsorption on the cell surface (Hurst, 1981) and/or better efficiency (Waites and Odgen, 1987; Monticello and

O'Connor, 1990). Afterwards, *Listeria* was able to grow even in the presence of 250–300 IU/g of nisin, probably because most of the molecules were preferably adsorbed onto fat and proteins resulting from extensive ripening of the cheese surface. During weeks 1–5, there was an overall increase of ca 4 log CFU/g in the core of the cheese, regardless of the presence of nisin.

The total counts (calculated results) in Nis<sup>+</sup> cheese at the end of ripening were ca 2.5 log units lower than in control ones, *ie* the difference was approximately the same as that observed after 2 weeks.

In cheese made from milk containing 10<sup>3</sup> *Listeria*/ml and Nis<sup>+</sup> lactococci, counts of *L. monocytogenes* were close to the predicted numbers (on the basis of data from 10<sup>5</sup> level) during the manufacture and then below the limit of detection by direct plating (*ie* less than 10/g), as expected. However, at the end of the ripening period (5 weeks), the counts were largely below the predicted numbers: less than 10 CFU/g in both core and crust instead of the expected 10<sup>4</sup> and 10<sup>3</sup>, respectively. This suggests that nisin was more efficient when less *Listeria* were present in milk as postulated by Mohamed *et al* (1984) and Monticello and O'Connor (1990).

#### *Cheeses made with low levels of Listeria*

With an initial level of 10<sup>1</sup> CFU/ml in milk used for making cheese with the Nis<sup>-</sup> starter, *Listeria* counts were, during the first 2 weeks, under the limit of detection by direct plating on Oxford agar, *ie* less than 10 *Listeria*/g. Afterwards, the bacteria resumed their growth and reached ca 10<sup>5</sup> CFU/g in rind and 10<sup>3</sup> in core of 6-week cheeses. In contrast, the counts in Nis<sup>+</sup> cheeses were under 10/g throughout the ripening period.

*L. monocytogenes* were not detected in 25 g of 1 or 2-week old cheeses made with Nis<sup>-</sup> *L. lactis*, although colonies of this bac-

terium were present on Oxford agar. Assuming that the enrichment broth was too inhibitory for *Listeria* sublethally injured by acidic conditions, the following modifications of the selective enrichment broth (SEB) were tested: i) omission of acriflavin and addition of an active phage to destroy the lactococci, ii) addition to SEB of 1.5% lithium chloride to inhibit the lactococci, iii) use of a buffer (HEPES 250 mmol/l (Sigma)) to prevent pH drop, iv) combination of i) and iii). In Nis<sup>-</sup> cheeses aged for 1 week, *Listeria* were detected after 1 day of incubation in all modified SEB, and in 2-week old cheeses, in all samples with modification (ii). This confirms the problem of recovery and detection of low densities of injured *Listeria* cells in cheese (Northolt, 1989). However, *Listeria* were never detected in Nis<sup>+</sup> cheeses aged for 1 or 2 weeks, regardless of the enrichment broth used, even after 2 days of incubation. Three reasons could be put forward to explain these results: firstly, the bacteria in Nis<sup>+</sup> cheeses were in worse physiological conditions than those in the control cheese and therefore, were killed during incubation of the enrichment broth secondly, the numbers of cells were too low to be detected after only 2 days of incubation; thirdly, the cell density was too low to allow a representative sampling. In fact, there were no *Listeria* detected in five 25-g test aliquots of cheese (a wedge of *ca* 8 g of cheese was taken from 3 different cheese wheels to make a comingled sample). The latter situation of low densities of *Listeria* would be consistent with the following view on the fate of *Listeria* during cheesemaking and ripening: if the initial count of *Listeria* is low, say 10/ml, there would only be a few surviving cells in Nis<sup>+</sup> cheese aged for 1 or 2 weeks, that is less than 10<sup>-2</sup>/g (10/kg) due to a reduction of at least 3 log units in the first 2 weeks (as seen in this study), and probably less than 0.25 *Listeria* per 25 g sample. Thus, the probability

of having a *Listeria*-free sample is very high together with the problem of recovery of few injured *Listeria* present with a tremendous number of lactococci. Lactococci should be inhibited in the enrichment broth to avoid any production of acid and/or nisin. It is not known at present if the selective agents chosen for the enrichment broth are totally harmless for injured *Listeria*.

Despite the points mentioned above, the data presented here have clearly demonstrated the usefulness of Nis<sup>+</sup> lactococci in killing *L. monocytogenes* in Camembert cheese (3 log units decrease based on direct enumeration). Data have also demonstrated their greater effectiveness, especially with lower levels of initial *Listeria* (10/ml) in cheese milk to result in the absence of *Listeria* in 25 g of cheese even after aging for 6 weeks. This, however, may not offer absolute assurance of safety because a single cell in the crust and core may result in a colony of 10<sup>7</sup> and 10<sup>5</sup> cells in the crust and core, respectively.

## CONCLUSIONS

Two main conclusions can be drawn from the present study. The first is that nisin-producing *Lactococcus lactis* (Prt<sup>+</sup> and Prt<sup>-</sup> strains combination) was found to be suitable as a starter for manufacture of Camembert cheese by providing the appropriate pH at cutting, hooping, turning and finished cheese stages and that Nis<sup>+</sup> starter are able to control *L. monocytogenes* during cheese making. The effectiveness of nisin was particularly great when low levels of *Listeria* (10<sup>3</sup> and 10<sup>1</sup> CFU/ml) were present in milk. It could be useful to prevent a possible post-pasteurization contamination of milk cheese or curd, and for making cheese from raw milk, provided there were low initial numbers of *Listeria*,

say less than 10 per ml. The system can be improved by selecting faster growing strains, developing "super nisin-producers" and/or adding extra nisin in milk so that enough nisin would be present when the pH was between 6.8 and 6.0 and the temperature was still around 30 °C for maximum bactericidal effect.

The second conclusion is that the nisin system has limits in preventing regrowth of survival *Listeria* during Camembert cheese ripening, as cheese pH increases. The same, would likely be true for other bacteriocins as these compounds by definition, are proteins, and are susceptible to proteolytic degradation. Presently, improvement of the nisin system seems to be the simplest way to obviate this problem.

Among the possible side-effects of using nisin-producing starters, the inhibition of the bacteria involved in flavor development and the emergence in the cheese factory environment of nisin-resistant (Nis<sup>®</sup>) strains of *L. monocytogenes* must be considered as the most serious. The former could be overcome by selecting Nis<sup>®</sup> strains of appropriate organisms. The second one could be minimized by controlling the level of *L. monocytogenes* in the dairy environment.

The problem of detecting a few sublethally injured *Listeria* in the presence of a tremendously dominant bacterial flora could be made more complicated by the use of bacteriocin-producing starters. The addition to the enrichment media of compounds adsorbing the bacteriocins or destroying them could be solutions to solve this problem.

If these findings concerning the destruction of *L. monocytogenes* V7 can be confirmed with other *Listeria* strains and that the use of Nis<sup>+</sup> *L. lactis* has no effect on the flavor development, the use of Nis<sup>+</sup> lactococci starter would be an effective, simple and economic means to control *Listeria* in

cheese made from raw milk. Good manufacturing practices are also important to minimize the initial contamination levels of *Listeria* and other psychrotrophic contaminants.

## ACKNOWLEDGMENTS

The assistance of G Pittel and L Vassal, INRA, Jouy-en-Josas, for cheese manufacture is gratefully acknowledged.

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