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Factors moderating *Listeria monocytogenes* growth in raw milk and in soft cheese made from raw milk

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Abstract – *Listeria monocytogenes* is a foodborne pathogenic bacterium sometimes found in raw milk. Raw milk contains natural bacterial inhibitors such as the lactoperoxidase system (LPS) and specific microflora. Six strains of *L. monocytogenes* isolated from raw milk in 1995 and 1996 in Normandy (France) were tested. The aim of the first part of this work was to evaluate the inhibitory effect of LPS on *L. monocytogenes* in milk. Kept at 15 °C for 65 h, in static conditions, populations of *L. monocytogenes* in pasteurized milk increased by 2 to 3.8 log depending on the strain. In raw milk, in the same conditions, populations increased by 0.8 to 2.3 log. Adding thiocyanate and hydrogen peroxide to raw milk (supplemented raw milk, SRM) enhanced its inhibitory effect. In SRM, three strains were unable to grow and the populations of the other strains increased by 0.7 to 1.3 log. The inhibitory effect of the LPS in milk was clearly demonstrated. The inhibitory effect of raw milk on *L. monocytogenes* was due to LPS, probably combined with the microbiological composition of raw milk. The aim of the second part of this work was to evaluate the inhibitory effect of using raw milk for making Camembert cheese (RMC). The results show that the growth of *L. monocytogenes* was about twice as slow in RMC as in Camembert made from pasteurized milk (PMC). The average lag phase (Lag) was 15 d in PMC and 34 d in RMC. Statistical analysis showed that the inhibitory effect of RMC on the growth of *L. monocytogenes* was mainly related to the microbiological composition of the raw milk, in terms of thermophilic *Lactobacillus* and yeast. Although our results did not clearly demonstrate an inhibitory effect of chemical composition of raw milk, inhibition of *L. monocytogenes* in RMC is probably due to the interrelationship between microbiological and chemical factors.

***Listeria monocytogenes* / inhibition / raw milk / lactoperoxidase system**

Résumé – Facteurs ralentissant la croissance de *Listeria monocytogenes* dans le lait cru et dans les fromages à pâte molle au lait cru. Six souches de *L. monocytogenes* isolées de lait cru en 1995 et 1996 (Normandie) sont utilisées. L'effet du système lactopéroxydase (LPS), inhibiteur de croissance microbienne naturellement présent dans le lait cru, sur *L. monocytogenes,* est évalué. Dans le lait pasteurisé incubé 65 h à 15 °C, la population de *L. monocytogenes*, augmente de 2 à 3,8 log selon les souches. Dans le lait cru, la population augmente de 0,8 à 2,3 log. L'ajout de thiocyanate et de péroxyde d'hydrogène au lait cru (SRM) accroît son effet inhibiteur. Dans le SRM, trois souches ne se développent pas ; pour les autres, la population augmente de 0,7 à 1,3 log. L'effet inhibiteur du lait cru sur *L. monocytogenes* est lié à la présence de LPS, et probablement à la composition microbiologique du lait cru. La croissance de *L. monocytogenes* dans le Camembert est également étudiée. Les résultats montrent que *L. monocytogenes* se développe environ deux fois plus lentement dans le Camembert au lait cru (RMC) que dans celui au lait pasteurisé (PMC). Les durées moyennes des

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phases de latence sont de 15 et 34 j, respectivement dans le PMC et le RMC. L'effet inhibiteur du RMC est principalement lié à la composition microbiologique du lait cru, en particulier la présence de lactobacilles thermophiles et de levures.

***Listeria monocytogenes* / inhibition / lait cru / lactopéroxydase**

1. INTRODUCTION

Listeria monocytogenes is a foodborne pathogenic bacterium that can cause listeriosis. It affects the young, the elderly, pregnant women and persons with weakened immune systems. *L. monocytogenes* has been implicated in meningitis, abortion, septicemia and infection with a lethality as high as 30% [14, 36]. It is a ubiquitous bacterium, able to grow in ready-to-eat foods including dairy products [13, 20, 23, 25, 31, 39]. *L. monocytogenes* is psychrotrophic and can grow in acid conditions [19]. The behavior of *L. monocytogenes* is related to its initial concentration and the pre-incubation conditions [17].

Raw milk can be contaminated with *L. monocytogenes*. Examples of the incidence of *L. monocytogenes* in raw milk cited in the literature are 2.3% in Canada, 3.2% in USA, 3.6% in Europe [38] and 2.4% in France [28]. Raw milk can be contaminated as a result of environmental hygiene conditions during milking, or can be contaminated directly by the cattle, which may sometimes shed *L. monocytogenes* in their milk as a result of listerial mastitis, encephalitis or abortion. In general, contamination due to environmental hygiene during milking is weak (below 3 cfu·mL^{-1} with most probable concentration 0.1 cfu·mL^{-1}, [28]). Direct contamination from dairy cattle can be higher than 10^3 cfu·mL^{-1} [38, 40].

Raw milk contains bacterial inhibitors such as the lactoperoxidase system (LPS) [1]. The LPS is composed of an enzyme, lactoperoxidase (LP); an oxidative agent, hydrogen peroxide (H_2O_2); and a substrate, the thiocyanate (SCN^-). The oxidation product ($OSCN^-$) can react with the amine and thiol groups of the enzymes essential for bacterial metabolism. LPS has a bacteriostatic effect on Gram-positive bacteria including *L. monocytogenes*, and is inactivated in pasteurized milk [32].

Soft cheese can become contaminated with *L. monocytogenes* owing to contaminated raw materials or lack of hygiene during manufacture, storage and distribution. Soft cheeses have been implicated in foodborne outbreaks [43]. The inhibitory effect of raw milk on several pathogens including *L. monocytogenes* [33] can indicate that *L. monocytogenes* grows more slowly in raw milk cheese than in cheese made from pasteurized milk. Factors that can limit growth are the LPS, lactic acid bacteria (which are sometimes bacteriocin producers), competition for nutrients, steric limitations, etc. [6, 14, 35]. Technological parameters such as low pH or a_w can also have an effect on the behavior of *L. monocytogenes* in cheese.

The study had two aims. The first was to evaluate the inhibitory effect of the LPS in milk. The second was to compare the growth of *L. monocytogenes* in artificially contaminated soft cheeses made from pasteurized and from raw milk, and determine factors pertaining to raw milk components, raw milk microbial flora and cheese-making technology that may have an inhibitory effect on *L. monocytogenes*.

2. MATERIALS AND METHODS

2.1. *L. monocytogenes* strains

2.1.1. Strains

Six strains of *L. monocytogenes* isolated from raw milk in 1995 and 1996 in Normandy (France) were used throughout the study. All strains were from a private collection (strains N° 315-734; 314-501; 360-248; 348-38; 346-209; and 307-163).With the aim of simplifying reading of this paper, the strains were recalled 1, 2, 3, 4, 5 and 6.

2.1.2. Typing of L. monocytogenes strains

The six strains were serotyped and phage-typed by the Centre National de Référence des *Listeria* (Institut Pasteur, Paris, France). For pulsed field gel electrophoresis (PFGE) analysis, Brain Heart Infusion Broth (BHI, Difco, Grenoble, France) was inoculated with a single colony. Cells were harvested from 8 mL of broth after overnight incubation at 37 °C. DNA isolation was performed in Low Melting Point agarose plugs (125 mmol·L^{-1}), as described by Brosch et al. [10], modified by the use of a lysis solution containing 0.5 mol·L^{-1} EDTA-0.5% Sarcosyl (Sigma, Saint-Quentin-Fallavier, France), 2 mg·mL^{-1} desoxycholic acid (Sigma), and 2.5 mg·mL^{-1} lysozyme (Appligene, Illkirch-Graffenstaden, France). The restriction endonuclease used for digestion was *Sma1* (Boehringer, Reims, France and Amersham, Orsay, France). The samples were eletrophoresed at 200 V and 15 °C in the CHEF DRIII system (Biorad, Ivry-sur-Seine, France). The pulsed times ranged from 1 to 12 s for 17 h. The gels were stained with ethidium bromide and photographed under UV transillumination. The PFGE patterns were analyzed with the Molecular Analyst software (Biorad). Similarities between macrorestriction patterns were expressed by Jaccard coefficient correlation by the UPGMA method (unweighted pair group using arithmetic averages) (minimum Profiling 5%, minimum Area 0.5%, position tolerance 1.2%).

2.2. Chemical analyses of milk

Chemical analyses were performed on the raw milk samples (before pasteurization or addition of thiocyanate and hydrogen peroxide) used throughout this study. Lactoperoxidase (LP) activity was determined according to the method described by Kumar and Bhatia [24]. The milk samples were analyzed in triplicate. The thiocyanate (SCN^-) concentration in the milk was determined using the method described by Hoogendoorn et al. [22]. Milk samples were analyzed in duplicate.

2.3. Microbiological analyses of milk

Twenty-five milliliters of each raw milk sample were analyzed for the presence of *L. monocytogenes*, according to the ISO 11290-1 standard [2]. To evaluate the microbiological quality of milk, enumerations of thermophilic *Lactobacillus*, mesophilic *Lactobacillus*, Enterobacteriaceae, *Enterococcus*, *Pseudomonas*, *Micrococcus*, *Coryneform* bacteria and yeast were performed. Table I summarizes the microflora enumeration techniques employed.

2.4. Behavior of *L. monocytogenes* in milk

2.4.1. Milk samples

Eight milk samples were used to study the behavior *of L. monocytogenes* in milk. Samples A, B and C were used raw. Samples D, E and F were pasteurized at 75 °C for 15 s in a small plate heat-exchanger type V8 (Vicarb, Fontanil-Cornillon, France). Chemical and microbiological analyses were performed on these three samples before pasteurization. The last two samples (G and H) and sample C were used raw with added H_2O_2 (Labogros, Buchs, France) and NaSCN (Sigma). The concentrations added were 0.25 mmol·L^{-1}, both for thiocyanate and for hydrogen peroxide. Chemical and microbiological analyses were performed before adding the thiocyanate and hydrogen peroxide to the raw milk. Different milk samples were used for the different situations (pasteurized, raw, SRM and different strains) because of experimental constraints.

2.4.2. Inoculum preparation

Ten microliters of frozen culture was prepared on slants of Tryptone Soya Agar (TSA, Oxoid, Dardilly, France) and incubated at 30 °C for 24 h. Bacteria were then inoculated into 10 mL of Tryptone Soya Broth (TSB, Oxoid) and incubated at 30 °C for 24 h. Then 0.1 mL of this culture was transferred into 10 mL of sterile TSB and incubated at 30 °C for 24 h. This incubation regime resulted in a stationary phase culture with approximately 10^9 cfu·mL^{-1}. Five hundred

Table I. Microbiological techniques used to analyze raw milk samples.

Microbiological flora	Technique and Medium	Incubation	
		Temperature (°C)	Time
Thermophilic *Lactobacillus*	Pour plate - M17 agar [2]	42	72 h
	Spread plate - MRS agar [1]	42	72 h
Mesophilic *Lactobacillus*	Pour plate - M17 agar [2]	30	72 h
	Spread plate - MRS agar [1]	30	72 h
Enterobacteriaceae	Pour plate - Violet Red Bile Glucose Agar (VRBGA) [2]	30	24 h
Enterococcus	Spread plate - Citrate Azide Tween Carbonate Agar (CATC agar) [3]	37	24 h
Pseudomonas	Spread plate - *Pseudomonas aeromonas* Selective Agar Base acc. to Kielwein (GSP agar) [3]	25	72 h
Micrococcus	Spread plate - Tryptone Soya Agar (TSA) + 3% NaCl agar [2]	30	72 h
Coryneform bacteria	Spread plate - TSA + 3% NaCl agar [2]	30	72 h
Yeast	Pour plate - Chloramphenicol Glucose Agar [2]	25	5 d

[1] Difco; [2] Biokar Diagnostics; [3] Merck.

milliliters of milk (in a 1-liter flask) were inoculated with 1 mL of diluted inoculum to reach the desired initial concentration (approximately 10^3 cfu·mL^{-1}).

2.4.3. Incubation temperature of L. monocytogenes in milk

The temperatures tested were 3 °C, 9 °C and 15 °C. Each experiment (one milk type, one temperature and one strain) was carried out in duplicate. The experimental design is presented in Table II.

2.4.4. Numeration of L. monocytogenes in milk

The six *L. monocytogenes* strains were inoculated separately in the eight milk samples. Plates of Palcam (Oxoid) were spread with 0.1 mL inoculum and incubated for 24 h to 48 h at 30 °C. Each milk sample was enumerated twice a day over a period of 65 h (values given were the mean of two independent experiments).

2.5. Main steps in the cheese-making

Eighteen milk samples were taken on different occasions (from April through August) from the same farm tank on the day of collection (after four milkings).

Eight raw milk samples were pasteurized (75 °C for 15 s) in a small plate heat-exchanger (Vicarb, type V8) to make pasteurized milk cheese (PMC) and ten were not, to make raw milk cheese (RMC). Milk samples used to make PMC and RMC were always different, because of cheese-making restraints. The milk was skimmed at 50–55 °C. Fat concentration was standardized to 28 g·L^{-1}, and the milk was put in four different 50-liter vats. One of these was used as control and was not inoculated with *L. monocytogenes*. The other three were inoculated with three different *L. monocytogenes* strains (5×10^{-1} cfu·mL^{-1}, one strain per vat). The milk was inoculated with mixed mesophilic lactic starter culture (MM100 and MM101, 0.6 U·100 mL^{-1}, Rhodia Food, Saint-Fons, France) and *Micrococcus* (MVA, 0.5 dose per 1000 L,

Table II. Experimental design used to study behavior of *L. monocytogenes* in milk.

Milk sample	Collecting date (day/month)	Milk type	Inoculated strains	Incubation temperatures (°C)
A	07/07	Raw	2, 3, 4, 5, 6	3, 9 and 15
B	15/07	Raw	1, 6	3, 9 and 15
C	24/08	Raw	1, 2, 3, 4, 5	3, 9 and 15
		SRM [a]	1, 2, 5, 6	3, 9 and 15
D	23/06	Pasteurized	1, 2, 3, 4	3, 9 and 15
E	21/07	Pasteurized	3, 4, 5, 6	3, 9 and 15
F	27/07	Pasteurized	1, 2, 5, 6	3, 9 and 15
G	04/08	SRM	1, 2, 3, 4	3, 9 and 15
H	10/08	SRM	3, 4, 5, 6	3, 9 and 15

[a] Supplemented raw milk: adding thiocyanate and hydrogen peroxide to raw milk.

Rhodia). The milk was cold-ripened at 12 °C for 15 h. It was then inoculated with *Penicillium camembertii* (Neige, 0.5 dose per 1000 L, Rhodia), *Geotrichum candidum* (Géo17, 0.5 dose per 1000 L, Rhodia) and mesophilic acid starter culture (MA011 and MA014, 0.35 U per 100 L, Rhodia). The milk was ripened for 20 min at 38 °C (warm ripening). The clotting agent (rennet extract Carlin, Rhodia) was added to the milk at 35 °C (pH = 6.35). Coagulation occurred in 40 min. The curd was cut into 2-cm cubes, healed for 25 min, drained and transferred to moulds. The moulds were turned after 1, 2 and 3 h. The cheeses were removed from the moulds and stored at 12 °C for 6 h before brining in a saturated sodium chloride solution (content of NaCl in cheese was 1.6% w/w). The cheeses were inoculated on their surface by spraying *Penicillium camembertii* (Neige, 1 dose per 500 mL) and *Geotrichum candidum* (Géo17, 0.5 dose per 500 mL). They were then drained and placed in a drying room for 24 h at 12 °C. They were then ripened at 11 °C with 85–95% relative humidity for 14 d, then packaged and stored at 4 °C for 40 d (i.e. until 55 d after manufacture).

The cheeses made from the control vat of milk were used to measure technological parameters during manufacture.

2.6. Behavior of *L. monocytogenes* in cheese

Inoculum preparation was the same as was used for inoculating *L. monocytogenes* in the milk samples. Strains were individually inoculated into milk after standardizing fat content. Initial bacterial concentration was approximately 5×10^{-1} cfu·mL^{-1} of milk. As three different *L. monocytogenes* strains were studied from one cheese production, ten cheese productions from raw milk enabled us to study five-fold the six *L. monocytogenes* strains. Eight cheese productions from pasteurized milk enabled us to study four-fold the six *L. monocytogenes* strains.

Enumeration of *L. monocytogenes* was performed during cheese manufacture, ripening and storage. *L. monocytogenes* was enumerated (i) at the beginning of the cold ripening of the milk, (ii) at the end of the warm ripening of the milk, (iii) when the curd was ladled into moulds, (iv) during destacking, (v) at the end of draining, (vi) after 7 d of ripening at 11 °C, (vii) at packaging, and six times during storage at 4 °C (after 5, 12, 19, 26, 33 and 40 d).

Enumeration of *L. monocytogenes* in the milk at the beginning of cold ripening (i) and at the end of the warm ripening (ii) was performed by the following method. Ten milliliters of milk were spread on five Palcam

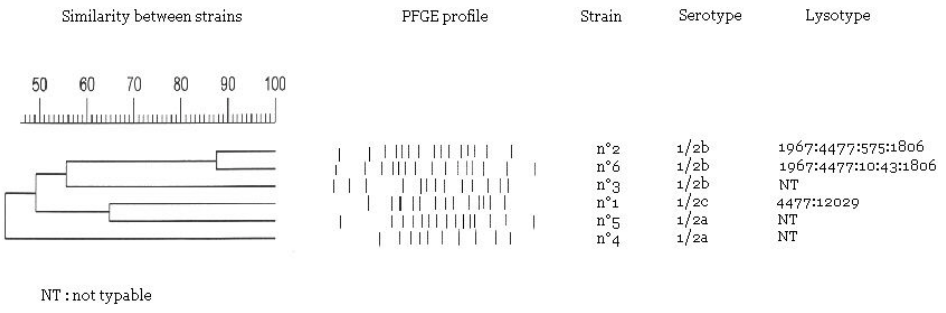

Figure 1. Pulsotype, serotype and lysotype of the six *L. monocytogenes* strains used throughout the study.

agar (Oxoid) Petri dishes (diameter 140 mm) and incubated at 37 °C for 48 h. The detection threshold was 10^{-1} cfu·mL^{-1}. Samples were enumerated in duplicate.

For enumeration of *L. monocytogenes* in the curd (during manufacture and ripening and at packaging, iii, iv, v, vi and vii), samples were enumerated in duplicate. Ten grams of cheese were diluted in 40 mL of Tryptone salt broth. One milliliter of this dilution was spread on five Palcam agar (Oxoid) Petri dishes (diameter 90 mm) and incubated at 37 °C for 48 h. The detection threshold was 5 cfu·mL^{-1}.

Enumeration *of L. monocytogenes* during storage was performed on 20 g of cheese diluted in 80 mL of Tryptone salt broth. One milliliter of this dilution (or 1 mL of the appropriate decimal dilution) was spread on 5 Palcam agar (Oxoid) Petri dishes (diameter 90 mm) and incubated at 37 °C for 48 h. The detection threshold was 5 CFU·mL^{-1}. The samples were enumerated in duplicate.

For each cheese production, 30 colonies of each *L. monocytogenes* strain were isolated at the end of the experimental period (56 d). PFGE profiles were determined to verify that the enumerated strains were those that had been inoculated into the milk.

2.7. Statistical analysis

The effect of raw milk composition (microbiological and chemical concentrations) on the behavior of *L. monocytogenes* in Camembert cheese was evaluated by principal components analysis with Statgraphics (Uniware, Bromley, UK). Chemical values were used directly and microbiological parameters were log transformed before analysis. Two distinguishing growth curve parameters were determined to characterize the growth of *L. monocytogenes* in cheese. These two parameters were the lag phase (Lag) and time to a 10^3 increase in population in the curd ($T10^3$). When no growth was observed during the 56 d of the experiment, Lag and $T10^3$ were arbitrarily set at 80 and 100 d, respectively. When growth was observed but $T10^3$ was longer than the 56-day experiment period, $T10^3$ was arbitrarily set at 80 d.

3. RESULTS

3.1. Characterization of *L. monocytogenes* strains

The six strains (all taken from raw milk) were characterized by their pulsotype, serotype and lysotype (Fig. 1). The results from serotype indicated that the strains belong to serotypes 1/2a (strains 4 and 5), 1/2b (strains 2, 3 and 6) and 1/2c (strain 1). The results from lysotype indicated that strains 2, 3 and 6 were different. The results from PFGE profiles and from similarity between strains indicated that strains 4 and 5 were different (less than 50% similarity). The results indicated that all six strains were different.

Table III. Lactoperoxidase and thiocyanate concentrations in milk samples used to evaluate effect of LPS on *L. monocytogenes* in milk. These analyses were performed on raw milk samples (before pasteurization or before addition of thiocyanate and hydrogen peroxide).

Milk sample	Collecting date (day/month)	Lactoperoxidase concentration ($\mu g \cdot mL^{-1}$)	Thiocyanate concentration ($\mu g \cdot mL^{-1}$)
A	07/07	38.9	5.94
B	15/07	28.5	4.32
C	24/08	36.1	5.03
D	23./06	21.7	4.94
E	21/07	25.8	4.54
F	27/07	33.0	4.43
G	04/08	48.6	6.53
H	10/08	40	5.17
Average		34.1	5.11

3.2. Chemical analysis of the eight milk samples used to evaluate effect of LPS in milk

The average value for lactoperoxidase concentration was 34.1 $\mu g \cdot mL^{-1}$ (Tab. III). Lactoperoxidase concentrations were found to differ widely from sample to sample. The minimum and maximum concentrations were 21.7 $\mu g \cdot mL^{-1}$ and 48.6 $\mu g \cdot mL^{-1}$, respectively. The minimum concentration was observed in a sample taken in June. The average value for thiocyanate concentration was 5.11 $\mu g \cdot mL^{-1}$ (range 4.32–6.53 $\mu g \cdot mL^{-1}$).

3.3. Microbiological analysis of the eight milk samples used to evaluate effect of LPS in milk

No *L. monocytogenes* cells were found in the 25-mL milk samples. Some differences were observed in the microflora composition (Tab. IV). The main microflora encountered in raw milk samples B, C and H was *Pseudomonas* (74.8%, 47.3% and 86%, respectively). In sample C, there were high proportions of coryneform bacteria and yeasts; 10.9% and 5.5%, respectively, against less than 0.15% and less than 0.4% in the other two samples (B and H). For milk samples A, D, E, F and G, percentages of *Pseudomonas* were 30.5%, 30.0%, 18.8%, 13.8% and 15.2%, respectively. In samples D, F and G, mesophilic *Lactobacillus* was the main microflora (more than 60%). For sample A, *Enterococcus* was the main microflora (55.6%). For sample E, the main microflora was represented by mesophilic *Lactobacillus* (28.8%), *Micrococcus* (28.8%) and thermophilic *Lactobacillus* (10.4%).

3.4. Behavior of *L. monocytogenes* in milk

In pasteurized milk (samples D, E and F, Tabs. III and IV) strains 1, 2, 5 and 6 were unable to grow at 3 °C for 65 h (Fig. 2A). Their populations increased by less than 0.5 log. The populations of strains 3 and 4 increased by 0.8 and 1.0 log during the 65 h of incubation. At 9 °C and 15 °C, all tested strains were able to grow in pasteurized milk. At 9 °C, populations increased by between 0.5 log (strain 3) and 1.8 log (strain 4). At 15 °C, the average population increase was 3.1 log during the incubation period. The range was between 2.7 log (strain 2) and 3.8 log (strain 6). Values given for each strain were the mean of two independent experiments.

In raw milk (samples A, B and C, Tabs. III and IV) all tested strains were unable to grow at 3 °C for 65 h (less than 0.5 log increase, Fig. 2B). At 9 °C, only strain 1 was

Table IV. Microbiological analysis of milk samples used to evaluate effect of LPS on *L. monocytogenes* in milk. These analyses were performed on raw milk samples (before pasteurization or before addition of thiocyanate and hydrogen peroxide).

Microbiological flora	Enumeration (cfu·mL^{-1}) and percentage[a] of each microbiological flora in raw milk samples							
	A	B	C	D	E	F	G	H
Thermophilic *Lactobacillus*	6.5×10^2 3.6%	1.7×10^2 0.3%	8.3×10^2 1.5%	1.3×10^2 0.5%	8.3×10^2 **10.4%** [b]	1.1×10^2 0.3%	2.0×10^3 3.8%	6.1×10^2 0.1%
Mesophilic *Lactobacillus*	8.3×10^2 4.6%	1.5×10^4 23.1%	1.6×10^4 29.1%	1.7×10^4 **60.7%**[b]	2.3×10^3 **28.8%** [b]	3.4×10^4 **78.2%** [b]	5.2×10^4 **78.8%** [b]	1.5×10^5 14.5%
Enterobacteriaceae	4.2×10^1 0.2%	1.0×10^2 0.2%	2.4×10^2 0.4%	1.5×10^2 0.5%	2.0 x10^2 2.5%	4.2×10^2 1.0%	3.0×10^2 0.5%	1.4×10^3 0.1%
Enterococcus	1.0×10^4 **55.6%** [b]	1.8×10^2 0.3%	1.1×10^3 2.0%	<10 < 0.03%	2.8×10^2 3.5%	2.5×10^2 0.6%	1.2×10^2 0.2%	1.7×10^4 1.7%
Pseudomonas	5.5×10^3 30.5%	4.8×10^4 **74.8%** [b]	2.6×10^4 **47.3%** [b]	8.4×10^3 30.0%	1.5×10^3 18.8%	6.0×10^3 13.8%	1.0×10^4 15.2%	8.6×10^5 **86.0%** [b]
Micrococcus	1.0×10^3 5.6%	1.1×10^3 1.7%	2.3×10^3 4.2%	2.4×10^3 8.6%	2.3×10^3 **28.8%** [b]	2.6×10^3 6.0%	1.5×10^3 2.3%	2.2×10^3 0.2%
Coryneform bacteria	$<10^2$ < 0.6%	1.0×10^2 0.15%	6.0×10^3 **10.9%** [b]	$<10^2$ < 0.4%	4.0×10^2 5.0%	$<10^2$ <0.2%	1.0×10^2 0.15%	3.0×10^2 0.03%
Yeast	3.9×10^2 2.0%	2.7×10^2 0.4%	3.0×10^3 **5.5%** [b]	2.5×10^2 0.9%	1.7×10^2 2.1%	2.0×10^1 0.05%	1.4×10^2 0.2%	4.0×10^2 0.04%

[a] Percentage of the amount of enumerated microflora; [b] main encountered microbiological flora in each raw milk sample.

able to grow (0.7 log increase). At 15 °C, the increase was between 0.8 log (strain 2) and 2.1 log (strain 6). The average value for the six strains was 1.4 log. Values given for each strain were the mean of two independent experiments.

The milk samples used as SRM (i.e. with added thiocyanate and hydrogen peroxide) were samples C, G and H (Tabs. III and IV). Thiocyanate concentration in SRM was about 20 µg·mL^{-1} (19.53 µg·mL^{-1} for sample C, 19.67 µg·mL^{-1} for sample H and 21.03 µg·mL^{-1} for sample G).

In SRM, no growth, or no significant growth, of *L. monocytogenes* was observed at 3 °C and 9 °C for all tested strains (Fig. 2C). At 3 °C, the populations of strains 1 and 4 decreased (1.5 log and 0.7 log, respectively). No difference between the beginning and the end of incubation was observed for other strains. At 9 °C, strain 1's population decreased (0.7 log), but for other strains no difference was observed between the beginning and the end of incubation. At 15 °C, the growth of strains 3 and 5 was completely inhibited compared with the increase observed in raw milk (1 log and 1.7 log, respectively). A decrease of 1.2 log was observed for strain 4. Strains 1, 2 and 6 were able to grow; the population increases were 0.7, 1.0 and 1.3 log for strains 6, 1 and 2, respectively. Values given for each strain were the mean of two independent experiments.

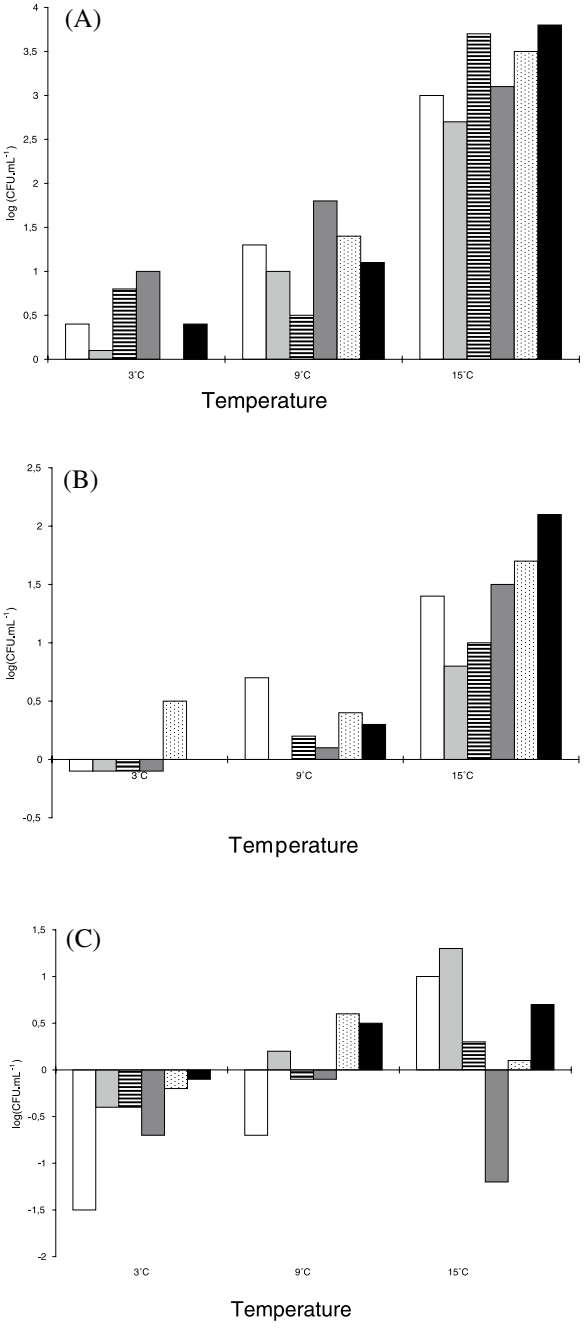

Figure 2. Difference in enumerated population of *L. monocytogenes* between the beginning and end of incubation (65 h) in pasteurized milk (A), in raw milk (B) and in supplemented raw milk (C). (□:strain 1; ▒:strain 2; ▤:strain 3; ▓:strain 4; □:strain 5; and ■:strain 6).

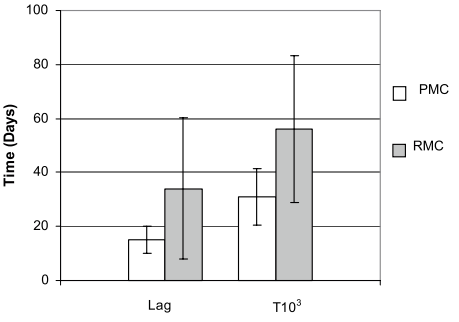

Figure 3. Average values for Lag phase and time to a 10^3-fold increase in population for the six strains of *L. monocytogenes* in Camembert made from pasteurized (PMC) and raw milk (RMC). The bars represent the standard deviation.

3.5. Behavior of *L. monocytogenes* in Camembert cheese

Three milk samples were naturally contaminated with *L. monocytogenes*. One of them was pasteurized before making the cheese. We considered that *L. monocytogenes* enumerated in cheeses made from this pasteurized milk was the strain artificially inoculated. The two other samples were used to make cheese from raw milk. For these two samples, analysis of control cheeses of these two cheese productions showed no *L. monocytogenes* contamination. This result could well be due to the very low contamination level of the raw milk. We therefore considered that all the *L. monocytogenes* enumerated in raw cheeses from these two cheese productions were those inoculated into the raw milk; this was confirmed by the results of the PFGE profiles. For the other cheese productions, the PFGE profiles also confirmed that enumerated strains were those inoculated into the milk (results not shown).

For each experiment, the growth curve of each strain of *L. monocytogenes* was plotted and Lag and $T10^3$ were determined.

L. monocytogenes grew more slowly in RMC than in PMC. Lag and $T10^3$ (Fig. 3) were longer in RMC than in PMC. In PMC, the average Lag was 15.1 ± 5.0 d, and average $T10^3$ was 31.1 ± 10.5 d. In RMC, the average Lag value was 34.1 ± 26.0 d and average $T10^3$ was 55.9 ± 27.2 d. For strain 1, $T10^3$ in RMC (60.9 d) was more than twice as long as in PMC (26.1 d). Growth of this strain of *L. monocytogenes*, in RMC was more than twice as slow as in PMC. For strains 3, 4 and 6, $T10^3$ in RMC (62.5, 57.5, and 51.4 d, respectively) was about twice as long as in PMC (34.1, 32.5 and 27.3 d, respectively). For strains 2 and 5, $T10^3$ was longer in RMC (52.6 and 50.7 d, respectively) than in PMC (35.3 and 30.4 d, respectively). The inhibitory effect of RMC seemed to be weaker for these strains than for the others.

In RMC, both Lag and $T10^3$ differed widely from cheese production to cheese production (Fig. 3). Standard deviation was 26.5 d in RMC. The minimal and maximal values observed for Lag in RMC were 7.1 and 80 d, respectively. The minimal and maximal values observed for $T10^3$ in RMC were 14.8 and 100 d, respectively. The results obtained in PMC (Fig. 3) showed less variation than in RMC. The standard deviation observed were 5.0 d for Lag and 10.5 d for $T10^3$. The minimal and maximal values observed for Lag in PMC were 6.9 and 29.0 d, respectively. The minimal and maximal values observed for $T10^3$ in PMC were 18.6 and 56.0 d, respectively. These results are probably related to the microbiological and chemical components of the raw milk used in the experiment. These components may have been partly destroyed during pasteurization of the milk used to manufacture PMC.

3.6. Effects of the variables studied on the growth of *L. monocytogenes*

Linear correlation analysis between parameters characterizing *L. monocytogenes* growth (Lag and $T10^3$) and the variables we studied (microbiological and chemical composition of raw milk) showed that Lag and $T10^3$ were well correlated (0.90, Tab. V). Lag and $T10^3$ were positively correlated to yeast (0.36 and 0.19, respectively) and thermophilic *Lactobacillus* (0.48 and 0.50, respectively). These results appear to

Table V. Linear correlation analysis between the variables studied (microbiological and chemical composition of raw milk) and parameters characterizing growth of L. monocytogenes in raw milk cheese.

Variable	Linear correlation coefficient with	
(used name on correlation circle, Fig. 4)	Lag [a]	$T10^3$ [b]
Contaminating microflora (Conta)	0.02	0.03
Lactococcus (Lc)	0.18	0.15
Streptococcus (Stept)	0.03	0.17
Pseudomonas (Pseudo)	0.10	0.24
Thermophilic *Lactobacillus* (Lbthermo)	**0.48** [c]	**0.50** [c]
Mesophilic *Lactobacillus* (Lbmeso)	–0.20	–0.25
Yeast	**0.36** [c]	**0.19** [c]
Coryneform bacteria (Coryne)	0.17	–0.02
Micrococcus (Microco)	–0.21	–0.13
Lactoferrin (Lactofer)	**0.22** [c]	0.17
M immunoglobulin (IgM)	–0.11	0.00
Lactoperoxidase (Perox)	0.17	**0.24** [c]
Thiocyanate (Thio)	0.07	–0.02
Lag	**1** [c]	**0.90** [c]
$T10^3$	**0.90** [c]	**1** [c]

[a] Lag phase; [b] time to a 10^3-fold increase in population in curd; [c] main values of the linear correlation coefficients.

indicate an inhibitory effect of thermophilic *Lactobacillus*, and perhaps of yeast, on the growth of *L. monocytogenes*. Some chemical variables may also affect behavior of *L. monocytogenes*, but to a lesser extent. The correlation coefficient between lactoferrin and lactoperoxidase and the growth parameters were about 0.2 (Tab. V).

The aim of the principal components analysis was to determine the interrelations between the microbiological and chemical composition of raw milk and the growth of *L. monocytogenes* in cheese. In our results, three factors (or axes) explained 61.4% of the total variance (Tab. VI). Major contributors to Factor 1 (25.2% of total variance, x axis in Figs. 4 and 5) were chemical variables (lactoferrin and thiocyanate). The correlation coefficients between these two variables and Factor 1 were higher than 0.8. Factor 2 (20.1% of total variance) was also correlated with a chemical variable: IgM. The correlation coefficient between this variable and Factor 2 (y axis in Figs. 4 and 5) was 0.75. *Streptococcus* was the main variable contributing to Factor 3 (16.1% of total variance) (Tab. VI). Lag and $T10^3$ were not well correlated with these three factors; the correlation coefficients were between 0.30 and 0.52 (Tab. VI). From the correlation circle, it was possible to determine what variables affect the growth of *L. monocytogenes*. To interpret the correlation circle, one looks at the position of all variables on the diagram. Two variables close together in the diagram (for example, Lag and $T10^3$) are correlated. The correlation circle (Fig. 4) indicates that thermophilic *Lactobacillus* (Lbthermo) had an inhibitory effect on the growth of *L. monocytogenes*. The higher the thermophilic *Lactobacillus* concentration in the raw

Table VI. Correlation coefficients from the Principal Components Analysis: correlation between factors and studied variables.

Variable	Factor 1 (25.2%)	Factor 2 (20.1%)	Factor 3 (16.2%)
Contaminating microflora	–0.61	–0.32	0.06
Lactococcus	–0.4	0.60	–0.15
Streptococcus	0.14	–0.50	**–0.77** [c]
Pseudomonas	–0.03	0.51	–0.52
Thermophilic *Lactobacillus*	–0.45	0.45	–0.49
Mesophilic *Lactobacillus*	0.16	–0.47	–0.12
Yeast	–0.65	0.26	0.59
Coryneform bacteria	–0.09	0.42	0.03
Micrococcus	0.48	–0.0.3	–0.64
Lactoferrin	**–0.87** [c]	–0.19	–0.06
M immunoglobulin	–0.19	**–0.75** [c]	–0.18
Lactoperoxidase	–0.66	–0.54	–0.38
Thiocyanate	**–0.81** [c]	–0.43	0.19
Lag [a]	**–0.52** [c]	0.43	–0.30
$T10^3$ [b]	**–0.45** [c]	0.36	–0.47

[a] Lag phase; [b] time to a 10^3 -fold increase in population in curd; [c] main values of the correlation coefficients.

milk, the longer were Lag and $T10^3$ (hence the slower the growth). In Figure 4, the values indicated on Factors 1 and 2 (or axes x and y) are the coefficient correlations between the studied variables and each factor.

From the principal components analysis, we drew a diagram of the distribution of individuals on RMC (Fig. 5). An "individual" corresponds to one strain and one cheese-making date. From the distribution of individuals, clusters of individuals close together on the diagram can be shown as groups. The different cheese productions made from a given strain of *L. monocytogenes* are not closely located on the diagram, but strains from the same cheese-making date are very close (Fig. 5). These results indicate that the six strains of *L. monocytogenes* studied do not really differ in their behavior in Camembert cheese. Factors affecting the growth of *L. monocytogenes* seem to be linked to the cheese production (i.e. milk sample and technological parameters). In Figure 5, the values indicated on Factors 1 and 2 (or axes x and y) are the coordinates of each point in the map designed by Factors 1 and 2.

4. DISCUSSION

4.1. Analysis of the eight milk samples used to evaluate effect of LPS in milk

The milk samples tested throughout this study show low lactoperoxidase concentrations compared with values from the literature (30 to 70 $\mu g \cdot mL^{-1}$, [1, 7]). Lactoperoxidase concentration is linked to lactation period [32]. Thiocyanate concentrations in the milk samples fell within the range described in the literature (between 1 and 15 $\mu g \cdot mL^{-1}$; [7, 15]). Thiocyanate concentration in milk

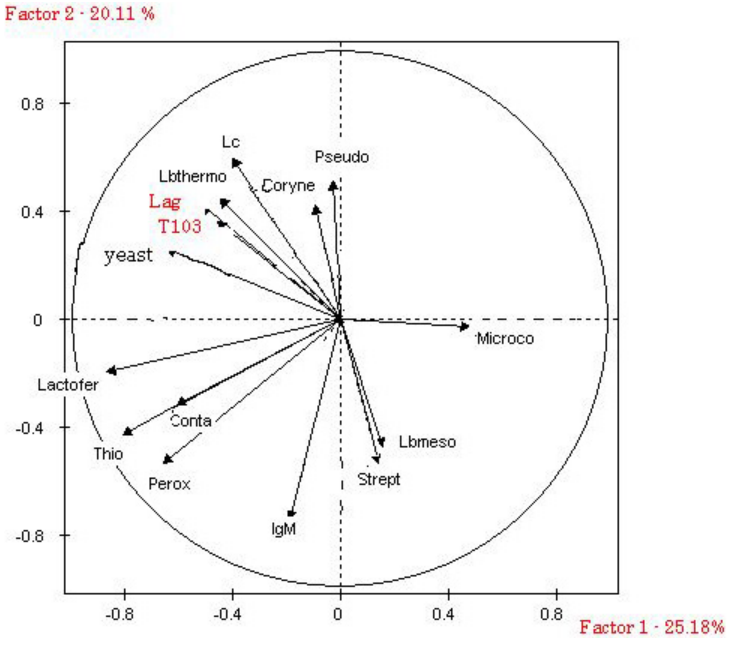

Figure 4. Correlation circle from the principal components analysis (see abbreviations Tab. V).

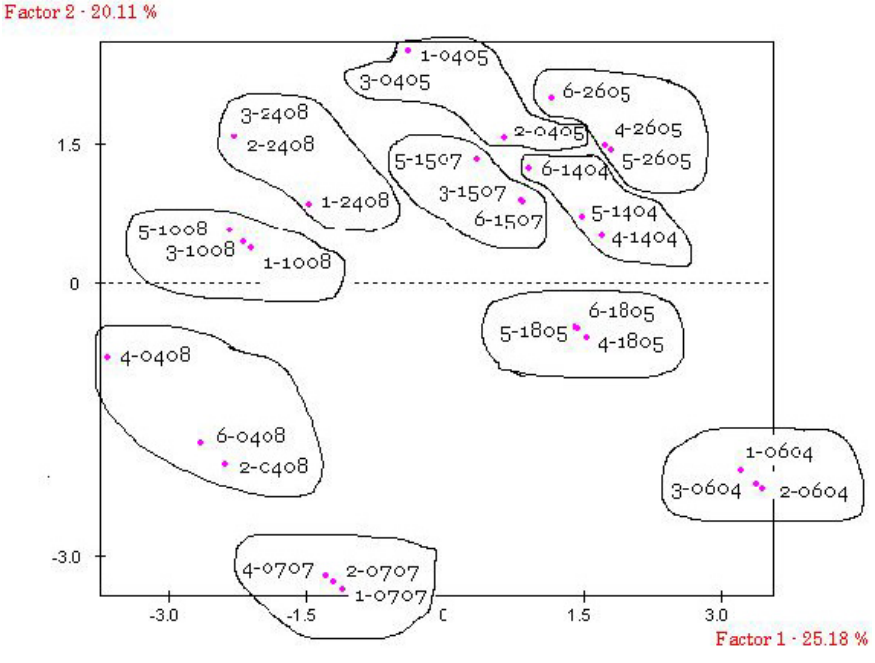

Figure 5. Individuals repartition from the principal components analysis.

depends on the animal's diet. Cruciferae such as cabbage and cauliflower are important sources of thiocyanate in milk.

4.2. Microbiological analysis of the eight milk samples used to evaluate effect of LPS in milk

Raw milk samples' composition varied greatly from sample to sample. For samples B, C and H, the microbiological composition agrees with results described by Champagne et al. [11]: the main psychrotrophic microflora encountered in raw milk was Gram-negative bacteria rods, with *Pseudomonas* comprising at least 50% of the genera. Observations for samples A, D, E, F and G differ from those described in the literature. Although some Gram-positive bacteria were present in the raw milk, they were present in much smaller numbers than the Gram-negative species [11].

4.3. Behavior of *L. monocytogenes* in milk

Growth of *L. monocytogenes* depended not only on temperature but also on milk composition. At 3 °C for 65 h, the *L. monocytogenes* population increase for strains 3 and 4 in pasteurized milk was very low (less than 1 log). No growth was observed in raw milk or SRM (Fig. 2B and 2C). At 9 °C for 65 h, all strains except strain 3 were able to grow in pasteurized milk (Fig. 2A). In raw milk, only strain 1 was able to grow (Fig. 2B) and in SRM growth was inhibited (Fig. 2C). At 15 °C for 65 h, population growth of *L. monocytogenes* strains in pasteurized milk was between 2.7 log and 3.8 log (Fig. 2A). In raw milk it was between 0.8 log and 2.1 log, and in SRM the maximum increase was 1.3 log. *L. monocytogenes* grew about twice as fast in pasteurized milk as in raw milk.

Pitt et al. [33] studied the effect of raw milk on one strain of *L. monocytogenes*. The pasteurized and raw milk samples used were stored at 37 °C for 72 h. The initial *L. monocytogenes* concentration was 10^4 cfu·mL^{-1}. Their results showed that *L. monocytogenes* was able to grow to almost 10^7 cfu·mL^{-1} in raw milk and 10^8 cfu·mL^{-1} in pasteurized milk after 16 h of incubation. During the remainder of the experimental period, the *L. monocytogenes* population in pasteurized milk declined to less than 10^5 cfu·mL^{-1}, while in raw milk *L. monocytogenes* became undetectable after 56 h of incubation at 37 °C. The effect of raw milk on *L. monocytogenes* could result from inhibitory products produced by activation of the milk LPS. In this study [33], raw milk had a bactericidal effect on *L. monocytogenes*. Our results show only an inhibitory effect of raw milk on *L. monocytogenes*. This could be due to the difference in incubation temperature, as in raw milk the LPS is activated by H_2O_2-producing lactic acid bacteria (H_2O_2-LAB) that are naturally present in raw milk. Production of H_2O_2 by H_2O_2-LAB may be faster at 37 °C than at 15 °C , related to the growth of these organisms.

Our results (especially with strain 1) agree with those obtained by Gaya et al. [18]. In that study, *L. monocytogenes* strain Scott A was unable to grow for 3 d in raw milk at 4 °C and 8 °C. In raw milk supplemented with thiocyanate and hydrogen peroxide, *L. monocytogenes* strain Scott A population decreased by 0.2 log and 0.7 log after 3 d at 4 °C and 8 °C, respectively.

Some authors [8, 35, 42, 44] have studied the effect of LPS on *L. monocytogenes* in sterilized skim milk at 20 °C [42], at 25 °C [8] and at 30 °C [44]. Their results show an inhibitory effect of the LPS on *L. monocytogenes*. At 20 °C, without LPS, *L. monocytogenes* strain Scott A populations had increased by about 6.2 log after 68 h [42]. In skim milk with LPS, the population increase was 2.9 log after 68 h. The difference in population increase between the control and activated skim milk was 3.3 log. Our results show a difference in population increase between pasteurized milk and SRM of 2.3 log at 15 °C. At 25 °C [8], addition of LPS inhibited the growth of *L. monocytogenes* strain ATCC 15313; no growth was observed for 50 h. At 30 °C, addition of lactoperoxidase inhibited the strain Scott A for 24 h (no growth observed). LPS also inhibited the *L. monocytogenes* strain Ohio at 30 °C, with a difference in population increase between activated and control milk of 3 log after 24h [44].

Results obtained by Garcia-Graells et al. [16] also showed that adding LPS inhibited the growth of *L. innocua* in milk: no growth was observed for 24 h at 20 °C.

Most earlier studies have demonstrated an inhibitory effect of LPS at mild temperatures (between 20 °C and 37 °C). Our results indicate an inhibitory effect of raw milk on *L. monocytogenes* at 3 °C, 9 °C and 15 °C. As SRM inhibits *L. monocytogenes* growth more strongly than raw milk, the inhibitory effect of raw milk could be linked to the LPS.

4.4. Behavior of *L. monocytogenes* in Camembert cheese

Studies on the behavior of *L. monocytogenes* in soft cheeses made from pasteurized milk have been published [4, 26, 39]. Maisnier-Patin et al. [26] studied the growth of *L. monocytogenes* strain V7 in pasteurized milk Camembert. The concentrations of *L. monocytogenes* in milk were 10^5 and 10^1 cfu·mL^{-1}. The observed lag phases were 7 and 14 d, respectively. In the present study, (with an initial *L. monocytogenes* concentration of 5×10^{-1} cfu·mL^{-1}), average lag values varied from 11 to 19.2 d depending on the strain. The Lag phase observed with a high initial concentration of *L. monocytogenes* (10^5 cfu·mL^{-1}) was shorter than those observed with low initial concentrations [26]. Some studies report the effect of inoculum concentration on the growth of *L. monocytogenes* [3, 12, 17]. Our results differed from one other study [4], where the initial concentration was 3×10^2 cfu·mL^{-1}, lag phase was between 10 and 15 d and population increased 10^3-fold over 40 d. The difference between these and our results may be related to the strains studied, all isolated from raw milk.

Other studies have investigated the behavior of *L. monocytogenes* in cheese made from raw cow's milk [34, 37] and from raw goat's milk [29]. *L. monocytogenes* did not grow in semi-hard raw cheese at 12 °C for 60 d [37]. One study [34] deals with the behavior of *L. monocytogenes* in pasteurized and raw Camembert. Ripening was at 12 °C for 10 d, and the cheeses were then stored at 2 °C for 50 d. The initial concentration of *L. monocytogenes* in the milk was 10^4 cfu·mL^{-1}. The lag phase was between 10 and 30 d in both raw and pasteurized cheese. The population increase at the end of the storage period was 1.84 and 2.08 log (cfu·g^{-1}) in raw and pasteurized cheese, respectively. In this study, the inhibitory effect of raw milk was less significant than our results show. This may be linked to the initial concentration of *L. monocytogenes* used and the microbiological and chemical composition of the raw milk. Morgan et al. [29] studied the behavior of *L. monocytogenes* in soft cheese made from raw goat's milk. Initial concentrations of *L. monocytogenes* in the milk were 10^1 and 10^2 cfu·mL^{-1}. The authors observed that when the initial concentration was 10^2 cfu·mL^{-1}, *L. monocytogenes* did not grow, but survived throughout the 42-day experiment. When the initial concentration was 10^1 cfu·mL^{-1}, *L. monocytogenes* survived for 7 d on the surface and for 42 d inside the cheese. This study showed that the initial concentration of *L. monocytogenes* in milk affects the behavior of the pathogen in cheese. The inhibition of *L. monocytogenes* in raw goat's soft cheese is probably related to pH. In the curd, pH was 4.25; after 21 d it increased to 6 on the cheese's surface. Inside the cheese the pH was less than 5.5 after 42 d. In Camembert cheese made from cow's milk, the curd pH was 4.5, increasing to 6 after 3 d on the surface and 28 d inside the cheese [26]. The behavior of *L. monocytogenes* in determined conditions was not only related to initial bacterial concentration but also to the physiological conditions of the cells [21].

To evaluate the behavior of *L. monocytogenes* in food, the initial concentration used should be as close as possible to concentrations found in naturally contaminated food. This is the case in our study (initial concentration of 0.5 cfu·mL^{-1}; the most probable concentration in collected raw milk is 0.1 cfu·mL^{-1}, [28]).

4.5. Effects of the variables studied on the growth of *L. monocytogenes*

The inhibitory effect of lactic acid bacteria (LAB) is well documented [11, 27,

37]. These studies refer to mesophilic and thermophilic LAB. Our results agreed with these studies, as thermophilic *Lactobacillus* inhibited the growth of *L. monocytogenes*.

Inhibition of *L. monocytogenes* growth by LPS (lactoperoxidase and thiocyanate) and lactoferrin is extensively described in the literature [5, 8, 9, 11, 30, 32, 44]. Bellamy et al. [5] showed that the growth of *L. monocytogenes* was affected by lactoferrin (concentrations between 0.3 and 150 $\mu g \cdot mL^{-1}$). Our results did not clearly demonstrate an inhibitory effect of the chemical composition of raw milk on subsequent growth of *L. monocytogenes* in cheese.

5. CONCLUSION

Our results demonstrate that *L. monocytogenes* grows more slowly in raw and in supplemented raw milk than in pasteurized milk. The inhibitory effect of raw milk is partly due to the LPS. Moreover, an inhibitory effect of RMC compared with PMC was observed. Growth of *L. monocytogenes* in RMC was about twice as long as in PMC. This inhibitory effect was mainly observed in the lag phase (Lag in RMC was 2.3 times as long as in PMC and $T10^3$ in RMC was 1.9 times as long as in PMC). The inhibitory effect of RMC on the growth of *L. monocytogenes* was mainly related to the microbiological composition of the raw milk, in terms of thermophilic *Lactobacillus* and yeast. High yeast concentrations in the raw milk mainly induced an increase in the lag phase. The inhibitory effect of thermophilic *Lactobacillus* was observed on both Lag and $T10^3$. The results obtained suggest that yeast has an inhibitory effect on *L. monocytogenes*. To our knowledge, inhibition of *L. monocytogenes* by yeast has not been previously described. There is a need to investigate the interrelationship between yeast and *L. monocytogenes*. To acquire a more thorough knowledge of the effects of chemical and microbiological factors on the behavior of *L. monocytogenes*, it would be useful to quantify these factors in cheese. Our results did not clearly demonstrate an inhibitory effect of the chemical composition of raw milk on the subsequent growth of *L. monocytogenes* in cheese.

The results of this study clearly demonstrate (i) the inhibitory effect of raw milk on the growth of *L. monocytogenes* and (ii) the inhibitory effect of raw milk cheese on the growth of *L. monocytogenes* compared with pasteurized milk cheese. The inhibitory effect of RMC was mainly related to the microbiological composition of the raw milk.

The inhibition of *L. monocytogenes* in Camembert-type surface-ripened cheese made from raw milk, as shown in the present paper, is probably a contributing factor to the food safety of this cheese [41].

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