Isolation of a bacteriocin-producing *Lactococcus lactis* subsp. *lactis* and application to control *Listeria monocytogenes* in Moroccan jben

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Aim: Use of a bacteriocin-producing lactococcal strain to control *Listeria monocytogenes* in jben.

Methods and Results: A *Lactococcus lactis* strain isolated from jben was shown, by the spot technique, to produce a bacteriocin different from nisin. Inhibitory activity of the bacteriocin-producing strain against *Listeria monocytogenes* was investigated in jben, made from cow’s milk fermented with the producer organism and contaminated with 10⁴ or 10⁷ cfu ml⁻¹. *Listeria* counts were monitored during manufacture, and during conservation at room and at refrigeration temperatures. Results showed that the pathogen was reduced by 2.7 logarithmic units after 30 h of jben processing when the initial inoculum of 10⁷ cfu ml⁻¹ was used. For the initial inoculum of 10⁴ cfu ml⁻¹, the bacterium was completely eliminated at 24 h. Furthermore, the use of the bacteriocin-producing starter culture extended the shelf-life of jben by 5 days.

Conclusions: *In situ* production of the lactococcal bacteriocin is an efficient biological means of controlling *L. monocytogenes* in jben and of allowing shelf-life extension.

Significance and Impact of the Study: The proposed technology will essentially benefit minimally processed dairy products and those made with raw milk.

INTRODUCTION

Jben is a popular Moroccan fresh cheese made with cow’s or goat’s raw milk. Its manufacture consists of two main steps: coagulation and whey draining. The inconsistency in jben processes results in a product with a variable hygienic quality, which may be a vehicle for pathogens responsible for serious food-borne diseases such as *Listeria monocytogenes*. Contaminated cheese may present a listeriosis risk even when stored at refrigeration temperature because of the psychrotrophic nature of the pathogen. Such risk is of more concern when cheeses are sold in the market place at ambient temperature usually exceeding 25 °C, as is the case for jben in Morocco. Also, use of non-pasteurized milk in traditional soft cheese processing, now being authorized in developed countries as a means of maintaining their sensory characteristics, provides the opportunity for *L. monocytogenes* to survive and grow throughout processing and storage.

Although no listeriosis cases have been officially associated with jben in Morocco, warnings concerning the occurrence of *L. monocytogenes* in the product have recently been addressed to a number of Moroccan dairy plants. Also, the absence of epidemiological studies concerning the disease in the country certainly accounts for the lack of reports on listeriosis cases.

The inhibitory effect of bacteriocins of lactic acid bacteria against *L. monocytogenes* in various food systems is well documented and suggests that bacteriocins have potential as biopreservatives to control *L. monocytogenes* in soft cheeses (Wan et al. 1997). Two main methods were recommended: (i) use of food additives inhibitory to the microorganism, such as nisin (Benkerroum and Sandine 1988; Delves-Broughton 1990), Microgard™ (Al-Zoreky et al.
and pediocin (Pucci et al. 1988), and (ii) use of bacteriocin-producing starter cultures in the fermentation (Berry et al. 1990).

The aim of the present study was to isolate bacteriocin-producing strains from Moroccan food products and to assess their effectiveness in the control of L. monocytogenes in jben when used as starter.

MATERIALS AND METHODS

Bacterial strains and media

Organisms used in this study were maintained as frozen stocks at −20 °C in the presence of 25% glycerol. Working cultures were propagated in appropriate broth media (Table 1).

Bacteria tested for bacteriocin production were isolated from raw milk, lben (a traditional Moroccan raw fermented milked, soured spontaneously and then churned to separate the lben from the butter) and commercial jben. Products were serially diluted in 0.85% saline and 0.1 ml aliquots were surface-plated on M17 agar (Terzaghi and Sandine 1975); they were then incubated for 24 h at 30 °C. After incubation, colonies were randomly selected, grown in MRS broth, Gram stained and tested for catalase production. Gram-positive and catalase-negative strains were tested for antimicrobial activity against Listeria monocytogenes ATCC 7644.

All incubations were at 30 °C unless otherwise indicated.

Bacteriocin activity detection

Bacteriocin producers were screened against L. monocytogenes using the spot method as described by Spelhaug and Harlander (1989). An overnight culture of the test organism grown in MRS broth supplemented with 2.5% yeast extract (MRSY) was diluted 10-fold in 10 mmol l⁻¹ Tris HCl (pH 7.0), and 2 μl aliquots were spotted onto M17 and MRS agar. Plates were incubated for approximately 24 h, until growth was evident, then overlaid with 5 ml Trypticase (Biokar) soft agar (0.7% agar) seeded with 0.1 ml of an overnight culture of L. monocytogenes.

Plates were incubated for an additional 18 h, then checked for clear zones around spots of the putative producers.

One strain was selected for further study and was identified using the following tests: ammonia production from arginine, CO₂ production from glucose in citrate-supplemented (0.2%) milk, dextran production, growth at different temperatures (10, 43 and 44 °C), milk coagulation within 16 h at 22 °C (fast cultures) and litmus reduction, growth at different pH values, resistance to sodium azide, reduction of triphenyltetrazolium chloride (TTC), growth

Table 1: Indicator bacteria, their origin and culture media used for their growth

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>Medium*</th>
<th>Sensitivity (Lc. lactis CCMM/IAC/BK2)</th>
<th>Nisaplin™</th>
</tr>
</thead>
<tbody>
<tr>
<td>Listeria monocytogenes</td>
<td>ATCC7644</td>
<td>TS†</td>
<td>++ +</td>
<td>++ +</td>
</tr>
<tr>
<td>Staphylococcus aureus SAD30</td>
<td>IAV Hassan II</td>
<td>TS</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Escherichia coli V517</td>
<td>OSU</td>
<td>TS</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Micrococcus flavus NCIB8166</td>
<td>OSU</td>
<td>TS</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lactococcus lactis subsp. lactis J</td>
<td>This study</td>
<td>MRS</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>IAV Hassan II</td>
<td>TS</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pseudomonas putida</td>
<td>OSU</td>
<td>TS</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Lactobacillus delbrueckii subsp. bulgaricus Y</td>
<td>IAV Hassan II</td>
<td>MRS</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Enterococcus faecium A2</td>
<td>IAV Hassan II‡</td>
<td>MRS</td>
<td>++ +</td>
<td>++ +</td>
</tr>
<tr>
<td>Lactococcus lactis subsp. lactis CCMM/IAC/BK2</td>
<td>This study‡</td>
<td>MRS</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Streptococcus salivarius subsp. thermophilus B</td>
<td>IAV Hassan II‡</td>
<td>MRS</td>
<td>–</td>
<td>++ +</td>
</tr>
</tbody>
</table>

‡ Spot diameter < diameter of inhibition zone ≤ 2 × spot diameter. ++ + : Diameter of inhibition zone ≥ 2 × spot diameter. –: No inhibition. ND = Not determined.

OSU = Oregon State University (Corvallis, USA). IAV Hassan II = Institut Agronomique et Vétérinaire Hassan II Rabat (Morocco).

⁎ Media were broth in the case of propagation and agar slants for storage.

†TS = Trypticase Soy.

‡ Deposited at the Collections Coordonnées de Micro-organismes Marocains (CCMM).

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at different NaCl concentrations, resistance to heat treatment (63°C, 30 min) and diacetyl production.

**Elimination of organic acids and hydrogen peroxide as inhibitory agents**

The effect of organic acids was eliminated by adjusting the pH of test supernatant fluids to 6.0 with 10 mol l⁻¹ NaOH. Neutralized supernatant fluids were filter-sterilized with a 0.22 μm Millipore filter membrane and tested by the well diffusion assay (Tagg and McGiven 1971) for persistence of the inhibition zone.

To exclude the effect of hydrogen peroxide, catalase (EC1.11.1.6, Sigma) was incorporated in the overlay agar without enzyme, and buffer with only the enzyme. After boiling, each sample was assayed for bacteriocin activity by a 1:1 mixture of culture supernatant fluid with buffer but concentration of 1 mg ml⁻¹ as described by Barefoot and Klaenhammer 1983).

**Action of proteases**

The cell-free supernatant fluids (CFS) of putative bacteriocin producers adjusted to pH 6 were treated separately with three different proteases. Each enzyme was obtained from Sigma except trypsin, which was from Serva: trypsin (EC 3.4.21.4), z-chymotrypsin (EC 3.4.21.1), pronase E (Type XXV) and pepsin (EC 34.23.1). They were dissolved in phosphate buffer (0-1 mol l⁻¹, pH 6) except for pepsin which was dissolved in HCl solution (0.02 mol l⁻¹, pH 2.0). Enzyme solutions were mixed with CFS (1:1) to a final concentration of 1 mg ml⁻¹. Incubation was at 37°C in a water-bath for 2 h. Then, samples were boiled for 3 min to stop the reactions. Controls included sterile MRS broth, a 1:1 mixture of culture supernatant fluid with buffer but without enzyme, and buffer with only the enzyme. After boiling, each sample was assayed for bacteriocin activity by the well diffusion method.

Nisin (Aplin and Barrett) at a final concentration of 36 × 10⁴ IU ml⁻¹ was used along with the CFS for comparison.

**Mode of action**

The bactericidal mode of action was demonstrated as described previously (Benkerroum et al. 1993). Briefly, M17 soft agar supplemented with glucose (0.5%) and seeded with 0.1 ml of an overnight Listeria culture was poured into a sterile Petri dish and incubated until growth was evident. The putative producer culture was then spotted onto the plate and incubation continued for an additional 24-72 h. Then, plates were checked for lysis zones around the spot.

**Spectrum of action**

The spectrum of activity against different bacteria was determined by the spot technique (Spelhaug and Harlander 1989). Cultures used as indicators are listed in Table 1. Producer micro-organisms were also tested against each other and against themselves.

**Manufacture of jben**

A bacteriocin-producing Lactococcus lactis CCMM/IAV/BK2 isolated from jben in the current study was used as a starter in jben manufacture to assess the effect of in situ production of bacteriocin on L. monocytogenes. Jben made with a non-producer L. lactis J isolated from a commercial sample was used as a control. The non-producer strain was identified and tested for anti-listerial activity, as was done for the producer.

For starter culture preparation, strains Lc. lactis subsp. lactis CCMM/IAV/BK2 and J were propagated in M17 broth at 30°C for 24 h. Flasks containing 90 ml sterile reconstituted milk (11%) were inoculated with 1% of the pre-incubated Lc. lactis CCMM/IAV/BK2 (Bac⁺) or Lc. lactis J (Bac⁻). Inoculated milk was then incubated overnight.

To prepare cheese samples, whole cow’s milk (9 l) was filtered with a strainer to remove hair and other debris, pasteurized at 70°C for 30 min and then cooled to 30°C in iced water. Pasteurized milk was divided into three batches of 31 each. The first batch was inoculated with the Bac⁺ starter culture (3%), and the second and third were inoculated with the Bac⁻ starter culture to serve as controls. Each of two trials of the first and second batches was contaminated with 10 or 0.5 ml of an overnight Listeria culture to give an approximate initial inoculum of about 10⁶ and 10⁵ cfu ml⁻¹, respectively. The third batch was the negative control and hence was not contaminated.

After inoculation and thorough mixing with a sterile glass rod, all trials were held at 30°C in water-baths to acidify. The pH was monitored at regular intervals and when a value of about 6-2 was reached, Chymosin (CHY-MAX Double Strength, Pfizer) diluted 1:19 with cold water was added at a level of 0.44 ml of the diluted solution l⁻¹ milk. The moulding was achieved at a pH value of 4.8–5.0 by transferring the curd into perforated plastic forms and allowing it to drain for 30 h at 30°C. The resulting cheese from each trial was divided into two portions (about 125 g each), placed into plastic boxes, covered with aluminium foil and stored at room (about 22°C) or refrigeration (about 7°C) temperatures.
Analysis of jben

Measurement of pH during fermentation and storage of cheese was done using a Jenway (model 3310, Jenway Ltd, UK) pH meter.

For bacteriological analysis, samples of milk, curd and cheese were taken at regular intervals and serially diluted to determine *L. monocytogenes* and lactococci populations by standard plate count methods. *Listeria* was enumerated on ASLM agar (Al-Zoreky and Sandine 1990) after incubation at 37 °C for 48–72 h. Black and catalase-positive colonies were considered to be *Listeria*. Lactococci were counted on M17 agar (Biokar) after 24–48 h of incubation at 30 °C.

Statistical analysis

All trials of jben making were repeated twice, and each determination was done in duplicate.

Statistical analysis (analysis of variance $\alpha=0.05\%$ and Student $t$-test) of data was done by computations using Statistical Analysis System (SAS, Institute, NC, USA) software.

RESULTS

Detection of LAB inhibitory to *L. monocytogenes*

Nine strains of lactococci isolated from different products exhibited an anti-listerial activity. They were identified according to physiological and morphological tests classically used for LAB identification (Table 2). One strain, designated *Lc. lactis* CCMM/IAV/BK2, was selected for further study for its greater inhibitory activity against *L. monocytogenes* and for being suitable for use as a starter culture in jben manufacture.

Nature of the inhibitory substance produced by *Lc. lactis* CCMM/IAV/BK2

The substance produced by *Lc. lactis* CCMM/IAV/BK2 was neither hydrogen peroxide nor organic acid. The inhibitory activity was not affected by catalase and was retained in neutralized supernatant fluid. Activity was sensitive to proteases (Table 3). The sensitivity pattern differed from nisin in that it was sensitive to $\alpha$-chymotrypsin. Furthermore, the inhibitory substance had a bactericidal mode of action as evidenced by the clearing around the spot of the producer culture on a pre-incubated lawn of the indicator strain (Fig. 1). The inhibitory substance was therefore considered as a bacteriocin, and further characterized by comparison with nisin and other bacteriocins produced by lactococci.

Spectrum of action

Table 1 shows the range of activity of the inhibitory substance against different bacterial strains. It was more active against Gram-positive than against Gram-negative bacteria. Its spectrum of action differed from nisin. Furthermore, the producer strain was inhibited by Nisaplin™.

Jben-making trials

Variations in pH in Bac$^+$ and Bac$^-$ samples were monitored during manufacture, and during storage at room and refrigeration temperatures. The corresponding results are reported in Fig. 2. The pH varied similarly both in Bac$^+$ and Bac$^-$ samples during fermentation ($P > 0.05$), to reach a final value of 4.6 after 30 h (Fig. 2a). During storage, the pH continued to decrease in some samples to attain a value of about 4.2 at 10 days at both storage temperatures. Then it increased in all samples until alteration was evident (Fig. 2b). Such alteration (surface discoloration, growth of

Table 2 Identification of lactic acid bacteria producing bacteriocins isolated from different products

<table>
<thead>
<tr>
<th>Strain</th>
<th>Identification</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCMM/IAV/BK1</td>
<td><em>Lactococcus lactis</em> subsp. <em>lactis</em></td>
<td>Lben</td>
</tr>
<tr>
<td>CCMM/IAV/BK2</td>
<td><em>Lactococcus lactis</em> subsp. <em>lactis</em></td>
<td>Lben</td>
</tr>
<tr>
<td>CCMM/IAV/BK4</td>
<td><em>Streptococcus salivarius</em> subsp. <em>thermophilus</em></td>
<td>Bakery yeast</td>
</tr>
<tr>
<td>CCMM/IAV/BK5</td>
<td><em>Streptococcus salivarius</em> subsp. <em>thermophilus</em></td>
<td>Bakery yeast</td>
</tr>
<tr>
<td>CCMM/IAV/BK8</td>
<td><em>Enterococcus faecium</em></td>
<td>Bakery yeast</td>
</tr>
<tr>
<td>CCMM/IAV/BK11</td>
<td><em>Enterococcus faecium</em></td>
<td>Bakery yeast</td>
</tr>
<tr>
<td>CCMM/IAV/BK3</td>
<td><em>Lactobacillus delbrueckii</em> subsp. <em>bulgaricus</em></td>
<td>Commercial yogurt</td>
</tr>
<tr>
<td>CCMM/IAV/BK7</td>
<td><em>Lactococcus lactis</em> subsp. <em>lactis</em></td>
<td>Lben</td>
</tr>
<tr>
<td>CCMM/IAV/BK6</td>
<td><em>Lactococcus lactis</em> subsp. <em>lactis</em></td>
<td>Lben</td>
</tr>
</tbody>
</table>

moulds and bad smell) was noticed 5 days later in all samples fermented with the Bac\textsuperscript{+} starter culture than in those fermented with the Bac\textsuperscript{−} starter culture when the higher inoculum was used (Fig. 5). In the samples contaminated with the lower level inoculum, the shelf-life extension was noticed only under ambient storage temperature (Fig. 4b).

Table 3  Action of proteolytic enzymes on the inhibitory activity of cell-free supernatant fluids of selected bacteriocin-producing strains against Listeria monocytogenes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Enzyme</th>
<th>(\alpha)-Chymotrypsin</th>
<th>Trypsin</th>
<th>Pronase</th>
<th>Pepsin</th>
<th>Catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCMM/IAV/BK1</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>CCMM/IAV/BK2</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>CCMM/IAV/BK5</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>Nisin</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>ND</td>
<td>R</td>
<td></td>
</tr>
</tbody>
</table>

\(R = \) resistant; \(S = \) sensitive; ND = Not determined.

**Effect of in situ production of bacteriocin on growth of L. monocytogenes**

The effect of bacteriocin produced \emph{in situ} on growth of \emph{L. monocytogenes} in cheeses was determined during fermentation and storage. The population of lactococci was also enumerated in Bac\textsuperscript{+} and Bac\textsuperscript{−} samples.

During fermentation, no significant difference \((P > 0.05)\) between numbers of lactococci in samples fermented with Bac\textsuperscript{+} and Bac\textsuperscript{−} starter cultures was observed (Fig. 3a). They increased steadily in all samples to reach \(2.8 \times 10^9\) cfu g\textsuperscript{-1} at the end of fermentation (30h). In contrast, \emph{L. monocytogenes} behaved differently during the fermentation period when the Bac\textsuperscript{+} or Bac\textsuperscript{−} starter was used, and depending on the initial level of contamination (Fig. 3). The Bac\textsuperscript{+} starter resulted in a reduction of the viable count of \emph{Listeria} by 2.7 log units at the higher challenge level \((10^7\text{ cfu ml}^{-1})\) while in samples with Bac\textsuperscript{−} starter, the number of \emph{Listeria} remained practically constant (Fig. 2b). In samples fermented with the Bac\textsuperscript{+} starter and contaminated with \(10^5\text{ cfu ml}^{-1}\), the bacterium was not detected at 24h of fermentation, whereas in cheese made with the Bac\textsuperscript{−} starter culture, a slight overall increase in \emph{Listeria} numbers (about 0.5 log units) was noted (Fig. 3a).

Results of viable counts of \emph{Listeria} during storage of cheese made from milk initially contaminated with \(10^4\) or \(10^7\text{ cfu ml}^{-1}\) are summarized in Figs 4 and 5, respectively. These results showed that no \emph{Listeria} were resuscitated from Bac\textsuperscript{+} samples initially contaminated with \(10^4\text{ cfu ml}^{-1}\) during storage at both temperatures, while in the Bac\textsuperscript{−} samples, growth of the pathogen was only slightly reduced (Fig. 4). At the contamination level of \(10^7\text{ cfu ml}^{-1}\), \emph{Listeria} was not eliminated from any of the samples after 15 and 20 days of storage for the Bac\textsuperscript{−} and Bac\textsuperscript{+} cheeses, respectively (Fig. 5). In fact, \emph{Listeria} populations decreased within the first 10 days of storage in all samples, and the destruction rate was higher in Bac\textsuperscript{+} than in Bac\textsuperscript{−}.

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Thereafter, re-growth of the pathogen occurred in cheeses initially contaminated with the higher level and stored at room temperature (Fig. 5b).

**DISCUSSION**

*Lactococcus lactis* CCMM/IAV/BK2 isolated from lben was found to produce an inhibitory substance fitting the definition of a bacteriocin (Klaenhammer 1988). The sub-

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**Fig. 2** pH variations of lben during processing (a) and storage (b) at refrigeration (☐, ■) or at room (○, ●) temperatures. Milk was fermented with Bac⁺ (solid symbols) or Bac⁻ (open symbols) samples. Thereafter, re-growth of the pathogen occurred in cheeses initially contaminated with the higher level and stored at room temperature (Fig. 5b).

**Fig. 3** Viable count of *Listeria monocytogenes* (○, ●) and lactococci (☐, ■) during fermentation of lben with Bac⁺ (solid symbols) or Bac⁻ (open symbols) starter culture. (a) Initial level of contamination: $10^4$ cfu ml⁻¹; (b) initial level of contamination: $10^7$ cfu ml⁻¹.
stance shares many properties with nisin but differs from it by two main characteristics: spectrum of action and sensitivity to proteases, namely \( \alpha \)-chymotrypsin, to which nisin is resistant. It needs to be pointed out, however, that there

Fig. 4 Viable counts of *Listeria monocytogenes* (○, ●) and lactococci (■, □) during storage of jben from milk contaminated with \( 10^5 \) listeriae ml\(^{-1} \) and fermented with a Bac\(^{+} \) (solid symbols) or Bac\(^{-} \) (open symbols) starter culture. (a) Storage at refrigeration temperature (about 7 °C); (b) storage at room temperature (about 22 °C)

Fig. 5 Viable counts of *Listeria monocytogenes* (○, ●) and lactococci (■, □) during storage of jben from milk contaminated with \( 10^5 \) listeriae ml\(^{-1} \) and fermented with a Bac\(^{+} \) (solid symbols) or Bac\(^{-} \) (open symbols) starter culture. (a) Storage at refrigeration temperature (about 7 °C); (b) storage at room temperature (about 22 °C)
are conflicting data about the sensitivity of nisin to \( \alpha \)-chymotrypsin; some authors found it sensitive (Hurst 1983) while others demonstrated its resistance (Daeschel and Klaenhammer 1985). In the present study, nisin (Aplin & Barrett) was resistant to \( \alpha \)-chymotrypsin. The fact that the producer strain is sensitive to nisin provides additional evidence that the bacteriocin produced by the strain isolated in this work is not nisin, as structural (nis A) and immunity (nis I) genes are located on the same operon (Venema et al. 1995). Geis et al. (1983) separated the inhibitory substances produced by \( Lc. \) lactis strains into three types on the basis of their inhibitory spectra and chemical properties. Our bacteriocin would not fit into any of these types. However, it would fit into the subclass IIA as defined by the latest classification of bacteriocins of lactic acid bacteria (Cenatiempo et al. 1996). In effect, the bacteriocin is antilisterial with a narrow spectrum of action and a molecular weight lower than 10 kDa as it passes through dialysis tubing of 8 kDa cut-off (data not shown). However, for more confirmation, the amino acid sequence needs to be established to determine the characteristic sequence of the subclass (i.e., YGNGVXC).

The total dry matter (TDM) of cheese samples after draining, and the pH decrease during cheese making, showed that the bacteriocin producing strain (\( Lc. \) lactis CCMM/IAV/BK2) was a suitable starter for cheese manufacture. No significant difference \((P > 0.05)\) between Bac\(^+\) and Bac\(^-\) samples was noted in pH drop during fermentation. The mean TDM of around 35\% met Moroccan regulatory standards for soft cheeses \((>23\%)\) and was consistent with those reported by other workers for jben (Ennahdi 1980; Bayi 1990). This value also indicates that both Bac\(^+\) and Bac\(^-\) starter cultures acidified milk sufficiently to allow good draining.

The inhibitory effect of the bacteriocin produced by \( Lc. \) lactis subsp lactis CCMM/IAV/BK2 against \( L. \) monocytogenes in the fresh cheese (jben) was dependent on the challenge level. The pathogen was completely destroyed in less than 24 h in Bac\(^+\) samples initially loaded with \( 10^4 \) cfu ml\(^{-1}\) while in samples contaminated with a higher level \((>10^3 \) cfu ml\(^{-1}\)) it survived through the whole period of storage at refrigeration and abuse temperatures (Fig. 5). Although, in the latter case, a decrease in \( Listeria \) counts was observed in all samples, there is no guarantee that the pathogen will eventually disappear. In fact, it can even resuscitate, resulting in an active re-growth as was shown here (Fig. 5b) and elsewhere (Motlagh et al. 1992; Wan et al. 1997). Such re-growth may be attributed to the emergence of resistant strains or to the inactivation of the bacteriocin by proteolytic enzymes. The development of resistant mutants of \( Listeria \) has been reported to occur for pediocin (Pucci et al. 1988), piscicolin (Ming and Daeschel 1993; Wan et al. 1997) and carnocin (Mathieu et al. 1994). According to Harris et al. (1991), nisin-resistant mutants occur at frequencies of \( 10^{-6} - 10^{-8} \). Such an observation may, in part, explain the re-growth upon an extended incubation period and when high levels of inoculation \((10^6 - 10^8 \) cfu ml\(^{-1}\)) are used. As for the action of microbial proteases on bacteriocins, Wan et al. (1997) showed that Camembert starter bacteria and moulds were responsible for the inactivation of piscicolin 126. Also, nisin-resistant strains of \( Staphylococcus \) aureus have been shown to produce a nisinase, a proteolytic enzyme which specifically inactivates the bacteriocin (Hurst 1981). Growth of proteolytic contaminants in our samples, as suggested by the pH increase starting from day 10 of storage, may account for such inactivation of the bacteriocin produced by \( Lc. \) lactis CCMM/IAV/BK2.

The challenge level used in the present study is regarded as very high considering the actual level of \( Listeria \) encountered in dairy products. Surveys have revealed that the level of \( L. \) monocytogenes in raw milk is generally less than 1 cfu ml\(^{-1}\) (Hayes et al. 1986). Therefore, the complete removal of listerias from samples contaminated at a challenge level of \( 10^3 \) cfu ml\(^{-1}\) within 24 h may be considered as a good performance of \( Lc. \) lactis CCMM/IAV/BK2 as an anti-listerial bacteriocin-producing strain. In this regard, some studies have shown that growth of \( Lc. \) monocytogenes was only reduced at a level of \( 10^3 \) cfu ml\(^{-1}\) in taleggio (Giraffa and Carminati 1997) and in milk (Parente and Hill 1992) in the presence of bacteriocinogenic strains.

The decline in \( Listeria \) counts during cheese manufacture with the Bac\(^+\) starter could be attributed to acid production. One advantage of using the Bac\(^+\) starter culture was that bacteriocin production, in combination with other fermentation end products, increases the inhibition of \( L. \) monocytogenes. The difference in the recovery of \( Listeria \) in the Bac\(^+\) and the Bac\(^-\) samples reflects the synergistic effect of lactic acid and bacteriocin on the pathogen.

The use of Bac\(^+\) starter allowed a 5 day extension of the shelf-life of fresh cheese at refrigeration as well as at room temperature. It should be emphasized, however, that such an extension might be possible only if proper sanitation and processing conditions are used to avoid contamination with resistant spoilage micro-organisms, such as Gram-negative bacteria, moulds and yeasts.

From the present study, it may be concluded that bacteriocin production \( in \) \( situ \) gives a protection factor against \( L. \) monocytogenes and may even prevent post-processing contamination of jben, especially during the first days of storage. In the new generation of minimally-processed dairy products, or those made with raw milk, it may be used as an additional hurdle along with acidification, refrigeration and/or other food grade additives, to inhibit or minimize microbial growth. In Third World countries where such products are usually made from raw milk and
stored at abuse temperature, it is particularly recommended to use the BacT starter to alleviate listeriosis risks.

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