Research Note

Behavior of *Listeria monocytogenes* during the Manufacture, Ripening, and Cold Storage of Afuega’l Pitu Cheese

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ABSTRACT

Afuega’l Pitu is an artisanal acid-coagulated cheese manufactured in Asturias (northern Spain) and mainly consumed between the third and the 30th day of ripening. Six cheese-making trials were performed in a pilot plant by using pasteurized whole milk inoculated with *Listeria monocytogenes* (strain L2 [serotype 1/2a], L39, or L41 [serotype 4b]) to ca. 2.7 log CFU/ml. A starter containing three strains, *Lactococcus lactis* subsp. *lactis* IPLA 947, *Lactococcus lactis* subsp. *diacetylactis* IPLA 838, and *Leuconostoc citreum* IPLA 616, grown separately in milk and combined in the volumetric proportion 3:1:1.3 was used. During the acidification *L. monocytogenes* counts increased 2.78- to 7.03-fold, depending on the strain, and remained within the curd; from this time counts decreased abruptly and were not detected in cheeses beyond the 7th day. The average pH in the curd was 4.43, and it decreased to around 4.0 in 5- to 7-day-old cheeses. These pH values were near the tolerance limit for *L. monocytogenes* and probably caused cell damage. Although moisture, a<sub>r</sub>, and NaCl levels were not limiting for the growth and survival of *L. monocytogenes*, salt content must be considered as a contributing factor in *L. monocytogenes* inactivation. Finally, the L2 strain grew better in curd and was slightly more resistant to low pH and refrigeration than strain L39 or L41. The manufacture of Afuega’l Pitu cheese from pasteurized milk and the design of a specific starter from the autochthonous lactic microbiota can lead to a safer product that can be consumed after very short ripening periods.

Key words: Cheeses, *Listeria monocytogenes*, manufacture, ripening

*L. monocytogenes* in cheese has been responsible for several outbreaks and sporadic cases of listeriosis in North America and Europe (12, 22, 32). Raw milk and postprocessing contamination is assumed to be the major source of contamination with this microorganism. A large number of publications deal with the behavior of *L. monocytogenes* in cheeses made from artificially inoculated milk, proving that growth and survival of this pathogen during cheese processing largely varied depending on the type of cheese (3, 5, 10, 16, 20, 21, 24, 25, 28, 30, 31). However, most cheese-related outbreaks demonstrated that surface-ripened soft cheeses generally represent a greater risk of pathogen transmission than do other cheeses. Poor acidification and short ripening periods are also risk factors that may favor *Listeria* stability and multiplication in cheeses (32). On the other hand, being a psychrotrophic microorganism may also favor the survival and growth of *L. monocytogenes* during cheese cold storage (4, 26, 31).

Afuega’l Pitu is an artisanal acid-coagulated cheese manufactured in Asturias (northern Spain) from raw milk; it is consumed over a period of 3 to 30 days (9). Recently, we reported that a high percentage of Afuega’l Pitu samples collected from several supermarkets in our region were contaminated with *L. monocytogenes* (17). The purpose of this study was to examine the potential of *L. monocytogenes* to grow and survive during manufacture, ripening, and cold storage of Afuega’l Pitu cheese.

MATERIALS AND METHODS

Bacterial cultures

Three *L. monocytogenes* strains were used separately in this study: L2 (serotype 1/2a, Afuega’l Pitu isolate), L39 (serotype 4b, Peñamellera 1 isolate) and L41 (serotype 4b, Afuega’l Pitu isolate) (unpublished data). Strains were cultured on tryptone soya agar and subcultured in tryptone soya broth (TSB) (Adsa-Micro, Barcelona, Spain) at 30°C. Cultures were washed with sterile distilled water, dispensed in ca. 200 ml of 11% pasteurized reconstituted skim milk (RSM) and added to pasteurized whole milk in the precise amount to obtain ca. 2.7 log *L. monocytogenes* CFU/ml of milk.

The starter culture used for cheese making contained three strains: *Lactococcus lactis* subsp. *lactis* IPLA 947, *Lactococcus lactis* subsp. *diacetylactis* IPLA 838, and *Leuconostoc citreum* IPLA 616, which were grown separately in milk and combined in the volumetric proportion 3:1:1.3. Lactococcal and *L. citreum* strains were propagated twice overnight in RSM at 30°C. The starter culture was prepared by mixing individual RSM cultures in the proportion described above to provide a 1% final inoculum (vol/vol).
Biochemical analyses

The pH, NaCl content, moisture, and water activity (aw) were determined as previously described (17). NaCl content was expressed as percentage of salt in moisture.

Manufacture and ripening of Afuega' l Pitu cheese

Three duplicate batches of the truncated-cone-shaped variety of Afuega' l Pitu cheese (9) were manufactured, each containing one of the three L. monocytogenes strains used in this study at a concentration of 2.7 log CFU/ml. Thirty-eight to 40 liters of pasteurized whole milk heated at 63°C for 30 min and cooled at 22°C were inoculated with the starter culture and the corresponding L. monocytogenes strain. Calf rennet (0.0025%) was added 2 h after the starter addition. At the end of the coagulation process (16 h) the mean acidity reached 0.716% ± 0.062% and the whey began to separate from the curd, which was carefully removed with a saucepan. The curd was ladled into truncated-cone-shaped molds and whey was allowed to drain at 15 to 16°C for over 24 h without turning the molds. Two-day-old drained pieces of cheese were then rubbed on the surface with a variable amount of dry salt (2 to 3 g) and molded again in smaller molds without turning them. After 24 h, the pieces of cheese were removed from the small molds and then ripened for a maximum period of 15 days in a well-ventilated room at 15 to 16°C and 85 to 90% relative humidity.

Microbiological analyses during cheese making and ripening

Samples of milk (10 ml), curd, and cheese (10 g) were aseptically taken in duplicate for the enumeration of L. monocytogenes and lactic acid bacteria during cheese making and ripening according to the following schedule: (i) pasteurized inoculated milk, (ii) curd at the end of the coagulation process (ca. 16 h), (iii) 1-day-old cheese, (iv) whey, and (v) 2-, 3-, 5-, 7-, and 15-day-old cheeses. Two types of samples, surface and interior, were taken for analysis. Surface samples consisted of ca. 0.5-cm thick slices from the surface, whereas interior samples were taken from the core of the cheese pieces. Samples were homogenized in 90 ml of a prewarmed sterile solution of 2% sodium citrate in a Stomacher Lab-Blender 400 (Seward Medical, London) for 1 min. Serial dilutions were made in quarter-strength Ringer's solution (Oxoid, Unipath LTD, Basingstore, Hampshire, England) and plated in duplicate on several media. Samples for total plate counts and lactococci were deep plated on PCA and M17-agar (Biokar diagnostics, Beauvais, France), respectively. Samples for total plate counts and Leuconostoc were spread plated on PALCAM agar (Merck, Darmstadt, Germany), respectively. Samples from cheeses aged over 2 days were also subjected to a selective enrichment in LEB (Merck) for 7 days at 30°C, streaked on PALCAM medium and analyzed for the presence of L. monocytogenes (17).

The effect of cold storage on L. monocytogenes was investigated in Afuega' l Pitu cheese at different stages of ripening. Cheese pieces ripened for 2, 3, 5, and 7 days were stored for 1 week in a refrigerated chamber (5 to 6°C). After this time cheese samples were analyzed as described above.

RESULTS AND DISCUSSION

Pilot plant Afuega' l Pitu cheese manufacture

The flavor, texture, and appearance of the curd and interior of pilot cheeses were similar to those of typical artisanal cheeses of the truncated-cone-shaped variety. However, the surface presented a poorly developed creamy layer of yeasts compared to the traditional cheeses. Table 1 shows microbiological and chemical analyses of all six batches only until the 7th day of ripening, since L. monocytogenes was not detected on cheese samples beyond this time (results shown below). The manufacture was reliable and our experimental cheeses were comparable to previous artisanal cheeses made elsewhere (in triplicate) from pasteurized (72°C, 15 s) milk with the same starter mixture (8). In curd and in the interior of 3- and 7-day-old cheeses, the mean pH values of pilot cheeses were 4.43, 4.09, and 4.09, and for the artisanal cheeses 4.46, 4.03, and 4.05, respectively. The mean moisture content in the same samples were 85.9, 68.97, and 61.89 for pilot cheeses and 85.88, 67.37, and 59.62 for artisanal cheeses. The average NaCl content of the interior part of 7-day-old cheeses was 1.11 and 1.39 for experimental and artisanal cheeses, respectively.

Behavior of Listeria monocytogenes during the manufacture and ripening of Afuega' l Pitu cheese

Results concerning the behavior of L. monocytogenes and the changes in pH and NaCl during the manufacture and ripening of Afuega' l Pitu cheese are shown in Figure 1.

**TABLE 1. Microbiological and chemical analysis during pilot Afuega’l Pitu cheese making contaminated with L. monocytogenes**

<table>
<thead>
<tr>
<th>Sample</th>
<th>CFU/g or ml (mean ± SD, n = 6)</th>
<th>Physicochemical parameters (mean ± SD, n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total plate counts</td>
<td>Lactococcus</td>
</tr>
<tr>
<td>Inoculated milk</td>
<td>7.21 ± 0.14</td>
<td>7.12 ± 0.15</td>
</tr>
<tr>
<td>Curd (16 h)</td>
<td>9.61 ± 0.15</td>
<td>9.55 ± 0.07</td>
</tr>
<tr>
<td>Cheese: 1 day</td>
<td>9.78 ± 0.11</td>
<td>9.73 ± 0.10</td>
</tr>
<tr>
<td>Salted cheese:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 days: surface</td>
<td>9.90 ± 0.13</td>
<td>9.73 ± 0.17</td>
</tr>
<tr>
<td>interior</td>
<td>9.94 ± 0.01</td>
<td>9.87 ± 0.07</td>
</tr>
<tr>
<td>3 days: surface</td>
<td>9.40 ± 0.48</td>
<td>9.10 ± 0.46</td>
</tr>
<tr>
<td>interior</td>
<td>9.85 ± 0.16</td>
<td>9.70 ± 0.12</td>
</tr>
<tr>
<td>5 days: surface</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>interior</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7 days: surface</td>
<td>6.71 ± 0.83</td>
<td>6.73 ± 0.90</td>
</tr>
<tr>
<td>interior</td>
<td>7.24 ± 0.47</td>
<td>6.85 ± 0.29</td>
</tr>
</tbody>
</table>

a ND, not determined.
After the coagulation process, *L. monocytogenes* was absent in whey samples whereas counts of the strains L2, L39, and L41 increased in curd 7.03-, 2.78-, and 3.62-fold, respectively. These increases were greater than the concentration factor of dry matter in curd (1.16-fold) indicating that the rise of *L. monocytogenes* counts was not only due to its retention and concentration in curd but also to active multiplication during the acidification process. The higher increase of L2 counts compared to L39 and L41 was probably due to some differences in generation times. Ryser and Marth (27) showed that different *L. monocytogenes* strains had also different generation times in filter-sterilized whey from Camembert cheese. The behavior of *L. monocytogenes* during the coagulation process of Afuega’l Pitu cheese is in agreement with the results reported by other authors. In most cases, they found that the presence of *L. monocytogenes* in milk was followed by the growth of the pathogen to a certain extent during the coagulation process, although it was retarded by lactic starter cultures. *L. monocytogenes* was also concentrated in the curd with only a small fraction of cells appearing in whey (22).

In the batches of Afuega’l Pitu cheese, the pH decreased to 4.43 at the end the coagulation process (16 h) and reached values around 4.0 on the 5th or 7th day of cheese making. During this time, counts of the three *L. monocytogenes* strains decreased abruptly so that the microorganism was not detected beyond the 7th day for strain L2 or the 5th day for strains L39 and L41 by either direct count or selective enrichment. The low pH values in curd and cheeses were near the tolerance limit for *L. monocytogenes* and probably caused cell damage. Although moisture, a*, and NaCl values (Table 1) were not limiting for the growth and survival of *L. monocytogenes* (11), salt content must be considered a contributing factor in *L. monocytogenes* inactivation even though the low pH alone is limiting. Most publications have reported that the minimum pH allowing growth of *L. monocytogenes* is around 4.3 (1, 6, 7, 13, 19) and more recently, Ito and Hutkins (15) indicated that the microorganism would survive under certain conditions at pH 3.5 for 24 h. On the other hand, *L. monocytogenes* inactivation in pilot cheeses may be caused by cell death or may be the result of an inability to detect acid-injured cells by the methods used (2, 23, 29).

As deduced from the results given above, the behavior of *L. monocytogenes* in Afuega’l Pitu cheese varied slightly depending on the strain (Figure 1). Strain L2 reached higher populations than L39 or L41 in curd and was the only one detected, although in very low numbers, in the core of 5-day-old cheeses. These data agree with our previous results which indicate that L2 was more resistant than L39 and L41 to lactic acid (data not shown). Ryser and Marth (25) also reported different susceptibilities of strains during ripening of Camembert cheese: the Scott A and Ohio strains reached populations of 5.11 to 7.01 log CFU/ml after 28 days of ripening whereas strains CA and V7 failed to grow significantly.

Counts of *L. monocytogenes* L2 and L41 were slightly higher in the interior than in the surface of Afuega’l Pitu cheese, probably due to the higher salt concentration and lower moisture percentage in the surface (Table 1). The pH values did not show significant variation between interior and surface samples. During Camembert and Brick cheese ripening, differences of pH values occurred between the
interior and the surface of cheeses. In these cases, the pathogen population was higher in the surface, perhaps because of their nearly neutral pH (18). Although the characteristic cheese-making process of Afuega’l Pitu makes comparison with other cheeses difficult, it is interesting to note that the inability of L. monocytogenes to grow during ripening of Cottage and Feta cheeses was attributed to high levels of lactic acid and low pH values (18, 28). In Feta cheese the pH of 2-day-old cheeses decreased to 4.6 and growth of L. monocytogenes stopped. However, the microorganism survived over 90 days even at the low pH, 4.30, of Feta cheese after the ripening period (20).

In spite of the restrictive environment of Afuega’l Pitu for Listeria development, 41% of samples collected from supermarkets in our region were positive for L. monocytogenes (17). A potential reason for these contradictory results may be that the pilot cheeses were made with a defined and fresh starter culture of high acidifying capacity, whereas the artisanal cheeses bought at the supermarket were made with commercial lyophilized cultures that have a longer lag phase. We do not know the ripening period of cheeses obtained at the supermarket but their mean pH values were at least 0.32 units higher than those of the pilot cheeses (17). On the other hand, repeated contamination with the same or closely related L. monocytogenes strains was detected in the artisanal Afuega’l Pitu cheese during a 2-year period (unpublished data), which could lead to an acid adaptation of L. monocytogenes, enhancing its survival in acidic environments (14). Finally, the consumption of cheeses with extremely short ripening periods may also contribute to the explanation of the presence of L. monocytogenes in the artisanal cheese samples.

Behavior of L. monocytogenes at refrigeration-temperature storage of Afuega’l Pitu cheese

Mechanical refrigeration maintains the temperature at 4 to 5°C, preserving many foods, including dairy products, and preventing the growth of several pathogenic microorganisms. However, the ability of L. monocytogenes to grow at refrigeration temperatures, while other competing microbiota cannot, might select for the pathogen, implying a risk for the consumer. To determine the behavior of L. monocytogenes in refrigerated Afuega’l Pitu cheese, samples aged for 2, 3, 5, and 7 days were stored for 1 week in a room at 5°C. After this period, strains L39 and L41 were not detected in any cheese sample, indicating that they did not survive in Afuega’l Pitu cheese under refrigeration temperatures. However, strain L2 was recovered by selective enrichment from both the surface and interior of one of the 5-day-old samples; due to the few positive samples analyzed, a greater resistance of strain L2 to refrigeration temperatures must be viewed cautiously. It is to be noted that Conner et al. (7) reported the effect of acid on L. monocytogenes inhibition to be greater at high (30°C) than at low (10°C) temperatures.

From the results presented above, it was concluded that the low pH of Afuega’l Pitu cheese creates restrictive environmental conditions that make the survival of L. monocytogenes difficult during manufacture and cold storage. Manufacture of cheeses from pasteurized milk and the design of a specific starter from the autochthonous lactic microbiota, which includes a highly acidifying Lactococcus lactis strain, can lead to the manufacture of a safe product that can be consumed after very short ripening periods (over 7 days).

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LISTERIA MONOCYTOGENES IN AFUEGA'L PITU CHEESE


