Effect of Cheese Water Activity and Carbohydrate Content on the Barotolerance of \textit{Listeria monocytogenes} Scott A

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ABSTRACT

High-pressure processing is an appropriate technique for improving the microbiological safety of packaged ready-to-eat foods. The effect of high-pressure treatment on \textit{Listeria monocytogenes} Scott A inoculated into fresh Hispánico-type cheese and ripe Mahón cheese was investigated. A 3.8-log reduction in the counts of \textit{L. monocytogenes} Scott A in fresh cheese was recorded after 3 min at 400 MPa and 12°C, whereas 12 min under the same conditions was required to obtain a 1-log reduction in ripe cheese. Dry matter values were 48.76% for fresh cheese and 58.79% for ripe cheese, and water activity ($a_w$) values were 0.983 and 0.922, respectively. In dehydrated fresh cheese (58.20% dry matter) in which 5% NaCl was added to achieve a 0.904 $a_w$ value, \textit{L. monocytogenes} Scott A counts were lowered by only 0.4 log after treatment for 10 min at 400 MPa. On the other hand, in a 60:40 mixture of ripe cheese:distilled water with a 0.976 $a_w$ value, the reduction under the same conditions was 3.9 log. Within the $a_w$ range of 0.945 to 0.965, \textit{L. monocytogenes} Scott A barotolerance was significantly higher in fresh cheese than in ripe cheese for equivalent $a_w$ values. Carbohydrate content was higher in fresh cheese than in ripe cheese. The addition of lactose at a concentration of 5 mg/g to an 85:15 mixture of ripe cheese:distilled water did not influence \textit{L. monocytogenes} Scott A barotolerance during treatment for 10 min at 400 MPa. Galactose at a concentration of 5 mg/g had a protective effect during high-pressure treatment, and glucose at a concentration of 5 mg/g favored \textit{L. monocytogenes} Scott A survival during refrigerated storage of pressurized samples at 8°C for 5 days.

\textit{Listeria monocytogenes}, a psychrotrophic pathogenic microorganism, has emerged as one of the major human foodborne pathogens (5). Although the incidence of listeriosis is low and the infection dose is high, illness can be severe in at-risk populations, such as immunocompromised people, elderly people, and fetuses or newborns (16). Substantial information is available concerning factors that affect \textit{L. monocytogenes} growth, such as temperature, pH, water activity ($a_w$), solutes, substrate state, and various combinations of these factors (7, 13, 19).

High-pressure processing (HPP), a nonthermal food preservation procedure, is particularly indicated for the elimination of postprocessing pathogen contamination in foods that might be disagreeably altered by thermal treatment. The barotolerance of \textit{L. monocytogenes} in broth (1, 17) and various food substrates (12, 15) is influenced by factors such as pH and temperature. Stationary-phase \textit{L. monocytogenes} cells are much more resistant to HPP than are exponential-phase cells (9). Also, a significant variability among \textit{L. monocytogenes} strains in response to HPP has been reported (10, 20).

The $a_w$ value of the substrate does affect the resistance of \textit{L. monocytogenes} to thermal treatment and pulsed electric fields (2, 18). A baroprotective effect associated with reduced $a_w$ was demonstrated for yeasts that can grow at reduced $a_w$ values (11). However, information about the effect of $a_w$ on \textit{L. monocytogenes} barotolerance is limited and somewhat contradictory. Reductions of 5 to 7 log in counts of several \textit{L. monocytogenes} strains were achieved by HPP at 600 MPa for 8 min in broth with $a_w = 0.96$ but were $<4$ log in broth with $a_w = 0.92$ (14). On the other hand, \textit{L. monocytogenes} cells grown in broth with $a_w = 0.96$ or higher showed a greater barotolerance than cells grown at lower $a_w$ values (8). In the present study, the effect of HPP on the survival of \textit{L. monocytogenes} in two types of cheese with different $a_w$ values was investigated, and the lethality of \textit{L. monocytogenes} with respect to factors related to substrate composition was also studied.

MATERIALS AND METHODS

Microorganism. \textit{L. monocytogenes} Scott A (CIP 103575) was obtained from the culture collection of Institut Pasteur (Paris, France) and kept frozen at $-80^\circ$C in treptic soy broth supplemented with 0.6% yeast extract (TSBYE; Biolife, Milano, Italy) with 15% glycerol added. A fresh culture of the microorganism in TSBYE at 30°C for 18 h was used to inoculate cheese samples.

Cheeses and HPP. Fresh Hispánico-type cheeses (approximately 250 g) were made as described previously (3) from pasteurized milk inoculated with a 1% lactic starter (MA016, Rhodia, Dangé-Saint-Romain, France) grown in sterile milk. After pressing, cheeses were salted for 1 h in a 15% NaCl brine solution, vacuum packaged, and kept at 4°C for a maximum of 36 h before use in experiments. Ripe (3-month-old) Mahón cheese was bought at a retail store, vacuum packaged, and kept at 4°C until use in experiments.

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To study the effect of the aw value on L. monocytogenes barotolerance in fresh cheese, unsalted fresh cheese was sliced, lyophilized, and mixed with unsalted fresh cheese by hand in stomacher bags to obtain a fresh cheese mixture with the same dry matter content as ripe Mahón cheese. Solid NaCl was added to aliquots of this mixture to obtain concentrations of NaCl that ranged from 0 to 5%. The mixtures were homogenized by hand. To study the effect of the aw value on L. monocytogenes barotolerance in ripe Mahón cheese, ripe cheese:distilled water mixtures in proportions ranging from 90:10 to 60:40 were prepared and homogenized by hand in stomacher bags. To study the effect of carbohydrates on L. monocytogenes barotolerance, solid lactose, glucose, and galactose were each added in individual trials to achieve a final concentration of 5 mg/g in an 85:15 mixture of ripe cheese:distilled water. Mixtures were homogenized by hand in stomacher bags.

Mixtures were vacuum packaged after preparation and kept overnight at 4°C to allow equilibration. On the following day, the aw and pH of the mixtures were determined. Aliquots (20 g) in stomacher bags were inoculated at 1% with a fresh culture of L. monocytogenes Scott A in TSBYE, homogenized by hand, and kept at 8°C. After 24 h at this temperature, HPP was carried out. One aliquot of each mixture was used to determine L. monocytogenes counts before HPP, another aliquot was used to determine counts 30 min after treatment, and a third aliquot was held at 8°C and used to determine counts after 24 h. In the experiment on the effect of carbohydrates on barotolerance, a fourth aliquot was used to determine counts after 5 days at 8°C. Uninoculated aliquots pressurized under the same conditions were used to determine pH and aw values.

HPP was performed in a high-pressure batch apparatus (model ACIP 6000, ACB, Nantes, France) with a 3.5-liter capacity and a 600-MPa maximum working pressure. All treatments were carried out at 12°C in triplicate. The initial temperature of the water that was used as pressure-transmitting fluid was 10°C and did not exceed 13°C during the process. The come-up time to reach 400 MPa was 3.3 min, and the depressurization time was 0.6 min.

Microbiological determinations. Cheese samples were homogenized in sterile 2% sodium citrate solution with a homogenizer (IUL, Barcelona, Spain). Decimal dilutions of the homogenate were prepared in sterile 0.1% peptone water. L. monocytogenes counts were determined on duplicate plates of PALCAM agar (Merck, Darmstadt, Germany) incubated at 37°C for 48 h. In the experiment on the effect of carbohydrates on barotolerance, counts were also determined on duplicate plates of ALOA (Chromogen Listeria) agar (Biomedics, Tres Cantos, Spain) prepared according to the instructions of the manufacturer. Interferences due to the growth of bacteria naturally present in cheese were discarded by plating dilutions of uninoculated samples on both media, PALCAM and ALOA.

For the determination of cell doubling time, L. monocytogenes Scott A was inoculated at approximately 10^6 CFU/ml in peptone aqueous solution (5 mg/ml), with concentrations of 1 mg/ml of filter-sterilized lactose, glucose, or galactose added when convenient. Cultures were incubated at 37°C. L. monocytogenes counts were determined at hourly intervals on duplicate plates of tryptic soy agar supplemented with 0.6% yeast extract and incubated at 37°C for 48 h.

Physicochemical determinations. The aw value was determined in triplicate with a series 3 Aqua Lab Water Activity Meter (Decagon Devices, Inc., Pullman, Wash.). Dry matter was determined in triplicate after drying to a constant weight in a vacuum oven at 100°C. Sodium chloride was determined in triplicate with

<table>
<thead>
<tr>
<th>Fresh cheese treatment (min)</th>
<th>Time at 8°C after treatment</th>
<th>Ripe cheese treatment (min)</th>
<th>Time at 8°C after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.22 G</td>
<td>0</td>
<td>7.13 G</td>
</tr>
<tr>
<td>3</td>
<td>3.40 C</td>
<td>3</td>
<td>7.09 G</td>
</tr>
<tr>
<td>6</td>
<td>2.65 B</td>
<td>6</td>
<td>6.91 FG</td>
</tr>
<tr>
<td>9</td>
<td>1.85 A</td>
<td>9</td>
<td>6.70 EF</td>
</tr>
<tr>
<td>12</td>
<td>6.38 DE</td>
<td>12</td>
<td>6.28 CD</td>
</tr>
<tr>
<td>15</td>
<td>6.20 D</td>
<td>15</td>
<td>5.96 BC</td>
</tr>
<tr>
<td>18</td>
<td>6.13 D</td>
<td>18</td>
<td>5.50 B</td>
</tr>
</tbody>
</table>

* Mean log counts (of triplicate experiments, in PALCAM) of 30-min samples (both cheeses) followed by the same letter are not significantly different (P > 0.05). The same meaning of letters applies to 24-h samples.

To study the effect of the aw value on L. monocytogenes barotolerance in fresh cheese, unsalted fresh cheese was sliced, lyophilized, and mixed with unsalted fresh cheese by hand in stomacher bags to obtain a fresh cheese mixture with the same dry matter content as ripe Mahón cheese. Solid NaCl was added to aliquots of this mixture to obtain concentrations of NaCl that ranged from 0 to 5%. The mixtures were homogenized by hand. To study the effect of the aw value on L. monocytogenes barotolerance in ripe Mahón cheese, ripe cheese:distilled water mixtures in proportions ranging from 90:10 to 60:40 were prepared and homogenized by hand in stomacher bags. To study the effect of carbohydrates on L. monocytogenes barotolerance, solid lactose, glucose, and galactose were each added in individual trials to achieve a final concentration of 5 mg/g in an 85:15 mixture of ripe cheese:distilled water. Mixtures were homogenized by hand in stomacher bags.

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Statistical treatment of data. One-way analyses of variance (SPSS Inc., Chicago, Ill.), with HPP conditions or cheese characteristics, were performed by means of the SPSS Win 9.0 software package. Differences between means were assessed by Tukey’s test with P < 0.05, as calculated by the same program.

RESULTS AND DISCUSSION

Survival of L. monocytogenes after HPP of fresh and ripe cheese. The numbers of surviving pathogen cells after HPP of fresh Hispánico-type cheese and ripe Mahón cheese inoculated with L. monocytogenes Scott A and processed at 400 MPa and 12°C for various times are shown in Table 1. HPP was much more effective in fresh cheese, in which a 3.8-log reduction of L. monocytogenes Scott A counts was recorded after 3 min at 400 MPa and 12°C, than in ripe cheese in which 18 min under the same conditions was required to obtain a 1-log reduction. Counts of L. monocy-
TABLE 2. Characteristics of fresh cheese and ripe Mahón cheese

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Fresh</th>
<th>Ripe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter (%)</td>
<td>48.96</td>
<td>58.79</td>
</tr>
<tr>
<td>NaCl (%)</td>
<td>1.54</td>
<td>3.67</td>
</tr>
<tr>
<td>a_w</td>
<td>0.983</td>
<td>0.922</td>
</tr>
<tr>
<td>Lactose (mg/g)</td>
<td>13.74</td>
<td>ND</td>
</tr>
<tr>
<td>Glucose (mg/g)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Galactose (mg/g)</td>
<td>0.21</td>
<td>0.10</td>
</tr>
</tbody>
</table>

*Mean values of triplicate determinations. ND, below detection limit.

Our study was considerably higher than in ripe Hispánico-type cheese (3), in which reductions of >6 log were recorded after treatment of 50-day-old cheese at 300 MPa for 10 min or at 500 MPa for 5 min.

Differences in a_w values and NaCl content might explain the higher barotolerance of L. monocytogenes Scott A in ripe Mahón cheese, which had higher dry matter and NaCl contents and lower a_w values than fresh Hispánico-type cheese (Table 2). Dry matter and NaCl content of the 50-day-old vacuum-packed Hispánico-type cheese examined in a previous study (3) should be substantially similar to the values obtained for the fresh cheese in the present study, although its a_w value might be slightly lower because of the formation of proteolysis products during ripening.

**Effect of cheese a_w on L. monocytogenes barotolerance.** To elucidate the influence that the a_w value of cheese had on the barotolerance of L. monocytogenes Scott A, the following experiment was conducted. A cheese mixture with 58.20% dry matter (data not shown) and a_w = 0.984 (Table 3) was prepared by mixing freeze-dried unsalted fresh cheese with unsalted fresh cheese. When increasing amounts of NaCl, up to 5% (wt/wt), were added to this cheese mixture, the a_w dropped to as low as 0.904 (Table 3). Variations in cheese pH values due to the addition of water or NaCl were below 0.1 pH (Table 3).

HPP of fresh cheese mixtures at 400 MPa for 10 min decreased L. monocytogenes Scott A counts by 0.4 log when a_w = 0.904 and by as much as 3.5 log when a_w = 0.984 (Table 3). A low a_w value had a clear baroprotective effect when L. monocytogenes Scott A was high-pressure treated in fresh cheese.

The diagrammatic presentation of log counts of surviving pathogens plotted against a_w values of dehydrated fresh cheese samples also seemed to follow a sigmoid curve (data not shown). The log count of L. monocytogenes Scott A after treatment of fresh cheese with a_w = 0.984 was not significantly different from that obtained after treatment of fresh cheese with a_w = 0.967 (Table 3), a fact that discards the influence of the physiological status of the cells on their barotolerance when a_w values rise above a certain value. On the other hand, the log count of L. monocytogenes Scott A after treatment of 50-day-old cheese at 300 MPa for 10 min did not achieve a proportionally higher reduction than fresh cheese, which had a_w = 0.904 (Table 3), a fact that discards the influence of the a_w on the barotolerance of this pathogen.

**TABLE 3. Log counts of Listeria monocytogenes Scott A in unsalted dehydrated (58.20% dry matter) fresh cheese and in ripe Mahón cheese: distilled water mixtures of different a_w values analyzed 30 min after high-pressure treatment at 400 MPa and 12°C for 10 min**

<table>
<thead>
<tr>
<th>Added NaCl (%)</th>
<th>a_w</th>
<th>pH</th>
<th>Log CFU/g</th>
<th>Cheese:water (%)</th>
<th>a_w</th>
<th>pH</th>
<th>Log CFU/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.984</td>
<td>4.96</td>
<td>3.61 BC</td>
<td>0</td>
<td>0.925</td>
<td>5.20</td>
<td>6.12 E</td>
</tr>
<tr>
<td>1</td>
<td>0.967</td>
<td>4.95</td>
<td>3.95 c</td>
<td>10</td>
<td>0.945</td>
<td>5.24</td>
<td>4.10 c</td>
</tr>
<tr>
<td>2</td>
<td>0.956</td>
<td>4.92</td>
<td>5.36 D</td>
<td>20</td>
<td>0.957</td>
<td>5.25</td>
<td>3.21 B</td>
</tr>
<tr>
<td>3</td>
<td>0.943</td>
<td>4.90</td>
<td>6.13 E</td>
<td>30</td>
<td>0.966</td>
<td>5.26</td>
<td>2.29 A</td>
</tr>
<tr>
<td>4</td>
<td>0.921</td>
<td>4.87</td>
<td>6.57 EF</td>
<td>40</td>
<td>0.976</td>
<td>5.29</td>
<td>3.27 B</td>
</tr>
<tr>
<td>5</td>
<td>0.904</td>
<td>4.85</td>
<td>6.69 F</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Mean log counts (of triplicate experiments, in PALCAM) followed by the same letter (comparison of samples from both cheeses) are not significantly different (P > 0.05). Mean log count of L. monocytogenes Scott A in inoculated cheeses before HPP was 7.12 log CFU/g.*
TABLE 4. Log counts of Listeria monocytogenes Scott A in PALCAM and ALOA media for samples analyzed 30 min, 1 day, and 5 days after high-pressure treatment of ripe Mahón cheese (with 15% water and different carbohydrates added) at 400 MPa and 12°C for 10 min<sup>a</sup>

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Log counts in PALCAM medium for HPP-treated samples</th>
<th>Log counts in ALOA medium for HPP-treated samples</th>
<th>Log counts in PALCAM medium for untreated samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min</td>
<td>1 day</td>
<td>5 days</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactose (5 mg/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (5 mg/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galactose (5 mg/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean log counts (of triplicate experiments) in the same column followed by the same letter are not significantly different (P > 0.05). Mean log count of <i>L. monocytogenes</i> Scott A in inoculated cheeses before HPP was 7.31 log CFU/g.

A decreased by only 0.4 log after treatment of fresh cheese with <i>a</i><sub>w</i> = 0.904 at 400 MPa for 10 min. The higher barotolerance recorded in <i.getZygosaccharomyces bailii</i> at low <i>a</i><sub>w</sub> values was attributed to cell shrinkage, inducing a reduction of membrane permeability and fluidity (11).

Ripe Mahón cheese <i>a</i><sub>w</sub> values increased from 0.925 to 0.976 when cheese:water mixtures in proportions of up to 60:40 were prepared (Table 3). This increase in <i>a</i><sub>w</sub> resulted in a decrease in <i>L. monocytogenes</i> Scott A barotolerance. Reductions in counts ranged from 1 log at <i>a</i><sub>w</sub> = 0.925 to 4.8 log at <i>a</i><sub>w</sub> = 0.966 (Table 3).

The decrease in log counts of surviving pathogens in ripe cheese was linear with respect to <i>a</i><sub>w</sub> values from 0.925 to 0.976 when cheese:water mixtures in proportions of up to 60:40 were prepared (Table 3). This increase in <i>a</i><sub>w</sub> resulted in a decrease in <i>L. monocytogenes</i> Scott A barotolerance. Reductions in counts ranged from 1 log at <i>a</i><sub>w</sub> = 0.925 to 4.8 log at <i>a</i><sub>w</sub> = 0.966 (Table 3).

In both fresh and ripe cheeses, the barotolerance of <i>L. monocytogenes</i> Scott A increased as the <i>a</i><sub>w</sub> values increased within the range of 0.925 to 0.965 (Table 3), thus explaining the results obtained in the previous experiment (Table 1). However, within the range of 0.945 to 0.965, the barotolerance of <i>L. monocytogenes</i> Scott A for an equivalent <i>a</i><sub>w</sub> value was significantly higher in fresh cheese than in ripe cheese, with differences in log counts of surviving pathogens of up to 2.2 log for <i>a</i><sub>w</sub> = 0.956. This result is in agreement with data obtained in a previous study at our laboratory (3), in which the effect of HPP on <i>L. monocytogenes</i> Scott A in fresh and ripe Hispánico-type cheeses was compared, and a higher barotolerance was found in the former cheese.

**Effect of carbohydrates on L. monocytogenes barotolerance.** The presence of carbohydrates in fresh cheese might influence both the lethality of <i>L. monocytogenes</i> Scott A during HPP treatment and the recovery of cells sublethally injured by HPP. The influence of cheese carbohydrate content on <i>L. monocytogenes</i> Scott A barotolerance was studied in the following experiment. Analysis of fresh and ripe cheeses for carbohydrates (Table 2) showed the presence of lactose only in fresh cheese and the presence of galactose at low levels in both cheeses; glucose was not detected.

In both fresh and ripe cheeses, the barotolerance of <i>L. monocytogenes</i> Scott A increased as the <i>a</i><sub>w</sub> values increased within the range of 0.925 to 0.965 (Table 3), thus explaining the results obtained in the previous experiment (Table 1). However, within the range of 0.945 to 0.965, the barotolerance of <i>L. monocytogenes</i> Scott A for an equivalent <i>a</i><sub>w</sub> value was significantly higher in fresh cheese than in ripe cheese, with differences in log counts of surviving pathogens of up to 2.2 log for <i>a</i><sub>w</sub> = 0.956. This result is in agreement with data obtained in a previous study at our laboratory (3), in which the effect of HPP on <i>L. monocytogenes</i> Scott A in fresh and ripe Hispánico-type cheeses was compared, and a higher barotolerance was found in the former cheese.

When samples were plated on ALOA 30 min after HPP treatment, <i>L. monocytogenes</i> counts were approximately 0.5 log higher than the respective counts on PALCAM (Table 4). It may thus be estimated that two thirds of the cells forming colonies on ALOA were not able to grow on PALCAM, most probably because of the sublethal injury caused by HPP treatment. <i>L. monocytogenes</i> counts in glucose samples plated on ALOA increased slightly from 30 min to day 1, and afterward, they remained at levels higher than...
in control or lactose samples and not significantly different from those in galactose samples. No growth of _L. monocytogenes_ Scott A occurred during refrigerated storage of untreated samples at 8°C, as shown by counts on PALCAM (Table 4), which were slightly lower on day 5 than on day 0.

The presence of galactose at 5 mg/g resulted in higher _L. monocytogenes_ counts than in control or lactose samples after HPP and throughout storage at 8°C, independent of the enumeration medium, indicating a baroprotective effect of galactose on _L. monocytogenes_ Scott A. On the other hand, the presence of glucose at 5 mg/g had no baro-protective effect during HPP but showed a beneficial effect on the survival of _L. monocytogenes_ Scott A during refrigerated storage following HPP, with significantly higher levels on day 5 for glucose samples than for control or lactose samples, independent of the enumeration medium.

However, the higher barotolerance of _L. monocytogenes_ Scott A in fresh cheese than in ripe cheese at equivalent aw values, within the 0.94 to 0.97 aw range, cannot be ascribed to the carbohydrates present in fresh cheese on the basis of the above results. The concentration of galactose used in our experiment (5 mg/g) was 24-fold that present in fresh cheese (0.21 mg/g); galactose was also present (0.10 mg/g) in ripe cheese. Additionally, no baro-protective effect with respect to lactose on _L. monocytogenes_ that could have explained its higher barotolerance in fresh cheese than in ripe cheese was observed.

_L. monocytogenes_ has various mechanisms of resistance to stress. Exposure to a certain stress may confer resistance to other stresses. Thus, it has been shown that cold-shocked _L. monocytogenes_ cells were 100-fold more baro-resistant than cells growing exponentially at 37°C (22). Also, it has been shown that exposure of _L. monocytogenes_ to pH 4.5 provided protection against HPP by preloading the pathogen cells with σD-dependent general stress proteins (23). Both stresses, i.e., low temperature and acidic conditions, were present in the fresh and ripe cheeses used in our experiments. Therefore, these stresses cannot, by themselves, explain the differences in _L. monocytogenes_ Scott A baroresistance found between fresh and ripe cheeses.

The adaptability of _L. monocytogenes_ to osmotic stress seems to depend on the ability of the microorganism to accumulate solutes, such as amino acids, peptides, betaine, and carnitine (6). It has been suggested that the di- and tripeptide transport system found in _L. monocytogenes_ Scott A, with its high affinity for proline-containing peptides, plays an important role in osmoregulation (21). The higher barotolerance of _L. monocytogenes_ Scott A in fresh cheese than in ripe cheese could be due to the different accumulations of stress-related solutes by the pathogen in the two substrates.

**ACKNOWLEDGMENT**

Financial support of this research by INIA project OT 002-04 is acknowledged.

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