High Pressure Homogenization versus Heat Treatment: Effect on Survival, Growth, and Metabolism of Dairy Leuconostoc Strains

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ABSTRACT

The effect of high pressure homogenization (HPH) with respect to a traditional heat treatment on the inactivation, growth at 8°C after treatments, and volatile profile of adventitious Leuconostoc strains isolated from Cremoso Argentino spoiled cheeses and ingredients used for their manufacture was evaluated. Most Leuconostoc strains revealed elevated resistance to HPH (eight passes, 100 MPa), especially when resuspended in skim milk. Heat treatment was more efficient than HPH in inactivating Leuconostoc cells at the three initial levels tested. The levels of alcohols and sulfur compounds increased during incubation at 8°C in HPH-treated samples, while the highest amounts of aldehydes and ketones characterized were in heated samples. Leuconostoc cells resuspended in skim milk and subjected to one single-pass HPH treatment using an industrial-scale machine showed remarkable reductions in viable cell counts only when 300 and 400 MPa were applied. However, the cell counts of treated samples rose rapidly after only 5 days of storage at 8°C. The Leuconostoc strains tested in this work were highly resistant to the inactivation treatments applied. Neither HPH nor heat treatment assured their total destruction, even though they were more sensitive to the thermal treatment. To enhance the inhibitory effect on Leuconostoc cells, HPH should be combined with a mild heat treatment, which in addition to efficient microbial inactivation, could allow maximal retention of the physicochemical properties of the product.

Some species of Leuconostoc are very relevant in the dairy industry. Leuconostoc bacteria use lactose and citrate, producing lactic acid, acetate, CO2, ethanol, acetaldehyde, diacetyl, acetoin, and 2,3-butane diol, which contribute to the sensory (flavor and texture) characteristics of butter and cream and allow the openings in some soft and semihard cheeses (Edam and Gouda cheeses) (9, 18, 30). However, the production of CO2 and certain flavor compounds by Leuconostoc is not well received in other types of cheeses, where it leads to diminished quality of the product. The presence of Leuconostoc bacteria in milk and dairy products as adventitious microflora could be the consequence of contamination during milk collection and/or during the manufacture of the products. This could be favored by the great ability of Leuconostoc to survive on surfaces, tools, and pasteurizers for long periods of time, and in adverse environments as well. Wild-type Leuconostoc strains are also reported to be heat resistant and able to survive pasteurization (17). Many adventitious Leuconostoc strains were recently isolated and found to be the main reason for blowing defects in Cremoso Argentino cheeses (22). To face this problem, some dairy industries subject milk to a stronger thermal treatment. However, excessive thermal treatments can negatively affect the yield and sensory quality of cheeses as a consequence of protein denaturation, nonenzymatic browning, and loss of vitamins and volatile flavor compounds.

In recent years, the development of alternative, nonthermal processes has received considerable attention, since they combine efficient microbial reduction with a maximal retention of the chemical and physicochemical properties of products. Among them, one of the most interesting is high pressure homogenization (HPH) (4, 11, 21, 31). HPH technology can be used as an alternative to pasteurization for improved safety and better microbiological quality of many products, including milk, fermented milks, and cheeses (7, 13, 14, 19, 20, 29). Studies based on HPH technology have been mostly focused on the inactivation of more common spoilage and pathogenic microorganisms (5, 16, 24, 28, 31). Information concerning the effect of HPH on Leuconostoc bacteria is almost nonexistent, probably because the genus is traditionally known for its positive role in the dairy field (31).

The principal aim of this work was to evaluate the effect of HPH in comparison to the effect of traditional heat
treatment on the inactivation, growth after treatment, and volatile profiles of adventitious Leuconostoc strains isolated from Cremoso Argentino spoiled cheeses and ingredients used for their manufacture. In particular, the inactivation effectiveness of HPH was studied in relation to the suspension media and inoculation levels and the performance of laboratory-scale and industrial homogenizers was compared.

MATERIALS AND METHODS

Strains and growth conditions. Fourteen Leuconostoc strains previously isolated at the Instituto de Lactología Industrial (INLAIN, UNL-CONICET, Facultad de Ingeniería Química, Santa Fe, Argentina) (Table 1) and characterized at the biochemical, genetic, and technological levels (2) were used in this study. They were routinely grown until stationary stage (overnight, 16 to 18 h) in de Man Rogosa Sharpe (MRS; Oxoid, Basingstoke, Hampshire, England) broth at 32°C and stored at −80°C in MRS supplemented with glycerol (15%, vol/vol). For the enumeration of Leuconostoc strains, MRS agar was used, and the plates were incubated in microaerophilic atmosphere at 32°C for 48 h.

HPH treatments. For laboratory-scale trials, a PANDA continuous high pressure homogenizer (Niro Soavi, Parma, Italy), previously sterilized according to the manufacturer’s recommendations, was used for all the homogenizing treatments. The machine was supplied with a homogenizing pressure relief–type valve with a flow rate of 10 liters/h. The valve assembly included a ball-type impact head made of ceramic, a stainless steel large- inner-diameter impact ring, and a tungsten carbide passage head. A machine was supplied with a homogenizing pressure relief–type valve with a flow rate of 10 liters/h. The valve assembly included a ball-type impact head made of ceramic, a stainless steel large- inner-diameter impact ring, and a tungsten carbide passage head. A water refrigeration system (Niro Soavi) was used to counterbalance inner-diameter impact ring, and a tungsten carbide passage head. A water refrigeration system (Niro Soavi) was used to counterbalance inner-diameter impact ring, and a tungsten carbide passage head. A machine was supplied with a homogenizing pressure relief–type valve with a flow rate of 10 liters/h. The valve assembly included a ball-type impact head made of ceramic, a stainless steel large- inner-diameter impact ring, and a tungsten carbide passage head.

TABLE 1. Identification and origin of Leuconostoc strains used in this study

<table>
<thead>
<tr>
<th>Classification and strain</th>
<th>Origin, isolation date (mo/yr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. mesenteroides subsp. mesenteroides</td>
<td>D2 Cremoso Argentino cheese, 06/2008</td>
</tr>
<tr>
<td></td>
<td>D3 Pasteurized milk, 07/2008</td>
</tr>
<tr>
<td></td>
<td>D4 Pasteurized milk, 07/2008</td>
</tr>
<tr>
<td></td>
<td>D8 Whey cream, 09/2008</td>
</tr>
<tr>
<td></td>
<td>D10 Whey protein concentrate, 10/2008</td>
</tr>
<tr>
<td></td>
<td>D11 Cremoso Argentino cheese, 10/2008</td>
</tr>
<tr>
<td>L. pseudomesenteroides</td>
<td>D6 Pasteurized milk, 07/2008</td>
</tr>
<tr>
<td></td>
<td>D7 Cremoso Argentino cheese, 09/2008</td>
</tr>
<tr>
<td></td>
<td>MB2 Cremoso Argentino cheese, 09/2008</td>
</tr>
<tr>
<td></td>
<td>MB3 Cremoso Argentino cheese, 09/2008</td>
</tr>
<tr>
<td></td>
<td>MB4 Cremoso Argentino cheese, 09/2008</td>
</tr>
<tr>
<td>L. garlicum</td>
<td>D1 Pasteurized milk, 05/2008</td>
</tr>
<tr>
<td></td>
<td>D5 Whey cream, 07/2008</td>
</tr>
<tr>
<td>L. citreum</td>
<td>MB1 Cremoso Argentino cheese, 09/2008</td>
</tr>
</tbody>
</table>

a Classification according to sequencing of the hypervariable region (first 500 bp) in the 5′ end of the 16S rRNA gene (2). b Used for Cremoso Argentino cheese manufacture.

Volatile molecule profiles of HPH- and thermal-treated skim milk previously inoculated with Leuconostoc cells. To determine the volatile aromatic compounds produced by the survivor cells of L. mesenteroides subsp. mesenteroides D11 inoculated into skim milk at an initial level of 10^7 CFU/ml and subjected to HPH or heat treatment, a gas chromatography–mass spectrometry analysis coupled with solid-phase microextraction technique was used. For each condition, aliquots of 5 g of sample were sealed in sterilized vials. Samples were heated at 40°C for 10 min, and volatiles adsorbed for 50 min on fused-silica fiber covered by carboxen polydimethyl siloxane (75-μm Supelco, Sigma-Aldrich Chemie GmbH, Munich, Germany). Adsorbed molecules were desorbed in the gas chromatograph for 5 min. For peak detection, a gas chromatograph (Agilent 6890GC, Agilent Technologies, Milan, Italy) equipped with a mass spectrometry detector (5970 MSD, Agilent) and a fused-silica capillary column (50-m length, 0.32-mm inside diameter) coated with a 1.2-μm polyethylene glycol film (CP-Wax 52 CB, Chrompack, Middleburg, The Netherlands) as stationary phase were used. The conditions were as follows: injection temperature, 220°C; detector temperature, 220°C; carrier gas (He) flow rate, 1.5 ml/min; splitting...
ratio, 1:20 (vol/vol). The oven temperature was programmed as follows: from 45°C to 100°C, increasing at 2.5°C/min; from 100 to 200°C, increasing at 6.5°C/min, and then holding for 5 min. Volatile peak identification was carried out by computer matching of mass spectral data with those of the compounds contained in the NIST '98 Mass Spectral Library (No. 1A, version 1.6, 1998, National Institute of Standards and Technology–U.S. Environmental Protection Agency–National Institutes of Health) and the Registry of Mass Spectral Data (1998, Wiley, New York).

**Effect of single-pass HPH treatments up to 400 MPa using prototype industrial-scale equipment on *Leuconostoc* viability and recovery during storage.** One hundred liters of commercial ultrahigh-temperature–treated skim milk (Granarolo, Italy) was inoculated with a fresh culture of *L. mesenteroides* subsp. *mesenteroides* D11 (16 to 18 h at 32°C) to an initial cell load of approximately 10^6 CFU/ml. The inoculated milk was subjected to single-pass treatments at 100, 200, 300, and 400 MPa using the prototype industrial-scale equipment (Niro Soavi). Viable cell counts in MRS agar (48 h at 32°C) were carried out before (control, cells without treatment) and immediately after (time zero) HPH treatments and at different intervals of time up to 15 days during the storage (8°C).

**Statistical analysis.** Experiments were replicated three times. All data were analyzed using the one-way analysis of variance procedure in Statgraphics Plus software (version 3.0, Statistical Graphics Corp., Warrenton, VA). Differences among means were detected by Duncan’s multiple range test. Differences were considered significant at a *P* value of <0.05.

**RESULTS**

HPH inactivation of *Leuconostoc* strains in relation to suspension media and number of passes at 100 MPa. The fourteen *Leuconostoc* strains subjected to HPH treatment at 100 MPa for up to eight passes revealed different susceptibilities when suspended in MRS broth or skim milk. Inactivation was significantly greater when


TABLE 2. Evolution of different initial cell loads of Leuconostoc mesenteroides subsp. mesenteroides D11 in reconstituted skim milk immediately after high pressure homogenization treatments and thermal treatment and during various times of storage at low temperature.

<table>
<thead>
<tr>
<th>Days of storage at 8°C</th>
<th>Cell load (log CFU/ml) following indicated treatment of skim milk inoculated with initial cell load of (10^5) CFU/ml</th>
<th>HPH</th>
<th>HPH</th>
<th>HPH</th>
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<tbody>
<tr>
<td></td>
<td>10^5 CFU/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C 1 3 5 8 HT C 1 3 5 8 HT C 1 3 5 8 HT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7.16 b A 6.9 A 6.81 a A 6.60 a A 6.49 a A</td>
<td>5.18 c A 5.09 c A 4.82 b c A 4.53 b A 4.35 b A</td>
<td>4.14 A A</td>
<td>3.98 b A 3.93 b A 3.42 b A 3.23 A A 2.94 A A</td>
</tr>
<tr>
<td>1</td>
<td>7.90 c b 7.96 c b 7.92 c b 7.36 b c 7.30 b A 3.23 A A</td>
<td>6.53 c b 6.45 c b 6.09 b b 5.95 b b 5.22 b ab 5.22 b ab</td>
<td>4.19 A A 1.89 A A 5.36 c A 5.19 c b 4.58 b b 4.44 ab b 4.33 A A</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>8.42 b c 8.66 c b 8.41 b c 8.44 b c 8.29 b b 4.41 a b 1.89 A A</td>
<td>8.34 b c 8.24 b c 8.45 b c 8.21 b c 8.94 c c 1.89 A A</td>
<td>7.56 c c 7.62 c c 7.41 b c 7.22 b c 6.92 A c 6.92 A c</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>8.58 b c 8.69 b c 8.72 b c 8.80 b c 8.61 b b 7.98 a c 3.34 a b 4.50 c</td>
<td>8.87 d b 8.57 b c 8.41 b c 8.61 b c 8.72 c d 3.34 a b 4.50 c</td>
<td>8.51 a d 8.41 a d 8.34 a d 8.53 a d 8.28 a d</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>ND ND ND ND ND 8.0 c 4.50 c</td>
<td>ND ND ND ND ND ND ND ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>ND ND ND ND ND ND ND ND</td>
<td>ND ND ND ND ND ND ND ND</td>
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</table>

\(\alpha\) C, control Leuconostoc cells without any treatment; HT, thermal treatment of 63°C for 30 min; HPH, high pressure homogenization treatment with laboratory-scale equipment at 100 MPa for indicated number of passes; ND, not determined. Dashes indicate viable cell counts of < 1 CFU/ml (detection limit). Within a row and for each initial cell load, means with different lowercase letters are significantly different (P < 0.05). Within a column, means with different uppercase letters are significantly different (P < 0.05).
damage (i.e., hexanal, nonanal, furfural, heptanal, decanal, and 2-furanmethanol) with respect to the amounts in untreated/uninoculated skim milk. Among heat-treated or HPH-treated skim milk samples, no significant difference in oxidation compounds was detected. In addition, HPH-treated samples revealed the presence of high levels of ethanol, while heated ones were characterized by higher content of short-chain fatty acids (data not shown). The levels of alcohols, such as ethanol, hexanol, heptanol, octanol, and nonanol, as well as sulfur compounds, increased over time (6 days) in the inoculated HPH-treated samples (Fig. 3). In contrast, higher amounts of aldehydes and ketones characterized the samples obtained from inoculated heat-treated skim milks. According to the data shown in Figure 3, the uninoculated HPH- and heat-treated skim milk samples were characterized by the same volatile molecules detected after 24 h at 8°C. However, the storage time increased the accumulation of ketones, furanes, and to a minor extent, aldehydes.

**Effect of single-pass HPH treatments up to 400 MPa using prototype industrial-scale equipment on Leuconostoc viability and recovery during storage at 8°C.**

After HPH treatments at 100 and 200 MPa, no reduction in viable cell loads was observed (Fig. 4). However, when *L. mesenteroides* subsp. *mesenteroides* D11 cells were subjected to 300 and 400 MPa, reductions of more than 5 log in comparison to the viable cell count in the control (cells without treatment) were observed. After 2 days of storage of the homogenized samples at 8°C, a rise of about 2 log was observed for those subjected to 100 and 200 MPa, and the increase was greater than 3 log for those subjected to 300 and 400 MPa. For all HPH-treated samples, the maximum viable cell loads were demonstrated after 5 days of refrigerated storage.

**DISCUSSION**

Adventitious *Leuconostoc* strains can cause commercial losses in many food industries, including dairy fermentation. In our region, some *Leuconostoc* species induced spoilage by producing excessive amounts of CO₂, which caused blowing defects in Cremoso Argentino cheese (22). *Leuconostoc* species have been identified as resistant to thermal treatments applied to milk, but they are also capable of growing at temperatures as low as 8°C (2) and 4°C (8), allowing them to grow during cheese ripening and causing early blowing by gas production. This fact makes it relevant to study their behavior at refrigeration temperatures. The control of adventitious *Leuconostoc* by cheesemakers is generally achieved by intensifying thermal treatments applied to the raw milk, which also can destroy heat-labile components of the milk and decrease the quality of the product. As a result, there has been high interest in nonthermal processes, which combine efficient microbial reduction with a maximal retention of the chemical and physicochemical properties of the product (31). In the present work, the effectiveness of nonthermal HPH technology was compared with that of a traditional thermal process, focusing on cell viability and recovery during refrigerated storage and differences in the volatile profiles of HPH- and heat-treated samples inoculated with spoilage *Leuconostoc* strains.

Fourteen *Leuconostoc* strains were subjected to HPH treatments at 100 MPa for up to eight passes, using MRS
Leuconostoc

Viable cell counts (log CFU per milliliter) obtained studied the inactivation of foodborne (6).

\( D_11 \) cells the inactivation was greater when the number of mesenteroides contributing to the observed increase

\( u \) )

reported that subsp. Listeria innocua \( u \) )

\( u \) and total bacteria in a \( L. \) broth and reconstituted skim milk as suspension media. According to our results, the inactivation of \( Leuconostoc \) cells was dependent on the suspension medium used, as it was remarkably greater in MRS broth than in skim milk. As can be expected and as was also noticed by other authors (3, 27, 28), the inactivation was greater when the number of passes was higher (from one to eight). The resistance of \( Leuconostoc \) cells in skim milk may be due to some milk constituents which would provide a protective effect against high pressure treatments. In this regard, Vachon and colleagues (28) studied the inactivation of foodborne pathogens and concluded that, regardless of the high pressure treatment applied, the bacteria tested were more resistant in milk than in phosphate buffer.

Among the fourteen \( Leuconostoc \) strains tested, \( L. \) mesenteroides subsp. mesenteroides D11 was chosen to perform the second stage of our experiments. According to our results, thermal treatment was more effective than the HPH treatment used for all the initial cell loads tested, as shown by undetectable cell counts immediately after thermal treatments and delayed growth during refrigerated storage as well. Likewise, Kheadr and coworkers (10) reported that milk pasteurization produced the greatest reduction in viable cell counts of \( Listeria \) innocua and total bacteria in a comparison to high pressure–treated milk. Our study showed that during the refrigerated storage (8°C), HPH-treated samples reached maximum cell counts in a shorter period than heat-treated ones. Even though heat treatment remained more effective than HPH to inactivate \( Leuconostoc \) cells, it could not assure the total inactivation of cell populations, as demonstrated by the maximum viable cell counts after a short period of storage at 8°C for samples with \( 10^7 \) and \( 10^8 \) CFU/ml initial cell loads. The cell counts of heated samples remained undetectable during 15 days only for the lowest initial inoculum tested (\( 10^7 \) CFU/ml). Still, undetectable counts do not assure irreversible destruction of bacterial cells but could be a consequence of reversible damage that keeps the cells in a noncultivable state on the usual growth media. If this was the case, some special enriched medium should be necessary in order to observe bacterial growth.

The volatile profiles showed the occurrence of high levels of aldehydes and hydrocarbon- and furan-derived compounds both in HPH- and heat-treated samples. These compounds are originated mainly from lipid oxidation (26), indicating that the thermal damage is similar for both treatments applied. Although HPH is regarded as a nonthermal technology, during this treatment, the temperature increases due to frictional heating in the homogenization valve (4). The temperature rise depends on several factors (inlet temperature, pressure level, number of passes, matrix, valve geometry, and temperature exchanger). In the present work, the use of a thermal exchanger limited the temperature rise, and after eight passes, the outlet temperature did not exceed 25°C. However, repeated passes at 100 MPa seem to induce, independent of inoculation, a matrix of thermal stress able to increase the levels of furans and aldehydes. The data obtained after 24 h showed that HPH-treated samples also had fatty acids, although in lower amounts than heat-treated ones. These fatty acids, absent in the uninoculated controls, can be attributed to the release of fatty acids from the disrupted microbial cells, while the increase over time is probably due to the increased milk fat susceptibility to lipolysis and lipid oxidation (26) of skim milk, characterized by a fat content of 0.2%. Moreover, HPH treatment is reported to activate microbial enzymes, including lipase (15), contributing to the observed increase of fatty acids during storage. After 6 days of storage at 8°C, HPH-treated samples were characterized by metabolites such as ethanol, hexanol, heptanol, and octanol due to faster microbial cell recovery and the microbial detoxification system. In fact, hexanol is the detoxification product of hexanal which has antimicrobial activity (12). The high amounts of ethanol after 24 h can be attributed to its extraction, due to cavitation phenomena, from the heterofermentative microbial cells. The ability to strip ethanol from yeasts has been reported previously (6). In the present study, the aldehyde content was similar in HPH-treated and heated samples after 24 h, while a marked increase in their content was observed for heated samples during storage, confirming the data reported by Rerkrai and colleagues (23). These authors reported a general increase in aldehyde content during storage of thermal-treated milk, attributing this behavior to oxygen availability and storage temperature. After 6 days of storage, heated samples were also characterized by higher amounts of ketones originated by milk fat oxidation. The differences in the volatile profiles of HPH-treated and heated skim milk inoculated with \( Leuconostoc \) bacteria would probably be expressed as different off-flavor patterns in the final dairy product.

When \( L. \) mesenteroides subsp. mesenteroides D11 cells suspended in commercial skim milk were subjected to one single-pass HPH treatment at up to 400 MPa using the industrial-scale equipment, the reductions in viable cell counts were only more than 5 log when 300 and 400 MPa were applied. However, these treatments were ineffective in...
completely destroying the bacteria, since remaining *Leuconostoc* cells were capable of growing rapidly after only 5 days of refrigerated storage at 8°C.

Many works have established that gram-positive bacteria are generally more resistant to HPH than gram-negative bacteria (27, 28, 31), suggesting a correlation between cell wall structure and high pressure resistance. It was postulated that HPH kills vegetative bacteria suspended in a liquid especially through mechanical destruction of the cell integrity caused by hydrodynamic cavitation, impingement against static surfaces, high turbulence, and fluid shear (31). In gram-positive bacteria, the robust peptidoglycan layer provides great structural strength which protects them from these phenomena. Other than the layer structure, some authors postulated that cell shape can be an additional factor contributing to susceptibility to high pressure treatments; smaller and/or spherically shaped microorganisms are expected to be more resistant than rod-shaped ones (1, 25). Wuytack et al. (31) could not confirm this hypothesis, since the coccus *Leuconostoc dextranicum* strain tested was more sensitive to HPH than the rod *Lactobacillus plantarum*. In despite of this, the characteristics of *Leuconostoc* cells tested in our work could explain, at least partially, their high resistance to HPH treatments.

The *Leuconostoc* strains tested in this work were highly resistant to the inactivation treatments applied. Neither HPH nor heat treatment assured their total destruction, even if they were more sensitive to the thermal treatment. To enhance the inhibitory effect on *Leuconostoc* cells, HPH should be combined with a mild heat treatment which, besides efficient microbial inactivation, could allow maximal retention of the physicochemical properties of the product.

ACKNOWLEDGMENTS

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