Fate of *Staphylococcus aureus* in Cheese Treated by Ultrahigh Pressure Homogenization and High Hydrostatic Pressure

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ABSTRACT

We evaluated the influence of ultrahigh pressure homogenization (UHPH) treatment applied to milk containing *Staphylococcus aureus* CECT 976 before cheese making, and the benefit of applying a further high hydrostatic pressure (HHP) treatment to cheese. The evolution of *Staph. aureus* counts during 30 d of storage at 8°C and the formation of staphylococcal enterotoxins were also assessed. Milk containing approximately $7.3 \log_{10}$ cfu/mL of *Staph. aureus* was pressurized using a 2-valve UHPH machine, applying 330 and 30 MPa at the primary and the secondary homogenizing valves, respectively. Milk inlet temperatures (T_{in}) of 6 and 20°C were assayed. Milk was used to elaborate soft-curd cheeses (UHPH cheese), some of which were additionally submitted to 10-min HHP treatments of 400 MPa at 20°C (UHPH+HHP cheese). Counts of *Staph. aureus* were measured on d 1 (24 h after manufacture or immediately after HHP treatment) and after 2, 15, and 30 d of ripening at 8°C. Counts of control cheeses not pressure-treated were approximately $8.5 \log_{10}$ cfu/g showing no significant decreases during storage. In cheeses made from UHPH treated milk at T_{in} of 6°C, counts of *Staph. aureus* were $5.0 \pm 0.3 \log_{10}$ cfu/g at d 1; they decreased significantly to $2.8 \pm 0.2 \log_{10}$ cfu/g on d 15, and were below the detection limit ($1 \log_{10}$ cfu/g) after 30 d of storage. The use of an additional HHP treatment had a synergistic effect, increasing reductions up to $7.0 \pm 0.3 \log_{10}$ cfu/g from d 1. However, for both UHPH and UHPH+HHP cheeses in the 6°C T_{in} samples, viable *Staph. aureus* cells were still recovered. For samples of the 20°C T_{in} group, complete inactivation of *Staph. aureus* was reached after 15 d of storage for both UHPH and UHPH+HHP cheese. Staphylococcal enterotoxins were found in controls but not in UHPH or UHPH+HHP treated samples. This study shows a new approach for significantly improving cheese safety by means of using UHPH or its combination with HHP.

Key words: *Staphylococcus aureus*, cheese, ultrahigh pressure homogenization, high hydrostatic pressure

INTRODUCTION

In the dairy industry, there is a growing interest in mild nonthermal processes, which combine efficient microorganism reduction with a maximum retention of the physicochemical properties of the product. High-pressure treatment of milk and dairy products is considered to be the most promising alternative to traditional thermal treatments (Trujillo et al., 2002; Hayes and Kelly, 2003). Over the last 15 yr, many aspects concerning the effects of high hydrostatic pressure (HHP) on cheese and dairy products have been investigated (O'Reilly et al. 2001; Patterson, 2005). Ultrahigh pressure homogenization (UHPH, also known as high pressure homogenization or dynamic high pressure) works at pressures greater than 200 MPa (Hayes and Kelly, 2003; Thiebaud et al., 2003) and is currently the subject of intensive research. The UHPH machines incorporate important technological developments to conventional homogenization, such as high pressure pumps and intensifiers, specifically designed high pressure valves made of ceramic material known to withstand extremely high pressure levels (up to 400 MPa), and modified geometry compared with classical homogenizing valves (i.e., APV-Gaulin homogenizers), leading to differences in fluid flow direction and narrower gaps between the valve and the seats (Thiebaud et al., 2003; Floury et al., 2004). Ultrahigh pressure homogenization can be used to make fine food emulsions, to disrupt dense cell microbial cultures and subsequently recover intracellular metabolites, to inactivate bacteriophages, and to modify functional properties of hydrocolloids (Middelberg, 1995; Floury et al., 2002; Moroni et al., 2002; Hayes et al., 2004). In consequence, UHPH is now attracting increasing interest in the food area.

With both HHP and UHPH techniques, microorganisms are subjected to high pressures. Whereas the time of exposure is usually in the order of few minutes or more in HHP treatments, the residence time of the fluids in the high-pressure section is in the order of seconds in most cases in current UHPH machines (Thie-

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baud et al., 2003). Microbial cells are destroyed by HHP, which induces changes in the morphology, the cell membrane and the wall of microorganisms, and modifies biochemical reactions and genetic mechanisms (Patterson, 2005). Inactivation of bacteria in high-pressure homogenization processes is achieved by pressure, exposure to hydrodynamic cavitations, impingement against static surfaces, and high turbulence and fluid shear. The temperature increase due to heat dissipation of kinetic energy in the high-pressure valve also contributes to inactivation (Wuytack et al., 2002; Hayes and Kelly, 2003; Thiebaud et al., 2003). However, the residence time of the fluid at the temperature reached after it passes the high-pressure valve can be less than 1 s if the heat-exchanger devices are placed immediately after this valve (Thiebaud et al., 2003; Picart et al., 2006). For both kinds of high-pressure treatments, the initial load of vegetative cells and the different matrixes holding the target bacteria also influence the resulting inactivation (Patterson et al., 1995; Vachon et al., 2002).

In general, resistance to pressure diminishes from spores to gram-positive and gram-negative bacteria (Wuytack et al., 2002). One of the most HHP- and UHPH-resistant nonsporulating gram-positive bacteria is *Staphylococcus aureus* (Patterson et al., 1995; Wuytack et al., 2002). *Staphylococcus aureus* is commonly found in milk and dairy products, particularly in cheeses made either from raw or pasteurized milk (Coveney et al., 1994), due to it being among the most important etiological agents of bovine mastitis and because it is extensively carried by food industry workers (Younis et al., 2003). *Staphylococcus aureus* is also one of the main agents of food intoxication caused by milk and dairy product consumption in France, Spain, and the United Kingdom (Brisabois et al., 1997; European Union, 2003). It is still one of the leading causes of foodborne illness worldwide and the second most commonly reported cause in the United States (Bunning et al., 1997; Balaban and Rasooly, 2000; Jablonski and Bohach, 2001; Younis et al., 2003). The most notable virulence factors associated with *Staph. aureus* are staphylococcal enterotoxins (StE). They are heat-stable proteins that are produced by approximately 25% of the *Staph. aureus* isolated from foods. They function as potent gastrointestinal toxins; in susceptible individuals, they may produce nausea, vomiting, diarrhea, abdominal cramps, and malaise 3 to 10 h after consumption. Staphylococcal enterotoxin A is the most common StE found in food-poisoning outbreaks in the United States (Balaban and Rasooly, 2000; Cenci-Goga et al., 2003).

Inactivation of *Staph. aureus* in milk and dairy products by HHP has been extensively studied (Patterson et al., 1995; Gervilla et al., 1999; López-Pedemonte et

al., 2007). In contrast, few studies of inactivation of *Staph. aureus* using UHPH have been performed. *Staphylococcus aureus* reduction in PBS (10 mM potassium phosphate, pH 7.0; 8.4 g/L of NaCl) under UHPH up to 300 MPa are barely significant at temperatures lower than 45°C (Wuytack et al., 2002; Diels et al., 2003). Wuytack et al. (2002) also observed that consecutive rounds of HHP or UHPH have an additive effect on the viability reduction of bacteria. However, no published reports have been found involving the use of a combination of both treatments. Microorganisms are inactivated by HHP and UHPH by different mechanisms. The successive application of both technologies can be seen as additional hurdles against pathogen cells. Examples can be found in previous works involving combinations of temperatures higher than 50°C with HHP or pulsed electric fields (Gervilla et al., 1999; Rowan et al., 2001) and HHP treatments applied to foods containing bacteriocins (López-Pedemonte et al., 2003; Arqués et al., 2005).

Several studies with food as the assay medium have demonstrated the influence of the food matrix on microbial sensitivity to HHP (Patterson et al., 1995; Patterson, 2005) and thus the importance of choosing the right matrix. Model cheeses are more similar to real cheese than slurries and tube tests. They offer the possibility of testing single strains in a real cheese environment and they allow us also to use pathogenic strains and to keep them confined within a controlled laboratory. The objective of this work was to study the inactivation of *Staph. aureus* in model cheeses made from inoculated UHPH-treated milk. The benefit of adding a further HHP treatment 24 h after cheese production, the evolution of *Staph. aureus* counts during 30 d of ripening at 8°C, and the probable formation of StE were also evaluated.

Experimental conditions for this study were chosen taking into consideration previous studies in which UHPH and HHP treatments were applied to milk and cheese samples. The aim was to test the performance of UHPH treatment previous to cheese making as an alternative to traditional thermal treatments. Recently, Briñez et al. (Universitat Autònoma de Barcelona, Barcelona, Spain, personal communication) found reductions of approximately 3 to 4 log₁₀ cfu/mL of *Staph. aureus* in milk, applying 300 MPa with a milk inlet temperature of 20°C. Hence, it seemed reasonable to apply the highest pressure treatment the equipment can reach without excessive fluctuation (330 MPa). Two UHPH milk inlet temperatures in the feeding tank (T_{in}) were selected: 6 and 20°C. Temperatures of 6°C can often be encountered in raw milk bulk tanks of dairy farms and processing industries, and UHPH treatment at this temperature would permit its incorporation to

milk continuous processing. Although not all milk lipids are in liquid state at 6°C, milk is fluid enough to circulate through the homogenizer. This T_{in} is thought to produce a lower milk temperature after the high-pressure valve. On the contrary, a T_{in} of 20°C would produce a higher milk temperature after the high-pressure valve, and thus was chosen to increase the thermal contribution to *Staph. aureus* inactivation. The aim of applying an HHP treatment after UHPH was to cause additional destruction to the UHPH-treated cells. This additional HHP treatment was applied to some 24-h cheeses (before the 30-d storage period), aiming to benefit from the effects of the ripening process coupled to HHP treatment and to reduce *Staph. aureus* number below the level at which enterotoxin formation may occur (López-Pedemonte et al., 2007). Based upon studies with milk and Cheddar cheese (Gervilla et al., 1999; O'Reilly et al., 2000) and model cheese studies with the same strain of *Staph. aureus* (López-Pedemonte et al., 2007), 400 MPa and 20°C were selected as the optimum pressure and temperature HHP treatment conditions. Higher pressures are expected to produce higher levels of inactivation, but they may also adversely affect cheese physicochemical characteristics and alter, if not arrest, its normal ripening (Malone et al., 2003; Juan et al., 2004; Wick et al., 2004).

MATERIALS AND METHODS

Pasteurization of Milk

Bovine raw milk was collected from a local farm and transported under refrigerated conditions to the laboratory in less than 1 h. It was then pasteurized (30 min at 65°C and rapidly cooled) to avoid the interference of natural *Staph. aureus* or competitive bacteria, and stored below 4°C until cheese manufacture.

Bacterial Strains

Staphylococcus aureus CECT 976 is an enterotoxin-A-producing strain and has been involved in one food poisoning incident (Belay and Rasooly, 2002). It was also chosen because of its high baroresistance shown in previous experiments (Patterson et al., 1995; López-Pedemonte et al., 2007). It was obtained as a freeze-dried culture in thermo-sealed vials from the Colección Española de Cultivos Tipo (CECT, University of Valencia, Valencia, Spain) and corresponds to number 13565 of the American Type Culture Collection (Manassas, VA). Freeze-dried cultures were rehydrated in tryptone soy broth (Oxoid Ltd., Basingstoke, UK) at 37°C for 18 h. Subsequently, these broths were used to inoculate tryptone soy agar plates (Oxoid), and individual colonies were collected to prepare cryobeads (Nalgene Sys-

tem 100 Laboratories, Microkit Iberica S.L., Madrid, Spain). They were then kept at -20°C to provide stock cultures for the assays.

Preparation of *Staph. aureus* Suspension and Inoculation of Milk

Before each experiment, 10 mL of tryptone soy broth was inoculated with *Staph. aureus* cells adsorbed on one cryobead and incubated at 37°C for 20 h. After incubation, the broth was spread on tryptone soy agar inside a tube and left at 37°C for 20 to 24 h to obtain cells in the stationary phase of growth. Subsequently, cell suspensions were prepared in 11 mL of tryptone NaCl solution (1 g/L of tryptone pancreatic casein digestion and 8.5 g/L of NaCl) to obtain 9.0 to 9.5 log₁₀ cfu/mL. One milliliter of cell suspension was used to determine the concentration by means of absorbance at 405 nm using a spectrophotometer (Cecil 9000 series, Cecil Instruments, Cambridge, UK). Thereafter, 10 mL of each cell suspension was inoculated into milk at room temperature. Milk-inoculated samples were left to stand for 70 min at 6 ± 1°C or 20 ± 1°C before pressure treatments. Milk destined to make cheese control samples (inoculated but not pressure treated) was inoculated at the same time as milk destined to be submitted to UHPH. The final concentration of cells in milk was approximately 7 log₁₀ cfu/mL.

UHPH Treatment of Milk

The UHPH treatment was applied to samples using a Stansted high pressure homogenizer (model DRG FPG 7400H:350, Stansted Fluid Power Ltd., Essex, UK). This machine has a high-pressure valve made of resistant ceramics and is able to support up to 350 MPa. A second pneumatic valve located after the first one is able to support 50 MPa. The high-pressure system consists of 2 intensifiers (80 mL useful volume) driven by a hydraulic pump. To avoid loss of homogenization performance due to temperature increase and rapid expansions or contractions of the first stage valve, it is refrigerated by constant circulation of water at ambient temperature in an external jacket built around it. Milk samples were subjected to a single-pass UHPH treatment of 330 MPa on the primary homogenizing valve and 30 MPa on the secondary valve. The flow rate of the milk in the homogenizer was relatively constant (approximately 16 L/h) at the pressure assayed. Inlet temperatures of 6 and 20°C were assayed. The homogenized milk was rapidly cooled. It reached 16 to 18°C by means of another external jacket built around the pipeline connecting the first homogenizing valve and the second one, and a spiral type heat exchanger (BCI/

2843 type, Occo Cooler Ltd., Telford, UK) located after the second valve. For the different experiments carried out at each temperature, 10 L of inoculated milk was used. The majority of this volume was processed through the homogenizer to ensure temperature and pressure equilibration. Afterwards, milk was collected in sterilized 1-L bottles. Cleaning and disinfection of the UHPH equipment was made according to Briñez et al. (2006).

Preparation of the Starter Culture

A mixture of commercial lyophilized *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *cremoris* (Ezal MAO 11, Rhodia Iberia S.A., Madrid, Spain), known as a nonbacteriocin producer, was used as a starter culture for the washed-curd cheese manufacture. The culture was revived by placing 0.015 g of the mixture in 1,000 mL of commercial sterilized skimmed milk and incubated at 30°C for 24 h. A volume of 50 mL was used to prepare a subculture in 200 mL of sterilized skimmed milk, which was also incubated at 30°C for 24 h. The final concentration was approximately $9 \log_{10}$ cfu/mL.

Manufacture of Cheese

Soft-curd cheeses of approximately 30 g were manufactured under controlled microbiological conditions following a modification of the procedure of Shakeel-Ur-Rehman et al. (1998) as described by López-Pedemonte et al. (2003). Milk already inoculated with *Staph. aureus* and UHPH treated was brought to 31°C in a water bath and 2% (vol/vol) starter culture was added together with 0.01% (vol/vol) of a 35% (wt/vol) CaCl solution (Arroyo, Santander, Spain) to improve coagulation. A liquid rennet extract (0.02%, vol/vol) of calf origin (520 mg/L active chymosin, Arroyo) was used as coagulating agent. Milk was poured into previously sterilized, 225-mL long-necked centrifuge bottles. Coagulation took place at 31°C. After 45 min curds were gently cut with sterile stainless steel tools and heated for 15 min at 37°C. About 40% of whey was discarded and replaced by sterile tap water. Bottles were centrifuged at $7,000 \times g$ for 40 min at 20°C. Then they were kept in the water bath at 37°C until pH reached 5.5. The whey was discarded and 20% sterile brine (200 g of NaCl/L of tap water) was added directly into each bottle. After 15 min, the brine was removed and cheeses were taken out of the bottles and dried with sterile paper. They were vacuum packed in plastic bags (bb4.l, Cryovac Packaging, Sant Boi de Llobregat, Spain) and stored at 8°C for 30 d. A series of cheeses made with pasteurized milk (neither inoculated nor UHPH-pro-

Figure 1. Flow chart showing the procedure for the production of the different series of cheeses made with milk treated with ultrahigh pressure (UHPH) and UHPH + high hydrostatic pressure (HHP).

cessed) was included to obtain blank cheeses. Figure 1 demonstrates the manufacturing of the different cheese series. Cheese making procedure was adjusted with cheese made of pasteurized milk to obtain a moisture content of approximately 45% and a pH of 5.5 after manufacture (López-Pedemonte et al., 2003). For cheese made of UHPH-treated milk, exactly the same procedure conditions were followed; thus in UHPH cheeses moisture is approximately 10% higher.

HHP Treatments of Cheeses

As can be seen in Figure 1, some of the samples were HHP-treated 24 h after manufacture using discontinuous HHP equipment (model S-FL-850-9-W, Stansted Fluid Power). This plunger-press-style vessel with internal high pressure intensifier has a pressure chamber with usable dimensions of 37 mm bore internal diameter $\times$ 245 mm long and houses inside the product loading canister. The vessel body and pressure-transmitting fluid inside (water) were held at treatment temperature by circulating water through an integral heat transfer jacket fitted to the outside of the high-pressure barrel assembly. The temperature was monitored with a thermocouple positioned in the transmission fluid in the upper part of the chamber. On each run, 2 vacuum-packed cheese samples (approximately 100 cm³) were placed inside the product canister. They were stabilized at $18 \pm 1^\circ\text{C}$ and then immediately submitted to a 10-

min HHP treatment at 400 MPa (as measured on the digital and analog pressure indicators). Average time of pressure increase was 90 s and average decompression time was 35 s. Limit temperatures of the pressurizing fluid were as follows: maximum temperature under pressure, 28 to 30°C; minimum temperature under pressure, 20 to 21°C.

Microbiological Analysis

Microbiological milk analysis was carried out before and after UHPH treatment. Decimal dilutions were prepared in peptone water (10 g/L of peptone and 5 g of NaCl/L, Oxoid) and spread onto tryptone soy agar supplemented with 6 g/L of yeast extract powder (Oxoid). Plates were then incubated at 37°C for 48 h.

Microbiological cheese analysis was performed 24 h after manufacture or immediately after HHP treatment as appropriate (d 1). Cheeses were also analyzed after 2, 15, and 30 d of storage at 8°C by homogenizing 10 g of sample in 90 mL of tryptone soy broth supplemented with 6 g/L of yeast extract powder in an electromechanical blender (BagMixer, Interscience, France). Decimal dilutions in peptone water were spread onto the surface of Baird Parker agar (Oxoid) and incubated at 37°C for 24 to 48 h. On each analysis day, the remaining first dilutions in tryptone soy broth supplemented with yeast extract of every sample were incubated for 18 h at 32°C. A loopful of this culture was streaked onto a plate of Baird Parker agar supplemented with rabbit plasmin fibrinogen (Oxoid) and incubated at 37°C to determine whether complete inactivation of *Staph. aureus* was achieved or not. When counts of *Staph. aureus* were below the detection limit ($1.0 \log_{10}$ cfu/g) but viable cells were recovered after the enrichment step, the assigned value was $0.99 \log_{10}$ cfu/g. When no recovery was found, the assigned value was zero. Blank cheese samples (not inoculated with *Staph. aureus*) were included to assess the efficacy of the pasteurization and manufacturing processes. Inoculated model cheeses not submitted to any pressure treatment were called control cheeses (as shown in Figure 1).

Reductions were calculated by comparing counts of control samples (N_0) with those of pressure-treated samples (N) as follows: Reduction ($\log_{10}$ cfu/g) = $\log_{10} N_0 - \log_{10} N$.

StE Detection

The presence or absence of StE was determined following the protocol for dairy samples of the VIDAS Staphylococcal Enterotoxin System II (SET2, enzyme-linked fluorescent assay provided by bioMérieux s.a., Marcy L'Etoile, France). According to the manufac-

turer, the sensitivity of this test in the detection of StE A in food samples is over 1.0 ng/mL. Assays were made in d 1 cheeses and in cheeses stored 30 d at 8°C.

Statistical Analysis

Each HHP experiment was performed 3 separate times with duplicate analysis in each replicate. The GLM procedure as implemented in SPSS 12.0 for Windows (SPSS Inc., Chicago, IL) was used to test effects on the colony count logarithm of *Staph. aureus* of the following factors: pressure treatment; T_{in} ; and storage day. A posthoc Tukey test was used to obtain paired comparisons among sample means on each storage day. A one-way repeated-measures ANOVA was performed with storage day as factor for testing the main effect on counts of *Staph. aureus* on every combination of pressure treatments (UHPH alone, UHPH + HHP, no pressure treatment) and T_{in} (6 and 20°C). Level of significance was $P < 0.05$.

RESULTS

Reduction of Counts of *Staph. aureus* in Milk

Initial *Staph. aureus* counts of inoculated milk destined to be UHPH treated at T_{in} of 6 and 20°C can be seen in Figure 2. Reductions of *Staph. aureus* counts in these milk samples as a consequence of UHPH treatments at 6°C T_{in} were inferior to those obtained at 20°C T_{in} (2.1 ± 0.1 vs. $3.6 \pm 0.2 \log_{10}$ cfu/g, respectively, $P = 0.003$).

Initial Counts of *Staph. aureus* in Cheese

Figure 3 shows the counts of *Staph. aureus* in control and in UHPH cheeses. Counts of *Staph. aureus* in control cheeses were higher than those found in inoculated milk, as normally occurs. When vegetative cells of a certain microorganism are inoculated into milk destined for cheese making, their counts in the obtained cheese are higher. This is mainly due to the concentration process that takes place and to their growth at moderate temperatures (in this case 31 to 37°C for 3 h).

As reflected in these initial figures, T_{in} affected the counts obtained in UHPH cheeses ($P < 0.001$). The initial differences between counts of *Staph. aureus* in control and UHPH cheeses were 3.7 ± 0.1 and $7.6 \pm 0.1 \log_{10}$ cfu/g for 6 and 20°C T_{in} , respectively. For both temperatures assayed, these values were higher than the reductions of *Staph. aureus* found in milk before and after UHPH treatment.

In UHPH+HHP cheeses for T_{in} of 6°C, the additional HHP treatment increased the reduction of counts of *Staph. aureus* from 3.7 ± 0.1 to $7.0 \pm 0.5 \log_{10}$ cfu/g. This

Figure 2. Counts of *Staphylococcus aureus* CECT 976 (expressed as means of 3 replications $\pm$ confidence interval) in milk before and after ultrahigh pressure homogenizing treatment of 330 MPa (first valve) and 30 MPa (second valve), at inlet temperatures of 6 and 20°C. No = initial counts of *Staph. aureus* in milk samples; N = counts of *Staph. aureus* in the same milk after ultrahigh pressure homogenizing treatment. Different letters above bars indicate significant difference among the means ($P < 0.05$).

difference between UHPH and UHPH+HHP cheeses reflected the effect of HHP. This effect was not seen for 20°C T_{in} because with the UHPH treatment alone, *Staph. aureus* counts were below the detection limit of our technique.

Evolution of Counts of *Staph. aureus* During 30 d of Storage at 8°C

Counts of *Staph. aureus* in control cheeses for both T_{in} did not differ during storage ($P = 0.174$ and $P = 0.093$ for 6 and 20°C, respectively) as can be seen in Figure 3. In contrast, counts of most pressure-treated samples decreased with storage time.

The counts of *Staph. aureus* during the 30-d storage period at 8°C (i.e., counts on d 2, 15, and 30 relative to d 1 counts) in cheeses made from milk UHPH treated at T_{in} of 6°C diminished from d 2 to 30 of storage ($P < 0.001$). The difference in *Staph. aureus* counts between these cheese samples reached 3.9 ± 0.3 log₁₀ cfu/g. For samples that received the additional HHP cycle of 400 MPa, counts were below the detection limit on d 1 and did not vary during their storage at 8°C ($P = 0.190$). Nevertheless, after incubating the initial dilution of the samples for 18 h at 32°C and streaking onto selective agar (Baird Parker supplemented with rabbit plasmin fibrinogen), characteristic colony growth showed that some *Staph. aureus* cells were able to recover.

In both UHPH and UHPH+HHP cheeses made from milk treated at T_{in} of 20°C, counts of *Staph. aureus*

Figure 3. Evolution of counts of *Staphylococcus aureus* CECT 976 (expressed as means of 3 replications $\pm$ confidence interval) in control cheeses (♦), ultrahigh pressure homogenization (UHPH) cheeses (▲), and in UHPH + high hydrostatic pressure cheeses (●) stored at 8°C. A) Counts at UHPH inlet temperature of 6°C; B) Counts at UHPH inlet temperature of 20°C. When interval bars are not visible they fall within plot symbols.

were already below the detection limit on d 1. After 15 d of storage at 8°C, complete inactivation was achieved for both samples, as *Staph. aureus* was not recovered after incubating the initial dilution of the samples for 18 h at 32°C and streaking onto Baird Parker supplemented with rabbit plasmin fibrinogen.

Evaluation of StE Formation

As can be seen in Table 1, StE were positively detected in all our control cheese samples but were not detected in any UHPH or UHPH+HHP cheese made from milk treated at both T_{in} .

Table 1. Staphylococcal enterotoxin detection in cheeses made with milk treated with ultrahigh pressure homogenization (UHPH) and UHPH + high hydrostatic pressure (HHP)

Cheese	Day 1 of storage at 8°C	Day 30 of storage at 8°C
Blank	ND ¹	ND
Control	Positive	Positive
UHPH	ND	ND
UHPH + HHP	ND	ND

¹ND = Not detectable.

DISCUSSION

The reductions of *Staph. aureus* counts obtained in UHPH treated milk at both T_{in} were higher than those obtained by Wuytack et al. (2002) and Diels et al. (2003) in PBS (10 mM, pH 7.0) after 300 MPa at T_{in} of 25°C (less than 1 log₁₀ cfu/mL) using an Emulsiflex C5 (Avestin, Ottawa, Canada). This finding could be explained by differences between food matrices and among UHPH equipment designs (Briñez et al., 2006).

Reductions of *Staph. aureus* counts in milk samples as a consequence of UHPH treatments at 6°C T_{in} were inferior to those obtained at 20°C T_{in} , probably due to the higher temperature reached after the high-pressure valve. This phenomenon was extensively described in the work of Thiebaud et al. (2003) in which it was demonstrated that for T_{in} of 4 and 24°C, the temperatures measured immediately after the high-pressure valve were 64.5 ± 1.0 and 78.0 ± 1.9°C, respectively. The authors also provide an estimation of the temperature increase due to pressure build-up and to the temperature increments after the high-pressure valve (i.e., 3.0 ± 0.1 and 15.5 ± 0.4°C/100 MPa for T_{in} of 24°C). As reported by Picart (2004) for pressures of 300 MPa, the use of a second 30-MPa pressure stage in the same homogenizer reduces the temperature after the high-pressure valve by approximately 5°C. Putting all this information together and taking T_{in} values of 4°C for our T_{in} of 6°C, and T_{in} values of 24°C for our T_{in} of 20°C, we estimated a maximum temperature reached after the high-pressure valve of approximately 65 and 75°C, respectively (differences in heat transfer between the different machines may certainly exist but cannot be estimated accurately). It should be remembered that the time the fluid remains at the maximum temperature is considered to be less than 1 s when refrigeration systems are working (Thiebaud et al., 2003; Picart et al., 2006).

Previous and current studies show that UHPH treatments are useful to reduce regular microflora normally present in raw milk (Hayes et al., 2003; Thiebaud et al., 2003; Pereda et al., 2006; Picart et al., 2006) and indicate the possible use of UHPH as a substitute for

continuous pasteurization systems. However, inactivation studies carried out in buffer suspensions or inoculated sterilized milk reveal that reductions of resistant pathogenic cells rarely surpass 3.5 log₁₀ cfu/mL (without recycling) at moderate inlet temperatures (Vachon et al., 2002; Wuytack et al., 2002, 2003; Diels et al., 2003). It is questionable whether these values are adequate to prevent foodborne intoxications caused by accidentally higher loads of pathogens inside raw milk. Furthermore, Briñez et al. (Universitat Autònoma de Barcelona, Barcelona, Spain, personal communication) have shown that *Staph. aureus* cells were able to maintain their counts during milk storage at 4°C (9 d), which is contrary to what happens with more acidic liquid foods such as orange juice (Briñez et al., 2006). Concerns have already been raised (Smiddy et al., accepted) that suggest the need for further studies as well as for new developments in UHPH equipments.

For both T_{in} assayed, the difference between counts of *Staph. aureus* in control and UHPH cheese were significantly higher than the reductions of *Staph. aureus* found in milk before and after UHPH treatment. Taking into consideration that temperatures higher than 37°C were never reached during the cheesemaking process, it seems that the interaction of pressurized *Staph. aureus* with healthy starter cells and relatively low pH somehow helped to achieve higher inactivation in cheese samples compared with milk samples.

As stated previously, *Staph. aureus* in control cheese samples showed its ability to survive and to not reduce in count. In pressurized samples, UHPH capability for causing damage to bacterial cells was probably coupled with the effect of the ripening process that takes place. The significant decreases of *Staph. aureus* counts during storage of UHPH cheese lead us to consider the existence of sublethal injury to cells as a consequence of this treatment. Sublethal injury has been studied by Wuytack et al. (2002, 2003) for several nonthermal treatments including UHPH; they did not find significant UHPH sublethally injured cells of *Staph. aureus* and *Salmonella enterica* serovar Typhimurium in phosphate buffered saline (10 mM potassium phosphate, pH 7.0, 8.4 g/L of NaCl) using culture medium supplemented with NaCl (0 to 6%), or SDS (0 to 100 mg/L) or with an adjusted pH (5.5 to 7.0). This could not be done with our cheese samples because of the interference of starter cells in nonselective media. Nevertheless, the existence of *Staph. aureus* cells sublethally injured following UHPH in our cheeses cannot be discounted. The presence of these cells should be assessed by means other than growing medium criteria.

In UHPH+HHP cheese, the HHP treatment caused an additional reduction of *Staph. aureus* of 3.3 log₁₀ cfu/g compared with UHPH cheese at T_{in} of 6°C. This

value was higher than the $1.4 \pm 0.2 \log_{10}$ cfu/g obtained by López-Pedemonte et al. (2007) after applying the same HHP treatment to the same *Staph. aureus* strain in a pasteurized model cheese. These figures provide additional support to the existence of cells sublethally damaged by UHPH and to the hypothesis of obtaining a synergistic effect by combining both technologies. After the 30-d storage period, for both UHPH and UHPH+HHP samples, similar reductions of 7.3 ± 0.2 and $7.2 \pm 0.2 \log_{10}$ cfu/g, respectively, were found. Nevertheless, the combination of a HHP treatment caused a sharper decrease of counts of *Staph. aureus*, which can be seen comparing the counts on d 15 ($P < 0.001$).

For UHPH samples, total inactivation of cells at T_{in} of 20°C was achieved on d 15 (unlike for T_{in} of 6°C), which meant a reduction of more than $8.0 \log_{10}$ cfu/g for UHPH treatment alone. Taking all this into account, the advantage of adding the HHP treatment has to be evaluated in view of the desired T_{in} and ripening time. The combination of both pressure treatments implies higher costs (higher energy, time consumption, and equipment investment) as well as increased modification of the original cheese matrix.

Staphylococcal enterotoxins were detected in all our control cheese samples. Conditions under which StE can be produced are as follows: 10 to 48°C temperature, pH 4.0 to 9.6, and water activity of 0.84 to 0.99 (Asperger, 1994; Belay and Rasooly, 2002; European Union, 2003). A *Staph. aureus* load of $5 \log_{10}$ cfu/g of food has been suggested as an amount that would allow enough enterotoxin formation to cause illness, probably within 2 h (Belay and Rasooly, 2002). All these conditions could be met in our model cheeses, except for the fact that the storage temperature was below 10°C. This temperature was chosen as a common ripening temperature of some Spanish cheeses and to prevent StE production during ripening as well. In this experiment, the initial *Staph. aureus* load was higher than $7 \log_{10}$ cfu/mL of milk. The UHPH treatment reduced its counts to $3.6 \pm 0.3 \log_{10}$ cfu/mL for T_{in} of 20°C but only to $5.5 \pm 0.1 \log_{10}$ cfu/mL for T_{in} of 6°C. Even when the cheese-making temperature was around 37°C for more than 3 h (and never surpassed it), StE could not be detected in any UHPH or UHPH+HHP cheese.

This study shows a new approach for improving cheese safety by using UHPH to treat inoculated milk before cheese making and by additionally applying HHP to cheese as an alternative. Raw milk used to make cheese is expected to contain no more than $5.5 \log_{10}$ cfu/mL of aerobic bacteria and no more than $3.3 \log_{10}$ cfu/mL of *Staph. aureus* (European Union, 1992). Provided that at least $7 \log_{10}$ cfu/g reductions were achieved and StE formation prevented, it seems reasonable to suppose that UHPH, and UHPH combined with

HHP, can be successfully used to improve the safety of soft-curd cheeses made from raw milk. Inlet UHPH milk temperatures can be in the 6 to 20°C range when the initial load of bacteria is not extremely elevated. Nevertheless, the choice of T_{in} must take into consideration the impact on whey protein denaturation, modification of structural properties of casein micelles, the rheological characteristics of the curds obtained, alteration of enzymatic activities (plasmin, alkaline phosphatase, lactoperoxidase, lipase), and fat globule size reduction (Thiebaud et al., 2003; Hayes et al., 2004; Datta et al., 2005; Sandra and Dalgleish, 2005; Lanciotti et al., 2006; Picart et al., 2006).

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