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Populations of Aerobic Mesophils and Inoculated *E. coli* during Storage of Fresh Goat's Milk Cheese Treated with High Pressure

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ABSTRACT

Pasteurized goat's milk inoculated with *Escherichia coli* 405 CECT was manufactured into cheese containing 10^8 CFU/g. The fresh cheese was treated by combinations of pressure (400, 450, and 500 MPa), temperature (2, 10, and 25°C) and time (5, 10, and 15 min). Once treated, cheeses were stored at 2 to 4°C. Counts of surviving *Escherichia coli* and aerobic mesophilic bacteria were determined 1, 15, 30, and 60 days after treatment. No colonies of surviving *E. coli* were detected 1 day after pressurization, except in samples treated for 5 min at 25°C at pressures of 400 and 450 MPa. No surviving *E. coli* were detected at 15, 30, or 60 days in any case. Aerobic mesophilic bacteria counts after treatment were between 2 and 3 log CFU/g in most cases and only a slight increase during refrigerated storage could be detected in samples treated at 400 MPa.

Key words: High hydrostatic pressure, *Escherichia coli,* mesophilic bacteria, fresh cheese, Mató cheese

High hydrostatic pressure treatment can be applied as a nonthermal food-preservation process when minimally heat-processed products have to be obtained *(23).* Inactivation of microorganisms using high pressure has been studied in a variety of foods to increase the efficacy of preservation processes. In raw milk, Timson and Short *(37)* observed that about 0.05% of the microorganisms, mainly bacterial spores, were resistant to pressures near 150,000 psi. A method for ripening cheddar cheese under high pressure has been patented *(40)* and there is a study on the production and characterization of a pressure-induced gel *(18).*

This study involved the traditional cheese from Catalonia (Spain) named Mató. It is made from goat's milk which has received a high-temperature pasteurization treatment with the purpose of denaturing whey proteins to incorporate them into the curd *(2).* Enzymatic coagulation without addition of lactic cultures and nonsalting of the curd results in high pH and a_w values in this type of cheese, conditions which are very favorable to the growth of most microorganisms. Procedures to prevent growth of pathogenic or spoilage bacteria, such as inoculation of lactic starters *(28)* or modified-atmosphere packaging *(20, 33),* are being applied and studied in cheeses similar to Mató, such as Burgos and fresh cheeses from goat's milk, which are often salted.

The presence of species of bacteria from the coliform group in pasteurized milk or in milk products is frequently used as an indicator of inadequate processing or postprocess contamination *(39).* A survey carried out by the Health Protection Branch, Health and Welfare Canada, between 1974 and 1976 to establish microbiological standards for cheese *(7)* showed the need for improvement in sanitation practices since high levels of total and fecal coliforms were found. Other studies on different types of cheeses from pasteurized milk *(8, 9, 25, 26, 27)* reveal, in most cases, coliform or *Enterobacteriaceae* population levels far above satisfactory standards of hygiene. The mean of coliform levels in 94 market samples of Spanish white cheese (Burgos) made from pasteurized milk was of 5.01 log CFU/g *(6).* These counts exceed the Spanish microbiological standard for cheeses made from pasteurized milk, established at $m = 1{,}000$ and $M = 10{,}000$ ($n = 5$ and $c = 2$) for *Enterobacteriaceae* per g and for the presence of *Escherichia coli* per g at $m = 100$ and $M = 1{,}000$ (for $n = 5$ and $c = 2$),where n is the number of subsamples for analysis, c is the number of subsamples permitted to exceed acceptable values, m is the level of acceptable contamination, and M is the level of organisms considered entirely unacceptable.

In our study, high-pressure treatments were applied to cheese to eliminate the contamination due to the cheese-making process. They are not substitutes for the thermal treatment that has to be applied to the milk. The aim of this study was, on the one hand, to study the effect of different pressures, times, and temperatures on the lethality of *Escherichia coli* inoculated in white cheese from goat's milk, and on the other hand, to observe the microbiological changes in these pressurized cheeses stored in refrigeration. For this reason, the results concerning lethality of aerobic mesophilic microorganisms are included.

MATERIALS AND METHODS

Cheese making

Murciano-granadina goat's milk collected from the herd of the Universitat Autònoma de Barcelona farms was

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heat-treated at 95 ± 2°C for 2 min in the continuous tubular heat exchanger of our pilot plant. Pasteurized milk was inoculated with *Escherichia coli* 405 CECT at a rate of 10^6 CFU/ml. $CaCl_2$ (35%, 0.2 ml/liter of milk) and rennet (520 mg chymosin/liter, 0.2 ml/liter of milk) were added to inoculated milk. Coagulation took place at 32°C. After 45 min, the curd was cut in cubes of 2 cm^3. Ten minutes after cutting, curd was left to drain in metallic baskets which were covered with cotton gauzes. A pressure of 0.02 kg/cm^2 was applied to the cheeses for 1.5 h during whey drainage, which was performed at room temperature for 3 h. During this period of time the cheeses were turned twice. The cheeses were divided in four pieces of 250 ± 25 g. These portions were vacuum packaged and kept under refrigeration until they reached the treatment temperature (2, 10, or 25°C). Portions that were used as controls were kept under refrigeration until they were analyzed.

Throughout the whole process of cheese making rigorous hygiene measures were applied to ensure that no additional enterobacteria or other contaminating microorganisms could contaminate the cheeses. To ensure this, a cheese made from pasteurized milk without inoculated enterobacteria was made simultaneously and tested as a control cheese from an uninoculated milk sample (CCUM).

Physicochemical analyses

Total solids content was determined by drying the cheese in a 102 ± 2°C oven until a constant weight was reached *(13)*. Gravimetric analysis for ash content was performed after the sample was calcinated in an oven at 550°C *(14)*. The Gerber method for fat content was applied, with Van Gulik modification for cheeses *(15)*. For quantitative analysis of total nitrogen, the digestion block method involving a modification of the Kjeldahl method was used *(16)*. A potentiometric measurement of pH was performed with a penetration pH meter CRISON micro-pH 2001 combination electrode *(32)*.

Microbiological analyses

Ten grams of cheese sample were homogenized in 90 ml of a 2% sodium citrate solution *(30)* at 45°C for 2 min in an electromechanical blender *(32)*. Decimal dilutions were prepared in Ringer solution. The number of aerobic mesophilic bacteria was determined with plate count agar (PCA) (Oxoid CM325, Basingstoke, U.K.). Plates were incubated at 30°C for 48 h *(12)*. Violet red bile agar (VRBA) (Oxoid CM107) was used to determine the numbers of *Escherichia coli* in inoculated milk, in control cheeses, and in pressurized cheeses. Purple colonies with a halo of the same color were counted. Results are expressed as the logarithm of colony-forming units (CFU) per gram or milliliter. Lethality was calculated as the logarithm of the initial count minus the logarithm of survivors after pressure treatment.

To verify the total destruction of *E. coli,* samples of treated cheeses were collected randomly. We considered the cheese to be the best medium to allow the repair of injured cells, in view of the high values of pH and a_w of cheese samples. Moreover, the microorganisms had a sufficient source of carbon (lactose) because the drainage of whey had not been extensive. The whole portion of pressurized cheese was incubated at 37°C for 18 h to permit the repair of sublethally injured microorganisms. Serial dilutions of the incubated samples were plated in violet red bile agar to determine the presence of *E. coli.*

High-pressure treatment

The equipment used was a discontinuous isostatic press from ACB (Nantes). The time needed to achieve the treatment pressure was between 3 and 4 min; depending on the pressure required, the decompression time was between 90 and 120 s. The pressure chamber and the water inside were cooled or heated to the treatment temperature with the constant flow of an ethylene glycol-water mixture.

Ultra-high pressure (400, 450, and 500 MPa), temperature (2, 10, and 25°C), and time (5, 10, and 15 min) combinations were assayed in cheese samples from inoculated milk after they had reached the treatment temperature. Each treatment was applied to 4 portions of cheese at the same time. These samples were designated pressurized cheeses from inoculated milk (PCIM) and were used to follow the population changes 1, 15, 30, and 60 days after treatment. Each pressurization treatment was performed three times. From each production, 4 portions that were not treated were considered control cheeses from inoculated milk (CCIM) samples. During the population study control and pressurized cheeses were stored at 2 to 4°C.

RESULTS AND DISCUSSION

The composition of experimental Mató cheese is shown in Table 1. Fresh commercially available cheeses *(21, 29)* show total solids values between 33 and 45.3%. The content of fat and nitrogen depends on the source of the milk but, in general, values for commercial cheeses are close to the values of our experimental cheeses. The pH depends on the addition of preservatives during cheese making; pH values were between 5 and 6.7 *(10, 21)*. Our pH values were measured 24 h after cheese making; all of them were within the optimal pH range for the growth of pathogens *(17)*. The content of NaCl of commercial cheeses varies from 0.3 to 1.51, and a_w values between 0.981 and 0.999 *(10, 21)*. Water activity was not measured, but the high moisture content, the absence of added salt, and the fact that this is not a ripened cheese imply that the a_w values of this cheese are optimum for the growth of most microorganisms.

In Table 2, microbial counts of uninoculated milk (UM), inoculated milk (IM), control cheese from uninoculated milk (CCUM) and control cheese from inoculated milk (CCIM)

TABLE 1. *Physicochemical characteristics of Mató cheese*

	Mean (n = 8)	SD
% Total solids	37.62	1.701
% Fat	21.60	1.161
% Total nitrogen	2.05	0.120
% Ash	1.51	0.098
pH	6.52	0.068

TABLE 2. *Effect of high pressure at 10 and 25°C on microbial counts in Mató cheese assayed on plate count agar (PCA) or on violet red bile agar (VRBA)*

	log CFU per ml or g					
	PCA			VRBA		
Sample[a]	Mean[b]	SD	Range	Mean[b]	SD	Range
UM	3.27	0.78	4.6×10^1–1.0×10^4	n.d.[c]	—	—
CCUM	4.70	1.27	3.9×10^2–5.9×10^5	n.d.	—	0–1[d]
PCUM	2.59	0.58	2.0×10^1–1.2×10^3	n.d.	—	0–1[d]
IM	6.00	0.39	3.0×10^5–3.3×10^6	6.14	0.35	4.1×10^5–3.3×10^6
CCIM	8.48	0.21	1.3×10^8–5.4×10^8	8.46	0.17	1.4×10^8–3.9×10^8
PCIM	2.53	0.52	0–1.9×10^3	—	—	0–7.6×10^2[e]

[a] UM, uninoculated milk; CCUM, control cheese from uninoculated milk; PCUM, pressurized cheese from uninoculated milk; IM, inoculated milk; CCIM, control cheese from inoculated milk; PCIM, cheese from inoculated milk.
[b] $n = 9$ for IM and UM; 12 for CCUM; 18 for CCIM; 16 for PCUM; and 54 for PCIM.
[c] n.d.: Counts not detectable.
[d] Counts not detectable; 1 CFU in some plates.
[e] Counts not detectable in 98.1% of samples.

TABLE 3. *High-pressure effects on the survival of* E. coli *inoculated into fresh cheese*

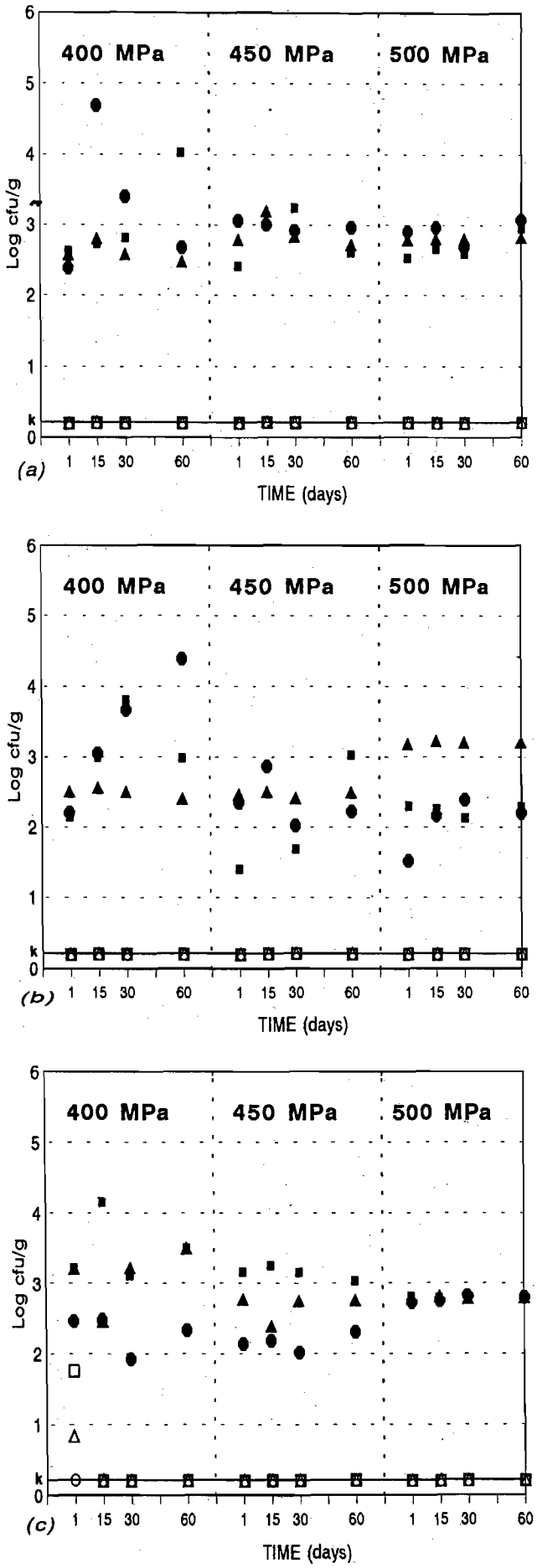
	log CFU/g								
	400 MPa Time (min)			450 MPa Time (min)			500 MPa Time (min)		
Temp./ Sample[a]	5	10	15	5	10	15	5	10	15
2°C/CCIM	7.65	7.65	7.98	7.98	8.46	8.72	7.65	8.46	8.72
PCIM	n.d.[b]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10°C/CCIM	8.65	8.74	8.65	8.22	8.74	8.70	8.70	8.45	8.22
PCIM	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
25°C/CCIM	8.45	8.45	8.11	8.45	8.64	8.11	8.64	8.64	8.64
PCIM	1.71	0.77	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
L[c]	6.74	7.68							

[a] CCIM, control cheese from inoculated milk; PCIM, pressurized cheese from inoculated milk.
[b] n.d., not detectable.
[c] Lethality, L: CCIM − PCIM.

TABLE 4. *High-pressure effects on aerobic mesophilic bacteria in fresh cheese inoculated and uninoculated with* Escherichia coli

	log CFU/g								
	400 MPa Time (min)			450 MPa Time (min)			500 MPa Time (min)		
Temp./ Sample[a]	5	10	15	5	10	15	5	10	15
2°C/PCIM	2.63	2.58	2.39	2.40	2.79	3.06	2.53	2.79	2.90
10°C/CCUM		2.60		3.13	2.60			5.77	3.13
PCUM		2.32		1.30	2.48	2.46	2.30	3.27	1.30
PCIM	2.14	2.50	2.20	1.40	2.46	2.35	2.29	3.17	1.52
L_a		0.28		1.83	0.12			2.50	1.83
L_b		0.10		1.73	0.14			2.60	1.61
25°C/CCUM	5.77	5.77	4.24	5.77	5.70	4.24	5.70	5.70	5.70
PCUM	3.10	3.04	2.88	3.04	2.79	2.69	2.68	2.71	2.66
PCIM	3.22	3.20	2.46	3.16	2.77	2.14	2.81	2.78	2.73
L_a	2.67	2.73	1.36	2.73	2.91	1.55	3.02	2.99	3.04
L_b	2.55	2.57	1.78	2.61	2.93	2.10	2.89	2.92	2.97

[a] CCUM, control cheese from uninoculated milk; PCUM, pressurized cheese from uninoculated milk; PCIM, pressurized cheese from inoculated milk.
[b] Lethality: L_a, CCUM − PCUM; L_b, CCUM − PCIM.



can be compared. Between uninoculated milk and cheese made from this milk an increase of 1.4 logarithmic units in aerobic mesophilic counts is observed. An increase of more than 2 logarithmic units in either aerobic mesophilic counts or VRBA counts is observed when we compare inoculated milk (IM) and cheese made with inoculated milk (CCIM). Spahr and Url *(35)* explain that the increase of 1 log unit in the bacterial counts during the first steps of cheese making is due to the physical concentration phenomenon resulting from syneresis of the curd rather than to bacterial growth. Due to the stringent hygienic measures and controlled conditions of cheesemaking in our study, we attribute the increase of counts from uninoculated milk (UM) to cheese (CCUM) to this phenomenon of concentration.

Studies that have examined the behavior of pathogenic *E. coli* during the manufacture of hard cheese have demonstrated that *E. coli* counts can show a remarkable increase during the manufacture of cheese, up to three orders of magnitude *(35)*. Most studies that demonstrate the growth of different species of pathogenic microorganisms during the cheese-making process have been found to involve inocula grown in favorable conditions prior to addition to pasteurized milk *(17)*. Johnson et al. *(17)* state that these circumstances differ from factory conditions, where microorganisms that survive pasteurization are probably not in the same optimal state. The increase of about 2.4 logarithmic units of inoculated bacteria from inoculated milk (IM) to control cheese from inoculated milk (CCIM) could be considered a consequence of physical concentration and growth of the inocula during cheese making (Table 2).

Growth of typical colonies in VRBA was not detectable in uninoculated milk (UM) or in cheese samples made from uninoculated milk (CCUM) (Table 2). It may therefore be concluded that all the microorganisms cultured on VRBA plates from inoculated milk (IM) samples and cheeses made from this milk (CCIM) are from the initial population of *E. coli* inoculated into pasteurized milk. For this reason no further identification of colonies that developed in VRBA was undertaken. Moreover, there were no differences between PCA counts and VRBA counts in inoculated milk (IM) and control cheeses made from inoculated milk (CCIM) and on the other hand the increase of counts from inoculated milk to cheese is the same in both PCA and VRBA.

From the data in Table 2 we can infer that pressurization treatment kills all of the inoculated microorganisms, and also some of the microorganisms that survived the pasteurization treatment.

In Table 3, counts in VRBA for all treatments are shown. In two of the treatments performed at 2°C (400 MPa, 5 min, 450 MPa, 15 min) 1 colony appeared on one of the 6 VRBA plates of the first dilution. In all the other treatments no colonies were detected on any plate. At 10°C no colonies

FIGURE 1. *Log CFU/g of aerobic mesophilic bacteria (■: 5 min, ▲: 10 min, ●: 15 min) and inoculated Escherichia coli (□: 5 min, △:10 min, ○:15 min) surviving high hydrostatic pressure treatments (a) 2°C, (b) 10°C, (c) 25°C:* indicates the limit for bacterial enumeration.

were detected on any plates, except for one treatment (450 MPa, 10 min), in which 2 and 5 colonies were found on 2 of the 6 plates from the first dilution. At 25°C it is possible to observe detectable survivors growing in treatments at 400 MPa, 5 min and 400 MPa, 10 min. In view of these results, lethality achieved with the treatments assayed is between 7.6 and 8.7 logarithmic units, except for one case at 6.7. In the literature, counts of coliforms and enterobacteria in similar types of cheeses *(1, 6, 10, 22, 24, 25, 33, 38)* in most cases exceed 10^4 CFU/g. These cheeses are made from pasteurized milk without the addition of starter cultures and most of them are salted, while Mató is not salted. This difference suggests a high probability of finding higher counts of enterobacteria in Mató than in the cheeses in studies cited. Our results indicate that high-pressure treatment of Mató cheese is able to inactivate between 10^7 and 10^8 CFU/g of *E. coli.*

At 25°C numbers of surviving *E. coli* following high-pressure treatment were detected in this study. This reduced inactivation of *E. coli* at room temperature has been observed previously by other authors working with nutrient, buffered, or physiological saline solutions *(19, 34, 36).* Carlez et al. *(5)* extensively inactivated uninoculated coliforms in chilled minced beef, working at 450 MPa and 20°C for 20 min. In minced beef another coliform (*Citrobacter freundii*) has been studied *(4);* extensive destruction was achieved at 300 MPa and 20°C for 20 min. They also observed that working at lower or higher temperatures than room temperature enhanced the destructive effect of pressure.

The decimal reduction of aerobic mesophilic counts has been calculated in some treatments (Table 4). It was not possible to inactivate all bacteria due to the presence of bacterial spores that survived the pasteurization treatment and which are resistant to the pressure treatments applied *(3, 11, 31).* Other groups of microorganisms that could be present in cheese are *Micrococcaceae,* which can survive pasteurization, and lactic acid bacteria that contaminated the cheese during manufacturing. These two groups of microorganisms would be inactivated by some of the high-pressure treatments applied, while bacterial spores would survive and be detected in PCA counts of cheese after pressure treatment. Final counts of aerobic mesophilic bacteria in cheese must be regarded in absolute values since we have not identified the microbial groups initially present in the cheese and have not established their frequency of distribution. Great variability in either initial or final counts can be expected because of varying factors in milk production and handling. The difficulty of identifying all the microbial groups present in cheese and their frequency of distribution makes necessary work on individually controlled strains of microorganisms inoculated into cheese.

Figures 1a, 1b, and 1c show the development of uninoculated aerobic mesophilic bacteria and *E. coli* which survived high-pressure treatments at 2, 10, and 25°C respectively. Counts were determined after 15, 30, and 60 days. A maintenance of the initial population is observed in most cases. In three of the lower-pressure treatments we have applied (see Figure 1) a small increase of surviving counts is observed from the first day after treatment. It can be attributed to growth of vegetative cells that were not inactivated by the pressure treatment. With the most vigorous treatments applied, no growth during refrigerated storage has been observed, so we assume that only bacterial spores would have survived.

Fifteen days after treatment no survivors of *E. coli* were detected even with cheeses that showed survivors counts immediately after treatment. One colony-forming unit appeared on 1 or 2 of the plates inoculated with the first dilution. Carlez et al. *(5)* studied the development of total coliforms surviving 300 MPa and 20°C for 20 min during chilled storage of minced beef and they found that when total coliforms are not completely inactivated their initial level is restored after 13 days during storage under vacuum but that counts did not increase during storage in air. Our results indicate that although some *E. coli* cells survived the high-pressure treatment they were not capable of surviving refrigeration, as no colonies were detected on VRBA plates starting from 15 days after treatment.

Experiments to confirm the total destruction of *E. coli* were performed with cheeses treated at 500 MPa and 10 min for 10°C and 400 MPa and 5 min for 25°C 1 day after treatment, and with cheeses treated at 400 MPa and 5 min for 25°C and 450 MPa and 5 min for 25°C 15 days after treatment. In all cases no growth on VRBA was detected.

To conclude, we think that the effect of high pressure on the bacterial flora of cheese may serve to significantly extend the refrigerated storage life of fresh Mató cheese. Studies on other microbial populations and organoleptic and physicochemical characteristics are being undertaken to obtain more information about the effects of these treatments on fresh cheese from goat's milk.

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