

Persistence of *Mycobacterium paratuberculosis* during Manufacture and Ripening of Cheddar Cheese

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Model Cheddar cheeses were prepared from pasteurized milk artificially contaminated with high 10^4 to 10^5 CFU/ml and low (10^1 to 10^2 CFU/ml) inocula of three different *Mycobacterium paratuberculosis* strains. A reference strain, NCTC 8578, and two strains (806PSS and 796PSS) previously isolated from pasteurized milk for retail sale were investigated in this study. The manufactured Cheddar cheeses were similar in pH, salt, moisture, and fat composition to commercial Cheddar. The survival of *M. paratuberculosis* cells was monitored over a 27-week ripening period by plating homogenized cheese samples onto HEYM agar medium supplemented with the antibiotics vancomycin, amphotericin B, and nalidixic acid without a decontamination step. A concentration effect was observed in *M. paratuberculosis* numbers between the inoculated milk and the 1-day old cheeses for each strain. For all manufactured cheeses, a slow gradual decrease in *M. paratuberculosis* CFU in cheese was observed over the ripening period. In all cases where high levels ($>3.6 \log_{10}$) of *M. paratuberculosis* were present in 1-day cheeses, the organism was culturable after the 27-week ripening period. The *D* values calculated for strains 806PSS, 796PSS, and NCTC 8578 were 107, 96, and 90 days, respectively. At low levels of contamination, *M. paratuberculosis* was only culturable from 27-week-old cheese spiked with strain 806PSS. *M. paratuberculosis* was recovered from the whey fraction in 10 of the 12 manufactured cheeses. Up to 4% of the initial *M. paratuberculosis* load was recovered in the culture-positive whey fractions at either the high or low initial inoculum.

Mycobacterium paratuberculosis is the etiologic agent of paratuberculosis (Johne's disease), a chronic granulomatous enteritis in ruminants and other animals (7, 8). The organism is excreted in the feces of animals with the pluribacillary form of the disease; colostrum and milk can also be infected systemically (40, 43) or contaminated with feces during the milking process. Crohn's disease, an intestinal inflammatory disorder that affects humans, shows many similarities to Johne's disease, both affecting the ileum and causing chronic diarrhea and weight loss. *M. paratuberculosis* has been isolated from humans suffering from Crohn's disease (4, 5, 6, 12, 44). However, when Johne's and Crohn's diseases are compared clinically and pathologically, several significant differences can be seen. These similarities and differences have been interpreted both in favor and in opposition to the view that *M. paratuberculosis* is a cause of Crohn's disease (13). In response to this, the United Kingdom government has advocated a precautionary approach and has supported actions to minimize exposure of the public to *M. paratuberculosis*.

Raw milk from an *M. paratuberculosis*-infected dairy herd is likely to be contaminated with *M. paratuberculosis* through direct excretion into the milk or via fecal contamination (32). Sweeney et al. (43) reported low levels (2 to 8 CFU/50 ml) of viable *M. paratuberculosis* cells present in milk from cows with clinical Johne's disease or asymptotically infected cows in

the latter stages of infection, whereas clinically infected animals can shed up to 10^{12} cells per day in feces (9). Therefore, raw cows' milk may act as a source of the bacterium for humans if control measures such as pasteurization are ineffective in killing this pathogen.

M. paratuberculosis is more resistant to adverse conditions such as low pH, high temperature, and salt than most other pathogenic bacteria (42). Several studies investigating the heat resistance of *M. paratuberculosis* have demonstrated the ineffectiveness of standard pasteurization regimens, including high-temperature, short-time pasteurization, to totally kill *M. paratuberculosis* in artificially infected raw milk (7, 15, 16, 23, 26, 30, 38, 41). *M. paratuberculosis* has also been cultured from naturally infected cows' milk after commercial-scale pasteurization (19). Furthermore, a more recent study has provided firm evidence of the presence of viable *M. paratuberculosis* in commercially pasteurized cows' milk manufactured for retail sale (18). Therefore, if *M. paratuberculosis* is present in raw milk and is not effectively inactivated by pasteurization, there is a possibility that the bacterium may exist in a viable form in other dairy products, such as cheese, manufactured from such milk.

Since the inefficacy of commercial high-temperature, short-time pasteurization to completely inactivate this potentially human-pathogenic mycobacterium has been demonstrated, efforts have concentrated on modifying the time and temperature parameters of pasteurization to improve its lethality for *M. paratuberculosis* in liquid milk (19, 22, 28). However, these modifications, while appropriate for liquid milk, may affect Cheddar cheese yield and textural properties (14), and therefore it is likely that standard pasteurization conditions (72°C

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for 15 s) or perhaps even those with a lower lethality will continue to be used for milk used in Cheddar cheese production.

In addition, *M. paratuberculosis* cells typically occur as clumps (20), which are dispersed to some extent during homogenization of retail milk prior to pasteurization (28). It has been suggested that declumped *M. paratuberculosis* was more susceptible to heat treatment (17). However, during Cheddar cheese making, liquid milk is not normally homogenized prior to pasteurization, which could increase the likelihood of clumped *M. paratuberculosis* cells being present in cheese milk. Also, during hard cheese manufacture, a major portion of the microbial load will be entrapped in the milk coagulum and during syneresis will be further concentrated in the cheese curd (35). Therefore, if viable *M. paratuberculosis* cells were to survive pasteurization, they would be concentrated in the cheese curd.

The inactivation of *M. paratuberculosis* in cheese milk will also depend on the environmental conditions of Cheddar manufacture and maturation vis à vis cheese cooking temperature, low moisture, low pH, salt content, prolonged low-temperature ripening, and the antagonistic effect of cheese starter and non-starter microflora. The in vitro effect of these parameters on *M. paratuberculosis* survival cannot predict the persistence of *M. paratuberculosis* during the dynamic of Cheddar manufacture and ripening. To assess the cumulative effect of these conditions on the persistence of *M. paratuberculosis*, it is necessary to manufacture Cheddar cheese on a laboratory scale to simulate commercial production. There has been no published research on the survival of *M. paratuberculosis* in Cheddar cheese, the major hard cheese produced worldwide.

To our knowledge, only two studies have investigated *M. paratuberculosis* survival in cheese (35, 42). One prepared a soft Hispanic-style cheese under laboratory conditions (35), and the former used a pilot-scale process to manufacture model hard (Swiss Emmentaler) and semihard (Swiss Tisliter) cheese (42). Both of these cheeses have distinct characteristics that differ from Cheddar. The Hispanic-style soft cheese has higher water content, higher pH, and a lower maximum temperature during manufacture than Cheddar. The Swiss Emmentaler cheese, although a hard cheese, differs from Cheddar through lower salt content, higher cheese cooking temperature, and the inclusion of propionic acid bacteria in its manufacture. The calculated *D* value for the Hispanic-style soft cheese was 59.9 days compared to 27.8 days for Swiss hard cheese and 45.5 days for Swiss semihard cheese.

The aim of this work was to study the persistence of *M. paratuberculosis* during the manufacture and ripening of Cheddar cheese with a laboratory-scale Cheddar cheese production system, simulating commercial Cheddar production.

MATERIALS AND METHODS

***M. paratuberculosis* strains.** Three strains of *M. paratuberculosis* were tested: type strain NCTC 8578 (obtained from the National Collection of Type Cultures, London, United Kingdom) and strains 806PSS and 796PSS, which were originally isolated from pasteurized cows' milk (17) (kindly provided by I. Grant, Department of Food Science, Queens University of Belfast, Belfast, United Kingdom). The field isolates originated from different areas within the United Kingdom, and although they showed similar pulsed-field gel electrophoresis profiles, the strains were distinguishable by restriction fragment length polymorphism typing (I. Grant, personal communication).

Preparation of inoculum for cheese making. Each strain was cultured in Middlebrook 7H9 broth medium containing 10% (vol/vol) Middlebrook OADC (oleic acid-albumin-dextrose-catalase; Becton Dickinson United Kingdom Ltd., Oxford, United Kingdom), 0.5% (vol/vol) Tween 80 (Sigma, Dorset, United Kingdom), and 0.0002% (wt/vol) mycobactin J (Synbiotics Europe SAS, Lyon, France) from 6-week-old (37°C) colonies grown on Herrold's egg yolk medium (HEYM) containing 2 µg of mycobactin J/ml (Synbiotics Europe SAS, Lyon, France). Culture broths (100 ml) were incubated at 37°C for 8 to 10 weeks. Cell suspensions were agitated with a Vortex Genie 2 (Scientific Industries Inc., Bohemia, N.Y.) for 3 min to disperse large clumps of *M. paratuberculosis* and decimally diluted in maximum recovery diluent (Oxoid, Basingstoke, United Kingdom) to achieve the desired inoculation level. Agitation of the cell suspension and its subsequent dilution were performed immediately before inoculation of the pasteurized cheese milk.

Origin of milk and testing. A mixture of evening and morning raw bovine milk from a local dairy farm was used for cheese making. All milk was pasteurized (72°C for a minimum of 15 s) on-farm and poured into sealed pint glass bottles. Cheese making with the whole-milk samples was performed on the same day as pasteurization. Milk samples were maintained at or below 5°C between pasteurization and commencement of cheese making. All milk was subjected to phosphatase testing by the Aschaffenberg-Mullen method (1) to verify pasteurization and tested for the presence of inhibitory substances with the Delvotest SP (DSM Food Specialties, Delft, The Netherlands). The titratable acidity of milk and whey was used to monitor the manufacturing process (34). Milk samples were tested for the presence of *M. paratuberculosis* on the test media discussed below.

Cheddar cheese-making facility. Cheddar cheeses were manufactured according to traditional procedures (34) with the cheese-making apparatus described by Banks and Muir (3). A rectangular stainless steel vat (305 by 200 by 200 mm, 10-liter capacity), with a centrally sloping floor, was fitted with a motorized paddle to stir the milk during cheese making. The vat was completely enclosed within a stainless steel jacket with a uniform 25-mm space all round through which water was circulated by an electronically controlled flow heater (Julabo F34; Jencons, Leighton Buzzard, United Kingdom). Cheese knives and a cheddaring box similar to those used by Banks and Muir (3) were used for the coagulum cutting and cheddaring processes, respectively. Strict safety precautions were taken to prevent any infection of personnel, contamination of the environment, and cross-contamination between trials. Cheddar manufacture and ripening were performed in a containment level 2 laboratory, and the cheese vat was located in a class 1 biological safety cabinet (200 by 70 by 60 cm; Rayair, Mach-Aire, Bolton, United Kingdom).

Manufacture and ripening of cheese. A total of 12 *M. paratuberculosis*-spiked Cheddar cheeses were manufactured. For each of the three *M. paratuberculosis* strains, duplicate cheeses were prepared at high (10^4 to 10^5 CFU/ml) and low (10^1 to 10^2 CFU/ml) inoculation levels. Two control cheeses were prepared without artificial *M. paratuberculosis* contamination. Eight liters of pasteurized milk (4°C) was added to the cheese-making vat and inoculated with 8 ml of *M. paratuberculosis* culture. The milk was warmed to 32°C over a 45-min period, and the mesophilic/thermophilic homofermentative starter culture YY80 (Chr. Hansen, Berkshire, United Kingdom) was added to a final concentration of 0.009% (vol/vol). Starter inoculum was prepared by dispersing 10 g of frozen direct vat set culture in 90 ml of maximum recovery diluent. Rennet (Hannilase XL 205; Chr. Hansen) was added, after appropriate acid development, to induce coagulation. Curds were cut into cubes (approximately 4 by 4 by 8 mm), and the temperature was increased linearly to 40°C over 40 min. The temperature of the vat was maintained at 40°C for a further 60 min.

The cheese curd was allowed to settle (pitched), and the whey was drained. Cheddaring of the curd was performed first by manual folding of the curd mass, followed by progressive application of weights up to 16 kg over 60 min. The curd was milled by cutting into cubes (30 by 20 by 20 mm) and salted (2.5%, wt/wt). Cheeses were pressed in a 10-cm-diameter form in a Moorlands cheese press (type S2; Moorlands Cheesemakers, Somerset, United Kingdom) at a pressure of 50 kPa for 16 h at 10°C. The pressed curd was removed from the molds, sampled for microbiological analysis (see below), vacuum sealed in food-compatible polyamide-polyethylene pouches (Somerville Packaging, Lisburn, N. Ireland), vacuum packed (Mini Jumbo; Henkelman Vacuum Systems, Hertogenbosch, The Netherlands), and stored at 10°C for up to 27 weeks.

Sampling, culture, and enumeration. A 50-ml portion of pasteurized cheese milk was taken before adding the *M. paratuberculosis* inoculum to check for natural contamination of the milk with *M. paratuberculosis*. The presence or absence of viable cells was confirmed by the decontamination and culture method, while immunomagnetic separation-PCR was used to confirm the presence or absence of live or dead cells in the pasteurized cheese milk (18).

During cheese manufacture, 10-ml aliquots of contaminated pasteurized milk

TABLE 1. Compositional analysis of model Cheddar cheese artificially contaminated with *M. paratuberculosis*

Parameter ^a	Value by <i>M. paratuberculosis</i> strain					Value by inoculum level		
	806PSS	796PSS	NCTC 8578	Control	SEM ^b	High	Low	SEM
Yield (g)	761	778	791	781	11.8 NS	778	775	9.6 NS
pH	5.20	5.20	5.21	5.21	0.017 NS	5.21	5.20	0.014 NS
Moisture (%)	37.5	37.4	37.5	38.0	0.24 NS	37.1	37.8	0.20*
Fat (%)	31.0	32.5	32.8	31.8	0.40 NS	32.3	31.9	0.31 NS
Salt (%)	1.53	1.51	1.61	1.52	0.03 NS	1.55	1.55	0.02 NS
Nonfat (%)	69.3	67.5	67.3	68.1	0.43*	67.7	68.3	0.35 NS
Dry matter (%)	62.3	63.1	63.0	62.0	0.42 NS	62.9	62.7	0.34 NS
% Salt:moisture	4.1	4.0	4.3	4.0	0.07 NS	4.2	4.1	0.06 NS
% Moisture nonfat substance	54.1	55.4	55.7	55.7	0.35*	54.7	55.4	0.28 NS
% Fat:dry matter	49.7	51.5	52.0	51.3	0.65 NS	51.4	50.8	0.53 NS

^a For % salt:moisture, the suggested range of percentages for first-grade cheeses was 52 to 54%; for second-grade cheeses, 50 to 56%. For % moisture nonfat substance, the suggested range of percentages for first-grade cheeses was 4.7 to 5.7%; for second-grade cheeses, 4.0 to 6.0%. For % fat:dry matter, the suggested range of percentages for first-grade cheeses was 52 to 56%; for second-grade cheeses, 50 to 57%.

^b $P > 0.05$ was considered not significant (NS). *, $P < 0.05$; **, $P < 0.01$.

and drained whey were used to determine the contamination level of the cheese milk and whey. Decimal dilutions of milk were prepared in maximum recovery diluent and inoculated onto the media described below.

Cheese samples were taken after 1 day and 3, 10, 18, and 27 weeks of ripening. The vacuum-sealed cheeses were unpacked, and two 2.5-g samples were taken with a cheese borer (1 by 10 cm). Cheeses were vacuum sealed again within 10 min. Five grams of cheese plug was aseptically transferred into a sterile stomacher bag containing 45 ml of cheese diluent. The cheese diluent comprised 0.5% (wt/vol) sodium chloride, 1% (wt/vol) Casitone (Difco, Detroit, Mich.), and 2.0% (wt/vol) sodium citrate. The mixture was homogenized in a Stomacher peristaltic Lab Blender 400 (Seward Medical, London, United Kingdom) for 2 min, heat sealed, and incubated for 1 h submerged in a water bath at 37°C. The resultant suspension was decimally diluted in maximum recovery diluent prior to microbiological analysis.

All liquid samples were inoculated (200 μ l) onto two slopes of each of the following media: HEYM supplemented with an antibiotic cocktail containing vancomycin (8.4 μ g/ml), amphotericin B (16.8 μ g/ml), and nalidixic acid (25 μ g/ml) (all from Sigma) (HEYM-VAN); 7H10-PANTA agar consisting of Middlebrook 7H10 agar base (Difco) supplemented with 10% (vol/vol) Middlebrook OADC enrichment, 2.0% (vol/vol) reconstituted PANTA Plus antibiotic supplement (polymyxin, amphotericin B, nalidixic acid, trimethoprim, and azlocillin; Becton Dickinson United Kingdom Ltd., Oxford, United Kingdom), and 0.5% glycerol. Two 0.5-ml aliquots of the cheese suspension were also injected into vials of Bactec 12B radiometric medium supplemented with 50 μ l of reconstituted PANTA Plus antibiotic supplement (both from Becton Dickinson United Kingdom Ltd.). All culture media were supplemented with 2 μ g mycobactin J ml⁻¹. All media were incubated at 37°C for up to 12 weeks, and colonies typical of *M. paratuberculosis* on the agar medium were counted. A representative number of typical and atypical colonies were confirmed by Ziehl-Neelsen acid-fast staining and IS900 PCR (32). Bactec vials were read regularly on a Bactec 460TB instrument (Becton Dickinson United Kingdom), and growth index values were recorded.

Starter lactic acid bacteria were enumerated with MRS agar (Oxoid, Basingstoke, United Kingdom), and Rogosa agar (Oxoid) was used to monitor changes in lactobacilli throughout the ripening period. Both media were incubated at 30°C and 5% CO₂ for a minimum of 48 h.

Cheese compositional analysis. Standard procedures as described by Kirk and Sawyers (27) were used to analyze cheeses for pH, moisture, salt, and fat (Gerber method). Cheese plugs were decontaminated by irradiation prior to chemical analysis to ensure the safety of personnel undertaking the chemical analysis. Irradiation was performed to a dose of 25 kGy with a Gammabeam 650 facility (Nordion International Inc. Canada) available on site.

Statistical analysis. Survival *D* values were calculated from the slope of the trend analysis line generated in Microsoft Excel (version 9.0) by plotting the log₁₀ number of *M. paratuberculosis* per gram of cheese versus the ripening time. Analysis of variance was performed with Genstat Release 6.1 (for Windows 2000).

RESULTS

The manufactured Cheddar cheeses were similar in salt, moisture, and fat composition to commercial Cheddar (Table 1). The average pH value (5.21) was also similar to that of the commercial product. The design and health and safety measures in place for the laboratory-scale manufacturing process prevented cross-contamination between batches. The contamination of cheese milk with *M. paratuberculosis* did not appear to affect intrinsic Cheddar properties such as pH, fat, and moisture or the yield of cheese produced. All manufactured cheeses were within the specified parameters for first- or second-grade Cheddar. Starter lactic acid bacteria decreased slowly over the 27-week ripening period for all manufactured cheeses (Fig. 1). The titratable acidity values and cooking time and temperature parameters (Fig. 2), monitored throughout the manufacturing process were similar to those of commercial Cheddar manufacture.

Although all cheeses and cheese milk samples were inoculated onto HEYM/VAN and 7H10/PANTA agar media without a decontamination step, only the former was used to enumerate *M. paratuberculosis*. Counts of *M. paratuberculosis* could not be obtained from a number of 7H10/PANTA slopes

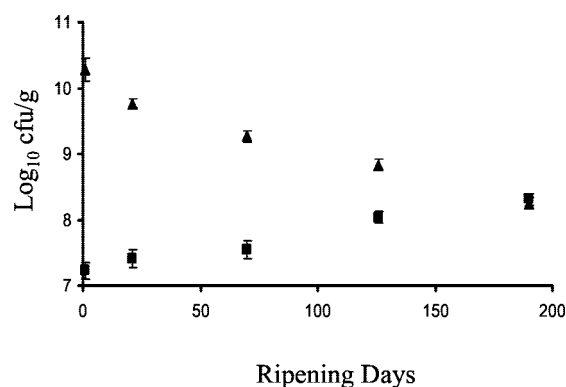


FIG. 1. Changes in lactic acid bacteria (triangles) and lactobacilli (squares) numbers during Cheddar cheese ripening. Error bars show standard deviation.

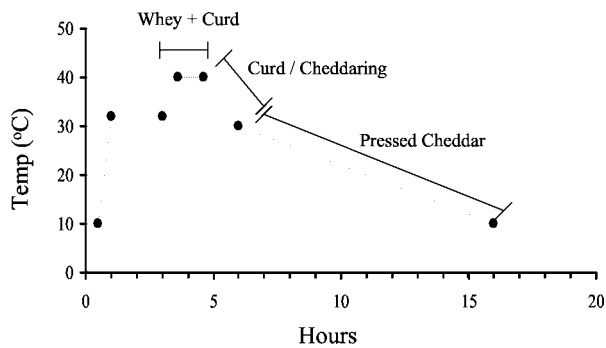


FIG. 2. Temperature changes during the first 16 h of Cheddar cheese ripening.

due either to contamination within 6 weeks of incubation or to the absence of growth. In both cases, *M. paratuberculosis* could be enumerated on the corresponding HEYM/VAN slopes. Where growth occurred on both agar media, colony counts were similar. On HEYM/VAN slopes, CFU were countable at 6 to 8 weeks, and the numbers did not change with prolonged incubation.

The number of *M. paratuberculosis* in the 1-day-old cheeses (per gram) was greater than the levels detected in the inoculated milk for all strains tested (Table 2). This concentration occurred when both high (10^4 to 10^5 CFU/ml) and low (10^1 to 10^2 CFU/ml) levels were present in the pasteurized cheese milk. This increase in counts, due to the physical concentration of bacteria in the cheese curd, was significantly ($P < 0.05$) higher for cheese contaminated with strains 806PSS and 796PSS compared to NCTC 8578. The average concentration effect for strains 806PSS, 796PSS, and NCTC 8578 are 11.7-, 9.3-, and 4.2-fold, respectively.

Figure 3 shows the survival of each of the three *M. avium* subsp. *paratuberculosis* strains at both high and low initial inocula over a 27-week ripening period. For all manufactured cheeses, a slow gradual decrease in *M. paratuberculosis* CFU/g of cheese was observed over the ripening period. In all cases where high levels ($>3.6 \log_{10}$) of *M. paratuberculosis* were present in 1-day cheeses, the organism was culturable after the 27-week ripening period. *D* values were calculated for cheeses spiked with high levels of *M. paratuberculosis*. The *D* values for strains 806PSS, 796PSS, and NCTC 8578 were 107, 96, and 90 days, respectively. However, the correlation of the trend anal-

TABLE 2. Changes in organism numbers^a

Strain inoculated or inoculum level	<i>M. paratuberculosis</i> count (\log_{10} CFU/ml or g)		Concn effect (\log_{10})
	Inoculated milk	Cheese (24 h)	
Strain			
806PSS	3.0	4.0	1.0
796PSS	3.1	4.1	0.9
NCTC 8578	2.6	3.1	0.6
SEM	0.70 NS	0.12 NS	0.11*
Inoculum			
High	4.0	4.7	0.8
Low	1.8	2.7	0.9
SEM	0.05 NS	0.10 NS	0.09 NS

^a See Table 1, footnote b.

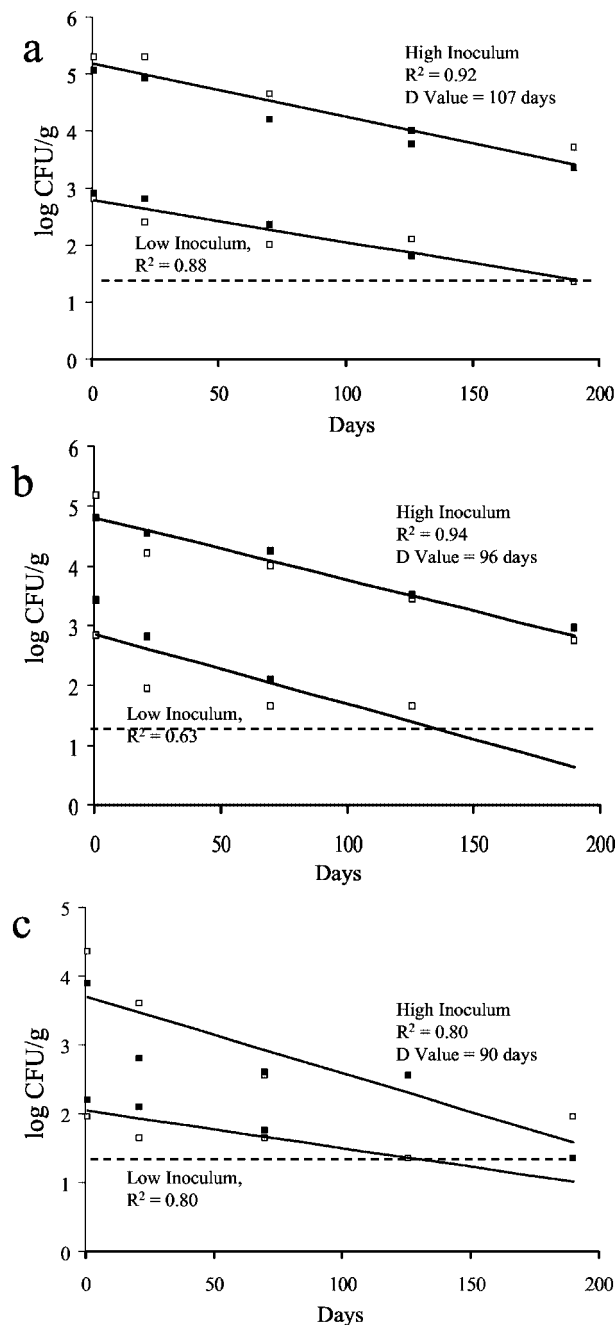


FIG. 3. Survival of *M. paratuberculosis* strains 806PSS (a), 796PSS (b), and NCTC 8578 (c) during Cheddar cheese ripening. Data are for each single model cheese at high and low initial inocula with trend analysis line applied. Open symbols, cheese 1; solid symbols, cheese 2. The dashed line indicates the detection limit.

ysis line for strain NCTC 8578 was poorer than that of the other strains at the high inocula. At these inocula, *M. paratuberculosis* was confirmed in the cheeses throughout the ripening period by the radiometric culture method.

At low levels of contamination, *M. paratuberculosis* was only culturable from 27-week-old cheese spiked with strain 806PSS. Despite the fact that cheeses containing low levels of strain 796PSS or strain 806PSS had similar levels of *M. paratubercu-*

TABLE 3. Recovery of *M. paratuberculosis* from the whey fraction released during Cheddar cheese manufacture

Strain (inoculum level)	<i>M. paratuberculosis</i> in whey ^a	
	HEYM/VAN, log ₁₀ CFU/ml (SEM)	Bactec 12B
806PSS (high)	2.7 (0.07)	+
806PSS (low)	0.4 (0.00)	+
796PSS (high)	2.2 (0.11)	+
796PSS (low)	0.8 (0.11)	+
NCTC8578 (high)	1.0 (0.29)	+
NCTC8578 (low)	ND	+

^a +, gradual increase in growth index value observed. ND, not detected.

At the start of the ripening period, counts of the former fell below the detection limit of 1.35 log₁₀ CFU/ml after 27 weeks of ripening. The number in cheeses contaminated with low levels of the reference strain NCTC 8578 also fell below the detection limit after 27 weeks of ripening, although starting levels in 1-day-old cheeses were lower than in both the other strains. At 27 weeks of ripening, one of the cheeses containing low levels of NCTC 8578 produced a growth index reading on Bactec 12B radiometric culture medium, indicative of *M. paratuberculosis*, but a positive identification could not be confirmed by subculture and growth on HEYM. Negative results from the radiometric culture were consistent with the absence of *M. paratuberculosis* in parallel samples that were cultured. After 18 weeks of ripening, strain NCTC 8578 (at the low inoculum) was detectable at the minimum level of detection in the replicate cheeses. *M. paratuberculosis* was only recovered from one of the cheeses contaminated with strain 796PSS at low levels after 18 weeks of ripening.

M. paratuberculosis was recovered from the whey fraction in 10 of the 12 cheeses manufactured from spiked milk (Table 3). The organism was not culturable from whey released from either of the cheeses contaminated with low levels of the reference strain NCTC 8578 but was detectable in both cheeses by the radiometric culture method. For both cheeses contaminated with high levels of this strain, less than 1% of the initial *M. paratuberculosis* load in cheese milk was recovered in the whey fraction. In the remaining manufactured cheeses, 1 to 4% of the initial *M. paratuberculosis* load was recovered in the whey fraction.

DISCUSSION

A laboratory-scale Cheddar cheese-making process was established which produced cheese with microbial and chemical composition similar to that of commercially produced Cheddar with recognized parameters. This process was used to monitor the persistence of *M. paratuberculosis* inoculated postpasteurization into the cheese milk throughout the manufacturing stage and during a 27-week ripening period (10°C). Raw milk for Cheddar cheese production is generally subject to the same pasteurization regimen as milk for retail sale, but occasionally a lesser heat treatment is used (24). Grant et al. (18) recovered low levels of viable *M. paratuberculosis* from pasteurized cows' milk for retail sale. Therefore, this study chose to artificially contaminate milk postpasteurization with low levels of *M. paratuberculosis*, given detection limitations, to reflect the possible

numbers surviving pasteurization. Higher inocula were also used in order to determine the kinetics of inactivation throughout the manufacturing and ripening stages, considering the insensitivity of viable quantitation methods.

There was an approximately 10-fold concentration of *M. paratuberculosis* strains 806PSS and 796PSS, originally isolated from pasteurized milk, from cheese milk to cheese curd. This increase was expected, as our cheese yield (9.7%) suggests that each liter of cheese milk produces approximately 100 g of curd following entrapment and syneresis. Although many pathogenic bacteria, including *Aeromonas hydrophila*, *Campylobacter jejuni*, *Escherichia coli*, *Salmonella enterica* serovar *Typhimurium*, and *Listeria monocytogenes*, do not survive the manufacturing process of hard cheese (2), the survival of *M. paratuberculosis* is not surprising given the previous high recovery of this organism from the curd of Swiss hard cheese (35).

The reference strain NCTC 8578 showed a lesser concentration in numbers from milk to curd. This reduction in numbers of the reference strain could have been due, inter alia, to the loss of this strain in the whey fraction or the partial deactivation of the *M. paratuberculosis* load during the manufacturing process. Although *M. paratuberculosis* was recovered from the whey fraction of cheese artificially contaminated with this strain, the levels were lower than that recovered in cheeses containing strains 806PSS and 796PSS. This would suggest no excessive partitioning of this strain in the whey fraction but rather loss during the manufacturing process. The parameters affecting bacterial survival during the manufacturing process include starter culture composition, curd cooking temperature, pH and NaCl concentration, and, most likely, a combination of all of these factors. Given the fact that all manufactured cheeses used the same starter culture and had similar process control and subsequent compositional analysis, the loss of numbers for the reference strain between contaminated milk and cheese curd may be due to a difference in strain resistance to the manufacturing parameters. Differences in *M. paratuberculosis* strain thermal tolerance (41) and combined pH and NaCl concentrations (42) have been observed previously.

To our knowledge, this study provides the first data on the persistence of *M. paratuberculosis* during Cheddar cheese manufacture and ripening. Hamer and Babel (21) reported Cheddar cheese contaminated with *Mycobacterium tuberculosis* to be infective after 220 days, while Kastli and Binz (25) determined survival times of 22 and 305 days for *Mycobacterium bovis* in Swiss hard and semihard cheeses, respectively.

The *D* values estimated for each of the three *M. paratuberculosis* strains (107 days for strain 806PSS, 96 days for strain 796PSS, and 90 days for strain NCTC 8578) in our Cheddar cheeses were much higher than those reported previously, even compared to Swiss Emmentaler, a hard cheese having a moisture content similar to that of Cheddar (35). *D* values of 45.5 and 27.8 days were calculated for semihard (Tilsiter) and hard (Emmentaler) Swiss cheeses, respectively, made from spiked raw milk, indicating a faster rate of death of *M. paratuberculosis* in the progressively harder cheeses. A *D* value of 59.9 days has been determined for the survival of *M. paratuberculosis* in a Hispanic-style soft cheese made from spiked pasteurized milk (42). The Hispanic-style soft cheese was produced under laboratory conditions and contained 2% (wt/vol) NaCl, pH

6.15, with a maximum temperature of 37°C reached during manufacture.

In addition to moisture content, a number of other factors are likely to contribute to the inactivation of *M. paratuberculosis* in cheese, including temperature, pH, salt, and the antagonistic effect of lactic acid bacteria (starter and nonstarter microflora) with their concomitant lactic acid production (35). Sung and Collins (41) investigated the in vitro effect of pH and NaCl and reported a *D* value of 19 days in acetate buffer (pH 5.0), with *D* values increasing with increased pH. After 24 h, our model cheeses reached pH 5.1 to 5.3. This pH remained constant throughout the ripening period and was lower than those encountered during the later stages of Swiss Emmentaler ripening (120 days). A more bactericidal effect on *M. paratuberculosis* would have been expected with the lower pH encountered in Cheddar ripening.

Although high levels of salting can affect the survival rates of microorganisms in some cheeses (36), NaCl concentrations from 2 to 6% had little or no effect on the survival rate of *M. paratuberculosis* under in vitro conditions at pH 4 to 6.8 (42). Since the average salt content in our model cheeses (1.6%) was lower, we conclude that salt content contributed little to the death of *M. paratuberculosis* during Cheddar ripening. A similar negligible detrimental effect was reported for Swiss Emmentaler cheese containing 0.4% salt.

The cheese temperature and exposure time experienced during production vary depending on the variety manufactured. During our model Cheddar production, the maximum curd cooking temperature was 40°C (applied for 2 to 3 h), compared to 53°C applied for 45 min in Swiss hard cheese (Emmentaler) (35). The ripening temperature of Swiss hard cheese was comparatively high, 10 days at 12°C, then 60 days at 22°C, and 50 days at 12°C. In contrast, the model Cheddar cheeses in this study were ripened for 190 days at 10°C. Spahr and Schafroth (35) reported that curd cooking temperature had little effect on the behavior of *M. paratuberculosis*. However, it has been reported that pathogens surviving the manufacturing process show a more rapid rate of death at higher ripening and storage temperatures (10). In an earlier study, Keswani and Frank (26) observed minimal thermal inactivation of *M. paratuberculosis* at 55°C, albeit over a much shorter time of 12 min. Therefore, we conclude that the curd cooking temperature during Cheddar manufacture coupled with a low ripening temperature contributed little to the inactivation of *M. paratuberculosis* during the production process.

The survival times of *M. paratuberculosis* in Cheddar cheeses cannot be directly compared with their in vitro behavior against the parameters of temperature, pH, and salt (42). Furthermore, there are key differences, other than those alluded to above, between this study and the only study available on *M. paratuberculosis* survival in hard cheese (35). In Swiss Emmentaler manufacture, raw milk was spiked with *M. paratuberculosis* cells with no subsequent heat treatment, and thus raw milk microflora entered the manufacturing process, where they were supplemented with a starter culture (not named in the study) and propionic acid bacteria as ripening bacteria. In our study, *M. paratuberculosis* was added postpasteurization along with a mesophilic-thermophilic starter culture. The possible contribution of raw milk microflora, the active antimicrobial enzyme systems of fresh raw milk, antagonistic starter culture

flora, and propionic acid-producing ripening bacteria on the reduction of *M. paratuberculosis* and perhaps unknown factors can only be speculated upon. However, Meyer et al. (29) suggested that rapidly growing propionic acid bacteria, characteristic of Swiss Emmentaler production, may be a factor responsible for the inactivation of mycobacteria. Also, this study used *M. paratuberculosis* strains previously isolated from commercially pasteurized milk. There is the possibility that such strains were more thermotolerant due to their passage through the pasteurization process.

This study has also shown the release of up to 4% of the *M. paratuberculosis* load from the cheese milk into the whey fraction during the manufacturing process. The previous studies investigating the persistence of *M. paratuberculosis* in Hispanic soft cheese (42) and Swiss semihard and hard cheeses (35) did not examine whey for the recovery of *M. paratuberculosis*. In this study, whey was sampled and analyzed immediately upon draining (pH 5.6 to 6.0) and therefore was not exposed to this pH for a considerable time, possibly preventing any inactivation of *M. paratuberculosis* that may occur in whey. The long-term survival of *M. paratuberculosis* in whey may be an important factor to consider when evaluating the use of whey as a cheese processing by-product or the effectiveness of wastewater treatment processes.

In conclusion, this study demonstrates that laboratory-grown *M. paratuberculosis* cells inoculated into milk postpasteurization were concentrated (approximately 10-fold) within the cheese curd and decreased slowly over a 27-week ripening period. *M. paratuberculosis* was also recovered from the whey fraction in the majority of the manufactured cheeses. Although the manufactured cheese closely resembled the commercial product in pH, moisture, fat, and salt content, the behavior of *M. paratuberculosis* may have been affected by the limitations of our experimental design, i.e., the inability to use naturally infected milk.

The number of *M. paratuberculosis* cells, if any, entering the cheese manufacturing process will depend on the frequency and degree of contamination of raw milk used and also the heat treatment applied to milk for cheese production. Studies have suggested infrequent and low levels of *M. paratuberculosis* in raw milk used for cheese making (15, 18, 28, 37, 39, 43), although rates of isolation may be influenced by the detection regimens used by different research groups (11). With regard to the effectiveness of raw milk heat treatment, a number of studies with pilot-scale commercial simulation of high-temperature, short-time pasteurization have not recovered *M. paratuberculosis* when milk was inoculated with 10^2 to 10^6 CFU/ml of different strains (23, 37, 38). Other studies have reported more than a 4 log₁₀ reduction in *M. paratuberculosis* numbers under similar conditions (28, 33). However, in one of these studies, very small numbers of *M. paratuberculosis* were recovered from milk heat treated at 72°C for 15 s (28).

M. paratuberculosis has been recovered after simulated high-temperature, short-time pasteurization of naturally infected milk (19) and has been cultured from pasteurized milk for retail sale (18). Therefore, the possibility exists that cheese milk for Cheddar production may contain viable *M. paratuberculosis* cells. The results of our study, with a reference strain and field isolates, suggest minimal inactivation during the manufacturing process and up to 10-fold concentration following

microbial entrapment and syneresis of the curd. Subsequently, a minimum of 3 months of low-temperature ripening is required to achieve a 1 log₁₀ reduction in *M. paratuberculosis* levels.

Cheddar cheese is sold for consumption as mild, medium, or mature depending on the length of ripening. In the United Kingdom, mild Cheddar is usually ripened for up to 4 months (16 weeks), while the mature variety is ripened up to and beyond 12 months. A larger margin of safety, in relation to *M. paratuberculosis*, may be provided by medium or mature Cheddar ripened longer than 16 weeks.

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