Survival of Mycobacterium avium subsp. paratuberculosis in raw milk cheese

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Keywords: behavior, food, cheese, Johne's disease, Crohn's disease.

SUMMARY

Since high-temperature short-time (HTST) pasteurization (71.7 °C for 15 s) on Mycobacterium avium subsp. paratuberculosis (Map) does not seem to be fully effective, it is obvious that any treatment of raw milk below the minimal HTST pasteurization requirement will not be effective in eliminating this potentially human-pathogenic mycobacterium. As a consequence cheese and other milk products made from either raw or pasteurized milk may harbor survivors which could pose a risk to consumers.

Raw milk was artificially contaminated with declumped cells of Map at a concentration of 10⁴ to 10⁵ CFU/ml and used to manufacture model hard (Swiss Emmentaler) and semi-hard cheese (Swiss Tisliter). Two different strains of Map were tested and for each strain two model hard and semi-hard cheeses were produced. The survival of Map was monitored over a ripening period of 120 days by plating out homogenized cheese samples onto 7H10-PANTA-agar.

In both the hard and the semi-hard cheese counts decreased steadily but slowly during cheese ripening. Nevertheless viable cells could still be detected in 120 day cheese. D-values were calculated at 27.8 days for hard and 45.5 days for semi-hard cheese. The most important factors responsible for the death of Map in cheese were the temperatures applied during cheese manufacture and the low pH at the early stages of cheese ripening. Since the ripening period for these raw milk cheeses lasts at least 90 to 120 days the D-values found indicate that 10³ to 10⁴ cells of Map per g will be inactivated.

INTRODUCTION

Crohn's disease is a chronic inflammatory bowel disease that primarily affects the ileum in humans and bears considerable similarity to the histopathology of cattle with Johne's disease (2). Recent studies have shown that a high percentage of people with Crohn's disease are infected with Mycobacterium avium subsp. paratuberculosis (Map). Whether the association of Map and Crohn's disease is causal or coincidental is not known (4). However, the similarities between Johne's and Crohn's disease have raised the question, whether milk, among other factors, could be a vector to transmit Map from cattle to humans.

It has been documented that cows with clinical Johne's disease or asymptptomatically infected cows in the latter stages of infection shed viable Map into their milk albeit at low concentrations, i.e. 2 to 8 CFU/50 ml of milk (20, 23, 24). Since fecal material from clinically infected cows may contain as high as 10⁹ CFU of Map per gram of feces fecal contamination of raw milk may provide a much larger contribution of this microorganism rather than actual shedding of the bacteria directly into milk by clinically infected cows (15).

Chiodini and Hermon-Taylor reported laboratory studies demonstrating that Map was more heat resistant than Mycobacterium bovis, one of the bacterial pathogens used to define pasteurization time and temperature standards (3). Their findings led several research groups to investigate the death of Map under different pasteurization conditions (5-9, 11, 14, 17-19, 21). The HTST pasteurization method (71.7 °C for 15 s) resulted in a high death rate, but according to some reports, survivors may occur. Since the effect of pasteurization on Map seems to be limited, it is obvious that any treatment of raw milk below the minimal HTST pasteurization requirements will not be effective in eliminating this potentially human-pathogenic mycobacterium. As a consequence cheese and other milk products made from either raw or pasteurized milk may harbor survivors which could pose a risk to consumers.

The aim of this work was to study the length of time that Map may survive in two different Swiss types of hard and semi-hard cheese made from raw milk under conditions...
similar to traditional cheese-manufacture. The findings will be a basis to estimate the possible risk that such cheese may pose to consumers.

MATERIALS AND METHODS

Map strains. Two strains of Map were tested: ATCC 19698 (obtained from DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany) and strain Nießßl, originally isolated from raw milk (kindly provided by P. Hammer, Federal Dairy Research Centre, Institute for Hygiene and Food Safety, Kiel, Germany).

Cultures were maintained on Middlebrook and Cohn 7H10 agar slants containing 10% (vol/vol) Middlebrook OADC (oleic acid-dextrose-catalase) enrichment (Becton Dickinson Microbiology Systems, Sparks, Md., U.S.A.), 0.5% glycerol (Fluka, Buchs, Switzerland), and 0.0002% (wt/vol) mycobactin J (Synbiotics Europe SAS, Lyon Cedex, France). The slants were incubated at 37 °C for 4 weeks and then held at 5 °C.

Fluid cultures were grown similar to the procedures used by Sung and Collins (21), and Keswani and Frank (11) using Middlebrook 7H9 broth medium containing 10% (vol/vol) Middlebrook ADC enrichment (albumin-dextrose-catalase; Becton Dickinson Microbiology Systems, Sparks, Md., U.S.A.), 0.2% (vol/vol) glycerol (Fluka, Buchs, Switzerland), and 0.0002% (wt/vol) mycobactin J (Synbiotics Europe SAS, Lyon Cedex, France). The Map strains were inoculated in 50 ml medium in 100 ml glass bottles and incubated at 37 °C for 3 months with constant gentle shaking.

Preparation of cell suspensions for cheese making. Aliquots of 25 ml of Map fluid cultures were centrifuged at 4,000 × g for 20 min and resuspended in 2.5 ml Middlebrook 7H9 broth. To break up clumps of Map the suspensions were syringed 20 times as was described by O’Connor et al. (16): the suspensions were drawn up by the syringe and expelled in less than 2 s through a 0.8 x 40 mm needle (Becton Dickinson Microbiology Systems, Sparks, Md., U.S.A.). Sterile glycerol was added to a final concentration of 10% (vol/vol). The cell suspensions were stored at -40°C before being used as inoculum in cheese making.

Manufacture and ripening of cheese. Cheeses were manufactured according to traditional procedures as previously published (1). With each strain two model hard (Swiss Emmentaler) and two model semi-hard (Swiss Tilsiter) cheeses were manufactured in a special cheese-making system that was self contained. Raw bovine milk made up of a mixture of evening and morning milk was used: 90 liters for hard cheese and 70 liters for semi-hard cheese. The milk was inoculated with lactic starter culture and at the same time with the Map test strain to obtain 10⁵ to 10⁷ CFU/ml vat milk. In addition a culture of propionic acid bacteria was added for hard cheese. Rennet was added to induce coagulation within 35 min. Curds were heated and maintained at 53 °C for 45 min (hard cheese) and 44 °C for 10 min (semi-hard cheese), respectively. For ripening the cheese were vacuum sealed and stored at temperatures usually applied in traditional cheese making, i.e. for hard cheese 10 days at 12 °C; then 60 days at 22 °C, and 50 days at 12 °C. Semi-hard cheese was ripened for 120 days at 14 to 15 °C.

Sampling. Cheese samples were taken after 1, 7, 30, 60, 90, and 120 days of ripening. The vacuum sealed cheeses were unpacked and samples were taken with a cheese borer of 20 mm diameter. Then cheeses were vacuum sealed again in a new plastic foil.

Enumeration method. Two grammes of cheese plugs were aseptically transferred into a sterile stomacher bag. Eighteen ml of pre-warmed (40 °C) sterile peptone water consisting of 0.5% (wt/vol) sodium chloride, 1.0% (wt/vol) caseine peptone, and 2.0% (wt/vol) sodium citrate was added. The mixture was homogenized in a Stomacher peristaltic lab-blender 400 (Seward medical, London, UK) for 3 min. The resulting suspension was diluted 1:10 in 0.8% (wt/vol) sodium chloride containing 0.1% (wt/vol) caseine peptone and plated on 7H10-PANTA agar. This agar was made up of Middlebrook and Cohn 7H10 agar base supplemented with 10% (vol/vol) Middlebrook OADC (oleic acid-dextrose-catalase) enrichment, 5.0% (vol/vol) PANTA PLUS antibiotic supplement (polymyxin B-amphotericin B-nalidixic acid-trimethoprim-azlocillin; Becton Dickinson Microbiology Systems, Sparks, Md., U.S.A.), 0.5% glycerol, and 0.0002% (wt/vol) mycobactin J (Synbiotics Europe SAS, Lyon Cedex, France).

All agar plates were packed in sterile plastic bags and incubated at 37 °C. After 21 days of incubation colonies typical for Map were counted. A representative number of typical colonies and all colonies of uncertain appearance were confirmed by Ziehl-Neelsen acid fast staining (Difco, Detroit, Mich., U.S.A.).

Decontamination of cheese samples. To decontaminate the cheese samples from bacteria originating from the raw milk flora that might...
grow on 7H10-PANTA-agar the NALC-NaOH method as described in the Manual of Clinical Microbiology (12) was applied. **D-value.** The D-value was calculated from the slope of the linear regression line generated with the SYSTAT program (release 9.0, SPSS Inc., Chicago, II, U.S.A.) by plotting the \( \log_{10} \) values of Map survivors/g cheese versus the ripening time.

**RESULTS**

Table 1 shows the survival of Map in all eight individual model cheeses as determined by enumeration of non-decontaminated samples. For both hard cheeses and semi-hard cheeses a slow but constant decrease in CFU numbers over the whole ripening period was observed. Within the first 30 days of ripening the decrease was most rapid. After 90 and 120 d, when ripening was complete, Map counts in two hard cheeses fell below the detection limit of 10 CFU/g. In semi-hard cheeses the CFU numbers were well above the detection limit.

The relatively short time of incubation of 21 d was sufficient to produce countable colonies. Incubation for an additional 5 weeks did not result in more colonies but increased the risk of growth of contaminating flora.

The decontamination of cheese samples with NALC-NaOH was shown not to be necessary. The raw milk flora did not affect the enumeration of Map. Only a few colonies on 7H10-PANTA-agar originated from the raw milk flora but could easily be distinguished from mycobacteria colonies, either by their macroscopic appearance or by acid-fast staining. All bacterial species used as starter cultures did not grow on 7H10-PANTA-agar. Furthermore we observed a loss of about 60 % in CFU of Map in decontaminated samples compared to non-decontaminated samples. As a consequence we discontinued sample decontamination after 30 days of ripening and did not use the enumeration results of decontaminated samples for Table 1.

Figure 1 shows the inactivation curves for Map over the whole ripening period. The mean of the log CFU/g of the four cheeses for each of the hard and semi-hard cheese was calculated and plotted versus the ripening time starting with the values for the 24 h cheese. The regression lines show a good correlation. The D-value for Map in hard cheese was 27.8 days, for semi-hard cheese 45.5 days.

![Figure 1. Inactivation curves for Map in hard cheese (Swiss Emmentaler) and semi-hard cheese (Swiss Tilsiter) during 120 days of ripening.](image)

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Table 1. log CFU of Map in all individual model hard and semi-hard cheeses. n.d. = not detectable.
DISCUSSION

Given the fastidious growth requirements of Map this organism will not multiply in cheese. This fact could be demonstrated very clearly with both the reference strain ATCC 19698 and the wild strain Niebüll which was originally isolated from raw milk. In both cheese types tested Map decreased slowly over the ripening period of 120 days. The decrease was slower in semi-hard cheese than in hard cheese. The long survival of Map in hard cheese is rather surprising. Compared to other Gram-positive pathogenic bacteria possibly present in raw milk Map differs greatly in its capacity to survive cheese manufacture and ripening. In a recent study Staphylococcus aureus was the only pathogenic bacterium which survived more than 24 hours. But it was not detectable after 7 days of ripening (1).

The survival times of Map in our model cheeses corresponds with the findings of Kästli and Binz (10) who determined survival times for M. bovis of 305 days for Swiss Tilziter and between 7 and 22 days for Swiss Emmentaler. In their experiments with naturally contaminated raw milk the initial level of contamination was estimated to be 1 to 10 CFU/ml, compared to the 10^2 to 10^3 CFU/ml in our study, which would explain the shorter survival time of M. bovis in Swiss Emmentaler.

Sung and Collins (22) previously published D-values for Map ATCC 19698 in cheese made of spiked milk. They produced a Hispanic-style soft white cheese (Queso fresco) under laboratory conditions with characteristics (2 % NaCl, pH 6.15, maximum temperature during manufacture was 37.7 °C) that differed greatly from our hard and semi-hard cheeses. The D-value found in Hispanic-style soft cheese was 59.9 days.

Of all the different factors that may account for the decrease in numbers of Map in cheese the most important are temperature and pH. Also salt content, lactic acid, and propionic acid may contribute to some extent. Mycobacteria are rather temperature resistant microorganisms. Most of the published data on heat sensitivity deal with pasteurization temperatures. Data on temperatures applied in cheese manufacture are limited. In a recent study Keswani and Frank (11) demonstrated only minimal thermal inactivation of Map at a temperature of 55 °C for 12 min. In our cheese manufacture the curd cooking temperature for hard cheese was 53 °C (applied for 45 min) suggesting a limited contribution to the reduction of CFU. In semi-hard cheese the cooking temperature only reached 44 °C, a temperature far below a value that might have had an influence on the survival of Map.

Map tolerates very low pH-values for several hours. Sung and Collins (22) reported the effect of low pH on survival when suspended in acetate buffer (50 mM) containing 1 % (vol/vol) lactic acid (50 mM). The average D-values for two strains tested at pH 4.0, 5.0, and 6.0 in acetate buffer supplemented with 1 % (vol/vol) lactic acid were 10.0 ± 2.5, 19.0 ± 3.9, and 33.3 ± 4.4 days, respectively. Our model cheeses reached their lowest pH-values within the first 24 h after cheese manufacture, when pH was 5.3 in hard cheese and 5.2 in semi-hard cheese. But as cheese ripening progresses the pH-value rises again reaching 5.7 in hard cheese and 5.8 in semi-hard cheese after 120 days. A pH-value around 5.2 to 5.4 with an estimated D-value of about 15 days only lasts for a relative short time, 10 days for hard cheese and 25 days for semi-hard cheese. This would suggest that the low pH in the cheese may account for a reduction of 1 to 2 logs within the first month of ripening. With the pH rising towards 6.0 during the later ripening the bacteriocidal contribution of the pH will be reduced resulting in an estimated D-value of approximately 30 days which corresponds well with previously published data (33.3 days observed at pH 6.0 under in vitro conditions) (22). The calculated D-values over the whole ripening period of 120 days was 27.8 days for hard cheese and 45.5 days for semi-hard cheese. This indicates that the contribution of pH to the reduction of Map is in fact lower. The influence of pH may be limited but its contribution to the destruction of Map seems to be more important than that of the temperature.

Salting of cheese only affects the survival rates of microorganisms in cheese types with very high salt content (17). Under in vitro conditions NaCl concentrations from 2 to 6 % had little or no effect on Map survival rates at pH 4 to 6.8 (22). Since the salt content in our model cheese was much lower (0.4 % for Swiss Emmentaler and 1.6 % for Swiss Tilziter) we conclude that no or only negligible detrimental effect can be attributed to salt.

In hard cheese of Swiss Emmentaler-type propionic acid bacteria play an important role in the ripening process by contributing to the typical flavor of this cheese. Meyer et al. (13) suggested that rapidly growing propionic acid bacteria might be a factor responsible for the death of mycobacteria. Unfortunately there is no information available on the effect of propionic acid on Map or other mycobacteria leaving this suggestion open to further investigations.

In conclusion the decrease of Map in cheese can not be attributed to one distinct factor. Of all the factors discussed above, pH and
temperature seem to be the most important. Since these factors are not constant but behave dynamically during the manufacture and ripening of cheese it will be very difficult to elucidate their individual contribution to the destruction of Map. However, in cheese manufacture and ripening these factors act together in an ideal and unique combination resulting in a combined bacteriocidal effect that is stronger than could be expected for each single factor determined on the basis of in vitro results.

In their experiments with similar contamination levels Sung and Collins (22) concluded that cheese production using pasteurized milk and a 60-days curing period will largely eliminate the predicted level of Map contamination. Based on our results this conclusion is also true for hard cheese and to some extent for semi-hard cheese made from raw milk. There is strong evidence that the manufacture and ripening conditions of hard cheese from raw milk are in their effect equivalent to pasteurization as regards Map.

ACKNOWLEDGMENTS

We express our thanks to Philipp Hammer, Federal Dairy Research Centre, Institute for Hygiene and Food Safety in Kiel, Germany for providing the Map strain Niebüll and Thomas Bodmer, Institute for Infectious Diseases, Division of Clinical Microbiology, University of Bern, Switzerland, for his advice and support.

REFERENCES


