Inactivation of *Mycobacterium bovis* ssp. *caprae* in high-temperature, short-term pasteurized pilot-plant milk

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ABSTRACT

Experiments to determine the efficacy of high temperature, short time (HTST) pasteurization of milk in terms of inactivation of pathogenic microorganisms were mainly performed between 1930 and 1960. Among the target organisms were *Mycobacterium bovis* and *Mycobacterium tuberculosis*. As a result, the Codex Alimentarius prescribes that HTST treatment of milk should lead to a significant reduction of pathogenic microorganisms during milk pasteurization. Due to the development of improved methods for the detection of survivors and of more advanced heating technology, verification of this requirement seemed to be necessary. To address recent outbreaks of tuberculosis in cattle caused by *M. bovis* ssp. *caprae* (*M. caprae*) in the southern regions of Germany, this organism was tested and compared with *M. bovis* ssp. *bovis* (*M. bovis*). Experiments were performed in a pilot plant for HTST pasteurization of milk with 3 strains of *M. caprae* and 1 strain of *M. bovis*. In preliminary trials at a fixed holding time of 25 s, the temperature at which significant inactivation occurred was 62.5°C for all strains. To determine D-values (decimal reduction times) for the inactivation kinetics, the strains were tested at 65, 62.5, and 60°C at holding times of 16.5, 25, and 35 s. At 65°C, the D-values of all strains ranged from 6.8 to 7.8 s, and at 62.5°C, D-values ranged from 14.5 to 18.1 s. Low inactivation was observed at 60°C. When the low slope of the inactivation curve allowed calculation of a D-value, these ranged from 40.8 to 129.9 s. In terms of log₁₀ reductions, the highest values for all strains were 4.1 to 4.9 log at 65°C, with a holding time of 35 s. The tested strains of *M. caprae* and *M. bovis* showed similar low resistance to heat. Standard HTST treatment should result in a high reduction of these organisms and thus the requirements of the Codex Alimentarius for inactivation of pathogens by this process are far exceeded.

Key words: *Mycobacterium caprae*, milk, heat inactivation, pilot plant

INTRODUCTION

Experiments to determine the efficacy of HTST pasteurization of milk in terms of inactivation of pathogenic microorganisms were mainly performed between 1930 and 1960. Among the target organisms were *Mycobacterium bovis* and *Mycobacterium tuberculosis*. Standards for pasteurization established during that time were based on approximately 100 scientific reports, and they are still partly valid today (Stumbo, 1973; Hammer, 2004). *Mycobacterium bovis* ssp. *caprae* (*M. caprae*) is of special significance in this concern because it was determined that one-third of human cases of *M. bovis*-associated tuberculosis cases in Germany have been caused by this organism (Kubica et al., 2003). Worldwide, 13 to 31% of zoonotic human tuberculosis cases can be attributed to *M. caprae* (Pérez-Lago et al., 2014). A strong likelihood of direct transmission from cattle to humans via consumption of raw milk was shown in Croatia (Cvetnic et al., 2007). Thus, *M. caprae* may be regarded as an emerging zoonotic pathogen in cattle (McDaniel et al., 2014). The Codex Alimentarius (2009) prescribes that HTST treatment of milk should lead to a reduction of pathogenic microorganisms to a level at which they do not constitute a significant hazard for human health. In detail, a reduction of at least 5 log₁₀-steps of *Coxiella burnetii* is required during a treatment at 72°C for 15 s (or at 63°C for 30 min during holder treatment). Because of the development of improved methods for the detection of survivors and of more advanced heating technologies, a verification of this requirement for the reduction of pathogenic mycobacteria seemed to be necessary.

Because of recent series of outbreaks of tuberculosis in cattle, in which 33 of 43 cases were caused by *M. caprae* in the southern regions of Germany (Ministry of Nutrition and Agriculture, 2013), this organism was tested in a pilot plant for HTST treatment of milk compared with *M. bovis* ssp. *bovis* (*M. bovis*). The aim of the study was to determine kinetic inactivation data to

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ensure that *M. caprae* is inactivated during heat treatment of milk to an extent that the requirements of the Codex Alimentarius are fulfilled.

MATERIALS AND METHODS

Strains and Media

For the experiments, 3 strains of *M. caprae* (7140/99 of human origin; 7411/06 and 9681/07 of bovine origin) and 1 strain of *M. bovis* (7540/01 of bovine origin) were chosen, all from the strain collection of the National Reference Center for Mycobacteria (Borstel, Germany).

Direct streaking onto Stonebrink medium supplemented with polymyxin B, amphotericin B, carbenicillin, trimethoprim (PACT; Heipha Dr. Müller GmbH, Eppelheim, Germany) was applied for detection and enumeration of surviving mycobacteria. In addition, for growth of inocula, modified Dubos medium (Giese et al., 1996) supplemented with polymyxin, amphotericin B, nalidixic acid, trimethoprim and azlocillin (PANTA; Becton Dickinson Diagnostics, Heidelberg, Germany) was used.

Experimental Setup and Performance

The pilot-plant pasteurizer used for the experiments has been used for similar studies, and its operation was described previously (Hammer et al., 2002, 2014; Peng et al., 2013).

Each *Mycobacterium* strain was tested once at 72, 70, 67.5, 65, 62.5, and 60°C to determine the temperature at which a considerable reduction occurred using a fixed holding time of 25 s. Experiments to determine the decimal reduction time (**D-value**) were performed in triplicate for all strains at temperatures of 65, 62.5, and 60°C, at holding times of 35, 25, and 16.5 s for each temperature. Retail whole UHT milk (3.5% fat) was inoculated with the test strains.

For inoculation of UHT milk, each *Mycobacterium* strain was grown for 4 wk in 3×400 mL of modified Dubos medium at 37°C. The bacteria were removed from the growth medium by centrifugation for 10 min at $14,000 \times g$, and the respective pellets were resuspended in approximately 100 mL of UHT milk and thoroughly mixed. This suspension was added to 28 L of UHT milk in the raw milk tank of the pilot plant and further mixed for 15 min using a magnetic stirrer. Stirring was continued during the entire heating run. Colony counts of the inoculated milk were determined by spread count on Stonebrink slant medium. For this purpose, the milk was decimally diluted in 0.85% NaCl (wt/vol) up to 10^{-6} , and 2 aliquots of 0.1 mL each were streaked onto 2 vials of the medium. To ensure equal

distribution of colonies on the medium, slants were incubated for 1 wk in the horizontal position until all sample fluid dried off. Only slants with colony numbers <60 were used for evaluation. The inoculation of UHT milk with *Mycobacterium* strains should result in 10^4 to 10^5 cfu/mL.

After heat treatment, milk samples of 5 mL were collected by penetrating a rubber-stoppered sampling valve located directly after the cooling section of the pilot plant utilizing a sterile one-way syringe and needle. The sample temperature at collection was <12°C. Until processing (up to 1 h delay), the samples were stored at 4°C in a refrigerator.

To determine the colony counts of survivors, the heated milk samples were decimally diluted in 0.85% NaCl (wt/vol) to 10^{-5} , streaked onto Stonebrink medium (see above), and incubated at 37°C. In addition, for detection of low numbers of survivors, 5 samples of undiluted milk (0.2 mL each) were streaked onto 5 vials of the medium. After 4 and 6 wk of incubation, the slants were evaluated.

One isolate from each experiment was picked randomly to verify that tuberculosis-causing mycobacteria were detected and not a contaminating species of the genus. For this purpose, a reverse line blot assay was applied (GenoType Mycobacterium CM/AS, Hain Life-science, Nehren, Germany).

Evaluation of Data

To determine the D-values, a log-linear regression curve was calculated using Excel 2010 (Microsoft Corp., Redmond, WA) by plotting $\log_{10} N_t$ (count of surviving bacteria) divided by $\log_{10} N_0$ (initial count) against holding time. The D-value was calculated as the divisor of 1 and the slope of the regression curve. To analyze the fit of the model, the coefficient of determination (R^2) was calculated for each curve; D-values from triplicate experiments were averaged and the standard deviation was calculated. To determine the z-values (temperature enhancement needed for a 10-fold reduction of the D-value), the procedure described above was applied by plotting the $\log_{10}$ -transformed averaged D-values against heating temperature.

For the statistical evaluation of the log reduction, the statistical package SAS (version 9.4; SAS Institute Inc., Cary, NC) was used. After $\log_{10}$ transformation of the bacterial counts, a variance analysis was performed. The calculation was carried out using the general linear model (GLM) procedure, which performs ANOVA by using least squares regression to fit general linear models. In the procedure, the following factors were considered for the estimation of the reduction: *Mycobacterium* strain, temperature, and holding time.

RESULTS AND DISCUSSION

D- and z-Values

To our knowledge, this is the first study presenting kinetic inactivation data for *M. caprae* in milk derived from heating experiments with a pilot plant closely mimicking modern commercial systems. The aim of this experimental setup was to generate data for risk considerations in practice.

Thus, one intention was to use raw milk for the heating experiments to reflect the natural situation. However, in preliminary trials, we found that the Stonebrink medium was completely lysed during long-term incubation (>3 wk) with samples containing raw milk. Most likely, this result was due to the protease plasmin, which is naturally present in milk and is inactivated in UHT milk.

From each experiment, 1 isolate of the survivors was picked randomly to verify that mycobacteria were detected and not a contaminating species of the genus. Colonies of the test strains had typical morphology on Stonebrink medium. No contaminants, neither mycobacteria nor of any other species, were detected (data not shown). As UHT milk was used for the experiments, the pilot plant was decontaminated by circulating water heated to 98°C for 30 min before starting the runs (Hammer et al., 2002, 2014; Peng et al., 2013), and the raw milk tank was autoclaved before use, all work was performed in a nearly sterile system (which was also necessary to meet biosafety-level 3 requirements). Therefore, the random picking was performed only as an additional safety measure to ensure that mycobacteria were present after heating.

Each *Mycobacterium* strain was tested once at 72, 70, 67.5, 65, 62.5, and 60°C using a fixed holding time of 25 s to determine the temperature at which a considerable reduction occurred. This reduction was observed at 65 and 62.5°C. No survival was detected above 65°C. Considering this result, 65, 62.5, and 60°C were selected as target temperatures for the determination of D-values.

At 65°C, the D-values of all strains ranged from 6.8 to 7.8 s, and at 62.5°C, the D-values were between 14.5 and 18.1 s. Weak inactivation was observed at 60°C. If the low slope of the inactivation curve allowed calculation of D-values, they were between 40.8 and 129.9 s and showed a large deviation if more than one value was obtained (*M. bovis* 7540/01; Table 1). For the one D_{60°C}-value obtained for *M. caprae* 9681/07, the coefficient of determination (R^2) was <0.7, which indicates a weak fit with the log-linear regression model. However, the z-value calculated for this strain showed a very strong fit ($R^2 = 1.00$; Table 1). To demonstrate the quality of the D-values in the table, the lowest R^2 from the triplicate experiments is given. In total, 28 values for R^2 were obtained, 18 of which were ≥ 0.9 , 7 were ≥ 0.8 , 2 were ≥ 0.7 , and 1 was <0.7, as already stated. This shows that the inactivation could, in most cases, be well described with the log-linear model.

For *M. caprae* 7140/99, no z-value could be calculated because only 2 D-values were obtained. The z-values for the other strains, including *M. bovis*, were between 4.1 and 7.1°C and showed a very good fit with the log-linear regression model.

Table 1. D (decimal reduction time; seconds)- and z (temperature change required for 1 log reduction in D)-values for the inactivation of 3 *Mycobacterium bovis* ssp. *caprae* and 1 *Mycobacterium bovis* ssp. *bovis* strain in milk

Strain	Temperature (°C)	D-value ¹	SD	R^2	z-value (°C)	R^2
<i>M. caprae</i> 7140/99	65	7.5	0.8	0.90	ND ²	—
	62.5	17.6	1.7	0.85		
	60	NR ³	—	—		
<i>M. caprae</i> 7411/06	65	7.8	0.4	0.94	4.1°C	0.95
	62.5	18.1	2.0	0.97		
	60	129.9 ⁴	—	0.81		
<i>M. caprae</i> 9681/07	65	8.1	1.0	0.79	7.1°C	1.00
	62.5	17.4	2.9	0.81		
	60	40.8 ⁴	—	0.57 ⁵		
<i>M. bovis</i> 7540/01	65	6.8	0.1	0.87	5.7°C	0.98
	62.5	14.5	0.9	0.92		
	60	52.1 ⁶	22.5	0.85		

¹The D-values are mean values of 3 independent experiments, and the lowest coefficient of determination (R^2) of the log-linear regression model used for evaluation is given.

²Not determined because only 2 D-values were available.

³No reduction.

⁴One value only (2 showed no reduction).

⁵An $R^2 < 0.7$ shows a weak fit with the log-linear regression model.

⁶Two values (1 showed no reduction).

In the scientific literature, there are few reports on the elaboration of kinetic data for the inactivation of mycobacteria as described herein. Most of the work has been performed in a purely empirical manner; for example, testing whether a certain heat treatment with defined temperature and time conditions is effective (Hammer, 2004). The monograph of Stumbo (1973) reported a $D_{65.6^{\circ}\text{C}}$ -value (150°F) of 0.3 to 0.4 min and a corresponding z -value of 4.4 to 5.6°C (8 – 10°F) for *M. tuberculosis*. That z -value is similar to our data, whereas the reported D -value is approximately 3-fold higher. However, it is not apparent what equipment was used to determine the data reported by Stumbo (1973).

Log₁₀ Reduction

Values for log₁₀ reduction were determined from direct colony counts on Stonebrink medium. If no count was obtained, the initial count was used to estimate the minimum reduction achieved, although the actual reduction could have been even higher (worst case).

According to the variance analysis, the *Mycobacterium* strain, temperature, and holding time all had highly significant ($P < 0.001$) effects on the reduction. The reduction increased with increasing temperature and holding time. The model explained 93% (R^2) of the total variance, which was 86% for the temperature, 6% for the holding time, and 1% for the strain. In Table 2, additive constants in log₁₀ for the tested factors are summarized.

Among the possible interactions, only the interaction temperature $\times$ holding time was significant ($P < 0.001$). The interaction explained an additional 1% of the variance; thus, there was only a slight increase (94%) of the explained variance through the model. The estimated least squares means did not change; only the standard errors were slightly reduced (data not shown).

A box-and-whisker plot (Figure 1) shows the dependence of the log₁₀ reduction on temperature and hold-

ing time for all strains using the raw data. This result highlights the strong dependence of the inactivation on temperature. As the strains contributed only 1% to the explanation of variance, they were not regarded separately in the graph. The whiskers display the lowest and highest values within $1.5 \times$ interquartile range (25th–75th percentile) and the dots outside this range are suspected outliers.

The averaged highest values for log₁₀ reduction of all strains were between 4.1 and 4.9 log₁₀ at 65°C and a holding time 35 s. At 25 s, the averaged highest values were 3.7 to 4.9 log₁₀, and at 16.5 s, they were 2.3 to 3.9 log₁₀. This result shows that a reduction of more than 2 log₁₀ could be achieved at a holding time of 16.5 s at 65°C , which is also reflected in the D -values of 6.8 to 8.1 s determined for all strains. Regarding the requirement of the Codex Alimentarius (2009) that pathogens should be reduced by at least 5 log₁₀ during HTST treatment for 15 s at 72°C , a reduction of >18 log-cycles can be extrapolated from the present data. In a worst-case scenario for all strains, this can be calculated as follows: Adding the highest determined z -value (7.1°C) to 65°C would reduce the highest measured D -value (8.1 s) by a factor of 10, to 0.81 s. Dividing the target holding time of 15 s for HTST by 0.81 s results in the value of >18 log-cycles given above.

Compared with those for *M. caprae*, recent data for *M. avium* ssp. *paratuberculosis* are available that clearly indicate that this organism is more heat resistant: *M. paratuberculosis* showed survival of small numbers at HTST conditions (Grant et al., 2002; Hammer et al., 2002, 2014; McDonald et al., 2005; Van Brandt et al., 2011). In their quantitative risk assessment on the survival of *M. paratuberculosis* in 3 milk pasteurization plants in Italy, Serraino et al. (2014) proposed a 4- to 7-log reduction based on the results from industrial-scale experiments. As results for inactivation of *M. paratuberculosis* achieved with our pilot plant meet this proposal very well (Hammer et al., 2002, 2014), we estimate that the inactivation data obtained for *M. caprae*

Table 2. Variance analysis: additive constants (in log₁₀) for factors and their influence on the reduction of *Mycobacterium* spp. (overall mean $\mu = -4.409$)

Factor	Level	Constant	LSM	SE
Strain	<i>M. caprae</i> 7140/99	0.212	−1.655 ^a	0.092
	<i>M. caprae</i> 7411/06	0.314	−1.553 ^a	0.092
	<i>M. caprae</i> 9681/07	0.000	−1.867 ^{ab}	0.092
	<i>M. bovis</i> 7540/01	−0.218	−2.085 ^b	0.092
Temperature ($^{\circ}\text{C}$)	60	3.848	−0.012 ^a	0.079
	62.5	2.361	−1.498 ^b	0.079
	65	0.000	−3.860 ^c	0.079
Holding time (s)	15	1.012	−1.251 ^a	0.079
	20	0.406	−1.857 ^b	0.079
	25	0.000	−2.263 ^c	0.079

^{a–c}Values with different superscripts within the same factor show significant differences ($P < 0.05$).

Figure 1. Box-and-whisker plot of the dependence of the $\log_{10}$ reduction on temperature and holding time for all strains included. The upper margin of the box shows the 75th percentile, the lower margin the 25th percentile, the line in the box the median value, and the cube the mean value. The whiskers display the lowest and highest values within $1.5 \times$ interquartile range (25th–75th percentile). The dots outside this range are suspected outliers.

with this equipment reflect the situation in practice. The high initial count in our experiments represents a worst-case situation; however, it allowed us to gain measured values at the chosen temperatures. Regarding the extrapolation example given above, it would be rather unlikely that, under HTST conditions, any measured value could be obtained.

Presently, no data are published on the natural occurrence of *M. caprae* in milk of infected animals. Such data are needed for proper exposure assessment in the framework of risk assessment (Codex Alimentarius, 1999). In addition, it might be necessary to expand the database of inactivation results by further tests with strains of caprine origin. It will be necessary to elaborate an international database with comparable results, harmonization of testing equipment, detection methods for survivors, number of organisms to be tested, and adaptation of these to the heating menstruum (Condron et al., 2015).

CONCLUSIONS

The tested strains of *M. caprae* and *M. bovis* showed similar low resistance to heat. The standard HTST

treatment should result in substantial reduction of the organisms and thus will far exceed the requirements of the Codex Alimentarius for inactivation of pathogens by this process. Even at the subpasteurization temperature of 65°C, a reduction of at least 4 $\log_{10}$ can be achieved during continuous flow treatment for 35 s.

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