Inactivation of *Mycobacterium avium* subsp. *paratuberculosis* in whole milk, skim milk and cream in a pilot plant pasteurizer

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1. Introduction

Heating experiments reported in the literature show differing results for the heat resistance of *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) in milk. There is evidence that small numbers may survive high temperature short time (HTST) treatment (reviewed by Gould et al. 2005) (1), and reports with special reference to commercial practices indicate that *M. paratuberculosis* can be present in commercial dairy products (Table 1). Results obtained by PCR and related techniques often appear to show a much higher presence of *M. paratuberculosis* DNA in the samples. In Table 1 only results after cultural confirmation are presented.

Tab. 1: Cultural detection of *M. paratuberculosis* in commercial dairy products

Product	processing conditions	samples tested/**positive**	Reference
whole milk	min. 71.7 °C for 15 s	244/**4**	(2)
whole milk	"pasteurized", no details given	702/**20**	(3)
whole milk	72-74 °C for 15-28 s	228/**3**	(4)
semi skim milk	72-75 °C for 14-25 s	179/**5**	(4)
skim milk	72 °C for 15-17 s	160/**2**	(4)
whole and skim milk	"HTST", no details given	124/**2**	(5)
milk	"pasteurized", no details given	18/**1**	(6)
milk	138 °C for 30 s	30/**1**	(6)
soft cheese*)	feta cheese, no details given	42/**1**	(7)
powdered infant milk	no information**)	51/**1**	(8)

*) no information given, whether processed from raw or heat-treated milk

**) milk powder for this type of product is made from HTST-treated milk, which has undergone subsequent further heat treatments during evaporation and spray drying

Heating experiments performed with commercial-scale pasteurizers confirm that the results listed in Table 1 reflect reality. Heat treatment of naturally infected cow's milk at a temperature of 73 °C and holding times of 15 and 25 s in a pasteurizer of a capacity of 2000l/h resulted in cultural detection of survivors in 10 of 144 samples (9). In that report a positive effect of homogenization on inactivation was postulated, although not statistically significant. A second report from the same group on application of homogenization in combination with high temperature short time (HTST) treatment in a pilot plant pasteurizer showed a significant effect of homogenization on the inactivation of *M. paratuberculosis* (10). Heat treatment of experimentally contaminated milk (10^3-10^4 organisms/ml) in a pasteurizer with a capacity of 3000 l/h at temperatures of 72, 75 and 78 °C at holding times of 15, 20 and 25 s resulted in a 4-6 log reduction of *M. paratuberculosis*. In three of 20 runs, (i) 72 °C/15 s, (ii) 75 °C/25 s, (iii) 78 °C/15 s, low levels of survivors were detected (0.002-0.004 colony forming units (cfu) ml^{-1}) (11).

Most experiments reported in the literature were performed with whole milk, whereas in the dairy industry skim milk and cream are very often subjected to different heating processes. The objective of this study was to apply a heating technology as comparable as possible with commercial systems, and to examine the heat resistance of *M. paratuberculosis* in raw whole milk, raw skim milk and cream. A wide range of temperature-time combinations was applied to reflect process situations occurring in industrial practice. The main objective of the project was to find temperature-time combinations in the different substrates that lead to a destruction of *M. paratuberculosis* under practical conditions. In addition, the efficacy of homogenization in combination with heat treatments was investigated.

2. Material and Methods

Materials and methods are listed briefly here except those parts differing from the more detailed descriptions given in (12).

2.1 Bacterial strains

For the heating experiments a cocktail of the following *M. paratuberculosis* strains was used:

Strain-no.	type	origin
DSMZ 44133	reference strain	bovine
OL	field strain	bovine, faeces
Niebuell	field strain	bovine, milk
ATCC 43015	reference strain	human (Linda)
M100/C	field strain	caprine

Field strains of bovine origin were identified by Ziehl-Neelsen stain, mycobactine J dependency and IS 900-based PCR. The Niebuell strain was isolated from an aseptically collected milk sample of a clinically diseased cow, and comes from an affected udder rather than from faecal contamination of the milk. The caprine strain was kindly supplied by Dr. Karen Stevenson, Moredun Research Institute, Scotland UK; and the OL strain by the Veterinary State Laboratory of Lower Saxony, Oldenburg, Germany. Strains were kept

on Herrold's egg yolk medium (HEYM) in permanent culture and subcultured every eight weeks. In addition stock cultures of each strain were stored at -80 °C in Dubos medium + 10% glycerol.

2.2 Pilot plant pasteurizer

2.2.1 Design

Exact calculations on the basic dimensions of the pilot plant used are given in (12). The pasteurizer was extended and improved for this study to allow heat treatments at UHT conditions. For this purpose new exchangeable holding sections and an additional heat exchanger for the process water heating was included. The water was heated by steam, regulated by an electronic valve driven by air pressure, which allowed very precise and stable holding of the selected temperature. Compared with the previous pilot plant, counterflow of heat-treated and non-heated milk is avoided throughout the entire product flow, contributing to avoiding cross contamination e.g., through micro leaks of the plates. Cooling of heated product was achieved in a separate plate exchanger supplied with ice water. The construction scheme is given in Figure 1.

Basic technical data are as follows:

- sample volume: up to 25 l
- holder for HTST: spiral shaped, inside tube diameter 7 mm
  a) total volume 0.223 l, tube length 3.5 m, for holding up to 30 s
  b) total volume 0.770 l, tube length 16.0 m, for holding up to 60 s
- holder for UHT: tube, inside diameter 5 mm, total volume 27 ml
- heating: 60-135 °C, adjustable at 0.1 °C steps
- flow rate: 18-80 l/h, adjustable at 1 l/h steps
- holding time: 2-60 s average holding time, adjustable by change of flow rate (average holding time = $\frac{\text{holder volume} \times 3600}{\text{flow rate}}$)

Data regarding the distribution of residence times (see Fig. 2) for the three holding sections are given below (Table 2). The "maximum residence time scattering" in this context means the time interval between the most rapid and the slowest milk particle at their passing through the outlet of a holding section. In Table 2 calculations for typical experiments are given as examples. In addition the respective Reynolds numbers are given to show that turbulent flow was achieved under all experimental conditions. These calculations were not made for every experiment.

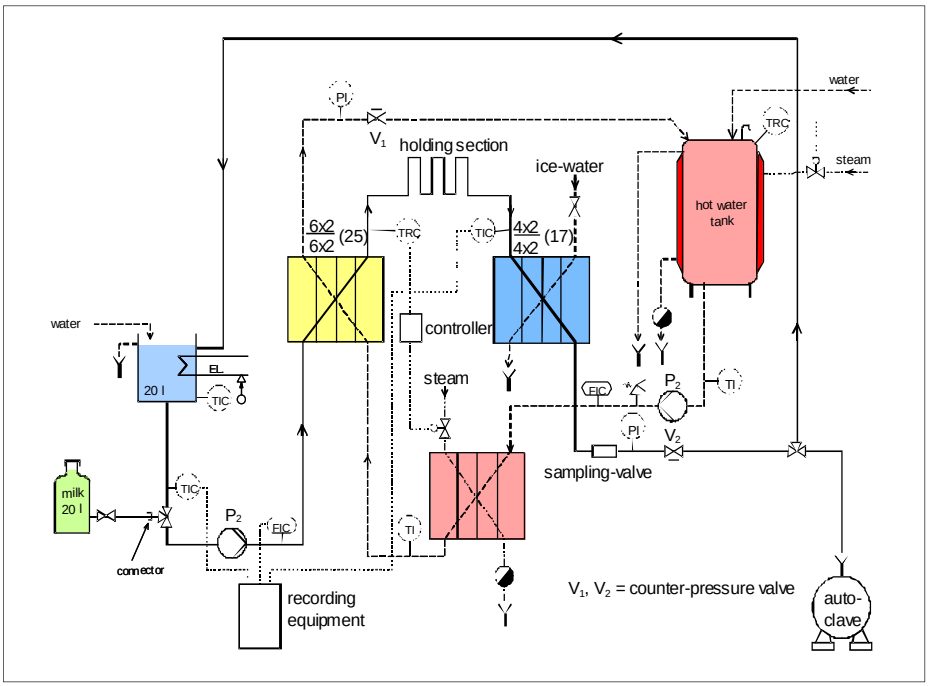

Fig. 1: Technical drawing of the extended pilot plant

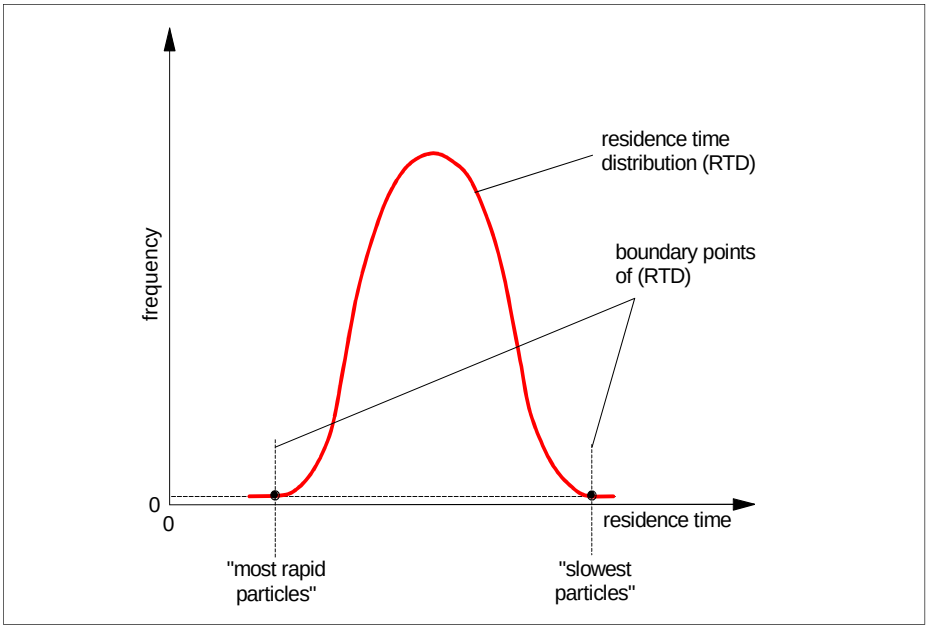

Fig. 2: Curve showing a residence time spectrum after fixing boundary points for determining the residence time scattering.

Tab. 2: Examples of residence time distributions for the holding sections of the pilot plant

holding section	average residence time	minimum residence time	standard deviation (%)	Reynolds-no.	experiment-no.
223 ml	15.0 s	12.1 s	19.4	4926	237
	25.1 s	20.1 s	20.0	2950	254
770 ml	47.0 s	41.1 s	12.6	5423	278
	62.2 s	54.4 s	12.6	5636	306
27 ml (UHT)	2.0 s	1.2 s	38.1	7005	439
	5.0 s	2.8 s	44.8	2816	450

2.2.2 Exclusion of cross-contamination

In these experiments, extremely low numbers of surviving bacteria were expected. Therefore, much of the experimental design was reviewed to avoid cross-contamination by all means possible.

Cross-contamination in the pilot plant

At the pasteurizer site the following preventive measures were taken:

(1) extended pressure at the product side (1-6 bar), at least 1 bar above the pressure at the process water side

(2) starting each run with the highest temperature

(3) within runs intermediate flushing with > 3 l water (total volume of the unit 1.4 l). Before "counting" the water flush, the milk-water mixture was removed from the unit until the flush water was completely clear.

(4) cleaning and sterilization between runs (after a run: 30 min alkaline at 80-90 °C, 10 min acid at 60 °C, 30 min potable water at 98 °C; before a run: 30 min potable water at 98 °C). The sample tanks were disconnected and autoclaved separately at 121 °C for 20 min.

(5) flush water, cooling water and process water check (0.2 µm filtration of 5 l water, culture, PCR and staining from filter retentates)

(6) leak control of the plate apparatuses for heating and cooling (7 bar overpressure, overnight)

Cross-contamination in the laboratory

Laboratory cross-contamination may, hypothetically, result from either poor handling techniques or the ingredients used. Therefore, since July 2003 (experiment no. 385, experiments no. 235-689 are included in this report) each day one uninoculated, and not heat-treated, milk sample was placed randomly among the samples from the heating experiments. This blank was treated in the same way as the other samples in terms of centrifugation, decontamination, inoculation steps and incubation time. In none of these samples was growth of *M. paratuberculosis* subsequently observed.

Another hypothetical source for contamination is that the mycobactine J, necessary as a growth factor in the culture media, is usually made from *M. paratuberculosis*. The manufacturer of the mycobactine (Synbiotics Europe) certifies that this protein is purified and chemically sterilized.

2.3 Homogenizer

The homogenizer used for the experiments is commercial, small-scale equipment, not in line with the pilot plant. The plunges are surrounded by a steam chest, which is filled with floating steam during operation. The instrument is a so-called aseptical homogenizer. During operation the sample tanks used in the pilot plant were connected to the homogenizer and the contents stirred continuously. The time delay between treatment in the pilot plant and with the homogenizer, derived from connecting and disconnecting sample tanks, was less than 10 min. For cleaning purposes the homogenizer was connected to the pilot plant. In addition to the cleaning procedure described for the pilot plant the homogenizer was sterilised after cleaning by application of floating steam for 10 min.

Basic technical data are as follows:

- manufacturer: APV Gaulin bv, Hilversum, NL
- type: LAB 60-10 TBSX
- flow rate: fixed at 60 l/h
- pressure: up to 700 bar, exactness +/- 10 bar

2.4 Sample preparation

For inoculation of raw milk, skim milk and cream each *M. paratuberculosis* strain was grown for 8-10 weeks in 400 ml modified Dubos medium. Bacteria were harvested from the growth medium by centrifugation for 15 min at 2200 x g, and pellets resuspended in 0.85 % NaCl by gentle shaking, mixed, but not homogenized prior to further use. This procedure results in a cell suspension, containing single cells and cells in clumps of different sizes, similar to those found in faecal matter of infected cows. The raw milk was bulk-tank milk from the experimental farm of the Federal Research Centre for Nutrition and Food. Skim milk was produced from that raw milk in the Centre's experimental dairy. Cream was purchased from a local supplier (30 % fat, heated 15-20 s at 105 °C). Samples were inoculated directly with the cocktail of strains and stirred continuously with a magnetic stirrer in the respective tank of the pilot plant during the heating run. Colony counts prior to heating were estimated by direct plating onto HEYM. The counting technique can only deliver an estimate of the true number of cells because each clump, irrespective of its size, will be measured as one cfu.

2.5 Heating experiments

The experiments performed are listed in Table 3. One experiment within a series is defined by a certain time-temperature combination of heating with a cocktail of *M. paratuberculosis* strains and a certain inoculum level (usually 10^3-10^5 cfu/ml). Three independent replicate experiments were performed for each time-temperature combination. Regarding the relationship between the inoculum level and cfu's determined after heating, it must be recognized that a cfu may represent a very small or a very large clump

of *M. paratuberculosis* cells. In addition, clumps can be divided into smaller units by mechanical forces during processing in the pilot plant and handling in the laboratory, each of which may result in a cfu.

Tab. 3: Heating experiments with *M. paratuberculosis*

series	no. of experiments	treatment	heating temperature (°C)	holding time (s)	substrate
A	93	HTST	67, 72, 77, 82, 87, 90	15, 30, 45, 60	skim milk
B	30	HTST	95, 100	15, 30, 45, 60	whole milk
C	57	HTST	85, 90, 95, 100	15, 30, 45, 60	cream
D	42	UHT	105, 110, 115, 120, 125, 130, 135	2, 5	whole milk
D	42				skim milk
D	42				cream
E	16	double HTST	15 s/72 °C + 15 s/72 °C 15 s/72 °C + 5 s/90 °C		whole milk
F	99	HTST + homo-genization	72, 75	15, 30 s at 200-700bar	whole milk

2.6 Detection of *M. paratuberculosis* in heat-treated milk

A modified Dubos medium was used for resuscitation and enrichment of heat injured *M. paratuberculosis*. For direct plating HEYM slant agar was used. The exact composition for both media is given in (12).

Milk samples were collected from the outlet of the equipment. For each experiment 300 ml were collected and split into three sub-samples of 100 ml, which were processed independently. The splitting has the advantage that, if there are surviving bacteria other than *M. paratuberculosis*, at least some of the subsequent cultures are not contaminated. This proved useful during the detection and isolation of the agent because no decontamination procedure of the heat-treated samples was done to avoid decreasing the viability of the surviving mycobacteria. Especially raw milk may contain e.g. sporeformers, which can spoil the subsequent isolation procedure of *M. paratuberculosis*. The sample temperature at collection was below 12 °C. Further cooling was not applied. Directly after collection (max. 1 h delay), samples were centrifuged at 4 °C in a refrigerated centrifuge at 14,000 x g for 10 min. The resulting pellets were suspended in 2 ml 0.85 % NaCl. For each sample 3 HEYM slant agar tubes and 3 vials of modified Dubos medium were inoculated with equal parts of the 2 ml suspension. The Dubos medium was used for resuscitation and enrichment of heat injured bacteria. Cultures on HEYM agar were evaluated monthly and finally after incubation for 6-8 months at 37 °C, bacteria taken from visible colonies were identified as *M. paratuberculosis* by an auramine staining and an IS 900-based PCR. The Dubos vials were incubated for the same time period, pooled, centrifuged and the pellets were streaked onto 2 HEYM slant agar tubes. If heavy contamination of the Dubos medium was detected, decontamination with N-acetyl-L-cysteine (NALC) was applied prior to culture on HEYM agar. After 3-6 months of incubation, bacteria from visible colonies were identified as described above.

2.7 PCR for identification of *M. paratuberculosis*

The PCR is based on the detection of an insertion sequence (IS 900), which is specific for *M. paratuberculosis*. DNA extraction was performed by boiling of one colony for 10 min at 100 °C. For the PCR primers according to (13) were used in a standard protocol (see (12)) resulting in a 400 bp band in agarose gel electrophoresis. Primer:

Para-Tb 4: 5'-GTT CGG GGC CGT CGC TTA GG-3'

Para-Tb 5: 5'-GAG GTC GAT CGC CCA CGT GA-3'

3. Results

In total 437 experiments (numbered 235-689) were done. Due to the extended incubation period, a final evaluation was only possible approximately 1 to 1 ½ year after each experiment.

3.1 Heating skim milk at 67-90 °C for 15-60 s (series A)

A summary of the distribution of results achieved via enrichment and/or via direct culture (qualitative) is given in Table 4. In Table 5 the results from direct culture are displayed separately and in detail, showing very low numbers of survivors. Compared with the cfu in the inoculum, these values correspond to a 3-6 $\log_{10}$ reduction. It should be noted that, because total volumes of 300 ml were used for quantification, calculated numbers of survivors may result in figures < 1. In addition, detection of survival by direct culture does not necessarily result in a colony count because sometimes only a homogenous film, containing acid-fast rods, was observed at the agar surface. By sub-culturing from this film typical colonies were obtained.

The reduction in viable numbers in the samples positive only after enrichment is between 5-7 $\log_{10}$ cycles, from inocula between 10^3-10^5 cfu ml^{-1}. For samples with no detection of surviving *M. paratuberculosis*, the log reduction is even greater.

Tab. 4: Inactivation of *M. paratuberculosis* in skim milk at 67-90 °C – qualitative results

n samples:	93		detection via direct culture:		21	
positive:	53		detection via enrichment:		39	
negative:	40		detection via both:		7	
	temperature (°C)			positives/total		
time (s)	67	72	77	82	87	90
15	3/3	1/3	2/3	1/3	1/3	0/3
30	5/5	4/5	4/7	5/7	6/6	4/9
45	2/3	1/3	2/3	1/3	0/3	3/3
60	2/3	1/3	2/3	2/3	0/3	1/3

From the qualitative results displayed in a scatterplot (Fig. 3), it is evident, that, visually, there seems to be no correlation between holding time and heating temperature. This was also due for the inoculum size (data not shown).

Tab. 5: Inactivation of *M. paratuberculosis* in skim milk at 67-90 °C – quantitative results

experiment-no.	temperature (°C)	holding time (s)	inoculum cfu ml^{-1}	survivors cfu ml^{-1}	log reduction
240	66.5	15.6	4.9 x 10 ^{5}	0.90	6
246	67.0	15.1	7.0 x 10 ^{5}	2.80	5
252	67.0	15.4	6.0 x 10 ^{3}	1.80	3
262	76.9	31.9	1.9 x 10 ^{4}	0.02	6
263	71.9	31.8	4.0 x 10 ^{4}	0.08	6
265	82.4	31.4	4.0 x 10 ^{4}	0.02	6
266	82.3	31.4	4.0 x 10 ^{4}	0.04	6
270	67.0	31.6	4.4 x 10 ^{4}	4.00	4
281	72.2	58.8	4.4 x 10 ^{4}	0.02	6
282	67.2	58.2	4.4 x 10 ^{4}	0.06	6
283	90.0	57.7	4.2 x 10 ^{4}	0.08	6
363	81.9	44.1	5.8 x 10 ^{3}	0.04	5

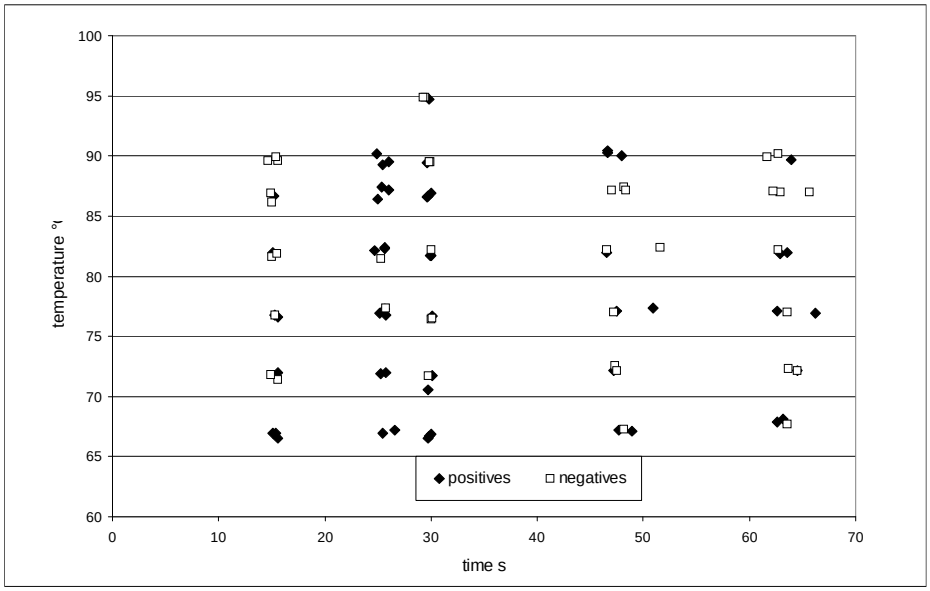

Fig. 3: Inactivation of *M. paratuberculosis* in skim milk at 67-90 °C – qualitative detection results

3.2 Heating whole milk at 95 and 100 °C for 15-60 s (series B)

A summary of the qualitative results is given in Table 6, and quantitative results are displayed in Table 7. Again, the results from direct culture show very low numbers of survivors. Compared with the cfu in the inoculum, these values indicate a 5-6 $\log_{10}$ reduction. In samples positive only after enrichment, a 5-6 $\log_{10}$ reduction was achieved from inocula between 10^3-10^4 cfu ml^{-1}.

Tab. 6: Inactivation of M. paratuberculosis in whole milk at 95 and 100 °C – qualitative results

n samples:	30	detection via direct culture:	4
positive:	8	detection via enrichment:	4
negative:	22	detection via both:	0

	temperature (°C)	positives/total
time (s)	95	100
15	0/3	1/3
30	4/6	2/6
45	0/3	0/3
60	1/3	0/3

Tab. 7: Inactivation of *M. paratuberculosis* in whole milk at 95 and 100 °C – quantitative results

experiment-no.	temperature (°C)	holding time (s)	inoculum cfu ml^{-1}	survivors cfu ml^{-1}	log reduction
289	99.3	15.5	5.9×10^4	0.90	6
300	94.5	25.6	5.9×10^4	2.80	5

3.3 Heating cream at 85-100 °C for 15-60 s (series C)

A summary of the distribution of results achieved via enrichment or via direct culture is given in Table 8. Only from one experiment were surviving *M. paratuberculosis* detected via direct culture, showing a reduction of 6 $\log_{10}$ cycles during heating at 95 °C for 15 s. In samples requiring enrichment for the detection of survivors the reduction was 5-7 $\log_{10}$.

Tab. 8: Inactivation of *M. paratuberculosis* in cream at 85-100 °C – qualitative results

n samples:	57	detection via direct culture:	5
positive:	9	detection via enrichment:	4
negative:	48	detection via both:	0

time (s)	temperature (°C)			positives/total
	85	90	95	100
15	1/3	0/3	1/3	1/3
30	1/3	1/6	0/6	1/6
45	1/3	0/3	0/3	1/3
60	0/3	0/3	0/3	1/3

3.4 Heating whole milk, skim milk and cream at 105-135 °C for 2-5 s (series D)

A summary of the qualitative results is given in Tables 9-11. Quantitative results were obtained in two experiments as displayed in Table 12. From an inoculum of 4.0×10^3- 1.4×10^5 cfu ml^{-1} , the reduction was between 5-7 $\log_{10}$.

Tab. 9: Inactivation of *M. paratuberculosis* in whole milk at 105-135 °C – qualitative results

n samples:	42			detection via direct culture:	3		
positive:	23			detection via enrichment:	20		
negative:	19			detection via both:	0		
time (s)	temperature (°C)				positives/total		
	105	110	115	120	125	130	135
2	2/3	1/3	2/3	3/3	2/3	2/3	0/3
5	3/3	2/3	2/3	1/3	1/3	0/3	2/3

Tab. 10: Inactivation of *M. paratuberculosis* in skim milk at 105-135 °C – qualitative results

n samples:	42			detection via direct culture:	0		
positive:	4			detection via enrichment:	4		
negative:	38			detection via both:	0		
time (s)	temperature (°C)				positives/total		
	105	110	115	120	125	130	135
2	1/3	1/3	0/3	0/3	0/3	0/3	0/3
5	0/3	0/3	0/3	0/3	0/3	1/3	1/3

Tab. 11: Inactivation of *M. paratuberculosis* in cream at 105-135 °C – qualitative results

n samples:	42			detection via direct culture:	1		
positive:	11			detection via enrichment:	10		
negative:	31			detection via both:	0		
time (s)	temperature (°C)				positives/total		
	105	110	115	120	125	130	135
2	1/3	2/3	2/3	2/3	2/3	0/3	1/3
5	1/3	0/3	0/3	0/3	0/3	0/3	0/3

Tab. 12: Inactivation of *M. paratuberculosis* in whole milk and cream at 105-135 °C – quantitative results

experiment-no.	temperature (°C)	holding time (s)	inoculum cfu ml^{-1}	survivors cfu ml^{-1}	log reduction
458 whole milk	104.9	4.9	4.6×10^4	0.04	6
521 cream	104.5	2.0	1.4×10^5	0.02	7

3.5 Double pasteurization of whole milk (series E)

In total 16 experiments applying double pasreurization were performed, with five showing survival of *M. paratuberculosis* (Table 13). No quantitative results were obtained. The reduction detected either by direct culture (4 results) or after enrichment (1 result) was between 5-7 $\log_{10}$.

Tab. 13: Inactivation of *M. paratuberculosis* in whole milk during double pasteurization – all experiments

inoculum, cfu ml^{-1}	first treatment			second treatment			total log reduction
	temp. (°C)	holding time (s)	result[1)]	temp. (°C)	holding time (s)	result	
2.8 x 10 ^{3}	72.0	16.0	+[2)]	89.9	17.6	+	5
				71.8	15.7	+	5
4.0 x 10 ^{3}	71.6	15.7	-	89.4	17.6	+	5
				72.4	16.0	-	>5
1.0 x 10 ^{4}	71.8	12.8	-	90.0	18.1	-	>6
				71.5	12.6	-	>6
1.0 x 10 ^{4}	71.9	12.8	-	89.9	17.9	-	>6
				71.4	12.8	-	>6
1.0 x 10 ^{4}	71.8	12.7	-	89.8	17.6	-	>6
				71.6	12.8	-	>6
2.2 x 10 ^{4}	72.1	14.7	-	90.0	5.0	-	>6
				72.0	15.0	-	>6
2.0 x 10 ^{5}	71.7	15.1[2)]	-	90.1	5.0	+	7
				72.0	15.0	-	>7
7.0 x 10 ^{4}	72.2	14.4	+	90.3	5.0	+	6
				72.2	15.0	-	>6

[1)] experiments were performed in pairs

[2)] survival detected in sample after first treatment at 6.6 cfu ml^{-1}, corresponds to a reduction of 2 $\log_{10}$ cycles

3.6 HTST in combination with homogenization (series F)

Heating experiments in combination with homogenization were performed with homogenization applied either prior to heating (upstream) or after heating (downstream). For downstream homogenization pressures up to 700 bar were applied, although in industrial practice the process is run at approximately 200 bar.

Tab. 14: Inactivation of *M. paratuberculosis* in whole milk during HTST treatment in combination with homogenization

heating conditions	homogenization pressure (bar) positives/total							
	0	200[1)]	200[2)]	300[2)]	400[2)]	500[2)]	600[2)]	700[2)]
72 °C/15 s	0/3	1/3	0/3	0/3	0/3	1/3	0/3	0/3
72 °C/30 s	0/3	2/3	0/3	0/3	0/3	0/3	0/3	0/3
75 °C/15 s	0/3	1/3	1/3	1/3	1/3	0/3	0/3	0/3
75 °C/30 s	0/3	2/3	1/3	2/3	1/3	2/3	2/3	1/3

[1)] homogenization upstream

[2)] homogenization downstream

3.7 Statistics

Heating skim milk at 67-90 °C for 15-60 s (series A)

A logistic regression equation was applied to evaluate the possible influence of heating temperature, holding time and initial count on the variance of the results. Due to the relatively small database for skim milk the fit was poor (R^2 = 0.19). Dependent on the heating temperature, the number of tests with survivors detected decreased with increasing temperatures, and the same tendency was observed for increasing holding times, but neither significantly. The initial count showed a significant dependency in terms of a lower chance of detecting survivors at higher initial counts (Table 15). This contrasts with observations in all other series of experiments and cannot be explained.

Tab. 15: Survival of *M. paratuberculosis* during heat treatments in skim milk – logistic regression

Parameter	estimate	p (0.05/0.01)[1)]	odds ratio
intercept	21.998	0.123	
holding time	-0.204	0.163	0.816
temperature	-0.145	0.058	0.865
holding time*temperature	0.002	0.308	1.002
initial count	-1.758	0.002	0.172

[1)] probability of error 5% (significant) or 1% (highly significant)

Heating whole milk at 95 and 100 °C for 15-60 s (series B) and heating cream at 85-100 °C for 15-60 s (series C)

For both series of experiments the database was too small for statistical evaluation.

Heating whole milk, skim milk and cream at 105-135 °C for 2-5 s (series D)

Within series D a check was made whether differences in survival depended on the sample substrate. For this purpose Fisher's exact left-sided test was applied. Compared with skim milk, there was a 4 fold increased chance of detecting survivors in cream and a 10 fold increase in whole milk (Table 16).

Tab. 16: Dependency of the survival of *M. paratuberculosis* on the sample substrate

combination	Fisher's exact left sided test	odds ratio	confidence limits
cream – raw milk	p < 0.01	0.293	0.11 - 0.80
cream – skim milk	p < 0.05	3.371	0.87 - 15.75
skim milk – raw milk	p < 0.001	0.087	0.02 - 0.31

In addition, logistic regression was applied to evaluate the possible influence of heating temperature, holding time, initial count (log) and substrate on the probability of detecting survivors. The result of predictive power as generalized R^2 was 0.22. Dependent on

heating temperature the number of results with survivors detected decreased with increasing temperatures ($p < 0.05$). The same was observed at prolonged holding times ($p < 0.05$). Although both values were significant, the low predictive power of the model must also be considered. The inoculum showed no influence on the number of positive results. A highly significant influence was detected for the substrates ($p < 0.001$). Especially skim milk showed a significant lower odds ratio than raw milk (odds ratio=0.071) of yielding positive results (Table 17), meaning that the chance of detecting survivors in raw milk is 14 fold higher than in skim milk.

Tab. 17: Dependency of survival of *M. paratuberculosis* from different parameters – logistic regression

Parameter	estimate	p (0.05/0.01)[1)]	odds ratio
intercept	5.7963	0.1231	
holding time	-0.3363	0.0337	0.714
temperature	-0.0465	0.0423	0.955
initial count	-0.0389	0.9467	0.962
substrate cream	-0.0245	0.9393	0.258[2)]
substrate skim milk	-1.3074	0.0012	0.071[2)]

[1)] probability of error 5% (significant) or 1% (highly significant)

[2)] compared to raw milk

Double pasteurization of whole milk (series E)

During double pasteurization, more positive results occurred after the second treatment. According to Fisher's exact left sided test, this difference was not significant (p=0.57). In addition, among the samples positive after the second treatment, more positives occurred after treatment at 90 °C than after treatment at 72 °C. However, the database was too small to establish statistical significance.

HTST in combination with homogenization (series F)

The effects of homogenization treatments, upstream or downstream, at 200 bar working pressure, on inactivation of *M. paratuberculosis* cells were compared using Fisher's exact left sided test. Although fewer survivors were detected after downstream homogenization, the difference was not significant (p= 0.096).

A logistic regression was used to test whether the initial count, homogenization pressure, heating temperature and holding time influenced inactivation, including all homogenized and non-homogenized samples from this series of experiments. Homogenization had no influence on inactivation but heating temperature had a significant influence. There was a 167-fold higher chance of detecting survivors after heating the sample at 75 °C than at 72 °C (Table 18).

Tab. 18: Dependency of survival of *M. paratuberculosis* on different parameters, including homogenization – logistic regression

Parameter	p (0.05/0.01)[1)]	odds ratio
initial count	0.1715	0.141
homogenization	0.9223	
temperature, 72 vs. 75 °C	0.0074	0.006
holding time, 15 vs. 30 s	0.2272	0.397

[1)] probability of error 5% (significant) or 1% (highly significant)

4. Discussion and conclusions

Heating experiments reported in the literature show differing results for the heat resistance of *M. paratuberculosis* in milk but it has been confirmed that small numbers may survive HTST treatment. Most of the experiments reported in the literature were performed with whole milk, whereas in practice skim milk and cream are very often subjected to different heating processes. Our objective was to apply heating technology as comparable as possible with commercial systems, and examine the heat resistance of *M. paratuberculosis* in raw whole milk, skim milk and cream, to identify processes where surviving bacteria would not be detectable. A wide range of temperature-time combinations was applied to reflect the process situations occurring in industrial practice. In addition, the efficacy of homogenization in combination with heat treatments was investigated. Our earlier studies (12) and this study confirm that the heat resistance of *M. paratuberculosis* is exceptional compared with other vegetative bacteria. However, it is necessary to consider the experimental design, the heating technology, and even possible bias due to cross-contamination. Other factors influencing the heat resistance, such as effects of clumping, mechanisms of heat inactivation and physiological state of the cells also need to be considered. Although surviving *M. paratuberculosis* cells can be detected after heating at time-temperature combinations ranging from 15 s at 67 °C up to 5 s at 135 °C, a substantial reduction in numbers can be achieved. Whether survival of a few organisms is of public health significance was not a part of this study.

4.1 Experimental design

For the heating experiments a “cocktail” of different *M. paratuberculosis* strains was used to test the selected treatment procedures under conditions of practical relevance. The aim was not to show that different strains may have different heat resistances, but to find the most effective inactivation procedure. Hence, the results do not identify which strain survived a particular treatment, or whether it was the same strain under all conditions tested.

The pilot plant was constructed to reflect industrial practice as closely as possible. To avoid cross-contamination, the regenerator section was replaced by a plate apparatus cooled by ice-water. Due to the small volume being processed and partly relatively low flow rates, which were the consequence of the selected holding time and the dimension of the used holding section, Reynolds numbers varied from approx. 2,800 to 7,000 (Table 2). Even the lowest number indicates turbulent flow, though it cannot be fully compared to Reynolds numbers achievable in high throughput equipment, sometimes larger than 62,000 (11). However, the most important factor in a heating process is the residence time

and the residence time distribution, which depends on the Reynolds number. These figures were calculated for each holding section (Table 2). In the smallest holding section, used for treatments up to the UHT level at holding times of 2 and 5 s, the residence time distribution is approx. 40 %, resulting in a minimum residence time of 1.2 s for a selected holding time of 2 s.

4.2 Cross contamination

The fact that survival seems to be independent of the initial count and of heating conditions suggests that cross-contamination has occurred. Therefore, measures to minimise and, hopefully, prevent cross-contamination in the pilot plant and during treatment of the heated samples in the laboratory, as listed in chapter 2.2.2, were crucial throughout all experiments. Although cross-contamination can never be completely excluded, in view of the listed measures it was very unlikely. This conclusion is supported in addition by the observation that, due to the use of raw whole milk and skim milk and the absence of decontamination, many cultures on HEYM agar and in Dubos medium were spoiled by contaminating flora during the examinations. This was not observed with samples heated above 120 °C, establishing that (i) cross contamination during and after processing did not occur and (ii) heating conditions were stringent enough to destroy the contaminating flora.

4.3 Effect of clumping

M. paratuberculosis cultures were not homogenized prior to heat treatments. This may affect the results for several reasons.

M. paratuberculosis is known to form clumps of cells in fluid culture and in its natural habitat, the gut. The size of these clumps may influence the apparent heat resistance because colony forming units, as recorded in the colony count of the initial inoculum, may be formed by rather small, or rather large, bacterial clumps, making it almost impossible to estimate the “true number” of bacterial cells subjected to the heat treatment. Statistical considerations led Klijn et al. (14) to postulate that, dependent on the fraction of large clumps present in a sample, a “tailing” of thermal death curves will occur. The more cells present in clumps, when each clump counts as a cfu, the more pronounced the tailing effect, because the true number of cells undergoing the heat treatment is underestimated. For these calculations a clump size of up to 10^4 cells was assumed. ”Tailing” is unlikely to be a consequence of slow heat penetration because calculations suggest that the time to reach equilibrium temperatures in bacterial clumps during heating is of the order of a few hundredths of a second (15). In relation to commercial situations, the size of clumps occurring in faeces should be considered, because the faecal route is the most important for contamination of milk. Herman et al. (16) observed clumps of only 10 cells at a maximum in faeces of infected dairy cattle. Our own observations with faeces of infected cattle showed clumps of up to several hundred cells, with the cells present, more or less, in a monolayer. Those observations were confirmed by a routine laboratory for the diagnosis of paratuberculosis (Veterinary State Laboratory, Oldenburg, personal communication). It should be noted, that clumps from liquid culture are very dense and of a globule-like shape with many layers of cells.

Currently the significance of the size and shape of clumps cannot be assessed. The sample to be heated may contain a mixture of small and large clumps. That sample is stirred during the entire experimental heating process, leading to a random feeding of the pilot plant with clumps of different sizes.

4.4 Mechanism of heat inactivation

Surviving *M. paratuberculosis* were detected in all types of experiments performed. In these and in earlier investigations (12), survival of low numbers of microorganisms appeared to be independent of heating temperature, holding time and initial count. From visual inspection of the scatterplot in Figure 3 it might even be postulated that the experimental conditions had no influence on survival.

Although no inactivation curve could be produced, the more or less random detection of survivors may partly be explained by the so-called “vitalistic theory or principle of inactivation” (17, 18, 19). Van Boeckel (17), after evaluation of 55 studies from the literature, concluded that the classical first order kinetics approach for the inactivation of microorganisms is the exception rather than the rule because the majority of survivor curves for vegetative cells evaluated were non-linear. The vitalistic theory is based on a probabilistic approach and that for each single cell there is a certain probability that it dies due to a lethal cause (i.e. heat). This leads to a distribution of inactivation times, even in a pure culture, because of the probability of death is different for each cell. The inactivation curve for the whole population is the cumulative distribution function of the respective probability density function of individual inactivation times (17). Existence of an exceptionally resistant and very small fraction of cells within the population of *M. paratuberculosis* cells would, to some extent, explain the reported results. Some evidence for the existence of at least different cell fractions can be seen in earlier examinations based on viability stainings (20,12) and the observations in old stationary cultures of *M. smegmatis* (21) (see chapter 4.5).

Further evidence for the possible presence of an extremely resistant fraction of cells can be found in results from several publications where authors reported the occasional detection of survivors at temperatures and holding times above usual pasteurization conditions (Table 19).

Tab. 19: Survival of *M. paratuberculosis* at time temperature combinations exceeding standard pasteurization conditions

Substrate	heating technology	temperature (°C)	holding time	reference
retail whole milk	commercial	138.0	30 s	(6)
whole milk	pilot plant	82.5	60 s	(10)
whole milk	commercial	78.0	15 s	(11)
whole milk	pilot plant	90.0	60 s	(12)
whole milk	laboratory (not specif.)	100.0	10 min	(16)
whole milk	Franklin apparatus	90.0	15 s	(22)
whole milk		134.9	5 s	present
skim milk	pilot plant	134.9	5 s	study
cream (30%)		135.2	2 s	

In agreement with our present and earlier studies, other authors failed to observe any effect of holding time on survival (12, 16, 22). In addition, little dependence of survival on the initial count has been reported; survivors being detected even after heating samples containing less than 10 cfu ml^{-1} (12, 16).

Another possible mechanism of survival at extreme heating conditions is the hypothesis of heat activation, which is supported by (i) earlier detection of survivors during recultivation and (ii) detection of survivors after heating at relatively severe temperatures.

Herman et al. (16) demonstrated significant heat activation, based on the observation of earlier detection than normal during isolation of *M. paratuberculosis* from heated milk samples.

Grant et al. (10) proposed heat activation based on the preferred detection of survivors after heating at elevated temperatures but were unable to confirm it statistically. We previously reported a significantly higher number of samples containing survivors after heating at elevated temperatures (12), while in the present study this occurred after heating raw milk ($p = 0.0158$), but not cream or skim milk. During double pasteurization more positive results occurred after a second treatment at 90 °C compared with a second treatment at 72 °C, but the database was too small to establish significance. Statistical evaluation of the homogenization experiments showed a high influence of heating temperature on survival, with a 167-fold greater chance of detecting survivors if the sample was heated at 75 °C rather than at 72 °C. Summarising and reanalysing these and our earlier observations, and additional data from the literature, with respect to the hypothesis of heat activation, this should be considered as an item of further research.

4.5 Physiological properties of *M. paratuberculosis*

No physiological properties of *M. paratuberculosis* that would explain the exceptional heat resistance are apparent. Hypotheses regarding effects of a steady state metabolic activity, dormancy, or even spore-like properties, as already discussed (12), may need to be reconsidered.

With respect to metabolic activity, it was shown in *M. smegmatis*, via gene expression studies, that old stationary cultures (> 128 d) contained a small proportion of very active cells, while most of the other cells were completely inactive. The ratio of active to inactive cells was estimated as 1:1000 (21). A similar observation had previously been made for *M. paratuberculosis* by application of a viability staining (12). Whether the inactive cell fraction can survive strong heat treatments, or whether they can even be activated by heat needs further investigations.

For *M. tuberculosis*, a dormancy operon containing 48 genes has been identified (25). The status of dormancy of *M. tuberculosis* is characterized by a shift from rapid to slow replication, and finally a shutdown of replication. Markers for this process are e.g. growing tolerance to anaerobic conditions, production of unique antigens, tenfold increase of glycine dehydrogenase production, and induction of expression of the sigF gene (encodes a stress-response sigma factor) (26). It would be of interest to investigate whether similar observations can be made for *M. paratuberculosis*.

As stated in a report of an ILSI working group on "*M. paratuberculosis* and the food chain", presently the heat resistance of *M. paratuberculosis* cannot be explained with any reason. The final sentence on that topic in that report is:

"If such mechanisms do not withstand experimental scrutiny we would be forced to reconsider the possibility that, despite the great care taken by experimentalists, there is some unknown property of M. paratuberculosis that prevents some cells receiving the intended heat treatment in pasteurizers or submerged ampoules"(1).

4.6 Efficacy of heat treatment

Despite all uncertainties regarding the complete inactivation of *M. paratuberculosis* during heat treatment and possible reasons for survival, a reduction of at least 5 to 7 $\log_{10}$ cycles could be demonstrated in whole milk, skim milk and cream.

In the framework of the Codex Alimentarius for pasteurized milk this should be fully sufficient. Experimental work performed all over the world, mainly in the years 1930 to 1960, led to the common acceptance that holder pasteurization and high temperature short time pasteurization are sufficient to inactivate the classical mycobacteria *M. tuberculosis* and *M. bovis* in milk to an extent that they no longer harm human health. The standards developed during that time for both types of heat treatment, which are still valid today, were based upon approx. 100 scientific reports (23).

Slightly more stringent heating conditions were introduced for the inactivation of *Coxiella (C.) burnettii*. Citation from Codex Alimentarius:

"As C. burnettii is the most heat-resistant non-sporulating pathogen likely to be present in milk, pasteurization is designed to achieve at least a 5 log reduction of C. burnettii in whole milk (4% milk fat). According to validations carried out on whole milk, the minimum pasteurization conditions are those having bactericidal effects equivalent to heating every particle of the milk to 72 °C for 15 seconds (continuous flow pasteurization) or 63 °C for 30 minutes (batch pasteurization)" (24).

For UHT-treatment the situation is completely different. According to the definition of the Codex Alimentarius, this process should result in a commercial sterile product:

"*UHT-treatment of milk and liquid milk products is the application of heat to a continuously flowing product using such high temperatures for such time that renders the product commercially sterile at the time of processing. When the UHT treatment is combined with aseptic packaging, it results in a commercially sterile product. UHT-treatment is normally in the range of 135 to 150 °C in combination with appropriate holding times necessary to achieve commercial sterility"* (24).

In series D (chapter 3.4) most tested temperatures were below 135 °C. Though in the range above 120 °C contaminants did not occur in cultures from samples heated above this temperature (see chapter 4.2), however, *M. paratuberculosis* was still detectable.

Regarding the relatively low numbers of *M. paratuberculosis* that can be expected in naturally contaminated milk, heating processes according to the time-temperature combinations tested will lead to a significant reduction of all cells. However, the results reported here show that complete inactivation cannot always be achieved. Results reported in the literature (Table 1) show, that surviving *M. paratuberculosis* can be expected in pasteurized milk and even in milk treated at UHT-level (6), and in other dairy products at retail level. Those findings are supported by the experiments performed with commercial pasteurizers, which resulted in occasional survival of *M. paratuberculosis* at very low levels (9, 11).

In terms of a risk-based approach the main question is, how many surviving cells are acceptable in a product with respect to public health issues. Not regarding the still open discussion whether *M. paratuberculosis* is a human pathogen, a 5-7 $\log_{10}$ cycles reduction was achieved.

4.7 Efficacy of homogenization

In contrast to the results of Grant et al. (10), no significant effects of homogenization, either upstream or downstream, were observed. A tendency to a greater efficacy of

downstream homogenization was not significant. In earlier experiments homogenization without heat treatment, even at homogenization pressures up to 700 bar, had no significant effect on colony counts (data not shown).

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6. Summary

Hammer, P., Kiesner, C., Walte, H.-G., Teufel, P.: **Inactivation of *Mycobacterium avium* subsp. *paratuberculosis* in whole milk, skim milk and cream in a pilot plant pasteurizer**. Kieler Milchwirtschaftliche Forschungsberichte **58** (1) 17-40 (2006)

06 Veterinary medicine and hygiene (*mycobacterium avium* ssp. *paratuberculosis*, heat resistance, pilot plant pasteurizer, whole milk, skim milk, cream)

Heating experiments reported in the literature show differing results about the heat resistance of *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) in milk. Presently it seems to be clear that small numbers may survive HTST treatment.
Recent reports with special relationship to practice show that *M. paratuberculosis* may indeed occur in commercial dairy products. Most of the experiments reported in the

literature were performed with whole milk, whereas in practice skim milk and cream are very often subjected to separate heating processes. The goal of this study was to apply a heating technology – as comparable as possible to commercial systems – to examine the heat resistance of *M. paratuberculosis* in raw whole milk, skim milk and cream and to find cutoff points where surviving bacteria would be no longer detectable. A wide range of temperature-time combinations was applied to reflect the process situations occurring in industrial practice. In addition, the efficacy of homogenization in combination with heat treatments and double pasteurization was investigated.

Raw whole milk, skim milk and cream were inoculated with a cocktail of five *M. paratuberculosis* strains of bovine, caprine and human origin at colony counts between 10^3-10^5 cfu ml^{-1}. All substrates were subjected to heat treatment in a pilot plant pasteurizer. Within the experimental setup avoidance and exclusion of cross-contamination were a main topic. Skim milk was heated between 67-135 °C, whole milk between 95-135 °C and cream between 85-135 °C at holding times between 2-60 s. Double pasteurization and homogenization experiments were done with raw whole milk at heating temperatures between 72-90 °C. Cultural detection of surviving *M. paratuberculosis* was performed by application of 6 month resuscitation/enrichment in modified Dubos medium and subsequent culture onto Herrolds egg yolk medium for another 6 months. Survivors were identified by acid fast staining and IS900 based PCR.

Low levels of surviving *M. paratuberculosis* were detected occasionally in all types of experiments performed, even at time-temperature combinations of UHT-treatment. Neither *M. paratuberculosis* could be excluded with the applied heat treatments nor be reduced below the detection limit. According to the statistical analysis survival was dependent on the heating temperature and the holding time but not on the initial bacterial count. The best chance to detect survivors was in whole milk followed by cream and skim milk.

Neither additional upstream nor downstream homogenization led to a significant inactivation during heat treatment. The same applied to double pasteurization.

Presently no physiological properties of *M. paratuberculosis* are known to explain the exceptional heat resistance. Despite the uncertainties about single cells surviving the heat treatment, a reduction of up to 7 log cycles could be demonstrated.

Zusammenfassung

Hammer, P., Kiesner, C., Walte, H.-G., Teufel, P.: **Inaktivierung von *Mycobacterium avium* ssp. *paratuberculosis* in Vollmilch, Magermilch und Rahm in einer Pilotanlage**. Kieler Milchwirtschaftliche Forschungsberichte **58** (1) 17-40 (2006)

06 Veterinärmedizin und Hygiene (*Mycobacterium avium* ssp. *paratuberculosis*, Hitzeresistenz, Pilotanlage,Vollmilch, Magermilch, Rahm)

In der wissenschaftlichen Literatur aufgeführte Experimente zur Hitzeresistenz von *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) in Milch zeigen unterschiedliche Ergebnisse. Derzeit scheint es aber klar zu sein, dass geringe Keimzahlen die Kurzzeiterhitzung überleben können. Neuere Berichte von besonderer Relevanz für die Praxis zeigen, dass *M. paratuberculosis* tatsächlich in Molkereiprodukten aus dem Handel vorkommen kann. Für die meisten der bisher durchgeführten Erhitzungsversuche wurde Vollmilch verwendet, obwohl in der Praxis Magermilch und Sahne sehr

oft getrennt wärmebehandelt werden. Ziel dieser Studie war es, mit einer den Bedingungen in der Praxis weitestgehend vergleichbaren Technologie, die Hitzeresistenz von *M. paratuberculosis* in roher Voll- und Magermilch sowie in Sahne zu untersuchen. Insbesondere sollten Temperatur-Zeit-Kombinationen gefunden werden, bei denen überlebende Erreger nicht mehr nachweisbar sind. Hierfür wurde eine Vielzahl von in der Praxis angewendeten Prozessbedingungen überprüft. Zusätzlich wurden auch durch eine Homogenisierung und eine Doppelpasteurisierung zu erzielenden Effekte mit untersucht.

Rohe Voll- und Magermilch sowie Sahne wurde mit einem Cocktail aus fünf *M. paratuberculosis* Stämmen boviner, capriner und humaner Herkunft beimpft. Die Keimzahl in der Milch betrug danach 10^3-10^5 KbE ml^{-1}. Alle Substrate wurden in einer Pilotanlage einer Hitzebehandlung unterzogen, wobei größter Wert auf Verhinderung und Ausschluss von Kreuzkontaminationen gelegt wurde. Die Magermilch wurde zwischen 67-135 °C, die Vollmilch zwischen 95-135 °C und die Sahne zwischen 85-135 °C bei Heißhaltezeiten von 2-60 s erhitzt. Experimente zur Doppelpasteurisierung und Homogenisierung wurden nur mit Vollmilch bei Erhitzungstemperaturen von 72-90 °C durchgeführt. Der kulturelle Nachweis von überlebenden *M. paratuberculosis* wurde nach sechsmonatiger Anreicherung bzw. Resuszitation in einem modifizierten Dubos-Medium und nachfolgendem Ausstrich auf Herrolds Eigelbmedium und Bebrütung für weitere sechs Monate geführt. Die Identifizierung erfolgte über eine Färbung auf Säurefestigkeit und eine auf dem Nachweis des Insertionssegmentes 900 basierende PCR.

In allen durchgeführten Experimenttypen wurden niedrige Keimzahlen überlebender *M. paratuberculosis* nachgewiesen, sogar bei Temperatur-Zeitkombinationen die einer UHT-Behandlung entsprechen. Mit keiner der angewendeten Hitzebehandlungen war es möglich, die Abwesenheit von Überlebenden zu garantieren, bzw. die entsprechende Anzahl auf ein Niveau unterhalb der Nachweisgrenze zu reduzieren. Die statistische Auswertung zeigte eine Abhängigkeit des Überlebens von Erhitzungstemperatur und Heißhaltezeit, aber nicht von der Ausgangskeimzahl. Bezogen auf das Substrat war die Wahrscheinlichkeit in Vollmilch Überlebende zu finden am größten, gefolgt von Sahne und Magermilch.

Durch zusätzliche Homogenisierung während der Hitzbehandlung konnte weder im Zulauf noch im Ablauf die Inaktivierungsrate signifikant beeinflusst werden. Das Gleiche galt für die Doppelpasteurisierung.

Derzeit gibt keine Erklärung für die beobachtete Hitzeresistenz von *M. paratuberculosis*. Unabhängig davon ob einzelne Zellen eine Hitzbehandlung überstehen, konnte insgesamt eine Reduzierung der Ausgangskeimzahl um bis zu 7 log-Stufen erreicht werden.

Résumé

Hammer, P., Kiesner, C., Walte, H.-G., Teufel, P.: **Inactivation de *Mycobacterium avium* subsp. *paratuberculosis* dans du lait entier, du lait écrémé et dans de la crème fraîche dans un pasteurisateur pilote** .Kieler Milchwirtschaftliche Forschungsberichte **58** (1) 17-40 (2006)

06 Médecine vétérinaire et hygiène (*mycobacterium avium ssp. paratuberculosis*, résistance à la chaleur, pasteurisateur pilote, lait entier, lait écrémé, crème fraîche)

Les essais faits sur la résistance à la chaleur de *Mycobacterium avium* subsp. *paratuberculosis* (*M. paratuberculosis*) mentionnés dans la littérature aboutissent à des résultats différents. Actuellement il parait être clarifié que des charges bactériennes

réduites peuvent survivre à la pasteurisation brève. Des rapports plus récents, étant d'un grand intérêt pour la pratique, démontrent que *M. paratuberculosis* peut en effet se produire dans des produits laitiers usuels. Bien qu'en pratique le lait écrémé et la crème subissent des traitements thermiques séparés, la plupart des essais thermiques se faisait jusqu'à présent avec du lait entier. Le but de cette étude était d'examiner la résistance à la chaleur de *M. paratuberculosis* dans du lait entier cru et dans la crème sous des conditions à peu près comparables à celles de la pratique. En particulier il s'agissait de trouver des combinaisons température-temps sous lesquelles il n'est plus possible de détecter des agents pathogènes survivants. C'est ainsi qu'un grand nombre de conditions procédurales appliquées dans la pratique ont été analysées. Les effets qu'on attend d'une homogénéisation et d'une pasteurisation double ont également été analysés.

Du lait entier cru et du lait écrémé ont été inoculés avec un cocktail de cinq souches de *M. paratuberculosis* d'origine bovine, caprine et humaine. La teneur bactérienne dans le lait comptait par après 10^3-10^5 ufc ml^{-1}.Tous les substrats étaient soumis à un traitement thermique dans un équipement pilote. Une attention particulière a été attribuée à l'empêchement et l'exclusion de contaminations croisées. Le lait écrémé était chauffé à 67-135 °C, le lait entier à 95-135 °C et la crème à 85-135 °C avec des durées de chambrage de 2-60 s. Des expériences sur la pasteurisation double et l'homogénéisation étaient uniquement réalisées avec du lait entier à des températures d'échauffement de 72-90 °C. La détection de *M. paratuberculosis* survivants était réalisée après un enrichissement de six mois, respectivement une ressuscitation dans un milieu Dubos modifié et après un frottis ultérieur sur gélose milieu de Herrolds au jaune d'œuf et une incubation ultérieure de six mois. Les *M. paratuberculosis* survivants étaient identifiés par coloration résistant à l'acide et par IS900 basée sur PCR.

Dans tous les genres d'essais réalisés des teneurs bactériennes minimales en *M. paratuberculosis* étaient occasionnellement détectées, même à des températures correspondant à un traitement UHT. Aucun des traitements thermiques n'a pu garantir l'absence de survivants ou bien leur réduction à un niveau en-dessous de la limite de détection. L'évaluation statistique démontrait une dépendance de la survie des températures d'échauffement et de chambrage mais non de la teneur bactérienne initiale. Par rapport au substrat, la probabilité de trouver des survivants dans du lait entier était la plus grande, suivie par la crème et le lait écrémé.

Une homogénéisation supplémentaire ne pouvait, ni à l'entrée ni à l'écoulement, influencer de manière signifiante le taux d'inactivation. Ceci vaut également pour la pasteurisation double.

A présent, il n'a pas d'explication pour la résistance thermique observée de *M. paratuberculosis*. Indépendamment du fait que des cellules individuelles pouvaient survivre à une traitement thermique, une réduction de la teneur bactérienne initiale jusqu'à 7 cycles log a pu être obtenue.