Efficacy of dairy on-farm high-temperature, short-time pasteurization of milk on the viability of *Mycobacterium avium* ssp. *paratuberculosis*

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ABSTRACT

Feeding pasteurized milk to suckling calves is a popular practice used increasingly on dairy farms. Waste milk is frequently fed to calves because of its high nutritional value and economic benefits compared to milk replacement products. However, one of the disadvantages of feeding waste milk is the potential for exposure to a high number of bacterial contaminants, which may lead to serious illnesses or infections in calves. One of these contaminants is *Mycobacterium avium* ssp. *paratuberculosis* (MAP), the causative agent of Johne's disease (paratuberculosis). The transmission and distribution of paratuberculosis in dairy herds occurs mostly through the feeding newborn calves with contaminated colostrum or milk, because this age group is believed to be most susceptible to infection. To reduce the risk of transmission of pathogens, on-farm pasteurization of milk has become increasingly popular. In this study, we analyzed the efficacy of a new commercial high-temperature, short-time pasteurizer (73.5°C for 20 to 25 s) in terms of MAP inactivation under experimental on-farm conditions. The pasteurizer uses a newly developed steam-heating technique, allowing for the pasteurization of the transition milk without clumping. In 3 independent trials, we spiked fresh raw milk samples to a level of 10^7 or 10^4 viable MAP cells/mL before pasteurization. We examined the thermal inactivation and viability of MAP using culture and a D29 bacteriophage-based assay. To verify the identity and number of MAP cells, we also performed PCR assays. Pasteurization of the inoculated milk (10^7 and 10^4 MAP cells/mL) resulted in a remarkable reduction in viable MAP cells. The mean inactivation rate of MAP

ranged from 0.82 to 2.65 $\log_{10}$ plaque-forming units/mL, depending on the initial MAP amount inoculated and the addition of conservative agents to the pasteurized milk. Nevertheless, approximately 10^3 MAP cells/mL remained viable and could be transferred to calves after high-temperature, short-time pasteurization of milk.

Key words: *Mycobacterium avium* ssp. *paratuberculosis*, pasteurization, phage assay, waste milk

INTRODUCTION

Since the 19th century, pasteurization has been the standard method used to preserve milk for human consumption. This thermal procedure inactivates most pathogenic microorganisms, preventing the oral transmission of milk-borne diseases. In addition, pasteurization increases the shelf life of milk by inactivating spoilage organisms while maintaining the original characteristics and properties of milk and milk products (Holsinger et al., 1997). Alongside the pasteurization of milk for human consumption, the feeding of milk pasteurized on-farm to calves has become increasingly popular. Because of the economic benefits, non-saleable milk, so-called waste milk, is often fed to suckling calves (Stabel et al., 2004), although it presents an increased potential risk of infection. Waste milk is milk that cannot be used for human consumption for various reasons: for example, colostrum milk, milk from cows with mastitis, milk from animals undergoing medical treatment, or milk with high SCC content (Aust et al., 2013). These types of milk contain valuable nutrients for suckling calves but are often contaminated with bacteria that may be pathogenic for the calves and their naïve immune systems (Selim and Cullor, 1997). Bacterial contamination of milk is caused predominantly by direct shedding of pathogenic microorganisms from dairy cows through the udder, or by contact with pathogens from the environment (e.g., fecal contamination; Oliver et al., 2005). One of the most important pathogens for calves is *Mycobacterium avium* ssp. *paratuberculosis* (MAP), which can be transmitted by contaminated

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milk and causes Johne's disease (paratuberculosis). Infection with MAP results in severe enteritis, profuse diarrhea, and progressive weight loss, not in calves but in adult ruminants (Harris and Barletta, 2001). Although symptoms may appear at a later stage, reduced milk production and decreased fertility among dairy cows at the subclinical stage of MAP infection also have a substantial economic impact on the dairy industry (Raizman et al., 2009), in addition to premature culling. Neonatal calves are affected mostly by transmission through colostrum and milk from infected cows (Taylor et al., 1981; Streeter et al., 1995), or from milk or colostrum contaminated with MAP-containing feces (Rademaker et al., 2007). However, feeding calves with pasteurized milk reduces the risk of infection and is suggested to have an immensely positive impact on the health status of suckling calves compared to non-pasteurized milk or milk replacer (Jamaluddin et al., 1996; Godden et al., 2005). To evaluate the efficacy of thermal MAP inactivation in raw milk, we investigated a new on-farm HTST pasteurizer. Its crucial technical difference compared with existing on-farm milk pasteurizers is its newly developed steam-heating system, which enables the pasteurization of transition milk. Under experimental on-farm conditions, fresh raw, bulk milk was inoculated to levels of 10^7 and 10^4 MAP cells/mL. To determine the viability of MAP before and after pasteurization of the inoculated milk, and also in the machine's rinsing and washing water, we carried out culture-based procedures, a phage-based assay, and PCR assays. We performed the present study to investigate the reduction of viable MAP cells by pasteurization as a way of preventing the transmission of paratuberculosis to calves consuming waste milk.

MATERIALS AND METHODS

Study Design and Sample Preparation

Between the end of July and the beginning of November 2017, we obtained fresh raw bulk milk from a local dairy farm directly after machine milking of mid-lactation animals in the morning (before transfer to milk tanks used for human consumption). The milk was then transported to the Institute of Microbiology and Animal Hygiene (Göttingen, Germany) under uncooled conditions to resemble on-farm circumstances (duration of approximately 20 min). The farm's MAP status prior to milk sampling was unknown. The farm houses more than 100 dairy cows, which are milked using a parlor system. The udders of the animals were wiped before milking. The on-farm pasteurizer (HT250; Martin Förster GmbH, Engen, Germany) was placed in an experimental animal stable belonging to the division of

Microbiology and Animal Hygiene (University of Göttingen, Germany). The machine is licensed to prepare milk for calf feeding only, not for human consumption, because a small amount of steamed water is mixed with the milk during pasteurization. The pasteurizer performs continuous-flow milk pasteurization using an HTST method, so that the milk can be directly transferred to the calf feeder. In detail, the inoculated milk was heated by the heat exchanger to 50 to 60°C, pasteurized with steam at 73.5°C for 20 to 25 s, and immediately cooled by the heat exchanger to 40°C. Three independent trials for inoculated milk were carried out for all experiments. For each trial, 40 L of raw milk were collected in 50 L sterile plastic containers. Then, 2 L of fresh raw bulk milk was taken to analyze the characteristics of the original milk from the farm (sample **M**). Then, 28 L of the raw milk was spiked with MAP to a final amount of 10^7 or 10^4 cells/mL (sample **M+**; 2 L of the inoculated milk was also taken for analysis), as described below. Containers were shaken thoroughly to evenly distribute the MAP in the milk. To ensure that no stagnant water was mixed with the milk samples to be analyzed after pasteurization, a milk volume of 12 L was required for pasteurization, because the pasteurizer has a flow-through capacity of 5 to 6 L. Then, each 12 L of inoculated milk was pasteurized (sample **P**). Then, another 12 L of spiked milk was pasteurized, but this time, a conservation agent (Bergo Stabilomix S; Bergophor Futtermittelfabrik Dr. Berger, Kulmbach, Germany) was automatically added after pasteurization at a concentration of approximately 2 mL/L (sample **C**). Conservative agents are often used to stabilize milk and increase its durability. In each trial, 10 of the 40 L milk was left over and used as retention samples.

After each pasteurization process, the pasteurizer performed a rinsing step using tap water (sample **R**). Rinsing took 6 min and used approximately 5 L of tap water per minute. Next, thorough cleaning was performed using 150 mL of detergent (HyClean A70 Pasteurizer Detergent; Förster-Technik GmbH, Engen, Germany). During this step, the machine was rinsed continuously with a disinfecting detergent for 15 min and then a further 15 min with tap water (sample **W**). For analysis of the milk samples (**M**, **M+**, **P**, **C**) or water samples (**R**, **W**), 2 L were collected in sterile glass bottles. All samples were analyzed on the day of collection. To ensure proper handling, conserving, and washing processes, we measured the temperature and pH values of all samples obtained from the first experiment (3 independent trials with milk spiked with 10^7 MAP cells/mL). The pH value of **M+** was not measured to prevent contamination of the pH meter. We also evaluated the pasteurization of all samples by testing the activity of alkaline phosphatase using the Lactognost

test (HEYL Chemisch-pharmazeutische Fabrik GmbH and Co. KG, Berlin, Germany) and peroxidase using the MQuant test (Merck KGaA, Darmstadt, Germany). Both enzymes are known to occur naturally in milk and have different heat stabilities. The absence of alkaline phosphatase activity indicates pasteurization conditions above 72°C for 16 s; the presence of peroxidase confirms conditions below 80°C (Rampling et al., 2004; Sharma and Rajput, 2014).

We determined the presence of MAP in all samples (M, M+, P, C, R, W) derived from milk spiked with 10^7 or 10^4 cells/mL by culture, D29 bacteriophage-based assay, and real-time quantitative PCR (qPCR). All experiments were performed in the laboratory of the division of Microbiology and Animal Hygiene (University of Göttingen), accredited according to the standard DIN EN ISO/IEC 17025:2005.

Inoculation of Raw Milk

Raw milk was spiked with MAP to final amounts of 10^7 and 10^4 cells/mL to imitate concentrations of MAP likely to occur in practice. The MAP reference strain we used (DSMZ ID 44133, Type-II) was from the Leibniz Institute DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). It was cultured in BD Difco Middlebrook 7H9 Broth containing 10% BD BBL Middlebrook OADC Enrichment (BD, Heidelberg, Germany), 0.2% glycerin (Carl Roth GmbH and Co. KG, Karlsruhe, Germany), and 2 µg/mL Mycobactin J (ID.vet, Grabels, France). The MAP was grown for 4 to 6 wk at 37°C under aerobic conditions in a shaker incubator at 100 rpm. For inoculation of the raw milk, the MAP culture solution was ultrasonicated twice for 2 min at 20 kHz (Sonoplus HD 2200; Bandelin Electronic GmbH and Co. KG, Berlin, Germany) to overcome bacterial clumps and adjusted to 10^9 or 10^7 MAP cells/mL by optical density measurement (BioPhotometer; Eppendorf AG, Hamburg, Germany) as described by Janagama et al. (2006). In total, we used 280 mL of the solution containing 10^9 MAP cells/mL to produce inoculated milk containing approximately 10^7 MAP cells/mL in 28 L of raw milk. To obtain milk with 10^4 MAP cells/mL, we used 28 mL of the MAP culture solution containing 10^7 cells/mL to inoculate fresh raw milk. Containers were mixed by shaking, and pasteurization was started immediately.

Detection of Viable MAP by Culture

All samples (M, M+, P, C, R, W) of both tested concentrations (10^7 and 10^4 MAP cells/mL) were prepared for culture. First, 10 mL of the samples were suspended

in 40 mL of 0.75% RonaCare CPC (Merck KGaA, Darmstadt, Germany) for a 1:5 ratio, mixed for 30 min at 250 rpm, and incubated for 24 h at room temperature. Then, the solutions were centrifuged at $3,000 \times g$ for 30 min. The supernatant was decanted, and the remaining cell pellet was suspended in 1.25 mL of 0.9% sterile NaCl solution. Each 0.25 mL of the suspension was cultured on 3 Herrold's egg yolk medium (HEYM) slants. Preparation of HEYM was in accordance with the official manual of diagnostic procedures published by the Friedrich-Loeffler-Institut of the German Federal Research Institute of Animal Health (Friedrich-Loeffler-Institut, 2014). The inoculated HEYM slants were incubated at 37°C. After 4 wk, bacterial growth was monitored for the first time and then regularly for up to 16 wk. Growth of MAP was verified by a MAP-specific semi-nested PCR (snPCR) as described below.

Detection of Viable MAP by D29 Bacteriophage-Based Assay

We also conducted D29 bacteriophage-based assays according to Foddai et al. (2009). To obtain the detection limit of the D29 bacteriophage-based assay for MAP in milk samples, a decimal dilution series ranging from 10^8 to 10^0 MAP cells/mL milk was performed and analyzed. Before the test, 1 mL of each dilution or 10 mL of each sample (M, M+, P, C, R, W) was centrifuged for 30 min at $3,000 \times g$. The supernatant was decanted and the remaining cell pellet was resuspended in 1 mL of a 0.9% sterile NaCl solution. Then, 100 µL of the D29 bacteriophage suspension containing 10^{10} pfu was added to the samples in each assay. Propagation of the D29 bacteriophage was carried out as described by Foddai et al. (2009). The suspension was incubated for 3 h at 37°C and 100 rpm. After the first incubation, the remaining extracellular bacteriophages were inactivated by virucide treatment with 100 µL of sterile 100 mM ammoniumferrous (II) sulfate-hexahydrate for 5 min without shaking, followed by 5 min of inversion. Afterward, the virucide was neutralized by adding 5 mL of Middlebrook 7H9 Broth medium containing 10% Middlebrook OADC Enrichment and 2 mM CaCl_2 . Samples were incubated for 2 h at 37°C under continuous inversion. All suspensions were diluted in decimal dilution series up to 10^{-5} in Middlebrook 7H9 Broth medium. Then, 1 mL of the suspension, its remaining residue, and 100 µL from each dilution were mixed in Petri dishes with 1 mL of *Mycobacterium smegmatis* laboratory strain (MC² 155) fluid culture, 5 mL of Middlebrook 7H9 Broth medium containing 10% Middlebrook OADC Enrichment, 2 mM CaCl_2 , and a further 5 mL of Middlebrook 7H9 agar. All samples were mixed

carefully by panning. After cooling for approximately 15 min, the agar plates were incubated at 37°C for 24 h.

As positive control, we used a MAP culture solution with approximately 10^8 or 10^5 cells as a reference assay, prepared simultaneously during all assays. We confirmed the functionality of the assay with different control trials; that is, virus activity and inactivation and the activity of *M. smegmatis* MC² 155 as a sensor cell were checked separately in each run. Further, to ensure sterile sample preparation, single nutrition media—nutrition media containing only bacteriophages and media mixed with *M. smegmatis* MC² 155 (independent or in combination with virus) were prepared separately for all assays. Plaques (pfu/mL) on agar plates were counted, taking only plates with 5 to 300 plaques into account. Calculation of plaques [$\pm$ standard deviation (SD)] was carried out based on the assumption that 1 mycobacterial cell in the original sample represented 1 pfu (Foddai et al., 2010). We also calculated the mean reduction from milk spiked with MAP (M+) to pasteurized milk (P) and from M+ to pasteurized milk with conservative agents (C). The number of bacteria in M+, P, and C samples for each trial, as were detected by the D29 phage assay, were logarithmized to base 10. We calculated the reduction of pfu/mL for each trial, and then the mean reduction overall from M+ to P and M+ to C ($\pm$ SD). All calculations were performed using Excel (Microsoft, Redmond, WA).

To exclude the detection of other mycobacteria in milk samples before inoculation of MAP (M), we performed a *Mycobacterium avium* complex multiplex PCR of the DNA extracted from the plaques or agar pieces as described below.

Detection of MAP by Quantitative Real-Time PCR

For qPCR analysis, we extracted DNA from each sample (M, M+, P, C, R, and W derived from milk spiked with of both MAP contents of 10^7 and 10^4 cells/mL) using a modified protocol from the InnuPREP MP Basic Kit A (Analytik Jena AG, Jena, Germany). First, 10 mL of each sample was centrifuged at $3,000 \times g$ for 30 min. The supernatant was decanted, and the cell pellet resuspended in 1 mL of 0.9% NaCl solution. Each sample was ultrasonicated twice for 2 min at 20 kHz and transferred to a Precellys tube (Soil grinding SK38, Precellys Lysing Kit; Bertin Instruments, Montigny-le Bretonneux, France). Then, 250 μ L of MagNA Pure Bacteria Lysis Buffer (Roche Molecular Diagnostics, Pleasanton, CA) was added and the samples were homogenized at 6,500 rpm for 30 s in a Precellys 24 homogenizer (Bertin Instruments). After mixing with 60 μ L of proteinase K solution (20 mg/mL; Carl Roth GmbH + Co. KG), the samples were incubated in a

shaker at 900 rpm and 56°C for 60 min (Bioer Mixing Block MB- 102; Bioer Technology, Hangzhou, China). Samples were transferred into new sterile tubes, 450 μ L of Lysis Solution RL were added, and then the samples were mixed and incubated for 15 min at room temperature with continuous shaking at 250 rpm on a thermomixer. The suspension was centrifuged briefly before the supernatants were transferred into new tubes and 450 μ L of Binding Solution B and 20 μ L of MAG Suspension M were added. Next, the suspensions were mixed, incubated for 5 min at room temperature, and washed according to the kit manual. The DNA samples were eluted with 100 μ L of DNase-free and RNase-free water and stored at -80°C before use. In each DNA extraction, we included a negative control of 1 mL of sterile 0.9% NaCl solution and a positive control of 1 mL of pure MAP culture (DSMZ ID 44133) with a content of 10^8 cells/mL.

The qPCR was performed as described by Fechner et al. (2017). A MAP DNA standard curve (DSMZ ID 44133) was titrated in $\log_{10}$ steps (1 ng/ μ L to 1 fg/ μ L) for quantification of MAP DNA concentration, as well as a positive control. We calculated MAP genome equivalents (MAP-GE) detected by qPCR as described elsewhere (Fechner et al., 2017), assuming a DNA concentration of 5.29 fg/MAP cell. The qPCR had a detection limit of 50 fg/reaction (approximately 10 MAP-GE per reaction) and a quantification limit of 5 pg of MAP DNA per reaction (approximately 950 MAP-GE per reaction; Fechner et al., 2017).

Verification of MAP Growth by Qualitative snPCR

Growth of MAP on HEYM slants was verified by snPCR. Pure colonies on the HEYM slants were suspended in 100 μ L of sterile 0.9% NaCl solution and incubated at 100°C and 300 rpm. After 20 min, the suspensions were centrifuged at $14,000 \times g$ for 10 min, and the supernatants were collected and centrifuged again. The extracted DNA was used as the DNA template. The snPCR was performed as described elsewhere (Münster et al., 2011). We used sterile distilled water as a negative control and extracted MAP DNA (10 ng/ μ L; DSMZ ID 44133) as a positive control in each PCR run. We carried out DNA isolation, preparation of master mixes, addition of template DNA, and electrophoresis in separate laboratories to avoid cross-contamination.

Verification of MAP in Plaques of the D29 Bacteriophage Assay by *M. avium* Complex Multiplex PCR

To exclude the detection of other mycobacteria in milk samples before inoculation with MAP (M), we

extracted DNA from the center of the plaques using the MiniElute Gel Extraction Kit (Quiagen, Hilden, Germany) after D29 bacteriophage-based assay performance. The DNA was used for *M. avium* complex multiplex PCR as described by Shin et al. (2010). In the case of no plaque formation, agar pieces were controlled equally. *M. avium* complex multiplex PCR allowed for the differentiation of mycobacteria species. In each PCR run, sterile distilled water was used as a negative control, and *M. avium* ssp. *avium* DNA extracted from the reference strain DSMZ ID 44156 (Leibniz Institute DSMZ German Collection of Microorganisms and Cell Cultures) was used as a positive control.

RESULTS

Enzyme Activity, pH Value, and Temperature Tests

All milk samples (M, M+, P, C) of the 3 trials (12 samples in total) initially spiked with 10⁷ MAP cells/mL were positive by peroxidase test, and positive by alkaline phosphatase test only in milk samples before pasteurization (M, M+). This finding confirmed the proper pasteurization process. The temperature of the milk samples before pasteurization (M, M+) ranged from 24.5°C to 30°C, and the temperature of the milk samples after pasteurization (P, C), as well as the water samples (R, W) ranged from 43°C to 48.5°C (Table 1). The pH values of milk that was not inoculated and pasteurized ranged from 6.6 to 6.7. The pH values of milk samples with a conservative agent (C) were ap-

proximately 6.0. The pH values of the rinsing water and washing water samples (R, W) ranged from 7.1 to 7.5 and 12.1 to 12.6, respectively.

Detection Limit of D29 Bacteriophage-Based Assay in Raw Milk

We performed a decimal dilution series ranging from 10⁸ to 10⁰ MAP cells/mL of milk and analyzed it to determine the detection limit of the phage-based assay for milk samples (Figure 1).

In raw milk samples before inoculation with MAP cells, no plaque-forming units were detected, verifying the absence of naturally occurring mycobacteria. In spiked milk samples (10⁸ to 10⁰ MAP cells/mL), plaque-forming units were detected up to 10² MAP cells/mL of milk. We calculated the MAP content from each dilution in triplicate, assuming that 1 pfu corresponded to 1 MAP cell (Foddai et al., 2010).

Detection of Viable MAP by D29 Bacteriophage-Based Assay

To detect viable MAP by D29 bacteriophage-based assay, all samples (M, M+, P, C, R, W) derived from milk spiked with 10⁷ and 10⁴ MAP cells/mL were analyzed in triplicate and in 3 independent trials (Figures 2 and 3). In 2 raw milk samples before inoculation (M), MAP was detected by D29 bacteriophage-based assay and verified by MAP-PCR. The detected counts of plaque-forming units (pfu; ±SD) were 2.29 × 10³ ±

Table 1. Temperature, pH value, and detection of peroxidase and alkaline phosphatase for each milk sample with an initial number of 10⁷ *Mycobacterium avium* ssp. *paratuberculosis* cells/mL in 3 independent trials (n = 3)

Sample ¹	Trial	Temperature, °C (±0.5)	pH (±0.2)	Peroxidase	Alkaline phosphatase
M	1	27	6.6	+	+
	2	25	6.7	+	+
	3	30	6.6	+	+
M+	1	26	—	+	+
	2	24.5	—	+	+
	3	28.5	—	+	+
P	1	44	6.7	+	—
	2	43	6.7	+	—
	3	44.5	6.6	+	—
C	1	43	6.0	+	—
	2	43	6.0	+	—
	3	43.5	5.9	+	—
R	1	45	7.4	—	—
	2	44.5	7.5	—	—
	3	45	7.1	—	—
W	1	43.5	12.6	—	—
	2	48	12.2	—	—
	3	48.5	12.1	—	—

¹M = original raw milk; M+ = artificially spiked milk; P = pasteurized milk; C = pasteurized milk containing conservation agent; R = rinsing water; W = washing water.

Figure 1. Detection limit of the D29 bacteriophage-based assay in raw milk samples containing artificially spiked *Mycobacterium avium* ssp. *paratuberculosis* (MAP) cells in log₁₀ stages from 10⁰ to 10⁸ per mL. Liquid culture solutions were used as a positive control (PC). Samples were repeated 3 times independently (n = 3). Calculation of MAP was performed, assuming that 1 pfu corresponded to 1 MAP cell. Values are given as means and standard deviations for each sample.

1.49×10^3 and $2.33 \times 10^3 \pm 8.22 \times 10^2$ pfu/mL (Figure 2). In all other raw milk samples, we did not observe formation of plaques.

Milk was spiked with MAP to 10⁷ MAP cells/mL of milk (Figure 2). The level of plaque-forming units in M+ samples ranged from $1.06 \times 10^6 \pm 4.06 \times 10^5$ to $4.48 \times 10^6 \pm 4.27 \times 10^6$ pfu/mL. In P and C samples, we found MAP amounts of $3.24 \times 10^3 \pm 9.07 \times 10^2$ to $9.73 \times 10^4 \pm 1.49 \times 10^4$ pfu/mL. The calculated content in rinsing water samples ranged from $3.36 \times 10^2 \pm 5.4 \times 10^1$ to $6.23 \times 10^2 \pm 1.23 \times 10^2$ pfu/mL. In W samples, values ranged from $7.33 \times 10^2 \pm 3.7 \times 10^1$ to $9.78 \times 10^3 \pm 7.06 \times 10^3$ pfu/mL. The mean reduction of viable MAP in P samples was 1.57 log₁₀ pfu/mL, and in C samples was 2.65 log₁₀ pfu/mL.

We observed the effect of pasteurization on the number of MAP commonly found in raw milk samples from naturally infected cows by reconstructing milk samples with a lower but more realistic level of 10⁴ MAP cells/mL milk (Figure 3). In 3 independent trials, the MAP amount in inoculated milk samples (M+) ranged from $1.53 \times 10^4 \pm 1.82 \times 10^3$ to $2.51 \times 10^4 \pm 1.23 \times 10^4$ pfu/mL. Following pasteurization (P) and pasteurization with additionally added conservative agent (C), $1.09 \times 10^3 \pm 3.1 \times 10^1$ to $4.36 \times 10^3 \pm 3.28 \times 10^2$ pfu/mL

Figure 2. Overview of the detection of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) in original raw milk (M), in milk artificially spiked to achieve 10⁷ MAP cells/mL (M+), after pasteurization (P), after pasteurization and conservation (C), and in rinsing (R) and washing water (W) using culture (+/-), phage-based assays (gray bars), and quantitative real-time PCR (white bars). Samples were repeated 3 times independently (n = 3 trials). Values are given as mean and standard deviations for each sample. Dashed line indicates the extrapolated MAP genome equivalent (MAP-GE) values below the quantification limit. Asterisks indicate extrapolated values, due to quantification limit.

Figure 3. Overview of the detection of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) in original raw milk (M), in milk artificially spiked to achieve 10⁴ MAP cells/mL (M+), after pasteurization (P), after pasteurization and conservation (C), and in rinsing (R) and washing water (W) using culture (+/–), phage-based assays (gray bars), and quantitative real-time PCR (white bars). Samples were repeated 3 times independently (n = 3 trials). Values are given as mean and standard deviations for each sample. Dashed line indicates the extrapolated MAP genome equivalent (MAP-GE) values below the quantification limit. Asterisks indicate extrapolated values, due to quantification limit.

were detected. In R and W samples, we observed values ranging from $2.11 \times 10^0 \pm 1.00 \times 10^0$ to $1.14 \times 10^1 \pm 3.53 \times 10^0$ pfu/mL. The mean reduction of viable MAP in P samples was 1.24 log₁₀ pfu/mL, and in C samples was 0.82 log₁₀ pfu/mL.

Culture Investigation

We did not detect MAP growth by culture in any original raw milk samples before inoculation (M), or in milk spiked with 10⁷ MAP cells/mL (Figure 2) or 10⁴ MAP cells/mL (Figure 3). In all cultures of M+ samples, growth of MAP was confirmed by snPCR. As well, in P, C, and R samples, MAP growth was observed. No growth of MAP was observed in any other sample.

Detection of MAP DNA by Quantitative Real-Time PCR

In addition to the detection of viable MAP by D29 bacteriophage-based assay and culture, we monitored the content of MAP DNA using qPCR. All samples (M, M+, P, C, R, W) of all trials were tested in 3 independent qPCR runs. Two milk samples before inoculation (M) tested positive for MAP DNA, but because of the

quantification limit, we could not quantify MAP-GE exactly; instead, we extrapolated them from the data (Figure 2).

In all 3 milk samples independently inoculated to a final number of 10⁷ MAP cells/mL (M+; Figure 2), the recovery rate of MAP-GE ranged from 6.03×10^3 to 2.30×10^5 per mL. The MAP DNA content in P and C samples was comparable to amounts detected in M+ samples, and ranged from 2.92×10^3 to 1.96×10^5 MAP-GE/mL. All R samples and 2 W samples tested positive for MAP DNA. However, MAP genome quantification failed here, because the numbers were below the quantification limit of the qPCR. The W sample from the second trial tested negative.

All milk and water samples obtained from raw milk with a final number of 10⁴ MAP cells/mL were also tested by qPCR (Figure 3). We found MAP DNA in milk samples (M+, P, C), but could not quantify them because of the quantification limit. In all water samples (R, W) MAP DNA was not detectable.

DISCUSSION

For farmers, it is of great economic interest to use milk for rearing calves instead of purchasing milk replacers, particularly because the nutritional value of

milk is higher than that of many milk replacers (Blome et al., 2003). However, the feeding of “waste milk” to young calves puts them at risk of germ transmission and infection. To reduce high bacterial counts, on-farm pasteurization is becoming increasingly popular. In the present study, we investigated the stability of MAP bacteria after HTST pasteurization under experimental on-farm conditions. The data showed that viable MAP cells were detectable in spiked milk after pasteurization and even in the rinsing and washing water of the pasteurizer. In contrast to conventional pasteurization methods, the device we used is based on a recently developed steam-heating system. It prevents clumping during pasteurization of the transition milk. The first colostrum after calving could not be used without technical difficulties.

The literature is contradictory with respect to the efficacy of pasteurization in eradicating MAP from milk samples. Some studies have demonstrated that MAP can survive under simulated pasteurization conditions (Grant et al., 1996; Foddai et al., 2010) or in pasteurized milk or bulk milk samples directly received from dairy farms (Ayele et al., 2005; Ellingson et al., 2005; Gerrard et al., 2018). Other researchers have shown that no viable MAP cells could be detected by culture using commercial on-farm HTST pasteurizers, although that milk contained 10^2 to 10^6 MAP/mL before pasteurization (Stabel, 2001; Stabel et al., 2004). These studies differed not only with respect to temperature ranges, time scales, and the way heat was applied, but also in methods used for MAP detection. For the detection of most microorganisms, culture of viable MAP cells is considered the gold standard diagnostic test (Stabel et al., 2002). However, MAP is a slow-growing bacteria with a long generation time (Lambrecht et al., 1988), and the culture period required is up to 16 weeks (Stabel et al., 2002). In addition, overgrowth of cultures by contamination of fungi or competing fast-growing bacteria and necessary sample decontamination further affects the detectability of MAP in culture through the loss of bacterial viability (Bradner et al., 2013). Furthermore, MAP can enter a state of dormancy in which they are viable but non-culturable (Lamont et al., 2012). Therefore, the detection of MAP from animal samples using culture is highly limited. Studies have shown that the detection limit of MAP culture from milk samples is approximately 10^3 MAP cells/g (Messelhäuser et al., 2012). The results of the present study show also that a feasible detection of MAP by culture depends on a minimum number of 10^3 MAP cells/mL (Figures 2 and 3). Given these findings, we used an additional D29 bacteriophage-based assay (Foddai et al., 2009) in this study to detect and enumerate viable MAP cells more quickly and efficiently. We observed that the detection

limit of the D29 bacteriophage-based assay in milk was approximately 10^2 MAP cells/mL, suggesting that this assay was more sensitive for detecting viable MAP compared with culture. The specificity of the phage assay is based on subsequent analysis of plaques by specific PCR methods. A recent study (Gerrard et al., 2018) investigated the occurrence of MAP in retail pasteurized milk ($n = 368$), using the same phage-based assay. Compared to previous studies, higher percentages of viable MAP cells (10.3%) were detected by the phage-based assay in the collected samples. This may indicate that the phage-based assay can detect lower numbers of MAP compared to culture and direct PCR (Gerrard et al., 2018). In the present study, we did not observe growth of MAP colonies in any of the original raw milk cultures, but the phage-based assay showed 2 positive results (M samples, first and third trial; Figure 2). Furthermore, the results of the qPCR, which we performed to detect and calculate MAP-GE, underline the difficulty of detecting MAP at low levels. Although MAP was detectable by qPCR in 2 out of 3 trials in the raw milk (M, second and third trials), the number of MAP-GE in the samples was too low to measure (Figure 2). In the first trial, MAP DNA was not measurable, probably because of inefficient DNA extraction procedures (Mumy and Findlay, 2004). Overall, DNA-based analysis of contaminants in milk is difficult, because milk components such as calcium ions can inhibit the PCR reaction and lead to underestimated recovery rates (Bickley et al., 1996). This factor may also explain the heterologous recovery rates of MAP-GE by qPCR in spiked milk samples (M+) we found in the present study. Although qPCR showed a positive result in the original raw milk sample of the second trial (M), viable MAP cells were not detectable in this sample by phage-based assay or culture. However, it is important to bear in mind that PCR does not distinguish between live and dead cells, and culture or phage-based assays detect only viable cells. Considering the results of each method used in the present, it is most likely that the number of viable MAP cells in the original raw milk sample was moderate, ranging between 2 and 10^3 MAP cells/mL, reaching the detection limit of the methods used.

Besides technical and methodological differences or problems in experimentation, the presence of cell clumps must be considered in relation to MAP detection in pasteurization experiments (Klijn et al., 2001). It has been established that MAP forms clumps containing a high number of cells during growth in culture (Lambrecht et al., 1988) or occurs naturally in fecal samples or contaminated milk (Klijn et al., 2001). This clumping of cells has a great effect on heat resistance through pasteurization and MAP survival (Klijn et al.,

2001). In the present pasteurization study, we observed a mean reduction of $0.82 \log_{10}$ to $2.65 \log_{10}$ pfu/mL, depending on the initial amount or addition of conservation agent, with the phage-based assay. Independent of the final MAP content before pasteurization (10^7 or 10^4 MAP cells/mL), a minimum number of 10^3 viable MAP cells/mL remained measurable by bacteriophage-based assay. Therefore, we assume that MAP survival is caused by a fraction of clumps additional to the heat-resistant single-cell fraction as described by Klijn et al. (2001). Cells inside the clumps may not be inactivated by heating because they are protected by the outer cells of the clumps. In our study, the culture solution was ultrasonicated before milk inoculation to disrupt cell clumps. However, prepared microscopic slides of ultrasonicated MAP culture solution showed that complete disruption of the clumps was not possible by ultrasonication, and that only a reduction of cell clumps was achieved in the current study. Therefore, it is reasonable to conclude that the presence of MAP cell clumps cannot be excluded in our study.

Even if milk was contaminated with a low bacterial dose, MAP survival after a short heat treatment cannot be excluded. At this point, we know little about the minimum oral infective dose of MAP in cattle. Sweeney et al. (2006) demonstrated a successful infection of young calves with a dose of 1.5×10^6 cfu. However, in sheep and red deer, the minimum oral infective dose of MAP is estimated to be approximately 10^3 cfu (Brotherston et al., 1961; Mackintosh et al., 2007). Therefore, one could presume that milk containing 10^3 MAP cells/mL might also be infectious for dairy calves. In previous studies, MAP was shed at low numbers, of only 2.0×10^0 to 8.0×10^0 cfu/50 mL (Sweeney et al., 1992) and $2.4 \times 10^1 \pm 1.2 \times 10^1$ cfu/mL in milk from asymptomatic cows (Stabel et al., 2014). However, the number of MAP cells shed in milk by naturally infected cows showing clinical signs of Johne's disease (paratuberculosis), is estimated to be up to $1.32 \times 10^3 \pm 3.1 \times 10^2$ cfu/mL (Stabel et al., 2014). As well, MAP has been detected in contaminated feces at levels of up to 10^8 cfu/g (Cocito et al., 1994), which can lead to high levels of contaminated milk. Accordingly, the pasteurization of MAP-contaminated milk used for calves can reduce the transmission of infection. Overall, contaminated milk cannot be excluded as a source of distribution. If the dairy herd becomes infected with MAP, it is advisable to avoid feeding MAP-contaminated milk. Regular examinations of milk samples, pasteurized or not, may help to prevent the spread of infection in a cattle herd.

In addition to milk-borne transmission, the spread of MAP into the farm environment via the washing water from the pasteurizer must also be considered. As the

present study shows, disinfecting the pasteurizer was not an effective means of entirely inactivating MAP. Although very low amounts of MAP were measured, viable MAP cells were still detected and calculated in the rinsing and washing water of the pasteurizer by phage-based assay. Low cell numbers ranging from 2.11×10^0 to 1.14×10^1 viable MAP cells/mL in rinsing and washing water were found after milk containing 10^4 MAP cells/mL was pasteurized. However, higher amounts of 3.89×10^0 to 9.78×10^3 viable MAP cells per mL washing water were found after pasteurization of milk containing 10^7 MAP cells/mL. Because these numbers were quite similar to those of pasteurized milk, these findings showed that the disinfection detergent we used was not effective in inactivating MAP. It has been documented that MAP remains viable and infectious for months outside the host in MAP-contaminated soil (Whittington et al., 2004). The pasteurization of milk with a high level of MAP contamination can therefore lead to a disturbance of the environment by releasing washing water. This is negligible in the case of direct infection, but probably leads to an accumulation of MAP in the farm environment.

We do not recommend feeding waste milk to newborn calves because of hygienic and veterinary concerns. However, given the results of the present study and findings from other studies, we can recommend that pasteurization of colostrum or milk become a standard on-farm procedure before feeding to suckling calves. This practice may promote a healthier herd by minimizing the risk of disease transmission.

In future, the effect of HTST pasteurization, including the newly developed steam-heating system, on colostrum quality and in particular on the composition of immunoglobulins, must be investigated, especially because Stabel et al. (2004) observed that colostrum pasteurization reduces the number of colostral immunoglobulins. Nevertheless, studies have indicated that feeding pasteurized colostrum instead of non-pasteurized colostrum to rearing calves results in better calf performance, lower mortality rates, and increased mean weight gain, conferring an economic advantage (Jamaluddin et al., 1996; Godden et al., 2005).

CONCLUSIONS

Although we detected a reduction in viable MAP cells in this study, MAP was able to survive pasteurization and can remain a problem in terms of transmission to young calves. However, in the case of waste milk feeding to calves, on-farm decontamination by pasteurization is highly recommended. Nevertheless, the distribution of paratuberculosis as a result of consuming pasteurized waste milk cannot be completely excluded.

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