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Milk acidification to control the growth of *Mycoplasma bovis* and *Salmonella* Dublin in contaminated milk

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ABSTRACT

Bacterial contamination of milk fed to calves compromises calf health. Several bacterial pathogens that infect cows, including *Mycoplasma bovis* and *Salmonella enterica* ssp. *enterica* serovar Dublin, are shed in milk, providing a possible route of transmission to calves. Milk acidification lowers the milk pH so that it is unsuitable for bacterial growth and survival. The objectives of this study were to (1) determine the growth of *M. bovis* and *Salmonella* Dublin in milk, and (2) evaluate the efficacy of milk acidification using a commercially available acidification agent (Salstop, Impextraco, Heist-op-den-Berg, Belgium) to control *M. bovis* and *Salmonella* Dublin survival in milk. For the first objective, 3 treatments and a positive control were prepared in 10 mL of milk and broth, respectively, and inoculated with *M. bovis* or *Salmonella* Dublin to an approximate concentration of 10^4 cfu/mL. Each treatment was retained at 5, 23, or 37°C with the positive control at 37°C. Aliquots were taken at 4, 8, 24, 28, 32, 48, 52, and 56 h after inoculation and transferred onto agar medium in triplicate following a 10-fold dilution series in sterile phosphate-buffered saline. All plates were incubated and colonies counted. For the second objective, 4 treatments and a positive control were prepared with 100 mL of milk and inoculated with *M. bovis* or *Salmonella* Dublin to an approximate concentration of 10^6 cfu/mL. With the use of Salstop, treatments were adjusted to an approximate pH of 6, 5, 4, or 3.5. The positive control was left untreated. At 1, 2, 4, 6, 8, and 24 h after treatment, triplicate aliquots were taken, the pH measured, and then the aliquots were transferred onto agar medium and into broth for enrichment. Following incubation, agar colonies were counted, while broths were plated and incubated prior to colonies being counted. All trials were repeated. *Mycoplasma bovis* did not grow in milk, but *Salmonella* Dublin proliferated. The pH of all acidification treatments remained stable for 24 h. No viable *M. bovis* organisms were detected at 1 h of exposure to pH 3.5 and 4 or at 8 h of exposure to pH 5. Following 24 h of exposure to pH 6 *M. bovis* remained viable. No viable *Salmonella* Dublin organisms were detected at 2 and 6 h of exposure to pH 3.5 and 4, respectively. *Salmonella* Dublin remained viable following 24 h of exposure to pH 5 and 6. These results demonstrate that milk acidification using Salstop is effective at eliminating viable *M. bovis* and *Salmonella* Dublin organisms in milk if the appropriate pH and exposure time are maintained.
Key words: milk acidification, calf, dairy, *Salmonella*, mycoplasma

INTRODUCTION

Mycoplasma bovis can cause severe disease in cattle of all ages, and it is most commonly associated with mastitis and arthritis in adults (Wilson et al., 2007) as well as pneumonia, arthritis, and otitis media in calves (Maunsell and Donovan, 2009). Animals affected with clinical and subclinical mycoplasma mastitis can shed the organism through their milk at concentrations $\geq 10^8$ and $\leq 10^6$ cfu/mL, respectively (Byrne et al., 2005). Cow-to-calf transmission of *M. bovis* can occur through the ingestion of infected milk (Maunsell et al., 2012). Because of the organism's highly contagious nature, unresponsiveness to antimicrobial treatment, and the role of subclinical carrier animals, elimination is difficult, and therefore, the focus is on preventing pathogen transfer (Maunsell et al., 2011).

Salmonella enterica ssp. *enterica* serovar Dublin is one of the most common *Salmonella* serotypes isolated from cattle, causing acute and subclinical disease in calves aged 2 wk to 3 mo (Wray and Davies, 2000). Clinical symptoms in calves include fever, ill thrift, depression, pneumonia, diarrhea, septicemia, and

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death (Mohler et al., 2009). *Salmonella* Dublin is host adapted to cattle and has a propensity to cause chronic subclinical infections. Calves infected with *Salmonella* Dublin that fail to clear the infection can remain as carrier animals within the herd, shedding the organism in feces and milk (Smith et al., 1989; House et al., 1993). As a result, approximately 50% of dairy herds that experience a *Salmonella* Dublin outbreak become persistently infected (Veling, 2004). Oral ingestion is the most common route of infection with a dose of 10^6 cfu or greater leading to clinical symptoms (Wray and Sojka, 1977).

Current options to minimize exposure of calves to bacterial pathogens in milk include feeding milk replacer to eliminate access to contaminated milk, milk acidification, and pasteurization. Pasteurization may be achieved via heat or UV irradiation. Heat pasteurization of waste milk improves weight gain and reduces morbidity and mortality compared with feeding unpasteurized waste milk (Jamaluddin et al., 1996). Heat pasteurization is also an effective method of eliminating *M. bovis* and *Salmonella* Dublin to enable effective utilization of contaminated milk (Butler et al., 2000; Stabel et al., 2004). Despite this, the cost of purchasing an effective pasteurization unit is significant, with an economic analysis proposing a break-even point of 315 calves on milk per day, which equates to a herd milking 1,260 cows year-round (Jamaluddin et al., 1996). Treatment of waste milk by UV irradiation is less effective at reducing bacterial counts compared with heat pasteurization (Teixeira et al., 2013).

Although feeding of milk replacer avoids an initial capital outlay, it can be costly over time, and past evaluations have suggested that routine feeding with it may result in a poorer nutrient intake compared with whole pasteurized milk (Godden et al., 2005). Although both options provide a liquid feed that is initially free from viable *M. bovis* and *Salmonella* Dublin, both have the potential to become contaminated if placed into contaminated storage or feeding equipment. An alternative treatment approach is milk acidification, which involves lowering the pH of milk to a level that is unsuitable for bacterial growth and survival but still of nutritional benefit to calves (Anderson, 2008). A continued preservative effect persists while the pH remains at the effective level, and milk acidification is an economical alternative for smaller producers. A pilot trial in 2005 indicated that the total bacterial count in raw bulk tank milk (**BTM**) is reduced when the pH is lowered to 4.1 with the addition of formic acid (Anderson, 2005b). However very little information is available on specific contact times required to inactivate particular bacterial species. Furthermore, formic acid has substantial work health and safety hazards associated with its use. Recently in light of these work health and safety issues, powdered forms have been made commercially available utilizing a combination of acids, but these have yet to be evaluated thoroughly for their efficacy.

The first objective of this study was to determine the growth and survival of *M. bovis* and *Salmonella* Dublin in inoculated milk over the course of 56 h at various incubation temperatures. The second objective of this study was to evaluate the efficacy of milk acidification using a commercially available feed acidification agent (Salstop, Impextraco, Heist-op-den-Berg, Belgium) to inhibit the growth and survival of *M. bovis* and *Salmonella* Dublin in inoculated milk over a period of 24 h. In addition, the pH stability of "hospital herd" waste milk with high levels of bacterial contamination was evaluated following acidification using Salstop.

MATERIALS AND METHODS

Acidifying Agent

The commercially available product Salstop SD (Impextraco) was used as the acidifying agent throughout the trial. Salstop SD was selected based on its availability, feed grade status, and work health and safety characteristics (powder vs. liquid). According to the product information insert provided by the manufacturer, Salstop SD is a dry white powder preservative used to control *Salmonella* species and other pathogenic bacteria in raw materials and finishing feeds, and it prevents the recontamination of these materials. It contains a mixture of propionic, acetic, formic, sorbic, and lactic acids on a silica carrier.

Preparing Bacterial Cultures

Mycoplasma bovis type strain (ATCC 25523) was inoculated onto Mycoplasma agar [Mycoplasma agar base (Oxoid CM0401; Oxoid Inc., Basingstoke, UK); distilled water; 0.2% wt/vol calf thymus DNA (Sigma D1501, Sigma-Aldrich, St. Louis, MO); Mycoplasma Selective Supplement G (Oxoid SR0059C); prepared by Elizabeth Macarthur Agricultural Institute (**EMAI**), NSW Department of Primary Industries, NSW, Australia] and incubated at 37°C in candle jars with elevated CO_2 levels for 5 to 10 d. Following positive growth, several colonies were selected and subcultured into 2 mL of Mycoplasma broth [Mycoplasma broth base (Oxoid CM0403); Milli-Q water; 0.2% wt/vol calf thymus DNA (Sigma D1501); Mycoplasma Selective Supplement Q (Oxoid SR0059C); 0.4% phenol red (Sigma P-3532); prepared by EMAI] at 37°C for 48 h. After 48 h of incubation, *M. bovis* growth reached a concentration of approximately 10^9 cfu/mL (data not shown).

Salmonella Dublin strain 380, a kanamycin-resistant field isolate collected from the feces of a calf with scours (Izzo et al., 2011), was chosen for use in this study. The isolate allowed for the addition of kanamycin to the agar medium to prevent the growth of unwanted organisms that may have made plate reading difficult. *Salmonella* Dublin was inoculated onto xylose lysine deoxycholate (**XLD**) agar with kanamycin (50 μg/mL; EMAI) and incubated at 37°C for 24 to 48 h. Following positive growth, several colonies were selected and subcultured into 2 mL of Luria broth (BD 241420) and incubated at 37°C for 24 h. After 24 h of incubation, *Salmonella* Dublin 380 growth reached a concentration of approximately 10^9 cfu/mL (data not shown).

Milk Collection and Heat Treatment

The milk used for all trials was BTM collected from The University of Sydney dairy. The University of Sydney dairy had no known history of *M. bovis* infection, but it did have a history of *Salmonella* Dublin infection. To reduce any existing bacterial contamination, the collected BTM was heat treated to 63 ± 2°C for 30 min. All BTM was cultured for *Mycoplasma* spp. and *Salmonella* spp. as described above, before and after heat treatment. For all trials, no *Mycoplasma* spp. or *Salmonella* spp. growth was observed before or after heat treatment. However, because of the known history of *Salmonella* Dublin infection in this herd, a negative control was included for the *Salmonella* Dublin milk acidification trials.

Bacterial Proliferation in Inoculated Milk

Trials were performed independently for each organism. For all experiments involving *M. bovis*, Mycoplasma agar and Mycoplasma broth were used as described above. For all experiments involving *Salmonella* Dublin, XLD + kanamycin agar and Luria broth were used as described above.

Three treatment groups and a positive control broth were prepared in sterile 15-mL polypropylene tubes (Biologix, Jinan, China) with 10 mL of milk (heat treated to 63 ± 2°C for 30 min) and broth, respectively. Each treatment and the positive control were inoculated with a volume of prepared organism broth culture to achieve a starting concentration of approximately 10^4 cfu/mL. To estimate the starting concentration of each treatment and control, an aliquot from each was removed and a 10-fold serial dilution in sterile PBS was performed. Each dilution was plated out in triplicate 10-μL volumes onto the appropriate agar and incubated as previously described, followed by colony counting. Each treatment group was maintained at their assigned temperature of 5°C (refrigerator), 23°C (bench top), or 37°C (incubator), with the positive control broth placed in the incubator at 37°C. Following inoculation and treatment, sampling occurred at 4, 8, 24, 28, 32, 48, 52, and 56 h. At each sampling interval, each treatment and control was subjected to a sampling protocol that involved vortexing followed by removal of 200 μL, which was subjected to a 10-fold serial dilution in sterile PBS, with each dilution inoculated onto the appropriate agar in triplicate 10-μL volumes. All plates were incubated under the appropriate conditions for that bacterial species followed by colony counting. Each trial was repeated, and the results are reported as the mean of the replicated trials.

Milk Acidification to Reduce the Bacterial Load in Milk

Trials were performed independently for each organism. For trials with inoculated *M. bovis* or *Salmonella* Dublin, heat-treated milk was used (63 ± 2°C for 30 min). For trials involving *M. bovis*, Mycoplasma agar and Mycoplasma broth were used as previously described, with the broth incubated for 4 d. For trials involving *Salmonella* Dublin, XLD + kanamycin agar was used as previously described, and mannitol selenite broth, which was incubated at 37°C for 24 h. To ensure that *Salmonella* Dublin was not already present within the milk, a negative treatment control was included containing 100 mL of heat-treated milk (63 ± 2°C for 30 min), which was not inoculated with *Salmonella* Dublin or treated with Salstop.

Four treatment groups and a positive control were prepared in sterile glassware with 100 mL of heat-treated milk. For each treatment and control, the milk was inoculated with the prepared organism in broth culture to achieve a starting concentration of approximately 10^6 cfu/mL. To estimate the starting concentration in each treatment and control tube, an aliquot from each was removed and a 10-fold serial dilution in sterile PBS was performed. Each dilution was plated out in triplicate 10-μL volumes onto the appropriate agar and incubated under the appropriate conditions for each bacterial species followed by colony counting. For each treatment and control tube, three 2-mL aliquots were also removed to measure the starting pH with a benchtop pH meter (labCHEM-pH, TPS, Brendale, QLD, Australia). Small increments of Salstop were added to each of the 4 treatment tubes followed by gentle but thorough mixing to ensure the entire additive was dissolved. A 2-mL aliquot was removed and the pH measured. This process was repeated on each of the 4 treatment tubes until they reached their approximate desired starting pH of 6, 5, 4, and 3.5. Once the desired pH was achieved, the pH was measured in triplicate 2-mL aliquots. The control tube remained untreated. All treatments and the control were placed on a benchtop at ambient temperature after which they were sampled following 1, 2, 4, 6, 8, and 24 h of pH treatment exposure. At each sampling interval, the air temperature was recorded, and the following procedures were performed for each treatment and control. Visual observations of each milk treatment and control were noted. Milk was thoroughly mixed by gentle swirling of the tube and three 2-mL aliquots were removed. To evaluate growth and viability of the organism, 10 μL of each aliquot was inoculated onto the appropriate agar. To confirm the organism's viability and ensure that the concentration was not below the limit of detection by agar alone, a broth enrichment step was also included. This step involved transferring 10 μL of each aliquot into 4 mL of the appropriate broth and incubating the mixture under appropriate conditions for each bacterial species. The pH of each aliquot was measured. Following incubation, each broth was inoculated onto the appropriate agar in 10-μL volumes and incubated. Following incubation, colony counting was performed on all plates where possible or otherwise determined as "too many to count." The trial was repeated and results reported as the combined replicate trials.

Stability of pH in Acidified High-Bacteria-Count Milk

For total plate count trials to assess the pH stability of milk with a high bacterial load of mixed organisms, the method described in the previous section was used with the following modifications. Bulk hospital herd waste milk collected from the University of Sydney dairy was used. This milk was inoculated onto sheep blood agar (**SBA**; MicroMedia MM1337, Moe, VIC, Australia), but a broth enrichment step was not performed. At 24 h all treatments and the control had an aliquot of milk removed, which underwent a 10-fold serial dilution in sterile PBS followed by inoculation onto SBA in triplicate 10-μL volumes. All SBA plates were incubated at 37°C for 24 h before analysis.

Statistical Analysis

For statistical analysis of bacterial proliferation in inoculated milk, a REML (GenStat 16th edition, VSN International, Hemel Hempstead, UK) analysis was performed on bacterial growth ($\log_e$) with trial as a random effect. Statistical significance was declared at $P < 0.05$.

For milk acidification trials, bacterial growth results were converted to binary data as either growth (1) or no growth (0). A generalized linear mixed model (GenStat) analysis was performed on bacterial growth for time and treatment separately with trial as a random effect. This analysis was completed on bacterial growth before the enrichment broth and after enrichment broth for *M. bovis* and *Salmonella* Dublin. For *Salmonella* Dublin, the negative control data were excluded from analysis because no growth occurred at any sample time point.

RESULTS

Bacterial Proliferation in Inoculated Milk

For the *M. bovis* type strain (ATCC 25523) trials, the mean temperatures (±SE) were 36.91°C (± 0.07) for the control and incubated milk, 22.94°C (± 0.17) for milk held at ambient temperature, and 5.77°C (± 0.23) for the refrigerated milk. The mean starting concentration (± SE) of *M. bovis* for all treatment groups was 5.30×10^3 cfu/mL (± 4.56×10^3). Results of *M. bovis* proliferation in milk and broth are shown in Figure 1. The medium (milk or broth), temperature treatment, and time had a significant effect on bacterial growth and survival ($P < 0.001$). Proliferation occurred in the control broth tube incubated at 37°C, achieving a peak mean concentration (± SE) of 4.19×10^9 cfu/mL (± 4.43×10^8) after 52 h. Milk treatment tubes incubated at 37 or 23°C had no viable organisms after 24 h. For the milk treatment tubes refrigerated at 5°C, the concentration of viable *M. bovis* organisms declined gradually over time but remained detectable at 56 h at a mean concentration (± SE) of 2.5×10^2 cfu/mL (± 1.83×10^2). This amount is a 1.18-$\log_{10}$ reduction from the starting concentration.

For the *Salmonella* Dublin strain 380 trials, the mean temperatures (± SE) were 37°C (± 0.11) for the control and incubated milk, 23.3°C (± 0.18) for milk held at ambient temperature, and 6.2°C (± 0.05) for the refrigerated milk. The mean starting concentration (± SE) of *Salmonella* Dublin for all treatment groups was 5.90×10^3 cfu/mL (± 4.17×10^3). Results of *Salmonella* Dublin proliferation in milk and broth are shown in Figure 2. The temperature treatment and time had a significant effect on bacterial growth ($P < 0.001$). Proliferation of *Salmonella* Dublin was substantial in the control broth tubes incubated at 37°C, as well as milk treatments held at 37 and 23°C. For the control broth tubes incubated at 37°C, the mean peak concentration of *Salmonella* Dublin (± SE) was reached at 24 h with a concentration of 9.14×10^8 cfu/mL (± 1.19×10^8). This concentration remained stable, with a final concentration of 8.02×10^8 cfu/mL (± 1.43×10^8) at 56 h. For the milk treatment tubes incubated at 37°C the mean peak concentration of *Salmonella* Dublin (±

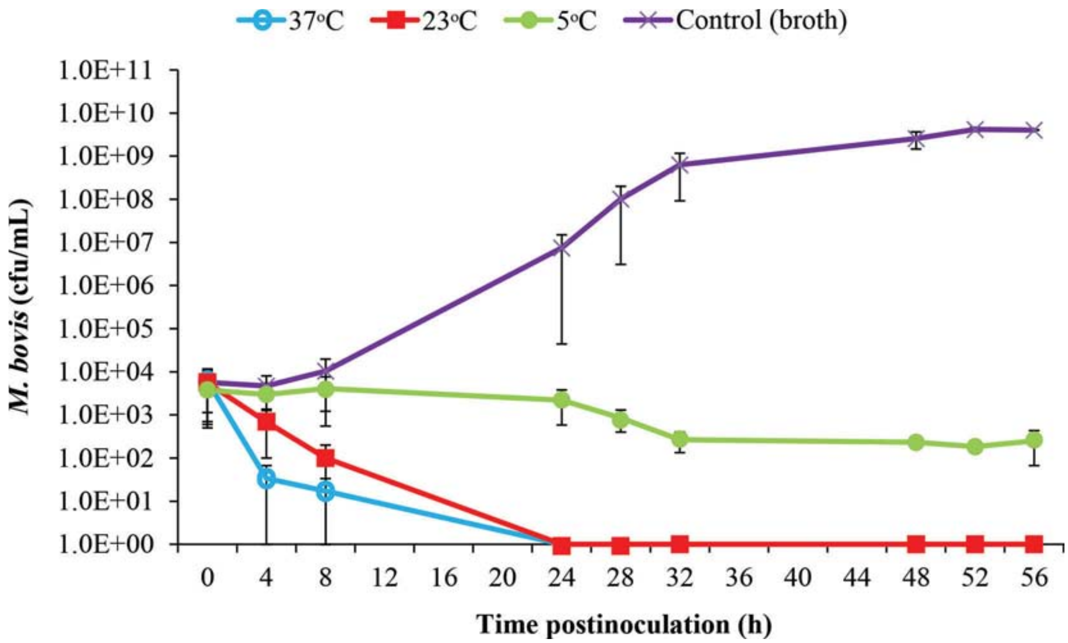

Figure 1. *Mycoplasma bovis* proliferation in milk at varying incubation temperatures over 56 h. Results are expressed as the mean counts (cfu/mL; ±SE) of triplicates from 2 independent experiments.

SE) of 2.03×10^9 cfu/mL ($\pm 1.41 \times 10^9$) was reached at 28 h with a decline in concentration to 3.25×10^6 cfu/mL ($\pm 2.29 \times 10^6$) at 56 h. For milk treatment tubes incubated at 23°C, the mean peak concentration of *Salmonella* Dublin (± SE) of 9.89×10^9 cfu/mL ($\pm 6.04 \times 10^9$) was reached at 52 h. For the milk treatment tubes refrigerated at 5°C, the concentration of *Salmonella* Dublin remained stable throughout the 52-h treatment period, with a final mean concentration (± SE) of 5.30×10^3 cfu/mL ($\pm 7.67 \times 10^2$).

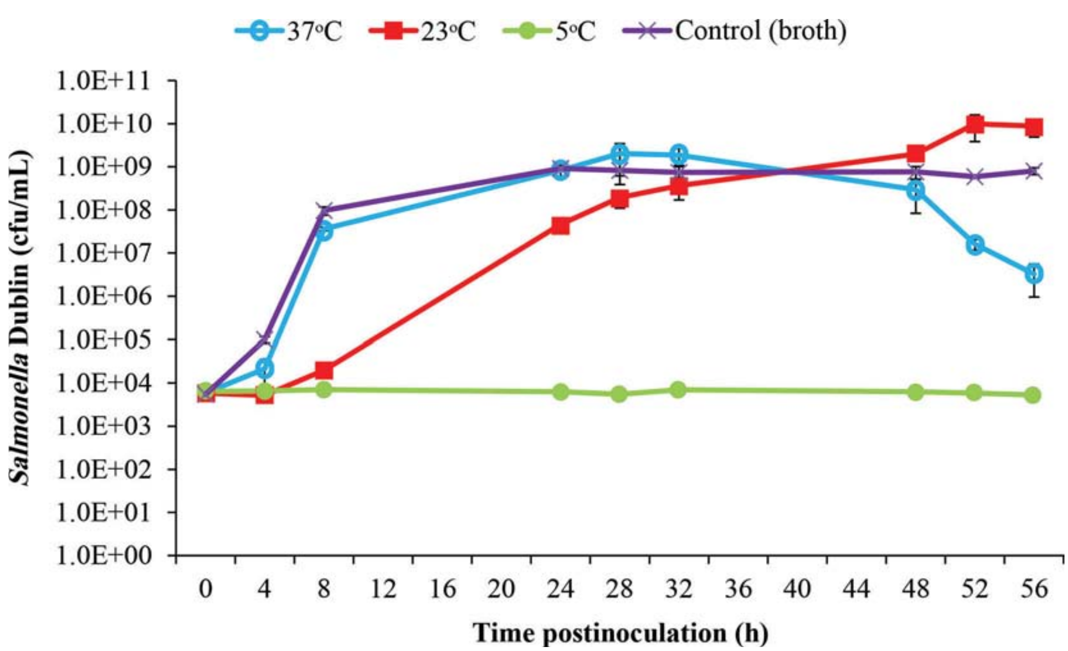

Figure 2. *Salmonella* Dublin proliferation in milk at varying incubation temperatures over 56 h. Results are expressed as the mean counts (cfu/mL; ±SE) of triplicates from 2 independent experiments.

Milk Acidification to Reduce the Bacterial Load in Milk

For the *M. bovis* type strain (ATCC 25523) trials, the mean ambient temperature (± SE) for 24 h for the treatment tubes placed on the laboratory benchtop was 23.6°C (± 0.03). The pH of all treatment groups remained stable throughout 24 h to give a mean pH (± SE) of 7.13 (± 0.05) for the positive control tubes and 5.99 (± 0.03), 5.18 (± 0.11), 4.08 (± 0.02), and 3.65 (± 0.03) for the different treatment groups. The mean starting concentration (± SE) of *M. bovis* for the control and all treatments was 1.36×10^6 cfu/mL (± 2.8×10^4). A significant difference existed in *M. bovis* survival between pH treatment groups before broth enrichment ($P < 0.001$) and following broth enrichment ($P < 0.001$). Results are shown in Table 1 and are reported as either growth or no growth. For milk treated to pH 4 and pH 3.5, no *M. bovis* growth was detected after 1 h of exposure time. For milk treated at pH 5, no *M. bovis* growth was detected at 8 h. Milk treated to pH 6 had no *M. bovis* growth detected at 24 h, but viable *M. bovis* organisms remained detectable at 24 h in nonacidified milk (positive control). Following enrichment in Mycoplasma broth, *M. bovis* viability was confirmed for all treatment tubes with the exception of pH 6 (Table 1). Milk adjusted to pH 6 showed no growth with direct inoculation onto Mycoplasma plates at 24 h; however, following broth enrichment, viable *M. bovis* organisms were recovered.

For the *Salmonella* Dublin strain 380 trials, the mean ambient temperature (± SE) for the treatment tubes placed on the laboratory benchtop for 24 h was 23.54°C (± 0.01). The mean pH (± SE) over 24 h was 7.13 (± 0.04) (negative control), 7.05 (± 0.14) (positive control), 6.19 (± 0.03), 5.13 (± 0.03), 4.05 (± 0.03), and 3.67 (±0.03) for the treatment groups. The pH remained stable for 24 h for all treatment groups with the exception of the positive control, which experienced a slight decrease in pH at 24 h to 6.59. The mean starting concentration (± SE) of *Salmonella* Dublin for all inoculated treatment tubes was 6.83×10^5 cfu/mL (± 6.33×10^3). A significant difference existed in *Salmonella* Dublin survival between pH treatment groups before broth enrichment ($P < 0.001$) and following broth enrichment ($P < 0.008$). Results are shown in Table 2 and are reported as either growth or no growth. No *Salmonella* spp. were isolated from the negative control tubes. Milk adjusted to pH 3.5 showed no *Salmonella* Dublin growth at 2 h. Milk adjusted to pH 4 showed no *Salmonella* Dublin growth at 6 h. *Salmonella* Dublin growth was still detected at 24 h in milk adjusted to pH 5; however, a reduction in the heaviness of growth was noted through visual observations. Milk adjusted to pH 6 and the positive control had *Salmonella* Dublin growth detected at all sampling time points. The positive control had visibly heavier growth at 8 h as compared with 0 h. Following enrichment in mannitol selenite broth, *Salmonella* Dublin viability was confirmed for all pH treatments with the exception of pH 5 at 24 h, which showed no growth with direct inoculation onto XLD + kanamycin plates; however, viable *Salmonella* Dublin organisms were recovered following broth enrichment (Table 2). No growth of *Salmonella* spp. was observed in the negative control tubes at any sampling points.

Stability of pH in Acidified High-Bacteria-Count Milk

For the trials involving hospital herd waste milk to assess the pH stability of milk with a high initial

Table 1. Viability of *Mycoplasma bovis* in milk over 24 h following pH treatment with the commercially available milk acidifier Salstop (Impextraco, Heist-op-den-Berg, Belgium)[1]

	Duration of pH treatment (h)						
Treatment	0	1	2	4	6	8	24
Growth following acidification treatment[2]							
Positive control	G	G	G	G	G	G	G
pH 6	G	G	G	G	G	G	NG
pH 5	G	G	G	G[3]	G[3]	NG	NG
pH 4	G	NG	NG	NG	NG	NG	NG
pH 3.5	G	NG	NG	NG	NG	NG	NG
Growth following acidification treatment and broth enrichment[2]							
Positive control	G	G	G	G	G	G	G
pH 6	G	G	G	G	G	G	NG[3]
pH 5	G	G	G	G[3]	G[3]	NG	NG
pH 4	G	NG	NG	NG	NG	NG	NG
pH 3.5	G	NG	NG	NG	NG	NG	NG

[1]Results are from triplicates of 2 independent trials and are represented by trial 1.

[2]Colonies grown on Mycoplasma agar: G = growth; NG = no growth.

[3]Results that differed between trial 1 and 2.

Table 2. Viability of *Salmonella* Dublin in milk over 24 h following pH treatment with the commercially available milk acidifier Salstop (Impextraco, Heist-op-den-Berg, Belgium)[1]

Treatment	Duration of pH treatment (h)						
	0	1	2	4	6	8	24
Growth following acidification treatment[2]							
Negative control	NG	NG	NG	NG	NG	NG	NG
Positive control	G	G	G	G	G	G	G
pH 6	G	G	G	G	G	G	G
pH 5	G	G	G	G	G	G	NG[3]
pH 4	G	G	G	G[3]	NG	NG	NG
pH 3.5	G	G[3]	NG	NG	NG	NG	NG
Growth following acidification treatment and broth enrichment[2]							
Negative control	NG	NG	NG	NG	NG	NG	NG
Positive control	G	G	G	G	G	G	G
pH 6	G	G	G	G	G	G	G
pH 5	G	G	G	G	G	G	G
pH 4	G	G	G	G	NG	NG	NG
pH 3.5	G	G	NG	NG	NG	NG	NG

[1]Results are from triplicates of 2 independent trials and are represented by trial 1.

[2]Colonies grown on xylose lysine deoxycholate agar (XLD agar) with kanamycin: G = growth; NG = no growth.

[3]Results that differed between trial 1 and 2.

bacterial load of mixed organisms, the mean ambient temperature (± SE) for the treatment tubes placed on the laboratory benchtop for 24 h remained stable at 23.18°C (± 0.12). The mean starting concentration (± SE) of total colony counts in the hospital herd milk was 8.53×10^5 cfu/mL (± 8.93×10^4). For sampling time points of 1 through 8 h, the total numbers of colonies grown were too many to count. At 24 h, when the 10-fold serial-dilution in PBS was performed, the mean concentration (± SE) for the positive control was 1.82×10^{10} cfu/mL (± 1.40×10^{10}), and for each treatment group was 1.35×10^{10} cfu/mL (± 1.18×10^{10}), 1.16×10^7 cfu/mL (± 3.92×10^5), 3.48×10^5 cfu/mL (± 8.17×10^4), and 7.35×10^3 cfu/mL (± 4.35×10^3) for pH 6, pH 5, pH 4, and pH 3.5, respectively. The mean pH (± SE) of milk over the course of 24 h following pH treatment using Salstop remained stable for 8 h, with a mean pH (± SE) of 6.75 (± 0.00) (positive control), 6.20 (± 0.1), 5.09 (± 0.07), 4.22 (± 0.09), and 3.55 (± 0.07). At 24 h, pH 5, 4, and 3.5 remained stable; however, the pH of milk treated to an initial pH of 6 and the positive control decreased to a mean pH (± SE) of 4.58 (± 0.10) and 4.36 (± 0.10), respectively.

Visual Observations of Milk Quality

For all the milk acidification trials, treatments less than or equal to pH 5 experienced milk separation with an obvious clear liquid top layer after 1 h of exposure. However, gentle swirling of the tube by hand returned the milk to a homogenous solution. Where the milk came into contact with the inside of the glassware during swirling, a thin film of fat adhered to the sides. Milk treated to pH 3.5 was visibly thicker with a yogurt-like consistency, which was not evident in the other treatment groups.

DISCUSSION

Analysis of the growth of *M. bovis* type strain (ATCC 25523) in milk at 3 different temperatures (5, 23, and 37°C) demonstrated the organism's inability to proliferate in milk. When milk inoculated with *M. bovis* to a mean concentration (± SE) of 3.79×10^3 cfu/mL (± 3.29×10^3) was refrigerated at 5°C, a slight decline in viable organisms was observed; however, *M. bovis* could still be recovered from the milk at 56 h. This latter finding is consistent with previous reports that demonstrated the ability of *M. bovis* to survive in milk refrigerated at 5°C, with colony counts reduced by approximately 0.3 $\log^{10}$ cfu/mL in 5 d (Boonyayatra et al., 2010) and 0.46 $\log^{10}$ cfu/mL in 5 wk (Vyletelova, 2010). In contrast, in the present study, milk maintained at 23°C and incubated at 37°C saw a rapid decline in *M. bovis* growth with no viable organisms detectable at 24 h. This trend was also observed in the *M. bovis* acidification trial with a higher starting concentration, whereby the concentration of viable *M. bovis* organisms decreased in the positive control over the course of 24 h. This finding highlights the importance of appropriate storage and handling conditions for samples collected for diagnostic culture for *M. bovis* in the laboratory and supports the current recommendation that samples should be maintained at 4°C and transported to the laboratory as soon as possible if microbiological culture is to be performed (Maunsell et al., 2011). This observation also demonstrates that although milk is an adequate transport medium, it is not a sufficient nutrient source for *M. bovis* growth, a finding that is interesting in light of other studies that have suggested that *Mycoplasma* spp. could survive for up to 8 mo in sand bedding, with the ideal temperature for survival being 15 to 20°C and the organism replicating at 4°C (Justice-Allen et al., 2010).

In contrast to *M. bovis* type strain (ATCC 25523), *Salmonella* Dublin strain 380 proliferated in milk at 23 and 37°C, with maximum concentrations of 9.89×10^9 cfu/mL ($\pm$ 6.04×10^9) and 2.03×10^9 cfu/mL ($\pm$ 1.41×10^9) reached, respectively, while survival remained stable at 5°C. Therefore, although storage of milk at $\geq$ 23°C may result in a decline in viable *M. bovis* organisms, the opposite effect was observed for *Salmonella* Dublin growth. Furthermore, although results suggest that *M. bovis* is unable to survive in milk for prolonged periods of time when left unrefrigerated, this study was conducted using milk that had been previously heat-treated to reduce the existing bacterial load before the inoculation of *M. bovis*, as well as using sterile glassware. Previous studies involving contaminated sand bedding have suggested the possibility of *Mycoplasma* spp. biofilm formation, with a positive association found between *Mycoplasma* spp. survival and the growth of gram-negative bacteria (Justice-Allen et al., 2010). Therefore, the use of heat-treated milk and sterile glassware may have affected the ability of *M. bovis* to survive in milk. As such, under normal farm conditions where it is likely that the milk being collected and fed to calves contains a mixed bacterial load and the containers used for storage and feeding of the milk may not be sterile (Stewart et al. 2005), the ability of *M. bovis* to survive in untreated milk may be altered. Although *M. bovis* was not shown to proliferate in milk, its ability to remain viable in milk for up to 8 h at ambient temperature explains how contaminated milk is able to infect calves because milk is often fed within a couple of hours of collection. This finding, in combination with the observed increase in *Salmonella* Dublin concentration over time at ambient temperature, means that seeking milk treatment options to reduce the bacterial load before feeding is warranted. Although treatment methods including heat pasteurization and UV treatment may reduce the total bacterial load of milk initially (Butler et al., 2000; Godden et al., 2006; Gelsinger et al., 2014; Pereira et al., 2014), the milk has the potential to become reinoculated once placed into nonsterile collection and feeding equipment (Stewart et al., 2005), allowing further proliferation of bacteria and as such limiting the health benefit of such treatments. Therefore, acidification of the milk has benefits in providing a continued preservative effect when combating the challenging issue of bacterial contamination commonly experienced when feeding calves.

Milk acidification against *M. bovis* type strain (ATCC 25523) using Salstop to pH 3.5 and pH 4 led to elimination of viable *M. bovis* after just 1 h of exposure time. This result may not be surprising given the bacteria's lack of cell wall, as well as its fastidious growth requirements with an ideal pH for the growth of *M. bovis* in broth being 7.6 (Nicholas et al., 2008). The sensitivity of *Mycoplasma* spp. to changes in pH was highlighted in an earlier study looking at porcine *Mycoplasma hyorhinis*, with significantly less growth found when the broth pH was reduced to just 6.5 (Dinter and Taylor-Robinson, 1969). For *Salmonella* Dublin strain 380, elimination of the organisms at pH 3.5 and pH 4 was slower and was not observed until 2 and 6 h of exposure, respectively. This outcome is similar to a previous trial that evaluated total aerobic colony counts of bacteria following acidification of BTM with formic acid, with no bacterial growth observed after 3 to 21 h of contact at a pH of 4.2 (Anderson, 2005b). Our results indicate that *M. bovis* is more sensitive to changes in pH than other bacterial species commonly found in milk.

For milk treated to pH 5, slight differences were observed in results between replicate trials 1 and 2 for *M. bovis* type strain (ATCC 25523). In trial 1, growth decreased at 6 h, with no growth from 8 h onwards. However in trial 2, growth decreased earlier, at 2 h, with no growth after 4 h. This pattern may have been due to the slight difference in the actual mean pH for each trial. Trial 1 had a slightly higher mean pH of 5.29, while trial 2 had a mean pH of 5.07. Although this difference in pH is only minor, it suggests that pH 5 may be the critical level at which only slight variances can cause changes in the necessary exposure time required to affect *M. bovis* growth and viability. From our data looking at *M. bovis* type strain (ATCC 25523) and *Salmonella* Dublin strain 380, acidification of milk to pH 4 would be necessary to ensure elimination of viable *M. bovis* organisms after 1 h of exposure. Dropping milk to pH 4 has the added benefit of eliminating viable *Salmonella* Dublin organisms after 6 h of exposure. However, because these trials were only performed on 1 strain of each pathogen, it is possible that not all strains would behave the same and, as such, some variation in responses may be seen. While beyond the scope of this paper, future studies could be directed at investigating strain variation.

During the milk acidification process, slight milk separation was observed for treatments pH 5 and lower. However, gentle mixing returned the milk to a homogenous solution with some fat remaining fixed to the inside of the glassware. Separation occurs as the pH of milk is reduced because of the coagulation of casein into a solid mass (Kruif, 1996). Casein is a protein that makes up 82% of total milk proteins, with 18% of total proteins remaining in the whey (Fox et al., 2015). Total separation to the point of a "cottage cheese–like" consistency that cannot be resuspended into solution has been reported in acidification of warm or hot milk (Anderson, 2008) and in preliminary trials conducted as part of this study in which constant agitation of the acidified milk occurred (results not shown). This modification to the milk components may affect calf nutrition because of the possibility of calves only consuming the milk whey. Therefore, if milk acidification is being considered as a treatment option for calf milk, managing its preparation and delivery to calves to avoid complete milk separation to the point that it cannot be returned to a homogenous solution is very important. For example, piping acidified milk over long distances may cause excessive agitation and milk separation, with the milk solids coating the inside of the pipes and only the whey being received and consumed by the calves. A much simpler system involving preparation of milk in buckets that are directly transported to calf feeders may therefore be necessary. As such, from a practical viewpoint, milk acidification may be more suitable for smaller dairy systems. Apart from physical separation, little information is available on the direct effect of acidification on the nutritional value of milk as a whole. However, studies assessing its impact on calf performance attributes including weight gain, feed intake, and feed efficiency have found no significant difference between calves fed acidified and normal milk (Jaster et al., 1990; Guler et al., 2006; Metin et al., 2006). These studies did provide positive outcomes including the reporting of significantly lower fecal consistency scores and a significantly lower incidence of diarrhea for calves receiving acidified milk.

Throughout the 24-h sampling period for milk acidification trials against *M. bovis* type strain (ATCC 25523) and *Salmonella* Dublin strain 380 in heat-treated milk, the pH remained stable for each treatment group once the desired pH was achieved, with the exception of the *Salmonella* Dublin positive control, which showed a slight decline at 24 h. This outcome is an important aspect for 2 reasons. First, it has been suggested that with a pH below 4, calves find acidified milk less appealing (Anderson, 2005a). It is therefore important that the pH does not continue to decrease once the milk has been adjusted to the desired pH. Second, if the pH increases over time, this will affect the ability to eliminate viable *M. bovis* and *Salmonella* Dublin should the milk become contaminated following treatment. The stability of pH at 3.5, 4, and 5 was confirmed in acidified hospital herd milk containing a mixed bacterial load. However, the control hospital herd waste milk and that acidified to a pH of 6 experienced a sharp decline in pH at 24 h, consistent with microbial fermentation and production of lactate. Therefore, if it is necessary to feed acidified waste milk with an initial high mixed bacterial load, ensuring an adequately low starting pH is essential for pH stability.

CONCLUSIONS

This study is the first of its kind to evaluate the use of milk acidification at various pH values and exposure times to eliminate viable *M. bovis* and *Salmonella* Dublin organisms in infected milk for type strains ATCC 25523 and strain 380, respectively. Although *M. bovis* was unable to proliferate in milk, its viability was dependent on the concentration of organisms and storage temperature conditions. Conversely, *Salmonella* Dublin was able to exponentially proliferate in milk at 23 and 37°C. Therefore, treating milk infected with *M. bovis* and *Salmonella* Dublin is necessary before calf consumption to eliminate viable organisms and to assist in preventing possible disease transmission via this route. Although the safest and recommended option is to not feed waste milk to calves, on farms where it may be necessary, acidification of milk using the acidifying agent Salstop is effective at eliminating viable *M. bovis* and *Salmonella* Dublin organisms in milk if the appropriate pH and exposure times are maintained. This trial provides evidence to support that the ideal pH to achieve these results is pH 4 with an exposure time of 1 h for *M. bovis* and 6 h for *Salmonella* Dublin, with the pH remaining stable over a period of 24 h.

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