Evaluation of lime treatments to control *Mycobacterium avium subsp. paratuberculosis* (*Map*) survival on dairy slurry storage

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Introduction

The application of animal slurry to the soil is a common agricultural management practice due its contribution of nutrients and organic matter. An important disadvantage of the use of slurry as fertilizer could be its high content of pathogens that can put both animals and public health at risks. Livestock slurry can carry a variety of bacterial, viral and protozoan pathogens [e.g. 1] resulting in a potential contaminant of surface and ground water. Indirect transmission of an infectious agent may occur through contamination of water, pasture, feed or food fertilized with insufficiently treated manure.

Among fecal-oral transmission pathogens there is low published information on the persistence of members of the Mycobacteria Genus, such us *Mycobacterium avium subsp. paratuberculosis* (*Map*) in slurry [e.g. 1]. This bacterium is the cause of paratuberculosis (Johne's disease), a chronic intestinal mycobacterial infection which is considered of worldwide occurrence in domestic ruminants.

To control the spread of *Map* infection in cattle, there are important factors to consider, among others the ability of the bacterium to survive for long periods outside its host. It has been reported, that after application of contaminated slurry with *Map* on agricultural soil, the bacterium tends to remain on the upper layers of soil rather than moving down through soil profile [e.g. 2]. The latter could suggest an infection risk for grazing livestock and potentially a contamination pathway for surface water. In order to reduce or control pathogens in slurry different options has been evaluated. Physical processes applied to stored dairy manure has been described [e.g. 1] which has been shown to be effective to eliminate pathogens in slurry material. Slurry treatment to control the spread of fecal-oral transmission agents, must consider practical aspects such as adaptation to the current system of slurry storage, and cost, in response to farmers adoption.

It has been suggested that the use of alkaline materials such as quicklime and slaked lime in the slurry represent a practical alternative to reduce pathogens load in the slurry material, because they can be easily obtained from farm providers, besides they will also contribute to increase soil pH in acid soils, and mainly because its use as disinfectant in organic wastes [e.g. 3].

The aim of the present study was to evaluate the effect of limes treatments, calcium oxide and hydroxide in two different concentrations 5% and 15%, and a control (no lime treatment) on *Map* survival in dairy slurry.

Material and Methods

Field experiment

Slurry sampling. Five litres of dairy slurry were collected from storage on a commercial dairy farm located in Los Ríos Region, Chile. Microbiological analysis of this material was previously evaluated, indicating a natural Map concentration in the slurry of 329 bacteria/mL, as well as total coliform (TC) of 6,600 CFU/mL.

Treatments

Two types of limes were used to evaluate the effect of chemical treatments on Map survival in the slurry, consisting of quicklime CaO (Soprocal, Ltda., Santiago, Chile) in concentration of 5 and 15%, and availability of 70%, and slaked lime (Ca(OH)2 (Quality Pro Ltda., Santiago, Chile) in concentration of 5 and 15%, and availability of 95%. In addition, a control treatment was considered, with no lime added.

Slurry samples were spiked with ATCC Map strain 19698. The bacteria were grown in 7H9 liquid medium supplemented with 10% OADC and 2 mg/mL of mycobactin, incubated for 1 month at 37 ° C in culture flasks with 40 mL of culture medium for an optimal growth. Based on published data (Sung et al. 2007), an optical absorbance of 0.6 nm as the midpoint was established as the exponential phase of growth in this culture system. With the aim to minimize the amount of dead cells which could lead to data inaccuracies, the liquid culture was

evaluated on weekly base in a spectrophotometer to verify the exponential growth phase of *Map*. The slurry was thoroughly mixed to achieve an final concentration of at least 106 CFU/ml of *Map*.

Experimental design
A laboratory scale experiment using a randomized block design with 5 treatments and 4 replicates was used to evaluate the effect of lime chemical treatments on Map survival in dairy slurry. A 100 mL of slurry was stored in a 400 mL sterilized glass beaker for each treatments and replicate, which were maintained at room temperature for the evaluation period. The samples were collected for both pH measurement and microbiological analysis at 24, 48 and 72 h after treatment.

Microbiological analysis
In each sampling period, slurry was fully mixed for the different glass beakers, before collecting a 2 mL sample, which was used to determine the presence and concentration of *Map* after treatment and also before treatment. In parallel, 1 mL of slurry was used for total coliforms identification and quantification after treatment.

Detection of Map
Slurry samples were processed as for culture of bovine feces in the BACTEC MGIT 960 liquid culture system (BD Diagnostic Systems, Franklin, NJ), according to manufacturer's protocol. Briefly, the slurry sample was conducted to a germination/decontamination step. The processed sample was then inoculated into tubes of MGITParaTB™ medium (Becton Dickinson, Sparks, MD) with supplement and antibiotics according to the manufacturer's protocol. Each MGIT tube contained 7 ml of modified Middlebrook 7H9 broth base with mycobactin J and a fluorescent oxygen indicator embedded in silicon at the bottom of the tube. Then, 500 µL of egg yolk suspension (Becton Dickinson, Sparks, MD) and 100 µL of VAN cocktail (vancomycin, nalidixic acid, and amphotericin, Sigma-Aldrich) were added to each tube of MGIT ParaTB™medium (Becton Dickinson, Sparks, MD), resulting in final concentrations of 10 g/mL vancomycin, 40g/mL amphotericin B, and 60 g/mL nalidixic acid. Each inoculated MGIT tube was entered into the MGIT 960 instrument and incubated at 37°C for 49 days. Tubes signaling positive by day 49 were removed and tested for presence of Map. Tubes not signaling positive by that time were considered not to have the organism.

DNA extraction
After a positive signal in the liquid culture system, the positive MGIT tube was removed and was inverted three times in order to mix the contents and was thereafter briefly vortexed. From the middle of the tube, an aliquot of 200 µl was transferred aseptically to 1.5 ml centrifuge tubes, which were then centrifuged at 5000 g at room temperature for 5 min. The supernatant of each tube was discarded. The pellet was broken up by pipetting with a mixture of 500 µl lysis buffer (2 mMEDTA, 400 mMNaCl, 10 mMTris-HCL pH 8.0, 0.6% SDS) and 2 µl proteinase K (10 µg/µl), and was then transferred to a bead beating tube (BioSpec Products, Inc., Bartlesville, OK, USA), containing 200 µl of 0.1 mm zirconia/silica beads (BioSpec Products, Inc). The tubes were incubated in a heating block (Eppendorf) at 56ºC for 2 hours. Following the latter, the tubes were shaken in a cell disrupter (MiniBeadbeater-8TM, BioSpec Products) at 3,200 rpm for 60 s and then put on ice to cool for 10 min. In order to remove foam and beads from the inner walls of the tubes, tubes were centrifuged at 5000 g for 30 seconds. A brief vortexing of the samples made sure that any DNA stuck to small solid particles was not lost when the lysate was transferred. The whole liquid volume (500 µl) from the bead beating tube was transferred to 1.5 mL microcentrifuge tubes and 500 µl of ethanol 100% was added. The tube was left to stand at room temperature for 2 min and was then vortexed for 5 s and centrifuged (Eppendorf) at 18,000 g at room temperature for 5 min. Supernantant was discarded and the resulting pellet was resuspended in 200 µl 70% ethanol by resuspension (pipetting) and centrifuged at 18,000 g for 5 minutes. The supernatant was removed and the tube was left open for 10 min at room temperature. The pellet re-suspended in 50 µl of sterile distilled DNAase-free water and incubated in a dry heating block at 100°C for 5 min (with the lid closed). Finally, samples were spun at 18,000 g for 30 seconds to remove solids. A 25 µl aliquot of the supernatant was transferred to a new 1.5 mL tube and stored at -20°C to be used as a template for PCR.

Real-time PCR
The main target was the insertion element IS900. The PCR total reaction was 20 µl, from which 5 µl was DNA template, 10 µl were 2x TaqMan Universal MasterMix (Roche), 0.2 µM primers. Sequence for IS900 primers which amplified a 63 nucleotide fragment of the IS900 gene target was 5'-gacgcgatgatcgaggag-3' (L) and 5'-gggcatgctcaggatgat-3' (R). The reactions were run in a Roche LightCycler 2.0 system under the following standard conditions: one cycle to 95 °C for 10 min, 45 cycles with three steps of 95 °C for 10 sec., 60 °C for 30 sec. and 72 °C for 1 sec, and a final cooling step at 40°C for 30 sec. Negative and positive (Mycobacterium

avium subsp. paratuberculosis ATCC 19698) PCR controls were included, as well as a DNA extraction negative and positive control.

Quantification of Map
Map quantification was performed according to the time to detection results (TTD) provided by the software of the BACTEC- MGIT 960 liquid culture system. The relationship between *Map* concentration and TTD was evaluated considering the estimation of the bacteria concentration.

Detection of Total Coliform (TC)
To evaluate the effect of chemical treatments on the viability of TC, the slurry samples were processed through the use of Petrifilm commercial culture medium (3M Microbiology Products, USA). Thus, 1 mL of each sample of slurry treated with chemicals was mixed with sterilized water to obtain a final dilution of 10-2. The samples were incubated at 37 ° C for 24 h to examine the presence of coliform colonies.

Quantification of TC and pH
To estimate TC concentration in the slurry, the colonies grown in the culture medium were visually counted and the results were expressed as CFU/mL of sample. The non-detection of coliforms was reported as <100 CFU/mL of sample. A direct measurement of pH was taken for each replicates using a Litmus paper.

Statistical analysis
To evaluate the effect of chemical treatments on *Map* survival, ANOVA was used to demonstrate significant differences between chemical treatments on bacterial survival. Because results did not show homocedasticity and were not normally distributed, the ANOVA Kruskal-Wallis test was used, followed by the multiple comparative Dunn test. T test was used to determine differences of Map load between treatments and between one single treatment and control. All analysis was performed using the program Statistix 8.0.

Results
The use of limes treatments on dairy slurry had an important effect on its microbiological and physical composition, where total coliform were totally eliminated in limes treatments and Map was partially controlled, mainly using the high concentration (15%) for both CaO and Ca $(OH)_2$.

The initial slurry pH observed was representative of dairy slurry in Southern Chile, characterized as slightly alkaline (pH 7.7) (Salazar et al. 2007). The lime treatments increased slurry pH up to 12 within 24h and it was maintained to the end of the experiment (72h), while in the control treatments pH did not change. Limes have been widely used for their alkaline effect for different waste and as soil treatments [3].

After the slurry spiking and prior to treatments addition, a time to detection (TTD) of 10.65 days for a positive culture signal result was recorded; this was equivalent to 64,383 bacteria/mL. Highly significant differences ($p = 0.0001$) were found among treatments regarding Map viability. The Dunn test indicated that the statistical difference between treatments is based on CaO and $Ca(OH)_2$ at 15% treatments versus control. Thus, at 24 h and 48 h, CaO 15% showed lowest available organism data relative to all other treatments ($p<0.05$%; Table 1). At 72 h only control samples showed homogeneous bacterial counts and they were significantly different than CaO and Ca $(OH)_2$ treatments ($p\leq 0.05$).

The TC quantification result for the control treatment ranged from 4,000 to 10, 400 CFU. CaO and Ca $(OH)_2$ at 5 and 15% treatments reduced significantly the concentration in 24h, where no CFU was recorded, and continue to the end of the experiment of TC in the slurry samples in relation to the control ($P = 0.0001$; Table 2).

The significant effect of alkaline treatments in reducing *Map* viability could be associated to the extreme alkalinity generated. This disinfectant effect would generate the denaturation of microorganism's structure proteins [4]. Furthermore a cell wall exposed to a strong alkalization could result in chemical changes such as saponification [5]. The addition of alkaline compounds to the slurry can also release NH_3, which is considered another method of disinfection associated with high pH in the slurry [3].

Conclusion and perspectives
Under experimentally conditions, this study provides preliminary evidence of 100% control of total coliform in dairy slurry using alkaline materials. In addition, a significant reduction ($p<0.05$) on the survival rate of *Map* was observed in limed treatments compared to non treated slurry. However, *Map* showed resistance to lime

treatments and higher concentrations of chemicals were required to produce a significant reduction or elimination of the bacterium in the slurry. This information should be considered for future management plans for infection control in susceptible animal populations as a Best Management Practice to reduce pathogens for slurry management on dairy farms. Further studies will be required to evaluate the optimum concentration and the secondary effects of these treatments on slurry physical and chemical composition, as well as its advantages and disadvantages from a biological, environmental, economical and practical point of view.

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Table 1. Quantification of *Map* (bacteria / mL) in contaminated slurry samples at 24, 48 and 72 h.

Treatment	24 h	48 h	72 h
Control (SD)	71,925 (14,686)	25,944 (1,711)	43,322 (16,214)
Ca5% (SD)	3,317,3 (3,529	7,103 (13,138)	10,631 (21,019)
CaO 15% (SD)	38 (76)	0	52 (68.7)
Ca $(OH)_2$ 5% (SD)	2,270 (876)	422 (351.4)	164 (267.4)
Ca $(OH)_2$ 15% (SD)	87 (77.2)	279.8 (372)	266.5 (503.4)

Table 2. Total Coliform (UFC/mL) quantification in different treatments during the study period.

Treatment	24 h	48 h	72 h
Control (SD)	6,775 (2,367)	7,550 (2,323)	4,775 (750)
CaO 5%	< 100	< 100	< 100
CaO 15%	< 100	< 100	< 100
$Ca(OH)_2$ 5%	< 100	< 100	< 100
$Ca(OH)_2$ 15%	< 100	< 100	< 100