NNY Agricultural Development Program
2008 Project Report

The Effect of the Silage Fermentation Process on Johne's Disease Organisms: *Mycobacterium avium* subsp. *paratuberculosis*

Project Leader(s): Sally A. Flis, Catherine S. Ballard, Everett D. Thomas, Heather M. Dann, and Jeffrey W. Darrah**,** *W.H. Miner Agricultural Research Institute, Chazy, NY*

Collaborator: Kimberly Cook, *USDA-ARS Bowling Green, KY*

Background: Johne's disease, which is caused by Mycobacterium avium subsp. paratuberculosis (MAP), is a chronic, progressive, enteric disease of ruminants. Cattle generally become infected with MAP as calves and often do not show clinical signs until 2 to 5 years of age. The primary route of infection is through the ingestion of fecal material, often from contaminated feed or water, milk, or colostrum containing MAP (Stabel, 1998; Manning and Collins, 2001). Multiplication of MAP occurs only in a susceptible host within a macrophage, making it an obligate pathogenic parasite (Collins, 2003). As a result of this, MAP cells that are shed in the feces or milk of a cow are extremely tolerant to environmental stresses.

Heat tolerance was demonstrated when MAP was recovered after pasteurization treatments of up to 140°F (60.1°C), however no MAP was recovered when temperatures exceeded 150°F (65.5°C) (Stabel, 2001). Further, it has been reported that pasteurization at 161°F (71.7°C) for 15 to 20 seconds achieved complete kill of MAP (Stabel et al., 2004). *Mycobacterium paratuberculosis* is also very cold tolerant. Richards and Thoen (1977) reported that even with a 1:10,000 dilution of feces contaminated with MAP they were able to recover the organism after 15 weeks of being frozen at -94°F (-70°C).

The MAP cells in excreted feces can remain alive for more than a year, depending on the conditions (Collins, 2003). Therefore, health programs such as New York State Cattle Health Assurance Program (NYSCHAP) strongly recommend no manure application to grass or alfalfa fields until the final crop has been harvested, especially forages intended for cattle less than 24 months of age. Those farms participating in the NYSCHAP program find it challenging to adhere to these restrictions on manure application. In addition, little research has been conducted to determine the impact of ensiling forages on the survivability of MAP.

In past studies at Miner Institute, the maximum temperature reached during the fermentation process for grass/alfalfa forages was only 103.8°F (39.9°C), suggesting that temperature will not be a factor in killing MAP during silage fermentation. The impact of pH during fermentation was evaluated by ensiling 100 g of re-hydrated grass hay in a vacuum bag system (Katayama et al., 2000). This study found that when pH was less than 4.8, MAP did not survive. Another study reported that MAP will survive pH levels between 5.3 and 8.0. (Manning and Collins, 2001). In grass/alfalfa silage at Miner

Institute the average pH after fermentation was 4.7 and 4.5 in 2004 and 2005, respectively. Silage pHs below 4.8 indicates that MAP excreted in feces and applied to forages at Miner Institute may be killed during the fermentation process.

Results from a preliminary study in which 4 replications of 600g of grass/alfalfa silage were inoculated with 5.5 x 10^6 cells of MAP per gram of forage and placed in vacuum sealed bags for 30 days showed an average decrease of 97% in the number of MAP cells per gram of forage as determined by polymerase chain reaction (PCR) which is a rapid and sensitive test able to detect MAP DNA in fecal samples. These results indicated that there is a potential to decrease the contamination of MAP through fermentation of grass forages. However, there was still a detectable amount of viable MAP DNA in the sample.

Therefore, the objective of this study was to determine how many days of ensiling of grass/alfalfa forages should be recommended to eliminate MAP DNA from the fermented feed. This study's findings may warrant a follow-up investigation conducted on a more practical, farm-scale system.

Methods:

Fecal Inoculant:

Fecal samples were taken from cows in the dairy herd at Miner Institute that were identified as Johne's positive by the dairy herd's management protocol (500 ml per cow). Half of each sample was shipped overnight to the USDA-ARS Animal Waste Management Research Unit (Bowling Green, KY) for determination of MAP cells per gram of manure by PCR. The other half of the sample was frozen at -112°F (-80°C). Manure from infected animals averaged 2.0 X 10^6 MAP cells/g manure, resulting in the diluted infected manure mix having a concentration of 1.0 X 10^5 MAP cells/g manure.

Forage Sample, Inoculation, Fermentation, and Sampling:

Fresh grass/alfalfa forage was collected at harvest from a field that had not received manure application in the 2007 crop season. The unfermented forage was frozen at -4°F (-20°C). After thawing, dry matter (DM) was determined for each sample and used to calculate the amount of water that could be added to the forage to achieve an ideal DM for fermentation. Additionally, a sample was taken before and after inoculation for MAP determination at the USDA-ARS Animal Waste Management Research Unit (Bowling Green, KY).

Six replications were prepared for sampling at 30, 45, 60, 75, and 90 days after ensiling. For each replication, 700 g of forage was inoculated with the diluted manure and mixed. From the inoculated silage, 600 g was placed in a vacuum-sealed bag and labeled with the number of days of ensiling until sampling. All bags were placed in a dark room at room temperature (68°F or 20°C) until final sampling. For each replication, the remaining inoculated unfermented forage was sent for MAP determination by PCR at the USDA-ARS Animal Waste Management Research Unit (Bowling Green, KY).

After the assigned number of days of ensiling the samples were removed from the bags. The pH, DM, and organic acid concentration were determined on each replication (Dairy One Forage Testing Lab, Ithaca, NY) to examine the quality of the sample fermentation.

A sub-sample from each bag was frozen and sent for MAP determination by PCR at the USDA-ARS Animal Waste Management Research Unit (Bowling Green, KY).

Statistical analysis was done to determine means of the silage fermentation characteristics for each sampling day.

Results:

Forage Fermentation:

Forage fermentation profiles of MAP inoculated forage did not differ from control bags (Table 1). Further, fermentation profiles of all days were within the range for the sample average reported by Dairy One Forage Lab (Ithaca, NY; Table 1). An interesting finding of this research was that the acetic acid concentration of the samples was higher for the 45, 60, 75, and 90 days than at 30 days of fermentation ($P < 0.05$, Table 1). Although visually the forage appeared to ensile properly, perhaps freezing the forage prior to fermentation impacted fermentation.

MAP Concentrations:

Control samples had MAP concentrations below the detection limit of the PCR test, which indicated the forage did not have any background contamination of MAP prior to dilute manure application. Total bacterial counts of the silage were similar between control samples and MAP inoculated samples at all samplings (Figure 1), which indicate similar bacterial populations during fermentation for both control and treated samples.

The MAP concentrations persisted in the silage for the duration of the experiment (90 days; Figure 1) although target sequences were decreasing from day 60 forward. The levels detected at all ensiling times were adequate to infect susceptible animals ($1.9 \pm 0.87 \text{ X } 10^5$ cells/g silage.

Conclusions/Outcomes/Impacts:

Unfortunately, the fermentation of forage through ensiling did not eliminate the presence of MAP in contaminated forage. Previous research conducted showed a much larger decrease in MAP populations after silage fermentation. The levels detected at all ensiling times for the current study were adequate to infect susceptible animals ($1.9 \text{ x } 10^5$ cells/g silage). While the use of PCR to detect MAP DNA is a quick and reliable method, it is not able to discern between live and dead cells. The question that arose from these findings was if the ensiling process preserves the target DNA sequence measured by the PCR method, keeping the counts higher than expected on all sampling days.

Future Investigations:

Based on these study results, a second research project was started in October 2008 where 3 treatments were used: a control (no MAP inoculated), forage inoculated with live MAP, and forage inoculated with dead MAP. These treatments will provide information on how long a dead versus live MAP will persist in fermented forage. This finding will help determine if the use of PCR for MAP detection is a suitable methodology for examining MAP presence in ensiled forage. If it is found to be unsuitable, the traditional bacterial culture method would need to be used for future studies evaluating the survivability of MAP during ensiling. Unfortunately, the culturing method takes months

to determine MAP presence. The follow-up study will be published in the 2009 NNYADP Report.

Outreach:

Data will be summarized and presented at local meetings (Miner Institute Corn Congress and/or Dairy Day). In addition, articles summarizing the results will be published in Miner Institute's *Farm Report* and other popular press publications.

Reports and/or articles in which results of this project have already been published:

The results of this project were presented as a poster presentation and published as an abstract for the Joint Meeting of the Geological Society of America, and the ASA-CSSA-SSSA in Houston, Texas. Survival of bovine *Mycobacterium avium* subsp. *Paratuberculosis* during grass silage forage fermentation. *S. A. Flis, K. L. Cook, C. S. Ballard, E. D. Thomas, and H. M. Dann.*

For more information:

Catherine S. Ballard
P.O. Box 90, Chazy, NY 12921
518-846-7121 x 112, ballard@whminer.com.