DYNAMICS OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* IN DAIRY HERDS: INSIGHTS INTO TRANSMISSION RISKS, BULK-MILK CONTAMINATION, AND ASSOCIATIONS BETWEEN DIAGNOSTIC TESTS

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DYNAMICS OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* IN DAIRY HERDS: INSIGHTS INTO TRANSMISSION RISKS, BULK-MILK CONTAMINATION, AND ASSOCIATIONS BETWEEN DIAGNOSTIC TESTS

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Johne's disease, a severe granulomatous enteritis of ruminant animals, is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). MAP infections have detrimental consequences for animal health and reduce dairy-herd productivity. Bacterial fastidiousness and slow generation time encumber diagnostic testing strategies. MAP is also a potential etiologic agent of human Crohn's disease, with the bulk-milk supply serving as a possible transmission vector. The objective of this dissertation is to explore MAP infection dynamics on dairy farms, with an emphasis on the routes of bulk-milk contamination, transmission risk across production type, and the interplay between diagnostic testing outcomes. Accordingly, we have applied statistical and mathematical approaches to both cross-sectional and longitudinal datasets.

Using questionnaire data from 292 U.S. dairies, we conducted a comparative risk assessment of organic vs. conventional management and determined that organic herds were at higher risk for new MAP infections. We concluded, empirically, that organic farms were more susceptible to a synergism of risk factors within the maternity pen and should improve calving-area hygiene if electing to permit cow-calf contact. Bulk-milk testing was also conducted for these herds. Most high ELISA tanks were PCR negative, implying that ELISA is not a perfect predictor of bulk-milk MAP status; for accurate risk assessment, bulk-milk ELISA should be used in tandem with PCR. A combination of ELISA and PCR may also aid in determining the specific route of bulk-milk contamination (either environmental or direct shedding).

To extend the investigation to individual animals, longitudinal data were obtained from 14 MAP-positive cows in 2 low-prevalence herds. Robust relationships between culture, fecal qPCR, and milk ELISA were revealed, using mixed linear modeling to adjust for cow characteristics. We explored temporal relationships and observed that spikes in fecal shedding were predictive of subsequent high milk ELISA results. We also noted that disease "Progressors," (infected animals with increasing fecal MAP CFU over time) had higher antibody titers overall. Interestingly, the paucity of positive milk samples, from both individual and bulk-tank sources, suggests that milk contamination is not a chief concern in low-prevalence herds.

Armed with insights from these studies, in addition to published literature, we developed a mathematical model to explore the interaction between categories of infection, environmental MAP burden, and bulk-tank contamination. Direct shedding into milk accounted for < 1% of the MAP CFU in the tank, with environmental contamination from high shedders as the primary driver of bulk-milk MAP burden. Culling of high shedders, cleaning of the maternity pen, and adherence to milking parlor cleanliness each had a strong influence on lowering the bulk-milk MAP load. A combination of these initiatives served to drive the MAP level below an acceptable threshold ($< 10^3$ CFU/L). While complete elimination of MAP may be an unrealistic target for high-prevalence herds, the production of bulk milk with a low MAP load appears feasible.

In this work, we assess the significance of a variety of contamination routes, transmission risks, and intervention strategies. These efforts are directed toward improved understanding of testing schemes and an ultimate refinement of control measures and milk quality programs. The conclusions from the studies presented in this dissertation may be applied to mitigate the spread of MAP in dairy herds, reduce prevalence, and lower or eliminate MAP in the bulk-milk supply.

BIOGRAPHICAL SKETCH

Annabelle Beaver grew up in Brooklyn, New York and attended an art-oriented school for twelve years that emphasized poetry, music, theater, and the Classics. Her undergraduate education at Princeton University also focused on the arts: she majored in English, earned Certificates in Theater and Creative Writing, and won approval to submit a Creative Writing Thesis in poetry. Annabelle received a number of honors in these disciplines and was also the harpist in the University orchestra for four years.

In 2011, after spending a year in Paris studying drama at the renowned L'École Internationale de Théâtre Jacques Lecoq, Annabelle became aware of a rigorous two-year Master's Degree Program in Animal Behavior and Conservation at Hunter College in New York City and gained admittance by taking several qualifying courses. There, she was introduced to a broad range of research perspectives, gained a solid background in statistics, and enjoyed her first teaching assistant role. Her first-year performance in this Master's program enabled her to secure a summer position as a Visiting Researcher at the University of British Columbia's Dairy Education and Research Centre. This experience cemented her interest in farmed animals, cows in particular, and gave rise to her Master's Thesis titled: *How Nutritional Dependency on the Dam Impacts Dairy Calf Weight and Behavior*.

Annabelle graduated in 2013 with her Animal Behavior and Conservation Master's, after completing forty-four credit hours with a GPA of 4.3. Based on this achievement, she was accepted into the Cornell University Animal Science Ph.D. Program as a Graduate Research Assistant. For the past four years, she has been working in the Schukken Lab, within the Department of Population Medicine and Diagnostic Sciences, immersed in laboratory and field research, investigating MAP bacteria (*Mycobacterium avium* subsp. *paratuberculosis*). In this

effort, she has collaborated with researchers at the Universities of Maryland, Pennsylvania, Wisconsin, and Penn State. Much of her dissertation research has been published in peer-reviewed journals, and she has presented her findings to researchers in Belgium, Norway, and the Netherlands. She has completed her Cornell course work in Animal Science and in her minor fields of study, Epidemiology and Microbiology, achieving a GPA of 4.2. She also undertook three graduate teaching assignments in the fields of Clinical Biostatistics, Dairy Cattle Genetics, and Applied Animal Welfare.

In 2016, she was named a Fulbright Scholar, and this grant enabled her to spend nine months in the Netherlands at Wageningen University in the Quantitative Veterinary Epidemiology research group, within the Department of Animal Sciences. As a Fulbright Scholar, she gave many presentations to student populations about her background and research. She also traveled to many neighboring countries, as well as within the Netherlands itself.

Annabelle has assembled a background in several interdisciplinary fields: Animal Science, Epidemiology, Microbiology, Statistics, Math Modeling, Animal Behavior, and Animal Conservation. She aspires to a research career devoted to improving the quality of life for dairy cattle. In her spare time, Annabelle also hopes to continue to write and submit poetry for publication, and to perform as a Shakespearian actor.

POSITIONS AND HONORS

2005	Advanced Placement Scholar with Honors
2005-2009	Princeton University Undergraduate Degree Candidate
2006	Finalist, English Prize for Academic Freshman
2008	E. E. Cummings Poetry Prize of the Academy of American Poets
2008	Lewis Center for the Arts Award: outstanding work by a junior
2009	Bain-Swiggett Poetry Prize (highest English Dept. Poetry Award)
2009	Creative Writing Thesis, selective application process
2009	AB Degree in English, Princeton University
2011-2013	Hunter College, City University of New York: Master's Degree Candidate Animal Behavior and Conservation Program
2012	Visiting Researcher, University of British Columbia Dairy Education and Research Centre
2013	Adjunct Lecturer, Research Methodology: Hunter College
2013	Master's Degree in Animal Behavior and Conservation Thesis: *How Nutritional Dependency on the Dam Impacts Dairy Calf Weight and Behavior* (GPA 4.3)
2013-2017	Cornell University PhD Candidate, Animal Science with minors in Epidemiology and Microbiology
2014	Graduate Teaching Assistant, Clinical Biostatistics
2015	Graduate Teaching Assistant, Dairy Cattle Genetics
2016-2017	Fulbright Scholar, Wageningen University, the Netherlands
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PUBLICATIONS

Beaver, A., R.W. Sweeney, E. Hovingh, D. Wolfgang, Y.T. Gröhn, and Y.H. Schukken. 2017. Longitudinal relationship between fecal culture, fecal quantitative PCR, and milk ELISA in Mycobacterium avium subsp. paratuberculosis-infected cows from low-prevalence dairy herds. J. Dairy Sci. 100:7507–7521.

Beaver, A., C.L. Cazer, P.L. Ruegg, Y.T. Grohn, and Y.H. Schukken. 2016. Implications of PCR and ELISA results on the routes of bulk-tank contamination with *Mycobacterium avium* subsp. *paratuberculosis.* J. Dairy Sci. 99:1391–1405.

Beaver, A., P.L. Ruegg, Y.T. Grohn, and Y.H. Schukken. 2016. Comparative risk assessment for new cow-level *Mycobacterium avium* subspecies *paratuberculosis* infections between 3 dairy production types: Organic, conventional, and conventional-grazing systems. J. Dairy Sci. 99:9885–9899.

Johnsen, J. F., **A. Beaver**, C.M. Mejdell, J. Rushen, A.M. de Passillé, and D.M. Weary. 2015. Providing supplementary milk to suckling dairy calves improves performance at separation and weaning. J. Dairy Sci. 98:4800–4810.

Johnsen, J. F., A.M. de Passille, C.M. Mejdell, K. Bøe, A.M. Grøndahl, **A. Beaver**, J. Rushen, and D.M. Weary. 2015. The effect of nursing on the cow–calf bond. Appl. Anim. Behav. Sci., 163:50–57.

Schukken, Y.H., R.H. Whitlock, D. Wolfgang, Y.T. Grohn, **A. Beaver**, J. VanKessel, M. Zurakowski, and R.M. Mitchell. 2015. Longitudinal data collection of *Mycobacterium avium* subsp. *paratuberculosis* infections in dairy herds: the value of precise field data. Vet. Res. 46:65–75

MANUSCRIPTS IN PREPRATION

Beaver, A., and Y.H. Schukken. 2017. Mathematical model representing the infection dynamics of *Mycobacterium avium* subsp. *paratuberculosis*: the dairy-farm environment and the bulk-tank milk supply.

Beaver, A*., S. Rani*, Y.H. Schukken, and A.K. Pradhan. 2017. Modeling the effects of infection status and hygiene practices on *Mycobacterium avium* subsp. *paratuberculosis* contamination in bulk-tank milk.
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Johnsen, J.F., C.M. Mejdell, **A. Beaver**, A.M. de Passille, J. Rushen, and D.M. Weary. 2017. Behavioural responses to cow-calf separation: the effect of nutritional dependence.

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During my Master's program, I was also afforded the opportunity to spend a summer at the University of British Columbia's Dairy and Education Research Centre. There, under the exemplary guidance of Dr. Dan Weary, Chair of Dairy Cattle Research, and Norwegian DVM and later friend, Julie Føske Johnsen, I was able to experience hands-on animal research, a pivotal step in my education process. I returned to Hunter for my second year armed with a battery of knowledge and a keen sense of direction.

I especially express my deep appreciation to many Cornell Faculty members and friends, in particular the members of my Doctoral Committee, Drs. Ynte Schukken, Michael Thonney, Yrjö Gröhn, and David Russell, for their support and stewardship. While in Dr. Schukken's Lab over my first three years, I had the opportunity to benefit greatly from his internationally-recognized expertise in the fields of veterinary epidemiology, bovine infectious diseases, and the application of the tools of mathematical modeling. I also express my appreciation for the assistance of the members of the Schukken Lab, Anja Sipka, Suzanne Klaessig, and Brianna

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Additional specific acknowledgments follow each research chapter.

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INTRODUCTION

1. Johne's disease: Definition and Etiology

Johne's disease, also known as Paratuberculosis, is a granulomatous enteritis of ruminant animals that is both chronic and progressive. In cattle, the initial clinical manifestations are weight loss and intermittent diarrhea without an associated reduction in appetite (Whitlock and Buergelt, 1996). Advanced clinical disease is marked by lethargy and emaciation, and animals often display intermandibular edema (a soft swelling under the jaw) resulting from hypoproteinemia. If left to progress, the disease may have a terminal outcome due to nutrient malabsorption, dehydration, and cachexia (Whitlock and Buergelt, 1996).

The etiologic agent of Johne's disease is *Mycobacterium avium* subsp. *paratuberculosis* (MAP), a resilient and slow-growing bacterium characterized by lengthy incubation periods *in vivo* and a fastidious nature *in vitro.* Paratuberculosis predominately affects wild and domestic ruminants, although other non-ruminant animals such as badgers, weasels, rabbits, and even crows, have been observed to harbor the bacterium (Beard et al., 2001).

1.1 Mycobacteria

MAP shares the genus *Mycobacterium* with several notable human pathogens including *M. tuberculosis*, *M. leprae*, and the zoonotic *M. bovis* (Cosma et al., 2003). Mycobacterial infections are notoriously difficult to treat due to a characteristic hardiness of the cell wall, which is composed of layers of peptidoglycan, arabinogalactan (a polysaccharide of arabinose and galactose subunits), and mycolic acids (Niederweis et al., 2010). The mycolic acids, long fatty acids each containing 60 to 90 carbon atoms, lead to a fungus-like growth pattern observable in culture; indeed, the prefix *myco* is derived from the Ancient Greek for fungus (Goodfellow and Magee, 1998). Mycobacteria are aerobic, largely non-motile, and are often classified as Gram-

positive. Despite this classification, mycobacteria only weakly display the crystal violet stain and have been shown to possess an outer membrane with a bilayer structure (Niederweis et al., 2010; Lambert, 2002). Thus, a positive result in acid-fast (Ziehl-Neelsen) staining is typically used as confirmation of mycobacterial presence (Koch and Cote, 1965).

2. Human Crohn's Disease

MAP has been implicated as a potential cause of Crohn's Disease (CD) in humans, although causation has not been conclusively ascertained (Over et al., 2011). In general, MAP has been more frequently identified in tissue samples of patients with CD than in control samples (Feller et al., 2007). Because this association has been established via retrospective case-control studies, it has not been possible to determine whether MAP is present prior to CD onset. Conceivably, MAP may be involved only in secondary infection in CD patients (Feller et al., 2007), although its potential as a causative agent cannot be dismissed (Kuenstner et al., 2017).

2.1 MAP and the Bulk Milk Supply

Contaminated dairy products represent the primary source of MAP transmission from cattle to humans (Streeter et al.,1995), and MAP has been documented to survive commercial pasteurization when its initial concentration exceeds 10^4 CFU/L (Grant et al., 2005). Thus, from a public health standpoint, it becomes important to monitor bulk milk destined for human consumption (Eltholth et al., 2009). MAP may enter the bulk tank by means of 2 distinct routes: the "internal route" or the "environmental route." In the internal route, infected animals shed MAP directly into milk. These animals are typically in later infection stages and may shed MAP as a consequence of supramammary lymph node infection or bacteremia (Sweeney et al., 1992). In the environmental route, MAP enters the bulk milk supply from a contaminated environment,

primarily via fecal contamination of udders or teats. However, MAP may also enter the tank by means of adulterated water sources or airborne particles (Eisenberg et al., 2013)

3. Johne's disease and the Dairy Industry

3.1 History and Current Prevalence Estimates

In 1895, German pathologist H.A. Johne and his fellow, L. Frothingham, described the presence of acid-fast bacilli in the intestinal mucosa of cattle affected with an unusual and fatal form of chronic enteritis. In the early 20th century, the disease was termed Johne's disease or "pseudo-tuberculosis," and was reported in cattle in Scandinavia, Western Europe, and North America (Chiodini et al., 1984). The disease has continued to spread worldwide, and current prevalence estimates suggest that approximately 70% of dairy herds in the U.S. harbor at least one MAP-positive animal (Lombard et al., 2013). Dairy herd-level prevalence is similarly high in several other countries with endemic MAP infections, such as the Netherlands (31-71%, Muskens et al., 2000), Denmark (47%, Nielsen et al., 2009), and Canada (28-57%, Tiwari et al., 2006). Within an infected U.S. dairy herd, the cow-level prevalence is reported to be 5 to 10% on average (Lombard, 2011), although recent data including *post mortem* culture results suggests that this proportion may be vastly underestimated (Schukken et al., 2015).

3.2 Challenges to Infection Mitigation in Dairy Herds

Although Johne's disease is now recognized as one of the dairy industry's most serious infectious diseases, efforts to curtail infection and reduce prevalence have not been straightforward. MAP infections represent hidden threats on dairy farms owing to lengthy subclinical phases, transient bacterial shedding (Whitlock and Buergelt, 1996), and imperfect yet costly diagnostic testing options (Collins, 1996). The incubation period from infection until the onset of clinical symptoms typically ranges from 2 to 10 years (Whitlock and Buergelt, 1996;

Manning and Collins, 2010); during the subclinical phase, cattle, including young stock, may shed MAP into their manure, thus distributing the bacteria throughout the farm environment. Adult animals may also shed MAP directly into milk. Initial signs of MAP infection include roughening of the hair coat, and reduced fertility, milk production, or body condition score (Whitlock and Buergelt, 1996). Because these signs are subtle and attributable to a wide variety of conditions, MAP infections often remain undetected. In fact, animals are typically removed from the herd before the appearance of clinical disease (Smith et al., 2010).

MAP cannot replicate in the environment, yet the bacteria may survive outside the host for extended periods of time and under a wide variety of environmental conditions (Raizman et al., 2004). MAP has been documented to persist in soil for more than a year in dry, shaded areas, and for 6 months in fully-shaded grass. To date, no upper limit on environmental survival time has been identified (Whittington et al. 2004). MAP may also endure sub-zero temperatures (Richards and Thoen, 1977) and is hypothesized to survive within protists (Pickup et al., 2006). The presence of MAP in other relevant substances has been documented, including slurry, river water, lake sediment, and dust (Jørgensen, 1977; Pickup et al., 2006; Eisenberg et al., 2013).

MAP's environmental resilience is problematic for infection control: the primary method of transmission is the fecal-oral route, which may occur via a contaminated environment. Calves under 6 months exhibit a heightened susceptibility to MAP infection, primarily through contact with adult cow manure (Lombard, 2011; Doré et al., 2012). Milk and colostrum represent additional infection sources. Calves are born agammaglobulinemic, and the ruminant gut allows for increased absorption of immunoglobulins and other macromolecules during the first 24 hours of life. It is hypothesized that MAP bacteria also have greater access to the intestinal mucosa during this "open gut" phase via a barrier with reduced selectivity (Olsen et al., 2002; Sweeney,

2011). Finally, intrauterine transmission has been documented, although this mechanism does not appear to be responsible for MAP infection persistence in dairy herds (Mortensen et al., 2004).

The economic loss attributable to Johne's disease is substantial. For the US dairy industry, the financial deficit has been estimated at upwards of 200 million dollars annually (Ott et al., 1999). Costs amass because infected, yet subclinical, animals show heightened susceptibility to other diseases and may have reduced production, fertility, or feed efficiency. These consequences may lead to spikes in veterinary and replacement costs, in addition to a higher overall cull rate (Ott et al., 1999; Chiodini et al., 1984).

3.3 Stages of MAP Infection

Following natural infection with MAP, calves typically enter a transient shedding stage in which MAP is shed in small quantities in the absence of clinical signs (Whitlock et al., 2000; Cho et al., 2012). Infected animals then enter a lengthy latent stage in which no shedding occurs. The subsequent phases of infection have been divided into low and high shedding categories, which ensue at approximately 3 and 5 years of age, respectively. Only 7% of infected animals ever become high shedders; however, once an animal reaches this stage, there is a 95% chance of death or removal from the herd within the year (Mitchell et al., 2015). The duration of latency to reach the high-shedding phase may be impacted by the age of infection: recent evidence suggests that adult cows can also become infected with MAP (Schukken et al., 2015), but such animals are unlikely to advance to the low or high shedding phases or to show clinical disease (Mitchell et al., 2015). Finally, using extensive longitudinal data, Schukken et al. (2015) noted 2 distinct shedding patterns among infected animals. *Progressors* demonstrated a steady increase in MAP CFU in fecal culture over time. On the other hand, for *Non-progressors,* fecal culture results did

not increase over time and were often negative, suggesting intermittent rather than consistent MAP shedding.

4. Summary of Pathogenesis and Immunology of MAP Infection

The host immune response to MAP infection is characterized by high levels of cell-mediated responses during the subclinical phase, followed by increased humoral immunity in later stages of disease (Stabel et al., 2000). Once MAP reaches the ileum, it is transcytosed through microfold cells covering the Peyer's patches and taken up by macrophages within the stroma (Tessema et al., 2001). MAP is a facultative intracellular pathogen and has been shown to overcome host defenses within the macrophage. The mechanisms underlying MAP survival within macrophages are varied, and MAP is often described as capable of modulating the innate immune response of the host (Weiss and Souza, 2008; Tessema et al., 2001). This intercellular survival may be attributed to the bacterium's ability to hinder macrophage activation, inhibit phagosome maturation and acidification, and reduce antigen presentation. In fact, certain mycobacterial products are able to resist degradation by scavenging reactive oxygen and nitrogen intermediates (Weiss and Souza, 2008).

Phagocytosis of MAP by macrophages has been shown to give rise to decreased expression of Major Histocompatibility Complex (MHC) molecules (Singh et al., 2013). Toll-like receptor 2 (TLR2) has been implicated as a key initiator of this downregulation by binding to MAP and triggering the mitogen-activated protein kinase (MAPK)-p38 pathway and, likely, the nuclear factor (NF)-kB pathway (Hussein et al., 2016). These pathways result in excessive expression of Interleuken 10 (IL-10) which in turn inhibits MHC class-II factor expression. Thus, it is not surprising that mutations in TLR2 and MHC genes have been identified as potential candidates for differential susceptibility to ruminant MAP infection (Koets et al., 2010;

Reddacliff et al., 2005). Other implicated genes include SLC11A1 and CARD15, which are involved in nitric oxide synthesis by macrophages and pathogen-associated molecular pattern (PAMP) recognition, respectively (Pinedo et al., 2009).

By evading host defenses, MAP proliferates silently until eventual macrophage rupture. The bacteria are then released into neighboring tissue where they are engulfed by other macrophages, re-initiating the cycle. Initially, a Th1-type response predominates, characterized by host production of interferon gamma (IFN-γ) and other pro-inflammatory cytokines (Singh et al., 2013). Eventually, this response is overshadowed by a Th2-type response, characterized by production of cytokines associated with antibody production, such as IL-5, IL-5, and IL-6. These Th2 regulatory cytokines are involved in triggering the humoral immune response by supporting B cell proliferation and subsequent immunoglobulin secretion (Stabel, 2006). During this shift, the animal begins to shed MAP in increasing amounts, and the infection may spread to other tissues. The granulomatous consequence of infection occurs when the immune system responds by recruiting additional monocytes and lymphocytes, which fuse together into multinucleated giant cells and epithelioid cells. This process eventually results in visible thickening of the intestinal mucosa, which inhibits nutrient absorption and leads to clinical symptoms (Sweeney, 2011).

5. Diagnostic Testing

A variety of diagnostic tests are available for the detection of MAP bacteria and the associated antibodies. The main diagnostic matrices for *ante-mortem* testing are manure, milk, and serum (Clark et al., 2008), while *post-mortem* diagnoses are typically obtained via bacterial culture of tissues from intestinal regions such as the ilium, ileocecal junction, ileocecal lymph nodes, or mesenteric lymph nodes (Huntley et al., 2005; Schukken et al., 2015). Variants of the

ELISA test (e.g., direct or indirect ELISA, sandwich ELISA, and kinetic ELISA (KELA)), or AGID tests, are used to detect MAP antibodies. Techniques such as culture and PCR target MAP itself. Each diagnostic test has its drawbacks and advantages with respect to efficiency, cost, practicality, and test characteristics. In addition, among the test characteristics, there is often a tradeoff between test sensitivity and specificity (Collins, 1996).

5.1 ELISA

ELISA is commonly performed on serum or milk samples, and estimates of sensitivity and specificity depend upon the class of ELISA (Collins et al., 2005; Cazer et al., 2013) and stage of disease. ELISAs have reduced sensitivity to detect infection in early stages of MAP infection, as measurable antibody production in serum is typically absent in newly-infected animals (Stabel, 1997). Sensitivity of serological testing is therefore known to improve as animals progress from subclinical to clinical stages (Whitlock et al., 2000). The onset of fecal shedding is generally accepted to precede antibody production in serum (van Schaik et al., 2003; Magombedze et al., 2017), yet little is known regarding the time of onset for antibody production in milk.

5.2 Culture

MAP culture of fecal samples is highly specific, with most reports citing a 100% specificity corresponding to a complete absence of false positives (Sockett et al., 1992; Whitlock et al., 2000). A potential stipulation to this estimate is the so-called “pass-through phenomenon” in which an animal ingests and sheds MAP without becoming truly infected (Sweeney et al., 1992; Whitlock et al., 2000). Thus, in rare instances, a negative animal may provide a fecal-culture positive result.

The high specificity of fecal culture may in part be linked to MAP's fastidious nature: MAP is typically cultured on Herrold's Egg Yolk Medium (HEYM) or 7H10 medium, and it requires ferric Mycobactin J, an iron chelate, for growth. Mycobactin dependency may be assessed, in addition to acid-fast staining, to confirm the presence of MAP (Chiodini, 1984; Chen et al., 2012). Despite its high specificity, the utility of the fecal culture technique is encumbered by a poor sensitivity, which has been estimated at 30 to 50% (McNabb et al., 1991). More recent research suggests that the false-negative rate for fecal culture may be even higher: using 10 years of longitudinal data, Schukken et al. (2015) determined that a 2% positive rate in *ante-mortem* fecal culture corresponded to nearly a 17% *post-mortem* tissue prevalence. Fungal overgrowth and competing bacterial contamination may also hamper accurate MAP CFU estimations (Stabel et al., 2002). In addition, fecal culture is costly and time consuming, with conventional methods typically requiring between 8 and 16 weeks for full growth of the organism (Collins, 2011). MAP culture of milk is occasionally performed but often results in low yield from both spiked samples and field samples (Stabel et al., 2002; Donaghy et al., 2008).

5.3 PCR

PCR techniques such as quantitative PCR (qPCR) and immunomagnetic PCR have been optimized for use in fecal samples, and more recently, in milk samples (Khare et al., 2004). PCR methods provide several benefits over culture, namely, increased sensitivity and rapid availability of results. The most widely-used molecular target is the insertion sequence IS900 (present in 14 to 17 copies within the MAP genome); however, IS900-like sequences have been found in other mycobacterial species, leading to false positive results (see Donaghy et al., 2010). Other gene targets include ISMav2 (present in 3 copies), ISMAP02 (present in 6 copies), ISMAP04 (present in 4 copies), F57 (present as a single copy), and the heat shock protein X

(*hspX*) gene (present as a single copy) (Stabel and Bannantine, 2005; Li et al., 2005; Singh et al., 2013). Single-copy sequences have reduced sensitivity compared to multiple-copy elements, but it is easier to determine the initial quantity of the target sequence. Like culture, PCR is often costly (Collins, 1996), and PCR methods alone are not sufficient to differentiate MAP DNA isolated from live vs. dead cells. Follow-up techniques, such as reverse-transcription PCR of RNA targets, or phage amplification assays, are needed to overcome this constraint (Stanley et al., 2007).

5.4 Herd-level testing strategies

As previously described, individual-based testing methods are often accompanied by limitations in practicality, timeliness, and cost. Herd-level testing strategies have therefore become important tools to identify the presence and prevalence of MAP in dairy herds. A common herd-level testing method involves pooling fecal samples from the farm environment or from individual animals for testing via culture or PCR (Collins, 2011). Yet such methods are subject to many of the same restrictions as individual-based diagnostics. Thus, there are demands for a simple, inexpensive, and rapid herd-level tests that could provide producers with an estimate of MAP prevalence (Clark et al., 2008; Cazer et al., 2013). Some bulk-milk PCR diagnostics have been implemented and described, but a more sensitive, commercial test, optimized for use in milk, is needed. DNA extraction from milk is complicated by MAP's robust cell wall and propensity to clump; additionally, the bacteria have been shown to fractionate to both the cream and pellet layers (Herthnek et al., 2008).

6. Management Strategies

The Voluntary Bovine Johne's Disease Control Program of the United States (VBJDCP) has been in effect since 1999 and provides management guidelines for both dairy and beef herds

(USDA, 2010). The VBJDCP highlights preventative strategies to control MAP spread between and within herds, with an emphasis on calving-pen management, environmental cleanliness, and safe introduction of replacement stock. The risks are categorized according to animal age and housing environment.

6.1 Management of the Calving Area

Poor management of the maternity area may result in the highest level of risk for new MAP infections, due to the increased susceptibility of young calves (Lombard, 2011). Hygiene of periparturient animals is paramount, since studies have shown that fecal contamination of udders is associated with higher odds of MAP positivity within the herd (Ansari-Lari et al., 2009). Moreover, the MAP strains isolated from contaminated teat skin have often been linked to other adult cows in the herd, rather than to the donor animal (Pithua et al., 2011). The presence of multiple groups of animals in the maternity pen, such as sick animals or even other lactating cows, has been associated with increased MAP prevalence (Pithua et al., 2011).

The VBJDCP, along with other national control programs (e.g., the Three Step Calf Rearing Plan in Australia (Animal Health Australia, 2016)) recommend immediate separation of cow and calf following parturition; however, there is a limited amount of scientific research pointing to decreased MAP prevalence following implementation of this practice (McAloon et al., 2015). In reference to milk source contamination, there is some evidence that calves fed colostrum from the dam in comparison to pooled colostrum have decreased odds of testing MAP positive (Nielsen et al., 2008) and that herd seronegativity is associated with the provision of milk replacer (Muskens et al., 2003). In contrast, recent research has identified viable MAP bacteria in commercial milk replacers (a 28.9% positive rate out of 83 samples), presumably due to the presence of milk by-products such as casein, whey, and milk powder (Grant et al., 2017).

6.2 Pasture Management

Spreading manure on pasture is linked to a higher infection risk (Obasanjo et al., 1997), as is the use of surface water due to runoff from contaminated pasture (Whittington et al., 2005; Pickup et al., 2006). In addition, a shared pasture between heifers and adult cows may lead to increased contact of susceptible heifers with older animals, resulting in new heifer infections. The contact with adult cow manure would also be expected to be heightened, which is particularly concerning given the high environmental survival rate of MAP on pasture (93% survival per week, Marcé et al., 2011).

6.3 Testing Programs

Despite the many limitations associated with diagnostic testing (described in section 5), test-and-cull programs may be useful interventions for MAP-positive herds. Using stochastic simulations, Lu et al. (2010) uncovered a robust relationship between time to culling and likelihood of infection fadeout; however, it is likely not economically feasible to remove all infected animals (Collins et al., 2005), and culling of heavy shedders exclusively is not usually effective in completely eliminating MAP from a dairy herd (Lu et al., 2010; Slater et al., 2016). Thus, test-and-cull programs should not serve as a substitute for hygiene and proper management of indoor and outdoor housing environments.

Screening of cattle for MAP negativity before being introduced into the herd, or sourcing animals from MAP-negative farms is also critical in limiting the introduction of MAP bacteria. Wells and Wagner (2000) concluded that the proportion of cows introduced from other herds was strongly related to the odds of MAP infection. Similarly, Orpin et al., (2005) found that farms obtaining animals from multiple other herds had an increased chance of testing positive for MAP compared to farms purchasing animals from a single source.

6.4 Vaccination

For a variety of reasons, vaccination against MAP in dairy cattle has not been widely accepted as a preventative strategy. The approved North American MAP vaccine exhibits cross-reactivity with *Mycobacterium bovis* and may cause granuloma formation at the injection site (Kalis et al., 2001; Nedrow et al., 2007). In addition, it is impossible to differentiate vaccinated and truly infected animals; vaccination can thus an impediment to accurate serological testing and may interfere with national testing programs (Bastida and Juste, 2011). Although vaccination can successfully forestall clinical disease, some evidence suggests that it cannot eradicate infection entirely (Kalis et al., 2001). The proportion of silently infected animals may consequently increase, and MAP-positive cows may be retained for longer periods of time, arguably increasing overall herd prevalence and infectivity. Kalis et al. (2001) found no difference in fecal culture of vaccinated vs. control herds, but noted that the control herds were more likely to follow preventive management strategies. Despite these shortcomings in cattle, MAP vaccination has shown more promise in other ruminant species, such as young goats and sheep (Benedictus and Kalis, 2003; Reddacliff et al., 2006).

7. Mathematical Modeling of MAP

Due to imperfect test sensitivity and inconsistent bacterial shedding, extensive longitudinal studies are often necessary to accurately determine the infection statuses of animals in a dairy herd. Such studies may not always be practical based upon economics and resource requirements. Mathematical modeling is useful for exploring the infection dynamics of MAP under different scenarios that may not be feasible for real-world implementation. In 2008, Mitchell et al. developed a formative multi-group compartmental model of MAP transmission dynamics on dairy farms. The model was structured to include a variety of age classes and

categories of infection (transient, latent, low, and high shedding cows). Using this model as a stepping stone, several generations of models have been developed to assess the impact of control strategies on herd prevalence, such as culling infected animals (Lu et al., 2010) or implementing a vaccination protocol (Lu et al., 2013).

8. Research Needs

8.1 The Risk for MAP Infections in Organic vs. Conventional Dairy Operations

There is an absence of research evaluating Johne's disease risk factors on organic and conventional dairy farms in the United States. In conjunction with the increased consumer demand for organic products (Organic Trade Association, 2016), an extensive appraisal of the safety of organic management procedures is necessary. In addition, empirical research is required to place conventional herds implementing organic practices (such as pasture grazing) within the spectrum of risk. In keeping with our understanding of the infection dynamics of MAP bacteria, it seems reasonable to hypothesize that the interaction between certain risky management practices could have a synergistic effect upon the likelihood of new MAP infections. This interaction has not received much attention in recent literature and may provide a useful addition to control programs specifically tailored to production type (organic versus conventional).

In Chapter 2, using cross-sectional questionnaire data from 292 farms in 3 U.S. states, we aimed to develop a risk assessment model for new MAP infections between organic, conventional-grazing, and conventional non-grazing farms. We sought to determine whether a given production type demonstrated a higher risk of MAP transmission based upon common management decisions. We aimed to clarify the area of greatest risk by dividing the risk factors into categories (such as calving area management, pre-weaned calf management, and post-weaned heifer management.) Each management practice was weighted by risk potential

according to the literature. We also explored whether certain farm types were more susceptible to a synergism of transmission risk resulting from the interaction between multiple management practices.

8.2 The Routes and the Risk of Bulk-milk Contamination

The high percentage of U.S. dairy operations with detectable levels of MAP in their on-farm environments (70%, see Lombard et al., 2013) demands the optimization of rapid diagnostics to evaluate the level of MAP contamination in the bulk-tank milk supply. This need is compounded by MAP's potential as a zoonotic organism (Over et al., 2011) and its ability to survive commercial pasteurization (Grant et al., 2005). Cross-sectional data is necessary to assess the overall prevalence of positive bulk tanks in U.S. dairy herds in addition to the level of contamination and potential associations with herd characteristics. Longitudinal data are required to determine whether bulk milk contamination presents a risk for low-prevalence herds over time.

The distinction between the internal and the environmental routes of contamination of bulk milk and their relative importance has not previously been described. An understanding of these routes is a prerequisite for a reduction of MAP CFU in the bulk milk supply. Since PCR tests are used to detect the causal organisms while ELISA identifies associated antibodies, these tests can be used in combination to pinpoint likely contamination mechanisms. High ELISA OD in milk is indicative of the presence of MAP antibodies and is associated with direct excretion of MAP into milk (Singh et al., 2007). ELISA OD values may only be indirectly linked to environmental contamination. Therefore, patterns of ELISA and PCR results can provide useful information regarding the ability of U.S. dairy operations to temper environmental-route contamination of bulk milk and maintain satisfactory on-farm hygiene. Studies addressing

concordance of PCR and ELISA in the bulk tank have predominately compared binary outcomes (such as Wilson et al., 2010); however, positive-negative ELISA classifications are not validated for bulk milk, since cutoffs are based upon thresholds for individual cows.

In Chapter 3, an optimized template preparation method for bulk-tank milk is presented, followed by an evaluation of commercial extraction and PCR kits targeting the *hspX* gene. Using these techniques, we determined the overall prevalence of MAP-positive bulk tanks from 292 dairy farms in 3 U.S. states. We compared continuous-scale ELISA results with PCR results from the same farm, using logistic and linear regression to gain insight into the routes of contamination. In Chapter 4, we tracked the level of bulk tank contamination in 2 low-prevalence herds over time using both ELISA and PCR tests. We also considered the contribution of the environmental and internal routes to MAP contamination of individual milk samples.

8.3 Relationship between Diagnostic Outcomes in Known MAP-positive Cows

Certain facets of the relationship between diagnostic testing outcomes for MAP have not yet been explored and require repeated measurements from known MAP-infected cattle. Much of the research pertaining to diagnostics for MAP has been cross-sectional and evaluates the level of agreement of 2 dichotomous outcomes (e.g., Pinedo et al., 2008). These approaches, although meaningful, may not account for subtleties in the association between diagnostic tests, as there is an information loss inherent in dichotomization. Statistical mixed models are valuable tools for studying these associations: multiple, continuous-scale diagnostic tests can be taken into consideration while adjusting for variables (such as individual cow and herd characteristics) that may impact the interrelationships under study. In addition, there is new information pertaining to the shedding levels of infected animals: the insight regarding infection Progressors and Non-

progressors should be included in statistical models to fully grasp the association between diagnostic tests.

In Chapter 4, we obtained frequent samples from 14 MAP-infected cows in 2 low-prevalence dairy herds to elucidate longitudinal associations between milk ELISA, milk PCR, fecal PCR, and fecal culture. Where possible, we have considered these variables on a continuous scale and adjusted for subject, herd, and cow characteristics. In this chapter, we aimed to understand the temporal relationship between fecal shedding and antibody titer and shed light on potential predictive abilities.

8.4 Mathematical Modeling: the Dairy-farm Environment and the Bulk Tank.

Current mathematical models for MAP must be updated to include new and unexplored insights into infection dynamics. The vast majority of MAP infection models in dairy herds have not considered indirect transmission via a contaminated environment (see Marcé et al., 2010), despite the long-term survival of MAP outside of its host (Lovell et al., 1944) and the documented importance of fecal-oral transmission (Doré et al., 2012; Whittington and Windsor, 2009). The recent discovery of a non-linear relationship between infectivity and environmental MAP load (Slater et al., 2016) has provided key information regarding the risk of this environmental transmission.

Additionally, existing models have generally assumed age-acquired resistance to infection (as in Mitchell et al., 2008; Lu et al., 2010). Recently, there has been evidence of newly-acquired infection in adult cows, which was documented using longitudinal data (Schukken et al., 2015). Mathematical models should therefore be modified to include adult-adult transmission. Finally, milk-quality models measuring the level of MAP contamination in the bulk tank are typically presented separately, that is, distinct from within-herd transmission

models. A comprehensive model including both transmission and milk contamination is necessary to understand the intricate relationship between herd prevalence, environmental burden, direct shedding into milk, and the force of infection.

In Chapter 5, we developed a multi-group compartmental mathematical model with stochastic elements to study the infection dynamics of MAP in a representative U.S. dairy herd. We included the previously unexplored dimensions of adult cow infection, non-linear environmental burden, and internal and environmental contamination of the bulk-tank milk supply. To parametrize this model, we made use of data from the cross-sectional and longitudinal studies described in Chapters 2 to 4 in addition to published literature. We explored factors influencing bulk tank MAP load, environmental burden, and prevalence, using simulations in addition to sensitivity and scenario analyses. We aimed to identify key intervention strategies to lower the concentration of MAP in the bulk tank while understanding the relationship of these interventions to herd prevalence and environmental burden.

9. Conclusion

The overall objective of this dissertation is to address and fulfill the aforementioned research needs. We aimed to accomplish this goal through the implementation of field studies, laboratory methods, and statistical and mathematical data-analysis techniques. The chapters presented in this work address the significance of bulk-milk contamination routes, MAP transmission risks, intervention strategies, and the relationship between diagnostic tests. The results may be useful in refining testing schemes and improving milk-quality initiatives.

10. References

Animal Health Australia. 2016. Three Step Calf Rearing Plan. Available at: https://www.animalhealthaustralia.com.au/what-we-do/endemic-disease/johnes-disease/jd-and-dairy-cattle/three-step-calf-rearing-plan/ Accessed September 18, 2017.

Ansari-Lari, M., M. Haghkhah, A. Bahramy, and A. M. N. Baheran. 2009. Risk factors for *Mycobacterium avium* subsp. *paratuberculosis* in Fars Province (southern Iran) dairy herds. Trop. Anim. Health Prod. 41:553–557.

Bastida, F., and R. A. Juste. 2011. Paratuberculosis control: A review with a focus on vaccination. J. Immune Based Ther. Vaccines. 9:8–24.

Beard, P. M., M. J. Daniels, D. Henderson, A. Pirie, K. Rudge, D. Buxton, S. Rhind, A. Greig, M. R. Hutchings, I. McKendrick, K. Stevenson, and J. M. Sharp. 2001. Paratuberculosis infection of nonruminant wildlife in scotland. J. Clin. Microbiol. 39:1517–1521.

Benedictus, G., and C. Kalis. 2003. Paratuberculosis: Eradication, control and diagnostic methods. Acta Vet. Scand. 44:231–241.

Cazer, C. L., R. M. Mitchell, K. M. Cicconi-Hogan, M. Gamroth, R.M. Richert, P. L. Ruegg, and Y. H. Schukken. 2013. Associations between *Mycobacterium avium* subsp. *paratuberculosis* antibodies in bulk tank milk, season of sampling and protocols for managing infected cows. BMC Vet. Res. 9:234–240.

Chiodini, R. J., H. J. van Kruiningen, and R. S. Merkal. 1984. Ruminant paratuberculosis (Johne's disease): The current status and future prospects. Cornell Vet. 74:218–262.

Cho, J., L. W. Tauer, Y. H. Schukken, M. I. Gomez, R. L. Smith, Z. Lu, Y. T. Grohn. 2012. Economic analysis of *Mycobacterium avium* subsp. *paratuberculosis* vaccines in dairy herds. J. Dairy Sci. 95:1855–1872

Chen, P., M. Shi, G. D. Feng, J. Y. Liu, B. J. Wang, X. D. Shi, L. Ma, X. D. Liu, Y. N. Yang, W. Dai, T. T. Liu, Y. He, J. G. Li, X. K. Hao, and G. Zhao. 2012. A highly efficient Ziehl-Neelsen stain: Identifying de novo intracellular mycobacterium tuberculosis and improving detection of extracellular M. tuberculosis in cerebrospinal fluid. J. Clin. Microbiol. 50:1166–1170.

Clark, D., J. Koziczkowski, R. Radcliff, R. Carlson, and J. Ellingson. 2008. Detection of *Mycobacterium avium* subsp. *paratuberculosis*: Comparing fecal culture versus serum enzyme linked immunosorbent assay and direct fecal polymerase chain reaction. J. Dairy Sci. 91:2620–2627.

Collins, M. T. 1996. Diagnosis of paratuberculosis. Vet. Clin. N. Am. Food Anim. Pract. 12:357–371.

Collins, M. T. 2011. Diagnosis of paratuberculosis. Vet. Clin. N. Am. Food Anim. Pract. 27:581–591

Collins, M. T., S. J. Wells, K. R. Petrini, J. E. Collins, R. D. Schultz, and R. H. Whitlock. 2005.

Evaluation of five antibody detection tests for diagnosis of bovine paratuberculosis. Clin. Diagn. Lab. Immunol. 12:685–692.

Cosma, C. L., D. R. Sherman, and L. Ramakrishnan. 2003. The secret lives of the pathogenic mycobacteria. Annual Reviews in Microbiology. 57:641–676.

Donaghy, J., J. Johnston, and M. Rowe. 2011. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in cheese, milk powder and milk using IS900 and f57-based qPCR assays. J. Appl. Microbiol. 110:479–489.

Donaghy, J., M. Rowe, J. Rademaker, P. Hammer, L. Herman, V. De Jonghe, B. Blanchard, K. Duhem and E. Vindel. 2008. An inter-laboratory ring trial for the detection and isolation of *Mycobacterium avium* subsp. *paratuberculosis* from raw milk artificially contaminated with naturally infected faeces. Food Microbiol. 25:128–135.

Doré, E., J. Paré, G. Côté, S. Buczinski, O. Labrecque, J. Roy, and G. Fecteau. 2012. Risk factors associated with transmission of *Mycobacterium avium* subsp. *paratuberculosis* to calves within dairy herd: A systematic review. J. Vet. Intern. Med. 26:32–45.

Eisenberg, S. W., R. Chuchaisangrat, M. Nielen, and A. P. Koets. 2013. Relationship between presence of cows with milk positive for *Mycobacterium avium* subsp. *paratuberculosis*-specific antibody by enzyme-linked immunosorbent assay and viable *M. avium* subsp. *paratuberculosis* in dust in cattle barns. Appl. Environ. Microbiol. 79:5458–5464.

Eltholth, M., V. Marsh, S. Van Winden, and F. Guitian, 2009. Contamination of food products with *Mycobacterium avium paratuberculosis*: A systematic review. J. Appl. Microbiol. 107: 1061–1071.

Feller, M., K. Huwiler, R. Stephan, E. Altpeter, A. Shang, H. Furrer, G. E. Pfyffer, T. Jemmi, A. Baumgartner, and M. Egger. 2007. *Mycobacterium avium* subsp. *paratuberculosis* and Crohn's disease: A systematic review and meta-analysis. Lancet Infect. Dis.7:607–613.

Grant, I.R., A.C. Foddai, J.C. Tarrant, B. Kunkel, F.A. Hartmann, S. McGuirk, C. Hansen, A.M. Talaat, and M.T. Collins. 2017. Viable *Mycobacterium avium* subsp. *paratuberculosis* isolated from calf milk replacer. J Dairy Sci. 100:9723–9735

Grant, I. R., A. G. Williams, M. T. Rowe, and D. D. Muir. 2005. Efficacy of various pasteurization time-temperature conditions in combination with homogenization on inactivation of *Mycobacterium avium* subsp. *paratuberculosis* in milk. Appl. Environ. Microbiol. 71:2853–2861.

Herthnek, D., S. S Nielsen, A. Lindberg, and G. Bolske. 2008. A robust method for bacterial lysis and DNA purification to be used with real-time PCR for detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk. J. Microbiol. Methods 75:335–340.

Hussain, T., S. Z. A. Shah, D. Zhao, S. Sreevatsan and X. Zhou. 2016. The role of IL-10 in *Mycobacterium avium* subsp. *paratuberculosis* infection. Cell Communication and Signaling. 14:29–42.

Jorgensen, J. B. 1977. Survival of *Mycobacterium paratuberculosis* in slurry. Nord. Vet. Med. 29:267–270.

Kalis, C. H., J. W. Hesselink, H. W. Barkema, and M. T. Collins. 2001. Use of long-term vaccination with a killed vaccine to prevent fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* in dairy herds. Am. J. Vet. Res. 62:270–274.

Khare, S., T. A. Ficht, R. L. Santos, J. Romano, A. R. Ficht, S. Zhang, I. R. Grant, M. Libal, D. Hunter, and L. G. Adams. 2004. Rapid and sensitive detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine milk and feces by a combination of immunomagnetic bead separation-conventional PCR and real-time PCR. J. Clin. Microbiol. 42:1075–1081.

Koch, M. L., and R. A. Cote. 1965. Comparison of fluorescence microscopy with Ziehl-Neelsen stain for demonstration of acid-fast bacilli in smear preparations and tissue sections. Am. Rev. Respir. Dis. 91:283–284.

Koets, A., W. Santema, H. Mertens, D. Oostenrijk, M. Keestra, M. Overdijk, R. Labouriau, P. Franken, A. Frijters, and M. Nielen. 2010. Susceptibility to paratuberculosis infection in cattle is associated with single nucleotide polymorphisms in toll-like receptor 2 which modulate immune responses against *Mycobacterium avium* subsp. *paratuberculosis*. Prev. Vet. Med. 93:305–315.

Kuenstner, J.T., S. Naser, W. Chamberlin, T. Borody, D.Y. Graham, A. McNees, J. Hermon-Taylor, A. Hermon-Taylor, C.T. Dow, W. Thayer, J. Biesecker, et al. 2017. The Consensus from the *Mycobacterium avium* subsp. *paratuberculosis* (MAP) Conference 2017. Front. Public Health. 5:208–212.

Lambert, P. 2002. Cellular impermeability and uptake of biocides and antibiotics in Gram-positive bacteria and mycobacteria. J. Appl. Microbiol. 92:46S–54S.

Li, L., J. P. Bannantine, Q. Zhang, A. Amonsin, B. J. May, D. Alt, N. Banerji, S. Kanjilal, and V. Kapur. 2005. The complete genome sequence of *Mycobacterium avium* subsp. *paratuberculosis*. Proc. Natl. Acad. Sci. USA 102:12344–12349.

Lombard, J. E. 2011. Epidemiology and economics of paratuberculosis. Vet. Clin. North Am. Food Anim. Pract. 27:525–535.

Lombard, J. E., I. A. Gardner, S. R. Jafarzadeh, C. P. Fossler, B. Harris, R. T. Capsel, B. A. Wagner, and W. O. Johnson. 2013. Herd-level prevalence of *Mycobacterium avium* subsp. *paratuberculosis* infection in United States dairy herds in 2007. Prev.Vet. Med. 108:234–238.

Lu, Z., Y. H. Schukken, R. L. Smith, and Y. T. Grohn. 2010. Stochastic simulations of a multi-group compartmental model for Johne's disease on US dairy herds with test-based culling intervention. J. Theor. Biol. 264:1190–1201.

Lu, Z., Y. H. Schukken, R. L. Smith, R. M. Mitchell, and Y. T. Gröhn. 2013. Impact of imperfect *Mycobacterium avium* subsp. *paratuberculosis* vaccines in dairy herds: A mathematical modeling approach. Prev. Vet. Med. 108:148–158.

Magombedze, G., T. Shiri, S. Eda, and J.R. Stabel. 2017. Inferring biomarkers for *Mycobacterium avium* subsp. *paratuberculosis* infection and disease progression in cattle using experimental data. Sci. Rep. 7:1–15

Manning, E. J., and M. T. Collins. 2010. Epidemiology of paratuberculosis. Paratuberculosis: Organism, Disease, Control. 22–28.

Marcé, C., P. Ezanno, H. Seegers, D. Pfeiffer, and C. Fourichon. 2011. Within-herd contact structure and transmission of *Mycobacterium avium* subsp. *paratuberculosis* in a persistently infected dairy cattle herd. Prev. Vet. Med. 100:116–125.

Marcé, C., P. Ezanno, M. Weber, H. Seegers, D. Pfeiffer, and C. Fourichon. 2010. Invited review: Modeling within-herd transmission of *Mycobacterium avium* subsp. *paratuberculosis* in dairy cattle: A review. J. Dairy Sci. 93: 4455–4470.

McAloon, C. G., P. Whyte, S. J. More, L. O'Grady, and M. L. Doherty. 2015. Development of a HACCP-based approach to control paratuberculosis in infected Irish dairy herds. Prev. Vet. Med. 120:152–161.

McNab, W. B., A. H. Meek, J. R. Duncan, B. W. Brooks, A. A. Van Dreumel, S. W. Martin, K. H. Nielsen, E. A. Sugden and C. Turcotte. 1991. An evaluation of selected screening tests for bovine paratuberculosis. Can. J. Vet. Res. 55:252–259.

Mitchell, R., R. Whitlock, S. Stehman, A. Benedictus, P. Chapagain, Y. Grohn, and Y. Schukken, 2008. Simulation modeling to evaluate the persistence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) on commercial dairy farms in the United States. Prev. Vet. Med. 83, 360–380.

Mortensen, H., S. S. Nielsen, and P. Berg. 2004. Genetic variation and heritability of the antibody response to *Mycobacterium avium* subsp. *paratuberculosis* in Danish Holstein cows. J. Dairy Sci. 87:2108–2113.

Muskens, J., H. Barkema, E. Russchen, K. Van Maanen, Y. Schukken, and D. Bakker. 2000. Prevalence and regional distribution of paratuberculosis in dairy herds in the Netherlands. Vet. Microbiol. 77:253–261.

Muskens, J., A. Elbers, H. van Weering, and J. Noordhuizen. 2003. Herd management practices

associated with paratuberculosis seroprevalence in Dutch dairy herds. J. Vet. Med. B. Infect. Dis. Wet. Public Health 50:372–377.

Nedrow, A., J. Gavalchin, M. Smith, S. Stehman, J. Maul, S. McDonough, and M. Thonney. 2007. Antibody and skin-test responses of sheep vaccinated against Johne's disease. Vet. Immunol. Immunopathol. 116:109-112.

Niederweis, M., O. Danilchanka, J. Huff, C. Hoffmann, and H. Engelhardt. 2010. Mycobacterial outer membranes: In search of proteins. Trends Microbiol. 18:109–116.

Nielsen, S. S., H. Bjerre, and N. Toft. 2008. Colostrum and milk as risk factors for infection with *Mycobacterium avium* subsp. *paratuberculosis* in dairy cattle. J. Dairy Sci. 91:4610–4615.

Nielsen, S. S., and N. Toft. 2009. A review of prevalences of paratuberculosis in farmed animals in Europe. Prev. Vet. Med. 88:1–14.

Obasanjo, I. O., Y. Grohn, and H. O. Mohammed. 1997. Farm factors associated with the presence of *Mycobacterium paratuberculosis* infection in dairy herds on the New York state paratuberculosis control program. Prev. Vet. Med. 32:243–251.

Olsen, I., Ó. Sigurðardóttir, and B. Djønne. 2002. Paratuberculosis with special reference to cattle. A review. Vet. Q. 24:12–28.

Organic Trade Association. 2016. U.S. Organic: State of the industry. Available at: http://ota.com/sites/default/files/indexed_files/OTA_StateofIndustry_2016.pdf Accessed Apr. 2, 2016.

Orpin, P., S. Duthie, and D. H. Grove-White. 2005. The use of targeted sampling and risk factor analysis to investigate the presence of Johne's disease in dairy herds. Cattle Pract. 13:219–225.

Ott, S. L., S. J. Wells, and B. A. Wagner. 1999. Herd-level economic losses associated with Johne's disease on US dairy operations. Prev. Vet. Med. 40:179–192.

Over, K., P. G. Crandall, C. A. O'Bryan, and S. C. Ricke. 2011. Current perspectives on *Mycobacterium avium* subsp. *paratuberculosis*, Johne's disease, and Crohn's disease: A review. Crit. Rev. Microbiol. 37:141–156.

Pickup, R. W., G. Rhodes, T. J. Bull, S. Arnott, K. Sidi-Boumedine, M. Hurley, and J. Hermon-Taylor. 2006. *Mycobacterium avium* subsp. *paratuberculosis* in lake catchments, in river water abstracted for domestic use, and in effluent from domestic sewage treatment works: Diverse opportunities for environmental cycling and human exposure. Appl. Environ. Microbiol. 72:4067–4077.

Pinedo, P. J., C. D. Buergelt, G. A. Donovan, P. Melendez, L. Morel, R. Wu, T. Y. Langaee, and

D. O. Rae. 2009. Candidate gene polymorphisms (BoIFNG, TLR4, SLC11A1) as risk factors for paratuberculosis infection in cattle. Prev. Vet. Med. 91:189–196.

Pinedo, P. J., D. Rae, J. Williams, G. Donovan, P. Melendez, and C. Buergelt. 2008. Association among results of serum ELISA, faecal culture and nested PCR on milk, blood and faeces for the detection of paratuberculosis in dairy cows. Transbound. Emerg. Dis. 55:125–133.

Pithua, P., S. J. Wells, and S. M. Godden. 2011. Evaluation of the association between fecal excretion of *Mycobacterium avium* subsp *paratuberculosis* and detection in colostrum and on teat skin surfaces of dairy cows. J. Am. Vet. Med. Assoc. 238:94–100.

Raizman, E. A., S. Wells, S. Godden, R. Bey, M. Oakes, D. Bentley, and K. Olsen. 2004. The distribution of *Mycobacterium avium* subsp. *paratuberculosis* in the environment surrounding Minnesota dairy farms. J. Dairy Sci. 87:2959–2966.

Reddacliff, L., K. Beh, H. McGregor, and R. Whittington. 2005. A preliminary study of possible genetic influences on the susceptibility of sheep to johne's disease. Aust. Vet. J. 83:435–441.

Reddacliff, L., J. Eppleston, P. Windsor, R. Whittington, and S. Jones.2006. Efficacy of a killed vaccine for the control of paratuberculosis in Australian sheep flocks. Vet. Microbiol. 115:77–90.

Richards, W. D., and C. O. Thoen. 1977. Effect of freezing on the viability of mycobacterium paratuberculosis in bovine feces. J. Clin. Microbiol. 6:392–395.

Schukken, Y. H., R. H. Whitlock, D. Wolfgang, Y. T. Grohn, A. Beaver, J. VanKessel, M. Zurakowski, and R. Mitchell. 2015. Longitudinal data collection of *Mycobacterium avium* subsp. *paratuberculosis* infections in dairy herds: The value of precise field data. Vet. Res. 46:65–75.

Singh, S. V., A. V. Singh, A. Kumar, P. K. Singh, R. Deb, A. K. Verma, A. Kumar, R. Tiwari, S. Chakraborty, and K. Dhama. 2013. Survival mechanisms of mycobacterium avium subspecies paratuberculosis within host species and in the environment—a review. Natural Science. 5:710–723.

Slater, N., R. M. Mitchell, R. H. Whitlock, T. Fyock, A. K. Pradhan, E. Knupfer, Y. H. Schukken, and Y Louzoun. 2016. Impact of the shedding level on transmission of persistent infections in *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Vet. Res. 47:38–50.

Smith, R., R. Strawderman, Y. Schukken, S. Wells, A. Pradhan, L. Espejo, R. Whitlock, J. Van Kessel, J. Smith, and D. Wolfgang. 2010. Effect of Johne's disease status on reproduction and culling in dairy cattle. J. Dairy Sci. 93: 3513–3524.

Sockett, D. C., D. J. Carr, W. D. Richards, and M. T. Collins. 1992. A repository of specimens

for comparison of diagnostic testing procedures for bovine paratuberculosis. Journal of Veterinary Diagnostic Investigation. 4:188-191.

Stabel, J.R. 2006. Host responses to *Mycobacterium avium* subsp. *paratuberculosis*: a complex arsenal. Anim. Health Res. Rev.7:61–70.

Stabel, J.R. 2000. Transitions in immune responses to *Mycobacterium paratuberculosis*. Vet. Microbiol. 77:465–473.

Stabel, J. R. 1997. An improved method for cultivation of *Mycobacterium paratuberculosis* from bovine fecal samples and comparison to three other methods. Journal of Veterinary Diagnostic Investigation. 9:375–380.

Stabel, J. R., S. J. Wells, and B. A. Wagner. 2002. Relationships between fecal culture, ELISA, and bulk tank milk test results for Johne's disease in US dairy herds. J. Dairy Sci. 85:525–531.

Stabel, J. R., and J. P. Bannantine. 2005. Development of a nested PCR method targeting a unique multicopy element, ISMap02, for detection of *Mycobacterium avium* subsp. *paratuberculosis* in fecal samples. J. Clin. Microbiol. 43:4744–4750.

Stanley, E. C., R. J. Mole, R. J. Smith, S. M. Glenn, M. R. Barer, M. McGowan, and C. E. Rees. 2007. Development of a new, combined rapid method using phage and PCR for detection and identification of viable *Mycobacterium paratuberculosis* bacteria within 48 hours. Appl. Environ. Microbiol. 73:1851–1857.

Streeter, R. N., G. F. Hoffsis, S. Bech-Nielsen, W. P. Shulaw, and D. M. Rings. 1995. Isolation of *Mycobacterium paratuberculosis* from colostrum and milk of subclinically infected cows. Am. J. Vet. Res. 56:1322–1324.

Sweeney, R. W. 2011. Pathogenesis of paratuberculosis. Vet. Clin. North Am. Food Anim. Pract. 27:537–46

Sweeney, R. W., R. H. Whitlock, and A. E. Rosenberger. 1992. *Mycobacterium paratuberculosis* cultured from milk and supramammary lymph nodes of infected asymptomatic cows. J. Clin. Microbiol. 30:166–171.

Tessema, M., A. Koets, V. Rutten, and E. Gruys. 2001. Bacteriology: Review paratuberculosis: How does *Mycobacterium avium* subsp. *paratuberculosis* resist intracellular degradation? Vet. Q. 23:153–162.

Tiwari, A., J. A. VanLeeuwen, S. L. McKenna, G. P. Keefe, and H. W. Barkema. 2006. Johne's disease in canada part I: Clinical symptoms, pathophysiology, diagnosis, and prevalence in dairy herds. Can. Vet. J. 47:874–882.

USDA Animal and Plant Health Inspection Service. 2010. Uniform Program Standards for the

Voluntary Bovine Johne's Disease Control Program. Available at: https://www.aphis.usda.gov/animal_health/animal_diseases/johnes/downloads/johnes-ups.pdf. Accessed July 21, 2017.

van Schaik, G., Y. H. Schukken, C. Crainiceanu, J. Muskens, and J. A. VanLeeuwen. 2003. Prevalence estimates for paratuberculosis adjusted for test variability using Bayesian analysis. Prev. Vet. Med. 60:281–295.

Wells, S. J., and B. A. Wagner. 2000. Herd-level risk factors for infection with *Mycobacterium paratuberculosis* in US dairies and association between familiarity of the herd manager with the disease or prior diagnosis of the disease in that herd and use of preventive measures. J. Am. Vet. Med. Assoc. 216:1450–1457.

Weiss, D., and C. Souza. 2008. Modulation of mononuclear phagocyte function by *Mycobacterium avium* subsp. *paratuberculosis*. Vet. Pathol. 45:829–841.

Whitlock, R. H., S. Wells, R. W. Sweeney, and J. Van Tiem. 2000. ELISA and fecal culture for paratuberculosis (Johne's disease): Sensitivity and specificity of each method. Vet. Microbiol. 77:387–398.

Whitlock, R. H., and C. Buergelt. 1996. Preclinical and clinical manifestations of paratuberculosis (including pathology). Veterinary Clinics of North America: Food Animal Practice 12, 345–356.

Whittington, R. J., D. J. Marshall, P. J. Nicholls, I. B. Marsh, and L. A. Reddacliff. 2004. Survival and dormancy of *Mycobacterium avium* subsp. *paratuberculosis* in the environment. Appl. Environ. Microbiol., 70:2989–3004.

Whittington, R. J., I. B. Marsh, and L. A. Reddacliff. 2005. Survival of *Mycobacterium avium* subsp. *paratuberculosis* in dam water and sediment. Appl. Environ. Microbiol. 71:5304–5308.

Whittington, R. J., and P. A. Windsor. 2009. In utero infection of cattle with *Mycobacterium avium* subsp. *paratuberculosis*: A critical review and meta-analysis. Vet. J. 179: 60–69.

Wilson, D. J., K. Rood, P. Biswas, and T. M. Byrem. 2010. Herd-level prevalence of Johne's disease in Utah and adjacent areas of the intermountain west as detected by a bulk-tank milk surveillance project. J. Dairy Sci. 93:5792–5797.

CHAPTER 2:
COMPARATIVE RISK ASSESSMENT FOR NEW COW-LEVEL *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* INFECTIONS BETWEEN 3 DAIRY PRODUCTION TYPES: ORGANIC, CONVENTIONAL, AND CONVENTIONAL-GRAZING SYSTEMS

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KEYWORDS: Johne's disease, organic management, conventional management, production type

ABSTRACT

Johne's disease, a granulomatous enteritis of ruminant animals, is a hidden threat on dairy farms, adversely impacting animal welfare as well as herd productivity. Control programs in the U.S. advocate for specific management practices to temper the spread of the causal organism (*Mycobacterium avium* subsp. *paratuberculosis* (MAP)), such as improving calving area hygiene and limiting introduction of replacement stock with unknown infection status. There remains a need for direct exploration of Johne's disease prevention strategies in the U.S. with respect to production type. Alongside the growing demand for organic products, the safety of organic dairy practices with respect to MAP control is warranted. Furthermore, conventional herds employing organic practices such as pasture grazing should be situated within the risk spectrum.

We developed a risk assessment model using the U.S. Voluntary Bovine Johne's Disease Control Program as a framework, with the goal of evaluating the risk of new cow-level MAP infections. A total of 292 organic and conventional farms in 3 states were surveyed on management practices, and an overall analysis was conducted in which each farm was first scored on individual practices using a range of "no risk" to "high risk," according to the literature. The sum of all risk factors was then analyzed to quantify and compare the risk burden for each production type. Organic herds received higher overall risk scores compared to both conventional grazing and non-grazing subtypes.

In order to identify which factors contributed to the overall increased risk for organic herds, the management practices were categorized and evaluated by logistic regression. We determined that the increased risk incurred by organic herds was predominantly due to decisions made in the calving area and pre-weaned calf group. However, while certain individual risk

factors related to calf management are commonly involved in prevention strategies (e.g. cow/calf separation), and were thus included in the overall risk assessment, empirical evidence linking them to the spread of MAP is lacking. Instead, these factors are problematic when executed with other management decisions, leading to a hypothesized synergism of transmission risk.

To this end, we developed a set of compound risk factors, which were also evaluated as outcomes in logistic regression models, with production type serving as the predictor of interest. Organic farms in our study were more susceptible to risks associated with the synergism of study variables. Notably, organic producers were most likely to allow calves to spend extended time with the dam while also lacking a dedicated calving area. Additionally, calves in organic herds were more often permitted to nurse even with poor udder hygiene on farm. A heightened vigilance towards calving area hygiene is therefore indicated for these herds.

1. Introduction

1.1. Johne's disease: cause, implications, and management initiatives

Johne's disease, which primarily affects cattle and other ruminant species, is a chronic infection of the gastrointestinal tract caused by the bacterium *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Calves under 6 months are most susceptible to MAP infection (Lombard, 2011). The disease is characterized by a prolonged incubation period (typically 1-6 years) prior to the onset of clinical signs, during which infected yet asymptomatic animals may shed MAP bacteria into manure and milk. Initial signs, such as decreased milk production, low fertility, reduced body condition, or roughening of the hair coat, may be subtle, and animals are often culled from dairy herds before the occurrence of clinical disease. Clinical signs are

progressive and typically manifest as weight loss and diarrhea, eventually leading to severe dehydration and cachexia (Whitlock and Buergelt., 1996; Manning and Collins, 2001).

The primary means of MAP transmission is the fecal-oral route. In particular, calf contact with manure from adult cows presents an unparalleled risk for vertical MAP transmission (Doré et al., 2012). MAP shed into manure may lead to environmental contamination and subsequent contamination of teats and udders; the milk or colostrum of uninfected animals may thus become adulterated. MAP may also be shed directly into the milk and colostrum of infected animals (Sweeney et al., 1992). Unpasteurized sources of milk present a risk to calves, yet MAP has also been shown to survive pasteurization at initial concentrations exceeding 10^4 cells/L (Grant et al., 2005). Intrauterine transmission is also possible, although it does not appear to play a major role in the spread of MAP (Mortensen et al., 2004).

The presence of MAP may be detected in serum, milk, or fecal samples from individual animals via ELISA (detection of MAP antibodies), PCR, or culture methods (detection of the causal organism) (Wilson et al., 2010). More recently, ELISA and PCR techniques have been applied to bulk-tank milk to evaluate MAP presence and prevalence at the herd level (Cazer et al., 2013; Beaver et al., 2016; Slana et al., 2009; van Weering et al., 2007). The dairy industry suffers substantial economic losses due to MAP, based on factors such as increase in overall cull rate and replacement costs, decreased revenue from lower milk production, and increased veterinary expenses. Ott et al. (1999) estimated that lost productivity as a result of Johne's disease is responsible for an annual 200 to 250 million dollar loss for the U.S. dairy industry. From a human-health standpoint, on-farm risk mitigation is also a valuable initiative, since MAP has been implicated as a potential cause of human Crohn's disease (see Feller et al., 2007). In the U.S., dairy producers may choose to participate in the Voluntary Bovine Johne's Disease Control

Program (VBJDCP). The program focuses on controlling the spread of MAP between and within herds, and has been in effect since 1999. A crucial part of VBJDCP is identifying and implementing preventative strategies focused on calf management, curtailing environmental contamination, and restricting introduction of potentially infected animals into the herd.

1.2. The Relationship between Johne's disease Risk Factors and Production Type

Key research has been conducted to address differences between organic and conventional farming, but the focus has typically been on economic and environmental consequences (Pieper et al., 2014). However, several studies have dealt with management implications related to disease prevention. Stiglbauer et al. (2013) compared management on organic and conventional farms and concluded that outside resources (such as nutritionists and veterinarians) were less-often employed on organic farms, and vaccinations were less-often administered. Richert et al. (2013) investigated perceptions and definitions of mastitis, ketosis, and pneumonia between conventional and organic herds, in addition to rate of recorded cases. Disease perceptions were largely similar between the production types and clearly influenced the rate of farmer-identified cases.

The safety of organic practices with respect to Johne's disease merits extensive evaluation, particularly in light of the dramatic increase in consumer demand for organic products over the last decade (Organic Trade Association). Few studies have sought to compare management practices of organic and conventional farm types in a risk-assessment type analysis. According to Zwald et al. (2004), conventional farms were more likely to report positive a Johne's diagnosis in their herds (48.5%, compared to 25% of organic farms). However, the basis of these diagnoses is not described further, and the herd-sizes of the conventional farms tended to be larger. Pieper et al. (2014) reviewed requirements for organic farming in Canada and

developed a conceptual analysis regarding the impact of these practices on Johne's disease transmission. The authors acknowledged the necessity for empirical research, not only with regard to organic farms, but also for conventional farms implementing relevant organic practices.

In the present large-scale study of 292 farms, we review the management practices from 3 types of U.S. dairy production systems in light of the risk of new cow-level MAP infections. The production types considered are organic (ORG), conventional non-grazing (CON-NG), and conventional grazing (CON-GR). This is the first study to evaluate Johne's risk factors with respect to production type and grazing on U.S. dairy farms, and the study design included herd-size matching and location matching from 3 regions of the U.S.

2. Materials and Methods

2.1. Questionnaire

The questionnaire results were obtained from a multi-institutional study of 292 organic and conventional farms from 3 states of the USA: Oregon, New York, and Wisconsin. Herd recruitment has been described previously (Bergman et al., 2014; Stiglbauer et al., 2013; Ciconni-Hogan et al., 2013; Richert et al., 2013). In summary, farms with ≥ 20 cows having sold milk commercially for ≥ 2 years were eligible for inclusion. To be classified as an organic herd, farms must have shipped certified organic milk for ≥ 2 years prior to study enrollment. Conventional herds were size-matched to organic herds based upon the total number of lactating and dry cows, divided into 4 categories (<100, 100-199, 200- 299, and ≥ 300). The number of conventional herds in each state was selected based on organic-to-conventional state ratios. In total, 97 farms were selected from NY, 48 from OR and 147 from WI, with organic-to-conventional ratios of 3:1 for NY, 1:1 for OR, and 2:1 for WI. For the analysis, conventional farms were divided into non-grazing (CON-NG, n= 64) and grazing (CON-GR, n= 36) for

comparison to organic (ORG, n= 192) farms. CON-GR herds were defined as those farms with at least 1 month per year of daily access to a pasture providing ≥ 30% of dry matter intake.

The questionnaire has been described in detail by Richert et al. (2013) and is available online at http://milkquality.wisc.edu/organic-dairies/project-c-o-w/. A herd visit was conducted at each farm, and questionnaires were administered at the time of the visit. The questions concerned implementation of on-farm management procedures, and included several direct questions relating to Johne's disease status, Johne's testing, and Johne's management protocols. Additionally, during these herd visits, udder hygiene was scored on each farm (for all animals in herds with ≤ 50 cows, and for a representative sample of 20% of cows for larger herds) using a scoring system outlined by Schreiner and Ruegg (2003).

2.2. Variable Selection

Variables from the questionnaire were selected based upon potential association with MAP prevalence and organized according to the nature of the risk, using the handbook for the United States Voluntary Bovine Johne's Disease Control Program (VBJDCP) as a framework. The management practices in our study were divided into 2 major categories: Within-herd Transmission Risk and Introduction Risk. Within-herd Transmission was further partitioned into the subcategories *Calving Area*, *Pre-weaned Calf Group*, and *Post-weaned Heifer Group*. Introduction Risk was defined based upon categories of *Additions and Replacement* stock.

As outlined in the VBJDCP manual, the risk in the *Calving Area* is assessed based upon the potential for neonatal calves to ingest manure from mature cows. We categorized the type of calving area on each farm according to several parameters, including whether a dedicated calving area was used, and if not, whether sick animals or other lactating cows were permitted in the area. For calves nursing the dam, time spent in the calving area was recorded and categorized

into 3 time frames: immediate removal, between 1 and 6 hours of contact, and ≥ 6 hours of contact. The source of the colostrum was classified as fresh or stored, and as single source or pooled. Finally, several hygiene precautions taken in the peri-parurient period were considered: checking fresh cows for dirt, udder clipping, and footbaths for both dry and fresh cows.

The risk for *Pre-weaned Calves* was also assessed based upon the potential for this group to ingest MAP-contaminated manure from adult cows. Producers were asked about their milk feeding practices (e.g., whether whole milk was fed, and whether the source was pasteurized.) Although not included in the VBJDCP manual, the housing of pre-weaned calves was incorporated into our risk assessment due to the findings that calves may begin shedding MAP in a short time period following infection (van Roermund et al., 2007; Mortier et al., 2014). We also considered the presence of calf scours and treatment protocols for scours cases. For *Post-Weaned Heifers*, we focused primarily on pasture movements (for ORG and CON-GR grazing systems), manure spreading, loader management, and primary water sources. Regarding the Introduction Risk, all categories of *Additions and Replacement* animals were considered: pre-weaned and weaned heifers, dairy cows, bulls, and other livestock. Additionally, sourcing these animals from multiple different farms was considered a risk factor.

As an auxiliary point of interest, we evaluated variables directly related to Johne's disease testing and management of known MAP-positive animals. Variables directly related to Johne's disease testing were not included in the main analysis due to a hypothesized increased likelihood that farms with MAP positive animals would conduct testing and participate in control programs. Further, the primary goal of the study was to evaluate the risk of new cow-level MAP infections. Lack of diagnostic testing cannot accurately be described as a transmission risk, and there is also some level of self-prediction inherent in using test results to predict new MAP

infections. However, these statistics are reported since they provide valuable supplementary information and have not previously been compared between U.S. production types.

Variables representing "compound risks" were generated by combining and dichotomizing the results from two risk factor variables if they were hypothesized to have a synergistic impact on the level of risk. Compound risks were assessed within and between the risk factor categories (e.g. *Calving Area Management* and *Additions/Replacement Group*). A summary of risk combinations follows. Farms permitting calves to nurse or allowing extended cow/calf contact (≥ 6 hours) were evaluated for an inclination to simultaneously conduct any of the following procedures: lacking a dedicated calving area, permitting sick or lactating cows in the calving area, failing to provide footbaths or clip udders of peri-parturient cows, having a poor average udder hygiene (> 2.5), failing to check fresh cows for dirt, and keeping Johne's positive animals until after calving. Several additional biologically-relevant combinations of these variables were also evaluated, such as lack of foobaths and lack of a dedicated calving area.

Herds permitting group calf housing were similarly evaluated for a propensity to allow new pre-weaned calves into the herd, and for the presence of treated or untreated scours. Farms allowing new dairy animals into the herd were assessed for: lack of a dedicated calving area or presence of lactating or sick cows in the area, pasture sharing between heifers and cows, spreading manure, and feeding of whole milk or waste milk to calves. The milk feeding was also evaluated alongside the bulk tank testing variable. Finally, the combination of surface water use and shared pasture between heifers and cows was tested.

2.3. Statistical Analyses

An overall risk assessment was conducted as follows: for each farm, key risk factors were individually scored on a scale of 1 to 3, with 1 representing low risk decisions, 2 representing

moderate risk, and 3 representing high risk. The number assignments for the risk factors and the rationale for the scoring decisions are provided in *Table 1*.

Table 1. *Assigned Risk Scores for the Overall Risk Assessment.* Practices that do not contribute to within-herd MAP proliferation or its introduction into the herd receive a score of 0. Low, medium, and high risks are assigned 1, 2, or 3, respectively. A score of 3 is given to practices with a direct relationship to the spread of MAP and a strong likelihood of occurrence given the presence of MAP. Supporting literature is referenced, with a summary of main conclusions.

Variable	*Score*	*Literature*	*Key conclusions*
Procedure for MAP+ Cows			
Cull immediately	0	Lu et al. (2010)	Strong relationship between time to culling and infection fadeout
Cull after calving	2		
Keep	3		
Calving Area			
Dedicated Calving area	0	Tiwari et al. (2009); Wells and Wagner (2000)	Presence of multiple cows in maternity associated with higher MAP prevalence; group housing for peri-parturient cows is risk factor for herd infection status
Separate from lactating cows	1		
With sick or lactating cows	3		
Average Udder Hygiene			
< 2.5	0	Pithua et al. (2011)	MAP strains on teat skin traced to sources other than the donor cow
≥ 2.5	1		
Check Fresh Cows for Dirt			
Yes	0	Ansari-Lari et al. (2009)	Dirt contamination of peri-parturient udders associated with higher odds of MAP + herd status
No	1		
Clip Udders			
Yes, dry cows	0	Elmoslemany et al. (2009)	Positive association between clipping udders and overall teat cleanliness
Yes, fresh cows	0		
Do not clip dry cows	1		
Do not clip fresh cows	1		
Use of Footbaths			
Yes, dry cows	0	Thomsen et al. (2012)	Automatic hoof washing results in improved hoof hygiene (thus reducing the amount manure tracked into the calving area)
Yes, fresh cows	0		
No footbaths for dry cows	1		
No footbaths for fresh cows	1		
Calves fed Stored Colostrum			
No	0	Nielsen et al. (2008); Stewart et al. (2005)	Calves fed colostrum from the dam had lower odds of positivity vs. calves fed pooled colostrum; buckets and containers represent contamination sources
Yes, from a single source	1		
Yes, from pooled sources	3		
Calves fed Unpasteurized Milk			
No	0	Grant et al. (2005); Muskens et al. (2003)	Pasteurization eradicates 10^4 MAP cfu/L; herd sero-negativity related to offering milk replacer.
Yes	3		
Calves Remain with dam			
No	0	Windsor and	Neonatal calves are most

1-5 hours	1	Whittington (2010)	susceptible to MAP; increased
≥ 6 hours	2	(vs. Johnson-Ifearulundu and Kaneene (1998))	risk of herd positivity with late cow-calf separation (although conflicting evidence is prevalent)
Entering Animals			
No entering animals	0	Wells and Wagner (2000)	The percentage of cows sourced from outside farms is related to the odds of MAP infection
Pre-weaned heifers	1		
Weaned heifers	1		
Dairy cattle	1		
Non-dairy cattle	1		
Bulls	1		
Other animals	1		
Source of Entering Animals			
Single farm	0	Orpin et al. (2005)	Farms purchasing from multiple herds have higher odds of testing positive compared to farms making single-herd purchases
Multiple farms	1		
Movement on Pasture			
Heifers do not share pasture	0	Marcé et al. (2011)	93% of MAP shed by infected cows persists on pasture each week; Contaminated pasture may result in new infections if younger animals are in contact with cow manure
Heifers share pasture with cows	2	Chiodini et al. (1984)	
Pre-weaned Calf Housing			
Calves never group housed	0	van Roermund et al. (2007); Windsor and Whittington (2010); Marcé et al. (2011) Wells and Wagner (2000)	Calves begin shedding in a short time period after infection; Calves are most susceptible to MAP; The longer calves spend in individual hutches, the lower the mean prevalence of infectious adults
Calves group housed for ≥ 1 Season	3		
Presence of Calf Scours			
No	0	Sorge et al. (2012)	Association between test-negative herds and a lower incidence of calf scours
Yes, ≥15% of cases treated	1		
Yes, < 15% of cases treated	2		
Same Loader, Feed & Manure			
No	0	Johnson-Ifearulundu and Kaneene (1998)	Reducing contact with contaminated equipment, feed, and manure, is crucial to prevent fecal-oral transmission to calves.
Yes	3		
Loader not washed/sanitized	+1		
Spreading Manure			
No	0	Obasanjo et al. (1997)	Spreading manure and harvesting resulting forage associated with risk of infection
Yes	1		
Water Source			
Municipal or Well water	0	Whittington et al. (2005) Pickup et al. (2006)	Surface water may be a significant reservoir for MAP; MAP enters surface water as runoff from contaminated pasture
Surface Water	2		

The risk scores for these variables were then tallied for each farm. A one-way ANOVA was conducted to evaluate the distribution of risk scores for the three farm types (ORG, CON-NG, and CON-GR). The omnibus test was followed up with Tukey HSD *post-hoc* comparisons. Additionally, ANOVA and Tukey HSD tests were conducted on each separate risk category.

In order to gain a more nuanced understanding of the results of the overall risk assessment, each individual risk factor was modeled as the outcome in a logistic regression, with *Production Type* (CON-NG, CON-GR, and ORG) as the predictor of interest. Due to the importance of *Herd Size* and *State* in the study design, these variables were included as additional predictors in all models. The *Herd Size* variable was represented as categorical (< 100, 100-200, or > 200). The general form of the logistic model may be expressed as:

$$\ln\left(\frac{Y}{1-Y}\right) = \alpha + \beta_1 Production_{ORG} + \beta_2 Production_{CON-GR} + \beta_3 Herd\ Size_{\ 100-200} + \beta_4 Herd\ Size_{\ >200} + \beta_5 State_{NY} + \beta_6 State_{OR}$$

where $\ln\left(\frac{Y}{1-Y}\right)$ represents the natural log of the odds for a given outcome variable, α is the intercept, and β_1 through β_6 represent the parameter estimates for the included predictors. Specifically, β_1 and β_2 are the slope estimates for the variable *Production Type*, with CON-NG serving as the reference level; β_3 and β_4 are the estimates for *Herd Size,* with a herd size of < 100 as the reference level, and β_5 and β_6 are the estimates for *State,* with WI as the reference level. As with the individual risk factors, the compound risks were modeled as outcomes in logistic regressions with production type, herd size, and state as predictor variables. If necessary, Firth's bias correction was employed for models that did not produce a reliable maximum likelihood estimate due to quasi-separation of data. Tukey HSD adjustments were made for the odds ratio comparisons. All statistical analyses were carried out using SAS (ver 9.4, SAS Institute, Cary, NC), and the figures were generated using GraphPad Prism (ver. 6, La Jolla, CA).

3. Results

3.1. Overall Risk Assessment

Risk scores for all farms ranged from 5 to 26, with a mean ± SD of 15.04 ± 4.13. The ANOVA yielded a significant result ($F_{(df = 2, 289)} = 14.60$, $P < 0.0001$). Specifically, Tukey HSD comparisons revealed differences in the mean risk scores between ORG and CON-GR herds ($P = 0.019$) and between ORG and CON-NG herds ($P < 0.0001$), with the higher score means, and thus higher risks, attributed to ORG herds. There was no significant difference in the risk scores between conventional subtypes. The results are presented graphically in *Figure 1*.

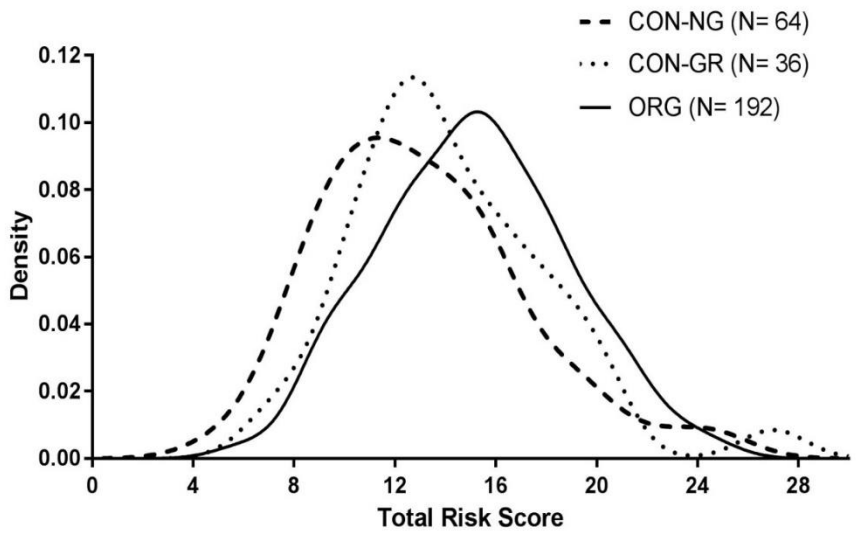

Figure 1. *Overall Risk Assessment. Figure 1* is a density plot depicting the relationship between *Production Type* (CON-GR, CON-NG, and ORG) and total risk score. Total Risk Score is shown on the X axis, and density of values on the Y axis. The 3 curves estimate the density for each production type.

When the overall risk assessment was subdivided into risk categories, ORG herds had higher mean risk scores than CON-NG and CON-GR in the *Calving Area* ($P = 0.013$ and $P = 0.014$, respectively) and in the *Pre-weaned Calf Group* ($P < 0.001$ and $P = 0.013$, respectively).

Additionally, CON-GR herds had higher risk scores than CON-NG herds in the *Pre-weaned Calf Group* (P = 0.033). Results are shown in *Figure 2.*

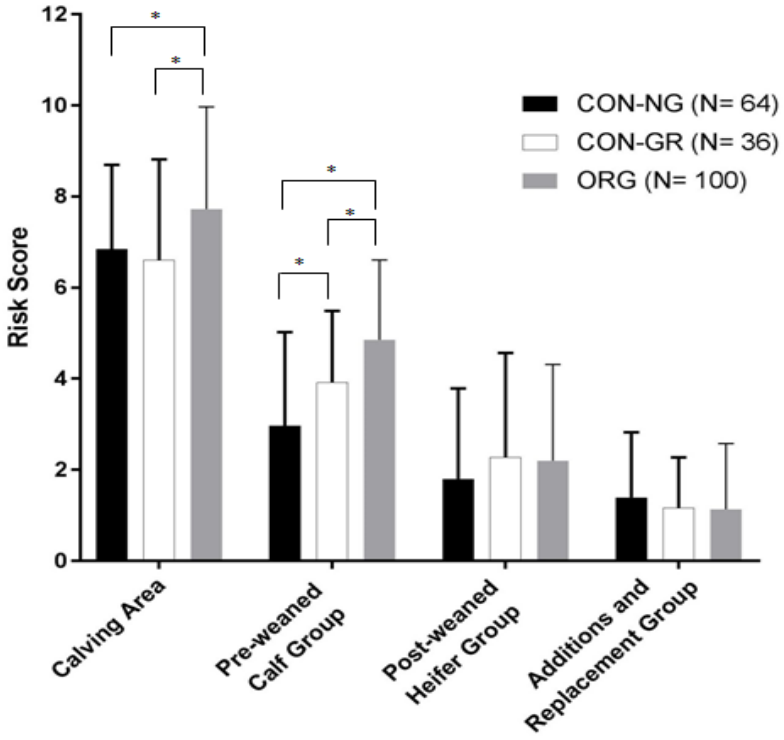

Figure 2. *Overall Risk Assessment Stratified by Risk Category.* The individual risk categories (*Calving Area, Pre-weaned Calf Group, Post-weaned Heifer Group,* and *Additions and Replacement group)* are shown on the X axis. The average risk score for each grazing type in each risk category is presented. Significant differences (P < 0.05) between the production types are marked with asterisks. Error bars represent the within-group SD.

3.2. Individual Risk Factors

The results of the logistic regression analyses, in addition to descriptive statistics, are presented in *Tables 2* to *5*. Significance levels for odds ratios reflect Tukey HSD adjustments.

i. Calving Area Management

More ORG farms allowed calves to nurse colostrum (40%) compared to CON-NG herds (9%, OR = 6.7, P = 0.0001). Similarly, ORG herds permitted extended contact (≥ 6 hours) between cows and calves at an increased frequency compared to CON-NG herds (OR = 8.9, P =

0002). Although a higher percentage of ORG farms allowed nursing and extended contact compared to CON-GR farms, this difference was not significant after the adjustment for multiple comparisons. With regard to calving area hygiene, CON-GR farms had a higher likelihood of clipping the udders of fresh cows after calving than did ORG farms (28% vs. 12%, OR = 3.5 P = 0.036), and CON-NG farms had a 2.5 times higher likelihood (P = 0.044) of providing footbaths after calving (38%) compared to ORG herds (17%). There was no significant difference in the number of farms in each group using dedicated calving areas, allowing sick or lactating cows into the calving area, or provisioning stored colostrum to calves (from pooled or single sources).

Table 2. *Calving Area Management.* Results are presented for the main predictor of interest, *Production Type,* with adjustments for *Herd Size* and *State.* Raw numbers of herds responding "yes" in each production type (CON-NG, CON-GR, and ORG) are followed by parenthetical percentages. Superscript letters represent the logistic regression results: for each variable, groups with significantly different odds ratios (P < 0.05 with Tukey HSD adjustment) are marked with different letters. Non-significant pairs share the same letters. The P value (Type III Sum of Squares) for *Production Type* is shown, with an asterisk indicating significance at P < 0.05.

Variable				***P Value (Production Type)***
	CON-NG (N= 64)	**CON-GR** (N= 36)	**ORG** (N= 192)	
Description of Calving area				
Lack of a dedicated calving area	44 (69)	20 (56)	140 (73)	0.202
Calving area also houses lactating cows	36 (56)	16 (44)	98 (51)	0.856
Calving area also houses sick cows	10 (16)	3 (8)	27 (14)	0.307
Colostrum Feeding Practices				
Calves nurse colostrum	6 (9)^{A}	9 (25)^{A}	76 (40)^{B}	<0.0001*
6 or more hours spent with dam	4 (67)	8 (89)	68 (89)	
1-5 hours spent with dam	2 (33)	1 (11)	8 (11)	
Use of stored colostrum	35 (55)	18 (50)	99 (52)	0.661
Use of pooled colostrum	3 (5)	3 (8)	6 (3)	0.587
Hygiene of Close-up Cows				
Clip Udders				
During dry period (before calving)	5 (8)	4 (11)	20 (10)	0.931
Fresh cows (after calving)	9 (14)^{AB}	10 (28)^{A}	22 (12)^{B}	0.031*
Footbaths				
During dry period (before calving)	8 (13)^{A}	5 (14)^{AB}	10 (5)^{B}	0.040*
Fresh cows (after calving)	24 (38)^{A}	11 (31)^{AB}	32 (17)^{B}	0.023*
Fresh Cows are checked for dirt	26 (41)	14 (39)	45 (23)	0.061

ii. Pre-weaned Calf Group

Waste milk was more routinely fed on ORG (71%) and CON-GR farms (61%) compared to CON-NG farms (36%); the odds of provisioning waste milk to calves was 5.9 times higher for ORG herds ($P < 0.0001$) and 3.4 times higher for CON-GR herds ($P = 0.026$). Similarly, ORG and CON-GR farms had a higher probability of feeding unpasteurized waste milk (61% and 58%, respectively) compared to CON-NG systems: the likelihood was 3.1 times higher for CON-GR herds ($P = 0.032$) and 4.0 times higher for ORG herds ($P < 0.0001$). A larger percentage of ORG farms fed calves whole milk (88%) vs. 64% of CON-GR (OR = 4.4, $P = 0.002$) and 42% of CON-NG herds (OR= 9.1, $P < 0.0001$). Finally, a larger percentage of ORG farms (82%) fed unpasteurized whole milk to calves, in contrast with 61% of CON-GR (OR= 3.5, $P = 0.008$) and 36% of CON-NG herds (OR = 7.0, $P < 0.0001$). With respect to calf housing, pre-weaned calves were group-housed at a 3.8 times lower frequency on CON-GR farms (22%) compared to ORG farms (48%, $P = 0.005$).

Table 3. *Pre-weaned Calf Group.* Results are presented for the main predictor of interest, *Production Type*, with adjustments for *Herd Size* and *State.* Raw numbers of herds responding "yes" in each production type (CON-NG, CON-GR, and ORG) are followed by parenthetical percentages. Superscript letters represent the logistic regression results: for each variable, groups with significantly different odds ratios at ($P < 0.05$ with Tukey HSD adjustment) are marked with different letters. Non-significant pairs share the same letters. The P value (Type III Sum of Squares) for *Production Type* is shown, with an asterisk indicating significance at $P < 0.05$.

Variable				***P Value (Production Type)***
	CON-NG (N= 64)	**CON-GR** (N= 36)	**ORG** (N= 192)	
Milk Feeding Practices				
Waste Milk (any type)	23 (36)^{A}	22 (61)^{B}	136 (71)^{B}	<0.0001*
Unpasteurized Waste Milk	18 (28)^{A}	21 (58)^{B}	119 (61)^{B}	<0.0001*
Whole Milk (any type)	27 (42)^{A}	23 (64)^{A}	169 (88)^{B}	<0.0001*
Unpasteurized Whole Milk	23 (36)^{A}	22 (61)^{A}	158 (82)^{B}	<0.0001*
Group Housing of Calves	16 (25)^{A}	8 (22)^{B}	91 (48)^{A}	0.004*
Presence of scours	62 (98)	33 (92)	170 (89)	0.156
Untreated scours (≥ 15% cases left untreated)	60 (95)	33 (92)	169 (88)	0.450

iii. Post-weaned Heifer Group

Intuitively, manure spreading on pasture was significantly more common for grazing systems (48% of ORG and 47% of CON-GR farms) compared to CON-NG farms (19%), with 5.4 and 3.3 multiplicative increases in the odds, respectively ($P < 0.0001$). CON-GR herds were 5.1 times less likely than ORG herds to test water for nitrates ($P = 0.001$): 75% of CON-GR herds did not test, versus 37% of ORG herds. With respect to primary water source, there were no significant differences in surface water usage. More CON-GR herds used municipal water compared to ORG herds (OR = 11.9, $P = 0.020$).

Table 4. *Post-weaned Heifer Group.* Results are presented for the main predictor of interest, *Production Type,* with adjustments for *Herd Size* and *State.* Raw numbers of herds responding "yes" in each production type (CON-NG, CON-GR, and ORG) are followed by parenthetical percentages. Superscript letters represent the logistic regression results: for each variable, groups with significantly different odds ratios at ($P < 0.05$ with Tukey HSD adjustment) are marked with different letters. Non-significant pairs share the same letters. The P value (Type III Sum of Squares) for *Production Type* is shown, with an asterisk indicating significance at $P < 0.05$.

Variable				***P value (Production Type)***
	CON-NG (N= 64)	**CON-GR** (N= 36)	**ORG** (N= 192)	
Pasture				
Heifers share pasture with cows	N/A	13 (28)	58 (30)	0.294
Heifers graze multiple pastures	N/A	18 (58)^{A}	139 (72)^{B}	0.041*
Manure spreading on pasture	12 (19)^{A}	17 (47)^{B}	93 (48)^{B}	< 0.001*
Use of same loader for feed and manure	18 (28)	8 (22)	44 (23)	0.377
Do not wash or disinfect loader	1 (5)	0 (0)	7 (16)	
Rinse loader with water only	11 (55)	7 (78)	26 (58)	
Water				
Primary source of drinking water				
Municipal water	3 (5)^{AB}	6 (17)^{B}	2 (1)^{A}	0.027*
Surface water	5 (8)	7 (19)	25(13)	0.611
Well water	56 (88)	23 (64)	165 (86)	0.114
Water is not tested for nitrates	31 (48)^{AB}	27 (75)^{A}	71 (37)^{B}	< 0.001*

iv. Additions and Replacement Group

A larger percentage of CON-GR herds (14%) accepted new pre-weaned heifers into their herds relative to 2% of ORG herds (OR = 7.5, P = 0.018). A larger percentage of ORG herds accepted new bulls (27% compared to 11% of CON-NG farms, OR = 3.8, P = 0.009). The source of entering animals (single vs. multiple farms) did not differ between the 3 herd types.

Table 5. *Additions and Replacement Group.* Results are presented for the main predictor of interest, *Production Type*, with adjustments for *Herd Size* and *State.* Raw numbers of herds responding "yes" in each production type (CON-NG, CON-GR, and ORG) are followed by parenthetical percentages. Superscript letters represent the results of the logistic regression: for each variable, groups with significantly different odds ratios (at P < 0.05 with Tukey HSD adjustment) are marked with different letters. Non-significant pairs share the same letters. The P value (Type III Sum of Squares) for *Production Type* is shown, with an asterisk indicating significance at P < 0.05.

Variable				***P Value (Production Type)***
	CON-NG (N= 64)	**CON-GR** (N= 36)	**ORG** (N= 192)	
Entering Animals				
Pre-Weaned Heifers	2 (3)^{AB}	5 (14)^{B}	4 (2)^{A}	0.020*
Weaned Heifers	17 (27)^{A}	9 (25)^{AB}	22 (12)^{B}	0.031*
Dairy Cows	11 (17)	6 (17)	18 (9)	0.115
Bulls	7 (11) ^{A}	6 (17) ^{AB}	52 (27) ^{B}	0.010*
Other Livestock	8 (13)	5 (14)	27 (14)	0.967
Any entering animal	28 (44)	21 (58)	75 (39)	0.122
Source of entering animals (multiple farms v. single farm)	5 (16)	3 (17)	17 (21)	0.571
Number of entering animals ‡	7.69	5.73	8.72	0.898

‡ Assessed using linear (rather than logistic) regression. LS means are provided.

3.3. Supplementary Analysis: Johne's Disease Testing and Management

Results for Johne's disease testing and management variables are shown in *Table 6.* In summary, well over half of producers for each production type reported a history of Johne's disease testing. Although there was no significant difference in overall level of testing for Johne's disease, organic herds were more likely to test the bulk tank milk supply.

Table 6. *Johne's Disease Testing and Management.* Results are presented for the main predictor of interest, *Production Type* with adjustments for *Herd Size* and *State.* Raw numbers of herds responding "yes" in each production type (CON-NG, CON-GR, and ORG) are followed by parenthetical percentages. Superscript letters represent logistic regression results: for each variable, groups with significantly different odds ratios (at $P < 0.05$ with Tukey HSD adjustment) are marked with different letters. Non-significant pairs share the same letters. The P value (Type III Sum of Squares) for *Production Type* is shown, with an asterisk indicating significance at $P < 0.05$.

Variable				***P Value (Production Type)***
	CON-NG (N= 64)	**CON-GR** (N= 36)	**ORG** (N= 192)	
Johne's Disease Testing				
Bulk tank milk	0 (0)^{A}	2 (6)^{AB}	40 (21)^{B}	0.016*
Milk (individual)	4 (6)	3 (8)	21 (11)	0.118
Blood	34 (53)	12 (33)	70 (37)	0.123
Fecal	17 (27)	14 (39)	43 (23)	0.253
Any type	44 (69)	24 (67)	144 (75)	0.144
Johne's Disease Status (self-reports)				
Never observed clinical Johne's	19 (30)	6 (17)	55 (29)	0.071
Clinical Signs observed on-farm:				
Poor body condition	26 (41)	13 (36)	79 (41)	
Loose Manure	34 (53)	21 (58)	100 (52)	
Confirmation via vet diagnosis	0 (0)	1 (3)	5 (3)	
Johne's Disease Management				
Written plan	12 (19)	10 (28)	25 (13)	0.155
Participation in Johne's control program	24 (38)	15 (42)	42 (22)	0.084
Procedure for Managing MAP+ cows				
Cull Immediately	30 (47)	27 (75)	110 (57)	0.547
Cull after calving	10 (16)	2 (6)	9 (5)	0.081
Keep	5 (8)	1 (3)	18 (9)	0.403
Keep or cull after calving vs. immediately	15 (24)	3 (9)	27 (14)	0.071

3.4. Compound Risk Factors

All two-level compound risk factors were evaluated, and any significant odds ratios from these logistic regressions are presented in *Table 7.* ORG herds were most susceptible to synergism of risk, with inferior performance to CON-NG herds with respect to all significantly-different compound risk factors.

Table 7. *Compound Risk Factors.* The odds ratios and confidence intervals for significant (P < 0.05) compound risk factors are presented. Non-significant odds ratios are marked with "NS." For each variable, the rightmost column lists the risk categories interacting.

Variable	***Odds ratios (Tukey HSD corrected CI)***			***Categories Interacting***
	ORG vs. CON-NG	**CON-GR vs. ORG**	**CON-GR vs. CON-NG**	
Calves spend ≥ 6 hours in maternity AND Sick or lactating cows in calving area	35.8 (1.3, 965.0)	NS	NS	Calving Area Management (multiple factors)
Sick or lactating cows in calving area AND calves nurse colostrum	43.7 (1.6, 692.0)	NS	NS	Calving Area Management (multiple factors)
Lack of footbaths for peri-parturient cows AND calves spend ≥ 6 hours with the dam	11.2 (2.3, 53.2)	NS	NS	Calving Area Management (multiple factors)
Lack of a dedicated calving area AND calves spend ≥ 6 hours with the dam	17.5 (2.5, 125.0)	NS	NS	Calving Area Management (multiple factors)
Calves nurse colostrum AND Udders of fresh cows not clipped	6.3 (1.1, 7.4)	NS	NS	Calving Area Management (multiple factors)
Calves nurse colostrum AND Average udder hygiene on farm is ≥ 2.50	13.0 (1.1, 150.2)	NS	NS	Calving Area Management (multiple factors)
Manure is spread on pasture AND Any entering animal	NS	NS	4.1 (1.1, 15.5)	Post-weaned Heifer Group Additions/Replacement Group
No bulk milk testing AND Calves fed unpasteurized waste milk	2.4 (1.0, 6.0)	NS	NS	Johne's Disase Management Pre-weaned Calf Group
No bulk milk testing AND Calves fed unpasteurized whole milk	3.4 (1.4, 8.1)	NS	NS	Johne's Disease Management Pre-weaned Calf Group

Compared to CON-NG herds, ORG herds allowing ≥ 6 hours of contact between the calf and the dam were 17.5 times less likely to have a dedicated calving area, 35.8 times more likely to allow sick or lactating cows into the calving area, and 11.2 times less likely to provide footbaths to peri-parturient cows. Compared to CON-NG herds, ORG herds allowing calves to nurse colostrum were 13.0 times more likely to have a poor average udder hygiene (≥ 2.5), 6.3 times less likely to clip the udders of fresh cows, and 43.7 times more likely to allow sick or lactating cows into the calving area. ORG herds not performing bulk tank testing were 2.4 times more likely to feed unpasteurized waste milk and 3.4 times more likely to feed unpasteurized whole milk compared to CON-NG herds.

4. Discussion

Via an overall risk assessment analysis, we evaluated risk factors and common management decisions and compared the overall level of risk for new cow-level MAP infections between 3 dairy production types. The risks were quantified according to literature on the pathobiology of MAP infection and modes of transmission. Organic herds demonstrated a significantly higher burden of risk than did both conventional grazing and non-grazing herds (see *Figure 1*.) The overall risk assessment is based upon an amalgamation of individual, differentially-important management choices; thus, we may refine our understanding of the increased risk for organic herds by homing in on categories contributing to the overall score, and then on individual risk factors. As apparent in *Figure 2*, the *Calving Area* was a main area in which organic farms demonstrated an increased risk, in addition to *Pre-weaned calf* management. In both categories, organic herds displayed higher risk scores compared to both conventional subgroups. There was no significant difference in the Introduction Risk between

the production types; therefore the main focus of control strategies for organic herds may be refined to mitigating Within Herd Transmission, specifically for young calves

According to the VBJDCP, management decisions in the calving area have the potential to accrue the highest level of risk, due to the heightened susceptibility of calves to MAP infections (see Lombard, 2011). Based on consideration of individual management factors, organic herds tended to be less rigorous regarding the hygiene of peri-parturient cows. Specifically, organic herds exhibited a decreased likelihood of providing footbaths to dry and fresh cows (compared to non-grazing herds) and a decreased likelihood of clipping udders of fresh cows (compared to conventional grazing herds). There is published evidence to suggest that automatic washing of hooves results in improved hoof hygiene (Thomsen et al., 2012). The use of footbaths may therefore reduce the amount of manure tracked into the calving area by close-up animals. Similarly, udder cleanliness is an important management objective to reduce calf contact with cow manure; udders are recurrently contaminated with manure from other animals and from the environment (McAloon et al., 2015), and researchers have noted a positive association between udder clipping and teat cleanliness (Elmoslemany et al., 2009). Indeed, it was observed that more than 80% of MAP from colostrum and teat-skin tests could be traced to sources distinct from the donor cow (Pithua et al., 2011), highlighting the importance of teat skin cleanliness during the colostrum period, particularly for herds permitting suckling postpartum. Organic herds in our study more often allowed the calf to nurse, and likewise, a higher number of organic farms permitted 6 or more hours of contact between cow and calf. The VBJDCP recommends immediate separation of the calf from the dam and prevention of nursing, with calves remaining in the maternity pen for more than 6 hours receiving the highest risk score.

Organic herds demonstrated increased risks in the pre-weaned calf group as well (*Figure 2*), with a greater tendency to provide unpasteurized whole milk to calves and to group-house calves (compared to conventional grazing herds). Wells and Wagner (2000) determined that group-housing for pre-weaned calves is associated with an increased risk of MAP-positive herd status, and Tiwari et al. (2009) concluded that group-housing for pre-weaned calves, at least during the winter, was positively associated with the number of MAP seropositive cows. These findings substantiate calf-to-calf contact as a source of MAP transmission, though this parameter is not included as a risk factor in the VBJDCP. Additional evidence is derived from the observation of horizontal MAP transmission in a group of calves (Van Roermund et al, 2007). Because of the brief interval between infection and fecal shedding, it has been hypothesized that transmission risk between calves may be intensified in a group housing system (Mortier et al., 2014). The performance of organic herds in the overall risk assessment reflects the sum of these aforementioned management decisions.

There were several parameters worth noting for which conventional farms had an inferior performance relative to organic herds, although these individual factors were not prominent enough to impact the outcome of the overall risk assessment. Firstly, 75% of conventional-grazing herds did not test their water sources for nitrates, whereas significantly more organic farms conducted nitrate testing. Nitrate pollution of surface water on farms may be indicative of fertilizer runoff (see Singh and Sekhon, 1979). Because manure ingestion by young animals is the primary means of MAP transmission (Doré et al., 2012), testing water for nitrates and subsequent actions to reduce manure pollution in the case of high nitrate values, may be an important control measure. However, this practice seems important primarily for farms using surface water as a main source.

Secondly, conventional herds did not outperform organic herds when it came to allowing certain groups of animals into the herd. Wells and Wagner (2000) confirmed that the percentage of cows sourced from outside farms was related to the odds of MAP-positive herd status, and Tiwari et al. (2009) found that increased MAP sero-prevalence was related to the purchase of heifers during the previous year. Although organic herds purchased more bulls from outside sources, conventional grazing-herds were more likely to purchase pre-weaned heifers, and non-grazing herds were more likely to purchase weaned heifers. There was no difference between the production types in the percentage of herds sourcing animals from multiple farms.

Finally, it is important to consider that several of the calving area risk factors contributing to the heightened risk for organic herds may not present an increased risk if executed alone. For example, although official MAP programs recommend immediate cow/calf separation, there is little empirical evidence to suggest that this practice leads to a demonstrable reduction in MAP infections. McAloon et al. (2015) asserted that empirical evidence supporting prompt calf removal as a preventative measure, was relatively weak. Indeed, Wells and Wagner (2000) and Johnson-Ifearulundu and Kaneene (1998) reported no significant increase in MAP positivity when the calf was permitted to remain with the dam for an extended period of time. Another related practice involves permitting the calf to nurse. In one study (Stewart et al., 2005), bacterial contamination resulted from milking colostrum into a bucket prior to transfer to a sterile sampling container. On the other hand, bacterial counts from directly-stripped colostrum were comparatively low. Stewart et al. (2005) concluded *harvesting* colostrum was the stage most prone to contamination, due to a soiled udder, milking machine, or improperly-sanitized bucket. The means of storage and number of storage containers could impact colostrum quality, and pooling colostrum could increase the risk of exposure to pathogens (Godden, 2008). High

bacterial counts may be indicative of fecal contamination. Therefore, the act of nursing the dam may not represent an increased risk of MAP transmission compared to other means of provisioning colostrum.

Nevertheless, the calving area is ripe for the synergism of risk factors, particularly due to increased susceptibility of neonatal calves. Goodger et al. (1996) present a compelling case for "multifactorial" risks in MAP transmission by evaluating cumulative risk scores against herd prevalence. Care of newborn calves significantly interacted with several other categories, including manure handling and environmental conditions; thus, certain variables appear to have a different relationship to prevalence when combined. Moreover, validation is provided to the notion that newborn calf care is particularly important in reference to risk synergy. From the current data, it does not appear that organic farms take precautions to avoid the synergistic relationship of risk factors. Because nursing and prolonged dam/calf contact is more often permitted on organic farms, hygiene of fresh cows is paramount. However, organic farms were 6 times more likely than conventional non-grazing herds to allow calves to nurse without clipping the udders of peri-parturient cows. Farms with poor udder hygiene (average score ≥ 2.50) allowing the calf to nurse from the dam were 13 times more likely to be organic rather than non-grazing. Organic management was also significantly less likely conventional non-grazing herds to provide footbaths while allowing extended dam/calf contact (≥ 6 hours). As previously mentioned, footbaths are important in maintaining hoof hygiene (Thomsen et al., 2012) and a calving area free of manure.

Tiwari et al. (2009) concluded that the presence of multiple cows in the maternity area was associated with a higher MAP sero-prevalence, and Wells and Wagner (2000) asserted that group housing for peri-parturient cows was a main risk factor for MAP-positive herd status.

Compared to conventional non-grazing herds, organic dairy farms were significantly more likely to lack a dedicated calving area while simultaneously allowing calves to spend 6 or more hours in that environment. Moreover, organic herds more often permitted calf nursing while allowing sick animals or other lactating cows to be present in the calving area.

In a study by Muskens et al. (2003), providing milk replacer to calves was found to be related to herd sero-negativity in a univariable analysis; thus routine MAP testing of bulk milk to assure its negativity could ameliorate milk-source transmission risk. Pasteurization may also be used as a safeguarding tool for bulk milk when MAP concentrations are below 10^4 cells/L (Grant et al., 2005). The increased level of bulk milk testing in organic herds (see *Table 6*) may be in response to the more common practice of feeding unpasteurized whole milk to calves. Indeed, the absence of a commercially available organic milk replacer (Stiglbauer et al., 2013) is likely a contributing factor to an increased MAP infection risk and may prompt organic farms to increase MAP testing of their milk. However, organic herds were still over 3 times more likely to feed calves a combination of unpasteurized, untested whole milk compared to non-grazing herds.

It is important to note that certain management practices more commonly executed by organic farms may be beneficial from an animal welfare standpoint, such as contact between a dam and her calf and group housing for calves. In the present study, approximately 40% of organic farms permitted calves to nurse. Consumers purchasing organic products may envision this type of practice, as they have expressed a preference for more natural calf rearing (Vasseur et al., 2010). Although there is some evidence to the contrary (see McGuirk and Collins, 2004), cow and calf contact and nursing have been shown to have health, welfare and production benefits. Bar-Peled et al. (1997) concluded that heifer calves permitted to nurse had higher average daily gains than bucket-fed calves and showed a higher conception rate and milk

production later in life. The presence of the dam may even increase calf immunoglobulin absorption (Selman et al., 1970; Stott et al., 1979). Additionally, there appear to be welfare benefits for group-housed calves, as calves isolated at birth have demonstrated increased behavioral and physiological indicators of stress (Creel and Albright, 1988) and have shown impaired performance on cognitive tests (Gaillard et al., 2014). The MAP transmission risk for group-housed calves may be mitigated if the probability of MAP transmission to calves in the calving area is diminished. The trends in our data suggest that organic farms would do well to improve hygiene in the calving area to better accommodate such practices. Initiatives may include clipping and cleaning udders of peri-parturient cows, providing footbaths, and increasing overall udder hygiene. A dedicated calving area with minimal manure contamination appears to be of great importance in keeping the risk of MAP spread to a minimum, particularly if calves spend an extended period of time within this area.

4.1. Study Limitations and Future Directions

Differences in general management practices on conventional-grazing, non-grazing, and organic herds are presented in Stiglbauer et al. (2013), with a small degree of overlap with the present study. In order to present a complete picture of the risk factors, it was necessary to re-analyze several variables; however, Stiglbauer et al. (2013) focused on general management, so implications of management practices on the spread of MAP were not discussed.

Conclusions regarding organic management practices in the U.S. are difficult to extend to other countries, since practices for organic management differ substantially between countries (Stiglbauer et al., 2013). Future work will be necessary to uncover disparities in Johne's disease risk between production types in other countries where the standards for organic production vary. For example, in most EU countries, calf nursing is advocated by some organic producers but is

not compulsory. On the other hand, nursing during at least a portion of the colostrum period is a requisite for organic dairy production in Sweden, Norway and Denmark (see Johnsen et al., 2015). Although Johne's disease appears to be well-controlled in Norway, no country has published enough evidence to claim zero or near-zero MAP prevalence (Nielsen and Toft., 2009). Initially, questionnaires similar to the one employed in our study could be used to glean an understanding of country or region-specific organic management practices and MAP risk factors. Intervention studies aimed at reducing risk on organic farms could subsequently be designed based upon common organic management practices in a given area. Better prevalence and incidence estimates are necessary in some countries (Nielsen et al., 2000; Nielsen and Toft, 2009), in addition to a breakdown of prevalence on organic vs. conventional farms.

There is some evidence that participation of organic herds in Johne's disease control programs is diminished relative to participation of conventional herds. This is particularly concerning in countries where the herd-level prevalence is high. In the Netherlands, where overall herd-level prevalence ranges from 31 to 71% (Muskens et al., 2000), it has been noted that few organic farmers choose to participate in the voluntary national prevention program (Kijlstra and Eijck., 2009). In the U.S. (70.4% prevalence, Lombard et al., 2013), there is also some evidence that fewer organic herds choose to participate in control programs (Beaver et al., 2016). This resistance may in part be due to the consistent recommendations of such programs to avoid practices seen as important to the organic dairy farmer. Management initiatives could therefore be tailored specifically to organic herds, with the goal of better accommodating rather than eliminating such practices, and safeguarding against synergism of risk factors.

5. Conclusion

In an overall risk assessment, organic herds demonstrated significantly higher risk of MAP transmission than both conventional grazing and non-grazing herds. Decisions regarding post-weaned heifer management and purchase of additional animals seem to be comparably executed across the 3 production types; thus the heightened risk for organic herds appears primarily due to management in the calving area and subsequent housing of pre-weaned calves. However, certain management practices traditionally cited as risk factors (e.g., prolonged cow/calf contact and nursing) lack a supporting body of empirical evidence to associate them with increased MAP prevalence. Such practices may even be beneficial for animal welfare. Nonetheless, these practices have the potential to interact with other management decisions and act synergistically on the risk of MAP transmission. An increased awareness of hygiene in the calving area is therefore necessary for farms electing to permit cow/calf contact, nursing, and subsequent group housing of calves. The organic herds in our study did not appear to take these extra precautions with regard to hygiene; consequently, an increased vigilance is recommended to mitigate an increased risk.

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7. References

Ansari-Lari, M., M. Haghkhah, A. Bahramy, and A. M. N. Baheran. 2009. Risk factors for *Mycobacterium avium* subsp. *paratuberculosis* in Fars Province (southern Iran) dairy herds. Trop. Anim. Health Prod. 41:553–557.

Bar-Peled, U., B. Robinzon, E. Maltz, H. Tagari, Y. Folman, I. Bruckental, H. Voet, H. Gacitua, and A. Lehrer. 1997. Increased weight gain and effects on production parameters of Holstein heifer calves that were allowed to suckle from birth to six weeks of age. J. Dairy Sci. 80:2523–2528.

Beaver, A., C. Cazer, P. Ruegg, Y. Grohn, and Y. Schukken. 2016. Implications of PCR and ELISA results on the routes of bulk-tank contamination with *Mycobacterium avium* subsp. *paratuberculosis*. J. Dairy Sci. 99:1391–1405.

Bergman, M. A., R. M. Richert, K. M. Cicconi-Hogan, M. J. Gamroth, Y. H. Schukken, K. E. Stiglbauer, and P. L. Ruegg. 2014. Comparison of selected animal observations and management practices used to assess welfare of calves and adult dairy cows on organic and conventional dairy farms. J. Dairy Sci. 97:4269–4280.

Cazer, C. L., R. M. Mitchell, K. M. Cicconi-Hogan, M. Gamroth, R.M. Richert, P. L. Ruegg, and Y. H. Schukken. 2013. Associations between *Mycobacterium avium* subsp. *paratuberculosis* antibodies in bulk tank milk, season of sampling and protocols for managing infected cows. BMC Vet. Res. 9:234–240.

Chiodini, R. J., H. J. van Kruiningen, and R. S. Merkal. 1984. Ruminant paratuberculosis (Johne's disease): The current status and future prospects. Cornell Vet. 74:218–262.

Cicconi-Hogan, K. M., M. Gamroth, R. Richert, P. L. Ruegg, K. E. Stiglbauer, and Y. H. Schukken. 2013. Associations of risk factors with somatic cell count in bulk tank milk on organic and conventional dairy farms in the United States. J. Dairy Sci. 96:3689–3702.

Creel, S., and J. Albright. 1988. The effects of neonatal social isolation on the behavior and endocrine function of Holstein calves. Appl. Anim. Behav. Sci. 21:293–306.

Doré, E., J. Pare, G. Cote, S. Buczinski, O. Labrecque, J. Roy, and G. Fecteau. 2012. Risk factors associated with transmission of *Mycobacterium avium* subsp. *paratuberculosis* to calves within dairy herd: A systematic review. J. Vet. Intern. Med. 26:32–45.

Elmoslemany, A. M., G. P. Keefe, I. R. Dohoo, and B. M. Jayarao. 2009. Risk factors for bacteriological quality of bulk tank milk in Prince Edward Island dairy herds. Part 1: Overall risk factors. J. Dairy Sci. 92:2634–2643.

Feller, M., K. Huwiler, R. Stephan, E. Altpeter, A. Shang, H. Furrer, G. E. Pfyffer, T. Jemmi, A. Baumgartner, and M. Egger. 2007. *Mycobacterium avium* subsp. *paratuberculosis* and Crohn's disease: A systematic review and meta-analysis. Lancet Infect. Dis.7:607–613.

Gaillard, C., R. K. Meagher, M. A. von Keyserlingk, and D. M. Weary. 2014. Social housing improves dairy calves' performance in two cognitive tests. PLoS ONE 9:e90205.

Godden, S. 2008. Colostrum management for dairy calves. Vet. Clin. North Am. Food Anim. Pract. 24:19–39. Goodger, W. J., M. T. Collins, K. V. Nordlund, C. Eisele, J. Pelletier,

C. B. Thomas, and D. C. Sockett. 1996. Epidemiologic study of on-farm management practices associated with prevalence of *Mycobacterium paratuberculosis* infections in dairy cattle. J. Am. Vet. Med. Assoc. 208:1877–1881.

Grant, I. R., A. G. Williams, M. T. Rowe, and D. D. Muir. 2005. Efficacy of various pasteurization time-temperature conditions in combination with homogenization on inactivation of *Mycobacterium avium* subsp. *paratuberculosis* in milk. Appl. Environ. Microbiol. 71:2853–2861.

Johnsen, J. F., A. M. de Passille, C. M. Mejdell, K. E. Boe, A. M. Grondahl, A. Beaver, J. Rushen, and D. M. Weary. 2015. The effect of nursing on the cow–calf bond. Appl. Anim. Behav. Sci.163:50–57.

Johnson-Ifearulundu, Y. J., and J. B. Kaneene. 1998. Management related risk factors for *M. paratuberculosis* infection in Michigan, USA, dairy herds. Prev. Vet. Med. 37:41–54.

Kijlstra, A., and I. Eijck. 2006. Animal health in organic livestock production systems: A review. NJAS-Wageningen J. Life Sci. 54:77–94.

Lombard, J. E., I. A. Gardner, S. R. Jafarzadeh, C. P. Fossler, B. Harris, R. T. Capsel, B. A. Wagner, and W. O. Johnson. 2013. Herd-level prevalence of *Mycobacterium avium* subsp. *paratuberculosis* infection in United States dairy herds in 2007. Prev. Vet. Med. 108:234–238.

Lombard, J. E. 2011. Epidemiology and economics of paratuberculosis. Vet. Clin. North Am. Food Anim. Pract. 27:525–535.

Lu, Z., Y. H. Schukken, R. L. Smith, and Y. T. Grohn. 2010. Stochastic simulations of a multi-group compartmental model for Johne's disease on US dairy herds with test-based culling intervention. J. Theor. Biol. 264:1190–1201.

Manning, E. J., and M. T. Collins. 2001. *Mycobacterium avium* subsp. *paratuberculosis*: Pathogen, pathogenesis and diagnosis. Rev. Sci. Tech. 20:133–150.

Marcé, C., P. Ezanno, H. Seegers, D. Pfeiffer, and C. Fourichon. 2011. Within-herd contact structure and transmission of *Mycobacterium avium* subsp. *paratuberculosis* in a persistently infected dairy cattle herd. Prev. Vet. Med. 100:116–125.

McAloon, C. G., P. Whyte, S. J. More, L. O'Grady, and M. L. Doherty. 2015. Development of a HACCP-based approach to control paratuberculosis in infected Irish dairy herds. Prev. Vet. Med. 120:152–161.

McGuirk, S. M., and M. Collins. 2004. Managing the production, storage, and delivery of colostrum. Vet. Clin. North Am. Food Anim. Pract. 20:593–603.

Mortensen, H., S. S. Nielsen, and P. Berg. 2004. Genetic variation and heritability of the

antibody response to *Mycobacterium avium* subsp. *paratuberculosis* in Danish Holstein cows. J. Dairy Sci. 87:2108–2113.

Mortier, R. A., H. W. Barkema, K. Orsel, R. Wolf, and J. De Buck. 2014. Shedding patterns of dairy calves experimentally infected with *Mycobacterium avium* subsp. *paratuberculosis*. Vet. Res. 45:71–79.

Muskens, J., H. Barkema, E. Russchen, K. Van Maanen, Y. Schukken, and D. Bakker. 2000. Prevalence and regional distribution of paratuberculosis in dairy herds in the Netherlands. Vet. Microbiol. 77:253–261.

Muskens, J., A. Elbers, H. van Weering, and J. Noordhuizen. 2003. Herd management practices associated with paratuberculosis seroprevalence in Dutch dairy herds. J. Vet. Med. B. Infect. Dis. Wet. Public Health 50:372–377.

Nielsen, S. S., H. Bjerre, and N. Toft. 2008. Colostrum and milk as risk factors for infection with *Mycobacterium avium* subsp. *paratuberculosis* in dairy cattle. J. Dairy Sci. 91:4610–4615.

Nielsen, S. S., S. M. Thamsborg, H. Houe, and V. Bitsch. 2000. Bulk tank milk ELISA antibodies for estimating the prevalence of paratuberculosis in Danish dairy herds. Prev. Vet. Med. 44:1–7.

Nielsen, S. S., and N. Toft. 2009. A review of prevalences of paratuberculosis in farmed animals in Europe. Prev. Vet. Med. 88:1–14.

Obasanjo, I. O., Y. Grohn, and H. O. Mohammed. 1997. Farm factors associated with the presence of *Mycobacterium paratuberculosis* infection in dairy herds on the New York state paratuberculosis control program. Prev. Vet. Med. 32:243–251.

Organic Trade Association. 2016. U.S. Organic: State of the industry. Available at: http://ota.com/sites/default/files/indexed_files/OTA_StateofIndustry_2016.pdf Accessed Apr. 2, 2016.

Orpin, P., S. Duthie, and D. H. Grove-White. 2005. The use of targeted sampling and risk factor analysis to investigate the presence of Johne's disease in dairy herds. Cattle Pract. 13:219–225.

Ott, S. L., S. J. Wells, and B. A. Wagner. 1999. Herd-level economic losses associated with Johne's disease on US dairy operations. Prev. Vet. Med. 40:179–192.

Pickup, R. W., G. Rhodes, T. J. Bull, S. Arnott, K. Sidi-Boumedine, M. Hurley, and J. Hermon-Taylor. 2006. *Mycobacterium avium* subsp. *paratuberculosis* in lake catchments, in river water abstracted for domestic use, and in effluent from domestic sewage treatment works: Diverse opportunities for environmental cycling and human exposure. Appl. Environ. Microbiol. 72:4067–4077.

Pieper, L., U. Sorge, A. Godkin, T. DeVries, K. Lissemore, and D. Kelton. 2014. Management practices and their potential influence on Johne's disease transmission on Canadian organic dairy farms—A conceptual analysis. Sustainability. 6:8237–8261.

Pithua, P., S. J. Wells, and S. M. Godden. 2011. Evaluation of the association between fecal excretion of *Mycobacterium avium* subsp *paratuberculosis* and detection in colostrum and on teat skin surfaces of dairy cows. J. Am. Vet. Med. Assoc. 238:94–100.

Richert, R. M., K. M. Cicconi, M. J. Gamroth, Y. H. Schukken, K. E. Stiglbauer, and P. L. Ruegg. 2013. Risk factors for clinical mastitis, ketosis, and pneumonia in dairy cattle on organic and small conventional farms in the United States. J. Dairy Sci. 96:4269–4285.

Schreiner, D. A., and P. L. Ruegg. 2003. Relationship between udder and leg hygiene scores and subclinical mastitis. J. Dairy Sci. 86:3460–3465.

Selman, I. E., A. D. McEwan, and E. W. Fisher. 1970. Studies on natural suckling in cattle during the first eight hours post partum II. Behavioural studies (calves). Anim. Behav. 18:284–289. Singh, B., and G. Sekhon. 1979. Nitrate pollution of groundwater from farm use of nitrogen fertilizers—A review. Agric. Environ.4:207–225.

Slana, I., M. Liapi, M. Moravkova, A. Kralova, and I. Pavlik. 2009. *Mycobacterium avium* subsp. *paratuberculosis* in cow bulk tank milk in Cyprus detected by culture and quantitative IS900 and F57real-time PCR. Prev. Vet. Med. 89:223–226.

Sorge, U. S., K. Lissemore, A. Godkin, J. Jansen, S. Hendrick, S. Wells, and D. F. Kelton. 2012. Risk factors for herds to test positive for *Mycobacterium avium* subsp. *paratuberculosis*-antibodies with a commercial milk enzyme-linked immunosorbent assay (ELISA) in Ontario and western Canada. Can. Vet. J. 53:963–970.

Stewart, S., S. Godden, R. Bey, P. Rapnicki, J. Fetrow, R. Farnsworth, M. Scanlon, Y. Arnold, L. Clow, and K. Mueller. 2005. Preventing bacterial contamination and proliferation during the harvest, storage, and feeding of fresh bovine colostrum. J. Dairy Sci. 88:2571–2578.

Stiglbauer, K. E., K. M. Cicconi-Hogan, R. Richert, Y. H. Schukken, P. L. Ruegg, and M. Gamroth. 2013. Assessment of herd management on organic and conventional dairy farms in the United States. J. Dairy Sci. 96:1290–1300.

Stott, G. H., D. B. Marx, B. E. Menefee, and G. T. Nightengale. 1979. Colostral immunoglobulin transfer in calves. IV. Effect of suckling. J. Dairy Sci. 62:1908–1913.

Sweeney, R. W., R. H. Whitlock, and A. E. Rosenberger. 1992. Mycobacterium paratuberculosis cultured from milk and supramammary lymph nodes of infected asymptomatic cows. J. Clin. Microbiol. 30:166–171.

Thomsen, P. T., A. K. Ersboll, and J. T. Sorensen. 2012. Short communication: Automatic

washing of hooves can help control digital dermatitis in dairy cows. J. Dairy Sci. 95:7195–7199.

Tiwari, A., J. Vanleeuwen, I. Dohoo, G. Keefe, J. Haddad, H. Scott, and T. Whiting. 2009. Risk factors associated with *Mycobacterium avium* subsp. *paratuberculosis* seropositivity in Canadian dairy cows and herds. Prev. Vet. Med. 88:32–41.

van Roermund, H. J., D. Bakker, P. T. Willemsen, and M. C. de Jong. 2007. Horizontal transmission of *Mycobacterium avium* subsp. *paratuberculosis* in cattle in an experimental setting: Calves can transmit the infection to other calves. Vet. Microbiol. 122:270–279.

van Weering, H., G. van Schaik, A. van der Meulen, M. Waal, P. Franken, and K. van Maanen. 2007. Diagnostic performance of the pourquier ELISA for detection of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in individual milk and bulk milk samples of dairy herds. Vet. Microbiol. 125:49–58.

Vasseur, E., F. Borderas, R. I. Cue, D. Lefebvre, D. Pellerin, J. Rushen, K. M. Wade, and A. M. de Passille. 2010. A survey of dairy calf management practices in Canada that affect animal welfare. J. Dairy Sci. 93:1307–1315.

Wells, S. J., and B. A. Wagner. 2000. Herd-level risk factors for infection with *Mycobacterium paratuberculosis* in US dairies and association between familiarity of the herd manager with the disease or prior diagnosis of the disease in that herd and use of preventive measures. J. Am. Vet. Med. Assoc. 216:1450–1457.

Whitlock, R. H., and C. Buergelt. 1996. Preclinical and clinical manifestations of paratuberculosis (including pathology). Vet. Clin. North Am. Food Anim. Pract. 12:345–356.

Whittington, R. J., I. B. Marsh, and L. A. Reddacliff. 2005. Survival of *Mycobacterium avium* subsp. *paratuberculosis* in dam water and sediment. Appl. Environ. Microbiol. 71:5304–5308.

Wilson, D. J., K. Rood, P. Biswas, and T. M. Byrem. 2010. Herd-level prevalence of Johne's disease in Utah and adjacent areas of the intermountain west as detected by a bulk-tank milk surveillance project. J. Dairy Sci. 93:5792–5797.

Windsor, P. A., and R. J. Whittington. 2010. Evidence for age susceptibility of cattle to Johne's disease. Vet. J. 184:37–44.

Zwald, A. G., P. L. Ruegg, J. B. Kaneene, L. D. Warnick, S. J. Wells, C. Fossler, and L. W. Halbert. 2004. Management practices and reported antimicrobial usage on conventional and organic dairy farms. J. Dairy Sci. 87:191–201.

CHAPTER 3:
IMPLICATIONS OF PCR AND ELISA RESULTS ON THE ROUTES OF BULK-TANK CONTAMINATION WITH *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS*

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KEYWORDS: *Mycobacterium avium* subsp. *paratuberculosis*, Johne's disease, bulk-tank milk, PCR, ELISA

ABSTRACT

Mycobacterium avium subsp. *paratuberculosis* (MAP), the etiologic agent of Johne's disease in dairy cattle, may enter the bulk tank via environmental contamination or direct excretion into milk. Traditionally, diagnostics to identify MAP in milk target either MAP antibodies (by ELISA) or the organism itself (by culture or PCR). High ELISA titers may be directly associated with excretion of MAP into milk but only indirectly linked to environmental contamination of the bulk tank. Patterns of bulk-milk ELISA and bulk-milk PCR results could therefore provide insight into the routes of contamination and level of infection or environmental burden. Coupled with questionnaire responses pertaining to management, the results of these diagnostic tests could reveal correlations with herd characteristics or on-farm practices that distinguish herds with high and low environmental bulk-tank MAP contamination.

A questionnaire on hygiene, management, and Johne's specific parameters was administered to 292 dairy farms in New York, Oregon, and Wisconsin. Bulk-tank samples were collected from each farm for evaluation by real-time PCR and ELISA. Before DNA extraction and testing of the unknown samples, bulk-milk template preparation was optimized with respect to parameters such as MAP fractionation patterns and lysis.

Two regression models were developed to explore the relationships among bulk-tank PCR, ELISA, environmental predictors, and herd characteristics. First, ELISA optical density (OD) was designated as the outcome in a linear regression model. Second, the log odds of being PCR positive in the bulk tank were modeled using binary logistic regression with penalized maximum likelihood. The proportion of PCR-positive bulk tanks was highest for New York and for organic farms, providing a clue as to the geographical patterns of MAP-positive bulk-tank samples and relationship to production type. Bulk-milk PCR positivity was also higher for large

relative to small herds. The models revealed that bulk-milk PCR result could predict ELISA OD, with PCR-positive results corresponding to high bulk-milk ELISA titers. Similarly, ELISA was a predictor of PCR result, although the association was stronger for organic farms. Despite agreement between high bulk-milk ELISA titers and positive PCR results, a large proportion of high ELISA farms had PCR-negative bulk tanks, suggesting that farms are able to maintain satisfactory hygiene and management despite a presence of MAP in these herds.

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of Johne's disease, a granulomatous enteritis that is both chronic and progressive; MAP predominately affects ruminants and has been cultured from the milk and feces of both clinically infected and asymptomatic animals. Because MAP-positive animals may fail to manifest clinical signs, testing at the herd level remains an important tool to evaluate on-farm MAP presence and prevalence (Manning and Collins, 2001).

According to a study conducted by the National Animal Health Monitoring System (Lombard et al., 2013), the proportion of US dairy herds with MAP in their on-farm environments exceeds 70%. This high prevalence, coupled with MAP's ability to survive common pasteurization practices (Sung and Collins, 1998; Grant et al., 2002, 2005), necessitates the optimization of simple, rapid diagnostic tests to determine herd MAP status and progression of MAP infection at the herd level. Current herd-level testing strategies commonly involve pooling fecal samples throughout the farm environment or from a large number of animals for evaluation by culture or PCR (Collins, 2011). Several of these strategies present certain drawbacks; for example, individual animal sampling may not be labor efficient and the fecal

culture method is not timely, with results typically reported after 8 to 16 weeks of incubation. Optimization of bulk-tank milk testing would facilitate timely response to MAP contamination through alteration of critical on-farm management and hygiene practices (Cazer et al., 2013).

Viable MAP identified by culture methods has been found in both raw and pasteurized pooled milk fed to calves (Ruzante et al., 2008), thus conceivably perpetuating the cycle of infection. Additionally, milk and other dairy products may represent a route of transmission of MAP from cattle to humans. This transmission is particularly concerning because MAP has been implicated in the development of Crohn's disease in humans. Although MAP has not been definitively identified as a cause of Crohn's disease (Over et al., 2011), investigation of its potential as a zoonotic organism is of sufficient public health concern to justify the monitoring of bulk milk intended for consumers (Eltholth et al., 2009).

An "acceptable" threshold for MAP concentration in the bulk tank is unknown, mainly due to an absence of quantitative information on the likelihood of human disease as a consequence of MAP exposure (Weber et al., 2008) and the lack of confidence in pasteurization as a safeguarding tool to remove all viable MAP. *Mycobacterium avium* subsp. *paratuberculosis* bacteria in concentrations exceeding 10^4 cells/L have been documented to survive HTST pasteurization (Grant et al., 2005). Thus, Weber et al. (2008) defined low-risk farms as those with a certain probability (approaching 100% in simulated test schemes) of having a concentration $<10^3$ MAP organisms/L of milk. The threshold was selected because there is no evidence to suggest that MAP can survive HTST pasteurization at this initial concentration. The goal of risk mitigation and bulk-milk quality assurance as outlined by Weber et al. (2008) is to focus efforts on reduction of MAP concentration in the bulk tank rather than on complete eradication of the pathogen from the tank or farm environment.

The routes of bulk-milk contamination with MAP may be categorized as either internal or environmental. In the internal route, MAP is shed directly into milk by an infected host, such as through mobilized, MAP-positive macrophages resulting from supramammary lymph node infection or bacteremia (Sweeney et al., 1992). If the source originates outside the mammary gland, the route of contamination is said to be environmental, and MAP may enter the bulk milk by means of fecal contamination or airborne particles (Eisenberg et al., 2013). Additionally, several studies have noted the survival of MAP in a variety of water sources traced to on-farm usage (e.g., Whan et al., 2001; Whittington et al., 2005; Pickup et al., 2006). Contaminated water supplies may represent a source of MAP transmission from cattle to humans, by direct consumption, food incorporation, or the washing of areas contacting food (Whan et al., 2001). It seems plausible that wash water could represent another means of environmental contamination of bulk milk; if the water used for cleaning milking equipment (or udders before milking) contains MAP, the bulk tank may become adulterated.

Traditionally, diagnostics to identify MAP in milk target either MAP antibodies (ELISA) or the organism itself (culture and PCR). An ELISA-positive status likely indicates some type of internal-route shedding into the bulk tank: either infected animals are shedding antibodies alone or are contributing both antibodies and MAP itself. Singh et al. (2007) recorded 84.6% agreement between individual milk ELISA and milk culture results from the same animals; hence, a positive ELISA is often indicative of internal-route MAP shedding. However, a bulk tank PCR-positive status may represent internal, environmental, or combined routes of contamination.

Of particular interest, then, are farms presenting discordant bulk-milk PCR and ELISA results; on such farms, conclusions may be drawn regarding the routes of contamination. In a

recent study, Wilson et al. (2010) noted only moderate agreement between ELISA and PCR in bulk-milk samples, with 113 of 241 samples taken from MAP-positive herds presenting discordant results. Although paired high bulk-tank ELISA and positive PCR results suggest the internal route, possibly with high within-herd prevalence, the effect of environmental contamination cannot be excluded. Bulk tanks that are PCR-negative with high ELISA titers may reveal herds with sufficient hygienic practices in place to restrict environmental contamination despite MAP-positive herd status. Conversely, a low bulk-milk ELISA and positive PCR may imply environmental contamination rather than internal-route shedding of MAP. We hypothesize that herds with this latter combination are those with only a few MAP-positive cows that nevertheless contaminate the bulk tank via the environmental route. Differences in management between these categories of farms may identify specific practices that could be useful in developing control programs to reduce MAP bacterial load in the bulk tank. An enhanced understanding of the routes of bulk-milk contamination and their interrelationship is an essential preliminary step in the reduction of MAP concentration in the bulk tank.

To date, little is known about the relationship between PCR and ELISA results in bulk milk, especially as it relates to management factors and environmental parameters. The objective of this study was to elucidate the relationship between PCR result and ELISA titer using bulk-tank samples and corresponding cross-sectional management data collected from 292 farms in 3 US states.

2. Materials and Methods

2.1 Herd Recruitment, Sample Collection, and Questionnaire Data

Bulk-tank milk samples and questionnaire results originated from a multi-institutional, collaborative study aimed at obtaining cross-sectional data on MAP risk factors. Samples and

complete questionnaires were collected from a total of 292 organic and conventional farms from 3 US states: New York (NY), Oregon (OR), and Wisconsin (WI). The recruitment of these herds has been described previously (Cazer et al., 2013; Cicconi-Hogan et al., 2013; Richert et al., 2013). Briefly, 192 organic farms were chosen with the help of extension agents in each county as well as organizations specializing in organic certification. Licensed conventional farms were sourced from state-specific lists provided by state departments of agriculture and 100 of these were selected based upon proximity to the organic farms. Matching was conducted according to herd size (divided into 3 categories: 20–99, 100–199, or ≥ 200 adult cows) and organic-to-conventional state ratios. Farms were eligible for inclusion if they had a minimum of 20 lactating cows and if they had been shipping milk (certified organic milk in the case of organic farms) for at least 2 years before the onset of the study.

The questionnaire, described in detail by Richert et al. (2013) and available online (http://milkquality.wisc.edu/organic-dairies/project-c-o-w/), included elements pertaining to general herd management and hygiene practices, as well as to Johne's-specific management factors. The farms were visited between 2009 and 2011, and bulk-tank samples were collected by project personnel (consisting of researchers from Cornell University, Oregon State University, and the University of Wisconsin). As described in Cicconi-Hogan et al. (2013), the bulk tanks were first agitated for a minimum of 5 min before sample collection. Bulk-milk samples were obtained directly from the tank using sterile dippers and sampling containers and immediately placed on ice. Samples from all 3 states were transported or shipped in coolers to Cornell University (Ithaca, NY) for laboratory analysis. At Cornell, samples were frozen at −20°C until testing could be conducted.

Of the 292 farms included in the study, 2 did not approve of Johne's testing of their bulk milk, and the bulk-milk samples from an additional 3 farms had been used up in previous diagnostics. One additional farm had missing data for a covariate under consideration. The total number of analyzed bulk-tank samples was therefore 286: of these, 97 were from NY, 46 from OR, and 143 from WI.

2.2 Extraction of DNA from Milk Samples

The Tetracore VetAlert Real-Time PCR and extraction kits (Tetracore Inc., Rockland, MD) have previously been validated for use in fecal samples, and preliminary results (unpublished material) suggest that this assay may also function well in milk. We conducted optimization experiments using MAP-spiked negative milk, for both the template preparation and quantitative PCR (qPCR) stages to maximize detection potential. The MAP isolates were kindly provided and cultured by the Sweeney Laboratory at The University of Pennsylvania School of Veterinary Medicine (Kennett Square, PA). We evaluated a variety of beadbeating durations, centrifugation speeds, and milk fractionation patterns. The efficacy of several heat treatments and lysis buffers was also considered. Finally, using spiked milk samples and the optimized extraction protocol, we determined the limit of detection for the qPCR. The final optimized protocol, based upon steps outlined by Herthnek et al. (2008) is described here in detail.

Frozen samples were stored overnight at 10°C. Once thawed, a 15-mL volume from each sample was pipetted into a sterile 50-mL tube and centrifuged at 2,547 × g for 30 min at 10°C. The whey fractions were discarded and the remaining cream and pellets subjected to 1.5 mL of lysis buffer (prepared using 2 mM EDTA, 400 nM NaCl, 10 mM Tris at pH 8, and 0.6% SDS; Sigma-Aldrich, St. Louis, MO), 10 s of vortexing, and 3 μL of 10 μg/μL proteinase K (Sigma-

Aldrich) to dissolve the cream. The mixture was then transferred to disruption tubes (2-mL microcentrifuge tubes with glass beads; Tetracore Inc.), which were filled to the top of the grooves to allow for a reduction in volume following incubation. Disruption tubes were incubated at 56°C for 1 h, followed by beadbeating for 5 min at three-fourths power (approximately 2,700 rpm) using a Mini-Beadbeater 8 (BioSpec Products, Bartlesville, OK), and then centrifuged at 16,000 × g for 10 min. The supernatant and cream fractions were transferred to 2-mL microcentrifuge tubes containing 100 μL of nucleic acid buffer (Tetracore Inc.), taking care to avoid transferring disruption beads. Samples were vortexed and centrifuged at 1,500 × g for 3 min.

Purification of DNA was conducted beginning with the removal of the supernatant and addition of 560 μL of Binding buffer (Tetracore Inc.). The full extraction protocol was followed according to the manufacturer's instruction. If necessary, extracted DNA was stored at 10°C for less than 48 h. The protocol was implemented on the 286 milk samples.

i. Negative and Positive Extraction Controls

A MAP-negative milk sample was included in each extraction and qPCR and fully processed using a procedure identical to that used for the unknown samples. Strain K-10 [sequenced by Li et al. (2005) and sequence revised by Wynne et al. (2010)] was used as the positive control and was also extracted according to protocol. The K-10 isolates were kindly provided by the Kapur Laboratory at Pennsylvania State University (University Park, PA). The strain was grown on Herrold's egg yolk slants containing mycobactin J (Fisher Scientific, Pittsburgh, PA) and incubated at 37°C. Colony growth was assessed weekly; after 12 weeks, colonies were transferred to tubes containing 7H9 broth (Becton Dickinson, Franklin Lakes, NJ) with Middlebrook Oleic Albumin Dextrose Catalase (Hardy Diagnostics, Santa Maria, CA),

cycloheximide, Tween 80 (Sigma-Aldrich), and Mycobactin J (Allied Monitor Inc., Fayette, MO) and incubated at 37°C. Contamination checks were conducted weekly by inoculating a loop-full of suspension to chocolate agar plates (Thermo Scientific, Waltham, MA) and monitoring daily for 3 days for any bacterial growth before discarding. The optical density (OD) at 600 nm of the K-10 suspension was measured twice weekly following repeated passage of 1 mL of the surface layer of broth through a 25-gauge needle. Once the OD reached 0.04, the presence of MAP was confirmed using acid-fast staining. When used in spiking experiments, 1 mL of the K-10 suspension was added to 10 mL of milk. The K-10 DNA was extracted following the procedure described above and diluted 1:100.

2.3 Real-Time PCR

The commercial VetAlert Johne's Real-Time PCR (Tetracore Inc.) was used to quantify MAP in the unknown survey samples. The kit includes a premade master mix containing forward and reverse oligonucleotide primers to amplify the *hspX* gene, a FAM-labeled probe to generate a fluorogenic signal, Taq polymerase, and facilitating buffers. The positive control included is a synthetic template in liquid form containing a portion of the target *hspX* sequence at 25,000 copies per 2.5 µL. Tris-EDTA buffer (1×; Affymetrix, USB Corporation, Cleveland, OH) was used as the no-template control, consistent with the manufacturer's recommendation.

The samples, in addition to positive and negative PCR and extraction controls, were tested using eleven 96-well plates (Life Technologies, Grand Island, NY). The test procedure involved pipetting 22.5 µL of master mix into each well in addition to 2.5 µL of the appropriate template; positive and negative PCR and extraction controls were added to duplicate wells, and the unknown samples were run in triplicates. The cycling program, executed using a StepOne

Real-Time PCR System (Life Technologies), included a 10-min enzyme activation step at 95°C followed by a 2-step PCR, which consisted of 45 cycles (95°C × 15 s, 62°C × 60 s).

Samples were considered positive at a cycle threshold (Ct) of ≤ 38. Any samples crossing the threshold after this cutoff value were retested in triplicate; such samples were considered negative unless at least 1 retested replicate was positive at an equal or smaller Ct and at a higher concentration than the standard curve boundary (i.e., 1 gene copy). Runs were considered valid according to Tetracore if the Ct values for the positive PCR control at 25,000 copies fell within 20 and 26 and if the no-template control did not cross the threshold during the run. Because qPCR will be analyzed as dichotomous (positive or negative) rather than by copy number, this variable will hereafter be referred to as "PCR" in the statistical analysis and related discussion. This distinction allows for more general discussions that are less directly dependent on the specific methodology used in this particular experiment.

2.4 ELISA

Samples were tested using the commercial ParaChek ELISA kit (Prionics, Zurich, Switzerland) according to the manufacturer's protocol. The ELISA OD for each bulk-tank sample was "corrected" by subtracting the value of the average negative control for the corresponding plate to adjust for interplate variation. The protocol is described in more detail by Cazer et al. (2013).

Positive and negative classifications for ELISA titers are not validated for bulk-tank samples because the controls and cutoffs represent standards for individual animals. The ELISA results in the bulk tank are therefore interpretable as a continuous measurement and may serve as a proxy for the average MAP infection status in the herd. A high titer may indicate either elevated herd-level antibody production, and thus an overall high prevalence of MAP, or a small

number of infected cows that nevertheless contribute high concentrations of antibodies to the bulk tank (Cazer et al., 2013). Thus, ELISA OD in the relative scale may be compared with positive and negative PCR outcomes to identify concordance or discordance of results.

2.5 Statistical Analyses

To fully explore the relationship between bulk-tank ELISA (continuous) and bulk-tank PCR (dichotomous), we generated 2 regression models; the questions we sought to answer with each model were related but distinct. First, we hoped to determine whether bulk-tank PCR result could predict ELISA titer after accounting for other variables already known to be related to ELISA, such as season of sampling and protocols for managing MAP-positive cows (Cazer et al., 2013). To address this question, we developed a linear regression model using bulk-tank ELISA OD as the outcome. Second, we attempted to evaluate how management factors affected bulk-milk MAP contamination via the environmental route and whether bulk-tank ELISA titer significantly predicted PCR once environmental parameters were accounted for. For this objective, we treated PCR as the outcome in a binary logistic regression model. All statistical testing was executed using SAS (version 9.4, SAS Institute Inc., Cary, NC). Figures were generated using JMP Pro (version 10, SAS Institute Inc.).

i. Linear Regression Model

Corrected ELISA OD was modeled as the outcome in a multivariable linear regression using a backward, stepwise selection method (PROC GLMSELECT). In addition to PCR result (positive or negative), seasonality and management procedures for Johne's positive cows were included as potential predictors, based upon a previous published model developed by Cazer et al. (2013). Consistent with this model, the *Seasonality* variable was created using the function $Cos(2\pi\frac{Day}{365})$ with "Day" representing the day of the year as a continuous variable ranging from 1

to 365. The *Protocols for managing MAP-positive cows* variable was categorized as either "cull immediately," "cull after dry off or calving," "keep," or "never had a positive Johne's test or clinical Johne's disease."

The variables *State* (New York, Oregon, or Wisconsin), *Herd Size* (> 200, 100–200, or < 100), and *Production Type* (conventional or organic) were forced to remain in the final model due to the importance of these parameters in determining the study population. The herd size variable was treated as purely categorical, rather than ordinal, to allow for the possibility of a nonlinear relationship between the size of a herd and its bulk-tank MAP status. Parameters were generated using reference coding, and the standard assumption that ε_{ij} are $N(0, \sigma^2)$ was assessed. The model was of the form:

$$\hat{Y}_{ELISA} = \alpha + \hat{\beta}_1 X_{\substack{herdsize \\ >200}} + \hat{\beta}_2 X_{\substack{herdsize \\ 100-200}} + \hat{\beta}_3 X_{NY} + \hat{\beta}_4 X_{OR} + \hat{\beta}_5 X_{Conventional} + \hat{\beta}_6 X_{PCR} + \hat{\beta}_k X_{\mathrm{k}}$$

where α is the intercept, $\hat{\beta}_1$ through $\hat{\beta}_k$ are the least-squares parameter estimates for the included covariates, and $\hat{Y}$ is the predicted value of ELISA based upon the values for the resulting model coefficients. $\hat{\beta}_1$and $\hat{\beta}_2$ are the corresponding parameter estimates for herd size (of > 200 and 100-200, respectively, with a herd size of < 100 serving as the reference level). $\hat{\beta}_3$ and $\hat{\beta}_4$correspond to State (NY and OR, respectively, with WI as the reference level), and $\hat{\beta}_5$ corresponds to production type (specifically to conventional farms, with organic farms as the reference level.) $\hat{\beta}_6$ is the parameter estimate for positive PCR result (with a negative result serving as the reference.) The X_k term represents additional significant predictor variables and their associated coefficients ($\hat{\beta}_K$). For the linear model, these potential predictors were *Season of sampling* and *Protocols for managing MAP-positive cows.*

ii. Logistic Regression Model

Variables from the management questionnaire were chosen based upon a potential association with MAP prevalence. Univariable analyses were then conducted as a method of variable selection for the logistic model: the relationship of bulk-tank PCR to continuous variables was evaluated using t-tests (PROC TTEST), whereas the relationship of bulk-tank PCR to binary or polychotomous categorical variables was evaluated using Chi square or Fisher's exact tests (PROC FREQ). Variables associated with PCR and demonstrating statistical significance at the $P < 0.05$ level were marked for inclusion in the multivariable model. No adjustments were made for multiple testing to provide a sufficiently liberal threshold for inclusion. As in the linear model, state (NY, OR, or WI), herd size (> 200, 100–200, or < 100), and production type (conventional or organic) were forced to remain in the model due to the role of these variables in farm selection. Bulk-tank PCR was considered as a binary variable, and the log odds of "PCR positive" were modeled using logistic regression (PROC LOGISTIC) with a penalized maximum likelihood function (Firth's bias adjustment) to correct for quasi-separation of data. Two-way interactions were tested and included in the model providing they offered significant improvement to the model fit (as evaluated using likelihood ratio tests.) The overall fit of the model was evaluated using the Hosmer-Lemeshow goodness-of-fit test. A biologically relevant increase in ELISA OD of 0.1 units was used in odds ratio calculations by exponentiating 0.1 times reference-coded parameter estimates. The form for the logistic model was as follows:

$$ln\left(\frac{P_{\text{PCR positive}}}{P_{\text{PCR negative}}}\right) = \alpha + \hat{\beta}_1 X_{\substack{herdsize \\ >200}} + \hat{\beta}_2 X_{\substack{herdsize \\ 100-200}} + \hat{\beta}_3 X_{NY} + \hat{\beta}_4 X_{OR} + \hat{\beta}_5 X_{Conventional} + \hat{\beta}_6 X_{ELISA} + \hat{\beta}_k X_k$$

where $ln\left(\frac{P_{\text{PCR positive}}}{P_{\text{PCR negative}}}\right)$ represents the natural log of the odds of PCR positivity in the bulk tank (the probability of being PCR positive divided by the probability of being PCR negative) based

upon the values for the logistic model parameters. α is the intercept, and $\hat{\beta}_1$ through $\hat{\beta}_k$ are the Firth-adjusted parameter estimates for the included covariates, estimated by maximum likelihood. $\hat{\beta}_1$and $\hat{\beta}_2$ are the corresponding parameters for *Herd Size* (of > 200 and 100-200, respectively, with a herd size of < 100 serving as the reference level). $\hat{\beta}_3$ and $\hat{\beta}_4$correspond to *State* (NY and OR, respectively, with WI as the reference level), and $\hat{\beta}_5$ corresponds to *Production Type* (specifically to conventional farms, with organic farms as the reference level.) $\hat{\beta}_6$ is the parameter estimate for corrected *ELISA OD* in the bulk tank. The X_k term represents any additional significant predictor variables or interactions between included predictor variables, and their associated coefficients ($\hat{\beta}_k$). In the logistic model, these potential predictors were *Nutritionist use, Fall housing of pre-weaned heifers, Spring housing of pre-weaned heifers, Presence of scours in calves,* and *Feeding calf starter*.

3. Results

3.1 Optimization of Template Preparation and Real-Time PCR

We conducted a variety of optimization experiments using MAP-spiked negative milk. First, 3 beadbeating durations were compared using a Mini-Beadbeater and followed up by DNA purification and qPCR. The durations tested were 1 min (as in Herthnek et al., 2008), 5 min (as in Odumeru et al., 2001), and an intermediate duration of 2 min. A range of OD values (0.04, 0.13, 0.24, 0.34, and 0.43) was implemented for each duration to account for the potential influence of quantity on the latency to disrupt the cells. The differences between the durations were found to be significant in a mixed-design ANOVA (P = 0.0002). For all OD values tested, the Ct values for 1 min were universally higher (corresponding to lower quantities of MAP) than those for 2 and 5 min in the corresponding samples (P = 0.003 and P = 0.0002, respectively, in Tukey's HSD *post-hoc* tests). There was no statistical difference between the 2- and 5-min

durations. Presented with the choice between 2 and 5 min, we selected 5 min of beadbeating; this decision was based upon a numerically lower Ct average and the use of 5 min in several studies in which the cream and pellet were combined (Odumeru et al., 2001; Gao et al., 2005).

Consistent with the findings of other studies (notably Gao et al., 2005, 2007; and Herthnek et al., 2008), we found detectable levels of MAP in both the cream and pellet layers and the best overall signal when the pellet and cream were pooled during template preparation. Both hexadecylpyridinium chloride (Sigma-Aldrich) and a lysis buffer (containing EDTA, NaCl, Tris, and SDS) in combination with proteinase K (Sigma-Aldrich) were evaluated for their ability to maximize recovery of MAP from the cream, with the latter combination providing the best signal. Several heat treatments were also assessed, although none provided a consistent improvement.

In optimization studies using MAP-spiked negative milk, the qPCR performed well and was able to detect dilutions of $10^{-3.5}$ (corresponding to 82.3 CFU/mL) in all plates tested, with an average reported copy number of 3.655 ± 0.028. A dilution of $10^{-4.5}$ (8.23 CFU/mL) amplified in approximately half of the wells tested, although this concentration fell outside of the boundaries established by the standard curve (i.e., the copy number was < 1).

3.2 Questionnaire

With regard to self-reported Johne's-related responses and perception of Johne's disease, 56.5% of farms in the study had observed at least one "clinical Johne's case" since the year 2000; however, only 2.4% of these farms reported confirmation of these cases by veterinary diagnosis. Clinical cases were most often identified by farm staff if the animal showed signs of loose manure (89.7%) or poor body condition (69.1%). At the time of sample collection, 27.7% of farms were participating in a Johne's control program and 16.1% had a written plan for

Johne's disease management. Conventional farms were more likely to participate in a Johne's control program compared with organic farms ($\chi^2_{(df = 1)} = 9.366$, P = 0.002) and were more likely to have a written plan for Johne's disease management ($\chi^2_{(df = 1)} = 3.925$, P = 0.048)

3.3 ELISA

The ELISA tests were conducted on 288 samples. Corrected optical densities (OD_{600}) ranged from −0.098 to 0.368 after subtraction of the negative control, with an average OD of −0.023 ± 0.003. The distribution of ELISA OD results is shown in *Figure 3*.

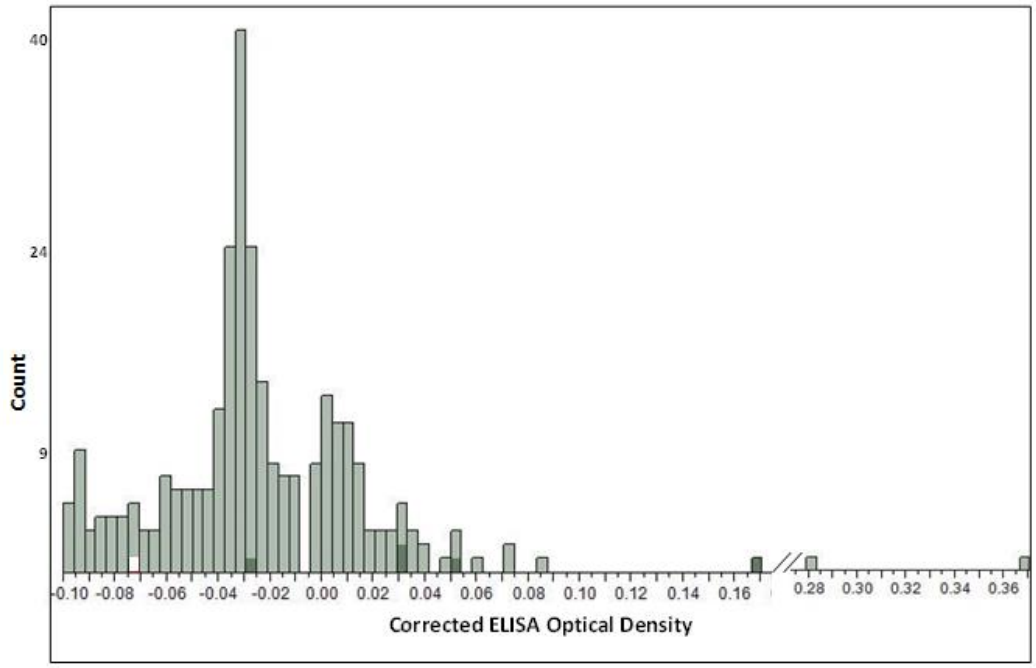

Figure 3. *Frequency Histogram of ELISA OD in Bulk Milk*. Corrected ELISA OD values are shown on the x-axis, with count on the y-axis. Farms with PCR-positive bulk milk are marked in dark gray (organic farms) or white (conventional farm); PCR-negative farms are represented by light gray.

3.4 Real-Time PCR

Real-time PCR was conducted on 286 samples. All runs were valid according to the criteria specified by Tetracore (www.Tetracore.com), and the negative extraction control did not

amplify during any of the runs. The mean R^2 and efficiency values were 0.992 ± 0.002 and 96.441 ± 0.812%, respectively. There were 6 positive samples corresponding to a mean copy number of 3.660 ± 1.522.

The farms with PCR-positive bulk tanks may be categorized in terms of their production type, herd size, and state. Five were organic: 3 from NY with herd sizes < 100, 1 from NY with herd size > 200, and 1 from WI with a herd size of < 100. There was an additional PCR-positive bulk tank from an NY herd, which was a nongrazing conventional farm with a herd size of > 200. The relationship between bulk-tank PCR positivity and bulk-milk ELISA titer is illustrated in *Figure 3*, which is coded according to production type.

3.5 Linear Regression Model

The final linear regression model with corrected ELISA OD as the outcome variable was significant overall ($F = 13.07$, $P < 0.0001$), indicating a rejection of the global null hypothesis that no β estimate is different from 0. The residuals were approximately normally distributed and centered around 0, although there were several outliers. In any case, the GLMSELECT procedure may provide valid approximations in spite of minor normality violations (SAS User's Guide; SAS Institute Inc.). None of the variables forced into the model (*Herd Size*, *Production Type,* or *State*) was a significant predictor of ELISA. The cosine seasonality curve remained highly significant ($P < 0.0001$), retaining the peak in OD in the summer months consistent with the previous model developed by Cazer et al. (2013). The variable *Protocols for managing MAP-infected cows* was no longer significantly associated with ELISA OD and was removed from the model. Notably, *PCR* was significant ($P = 0.002$) and therefore conserved in the final model, which may be expressed as:

$$\hat{Y}_{ELISA} = \alpha + \hat{\beta}_1 X_{\substack{herdsize \\ >200}} + \hat{\beta}_2 X_{\substack{herdsize \\ 100-200}} + \hat{\beta}_3 X_{NY} + \hat{\beta}_4 X_{OR} + \hat{\beta}_5 X_{Conventional} + \hat{\beta}_6 X_{\cos\_season} + \hat{\beta}_7 X_{PCR}$$

The values for the intercept and for $\hat{\beta}_1$ through $\hat{\beta}_7$ and their associated significance levels are provided in *Table 8*.

Table 8. *Linear Regression Model with Corrected Bulk-milk ELISA OD as the Outcome.* Reference-coded parameter estimates, associated standard errors, t ratios, and significance levels are shown. Each significant P value (P < 0.05) is marked with an asterisk.

Term	$\hat{\beta}$	*Std Error*	*t Ratio*	*P>|t|*
Intercept	-0.028	0.004	-6.85	<0.001*
State				
New York	-0.001	0.006	-0.15	0.884
Oregon	-0.001	0.008	-0.15	0.883
Wisconsin	Reference			
Herd size				
>200	-0.012	0.008	-1.43	0.154
100-200	-0.007	0.007	0.88	0.381
<100	Reference			
Production type				
Conventional	0.005	0.005	0.89	0.374
Organic	Reference			
PCR result				
Positive	0.054	0.017	3.13	0.002*
Negative	Reference			
Seasonality				
$Cosine\left(2\pi\frac{day}{365}\right)$	-0.029	0.003	-8.62	<0.0001*

3.6 Univariable Analyses

The variables found to be significantly associated with PCR result following univariable analyses were *ELISA* (corrected OD), *Presence of scours in calves*, *Housing for pre-weaned heifers* (in the fall and spring), *Feeding calf starter*, and *Nutritionist use*. These variables were considered appropriate potential predictors for the logistic regression model because of their previously documented relationship to MAP prevalence. Significance levels from the preliminary univariable analyses are shown in *Table 9*.

Table 9. *Candidate Predictors for the Logistic Regression Model.* The variables shown below were significantly related to bulk-milk PCR result ($P < 0.05$) via the specified univariable analyses and considered eligible for inclusion in the logistic regression model.

Variable	*Description*	*Variable Type*	*Test*	*P value*
Nutritionist	Use of nutritionist (yes/no)	Binary	Fisher's Exact	0.028
Fall Housing	Pre-weaned heifers housed in free stalls, hutches, individual-animal area, multiple-animal area, pasture/drylot, or tied in a barn	Nominal	Chi-square	0.005
Spring Housing	Pre-weaned heifers housed in a group area (multiple animal area, pasture/drylot) compared to an individual or restricted area (hutches, individual animal area, or tied in a barn)	Binary	Fisher's Exact	0.012
Scours	Presence of scours in calves (yes/no)	Binary	Fisher's Exact	0.036
Calf Starter	Calves are provided with calf starter (yes/no)	Binary	Fisher's Exact	0.047
ELISA	Corrected ELISA optical density	Continuous	*t* Test	*P*< |t| 0.004

3.7 Logistic Regression Model

The variables selected in the univariable analyses were considered eligible predictors for inclusion in the Firth-corrected, binary logistic regression model with the logit of PCR positive as the outcome. The variables *Herd Size* (< 100, 100–200, or > 200), *Production Type* (organic or conventional), and *State* (NY, OR, or WI) were forced to remain in the model.

The corrected parameter estimates are shown in *Table 10* in addition to associated significance levels, odds ratio estimates, and 95% confidence intervals. The overall model was significant according to the likelihood ratio test, ($\chi^2_{(df\ 7)} = 24.477$, $P < 0.001$), suggesting that an improved fit is provided relative to a model containing the intercept alone. The model fit the data well as indicated by the Hosmer and Lemeshow goodness-of-fit test ($\chi^2_{(df\ 8)} = 2.579$, $P = 0.958$), and the quasi-nonconvergence was successfully controlled by means of the bias correction.

Table 10. *Firth-corrected Parameter Estimates for the Logistic Regression Model.* Bulk-tank PCR result is the outcome. Specifically, the log odds of being PCR positive in the bulk tank are modeled. Also shown are the standard errors of the estimate, Wald Chi Square statistics with significance levels, and odds ratio estimates (**$\widehat{OR}$**) with 95% Wald confidence intervals (**CI**).

Term	*Parameter Estimate*	*SE*	*Wald* χ^2	*P>* χ^2	$\widehat{OR}$	*95% Wald CI for* $\widehat{OR}$
Intercept	-5.293	1.153	21.063	<0.001	-	-
State						
New York	2.450	1.116	4.819	0.028*	11.591*	1.300, 103.321
Oregon	-0.277	1.726	0.026	0.873	0.758	0.026, 22.341
Wisconsin	Reference	-	-	-	-	-
Herd size						
>200	2.333	0.992	5.530	0.019*	10.305*	1.475, 72.008
100-200	0.284	1.449	0.038	0.845	1.328	0.078, 22.720
<100	Reference	-	-	-	-	-
Production Type						
Conventional	-0.454	0.905	0.252	0.616	-	- [1]
Organic	Reference	-	-	-	-	-
ELISA						
(corrected OD)	36.312	10.356	12.295	0.001*	-	- [1]
ELISA x Production Type						
Conventional						
Organic	-31.609	12.529	6.365	0.012*	1.600 [2]	0.429, 5.966
	Reference	-	-	-	37.756*[2]	4.960, 287.391

*Considered significant at P < 0.05. Odds ratios, computed based upon specified reference levels, are significant if the corresponding Wald CI does not include 1.00

[1]The presence of a significant interaction precludes computation of main-effect odds ratios.

[2]The odds ratios for the interaction were calculated based upon a biologically-relevant 0.1 unit increase in ELISA OD.

Of the forced-entry predictors, there was a significant effect of *State*; specifically, the model demonstrated an increase in the odds of being PCR positive in the bulk tank for farms in NY compared with WI. There was also an effect of *Herd Size*, with the largest herd size category (> 200) showing increased odds relative to the smallest (< 100). Of the other candidate predictors, *ELISA OD* was retained and an interaction was uncovered between *Production Type* and *ELISA OD*. The interaction provided a significant improvement to the model fit, as evaluated using a likelihood ratio test for nested models.

The interaction between bulk-milk ELISA and production type is depicted in *Figure 4.* as ELISA titer increases in organic herds, so too does the value of the linear predictor. This linear predictor is directly related to the probability of bulk-milk PCR positivity (p(PCR +) = $\frac{1}{1+e^{-(linear\ predictor)}}$.) The figure also shows a weaker relationship between the linear predictor and bulk-tank ELISA titer on conventional farms.

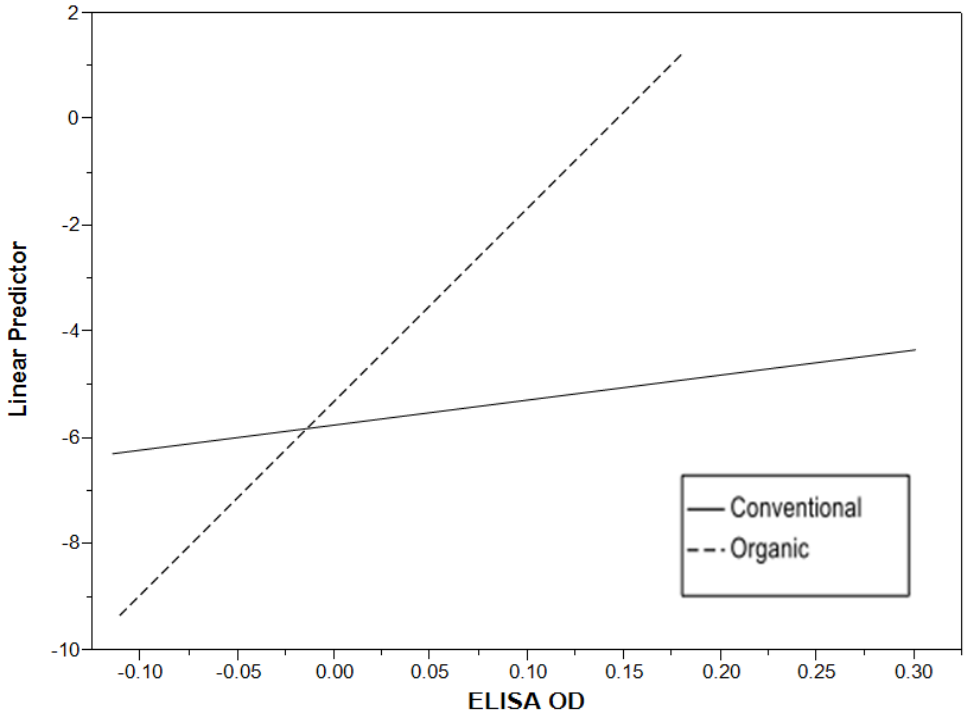

Figure 4. *Representation of the Interaction between Bulk-milk ELISA OD and Production Type (organic vs. conventional) on Bulk-milk PCR Status.* The figure shows the linear predictor on the Y axis and ELISA OD on the X axis. Values of the linear predictor were computed for the reference levels Wisconsin and herd size < 100 at the observed ELISA titers.

The final model, as detailed in *Table 10*, was of the form:

$$ln\left(\frac{P_{\text{PCR positive}}}{P_{\text{PCR negative}}}\right) = \alpha + \hat{\beta}_1 X_{herdsize}_{>200} + \hat{\beta}_2 X_{herdsize}_{100-200} + \hat{\beta}_3 X_{NY} + \hat{\beta}_4 X_{OR} + \hat{\beta}_5 X_{Conventional} + \hat{\beta}_6 X_{ELISA} + \hat{\beta}_7 X_{ELISA*Conventional}$$

where $\hat{\beta}_7$ represents the coefficient for an interaction between bulk-milk *ELISA* and *Production Type* (with organic farms again serving as the reference level for the interaction).

4. Discussion

Our data demonstrate a strong relationship between ELISA and PCR results in bulk-tank milk. In the logistic model, ELISA was an important predictor of PCR status, particularly on organic farms, even after consideration of more than 300 relevant environmental and management factors. Additionally, and as expected, PCR significantly predicted ELISA in the linear model after controlling for seasonality.

Specifically, these results point to a substantial level of concordance between positive PCR outcomes and high ELISA OD in the same bulk-tank samples. Although the possibility of environmental contamination cannot be excluded on these farms, paired high bulk-tank ELISA and positive PCR results allude to the internal route, likely with a high prevalence. Direct shedding by late-stage MAP-infected cows may therefore be the dominant source of bulk-milk contamination on these farms, because antibody production occurs mainly in Progressors (Schukken et al., 2015) and in the later stages of infection when MAP is systemically present (Streeter et al., 1995). It may be assumed that such late-stage MAP-infected animals are shedding MAP directly into their milk, because there is a strong trend for an increased ELISA titer with an increased shedding category (Schukken et al., 2015) and a reported high agreement between milk ELISA results and milk culture from individual animals (Singh et al., 2007).

Although studies such as Wilson et al. (2010) have called attention to a certain level of discordance between PCR and ELISA diagnostics in bulk-tank milk samples, this relationship hinges on a positive/negative designation for ELISA results. Because the standards on ELISA plates are based upon individual-animal levels, antibodies in the bulk tank may be diluted

beyond the “positive” threshold yet still indicate a low prevalence of MAP antibody-positive cows.

Nevertheless, the concordance in our data does not preclude discordance on some farms; despite the demonstrable agreement between PCR positives and high ELISA titers in our bulk-tank samples, the majority of high ELISA farms were PCR negative. Although falsely high bulk-milk ELISA cannot be completely ruled out, the high ELISA–negative PCR profile suggests a general presence of MAP in these herds but little direct excretion into the bulk tank. Importantly, high ELISA farms that are PCR negative indicate an apparent lack of environmental contamination; the implication is that farms in NY, OR, and WI are largely able to maintain proper hygiene and management practices to ensure a low bacterial load in the bulk tank and thus low-risk milk, despite an estimated high prevalence.

The slight positive skew of the corrected bulk-milk ELISA titers (as apparent in *Figure 3*) suggests that the majority of farms have ELISA values at the lower end of the spectrum. The low ELISA farms are almost universally PCR negative in the bulk tank, a combination that is ideal with respect to bulk-milk status. An anomaly in the data set was a bulk-tank PCR-positive farm with a relatively low ELISA titer of −0.072 (roughly the 10th percentile). Farms with this PCR positive–low ELISA profile may house a small number of infected cows that nevertheless contaminate the bulk tank by means of the environmental route (Cazer et al., 2013), because internal-route contamination with MAP would likely be accompanied by antibody secretion. It is conceivable that early-stage, intermittently shedding cows may be shedding the bacteria directly into milk before the development of detectable antibody levels. However, this effect would be more plausibly observed in individual animals rather than at the herd level. In order for such a herd-level effect to be apparent, the majority of the lactating herd would need to be in the same

phase of infection and exhibit similar shedding patterns into milk. This likelihood seems low, as van Schaik et al. (2003) noted that kinetics ELISA results (for serum) were highly variable at the cow level. The between-cow variability is likely present with respect to milk antibodies as well. Although a positive fecal culture is generally understood to precede a positive ELISA (van Schaik et al., 2003), the latency to develop an antibody response following the onset of shedding is usually restricted to several months. Little information exists on the sequential relationship of direct shedding of MAP into milk and the humoral immune response, but it is improbable that the animals in a herd would be in precisely the same phase relative to milk antibody production and direct shedding into milk.

Bulk-milk ELISA results appear to be beneficial for identifying high-risk farms for potential zoonotic transmission (particularly in the event that further evidence links MAP to Crohn's disease development). However, ELISA is not a perfect predictor of bulk-milk contamination, as demonstrated in previous work (Van Weering et al., 2007) and as evidenced by the farms in the current study displaying high levels of MAP antibodies in the bulk tank without the apparent presence of the pathogens themselves. A combination of bulk-tank ELISA and PCR results should therefore provide a better tool for potential zoonotic risk assessment than ELISA alone. The Tetracore VetAlert Real-Time PCR and extraction kits (www.Tetracore.com) appear to be useful diagnostics that function well not only for fecal samples (Alinovi et al., 2009) but also for bulk-tank milk samples. Successful optimization of the template preparation methods and beadbeating durations relied on the observation that MAP fractionates to both the pellet and cream layers (Gao et al., 2007; Herthnek et al., 2008). Following optimization, we were able to use the Tetracore kits to reliably detect MAP concentrations > $10^{-4.5}$ (corresponding to 8.23

CFU/mL) in bulk-tank samples, with the caveat that optimization was conducted using spiked milk rather than with positive farm samples.

Variation exists regarding the rate of bulk-tank positivity as detected by PCR. At one end of the spectrum, Khol et al. (2013) found a 0% positive rate in bulk-tank samples throughout their study of low-prevalence Austrian herds. The rate of 2.10% sample positivity for our PCR results (with a 5.16% rate for NY farms) appears consistent with rates reported in numerous other studies, including Bosshard et al. (2006; 3.00%) and Cetinkaya et al. (1996; 3.50%), although several studies have reported a higher prevalence based on bulk-tank PCR. For example, Corti and Stephan (2002) reported a 19.7% positive rate using IS900 PCR for bulk-tank samples collected from different regions in Switzerland, with a region-dependent prevalence ranging from 1.7 to 49.2%.

The bulk-tank PCR-positive rate is undoubtedly affected by region, method, and gene target used for detection. There are specificity issues linked to PCR based on the IS900 target because highly homologous sequences have been identified in other environmental mycobacteria (Bosshard et al., 2006; Slana et al., 2008). The Tetracore qPCR targets the *hspX* gene, which is thought to be involved in intracellular survival and occurs as a single copy in the MAP genome; its presence in single-copy form facilitates quantification. Compared with IS900, the *hspX* target is less prone to false-positive results, improving reliability (Slana et al., 2008). When used on fecal samples, the Tetracore test has a specificity of 97% (Alinovi et al., 2009). Because the test is commercially available, it fits the criteria of being a simple and rapid option for herd-level testing.

Milk-filter testing has also been explored as a potential herd-level indicator of MAP presence. Slana et al. (2012) concluded that qPCR testing of milk filters could be beneficial for

identifying MAP, in addition to providing a rough prevalence estimate for a given farm. This technique could be classified as both inexpensive and simple, because commercial test kits may be used and the filter is readily accessible. Yet, the presence of MAP on the milk filter likely does not correlate perfectly with its presence in bulk milk. Given the practice of feeding waste milk to calves and the potential relationship of MAP and human Crohn's disease, evaluation of the bulk milk itself may prove more informative.

Used in combination with bulk-milk ELISA, bulk-milk PCR results may provide a useful indication of the MAP status of the herd and the routes of contamination on a given farm. Although ELISA OD has been considered an imprecise tool to evaluate the status of individual animals, the titers do indeed appear to be useful indicators of overall herd MAP shedding level. Lombard et al. (2006) found that the herd-level sensitivity for the Parachek ELISA was comparable to that of serum ELISA and ranged from 56 to 83% (when fecal culture was used as a reference for infection status). The application of ELISA for bulk-tank milk has not yet been extensively explored, but some information can be obtained on the sensitivity and specificity of bulk-milk diagnostics. Van Weering et al. (2007) were able to test these parameters for the Pourquier ELISA using bulk-tank samples from herds with known seroprevalence. In herds with 3% or greater seroprevalence, the sensitivity was 85% and the specificity 96% (using a cutoff revised for bulk-tank samples.) Thus, the positive and negative predictive values were, respectively, 67 and 94%. Nielsen et al. (2000) reported 97.1% sensitivity and 83.3% specificity for a milk ELISA (using antibodies from Allied Monitors, Fayette, MO) adapted for bulk-tank use. Despite the high sensitivity, Nielsen et al. (2000) argued that the diagnostic test had limitations due to the ability of small variations in the cutoff to dramatically affect the estimated prevalence. This observation provides support for the interpretation of bulk-tank ELISA as a

continuous variable to scale the risk of MAP infection in the herd, rather than in relation to a cutoff level.

There is some reported variation in the sensitivity and specificity of milk ELISA based upon the method used, and sensitivity may be increased through the use of modified protocols (Cazer et al., 2013). McKenna et al. (2005) compared absorbed and unabsorbed indirect assays evaluated against tissue and fecal cultures. The unabsorbed ELISA had increased sensitivity but this advantage came at the expense of lower test accuracy and specificity. The negotiation and compromise between sensitivity and specificity can be obviated in part by interpreting the ELISA values as numerical rather than dichotomous, indicative of the probability of herd MAP infection. However, it is difficult to completely avoid the sources of variability resulting from the kit or method used for milk ELISA, particularly with respect to a disease such as Johne's, because no "gold standard" exists for reference.

Bulk-tank PCR results on high ELISA farms may depend upon whether the farm is organic or conventional: we uncovered a potential interaction in the logistic model between ELISA and production type. The interaction revealed a strong linear relationship between ELISA titer and the logit of PCR positivity for organic farms and essentially an absence of a relationship between bulk-milk ELISA titer and PCR result for conventional farms. According to the logistic model, a biologically relevant increase of 0.1 units in ELISA OD on organic farms resulted in a 37.76 multiplicative increase in the odds of being PCR positive in the bulk tank. For conventional farms, the same 0.1-unit change would yield instead an increase of only 1.60. This may suggest that, in our data, conventional farms with a high MAP prevalence are better equipped to temper environmental contamination. Indeed, conventional farms in our study were

more likely to participate in a Johne's control program and have a written plan for Johne's disease management, compared with organic farms.

Another possible explanation may involve the age and retention rate of animals. Stiglbauer et al. (2013) concluded that the mean percentage of first-lactation animals was lower on organic compared to conventional farms. This study appears to affirm the notion that organic farms tend to retain older animals and perhaps have an increased risk of age-related disease (Stiglbauer et al., 2013). As Johne's disease is progressive, subclinically infected cows retained for a longer period may become heavy shedders or develop clinical Johne's and thus increase the odds of both direct shedding of MAP into milk and environmental contamination. The interaction is graphically represented in *Figure 4*. For organic farms, bulk-tank ELISA titer increased alongside values of the linear predictor, which were related to the probability of PCR positivity in the bulk tank. On conventional farms, on the other hand, the values for the linear predictor, and thus the associated probabilities, remained relatively constant across the differing levels of ELISA titer. Consequently, a high ELISA titer seems to better predict positive bulk-tank PCR status on organic compared with conventional farms.

The interaction was included in the model due to its significance level; however, as shown in *Figure 3*, all but one of the PCR-positive bulk tanks were from organic farms. The absence of a relationship between bulk-milk ELISA and PCR results for conventional farms may reflect a lack of data on this subgroup rather than a true difference. There was nevertheless a strong relationship between ELISA titer and PCR status in bulk milk, at least with respect to organic farms, and the possibility of a true interaction cannot be excluded. Moreover, this is not a case of opposing PCR–ELISA relationships observed for the different farm types: the relationship between bulk-tank ELISA and PCR for both conventional and organic farms

manifests in the same direction (i.e., both slopes are positive). Therefore, the main conclusions regarding the PCR–ELISA relationship in the bulk tank remain the same regardless of whether the interaction is included in the model.

As indicated, all but one PCR-positive bulk tanks were from organic farms. Although the main effect of production type was nonsignificant in our logistic model, it should not necessarily be interpreted as such, due to the presence of an interaction. Conventional farms may be able to keep prevalence low by the use of milk replacer rather than pasteurized or unpasteurized milk from the herd. There is no approved commercially available organic milk replacer (Stiglbauer et al., 2013). Stiglbauer et al. (2013) noted that management on conventional and organic dairies differed insofar as conventional farms made use of outside resources such as the DHIA and veterinarians (also observed by Zwald et al., 2004) much more frequently than did organic farms. Additionally, conventional farms more often called in nutritionists, and cattle on these farms were fed almost twice as much grain as were cattle on organic dairies (Stiglbauer et al., 2013). In our study, the univariable analyses seemed to suggest a relationship between nutrition (in particular, the use of a nutritionist and the provisioning of calf starter) and negative bulk-tank PCR status. It has been suggested that calves are more likely to sustain MAP infection if they have not been provided with adequate nutrition (Doyle, 1956). Thus, Sorge et al. (2012) hypothesized that the combination of poor nutrition, which would heighten calf susceptibility, and exposure to fecal pathogens may lead to an increase in herd MAP prevalence. The potential connections between MAP infection, nutrition, and production type are interesting to consider and may certainly be explored further.

With respect to geographical distribution, all but one of the farms in our data set with PCR-positive bulk milk were located in New York State. Moreover, the NY location was

significantly related to bulk-tank PCR status according to the logistic model. When compared with WI herds, farms in NY had 11.59 times greater odds of being PCR positive in the bulk tank. This finding does not align with results of previous studies indicating that a positive MAP status (Wells and Wagner, 2000) or environmental contamination with MAP (Lombard et al., 2006) is more common in Midwestern herds. However, the diagnostics used by Wells and Wagner (2000) were serological ELISA on individual animals, paired with prior knowledge regarding clinical signs in the herd. Although Lombard et al. (2006) found a regional difference in environmental-sample culture results, no such difference was uncovered according to fecal culture or serum and milk ELISA. Certainly, more information is needed regarding the geographic distribution of bulk-milk MAP contamination. The findings of our study may provide the first clue regarding the geographical patterns of MAP-positive bulk-tank samples from US herds.

Although several groups have found that the effect of herd size on infection prevalence may be small and region-dependent (Collins et al., 1994) or negligible (van Schaik et al., 2003), there is much supporting evidence to corroborate an increased MAP prevalence among larger herds (e.g., Wells and Wagner, 2000; Crossley et al., 2005). Although no effect of herd size was uncovered in our linear model, farms with a herd size of > 200 (compared with < 100) had significantly higher odds in the logistic model of being PCR positive in the bulk tank. The lack of difference between small and medium-sized farms suggests a nonlinear relationship and the possibility of a threshold level to breach. Descriptively, large farms accounted for only 13.36% of farms in the study, but 33.34% of PCR positive farms were in this large herd-size category. Hypotheses for why a large herd size might be related to higher MAP levels are generally speculative. According to Crossley et al. (2005), the probability of having a subclinically infected cow in the herd is greater for larger farms, and the infection status of larger herds may

be more difficult to monitor. Additionally, because larger herds may have increased stocking densities, MAP exposure based on relative contact could be heightened.

Wells and Wagner (2000) noted an association between the housing style for pre-weaned heifer calves and herd-level MAP prevalence. In the univariable analysis stage, we identified an association between fall housing of pre-weaned heifers in a multiple-animal pen and a PCR positive bulk-tank status. For spring housing, the difference was not as pronounced; however, a positive bulk-milk PCR was associated with farms on which the housing system implied calf-calf or cow-calf contact (i.e., multiple animal areas, or pasture/drylot) compared with farms practicing individual or restrictive housing (i.e., individual animal area, individual hutch, or tied in a barn).

Another variable found to be significant at the univariable stage was presence of scours in calves. Sorge et al. (2012) reported a lower incidence of calf scours among herds that tested negative for MAP antibodies (via serum ELISA) compared with test-positive herds. This finding is consistent with observations of van Roermund et al. (2007), who noted that calves, once infected, are able to horizontally transmit the infection to other calves in a relatively short period, likely by the fecal–oral route. In a study by Mortier et al. (2014), calves (1 year of age and younger) experimentally inoculated with MAP showed a peak in MAP fecal shedding 2 months after inoculation, with the first fecal positive at 0.5 months, supporting the hypothesis of calf-to-calf transmission. The researchers posited that transmission potential between calves would understandably be heightened in a group-housing system. Although these types of relationships were not found to be significant in our multivariable logistic model, perhaps due to the necessary inclusion of several study design variables, they seem worth considering as potential risk factors for bulk milk contamination.

To fully capture the effect of environmental predictors on bulk-tank MAP status, more in-depth investigation of within-herd prevalence may be required. An advantage to a cross-sectional study such as this one is that it provides a snapshot of a variety of parameters from a large number of distinct farm environments; however, data of this type do not allow for investigation of within-herd dynamics over time.

Data obtained in a cross-sectional manner, without the benefit of repeated sampling, may be more susceptible to misclassification, particularly in low-prevalence herds (Lavers et al., 2013). Hence, longitudinal research on the association between within-herd prevalence, environmental load, bulk-milk ELISA, and bulk-milk PCR, is warranted. Lavers et al. (2013) longitudinally investigated the relationship between within-herd prevalence (determined by pooled fecal culture) and environmental culture results in a subset of Canadian herds; logically, the sensitivity of the environmental cultures increased alongside the level of within-herd fecal culture positives. Equivalent conclusions have been reached with respect to sensitivity of milk ELISA testing and fecal culture; specifically, as within-herd prevalence increases, so too does herd-level sensitivity for individual milk ELISA tests (McKenna et al., 2005; Lavers et al., 2014). How the relationship between bulk-tank PCR and ELISA results might differ based upon the within-farm prevalence and level of environmental burden has not yet been investigated. Additionally, it remains to be explored how the bulk-tank PCR–ELISA relationship could shift as herds alter management practices and change subtly in prevalence over time.

5. Conclusions

A substantial level of concordance between bulk-milk MAP ELISA and PCR in 286 samples was corroborated by fitting 2 regression models. Despite the agreement between high ELISA titers and positive PCR results in the bulk tank, the majority of high-ELISA farms were

PCR negative, suggesting that such farms are largely able to maintain satisfactory hygiene and management despite an on-farm presence of MAP. Therefore, ELISA is not a perfect predictor of bulk-milk contamination with MAP pathogens themselves and is best used in combination with bulk-milk PCR to identify high-risk farms. An interaction may exist between production type and ELISA result on PCR results: organic farms demonstrated a higher probability of PCR-positive status with increasing ELISA titer compared with conventional farms. Additionally, a PCR-positive bulk tank was associated with large herd sizes and with a New York State location.

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7. References

Alinovi, C. A., M. P. Ward, T. L. Lin, G. E. Moore, and C. C. Wu. 2009. Real-time PCR, compared to liquid and solid culture media and ELISA, for the detection of *Mycobacterium avium* subsp. *paratuberculosis*. Vet. Microbiol. 136:177–179.

Bosshard, C., R. Stephan, and T. Tasara. 2006. Application of an F57 sequence-based real-time PCR assay for *Mycobacterium paratuberculosis* detection in bulk tank raw milk and slaughtered healthy dairy cows. J. Food Prot. 69:1662–1667.

Cazer, C. L., R. M. Mitchell, K. M. Cicconi-Hogan, M. Gamroth, R. M. Richert, P. L. Ruegg, and Y. H. Schukken. 2013. Associations between *Mycobacterium avium* subsp. *paratuberculosis* antibodies in bulk tank milk, season of sampling and protocols for managing infected cows. BMC Vet. Res. 9:234–240.

Cetinkaya, B., K. Egan, D. A. Harbour, and K. L. Morgan. 1996. An abattoir-based study of the

prevalence of subclinical Johne's disease in adult cattle in south west England. Epidemiol. Infect. 116:373–379.

Cicconi-Hogan, K. M., M. Gamroth, R. Richert, P. L. Ruegg, K. E. Stiglbauer, and Y. H. Schukken. 2013. Associations of risk factors with somatic cell count in bulk tank milk on organic and conventional dairy farms in the United States. J. Dairy Sci. 96:3689–3702.

Collins, M. T. 2011. Diagnosis of paratuberculosis. Vet. Clin. North Am. Food Anim. Pract. 27:581–591.

Collins, M. T., D. C. Sockett, W. J. Goodger, T. A. Conrad, C. B. Thomas, and D. J. Carr. 1994. Herd prevalence and geographic distribution of, and risk factors for, bovine paratuberculosis in Wisconsin. J. Am. Vet. Med. Assoc. 204:636–641.

Corti, S., and R. Stephan. 2002. Detection of *Mycobacterium avium* subsp. *paratuberculosis* specific IS900 insertion sequences in bulk-tank milk samples obtained from different regions throughout Switzerland. BMC Microbiol. 2:15–21.

Crossley, B. M., F. J. Zagmutt-Vergara, T. L. Fyock, R. H. Whitlock, and I. A. Gardner. 2005. Fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* by dairy cows. Vet. Microbiol. 107:257–263.

Doyle, T. M. 1956. Johne's disease. Vet. Rec. 68:869–878.

Eisenberg, S. W., R. Chuchaisangrat, M. Nielen, and A. Koets. 2013. Relationship between presence of cows with milk positive for *Mycobacterium avium* subsp. *paratuberculosis*-specific antibody by enzyme-linked immunosorbent assay and viable *M. avium* subsp. *paratuberculosis* in dust in cattle barns. Appl. Environ. Microbiol. 79:5458–5464.

Eltholth, M. M., V. R. Marsh, S. Van Winden, and F. J. Guitian. 2009. Contamination of food products with *Mycobacterium avium paratuberculosis*: A systematic review. J. Appl. Microbiol. 107:1061–1071.

Gao, A., L. Mutharia, M. Raymond, and J. Odumeru. 2007. Improved template DNA preparation procedure for detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk by PCR. J. Microbiol. Methods 69:417–420.

Gao, A., J. Odumeru, M. Raymond, and L. Mutharia. 2005. Development of improved method for isolation of *Mycobacterium avium* subsp. *paratuberculosis* from bulk tank milk: Effect of age of milk, centrifugation, and decontamination. Can. J. Vet. Res. 69:81–87.

Grant, I. R., H. J. Ball, and M. T. Rowe. 2002. Incidence of *Mycobacterium paratuberculosis* in bulk raw and commercially pasteurized cows' milk from approved dairy processing establishments in the United Kingdom. Appl. Environ. Microbiol. 68:2428–2435.

Grant, I. R., A. G. Williams, M. T. Rowe, and D. D. Muir. 2005. Efficacy of various

pasteurization time-temperature conditions in combination with homogenization on inactivation of *Mycobacterium avium* subsp. *paratuberculosis* in milk. Appl. Environ. Microbiol. 71:2853–2861.

Herthnek, D., S. S. Nielsen, A. Lindberg, and G. Bolske. 2008. A robust method for bacterial lysis and DNA purification to be used with real-time PCR for detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk. J. Microbiol. Methods 75:335–340.

Khol, J. L., M. Wassertheurer, E. Sodoma, S. Revilla-Fernandez, J. Damoser, E. Osterreicher, M. Dunser, U. Kleb, and W. Baumgartner. 2013. Long-term detection of *Mycobacterium avium* subsp. *paratuberculosis* in individual and bulk tank milk from a dairy herd with a low prevalence of Johne's disease. J. Dairy Sci. 96:3517–3524.

Lavers, C. J., H. W. Barkema, I. R. Dohoo, S. L. B. McKenna, and G. P. Keefe. 2014. Evaluation of milk ELISA for detection of *Mycobacterium avium* subsp. *paratuberculosis* in dairy herds and association with within-herd prevalence. J. Dairy Sci. 97:299–309.

Lavers, C. J., S. L. McKenna, I. R. Dohoo, H. W. Barkema, and G. P. Keefe. 2013. Evaluation of environmental fecal culture for *Mycobacterium avium* subsp. *paratuberculosis* detection in dairy herds and association with apparent within-herd prevalence. Can.Vet. J. 54:1053–1060.

Li, L., J. P. Bannantine, Q. Zhang, A. Amonsin, B. J. May, D. Alt, N. Banerji, S. Kanjilal, and V. Kapur. 2005. The complete genome sequence of *Mycobacterium avium* subsp. *paratuberculosis*. Proc. Natl. Acad. Sci. USA 102:12344–12349.

Lombard, J. E., I. A. Gardner, S. R. Jafarzadeh, C. P. Fossler, B. Harris, R. T. Capsel, B. A. Wagner, and W. O. Johnson. 2013. Herd-level prevalence of *Mycobacterium avium* subsp. *paratuberculosis* infection in United States dairy herds in 2007. Prev.Vet. Med. 108:234–238.

Lombard, J. E., B. A. Wagner, R. L. Smith, B. J. McCluskey, B. N. Harris, J. B. Payeur, F. B. Garry, and M. D. Salman. 2006. Evaluation of environmental sampling and culture to determine *Mycobacterium avium* subsp. *paratuberculosis* distribution and herd infection status on US dairy operations. J. Dairy Sci. 89:4163–4171.

Manning, E. J., and M. T. Collins. 2001. *Mycobacterium avium* subsp. *paratuberculosis*: Pathogen, pathogenesis and diagnosis. Rev. Sci. Tech. 20:133–150.

McKenna, S. L. B., G. P. Keefe, H. W. Barkema, and D. C. Sockett. 2005. Evaluation of three ELISAs for *Mycobacterium avium* subsp. *paratuberculosis* using tissue and fecal culture as comparison standards. Vet. Microbiol. 110:105–111.

Mortier, R. A., H. W. Barkema, K. Orsel, R. Wolf, and J. De Buck. 2014. Shedding patterns of

dairy calves experimentally infected with *Mycobacterium avium* subsp. *paratuberculosis*. Vet. Res. 45:71–80.

Nielsen, S. S., S. M. Thamsborg, H. Houe, and V. Bitsch. 2000. Bulk tank milk ELISA antibodies for estimating the prevalence of paratuberculosis in Danish dairy herds. Prev. Vet. Med. 44:1–7.

Odumeru, J., A. Gao, S. Chen, M. Raymond, and L. Mutharia. 2001. Use of the bead beater for preparation of *Mycobacterium paratuberculosis* template DNA in milk. Can. J. Vet. Res. 65:201–205.

Over, K., P. G. Crandall, C. A. O'Bryan, and S. C. Ricke. 2011. Current perspectives on *Mycobacterium avium* subsp. *paratuberculosis*, Johne's disease, and Crohn's disease: A review. Crit. Rev. Microbiol. 37:141–156.

Pickup, R. W., G. Rhodes, T. J. Bull, S. Arnott, K. Sidi-Boumedine, M. Hurley, and J. Hermon-Taylor. 2006. *Mycobacterium avium* subsp. *paratuberculosis* in lake catchments, in river water abstracted for domestic use, and in effluent from domestic sewage treatment works: Diverse opportunities for environmental cycling and human exposure. Appl. Environ. Microbiol. 72:4067–4077.

Richert, R. M., K. M. Cicconi, M. J. Gamroth, Y. H. Schukken, K. E. Stiglbauer, and P. L. Ruegg. 2013. Management factors associated with veterinary usage by organic and conventional dairy farms. J. Am. Vet. Med. Assoc. 242:1732–1743.

Ruzante, J. M., I. A. Gardner, J. S. Cullor, W. L. Smith, J. H. Kirk, and J. M. Adaska. 2008. Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from waste milk delivered to California calf ranches. Foodborne Pathog. Dis. 5:681–686.

Schukken, Y. H., R. H. Whitlock, D. Wolfgang, Y. T. Grohn, A. Beaver, J. VanKessel, M. Zurakowski, and R. Mitchell. 2015. Longitudinal data collection of *Mycobacterium avium* subsp. *paratuberculosis* infections in dairy herds: The value of precise field data. Vet. Res. 46:65–75.

Singh, S. V., A. V. Singh, R. Singh, K. S. Sandhu, P. K. Singh, J. S. Sohal, V. K. Gupta, and V. S. Vihan. 2007. Evaluation of highly sensitive indigenous milk ELISA kit with fecal culture, milk culture and fecal-PCR for the diagnosis of bovine Johne's disease (BJD) in India. Comp. Immunol. Microbiol. Infect. Dis. 30:175–186.

Slana, I., P. Kralik, A. Kraklova, V. Babak, and I. Pavlik. 2012. Short communication: Examination of milk filters by real-time PCR as a herd-level indicator of the presence of *Mycobacterium avium* subsp. *paratuberculosis* in dairy herds. J. Dairy Sci. 95:1162–1165.

Slana, I., F. Paolicchi, B. Janstova, P. Navratilova, and I. Pavlik. 2008. Detection methods for

Mycobacterium avium subsp. *paratuberculosis* in milk and milk products: A review. Vet. Med. Czech 53:283–306.

Sorge, U. S., K. Lissemore, A. Godkin, J. Jansen, S. Hendrick, S. Wells, and D. F. Kelton. 2012. Risk factors for herds to test positive for *Mycobacterium avium* subsp. *paratuberculosis*-antibodies with a commercial milk enzyme-linked immunosorbent assay (ELISA) in Ontario and western Canada. Can. Vet. J. 53:963–970.

Stiglbauer, K. E., K. M. Cicconi-Hogan, R. Richert, Y. H. Schukken, P. L. Ruegg, and M. Gamroth. 2013. Assessment of herd management on organic and conventional dairy farms in the United States. J. Dairy Sci. 96:1290–1300.

Streeter, R. N., G. F. Hoffsis, S. Bech-Nielsen, W. P. Shulaw, and D. M. Rings. 1995. Isolation of *Mycobacterium paratuberculosis* from colostrum and milk of subclinically infected cows. Am. J. Vet. Res. 56:1322–1324.

Sung, N., and M. T. Collins. 1998. Thermal tolerance of *Mycobacterium paratuberculosis*. Appl. Environ. Microbiol. 64:999–1005.

Sweeney, R. W., R. H. Whitlock, and A. E. Rosenberger. 1992. *Mycobacterium paratuberculosis* cultured from milk and supramammary lymph nodes of infected asymptomatic cows. J. Clin. Microbiol. 30:166–171.

van Roermund, H. J. W., D. Bakker, P. T. J. Willemsen, and M. C. M. De Jong. 2007. Horizontal transmission of *Mycobacterium avium* subsp. *paratuberculosis* in cattle in an experimental setting: Calves can transmit the infection to other calves. Vet. Microbiol. 122:270–279.

van Schaik, G., Y. H. Schukken, C. Crainiceanu, J. Muskens, and J. A. VanLeeuwen. 2003. Prevalence estimates for paratuberculosis adjusted for test variability using Bayesian analysis. Prev. Vet. Med. 60:281–295.

Van Weering, H., G. Van Schaik, A. Van Der Meulen, M. Waal, P. Franken, and K. Van Maanen. 2007. Diagnostic performance of the Pourquier ELISA for detection of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* in individual milk and bulk milk samples of dairy herds. Vet. Microbiol. 125:49–58.

Weber, M. F., M. Nielen, A. G. Velthuis, and H. J. van Roermund. 2008. Milk quality assurance for paratuberculosis: Simulation of within-herd infection dynamics and economics. Vet. Res. 39:12–31.

Wells, S. J., and B. A. Wagner. 2000. Herd-level risk factors for infection with *Mycobacterium paratuberculosis* in US dairies and association between familiarity of the herd manager with the disease or prior diagnosis of the disease in that herd and use of preventive measures. J. Am. Vet. Med. Assoc. 216:1450–1457.

Whan, L. B., I. R. Grant, H. J. Ball, R. Scott, and M. T. Rowe. 2001. Bactericidal effect of chlorine on *Mycobacterium paratuberculosis* in drinking water. Lett. Appl. Microbiol. 33:227–231. Whittington, R. J., I. B. Marsh, and L. A. Reddacliff. 2005. Survival of *Mycobacterium avium* subsp. *paratuberculosis* in dam water and sediment. Appl. Environ. Microbiol. 71:5304–5308.

Wilson, D. J., K. Rood, P. Biswas, and T. M. Byrem. 2010. Herd-level prevalence of Johne's disease in Utah and adjacent areas of the intermountain west as detected by a bulk-tank milk surveillance project. J. Dairy Sci. 93:5792–5797.

Wynne, J. W., T. Seemann, D. M. Bulach, S. A. Coutts, A. M. Talaat, and W. P. Michalski. 2010. Resequencing the *Mycobacterium avium* subsp. *paratuberculosis* K10 genome: Improved annotation and revised genome sequence. J. Bacteriol. 192:6319–6320.

Zwald, A. G., P. L. Ruegg, J. B. Kaneene, L. D. Warnick, S. J. Wells, C. Fossler, and L. W. Halbert. 2004. Management practices and reported antimicrobial usage on conventional and organic dairy farms. J. Dairy Sci. 87:191–201.

CHAPTER 4:
LONGITUDINAL RELATIONSHIP BETWEEN FECAL CULTURE, FECAL QUANTITATIVE PCR, AND MILK ELISA IN *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS*-INFECTED COWS FROM LOW-PREVALENCE DAIRY HERDS

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KEYWORDS: *Mycobacterium avium* subsp. *paratuberculosis*, Johne's disease, milk ELISA, fecal quantitative PCR, fecal culture

ABSTRACT

Mycobacterium avium subsp. *paratuberculosis* (MAP), the causative agent of ruminant Johne's disease, presents a particular challenge with regard to infection mitigation on dairy farms. Diagnostic testing strategies to identify and quantify MAP and associated antibodies are imperfect, and certain facets of the relationship between diagnostic tests remain to be explored. Additional repeated-measures data from known infected animals are needed to complement the body of cross-sectional research on Johne's disease testing methods. Statistical models that accurately account for multiple diagnostic results, while adjusting for the effects of individual animals and herds over time, can provide a more detailed understanding of the interplay between diagnostic outcomes. Further, test results may be considered as continuous wherever possible, so as to avoid the information loss associated with dichotomization.

To achieve a broader understanding of the relationship between diagnostic tests, we collected a large number of repeated fecal and milk samples from 14 infected cows, in addition to bulk milk samples, from 2 low-prevalence dairy herds in the Northeast United States. Predominately through the use of mixed linear modeling, we identified strong associations between milk ELISA optical density, fecal qPCR, and fecal culture in individual animals, while concurrently adjusting for variables that could alter these relationships. Notably, we uncovered subtleties in the predictive abilities of fecal shedding level on milk ELISA results, with animals categorized as disease "Progressors" reaching higher ELISA optical density levels. Moreover, we observed that spikes in fecal shedding could predict subsequent high ELISA values up to 2 months later. We also investigated the presence of MAP in individual milk samples via PCR and noted an association between poor udder hygiene and MAP positivity in milk, suggesting some level of environmental contamination. The paucity of positive milk samples and the complete

absence of detectable MAP in the bulk tank throughout the study period indicate that contamination of milk with MAP may not be of chief concern in low-prevalence herds. An enhanced understanding of the interrelationships between diagnostic tests can only benefit the development of testing strategies and objectives, which in turn may lessen MAP infection prevalence in dairy herds.

1. Introduction

Johne's disease is a progressive enteritis of ruminant animals caused by the bacterium *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The primary clinical manifestations of Johne's disease in cattle are loose manure and weight loss, leading to nutrient malabsorption, dehydration, and severe wasting (see Manning and Collins, 2001). MAP is of particular import on dairy farms where subclinical animals may escape detection for a period of years due to lengthy incubation periods and intermittent bacterial shedding (Whitlock and Buergelt, 1996). During the subclinical phase, infected cows may shed MAP into both manure and milk, thus spreading bacteria throughout the dairy farm environment.

A variety of diagnostic tests are available to detect MAP infections, with serum, manure, and milk representing the primary ante-mortem sources. Testing strategies such as ELISA target MAP antibodies, whereas culture and PCR techniques focus on detection of the causal organism. Each diagnostic is accompanied by its own set of advantages and limitations, such as cost, efficiency, and the tradeoff between test sensitivity and specificity (Collins, 1996). For example, fecal culture is extremely specific, but may fail to detect a high percentage of infected animals. On the other hand, ELISAs can produce false positives and may also have reduced sensitivity when infected animals have not yet reached a phase of antibody production (Whitlock et al., 2000). Although the relationship between diagnostic tests has been explored in previous studies,

much of this research has been cross-sectional and has largely centered around dichotomous comparisons and levels of concordance between binary outcomes (e.g., Pinedo et al., 2008; Taddei et al., 2004).

A small number of longitudinal studies have been conducted with the goal of elucidating associations between different diagnostics testing strategies. These studies have predominately focused on the relationship between serum ELISA and fecal culture (e.g., Sweeney et al., 2006; Schukken et al., 2015; Van Schaik et al., 2003) and have commonly employed infrequent sampling schemes across long durations. Nielsen et al. (2002) have advocated for further studies that draw upon repeated measurements from individual cows, in addition to a continuous-scale ELISA measurement, to clarify shifts in the nature of the immune response. It has also been suggested that different levels of interpretation for test results should be employed based upon diagnostic objectives (Nielsen et al., 2002).

According to van Schaik et al. (2003), kinetic ELISA results in serum showed a high variability at the cow level over time; it is reasonable to hypothesize that this fluctuation could be traced to changes in fecal shedding levels, which were also shown to be highly variable. Although the onset of fecal shedding is generally understood to precede a positive antibody response in serum (van Schaik et al., 2003), little is known about the ability of heightened fecal shedding to predict subsequent ELISA optical density (OD) values in milk. This concept should be empirically evaluated by means of a frequent sampling scheme; detection of spikes in fecal shedding could facilitate prediction of a future high milk ELISA result.

Our knowledge of infection dynamics and shedding patterns of MAP-positive animals is continually expanding, and these new insights must be taken into consideration when evaluating efficacy of various diagnostic tests and associations among them. In a recent longitudinal study,

Schukken et al. (2015) observed 2 distinct shedding patterns in MAP infected dairy cows: *Progressors* demonstrated a temporal increase in MAP colony count by fecal culture, while *Non-progressors* showed no demonstrable increase over time. Moreover, Progressors were consistently fecal culture positive, whereas Non-progressors were often culture negative, suggesting intermittent rather than constant fecal shedding (Schukken et al., 2015). It is certainly plausible that Progressors and non-progressors could have differing associations with other variables, such as the change in ELISA OD over time.

Several questions regarding MAP positivity in milk also remain unanswered. Particularly with respect to low-prevalence herds, it is unclear at what frequency individual milk samples from known infected cows test positive for MAP. This question is complicated by the multiple mechanisms linked to MAP positivity in milk; specifically, MAP may be shed directly (as first discussed by Sweeney et al., 1992), or may enter the milk via fecal contamination or airborne particles (Eisenberg et al., 2013). These two distinct mechanisms of milk contamination have been designated as the "internal route" and the "environmental route," respectively (Beaver et al., 2016a). The relative potential of milk ELISA, fecal PCR, and udder or leg hygiene to predict MAP positivity in milk could provide some insight into the likelihood of contamination via the internal and environmental routes.

Finally, there remains a need for statistical models that accurately account for multiple diagnostic results while simultaneously adjusting for individual animals, herds, and temporal relationships; such models can help clarify the interplay between multiple variables and their relative influence on diagnostic outcomes. This type of modeling also permits adjustment of cow-specific characteristics such as parity and stage of lactation, which have been shown to

impact antibody titers (Nielsen et al., 2002) and may plausibly alter the relationship between diagnostic test results.

In the current study, we sought to investigate the multifaceted relationship between milk ELISA, fecal qPCR, and fecal culture. To this end, we obtained repeated samples from known MAP-infected cows in 2 low-prevalence dairy herds in the northeast United States. The relative agreement between fecal PCR, fecal culture, and milk ELISA diagnostics may be better understood by tracking results numerically over time rather than relying exclusively on binary comparisons and simultaneous samples to evaluate concordance. We also explored potential predictors of MAP positivity in individual milk samples. As an additional point of interest, we tracked the bulk-tank MAP status over time in both herds, by means of PCR and ELISA diagnostics. Certainly, the interplay between these diagnostic variables merits further consideration and may provide additional insights into MAP infection dynamics. A nuanced understanding of these interrelationships can only improve testing strategies and objectives, with the ultimate goal of infection mitigation in dairy herds.

2. Materials and Methods

2.1 Herd Selection

Two conventional dairy herds with a history of low MAP prevalence were selected for enrollment in a collaborative study with Cornell University (Ithaca, NY), Pennsylvania State University (State College, PA), and the University of Pennsylvania (New Bolton Center, Kennett Square, PA). One of the study herds was located in Pennsylvania (PA herd) and the other in New York State (NY herd). At the time of study enrollment, the NY herd and the PA herd were comprised, respectively, of 175 and 460 Holstein cows, not including young-stock.

2.2 Full-herd Fecal Sample Collection

Updated prevalence estimates were confirmed by full-herd fecal culture conducted in September 2015 (for the PA herd) and December 2015 (for the NY herd). Sampling at the PA herd was overseen by personnel from Pennsylvania State University and sampling at the NY herd by Cornell University and Quality Milk Production Services (Cobleskill, NY). Fecal samples were obtained rectally from each adult cow using individual palpation sleeves and transferred on-site to 50-mL conical tubes uniquely labeled with cow ID. Samples were immediately shipped on ice to the University of Pennsylvania for culture.

2.3 Full-herd Fecal Culture Protocol

Upon arrival at the University of Pennsylvania, samples were processed fresh for culture using the procedure previously described in Whitlock and Rosenberger (1994). Briefly, 2 g of fecal sample were placed in a sterile 50-mL tube and suspended in 35 mL sterile water. Samples were vortexed, agitated for 30 mins, and allowed to rest for an additional 30 mins to permit large particulate matter to settle. The top 5 mL were then transferred to Brain Heart Infusion Broth containing hexadecylpyridinium chloride and processed using a centrifugation-double incubation technique to obtain an inoculum. The inoculum was added to 4 tubes of Herrold's Egg Yolk Medium with Mycobactin J and incubated for 16 weeks at 37°C. Because MAP colonies may originate either from a single cell or from a group of cells, counts per tube are expressed as "colony forming units" (CFU). At 16 weeks, the CFU in each tube was recorded, and the average CFU across the 4 tubes was used in subsequent analyses. Animals with an average CFU of < 10 were categorized as *low shedders*, those with 11-74 CFU as *moderate shedders*, and those with >75 as *high shedders*. Animals with more than 300 CFU per tube were considered to be *super shedders*. After the 16-week mark, colonies were sub-cultured on Herrold's Egg Yolk Medium

without Mycobactin J to assess mycobactin dependency and colony morphology and thus confirm the presence of MAP in the samples. A final prevalence reading was available from the PA herd in January 2016 and in March 2016 for the NY herd. The fecal-culture positive animals from these full-herd samplings were marked for inclusion in the longitudinal study.

2.4 Longitudinal Fecal and Milk Sample Collection

Based upon the results from the full-herd samplings, culture-positive animals were tracked over the course of approximately 5 months, from March-August 2016. At the NY herd, positive cows were sampled on a weekly basis. Due to a higher volume of positive animals, cows from the PA herd were sampled approximately once every 2 weeks. At each visit, a composite milk sample and a fecal sample were obtained from each animal.

The milk samples were taken in the milking parlor during the morning milking, following the pre-dipping phase. At each visit, a bulk-tank sample was also collected directly from the tank, using a sterile dipper attached to a 50-mL tube. All milk samples were transported or shipped immediately on ice to Cornell University for qPCR and ELISA testing.

Fecal samples were collected using the methods previously described. Each fecal sample was then divided into 2 sampling containers: one set (containing half a sample from each cow) was shipped on ice to the University of Pennsylvania for MAP culture, and the other set was transported directly, or shipped on ice, to Cornell University for qPCR testing. Following fecal sample collection, the positive animals were scored on udder and leg hygiene using the 4 point method described by Schreiner and Ruegg (2002); in summary, scores ranged from 1 (completely clean) to 4 (completely covered in dirt or manure).

Clearly, milk samples could not be obtained from dry cows, and fecal samples could not feasibly be obtained in certain isolated instances when cows were pastured for a portion of the

dry period. In total, 183 fecal samples and 133 milk samples from 14 known positive animals were collected over the course of 142 days. Milk and fecal samples were also taken from a MAP-negative cow at each visit to serve as the study's negative control. One positive cow in the PA herd was culled after a single week of longitudinal sampling and was therefore excluded from the statistical analyses due to an absence of repeated measurements. Additionally, one positive cow in the NY herd was dry for the majority of the sampling period, with a single measurement obtained from milk. Consequently, this animal was excluded from any analyses that included longitudinal milk results.

2.5 Fecal and Milk Testing Procedures

For the individual-cow and bulk-tank milk samples, the Tetracore VetAlert Extraction System and Real-Time PCR kit (Tetracore Inc., Rockland, MD) were employed. The template preparation procedure for the milk samples and the use of controls has been previously described in detail in Beaver et al. (2016a). Strain K-10 was used as the positive control (see Li et al., 2005; Wynne et al., 2010). Once the OD_{600} of a K-10 culture suspension reached 0.04, 1 mL was spiked into 10 mL of milk, extracted according to protocol, and diluted 1:100. For the unknown samples, frozen aliquots of 15 mL were thawed overnight then centrifuged at 2,547 × *g* for 30 min at 10°C. The whey fractions were discarded and the cream and pellets treated with lysis buffer and proteinase K (see Beaver et al., 2016a). This mixture was transferred to disruption tubes, incubated for 1 hours at 56°C, and beadbeat for 5 mins at approximately 2,700 rpm (Mini-Beadbeater 8, BioSpec Products, Bartlesville, OK). The purification steps are enumerated in the manufacturer's protocol.

Extracted DNA was stored at 10°C for < 48 hours if qPCR could not be conducted immediately. The cycling program for the qPCR consisted of a 10-min enzyme activation step at

95°C followed by 45 cycles of (95°C × 15 s, 62°C × 60 s) using a StepOne Real-Time PCR System (Life Technologies, Grand Island, NY). Samples were considered positive if the cycle threshold (Ct) value was ≤ 38 and if the number of gene copies was ≥ 1. Valid runs were defined based on the Ct of the included standard (between 20 and 26) and by the lack of amplification of the no-template control (for which 1x Tris-EDTA buffer (Affymetrix, USB Corporation, Cleveland, OH) was utilized.) The average copy number across the sample wells, run in triplicate, was used in subsequent statistical analyses.

At the University of Pennsylvania, fecal samples were cultured as previously described. At Cornell University, MAP DNA was extracted from the fecal samples and quantified using qPCR methods. For this purpose, the Tetracore Extraction System and Real-Time PCR kits were again used, adhering to the same cycling program as described for the individual and bulk milk samples. The Two Gram Protocol and the Mini Beadbeater Protocol were followed according to the manufacturer's instructions.

At the Animal Health Diagnostic Center at Cornell University, ELISA was conducted on the bulk-milk and individual-milk samples using the Parachek 2 ELISA kit (Thermo Fisher Scienfitic, Waltham, MA). Samples were tested in duplicate wells. The positive or negative designation was determined for each sample by the following equation:

$\frac{Mean\ OD - Mean\ negative\ OD}{(Mean\ positive\ OD - Mean\ negative\ OD)} = corrected\ OD$, with OD ≥ 0.100 used as the cutoff for positive samples.

2.6 Statistical Analyses

i. Descriptive Statistics

As a preliminary stage of analysis, each variable was studied descriptively and graphically to gain an understanding of the distributions and frequencies of the available data.

Because the distributions of *Fecal Culture CFU* and *Fecal qPCR,* were positively skewed, these variables were log transformed (ln(value+1)). The *Milk ELISA OD* distribution was also strongly right skewed, so this variable was log-log transformed (as in Plikaytis et al., 1991) to achieve normality. For simplicity of interpretation, the transformed variables will henceforth be referred to as *Fecal Culture (L), Fecal qPCR (L),* and *Milk ELISA OD (LL).* Univariable analyses were then conducted on relevant pairs of variables: Pearson Correlations (PROC CORR) for continuous variables or ANOVA (proc GLM) for pairs including one categorical variable. Relevant relationships were graphed, and results of these analyses were taken into consideration in the development of the mixed-effect models.

ii. Progressor and Non-progressor Classification

Using the observations of Schukken et al. (2015) as a framework, we sorted known MAP-infected animals in the present study into *Progressor* and *Non-progressor* categories based upon fecal qPCR copy numbers. Animals with a positive slope in regression over time were classified as *Progressors,* provided they shed ≥ 100 copies of MAP at some time over the course of the study. Animals with no observed increase in qPCR quantity over time, those exhibiting intermittent fecal shedding, or those consistently shedding below 100 copies of MAP were considered to be *Non-progressors.* This variable was designed to serve as a predictor of interest in our models and as a general point of comparison.

iii. Mixed-Effects Models

In order to adjust for individual animals, the association between multiple predictors, and the repeated effects of week, 3 mixed-effect, linear regression models were constructed. For repeated measures data, PROC MIXED permits adequate flexibility to determine a representative covariance structure and is able to account for time-dependent correlations within subjects.

Mixed models are also better equipped to handle missing values than conventional methods (Wang and Goonewardene, 2004). The main objective of these mixed-effects analyses was to model the relationship between *Fecal culture* (mean CFU), *Fecal qPCR* (mean copy number), and *Milk ELISA* (mean optical density) over time. Each of the 3 variables served as the outcome in the 3 mixed-effect regressions, and all were considered as predictors for the models in which they were not outcome variables. The following additional variables were considered as potential predictors in all models: *Days in Milk (DIM)*, *Lactation Stage* (fresh (0-21 DIM), lactating (> 21 DIM), or dry), *Milk production* (in pounds per day), *Parity* ($\leq 2^{nd}$ or $\geq 3^{rd}$), *Pregnancy* (yes or no), *Seasonality* (month and day of sampling as a continuous variable, also considered after cosine or sine transformations), and *Cow age* (days). Whether or not the cow was a *Progressor* was evaluated as a predictor for the milk ELISA model (but was excluded from the other models since the level of fecal shedding was used to determine Progressor status). Because *Herd* was a design variable with only 2 levels, it was incorporated into all models as a fixed effect.

Models with repeated effects of week on individual cows were evaluated with and without the additional random intercept for each subject. A number of relevant covariance structures were also tested. The final models were selected based upon AICC and likelihood ratio tests. The basic form of each model may be expressed as follows:

$$Y_{ijcw} = \mu + \beta_i H + \tilde{\beta}_j \tilde{X}_J + d_c + R\varepsilon_{ijcw}$$

where Y_{ijcw} is the log-transformed MAP quantity (or the log-log transformed ELISA OD) measured for cow *c* in herd *i* on week *w*, with *j* fixed effects

μ is the overall mean

$\beta_i H$ is the fixed effect of herd i

d_c is the random effect of cow *c*, and $d_c \sim$ IID(0, σ_d^2)

$\tilde{\beta}_j \tilde{X}_j$ represents the vector of the included j fixed effects

R represents the correlation matrix of observations within a cow on different weeks, and $R\varepsilon_{ijcw}$ is the random error associated with cow *c* in week *w* in the *i*th herd with *j* fixed effects; $R\varepsilon_{ijcw} \sim$ IID(0, σ_ε^2)

iv. Delay in Antibody Production

Several analyses were conducted to explore the temporal relationship between spikes in fecal shedding and antibody production in milk. Two thresholds were considered for fecal shedding (based upon qPCR copy numbers): ≥ 40 MAP copies, representing a shedding level higher than approximately 75% of observations and ≥ 100 MAP copies, representing a shedding level higher than approximately 80% of observations (not including measurements of 0 copies). First, using chi-square statistics (PROC FREQ), ELISA was evaluated as a binary variable to determine whether a positive *Milk ELISA Result* corresponded to spikes in fecal shedding in the previous month. Next, Wilcoxon rank sum tests (PROC NPAR1WAY) were conducted to gauge whether antibody level in milk corresponded to spikes in fecal shedding in the month leading up to each *Milk ELISA OD* reading. Finally, in order to account for repeated measurements and to adjust for *Herd*, we used mixed linear models to evaluate the impact of these spikes in shedding, at both thresholds, on *Milk ELISA OD*. Based upon the results of these mixed models, several other temporal hypotheses were tested for spikes ≥ 100 copies. Namely, we used additional mixed models to evaluate spikes that occurred between 1 and 2 months prior to each *Milk ELISA OD* and between 2 and 3 months prior.

v. Predictors of Milk PCR Positivity

To determine potential predictors of a positive PCR status in individual milk samples from the NY herd, a simple logistic regression model was developed using milk PCR as the

outcome variable. Individual *Udder Hygiene* scores (from 1 to 4), *Leg Hygiene* scores (from 1 to 4), *Fecal qPCR quantity*, and *Milk ELISA OD* were evaluated as predictors in the model. Due to the paucity of positive milk qPCR observations, a repeated-measures component was not included to avoid overfitting the model relative to available data. In addition, Firth's bias correction was employed to provide penalized estimates for odds ratios and associated confidence intervals. This model may be expressed as:

$$\ln\left(\frac{Y}{1-Y}\right) = \alpha + \tilde{\beta}\tilde{X} + \varepsilon$$

Where α is the intercept, $\ln\left(\frac{Y}{1-Y}\right)$ represents the natural log of the odds for $\frac{milk\ qPCR\ positive}{milk\ qPCR\ negative}$, $\tilde{\beta}\tilde{X}$ is the vector of included predictors and Firth-adjusted slope estimates, and ε is the error.

3. Results

3.1 Descriptive Results for Diagnostic Tests

i. Prevalence Estimates

Based upon the full-herd fecal culture conducted on each farm, prevalence was confirmed to be 2.9% in the NY herd (5 positive/175 total) and 2.2% in the PA herd (10 positive/460 total).

ii. Longitudinal Fecal qPCR

During the study, a broad range of fecal qPCR counts were recorded, from 0 to 43,466 copies/ 2 grams, with an average ± SE of 660 ± 343. The total average shedding burden from a set of fecal samples was 9,318 ± 5,014 copies for the PA herd and 73 ± 24 for the NY herd. All 13 included cows had at least one fecal qPCR positive result during the study, and 5 cows were consistently positive at all time points.

iii. Longitudinal Milk qPCR

Over the course of the study, there were only 4 positive individual milk qPCR results, originating from 2 cows in the NY herd, with an average copy numbers of 5.3 ± 1.5. Due to the

low copy numbers and small number of positive samples, the results for this variable will henceforth be analyzed as "present" or "absent."

iv. Longitudinal Milk ELISA

There was a 15.8 % positive rate for all individual samples tested. The recorded values for corrected OD (i.e., $\frac{Mean\ OD - Mean\ negative\ OD}{Mean\ positive\ OD - Mean\ negative\ OD}$) ranged from -0.024 to 1.531, with a mean ± SE of 0.051 ± 0.015. Samples were considered positive at OD ≥ 0.10. At the cow level, 6/12 cows had at least one reported ELISA-positive result. Two cows were ELISA positive at the outset of the study, while the other 4 transitioned to a positive result over the course of the study.

v. Bulk Tank Milk

All bulk-tank milk samples were negative by both ELISA and qPCR. The average corrected OD ± SE for the bulk tank samples was -0.015 ± 0.001 for the PA herd and -0.018 ± 0.001 for the NY herd.

vi. Longitudinal Fecal culture

The rate of fecal-culture-positive samples was 47.8%, and 12/13 cows tested positive at some point during the study. 26.0% of the positive samples averaged < 1 CFU/tube (i.e., MAP did not grow on all of the tubes tested per sample, thus the average for the sample was < 1 copy). 51.9% of the samples had an average of 1-10 CFU/tube, 10.4% had an average of 11-50 CFU/tube, and 1.3% had an of 150-300 CFU/tube. The percentage of samples with an average of > 300 CFU/tube was 10.4%.

vii. Udder and Leg Hygiene

The average udder and leg hygiene scores ± SE for the NY herd were 3.00 ± 0.072 and 2.911 ± 0.070, respectively. For the PA herd, the udder and leg hygiene scores ± SE were 2.020 ± 0.111 and 2.592 ± 0.146, respectively.

viii. Relationships between Variables

In *Table 11a,* the binary associations between fecal culture, fecal PCR, and milk ELISA results are shown. In *Table 11b,* the results have been divided by Progressor status.

Table 11. *Contingency Tables. a)* Raw numbers of positive and negative results for 3 diagnostic tests (Fecal culture, Fecal PCR, and Milk ELISA). *b)* Raw numbers of positive and negative results for 3 diagnostic tests are compared (Fecal culture, Fecal PCR, and Milk ELISA) based upon Progressor status.

Table 11a

	Fecal PCR		**Milk ELISA**	
	Positive	Negative	Positive	Negative
Fecal Culture				
Positive	67	9	18	49
Negative	39	42	3	62
Milk ELISA				
Positive	21	0		
Negative	74	35		

Table 11b

Progressors				
	Fecal PCR		**Milk ELISA**	
	Positive	Negative	Positive	Negative
Fecal Culture				
Positive	34	1	18	12
Negative	10	3	2	10
Milk ELISA				
Positive	20	0		
Negative	18	2		

Non-progressors				
	Fecal PCR		**Milk ELISA**	
	Positive	Negative	Positive	Negative
Fecal Culture				
Positive	33	8	1	37
Negative	29	39	0	52
Milk ELISA				
Positive	1	0		
Negative	56	33		

Following biologically-relevant univariable analyses, graphical representations were produced for important relationships between variables and are shown in *Figures 5* and *6.* These results will be discussed further in the context of mixed regression models.

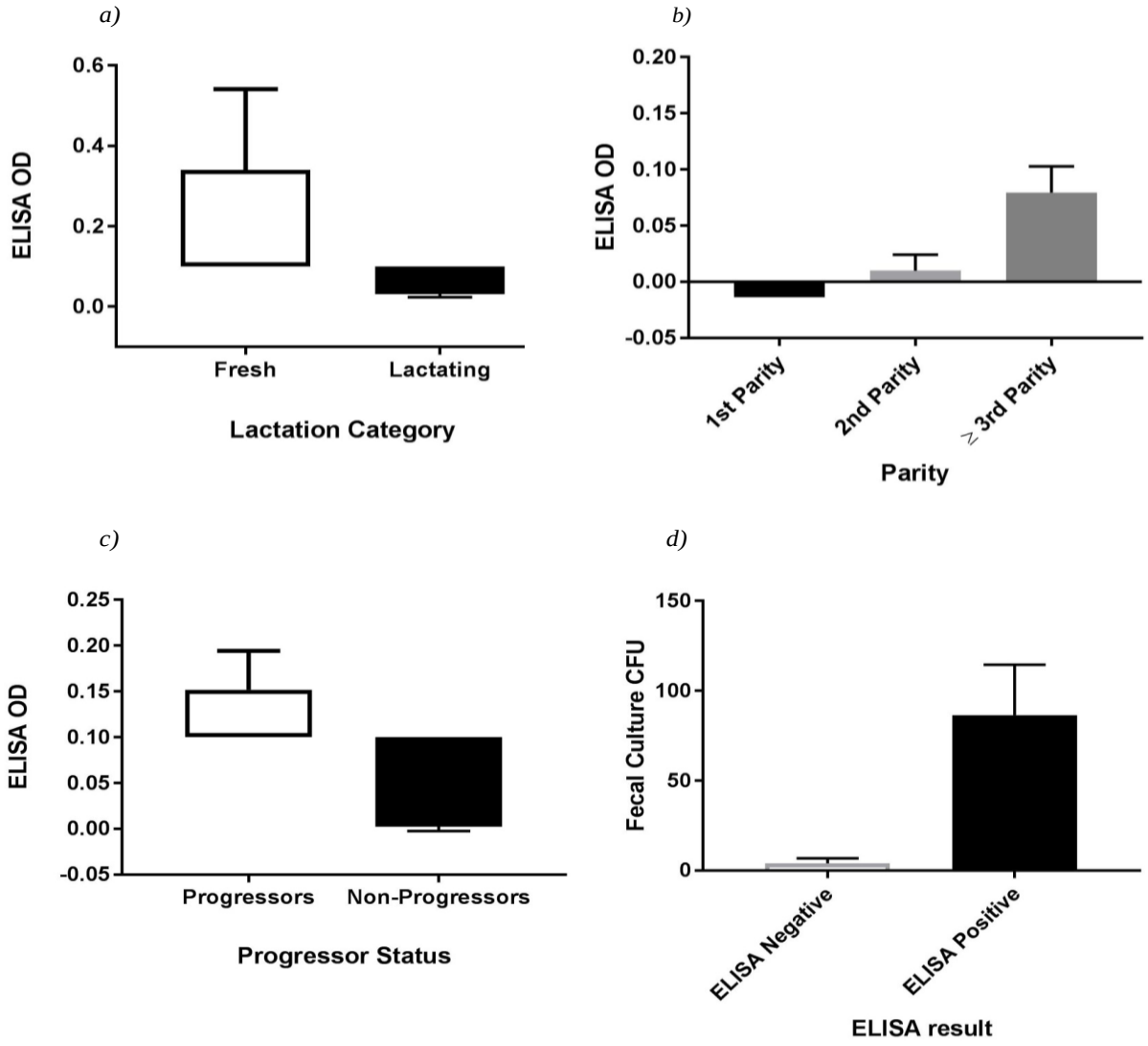


Figure 5. *Milk ELISA by Lactation Category, Parity, Progessor Status, and Fecal Culture.* In this figure, associations of *Milk ELISA OD* with a variety of important predictor variables are shown. In Panel *a), Milk ELISA OD* values are divided based upon *Lactation Category* (either fresh or lactating). In Panel *b), Milk ELISA OD* is sorted by *Parity* (either 1^{st}, 2^{nd}, or $\geq 3^{rd}$ parity). In Panel *c), Milk ELISA OD* by *Progressor Status* (either Progressor or Non-progressor) is shown. In Panel *d) Milk ELISA result* (yes/no) is evaluated with respect to corresponding *Fecal Culture CFU.* Error bars represent 1 SEM.

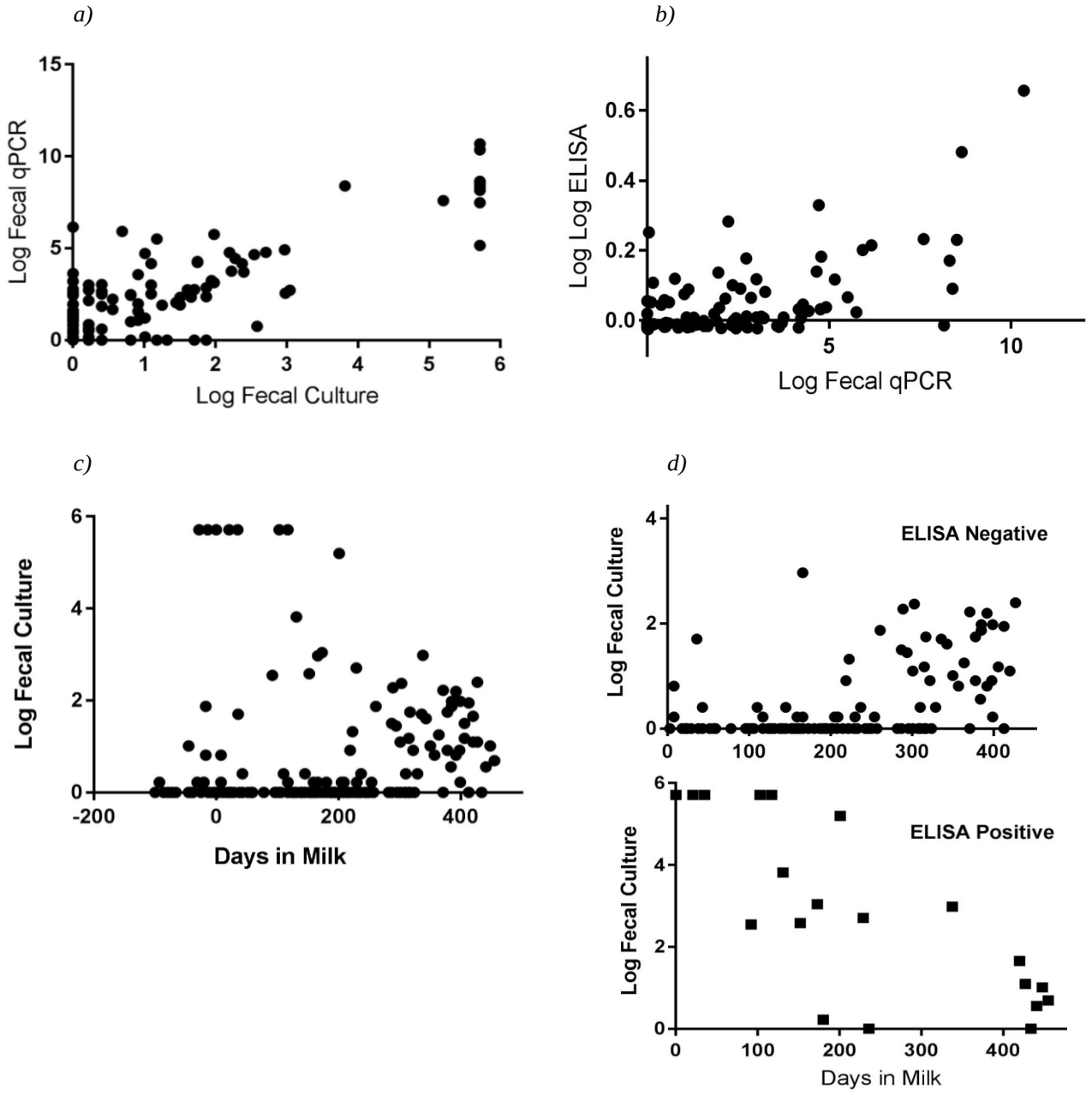


Figure 6*. Relationships between Continuous Variables.* Associations between continuous study variables are plotted. Panel *a)* shows the relationship between *Fecal Culture* CFU and *Fecal qPCR* copy number. Both variables have been log transformed (ln(value+1)) prior to plotting in order to represent their association graphically. Panel *b)* shows the correlation between *Fecal qPCR* copy number (log transformed) and *Milk ELISA OD* (log-log transformed). Panel *c)* shows the association between *Fecal Culture CFU* (log transformed) and *Days in Milk.* In Panel *d),* the relationship between *Fecal Culture CFU* and *Days in Milk* is divided by *Milk ELISA Result.*

3.2 Mixed Regression Models

i. Assumptions

In order to satisfy the assumptions of linearity and normality of model residuals, the log-transformed or log-log transformed variables were used as outcomes or predictors where necessary.

ii. Model Selection

Several plausible covariance structures were compared using AICC, and formal Chi-square difference tests were conducted using -2 Log Likelihood statistics. The first-order heterogeneous autoregressive structure (ARH(1)) provided an optimal fit for the repeated effect in the case of the *Fecal qPCR Model* and the *Fecal Culture Model.* ARH(1) is an extension of the first-order autoregressive structure (AR(1)) that allows for different variances across the diagonal of the covariance matrix. Such an arrangement permits specification of both the between-subject and within-subject covariance structures. The AR(1) structure provided the best fit for the repeated effect of week in the *Milk ELISA Model*. Further, models with both repeated and random effects were compared to models with only repeated effects, using AIC and Likelihood ratio tests. In the case of all 3 models, inclusion of both the random and repeated statements resulted in significant improvement to the model fit. Variance component structures were used for random statements in all models, since altering these structures did not offer significant improvement.

iii. The Fecal qPCR Model

Fecal qPCR (L) was significantly predicted by both *Fecal Culture* ($P < 0.0001$) and *Milk ELISA OD* ($P = 0.011$). In general, higher *Fecal qPCR (L)* copy numbers corresponded to higher

Milk ELISA titers and higher CFU in *Fecal Culture*. There was no significant effect of *Herd* (P = 0.839), and no additional variables were retained in the model. Results are shown in *Table 12.*

Table 12. *The Fecal qPCR Model.* Results from the Fecal qPCR mixed-linear model are shown. Natural log transformed fecal qPCR copy numbers served as the outcome in this model. Slope estimates, standard errors (SE), F Values from Type III analyses and associated P values are presented for each effect retained in the model. The PA herd served as the reference level for the *Herd* variable. Asterisks indicate significance at the P < 0.05 level.

Effect	*Estimate*	*SE*	*F Value (Type III)*	*P > F*
Intercept	1.504	0.522	--	--
ELISA OD	2.239	0.754	8.48	0.004*
Fecal Culture CFU	0.017	0.002	102.69	< 0.0001*
Herd				
NY	0.091	0.832	0.01	0.914
PA	--	--	--	--

iv. The Fecal Culture Model

Fecal Culture (L) CFU was significantly related to *Fecal qPCR (L)* ($p < 0.0001$), *DIM* ($p < 0.0001$), and *Milk ELISA Result* ($p < 0.0001$.) Specifically, a positive *ELISA Result* and high *Fecal qPCR (L)* quantities were both important predictors of high *Fecal Culture (L)* CFU values. *Fecal Culture (L)* values peaked at calving and decreased with higher *DIM* values, over the course of lactation. There was also a significant interaction between *DIM* and *Milk ELISA Result* on *Fecal Culture (L)* ($p < 0.0001$), suggesting that the relationship between *Fecal Culture (L)* and *DIM* was stronger for animals showing positive ELISA results. Continuous *Milk ELISA* OD was considered for inclusion in this model, but in this instance, its predictive ability was not as strong as that of the binary *ELISA Result*. *Herd* was not a significant variable in the model ($p = 0.570$). Results are shown in *Table 13.*

Table 13. *The Fecal Culture Model.* Results from the Fecal Culture mixed-linear model are shown. Natural log transformed fecal culture CFU counts served as the outcome in this model. Slope estimates, standard errors (SE), F Values from Type III analyses and associated P values are presented for each effect retained in the model. The PA herd served as the reference level for the *Herd* variable, while negative served as the reference level for *ELISA result.* Asterisks indicate significance at the $P < 0.05$ level.

Effect	*Estimate*	*SE*	*F Value (Type III)*	*P > F*
Intercept	2.713	0.415	--	--
Log Fecal qPCR	0.285	0.032	78.90	< 0.0001*
DIM	-0.007	0.001	28.25	< 0.0001*
ELISA				
Positive	2.161	0.293	54.32	< 0.0001*
Negative	--	--	--	--
DIM*ELISA				
Positive	-0.007	0.001	56.29	< 0.0001*
Negative	--	--	--	--
Herd				
NY	-0.275	0.483	0.32	0.570
PA	--	--	--	--

v. The Milk ELISA OD Model

Milk ELISA (LL) was significantly predicted by *Fecal qPCR* ($P < 0.0001$), *Stage of Lactation* ($P = 0.001$), *Progressor Status* ($P = 0.009$) and *Parity* ($P < 0.006$). Although a non-linear effect of *DIM* on milk ELISA OD was also observed, the model fit was improved by categorizing *DIM* into *Stages of Lactation*. In particular, fresh cows had higher values of *Milk ELISA (LL)* compared to cows in later stages of lactation. Animals that had been categorized as *Progressors* also had higher *Milk ELISA (LL)* titers compared to *Non-progressors.* The *Progressor* variable contributed significantly to the model beyond the contribution provided by the *Fecal qPCR.* Additionally, cows of a 3rd or higher *Parity* had significantly higher *Milk ELISA (LL)* titers compared to cows in their 1st or 2nd lactations. Herd was not a significant variable in the model ($P = 0.073$). Results are shown in *Table 14.*

Table 14. *The Milk ELISA Model.* Results from the Milk ELISA mixed-linear model are shown. ELISA OD values served as the outcome in this model after a natural log log transformation. Slope estimates, standard errors (SE), F Values from Type III analyses and associated P values are presented for each effect retained in the model. The PA herd served as the reference level for the *Herd* variable, Non-progressors served as the reference category for *Progressor Status,* lactating animals for *Stage of Lactation*, and ≥ 3rd parity for the *Parity* variable. Asterisks indicate significance at the $P < 0.05$ level.

Effect	*Estimate*	*SE*	*F Value (Type III)*	*P > F*
Intercept	0.106	0.021	--	--
Fecal qPCR	0.00001	2.1E-6	40.29	< 0.0001*
Stage of Lactation				
Dry	N/A	--	--	--
Fresh	0.089	0.026	11.35	0.001*
Lactating	--	--	--	--
Progressor Status				
Progressor	0.070	0.026	7.02	0.009*
Non-progressor	--	--	--	--
Parity				
1st or 2nd	-0.067	0.024	7.94	0.006*
≥ 3rd	--	--	--	--
Herd				
NY	-0.033	0.018	0.86	0.355
PA	--	--	--	--

3.3 Delay in Antibody Production

The results of the Chi-square analyses revealed that spikes in fecal shedding were more likely ($P < 0.0001$) to correspond to subsequent positive *Milk ELISA Results* ($\chi^2 = 51.41$ and $\chi^2 = 50.65$ for ≥ 100 and ≥ 40 copies, respectively). This relationship was confirmed by the Wilcoxon tests, which demonstrated that *Milk ELISA* OD on a given week was significantly, positively related to spikes in fecal shedding of ≥ 100 copies that occurred within the past month ($z = 6.208$, $P < 0.0001$) and also to smaller spikes of ≥ 40 copies ($z = 6.338$, $P < 0.0001$). After adjustment for herd and for repeated subject measurements, differences were no longer significant in reference to the ≥ 40 copy threshold ($P = 0.417$). On the other hand, differences remained significant in reference to the ≥ 100 copy threshold ($P = 0.003$). Consequently, we sought to test

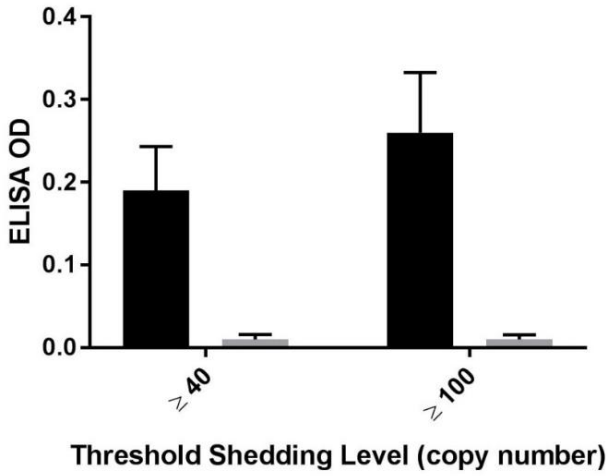
the boundary of this temporal relationship by considering ≥ 100 copy spikes occurring between 1 and 2 months prior to each ELISA, and between 2 and 3 months prior. The significance was upheld between 1 and 2 months (P = 0.023), but not between 2 and 3 months (P = 0.818). Complete results are shown in *Table 15.* Select results are depicted in *Figure 7.*

Table 15. *Delay in Antibody Production.* Results of 2 mixed linear models created by assessing spikes in fecal shedding at ≥ 40 copies and at ≥ 100 copies are shown. ELISA OD values served as the outcome in this model after a natural log-log transformation. Slope estimates, standard errors (SE), F Values from Type III analyses and associated P values are presented for each effect in the model. The PA herd represented the reference level for the *Herd* variable, whereas "No" was the reference level for the respective shedding variables. Asterisks indicate significance at the P < 0.05 level.

Spikes in Fecal Shedding ≥ 40 copies ***in the month leading up to each ELISA OD***				
Effect	*Estimate*	*SE*	*F Value (Type III)*	*P > F*
Intercept	0.096	0.043	--	--
Shedding ≥ 40 (Yes vs. No)	0.021	0.025	0.66	0.417
Herd (NY vs. PA)	-0.060	0.069	0.77	0.382

Spikes in Fecal Shedding ≥ 100 copies ***in the month leading up to each ELISA OD***				
Effect	*Estimate*	*SE*	*F Value (Type III)*	*P > F*
Intercept	0.145	0.038	--	--
Shedding ≥ 100 (Yes vs. No)	0.084	0.028	9.11	0.003*
Herd (NY vs. PA)	-0.053	0.055	0.93	0.337
Between 1 and 2 months prior to each ELISA OD				
Intercept	0.115	0.035	--	--
Shedding ≥ 100 (Yes vs. No)	0.061	0.027	4.99	0.023*
Herd (NY vs. PA)	-0.029	0.047	0.37	0.544
Between 2 and 3 months prior to each ELISA OD				
Intercept	0.045	0.039	--	--
Shedding ≥ 100 (Yes vs. No)	-0.007	0.032	0.05	0.818
Herd (NY vs. PA)	-0.0001	0.054	0.00	0.999

Figure 7. *Temporal Relationship between Milk ELISA OD and Fecal Shedding.* This figure illustrates the relationship between fecal shedding (*qPCR Copy Number*) and subsequent *Milk ELISA OD* values, observed up to a month later. On the X axis, two threshold shedding levels are shown (≥ 40 copies/2g and ≥ 100 copies 2/g). *Milk ELISA OD* is represented on the Y axis. Gray bars illustrate the average *Milk ELISA OD* values for cows consistently shedding below the respective threshold levels in the previous month. Black bars illustrate the average values for cows reaching the threshold shedding level at least once in the previous month. Error bars represent 1 SEM.



3.4 Predictors of Milk PCR Positivity

Out of the potential predictors for *Milk PCR Result*, only *Udder Hygiene* score was retained in the model. An increase in 0.5 units in this score (corresponding to a decrease in udder hygiene) represented a 5.72 times greater odds of a positive result by milk PCR. See *Table 16* for complete results.

Table 16. *The Milk PCR Model.* Results of the logistic regression model with Firth's bias correction are reported. The outcome variable was the log of the odds for PCR positivity in individual milk samples. Slope estimates and associated standard error, in addition to Wald Chi-square statistics and P values, are shown. The odds ratio and 95% Wald Confidence Interval have been calculated based upon a change of 0.5 units in the udder hygiene score.

Term	*Slope Estimate*	*SE*	*Wald* χ^2	*P>* χ^2	$\widehat{OR}$	*95% Wald CI for* $\widehat{OR}$
Intercept	-14.973	5.816	--	--	--	--
Udder Hygiene Score	3.487	1.506	5.364	0.021*	5.72	1.82, 66.55

4. Discussion

Through the analysis of this repeated-measures dataset, we have come to an enhanced understanding of the relationships and interrelationships between outcomes of important MAP diagnostic tests. In particular, we have reached several new conclusions regarding the relationship between fecal shedding and antibody production in milk. This correlation is shown in *Figure 5b.* With respect to longitudinal analyses, individual milk ELISA diagnostics have received limited attention in Johne's disease literature compared to serum ELISA (see Sweeney et al., 2006; Schukken et al., 2015; Van Schaik et al.,2003). Due to a variety of factors such as differing immune responses and individual cow characteristics, it has been argued that milk ELISA results should be examined beyond a positive/negative classification (Nielsen et al., 2002). On the surface, our dataset revealed a high number of animals with paired negative milk ELISA and positive fecal qPCR results (see *Table 11a*), an apparent discordancy; however, all ELISA-positive milk samples in our study had corresponding positive results from fecal qPCR, implying that a less stringent threshold may be indicated for some animals. When ELISA OD is treated as an infection likelihood for a given animal (Collins et al., 2005), it is difficult to justify the grouping of highly-negative OD values with those just shy of the cutoff.

We have therefore made use of frequent repeated-measurements to model ELISA OD as a continuous variable to gain a better understanding of within-host changes in immune response over time while adjusting for cow and herd characteristics. Our *Milk ELISA Model* revealed that qPCR copy number was a strong predictor of ELISA OD (LL), although these diagnostics target different indicators of MAP infection. Similarly, in our *Fecal qPCR Model,* ELISA OD was retained as a predictor even after the inclusion of fecal culture, suggesting that a knowledge of

ELISA titer can provide additional predictive information on corresponding fecal qPCR copy numbers.

We also sought to observe whether animals categorized as Progressors and Non-progressors had a similar development of milk ELISA OD values over time. Based upon observation, Schukken et al. (2015) ascertained that Non-progressors seemed to exhibit a virtual absence of humoral immune response as measured by serum ELISA. We aimed to quantify a similar concept using milk ELISA OD values. According to our *Milk ELISA Model*, animals categorized as Progressors showed significantly higher milk ELISA (LL) OD (see *Figure 5c).* This conclusion signifies a complexity to the relationship between milk ELISA and fecal shedding, where qPCR alone is not a perfect predictor of milk ELISA OD value. This study represents the first attempt to quantify the link between milk ELISA OD and Progressor status. The distinction between these two shedding patterns and their relationships to ELISA outcomes should certainly be included in future studies of infection dynamics and diagnostic test evaluations. Nielsen et al. (2002) advocate for multiple cut-off points for the use of ELISA in routine diagnostics, in order to more accurately adjust for individual animal characteristics. According to our *Milk ELISA Model*, knowledge of whether or not the animal is a Progressor can alter our understanding of how milk ELISA OD values will develop over time. This finding provides corroborating evidence for the assertion that a universal cutoff level is insufficient to encompass all diagnostic objectives.

There were several other variables in our dataset that were shown to impact milk ELISA OD (LL), namely, parity and lactation stage. The nature of mixed-model analyses facilitated incorporation of these variables while simultaneously evaluating the relationship between diagnostic tests. The significant effects of parity and lactation stage are consistent with the

findings of other studies (Nielsen et al., 2002); adjustments for these variables are therefore critical before consideration of the relationship between milk ELISA OD and fecal qPCR. As shown in *Figure 5a,* and as supported by our MILK *ELISA Model,* fresh cows had higher levels of milk ELISA (LL) compared to lactating animals. We did not find that milk production had a significant impact on ELISA OD, which seems to contradict certain findings (e.g., Lombard et al., 2005) but is consistent with others (e.g. Johnson et al., 2001). Additionally, variations in milk production may be sufficiently accounted for through the inclusion of lactation stage and parity in the model.

Prior to the mixed-modeling stage, we observed a trend for increasing ELISA OD by parity, with 3rd lactation animals showing highest ELISA OD values, as apparent in *Figure 5b.* This observation is logical considering the progressive nature of MAP infections (Whitlock and Buergelt, 1996). Because only one animal in our study remained in her 1st parity for the entirety of the sampling period, while 2 others transitioned to their 2nd lactations over the course of the study, we elected to classify 1st and 2nd lactation animals together for analysis. Since the study was comprised solely of known-positive animals (evaluated by fecal shedding), the small number of 1st parity animals in itself suggests decreased fecal shedding for this demographic. Our *Milk ELISA Model* demonstrated that being 3rd parity or higher could significantly predict an increased milk ELISA (LL), compared to 1st or 2nd parity. This conclusion arguably reflects more than the progressive nature of Johne's disease, since a parity effect remained significant even after the inclusion of fecal qPCR as a model predictor. Rather, our *Milk ELISA model* implies that as known positive cows age, the aggregate release of MAP from granulomas increases (Koets et al., 2015), potentially resulting in a cumulative exposure of the immune system to MAP.

In summary, there appears to be a direct relationship between fecal shedding and milk ELISA OD in a given week (as shown in both the *Milk ELISA* and the *Fecal qPCR* models); however, the chronological association between these diagnostic tests may be more complex. Because serum antibody production is understood to follow the onset of fecal shedding (van Schaik et al., 2003), we hypothesized that a temporal relationship may exist with respect to milk antibody production as well. In particular, we aimed to address the question of whether spikes in fecal shedding (as measured by qPCR) could predict subsequent milk ELISA OD values. We used several different methods to tackle this question; our results strongly indicate that previous levels of fecal shedding may predict future ELISA readings (see *Figure 7*). First, we examined milk ELISA results on a positive/negative basis, and although the cutoff value is necessarily arbitrary to some degree, our results demonstrated that a positive to negative conversion in milk ELISA may be related to a previous level of fecal shedding. More specifically, fecal shedding levels may be useful in predicting a change in milk antibody status for a given animal.

Secondly, we considered milk ELISA optical density values on a continuous scale and adjusted for herd and individual cow effects using mixed modeling. We concluded that spikes in fecal shedding that met or exceeded 100 copies/2g were very strongly related to milk ELISA optical density readings taken up to a month later. Additionally, this effect was also observable between 1 and 2 months prior to each ELISA reading, although the significance level was slightly diminished. When considering fecal shedding between 2 and 3 months prior to the milk ELISA titers, however, no effect was present.

These conclusions seem to fit with our current understanding of the immunopathology of MAP infections. That is, MAP bacteria are notorious for an ability to overcome host immune defenses within macrophages and thus evade detection in early infection stages (Whittington and

Sergeant, 2001). During this phase, the bacteria proliferate intracellularly in the Peyer's patches, effectively walled off from the humoral immune response, with the help of suppressed MHC antigen expression (Weiss et al., 2001). An important role of Th2 regulatory cytokines is to activate B-cell proliferation and ultimate antibody production; yet substantial shedding of MAP from the intestinal mucosa may be needed before this process can be initiated (Whittington and Sergeant, 2001). Once triggered, a shift from Th1 to Th2 dominance is not instantaneous and is now assumed to be more than a straightforward replacement (Begg et al., 2011; Whittington et al., 2012). Similarly, it is reasonable to hypothesize that B cell proliferation and the production of detectable levels of antibodies would not occur immediately following spikes in shedding, and would persist for a certain duration once initiated. Temporal investigations of antibody levels typically focus on the time between initial infection and serum IgG production (as in Bannantine et al., 2008; Stabel et al., 2011). Our findings address a slightly different question and provide a potential time frame for the peak in antibody production in milk, linked to a previous uncontrolled release of MAP.

In our *Fecal Culture Model*, we noted a direct relationship between DIM and fecal culture CFU, with CFU peaking at calving and decreasing over the course of lactation. This relationship is subtly apparent in *Figure 5c*, and became more pronounced after adjustments for herd and subject. Secondly, we observed an effect of ELISA result on Fecal Culture (L), as consistent with *Figure 5d.* Although the relationship between milk ELISA and DIM has been reported elsewhere (Nielsen and Toft, 2012), a multivariable relationship between Fecal Culture, ELISA, and DIM requires exploration. We uncovered a significant interaction between ELISA result and DIM on Fecal Culture (L) CFU, with a stronger relationship corresponding to positive ELISA results. This finding is rational, since, according to our model, positive milk ELISA

results are associated with high CFU counts. And, although there is little variation in CFU burden from low shedders, this variation is more pronounced in high shedders. Hence, differences in shedding levels (attributable to a variable such as DIM) can be more readily observed among high shedding animals.

The ability of fecal qPCR copy numbers to predict fecal culture CFU, and vice versa, is perhaps the most intuitive conclusion of our study, and yet this positive association has not played an important role in the literature to date. Pinedo et al. (2008) observed only a "fair" agreement between fecal PCR and fecal culture results based upon the cutoff for kappa coefficients. Similarly, when comparing 3 different PCR kits to fecal culture, Taddei et al. (2004) found "fair" agreement for one kit and "poor" agreement for the other two, according to kappa values. The conclusions of the current study do not contradict these findings, but may suggest that considering these variables as continuous in a repeated-measures context could provide additional information to their relative concordance. Indeed, relying on the negative and positive designations for these variables exclusively also resulted in low agreement between the tests for our study, as shown in the contingency table (*Table 11a*). This level of agreement, however, is enhanced by 11 percentage points for Progressors compared to Non-progressors, suggesting that shedding level itself plays a role in the relationship between the two diagnostic tests. *Figure 5a* reveals an apparent correlation between fecal qPCR and culture even before the adjustments for cow, week, and herd. Indeed, sensitivity improves as shedding level increases (Raizman et al., 2004), which, in turn, strengthens relative concordance. It is also important to consider whether extremely low positive values (particularly values < 1 resulting from averages across wells or plates) are truly to be considered discordant with negative results. When the principal goal is

detection, even a small positive value can be informative; on the other hand, concordance may be redefined when assessing the infectivity and infection progression for known positive animals.

The use of mixed modeling with both repeated and random effects has allowed us to study concordance on a continuous scale over time. Liu et al. (2007) noted that inclusion of an additional random component is more likely to improve model fit when the repeated measurements follow an autoregressive-type structure. As consistent with this observation, the addition of a random component resulted in significant improvement to our models. Herd was included as a predictor in all models in order to eliminate its potential confounding effect as a study design variable. It is worth noting that herd was not significant in any of our models, despite differences in herd size and location of the two farms. This suggests that results of the current study could potentially be applicable to other low-prevalence herds in the Northeast U.S; however, the limited number of herds and infected animals in our study makes it difficult to reach conclusions that hinge upon external validity, a necessary trade-off between precision and quantity. The relatively short time frame for sampling may also be considered a limitation, although the frequent sampling scheme has provided more precise windows into shifts in diagnostic outcomes.

Another important diagnostic test that demands further exploration is the detection of MAP in individual milk samples using PCR. An understanding of the routes of milk contamination is an important preliminary stage in curtailing this contamination and mitigating transmission (Beaver et al., 2016a). The hygiene at the PA farm was markedly better than for the NY farm, particularly in the case of udder cleanliness (an average score of 2.02 in PA compared to 3.00 in NY). As a result, it is perhaps not coincidental that all PCR positive milk samples originated in the NY herd, despite an overall lower shedding burden compared to PA. The

logistic regression demonstrated higher odds of a positive milk PCR result when udder hygiene from the corresponding animal was poor. These odds increased more than 5 fold for every half unit increase (worsening) in udder hygiene score. Leg hygiene was not a significant variable in the model, invoking the results of Schreiner and Ruegg (2003), who determined via the same scoring system that udder hygiene (compared to leg hygiene) was a more significant predictor of intramammary environmental pathogens.

Antibody production occurs chiefly in late-stage, systemically infected animals (Streeter et al., 1995) and in Progressors (according to this study). Because high ELISA titers are most often accompanied by the presence of MAP itself (Singh et al., 2007) it may be hypothesized that direct shedding of MAP into milk occurs mainly in the later stages of infection. Indeed, Sweeney et al. (1992) noted a higher likelihood of direct shedding among clinical animals. In our study, there was no significant relationship between the presence of MAP in milk and corresponding ELISA titers or fecal qPCR copy numbers. Thus, direct shedding is a less likely explanation for contamination in these samples. Moreover, no clinical animals were enrolled in our study, with only 2 animals producing substantial antibody levels at the study's outset. Although direct shedding into milk cannot be definitively ruled out, the relationship with poor udder hygiene (rather than high milk ELISA values or fecal copy numbers), suggests that environmental contamination has played at least some role. These results must be interpreted with caution due to the small number of milk samples positive by PCR; while the penalized parameter estimates produced by the Firth method are both finite and reliable (Firth, 1993), the paucity of positive samples does represent a caveat in the interpretation of results. Similarly, only one herd was considered for this analysis, so it remains to be determined whether these inferences would hold for other herds with differing hygiene scores and management practices. In any case, the small

number of positive samples is in itself informative, suggesting that even in herds with poor udder hygiene, environmental contamination of milk is rare and occurs in low levels (as indicated by the average copy number of < 6 copies/2g).

The contamination of individual milk samples is of further importance in relation to the bulk tank milk supply. MAP bacteria have been implicated as a potential cause of Crohn's Disease in humans (see Feller et al., 2007), with bulk tank milk as a conceivable vector. Further, both pasteurized and unpasteurized milk from the bulk tank is routinely fed to calves in 36 to 71% of U.S. dairy farms (Beaver et al., 2016b), which could result in new infections given the heightened susceptibility of calves (Lombard, 2011). Over the course of our study, no bulk tank milk sample ever tested positive by PCR; furthermore, bulk-milk ELISA OD values remained low and invariant. Although the possibility of false negatives cannot be excluded, the qPCR test used in the present study has been optimized for use in milk, with an ability to detect dilutions up to $10^{-3.5}$ (Beaver et al., 2016a). These results may therefore suggest that bulk-tank contamination is not of primary concern in low-prevalence herds. This finding is consistent with the cross-sectional work of Khol et al. (2013), who found a 0% positive rate among low-prevalence Austrian herds. The rate of positivity has been higher in other cross-sectional studies that included bulk milk samples from both high and low prevalence herds (ranging from approximately 3% (as in Bosshard et al, 2006) to 20% (as in Corti and Stephan, 2002)).

Importantly, the prevalence levels observed in the NY and PA herds (2.9% and 2.2%, respectively) actually reflect shedding levels rather than absolute prevalence, since it is not possible to identify latent animals using current *ante-mortem* diagnostic methods. Indeed, the classic iceberg phenomenon posits that for every 4-8 subclinical animals, there are 10-14 silently-infected animals present in a given herd (Whitlock and Buergelt, 1996). These ratios

imply that the herds in the present study have a true prevalence closer to 6-9%, and perhaps even higher. In fact, based upon longitudinal data, Schukken et al. (2015) found that a 2.2% positive prevalence in fecal samples corresponded to a subsequent 16.7% prevalence in tissue samples at slaughter, suggesting a much greater level of infection than is detectable by fecal culture. The lack of contamination in the bulk tank may therefore be considered in light of these absolute prevalence levels. It is notable that a higher shedding level or reduced hygiene (as inferred from the logistic regression on individual milk samples) would be necessary in order for bulk tank contamination to represent a substantial concern.

5. Conclusions

Multivariable analyses of repeated-measurements from known MAP positive cows have provided a more comprehensive outline of the relationship between several important diagnostic tests. This study has shown strong associations between milk ELISA, fecal qPCR, and fecal culture results, at least when these variables are considered on a continuous scale. The associations remain after adjustment for potentially confounding effects of herd and individual cow characteristics. In general, the predictive ability of fecal shedding level on milk ELISA OD is much greater and more complex than anticipated. Importantly, we have quantified the relationship between milk ELISA and the newly characterized Progressor status of MAP-shedding cows, with Progressors demonstrating significantly higher ELISA OD values. We have also provided a measure of clarification to the temporal relationship between fecal shedding and antibody production in milk, noting that spikes in fecal shedding, particularly those above a 100 copy/ 2g threshold, could predict high ELISA OD values up to 2 months later. Finally, we explored predictors of MAP positivity in milk and uncovered a potential relationship between poor udder hygiene and positive milk PCR results. The low overall positive rate of MAP in

individual milk samples and the absence of detectable MAP in the bulk tank throughout the study, suggest that milk contamination may not be of primary concern in low-prevalence herds.

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7. References

Bannantine, J. P., D. O. Bayles, W. R. Waters, M. V. Palmer, J. R. Stabel, and M. L. Paustian. 2008. Early antibody response against *Mycobacterium avium* subsp. *paratuberculosis* antigens in subclinical cattle. Proteome Sci. 6:5–16.

Beaver, A., C. Cazer, P. Ruegg, Y. Grohn, and Y. Schukken. 2016a. Implications of PCR and ELISA results on the routes of bulk-tank contamination with *Mycobacterium avium* subsp. *paratuberculosis.* J. Dairy Sci. 99:1391–1405.

Beaver, A., P. Ruegg, Y. Grohn, and Y. Schukken. 2016b. Comparative risk assessment for new cow-level *Mycobacterium avium* subsp. *paratuberculosis* infections between 3 dairy production types: Organic, conventional, and conventional-grazing systems. J. Dairy Sci. 99:9885–9899.

Begg, D. J., K. De Silva, N. Carter, K. Plain, A. Purdie, and R. Whittington. 2011. Does a Th1

over Th2 dominancy really exist in the early stages of *Mycobacterium avium* subsp. *paratuberculosis* infections? Immunobiology 216:840–846.

Bosshard, C., R. Stephan, and T. Tasara. 2006. Application of an F57 sequence-based real-time PCR assay for *Mycobacterium paratuberculosis* detection in bulk tank raw milk and slaughtered healthy dairy cows. J. Food Prot. 69:1662–1667.

Collins, M. T. 1996. Diagnosis of paratuberculosis. Vet. Clin. N. Am. Food Anim. Pract. 12:357–371.

Collins, M. T., S. J. Wells, K. R. Petrini, J. E. Collins, R. D. Schultz, and R. H. Whitlock. 2005. Evaluation of five antibody detection tests for diagnosis of bovine paratuberculosis. Clin. Diagn. Lab. Immunol. 12:685–692.

Corti, S., and R. Stephan. 2002. Detection of *Mycobacterium avium* subsp. *paratuberculosis* specific IS900 insertion sequences in bulk-tank milk samples obtained from different regions throughout Switzerland. BMC Microbiol. 2:15–21.

Eisenberg, S. W., R. Chuchaisangrat, M. Nielen, and A. P. Koets. 2013. Relationship between presence of cows with milk positive for *Mycobacterium avium* subsp. *paratuberculosis*-specific antibody by enzyme-linked immunosorbent assay and viable *M. avium* subsp. *paratuberculosis* in dust in cattle barns. Appl. Environ. Microbiol. 79:5458–5464.

Feller, M., K. Huwiler, R. Stephan, E. Altpeter, A. Shang, H. Furrer, G. E. Pfyffer, T. Jemmi, A. Baumgartner, and M. Egger. 2007. *Mycobacterium avium* subsp. *paratuberculosis* and Crohn's disease: A systematic review and meta-analysis. Lancet Infect. Dis. 7:607–613.

Firth, D. 1993. Bias reduction of maximum likelihood estimates. Biometrika 80:27–38.

Johnson, Y. J., J. Kaneene, J. Gardiner, J. Lloyd, D. Sprecher, and P. Coe. 2001. The effect of subclinical *Mycobacterium paratuberculosis* infection on milk production in Michigan dairy cows. J. Dairy Sci. 84:2188–2194.

Khol, J. L., M. Wassertheurer, E. Sodoma, S. Revilla-Fernandez, J. Damoser, E. Osterreicher, M. Dunser, U. Kleb, and W. Baumgartner. 2013. Long-term detection of *Mycobacterium avium* subsp. *paratuberculosis* in individual and bulk tank milk from a dairy herd with a low prevalence of Johne's disease. J. Dairy Sci. 96:3517–3524.

Koets, A. P., S. Eda, and S. Sreevatsan. 2015. The within host dynamics of *Mycobacterium avium* subsp. *paratuberculosis* infection in cattle: Where time and place matter. Vet. Res. 46:61–78.

Li, L., J. P. Bannantine, Q. Zhang, A. Amonsin, B. J. May, D. Alt, N. Banerji, S. Kanjilal, and V. Kapur. 2005. The complete genome se quence of *Mycobacterium avium* subsp. *paratuberculosis.* Proc. Natl. Acad. Sci. USA 102:12344–12349.

Liu, C., D. Cao, P. Chen, T. Zagar, and E. Lilly. 2007. Random and repeated statements—How to use them to model the covariance structure in Proc Mixed. Pages 1–11 in SAS Conference Proceedings: Midwest SAS User Group, Paper S02-2007. Eli Lilly & Company, Indianapolis, IN.

Lombard, J. E. 2011. Epidemiology and economics of paratuberculosis. Vet. Clin. N. Am. Food Anim. Pract. 27:525–535. Lombard, J. E., F. B. Garry, B. J. McCluskey, and B. A. Wagner. 2005. Risk of removal and effects on milk production associated with paratuberculosis status in dairy cows. J. Am. Vet. Med. Assoc. 227:1975–1981.

Manning, E. J., and M. T. Collins. 2001. *Mycobacterium avium* subsp. *paratuberculosis*: Pathogen, pathogenesis and diagnosis. Rev. Sci. Tech. 20:133–150.

Nielsen, S. S., C. Enevoldsen, and Y. T. Grohn. 2002. The *Mycobacterium avium* subsp. *paratuberculosis* ELISA response by parity and stage of lactation. Prev. Vet. Med. 54:1–10.

Nielsen, S. S., and N. Toft. 2012. Effect of days in milk and milk yield on testing positive in milk antibody ELISA to *Mycobacterium avium* subsp. *paratuberculosis* in dairy cattle. Vet. Immunol. Immunopathol. 149:6–10.

Pinedo, P. J., D. Rae, J. Williams, G. Donovan, P. Melendez, and C. Buergelt. 2008. Association among results of serum ELISA, faecal culture and nested PCR on milk, blood and faeces for the detection of paratuberculosis in dairy cows. Transbound. Emerg. Dis. 55:125–133.

Plikaytis, B. D., S. H. Turner, L. L. Gheesling, and G. M. Carlone. 1991. Comparisons of standard curve-fitting methods to quantitate *Neisseria meningitidis* group A polysaccharide antibody levels by enzyme-linked immunosorbent assay. J. Clin. Microbiol. 29:1439–1446

Raizman, E. A., S. Wells, S. Godden, R. Bey, M. Oakes, D. Bentley, and K. Olsen. 2004. The distribution of *Mycobacterium avium* subspecies *paratuberculosis* in the environment surrounding Minnesota dairy farms. J. Dairy Sci. 87:2959–2966.

Schreiner, D. A, and P. Ruegg. 2003. Relationship between udder and leg hygiene scores and subclinical mastitis. J. Dairy Sci. 86:3460–3465.

Schukken, Y. H., R. H. Whitlock, D. Wolfgang, Y. Grohn, A. Beaver, J. VanKessel, M. Zurakowski, and R. Mitchell. 2015. Longitudinal data collection of *Mycobacterium avium* subsp. *paratuberculosis* infections in dairy herds: The value of precise field data. Vet. Res. 46:65–75.

Singh, S. V., A. Singh, R. Singh, K. Sandhu, P. Singh, J. Sohal, V. Gupta, and V. Vihan. 2007. Evaluation of highly sensitive indigenous milk ELISA kit with fecal culture, milk culture and fecal-PCR for the diagnosis of bovine Johne's disease (BJD) in India. Comp. Immunol. Microbiol. Infect. Dis. 30:175–186.

Stabel, J. R., W. R. Waters, J. P. Bannantine, and K. Lyashchenko. 2011. Mediation of host immune responses after immunization of neonatal calves with a heat-killed *Mycobacterium avium* subsp. *paratuberculosis* vaccine. Clin. Vaccine Immunol. 18:2079–2089.

Streeter, R. N., G. F. Hoffsis, S. Bech-Nielsen, W. P. Shulaw, and D. M. Rings. 1995. Isolation of *Mycobacterium paratuberculosis* from colostrum and milk of subclinically infected cows. Am. J. Vet. Res. 56:1322–1324.

Sweeney, R. W., R. H. Whitlock, S. McAdams, and T. Fyock. 2006. Longitudinal study of ELISA seroreactivity to *Mycobacterium avium* subsp. *paratuberculosis* in infected cattle and culture negative herd mates. J. Vet. Diagn. Invest. 18:2–6.

Sweeney, R. W., R. H. Whitlock, and A. E. Rosenberger. 1992. *Mycobacterium paratuberculosis* cultured from milk and supramammary lymph nodes of infected asymptomatic cows. J. Clin. Microbiol. 30:166–171.

Taddei, S., C. Robbi, C. Cesena, I. Rossi, E. Schiano, N. Arrigoni, G. Vicenzoni, and S. Cavirani. 2004. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine fecal samples: Comparison of three polymerase chain reaction-based diagnostic tests with a conventional culture method. J. Vet. Diagn. Invest. 16:503–508.

van Schaik, G., C. R. Rossiter, S. M. Stehman, S. J. Shin, and Y. H. Schukken. 2003. Longitudinal study to investigate variation in results of repeated ELISA and culture of fecal samples for *Mycobacterium avium* subsp. *paratuberculosis* in commercial dairy herds. Am. J. Vet. Res. 64:479–484.

Wang, Z., and L. Goonewardene. 2004. The use of mixed models in the analysis of animal experiments with repeated measures data. Can. J. Anim. Sci. 84:1–11.

Weiss, D. J., O. A. Evanson, D. J. McClenahan, M. S. Abrahamsen, and B. K. Walcheck. 2001. Regulation of expression of major histocompatibility antigens by bovine macrophages infected with *Mycobacterium avium* subsp. *paratuberculosis* or *Mycobacterium avium* subsp. *avium.* Infect. Immun. 69:1002–1008.

Whitlock, R. H., S. Wells, R. W. Sweeney, and J. Van Tiem. 2000. ELISA and fecal culture for paratuberculosis (Johne's disease): Sensitivity and specificity of each method. Vet. Microbiol. 77:387–398.

Whitlock, R. H., and C. Buergelt. 1996. Preclinical and clinical manifestations of paratuberculosis (including pathology). Vet. Clin. N. Am. Food Anim. Pract. 12:345–356.

Whitlock, R. H., and A. E. Rosenberger. 1990. Fecal culture protocol for *Mycobacterium*

paratuberculosis: A recommended procedure. Proc. Annu. Meet. U.S. Anim. Health Assoc. 94:280–285.

Whittington, R. J., and E. Sergeant. 2001. Progress towards understanding the spread, detection and control of *Mycobacterium avium* subsp. *paratuberculosis* in animal populations. Aust. Vet. J. 79:267–278.

Whittington, R. J., D. J. Begg, K. de Silva, K. M. Plain, and A. C. Purdie. 2012. Comparative immunological and microbiological aspects of paratuberculosis as a model mycobacterial infection. Vet. Immunol. Immunopathol. 148:29–47.

Wynne, J. W., T. Seemann, D. M. Bulach, S. A. Coutts, A. M. Talaat, and W. P. Michalski. 2010. Resequencing the *Mycobacterium avium* subsp. *paratuberculosis* K10 genome: Improved annotation and revised genome sequence. J. Bacteriol. 192:6319–6320.

CHAPTER 5:
MATHEMATICAL MODEL REPRESENTING THE INFECTION DYNAMICS OF *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS:* THE DAIRY-FARM ENVIRONMENT AND THE BULK-TANK MILK SUPPLY

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KEYWORDS: mathematical modeling, *Mycobacterium avium* subsp. *paratuberculosis*, infection dynamics, bulk milk, environmental contamination

ABSTRACT

Johne's disease is a progressive granulomatous enteritis that predominately affects ruminants such as dairy cattle. The etiologic agent is *Mycobacterium avium* subsp. *paratuberculosis* (MAP), a complex and slow-growing pathogen that presents many challenges to effective diagnosis and intervention. Mathematical modeling provides an opportunity to study MAP transmission for long time periods and under a variety of scenarios that may not be feasible for real-world application. In this work, a stochastic, multi-group compartmental mathematical model has been developed to explore several uncharted mechanisms of MAP transmission and contamination in a dairy herd. First, the model includes indirect transmission of MAP via the environment, with a non-linear relationship between infection and environmental burden. The environment is partitioned into various compartments to mimic the layout of pens in a conventional dairy herd. Second, the bulk milk supply has been included to model routes of contamination and weigh their relative contributions to the overall MAP concentration in the bulk tank. These routes include direct shedding of MAP into milk as well as environmental contamination, and the burden of MAP in the bulk tank is permitted to influence the force of infection for calves. Monitoring of MAP contamination of bulk milk is also indicated if evidence is uncovered to link MAP to the development of human Crohn's Disease.

Using the model output, we determined that bulk-milk contamination is primarily a concern for high-prevalence herds in which the number of active shedders exceeds 12%. The environmental route (e.g., fecal contamination of teats and udders) was largely responsible for the observed MAP load in the tank, with direct shedding into milk accounting for less than 1% of the final MAP concentration. Based upon this information, several potential control programs

were selected, and their efficacy in reducing bulk-milk contamination and overall prevalence was evaluated.

Although recent evidence suggests that heavy shedders are not the drivers of infection persistence in dairy herds, they do appear to be influential in determining the concentration of MAP in bulk milk: annual culling of high shedders resulted in a 53% decrease in MAP CFU per liter of bulk milk. Pairing this initiative with rigorous cleaning of the maternity pen and strict milking-parlor hygiene resulted in milk that may be categorized as low-risk for human exposure; this risk assessment is based upon the known threshold of pasteurization and assumes normal consumption patterns. The conclusions from the mathematical model presented in this work may be used to tailor milk quality initiatives and control programs, with the objective of reducing herd prevalence and mitigating MAP contamination of the bulk-tank milk supply.

1. Introduction

Johne's disease is a chronic, progressive enteritis of ruminant animals caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). In cattle, the most common clinical manifestations are loose manure and weight loss, leading to nutrient malabsorption, dehydration, and cachexia (Manning and Collins, 2001). In addition to these severe health and welfare implications, MAP infections result in substantial economic losses for the U.S. dairy industry, upwards of 200 million dollars annually (Ott et al., 1999). Current estimates suggest that more than 68% of U.S. dairy farms house at least 1 MAP-infected animal (USDA, 2008).

From an epidemiological standpoint, MAP infections are highly complex, owing to lengthy latent periods that result in a large proportion of silent infections (Whitlock and Buergelt, 1996). In dairy herds, asymptomatic animals may shed MAP into manure and milk, often

unbeknownst to producers, thus spreading MAP throughout the farm environment. This environmental dissemination is particularly concerning, since the primary means of MAP infection is through the fecal-oral route from a contaminated environment (Whittington and Windsor, 2009). Even with testing schemes in place, subclinical animals may evade detection due to intermittent shedding and a myriad of limitations associated with diagnostic options. For example, fecal PCR is costly (Collins, 1996) and serum or milk ELISA may fail to detect animals in early infection stages (Whitlock et al., 2000). Fecal culture for MAP, traditionally referred to as the "gold standard," typically takes between 8 and 16 weeks and is not highly sensitive: recent research shows that even semi-annual fecal culture schemes are insufficient to detect the majority of infected animals in a herd (Schukken et al., 2015). Thus, in real-world scenarios, it is often difficult to accurately determine infection statuses of individual animals, and the feasibility of extensive longitudinal studies is limited by matters of cost and efficiency.

Due to these complexities, mathematical modeling of MAP in dairy herds is a useful tool for simulating a variety of different scenarios that may not be entirely feasible for field study. Adding years to mathematical model duration may correspond to an additional fraction of a second in computation time, depending on the program used. Moreover, altering the value of a model parameter is an attractive alternative to decades of herd monitoring to observe the true impact of a control strategy. Clearly, there is no substitute for real-world implementation; however, mathematical models may be regularly adapted and refined to incorporate new insights and mimic real-world scenarios. MAP models in particular must continue to develop alongside our evolving understanding of disease progression and transmission, since the model's value depends heavily on accurate representation of infection biology (Dorshorst et al., 2006, Mitchell et al., 2015b).

Several insights into MAP transmission have been gained recently and should therefore be incorporated into current mathematical models. Firstly, existing models of MAP on dairy farms have typically assumed complete age-acquired immunity in cattle greater than 1-year old (Mitchell et al., 2008; Lu et al. 2010). Recent research, however, supports the concept of a reduced susceptibility rather than complete resistance in older animals. Schukken et al. (2015) investigated strain-specific profiles of MAP-infected animals using 10 years of longitudinal data from 3 U.S. dairy farms; cows found to be infected with a given MAP strain at the time of slaughter were significantly more likely to have been exposed as adults to super-shedders infected with the same strain. This quantitative evidence suggests that new adult infections should be incorporated into our understanding of MAP transmission patterns.

Additionally, current mathematical models must be adapted to account for indirect MAP transmission via the dairy farm environment (Marcé et al., 2006). It is well-established that the primary means of MAP transmission is through the fecal-oral route (Doré et al., 2012; Whittington and Windsor, 2009) and that the bacteria may survive for several months in the environment (Lovell et al., 1944). Thus, bypassing environmental burden and modeling direct contact with MAP-shedding animals would almost certainly underestimate transmission rate, since MAP may persist in the environment even in the absence of contemporary shedding animals (Marcé et al., 2006). One informative model of environmental transmission in dairy herds has been developed (Marcé et al., 2011), but several new insights have since come to light. Slater et al. (2016) made use of extensive longitudinal data on shedding levels and persistent MAP infections in 4 dairy herds to investigate direct and environmental contribution to infection dynamics. The researchers applied a maximum likelihood approach and concluded that the primary contribution to average infectivity in each herd was from environmental transmission, as

a function of the number of actively shedding animals in the herd. Further, when the results from all herds were considered collectively, transmission from direct contact with infected animals made no contribution to infectivity (assuming a simplified density dependent method). Moreover, Slater et al. (2016) concluded that the relationship between environmental MAP burden and infectivity was non-linear. This insight may partially explain infection persistence in the absence of heavy shedders and the incongruously small contribution of these heavy shedding animals to overall infection dynamics. Current mathematical models of dairy herds often do not reflect these realities.

Lastly, mathematical models for MAP have not traditionally included the bulk tank milk supply as a model compartment. Bulk-milk contamination may factor into the force of infection, particularly if calves are fed unpasteurized milk from the herd (Stabel, 2001). Furthermore, MAP in the bulk tank may be considered a potential public health concern due to its hypothesized role in the development of human Crohn's Disease (Feller et al., 2007) and its documented survival in retail milk products (Ellingson et al., 2005). Although the association between MAP and Crohn's Disease has not been confirmed as causative, there is sufficient evidence to justify monitoring milk sources marketed for human consumption (Eltholth et al., 2009).

Recent research on routes of contamination of the bulk milk supply will allow us to model the status of the bulk tank as it correlates to environmental MAP concentration. There are 2 distinct routes of bulk-tank contamination, which have been classified as either "internal" or "environmental" (Beaver et al., 2016a); the "environmental" route results primarily from fecal contamination, whereas the "internal" route represents direct shedding of MAP by infected animals into their milk (Sweeney et al., 1992). To accurately model MAP burden in the bulk tank, the impact of each of these routes and the relationship of these two routes to overall

prevalence must be incorporated. The level of cleaning required to keep bulk-tank MAP below the pasteurization threshold is unknown, both with respect to hygiene of individual pens and hygiene during milking. Such questions may be explored following the addition of the bulk tank as a model compartment.

Certain model parameter values are well established, based upon MAP infection biology or dairy-herd structure, and do not require the flexibility to vary within the model. Such parameters include exit rates of various groups of animals (e.g., the death rate from clinical Johne's disease (Mitchell et al., 2008)). On the other hand, certain rates are likely to vary even on daily basis, such as fecal shedding levels for different infection classes (van Schaik et al., 2003) and the death rate of environmental MAP bacteria. Although deterministic models may be useful for assessing average results, they cannot account for such variance in study variables. The addition of stochastic elements will allow for flexibility in these parameters and provide a plausible range of potential outputs.

An understanding of the infection dynamics of MAP in dairy herds can help mitigate its spread. Development of a mathematical model that includes both the bulk tank and the farm environment may provide information allowing refinement of control programs and milk quality initiatives. The aim of our research was to study the impact of infection dynamics on the level of bulk-tank MAP, to understand the primary drivers of bulk-milk contamination, and to identify the most promising interventions. The ultimate objective of such a modeling approach is to contribute to a reduction in both herd prevalence and MAP contamination of the bulk-milk supply.

2. Methods

2.1 Model Description

The model was parametrized using cross-sectional and longitudinal data collected by our research group (Schukken et al., 2015; Beaver et al., 2016a, 2017) in addition to data from other published literature. The model was run using the Rosenbrock integration method in Berkeley Madonna (Berkeley, CA) by implementing daily time steps across a 100-year duration. Additional figures were created in GraphPad Prism (version 6, GraphPad, La Jolla, CA) based upon the obtained Berkeley Madonna data.

Using the basic framework established in the published model of Mitchell et al. (2008), we classified animals as Calves (under 1 year), Heifers (1-2 years), or Adult Cows (≥ 2 years). Likewise, we included the following infection states: Susceptible, Transient, Latent, Low Shedding, and High Shedding. Calves were classified as either susceptible (X_1) or, after becoming infected with MAP, transiently shedding (Tr_1). Heifers were classified as either susceptible (X_2), transient (Tr_2), or latent (H_2). Adult cows were partitioned into either susceptible (X_3), transient (Tr_3), and latent (H_3) compartments in addition to Low Shedding (Y_1) and High Shedding (Y_2) compartments. A constant total herd size of 100 conventionally-managed animals was used for the base model. Values for each age-compartment were determined using a representative dairy-herd age structure. Initial values were as follows: X_1 = 19; X_2 = 20; X_3 = 60. Infection was initiated in the herd at day 0 using a transient calf: Tr_1 = 1.

Our multi-group compartmental model of MAP transmission is depicted in *Figure 8* (generated using Lucidchart (Lucid Software, South Jordan, UT)).

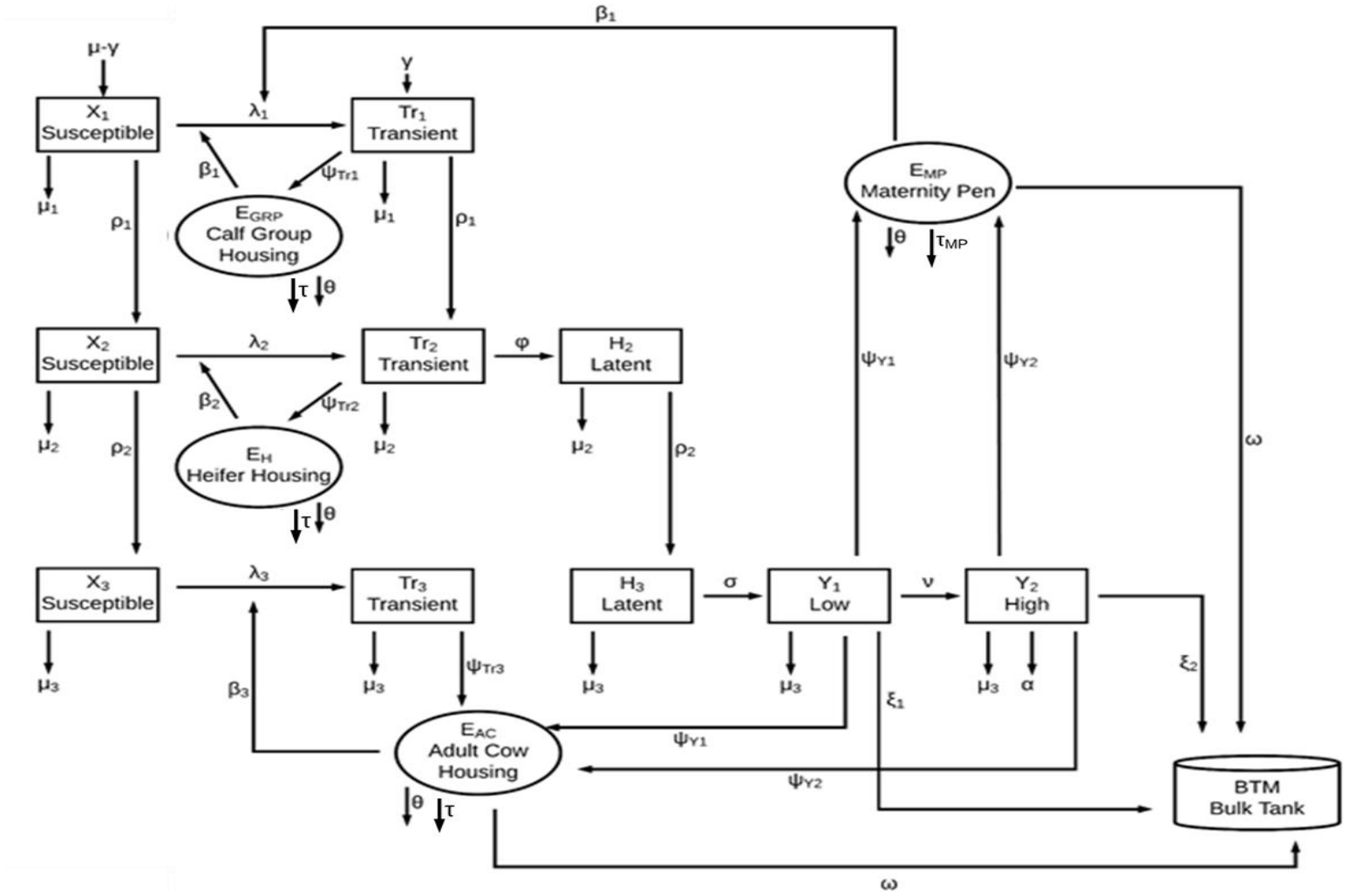

Figure 8. *Diagram Representing the Base Mathematical Model.* Groups of animals are represented by rectangular compartments, and animals move downward in the model as they age. The environmental MAP burden in each area of the dairy farm is represented by oval compartments, and the bulk tank is represented by a cylindrical compartment. For simplicity of representation, *Dry Cow Housing* is not shown in the diagram but may be considered a subset of *Adult Cow Housing.* Additionally, the relationship between bulk milk contamination and neonatal calf infection is not shown in the diagram but is included in the model equations.

The overall birthrate is given by μ, and the rate of vertical infection at birth is given by γ. Thus, susceptible calves are born at a rate of μ-γ. Based upon natural aging patterns, susceptible calves become susceptible heifers at a rate of ρ_1 and susceptible adult cows at a rate of ρ_2. Transient calves (born at a rate of γ) age into transient heifers at a rate of ρ_1, latent heifers at a rate of φ, and latent cows at a rate of ρ_2. Latent cows progress into the low shedding category at a rate of σ, subsequently into high shedding cows at a rate of ν. Cows infected as adults do not progress to latent stages but remain transient until removal from the herd (Mitchell et al., 2012). The removal rates for calves, heifers, and adult cows, are expressed as μ_1, μ_2, and μ_3 respectively. Additional death or removal due to clinical Johne's disease is represented by α.

The rate of environmental contribution in CFU/year is given by ψ and varies according to age, infection category, and lactation status. Adult cows were assumed to be dry for 60 days/year, with an age at first calving of 24 months (USDA, 2014). The removal rate of MAP bacteria after 1 year (a sum of the natural death rate and the efficiency of alleyway scrapers) is defined by $\theta + \tau$. Yearly cleaning of the Maternity Pen is parameterized by τ_{MP}.

λ_1, λ_2, and λ_3 represent the force of infection for calves, heifers, and adult cows, respectively. In previous models (Mitchell et al., 2008; Lu et al., 2010), λ_2, and λ_3 were assumed to be 0. In the current model, λ_1, λ_2, and λ_3 were parameterized in reference to the environment, which was partitioned into relevant compartments (see *Table 17* for important assumptions). The compartment E_{MP} represents the Maternity Pen, E_{GRP} represents group housing of calves, E_H represents the heifer housing environment, E_{AC} represents the adult cow environment, and E_{DC} represents the dry cow environment.

Table 17. *Environmental Compartments and Key Assumptions.* Each environment included in the model is listed, along with assumptions for the model herd.

	Description	*Number of Pens*	*Size per Pen*	*Time in Pen (days/year)*	*Manure (g/animals/day)*[†]	*Potential Occupants*
E_{MP}	Maternity Pen	1	10 m^2	1	75,200g [#]	X_3, Tr_3, H_3, Y_1, Y_2, X_1, Tr_1
E_{GRP}	Calf Group Housing	3	50 m^2	274*	12,400	X_1, Tr_1
E_H	Heifer Housing	3	50 m^2	305	24,500	X_2, Tr_2, H_2
E_{AC}	Adult Cow Housing	4	100 m^2	305	75,200g	X_3, Tr_3, H_3, Y_1, Y_2
E_{DC}	Dry Cow Housing	1	100 m^2	60	38,600g	X_3, Tr_3, H_3, Y_1, Y_2

*Calves were assumed to spend the first 3 months in individual housing (USDA, 2014). In this model, we also assumed calf hutches were cleaned between animals, thus making a negligible contribution to infection dynamics. The time spent in hutches is subtracted from the yearly Group Housing duration.

[†] Manure quantity estimated from Nennich et al., 2005

Reflects manure produced by adult animals only, as any manure produced by a calf in the Maternity Pen is assumed to have no impact on environmental MAP burden.

Animals are assumed to be exposed to a 10 m^2 area at a given time. Thus, λ_1, λ_2, and λ_3 may be defined as:

$$\lambda_1 = \beta_1\left(E_{MP} + 0.2(E_{GRP}) + 224(BT_{per_{liter}})\right)^{\eta} \quad [Force\ of\ infection\ for\ calves]$$

$$\lambda_2 = \beta_2(0.2(E_H))^{\eta} \qquad [Force\ of\ infection\ for\ heifers]$$

$$\lambda_3 = \beta_3(0.1(E_{AC} + E_{DC}))^{\eta} \quad [Force\ of\ infection\ for\ adult\ cows]$$

where β_1 is the transmission parameter for calves, β_2 is the transmission parameter for heifers, and β_3 is the transmission parameter for adult cows. η is a power term used to account for the non-linearity in the force of infection relative to MAP burden (Slater et al., 2016). In our model herd, calves are weaned at 8 weeks of age and are assumed to drink, on average, 4L of raw bulk milk per day until this time (USDA, 2014). The measurement unit $BT_{per_{liter}}$ represents the MAP concentration (CFU/L) in the bulk tank milk supply.

Since the bulk tank on dairy farms is regularly emptied and cleaned, typically on a daily basis, it is assumed that MAP bacteria do not accumulate across the time steps. Therefore, to

accurately represent the MAP burden at any given time, the bulk tank compartment in our model is not represented as a differential equation but as an instantaneous measurement. $BT_{per_{liter}}$ is expressed as follows:

$$BT_{per_{liter}} = \frac{\kappa\,(\omega(E_{AC} + E_{MP}) + 0.836(\xi_1(0.05\,Y_1) + \xi_2(0.19\,Y_2))}{0.836\,(32X_3 + 32Tr_3 + 34H_3 + 30Y_1 + 26Y_2)}$$

where ω is the rate at which MAP enter the bulk tank from the environment, such as through contaminated teats or udders. The efficiency of the milk filter at preventing bacteria from entering the bulk milk supply is 1-κ, thus the inefficiency of the filter is given by κ. The rate of direct shedding into milk by low shedders is represented as ξ_1, and ξ_2 is the rate of direct shedding by high shedders, with 5% of low shedders and 19% of high shedders assumed to be shedding directly into milk at any given time (Sweeney et al., 1992). The number of liters of milk produced per day by cows with different infection statuses was estimated according to Smith et al. (2009) and is accounted for in the denominator of the equation; 83.6% of adult cows are assumed to be lactating at a given time (USDA, 2014), and the remaining 16.4% are dry cows. The resulting bulk milk concentration is permitted to take on values ≥ 0. Additional parameters include ι (latency until removal of the calf from the calving pen) and δ (yearly rate of culling for heavy shedders during an intervention program). The input values for all model parameters are shown in *Tables 18* and *19.*

Table 18. *Population Parameters in the Base Model.* Relevant equations pertaining to the simulated herd are shown.

	Description	**Value**
N	Total population	$N_1+N_2+N_3$
N_1	Calf population	X_1+Tr_1
N_2	Heifer population	$X_2+Tr_2+H_2$
N_3	Adult cow population	$X_3+Tr_3+H_3+Y_1+Y_2$
Prevalence	Overall herd prevalence	$(Tr_1+Tr_2+H_2+Tr_3+H_3+Y_1+Y_2)/N$
Shedding Level	Percentage of shedding animals	$(Tr_1+Tr_2+Tr_3+Y_1+Y_2)/N$

Table 19*. Values for Parameters in the Base Model.* Unless otherwise noted, rates are expressed as animals or bacteria per year.

	Description	Value	Reference
Γ	Proportion of calves infected at birth	$0.08((Y_1+Y_2+Tr_3)/N)$	Unpublished data[1]
μ_1	Removal rate of calves	0.09	Mitchell et al., 2008
μ_2	Removal rate of heifers	0.01	Mitchell et al., 2008
μ_3	Removal rate of cows	$\mu_{3v} + \mu_{3inv}$	Mitchell et al., 2015a
μ_{3v}	Voluntary cull rate	$\mu_{3vol} - Y_2(\delta+\alpha)/N_3$	Mitchell et al., 2008
μ_{3inv}	Involuntary cull rate	0.05	Mitchell et al., 2008
μ_{3vol}	No adjustment for disease-related exit	0.3	Mitchell et al., 2008
Δ	Cull rate of Y_2 during intervention	0	No intervention in the base model
A	Removal or death rate from clinical JD	0.67	Smith et al., 2010
Σ	Exit rate from latent adult cows to low shedders	0.60	Mitchell et al., 2008
N	Exit rate from low to high shedders	0.22	Mitchell et al., 2015a[2]
β_1	Transmission rate to calves	0.01	Mitchell et al., 2015a
β_2	Transmission rate to heifers	0.005	Humphrey et al., 2006
β_3	Transmission rate to adult cows	0.001	Schukken et al., 2015
Ψ	MAP contribution to the environment (expressed as CFU/cow/year)[3]		
ψ_{Tr1}	Contribution from a transient calf	3.40×10^7	Grant et al., 2001; Beaver et al., 2017; Chiodini et al, 1984; Nennich et al., 2005; Mitchell et al., 2015b
ψ_{Tr2}	Contribution from a transient heifer	8.94×10^7	
ψ_{Tr3}	Contribution from a transient adult	2.29×10^8	
ψ_{Y1}	Contribution from a low shedder	1.15×10^9	
ψ_{Y2}	Contribution from a high shedder	3.65×10^{13}	
ψ_{Tr3}	Contribution from a transient dry cow	2.32×10^7	
ψ_{Y1D}	Contribution from low shedding dry cow	1.16×10^8	
ψ_{Y2D}	Contribution from high shedding dry cow	1.87×10^{13}	
Θ	Death rate for MAP in the environment	0.995	Humphry et al., 2006
T	Additional disappearance rate of MAP[4]	273.75	Expert opinion
τ_{MP}	Additional disappearance rate of MAP in the Maternity Pen[5]	1.00	
H	Power term for force of infection	0.116	Slater et al., 2016
ξ_1	CFU shed into milk by a low shedder (daily rate)	1,200	Sweeney et al., 1992; Smith et al., 2009
ξ_2	CFU shed into milk by a high shedder (daily rate)	2,600	
Ω	Rate at which environmental MAP enters bulk milk	0.0005	Unknown. Estimates from Beaver et al., 2016a, 2017
K	Milk filter inefficiency	0.33	Van Kessel et al., 2011
I	Days/year cow-calf pair spends in maternity pen	1	USDA, 2014

1. According to strain-typing data from the Regional Dairy Quality Management Alliance, 10 of 123 infected dams (8%) from a NY State dairy herd appeared to transmit MAP to their daughters.
2. Mitchell et al., 2015a calculated a probability of more than 0.0006 per day for continuous low shedders to transition to high shedding
3. Values for ψ have been calculated using the manure quantities and durations spent in each pen, as listed in *Table 17*.
4. Assuming the alley scrapers are successful at eliminating 75% of MAP on a daily basis
5. We assume that the bedded pack in the maternity pen is thoroughly cleaned once per year

2.2 Main Research Questions

Using our mathematical model, we sought to address the following research questions:

1) Which model parameters are most influential in determining a) bulk-milk MAP concentration, b) overall herd prevalence, and c) environmental burden?
2) Which parameters are unknown or require further data collection in real-world settings?
3) What is the relative impact of the environmental and internal routes on the concentration of MAP in the bulk tank?
4) What is the relationship between prevalence, percentage of active shedders, and level of bulk milk contamination? How does this insight relate to the threshold for MAP detection in the bulk tank and the threshold of pasteurization?
5) What interventions (individually and in combination) effectively eliminate bulk-tank MAP or drive it below these thresholds? How is herd prevalence affected by these interventions?

2.3 Stochastic Elements

In order to allow for natural variability in MAP shedding levels and the death rate of MAP bacteria, several parameters were permitted to vary at each time step. For MAP environmental death rate, θ, a normal distribution was assumed, with an average ± SD yearly death rate of 0.995 ± 0.01 (Humphrey et al., 2006). For the shedding levels (ψ_1, ψ_2, ψ_3, ψ_{3D}, ψ_{3L}, ψ_{3H}, ψ_{3LD}, and ψ_{3HD}), a normal distribution was again used. For each ψ, the average shedding level modeled corresponded to the parameter value reported in *Table 19,* and the SD was estimated to be the average shedding level reduced by 10 fold. Because fecal shedding levels, both between and within animals, are known to vary substantially on a day-to-day basis, the model was run using daily time steps for 100 years to understand slow-shifting herd dynamics and prevalence stability.

2.4 Model Validations

We ran the base (deterministic) model to evaluate it in terms of overall biological plausibility according to relevant literature. We aimed to ensure that the final age distributions and number of animals in each infection category were representative of real-world scenarios (for example, Schukken et al. 2015). Additionally, by means of the sensitivity analysis, we were able to identify and correct any unexpected associations between model inputs and outputs.

2.5 Sensitivity and Scenario Analysis

To evaluate several of our main research questions, we conducted one-factor-at-a-time sensitivity analyses. We aimed to address the impact of several key rates on the MAP concentration in the bulk tank, the level of environmental burden, and overall herd prevalence. Two boundary values were established for each tested rate, and six intermediate values were generated using arithmetic or geometric series. The tested input parameters and their boundary values are listed in *Table 20.* For these analyses, we applied a fourth-order Runge-Kutta integration method in Berkeley Madonna to both the stochastic and base models.

Table 20. *Input Parameters for One-factor-at-a-time Sensitivity Analyses.* Unless otherwise specified, the boundary values are shown as rates per year.

Variable	Description	Lower boundary	Upper boundary
β_2	Transmission rate to heifers	0.001	0.009
β_3	Transmission rate to adult cows	0.0005	0.005
ξ_2	Rate of direct shedding into milk by high shedders (CFU/day)	1,040	100,000
ω	Rate at which environmental MAP enters the bulk tank	0.0001	0.005
η	Power term for force of infection (no units)	0.020	0.200
κ	Milk filter inefficiency	0.200	0.400

Using information obtained from the sensitivity analyses, intervention strategies were evaluated individually and in succession (based upon 1,000 iterations per scenario), to

understand their influence on prevalence and bulk-tank MAP concentration. The following two interventions were modeled: 1) cleaning of the maternity pen following each calving ($\theta_{MP} = 58$, given 58 adult cows calving 1x each per year) and 2) annual and bi-annual culling of heavy shedders ($\delta = 1$ or 2, respectively).

3. Results

3.1 Model Validation

The base, deterministic model yielded biologically-plausible results. According to a snapshot measurement taken after 100 years, the herd was comprised of 20 calves, 22 heifers, and 58 adult cows, representative of a conventional U.S. dairy herd distribution. Of the 100 animals, 7 were transient shedders (3 calves, 2 heifers, and 2 adults), 4 were low shedders, 1 was a high shedder, and 8 were latently infected (4 heifers and 4 adults). These numbers (rounded to the nearest animal) are consistent with the iceberg phenomenon (Whitlock and Buergelt, 1996), which suggests that for every advanced clinical case, there are 15-25 cows with subclinical MAP infections present in the herd. With no added intervention, the prevalence (20%) and shedding level (12.2%) were reasonable and in agreement with values obtained in previous transmission models for MAP (e.g., Lu et al., 2010) and observational studies (Mitchell et al. 2015).

3.2 Bulk Milk MAP Concentration

Based upon 1,000 iterations of the stochastic model, the MAP concentration in bulk milk stabilized after approximately 30 years and reached a mean ± SD of 8,3010 CFU/L ± 211 after 100 years. The minimum and maximum values observed were 7,611 and 8916 CFU/L, respectively. *Figure 9* shows the relationship between the percentage of active shedders in the herd and the bulk-tank MAP concentration.

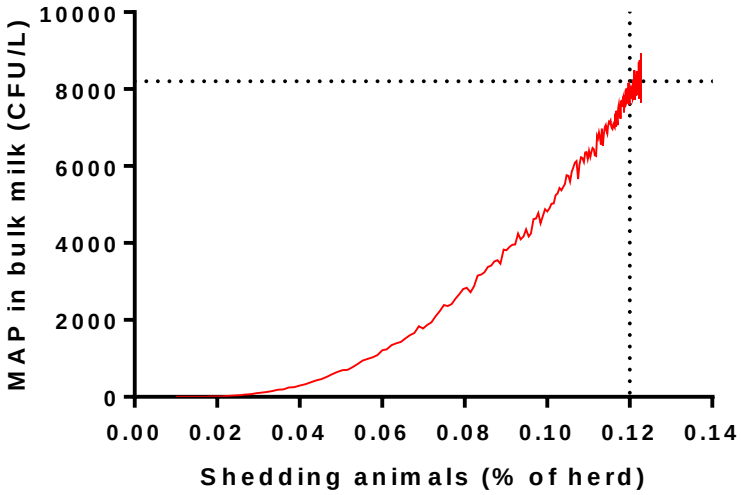

Figure 9. *Relationship between the Percentage of Active Shedders and the Bulk Milk Concentration.* Bulk milk MAP concentration (CFU/L) is shown on the Y axis and the percentage of active shedders in the herd is shown on the X axis. The detection threshold (8,200 MAP CFU/L) is given by a horizontal dotted line. The percentage of active shedders needed to reach this threshold is indicated by the vertical dotted line.

3.3 Sensitivity and Scenario Analysis

Graphical results from relevant one-factor-at-a-time sensitivity analyses are shown in *Figures 10-12*. In summary, changing the value of ω (permitted to vary between 0.005 and 0.0001) had the largest impact on bulk milk MAP concentration, resulting in a reduction of 81,131 CFU/L (*Figure 11a*). In *Figure 11c*, the small changes in bulk-milk MAP concentration, despite the wide range of input values for ξ_2, suggests that direct shedding into milk is not a main mechanism for bulk tank contamination. Changing the efficacy of milk filtration ($1-\kappa$) from 60% to 80% resulted in a reduction of 5,017 MAP CFU/L in the bulk tank. The greatest effect on herd prevalence was obtained by varying η between 0.02 and 0.2, which resulted in final prevalence of 2.50% and 74.98%, respectively (given no change in the corresponding values for β). Because of the large effect of η on prevalence, changing the value of η also had the largest impact on environmental MAP concentration, followed by alterations in θ and β_3.

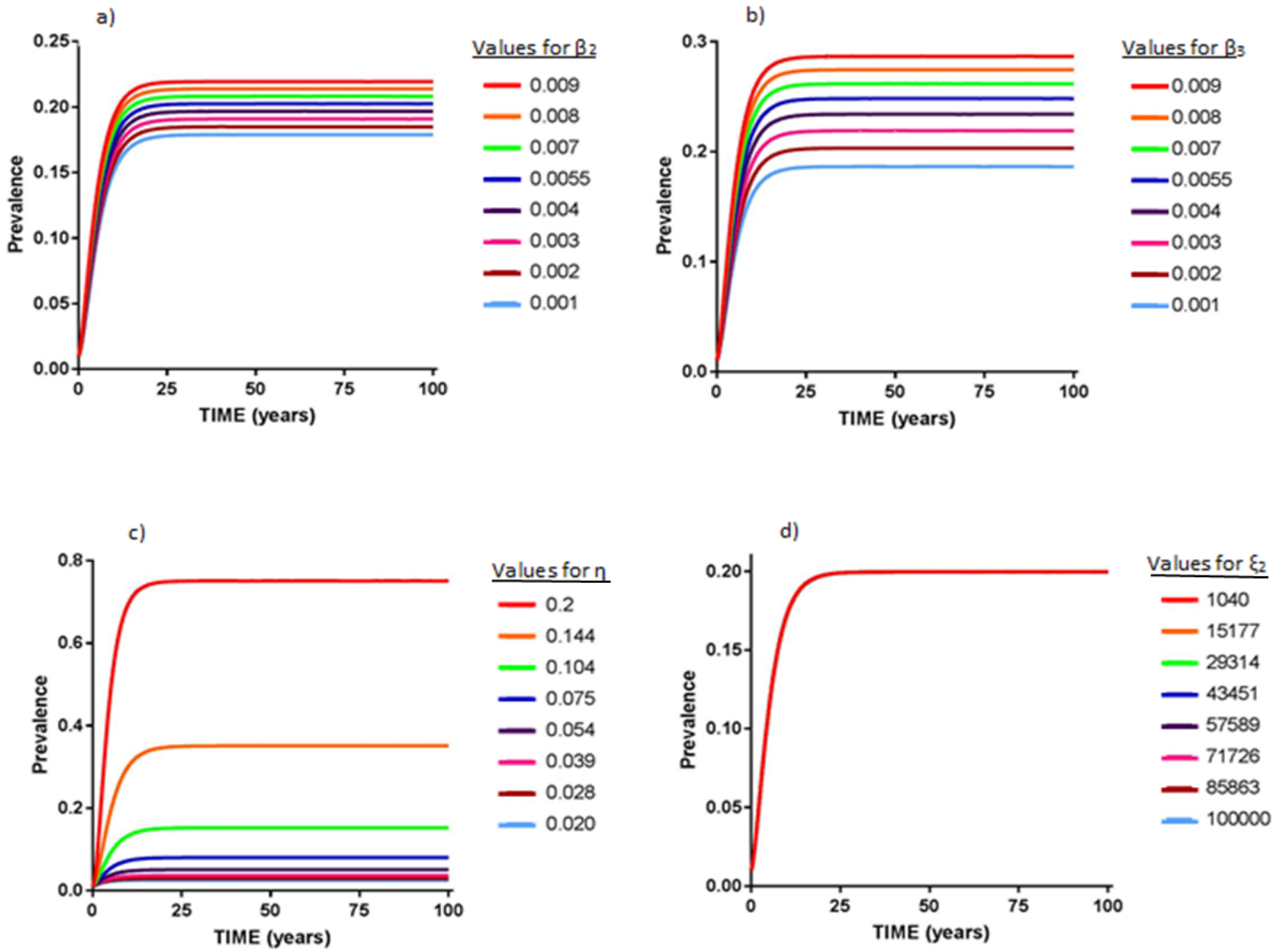

Figure 10. *Results of the Sensitivity Analysis on Overall Prevalence.* a) β_2 b) β_3 c) η d) ξ_2

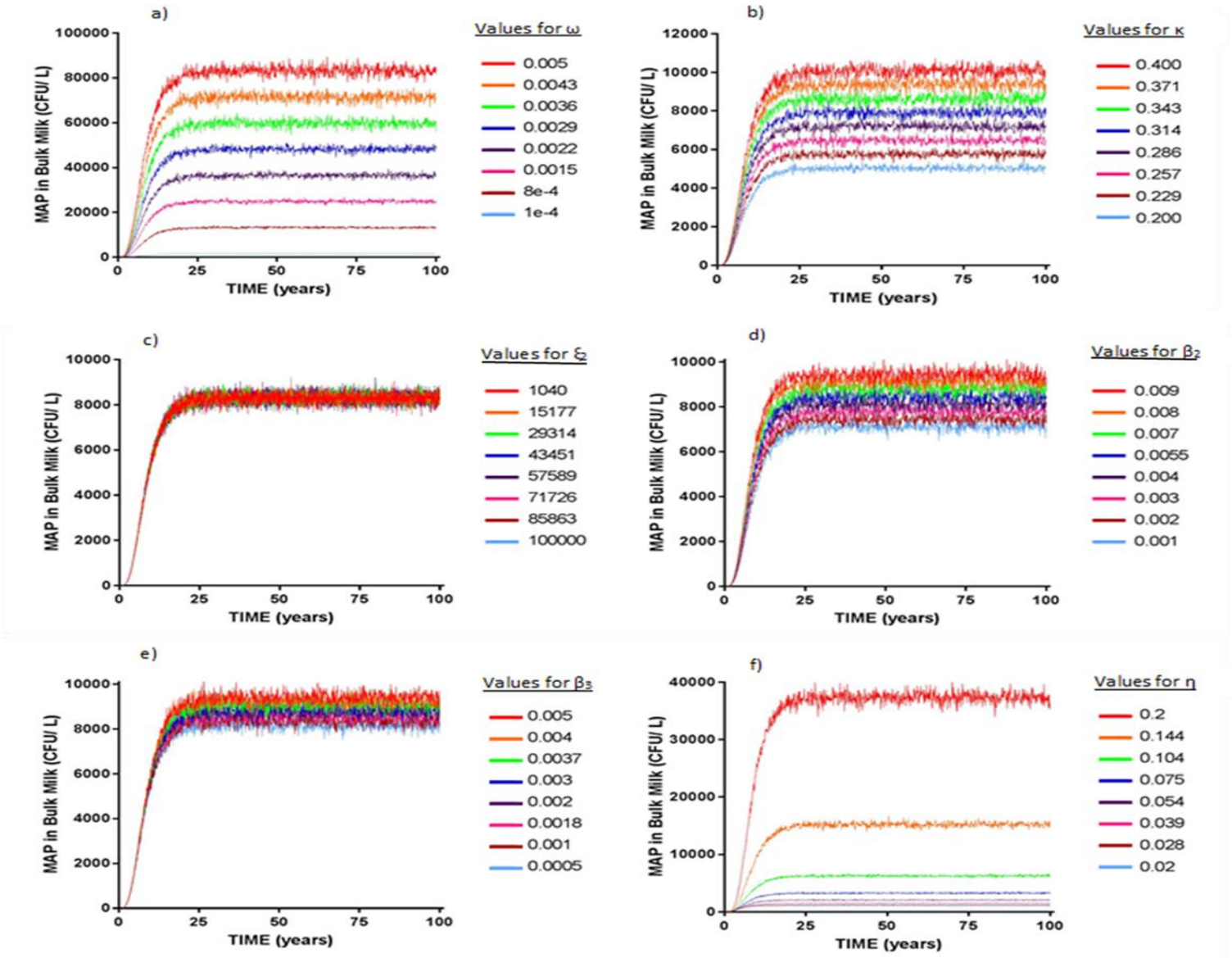

Figure 11. *Results of the Sensitivity Analysis on Bulk Milk MAP Concentration.* a) ω b) κ c) ξ_2 d) β_2 e) β_3 f) η

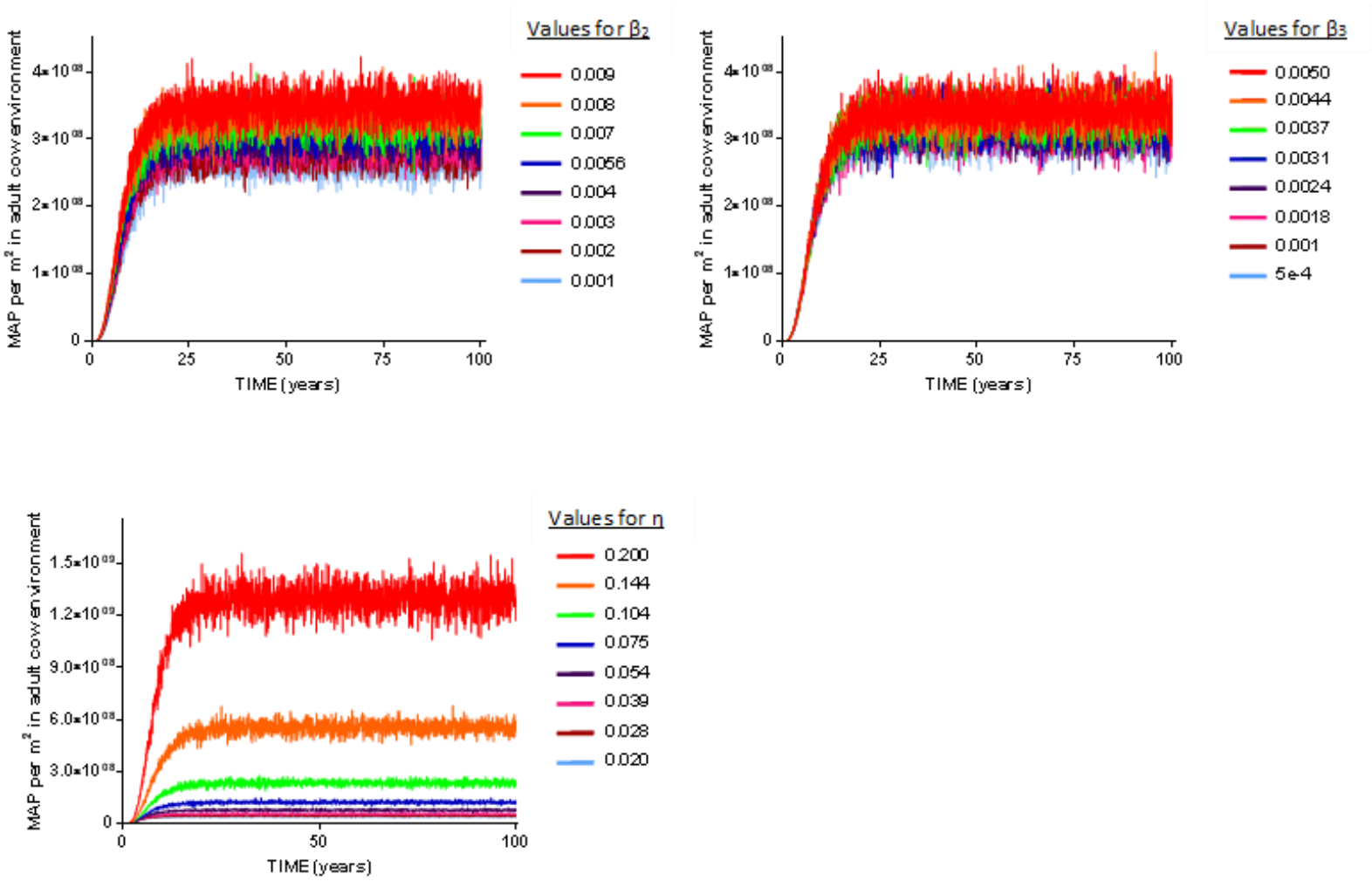

Figure 12. *Results of the Sensitivity Analysis on Environmental MAP load in the Adult Cow Environment* a) β_2 b) β_3 c) η

Compared to *Figure 10*, the results depicted in *Figures 11* and *12* display higher variance, which reflects a greater level of uncertainty. The difference in variance implies that overall herd prevalence is not as strongly impacted by the model's stochastic elements. This phenomenon may predominately be explained by the non-linear representation of the relationship between infection and environmental burden. The parameters permitted to vary within the model include the death rate of MAP bacteria and the level of shedding within each infection category; understandably, variation in fecal shedding directly impacts environmental burden. These stochastic elements would also be expected to have a substantial influence on the level of bulk milk contamination given that the environmental route represents the main source of bulk-milk contamination. On the other hand, due to the inclusion of the exponent η in the model, the prevalence varies only marginally alongside fluctuations in environmental burden.

Figure 13 is a graphical representation of interventions implemented in the study herd.

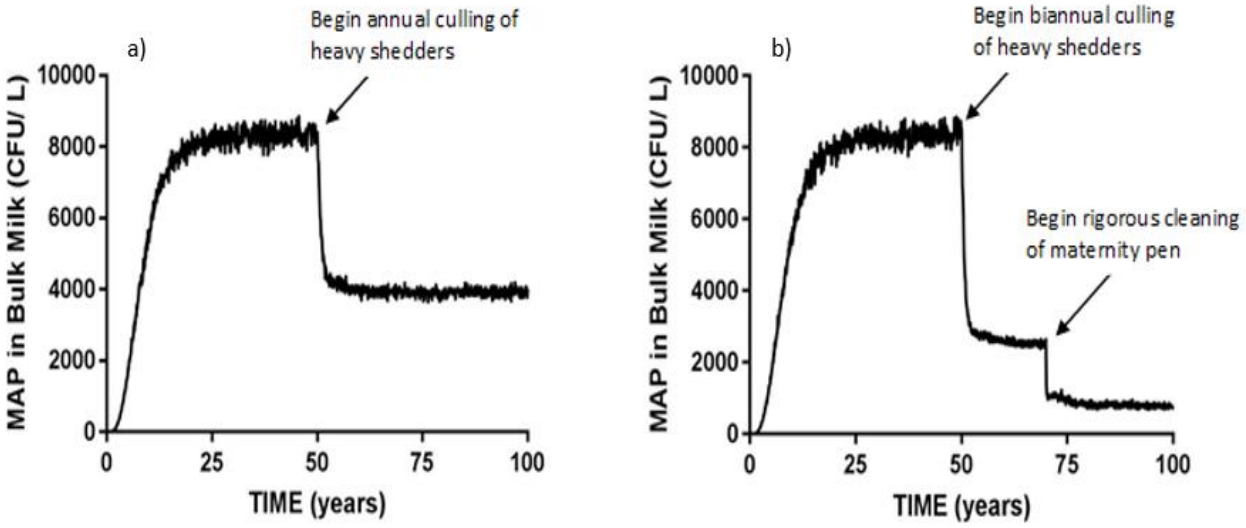

Figure 13. *The Impact of Interventions on Bulk-Milk MAP Burden.* a) The effect of culling high shedders 1x per year on bulk milk MAP concentration. In this hypothetical scenario, the intervention is initiated at 50 years b) The effect of culling high shedders 2x per year (initiated at 50 years) on bulk milk MAP concentration in combination with cleaning the maternity pen after each calving (initiated at 70 years).

Cleaning of the maternity pen following each calving resulted in a reduction of 5,700 ± 150 MAP CFU/L in bulk milk (corresponding to the mean and SD resulting from 1,000 simulations). Annual culling of heavy shedders reduced the bulk-milk MAP concentration by 4,379 ± 98 CFU/L, while biannual culling reduced it by 5,785 ± 62 CFU/L. The most effective intervention involved a combination of biannual culling of heavy shedders and rigorous maternity-pen cleaning, which collectively resulted in a 90.5% reduction of MAP in the bulk tank (corresponding to a reduction of 7,529 ± 47 MAP CFU/L).

The effect of these interventions (biannual culling of heavy shedders and rigorous cleaning of the maternity pen) as ω is systematically decreased, is shown in *Figure 14*. As ω is expected to decrease with improved udder and teat hygiene, it may be considered as a function of milking-parlor hygiene.

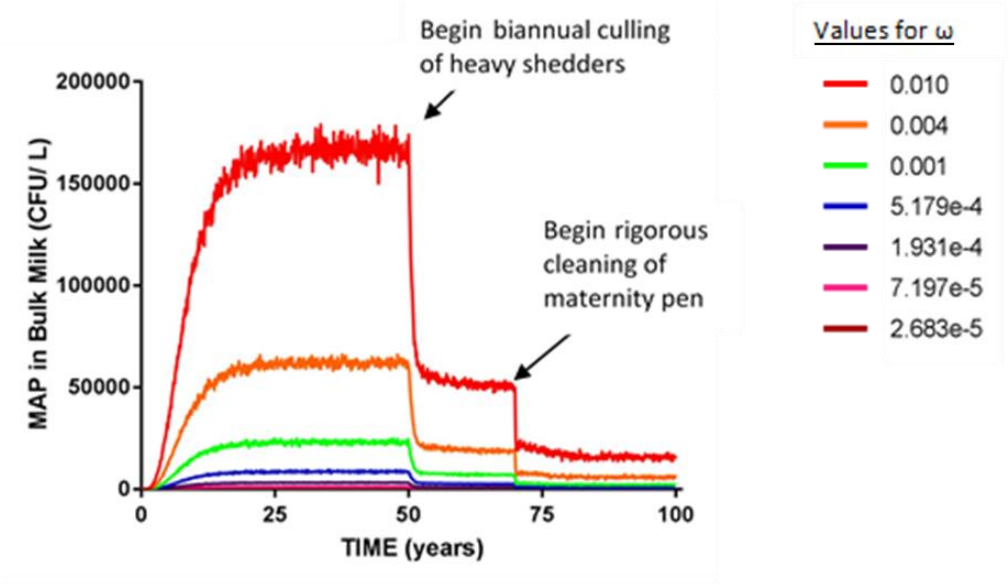

Figure 14. *The Impact of Interventions on Bulk-Milk MAP Burden, Given Different Levels of Milking Parlor Hygiene.* In this scenario, biannual culling of heavy shedders is initiated at 50 years and rigorous maternity-pen cleaning begins at 70 years. The colored lines correspond to differing levels of ω, as a function of milking-parlor hygiene.

4. Discussion

In this work, we developed a mathematical model for MAP transmission in dairy herds that reflects new or unexplored facets of MAP transmission, environmental burden, and bulk-milk contamination routes. To accomplish this objective, we relied upon field data and published literature to establish representative parameter values. We sought to evaluate the impact of a variety of factors on the overall herd prevalence and concentration of MAP bacteria in the bulk tank. Finally, we aimed to assess the ability of intervention strategies to mitigate bulk-tank contamination.

Based upon 1,000 iterations of the base stochastic model, without specific control programs in place, the MAP concentration in the bulk tank stabilized after approximately 30 years and reached an average of 8,310 CFU/L. In a previous study, we established the threshold of detection for MAP in milk using *hspX*-based qPCR and serial dilutions of MAP-spiked milk: samples with 82,000 MAP CFU/L were detected 100% of the time, while concentrations of 8,200 CFU/L were detectable in half of all samples tested (Beaver et al., 2016a). Thus, for our modeled herd, the MAP concentration in the bulk tank at 100 years hovers around the threshold of detection, with a likely detection rate of approximately 35.5%[1]

These bulk milk concentrations should be considered in light of overall herd prevalence and shedding levels. To date, there is little information pertaining to this association, and it may be valuable to determine the prevalence level necessary for a bulk tank to test positive for MAP. In our model, we define overall prevalence as the percentage of infected animals (latent, transient, low-shedding, and high-shedding) present in the herd at a given time. In the absence of any intervention, our hypothetical herd attained a prevalence of approximately 20%, with 12% of

[1] Calculation is based upon the 1,000 simulations and upon the assumption that MAP CFU/L must reach 8,200 for a 50% chance of detection.

animals shedding some quantity of MAP in feces. This result represents an above-average shedding level: in the United States, the average proportion of infected animals in a MAP-positive dairy herd is reported at 5 to 10% (Lombard, 2011). This estimation more plausibly represents an average number of shedders rather than true prevalence; latently-infected animals, by definition, are unlikely to test positive by fecal culture and may evade detection by ELISA as well if they have not reached a stage of antibody production (Streeter et al., 1995).

Figure 9 shows the relationship between the shedding level in our model and bulk-tank MAP. The detection threshold of 8,200 MAP CFU/L corresponds to a 12% shedding level (i.e., 12% of the herd actively shedding MAP). We may therefore hypothesize that substantial MAP contamination of bulk milk occurs predominately in herds with an above-average number of shedders. Indeed, our real-world data have indicated that detectable bulk-tank contamination with MAP is rare (according to a cross-section of U.S. dairy herds, Beaver et al., 2016a) and may not be a primary concern in low-prevalence herds (Beaver et al., 2017). Of course, the probability of bulk milk contamination depends heavily upon the rigorousness of hygiene practices throughout the farm environment and within the milking parlor (Galton et al., 1982). Because the threshold of detection is contained within our model's obtained values for bulk-tank MAP concentration, we are in a unique position to evaluate the likelihood of detection following implementation of a variety of control strategies.

In the Netherlands, a country with a 30-71% herd-level MAP prevalence (Weber et al., 2009), a Bulk Milk Quality Assurance Programme was initiated in 2006, with the goal of mitigating MAP contamination of the bulk tank (Weber et al., 2009). Dutch dairy farms testing positive for MAP (via individual milk ELISA) enter a control program and are advised to cull test-positive cattle. The animals targeted for culling in such a program would primarily be

expected to be high shedders, since antibody production (and thus a positive ELISA result) occurs predominately in the later stages of MAP infection (Streeter et al., 1995). Using our mathematical model, we were able to assess the efficacy of such a milk-quality program by simulating the removal of high shedders from the herd. We found that culling of high shedders led to a demonstrable reduction in bulk milk MAP concentration, and that the largest impact was achieved by pairing this initiative with other control measures. Targeting maternity pen hygiene in addition to annual culling of heavy shedders reduced the bulk tank MAP concentration from approximately 8,437 CFU/L to 2,900 CFU/L. Improving maternity pen hygiene and culling heavy shedders on a bi-annual basis further reduced the MAP concentration to 1,876 CFU/L (*Figure 13b*). Although removing high shedders may be valuable in lowering bulk milk MAP, this intervention alone is generally not effective in completely eradicating MAP from the herd (Slater et al., 2016; Lu et al., 2010). For example, in the current model, biannual culling of heavy shedders reduced the overall prevalence from 20% to 17.4%. Long-term testing, paired with improved hygiene and cleaning initiatives, therefore remains essential.

MAP in the bulk tank may survive high-temperature short-time (HTST) pasteurization when present at initial high concentrations ($> 10^4$ CFU/L, Grant et al., (1996, 2005)). Because the likelihood of human disease following MAP exposure is currently unknown, ensuring that MAP in the bulk tank remains well below this threshold seems important. Weber at al. (2008) aimed to simulate a quality-assurance program for bulk tank milk based upon the likelihood of pasteurization survival; herds with a high probability of bulk tanks at $< 10^3$ CFU/L concentrations were classified as low risk. All bulk milk concentrations observed in our model were below 10^4 CFU/L but above 10^3 CFU/L. In order to drive the bulk milk concentration below 10^3 CFU/L limit, it becomes necessary to improve hygiene practices in the milking parlor.

Pairing this initiative with culling of high shedders and cleaning of the maternity pen following each calving results in low-risk milk (see *Figure 14*).

Data are available on the efficacy of milk filters at preventing bulk milk contamination with salmonella (Van Kessel et al., 2011). According to this work[2], we estimated a 33% milk-filter inefficiency and used this value as a baseline in the model. Milk-filter inefficiency likely varies based upon the brand of filter, the frequency of filter change, and the type of bacterium under study. Because of MAP's tendency to form clumps (Grant et al., 2002), its ability to pass through a milk filter may not correspond exactly to the baseline value. We therefore conducted sensitivity analyses to explore the range of possible bulk tank concentrations when milk-filter efficiency was altered. With a 40% inefficiency, the concentration of MAP in the bulk tank reached an average of 10,291 CFU/L compared to 5,146 CFU/L when the inefficiency was only 20% (see *Figure 11f*). Differences in milk filter efficiency between farms with similar prevalence and shedding levels may partially explain why certain of these herds test consistently test positive for bulk tank MAP while others do not.

We also aimed to weigh the relative impact of the environmental and internal routes of transmission on bulk milk contamination with MAP bacteria. Sweeney et al. (1992) concluded that approximately 19% of heavy fecal shedding cows and 3 to 11% of low fecal-shedding cows secreted MAP directly into milk. The colony counts observed in individual milk samples ranged from 2 to 8 CFU/50 mL (Sweeney et al., 1992). Based upon the results of our sensitivity analyses, direct shedding into milk (i.e., the internal route) did not appear to be highly influential for the bulk-tank MAP concentration (as shown in *Figure 11b*). Given that high shedders produce 26 L of milk per day (Smith et al., 2009) the upper limit for direct shedding would

[2] 74 out of 111 dairy operations had culture-positive milk filters with corresponding culture-negative bulk tanks (Van Kessel et al., 2011)

amount to only 4,160 CFU/day per high shedding animal (Sweeney et al., 1992). Applying this upper limit to the model resulted in only a 0.001% increase in bulk-milk MAP compared to a complete absence of direct shedding. In the unlikely scenario in which high shedders contribute 5x the upper limit (20,800 CFU in milk per high shedder per day), the percent increase remained low, at 0.006%. Thus, using the current model, the environmental route may be pinpointed as the dominant route of bulk-tank contamination with MAP, accounting for more than 99% of the MAP burden in bulk milk.

What interventions may serve to effectively minimize calf exposure to adult cow manure? Our model allows us to address this question by evaluating the impact of various intervention strategies on environmental MAP burden and herd prevalence. In parallel, we may track bulk tank MAP concentration in response to these interventions. The Voluntary Bovine Johne's Disease Control Program of the United States counsels herds to make use of an individual calving pen that is clean, dry, and free of manure (USDA, 2010). Additionally, immediate separation of calves and dams is recommended. We found that a thorough cleaning of the maternity area following each calving resulted in a substantial reduction in environmental MAP burden (4.64×10^9 CFU/m^2 vs. 1.24×10^8 CFU/m^2). Cleaning of the maternity pen after each calving also resulted in a substantial reduction of bulk-milk MAP concentration (8,453 CFU/L vs. 6,084 CFU/L) and overall herd prevalence. In addition, we assessed the impact of prompt calf removal from the maternity pen (within the first 6 hours after birth) on the force of infection, compared to allowing the calf to remain in maternity for 5 full days (120 hours). In the absence of any additional hygiene practices, a prompt separation of cow and calf effectively prevented 1 new calf infection per year in our standard herd. If, however, the calving area was cleaned following each calving and heavy shedders are biannually culled, the effect of extended

cow-calf contact on prevalence is diminished, leading to only 1 new calf infection every 5 years. Thus, there is more flexibility to permit cow-calf contact if hygiene within the calving area is improved and other control measures are implemented (see also Beaver et al., 2016b). With high hygiene standards, the reported benefits of cow-calf contact in the sectors of health, welfare, and production (Bar-Peled et al., 1997; Stott et al., 1979) may outweigh the risks in the case of test-negative dams.

4.1 Model Limitations

The utility of mathematical models is dictated by the accuracy of parameter values, and thus by our understanding of disease pathobiology and epidemiology (Dorshorst et al., 2006; Mitchell et al., 2015b). Because of the complex nature of MAP infections, knowledge of these facets is continually expanding and evolving. Notably, the non-linear relationship between infectiousness and environmental MAP burden has only recently been presented (Slater et al., 2016) and the degree of concavity in this relationship appears to vary substantially across herds. Slater et al. (2016) noted a good fit when the power term (represented as η in our model) ranged from 0.1 to 0.35. This range would drastically affect the force of infection without a concurrent adjustment to the indirect transmission rate (β in our model). This observation is not necessarily a limitation, but rather a call for further research into the relationship between the exponent η and the indirect transmission rate β to determine the true impact of each and environmental factors potentially altering this relationship. Similarly, the required infectious dose for calves ingesting contaminated milk or colostrum is unknown, due to the limited number of challenge trials evaluating this threshold. In the absence of more concrete data, we approximated the non-linear relationship between bulk-milk contamination level and the force of infection using the same

power term, η. In reality, the power term adjustments for the bulk tank milk supply and for the environment may differ.

In addition, while there is some information regarding the rate MAP survival on pasture (Humphry et al, 2006; Whittington et al., 2003), there is little real-world data on MAP persistence in different bedding types or indoor conditions. Such data would likely be useful for incorporation in future mathematical models. Although our model herd certainly represents a reasonable U.S. dairy herd (see USDA, 2014), it cannot be representative of all U.S. dairy herds. The parameters permitted to vary or attain multiple values in our model were selected based upon the main study objectives; assumptions must be made for the parameters not under investigation in the model. Factors such as herd size, pasture access, and pen dimensions remained constant in our simulations, as variation in these elements is beyond the scope of this research. The current model may be used as a framework for future exploration of such variables.

5. Conclusion

We developed a multi-group compartmental mathematical model of MAP infection dynamics to investigate the relationship between MAP prevalence, environmental burden, and contamination of the bulk tank milk supply. We conclude that the prevalence required for detectable bulk milk contamination would be expected to exceed the national average (5 to 10% at the cow level). Direct shedding into milk did not make a substantial contribution to the MAP concentration in the bulk tank, and the environmental contamination route accounted for more than 99% of the total observed MAP burden in bulk milk. Culling of high shedders, routine cleaning of the maternity pen, and strict milking-parlor hygiene each had a strong impact in reducing bulk-milk MAP concentration, with the largest reduction achieved by combining these

3 initiatives. Such a combination of interventions appears to improve the likelihood of producing low-risk milk for human consumption (concentration of $< 10^3$ MAP CFU/L). These insights may allow for the refinement of control programs and milk quality initiatives to reduce herd prevalence and MAP burden in bulk milk. Although complete eradication of MAP is unrealistic for many herds, producing milk with little or no MAP appears to be an attainable goal.

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7. References

Bar-Peled, U., Robinzon, B., Maltz, E., Tagari, H., Folman, Y., Bruckental, I., Voet, H. Gacitua, H., Lehrer., A. 1997. Increased weight gain and effects on production parameters of Holstein heifer calves that were allowed to suckle from birth to six weeks of age. J. Dairy Sci. 80:2523–2528.

Beaver, A., C. Cazer, P. Ruegg, Y. Gröhn and Y. Schukken. 2016a. Implications of PCR and ELISA results on the routes of bulk-tank contamination with *Mycobacterium avium* subsp. *paratuberculosis*. J. Dairy Sci. 99:1391–1405.

Beaver, A., P. Ruegg, Y. Gröhn and Y. Schukken. 2016b. Comparative risk assessment for new cow-level *Mycobacterium avium* subsp. *paratuberculosis* infections between 3 dairy production types: Organic, conventional, and conventional-grazing systems. J. Dairy Sci. 99:9885–9899.

Beaver, A., R. Sweeney, E. Hovingh, D. Wolfgang, Y. Gröhn, and Y. Schukken. 2017. Longitudinal relationship between fecal culture, fecal quantitative PCR, and milk ELISA in *Mycobacterium avium* subsp. *paratuberculosis*-infected cows from low-prevalence dairy herds. J. Dairy Sci. 100:7507–7521.

Chiodini, R. J., H. J. Van Kruiningen, and R. S. Merkal, 1984. Ruminant paratuberculosis (Johne's disease): the current status and future prospects. Cornell Vet. 74:218–262

Collins, M. T. 1996. Diagnosis of paratuberculosis. Veterinary Clinics of North America: Food Animal Practice. 12:357–371.

Doré, E., J. Paré, G. Côté, S. Buczinski, O. Labrecque, J. Roy and G. Fecteau. 2012. Risk factors associated with transmission of *Mycobacterium avium* subsp. *paratuberculosis* to calves

within dairy herd: A systematic review. Journal of Veterinary Internal Medicine. 26:32–45.

Dorshorst, N. C., M. T. Collins and J. E. Lombard. 2006. Decision analysis model for paratuberculosis control in commercial dairy herds. Prev. Vet. Med. 75:92–122.

Ellingson, J. L., J. L. Anderson, J. J. Koziczkowski, R. P. Radcliff, S. J. Sloan, S. E. Allen and N. M. Sullivan. 2005. Detection of viable *Mycobacterium avium* subsp. *paratuberculosis* in retail pasteurized whole milk by two culture methods and PCR. J. Food Prot. 68:966–972.

Eltholth, M., V. Marsh, S. Van Winden and F. Guitian. 2009. Contamination of food products with *Mycobacterium avium paratuberculosis*: A systematic review. J. Appl. Microbiol. 107:1061–1071.

Feller, M., K. Huwiler, R. Stephan, E. Altpeter, A. Shang, H. Furrer, G. E. Pfyffer, T. Jemmi, A. Baumgartner and M. Egger. 2007. *Mycobacterium avium* subsp. *paratuberculosis* and Crohn's disease: A systematic review and meta-analysis. The Lancet Infectious Diseases. 7:607–613.

Galton, D., R. Adkinson, C. Thomas and T. Smith. 1982. Effects of premilking udder preparation on environmental bacterial contamination of milk. J. Dairy Sci. 65:1540–1543.

Grant, I. R., M. T. Rowe, L. Dundee and E. Hitchings. 2001. *Mycobacterium avium* subsp. *paratuberculosis*: Its incidence, heat resistance and detection in milk and dairy products. International Journal of Dairy Technology. 54:2–13.

Grant, I. R., H. J. Ball, S. D. Neill and M. T. Rowe. 1996. Inactivation of *Mycobacterium paratuberculosis* in cows' milk at pasteurization temperatures. Appl. Environ. Microbiol. 62:631–636.

Grant, I. R., E. I. Hitchings, A. McCartney, F. Ferguson and M. T. Rowe. 2002. Effect of commercial-scale high-temperature, short-time pasteurization on the viability of *Mycobacterium paratuberculosis* in naturally infected cows' milk. Appl. Environ. Microbiol. 68:602–607.

Grant, I. R., A. G. Williams, M. T. Rowe and D. D. Muir. 2005. Efficacy of various pasteurization time-temperature conditions in combination with homogenization on inactivation of *Mycobacterium avium* subsp. *paratuberculosis* in milk. Appl. Environ. Microbiol. 71:2853–2861.

Humphry, R., A. Stott, C. Adams and G. Gunn. 2006. A model of the relationship between the epidemiology of Johne’s disease and the environment in suckler-beef herds. The Veterinary Journal. 172:432–445.

Lombard, J. E. 2011. Epidemiology and economics of paratuberculosis. Veterinary Clinics of North America: Food Animal Practice. 27:525–535.

Lovell, R., M. Levi, and J. Francis, 1944. Studies on the survival of Johne's bacilli. J. Comp. Pathol. Therap., 54: 120–129.

Lu, Z., Y. H. Schukken, R. L. Smith and Y. T. Grohn. 2010. Stochastic simulations of a multi-group compartmental model for Johne's disease on US dairy herds with test-based culling intervention. J. Theor. Biol. 264:1190–1201.

Manning, E. J. and M. T. Collins. 2001. *Mycobacterium avium* subsp. *paratuberculosis*: Pathogen, pathogenesis and diagnosis. Rev. Sci. Tech. 20:133–150.

Marcé, C., P. Ezanno, M. Weber, H. Seegers, D. Pfeiffer and C. Fourichon. 2010. Invited review: Modeling within-herd transmission of *Mycobacterium avium* subsp. *paratuberculosis* in dairy cattle: A review. J. Dairy Sci. 93:4455–4470.

Marcé, C., P. Ezanno, H. Seegers, D. U. Pfeiffer and C. Fourichon. 2011. Predicting fadeout versus persistence of paratuberculosis in a dairy cattle herd for management and control purposes: A modelling study. Vet. Res. 42:36–48.

Mitchell, R. M., Y. Schukken, A. Koets, M. Weber, D. Bakker, J. Stabel, R. H. Whitlock and Y. Louzoun. 2015a. Differences in intermittent and continuous fecal shedding patterns between natural and experimental *Mycobacterium avium* subsp. *paratuberculosis* infections in cattle. Vet. Res. 46:66–75.

Mitchell, R. M., R. H. Whitlock, Y. T. Gröhn and Y. H. Schukken. 2015b. Back to the real world: Connecting models with data. Prev. Vet. Med. 118:215–225.

Mitchell, R. M., G. F. Medley, M. T. Collins and Y. H. Schukken. 2012. A meta-analysis of the effect of dose and age at exposure on shedding of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in experimentally infected calves and cows. Epidemiology & Infection. 140:231–246.

Mitchell, R., R. Whitlock, S. Stehman, A. Benedictus, P. Chapagain, Y. Grohn and Y. Schukken. 2008. Simulation modeling to evaluate the persistence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) on commercial dairy farms in the united states. Prev. Vet. Med. 83:360–380.

Nennich, T., J. Harrison, L. VanWieringen, D. Meyer, A. Heinrichs, W. Weiss, N. St-Pierre, R. Kincaid, D. Davidson and E. Block. 2005. Prediction of manure and nutrient excretion from dairy cattle. J. Dairy Sci. 88:3721–3733.

Ott, S. L., S. J. Wells and B. A. Wagner. 1999. Herd-level economic losses associated with Johne's disease on US dairy operations. Prev. Vet. Med. 40:179–192.

Schukken, Y. H., R. H. Whitlock, D. Wolfgang, Y. Grohn, A. Beaver, J. VanKessel, M. Zurakowski and R. Mitchell. 2015. Longitudinal data collection of *Mycobacterium avium* subsp. *paratuberculosis* infections in dairy herds: The value of precise field data. Vet. Res. 46:65–75.

Slater, N., R. M. Mitchell, R. H. Whitlock, T. Fyock, A. K. Pradhan, E. Knupfer, Y. H. Schukken and Y. Louzoun. 2016. Impact of the shedding level on transmission of persistent infections in *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Vet. Res. 47:38–50.

Smith, R., Y. Grohn, A. Pradhan, R. Whitlock, J. Van Kessel, J. Smith, D. Wolfgang and Y. Schukken. 2009. A longitudinal study on the impact of Johne's disease status on milk production in individual cows. J. Dairy Sci. 92:2653–2661.

Smith, R., R. Strawderman, Y. Schukken, S. Wells, A. Pradhan, L. Espejo, R. Whitlock, J. Van Kessel, J. Smith and D. Wolfgang. 2010. Effect of Johne's disease status on reproduction and culling in dairy cattle. J. Dairy Sci. 93:3513–3524.

Stabel, J. 2001. On-farm batch pasteurization destroys *Mycobacterium paratuberculosis* in waste milk. J. Dairy Sci. 84:524–527.

Streeter, R. N., G. F. Hoffsis, S. Bech-Nielsen, W. P. Shulaw and D. M. Rings. 1995. Isolation of *Mycobacterium paratuberculosis* from colostrum and milk of subclinically infected cows. Am. J. Vet. Res. 56:1322–1324.

Stott, G. H., Marx, D. B., Menefee, B. E., Nightengale, G. T. 1979. Colostral immunoglobulin transfer in calves. IV. Effect of suckling. J. Dairy Sci. 62:1908–1913.

Sweeney, R. W., R. H. Whitlock and A. E. Rosenberger. 1992. *Mycobacterium paratuberculosis* cultured from milk and supramammary lymph nodes of infected asymptomatic cows. J. Clin. Microbiol. 30:166–171.

USDA Animal and Plant Health Inspection Service. 2010. Uniform Program Standards for the Voluntary Bovine Johne's Disease Control Program. Available at: https://www.aphis.usda.gov/animal_health/animal_diseases/johnes/downloads/johnes-ups.pdf Accessed July 21, 2017.

USDA APHIS. 2008. APHIS Info Sheet. Johne's disease on U.S. dairies, 1991–2007. USDA-APHIS, Fort Collins, CO. Available at: https://www.aphis.usda.gov/animal_health/nahms/dairy/downloads/dairy07/Dairy07_is_Johnes.pdf Accessed August 4, 2017.

USDA National Animal Health Monitoring System. 2014. Dairy Cattle Management Practices in the United States, 2014. Available at: https://www.aphis.usda.gov/animal_health/nahms/dairy/downloads/dairy14/Dairy14_dr_PartI.pdf Accessed July 21, 2017.

Van Kessel, J. S., J. S. Karns, J. E. Lombard and C. A. Kopral. 2011. Prevalence of salmonella enterica, listeria monocytogenes, and escherichia coli virulence factors in bulk tank milk and in-line filters from US dairies. J. Food Prot. 74:759–768.

van Schaik, G., C. R. Rossiter, S. M. Stehman, S. J. Shin and Y. H. Schukken. 2003. Longitudinal study to investigate variation in results of repeated ELISA and culture of fecal samples for *Mycobacterium avium* subsp *paratuberculosis* in commercial dairy herds. Am. J. Vet. Res. 64:479–484.

Weber, M. F. and H. Groenendaal. 2009. Milk quality assurance for paratuberculosis: Effects of infectious young stock. Proceedings of the 10th International Colloquium on Paratuberculosis. 9–14.

Weber, M. F., M. Nielen, A. G. Velthuis and H. J. van Roermund. 2008. Milk quality assurance for paratuberculosis: Simulation of within-herd infection dynamics and economics. Vet. Res. 39:12–31.

Whitlock, R., S. Wells, R. W. Sweeney and J. Van Tiem. 2000. ELISA and fecal culture for paratuberculosis (Johne's disease): Sensitivity and specificity of each method. Vet. Microbiol. 77:387–398.

Whitlock, R. H. and C. Buergelt. 1996. Preclinical and clinical manifestations of paratuberculosis (including pathology). Veterinary Clinics of North America: Food Animal Practice. 12:345–356.

Whittington, R. J., D. J. Marshall, P. J. Nicholls, I. B. Marsh, and L. A. Reddacliff, 2004. Survival and dormancy of *Mycobacterium avium* subsp. *paratuberculosis* in the environment. Appl. Environ. Microbiol. 70:2989–3004.

Whittington, R. J. and P. A. Windsor. 2009. In utero infection of cattle with *Mycobacterium avium* subsp. *paratuberculosis*: A critical review and meta-analysis. The Veterinary Journal. 179:60–69.

8. Appendix. *Differential Equations for solving the base (deterministic) model:*

$$\frac{d}{dt}(X_1) = N(\mu - \gamma) - X_1(\lambda_1 + \rho_1 + \mu_1)$$

$$\frac{d}{dt}(X_2) = \rho_1 X_1 - X_2(\lambda_2 + \rho_2 + \mu_2)$$

$$\frac{d}{dt}(X_3) = \rho_2 X_2 - X_3(\lambda_3 + \mu_3)$$

$$\frac{d}{dt}(Tr_1) = \gamma N + \lambda_1 X_1 - Tr_1(\rho_1 + \mu_1)$$

$$\frac{d}{dt}(Tr_2) = \lambda_2 X_2 - \rho_1 Tr_1 - Tr_2(\mu_2 + \phi)$$

$$\frac{d}{dt}(Tr_3) = \lambda_3 X_3 - \mu_3 Tr_3$$

$$\frac{d}{dt}(H_2) = \phi Tr_2 - H_2(\rho_2 + \mu_2)$$

$$\frac{d}{dt}(H_3) = \rho_2 H_2 - H_3(\mu_3 + \sigma)$$

$$\frac{d}{dt}(Y_1) = \sigma H_3 - Y_1(\mu_3 + \nu + \delta_1)$$

$$\frac{d}{dt}(Y_2) = \nu Y_1 - Y_2(\mu_3 + \delta_2 + \alpha)$$

$$\frac{d}{dt}(E_{AC}) = \frac{\psi_{Tr3} Tr_3 + \psi_{Y1} Y_1 + \psi_{Y2} Y_2}{4} - E_{AC}(\theta + \tau)$$

$$\frac{d}{dt}(E_{DC}) = \psi_{Tr3D} Tr_3 + \psi_{Y1D} Y_1 + \psi_{Y2D} Y_2 - E_{DC}(\theta + \tau)$$

$$\frac{d}{dt}(E_H) = \frac{\psi_{Tr2} Tr_2}{3} - E_H(\theta + \tau)$$

$$\frac{d}{dt}(E_{MP}) = \frac{\iota(\psi_{Tr3} Tr_3 + \psi_{Y1} Y_1 + \psi_{Y2} Y_2)}{365} - E_{MP}(\theta + \tau_{MP})$$

$$\frac{d}{dt}(E_{GRP}) = \frac{\psi_{Tr1} Tr_1}{3} - E_{GRP}(\theta + \tau)$$

CHAPTER 6:
DISCUSSION

1. Introduction

Due to complexities surrounding the epidemiology and pathogenesis of MAP bacteria, certain dimensions of infection dynamics, transmission, and contamination routes remain poorly understood. The overall objective of this dissertation was to resolve some of these uncertainties and inform decisions regarding diagnostic testing, management, and milk-quality programs. The main findings of this work, and their interrelationships, will be discussed alongside practical applications and future research directions.

2. Production Type: Implications of Organic Management

The demand for organic dairy products has burgeoned in the past decade (Organic Trade Association, 2016), and Johne's disease maintains its longstanding status as one of the industry's most serious infectious diseases (Chiodini et al., 1984). Thus, an exploration of the specific MAP transmission risks for organic herds is overdue. As described in Chapter 2, pasture grazing likely impacts MAP transmission, depending upon water sources, contact between various dairy-herd age classes, and manure-spreading practices (Obasanjo et al., 1997; Whittington et al., 2005; Chiodini et al., 1984).

To provide a measure of resolution to this matter, we conducted a comparative risk assessment between organic, conventional non-grazing, and conventional grazing herds (Chapter 2). The comprehensive dataset used for this analysis was representative of a cross-section of U.S. dairy herds and had the advantage of accurate state ratios of organic to conventional management, and herd size and location matching. Through this risk assessment, we determined that organic herds (compared to both conventional subgroups) were at greater risk for new MAP infections.

A parallel conclusion was reached in Chapter 3, by fitting the bulk milk results from these same herds into a logistic regression model. We uncovered a potential interaction between bulk-milk ELISA OD and production type on bulk-milk PCR status. In organic herds, there was a very strong relationship between ELISA and PCR results in the bulk tank: an increase of 0.1 units in ELISA was associated with nearly 38 times higher odds of testing positive by PCR; in contrast, the relationship was much less pronounced for conventional herds. This finding may imply that high-prevalence conventional farms restrict environmental contamination more successfully than their organic counterparts. On a basic level, organic herds are less likely than conventional non-grazing herds to administer footbaths or clip the udders of fresh cows (Chapter 2); an absence of these practices may result in contamination of the bulk milk supply.

On the other hand, the perception of a heightened risk for organic herds may be oversimplified. One goal of the risk assessment conducted in Chapter 2 was to investigate whether risk factors commonly outlined in control programs were truly tied to increased MAP prevalence. In the VBJDCP, immediate cow-calf separation and prevention of nursing are recommended as a protective strategy, although the efficacy of this strategy is not well substantiated in the literature (see McAloon et al., 2015). Such practices may contradict both the consumer's perception of organic farming and the organic producer's philosophies. Perhaps in consequence, the participation of organic farms in control programs is reduced compared to conventional herds, and organic dairies are also less likely to make use of written Johne's disease management plans (Chapter 3).

Based upon the results from the overall risk assessment (Chapter 2), the bulk tank analysis (Chapter 3), and our mathematical model (Chapter 5), an improved approach for infection mitigation in organic herds may be proposed. The mathematical model suggests that,

relative to cow-calf separation, other control measures, such as rigorous cleaning of the maternity pen, are far more valuable in minimizing calf exposure to adult-cow manure and reducing prevalence over time. When such control measures are implemented, the benefits of immediate cow-calf separation on prevalence level are minimal (Chapter 5). Thus, with high standards for calving-area hygiene, the health and welfare benefits of cow-calf contact and nursing (Bar-Peled et al., 1997; Stott et al., 1979) may outweigh the risks in the case of test-negative dams.

Our math model not only showed a reduction in prevalence linked to maternity-pen cleanliness, but a substantial reduction in the environmental MAP burden and level of bulk-milk contamination as well (Chapter 5). Poor udder hygiene has also been tied to MAP contamination of individual milk samples (Chapter 4). Organic herds included in our comparative risk assessment did not, however, exhibit a heightened vigilance towards hygiene and environmental cleanliness, despite an increased susceptibility to risk synergism in the calving area. This conclusion was determined using logistic regression on important compound risk factors (Chapter 2). Therefore, organic herds wishing to permit cow-calf contact and/or nursing from test-negative dams should be counseled to target hygiene within the calving area. In addition to direct cleaning of the calving pen, specific control measures may include clipping and cleaning udders of periparturient cows, providing footbaths to both dry and fresh animals, and maintaining a dedicated maternity area. Such practices are associated with decreased MAP prevalence (Ansari-Lari et al., 2009; Pithua et al., 2011).

3. Associations between Diagnostic Outcomes

Although there has been substantial research on diagnostic testing for MAP, the intricacies of relationships between these diagnostics have not been explored in detail. This

deficit may, in part, be due to the exclusively cross-sectional nature of much of this research, or the simplification of results by means of dichotomization. In addition, milk ELISA results have not received as much attention in MAP research compared to serum ELISA.

At the herd level, we noted a strong association between PCR and ELISA results in bulk-tank milk samples, when ELISA was treated as a continuous variable (Chapter 3). On the surface, this concordance does not match the conclusions put forth by other researchers. For example, Wilson et al. (2010) characterized the agreement between dichotomized bulk-milk ELISA and PCR as "moderate." The binary classification for ELISA in the case of bulk-milk results is particularly problematic, since the cutoff levels provided with ELISA kits are standardized according to individual-animal levels. A "negative" result in the bulk tank contributes little information, since the bulk tank is a herd-level source, and antibodies from a small number of positive cows may be diluted by a large volume of milk. Rather, bulk-milk ELISA OD on a continuous scale can provide information on the likelihood of infection at the farm level.

At the individual-animal level, we had the benefit of acquiring repeated milk and fecal samples from 14 MAP-positive cows in 2 U.S. states (Chapter 4). With the help of mixed linear modeling, these longitudinal data allowed us to identify strong relationships among the results of individual fecal culture, fecal qPCR, and milk ELISA. At first glance, this high level of agreement is, again, inconsistent with conclusions presented in the literature (see Pinedo et al., 2008; Taddei et al, 2004); however, the results of our study imply that a less conservative threshold for a positive ELISA should be considered for some animals, since all ELISA-positive milk samples were also positive by qPCR. Nielsen et al. (2002) argued for flexibility in ELISA thresholds according to testing objectives. Pairing highly negative values with those just below

the threshold is not informative when interpreting ELISA titer as a MAP infection likelihood. Moreover, the agreement between qPCR and ELISA in our data showed vast improvement for Progressors compared to Non-progressors, suggesting that shedding level itself impacts the qPCR-ELISA association. Importantly, Chapter 4 represents the first attempt to quantify the relationship between Progressor status and milk ELISA OD.

There is an additional element to consider in evaluating the relationship between individual milk ELISA and fecal qPCR (as proxies for antibody production and shedding, respectively). Just as it may not be fully informative to assess binary outcomes, it may be equally limiting to consider an association at a single time point: the continuous-scale evaluation should be extended to the temporal sphere as well. Based upon our knowledge of MAP infection biology, it is plausible that an increase in fecal shedding could predict future high ELISA titers: extensive MAP shedding from the intestinal mucosa is thought to be required before B-cell proliferation is initiated (Whittington and Sergeant, 2001), and the production of detectible antibody levels would not be instantaneous. Indeed, we concluded that spikes in fecal shedding were predictive of high ELISA readings taken up to 2 months later (Chapter 4). To our knowledge, the complexities of the temporal relationship between milk ELISA and fecal qPCR have not previously been investigated. Rather, much of the research into temporality has instead focused on the latency between infection and serum antibody production (Bannantine et al., 2008; Stabel et al., 2011; van Schaik et al., 2003).

Our cross-sectional and longitudinal studies (Chapters 3 and 4, respectively) allowed for an improved understanding of the intricate relationships between diagnostic tests. Despite the high agreement between high bulk-milk ELISA and PCR results in the cross-sectional study, most high ELISA farms were PCR negative. A high ELISA result in the bulk tank suggests high

prevalence, with a sufficient number of positive cows contributing antibodies to raise the herd-level titer. The ELISA result alone, however, does not provide information regarding a given herd's ability to curtail environmental contamination. This information indicates that ELISA should not be used exclusively to determine the bulk-tank MAP status; more valuable diagnostic information can be gleaned from paired ELISA and PCR results. The conclusions of the longitudinal study have provided further support for the concept of tailoring testing schemes according to the specific goals of the herd. Diagnostic outcomes and the level of concordance between them may be interpreted differently when the primary herd goal is detection, versus an assessment of level of infectivity or progression of infection in a known-positive cow. Similarly, the ability to categorize an animal as a Progressor or Non-progressor allows us to predict the pattern of ELISA titers over time; the use of a single threshold for both categories would be insufficient to resolve all diagnostic objectives. When making culling decisions, it may be important to predict future ELISA results, since the shift to heightened levels of humoral immune response is generally associated with the onset of clinical disease.

4. Variables Related to Individual Animal or Herd-Level MAP Positivity

In this work, several important variables have been identified in conjunction with MAP positivity. These associations have primarily been in agreement with broader findings in the Johne's disease literature. For example, in Chapter 3, we noted that farms with large compared to small herd sizes were more likely to test positive by bulk-tank PCR. Indeed, the probability of subclinical infection increases with herd size, and larger herds are more difficult to screen and monitor (Crossley et al., 2005). Although some studies have found no significant relationship between herd size and MAP prevalence, the majority of research has supported this conclusion (e.g., Wells and Wagner, 2000; Crossley et al., 2005).

In our longitudinal study (Chapter 4), several variables were shown to influence individual milk ELISA results. We found that fresh cows produced higher antibody levels than did cows in later days in milk, representative of changes in antibody concentration over the course of lactation. This finding is consistent with the outcomes of cross-sectional studies (Nielsen et al., 2002). Nielsen et al. (2002) also uncovered an effect of parity on ELISA results, with animals in 2nd or higher parities showing an increased likelihood of testing ELISA positive in milk. We reached similar conclusions in our longitudinal work, noting that animals in 3rd or higher parity had greater ELISA OD values, in keeping with the progressive advancement of MAP infections. Interestingly, the effect of parity remained significant following the addition of fecal qPCR into our model. This observation could indicate a cumulative effect of MAP exposure on the bovine immune system as infected cows age (Chapter 4).

At the herd level, we found an effect of seasonality on bulk-milk MAP ELISA, with antibody production peaking in the summer and decreasing in the winter. The seasonality effect followed the pattern of a cosine curve across the years of testing (Chapter 3) and was in accordance with the previous published model of Cazer et al. (2013). At least at the univariable stage, we also observed a correlation between group housing of pre-weaned heifers and positive bulk-milk results by PCR, perhaps implying a higher overall prevalence. Horizontal MAP transmission between calves has been documented, and transmission potential would be expected to increase when young animals are housed together (van Roermund et al., 2007). Although this variable was not significant at the multivariable stage, it nonetheless seems advisable to evaluate it as a potential risk factor. As stated in Chapter 2, there are measurable welfare benefits to group housing for calves (Gaillard et al., 2014; Creel and Albright, 1988), so control focus may instead

be placed on maternity-pen hygiene to minimize the number of infected calves introduced into group housing systems later in life.

Finally, herds in NY State showed a greater likelihood of testing positive by bulk-milk PCR (Chapter 3), a conclusion that is not consistent with other studies noting increased prevalence in midwestern herds (Wells and Wagner, 2000; Lombard et al., 2006); however, it becomes difficult to determine whether our findings are truly incongruous, since these studies relied upon different diagnostics (such as serological ELISA and environmental culture) rather than bulk milk. Further data are therefore needed pertaining to geographical distribution of bulk-milk MAP status.

The significance of these ancillary variables suggests that diagnostic outcomes should not be evaluated without consideration of meaningful secondary factors. For example, when assessing the infection likelihood for a given herd, bulk-milk ELISA results taken in the summer from a large herd cannot be directly compared to winter results from a smaller farm. Similarly, individual diagnostics should be appraised in reference to parity and stage of lactation.

5. Bulk Milk Contamination and its Relationship to Prevalence and Shedding Levels

A primary goal of the multi-group compartmental mathematical model described in Chapter 5 was to evaluate the bulk-milk MAP contamination level alongside infection dynamics within the dairy herd. We sought to understand the interaction between environmental contamination, stages of infection, shedding levels, and the bulk-milk supply.

The prevalence required for detectable bulk-milk contamination, (assuming a standard ratio of infection categories (see Whitlock and Buergelt, 1996)) has not been previously investigated. The threshold of detection in the bulk tank (8,200 CFU/L, with a 50% detection rate) was established in Chapter 3, by conducting qPCR on serial dilutions of MAP-spiked milk.

In our mathematical model, this detection threshold aligns with an overall prevalence of 19.6%, corresponding to a shedding level of 12% (Chapter 5). Thus, with reasonable hygiene standards and a small herd size, approximately 12% of cows must be actively shedding MAP for bulk-milk contamination to occur in detectable amounts. In comparison, the average proportion of shedding (test-positive) animals in a MAP-positive U.S. dairy herd is estimated at 5 to 10% (Lombard, 2011). We may therefore expect that herds with detectable MAP in their bulk tanks exhibit a shedding level higher than the national average.

The real-world studies described in Chapters 3 and 4 affirm this conclusion. Using cross-sectional bulk milk samples from different U.S. regions, it was determined that detectable bulk-milk MAP contamination is rare. In Chapter 3, samples were sourced from both infected and non-infected herds, and bulk-tank contamination occurred in 6 of 286 tested samples (a 2.1% positive rate). Moreover, the majority of herds with high bulk-tank ELISA results were PCR negative, signifying that U.S. dairy farms are usually able to curtail bulk-milk MAP contamination despite a positive herd infection status. In Chapter 4, bulk-milk samples were collected consistently from 2 herds, each with approximately 3% shedders, over the course of 5 months. No bulk-tank sample ever tested positive for MAP, suggesting that bulk-milk contamination does not represent a chief concern in low-prevalence dairy herds.

Because PCR-positive results in the bulk tank are associated with high herd prevalence, an inexpensive and time-efficient commercial test such as Tetracore (Chapter 3) may be useful for preliminary herd screening to provide a general estimate of prevalence and identify problem herds. For a more comprehensive risk assessment, PCR should be paired with ELISA to provide indications of the most likely contamination routes, whether direct (internal) or indirect

(environmental). An understanding of the routes of contamination will inform the selection of control strategies to diminish bulk-tank MAP levels.

6. The Routes of Bulk Milk Contamination

The nomenclature for the "internal" and "environmental" routes of bulk-milk contamination were first defined in the grant proposal titled *On-farm intervention programs to reduce MAP bacterial load in milk* (Schukken et al., 2011), which was funded by the United States Department of Agriculture. Chapter 3 of this dissertation represents the first introduction of these terms into the Johne's disease literature. As described in Chapter 3, a combination of ELISA and PCR results from the bulk tank can often clarify the most likely contamination route(s).

Our mathematical model (Chapter 5) included internal-route shedding from infected cows in relevant infection categories. These rates were calculated according to the thresholds described by Sweeney et al. (1992) and adjusted for category-specific milk production (Smith et al., 2009). The environmental route was modeled by estimating the total CFU contribution from each infected animal, in relation to infection category and quantity of manure produced. Results of the sensitivity analysis revealed that the environmental route was responsible for more than 99% of the MAP burden in the bulk tank. Even when the level of internal-route shedding was artificially increased to a level 5 times higher than the upper limits reported in Sweeney et al. (1992), this route made a negligible contribution to the overall level of bulk-milk contamination.

A related conclusion was reached in Chapter 4, in which a relationship between udder hygiene and milk contamination was identified. Specifically, the odds of a positive PCR result in an individual milk sample decreased more than 5-fold for each half unit improvement in udder hygiene score. Moreover, there was no observable relationship between the presence of MAP in

individual milk samples and the corresponding ELISA result or fecal qPCR copy numbers. The likelihood of direct shedding into milk and antibody production are both shown to increase with infection category (Sweeney, 1992; Streeter et al., 1995). Thus, compared to the environmental route, the internal route represents a less probable explanation for MAP contamination in this study (Chapter 4). The results of these studies may be interpreted as a favorable: although it may not be possible to control direct shedding into milk, there are concrete measures by which to curtail environmental contamination, improve hygiene, and thereby reduce MAP contamination of raw milk.

7. Intervention Strategies to Minimize Milk Contamination

Recent Johne's disease research has focused on the contribution of heavy shedders to overall prevalence and MAP persistence within the herd (Slater et al., 2016; Mitchell et al., 2015). It has been determined that these animals are not the primary drivers of MAP persistence in dairy herds and that their contribution to the environment is not linearly related to new MAP infection risk in susceptible animals (Slater et al., 2016). This conclusion is in accordance with the observations of Lu et al. (2010): specifically, culling of heavy shedders alone is insufficient for complete MAP eradication from simulated herds. In our mathematical model (Chapter 5) we found that culling of heavy shedders reduced prevalence substantially, but not entirely. On the other hand, this intervention was very effective in reducing the MAP CFU in bulk-tank milk, providing a mean reduction of 4,379 CFU/L when conducted annually, and 5,785 CFU/L when conducted twice annually. Our model also highlighted the importance of milking parlor hygiene and rigorous maternity-pen cleaning to improve milk quality. As described in Chapter 2, automatic hoof washing and cleaning of udders of periparturient cows have been linked to improved hygiene and manure reduction (Thomsen et al., 2012; Elmoslemany et al., 2009). In

our mathematical model, the greatest reduction in bulk-milk MAP was achieved via a combination of the three interventions: biannual culling of heavy shedders, improved milking parlor hygiene, and systematic cleaning of the calving area. Even for high prevalence herds, lowering the bulk-milk MAP concentration to safe levels for human consumption ($< 10^3$, Weber et al., 2008) appears to be an attainable goal (Chapter 5).

8. Future Directions

Much of the work presented in this dissertation represents a necessary preliminary investigation of concepts that have, to date, been insufficiently addressed in the Johne's disease literature. Although new insights have been put forth, many have given rise to further questions and hypotheses that must be answered and tested in future research.

8.1 Production Type

It remains to be seen whether, and to what extent, the increased risks associated with organic management (described in Chapter 2) correspond to an increased cow-level MAP prevalence in organic U.S. dairy herds. This concept may be empirically tested using cross-sectional data. Such a study would require considerable resources and cooperation; however, it seems an essential step if the implications of production type on the risk of new MAP infections are to be fully understood. Compound risk factors and the synergistic relationship between management practices (Chapter 2) could be evaluated in conjunction with herd prevalence.

In addition, a case-control type study could be conducted, with the recruitment of known-MAP positive herds serving to increase the number of PCR positive results in the bulk tank. Such an exploration may allow for a reexamination of the interaction between production type and ELISA titer on bulk-tank PCR status (Chapter 3).

Finally, the Project COW questionnaire (Chapter 2) provided answers to many useful questions regarding Johne's disease management in organic and conventional production systems. Conclusions from the cross-sectional milk analysis, the longitudinal phase, and the mathematical model, have offered some indications of how to best expand upon this questionnaire. In particular, more detailed questions pertaining to Johne's disease testing strategies could prove to be a useful addition. For example, although producers were queried as to whether bulk-milk testing was conducted in their herds, the test category (e.g., ELISA, PCR, or both) and specific test frequency were not itemized.

Questions could also be designed to recognize the impact of the environmental route in MAP spread and in bulk-milk contamination. Given the importance of calving-pen hygiene on bulk-milk MAP concentration (Chapter 5), it may also be informative to enquire about bedding types used, and frequency and methods of cleaning. Along these lines, producers employing a "written plan for Johne's disease management" could be asked to describe the specific steps involved in preventative management on their respective farms.

8.2 Associations between Diagnostic Outcomes

One limitation of the longitudinal study presented in Chapter 4 is the relatively short duration of sampling, given the slow progression of ruminant MAP infections. The associations and temporal relationships described in this chapter are certainly substantiated and have the benefit of a frequent sampling scheme; however, it may be valuable to extend the study to explore temporal patterns over a longer time frame. There may be further subtleties in the relationships between diagnostic tests that require more extensive longitudinal data. In addition, it will be necessary to include a larger sample of herds from a variety of regions to improve external validity and extend conclusions to the subset of low-prevalence U.S. dairy herds in

general. Both moderate and high-prevalence herds could be recruited for future studies, particularly if the relationship between hygiene and individual milk contamination (Chapter 4) is to be investigated further. Such data could confirm or disprove the absence of a relationship between PCR positivity in individual milk samples and corresponding fecal qPCR counts.

8.3 Assessment of Intervention Strategies

Because of the rarity of positive bulk milk results in real-world data (see Chapters 3 and 4), identifying the exact hygiene practices necessary to mitigate bulk-milk contamination has not been straightforward. In Chapter 5, we conclude that the broad category of "milking parlor hygiene" is critical for mitigating bulk-milk contamination; however, certain hygiene practices may be more pertinent than others in this regard. For example, we may hypothesize that cleaning udders prior to milking will reduce the bulk-tank MAP concentration, based upon evidence from individual milk samples (Chapter 4), but more data are needed to definitively link this practice to decreased bulk-tank contamination. As described in Chapter 5, detectable levels of contamination are more likely to occur in high prevalence herds. Thus, the addition of longitudinal data from such herds could allow for the evaluation of specific hygiene practices. This information could, in turn, serve to refine the input values for certain parameters in our mathematical model. One such parameter is the rate at which MAP from the environment enters the bulk tank milk supply (denoted as ω in our model).

8.4 Mathematical Modeling

Several other model parameters could benefit from supplementary real-world data collection to provide the most accurate representation of infection biology. The non-linear association between environmental MAP burden and infection has only recently been introduced into the literature (Slater et al., 2016) and requires continued investigation. As stated in Chapter

5, the relationship between the exponent η (representing the non-linearity between infectiousness and environmental MAP burden) and the transmission rate β must be more clearly defined. The rate of MAP survival in various indoor conditions (such as potential differential survival in a variety of common bedding materials) may inform this relationship.

Chapter 4 provided further information regarding the newly classified Progressor and Non-progressor infection categories (Schukken et al., 2015). Additional data are needed to understand genetic and environmental factors that predispose certain animals to become Progressors. This understanding could allow for the incorporation of Progressor status into our mathematical model framework. The model could also benefit from additional data on infectious dose threshold in milk or colostrum and the efficacy of milk filtration (Grant et al., 2002).

Finally, there is flexibility within the model framework presented in Chapter 5 to address other research questions. Future models could investigate elements such as herd size, pen layout, and movement on pasture and their impact on infection dynamics, prevalence, and bulk-milk contamination level. For example, in Chapter 3, we noted an association between large herds and PCR-positive tanks; however, the association was nonlinear. Mathematical modeling could be used to determine a potential threshold for herd size and its influence on bulk-tank MAP status.

9. Conclusions

In this dissertation, we have explored the risks and routes of MAP transmission and contamination. By means of field work, laboratory methods, and statistical and mathematical modeling approaches, we have unraveled some of the uncertainties surrounding this complex pathogen. Several new insights have been presented regarding milk qPCR diagnostics, management strategies, milk quality initiatives, and the relationship between laboratory tests for MAP. The main findings are summarized in *Figure 15.*

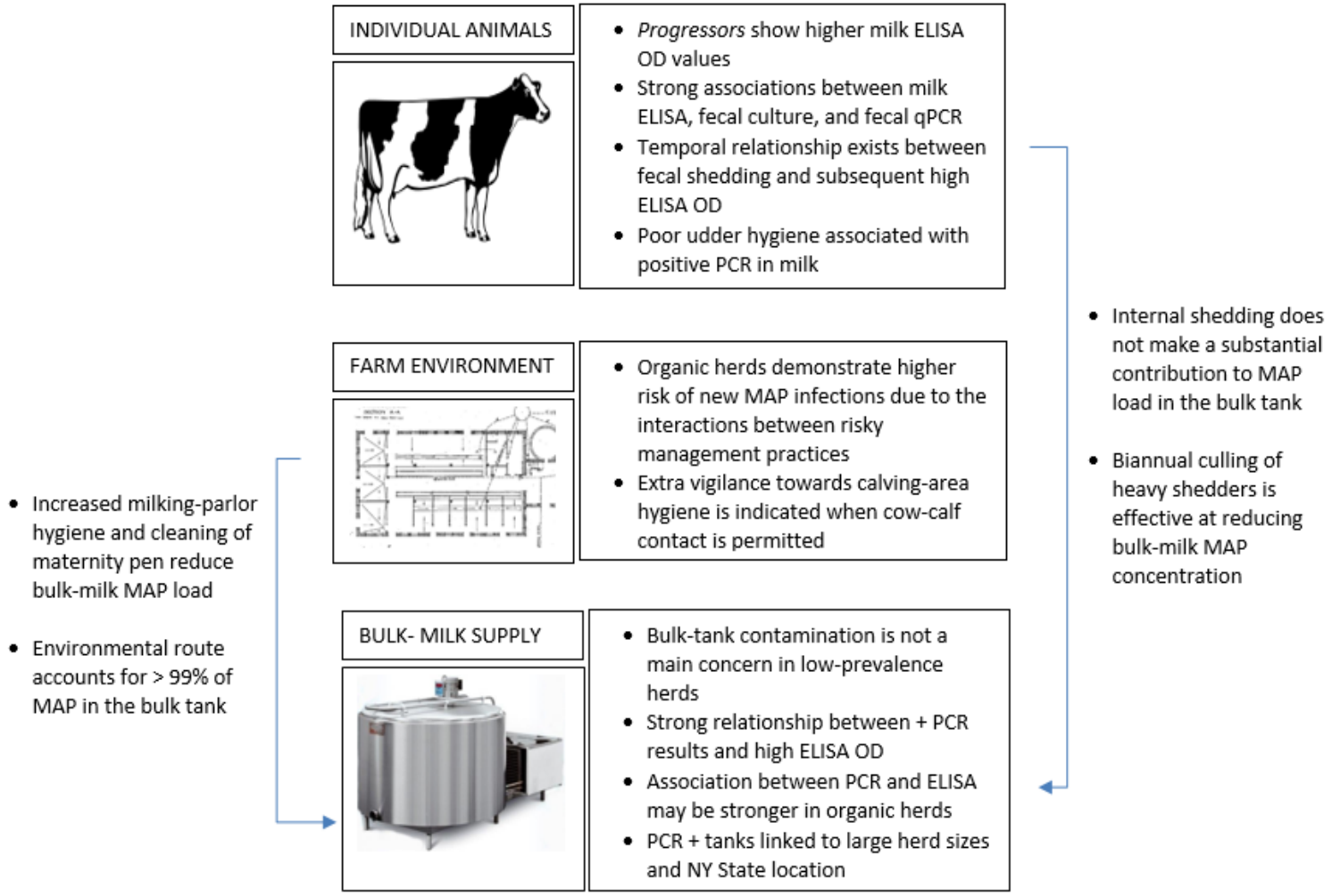

Figure 15. *Diagram Summarizing the Main Dissertation Conclusions.* Results are listed according to individual animals, the dairy farm environment, and the bulk-tank milk supply.

At the individual-animal level, we identified strong associations between milk ELISA, fecal culture, and fecal qPCR, when results were considered on a continuous scale. Importantly, we noted that the relationship between qPCR and ELISA diagnostics is quite nuanced: disease Progressors demonstrate higher milk ELISA values compared to Non-progressors, and spikes in fecal shedding may be used to predict future ELISA readings. The temporal relationship between fecal shedding and antibody production in milk has not been previously explored in detail. This information may be used to assess infectivity and to inform culling decisions.

At the herd level, we found that organic farms exhibit higher risk for new MAP infections based upon the interaction of management practices within the calving area. This study represented the first exploration of Johne's disease risk factors in different U.S. production types. Our research suggests that organic herds allowing cow-calf contact could improve hygiene within the maternity pen to avoid risk synergism. Moreover, our mathematical model revealed that meticulous cleaning of the calving area following each calving can lead to substantial reduction in bulk-milk MAP CFU. When this initiative is coupled with biannual culling of heavy shedders and improved milking-parlor hygiene, the concentration of MAP in the bulk tank can be reduced below a threshold safe for human consumption. Given the potential zoonotic risk of MAP, this is a very relevant and important finding.

The environmental route, which accounted for more than 99% of the MAP CFU in the bulk tank, is more conducive to such intervention strategies. Indeed, we found a direct correlation between individual udder hygiene and MAP contamination of milk samples, alluding to a concrete and feasible intervention. In addition, the bulk-milk results from the cross-sectional and longitudinal studies have largely been encouraging: the majority of the U.S. dairy herds sampled demonstrated an ability to temper environmental contamination and prevent MAP

contamination of the bulk tank. Moreover, bulk-milk contamination does not appear to affect low-prevalence herds to a measurable degree.

Through this work, we have come to an enhanced understanding of MAP infection dynamics and the interplay between individual animals, the environment, and the bulk-tank milk supply. Such information may be used to refine intervention strategies for infection mitigation and bulk-milk MAP reduction, and to stimulate future research in this challenging field.

10. References

Ansari-Lari, M., M. Haghkhah, A. Bahramy, and A. M. N. Baheran. 2009. Risk factors for *Mycobacterium avium* subsp. *paratuberculosis* in Fars Province (southern Iran) dairy herds. Trop. Anim. Health Prod. 41:553–557.

Bannantine, J. P., D. O. Bayles, W. R. Waters, M. V. Palmer, J. R. Stabel, and M. L. Paustian. 2008. Early antibody response against *Mycobacterium avium* subsp. *paratuberculosis* antigens in subclinical cattle. Proteome Sci. 6:5–16.

Bar-Peled, U., B. Robinzon, E. Maltz, H. Tagari, Y. Folman, I. Bruckental, H. Voet, H. Gacitua, and A. Lehrer. 1997. Increased weight gain and effects on production parameters of Holstein heifer calves that were allowed to suckle from birth to six weeks of age. J. Dairy Sci. 80:2523–2528.

Cazer, C. L., R. M. Mitchell, K. M. Cicconi-Hogan, M. Gamroth, R.M. Richert, P. L. Ruegg, and Y. H. Schukken. 2013. Associations between *Mycobacterium avium* subsp. *paratuberculosis* antibodies in bulk tank milk, season of sampling and protocols for managing infected cows. BMC Vet. Res. 9:234–240.

Chiodini, R. J., H. J. van Kruiningen, and R. S. Merkal. 1984. Ruminant paratuberculosis (Johne's disease): The current status and future prospects. Cornell Vet. 74:218–262.

Creel, S., and J. Albright. 1988. The effects of neonatal social isolation on the behavior and endocrine function of Holstein calves. Appl. Anim. Behav. Sci. 21:293–306.

Crossley, B. M., F. J. Zagmutt-Vergara, T. L. Fyock, R. H. Whitlock, and I. A. Gardner. 2005. Fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* by dairy cows. Vet. Microbiol. 107:257–263.

Elmoslemany, A. M., G. P. Keefe, I. R. Dohoo, and B. M. Jayarao. 2009. Risk factors for bacteriological quality of bulk tank milk in Prince Edward Island dairy herds. Part 1: Overall risk factors. J. Dairy Sci. 92:2634–2643.

Gaillard, C., R. K. Meagher, M. A. von Keyserlingk, and D. M. Weary. 2014. Social housing improves dairy calves' performance in two cognitive tests. PLoS ONE 9:e90205.

Grant, I. R., H. J. Ball, and M. T. Rowe. 2002. Incidence of *Mycobacterium paratuberculosis* in bulk raw and commercially pasteurized cows' milk from approved dairy processing establishments in the United Kingdom. Appl. Environ. Microbiol. 68:2428–2435.

Lombard, J. E., B. A. Wagner, R. L. Smith, B. J. McCluskey, B. N. Harris, J. B. Payeur, F. B. Garry, and M. D. Salman. 2006. Evaluation of environmental sampling and culture to determine *Mycobacterium avium* subsp. *paratuberculosis* distribution and herd infection status on US dairy operations. J. Dairy Sci. 89:4163–4171.

Lu, Z., Y. H. Schukken, R. L. Smith, and Y. T. Grohn. 2010. Stochastic simulations of a multi-group compartmental model for Johne's disease on US dairy herds with test-based culling intervention. J. Theor. Biol. 264:1190–1201.

McAloon, C. G., P. Whyte, S. J. More, L. O'Grady, and M. L. Doherty. 2015. Development of a HACCP-based approach to control paratuberculosis in infected Irish dairy herds. Prev. Vet. Med. 120:152–161.

Mitchell, R. M., Y. Schukken, A. Koets, M. Weber, D. Bakker, J. Stabel, R. H. Whitlock, and Y. Louzoun. 2015. Differences in intermittent and continuous fecal shedding patterns between natural and experimental Mycobacterium avium subsp. paratuberculosis infections in cattle. Vet. Res. 46:66–75.

Nielsen, S. S., C. Enevoldsen, and Y. T. Grohn. 2002. The *Mycobacterium avium* subsp. *paratuberculosis* ELISA response by parity and stage of lactation. Prev. Vet. Med. 54:1–10.

Obasanjo, I. O., Y. Grohn, and H. O. Mohammed. 1997. Farm factors associated with the presence of *Mycobacterium paratuberculosis* infection in dairy herds on the New York state paratuberculosis control program. Prev. Vet. Med. 32:243–251.

Organic Trade Association. 2016. U.S. Organic: State of the industry. Available at: http://ota.com/sites/default/files/indexed_files/OTA_StateofIndustry_2016.pdf Accessed Apr. 2, 2016.

Pinedo, P. J., D. Rae, J. Williams, G. Donovan, P. Melendez, and C. Buergelt. 2008. Association among results of serum ELISA, faecal culture and nested PCR on milk, blood and faeces for the detection of paratuberculosis in dairy cows. Transbound. Emerg. Dis. 55:125–133.

Pithua, P., S. J. Wells, and S. M. Godden. 2011. Evaluation of the association between fecal excretion of *Mycobacterium avium* subsp *paratuberculosis* and detection in colostrum and on teat skin surfaces of dairy cows. J. Am. Vet. Med. Assoc. 238:94–100.

Schukken. Y. H., Y. T. Grohn, R. M. Mitchell, D. Wolfgang, A. K. Pradhan, and R. W.

Sweeney. 2011. On-farm intervention programs to reduce MAP bacterial load in milk. United States Department of Agriculture, funded project USDA–06385.

Schukken, Y. H., R. H. Whitlock, D. Wolfgang, Y. Grohn, A. Beaver, J. VanKessel, M. Zurakowski, and R. Mitchell. 2015. Longitudinal data collection of *Mycobacterium avium* subsp. *paratuberculosis* infections in dairy herds: The value of precise field data. Vet. Res. 46:65–75.

Slater, N., R. M. Mitchell, R. H. Whitlock, T. Fyock, A. K. Pradhan, E. Knupfer, Y. H. Schukken, and Y Louzoun. 2016. Impact of the shedding level on transmission of persistent infections in *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Vet. Res. 47:38–50.

Smith, R., Y. Grohn, A. Pradhan, R. Whitlock, J. Van Kessel, J. Smith, and D. Wolfgang, Y. Schukken, 2009. A longitudinal study on the impact of Johne's disease status on milk production in individual cows. J. Dairy Sci. 92: 2653–2661.

Stabel, J. R., W. R. Waters, J. P. Bannantine, and K. Lyashchenko. 2011. Mediation of host immune responses after immunization of neonatal calves with a heat-killed *Mycobacterium avium* subsp. *paratuberculosis* vaccine. Clin. Vaccine Immunol. 18:2079–2089.

Stott, G. H., D. B. Marx, B. E. Menefee, and G. T. Nightengale. 1979. Colostral immunoglobulin transfer in calves. IV. Effect of suckling. J. Dairy Sci. 62:1908–1913.

Streeter, R. N., G. F. Hoffsis, S. Bech-Nielsen, W. P. Shulaw, D. M. Rings, 1995. Isolation of *Mycobacterium paratuberculosis* from colostrum and milk of subclinically infected cows. Am. J. Vet. Res. 56: 1322–1324.

Sweeney, R. W., R. H. Whitlock, and A. E. Rosenberger. 1992. *Mycobacterium paratuberculosis* cultured from milk and supramammary lymph nodes of infected asymptomatic cows. J. Clin. Microbiol. 30:166–171.

Taddei, S., C. Robbi, C. Cesena, I. Rossi, E. Schiano, N. Arrigoni, G. Vicenzoni, and S. Cavirani. 2004. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine fecal samples: Comparison of three polymerase chain reaction-based diagnostic tests with a conventional culture method. J. Vet. Diagn. Invest. 16:503–508.

Thomsen, P. T., A. K. Ersboll, and J. T. Sorensen. 2012. Short communication: Automatic washing of hooves can help control digital dermatitis in dairy cows. J. Dairy Sci. 95:7195–7199.

van Roermund, H. J., D. Bakker, P. T. Willemsen, and M. C. de Jong. 2007. Horizontal transmission of *Mycobacterium avium* subsp. *paratuberculosis* in cattle in an experimental setting: Calves can transmit the infection to other calves. Vet. Microbiol. 122:270–279.

van Schaik, G., C. R. Rossiter, S. M. Stehman, S. J. Shin and Y. H. Schukken. 2003. Longitudinal study to investigate variation in results of repeated ELISA and culture of fecal samples for *Mycobacterium avium* subsp *paratuberculosis* in commercial dairy herds. Am. J. Vet. Res. 64:479–484.

Weber, M. F., M. Nielen, A. G. Velthuis, and H. J. van Roermund. 2008. Milk quality assurance for paratuberculosis: Simulation of within-herd infection dynamics and economics. Vet. Res. 39:12–31.

Wells, S. J., and B. A. Wagner. 2000. Herd-level risk factors for infection with *Mycobacterium paratuberculosis* in US dairies and association between familiarity of the herd manager with the disease or prior diagnosis of the disease in that herd and use of preventive measures. J. Am. Vet. Med. Assoc. 216:1450–1457.

Whitlock, R. H., and C. Buergelt. 1996. Preclinical and clinical manifestations of paratuberculosis (including pathology). Vet. Clin. North Am. Food Anim. Pract. 12:345–356.

Whittington, R. J., I. B. Marsh, and L. A. Reddacliff. 2005. Survival of *Mycobacterium avium* subsp. *paratuberculosis* in dam water and sediment. Appl. Environ. Microbiol. 71:5304–5308.

Whittington, R. J., and E. Sergeant. 2001. Progress towards understanding the spread, detection and control of *Mycobacterium avium* subsp. *paratuberculosis* in animal populations. Aust. Vet. J. 79:267–278.

CHAPTER 7: ADDENDUM.
MODELING THE EFFECTS OF HYGIENE PRACTICES ON *MYCOBACTERIUM AVIUM* SUBSP. *PARATUBERCULOSIS* CONTAMINATION IN BULK-TANK MILK

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KEYWORDS: *Mycobacterium avium* subsp. *paratuberculosis*, Johne's disease, bulk-tank milk, infection status, milk filters, washing efficiency

ABSTRACT

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a pathogenic bacterium that causes Johne's disease, one of the most important endemic infectious diseases in dairy cattle. Contamination of the bulk tank with MAP can occur through direct shedding into milk by infected cows (internal route), fecal contamination (fecal route), or via the introduction of soil and water containing MAP (environmental route). Humans can be exposed to MAP from raw milk consumption; additionally, there are reports of MAP survival in milk after pasteurization. The risk of human consumption is particularly important due to an association between MAP and human Crohn's disease.

In the current study, we used a probabilistic modeling framework to predict the level of MAP contamination in the bulk tank and weigh the relative importance of each contamination route. Our model focused on several different infection statuses and the contribution of each group to environmental and fecal contamination, in addition to internal-route shedding. We assessed the influence of common hygiene practices, such as washing of udders before milking and the use of milk filters, on the concentration of MAP in bulk milk. We extracted parameters and distributions from national surveys and from published and unpublished literature.

Our base model, comprising all hygiene practices, provided an average estimate of 0.76 log CFU/L for the final concentration of MAP in bulk milk, with a maximum of 6.70 log CFU/L and a minimum of 0.035 log CFU/L. According to sensitivity analyses, the fecal route was responsible for approximately 93% of the total MAP contamination (a correlation of 0.71). Moreover, herd size was highly influential, with correlation coefficients of 0.48, and the prevalence of high shedders also showed a positive correlation of 0.06. Washing of udders prior to milking contributed to lowering the total contamination level, demonstrating a negative

correlation (0.42) with MAP concentration in bulk milk. This study emphasized the importance of good hygiene practices in maintaining the quality of raw milk in endemically-infected dairy herds.

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP), an obligate pathogenic bacterium, is the etiological agent of Johne's disease (JD), a chronic infectious gastroenteritis in ruminants and other domestic and wild animals (Over et al., 2011; Motiwala et al., 2006). JD is recognized as a severe animal health issue that has reduced the economy and yields of dairy industries worldwide, due to decreased milk production, premature culling, reduced meat quality, low fertility, and increased replacement rate (Ott et al., 1999; Lombard et al., 2005; Li et al., 2015). In the United States, the average prevalence of MAP-positive cows in an infected herd has been reported to be 3-10%, which is dependent on herd size (van Schaik et al., 2003). There has been an estimated loss of $250 million per annum in the U.S. dairy industry due to JD, and the percentage of herds with at least 1 MAP positive animal exceeds 68% (Smith et al., 2011). The prevalence of infected herds in the U.S. is region-specific and has been reported at 24.2% in the Midwest, 23.5% in the West, 17.2% in the Southeast, and 16.1% in the Northeast (Hirst et al., 2004).

MAP is also speculated to cause Crohn's disease (CD), a chronic inflammatory bowel disease, in humans (Feller et al., 2007). Recently, in some parts of the U.S., the incidence of CD is estimated to affect up to 7–8 per 10^5 populations per year (Loftus et al., 2007; Herrinton et al., 2008). The possible link between MAP and CD in humans has not been well established; however, there is evidence of some kind of association between MAP and at least some cases of

Crohn's disease (Waddell et al., 2008). If MAP is ever shown to have a role in Crohn's disease (causal or complicating infection) then there is sufficient evidence in the public domain indicating that cows' milk (raw or pasteurized) is a potential vehicle of transmission of the pathogens from cattle to humans (Grant, 2005).

Cattle may become infected with MAP by consuming grass or feed that has been cross-contaminated with MAP-positive feces (Mortier et al., 2014). The pathogen may be shed directly into feces of infected animals, and fecal concentrations of 10^6 to 10^8 CFU/g have been observed (Crossley et al., 2005; Chiodini et al., 1984; Whittington and Sergeant, 2001). MAP is highly resistant to environmental changes and can survive in soil and water for more than a year (Cerf et al., 2007). It has been observed that 3-19% of asymptomatic cows shed MAP directly into milk and 9-36% into colostrum (Sweeney et al., 1992; Streeter et al., 1995). Up to 35% of clinical cows shed the bacteria into milk, and the shedding level varies according to severity of disease (Taylor et al., 1981; Giese and Ahrens, 2000; Smith et al., 2009).

For modeling purposes, animals are typically divided into three age groups, based upon susceptibility to MAP infection: <1-year-old young stock, 1-2-year-old heifers, and >2-year-old adults; susceptible individuals usually become infected as calves and move into a transient infection as they age (Doré et al., 2012). Young calves are highly prone to infection from the fecal-oral route in an environment contaminated with adult cow manure, or from ingestion of MAP-positive milk, feed, or colostrum (Geraghty et al., 2014). In addition, horizontal transmission has been documented among calves housed in the same pen (van Roermund et al., 2007). Infection may also occur *in utero,* but this process has been reported to be quite rare (Whittington and Windsor, 2009).

Many control programs have been implemented to reduce MAP prevalence in various dairy-producing countries (Sorge et al., 2011). The control strategies typically include constraining the purchase of cattle from non-certified herd dealers, screening newly introduced cattle for infection, and removing the calf from the calving area as soon as possible after parturition (Wraight et al., 2000; Whittington and Sergeant, 2001). Another common intervention strategy involves a test-and-cull program for MAP-positive cows, which leads to economic losses for both dairy producers and the industry (Lu et al., 2010). The chronic nature of MAP infection, paired with a lack of reliable diagnostic tests, make the bacterium difficult to study (Nielsen, 2008; Norton et al., 2010).

Several mathematical models have been developed to assess the impact of different control measures on MAP infection dynamics in susceptible livestock species such as dairy cattle (Mitchell et al., 2008; Marcé et al., 2011; Kudahl et al., 2007), beef cattle (Humphry et al., 2006) and other animals (Ezanno et al., 2005; Whittington et al., 2000; Heuer et al., 2012). Additionally, a model framework called 'JohneSSim' has been built in the Netherlands to support MAP control strategies. The model is able to take into consideration further complexities of MAP infection by employing stochastic simulation to estimate the prevalence of MAP in three adult shedding categories and through six transmission routes (Groenendaal et al., 2002; Groenendaal et al., 2003; Groenendaal and Galligan, 2003). However, the JohneSSim and related models (such as 'PTB-Simherd') have proven difficult to replicate (Ostergaard et al., 2000; Kudahl et al., 2007).

A few mathematical models have incorporated indirect transmission of MAP infection through a contaminated environment (Marcé et al., 2011; Humphrey et al., 2006); however most current models account, exclusively, for direct transmission within a herd. The simulation

models mentioned above concluded that test and cull programs alone do not substantially lower MAP prevalence and should be paired with improved hygienic conditions. In the current work, we aim to develop a predictive model to provide an estimate of the level of bulk-milk MAP contamination resulting from poor hygiene practices. To accomplish this objective, we incorporated different infection stages, environmental factors, and hygiene practices to predict the concentration of MAP in bulk tank milk under different scenarios.

2. Methods

2.1 Model Framework

A simulation model was created to estimate concentrations of MAP in bulk-tank milk. In accordance with Mitchell et al. (2008) and Schukken et al. (2015), cows were categorized based upon infection status and partitioned into non-infected, latently infected, intermittently shedding (transient shedders), and Progressors. Progressors and intermittent shedders begin shedding MAP at similar ages, but Progressors continue to test positive in fecal samples and show an increasing number of colony forming units (CFU) over time. We further divided the Progressors into low-shedding and high-shedding categories. Intermittent shedders may shed anytime during their adult lifetimes, but do not shed consistently. We assumed that latent cows were not shedding MAP; therefore, for the purposes of our model, these animals were grouped together with non-infected animals.

Concerning routes of contamination, MAP may enter the bulk tank by means of fecal shedding (such as via fecally contaminated udders), internal (direct) shedding, or environmental contamination (such as from soil and water mixed with feces). The three contamination routes are explained in the conceptual model (*Figure 16*). Although the environmental route could be considered a subset of the fecal route, we elected to model these components separately, since

the concentration of MAP resulting from the environmental route is expected to be diminished, due to mixing with soil, grass, water, and other environmental vectors.

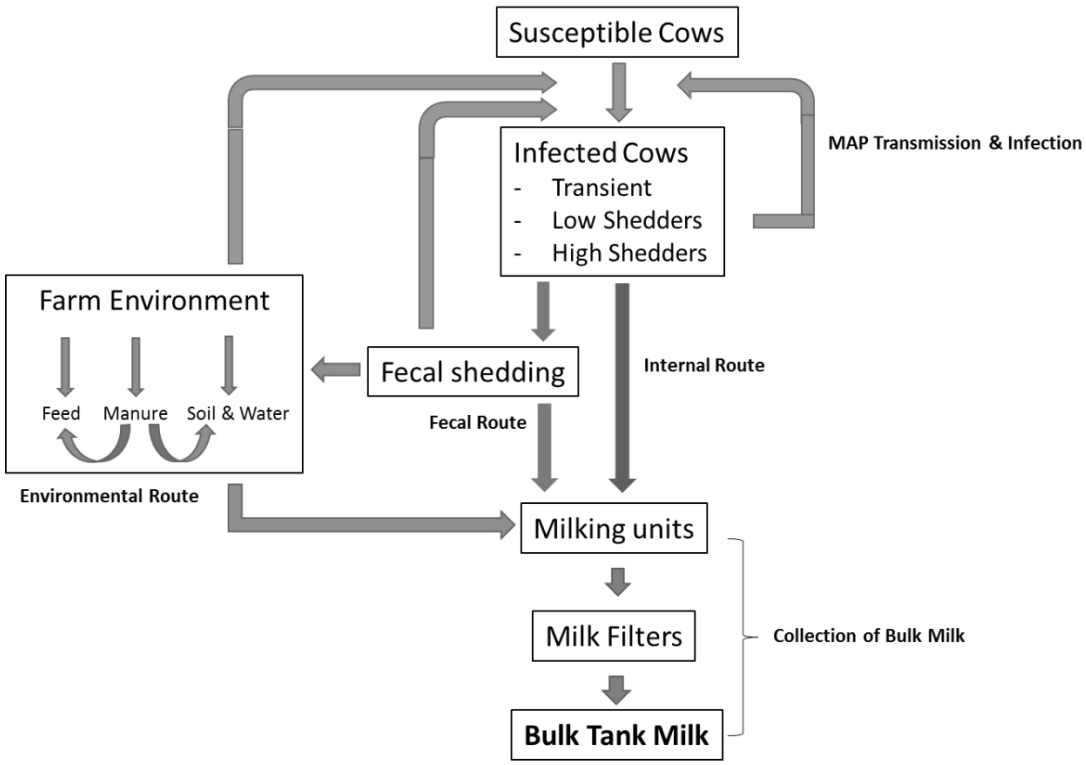

Figure 16*. Conceptual Model*. The diagram illustrates the spread of MAP infection in a dairy herd and the three routes of bulk-milk contamination.

Bulk tank milk contamination with MAP was taken to be the sum of these three routes: direct excretion into milk, fecal contamination, and indirect (environmental) contamination.

2.2 Model Assumptions

As described, our simulation model has been developed based on three contamination routes: internal shedding, fecal shedding, and environmental contamination. Since MAP is unlikely to multiply after it leaves the host (National Advisory Committee on Microbiological Criteria for Foods, 2010), this model does not include any growth module for MAP in milk or within the environment. The fecal contamination route was considered to result purely from

direct fecal contamination of milking parlor equipment or personnel. For the environmental route, we assumed that all cows graze on fields spread with manure containing some concentration of MAP. The udders and legs of grazing cattle may become contaminated with a combination of soil, water, and manure, which may enter the bulk tank through cross-contamination. Because the manure is mixed with these other environmental components, the concentration of MAP is assumed to be lower than that of direct fecal contamination. For simplicity, we will henceforth use the term "dirt" to refer to the elements of fecal contamination acquired on pasture.

In the model, we included the case of "no shedding," as it is assumed that a proportion of transiently-shedding cows are not shedding at a given time point. Therefore, each infection status, with the exception of high shedders, was distributed along 0 to its highest shedding concentration, in order to incorporate the above reasoning. All cows were assumed to be milked twice a day, and the total amount of milk produced daily is assumed to make up the bulk tank milk supply without any spillage or wastage. The usage of milk filters is fundamental in all dairy farms; additionally, other hygiene practices, such as washing of udders before milking, and cleaning of milking equipment, are also strongly recommended. In our model, we define "washing techniques" as the combined effect of milk filtration, washing of udders, and cleaning of milking equipment.

2.3 Model Parameters

The model was parameterized using existing data from surveys and peer-reviewed literature. Input parameters are described in detail in *Table 21*.

Table 21. *Description of parameters and variables used in the simulation model.*

Variable		***Distribution/Value***	***Reference***
Herd dynamics			
H_{size}	Herd size	Pert (40,176,550)	USDA census 2012
P_{non}	Prevalence, uninfected (%)	Uniform	Smith 2010a & b
P_{lat}	Prevalence, latent	Uniform (0.067,0.068)	Raizman et al., 2007; Smith et al., 2009
P_{trans}	Prevalence, transient	Triangle (0.037,0.038,0.041)	Raizman et al., 2007: Smith et al., 2009; Beate et al., 2005
P_{low}	Prevalence, low shedders	Triangle (0.014,0.0209,0.021)	Raizman et al., 2007: Smith et al., 2009; Beate et al., 2005
P_{high}	Prevalence, high shedders	Triangle (0.009,0.018,0.022)	Raizman et al., 2007: Smith et al., 2009; Beate et al., 2005
Milk production			
M_{non}	Milk produced by uninfected	34	Raizman et al., 2007
M_{lat}	Milk produced by latent cows	34.7	Smith et al., 2009
M_{trans}	Milk produced by transient	32	Raizman et al., 2007
M_{low}	Milk produced by low	29	Raizman et al., 2007
M_{high}	Milk produced by high	12	Raizman et al., 2007
ρ	Density of milk (kg/L)	Uniform (1.022,1.036)	Walstra et al., 2005
Fecal route contamination			
F_{non}	Fecal shedding, uninfected	0	Assumption
F_{lat}	Fecal shedding by latent cows	0	Assumption
F_{trans}	Fecal shedding by transient cows (log CFU/g)	Triangle (0,3,4.6, Truncate (3,4.6))	Raizman et al., 2007
F_{low}	Fecal shedding by low shedders (log CFU/g)	Triangle (0,4.6,5.1, Truncate (4.6,5.1))	Raizman et al., 2007
F_{high}	Fecal shedding by high shedders (log CFU/g)	Uniform (5.1,8.1)	Raizman et al., 2007; Hutchison et al., 2004
ε	Average fecal contamination of bulk milk (mg/L)	Triangle (2,40,300)	Stadhouders and Jorgensen 1990
σ	Fraction of feces in dirt	Triangle (0.8,0.975,1)	Boulais et al., 2011
λ	Efficiency of washing	Pert (0.45, 0.74, 0.96)	Boulais et al., 2011
Internal route contamination			
I_{neg}	Shedding in milk by	0	Assumption
I_{pos}	Shedding in milk by positive cows (log CFU/L)²	Triangle (0,2.2,3.3, Truncate (1.6,3.3))	Sweeney et al., 1992; Grant et al., 2002b
ϕ	Efficiency of Milk Filters	0.67	Van Kessel et al., 2008
Environmental route contamination			
μ	Concentration of MAP in manure (log CFU/g)	Uniform (1.92,3.20, Truncate (3,3.20))	Fecteau et al., 2013

N_{ls}	Amount of manure on lightly soiled cows (g)	10^Pert (0.5,1,1.5)	Boulais et al., 2011; Magnusson et al., 2007; Vissers et al., 2007
N_{ms}	Amount of manure on moderately soiled cows (g)	10^Pert (-0.75,0,0.75)	Boulais et al., 2011; Magnusson et al., 2007; Vissers et al., 2007
N_{hs}	Amount of manure on highly soiled cows (g)	10^Pert (-2.5,-1.5,-0.5)	Boulais et al., 2011; Magnusson et al., 2007; Vissers et al., 2007
α	Proportion lightly soiled cows	Triangle (0,0.025,0.05)	Boulais et al., 2011; Magnusson et al., 2007; Vissers et al., 2007
β	Proportion moderately soiled cows	Triangle (0,0.125,0.25)	Boulais et al., 2011; Magnusson et al., 2007; Vissers et al., 2007
γ	Proportion highly soiled cows	1-α-β	Boulais et al., 2011; Magnusson et al., 2007; Vissers et al., 2007

i. Herd Composition and Infection Statuses

According to USDA census 2012, the vast majority of U.S. dairy farms have less than 200 cows. In this study, herd size (H_{size}) was assumed to have a triangular distribution, with 176 representing the most likely number of cows. Based upon the database, the minimum herd size was taken as 40 cows and the maximum as 550 (*Table 21*).

The percentage of cows in each infection stage was estimated from data gathered from multiple studies and surveys (*Table 21*), and multiple values were fitted to obtain the distribution for a particular variable. In our model, the uninfected cows (P_{non}) accounted for about 85.88% of the total herd size, with a uniform distribution between 81.3% and 91.2%. The latent group (P_{lat}) accounted for 6.76% of the total herd size, which was established from a uniform distribution between 6.8% and 6.7% (Raizman et al., 2007) . The remainder (7.36%) was comprised of transient shedders (P_{trans}, 3.85%) and Progressors (P_{low}, 1.87%, and P_{high}, 1.64%) (Raizman et al., 2007; Smith et al., 2009; Wells et al., 2002; Vazquez et al., 2013; Smith et al., 2015; Schukken et al., 2015). The final prevalence of infection statuses was normalized to 100%.

ii. Milk Yield

Johne's disease status has been found to have a significant effect on milk production, and this effect is not uniform across the different infection categories (Smith et al., 2015). An uninfected cow, on average, produces 34 kg of milk per day (M_{non}) (Raizman et al., 2007). Milk production data indicate that latent animals produce more milk than uninfected animals, a difference that decreases over time in the latent infection state. Therefore, the average daily milk production by a latent cow in our model is 34.7 kg (M_{lat}) (Smith et al., 2015; Smith et al., 2009). Cattle in the low and high-shedding categories have meaningfully lower milk production than non-infected cows, with large decreases in production over time. Their average daily milk productions were estimated to be 32 kg (M_{trans}), 29 kg (M_{low}) and 12 kg (M_{high}) for intermittently, low, and high MAP-shedding cows, respectively (Raizman et al., 2007). The sum of the amount of milk produced by all cows, according to their infection statuses, comprised the total volume of bulk-tank milk (B_{milk}).

iii. Internal Shedding

The internal shedding route pertains to the amount of MAP directly secreted into milk. The MAP concentrations were estimated from a study that included 77 subclinical dairy cows, 9 of which directly excreted MAP into milk at concentrations of 1.6 to 2.2 log CFU/L (Sweeney et al., 1992a). 68 animals had no detectable excretion (i.e., below the detection limit of 1.6 log CFU/L) (Boulais et al., 2011). Several other studies reported the concentration of MAP from internal shedding in the range of 2.2 to 3.3 log CFU/L (Grant et al., 2002a; Grant et al., 2002b). The distribution for internal shedding was taken to be triangular, with 0 as the lowest bound, 3.3 log CFU/L as the highest bound, and 2.2 log CFU/L as the most likely (I_{pos}) (*Table 21*). The distribution was further truncated at the lower limit of detection of PCR (1.6 log CFU/L

according to Boulais et al., 2011). The uninfected and latent cows were assumed not to shed any MAP directly into milk (I_{neg}).

iv. Fecal Shedding

According to Raizman et al. (2007) and Boulais et al. (2011), colony counts for fecal cultures were recorded as 3 to 4.6 log CFU/g for intermittent shedders (F_{trans}), 4.6 to 5.1 log CFU/g for low shedders (F_{low}) and more than 5.1 log CFU/g for high shedders (F_{high}). The maximum level of fecal shedding was calculated to be 8.1 log CFU/g (Hutchison et al., 2004). As in the internal route, we included 0 as the minimum level of shedding and truncated the distribution at the detection level for culture techniques (i.e., 3 log CFU/L (Raizman et al., 2007)) (*Table 21*). We assumed no fecal shedding from uninfected and latent cows (F_{non} & F_{lat}).

v. Environmental Contamination

The average environmental "dirt" contamination (ε) was estimated at 40 mg/L with minimum and maximum values of 2 mg/L and 300 mg/L, respectively (Stadhouders, J. Jørgensen, 1978). We assumed a PERT distribution for obtaining the above estimate (*Table 21*). Concepts pertaining to the fraction of feces in dirt were adopted from studies on contamination of bulk milk with *Bacillus cereus* and butyric acid bacteria (Vissers et al., 2006; Magnusson et al., 2007). The percent of feces in the "dirt" mixture (σ) was calculated as 92.5% which was characterized by a triangular distribution of 0.8, 0.975 and 1 (*Table 21*).

Studies on predictive modeling of *Bacillus cereus* spores in milk (Vissers et al., 2007; Magnusson et al., 2007) provided many useful variables for adaptation in our model. For example, we categorized cows based upon the quantity of soil brought into the milking parlor after grazing ($\alpha =$ slightly soiled, $\beta =$ moderately soiled, and $\gamma =$ highly soiled). The total quantity of manure entering bulk milk was the sum of the amount of soil and manure mixture

along with the remaining dirt contamination ($1 - \sigma$), i.e., 7.5%. MAP bacteria have been observed to survive in soil and water for more than a year (Cerf et al., 2007). Based upon the results of Fecteau et al., (2013) we quantified the concentration of MAP in manure (μ), as 1.92 to 3.2 log CFU/g. A uniform distribution was assumed, with a truncation at 3 log CFU/g (the lower limit of detection for culture technique) (*Table 21*).

vi. Washing Efficiency

The efficiency of washing techniques depends upon how strictly and often these techniques are followed. In our model, the efficiency (λ) of washing and cleaning techniques (washing of udders and teats before milking and cleaning of milking equipment), was calculated as 71.67 % using a PERT distribution with a mode of 74%, a minimum of 45%, and a maximum of 96% (*Table 21*). We included the inefficiency of washing techniques ($1 - \lambda$) in our model to represent the percentage of feces and soil entering the bulk tank (Boulais et al., 2011).

vii. Milk Filter Efficiency

On a typical U.S. dairy farm, milk is run through a milk filter to prevent coarse foreign matter (e.g. feces, soil etc.) from entering the bulk tank. The filter may be textile or metal depending upon the milking devices and hygiene norms of the farms (Slana et al., 2012). Adapted from a study on the prevalence of *Salmonella* in bulk milk, and based on the number of herds with negative milk samples and positive milk filters, we calculated the success rate of milk filters (ϕ) as 0.67 (*Table 21*) (Van Kessel et al., 2008). Hence, the percentage of milk filters incapable of capturing the bacteria is 33% ($1 - \phi$).

viii. Final Concentration of MAP in bulk milk

The final concentration of MAP (MAP_{base}) in bulk milk in the base model was calculated by summing the respective amount of MAP at the herd level from the three aforementioned

contamination routes ($MAP_{feces}, MAP_{inter}, \& MAP_{envir}$) multiplied by the inefficiency of milk filters per unit liter of bulk milk. The equations involved in the calculation of the intermediate concentrations of MAP, prior to milk filtration, are shown in *Table 22.*

Table 22*. Equations Used to Calculate Model Outputs.* The equations involved in the calculation of the intermediate concentration of MAP for each route, before milk filtration, are presented.

Description	***Equation***
Liters of Bulk milk	$B_{milk} = H_{size} \times \sum_{1}^{5} \left(P_{stat} \times \frac{M_{stat*}}{\rho} \right)$
MAP from fecal shedding (log CFU)	$MAP_{feces} = H_{size} \times \sum_{3}^{5} (P_{stat} \times F_{stat}) \times \frac{(\varepsilon \times B_{milk} \times \sigma)}{1000}$
MAP from direct shedding (log CFU)	$MAP_{inter} = H_{size} \times \frac{1}{\rho} \times \{[I_{neg}((P_{non} \times M_{non}) + (P_{lat} \times M_{lat})) + [I_{pos}((P_{trans} \times M_{trans}) + (P_{low} \times M_{low}) + (P_{hvy} \times M_{hvy}))]\}$
MAP from environment (log CFU)	$MAP_{envir} = \mu \times [H_{size}((N_{ls} \times \alpha) + (N_{ms} \times \beta) + (N_{hs} \times \gamma)) + \frac{(\varepsilon \times B_{milk} \times (1-\sigma))}{1000}]$
Final concentration of MAP in bulk milk (log CFU/L)	$MAP_{base} = \frac{\left[\left(\left(MAP_{feces} \times (1-\lambda)\right) + MAP_{inter} + \left(MAP_{envir} \times (1-\lambda)\right)\right) \times (1-\phi)\right]}{B_{milk}}$

*Summation over stat (statuses of MAP infection), i.e. 1-non-infected, 2-latent, 3-transient, 4-low, 5-high shedders.

ix. Scenario and Sensitivity Analysis

Empirical distributions were adjusted with statistical distributions for each parameter using @RISK 7.5 (Palisade Corporation), and Monte Carlo simulations were run for 100,000 iterations. Tornado graphs were employed to depict the Spearman rank correlation between the MAP concentration and select parameters associated with the three contamination routes. We

assumed 2 distinct scenarios for hygiene practices in our model. In the first scenario (scenario 1), no washing of udders or teats and no cleaning of milking equipment are included. The second scenario (scenario 2) is a "worst-case" scenario in which no hygiene practices are followed (i.e., no washing of udders and no milk filters). For each scenario, the final MAP concentration in bulk milk was calculated and compared to that of the base model (in which all hygiene practices were included).

3. Results

Mean values, along with other descriptive statistics for bulk-milk MAP concentrations from the base model, and from the two scenarios, are summarized in *Table 23.*

Table 23. *Descriptive Statistics of Empirical Distributions of MAP Concentrations in Bulk Milk in Different Scenarios*. MAP_{base} includes both washing of udders and usage of milk filters (base model), MAP_{no_wash} indicates that udders were not washed prior to milking (scenario 1), and MAP_{worst} represents to MAP concentration when there is no washing of udders and no milk filter usage (scenario 2).

Parameter	***Empirical distribution***				
	Mean	*SD*	*5th percentile*	*50th percentile*	*95th percentile*
MAP_{base}(log CFU/L)	0.758	0.639	0.136	0.563	2.039
MAP_{no_wash}(log CFU/L)	2.570	1.936	0.463	2.046	6.469
MAP_{worst}(log CFU/L)	7.787	5.865	1.403	6.200	19.602

Based upon an average herd size of 216, the total volume of bulk milk was calculated to be 1858.55 US gallons (7035.38 liters) per day. The mean contributions of MAP to bulk-tank milk from the environmental and internal routes were generally low, with the fecal route contributing the most.

Probability distributions for the final concentration of MAP in bulk milk are depicted in Figure 17.

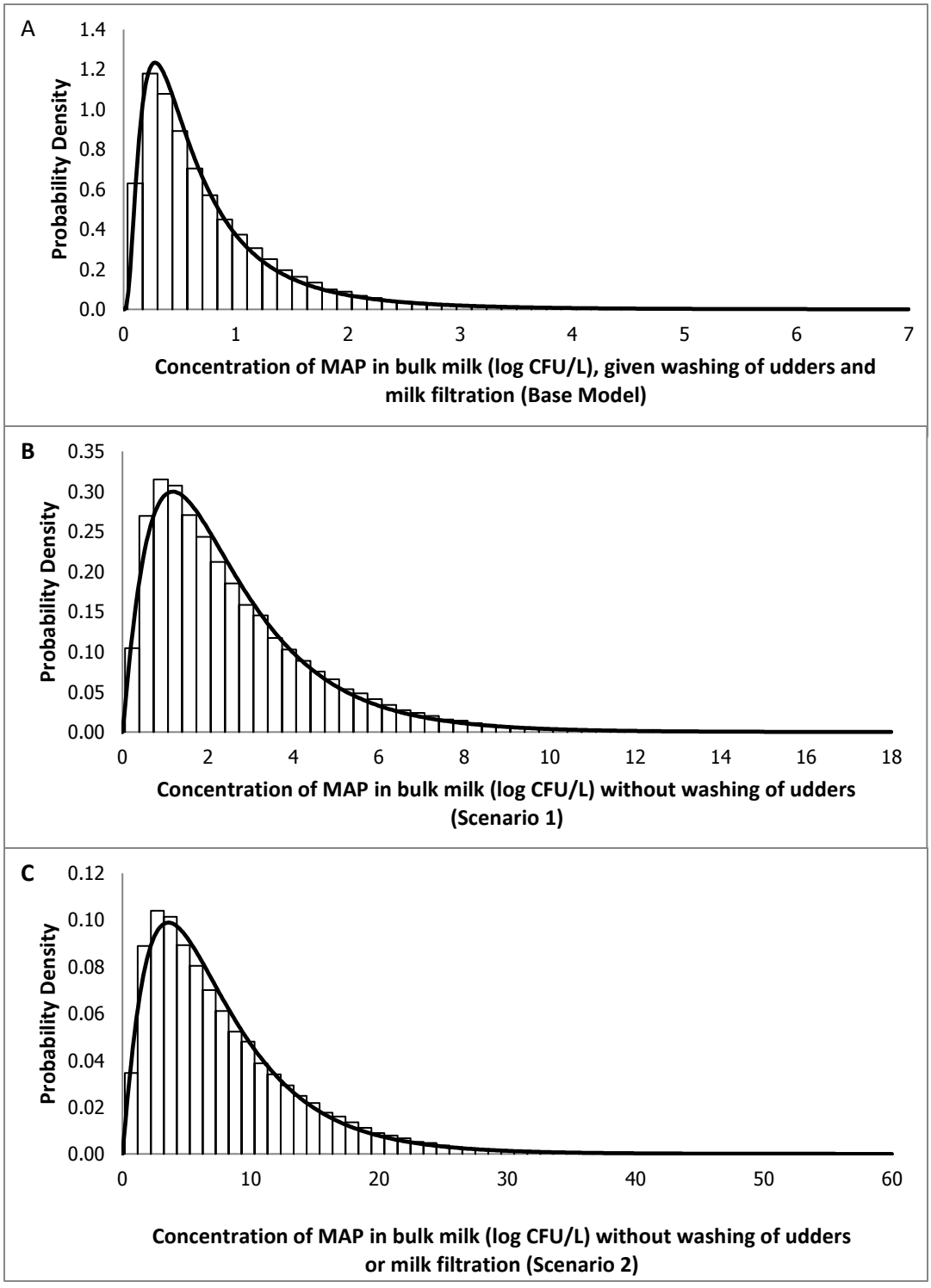

Figure 17. *Probability Distributions of MAP Concentrations.* A) the final concentration of MAP in bulk milk after the implementation of all hygiene practices (base model), B) final concentration of MAP in scenario 1 (no washing of udders), and C) final concentration of MAP in the worst-case scenario (scenario 2).

When washing of udders was conducted prior to milking (base model), the concentration of MAP in bulk-tank milk in our modeled herd reached a maximum of 6.70 log CFU/L with an average concentration of 0.76 log CFU/L (*Figure 17A*). The final concentration of MAP in the bulk tank was fitted to a Lognormal distribution with mean value of 0.775 and SD of 0.759.

In the base model, the amount of MAP from the fecal route contributed approximately 93% of the total MAP concentration in bulk milk, while the remainder was divided between the internal and environmental routes (6% and 1% respectively). The mean final MAP concentration in Scenario 1 was estimated as 2.57 log CFU/L and was fitted into a Pearson Type 6 distribution with shape parameters 2.119 (alpha 1) and 14.366 (alpha 2), and scale parameter 16.214 (beta) (*Figure 17B*). Similarly, in Scenario 2, the mean final MAP concentration was calculated to be 7.78 log CFU/L and was fitted into a Pearson Type 6 distribution with shape parameters 2.119 (alpha 1) and 14.366 (alpha 2), and scale parameter 49.134 (beta) (*Figure 17C*).

Figure 18 depicts the results from the sensitivity analysis and illustrates the relative importance of different variables in the final concentration of MAP in bulk milk.

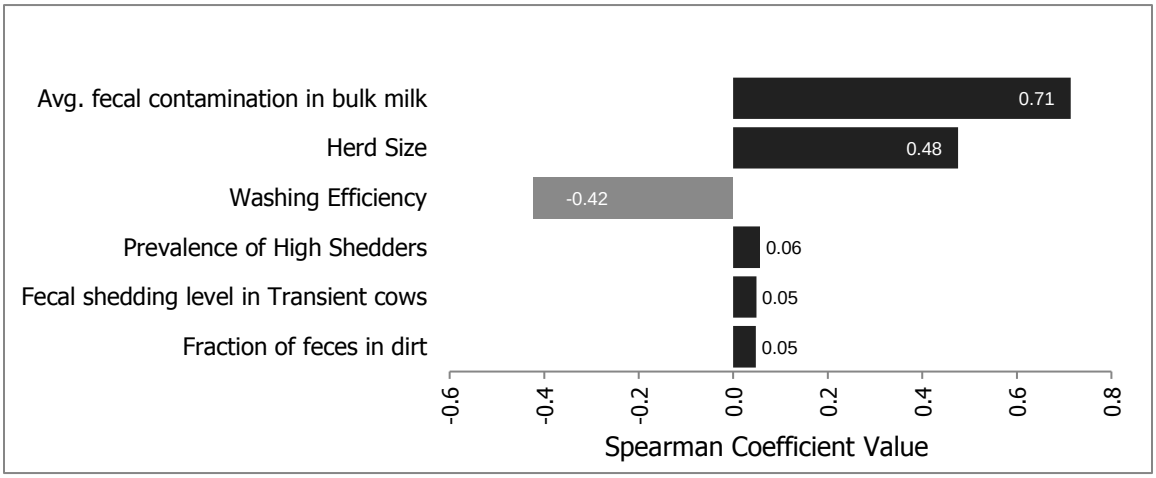

Figure 18. *Tornado Graph Depicting Results from the Sensitivity Analysis.* Sensitivity of analysis for different variables on the final concentration of MAP in bulk milk are shown (Spearman Rank Correlation).

The change in output (mean final MAP concentration) is measured across the range of input values of farm variables (Spearman Rank Correlation). According to the sensitivity analysis, the average fecal contamination of bulk milk was the most important factor in the contamination process, followed by herd size and prevalence of high shedders, with correlation coefficients of 0.71, 0.48 and 0.06 respectively. On the other hand, washing efficiency was negatively correlated (0.42) with the final concentration of MAP in bulk milk.

Figure 19 shows a comparison of cumulative probability densities of final MAP concentrations in bulk milk in the base model, scenario 1, and scenario 2.

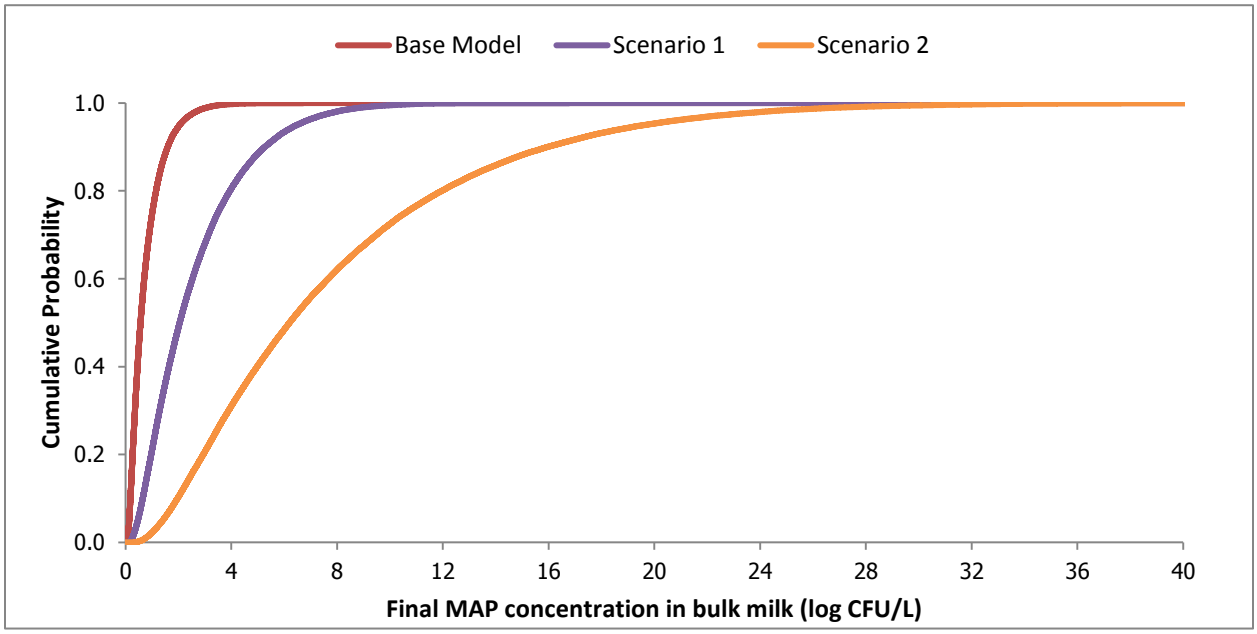

Figure 19. *Cumulative Probability Densities of Final MAP Concentrations in Bulk Milk.* The base model, scenario 1 (no washing of udders), and scenario 2 (no washing udders and no milk filtration) are compared.

In the base model, the final concentration of MAP in bulk milk was estimated at an average of 0.756 log CFU/L when all hygiene practices were implemented, (i.e., washing udders and teats, cleaning milking equipment, and milk filter usage.) The final MAP concentration in

bulk milk in the absence of washing practices (scenario 1) was 3.38 times the MAP concentration in the base model (*Figure 19*). The worst-case scenario of no hygiene practices and no milk filtration (scenario 2) resulted in a 7.02 log increase in the final average MAP concentration in bulk milk as compared with the base model (*Figure 19*).

4. Discussion

The objective of the current study was to predict the concentration of MAP in bulk tank milk under a variety of hygienic conditions. We also sought to determine the amount of MAP entering bulk milk via fecal, internal, and environmental routes, and to compare the relative contribution of each route to the overall MAP contamination level. In order to address these objectives, we developed a risk assessment model evaluating factors that incorporated a variety of MAP infection statuses. Infected animals were categorized as intermittent or Progressors (Schukken et al., 2015), a classification system that has not yet been included in risk assessment models. Progressors were further divided into low and high shedders. To the extent possible, uncertainties (resulting from a lack of perfect knowledge) and variabilities (based upon temporal, geographical, and/ or individual heterogeneity) associated with these infection statuses were incorporated into the model.

The model also included several herd management strategies and hygiene practices such as milk filtration and milking-parlor cleanliness. Strengths of this study include a simple design combined with comprehensive data on infection statuses, which enabled insights into the main contamination route. The pre-mature culling of infected cattle is a substantial issue for dairy producers (Groenendaal and Galligan, 2003); thus, studying how animals with particular infection statuses actually contribute to contamination of the bulk milk could provide insights

into control strategies. In addition, provisioning contaminated milk to newborn calves may lead to an increase in prevalence and subsequent horizontal transmission (Beaver et al., 2016).

Given the implementation of hygiene practices, the estimated MAP load in the bulk tank ranged from 0.035 to 6.7 log CFU/L with an average of 0.76 log CFU/L, depending on herd size and the ratio of infection statuses (*Figure 17A*). The mean concentration of MAP in bulk-tank milk was similar to concentrations reported in other models (Raizman et al., 2007; Boulais et al., 2011). Infected cows shed relatively little MAP directly into their milk while, most is shed into feces (Sweeney et al., 2011). Using our model, we were able to quantify the contribution of the fecal route on bulk-milk contamination and concluded that this route was responsible for 93% of the total MAP contamination in bulk milk. The remaining 7% stemmed from the internal and environmental routes (6% and 1%, respectively).

In our model, the prevalence of heavy shedders also showed substantial impact on MAP contamination in bulk milk. Although the number of heavy shedders in a herd is usually below 5, these animals sometimes shed up to 10^{12} MAP CFU/day in their feces (Chiodini et al., 1984). Washing efficiency was negatively correlated with bulk-milk contamination, a conclusion that emphasizes the importance of proper hygiene practices. With the help of properly implemented hygiene practices, the level of MAP contamination in bulk milk could be lowered by an average of 1.8 log CFU/L (*Figure 19*). We also quantified the role of milk filters in preventing MAP contamination of the bulk tank, concluding that the presence of such filters led to a reduction of up to 7 log MAP CFU/L (*Figure 19*).

Raw milk intended for consumers should be monitored, given the potential role of MAP in the development of human Crohn's disease and the legal sale of raw milk in several States in the U.S. (Sweeney et al., 2011). Moreover, MAP in bulk tank milk has been shown to survive

pasteurization when present in high concentrations. Specifically, MAP has been documented to survive HTST pasteurization when its initial concentration exceeds 10^4 cells/L (Grant et al., 1996; Grant et al., 2005). Thus, ensuring that MAP in the bulk tank remains below this threshold is crucial. Based upon the likelihood of pasteurization survival, Weber & Groenendaal (2009) classified herds with a high probability of bulk-milk MAP concentrations below 10^3 cells/L as low risk. According to our model, the results for MAP load in bulk milk could be classified as low risk, since the 95th percentile reaches 10^2 CFU/L. This is given an average herd size and overall prevalence of 7.36%. However, improper implementation of washing and cleaning practices could be problematic for public health, as the 95th percentile for the concentration of MAP in the bulk tank reached 10^6 CFU/L.

The average MAP loads in our model were generally low; however, the 95^{th} percentile, in some instances, was greater than the upper limit of biologically plausible microbial contamination (10^8 cells/L). These unrealistic counts may be due to the additive nature of intermediate distributions used in the equations to calculate final MAP concentration. The fecal contamination was calculated based on an average value, which could certainly be higher in practical situations. Additionally, we elected to carry out the simulations using daily average milk production and did not account for days in milk. Finally, adult-to-adult MAP transmission has recently been identified (Schukken et al., 2015) and could be an important component of future models.

5. Conclusion

Here we developed a risk assessment model to evaluate MAP contamination in bulk-tank milk. The impact of contamination routes and hygiene practices on the bulk-milk contamination

level were evaluated by the means of scenario and sensitivity analyses, and Monte Carlo simulations. MAP from the fecal-route comprised more than 90% of the total MAP load in the bulk tank, with the remainder divided between the internal and environmental routes. Determining the primary source of milk adulteration with MAP is a preliminary step in milk-quality initiatives and control programs. This study also concluded that herd size and prevalence of heavy shedders were positively correlated with MAP concentration in bulk milk. Based upon the results of the scenario analyses, our model estimated that the level of MAP contamination in the bulk tank could be lowered by an average of 1.8 log CFU/L given proper implementation of hygiene practices. Furthermore, in this scenario, even the 95^{th} percentile concentration (10^2 CFU/L) could be considered low risk for human consumption. On the other hand, without suitable cleaning techniques, the level of MAP contamination could exceed 10^6 CFU/L and thus become problematic for public heath, particularly if there is indeed a causative link to human Crohn's disease. These insights may be useful for developing initiatives to improve bulk-milk quality in endemically infected dairy herds.

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7. References

Beaver, A., P. Ruegg, Y. Gröhn, and Y. Schukken. 2016. Comparative risk assessment for new cow-level *Mycobacterium avium* subsp. *paratuberculosis* infections between 3 dairy production types: Organic, conventional, and conventional-grazing systems. J. Dairy Sci. 99:9885–9899.

Boulais, C., R. Wacker, R., J. C. Augustin, M. H. Cheikh, M. H. Ben, and F. Peladan. 2011. Modeling the occurrence of *Mycobacterium avium* subsp. *paratuberculosis* in bulk raw milk and the impact of management options for exposure mitigation. J. Food Prot. 74:1126–1136.

Cerf, O., M. Griffiths, and F. Aziza. 2007. Assessment of the prevalence of *Mycobacterium avium* subsp. *paratuberculosis* in commercially pasteurized milk. Foodborne Pathog. Dis.

4:433–447.

Chiodini, R. J., H. J. Van Kruiningen, and R. S. Merkal. 1984. Ruminant paratuberculosis (Johne's disease): the current status and future prospects. Cornell Vet. 74:218–262.

Crossley, B. M., F. J. Zagmutt-Vergara, T. L. Fyock, R. H. Whitlock, and I. A. Gardner, 2005. Fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* by dairy cows. Vet. Microbiol. 107:257–263.

Doré, E., J. Paré, G. Côté, S. Buczinski, O. Labrecque, J. Roy, and G. Fecteau. 2012. Risk factors associated with transmission of *Mycobacterium avium* subsp. *paratuberculosis* to calves within dairy herd: A systematic review. J. Vet. Intern. Med. 26:32–45.

Ezanno, P., G. van Schaik, M. F. Weber, and J. A. P Heesterbeek. 2005. A modeling study on the sustainability of a certification-and-monitoring program for paratuberculosis in cattle. Vet. Res. 36:811–826.

Fecteau, M. E., E. Hovingh, R. H. Whitlock, and R. J. Sweeney. 2013. Brief communication: Persistence of *Mycobacterium avium* subsp. *paratuberculosis* in soil, crops, and ensiled feed following manure spreading on infected dairy farms. Can. Vet. J. 54:1083–1085.

Feller, M., K. Huwiler, R. Stephan, E. Altpeter, A. Shang, H. Furrer, G. E. Pfyffer, T. Jemmi, A. Baumgartner, and M. Egger. 2007. *Mycobacterium avium* subsp. *paratuberculosis* and Crohn's disease: A systematic review and meta-analysis. Lancet Infect. Dis.7:607–613.

Geraghty, T., D. A. Graham, P. Mullowney, and S. J. More. 2014. A review of bovine Johne's disease control activities in 6 endemically infected countries. Prev. Vet. Med. 116:1–11.

Giese, S. B., and P. Ahrens. 2000. Detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk from clinically affected cows by PCR and culture. Vet. Microbiol. 77:291–297.

Grant, I. R. 2005. Zoonotic potential of *Mycobacterium avium* subsp. *paratuberculosis* : the current position. J. Appl. Microbiol. 98:1282–1293.

Grant, I. R., H. J. Ball, S. D. Neill, and M. T. Rowe. 1996. Inactivation of *Mycobacterium paratuberculosis* in cows' milk at pasteurization temperatures. Appl. Environ. Microbiol. 62:631–636.

Grant, I. R., H. J. Ball, and M. T. Rowe. 2002. Incidence of *Mycobacterium paratuberculosis* in bulk raw and commercially pasteurized cows' milk from approved dairy processing establishments in the United Kingdom. Appl. Environ. Microbiol. 68:2428–2435.

Grant, I. R., E. I. Hitchings, A. McCartney, F. Ferguson, and M. T. Rowe. 2002. Effect of commercial-scale high-temperature, short-time pasteurization on the viability of *Mycobacterium paratuberculosis* in naturally infected cows' milk. Appl. Environ. Microbiol. 68:602–607.

Groenendaal, H., and D. T. Galligan. 2003. Economic consequences of control programs for paratuberculosis in midsize dairy farms in the United States. JAVMA-J. Am. Vet. Med. A. 223:1757–1763.

Groenendaal, H., M. Nielen, and J. W. Hesselink. 2003. Development of the Dutch Johne's disease control program supported by a simulation model. Prev. Vet. Med. 60:69–90.

Groenendaal, H., M. Nielen, A. W. Jalvingh, S. H. Horst, D. T. Galligan, and J. W. Hesselink. 2002. A simulation of Johne's disease control. Prev. Vet Med., 54:225–245.

Herrinton, L. J., L. Liu, J. D. Lewis, P. M. Griffin, and J. Allison. 2008. Incidence and prevalence of inflammatory bowel disease in a Northern California managed care organization, 1996-2002. Am. J. Gastroenterol. 103:1998–2006.

Heuer, C., R. M. Mitchell, Y. H. Schukken, Z. Lu, C. Verdugo, and P. R. Wilson. 2012. Modelling transmission dynamics of paratuberculosis of red deer under pastoral farming conditions. Prev. Vet. Med. 106:63–74.

Hirst, H. L., F. B. Garry, P. S. Morley, M. D. Salman, R. P. Dinsmore, B. A. Wagner., K.D. McSweeney, and Goodell, G. M. (2004). Seroprevalence of *Mycobacterium avium* subsp. *paratuberculosis* infection among dairy cows in Colorado and herd-level risk factors for seropositivity. JAVMA- J. Am. Vet. Med. A., 225:97–101.

Humphry, R. W., A. W. Stott, C. Adams, and G. J. Gunn. 2006. A model of the relationship between the epidemiology of Johne's disease and the environment in suckler-beef herds. Vet. J. 172:432–445.

Hutchison, M. L., L. D. Walters, S. M. Avery, B. A. Synge, and A. Moore. 2004. Levels of zoonotic agents in British livestock manures. Lett. Appl. Microbiol., 39:207–214.

Kudahl, A. B., S. Ostergaard, J. T. Sørensen, and S. S. Nielsen, S. 2007. A stochastic model simulating paratuberculosis in a dairy herd. Prev. Vet. Med. 78:97–117.

Li, L., R. Katani, M. Schilling, and V. Kapur. 2015. Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis* on dairy farms. Annu. Rev. Anim. Biosci. 4:155-176.

Loftus, C. G., E. V. Loftus, W. S. Harmsen, A. R. Zinsmeister, W. J. Tremaine, L. J. Melton, and W. J. Sandborn. 2007. Update on the incidence and prevalence of Crohn's disease and ulcerative colitis in Olmsted County, Minnesota, 1940-2000. Inflamm. Bowel Dis. 13:254–261.

Lombard, J. E., F. B. Garry, B. J. McCluskey, and B. A. Wagner. 2005. Risk of removal and effects on milk production associated with paratuberculosis status in dairy cows. JAVMA- J. Am. Vet. Med. A. 227:1975–1981.

Lu, Z., Y. H. Schukken, R. L. Smith, and Y. T. Grohn. 2010. Stochastic simulations of a multi-group compartmental model for Johne's disease on US dairy herds with test-based culling intervention. J. Theor. Biol. 264: 1190–1201.

Magnusson, M., A. Christiansson, and B. Svensson. 2007. *Bacillus cereus* spores during housing of dairy cows: Factors affecting contamination of raw milk. J. Dairy Sci. 90:2745–2754.

Marcé, C., P. Ezanno, H. Seegers, D. U. Pfeiffer and C. Fourichon. 2011. Predicting fadeout versus persistence of paratuberculosis in a dairy cattle herd for management and control purposes: A modelling study. Vet. Res. 42:36–48.

Marcé, C., P. Ezanno, M. Weber, H. Seegers, D. Pfeiffer and C. Fourichon. 2010. Invited review: Modeling within-herd transmission of *Mycobacterium avium* subsp. *paratuberculosis* in dairy cattle: A review. J. Dairy Sci. 93:4455–4470.

Mitchell, R. M., R. H. Whitlock, S. M. Stehman, A. Benedictus, P. P. Chapagain, Y. T. Grohn, and Y. H. Schukken. 2008. Simulation modeling to evaluate the persistence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) on commercial dairy farms in the United States. Prev. Vet. Med. 83:360–380.

Mortier, R. A., H. W. Barkema, K. Orsel, R. Wolf, and J. De Buck. 2014. Shedding patterns of dairy calves experimentally infected with *Mycobacterium avium* subsp. *paratuberculosis*. Vet. Res. 45:71–79.

Motiwala, A. S., L. Li, V. Kapur, and S. Sreevatsan. 2006. Current understanding of the genetic diversity of *Mycobacterium avium* subsp. *paratuberculosis.* Microbes Infect. 8:1406–1418.

National Advisory Committee on Microbiological Criteria for Foods. 2010. Assessment of food as a source of exposure to *Mycobacterium avium* subsp. *paratuberculosis* (MAP). J. Food Prot. 73:1357–1397.

Nielsen, S. S. 2008. Transitions in diagnostic tests used for detection of *Mycobacterium avium* subsp. *paratuberculosis* infections in cattle. Vet. Microbiol., 132:274–282.

Nielsen, S. S., Y. T. Gröhn, and C. Enevoldsen. 2002. Variation of the milk antibody response to paratuberculosis in naturally infected dairy cows. J. Dairy Sci., 85:2795–2802.

Norton, S., W.O. Johnson, G. Jones, and C. Heuer. 2010. Evaluation of diagnostic tests for Johne's disease (*Mycobacterium avium* subsp. *paratuberculosis*) in New Zealand dairy cows. J. Vet. Diagn. Invest. 22:341–51.

Ostergaard, S., J. T. Sørensen, and A. R. Kristensen. 2000. A stochastic model simulating the feeding-health-production complex in a dairy herd. J. Dairy Sci. 83:721–733.

Ott, S. L., S. J. Wells, and B. A. Wagner. 1999. Herd-level economic losses associated with

Johne's disease on US dairy operations. Prev. Vet. Med. 40:179–192.

Over, K., P. G. Crandall, C. A. O'Bryan, and S. C. Ricke. 2011. Current perspectives on *Mycobacterium avium* subsp. *paratuberculosis*, Johne's disease, and Crohn's disease: A review. Crit. Rev. Microbiol. 37:141–156.

Raizman, E. A., J. Fetrow, S. J. Wells, S. M. Godden, M. J. Oakes, and G. Vazquez. 2007. The association between *Mycobacterium avium* subsp. *paratuberculosis* fecal shedding or clinical Johne's disease and lactation performance on two Minnesota, USA dairy farms. Prev. Vet Med. 78:179–195.

Schukken, Y.H., R.H. Whitlock, D. Wolfgang, Y.T. Grohn, A. Beaver, J. VanKessel, M. Zurakowski, and R.M. Mitchell. 2015. Longitudinal data collection of *Mycobacterium avium* subsp. *paratuberculosis* infections in dairy herds: the value of precise field data. Vet. Res. 46:65–75

Slana, I., P. Kralik, A. Kraklova, V. Babak, and I. Pavlik. 2012. Short communication: Examination of milk filters by real-time PCR as a herd-level indicator of the presence of *Mycobacterium avium* subsp. *paratuberculosis* in dairy herds. J. Dairy Sci. 95:1162–1165.

Smith, R., Y. Grohn, A. Pradhan, R. Whitlock, J. Van Kessel, J. Smith, D. Wolfgang and Y. Schukken. 2009. A longitudinal study on the impact of Johne's disease status on milk production in individual cows. J. Dairy Sci. 92:2653–2661.

Smith, R. L., Y. H. Schukken, and Y. T. Gröhn. 2015. A new compartmental model of *Mycobacterium avium* subsp. *paratuberculosis* infection dynamics in cattle. Prev. Vet. Med. 122:298–305

Smith, R. L., Y. H. Schukken, A. K. Pradhan, J. M. Smith, R. H. Whitlock, J. S. Van Kessel, D. R. Wolfgang, and Y. T. Grohn. 2011. Environmental contamination with *Mycobacterium avium* subsp. *paratuberculosis* in endemically infected dairy herds. Prev. Vet. Med. 102:1–9.

Sorge, U. S., K. Lissemore, A, Godkin, J. Jansen, S. Hendrick, S. Wells, and D. F. Kelton. 2011. Changes in management practices and apparent prevalence on Canadian dairy farms participating in a voluntary risk assessment-based Johne's disease control program. J. Dairy Sci. 94:5227–5237.

Stadhouders, J., and K. Jørgensen. 1978. Prevention of the contamination of raw milk by a hygienic milk production. *Bulletin of the International Dairy Federation*, 251:24–31.

Streeter, R. N., G. F. Hoffsis, S. Bech-Nielsen, W. P. Shulaw, and D. M. Rings. 1995. Isolation of *Mycobacterium paratuberculosis* from colostrum and milk of subclinically infected cows. JAVMA-J. Am. Vet. Med. A. 56:1322–1324.

Sweeney, R. W. 2011. Pathogenesis of paratuberculosis. Vet. Clin. North Am. Food Anim. Pract.

27:537–546

Sweeney, R. W., R. H. Whitlock, and A. E. Rosenberger. 1992. *Mycobacterium paratuberculosis* cultured from milk and supramammary lymph nodes of infected asymptomatic cows. J. Clin. Microbiol. 30:166–171.

Taylor, T. K., C. R. Wilks, and D. S. McQueen. 1981. Isolation of *Mycobacterium paratuberculosis* from the milk of a cow with Johne's disease. Vet. Rec. 109:532–533.

Van Kessel, J. S., J. S. Karns, D. R. Wolfgang, E. Hovingh, B. M. Jayarao, C. P. Van Tassell, and Y. H. Schukken. 2008. Environmental sampling to predict fecal prevalence of *Salmonella* in an intensively monitored dairy herd. J. Food Protect. 71:1967–1973.

van Roermund, H. J., D. Bakker, P. T. Willemsen, and M. C. de Jong. 2007. Horizontal transmission of *Mycobacterium avium* subsp. *paratuberculosis* in cattle in an experimental setting: Calves can transmit the infection to other calves. Vet. Microbiol. 122:270–279.

van Schaik, G., S. M. Stehman, Y. H. Schukken, C. R. Rossiter, and S. J. Shin. 2003. Pooled fecal culture sampling for *Mycobacterium avium* subsp. *paratuberculosis* at different herd sizes and prevalence. J. Vet. Diag. Invest.15:233–241.

Vazquez, P., J. M. Garrido, R. A. Juste, M. Price-Carter, and G. Lisle. 2013. Specific antibody and interferon-gamma responses associated with immunopathological forms of bovine paratuberculosis in slaughtered Friesian Cattle. Plos One. 8:1–9

Vissers, M. M., F. Driehuis, M. C. Te Giffel, P. De Jong, and J. M. G. Lankveld. 2006. Improving farm management by modeling the contamination of farm tank milk with Butyric Acid Bacteria. J. Dairy Sci. 89:850–858.

Vissers, M. M., M. C. Te Giffel, F. Driehuis, P. De Jong, and J. M. G. Lankveld. 2007. Minimizing the level of *Bacillus cereus* spores in farm tank milk. J. Dairy Sci. 90:3286–3293.

Waddell, L. A., A. Rajić, J. Sargeant, J. Harris, R. Amezcua, L. Downey, S. Read, and S. A. McEwen. 2008. The zoonotic potential of *Mycobacterium avium* subsp. *paratuberculosis*: A systematic review. C. J. Public Health 99:145-155.

Weber, M., and H. Groenendaal. 2009. Milk quality assurance for paratuberculosis: effects of infectious young stock. *Proceedings of the 10th International Colloquim on Paratuberculosis,* 9–14. http://www.paratuberculosis.info/images/stories/pdfs/429.pdf

Wells, S. J., R. H. Whitlock, B. Wagner, J. Collins, F. Garry, H. Hirst, J. Lawrence, W. J. Saville, and A. L. L. Naugle. 2002. Sensitivity of test strategies used in the Voluntary Johne's Disease Herd Status Program for detection of *Mycobacterium paratuberculosis* infection in dairy cattle herds. JAVMA-J. Am. Med. A. 220:1053–1057.

Whittington, R. J., S. Fell, D. Walker, S. McAllister, I. Marsh, E. Sergeant, C. A. Taragel, D. J. Marshall, and I. J. Links. 2000. Use of pooled fecal culture for sensitive and economic detection of *Mycobacterium avium* subsp. *paratuberculosis* infection in flocks of sheep. J. Clin. Microbiol. 38:2550–2556.

Whittington, R. J., and E. S. G. Sergeant. 2001. Progress towards understanding the spread, detection and control of *Mycobacterium avium* subsp. *paratuberculosis* in animal populations. Aust. Vet. J. 79:267–278.

Whittington, R. J., and P. A. Windsor. 2009. In utero infection of cattle with *Mycobacterium avium* subsp. *paratuberculosis*: A critical review and meta-analysis. Vet. J. 179:60–69.

Wraight, M. D., J. McNeil, D. S. Beggs, R. K. Greenall, T. B. Humphris, R. J. Irwin, S. P. Jagoe, A. Jemmeson, W. F. Morgan, P. Brightling, and G. A. Anderson. 2000. Compliance of Victorian dairy farmers with current calf rearing recommendations for control of Johne's disease. Vet. Microbiol. 77:429–442.