Resilience to infection and antibody responses associated with direct ileal inoculation of *Mycobacterium avium* subspecies *paratuberculosis* in calves

by

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ABSTRACT

RESILIENCE TO INFECTION AND ANTIBODY RESPONSES ASSOCIATED WITH
DIRECT ILEAL INOCULATION OF MYCOBACTERIUM AVIUM SUBSPECIES

PARATUBERCULOSIS IN CALVES

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Mycobacterium avium subspecies paratuberculosis (Map) is the causative agent of Johne’s disease (JD), a chronic enteritis of cattle and other ruminants. JD is widespread, affecting global dairy and beef industries, with significant associated economic burden. Clinical JD is invariably fatal, and control on farms is hindered by a lengthy subclinical period of infection where animals show no outward signs of disease but can still shed Map in their feces and spread the pathogen to other susceptible animals. The dynamics of Map infection are poorly understood, though some animals apparently clear the pathogen or display resilience to initial Map infection. Vaccination and treatment regimens show limited success in truly preventing or reversing infection, and diagnostic techniques for detection of Map infection are often poorly sensitive or cost-prohibitive for effective surveillance. In this thesis, we utilize a previously described model to examine the development and progression of experimental intestinal Map infection in the first 28 weeks following direct ileal inoculation. We further utilize this model to study how oral supplementation of monensin sodium affects establishment of Map infection and
development of subclinical disease. Finally, we examine *Map*-specific serum antibody responses in these calves, with a goal of characterizing *Map* proteins responsible for antibody development in the early post-inoculation period. We found that calves showed high levels of resilience to *Map* infection; this is a novel finding which significantly contributes to our understanding of early intestinal *Map* infection. We further show that oral monensin may be associated with increased resilience to persistent intestinal *Map* infection and decreased serum antibody responses in calves. Last, we demonstrate that *Map*-specific serum antibodies can be detected at 20 weeks post inoculation, and describe 25 proteins which may drive development of these antibody responses in the early post *Map*-inoculation period. Overall, this work contributes to our understanding of the pathogenesis and resilience of early intestinal *Map* infection in calves, and may be useful for future studies seeking to discover and develop novel diagnostic testing strategies and for understanding vaccine efficacy.
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LIST OF ABBREVIATIONS

AFB – Acid-fast bacilli
APC – Antigen presenting cell
BCG – Bacillus Calmette–Guérin
CD – Cluster of differentiation
CFU – Colony forming units
ELISA – Enzyme linked immunosorbent assay
G+C – Guanine and cytosine DNA content
GO – Gene ontology
GTP – Guanosine-triphosphate
HEYM – Herrold’s egg yolk medium
H&E – Hematoxylin and eosin staining
HTC – High titre cow
HRP – Horseradish peroxidase
IFN-γ – Interferon gamma
IgG – Immunoglobulin G
IgM – Immunoglobulin M
IL – Interleukin
IS – Insertion sequence
JAK-STAT – Janus kinase-signal transducer and activator of transcription
JD – Johne’s disease
LAM – Lipoarabinomannan
LpdC – Lipoamide dehydrogenase
MAC – *Mycobacterium avium* complex

Man-LAM – Mannosylated lipoarabinomannan

*Map* – *Mycobacterium avium* subsp. *paratuberculosis*

MAPK – Mitogen-activated protein kinase

MHC – Major histocompatibility complex

*Mtb* – *Mycobacterium tuberculosis*

ORF – Open reading frame

PIM – Phosphatidylinositol mannoside

PCR – Polymerase chain reaction

qPCR – Quantitative polymerase chain reaction

Rab – Ras-associated GTP-binding protein

RFLP – Restriction fragment length polymorphism

RILP – Rab-interacting lysosomal protein

SOCS – Suppressor of cytokine signaling

SDS-PAGE – Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SodA – Superoxide dismutase

S/P – Sample:Positive Control ratio

TACO – Coronin-1

Th – T helper lymphocyte

TNF-α – Tumour necrosis factor alpha

VBNC – Viable but not cultivatable

WCS – Whole cell sonicate
1 Review of the literature

1.1 Johne’s disease in cattle

1.1.1 Overview of Johne’s disease in cattle

Johne’s disease (JD) is a chronic progressive enteric infection in ruminants caused by the bacterium *Mycobacterium avium* subspecies *paratuberculosis* (*Map*). Susceptible animals, typically young calves, are infected through ingestion of contaminated feces, milk, or colostrum shed by an infected animal [1, 2]. If an animal cannot clear the infection, this initiates a lengthy subclinical period lasting months to years, where the animal may intermittently and asymptotically shed *Map* into the environment, thus increasing the spread of the pathogen [3]. *Map* causes diffuse granulomatous inflammation in the distal jejunum and ileum, marked thickening of the intestinal wall, chronic malabsorptive diarrhea, hypoproteinemia, edema, muscle wasting, and decreased milk production [3, 4]. Clinical JD is invariably fatal with death resulting from dehydration and cachexia, though most animals are culled because of decreased milk production or chronic unresponsive diarrhea long before natural death occurs [1, 5].

The disease was first described in Europe in 1895 and in North America shortly thereafter. In the 100+ years since its discovery, JD has been described in many countries around the globe [1]. It has been estimated that in Canada as many as 65% of herds test positive for the presence of at least one *Map* infected animal, which costs producers an estimated $15 million annually [4]. In the United States, economic losses have been estimated at over $200 million per year, though these figures are now over a decade old.
As such, there is a major effort from beef and dairy industries around the world to limit the spread of JD. No vaccination or therapeutic strategy has been shown to entirely prevent or control *Map* infection, so JD control strategies rely primarily on biosecurity measures such as identifying and removing infected animals from herds before the onset of fecal shedding and increased risk for transmission of *Map* to susceptible herdmates [1]. More recently, a link has been made between *Map* and Crohn’s disease in humans, though a causal link between *Map* infection and development of Crohn’s disease has not yet been clearly identified [6–8].

### 1.1.2 Stages of disease

JD has traditionally been divided into four stages of disease: silent infection, subclinical disease, clinical disease, and advanced clinical disease [1]. Silent infection refers to the initial period following *Map* infection; however, host-pathogen interactions including mechanisms of establishment of persistent enteric *Map* infection during this period are not fully established. Apparently not all animals exposed to *Map* will progress and perhaps some animals are able to prevent establishment of *Map* infection or even clear the pathogen before it progresses [9, 10]. Animals that are permissive to intestinal *Map* infection at this stage show no clinical signs of infection, and are undetectable by current diagnostic testing strategies, though low levels of *Map* may be intermittently shed in the feces [1, 3, 11].

Subclinical disease is the second stage of JD, and *Map*-infected animals probably spend the bulk of their lives in this stage of disease which may persist for several years [1]. After *Map* infection is established in the intestine, bacterial load increases and
additional macrophages are recruited to the site, resulting in the characteristic granulomatous inflammation which is initially multifocal in distribution but eventually spreads along the intestinal tract [3]. Intermittent fecal shedding increases in frequency and magnitude during the subclinical phase and may be detected with regular fecal bacterial culture or fecal PCR screening, though animals in this stage of disease typically remain asymptomatic [3]. Because subclinical disease may last for several years, animals are often sent for slaughter or culled for an unrelated reason before progression into clinical JD.

In clinical JD, increased *Map* levels within the intestine and associated infiltration of immune cells results in thickened intestinal mucosa and lymphadenitis of the associated mesenteric lymph nodes [12, 13]. During the clinical phase, it is common to see bouts of nonresponsive progressive malabsorptive diarrhea. Dehydration and loss of appetite become apparent later; however, affected animals undergo gradual muscle wasting, weight loss and decreased milk production [1].

As clinical JD continues to develop over weeks to months, animals progress to advanced clinical disease. This fourth stage of disease is characterized by progressive diarrhea, subcutaneous edema due to hypoproteinemia, muscle cachexia and weight loss. The health of the animal deteriorates rapidly and death results from dehydration and cachexia if the animal is not first sent to slaughter for salvage [1, 3].

More recently, efforts have been made to establish clear case definitions to better define the stages of *Map* infection, particularly when also considering a number of
recently developed experimental models of infection used to study paratuberculosis. Whittington et al (2017) described five key terms related to the *Map* infection process. Animals experimentally inoculated, or otherwise exposed to *Map*, are termed *Map*-exposed. Animals with confirmed presence of the bacterium *Map* within the intestinal tissues are termed *Map*-infected. Infected animals with evidence of disease, including histologic lesions or clinical signs, are termed *Map*-diseased. *Map*-exposed animals with no evidence of infection following a known exposure (including experimental exposure) are termed *Map*-resilient. And finally, animals which were previously classified as *Map*-infected, and have subsequently cleared the pathogen based on negative diagnostic tests and no detection of *Map* within tissues, are termed *Map*-recovered [14]. This terminology is extremely helpful and important, because it clearly defines two terms which have previously been lacking consistent usage in the literature: *Map*-resilient and *Map*-recovered. There is ample evidence supporting the idea of both resilience to and recovery from *Map* infection in cattle, and conceptually *Map*-resilience and *Map*-recovery fit well within our current knowledge of the prevalence, pathophysiology, and epidemiology of *Map* in endemic herds (described below) [15, 16]. However, as discussed below in section 1.4, detection of low-levels of *Map* within the relatively large intestinal tract remains a significant hurdle to overcome. Therefore, usage of the term recovery from *Map* infection is really a “best estimate” based upon multiple or repeated sampling throughout the intestinal tract.
1.1.3 Prevalence and economic impact

The JD prevalence in a herd is a measure of positive animals, in any stage of disease, at any given point in time. This is often assessed as animal level prevalence within a herd, or herd level prevalence within a geographical region [17]. However, the dynamics of enteric Map infections and limitations of diagnostic methods (diagnostic methods are discussed in detail in section 1.5 of this review) makes accurate estimation of disease prevalence challenging. Currently, the most commonly used screening test for prevalence studies is a commercial antibody ELISA assay targeting Map-specific antibodies in serum or milk. This test is very rapid and cost effective for large-scale screening; however, it has a low sensitivity of detection particularly in the early stages of Map infection/subclinical disease [4, 18]. ELISA assays are targeted toward detection of high titre cows (HTC), which are animals with very high serum levels of Map-specific antibodies. This typically limits detection of animals within one of the two “visible” stages of disease: the clinical stage and advanced clinical stage. Animals in the two “invisible” stages of disease, silent infection and subclinical JD, will often test negative because of low serum Map-specific antibody levels [1, 18–20].

The “iceberg” phenomenon is used to describe many endemic diseases, where the bulk of infected animals reside in an “invisible” or subclinical stage of disease, and what is seen as clinical positive animals is a small fraction of the total disease prevalence within a population [18]. Magombedze et al. (2013) applied this phenomenon to several longitudinal prevalence studies of natural Map infections, which show that while the subclinical phase can last from 2-10 years, calves may begin shedding Map
asymptomatically as early as one year of age. Their conclusion was that most animals progress through the silent phase quickly within the first year of infection, and spend the bulk of their time in the subclinical phase. Furthermore, progression through the silent phase is greatly affected by repeated *Map* ingestion and exposure, where continued high-level ingestion increases *Map* infection and progression to the subclinical phase of disease [18]. The implications of this seem to be that the bulk of animals “invisible” to ELISA screening are probably within the subclinical phase of disease, and can readily shed *Map* into the environment which increases spread of the pathogen within a herd and significantly alters the disease dynamics in susceptible cohorts.

A substantial amount of work has been done to estimate the prevalence and economic impact of JD, though much of this data is now over five years old. Dairy cattle appear to have a higher rate of positivity than beef cattle potentially a result of more confined living conditions and increased lifespan within a herd. Best estimates for North American beef and dairy producers place the individual animal prevalence at 1-9% on average, where the higher burden of disease occur in regions with a larger agricultural sector. This equates to a herd prevalence between 35-70% for herds with at least one positive animal, and approximately 45% for herds with multiple positive animals [2, 4, 17]. Using these figures, it is considered likely that the true prevalence is much higher due to the previously discussed iceberg phenomenon [17]. This extensive prevalence carries a total economic burden of roughly $15 million in Canada and $200-250 million in the United States each year due to loss in productivity as a result of Johne’s disease progression [4, 21].
1.1.4 *Map* infection prevention and control

Control of JD and *Map* infection within a herd has largely been focused on risk mitigation strategies, because antibiotics or other therapeutic alternatives have been shown to be ineffective, particularly once a diagnosis of JD is made in an animal. Infected animals are identified and typically removed from the herd as quickly as possible to eliminate or reduce sources of viable *Map* to susceptible herdmates, while susceptible animals such as younger calves are segregated from the herd to limit exposure [22–24]. As discussed below however, precise and reliable identification of infected animals, particularly subclinical *Map*-infected/diseased animals, poses a significant challenge to this practice. Environmental contamination with *Map*, and subclinical *Map* shedding further complicate matters. A recent oral *Map* infection model of calves demonstrated that fecal-oral transmission between co-housed calves also poses a major risk for infection to other calves, and that *Map*-exposed calves may passively and asymptptomatically shed the *Map* bacterium without showing any evidence of *Map* infection [25].

Vaccination strategies are largely ineffective at preventing or controlling *Map* infection. Some researchers have found that *Map* vaccination is associated with decreased fecal *Map* shedding, delayed or decreased development of clinical signs, and decreased tissue colonization, though they cannot prevent *Map* infection [24, 26–28]. Furthermore, there is a significant concern in the field that *Map* vaccines interfere with serodiagnostic testing, not only for JD but also for the diagnosis of bovine tuberculosis caused by the closely related bacterium *M bovis*. This also impedes use of *Map* vaccination strategies in herd management practices [29–31].
Usage of antibiotic or other chemotherapeutic approaches to treat or prevent *Map* infection have had only a limited research focus. Some antibiotics display mild efficacy (rifampin, isoniazid), though they have never been shown to definitively cure JD, and essentially represent at best a short-term therapeutic approach for valuable animals [32]. One key chemotherapeutic agent which has shown promise is monensin sodium, an ionophore of sodium and potassium which is commonly used as a feed additive in cattle for growth promotion and management of coccidiosis [33, 34]. Monensin has been shown to directly inhibit *Map* growth in culture and may also reduce number and severity of lesions [35, 36]. Monensin may also reduce fecal *Map* shedding and development of *Map*-specific serum and milk antibody responses [37, 38]. The literature on monensin supplementation in *Map* infection is reviewed in greater detail in chapter 3 of this thesis.

1.2 *Mycobacterium avium* subspecies *paratuberculosis*

1.2.1 Microbiology

*Mycobacterium* spp. are members of the phylum Actinobacteria, a group of Gram-positive bacteria with a high Guanine and Cytosine (G+C) DNA content. The genus *Mycobacterium* is the sole member of the family Mycobacteriaceae, in the order Actinomycetales [39]. Mycobacteria are generally described as aerobic, non-motile and non-sporulating short rods, which stain positive by Ziehl-Neelsen (acid fast) staining, and often give a positive Gram stain result, though Gram staining is unreliable with mycobacteria [40]. The genus includes a number of human and animal pathogens, including the well-known intracellular pathogens *M. tuberculosis*, *M. leprae* and *Map*, responsible for human tuberculosis, human leprosy, and ruminant paratuberculosis,
respectively. Despite classification as an Actinobacteria, physiologically mycobacteria are not strictly Gram-positive organisms, due to their very complex and unique cell wall structure [41]. In addition to a peptidoglycan layer surrounding the cytoplasmic membrane, the mycobacterial cell wall consists of a polysaccharide layer (arabinogalactan), a thick mycolic acid coat, an outer lipid layer, and cytoplasmic membrane bound lipoarabinomannans (LAMs) and phosphatidylinositol mannosides (PIMs) [42]. This thick cell wall makes mycobacteria extremely resistant to chemical and enzymatic lysis, as well as adverse environmental conditions [41, 43].

*Map* is an obligate intracellular pathogen of ruminant macrophages; *Map* has a prolonged doubling time of approximately 24 hours [44]. A characteristic trait of *Map* is the dependence on the iron-chelating agent mycobactin for growth in vitro. Iron-chelating agents are required for uptake of otherwise insoluble environmental iron [45]. The dependence on mycobactin is likely evidence of the adaptation to survival by *Map* in the intracellular environment, where iron can be scavenged from the host, negating the need for iron chelating agents. Historically, mycobactin dependence was used as the sole method of differentiation of *Map*, however with the advent of molecular biology genetic characterization became a far more reliable and accurate method of speciation [46].

Originally described in taxonomy as *M. paratuberculosis*, DNA-DNA hybridization showed a high degree of homology to *M. avium* and it was thus renamed to *Map* [46]. Now, *Map* belongs to the taxonomic group *Mycobacterium avium* complex (MAC), comprised of obligate and opportunistic pathogens of animals and humans, as well as common non-pathogenic environmental organisms [1, 47].
Culture of *Map* is difficult and time consuming, due to the complex nutritional requirements of the organism and the prolonged doubling time. Numerous solid and liquid media culture preparations have been described, one of the oldest being Herrold’s egg yolk medium (HEYM) supplemented with mycobactin J [1]. HEYM is a highly nutrient-rich medium, shown to yield excellent results in culturing nearly all strains of *Map*. Commercial medias, such as Middlebrook 7H11 for solid culture and Middlebrook 7H9 for liquid culture, are available for mycobacterial culture, though these media require additional nutritional supplementation for growth of *Map*. A nutrient cocktail consisting of albumin, oleic acid, dextrose, and catalase (OADC) is commonly used with mycobactin J to enhance *Map* growth in vitro [48, 49]. Antibiotic and antifungal cocktails may be added to reduce the risk of any contaminant growth during the prolonged incubation time. On 7H11 solid media, *Map* colonies are described as approximately 1-2 mm in diameter, circular, with a shiny white appearance [48, 50]. In 7H9 liquid media, *Map* forms large clumps of bacterial cells and appears as a fungi-like film along the surface of the media [40]. The detergent tween 80 is frequently added to prevent bacterial cell clumping; bacterial growth can be estimated by spectrophotometry and comparison to a growth curve [51].

*Map* strains were first characterized as either S-type or C-type based upon their host range: S-type *Map* strains were initially cultured from sheep, whereas C-type *Map* strains were cultured from cattle [1]. However as molecular techniques became more widely utilized, restriction fragment length polymorphism analysis (RFLP) of the IS900 element, discussed below, in the *Map* genome has allowed for division of the *Map* strains into three distinct clusters: C-type was reclassified as Type 2, whereas S-type was divided
into Type 1 and Type 3 [52]. Type 2 Map strains are substantially easier to culture than their counterparts and have a wider host range, having been isolated from cattle, goats, humans, and both ruminant and non-ruminant wildlife species. Types 1 and 3 are more difficult to isolate and maintain in culture and tend to have a narrower host range primarily infecting sheep and goats, though they may also be able to infect cattle [1, 52, 53].

1.2.2 Microbial genetics

Map K-10, a C-type strain isolated from a clinically-affected American dairy herd in 1990, was the first Map genome to be fully sequenced. The 4.8 million base pair genome contains more than 4500 open reading frames (ORFs) and has a G+C content of 69% [54]. Sequencing of another Map C-type genome, isolated from human breast milk of a Crohn’s disease patient, showed high similarity to Map K-10, whereas an S-type genome showed numerous major polymorphisms in the genome compared to Map K-10 [55].

With more genomic information available in recent years, researchers have been able to run in-depth analysis of Map virulence factors in order to gain a better understanding of the microbial processes which contribute to Map infection and JD. A key characteristic of the Map genome is the presence of a number of insertion sequences (IS) [54]. These insertion sequences are very short transposable elements, often spread from bacterium to bacterium or acquired directly from the environment. While generally non-coding sequences, ISs enter the host bacterium’s genome and cause genomic rearrangement. This is thought to play a major role in bacterial evolution through silencing or altering gene expression, with lethal mutations causing death of the bacterium prior to the next round of reproduction [56]. The Map genome has between 50 and 60 unique
insertion sequences depending on the strain. One of the best characterized is IS900, which is a multi-copy insertion sequence described in Map. The exact number of copies varies from strain to strain but has been estimated to range from 14-18 copies per bacterium [57]. IS900 has long been used for Map diagnostics and RFLP strain typing, as discussed above because IS900 was long regarded as a Map-specific genetic element. However, since its discovery there have been several instances of highly similar (termed ‘IS900-like’) elements identified in non-Map mycobacteria. While not 100% identical, they share a high level of homology and bring into question the validity of IS900 as a reliable Map identification tool [58, 59].

Analysis of the various Map genomes has revealed many other Map-specific genes which have been considered as ancillary tools for definitive identification of Map. One of these known as F57, is seen as a preferred choice as a diagnostic replacement for IS900. While the exact function of F57 remains unknown, it is a single copy gene which to date has no similar homology in any other bacterial species [60].

Many pathogens are known to alter their gene regulation within the host in order to maintain genetic fitness under altered nutritional availability; genes which help ensure survival within a host are up-regulated, whereas genes needed for survival outside of the host are down-regulated. Map gene expression profiles have been monitored during a transition from growth in bacterial culture to survival within macrophages. The first major change was an up-regulation in the genes required for cholesterol metabolism [61]. During macrophage infection, Map localizes to cholesterol-rich compartments within macrophages, and is unable to survive within cholesterol-poor environments [62]. This is
consistent with other pathogenic mycobacteria, which appear to preferentially utilize lipids as a carbon source instead of carbohydrates [63]. Sheep and cattle have been found to have increased serum cholesterol levels as a result of *Map* exposure [64]. Additionally, several *Map* genes showing homology to other bacterial stress response genes, such as *sodA* which encodes superoxide dismutase, were up-regulated in response to survival within a host environment. These stress response genes may play a significant role in the pathogenesis of *Map* by improving survival to reactive oxygen and nitrogen species produced by the host [61].

1.3 Pathogenesis

1.3.1 Establishing infection

The most important route of infection typically occurs in calves through ingestion of *Map* shed by an infected animal in contaminated colostrum/milk or feces. Upon ingestion, the thick cell wall allows ready passage of viable *Map* through the digestive tract, where it can invade the intestinal mucosa. The distal ileum has traditionally been hypothesized as the primary site of infection; however, experimental models have demonstrated that *Map* infection may also occur through invasion of the jejunum and the tonsillar crypts [65, 66]. Intestinal invasion is thought to be facilitated primarily via M cells overlying dome regions of the Peyer’s patches, where *Map* is eventually phagocytosed by resident macrophages within the intestinal tissues before becoming established in the lamina propria of the intestine [3].

Pathogens are eliminated within a macrophage by lysosomal digestion via fusion of the nascent phagosome with a series of lysozyme-containing endosomes. Briefly, the
nascent phagosome is sequentially coated with Ras-associated GTP-binding proteins (Rab) such as Rab-4 and Rab-7, essential for early and late endosome recruitment respectively. *Map*-containing phagosomes block late endosome recruitment, despite Rab-7 localization, through retention of the phagosomal membrane protein coronin-1 (TACO). The current hypothesis for this retention of TACO is a cholesterol-dependent interaction with the mycobacterial protein lipoamide dehydrogenase C (LpdC) [62, 67, 68]. A secondary mechanism for blocked endosome fusion is inhibited recruitment of Rab-interacting lysosomal protein (RILP), an effector protein essential for recruitment and fusion of endosomes [62]. *Map* is thus able to survive and replicate within cholesterol-rich phagosomes by blocking late endosome fusion, though it is readily eliminated from cholesterol-poor phagosomes [62, 68].

IFN-γ plays a major role in activation or priming of macrophages, to increase killing of intracellular pathogens. Cell signaling within the macrophage via the JAK-STAT signaling pathway has numerous downstream immune effects including increased production of reactive oxygen and nitrogen species which further contribute to enhanced ability of macrophages to kill the invading pathogen [69, 70]. In contrast, *Map*-infected macrophages have been shown by some work to be less responsive to IFN-γ, and this occurs by an increased expression of suppressor of cytokine signalling (SOCS) type 1 and 3, which blocks the JAK-STAT signaling pathway, or through decreased expression of surface IFN-γ receptors on the macrophage cell surface [1]. In addition, many of the stress response genes up-regulated by *Map* within the host act to mitigate the effects of
reactive oxygen and nitrogen species [61]. *Map* may be thus able to survive and replicate within host macrophages despite a normal IFN-γ response.

**1.3.2 Progression of disease**

JD can be subdivided broadly into a phase where the host is apparently able to control the *Map* infection (subclinical disease) which precedes the phase where the host is apparently unable to control the *Map* infection (clinical disease). The transition between these phases is thought to be driven by the host immune response, and *Map*’s ability to survive and replicate within host macrophages. Clinical disease is associated with a strong antibody-mediated immune response [12, 71]. A predominant T-helper 1 (Th1) type immune response driving cell-mediated immunity is seen during early infection and subclinical stages of JD. The Th1 type immune response is characterized by macrophages secreting pro-inflammatory cytokines TNF-α, IL-1β, and IL-8 to recruit and activate monocytes and other cells of the innate immune response to the site of infection [72, 73]. IL-12 is secreted to induce differentiation of naïve T cells into Th1 cells and stimulates production of high levels of TNF-α and IFN-γ [74–76]. Activated macrophages also secrete IL-4, a moderately anti-inflammatory cytokine which likely acts to maintain control over the escalating inflammatory response and limit further unnecessary tissue damage [77]. Recruitment of additional macrophages, T lymphocytes, and other associated immune cells results in chronic granulomatous inflammation at the site of *Map* infection, and this likely represents the hosts’ attempt to contain the pathogen [78].

Death of infected macrophages release *Map* into the extracellular space and surrounding tissue. This represents either an opportunity for *Map* passage into the gut
lumen and shedding into the environment, or these Map are phagocytosed by other macrophages within the tissue. A complex balance of the Th1 and Th2 immune responses are thought to take place to control spread of disease [77, 79]. Increasing levels of localized IFN-γ prime uninfected macrophages responding to sites of intestinal inflammation which lead to increased resistance to infection by Map, because the macrophages have increased killing capacity of phagocytosed Map [80]. However, an additional response to bacterial killing is a release of IL-10 by the macrophage. IL-10 is considered a T-helper 2 (Th2) type anti-inflammatory cytokine, and generally acts to control local inflammation and reduce tissue damage. It is also able to potently decrease IFN-γ production and response. Purified mannosylated lipoarabinomannan (Man-LAM) from the Map cell wall has been shown to transiently increase macrophage expression of IL-10. Man-LAM was also shown to decrease macrophage killing of Map through MAPK p38 inhibition, independently of IL-10 [81]. Overall, the careful balance of IL-10 and IL-12 may sufficiently decrease localized macrophage priming, which in effect increases local macrophage susceptibility to Map infection, and may thus contribute to progression of the infection through the tissue.

Traditionally, the development of clinical disease has been associated with a presumed switch from a Th1 dominant immune response in subclinical disease to a Th2 dominant immune response [72, 82, 83]. In recent years, this dogma has been questioned with researchers finding evidence that only a subset of animals seem to display the classical Th1-Th2 switch [71]. It is still widely held that Th2 anti-inflammatory cytokines, IL-10 and IL-4 may impede host control over Map. The resulting disease progression
involves development of severe granulomatous inflammation and thickening of the gut wall, culminating in severe malabsorptive diarrhea with extracellular Map shed in large numbers in the feces [3]. Several studies have employed computational modelling to examine the immune cell dynamics during the progression from subclinical to clinical disease. A recent publication has proposed that increasing numbers of bacteria within sites of inflammation in the gut leads to altered differentiation of Th cells due to increased antigen load, and subsequent competition for antigen between the Th1 and Th2 response [79]. Increased Map within the tissue would correlate with increased successful killing of Map by macrophages and other immune cells, leading to spikes in IL-10 production. With disease progression, this increasingly dominant IL-10 cytokine levels may be sufficient to induce a shift to a largely Th2-mediated immune response and initiate progression into clinical disease. This hypothesis is supported by data showing that increased Map dosage at time of infection reduces time spent in subclinical disease [18, 21].

As discussed above, not all animals exposed to Map develop disease. There is increasing evidence that some animals may display resilience to or recovery from Map infection, with these terms described below [15, 16, 21]. While the mechanism behind this phenomenon remains unknown, there has been a significant amount of research investigating the early clearance of other mycobacterial infections, particularly Mycobacterium tuberculosis (Mtb). Verrall et al. discuss an incident in Lübeck, Germany in 1929 where 252 human infants were accidentally inoculated with a virulent strain of Mtb in place of the intended BCG vaccine. In this disaster, a third of the exposed infants died, however the remainder developed only mild illness or showed no reaction at all.
This incident serves to demonstrate how even with identical exposure, there is a high degree of variation among the host responses [84]. Advocates of early clearance of *Mtb* suggest that effective killing of the pathogen by resident tissue macrophages before recruitment of monocytes and Th1 cells to the site of infection is a key step to escaping a latent *Mtb* infection [84]. Meanwhile, computer modelling for *Map* has shown that with heightened Th1 responses and minimal Th2 responses, the pathogen was readily cleared from the host. However, during the more traditional Th1-Th2 balanced scenario, persistent infection was predicted. An explanation for this, therefore, may be that an poor Th2 response, or dominant Th1 response, during early *Map* infection may in fact allow for clearance of the disease [79].

### 1.3.3 Antibody responses

Antibody-mediated (humoral) immune responses are initiated by phagocytosis of an antigen followed by antigen processing and presentation by a professional antigen presenting cell (APC), such as macrophages and dendritic cells. The processed antigen is presented on MHC-II molecules on the cell surface, and subsequently interacts with Th lymphocytes (CD4+) and B cells [85]. CD4+ Th lymphocytes can recognize and respond to these antigens, without prior host exposure, due to a process of clonal selection during development and maturation in the thymus. In a similar fashion, B cells undergo clonal selection during maturation in the bone marrow, and express IgM on their cell surface capable of specific recognition of conformational, or native, antigen, without prior host exposure [86, 87].
These general components for development of *Map*-specific humoral immune responses are present during *Map* infections. The predominating systemic antibody-mediated immune response isn’t seen until much later however, during the subclinical and early clinical stages of disease, where high levels of *Map*-specific IgG antibodies are observed in the sera of infected animals [3]. Several reports have documented the existence of IgG antibodies against purified *Map* proteins in animals as early as 70 days post oral *Map* challenge [88–90]. These antibody responses showed definitive response to the purified proteins in *Map* infected animals, but not in uninfected or *M. bovis* infected animals. The proteins had varying degrees of reactivity, the strongest being surface-associated antigens, and clearly demonstrate the existence of a *Map*-specific antibody response early in subclinical disease [88, 89]. Additionally, antigens secreted by *Map* have been used as additional serological biomarkers for early antibody responses in subclinical *Map* infected animals [91].

In cattle, IgG1 antibodies are associated with a Th2 immune response; whereas IgG2 antibodies are associated with a Th1 response. Research has shown that high concentrations of IFN-γ act to induce production of IgG2 and IgM antibodies, while IL-4 induces production of IgG1 [92]. With this concept in mind, researchers have hypothesized that in early infection and subclinical disease, a preference for IgG2 and IgM antibodies may be responsible for poor detection on commercial IgG1 immunoassays. In support of this, a study by Koets et al. (2001) found that in naturally *Map* infected animals, asymptomatic shedders showed a clear IgG2 antibody response dominance, while clinical animals exhibited the classical IgG1 dominance [19]. In another
study, researchers challenged animals with an oral dosage of \textit{Map} and observed the development of IgG1, IgG2, and IgM serum antibodies by flow cytometry. They detected little IgG1 or total IgG until 44 to 52 weeks post challenge, and these levels were significantly below the limits of detection of commercial immunoassays. While they observed little evidence of IgG2 or IgM antibody production, their assay also failed to detect any evidence of early antibody responses in the first few months post infection. They concluded that the absence of IgG2 and IgM antibodies does not necessarily reflect a lack of antibody production, but that these antibody subclasses may only be produced in levels below the limits of detection of their immunoassay [19].

Collectively considering the mechanisms driving development of early cellular and antibody-mediated responses during early \textit{Map} infection, it is evident that a portion of phagocytosed \textit{Map} is effectively eliminated by macrophages. During \textit{in vitro} macrophage infection studies, \textit{Map} numbers begin to significantly decrease after 24 hours, suggesting that macrophages are capable of efficiently killing \textit{Map} early [93]. In the host, as discussed above, high levels of IFN-γ or cholesterol-poor phagosomes contribute to rapid clearing of phagocytosed \textit{Map}. However, \textit{in vivo} work has found that \textit{Map} infection preferentially induced local mucosal antibody responses, instead of strong systemic antibody responses [65]. Furthermore, \textit{in vitro} studies have also shown that \textit{Map} infection is associated with reduced levels of MHC-I and MHC-II expression in the macrophage within the first 24 hours of macrophage infection. Furthermore, heat-killed \textit{Map} alone was capable of decreasing MHC expression, a result of cell-wall LAMs acting on the macrophage. These factors act to decrease the ability of macrophages to present
processed antigen and activate T-cells and may contribute to the weak early systemic Map-specific antibody responses in cattle, despite effective Map killing and antigen processing [94].

1.4 Diagnostic techniques

Effective diagnostic techniques are a crucial requirement in any JD surveillance program in order to accurately identify and remove infected animals from a herd, before they can spread the disease or contaminate the environment. As has been previously mentioned, a major challenge in JD diagnostics is the lengthy subclinical phase of disease, where intermittent, low level fecal Map shedding from the host may facilitate spread within the herd yet there are no clinical signs of disease. The major techniques used for JD diagnostics can be divided into four categories: 1) bacterial culture, 2) molecular detection, 3) serology, and 4) histology. Sensitivity, the ability to accurately identify positive samples, and specificity, the identification of negative samples as negative, are two key factors used to determine the optimal method, however cost and time are also important factors to consider.

1.4.1 Bacterial culture

Bacterial culture has long been considered to be the “gold standard” in JD diagnostics, and is used as the standard reference test by diagnostic laboratory and accreditation boards around the globe [48]. Culture can be performed on feces, colostrum/milk, or tissue samples. Standard Map culture techniques describe under section 1.2.1 require the addition of several decontamination steps in order to decrease the risk of contamination from fungi or other bacteria present in the sample. The most
commonly used decontamination method consists of a 24-hour incubation in ½ Brain-Heart Infusion broth supplemented with 0.9% hexadecylpyridinium chloride, a cationic quaternary ammonia compound with bactericidal action through disruption of the bacterial cell wall of contaminating organisms. Samples are then mixed with a cocktail of antifungals and antibiotics (common ones include amphotericin B, naladixic acid, polymixin B, and vancomycin) in order to further reduce the risk of background microbial contamination, and grown using mycobacterial media supplemented as previously described [95]. Automated Map culture systems, such as the BACTEC culture system, are frequently employed to simplify the workflow required to culture the organism, particularly in diagnostic laboratories, and function by measuring radiometric (BACTEC 460) or fluorometric (BACTEC 960) changes in their specialized media as an indicator of Map growth. Positive cultures, either through manual culture or with the BACTEC system, are confirmed by PCR for detection of the Map-specific IS900 element, as described below [96, 97].

A main benefit of bacterial culture and the reason why it is still widely regarded as a preferred diagnostic technique is that, unlike other diagnostic tests, culture detects only viable Map. The relevance is that animals which are fecal culture positive are actively able to spread viable Map and infect other members of the herd [98]. The most obvious disadvantage of culture is that eight weeks or more is needed in order to accurately report a sample as negative. An additional disadvantage of culture in the case of the BACTEC system is the high cost. The automated detection systems cost approximately $20 per sample in consumables and reagents, not including the cost of the machine and labour.
Individual cow samples will often be pooled in groups of four or five to reduce culture cost; however this results in significantly decreased sensitivity [48]. Furthermore, animals ingesting Map-contaminated feces may passively shed live bacteria in their feces without any true infection. This phenomenon may account for a higher reported incidence of fecal Map shedding on farms endemic with JD, and as a result the culling of healthy animals [21].

An additional concern is the presence of viable but not cultivatable (VBNC) Map, bacterial cells in a spore-like persister cell state in response to environmental stress. While still viable and able to return to an actively growing state when conditions are more favourable for growth, these Map take considerably longer than normal Map to be grown in culture; therefore samples containing VBNC Map may be falsely reported as negative [99]. One significant contribution to a VBNC state in Map is a response to freezing; the freezing of samples prior to culture has been demonstrated to significantly reduce test sensitivity. Taking these factors together, while culture does allow for detection of actively viable Map in a sample, the increased cost and time required for positive identification of Map-containing samples, combined with low sensitivity make this test unreliable, particularly with regards to diagnosis of subclinical animals, where shedding is intermittent and often below the level of detection.

1.4.2 Molecular techniques

Molecular techniques are becoming increasingly popular in Map diagnostics because of lower costs and decreased turnaround time. These tests can be performed directly on DNA extracted from feces or milk/colostrum; therefore standard culture of
viable *Map* is not required. Molecular techniques can also be performed on tissue DNA, primarily from the ileocecal region of the intestine and associated lymph nodes [49, 100]. Several commercial kits specific for *Map* are now available for DNA extraction from tissue or feces. These kits are designed for use on automated DNA extraction systems for high-throughput laboratories; however they have been shown to be less sensitive than some of the more time-consuming manual alternatives [101].

A common theme in DNA extraction techniques is the use of several DNA diluting steps prior to purification. This serves both to reduce the levels of PCR inhibitors, present in high levels in feces and tissues, and to prevent overloading of silica column/beads because of high levels of non-specific DNA. However with a target present in low numbers, such as *Map* during subclinical Johne’s disease, the *Map* genomic DNA can be easily diluted to extinction and lead to a false negative. Furthermore, as mentioned previously the mycobacterial cell wall is extremely resistant to lysis, and often a proportion of *Map* will not lyse during the DNA extraction, limiting detection. To help improve sensitivity, researchers are investigating the use of customized pre-treatment steps to effectively decontaminate the sample and remove a large portion of non-*Map* DNA along with soluble PCR inhibitors [100, 102–104]. Coupled with a modified DNA purification process to ensure optimum *Map* lysis, these protocols have increased sensitivity even with low numbers of *Map* in a sample. However, DNA extractions fail where bacterial culture excels in discriminating for detection of viable organisms. Indeed a main argument against molecular diagnostics is there is no evidence from the results that detected *Map* shedding from an animal is truly representative of *Map* infection or JD [98].
The most widely used molecular target for detection is the insertion sequence IS900. Because it is found in multiple copies within the genome, and is presumed to be *Map*-specific, this sequence provides an excellent high sensitivity and high specificity target for detection [54]. Detection is based largely on quantitative PCR (qPCR), which has increased sensitivity over conventional PCR and allows for quantitative analysis by measuring changes in fluorescence after each round of amplification, though quantification is not possible with IS900 because there are varied IS900 copy numbers across different *Map* strains [48]. Another approach is to use a modified conventional PCR assay, such as a nested or hemi-nested PCR reaction. In this assay, the product from an initial endpoint PCR reaction is used as the template for another, amplifying a target within the previously amplified region. The end result is an assay with enhanced sensitivity, where low copy numbers or poor PCR efficiency would otherwise not allow for detection, without the need to purchase expensive qPCR equipment [105]. However, the extra steps make the reaction more prone to contamination, and the increased number of cycles may allow non-specific products to accumulate to detectable limits.

The discovery of IS900-like elements in non-*Map Mycobacterium* spp. has cast some doubt over the use of IS900 as a *Map* detection target in recent years. To address this, considerable effort has been made to redesign IS900 primers to bind regions of enhanced variability between *Map* IS900 and IS900-like regions, or identify novel targets for *Map* detection. F57 is a single copy gene of unknown function and specific to *Map*, which has been used extensively in recent years both for *Map* detection and quantification [60, 95]. Multiplex PCR, a PCR technique with multiple primer sets to amplify multiple
targets simultaneously, and this technique has been proposed using IS900, F57, and/or other *Map*-specific genes [60]. These additional *Map*-specific gene sequences help to confirm that the observed amplification of IS900 is a true positive, and not due to an IS900-like sequence. However, poor PCR efficiency and low *Map* copy numbers in the sample may lead to preferential amplification of the IS900 element only, and be reported as a false negative.

1.4.3 Serology

By far the most cost-effective method for large-scale screening of *Map*-exposed animals has been by detecting *Map*-specific antibodies in serum of hosts using enzyme-linked immunosorbent assays (ELISA). The test does not detect the pathogen itself, but rather detects *Map*-specific antibodies in the hosts’ serum, indicative of a *Map* infection and subsequent immune response [48]. Sample serum is tested against specific *Map* antigens fixed on a surface, then detected by a colourimetric reaction which in samples containing the target antibody produces a colour change, while samples lacking antibody remain clear. ELISA assays have a high test specificity, but their sensitivity can be unacceptably low, particularly in early infection and subclinical disease where few *Map*-specific antibodies are produced by infected animals [48, 106].

Commercial *Map* ELISA kits are available and utilized heavily. These tests are designed to identify *Map*-specific antibodies which, as previously mentioned, are shown to be more commonly observed in the late subclinical and clinical stages of disease [92]. As previously discussed as the “iceberg” phenomenon for projecting herd prevalence of *Map* infection, it is important to remember that a majority of animals in a herd will likely
be in a subclinical stage of disease, with only low levels of serum *Map*-specific antibody production and thus undetectable by commercial ELISAs [18, 65].

### 1.4.4 Histology

Animals presenting with clinical JD will often present with gross lesions during post mortem examination, and these lesions combined with clinical signs are highly suggestive for a tentative diagnosis of JD in an animal [3]. Fresh and frozen gut tissue and lymph node can be used for bacterial culture and molecular detection of *Map*, as described above. Hematoxylin and eosin (H&E) stained tissues are examined histologically for confirmation of the diagnosis. In *Map*-infected animals, granulomatous inflammation can be recognized histologically, even in the absence of gross lesions during subclinical and clinical stages of disease [48]. However, the discrepancy between the size of the bovine gut and the amount of tissue used for histology can in some cases greatly reduce sensitivity of this test. Except in advanced JD cases, histologic evaluation of the bovine intestine is probably best utilized as one part of a panel of tests with the goal of improving the overall ability to diagnose JD [107].

*Map*, as with all mycobacteria stain positive by Ziehl-Neelsen (acid-fast) staining [40]. The principle of this test is that after an initial stain the thick *Map* cell wall is resistant to acid de-colourization, whereas host cells and other bacterial cells are de-colourized. Tissues can be assessed for the presence of acid-fast bacilli as a confirmatory test for enteric *Map* infection. While acid-fast staining is less subjective then H&E, the same issue of tissue sampling in relationship to the size of the intestinal tract makes this test poorly sensitive on its own, particularly during subclinical disease [48].
1.5 Research objectives

The focus of this thesis was to examine three key factors or knowledge gaps regarding enteric *Map* infection in young calves. First, in chapter 2, we aimed to utilize a previously described model of enteric *Map* infection in calves to study the progression and development of *Map* infection in the intestine and draining lymph nodes over a period of 28 weeks. Our hypothesis was that the use of a high dosage of viable *Map* injected directly into the distal ileum would accelerate development and progression of disease, and allow us to examine the progression of initial enteric *Map* infection as well as the transition between subclinical and clinical disease.

In chapter 3, we sought to examine how oral monensin sodium supplementation of calves prior to *Map* infection and during early experimental *Map* intestinal infection affects establishment and progression of *Map* infection in the calf intestine. Based on the literature, our original hypothesis for this chapter was that calves supplemented with monensin would show decreased progression of *Map* infection compared with our monensin-free animals (chapter 2). We thus hypothesized that monensin-supplemented calves would have decreased lesion severity, fecal *Map* shedding, and detectable *Map*-specific serum antibodies. However, given our early data from the monensin-free calves, which identified resilience to enteric *Map* infection (ie they did not develop persistent intestinal infection, chapter 2), our hypothesis for this work was modified. The updated hypothesis for chapter 3 was that monensin-supplementation would increase resilience to intestinal *Map* infection in an experimental calf infection model.
Finally, in chapter 4, we examined the *Map*-specific antibody responses in the serum of calves following experimental enteric *Map* inoculation, using custom whole *Map* cell sonicate-based ELISA, western blotting, and protein sequencing to identify novel serum *Map*-specific antibodies. Our hypothesis for this chapter was that *Map*-specific serum antibodies are detectible in calf serum early after intestinal *Map* infection. We hypothesize that these antibodies may be useful to further our understanding of early host-pathogen interactions, and may be beneficial for future development of improved serodiagnostic techniques for enteric *Map* infection.

### 1.6 Animal Study Design

Due to limitations in the ability to acquire and house calves in this study, the overall design used was a block cohort study. A total of eight blocks of six calves each (48 calves in total) were used, with each block containing four experimentally *Map*-inoculated calves and two un-inoculated control calves. The first four groups were monensin-free, with *Map*-inoculation performed at approximately one month of age, after a one week long adjustment period for calves within the animal isolation facility. Blocks of calves were euthanized at 4 weeks post inoculation (wpi), 12 wpi, 20 wpi, and 28 wpi. The remaining four blocks underwent an identical sourcing, transportation, housing, inoculation, and euthanasia schedule; however these calves were supplemented with monensin sodium, as described below (chapter 3). Data from the monensin-free calves is discussed in chapter 2, and data from the monensin-supplemented calves (as well as comparison between these groups) is discussed in chapter 3.
Animal group sizes were based on a sample size calculation \( n = \left( \frac{Z^2 \sigma^2}{L^2} \right) \), where \( Z \) = desired confidence level, \( \sigma \) = desired variance, and \( L \) = desired precision), using estimates of variance and precision based on data generated from previous work using this model [77]. This work, and other unpublished studies, showed consistent induction of enteric *Map* infection by 12 wpi, with minimal variance between animals. As such, sample size was calculated to be four calves per block, for a 95% confidence interval, based on an estimated precision of 10%, and an anticipated variance in groups of 10%. Two control calves per block was chosen with the desire to limit unnecessary animal numbers but also in light of the requirement to house animals in pairs, based upon animal care recommendations. All animal work presented in this thesis was reviewed and approved by the University of Guelph Animal Care Committee.

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2 Resilience to infection by *Mycobacterium avium* subspecies *paratuberculosis* following direct intestinal inoculation in calves
Resilience to infection by *Mycobacterium avium* subspecies *paratuberculosis* following direct intestinal inoculation in calves

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2.1 Abstract

*Mycobacterium avium* subspecies *paratuberculosis* (*Map*) is the cause of Johne’s disease (JD), a chronic enteritis of cattle. A significant knowledge gap is how persistence of *Map* within the intestinal tract after infection contributes to progression of disease. To address this, we exposed calves to *Map* by direct ileocecal Peyer’s patch injection. Our objective was to characterize the persistence of *Map* in tissues, associated intestinal lesions, fecal *Map* shedding, and serum antibody responses, through the first 28-weeks post-inoculation (wpi). Previous work using this model showed 100% rate of *Map* infection in intestine and lymph node by 12 wpi. We hypothesized that direct inoculation of *Map* into the distal small intestine would induce intestinal *Map* infection with local persistence and progression towards clinical disease. However, our data show decreased persistence
of Map in the distal small intestine and draining lymph nodes. We identified Map in multiple sections of distal ileum and draining lymph nodes of all calves at 4 and 12 wpi, but then we observed reduced Map in distal ileum at 20 wpi, and by 28 wpi we found that 50% of animals had no detectable Map in intestine or the lymph node. This provides evidence of resilience to Map infection following direct intestinal Map inoculation. Further work examining the immune responses and host-pathogen interactions associated with this infection model are needed to help elicit the mechanisms underlying resilience to Map infection.

2.2 Introduction

Johne’s disease (JD) is a chronic progressive enteric infection of cattle caused by the bacterium Mycobacterium avium subspecies paratuberculosis (Map). Susceptible animals, typically young calves, become infected by ingesting contaminated colostrum, milk, or feces [1]. Map invades the intestinal mucosa via M cells overlying Peyer’s patches, where it is taken up by and replicates within resident macrophages. A potent inflammatory response drives recruitment of macrophages and development of granulomatous inflammation at the site of infection [2]. Infected animals then enter a lengthy subclinical period lasting up to 10 years; though these animals often show no clinical signs of infection for several years, they eventually shed Map via their feces into the environment and thus perpetuate its spread to susceptible herdmates [2, 3]. Only a small portion of infected animals eventually progress to the invariably fatal clinical disease characterized by malabsorptive diarrhea due to chronic inflammation and thickening of the intestinal mucosa. Diseased animals experience decreased milk production, reduced
reproductive efficiency, weight loss and wasting as clinical disease progresses [4]. The mechanisms underlying the early progression of intestinal *Map* infection remain underexplored and largely unknown.

It is difficult to estimate accurately the number of infected herds due to poor sensitivity of screening tests, especially in subclinical animals; however, it is estimated that in North America between 10-70% of herds have at least one *Map*-infected animal [5–7]. There is significant economic burden associated with JD of $250 million USD per year in the United States alone, due to loss of productivity and early culling, though these estimates are nearly two decades old [8]. Subclinically diseased animals shed *Map* sporadically and clinically diseased animals shed *Map* progressively; both contribute to environmental contamination and exposure of susceptible calves to contaminated milk, colostrum, and feces [7, 9, 10]. Recent experimental models of calf-to-calf transmission indicated that 50% of contact-exposed animals showed demonstrable infection within 3 months of exposure [11]. Despite routine *Map* exposure, individual animal prevalence in endemic herds ranges between only 5-20% [5, 6]. Thus, a major question exists of why in endemic herds, where susceptible calves are routinely exposed to *Map*, do so few animals ever show evidence of disease? Whittington et al. recently proposed the classification of “resilient” for susceptible animals exposed to *Map*, which show no signs of infection [12]. It remains unclear if such resilience is derived primarily from improved mucosal barrier function, enhanced early pathogen clearance, other factors, or a combination of these factors.
The purpose of this study was to examine the early post Map-infection phase within the small intestine of calves to more fully understand persistence of Map in intestine and lymph nodes, and development of localized granulomatous lesions following direct experimental inoculation. Use of direct ileal Peyer’s patch inoculation with a measured dose of Map allows for further exploration of localized intestinal Map infection in calves, an aspect of this disease that remains underexplored. The model has been shown to consistently induce enteric Map infection and is unique in its ability to reliably recapitulate the histologic lesions, patterns of fecal Map shedding, and immunologic responses consistent with natural subclinical JD [13]. Previous work utilizing this model has only examined the first 12 weeks post-experimental inoculation; thus, new work examining the progression of infection beyond 12 weeks is warranted, to help construct an improved understanding of early enteric Map infection.

This model is not intended to investigate the process of Map tissue invasion and infection, but instead attempts to recreate an established enteric infection through introduction of a measured dose of Map directly into the distal ileum. Studies using mathematical modelling for progression of JD following Map infection have shown that antigen load within the intestinal tissues plays a crucial role in driving progression from subclinical to clinical disease [14]. Therefore, our initial hypothesis was that calves directly inoculated with Map at the primary site of natural infection would develop persistent localized Map infection and then progress towards clinical disease. A more complete understanding of early progression of enteric Map infection with this model is likely to have significant impact on understanding and limiting naturally infections.
2.3 Methods

2.3.1 Bacterial strain and growth conditions

The *Map* strain gc86 was used for inoculation in this study; this is a field strain isolated from the feces of a cow with clinical JD in Ontario, Canada [15]. This *Map* strain has been utilized for a number of JD studies, both in vitro and in vivo [16–19]. Prior to each surgical inoculation, a frozen stock of *Map* gc86 was recovered into Middlebrook 7H9 broth (Becton Dickinson, Oakville, Ontario, Canada) supplemented with 10% OADC (oleic acid, albumin, dextrose, catalase; Becton Dickinson Canada), 0.05% Tween 80 (Sigma-Aldrich, Oakville, Ontario, Canada), and 2 mg/L Mycobactin J (Allied Monitor Inc, Fayette, Missouri, USA), and then grown in a stationary flask at 37 °C with 5% CO₂. Colony forming units were approximated by measuring light absorbance using spectrophotometry at 540 nm and comparison to a previously described standard curve (validated for use with gc86 by confirmation with quantitative culture) [13, 20, 21].

2.3.2 Inoculum preparation

Following quantification, the *Map* for inoculation of calves was collected by centrifugation of $6 \times 10^9$ CFU of *Map* at $1500 \times g$ for 20 min. The bacterial pellet was re-suspended in 1.5 mL of sterile phosphate-buffered saline (PBS) to a final concentration of $1 \times 10^9$ CFU per 250 µL, and briefly pulse sonicated to disperse bacterial clumps (confirmed via microscopy, and flow cytometry scatter plot). Tuberculin syringes were loaded with 250 µL (representing a total of $10^9$ CFU *Map*) of inoculum and stored at 4 °C until time of surgery (within 12 h of preparation).
Prior to inoculation, the viability of the Map contained within inoculum was determined by fluorescein diacetate (Sigma-Aldrich) staining, with analysis by flow cytometry, as previously described [18, 22]. A minimum of 90% viability of Map was confirmed prior to animal inoculation.

2.3.3 Animal trial design
Castrated male Holstein calves between 3-4 weeks old were sourced from the University of Guelph Elora dairy research and teaching farm, a farm considered Johne’s disease free based on voluntary participation in a Johne’s disease surveillance program. There have been no cases of clinical Johne’s disease for over 15 years and continuous negative on herd and individual animal level by regular milk and serum ELISA testing. Calves were transported to and housed for the duration of the experiment in an animal biosafety level 2 facility on the main campus of the University of Guelph in Guelph, Ontario. Based upon animal welfare concerns, calves were co-housed in groups of two or four animals per room, with uninfected animals housed separately in dedicated control rooms. Personnel entering the rooms donned clean coveralls, boots, surgical mask, hairnet, and gloves within the antechambers of each animal room. Re-entry into rooms housing control animals was not permitted following contact with Map-exposed animals, to eliminate the risk of cross contamination. Animals were maintained on standard non-medicated diets for the duration of the study.

A total of 24 calves were used for this experiment. Calves were randomly assigned into four groups based on pre-determined termination/euthanasia time points and calf availability. Each group contained two control and four Map-exposed calves. A one-week
period was given to all calves after arriving into the animal housing facility to allow for environmental acclimation and to ensure health prior to Map inoculation. Calves were euthanized by intravenous barbiturate overdose according to their pre-assigned groups, by time following Map inoculation: 4 wpi, 12 wpi, 20 wpi, or 28 wpi. All live animal research protocols for this study were pre-approved by the University of Guelph Institutional Animal Care Committee.

2.3.4 Surgical Map inoculation

Calves were inoculated with live Map using a surgical inoculation method previously described [13]. Briefly, a 10-centimeter skin incision was made in the right paralumbar fossa and then extended through the abdominal wall muscle and peritoneum into the abdominal cavity. The distal ileum and cecum were exteriorized, the Peyer’s patches of the distal ileum were visually identified, and then $1 \times 10^9$ CFU live Map in PBS was injected into the subserosal Peyer’s patch rich region on the anti-mesenteric surface of the distal ileum. Following Map injection, 250 µL of sterile diluted India ink was subserosally injected into the distal ileum approximately 5cm proximal to the site of Map injection in order to facilitate localization of the Map-inoculation site during tissue collection at post-mortem examination. The distal ileum was then replaced into the abdomen prior to closure of the muscle and skin incisions using standard suture pattern and technique. Calves were not administered antibiotics at any point during the study. A single therapeutic dose (0.5 mg/kg body weight, by intramuscular injection) of meloxicam was administered immediately after surgical Map-inoculation and the incision site was treated with topical povidone-iodine to reduce the risk of secondary bacterial infection at
the surgical site. No major complications were observed in any calf after surgical inoculation of *Map*. Some animals developed mild localized inflammation at the incision site in the week following surgery; these wounds were treated topically using antiseptic solution until proper healing occurred.

2.3.5 Feces and blood collection

Feces were collected per rectum prior to *Map* inoculation and bi-weekly thereafter until the end of the study. Feces were immediately stored in 15 mL cryovials at −80 °C until processing. Serum was collected into serum separator tubes via jugular venipuncture prior to *Map*-inoculation and monthly thereafter. Serum was separated from cells and stored at −80 °C until processing.

2.3.6 Euthanasia, post-mortem examination, and tissue collection

Calves were euthanized by intravenous barbiturate overdose, and a thorough post-mortem examination was performed immediately following euthanasia. Four segments of the distal small intestine including ileocecal valve (A), and three sections of ileum proximal to the ileocecal valve at 5 cm intervals (B-D), all of which contained both Peyer's patch and non-Peyer's patch regions, and the ileocecal (draining) lymph node were collected. Serial sections of each intestine and lymph node tissue samples described above were preserved in 10% neutral buffered formalin for histologic assessment, snap frozen in OCT compound, and snap frozen in cryovials for storage at −80 °C.
2.3.7 Histologic lesion scoring

After 24 h immersed in 10% neutral buffered formalin, tissues were trimmed for size, placed into cassettes, dehydrated, and embedded in paraffin. Serial sections were cut at 8 μm and then stained by routine hematoxylin and eosin (H&E) and Ziehl-Neelsen (ZN) according to standard protocols in the Animal Health Laboratory (Lab Services Division, University of Guelph). All tissue sections were visually scored for inflammation as described below, and all ZN-stained sections were scored for the presence or absence of acid-fast bacilli (AFB) by a board-certified veterinary pathologist (BLP), who was blinded to calf ID, experimental Map inoculation status and time point after Map inoculation of calves for each tissue at the time of scoring.

The scoring system for inflammation in the intestine and draining lymph node (Table 1) was modified slightly from several previously published scoring systems in ruminant JD studies [23–25]. Briefly, scores from 0-5 were assigned based on the severity and distribution of granulomatous inflammation in each tissue section individually. Scores ranged from a minimum of 0 (no lesions, histologically normal) to a maximum of 5 (severe coalescing to diffuse granulomatous inflammation in the intestinal mucosa or lymph node parenchyma and extending into the submucosa of the small intestine or into afferent or efferent lymphatics of the node). Representative images illustrating the scoring system are shown in Figure 1. Given the range of inflammatory cells in the intestine of normal calves and for the purpose of this study, lesion scores of 0 were considered within normal limits. Scores of 1 were defined as mild lesions, scores of 2-3 were defined as moderate lesions, and scores of 4-5 were defined as severe lesions.
2.3.8 Tissue DNA extraction

DNA was extracted from all intestinal and lymph node tissue samples (five samples per calf, tissues A-E as described above) using the DNEasy Blood and Tissue Kit (Qiagen, Toronto, Ontario, Canada), with several modifications. Briefly, 100 mg of frozen tissue per site, compared to 20 mg standard in commercial tissue DNA extraction kits, was digested in 360 μL of buffer ATL and 40 μL of proteinase K overnight at 56 °C with mixing at 1000 RPM on a Thermomixer (Eppendorf Canada, Mississauga, Ontario, Canada). Cell lysis was performed in 400 μL of buffer AL at 95 °C for 5 min. DNA was precipitated with 400 μL of ice cold 100% ethanol at −20 °C for 5 min. The entire sample volume was then loaded onto spin columns in two stages (600 μL at a time), which was followed by two wash steps (each) with buffers AW1 and AW2. DNA was eluted with 50 μL of buffer AE, and stored at −80 °C for downstream processing. Every tissue section which tested negative by PCR (described below), was re-extracted and re-processed, to maximize sensitivity of the diagnostic assay.

2.3.9 Fecal DNA extraction

Fecal samples for DNA extraction were first subjected to a pre-treatment technique modified from the Mississippi Veterinary Research and Diagnostic Laboratory, with the goal of reducing normal fecal microbiota and increased sensitivity for detection of Map [26]. Briefly, 1g of frozen feces was thawed and diluted in 17.5 mL of pre-lysis buffer (30 mM Tris-HCL pH 8, 0.02N NaOH, 0.1% SDS; final pH 9.65), vortexed for 30 s, and allowed to settle for 30 min. The top 15 mL of supernatant was collected and transferred to a new tube, then pelleted by centrifugation at 1500 × g for 20 min. This pellet obtained
after pre-treatment was used as the starting point for the DNA extraction using the Stool DNA isolation kit (Norgen Biotek, Thorold, Ontario, Canada). Briefly, DNA was extracted according to the manufacturer’s recommendation, with inclusion of an optional 10-min incubation at 65 °C following pellet resuspension in Lysis Buffer L and Lysis Additive A, prior to bead beating at 30 hertz for 1 min (TissueLyser II, Qiagen, Toronto, Ontario, Canada).

2.3.10 Map PCR

Map was detected by PCR using a two-step hemi-nested PCR reaction for the Map IS900 element. Accustart PCR Toughmix II (Quanta Biosciences, Beverly, Massachusetts, USA) was used as the PCR master mix, with the addition of 2.5 μg of molecular grade bovine serum albumin. Both rounds of PCR utilized the same cycling conditions, consisting of a 5-min initial denaturation step at 95 °C followed by 35 cycles of: 30 s at 95 °C, 30 s at 68 °C, and 60 s at 72 °C. This was followed by a final 5-min extension at 72 °C. Products were then loaded onto a 1% agarose gel in a Tris-EDTA-Acetic acid buffer, and stained using ethidium bromide. The first round of PCR utilized the forward primer IS900.F1 (5’- CTTGAGTTGATTGCGGCGG-3’), and the conserved reverse primer IS900.R (5’TGGTTGCGGGGTGGTAGAC-3’) for a 1064 base pair product. The second round of PCR utilized the forward primer IS900.F2 (5’-GATGCGCCACGACTTGCA-3’) and IS900.R, for an 839 base pair product.

2.3.11 Validation of molecular assays

PCR primers were designed based on sequence alignment of IS900 and non-Map IS900-like elements, available on NCBI GenBank, and targeted to regions conserved in
Map sequences, but not in non-Map sequences. Primers were also selected with a high annealing temperature (68 °C), to reduce the risk of non-specific priming. Together, these factors help reduce the risk of low specificity often attributed to nested PCR methods. The primers were validated against Map gc86 using a serial dilution of DNA, with repeatable detection down to 0.025 pg/5 µL, and occasional detection down to 0.0025 pg/5 µL (approximately 5 and 0.5 genomic equivalents respectively, based upon 4 830 000 base pairs). The primers were tested against Map K10, and Mycobacterium smegmatis, with positive detection of K10 and no detection of M. smegmatis. Agarose gel analysis showed one or two bands (two bands present in higher DNA concentrations only), which Sanger sequencing identified as first and second round PCR products. As a comparison, the same serial dilution was run on the Vet Alert commercial Map qPCR assay (Tetracore, Rockville, Maryland, USA), with reliable detection to the same lower limit of 5 genomic equivalents.

To further evaluate the PCR assay, and to validate the tissue DNA extraction, DNA was extracted from two segments of intestinal tissue (ileum) and one segment of lymph node taken from a cow diagnosed with clinical JD (based upon clinical presentation, histopathologic lesions). The hemi-nested PCR assay, combined with the tissue DNA extraction method described here, was able to detect Map in these tissues down to a 1/100 000 dilution, while the same dilution series using the Tetracore qPCR kit showed detection only to 1/10 000.
Lastly, fecal DNA extraction was validated by spiking feces with a serial dilution of *Map* gc86, with repeatable detection to a dilution of approximately 5 CFU/g (CFU approximate, determined by spectrophotometry).

### 2.3.12 Serum ELISA

*Map*-specific serum antibodies were measured in individual calf sera collected just prior to euthanasia, using a commercially-available *Map* antibody ELISA assay (IDEXX Canada, Markham, Ontario, Canada) at the Animal Health Laboratory, University of Guelph. The IDEXX *Map* antibody ELISA test determines seropositivity of individual samples based on the sample: positive control absorbance ratio (S/P). Samples with an S/P ratio below 0.45 were considered negative, while an S/P ratio above 0.55 was considered positive. An S/P ratio between 0.45 and 0.55 was considered suspect, according to the assay’s guidelines.

### 2.3.13 Animal infection status

Animals were defined as exposed, infected, diseased, or resilient based upon clinical case definitions described by Whittington et al. [12]. All animals that underwent surgical *Map* inoculation were considered *Map*-exposed. Calves with positive detection of *Map* in tissues either by PCR or by ZN staining, were considered *Map* infected. Animals with *Map*-induced histologic lesions in ileum and/or lymph nodes were considered diseased. Finally, *Map*-exposed animals but with no *Map* detected in tissues at the time of euthanasia, were considered resilient to *Map* infection. Fecal shedding and presence of *Map*-specific serum antibodies were not included in defining animal infection status, due to the possibility of transient fecal shedding in either unexposed or resilient uninfected
animals, or development of antibody responses in resilient animals, as demonstrated in a recent sheep infection study [27].

2.3.14 Statistical inferences

Statistical significance was calculated using R Studio, by generalized linear mixed effect regression modelling using the lme4 package’s “glmer” function. Modelling was designed to examine number of PCR positive tissues by time-point (logistic regression), number of ZN positive tissues by time-point (logistic regression), and mean histologic lesion scores by time point (poisson distribution). Animal ID and tissue ID were included as nested random effects, and time-point as a fixed effect with slope and intercept dependent on animal ID and tissue ID. The general formula for this regression was: FACTOR ~ time-point + (0 + time-point|animal/tissue), where FACTOR represents PCR data, ZN data, or histologic lesion scores [28]. Significance was reported as odds ratios with a significance level of α = 0.05. Marginal effects plots were generated using the sjPlot’s “plot_model” function for models which reported significant odd’s ratios.

2.4 Results

2.4.1 Gross lesions

At post-mortem examination, the location of initial *Map* inoculation site in each calf was confirmed visually based on the presence of India ink. Ink was observed in Peyer’s patches region of the distal ileum 5-8 cm proximal to the ileocecal valve, and frequently in the draining ileocecal lymph node of *Map*-exposed calves. Ileocecal lymph node enlargement was observed in some *Map*-exposed calves; however, lymph node size was variable between calves and not significantly different between *Map*-exposed and control
calves. Some calves had fibrous adhesions between the serosal surface of the distal ileum and the omentum or rarely the parietal peritoneum; however, the small intestinal mucosa in all calves was grossly normal and significant gross lesions attributable to \textit{Map} infection were not observed in any animal.

\textbf{2.4.2 Histologic lesions and scoring}

Histologic lesions, lesion scores, and the presence of AFB in intestinal and lymph node segments, respectively, are shown in Figures 1 and 2 with a data summary in Table 2. No calves in control groups (not \textit{Map}-exposed) had evidence of granulomatous inflammation or AFB in any tissues at any time-point in this study. Within the small intestine, granulomatous inflammation attributed to \textit{Map} and AFB were observed predominantly within the lamina propria. The maximum score for granulomatous inflammation in any intestine or lymph node tissue segment in this study was 3.

Experimentally \textit{Map}-inoculated calves in the 4 wpi group had mild \((n = 3)\) or moderate \((n = 2)\) granulomatous inflammation in 25\% (5/20) of all tissue segments. The maximum lesion score observed was 2 \((n = 2)\) with a mean lesion score of 0.35 (95\% CI of 0.05–0.64). Acid fast bacteria were directly observed following ZN staining in 15\% (3/20) of tissue segments.

At 12 wpi, granulomatous inflammation was observed in 55\% (11/20) of all tissue segments from \textit{Map}-exposed animals, with eight mild lesions, and three moderate lesions. The maximum lesion score was 3 \((n = 3)\), with a mean lesion score of 0.85 (95\%
CI of 0.39–1.31). Acid fast bacteria were directly observed following ZN staining in 25% (5/20) of tissue segments.

At 20 wpi, granulomatous inflammation was observed in 35% (7/20) of all tissue segments from Map-exposed animals, with five mild lesions, and two moderate lesions. The maximum lesion score was 2 ($n = 2$), with a mean lesion score of 0.45 (95% CI 0.15–0.75). Acid fast bacteria were directly observed following ZN staining in 15% (3/20) of tissue segments.

Finally, at 28 wpi, granulomatous inflammation was observed in 40% (8/20) of all tissue segments from Map-exposed calves, with seven mild lesions, and one moderate lesion. The maximum lesion score was 3.0 ($n = 1$), with a mean lesion score of 0.5 (95% CI 0.17–0.83). Acid fast bacteria were not directly observed following ZN staining in any of the tissue segments from animals in this time group.

Statistical analysis of tissue inflammation scores and the number of tissues with observed AFB following ZN staining were not significantly different between time-points. Tissue inflammation scores had an odds ratio of 1.22 (95% CI 0.78–1.91, $p = 0.386$), while ZN positive tissues had an odds ratio of 0.61 (95% CI 0.33–1.14, $p = 0.120$).

### 2.4.3 Tissue PCR

Map specific tissue PCR data are shown in Figure 2. At all time-points in this study, no intestine or lymph node tissue segments from control (not Map-exposed) calves tested positive for Map by PCR.
At 4 wpi, *Map*-exposed animals were positive for *Map* by PCR in 65% (13/20) of intestine and lymph node tissue segments. All calves in the 4 wpi group tested positive for *Map* by PCR in two or more intestinal or lymph node tissue segments. At 12 wpi, *Map*-exposed calves were positive for *Map* by PCR in 50% (10/20) of tissue segments. All *Map*-exposed calves at 12 wpi tested positive for *Map* by PCR in two or more sections of intestine or lymph node. At 20 wpi, *Map*-exposed calves were positive for *Map* by PCR in 25% (5/20) of tissue segments. All calves at this time point tested positive for *Map* by PCR in at least one tissue, and only one animal tested positive in two tissues. Finally, for calves euthanized at 28 wpi, 20% (4/20) of all tissue segments were positive for *Map* by PCR. Only two calves in the 28 wpi group tested positive for *Map* by PCR in at least one tissue segment: one calf tested positive for *Map* by PCR in a single segment of small intestine, and the other calf was positive for *Map* by PCR in two segments of small intestine and the draining ileocecal lymph node. The remaining two *Map*-exposed animals in the 28 wpi group were negative in all tissue segments tested, despite repeated extraction and processing of DNA from all segments of tissue negative for *Map* by PCR, as described above.

Analysis of *Map* positive tissue segments at each time point identified statistically significant differences between study groups. The logistic model calculated an odds ratio of 0.36 (95% CI 0.18-0.70, *p* = 0.003), suggesting a significant protective effect of time on the probability of *Map* positive tissue segments by PCR (Figure 3).
2.4.4 Fecal *Map* PCR

Bi-weekly fecal *Map* PCR data for all animals are shown by time point in Figure 4. Shedding of *Map* in the feces of control calves (not *Map*-exposed) was not detected in this study. *Map* shedding in the feces of *Map*-exposed calves was detected only sporadically throughout this study. As shown in Figure 4A, the 4 wpi group had no detected instances of *Map* shedding in the feces of *Map*-inoculated calves. In the 12 and 20 wpi groups (Figures 4B and 4C, respectively), *Map* shedding in the feces was detected in 50% (2/4) of the *Map*-exposed animals, but only at two individual time points; intermittent fecal shedding of *Map* in the feces was not detected in these groups. At 28 wpi (Figure 4D), shedding of *Map* in the feces was detected in 75% (3/4) of the animals, and each individual calf shed *Map* in the feces on multiple occasions. In one animal (calf ID 1073), intermittent shedding of *Map* in the feces was detected.

2.4.5 Serum ELISA

ELISA detecting *Map*-specific serum antibodies at the time of euthanasia in *Map*-exposed calves is shown as S/P ratios in Table 3. Briefly, sera from all calves were negative by ELISA for *Map*-specific antibodies at the termination points of the study, except for a single calf from the 28 wpi group with an S/P ratio of 0.461. This calf was classified as suspect based on the manufacturers recommendation. Control animals all had no detectable *Map*-specific serum antibodies.

2.4.6 Animal infection status

In the 4, 12, and 20 wpi groups, all *Map*-exposed calves were confirmed to have *Map* infection, based on detection of *Map* by PCR and/or ZN staining in at least one tissue
segment. All but one of these calves (1131, 20 wpi) were confirmed to be Map infected based on PCR findings in multiple tissues, or PCR findings alongside visual detection of Map by ZN staining. However, at 28 wpi, two calves (1076 and 1082) tested negative for Map in all tissue segments by both PCR and ZN staining and are thus classified as resilient. One of these calves (1082) had mild sterile granulomatous inflammation in three tissue segments, while the other calf (1076) lacked histologic evidence for Map-induced inflammation in all tissues.

2.5 Discussion

It is widely assumed that on endemic Map-infected farms the Map exposure rate is high, yet only a small number of exposed animals ever show evidence of infection or disease, though experimental evidence has documented a high rate of transmission between exposed and susceptible animals [5, 6, 11]. The question of why Map persists and progresses in some individual animals while others do not remains unanswered. The purpose of this study was to examine persistence of Map within tissues of calves following direct experimental intestinal inoculation. Based on our previous work, we hypothesized that injection of a large measured dose of Map directly into the Peyer’s patches of the distal ileum would induce localized and persistent intestinal Map infection, similar to naturally occurring subclinical JD. We also hypothesized that given the route of Map exposure the model could be useful for studying the progression of subclinical to clinical JD.

Map-exposed animals in this study were examined for evidence of localized Map persistence within the tissue using multiple diagnostic tests that are frequently employed
for diagnosis of naturally-occurring JD in the field. These data were then used to estimate *Map* persistence, based on both direct (detection of the pathogen by PCR and ZN staining in intestine and draining lymph nodes collected during post-mortem examination) and indirect (patterns of fecal *Map* shedding, granulomatous enteritis, and serological responses) evidence. A major finding in this model was that in the 28 wpi group, 50% (2/4) of the *Map*-exposed animals had no detectable *Map* in the small intestine or draining ileocecal lymph node tissues. Based on the infection status classifications defined above, these animals could reasonably be classified as resilient (no evidence for persistent *Map* tissue infection following direct injection of a large known dose of viable *Map*). The idea of resilience or resistance to *Map* infection is not new and is based on significant experimental and clinical evidence [27, 29–32]. However, the majority of work on the subject of resilience has utilized oral infection models, or naturally-infected animals, where resilience may be at least in part attributable to prevention of *Map* uptake across the mucosal barrier. In the present study, mucosal uptake was bypassed through direct injection of *Map* into the Peyer’s patch rich region of the distal ileum, and this effectively removes mucosal uptake as a factor in understanding persistence in tissue and pathogenesis of early *Map* infection in a calf model. This approach thus adds significant data supporting the idea that with respect to the pathogenesis of the *Map* in calves, resilience to *Map* infection is due, at least in part, to host factors following tissue infection with *Map*.

In our study, calves examined at 28 wpi had a wide variation in the outcome following direct *Map* inoculation. In addition to the two calves classified as resilient, one
calf (1075) had evidence for persistent intestinal *Map* infection, with *Map* positively identified by PCR in multiple sections of intestine and the draining lymph node. Based on the definitions of paratuberculosis cases, this calf can be considered subclinically diseased, due to mild granulomatous enteritis attributable to *Map* in three segments of small intestine as well as moderate granulomatous lymphadenitis in the draining ileocecal lymph node [12]. In addition, this particular calf had two instances of fecal *Map* shedding by direct fecal *Map* PCR, as well as evidence of serum *Map*-specific antibodies using a commercial *Map* ELISA test.

Of the two calves classified in this study as resilient, one had mild granulomatous inflammation in two intestinal tissue segments and the draining lymph node, but with no detectable *Map* by PCR or ZN staining. Given the previously reported high success of *Map* infection using direct ileal inoculation (100% at 12 wpi), we believe that this is consistent with residual inflammation due to recovery from intestinal *Map* infection [13]. Unfortunately, one significant limitation to this study is that individual animals were not sampled repeatedly over the time course of the study to properly establish that these animals were truly initially *Map* infected. While this *Map* model has been shown in this and prior studies to reliably induce enteric *Map* infection, classification of an animal as “recovered” requires repeat tissue sampling within the same animal over time, to show evidence of tissue infection at an earlier time point that has subsequently been cleared from the animal [12]. The other calf classified in this study as resilient was histologically normal at 28 wpi, but had two instances of fecal *Map* shedding, at four and six wpi. The fecal shedding patterns observed in this study, particularly in the 28 wpi group, are
consistent with findings from other experimental infection studies [11, 27, 32-33]. However, it is well accepted that fecal shedding can be a transient phenomenon and thus not truly indicative of tissue *Map* infection.

Tissue PCR was the most significant measure of *Map* infection in this study, as statistical analysis identified a significant protective effect of time on the probability of *Map* PCR positive tissues in each infection group. This suggests that in the present infection model, the prevalence of *Map* in tissues decreases over time. Another important finding was the translocation of *Map* from the inoculation site in the distal ileum to the draining ileoceleal lymph node. Numerous publications have demonstrated the persistence of *Map* in lymph nodes, suggesting that the draining lymph nodes are a preferred tissue target for improved sensitivity of diagnostic tests during post-mortem examination [34–38]. Given the route of inoculation in this study, there is a possibility that subserosal injection results in increased draining to the local lymph node, and subsequently dissemination outside of the intestinal tract, though site of infection and draining lymph node are likely to remain key sentinel sites for monitoring persistence of *Map* within an animal.

The histologic data in this study were less predictive. Neither the mean histologic lesion scores based on H&E stained tissue segments nor the probability of positive acid-fast bacteria in ZN-stained tissue segments showed any significant differences between groups, as an effect of time. This is likely due to both the low sample size of the study, as well as the generally low mean histologic lesion scores, and number of AFB detected in each infection group.
There is often a question of relevance of the direct inoculation model compared to an oral infection model, and how they relate to natural *Map* infection. Specifically, the subserosal injection utilized in this infection model may show disproportionate delivery of *Map* to the submucosa, which may result in altered host responses, and thus not indicative of natural infection. Our histologic examination of the tissue included examination of the submucosa and subserosa, and we observed lesions attributable to *Map* predominantly within the lamina propria, which is consistent with natural infection. Our data examining *Map* persistence after direct intestinal *Map* inoculation over seven months are directly comparable to a recent study by Begg et al. where *Map* persistence was examined by intestinal biopsy and then post-mortem examination over a several year period after oral exposure to *Map* [32].

Taken together, this study provides experimental evidence of resilience to *Map* infection following direct inoculation of the bacteria into the distal small intestine, considered the primary site of *Map* infection in natural disease. These data suggest that early localized tissue clearance of *Map* occurs first from the intestinal tract, then from the draining lymph node. The varied outcomes following *Map* exposure in this study show similarities with patterns of *Map*-induced disease observed in the oral model and during natural exposure in endemic herds, but in a much shorter period of time. Consequently, further investigation is required examining host-pathogen interactions in *Map* infection, and how these factors relate to *Map* persistence, and resilience to infection observed within this experimental model.
2.6 Acknowledgements

This work could not be completed without the support of the staff of the University of Guelph animal isolation facility, central animal facility, and Ponsonby dairy research station. We would also like to thank the undergraduate and DVM students who volunteered their time assisting with sample collection and processing work.

2.7 References


effects on maturation of the phagosomes containing *Mycobacteria avium* subspecies *paratuberculosis* in J774 cells. FEMS Immunol Med Microbiol 34:127–134


2.8 Figures and Tables

Figure 2.1 Histologic lesions identified in small intestine and lymph node.

Panel A is ileum, score = 1 due to focal granulomatous inflammation with multinucleated giant cells (*); panel B is ileum, score = 3 due to coalescing granulomatous inflammation in lamina propria (*) and submucosa (not shown), with multinucleated giant cells (**) ; panel C is lymph node cortex, score = 1, due to focal granulomatous inflammation in the subcapsular cortex (*); and panel D is lymph node cortex, score = 3 due to coalescing granulomatous inflammation in subcapsular cortex (*) and extending throughout the node (not shown). All images are H&E stained, viewed at 100X magnification.
Figure 2.2 Lesion scores, ZN, and PCR results for *Map*-exposed calves, by weeks post-inoculation (wpi).

Histologic lesions were classified as negative if no lesions were detected, mild (light gray) for lesion scores of 1, or moderate (dark gray) for lesion scores of 2 and 3. No severe histologic lesions were observed. ZN data shows detection of acid fast bacilli (dark gray). Lastly, PCR shows detection of *Map* DNA in tissue by PCR (dark gray). Time points were 4 (A), 12 (B), 20 (C), and 28 (D) wpi, examining four ileal tissues (A-D), and the draining ileocecal lymph node (E).
Figure 2.3 Marginal effects plot of the PCR statistical model, depicting the probability of PCR positive tissues as a factor of time-point.

The calculated probability is depicted as the solid line, with the confidence interval depicted as the shaded region. The statistical model identified a significant effect of time on the probability of PCR positive tissues, with an overall odd's ratio of 0.36 (95% CI 0.18–0.70, \( P = 0.003 \)).
Figure 2.4 PCR data for the presence of *Map* in feces for all *Map*-exposed animals by weeks post-inoculation (wpi), including 4 (A), 12 (B), 20 (C), and 28 (D).

Feces collected before inoculation and then at two-week intervals following inoculation until euthanasia, as described above. Data are shown as positive (+, light grey) or negative (-). Time points not measured are marked N/A and shaded dark grey.
Table 2.1 Scoring system for identification of histologic lesions by H&E staining.

<table>
<thead>
<tr>
<th>Score</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>None</td>
</tr>
<tr>
<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Focal granulomatous inflammation (lamina propria or subcortical sinus, cortex only)</td>
</tr>
<tr>
<td>2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Multifocal to coalescing granulomatous inflammation (lamina propria or subcortical sinus or cortex only)</td>
</tr>
<tr>
<td>3</td>
<td>Coalescing/diffuse granulomatous inflammation (lamina propria, subcortical sinus, cortex)</td>
</tr>
<tr>
<td>4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Coalescing/diffuse granulomatous inflammation (extending into submucosa or medulla)</td>
</tr>
<tr>
<td>5</td>
<td>Coalescing/diffuse lesions extending deeply into submucosa +/- lymphangitis</td>
</tr>
</tbody>
</table>

<sup>a</sup> Scores of 0 were classified as within normal limits.

<sup>b</sup> Scores of 1 were classified as mild lesions.

<sup>c</sup> Scores of 2-3 were classified as moderate lesions.

<sup>d</sup> Scores of 4-5 were classified as severe lesions.
Table 2.2 Summary of histologic lesion scores (H&E) and acid fast bacteria (ZN) positive tissues by infection group (time-point).

<table>
<thead>
<tr>
<th>Infection Group</th>
<th># Mild Lesions</th>
<th># Moderate Lesions</th>
<th># Severe Lesions</th>
<th>Mean Lesion Score (95% CI)</th>
<th>Maximum Lesion Score</th>
<th># ZN Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 wpi</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0.35 (0.05–0.64)</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>12 wpi</td>
<td>8</td>
<td>3</td>
<td>0</td>
<td>0.85 (0.39–1.31)</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>20 wpi</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>0.45 (0.15–0.75)</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>28 wpi</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>0.5 (0.17–0.83)</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 2.3 *Map*-specific serum ELISA results.

<table>
<thead>
<tr>
<th>Infection Group</th>
<th>Animal ID</th>
<th>S/P Ratio</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 wpi</td>
<td>1115</td>
<td>0.077</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1116</td>
<td>0.029</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1117</td>
<td>0.125</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1120</td>
<td>0.062</td>
<td>-</td>
</tr>
<tr>
<td>12 wpi</td>
<td>1077</td>
<td>0.025</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1079</td>
<td>0.031</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1080</td>
<td>0.045</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1091</td>
<td>0.021</td>
<td>-</td>
</tr>
<tr>
<td>20 wpi</td>
<td>1130</td>
<td>0.06</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1131</td>
<td>0.181</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1132</td>
<td>0.067</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1133</td>
<td>0.061</td>
<td>-</td>
</tr>
<tr>
<td>28 wpi</td>
<td>1073</td>
<td>0.391</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1075</td>
<td>0.461</td>
<td>(+)\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>1076</td>
<td>0.086</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1082</td>
<td>0.166</td>
<td>-</td>
</tr>
</tbody>
</table>
3 A preliminary investigation of the effects of oral monensin sodium in an enteric *Mycobacterium avium* subspecies *paratuberculosis* infection model of calves
A preliminary investigation of the effects of oral monensin sodium in an enteric *Mycobacterium avium* subspecies *paratuberculosis* infection model of calves

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3.1 Abstract

*Mycobacterium avium* subspecies *paratuberculosis* (*Map*) is the causative agent of Johne’s disease (JD), an enteric infection of ruminants causing significant economic burden for dairy and beef producers. Efforts to control *Map* in endemic herds typically focus on herd management practices such as limiting exposure or early culling of infected animals, and occasionally vaccination. The ionophore monensin sodium is thought to have protective effects against *Map*, both *in vivo* and *in vitro*; however, this has not been
thoroughly evaluated experimentally. Using a direct intestinal *Map* challenge model, we recently observed similarities regarding persistence of *Map* in tissues and resilience to infection compared with experimental oral infection or in natural disease. Here we sought to investigate the effects of oral monensin supplementation in experimentally *Map*-infected calves. We examined the persistence of *Map* in the intestinal tissues, *Map*-induced intestinal inflammation, fecal *Map* shedding, and seroconversion using a commercial serologic assay. We show that monensin-supplemented *Map*-infected calves demonstrated resilience to *Map* infection earlier than the monensin-free *Map*-infected calves. Monensin-supplemented calves also demonstrated significant reduction in mean histologic lesion scores as an effect of time. However we were unable to demonstrate statistically that observed difference between monensin-supplemented and monensin-free calves was in fact due to monensin supplementation, compared with variance between animals. This preliminary study provides evidence to suggest that oral monensin sodium is beneficial to the host during early intestinal *Map* infection, and that the mechanism should be further investigated.

### 3.2 Introduction

Johne’s disease (JD) is a chronic enteritis of ruminants, and has significant economic impact on North American dairy and beef producers [1, 2]. JD is caused by the bacterium *Mycobacterium avium* subspecies *paratuberculosis* (*Map*), which is ingested by young animals via contaminated feces, colostrum, or milk. *Map* invades the intestinal mucosa of the small intestine overlying Peyer’s patches, and survives within resident macrophages [3]. Infected animals may show no outward evidence of infection for several
years (subclinical JD); however, *Map* is sporadically shed in their feces, which perpetuates and disseminates the pathogen to susceptible herdmates [4]. A subset of infected animals may develop clinical JD, which is characterized by inflammation and thickening of the small intestinal mucosa leading to chronic malabsorptive diarrhea, dehydration, wasting, and eventually death. Clinical JD is generally considered fatal, and infected animals intermittently and increasingly shed viable *Map* in their feces [3, 4].

Control of JD on farms is largely focused on surveillance techniques seeking to identify *Map*-infected animals and remove them from the herd [5, 6]. Other management practices, such as separating susceptible calves from shedders of *Map* also play a role in prevention of *Map* exposure; however, JD remains a very challenging disease to control—particularly in endemically infected herds [6, 7]. Thus far, attempts to develop an efficacious JD vaccine have shown some success, though while vaccines may reduce fecal *Map* shedding and slow disease progression, they do not prevent new infections [8–10]. Vaccination also causes false positive reactions in commercial serologic tests for *Map* (ELISA), which are commonly used in diagnostics and herd management around the world [11–13].

One control strategy that has not been thoroughly investigated is use of chemoprophylactic or antimicrobial agents for preventive or therapeutic purposes. The ionophore monensin sodium has been studied *in vivo* and *in vitro*, for its hypothesized beneficial effects against *Map*. Monensin is widely used in North American ruminants for control of coccidiosis, for feed efficiency, and ketosis prevention (in dairy cattle) with no milk or meat withdrawal time [14–17]. Monensin is also on label in Canada, as a controlled
release capsule, for the reduction of fecal *Map* shedding in mature cattle from high-risk herds, as part of a multi-component JD control program (Kexxtone, Elanco Animal Health, Guelph, Ontario, Canada). Data suggesting efficacy of oral monensin as therapeutic or prophylactic against *Map*-associated disease in cattle is reviewed extensively by Fecteau and Whitlock, 2011 [18]. The data show that *Map* is susceptible to monensin *in vitro*, with a dose-dependent inhibitory effect on *Map* grown in liquid and radiometric culture systems [19, 20]. Monensin has also been shown to provide a prophylactic protective effect in mice experimentally inoculated with *Map* via intraperitoneal injection [19, 21]. In the mouse model, there was significant reduction of hepatic granulomas in monensin-supplemented *Map*-infected mice compared with control mice. In naturally *Map*-infected cattle, oral monensin administration was associated with reduced severity of histologic lesions and decreased fecal *Map* shedding, suggesting that monensin slowed or reversed progression of disease [22, 23]. Adult dairy cows receiving oral monensin were less likely to test positive for *Map* antibodies by ELISA, supporting reports of immunomodulatory functions of monensin [24]. Despite these data suggesting beneficial effects of monensin during various aspects of intestinal *Map* infection, no studies of the effects of oral monensin during experimental *Map* infection of calves have been published.

Our data using the direct intestinal *Map* inoculation model suggest that many calves are resilient to *Map* infection which is most likely due to early clearance of the pathogen, consistent with naturally-occurring JD and a long-term oral *Map*-exposure model [25–27]. In this study, we investigated the effects of oral monensin supplementation during the early post *Map*-exposure period. We examined histologic intestinal lesions, fecal *Map*
shedding, and serum Map-specific antibody responses in monensin-supplemented calves following experimental Map inoculation into the Peyer’s patch-rich region of the distal ileum [25]. We hypothesized that monensin-supplemented calves would have increased resilience against early Map colonization based on decreased severity of histologic lesions, reduced frequency of fecal Map shedding, and decreased serum Map-specific antibody responses. Monensin is not approved for control of Map in calves; however, this study is immediately relevant to field control of Map because calves are routinely administered oral monensin sodium for alternate approved reasons.

3.3 Methods

3.3.1 Animal infection model

The animal infection model was performed as previously described [25]. This study was designed as a block cohort study with 2 large groups of monensin-supplemented and non monensin-supplemented calves (n=24 each). Data from the non monensin-supplemented calves have been previously published, and are compared directly with the monensin-supplemented calves in this study [25]. For the monensin-supplemented calves reported in this study, 24 one-week-old castrated male Holstein calves were obtained from the Elora Research Station – Dairy Facility, University of Guelph. Calves were randomly assigned to one of four groups of six animals each, and acclimated to the facility for 1 week. Groups contained four Map-exposed and two unexposed control animals for each timepoint, and were housed in an animal biosafety level 2 unit in pairs; control animals were housed separately from Map-exposed calves. Each Map-exposed calf was inoculated with $10^9$ colony forming units (CFU) of live Map, prepared as
previously described, by sub-serosal injection into the anti-mesenteric surface of the distal ileum approximately 5cm proximal to the ileocecal valve [25]. Feces were collected pre-exposure and bi-weekly thereafter; serum was collected pre-exposure and monthly thereafter. Groups of animals were euthanized at 4, 12, 20 or 28 weeks post inoculation (wpi), when full post-mortem examinations were performed. Tissues were collected from the ileocecal valve (tissue A), three sections of distal ileum with 5 cm separation (tissues B-D), and the draining ileocecal lymph node (tissue E). Sections of each tissue were snap frozen in liquid nitrogen for future DNA extraction and fixed in 10% neutral buffered formalin. All animal work was performed under an approved animal utilization protocol, reviewed by the University of Guelph Animal Care Committee.

3.3.2 Oral monensin supplementation

Animals were supplemented with oral monensin sodium as described below beginning one week prior to surgical Map exposure, and continuing throughout the duration of the study. For calves prior to weaning, a liquid monensin sodium formulation (Rumensin Max, Elanco Animal Health, Manukau, New Zealand; 50mg/head/day) in water was administered orally once daily to each calf by dose syringe. At six wpi, calves were weaned and transitioned to a granular formulation of monensin sodium premix (Rumensin, Elanco Animal Health Canada; 200g/kg) in a pelleted calf starter grain ration at a dose calculated to deliver 22 mg/kg body weight on a dry matter intake basis per day, which is the approved dose for oral monensin sodium for control and prevention of coccidiosis in dairy cattle.
3.3.3 Tissue histology

After 24 hours of fixation in formalin, all tissue sections were trimmed, paraffin embedded, sectioned at 5 microns, stained with haematoxylin and eosin (H&E), and cover slipped. All sections were examined histologically for the presence of granulomatous inflammatory lesions attributable to Map infection. Serial sections of all tissues were also stained per standard protocol with Ziehl Neelsen (ZN) for direct detection of acid-fast bacilli (AFB) within the tissue. All staining was performed by the histology laboratory personnel at the Animal Health Laboratory, University of Guelph. Histologic lesions were blindly scored as previously described on a scale of 0-5 [25]. Briefly, a score of 0 was assigned if no inflammation was present; a score of 1 was assigned to for lesions of focal granulomatous inflammation (mild lesions); a score of 2-3 was given to tissues with multifocal granulomatous inflammation (moderate lesions). A score of 4-5 was given to tissues with coalescing granulomatous inflammation (severe lesions).

3.3.4 Molecular detection of Map

Map detection in tissues and feces was performed as previously described [25]. Briefly, tissue DNA was extracted from 100 mg of flash-frozen tissue (tissues A-E, described above) using the Qiagen DNEasy Blood and Tissue kit (Qiagen Canada, Toronto, Ontario, Canada) with the protocol modified for increased volume of tissue. Any tissues where Map was not detected by PCR were re-extracted and re-processed. Fecal DNA was extracted from 1g wet weight of feces utilizing a pre-treatment step designed to reduce background fecal microbiota before extraction using the Norgen Biotek Stool DNA
Extraction Kit (Norgen Biotek, Thorold, Ontario, Canada). PCR amplification was performed using the Quanta PCR Toughmix II (Quanta Biosciences, Beverley, Massachusetts, United States) in a hemi-nested reaction targeting the IS900 gene.

3.3.5 Serum ELISA

*Map*-specific antibodies were detected using the IDEXX *Map* antibody ELISA assay (IDEXX Canada, Markham, Ontario, Canada), a commercially available assay widely utilized for JD diagnostics. Samples were scored as a sample: positive Control (S/P) ratio, where an S/P ratio below 0.45 was considered negative, between 0.45 and 0.55 considered suspect, and above 0.55 considered positive, according to the manufacturer’s guidelines.

3.3.6 Animal infection status

Animals were classified as exposed, infected, diseased, or resilient based upon the classification scheme developed by Whittington et al. 2017, and as previously described [25, 28]. By this classification system, animals which undergo surgical *Map* inoculation were considered *Map*-exposed. *Map*-exposed animals with detectable *Map* organisms in intestinal tissue based upon PCR or ZN detection were classified as *Map*-infected. Infected animals with moderate or severe granulomatous inflammation (scores > 2, as outlined above) were classified as diseased. Lastly, any *Map*-exposed animals with no detectable *Map* in tissue at euthanasia were classified as resilient. As noted in our previous work, direct surgical Peyer’s patch inoculation of *Map* in our lab has been 100% effective at inducing demonstrable *Map* intestinal infection in calves by 12 wpi, which suggests that animals classified as resilient after 12 wpi are likely recovered,
though this would require serial biopsy sampling of intestinal tissues from individual calves to confirm *Map* infection at earlier time points [25, 28, 29].

3.3.7 Statistics

Statistical inference was performed as previously described, using R Studio, and the lme4 package for generalized mixed effect regression modelling, with the “glmer” function [25]. Effects of time-point on the number of PCR positive tissue segments (logistic regression), number of ZN positive tissue segments (logistic regression), and the mean histologic lesion scores (poisson distribution) were expressed using the formula: FACTOR ~ time-point + (0 + time-point|animal/tissue), where FACTOR represents PCR data, ZN data or mean lesion scores. This method was also utilized to examine the effect of monensin supplementation in this study with the baseline monensin-free animals previously reported, using the formula: FACTOR ~ time-point + monensin + (0 + time-point|animal/tissue). Main effects were reported as odds ratios with corresponding 95% confidence intervals (CI), and a significance level of α=0.05 was used.

3.4 Results

3.4.1 Gross lesions

During post-mortem assessment, India ink was observed in tissue approximately 5-8cm proximal to the ileocecal valve and within the draining ileocecal lymph node of *Map*-exposed calves, confirming the site of *Map* inoculum. There was mild ileocecal lymph node enlargement in the *Map*-exposed animals compared with controls; however, this was not significantly different between groups. Fibrous adhesions were occasionally observed between the serosal surface of the ileum at the site of injection and the
omentum. No gross pathologic changes consistent with JD were observed in the small intestine of any Map-exposed animals during post-mortem examination. One Map-exposed calf assigned to the 20 wpi group (calf ID 1228) developed severe pneumonia three weeks after surgical Map exposure and was euthanized.

### 3.4.2 Histologic lesion scores

Histologic lesion scores and observed AFB by ZN staining are shown in Figure 3.1 and summarized in Table 3.1. Control calves had no detectable granulomatous inflammation in intestine or lymph node sections, and no AFB detected in ZN-stained tissue segments. Granulomatous inflammation in the intestine of Map-exposed calves was located primarily within the lamina propria, and we observed only occasional lesions within the submucosa or the serosa; this was similar to our previous study in monensin-free calves. The maximum histologic lesion score for inflammation attributable to Map observed in any tissue section in this study was 3.

At 4 wpi, Map-exposed calves had a mean lesion score of 1.05 (95% CI 0.56-1.54), with a maximum lesion score of 3 (n=2). Lesions were observed in 60% (12/20) of tissue segments, with five mild lesions and seven moderate lesions. AFB were observed in 30% (6/20) of ZN-stained tissue segments.

At 12 wpi, Map-exposed calves had a mean lesion score of 0.85 (95% CI 0.28-1.42), with a maximum lesion score of 3 (n=3). Lesions were observed in 35% (7/20) of tissue segments, all of which were scored as moderate lesions. AFB were observed in 5% (1/20) of ZN-stained tissue segments.
At 20 wpi, *Map*-exposed calves had a mean lesion score of 0.27 (95% CI -0.06- 0.6), with a maximum lesion score of 2 (n=1). Lesions were observed in 20% (3/15) of tissue segments, with two mild and one moderate lesion. AFB were not observed in any of the ZN-stained tissue segments.

At 28 wpi, *Map*-exposed calves had a mean lesion score of 0.25 (95% CI 0.04-0.46), with a maximum lesion score of 1 (n=5). Lesions were observed in 25% (5/20) of tissues, all demonstrating mild lesions. AFB were not observed in any of the ZN-stained tissue segments.

There was a significant protective effect of time on the mean histologic lesion scores with an odds ratio of 0.47 (95% CI 0.30-0.73, P<0.001), depicted as a marginal effects plot in Figure 3.2. For detection of AFB, the model also indicated a statistically significant protective effect of time; however, the model flagged convergence errors. This is likely largely due to the small number of ZN positive tissues at time points other than the earliest time of 4 wpi. Therefore, statistical modelling to predict the probability of ZN positive tissues over time was discarded from this study.

### 3.4.3 Tissue PCR

PCR detection of *Map* in intestinal tissues is shown in Figure 3.1. At all time-points, all sections of intestine and lymph node tissue segments from unexposed control calves tested negative for *Map* by PCR. At 4 wpi, 50% (10/20) of all tissues tested positive for *Map* by PCR. The number of positive tissues per calf varied between one and four, with *Map* detected in at least one tissue of every calf and in the draining lymph node of all
*Map*-exposed animals. At 12 wpi, 40% of all tissue segments tested positive for *Map* by PCR. All *Map*-exposed calves at this time-point had between one and three positive tissue segments, and all of these calves tested positive for *Map* by PCR in the draining lymph node. At 20 wpi, *Map* was detected in 27% (4/15) of all tissues from *Map*-exposed calves. One calf at 20 wpi had no *Map* detectable by PCR in any tissue, while the other two calves were *Map*-positive by PCR in two tissues including one section of intestine and one of draining lymph node. At 28 wpi, *Map* was detected by PCR in only 10% (2/20) of all tissues from *Map*-exposed calves. At this time point, only two of four calves had *Map* detectable in any tissues, and in both of these calves, *Map* was detected in the draining ileocecal lymph node only.

There was a significant protective effect of time on the probability of positive tissue *Map* PCR segments, with an odds ratio of 0.12 (95% CI 0.02-0.67, P=0.016), shown as marginal effects plot (Figure 3.3).

3.4.4 Fecal *Map* PCR

Results for detection of *Map* in feces by PCR are shown in Figure 3.4. All unexposed control calves tested negative by fecal PCR at all time-points throughout the study. *Map* was detected in feces of 10% (14/133) of all fecal samples from *Map*-exposed calves, and 67% (10/15) of all calves tested positive for *Map* in feces by PCR at least once during the study while 27% (4/15) tested positive in two consecutive samples. In the 4 wpi group, *Map* shedding was only observed in a single calf, and only at a single time-point. In the 12 wpi group, all four animals tested positive for *Map* in feces, with two of these animals showing consecutive shedding over two time-points. In the 20 wpi group,
two calves tested positive for *Map* in feces, with one having consecutive shedding over two time-points. Lastly, in the 28 wpi group, three of the calves had detectible fecal *Map*, with one showing consecutive shedding over two time-points and the latest incident of fecal shedding of *Map* detected by PCR occurred at 8 wpi. Intermittent shedding was not detected in any of the *Map*-exposed calves in this study.

### 3.4.5 Serum ELISA

Detection of *Map*-specific antibodies by commercial ELISA assay identified all calves, both *Map*-exposed and control animals, as negative. S/P ratios ranged from 0.00 to 0.077, well below the manufacturer recommended cut-off threshold of 0.45, which is required for classification as suspect.

### 3.4.6 Animal infection status

All *Map*-exposed animals at 4 and 12 wpi were classified as *Map*-infected based on detection of the organism within tissue by PCR and/or ZN staining. All but one (calf ID 1166, 4 wpi) of the calves at these two time-points were also classified as diseased, based upon the presence of moderate or severe *Map*-induced granulomatous inflammation (score of 2 or greater) in at least one tissue section. At 20 wpi, two animals were classified as *Map*-infected; one of these was also classified as diseased but the other calf had no detectable *Map* in tissue and was thus classified as resilient. At 28 wpi, two calves were classified as *Map*-infected based on a single *Map*-positive tissue section (lymph node) by PCR; however, the other two calves had no detectable *Map* in any tissue and were thus classified as resilient.
3.4.7 Effects of monensin

All of the data from monensin-supplemented calves in this study were then compared with our monensin-free calf data already published in full as part of the overall block cohort study, in order to investigate the effect of oral monensin sodium on *Map* persistence and markers of infection in this model [25]. Table 3.2 summarizes the PCR, ZN, and H&E data from both studies by time-point along with animal infection classification (diseased/infected with no evidence of disease/resilient). Statistically, monensin supplementation did not have a significant effect on mean inflammatory lesion scores (P=0.520), the presence of AFB in tissues by ZN staining (P=0.336), or on the number of *Map*-positive tissues by PCR (P=0.177). The statistical analyses of data from monensin-free and monensin-supplemented calves, analyzed independently and combined, are summarized and presented in Table 3.3.

3.5 Discussion

JD poses several challenges with regards to prevention and control measures within affected herds. Diagnostic methods with poor sensitivity, especially during subclinical disease, make identification of infected animals difficult. Vaccination has shown partial success in reducing fecal *Map* shedding, but still fails to prevent infection, therefore alternative approaches to aiding producers with management of *Map* infection on farms is needed. With *in vivo* and *in vitro* evidence supporting putative beneficial effects of oral monensin sodium against *Map* infection in cattle, we set out to utilize an experimental model of intestinal *Map* infection to investigate the effects of oral monensin supplementation of young calves on persistence of *Map* within tissues, *Map*-induced
lesions, fecal *Map* shedding, and *Map*-specific antibody responses. We hypothesized that oral monensin supplementation of young calves during the early phases of intestinal *Map* infection would result in reduced persistence of *Map* within the intestinal tissue, reduced severity of lesions, fecal *Map* shedding, *Map*-specific antibody responses, and improve overall host resilience to *Map* infection.

Our previous publication investigated the use of the direct Peyer's patch injection model in monensin-free calves, which were the first cohorts of this large block animal study [25]. Those results were comparable to the findings of a recent oral *Map* exposure study in cattle of five years in duration, which also demonstrated resilience and recovery from *Map* infection [27]. As summarized in Table 3.2, calves in our previous published study demonstrated apparent localized *Map* clearance, characterized by significantly reduced PCR positive tissues as an effect of time; however, there were no significant changes in mean histologic lesion scores or number of ZN positive tissues. Compared with the monensin-free calves, monensin-supplemented calves demonstrated resilience to *Map* infection eight weeks earlier compared to monensin-free calves (20 wpi compared to 28 wpi) [25]. One calf at 28 wpi in the monensin-free study demonstrated evidence of progression to subclinical disease characterized by the presence of *Map* by PCR and *Map*-induced inflammation in multiple segments of intestine and draining lymph node, as well as evidence of seroconversion based on the commercial serum *Map* antibody ELISA. Comparatively, none of the monensin-supplemented calves at 28 wpi showed any evidence of disease, and no monensin-supplemented calf at any time-point showed evidence of seroconversion.
Statistical analysis found no significant effect of monensin observed on PCR, ZN, or mean histologic lesion scores, however several other distinctions were observed. The monensin-supplemented calves had a comparatively greater number of histologic lesions, and a higher mean histologic lesion score at 4 wpi, compared with monensin-free calves (Table 3.2). Furthermore, we observed a significant reduction of mean histologic lesion scores as an effect of time in the monensin-supplemented calves, but not in the monensin-free calves (Table 3.3). The reduction of Map in tissues, as evidenced by the PCR results, was statistically significant in both studies, with comparable protective effects of time in both monensin-free and monensin-supplemented calves. Fecal Map shedding was similar overall between the two studies, with a few minor differences. The monensin-supplemented calves had a greater number of positive fecal samples (10% versus 7%); however, no Map was detected after 8 wpi, while we detected Map shedding in the monensin-free calves at 12 wpi [25].

In summary, this preliminary study examining the effects of oral monensin supplementation on early enteric Map infection in calves identified some changes in resilience to Map infection following experimental inoculation, though no statistically significant effect of monensin was observed. Of interest, the combined statistical model identified a statistical effect of time on all three measured factors (Map tissue PCR positivity, positive ZN tissues, mean histologic lesion scores) not observed in either the monensin-free calves or the monensin-supplemented calves when analyzed independently. The inter-animal variance observed in both the monensin-free and monensin-supplemented calves highlight the small group sizes as a limitation to this
study. And because of this, we were unable to identify a statistically significant effect of monensin supplementation. Nevertheless, there is still some evidence to suggest a positive effect of oral monensin during early enteric *Map* infection of calves, and warrants further investigation on a larger scale to more fully understand if monensin is a key contributor to resilience or recovery from experimental *Map*-infection.

### 3.6 Acknowledgements

This work could not be completed without the support of the staff of the University of Guelph animal isolation facility, central animal facility, and University of Guelph Elora dairy research station. We would also like to thank the undergraduate and DVM students who volunteered their time assisting with sample collection and processing work. In addition, special thanks to Sarah Schorno, and Ryan Horricks, for their insight in developing the statistical inference methods utilized in both this study, and in our monensin-free study.

### 3.7 References


3.8 Figures and Tables
Histologic lesions were considered within normal limits (score of 0), mild lesions (score of 1, light grey), or moderate lesions (score of 2 or 3, dark gray). ZN results were based on detection of AFB in the tissue segment (positives shown in dark grey). PCR results were based on detection of Map by PCR in the tissues (positives shown in dark grey). The four panels represent study time-points in weeks post inoculation (wpi): 4 wpi (A), 12 wpi (B), 20 wpi (C) or 28 wpi (D), with examination of four ileal tissues (A-D) and the ileocecal lymph node (E) for each animal. Calf ID 1228 at 20 wpi (Panel C) was euthanized early and discarded from the study, due to illness shortly after surgical Map inoculation.
**Figure 3.2 Marginal effects plot of the histologic lesion score statistical model.**

Figure depicts the predicted mean lesion score in tissues of *Map*-exposed calves as a factor of time, in weeks post inoculation (wpi). The calculated probability is depicted as the solid line, with the 95% confidence interval shown as the shaded region. The statistical model identified a significant protective effect of time on the predicted mean histologic lesion score, with an odd’s ratio of 0.47 (95% CI 0.30-0.70, P<0.001).
Figure 3.3 Marginal effects plot of the *Map*-positive tissues by PCR statistical model.

Figure depicts probability of *Map*-positive tissues in *Map*-exposed calves as a factor of time, in weeks post inoculation (wpi). The calculated probability is depicted as the solid line, with the 95% confidence interval shown as the shaded region. The statistical model identified a significant protective effect of time post inoculation on the probability of intestinal tissues testing positive for *Map* by PCR, with an odd’s ratio of 0.12 (95% CI 0.02-0.67, P=0.016).
Figure 3.4 PCR detection of *Map* in feces of *Map*-exposed calves, by weeks post inoculation (wpi).

Panels show calves in different time-points at 4 (A), 12 (B), 20 (C), and 28 (D) weeks post inoculation (wpi), samples pre-inoculation and biweekly thereafter. Positive results are highlighted grey, with unmeasured time-points marked as N/A and shaded dark grey. Calf 1228 in the 20 wpi group (Panel C) was euthanized due to illness shortly after inoculation, and was excluded from the trial.
Table 3.1 Summary of histologic lesion scores and ZN positive tissues in *Map*-exposed calves, by time-point in weeks post inoculation (wpi).

<table>
<thead>
<tr>
<th>Timepoint (wpi)</th>
<th># Mild Lesions</th>
<th># Moderate Lesions</th>
<th># Severe Lesions</th>
<th>Mean Lesion Score (95% CI)</th>
<th>Maximum Lesion Score</th>
<th># ZN Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>5</td>
<td>7</td>
<td>0</td>
<td>1.05 (0.56-1.54)</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>12</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0.85 (0.28-1.42)</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0.27 (-0.06-0.6)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>28</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0.25 (0.04-0.46)</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3.2 Comparison of histologic lesions, ZN data, *Map* PCR data, and animal infection status between the monensin-supplemented calves, and the monensin-free calves [25].

<table>
<thead>
<tr>
<th>Group</th>
<th>Timepoint (wpi)</th>
<th>Mean Lesion Score (95% CI)</th>
<th># Lesions (Any Grade)</th>
<th># ZN Positive Tissues</th>
<th># PCR Positive Tissues</th>
<th>Infection Status²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monensin- Supplemented</td>
<td>4</td>
<td>1.05 (0.56-1.54)</td>
<td>12</td>
<td>6</td>
<td>10</td>
<td>3/1/0</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.85 (0.28-1.42)</td>
<td>7</td>
<td>1</td>
<td>8</td>
<td>4/0/0</td>
</tr>
<tr>
<td></td>
<td>20²</td>
<td>0.27 (-0.06-0.6)</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>1/1/1</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0.25 (0.04-0.46)</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>0/2/2</td>
</tr>
<tr>
<td>Monensin- Free</td>
<td>4</td>
<td>0.35 (0.05 – 0.64)</td>
<td>5</td>
<td>3</td>
<td>13</td>
<td>2/2/0</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0.85 (0.39 - 1.31)</td>
<td>11</td>
<td>5</td>
<td>10</td>
<td>2/2/0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.45 (0.15 - 0.75)</td>
<td>7</td>
<td>3</td>
<td>5</td>
<td>2/2/0</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0.5 (0.17 – 0.83)</td>
<td>8</td>
<td>0</td>
<td>4</td>
<td>1/1/2</td>
</tr>
</tbody>
</table>

1. Timepoint shown as weeks post inoculation (wpi)
2. Infection status is shown as number of diseased animals, infected animals with no evidence of disease, and resilient animals, respectively, as described. (diseased/infected/resilient)
3. One animal in the 20 wpi group was euthanized early and removed from the study due to illness. As such, there is a maximum of 15 tissues at this time-point, versus 20 tissues in all others time-points.
Table 3.3 Summary of statistical model results for the monensin-free calves and the monensin-supplemented calves [25].

<table>
<thead>
<tr>
<th>Group</th>
<th>Effect</th>
<th>Mean lesion score</th>
<th>ZN</th>
<th>Map PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monensin-supplemented</td>
<td>Timepoint</td>
<td>0.47 (95% CI 0.30-0.73, P&lt;0.001)*</td>
<td>N/A³</td>
<td>0.12 (95% CI 0.02-0.67, P=0.016)*</td>
</tr>
<tr>
<td>Monensin-Free</td>
<td>Timepoint</td>
<td>1.22 (95% CI 0.78-1.91, P=0.386)</td>
<td>0.61 (95% CI 0.33-1.14, P=0.120)</td>
<td>0.36 (95% CI 0.18-0.70, P=0.003)*</td>
</tr>
<tr>
<td>Combined</td>
<td>Timepoint</td>
<td>0.73 (95% CI 0.58-0.91, P=0.005)*</td>
<td>0.42 (95% CI 0.24-0.74, P=0.002)*</td>
<td>0.32 (95% CI 0.18-0.58, P&lt;0.001)*</td>
</tr>
<tr>
<td></td>
<td>Monensin</td>
<td>1.15 (95% CI 0.76-1.73, P=0.520)</td>
<td>0.60 (95% CI 0.21-1.70, P=0.336)</td>
<td>0.57 (95% CI 0.25-1.29, P=0.177)</td>
</tr>
</tbody>
</table>

1. The measured fixed effect(s) of the generalized linear mixed effect regression models, examining effect of time-point and monensin where applicable.

2. Results for mean histologic lesion scores (lesions), probability of positive ZN tissues (ZN), and probability of Map PCR positive tissues (Map PCR) shown as Odd’s Ratios, with 95% Confidence Interval and P-value in parenthesis. Statistically significant findings are marked with an *.

3. Model was statistically significant, however failed to converge and was thus excluded from the trial.
4 Serum antibody responses after direct enteric inoculation of *Mycobacterium avium* subspecies *paratuberculosis* in calves
Serum antibody responses after direct enteric inoculation of
Mycobacterium avium subspecies paratuberculosis in calves

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4.1 Abstract

The antibody responses attributed to early Map infection have significant implications for the field of serodiagnostics for Johne’s disease. Serum ELISA assays detecting Map-specific antibody responses are widely utilized in the field to detect Map exposure. They are a relatively inexpensive strategy to detect Map-infected animals; however, the test suffers from poor sensitivity, especially early in the disease process. Researchers have sought to identify novel immunogenic proteins that may have value as serodiagnostic biomarkers of infection; however, most research efforts have largely focused on examination of serum from cows with naturally occurring clinical Johne’s disease. In this study, we examined sera with the goal to identify Map-specific antibodies in the first 28 weeks following experimental direct ileal Map inoculation. We detected increased Map-specific antibody responses in a small subset of these calves using a custom ELISA. We identified 25 proteins by western blotting and protein mass spectrometry, which may be of interest as markers of early intestinal Map infection. These proteins were further characterized using gene ontology mapping and were largely identified as cytosolic proteins associated with various metabolic pathways, and not
intrinsically associated with the *Map* cell envelope. In addition, we examined the effects of oral monensin sodium supplementation in calves on the development of these antibody responses and found that monensin supplementation was associated with reduced serum antibodies. Overall, this research provides insight into the antibody responses associated with early enteric *Map* infection and provides the foundation for research into novel *Map* serodiagnostic testing.

### 4.2 Introduction

Johne’s disease (JD) is a chronic enteritis of cattle caused by the bacterium *Mycobacterium avium* subspecies *paratuberculosis* (*Map*) [1]. The disease is widespread across North America and is associated with significant economic losses in the dairy and beef cattle industries [2]. One key challenge of controlling *Map* infection is the poor sensitivity of diagnostic tests, particularly for tests available for use during the early and subclinical stages of JD. During these stages, animals show no outward signs of infection, and test negative using commercial serological testing strategies for *Map*-specific antibodies [3, 4]. Early detection of *Map* infection in cattle is important to both prevention and control because subclinical *Map*-infected cattle shed *Map* sporadically in their feces, and there is significant risk of propagating *Map* infection due to spread of live *Map* to susceptible herdmates [5].

A clear limitation to ELISA-based serum antibody tests for detecting *Map* infection during early and subclinical stages is that development of *Map*-specific antibodies occurs primarily in the late subclinical and clinical stages of disease [6]. *In vitro* data suggest that *Map* inhibits antigen presentation by macrophages, which could explain a delay in the
development of *Map*-specific antibodies [7]. There are, however, several studies examining early serum antibody development in calves following either experimental or natural *Map* infection, though antibody levels detected are far below the cutoff threshold of commercially available assays [8–11].

A significant challenge with respect to examination of early antibody responses during mycobacterial infection in any species is identifying antigens that are both sensitive and specific to the target pathogen. An important goal when searching for potential serodiagnostic biomarkers is selection of antigens displaying strong sensitivity while still retaining high specificity. Cell surface antigens such as *Map* lipoarabinomannans have been a frequent target for studies seeking to identify specific antibody responses in cattle; however there are an array of other bacterial antigens that have not been specifically investigated as potential serodiagnostic biomarkers with specificity for *Map*. For example, among secreted antigens of *Map* (the *Map* secretome), there are an abundance of proteins hypothesized to be important in antigen recognition during early *Map* infection, and the secretome has been a growing focus of study as potential vaccine candidates and serodiagnostic markers [12–15]. It has been observed that many of these proteins in the *Map* secretome are primarily associated with the cytoplasm and not the cell wall. This same finding has also been observed in proteomic studies of the *Map* cell envelope, and in the related human pathogen *Mycobacterium tuberculosis* (*Mtb*) [16–20]. Based on these data, it is reasonable to hypothesize that during early mycobacterial infections the host may be exposed to non-cell wall-associated antigens (i.e. secreted antigens), that
these proteins may be immunogenic, and thus they may hold significant potential for use as serodiagnostic biomarkers during early \textit{Map} infection.

Using a direct ileal inoculation model of \textit{Map} to examine the pathogenesis of early enteric \textit{Map} infection, we recently showed that calves generally achieve localized bacterial clearance without systemic serologic evidence of disease, based on commercial ELISA [21]. In the present study, we set out to examine \textit{Map}-specific serum antibody responses in young calves with confirmed \textit{Map} intestinal infection, using a customized enzyme-linked immunosorbent assay (ELISA) and western blotting with \textit{Map} whole cell sonicate (WCS) as the antigen source. Our objective was first to compare serum antibody responses during early intestinal \textit{Map} infection with serum antibodies in uninfected control calves, and then identify and sequence a selection of \textit{Map} protein targets that could be potentially useful as specific serodiagnostic biomarkers during early enteric \textit{Map} infection. Further, we had previously shown that \textit{Map}-infected calves in this model receiving oral monensin sodium during the early infection period show evidence of increased resilience to colonization of tissue by \textit{Map} (Stinson et al 2018b, in preparation). Therefore, antibody responses between monensin-supplemented and monensin-free calves were also examined, to examine any effects of monensin on development of serum antibody responses, as previously hypothesized [22].

4.3 Methods

4.3.1 Bacterial strain and growth conditions

Cultivation of \textit{Map} strain gc86 was performed as previously described [21]. Briefly, \textit{Map} was recovered from frozen into Middlebrook 7H9 broth (Becton-Dickinson, Oakville,
Ontario, Canada). The broth was supplemented with 10% OADC (oleic acid, albumin, dextrose, catalase; Becton-Dickinson, Oakville, Ontario, Canada), along with 0.05% Tween 80 (Sigma-Aldrich, Oakville, Ontario, Canada) and 2mg/L Mycobactin J (Allied Monitor Inc, Fayette, Missouri, USA). Bacterial growth was performed in stationary flasks at 37°C, with 5% CO₂.

4.3.2 Whole cell sonicate preparation

*Map* WCS was prepared by centrifugation of liquid *Map* culture at 1500xg for 20 minutes, at 4°C. Pellets were washed twice in phosphate buffered saline (PBS, pH 7.4), and resuspended in PBS. The *Map* suspension was sonicated using a sonic dismembrator (Model 120, Fisher Scientific, Mississauga, Ontario, Canada) on ice, at 60% amplitude for three 10-minute cycles, with 10 minutes of rest on ice between cycles. After sonication, the suspension was centrifuged at 14,000xg for 5 minutes to pellet insoluble cell debris, and the resultant supernatant was stored at -80°C until use. Protein concentration of the WCS was determined using the Pierce 660 nm protein assay kit (Thermo-Fisher Scientific, Mississauga, Ontario, Canada).

4.3.3 Collection of serum

Serum samples from clinical cases of naturally-occurring JD were acquired from several adult cattle in Ontario after confirmation of the diagnosis in each animal clinically and by the Animal Health Laboratory at the University of Guelph (fecal *Map* positive and serum ELISA positive). The positive serum control (PSC) sample used in this study was generated by pooling serum samples from six individual cows with clinical JD. Serum
samples from all experimentally *Map*-exposed calves using the direct Peyer’s patch serosal *Map* injection model were sourced from our previous infection studies, chapters 2 and 3 of this thesis. Samples were collected pre-exposure and then every four weeks thereafter [21].

### 4.3.4 Immune response (HIR © technology) testing

A subset of calves from each of the monensin-free and monensin-supplemented groups underwent immune response testing using the HIR technology for classification of individual animal status for antibody-mediated and cell-mediated immune response (AMIR, CMIR, respectively) as previously described [23, 24]. Classification of immune responses as low, average, or high AMIR or CMIR responders have previously been shown to correlate with resilience to various infectious disease, and the purpose of testing calves in the present study was to confirm that these calves were not genetic immune outliers which may help to explain variation in the response to this experimental *Map* infection model. This testing was performed on all calves within the 20 and 28 wpi groups of the monensin-free calves, and the 12, 20 and 28 wpi groups of the monensin-supplemented calves. Testing was not performed on the monensin-free 12 wpi group due to timing of the study and availability of the required test antigens. Testing was not performed on any of the 4 wpi groups because of age and the assumption that high maternal antibodies in young calves interfere with results.

### 4.3.5 WCS serum ELISA

*Map*-specific serum antibodies were detected by a custom in-house indirect ELISA using *Map* WCS as the antigen and sera from individual test animals as the primary
antibody. The assay was optimized using a standard checkerboard titration approach of both the WCS and the positive JD cow control sera. Briefly, WCS was bound to a flat-bottom Immulon 2 HB 96-well microtitre plate (Thermo-Fisher Scientific, Mississauga, Ontario, Canada) in 50 µL of 100 mM bicarbonate/carbonate coating buffer (coating buffer, pH 7.4), with a concentration of 20 µg/mL in column 1 and the checkerboard was completed using a 2-fold serial dilutions in subsequent columns 2-11. Column 12 contained coating buffer only with no WCS. Antigen binding to the plate was performed at room temperature for 2 hours after which plates were washed three times with PBS containing 5% Tween 20 (PBST, pH 7.4). Plates were then blocked using 200 µL PBST containing 5% sheep serum (blocking buffer) overnight at 4°C with gentle agitation. The PSC was bound in blocking buffer, with a dilution of 1/20 in row A and the checkerboard was completed using a 2-fold dilution series in subsequent rows B-G. Row H had no sera added and was instead filled with an equal volume of blocking buffer. Binding of the PSC was allowed to occur at room temperature for 2 hours with gentle agitation. The plate was then washed three times with PBST and bound with HRP-tagged sheep-anti-bovine IgG (heavy and light chain, polyclonal, catalog A10-115P, Bethyl Laboratories, Montgomery, Texas, United States), diluted 1/50,000 in blocking buffer for 2 hours at room temperature with gentle agitation. The plate was again washed three times in PBST, and coated with 50 µL of 1-Step Ultra TMB-ELISA solution (Thermo-Fisher Scientific, Mississauga, Ontario, Canada), and allowed to develop for 30 minutes. The reaction was stopped by adding 50 µL of 2M H₂SO₄, and immediately read by absorbance at 450 nm.
Optimum antigen and primary antibody concentrations were determined by graphing the optical density data acquired following completion of the checkerboard titration protocol, and selected based upon a point which provided maximum primary antibody binding with minimal background signal. The optimum concentrations for WCS and PSC were selected as 0.3125 µg/mL and 1/80 respectively. The above workflow was utilized for all custom serum ELISA work, at these concentrations. ELISA on experimental samples were run in quadruplicate, with the 96 well plate divided into four replicate quadrants to minimize edge effect. ELISA data were normalized by running a dilution series of the PSC (dilutions of 1/80, 1/160, 1/320, 1/640, and 0) in each quadrant, followed by calculation of a relative antibody titre of the experimental calf serum against the PSC, reported as sample:positive control value (S/P). A significance cutoff was calculated to be a value of 0.15, determined by taking the maximum observed value in serum from a control animal, plus two standard deviations of observed values across all negative controls (uninoculated/non-

4.3.6 SDS-PAGE and protein transfer

To visually detect Map-specific antibodies within experimental calf serum, SDS-PAGE of Map WCS was performed using the Criterion midi protein gel system (Bio-Rad, Mississauga, Ontario, Canada), with the Criterion TGX 4-15% precast protein gels with a single IPG/Prep well (Bio-Rad, Mississauga, Ontario Canada). Map WCS diluted in 2x Laemmli sample buffer (Bio-Rad, Mississauga, Ontario Canada) with 5% β-mercaptoethanol (Sigma-Aldrich, Oakville, Ontario, Canada) at a final protein
concentration of 50 µg/mL. The sample was heat denatured at 95°C for 5 minutes, recovered on ice for 1 minute, and loaded on the pre-cast SDS-PAGE gels. Gels were run at constant 200v in TGS buffer (Bio-Rad, Mississauga, Ontario, Canada). When the dye front reached the bottom of the SDS-PAGE gel, the gel was removed from the Criterion system, and transferred to a polyvinylidene difluoride (PVDF) membrane using the Trans-blot turbo system (Bio-Rad, Mississauga, Ontario, Canada) and the TGX turbo midi protocol (25v constant, 7 minutes). After transfer, the PVDF blots underwent periodate oxidation using 5 µM sodium periodate (Sigma-Aldrich, Oakville, Ontario, Canada) in 50 mM sodium acetate (Sigma-Aldrich, Oakville, Ontario, Canada; pH 4.5) to remove carbohydrate contamination of the blot as previously described [25, 26]. Blots were dried and stored at room temperature for up to one week prior to use.

4.3.7 Western blotting

Protein blots were re-activated in methanol, rinsed in PBS, and blocked for 1 hour at room temperature in blocking buffer (PBST with 5% sheep serum). The blot was then cut into 1cm strips prior to incubation in different primary antibodies, consisting of individual calf sera. To reduce background antibody binding, the experimental calf sera underwent four rounds of absorption against 20 µg/mL *Mycobacterium smegmatis* WCS, in a 96 well microtitre plate for 2 hours at room temperature with gentle agitation. Protein blot strips were then added to the pre-absorbed experimental serum diluted 1/100 with blocking buffer and incubated at 4°C overnight with gentle agitation. Strips were washed three times by incubating in PBST at room temp with gentle agitation and subsequently incubated for one hour at room temperature in secondary antibody (sheep-anti-bovine
IgG, described above, 1/10000 in blocking buffer). After secondary incubation, the blots were washed three times in PBST and detection was performed by chemi-luminescence using Western Lightning ECL Pro (Perkin-Elmer Health Sciences Canada, Woodbridge, Ontario, Canada). Bands were visualized and photographed using the ChemiDoc XRS+ imaging system (Bio-Rad, Mississauga, Ontario, Canada).

4.3.8 Protein sequencing

Sera from all calves at the following time points were processed by western blotting: pre-inoculation, 4 weeks post inoculation (wpi), 12 wpi, 20 wpi, and 28 wpi. Bands of interest from western blots were visually selected based upon their presence in Map-exposed calves post-inoculation, but not in control calves or at pre-inoculation time-points. Selected bands containing proteins of interest were excised from the protein gel, and submitted to the University of Guelph Advanced Analysis Center for in-gel tryptic digestion and subsequent protein identification by high-performance liquid chromatography and tandem mass spectrometry (HPLC-MS/MS) [27]. Peptide sequences were filtered using a 1% false discovery rate threshold, and a minimum peptides threshold of two.

4.3.9 Protein identification and gene ontology

Peptide sequences from mass spectrometry were identified by protein BLAST search, using the NCBI blastp suite with the non-redundant protein sequences database. The full-length protein sequences for identified proteins were analyzed for gene ontology (GO) terms using InterProScan (EMBL-EBI, Hinxton, United Kingdom), with protein
classification based on biological process and cellular component [28]. GO terms were summarized using REVIGO, using Mtb as a gene ontology reference database [29].

4.4 Results

4.4.1 Immune response testing

HIR testing of calves in this study demonstrated that all calves tested were consistent with average immune responders, both for AMIR and CMIR traits, when compared with the large cohort of animals tested with this technology.

4.4.2 WCS serum ELISA

The custom WCS serum ELISA data for the monensin-free and monensin-supplemented calves are shown in Figures 4.1-4.4 and 4.5-4.8 (respectively), panels A-D. At the 20 and 28 wpi time-points in the monensin-free calves (Figures 4.3 and 4.4), S/P values above the calculated significance threshold of 0.15 were observed in 50% (2/4) of Map-inoculated calves (1131 and 1133; 1075 and 1076, respectively). No other calves showed S/P values above the significance threshold of 0.15 at any time-point in this study. No monensin-supplemented calves in either the Map-inoculated or control groups had S/P values above the significance threshold at any time-point in this study.

4.4.3 Western blotting

Figures 4.9 and 4.10 show representative western blots from a subset of paired calf serum including monensin-free (Figure 4.9) and monensin-supplemented (Figure 4.10) calves at pre-inoculation and at 28 wpi. Though pre-absorption reduced the background considerably, there was significant background observed in all of the western
blots. In particular we noted several conserved reactive bands present in both uninfected controls and Map-inoculated calves, likely due to exposure to conserved environmental mycobacteria or other related bacterial antigens. Western blot strips were aligned visually after imaging to allow for more accurate selection of bands uniquely present in Map-inoculated calves compared to uninoculated control calves. Faint bands were detected in the Map-inoculated calves post-inoculation but not pre-inoculation, and were absent from the control calves at all time-points; however, these bands were not consistently observed in all Map-inoculated calves. There was also a mild, though subjective, overall decrease in bands in the monensin-supplemented calves compared with the monensin-free calves.

4.4.4 Band selection, protein identification, and gene ontology

A total of eight bands from Map-inoculated calves at the 28 wpi time-point were visually selected for further investigation by protein sequencing as shown in Figure 4.3. These bands were selected based on observations in the monensin-free 28 wpi group, and based on their presence in the Map-inoculated calves at 28 wpi, but not detected in the uninoculated control calves or in any of the calves at the pre-infection time-point. None of these bands identified were consistently seen in all Map-inoculated calves at 28 wpi in this study. From the eight selected bands from western blots (Figure 4.9) for sequencing by HPLC-MS, a total of 25 distinct proteins were subsequently identified. With one exception (hypothetical membrane protein MAP3291c), none of these proteins identified were intrinsically associated with the mycobacterial cell wall, though some have previously been identified in these locations as a secondary biological function (moonlighting function; locations and functions discussed below). The 25 proteins
identified are listed in Table 4.1. Also in Table 4.1, references are provided to previous studies which have identified these proteins either as a part of other mycobacterial protein or antigen studies.

GO terms defining protein biological process for each of the 25 proteins identified from the 8 bands selected in this study were identified using InterProScan, and are summarized in Table 4.2. The REVIGO tree map of biological terms clustered by parent classification is shown in Figure 4.11. The size of each term’s box represents the relative abundance, based on number of peptides identified by MS for the protein, and the number of proteins associated with each GO term. Proteins identified in this study were primarily associated with various metabolic processes, subdivided into three categories: metabolic process, nucleic acid metabolic process, and protein metabolic process. Response to stimulus (iron or stress) was the next largest category of biological process for proteins identified by MS in this study, followed by homeostatic process (iron homeostasis), and unclassified biological processes. Analysis of cellular localization of proteins discovered by MS from calves in this study is shown in Figure 4.12. The majority of proteins were localized to the cytosol (42.5%), with the next highest classification being plasma membrane (27.5%). Six of the proteins identified were associated with multiple cellular compartments, likely reflecting activity in multiple cellular locations.

4.5 Discussion

In this study we set out to determine whether Map-specific antibodies could be detected in the serum of calves early after experimental intestinal Map-inoculation. We then sought to identify Map proteins with potential for use as serodiagnostic biomarkers.
in calves with early intestinal Map infection. We also investigated the effects of oral monensin supplementation altered development of early serum antibody responses in these Map-inoculated calves.

Our custom WCS ELISA identified four Map-inoculated calves with significantly increased S/P ratios, based on the specific criteria outlined above. Each of these calves was experimentally Map-inoculated, and we observed the increase in S/P ratios only at the 20 or 28 wpi time-point (Figures 4.3 and 4.4). Based upon data taken from our previous study examining the progression of Map infection in these calves, we can make direct comparisons of the significance of these increased S/P ratios and Map infection status for these animals [21]. In the 20 wpi group, the two calves with a significantly elevated S/P ratio by our custom Map ELISA (1130 and 1133) were both classified as Map infected, as previously described [21, 30]. Calf 1130 was further classified as subclinically diseased, based upon the presence of granulomatous lesions in the intestine and lymph node tissues. Both of these animals tested as Map-negative using a commercial ELISA test, and thus would not be identified as suspects and flagged for culling from a herd during natural Map infection. Of the 28 wpi group Map-exposed calves with significantly increased S/P ratio in the present study, one of these calves (1075) was classified as Map-infected and subclinically diseased. Additionally, this calf was identified as a “suspect” by the commercial Map ELISA [21]. The other calf (1076) was classified as resilient and was Map-negative by the commercial ELISA.

Based on our ELISA work, we can conclude that a subset of Map-inoculated calves in our study demonstrated significant increase in Map-specific serum antibody responses,
in comparison with the non-*Map*-inoculated control calves. This supports the hypothesis that some calves develop *Map*-specific serum antibodies after experimental intestinal *Map* exposure/inoculation, and that at least some calves develop significant amounts of *Map* antibodies which can be observed as early as 20-28 wpi. Furthermore, we also observed that in 3/4 of these calves with significantly increased *Map* antibodies in our custom ELISA, the antibody titres were not detected by a commercial ELISA (chapters 2 and 3). This highlights the poor sensitivity of the currently available serodiagnostic tests, and the need to identify *Map* proteins that induce antibody responses during early enteric infection.

Examination of the western blots revealed numerous bands reacting with serum samples from many animals, which created a substantial challenge in identification of unique proteins reacting with serum from *Map*-exposed but not negative non-exposed control calves. This is not surprising, given that WCS contains an abundance of proteins which share homology with other bacterial or environmental mycobacterial proteins, and thus have conserved epitopes. To simplify selection of bands in this study, we focused our search to the 28 wpi monensin-free calves because they had the widest variation in response to *Map*-inoculation in our first infection study, and represented the longest time course in the monensin-free calves [21]. From this subsection of blots (Figure 4.9), there were a number of distinct bands reacting with sera from *Map*-inoculated calves at 28 wpi but not prior to *Map*-inoculation, or in the sera of uninfected control calves. There was no clear link observed between the infection status of calves including *Map* PCR or lesion
scores (from our previous study), or the results of the WCS ELISA on presence of distinct protein bands in the western blots.

From the eight sequenced bands, we detected 25 mycobacterial proteins based on the cut-off thresholds described above. As shown in Table 4.1, 10 of the proteins identified were also identified in recent studies examining and characterizing the contents and production of Map membrane vesicles, and five of the other proteins identified in the present study were also identified in a study characterizing the Map secretome [13, 31]. From those 15 proteins, only one was previously identified as an immunogenic protein (Bacterioferritin, MAP1595) when tested against Map-ELISA positive cows. Nine of the 25 proteins identified from our western blots have been previously characterized in purified protein derivatives (PPD) or cell envelope proteomic studies, with two having previously been identified as immunogenic in cows with clinical JD: GrpE (MAP3841) in PPD, and again bacterioferritin (MAP1595) in the cell envelope [20, 32, 33]. Interestingly, the data by Leite et al (2015) showed that while bacterioferritin may be considered an immunogenic protein, it showed poor specificity where 60% of uninfected control sera (ELISA-negative cows) also were immunoreactive to the same protein [20].

Comparing our data with a major proteomic study examining the cell envelope of Mtb, 17 of the proteins identified in the present study were also identified and localized to the Mtb cell envelope [19]. Of particular note, enolase (MAP0990) is a protein involved with the glycolytic pathway and was identified in the Mtb cell envelope. The authors of that study hypothesized that in this context, enolase acts as a cell surface plasminogen binder, and may confer protective immunity to Mtb infection [34]. Plasminogen binding by
enolase is quite well known amongst pathogenic bacteria, where it plays a crucial role in
tissue invasion and cell entry, though no data on enolase activity or plasminogen binding
as a factor of pathogenesis are available for Map [35].

GO analysis revealed that the majority of these Map proteins were primarily
localized to the cytosol (42.5%) or plasma membrane (27.5%). Six of the Map proteins
sequenced in our study had multiple (three or four) identified cellular components. This is
likely representative of moonlighting activities, such as enolase (MAP0990) as a putative
plasminogen binding protein, or aconitase (MAP1201c) which is found to play a role in
iron ion homeostasis in Mtb [36]. With regards to biological processes (Figure 4.11), the
majority of Map proteins sequenced in this study are likely involved with some form of
metabolic process, which includes proteins involved with DNA replication, transcription,
and translation. The GO analysis of these proteins suggests that these proteins are
primarily associated with intracellular processes and are not integral membrane proteins,
with the notable exception of MAP3291c, which is a hypothetical integral membrane
protein. This is consistent with other studies discussed above, which have sought to
identify novel immunogenic proteins in the Map secretome, membrane vesicles, cell
envelope, and PPD.

In the ELISA testing of the monensin-supplemented calves, no single calf showed a
significant increase in S/P ratio. This is consistent with our data from the serum western
blots of monensin-supplemented calves, which appear to have a minor, albeit subjective,
decrease in number and intensity of protein bands. This may support the concept of an
immunomodulatory effect of monensin as previously hypothesized, however there are
several contributing factors which may account for overall decreased seropositivity by custom ELISA observed in both the monensin-free and monensin-supplemented groups in this study. WCS was used in our custom ELISA, and this is highly complex antigen source with a large number of cross-reactive or non-immunogenic antigens, and therefore, may be not useful for detecting low levels of *Map*-specific antibodies in serum due simply to dilution of *Map*-specific immunogenic antigens bound to the microtitre plate well. Second, as has been discussed previously in the literature, it is possible that the time-points observed in the present study are too early to detect significant antibody production in the serum of *Map*-inoculated calves [11]. Contrary to our original hypothesis (that calves would become infected and demonstrate progress of intestinal *Map* infection with this model), most calves of both groups (monensin-supplemented and monensin-free groups) had no evidence of persistent enteric *Map* infection at the later timepoints (20 and 28 wpi). Therefore, it is not surprising to not detect *Map* specific serum antibody in these calves; however, calves in the monensin-supplemented groups did not show any significant increase in ELISA S/P ratios at any time point.

The inclusion of HIR testing on a subset of calves in this study was utilized to confirm that all calves in this study were considered average immune responders and to identify any animals which may have altered immune responses. This has been utilized previously as a means of identifying animals which may be more susceptible or resilient to disease. It was also a means of identifying explanations for altered antibody responses in this study. Since all animals tested were classified as average responders in both AMIR and CMIR (as expected), we conclude that these calves have average/normal antibody
responses (not enhanced or reduced), and that these data are thus representative of Holstein calf population at large.

In conclusion, this study sought to characterize early \textit{Map}-specific antibody responses in calves following experimental inoculation. We have shown that early serum antibody detection using a custom \textit{Map} WCS ELISA in experimental intestinal \textit{Map} infection of calves is detectable as early as 20 wpi, and detection of low levels of antibody responses in \textit{Map}-infected calves is possible prior to development of seropositivity based on a commercially available ELISA. This led us to attempt to identify novel immunogenic antigens which may have potential as serodiagnostic biomarkers of infection. We identified 25 proteins by western blotting and protein sequencing which may be immunogenic, and compared these with what has previously been observed in \textit{Map} and \textit{Mtb} studies. The proteins identified in WCS share many similarities with proposed early biomarkers from \textit{Map} and \textit{Mtb}; however, to our knowledge this is the first study which has sought to characterize these proteins in calves with early experimental intestinal \textit{Map}-inoculation. As is evident from data on \textit{Map} membrane vesicles and the \textit{Map} secretome, it is possible that these proteins are released from \textit{Map} during early infection and provide an antigen source for early antibody development. Lastly, we found that oral monensin supplementation is associated with decreased or delayed serum antibody responses in calves. This may merely represent reduced antigen exposure in these calves, or it may represent an immunomodulatory effect of monensin, though the mechanism for this remains unknown [22].
Future work is required to further purify, separate and confirm that the sequenced proteins identified in this study are immunogenic and thus responsible for the specific antibody bands observed on western blots. In addition, characterization of their specificity and sensitivity at various stages of intestinal *Map* infection of cattle is required in order to determine their potential use in diagnostic testing. As a major limitation to the study, the use of WCS as an antigen source resulted in significant background cross-reactivity, as evident on the western blots. This impeded the ability to clearly identify antibody responses on western blotting which were directly attributable to *Map* infection, and may have negatively impacted the sensitivity of the ELISA. In addition, the use of a direct ileal *Map*-inoculation method of experimental infection may result in antibody responses which are not observed in natural infection by *Map*; this should be further investigated, and so additional work is required to confirm the relevance of these findings.

4.6 Acknowledgements
The authors would like to thank the University of Guelph Mass Spectrometry Facility, particularly Dr. Dyanne Brewer, for their help in developing the proteomics workflow. We would also like to thank Dr. Sarah Schorno for her assistance with the gene ontology workflow.

4.7 References


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4.8 Figures and Tables

**Monensin-Free 4 wpi**

![Graph](image)

**Figure 4.1** WCS ELISA results of the monensin-free 4 week post inoculation (wpi) calves.

The figure depicts the S/P ratio of the calves pre-inoculation, and every 2 weeks thereafter. *Map*-inoculated calves are shown in red, while control calves are shown in blue. The black line at a S/P ratio of 0.15 represents the statistical cutoff value for significant increase in antibody responses.
Figure 4.2 WCS ELISA results of the monensin-free 12 week post inoculation (wpi) calves. The figure depicts the S/P ratio of the calves pre-inoculation, and every 4 weeks thereafter. *Map*-inoculated calves are shown in red, while control calves are shown in blue. The black line at a S/P ratio of 0.15 represents the statistical cutoff value for significant increase in antibody responses.
Figure 4.3 WCS ELISA results of the monensin-free 20 week post inoculation (wpi) calves.

The figure depicts the S/P ratio of the calves pre-inoculation, and every 4 weeks thereafter. *Map*-inoculated calves are shown in red, while control calves are shown in blue. The black line at a S/P ratio of 0.15 represents the statistical cutoff value for significant increase in antibody responses.
Figure 4.4 WCS ELISA results of the monensin-free 28 week post inoculation (wpi) calves.

The figure depicts the S/P ratio of the calves pre-inoculation, and every 4 weeks thereafter. Map-inoculated calves are shown in red, while control calves are shown in blue. The black line at a S/P ratio of 0.15 represents the statistical cutoff value for significant increase in antibody responses.
Figure 4.5 WCS ELISA results of the monensin-supplemented 4 week post inoculation (wpi) calves.

The figure depicts the S/P ratio of the calves pre-inoculation, and every 2 weeks thereafter. *Map*-inoculated calves are shown in red, while control calves are shown in blue. The black line at a S/P ratio of 0.15 represents the statistical cutoff value for significant increase in antibody responses.
Figure 4.6 WCS ELISA results of the monensin-supplemented 12 week post inoculation (wpi) calves.

The figure depicts the S/P ratio of the calves pre-inoculation, and every 4 weeks thereafter. *Map*-inoculated calves are shown in red, while control calves are shown in blue. The black line at a S/P ratio of 0.15 represents the statistical cutoff value for significant increase in antibody responses.
Figure 4.7 WCS ELISA results of the monensin-supplemented 20 week post inoculation (wpi) calves.

The figure depicts the S/P ratio of the calves pre-inoculation, and every 4 weeks thereafter. Map-inoculated calves are shown in red, while control calves are shown in blue. The black line at a S/P ratio of 0.15 represents the statistical cutoff value for significant increase in antibody responses.
Figure 4.8 WCS ELISA results of the monensin-supplemented 28 week post inoculation (wpi) calves.

The figure depicts the S/P ratio of the calves pre-inoculation, and every 4 weeks thereafter. *Map*-inoculated calves are shown in red, while control calves are shown in blue. The black line at a S/P ratio of 0.15 represents the statistical cutoff value for significant increase in antibody responses.
Figure 4.9 Western blots of the monensin-free 28 week post inoculation (wpi) calves.

Each calf was tested pre-inoculation (left strip) and at 28 wpi (right strip). Calves 1050 and 1078 were un-inoculated control animals, while calves 1073, 1075, 1076, and 1082 were Map-inoculated. In addition, the eight bands selected for protein mass spectrometry are indicated.
Figure 4.10 Western blot of the monensin-supplemented 28 week post inoculation (wpi) calves.

Each calf was tested pre-inoculation (left strip) and at 28 wpi (right strip). Calves 1265 and 1266 were un-inoculated control animals, while calves 1260, 1261, 1262, and 1264 were Map-inoculated.
Figure 4.11 Gene ontology (GO) terms associated with biological process for proteins sequenced via mass spectrometry.

Terms are grouped by the primary term (seen as individual boxes), and by common parent term (represented by colours). The size of each term is a representation of frequency observed, both by number of peptides observed in a sample for each protein, and the number of proteins across all samples with the associated GO term.
Figure 4.12 Proportion of gene ontology (GO) terms associated with cellular component.

GO ID shows subcellular location, based on known location of function for proteins identified via mass spectrometry. A classification as cellular component refers to a protein where the subcellular localization is unknown or has not been characterized.
Table 4.1 Proteins identified by mass spectrometry, with associated reference to previous studies characterizing the protein.\textsuperscript{1,2}

<table>
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<th>Mass</th>
<th># Peptides</th>
<th>Protein</th>
<th>Map Gene ID</th>
<th>Map MV and Secretome</th>
<th>Map PPD and Cell Envelope</th>
<th>Mtbp Studies</th>
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<td>MV + Secretome</td>
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<td>Membrane (Gu, 2003)</td>
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<td>MV</td>
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<td>MV + Secretome</td>
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1. \textit{Map} studies highlighted in red indicate that the protein was characterized as immunogenic in the associated research.

2. \textit{Map} secretome study and MV study refer to Facciuolo (2013) and Martin (2016), respectively.
Table 4.2 Gene ontology (GO) terms for Biological Process (GO:BP) and Cellular Component (GO:CC) for proteins identified via mass spectrometry.

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<th>Protein Name</th>
<th>Map</th>
<th>Gene ID</th>
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<th>Biological Process</th>
<th>GO:CC</th>
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<td>N-acetyl-gamma-glutamyl-phosphate reductase</td>
<td>MAP1361</td>
<td>GO:006526</td>
<td></td>
<td>Arginine Biosynthetic Process</td>
<td>GO:0044445</td>
<td>Cytosol</td>
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</table>
5 General Discussion

In this thesis, we sought to examine three key factors of enteric \textit{Map} infection, utilizing the previously described direct ileal Peyer’s patch \textit{Map} inoculation calf model. In chapter 2, we examined the outcomes of direct intestinal \textit{Map} inoculation of young calves to determine how intestinal \textit{Map} infection progresses over time, with the objective of studying how tissue and lymph node colonization with \textit{Map} affects development of disease. In chapter 3, we used the enteric \textit{Map} model to investigate the potential beneficial effects of oral monensin sodium as a chemotherapeutic agent on the persistence and progression of intestinal \textit{Map} infection. Lastly, in chapter 4, we sought to identify \textit{Map}-specific host antibody responses in serum of calves following experimental intestinal \textit{Map} infection and to characterize immunogenic proteins which have potential use as biomarkers of early enteric \textit{Map} infection.

5.1 Chapter 2: Modelling disease progression

Our hypothesis for chapter 2 was that following induction of enteric \textit{Map} infection by direct intramural ileal inoculation of a large dose of live \textit{Map} bacteria, calves would progress rapidly through early and subclinical phases of \textit{Map} infection toward clinical disease. Thus, we wanted to utilize the model to study the poorly understood early infection period as well as the so-called transition phase between subclinical and clinical \textit{Map} infection. This hypothesis was driven by our previous data gained from the model, which showed that 100% of calves showed persistent enteric infection at 90 days post \textit{Map}-inoculation and was supported by mathematical modelling work done by Magombedze and colleagues in 2014 showing how progression of intestinal \textit{Map} infection
and eventual development of JD was directly impacted by Map bacteria/antigen load within intestinal tissues [1, 2]. These data suggested that a subserosal injection of a high dose of live Map into the distal ileum would not only result in early local colonization and establishment of Map infection within the intestine, but also more rapid progression from localized infection into subclinical and clinical disease.

However, instead of rapid progression from early enteric Map infection toward clinical disease, we discovered that a majority of calves showed a remarkable resilience to Map infection, terminology recently described by Whittington et al, who developed clear case definitions for Map infection and JD [3]. We only followed calves in our study to 28 weeks post inoculation (wpi); however, we found that 50% (2/4) calves showed evidence of resilience to Map infection, while 1/4 calves had evidence of residual Map infection in tissues (a single intestinal tissue positive by PCR but with no evidence of intestinal inflammation or disease). In only 1/4 calves (1075) was there evidence for sustained Map infection in tissues (granulomatous inflammation in intestine and lymph node, Map PCR positive in multiple tissues, presence of Map-specific serum antibodies by commercial ELISA test). Based upon what we observed in calf 1075, we predict that this would be the only calf to continue to progress towards clinical JD, while the remaining three calves in the 28 wpi group would not progress, though the timeline for this transition remains unknown.

The data we report using the direct intestinal Map challenge model in chapter 2 of this thesis are consistent with data reported by Begg et al (2018), which was the first group to report resilience and recovery in calves after Map infection [4]. In their study, the
researchers examined calves after oral \textit{Map} inoculation for a maximum period of five years. At the termination point of that study, 35\% of calves were classified as \textit{Map} infected and 10\% of calves were classified as sub-clinically diseased, based upon detection of \textit{Map} and associated granulomatous lesions within the intestinal tissues. These numbers are also consistent with what is observed in natural \textit{Map} infection in endemic \textit{Map}-infected herds where although the exposure rate is very high, the individual infection rate remains quite low. In endemic herds, only a small portion of calves exposed to \textit{Map} are ever considered \textit{Map} infected, and an even smaller proportion of individual animals ever have evidence of clinical Johne’s disease \cite{5–7}. As we observed and discussed in chapter 2 of this thesis, we have effectively recapitulated patterns of \textit{Map} resilience and susceptibility in calves within a 28 week period that are highly similar to what is observed not only in natural infection and in oral infection models over a 5 year period. This is significant, because it allows the direct Peyers patch \textit{Map} challenge model to be applied to fields such as vaccine or chemotherapeutic research, where the effects of a preventive or therapeutic intervention could potentially be examined and studied in a relatively short period of time, though larger groups of animals would certainly be required for future studies.

A significant limitation of the \textit{Map} infection model utilized and discussed in this thesis is a lack of confirmatory testing in the early post \textit{Map}-inoculation time-points of calves to confirm establishment of \textit{Map} infection within the distal small intestine. The terminology developed by Whittington et al \cite{2017}, which was published well after the conception of this thesis work, defines the appropriate terms for \textit{Map} recovery as apparent resilience in
a calf which has previously been confirmed as \textit{Map} infected [3]. As discussed in chapter 2, what we report as resilience is most likely true recovery from \textit{Map} infection, though without multiple sequential sampling time-points in the same calves we are unable to definitively affirm true recovery. In support of this, 100\% of calves in the original paper describing the model were confirmed as \textit{Map} infected within 90 days post inoculation [1]. This high rate of \textit{Map} infection in this model is consistent with our data seen in the present body of work at 4 and 12 wpi (chapters 2 and 3 of this thesis), and other studies in which the same direct Peyer’s patch \textit{Map} inoculation model was used to study early \textit{Map} infection [8]. It is therefore probable that the calves classified as resilient in this study were in fact initially \textit{Map}-infected, and are true examples of recovery from \textit{Map} infection.

What remains unknown are specific cellular and molecular host mechanisms in the intestinal mucosa at the site of \textit{Map} infection within individual animals that contribute to or determine the ultimate outcome of \textit{Map} infection. Mathematical modeling has predicted that subsets of animals are capable of clearing an established \textit{Map} infection, depending on the host immune response [2]. In that study, which supports data looking at varied immune responses in sheep, the researchers found that a strong cell-mediated immune response can readily clear a local \textit{Map} infection [2, 9]. While the present thesis did not examine specific immune responses locally or systemically in calves during early \textit{Map} infection (that work is currently being performed as part of another thesis), a subset of calves underwent immune response classification using the well validated HIR testing strategy developed by the Mallard lab at the University of Guelph, as described in chapter 4, which characterizes a calf’s cell-mediated and antibody-mediated immune responses.
All calves tested were classified as standard immune responders for both cellular and humoral immune responses. These data strengthen our findings in chapter 2, because the resilience to *Map* infection observed in some calves in this study cannot be entirely attributed to a particular subset of calves with a genetic predisposition for a stronger, more effective immune response.

Overall, the data generated in studies described in chapter 2 of this thesis contribute to the overall understanding of resilience to intestinal *Map* infection in calves. This is the first work examining the phenomenon of resilience or recovery following direct intestinal inoculation of *Map* in lieu of oral infection. Given that we directly injected a large dose of live *Map* directly into tissues of the distal ileum in this model, we believe that resilience to *Map* infection is not merely a factor of reduced *Map* uptake across the mucosal barrier, which can be a major limitation in oral infection models where the pathogen must cross the barrier and then persist locally in the intestine. Rather, we hypothesize that true resilience or recovery from intestinal *Map* infection is an active process after *Map* crosses the intestinal barrier and is governed by some as yet unknown localized host responses.

### 5.2 Chapter 3: Oral monensin supplementation

The work outlined and discussed in chapter 3 of this thesis was designed to examine the role of oral monensin sodium on progression of *Map* infection in intestinal tissues of experimentally infected calves. We used the direct Peyer’s patch *Map* inoculation model to study the effects of oral monensin supplementation during early intestinal *Map* infection with direct comparison to non-monensin supplemented calves (chapter 2). As reviewed in earlier chapters, monensin is an ionophore feed additive used in the beef and dairy
industries for prevention of coccidiosis and as a growth promoter [11, 12]. Further, there is also a label claim in Canada for use of monensin sodium in the format of a controlled release capsule (CRC) in adult dairy cows to control fecal *Map* shedding in adult cattle [13, 14]. It has also been reported in numerous studies that monensin reduces *Map*-associated lesions and their progression, antibody responses, and fecal *Map*-shedding in adult dairy cows naturally infected with *Map* (reviewed in chapters 1 and 3). Based upon these previous studies, we hypothesized that oral monensin supplementation would reduce persistence and progression *Map* infection in calves and set out to answer this question using our direct intestinal *Map* inoculation model. However due to the initial data observed in these calves which showed resilience/recovery from experimental *Map* infection rather than persistence and progression of disease, our hypothesis for chapter 3 was modified. We then hypothesized that monensin supplementation would enhance resilience to infection and early *Map* clearance, evidenced by decreased *Map*-induced intestinal lesions, reduced fecal *Map* shedding, and reduced serum antibody responses.

We found that resilience to persistent intestinal *Map* infection was first observed in 1/3 monensin-supplemented calves at 20 wpi, eight weeks earlier than in the monensin-free calves. At 28 wpi, while the same proportions of *Map*-inoculated calves were classified as resilient (2/4), none of the monensin-supplemented calves showed evidence of subclinical disease, compared with one calf (1076) from the monensin-free study (chapter 2). There was also no evidence of increased antibody titres or seroconversion in any monensin-supplemented calves based on results from the commercial ELISA test.
Based upon these data, none of the monensin-supplemented calves in the 28 wpi time-point of this study appear to be progressing towards clinical JD.

The data on monensin supplementation in calves during *Map* infection has frequently shown that monensin acts to reduce the number of *Map*-associated granulomatous lesions [15–17]. While this is consistent with what was observed in our 20 and 28 wpi groups, intestinal lesion scores at both the 4 and 12 wpi time-points in monensin-supplemented calves were significantly higher than intestinal lesion scores in the monensin-free calves at the same time points. The mechanisms or reasons for this are not clear, but in addition to its antimicrobial effects, monensin has been reported to have immunomodulatory effects [18, 19]. In cattle, oral monensin supplementation was associated with significantly reduced *Map*-specific serum antibodies as measured by commercial ELISA, compared with monensin-free animals [20]. It is possible that monensin is acting to enhance early immune responses, associated with more pronounced lesions in the early time-points, and more effective killing of *Map* at the site of infection. Some reports suggest that monensin modifies conditions within the phagosome responsible for blocking intracellular survival of *Brucella suis* and *Legionella pneumophila*, though the specific mechanisms responsible are unclear, and may not be applicable to *Map* infection in vivo within the bovine intestine [21, 22].

These potential immunomodulatory effects of monensin complement other reports of direct bacteriocidal capacity of monensin against *Map* in vitro [16, 23]. It is possible that monensin acts upon multiple pathways within the calf intestine, which synergistically provide a beneficial effect to the host against *Map* infection as we have observed in young
calves in this study; however work should be done to understand the mechanisms involved.

Overall, chapter 3 identified that oral monensin-supplementation may result in increased resilience or resistance to localized experimental enteric Map infection in calves even though several of the monensin supplemented calves in this study had more severe Map-induced granulomatous enteritis in the early time points following experimental Map inoculation. One significant limitation of this study, as discussed in depth in chapter 3, was the low sample size, and as a result a specific statistical effect of monensin on histologic lesion scores and detection of Map by PCR and ZN staining was not observed. Immune response classification via high immune response (HIR) technology was performed on a subset of the monensin supplemented calves (chapter 4), and all were classified as average immune responders for both cellular and humoral immune responses, comparable with what was observed in the monensin-free calves. We observed a consistently high infection rate using this model (100%) in calves at 4 and 12 wpi, which further supports the hypothesis discussed above, that what was reported as resilience in chapters 2 and 3 is in fact true recovery from Map infection.

5.3 Chapter 4: Early antibody responses

The objective of chapter 4 of this thesis was to identify and characterize the Map-specific serum antibody responses to early experimental intestinal Map infection in calves. We set out to accomplish this using custom designed ELISA and western blot assays. We then selected several distinct bands identified on western blotting and sought to identify, through protein mass spectrometry, specific serum antibody-inducing Map
proteins in these calves. Previous work from our collaborators and other proteomic studies of Map have identified numerous cytosolic Map proteins secreted by the bacterium (the so-called secretome), within membrane vesicles, and/or as components of Map cell wall preparations [24–28]. Furthermore, a number of these proteins have been identified as immunogenic because they were linked with specific antibody reactivity in the serum of cows with naturally-occurring clinical Johne’s disease. We hypothesized that many of these same cytosolic proteins would be identified as immunogenic proteins in our intestinal Map-inoculated calves. Given the study design, we also sought to evaluate how oral monensin affected serum antibody development during early experimental Map infection. Based upon our previous work in chapters 2 and 3 of this thesis, we hypothesized that monensin-supplemented calves would have decreased serum antibody responses which would correlate with reduced Map persistence and early localized Map resilience, when compared with monensin-free calves.

By ELISA, we identified four calves with S/P ratios above our calculated significance cut-off threshold. All four of these calves were Map-inoculated monensin-free animals. Specifically, we observed two (2/4) of these calves at 20 wpi and two (2/4) in the 28 wpi group. This included one calf which tested as suspect on the commercial ELISA (1075, monensin-free 28 wpi group). Of these four calves with increased S/P ratios, one was classified as resilient (1076, monensin-free 28 wpi group) which raises the question of how well active Map infection can be differentiated from Map-exposure by serological testing.
Using western blots to identify *Map*-specific antibodies by visible banding patterns, we observed significant background (non-specific) protein binding which was present in un-inoculated control calves. This limited our ability to identify unique bands of interest. We attempted to reduce the background binding by removing common mycobacterial antigenic epitopes by pre-absorbing the calf serum samples with *M. smegmatis* (a closely related environmental mycobacterial species). While this did greatly reduce the background signal (data not shown), there were still a large number of non-specific bands which challenged our ability to make significant comparisons between groups. The 28 wpi group from the monensin-free calves was selected as representative, given that this was the longest monensin-free time-point and had the widest variations in responses to *Map*-inoculation. We selected eight distinct and unique bands, and identified 25 proteins by mass spectrometry, which were found to be present in a subset (but not all) of the *Map*-inoculated calves in this project. These proteins were primarily identified as cytosolic proteins associated with various metabolic processes. In fact, 14/25 had previously been identified in *Map* proteomic work discussed in chapter 4, though only two of these 14 were previously reported to be immunogenic in adult cows with subclinical *Map* infection. The identification of these proteins highlights the potential significance of non-surface proteins as potential antigenic peptides during early *Map* infection.

None of the monensin-supplemented calves in this study had significantly increased serum antibody responses by ELISA as measured by S/P ratios. Furthermore, monensin-supplemented calves had a clear, albeit subjective, reduction in the abundance of protein
bands detected by western blotting. This suggests that monensin may decrease serum ELISA responses in Map-infected calves, as has previously been hypothesized [20].

Overall, chapter 4 shows that limited Map-specific antibodies are generated during the first several months following experimental enteric Map infection of calves. Further characterization of these antibody responses may support future additional work with the goal of understanding specifically which Map proteins are antigenic during the early enteric Map infection period in calves, a period which remains poorly understood but may yield significant knowledge in the area of detecting early exposure and infection. It has previously been observed that Map inhibits antigen presentation and serum antibody development during early infection [29]. Therefore, the secreted proteins highlighted in this study (as opposed to conventionally processed and presented Map antigens derived from the cell wall of the bacterium) are an attractive target for study. Secreted proteins from viable Map could presumably stimulate host antibody responses earlier during localized persistent enteric Map infection, and are therefore an excellent candidate biomarker for early Map infection. We identified 25 putatively immunogenic proteins in chapter 4 of this thesis, some of which had previously been identified and recognized as components of the Map secretome or of membrane vesicles.

5.4 Limitations of this work

We identified the same major limitations in both chapters 2 and 3 of this thesis. First, the inability to confirm an animal as Map infected as previously defined following Map inoculation limits our ability to identify an animal as truly resilient or recovered from Map infection (i.e. repeated measures from the same animal are required). While we believe
we have provided substantial evidence to support the validity of our model in reliably inducing enteric \textit{Map} infection, future work using this experimental model would benefit from inclusion of an early tissue intestinal biopsy to confirm \textit{Map} infection early after experimental inoculation. While this presents a significant challenge with regards to technical aspects of the procedure and animal welfare, the information gained in this model would provide significant advantage and clarify early post-inoculation events in these calves.

A second major limitation in chapters 2 and 3 is the low number of calves in each infection group. Calculations to determine group sizes, as discussed in section 1.6, were based on previous work where this model was found to be highly repeatable, with 100\% infection efficiency at 12 wpi and little variance in tissue or systemic immune responses. Due to the highly varied responses to \textit{Map} inoculation we observed in timepoints after 12 wpi, there was a loss of statistical power based on classification and grouping of calves based on the factors measured in this study. In retrospect, increasing the expected variance and precision would have demanded that we use larger group sizes over fewer time-points, which would have allowed for greater power when examining effects of early \textit{Map} infection and monensin supplementation. This limitation is best observed in data from chapter 3, where no statistical significance of monensin on \textit{Map} infection was observed, despite several major distinctions between the monensin-free and monensin-supplemented calves. In addition, we were limited in our ability to acquire and house large numbers of animals, which required us to run the infection study as a block cohort over a
period of time, which may have further contributed to increased variance in responses of calves.

A major limitation in chapter 4 was that the *Map* whole cell sonicate (WCS) was utilized in this study because we were seeking the broadest possible range of antigen reactivity; however, WCS is an extremely complex antigen source for examining early antibody responses. In the custom ELISAs, this could result in increased background and decreased sensitivity. Because a microtitre plate has a finite capacity to bind protein, increasing the number of non-specific proteins in a sample by using highly complex antigens as in our study could dilute the binding sites of highly antigenic proteins, and thus compromise the accuracy of the assay. This was also evident in the western blot analysis, where a large number of background protein bands were consistently detected in all calves, despite pre-absorption of individual calf serum with the closely related but non-pathogenic *M. smegmatis*. While the use of WCS did identify a number of putatively antigenic proteins in our study, bands processed by mass spectrometry all identified more than one protein per band, which may not have all been antigenic. A less complex antigen source, such as cell membrane preparations or *Map* secretome, may have enhanced the interpretation of data generated in these western blots.

### 5.5 Future directions

There are three primary questions that rise from this thesis. First, the specific host factors responsible for determining or driving host resilience and/or apparent recovery from *Map* infection in calves remain unknown. While we discussed the potential of host immune responses playing a role in determining the fate of *Map* post-inoculation, we have
not yet completed the ongoing work examining cytokine profiles and cellular immune responses within the local intestinal mucosal tissues (the infection site) of these calves. This work is currently underway as part of another thesis. However, now that we have observed resilience to Map in this experimental model, a follow-up study with a larger cohort at 28 wpi, or longer designed to examine host immune responses at the site of inoculation over time may contribute to our understanding of the factors responsible for resilience and recovery from Map inoculation, and what factors permit colonization, persistence and progression of Map infection and disease. It would also be beneficial to compare infection using different Map strains to determine if the data in this study are specific for the Map inoculum used in this study (gc86, chapter 2).

A second remaining knowledge gap is to clarify the role of oral monensin supplementation during intestinal Map infection. While we presented some evidence suggesting that monensin-supplemented calves have increased resilience to Map infection, we were unable to demonstrate a statistical effect of monensin on key diagnostic markers of Map infection examined in this study (Map PCR, AFB by ZN staining, and histologic lesions observed in H&E stained tissues, described in chapters 2 and 3). As previously discussed, larger cohorts would greatly improve the ability to statistically examine the effects of oral monensin supplementation on experimental Map inoculation.

Finally, there remains a significant potential for further work investigating the Map-specific antibody responses during early enteric infection in these calves. While we have already discussed the advantages that more focused and novel purified protein
preparations (of microvesicles or secreted proteins in particular) may have on sensitivity and specificity of ELISA and western blot experiments, there are other avenues which are of interest for future investigation. We have explored some preliminary attempts using two-dimensional SDS-PAGE gels to help improve protein resolution using the crude Map WCS as capture antigen, which may allow us to better identify and characterize immunogenic Map proteins. We have also done some preliminary experiments to examine intestinal mucosal antibodies specific for Map; samples were obtained from some animals in this study by scraping the intestinal mucosa for use as primary antibody in a custom Map ELISA. Work using other models of experimental Map-inoculation has found that mucosal antibody responses may be observed much earlier in the post-inoculation period, compared with serum antibody responses [30]. This has shown some promising early results in identifying Map-inoculated calves, though there is insufficient evidence to comment on the reproducibility or ultimate value of this approach.

Johne's disease is an extremely fascinating example of the complexities of mycobacterial infections. Because the bacterium is difficult to therapeutically address, research seeking to understand host resilience and recovery from Map infection are a major area for future studies and will lead to novel methods to control and manage this disease in the field. Unfortunately, the specific factors that govern resilience and recovery versus Map persistence and progression remain a mystery. However, this thesis has provided significant insight into the fate of Map following direct ileal inoculation, the efficacy of potentially useful chemotherapeutic agents, and identification and limited characterization of Map antibodies that develop in serum during early intestinal Map
infection. The infection model utilized in this thesis is reproducible in establishing mucosal Map infection in calf intestine, which other models including oral, intestinal loop, and lymphatic inoculation models do not achieve. Therefore, we believe the model holds promise for use in evaluating novel JD diagnostics, therapeutics, and preventive strategies of persistence. Our hope is that this thesis helps to build a foundation upon which these advancements can be built.

5.6 References


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