Investigation of cellular and whole tissue infection, proliferation, and shedding of *Coxiella burnetii* in bovine mammary gland tissue.

Lucy Therese O'Shannessy

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Declaration

This thesis is submitted to The University of Sydney in fulfillment of the requirements for the Degree of Doctor of Philosophy.

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text.

I hereby declare that I have not submitted this material, in either full or in part, for a degree at this or any other institution.

Lucy Therese O'Shannessy

BAnVetBioSc (Hons I)

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Abstract

Coxiella burnetii is a gram negative obligate intracellular bacterium that is the causative agent of Q fever in humans (a debilitating long-term illness) and coxiellosis in animals. Cattle are one of the reported reservoirs for human infection, and while cattle infection and excretion of the bacteria is commonly believed to be largely asymptomatic, reproductive loss can manifest (such as sporadic abortions and stillbirths). The pathogenesis of *C. burnetii* infection in cattle is poorly defined. The bovine mammary gland may have a key role in pathogenesis as *C. burnetii* can be excreted persistently in milk throughout a lactation and, *in vitro*, *C. burnetii* displays a replicative tropism for bovine mammary epithelial cells (compared to other epithelial cell types). It is important to understand the dynamics of infection in the mammary gland as persistent bacterial excretion in milk may contaminate the environment which poses an ongoing zoonotic transmission risk, while *C. burnetii* infection of the udder may impact milk production which has economic consequences for the dairy industry. Therefore, this thesis aimed to firstly, investigate *C. burnetii* excretion in milk and secondly, disease manifestation in the mammary gland. Such information may ultimately aid development of effective control of *C. burnetii* excretion from cows and estimation of the economic and welfare impact of coxiellosis to the dairy industry.

A synthesis of the existing literature on *C. burnetii* infection dynamics in cattle and the mammary gland was provided in Chapter 1 to identify both the knowledge gaps, and motivations, for further research into this topic. The host immune response was identified as a key factor potentially influencing infection outcomes in cattle but as there was a paucity of knowledge on application of suitable tools to measure cell mediated immune (CMI)

response in cattle, Chapter 2 entailed optimisation of a whole blood cytokine recall assay specifically for use with cattle samples. In Chapter 3, a longitudinal study was conducted in a *C. burnetii* endemically infected Australian dairy cow herd to describe bacterial excretion (by multiplex qPCR) and humoral (*C. burnetii* immunoglobulin (Ig) G antibody by Enzyme-linked immunosorbent assay [ELISA]) and cellular immune (using the cytokine recall assay optimised in Chapter 2) responses. Throughout this approximately seven-month study, a variety of samples (blood, placenta, faeces, vaginal fluid, milk) were collected from cows at five timepoints, beginning at approximately three weeks prior to calving until approximately 200 days in milk (DIM) (mid-lactation). In Chapters 4 (longitudinal) and 5 (cross-sectional), the impact of *C. burnetii* infection on milk production in cattle was explored using the bacterial excretion and immune responses described in Chapter 3 as exposure variables, and milk volume, total solids, and somatic cell count (SCC) as outcome variables, as these measures are drivers of dairy herd profitability.

A variety of *C. burnetii* excretion routes and immune responses were described in the endemically infected herd, with excretion of *C. burnetii* occurring through milk at 200 days after calving in 13/133 sampled cows (termed *C. burnetii* milk shedding cows). A focus on individual cow patterns revealed these 13 cows may be persistently infected, as while only some had also excreted *C. burnetii* at calving, they all had persistently high antibodies titres throughout the entire seven-month sampling period which, at early lactation, were significantly higher compared to the non-milk shedding cows at 200 DIM. Together with the low Interferon gamma (IFNγ) response (an important T helper 1 [TH1] cytokine) from the *C. burnetii* milk shedding cows, persistent infection in cows may be driven by a T helper 2 (TH2)

preference. Optimisation of the cytokine recall assay for use with cattle samples may help encourage future cytokine investigations in cattle to determine which specific factors govern *C. burnetii* persistence.

The investigation into the impacts of *C. burnetii* infection in cattle on milk production provided evidence that coxiellosis may be an important disease to the dairy industry. An intrauterine infection was associated with milk production loss, specifically, cows with a placental infection produced 2.4 L/d less volume and cows with *C. burnetii* detected in the vaginal fluid produced 0.2 kg/d less total solids, compared to cows without *C. burnetii* detected in the placenta and vaginal fluid, respectively. Furthermore, the presence of *C. burnetii* in gland quarter milk was associated with significantly higher quarter SCC compared to quarters without *C. burnetii*. An exploration of causation (using directed acyclic graphs) in Chapter 5 led to the development of two competing plausible causal hypotheses to explain this association, namely that *C. burnetii* causes an increased SCC and that a raised SCC increases presence of *C. burnetii* in milk. Ultimately, this thesis helps expand understanding of *C. burnetii* pathogenesis in cattle, including an improved understanding of the different infection states in cows and the impact of these for the public health and dairy industry sectors.

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List of Abbreviations

BCB	Bovine cytochrome b
BMSCC	Bulk milk somatic cell count
CB	*Coxiella burnetii*
CI	Confidence interval
CMI	Cell mediated immunity
CO_2	Carbon dioxide
DAG	Directed acyclic graph
DIM	Days in milk
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FISH	Fluorescence In Situ Hybridization
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IMI	Intramammary infection
L1 – L5	Longitudinal time point 1-5
LPS	Lipopolysaccharide

MALDI-TOF	Matrix Assisted Laser Desorption Ionization-time of Flight
MAP	*Mycobacterium avium* subspecies *paratuberculosis*
OD	Optical density
OR	Odds ratio
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PWM	Pokeweed mitogen
qPCR	Quantitative PCR
RT	Room temperature
SCC	Somatic cell count
SD	Standard deviation
S/P	Sample to positive ratio
TH1	T helper 1
TH2	T helper 2
TNF	Tumor Necrosis Factor
USA	United States of America

Contribution to Conference Proceedings

Authors	Presentation title	Contribution	Conference name and date
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Lucy O'Shannessy, Katrina Bosward, John House, Sam Rowe, Paul Sheehy.	Exploring the causal relationship between *Coxiella burnetii* presence in dairy cow milk and somatic cell count using directed acyclic graphs.	Conference abstract and oral presentation	Sydney School of Veterinary Science Postgraduate and Staff Research Conference, Sydney, Australia, 2023
Lucy O'Shannessy, Katrina Bosward, Ruth Zadoks, Samuel Rowe, Paul Sheehy, John Morton, Victoria Brookes, Bridgette Logan, Mark Westman, Mei Zheng, John House.	Exploring possible underlying causal relationships to explain the association between *Coxiella burnetii* presence in dairy cow milk and somatic cell count using directed acyclic graphs (DAGs).	Conference abstract and poster	International Symposium on Veterinary Epidemiology and Economics, Sydney, Australia, 2024
Lucy O'Shannessy, Katrina Bosward, Paul Sheehy.	Developing a quantitative three-dimensional bovine mammary gland cell model using an automated, high-content confocal imaging platform to investigate *Coxiella burnetii* pathogenesis.	Conference abstract and poster	Australian Society for Microbiology Annual Scientific Meeting, Brisbane, Australia, 2024
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Disclosure and Author Contribution Statement

All chapters have been written to be individually published in peer-reviewed journals. Chapter 2 is currently under review in the Journal of Veterinary Diagnostic Investigation. I personally contributed to this manuscript though the research conceptualisation, sample collection, laboratory testing of samples, statistical analyses, interpretation of results, and writing and preparation of the original and final manuscript.

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Lucy O'Shannessy

20th February 2025

Paul Sheehy (primary supervisor)

21st February 2025

Chapter 1 Literature Review

1.1 Discovery of *Coxiella burnetii*

During 1935, medical pathologist, Edward Derrick, first started investigating an outbreak of febrile illness with unknown cause in abattoir workers in Brisbane, Australia (Derrick, 1937). Using samples from infected humans, he could replicate the disease in guinea pigs but was unable to visualise or isolate a causative agent, leading him to theorise a virus was causing the mystery illness that had been termed "Q" fever (Derrick, 1937). Through collaboration with researchers in Melbourne, Australia, "rickettsia-like" organisms were visualised in splenic samples from infected mice (Derrick, 1937). In America, similar investigations by a rickettsia disease ("Rocky Mountain Fever") research team, that included Herald Cox and Gordon Davis, were taking place into an agent discovered in ticks that was causing febrile illness in guinea pigs and that was observed to have bacterial and viral properties (Davis et al., 1938). By 1938 both the Australian and American groups realised they were investigating the same new bacterium and the two research teams proposed the names *Rickettsia burneti* and *Rickettsoa diaporcia*, respectively. In later years, further investigation into the agent led to an order reclassification (Legionellales) and renaming to *Coxiella burnetii* (Stein et al., 1993).

Today, the febrile illness first investigated in abattoir workers and guinea pigs is still known as Q fever, and the causative agent, *C. burnetii*, is classed by the Unites States of America (USA) Centre for Disease Control and Prevention as a category B bioterrorism agent due to

its high infectivity, aerosol transmission, and potential for debilitating disease (Eldin et al., 2017).

1.2 *Coxiella burnetii* classification and morphology

Coxiella burnetii is an obligate intracellular bacterium that replicates within eukaryotic cells (Coleman et al., 2004). It is classified as gram-negative based on cell wall structure and composition, however, *C. burnetii* stains poorly using the Gram technique. The Gimenez staining method is preferred for visualisation of *C. burnetii* (McCaul and Williams, 1981). There are two morphologically distinct forms of *C. burnetii* that make up its biphasic lifecycle, the small cell variant and the large cell variant (McCaul and Williams, 1981). The small cell variant is approximately 0.2 – 0.5 µm and rod shaped with condensed chromatin that is spore-like as it is metabolically inactive and highly resistant to extreme heat, osmotic pressure and mechanical disruption, allowing it to be environmentally stable (Maurin and Raoult, 1999, McCaul and Williams, 1981). The large cell variant is pleomorphic (commonly round and >0.5 µm), with dispersed chromatin and is the metabolically active replicative form (Maurin and Raoult, 1999, McCaul and Williams, 1981).

1.3 *Coxiella burnetii* genome

In 2003, the first complete sequence of the *C. burnetii* genome was published for the strain Nine Mile phase 1 RSA493 which revealed a 1,995,275 base pair chromosome and a QpH1 plasmid, along with identification of some other key features (Seshadri et al., 2003). For

example, the presence of 32 insertion sequences in the chromosome indicated genomic plasticity, while identification of sodium/ion proton exchangers and drug-efflux systems provided a better understanding of the reasons for pathogen success within the hostile phagolysosome (by maintaining pH haemostasis and removing host-produced antimicrobials, respectively) (Seshadri et al., 2003). The ability to grow *C. burnetii* in axenic culture (for host cell free propagation) and advancements in genetic technologies allowed further *C. burnetii* genetic discoveries (Omsland et al., 2008, van Schaik et al., 2013), including identifying the strong genomic homogeneity between *C. burnetii* strains (Beare et al., 2009). *Coxiella burnetii* strains are divided into six groups based on genetic similarity, with some trends having been identified within groups such as isolates from Europe belonging to group I to IV while isolates from North America fall mainly in group V and VI (Hemsley et al., 2019, Hendrix et al., 1991). Genomic research comparing *C. burnetii* strains to *Coxiella*-like endosymbionts found in ticks suggests *C. burnetii* may have evolved from a tick endosymbiont ancestor to infect vertebrate hosts after a relatively recent gain of virulence factors (Duron et al., 2015). Modern genotyping techniques, including multispacer sequence typing (MST), multiple locus variable number of tandem repeats analysis (MLVA), and single nucleotide polymorphisms (SNP), are important tools for outbreak and ancestral investigations (van Schaik and Samuel, 2012). The selection of the appropriate technique is determined by the aim of the investigation as each has trade-offs in sensitivity, cost, and speed of analysis (van Schaik and Samuel, 2012).

1.3.1 *Coxiella burnetii* phase variation

Coxiella burnetii can exist in one of two antigenic phases: the virulent phase 1 cell that is found in natural infection and which contains a full-length lipopolysaccharide (LPS); and the avirulent phase 2 cell which has a truncated LPS due to a deletion in the O-antigen region and that arises after serial passage in culture (Amano and Williams, 1984, Eldin et al., 2017). The full length LPS is thus one of the few known *C. burnetii* virulence factors which is theorised to aid in evasion of the host cell immune response by preventing binding of antibodies and activation of Toll-like Receptor 4 (Eldin et al., 2017). While the phase 2 cell is avirulent and only produced in laboratory settings (possibly due to a loss of host related pressures) it has still proved useful in expanding understanding of *C. burnetii* pathogenesis by phase comparison studies to highlight mechanisms of virulence used by the phase 1 cell (Capo et al., 1999). Furthermore, others have confirmed similarities between certain intracellular kinetics of the phases and therefore suggest under some circumstances the phase 2 cell is a good model for deriving relevant results to natural infection due to the lower biosafety level requirements compared to the virulent phase 1 (biosafety level two and three standard, respectively) (Howe et al., 2010).

1.4 Lifecycle within vertebrates

Coxiella burnetii can infect a wide range of vertebrates including humans, ruminants, companion animal species (for example, dogs, cats and horses), rodents, marine mammals, wildlife, and birds (Eldin et al., 2017, Psaroulaki et al., 2014).

1.4.1 Uptake: host cell infection

During primary infection this bacterium targets monocytes and macrophages (Amara et al., 2012, Stein et al., 2005). The process of *C. burnetii* internalisation into host cells is considered passive, whereby the bacterium does not actively stimulate its own uptake. Instead, endocytic entry is speculated, facilitated by binding of bacterial ligands to host cell receptors, which initiates a signalling cascade that promotes actin rearrangement of the cytoskeleton and subsequent organism engulfment (Meconi et al., 1998, Rosales et al., 2012). This idea was proposed after it was demonstrated that live and inactivated *C. burnetii* did not differ in their uptake efficiency by mice fibroblasts (Baca et al., 1993). In phagocytes, there has been focused investigations into host-bacterial interactions, which indicate $\alpha_v\beta_3$ integrin is the predominate cellular receptor mediating internalisation (Capo et al., 1999). Furthermore, unlike the phase 2 cell that additionally requires binding to Complement Receptor 3 for entry, the phase 1 cell is speculated to inhibit Complement Receptor 3 activation, which reduces uptake efficiency but improves subsequent internal survival (Capo et al., 1999). In non-professional phagocytes, such as epithelial cells, this bacterial uptake strategy is referred to as a 'zippering' mechanism, although the cellular receptor/s governing *C. burnetii* entry into non-professional phagocytes have not yet been identified (van Schaik et al., 2013). Outer Membrane Protein A is the first identified bacterial surface factor mediating the *C. burnetii* uptake process into non-phagocytic cells (Martinez et al., 2014).

1.4.2 Cellular replication and tissue dissemination

Once *C. burnetii* is internalised within the host cell it manipulates endosomal pathways to allow for maturation of the Coxiella containing vacuole, a phagolysosome-like compartment where bacterial replication occurs (Howe and Mallavia, 2000, van Schaik et al., 2013). Within the Coxiella containing vacuole, the small cell variants morph into the large cell variants which then replicate exponentially by binary fission and by day six post-infection the Coxiella containing vacuole occupies a considerable portion of the cell. Despite its size, the Coxiella containing vacuole does not affect host cell viability as *C. burnetii* prevents apoptotic pathways and activates pro-survival proteins (van Schaik et al., 2013, Voth et al., 2007). At this point, the stationary phase is reached, and large cell variants begin morphing back into small cell variants (Coleman et al., 2004). While the mechanisms of bacterial spread to susceptible cells is not fully characterised, induction of apoptosis at later states of cellular infection may enable spread of the small cell variants (van Schaik et al., 2013, Zhang et al., 2012). Localisation of infection at specific tissue sites can vary greatly and is influenced by a number of factors such as the host species, *C. burnetii* strain and route of infection (Eldin et al., 2017). For example, in guinea pigs, inoculation via intraperitoneal injection lead to diffuse infection of tissue but not presence in the lungs (Baumgärtner et al., 1993), while inoculation via inhalation lead to less diffuse infection of tissue but presence in the lungs (Stein et al., 2005). In ruminants, *C. burnetii* shows a tropism for reproductive tissue, which is expelled during parturition and is one of the ways the bacterium can enter the environment to continue the lifecycle (Roest et al., 2012).

1.5 Q fever

As mentioned in Section 1.1, *Coxiella burnetii* is the causative agent of Q fever, a zoonoses diagnosed in people globally that can have debilitating long-term and sometimes fatal manifestations (Eldin et al., 2017). Q fever outbreak patterns vary from sporadic cases and single-source outbreaks to large scale Q fever epidemics such as that which occurred from 2007 – 2010 in the Netherlands. The latter began from infected goat dairy farms, that resulted in approximately 4000 human infections and the slaughter of approximately 50,000 pregnant goats and sheep (Karagiannis et al., 2009, Van Asseldonk et al., 2013). In Australia, a livestock related occupation is considered the major risk factor for Q fever and includes specific occupations such as abattoir workers, producers, and large animal veterinarians (Sloan-Gardner et al., 2017). A human vaccine, called Q-Vax® (Seqirus), is available in Australia (formalin fixed whole cell *C. burnetii* phase 1 Henzerling strain preparation) which is effective in preventing disease but can have adverse side effects in people that have been previously exposed, and therefore, a pre-screening step is required to assess humoral and cell mediated immune response before administration (Gidding et al., 2009).

1.5.1 Transmission

Coxiella burnetii is very infectious to humans, with transmission occurring most commonly through inhalation of contaminated aerosols or dust (Eldin et al., 2017, Tan et al., 2024). Humans can directly inhale the bacteria from infected animal material (excreted fluids and

tissues) or indirectly from aerosol dissemination of bacteria, for example by the wind. In most countries, domestic ruminants are the primary reservoirs for human infection and while sheep are reported as the species most associated with human outbreaks worldwide, in Australia, cattle are more frequently reported as the source in association with human infection (Graves and Islam, 2016, Tan et al., 2024). Infected cattle, sheep, and goats can excrete *C. burnetii* in large quantities in reproductive tissue, with up to 10^9 organisms per gram having been identified in a naturally infected cow's placenta (Hansen et al., 2011). *Coxiella burnetii* can also be excreted in ruminant milk but ingestion is considered an inefficient route of transmission. One penitentiary study observed some seroconversion from male prisoners who drank unpasteurised milk containing viable *C. burnetii*, however, this was not associated with illness (Benson et al., 1963). While some epidemiological studies investigating sources of Q fever cases have found consumption of unpasteurised milk was associated with infection (Signs et al., 2012), it is difficult to rule out inhalation as the route of entry. In saying this, inoculation of immunocompetent BALB/c mice by oral gavage did lead to bacterial dissemination to spleen, lung, and liver tissue (Miller et al., 2020). Transmission by tick bites and sexual transmission between humans have also been reported in the literature but are thought rare (Graves et al., 2020, Milazzo et al., 2001).

1.5.2 Clinical presentations and management

Approximately 50% of human exposures will result in asymptomatic serological conversion (Anderson et al., 2013). Clinical illness may occur after an incubation period of two to five weeks (Million and Raoult, 2015), with Q fever manifestations broadly classified into three

categories: acute Q fever (affecting approximately 50% of exposed individuals), persistent focalised infection (also known as chronic Q fever), and Q fever fatigue syndrome. Symptomatic acute Q fever presents as a usually self-limiting flu like illness with non-specific symptoms such as persistent headaches, fever, and fatigue (Eastwood et al., 2018). Other more severe manifestations such as hepatitis and pneumonia may occur, with the former more common in Australia and the latter more common in countries like France and Canada, possibly due to strain differences (Eldin et al., 2017, Graves and Islam, 2016). Approximately 80% of acute Q fever infections resolve within three months (Anderson et al., 2013, Melenotte et al., 2020), however, approximately 20% will go on to experience Q fever fatigue syndrome, and up to 5% will develop persistent focalised infection (Anderson et al., 2013, Morroy et al., 2016). The different forms of Q fever can be diagnosed based on clinical presentation and indirect (serological) and direct (molecular) measures (Melenotte et al., 2020). Serological diagnosis relies on known patterns of antibody temporal responses (Dupont et al., 1994, Peacock et al., 1983). Approximately 10 days after onset of symptoms Phase 2 IgM antibodies arise, followed shortly after by Phase 2 IgG antibodies (Dupont et al., 1994, Seqirus, 2021). At 20 days after symptom onset Phase 1 IgM antibodies and finally approximately 40 days after onset of infection Phase 1 IgG antibodies rise (Dupont et al., 1994, Seqirus, 2021). Acute Q fever can be diagnosed by certain established criteria, including seroconversion, phase 2 IgG antibody titre over 200 and phase 2 IgM antibody titre over 50, and/or detection of *C. burnetii* by PCR in blood or serum without infection of the heart valves (Melenotte et al., 2020). The recommended treatment for symptomatic acute Q fever is doxycycline for 14 days, while for acute Q fever endocarditis, doxycycline and hydroxychloroquine are recommended for 18 months (Melenotte et al., 2020). Prophylactic antibiotic treatment for acute Q fever patients with valvulopathy (which is a

risk factor for Q fever endocarditis) may prevent evolution to the chronic form of Q fever endocarditis (Melenotte et al., 2020).

Persistent focalised infection (also known as chronic Q fever) is the clinical manifestation of symptoms for several months to years and can lead to considerable morbidity and mortality if untreated (Melenotte et al., 2020). Q fever endocarditis is the most common manifestation of persistent focalised infection, making up 76% of cases in this category (Melenotte et al., 2018). These patients have persistent colonisation of bacteria in heart valves which can lead to valvular inflammation, fibrosis and calcification, and ultimately impaired heart valve function (Lepidi et al., 2003). Other forms of persistent focalised infection include vascular infections (19%) and osteoarticular infection (7% of persistent focal cases) (Melenotte et al., 2018). Persistent *C. burnetii* infection is thought be due to an inability of the host to clear the infection, with risk factors including pre-existing valvulopathy, being over the age of 40, and being male (Melenotte et al., 2018, Million et al., 2013). Infection during pregnancy is also considered a risk factor for chronic infection (Melenotte et al., 2020). The diagnosis of persistent focalised *C. burnetii* infection varies by the manifestations which are diverse (Melenotte et al., 2020). For example, Q fever endocarditis is diagnosed by detection of *C. burnetii* in a cardiac valve material (culture, PCR, Fluorescence In Situ Hybridization [FISH], immunochemistry) (Melenotte et al., 2020). It may also be diagnosed by either the presence of *C. burnetii* detection in blood (by culture or PCR) or an IgG antibody titre greater than 6400, with endocardial involvement (detected, for example, by an echocardiogram) or valvulopathy (Melenotte et al., 2020). To treat persistent focalised infection, doxycycline together with hydroxychloroquine for 18 months

is recommended, with progress monitored during this period by imaging (of the affected area) and serology follow-ups (Melenotte et al., 2020).

Q fever fatigue syndrome is a debilitating long-term fatigue that usually lasts more than a year but may persist for up to 10 years. Along with profound fatigue, patients experience sleep disturbances, night sweats, nausea, and alcohol intolerance (Ayres et al., 1998, Morroy et al., 2016). The pathogenesis of Q fever fatigue syndrome is unclear, but some studies suggest the persistence of *C. burnetii* antigen (not viable cells) may lead to ongoing immune stimulation (Sukocheva et al., 2010). Diagnosis of Q fever fatigue syndrome is more challenging, and is usually based on persistence of symptoms for more than one year after acute infection (without evidence for persistent focalised infection) (Anderson et al., 2013). There is also no universal standard for treatment of Q fever fatigue syndrome with the effectiveness of antibiotics having been sparsely reported (Anderson et al., 2013, Morroy et al., 2016).

1.6 Overview of *Coxiella burnetii* infection in cattle

Based on seroprevalence studies, *C. burnetii* exposure has been detected in cattle from around the globe (except for New Zealand) (Guatteo et al., 2011, Rabaza et al., 2021). Natural infection is often asymptomatic in cattle (Agerholm, 2013, Freick et al., 2017, Gisbert et al., 2024, Rodolakis et al., 2007), but disease (termed coxiellosis) can manifest, most commonly in the form of reproductive loss (Agerholm, 2013, Dobos and Fodor, 2021,

Gisbert et al., 2024, Guatteo et al., 2012). Infection and/or disease in animals is reportable according to the World Organisation for Animal Health (World Organisation for Animal Health).

1.6.1 Transmission in cattle

As with humans, inhalation of *C. burnetii* has been speculated as the most likely route of infection in cattle (Woldehiwet, 2004). Inhalation as a form of effective transmission has been demonstrated in ruminants, with intranasal inoculation of pregnant and non-pregnant goats leading to seroconversion and the presence of *C. burnetii* in tissue (Roest et al., 2012, Roest et al., 2020). Limited research has been conducted to investigate other routes of transmission in cattle. One *C. burnetii* experimental infection of cattle study in the United States showed inoculation of non-pregnant lactating cows via the intramammary (six cows over two experiments) and vaginal (one cow) routes successfully led to establishment of infection in all cows (as determined by presence of *C. burnetii* antibodies and excretion) (Bell et al., 1949). Additionally, of two calves fed *C. burnetii* infected milk, one calf showed clinical signs and presence of *C. burnetii* antibodies. The results of these experiments should be interpreted with caution due to the small sample sizes and the possibility that the *C. burnetii* inoculum could have become aerosolised during inoculation, for example, by splashing of milk during calf feeding. In saying this, in one of two intramammary inoculation experiments within this study, 2/2 infected cows excreted *C. burnetii* only in milk (for a period between 17 and 200 days) without any evidence of *C. burnetii* presence in faeces, urine, nasal wash and blood. Transmission of *C. burnetii* between cattle and ticks has been

found to be possible under experimental infection conditions (Derrick et al., 1942), and *C. burnetii* has been found in semen suggesting sexual transmission is also possible (Kruszewska and Tylewska-Wierzbanowska, 1997). In-utero transmission by spread of infection from the placenta to the foetus has also been theorised (Agerholm, 2013), as *C. burnetii* has been detected in the stomachs of aborted foetuses (Cantas et al., 2011) and tissues of a stillborn calf (Behymer et al., 1976).

1.6.2 Clinical presentation and management in cattle

Infection in cattle is reported in the literature as usually subclinical, as cattle have been shown to excrete the bacterium in the placenta, vaginal mucus, faeces, and milk without any recorded clinical symptoms (Freick et al., 2017, Rodolakis et al., 2007). Acute infection is not well defined in naturally infected cattle, however, intradermal experimental infection of non-pregnant eight – 11-month-old heifers led to seroconversion from day six to 13, pyrexia 24 – 36 hours following inoculation (that resolved within one week), and self-limiting pneumonia (Plommet et al., 1973). In contrast, intranasal experimental infection of pregnant goats reported no acute clinical signs of disease, including no loss of appetite or changes in rectal temperature (Roest et al., 2012), with the difference in symptoms compared to the cattle studies possibly due to variations in *C. burnetii* route of infection and strain, host immune response, and animal management practices.

Coxiella burnetii infection in cattle has also been associated in both observational (Guatteo et al., 2012, Clemente et al., 2009) and experimental infection (Plommet et al., 1973, Behymer et al., 1976) studies with reproductive loss, specifically sporadic abortion and still-birth. However as mentioned above, infected cows can also show no clinical signs, with one review of reproductive disorders in *C. burnetii* infected ruminants, having attempted to explain the inconsistencies in foetal loss outcomes between cows with *C. burnetii* DNA detected in the placenta and/or vaginal swabs (Agerholm, 2013). They suggested that following *C. burnetii* colonisation of the placental tissue, infection will become either latent or active, with only an active infection leading to abnormal offspring (Agerholm, 2013). While placentitis due to *C. burnetii* placental infection is considered rare, an association between *C. burnetii* presence in aborted placentas from naturally infected cows and inflammation has still been reported (Agerholm, 2013, Bildfell et al., 2000, Muskens et al., 2012).

Diagnosis of *C. burnetii* infection in cattle is usually only employed for epidemiological studies following human Q fever notifications or for confirmation of reproductive disorder aetiology on affected farms. Specific diagnostic approaches in cattle vary depending on the purpose, for example, molecular detection of *C. burnetii* by PCR in bulk tank milk (BTM) samples has been used to monitor active infection at the herd level (Taurel et al., 2014), while visualisation of *C. burnetii* by immunohistochemistry in aborted placental samples has been used when investigating if *C. burnetii* may be the causative agent (Bildfell et al., 2000). Serology as a tool for epidemiological studies or diagnosis of *C. burnetii* infection in cattle has predominately been limited to combined IgG phase 1 and 2 assessment using ELISA, but

some attempts have been made to progress utilisation of *C. burnetii* antibody temporal patterns and titres to differentiate states of infection in cattle (which is expanded on in a section below) (Böttcher et al., 2011, Lucchese et al., 2015, Serrano-Pérez et al., 2015, Wood et al., 2019).

Vaccination using Coxevac® (Ceva) (formalin fixed whole cell *C. burnetii* Nine Mile Phase 1 preparation) is sometimes conducted in European countries to reduce public health risk by decreasing bacterial shedding and environmental contamination, and to minimise reproductive losses. While there has been some variation in the effectiveness of this approach, possibly due to influence from factors like pregnancy state at the time of vaccination or vaccination coverage of the herd, shedding and reproductive outcomes have been improved in some cases (Garcia-Ispierto et al., 2015, Taurel et al., 2012, Taurel et al., 2014). Prophylactic vaccination of heifers has been demonstrated to be effective in preventing shedding compared to unvaccinated controls (Guatteo et al., 2008). However, the cost of potential adverse reactions to *C. burnetii* vaccination (swelling at the injection site) must also be considered on commercial dairy farms as an increase in body temperature and reduction in milk yield in vaccinated compared to unvaccinated control cows was reported in one herd, although it was suggested that such an outcome may be avoided by vaccinating heifers (Schulze et al., 2016). The effect of antibiotics on *C. burnetii* shedding has been rarely investigated but there is some evidence to suggest treatment at drying off may prevent vaginal shedding around parturition, while treatment of already shedding cows has no effect (Taurel et al., 2012).

1.7 Shedding routes from cattle

1.7.1 Overview

Coxiella burnetii DNA has been detected in a variety of ruminant excretions by PCR which this thesis will henceforth refer to as *C. burnetii* shedding. Some shedding routes display distinct characteristics in cattle. The highest quantities of bacteria are shed in birth products during parturition, where up to 10^9 organism per gram can be evident in ruminant placental samples (Hansen et al., 2011, Howard and Omsland, 2020, Welsh et al., 1951). Persistent shedding of *C. burnetii* can occur through milk which is a common excretion route in dairy cattle and goats, whereby bacteria may be shed either frequently or intermittently, sometimes over consecutive lactations (Biberstein et al., 1974). Furthermore, cattle may additionally excrete *C. burnetii* in vaginal mucus and faeces, while there exists limited evidence for bacterial presence in urine and semen (Guatteo et al., 2006, Kruszewska & Tylewska-Wierzbanowska 1997, Rodolakis et al., 2007).

The specific kinetics of shedding patterns are complex, and details surrounding onset, persistence and concomitancy remain misunderstood, in part due to contradictory results. Overall, shedding manifestation is likely an outcome of host, pathogen and environment interactions, with the potential role of specific factors such as transmission route, infective dose, bacterial strain, as well as host immunological and physiological status (pregnant vs non-pregnant) discussed in more detail in the pathogenesis section (Section 1.9).

1.7.2 Faecal and vaginal shedding in livestock

In small ruminant (specifically goat and sheep) studies, faecal and vaginal shedding routes are more frequently reported at the herd and individual level, and with higher bacterial concentrations, in comparison to cattle (Álvarez-Alonso et al., 2018, Canevari et al., 2018, Guatteo et al., 2007, Muskens et al., 2011, Rodolakis et al., 2007, Turcotte et al., 2021). While some studies have indicated vaginal mucus shedding may occur frequently in cattle herds in the period immediately following parturition, this is generally brief and not associated with the high bacterial titres observed in placental samples (Freick et al., 2017, Guatteo et al., 2012, Guatteo et al., 2007). In contrast, shedding in faeces has been described as sporadic and scarce and is infrequently reported for cattle (Garcia-Ispierto et al., 2013, Guatteo et al., 2007, Rodolakis et al., 2007). In saying this, one cross-sectional study identified *C. burnetii* by PCR in similar proportions in faecal, vaginal mucus and milk excretions from 242 cows (from 31 herds) that were either seropositive and had either aborted or calved in the preceding 45 days (Guatteo et al., 2006). A potential reason for discrepancies between studies may be owing to the presence of Taq polymerase inhibitors, common to faecal samples, which when not inactivated can lead to false negatives in PCR detection (Guatteo et al., 2006).

However, there have also been questions surrounding the dynamics of the faecal and vaginal shedding routes entirely, with the possibility that PCR positive samples may be the product of environmental contamination as opposed to active shedding from these excretion sites (Roest et al., 2012, Welsh et al., 1958). This idea was suggested based upon the inability of Roest et al. (2012) to find evidence of *C. burnetii* replication in the gut and

genital tract from experimentally infected euthanised goats at various time points, despite PCR detection of bacteria from faecal and vaginal samples following parturition. Therefore, the large quantities of *C. burnetii* that can be shed during parturition may have directly contaminated samples (Roest et al., 2012) or have resulted in passive shedding whereby organism passes through the gastrointestinal tract from bacterial ingestion during grazing (Bauer et al., 2020). However, in saying this, detection of *C. burnetii* by PCR was reported in the genital tract tissue of 25% of 20 goats randomly sampled at a French abattoir, while Bouvery et al. (2003) found evidence of *C. burnetii* in goat faecal samples prior to parturition in an observational study (Alsaleh et al., 2011). Also in favour, Bauer et al. (2020) found high concentrations of *C. burnetii* in goat rectal swabs, exceeding that of vaginal mucus swabs, which they proposed indicated some degree of replication must be taking place in the gastrointestinal tract prior to ruminant excretion. Despite these varying results, in reviews of *C. burnetii* infection in ruminants, vaginal and faecal shedding are generally recognised as cattle excretion routes.

1.7.3 Milk shedding from livestock

Milk is an established shedding route of particular importance in cattle due to the relative persistence that can be displayed over prolonged periods, compared with faecal and vaginal shedding, in both clinical and subclinical cows (Biberstein et al., 1974, Guatteo et al., 2007). In sheep, persistent shedding in milk is infrequently observed while persistence in goats varies between studies (Alverez-Alonso et al., 2018, Bauer et al., 2020, Rodolakis et al., 2007, Roest et al., 2012). On *C. burnetii* infected cattle farms, the within herd proportion of

milk shedders has varied between investigations, (for example 0% to 45%) however, due to the intermittent nature of shedding it can be difficult to distinguish whether animals/herds are not shedding in milk or just not shedding in milk at the time of sampling (Barlow et al., 2008, Khatun et al., 2022). Within a herd, two studies have found the proportion of shedders to increase significantly with increasing days in milk, while a regression analysis of 12 herds found parity to be a significant variable but not days in milk (Angen et al., 2011, Barlow et al., 2008, Freick et al., 2017). Shedding kinetics, including duration, onset, bacteria load, and intermittence, also display differences which will be discussed in more detail in later sections (Section 1.9) (Barlow et al., 2008, Freick et al., 2017, Guatteo et al., 2007). Such differences can be difficult to compare owing to the variations between investigations, including breeds, physiological stages, sample sizes/strategies, production systems, and presumably *C. burnetii* strains, however, this route is still reported as most prevalent over time at the herd and individual level in cattle (Freick et al., 2017, Guatteo et al., 2007, Guatteo et al., 2012, Rodolakis et al., 2007). In aborting cows, *C. burnetii* milk shedding was identified as the most frequent route (compared to vaginal shedding) throughout a four-week sampling period following abortion (Guatteo et al., 2012), a finding also supported for asymptomatic cows by multiple longitudinal investigations (Freick et al., 2017, Guatteo et al., 2007, Rodolakis et al., 2007). More specifically in asymptomatic cattle, milk shedding has been shown to occur persistently for up to three months after calving and intermittently over consecutive lactations (up to 13 months) (Biberstein et al., 1974).

There have only been a modest number of studies reporting *C. burnetii* quantifications in individual milk samples, with the upper range reported at 10^4 cells/ml (Kim et al., 2005).

However, comparing between studies is challenged by diverse PCR methodology (Angen et al., 2011, Freick et al., 2017, Guatteo et al., 2007, Guatteo et al., 2012, Kim et al., 2005). Firstly, there has been a variety of reported outputs, including, cycle thresholds (Freick et al., 2017, Guatteo et al., 2007, Guatteo et al., 2012), log scale titres (Guatteo et al., 2007) and concentrations (cells/ml) (Angen et al., 2011, Kim et al., 2005). Secondly, different genes are targeted in these respective PCRs, and while IS*1111* is a popular choice due to multiple copy number increasing the test sensitivity, this number varies between genotypes potentially influencing validity of quantity comparison between and within studies (Biberstein et al., 1974, Guatteo et al., 2007, Kim et al., 2005, Rodolakis et al., 2007). Furthermore, there is no standardised approach for *C. burnetii* DNA extraction from milk with the milk fraction often not stated in the method section or varying between the fat (Angen et al., 2011, Berri et al., 2003, Rodolakis et al., 2007), whole milk (Angen et al., 2011, Berri et al., 2003, Bottcher et al., 2013, Guatteo et al., 2007, Guatteo et al., 2006, Guatteo et al., 2012, Muskens et al., 2011, Pearson et al., 2014, Rodolakis et al., 2007) or cell pellet (Berri et al., 2003, Esmaeili et al., 2019, Gyuranecz et al., 2012, Loftis et al., 2010, Rodolakis et al., 2007). Some optimisations have been published within studies, with Angen et al. (2011) finding no statistical difference in the cycle thresholds or number of positives from 55 milks samples belonging to a herd with previously established high prevalence, while Rodolakis et al. (2007) observed more positive results when using the cream or full milk compared to the cell pellet from two herds tested over five time points. Determining the location of *C. burnetii* within milk components (whether it be in cells or non-cell associated), may allow informed selection of milk fraction for optimal sensitivity. Finally, the criteria for classifying samples as PCR-positive is not always stated or the cut-off cycling threshold is not

justified using the assay limit of detection (Guatteo et al., 2007, Guatteo et al., 2006, Guatteo et al., 2012, Kim et al., 2005, Rodolakis et al., 2007).

The persistent and prolonged shedding of *C. burnetii* in milk displayed by dairy cows has been proposed as evidence of a chronic form of infection in multiple studies, with Freick et al. (2017) suggesting milk shedding may be an indicator of persistent infection (Arricau-Bouvery & Rodolakis 2005, Kim et al., 2005, Sobotta et al., 2017). This conclusion was based upon their distinct timing observations of shedding routes, whereby heifers that were seronegative prior to parturition on an endemic farm, first displayed transient vaginal mucus shedding at calving followed by onset of milk shedding which increased in the herd as lactation progressed (Freick et al., 2017). However, studies that have included longitudinal data on individual cows have found not every animal that sheds in milk will do so until the final time point of the study and while this may be due to intermittence, it also may highlight the cessation of milk shedding and therefore cannot alone be used as an indicator of chronic disease (Freick et al 2017, Guatteo et al., 2012). In humans, chronic Q fever (which is associated with severe complications) can be determined based on a phase-specific antibody response, but the current commercially available ELISA Q fever kits for ruminants only contain combined phase 1 and phase 2 antigen coated wells and thereby cannot distinguish different forms of infection (Wood et al., 2019). A small number of studies have investigated phase-specific antibody trends within cattle herds with phase specific ELISAs (Bottcher et al., 2013, Lucchese et al., 2015) and immune-fluorescence assay (Wood et al., 2019), although limited understanding still remains around whether ruminant immunological response patterns are associated with acute and chronic conditions. Given

that it has been suggested that persistent *C. burnetii* shedding, which is observed mostly in milk, may be associated with 'heavy shedder' animals, further investigation into diagnosis and impacts of chronic disease may be valuable (Guatteo et al., 2007). Furthermore, such persistence of *C. burnetii* in the mammary gland has also been proposed as a mechanism to explain placental shedding in animals at consecutive parturitions, as this may accommodate re-infection of reproductive tissue following the non-pregnant period (Roest et al., 2020). Shedding in the placenta can lead to release of large numbers of *C. burnetii* organisms so an interaction between these routes may also be of interest. Overall, on review, prolonged shedding of *C. burnetii* in milk may serve a critical epidemiological role in maintaining infection within the herd (Freick et al., 2017) and, moreover, pose a potentially significant source of human infection by environmental contamination, as discussed in the next section (Kim et al., 2005).

1.8 Milk shedding from ruminants: potential concerns for animal and human health

1.8.1 Transmission

Consideration of the role of milk in transmission of *C. burnetii* to humans and other animals and subsequent development of clinical disease is often limited to the debated ingestion route (Benson et al., 1963). However, there is also the potential for aerosolisation of milk contaminated with *C. burnetii* and therefore transmission via inhalation. As there has been a paucity of research conducted into the relative contribution of each shedding route to human or animal aerosol inhalation, the importance of milk shedding in this regard is unknown. The characteristic persistence of *C. burnetii* shedding in bovine milk may present

a unique risk to human and animal health compared with bacterial excretion from other routes. Results of a systematic review of studies investigating environmental presence of *C. burnetii* (using dust, air, soil, and liquid samples), concluded that positive samples were more likely to be detected in endemic, as opposed to outbreak, ruminant settings, while Welsh et al. (1958) found *C. burnetii* in air samples in pens immediately following ewe partition but not in air samples from all pens beyond 14 days post-partum (Abeykoon et al., 2021). When taken together and considering that air sampling is deemed an assessment of current bacterial presence, it appears an ongoing contamination of the environment may be needed to continually generate *C. burnetii* aerosols. Therefore, due to its distinctive persistence, milk shedding may provide a source of sustained risk to human infection and ongoing within-herd transmission (Freick et al., 2017, Kim et al., 2005).

Alternatively, the physical generation of pre-existing *C. burnetii* deposits from the various shedding routes is also a possible source of aerosolisation, as locations or periods with high ruminant movement have been linked to an increased chance of identifying positive air and inhalable dust samples (Hogerwerf et al., 2012). The inherent conditions of bovine milking sheds, including high movement and surfaces prone to build-up of dust (a sample type associated with high quantities of *C. burnetii* compared with air, soil, and liquid), make such locations pre-disposed to bacterial aerosol presence and thereby potentially heightened transmission risk (Abeykoon et al., 2020). In saying this, low humidity and precipitation have also been associated with increased *C. burnetii* environmental detection, two conditions likely uncommon to the majority of milking sheds, meaning environmental sampling at bovine dairies would be required for a true sense of location risk (De Rooij et al., 2016).

Despite the limited dedicated study into the role of *C. burnetii* infected milk in inhalation transmission, assessment of the currently available *C. burnetii* environmental studies suggests the infection threat of ongoing bacterial excretion in bovine milk may be enhanced by conditions suited to aerosol generation in milking sheds.

Furthermore, the persistent shedding pattern has, in one study, been associated with animals shedding high quantities of *C. burnetii*, or animals termed 'heavy shedders' (Guatteo et al., 2007). The importance of heavy-faecal shedders to within-herd cattle transmission of *Escherichia coli* and *Mycobacterium avium* subsp. *paratuberculosis* (MAP) has been previously established, with Omisakin et al. (2003), finding 9% of animals in a herd were contributing to 97% of environmental *E. coli* O157 (Matthews et al., 2006, Omisakin et al., 2003, van Schaik et al., 2003). The threat of persistent and heavy *C. burnetii* shedders is accentuated by the asymptomatic presentation that can accompany milk shedding, allowing active excretion to occur undetected in the herd and thus complicate effective control, as occurs in various other livestock diseases, including salmonella and Johne's disease (Freick et al., 2017, Guatteo et al., 2007, Gopinath et al., 2012, Rodolakis et al., 2007). While the dynamics of within-herd transmission of *C. burnetii* are still somewhat uncertain, experimental inoculation of non-pregnant goats detected *C. burnetii* only in one of ten goats within the mammary gland tissue, associated lymph nodes and colostrum (Roest et al., 2020). As such, the authors concluded that following breeding and parturition, these animals may contaminate the environment with infected milk providing a source of infection for susceptible pregnant goat does that could subsequently shed huge quantities in their placentas. An influence of milk shedding on placental shedding has also been

proposed to explain positive placentas in a single animal at consecutive parturitions (Berri et al., 2007, Roest et al., 2020). A persistent infection in the mammary gland may accommodate re-infection of reproductive tissue following the non-pregnant period, considering it would seem unlikely a previously infected animal would clear the infection and become re-infected unless different strains were involved with little cross-protection (Berri et al., 2007, Roest et al., 2020).

Critical to the transmission risk associated with milk is the infective capacity of *C. burnetii* excreted in contaminated milk, with a multitude of studies having confirmed viable bacteria in dairy products, including bovine milk and derived cream, butter, and cheese (Enright et al., 1957, Jellison et al., 1948a, Loftis et al., 2010, Stewart et al., 2015). Of additional concern, *C. burnetii* may remain viable for extended periods in dairy products, with bacteria having been demonstrated as infectious for up to 42 days in ripening bovine cheese and eight months in goat cheese (Barandika et al., 2019, Sipka, 1958). Interestingly, other studies have detected non-viable *C. burnetii* in bovine dairy products (Eldin et al., 2013, Hirai et al., 2012), potentially suggesting that strain variation or bacterial load may influence viability (Galiero et al., 2016). The process of determining such viability is vital for risk classification but is laborious, involving inoculation of bacteria into cell culture or animal models under physical containment level three conditions. While analysis of individual and BTM samples using PCR does not commonly distinguish between viable and unviable bacteria, it does provide a measure of *C. burnetii* DNA prevalence and thereby a comparatively more convenient alternative for determining potential risk from contaminated milk.

Detection of *C. burnetii* DNA is a requirement for determining active infection within a herd or individual, as positive serology may not necessarily correlate with bacterial shedding in infected animals (Berri et al., 2001). Using individual milk samples for PCR analysis allows for assessment of infection at the individual animal level, whereas BTM may be used for the ascertaining herd level active infection status. Several studies have attempted to validate the use of BTM samples for also determining the number of within herd shedders and heavy shedders, by correlating bacterial quantity in the BTM sample with the proportion of positive individual milk samples per herd, however, standardisation is complicated and results in varying levels of success (Guatteo et al., 2007). Nevertheless, BTM remains a simple and non-invasive sample for *C. burnetii* infection screening purposes. To date, no Australian study has been conducted on the prevalence of *C. burnetii* in bovine BTM samples. Although, recently, BTM samples from 49 of the 61 commercial goat dairies in Australia identified three positive farms, suggesting that *C. burnetii* prevalence in Australian goat dairies is low (Hou et al., 2022). Since milk shedding patterns between caprine and bovine herds is not always similar, the relevance of these findings to bovine dairies may be limited, and moreover the cross-sectional nature of the work may not account for the possibility of intermittent shedding. In contrast, a multitude of prevalence studies using bovine BTM samples have been conducted overseas. For example, in the USA, *C. burnetii* prevalence estimates in bovine dairy herds based on BTM sampling vary (likely due to differences in years conducted and testing methods), however, infection is thought to be widespread, with Kim et al. (2005) reporting <90% of samples collected over a three-year period throughout the country were PCR positive.

It is however important to note that concerns regarding the potential Q fever transmission associated with bovine milk may be influenced by *C. burnetii* strain and geographic region. The predominate strain isolated from milk in the USA, ST20 (genomic group III), has not been reported in a USA human case despite its widespread presence in BTM samples obtained from USA dairy herds (Kim et al., 2005, Pearson et al., 2014). Similarly while the strain ST20 has been shown to be prevalent in cattle samples obtained from other geographical areas, including Spain, Portugal, Hungary, Germany, and The Netherlands, there are only limited reports that it was implicated in human Q fever cases, including chronic Q fever cases from France in the 1990's as well as a recent outbreak in the UK traced to a goat dairy (Glazunova et al., 2005, Reichel et al., 2012, Santos et al., 2012, Sulyok et al., 2014, Tilburg et al., 2012). However, as Q fever in humans is considered underreported, due to non-specific and/or asymptomatic disease manifestations, linking *C. burnetii* genotypes with clinical cases of Q fever occurs infrequently (Dahlgren et al., 2015, Kaufman et al., 2018). In saying this, the genotype ST8 (genomic group IV) that was found in caprine samples in a USA goat dairy was linked to a human outbreak in 2011 in Washington (Kersh et al., 2013). Indeed, in many geographical regions, goats are more frequently associated with human Q fever than cattle (Tan et al., 2024). One proposed explanation for this, is that strains isolated from cattle stimulate a greater inflammatory immune response which is protective against establishment of infection. This theory is supported by *in vitro* studies that demonstrated a greater production of pro-inflammatory cytokines (TNFa and IL-1b) by human peripheral blood mononuclear cells (PBMCs) in culture following stimulation with a variety of cattle strains compared to that elicited by goat strains (Ammerdorffer et al., 2017). In contrast, in Australia, results of a survey study found cattle are the most

common animal associated with Q fever infection which may potentially indicate a strain specific virulence between bovine isolates from the USA and Australia (Graves & Islam 2016). This idea is supported by a study that found *C. burnetii* strains from Australian acute Q fever patients are genetically distinct (due to single locus variations) to those from other parts of the world (Vincent et al., 2016).

While pasteurisation has been shown to effectively inactivate *C. burnetii*, dairy producers, milk processers, veterinarians, farm contractors, and the herd nonetheless remain at risk of infection, and indeed any person or susceptible animal that may come into contact with contaminated raw milk directly or indirectly (Cerf and Condron, 2006, Enright et al., 1957). Recently, the significance of this later scenario has been emphasised for the broader rural community by serological studies that have increasingly found high proportions of positive respondents reporting no known animal exposure, and thus the conclusion that *C. burnetii* infection may not purely be an occupational hazard (Gidding et al., 2020, Tozer et al., 2011). Furthermore, the potential Q fever risk to the public from unpasteurised dairy products remains unclear due to poor characterisation of the ingestion transmission route but requires vigilance as these products continue to gain popularity in society, especially as previous work has highlighted 42.9% of commercial raw milk samples from 12 USA states contained viable *C. burnetii* (Loftis et al., 2010). The reports of a Q fever cluster associated with unpasteurised milk consumption in Michigan (Signs et al., 2012), and the successful infection of mice from inoculation of *C. burnetii* into the stomach in a dose dependant manner, indicate the risk of Q fever following ingestion may not be as negligible as is frequently believed (Miller et al., 2021). When considering that groups vulnerable to severe

and deadly manifestations of Q fever, namely pregnant women, children, and immunocompromised people, have been identified as consumers of raw milk, there is a need for more focused understanding and risk assessment of ingestion transmission. Ultimately, to accurately define the role of milk as a source of *C. burnetii* in Q fever cases (whether it be by inhalation or ingestion), implementation of a human surveillance system that is animal (and where possible excretion) specific, state/country consistent, and genotype inclusive is necessary.

1.8.2 Impact of *Coxiella burnetii* infection on animal production

1.8.2.1 Mastitis

Mastitis is the largest cause of economic loss in the dairy industry globally, arising from a drop in milk quality and/or quantity due to inflammation in the mammary gland. Given that *C. burnetii* antibodies have been identified in bovine milk, and organism has been detected in both mammary gland tissue and milk, naturally, udder specific manifestations of *C. burnetii* infection have been investigated. The first reports of *C. burnetii* associated with bovine mastitis arose in the 1940's with separate documentations of acute and chronic presentations in cows experimentally and naturally infected, respectively, though notably, both studies did not also test for potential presence of common mastitis pathogens (Bell et al., 1949, Jellison et al., 1948b). Nevertheless, *C. burnetii* was subsequently included as an uncommon mastitis pathogen in one review of the disease in 1975 (Philpot and Pankey, 1975). Since then, a variety of studies have explored whether *C. burnetii* may be a contagious mastitis pathogen and as such increase somatic cell count in milk, however, a

definitive conclusion from observational studies is complicated by several factors (Agger & Paul 2014, Angen et al., 2011, Barlow et al., 2008, Freick et al., 2017, Radinovic et al., 2011, Saegerman et al., 2015, To et al., 1998). Firstly, *C. burnetii* has been demonstrated to replicate intracellularly and to have a tropism for mammary epithelial cells, so it is difficult to distinguish whether *C. burnetii* is the causative agent of increased somatic cell count or merely present in sloughed epithelial cells normally found in milk samples (Sobotta et al., 2017). Secondly, while the presence of *C. burnetii* antibodies in milk does indicate an immunogenic response from the host against the bacterium, it is unclear whether this is a product of a localised udder lymphoid response to infection in the udder or serum antibody crossing the blood-milk barrier representing a response to systemic infection (Telemo and Hanson, 1996).

Histopathological study of udder tissue infected with known mastitis pathogens commonly reveals various degrees of microscopic damage, including necrosis or fibrosis. This can arise even in subclinical manifestations where gross clinical signs are absent, as seen in some *Staphylococcus aureus* mastitis cases, however, similar attempted work for *C. burnetii*-infected bovine udder tissue, has yielded inconclusive results (Benites et al., 2002, Hensen et al., 2000, Jellison et al., 1948b). Histological analysis from one cow known to be shedding *C. burnetii* in milk found 'subacute focal inflammation with suppuration' in udder tissue but was unable to visualise organism despite inoculation of guinea pigs with tissue obtained from the udder leading to successful infection (Jellison et al., 1948b). The study's inability to visualise organism may be due to the fact that light microscopy can lack sensitivity in detecting organisms in low concentrations, and moreover the sample may have been

unrepresentative as the number of sections analysed was not disclosed and tissue sections from a single udder can vary in their pathology as previously reported in a *Staphylococcus aureus* mastitis investigation (Hensen et al., 2000). A similar inability to visualise *C. burnetii* in goat mammary gland tissue that was PCR-positive has also been observed in two experimental infection studies, including one study in which a single mammary gland exhibited extensive accumulation of lymphocytes in the interstitial fluid (Sanchez et al., 2006). More recent work visualised *C. burnetii* by FISH in the mammary epithelial cells of one goat shedding *C. burnetii* in milk, and while granulomatous inflammation was also identified in this goat's udder tissue, the authors suggested this may more likely be a reaction to a foreign body than to *C. burnetii* (Bauer et al., 2024). Further work is needed to analyse tissue samples for presence of organism and potential damage patterns using a greater number of samples.

Somatic cell count is another indicator of udder health and is often used in mastitis diagnosis. Barlow et al. (2008) performed a case/control and cross-sectional study to establish a link between *C. burnetii* shedding in milk and chronic subclinical mastitis in a commercial American dairy herd known to be free of *Streptococcus agalactiae* and *Mycoplasma* spp. intramammary infections (IMIs) (Barlow et al., 2008). A study design advantage of this work was the ongoing testing of each individual animal for major and minor mastitis pathogens by aerobic bacteriology (*Staphylococcus aureus*, *Streptococcus* spp., *Arcanobacterium pyogenes*, coliform, coagulase negative staphylococci, and *Corynebacterium* species), and, as such, the ability to interpret *C. burnetii* shedding results in light of the potential influence of existing IMIs (Barlow et al., 2008). The case/control

component of this study compared *C. burnetii* milk quarter shedding as detected by PCR between cows with either an elevated SCC (greater than 200,000 cells/ml) or normal SCC for two consecutive months and which had all tested negative for the common intramammary infectious agents (Barlow et al., 2008). A significant association was found between the number of *C. burnetii* positive milk quarters per cow and cow level chronically elevated SCC (Barlow et al., 2008). Subsequently, a cross-sectional study was completed with all lactating cows in the herd (n= 351) to assess a correlation between the previous three-month average SCC and *C. burnetii* milk shedding status (Barlow et al., 2008). Linear regression modelling determined that for cows free of common IMIs, *C. burnetii* positivity led to a higher median SCC compared with *C. burnetii* negativity (229,000 cells/ml vs 82,000 cells/ml), while logistic regression modelling showed cows shedding *C. burnetii* were 3.92 times more likely to have an elevated three-month SCC (Barlow et al., 2008). The former finding is of particular importance, as a SCC of 200,000 cells/ml is the threshold for defining disease that subsequently triggers economic loss via treatment, culling and milk penalties (Schukken et al., 2003). Interestingly, while not reaching statistical significance, the average SCC was also higher in cows that were positive for both an IMI caused by a mastitis pathogen and *C. burnetii* compared to those that were *C. burnetii* negative, potentially indicating a synergistic role of this bacterium with other mastitis pathogens in mastitis development (Barlow et al., 2008). Therefore, despite not testing for all possible mastitis pathogens or including sample size justifications, this work does provide evidence in support of *C. burnetii* being associated with chronic subclinical mastitis (Barlow et al., 2008). Various other studies have reported results both in agreement (Khatun et al., 2022, Radinović et al., 2011, TO et al., 1998) and disagreement (Agger and Paul, 2014, Angen et al., 2011, Freick et al., 2017, Saegerman et al., 2015) with this conclusion, although, each of these studies has

limitations associated with their methodologies or reporting of results. These limitations may have stemmed from a broad investigative scope, with all studies investigating the role of *C. burnetii* in relation to SCC having several aims (the exception being Radinovic et al., [2011]), which restricts feasible inclusion of methodology encompassing relevant biological aspects of mastitis (Agger & Paul 2014, Angen et al., 2011, Freick et al., 2017;, Radinovic et al., 2011, Saegerman et al., 2015, To et al., 1998). For example, the presence of common mastitis pathogens can increase SCC, which is an important factor to consider when interpreting *C. burnetii* results from milk as it may influence the validity of conclusions, though despite this, none of the aforementioned investigations included testing for existing IMIs. Furthermore, several studies excluded negative controls, utilised small sample sizes without statistical justification, and grouped mastitis with other reproductive disorders when reporting results, all of which may reduce the reliability and validity of their respective mastitis findings (Radinovic et al., 2011, To et al., 1998). Other studies compared *C. burnetii* antibody levels in BTM samples with SCCs, finding no association between these two variables, and while serology is an important screening tool in *C. burnetii* epidemiological studies, it likely gives little insight into *C. burnetii*'s role in current mastitis cases as antibody presence can lack correlation to active infection/shedding of organism (Agger & Paul 2014, Berri et al., 2001, Guatteo et al., 2012, Saegerman et al., 2015). Nevertheless, when unable to detect *C. burnetii* DNA by PCR in any sample, Khatun et al., (2022) used the antibody against *C. burnetii* in milk obtained from individual udder quarters from cows with mastitis in a linear mixed model to find antibody positive quarters had a significantly higher SCC compared to negative *C. burnetii* antibody control quarters.

Finally, upon review of these studies, it is interesting to note the study geographical location skew towards European countries (five from seven), and the potential influence or bias this may have on the current understanding of *C. burnetii* as a mastitis pathogen. Of the European studies, four from five found no association between *C. burnetii* and increased SCC and/or mastitis (Agger & Paul 2014, Angen et al., 2011, Freick et al., 2017, Radinovic et al., 2011, Saegerman et al., 2015), while in contrast, the two non-European investigations (USA and Japan) did find correlations (Barlow et al., 2008, To et al., 1998). Despite varying methodical approaches, the general agreement between studies from similar geographic regions may indicate a location-specific disease manifestation, potentially resulting from varying *C. burnetii* strains. While undoubtedly a variety of host factors impact the course of infection and disease, it has long been hypothesised that *C. burnetii* virulence is pathotype-specific, meaning that strains can be grouped by pathogenicity as determined by their genome content (Hendrix et al., 1991, Long et al., 2019, Russell-Lodrigue et al., 2009). For example, the hypervirulent epidemic *C. burnetii* strain, Cb 175, that causes one of the greatest occurrences of community-acquired pneumonia, has a genetic deletion that is thought to be a possible cause of virulence (D'Amato et al., 2015, Eldin et al., 2017). Therefore, it may be possible that specific *C. burnetii* strains could also be associated with mastitis, and, as such, provide explanation for the contradictory reports in the literature. Ultimately establishing mastitis causation necessitates controlled experimentation, though physical containment level three requirements for experimental infection of animals with *C. burnetii* has limited most udder health studies to being observational in nature (Agerholm et al., 2013). Alternatively, intervention of natural *C. burnetii* infection with either antimicrobial treatment or culling followed by a reduction in bulk tank milk somatic cell count (BMSCC) may be possible approaches to better defining the association between *C.*

burnetii infection and SCC. An observational long term-investigation with a large sample size may be able to opportunistically detect seroconversion of a cow followed by shedding and an increased SCC in the absence of a known mastitis pathogen.

1.8.2.2 Other milk quality parameters impacted by *Coxiella burnetii* infection

Coxiellosis studies have also been directed towards establishing if a link exists between bovine *C. burnetii* shedding and/or seroprevalence and impacts upon a variety of milk quality parameters. In one study of a single cattle herd, vaginal shedding at parturition and seroconversion by 42 days in milk was associated with decreased milk fat yield (Freick et al., 2017). The results of a multivariable regression analysis of 24 Danish dairy herds (10 BTM *C. burnetii* antibody positive, 10 BTM *C. burnetii* antibody negative and four BTM *C. burnetii* antibody intermediate) also found a link between *C. burnetii* and milk fat yield, specifically that the odds of being seropositive were decreased for cows with high milk fat (Paul et al., 2012). The same was true for milk yield, while there was increasing odds for seropositivity in cows with high milk protein content (Paul et al., 2012).

In a caprine herd, does with high bacterial concentrations in vaginal swabs, had daily milk yield reduced by 17% compared to goat does without *C. burnetii* detected in vaginal swabs (Canevari et al., 2018). Conversely, an increase in milk production of 0.276L per day was found in goat does that seroconverted to only IgG phase 1 compared to does that did not seroconvert with the authors of the study proposing that the former had a fully developed humoral immune response, which was protective against effects of acute *C. burnetii*

infection (as phase 1 antibodies are produced after phase 2) (Muleme et al., 2017). However, the researchers also found similar milk yields between goat does that did not seroconvert and those that seroconverted to only to IgG phase 2 (possibly indicating an acute infection) and seronegative goat does. As milk yields were only measured over the first nine weeks of lactation, they suggested the effects of an acute infection may become apparent at later days in milk (Muleme et al., 2017). The relevance of these findings to dairy cows is unknown considering the species and potential genotype differences.

1.8.2.3 Mammary gland health

The cellular microenvironment of the mammary gland is complex, and disease manifestation is the result of various host and pathogen interactions. Thus far, *C. burnetii* research in cattle has focused on identifying whether *C. burnetii* may independently cause an increase in SCC, which is evidently still unclear. However, there are many roles for pathogens in the mammary gland and despite not all directly increasing SCC, their influence on udder health is of importance.

1.8.2.3.1 Mastitis pathogens

Mastitis pathogens can be divided into major and minor groups based on their effect on mammary gland health (Cobirka et al., 2020). Major mastitis pathogens, including *Streptococcus agalactiae*, *Mycoplasma* spp., coliform, *Staphylococcus aureus*, and other

Streptococcus spp., are considered most concerning to farm production due to increased virulence and more severe clinical mastitis (Cobirka et al., 2020). Minor pathogens (such as coagulase negative staphylococci and *Corynebacterium* spp.), are more commonly associated with moderate subclinical mastitis (Cobirka et al., 2020). The exact role of the latter class of pathogens in mastitis is debated, with some evidence that minor mastitis pathogens may actually induce a protective effect against the establishment of IMIs with major pathogens (Lam et al., 1997, Matthews et al., 1991, Michel et al., 2011, Schukken et al., 1989). The underlying mechanisms of this potential protective effect are not known but it has been theorised that a relatively small increase in SCC induced by minor IMIs may prime the immune response to more efficiently clear new IMIs from a major pathogen (Bradley, 2002, Schukken et al., 1999). An alternate theory is suggested by *in vitro* work demonstrating competition between coagulase negative staphylococci and major mastitis pathogens (*Staphylococcus aureus* and *Streptococcus agalactiae*), leading to the possibility that some minor pathogens may produce inhibitory substances, such as bacteriocins, that make the cellular environment unsuitable for the survival of other pathogens (Bradley, 2002, De Vliegher et al., 2004, dos Santos Nascimento et al., 2005). In contrast, other studies have found that the presence of minor pathogens in the udder leads to increased risk of major IMI (Compton et al., 2007, Parker et al., 2007). Possible reasons behind this include disruption to normal teat defences (such as the keratin plug) which improve the chances of major pathogens penetrating udder tissue, or the release of nutrients that aid major pathogen growth (Reyher et al., 2012). Interestingly, it has been reported that an existing IMI with coagulase negative staphylococci could provide either a protective or detrimental effect on establishment of *Staphylococcus aureus* and *Streptococcus agalactiae* IMI respectively, following experimental challenge (Nickerson and Boddie, 1994). To explain

such differing results, several authors have suggested a selective protective effect that is dependent on the species of major mastitis pathogens, and that moreover, this effect may only occur at specific times during the production cycle (Bradley 2002; Reyher et al., 2012). Additionally, a systematic review of the role of minor mastitis pathogens in new IMI development, identified that the method of major mastitis pathogen challenge (infusion vs immersion) influenced protective or detrimental outcomes in animals with existing minor mastitis pathogen infections (Reyher et al., 2012). Challenge by immersion, which requires major pathogens to enter via the teat canal, was more likely to identify increased risk of development of a new IMI compared with infusion, which bypasses teat defences. As such, they theorised that minor pathogens may have differing effects on major pathogens depending on their location within the mammary gland.

1.8.2.3.2 Non-mastitis pathogens

Non-mastitis pathogens are agents which, though not recognised to cause mastitis, may influence mammary gland health. Historically, mastitis research has focused on bacterial aetiologies, however, experimental intramammary inoculation with viruses such as Bovine Herpesvirus 4 and Bovine Herpesvirus 1 has been documented to cause clinical and subclinical mastitis in cows (Greig and Bannister, 1965, Wellenberg et al., 2002b, Wellenberg et al., 2002a). Therefore, while it is clear the mammary tissue is susceptible to viral infection and, in some cases, capable of directly inducing mastitis, the significance of the intramammary route of entry in natural viral pathogenesis is unknown. Thus far, mammary gland colonisation from experimental viral challenge by the natural route of infection has

only been demonstrated for foot and mouth disease virus (oronasal route), which lead to mammary epithelial cell necrosis but no significant change in immune response and SCC (Blackwell and Yilma, 1981, Blackwell et al., 1983). In saying this, there are various examples of viruses having been isolated from mammary tissue and milk of natural clinical and subclinical mastitis cases (Wellenberg et al., 2000, Yoshikawa et al., 1997), as well as epidemiological studies which have identified significant associations between BTM viral antibody levels and BTM SCC (Emanuelson et al., 1992). However, these findings do not prove direct causation, and several reviews have instead suggested viral associations with mastitis are more likely due to an indirect role, whereby their presence in the mammary gland from systemic infection encourages establishment of secondary bacterial udder infections (Wellenberg et al., 2002b). This idea is founded on certain well-recognised characteristics of viruses which can weaken the host udder defences against mastitis pathogens, including immunosuppression (Bovine Herpesvirus 1, Bovine Immunodeficiency Virus, and Bovine Leukaemia Virus) and induction of teat lesions (Bovine Herpesvirus 2, Cowpox, Foot and Mouth Disease Virus and Bovine Papillomavirus).

Viruses are not the only type of non-mastitis pathogen that may affect mammary gland health, with the causative agent of Johne's Disease, MAP, also having been associated with increased risk of clinical and subclinical mastitis in cattle (Dieguez et al., 2008, Rossi et al., 2017). *Mycobacterium avium* spp. *paratuberculosis* is a facultative intracellular bacterium that is predominately transmitted via the faecal-oral route but also inhalation of aerosols, (Eisenberg et al., 2010). Due to the well-established financial burden of Johne's Disease to

the dairy industry, milk effects have been more extensively explored compared to *C. burnetii* (Ott et al., 1999).

At the herd level, dairy farms positive for Johne's Disease have reported higher incidence of clinical mastitis and increased BMSCC compared with Johne's Disease negative farms (Dieguez et al., 2008). This relationship may be owing to the effect Johne's Disease exhibits on culling rates, as MAP ELISA-positive cows have been identified as 1.4 times more likely to be culled than ELISA-negative cows, which thereby reduces the opportunity to select animals based on SCC/clinical mastitis (Tiwari et al., 2005). Likewise, an individual cow level study found MAP ELISA-positive cows displayed higher incidence of clinical mastitis compared with ELISA-negative cows, a finding that was irrespective of specific Johne's Disease manifestation (latent, high shedding, low shedding), possibly due to insufficient statistical power from the small sample sizes of these groups (Rossi et al., 2017). Important to interpreting these findings, is that MAP infection in the bovine mammary gland does not result in severe inflammation, and rather it has been proposed this site may be a reservoir for replication in the host (Rossi et al., 2017). As such theories for clinical mastitis associations with MAP have centred on an indirect role for this pathogen. Comparisons have been drawn to the bacterium *Mycoplasma arginine* which has displayed an inability to increase SCC independently, though during co-infection with *Streptococcus dysgalactiae* infected cows presented with more severe clinical mastitis (Stipkovits et al., 2013). A differing concept is that pre-existing mastitis may influence MAP shedding and as such predispose cows with mastitis to MAP presence in their milk. *In vitro, E. coli* infection of bovine mammary epithelial cells facilitates translocation of MAP to the apical surface of the

polarised cell that secrete into the lumen, thereby providing one possible mechanism for increased MAP presence in mastitic milk (Schwarz et al., 2018). MAP has also shown potential ability to increase permeability of intestinal epithelium (Pott et al., 2009) and if able to replicate this characteristic in mammary gland epithelium could increase the volume of SCC accessing the lumen from the blood stream of an active clinical or subclinical mastitis case.

Subclinical mastitis to a lesser degree has also been associated with MAP seropositivity at the herd and individual cow level, and as such, *in vitro* work has attempted to assess co-infection with contagious mastitis pathogens (McNab et al., 1991, Pena et al., 2020). Pena et al. (2020) found no significant interactions between MAP and *Streptococcus agalactiae*, while conversely, introducing *Staphylococcus aureus* to MAP-infected bovine mammary epithelial cells facilitated increased internalisation. The ability of MAP to up-regulate the membrane glycoprotein ICAM-1 (Pott et al., 2009), that *Staphylococcus aureus* binds to for internalisation, has been proposed as the mechanism increasing *Staphylococcus aureus* internalisation (Pena et al., 2020), providing initial evidence of a synergistic effect which requires further validation by experimental animal infection studies.

Thereby it is clear there are diverse roles for pathogens of the mammary gland and unravelling a singular effect on mastitis in a microenvironment of multi-faceted co-infection interactions is complex. It is difficult to place *C. burnetii* in the context discussed above, owing to the limited amount of work conducted into the effect on mammary gland health. In saying this, in cows free of common intramammary infectious agents, shedding *C. burnetii*

in milk has been associated with significantly higher SCC compared to non-*C. burnetii* milk shedders, thereby providing foundational evidence that this bacterium may have a direct effect on subclinical mastitis (Barlow et al., 2008). Likewise, in a study of cows with mastitis, the SCC of *C. burnetii* antibody positive quarters were significantly higher than negative control quarters (Khatun et al., 2022). Furthermore, cow milk positive for both *C. burnetii* and a common mastitis pathogen has also recorded higher average SCC compared with a common mastitis pathogen alone. A similar finding was determined for *C. burnetii* antibody positive quarters and gram-positive bacteria (Barlow et al., 2008; Khatun et al., 2022). While this may be due to both pathogens independently increasing SCC, it also cannot be ruled out that *C. burnetii* could exhibit an indirect role in mastitis, similar to synergistic interactions suggested for some minor mastitis pathogens. Interestingly, *in vitro* infection of *C. burnetii* in bovine mammary epithelial cells fails to induce a substantial pro-inflammatory response (IL-1β, IL-6 and TNF-α), despite these cells strongly reacting to positive control LPS from *E. coli*, possibly signalling *C. burnetii* may utilise immunosuppression in the mammary gland for effective replication. Although, in doing so, it may also incidentally increase susceptibility of the host to establishment of secondary bacterial infections, an interaction that has been suggested to explain associations between various viruses and mastitis (Sobotta et al., 2017). Alternatively, due to the presence of a type IV secretion system encoded in the *C. burnetii* genome (with unknown function), this bacterium may synergistically interact with mastitis pathogens via production of substances which modulate the cellular microenvironment in favour of their survival. On the other hand, *C. burnetii* could indirectly effect SCC through influence on the host as opposed to the mastitis causing pathogen itself. Epithelial cells have been suggested as a target at excretion sites to facilitate shedding and while *in vitro, C. burnetii* replication maintains cell viability, functional changes to the

epithelium have not been examined *in vitro* (Sobotta et al 2017). An increase of the blood constituents, lactate dehydrogenase, serum albumin and total immunoglobulin G, were found in the milk of quarters with *C. burnetii* antibody compared to those without, potentially signifying that past exposure may lead to blood-milk barrier damage (Khatun et al., 2022). Any changes to epithelial permeability could influence paracellular transport of somatic cells through the blood-milk barrier and as such the mastitis severity classification of an existing case (Zhao and Lacasse, 2008).

Advancing understanding of *C. burnetii* pathogenesis in the mammary gland will refine such discussed theories. Moreover, increasing feasibility of on-farm multiplex PCR-based methods in mastitis diagnosis may allow for the impact and epidemiology of aerobically unculturable pathogens to be elucidated (Ashraf and Imran, 2018). When considering it is a common occurrence in mastitis studies for a proportion of total samples to have unknown aetiology (for example 20 – 31%) (Ganda et al., 2016, Petrovski et al., 2009), there is incentive to continue investigation into the effect lesser understood pathogens may exhibit on mammary gland health.

1.9 *Coxiella burnetii* pathogenesis in the mammary gland

1.9.1 Factors influencing shedding

1.9.1.1 Shedding route

The specific kinetics of shedding patterns are complex, and details surrounding onset, persistence and concomitancy remain misunderstood, in part due to contradictory results. It has been theorised that following inhalation of *C. burnetii* and dissemination within the host, shedding may occur due to localisation of infection in the epithelial cells of the excretion sites (Sobotta et al., 2017). This hypothesis was based on molecular and visual detection of *C. burnetii* replicating in placental epithelial cells in goats just prior to expulsion of the placenta (Roest et al., 2012, Sánchez et al., 2006). As such the varying availability of tissue to bacteria between animals based on host factors such as specific physiological stages (placental presence, hormone profiles), or immune responses, may explain the reported shedding variability. In experimentally infected goats, pregnant does were reported to have *C. burnetii* positive placentas at parturition (Sanchez et al., 2006), whereas experimentally infected non-pregnant goat does did not, despite positive mammary gland and milk samples from one goat doe (Roest et al., 2020).

Other factors which are often unknown in studies, including transmission route and bacterial strain, may influence tissue preferences. In humans, pathogenicity of *C. burnetii* can be linked to genotype (D'Amato et al., 2015), which in animals may similarly influence shedding route. In animal models, transmission routes can influence disease symptoms, for example, in mice experiments, intranasal injection caused pneumonia while intraperitoneal inoculation resulted in hepatosplenomegaly and pneumonia, and therefore, may likewise impact shedding manifestation (Marrie, 1995). This theory could be used to explain the two distinct shedding patterns observed in a goat herd that had experienced an abortion outbreak, whereby faecal and vaginal shedding occurred concomitantly in 81% of cases but

milk shedding occurred with another route on only 49% of cases (Rousset et al., 2009). If *C. burnetii* was to enter through the mammary gland, the infection may remain limited to this organ, however, if transmission by inhalation was to occur, a systemic infection and dissemination to all organs may be more likely.

Although not all findings support the theory that tissue localisation is critical to shedding, as Roest et al., (2012) identified bacteria in milk from goats with PCR negative mammary gland tissue. One explanation may be that *C. burnetii* could be excreted within immune cells from systemic infection which has been proposed as a mechanism by which organisms from the human gut enter colostrum (Qutaishat et al., 2003). Indeed, *C. burnetii* has been detected in bovine mammary lymph node tissue (confirmed by injection of sample into guinea pigs) as well as by PCR in experimentally infected goats in three studies (Bouvery et al., 2003, Jellison et al., 1948b, Roest et al., 2012, Roest et al., 2020, Sanchez et al., 2006). However, it also must be considered, in large organs such as the mammary gland, bacterial DNA may not be uniform throughout tissue, meaning non-representative samples for PCR could influence validity of conclusions (Hensen et al., 2000).

Identifying the relative location of *C. burnetii* in excretions or tissue may aid understanding of shedding dynamics but while histology of excreted placentas has highlighted *C. burnetii* in the trophoblasts, similar work has not been conducted on vaginal mucus, milk and faecal samples (Roest et al., 2012, Sanchez et al., 2006). Interestingly, histological analysis of tissue from cow and goat reproductive tracts, mammary glands and digestive tracts has thus far failed to identify *C. burnetii*, despite positive PCR results in these tissues (Jellison et al.,

1948b, Sanchez et al., 2006). This may reflect a lack of immunohistochemistry sensitivity to small quantities or particular structures that vary between the *C. burnetii* developmental stages (small and large cells variants) which have previously shown differences in their staining patterns and antigenic profiles (McCaul et al., 1991). More recently, *C. burnetii* was visualised by FISH in the mammary epithelial cells of one naturally infected goat shedding *C. burnetii* through milk (Bauer et al., 2024).

1.9.1.2 Shedding onset

The timing between initial infection and onset of shedding is also still unclear. Several seroconversion studies have identified first detection of infection around parturition, even on endemic farms where presumably animals would have been exposed to *C. burnetii* since birth (Muleme et al., 2017, Freick et al., 2017). This may be due to the high bacterial load in the environment following expulsion of placentas and the lower immune response of periparturient cows or, reflect a lack of sensitivity of ELISA to detect antibodies in small quantities. While shedding in heifers that seroconverted in the pre-partum period has been identified at parturition in vaginal mucus and milk, shedding has not been monitored prior to calving to establish timing of onset (Freick et al., 2017). However, in two separate intranasal experimental infection studies with pregnant and non-pregnant goats, excretion in faeces and vaginal mucus did not begin until parturition, despite inoculation taking place at 76 days gestation and prior to breeding, respectively (Roest et al., 2012, Roest et al., 2020). While this may be due to a normal lag period between infection and shedding (which

is not well defined), there could also be a potential trigger for shedding related to parturition. Furthermore, histopathological analysis of placental tissue from experimentally infected goats throughout gestation and at abortion, found considerable bacterial multiplication just prior to abortion (Sanchez et al., 2006).

Various physiological changes occur approaching parturition, including immunity and hormone profiles, which could also influence *C. burnetii* replication and perhaps shedding. *In vitro, C. burnetii* replication is inhibited by addition of progesterone in axenic culture as well as within the JEG-3 cell line (human placental tumour cell line that produces and responds to progesterone) (Howard & Omsland 2020). The finding that progesterone directly inhibited *C. burnetii* growth without killing the bacteria lead Howard & Omsland (2020) to hypothesise, *C. burnetii* may target progesterone producing tissue such as the placenta, to limit growth during gestation and remain undetected until the placenta reaches maximal size at parturition. By this time progesterone production has dropped off, allowing for rapid bacterial replication (which has been observed in histological slides of goat placentas) of the fully developed placenta, and thereby optimal transmission risk following expulsion (Howard & Omsland 2020, Sanchez et al., 2006). Furthermore, the sex hormones, estrodial and testosterone have also been shown to effect disease severity and cellular response in mice, while *C. burnetii* seropositivity in gestational cows and goats was found to be associated with blood cortisol, progesterone, and pregnancy associated glycoproteins (Bauer et al., 2021, Garcia-Ispierto et al., 2010). No work has yet been done to assess an effect of such hormones on the other shedding routes, including the mammary gland cells

nor included assessment of other pregnancy hormones such as oxytocin and Gonadotropin-releasing hormone.

On the other hand, a controlled study detected faecal shedding prior to parturition, up to three weeks prior to parturition in some does (Bouvery et al., 2003), although notably the infection route (subcutaneous, which is likely uncommon in natural infection) and strain in this study differed to the previously mentioned goat inoculation investigations. Similarly, Garcia-Ispierto et al. (2013), found positive milk samples in cows at day 171-177 of gestation. However, when given the observational study type, it is not possible to accurately determine infection time, leaving open the possibility animals may be shedding following on from their previous parturition.

A controlled inoculation experiment has not yet explored milk shedding prior to parturition but *C. burnetii* DNA has been detected by PCR in udder tissue of gestational goats as soon as 26 days post infection (first sampling point) (Roest et al., 2012). Within a herd of 96 heifers who seroconverted predominately from parturition until 42 DIM, the proportion of shedders increased significantly with increasing days in milk, being significantly higher at 100 DIM (which remained high at 150 DIM) compared to parturition, 21 DIM, and 42 DIM. While intermittent shedding complicates the ability to make conclusions, this may indicate an onset as lactation progresses, possibly due to an increasing chance to become infected over time or again may be the result of a physiological change occurring throughout lactation (and in the case of commercial dairy cows as gestation progresses towards the next parturition) (Freick et al., 2017). For example, the number of mammary epithelial cells

in the bovine udder progressively decreases as lactation advances due in part to the shedding of viable mammary epithelial cells from the epithelium into milk (Herve et al., 2016). Therefore, as *C. burnetii* has shown ability to infect this cell type, its presence in milk could theoretically be determined by cell shedding rate and thus increase the chance of detecting presence in milk as lactations progresses (Sobotta et al., 2017). It should be noted that a small number of heifers were found to be shedding in milk on the day of parturition, but as no individual cow data was included in the study that would allow these shedders to be matched to their serostatus, an assessment of shedding relative to seroconversion is not possible (Freick et al., 2017). Using linear regression models, one cross-sectional study similarly found DIM to be a significant variable influencing milk shedding, with the highest proportion of shedders found in the 270+ DIM group (compared with 1-90 DIM, 91-180, 181-270), while another study using individual milk samples from 12 herds found parity to be a significant variable influencing shedding but not days in milk (Angen et al., 2011).

Complicating understanding is that even within one infection there may be potential for multiple shedding onsets due to the intermittent shedding pattern. Intermittent shedding is observed in dairy ruminants (Guatteo et al., 2007), particularly for milk, but it is unknown whether the varying presence in milk could be due to reactivation of a latent infection, or a chronic infection influenced by host physiological/bacterial factors. Understanding dynamics of infection in the mammary gland may help elucidate shedding onsets.

1.9.1.3 Shedding duration

The duration of *C. burnetii* shedding varies between routes and species. A persistent bacterial excretion pattern has been observed in the milk of dairy cattle and goats. Bovine *in vitro* work has indicated *C. burnetii* displays highest replication in mammary epithelial cells compared with lung, placental, and intestinal epithelia while failing to trigger a strong pro-inflammatory response (IL-1β, IL-6 and TNF-α) (Sobotta et al., 2017). Thus, *C. burnetii* may be able to persist undetected in the udder, enabling chronic infection/shedding from milk. In experimentally infected non-pregnant goats, *C. burnetii* was only found in one of ten goats in the mammary gland, associated lymph nodes, and colostrum, which also points towards a preference for this tissue, as samples were collected from many tissues including the liver, spleen, kidney, lung, bone marrow, ovaries, blood, feaces, and cotyledons and all were PCR-negative (Roest et al., 2020).

It is interesting to speculate whether the persistent but intermittent shedding observed in milk is the result of a true chronic infection or a latent infection that keeps recrudescing. When considering the differing viability of *C. burnetii* that has been found in milk between studies, there may be evidence for a persistent but non-infectious disease state in ruminants (Eldin et al., 2013, Hirai et al., 2012). Q fever latency has been investigated in rodent models using guinea pigs and mice previously infected with *C. burnetii* and found that suppression of the immune system by cortisone steroids and irradiation led to infection reactivation, as determined by the number of infective tissues compared to control animals (Sidwell et al., 1964a, Sidwell et al., 1964b). Rodent models have also been used to assess chronic infection, finding athymic mice (which lack a thymus) display chronic infection following phase 1 *C. burnetii* challenge, as opposed to euthymic mice that resist infection,

thereby indicating the T-cell response influences persistence in mice (Kishimoto et al., 1978).

Cell mediated immune responses are essential to clearance of intracellular pathogens, with human and mice studies having assessed the relationship between cytokines and *C. burnetii* infection outcomes, as cytokines are moderators of cell mediated immunity. For example, IFNγ is a TH1 cytokine that was found (*in vitro*) to be involved with *C. burnetii* killing within human monocytes by apoptosis (Dellacasagrande et al., 1999). Interleukin (IL)-10 is an anti-inflammatory cytokine that dampens CMI responses, and *in vitro*, monocytes from chronic Q fever patients displayed elevated IL-10 production compared to acute Q fever patients (Capo et al., 1996). Furthermore, the addition of exogenous IL-10 to *C. burnetii* infected human monocytes has been shown to increase bacterial replication via an interaction with TNFa but it remains unclear whether bacterial factors could upregulate IL-10 in chronic patients or whether these patients may have an impaired response that facilitates chronic *C. burnetii* infection (Capo et al., 1996, Ghigo et al., 2001). A host genetic influence on Q fever persistence has previously been suggested based on two species of mice reacting differently to immune suppression following a previous *C. burnetii* challenge, with laboratory mice displaying increase in infective tissue while Deer mice had no change observed (Sidwell et al., 1964a).

Despite the perceived importance of CMI responses to *C. burnetii* infection in humans and mice, only a small number of studies have assessed these responses in infected cattle. Małaczewska et al. (2018) found, within an endemically infected herd in Poland, significantly

higher IFNγ and lower IL-10 serum levels from seropositive non-shedders compared to seropositive shedders based on a single time point assessment (prior to vaccination) that included assessment of shedding from either the vaginal and/or milk route. However, given that cytokine levels were measured directly from the serum, it is not possible to know whether the reported levels were the result of *C. burnetii* infection or otherwise influenced by a different pre-existing or resolved pathogen infection. Use of the interferon gamma recall assay by Bottcher et al. (2017) allowed more specific assessment of cytokine response in an endemic cattle herd, considering in this assay, *C. burnetii* antigen is added to the blood to stimulate a response before the ELISA is conducted. The shedding response of the herd had been monitored over two years and after grouping the cows by shedding pattern, it was found that shedding in both vaginal mucus and milk at calving, had insignificantly ($p < 0.07$) lower IFNγ compared to the group shedding only in vaginal mucus at calving. Furthermore, significantly higher proportion of the group shedding in vaginal mucus at calving were positive for IFNγ compared to the group shedding only in milk. They theorised that the lower IFNγ levels observed in milk shedders, compared to those in vaginal mucus, may be the result of varying infection routes causing a difference in immune response, as infection route was found to determine clinical manifestation of acute Q fever in mice (Marrie et al., 1996). However, it should be noted to compare these groups, a phase 2 *C. burnetii* antigen was used which is an avirulent cell that is only produced after serial passage in a laboratory setting, and while the truncated LPS may allow for exposure of more immunogenic surface proteins, this would not be a structure an animal would be exposed to in a natural infection. Additionally, an IL-10 monoclonal antibody was added during incubation of blood with the antigen to boost IFNγ production (as IL-10 levels have been found to reduce IFNγ

production), which may be a useful approach in a diagnostic context, as this increased the sensitivity of interferon gamma release assay diagnosis for MAP (Buza et al., 2004).

Interestingly, while cattle and goats have both shown milk shedding persistence over months and multiple lactations in observational studies, experimental infection studies in goats have thus far failed to replicate these field observations (Berri et al., 2007, Biberstein et al., 1974, Canevari et al., 2018, Rodolakis et al., 2007). Roest et al. (2012) could not detect milk shedding in four pregnant intranasally infected (strain: CbNL01, dose: 10^6) goats beyond 35 days post parturition, despite positive faecal and vaginal swabs being found during the weekly testing until 95 days post parturition. Sanchez et al. (2006) found the average milk shedding duration from six subcutaneously infected goats (10^4 dose) was 16.8 +/- 9.6 days after abortion, with the two goats killed on day eight post abortion having PCR positive mammary glands, but the two goats killed on 120 days post abortion both being PCR negative. Using this same inoculation method (strain CbC1, dose: 10^4, method: subcutaneous), Bouvery et al. (2003) could only detect milk shedding 20-27 days after abortion in seven goats, however goats inoculated with 10^6 and 10^8 (six goats in each group) were shedding in milk up until the last day of their study at 52 days after abortion.

Between experimental infection studies and observational studies there are a variety of factors that may differ including inoculation dose, transmission route, and bacterial strain, all of which could explain the divergent results. Dosage has previously been shown to influence the efficiency of transmission in mice inoculated via oral gavage, with bacterial dissemination and seroconversion occurring at 10^8 but not 10^5 (Miller et al., 2021) and may

likewise impact shedding persistence. The same could be speculated for transmission route, as while it is known *C. burnetii* presence in the mammary gland and milk can occur following inhalation, the localisation of the udder following inoculation directly into the mammary gland has also been proven (Bell et al., 1949). The rate of occurrence for this transmission route in a natural setting is unknown but considering viable bacteria are excreted in milk, it is plausible *C. burnetii* could be spread contagiously during milking via the teat canal or by cuts in the skin. Furthermore, in this study, one cow had three of their quarters inoculated using different methods, that being teat canal, subcutaneous, and glandular, with shedding persistence even varying between these quarters. While this data is difficult to interpret, because quarters can act independently depending on interactions with other pathogens, such differences may still be relevant to include.

1.10 Focus of research

This review of the literature on *C. burnetii* infection in cattle, demonstrates there may potentially be a zoonotic transmission risk associated with cattle shedding *C. burnetii* in milk, however, it is unclear whether cattle infection (especially in the mammary gland) may lead to milk production losses. Therefore, this thesis aimed to further elucidate the factors that govern *C. burnetii* shedding in bovine milk (to ultimately help reduce human and animal infection) and to measure milk production in infected cattle to assess the potential impact of coxiellosis to the dairy industry. As CMI responses were only investigated in a limited number of cattle *C. burnetii* investigations but have been identified as an important component to clearance of infection in humans and mice, the first objective of this thesis

was to optimise an assay capable of measuring *C. burnetii*-specific CMI responses in cattle. Next, a longitudinal study was conducted in an endemically infected Australian dairy herd to investigate the influence of the *C. burnetii* cell mediated and humoral immune responses on shedding throughout the reproductive and lactation cycle. Then, using the described *C. burnetii* shedding and immune responses as exposure variables, the impact of *C. burnetii* on milk production (volume, total solids, fat, and protein) was explored over a lactation. Finally, the mammary gland health of cows shedding *C. burnetii* in milk was investigated by assessing the relationship between gland quarter level presence of *C. burnetii* and SCC.

Chapter 2 Optimisation of a bovine whole blood cytokine recall assay for the detection of interferon-gamma and interleukin-10 following stimulation with *Coxiella burnetii* antigens.

The content within Chapter 2 is under review for publication in the Journal of Veterinary Diagnostic Investigation.

2.1 Abstract

The intracellular bacterium, *Coxiella burnetii*, causes Q fever in humans and coxiellosis in animals. Cell mediated immune responses have been shown to be important in Q fever however there is a paucity of studies investigating their role in coxiellosis in cattle. Therefore, the present study aimed to optimise a whole-blood cytokine recall assay and to evaluate *C. burnetii*-specific IFNγ and IL-10 responses in naturally infected cattle. Duplicate blood samples were collected from cows in an endemically infected dairy herd and subsequently transported at ambient temperature or on-ice prior to stimulation with phase 1 and 2 *C. burnetii* antigen at varying concentrations for 24 and 48 hours and IFNγ and IL-10 were measured in supernatant by ELISA. Using the optimised assay methodology, the cytokine response was measured at calving and approximately eight weeks post calving. The effect of media preparation technique, sample storage temperature, co-incubation time, antigen concentration, sampling time, and *C. burnetii* phase on IFNγ and IL-10 response was assessed using generalised linear models and Spearman's correlation coefficient (ρ). The greatest mean cytokine responses were elicited when blood samples were transported at

ambient temperature and stimulated with the highest *C. burnetii* antigen concentration for 48 hours. There was a significantly greater mean IFNγ (2.04 times) and IL-10 (1.86 times) response at eight weeks post calving compared to calving. At eight weeks post calving, the correlation between cytokine response to phase 1 and 2 *C. burnetii* was ρ = 0.84 for IFNγ and ρ = 0.91 for IL-10. Ultimately this optimisation study will guide informed use of the cytokine recall assay in cattle research, allowing effective investigation of CMI response in coxiellosis.

2.2 Introduction

Coxiella burnetii is the gram-negative, intracellular bacterial pathogen responsible for the zoonotic disease, Q fever in humans and coxiellosis in animals. In humans, infection typically manifests as an acute flu-like illness but can also present as a persistently focal infection, such as endocarditis, which may result in death in a small percentage of patients (Maurin and Raoult, 1999). Domestic ruminants (cattle, sheep and goats) are the most important reservoir for human infection with the bacterium shed primarily in the products of conception (placenta and foetal fluids) however it has also been demonstrated to be shed in faeces and sporadically in milk throughout lactation (Hansen et al., 2011, Rodolakis et al., 2007). For the most part, animal infections with *C. burnetii* are considered subclinical. If clinical disease does manifest in animals, it is usually in the form of reproductive failure, with *C. burnetii* being a well-accepted cause of abortions in goats and to a lesser extent in sheep (Arricau-Bouvery and Rodolakis, 2005, Sanford et al., 1994, Van den Brom et al., 2015). However, the disease manifestations in cattle are less clearly defined and, as such, further research is required to understand *C. burnetii* pathogenesis and impacts in this species to enable the development of informed and effective control measures such as vaccination.

Being an intracellular pathogen, cell mediated immune responses are integral in the containment of infection by the host (Andoh et al., 2007). Cytokines are important moderators of the CMI response. The T helper 1 pro-inflammatory cytokine, IFNγ, has been demonstrated to be necessary for *C. burnetii* infection clearance in mice and dysregulation of IFNγ may have a role in chronic disease progression in humans (Andoh et al., 2007, Pennings et al., 2015, Schoffelen et al., 2017). Interleukin 10 (IL-10) is a complex cytokine, with potent anti-inflammatory function but is also stimulatory for the humoral response. Interleukin 10 has been demonstrated to be required for *C. burnetii* replication in human monocytes *in vivo* and its spontaneous release from chronic Q fever patient PBMCs was elevated compared to healthy human controls (Capo et al., 1996, Ghigo et al., 2001).

There are various ways to measure cytokine responses, including use of the cytokine recall assay. Cytokine recall assays are preferred over other assays, such as measurement of gene expression and the unstimulated determination of cytokine levels in serum, because *ex vivo* stimulation of patient cells or whole blood samples with the specific antigen/s of interest can determine pathogen-specific cytokine secretion. In Q fever research, the assay has been used to investigate how CMI responses may influence acute and chronic (persistently infected) *C. burnetii* infection states in humans and, more recently, the assay has been proposed as a method of diagnosis and alternative to the skin test for pre-vaccination screening (Schoffelen et al., 2017, Schoffelen et al., 2013a, Scholzen et al., 2021).

Most *C. burnetii* research in cattle has historically focused on measuring the humoral response and, while this is valuable for determining herd level exposure, information

regarding CMI responses is vital to understanding the pathogenesis of this intracellular pathogen (Freick et al., 2017, Guatteo et al., 2007, Guatteo et al., 2012). In cattle studies, cytokine recall assays have been used to gain insights into pathogenesis and diagnosis of other intracellular bacterial species, such as MAP, which suggests that this assay may also be useful in investigations of *C. burnetii*, and its use has been described in a small number of studies employing methodology similar to that used in human studies (Boettcher, 2017, Waters et al., 2003). However, the cytokine recall assay has likely been under-utilised to date, in part due to logistical constraints such as the requirement to stimulate samples within 18 hours of collection and a paucity of information regarding its optimal use with cattle samples.

The aim of this study was therefore to optimise the whole blood cytokine recall assay specifically for use with *C. burnetii* antigens for the detection of the cytokines IFNγ and IL-10 in cattle. Technical variables were adjusted including media preparation technique, storage temperature of samples during transportation and the duration of sample incubation with *C. burnetii* antigens at varied concentrations to optimise production of IFNγ and IL-10 in a whole blood cell assay. The assay was further evaluated for informed use within the context of dairy management systems by assessing the impact of sampling time relative to calving on cytokine responses.

2.3 Materials and Methods

2.3.1 Ethics Approval

Approval for the study was granted by the University of Sydney Animal Ethics Committee (Ethics Approval number: 2021/2014).

2.3.2 Animals and sample collection

Samples for the experiments in this study were derived from dairy cows on a farm located in New South Wales, Australia. Lactating cows were fed a total mixed ration diet comprised of corn and alfalfa silage, canola meal, brewers grain, wheat, citrus pulp, and almond hulls. *Coxiella burnetii* infection was verified as endemic in the herd through serological evidence of antibody against *C. burnetii* in both serum samples from individual cows and BTM samples, molecular evidence of *C. burnetii* DNA via PCRs in milk, placenta, feces, and vaginal swabs, and growth of *C. burnetii* in Vero cell cultures, from milk, serum, placenta, and vaginal swabs as part of another study (unpublished data). Optimisation of the cytokine recall assay for use with cattle samples was conducted according to the study design summarized in Table 2.1. For the experiments for optimisation of technical variables, 6 cows between 42 – 270 DIM were selected for testing the media preparation technique and the sample storage temperature during transport, while 4 cows between 106 – 116 DIM were used to test co-incubation time and *C. burnetii* antigen concentration. For experiments of evaluation of the assay within the lactation cycle, 46 cows were samples at 1– 3 days post calving and at approximately 8 weeks post calving (45 – 60 DIM) to test the effect of sampling time. To assess the effect of *C. burnetii* phase 169 cows at approximately 8 weeks

post calving (45 – 60 DIM) were used. Specific details for each variable that was assessed are provided with the corresponding results.

Table 2.1. Study design for optimisation of the cytokine recall assay for use with Australian dairy cattle.

	Variables assessed	Levels	Number of animals[c]	Stage of lactation
Optimisation of technical variables	Media preparation technique[a]	Fresh Frozen	6	42 – 270 days in milk (DIM)
	Sample storage temperature during transport[a]	On-ice Ambient	6	42 – 270 DIM
	Co-incubation time[b]	24 hours 48 hours	4	106 – 116 DIM
	C. burnetii antigen concentration[b]	5 µg/ml 0.5 µg/ml 0.05 µg/ml	4	106 – 116 DIM
Optimisation of assay within the lactation cycle	Sampling time	Calving Eight weeks post calving	46	1– 3 days post calving and approximately 8 weeks post calving (45 – 60 DIM)
	C. burnetii phase	Phase 1 Phase 2	169	Approximately 8 weeks post calving (45 – 60 DIM)

[a] Variables assessed in the same experiment

[b] Variables assessed in the same experiment

[c] Total number of animals in each experiment

Blood (10 mls) was collected from the coccygeal vein (located in the tail) of cattle into commercially available vacuum sealed polyethylene terephthalate blood collection tubes, containing 158 USP units of spray dried lithium heparin (BD Vacutainer, Becton Dickinson). Care was taken to ensure the method of blood collection did not result in the formation of microclots within the sample, with the lithium heparin vacuum sealed blood collection tube filled first if other tubes (for example, for serum) were also collected concurrently. Following collection, blood tubes were gently inverted to incorporate the anticoagulant and transported to the laboratory at ambient temperature or on ice until processing.

2.3.3 Whole blood stimulation assay

The whole blood stimulation assay was adapted from previously published methodology utilised in human (Schoffelen et al., 2013b) and ruminant (Boettcher, 2017) *C. burnetii* IFNγ studies and from an IFNγ recall assay using MAP antigen (Begg et al., 2017, Jungersen et al., 2002).

2.3.3.1 Culture plates and media

All experiments were conducted in tissue culture treated 48 well polystyrene plates (Corning Costar, Corning). Complete culture medium used for all experiments consisted of RPMI medium 1640 (Sigma-Aldrich), containing 10% fetal bovine serum (FBS; Invitrogen), 2mM L-glutamine (Sigma), and 100 µg/ml penicillin-streptomycin (Invitogen). All plates were pre-

loaded with 300 µl of media only, or media to which mitogens or antigens were added (details provided in section 2.3.3.2 and 2.3.3.3), and frozen at -45 °C until use, except for the experiments which compared outcomes with frozen and fresh media. For the latter, complete media was made up immediately prior to loading the whole blood onto the plates.

2.3.3.2 Antigens

Commercially available formaldehyde inactivated whole cell Nine Mile strain *C. burnetii* phase 1 and phase 2 antigens (B1CF1; B1CF2; Dolfinin) were used in all experiments in this study. According to the manufacturer, the product is supplied at an antigen concentration of 1 mg/ml (data on genomic equivalents was not available). In the optimisation experiments of technical assay variables (Table 2.1), three final concentrations were tested: 5 µg/ml, 0.5 µg/ml, and 0.05 µg/ml. For the optimisation experiments through the lactation cycle (Table 2.1), a final concentration of 5 µg/ml was used. Working solutions at each concentration were prepared (diluted in media) and aliquoted into duplicate wells for each blood sample for the initial technical optimisation, and into singular wells for each blood sample for the subsequent optimisations with larger animal numbers. Interferon gamma and IL-10 responses to antigen were assessed for each blood sample.

2.3.3.3 Controls: mitogen and lipopolysaccharide

Pokeweed mitogen (PWM; Sigma) at a final concentration of 5 µg/ml was used as a non-specific T and B cell positive control, to ensure that the cells in each sample were viable and capable of responding to antigenic stimulation. A rough form lipopolysaccharide (LPS) (which lacks the O-antigen region) derived from *Escherichia coli* K12 (LPS-EK Ultrapure; Invitrogen) at 0.01 µg/ml was also included as an innate immune response positive control. Working solutions of each were prepared and aliquoted into duplicate wells for each blood sample for the initial technical optimisation and into singular wells for each blood sample for the subsequent optimisations with larger animal numbers. Interferon gamma and IL-10 responses to mitogen and LPS were assessed for each blood sample.

2.3.4 Stimulation assay

Pre-loaded plates (see section 2.3.3.1), previously stored at -45 °C were thawed in an incubator set at 37 °C (HERAcell 150, Kendro Laboratory). Plates loaded with freshly prepared media (300 µl) were also brought to 37°C in an incubator prior to loading blood into the wells. Blood samples were loaded onto both freshly prepared and pre-prepared thawed plates within 18 hours following collection. Blood tubes were gently mixed on a rotating homogeniser, and 300 µl was added to each well of the 48 well plate such that the ratio of media (+/- additives) to blood was 1:1. Each plate was then placed into the incubator set at 37 °C with 5% CO_2 for 24 or 48 hours for the initial optimisation experiments or 48 hours for the subsequent optimisation experiments. Following this incubation, 300 µl of assay supernatant was transferred from each well to individual (polypropylene) tubes,

capped and placed in a 96-tube racked storage system (SSIbio) and stored at -45 °C until use in an ELISA.

2.3.5 Interferon gamma ELISA

Cytokine detection was performed on assay supernatant samples from the whole blood stimulation assay using a sandwich ELISA. Microplates (96 well; Nunc MaxiSorb, Thermo Scientific) were coated with monoclonal mouse anti-bovine IFNγ (clone IFN 6.19, a generous gift from Dr G. Jungersen) at a concentration of 1.5 µg/ml diluted in PBS and incubated at 4 °C overnight. The plates were washed three times in wash buffer (0.05% Tween20 in reverse osmosis [RO] water) prior to the addition of 50 µl of undiluted assay supernatant sample or IFNγ standards (standard curve ranged from 0 ng/ml to 20 ng/ml) (Kingfisher Biotech) diluted 1:2 in PBS. Following incubation for 1 hour at room temperature (RT), each plate was washed three times in wash buffer before adding 50 µl per well of a biotin-conjugated secondary antibody (mouse anti-bovine IFNγ clone CC302-Biotin; Serotec) at a concentration of 0.5 µg/ml in PBS. The plates were then incubated for 1 hour at RT and then washed three times with wash buffer prior to the addition of 50 ml per well of HRP-Streptavidin (KRL Laboratories) to each well at 0.167 µg/ml. Following incubation at RT for 20 minutes, each plate was washed five times prior to the addition of 100 µl per well of tetramethylbenzidine substrate set (BD OptEIA, BD Biosciences) followed by incubation in the dark. The development of the plates was monitored using the 620 nm wavelength on an absorbance plate reader (Multiskan Ascent, Thermo Electron Corporation) until the 20 ng/ml standard reached an optical density (OD) of 0.45 to 0.5 or the negative control exceeded OD

0.05 at which point the reaction was stopped by adding 100 µl per well of 2M sulfuric acid. All samples and standards were run in duplicate (technical replication) and OD were measured on an absorbance plate reader at 450 nm. An average optical density for each sample was obtained from the duplicates and values were converted to ng/ml based on the standard curve. The cytokine response is reported with each cow's unstimulated control (media background) subtracted, unless otherwise stated. For the analyses where the purpose was to compare result presentation, sample to positive ratios (S/P ratio [%]) were also calculated: ([OD *C. burnetii* stimulation – OD media background control]/ [OD stimulation positive control (PWM) – OD media background]) x 100.

2.3.6 Interleukin 10 ELISA

Interleukin 10 detection was performed on assay supernatant samples from the whole blood stimulation assay using a sandwich ELISA. Microplates (96 well; Nunc MaxiSorb,) were coated with mouse anti-bovine IL-10 antibody (clone CC318; Bio-Rad) at a concentration of 2.5 µg/ml diluted in wash buffer (0.05% Tween20 in RO water) and incubated at 4 °C overnight. Plates were washed three times with wash buffer prior to the addition of 50 µl of undiluted assay supernatant sample or undiluted IL-10 standards (standard curve ranged from 0 ng/ml to 25 ng/ml) (bovine IL-10 Yeast-derived Recombinant Protein; Kingfisher Biotech). Following incubation for 1 hour at RT, each plate was washed three times with wash buffer before adding 50 µl per well of a biotin-conjugated secondary antibody (mouse anti bovine IL-10 clone CC320-Biotin; Biorad) at a concentration of 0.5 µg/ml in wash solution. The plates were then incubated for 1 hour at RT and then washed three times with

wash buffer prior to the addition of 50 µl per well of HRP-Streptavidin (KRL Laboratories) to each well at 0.333 µg/ml. Following incubation at RT for 30 minutes, each plate was washed five times with wash buffer prior to the addition of 100 µl per well of tetramethylbenzidine substrate set (BD OptEIA, BD Biosciences) followed by incubation in the dark. The development of the plates was monitored using the 620 nm wavelength on an absorbance plate reader until the 25 ng/ml standard reached OD 0.45 to 0.5 or the negative control exceeded OD 0.05 at which point the reaction was stopped by adding 100 µl per well of 2M sulfuric acid. All samples and standards were assayed in duplicate, and OD were measured on an ELISA plate reader (Multiskan Ascent, Thermo Electron Corporation) at 450 nm. An average optical density for each sample was obtained from the duplicates and values were converted to ng/ml based on the standard curve. The cytokine response is reported with each cow's unstimulated control (media background) subtracted, unless otherwise stated. As a comparison, S/P ratios were also calculated: ([OD *C. burnetii* stimulation – OD media background control]/ [OD stimulation positive control (PWM) – OD media background]) x 100.

2.3.7 Statistical Analyses

The effects of media preparation technique, sample storage temperature, co-incubation time, antigen concentration, sampling time, and *C. burnetii* phase on IFNγ and IL-10 response was determined using generalised linear models with a gaussian distribution (based on distributions of Pearson residuals) and a log link and cow fitted as a fixed effect (as when fitted as a random effect some models would not converge). Exponentiated

coefficients from these models estimate ratios of arithmetic means for the variable category of interest relative to the comparison category (i.e. the reference category) of that variable. Interactions were explored for experiments with multiple variables, with inclusion of any interaction terms in the models specified in the results section. Consideration of interaction terms was based on scientific knowledge which were co-incubation time by antigen concentration, co-incubation time by *C. burnetii* phase, antigen concentration by *C. burnetii* phase, and sampling time by blood stimulant type, with inclusions of interaction terms (variable selection) was decided based on a combination of sample size, data visualisation, and P-values (Wald p values) for interaction terms. As there was no interaction between *C. burnetii* phase and other variables, phase 1 and 2 data were pooled for statistical analysis. If there was no reasonable basis for assuming interaction, only the main effects of covariates were fitted (main effects once fitted were not removed [because those effects were of interest regardless of P-values]). Assessment of the effect of sampling time on cytokine response for the positive control pokeweed mitogen was conducted using a tobit model to account for right censored data, as 20 ng/ml was the highest possible result. With this model, standard errors were adjusted for clustering by cow (using the robust [sandwich] estimator of variance that allows for intragroup correlation). Only descriptive statistics were used to assess the effect of cytokine response from the control mitogens in the initial technical variable optimisation due to restricted sample sizes. Correlation between cytokine response to phase 1 and 2 *C. burnetii* and presenting cytokine data as a S/P% and in ng/ml were determined using the Spearman's correlation coefficient (ρ). Statistical analyses were performed using Stata (StataCorp; version 18) and figures were created in RStudio (R Core Team, 2023).

2.4 Results

2.4.1 Effect of media preparation method and sample storage temperature during transportation on cytokine responses

The effect on IFNγ and IL-10 responses of varying the temperature at which the whole blood was kept throughout the time from collection until being placed in the stimulation assay, was determined using blood collected from six cows (Figure 2.1; Figure 2.2). A greater mean cytokine response to the positive control, PWM, and to *C. burnetii* phase 1 and 2 antigen was obtained from blood transported and stored at ambient temperature versus when blood was chilled on ice (Table 2.2). For IFN-γ, the response to PWM was 1.92 times higher (95% confidence intervals [95% CI]: 1.59 – 2.30; $p < 0.001$), and to *C. burnetii* was 2.46 times higher (95% CI: 2.01 - 3.02; $p < 0.001$) with ambient storage (Table 2.2). While for IL-10, the response to PWM was 1.85 times higher (95% CI: 1.46 – 2.35; $p < 0.001$), and to *C. burnetii* was 1.12 times higher (95% CI: 1.01 - 1.24; $p = 0.032$) (Table 2.2).

The effect of pre-preparing media and storing at -45°C prior to use in the stimulation assay versus using freshly prepared media (that had not been frozen prior to use) on IFNγ and IL-10 responses in whole blood obtained from six cows was examined (Figure 2.1; Figure 2.2; Table 2.2). There was no significant difference in the mean IFNγ response between the two treatment groups to stimulation with the positive control PWM (95% CI: 0.63 – 1.21; $p = 0.414$) or with *C. burnetii* antigen (95% CI: 0.81 –1.49; $p = 0.532$) (Table 2.2). There was some limited evidence to suggest IL-10 response was lower when using the pre-prepared media

compared to the freshly prepared media for PWM (95% CI: 0.52 – 1.00; p = 0.053) and *C. burnetii* antigen (95% CI: 0.81 –1.00; p = 0.058) (Table 2.2).

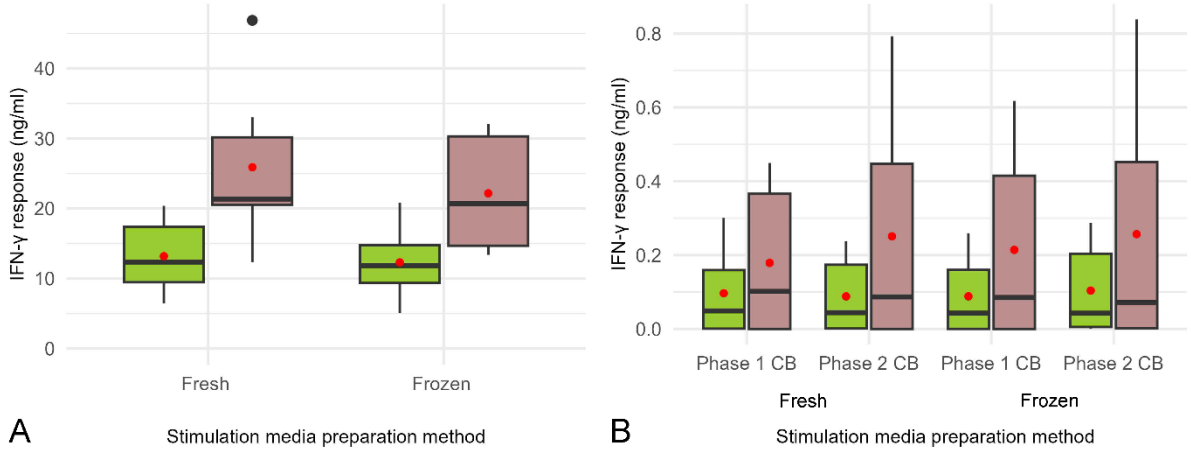

Figure 2.1. Box and whisker plot depicting the effect of media preparation method and sample storage temperature during transportation on IFNγ response to positive control pokeweed mitogen (PWM) and *Coxiella burnetii* antigen stimulation. Whole blood (n = six Australian cows) was transported either on-ice (green) or at ambient temperature (brown) then stimulated *ex vivo* for 48 hours with PWM (**A**) or *C. burnetii* antigen phase 1 (Phase 1 CB) or 2 (Phase 2 CB) (**B**). Cytokine response was determined using an ELISA and presented as ng/ml. Bolded black lines represent medians, red dots represent means.

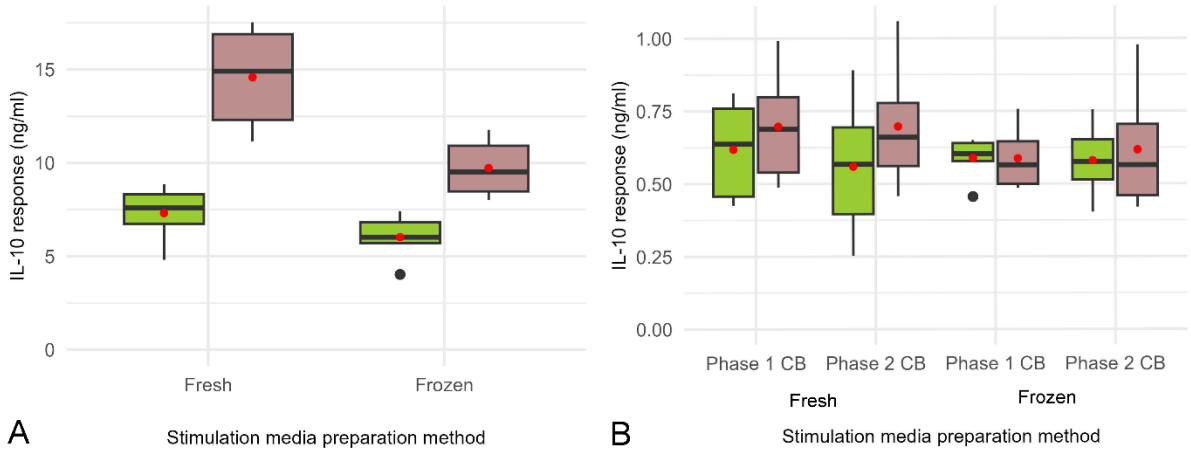

Figure 2.2. Box and whisker plot depicting the effect of media preparation method and sample storage temperature during transportation on IL-10 response to positive control pokeweed mitogen (PWM) and *Coxiella burnetii* (CB) antigen stimulation. Whole blood (n = six Australian dairy cows) was transported either on-ice (green) or at ambient temperature (brown) then stimulated *ex vivo* for 48 hours with PWM (**A**) or *C. burnetii* antigen phase 1 (Phase 1 CB) or 2 (Phase 2 CB) (**B**). Cytokine response was determined using an ELISA and presented as ng/ml. Bolded black lines represent medians, red dots represent means. Note: y-axis scales for A and B are different.

Table 2.2. Effect of media preparation method and sample storage temperature during transportation on IFNγ and IL-10 response to positive control pokeweed mitogen (PWM) and *Coxiella burnetii* antigen stimulation (using generalised linear models). Whole blood (n = six Australian dairy cows) was stimulated *ex vivo* with PWM or *C. burnetii* antigen for 48 hours. Cytokine response was determined using an ELISA and presented as ng/ml. Results from stimulation with phase 1 and 2 *Coxiella burnetii* are pooled.

Cytokine	Comparison	Variable	Number of results	Mean (sd)[a]	Coefficient[b]	CI	P value
IFN-γ	PWM						
	Media preparation method	Fresh	12	19.53 (11.20)	Reference category		
		Frozen	12	17.22 (8.68)	0.87	0.63 – 1.21	0.414
	Sample temp during transport	On-ice	12	12.74 (5.24)	Reference category		
		Ambient temp	12	24.02 (10.33)	1.92	1.59 – 2.30	<0.001
	C. burnetii						
	Media preparation method	Fresh	24	0.15 (0.21)	Reference category		
		Frozen	24	0.17 (0.24)	1.1	0.81 – 1.49	0.532
	Sample temp during transport	On-ice	24	0.09 (0.11)	Reference category		
		Ambient temp	24	0.23 (0.28)	2.46	2.01 – 3.02	<0.001
IL-10	PWM						
	Media preparation method	Fresh	12	10.95 (4.34)	Reference category		
		Frozen	12	7.88 (2.34)	0.72	0.52 – 1.00	0.053
	Sample temp during transport	On-ice	12	6.67 (1.45)	Reference category		
		Ambient temp	12	12.15 (3.31)	1.85	1.46 – 2.35	<0.001
	C. burnetii						
	Media preparation method	Fresh	24	0.64 (0.20)	Reference category		
		Frozen	24	0.59 (0.13)	0.90	0.81 – 1.00	0.058
	Sample temperature during transport	On-ice	24	0.59 (0.15)	Reference category		
		Ambient temp	24	0.65 (0.18)	1.12	1.01 – 1.24	0.032

CI = confidence intervals; sd = standard deviation; temp = temperature. [a] Units are ng/ml. [b] Estimated ratio of arithmetic means (adjusted for cow as a fixed effect).

2.4.2 Effect of *Coxiella burnetii* antigen concentration and incubation time on interferon gamma and interleukin 10 responses

The effect of *C. burnetii* antigen concentration and incubation time on IFNγ response was measured in whole blood samples from four cows. The greatest median IFNγ response to *C. burnetii* stimulation was elicited with the highest antigen concentration (5 µg/ml) for both phase 1 and phase 2 *C. burnetii* and after the longer (48 hours) incubation period (Figure 2.3). There was a statistically significant interaction between the variables: co-incubation time and antigen concentration, and therefore the effects of time was estimated separately for each concentration (Table 2.3), and the effect of concentration was estimated separately for each time (Table 2.4), resulting in a significant effect of co-incubation time on IFNγ response when the highest antigen concentration was used (Table 2.3). There was also a significant increase in IFNγ response with increasing antigen concentrations at both co-incubation times (Table 2.4). At 5 µg/ml, IFNγ response to *C. burnetii* increased from 0.04 ng/ml (+/- 0.03 ng/ml) after a 24-hour incubation, to 0.11 ng/ml (+/- 0.12 ng/ml) after a 48-hour co-incubation ($p < 0.001$) (Table 2.3).

The mean IFNγ response to media (unstimulated control) after 24-hour co-incubation was 0.01 ng/ml (when rounded to two decimal places) (+/- 0.03 ng/ml) and after 48-hour co-incubation was 0.01 ng/ml (when rounded to two decimal places) (+/- 0.01 ng/ml) (Figure 2.3). The mean IFNγ response to LPS after 24-hour co-incubation was 0.01 ng/ml (+/- 0.02 ng/ml) and after a 48-hour incubation was 0.02 ng/ml (+/- 0.03 ng/ml) (Figure 2.3). The

mean response to PWM (positive control) after a 24-hour co-incubation was 2.39 ng/ml (+/- 0.68 ng/ml) and after 48-hour co-incubation was 4.36 ng/ml (+/- 0.85 ng/ml) (Figure 2.3).

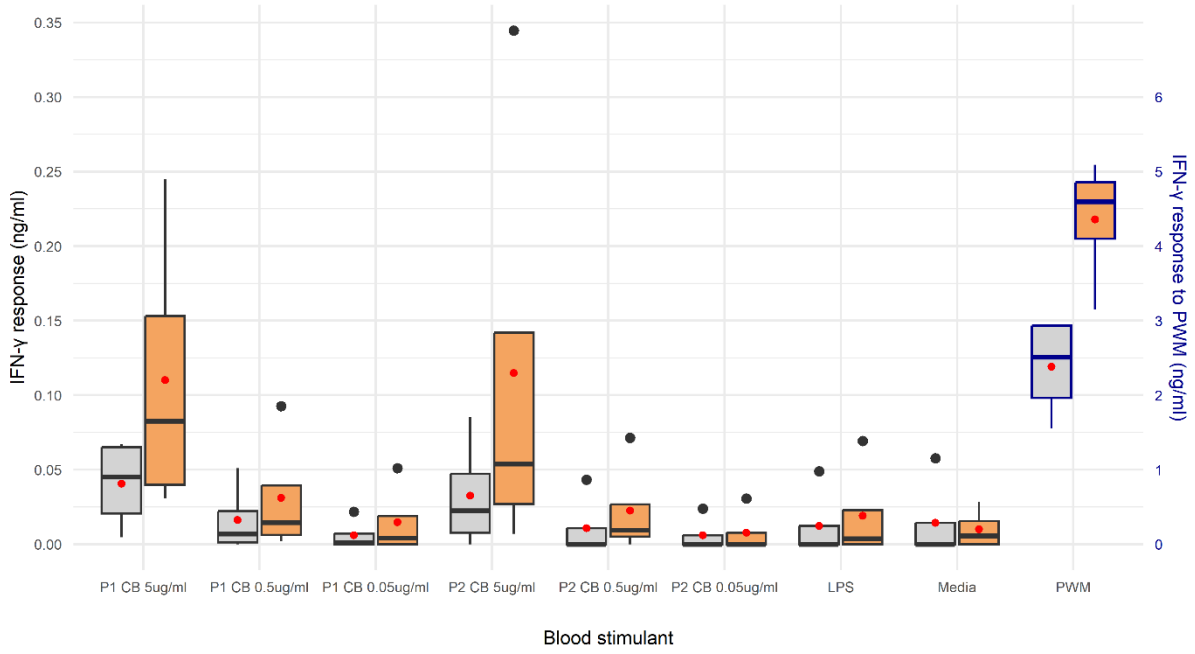

Figure 2.3. Box and whisker plot depicting the effect of IFNγ response to varying incubation times with *Coxiella burnetii* antigen and controls as well as *Coxiella burnetii* antigen at varying concentrations in Australian dairy cattle whole blood (n = results from four cows in each box and whisker plot). Whole blood was stimulated *ex vivo* with *C. burnetii* phase 1 (P1 CB) and 2 (P2 CB) at concentrations of 5 µg/ml, 0.5 µg/ml or 0.05 µg/ml and incubated for 24 hours (grey) or 48 hours (orange). Other stimulants included lipopolysaccharide from rough form *Escherichia coli* (LPS), media (unstimulated control) and pokeweed mitogen (PWM; positive control). Cytokine response was determined using an ELISA and presented as ng/ml. Bolded black lines represent medians, red dots represent means, black dots represent outlier values. IFNγ response to PWM is presented on the secondary y-axis.

The effect of *C. burnetii* concentration and incubation time on IL-10 response was measured in whole blood cells collected from four cows. The greatest mean IL-10 response to *C. burnetii* was elicited with the highest antigen concentration (5 µg/ml) for both phase 1 and phase 2 *C. burnetii* after stimulation for 48-hours prior to harvesting the supernatant (Figure 2.4). A statistical interaction was found between the variables: co-incubation time and antigen concentration, and therefore this study estimated the effect of time separately for each concentration (Table 2.3), and the effect of concentration was estimated separately for each time (Table 2.4). The effect of co-incubation time was significant at all concentrations (Table 2.3). There was a significant increase in IL-10 response with increasing antigen concentrations at both co-incubation times (Table 2.4). At 5 µg/ml, IL-10 response to *C. burnetii* increased from 0.53 ng/ml (+/- 0.16 ng/ml) after a 24-hour incubation, to 0.58 ng/ml (+/- 0.18 ng/ml) after a 48-hour co-incubation (p = 0.027) (Table 2.4).

The mean IL-10 response to media (unstimulated control) after a 24-hour co-incubation was 0.18 ng/ml (+/- 0.08 ng/ml) and after 48-hour co-incubation was 0.23 ng/ml (+/- 0.08 ng/ml) (Figure 2.4). The mean IL-10 response to LPS after 24-hour co-incubation was 0.46 ng/ml (+/- 0.1 ng/ml) and after 48-hour co-incubation was 0.51 ng/ml (+/- 0.12 ng/ml) (Figure 2.4). The mean response to PWM (positive control) after a 24-hour co-incubation was 3.10 ng/ml (+/- 1.27 ng/ml) and after 48-hour co-incubation was 5.37 ng/ml (+/- 1.64 ng/ml) (Figure 2.4).

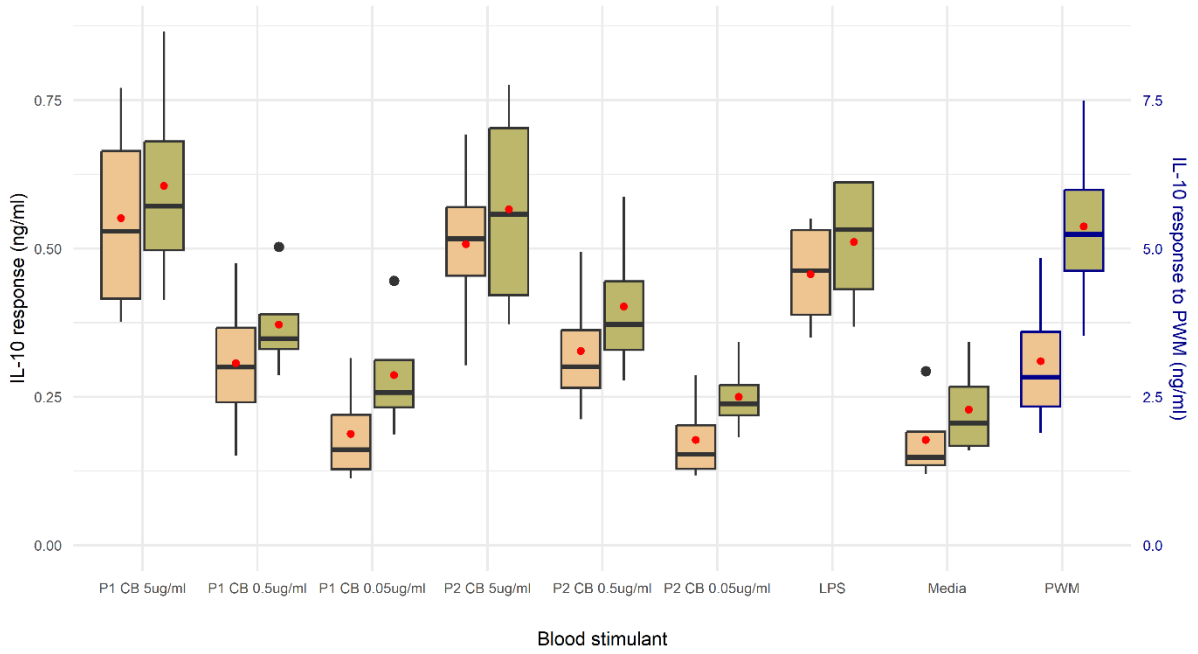

Figure 2.4. Box and whisker plot depicting the distribution of IL-10 response to varying incubation times with *Coxiella burnetii* antigen and controls as well as *Coxiella burnetii* antigen at varying concentrations in Australian dairy cattle whole blood (n = results from four cows in each box and whisker plot). Whole blood was stimulated *ex vivo* with *C. burnetii* phase 1 (P1 CB) and 2 (P2 CB) at concentrations of 5 µg/ml, 0.5 µg/ml or 0.05 µg/ml and incubated for 24 hours (yellow) or 48 hours (green). Other stimulants included lipopolysaccharide from rough form *Escherichia coli* (LPS), media (unstimulated control) and, pokeweed mitogen (PWM; positive control). Cytokine response was determined using ELISA and presented as ng/ml. Bolded black bars represent medians, red dots represent means. Cytokine response to PWM is presented on the secondary y-axis.

Table 2.3. Effect of incubation time on IFNγ and IL-10 response (using generalised linear models). Whole blood (n = four Australian dairy cows) was stimulated *ex vivo* with phase 1 and 2 *Coxiella burnetii* at concentrations of 5 µg/ml, 0.5 µg/ml or 0.05 µg/ml and incubated for 24 hours or 48 hours then cytokine response was measured by ELISA and presented as ng/ml. Results from stimulation with phase 1 and 2 *C. burnetii* are pooled.

Cytokine	***C. burnetii* conc, µg/ml**	**Incubation time, h**	**Number of results**	**Mean (sd)[a]**	**Coefficient[b]**	**CI**	**P value**
IFN-γ	5	24	8	0.04 (0.03)	Reference category		
		48	8	0.11 (0.12)	3.53	2.68 – 4.65	<0.001
	0.5	24	8	0.01 (0.02)	Reference category		
		48	8	0.03 (0.04)	1.82	1.00 – 3.29	0.48
	0.05	24	8	0.01 (0.01)	Reference category		
		48	8	0.01 (0.02)	1.80	0.52 – 6.23	0.353
IL-10	5	24	8	0.53 (0.16)	Reference category		
		48	8	0.58 (0.18)	1.11	1.01 – 1.22	0.027
	0.5	24	8	0.32 (0.12)	Reference category		
		48	8	0.39 (0.11)	1.19	1.03 – 1.38	0.018
	0.05	24	8	0.18 (0.08)	Reference category		
		48	8	0.27 (0.09)	1.43	1.12 – 1.81	0.003

CI = confidence intervals; conc = concentration; h = hours; sd = standard deviation

[a] Units are ng/ml

[b] Estimated ratio of arithmetic means (adjusted for cow as a fixed effect).

Table 2.4. Effect of antigen concentration on IFNγ and IL-10 response (using generalised linear models). Whole blood (n = four Australian dairy cows) was stimulated *ex vivo* with phase 1 and 2 *Coxiella burnetii* at concentrations of 5 µg/ml, 0.5 µg/ml or 0.05 µg/ml and incubated for 24 hours or 48 hours then cytokine response was measured by ELISA and presented as ng/ml. Results from stimulation with phase 1 and 2 *C. burnetii* are pooled.

Cytokine	Incubation time, h	*C. burnetii* conc, µg/ml	Number of results	Mean (sd)[a]	Coefficient[b]	CI	P value
IFN-γ	24	5	8	0.04 (0.03)	Reference category		
		0.5	8	0.01 (0.02)	0.51	0.28 – 0.91	0.023
		0.05	8	0.01 (0.01)	0.24	0.08 – 0.74	0.013
	48	5	8	0.11 (0.12)	Reference category		
		0.5	8	0.03 (0.04)	0.26	0.19 – 0.35	<0.001
		0.05	8	0.01 (0.02)	0.12	0.07 – 0.23	<0.001
IL-10	24	5	8	0.53 (0.16)	Reference category		
		0.5	8	0.32 (0.12)	0.61	0.54 – 0.70	<0.001
		0.05	8	0.18 (0.08)	0.35	0.29 – 0.44	<0.001
	48	5	8	0.58 (0.18)	Reference category		
		0.5	8	0.39 (0.11)	0.66	0.59 – 0.74	<0.001
		0.05	8	0.27 (0.09)	0.45	0.39 – 0.53	<0.001

CI = confidence intervals; conc = concentration; h = hours; sd = standard deviation

[a] Units are ng/ml

[b] Estimated ratio of arithmetic means (adjusted for cow as a fixed effect).

2.4.3 Interferon gamma and interleukin 10 response to phase 1 *Coxiella burnetii* in dairy cattle through lactation from calving to approximately eight weeks post calving

The IFNγ and IL-10 response was compared in whole blood collected from cows (n = 46) at two time points during their lactation (i.e. one-three days post calving and at approximately eight weeks post calving [or 45 – 60 days in milk]) (Figure 2.5; Figure 2.6). Only the response to phase 1 *C. burnetii* was addressed due to logistical constraints. There was evidence of an interaction between blood stimulant type and sampling time and therefore effects of sampling time were estimated separately for each blood stimulant type (Table 2.5) and effects of blood stimulant type were estimated separately for each sampling time (Table 2.6). The mean IFNγ response to *C. burnetii* phase 1 antigen stimulation increased from 0.04 ng/ml (+/- 0.11 ng/ml) at calving to 0.14 ng/ml (+/- 0.16 ng/ml) at 8 weeks post calving, which represented a significant 2.04 times increase between the sampling time points (95% CI: 1.45 ng/ml – 2.90 ng/ml; $p < 0.001$) (Table 2.5). For LPS and media, the median IFNγ response at calving and eight weeks post calving were similar in Figure 2.5, though there were more extreme high values (based on data visualisation) for both LPS and media at calving and a significant decrease in mean response between the two time points (Table 2.5). For PWM, there was a 4.13 ng/ml reduction in mean IFNγ response between calving and eight weeks post calving (95% CI: -6.92 ng/ml to -1.35 ng/ml; $p = 0.004$) (Table 2.5).

When examining the effect of stimulant type within each timepoint, at calving, there was no significant difference between the IFNγ response to *C. burnetii* phase 1 and the media ($p = 0.083$) (Table 2.6). The median response to *C. burnetii* and LPS were also similar at calving

(Figure 2.5), though there were more extreme high values for LPS (based on data visualisation) (Figure 2.5), and a significantly higher mean response to this stimulant compared to *C. burnetii* (Table 2.6). In contrast, by eight weeks post calving the response to *C. burnetii* was significantly higher compared to media and LPS, which represents a five- and 10-fold reduction in the mean response to media and LPS compared to *C. burnetii* (Table 2.6).

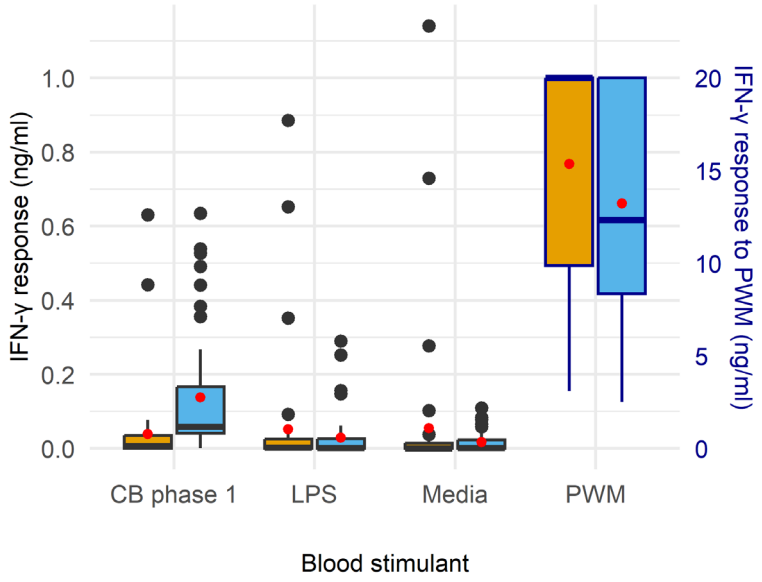

Figure 2.5. Box and whisker plot depicting the distribution of IFNγ response (ng/ml) of whole blood collected from 46 Australian dairy cows sampled at calving (yellow) and approximately eight weeks post calving (blue). Whole blood was stimulated *ex vivo* with *Coxiella burnetii* phase 1 (CB phase 1), lipopolysaccharide from rough form *Escherichia coli* (LPS), pokeweed mitogen (PWM; positive control) and media (unstimulated control). Cytokine response was determined using ELISA and presented as ng/ml. Thick bars represent medians, red dots represent means. Cytokine response to PWM is presented on the secondary y-axis.

For IL-10 the mean IL-10 response to *C. burnetii* phase 1 antigen stimulation increased between the sampling time points by 1.86 times from 0.31 ng/ml (+/- 0.21 ng/ml) at calving to 0.62 ng/ml (+/- 0.27 ng/ml) at eight weeks post calving (95% CI: 1.54 ng/ml – 2.25 ng/ml; $p < 0.001$) (Table 2.5). The mean IL-10 response to LPS was significantly higher at calving (0.88 ng/ml [+/- 0.38 ng/ml]) compared to eight weeks post calving (0.45 ng/ml [+/- 0.15 ng/ml]), which corresponded to a 0.48 times reduction between the sampling time points (95% CI: 0.42 ng/ml – 0.56 ng/ml; $p < 0.001$) (Table 2.5). The mean IL-10 response to media at calving and eight weeks post calving decreased between the two time points ($p = 0.027$) (Table 2.5). There was a 0.82 times reduction in mean IL-10 response to PWM between calving and eight weeks post calving (95% CI: 0.74 ng/ml – 0.91 ng/ml; $p = <0.001$) (Table 2.5).

At calving, there was no significant difference between the IL-10 response to *C. burnetii* and media only ($p = 0.659$) (Table 2.6). The mean response to LPS was 2.75 times higher compared to *C. burnetii* at calving (95% CI: 2.31 – 3.28 ng/ml; $p <0.001$) (Table 2.6). In contrast, by eight weeks post calving the response to *C. burnetii* was significantly higher compared to media and LPS (Table 2.6).

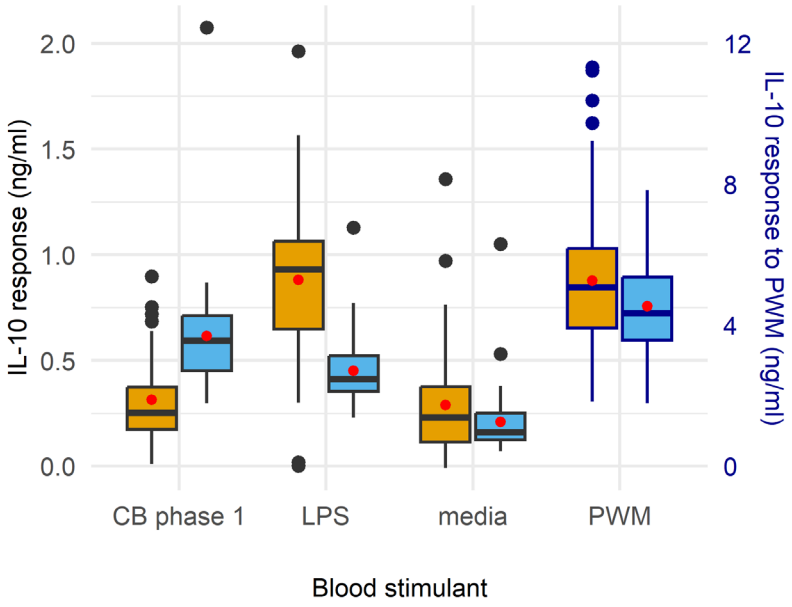

Figure 2.6. Box and whisker plot depicting the distribution of IL-10 response (ng/ml) of whole blood collected from 46 Australian dairy cows sampled at calving (yellow) and approximately eight weeks post calving (blue). Whole blood was stimulated *ex vivo* with *Coxiella burnetii* phase 1 (CB phase 1), lipopolysaccharide from rough form *Escherichia coli* (LPS), pokeweed mitogen (PWM; positive control) and media (unstimulated control). Cytokine response was determined using ELISA and presented as ng/ml. Thick bars represent medians, red dots represent means. Cytokine response to PWM is presented on the secondary y-axis.

Table 2.5. Effect of sampling time on IFNγ and IL-10 response from each blood stimulant for 46 Australian dairy cows sampled at calving and approximately eight weeks post calving. Whole blood was stimulated *ex vivo* with *Coxiella burnetii* phase 1, lipopolysaccharide from rough form *Escherichia coli* (LPS), pokeweed mitogen (PWM; positive control) and media (unstimulated control). Cytokine response was determined using ELISA and presented as ng/ml. Effects were analysed using generalized linear models except for PWM where a tobit model for right censored data was used.

Cytokine	Blood stimulant	Sampling time	Mean (sd)[a]	Coefficient[b]	CI	P value
IFN-γ	*C. burnetii* phase 1	Calving	0.04 (0.11)	Reference category		
		Eight weeks post calving	0.14 (0.16)	2.04	1.45 – 2.90	<0.001
	LPS	Calving	0.05 (0.17)	Reference category		
		Eight weeks post calving	0.03 (0.06)	0.26	0.12 – 0.54	<0.001
	Media	Calving	0.05 (0.20)	Reference category		
		Eight weeks post calving	0.02 (0.03)	0.14	0.03 – 0.63	0.01
	PWM	Calving	15.36 (6.13)	Reference category		
		Eight weeks post calving	13.22 (5.43)	-4.13[c]	-6.92 to -1.35	0.004
IL-10	*C. burnetii* phase 1	Calving	0.31 (0.21)	Reference category		
		Eight weeks post calving	0.62 (0.27)	1.86	1.54 – 2.25	<0.001
	LPS	Calving	0.88 (0.38)	Reference category		
		Eight weeks post calving	0.45 (0.15)	0.48	0.42 – 0.56	<0.001
	Media	Calving	0.29 (0.26)	Reference category		
		Eight weeks post calving	0.21 (0.16)	0.71	0.53 – 0.96	0.027
	PWM	Calving	5.47 (1.94)	Reference category		
		Eight weeks post calving	4.63 (1.33)	0.82	0.74 – 0.91	< 0.001

CI = confidence intervals; sd = standard deviation

[a] Units are ng/ml

[b] Estimated ratio of arithmetic means (adjusted for cow as a fixed effect)

[c] raw regression coefficient from the tobit model (can be interpreted as difference between means)

Table 2.6. Effect of stimulant type on IFNγ and IL-10 response within each sampling time, from 46 Australian dairy cows sampled at calving and approximately eight weeks post calving (using generalised linear models). Whole blood was stimulated *ex vivo* with *Coxiella burnetii* phase 1, lipopolysaccharide from rough form *Escherichia coli* (LPS), pokeweed mitogen (PWM; positive control) and media (unstimulated control). Cytokine response was determined using ELISA and presented as ng/ml values presented.

Cytokine	Sampling time	Blood stimulant	Mean (sd)[a]	Coefficient[b]	CI	P value
IFN- γ	Calving	*C. burnetii*	0.04 (0.11)	Reference category		
		LPS	0.05 (0.17)	1.64	1.14 – 2.36	0.007
		Media	0.05 (0.20)	1.43	0.95 – 2.14	0.083
	Eight weeks post calving	*C. burnetii*	0.14 (0.16)	Reference category		
		LPS	0.03 (0.06)	0.21	0.10 – 0.43	<0.001
		Media	0.02 (0.03)	0.10	0.02 – 0.43	0.002
IL-10	Calving	*C. burnetii*	0.31 (0.21)	Reference category		
		LPS	0.88 (0.38)	2.75	2.31 – 3.28	<0.001
		Media	0.29 (0.26)	0.95	0.75 – 1.20	0.659
	Eight weeks post calving	*C. burnetii*	0.62 (0.27)	Reference category		
		LPS	0.45 (0.15)	0.72	0.62 – 0.83	<0.001
		Media	0.21 (0.16)	0.36	0.28 – 0.47	<0.001

CI = confidence intervals; sd = standard deviation

[a] Units are ng/ml

[b] Estimated ratio of arithmetic means (adjusted for cow as a fixed effect).

2.4.4 Correlation between cytokine response to *Coxiella burnetii* phase 1 and phase 2 stimulation

The correlation between the cytokine response for 169 cows in the whole blood assay following stimulation with phase 1 and 2 *C. burnetii* was examined at the approximately eight weeks post calving sampling timepoint (or 45 – 60 days in milk) (Figure 2.7A and 2.7B).

There was a positive correlation between IFNγ responses to phase 1 and 2 *C. burnetii* (ρ = 0.84; 95% CI: 0.79 - 0.89; p <0.001) (Figure 2.7A). There was a close positive linear correlation between IL-10 response to phase 1 and phase 2 *C. burnetii* stimulation (ρ = 0.91; 95% CI: 0.89 - 0.94; p <0.001) (Figure 7B).

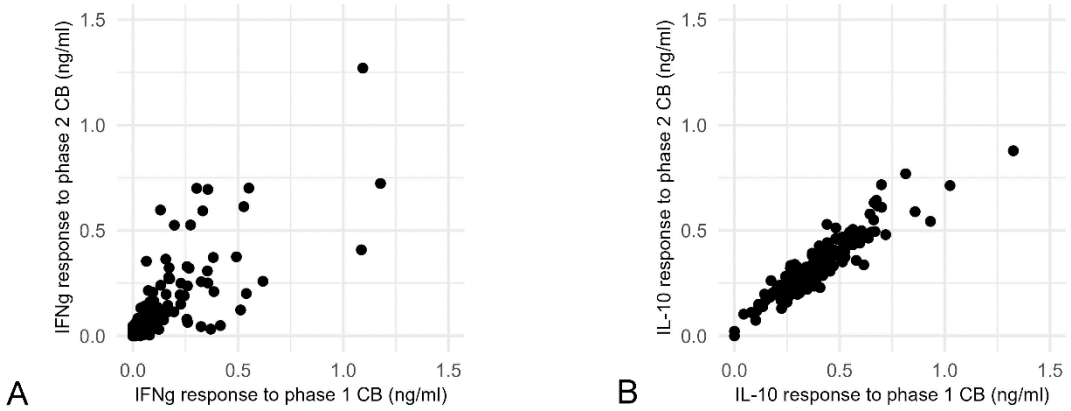

Figure 2.7. Scatter plots to depict associations between cytokine response (ng/ml) from cattle whole blood stimulated *ex vivo* with *Coxiella burnetii* phase 1 (Phase 1 CB) or phase 2 (Phase 2 CB) for 169 Australian dairy cows measured at approximately eight weeks post calving. **A.** IFN-γ. **B** IL-10.

2.4.5 Correlations between cytokine response to *Coxiella burnetii* when described as sample to positive ratio and as ng/ml

There were close positive linear relationships between IFNγ response when described as an S/P ratio (%) and as ng/ml to *C. burnetii* phase 1 (ρ = 0.97; 95% CI: 0.95 - 0.98; p <0.001) and phase 2 (ρ = 0.97; 95% CI: 0.95 - 0.98; p <0.001) (Figure 2.8A and 2.8B). There were also close positive linear relationships between IL-10 response when described as an S/P

ratio[%]) and as ng/ml to *C. burnetii* phase 1 (ρ = 0.83; 95% CI: 0.76 - 0.90; p <0.001) and phase 2 (ρ = 0.80; 95% CI: 0.72 - 0.88; p <0.001) (Figure 2.8C and 2.8D).

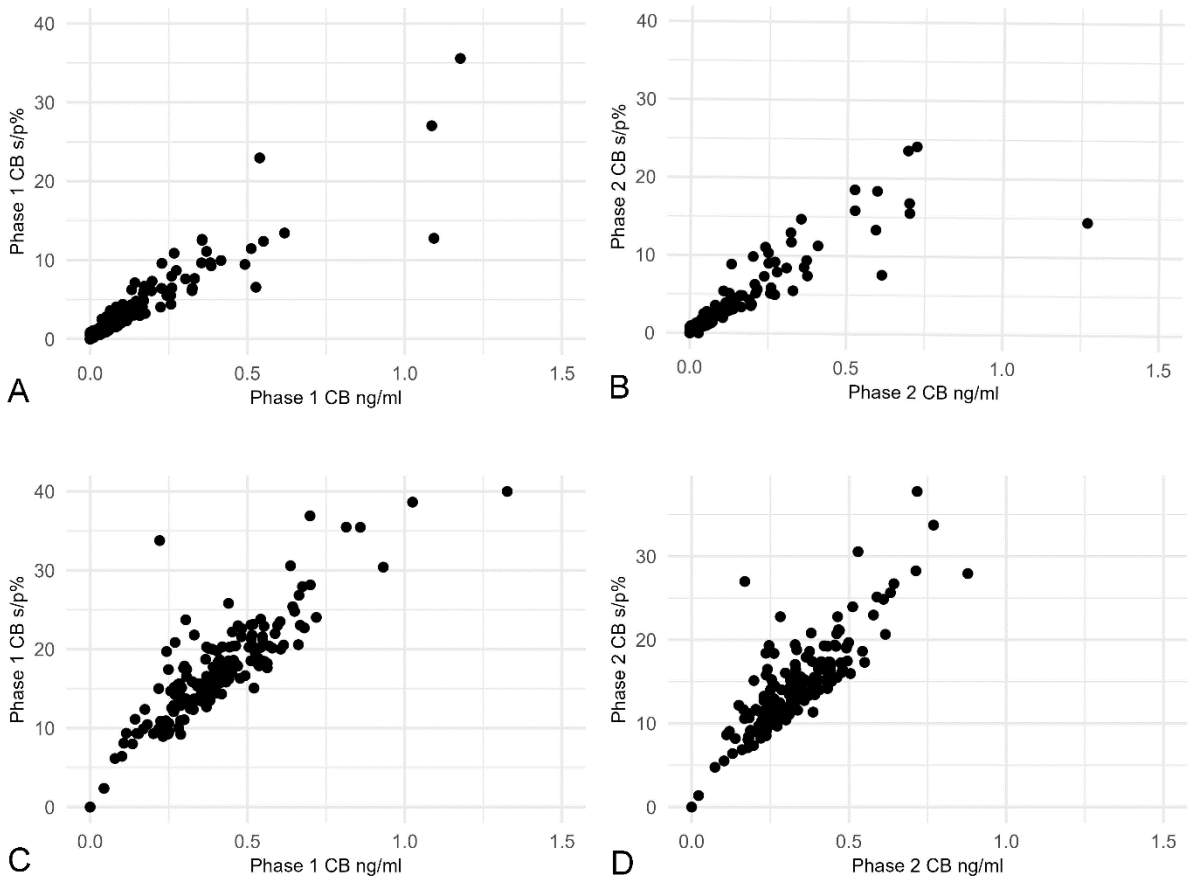

Figure 2.8. Scatter plots to depict associations between cytokine response to *Coxiella burnetii* phase 1 (Phase 1 CB) and 2 (Phase 2 CB) when presented as a sample to positive ratio (S/P ratio [%]) or in ng/ml for: **A/B** IFNγ and **C/D** IL-10 from 169 Australian dairy cows sampled at eight weeks post calving. S/P ratio (%) calculated as: ([OD *C. burnetii* stimulation – OD media background control] / [OD stimulation positive control (PWM) – OD media background]) x 100. Values in ng/ml were obtained by transforming OD values according to a standard curve and calculated as: ng/ml *C. burnetii* stimulation – ng/ml unstimulated control.

2.5 Discussion and conclusions

The cytokines IFNγ and IL-10 have been shown to be important in Q fever pathogenesis, diagnosis and vaccine efficacy research in humans but there are few studies investigating their role in the pathogenesis of coxiellosis in cattle (Boettcher, 2017, Schoffelen et al., 2013a, Schoffelen et al., 2017, Scholzen et al., 2021). Use of the whole blood cytokine recall assay in cattle has been limited in part by a paucity of literature describing the optimal methodology for use with ruminant samples. This study assessed the potential for variation in technical variables to impact the IFNγ and IL-10 response to stimulation, with a greater response to PWM and *C. burnetii* antigen obtained when samples were stored at ambient temperature during transportation (Figure 2.1; Figure 2.2). Similarly, a greater response was obtained when samples were co-incubated with the highest concentration of *C. burnetii* utilised in the study for a period of 48 hours (Figure 2.3; Figure 2.4). This methodology was then applied to investigate the use of the assay at time points in a typical lactation within a dairy management system, with the average cytokine response following *C. burnetii* stimulation for both IFNγ and IL-10 being dampened periparturiently and then increasing into lactation (Figure 2.5; Figure 2.6). At eight weeks post calving there was a close correlation for both the IFNγ and IL-10 responses to phase 1 and phase 2 *C. burnetii* antigen (Figure 2.7).

Previous Q fever work utilizing samples collected from humans highlighted that the magnitude of a cytokine response can be manipulated by adjusting assay conditions, but the

response can vary according to the stimulant (Schoffelen et al., 2013b, Schoffelen et al., 2014). Increasing the detectable cytokine response improves assay sensitivity and enables enhanced identification of variability between animals. This study investigated the effect of adjusting technical assay factors for optimal use of the assay with cattle samples to detect IFNγ and IL-10, including factors such as sample temperature during transport, length of co-incubation with stimulants and *C. burnetii* concentration. Maintaining cell viability from whole blood samples during transport is critical for this assay but can be challenging in the livestock context as farms are often located long distances away from laboratories.

This study found a higher IFNγ and IL-10 response to the positive control PWM and *C burnetii* antigen from samples that were transported at ambient temperature compared to transportation on ice, possibly indicating the lower temperature impacts cell viability (Figure 2.1; Figure 2.2). Refrigeration before and after transportation on ice is logistically difficult, more costly, and likely holds little benefit in preventing bacterial overgrowth given blood is generally considered a sterile sample. Additional temperature ranges have been tested in the MAP IFNγ recall assay in sheep and identified that storing samples at 37°C during transport can lead to a greater antigen-specific response but only if the antigen is immediately added after collection (Bosward et al., 2010). It is not known if similar responses will also occur in cattle. The addition of stimulants on-farm and warming of samples during transport was beyond the scope of the current study.

As another means of alleviating time pressure and decreasing the logistical demand of the assay, this study evaluated the impact of using pre-prepared and frozen media compared to

that made up freshly prior to stimulation. While the IFNγ response to the positive control (PWM) and *C. burnetii* antigen was not significantly different when using frozen compared to freshly made media, for IL-10 however, the response was higher when media was prepared fresh (Figure 2.1; Figure 2.2). The logistical demands of this assay meant the present study was obligated to use pre-prepared frozen media especially when assessing larger numbers of samples, however if media can be prepared fresh this may be the preferred approach for the IL-10 assay.

This study also evaluated whether the length of incubation of whole blood samples with stimulants impacted the cytokine response, and it was found that a 48 hour co-incubation led to a higher PWM-induced and *C. burnetii*-induced IFNγ and IL-10 response compared to a 24 hour co-incubation, while the same trend was also apparent for the LPS-induced IL-10 response (Figure 2.3 & 2.4). Incubation times beyond 48 hours were not investigated in the current study, however Johne's disease work in sheep found a decrease in IFNγ response to MAP antigen and positive control Concanavalin A (ConA) between 48h and 72h (Bosward et al., 2010). Similarly in humans, longer co-incubations lead to higher IFNγ response following PWM but not *C. burnetii* antigen stimulation (Schoffelen et al., 2014). While longer incubations may further increase the detectable cytokine response, the potential benefit must be weighed against the additional time required to acquire results, a matter of particular importance if adapting this assay for diagnostic application.

Antigen concentration was the final technical aspect to be investigated with an increased IFNγ and IL-10 response being obtained when using *C. burnetii* at 5 µg/ml (Figure 2.3; Figure

2.4). This was the highest concentration of antigen able to be used in the current study due to the cost of the commercial antigen and the large number of animal samples to be tested and it is not known whether higher concentrations would result in a further increased cytokine response. The *C. burnetii* antigens used in this assay were produced by the manufacturer for use in complement fixation assays, and according to the manufacturer of the antigen, the concentration supplied was 1 mg/ml. While this gives some indication of how much antigen was present, ideally genomic equivalents would allow comparison between antigen sources.

Various immunological changes occur throughout gestation, parturition, and lactation, and as such, this study sought to investigate the effect of the stage of lactation on cytokine response in cattle to optimise sampling time for pathogenesis investigation (Vlasova and Saif, 2021). The IFNγ and IL-10 response from 46 cows was measured at two time points through lactation: at calving (one to three days post-calving) and at eight weeks post-calving (prior to re-breeding), with a higher IFNγ and IL-10 response to *C. burnetii* in the blood samples collected at eight weeks post-calving compared to that in the periparturient samples (Figure 2.5; Figure 2.6). At calving the difference between the mean response to *C. burnetii* and the unstimulated control was negligible for both IFNγ and IL-10 despite a large response to the PWM control confirming cellular viability (Figure 2.5; Figure 2.6). This finding is consistent with studies that have shown a down-regulation of cell mediated immunity around parturition in dairy cows (Vlasova and Saif, 2021). However, in contrast to the responses to *C. burnetii* stimulation, the mean IFNγ and IL-10 response to the positive control T and B cell stimulant, PWM, were significantly higher at calving compared to eight weeks post-calving

(Figure 2.5; Figure 2.6), and the IL-10 response to the innate immune system mitogen, LPS, was higher at calving (Figure 2.6). Further research is required, but these results may indicate that the T cell, B cell, and innate immune system responses are not dampened at parturition relative to eight weeks post calving and suggest it is possible there is a *C. burnetii*-specific downregulation in some pro-inflammatory cytokines at calving. This could potentially be due to a delay in recovery of cell mediated responses specifically to *C. burnetii,* if onset of infection occurred during gestation which is dominated by humoral immunity or, inadequate time to develop a strong memory response to stimulation if infection occurred near to calving where there is more likely to be a higher environmental load. The latter is supported by the findings of a study in goats whereby only after day 35 post infection was there an increase in IFNγ protein detected in *C. burnetii* stimulated whole blood from pregnant infected goats compared to naïve goats (Ammerdorffer et al., 2014). Alternatively, the mitogens may induce an artificial response that is suitable as a positive control for determining cell viability but not for capturing physiologically driven changes in immune response. Though in saying this, work in humans has found a reduction in T cell proliferation and IFNγ production to the mitogens ConA and phytohemagglutinin in samples from pregnant compared to non-pregnant women (Luppi, 2003). Future assessment of additional timepoints throughout lactation than the two investigated in this study may shed further light on the dynamics of *C. burnetii* response.

As both phase 1 and 2 *C. burnetii* antigens have been used throughout human Q fever research, both antigens were tested as part of the optimisation of the cytokine recall assay for use with cattle whole blood samples. The phase 1 cell is the only phase found in natural

infection of immunocompetent hosts (Eldin et al., 2017) and is used in the cell mediated diagnostic assay, Q-detect (Innatoss Laboratories) that is commercially available for use in humans. The phase 2 cell is considered avirulent due to a truncation in the LPS O-antigen region following serial passage of phase 1 cells in culture (Amano and Williams, 1984) but has still been used in cattle and human studies, with some studies reporting a stronger IFNγ response following stimulation with phase 2 antigen (Izzo and Marmion, 1993, Limonard et al., 2012, Roest et al., 2013). The present study sought to directly compare the cytokine response following antigenic stimulation of whole blood with both phases on a group and individual animal basis in 169 cows at eight weeks post calving to determine their respective potential use for understanding the pathogenesis of infection in cattle. While there was a close correlation between the IFNγ response to phase 1 and phase 2 antigen in each cow, the minor differences suggest that the variation in the O-antigen region of LPS may influence the response as has also been proposed in previous human work assessing individual IFNγ responses (Figure 2.7A) (Izzo and Marmion, 1993). To better understand the role of LPS in the cytokine response, the rough *E. coli* LPS control was assessed and found to result in a lower mean IFNγ response compared to whole cell *C. burnetii* (Figure 2.5). A minimal response to the region of LPS conserved across bacterial species (i.e. lipid A and core) was unsurprising given the predominate cells producing IFNγ in the recall assay in human studies are T cells (mainly CD4 T cells), and this is thought to be resulting from recognition of highly specific *C. burnetii* cell surface proteins (Raju Paul et al., 2023, Scholzen et al., 2021). However, the O-antigen region of *C. burnetii* LPS could still indirectly contribute to the cytokine response, including by influencing the level of exposure of antigenic surface proteins, since the truncation of the O antigen side chains in phase 2 LPS may allow for increased accessibility to proteins in the bacterial cell wall (Hackstadt, 1990). Secondly, the

virulent phase 1 LPS may help down-regulate pro-inflammatory cytokines, a theory that is supported by *in vitro* studies which have shown that the two phases bind different human macrophage uptake receptors, subsequently leading to short term intracellular survival of phase 2 compared to phase 1 (Capo et al., 1999). Cows may vary in their response to these different antigenic phases which could explain the minor differences between response to phase 1 and 2 *C. burnetii* on the individual level (Figure 2.7A). Interestingly during the development of the *C. burnetii* enzyme-linked immunosorbent spot, it was suggested that the Q fever disease state (i.e. acute or chronic) may influence the human response to phase 1 and phase 2 *C. burnetii* (Limonard et al., 2012). Future research should be directed towards further understanding the physiological relevance of using the artificially laboratory produced phase 2 cellular antigen to assess responses in natural *C. burnetii* infection in cattle, as this may prove useful in distinguishing different disease states when used in conjunction with phase 1 or in assessing responses to vaccines that rely on one or the other *C. burnetii* phases.

For IL-10, there was a close correlation between the responses to phase 1 and 2 *C. burnetii* and a stronger response to the LPS control compared to IFNγ (Figure 2.7B; Figure 2.6). In combination these results indicate the most highly conserved region of the LPS (lipid A and core), present on both *C. burnetii* phases and in the LPS control, is likely contributing in a non-specific manner to IL-10 production, possibly via binding to toll-like receptor 4 on monocytes as part of the innate immune system (Schramek and Mayer, 1982). Indeed, previous human Q fever research identified monocytes as the most common producers of IL-10 following stimulation of PBMCs with *C. burnetii* (Raju Paul et al., 2023). While the exact

reason for the strong LPS-induced IL-10 response of the cattle is unknown, significant systemic increase in IL-10 response to cows intramammary-challenged with *E. coli* LPS was suggested to reflect a protective mechanism against excessive inflammation (Choudhary et al., 2024). Studies that investigate pathogenesis of coxiellosis in cattle utilising IL-10 assays should include an LPS control due to the ubiquitous exposure to gram negative bacteria in the dairy setting and, as such, the potential for cross reactivity. The contrast in results for IL-10 compared to IFN-γ, highlight the importance of optimising assays for each cytokine and the value in understanding the whole blood dynamics that govern immune responses when interpreting results. Previously published studies using the cytokine recall assay for measuring IFNγ response to both *C. burnetii* and MAP antigen in cattle successfully increased the IFNγ response by blocking the activity of IL-10 with an anti-IL-10 monoclonal antibody (Boettcher, 2017, Buza et al., 2004). In the present study, however, there was interest in monitoring the actual IFNγ response of cows over time and how this response could contribute to the pathogenesis of coxiellosis, and therefore, it was decided not to block the activity of IL-10 and instead to measure the natural IL-10 response.

Finally, the present study compared the presentation of results in either ng/ml or as a sample to positive control ratio (S/P%) because both have been used previously in Q fever and coxiellosis literature (Boettcher, 2017, Schoffelen et al., 2014) (Figure 2.8). The presentation of results as a S/P% directly accounts for the cells' ability to produce a cytokine by having the *C. burnetii* response relative to a positive control response such as PWM. The presentation of results in ng/ml represents the total response to *C. burnetii*, with response to PWM being used as an adjunctive confirmation of cell viability. This study found a close

correlation per cow when presenting IFNγ and IL-10 response to *C. burnetii* as an S/P% and as in ng/ml thus enabling data to be presented in the format most useful to the purpose of the investigation (Figure 2.8). For example, the S/P% allows for standardization of the cytokine response to *C. burnetii* antigen stimulation which may be important in a diagnostic context as it can increase sensitivity for detecting cows with a low response compared to the mitogen control. Presenting the results in total ng/ml may be more useful when comparing disease outcomes between animals as this allows independent monitoring of the response to controls and mitogens and thus increased scope for interpretation.

Future studies could be directed at investigating the effect of additional technical adjustments on cytokine response, including *C. burnetii* antigen inactivation method and the *C. burnetii* strain. The present study utilised formaldehyde inactivated antigen, however, an increased sensitivity in the IFNγ recall assay was previously reported when using antigen that was heat inactivated compared to formaldehyde inactivated, possibly due to antigen loss from cross-linking (Schoffelen et al., 2013b). In addition, different strains have been demonstrated to be more virulent in terms of disease manifestation in mice models (Eldin et al., 2017). The strain used in this study was Nine Mile, which has been found to be genetically distinct from strains isolated from Australian Q fever patients, and therefore the use of the strains more closely related to the ones infecting the cattle in this study may have had a differing effect (Vincent et al., 2016).

Chapter 3 Longitudinal study of *Coxiella burnetii* shedding patterns and immune responses from the dry period through the first seven months of lactation in a naturally infected Australian dairy herd.

3.1 Abstract

Coxiella burnetii is the causative intracellular bacterium of the zoonotic disease Q fever. Domestic ruminants are reservoirs for human infection, and some may excrete the bacterium (shedding) for prolonged periods in milk. The cell mediated immune response effects bacterial clearance in humans and mice but is poorly characterised in cattle. This study investigated *C. burnetii* shedding and immunological variability of 192 Australian dairy cows in a *C. burnetii* endemically infected herd to provide a more holistic understanding of infected animals. Blood, placenta, faeces, vaginal mucus, and milk were sampled at five time points over approximately seven months, including the dry period, calving, early lactation, and mid lactation. *Coxiella burnetii* DNA was detected in samples using multiplex qPCR; *C. burnetii* antibody levels in serum using ELISA; and IFNγ and IL-10 response to *C. burnetii* antigen stimulation using whole blood cytokine recall assay. The association between antibody or cytokine response and shedding was assessed using logistic regression models, while differences in the prevalence of PCR positive milk samples between sampling time points were assessed using generalised estimating equations. At calving, 52% of cows had *C. burnetii* DNA detected in their placenta, and while shedding decreased into early lactation, there was a significant increase in the prevalence of milk shedding at mid-lactation (10%) (200 DIM) predominately through milk, of which 62% of these 10% of cows had also shed at

calving or early lactation. Antibody titre at early lactation was significantly associated with *C. burnetii* shedding in milk at mid-lactation, and for every 10 unit (S/P%) increase in antibody titre, odds of shedding increased by 2.14 times (95% CI: 1.37 – 3.34; p = 0.001). Cows with *C. burnetii* detected in milk at mid lactation were not associated with IL-10 response, but all had low IFNγ response at early lactation. A strong IFNγ response was associated with 31% lower odds of shedding at mid lactation compared to a low response but this estimate was imprecise and not significant (OR: 0.69; 95% CI: 0 – 4.85; p = 0.75), given there were also cows with low IFNγ that did not shed at mid lactation, despite some of them having been infected at calving. These findings suggest cows that preference a TH2 response, marked by high antibody and low IFN-γ, favour persistent infection, evident by *C. burnetii* presence in milk approximately 200 days after calving. Future work may determine whether the significant association of antibody with milk shedding is causal in this outcome.

3.2 Introduction

Coxiella burnetii is a gram-negative intracellular bacterium that causes the globally important zoonotic disease Q fever in humans and coxiellosis in animals (Maurin and Raoult, 1999). Domestic ruminants are the main reservoirs for human infection with sheep being responsible for the majority of Q fever outbreaks around the world (Tan et al., 2024). In Australia, annual Q fever notifications have ranged from 475-722 cases over the last 10 years, with cattle cited in some reports as the species most linked to human exposure (Graves and Islam, 2016). At parturition, cattle shed *C. burnetii* in their placenta (occasionally in very large quantities), vaginal mucus, faeces, and colostrum, and after the periparturient

period shedding may continue in the vaginal mucus, faeces, and milk to a variable extent (Freick et al., 2017, Guatteo et al., 2012). The detection of *C. burnetii* DNA in milk can occur throughout a cow's entire lactation, further contributing to environmental contamination (Freick et al., 2017, Guatteo et al., 2007). *Coxiella burnetii* infection in animals is thought to be largely subclinical, however, clinical disease manifestations such as sporadic abortion, stillbirths, and weak offspring can occur and an association between milk shedding and subclinical mastitis has also been reported (Agerholm, 2013). Pathogenesis of infection with *C. burnetii* is complex and the host, pathogen, and environmental factors that contribute to bacterial shedding and disease outcomes in infected cattle are poorly understood. However, such information is vital from a public health perspective to allow development of informed shedding control measures and from a dairy industry perspective to allow for the effective assessment of the potential financial burden resulting from production losses.

There have been a relatively small number of studies conducted that shed light on patterns of *C. burnetii* infection in cattle. Cross-sectional studies are limited by their single time point of analysis (Guatteo et al., 2006), while the longitudinal studies have taken various methodological approaches when investigating shedding and the factors that contribute to infection outcomes, some of which have limitations. For example, the PCR criteria used to classify presence of *C. burnetii* DNA is vital to interpreting and synthesising results, however, assessment based solely on the single copy transposon gene, IS*1111*, and the exclusion of PCR cycle cut-offs relative to technical assay sensitivity, may reduce data robustness (Guatteo et al., 2007, Mathews et al., 2024). Furthermore, placental shedding has rarely been measured (García-Ispierto et al., 2013, Guatteo et al., 2012, Serrano-Pérez et al., 2015),

likely due to the logistical challenges of collecting samples at the time of parturition. However, it is an important factor in infection dynamics, as evidence suggests that *C. burnetii* targets placental cells following intranasal experimental infection of pregnant goat does (Roest et al., 2012), and placental involvement is a key feature of the reproductive disease manifestations of coxiellosis in ruminants (Agerholm, 2013).

Another potential influential factor in *C. burnetii* pathogenesis that has been rarely assessed in cattle is the cell mediated immune response (Boettcher, 2017, Małaczewska et al., 2018). The CMI responses are essential to effective clearance of intracellular pathogens, however, most studies have focused on the humoral immune response by measuring antibodies (Andoh et al., 2007, Freick et al., 2017, García-Ispierto et al., 2013, Guatteo et al., 2007, Rodolakis et al., 2007). One cattle study investigating CMI via unstimulated serum levels of a range of cytokines in a *C. burnetii* endemically infected dairy herd in Poland, found IFNγ and IL-10 were associated with milk shedding in seropositive cows (prior to vaccination) (Małaczewska et al., 2018). In observational studies, where cytokine levels may be influenced by a range of pathogens, it may be informative to measure *C. burnetii*-specific cytokines levels using the whole blood cytokine recall assay, which was previously optimised (Chapter 2). Finally, the utility of pathogenesis findings from studies conducted in different countries must be considered, as strains can vary genetically by geographic location, such as those found in Australia, which could impact virulence (Vincent et al., 2016).

The aim of the study was to conduct a longitudinal investigation into the *C. burnetii* shedding patterns and humoral and cellular immune responses in a naturally infected Australian dairy

herd to enable further understanding of the pathogenesis of this bacterial infection. It was anticipated that this data would also inform future studies aimed at understanding the impacts of coxiellosis on milk production and reproductive performance, as well as aid in the understanding of public health risks.

3.3 Materials and methods

3.3.1 Ethics

Approval for the study was granted by the University of Sydney Animal Ethics Committee (Ethics Approval number: 2021/2014).

3.3.2 Animals and study design

A prospective cohort study was conducted in a naturally infected Holstein Friesian dairy cow herd located in New South Wales, Australia from May through to December, 2022. Lactating cows were fed a total mixed ration diet comprised of corn and alfalfa silage, canola meal, brewers grain, wheat, citrus pulp, and almond hulls. Prior to the study commencing, *C. burnetii* infection was verified as endemic in the herd through serological evidence of antibody against *C. burnetii* in both serum samples from individual cows and BTM samples, molecular evidence of *C. burnetii* DNA via PCRs in milk, placenta, faeces, and vaginal swabs, and growth of *C. burnetii* in Vero cell cultures from milk, serum, placenta, and vaginal swabs as part of a previously unpublished study.

The original sample size was calculated to identify differences in seropositivity across the study time points. It was estimated that 400 cows would need to be enrolled for the comparison of seroprevalence at two time points (L2 and L4) with 80% power, a significance level of 0.05, and expected drop-out of 50% of enrolled subjects if the true difference in apparent prevalences is 11% (0.1 and 0.21). Drop-out was anticipated to be due to cows not calving during the required time window to allow ongoing sampling.

3.3.3 Cow data collection

Cow parity and pregnancy test results recorded during routine herd management practices were downloaded from the herd management software (Dairy Comp 305, Valley Ag Software, Tulare, CA).

3.3.4 Samples

The samples collected at each time point from animals enrolled in the study are outlined in Figure 3.1. The sampling time points were selected to capture physiologically distinct points in the dairy cow calving and lactation cycle, including: the dry period, calving, early lactation, and mid lactation. These points were defined as: 240 – 270 days pregnant (L1; approximately three weeks before calving), calving (1 – 3 days post calving for serum samples and

immediately post-partum [up to a maximum of approximately 1 hour post-calving] for all other sample types) (L2), 17 - 32 DIM (L3), 45 - 60 DIM (L4), and 178 - 201 DIM (L5).

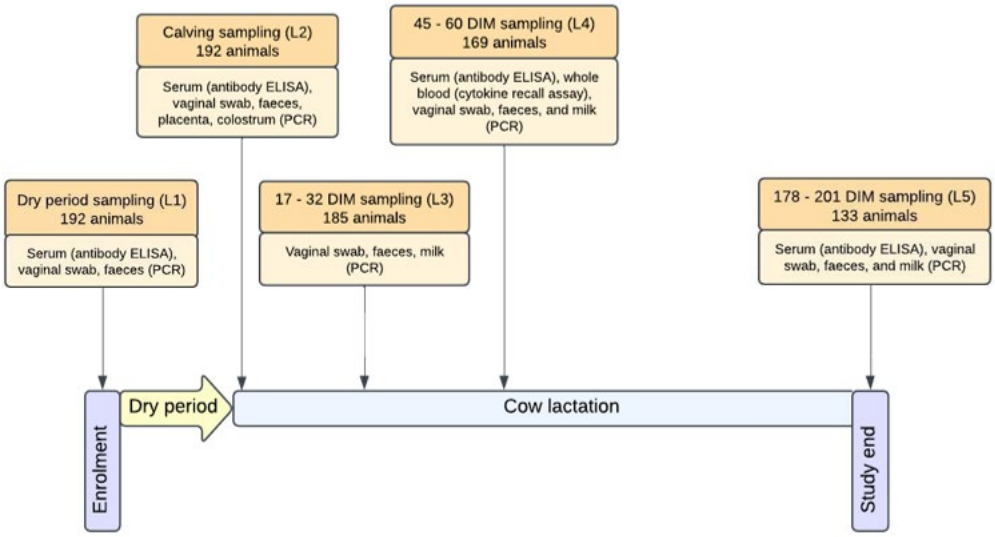

Figure 3.1. Sampling time and animal number for longitudinal study investigating *Coxiella burnetii* shedding and immune responses in a naturally infected Australian dairy herd. For L2, serum samples were collected 1 -3 days post calving while all remaining sample types were collected immediately post-partum. L = longitudinal time point.

3.3.4.1 Blood samples

Blood collection and processing for cytokine analysis was as outlined previously in Chapter 2 Section 2.3.2. Blood samples for use in the cytokine recall assay were transported at ambient temperature. For serology, blood (10 mls) was collected from the coccygeal vein using an 18 G needle into clot activator tubes. Following collection, blood tubes for serology were transported to the laboratory on ice following which blood was centrifuged at 2,200 x*g* for 15 minutes and the serum layer removed and stored in aliquots at -45°C until use.

3.3.4.2 Vaginal swab, faeces, placenta, milk samples

One flocked swab (FLOQSwabs; Copan) was inserted into the vagina and gently rotated against the vaginal mucosal wall at L1-L5 sampling time points, then placed into tubes containing 5 mls PBS. Faeces were collected from the rectum at L1-L5 sampling points and placed into a sterile polypropylene jar (TECHNO-PLAS). An approximately 50 g piece of placental membrane was aseptically subsampled from the whole placenta (selected by sample accessibility within the cow) prior to expulsion immediately post-partum (sampling timepoint L2) and placed into a 70ml sterile jar. Colostrum and milk samples were collected from all functional mammary quarters at L2 and L3 - L5 sampling time points, respectively (Figure 3.1). Samples were collected by cleaning each teat with a wipe soaked in 70% ethanol and then expressing approximately 10mls of milk per teat into sterile polypropylene jars. At L2, a composite sample was collected by expressing milk from all four quarters into one jar while at L3 – L5 milk from each individual quarter was expressed into separate jars with a composite sample formed at the lab. Swabs, faeces, placenta, colostrum, and milk samples were transported on ice then stored at -20 or -45˚C until use.

3.3.5 *Coxiella burnetii* molecular detection

3.3.5.1 DNA extraction (blood, milk, faeces, vaginal swabs)

All DNA extractions were undertaken in biosafety cabinets within physical containment 2 laboratories. DNA was extracted using the Biosprint® 96 One-For-All Vet Kit (Qiagen) according to the manufacturer's protocol for purification of viral nuclei acids and bacterial DNA from animal tissue homogenates, serum, plasma, other body fluids, swabs, and washes. Faecal samples were extracted using the Applied Biosystems™ MagMAX™ CORE Nucleic Acid Purification Kit (Applied Biosystems™), according to the manufacturers protocol. For milk samples, prior optimisation experiments determined that using the washed cell pellet from milk was the most sensitive in detecting *C. burnetii* DNA compared to the using the supernatant or whole milk (data not shown). Milk samples were centrifuged at 1,700 x*g* for 15 minutes to pellet cells, and then the pellet was resuspended in RLT buffer (Qiagen). For vaginal swabs, the swab tip was homogenised in the PBS solution by vortexing 10 times for three seconds, after which 160 µl of the sample was added to 40 µl of proteinase K and incubated overnight in a dry block heater (Ratek). For placental samples, a randomly selected approximately 25 mg piece of tissue was homogenised in 1 ml of Buffer RTL supplied in the kit and homogenised in a high throughput bead mill with five mm stainless steel beads (TissueLyser) at 25 Hz. Next, 160 µl of the tissue sample was added to 40 µl of proteinase K and incubated in a dry block heater at 56°C overnight. For faecal samples, 1 ml of PBS was added to 0.2 – 0.3 g of faeces and vortexed for three minutes, followed by centrifugation at 100 x *g* for 1 min. Next, 200 µl of faecal sample was added to 100 µl of proteinase K and incubated at 55°C for 30 minutes and then centrifuged at 15,000 x *g* for 2 minutes. Following sample preparation, 140 µl of each aliquot from milk, faeces, placenta, and the swab eluate was loaded directly into the corresponding lysate S-block according to the Biosprint® 96 One-For-All Vet Kit (Qiagen) manufacturer's instructions. Negative

extraction controls which contained only RTL buffer and Proteinase K were included for every eight – 12 samples.

3.3.5.2 Quantitative PCR detecting host species DNA (endogenous control) and *Coxiella burnetii* DNA

Primers targeting the mitochondrial DNA gene, bovine cytochrome b (BCB), were designed as an endogenous control to confirm the presence of DNA and to confirm DNA integrity (Table 3.1). For the BCB assay, reactions were similarly set up comprising 5 µl 1X SensiFAST™ Probe Lo-ROX kit (Bioline), primers and probes (volumes in Table 3.1), 2.55 µl nuclease free water, and 2 µl of DNA to make a total volume of 10 µl. Two no template control wells, containing nuclease free water instead of DNA, were used per run to ensure reagents were contamination free. The positive control used was DNA extracted from a bovine sample obtained from a previous study. The reactions were performed using a Bio-Rad CFX96 Touch Real-Time PCR detection system (Bio-Rad Laboratories) for 40 cycles involving an initial 3 minutes of denaturation at 95°C and 40 seconds of annealing and extension at 60°C, followed by 39 cycles of 10 seconds of denaturation at 95°C, and 40 seconds of annealing and extension at 60°C. The results were viewed and exported from Bio-Rad CFX Manager (Bio-Rad Laboratories). Any sample without BCB detected was re-extracted and if the repeated PCR was negative then the sample was excluded from further analysis.

Detection and quantification of *C. burnetii* DNA in extracted samples was performed using an optimised multiplex qPCR assay targeting the two single copy genes: groEL (heat shock operon; *htp*AB) and *com1* (the outer membrane protein-coding gene), and the multicopy insertion sequence gene: IS*1111*. Primers and PCR conditions are shown in Table 3.1. Each reaction contained 5µL 1X SensiFAST Probe No-ROX Kit (BioLine), primers and probe (volumes in Table 3.1), 2µL DNA and 1.4 µL nuclease free water in a total volume of 10µL. Amplification and fluorescence detection was performed in a Bio-Rad-CFX Real-Time PCR Thermocycler (Bio-Rad laboratories Pty Ltd) according to the following cycling parameters: initial denaturation at 95°C for 3 minutes followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing and extension at 60°C for 40 seconds. Each qPCR run included no template controls and positive controls containing 1,100, 110 and 11 copies of the *C. burnetii* genome per reaction (Amplirun® Vircell). The lower limit of detection for these qPCR assays was determined to be 11 copies of the *C. burnetii* genome per reaction which corresponded to a cycling or quantification threshold of approximately 34, 36, and 35 for IS*1111*, *com1* and *htp*AB, respectively. PCR reactions for placental samples were all repeated in triplicate while for all other sample types, any sample producing amplification for any *C. burnetii* gene target was subsequently repeated once. For a sample to be considered 'positive' it required 11 or more copies of IS*1111*, and 11 or more copies of either *com1* or *htp*AB. Samples with 11 or more copies of IS*1111* but less than 11 copies of *com1* or *htp*AB (that is, IS*1111* only) were considered 'suspect' and samples with less than 11 copies of IS*1111* were deemed 'negative'. 'Suspect' samples were recorded as 'positive' for any analysis.

Table 3.1. Sequence, product lengths, and concentrations of *Coxiella burnetii* and endogenous control gene primers used in the multiplex PCR (adapted from Mathews, 2022).

Gene target	Primer/ probe	Primer sequences (5'-3')	Concentration (nM), volume (ul)	Product length (bp)	Reference
IS*1111*[a]	Forward primer	CGCAGCACGTCAAACCG	300, 0.3	146	(De Bruin et al., 2011)
	Reverse primer	TATCTTTAACAGCGCTTGAACGTC	300, 0.3		
	Probe	[e]FAM-ATGTCAAAAGTAACAAGAATGATCGTAAC-BHQ1[f]	200, 0.2		
*htp*AB[b]	Forward primer	GTGGCTTCGCGTACATCAGA	300, 0.3	114	Designed in-house by Sullivan Nicolaides Pathology (Brisbane Queensland) using accession number: M20482 (Shapiro et al., 2020)
	Reverse primer	CATGGGGTTCATTCCAGCA	300, 0.3		
	Probe	[g]HEX-AGCCAGTACGGTCGCTGTTGTGGT-BHQ1	200, 0.2		
com1[c]	Forward primer	AAAACCTCCGCGTTGTCTTCA	400, 0.4	76	(Lockhart et al., 2011)
	Reverse primer	GCTAATGATACTTTGGCAGCGTATTG	400, 0.4		
	Probe	[h]Cy5-AGAACTGCCCATTTTTGGCGGCCA-BHQ2[i]	200, 0.2		
BCB[d]	Forward primer	GAGGCGGATTCTCAGTAGACAAAG	300, 0.3	121	*Bos taurus* Mitochondrial Cytochrome B gene (Genbank accession no. GQ358783.1)
	Reverse primer	GAGCCTGTTTCGTGGAGGAATA	300, 0.3		
	Probe	Quasar 670 - CCCTTACCCGATTCTTCGCTTTCCA-BHQ2	150, 0.15		

[a] *C. burnetii* multi-copy insertion sequence 1111 (IS*1111*), [b] *C. burnetii* single copy heat shock operon (*htp*AB), [c] *C. burnetii* single copy outer membrane protein gene (*com1*), [d] Bovine cytochrome B (BCB), included as DNA extraction control, [e] 6-Carboxyfluorescein, [f] Black Hole Quencher-1,[g] HEX™ Dye Phosphoramidite, [h] Cyanine Dye 5, [i]Black Hole Quencher-2.

3.3.6 *Coxiella burnetii* antibody detection

Antibody detection was performed on serum samples run in duplicate using a commercially available ELISA kit (IDEXX Q Fever Antibody Test Kit; IDEXX Laboratories) following the manufacturer's instructions. All reagents and control sera for the ELISA were provided as a part of the kit. The microplate supplied with the kit is coated with combined phase 1 and phase 2 *C. burnetii* Nine Mile strain antigen. The serum samples were diluted 1:400 with wash buffer and 100 µl of serum was added to the wells of each plate in duplicate and incubated for 1 hour in a humid chamber at 37°C. The plate was washed three times using an automated plate washer (Stat Fax 2600, Block Scientific) before adding peroxidase labelled anti-ruminant IgG conjugate to the wells, followed by a further 1 hour incubation at 37°C. The plate was again washed three times. The tetramethylbenzidine substrate was then added, followed by incubation at RT away from direct light before the stop solution was applied. After completion of the ELISA protocol, the optical densities were read using a spectrophotometer at a wavelength of 450nm (Polar Star Optima, BMG LabTech) and analysed using Mars Data Analysis Software (BMG LabTech). A cut-off of sample to positive ratio (S/P%) ≥ 40% was used for classifying positives, samples with S/P% < 30% were negative, and samples with S/P% ≥ 30% but < 40% were suspect.

3.3.7 Cytokine recall assay

The whole cell blood stimulation assay was conducted as outlined previously in Chapter 2. Briefly, the optimised method involved *ex vivo* stimulation of whole blood collected at the L4 timepoint using a series of six pre-prepared RMPI culture treatments, including positive control pokeweed mitogen (5 µg/ml), lipopolysaccharide (0.01 µg/ml; LPS-EK Ultrapure), phase 1 and 2 *C. burnetii* antigen (5 µg/ml; B1CF1; B1CF2), and RPMI media control. Whole blood was transported and stored at ambient temperature within approximately 16-18 hours following collection, then co-incubated with the culture treatments for 48 hours at 37°C and 5% CO2. The assay supernatant was collected and interferon-gamma and interleukin 10 response determined by the ELISAs described in Chapter 2. Cytokine response was recorded in ng/ml and was also categorized into three levels based on visual analysis of the distribution and expert opinion. Level ranges for IFNγ were 0 – 0.2 ng/ml for level 1, 0.2 – 0.4 ng/ml for level 2, and >0.4 ng/ml for level 3. Level ranges for IL-10 were 0 – 0.2 ng/ml for level 1, 0.2 – 0.6 ng/ml for level 2, and >0.6 ng/ml for level 3.

3.3.8 Statistical analysis

All figures were developed in R Studio (R Core Team, 2023) and statistical testing was conducted in Stata (StataCorp; version 18). For any analysis involving PCR results, suspect samples were classed as positives. Prevalence of PCR positivity was compared between timepoints using generalised estimating equations that included random effect of cow, autoregressive structure for correlations in residuals between timepoints within cow, a logit

link, binomial error distribution and timepoint as a fixed effect. An exploratory analysis to evaluate the extent of agreement between the exposure variables, vaginal swab and placenta samples, was conducted using Cohen's kappa (Landis and Koch, 1977). Correlation between antibody titres at each timepoint was assessed by pairwise correlation using the Pearson correlation coefficient. The associations between L5 milk PCR result (binary outcome variable) and each of L4 antibody level and cytokine responses (exposure variables) were assessed using separate logistic regression models. Antibody response was assessed as a continuous exposure variable (divided by 10 to attain the odds for every 10 unit increase in titre), while cytokine response was assessed as both a continuous and categorical variable (with three ordinal levels) to increase investigative flexibility given the limited prior knowledge on the type of expected effect. Linearity in the logit was assessed for exposure variables fitted as continuous data by inspecting loess plots and using fractional polynomials. Ordinary logistic regression models were used, except for when IFNγ was the categorical exposure variable, as an exact logistic regression model was required in this instance because there were no exposed cows in the level 3 group (Cox, 2018). The relationships between placental PCR result at L2 (exposure variable) and subsequent antibody and cytokine response at L4 (outcome variables) were also assessed using regression models. When the outcome was encoded as a continuous variable, a linear regression model was used for IL-10, while generalised linear models with log link and Poisson residual distribution were used for the IFNγ and antibody variables as the residuals were markedly right skewed (assessed using histograms). When cytokine responses were encoded as categorical outcomes (with three ordinal levels), multinomial logistic models were used.

3.4 Results

3.4.1 *Coxiella burnetii* molecular detection

The proportion of the herd with *C. burnetii* DNA detected in body tissues and fluids, as measured by PCR, changed over the sampling period (Table 3.2).

No vaginal mucus (0/192) or faecal (0/191) samples were positive on PCR at the first (L1) sampling point, three weeks prior to calving. At calving, 34% (66/192) of cows returned a positive placenta result for *C. burnetii* DNA on PCR. A further 18% (34/192) of cows returned a suspect positive result for their placenta sample, as per the criteria for PCR classification. At calving, 16% (30/191) of the cows had a positive colostrum sample, 13% (25/190) returned a positive faecal sample and 11% (20/190) returned a PCR positive vaginal swab. At calving, 18/20 vaginal swab positive cows were also placenta positive which corresponded to a crude agreement of 56% and kappa value of 0.15. At L3 (17 - 32 DIM), 4% (8/185) of cows returned a positive faecal sample, while 0.6% (1/163) of vaginal swabs and 0.5% (1/185) of milk samples were PCR positive. No animals returned PCR positive results in any tissues or fluids sampled at L4. At L5 (178 – 201 DIM), 10% (13/133) of cows returned a positive milk sample while 0.8% (1/128) of cows returned a positive faecal sample, however, no *C. burnetii* DNA was detected in swabs of vaginal mucus (0/132) of any cow.

Table 3.2. *Coxiella burnetii* DNA and antibody as detected by PCR in placenta, faeces, vaginal swabs, colostrum, and milk, or serum respectively, collected from Australian dairy cows in a naturally infected herd at five time points through the dry period to mid-lactation. L1- three weeks prior to calving (240 – 270 days pregnant; dry-period); L2- calving; L3- 17 – 32 days in milk (DIM); L4- 45 – 60 DIM; L5- 178 - 201 DIM.

		Sampling time point				
Sample type	**PCR positive classification**	**L1**	**L2**	**L3**	**L4**	**L5**
Placenta	Total positives	NA[a]	100/192 (52.1%[d])	NA	NA	NA
	Positive[b]		66/192 (34.4%)			
	Suspect positive[c]		34/192 (17.7%)			
Milk	Total positives	NA	30/191 (15.7%)	1/185 (0.5%)	0/169 (0%)	13/133 (9.7%)
	Positive[b]		7/191 (3.7%)			11/133 (8.3%)
	Suspect positive[c]		23/191 (12.0%)	1/185 (0.5%)		2/133 (1.5%)
Vaginal mucus	Total positives	0/192 (0%)	20/191 (10.5%)	1/163 (0.6%)	0/151 (0%)	0 (0%)
	Positive[b]		7/191 (3.6%)			
	Suspect positive[c]		13/191 (6.8%)	1/163 (0.6%)		
Faeces	Total positives	0/191 (0%)	25/190 (13.2%)	8/185 (4.3%)	0/169 (0%)	1/128 (0.8%)
	Positive[b]		2/190 (1.1%)			
	Suspect positive[c]		23/190 (12.1%)	8/185 (4.3%)		1/128 (0.8%)
Serology	Total positive	77/192 (40%)	42/189 (22%)	NA	56/169 (33%)	36/132 (27%)
	Positive[e]	68/192 (35.4%)	41/189 (21.7%)		52/169 (30.8%)	36/132
	Suspect positive[f]	9 (4.7%)	1 (0.5%)		4 (2.4%)	

[a] sample type not collected at this sampling time; [b] PCR positive sample; [c] suspected PCR positive sample.

[d] % rounded to one decimal place; [e] *C. burnetii* antibody serum titre above the IDEXX ELISA kit positive threshold of S/P%>40; [f] *C. burnetii* antibody serum titre S/P% ≥ 30% but < 40%.

3.4.2 Increase in proportion of PCR positive milk samples at L5

There was no significant difference in the prevalence of PCR positive colostrum samples at calving (L2, 16%) and PCR positive milk samples at mid lactation (L5, 10%) (odds ratio [OR]: 0.65; 95% CI: 0.32 – 1.33; p = 0.243), however, there was a significant increase in the prevalence of PCR positive milk samples from early stage to mid stage lactation (L3 [0.5%] to L5 [10%]; OR: 19.86; 95% CI: 2.56 – 153.84; p = 0.004; and L4 [0%] to L5 [10%]; OR: 18.11; 95% CI: 2.32 – 141.14; p = 0.006) (Table 3.2). The individual PCR patterns from the 13 cows with PCR positive milk at L5 are outlined in Table 3.3. Of the 13 cows that had PCR positive milk samples at L5, six were also PCR positive at L2 and two were PCR positive at L3, while five returned a positive PCR only in milk at the L5 timepoint.

Of the 133 animals sampled at L5, 12% were heifers (parity one), 40% were parity two, 22% were parity three, 11% were parity four, 11% were parity five, 1.5% were parity six, and 2% were parity seven. Of the 13 cows with PCR positive milk at L5, 54%, were parity two, 23% were parity three and 23% were parity four. There was no uniform pregnancy state in cows with PCR positive milk at the L5 sampling. Based on conception dates, of the 13 cows with PCR positive milk at L5, eight were pregnant (61.2%) and five were non-pregnant (38.5%), while for the cows with PCR negative milk at L5, 99 were pregnant (82.5%) and 21 were non-pregnant (17.5%) during the L5 milk sampling dates.

Table 3.3. Patterns of *Coxiella burnetii* DNA presence in all sample types from 133 Australian dairy cows sampled at five time points during a seven-month longitudinal study with results summarised according to their milk PCR result at the final sampling time point (L5). L1- three weeks prior to calving (240 – 270 days pregnant; dry-period); L2- calving; L3- 17 – 32 days in milk (DIM); L4- 45 – 60 DIM; L5- 178 – 201 DIM.

Sampling time point	Pattern 1	Pattern 2	Pattern 3	Pattern 4
L1 PCR positive sample (vaginal swab, faeces)	-[a]	-	-	-
L2 PCR positive sample (vaginal swab, faeces, placenta, colostrum)	+[b]	-	+	-
L3 PCR positive sample (vaginal swab, faeces, milk)	-	-	+	+
L4 PCR positive sample (vaginal swab, faeces, milk)	-	-	-	-
Number of L5 milk PCR positive cows following this pattern (13 cows)	6 (46%)	5 (38%)	1 (8%)	1 (8%)
Number of L5 milk PCR negative cows following this pattern (120 cows)	65 (54%)	50[c] (42%)	4 (3%)	1 (1%)

[a] sample was negative for *C. burnetii* DNA, as measured by PCR.

[b] sample was positive for *C. burnetii* DNA, as measured by PCR.

[c] one cow within 50 had a PCR positive sample at L5 in their faeces.

3.4.3 Antibody response

The proportion of cows that were seropositive peaked at 40% (77/192) three weeks prior to calving (L1), before falling to 22% (42/189) at calving (L2), as shown in Table 3.4. The proportion of seropositive cows then rose to 33% (56/169) by 45-60 DIM (L4). By L5 the seroprevalence had fallen to 27% (36/132). From these results patterns of serostatus across all time points in individual cows were determined (Table 3.4). The two most common patterns were those in which the antibody titre was maintained over time. That is, 53%

(69/130) of cows remained seronegative at every sampling point and 21% (27/130) of cows returned a seropositive result at every sampling timepoint. The third largest pattern comprised 12% (15/130) of cows that were seropositive at L1, three weeks prior to calving, and then seronegative at the rest of the samplings (Table 3.4).

Table 3.4. Patterns of antibody against *Coxiella burnetii* in 130 Australian dairy cows across four sampling time points through the first 7 months of lactation. L1- three weeks prior to calving (240 – 270 days pregnant; dry-period); L2- calving; L4- 45 – 60 DIM; L5- 178 – 201 DIM. Serology was determined by ELISA (IDEXX). Only cows that were sampled at all time points were included in the patterns.

	L1	**L2**	**L4**	**L5**	**Number of cows per pattern; %[d]**	**Cow classification**	
						Cows, n	Heifers, n
Pattern 1	-[a]	-	-	-	69/130; 53.1%	59	10
Pattern 2	+[b]	+	+	+	27/130; 20.8%	26	1
Pattern 3	+	-	-	-	10/130; 7.7%	10	0
	sus[c]	-	-	-	5/130; 3.8%	5	0
Pattern 4	-	-	+	-	4/130; 3.1%	2	2
	-	-	sus	-	2/130; 1.5%	2	0
Pattern 5	+	-	+	+	4/130; 3.1%	4	0
Pattern 6	+	-	+	-	3/130; 2.3%	2	1
	sus	-	+	-	1/130; 0.8%	1	0
Pattern 7	+	+	+	-	1/130; 0.8%	1	0
Pattern 8	+	+	-	+	1/130; 0.8%	1	0
Pattern 9	-	-	+	+	2/130; 1.5%	2	0
Pattern 10	-	-	-	+	1/130; 0.8%	1	1
Seroprevalence	77/192 (9 sus) 40%	42/189 (1 sus) 22%	56/169 (4 sus) 33%	36/132 (0 sus) 27%			

[a]– is antibody serum titre below the threshold S/P%<40.

[b]+ is antibody serum titre above the IDEXX ELISA kit positive threshold of S/P%>40.

[c]sus is antibody serum titre S/P% ≥ 30% but < 40%.

[d] percentage rounded to one decimal place.

3.4.4 Relationship between *Coxiella burnetii* serology and milk PCR result

All cows that returned a PCR positive milk sample at L5 (n=13) were serologically positive at all time points measured (pattern 2; Table 3.4). There was a close positive linear correlation between each individual cows' antibody titres at each timepoint (r >0.8; Table 3.5), with the majority of cows with PCR positive milk at L5 (11/13) having antibody titres that remained above an S/P% of 100 throughout the entire seven-month sampling period (Figure 3.2). Furthermore, cows that returned a PCR positive milk sample result at L5 and also in their colostrum (L2) generally had persistently higher antibody titres across the sampling time points than cows that only returned a PCR positive result in their colostrum at L2 (Figure 3.3).

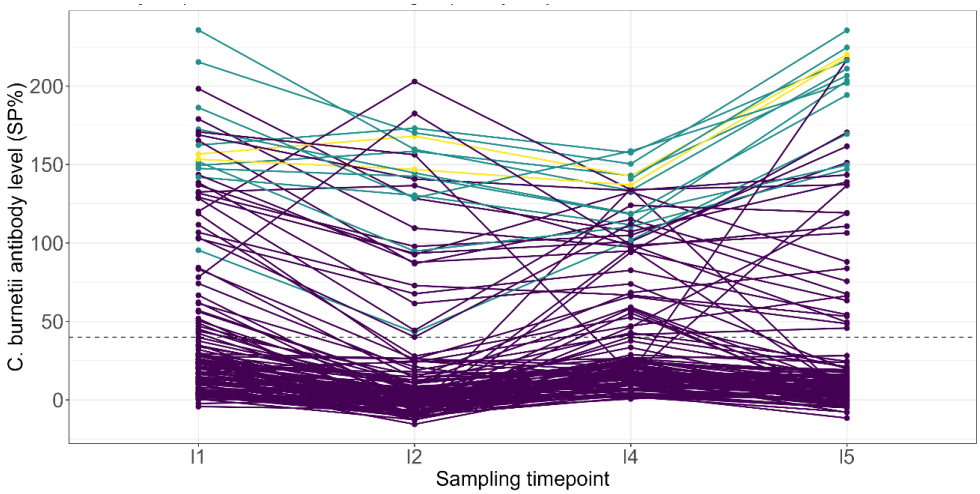

Figure 3.2. *Coxiella burnetii* antibody response in Australian dairy cows sampled across four sampling time points (n=130) in longitudinal study of the first seven-months of lactation, grouped by *C. burnetii* DNA detection in milk at L5. L1- three weeks prior to calving (240 – 270 days pregnant; dry-period); L2- calving; L4- 45 – 60 DIM; L5- 178 – 201 DIM. Navy lines = PCR negative milk at L5, green lines = PCR positive milk at L5, yellow lines = PCR suspected positive milk at L5. Each line represents one cow. Dashed line represents IDEXX ELISA seropositive cutoff (S/P% = 40).

Table 3.5 Pairwise correlation coefficients (Pearson's) for individual cow serum *Coxiella burnetii* antibody titres at each measured time-point from longitudinally sampled Australian dairy cows over a period of approximately seven months. L1- three weeks prior to calving (240 – 270 days pregnant; dry-period) (n = 192); L2- calving (n = 192); L4- 45 – 60 DIM (n = 169); L5- 178 – 201 DIM (n = 133). P values for all correlation coefficients were less than <0.001.

	L1	**L2**	**L4**	**L5**
L1	1.00			
L2	0.88	1.00		
L4	0.83	0.83	1.00	
L5	0.84	0.85	0.83	1.00

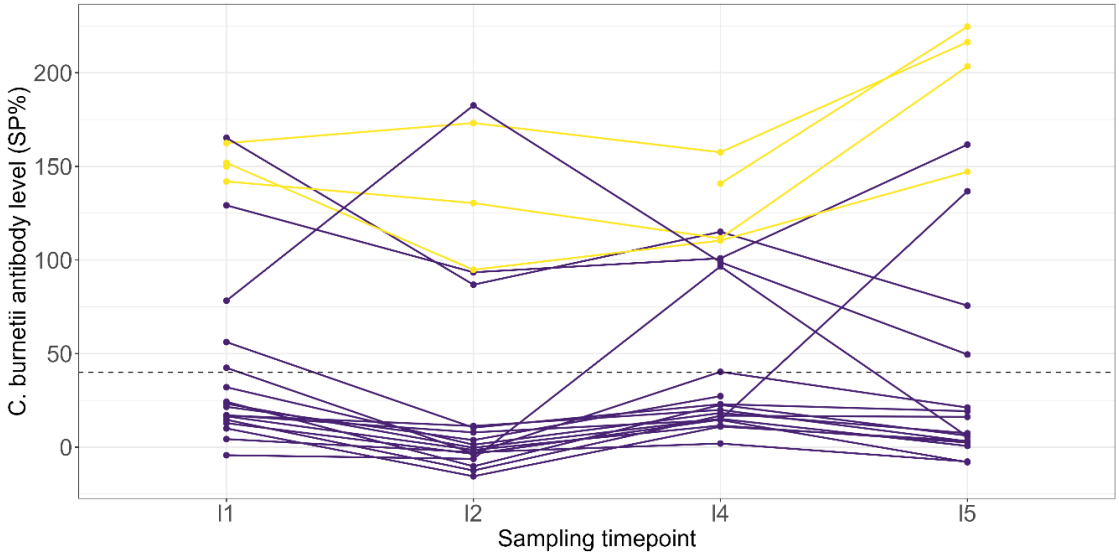

Figure 3.3. Antibody response over time of 21 Australian dairy cows with *Coxiella burnetii* DNA detected in their colostrum at calving (L2) grouped by their *C. burnetii* DNA status in milk at L5 (178 – 201 DIM). L1- three weeks prior to calving (240 – 270 days pregnant; dry-period); L2- calving; L4- 45 – 60 DIM; L5- 178 – 201 DIM. Yellow line = cows with PCR positive colostrum at L2 and milk at L5 (n = 4), navy lines = cows with only PCR positive colostrum at L2 (n = 17). Dashed line represents IDEXX ELISA seropositive cutoff (S/P% = 40). Note: one cow has an incomplete line as it did not have an ELISA result recorded at L2.

3.4.5 Cytokine distribution

The IFNγ and IL-10 responses measured from all cows sampled at L4 are presented in Figure 3.4. The distrubtions varied between the two cytokines with IFNγ having a minumun and maximun response of 0 ng/ml and 1.18 ng/ml respectively, and median of 0.05 ng/ml while IL-10 had a minimun and maximun response of 0 ng/ml and 1.33 ng/ml respectively, median of 0.40 ng/ml.

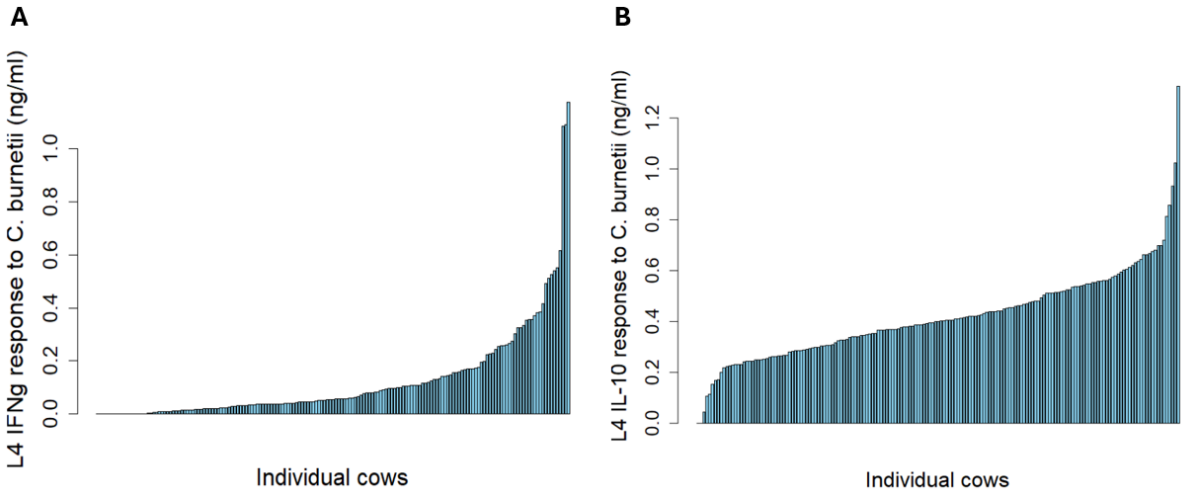

Figure 3.4. Distribution of individual Australian dairy cow (n = 169) cytokine responses at L4 to *ex vivo* stimulation of whole blood with *Coxiella burnetii* antigen. **A**: IFNγ response and **B**: IL-10 response. Cytokine response was measured using the whole blood cytokine recall assay. L4- 45-60 days in milk days in milk.

3.4.6 Immune profile considering cytokine and antibody response at early lactation

A scatter plot of *C. burnetii* antibody level versus IFNγ (Figure 3.5A) or IL-10 (Figure 3.5B) response at L4 is presented in Figure 3.5, using different coloured dots to identify the *C.*

burnetii PCR result in milk at L5. Visual assessment of the data found no cows in the study returned both a high antibody and cytokine response at L4 (Figure 3.5). Cows with PCR positive milk (represented in Figure 3.5 by green and yellow dots) at L5 tended to return a higher antibody level and lower IFNγ response at L4 (Figure 3.5A). Statistical analysis found antibody level at L4 was associated with subsequent PCR positive milk at L5, and for every 10 unit increase in antibody titre, the odds of being PCR positive in milk at L5 increased by 2.1 times (95% CI: 1.4 – 3.3; p = 0.001) (Table 3.6). IFNγ response at L4 was not significantly associated with milk PCR status at L5 when cytokine response was assessed as a continuous or categorical variable (Table 3.6). There was some evidence that cows with higher IFNγ responses had lower odds of having PCR positive milk compared to low level responders, as mid-level responses had 24% lower odds (0.76; 95% CI: 0.02 – 6.15) and high-level responses had 31% lower odds (0.69; 95% CI: 0 – 4.85), but these estimates were imprecise (Table 3.6). No statistical associations or visual trends were identified between IL-10 response at L4 and PCR milk status at L5 (Table 3.6; Figure 3.5B).

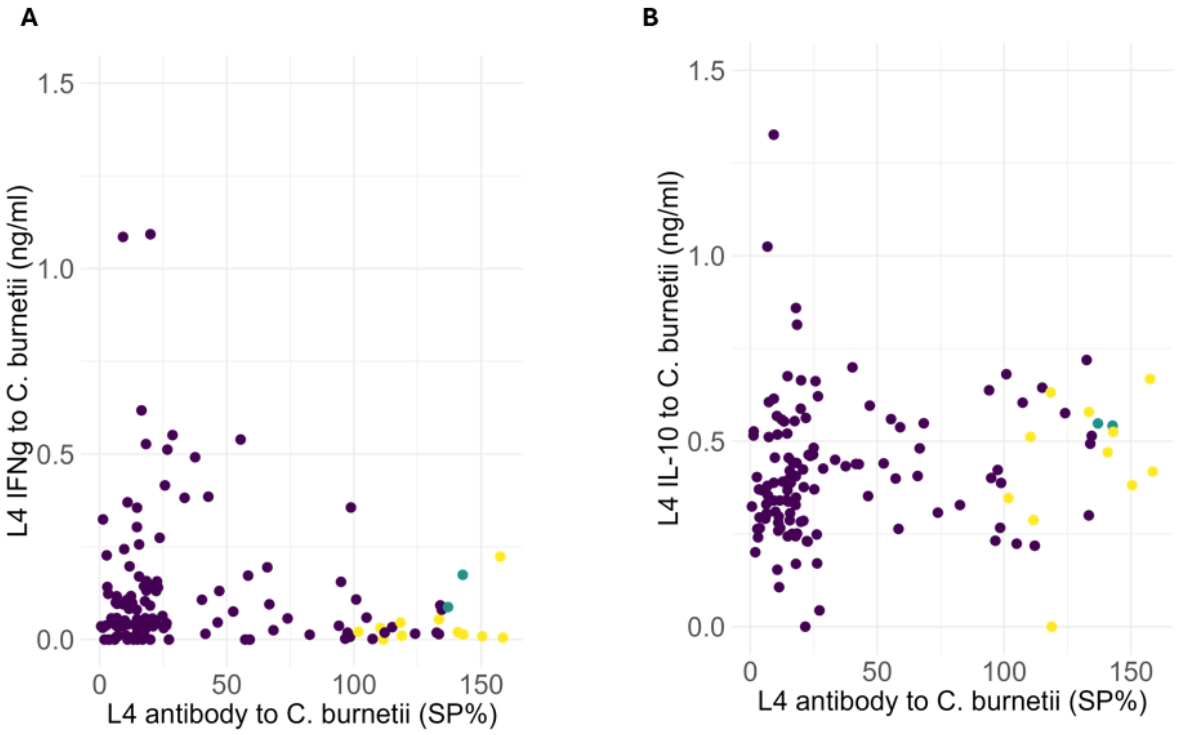

Figure 3.5. Scatterplots depicting Australian dairy cow *Coxiella burnetii* antibody (IgG) response against IFNγ, and IL-10 response to stimulation with *C. burnetii* antigen at (L4), grouped by L5 milk PCR status (n = 133). **A:** Antibody levels and IFNγ response. **B:** Antibody levels and IL-10 response. L5 milk PCR status was determined by testing milk samples in a *C. burnetii* multiplex qPCR. Antibody was measured in serum using the commercially available IDEXX ELISA and cytokine response was measured using the whole blood cytokine recall assay. Navy = cows with PCR negative milk at L5, green = green with suspect PCR positive milk at L5, yellow = cows with PCR positive milk at L5. L4- 45-60 days in milk (DIM); L5- 178 – 201 DIM. Note: cytokine level ranges for categorisation of IFNγ were 0 – 0.2 ng/ml for level 1, 0.2 – 0.4 ng/ml for level 2, and >0.4 ng/ml for level 3. Level ranges for IL-10 were 0 – 0.2 ng/ml for level 1, 0.2 – 0.6 ng/ml for level 2, and >0.6 ng/ml for level 3.

Table 3.6. Associations between *Coxiella burnetii* serum antibody level, IFNγ and IL-10 response at L4 (exposure variables) and *C. burnetii* DNA in milk at L5 (outcome variable) from Australian dairy cows enrolled in a longitudinal study as determined by logistic regression models. Antibody titre was determined by measuring *C. burnetii* specific IgG in serum by ELISA. IFNγ and IL-10 were measured by an ELISA from whole blood stimulated *ex vivo* with *C. burnetii* antigen. *Coxiella burnetii* DNA was determined by testing milk samples in a multiplex qPCR. L4- 45-60 days in milk (DIM).

Immune marker (exposure)	Variable type (exposure)	Level	Number of cows	Estimate	Confidence intervals	p value
ELISA	Continuous	NA	133	2.14[b]	1.37 – 3.34	0.001
IFNγ	Continuous	NA	133	0.004[b]	1.06e-06 – 11.92	0.173
	Categorical[a]	Low	112	Reference category		
		Medium	12	0.76[c]	0.02 – 6.15	1.00
		High	9	0.69[c]	0 – 4.85	0.75
IL-10	Continuous	NA	133	2.81[b]	0.16 – 50.94	0.484
	Categorical	Low	7	Reference category		
		Medium	108	0.61[d]	0.07 – 5.61	0.664
		High	18	0.75[d]	0.06 – 9.87	0.827

[a] exact logistic regression.

[b] estimate is odds ratio for being PCR positive for every one unit (S/P% for ELISA and ng/ml for cytokines) increase in predictor.

[c] estimate is adjusted odds ratio (for the exact likelihood) for being PCR positive within a given category of the exposure variable compared to the reference category.

[d] odds ratio for being PCR positive within a given category of the exposure variable compared to the reference category.

The immune response from cows with DNA detected in their placenta at calving were also visually assessed by scatterplots which did not identify any trends in their subsequent antibody titre, IFNγ or IL-10 response at L4 (Figure 3.6). Statistical modelling of the relationship between PCR status at calving (L2) and immune response at early lactation (L4)

as continuous variables reported no significant associations with estimated average differences in immune response between PCR placenta positive and negative cows (Table 3.7). Analysis of the cytokines as categorical variables found cows with a positive placenta at L2 had 3.03 times higher relative risk (95% CI: 1.04 – 8.83; p = 0.041) of having a mid-level IFNγ response at L4, compared to a low level response, and 1.52 times higher relative risk (95% CI: 0.41 – 5.63; p = 0.529) of having a high-level IFNγ response at L4 compared to a low level response (Table 3.7). For IL-10, cows with a PCR positive placenta at L2 had 0.37 times lower relative risk (63% lower) (95% CI: 0.07 – 1.89; p = 0.231) of having a mid-level response at L4, and 0.33 times lower relative risk of having a high-response (67% lower) (95% CI: 0.05 – 2.07; p = 0.238) compared to compared to low level responses (Table 3.7).

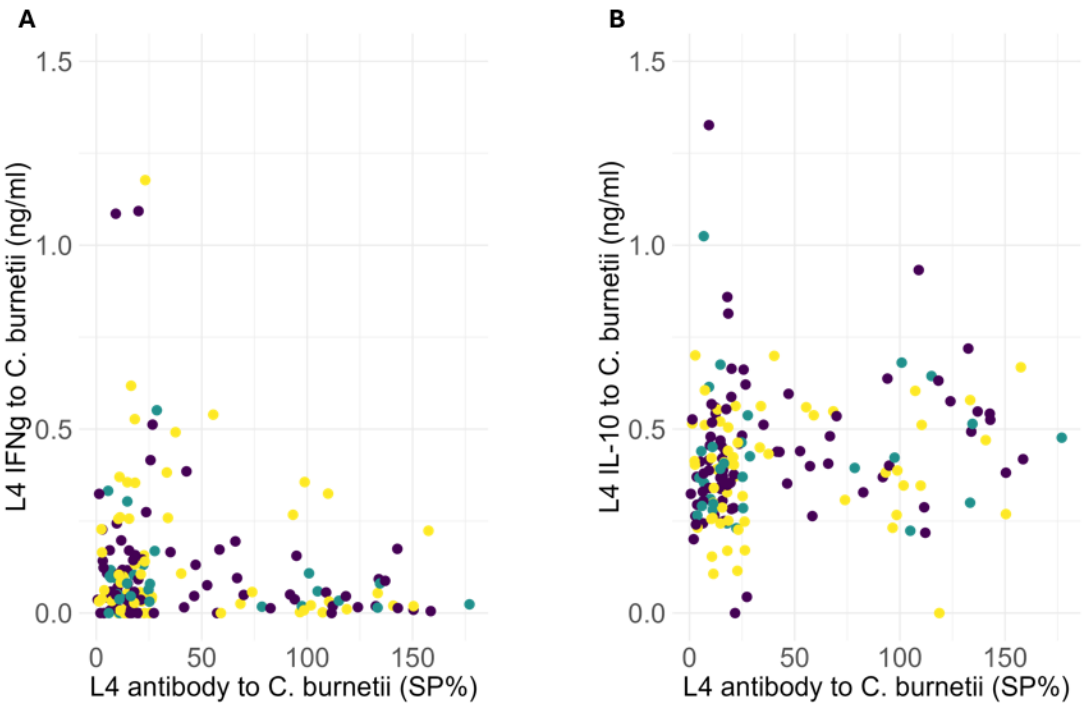

Figure 3.6. Scatterplots of Australian dairy cows' *C. burnetii* antibody (IgG), IFNγ, and IL-10 response at L4, grouped by L2 placenta PCR status (n = 169). **A:** Antibody levels and IFNγ response**. B:** Antibody levels and IL-10 response. L2 placenta PCR status was determined by testing placental samples in a *C. burnetii* multiplex qPCR. Antibody was measured in serum using the commercially available IDEXX ELISA and cytokine response was measured using the whole blood cytokine recall assay. Navy = cows with PCR negative placenta, green = cows with a suspect PCR positive placenta, yellow = cows with PCR positive placenta. L2- calving; L4- 45-60 days in milk (DIM). Note: cytokine level ranges for categorisation of IFNγ were 0 – 0.2 ng/ml for level 1, 0.2 – 0.4 ng/ml for level 2, and >0.4 ng/ml for level 3. Level ranges for IL-10 were 0 – 0.2 ng/ml for level 1, 0.2 – 0.6 ng/ml for level 2, and >0.6 ng/ml for level 3.

Table 3.7 Associations between *Coxiella burnetii* DNA in placental samples at L2 (exposure variable) and subsequent *C. burnetii* serum antibody level, IFNγ and IL-10 response at L4 (outcome variables) from Australian dairy cows enrolled in a longitudinal study. Linear regression models were used for continuous outcome variables while multinomial logistic regression models were used for categorical outcome variables. *Coxiella burnetii* DNA was determined by testing placental samples in a multiplex qPCR. Antibody titre was determined by measuring *C. burnetii* specific IgG in serum by ELISA. IFNγ and IL-10 were measured by an ELISA from whole blood stimulated ex vivo with *C. burnetii* antigen. L4- 45-60 days in milk (DIM).

Immune marker (outcome)	Variable type (outcome)	Level	Number of cows	Estimate	Confidence intervals	p value
ELISA	Continuous	NA	169	1.02[a]	0.97 – 1.07	0.373
IFNγ	Continuous	NA	169	1.17[a]	0.49 – 2.79	0.718
	Categorical	Low	139	Reference category		
		Medium	20	3.03[b]	1.04 – 8.83	0.041
		High	10	1.52[b]	0.41 – 5.63	0.529
IL-10	Continuous	NA	169	-0.04[c]	-0.09 – 0.01	0.123
	Categorical	Low	8	Reference category		
		Medium	141	0.37[b]	0.07 – 1.89	0.231
		High	20	0.33[b]	0.05 – 2.07	0.238

[a] estimate is ratio of arithmetic means in outcome (S/P% for ELISA and ng/ml for IFNγ) between PCR placenta positive and negative group (exposure variable).

[b] estimate is the relative risk ratio for PCR positive cows being in the given level of the categorical outcome compared to the reference category relative to the PCR negative cows.

[c] estimate is difference between means in outcome (ng/ml for IL-10) between PCR placenta positive and negative group (exposure variable).

3.5 Discussion and conclusions

This longitudinal study investigated the patterns of bacterial shedding and immunological responses in cows in a *C. burnetii* endemic Australian dairy herd prior to calving and into lactation. From a zoonotic and within herd transmission perspective, identifying and understanding persistent shedders (as measured by detection of *C. burnetii* DNA by PCR) that are unable to clear *C. burnetii* infection is of importance, as they may pose a recurring zoonotic and within herd transmission risk from milk aerosolization. The present study found a large proportion of cows were infected and shedding at parturition, with 52% having *C. burnetii* DNA detected in their placenta at calving (Table 3.2). Shedding decreased into early lactation but there was a significant increase in the proportion shedding at mid-lactation (approximately 200 DIM; L5) predominately through milk, of which 62% of these cows had also shed at calving or early lactation (Table 3.2; Table 3.3). All cows shedding in milk at mid-lactation (L5) generally had a persistently high antibody titre, with increasing antibody titre at early lactation (L4) being significantly associated with increased odds of shedding in milk at mid-lactation (L5) (Figure 3.2; Figure 3.5). While the IFNγ response at early lactation (L4) from cows shedding in their milk at 200 DIM (L5) was low, this finding was not significant (Figure 3.5). In contrast to the cows shedding in milk at mid-lactation (L5), the cows shedding in placenta at calving (L2) were not significantly associated with antibody or cytokine responses at early lactation (L4) (Figure 3.6).

3.5.1 Variation in shedding response from cattle in an endemically infected herd

Existing literature shows cattle infected with *C. burnetii* display variation in shedding routes and persistence on an individual and herd level. This study is the first to monitor *C. burnetii* shedding in Australian dairy cows, and found that cows did not shed into faeces or vaginal mucus prior to calving, which is a finding that aligns previous caprine experimental infection studies (Table 3.2) (Roest et al., 2012). The finding that the highest prevalence of shedding during the reproductive/lactational cycle occurred at calving (Table 3.2), also aligned with previous cattle observational studies (Freick et al., 2017). At calving, 52% of cows had *C. burnetii* DNA detected in their placenta, with the robustness of this result aided by the sampling and testing methodological choices. As the high environmental load of *C. burnetii* (Bauer, 2024), increased the risk of contamination of placental samples at collection, every effort was taken to collect the samples prior to uterine expulsion and using aseptic technique. In addition, rigorous criteria were used to classify placental samples with detected DNA as either positive (33%) or suspected positive (18%). A multiplex qPCR utilising three *C. burnetii* genes was utilised with a stringently determined cut off, as while the multicopy transposon gene IS*1111* is a commonly used target, when assessed alone it could lead to false positive results, as it is also contained within *Coxiella*-like endosymbionts (Table 3.2) (Mathews et al., 2024). Interestingly, at calving, only 11% (20/191) of cows were shedding in their vaginal mucus, which was unexpectedly low considering the high proportion of cows with a positive placenta (52%) (Table 3.2). Higher proportions of positive vaginal swabs have been reported at calving in other cattle observational studies, including 30% in heifers from an endemically infected German herd (Freick et al., 2017) and 100%

(24/24) in aborting cows from 23 herds in France (Guatteo et al., 2012) which may be due to either differences in level of infection or detection limits and interpretation. To help confirm the current findings, longer vaginal swabs and infusettes that collect mucus were also tested in different cows but they continued to be poorly correlated with placental samples (data not shown).

Beyond calving, the proportion of the cohort shedding in each sample type reduced (Table 3.2). Faecal shedding is known to be sporadic and reduced from 4% (L3) to 0% (L4) to 0.8% (L5), with some studies having even suggested this route may be the result of passive shedding, as *C. burnetii* DNA has not been detected in gastrointestinal tissue (Table 3.2) (García-Ispierto et al., 2013, Guatteo et al., 2007, Rodolakis et al., 2007, Roest et al., 2012). For milk shedding, the highest proportion of cows were also identified at L2 in colostrum (16%), which was followed by a significant reduction at L3 (0.5%) and L4 (0%), however, there was a significant rise in the proportion (10%) shedding at L5 (approximately 200 DIM) (Table 3.2). There is previous evidence both supporting (Barlow et al., 2008, Freick et al., 2017), and not supporting (Angen et al., 2011), a higher proportion of cows shedding in milk at later stage lactation compared to early lactation, with the difference in findings to Angen et al. (2011) possibly due to method and analysis variations. For example, in their study, 12 dairy cow herds were included and the influence of days in milk on *C. burnetii* detection was assessed in a regression model, whereas in the present study, proportions from set timepoints were compared. However, a key difference of the present study to previous research was the additional assessment of individual shedding patterns, which can provide nuanced insight into the infection dynamics in individuals.

This study considered whether the increase in shedding at L5 compared to early lactation (L3 and L4) was due to cows shedding for the first time at L5 or whether they had also been shedding at calving (L2) and for some reason may have stopped shedding during early lactation (L3 and L4). Both scenarios were observed, with the most common pattern being shedding at L2 and L5 only (Table 3.3). While the inability to detect PCR positive samples at L3 and L4 from most cows may have been due to the intermittent nature of shedding (that is, they were not shedding on the day of sampling but may have been shedding on other days around that time point), it could also be that the lack of shedding at early lactation was a real result (possibly indicating infection latency). The latter scenario is consistent with the hypothesis that a hormonal effect could cause a recrudescence in shedding from the mammary gland at later stage of lactation. In the normal production cycle in dairy cow herds, later stage lactation would likely coincide with early-stage pregnancy (a time of increased progesterone) with *in vitro* and *in vivo* studies having found stage of pregnancy and progesterone may impact placental shedding dynamics in ruminants and humans, respectively. More specifically, a human placental cell line inoculated with *C. burnetii* showed a significant reduction in bacterial replication when progesterone was added (Howard and Omsland, 2020), while experimental infection (intraperitoneal) of pregnant goats found histopathological evidence of bacterial replication right before parturition (Sánchez et al., 2006) (when progesterone levels would be reducing). However, investigation of the pregnancy status of the L5 shedding cows in this study, showed that only approximately half the cows were pregnant during this sampling point. For a clearer understanding of the effect of progesterone on milk shedding, it may also be important to consider the oestrus cycle of the non-pregnant cows as progesterone also increases during the luteal phase (Ball and

Peters, 2008). Nevertheless, all cows shedding in milk at L5 were at the same stage of lactation and so investigation of the effect of hormones that mediate mammary gland development over lactation on *C. burnetii* in mammary epithelial cells may be warranted, as well as other local mammary gland factors that change over lactation, including cytokine profiles and somatic cell count. Another aspect to consider is the influence of age on shedding at later lactation, as in the present study, all cows shedding at L5 were parity two or greater, with a positive association between *C. burnetii* in milk and increasing parity number having previously been reported by one study assessing 12 dairy herds (Angen et al., 2011). While older cows may just be more likely to become infected from a previous pregnancy and increased time spent within the endemic environment, they could also have some specific pre-disposition towards milk shedding compared to younger heifers, such as leakier epithelial cell barriers or higher somatic cell counts (Bradley and Green, 2005).

3.5.2 Host immune preference of the cow may determine establishment of persistent infection

Persistent focalised infection is a chronic sequalae of acute Q fever in approximately 1-5% of human cases (Melenotte et al., 2020), and while some cattle studies have shown *C. burnetii* shedding can occur persistently in milk, there is very limited understanding of the pathogenesis of persistent *C. burnetii* infection in ruminants. The present study hypothesised that the 10% of cows that were shedding at L5 in milk may represent persistently infected animals as opposed to susceptible animals that were recently infected given their antibody titres remained continually high throughout the entire sampling period

of almost eight months (Figure 3.2). There are however several possible reasons that animals may have remained seropositive throughout the sampling period. Firstly, antibody titres following a primary *C. burnetii* infection may normally persist in ruminants, as experimental infection studies of pregnant and non-pregnant female goats have found average antibody titres remain elevated after infection (Bouvery et al., 2003, Roest et al., 2013, Roest et al., 2020). For example, *C. burnetii* intranasally-infected pregnant yearling goats (day 76 of pregnancy) (that either aborted or delivered a mix of healthy and ill offspring between week seven and ten post infection), had *C. burnetii* IgG phase 1 and 2 titres that increased starting from week six- and two-post infection, respectively, and remained steady at an S/P% of ~80 up until the end of the trial at 12 weeks (three months) post infection (Roest et al., 2013). Secondly, the significant *C. burnetii* environmental load that was found at the study farm by PCR results from dust samples, could cause re-exposures leading to continuing stimulation of the immune response and maintained seropositivity (Bauer, 2024). However, despite the high *C. burnetii* environmental load, the third largest antibody pattern was cows that were seropositive three weeks prior to calving (L1) and then seronegative at subsequent timepoints at calving and post calving (Table 3.4). In humans, when *C. burnetii* interacts with macrophages they will polarise to either a classically activated M1 macrophage type or alternatively activated M2 macrophage type that subsequently leads to either TH1 or TH2 dominated immunity respectively, with the latter scenario thought to contribute to a chronic infection outcome (Benoit et al., 2008, Sam et al., 2023). Under the assumption that the calving area is a high-risk exposure site occupied by all cows in this study, these cows with a positive to negative serostatus may present the alternate response to the persistent group above and preference a TH1 dominated response.

A very high antibody titre can be an indicator of chronic Q fever in humans, possibly as these patients are unable to clear infection (meaning their immune system is continually stimulated) or because of the potential *C. burnetii*-induced M2 polarisation described above, and has also been observed in milk shedding cows over sampling periods of 30 and 90 days (Eldin et al., 2017, Guatteo et al., 2012, Guatteo et al., 2007, Boettcher et al., 2013). The current study found antibody titre at L4 was a significant predictor for milk shedding at L5, with odds of being PCR positive doubling for every ten unit increase in titre at L4 (Table 3.6). While antibody is generally thought of as a measure of exposure, it is also possible the association could have a causal influence on the outcome. Currently the role of antibody in human Q fever infection clearance is still unclear but it has been suggested that the excessive antibody in chronic patients may be a potential mechanism to promote bacterial replication, as opsonised *C. burnetii* show increased propagation inside macrophages compared with non-opsonised *C. burnetii* (Desnues et al., 2009). Alternatively, the high antibody titre of milk shedding cows may only signify a strong TH2 humoral response which in turn prevents a strong TH1 CMI response. The antagonism between the TH1 and TH2 immune response is an immunological principle highlighted in the present study by the distribution of cows in Figure 3.5A, as no cow had both a strong antibody level and strong TH1 IFNγ response. The TH1 immune response is especially vital in coxiellosis pathogenesis as CMI controls microbicidal and apoptotic clearance of *C. burnetii* living within infected human cells/monocytes (Sam et al., 2023). Indeed, all cows shedding in milk were grouped by high antibody levels and low IFNγ response, which highlights the likely importance of the immune profile in shedding outcome (Figure 3.5A). Conversely, a similar antibody/cytokine pattern was not observed in relation to shedding of *C. burnetii* in the placenta of cows at

calving (Figure 3.6A; Table 3.7), suggesting that the immune response following infection may determine a persistent infection state (that in this study could be detected as milk shedding at later stage lactation). The humoral immune response only represents part of the immunological story and while many of the previous studies have focussed on measuring antibody, one of the key features of this study was the measurement of cytokines in these animals.

Interferon-gamma is an important TH1 pro-inflammatory cytokine of the cell mediated immune response, that has been shown to promote *C. burnetii* killing within human monocytes by apoptosis *in vitro* and is vital to clearance of infection in mice (Dellacasagrande et al., 1999). However, its role in chronic Q fever disease progression in humans is still unclear due to conflicting findings between studies. This study investigated *C. burnetii*-specific IFNγ response using the cytokine recall assay at eight weeks post calving, as previous optimisation experiments identified that IFNγ responses were reduced at calving (Chapter 2). The assay works by stimulating cattle whole blood *ex vivo* with whole cell phase 1 and 2 *C. burnetii*, with the level of cytokine detected reflecting a combination of the cow's inherent ability to produce the cytokine as well as the number of, and time since, *C. burnetii* exposures. In the present study, while all cows shedding in milk returned a low IFNγ response (Figure 3.5A) the association was not statistically significant, likely because of the considerable number of cows with a low IFNγ response that did not subsequently shed at L5 (Table 3.6). It was confirmed that these cows did include those that had been infected, as some were shedding *C. burnetii* in their placenta at calving, which is identified by the green and yellow dots in the bottom left corner of Figure 3.6A. The reason why all exposed cattle

with a low IFNγ response measured at L4 did not become persistently infected is still uncertain. One explanation is that some cows may produce a strong IFNγ response at the onset of infection, that contributes to adequately clearing *C. burnetii*, but which subsequently reduces to low levels upon recall when measured at L4. Likewise, human Q fever studies that found no significant difference in the ability of chronic and healthy individuals to respond to *C. burnetii* stimulation suggest to fully understand the role of IFNγ, it would need to be measured during these patient's initial acute stage (not when they are already chronic) (Schoffelen et al., 2017). Measuring the cytokine response at the time of infection is a challenging task in an observational setting but may be possible during a controlled experimental infection trial. It would additionally be interesting to measure the IFNγ response from the mammary gland tissue to assess a more local effect, for example, by adapting the cytokine recall assay to milk samples or gene expression from mammary epithelial cells in the mammary gland of shedding and non-shedding animals. Another explanation is that IFNγ in itself is not deterministic in *C. burnetii* persistence but that the low IFNγ response signifies a low TH1 response which could mediate disease progression through a variety of mechanism as the immune system is hugely complex. For example, IL-2 was found to be significantly lower in chronically infected human Q fever patients, compared to seropositive healthy controls, and as this cytokine directs T cells, helping to balance tolerance and immunity, it could be a target for future investigation (Schoffelen et al., 2014).

This study also monitored IL-10 response to *C. burnetii* antigen stimulation, as IL-10 is traditionally recognised as an anti-inflammatory cytokine and has been linked to human Q fever infection progression. Interlukin-10 was found to be necessary for *C. burnetii*

intracellular replication in monocytes *in vitro* (Ghigo et al., 2001) and was spontaneously released in elevated levels from PBMCs of chronic Q fever patients compared to healthy human controls (Capo et al., 1996). This is the first ruminant study to measure *C. burnetii* specific IL-10 using the recall assay and no significant associations or visual trends between IL-10 response and cows shedding at L5 were found (Figure 3.5B; Table 3.6). Given the high antibody titres from these cows, this was somewhat surprising as IL-10 (which is a complex cytokine produced by most leukocytes and has multiple target cells) is considered stimulatory for the humoral response via action on B cells (Saxena et al., 2015). The limited research into IL-10 in ruminants thus far has been contradictory. One study found IL-10 mRNA expression was downregulated in PBMCs from experimentally *C. burnetii*-infected pregnant goats compared to non-infected animals (Ammerdorffer et al., 2014). While they did not identify chronically infected goats for direct comparison to human work, they speculated key roles for cytokines may differ between these species considering the vastly different disease manifestations. Contrastingly, the unstimulated serum levels of a range of cytokines were monitored by ELISA in a *C. burnetii* endemically infected Polish dairy herd (n = 68) experiencing reproductive disorders (prior to vaccination), with IL-10 levels from cows shedding in milk significantly higher compared to seropositive non-shedding cows (Małaczewska et al., 2018). The difference in IL-10 levels between shedding and seropositive non-shedding cows can be interpreted in various ways. The increased IL-10 could be either encouraging persistence in the shedding group or helping to provide a balanced host response by preventing excessive pro-inflammation in actively infected animals, so that they may subsequently join the non-shedding seropositive animal group. Again, without further insight into the timing of infection it is difficult to speculate.

Therefore, despite some remaining uncertainties around the specifics of the role of IFNγ and IL-10 in *C. burnetii* infection in cattle, this study demonstrates that a TH2 immune preference (or predominance) is associated with persistent infection. Furthermore, the antibody titres remaining high and closely correlated over the seven-month sampling period ($r > 0.8$; Table 3.5; Figure 3.2) may make the combined phase 1 and 2 commercial ELISA a convenient way of consistently identifying persistently infected animals at various timepoints. It may be also interesting to further explore cows that showed a more variable response with such analysis perhaps an opportunity to also more robustly define consistently high scorers. Future work may be directed at determining why some cows favour this immune profile, including the effect of *C. burnetii* dose, host genetics, and time of infection. Previous research has suggested the route and/or site of *C. burnetii* infection may be responsible for clinical outcome, and that cows shedding in their milk become persistently infected due to an insufficient immune response in the teat canal compared to those infected via inhalation (Boettcher, 2017). It has even been proposed that milk shedding in cattle may be a symptom of chronic infection (Freick et al., 2017). Given bovine mammary epithelial cells showed a weaker pro-inflammatory cytokine response compared to lung, placental, and gastrointestinal epithelial cells, it is plausible the mammary gland provides a site for undetected replication of *C. burnetii* (Sobotta et al., 2017). Furthermore, chronic endocarditis is found almost exclusively in human patients with pre-existing conditions such as valvopathies and the immunocompromised (Maurin and Raoult, 1999). However interestingly, this study identified that of all the cows shedding in their colostrum at L2, cows that also shed at L5 had higher antibody titres than cows that only shed at L2 (Figure 3.3). While this could not be statistically tested due to the small sample sizes in the two groups, it may perhaps indicate that presence of the bacteria in the mammary gland does not alone

dictate the immune response and subsequent establishment of a persistent state of infection (Capo et al., 1998). Future studies should confirm this theory by visualising mammary gland tissue from milk shedding cows. Research should also be directed towards establishing if milk shedding cows have an impacted production. Indeed, the diverse variables presented in this study to describe *C. burnetii* infection, including shedding, humoral, and cell mediated immunity, should be used as exposures to investigate the impact of infection on production.

Chapter 4 Hypothesis screening field study into the potential impacts of *Coxiella burnetii* exposure on health and productivity of dairy cows: test day milk yield, fat, and protein

4.1 Abstract

Coxiella burnetii is the gram-negative intracellular bacterium that causes the disease coxiellosis in ruminants, which most commonly manifests as reproductive loss (including abortion and stillbirth). Reproductive loss from *C. burnetii* infection is considered to occur sporadically in cattle but there is incomplete understanding about whether *C. burnetii* infection could lead to milk production losses in cattle. The aim of this longitudinal study was to investigate if *C. burnetii* infection was associated with reductions in average daily milk volume, total solids, and fat and protein yield and concentration (using herd monthly milk test data). Initially, 396 cows, that were approximately three weeks out from calving, were enrolled into the study, then subsequently sampled throughout their reproductive and lactation cycle (three weeks prior to calving, calving, early lactation and mid-lactation). The samples collected were blood, vaginal swabs, faeces, milk, and the placenta (only at calving), which were used to assess *C. burnetii* antibody levels in serum (by ELISA), *C. burnetii* specific IFNγ and IL-10 response (by cytokine recall assay), and *C. burnetii* shedding in vaginal mucus, faeces, milk, and the placenta (by PCR). Generalised estimating equations were used to explore associations between *C. burnetii* exposure variables and milk production variables. Cows with *C. burnetii* DNA detected in placental samples produced on average 2.4 L/day less milk volume over the lactation and cows with *C. burnetii* DNA detected by a vaginal swab at

calving produced on average 0.2 kg/d lower total solids over the lactation compared to cows without *C. burnetii* DNA detected in the placenta and vaginal swab, respectively. These findings suggest *C. burnetii* intrauterine infection in cattle may have economic consequences for the dairy industry and future work should be directed towards establishing effective intervention strategies such as vaccination.

4.2 Introduction

Efficient milk production is one of the main drivers of dairy cow herd profitability, with producers being paid for milk primarily based on either volume or total solids (fat and protein yield). Many factors can affect milk volume and solids, including management practices (for example, nutrition and dry period length), and cow genetics and health (Abdelsayed et al., 2014). The mechanisms by which diseases may lead to milk production loss are varied, for example, health disorders such as diarrhea, mastitis, and milk fever can reduce dry matter and energy intake (Bareille et al., 2003), while mastitis causes mammary gland inflammation which may damage milk-producing tissue (Akers and Nickerson, 2011).

Infection with the gram-negative, intracellular bacterium, *Coxiella burnetii,* has been hypothesised to negatively affect milk production in dairy cows (Angen et al., 2011, Freick et al., 2017). However, studies have reached varying conclusions as to whether *C. burnetii* exposure or infection affects milk volume, and fat and protein yields. For example, a German prospective cohort study in one herd found that cows with *C. burnetii* detected by vaginal swabs at calving had significantly lower 305-day fat yield, when compared to

negative cows, but there was no evidence of a marked reduction in milk volume or protein yield (Freick et al., 2017). Furthermore, there was no evidence of marked differences in milk volume, protein or fat yield between cows with and without *C. burnetii* detected in milk at mid lactation (Freick et al., 2017). In contrast, investigation of daily milk volume, and fat and protein concentration by a Danish cross-sectional study of milk samples from 12 dairy herds found higher shedding loads of *C. burnetii* in milk were only associated with higher protein concentration (Angen et al., 2011). Associations between antibody in milk or serum as measures for *C. burnetii* exposure and milk production have also been assessed and protein concentration was higher (Khatun et al., 2022) and fat yield lower (Freick et al., 2017) in antibody-positive cows. No bovine study has identified either a meaningful increase or decrease in milk volume in association with *C. burnetii* exposure or infection (Angen et al., 2011, Freick et al., 2017, Khatun et al., 2022, Paul et al., 2012).

Therefore, while these studies indicate there may be an influence of *C. burnetii* exposure or infection on milk fat and protein composition, the translation of this to a potential economic impact through effects on milk volume or solids remains unclear. Furthermore, there has been limited discussion about potential biological mechanisms behind identified associations, which is probably due to the knowledge gaps in the areas of *C. burnetii* diagnosis, ecology, and pathogenesis in cattle. In addition, estimated effects based on some exposure variables may not reflect the true impact of *C. burnetii* as for example, antibody 'positive' cows can include individuals with active and resolved infections (Guatteo et al., 2012), while *C. burnetii* shedding in milk can be intermittent (Guatteo et al., 2007) and non-differential misclassification of cows' milk shedding status would be expected to result in underestimation of the true strength of association. Exposure variables (also known as

independent or predictor variables) that reflect cell mediated immunity have not been explored and may improve understanding of effects of infection on milk production as CMI is integral to elimination of intracellular pathogens and influences outcomes in human and mice *C. burnetii* infection and disease (Q fever) (Andoh et al., 2007, Pennings et al., 2015). Finally, effects of infection on milk production may vary by geographic region due to spatial differences in virulence of *C. burnetii* strains; Australia has genetically distinct *C. burnetii* strains that may have different virulence to those found in Europe, where most cattle milk production research has been focused (Vincent et al., 2016).

This study investigated associations between *C. burnetii* and milk production in Australian dairy cattle using exposure variables that describe *C. burnetii* shedding, antibody, and CMI response.

4.3 Materials and methods

4.3.1 Study subjects

A prospective cohort study was conducted from 2022-05-12 to 2024-01-25 in the single *C. burnetii* endemically infected commercial dairy cow herd described in Chapter 2 and 3. Lactating cows were fed a total mixed ration diet comprised of corn and alfalfa silage, canola meal, brewers grain, wheat, citrus pulp, and almond hulls. The original sample size was calculated to investigate a different question, namely whether there was a difference in seropositivity at two timepoints of a lactation (Chapter 3). It was estimated that 400 cows would need to be enrolled for the comparison of seroprevalence at two time points (L2 and

L4) with 80% power, a significance level of 0.05, and expected drop-out of 50% of enrolled subjects if the true difference in apparent prevalences is 11% (0.1 and 0.21). The main source of drop-out was expected to be an inability to resample cows that calved outside the required sampling period time (more details below).

4.3.2 Enrolment of subjects

From [12/05/22] to [14/05/22], cows (nulliparous and parous) between 240 and 270 days of pregnancy were eligible for enrolment into the study. Cows that did not calve during the calving sampling period (a period of 11 – 34 days after cow enrolment dates) were excluded from further sampling to determine exposure or infection status but were included in analyses that used exposure or infection status at enrolment. Biological samples were also collected throughout lactation, and cows that were not sampled at a particular sampling time point were excluded from subsequent samplings.

4.3.3 Measurement of exposure or infection status

The sampling time points of this longitudinal study were selected to capture physiologically distinct timepoints in the dairy cow lactation. These points included the dry period (240 – 270 days pregnant; L1), calving (L2), early lactation (17 – 32 days in milk (DIM); L3 and 45 – 60 DIM; L4), and mid lactation (178 – 201 DIM; L5) (Figure 4.1). The *C. burnetii* exposure or infection status of cows was defined by each sample type at each sampling timepoint according to either *C. burnetii* DNA (in placenta, vaginal swabs, faeces, and milk), antibody

(in serum) or cytokine response (from whole blood) (Figure 4.1). Serostatus was considered as a measure of exposure to *C. burnetii* while detection of *C. burnetii* DNA was considered as a measure of infection. Samples were collected and analysed according to previously described methods (Chapter 3). The use of the diagnostic assays used to measure exposure or infection status are summarised in Table 4.1 and briefly described below.

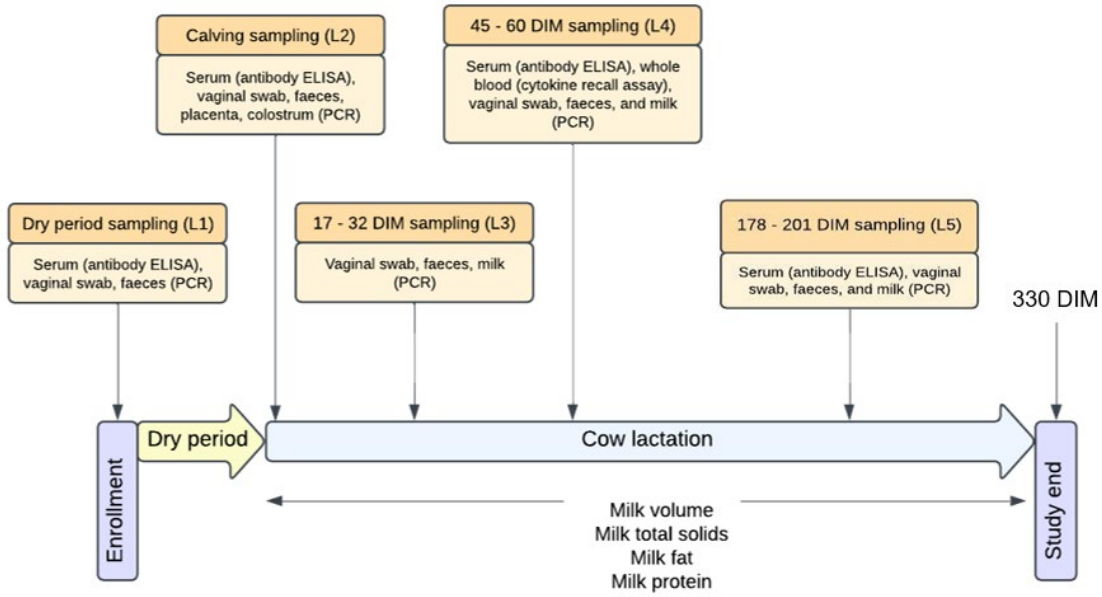

Figure 4.1. Summary of the longitudinal sampling design in an Australian dairy herd, including the types of samples collected at each time point (to determine *C. burnetii* exposure or infection status) and the follow-up period for measuring outcomes (milk volume, total solids, fat, and protein). Exposure or infection statuses were defined according to shedding (PCR), serology (antibody ELISA), and cytokine response (cytokine recall assay for IFNγ and IL-10). Outcomes were milk volume (L), fat (% and solids [kg]), protein (% and solids [kg]), and combined total solids (kg fat and protein) derived from monthly herd-test data.

Table 4.1. Overview of diagnostic assays used to measure *C. burnetii* exposure or infection status in Australian dairy cows enrolled in a longitudinal study.

Assay (specimens)	Description	Coding structure/s
Antibody ELISA (serum)	Measurement of *C. burnetii* specific IgG levels in serum	Categorisation of S/P ratios SP ratios < 30 = "Negative" SP ratios > 29 = "Positive"
Multiplex qPCR (placenta, vaginal swabs, faeces, milk)	Measurement of *C. burnetii* DNA in a range of samples	Cycle thresholds < equivalent 11 copies of IS*1111* (approx. ct: < 34)= "Negative" Cycle thresholds = > equivalent 11 copies of IS*1111* (approx. ct: => 34) but < equivalent of 11 copies of either *com1* (approx. ct: < 36) or *htp*AB (approx. ct: < 35) = "Intermediate" Cycle thresholds > equivalent 11 copies of IS*1111* (approx. ct: >34) and > equivalent of 11 copies of either *com1* (approx. ct: > 36) or *htp*AB (approx. ct: >35) = "Positive"
Cytokine stimulation assay (whole blood)	Measurement of IFNγ or IL-10 production in ng/ml when stimulated with phase 1 (p1) or 2 (p2) *Coxiella burnetii,* minus background well	Treated as a continuous variable
		Categorisation of cytokine measurements (ng/ml) using the rules outlined below: L4 IFNγ p1: 1 (0 – 0.2), 2 (0.2 - 0.4), 3 (above 0.4) L4 IFNγ p2: 1 (0 – 0.2), 2 (0.2 - 0.4), 3 (above 0.4) L4 IL-10 p1: 1 (0 – 0.2), 2 (0.2 - 0.6), 3 (above 0.6) L4 IL-10 p2: 1 (0 – 0.2), 2 (0.2 - 0.6), 3 (above 0.6)

Ct: cycle threshold; L4: longitudinal sampling timepoint 4 (45 – 60 DIM); SP ratio: sample to positive ratio

4.3.3.1 *Coxiella burnetii* molecular detection

DNA was extracted using the Biosprint® 96 One-For-All Vet Kit, according to the manufacturer's protocol and as described in Chapter 3, for purification of viral nuclei acids and bacterial DNA from animal tissue homogenates, serum, plasma, other body fluids, swabs, and washes. To confirm DNA integrity, a qPCR assay targeting the mitochondrial DNA (mtDNA) gene, BCB, was conducted as an endogenous control, as previously described (Chapter 3). Detection and quantification of *C. burnetii* DNA was performed using an

optimised multiplex qPCR assay targeting the two single copy genes: groEL (heat shock operon; *htp*AB) and *com1* (the outer membrane protein-coding gene) and the multicopy insertion sequence gene: IS*1111*, as previously described (Chapter 3). The reactions were performed using a Bio-Rad CFX96 Touch Real-Time PCR detection system. Each PCR run included a no template control and positive control which for the BCB PCR was DNA previously extracted from a bovine sample and for the *C. burnetii* PCR was 1,100, 110 and 11 copies (lower limit of detection) of the *C. burnetii* genome (Amplirun® Vircell, Granada, Spain). For a sample to be considered 'positive' it required 11 or more copies of IS*1111*, and 11 or more copies of either *com1* or *htp*AB. Samples with 11 or more copies of IS*1111* but less than 11 copies of *com1* or *htp*AB (that is, IS*1111* only) were considered 'suspect' and samples with less than 11 copies of IS*1111* were deemed 'negative'. 'Suspect' samples were recorded as 'positive' for the analysis (Table 4.1).

4.3.3.2 *Coxiella burnetii* antibody detection

Antibody detection was performed on serum samples using a commercially available ELISA kit (IDEXX Q Fever Antibody Test Kit) following the manufacturer's instructions and as previously described (Chapter 3). After completion of the ELISA protocol, the optical densities were read using a spectrophotometer at a wavelength of 450nm and analysed using Mars Data Analysis Software. A cut-off of sample to positive ratio (S/P%) < 30% was used for classifying 'negatives', and samples with S/P% ≥ 30% but < 40% were 'suspect'. All suspect samples were then tested again, and their new result recorded. For the analysis, samples with an S/P% > 29 were classed as 'positive' (Table 4.1).

4.3.3.3 Cytokine Recall Assay

To assess the CMI response, the whole blood stimulation assay was performed as previously described (Chapter 3) to detect the IFNγ and IL-10 response. Briefly, within 16-18 hours of collection, 300 µl of whole blood in 48 well plates was co-incubated with 300 µl of culture media containing *C. burnetii*, antigen, positive control mitogen (pokeweed mitogen) or negative control (culture media only) for 48 hours at 37°C and 5% CO2. Next, 300 µl of plasma supernatant was transferred from each well and stored at -20 °C until use in the IFNγ and IL-10 sandwich ELISA. After completion of the ELISA protocol, optical densities were read using a spectrophotometer at 450nM. The OD values were converted to ng/ml based on the standard curve. Cytokine data was analysed as a continuous and categorical variable to increase flexibility in assessing the relationship given there was not prior knowledge on the type of expected effect (Table 4.1). Categories were formed based on visual data analysis and antibody levels (Table 4.1).

4.3.4 Coding of outcome status

Monthly herd test data, subject demographic information and events were extracted from the herd management software (Dairy Comp 305, Valley Ag Software, Tulare, CA). The outcomes evaluated were routine monthly herd test day (i.e. 24-hour estimated) milk volume (L), fat (% and yield [kg]), protein (% and yield [kg]), and combined total solids (kg fat and protein) during the lactation following enrolment. Monthly herd test day data were

truncated at 330 DIM to minimise the influence of variable lactation lengths amongst subjects on results. The distribution of test day data was also assessed to check for outliers, with any implausible values excluded based on expert knowledge.

4.3.5 Statistical analysis

The objective of statistical analysis was to explore the data to identify evidence that *C. burnetii* exposure or infection (measured using various biological specimens and assays) may impact any of the milk production variables over a single lactation. Analysis was conducted in R programming environment (R Core Team, 2018) and Stata version 18. Loess smooth functions were used to depict the unadjusted relationships between *C. burnetii* exposure variables and milk production outcomes from 1 to 330 DIM. To explore associations between *C. burnetii* exposure variables and milk production variables, generalised estimating equations, with gaussian residual distribution and identity link (i.e., linear models) were used. Clustering was specified at the subject- (i.e. lactation-) level with an autoregressive covariance structure to account for correlations in residuals between time-ordered herd tests within lactations. Continuous exposures variables were evaluated for linear and quadratic associations with the outcome variable, with only the linear relationship reported if the quadratic relationship was not statistically significant ($p < 0.05$). Parity was included as a categorical covariate (lactation number = 1, 2, 3, ≥4) to minimise potential confounding by this variable. Days in milk was also included in models, which was parameterised using fractional polynomials (selected using the -fp- command in Stata). Other covariates were not included for control of confounding based on the judgement of

collaborating subject matter experts and epidemiologists. Exposure variables were treated as time-fixed, even though exposure variables were measured at various time points (due to the broad study scope and exploratory nature of the investigation).

4.4 Results

4.4.1 Descriptive results

There were 396 animals enrolled at late gestation (L1), 187 of which only had samples for L1 exposure variables due to calving outside the pre-specified window (n = 171) or for whom a placental sample was not able to be collected at calving (n = 16) and 17 of which were subsequently excluded from the study due to labelling errors. In total, 192 cows or 48% had a calving sample collected, which was an expected proportion lost to follow-up given the study design. At the subsequent time points, seven cows were removed due to loss to follow up between calving and L3 (17-32 DIM) (leaving 185 cows), a further 16 were lost to follow up between L3 and L4 (45 – 60 DIM) (leaving 169 cows), and a further 36 were lost to follow up between L4 and L5 (178 – 201 DIM) (leaving 133 cows). A detailed assessment of the *C. burnetii* antibody, shedding, and cytokine responses (that are used as exposure variables in the present Chapter) from cows at each timepoint was provided in Chapter 3. Due to low prevalence (<5%), some sample types were excluded from analysis as exposure variables; these were faecal results at all timepoints, vaginal results outside of calving, and milk results at early lactation.

4.4.2 Associations between *Coxiella burnetii* exposure variables and milk volume and total solids

The findings from models investigating the association between *C. burnetii* exposure variables, measured by *C. burnetii* shedding (as determined by PCR) and antibody status, and the outcomes, milk volume and total solids, are presented in Table 4.2. Cows with *C. burnetii* DNA detected in their placenta (100/192) had lower test-day milk yield than cows without DNA detected in the placenta, producing 2.38 L per day less milk (95% CI: -4.39 L/cow/day to -0.37 L/cow/day; p = 0.02), but had similar total solids (-0.03 kg/cow/d [95% CI: -0.18 kg/cow/day - 0.12 kg/cow/day]; p = 0.69 Table 4.2; Figure 4.2). Cows with *C. burnetii* DNA detected in vaginal swabs at calving (20/191) had lower total solids (-0.21 kg/cow/d [95% CI: -0.41 kg/cow/day to -0.01 kg/cow/day]; p = 0.039 Table 4.2; Figure 4.3). Conversely, there was some evidence that cows with *C. burnetii* DNA detected in colostrum at calving (30/191) or milk at mid lactation (13/133) had higher milk volume and total solids than cows where *C. burnetii* DNA was not detected, however, the estimated effects were imprecise (and not statistically significant), as evidenced by wide confidence intervals (Table 4.2). For example, at mid lactation, cows with *C. burnetii* DNA detected in milk were estimated to produce 2.54 L/day more but the 95% confidence interval was wide, ranging from -1.07 L/cow/day to 6.15 L/cow/day (p = 0.168; Table 4.2; Figure 4.4). Similarly, there was some limited evidence that cows that were seropositive for *C. burnetii* had higher milk volume and total solids relative to seronegative cows but estimated effects were imprecise (and not statistically significant) (Table 4.2). When assessing CMI by cytokine response to *C. burnetii* antigen stimulation, the effect estimates were also imprecise (and not statistically

signficant) (Table 4.3). Visual assessment of loess smoother plots for the relationship between CMI and milk production did not identify evidence of an association (Figure 4.5).

Table 4.2. Associations between *Coxiella burnetii* seropositivity and shedding (exposure variables) and test-day milk volume and solids production in Australian dairy cows enrolled in a longitudinal study. Serostatus was determined by using serum samples in an IgG ELISA while shedding was determined by testing placental, vaginal swab, colostrum, and milk samples in a multiplex qPCR. L1- three weeks prior to calving (240 – 270 days pregnant; dry-period); L2- calving; L4- 45 – 60 days in milk (DIM); L5- 178 – 201 DIM. Number of cows sampled at each timepoint: L1 = 396; L2 = 192; L3 = 186; L4 = 169; L5 = 133.

	Milk volume (L/cow/day)		**Milk solids (kg/cow/day)**	
Exposure variable	**Estimate [a] (95% CI)**	**P**	**Estimate (95% CI)**	**P**
Seropositive vs negative				
Pre-calving (L1)	1.26 (-0.69 to 3.21)	0.204	0.07 (-0.07 to 0.21)	0.305
Calving (L2)	0.76 (-1.83 to 3.35)	0.566	0.11 (-0.08 to 0.29)	0.26
Early lactation (L4)	1.59 (-0.75 to 3.93)	0.183	0.07 (-0.09 to 0.23)	0.386
Mid lactation (L5)	1.65 (-1.07 to 4.36)	0.234	0.16 (-0.03 to 0.34)	0.099
Shedding (PCR positive vs negative)				
Placenta at calving (L2)	-2.38 (-4.39 to -0.37)	0.020	-0.03 (-0.18 to 0.12)	0.69
Colostrum at calving (L2)	1.96 (-0.87 to 4.78)	0.174	0.04 (-0.17 to 0.25)	0.724
Vaginal swab at calving (L2)	-1.75 (-4.9 to 1.41)	0.277	-0.21 (-0.41 to -0.01)	0.039
Milk in mid lactation (L5)	2.54 (-1.07 to 6.15)	0.168	0.23 (-0.07 to 0.53)	0.133

[a] Estimates were adjusted for clustering of measurements within subjects and hypothesised confounders (parity and days in milk at herd test) in a multivariable generalised estimating equations model (autoregressive covariance structure).

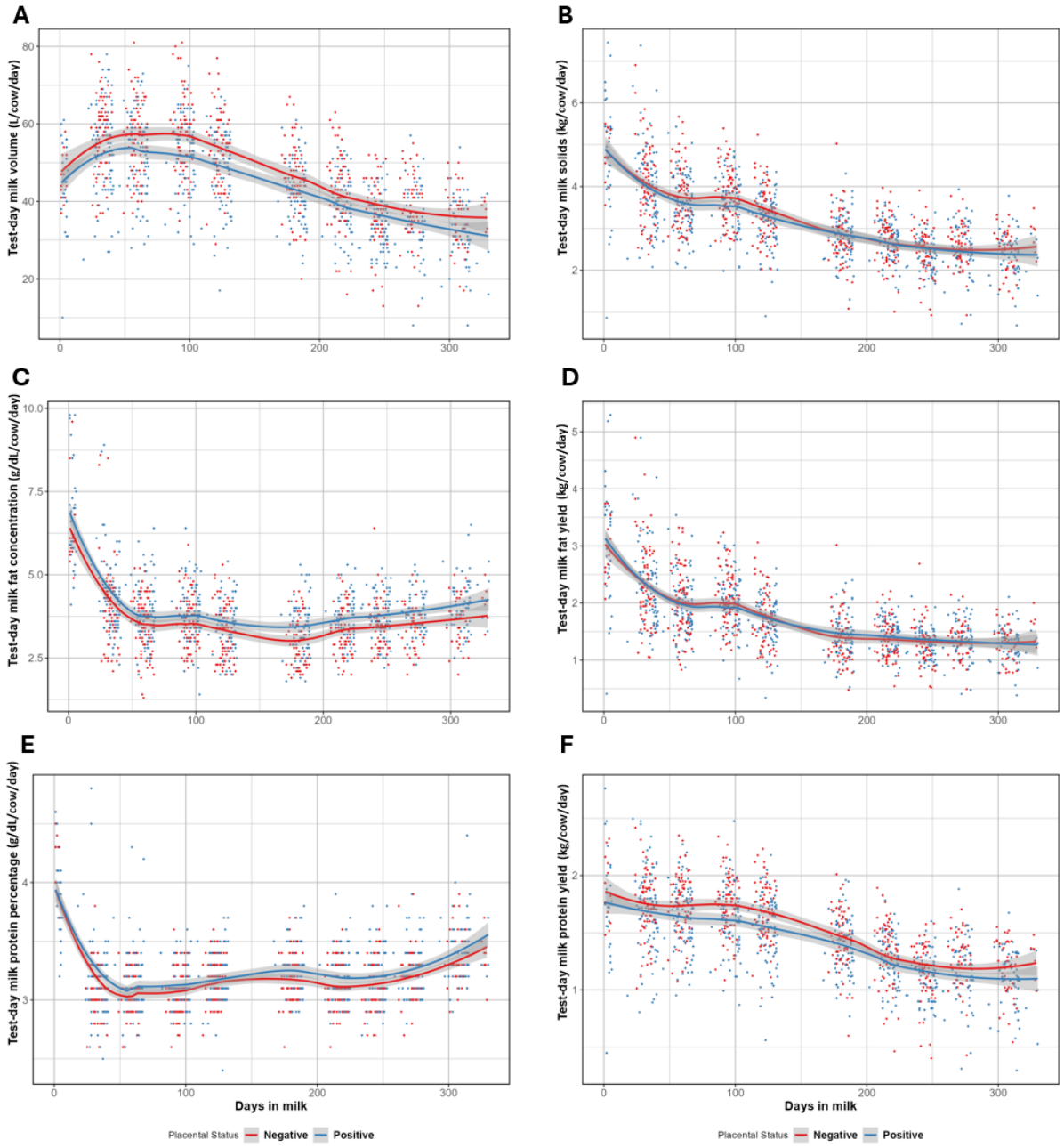

Figure 4.2. Crude relationships summarised as loess curves of test-day milk volume (L/cow/d), fat (% [g/dL/cow/d] and solids [kg/cow/d]), protein (% [g/dL/cow/d] and solids[kg/cow/d]), and combined total solids (kg fat and protein combined/cow/d) from 192 Australian dairy cows enrolled in a longitudinal study, grouped according to their *C. burnetii* placental shedding status. Each scatter-point indicates one test-day result for one cow. Standard errors shown as shaded areas around curves. Test-day data were truncated at 305 days in milk (DIM). Shedding was determined in placental samples by a multiplex qPCR. Blue = exposed; red = not exposed. **A** test-day milk volume (L). **B** combined total solids (kg). **C** fat concentration (%). **D** fat yield (kg). **E** protein concentration (%). **F** protein yield (kg)

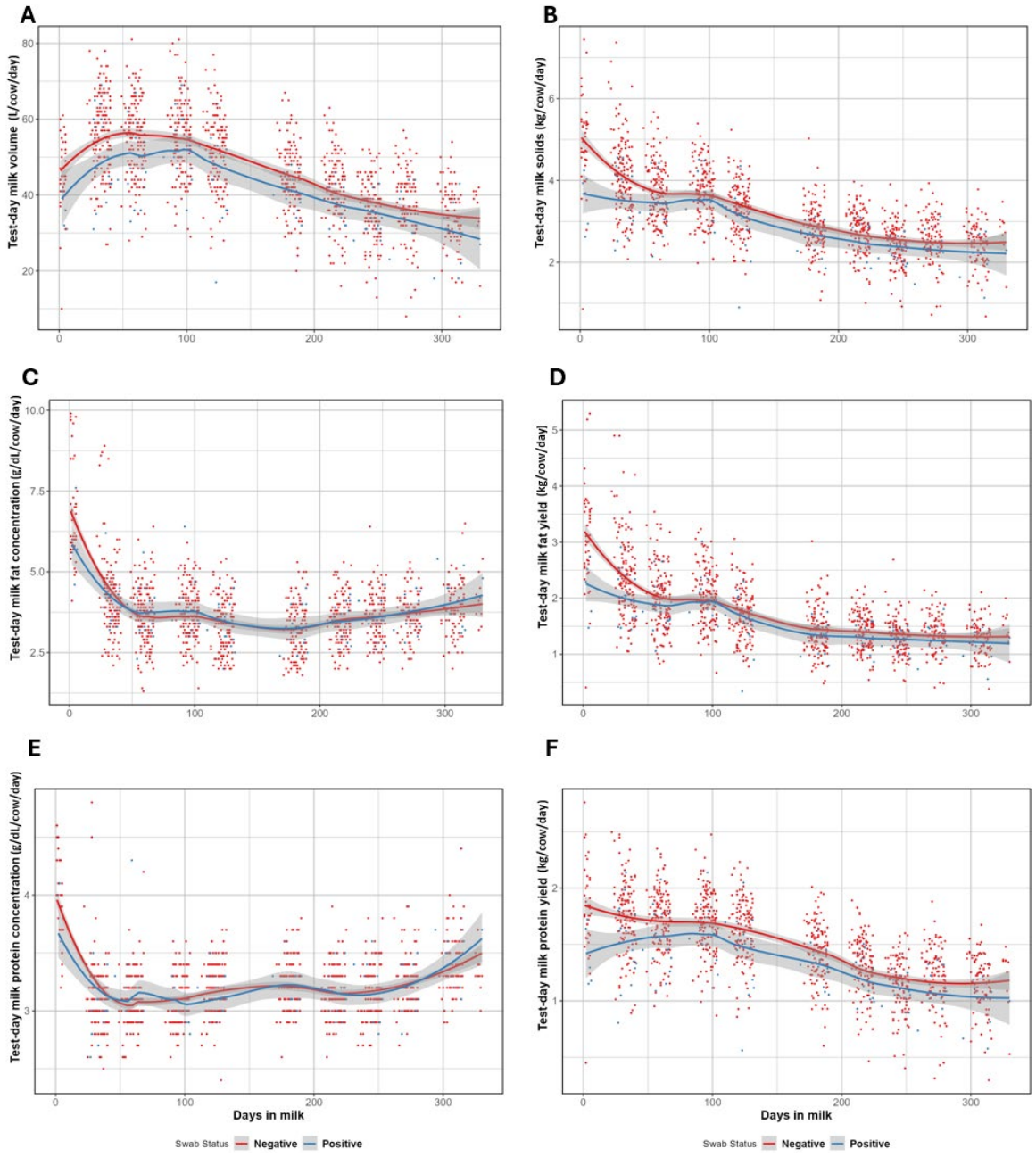

Figure 4.3. Crude relationships summarised as loess curves of test-day milk volume (L/cow/d), fat (% [g/dL/cow/d] and solids [kg/cow/d]), protein (% [g/dL/cow/d] and solids[kg/cow/d]), and combined total solids (kg fat and protein combined/cow/d) from 192 Australian dairy cows enrolled in a longitudinal study, grouped according to their *C. burnetii* vaginal swab status at calving. Each scatter-point indicates one test-day result for one cow. Standard errors shown as shaded areas around curves. Test-day data were truncated at 330 days in milk (DIM). *C. burnetii* DNA was determined in vaginal swab samples by a multiplex qPCR. Blue = exposed; red = not exposed. **A** test-day milk volume (L). **B** combined total solids (kg). **C** fat concentration (%). **D** fat yield (kg). **E** protein concentration (%). **F** protein yield (kg)

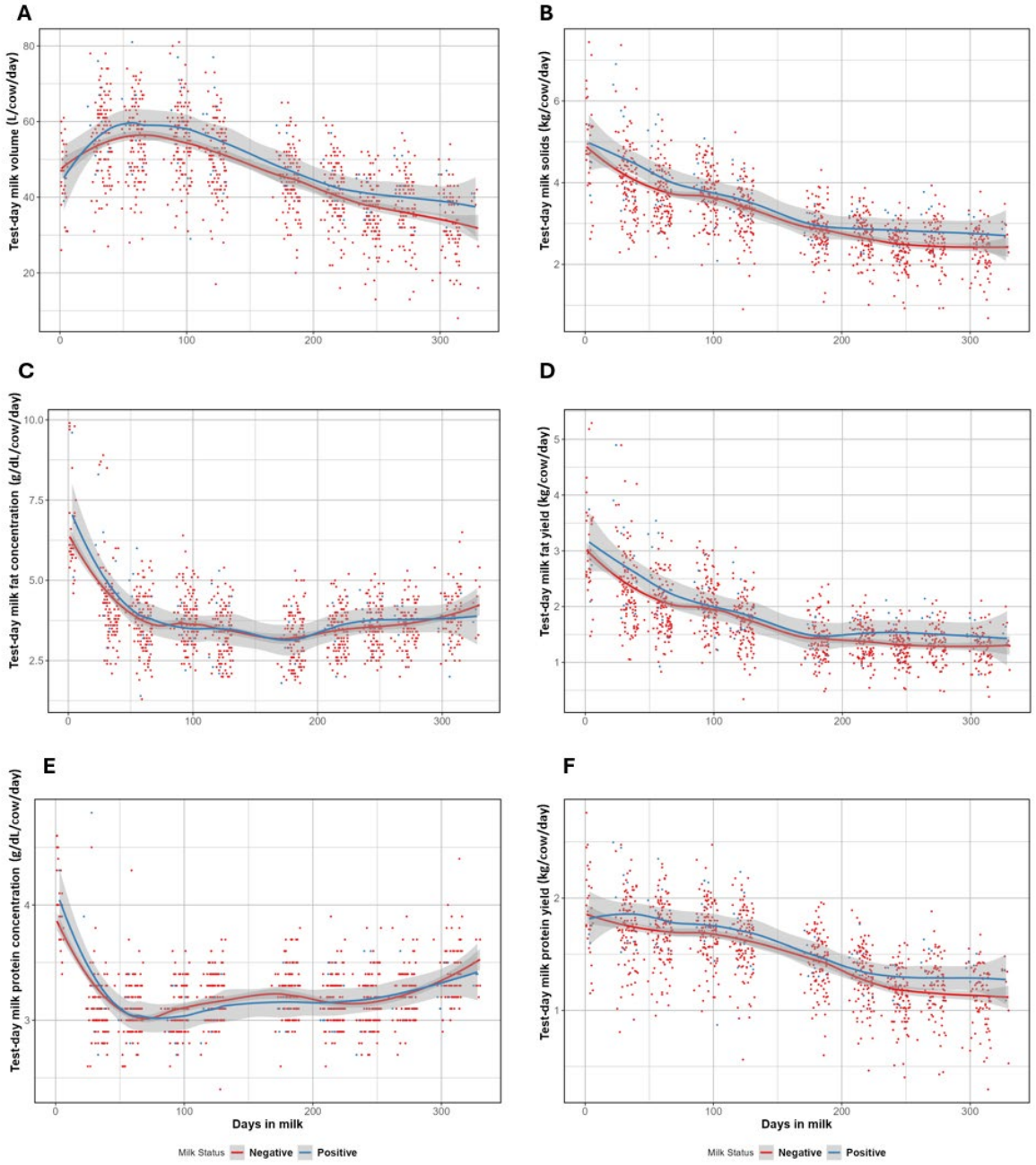

Figure 4.4. Crude relationships summarised as loess curves of test-day milk volume (L/cow/d), fat (% [g/dL/cow/d] and solids [kg/cow/d]), protein (% [g/dL/cow/d] and solids[kg/cow/d]), and combined total solids (kg fat and protein combined/cow/d) from 192 Australian dairy cows enrolled in a longitudinal study, grouped according to their *C. burnetii* milk shedding status at mid lactation. Each scatter-point indicates one test-day result for one cow. Standard errors shown as shaded areas around curves. Test-day data were truncated at 330 days in milk (DIM). Shedding was determined in milk samples by a multiplex qPCR. Blue = exposed; red = not exposed. **A** test-day milk volume (L). **B** combined total solids (kg). **C** fat concentration (%). **D** fat yield (kg). **E** protein concentration (%). **F** protein yield (kg).

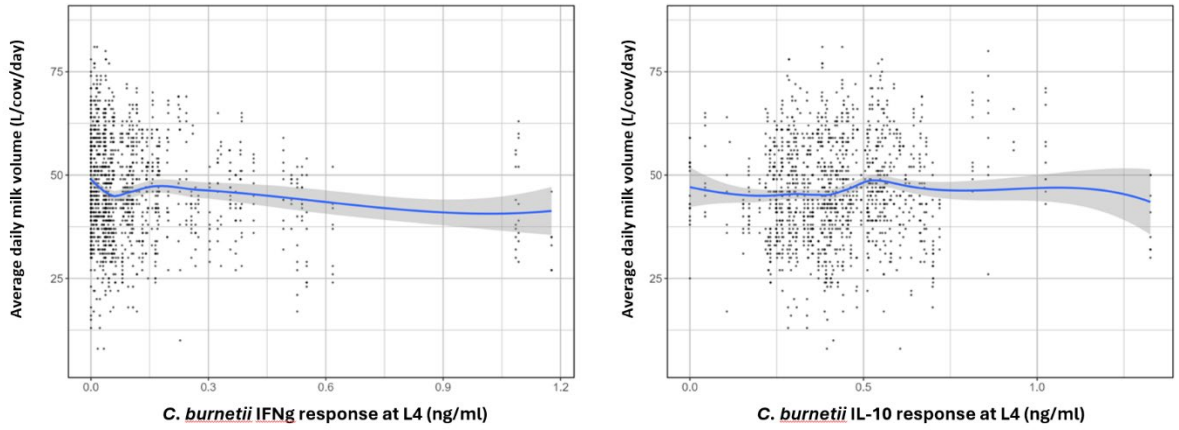

Figure 4.5. Loess curves demonstrating the relationship between cytokine response at early lactation and average daily milk volume (L/cow/day) in Australian dairy cattle (n = 169). Standard errors shown as shaded areas around curves. **A** IFNγ. **B** IL-10. IFNγ and IL-10 were measured by an ELISA from whole blood stimulated *ex vivo* with *C. burnetii* antigen.

Table 4.3. Associations between IFNγ and IL-10 responses to *Coxiella burnetii* stimulation (exposure variables) measured at early lactation (L4; 45 – 60 days in milk) and test-day milk volume and solids production in 169 Australian dairy cows enrolled in a longitudinal study. IFNγ and IL-10 were measured by an ELISA from whole blood stimulated *ex vivo* with *C. burnetii* antigen.

		Milk volume (L/cow/day)		**Milk solids (kg/cow/day)**	
Exposure variable[a]	**Level**	**Estimate[b] (95% CI)**	**P**	**Estimate (95% CI)**	**P**
IFNγ to phase 1 at early lactation (continuous[a])		-3.35 (-7.89 to 1.19)	0.148	-0.08 (-0.49 to 0.32)	0.682
IFNγ to phase 1 at early lactation (categorical)	1	Reference category		Reference category	
	2	-1.49 (-4.82 to 1.85)	0.382	-0.09 (-0.37 to 0.19)	0.525
	3	-2.19 (-5.73 to 1.35)	0.226	-0.04 (-0.3 to 0.22)	0.751
IFNγ to phase 2 at early lactation (continuous)		-2.26 (-6.78 to 2.25)	0.326	0.03 (-0.32 to 0.37)	0.884
IFNγ to phase 2 at early lactation (categorical)	1	Reference category		Reference category	
	2	-1.96 (-5.31 to 1.38)	0.25	0.02 (-0.27 to 0.31)	0.902
	3	-2.68 (-5.92 to 0.56)	0.104	-0.15 (-0.35 to 0.06)	0.165
IL-10 to phase 1 at early lactation (continuous)		2.82 (-2.68 to 8.32)	0.315	0.2 (-0.2 to 0.6)	0.321
IL-10 to phase 1 at early lactation (categorical)	1	Reference category		Reference category	
	2	-0.01 (-2.51 to 2.5)	0.996	-0.22 (-0.57 to 0.13)	0.223
	3	-1.03 (-5 to 2.94)	0.61	-0.21 (-0.6 to 0.18)	0.284
IL-10 to phase 2 at early lactation (continuous)		2.28 (-5.06 to 9.62)	0.543	0.06 (-0.49 to 0.61)	0.832
IL-10 to phase 2 at early lactation (categorical)	1	Reference category		Reference category	
	2	-0.78 (-3.33 to 1.76)	0.547	-0.16 (-0.41 to 0.1)	0.221
	3	-1.63 (-6.49 to 3.23)	0.51	-0.16 (-0.49 to 0.18)	0.357

[a] Continous IFNγ and IL-10 exposure variables were measured in units of ng/ml.
[b] Estimates were adjusted for clustering of measurements within subjects and hypothesised confounders (parity and days in milk at herd test) in a multivariable generalised estimating equations model (autoregressive covariance structure).

4.4.3 Association between *Coxiella burnetii* exposure variables and milk fat concentration and fat yield

Cows with *C. burnetii* DNA detected in their placenta (100/192) had significantly higher test-day fat concentration than cows without detected DNA, producing milk that was estimated to have, on average, 0.29 g/dL higher daily milk fat concentration (95% CI: 0.1 g/dL/cow/day to 0.48 g/dL/cow/day; p = 0.003) (Table 4.4; Figure 4.2). However, there was a similar overall total daily fat yield (kg) (0.03 kg/cow/day [95% CI: -0.07 kg/cow/day - 0.13 kg/cow/day]; p = 0.54) (Table 4.4). On the other hand, *C. burnetii* DNA detected in a vaginal swab (20/191) was associated with an estimated lower daily fat yield of 0.12 kg/cow/day less (though this was not statistically significant) (95% CI: -0.25 kg/cow/day to 0.01 kg/cow/day; p = 0.066). No associations were evident between *C. burnetii* antibody and cytokine exposure variables and either fat concentration (%) or yield (kg) (Table 4.4; Table 4.5).

Table 4.4. Associations between *Coxiella burnetii* seropositivity and shedding (exposure variables) and test-day milk fat percentage (g/dL) and total yield (kg) in Australian dairy cows enrolled in a longitudinal study. Serostatus was determined by using serum samples in an IgG ELISA while shedding was determined by testing placental, vaginal swab, colostrum, and milk samples in a multiplex qPCR. L1- three weeks prior to calving (240 – 270 days pregnant; dry-period); L2- calving; L4- 45 – 60 days in milk (DIM); L5- 178 – 201 DIM. Number of cows sampled at each timepoint: L1 = 396; L2 = 192; L3 = 186; L4 = 169; L5 = 133.

	Milk fat percentage (g/dL/cow/day)		**Milk fat yield (kg/cow/day)**	
Exposure variables	**Estimate[a] (95% CI)**	**P**	**Estimate (95% CI)**	**P**
Seropositive vs negative				
Pre-calving (L1)	-0.03 (-0.19 to 0.13)	0.705	0.04 (-0.05 to 0.13)	0.394
Calving (L2)	0.1 (-0.15 to 0.35)	0.428	0.09 (-0.04 to 0.21)	0.191
Early lactation (L4)	-0.07 (-0.28 to 0.15)	0.543	0.04 (-0.07 to 0.15)	0.447
Mid lactation (L5)	0.08 (-0.16 to 0.32)	0.528	0.11 (-0.01 to 0.24)	0.068
Shedding (PCR positive vs negative)				
Placenta at calving (L2)	0.29 (0.1 to 0.48)	0.003	0.03 (-0.07 to 0.13)	0.54
Colostrum at calving (L2)	-0.17 (-0.48 to 0.15)	0.295	0 (-0.15 to 0.15)	0.994
Vaginal swab at calving (L2)	-0.11 (-0.39 to 0.16)	0.429	-0.12 (-0.25 to 0.01)	0.066
Milk in mid lactation (L5)	0.07 (-0.36 to 0.5)	0.756	0.14 (-0.07 to 0.36)	0.198

[a] Estimates were adjusted for clustering of measurements within subjects and hypothesised confounders (parity and days in milk at herd test) in a multivariable generalised estimating equations model (autoregressive covariance structure).

Table 4.5. Associations between cellular IFNγ and IL-10 responses to *Coxiella burnetii* stimulation (exposure variables) measured at early lactation (L4; 45 – 60 days in milk) and test-day milk fat percentage (g/dL) and total yield (kg) in 169 Australian dairy cows enrolled in a longitudinal study. IFNγ and IL-10 were measured by an ELISA from whole blood stimulated *ex vivo* with *C. burnetii* antigen.

		Milk fat percentage (g/dL/cow/day)		**Milk fat yield (kg/cow/day)**	
Exposure variables[a]	**Level**	**Estimate[b] (95% CI)**	**P**	**Estimate (95% CI)**	**P**
IFNγ to phase 1 at early lactation (continuous)		0.26 (-0.18 to 0.7)	0.242	-0.04 (-0.32 to 0.24)	0.778
IFNγ to phase 1 at early lactation (categorical)	1	Reference category		Reference category	
	2	0.03 (-0.31 to 0.37)	0.879	-0.04 (-0.23 to 0.15)	0.686
	3	0.23 (-0.08 to 0.55)	0.148	-0.01 (-0.18 to 0.16)	0.91
IFNγ to phase 2 at early lactation (continuous)		0.33 (-0.13 to 0.8)	0.163	0.07 (-0.17 to 0.3)	0.57
IFNγ to phase 2 at early lactation (categorical)	1	Reference category		Reference category	
	2	0.26 (-0.05 to 0.58)	0.105	0.06 (-0.14 to 0.26)	0.569
	3	0.08 (-0.3 to 0.46)	0.67	-0.07 (-0.22 to 0.07)	0.309
IL-10 to phase 1 at early lactation (continuous)		0.18 (-0.41 to 0.78)	0.546	0.16 (-0.12 to 0.44)	0.262
IL-10 to phase 1 at early lactation (categorical)	1	Reference category		Reference category	
	2	-0.28 (-0.75 to 0.19)	0.248	-0.15 (-0.4 to 0.11)	0.256
	3	-0.07 (-0.6 to 0.45)	0.784	-0.11 (-0.38 to 0.16)	0.419
IL-10 to phase 2 at early lactation (continuous)		-0.07 (-0.81 to 0.67)	0.853	0.03 (-0.35 to 0.41)	0.869
IL-10 to phase 2 at early lactation (categorical)	1	Reference category		Reference category	
	2	-0.16 (-0.48 to 0.17)	0.339	-0.11 (-0.29 to 0.08)	0.26
	3	-0.01 (-0.48 to 0.45)	0.954	-0.08 (-0.31 to 0.15)	0.488

[a] Continuous IFNγ and IL-10 exposure variables were measured in units of ng/ml.
[b] Estimates were adjusted for clustering of measurements within subjects and hypothesised confounders (parity and days in milk at herd test) in a multivariable generalised estimating equations model (autoregressive covariance structure).

4.4.4 Association between *Coxiella burnetii* exposure variables and milk protein concentration and protein yield

Cows with *C. burnetii* DNA detected in their vaginal swab at calving (20/191) were estimated to have a 0.08 kg lower protein yield per day (95% CI: -0.16 kg/cow/day to 0.01 kg/cow/day; p = 0.068) than cows without DNA detected in vaginal swabs (though this was not statistically significant) (Table 4.6). Conversely, cows with *C. burnetii* DNA detected in milk at mid lactation (13/133), were estimated to produce 0.1 kg more protein per day (95% CI: -0.01 kg/cow/day to 0.2 kg/cow/day; p = 0.077) compared to cows without detected DNA (this was also not statistically significant) (Table 4.6). The estimates for the effect of *C. burnetii* antibody exposure variables on protein production were imprecise (and not statistically significant) and no trends were apparent (Table 4.6). There were no significant associations evident between cytokine response to stimulation with *C. burnetii* antigen and protein yield and concentration (Table 4.7).

Table 4.6. Associations between *Coxiella burnetii* seropositivity and shedding (exposure variables) and test day milk protein percentage (g/dL) and yield (kg) in Australian dairy cows enrolled in a longitudinal study. Serostatus was determined by using serum samples in an IgG ELISA while shedding was determined by testing placental, vaginal swab, colostrum, and milk samples in a multiplex qPCR. L1- three weeks prior to calving (240 – 270 days pregnant; dry-period); L2- calving; L4- 45 – 60 days in milk (DIM); L5- 178 – 201 DIM. Number of cows sampled at each timepoint: L1 = 396; L2 = 192; L3 = 186; L4 = 169; L5 = 133.

Exposure variables	**Milk protein percentage (g/dL/cow/day)**		**Milk protein yield (kg/cow/day)**	
	Estimate[a] (95% CI)	**P**	**Estimate (95% CI)**	**P**
Seropositive vs negative				
Pre-calving (L1)	0.01 (-0.04 to 0.05)	0.815	0.04 (-0.02 to 0.09)	0.185
Calving (L2)	0.01 (-0.06 to 0.08)	0.866	0.03 (-0.05 to 0.1)	0.485
Early lactation (L4)	-0.03 (-0.08 to 0.02)	0.249	0.03 (-0.03 to 0.1)	0.32
Mid lactation (L5)	-0.01 (-0.08 to 0.06)	0.77	0.05 (-0.03 to 0.13)	0.247
Shedding (PCR positive vs negative)				
Placenta at calving (L2)	0.04 (-0.01 to 0.1)	0.125	-0.05 (-0.11 to 0.00)	0.072
Colostrum at calving (L2)	-0.04 (-0.12 to 0.04)	0.364	0.04 (-0.04 to 0.12)	0.328
Vaginal swab at calving (L2)	-0.04 (-0.13 to 0.04)	0.347	-0.08 (-0.16 to 0.01)	0.068
Milk in mid lactation (L5)	0.03 (-0.1 to 0.15)	0.654	0.1 (-0.01 to 0.2)	0.077

[a] Estimates were adjusted for clustering of measurements within subjects and hypothesised confounders (parity and days in milk at herd test) in a multivariable generalised estimating equations model (autoregressive covariance structure).

Table 4.7. Associations between cellular IFNγ and IL-10 responses to *Coxiella burnetii* stimulation (exposure variables) measured at early lactation (L4; 45 – 60 days in milk) and test-day milk protein percentage (g/dL) and yield (kg) in 169 Australian dairy cows enrolled in a longitudinal study. IFNγ and IL-10 were measured by an ELISA from whole blood stimulated *ex vivo* with *C. burnetii* antigen.

		Milk protein percentage (g/dL/cow/day)		**Milk protein yield (kg/cow/day)**	
Exposure variables [a]	**Level**	**Estimate**[b] **(95% CI)**	**P**	**Estimate (95% CI)**	**P**
IFNγ to phase 1 at early lactation (continuous[a])		0.16 (0.06 to 0.26)	0.002	-0.04 (-0.19 to 0.1)	0.547
IFNγ to phase 1 at early lactation (categorical)	1	Reference category		Reference category	
	2	-0.01 (-0.08 to 0.07)	0.876	-0.05 (-0.16 to 0.06)	0.368
	3	0.11 (0.02 to 0.19)	0.018	-0.03 (-0.14 to 0.08)	0.613
IFNγ to phase 2 at early lactation (continuous)		0.09 (-0.04 to 0.22)	0.177	-0.03 (-0.18 to 0.11)	0.633
IFNγ to phase 2 at early lactation (categorical)	1	Reference category		Reference category	
	2	0.04 (-0.04 to 0.12)	0.316	-0.04 (-0.14 to 0.07)	0.475
	3	0.05 (-0.06 to 0.16)	0.345	-0.07 (-0.17 to 0.04)	0.209
IL-10 to phase 1 at early lactation (continuous)		-0.08 (-0.25 to 0.1)	0.397	0.03 (-0.13 to 0.2)	0.707
IL-10 to phase 1 at early lactation (categorical)	1	Reference category		Reference category	
	2	-0.17 (-0.34 to -0.01)	0.043	-0.08 (-0.19 to 0.04)	0.193
	3	-0.15 (-0.33 to 0.04)	0.115	-0.11 (-0.25 to 0.04)	0.144
IL-10 to phase 2 at early lactation (continuous)		-0.08 (-0.31 to 0.15)	0.508	0.02 (-0.2 to 0.24)	0.852
IL-10 to phase 2 at early lactation (categorical)	1	Reference category		Reference category	
	2	-0.06 (-0.16 to 0.05)	0.278	-0.05 (-0.14 to 0.04)	0.246
	3	-0.04 (-0.2 to 0.12)	0.6	-0.08 (-0.22 to 0.05)	0.225

[a] Continuous IFNγ and IL-10 exposure variables were measured in units of ng/ml.
[b] Estimates were adjusted for clustering of measurements within subjects and hypothesised confounders (parity and days in milk at herd test) in a multivariable generalised estimating equations model (autoregressive covariance structure).

4.5 Discussion and conclusions

Milk production loss in dairy cow herds carries a financial cost for producers and a welfare cost for cows (via culling). Infectious diseases can lead to production losses, however, the impact of infection with *C. burnetii* in cattle is not well understood. This exploratory study investigated associations between *C. burnetii* exposure variables (*C. burnetii* shedding, *C. burnetii* antibody, and cell mediated immune responses) and test day (i.e. 24-hour) milk production variables (volume, total solids, and fat and protein concentrations and yields). *Coxiella burnetii* detection in the placenta and vaginal fluid (by PCR) were associated with lower average daily milk volume and total solids, respectively.

4.5.1 Coxiella burnetii infection was associated with production loss (volume and total solids)

Milk volume and total solids are important for dairy producers, because they are the main drivers of milk income. This study found that cows shedding *C. burnetii* in their placenta at calving produce less milk per day on average (estimated as 2.4 L/cow/d less), while cows with *C. burnetii* DNA detected in their vaginal fluid at calving produce less milk solids per day (estimated as 0.2 kg/cow/d less) (Table 4.2). Both vaginal swabs and placental samples were speculated to be indicative of infection of the products of conception, with the lochia cleared via the vagina following calving (rather than colonisation of the vaginal mucosa per se), with the slight differences in estimated effects on volume and total solids between these exposure variables possibly due to sampling variation and consequent imprecision of estimates. For example, the lower prevalence of PCR positive vaginal swabs (11%) compared

to placental samples (52%) (possibly due to differences in sampling and laboratory methodology lowering sensitivity of vaginal swabs to detect infection of the products of conception compared to placental tissue) presumably reduced the precision of the estimated association between vaginal swab status and volume. Alternatively, the slight agreement (kappa = 0.15, Chapter 3) between these two exposure variables could be because these variables identify cows with different infection states. For example, a vaginal swab may only detect *C. burnetii* when it is present in very high loads whereas a placental sample may be more sensitive for detecting smaller bacterial loads. Nevertheless, reductions in both volume and total solids from cows with *C. burnetii* DNA detected in placental tissue and vaginal swabs at calving, indicate coxiellosis in dairy cattle may have an economic cost for producers, which could be considerable in herds such as the study herd given the ~50% prevalence of placental infection in cows in this study. A critical review of global studies reporting *C. burnetii* prevalence in cattle found the median animal level prevalence from 36 studies was 19.4% (interquartile range: 6.6% to 39.3%) and the median herd level prevalence from 27 studies was 37.7% (interquartile range: 19.3% to 69.7%), however, they did note there were frequent limitations with study designs (Guatteo et al., 2011). A similar loss in production has also been found in a study in four goat herds in Victoria, Australia, that estimated high *C. burnetii* shedding does at kidding (detected by vaginal swabs) produced 0.53 L/day less milk yield than non-shedding does (after adjusting for the effect of farm, DIM, parity, and kidding season) (Canevari et al., 2018). While total solids have not been previously measured in ruminant studies, an association with reduced fat yield was reported from cows with *C. burnetii* DNA detected by PCR of vaginal swabs (Freick et al., 2017). In the present study, a breakdown of the total solids from cows with *C. burnetii* detected in their vaginal fluid identified a reduction in both daily fat (-0.12

kg/cow/d; 95% CI: -0.25 to 0.01 kg/cow/d; p = 0.066) and protein (-0.08 kg/cow/d; 95% CI: -0.16 to 0.01 kg/cow/d; p = 0.068) production (Table 4.4 and Table 4.6).

4.5.2 No associations between milk production and either *Coxiella burnetii* milk shedding or host immune response exposure variables

There was no compelling evidence for associations between exposure variables, *C. burnetii* shedding in colostrum, *C. burnetii* shedding in milk, *C. burnetii* antibody, and *C. burnetii* specific IFNγ and IL-10 response, and either milk volume or total solids (Table 4.2). In contrast to the production loss associated with presence of *C. burnetii* in the placenta and reproductive tract at calving, *C. burnetii* shedding in colostrum at calving and milk at mid-lactation were not strongly associated with milk volume or total solids. A previous observational study of cattle in Germany also reported no marked differences in milk volume or fat and protein yield in cows with *C. burnetii* DNA detected in milk at 100 and/or 150 DIM (Freick et al., 2017). However, there is also evidence in the literature that intramammary inoculation with *C. burnetii* leads to lower milk volume (Ormsbee, 1951). Experimental infection of four first lactation cows (and two controls) by injection of *C. burnetii* infected yolk-sac culture into the left front and rear quarters via the lacteal duct led to a fall in milk volume from infected quarters (compared to uninfected control quarters) that began to return to normal levels by four days after inoculation but which was reported to not have reached pre-inoculation levels (Ormsbee, 1951). The author noted control quarters that were injected with non-*C. burnetii* infected yolk-sac culture also had reduced milk volume following inoculation, likely due to a reaction to the foreign protein, but did still conclude this volume reduction was of lower magnitude and increased transience compared

to the treatment quarters (Ormsbee, 1951). The relevance of this result to natural infection is unclear, with the difference in findings between this experimental infection study and the present observational study possibly a reflection of variation in *C. burnetii* dose, route of infection, host factors (for example, genetics), or *C. burnetii* strain. Furthermore, while shedding of bacterial species in milk is usually indicative of a mammary gland infection, it is possible that as an intracellular pathogen *C. burnetii* enters the milk from naturally infected cows within immune cells through the blood-milk barrier. Histopathological investigation of mammary gland tissue from cows shedding *C. burnetii* in milk (detected by PCR) would be required to confirm this.

There was no evidence of large reductions in milk volume or total solids in *C. burnetii* seropositive cows relative to seronegative cows (Table 4.2). In contrast, goat does that seroconverted to phase 1 *C. burnetii* had higher milk volumes over the first nine weeks of lactation compared to goats that did not seroconvert (Muleme et al., 2017). In the current study, a combined *C. burnetii* phase 1 and 2 ELISA was used. The antibody phase responses have been used clinically to distinguish acute from persistent infections in humans, with antibodies to phase 2 appearing before antibodies to phase 1 (Dupont et al., 1994, Roest et al., 2013). Therefore, in the goat study it was speculated that a fully developed *C. burnetii* humoral response in goats (evident by presence of antibody to phase 1 *C. burnetii*) may protect against adverse effects of acute *C. burnetii* infection on milk production (Muleme et al., 2017).

The current study is the first to measure the association between milk production and *C. burnetii*-specific IFNγ and IL-10 responses of cows at early lactation. This was determined to

be an important component of the study as cytokines are moderators of the cell mediated immune responses which are important to clearance of intracellular pathogens (Andoh et al., 2007). More specifically, IFNγ promotes cell mediated immune responses and is needed for intracellular killing of *C. burnetii* in human monocytes (via apoptosis) (Dellacasagrande et al., 1999), while IL-10 has strong anti-inflammatory functions that dampen cell mediated immune responses and is hypothesised to progress the persistent form of *C. burnetii* infection in humans (Capo et al., 1996, Ghigo et al., 2001). The cows' cytokine responses to *C. burnetii* antigen stimulation were not strongly associated with milk volume or total solids as the effect estimates were imprecise (Table 4.3). The scope of the analysis in the current study was limited by sample size and time, however, future studies could be directed at assessing both more complex non-linear relationships (over those that can be accounted for by linear and quadratic terms) as well as interactions between *C. burnetii* variables to better capture the complexity of the immune response.

4.5.3 Pathophysiology of *Coxiella burnetii* remains poorly understood

Considering plausible explanations for identified associations is important when assessing whether observed associations reflect causal links between *C. burnetii* infection and milk production, and for guiding future studies. There are currently numerous potential mechanisms for the findings observed in this study. Specific examples of the uncertainty surrounding the biological significance of some exposure variables has been discussed above, including whether *C. burnetii* DNA in placental and vaginal swab samples represents the same infection state as well as whether milk shedding is indicative of an intramammary

infection. Therefore, it is possible to speculate only on the mechanisms by which an infection of the reproductive tissue could have caused a reduction in milk volume and solids. A basic model of normal milk production was used to structure thinking for ad-hoc development of hypotheses that may explain the outcome responses from each exposure variable (Figure 4.6).

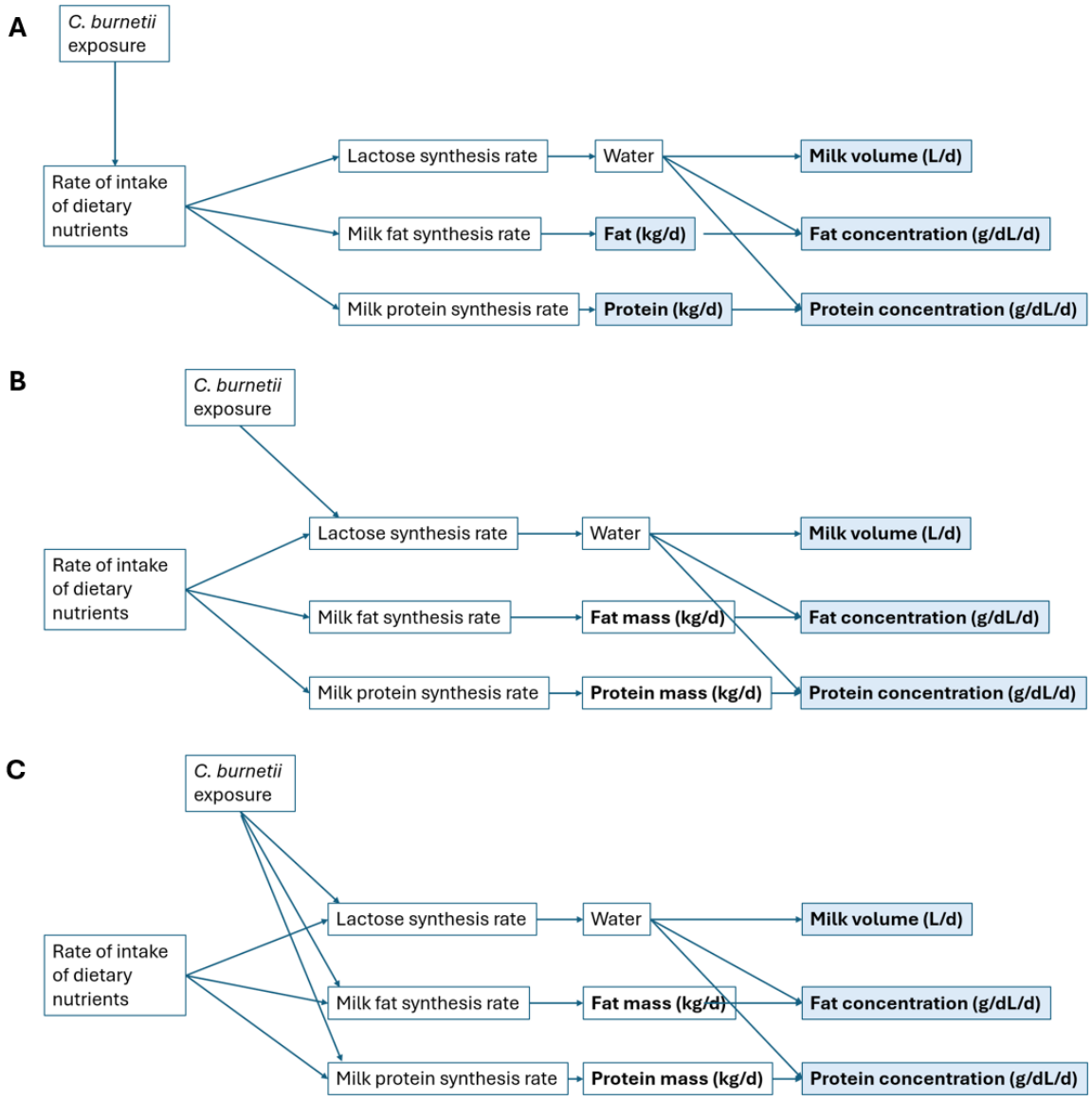

Figure 4.6. Basic model of milk production with proposed mechanisms by which *C. burnetii* could impact measured outcomes. Blue boxes = outcome affected by *C. burnetii*. Bold font = outcomes measured in the study. *Coxiella burnetii* exposure or infection was determined by shedding, serology, and cell mediated immunity. Serostatus was determined by using serum samples in an IgG ELISA. Shedding was determined by testing placental, vaginal swab, colostrum, and milk samples in a multiplex qPCR. IFNγ and IL-10 were measured by an ELISA from whole blood stimulated ex vivo with *C. burnetii* antigen. The outcomes evaluated were test day milk volume (L), fat (% and solids [kg]), protein (% and solids[kg]), and combined total solids (kg fat and protein). The 'water' node is water content in milk. All rates are on a per day basis.

As *C. burnetii* detection in a vaginal swab at parturition was associated with lower total solids as well as lower estimates for volume, and fat and protein yield (Figure 4.3), it was theorised that *C. burnetii* may reduce peri-parturient (very early lactation) dietary intake of nutrients in *C. burnetii* exposed compared to unexposed cows (Figure 4.6A). This would reduce not just milk volume, but also fat and protein yields, while fat and protein concentrations would remain unchanged if the proportional change in rate of volume synthesis equals the proportional change in rate of fat and protein mass synthesis in exposed cows. The plausibility of this hypothesised mechanism is supported by research that shows *C. burnetii* can cause physiological changes in ruminants which could ultimately lead to changes in appetite. For example, experimental infection of *C. burnetii* by the intradermal route in non-pregnant heifers (Plommet et al., 1973) and the subcutaneous route in pregnant goats can raise body temperature (dependent on dose) in the week of inoculation (Bouvery et al., 2003). The timing of a physiological change would also be important to consider given the present study identified a reduction in volume from the cows shedding *C. burnetii* in their placenta at calving, which lasted throughout the entire lactation (Figure 4.2). Intraperitoneal experimental infection of goats at day 90 of pregnancy found considerable bacterial replication (determined by PCR and immunohistochemistry) occurred in the placenta between day 116 and 130 of gestation, which is towards the end of pregnancy (full gestation of does is 150 days) (Sánchez et al., 2006). It is therefore possible that a similar replication of *C. burnetii* in infected cows could occur during the transition period in dairy cows, which is a critical stage in the bovine lactation cycle. A sudden shift in uterine infection dynamics could induce a systemic inflammatory response and consequently reduce dry matter intake, which has been shown to trigger metabolisable energy imbalances (Pérez-Báez et al., 2019) and ultimately reduce the availability of

nutrients required for synthesis of milk components during lactation. In the context of the present study, if there was a decrease in dry matter intake around calving, the most pronounced drop in milk production would likely occur during early lactation, as was evident in the lactation curves from cows with *C. burnetii* DNA detected in their vaginal swab (Figure 4.3A and 4.3B). Future studies could be directed at measuring and comparing dry matter intake and markers of metabolisable energy prior to and after parturition in cows and correlating with *C. burnetii* infection status, particularly as measured by PCR of vaginal swabs.

In contrast, placental shedding was associated with lower daily milk volume but higher fat concentration (Figure 4.2), and as such, a different causal hypothesis may better explain these observations. In this case, *C. burnetii* may decrease rates of synthesis of the key drivers of volume, including lactose, in *C. burnetii* placental shedding compared to non-*C. burnetii* placental shedding cows but not affect rate of fat synthesis (Figure 4.6B). This mechanism could reduce milk volume but leave fat yield unaffected, thereby increasing fat concentration, as fat concentration = fat yield/milk volume. To investigate this causal hypothesis, future studies could longitudinally measure and compare lactose in milk from cows shedding and not shedding *C. burnetii* in the placenta at calving. It may also be helpful to measure lactose synthesis *in vitro* from bovine mammary epithelial cells infected with *C. burnetii* to directly determine if this bacterium can impact lactose synthesis. However, it is also essential to note that the imprecision (95% confidence intervals) in the present study's outcome estimates prevents any certainty in explanatory hypotheses. For example, while for the placental shedding exposure variable the estimated differences for fat yield (kg) were not statistically significant between *C. burnetii* exposed and unexposed cows, the

estimate was also imprecise, with the 95% confidence intervals ranging from -0.07 kg/cow/day to 0.13 kg/cow/day (Table 4.4). Therefore, the true effect may have been a reduction in this variable, in which case Figure 4.6A (*C. burnetii* reduces dietary intake of nutrients) could alternatively explain the observed relationships. Furthermore, the mechanisms described so far involve *C. burnetii* impacting a single milk production pathway, however, the bacterium could influence multiple pathways concurrently (Figure 4.6C), including in different directions (that is, increasing or decreasing synthesis) or by different amounts, which further complicates interpretation. Ultimately the simplified model approach utilised here can help in structuring thinking to develop plausible mechanistic theories in order to guide future targeted investigations.

Interpretation of the findings from this study must also be considered in the context of the study's limitations. Due to the exploratory aim of this investigation and as such the subsequent prioritization of sensitivity for detecting associations of interest, multiple comparisons corrections were not utilised during statistical analyses, meaning that some statistically significant findings may have been due to chance and not indicative of a true biological process. Confounding and misclassification bias could also influence validity of these results as the knowledge gaps surrounding *C. burnetii* and milk production challenge the ability to address such issues. For example, there may be factors independently associated with both the exposure variables and outcomes variables that were not accounted for during modelling, thus confounding the causal effect of the exposure variable. Co-infection may be an important variable to measure in future studies. Furthermore, it is possible that information bias could occur due to imperfect measurement of the underlying causal exposure statuses (Wood et al., 2019). Another limitation is that

the sample size was not set for the objectives of this study, which means there may have been insufficient statistical power for detecting biologically and economically important effects. In saying this, the enrolment of 400 cows was justifiable given the exploratory aim and the largely unknown effects of *C. burnetii* infection in cattle on volume and solids. Finally, despite contextualizing the findings from this single herd within the existing body of research on the topic, the external validity of the results using for the first time placental infection as an exposure variable, and total solids as an outcome, is unknown.

4.5.5 Future Research

Having observed an association between *C. burnetii* infection and each of lower daily milk volume and total solids in an Australian dairy herd, further work is needed to determine the generalisability of these findings in diverse countries and production systems. While logistically difficult and expensive to perform, experimental infection studies would also be useful in clarifying whether *C. burnetii* causes milk production loss (and if so under what mechanisms) as they allow assessment within a controlled environment. If the association with milk production loss in this study does prove causal, future work should be directed towards identifying risk factors for infection, to effectively guide subsequent intervention strategies such as vaccination and biocontainment practices.

Chapter 5 *Coxiella burnetii* is associated with an increase in somatic cell count in dairy cow milk: an exploration of causality using directed acyclic graphs.

5.1 Abstract

The bacterium *Coxiella burnetii* is well known as the cause of the zoonotic disease Q fever, but the disease manifestations in animal reservoirs are less well understood. In cows, the bacterium can be shed in milk, but reports differ regarding association with subclinical mastitis. This study used a cross-sectional design to assess the association between *C. burnetii* presence and somatic cell count at the individual mammary quarter level followed by exploration of possible causal relationships using directed acyclic graphs (DAGs) informed by multidisciplinary expertise. A cross-sectional study was devised which involved aseptic sampling of 532 individual quarter milk samples from 133 mid-lactation dairy cows from one herd after routine milking. Samples were tested for *C. burnetii* DNA by PCR and for intramammary pathogens by microbiological culture and Matrix Assisted Laser Desorption Ionization-Time of Flight mass spectrometry (MALDI-TOF). Somatic cell counts from 196 of the quarter samples (from 49 cows) were measured by a portable cell counter. Careful thought is required when inferring causal relationships based on associations from cross-sectional studies as the temporal sequence of disease progression relative to infection cannot be determined. Current knowledge of *C. burnetii* milk shedding dynamics and somatic cell counts in cows informed development of multiple DAGs to depict various hypothesised underlying causal relationships, which then informed regression modelling to estimate possible causal relationships between somatic cell count and quarter PCR status

(positive/negative). The DAGs represented two plausible causal hypotheses: 1) *C. burnetii* causes an increase in SCC and 2) increased SCC causes increased probability of this obligate intracellular bacterium being in milk. From generalised linear mixed effects modelling informed by the DAG that represented hypothesis 1, the mean SCC was estimated as increasing by a factor of 2.84 from *C. burnetii*-positive quarters compared to *C. burnetii*-negative quarters. Mixed effects logistic regression modelling informed by the DAG that represented hypothesis 2 also indicated a positive association between *C. burnetii* and SCC, whereby for every 100,000 cells/ml increase in SCC, the odds of the gland quarter being *C. burnetii*-positive increase by a factor of 1.14 times. This study is the first to find a positive association between *C. burnetii* presence and SCC at quarter level and presents two plausible but competing hypotheses to explain this relationship. With cross-sectional study designs, it is not possible to identify direction of causation from data alone. To determine which hypothesis is causally correct, future longitudinal or experimental studies are required.

5.2 Introduction

Coxiella burnetii is an obligate intracellular bacterium and the causative agent of Q fever in humans (Eldin et al., 2017). Cattle are one of the reservoirs for human infection (Tan et al., 2024) and while infection is mostly considered subclinical, disease (coxiellosis) manifestations such as abortion and stillbirth can occur (Agerholm, 2013). Previous studies have provided evidence supporting (Barlow et al., 2008, Khatun et al., 2022) and not supporting (Agger and Paul, 2014, Angen et al., 2011, Freick et al., 2017) an association

between *C. burnetii* DNA or antibody presence in cattle and elevated somatic cell count and subclinical mastitis. Subclinical mastitis is a disease characterised by inflammation of the mammary gland that can lead to reduction in milk quality, quantity or both, and is commonly diagnosed by elevated SCC in milk (Batavani et al., 2007). In a report from one herd, cows with *C. burnetii* detected in milk were 3.92 times more likely to have chronic high SCC than cows without *C. burnetii* in milk (Barlow et al., 2008), and similarly, cows challenged via the intramammary route with *C. burnetii* subsequently developed acute mastitis that spontaneously resolved after eight days (Bell et al., 1949). In contrast, other studies have not found compelling evidence for associations between SCC and each of *C. burnetii* presence in milk or vaginal swabs (as detected by PCR) (Freick et al., 2017, Angen et al., 2011) or serum and milk *C. burnetii* antibody (Freick et al., 2017, Agger and Paul, 2014). Given the economic importance of subclinical mastitis in the dairy industry, it is valuable to understand whether *C. burnetii* is a cause of subclinical mastitis and increased SCC.

There have been a variety of sampling and analytical approaches employed in previous studies to investigate the association between the presence of *C. burnetii* and SCC, each with their strengths and limitations. This variation in approach is in part due to the substantial knowledge gaps in relation to *C. burnetii* infection dynamics in the mammary gland and shedding into milk. In the field of mastitis research however, there are established best practice methodologies for subclinical mastitis diagnosis, including the detection of the agent causing the intramammary infection and measurement of SCC. In general, the relationship between IMI and SCC is most accurate at the individual mammary quarter level (because quarters are relatively independent biological units) (Schukken et al.,

2003) but thus far in *C. burnetii* research, SCCs have been measured at udder level rather than quarter level and quarter milk samples have only been used by a single study for detecting *C. burnetii* (Barlow et al., 2008). The use of udder-level milk samples (that is, composite milk samples collected from all four quarters), while logistically expedient and less expensive, can reduce analytical and possibly diagnostic sensitivities due to dilution of the pathogen count and/or SCC in affected quarters by milk from 'healthy' quarters (Reyher and Dohoo, 2011, Souza et al., 2016). Furthermore, intermittent shedding of *C. burnetii* (Guatteo et al., 2007) may influence diagnostic sensitivity for detection especially as single sample cross-sectional PCR studies have been common as opposed to parallel interpretation of repeated sampling over time, which can increase sensitivity of IMI diagnosis in mastitis research (Dohoo et al., 2011). Additionally, there has been considerable variation in approaches to minimising possible sources of bias (Angen et al., 2011, Barlow et al., 2008, Freick et al., 2017). For example, the potential influence of an existing IMI from a known mastitis pathogen has rarely been considered in design of studies focussed on *C. burnetii* in quarters (Barlow et al., 2008, Khatun et al., 2022). Without clarity on inclusion or exclusion of model covariates, it is difficult to fully scrutinise and synthesise results from, and between, the various studies. Finally, when it comes to interpretation of findings, of the studies that have found a positive association between *C. burnetii* and SCC, there has been limited discussion of the underlying causal relationship (Barlow et al., 2008, Khatun et al., 2022). This is particularly important considering the poorly understood infection dynamics of *C. burnetii* in the mammary gland including how *C. burnetii* enters milk

Causation, in a disease context, is defined as “an event, condition, or characteristic that preceded the disease event and without which the disease event either would not have occurred at all or would not have occurred until some later time” (Rothman and Greenland, 2005). Temporality is a key principle for assessing causality, and for an event, condition, or characteristic to be causal, the subject must have been exposed to it before onset of the outcome event or status (Lash et al., 2021). Thus, particular care is required when interpreting results from cross-sectional studies investigating associations between *C. burnetii* and SCC as neither variable is permanent and both are assessed at the same timepoint, so the temporal sequence of events cannot be identified under this design. Causal diagrams, such as directed acyclic graphs, are epidemiological tools for study design and analysis that may be useful in overcoming some of the aforementioned barriers preventing understanding about the causal relationship between *C. burnetii* and SCC (Poppe et al., 2024). DAGs are schematics that visually depict hypothesised causal interrelationships between a set of variables. Causal pathways in DAGs are depicted as arrows. Pathways must be unidirectional, and DAGs must be acyclic, that is, a variable must not directly or indirectly affect itself. Directed acyclic graphs can explicitly inform the identification of potential sources of bias and so can be used to guide study design and analyses as well as promote transparency and careful thought about causal hypotheses within a field of research (Poppe et al., 2024).

Taking all these considerations into account, the aim of this study was to A) conduct a cross-sectional study to assess the association between *C. burnetii* and SCC in lactating dairy cows at the individual mammary gland quarter level and B) interpret the findings in a causation

context using DAGs informed by multidisciplinary expertise. This process was undertaken to both expand understanding of *C. burnetii* pathogenesis in the mammary gland and provide demonstration of the application of DAGs as a robust tool for *C. burnetii* researchers to use for causal thinking during observational study design, analysis, and interpretation.

5.3 Materials and methods

The workflow for this study involved implementing a cross-sectional study in a dairy cow herd, DAG development, and statistical analyses and interpretation.

5.3.1 Study design and animal sampling

A cross-sectional study was conducted in a single dairy cow herd confirmed to be *C. burnetii*-endemically infected located in New South Wales, Australia (Chapter 2, 3, and 4). Sera and individual quarter milk samples were collected from 133 mid-lactation (approximately 200 DIM) cows (which had been selected as part of a longitudinal study) after routine milking and analysed in the laboratory as detailed in Figure 5.1.

Serum was utilised to determine cow serostatus using a *C. burnetii* ELISA. All individual quarter milk samples were analysed for detection of an IMI by aerobic microbiological culture (which also included identification of contaminated samples [that is, samples with three or more colony types identified during microbiological culture, detailed in Section

5.3.4]). Aliquots of quarter milk samples were pooled to create a composite sample for each individual cow which was then used to screen for presence of *C. burnetii* DNA in milk by multiplex qPCR. All quarters from cows returning positive composite samples were then analysed individually by PCR.

Next, a subset of quarter milk samples were selected for SCC analysis (details on SCC analysis provided below in Section 5.3.6) based on selection at the cow level. The sample selection process for SCC testing was conducted assuming all samples from a selected cow would be analysed regardless of individual quarter status. Sample size calculations were performed assuming SCC would be the outcome (i.e. dependent) variable and quarter *C. burnetii* PCR status (positive or negative) would be the exposure (i.e. independent) variable. Clustering of SCCs between quarters within cows was disregarded. From these calculations, with 24 PCR positive quarters and 72 PCR negative quarters, the study would have 83% power for detecting a difference in SCC between these groups at the 0.05 level if the true ratio of geometric mean SCCs was two (Barlow et al., 2008) and the within-exposure group SCC standard deviation was one (Bansal et al., 2005, Barlow et al., 2008, Berning et al., 1987, Schepers et al., 1997). For cows without *C. burnetii* positive quarters: any cow with a contaminated quarter was excluded from further analyses. The remaining *C. burnetii*-negative cows were stratified by their serum antibody status to balance for cow's serological result. Then, all quarters from the 18 seropositive cows and 18 randomly selected seronegative cows were analysed for SCC. For cows with one or more *C. burnetii* positive quarter: all cows were enrolled and all their quarters analysed even if any of their

quarters' samples were characterised as contaminated. All cows with one or more *C. burnetii* positive quarters were seropositive.

All quarter milk samples selected for SCC testing also had any bacterial isolates detected from their sample during microbiological culture speciated by MALDI-TOF.

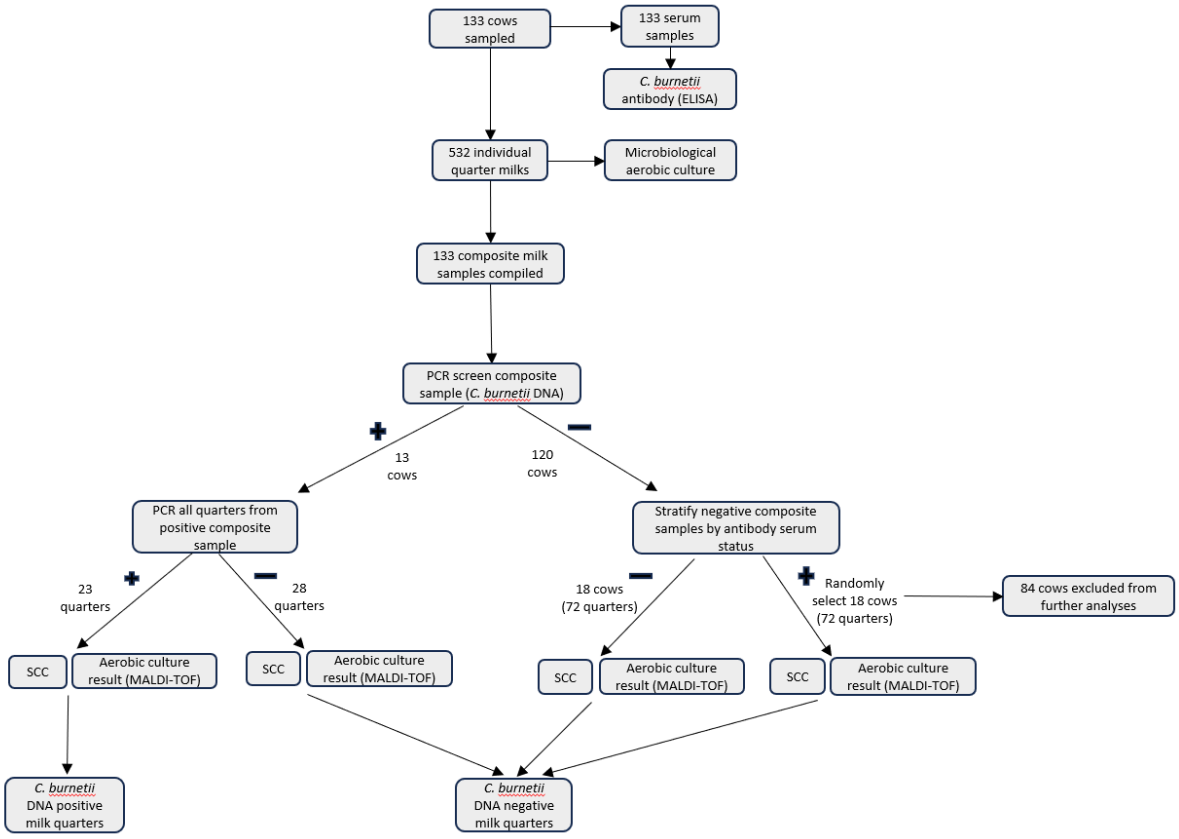

Figure 5.1. Cross sectional study design overview. Serum and individual quarter milk samples were collected from 133 Australian dairy cows at approximately 200 days in milk (DIM). Serum samples were tested for *C. burnetii* antibody level (ELISA) and individual milk quarters were used in aerobic microbiological culture to determine any intramammary infections (from non-*C. burnetii* organisms). Composite milk samples were compiled from each cows' quarter samples to screen for presence of *C. burnetii* DNA in milk (PCR). For any composite sample that was PCR positive (n = 13), all individual quarters making up that composite sample were individually tested for presence of *C. burnetii* DNA (n = 52). Somatic cell counts (SCC) and speciation (by MALDI-TOF) of any microbiological growths from aerobic culture of milk samples was conducted for all quarter samples from cows that had a

PCR positive composite sample (excluding 1 due to an inconclusive PCR result; n = 51). Cows with a *C. burnetii* PCR negative composite sample were stratified by their *C. burnetii* antibody serum status. All seropositive *C. burnetii* milk PCR negative cows (18 total) had their quarter milk samples (n = 72) measured for SCCs and any microbe growth from aerobic culture speciated by MALDI-TOF. The same occurred for the quarters from the 18 of the randomly selected seronegative *C. burnetii* milk PCR negative cows (n = 72). Somatic cell counts were compared between the *C. burnetii* PCR positive and negative milk quarters.

5.3.2 Sample collection and laboratory analysis

Blood was collected from the tail vein using a 10 ml clot tube as previously described (Chapter 3). The samples were chilled during transport to the laboratory where the tubes were centrifuged, serum harvested and stored at -45°C. Individual quarter milk samples were collected aseptically by cleaning each teat with an alcohol wipe, discarding foremilk and then expressing approximately 10 mls of milk (per quarter) into separate sterile collection vessels. Milk samples were chilled during transport to the laboratory then on arrival were aseptically split into three aliquots for PCR, aerobic culture and SCC. Samples for use in PCR were stored at -20°C while samples for microbiological culture and somatic cell count were stored at -80°C. Samples for microbiological culture were stored for less than one week before use.

5.3.3 *Coxiella burnetii* antibody detection

Antibody detection was performed on serum samples in duplicate using a commercially available ELISA kit (IDEXX Q Fever Antibody Test Kit) following the manufacturer's

instructions and as previously described (Chapter 3). Optical densities for duplicates were averaged and analysed using Mars Data Analysis Software (BMG LabTech). A cut-off of sample to positive ratio (S/P%) ≥ 40% was used for classifying positives, samples with S/P% < 30% were negative, and samples with S/P% ≥ 30% but < 40% were suspect. All serum samples at L5 were either positive or negative (no suspect results) (Chapter 3).

5.3.4 Microbiological culture

Milk samples were thawed at room temperature and streaked onto Columbia sheep blood agar plates (Edwards) for detection of intramammary microorganisms (other than *C. burnetii*) by microbiological culture and to identify any sample contamination. These plates were placed in an incubator at 37°C and assessed for growth after 24 and 48 hours. A growth was defined as three colonies of the same species (= 300 colony forming units/ml) from a non-contaminated sample. Contaminated samples were defined as three or more colony types (Rowe et al., 2024). One colony per morphology type was streaked onto a new sheep blood agar plate and incubated for 24 hours at 37°C for isolate purification. Isolates were stored in tubes (Nalgene, Thermo Scientific) in brain heart infusion broth (Edwards) with 10% glycerol at -80°C for subsequent analysis.

Bacterial isolates from cows selected for analysis were speciated using MALDI-TOF, as summarised in Figure 5.1. Isolates were thawed and streaked onto Columbia sheep blood agar plates using sterile plastic loops then incubated at 37°C for 48 hours. A sample from a

colony was smeared onto a MALDI-TOF plate using a sterile toothpick followed by addition of 1 µl of formic acid. Samples speciation was performed by MALDI-TOF at New South Wales Department of Primary Industries.

5.3.5 *Coxiella burnetii* molecular detection

5.3.5.1 DNA extraction (milk)

DNA extraction was performed on the cell pellet milk using the Biosprint® 96 One-For-All Vet Kit as previously outlined (Chapter 3) and according to the manufacturer's instructions.

5.3.5.2 Quantitative PCR detecting host species DNA (endogenous control) and *Coxiella burnetii* DNA

To confirm the DNA integrity, qPCR assay targeting the mitochondrial DNA (mtDNA) gene, BCB, were conducted as an endogenous control as previously described (Chapter 3). Detection and quantification of *C. burnetii* DNA in extracted samples was performed as previously described (Chapter 3) using an optimised multiplex qPCR assay targeting the two single copy genes: groEL (heat shock operon; *htp*AB) and *com1* (the outer membrane protein-coding gene) and the multicopy insertion sequence gene: IS*1111*. Each qPCR run included no template controls and positive controls containing 1,100, 110 and 11 copies of the *C. burnetii* genome per reaction (Amplirun® Vircell). The lower limit of detection for

these qPCR assays was determined to be 11 copies of the *C. burnetii* genome per reaction which corresponded to a cycling or quantification threshold of approximately 34, 36, and 35 for IS*1111*, *com1* and *htp*AB, respectively. For a sample to be considered positive it required 11 or more copies of IS*1111*, and 11 or more copies of either *com1* or *htp*AB. Samples with 11 or more copies of IS*1111* but less than 11 copies of *com1* or *htp*AB (that is, IS*1111* only) were considered "suspect" and samples with less than 11 copies of IS*1111* were deemed "negative".

5.3.6 Somatic cell counts

Milk aliquots were defrosted at 37°C and inverted to mix. Numbers of somatic cells/µl of milk in each sample were counted using a portable cell counter (DeLaval Cell Counter, DeLaval). According to the manufacturer's instructions, approximately 60 µl of milk was drawn up into a cassette that contained dye to stain cell nuclei. Cassettes were inserted into the portable cell counter and cells counted by image analysis.

5.3.7 Cow data collection

Cow parity and DIM on the sampling date were downloaded from the dairy herd management computer program, Dairy Comp 305 (DC 305; Valley Agricultural Software, Tulare, CA)

5.3.8 Development of directed acyclic graphs

5.3.8.1 Compiling knowledge

Current literature and multidisciplinary expertise on *C. burnetii* milk shedding dynamics and mastitis informed development of multiple DAGs to depict various hypothesised underlying causal relationships between *C. burnetii* and SCC at individual quarter level.

5.3.8.2 Graph formation

All DAGs were drawn using the online software DAGitty (Textor et al., 2016). Firstly, the independent (exposure) and dependant (outcome) variables were identified. Then a list of variables known or hypothesised to be caused by and/or causing each of the exposure and outcome was developed, to identify both potential confounding pathways and mediating variables for the causal relationship between the exposure and outcome variables. A confounding pathway is present if a variable influences both the exposure and outcome, and a mediator is a variable lying on the causal pathway between the exposure and outcome. Interaction terms were included if it was hypothesised that the magnitude and/or direction of the causal relationship between the exposure and outcome variables vary depending on the value of a third variable, and were depicted according to previously published methodology (Attia et al., 2022). Each final DAG included a combination of direct and indirect hypothesised causal pathways and confounding pathways. Generally, if it is unclear whether a confounding path exists between the exposure and outcome variable, it is better

to include the variable causing this pathway than risk confounded effect estimates (Poppe et al., 2024). Multiple DAGs were developed to model the data each way (with and without conditioning for confounders [that is, multivariable and univariable analysis]) to highlight any influence on results. All paths, and absence of paths, were based on previously published scientific evidence and expert hypothesis.

5.3.9 Statistical analyses

If a DAG is correct, regression analysis informed by that DAG will result in an estimate for the magnitude and direction of the effect of the exposure variable on the outcome variable that is not confounded. The DAG is used to identify minimal sufficient adjustment sets (sets of covariates that if accounted for would block all confounding pathways). If the effect estimate is adjusted for all variables in a minimal sufficient adjustment set (for example, by fitting all of those variables as covariates in the regression model), that estimate will not be confounded (assuming the DAG has identified all confounding pathways).

Minimal sufficient adjustment sets can differ depending on whether the aim is to estimate the total effect of the exposure on the outcome or just the direct effect. The total effect is the sum of all pathways from the exposure to the outcome including pathways through other variables whereas the direct effect is just the effect represented by the arrow directly from exposure to outcome.

Minimal sufficient adjustment sets for the total effect based on each DAG were derived by DAGitty. For continuous variables, fractional polynomial regression was used to determine the shape of the relationship between that variable and the outcome variable. Based on this, parity (when included) was assumed to have a linear relationship with the outcome and so was incorporated as continuous non-transformed data. When included in models, milk culture result (non-*C. burnetii* IMI) was incorporated as a categorical covariate while DIM was controlled for by restriction whereby all the cows in the study were at similar DIM. Clustering of the outcome variable between quarters within cow were accounted for in the model by fitting cow as a random effect.

In some DAGs, *C. burnetii* presence was the exposure and SCC was the outcome, while other DAGs included the reverse scenario (that is, SCC was the exposure and *C. burnetii* presence was the outcome). Generalised linear mixed effects models with a gaussian residual distribution were used when the exposure variable was *C. burnetii* and the outcome variable was SCC, and the log link function was used as the SCC data were markedly right skewed. Coefficients were exponentiated and as such, were estimates of the ratio of arithmetic means. Mixed effects logistic regression models with a binomial distribution were used when the exposure variable was SCC, and the outcome variable was *C. burnetii*. The exposure variable, SCC, was first divided by 100 so that the effect estimate was expressed per 100 cells/ml increase in SCC, as a one cell unit increase is not a meaningful difference in the mastitis field. All statistical analyses were performed in Stata (StataCorp; version 18).

5.4 Results

5.4.1 Cross-sectional descriptive results

The prevalences of *C. burnetii* and aerobically culturable microorganisms in milk from quarters selected in the study as measured by multiplex qPCR and aerobic microbiological culture, respectively, are summarised in Table 5.1. A more detailed summary of the identification of the species grown during microbiological culture (as determined by MALDI-TOF) is also provided in Table 5.3. The distribution of cell counts was right skewed, with most samples having a SCC below 250 cells/ml (Figure 5.2). *C. burnetii* presence in the quarter was highly clustered within cow (intra class correlation coefficient: 0.65; 95% CI 0.33 – 0.88) (Table 5.2).

Table 5.1. Summary of prevalence of *Coxiella burnetii* and non-*C. burnetii* microbial growth from 196 quarter milk samples from 49 mid-lactation Australian dairy cows after routine milking. Presence of *C. burnetii* was determined by multiplex qPCR while microbiological growth was determined by aerobic culture on Columbia sheep blood agar plates.

Quarter status	***C. burnetii* DNA presence**	**Microbiological growth**
Negative	172	164
Positive	23	30
NA	1[a]	2[b]
Total	196	196

[a] Inconclusive PCR result.

[b] Sample for aerobic microbiological culture was contaminated due to more than three colony growths on sheep blood agar plate.

Table 5.2. Summary of the actual and expected clustering of *Coxiella burnetii* in milk quarters within cow from 49 Australian dairy cows sampled after routine milking at mid lactation. Presence of *C. burnetii* was determined by multiplex qPCR. The expected numbers of cows with each of 0, 1, 2, 3, 4 (if there was no clustering) were calculated by binomial probabilities assuming the probability of a quarter being PCR positive was 0.133 (the observed apparent prevalence).

Number of *C. burnetii* positive quarters	**Actual number of cows**	**Expected number of cows (if no clustering)**
0	36	27.59
1	6	17.04
2	5	3.95
3	1	0.41
4	1	0.02

Table 5.3. Summary of the distribution of the 193 quarter milk samples (from 49 Australian dairy cows) with both a *Coxiella burnetii* and non-*C. burnetii* microbial growth result (status defined by positive or negative). Rows represent presence (positive) or absence (negative) of *C. burnetii* DNA as determined by PCR. Columns represent presence (positive) or absence (negative) of microbiological growth as determined by aerobic culture on Columbia sheep blood agar plates. Numeric values in cells represent the number of milk quarters in each category. For the quarters with a microbiological growth, the identified species (by MALI-TOF) are listed.

		Microbiological growth quarter status		
		Negative (-)	Positive (+)	
				Species[a]
***Coxiella burnetii* quarter status**	Negative (-)	147	24	*Staphylococcus simulans* 11/24
				Pseudomonas spp. 3/24
				Corynebacterium bovis 2/24
				Staphylococcus epidermidis 1/24
				Staphylococcus chromogenes 1/24
				Streptococcus dysgalactiae 1/24
				Klebsiella pneumoniae 1/24

			Enterococcus saccharolyticus 1/24
			Staphylococcus haemolyticus 1/24
			Corynebacterium xerosis 1/24
			Corynebacterium amycolatum 1/24
			Candida glabrata 1/24
			Lactococcus garvieae 1/24
	Positive (+)	17	5	*Staphylococcus simulans* 4/5
				Staphylococcus haemolyticus 2/5
				Staphylococcus epidermidis 1/5
				Pseudomonas spp. 1/5

[a] some quarters had two microbial growths.

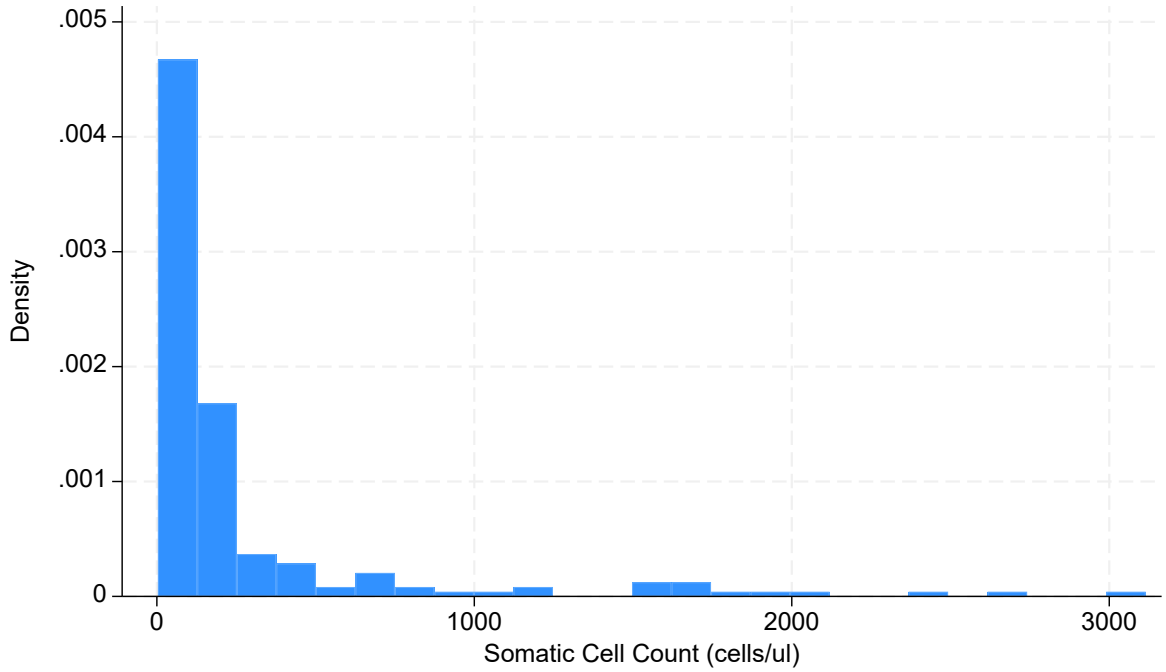

Figure 5.2. Histogram summarising the distribution of somatic cell count (SCC) from 196 quarter milks sampled from 49 Australian dairy cows at mid-lactation after routine milking. SCC was measured in cells/µl using a cell counter (Delaval Cell Counter). Bin width was determined using the Freedman-Diaconis Rule.

5.4.2 Hypothesised directed acyclic graphs

Two basic competing hypotheses were proposed: 1) the presence of *C. burnetii* causes an increased SCC in individual mammary gland quarters and 2) increased SCC causes increased probability of *C. burnetii* being in milk. Both hypotheses were considered plausible.

The underpinning argument for hypothesis 1 was that *C. burnetii* may cause an IMI leading to the host response of inflammation, which is the most common mechanism for subclinical mastitis, and is detected as a measurable increase in SCC in milk samples (Gonçalves et al., 2018, Schukken et al., 2003). This hypothesis is supported by the study describing the visual identification of *C. burnetii* infecting alveolar mammary epithelial cells in one goat doe's mammary gland by FISH (Bauer et al., 2024). Furthermore, *C. burnetii* is known to cause inflammation in other host tissues such as in heart valves of Q fever endocarditis patients (Lepidi et al., 2003). *Coxiella burnetii* presence in aborted placental tissue has also been associated with placentitis in naturally infected cattle and caused placentitis in experimentally infected (subcutaneous) pregnant goat does (Bildfell et al., 2000, Sánchez et al., 2006). Finally, experimental intramammary inoculation of four cows (and two controls) with *C. burnetii* by "large amounts of a 10 per cent suspension of an infected yolk-sac culture" resulted in development of severe acute clinical mastitis (Bell et al., 1949).

Hypothesis 2 was that when a cow is systemically infected with *C. burnetii* and independently of this, a quarter is inflamed, more immune cells move from blood vessels to the quarter per unit of time (Aitken et al., 2011) and given presence of *C. burnetii* within

those immune cells, *C. burnetii* will also enter the quarter which, in turn, increases the probability that it will be present in the quarter. Evidence in the literature supporting this hypothesis was reports indicating that *C. burnetii* has high tropism for, and replicates within, macrophages (and monocytes) (Eldin et al., 2017), and macrophage numbers increase in absolute number in milk from cows with subclinical mastitis (Gonçalves et al., 2017). Furthermore, *C. burnetii* was not visualised histologically infecting udder tissue in experimentally infected goats (Sánchez et al., 2006) and naturally-infected cows (Jellison et al., 1948b), despite these animals having PCR positive milk. This may be either due to the lower sensitivity of immunohistochemistry and histopathology compared to PCR, or because *C. burnetii* was not causing an IMI. There is also some evidence to suggest other pathogens may be present in milk by migration from other parts of the body, for example, after thorough laboratory investigation, the presence of *Salmonella enterica* serotype Typhimurium in human breast milk (that lead to transmission to a neonate) was concluded to have originated from a maternal systemic infection (not environmental contamination) (Qutaishat et al., 2004). The authors suggested it was biologically plausible that *Salmonella enterica* serotype Typhimurium originating from the gut may have been transported within macrophages (which, like *C. burnetii*, they can replicate in) to the mammary gland during immune cell colonisation of the mammary gland during lactation (Qutaishat et al., 2004).

5.4.2.1 Hypothesis 1 directed acyclic graph

In the DAG developed to capture Hypothesis 1, *C. burnetii* IMI (the exposure variable) causes an increase in SCC in milk (the outcome variable) (Figure 5.3). Parity, DIM, and presence of

IMI by mastitis pathogens are well understood to influence SCC. However, to the authors' knowledge, there is limited understanding about the causal relationship between these variables and *C. burnetii* infection. However, *C. burnetii* prevalence in milk has been reported as being positively correlated with both parity and DIM (Angen et al., 2011, Barlow et al., 2008). Therefore, the DAGs depict both parity and DIM as causes of the exposure, *C. burnetii* quarter infection (Figure 5.3). One study included assessment of correlation between the presence of *C. burnetii* DNA and mastitis pathogens in milk and reported no correlation (Barlow et al., 2008), while another study found evidence for a possible association between presence of *C. burnetii* antibody in quarter milk and Coagulase positive *Staphlococcus* and *Streptococcus uberis* (Khatun et al., 2022). However, to the authors knowledge no study has assessed whether presence of *C. burnetii* in a quarter predisposes the quarter to subsequent IMI by mastitis pathogens or the reverse (i.e. presence of mastitis pathogens in the quarter predisposes the quarter to subsequent *C. burnetii* entry and/or persistence). Accordingly, three DAGs capturing various scenarios under Hypothesis 1 were developed (Figure 5.3). These were, no causal relationship between IMI by a microorganism (other than *C. burnetii*) and an IMI by *C. burnetii* (Figure 3A), an IMI by a microorganism (other than *C. burnetii*) causing *C. burnetii* IMI (Figure 5.3B), and *C. burnetii* IMI causing establishment of an IMI with a microorganism (other than *C. burnetii*) (Figure 5.3C).

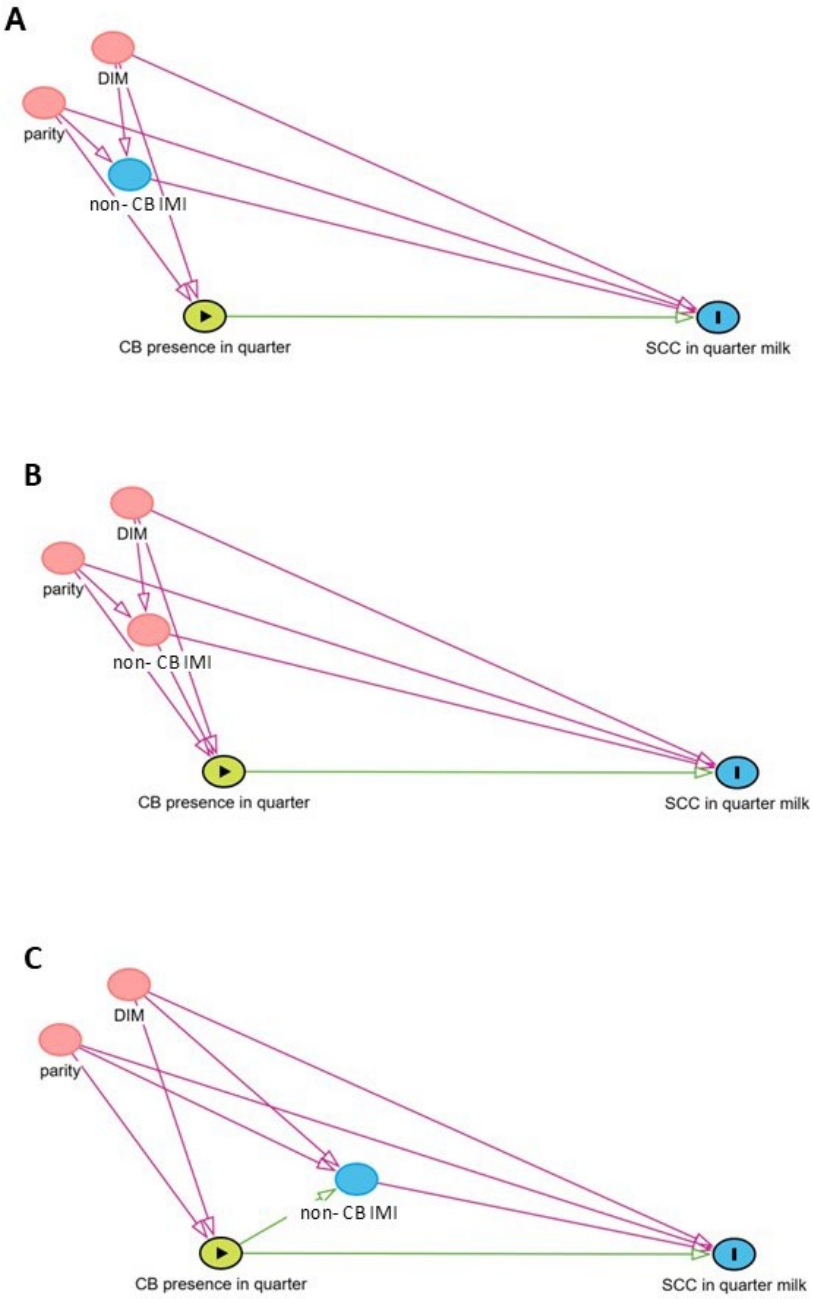

Figure 5.3. DAGs that capture Hypothesis 1, that *C. burnetii* (CB; exposure) causes an increase in SCC (outcome). DIM = days in milk and non-CB IMI = non-*C. burnetii* intramammary infection. Green variable with triangle represents the exposure; blue variable with I represents outcome; blue variables represent ancestors to the outcome; pink variables represent variables on confounding pathways between exposure and outcome variables. Green arrow pathways represent causal pathways from the exposure of interest to the outcome and pink arrow pathways represent confounded pathways. **A-C** represent the different potential causal relationships between variables: **A** The variable, 'non-CB IMI', only causes the outcome (and not the exposure). **B** 'non-CB IMI' causes both the exposure and

outcome so is a confounding variable. **C** The exposure variable causes a 'non-CB IMI' which then causes an increase in SCC so 'non-CB IMI' is a mediator.

5.4.2.2 Hypothesis 2 directed acyclic graph

DAGs were also developed to capture Hypothesis 2, that postulated that raised SCC (the exposure variable) causes an increased probability of *C. burnetii* in milk in the quarter (the outcome variable) (Figure 5.4). Given the basis for this hypothesis (described above), this effect of SCC on probability of *C. burnetii* in milk in the quarter can only occur if *C. burnetii* is present elsewhere in the cow. Accordingly, to capture this causal process in the DAG, an interaction term was utilised, whereby the variables 'SCC in quarter milk' (the exposure variable) and '*C. burnetii* circulating in immune cells' can only cause the outcome (*C. burnetii* in milk in the quarter) when present together. The factors, parity, DIM, and IMI due to a microorganism aside from *C. burnetii*, were depicted according to the same rules outlined for Hypothesis 1 (Figure 5.4). The development of a temporal DAG was required to capture the scenario that *C. burnetii* causes a non-*C. burnetii* IMI to avoid creation of a cyclic relationship (Figure 4C). SCC = somatic cell count; CB = *C. burnetii*.

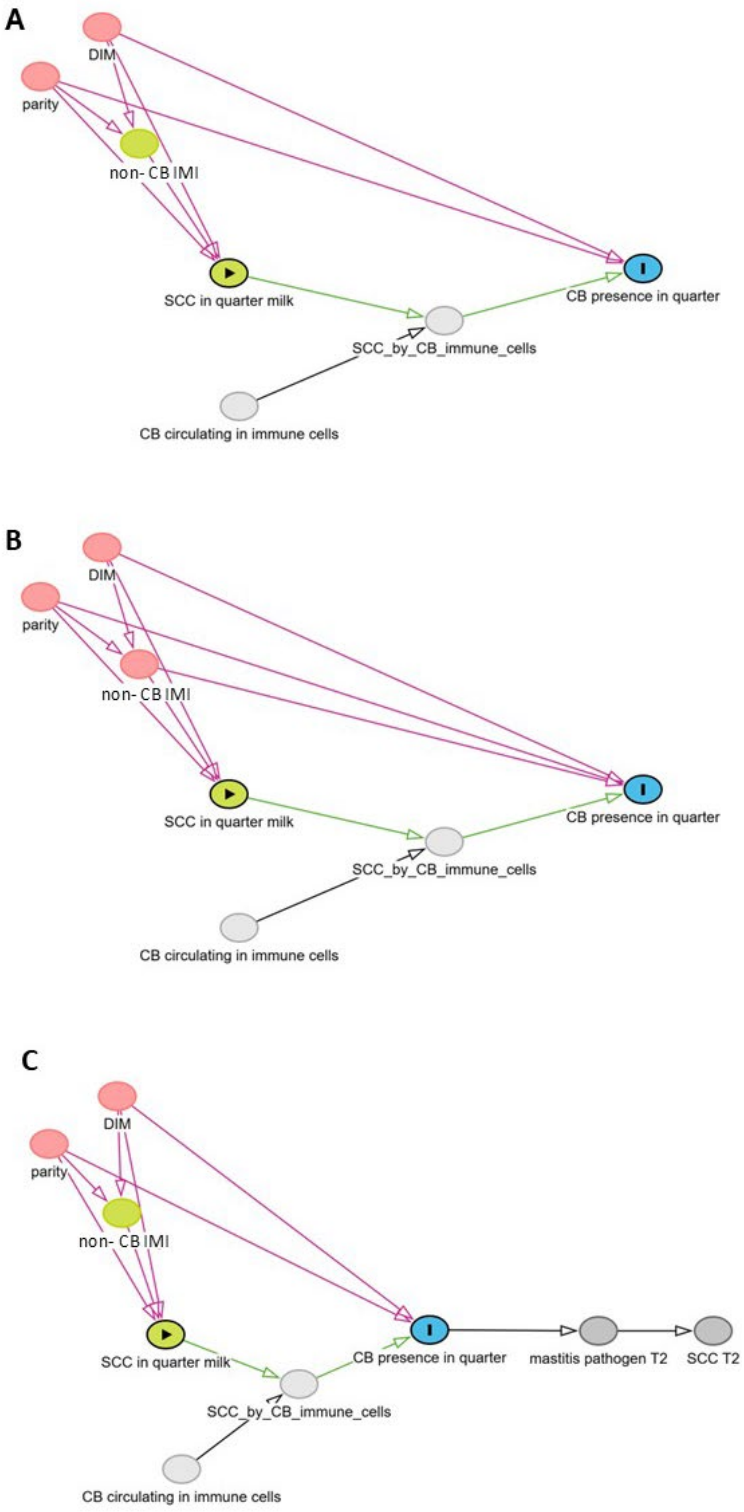

Figure 5.4. DAGs that capture Hypothesis 2, that an increase in quarter SCC (the exposure variable) causes *C. burnetii* (CB) presence (the outcome variable) in quarter milk. DIM = days in milk and non-CB IMI = non-*C. burnetii* intramammary infection. Green variable with triangle represents the exposure; blue variable with I represents outcome; green variables represent ancestors to the exposure; pink variables represent variables on confounding

pathways between exposure and outcome variables; grey variables represent unmeasured variables. An interaction term is depicted on the causal path between exposure and outcome (green line) as the effect of 'SCC in quarter milk' on the outcome can only occur if *C. burnetii* are circulating in immune cells in the blood. Green arrow pathways represent causal pathways, pink arrow pathways represent confounded pathways and grey arrows represent pathways to and from unmeasured variables. **A-C** represent the different potential causal relationships between variables: **A** The variable, 'non-CB IMI, only causes the exposure. **B** 'non-CB IMI' causes both the exposure and outcome so is a confounding variable. **C** The exposure variable causes the outcome only through an interaction term which then causes 'non-CB IMI' which then causes an increase in SCC at time 2 (T2) so 'non-CB IMI' is a mediator. SCC = somatic cell count; CB = *C. burnetii*.

5.4.3 Statistical associations between quarter level *Coxiella burnetii* and somatic cell count

Results of the statistical modelling informed by each DAG for Hypothesis 1 and 2 are shown in Tables 5.2 and 5.3, respectively. Days in milk was not included as a covariate in any model as it had been controlled for by restriction due to cows being sampled at the same stage of lactation. Firstly, it was evident that the addition of covariates changed the results by influencing the magnitude of the effect. For example, *C. burnetii* was estimated as increasing the arithmetic mean SCC by factors of 2.24 and 2.84 when modelled by univariable analysis and by DAG-informed analysis (that included the covariates: parity and non-*C. burnetii* IMI), respectively (Table 5.4; Figure 3B). Secondly, associations between *C. burnetii* and SCC were present when both causal hypotheses were modelled (Table 5.4; Table 5.5).

Table 5.4. Results from generalised linear mixed effects modelling the relationship between the exposure, *C. burnetii* presence in milk quarter from 49 Australian dairy cows (193 quarters), and the outcome, somatic cell count (SCC) in milk quarters (Hypothesis 1).

DAG identity number	Fitted exposure variables (and covariates)	Exp (β)*	Confidence Interval	P value
Univariable analysis	*C. burnetii* presence	2.24	1.3 – 4.0	0.006
Total effect of CB given DAG in Figure 3A	*C. burnetii* presence (parity)	2.19	1.24 – 3.88	0.007
Total effect of CB given DAG in Figure 3B	*C. burnetii* presence (non-*C. burnetii* IMI, parity)	2.84	1.53 – 5.28	0.001
Total effect of CB given DAG in Figure 3C	*C. burnetii* presence (parity)	2.19	1.24 – 3.88	0.007

*****estimated ratio of arithmetic means.

Table 5.5. Results from mixed effects logistic regression modelling the relationship between the exposure, somatic cell count (SCC) in milk quarter, and the outcome, *C. burnetii* presence in quarter milk from 49 Australian dairy cows (193 quarters) (Hypothesis 2).

DAG identity number	Fitted exposure variables (and covariates)	Odds ratio*	Confidence Interval	P value
Univariable analysis	SCC	1.15	1.00 – 1.31	0.043
Total effect of CB given DAG in Figure 4A	SCC (parity)	1.14	1.00 – 1.31	0.046
Total effect of CB given DAG in Figure 4B	SCC (non-*C. burnetii* IMI, parity)	1.14	1.00 – 1.31	0.058
Total effect of CB given DAG in Figure 4C	SCC (parity)	1.14	1.00 – 1.31	0.046

*Odds ratios estimate the proportional change in odds of *C. burnetii* presence in quarter for each additional 100 somatic cells/µl milk.

Predicted values from models based on DAGs 3B and 4B are graphed below (Figure 5.5; Figure 5.6). For Hypothesis 1, *C. burnetii* quarters had 2.84 (95% CI: 1.53 – 5.28) times higher SCCs which, for the study population, corresponded to average SCCs of 655,000 and 231,000 cell/ml for *C. burnetii* positive and negative quarters, respectively (Table 5.4; Figure 5.5). For Hypothesis 2, for every 100,000 cells/ml increase in SCC, the odds of the quarter being *C. burnetii*-positive were estimated as being 1.14 times higher (Table 5.5) which, for the study population, corresponded to the probabilities in Figure 6.

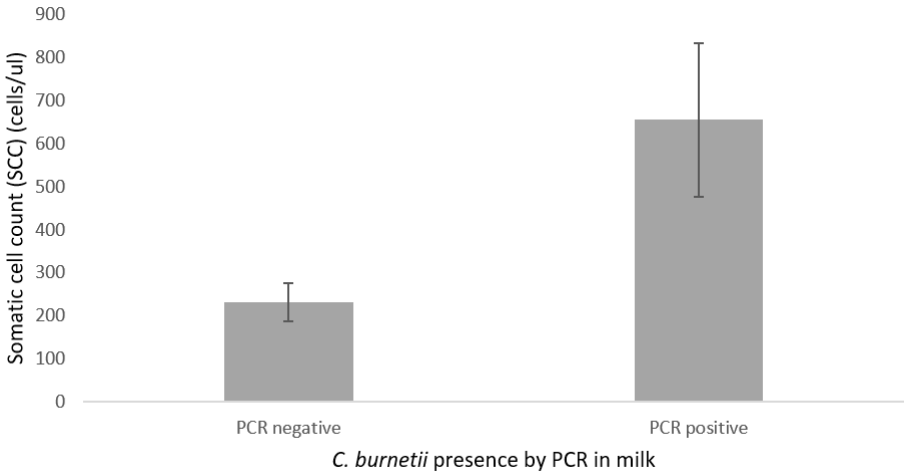

Figure 5.5. Predicted average somatic cell counts from 193 quarter level milk samples collected from 49 Australian dairy cows at approximately 200 days in milk grouped by *Coxiella burnetii* presence based on directed acyclic graph-informed modelling (covariates: parity and intramammary infection from a non-*C. burnetii* microbe). Presence of *C. burnetii* was determined by multiplex qPCR and somatic cell count was measured by a Delval Cell Counter. Error bars are 95% confidence intervals.

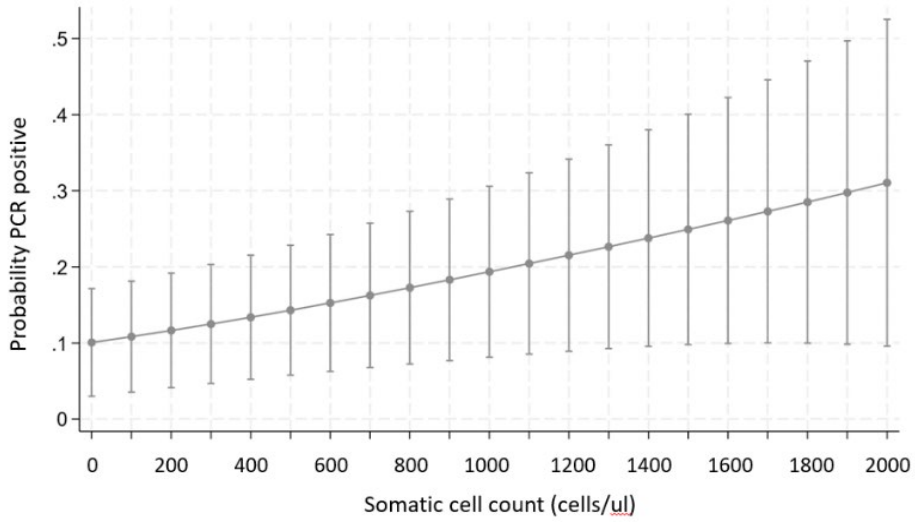

Figure 5.6. Predicted average probabilities of having *Coxiella burnetii* present in 193 quarter level milk samples collected from 49 Australian dairy cows at approximately 200 days in milk for every 100 somatic cells/ul increase, based on directed acyclic graph-informed modelling (covariates: parity and intramammary infection from a non-*C. burnetii* microbe). Presence of *C. burnetii* was determined by multiplex qPCR and somatic cell count was measured by a Delval Cell Counter. Error bars are 95% confidence intervals.

5.5 Discussion and conclusions

Contrasting observations are reported regarding the association between *C. burnetii* infection and SCC in bovine milk (Angen et al., 2011, Barlow et al., 2008, Freick et al., 2017). Additionally, there has been limited discussion focussed on the underlying causal association within the context of current knowledge of *C. burnetii* pathophysiology in the mammary gland. In this cross-sectional study, dairy cow quarter milk was sampled to measure *C. burnetii* DNA and SCC, then DAGs were utilised to describe hypothesised causal relationships and inform statistical analysis. Positive associations between quarter level *C. burnetii* and quarter level SCC were evident using analyses that accounted for potential confounding. A

key difference in this study from previous studies was the exploration of causality, and a demonstration of the use of careful, explicit thought when analysing data and interpreting results from cross-sectional studies. Here it is shown that DAGs are a helpful epidemiological tool to improve understanding of the role of *C. burnetii* in subclinical mastitis.

5.5.1 The presence of *Coxiella burnetii* in a bovine mammary gland quarter may cause increase somatic cell count in the quarter milk

Given there is disagreement in the literature on an association between *C. burnetii* infection in cattle and raised SCC (Angen et al., 2011, Barlow et al., 2008, Freick et al., 2017), this study sought to provide clarity on this association, as subclinical mastitis is an important disease of dairy cows. In the majority of bovine *C. burnetii* research investigating mammary gland infection and SCC, the researchers have viewed the relationship from the perspective that *C. burnetii* may act as a classic mastitis pathogen and cause a raised SCC (as *C. burnetii* is the exposure variable and SCC is the outcome) (Barlow et al., 2008, Freick et al., 2017, Khatun et al., 2022). To capture this hypothesis, DAGs were developed informed by explicit thinking and multidisciplinary expertise in the fields of *C. burnetii*, mastitis, and epidemiology. Importantly, through the DAG development process, this study identified potential confounding pathways (for the relationship between exposure and outcome) requiring subsequent conditioning in models (that is, inclusion of identified confounding variables). DAG-informed statistical modelling (that included the covariates parity and IMI from a non-*C. burnetii* microbe) found a positive association between *C. burnetii* and SCC, with *C. burnetii* positive quarters having 2.84 times higher cell counts than negative quarters

(Table 5.4). A positive association between *C. burnetii* and SCC was also found by Barlow et al. (2008) whereby *C. burnetii* presence in milk was a significant predictor for the previous three-month average SCC in composite milk from a North American dairy herd (when also accounting for udder health status as assessed by aerobic bacteriological culture). While *C. burnetii* antibody in quarter milk was also associated in one study with elevated SCC in quarter milk, it is difficult to characterise the role of *C. burnetii* in this context, as *C. burnetii* antibody can lack sensitivity for detecting active infection and/or shedding, and rather likely presents a group comprised of currently infected and previously infected individuals (Khatun et al., 2022).

A possible reason for the contrasting results between the present study and studies that have found no association between *C. burnetii* presence in milk and SCC, may be the variation in methodological approaches. For example, the studies that have found no association used composite milk samples to measure *C. burnetii* presence and SCC (Angen et al., 2011), and while this study initially screened *C. burnetii* presence in samples on the composite level (which could have led to misclassification), it may have had more statistical power for detecting an association between *C. burnetii* and SCC because the association was assessed using quarter level samples. The most accurate relationship between IMI and SCC exists at the quarter level and as composite samples measure an average effect across four relatively independent biological units, they have reduced sensitivity in detecting a pathogen or inflamed quarter due to dilution of milk from infected quarters by milk from the healthy quarters (Reyher and Dohoo, 2011, Souza et al., 2016). Another difference between studies is the duration and timing of SCC measurement, relative to *C. burnetii* detection, which is an

important factor to consider as mastitis pathogens affect SCCs in certain periods during and after infection. Unfortunately, in *C. burnetii* research, this information is not always clear which makes it difficult to fully scrutinise derived results. For example, if finding an absence of association between *C. burnetii* presence and SCC based on measurement of SCC at time 1 and measurement of *C. burnetii* presence at time 2, this could be simply because *C. burnetii* had not yet infected the cow during the time when SCC was measured. Alternatively, measuring SCC over a whole lactation based on presence or absence of *C. burnetii* in milk at parturition could dilute out an effect on SCC as the duration period of a potential effect of *C. burnetii* on SCC is not currently known. Overall, in comparing studies, it is evident this work has built upon the existing research, by using both analysis that accounts for bias and quarter level samples, thereby strengthening the evidence for a positive association between *C. burnetii* and SCC. However, this study cannot conclude that *C. burnetii* causes a raised SCC, as a second competing plausible causal hypothesis was also developed to explain the association, which highlights an important conclusion, that being, cross-sectional studies cannot be used to determine the direction of causation from the data alone.

5.5.2 A raised somatic cell count in quarter level bovine milk may cause increased probability of *Coxiella burnetii* being in the quarter milk

The second hypothesis to explain the underlying causal relationship for a positive association between *C. burnetii* and SCC was that an increased SCC causes increased probability of *C. burnetii* presence in milk (Figure 5.4). DAG-informed mixed effects logistic regression modelling found evidence for a positive association between *C. burnetii* and SCC, and it was estimated that for every 100,000 cells/ml increase in SCC, the odds of the quarter being *C. burnetii*-positive increase by a factor of 1.14 times (95% CI: 1.00 – 1.31; p = 0.058; Table 5.4). One study of 12 Danish dairy herds also investigated the relationship between *C. burnetii* shedding in milk and SCC by using SCC as the exposure variable and *C. burnetii* presence in milk as the outcome variable (Angen et al., 2011). However, theses researchers did not discuss the possibility that a raised SCC could cause an increased presence of *C. burnetii*, instead focusing on generally exploring whether an association existed between *C. burnetii* and a range of intrinsic cow variables that also included parity and DIM (Angen et al., 2011). Unlike the present study, the Danish study did not find an association between SCC and *C. burnetii* in milk, with the difference in results possibly in part due to some of the method variations discussed above, including their use of composite milk samples and an ambiguous SCC measurement period. Alternatively, while findings from a single herd and from cows at a similar stage of lactation may remove potential confounding by these factors, it also limits generalisability or external validity, as for example, *C. burnetii* strain virulence and management practices may differ between herds. Such variation may have been captured from enrolment of cows from 12 herds and highlights the possibility that the association found in the present study may only exist under some circumstances. The present study's methodology should be repeated in diverse environments to determine if, and under what, circumstances an association between *C. burnetii* and SCC is present.

5.5.3 Future research opportunities to determine causality

It is important to establish which hypothesis correctly explains the underlying causal mechanism of the association between *C. burnetii* and SCC because if *C. burnetii* does cause a raised SCC this could have economic consequences for the dairy industry through penalties for producers with elevated BTM SCCs or management costs associated with threshold SCCs. As this study collected quarter strippings milk (post milking collection which have higher SCCs (Dohoo and Meek, 1982)), the extracted average SCCs from the exposed and unexposed group (Figure 5.5) could not be directly compared to commonly agreed upon SCC cut-offs for intervention (> 200,000 cells/ml) that are derived from composite samples collected during routine milking (Schukken et al., 2003). Therefore, future work seeking to assess the potential economic impact of *C. burnetii* to the dairy industry should also analyse samples collected during routine SCC testing on dairy farms. Nevertheless, when considering together the observed estimated SCC increase (from Hypothesis 1; Table 5.4) and the 10% prevalence of *C. burnetii* shedding in milk from at least one quarter within cow in the cross-sectional study in this herd (Chapter 3), there may be economic benefits for dairy industries if the causation that explains this association was understood, especially given some studies have up to approximately 30% of clinical mastitis cases with undetermined aetiologies (Ganda et al., 2016). To determine causality, the sequence of events between *C. burnetii* and SCC must be established using longitudinal studies.

The methodological considerations for effective investigation of causation in longitudinal studies under observational and experimental conditions may vary. Future observational

studies should endeavour to conduct daily serum and quarter milk sampling for a more accurate understanding of *C. burnetii* exposure and shedding, relative to any changes in SCC. A key point to determine is whether *C. burnetii* presence in milk is followed by a raised SCC without the presence of another mastitis pathogen prior to or during the raised SCC measurements. While in the present study there were *C. burnetii* positive quarters without an IMI from another microbe (Table 5.3), the single timepoint of analysis prevents confirmation that an IMI from other microbes was not present, as intermittent shedding can occur. Furthermore, inflammation of the udder can arise due to other factors, aside from the aerobically culturable pathogens accounted for in the present study, such as viruses or physical trauma, which should also be monitored in future studies for more robust conclusions. Investigation of the relationship between *C. burnetii* and SCC using experimental infection of cattle via the inhalation transmission route has not previously been conducted but may allow for testing of specific questions in a controlled manner with improved relevancy of results to natural infection. Under this approach it could first be determined whether *C. burnetii* infection causes an increased SCC, with reduced influence of external causes of inflammation (compared to observational studies). Another element could additionally include inoculation of cows via the intramammary route with a range of minor or major mastitis pathogens to see if this changes the outcome.

An important consideration in any future research on this topic, is that the conclusions are conditional on a variety of factors that may influence the effect estimate for the relationship of interest, particularly regarding potential biasing pathways (Poppe et al., 2024). As a logical consequence, researchers must think carefully about causality in the complex udder

microenvironment to correctly interpret their results. The gold standard approach for choosing covariates to condition confounding pathways in statistical models is based on knowledge from the literature (Poppe et al., 2024) but in the context of *C. burnetii* and the mammary gland there are many unknowns, so researchers do not always justify sampling or analysis decisions related to potential confounding variables. In the present study, within each hypothesis statistical models reflected different DAGs, and indeed it was found covariate selection changed the p-values and estimated magnitude of the results somewhat (Table 2; Table 3). For example, in DAG 5.3A (Figure 5.3) where there were no confounding pathways, the estimated effect size was 2.24 (95% CI: 1.3 – 4.0; p = 0.006), whereas in DAG 5.3B (Figure 5.3) where parity and non-*C. burnetii* IMI were depicted causing *C. burnetii* presence (exposure) and SCC (outcome) the estimated effect size (that included the covariates parity and IMI by a non-*C. burnetii* microbe) was 2.84 (95% CI: 1.53 – 5.28; p = 0.001). This reinforces the challenges of interpreting and comparing findings in the literature when different methodological and analytical approaches are used, which is escalated by the absence of justification. Therefore, greater insight on the role of *C. burnetii* in subclinical mastitis may be aided by DAGs as the explicit thinking helps researchers with appropriate covariate selection and readers with clear interpretation of the results under the context of any assumptions.

Chapter 6 General Discussion

Coxiella burnetii is a gram-negative intracellular bacterium that causes the zoonotic disease Q fever in humans and coxiellosis in animals (Eldin et al., 2017). Q fever manifests in humans broadly as an acute infection, a persistent focalised infection (also known as chronic Q fever), or Q fever fatigue syndrome. Persistent focalised Q fever can be life threatening, while Q fever fatigue syndrome is a debilitating chronic fatigue that can last up to 10 years (Anderson et al., 2013). People in contact with livestock, such as producers and veterinarians, are at particular risk of *C. burnetii* exposure, as domestic ruminants are the main reservoirs for human infection (Graves and Islam, 2016). In Australia, cattle are the species most associated with human infection, with transmission occurring via inhalation from *C. burnetii* infected animal material, which includes reproductive tissue (at parturition) as well as milk, vaginal mucus, and faeces through lactation (Guatteo et al., 2007, Guatteo et al., 2012). Cattle may also suffer disease from *C. burnetii* infection, predominately reported as sporadic cases of reproductive loss, such as abortion and weak offspring, while there have been findings from different studies that are equivocal with respect to an association with subclinical mastitis (Agerholm, 2013). There are currently still important gaps in *C. burnetii* pathogenesis knowledge in cattle, namely what factors influence the variation observed in both shedding routes and persistence as well as disease manifestations. The bovine mammary gland may play a role in *C. burnetii* pathogenesis in cattle as the bacterium can be shed for prolonged periods in milk and, *in vitro*, has been observed to have high replicative tropism in bovine mammary epithelial cells, in comparison to other epithelial cell types (Sobotta et al., 2017). It is important to improve understanding about how *C. burnetii* interacts with bovine mammary tissue from both a public health and dairy industry

perspective, as prolonged shedding in milk may contaminate the environment leading to ongoing zoonotic and within herd transmission, while purported mammary gland infection could impact milk production.

Therefore, this thesis investigated *C. burnetii* infection and persistence of shedding in cattle, with a focus on the role of the bovine mammary gland but within the context of the whole animal by drawing together herd, individual animal, and gland observations. Through this process, a more holistic understanding of *C. burnetii* infection and shedding dynamics was developed, including the relevant host factors and responses and the impacts on subsequent milk production in dairy cattle. An initial review of the literature (Chapter 1) consolidated knowledge relevant to an understanding of *C. burnetii* and the udder tissue of lactating animals and identified gaps, some of which were then investigated in the following chapters. Chapter 2 outlined optimisation of the cytokine recall assay to measure *C. burnetii*-specific CMI responses in cattle (a host immune response marker) that was then utilised in a longitudinal study of a larger cohort of dairy cows to investigate shedding dynamics (Chapter 3) and subsequent detailed evaluation of milk production impacts (Chapter 4) in an endemically infected Australian dairy herd. The final investigation included a targeted assessment of the impact of *C. burnetii* on mammary gland health and was conducted by measuring the SCC in individual quarter milk samples to investigate any potential relationship between the presence of *C. burnetii* within lactating mammary tissue and a somatic tissue response (Chapter 5). In this section (Chapter 6), a synthesis of key findings from the investigative chapters, limitations to understanding, and opportunities for further

investigation are discussed to contextualise the contribution of the research to the field and identify the opportunities for future studies.

6.1 Description of *Coxiella burnetii* shedding and immune responses in an endemically infected Australian dairy herd.

As there are knowledge gaps surrounding *C. burnetii* infection dynamics in cattle, a longitudinal study investigating organism shedding (by detection of *C. burnetii* DNA in placental, vaginal, faecal, and milk samples) and immune responses (humoral and cell mediated) was conducted (Chapter 3). In this study, 192 cows were initially enrolled and then sampled over approximately seven months at five timepoints: dry period (240 – 270 days pregnant; L1), calving (L2), early lactation (17 – 32 DIM; L3), early lactation (45 – 60 DIM; L4) and mid lactation (178 – 201 DIM; L5). The acquired data can aid development of a more detailed understanding of pathogenesis, that could in turn assist the development of effective protection strategies against transmission and be utilised as measures of exposure and host response when assessing the impact of infection on milk production (Chapter 4 and 5).

The longitudinal study described in Chapter 3, found that the highest proportion of the study population were shedding *C. burnetii* at calving, with 52% of placental tissues returning a PCR positive result at L2 (Table 3.2). At calving, shedding via the vaginal, faecal, and milk routes were at their highest route-respective prevalences (compared to all other timepoints), which was followed by a decline in organism shedding at L3 and an absence at

L4 (Table 3.2). However, an absence of shedding was not maintained throughout the whole lactation, as there was a significant increase in the prevalence of cows shedding in milk at L5 (10%; 13/133), compared to L3 and L4 ($p \leq 0.006$; Table 3.2). While only some of the cows shedding in milk at L5 were also observed to be shedding organism at calving (6/13), the majority (11/13) had persistently high *C. burnetii* antibody titres (S/P% > 100) throughout the entire seven-month sampling period (Figure 3.2). A very high *C. burnetii* specific antibody titre is also observed in persistently infected human Q fever patients (Eldin et al., 2017, Melenotte et al., 2020), which may be because their immune system is continually exposed to the bacterium or due to *C. burnetii* manipulation of macrophage towards the M2 type (explained in more detail in Chapter 3) (Benoit et al., 2008). Furthermore, previous *C. burnetii* cattle research in naturally infected European herds had identified persistently high antibody titres in milk shedding cows up to a maximum period of three months (Boettcher, 2017, Guatteo et al., 2012). In combination, these findings add weight to a persistent infection state in cows, whereby following infection, some animals may be unable to clear *C. burnetii*, which in the present study was evident by shedding in milk approximately 200 days after calving. However, it must also be noted that given the endemic setting, the possibility of environmental re-exposure leading to the persistently high antibody titre and shedding at later stage lactation cannot be completely dismissed. Noting this observation, the present study favours the persistence hypothesis over the re-exposure hypothesis to explain the shedding at L5 from cows with persistently high antibody titres, consistent with the observation that despite the high environmental load detected at the property (Bauer, 2024) and all cows enrolled having had a relatively similar chance of re-exposure, only 10% followed this specific pattern. Analysis of *C. burnetii* IgG and IgM antibody patterns may hold value in distinguishing antibodies that are present during an ongoing infection from those

that arise from a recent re-exposure. This would potentially align with a goat experimental infection study, where IgM antibody titres rose and fell within three weeks of infection while IgG antibody titres were maintained up till the end of the study at 13 weeks post infection (Roest et al., 2013). However, whether the transience of IgM antibody observed during naïve goat experimental infection would also occur during a new infection from a previously infected animal (re-exposure) in an endemic setting is unclear.

For a more detailed understanding of the pathogenesis of the possible *C. burnetii* persistence in cattle, it is important to also consider what may have triggered *C. burnetii* shedding in milk at later stage lactation. Possible explanations for the shedding in milk at L5 include a recrudescence of infection or simply an increased technical ability to detect *C. burnetii* due to change in milk volume and, or, increased cellular constituents within the milk sampled. There are various changes that occur around the L5 time point of lactation (approximately 200 DIM) that may support either scenario. Regarding the recrudescence of infection scenario, the L5 period (approximately 200 DIM) often coincides with the onset of pregnancy in dairy cattle herds, and as pregnancy is considered a risk factor for chronic infection in humans (Maurin and Raoult, 1999), it was theorised pregnancy could have triggered shedding at L5. However, in the present study, not all cows shedding in milk at L5 were pregnant and therefore the rise in shedding from early (L3 and L4) to mid-lactation (L5) could not be explained by this factor (Chapter 3). Alternatively, in general, SCC (a combination of immune derived and mammary epithelial cells) increases as lactation progresses, and if *C. burnetii* were inside cells shed into milk, there may be an increased detection sensitivity at L5 (compared to early lactation [L3 and L4]). Indeed, within the L5

timepoint, *C. burnetii* presence in quarter milk (Chapter 5) was associated with a higher quarter SCC, with an association between *C. burnetii* presence in milk and increased SCC having also been identified in cattle before (Barlow et al., 2008). However, to determine if increased cell count was associated with an increased ability to detect *C. burnetii* in milk at L5, the SCC from milk shedding cows at L5 would need to be compared to their SCCs at earlier timepoints when they weren't shedding in milk.

To confirm and further characterise the potential persistent *C. burnetii* infection state in cattle, future targeted research into this question is needed, which is important considering such animals may pose a unique threat to zoonotic transmission from ongoing environmental contamination. The observational style of this study was valuable as it allowed investigation of natural *C. burnetii* infection, however, unknowns surrounding the time since infection and the potential re-exposures prevent certainty of persistence conclusions. Confirmation that *C. burnetii* from a single infection was present within the host during the seven-month period would be of benefit but even if daily sampling had been conducted in the present study, the potential that multiple *C. burnetii* exposures/infections explained ongoing detection in samples could not be ruled out. A further limitation is that an absence of shedding during a sampling would not necessarily mean the bacterium was not persisting within the host. Therefore, cattle experimental infection studies may be the preferred approach to investigate *C. burnetii* persistence, as the controlled environment allows interpretation of results considering a known *C. burnetii* inoculation date and an uncontaminated (non-endemic) environment. Confirmation of bacterial presence in the host from the single infection could be achieved by monitoring *C. burnetii* presence in excretion

routes or within tissue from sacrificed animals. As such, while visualisation of *C. burnetii* within the tissue would not be a requirement to determining persistence in cattle, it would aid understanding of the pathogenesis. However, visualisation is a challenging task as despite multiple ruminant studies having identified *C. burnetii* PCR positive mammary gland tissue, the bacterium has only once been visualised infecting cells of the mammary gland (using FISH), which is likely, in part, because visualisation assays can only scan small areas of tissue. An additional limitation to using experimental approaches to investigate *C. burnetii* persistence in cattle, is the likely difficultly of establishing persistent infection in an artificial environment, as the mechanisms by which this occurs naturally in cattle is unknown and may be influenced by a combination of complex factors, including the *C. burnetii* strain, the *C. burnetii* dose during transmission, pregnancy status or individual animal immune response preference. Chronic *C. burnetii* infection has previously been established in athymic mice models (Kishimoto et al., 1978) but the relevance of results derived from such models to natural infection is uncertain. Therefore, progress on the understanding of *C. burnetii* persistence in cattle will be made by a combination of both observational and experimental study types. Opportunities to use observational cattle studies to build on the current and existing observational work could include further investigation of *C. burnetii* phase specific antibody patterns to determine whether persistently infected cows may display similar patterns to chronically infected humans, thereby providing further support to the cattle persistence hypothesis. More specifically, in humans, chronic Q fever can be diagnosed based on immune profiles such as *C. burnetii* phase specific antibody response, whereby affected individuals exhibit higher antibody titres against phase 1 antigen compared to acute cases (Melenotte et al., 2020). Similarly, there is evidence, albeit from a limited number of studies, to suggest cows shedding in milk also display a higher average phase 1 antibody

response compared to non-shedding animals, but this varies on the individual level and therefore requires further investigation (Boettcher et al., 2017). Finally, if feasible, future observation studies should more frequently collect milk samples from cows to avoid misclassifying milk shedding cows, as *C. burnetii* is known to be shed intermittently in milk (Guatteo et al., 2007).

6.2 Investigation of immune factors influencing *Coxiella burnetii* shedding persistence in cows

The impact and progression of potential pathogen infection is determined by the interaction of host, pathogen, and environment factors. In humans, persistent focalised Q fever infection patients are unable to clear infection, and while the mechanisms behind this are poorly understood, the cell mediated immune response is likely integral due to the obligate intracellular nature of *C. burnetii*. However, very few studies have attempted to measure CMI in ruminants within this specific disease context, likely due to several factors, including a limited knowledge base on the application of suitable approaches to measure CMI response, especially for observational *C. burnetii* infection studies in cattle. Informed use of assays is essential to effective investigation, with the steps taken to optimise the methodology of a cytokine recall assay for assessment of *C. burnetii* specific production of IFNγ and IL-10, outlined in Chapter 2. Key findings included a significantly stronger cytokine response from samples collected at approximately eight weeks post calving compared to the calving timepoint (2.04- and 1.86-times higher for IFNγ and IL-10 respectively; Table 3.5). This study also optimised technical assay variables, finding storage of samples at ambient temperature

during transport, and a 48-hour co-incubation of stimulation media and blood samples, resulted in an increased cytokine response (Table 3.2 and Table 3.3). However, the IFNγ and IL-10 response was not the same for all tested variables, likely as these cytokines are produced through varying mechanisms, which highlights the importance of optimising conditions whenever a different cytokine is measured with this assay. For example, using freshly prepared stimulation media led to a higher IL-10 responses to *C. burnetii* compared to pre-prepared media, whereas for IFNγ there was no difference between the media preparation methods (Table 2.2), with the ultimate decision to use pre-prepared stimulation media also considering the need for scalability. The assay conditions optimised in the present study were then applied within the longitudinal study to effectively investigate the impact of the CMI response on pathogenesis, particularly whether IFNγ or IL-10 may have influenced the shedding outcomes (Chapter 3) and milk production outcomes (Chapter 4).

IFNγ is an important TH1 cytokine that aids in intracellular death of *C. burnetii* (via apoptosis of mammalian cells) and may have a role in pathogen persistence outcomes in infected individuals (Dellacasagrande et al., 1999). For example, in one human study (Pennings et al., 2015), unstimulated serum levels of INFγ in infected patients with chronic vascular foci were lower compared to patients with acute Q fever, while low IFNγ levels have also been significantly associated with milk shedding in two separate cattle studies of endemically infected herds in Poland and Germany (Boettcher, 2017, Małaczewska et al., 2018). However, the role of IFNγ is complex, as in another study (Schoffelen et al., 2017) there was no dysregulation in the ability of cells from chronically infected human patients to produce IFNγ in response to *C. burnetii* antigen stimulation compared to healthy individuals, possibly

indicating that an upstream factor downregulated IFNγ production in the former research. The findings from the present study accentuate the complexity of the relationship between IFNγ and *C. burnetii* persistence, as while L4 IFNγ response was low in all cows that subsequently shed in milk at L5, there was no significant association between these two variables likely because there were also cows with a low IFNγ response at L4 that did not subsequently shed in milk at L5 (Figure 3.5A). To further understand the role of IFNγ in cattle, measurement of this cytokine should occur at the onset of *C. burnetii* infection in cows that subsequently become persistently infected and also within their milk or udder tissue to examine any local mammary gland effect. There may also be value in focusing on the influence of other cytokines as they may impact persistence of infection and shedding. The present study was the first to measure *C. burnetii* specific IL-10 production in cattle using the cytokine recall assay but found IL-10 response at L4 was not associated either statistically or by visual trends with either milk shedding at L5 (Figure 3.5B) or placental infection at L2 (Figure 3.6B).

Therefore, while the current study observed the low IFNγ response from L5 shedding cows was not specifically a determining factor in persistence of infection (given this response was also reported in non-persistently infected cows), it does still more broadly indicate there may be a deficiency in their TH1 immune response. When combined with the persistently high antibody titres and the significant association between antibody titre at L4 and *C. burnetii* shedding at L5 (Table 3.6), there is evidence for a TH2 immune response dominance in these cows. Evidence that a TH2 dominated immune response profile may be deterministic in pathogen persistence is strengthened by the uniqueness of the pattern, as

cows with an infected placenta (at calving) displayed a varied IFNγ and antibody response at L4 (Figure 3.6). The low TH1 immune response from L5 shedding cows may fail to effectively clear intracellular *C. burnetii*, while the high antibody titres could potentially aid the bacterial pathogenesis as an *in vitro* study found opsonised (IgG) *C. burnetii* exhibit higher replication inside human macrophages compared to non-opsonised *C. burnetii*, and the vacuoles with opsonised bacterium did not convert to phagolysosomes (by day nine post infection) (Desnues et al., 2009).

6.3 Opportunities to influence persistent infection in cattle: future research directions

An opportunity to further enhance knowledge of the relationships between the host immune response and *C. burnetii* infection and persistence of infection might include development of studies to determine why the cows that are shedding *C. burnetii* in milk at L5 might favour a TH2 response, with some proposed variables of interest outlined in Table 6.1. It is important to understand the risk factors for ineffective bacterial clearance by the host as this knowledge may assist in the prevention or treatment of *C. burnetii* persistent infection and thereby reduce the associated zoonotic risk from ongoing shedding. Vaccination of heifers is one strategy that has been shown to prevent *C. burnetii* shedding (Guatteo et al., 2008), as this process is underpinned by enhancing the host immune response to control *C. burnetii* based on an optimised dose, route of administration, and antigen structure. However, animal approved vaccines against *C. burnetii* are not available in all countries (such as Australia). Therefore, there is value in understanding how persistence arises to enable more targeted and hence effective control solutions. For example, human

research has revealed genetic variation in cytokine genes between Q fever fatigue syndrome or Q fever endocarditis patients and unexposed individuals which may indicate genetically controlled immune responses determine *C. burnetii* persistence in the host (Helbig et al., 2005). In this instance, prevention of persistent infection in dairy cattle may be possible by selective breeding to avoid maintaining genetics that favour *C. burnetii* persistence in a herd. However, regardless of the causal mechanisms, in any animal where treatment is required, accurate detection of persistently infected cows would be essential, with findings from Chapter 3 indicating a S/P% of approximately 100 using the commercially available combined phase 1 and 2 *C. burnetii* IDEXX ELISA kit may hold value in distinguishing such animals over the duration of lactation.

There are a variety of factors (Table 6.1), that could pre-dispose individual animals to persistent *C. burnetii* infection, such as the site of infection, which was briefly explored in Chapter 3. The udder microenvironment was speculated to facilitate persistence given that shedding reappeared at later stages of lactation predominately in milk, and that *in vitro*, bovine epithelial cells from the mammary gland had lower pro-inflammatory cytokine response to *C. burnetii* infection compared to the lung, placenta, and intestine (Sobotta et al., 2017). However, in the current study, it was observed that there were cows from the study herd shedding *C. burnetii* in their milk at calving that did not go on to shed at 200 DIM, nor exhibit a persistently high antibody titre. While this suggests mammary gland infection (here measured at time of calving) is not the sole determining factor in persistence (and later shedding) within a cow, a lack of understanding about whether shedding in milk correlates to mammary gland infection or systemic infection, limits the utility of this

observation (Chapter 5). It is important to establish in which tissue or compartment *C. burnetii* could be persisting in chronically infected cows, whether this be the mammary gland or another site such as heart valves, as this knowledge helps refine causal mechanisms. For example, *C. burnetii* persistence in heart valves of endocarditis patients has been associated with the risk factor of pre-exiting valve pathology (Eldin et al., 2017). To further progress understanding of the mechanisms behind persistence of *C. burnetii* infection in dairy cattle, the factors outlined in Table 6.1 could be addressed in future longitudinal experimental or observational studies.

Table 6.1. Summary of potential reasons for T Helper 2 (TH2) immune preference in persistently infected cows categorised according to host, pathogen or environment factors and brief outline of possible future experimental investigations.

Category	Variable	Explanation	Experiment
Host	Time of infection	The host immune response and availability of tissue can change throughout a dairy cow lactation cycle, for example, pregnancy induces a TH2 dominance while placental tissue is only present during pregnancy (Vlasova and Saif, 2021).	Experimental infection to allow controlled inoculation of *C. burnetii* at different time points, for example, before and during stages of pregnancy.
	Site of infection	The microenvironment of tissues from different sites in the host can vary, including available nutrients and immune response from cells which may influence *C. burnetii's* ability to persist.	Experimental infection or observational to determine where *C. burnetii* resides within the persistently infected cattle host using visualisation techniques such as immunohistochemistry or FISH.
	Genetics	Absence or presence of genetic trait may prevent effective host immune response against *C. burnetii*, with variation in cytokine genes having been observed between chronically infected (Q fever endocarditis patients) and unexposed humans (Helbig et al., 2005).	Experimental infection or observational to measure immune factors such as cytokines (additional to IFNγ and IL-10) using the scaffold outlined in Chapter 2 or other methods such as gene expression analysis or whole genome sequencing.
Pathogen	*C. burnetii* strain	Pathogenicity may vary by *C. burnetii* strain due to presence or absence of certain genes that may manipulate host cellular immune response. For example, in the hypervirulent epidemic *C. burnetii* strain, Cb 175, a genetic deletion (and thus genome reduction compared to the Nine Mile strain) is thought to be a possible cause of virulence (D'Amato et al., 2015).	Experimental infection or observational to characterise *C. burnetii* strains from persistently and non-persistently infected cows.
Environment	*C. burnetii* infective dose	Smaller or larger doses of *C. burnetii* (during transmission) may influence the host immune response (Miller et al., 2021), for example, low doses may not trigger effective immunity to clear the bacteria.	Experimental infection to allow controlled inoculation of *C. burnetii* at different doses.
	Route of infection	Different transmission routes may influence the immune response to *C. burnetii* infection (Miller et al., 2021), for example, the number and type of immune cells may differ upon entry via inhalation compared to the teat canal or ingestion.	Experimental infection to allow controlled inoculation of *C. burnetii* by different routes, for example by inhalation, ingestion, intramammary.

6.4 Relationship between *Coxiella burnetii* milk shedding, mammary gland health, and milk production.

The study described in Chapter 4, sought to determine whether the infection states and exposures described in Chapter 3 were associated with milk production losses, thereby adding an additional knowledge layer to *C. burnetii* pathogenesis within the dairy production context, and exploring the potential economic impact of *C. burnetii* infection. Coxiellosis can cause reproductive loss in cattle (Agerholm, 2013), but it is unclear whether natural infection may impact on factors influencing milk production and milk quality, including milk volume and total milk solids. In the current study, there was limited evidence for cows shedding *C. burnetii* in milk at L5 having significantly different daily volume (estimate= 2.54 L/cow/day; 95% CI: -1.07 to 6.15) and daily total solids (estimate = 0.23 kg/cow/day; 95% CI: -0.07 to 0.53), compared to cows not shedding in milk at L5 (Figure 4.4). This finding agreed with the research by Freick et al. (2017) that also found no markedly different average 305-day milk volume, fat or protein responses between cows shedding and not shedding in milk at 100 and/or 150 DIM. However, a drop in milk production in cows challenged via intramammary inoculation of *C. burnetii* infected yolk-sac culture has been reported in one previous study (Ormsbee, 1951) but the relevance of this inoculation method to natural infection is unclear. Furthermore, whether the cows shedding in milk in the present study had an established *C. burnetii* IMI is unknown because, as discussed in Chapter 5, there are two scenarios by which *C. burnetii* could be present in the milk. Firstly, *C. burnetii* may colonise the mammary gland and infect mammary epithelial cells which is supported by work visualising *C. burnetii* in the mammary epithelial cells of a goat doe that was shedding in milk via FISH methodology (Bauer et al., 2024). The second scenario is that *C. burnetii* may reach the milk

compartment inside immune cells that pass from circulation to the mammary gland via the blood-milk barrier. Previous human research into *Salmonella enterica* serotype Typhimurium similarly speculated this bacterium's presence within milk may have been due to paracellular transportation through the blood milk barrier within macrophages originating from the gut (Qutaishat et al., 2003). Therefore, given the uncertainty about *C. burnetii*'s interaction with mammary gland tissue, an investigation of quarter level SCC from L5 samples was conducted (Chapter 5) to further characterise the relationship between *C. burnetii* and the mammary gland, as SCC is used a measure of mammary gland health in the dairy industry.

Conventionally in the scientific literature of bovine mastitis, a raised SCC arises due to an IMI with a pathogen causing an inflammatory response in the udder (which is often accompanied by shedding in milk of that pathogen). The present study therefore investigated whether *C. burnetii* could cause a raised SCC, and found *C. burnetii* presence in quarter milk at L5 was associated with a 2.84 times higher quarter SCC, compared to quarter samples without *C. burnetii* present (95% CI: 1.5 – 5.3; p = 0.001) (Table 5.4). A similar association was also reported by Barlow et al. (2008) that reported a positive association between *C. burnetii* in composite milk and SCC. The potential for a local immunogenic effect of *C. burnetii* infection in the mammary gland is supported by evidence showing *C. burnetii* infection of the placenta can cause placentitis (immune inflammation of the placenta) in experimentally inoculated pregnant goat does (Sánchez et al., 2006). Furthermore, a scenario where *C. burnetii* could raise SCC (Chapter 5) without affecting milk volume or solids (Chapter 4) may also be plausible, as *C. burnetii* has been associated with low levels of inflammation in heart valves of Q fever endocarditis patients (Lepidi et al., 2003), which if

mirrored in the mammary gland could raise SCC without damaging milk producing tissue. However, an alternate hypothesis for the raised SCC and unaffected milk volume and solids was also developed. Namely, that *C. burnetii* was not infecting the mammary epithelial cells (thereby not damaging the milk producing tissue) and was only detected in milk with raised SCC as it was within immune cells derived from other tissues or compartments. Analysis of the data assuming this hypothesis (whereby SCC was the exposure variable, and *C. burnetii* was the outcome variable) also reported a significant association (Table 5.4).

The ultimate ability to conclude which causal hypothesis correctly explains the positive association between *C. burnetii* and SCC requires establishment of the sequence of events, as temporality is a key requirement for causation. Therefore, it must be determined whether *C. burnetii* presence in milk is followed by a raised SCC or whether a raised SCC is first required before *C. burnetii* is present in milk. There is incentive to determining the answer, given there is an economic cost associated with raised SCC in bulk milk in developed dairy industries, whereby processors may penalise producers with elevated total SCCs. Any future studies (especially observational) aiming to investigate causation would benefit from utilising DAGs, as Chapter 5 demonstrated both their application and usefulness in navigating causal thinking about complex relationships during study design, analysis, and interpretation of results. Alternatively, experimental infection would allow assessment of the relationship with reduced potential confounding (such as from mastitis pathogens) but the limitations surrounding the relevance of results from this study type has already been discussed. There is opportunity to expand on the existing experimental studies by assessing SCC from cows challenged by the inhalation route.

6.5 *Coxiella burnetii* intrauterine infection in dairy cattle is associated with reduction in milk volume and total solids

An infection in the mammary gland is not the only mechanism by which milk production losses can occur, and therefore, the effect of other measures of *C. burnetii* exposure on milk production were also investigated. Cows with a placental infection at calving had lower average daily volume over the course of the subsequent lactation, estimated as 2.4 L/cow/d less (95% CI: -4.39 to -0.37), while cows with *C. burnetii* detected by a vaginal swab at calving had significantly lower daily total milk solids over the course of the subsequent lactation, estimated as 0.2 kg/cow/d less (95% CI: -4.9 to 1.41) (Table 4.2). An association between *C. burnetii* infection and volume and total solids reduction has not previously been reported in cattle. In goat does, high shedding in vaginal mucus was estimated to result in 0.53L/day lower volume than non-vaginal shedding cows (Canevari et al., 2018). As this study found cows shedding *C. burnetii* through milk did not have lower volume or solids, it may be hypothesised that *C. burnetii* does not directly impact milk producing mammary epithelial cells, and instead, the loss associated with intrauterine-infected cows at calving may arise due to a systemic effect. It is also important to consider that the loss in volume and solids was observed throughout the entire lactation (Figure 4.2 and 4.3) when hypothesising the causal mechanism behind the association. One suggested explanation was that if a sudden shift in intrauterine dynamics occurred around calving, an associated inflammatory response could potentially make the cow unwell during the vital transition period, thereby reducing dry matter intake or disrupting mammary gland development, which can influence milk production over the entire lactation. In the present study, neither antibody nor cytokine

response in early lactation were associated with production outcomes, which was also supported by the varied antibody, IFNγ, and IL-10 response from cows with a placental infection described in Chapter 3. Future work could assess these factors at calving, but the possible immune suppression at this time may make this difficult to discern for certain cytokines (Chapter 2), and therefore, acute phase proteins may be a valuable measure of inflammation at this time. Indirect factors such as core body temperature or dry matter intake of cows could be monitored over time to determine if there is a physiological change during the calving period associated with *C. burnetii* infection. Therefore, while the reason for milk production loss associated with *C. burnetii* infection of the products of conception remains unclear, these findings still show coxiellosis could have a significant economic impact for the dairy industry, and as such, should be replicated in diverse locations and production systems.

6.6 Summary of contribution of this thesis to the research field

At the onset, this thesis set out to fill knowledge gaps on *C. burnetii* infection dynamics in dairy cows, specifically to determine what factors influence shedding persistence and whether there is an impact of infection on milk production. An improved understanding around what causes certain cows to shed *C. burnetii* persistently would allow development of targeted effective prevention and treatment strategies which is important as ongoing shedding can contaminate the environment leading to zoonotic transmission. Likewise, defining the impact of *C. burnetii* on milk production is valuable as it allows assessment of

the potential cost of infection to the dairy industry which includes the financial and welfare consequences for affected producers and cows, respectively.

In agreement with the relatively few studies that have longitudinally investigated *C. burnetii* infection in endemically infected dairy cow herds, the present investigation found *C. burnetii* could be shed in milk considerably after the calving period. The additional description of individual cow patterns over approximately seven months was a strength of this work as it helped add support for a persistent infection state in cows, given the L5 shedding cows (13/133) generally had persistently high antibody titres over the entire sampling period and that half (6/13) had also shed at calving (through placenta, vaginal mucus, faeces, or colostrum). The CMI responses are not commonly described in cattle studies but add a valuable detail for infection dynamics given *C. burnetii* is an intracellular bacterium. Prior to subsequent shedding, the L5 milk shedding cows were found to produce a low amount of the important TH1 cytokine, IFNγ, which together with the strong antibody response indicated a TH2 dominance may be deterministic in the persistence outcome. However, unlike two previous European cattle studies that investigated cytokine responses in endemically infected herds (Boettcher, 2017, Małaczewska et al., 2018), the present work found no statistical relationship between IFNγ and milk shedding, with evidence that some cows with low IFNγ response at L4 (that had been confirmed infected at L2) did not become subsequent shedders at L5. The disagreement on the patterns of IFNγ for *C. burnetii* milk shedding cows between studies adds a different perspective to the current *C. burnetii* pathogenesis knowledge in cattle and highlights the need for further research into clarifying the role of IFNγ in infection persistence. It is anticipated that this study's optimisation of the

cytokine recall assay for use with cattle samples may provide more opportunities for others to investigate cytokine responses in this species.

The second main objective of this thesis was to investigate the production impact of *C. burnetii* infection, which provides both an additional layer to the pathogenesis of the above-described infection states as well as an assessment of the potential economic impact of infection. The persistently infected cows showed no difference in average daily volume or total solid compared to the non-infected group, but did have significantly higher SCC. In the literature there have been contrasting findings about an association between *C. burnetii* infection and elevated SCC (Angen et al., 2011, Barlow et al., 2008), and therefore, this study helped provide clarity on the topic by adding support for the presence of a positive association using quarter level samples and accounting for the influence of possible confounding variables. A key point of additional value in this study was the exploration of causation, as while most existing work on this topic has investigated the idea that *C. burnetii* may cause an increased SCC, here, two equally plausible causal hypotheses were proposed to explain the association, namely that *C. burnetii* causes an increased SCC and that a raised SCC increases presence of *C. burnetii* in milk. Raised SCC is an important issue for the dairy industry, with penalties for producers from high bulk tank SCC, but only the former hypothesis would make *C. burnetii* a potential problem in this regard, and therefore, future work is needed to confirm which hypothesis is true. Longitudinal studies are required to determine the sequence of events leading to the positive association between *C. burnetii* and SCC with a demonstration of the use of DAGs to navigate complex causal relationships having also been described in this thesis to help guide future related research.

Finally, this study also showed that coxiellosis could be an important disease for the dairy industry as an intrauterine infection was associated with milk production loss, specifically 2.4L/cow/d lower volume and 0.2kg/cow/d lower total solids from cows with a *C. burnetii* detected in the placenta and vaginal fluid respectively. An association between *C. burnetii* infection and reduction in volume has only previously been found in high vaginal shedding Australian goat does (compared to non vaginal shedding goat does) (Canevari et al., 2018), while this is the first study to find a reduction in total solids from ruminants. Given the potential economic importance of these findings, the robustness of the results should be determined by repeating the study, including in diverse dairy management systems and countries (where *C. burnetii* strains may differ). Following on from potential confirmation of results, research should then focus on interventions to prevent milk loss, such as prophylactic vaccination to prevent *C. burnetii* infection. Targeted treatment of intrauterine infected cows may also help reduce milk production loss, although the effectiveness of this option would be improved by understanding the causal mechanism, for example, if it was known that a reduced dry matter intake around calving caused the milk loss, these cows could be nutritionally supplemented.

Therefore, it is evident this thesis has helped further elucidate the *C. burnetii* infection dynamics in dairy cattle, with particular respect to the mammary gland and lactation, and directed greater awareness to the negative impacts of coxiellosis on milk production in this species. This thesis has also provided development and demonstration of tools, namely the

C. burnetii cytokine recall assay and DAGs, respectively, that can be applied in future studies to further enhance *C. burnetii* pathogenesis knowledge in cattle.

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