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Resilience on-farm: mechanisms, markers and applications

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Abstract

This project applied state-of-the-art technology in gastrointestinal immunology and genomics, transcriptomics and microbiome analysis to determine the impacts of co-infections with *Haemonchus contortus* (Hc) and *Mycobacterium avium* subspecies *paratuberculosis* (Mptb/MAP) on weight gain, gut microbiota and immune performance to elucidate determinants of efficient growth, reproduction and gastrointestinal disease resilience. In addition, a range of outstanding issues regarding vaccination, detection, diagnosis and control of Johne's disease (JD) in sheep and cattle were also investigated. Because of its scope, the project was split into five sub-projects.

While the combined impact of co-infection reduced weight gains and increased the severity of JD, suppressive drenching supported weight gains, but the Mptb pathology was also increased, possibly due to delayed immune responses. Hence "over-drenching" is not advisable.High levels of Hc infestation were prevented by monthly rotational grazing but compromised re-infection to consolidate resilience scores.

A resilience matrix was developed and genomic analyses were applied to animals in the co-infection trial and historical data from 2 previous OJD trials. While the small number of suitable animals (50) in the co-infection trial did not permit definitive markers to be determined, analysis of 17 susceptible and 637 resistant sheep from previous OJD studies revealed a region (5 SNPs) on chromosome 1 that was found significant for resilience in the P.PSH.0576 trial and one SNP on chromosome 7 was found significant for resistant/susceptible phenotype in the OJD.028 project. This requires validation in larger and more current datasets.

The project also confirmed that the interferon gamma (IFNγ) test and the IgG1 antibody ELISA can be used to screen adult sheep for potential exposure to MAP on nonvaccinating farms. In addition, several microRNA candidates were identified that have the potential to be used for routine diagnostic testing by PCR, for detecting animals exposed to MAP or resilient to MAP infection. These also require validation in Industry flocks.

Environmental risk factors of JD disease transmission were considered with respect to the spread of the different strain types of MAP. The outcomes recommend that advice be provided to producers regarding the potential risks of co-grazing cattle, particularly age-susceptible (<2 year old) cattle, with Gudair ® vaccinated sheep, and that Integrated Parasite Management (IPM) control of wormy pastures (from sheep) by grazing with cattle should be conducted with appropriate caution in ovine JD endemic regions. The HT-J test that has recently been evaluated for pooled testing in beef cattle (B.AHE.0322), and together with the strain-specific qPCR assays developed in this Sub-project could be conducted along with routine Johne's Beef Assurance Score (JBAS) testing of cattle through the further development of a multiplex assay, such that the full distribution of cross-species transmission can be assessed.

Mycobacterial evolutional genetics and virulence factors were examined using whole genome sequencing, creating a diagnostic database. Examination of the diversity in genotypes of *H. contortus* currently circulating in regions of Australia also produced an early compendium of sequence data that would be available via Genbank/SRA in the National Centre for Biotechnology Information (NCBI) and be available for studies on drench resistance.

Investigations on Gudair® use and cessation of vaccination were also conducted, revealing that 88% of sheep producers surveyed have continued to vaccinate their sheep with Gudair®, with continuation greater for predominantly Merino sheep flocks. Reasons for discontinuing vaccination stemmed from management, economic, or health concerns. However, it is gratifying that the results suggest that Gudair® is still widely used by Australian sheep producers and concerns about large-scale discontinuation are unfounded.

Executive summary

Precise, validated benchmarking tools for animal health and welfare ensures the ability to select, breed and produce animals "fit for purpose", underpinning the profitability and sustainability of livestock enterprises. It is well-documented that under endemic pathogen challenge, some individuals within and between breeds remain productive while others suffer. This "resilience" is the summation of the response of an animal to challenges associated with performance, plus genetic constitution that contributes to the overall productive measures. Resilience maybe a host trait rather than pathogen specific. The actual basis of resilience is not known but is considered an interaction between foraging ability, intake, food conversion efficiency and physiological responses to parasitism.

Complex interactions between chronic infections such as nematodiasis, fasciolosis, and *Mycobacterium avium* subspecies *paratuberculosis* (Mptb/MAP) on host immunity and productivity have been limited by technology which have required study on single entities using reductionist paradigms. New technology makes it possible to apply a systems approach to study the whole animal impact of nutrition and gastrointestinal pathogens.

This project applied state-of-the-art technology in gastrointestinal immunology and genomics, transcriptomics and microbiome analysis to determine the impacts of co-infections with pathogens and parasites on productivity, gut microbiota and immune performance to elucidate determinants of efficient growth, reproduction and gastrointestinal disease resilience. In addition, a range of outstanding issues regarding vaccination, detection, diagnosis and control of Johne's disease (JD) in sheep and cattle were also investigated. For efficient coverage of this broad scope, the investigation was conducted as five sub-projects. Detailed descriptions of materials and methods for each of the sub-projects are contained in the separate appendices to this report.

All trials in this project were conducted in accordance with guidelines prescribed by the University of Sydney Human and Animal Ethics Committee proposals 2017/824, 2017/1245 and 2017/1249.

Sub-project 1: Understanding gut pathogen inter-relationships

The sub-project utilised archival material from JD trials to enable a marker approach to define the resilience phenotype and conducted an **experimental co-infection trial** with *Haemonchus contortus* (Hc) and *Mycobacterium avium* subspecies *paratuberculosis* (Mptb/MAP) to assess resilience phenotypes. The samples and monitoring of the 94 sheep in treatment groups were used in this and in other sub-projects to assess the interaction between the pathogens to define resilience to infection at production, pathological, immunological and host genomic levels.

Inoculation with 800 *H. contortus* larvae (HcL3) produced the desired haemonchosis infection with individual variation in faecal worm egg count (FWEC), without requiring treatment of more than a few sheep with an anthelminthic. The management option to rotate paddocks at 4-week intervals through 7 paddocks, maintained a low but constant FWEC in sentinel control groups but limited phenotypic expression of resilience (unimpaired productivity, despite parasitosis) as treated sheep did not become significantly re-infected. Exposure of sheep to 3.03x 10^9 live Mptb in total across 3 doses, produced clinical paratuberculosis and evident wasting in groups infected with the

bacterium, although there was still a spectrum of disease seen across individuals in these groups. The combined co-infection group had reduced weight towards the end of the trial compared to the Hc exposed and control groups and results also confirmed that "suppressive" monthly drenching, without Mptb exposure, enabled the highest Average Daily Live Weight Gain (ADLWG) (37 g/day). Interestingly, suppressive drenching enhanced the development of clinical disease in the Mptb group, possibly due to an immunosuppressive effect that reduced antibacterial responses, though this requires further investigation. The practical manifestation of this finding is that "over-drenching" is not advisable and has the potential to exascerbate co-morbidities, in addition to the known negative consequences with regards to development of drench resistance. Sheep co-infected with both Hc and Mptb had the higher likelihood of faecal shedding compared to *Mptb* exposure alone, and also suffered greater pathology. Faecal samples collected at 5 weeks post treatment and at necropsy (1 year) identified rapid and ongoing changes to the gut microbiome arising from the co-infections.

While the successful coinfection allowed sampling and in-depth examination of the resilience phenotype and genotype in Merino weathers under Australian production conditions, the numbers of animals were insufficient to enable identification and validation of predictive markers (see SP2). This was anticipated both from the exceptionally efficient worm control which did not permit susceptible sheep to become reinfected from pasture after their first drench and the inability to obtain sufficient Haemonchus DNA from archived samples.

Therefore, for the further definition of markers for resilience it is recommended that larger trials be either conducted on farms with records for production (e.g. weight, desirable carcass traits, fleece weights) together with disease traits (e.g. using rams with ASBVs for parasite faecal worm egg counts-FWEC or OJD status). Those farms involved in the Merino Lifetime Productivity (MLP) program and those farms surveyed in SP5 should be suitable. Data can be gathered from these farms or collated from existing records and subjected to genomic analysis as performed in SP2 or marker validation for superior immune performance as detailed in allied MLADC project P.PSH.0816. By comparison, genomic and biomarkers along with new tests for resistance to OJD, with direct application for validation in disease management were developed from the co-infection trial together with archived datasets and samples (SPs 3,4 &5). These also require validation on farm.

Sub-project 2: Genetic markers for a resilience phenotype

The overall objectives of SP2 were to develop an index for resilience, and to identify genetic markers for resilience, including disease resistance. Resilience in the context of animal production relates to the capacity of an animal to continue producing in the face of physical or physiological stressors, including disease. To maximise the power of gene discovery, the information generated from the current and two previous experiments were included. This includes 1) the data from 50 sheep from the first three treatment groups from the current trial (P.PSH.0813) where animals were challenged with *H.contortus* and/or *M.paratuberculosis* or were kept as control; 2) the data on 279 sheep from a previous experiment (P.PSH.0576); and 3) the data on 654 animals from the experimental trial OJD.028. These consisted of 17 susceptible and 637 resistant sheep as diagnosed by histopathological screening.

A combined resilient index phenotype was developed by integrating three different phenotypes associated with disease burden and production gain viz. MAP infection, parasite load and

performance (weight gain) of the animal. For trial P.PSH.0576, only weight gain and ParaTb_score3 could be obtained from the available data. The samples from all the animals in the current trial (P.PSH.0813) and from the experiment OJD.028 were genotyped for 50K SNPs. The genotypes on 600K SNPs were available for the animals in trial P.PSH.0576. Genome-wide association analysis was implemented with a mixed model to identify genetic markers associated with susceptible/resistant binary phenotype (case control response variable) or the quantitative phenotype (Resilience Index).

This study developed a flexible working model for resilience in sheep (a resilience index). However, the current trial was not powerful enough for detecting statistically significant genetic markers associated with the resilience index. This needs to be applied to a larger cohort on farm to obtain a workable dataset. However, a region (5 SNPs) on chromosome 1 was found significant for resilience in project P.PSH.0576 and one SNP on chromosome 7 was found significant for resistant/susceptible phenotype in project OJD.028. The closest gene PSM3A on chromosome 7 is relevant to the bacterial immune response. Samples from farms involved in SP5 or faeces from endemic farms would be ideal to validate these markers.

Sub-project 3: Host response biomarkers

Sub-project 3 encompassed assessing the reliability of previously identified biomarkers for resilience and susceptibility to paratuberculosis as well as using new technologies to identify other novel biomarkers. Previously markers of paratuberculosis disease expression were identified using experimental challenge models of infection under controlled experimental conditions. Production outcomes can be impacted by multiple factors, pathogen burden being just one. Here, we aimed to evaluate if these were effective biomarkers for identifying disease resilience and susceptibility in commercial flocks, especially in flocks that are vaccinated against paratuberculosis, as well as in the experimental co-challenge model described in Sub-project 1. We have identified two tests that can be used to screen adult sheep for potential exposure to MAP on non-vaccinating farms: the interferon gamma (IFNγ) test and the IgG1 antibody ELISA. These measures can be used to determine freedom from exposure to paratuberculosis if the industry requires such a measure in individual non-vaccinated animals. While detection of MAP in faeces is an excellent farm-wide screening tool for diagnosing disease, these immunological measures are tools which can be used in individual animals for trade or breeding purposes. In addition, we have identified another immunological marker which requires further study in commercial operations as a potential biomarker for identifying MAP infected animals, regardless of vaccination status.

In Sub-project 3, new technologies such as Next Generation Sequencing and genetic manipulation in a relevant mycobacterial infection model were used. We screened our biobank of archived samples created from previous MLA-funded studies to broaden the scope of identifying other potential biomarkers for disease resilience and susceptibility. Several microRNA candidates were identified. These have the potential to be used for routine diagnostic testing by PCR, for detecting animals exposed to MAP or resilient to MAP infection were identified. We have explored the effect of a selection of these microRNA on their target mRNAs and downstream contribution to immunity using a zebrafish model for mycobacterial infection. The zebrafish model is a suitable economical alternative which can reduce the use of ruminants for some screening and validatory aspects of paratuberculosis research.

There is a lack of understanding of immune modulation mechanisms which ultimately impact disease manifestation, animal welfare and associated production losses in the face of multiple pathogen burden in sheep. Investing in such research programs will develop methods of early detection of animals with dysfunctional/inadequate immune systems and provide strategies to boost immune function and better outcomes. Rather than under controlled experimental conditions, evaluation should be conducted on farms which may be confronting production losses despite best practice control strategies with a view to identifying early predictive biomarkers for nonproductive animals (eg. some canvassed in SP5). Investment into basic research to expand knowledge of the functional aspects of miRNA in disease manifestation could lead to the development of therapeutics to drive better immune modulation and protection against disease.

Sub-project 4: Pathogen-host interactions

Sub-project 4 focused on the Pathogen and Environmental factors within the "Pathogen-Host-Environment" epidemiological triangle, focusing on two insidious pathogens of livestock; *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the causative agent of Johne's disease (JD), and *Haemonchus contortus.* Environmental risk factors of JD disease transmission were considered with respect to the spread of the different strain types of MAP, as well as broader aspects of mycobacterial pathogenicity. Mycobacterial evolutional genetics and virulence factors were examined using whole genome sequencing. The diversity in genotypes of *H. contortus* currently circulating in regions of Australia was also studied.

Historically, the various strains of MAP in Australia have been associated with their respective host species; MAP sheep (S) strain was isolated from sheep and MAP cattle (C) strain from cattle. With changes to the National Bovine JD (BJD) control program, there are ramifications of a positive diagnosis for an individual producer irrespective of the causative MAP strain type. This, as well as increasing report of S strain infections of Mptb in cattle, was the impetus for a study to evaluate risk factors associated with 'cross-species' infection and assess current diagnostic tools for the two main Mptb strain types (C and S strains) important to the Australian livestock industries. A retrospective study of 43 properties across South Eastern Australia with S strain MAP infection diagnosed in beef cattle identified: (i) a range of beef cattle breeds in which S strain MAP infections were reported (Shorthorn, Murray Grey, Hereford, Charolais, Angus) with multiple clinical cases in cattle aged from 3-8 years, (ii) mixed enterprise farms (running both sheep and cattle) comprised the majority for which relevant herd history data was available, with 70% of these producers reporting co-grazing sheep and cattle and/or a history of ovine JD on-farm, (iii) most producers that reported co-grazing cattle and sheep and/or ovine JD history had been vaccinating with Gudair®. The recommendations from this study are for producers to know the JD status of neighbours and trading partners in relation to all livestock species on-farm, for additional advice to be provided to producers regarding the potential risks of co-grazing cattle, particularly age-susceptible (<2 year old) cattle, with vaccinated sheep, and Integrated Pasture Management (IPM) control of wormy pastures (from sheep) through grazing with cattle should be conducted with appropriate caution in ovine JD endemic regions.

A study on diagnostic tests for identifying MAP strains identified a need for a rapid diagnostic test that could identify the strains present in culture, particularly in the case of a mixed infection (both C and S strain MAP infecting the same host). MAP strain-specific gene target assays were developed

and validated based on genes present only in C strain or in S strain MAP, with specificity confirmed bioinformatically using a global collection of 400 whole genome sequenced MAP isolates. A simulated mixed infection study confirmed that the growth of both C and S strains of MAP are supported within mixed cultures in M7H9C liquid culture media, and the new strain-specific qPCR assays could detect the growth of both C and S strains of MAP in these mixed cultures, which were unable to be distinguished using routine IS*1311* PCR REA testing. With most veterinary diagnostic laboratories shifting to qPCR-base diagnostics, these new strain-specific gene qPCR assays allow for rapid strain typing of MAP C and S strains using modern technologies, without the need for additional incubation and subculturing. There is an opportunity to develop a multiplex qPCR assay with these strain-specific genes combined with IS*900*, the target gene from MAP included in the High-Throughput Johne's (HT-J) test, to diagnose and strain type MAP simultaneously.

Whole genome sequencing of a range of mycobacterial species and *H.contorus* larval species was performed. This work applied cutting-edge next generation sequencing and built on collaborations within public health to develop expertise in whole genome sequence data analysis and bioinformatics pipelines for livestock animal health. Genetic studies such as have been undertaken in this collaboration expand the available sequences to enable the analysis of genetic diversity in mycobacterial and nematode species that will hopefully contribute to both human and animal health outcomes. While the focus of this project was markers for resilience, these additional tools tools can be further applied to aid in the understanding of mycobacterial and *H.contortus* genetic heterogeneity in the Australian setting, including genetic tests for anthelmintic resistance traits.

A detailed mycobacterial phylogenetic analysis showed an evolutionary split between rapid and slow growing species of mycobacteria, with rapid growing species being the ancestral species, as well as identifying a range of key genomic differences that shed light on the evolutionary path to become virulent pathogens. In addition, 47 MAP S strain isolates were successfully sequenced from long-term Gudair® vaccinating farms. To our knowledge, this is the largest number of MAP sheep strain genomes in the world. This identified a grouping of persistent isolates on the vaccinating farms, raising the question whether large-scale vaccination of sheep is leading to virulent strains that are persisting in flocks and may be spreading to cattle. It is unclear how virulent S strain MAP from vaccinated sheep is for cattle and whether this can become established within the species, with broader implications.

Based on these findings, wider research into the prevalence and virulence traits of S strain MAP in Australia should be undertaken. MAP strain typing is recommended to be conducted routinely on co-grazing farms or by producers purchasing animals from mixed farming enterprises to obtain a greater understanding of the prevalence, geographic distribution and risk of transmission of S strain MAP in cattle and whether mixed infections occur. The HT-J test has recently been evaluated for pooled testing in beef cattle (B.AHE.0322); the strain-specific qPCR assays developed in this Sub-project could be conducted along with routine Johne's Beef Assurance Score (JBAS) testing of cattle through the further development of a multiplex assay, such that the full distribution of cross-species transmission can be assessed and future applications discussed more widely. Pathogen genomics approaches developed in this sub-project can be applied to investigating farms where there is persistence of ovine JD or bovine JD despite vaccination or other control and management practices. This knowledge can also be applied to investigate the epidemiology and spread of MAP lineages.

Sub-project 5: Mptb shedding in vaccinates

Sub-project 5 examined the reasons and risk factors involved in decisions to cease vaccination with Gudair® and the continuing presence of shedding sheep on vaccinating properties. Vaccination with Gudair® has been a key strategy for controlling ovine Johne's disease in Australia since its approval in 2002. However, concerns have been raised about potential discontinuation of vaccination by producers after a decline in the incidence of clinical disease. Cessation of vaccination may not only compromise the management of this disease for individual producers but also jeopardise its control at a regional and national level. However, there is no objective information about the proportion of farmers who have discontinued Gudair® vaccination and their reasons for discontinuation. An online questionnaire was distributed to Australian sheep producers to identify the proportion of respondents discontinuing the Gudair® vaccine and reasons for discontinuation. Results revealed that 88% of sheep producers surveyed have continued to vaccinate their sheep with Gudair®, with continuation greater for predominantly Merino sheep flocks. Reasons for discontinuing vaccination stemmed from management, economic, or health concerns. These results suggest that Gudair® is still widely used by Australian sheep producers and concerns about large-scale discontinuation are unfounded. These findings have implications for ovine Johne's disease control programs in Australia.

Previous research conducted in Australia has demonstrated that Gudair® is quite effective in reducing sheep mortalities. While some farms have also been successful in reducing the prevalence of the disease in their flocks to undetectable levels, sheep in other flocks continue to shed Mptb in faeces even after an on-going vaccination program at the farm. A study was conducted to investigate the reasons for this differential effectiveness of the vaccine in different farms, specifically, to evaluate the management and husbandry factors that may be associated with Mptb faecal shedding in vaccinating flocks. We enrolled 64 sheep farmers and interviewed them to obtain information about their management and biosecurity practices. Pooled faecal samples were collected from sheep at each farm and cultured to create two outcome variables: Mptb positive (yes/no) and disease prevalence level (nil, <1%, ≥ 1%). Binary and ordinal logistic regression analyses were conducted to evaluate the association of management, husbandry and biosecurity factors with these outcome variables. Farms were more likely to have Mptb positive sheep and a higher disease prevalence in their flocks if they (a) provided supplementary feed on the ground (instead of in a trough), (b) had a greater number of neighbours with sheep, and (c) introduced rams from a greater number of sources. The results suggest the effectiveness Gudair® vaccination to control OJD can be improved if sheep producers maintain other risk management strategies and biosecurity practices. Extension agencies should advise farmers not to relax their biosecurity practices and to purchase rams from only low-risk sources, even if they are vaccinating their flocks with Gudair®. However, SP4 outcomes raised the possibility of shedding of S strains from vaccinated sheep "escape S strains" or "variants" which compromise individual control programs and cross-infect cattle. These may require "customised " vaccines, more intense testing using the HT-J test in combination with the strain-specific assays developed in this project, as well as updated recommendations as specified in sub-project 4 to limit co-grazing of pastures to control disease spread successfully.

Index of Abbreviations

Average daily liveweight gain: ADG

Basic Local Alignment Search Tool: BLAST

Bovine tuberculosis: BTB

Carboxyfluorescein diacetate succinimidyl ester: CFSE

Chemokine ligand 10: CXCL10

Faecal worm egg count: FWEC (alt. Faecal egg count: FEC)

False Discovery Rate: FDR

Genome-wide association analysis: GWAS

Genomic relationship matrix : GRM

Guanine-cytosine content: GC content (GC%)

Haemonchus contortus: Hc

High throughput Johne's Disease test (qPCR): HT-J

Immunoglobulin G1 specificity: IgG1 %SP

Interferon-gamma: IFNγ

Individual faecal culture: IFC

Johne's disease: JD

Mycobacterium avium paratuberculosis: Mptb, MAP

MAP sheep and cattle strains: S-strains, C-strains

Minor allelic frequency: MAF

Minimum Information for Publication of Quantitative Real-Time PCR Experiments: MIQE

Operational taxonomic unit: OTU

Peripheral blood mononuclear cells: PBMCs (predominantly, lymphocytes)

Phosphate-buffered saline: PBS

Protoplasmic antigen A: PPA

Purified protein (derivative) antigen: PP(D)A

Pokeweed mitogen, a non-specific immune cell stimulant: PWM

Restriction endonuclease analysis: REA

Single nucleotide polymorphism: SNP

Whole genome sequence: WGS

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Sub-project 1 – Understanding gut pathogen interrelationships through co-infections of gastrointestinal parasites and Ovine Johne's Disease (OJD)

1.1 Background

Precise, validated benchmarking tools for animal health and welfare ensures the ability to select, breed and produce animals "fit for purpose", underpinning the profitability and sustainability of livestock enterprises. It is well-documented that under endemic pathogen challenge, some individuals within and between breeds remain productive while others suffer. This "resilience" is the summation of the response of an animal to challenges associated with performance, plus genetic constitution that contributes to the overall productive measures. Resilience maybe a host trait rather than pathogen specific. The actual basis of resilience is not known but is considered an interaction between foraging ability, intake, food conversion efficiency and physiological responses to parasitism.

Livestock enterprises confront a range of endemic constraints to productivity; infectious and parasitic onslaughts comprise mixed infections. Most research for alternatives has been directed towards "quick-fix", broad-based, enhancement of innate immunity using a range of probiotics, neutraceuticals and various "immunostimulants". This approach alone does not capture the "growth gap" achieved by in-feed antibiotics and growth promotants.

Complex interactions between chronic infections such as nematodiasis, fasciolosis, and *Mycobacterium avium* subspecies *paratuberculosis* (*Mptb*) on host immunity and productivity have been limited by technology which have required study on single entities using reductionist paradigms. New technology makes it possible to apply a systems approach to study the whole animal impact of nutrition and gastrointestinal pathogens and unravel the processes that drive and modulate efficient physiology, growth, development and reproduction. This project was based on a long-term approach to understand how successful animals function. The plan focuses on the genetic and non-genetic mechanisms enabling productivity under adversity.

The project applied state-of-the-art technology in gastrointestinal immunology and genomics, and microbiome analysis to determine the impacts of co-infections with pathogens and parasites on productivity, gut microbiota and immune performance (Fig. 1.1) and thence elucidate determinants of efficient growth, reproduction and gastrointestinal disease resilience.

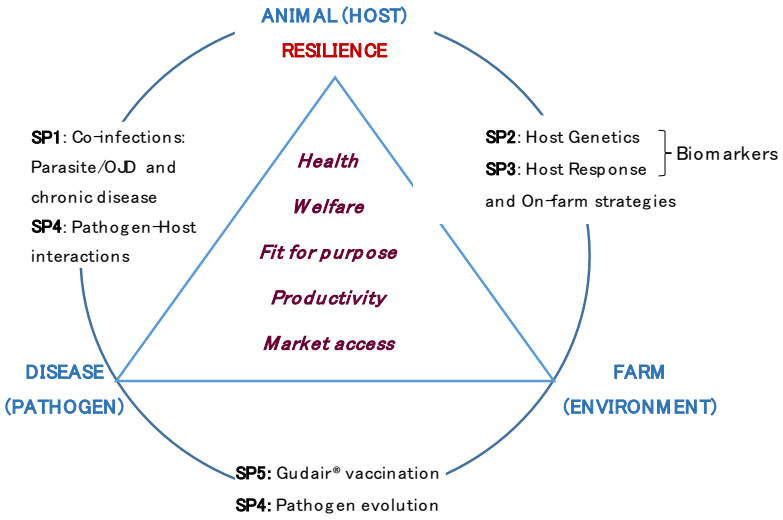

Fig. 1.1: Schematic diagram of the proposed research program (SP: Sub-project)

2.1 Objectives

To apply a systems approach to study the whole animal impact of gastrointestinal pathogens and unravel the processes that determine and modulate efficient physiology, growth, development and reproduction. This project undertook a long-term approach to understand how successful animals function.

To investigate gastrointestinal immunology and genomics, metabolomics and microbiome analysis during co-infections with pathogens and parasites to assess effects on productivity, gut microbiota and immune performance under adversity.

To elucidate determinants (incorporating genomic, phenotypic and biochemical markers) of efficient growth, reproduction and gastrointestinal disease resilience which may have application in field selection.

This subproject had two arms, on exploring archival samples from the P.PSH.0576 project and one based on an experimental co-infectional trial.

2.1.1 Sub-project 1 Objectives

a) Using archival material from JD trials conducted in previous project (P.PSH.0576), for which detailed JD outcomes are known, and overlaying information regarding the infection rate and management of intestinal parasites, leading to sub-project 2.
b) An experimental infection trial where sheep of the same breed are experimentally inoculated with both *Mptb* and gastrointestinal parasites with appropriate controls to identify markers for animals which performed under the co-infections. This resilience would be measured at

production, pathological, immunological and host genomic levels with pilot studies on the hosts microbiome. This would enable determination of disease outcomes and potential biomarkers andwould integrate with sub-project 3.

3.1 Methodology

3.1.1 Assessment of archival material

The development, optimisation and validation of a nematode quantitative PCR for frozen faeces can be found in Appendix 1.

3.1.2 Conduct of co-infection trial

A total of 90 weaner lambs from an OJD MN3 status flock at Armidale were infected alone or in combination with 800 *H.contortus* L3 (HcL3; Kirby Strain) and/or *M.avium. subspecies paratuberculosis* (Telford 9.2) (*Mptb*). The sheepMAP program monitors the presence of Mptb on a property through regular herd testing, to receive MN3 status, the flock must have 3 negative sample test (pooled faecal culture) over at least a 4 year period and have a Johne's disease flock management plan. Sheep were drenched on arrival and tested as Faecal Worm Egg Count (FWEC) negative prior to infection. The animals in groups 1 – 6 were run together (excepting 10 uninfected *Mptb* control group for quarantine purposes) and progressively rotated through 7 paddocks (where they were exposed to potential natural infestation with gastrointestinal parasites and infection with Mptb) at 4 weekly intervals to keep ahead of the life cycle of *H. contortus*. In contrast group 7 was maintained in a separate area and utilized as a paratuberculosis free control group for comparison of exposed and unexposed individuals.

The groups for the co-infection study are shown in Table 1.3.2.1. The HcL3 were administered orally in 10ml water containing 200 HcL3 at weekly intervals for 4 weeks (Groups 1 & 4). *M.paratb* Telford 9.2 (Freeze dry ID 2636-5) was administered in 10ml PBS orally (Groups 1 – 3). Three doses were given on weeks 1, 2, and 5 with each dose containing 1.13×10^9 (doses 1&2) and 7.70×10^8 (dose 3) live *Mptb* as determined post-inoculation by Helber counts. Groups 5 and 6 served as sentinels for parasitic pasture burden accounting for the two different drenching regimes.

Table 1.3.2.1 Groups in the co-infection trial

Group	Number	Treatment
1	20	*H.contortus* + *Mptb* (normal drenching[a])
2	15	*Mptb* (normal drenching[b])
3	15	*Mptb* (suppressive drenching)
4	20	*H.contortus* (normal drenching[a])
5	10	*H.contortus* controls (normal drenching[b])
6	10	*H.contortus* controls (suppressive drenching)
7	10	*Mptb* controls (uninfected)(suppressive drenching)

normal drenching: a) when FWECs exceed 5000 epg on 2 successive counts, b) when FWECs exceed 500 epg on 2 successive counts. Suppressive drenching prior to animals being moved to a new paddock, every 4 weeks.

Faecal egg counts were performed for the duration of the trial at 2-weekly intervals to monitor the establishment and progress of the infection. A drenching decision of >5000 epg on successive FWECs ("normal drenching") was established to avoid clinical disease and prevent over-contamination of paddocks for treatment groups experimentally inoculated with *H. contortus* (Groups 1 & 4). For treatment groups not experimentally inoculated with *H. contortus* and maintained under a 'normal drenching' regime, drenching was done at >500 epg (Groups 2 & 5). To optimise performances and minimise parasite effects, "suppressive" drenching was performed prior to animals being moved to a new paddock at 4-weekly intervals (Groups 3, 6 & 7). All animals were drenched using levamisole based on FWEC.

Sheep were weighed periodically throughout the trial to determine average daily gain (ADG) and as a marker of disease progression for paratuberculosis, with rapid weight loss an indicator of the onset of clinical disease. Blood was collected from animals at four timepoints throughout the trial to determine resilience and susceptibility at an immunological level (Fig. 1.3.2.2).

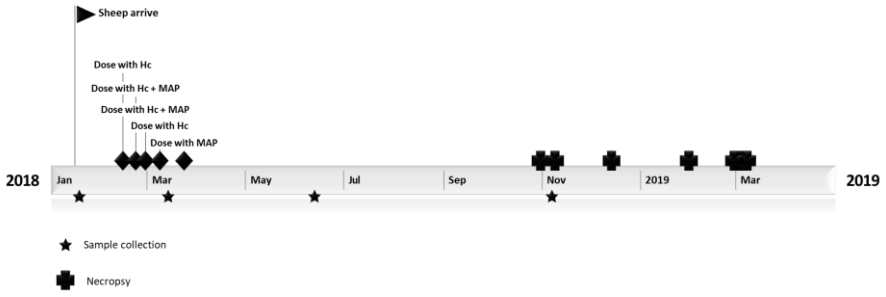

Fig. 1.3.2.2 Co-infection Trial timeline

3.1.3 Microbiome

Sample selection and DNA extraction

Five animals were randomly selected from each of the treatment groups 1, 2, 4 and 7 (MAP and *H. contortus*, MAP, *H. contortus* and control). Faecal samples were collected at five weeks post exposure and ileal content were collected at necropsy from the same animals (group 1 and 2 only). Collected samples were stored at -80°C prior to DNA extraction using commercial kits (DNeasy PowerLyzer PowerSoil Kit). DNA quality and quantity were verified by NanoDrop spectrophotometry.

DNA amplification and next-generation sequencing

PCR amplification of the V3-V4 region of the bacterial 16S rRNA gene, library preparation, and next-generation sequencing were performed by the Ramaciotti Centre for Genomics (University of New South Wales). PCR amplicons were sequenced on an Illumina MiSeq using 250 bp paired-end chemistry. The quality of the raw data was evaluated using FastQC (v0.11.8) upon receipt. Primer sequences were trimmed from the raw reads using Cutadapt (v1.8.3). Data processing and analysis were performed with QIIME2 (Quantitative Insights into Microbial Ecology 2) (v2.7.10).

Data analysis

Demultiplexed sequences were imported as a QIIME2 artefact, and quality trimming, paired-end read merging, dereplication, chimera filtering, and OTU generation were performed using the DADA2 pipeline. Twenty base-pairs were trimmed of the 3' end of the forward reads. The forward and reverse reads were truncated at 270 and 200 base-pairs, respectively. Taxonomy was assigned to each OTU using the Greengenes 13_8 99% OTU database. Representative sequences were aligned with PyNAST, the alignment was filtered to remove gaps and variable positions with Lane masking, and a phylogenetic tree was constructed with FastTree to support diversity analyses that required phylogenetic information.

Alpha and beta diversity were compared between samples from the two sample types (faecal and ileal contents). Data were rarefied to 160,000 reads per sample prior to alpha and beta diversity analysis. Alpha rarefaction curves were generated to determine whether or not the number of reads generated were sufficient to describe the true level of diversity in each group of samples. Alpha diversity in each group was assessed using Shannon's diversity index, Faith's Phylogenetic Diversity (PD), and Pielou's Evenness, and compared between groups using the non-parametric Kruskal-Wallis test. Beta diversity in each group was assessed using a Bray-Curtis, weighted UniFrac, and unweighted UniFrac distance matrices. Beta analysis was compared between groups using a permutational multivariate analysis of variance (PERMANOVA) with 999 permutations. Principle coordinate analysis (PCoA) of the distance matrices was undertaken and PCoA plots were generated and visualised in Emperor. The same analyses were performed to compare diversity between the control group and the three challenge groups (MAP only, *H. contortus* only, MAP + *H. contortus*).

3.1.4 Statistical Analysis

Statistical analysis for comparisons between group 1 to 4 was performed by Associate Professor Peter Thomson utilising the statistical and computing package R as detailed below.

Body weight
For this analysis, data from all seven treatments have been retained. A linear mixed model was used to analyses these repeated measures data, with sheep ID as a random effect. Fixed effects were trial day (as a spline), treatment, and their interaction, thus allowing for different shaped growth curves. The 'lmer' function was used for model fitting.

Faecal egg count
The FEC data were a longitudinal data series, i.e. repeated observation in each sheep. To accommodate this, a linear mixed model was used with a random sheep effect was included in the model. Fixed effects were treatment, day (fitted as a spline) as well as their interaction, to allow for a different shaped time course of FEC for each treatment. Data were analysed on a $y = \log_e(\text{FEC} + 12.5)$ scale (25 being the smallest non-zero FEC). These data were analysed using the 'lmer' function in the 'lme4' R package.

Total abomasal worm count
The worm count data were analysed in the same way as the that of the binary tissue culture variable except a (general) linear model was used to analyse the data, using the 'lm' function. Because of the extreme positive skew and unstable variance, the data were log-transformed, $y = \log_e(\text{Count} + 25)$, prior to analysis. (The value 25 was added to allow for zero counts, 25 being half the smallest non-zero value). Back-transformed means and approximate standard errors were directly calculated using the 'emmeans' package.

Faecal shedding
These data are in the form of longitudinal binary data series, so a logistic generalised linear mixed model (GLMM) was used for the analysis, with a random effect for each sheep ID. Fixed effects were specific for treatment, trial day and their interaction. Note that since only four distinct days were available for this trait (208, 266, 278 and 358 days), this term was specified as a four-level factor rather than as a spline. These data were analysed using the 'glmer' function in the 'lme4' R package, and again other aspects of the analysis are the same as described for previous traits.

Tissue culture status
The associations between each of the three tissue culture (LN, gut, liver) states (positive vs negative) and the four treatments was initially evaluated as frequency counts and percentages using Fisher exact tests on the 2×4 contingency tables, using the 'fisher.test' function. The effect of both average daily growth (ADG) as well as treatment on tissue culture status was then assessed using logistic regression using the 'glm' function in R, with the status as the binary outcome (positive = 1, negative = 0). To allow for a possible nonlinear response of ADG (on the log-odds scale), a smooth function (spline) of ADG was included in the model using the 'bs' function in the 'splines' R package. A Treatment × ADG interactions was assessed to allow for different nonlinear associations for each treatment. Model-base probabilities of a positive tissue culture for combinations of treatment and ADG were computed using the 'emmeans' function in the 'emmeans' R package, formal pairwise comparisons of means was conducted using the 'cld' function in the same package.

Data from this analysis are presented as the model-based mean to give a more accurate result accounting for the variables included in the statistical model for analysis.

3.1.5 Defining resilient, resistant and susceptible phenotypes

The methodology used to determine the resilience score of animals in groups 1 to 4 in the co-infection trial is presented in detail in sub-project 2 (section 3.2.2; p 47). Briefly scores were developed to distinguish the degree of disease for both paratuberculosis, taking into account bacterial shedding, tissue culture, histopathology and clinical manifestation of disease, and Haemonchosis, based on the average faecal egg count for individual animals prior to first drenching or conclusion of the trial (whichever occurred first). These scores along with productivity of individuals based on average daily weight gain, were used to give each sheep a resilience ranking.

4.1 Results – Determining resilience

4.1.1 Archival Samples

Results from analysis of previous trials can be found in appendix 1. Unfortunately, due to the strict parasite control regime utilised for previous ovine paratuberculosis projects, there was limited presence of *H. contortus* DNA in archival faecal samples and as such examination of concurrent disease was limited.

4.1.2 Production level

4.1.2.1 Weight

Weight gains (Table 1.4.1.1) of the weaners over the SP1 co-infection trial's 390 day period indicated that the various combinations of infection did not produce any major compromise to growth, although the weight gains fell short of industry benchmarks. The principal influence was due to the drought and reduced pasture feed, especially over the winter (Fig. 1.4.1.1).

Table 1.4.1.1 Mean body weight for each group.

Group 1* Mptb+Hc	Group 2 Mptb+N-Rx	Group 3 Mptb+S-Rx	Group 4 Hc	Group 5 Uninf N-Rx	Group 6 Uninf S-Rx	Group 7 Unexp Uninf
26.92 ± 0.70^{A}	28.06 ± 0.81^{A}	27.82 ± 0.81^{A}	28.7 ± 0.70^{A}	28.68 ± 0.99^{A}	27.73 ± 0.99^{A}	28.16 ± 1.02^{A}

*Mptb, Hc: induced infection/infestation; N-Rx normal drenching; S-Rx suppressive drenching
The value in each cell is the model-based mean± standard error of body weight.
Means are calculated at the mean value of Day (184 days).
Means with the same superscript are not significantly different from each other ($P > 0.05$).

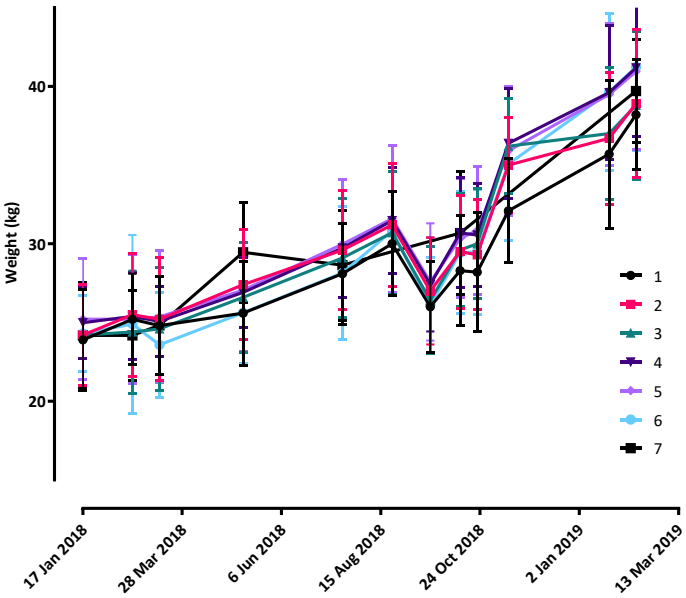

Fig. 1.4.1.1 Progressive liveweight gains (Kg +/- SD), within groups throughout the 390 days of the SP1 co-infection trial.

Closer inspection of mean body weight at selected timepoints (Table 1.4.1.2) indicated that the combined co-infection group had reduced weight towards the end of the trial compared to the Hc exposed and control groups (Group 4, 5 and 6) The "control" of the *H. contortus* infestation by rotational grazing also enabled Group 4 to keep in touch with higher performing Groups 5-7. The results also confirmed that "suppressive" monthly drenching, without Mptb exposure, enabled the highest ADLWG (37 g/day) in the co-grazed groups (which excluded Group 7).

Table 1.4.1.2 Mean body weight (kg) for each group at selected days since start of trial.

Day	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
0	23.64 ± 0.82^{A}	23.75 ± 0.94^{A}	23.69 ± 0.94^{A}	24.58 ± 0.81^{A}	24.59 ± 1.15^{A}	23.92 ± 1.15^{A}	23.24 ± 1.16^{A}
100	26.21 ± 0.76^{A}	27.24 ± 0.87^{A}	26.27 ± 0.87^{A}	26.71 ± 0.76^{A}	26.98 ± 1.07^{A}	25.48 ± 1.07^{A}	27.44 ± 1.09^{A}
200	27.33 ± 0.72^{A}	28.69 ± 0.83^{A}	28.23 ± 0.83^{A}	28.83 ± 0.72^{A}	28.94 ± 1.01^{A}	27.53 ± 1.01^{A}	29.42 ± 1.19^{A}
300	30.31 ± 0.73^{A}	31.97 ± 0.84^{AB}	32.25 ± 0.85^{AB}	33.43 ± 0.73^{B}	33.21 ± 1.03^{B}	32.52 ± 1.03^{AB}	32.65 ± 1.39^{AB}
390	37.32 ± 1.02^{A}	39.71 ± 0.99^{AB}	39.84 ± 1.07^{AB}	41.75 ± 0.85^{B}	41.28 ± 1.2^{B}	41.55 ± 1.2^{B}	39.47 ± 1.45^{AB}

The value in each cell is the model-based mean ± standard error of body weight.
Means with the same superscript are not significantly different from each other ($P > 0.05$).

Focusing on groups 1 to 4, the mean ADG was 31.2 g/day, with individual values ranging from -7.92 g/day to 62.05 g/day. There are moderate differences in ADG between the four groups. Table 1.4.1.3 shows the mean ADG for each treatment.

Table 1.4.1.3 Mean ADG for Groups 1 - 4.

Group 1 Mptb+Hc	Group 2 Mptb+N-Rx	Group 3 Mptb+S-Rx	Group 4 Hc
24.4 ± 3.5 ^{A}	33.7 ± 4.1 ^{AB}	30.8 ± 4.1 ^{AB}	36.4 ± 3.6 ^{B}

The value in each cell is the model-based mean ± standard error of ADG.
Means with the same superscript are not significantly different from each other ($P > 0.05$).

4.1.3 Pathological Level

4.1.3.1 Parasitism

Faecal Egg Count

Group FWEC data is displayed in Fig. 1.4.2.1 below, indicating that egg production was significantly higher in sheep from Groups 1 & 4 which were deliberately dosed with 800 *Hc*L3 in Feb 2018 ($p<0.05$). In contrast, cohorts in other groups co-grazing with infected animals showed consistently low FWEC, indicating that the drenching/monthly paddock rotational strategy controlled the level of pasture infestation and subsequent L3 ingestion by sentinel sheep in Groups 5 & 6 (and other *Mptb* infected groups) until February 2019. At that time the final FWEC indicated a rise in output in all groups.

While the worm control was both enlightening and confirmatory of integrated pest management (IPM) strategies, it also meant that the 23 sheep in Groups 1 & 4 that required treatment for high FWEC did not necessarily become re-infested over the remainder of the trial; they did not require further treatment but their productivity could only be assessed to the time of “normal” drenching. From late autumn (week 14), during the winter and a winter drought, egg counts declined and were maintained at low levels due to a combination of rotational grazing (every 4 weeks in IPM) and the prolonged drought period which required additional supplementary feeding with hay. It was during this period that weight gains reduced (Fig. 1.4.1.1).

It is also noted that *Mptb* control sheep in group 7, which were NOT co-grazed with the main cohort (i.e. outside of the quarantine area) exhibited the typical spring larval pickup with rainfall in early November. The subsequent peak in FWEC in Dec 2018 was treated with levamisole, but another rise occurred around the time of necropsy.

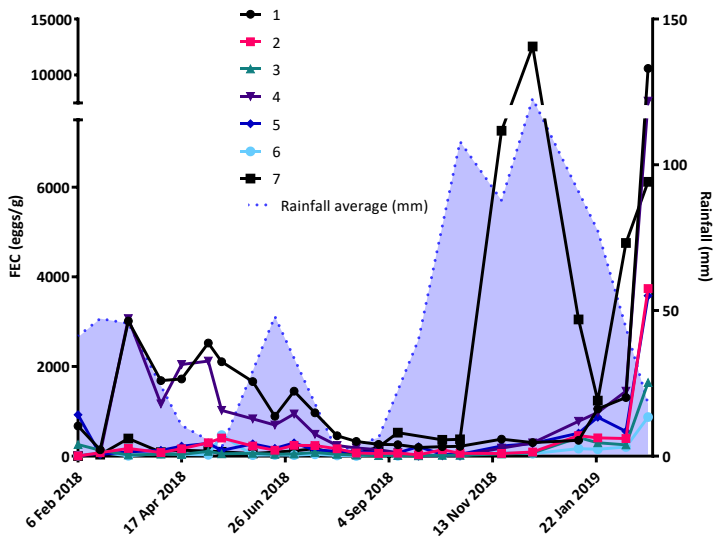

Fig. 1.4.2.1 Sequential mean faecal worm egg counts (FWEC; eggs/gm faeces) throughout the SP1 co-infection trial

Total abomasal worm count

Abomasal worm counts at necropsy are presented in Fig. 1.4.2.2. Amongst the co-grazed cohorts in groups 1-6, there was a tendency for higher mean counts in the deliberately infected groups 1 & 4 (mean worm counts of 227 and 487, respectively). Focusing analysis on groups 1 to 4 showed a significant reduction in total abomasal worms in group 3 (Mptb infection) compared to group 4 (Hc induced infestation) (Table 1.4.2.2). The outstanding “Mptb control” group 7, possessed higher worm counts at necropsy reflecting the higher contamination of the alternative area grazed by the group to ensure that they remained free of contact with Mptb.

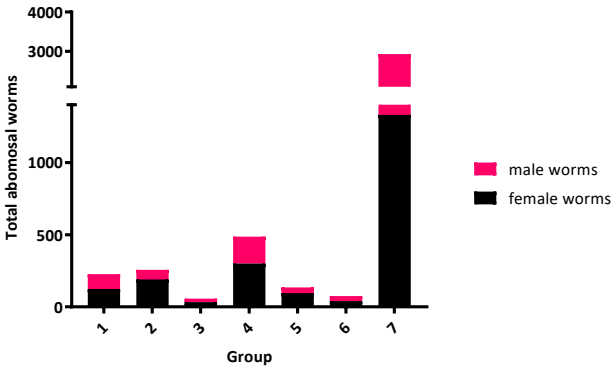

Fig.1.4.2.2 Group mean abomasal worm counts for *H. contortus* for animal groups within the SP1 co-infection trial.

Table 1.4.2.1 Mean total abomasal worm count for each group.

Group 1	Group 2	Group 3	Group 4
108.8 ± 36.3 ^{AB}	97.2 ± 34.3 ^{AB}	44.3 ± 15.5 ^{A}	126.9 ± 41.1 ^{B}

The value in each cell is the model-based mean ± standard error of the worm count.
Means are calculated at the mean value of ADG (31.2 g/day).
Means with the same superscript are not significantly different from each other ($P > 0.05$).

H. contortus is the only nematode parasite where FWEC correlates well with the worm burden. Since the commencement of the trial, fortnightly egg count data indicated that both the co-infected and singly infected groups (1 & 4) generated FWECs commensurate with moderate worm infestations. However, the correlation between female worms and final FWEC was disappointingly low at 0.26 (Fig. 1.4.2.3). Although for groups 1-6 there were no significant differences in worms counts, the within group variation in worm counts (range 0-950) allowed for analysis of individual performance data.

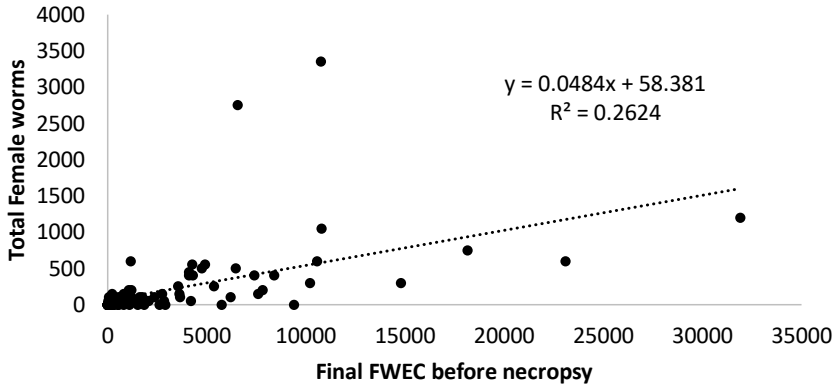

Fig. 1.4.2.3. Correlation between final faecal worm egg counts FWEC (Dec 2018 or Feb 2019) and abomasal female *H. contortus* worms at necropsy in the SP1 co-infection trial.

4.1.3.2 Paratuberculosis disease

The retrospective culture results from the dosages of *Mptb* indicated that the infective dose was around 10-fold higher than that used in previous trials. This had implications for the histopathology and performance outcomes late in the trial as recipients of the *Mptb* developed more severe lesions from the higher level of infection, requiring necropsy of 23/50 around 11 months after infection and 1-2 months before the end of the co-infection trial.

Faecal shedding

The level of faecal shedding based on HTJ screening was examined in groups 1 to 4. There was a significant difference in the probability of a positive HTJ results based on treatment group (Table 1.4.2.2). Sheep co-infected with both Hc and *Mptb* had the higher likelihood of faecal shedding compared to *Mptb* exposure alone. Interestingly the *Mptb* exposed group which was under the suppressive drenching regime (Group 3) had the highest probability of having a positive HTJ result compared to all other *Mptb* exposed groups including the co-infected sheep.

Table 1.4.2.2. Probability of a positive HTJ result for each group.

Group 1	Group 2	Group 3	Group 4
0.759 ± 0.221 [A]	0.217 ± 0.230 [B]	0.890 ± 0.130 [C]	0.000 ±0.000 [B]

The value in each cell is the model-based mean ± standard error of the FEC.
Probabilities are calculated averaging across levels of Day.
Means with the same superscript are not significantly different from each other ($P > 0.05$).

Tissue culture

Mptb infection status at conclusion of the trial was determined based on culture of live Mptb from sections from the intestine, gut associated lymph nodes (LN) and liver. Examination indicated a significantly higher probability of liver culture positive animals in the Mptb + Hc challenged group (Table 1.4.2.3) and a very high proportion of tissue culture positives in general (Table 1.4.2.4). Liver culture positivity correlates with disseminated disease and also seems to be correlated with higher rates of Mptb faecal shedding in this group. With these results, disseminated disease and high levels of faecal shedding was a criterion considered for susceptibility (see sub-project 2; 3.2.2).

Table 1.4.2.3. Probability of a positive tissue culture status for liver for each group.

Group 1	Group 2	Group 3	Group 4
0.730 ± 0.124 ^{A}	0.359 ± 0.144 ^{AB}	0.550 ± 0.144 ^{A}	0.052 ± 0.056 ^{B}

The value in each cell is the model-based probability ± standard error of a positive tissue culture results for gut.
Probabilities are calculated at the mean value of ADG (31.2 g/day).
Probabilities with the same superscript are not significantly different from each other ($P > 0.05$).

Table 1.4.2.4 Frequency and percentage of tissue culture status.

a) Liver

Liver Tissue Culture Status	Group 1	Group 2	Group 3	Group 4
Negative	5 (25%)	10 (67%)	7 (47%)	19 (95%)
Positive	15 (75%)	5 (33%)	8 (53%)	1 (5%)

Fisher exact test: $P < 0.0001$

b) Lymph nodes

LN Tissue Culture Status	Group 1	Group 2	Group 3	Group 4
Negative	2 (10%)	3 (20%)	3 (13%)	18 (90%)
Positive	18 (90%)	12 (80%)	13 (87%)	2 (10%)

Fisher exact test: $P < 0.0001$

c) Gut

Gut Tissue Culture Status	Group 1	Group 2	Group 3	Group 4
Negative	1 (5%)	2 (13%)	2 (13%)	16 (80%)
Positive	19 (95%)	13 (87%)	13 (87%)	4 (20%)

Fisher exact test: $P < 0.0001$

In sheep experimentally inculated with Mptb alone, the suppressive drenching regime (Group 3) showed higher rates of disease and liver positives compared to the regular drenching regime (Group 2), confirming the increased level of shedding in this group and the proposition that suppressive drenching does not support optimal development of immunity and may be immunosuppressive.

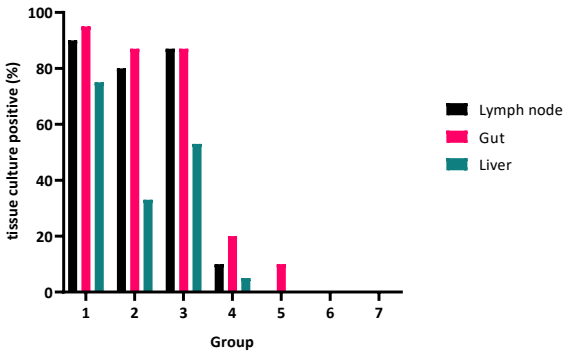

Fig. 1.4.2.2 Tissue culture results from gut, lymph node and liver taken at necropsy (groups 1-7 along X-axis). Results are expressed as the percentage of each group positive for Mptb infection.

4.1.4 Immune Level

Examination of resilience and susceptibility at the immune level in this cohort of animals is presented in sub-project 3.

4.1.5 Host Genome level

Examination of resilience and susceptibility at the host genome level in this cohort of animals is presented in sub-project 2.

4.1.6 Microbiome analysis

Group variation

Alpha diversity was significantly different between the faecal and ileal samples according to Shannon's diversity index ($P < 0.001$) and Pielou's Evenness ($P < 0.001$), but not according to Faith's PD ($P = 0.65$). Beta diversity was significantly different between the faecal and ileal samples according to the Bray-Curtis ($P = 0.001$), unweighted UniFrac ($P = 0.001$), and weighted UniFrac ($P =$

0.001) distance matrices (Figure 1.4.6.1). The alpha rarefaction curves indicated that there were sufficient numbers of reads to describe the true level of diversity in each group (Figure 1.4.6.2). As such, the faecal and ileal samples were analysed separately in all subsequent analyses.

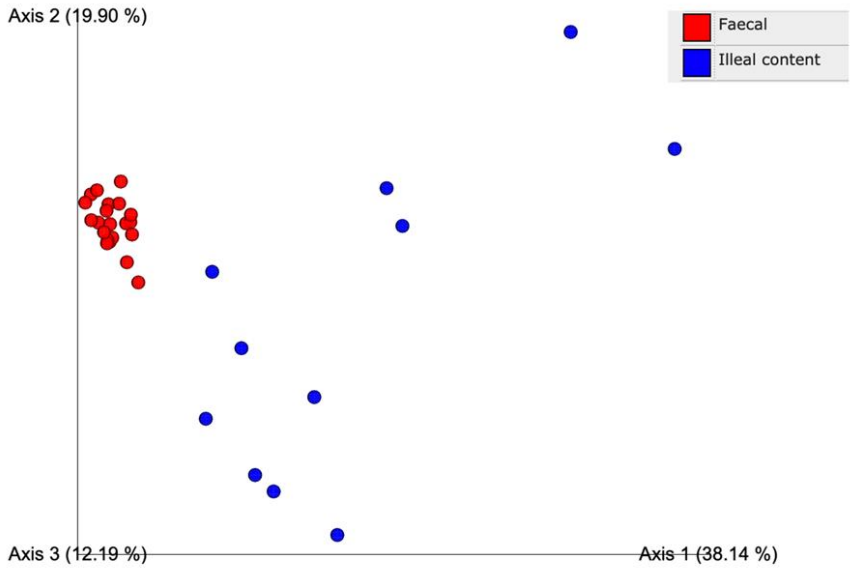

Fig. 1.4.6.1: PCoA plot based on the weighted UniFrac distance matrix.

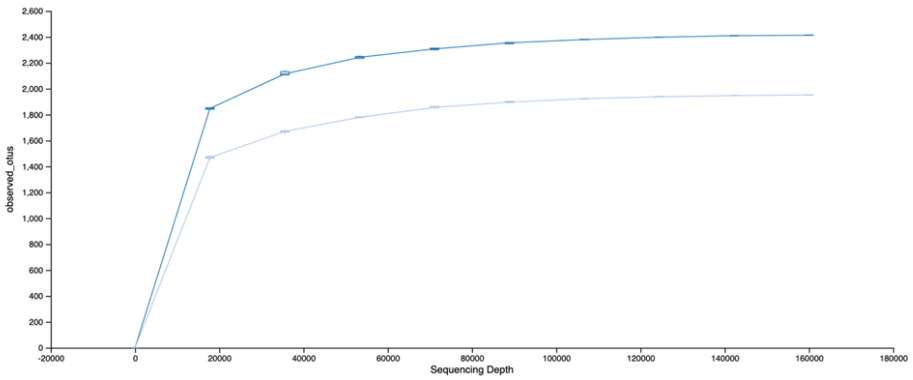

Fig. 1.4.6.2: Alpha rarefaction curve for combined data set (faecal and ileal samples).

Faecal sample analysis

The faecal samples were collected from the sheep five weeks post exposure to MAP, *H. contortus* or MAP and *H. contortus* and at this early stage in the infection process, beta diversity indicated significant difference across the four groups according to the Bray-Curtis (P = 0.001) distance matrix (Figure 1.4.6.3) but not the unweighted UniFrac (P = 0.284) or weighted UniFrac (P = 0.08) distance matrices (Figure 1.4.6.4).

Between individual groups, Faith's PD (P = 0.01) indicated significant difference between the Control group (group 7) and the group challenged with MAP + *H. contortus* (group 1). None of the other groups displayed significant differences according Shannon's diversity index (P > 0.05), Faith's PD (P = 0.65), and Pielou's Evenness (P > 0.05) however, there were trends in relation the relative abundance of each bacterial family as illustrated in figure 1.4.6.5. Regardless of treatment type, the sheep all returned abundance of bacteria from the families Ruminococcaceae, Bacteroidaceae, Lachnospiraceae, and Rikenellaceae however, sheep from Group 1 (MAP and H. contortis) additionally returned abundance (5.7%) of bacteria from the phylum Verrucomicrobia (family Verrucomicrobiaceae) in contrast to the other treatments.

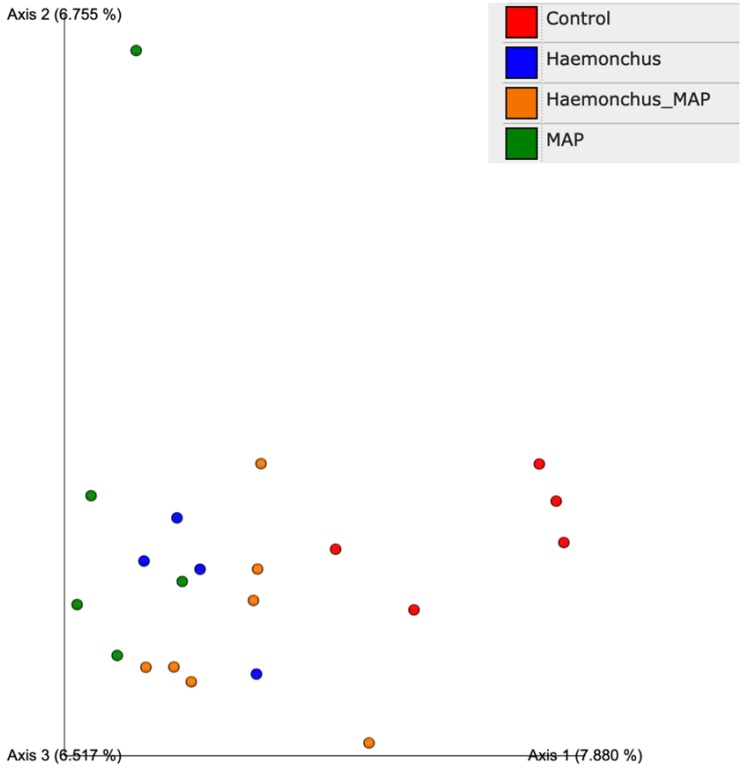

Fig. 1.4.6.3: PCoA plot generated from the Bray-Curtis distance matrix.

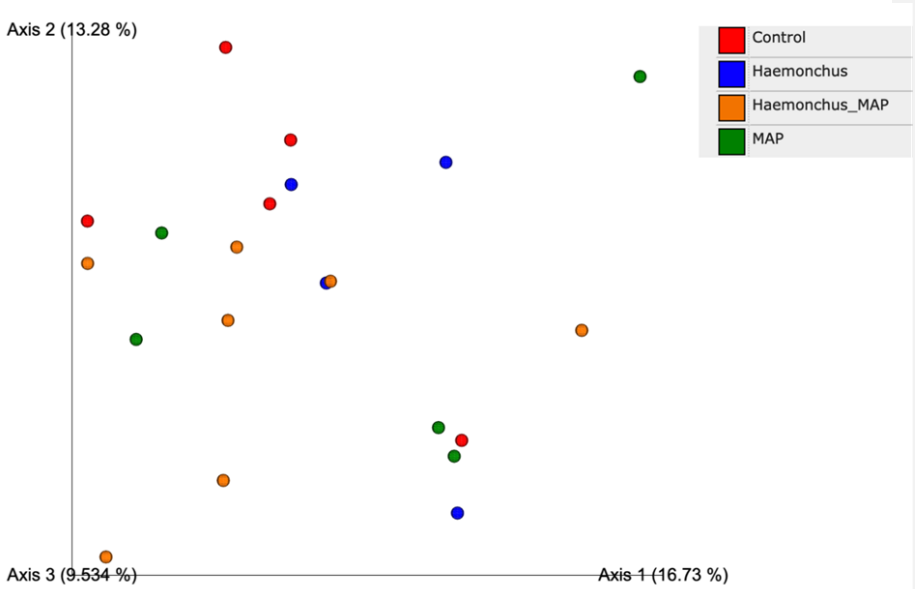

Fig. 1.4.6.4: PCoA plot generated from the weighted UniFrac distance matrix.

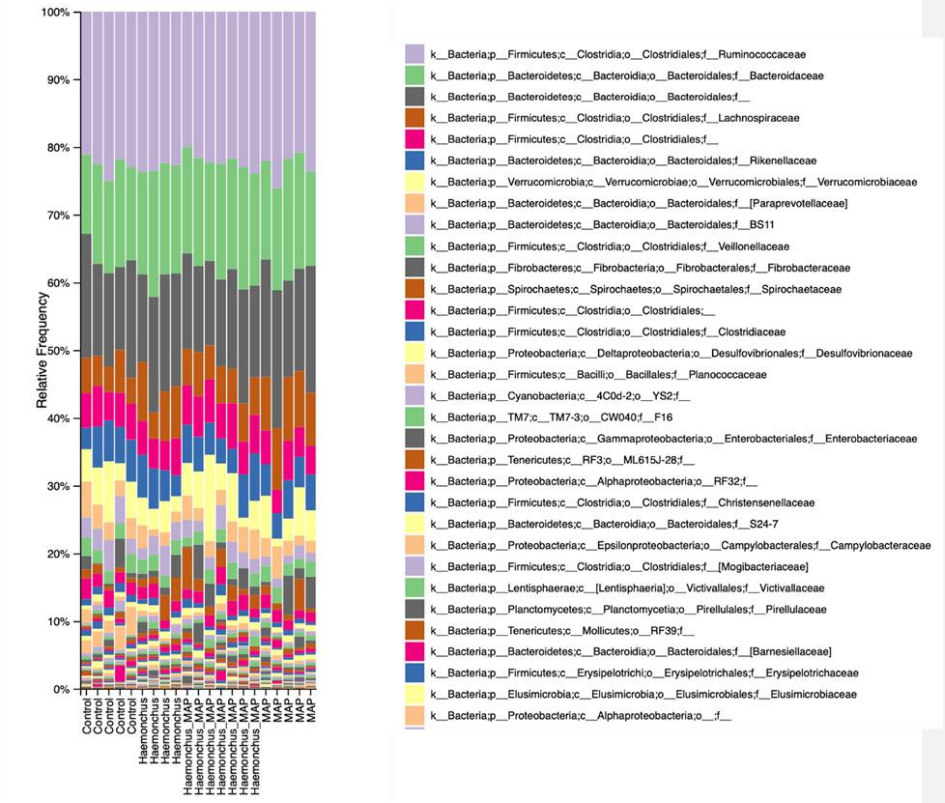

Fig. 1.4.6.5: Bar chart depicting the relative abundance of each bacterial family in the faecal samples collected from each challenge group.

Ileal sample analysis

No ileal samples were collected from the control sheep (group 7) or the sheep challenged with *H. contortus* (group 4) only; as such, only ileal samples collected from sheep challenged with MAP (group 2) and sheep challenged with MAP + *H. contortus* (group 1) were able to be compared. Alpha diversity differed significantly between the two groups according to Shannon's diversity index (P = 0.04), Faith's PD (P = 0.02), and Pielou's Evenness (P = 0.02). Beta diversity differed significantly accordingly to the Bray-Curtis (P = 0.03) and weighted UniFrac (P = 0.02) distance matrices (Figure 1.4.6.6), but not the unweighted UniFrac distance matrix (P = 0.13).

Clear variation in the relative abundance of bacterial families between the two groups was identified as illustrated in figure 1.4.6.7. Sheep from Group 1 (MAP and *H. contortis*) returned abundance of bacteria from the phylum Bacteroidetes (including family Bacteroidaceae) and Firmicutes (family Ruminococcaceae). The exact proportions of the dominant bacteria in the ileum of the co-infected sheep are: Bacteroidaceae (18.3%), Ruminococcaceae (17.1%), Enterobacteriaceae (7.1%), and Verrucomicrobiaceae (6.7%). In contrast the dominant bacterial families in the ileum of sheep

challenged with MAP only were Planococcaceae (phylum Firmicutes 37.8%), Enterobacteriaceae (phylum Proteobacteria 25%), Ruminococcaceae (5.4%), and Peptostreptococcaceae (3%)

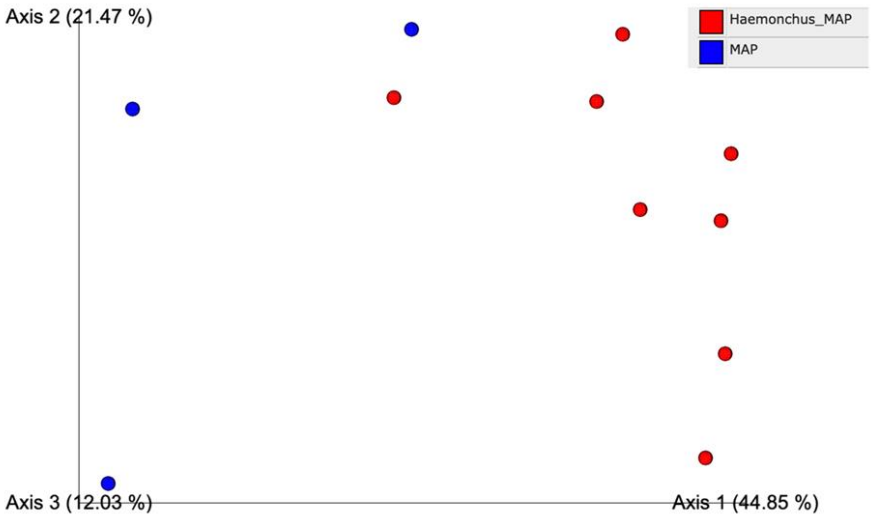

Fig. 1.4.6.6: PCoA plot generated from the weighted UniFrac distance matrix.

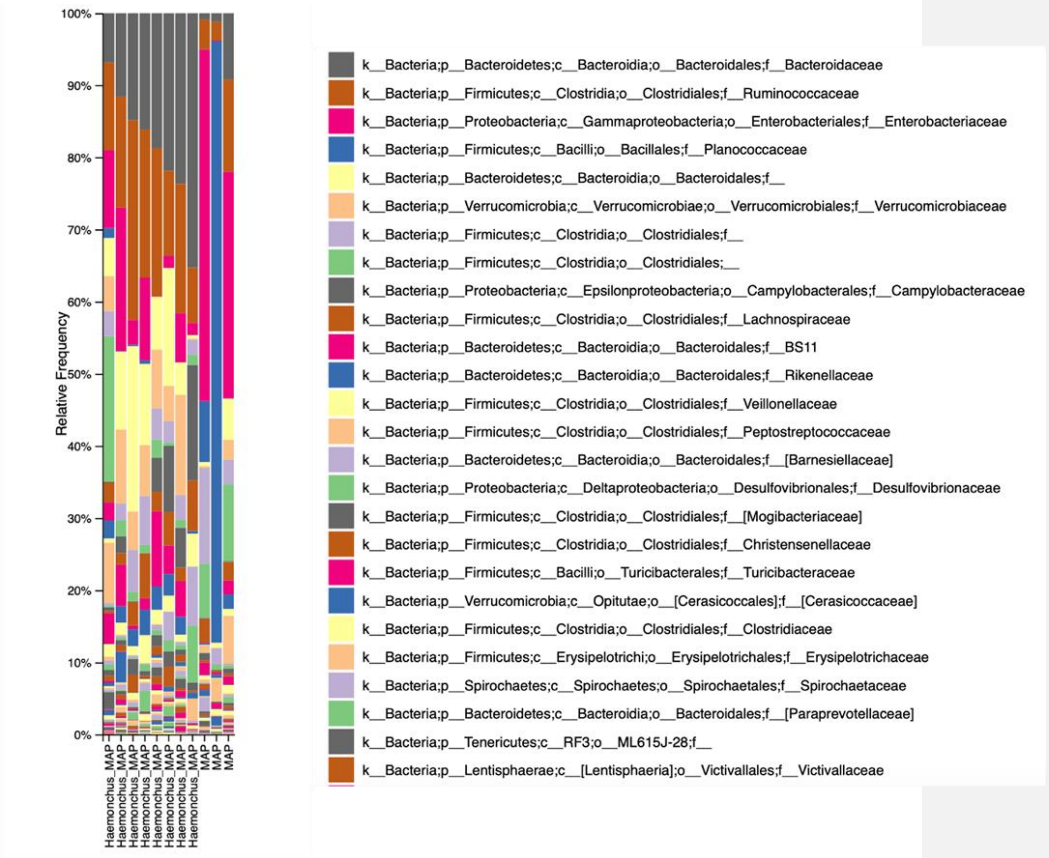

Fig. 1.4.6.7: Barchart depicting the relative abundance of each bacterial family in the ileal samples collected from each challenge group.

4.1.7 Determining resilience score

The resilience score for animals in groups 1 to 4 was determined as described in sub-project 2 (p 47). Unsurprisingly, animals in the dual challenge group (group 1) showed the lowest resilience rankings compared to all other groups. All groups with sheep experimentally exposed to *Mptb* had lower resilience scores compared to group 4, where sheep were experimentally exposed to Hc and had natural *Mptb* exposure (Figure 1.4.6.8a). Assessment of individual values shows that there is variability in resilience score within groups with some sheep within the dual challenge group with higher resilience scores compared to animals in the other groups (Figure 1.4.6.8b).

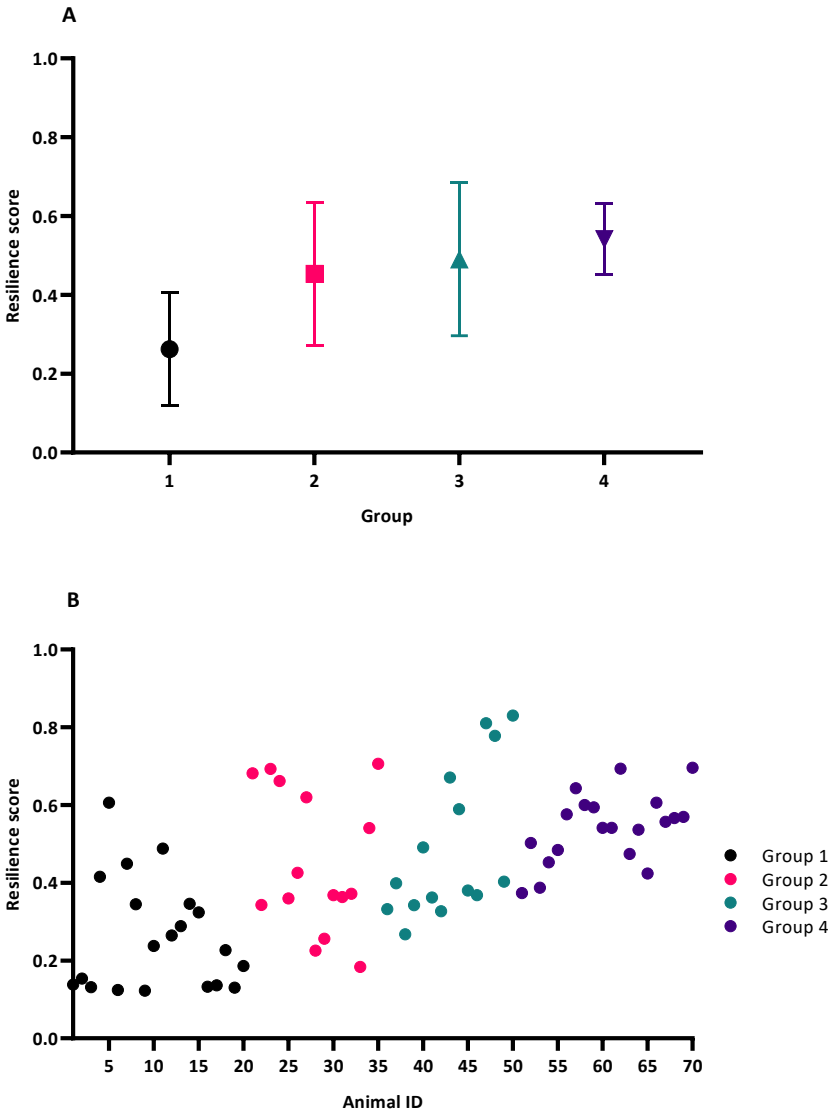

Fig. 1.4.6.1 Resilience score for sheep from groups 1 to 4 in the coinfection trial.

(A) Average resilience score for each group shown (+/- standard deviation). (B) Individual animal resilience score grouped by treatment and animal identification number.

5.1 Discussion

5.1.1 Effects of co-infection on productivity

Productivity is the key driver of profitability in any livestock enterprise. The ability to improve or ensure the highest productivity of livestock species relies on the interplay or disease, nutrition and genetics along with influences from husbandry and animal management (Thornton, 2010). Through determining the disease outcome and 'pressure' on an individual and the impact of this on productivity we are able to determine the resilience of an animal in a production setting, to specific factors (Berghof et al., 2019). The ability to then link the 'resilience' phenotype to genotype would provide producers with an invaluable tool to ensure productivity and profitability by using livestock selected and bred for resilience to a specific disease or generally more 'resilient' animals in certain production systems.

Direct effects of disease or concurrent diseases on productivity in a commercial system can be difficult to ascertain due to the interplay of other factors such as nutrition and management. The use of a co-infection trial has allowed detailed monitoring of the impacts of both paratuberculosis and haemonchosis and afforded us the ability to control and account for variables that might influence the measurement of productivity. In this way we can attribute variability in productivity directly to pathogen and individual animal resilience. This is the first trial that has successfully and intentionally coinfected sheep with *Mptb* and *H. contortus* running from February 2018 till March 2019. Infestation with 800 *H. contortus* larvae (HcL3) produced the desired haemonchosis burden with individual variation in FWEC, without requiring treatment of more than a few sheep with an anthelminthic, outside of the design of the trial. The management option to rotate paddocks at 4-week intervals through 7 paddocks, keeping pace with the earliest pre-patent period of *H. contortus*, maintained a low but constant FWEC in sentinel control groups (5 and 6). Exposure of sheep to 3.03x 10^9 live *Mptb* in total across 3 doses, produced clinical paratuberculosis and evident wasting in infected groups, although there was still a spectrum of disease seen across individuals in the infected groups. Therefore, the development of a successful coinfection trial methodology has allowed careful examination of the resilience phenotype and genotype in merino weathers under Australian production conditions. However, the higher infective dose of *Mptb* meant that the principal effects of the co-infection were driven by the response to the bacterium rather than by *Hc*.

Granting that the merino breed's primary purpose is wool production, weight gain was chosen as a marker of production performance for this trial. The ability to repeatedly measure weight gives a more detailed description of production impacts during concurrent disease progression. Furthermore weight loss, or wasting, is a common feature of clinical paratuberculosis in sheep (Marquetoux et al., 2018). The increased weight loss or lack or productivity seen in the co-infected animals was a new but not unexpected finding, confirming that additional pressure from concurrent disease causes additional production losses. Interestingly, although not statistically significant, weight gain was also reduced in paratuberculosis exposed sheep undergoing a suppressive drenching regime (i.e. theoretically, with fewer worms). Further examination of the immunological and molecular effects of suppressive drenching on disease progression and productivity is warranted in larger flocks or those with existing endemic OJD.

5.1.2 Effects of co-infection on disease progression and outcome

Co-exposure of sheep to *H.contortus* and Mptb resulted in increased rates of paratuberculosis shedding and infection, based on tissue culture, compared to animals exposed to *Mptb* alone and maintained under a 'normal' drenching regime. The co-exposed group also had a higher probability of disseminated disease, based on culture of live Mptb from the liver. This finding, although not unsurprising shed a light on the importance of integrating disease management programs for livestock. Additionally, these results provide insight into confounding factors inhibition control options for ovine paratuberculosis. The combination of reductions in ADG, increased disseminated disease and faecal shedding seen in the co-exposed group poses huge risk to producers. Therefore, further understanding of the impacts of co-exposure of sheep to paratuberculosis and *H.contortus* and the impacts of this on measures for disease control, especially vaccination against paratuberculosis, should be further explored.

Interestingly, exposure of sheep to Mptb alone coupled with a suppressive drenching regime lead to the highest likelihood of having animals shedding Mptb in their faeces, even compared to co-exposed sheep, and a larger than normal portion of sheep with disseminated disease. This outcome highlights the importance of assessing control measures and treatments for diseases in a holistic manner, as although the suppressive drenching regime was able to reduce the *Hc* burden, it resulted in more severe paratuberculosis, increased shedding and pasture contamination along with reductions in ADG. Therefore, co-exposure trials such as these are important in the process of understanding disease dynamics and control. Previously, suppressive drenching compromised the development of immunity to *Hc* (Barger, 1988), likely due to reduced exposure. In southern African buffalo, increased worm resistance (both genetic and temporal) reduced the host's ability to control bovine tuberculosis (BTB), but was alleviated somewhat by drenching (Ezenwa et al., 2021). This is also reminiscent of the reduced ability of inbred strains on mice with polarised proclivities for Th1 or Th2 immunity to control either *Leishmania* or intestinal worms (Mossman & Coffman, 1989) or cattle responding to chronic Fascioliasis or Babesiosis (Brown et al., 1998). However, anthelmintic treatment exacerbated OJD in this trial while alleviating clinical outcomes for BTB in buffalo (Ezenwa et al., 2021); the reason for the difference (immunity or parasiticide) remains unclear. What is emphasized is that any concentration on selection for resistance to a particular pathogen may be detrimental to overall fitness (or the ability to respond to other stressors), such that resilience was the principal focus of this project.

There were several limitations of the current co-exposure trial which have resulted in an inability to fully examine the impacts of concurrent disease on *Hc* infection. Firstly, the intense and prolonged drought during the time of the trial resulted in inhospitable conditions for maintaining Hc eggs and larvae on pasture for continued exposure. In addition, to keep up with pasture demands of the sheep in the trial, the rotational grazing interval had to be reduced, resulting in animals moved to new pasture every 4 weeks, before the pre-patent period of *Hc* and again reducing uptake from grazing and reinfection of drenched animals. This discounted our ability to use "drenching interval" as a measure of resilience (Morris et al., 2010). Furthermore, conditions in the middle of the trial necessitated supplementary feeding, which again reduced the likelihood of sheep ingesting worm larvae from the pasture. Climatic events, together with a higher than normal dose of Mptb in exposed groups, resulted in faster onset to clinical disease and animals reaching humane endpoints earlier than in previous trials (Begg et al., 2010). Although these factors have impacted the

reinfection of some animals with *Hc,* there was still a low level of strongyle type eggs in the faeces throughout the trial and an increase in faecal egg counts towards the end of the trial and aligns to commercial production systems that would experience the same seasonality of parasitism.

5.1.3 Co-infections and resilience

Resilience is principally the ability of the animal to maintain productivity under challenge, in the case of the current project, concurrent disease challenge (Berghof et al., 2019). In commercial production systems some animals perform better although they are subjected to the same stressors, such as disease, and management conditions. These animals are termed resilient, and ideally being able to identify these animals and understand what contributes to their improved productivity and resilience would increase profitability of livestock enterprises.

Exposure to both Mptb and *H. contortus* resulted in reduced average resilience score in sheep, compared to exposure to either of these agents alone. The small numbers of higher performing animals in different groups also reduced the power of the analysis of resilience (see also SP2). Interestingly differences were also observed between the single challenged paratuberculosis and haemonchosis groups. Inoculation of sheep with Mptb resulted in reduced average resilience score compared to inoculation with *H. contortus* alone. The differences in disease progression of paratuberculosis and haemonchosis, namely the cyclic nature of parasite infection compared to the progression of paratuberculosis could account for the differing level of disease 'pressure' between these groups as well as the "quantum of infection" and virulence of both pathogens. Resilience can be shaped by the animal's immune system which develops through multiple exposures to a pathogen, such as in the use of vaccination (Dantzer et al., 2018). Therefore, the cyclical and seasonal nature of parasite infection, coupled with the low reinfection rate in the current trial could act as a form of immune training that has led to the development of resilience in the single exposure groups (eg. Morris et al., 2010). Further examination on the development of resilience in larger groups of sheep with defined genotypes on farms under differing nutritional regimes and environmental conditions is needed to fully understand the interplay of immunity, nutrition, exposure and genetics in resilient animals.

Within treatment group variability in resilience score was high in all groups. As expected, large differences were seen in individual animal resilience regardless of the challenge. One animal in the duel challenge group had a higher resilience score than 40% of the sheep in the Hc challenge group, which had the highest average resilience scores of all treatments. The ability to detect differences in individuals with our scoring system allows alignment of this with genotypic and immunological data as in sub-project 2 and 3, getting closer to defining what determines resilience.

There is clearly a limit to an animal's resilience resources. This limit can be breached by a sufficiently large pathogen challenge, either singly, or in combination.

5.1.4 Co-infections and the microbiome

Recent advances in livestock research highlight the 'microbiome' (commensal microbial population) influences on health and predisposition to diseases. The microbiome within ruminants comprises of populations of bacteria, fungi and archaea within the rumen to generate energy from otherwise indigestible food sources. Cattle and other ruminants are dependent on their commensal gut microbes for feed fermentation and digestion (Bath et al., 2013; Guan et al., 2008). Microbe

populations produce volatile fatty acids (VFA's) used for energy and weight gain and manipulation of the microbes can maximise the energy ruminants are able to extract from their feed (Dijkstra et al., 1993). Exogenous adaptation of the gut microbiome profile can significantly influence feed conversion efficiency and thus impact production performance. There is evidence that the microbiome can also have a strong influence on the whole animal, including immunity and disease susceptibility, physiology, metabolism and overall body weight and condition (Ellison et al., 2014; Ellison et al., 2019; Koboziev et al., 2014; Wolff et al., 2017). This has potential economic significance in food producing animals. An animal which can be fed the same quantity but show increased feed conversion and increased resilience is of more value both economically and environmentally and the overall health of the host will also alter microbiome composition.

Both *H. contortus* and MAP colonize the gastrointestinal tract, sharing this environment with commensal microbiome bacteria. It is reasonable to assume there is disturbance to commensals with introduction of new organisms within the same environmental niche. There is growing evidence that *H. contortus* alters microbiome populations through an increase in anaerobic populations (El-Ashram et al., 2017; Mamun et al., 2020), as do other helminth infections (Cortes et al., 2020). There is some evidence to suggest exposure to MAP may alter microbiome composition however, this evidence is limited to cattle (Fecteau et al., 2016). MAP pathogenesis and progression differs significantly between cattle and sheep, warranting species specific investigation into pathogen facilitated microbiome alterations. Both pathogens are prevalent within Australian sheep populations resulting in significant economic costs from production losses.

This pilot study sought to explore whether experimental exposure of sheep to an infectious dose of MAP, *H. contortus* or a co-infection with both MAP and *H. contortus* would result in variation to the gut microbiome. The data presented above shows significant differences in the bacterial populations and relative abundances between treatment and control groups in both the faecal and the ileal derived samples. The faecal samples were collected five weeks post infection/infestation whereas the ileal samples were collected at necropsy (1 year) thus change of to the microbiome is rapid and ongoing.

Acknowledgements: The expert assistance of Dr. Andrew McPherson with the microbiome studies is greatly appreciated.

6.1 Conclusions/recommendations – Sub-project 1

Co-infection of merino wethers with Mptb and Hc can be achieved and sustained in a research setting allowing sampling to examine the interplay of these two pathogens. Animals within this trial could be assigned a resilience score based on degree of disease and impact on productivity. Most importantly, a large variability between sheep within infection/infestation groups was noted, suggesting that differences in inherent resilience to challenge can be identified through this system. The ability to identify variability will allow insight into changes in immunology and genetics that have led to this variability and possible biomarkers for a 'resilience' phenotype.

This study has demonstrated successfully that the co-exposure of merino wethers to Mptb and Hc resulted in reduced productivity, increased disease severity and dissemination. The additive effect of paratuberculosis and haemonchosis shows the need to implement concurrent disease management programs that are integrated for commercial properties. However, the negative impacts of a

suppressive drenching regime on sheep exposed to Mptb alone, stresses the need to better understand the interplay between pathogens, to arrive at the most beneficial animal health management approach. Emphasis must be placed on testing for disease such as paratuberculosis, especially in areas endemic for Hc, where use of suppressive drenching regimes may be commonplace and should be discouraged, as in IPM programs.

The overall results from the small-scale pilot study suggest adaptation of the microbiome in response to co-exposure of merino wethers to MAP and *H. contortus*. At this stage it is not clear if this is a causative factor in the subsequent progression to rapid disease susceptibility and further work is required to gain a clearer understanding of the pathogen impact on microbe composition. This may help identify adaptation to management e.g. diet modifications to combat adverse microbiome populations, increasing feed conversion, reduce disease susceptibility and reducing economic losses. Future studies would be possible through use of stored samples.

7.1 Key messages – Sub-project 1

- Co-infection of merino wethers with Mptb and Hc can be achieved and sustained as a research model for co-infection studies.
- Individual animal resilience scores can be assigned and show variability both between and within treatment
- Co-exposure results in increased disease severity, disseminated disease and reduced weight gain, as anticipated
- Suppressive drenching regimes in paratuberculosis infected sheep causes increased *Mptb* shedding and dissemination of disease
- The microbiome is altered following exposure to co-infection, as anticipated.

8.1 Bibliography - Sub-project 1

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Sub-project 2: Developing genetic markers for resilience phenotype index.

1.2 Background Sub-project 2

Resilience in sheep relating to one stressor (disease or environment) have been explored in a number of studies, however, resilience under multiple stressors such under co-infection with different diseases have not been explored.

Genomic tools using high-density genotyping have been used to discover genes and regions related to production, reproduction and disease phenotypes. In sheep, the use of genome-wide analysis has been applied to studies of wool and meat traits and disease (see e.g. Pickering *et al.* 2015). Genomic information can be used to discover markers and genomic regions associated with the resilience phenotype.

This Subproject explored experimental data to define resilience phenotype and perform genetic analysis of resilience in sheep. A combined resilient score phenotype was developed by studying the combined effect of MAP infection, parasite load and the performance of the animal. The ability to maintain growth rate (body weight) was used as a measure of performance. The data from the current experiment and from two previous experiments were included in the analyses as described below.

2.2 Objectives – Sub-project 2

The overall objectives of this sub-project were to develop an index for resilience, and to identify genetic markers for resilience, including disease resistance, that can be evaluated via Australian Sheep Breeding Values (ASBV).

3.2 Methods – Sub-project 2

3.2.1 Description of experimental datasets

The information generating from the current trial and two previous experiments have been included as described in the following sub-sections:

3.2.1.1 P.PSH.0813 - current experiment

A total of 95 sheep were included in this trial (Sub-project 1) where animals were challenged with *H.contortus* and/or *M.paratuberculosis* or were kept as unchallenged controls. In addition, the animals were subjected to two drenching regimens, as described in Table 3.2.1.1. and in the SP1 report. Briefly, the body weight of individual animals was recorded regularly between treatment day 0-300 (n=10). Measurement of faecal egg count was recorded 2-3 times per month (n=22). MAP was measured using a qPCR method (HTJ) on at two time points and with final a detailed histopathology examination. The animals were drenched when an animal reached FEC of 5000 in Hc challenged groups (1& 4) and 500 epg in groups 2 & 5. The data from 50 sheep from the first three groups, which were exposed to MAP and/or to Hc, were included for SNP association analysis.

Table 3.2.1.1. Animals included in generation of new data showing allocated groups and treatment

Group	Treatment	N
1	Hc + MAP, normal drenching	20
2	MAP, normal drenching	15
3	MAP, suppressive drenching	15
4	Hc, normal drenching	20
5	Controls for Hc, normal drenching	10
6	Controls for HC, suppressive drenching	10
7	Controls for MAP, separate paddock	10

3.2.1.2 P.PSH.0576 – previous experiment

In this project, 461 sheep from the Merino, Border Leicester, White Suffolk first cross Merino and Poll Dorset breeds were purchased from farms participating in the Australian Market Assurance scheme for Paratuberculosis for use in 11 separate MLA-funded projects, carried out at The University of Sydney between 2007 and 2015 (project numbers OJD.031, P.PSH.0311, and P.PSH.0576).

Genotypic data: The genotypic data on these animals were available from project P.PSH.0576 where the DNA samples of these animals were genotyped with the AgResearch OvineHD BeadChip 600K SNPchip (Anderson et al. 2014). Out of these seven samples with less than 95 per cent call rate were excluded from further analyses. The map positions of the SNPs are based on sheep assembly Oar_v3.1 (http://www.livestockgenomics.csiro.au/sheep/oar3.1.php) and were downloaded from http://bioinformatics.tecnoparco.org/SNPchimp/. A total of 43,040 SNPs was excluded because of low call rate (< 90 per cent) and low Minor Allelic Frequency (MAF < 0.01). There were 1,119 unmapped SNPs and 25,046 SNPs on the X-chromosome and these were excluded from the analysis. The remaining 534,784 SNPs mapped on the autosomes were used in the current SNP association analysis. Overall mean spacing between the adjacent SNPs is 4282 bp and mean minor allelic frequency (MAF) is 0.2429.

Out of these animals, the genotypic data and data required for computing the resilience score were available for 279 animals which were used for the final GWAS analysis presented in this report. The resilience score in this trial was computed based on ADG and PTb-score as described in the following section. These animals belong to four breed groups viz. Border Leicester (40), Merino (159), Poll Dorset (41) and White Suffolk X Merino (39). The breed, sex, vaccination status and trial were included as fixed effects in the model for conducting GWAS analysis.

3.2.1.3 OJD.028 – previous experiment

This dataset belongs to the genotypic data generated from the samples of archived samples from an earlier project OJD.028. The data from this trial on MAP exposure and infection status were available. These animals were also exposed to parasites under field conditions and controlled using standard detection and drenching procedures. The main objective to include samples from this trial was to increase the power of gene discovery for resilience to disease burden, modelled on MAP infection. This trial involved 840 female Merino sheep consisted of three age groups (lambs, hoggets and adult sheep) sourced from uninfected properties and tested negative for OJD. Four different levels of contamination were created by co-grazing the experimental sheep with infected sheep at different graded stocking rates. They remained under the trial for a 2.5-year period. Infection with Mptb was tested using histopathology and tissue culture techniques. Parasite burden in these animals was managed according to standard practises and FEC were kept below 500. These data were used to explore disease resistance for comparison with other experiments for resistance/resilience analysis, however data on ADG and FEC on individual animals were not available from this trial.

Description of the phenotype (OJD.028): A total of 654 animals from project OJD.028 were used for GWAS analysis included in this report. These consisted of 17 susceptible and 637 resistant sheep as diagnosed by histopathological screening. Two other resistant/susceptible phenotypes classified based on the following method were also analysed.

IFC (Individual faecal culture) positive: (consisted of 17 susceptible and 637 resistant sheep)

Tissue culture: (consisted of 19 susceptible and 635 resistant sheep). Only a proportion of susceptible animals were common across these three tests.

Individual faecal culture (IFC) test: Faecal and blood samples were collected from trial sheep at approximately three-monthly intervals for the duration of the trial. Pooled faecal culture (PFC) was undertaken and samples of faeces from individual sheep were stored at -80°C; when a positive result was obtained from a pool, the individual samples within that pool were then cultured so that positive culture results were attributed to individuals.

The fixed effects were “age of the animals” and “treatment groups”. There were 276, 204 and 174 animals in low, medium and high exposure groups, respectively. There were 3, 8 and 6 animals susceptible in these three groups, respectively, as diagnosed by histopathology examination. There 229, 222 and 203 in Adults, Hoggets and Lambs age groups, respectively. There were 3, 6 and 8 cases in these three age groups, respectively. The details of this experiment, including experiment groups, can be found in McGregor et al, 2012 and McGregor et al, 2015.

3.2.2 Development of resilience index

Resilience in the context of animal production relates to the capacity of an animal to continue producing in the face of physical or physiological stressors, including disease (de Silva *et al.* 2018). Generically, when considering response to disease, resilience is proportional to the total disease burden (Torres *et al.* 2016). A combined resilient index phenotype was developed by integrating three different phenotypes associated with disease burden and production gain. The phenotypic

traits were MAP infection, parasite load and performance (weight gain) of the animal. These three phenotypes were defined as follows:

Paratuberculosis (PTb)-score: A scoring system for MAP infection was developed and given a score between 0 and 100 according to the intensity of the MAP infection of each animal such that the most susceptible animals were given a score of 100. The detail of this scoring system, PTb-score, is provided in Table 3.2.2. This score was further rescaled on a 0 to 1 scale (PTb-score-01) such that the most resilient animals (with lowest infection) were scored as 1 and with highest infection as 0.

Table 3.2.2. Calculation of PTb-score

Component	Description	Scoring		
Histopathological lesions of the gut	Histopathological lesions were assessed at 2-4 sites along the small intestine and associated mesenteric lymph nodes, from the terminal ileum to the posterior jejunum	Intestine	Perez score	Points
			Lesion grade 3b	4
			Lesion grades 3a, 3c, 3d	3
			Lesion grade 2	2
			Lesion grade 1	1
			No lesions	0
		Mesenteric lymph nodes	Lesion grade 3	3
			Lesion grade 2	2
			Lesion grade 1	1
			No lesions	0
		Liver †	Lesions present	10
			No lesions present	0
		Add 1 point for every intestinal site with AFB score > 2		
		Add a further 1 point for every intestinal site with AFB score ≥ 4		
		Add 1 point for every mesenteric lymph node site with AFB score ≥ 2		
Maximum histopathology score for small intestine and associated lymph nodes at each region*				10
Maximum histopathology score across all sites = (10 x 4 regions of intestine/lymph nodes) + 10 (liver lesion)				50
Tissue culture	Tissue culture for MAP of multiple regions of the intestine, liver and associated lymph nodes*	Intestine (ileum and jejunum) - Positive		5
		Mesenteric lymph nodes (ileal and jejunal regions) -Positive		5
		Liver and/or associated lymph node † - Positive		10
		Maximum tissue culture score		20
Gross lesions	At necropsy, identification of gross lesions associated with paratuberculosis ‡			30
TOTAL SCORE				100

* This is assuming four regions along the small intestine are examined. If two regions only were examined (terminal ileum and mid-jejunum), all scores were multiplied by a factor of 2, to give a maximum score of 20 for the intestine and lymph node at each region assessed.

‡ This can include enlarged, reactive mesenteric lymph nodes, lymphatic cording, thickening of the small intestines and presence of oedema.

† Liver histopathological lesions and tissue culture positivity were heavily weighted as these are indicative of severe, disseminated infection.

Weight gain/change: Average daily weight gain (ADG) in g was computed:

$$ADG = \left(\frac{\text{Final weight of an animal } - \text{Initial weight of an animal}}{\text{Number of days}}\right) \times 1000$$

Where, ADG means average daily weight gain/loss in grams;

The final weight of an animal means weight of a sheep in kilograms at 48-50 weeks post inoculation (administration of the first dosage of Mptb suspension);

Initial weight of an animal means the weight of a sheep in kilograms at 38 weeks post inoculation (administration of first dosage of MPTB suspension);

The number of days refers to the duration between these two-time points.

The ADG was converted to 0 to 1 scale (ADG-01) such that the most resilient animals (with highest ADG) were score as 1 and with lowest ADG (or highest weight loss) as 0.

Parasite load (FEC): The parasitic load on the animals for Hc was estimated with faecal egg count (FEC) as described in SP1. These FEC counts were transformed to log scale to account for skewed distribution and then log (FEC) was converted to a scale of 0 to 1 scale (FEC-01) such that the most resilient animals (with lowest FEC) were score as 1 and with highest FEC as 0.

Computation of combined resilient score: The combined resilient score (Resilience Index) was computed as the mean of three scores (scaled to a range of 0 to 1):

Resilience Index= (FEC-01 + ADG-01 + PTb-score-01)/3

For trial P.PSH.0576, only ADG and ParaTb_score3 could be obtained from the available data,

hence the mean_res_score for these animals were computed as:

Resilience Index = (FEC-01 + ADG-01+ PTb-score-01)/2

3.2.3 Genotyping with 50K SNP chip

The samples from all the animals in the current project (P.PSH.0813) and from project OJD.028 were included for extraction of DNA for genotyping. A total of 757 DNA samples were genotyped with the ovine 50K SNP chip (a deliverable from the Sheep Genomics Program, jointly funded by AWI and

MLA, ca. 2003-2008; Thermo Fisher Scientific Axiom array) by a service provider (Xytogen Genotyping Australia Pty Ltd). The QC analysis suggested overall call rate (proportion of the SNP genotypes completed) for the samples was 98 % with only less than 2% of missing genotypes. The distribution of sample-wise and SNP-wise call rate is presented in Appendix Fig. 3.2.3.1 and Appendix Fig. 3.2.3.2, respectively.

Distribution of minor allelic frequency across 56,249 SNP genotyped presented in Fig. Appendix Fig. 3.2.3.3 indicated a slight over representation of SNPs with higher MAF. Appendix Table 3.2.3.4 shows the SNP are evenly distributed within chromosomes and across the genome indicated by the comparable mean spacing between the adjacent SNPs. As expected, the mean MAF of SNPs across different chromosomes are quite similar.

The genetic kinship among 767 animals were estimated using the genotypes and is presented in Fig. 3.2.3. The dark blocks on the diagonal indicate the presence of the close relationships with the dataset. This suggested that a mixed model approach including SNP based kinship in the model would be appropriate for conducting genome-wide association analysis.

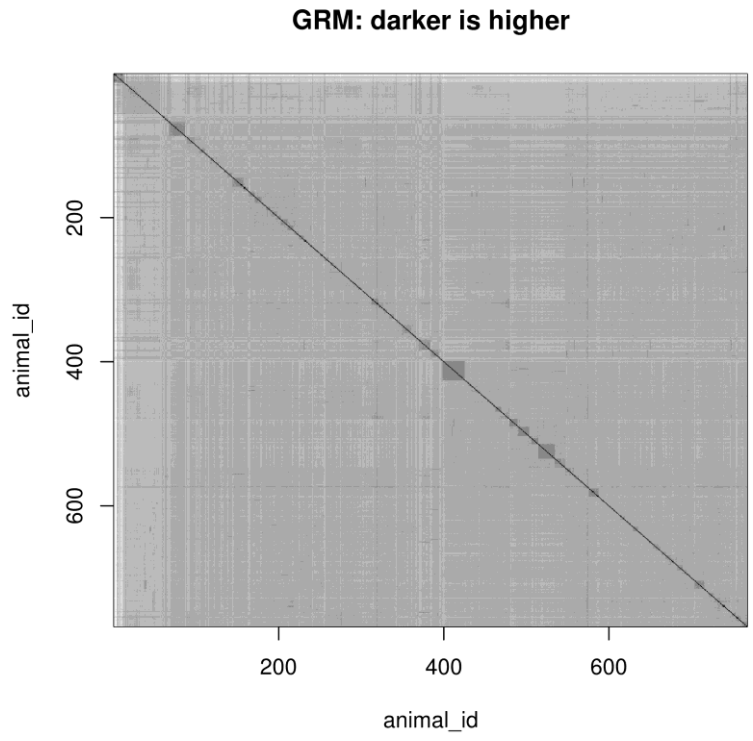

Fig. 3.2.3. Genetic kinship among 767 sheep computed based on SNP genotypes.

3.2.4 Genome-wide association analysis

A mixed model was used to test how susceptible/resistant binary phenotype (case control response variable) or the quantitative phenotype (Resilience Index) depends on the number of alleles of SNP genotype. All the SNPs across the genome were tested with one SNP in the model at a time. The model was fitted using GCTA software (Yang et al. 2011) using following model:

$y = a + bx + cz + g + e$

where y is the binary or quantitative phenotype, *a* is the mean term, *b* is the additive effect of a candidate SNP to be tested for association, *x* is the genotype indicator variable coded as 0, 1 or 2, g is the polygenic effect (random effect) i.e. the combined effect of all SNPs (captured by the genomic relationship matrix (GRM) calculated using all SNPs, Yang et al. 2011), *c* is effect of fixed effect *z*, and *e* is the residual.

The *p*-values and effect size for all the SNPs across the genome were estimated and compiled. The genome-wide false discovery rate were computed using the qvalue package (Storey & Tibshirani 2003) of Bioconductor. Chromosome-wise and genome-wide Manhattan plots of p-values were generated to identify key regions associated with different binary phenotypes or Resilience Index. The top SNPs for each phenotype were listed.

4.2 Results – Sub-project 2

4.2.1 Development of combined resilience phenotype

The Resilience Index and its correspondence with the three component phenotypes for the current project is presented in Fig. 4.2.1. The distributions of these traits are shown as histogram plots on the diagonal panel. The correlation estimates among component phenotypes and with the Resilient Index on the upper triangle of the scatterplot matrix show partial dependence of the final Resilience Index with PTb-score, AFEC and ADG.

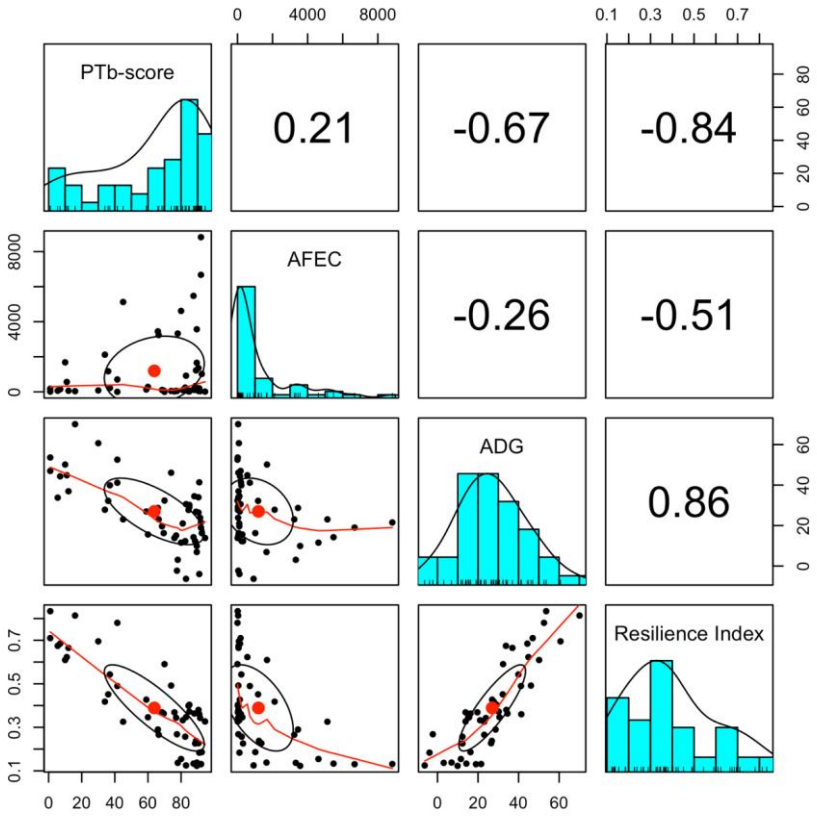

Fig. 4.2.1. The Resilience Index and its correspondence with the three component phenotypes. The names and distributions of original and computed traits are given on the diagonal. The scatterplots and the estimates of Pearson correlation coefficient are provided in lower and upper triangle of the matrix.

4.2.2 Marker associations from P.PSH.0813 – current project

A total of 50 sheep with the Resilience Index (as defined in methods) phenotype across three different experimental groups within the current trial 0813 were used in this GWAS analysis. The results of SNP associations with Resilience Index are presented in Fig. 4.2.2 in the form of a Manhattan plot. Out of 52,772 tested, there were 2681 significant SNPs, using a nominal *p*-value < 0.05. A summary of GWAS results including a list of 10 top significant SNPs is provided in Appendix File 4.2.2.1. The detailed chromosome-wise plots of these SNPs are provided as Appendix File 4.2.2.2. However, with such a small number of animals available in this trial, no SNP exceeded the more stringent False Discovery Rate (FDR) < 0.05 or a more relaxed FDR < 0.10.

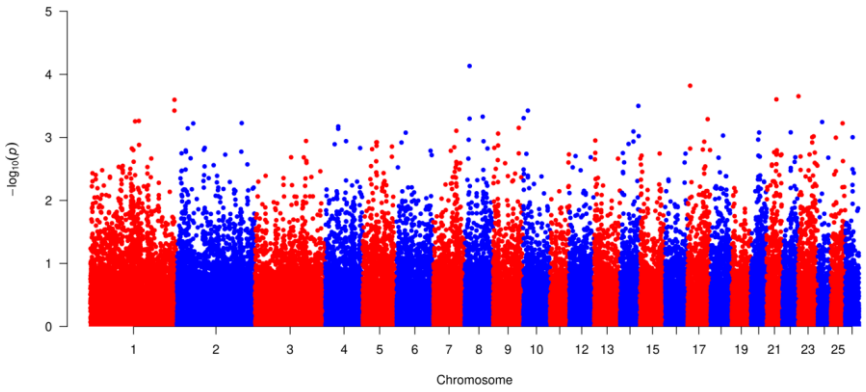

Fig. 4.2.2. Manhattan plot of genome-wise SNP association with Resilience index. Y-axis shows *p*-values on a -log10 scale. One dot of the figure represents an association between a SNP and Resilience index. No SNP was found significant after adjusting for FDR < 0.10.

4.2.3 Marker associations from project P.PSH.0576

There were 279 sheep with the Resilience Index (as defined in the Methods section) phenotype across four different trials within project P.PSH.0576 viz trial 576.1 (n=187), trial 31.6 (n=48), trial 311.1 (n=28), and trial 576.2 (n=16). The results of SNPs associations with Resilience Index are presented in Fig. 4.2.3 in the form of a Manhattan plot. Out of 534,784 tested, there were 23,973 significant SNPs using a nominal p-value < 0.05. A summary of GWAS results including a list of 10 top significant SNPs is provided in Appendix File 4.2.3.1. The detailed chromosome-wise plots of these SNPs are provided as Appendix File 4.2.3.2. After controlling for false discovery rate (qvalue < 0.1), five SNPs remained significant (Appendix File 4.2.3.1). All of the five SNPs (viz. oar3_OAR1_18537092, oar3_OAR1_18537159, oar3_OAR1_18537241, oar3_OAR1_21613485 and oar3_OAR1_18536960) were located in one genomic region at 18 Mb on chromosome 1 (between 18536960 to 21613485 bp).

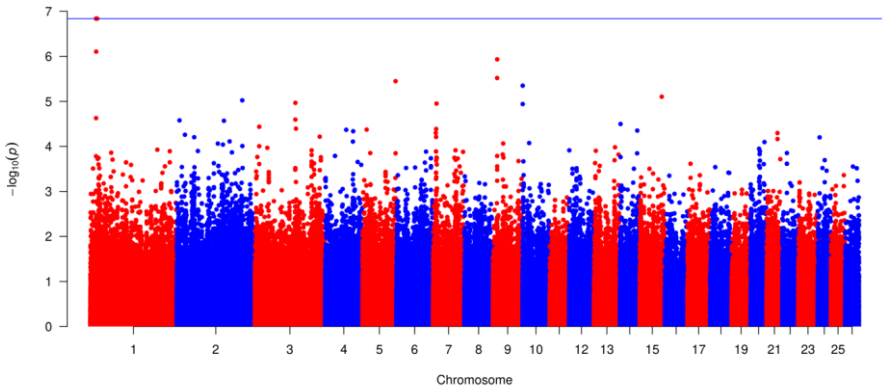

Fig. 4.2.3. Manhattan plot of genome-wide SNP association with Resilience index. Y-axis shows *p*-values on a -log10 scale. The horizontal blue line is the threshold significance at the *q*-value = 0.1.

4.2.4 Marker associations from project OJD.028

The results of SNPs associations with the resistant/susceptible phenotype diagnosed by histopathology are presented in Fig. 4.2.4 in the form of a Manhattan plot. There were 2500 significant SNPs using a nominal p-value <0.05. A list of 20 top significant SNPs is provided in Appendix File 4.2.4.1. The detailed chromosome-wise plots of these SNPs are provided as Appendix File 4.2.3.2.

After controlling for false discovery rate (qvalue < 0.05), one SNP remained significant (p-value=8E-10; Appendix File 4.2.4.1). This SNP, AX-123224357, is located on chromosome 7 at position 72674247 base pairs. The closest gene to this SNP is PSM3A, which is relevant to bacterial immune response.

The results for resistant/susceptible as diagnosed by tissue culture and IFC positivity were also analysed. There were a number of significant SNPs based on nominal P-values of 0.05. However, no SNP exceeded the more stringent FDR cutoff of 0.05 or 0.10. The results from these two phenotypes are not presented, but the detailed results for are available at : https://app.box.com/s/8bkpswx6n0mxzpofs5z3yppfxot0hq4k.

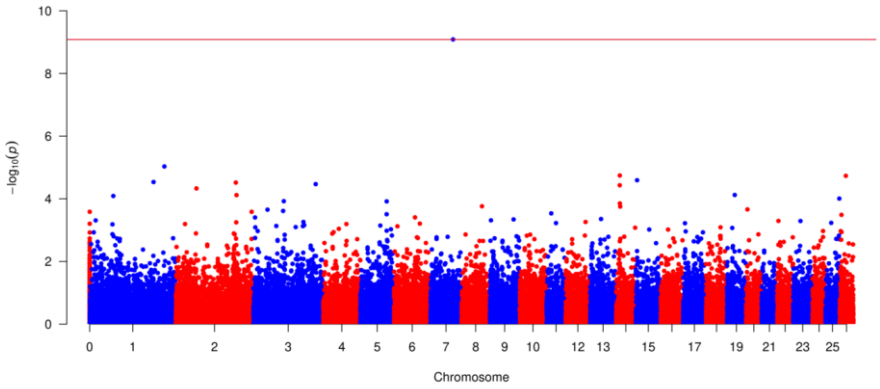

Fig. 4.2.4. Manhattan plot of genome-wise SNP association with Resilience index. Y-axis shows *p*-values on a -log10 scale. The horizontal red line is the threshold significance at the *q*-value = 0.1.

5.2 Discussion – Sub-project 2

The aim of this study was to develop an index for a resilience phenotype in sheep and to explore the genetic component of this resilience. The completion of a project in which sheep were intensively studied following experimental infection with MAP alone, or in combination with parasites, allowed the development of an empirical model incorporating components of disease burden and weight gain.

Resilience is a prevalent but broad concept that has different meanings in alternative contexts. Clearly a definition is important before we can investigate the extent to which resilience has a role in sheep production, and particularly in the case of this program, achieving the stated objectives. In this analysis, disease burden is a key determinant of outcome, since it imposes the most significant physiological stress that needs to be overcome by the animals in order to maintain a productive growth trajectory, and thereby demonstrate resilience.

The major advantage here was the detailed phenotypic information available for individual sheep. Each animal was monitored intensively, with frequent measurements of a range of phenotypes that identified the progressive response to treatment. The phenotypes were then modelled to arrive at a single Resilience Index that captured the essential components and the observed variances within the study. Parasitic infection and the response to MAP are very complex non-linear disease processes, but here we employed a simple additive model to approximate total disease burden. The final model used two components of disease burden (Mptb score and FWEC) and one component (weight gain) as the key production outcome.

Faecal egg count is a robust and simple measure of parasitic worm infection, and although it has some limitations as a proxy for worm burden, it has been shown to demonstrate a dose related

effect on production outcomes in sheep, and has a relatively small but significant genetic component (Al Kalaldeh et al. 2019). Computation of the second component of the Resilience index, PTb-score, required detailed information from histopathological examination of necropsy tissue and is considered to be the most conclusive method for defining the MAP phenotype. Despite its value in this study, histopathological examination in large-scale field applications is difficult. Considering this potential limitation, we explored other measures of disease that do not rely on highly invasive or post-mortem analysis. HT-J, is a rapid high-throughput faecal PCR-based laboratory assay (Plain et al., 2014) that is applicable in live animals. In recent times, it has been developed and adopted as a standardised measure to complement more laborious and impractical evaluation of MAP infection (Plain et al. 2014). We found that the HT-J phenotype is closely associated with the detailed PTb-score and also with Resilience Index, as shown in Fig. 5.2.1, which suggests that the PTb-score can be replaced with HT-J without much loss of information.

Genome-wide genotype data were available from three MLA-funded projects and collated here to explore association of markers with resilience, and any relationships between disease resistance and resilience. GWAS analysis of data from the experimental co-infection study suggested that there may be regions associated with the Resilience Index, but the nature of the trial required an intensive operation and sample number became a limiting factor, hence the association analysis was grossly underpowered. However, analysis based on a second trial identified the chromosome 1 genomic region associated with the Resilience Index. The SNP-based heritability estimate (0.48 ± 0.24) for the Resilience Index suggested a heritable component for this trait. In this trial, the level of parasitic infection was managed according to standard practices, and for the purpose of a Resilience Index calculation was assigned a value of zero. The samples from a third trial were also used to analyse disease resistance to MAP.

The region associated with the resilience phenotype covers a ~3Mbp segment on chromosome 1. The region is gene rich with at least 77 expressed genes. Deciphering the functional implications of this association may be useful to determine whether this is a robust candidate for sheep resilience. Disease resistance analysis suggested a candidate gene on chromosome 7 linked to the PMSA3 gene. This gene has a key role in macrophage function, which is consistent with the known cellular innate immune response to MAP and has been postulated as associated with the dichotomy of responses to worms and BTB in worm-resistant, African buffalo (Enezwa et al., 2021). Further exploration of structural or expression related variation affecting functional pathways in which the protein coded by this gene participates, are warranted.

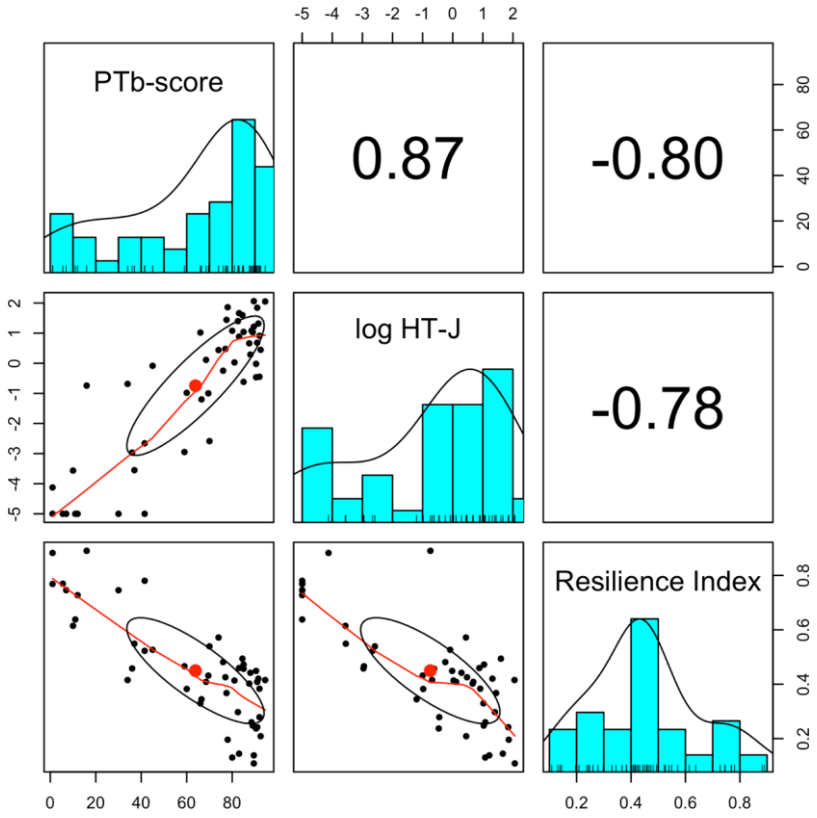

Fig. 5.2.1. A pair plot showing association of HT-J with PTb-score and Resilience Index. The names and distributions of original and computed traits are given on the diagonal of the plot matrix. The scatterplots and the estimates of Pearson correlation coefficient are provided in lower and upper triangle of the matrix.

6.2 Conclusion/Recommendations – Sub-project 2

A flexible working model for resilience in sheep (a resilience index) was developed. The model was validated using data from a coinfection trial, however this trial was not powerful enough for detecting statistically significant genetic markers associated with the index. This needs to be applied to a larger cohort on farm to obtain a workable dataset.

When applied to our existing data of Mptb in sheep, Resilience Index phenotype association analysis identified prospective genetic SNP markers for resilience on Chromosome 1.

A single SNP marker proximal to the PSM3A gene on Chr7 was associated with resistance to Mptb infection defined by the detailed phenotypic analysis of histopathology. Samples from farms involved in SP5 or faeces from endemic farms would be ideal to validate this marker.

Histopathological analysis of Mptb was shown to correlate closely with the more rapid and standardised PCR-based measure HT-J.

7.2 Key Messages – Sub-project 2

A component model for resilience in sheep (RI) was developed based on a coinfection trial of MAP and Hc.

Adapting the RI model to studies of infection with MAP and Hc identified prospective genetic markers for resistance to infection and to resilience.

The RI model is adaptable and may be incorporated into expanded studies of resilience in sheep.

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Sub-project 3: Host response biomarkers and on-farm management

1.3 Background – Biomarker discovery

1.3.1 Immunological biomarkers for disease susceptibility and resilience

Immunological biomarkers including cellular and humoral immune responses as well as a panel of gene expression markers were previously identified as markers of paratuberculosis disease expression under controlled experimental infections (de Silva et al. 2013; Pooley et al. 2019; Purdie et al. 2019). In this project, we aimed to evaluate if these were effective biomarkers for identifying disease resilience and susceptibility in commercial flocks, especially in flocks that are vaccinated against paratuberculosis. Vaccination and infection both generate immunological memory therefore, current tests do not differentiate between infected and vaccinated (DIVA) animals. While this discrimination is important for trade purposes, it is not relevant for disease management on-farm. The ability to identify or predict disease susceptibility would be a useful new tool to incorporate into on-farm management strategies.

1.3.2 Predictive markers in parasitic co-infections

Production outcomes can be impacted by multiple factors, pathogen burden being just one. Multiple pathogens can contribute to pathogen burden but often effect of diseases are studied in isolation. Little is known about the overall effect of a combination of pathogens on host immune responses and ultimately on resilience and susceptibility to disease. Utilising samples from the co-challenge trial in Sub-project 1, we have assessed immune response dynamics in sheep with a view to identifying biomarkers for resilience or susceptibility to disease in sheep exposed to multiple pathogens.

1.3.3 Novel biomarker discovery

To broaden the scope for identifying potential biomarkers, as well as to identify novel methodologies currently in use, a literature search was carried out to identify states of disease susceptibility and resilience in relation to mycobacterial diseases in general. This identified microRNA (miRNA) as potential biomarkers for predicting disease outcomes in paratuberculosis. The abundance and stability of miRNAs within circulating extracellular vesicles such as exosomes and microparticles make them ideal for this purpose. miRNAs are small non-coding RNA molecules that regulate a wide range of biological processes by post-transcriptionally regulating gene expression which ultimately leads to silencing of protein expression and affect biological functions. A biobank of archived samples from previous experimental trials was a valuable resource to screen for potential miRNA biomarkers for disease resilience.

To further understand the role of candidate miRNA biomarkers underpinning resilience or susceptibility to disease, we collaborated with the Centenary Institute to use a platform where genetic manipulation was feasible. Optical transparency of embryos, availability of gene editing tools and ability to visualise organism-wide disease make zebrafish an ideal model organism. The *M. marinum*-zebrafish model is a well-established model for mycobacterial diseases, allowing the study of disease processes in a natural host, but has not been used for paratuberculosis research. Alongside the low costs associated with maintenance and generation of animals, and the ability to

use high throughput methods, the use of zebrafish allows for the visualisation of infection which is not achievable in ruminants.

2.3 Project Objectives – Biomarker discovery

To broaden recommendations from project P.PSH.0576 and to adapt tools developed in that MAP project to provide farmers with information that can be used to minimise the risk and impact of disease to enable novel control strategies on-farm as well as biosecurity during trade. This sub-project will also extend biomarker discovery for mycobacterial diseases across species.

3.3 Methodology – Sub-project 3: Biomarker discovery

3.3.1 Immunological biomarkers for disease susceptibility and resilience

Detailed descriptions for this section can be found in Appendix 3A.

3.3.1.1 Animals

Blood and faecal samples were collected from sheep in commercial enterprises in NSW and VIC to identify host responses which could be used to inform on-farm management of ovine paratuberculosis. Table 3.1.1 summarises details for the farms selected.

Table 3.1.1 Details of farms selected for immunological biomarker studies

Flock exposure history	Gudair® vaccination*	No. of farms	Location	No. of animals per farm
Unexposed	None	3	NSW	30
Exposed	Vaccinating	4	VIC	30
Exposed	Vaccinating	1	NSW	50

*Within the survey respondents, there were no farms with a history of disease that were currently not vaccinating their flocks

3.3.1.2 IFNγ^{PLUS} Assay

Whole blood cultures and IFNγ ELISA were carried out as described in (Plain et al. 2012).

3.3.1.3 IgG1 ELISA

The IgG1 ELISA was as described in (Pooley, Begg et al. 2019). Initial analysis compared IgG1 %SP values calculated using positive and negative control IgG1 OD values to 316v antigen, PPA or PPDA using the following formula:

$$IgG1\ \%SP = \frac{OD\ sample - OD\ ELISA\ negative\ control}{OD\ ELISA\ positive\ control - OD\ ELISA\ negative\ control} x\ 100$$

3.3.1.4 Lymphocyte proliferation

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation and labelled with the vital tracking dye CFSE (carboxyfluorescein diacetate succinimidyl ester) prior to incubation at 37°C in 5% CO_2 for 5 days with and without MAP-specific antigen (316 v) (de Silva et al. 2010). Proliferation was calculated as:

% $CFSE^{dim}$ cells in the presence of MAP antigen –% $CFSE^{dim}$ cells in the presence of culture medium

3.3.1.5 Gene expression RT-PCR

Blood samples were collected into PAXgene® tubes and stored at -20 °C. RNA extraction was performed using the PAXgene® Blood RNA kit, according to manufacturer's instructions and stored frozen at -80°C prior to use. Complementary (c)DNA was synthesised using the SensiFAST cDNA synthesis kit (Bioline), according to the manufacturer's instructions,

Primer selection and design: Targets were identified from a previous study (Appendix 3). Primers were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) for each of the genes to be validated.Two previously validated reference markers were utilised following assessed by geNorm; this follows MIQE guidelines (Vandesompele et al. 2002).

Real-Time quantitative PCR (qRT-PCR): Selected RNA samples were DNase treated to remove genomic DNA, then reverse transcribed to cDNA using oligo(dt) primers and the AffinityScript qPCR cDNA synthesis kit (Stratagene, Agilent) according to the manufacturers' instructions. qRT-PCR was performed using an Mx3000P Real-time PCR system (Stratagene, Agilent) using the QuantiTect SYBR Green PCR kit (Qiagen). The specificity of the reaction was confirmed using melting curve analysis and standard curves were performed on each plate for each primer set.

3.3.1.6 Faecal MAP PCR

Faecal MAP DNA extraction and IS900 qPCR were as described in Plain et al. 2014. A positive faecal PCR test is presented as positive HT-J result based on validated parameters.

3.3.1.7 Statistical analysis

See Appendix 3A for details.

3.3.2 Predictive markers in parasitic co-infections

Detailed descriptions for this section can be found in Appendix 3B.

3.3.2.1 Animal trial

Details of the *Haemonchus contortus* (Hc) and MAP co-challenge trial and the different *in vivo* treatment groups are detailed in the report for sub-project 1. The groups relevant to this section are briefly described in Table 3.2.1.

Table 3.2.1 Treatment groups in co-challenge trial described in sub-project 1

Treatment group number	Treatment
1	Hc and MAP
2	MAP
4	Hc
7	No treatment*

* This group was held on a separate paddock to the other groups to ensure there was no passive uptake of Hc and MAP.

3.3.2.2 Haemonchus antigen preparation

L3 larvae were disintegrated using metal beads and subjected to freeze-thaw cycles. Protein quantification was by a Bradford assay (Biorad).

3.3.2.3 IFNγ assay

Whole blood stimulation and ELISA were carried out as described in section 3.3.1.2, excluding the addition of the cytokines IL-7 and IL-12 as the blood cultures were set up within a few hours of collection.

3.3.2.4 Lymphocyte proliferation

As described in Section 3.3.1

3.3.2.5 CXCL-10 (IP-10) assay

Culture supernatants from whole blood stimulations (Section 3.3.2.3) were also used for detecting the cytokine CXCL-10 by ELISA (Kingfisher Biotec) following manufacturer's instructions.

3.3.2.6 Cytokine array

A protein array with a panel of 20 ovine cytokines (Raybiotec) was used for biomarker discovery. These arrays use a multiplexed sandwich ELISA-based quantitative array platform which enables accurate determination of the concentration of multiple cytokines simultaneously.

3.3.2.6.1 Animals

A subset of animals was selected for biomarker discovery based on disease outcome characteristics. Diseased animals (n=4) were tissue culture positive (containing viable MAP in any tissue section examined), HT-J positive (in any one or more of the 3 sampling time points assessed) and reached the faecal egg count (FEC) threshold for its group. Resilient animals (n=3) were tissue culture negative in all sections tested, HT-J negative at all 3 time points assessed and did not reach the FEC threshold for its group.

3.3.2.6.2 Blood culture

The sampling point for this analysis was 4 months post exposure (4 months following the primary Hc dose and 3.75 months after the primary MAP dose). Blood was cultured with an equal volume of a stimulus (PWM, Hc or MAP 316v antigen) or medium alone for 2 days.

3.3.2.7 Statistical analysis

Multiple comparisons were by ANOVA. Detailed descriptions are in Appendix 3B.

3.3.3 Novel biomarker discovery

3.3.3.1 Identification of candidate biomarkers based on literature review

The search engines Pubmed (http://www.ncbi.nlm.nih.gov/pubmed/) and Web of Science (http://apps.webofknowledge.com) were used with the key terms: mycobacteria, biomarker, susceptibility, discovery, review in conjunction with a range of operator (and, or, not) combinations to refine the search. Outcomes of this evaluation have been peer-reviewed and published (Appendix 3D).

3.3.3.2 Screening archived samples for microRNA patterns of resilience and susceptibility

Detailed descriptions are in Appendix 3.

Archived ovine plasma samples from the MAP exposure trials run at the University of Sydney (P.PSH 0576.1) were used for miRNA sequencing. Plasma from whole blood collected via jugular venepuncture into EDTA tubes was used for RNA extraction and miRNA sequencing.

Animals were selected for the study based on disease status, histology classification, and vaccination status. Animals that were exposed to an infectious dose of MAP but failed to become infected were classified as resilient (de Silva et al. 2018). Resilient animals that did not succumb to disease showed evidence of exposure to MAP, however, infection was not able to be detected. Samples collected at 13 weeks post exposure (early timepoint) and 49 weeks post exposure (late timepoint) were selected for small RNA sequencing using Illumina NextSeq 500 and CLC Genomics Workbench for data analysis.

To investigate not only the miRNA response to infection but whole transcriptomic responses, gene lists of differentially expressed miRNA for each treatment were uploaded to IPA for further *in silico* analysis alongside previously published microarray data of MAP infected and resilient sheep (Purdie et al. 2019).

To validate gene expression changes observed in the miRNA-seq data, 8 differentially expressed genes were selected for validation using qPCR.

3.3.3.3 MicroRNA studies in zebrafish

This research was carried out in collaboration with Dr. Stefan Oehlers, Centenary Institute, Sydney. Further details can be found in Appendix 3C and 3F.

3.3.3.3.1 Zebrafish husbandry

Adult zebrafish (Table 3.3.3.1) were housed at the Centenary Institute and were generated by natural spawning (Sydney Local Health District AWC approval 2017-036).

3.3.3.3.2 Zebrafish infection

Embryos were collected immediately following fertilisation for guide RNA (gRNA) for gene knockout and antagomiRs (GenePharma, China) (AM) for gene knockdown. Embryos were dechorionated prior to microinjection of fluorescent *M. marinum*. Images were captured using the Leica Application Suite program and analysed in ImageJ.

Following initial optimisation experiments, gRNA oligos (Sigma-Aldrich, USA) for Crispr-Cas9 miRNA knockout were designed as previously described (Chen et al. 2016). Embryos obtained by natural spawning were injected with either miR-126a antagomiR, miR-206 antagomiR, or a scramble control at the single cell stage and incubated at 32°C. Embryos were further treated with either 50 nM of rapamycin or appropriate control, refreshed daily. Mortality was measured every 24 hours and static imaging conducted at 1, 3, and 5 days post infection (dpi) to analyse bacterial burden or neutrophil activity.

4.3 Results – Sub-project 3: Biomarker discovery

4.3.1 Immunological biomarkers for disease susceptibility and resilience

Detailed descriptions for this section can be found in Appendix 3A.

4.3.3.1 Johne's disease prevalence

Of the three farms self-reporting an absence of disease (Table 3.1.1), one provided a negative report based on routine faecal PCR testing of 1000 animals to support this claim. One farm consented to faecal testing and all 30 animals selected for this study were test negative. One producer did not consent to reveal the results of any such tests nor to have diagnostic testing carried out as part of the study. Farms with a history of paratuberculosis prevalence from Victoria (Table 3.1.2) were also sampled for sub-project 5.

Table 3.1.2 Prevalence of Johne's disease on-farm

Farm site ID	Prevalence on farm (n=325 or 350)	Prevalence in test cohort (n=30)	HTJ category for test cohort		
			Low	Medium	High
D	Not done	10%	1	1	1
E	1%	3%	1	0	0
F	4%	93%	16	11	1
G	1%	20%	2	1	3

The prevalence of paratuberculosis from the NSW farm with a history of paratuberculosis (Table 3.1.1) is unknown. Blood samples were collected from a random selection of 50 lambs at 5-6 months of age and faecal samples at 17-18 months of age. Weights were collected at both time points.

4.3.1.2 IFNγ response

4.3.1.2.1 Detecting MAP exposure by the IFNγ response

IFNγ results for the 3 non-vaccinating farms are shown in Figure 3.1.2.1. The specificity of the IFNγ response to MAP antigen, indicated that in 93% of samples, this response was likely due to exposure to other environmental *M. avium* species (MAP/PPDA <2). Therefore, the threshold for a positive result was set to 4.6 pg/mL which is the mean + 2 sd for the MAP-specific IFNγ response (Figure 3.1.1). We have previously shown that the IFNγ response is a marker for MAP exposure in sheep in the context of controlled experimental trials (de Silva et al. 2018). Together, these results support our hypothesis that on non-vaccinating farms the IFNγ^{PLUS} test can be used to detect exposure to

MAP and can provide supporting evidence when classifying farms as having freedom from paratuberculosis.

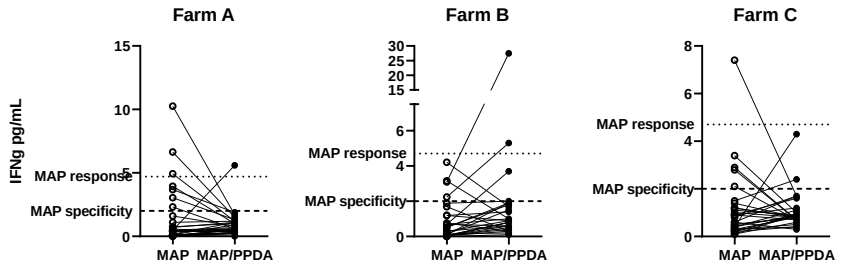


Fig. 3.1.2.1 Thresholds for the IFNγ response in sheep from farms with no history of paratuberculosis Thresholds for MAP specificity (MAP/PPDA >2) and an IFNγ response to MAP (4.6 pg/mL which is mean + 2 sd of the MAP-specific response) are shown.

4.3.1.2.2 The IFNγ response as a marker of disease in vaccinating farms

Prevalence of disease on farm as well as within the cohort selected for the IFNγ^{PLUS} test is shown in Table 3.1.2. Gudair® vaccination creates immunological memory of exposure to MAP. It is therefore expected that all vaccinated sheep will have a positive response in the IFNγ test, however the response is likely to wane over time. Other studies (Reddacliff et al 2006) have reported that the IFNγ response could be detected in about 40-60% of Gudair® vaccinated lambs at 2-3 years of age. A positive IFNγ response was detected in 66% of sheep. Vaccination also increased the PPDA response and as a result the MAP/PPDA IFNγ ratio was below threshold in most animals (91%) (Figure 3.1.2.2). Therefore, in vaccinating farms this value was not considered when defining a positive IFNγ response.

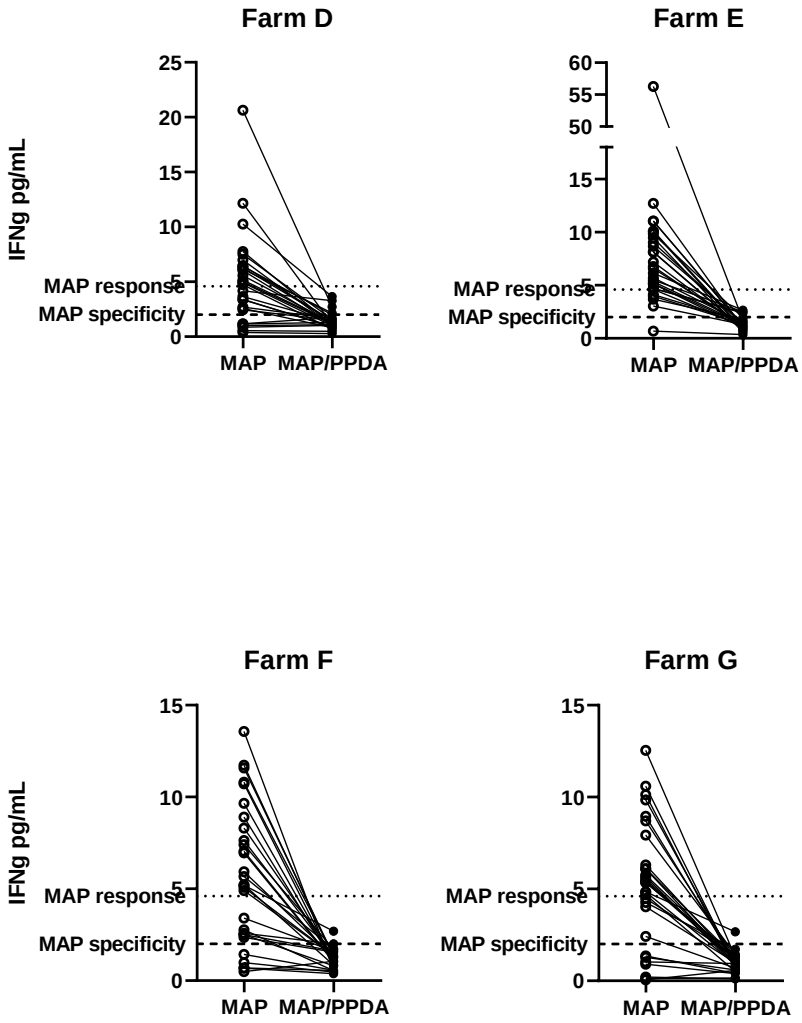


Fig. 3.1.2.2 IFNγ response to mycobacteria in vaccinated sheep from farms with a history of paratuberculosis
Thresholds for MAP specificity (MAP/PPDA >2) and an IFNγ response to MAP (4.6 pg/mL, which is mean + 2 sd of the MAP-specific response in sheep from farms without disease) are shown.

The chi-square test of independence showed that there was a significant association between IFNγ and HT-J ($p < 0.001$) with the IFNγ positive group having 7.49 times the odds of being HT-J positive than the IFNγ negative group (odds ratio 95% CI: 2.43, 23.10) (Table 3.1.3).

Table 3.1.3 Association of the MAP IFNγ and faecal PCR results in vaccinated sheep on farms with disease

Explanatory variable	Categories	MAP IFNγ		Total	Odds	P-value
		Positive	Negative		(95% CI)	
		n (%)	n (%)			
HT-J (Faecal PCR)	Positive	34	4	38	7.49	<0.001
		(44.7%)	(9.8%)		(2.43, 23.1)	
	Negative*	42	37	79		
		(55.3%)	(90.2%)			

*Reference category CI: Confidence interval.

Fig. 3.1.2.3 shows the MAP-specific IFNγ responders and non-responders in relation to faecal shedding in the animals tested (Table 3.1.2). The farms (E-G) and cohorts with low prevalence of faecal shedding had low numbers of IFNγ non-responders but there was no significant difference (p=0.059) between the numbers of non-responders and responders on farm.

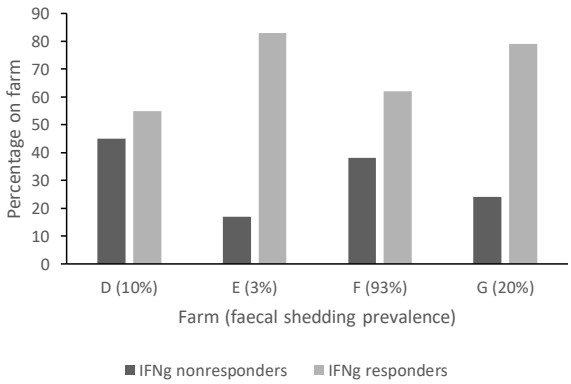


Fig. 3.1.2.3 Distribution of IFNγ responders on farms with varying prevalence of faecal shedding. The percentage of MAP-specific IFNγ responders (>4.6 pg/mL) and non-responders from four farms, vaccinating with Gudair® and a history of paratuberculosis are shown. The percentage of faecal shedders in the test cohort for each farm is shown on the x-axis. The on-farm prevalence for farm D is unknown and was 1% for farm E, 4% for farm F and 1% for farm G.

4.3.1.3 MAP-specific IgG1 antibodies as a biomarker

4.3.1.3.1 Detecting MAP exposure by IgG1 antibody response

The animals tested from nonvaccinating farms with no history of disease (Table 3.1.1) had no IgG1 response to MAP antigen (316v), PPA or PPDa (Figure 3.1.3.4). A value of 5% or greater was defined as a positive IgG1 response.

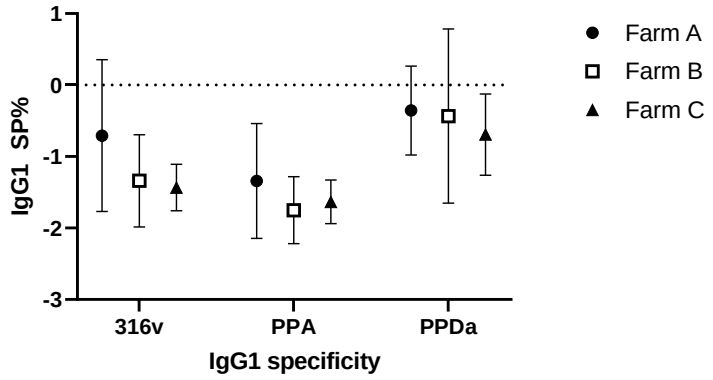


Fig. 3.1.3.4. Antigen specific IgG1 antibodies in non-Gudair®-vaccinated sheep from three farms with no history of paratuberculosis
Mean +/- sd are shown

4.3.1.3.2 The IgG1 response as a marker of disease in vaccinating farms

Similar to the IFNγ response, it was expected that Gudair® vaccination would create immunological memory of exposure to MAP and these sheep would test positive for an antibody response as shown in Figure 3.1.3.5.

The IgG1 antibody response to MAP antigen (316v) or PPDa was significantly different between farms ($p<0.05$) (Figure 3.1.3.5). Mean IgG1 SP% was significantly higher in the farm with the highest prevalence of faecal shedding compared to the other farms. However, a chi-square test of independence showed that there was no significant association between the HT-J result and the IgG1 result (Table 3.1.5). However, there were moderate associations when data analysis was based on faecal MAP DNA quantity (log10) rather than a positive or negative result (Table 3.1.6) for both IgG1 SP% or when a higher cut-off (SP%>100) for a positive IgG1 result was used.

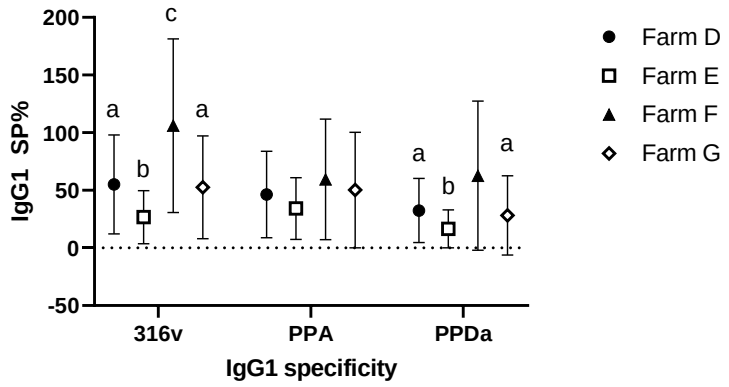


Fig. 3.1.3.5. Antigen specific IgG1 antibodies in Gudair®-vaccinated sheep from four farms with a history of paratuberculosis
Mean +/- sd are shown. Within each IgG1 specificity group, significant differences between farms are shown by different letters.

Table 3.1.5 Association of the MAP IgG1 and faecal PCR results in vaccinated sheep on farms with disease

Explanatory variable	Categories	MAP IgG1		Total	P-value
		Positive	Negative		
		n (%)	n (%)		
HT-J (Faecal PCR)	Positive	36	2	38	0.335
		30%	2%		
	Negative	72	10	82	
		60%	8%		

Table 3.1.6 Association of faecal MAP quantity with IgG1 response

Spearman r	Faecal MAP quantity (log_{10}) vs IgG1 MAP (SP%)	Faecal MAP quantity (log_{10}) vs IgG1 MAP Positive*	Faecal MAP quantity (log_{10}) vs IgG1 strong**
r	**0.4678**	0.174	**0.5237**
95% confidence interval	0.3101 to 0.6004	-0.01074 to 0.3473	0.3756 to 0.6458
P value			
P (two-tailed)	<0.0001	0.0573	<0.0001
P value summary	****	ns	****
Exact or approximate P value	Approximate	Approximate	Approximate
Significant (alpha = 0.05)	Yes	No	Yes
Number of XY Pairs	120	120	120

*SP%>5; **SP%>100

There was a moderate positive correlation between the level of faecal MAP and the IgG1 response ($r(116)=.53$, $p<0.05$) but not the IFNγ response to MAP ($r(116)=-.05$, $p>0.05$).

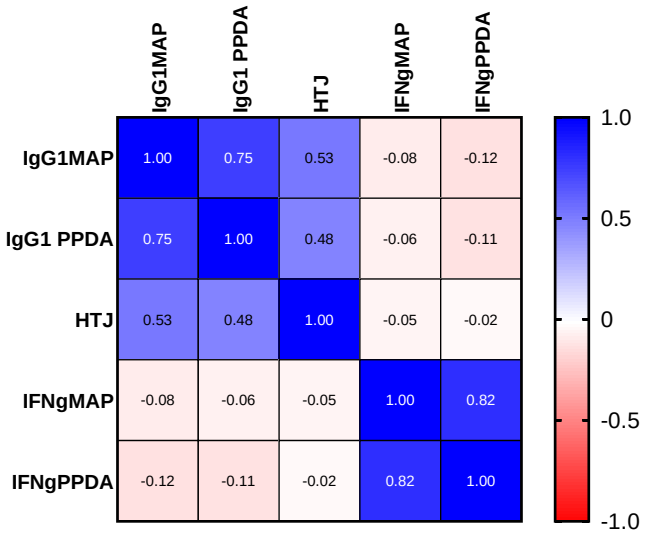


Fig. 3.1.3.6 Correlation of faecal shedding (HT-J) and immune responses in vaccinated sheep on farms with disease

4.3.1.4 Gene expression biomarkers to detect disease

We sought to determine if genes selected as differentially regulated in those sheep that were MAP exposed and vaccinated could be utilised as markers of resistance or susceptibility to disease. The trial that provided the samples for the transcriptomic study (Appendix 3) comprised of lambs exposed to MAP under a controlled experimental model of ovine JD established utilising a validated sheep infection model with reliable disease outcome (Begg et al. 2010). The samples for this study were sourced from vaccinating commercial properties with a history of persisting Johne's disease. In contrast to the experimental study, the sheep selected for sampling were mature animals with an average age of 3 years thus allowing for natural exposure and progression of disease.

Prevalence of disease (Table 3.2.1) within farm was variable. Limitations of the modified comparative Ct (ΔΔCt) method (Livak et al. 2001; Pfaffl 2001) to determine variation between outcome variables (vaccinated resilient and vaccinated susceptible) requires similar sample numbers for statistical relevance. Therefore, the data from all four commercial properties was collated for analysis (Table 3.1.7). The comparison of the qPCR derived fold changes of the selected genes with the reported transcriptomic findings (Appendix G) show close correspondence for 8 of the 10 genes of interest suggesting that these genes are suitable predictors of disease resilience in both vaccinated lambs and older sheep.

Table 3.1.7. qPCR derived fold change of genes of interest for non-infected vaccinated sheep (resilient) compared to the infected vaccinated sheep (susceptible) and matched comparison of the observed array expression.

Gene name	Accession #	Microarray Fold Change	qRT-PCR Fold Change	
			All farms	P-value
LXN	NM_001080340	-1.6	-1.5	0.001
RARRES1.2	NM_001075430	-2.1	-1.6	0.001
LYZ1.1	NM_180999	-2.8	-2.5	0.001
TNFRS21.2	NM_001076911	-1.5	-1.5	0.046
TET2.1	XM_001790146	-1.5	-1.6	0.001
HbF1.1	NM_001014902	-1.4	-1.1	0.400
C10H15orf48	XM_004010648	-1.4	-1.4	0.001
TES.2	NM_001046390	1.6	1.3	0.348
IP-10 (CXCL10)	NM_001046551	1.7	2.3	0.001
BOLA1a.1	NM_001038518	2.2	1.8	0.001

4.3.1.5 Validation of biomarkers in lambs

4.3.1.5.1 Weights

During the period of study, the variation in weight gain over 344 days of the animals selected for this study ranged from 1.6-20.2 kg. Weight gain was negatively correlated to starting weight but was not significant ($r(39)=-.33$, $p=.039$).

4.3.1.5.2 Faecal PCR

At 12 months post vaccination all sheep tested for faecal shedding of MAP by PCR were negative (n=36).

4.3.1.5.3 IFNγ response

The low IFNγ response to MAP antigen at 2 months post vaccination (Fig. 3.1.5.1) in the majority of animals is unexpected.

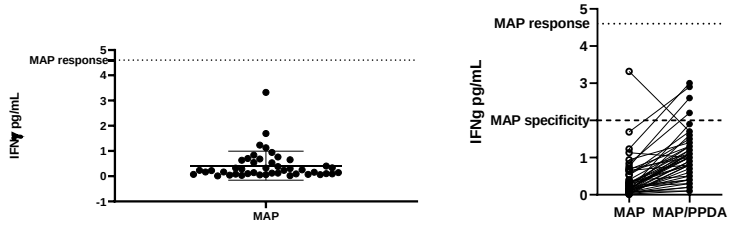


Fig. 3.1.5.1 MAP-specific IFNγ response in vaccinated lambs
IFNγ responses in 50 lambs at 2 months post Gudair® vaccination. The threshold for a positive IFNγ response to MAP (4.6 pg/mL) was selected based on responses to MAP antigen in animals from nonvaccinating, non-diseased farms.

4.3.1.5.4 IgG1 response

The IgG1 response (Fig. 3.1.5.6) in these vaccinated lambs was also significantly lower ($p<0.0001$) compared to vaccinated adult sheep.

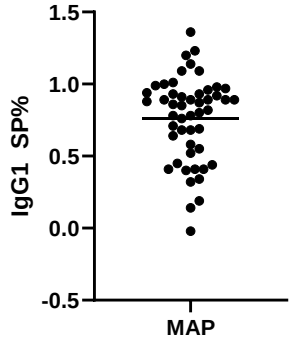


Fig. 3.1.5.2 MAP-specific IgG1 response in vaccinated lambs
Serum IgG1 was assessed in 50 lambs at 2 months post Gudair® vaccination.

4.3.1.5.5 Lymphocyte proliferation

There was a significant negative correlation between weight gain and the proliferative response to MAP antigen ($r(39)=-.33$, $p<0.05$), however the correlation between the two variables was low. There were no overt signs of disease in these sheep; they were all negative for faecal MAP and none in the cohort had signs of clinical disease.

4.3.1.6 CXCL10 as a candidate biomarker

Archived plasma supernatants from whole-blood stimulation cultures for sheep from a controlled experimental trial (Begg et al. 2017) were analysed. Outbred Merino lambs aged 3-7 months (n=76) had been randomly allocated into Gudair® vaccinated (n=30) and nonvaccinated (n=46) groups and then into MAP exposed (n=20 vaccinated and 36 nonvaccinated) and unexposed (n=10 in each) subgroups.

4.3.1.6.1 CXCL10 (IP-10) and IFN-γ in infected vs uninfected animals

CXCL10 and IFN-γ responses in all infected animals (clinical and subclinical), irrespective of their vaccination status, were tested in plasma supernatant for statistical significance between timepoints. Both CXCL10 and IFN-γ responses increased significantly between 4- and 6-months post exposure ($P<0.05$) (Figure 3.1.6.1). In addition, CXCL10 levels significantly increased between 6 and 12-months post exposure ($P<0.05$).

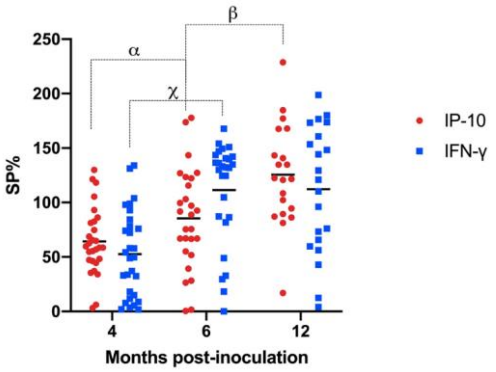


Fig. 3.1.6.1 CXCL10 (IP-10) and IFN-γ responses in infected sheep (irrespective of vaccination status). Significant differences: α, significant increase in CXCL10 response from 4 months to 6 months post-inoculation; β, significant increase in CXCL10 responses from 6 months to 12 months post-inoculation; χ, significant increase in IFN-γ levels from 4 months to 6 months post-inoculation.

CXCL10 levels of infected animals were significantly lower than uninfected animals (regardless of vaccination status) at 4 months post exposure, and significantly higher at 6- and 12-months post exposure (Figure 3.1.6.2A). IFN-γ levels in infected sheep were significantly lower than uninfected sheep at 4 months post exposure, but similar at later time points (Figure 3.1.6.2B).

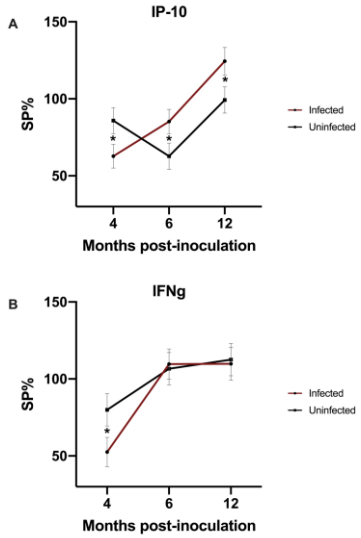


Fig. 3.1.6.2 Comparisons between CXCL10 (IP-10) (A) and IFN-γ (B) responses in infected and uninfected sheep using predicted means derived from the linear mixed model. * denotes significant differences between infected and uninfected at either 4, 6- or 12-months post MAP exposure.

4.3.1.6.2 Ability for CXCL10 and IFN-γ to predict severity of disease outcome

We sought to determine whether severity of disease at the end of the trial could be differentiated by CXCL10 and IFN-γ plasma supernatant responses at prior time points. For animals within the infected group that progressed to paucibacillary disease, the CXCL10 and IFN-γ responses at 4 months post exposure tended to be higher compared to those that progressed to multibacillary disease. . Sheep that progress to clinical disease two distinct types of lesions: paucibacilliary and multibacilliary. This classification is based on histological presentation cellular composition. Paucibacillary lesions contain many lymphocytes and few acid-fast bacilli where as multibacillary lesions mainly contain macrophages and are abundant in acid fast bacilli. While both lesion types are found in animals with clinical disease, it is likely that there is a progression from paucibacillary to multibacillary as disease advances. Multibacillary animals are also more likely to shed higher quantities of MAP. The advantage of early detection of differential CXCL10 or IFNγ responses is the potential for identifying those that are more likely to contribute to infecting the rest of the flock.

When disease outcome was regrouped as either ‘clinical’, ‘subclinical’ or ‘uninfected’, CXCL10 responses in uninfected MAP exposed sheep were significantly higher than clinical animals (Figure 3.1.6.4A). In the clinical and subclinical groups, the CXCL10 response increased with time. IFN-γ levels in clinical sheep were significantly lower than subclinical at 12 months PI (Figure 3.1.6.4B). In clinical sheep, IFN-γ increased significantly between 4 and 6 months, then decreased significantly from 6 to 12 months PI, while subclinical sheep had significantly higher IFN-γ levels at 12 months compared to 6 months.

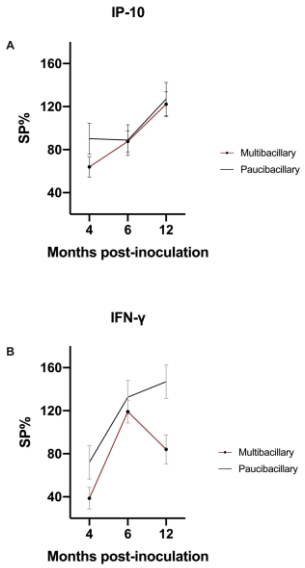


Fig. 3.1.6.3 CXCL10 (IP-10) (A) and IFN-γ (B) responses in infected sheep with either paucibacillary or multibacillary lesions.

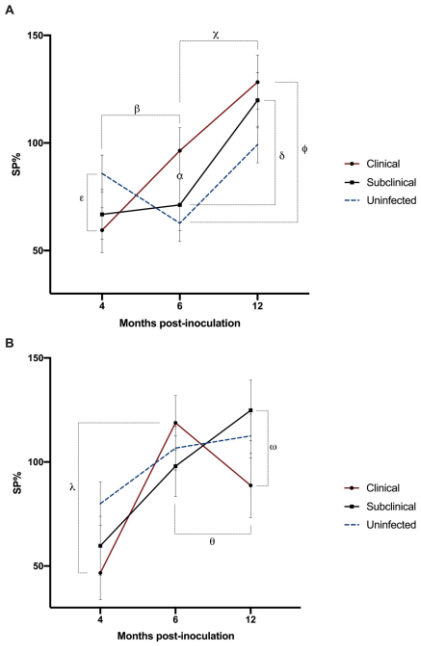


Fig. 3.1.6.4 Comparisons between CXCL10 (IP-10) (A) and IFN-γ (B) responses at 4, 6- and 12-months post-inoculation of MAP bacteria, in exposed sheep which either became clinically diseased, subclinically disease or cleared the infection (uninfected). A: Significant differences in CXCL10 responses (all $p<0.05$): α; lower CXCL10 response from subclinical animals than clinical, at 6 months post-MAP exposure; β; higher CXCL10 response in clinical sheep at 6 months compared to 4 months; χ; higher CXCL10 response from clinical sheep at 12 months compared to 6 months; δ, higher CXCL10 response in subclinical sheep at 12 months compared to 6 months; ε, higher CXCL10 response in uninfected compared to clinical sheep at 4 months; ϕ, lower CXCL10 response in uninfected compared to clinical sheep at 12 months. B: Significant differences in IFN-γ responses: λ, higher IFN-γ levels in clinical sheep at 6 months compared to 4 months; Θ, lower IFN-γ response in clinical sheep at 12 months compared to 6 months; ω, significantly higher IFN-γ response in subclinical sheep compared to clinical cases at 12 months post-inoculation.

4.3.1.6.3 CXCL10 as a predictor of vaccine efficacy

To determine the effects of vaccination on biomarker responses, we mapped CXCL10 and IFN-γ plasma responses in vaccinated and nonvaccinated animals that had not been exposed to MAP. While CXCL10 responses showed more variability and were significantly different between the two groups at 4- and 6-months post exposure (Figure 3.1.6.5A), IFN-γ responses were significantly different between vaccinated and unvaccinated animals at all 3 time points (Figure 3.1.6.5B).

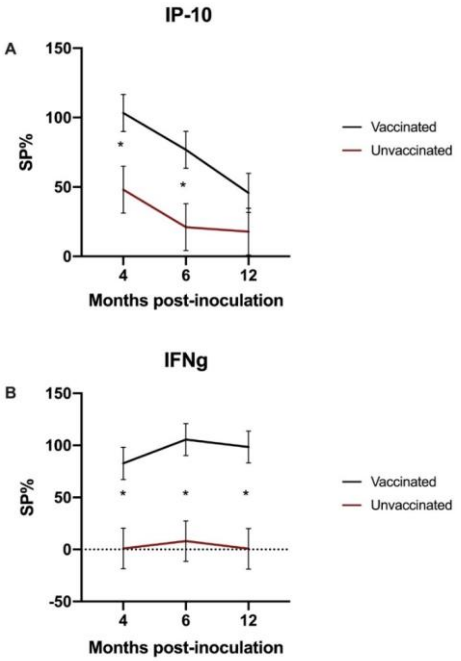


Fig. 3.1.6.5. Predicted average (generated from a table of predicted means in a linear mixed model CXCL10 (IP-10) (A) and IFN-γ (B) responses in unexposed animal which were either unvaccinated or vaccinated. Significant differences: * denotes significant differences between infected and uninfected sheep at 4, 6- or 12-months post-inoculation. Significant differences in IFN-γ responses between infected and uninfected sheep occurred at 4, 6- and 12-months post MAP exposure, while IP-10 responses differed between the two groups at 4 and 6 months post-MAP exposure.

To determine the potential for CXCL-10 (IP-10) to monitor vaccine efficacy, we analysed all exposed-vaccinated animals and classified them as either infected or uninfected (Fig. 3.1.6.6). Significant differences in CXCL-10 (IP-10) responses were observed ($P<0.05$, 95% CI) between infected and uninfected vaccinated sheep at 4 and 6 months post-inoculation. No significant differences were observed when IFN-γ was analysed as the response variate (data not shown).

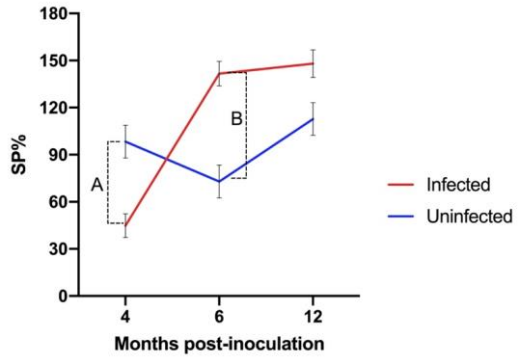


Fig. 3.1.6.6. Comparison of CXCL-10 (IP-10) responses (predicted averages and standard errors generated from a table of predicted means in a linear mixed model) elicited by sheep that were administered with the Gudair® vaccine and either were later identified to be infected or were uninfected at the conclusion of the trial. Significant differences: A, uninfected Gudair® vaccinated sheep had a significantly higher IP-10 response at 4 months post-inoculation compared to Gudair® vaccinated animals that were infected; B, at 6 months post-inoculation, the infected sheep had significantly higher IP-10 responses compared to uninfected sheep.

4.3.2 Predictive markers in parasitic co-infections

Detailed descriptions for this section can be found in Appendix 3B. Immunological markers in all animals from the co-challenge experiment in sub-project 1 are described in this section.

4.3.2.1 IFNγ response in co-challenge

At 4 months post exposure, the IFN-γ responses were significantly different between in vitro stimulus groups (Figure 3.2.1.2). Later on, at 9 months post exposure, the IFN-γ response in the groups with MAP exposure (Group 1 and 2) was significantly higher than in the group with no MAP exposure (Group 4) (Figure 3.2.1.2). Similarly, in Groups 1 and 2 the IFN-γ response to MAP antigen was significantly higher compared to the IFNγ response to Hc antigen in the corresponding group (Fig. 3.2.1.2).

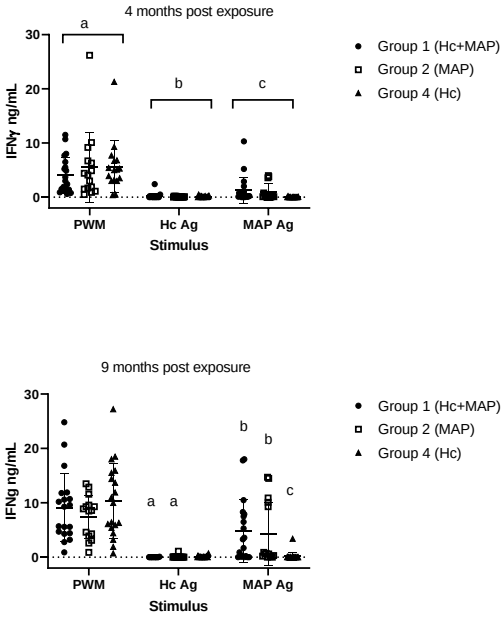
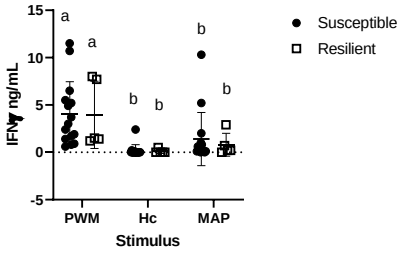
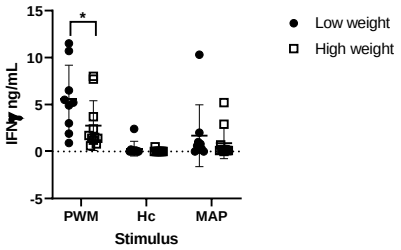


Fig. 3.2.1.2 IFNγ response to *in vitro* stimuli in animals from the different *in vivo* treatment groups The mean +/- sd of the IFNγ response is shown at 4- and 9-months post exposure. Different letters indicate significant differences between groups.

Sheep in the co-challenged group were classified for disease severity (Table 3.2.2 in SP2). There were significant differences in the PWM-IFNγ response compared to the other stimuli (Figure 3.2.1.3B) but none between Hc or MAP antigen responses between the two disease outcome groups (Figure 3.2.1.3 B). The PWM IFNγ response was significantly higher in the low weight group (Figure 3.2.1.3C). This suggests that the general capacity of blood cells to respond to a nonspecific stimulus is lower in animals that gain weight.



B.



C.

Fig. 3.2.1.3 IFNγ response at 4 months post exposure in co-challenged sheep at 4 months post exposure based on disease outcome (B) or weight (C) as a measure of productivity
*p<0.05; different letters indicate significant differences between groups.

4.3.2.2 Lymphocyte proliferation in co-challenge

The total lymphocyte proliferative response was not significantly different between groups for Hc or MAP antigens (Fig. 3.2.3).

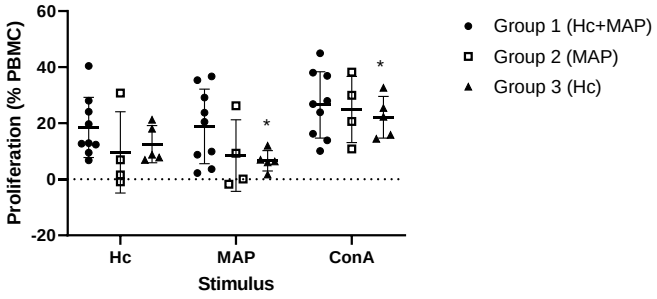


Fig. 3.2.2.1 Proliferative response at 4 months post challenge based on treatment group.
PBMC proliferation results to specific (Hc and MAP) antigens and a nonspecific T cell activator (ConA) for sheep from treatment groups 1 (Hc+MAP), 2 (MAP) and 4 (Hc) are shown (n=18). *p<0.05 between indicated groups

Using criteria described in Table 3.2.2 for disease outcome, there was a significant positive correlation between the Hc and MAP proliferative responses ($r(18)=0.73$, $p<0.05$) but no correlation between the disease score and proliferative response to either Hc or MAP antigens.

Proliferation to MAP antigen in Group 1 (Hc+MAP challenge) was significantly different only between CD8 T cells and B cells. Subset proliferation to Hc antigen was not significantly different to each other for Group 1. For Group 2 (MAP challenge) CD8 proliferation was significantly greater than CD4 and B cell proliferation and for Group 3 (Hc challenge) $\gamma\delta$ T cell proliferation was significantly greater than CD4 T cells and B cells. Disease score was negatively correlated to Hc-specific $\gamma\delta$T cell proliferation ($r(56)=-0.25$, $p=0.079$) and MAP-specific CD4 proliferation ($r(56)=-.26$, $p=0.072$) but was not significant. However, in animals with the most severe disease (score 75-100) as well those that were resilient (score 0), $\gamma\delta$ T cell proliferation to Hc antigen was significantly higher than for CD4 T cells.

4.3.2.3 CXCL10 (IP-10) response in co-challenge

The cytokine response to MAP antigen was significantly higher in the MAP exposed groups (both co-challenge and MAP alone) than the Hc alone group. In both MAP exposed groups, the recall response to MAP antigen was significantly higher than to Hc antigen. The MAP-specific response was not significantly different between disease outcome groups (Fig. 3.2.3.2).

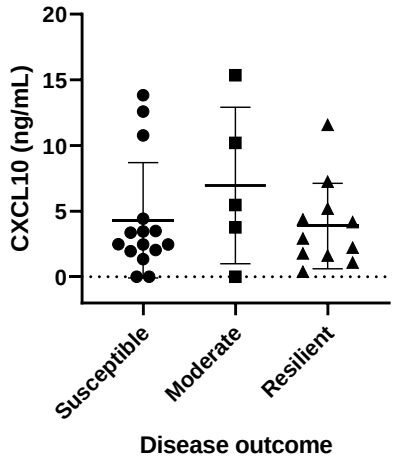


Fig. 3.2.3.2 MAP-specific CXCL10 response at 9 months post exposure in relation to disease outcome in MAP-exposed groups (Group 1 and 2).

4.3.2.4 Cytokine biomarker discovery

A subset of four diseased and three resilient sheep were selected for further biomarker discovery from sheep exposed to the dual challenge with MAP and Hc or with MAP alone. Overall results from

the protein array (Raybiotec QAO Cyt Array 1 and 2) which detects twenty ovine cytokines are shown in Table 3.2.4.1. Some cytokines were not detectable.

Table 3.2.4.1 Secretion of ovine cytokines in response to MAP, Hc or PWM stimulants

Cytokine	Result
IL-17A IL-21 CXCL-10 (IP-10) CXCL9 RANTES	Cytokines of interest in relation to stimulant or disease outcome

The IL-21 response to stimulation with MAP antigen was significantly higher in the resilient animals compared to the diseased group (Figure 3.2.4.2). While the response to Hc antigen was also elevated in the resilient group it was not significantly different to the diseased sheep.

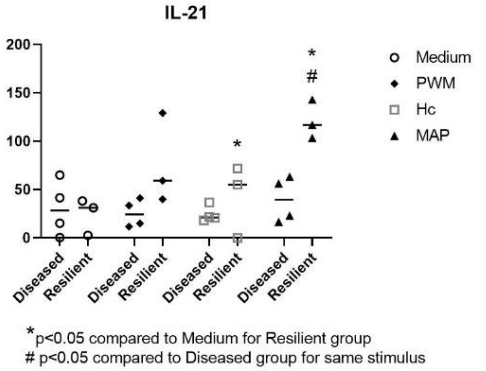


Fig. 3.2.4.2 Cytokine responses in sheep that were classified as resilient or disease at 4 months post exposure to Hc and MAP or MAP alone

4.3.3 Novel biomarker discovery

4.3.3.1 Identification of candidate biomarkers based on literature review

The publication based on this section is in Appendix 3D.

In the early stages following pathogen exposure, IFN-γ and proinflammatory responses are elevated and is coupled with a decrease in anti-inflammatory IL-10. During the subclinical infection stage, there is an increase in several proinflammatory cytokines. From here, the animal may successfully control the infection and eliminate the bacteria (termed resilience) or progress to clinical disease. An early, elevated IFN-γ and antibody response is observed in infected sheep that progress down a pathway of resilience to ovine Johne's disease. During clinical disease, the response is primarily anti-inflammatory, with a decrease in key proinflammatory cytokines and an increase in cytokines such as interleukin 10 (IL-10) and transforming growth factor beta (TGF-β). Elevated CXCL10 (IP-10) levels may be predictive of animals that will develop active disease.

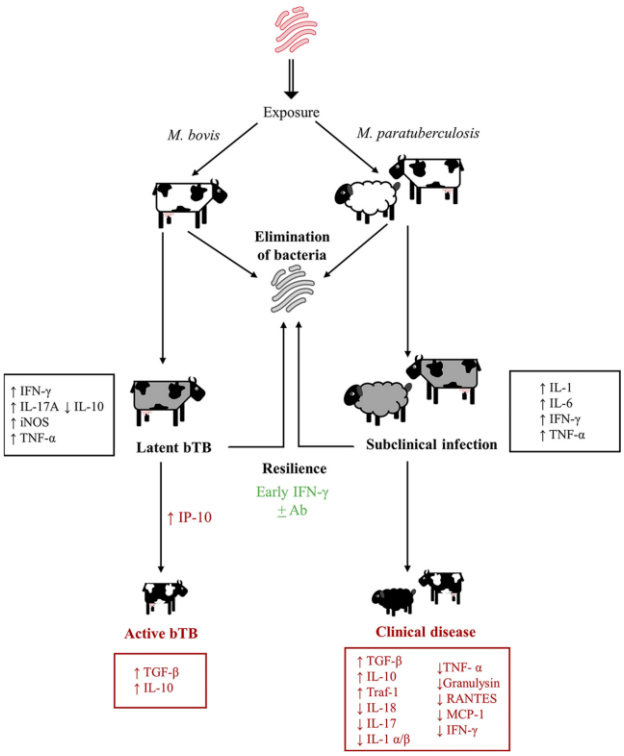


Fig. 3.3.1.1 Cytokine markers predictive or associated with stages of mycobacterial infection (Appendix 3D)

4.3.3.2 Screening archived samples for microRNA patterns of resilience and susceptibility

Detailed descriptions for this section can be found in Appendix 3C.

4.3.3.2.1 Small RNA sequencing uncovers distinct miRNA profiles of MAP infection and resilience in sheep

miRNA sequencing of archived plasma samples from MAP exposure trials confirmed the ability of next generation sequencing (NGS) to identify potential biomarkers of infection.

Differential expression analysis of samples collected at 13 weeks post MAP exposure (early timepoint) and 49 weeks post MAP exposure revealed distinct miRNA profiles between infected and resilient sheep (Figure 3.3.2.4). A total of 140 miRNA were differentially expressed between groups, and 83 miRNA either up or downregulated compared to controls. Regardless of the timepoint, 29 upregulated and 29 downregulated miRNA were specifically differentially expressed in infected animals compared to both resilient and controls, displaying a general miRNA MAP infection profile. Likewise, 10 upregulated and 15 downregulated miRNA were exclusively differentially regulated in resilient sheep when compared to control and MAP infected animals.

Within the infected and resilient miRNA groups, there were also time-specific profiles, with miRNA differentially regulated only in either early or late infection or exposure. Within the infected group, there were 15 upregulated and 16 downregulated miRNA in early infection only, and 19 upregulated and 10 downregulated miRNA in late infection only. In resilient exposed animals, early exposure showed 14 upregulated and 13 downregulated miRNA, and 6 upregulated and 12 downregulated miRNA specific to late exposure timepoint.

In addition, six miRNA were identified as being potentially regulated due to vaccination, and displayed differential regulation between control vaccinated, infected vaccinated, and resilient vaccinated groups.

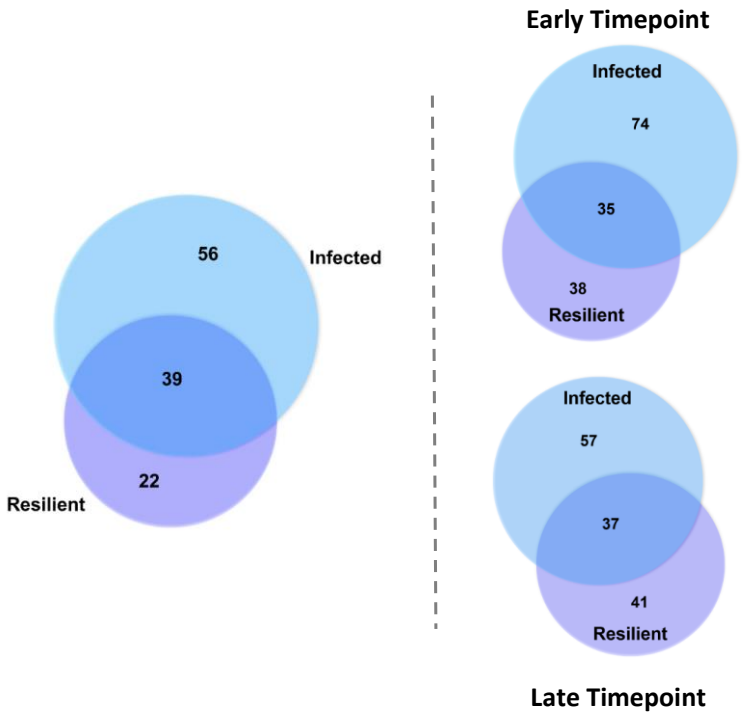


Fig. 3.3.2.4 Differential expression of miRNA in MAP infected and resilient sheep. Overlap between miRNA expression in MAP infected and resilient sheep shows both shared miRNA patterns and distinct profiles regardless of timepoint. The same is observed when groups are analysed based on timepoint (where 13 weeks post MAP exposure is early and 49 weeks post MAP exposure is late).

4.3.3.2.2 Microarray and small RNA sequencing dataset crossover

In the MAP infected cohort, 88.7% of miRNA identified as significantly differentially expressed and chosen for further analysis had either experimentally or moderate to high predicted mRNA targets that were also present in the mRNA infected cohort data. From the differentially expressed mRNA, 57.9% were targets of miRNA of interest. While all miRNA in the resilient animals had 100% of targets present in the mRNA dataset, only 28.9% of the resilient mRNA were targets of the miRNA selected for further analysis.

4.3.3.2.3 Integrated miRNA and mRNA analysis reveal biological pathways regulated following MAP exposure and infection

Pathway analysis of miRNA and their target mRNA provided immune snapshots at the early (13 weeks post MAP exposure) and late (49 weeks post MAP exposure) stages of infection and exposure

to MAP. Across both infected and resilient animals there were common pathways which may be indicative of exposure to or infection with MAP. Within the early timepoint cytokine-chemokine signalling pathways, T-cell activation pathways and immune related signalling pathways were all differentially regulated in both exposed resilient and infected animals compared to control groups. Similarly, in the late timepoint, cytokine/chemokine pathways and immune signalling pathways were dysregulated in both MAP exposed and infected animals.

4.3.3.2.4 Infected animals

Following exposure to MAP, infected animals displayed a distinct profile compared to control and MAP resilient exposed groups in the early timepoint. Infected sheep displayed a classical inflammatory response, with increased signalling through cytokine-chemokine pathways, increased engulfment of bacteria through phagocytosis, and increased T cell activity. Increased Th1 responses were evident in MAP infected animals with enhanced pro-inflammatory cytokine gene expression, as well as genes associated with leukocyte extravasation and chemotaxis towards bacteria stimuli. Further, genes involved in haematopoietic and chemotactic pathways were upregulated, indicating presence of bacterial infection following challenge. Immune signalling cascades were also enhanced, including LPS-stimulated MAPK signalling, natural killer cell signalling, and mTOR related signalling pathways, suggesting pathogen recognition and host responses to clear bacterial infection.

In the late stage of infection, a switch to an anti-inflammatory and pro-mycobacterial response is evident. A reduction in Th1 associated responses is observed, alongside decreasing antigen recognition and TCR signalling. T cell activation and responses were dampened in late infection, suggesting a chronic granulomatous infection. Decreases in inflammatory cytokine-chemokine pathways further imply a classic anti-inflammatory immune response in the later stages of MAP infection.

4.3.3.2.5 Exposed animals

Animals that were exposed to MAP and later appeared to be resilient were distinct from animals that were or became infected, suggesting a divergent, effective host response. In the early stages of exposure, resilient sheep exhibited a non-inflammatory immune response, with T cell receptor signalling decreased. Genes associated with cytokine-chemokine signalling were also downregulated. Of interest, production of reactive oxygen and nitrogen species was decreased, which may indicate a non-damaging or less destructive immune response. Similarly, the T cell exhaustion pathway was down regulated in resilient animals in the early stages post exposure. This pathway was not present in infected animals at either timepoint and may contribute to the observed resilience.

Conversely, at the late timepoint post exposure, resilient animals exhibited a classical inflammatory profile, similar to the observations in early infected animals. Antigen recognition and TCR signalling was increased, while T cell function and exhaustion and lymphocyte apoptosis were also increased suggesting bacterial recognition and active host responses. Further, inflammatory cytokine-chemokine associated genes were altered, resulting in an increase in signalling pathways.

4.3.3.2.6 qPCR validation of small RNA sequencing

Eight differentially expressed miRNA were analysed using qPCR and compared to the fold change provided by the sequencing analysis (Figures 3.3.2.5-6). In both early and late timepoint samples, biological variation in miRNA levels was more apparent in qPCR detection. However, despite outliers, the overall direction of expression was aligned to the observed sequencing fold change in most groups. This level of agreement between the sequencing and qPCR results for miRNA at both timepoints was expected due to the differences in sensitivity and data normalisation method used between the two methods.

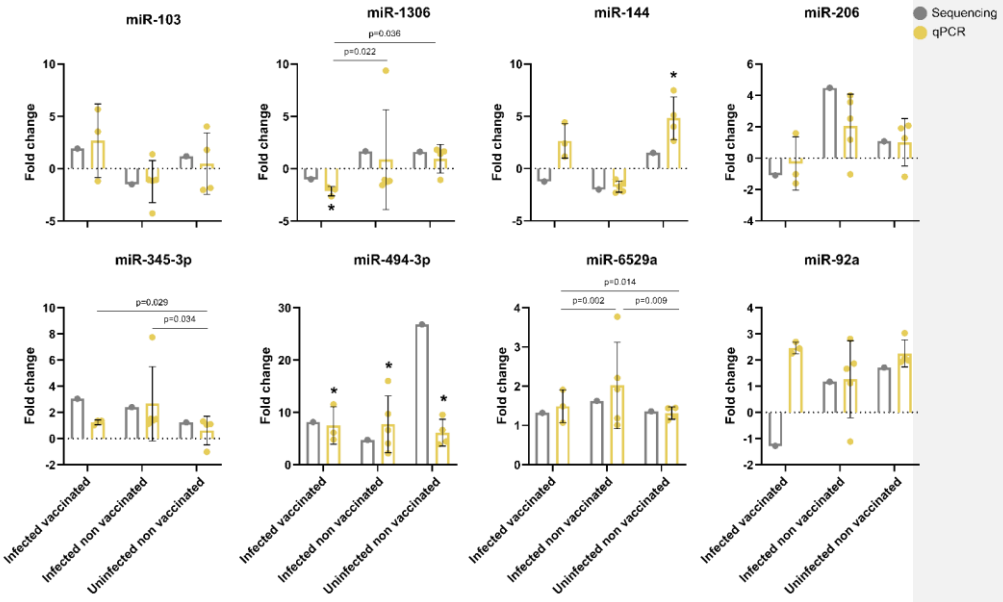


Fig. 3.3.2.5 Validation of early timepoint (14 weeks post MAP exposure) sequencing expression data. Fold change obtained from the sequencing data was compared to qPCR fold changes to analyse the degree of similarity in magnitude and direction of expression. Sequencing fold changes represent the analysis of the treatment group as a whole, while qPCR analysis was performed on individual animals from each group. Statistical analysis was performed on C(w)q values rather than fold change values due to the data transformation in the analysis.

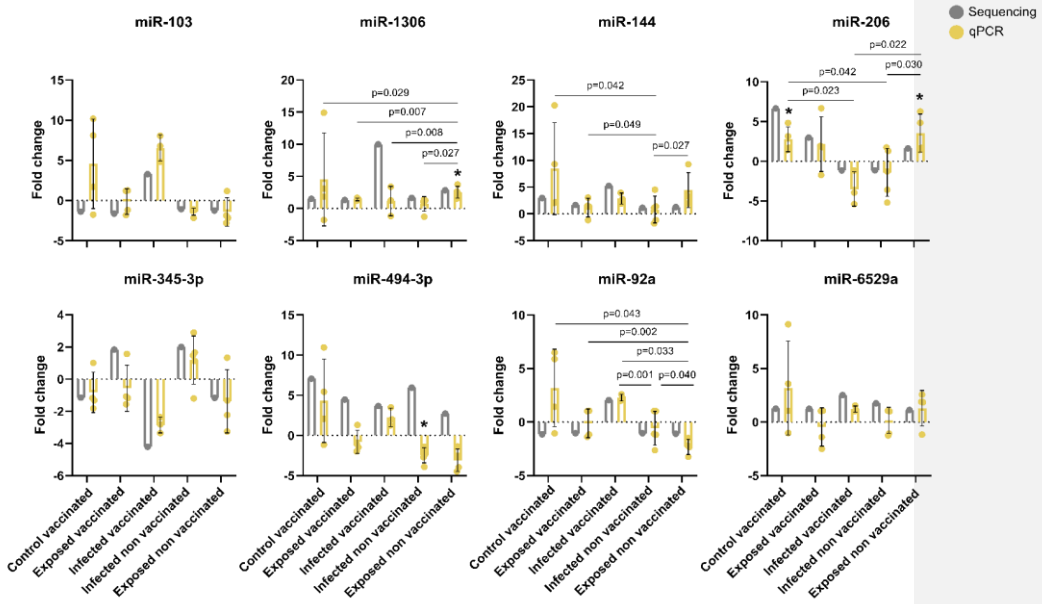


Fig. 3.3.2.6. Validation of late timepoint (49 weeks post MAP exposure) sequencing expression data. Fold change obtained from the sequencing data was compared to qPCR fold changes to analyse the degree of similarity in magnitude and direction of expression. Sequencing fold changes represent the analysis of the treatment group as a whole, while qPCR analysis was performed on individual animals from each group. Statistical analysis was performed on C(w)q values rather than fold change values due to the data transformation in the analysis.

4.3.3.3 MicroRNA studies in zebrafish

A selection of miRNA from Section 4.3.3.2 were initially assessed for their role in mycobacterial infections using a zebrafish-*M. marinum* infection model. More detailed studies were then carried out for the role of miR-206.

Detailed descriptions for this section can be found in Appendix 3C and 3F.

4.3.3.3.1 miRNA expression in *M. marinum* infected embryos

Infected embryos profiled at both 3 and 5 dpi displayed dysregulated expression of miRs -126a, -206, and -128a (Figure. 3.3.3.1). At 3 dpi all three miRNA were upregulated (FC ± 1.5) in comparison to uninfected control embryos. At 5 dpi, increased expression of miR-126 was sustained, while miRs -128 and -206 were strongly downregulated. Bacterial burden was increased significantly from 3 to 5 dpi ($p<0.0001$) (Figure 3.3.3.1).

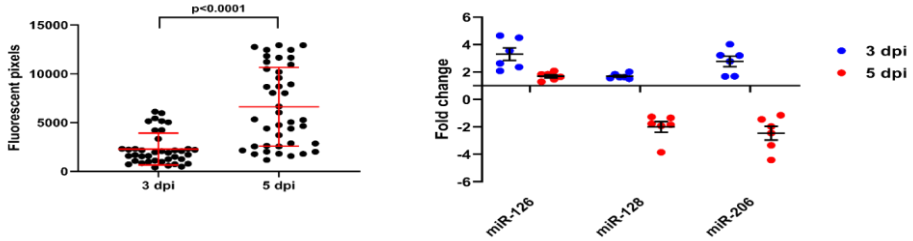


Fig. 3.3.3.1 Bacterial burden and miRNA expression in *M. marinum* infected embryos at 3- and 5-days post infection

4.3.3.3.2 miRNA knockdown during infection

At 1 dpi, all knockdown, *M. marinum* infected, and knockdown+*M. marinum* embryos displayed significantly reduced growth from scramble injected control embryos (p<0.0001) (Fig. 3.3.3.4.).

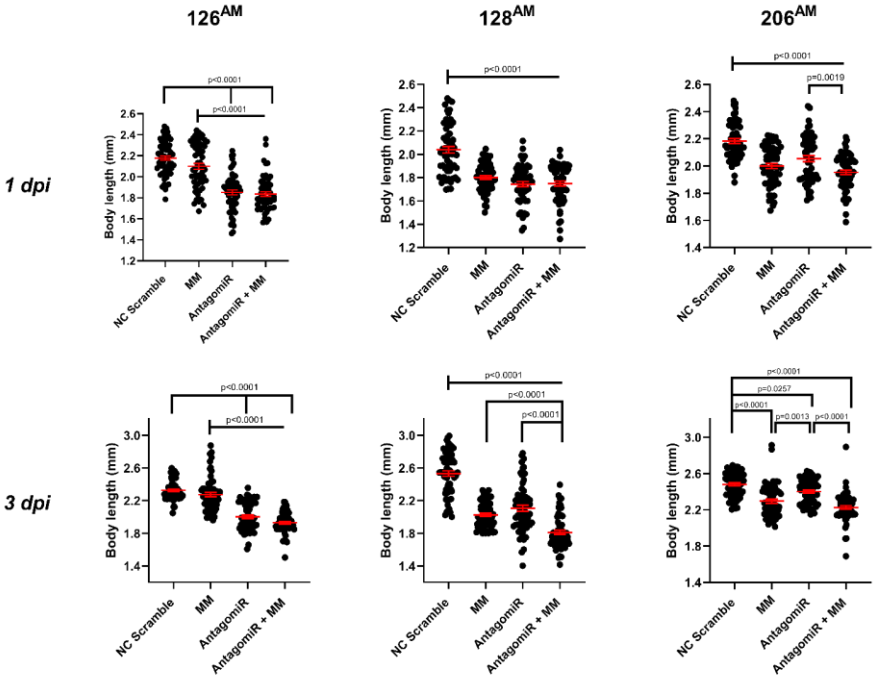


Fig. 3.3.3.4 Total body length of knockdown and infected embryos.
Embryos were injected with either a scramble control or miRNA specific antagomiR, and some further infected with *M. marinum* at 1 dpf. Measurements were taken at 1 and 3 dpi. Each data point represents a single measurement, with the mean and SEM shown in red.

The same trend was observed at 3 dpi for all miRNA knockdown embryos, while infection with *M. marinum* further reduced embryo development for miRs -126^{AM}, -128^{AM}, and -206^{AM} treatments (p=0.0082, p<0.0001, p<0.0001 respectively).

There was no difference between bacterial burden in control-infected and knockdown-infected embryos for miRs- 126 and -128 at 1 dpi, while miR-206 knockdown embryos had a marginally higher *M. marinum* burden (p=0.0019) (Figure 3.3.3.5.). Significant changes were measured at 3 dpi, with both miRs -206 and -128 knockdown embryos having lower bacterial burdens than the control infected groups (p=<0.0001). Conversely, miR-126 knockdown embryos had higher bacterial burdens than control infected embryos (p<0.0001). Between 1 and 3 dpi, bacterial burdens of miRs -126 and -128 knockdown embryos increased (p<0.0001), the level of bacteria in miR-206 knockdown embryos remained unchanged.

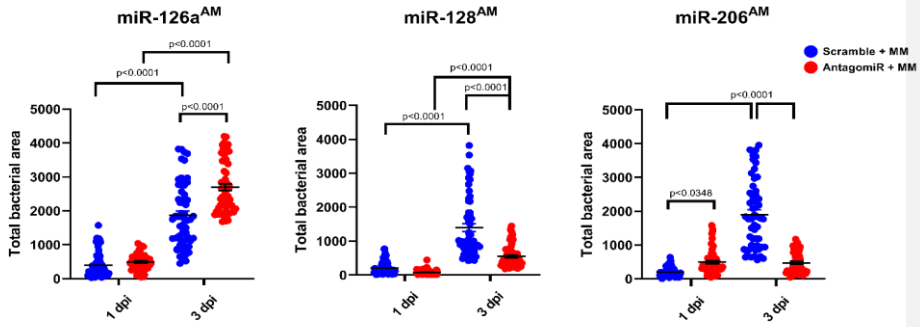


Fig. 3.3.3.5 Bacterial burden in miRNA knockdown embryos.
AntagomiR or scramble control injected embryos were imaged at 1 and 3 dpi, and bacterial burden quantified. Each data point represents a single measurement, with the mean and SEM shown in red.

Expression levels of each miRNA were analysed to measure the efficacy of the antagomiR and knockdown during *M. marinum* infection. At both 1 and 3 dpi, miRNA levels were decreased in antagomiR (AM) treated embryos and was sustained in antagomiR + *M. marinum* (AM+MM) groups for all 3 miRNA (Fig. 3.3.3.6.)

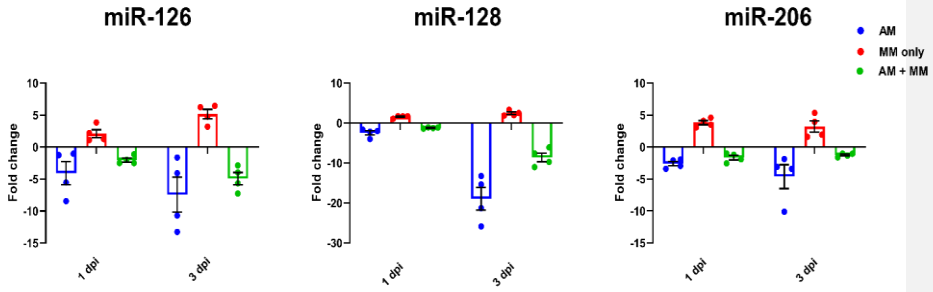


Fig. 3.3.3.6 miRNA expression following antagomiR treatment and *M. marinum* infection. Embryos were injected with either a scramble control or miRNA specific antagomiR, and some further infected with *M. marinum* at 1 dpf. miRNA expression was analysed at 1 and 3 dpi and compared to uninfected, scramble control embryos. Error bars and lines indicate the mean and SEM.

4.3.3.3.3 Validation of antagomir using miR-126a knock-out

Following injection of CRISPR, embryos were monitored for visible phenotypic defects. The relative expression of miR-126a in each of the knock out groups was measured, and a 40% reduction in expression was seen compared to control groups. For the establishment of a miR-126a knockout line, embryos from a robustly outcrossed line, fli1a:GFP, were chosen for injection and transferred to an aquarium system at 5 dpf. Knockout was confirmed using qPCR compared to dye injected control embryos.

4.3.3.3.4 miRNA regulation of neutrophil response

As both miRNA knockdowns resulted in elevated expression of *cxcl12a* and *cxcr4* genes which are involved in neutrophil migration and retention of cells at sites of infection and inflammation, we analysed the neutrophil response to *M. marinum* infection in antagomiR treated embryos.

miR-206^{AM} embryos had significantly more neutrophils at the site of infection for the first 2.5 hours of infection compared to control infected embryos (Figure 3.3.3.13). While the neutrophil response began to wane at approximately 12 hpi, the response in miR-206^{AM} embryos was sustained and maintained higher numbers of cells in comparison. When embryos were imaged at 5 dpi to investigate neutrophil reverse migration and retention, miR-206^{AM} embryos displayed similar numbers of neutrophils to the control infected group, however maintained higher numbers at the later stage of infection (approximately 124-125 hpi).

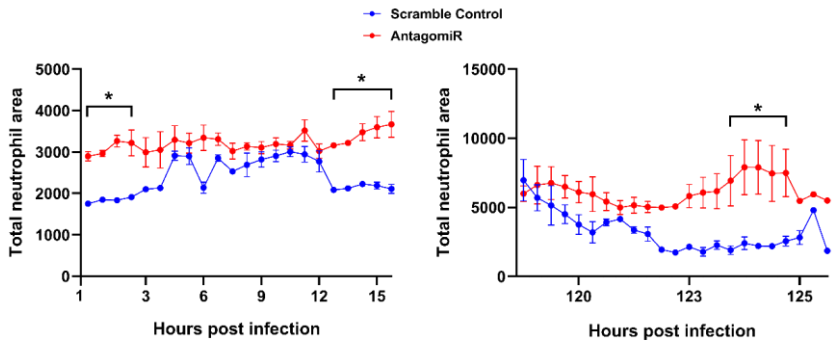


Fig. 3.3.3.13 Neutrophil response to infection in miR-206 antagomiR treated embryos.

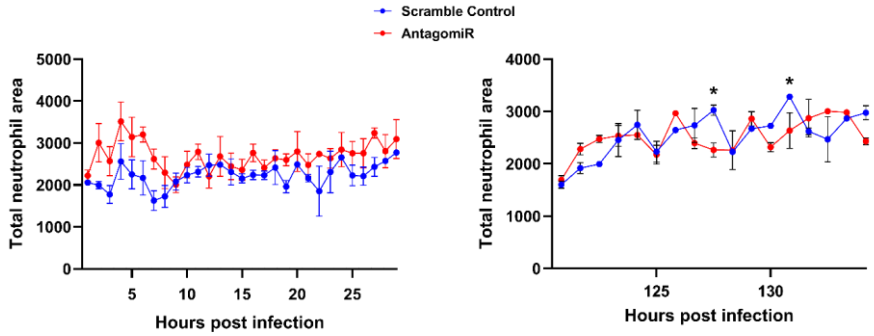


Fig. 3.3.3.15 Neutrophil response to infection in miR-126 antagomiR treated embryos.

In comparison, miR-126^{AM} embryos did not display any difference in their neutrophil migratory response compared to control infected embryos (Figure 3.3.3.15). At both 1 dpi and 5 dpi, miR-126^{AM} neutrophil response mirrored that of control embryos. Bacterial burden analysis also revealed that despite receiving the same bacterial dose, miR-126AM embryos had significantly higher bacterial loads at 1, 3, and 5 dpi.

Infection-induced miR-206 upregulation is driven by mycobacterial virulence factors

To investigate whether the decreased bacterial burden in miR-206 knockdown embryos was a general response to foreign pathogens or a more directed response, embryos were infected with either ΔESX1 *M. marinum* or uropathogenic *Escherichia coli* (UPEC). ΔESX1 *M. marinum* lack the key type VII secretion system and are far less virulent as they are unable to lyse host cell membranes to escape the phagosome. In comparison to mycobacteria, UPEC cause an acute sepsis infection and are an example an extracellular bacterium. Expression of miR-206 was analysed by qPCR in embryos infected with WT *M. marinum*, ΔESX1 *M. marinum*, or UPEC at 1 dpi (Figure 3.3.3.16D). Infection with ΔESX1 *M. marinum* increased miR-206 expression, however this response was less than the level induced by infection with virulent WT *M. marinum*. Conversely, miR-206 was decreased in

embryos infected with UPEC. ΔESX1 *M. marinum* infection burdens were unaffected by miR-206 knockdown at either 1 or 3 dpi (Figure 3.3.3.16E). Similarly, there was also no difference in UPEC burden levels in miR-206 knockdown embryos at either 6 hours post infection (hpi) or 1 dpi despite an increase in infection between timepoints (Figure 3.3.3.16F). These results indicate that the impact on bacterial burden in miR-206 knockdown embryos is driven by *M. marinum* virulence factors.

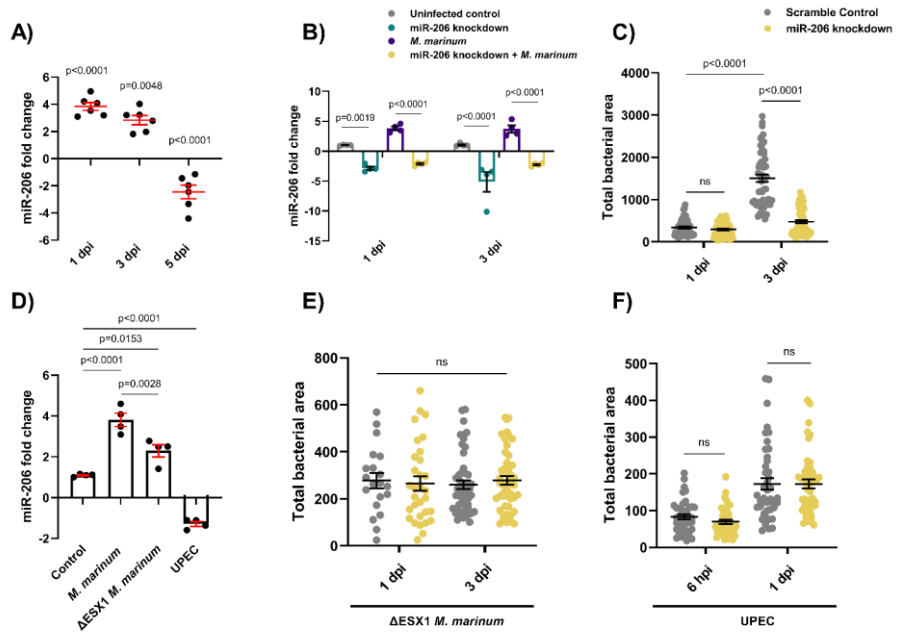


Fig. 3.3.3.16 Infection-induced miR-206 expression alters bacterial burden.
(A) Expression of miR-206 analysed by qPCR at 1, 3, and 5 dpi. (B) Expression of miR-206 in uninfected and infected antagomir-injected embryos. (C) M. marinum burden in antagomir-injected embryos at 1 and 3 dpi. (D) Expression of miR-206 at 1 dpi following infection with either WT M. marinum, ΔESX1 M. marinum, or UPEC. (E) ΔESX1 M. marinum burden in antagomir-injected embryos at 1 and 3 dpi. (F) UPEC burden in antagomir-injected embryos at 6 hpi and 1 dpi. Each data point represents a single measurement, with the mean and SEM shown. For qPCR analysis, each data point represents 10 embryos, and contain 2 biological replicates. Bacterial burden analysis data points represent individual embryos (n=40-50 embryos per group) and are representative of 2 biological replicates.

To confirm the hypothesised link between the observed increased transcription of *cxcr4b* and *cxcl12a* and reduced bacterial burden through an increased neutrophil response early in infection, both genes were targeted for knockdown by Crispr-Cas9. As both *cxcr4b* and *cxcl12* are involved in neutrophil migration and haematopoiesis, a reduction in their expression was expected to result in a

reduced neutrophil response to infection and therefore an increased bacterial burden, reducing the protective effect of miR-206 knockdown.

Addition of cxcr4b knockdown to miR-206 knockdown dampened the miR-206 knockdown-induced increase in neutrophil recruitment to a trunk infection and increased bacterial burden back to control levels. The effect observed in the double knockdown is consistent with a reduction in Cxcr4 and therefore the neutrophil response in infection via haematopoiesis and chemoattraction. This suggests the miR-206 associated increase in cxcr4b is contributing to the enhanced neutrophil migration and reduced bacterial burden.

5.3 Discussion – Sub-project 3: Biomarker discovery

5.3.1 Immunological biomarkers for disease susceptibility and resilience

Current MAP diagnostic tests, faecal PCR and antibody ELISA, provide producers with a measure for *freedom of infection* for their livestock. Paratuberculosis is a chronic infection with a prolonged subclinical phase and infected animals can remain undetected. Validation of the IFNγ test as a marker of exposure on animals from nonvaccinating farms indicates that this test can provide a measure for *freedom from exposure* to MAP. We have previously shown that around 30% or MAP exposed sheep have an IFNγ response by 10 weeks post exposure and 80% are responsive by 20 weeks post exposure. In addition, animals that are resilient to disease still retain an IFNγ response to MAP at 2 years post exposure (de Silva, Plain et al. 2018). Thus, the IFNγ test could be a valuable measure when certifying freedom from exposure to MAP for vendor declarations of individual, nonvaccinated animals. Similarly, the IgG1 response was low in nonvaccinated disease-free sheep and could be incorporated into a test when characterizing freedom from exposure to MAP. Given the lag in the development immune responses post exposure, a quarantine period and re-testing is recommended.

Interpreting the IFNγ test in vaccinated sheep is complicated. As expected, both infection and vaccination generate MAP-specific IFNγ responses. Initial efficacy studies carried out prior to registration of the Gudair® vaccine showed that IFNγ response to vaccination was strong in the first 12 months post vaccination (Reddacliff et al. 2006). On these farms, where disease prevalence was greater than 5%, there was no difference in the IFNγ response in vaccinates and non-vaccinates greater than 24 months after vaccination. The vaccinated animals assessed in the SP5 trial were 2-4 years old at the time of sampling. Overall, there was a tendency for fewer non-responders in the IFNγ test on farms where disease prevalence was low. This suggests that failure to generate an effective IFNγ response in some vaccinated individuals could be a factor in disease persisting on some farms.

We also investigated the potential of a new marker, CXCL10 (IP10), for diagnosis and prognosis of sheep experimentally infected with MAP. CXCL-10 is a chemo-attractive cytokine secreted by monocytes in response to IFNγ. We found CXCL10 to be a good indicator of vaccine efficacy within our experimental model. For gene expression, this marker was successfully validated in the samples obtained from commercial properties. In MAP infection, the strength of early IFN-γ responses is highly correlated with, and predictive of future disease outcome, where sheep with weaker early

IFN-γ responses were more likely to become infectious (de Silva et al. 2013). We observed a similar effect for IFN-γ, as uninfected sheep expressed significantly higher levels than infected animals at 4 months PI. The same was observed for CXCL10, and the differences between infected and uninfected sheep remained significant at 4, 6- and 12-months post MAP exposure. These findings demonstrate a greater diagnostic potential of CXCL10 compared to IFN-γ and prompted an analysis of the prognostic value of CXCL10.

In an on-farm setting, determining vaccine efficacy can allow producers to know which sheep are more likely to become infectious, as most sheep which do not respond to vaccine are highly likely to develop multibacillary lesions, and shed heavy loads of viable MAP in their faeces (Reddacliff et al. 2006). There are also implications of vaccine non-responders in live-sheep trade between farms, whereby the introduction of "false-negative" sheep into a naïve-unvaccinated flock can lead to widespread dissemination of MAP (Windsor 2015; Whittington et al. 2019). A method of assurance prior to sheep trade, which indicates that a vaccine recipient successfully elicited an immune response can substantially reduce the burden of disease in Australia and serve as protective measure to ensure farms can uphold JD-free status. Our findings that CXCL10 levels may be predictive of disease outcome can be applied in a similar fashion in relation to vaccine efficacy; the CXCL-10 (IP-10) levels at early timepoints post-vaccination (4 months) could discriminate sheep that had responded appropriately to vaccination, whereby the vaccine had elicited a protective immune response and these animals did not become infected. Thus, sheep with a greater likelihood of succumbing to clinical disease can be detected and removed from the flock prior to reaching a highly infectious disease state. Studies have shown that sheep can continue to shed viable MAP in their faeces for up to 6 years after commencing a yearly vaccination regime; MAP is also capable of long-term persistence in the environment (Dhand et al. 2013; Windsor 2015). As such, even with ongoing vaccination and extensive biosecurity measures, the eradication of JD from Australian flocks is unlikely without destocking of high-risk mobs (Windsor 2013), which can be expedited if a reliable, simple and cost-effective diagnostic tool is available.

The ability for CXCL10 to predict severity of disease outcome and vaccine efficacy in our experimental infection model is a conceptually promising finding, and warrants further investigation with greater sample size, and the use of a naturally infected cohort. With larger sample size, ROC curve analyses can be conducted to establish appropriate cut-off values for both IFN-γ and CXCL10. As simultaneous measurements of CXCL10 and IFN-γ have shown to improve sensitivity, it would be imprudent to neglect IFN-γ, particularly as IGRA are becoming increasingly common in mycobacterial diagnostics. Once the optimal diagnostic cut-off values have been established for both IFN-γ and CXCL10 and tested under experimental conditions, a field validation study would be the ideal future direction. Furthermore, our observations of prolonged CXCL10 expression at 6- and 12-months post exposure in sheep with paucibacillary and multibacillary lesions may suggest a central role of CXCL10 in granuloma formation, and further studies may shed light on the progression of lesions from paucibacillary to multibacillary.

5.3.2 Predictive immune markers in parasitic co-infections

In MAP challenged groups, the MAP-specific IFNγ response was significantly higher in MAP exposed compared to nonexposed controls and animals exposed to *Hc* alone. This demonstrates that the IFNγ

test can be used as a measure for MAP exposure (see section Sub-project 3 Part 1) even in sheep with a worm burden.

The MAP-IFNγ response between disease outcome groups was not significantly different. However, there was a significantly lower response to the non-specific antigen PWM in sheep that had higher weight gain, compared to the lower weight gain group. While this may seem counterintuitive it most likely relates to the reactivity of the immune response which may affect health overall, and as a result, production measures such as weight. Expending energy to fuel overreactive immune cell activity could be detrimental. It may also indicate that higher active responses may be occurring locally at the site of infection as these measures emanated from blood leukocytes. The hypothesis that the immune fitness of livestock affects productivity is being analysed in another MLA project (P.PSH.0816). This study has shown that in cases where sheep are subjected to multiple pathogen pressures, it is likely that it is the overall immune capacity (in this case seen as the response to PWM) that is important rather than pathogen-specific responses. This conclusion is supported in other co-infection models (Enezwa et al., 2021), emphasising that examination of the higher performers under co-infection (resilient animals) is relevant to improving overall profitability on farm.

$CD4^+$ T cells are known to be the first responders to MAP infection from amongst the adaptive immune cells (Stabel 2000). MAP-specific CD8 and γδ T cell responses do not appear until later, at around 18 months post exposure (Koo et al. 2004). CD8 T cells, also known as cytotoxic T cells, play a key role against intracellular bacteria including MAP. Sheep that are resilient to paratuberculosis have elevated MAP-specific CD8 and γδ T cell proliferation compared to diseased animals (de Silva, Plain et al. 2018). γδ T cells are 'unconventional' T cells; unlike other T cells they are not restricted by major histocompatibility complex (MHC) and recognise lipid antigens. The lack of clear distinctions between immune parameters displayed by sheep with differing clinical phenotypes was disappointing, as this could have enabled clear pathways to biomarker discovery for addressing resilience on-farm. However, this study has improved our knowledge on cellular responses to different pathogen challenges which forms a basis for manipulating and generating relevant immunological memory for useful vaccines for the future.

Assesment of 20 cytokines to discover other immunological biomarkers highlighted a cytokine not previously identifed in relation to paratuberculosis. IL-21 is a T-cell cytokine; it is expressed in activated $CD4^+$ T cells, and has a broad range of actions which regulates both innate and adaptive immune responses. The enhanced cytokine response to MAP in resilient sheep is further evidence supporting an important role for this as a biomarker as well as in protection against disease. Four other cytokines should also be investigated further as candidate biomarkers and are discussed in Appendix 3B.

5.3.3 Novel biomarker discovery

An ideal biomarker should have high specificity and sensitivity, be detectable by minimally invasive sampling procedures, and its detection should be indicative of a disease state. Advances in high throughput sequencing technologies have accelerated the use of transcriptional biomarkers to enable discovery of host biosignatures which are disease specific. Many studies have investigated gene expression in the pathogenesis of paratuberculosis. This has revealed that key functional

pathways such as antigen presentation and MHC processing and lipid metabolism are altered during mycobacterial infection.

The abundance and stability of miRNAs in circulating extracellular vesicles such as exosomes and microparticles make them ideal as potential biomarkers. miRNAs are small non-coding RNA molecules that regulate a wide range of biological processes by post-transcriptionally regulating gene expression which ultimately leads to silencing of protein expression. From the evaluation of the literature, miRNA was identified as having potential as biomarkers for predicting disease outcomes in paratuberculosis.

Screening of archived plasma samples from a past experimental challenge trial led to identification of candidate miRNA biomarkers capable of differentiating between sheep that are resilient or susceptible to paratuberculosis. By further analysing these putative miRNA biomarkers in conjunction with mRNA expression datasets from MAP exposed and infected sheep (Purdie, Plain et al. 2019), we applied bioinformatic analysis to further explore the biological relevance of these miRNA during infection and their potential contribution to disease resilience.

This combined analysis revealed evidence of a decrease in both miRNA and mRNA genes associated with T cell exhaustion at the early stage of MAP exposure; a finding not observed in clinical disease. This mechanism may be a significant contributor to the prevention of infection through a sustained successful T cell response. This situation, combined with the lack of inflammatory signals may ensure minimal tissue damage to the host while increasing T cell responses assist in clearing/containing bacteria, contributing to the protective effect seen in resilient animals. In the early stage post MAP exposure, there is also evidence of an increased protective IL-1 signalling response, possibly aiding in bacterial clearance through effective TNFα-mediated MAP killing. A unique response was observed in the miRNA profiles of resilient animals which was distinct from that of those animals progressing to clinical disease. There was a distinct lack of inflammatory response miRNA in early exposed sheep, contrary to typical host responses to invading pathogens. Since there were no increases in anti-inflammatory genes or pathways, this response was simply a non-inflammatory phenomenon. We contend that this may be beneficial to the host, reducing localised tissue damage and preventing the bacteria from hijacking the response, possibly contributing to the resilient phenotype of these animals. The more rapid development of protective responses which limit parasite numbers is demonstrated by worm-resistant sheep (Windon, 1996). MAP infected animals that progressed to clinical infection followed the accepted or conventional immune trajectory i.e. early pro-inflammatory response with increased cytokine production and immune cell infiltration, followed by a shift towards an anti-inflammatory profile allowing for persistent granulomatous infection to establish. These key genes and potential signalling pathways modulated by miRNA may be crucial factors in determining the host ability to clear bacteria and prevent establishment of an infection or conversely, whether they progress to disease.

The use of zebrafish-*M. marinum* model has proved a useful tool in the investigation of immune responses to MAP infection, in particular the role of miRNA in infection. Our studies involving zebrafish embryos have provided key insights into the functions of selected miRNA that have been observed as differentially regulated throughout disease progression in paratuberculosis.

miR-206 was found to be decreased in resilient animals at the early stage of infection (13 weeks post infection) and thus chosen for functional analysis to uncover its biological relevance. The stark

decrease in bacterial burden of miR-206 deficient embryos (generated by knocking down miR-206 expression) compared to control (wild type) infected suggests a protective role for this miRNA against mycobacteria. Through *in silico* target prediction and current experimentally observed functions, immune cell response genes were implicated as potential targets and were further investigated. Gene expression analysis revealed an increase in both cxcl12 and cxcr4b, a signalling axis known to be of key importance in mycobacterial infections (Feng et al. 2011; Torraca et al. 2017; Isles et al. 2019). As such, neutrophil responses to bacteria were analysed. In the absence of miR-206 expression in the miR-206 deficient embryos there was both an increase in the influx of neutrophils to the site of infection and a decrease in the reverse migration of the neutrophils was observed in miR-206 deficient -embryos. Thus, the lower miR-206 observed in resilient sheep could have enabled neutrophil movement into the site of infection and retained these cells there, ultimately enabling destruction of MAP.

As miR-206 was decreased in resilient animals, the use of a knockdown in embryos provides an insight into the conditions in sheep and which biological pathways are altered to potentially confer resilience to MAP infection. Due to the increase in Cxcl12 and therefore it's receptor Cxcr4b, we believe that the decreased miR-206 levels in animals resilient to MAP infection results in a stronger initial immune response to the bacterial presence, with a higher number of neutrophils being produced and chemotactically drawn to the site of infection. This initial neutrophil response also appears to be sustained for a longer period, potentially assisting resistance to the establishment of a chronic infection and the formation of gut granulomas.

The second miRNA investigated in the zebrafish model was miR-126, a vascular associated miRNA that was decreased in infected sheep at a late stage of infection (49 weeks post infection). In contrast to miR-206, there was a significant increase in the bacterial burden of miR-126 deficient embryos compared to control infected. Our work has implicated the mTOR pathway in the effect of miR-126 on infection, as the addition of rapamycin to infected embryos mirrored the effect of a reduction in miR-126.

A previous study uncovered a new, primarily non-vascular role for miR-126 in mediating the interferon-α response to pathogenic nucleic acids (Agudo et al. 2014). We propose this mechanism as the biological pathway through which miR-126 is acting. The perturbation in the interferon response and detection of MAP nucleic acids prevents an effective immune response and allows the infection to take hold as infected animals succumb to disease.

Assessing the biological pathways altered by the differing levels of these miRNA in infection alludes to an impaired or inappropriate immune response contributing to disease outcome following MAP infection. For resilient animals, an improved and sustained initial neutrophil response due to reduced miR-206 may be one way in which the host immune system is able to successfully clear the pathogen. In infected animals, a late reduction in miR-126 may be indicative of the modulation of host responses by MAP to shift towards an anti-inflammatory response through inhibiting mTOR signalling. These results provide further insight into the regulation networks of gene expression during infection, and further contribute to our understanding of the complex immune patterns observed in MAP infection.

While further validation studies are required to assess the repeatability and diagnostic potential of the putative miRNA biomarkers, these data provide strong evidence of miRNA regulation of host responses to MAP and their significance as regulators of host gene expression. It would be instructive to repeat such investigations on sheep with extreme resistance phenotypes following worm challenge.

6.3 Conclusions/Recommendations – Sub-project 3: Biomarker discovery

6.3.1 Immunological biomarkers for disease susceptibility and resilience

In adult sheep the IFNγ test or the IgG1 ELISA can be used to screen for exposure to MAP on nonvaccinating farms. These measures will be beneficial for determining freedom from exposure to paratuberculosis if the industry requires such a measure in individual nonvaccinated animals. While detection of MAP in faeces is an excellent farm-wide screening tool, these immunological measures are tools which can be used in individual animals for trade or breeding purposes.

For Gudair® vaccinated sheep, while there is a significant association of a positive IFNγ test with the HT-J result, the test alone does not provide useful information about the faecal shedding status of the animal. However, the IFNγ response to vaccination should be assessed over time to determine whether a booster vaccination is required, especially on farms where disease persists despite vaccination. This should initially be studied under controlled conditions to monitor safety and injection-site lesions as well accompany genomic analysis of persistent strains of MAP.

The cohort of 50 ewe lambs from the NSW farm should be monitored for a longer period beyond the duration of this project as it could provide valuable information in relation to vaccination. While this type of study has been carried out in the past, the finding that this cohort had uniformly low responses to vaccination requires further evaluation.

CXCL10 has the potential to be a valuable test for discriminating between infected and noninfected sheep, regardless of vaccine status, and should be evaluated as such in the context of commercial farms or a case study farm.

We have validated the expression of eight genes (LXN, RARRES, LYZ, TET2, C10H15orf48, CXCL10 and BOLA1a) as potential biomarkers of vaccine efficacy (disease resilience) in samples obtained from commercial flocks. The limitations of the conventional comparative Ct (ΔΔCt) method for evaluation of fold change analysis requires consistency of sample variable numbers and in flocks with high variability in disease expression, this limits the efficacy and utility of the analysis. A potential solution for this issue may be development of a universal standard expression sample set.

6.3.2 Predictive markers in parasitic co-infections

The MAP-specific IFNγ test described in Appendix 3A can be used successfully and reliably in sheep co-infected with MAP and *Haemonchus.*

Several cytokines should be assessed further as biomarkers for overall disease manifestation in sheep subjected to multiple pathogen burdens. Rather than under controlled experimental conditions, evaluation should be carried out in farms which may be confronting production losses

despite best practice control strategies with a view to identifying early predictive biomarkers for nonproductive animals.

6.3.3 Novel biomarker discovery

miRNA are suitable biomarkers for paratuberculosis. There is potential for a screening a panel of miRNA by PCR as a diagnostic tool which may also be able to discriminate between vaccinated and infected animals.

The zebrafish-*M. marinum* infection model is a suitable rapid, low cost alternative for initial validation of gene action in paratuberculosis research. While research in large ruminants cannot be removed completely, it provides a means of reducing the number of animals used for research purposes.

Investment into basic research to expand knowledge of the functional aspects of miRNA in disease manifestation could lead to the development of therapeutics to drive better immune modulation and protection against disease.

7.3 Key messages – Sub-project 3: Biomarker discovery

7.3.1 Immunological biomarkers for disease susceptibility and resilience

The IFNγ test can be used to provide a 'freedom from exposure' certification for individual sheep from nonvaccinating farms; it provides a benefit over detection of MAP in faeces which can be intermittent.

A novel immunological biomarker which can distinguish between infected and noninfected sheep was identified.

The immune responses in vaccinated sheep should be re-evaluated in farms with on-going paratuberculosis.

7.3.2 Predictive markers in parasitic co-infections

There is a lack of understanding of immune modulation mechanisms which ultimately impact disease manifestation, animal welfare and associated production losses in the face of multiple pathogen burden in sheep. These also include nutrition and other management activities. Investing in such research programs will develop methods of early detection of animals with dysfunctional immune systems and provide strategies to boost immune function and better outcomes.

7.3.3 Novel biomarker discovery

miRNA are suitable biomarkers for identifying susceptibility and resilience to paratuberculosis. A panel of miRNA markers should be tested on-farm for further validation, likely on some farms participating in SP5.

The zebrafish-*M. marinum* infection model is a suitable rapid, low cost alternative for paratuberculosis research.

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Sub-project 4: Pathogen-host interactions and pathogen evolution

1.4 Background – Sub-project 4

The interplay between pathogen, host and the environment is a conceptual epidemiological framework by which to understand infectious diseases (Fig. 1.4.1). This illustrates the interactions between pathogen-related, host(animal)-related and environmental factors that influence disease establishment. Examples of pathogen, host and environmental factors that may have an effect on the occurrence and severity of diseases of livestock include: pathogen virulence, host genetic susceptibility and environmental conditions allowing for disease occurrence.

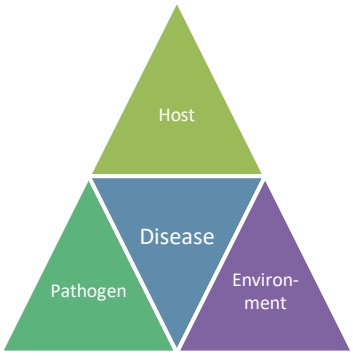

Fig. 1.4.1: Pathogen-Host-Environment epidemiological triangle of disease.

Sub-project 4 focuses on the Pathogen and Environmental factors within this triad. In relation to Johne's disease (JD), *Mycobacterium avium* subspecies *paratuberculosis* (MAP) virulence as well as environmental risk factors are considered with respect to the spread of the different strain types of MAP, as well as broader aspects of mycobacterial pathogenicity. The diversity in genotypes of *Haemonchus contortus* currently circulating in regions of Australia was considered in relation to the pathogenic features of this parasite.

Specific background relevant to each of these aspects is provided below.

1.4.1 Infection of cattle and sheep with differing MAP strains

Historically, the various strains of MAP in Australia have been associated with their respective host species; MAP sheep (S) strain was isolated from sheep and MAP cattle (C) strain from cattle (Whittington and Sergeant, 2001b). This is different to the situation in some other countries (Sevilla et al., 2005, Verdugo et al., 2014), where reports of cross-species infections are more common. The prevalence upper limit of cattle infected with MAP sheep (S) strain was estimated to be 0.08% in NSW in 2008 (Moloney and Whittington, 2008), but the incidence of S strain MAP infections in cattle is thought to be increasing in Australia (Sergeant, 2015). A simplified nomenclature of the current knowledge regarding MAP strain types commonly infecting sheep and cattle populations is shown in Fig. 1.4.2.

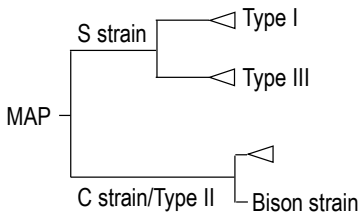

Fig. 1.4.2: Simplified phylogeny of strain types of MAP (based on Stevenson *et al.*, 2015, Bryant *et al.*, 2016). Type I and Type III strains lie within the broader MAP S strain grouping. MAP C strain and Type II classifications are synonymous, with the majority of C strains on one branch (including human isolates of MAP), with a sub-grouping of MAP Bison strains.

With changes to the National Bovine JD (BJD) control program, there are ramifications of a positive diagnosis for an individual producer irrespective of the MAP strain type. However, farm biosecurity practices need to take into consideration the species of livestock and strain type(s) of MAP present on the property, to effectively control the disease and to stop spread between farms and possibly between ruminant species on the same property. A fundamental step towards improving on-farm disease management is to better understand the circulating MAP strains in the Australian context, which can be linked to information regarding management risk factors.

From the available evidence, it appears that S strain MAP infections in cattle are no longer rare events but may be increasing in incidence. The reasons for this are not clear. The source of infection appears to be Ovine JD (OJD) infected sheep populations, however many infected producers now vaccinate their sheep with Gudair®. A report from Sergeant (2015) concluded that there was insufficient evidence to support or refute vaccinated sheep populations being involved in the transmission of S strain infections to cattle.

Based on a review of the literature, there were key knowledge and technical gaps identified concerning the diagnostic tests available to detect infection by different strains of MAP. The technical limitations of the IS*1311* PCR-REA for differentiation between MAP strains means this method is not an ideal. It has recently been identified from whole genome sequencing of MAP strains that the IS*1311* SNP discrimination may be equivocal (Bryant et al., 2016), though the situation in Australia is not yet known. This method is also not suitable for the identification of strains in cultures from animals with mixed infections. Currently, there is no single diagnostic test that is capable of rapidly and accurately distinguishing between the two MAP strains on DNA directly isolated from faeces or tissues.

Although bacteriological tests, such as faecal culture or PCR, are known to be more sensitive for the diagnosis of paratuberculosis, particularly in the subclinical stages of disease, serological assays are still used in some settings as a screening test for herd or flock level diagnosis, due to the low cost/animal of these types of diagnostic tests. These tests measure the host immune response to infection, in terms of the production of antibodies against MAP that are circulating in the blood. A commonly used serological test for paratuberculosis in Australia is the commercially available

enzyme-linked immunosorbent assay (ELISA) (IDEXX Paratuberculosis Screening Antibody Test), which can be used for a range of sample types (milk, serum, plasma) and species (cattle, sheep, goats). A further knowledge gap identified was the limited information regarding the diagnostic sensitivity of serum antibody ELISAs for the detection of MAP S strain infections in cattle.

The full literature review of the current situation in Australia with regard to the circulating MAP strain types, differences in virulence, cross-species infections and diagnostics tests available to detect these topics is provided in **Appendix A4.1.**

1.4.2 Mycobacterial genetics

The evolution of pathogenicity and virulence factors in mycobacterial species has not been fully elucidated. Some mycobacteria species are common environmental organisms, whereas others are significant pathogens of humans and animals. Mycobacteria are routinely classified as rapid or slow growers based on their *in vitro* growth characteristics (Kim et al., 2013). The slow growing phenotype has been associated with those mycobacteria that survive intracellularly and are pathogenic, while rapid growing species are mainly environmental. Pathogenic, slow growing mycobacterial species include *M.tuberculosis*, *M.bovis* and MAP. The genetic factors associated with the evolution of these two broad classes of mycobacteria are not well understood.

The Mycobacterium Avium Complex (MAC) is a group of mycobacteria traditionally consisting of two species, *M.intracellulare* and *M.avium*. These comprise a broad range of mycobacterial 'lifestyles', from obligate pathogens, to opportunistic pathogens and rapid-growing environmental species. Many MAC species may be found in the environment and can be a source of nosocomial infections or opportunistic pathogens in humans that are notoriously difficult to diagnose and treat (Ahmed et al., 2020, Santos et al., 2017, Siddiqui and Shakil, 2010). Within *M.avium*, four subspecies have been described; *Mycobacterium avium* subspecies *avium* (MAA), *Mycobacterium avium* subspecies *silvaticum* (MAS), *Mycobacterium avium* subspecies *hominissuis* (MAH) and MAP. MAA and MAS typically cause respiratory disease in avian species, whereas MAH causes localized legions in swine and occasional respiratory disease in immunocompromised humans (Tell et al., 2001, Kei-Ichi et al., 2017). Unusual infections of *M.avium* subspecies outside their typical host species have been described (Chiers et al., 2012, Yoshida et al., 2018). The lack of available tests able to rapidly distinguish *M.avium* to the species and subspecies level may reflect our poor understanding of their definition and taxonomy.

Heterogeneity of mycobacterial strains infecting an individual host has been identified in both human (Allix et al., 2004, Pérez-Lago et al., 2014) and animal (Davidson et al., 2016, Ahlstrom et al., 2015) mycobacterial infections. The presence of 'mixed infections' (more than one genotype infecting a single individual) can give pathogens the opportunity to adapt to their microenvironment. "Escape" variants may be selected by treatments such as antimicrobial therapy and lead to host adaptation or the emergence of drug resistant strains. In Johne's disease, mixed infections can exist at the group and individual animal level (Davidson et al., 2016, Ahlstrom et al., 2015).

The concern in JD is the sub-optimal performance of available vaccines. Several studies have demonstrated that current vaccines do not prevent infection or shedding of MAP (Stringer et al., 2013, Windsor et al., 2014). A trial in sheep found shedding prevalent in ~40% of properties after a decade of vaccination (Dhand et al., 2016). Like Gudair®, Silirum® vaccination of deer has been

shown to reduce the incidence of clinical disease but does not prevent infection or shedding (Stringer et al., 2013). Gudair® and Silirum® are both heat inactivated preparations of strain 316F, a C strain MAP isolate, which has been maintained in culture for decades (Bull et al., 2013). Genomic changes in this vaccine strain have been described (Bull et al., 2013), with some evidence that indigenous/endemic current strains may be more effective to reduce clinical and subclinical JD (Singh et al., 2013). This suggests the MAP strain used within the vaccine may influence vaccine efficacy.

In endemic disease scenarios, the pathogen and host co-evolve to survive. Poor vaccine efficacy may potentially drive selection of persistent phenotypes/genotypes of MAP. Over time, continual selection for these phenotypes may result in further reductions in vaccine efficacy and eventual vaccine failure. Vaccines are less vulnerable to pathogen evolution than antimicrobial drugs, however there is still the potential for vaccine-persistent phenotypes to be selected (Kennedy and Read, 2017). As more farmers take up vaccination to protect their animals, more intense selection pressure is applied that could potentially lead to reduced vaccine efficacy, as persistent or novel MAP phenotypes become more prevalent. This may also be a potential problem for cross-species infections.

1.4.3 Haemonchus contortus genetics

Helminthic parasites have a major impact on human and animal health, with wide ranging socioeconomic impacts. Livestock can be infected with a variety of helminths through ingestion of the larvae from pastures, with the most common parasites affecting livestock being the gastrointestinal nematodes and flukes. Control of these parasites is currently dependent on a range of anthelmintic drugs such as macrocyclic lactones (avermectins and milbemycins, such as ivermectin and moxidectin), salicylanilides (e.g. closantel), and benzimidazoles (e.g. triclabendazole).

Haemonchus contortus is one of the most important parasites of small ruminants internationally, with major economic and welfare impacts on the Australian sheep industry (Emery et al., 2016). Due to its high fecundity (between 5000–15,000 eggs per female worm daily) enabling rapid genetic variation and its short prepatent period, anthelmintic resistance is a major issue with this parasite (Kotze and Prichard, 2016). Resistance of *H.contortus* to organophosphates and benzimidazoles (BZs) was detected within 20 years of these drug classes being introduced in Australia. By the early 2000's, resistance to a broad range of anthelmintic drug classes had been reported, with multi-drug resistant strains (resistant to 3 or more classes of anthelmintic drug) of *H.contortus* also identified (Kotze and Prichard, 2016, van Wyk et al., 1997).

Genetic diversity in parasites is key to understanding the population-level variation and diversity. This variation can assist parasites to avoid eradication by the host-immune system and adapt resistance traits. Further, the life cycle of *H.contortus* includes both parasitic and free-living stages, each subject to different environmental pressures. Whole genome sequencing (WGS) has recently been applied to the study of helminth genomes (Laing et al., 2016, Gasser et al., 2016). The genomic landscape for this parasite is extremely complex; they are polyandryous (each female can mate with multiple males), and sequencing studies have shown a very high level of genetic diversity, with a high density of single nucleotide polymorphisms (SNP) and large numbers of insertions and deletions (indels) across the genome.

Molecular studies of this parasite are challenging at multiple levels; (i) DNA isolation and purification, (ii) the size of the genome (>250 Mbp), (iii) genome sequence assembly tools suitable for highly polymorphic genomes, (iv) and the availability of a completed genome for resequencing analyses. For DNA isolation, late-stage juvenile and adult parasites are usually used as they allow isolation of sufficient DNA for molecular analyses, however these stages of the parasite are almost always inaccessible in the live host, thus necessitating DNA extraction from excreted eggs once the infestation is patent. Immature life stages, such as those found in the environment, can be collected non-invasively however, these samples typically yield lower quantities of DNA, can be environmentally resistant, and are susceptible to contamination. Isolation of DNA from larval cultures, though complicated by the resistance of the outer cuticle, may offer an alternative approach to examine population-level genomic diversity across different geographic locations.

The level of genetic diversity in the *H.contortus* populations in NSW was investigated in B.AHE.0315 and this investigation aimed to augment these outcomesby developing methods and an analytical pipeline to assess the population level heterogeneity in Australia.

2.4 Objectives – Sub-project 4

The overall objectives of Sub-project 4 were to investigate host-pathogen interactions and pathogen evolution for MAP and *H. contortus*, utilising recent advances in sequencing approaches and publicly available genomes.

The broad objectives relating to Johne's disease were: (i) to evaluate risk factors associated with 'cross-species' infection, (ii) to assess current diagnostic tools, and (iii) to examine the genetic changes in MAP and mycobacterial evolution. The objectives in relation to *H.contortus* were to conduct a study to better understand population-level variation at the genetic level. These broad objectives were broken down into the specific objectives below:

2.4.1 Improved understanding of the potential drivers of risk of S strain infections in cattle through case reports and risk factor analysis.

2.4.2 Evaluate variance in diagnostic accuracy for animals infected with different strains of MAP at the level of i) detecting host immune responses and ii) pathogen detection (e.g. is C strain "out-growing" S strain in culture and thus masking the true level that co-infection actually occurs?).

2.4.3 Improved understanding of how MAP strains are changing over time and how this may affect on-farm infection by:

i. Whole genome sequencing (WGS) of divergent mycobacterial species from an evolutionary perspective in order to understand how common properties have arisen;

ii. WGS of a diverse collection of MAP isolates to understand common epidemiological situations, such as changes in the epidemiology of paratuberculosis in Australia over time (S, C and B strain) and possible effects of Gudair® vaccine on MAP host preference (S strain in cattle).

2.4.4 Extend our knowledge of the population-level heterogeneity of *H.contortus* through WGS of field isolates.

3.4 Methodology – Sub-project 4

The Methodology for each study is aligned to the Sub-project Objectives (Section 2.4). Where relevant, these refer to Appendices to the Final Report.

3.4.1 Improved understanding of the potential drivers of the risk of S strain infections in cattle

A study was conducted to improve understanding of the potential risk factors for S strain MAP infections in cattle. The changes to the BJD policy involving a shift to management of JD as one disease (including C and S strain MAP) had a major impact on how this aspect of the research was undertaken. This had implications for the future trade of animals by cattle producers with cattle previously diagnosed, or diagnosed in future, with S strain MAP, and mixed enterprises in OJD endemic regions. Initially, the proposed design involved a two-part case-control study; a survey was to be designed and distributed to farmers that ran mixed enterprises to report on current grazing and disease management practices on-farm. Farmers that met the study criteria would be enrolled and on-farm visits to collect faecal samples would take place for herd prevalence assessment and MAP strain typing. Discussions with key stakeholders during the planning stages for the risk factor survey identified a major challenge for participation as producers were risk averse due to the compulsory reporting requirement to DPI-NSW of any positive findings. It was predicted that farmers would not be motivated to participate, particularly mixed enterprises. This occurred and made the survey unachievable.

To improve the understanding of potential drivers and the current and future risks of S strain infections in cattle and to provide suggested farm management practice changes, a retrospective study was conducted. This involved the compilation of information regarding reports of cattle farms diagnosed with MAP S strain (n=43). All available information was compiled regarding previous reports of cattle farms diagnosed with MAP S strain infections, with permission granted to obtain a dataset of cases from Animal Health Australia. David Allan, former BJD councillor associated with the Financial non-financial assistance package (FNFAP) program, was involved as a consultant to maintain confidentiality in relation to the producer details. Data were categorised into general data on a whole herd level and individualised data about specific cases. Descriptive analyses were performed and interpreted to determine if existing biosecurity measures should be modified in relation to S strain MAP infections in beef cattle.

Detailed methods are provided in **Appendix A4.2.**

3.4.2 Diagnostic test accuracy for MAP infection with different strain types

In order to better understand MAP strain differences and how the strains may be changing over time, the ability to diagnose infection caused by different MAP strains in a range of species using the current test platforms was evaluated. This aspect of the project investigated the accuracy of 2 diagnostic tests for the identification of infection by the differing strain types of MAP most prevalent

in Australia (S and C strains), and the potential for under-reporting of cross-species and mixed infections.

Two studies were performed, examining serological and culture-based diagnostic test methods.

3.4.2.1 Serological case study to assess ELISA detection of S strain MAP infection of cattle.

Data were compiled from case study information collected as part of this project to assess risk factors for S strain infection in Australian beef cattle (Section 3.4.1), as well as previous reports. Using the data collected regarding serological results we determined if there was evidence of identification by serological testing of JD infected beef cattle farms that were infected with the S strain, rather than the C strain, of MAP. Further, the case information regarding clinical cases of cattle infected with S strain MAP was examined to determine if there was evidence of positive serum antibody ELISA responses in these cases.

3.4.2.2 MAP culture study, examining mixed cultures of S and C strain MAP

Current strain typing methodology post-culture and its limitations were assessed. Firstly, an assay was developed for the accurate identification of different strain types by quantitative PCR rather than the traditional IS*1311* PCR REA. Species-specific genes were selected for the MAP K-10 cattle and MAP S397 sheep strain (Bannantine 2012, Li 2005). Five genes were selected from each MAP strain, based on 100% sequence specificity to their specific strain. These were screened and two genes that performed well in the qPCR were taken forward for validation. Sequence homology was analysed for each of the final genes selected as the strain-specific gene targets, using basic local alignment search (BLAST) of 400 whole genome sequenced MAP isolates from around the world. These strain-type specific gene assays are termed 'C1' for the cattle strain-specific and 'S1' for the sheep strain-specific gene.

To examine the potential for differential growth of the two MAP strains in liquid culture media, mixed S and C strain MAP cultures were set-up and growth for each MAP strain at different timepoints was examined. The experimental design is shown in Figure 3.4.1.

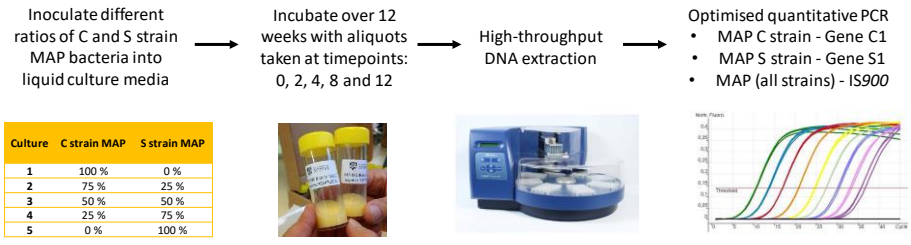

Fig 3.4.1: Replicate cultures (n=3 replicates/culture group) were set-up containing differing ratios of MAP C and S strain (Culture groups 1 – 5). These were cultured for 12 weeks, with aliquots taken at 0, 2, 4, 8 and 12 weeks of culture. DNA was extracted according to the method described by Plain et al., 2014. An optimised qPCR was performed on each culture sample to determine growth curves for the MAP strains present.

Full details of the strain-type specific gene assays are provided in **Appendix A4.3**.

3.4.2.3 Statistical analyses

The one-way ANOVA was conducted to compare between the different types of gene and mastermixes in the respective species. The interaction between culture, weeks, and genes were analysed using a restriction maximum-likelihood (REML) linear mixed model, using the log_{10}-transformed qPCR DNA quantities on R-studio. The fixed effects were culture, weeks and genes, with three interactions tested, whereas the random effects were the replicates of liquid culture. To account for repeated measures of different liquid culture replicates, serial correlation/auto-regression was included in the final REML model. Differences between the interaction of genes and week for each culture was tested using the least significant difference (LSD) derived from REML. Statistical difference was measured at level $p< 0.05$.

3.4.3 Whole genome sequencing of mycobacterial isolates

A full review was undertaken of current approaches for obtaining samples for mycobacterial genomic studies and the methods applicable to genomic comparisons of mycobacteria. This is reported in **Appendix A4.4.**

3.4.3.1 Evolutionary transitions in Rapid and Slow growing mycobacteria

A comparative genomics approach was applied to investigate the evolution of traits associated with growth of mycobacteria. Briefly, a whole genome-based phylogenetic analysis was undertaken of sequenced Mycobacterium species with high quality assemblies (n-157), available in the National Centre for Biotechnology Information database as of 31 January 2019. The genomes were uniformly re-annotated using the Broad Institutes' prokaryotic annotation pipeline to ensure a consistent annotation protocol. Clusters of orthologous gene groups (genes in different species derived from a common ancestral gene) were compared.

The full Methods are reported in the published paper (Bachmann et al., 2019) **(attached as Appendix A4.5).**

3.4.3.2 Whole genome sequencing of MAP and MAC isolates

MAP cultures were performed in liquid medium M7H9C as previously described (Whittington et al., 2013). The liquid cultures were put on modified 7H10 with mycobactin J (MJ) slopes and assessed for growth weekly. Individual colonies were selected from the slopes using a sterile loop and a secondary culture performed in M7H9C to expand. This was inoculated back onto 7H10+MJ slopes for 4-8 weeks prior to harvesting of the mycobacterial lawn in 1 x Tris EDTA buffer (TE; 10 mM Tris, 1mM EDTA).

Genomic DNA (gDNA) was extracted via a modified cetyltrimethylammonium bromide (CTAB) and phenol/chloroform isolation protocol (Choy et al., 1998). Frozen samples were thawed at room temperature prior to addition of 550 µL of TE buffer and brief vortexing. Declumping was achieved by drawing the suspension through a 25 gauge needle seven times before another 10 s vortex. Heat inactivation occurred at 85^{o}C for 30 min, followed by a beat beating step (frequency 30 for 1 min 40s twice, with a 180^{o}C rotation in between). To continue cell wall degradation, 60 µL of 200 mg/mL lysozyme was added and the samples then incubated for 2 h at 37^{o}C with gentle mixing. Upon completion, another 3 h incubation in the same conditions was done following the addition of 20µL 20mg/ml RNAse. To compete the breakdown of the mycobacterial cell wall, 10 µL (200 U) of

Mutanolysin (10,000 U/mL) was added and the tubes returned to the incubator overnight. The following day, 35 µL of Proteinase K solution (10 mg/mL) was added and mixed well prior to addition of 60 µL of 10% sodium dodecyl sulphate and the lysates returned to the incubator overnight. The following morning, 97.5 µL 5M NaCl and then 82.5 µL CTAB/ NaCl that had been pre warmed to 65°C were added to the lysates. The tubes were incubated at 65°C with gentle mixing for 10 min. Samples were then transferred to 2 mL microcentrifuge tubes and 700 µL of 25:24:1 phenol/ chloroform/ isoamyl alcohol added with mixing. The upper aqueous phase was collected after centrifuging 12,000 x g for 10 min. To remove excess phenol, an approximately equal volume of Chloroform:isoamyl alcohol (24:1) was added prior to repeating the centrifuge step. The upper aqueous layer was collected and mixed with 1200 µL of isopropanol via gentle inversion for one minute. DNA was pelleted via centrifuging for 15 min at 12,000 x g. Washing of the pellet was done twice using 70% ethanol at -20°C, followed by centrifugation for 15 min at 12,000 x g. Upon completion, the pellets were resuspended in 30 µL of 10mM Tris buffer pH 8.0. Re-solubilisation of DNA occurred at room temperature overnight with gentle mixing. On completion, samples are stored at -80°C.

Quality and quantity of DNA was assessed using a NanoDrop$_{TM}$ 2000 spectrophotometer (Thermo Fisher Scientific). Samples with $A_{230/280nm}$ ratios less than 1.3 were cleaned up by ethanol precipitation step. Any samples which had $A_{260/280nm}$ ratios below 1.8 or $A_{230/280nm}$ ratios below 1.3 after clean-up were discarded and re-isolated. A Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific) was used to more accurately quantify the DNA concentration for sequencing using the Qubit fluorimeter (Thermo Fisher Scientific).

Whole genome sequencing was carried out at the NSW Mycobacterium Tuberculosis Reference Laboratory at the Centre of Infectious Diseases and Microbiology, Westmead Hospital on the Illumina sequencing platform. A Nextera XT library preparation kit (Illumina, Scoresby, Victoria, Australia) was used to generate paired indexed libraries of 150 base pairs in length as per the manufacturer's instructions. Sequencing was done using the Illumina NextSeq platform.

Publicly available whole genome sequencing data and associated metadata for additional MAP and MAC species were downloaded from the National Centre for Biotechnology Information (NCBI) GenBank and Sequence Read Archive (SRA) databases.

Bioinformatic analyses were performed and the results visualised using a pipeline that involved the analytical programs: Trimmomatic (Bolger et al., 2014), SPAdes (version 3.12.0) (Nurk et al., 2013), Quast (version 5.0.2) (Gurevich et al., 2013), Prokka (version 1.13.3) (Seemann, 2014), Roary (version 3.12.0) (Page et al., 2015), iqTree (version 1.6.7) (Nguyen et al., 2015), iTol (Letunic and Bork, 2019), and ModelFinder (Kalyaanamoorthy et al., 2017).

Full details of the isolates included in the study and bioinformatic analyses performed are provided in **Appendices A4.5 and A4.6.**

3.4.4 Whole genome sequencing of *H.contortus* isolates

3.4.4.1 Samples

The drought conditions led to significant issues with obtaining suitable material to undertake this WGS study. Larval isolates were sourced with the help of Dr Keith Walker and the Elizabeth Macarthur Agricultural Institute (EMAI) parasitology laboratory. These have been de-identified and

only the location of collection has been included for epidemiological purposes (Table 3.4.1). These samples had an unknown concentration of larvae, derived from larval cultures performed for ID of nematode genera at EMAI. The majority were mixed cultures. In addition, two larval culture samples from Tasmania were obtained with the assistance of Graeme Knowles and Janine Davis from DPIPWE Animal Health Laboratories.

Table 3.4.1: Larval culture samples from EMAI.

Keylist	Host Species	Haemonchus %	Trichostrongylus %	Ostertagia %	Oesophago-stamum %	State	Location
SP1	-	100	0	0	0	-	L3 used in trial
Tas01*	Ovine	100	0	0	0	TAS	Nugent
Tas02*	Ovine	100	0	0	0	TAS	Lega
11	Ovine	100	0	0	0	NSW	Ural
47	Ovine	100	0	0	0	NSW	North Nar
4	Ovine	99	1	0	0	NSW	Oph
58	Ovine	99	0	1	0	NSW	Forest I
45	Ovine	98	0	2	0	NSW	Ober
46	Ovine	96	1	3	0	NSW	Wellin
56	Ovine	96	1	3	0	NSW	Vitto
59	Ovine	96	0	3	1	NSW	Vitto
43	Caprine	95	3	2	0	NSW	Bathurst
49	Ovine	95	5	0	0	NSW	Ballimore
33	Ovine	94	2	1	3	NSW	Murrumbateman
66	Ovine	94	3	3	0	NSW	Braidwood
54	Ovine	93	6	1	0	NSW	Cassilis
9	Ovine	92	3	3	2	NSW	Burraga
24	Ovine	92	5	2	1	NSW	Tarlo
37	Ovine	89	9	1	1	NSW	Murringo
41	Ovine	89	5	2	4	NSW	Narromine
38	Ovine	87	3	8	2	NSW	Cooma
67	Ovine	87	4	7	2	NSW	Piambong
70	Ovine	86	11	2	1	NSW	Bibbenluke
14	Ovine	84	9	7	0	NSW	Tarago
55	Ovine	84	9	7	0	NSW	Hillston
48	Ovine	83	17	0	0	NSW	Ballimore
29	Ovine	82	10	5	3	NSW	Aggan
34	Ovine	81	14	5	0	NSW	Murringo
61	Caprine	76	20	3	1	NSW	Lowther
31	Ovine	75	2	6	17	NSW	Elderslie
35	Ovine	75	17	6	2	NSW	Murringo
28	Ovine	74	16	9	1	NSW	Koonawatha
68	Ovine	74	19	7	0	NSW	Bibbenluke
32	Caprine	73	18	5	4	NSW	Lower Portland
39	Ovine	71	26	2	1	NSW	Dramana
16	Ovine	70	1	19	10	NSW	Ando
63	Ovine	67	14	19	0	NSW	Vittoria
26	Ovine	64	25	2	9	NSW	Ando
65	Ovine	60	21	18	1	NSW	Piambong
20	Ovine	59	40	1	0	NSW	Windellama

* These larval culture samples from sheep were kindly provided by the Parasitology Dept. Biosecurity Tasmania DPIPWE.

Commented [JS1]: Were these isolates from artificially infested donors? Or might the samples have been collected in summer from irrigated pastures?
With Hc being a subtropical parasite that favours summer rainfall conditions, two 100% samples from TAS under natural conditions is difficult to believe. Hc did get into northern Tassie several years ago in Feb (my Hc review when we accessed DV postcode data sets), but interesting no other worms in the samples. Karren?

Commented [KP2R1]: Samples from Tasmania were provided by Parasitology at Biosecurity Tasmania DPIPWE. I will include this as a footnote in the Table. They were from larval cultures from sheep. The samples were de-identified in order to maintain confidentiality regarding the producer details, so I don't have any further information on the origins of these samples.

3.4.4.2 DNA extraction from *H.contortus* larvae

An optimised method for DNA extraction from *H.contortus* larvae was developed to obtain sufficient quality and quantity of DNA for WGS. This method involved an enzymatic digestion with proteinase K followed by homogenisation by bead beating of whole nematode larvae in a lysis buffer. Isolation of DNA is performed using a commercial spin column nucleic acid purification kit followed by ethanol precipitation and purification of DNA in solution.

Buffer *ATL+* was prepared by adding Reagent DX (Qiagen) at 0.5% v/v to Buffer ATL (Qiagen) and stored at room temperature (RT). The larval samples were removed from -80°C freezer and allowed to thaw at RT, then centrifuged in the microfuge at 5,000 x *g* for 3 min. Most of the supernatant was removed by pipetting, leaving ~20 µL and the pelleted material. To this, 250 µL Buffer *ATL+* and 20 µL Proteinase K was added and incubated at 56°C overnight with rocking. The digested larval solution (250 µl) was added to a 2 ml sterile vial containing 200 µL silica zirconia beads and 2-4 3mm glass beads for bead beating, using low binding pipette tips (Axygen). The samples were mechanically lysed using the Tissuelyser (Qiagen) for 140 s at maximum speed, twice. The vial was centrifuged in a microfuge at 1,000 x g for 1 min to pellet beads and large cell debris. The supernatant was removed and transferred to a new 1.5 ml eppendorf tube. Larval integrity was assessed under a microscope to confirm that the DNA was released and larvae were no longer intact. DNA was extracted using the DNAeasy Blood and Tissue Spin-Column Kit (Qiagen), using the protocol for *Purification of Total DNA from Animal Tissues*. An ethanol precipitation of the eluted DNA was performed and the pellet was resuspend in a minimum of 25 µL 10mM Tris HCl. DNA purity was assessed using a Nanodrop 1000 spectrophotometer; acceptable quality metrics were $A_{260nm/280nm}$ > 1.8 and $A_{260nm/230nm}$ > 1.6. The DNA was quantified for genotyping using a Qubit fluorimeter and Quant-iT™ PicoGreen™ dsDNA Assay Kit (Thermo Fisher Scientific).

3.4.4.3 Whole genome sequencing

WGS was conducted at The Ramaciotti Centre for Genomics, University of NSW. A Nextera DNA Flex library preparation was performed for each larval DNA sample to be sequenced. Short-read next generation sequencing was performed using the Illumina NovaSeq 6000 S1 2x150bp flowcell. Resulting fastq files (400-500Gb) were assessed for read quality using FastQC version 0.11.3.

A number of bioinformatic pipelines were trialled in order to align the sequence read to a reference *H.contortus* genome.

1. Quality filtering of raw fastq reads to remove poor quality base calls from the dataset and trimming was done with Trimmomatic (version 0.36) (Bolger et al., 2014), with a sliding window of 4 bp and quality threshold of 20 and minimum length set to 30. Trimmed sequences were mapped with Bowtie2 version 2.2.5 (tool for aligning sequencing reads to long reference sequences) with default parameters, using a New Zealand reference genome (Palevich et al., 2019).

2. CLC Genomics Workbench (Qiagen) whole genome alignment workflow, using pre-trimmed files with Trimmomatic, as described above.

3. Quality filtering and trimming was done with Trimmomatic with a sliding window 4 and minimum quality score of 15 and a minimum read length of 40. Following this, the bioinformatic pipeline from the read alignment to variant calling was undertaken, as described and validated for *C.elegans* by

Smith and Yun (2017). The recommended workflow for variant analysis involved is shown in Figure 3.4.2.

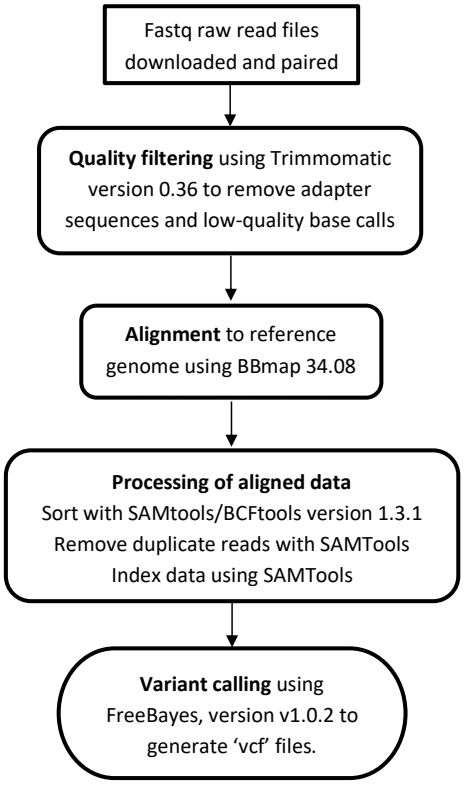

Figure 3.4.2: Recommended workflow for variant analysis, modified from Smith and Yun, 2017.

3.4.4.4 Reference genome selection

The reference genome used for genomic studies should ideally be genetically as close as possible to the field isolates. This ensures that any sequences in the field isolates that are not present in the genome are not overlooked or missed. Another consideration is the completeness of the genome data. Use of a high-quality reference genome improves the reliability of any mutations called in the field isolates and enables more options to be undertaken in the downstream analysis. Eukaryotic organisms do not have a pan-genome and all genes should be present in all organisms. Therefore, having a reference genome that is of the same species but from another country and potentially genetically unique, differing from the field isolates by a number of smaller mutations is acceptable.

For these reasons, we favoured a more complete *H.contortus* reference genome from Europe for this investigation, isolate ISEv4 (accession GCA_000469685.2), over a more geographically close reference sequence from New Zealand (accession GCA_007637855.2) or Australia (accession AUUS01).

4.4 Results – Sub-project 4

The Results for each study are aligned to the sub-project Objectives (Section 2.4). Where relevant, these refer to Appendices to the Final Report.

4.4.1 Improved understanding of the potential drivers of risk of S strain infections in cattle

A total of 43 properties across South Eastern Australia formed the initial dataset retrieved from Animal Health Australia as a part of the FNFAP program that met the inclusion criteria of an S strain MAP infection diagnosed in beef cattle. Of these, 23 properties were from Victoria, 9 from NSW, 8 from Tasmania, and 3 from South Australia. The geographic location of the properties (where data were available) was plotted on a choropleth map showing prevalence of OJD (2007-2013) (Cowled et al., 2016), showing that the majority of the properties were located in regions with the highest incidence of OJD (Fig. 4.4.1). It was evident that the beef cattle properties were mainly located in regions of moderate to high OJD prevalence.

Biosecurity and Animal Husbandry practices

Interaction with sheep: Information about sheep and associated management practices on the properties is summarised in Figure 4.4.2. Of the properties included in the study for which some herd history data was available (n=28/43), mixed enterprise farms comprised the majority (23/28), with 70% of these reporting co-grazing sheep and cattle and/or a history of OJD on-farm (Fig. 4.4.2A). The majority of the properties that reported co-grazing sheep and cattle and/or a history of OJD also reported that they were vaccinating their sheep with Gudair®, with only one property reporting that they did not vaccinate their sheep (Fig. 4.4.2B).

A more detailed analysis was possible for 16 properties in Victoria for which more comprehensive data were originally collected. Self-replacing and commercially run enterprises formed the majority of properties (Fig. 4.4.3). A key finding from the study was that a range of cattle breeds (Fig. 4.4.3).and ages (Fig. 4.4.4) were affected.

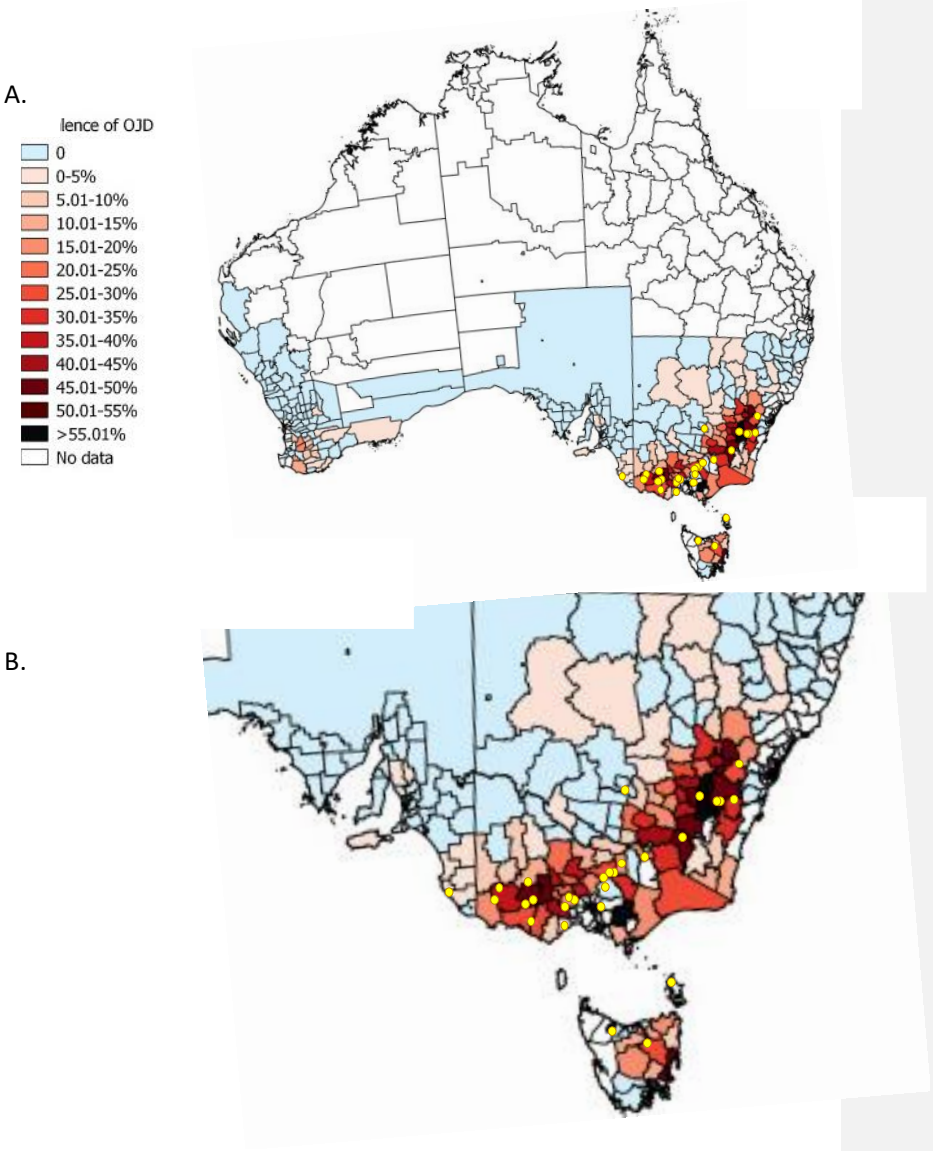

Fig. 4.4.1. Distribution of properties with an S strain *MAP* infection in beef cattle identified as a part of the FNFAP program conducted between 2004-2013: Choropleth map showing prevalence of OJD sourced from (Cowled et al., 2016) across Australia (expressed as the prevalence of OJD-test reactor positive properties per 100 properties surveyed, based on National Sheep Health Monitoring Project abattoir data from 2007–2013). Yellow dot markings show locations of properties. Some individual dots include more than one property, on occasions when these were located in the same postcode. A. Locations on map of Australia, demonstrating these are in the south-eastern corner. B. Map zoomed to show key region.

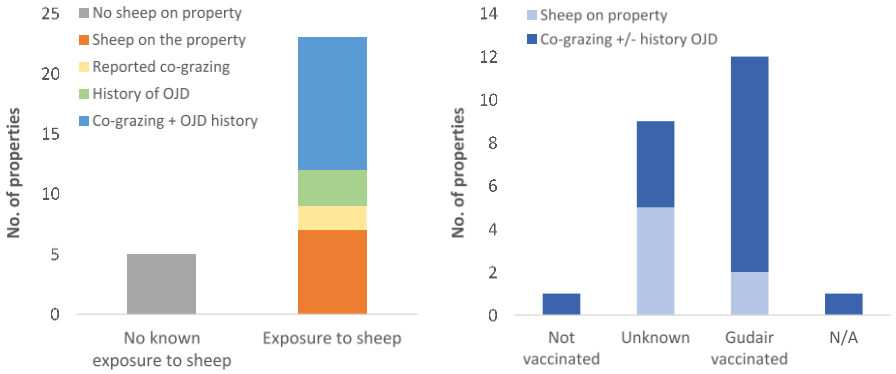

Fig. 4.4.2. Proportion of beef cattle properties with an S strain MAP diagnosis that had known exposure to sheep. Only properties with some herd history were included (n=28/43). A. Proportion of properties that reported a known exposure of their cattle to sheep versus no known exposure to sheep. Exposure history to sheep ranged from reporting having sheep on the property, a history of co-grazing with sheep, a history of OJD in their sheep or both co-grazing and OJD history. B. Proportion of properties with known exposure to sheep (total n=23) that were vaccinating with Gudair®. Gudair® vaccine use (where known) was sub-grouped by those producers that reported co-grazing and/or a history of OJD or simply reporting having sheep on the property. N/A: not applicable (this property previously had sheep on the property that were culled due to OJD, but the owner was no longer running sheep and hence not vaccinating).

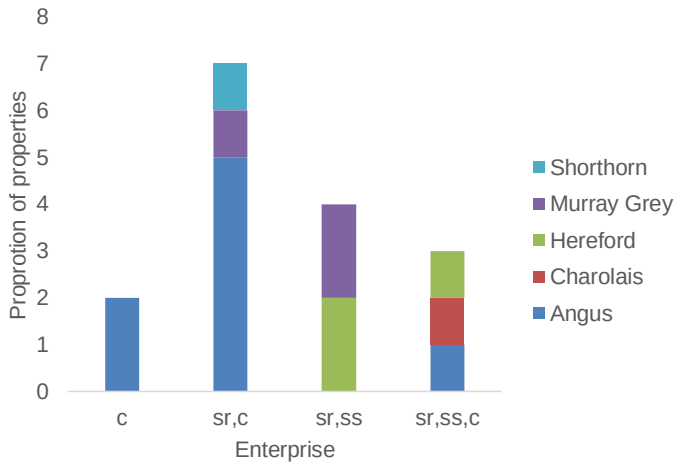

Fig. 4.4.3. Proportion of different enterprises (commercial, c; self-replacing, sr; seedstock, ss) run on properties with an S strain diagnosis in cattle, for which detailed herd history was available. The relationship between type of enterprise and breed of beef cattle (Shorthorn, Murray Grey, Hereford, Charolais or Angus) run on these properties is shown.

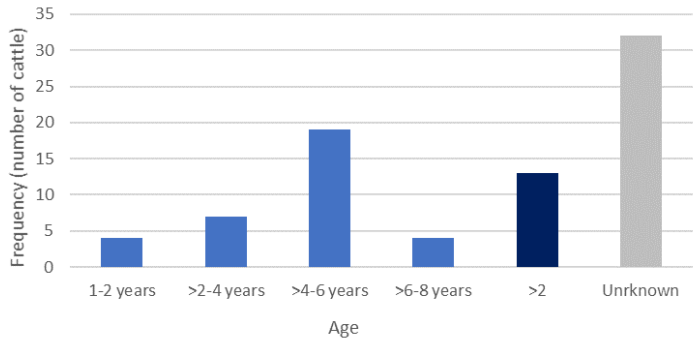

Fig 4.4.4. Represents data from 16 properties with detailed herd history and for which there were 78 individual cases diagnosed with S strain MAP infections. The age of the cattle at the time of diagnosis is shown.

Multiple clinical cases were identified of S strain MAP infection of beef cattle in animals aged from 3-8 years, with most presenting with weight loss and scours and some additionally having bottle jaw (Table 4.4.1). A range of cattle breeds were identified as clinical JD cases infected with S strain MAP.

Table 4.4.1. Cattle with clinical JD and confirmed to be infected with S strain MAP.

Herd ID *	Animal ID	Breed	Class	Age	Clinical signs	Serum Ab ELISA	Histo-pathology	Tissue culture	Faecal culture
A	1	Shorthorn	Cow	5	w, s	positive	positive	nt	nt
A	2	Shorthorn	Cow	5	w, s	positive	positive	nt	nt
A	3	Shorthorn	Cow	ns	w, s	nt	nt	nt	positive
A	6	Shorthorn	Cow	ns	w, s	nt	nt	nt	positive
A	28	Shorthorn	Steer	3	w, s	positive	positive	nt	nt
A	34	Angus	Bull	5	w,s	positive	positive	nt	positive
B	35	Angus	Cow	5	w,s	positive	positive	nt	nt
C	44	Angus	bull	5	w,s,bj	nt	positive	nt	nt
D	45	Hereford	bull	6	w,s	positive	nt	nt	nt
E	59	Angus	cow	8	w,s	nt	positive	nt	nt
F	60	Angus	cow	7	w,s	positive	positive	nt	nt
F	61	Angus	cow	7	w,s	positive	positive	nt	nt
G	62	Angus	cow	6	w,s	nt	positive	nt	nt
H	64	Murray Grey	cow	6	w,s,bj	positive	positive	nt	nt
I	65	Charolais	breeder	5	w,s	positive	positive	positive	positive
I	66	Charolais	cow	ns	w,s	nt	nt	nt	nt
I	67	Charolais	cow	ns	w,s	nt	nt	nt	nt
J	68	Angus	cow	5	w,s	positive	positive	positive	nt

ns=not stated, nt=not tested, Clinical signs: w=weight loss, s=scouring, bj=bottlejaw.
* Herds/properties were de-identified in order to maintain confidentiality.

The full Results for this study are reported in **Appendix A4.2.**

4.4.2 Results of the evaluation of diagnostic accuracy for different strains of MAP

The Results of studies examining culture-based and serological diagnostic test methods are provided below.

4.4.2.1 Results of the serological case study to assess ELISA detection of S strain MAP infection of cattle

Herd level diagnosis

The method of identification of JD infection on beef cattle properties subsequently identified as infected with S-strain MAP (see Section 4.4.1) is shown in Figure 4.4.5. At least 30% of these farms were identified by a positive serological test reactor. The means of identification of the farms as JD infected was not recorded for a further 30% of the farms in the dataset provided by Animal Health Australia; these were likely to have been identified within the Market Assurance Program or Test and Control Program (TCP2/3) using a serological test. The remainder of the properties had clinical cases or were trace forwards from properties already identified as infected.

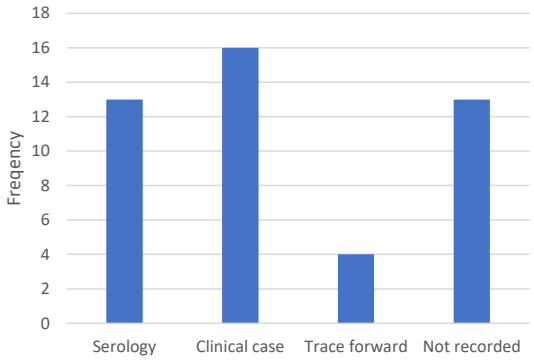

Fig. 4.4.5: Method of identification of JD infection on the beef cattle properties, subsequently identified as infected with S-strain MAP. Serological testing involved identification of seropositive animals in the Market Assurance Program maintenance test.

Individual cases

Individual animal diagnostic test information was available for a subset (16/43) of the cattle properties identified as infected with S strain. The diagnostic test information for individual animals on these properties is shown in Figure 4.4.6. Over 40 individual infected cattle from these properties were positive in serological tests.

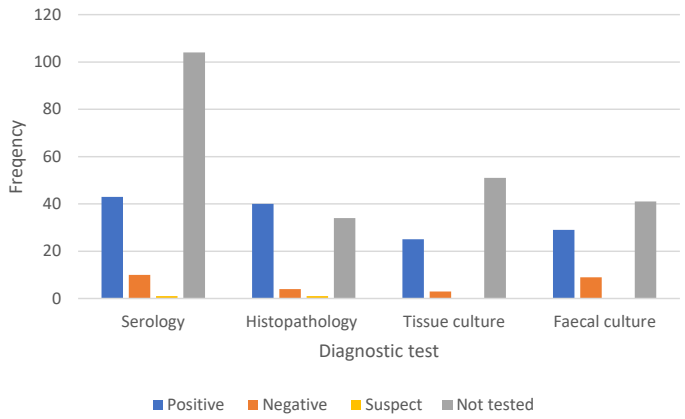

Fig. 4.4.6: Represents data from 16 properties in Victoria that had a combined 78 individual cases diagnosed with JD, for which case diagnosis information was known. The proportion of individual animals diagnosed with JD using a variety of diagnostic tests is shown.

In some cases, information was provided on clinical cattle cases with S strain MAP infections. These are shown in Table 4.4.1. All of the clinical cases that were tested with a serum antibody ELISA were positive (n=11).

4.4.2.2 MAP culture study, examining mixed cultures of S and C strain MAP

MAP species-specific gene target assays were initially tested in qPCR against a standard curve of either C strain MAP or S strain MAP, using two qPCR mastermixes; SensiMix SYBR low ROX qPCR (Bioline) and QuantiTect SYBR Green (Qiagen). There results for the four genes that were selected for further validation are shown in Figure 4.4.7.

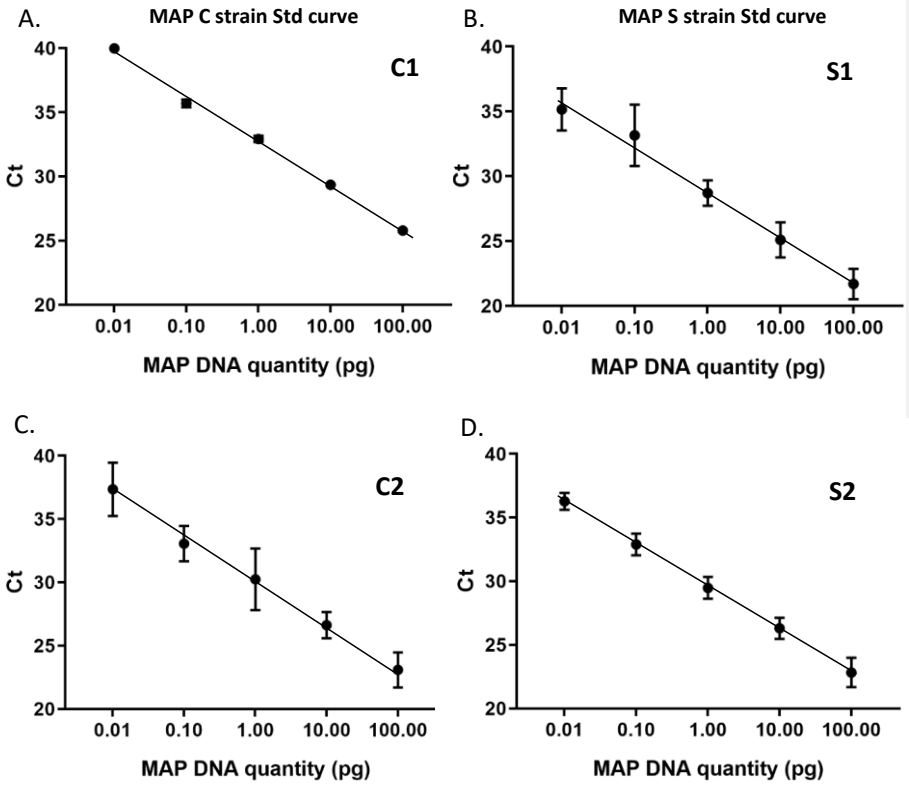

Fig. 4.4.7. Standard curves generated from MAP C strain (A and C) and MAP S strain (B and D), with qPCR assays targeting strain-specific gene C1 (A), S1 (B), C2 (C), and S2 (D). (Details of gene IDs are reported in Appendix A4.3).

Validation of the strain-specific gene qPCR assays was performed using a diverse range of MAP isolates from a previous study (Whittington et al., 2011). In brief, the 30 isolates originated from different geographic regions, such as Australia, Northern Ireland (United Kingdom), Spain and the United States, from a range of different hosts, such as, sheep, cattle, bison, oryx, gibbon, elk and humans. The isolates were all confirmed to be MAP by PCR and strain typed by IS1311 PCR REA, with whole genome sequencing performed as part of this project confirming the original strain typing result (Table 4.4.2). The results of the qPCR testing for these clinical isolates is shown in Figure 4.4.8.

Table 4.4.2. Source of MAP clinical sequenced isolates and IS*900* and IS*1311* molecular characterisation

Sample ID	Accession No.	Country	Isolate Source	IS*900*	IS*1311*	WGS
3435	VRS1065 (Vanderstock)	Australia	Sheep	MAP	S	Yes
3443	FD1104 (Sharwood)	Australia	Sheep	MAP	S	Yes
C3	CM00/416	Australia	Cow lymph node	MAP	C	Yes
3324	JD98/107-1 (17G ovi)	Spain	Sheep	MAP	S	Yes
3325	JD98/107-3 (938i ovi)	Spain	Sheep	MAP	C	Yes
3326	JD98/107-6 (22G ovi)	Spain	Sheep	MAP	S	Yes
3341	99/21-1	Northern Ireland	Sheep feces	MAP	C	Yes
3343	99/21-34	Northern Ireland	Sheep feces	MAP	C	Yes
3384	99/87-91 (14)	United States	Cow	MAP	C	Yes
3391	JD99/87-36 (30)	United States	Bison	MAP	B	Yes
3413	116/1 (Parfett)	Australia	Cow feces	MAP	S	Yes
3446	JD00/41-12	United States	Tule elk	MAP	C	Yes
3448	JD00/41 - 21	United States	Springbok	MAP	C	Yes
3449	JD00/41 - 24	United States	Tule elk	MAP	C	Yes
3450	JD00/41 - 25	United States	Oryx	MAP	C	Yes
3453	JD00/41 - 13	United States	Elk	MAP	C	Yes
3457	98/101	Australia	Namatode larvae	MAP	S	Yes
3460	CM00/426	Australia	Cow feces	MAP	C	Yes
4345	99/21-G5	Northern Ireland	Sheep milk	MAP	C	Yes
4350	JD00/41 - 1	United States	Gibbon	MAP	C	Yes
3318	ATCC 43015 Linda [CIP 103965]	United States	Human	MAP	C	Yes
3451	JD00/41 - 26	United States	Springbok	MAP	C	Yes
3360	99/87-87 (4)	United States	Bison	MAP	B	Yes
3428	99/233 (18I)	Spain	Sheep	MAP	S	Yes
3426	99/233 (19I)	Spain	Sheep	MAP	S	Yes
3447	JD00/41 - 14	United States	Elk	MAP	C	Yes

WGS: Whole genome sequenced. Some isolate information derived from Table 1 (Whittington et al., 2011).

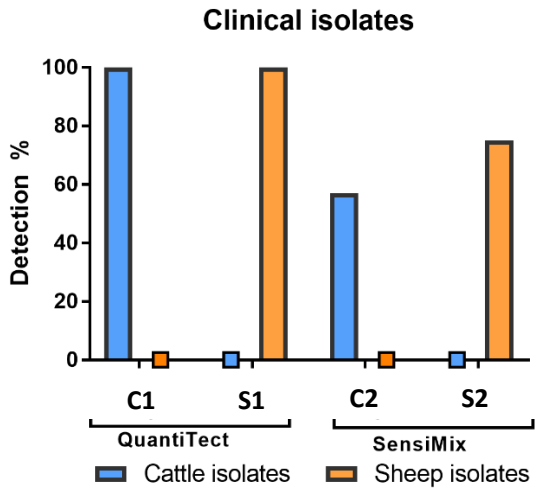

Fig. 4.4.8: Validation of MAP strain-specific genes on clinical isolates (n=30). Detection rate of the specific strain is shown on the y-axis.

From this validation, the gene targets C1 and S1 were considered optimal for the identification of the different MAP strain type. These strain-specific genes were validated bioinformatically using 400 whole genome sequenced MAP isolates and shown to hold true to strain type, representing two distinct clusters aligned with MAP cattle and sheep strains in a global phylogenetic tree.

The qPCR assays were used in a simulated mixed infection study, performed to determine if the new strain-specific qPCR assays could identify a potential mixed strain infection, where both the S and the C strain of MAP were infecting the same animal. It also examined the potential for differential growth of the MAP strains in culture media over time. The culture conditions are shown in Table 4.4.2 and the results are shown in Fig. 4.4.9.

Table 4.4.3. Mixed infection study culture conditions. Cattle strain (CM00/416) and sheep strain (Telford 9.2) MAP were inoculated at different ratios into M7H9C liquid culture media.

Culture No.	Cattle - CM00/416 (MAP)*	Sheep - Telford 9.2 (MAP)*
1	100%	0%
2	75%	25%
3	50%	50%
4	25%	75%
5	0%	100%
Negative Control	-	-
Positive control	0%	100%
Media control	-	-

* Total inoculum 1 x 10^2 MAP cells/culture

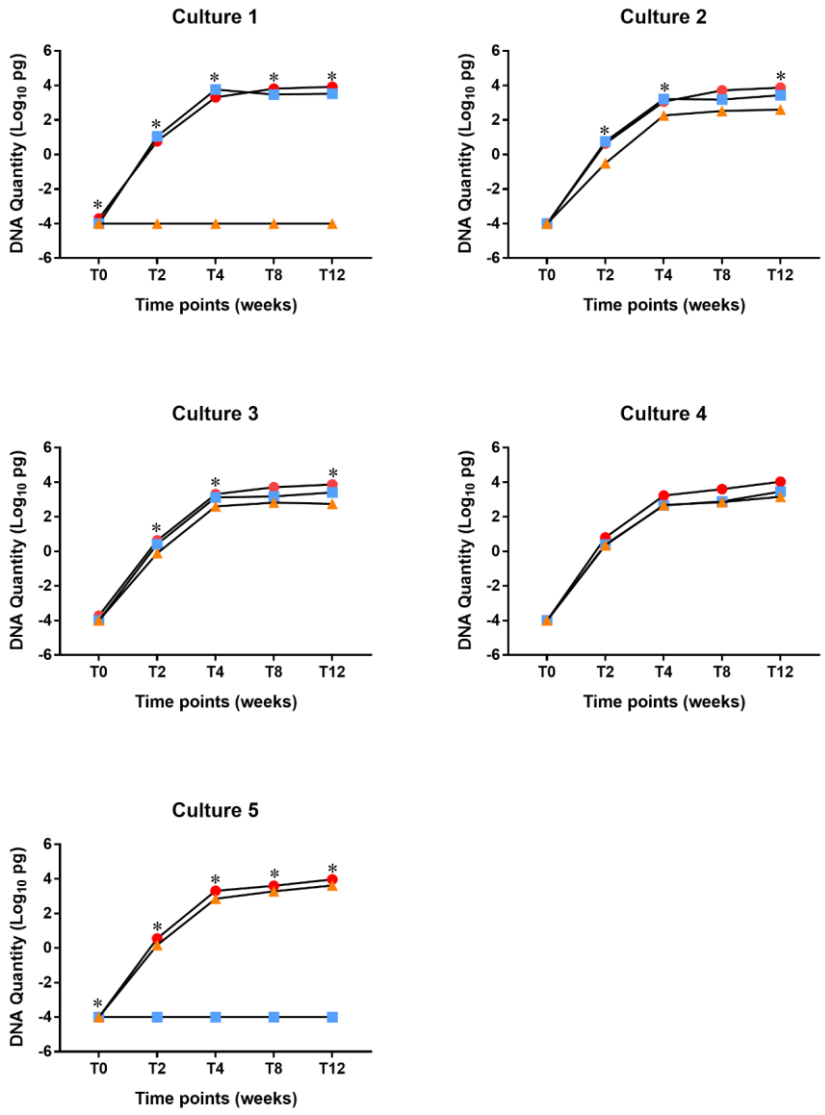

Fig.4.4.9. Simulated mixed infection cultures showing the detection of the growth of MAP C and S strain in liquid culture media over time. Standard IS*900* gene qPCR results are shown with the red circle, C strain-specific gene (C1) qPCR results are shown with the blue square, and S strain-specific gene (S1) qPCR results are shown with the orange triangle. Timepoints T0 to T12 refer to aliquots taken at 0 – 12 weeks of culture.
* Significant difference in the DNA quantity detected by the S-strain and C-strain gene qPCRs. See Table 4.4.3 for set-up conditions of each culture.

Overall, the results confirmed the specificity of the strain-specific qPCR assays (Fig. 4.4.9, culture 1 and 5). Further, it showed that both strains we able to grow together in the mixed cultures, although the C strain seemed to slightly out-grow the S strain in cultures where this comprised 50% or higher of the initial inoculum, based on DNA quantities detected over time. However, this had no impact on the ability to discern growth of both strains in these cultures. The different strains in these mixed cultures were unable to be identified using the routine IS*1311* PCR REA method (Fig. 4.4.10), with the three simulated mixed infection cultures (Lanes 2-4) having identical banding patterns as cultures that were 100% C-strain MAP.

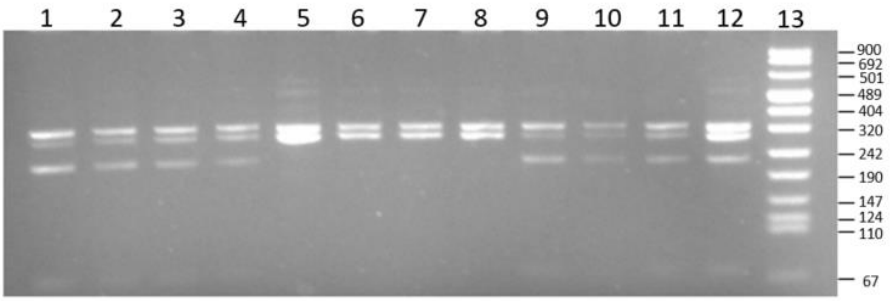

Fig. 4.4.10. IS*1311* PCR-restriction endonuclease analysis (REA) profiles for DNA extracted from culture. All lanes (1-13) are IS*1311* PCR products derived from DNA extracted from either pure MAP cultures or positive faecal culture samples, restricted with Mse I and Hinf I enzymes. Lanes 1 and 5 are IS*1311* PCR-REA products from pure MAP C-strain (Lane 1) or pure MAP S- strain (Lane 5) cultured in liquid culture media (M7H9C). Lanes 2-4 are IS*1311* REA products from simulated mixed infections of C- and S- MAP strains; Lane 2 was a culture comprising of 75% C- and 25% S-strain, Lane 3 was 50% C- and 50% S-strain, and Lane 4 was 25% C- and 75% S-strain, at the commencement of the culture. Lanes 6-8 are field samples (primary faecal cultures) from sheep, Lanes 9-12 are field isolates (primary faecal cultures) from cattle. Lane 13 is the MW marker.

Further experiments on mixed cultures of a range of field samples with MAP S and C strain present confirmed that these assays are able to detect the growth of MAP S and C strain in mixed cultures.

Full details of the strain-specific qPCR assays, validation data and results of the mixed infection studies are presented in Appendix A4.3.

4.4.3 Mycobacterial genomics Results

4.4.3.1 Evolutionary transitions in Rapid and Slow growing mycobacteria

An analysis of the genomes of 157 rapid and slow growing mycobacterial species using a core genome phylogenetic comparison showed that the rapid growing mycobacteria are the ancestral species, from which the slow growers (including pathogenic species *M.tuberculosis*, MAP, and *M.bovis*) evolved (Fig. 4.4.10). On the tree, *M.bovis* and *M.tuberculosis* can be seen within the cluster of closely-related *M.tuberculosis* complex species, whereas MAP (paratuberculosis) clusters with *M.avium*, *M.lepraemurium* and *M.bouchedurhonense*.

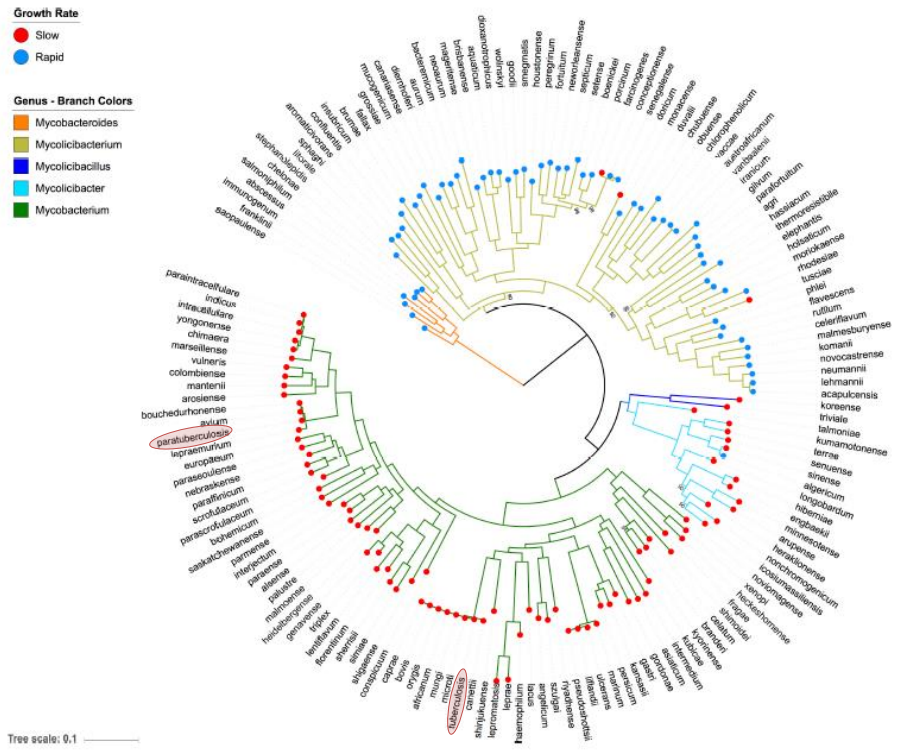

Fig. 4.4.11. Phylogenetic tree of all well-characterised *Mycobacterium* species. This Maximum likelihood tree of 157 well-characterized *Mycobacterium* species is based on nucleotide alignment of 304 single copy genes. It shows five distinct sub-genera and indicates that slow growers evolved from more ancestral fast-growing species. Bootstrap values are shown on nodes with less than 100% support. The location of *M.tuberculosis* and *M.avium* ssp. *paratuberculosis* on the tree has been highlighted within the Slow growers (Red).

There were 293 orthologous genes that were enriched in the rapid growers, and conversely 309 genes enriched in the slow growers. Functionally, genes related to amino acid transport and metabolism as well as gene transcription were highly enriched in the rapid growers. Genes associated with the ESX-5 Type VII secretion system, PPE family (containing Pro-Pro-Glu motifs and associated with virulence) and the mammalian cell entry (*mce*) operon were enriched in the slow growers.

The full results of this study have been published (Bachmann et al., 2019) and included in **Appendix A4.4.**

4.4.3.2 Whole genome sequencing of MAC isolates

An investigation was carried out that incorporated publicly available WGS data from Mycobacterium avium complex (MAC) genomes, as well as isolates from the University of Sydney collection and human patient-derived isolates, to investigate genetic relationships between species within the MAC.

Whole genome phylogeny revealed tight clustering of certain subspecies; MAP formed its own distinct cluster (Green arrow, Fig. 4.4.12) as did the two avian groups, *M. avium* ssp. *avium* and *M.avium* ssp. *silvaticum* (Blue arrow, Figure. 4.4.12). In addition, the core and accessory genomes were compared between the subspecies, as well as lists of subspecies-specific genes (genes that were present in one subspecies and not the other two were compared to each other).

The full Results of this study are reported in **Appendix A4.6** "The *Mycobacterium avium* complex"

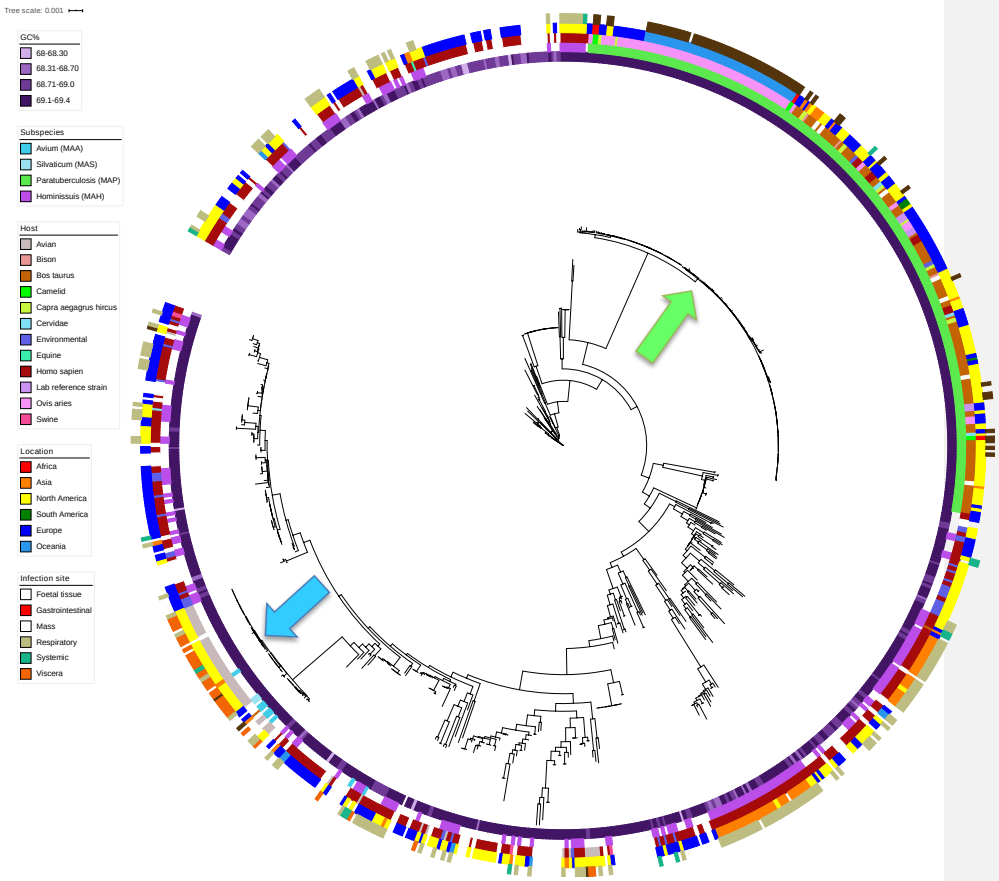

Fig. 4.4.12. Whole genome phylogenetic tree and associated metadata of 773 isolates. Note the tight clustering of subspecies paratuberculosis (green arrow) and subspecies avium (blue arrow) isolates. Innermost circle is GC content, second circle is reported subspecies, third circle is reported host species, fourth is location and the outermost circle represents their site of isolation.

4.4.3.3 Whole genome sequencing of MAP to study epidemiology

Three studies were performed to investigate the genetics of MAP strain in Australia.

Study 1 investigated isolates derived from archived samples from a 10-year vaccination longitudinal study of closed, OJD-endemic Merino flocks (MLA project P.PSH.0565; Dhand et al., 2016). This study aimed to compare genomic changes in MAP that may have occurred during this period. The hypothesis for the research was that the sub-optimal performance of the Gudair® vaccine, which does not completely arrest infection, may enable vaccine-selective persistence of more virulent S strain MAP genotypes in vaccinating flocks.

Among the study isolates there appeared to be clustering of isolates within locations and based on initial farm JD prevalence when the WGS data was mapped to the Telford S strain MAP reference (Fig. 4.4.13). For certain farms, grouping of the persistent isolates separately from the initial isolates is seen. For example, for farm M4, the two initial isolates (110b, 110c) are on one branch and the two persistent isolates (126a, 126b) are on a connected but separate branch. A similar pattern is seen with Farm L1 (isolates 111a,b,c and 127a,b,c). There is also an interesting grouping of just persistent isolates M1, L4 and L4. This suggests that there is a genetic relatedness amongst the persistent isolates, which is being explored in further detail.

The full Results of Study 1 are reported in **Appendix A4.6** "Vaccine driven selection of *Mycobacterium avium* subspecies *paratuberculosis*".

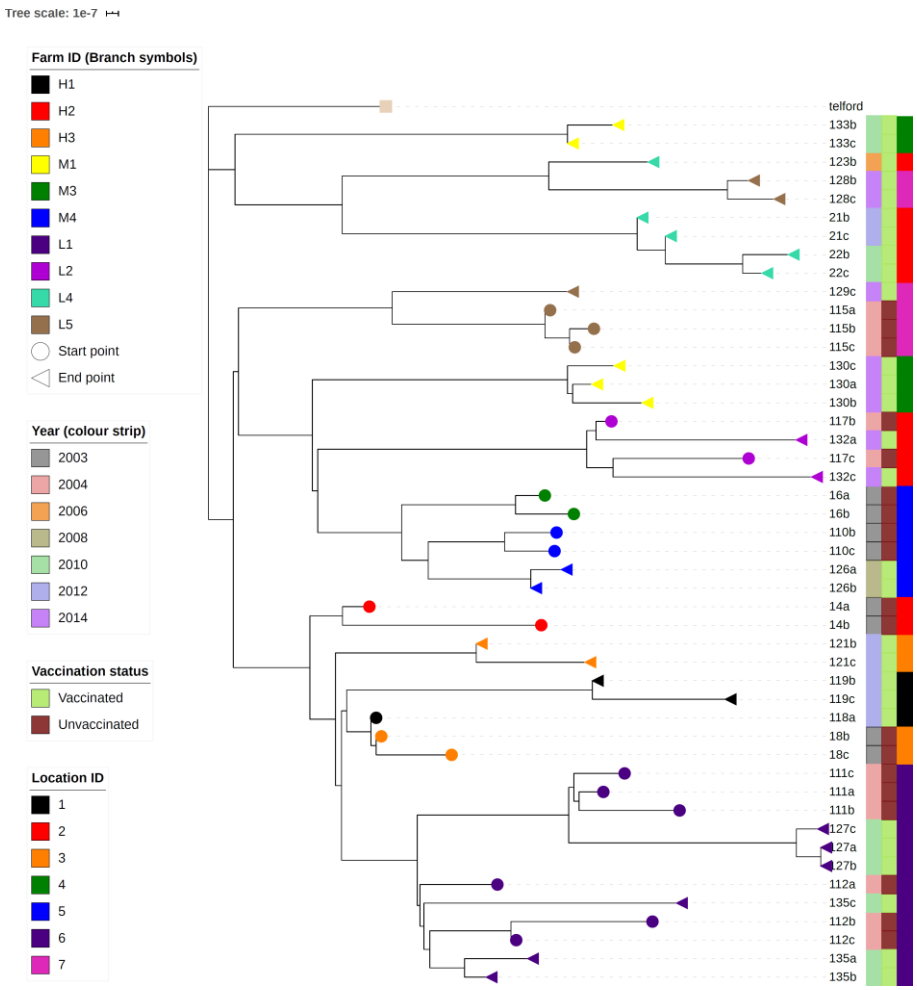

Fig. 4.4.13. Phylogenetic tree of sheep MAP isolates from the decade vaccine trial. Branch symbol colour indicates property ID and shape indicates whether the sample is from the start of the trial before vaccination (circle) commenced or the end of the trial (triangle) where vaccination had been practiced for several years. The first colour strip is the year which the isolates were collected, the second colour strip is the vaccination status of the property at the time of collection. The final strip is the location ID for each property, note that some properties are from the same region, thus they have the same location ID. Of the 12 properties from the original trial, isolates were only grown for 10 due to a lack of MAP positive samples for the full trial. Where possible, cultures were grown from two faecal pools for each farm at each time point and three colonies were sequenced from each culture.

Study 2 investigated the relationship between Australian and International MAP strains. This study involved WGS of cattle and sheep MAP strains from Australia and phylogenetic comparison with available sequenced strains globally. A global phylogenetic tree was generated from over 400 MAP genomes (Fig. 4.4.14). In this phylogenetic tree, two clear clusters could be identified; one contains only isolates which were either sheep MAP typed by IS*1311* PCR REA, had been PCR positive for the S strain MAP strain-type specific gene from Section 4.4.4.2, or were identified by the BLAST analysis of the S strain MAP strain-type specific gene. The other large clade contained genomes for cattle or bison MAP IS*1311* PCR REA typed isolates, or those which were positive for the C strain MAP strain-type specific gene (Section 4.4.4.2) via PCR or BLAST.

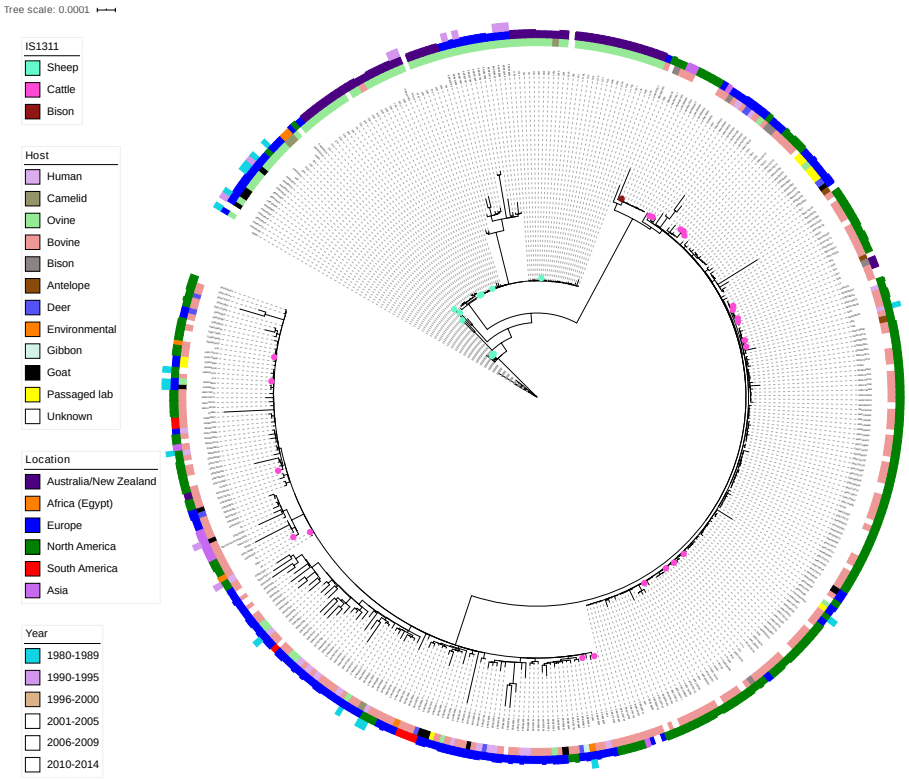

Fig. 4.4.14. Global MAP phylogenetic tree. This includes whole genome sequenced isolates from around the world aligned with the sequenced Australian MAP genomes from our study. The dots on the tree diagram indicate IS*1311* PCR REA typing results, while colours in the rings around the outside show host species that MAP was isolated from (inner ring), global location (middle ring) and year of isolation if known (this information is often not reported). Prior to tree generation, most ambiguous results were found to be either have poor sequence quality (>700 contigs and N50 >10,000) and could not be included in the core genome alignment to produce the tree, or were not MAP (n=4). For ease of readability, branch lengths were ignored.

The full Results of Study 2 are reported in **Appendix A4.6** "Phylogenetic relationship between Australian and International MAP strains".

Study 3 investigated the comparative genomics of *Mycobacterium avium* subspecies *paratuberculosis* Sheep strains. A clear distinction between the Type I and Type III (see Fig. 1.4.2) isolates was evident when the K10 reference was used as a tree root (Fig. 4.4.15). The Type I isolates were of Australian, New Zealand and European origin, with a distinct and very closely related Australia and New Zealand clade present. The Type III isolates appeared more diverse, both genetically as indicated by their branch lengths in the phylogenetic tree, and geographically.

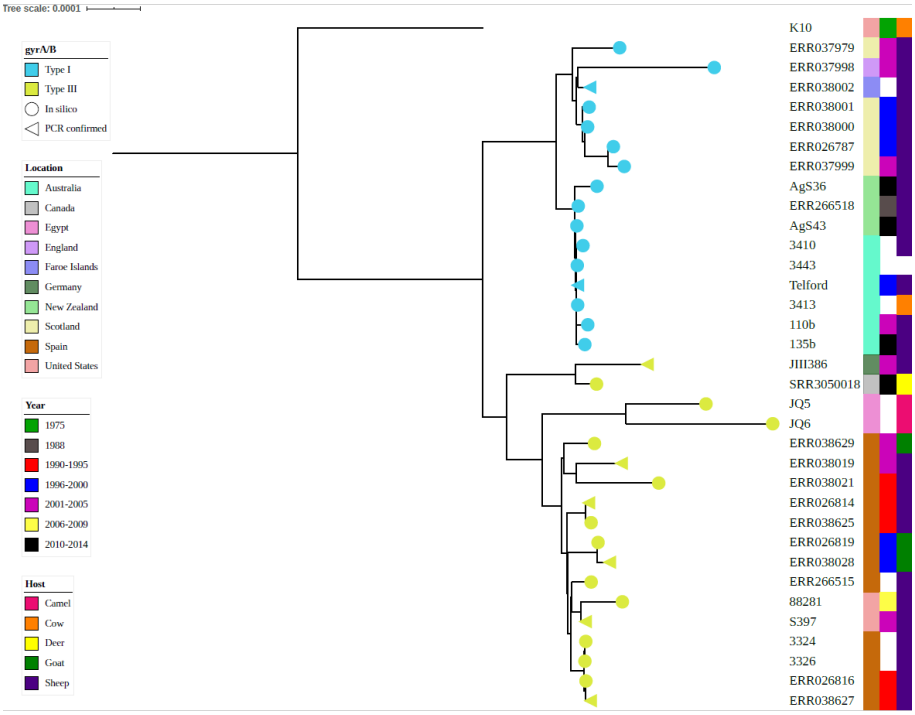

Fig. 4.4.15. Phylogenetic tree of the final dataset of 34 sheep MAP isolates. The cattle K10 reference genome used in the tree as a root was excluded from all further analyses. Test confirmed types (n=8) are annotated with a triangle, in silico results are represented by a circle, yellow is type III and blue is the type I. The colour strips from left to right are location, year of isolation and host species, respectively. White blocks indicate missing data.

A number of lineage-specific genes were identified between Type I and Type III S strains, as shown in Table 4.4.4. These were genes that were present in one lineage and absent from the other or had consistent lineage-specific variations. BLAST analysis demonstrated that the lineage-specific genes were present in all isolates but contained consistent SNP variations and this had resulted in different annotations for these genes.

Table 4.4.4. Lineage-specific genes and variation of type I and type III isolates.

Gene	Type	Annotation	Variations
Group 4585	I	Putative nuclear transport factor 2 family protein	5 variable mismatches in the Type III protein
Group 4593	I	MMPL family transporter	No significant protein hit in Type III isolates
*cinA*1	I	1,8-cineole 2-endo-monooxygenase	Type III protein has 8-17 mismatches, length is identical between lineages
*mhpA*2	I	3-(3-hydroxy-phenyl)propionate/3-hydroxycinnamic acid hydroxylase	Type I isolate protein sequences are 62 amino acids longer and Type III isolates have a single mismatch
Group 4493	I	Hemolysin III family protein	Type III protein is 54 amino acids longer and contains 9 mismatches to the Type I version
Group 4363	III		
Group 4592	I	MMPL family protein	No significant protein hit in Type III isolates
Group 1815	I	Hypothetical protein	1-2 mismatches in Type III protein and Type III is 22 amino acids shorter
Group 4617	I	*TetR/AcrR* family transcriptional regulator	11 amino acid mismatches and the Type I protein is three amino acids shorter
Group 4778	III		
Group 4500	I	Nitroreducatase family protein	Type I protein is 185 amino acids long and Type III is 171. Contains 11 mismatches
Group 4772	III		
Group 4781	III	Hypothetical protein	Type I isolates 37-164 amino acids long with 24-105 mismatches. Type III are all 299 amino acids long with a single mismatch present in four isolates

Where Prokka annotated the gene as a hypothetical protein but BLASTx was able to provide a putative annotation, the BLASTx annotation was used. Variations are from BLASTp results. *bp = base pairs.

The full Results of Study 3 are published in (Mizzi et al., 2021) and included as Appendix A4.7.

4.4.4 *H.contortus* whole genome sequencing Results

4.4.4.1 Larval sample details and regional location of submissions

Larval cultures were sourced from faecal submissions sent to EMAI for larval culture and genera identification (Table 3.4.1). The samples selected for WGS were those with the highest proportion of *H.contortus* that represented the widest geographic distribution across NSW (Fig. 4.4.16). Additionally, the L3 larvae used to inoculate sheep in Trial SP1 were included for WGS.

The proportional composition of *H.contortus* larvae in these samples was 87%, with the major alternate genus being *Trichostrongylus*.

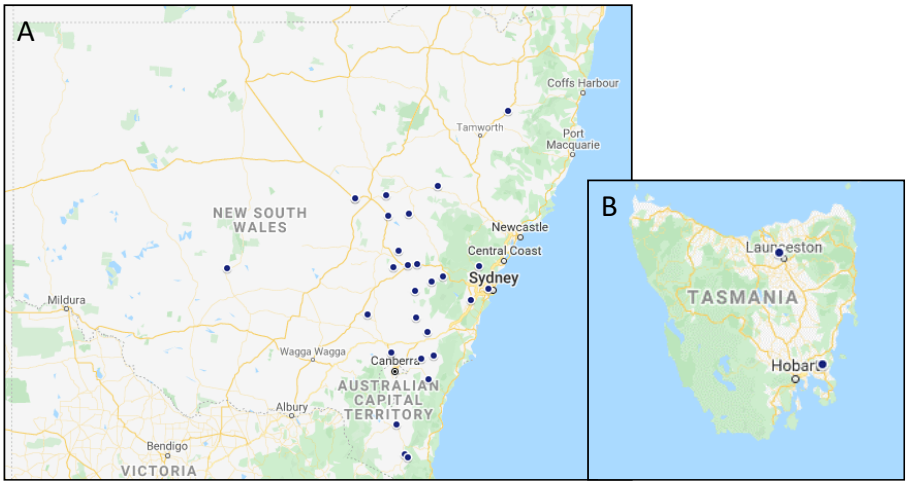

Fig. 4.4.16. Regional location of the source of the samples for larval culture are shown by a blue dot.

4.4.4.2 Descriptive Statistics of the raw data

Forty one *H.contortus* larval isolates were sequenced. The sequencing data generated from each larval isolate was highly variable and initially the raw data contained a high proportion of low-quality reads. Trimming was undertaken to remove low-quality data. Post-trim, the average number of reads per isolate was 51,868,048 and average GC% of 45.5% (Table 4.4.5), with all reads between 40 and 151 base pairs. This GC content was consistent with known statistics from *H.contortus* reference genomes, which have 43.1% GC. Variation from this reference GC% is likely due to contamination with DNA from other larval species in the samples or bacterial contamination.

Table 4.4.5. Total number of reads and GC% of the trimmed, paired fastq files.

Keylist	Total number of reads	GC%	Host Species	Haemonchus %
SP1	59611333	46	-	100
Tas01	46831985	43	Ovine	100
Tas02	49376914	46	Ovine	100
11	57398644	47	Ovine	100
47	60280974	43	Ovine	100
4	51530970	50	Ovine	99
58	39723089	44	Ovine	99
45	48853130	44	Ovine	98
46	47159755	44	Ovine	96
56	43739081	44	Ovine	96
59	53077543	45	Ovine	96
43	48068774	44	Caprine	95
49	54041182	44	Ovine	95
33	39889015	44	Ovine	94
66	57848438	45	Ovine	94
54	49594976	44	Ovine	93
9	61838542	55	Ovine	92
24	52216950	45	Ovine	92
37	50909646	45	Ovine	89
41	58065835	45	Ovine	89
38	44044577	44	Ovine	87
67	50817023	44	Ovine	87
70	53401778	46	Ovine	86
14	62619168	59	Ovine	84
55	48601732	45	Ovine	84
48	47126886	43	Ovine	83
29	51098528	43	Ovine	82
34	49820776	45	Ovine	81
61	50799995	46	Caprine	76
31	55265405	43	Ovine	75
35	54353307	44	Ovine	75
28	49288709	47	Ovine	74
68	50241486	44	Ovine	74
32	56519626	46	Caprine	73
39	42018318	45	Ovine	71
16	60011329	49	Ovine	70
63	62863916	46	Ovine	67
26	57141569	44	Ovine	64
65	52127676	44	Ovine	60
20	46503350	45	Ovine	59

4.4.4.3 Alignment to the *H.contortus* reference genome

Bioinformatic Pathway 1

This method is recommended for long reference sequences such as the *H.contortus* genome. The success of the alignment was not able to be determined, hence an alternative approach was attempted.

Bioinformatic pathway 2

Using the CLC genomics workbench analytical method, sequence reads were able to be successfully mapped to the reference genome. This was able to be visualised in CLC Genomics, as shown in Figure 4.4.17. However, variant calling (SNPs, indels) within the program was not successful. Thus, an alternative approach was sought for analysing the degree of genetic variability of the larval samples to the reference genome.

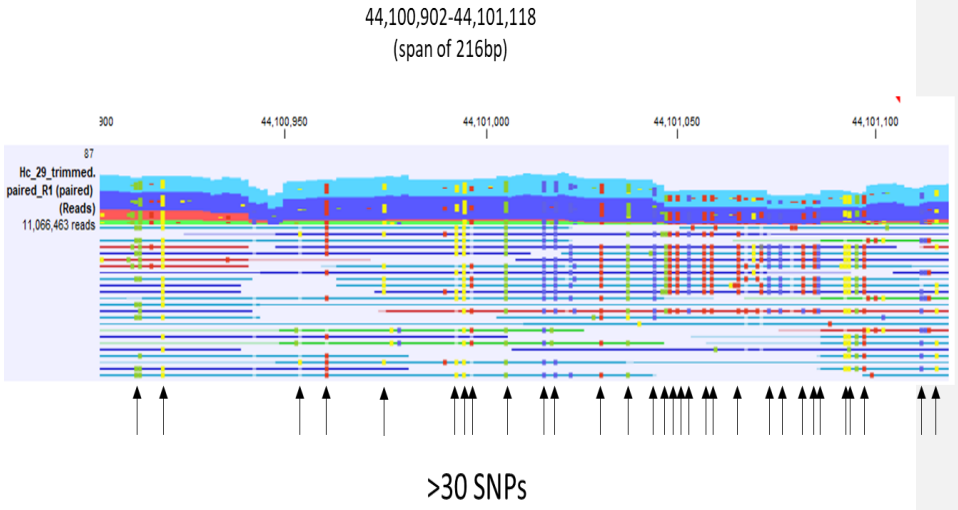

Fig. 4.4.17. Example of the alignment of *H.contortus* reads from sample keylist number 29 to the reference genome. A short section (216bp) of the aligned sequence is shown. The location of the SNPs is indicated by arrows.

Bioinformatic Pathway 3

Alignment of reads to the reference genome sequence ISEv4 (accession GCA_000469685.2) was achieved. This reference genome was chosen since it was the most completely assembled and well annotated *H. contortus* genome available at the time. This analysis pipeline was successful in generating variant call format (vcf) files. These files were very large, due to the high degree of variation between the WGS isolates and the reference genome and were unable to opened locally. Although the analysis of this large volume of data was problematic, this did not affect the overall outcome of the project.

5.4 Discussion – Sub-project 4

5.4.1 Improved understanding of the potential drivers of risk of S strain infections in cattle

It is evident that a number of factors may be linked to the spread and transmission of S strain MAP infections in beef cattle. Attention to vaccination programs and grazing management practices is critical.

Qualitative risk factors for S strain MAP diagnoses in cattle from this study and practical implications of this for the industry are provided below.

Beef cattle properties located in regions with a high prevalence of OJD.

When cases of S strain MAP infection in cattle were mapped, it was apparent that the majority of the affected properties were confined to the South Eastern parts of Australia. This is unsurprising considering the favourable temperate climate, which results in high intensity grazing of livestock compared to northern production systems where cattle and sheep are grazed extensively at low stocking densities (Greenwood et al., 2018). Mapping of the Victorian properties identified that most were located in the South West region of the state. Overlaying these postcodes with the previous zoning map of OJD, shows many properties were located in medium to high OJD prevalence zones (Cowled et al., 2016). Properties in NSW were located in the Wheat-sheep zone where OJD prevalence is high and the two Tasmanian properties were located in low prevalence OJD zones. This is indicative of the mixed enterprise farming that is common in South Eastern parts of Australia, where sheep and cattle are reared under high stocking densities.

The risk factor analysis did not identify any particular breed of cattle, which is consistent with previous studies that show S strain MAP can establish in any apparently healthy beef breed if proper management practices are not enforced to control spread (Larsen et al., 2012).

Mixed enterprise properties and co-grazing.

Co-grazing with sheep that had a history of OJD, even if the sheep were Gudair® vaccinated, was commonly reported in properties that had been diagnosed with S strain MAP infections in cattle. There is previously reported evidence from Australia and Iceland that co-grazing of OJD infected sheep with cattle is a common factor facilitating transmission (Sergeant, 2015, Whittington and Sergeant, 2001a). Results from an epidemiological study in New Zealand suggested that the high similarity of subtypes (percentage similarity index (PSI) = 0.82) was related to close grazing between species, facilitating direct transmission (Verdugo et al., 2014).

One example highlights the potential risks of co-grazed cattle being exposed and infected with S strain MAP. This was a property that had recently implemented a Gudair® vaccination program (within 3 years). It was surmised that the transmission was possibly due to being exposed to an older cohort of unvaccinated sheep. It was also reported from this farm that three neighbouring properties diagnosed with OJD had drainage that flowed onto the property. Contaminated run-off potentially further increased the transmission risk for both sheep and cattle through high infectious doses of MAP.

Vaccinated sheep and co-grazing

A high proportion of the properties diagnosed with S strain infections in cattle, that had reported co-grazing with sheep and/or a prior history of OJD on their farms, had instituted a vaccination program for their sheep flocks. One property reported a history of OJD infection in their Merino flock, with evidence of chronic OJD determined from multiple deaths, despite vaccinating lambs since 2003. Thus, the likely transmission route was from vaccinates. From the evidence provided from this property and others in the retrospective study, it appears that vaccinated sheep pose a transmission risk to cattle This is a possibility that may or may not be recognised by producers, and with more stringent MAP reporting introduced for both cattle and sheep, was a plausible reason for the reluctance of many mixed-enterprise producers to participate in our original survey.

It is known that Gudair® vaccination does not completely prevent MAP faecal shedding (Reddacliff et al., 2006). Continued shedding despite vaccination with Gudair® has been reported in previous sheep studies, indicating that vaccination reduced shedding and mortality rates by 90% but did not stop infection, meaning faecal contamination of pastures is still likely if an animal has a high infectious dose (Windsor et al., 2014). We have seen in our experimental infection model that a small proportion (5-10%) of Gudair® vaccinated sheep still become infected; some of these infected sheep developed clinical JD and are highly infectious super-shedders (Pooley et al., 2019). Continued MAP shedding by Gudair® vaccinated sheep is further evidenced by the PFC results from Sub-project 5 of this research program.

In the pathogen genomics aspect of this sub-project, we investigated the potential for vaccine-driven persistence of MAP in sheep and the potential that this may be associated with increased virulence, which could relate to the ability of S strains of MAP derived from vaccinated sheep to infect cattle.

Age-susceptibility and co-grazing

One property reported historic grazing of heifer weaners in paddocks where known infected sheep grazed. Subsequent positive detection of S strain MAP infection in breeders >2 years of age indicates that first exposure occurred as calves or weaners. This observation makes transmission through direct or indirect contact with sheep more probable if the cattle are first exposed <2 years of age, as these animals are more susceptible to infection than individuals exposed >2 years age (McGregor et al., 2012, Windsor and Whittington, 2010). Previous reports of historical S strain MAP infection in cattle in Australia and Iceland found that, in each individual case, there had been direct or indirect contact of calves with OJD infected sheep (Whittington et al., 2001).

Bringing in breeding animals from properties located in regions with a high prevalence of OJD.

There was limited evidence from trace-back investigations that MAP S strain infections may not only be transferred between species (from sheep to cattle) but may also occur between cattle.

The properties investigated in the study ran a variety of operations: self-replacing, commercial and/or seedstock. Until recently it was not recognised that the type of operation could play a potential role in S strain MAP transmission in cattle. In this study, at least three properties were found to have bought S strain MAP infected cattle that were traced back to properties either known to have an infected status or located in previously recognised high OJD prevalence regions.

Seedstock properties make up approximately 30% of the industry (ARCBA, 2017), and despite this relatively low proportion, the system of selling breeding cattle to commercial herds could increase

the likelihood of S strain MAP being introduced into an unexposed herd if this can spread from cow to cow. For example, in this study one property that operated a self-replacing commercial herd was identified, that purchased a 2-year-old bull in 2012 that presented clinical signs of JD three years later. Trace-back investigations identified the bull originally came from a property in the South West region of Victoria. Weaner and yearling heifer replacements could have been exposed to the bull and were subsequently vaccinated. Another property also traced its index case back to the same postcode, though it was not clear if this case was from the same property. Other cases were also identified on this property. This could indicate the potential for S strain MAP to spread between cattle. Currently, there is limited evidence in the literature to confirm this possibility. A previous study could not identify whether S strain MAP could establish endemic infections in cattle (Whittington and Sergeant, 2001a).

Under-reporting/lack of testing.

The prevalence level of bovine JD in beef cattle in 2012 was considered to be low, with a prevalence in Victoria of 0.2% and an overall Australian prevalence of 0.05% (Sergeant, 2015). A previous study estimated the prevalence of S strain MAP infection in beef cattle exposed to high OJD prevalence zones was 0.08% (upper limit) in NSW, with the emphasis that transmission risk of S strain MAP infections for beef cattle is also low (Moloney and Whittington, 2008, Whittington and Sergeant, 2001a).

All of the properties with a MAP S strain diagnosis in cattle were practising some version of disease management related to JD control. Half of the properties participated in CattleMAP to determine their disease status and to achieve a high level of assurance (MN3) to limit trading impacts. Only three properties (19%), had a MAP assurance of MN3, with one property undergoing MAP testing for 6 years prior to the first diagnosis of S strain MAP in cattle. It was reported that this property had a history of OJD infection in sheep, with heifer weaners run in paddocks where infected sheep had previously grazed. Though the sheep on this property had been vaccinated with Gudair®, the time frame of the vaccine program was unreported.

There may be additional disincentives to testing for JD in the beef cattle industry under the changes to the BJD plan nationally in relation to trade and MAP strain type. This was encountered when originally planning for the risk analysis survey. This, coupled with sporadic evidence of S strain MAP infection in beef cattle, suggests that there may be under reporting of cases, which has hampered progress into the identification of potential factors that facilitate transmission of S strain MAP infection in cattle.

MAP control and Integrated Parasite Management (IPM)

In IPM programs for reducing pasture levels of infective larvae (L3) for worm control, one strategy in the toolkit of grazing management for mixed enterprises is to graze “wormy pasture” with adult cattle. In MAP endemic regions OR even with Gudair® vaccination of sheep, this strategy should be practiced with caution to prevent transmission of S strain MAP.

Success in achieving the project objectives

Objective: Compile information on cattle farms diagnosed with MAP sheep (S) strain and undertake an epidemiological risk factor survey of farms with MAP infection to help understand the drivers for S strain in cattle.

A clear limitation of this project was the study design, in that the initial survey could not be undertaken as planned. The study design was altered to report on archival data from the FNFAP where investigations contained information on unreported S strain MAP in beef cattle. In this way, the project objectives to help understand the drivers for S strain in cattle were able to be met successfully.

It is not clear from this study how virulent S strain MAP from vaccinated sheep is for cattle and whether this can become established within the species, with broader implications. Therefore, wider research into the prevalence of S strain MAP in Australia should be undertaken.

5.4.2 Evaluate variance in diagnostic accuracy for animals infected with different strains of MAP

The retrospective study confirmed that current serological tests for paratuberculosis can diagnose BJD in cattle, including clinical cases, even if this was caused by the S strain of MAP,. The diagnostic sensitivity is unknown due to low numbers of reported cases and limited case history information.

A strain-specific qPCR assay was developed as part of the study to investigate detection of varying MAP strains in culture. Quantitative PCR assays were developed for 10 species-specific genes and the best performing genes were validated across a panel of known MAP strains; whole genome sequencing was conducted on isolates from a range of hosts, and field samples from OJD endemic properties in Australia. Mixed infections were simulated and the species-specific qPCR assays were used to 'diagnose' mixed infection. The gene selection was based on the early publications of WGS analysis of C-strain and S-strain MAP (Li et al., 2005; Bannantine et al., 2012). Based on a panel of 400 global MAP isolates, these genes were 100% specific to the respective strain type of MAP. The strain-specific gene assays were validated. It was demonstrated that the growth of both C- and S-strains of MAP are supported within mixed cultures in liquid culture media. These simulated mixed infections were not able to be discriminated by the conventional IS1311 PCR-REA strain typing technique. However, the new strain-specific gene assays were able to distinguish the two strains in culture, including simulated mixed infections.

The techniques used for the IS*1311* PCR-REA are outdated, with most veterinary diagnostic laboratories shifting to qPCR-base diagnostics. Therefore, the strain-specific gene qPCR assays allow for rapid strain typing of MAP C- and S- strains using modern technologies, without the need for additional incubation and subculturing. Further, unlike the IS*1311* PCR-REA which is based on a single nucleotide change in the IS*1311* gene (Marsh et al., 1999), the new qPCR assays are based on an entire gene that is either present or absent in the respective MAP strain.

With the increased detection of cross-species transmission of MAP in Australia, it would not be surprising if mixed infections were common, yet not reported due to the lack of appropriate strain typing tools available. Our study demonstrated that mixed infections, if they were to occur, could be identified in a culture using M7H9C liquid culture media, as this supported the growth of both strains

in one culture. The strain-specific genes identified in this study were able to detect MAP C- and S-strains in mixed infection cultures as early as four weeks, providing opportunities for farmers to strain type their herd or flock and implement appropriate management strategies for control of JD.

Success in achieving the project objectives

Objectives: Evaluate variance in diagnostic accuracy for animals infected with different strains of MAP at the level of i) detecting host immune responses and ii) pathogen detection (eg. is C strain "out-growing" S strain in culture and thus masking the true level that co-infection actually occurs?).

This aspect of the sub-project has achieved the objectives. It has been shown that a host immune assay (serum antibody ELISA) can detect infection with different MAP strains. In relation to part (ii) pathogen detection, it was shown that C strain MAP does not "out-grow" S strain MAP in culture and that the growth of both strains of MAP is supported in M7H9C liquid culture media and hence detectable in the case of a mixed infection.

The second part of this objective was achieved with the validation of a new diagnostic tool that involved species-specific genes and differentiated between the C and S strains of MAP, including in a mixed infection. This rapid, qPCR-based strain typing diagnostic assay has broader practical applications. There is an opportunity to develop a multiplex qPCR assay with these strain-specific genes to diagnose and strain type MAP simultaneously. An Invention Disclosure has been submitted to the University of Sydney following joint discussions with MLA, to investigate the commercial feasibility and patentability of this finding. The availability of this method may encourage producers to strain type their herd or flock following a MAP diagnosis.

5.4.3 Mycobacterial genomics

5.4.3.1 Whole genome sequencing (WGS) of divergent mycobacterial species from an evolutionary perspective

A detailed mycobacterial phylogenetic analysis showed an evolutionary split between rapid and slow growing species of mycobacteria, with rapid growing species being the ancestral species (reported in (Bachmann et al., 2019)). A range of key genomic differences were identified that elucidate the evolutionary path to become virulent species. One such region was the mammalian cell entry (mce1) operon, which is involved in a key virulence mechanism of host macrophage invasion (Hemati et al., 2019, Zhang and Xie, 2011). However, homologs of these genes are also found in rapid growing species such as *M. smegmatis* (Kumar et al., 2005). This study showed that the mce1 operon from rapid and slow growing species grouped into two ortholog clusters, suggesting that these genes may have had a different function in the ancestral rapid growing species, possibly associated with the ability to enter amoeba cells.

A WGS approach was also applied to investigate the degree of similarity and genetic relationships between members of the *M.avium* complex. Isolates were derived from a variety of sources (SRA, NCBI, University of Sydney collection and human clinical samples). Clusters of isolates within the pan-genome analysis were compared to demonstrate the genomic differences between subspecies. The *M avium* serovars sequenced in this study represent a unique data set that will be explored in greater detail. These will be compared to additional publicly-available sequences to further interrogate the taxonomic definitions within the MAC, which are currently based on results from

traditional and *in-silico* typing methods. Genetic studies such as have been undertaken in this collaboration expand the available sequences to enable the analysis of genetic diversity in this family and will hopefully contribute to both human and animal health outcomes.

5.4.3.2 WGS of a diverse collection of MAP isolates to understand epidemiology

Study 1 successfully sequenced 47 MAP sheep strain isolates from long-term vaccinating farms and identified grouping of persistent isolates. To our knowledge, this is the largest number of MAP sheep strain genomes in the world. The study found large differences between these isolates and the vaccine strain and K10 reference, which was expected due to the study isolates being S strains, whereas the vaccine strain and K10 reference are C strain MAP. Common differences between the Telford strain and the current collection of isolates were also identified, which may reflect genetic drift over time.

One of these genetic differences was within siderophore genes. Mycobacterial siderophores are involved in iron homeostasis (Sritharan, 2016). Iron is a critical micronutrient for most aerobic bacteria, including mycobacteria. Mycobactins are siderophores produced by a range of pathogenic and non-pathogenic mycobacteria except MAP. WGS of MAP K10 identified that a key gene in the mycobactin biosynthesis pathway was truncated, potentially explaining the dependence for MAP *in vitro* growth on exogenous mycobactin J (Li et al., 2005). The siderophore exporter MmpL4 is involved in membrane transport to avoid toxic accumulation of siderophores and is a virulence factor for *M.tuberculosis*, which synthesises its own siderophores under iron limiting conditions (Wells et al., 2013). We can only speculate regarding how this loss-of-function mutation may impact the MAP sheep strain field isolates compared to MAP Telford strain. It is possible that a loss of siderophore export may enable greater iron acquisition *in vivo* but be detrimental to *in vitro* culture in the presence of exogenous Mycobactin J, due to toxic accumulation of this in the mycobacterial cell. This could be further explored in virulence assays conducted in the laboratory.

Study 2 was a major study examining over 400 MAP genomes from Australia and internationally. To our knowledge, this is the largest global whole genome study of MAP where a large proportion of the isolates were of Australian origin. Australian MAP C strain isolates were seen to be interspersed on the phylogenetic tree among isolates from around the world. However, for the MAP S strain isolates, there was substantial delineation between these and other S strain MAP isolates from different regions of the world. This may reflect the original routes of transmission, though this would need to be explored in more detail.

Study 3 focused on S strains of MAP from around the world. Prior research had predominantly focused of C strains of MAP, however in countries where sheep industries are more prevalent, such as Australia and New Zealand, ovine JD is a substantial burden. Within the S strains, two sub-lineages, type I and type III MAP, exist but little is known of their relative significance in terms of epidemiology and pathogenicity. This study addressed the epidemiological and genomic differences between these sub-types and revealed lineage-specific mutations and global patterns. Lineage-specific markers can be exploited in future for new diagnostic tests and aid in future vaccine development. Further, an improved knowledge of strain-specific characteristics may reveal insights on mechanisms of host tropism and transmission of the disease. Understanding the global phylogeny and population structure of these sub-types assists with categorising new isolates in an international context. In the event of an outbreak in livestock, or the occurrence of human disease cases, isolates

can be more efficiently traced and potential sources or biosecurity issues identified. Together, these outcomes will potentially lead to better control in animals and assist public health efforts.

Success in achieving the project objectives

Objectives: Improved understanding of how MAP strains are changing over time and how this may affect on-farm infection. (i) WGS of divergent mycobacterial species from an evolutionary perspective in order to understand how common properties have arisen; and (ii) WGS of a diverse collection of MAP isolates to understand common epidemiological situations, such as changes in the epidemiology of paratuberculosis in Australia over time (S, C and B strain) and possible effects of Gudair® vaccine on MAP host preference (S strain in cattle).

All of the objectives have been achieved in this aspect of the sub-project. The knowledge base created will be available to current and future researchers and the findings have elucidated key aspects of mycobacterial evolution, pathogenesis and potential vaccine-driven persistence or emergence of new strains.

5.4.4 *H.contortus* WGS of field isolates

In this study, whole genome re-sequencing and alignment of the sequence data to a reference genome was successfully completed for over 40 *H.contortus* larval samples.

The genetic tools are still being developed to conduct a deeper analysis of *H.contortus* sequencing data. Genome-wide assessment of signatures of Ivemectin resistance recently revealed a number of candidate genes in addition to the one already known (HCON_00148840, glc-3) (Khan et al., 2020). In November 2020, the first fully assembled genome for *H.contortus* was published; this identified a genome size that was smaller than previous reference genomes (283.4 Mbp) and included chromosome-scale genome assembly including the sex chromosome (Doyle et al., 2020). This paper found a high degree of similarity between *H.contortus* and *C.elegans* in terms of conserved chromosomal elements, validating the use of Bioinformatic pipeline 3 (based on *C.elegans* WGS data analysis) for the assessment of variation at the genomic level for this parasitic worm. The assessment of the degree of variability between isolates and its impact on phenotype are suggested as future research avenues, utilising appropriate analytical pipelines as they become available.

Success in achieving the project objectives

Objectives: Extend our knowledge of the population-level heterogeneity of *H.contortus* through WGS of field isolates.

The objectives were successfully achieved. A WGS library of over 40 *H.contortus* larval field isolates has been established. These isolates showed a high level of heterogeneity compared to the reference genome. The WGS data generated will facilitate the application of newly developed genetic tools and in-depth analysis of new candidate genes and regions to assess phenotypic variants.

6.4 Conclusion/Recommendations – Sub-project 4

6.4.1 Recommendations from the risk factor analysis survey for MAP S strain in cattle

This study indicates that there is potential for improvements on current biosecurity and control programs. Programs can be expanded to include the below recommendations to better enable farmers to manage JD efficiently on-farm and to break the route(s) of transmission.

Recommendation 1: As per existing advice for trade, producers should be aware of the type of operations they buy cattle from and ensure that they trade with low-risk JD farms or farms with a similar J-BAS status. Knowing the JD status of neighbours and of properties the producer is involved with for trading is important for effective biosecurity programs to managing the disease on-farm; this includes **all** species (cattle and sheep) on the farm.

Recommendation 2: The more widely accepted transmission route for S strains is through co-grazing, where age-susceptible livestock are grazed concurrently with suspected or infected sheep. The importance of continued awareness of MAP spread when sheep are vaccinated with Gudair® has not been highlighted previously. Additional advice should be provided to producers regarding the potential risks of co-grazing cattle, particularly age-susceptible (<2 year old) cattle, with vaccinated sheep.

The IPM control of wormy pastures (from sheep) through grazing with cattle should be conducted with appropriate caution in MAP endemic regions.

Recommendation 3: Undertake additional research to obtain a greater understanding of the prevalence, geographic distribution and risk of transmission of S strain MAP in cattle. Working closely with farmers, new testing methods (see Section 6.1.2) could be employed to determine the full distribution of cross-transmission spread.

6.4.2 Recommendations from the evaluation of diagnostic accuracy for animals infected with different strains of MAP

Recommendation 4: Strain typing is recommended to be conducted routinely on co-grazing farms or by producers purchasing animals from mixed farming enterprises to assess the prevalence of cross-species transmission and whether mixed infections (both C and S strain MAP infecting the host) occur. Mixed infection would have gone undiagnosed previously as these cannot be distinguished using the existing strain-typing method (IS*1311* PCR REA).

The HT-J test has recently been evaluated in terms of the appropriate pooling rate for testing of beef cattle for the J-BAS. This test is able to detect both MAP strain types (MLA project B.AHE.0322 "Development and evaluation of approaches for cost-effective testing of beef herds for Johne's disease"). The strain-specific gene assays would enable rapid, concurrent strain typing of MAP to gain a better understanding of the prevalence of cross-species transmission of MAP.

Ideally, it would be advantageous if strain typing could be conducted simultaneously with the diagnosis of JD, as this would reduce the number of qPCR tests required. A multiplex assay, including the strain-type specific gene assays and MAP IS900 as included in the HT-J, would be a valuable new diagnostic tool. Further development and validation of a multiplex assay could be achieved using the existing sample panel established during this study.

6.4.3 Conclusions and Recommendations from Mycobacterial Genomics

Within the broad Mycobacterial species and sub-lineages, there are major evolutionary genetic 'steps' that relate to the pathogenicity of these bacteria. This is evident in the differences between slow and fast growing species, the M.avium species and within sublineages of MAP. There also appear to be distinct regional clusters, such as the Australian-New Zealand group within the Type I lineage.

Within species and lineages, there is evidence for lineage-specific genes and gene variants, some of which are associated with virulence in mycobacteria. This knowledge can be applied to field isolates, such as those derived from vaccinating farms. Pathogen genomic approaches developed in this sub-project can be applied to investigating farms where there is persistence of OJD or BJD despite vaccination or other control and management practices. This knowledge can also be applied to investigate the epidemiology and spread of MAP lineages, without the need for full genome sequencing in future studies. Further *in vitro* work is recommended to assist in identifying the functional impacts of the genes identified and reveal how genetic differences relate to virulence and host adaptation. It is important to be aware of any changes in the genetic landscape for these pathogens to facilitate future diagnosis and control.

6.4.4 Conclusions and Recommendations from *H.contortus* Genomics

Genetics for *H.contortus* and other nematodes is a developing field. New analytical tools and a completed genomic sequence for this parasite have recently become available. With the resources created in this project, these tools can be further applied to aid in the understanding of *H.contortus* genetic heterogeneity in the Australian setting. This could include genetic tests for anthelmintic resistance traits.

7.4 Key Messages – Sub-project 4

1. **As per 6.4; Recommendations 1-3 above**.

2. **MAP strain typing is recommended to be conducted routinely on co-grazing farms** or by producers purchasing animals from mixed farming enterprises to assess the prevalence of cross-species transmission and whether mixed infections (both C and S strain MAP infecting the host) occur. The HT-J test has recently been evaluated for pooled testing in beef cattle (B.AHE.0322) and the strain-specific qPCR assays developed in this study could be used in conjunction with the HT-J to detect both MAP strain types.

3. **Pathogen genomic approaches developed in this sub-project can be applied** to investigating farms where there is persistence of OJD or BJD despite vaccination or other control and management practices. This knowledge can also be applied to investigate the epidemiology and spread of MAP lineages.

8.4 Bibliography – Sub-project 4

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Sub-project 5: Why Mptb shedding persists in some flocks despite vaccination.

1.5 Background – Sub-project 5

Ovine Johne's disease (OJD) is a chronic wasting disease of sheep caused by *Mycobacterium avium* subsp. *paratuberculosis* (Mptb) and characterised by progressive emaciation, chronic diarrhoea and eventually death. First reported in the late 19th century in Denmark (Johne and Frothingham 1895), the disease spread across the globe during the past century and is now present in most countries. Although primarily a disease of domestic ruminants, it has also been reported in wild ruminants and non-ruminants (Beard *et al.* 2001a; Beard *et al.* 2001b; Daniels *et al.* 2003). In addition, there are speculations over the involvement of Mptb in Crohn's disease in humans (Greenstein 2003; Greenstein and Collins 2004).

In Australia, paratuberculosis in cattle has been known to be present since 1925, but the disease in sheep was not reported until the early 1980s (McCausland 1980; Seaman *et al.* 1981; Seaman and Thompson 1984). Since then, OJD has spread across most states and caused considerable economic losses to sheep farmers. This was reflected in the findings of a study conducted in the southern tablelands of New South Wales (NSW) that estimated a decrease in farm gross margins ranging from 2.2% to 15.4% on 12 infected farms in 2002 (Bush *et al.* 2006).

The heat inactivated vaccine, Gudair®, is a key element in the control OJD in Australia. Control with vaccination was recommended in 2002 after successful testing of this vaccine in NSW. Research conducted in the past 15 years since the commencement of vaccination in Australia has shown that vaccination with Gudair® substantially reduces mortalities (Windsor *et al.* 2014; Dhand *et al.* 2016b). Some farms have also been able to eliminate Mptb shedding or at least reduce shedding to below detectable levels, but animals from other Gudair® vaccinated flocks continue to shed Mptb organisms in their faeces and remain infectious for several years (Eppleston *et al.* 2005; Reddacliff *et al.* 2006; Dhand *et al.* 2013; Dhand *et al.* 2016b). The reasons for these differences in vaccine effectiveness between flocks remain unclear, however, differences in management, biosecurity practices, host genome or pathogens infecting these flocks may contribute to these differences.

The first component of this sub-project was conducted to investigate the differential effectiveness of the Gudair® vaccine in flocks, i.e. to understand why sheep on some farms continue to shed Mptb in their faeces despite vaccination. Specifically, the study was conducted with the objective to evaluate management factors that may be associated with the detection of Mptb faecal shedding in sheep and the prevalence of OJD in sheep flocks that had reported vaccinating with Gudair® for at least five years. The identification of these factors would enable farmers to make appropriate changes in their husbandry, management and biosecurity practices to reduce the incidence of the disease. This will also enable animal health policymakers to develop guidelines for veterinarians, farmer consultants and the industry to improve the effectiveness of the vaccine.

Vaccination with Gudair® contributes to a delayed onset of OJD, decreases mortalities and decreases faecal shedding of *Mptb* [1,2]. Although the vaccine is very effective in reducing the incidence of clinical disease, animals on some farms continue to shed *Mptb* in their faeces despite ongoing vaccination in

the flocks for over a decade [3,4]. Thus, there is concern in the scientific community and animal health policymakers of a risk of OJD resurgence if farmers cease to vaccinate following a lack of observable clinical cases after a few years, due to the assumption that they have eradicated the disease from their farm [4–6]. There have also been considerations by farmers of not vaccinating wether lambs for economic reasons [7], concurrent with concerns about the human safety aspects of Gudair® [8]. Cessation of vaccination may not only compromise the management of this disease for individual producers but also jeopardise its control at a regional and national level [7]. However, there is no objective information about the proportion of farmers who have discontinued Gudair® vaccination and their reasons for discontinuation. Therefore, the second component of this sub-project was conducted to estimate the proportion of producers ceasing to vaccinate their animals with Gudair® and to identify the reasons for such discontinuation.

2.5 Objectives – Sub-project 5

This study was conducted to understand the differential effectiveness of Gudair® vaccine in flocks, i.e. to understand why sheep on some farms and not the other continue to shed Mptb in their faeces despite vaccination. Specifically, the project was conducted with the following objectives:

- To evaluate management factors associated with the presence of Mptb in sheep faeces and the prevalence of OJD in sheep flocks vaccinating for at least five years.
- To estimate the proportion of farmers who discontinue vaccination and identify drivers for vaccine cessation.

3.5 Methodology – Sub-project 5

3.5.1 Study 1: Reasons for the differential effectiveness of the Gudair® vaccine

Detailed methods are presented below but in brief, 64 sheep farms that have been vaccinating lambs with Gudair® for some years were enrolled. Presence or absence of shedding in these flocks was measured using pooled faecal culture (PFC) and information about their management and biosecurity practices was obtained using face-to-face questionnaires. Data were analysed to compare management and biosecurity practices between OJD positive and negative farms.

Target population and eligibility criteria

The target population for this study was the OJD infected flocks in NSW and Victoria. The farms had to meet the following criteria to qualify for the study: (a) farmers were willing to participate in the study, (b) flocks had at least one positive OJD diagnosis, (c) flocks had sufficient numbers of sheep to sample (n ≥ 350) and (d) flocks had been reported by the farmer as being consistently vaccinated with Gudair® for at least five years.

Selection of producers

Initially, an online survey was conducted to assess the eligibility of farmers in terms of the above criteria. Farmers were enrolled from the NSW Government Local Land Services database, NSW sheep farmer association database, Meat and Livestock Australia associated Integrity Systems Company's database and by referrals from sheep farmers and district veterinarians. A total of 182 farmers participated in this online survey and a further 13 responses were obtained by mail. Of these farmers, 74 (56 in NSW and 18 in VIC) met the eligibility criteria of the project and were mailed letters containing a participant information statement and a cover letter outlining the study. They were then telephoned to confirm their participation and to establish a date for faecal sampling and conducting an interview.

Questionnaire design

A five-page questionnaire was developed with 77 questions to collect data from the studied farms for potential husbandry and biosecurity factors that affect the prevalence of OJD. The questions covered aspects including farming enterprise (property description, environment and management), OJD infection history (first OJD diagnosis, Gudair® vaccination commencement and annual clinical signs/losses encountered), biosecurity (risk posed by neighbours, sheep purchases/introductions, water management and wildlife risks), and OJD control (stock management and overall animal health). All farmer interviews were conducted face-to-face by four people, except for one which was emailed in by the farmer.

Sample collection and farmer interviews

Faecal samples were planned to be collected from 350 sheep at each farm to enable testing of 14 pools, each containing one pellet from 25 sheep. This samples size provides 95% confidence of detecting a prevalence of 2% assuming 50% test sensitivity and a perfect specificity (Sergeant *et al.* 2001). Faecal samples were collected per rectum from three-, four- and five-year-old ewes and wethers and pooled into 14 groups of 25 sheep per vial.

Laboratory methods

Pooled Faecal Culture

The PFC method was based on the Australian New Zealand Standard Diagnostic Protocols for Johne's disease (Eamens 2015). Firstly, the pooled faeces were homogenised with care to minimise the risk of sample-to-sample cross-contamination, by using a Waring commercial blender base, with 250 mL steel blenders without saline. Homogenised pools (2 g) were resuspended in 10 mL of saline, with 3-5 mL of the supernatant removed following sedimentation for 30 min. The supernatant was then

decontaminated and added to 20 mL of 0.9% hexadecyl pyridinium chloride (HPC) (Sigma Chemicals) in half-strength brain heart infusion (BHI) (Becton Dickson) broth. After incubating at 37°C for 24 h, the mixturewas centrifuged at 1000 x *g* for 30 min at 8°C. The supernatant was discarded, and the pellet was resuspended in 1 mL of an antibiotic mixture of vancomycin, amphotericin B and nalidixic acid (VAN) (Sigma Health) and incubated at 37°C for 72 h. Following this, 0.1 mL of the suspension was added to M7H9C liquid culture medium and incubated for 12 weeks at 37°C, as previously described (Whittington *et al.* 2013).

DNA isolation from liquid culture media

Following incubation of cultures for 12 weeks, bead beating followed by a semi-automated magnetic bead isolation technique was used to isolate the Mptb DNA from the liquid culture using a previously validated method (Plain *et al.* 2015). In the bead-beating step, 200 µL of the liquid culture medium was added to 200 µL Buffer AL (Qiagen), and then transferred to a 2 mL tube containing 0.3 g of zirconia/silica beads (BioSpec Products, Inc., Daintree Scientific) and disrupted using a Fast Prep-24 bead beater (MP Biomedicals) at 6.5ms^{-1} for 60 seconds, twice. The tubes were centrifuged at 16,000 x *g* for 2 min, and 100 µL of the homogenate was added to a deep 96-well plate. DNA was then isolated using the BioSprint® 96 One-For-All Vet Kit (Qiagen) and a MagMAX-96 automated magnetic processor (Applied Biosystems), according to the protocol for tissue homogenates using Buffer RLT, following the manufacturer's instructions.

IS900 PCR to detect MPTB

The IS*900* qPCR was performed on the Mx3000P real-time PCR instrument (Stratagene, Agilent) to detect Mptb (Kawaji *et al.* 2007; Plain *et al.* 2014). Briefly, the reaction mixtures contained 5 µL template DNA, 250 nM forward and reverse primers: MP10 and MP11, and SensiMix SYBR low-ROX qPCR master mix (Bioline) (Kawaji *et al.* 2007). Cycling conditions included an initial denaturation at 95°C for 30 s, and annealing/extension at 68°C for 60 s with fluorescence acquisition at the end of the annealing/extension step, followed by a melt curve analysis from 65°C to 95°C. A five-step standard curve of Mptb genomic DNA was included in every qPCR as a reference standard to normalise results between experiments. Growth was confirmed for the DNA quantity as previously described (Plain *et al.* 2015).

Data management and analysis

Data were entered into an MS Excel spreadsheet and were cleaned to double-check typos, missing values and implausible values. Farmers were phoned/emailed to obtain some missing data after preliminary checks.

Data from MS Excel were imported into the SAS statistical program which was used for all statistical analyses (version 9.4 © 2016 by SAS Institute Inc., Cary, NC, USA). Two outcome variables were created to evaluate management and biosecurity factors responsible for the differential effectiveness of the Gudair® vaccine in sheep flocks: Mptb positive (yes/no) and OJD prevalence level (nil, <1%, ≥ 1%). A flock was considered to be Mptb positive if any pool sourced from the flock was positive in PFC. To create the second outcome, the number of positive pools and the total number of pools collected from each flock were used to calculate the disease prevalence in each flock using a pooled prevalence calculator for variable pool sizes

(https://epitools.ausvet.com.au/ppvariablepoolsize). The prevalence was then categorised to create an ordinal outcome variable, OJD prevalence level (nil, <1%, ≥ 1%).

Descriptive analyses were conducted to make a preliminary assessment of the association of the explanatory variables with the two outcome variables. Contingency tables of categorical variables and summary statistics of quantitative explanatory variables by the outcome variables were created.

The binary and ordinal outcomes were used to conduct binary and ordinal logistic regression analyses, respectively. Univariable logistic regression analyses were first conducted to evaluate the unconditional association of the explanatory variables with the outcome variables. Explanatory variables with p-values < 0.2 were shortlisted for multivariable analyses. Collinearity analyses between shortlisted variables were then conducted to identify pairs of highly collinear variables. One of a pair of collinear variables was excluded from further analyses. Similarly, variables with more than 20% missing values were excluded. The remaining variables were included in multivariable models using a forward stepwise approach. Interactions between variables in the final model were tested and retained if significant (p-value <0.05). The goodness of fit of the binomial logistic regression model was tested using Hosmer and Lemeshow goodness-of-fit test. The assumption of proportional odds for the ordinal logistic model was tested using the Score test.

3.5.2 Study 2: Reasons for vaccine discontinuation

For investigating the factors related to cessation of Gudair®, we designed a questionnaire and obtained ethics approval. Procedures of the study were approved by the University of Sydney Human Ethics Committee.

A flyer and a one-page questionnaire were designed to enrol producers to obtain information about cessation of vaccination. The questionnaire comprised a total of four main questions, relating to: 1) sheep flock size; 2) predominant breed of sheep on farm; 3) previous and/or current cases of OJD on farm; and 4) Gudair® vaccination commencement and (dis)continuation. The questionnaire was distributed via newsletters and emails to Australian sheep producers in 2018 with the help of Meat and Livestock Australia and other industry bodies.

Data were collated in Microsoft Excel, with R Studio v 1.3.1073 [9], an integrated development environment for R v 4.0.2, used for subsequent data cleaning and analyses. Descriptive analyses were conducted by creating frequency and contingency tables, with graphs designed using ggplot2 [10]. Logistic regression models were fitted, with the binary outcome "have you continued to vaccinate with Gudair®?" to characterise producers continuing/discontinuing vaccination. The categorical explanatory variables tested included flock size (< 2500, between 2500 and 4999, ≥ 5000), predominant sheep breed (Merino vs others), farm location (NSW, VIC, other states), and previous OJD detection on farm (yes/no). In addition, a numerical explanatory variable 'number of years since vaccination commencement' was created by subtracting the vaccine start year from the year of survey (2018) and tested using logistic regression. A likelihood ratio chi-square P-value of < 0.05 was considered statistically significant.

4.5 Results – Sub-project 5

4.5.1 Study 1: Reasons for the differential effectiveness of the Gudair® vaccine

Farm visits to perform sampling for PFC and to administer the questionnaire were completed between 27 March 2018 and 21 February 2019. A total of 64 farms from 41 postcodes participated in the study of which 48 (75%) were from NSW and 16 (25%) from Victoria. Mptb faecal shedding was detected 19/48 flocks in NSW (39.6%), 8/16 flocks in Victoria (50.0%) and 27/64 flocks in total (42.2%). Overall, Mptb shedding was not detected in 37/64 flocks, and the prevalence was <1% in 19/64 (29.7%) flocks and ≥ 1% in 8/64 flocks (12.5%). Of the 48 flocks in NSW, 16 (33.3%) had <1% prevalence and 3 (6.3%) had ≥ 1% prevalence whereas of the 16 flocks in Victoria, the prevalence was <1% in 3 (18.8%) and ≥ 1% in 5 (31.3%) flocks.

4.5.1.1 Property description and enterprises

The average area of the enrolled properties was 1167 ha of which 89% was grazed on the average (Table 5.1). The properties were located between 110 and 1160 m above sea level. Most of the properties had an undulating topography (Table 5.2).

Table 5.1. Description of the surveyed properties.

Variable Name	**Mean**	**SD**	**Min**	**Q1**	**Median**	**Q3**	**Max**	**N**
Total area (ha)	1204	845	96	600	962	1600	4000	64
Altitude (m)	535	278	110	300	540	703	1160	64
Average rainfall (mm)	634	149	26	600	650	687	900	64
% area grazed	89	16	35	80	99	100	100	64
Adult micron	19	3	16	17	18	19	29	59
Area cropped (ha)	403	336	19	200	340	500	1450	24

SD: Standard deviation; Min: Minimum; Q1: First quartile; Q3: Third quartile; N: Number of flocks

Table 5.2. Location of the surveyed properties.

Variable	**Categories**	**Frequency**	**Percent**
Topography	Flat	8	12.5
	Undulating	42	65.6
	Mixed	14	21.9
	Total	64	100.0
Soil	Granite	25	39.7
	Basalt	12	19.0
	Mixed	26	41.3
	Total	63	100.0
State	NSW	48	75.0
	VIC	16	25.0
	Total	64	100.0

The enterprises of the producers are presented in Table 5.3. Most of the flocks were self-replacing Merino flocks although some also reared cross-bred ewes or engaged in sheep trading. Stock numbers are presented in Table 5.4.

Table 5.3. Enterprises of the surveyed producers.

Variable	**Categories**	**Frequency**	**Percent**
Merino	No	7	10.9
	Yes	57	89.1
	Total	64	100.0
Self-replacing flock	No	3	4.8
	Yes	59	95.2
	Total	62	100.0
	Missing	2	3.1
Cross-bred ewes	No	42	65.6
	Yes	22	34.4
	Total	64	100.0
Sheep trading	No	60	93.8
	Yes	4	6.3
	Total	64	100.0
Cropping	No	38	59.4
	Yes	26	40.6
	Total	64	100.0

Table 5.4. Stock numbers in the properties included in the study.

Enterprises	**Groups**	**Mean**	**SD**	**Min**	**Q1**	**Median**	**Q3**	**Max**	**N**
Merino	Ewes	3963	4849	0	1400	2500	4300	32000	57
	Wethers	1223	1528	0	20	600	1950	6500	57
	Young	2678	3731	0	790	1580	2825	25000	57
	Adult	2508	2122	0	900	1600	3300	9011	57
	Total	5186	5341	200	1900	3060	7200	32000	57
Cross-bred	Ewes	2359	3027	0	200	1225	2730	10700	22
	Young	1135	1418	0	150	643	1300	4500	22
	Adult	1373	1996	0	0	705	2300	8000	22
	Total	2509	3022	10	350	1225	4000	10700	22
Cattle	Total	191	171	26	60	150	257	680	21

SD: Standard deviation; Min: Minimum; Q1: First quartile; Q3: Third quartile; N: Number of flocks

4.5.1.2 OJD infection history

The flocks had been diagnosed with OJD for 17 years and had been vaccinating for 15 years on the average (Table 5.5). The median loss prior to the commencement of the Gudair® vaccination was 5% which has reduced to negligible after vaccination. About half of the producers reared cattle and about a third run cattle and sheep together (Table 5.6).

Table 5.5. OJD Infection history.

Variable Name	Mean	SD	Min	Q1	Median	Q3	Max	N
Number of years since diagnosed	16.8	6.3	2	14	18	21	31	60
Number of years since suspected	20.7	8.3	4	14	20	25	44	52
Number of years since vaccinating	15.4	4.2	5	12	17	18	24	63
Annual losses prior to vaccination commencement	5.4	6.3	0	1	4	7	30	58
Percentage of clinicals in the last 1 year	0.07	0.2	0	0	0	0.019	0.2	64

SD: Standard deviation; Min: Minimum; Q1: First quartile; Q3: Third quartile; N: Number of flocks

Table 5.6. Cattle enterprises, BJD and cattle strain.

Variable	Categories	Frequency	Percent
Mix cattle and sheep?	No cattle	34	53.1
	Mix cattle with sheep	22	34.4
	Don't mix cattle with sheep	8	12.5
	Total	64	100.0
BJD	No	6	20.7
	Yes	3	10.3
	Don't know	20	69.0
	Missing information	35	54.7
	Total	64	100.0
Strain	Cattle and sheep	1	14.3
	Sheep	6	85.7
	Total	7	100.0

4.5.1.3 Biosecurity practices

Biosecurity practices varied among producers (Table 5.7). Most of the producers introduced rams in the past years (60/64; 94%) but only 30% (19/64) introduced ewes. Further details about the number of rams and ewes introduced and their sources are presented in Table 5.8.

Feral animals were present on most of the properties (Table 5.9).

Table 5.7. Property biosecurity – categorical variables.

Variable	**Categories**	**Frequency**	**Percent**
Sharing and straying[1]	Negligible	17	26.6
	Occasional	13	20.3
	Frequent	17	26.6
	Very frequent	17	26.6
	Total	64	100.0
Introduced rams over the last 5 years?	Yes	60	93.7
	No	4	6.3
	Total	64	100.0
Introduced ewes over the last 5 years?	No	45	70.3
	Yes	19	29.7
	Total	64	100.0
Water source[2]	Negligible risk	11	17.2
	Low risk	14	21.9
	Medium risk	27	42.2
	High risk	12	18.8
	Total	64	100.0

[1.] An index created from questions about sharing of rams/sheds/yards/roads with neighbours and straying of sheep between properties.
[2.] An index created based on questions about run-off water received by the property, whether the run-off is from OJD infected properties, sources of water, sources of water in the lambing and weaning paddocks.

Table 5.8. Property biosecurity – numeric variables.

Variable Name	Mean	SD	Min	Q1	Median	Q3	Max	N
Num of neighbours with sheep	4.8	3.4	0	3	4	6	18	64
Num neighbouring properties known OJD infected	1.8	2.5	0	0	1	3	11	47
Proportion of neighbouring properties infected	0.6	0.4	0	0.13	1	1	1	58
Number of rams introduced in the past five years	39	38	1	15	30	45	200	60
Number of sources from which rams were introduced	2.7	1.6	1	2	2	3	8	60
Number of ewes introduced in the past five years	1055	1163	22	200	513	2000	4000	18
Number of sources from which ewes were introduced	2.6	2.1	1	1	1.5	5	7	18

SD: Standard deviation; Min: Minimum; Q1: First quartile; Q3: Third quartile; N: Number of flocks

Table 5.9. Prevalence of wildlife on the property.

Variable	**Prevalence**	**Frequency**	**Percent**
Kangaroos	Negligible	1	1.6
	Low	19	29.7
	Medium	22	34.4
	High	22	34.4
	Total	64	100.0
Feral goat or deer	Negligible	45	70.3
	Low	10	15.6
	Medium	7	10.9
	High	2	3.1
	Total	64	100.0
Rabbits	Negligible	7	10.9
	Low	49	76.6
	Medium	7	10.9
	High	1	1.6
	Total	64	100.0
Foxes	Negligible	42	65.6
	Low	12	18.8
	Medium	9	14.1
	High	1	1.6
	Total	64	100.0
Wildlife risk[1.]	Negligible risk	11	17.2
	Low risk	20	31.3
	Medium risk	12	18.8
	High risk	21	32.8
	Total	64	100.0

[1.] An index created from the prevalence of kangaroos, goats, deer, rabbits and foxes on the property.

4.5.1.4 OJD management

About 60% of the producers did not vaccinate all lambs with Gudair® and mixed lambs and weaners with unvaccinated stock (Table 5.10). The median age of vaccination was 6 months (Table 5.11).

Table 5.10. OJD vaccination and flock management – categorical variables.

Variable	Categories	Frequency	Percent
Do you vaccinate all lambs with Gudair®?	No	40	62.5
	Yes	24	37.5
	Total	64	100
Do you mix lambs/weaners with unvaccinated animals?	No	30	46.9
	Yes	34	53.1
	Total	64	100
Average ewe fat score at last joining	<3	16	25.4
	=3	32	50.79
	>3	15	23.81
	Total	63	100.0
Are lambs mulesed at marking?	No	26	40.6
	Yes	38	59.4
	Total	64	100.0
Are weaners kept separate from adult sheep?	No	1	1.6
	Yes	63	98.4
	Total	64	100.0
Are maiden ewes kept separate from adult sheep?	No	8	12.5
	Yes	56	87.5
	Total	64	100.0
Supplementary feed	Only on ground	36	56.3
	Both ground and trough	22	34.4
	Only trough	6	9.4
	Total	64	100.0

Table 5.11. OJD vaccination and property management - numeric variables.

Variable Name	Mean	SD	Min	Q1	Median	Q3	Max	N
Age of Gudair vaccination (weeks)	7.6	3.1	2	6.0	6.0	8.0	20.0	64
Marking percent	102.2	22.7	60	85.0	98.0	110.0	180.0	63
Age culled	6.1	1.0	4	5.5	6.0	6.5	9.0	63
Stocking rate in lambing paddocks	9.2	6.7	0	5.0	6.8	12.5	36.0	62
Stocking rate in weaning paddocks	13.5	8.7	3	7.1	11.5	17.0	38.9	56

SD: Standard deviation; Min: Minimum; Q1: First quartile; Q3: Third quartile; N: Number of flocks

4.5.1.5 Management and biosecurity factors associated with OJD

Contingency tables for categorical variables and summary statistics for numeric variables are presented in Tables 5.12 to Table 5.15 (Appendix 5.1).

In total, 33 variables were tested in univariable models of which eight variables had a p-value of <0.20 in the models for both the binary outcome 'Mptb positive' (Table 5.16 - Appendix 5.1) and the ordinal outcome 'OJD prevalence level' (Table 5.17 - Appendix 5.1). Two of these variables were excluded as they had more than 20% missing values. None of the pairs of variables was highly collinear. Finally, six variables were tested in the multivariable model, of which three were significant (p-value <0.05) in both models.

The final model results for the binary outcome are presented in Table 5.18 and for the ordinal outcome in Table 5.19. Farms providing supplementary feed in troughs instead of on the ground were less likely to have Mptb positive sheep and had a lower prevalence of OJD. Farms with a greater number of neighbours with sheep and those introducing rams from a greater number of sources were more likely to be Mptb positive and had a higher OJD prevalence. In addition, farms with a greater number of neighbours known to be OJD positive and purchasing ewes from a greater number of sources were at a higher risk, but these variables had to be excluded from multivariable modelling due to a large number of missing values. None of the interactions was significant. The Hosmer and Lemeshow goodness-of-fit test indicated that the fit of the binomial logistic regression model was good (p-value: 0.15). The proportional odds assumption of the ordinal model was met (Score test p-value: 0.84).

Table 5.18. Final multivariable binomial logistic regression model for the outcome variable Mptb positive (yes/no) based on data collected from 60 sheep flocks in Australia in 2018-2019.

Variables	Categories	b	SE	Odds-ratios	95% CL	LR P-value
Intercept		-1.9	0.92			
Supplementary feed	Only on ground	0.00		1.00		0.004
	Both on ground and trough	-2.29	0.81	0.10	0.02, 0.42	
	Only on trough	-1.13	1.21	0.32	0.02, 2.74	
Number of neighbours with sheep	-	0.23	0.11	1.26	1.02, 1.60	0.03
Number of ram sources	-	0.46	0.22	1.58	1.06, 2.52	0.004

b: parameter estimate; SE: Standard error; CL: Confidence limits; LR: Likelihood ratio.

Table 5.19. Final multivariable ordinal logistic regression model for the outcome variable OJD prevalence level (nil, <1%, ≥ 1%) based on data collected from 60 sheep flocks in Australia in 2018-2019.

Variables	Categories	b	SE	Odds-ratios	95% CL	LR P-value
Intercept		-3.61	0.95			
		-1.54	0.82			
Supplementary feed	Only on ground	0.00		1.00		0.005
	Both on ground and trough	-2.04	0.72	0.13	0.03, 0.48	
	Only on trough	-1.23	1.21	0.29	0.01, 2.33	
Number of neighbours with sheep	-	0.18	0.10	1.20	1.0, 1.46	0.051
Number of ram sources	-	0.38	0.18	1.46	1.03, 2.12	0.001

b: parameter estimate; SE: Standard error; CL: Confidence limits; LR: Likelihood ratio.

4.5.2 Study 2

A total of 195 sheep producers across Australia participated in this survey (Fig. 5.1). Of these, 182 responses were obtained online and 13 by post. Detailed results of the association of explanatory variables with the outcome are presented in Table 5.20.

The respondents predominantly owned Merino sheep, with about half running flocks of less than 2500 sheep. More than half of the respondents reported that OJD was previously detected on their farm, with a median of 15.5 years since first OJD detection and over half no longer seeing OJD on farm (56/104, 53.9%).

Most of the producers reported using Gudair® for vaccinating lambs (163/195; 83.6%), with a median of 12.0 years since vaccine commencement. Of these producers, 162 completed the rest of the questionnaire, with 88.3% reporting that they have continued to vaccinate their lambs. By contrast, almost 12% of producers disclosed that they have partially (7/162, 4.3%) or fully (12/162, 7.4%) discontinued vaccinating their sheep, with partial discontinuation relating to vaccine cessation for certain flock members such as wethers likely to be sold within the year, or lambs destined for slaughter.

For producers that discontinued vaccination, the median time using the Gudair® vaccination was 6.0 years. Vaccine discontinuation was attributed to management (n = 8, e.g. lambs to be slaughtered, breeding for overseas exportation, farm mismanagement or wanting to reduce time commitments), economic (n = 5, e.g. cost of vaccine, no financial gain or inability to purchase small quantities), and health reasons (n = 3, e.g. risk of self-inoculation, no perceived livestock benefits or concerns about efficacy). These reasons were not mutually exclusive.

Results of the logistic regression analyses presented in Table 5.1 revealed that continuation of vaccination was greater for producers that farm Merino sheep than those where the predominant breed is other than Merino (P = 0.03). Flock size, farm location, previous OJD detection on farm and years since vaccine commencement (P = 0.99) were not significantly associated with vaccine continuation. Multivariable analyses were not conducted as only one variable was significant in univariable analyses.

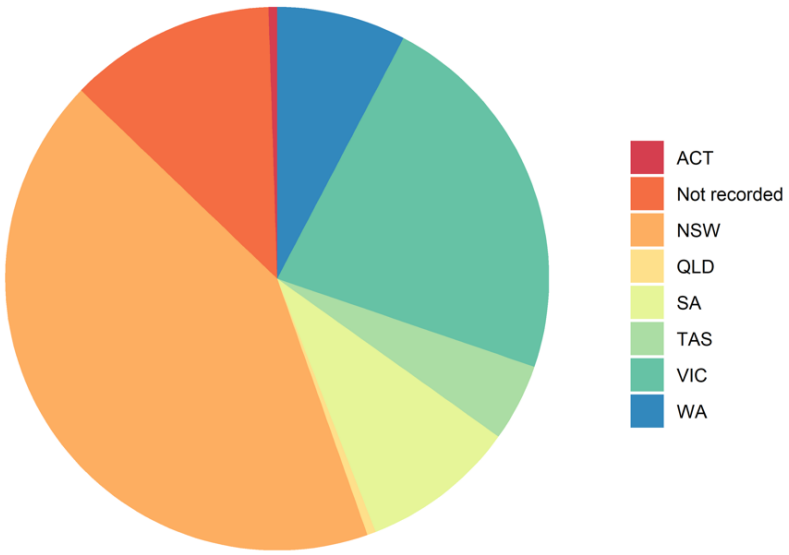

Fig. 5.1. Regional location of the 195 survey respondents according to state in Australia (NSW = 83, VIC = 44, SA = 18, WA = 15, TAS = 9, QLD = 1, Not recorded = 24).

Table 5.20. Descriptive and univariable logistic regression results for the explanatory variables tested for evaluation of their association with the outcome variable 'have you continued to vaccine with Gudair®?' based on the sheep producer survey conducted in Australia in 2018. Significant explanatory variables (P < 0.05) are bolded.

Categorical variables	**Categories**	**N (%)**	**Have you continued to vaccinate with Gudair®? N (Row %)**		**Estimate**	**SE**	**OR**	**95% CI**		**P-value**
			Yes	**No**						
Flock size	< 2500[1]	100 (51.28)	70 (70.00)	11 (11.00)	0.00		1.00			0.76
	2500 – 4999	49 (25.12)	38 (77.55)	4 (8.16)	0.40	0.62	1.49	0.47	5.68	
	≥ 5000	46 (23.59)	35 (76.09)	4 (8.70)	0.32	0.62	1.38	0.44	5.24	
Predominant breed of sheep	Other[1]	72 (36.92)	46 (63.89)	11 (15.28)	0.00		1.00			**0.03**
	Merino	123 (63.07)	97 (78.86)	8 (6.50)	1.07	0.50	2.90	1.10	7.96	
Farm location (Australia)	NSW[1]	83 (48.53)	65 (78.31)	8 (9.64)	0.00		1.00			0.29
	VIC	44 (25.73)	39 (88.64)	2 (4.55)	0.88	0.82	2.40	0.57	16.44	
	Other states	44 (25.73)	27 (61.36)	5 (11.36)	-0.41	0.61	0.67	0.20	2.37	
Previous OJD detection on farm?	No[1]	89 (45.64)	53 (59.55)	9 (10.11)	0.00		1.00			0.39
	Yes	106 (54.36)	90 (84.91)	10 (9.43)	0.42	0.49	1.53	0.57	4.03	

1: parameter estimate; SE: Standard error; CL: Confidence limits; LR: Likelihood ratio.

5.5 Discussion – Sub-project 5

5.5.1 Study 1 - Reasons for differential effectiveness of the vaccine

This study was conducted to understand the reasons for the previously observed differential effectiveness of the Gudair® vaccine in eliminating OJD infection in long term vaccinating flocks. We enrolled 64 sheep farmers and interviewed them to obtain information about their management and biosecurity practices. Data from the questionnaire and PFC results from approximately 350 sheep from each flock were analysed to identify the practices associated with the continued presence of OJD and its prevalence on the farm. The results suggest that some management and biosecurity practices were associated with the disease status and disease prevalence in the study flocks.

Despite vaccination against OJD for at least five years, sheep at 42.2% of the flocks in this study were shedding Mptb organisms in their faeces. Although the animal level prevalence was <1% in a majority of the flocks, 12.5% of the flocks did have ≥ 1% prevalence. This finding is consistent with our previous research that showed that sheep shedding Mptb were present in 30/37 flocks vaccinating for at least five years and 3/8 flocks vaccinating for a decade (Windsor *et al.* 2014; Dhand *et al.* 2016b). This led to the conclusion that vaccination did not eliminate shedding (see SP4, p135). However, the prevalence of clinical sheep in the flocks had dropped considerably from an average of 5.4% prior to vaccination to negligible currently. This confirms the findings of our previous studies which indicated that vaccine is very effective in reducing mortalities and the incidence of clinical disease but sheep on many properties continue to shed Mptb organisms in their faeces (Windsor *et al.* 2011; Windsor *et al.* 2014; Dhand *et al.* 2016a; Dhand *et al.* 2016b). These findings suggest that vaccination would have to be complemented with biosecurity and OJD control strategies for a successful control/ elimination of the disease. Further, it suggests that it is possible to introduce/ reintroduce disease by introducing sheep from a vaccinated flock.

Supplementary feeding is often required in sheep-grazing systems when nutrient supply falls short of demand, aiming to fulfil the nutritional requirements of sheep. In this study, the flocks that were provided with supplementary feed on the ground were more likely to be Mptb positive and to have a higher prevalence of OJD, compared to flocks that were trough fed, or fed both on the ground and in the trough. Providing supplementary feed on the ground could potentially increase the risk of faecal-oral transmission as it is known that infected sheep are capable of shedding a large number of Mptb organisms in their faeces (up to 10^8 –10^{10} Mptb per gram of faeces), resulting in extensive contamination of the environment (Whittington *et al.* 2000; Lugton 2004). Some vaccinated animals can also develop the clinical disease (multibacillary lesions) and excrete large quantities of Mptb bacilli, similar to the quantities of Mptb excreted by non-vaccinated animals (Reddacliff *et al.* 2006), which can further be exacerbated by malnutrition (Lugton 2004; Dhand *et al.* 2007). In addition, Mptb can survive in harsh Australian environments, therefore prolonging environmental contamination of Mptb bacilli (Whittington *et al.* 2004). While distributing supplementary feed on the ground may be more convenient for farmers, it may facilitate the ingestion of bacteria along with the feed, as they graze closer to the ground, thereby potentially reducing the effectiveness of the vaccine. Alternative supplementary feeding methods such as offering grain or pellet feed in elevated troughs or roughage in hay racks, where faeces are less likely to contaminate, should be explored as a preventative measure to prevent the spread of OJD in vaccinated flocks. This is

especially important in countries such as Australia, where extensive drought feeding of sheep is common, particularly given the recent uptake of confinement feeding in drought lots. This appears to be a novel finding of this study as we are not aware of the association of supplementary feeding on the ground with increased OJD prevalence or reduced effectiveness of vaccination in any other study even though it makes biological sense. Interestingly, a strong negative relationship was observed between supplementary feeding and sheep live weight in a study conducted in 2002 to investigate exposure factors for OJD (Abbott *et al.* 2004). The authors attributed this to low levels of pasture availability in those paddocks, but could this be partly due to the association observed between feeding on ground and OJD in this study?

Farms introducing rams or ewes from several sources in the past five years prior to sampling were at a higher risk of Mptb positivity and a higher OJD prevalence, suggesting that re-introduction of the disease potentially occurred with the introduction of these animals. A previous study found that vaccinating properties were more likely to test positive for OJD and had a greater risk of having maintained or increased the OJD prevalence levels if animals were regularly introduced into the flock (Windsor *et al.* 2014). In the present study, some farms introduced unvaccinated sheep from properties with unknown OJD status, and some were not vaccinated on arrival. Most farmers justified this practice by stating that they had introduced less susceptible animals or introduced animals intended to be sold prior to the age of shedding. However, in some cases, the lambs were sold at ages older than eight months, which is the age that Mptb shedding can occur in unvaccinated infected animals (Reddacliff *et al.* 2006; McGregor *et al.* 2012). The risk of introducing unvaccinated sheep from properties with unknown OJD status or not vaccinating animals on arrival can compromise existing vaccination efforts. Previous research has demonstrated that farmers that actively chose not to vaccinate their wethers due to economic concerns, had a 6-fold greater shedding prevalence among their unvaccinated animals, compared to vaccinates (Eppleston *et al.* 2011). This consequently resulted in an overall increase in Mptb excretion in the faeces, resulting in high levels of environmental contamination and exposure risk to lambs (Eppleston *et al.* 2011).

Moreover, the introduction of livestock from a number of sources, regardless of vaccination history or OJD status, may reflect sheep farmers' expectation that the adoption of vaccination will eliminate the need for additional OJD risk management strategies. In Australia, sheep farmers are advised to purchase either vaccinates or sheep from properties participating in SheepMAP, the national market assurance program to ensure that the sheep purchased are a low risk to their vaccination program (Sergeant 2001). Thus, sourcing animals from flocks of unknown OJD risk could jeopardise the efficacy of OJD vaccination programs, increasing the lateral spread of the pathogen and the prevalence of OJD on farms, despite vaccination of all home-bred sheep. These findings suggest that farmers should continue to implement appropriate biosecurity measures and appropriate OJD risk management strategies, despite ongoing Gudair® vaccination.

In a similar finding, farms surrounded by a greater number of sheep flocks or a greater number of infected flocks were more likely to be positive for Mptb. These flocks could have potentially got reinfected from neighbouring farms, thus reducing the effectiveness of the vaccine. While almost all farmers reported having a sheep proof fence around their property, many admitted to having faults in their fences, sometimes with long periods of time elapsing prior to repairs being made. The movement of sheep between neighbouring properties or sharing of resources between farms can introduce Mptb pathogen into the flock, even if the sheep are vaccinated, as the Gudair® vaccine

only delays the onset of disease and shedding, rather than preventing infection (Reddacliff *et al.* 2006). Furthermore, this is a general biosecurity risk for a range of disease. Surprisingly, sharing and straying practices were not found to be different between infected and non-infected farms, meaning that further investigation of this issue is required. Regardless, the association of the number of infected neighbouring flocks with OJD positive status suggests that strict biosecurity measures, including increased farm containment, should be maintained to prevent straying sheep from entering the farm. It also suggests that a group approach will improve the efficacy of OJD control by vaccination at regional and national levels (Eppleston *et al.* 2011).

In this study, a range of wildlife including kangaroos, deer, foxes, and rabbits were found to be present in varying prevalence levels on the sampled properties. However, they were not associated with increased risk of OJD. Although Mptb can infect a wide range of wild ruminant species, our results align with those of Dhand *et al.* (2007) and Corn *et al.* (2005) who similarly showed that wildlife did not contribute to the lateral spread of OJD through Mptb pasture contamination, especially in flocks that were already infected. Similarly, poor water management is often considered as a mechanism for the spread of diseases between properties, but there was no significant association between runoff water and OJD prevalence in this study. Conversely, water has previously been shown to be a significant reservoir of Mptb infection, posing a greater risk than pasture or soil environments for the long-term persistence of Mptb (Whittington *et al.* 2005). Mptb has been reported to remain viable in dam and trough water environments for 36 to 48 weeks, for semi-exposed and shaded environments, respectively, acting as a lasting source of infection (Whittington *et al.* 2005). A previous study also showed that farms with a greater proportion of their boundary receiving runoff water were associated with a lower prevalence of OJD (Dhand *et al.* 2007). The differences in findings suggest that further work is necessary to uncover when Mptb is present in water sources on the farm and when it can pose as a significant transmission risk of OJD. In spite of our negative results, it is still important to acknowledge that the provision of water to stock is another biological plausible factor that may affect OJD prevalence on the farm.

Our study had several strengths and limitations. We used a systematic approach to select farmers. They were also interviewed in person instead of asking them to self-complete questionnaires, thus minimising the chances of misclassification bias. Further, we collected and tested faecal samples in the laboratory to create the two outcome variables used in the study, rather than relying solely on farmer reported prevalence which substantially reduced the potential for information bias for outcome variables in the study. Further, the reduction in the pool size from the previous recommended pool size of 50 to 25 in our study enabled us to increase the number of pools from seven to 14. Although the total sample size per farm remained the same as the recommended sample size of 350, this approach of reducing the pool size increased the flock sensitivity (Dhand *et al.* 2010), thus enabling us to detect Mptb from pooled faeces of flocks with very low disease prevalence levels using both PFC and Mptb IS*900* qPCR. Nonetheless, a cross-sectional questionnaire study like this one makes it difficult to completely remove bias associated with the explanatory variables (Dhand *et al.* 2007; Windsor *et al.* 2014). The questionnaire covered a range of topics, such as farming enterprise, OJD infection history, control and biosecurity. The responses of individual farmers to each of the topics was dependent on farmer recall, potentially causing misclassification bias. In addition, this analysis was conducted at a population level from sheep farmers located in the south-eastern region of Australia (NSW and VIC) that utilised Gudair® as a control strategy, thus the results should be extrapolated for individual farms with caution.

For the second study, although we achieved a good sample size of 195 producers, the response rate is likely to be low as a much larger number of producers had the opportunity to respond. If those continuing to vaccinate were more likely to respond to the survey, this could have caused a selection bias and inflated the proportion of producers continuing to vaccinate. Further, as the survey was circulated via industry bodies, the study population of producers included members of industry bodies or those who pay a levy to Meat and Livestock Australia. These groups are more likely to be aware of recommendations regarding OJD control and are more likely to continue vaccinating than the general population of sheep producers in Australia. Therefore, we acknowledge that the proportion of producers found to be continuing to vaccinate in this study could be a slight overestimate.

Alternative explanatory variables for the differences in OJD prevalence between vaccinating flocks include host and pathogen factors which were not the focus of this study. As Gudair® vaccine does not prevent infection, there is the potential for persistence of Mptb or selection of strains with increased virulence. Evolution of more virulent pathogen strains in resistant populations has been reported for viral diseases. Positive selection of virulent strains of other mycobacteria has been reported, though a similar virulence distinction between Mptb strains has not been identified, other than the significant differences between Sheep (Type I and III) and Cattle (Type II) strain types. Thus, further studies are required to investigate whether vaccination can lead to the persistence of more virulent Mptb strains, as population-level host resistance increases.

6.5 Conclusion/Recommendations – Sub-project 5

The long-term implementation of the Gudair® vaccine is known to reduce OJD prevalence level but the effectiveness of the vaccine varies between farms. This study was conducted to investigate the reasons for this differential effectiveness of the vaccine between flocks. The study found that farming practices such as supplementary feeding the animals on the ground and introducing animals from multiple properties or properties with unknown OJD status can contribute to an OJD positivity status and prevalence for farms participating in the vaccination program. Therefore, sheep farmers adopting vaccination to control OJD should also be advised to continue to implement other risk management strategies. In particular, they should provide supplementary feed off the ground in troughs and to source replacement sheep including rams from reliable low-risk providers. The findings from this study are expected to inform decision making and implementation of appropriate biosecurity measures by farmers. The results will also assist veterinarians in providing the best advice to farmers in order to minimize the risk of transmission of Mptb from purchasing new sheep of multiple origins.

It is encouraging to note that a vast majority of sheep producers are continuing to vaccinate their sheep with Gudair®. Ongoing vaccination of new flock members is the current best-practice to ensure that the disease is well-managed and to help alleviate the financial burdens associated with production losses from OJD for Australian sheep farmers. [11]

7.5 Key Messages – Sub-project 5

- **Continuation of the vaccination program is critical**
  It is encouraging to note that 88% of sheep producers surveyed had continued to vaccinate their sheep with Gudair ®. The study confirmed that the Gudiar® vaccine substantially reduces clinical disease but animals on some farms continue to shed Mptb in faeces. Therefore, it is critical for producers to continue to vaccinate their sheep.

- **Discourage supplementary feeding on the ground**
  In this study, the flocks that were provided with supplementary feed on the ground were more likely to be Mptb positive and to have a higher prevalence of OJD, compared to flocks that were trough fed. Therefore, farmers should be discouraged to provide supplementary feed on the ground. Further investigations should be conducted to confirm this association in an experimental study.

- **Encourage producers to source replacement sheep from reliable low-risk providers**
  In this study, farms introducing rams or ewes from several sources in the past five years prior to sampling were at a higher risk of Mptb positivity and a higher OJD prevalence. The risk of introducing unvaccinated sheep from properties with unknown OJD status or not vaccinating animals on arrival can compromise existing vaccination efforts. Therefore, producers should be advised to source replacement sheep, including rams, from reliable low-risk providers.

- **Vaccination should be complemented with biosecurity and other OJD control strategies**
  Our study suggests that vaccination should be complemented with biosecurity and OJD control strategies for a successful control/ elimination of the disease. This message should be communicated to producers and industry bodies for an effective control of OJD in Australia.

8.5 Bibliography – Sub-project 5

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Appendices

1 Sub-project 1: Understanding gut pathogen inter-relationships

2 Sub-project 2: Genetic markers for a resilience phenotype

Fig. 3.2.3.1 Sample-wise call rate
Fig. 3.2.3.2 SNP-wise call rate
Fig. 3.2.3.3 MAF frequency distribution across 56,249 SNPs
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Summary 4.2.2.1 GWAS summary and top 10 SNP associations with Resilience Index (P.PSH.0813)
Plots 4.2.2.2 Chromosome-wise plots of SNP associations with Resilience Index (P.PSH.0813)
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Summary 4.2.4.1 GWAS summary and top 10 SNP associations with Resilience Index (OJD.028)
Plots 4.2.4.2 Chromosome-wise plots of SNP associations with Resilience Index (OJD.028)

3 Sub-project 3: Host response biomarkers

Appendix 3A Immunological biomarkers for disease susceptibility and resilience
Appendix 3B Predictive markers in parasitic co-infections
Appendix 3C Novel biomarker discovery
Appendix 3D Biomarkers for resilience (publication)
Appendix 3E miRNA isolation & quantification
Appendix 3F miRNA-206 expression is immunosuppressive
Appendix 3G Correlates of vaccine protection

4 Sub-project 4: Pathogen-host interactions

Appendix 4.1 – Lit Review
Appendix 4.2 – S strain in cattle
Appendix 4.3 – Mixed infection
Appendix 4.4 – Mycobacterial WG
Appendix 4.5 – Bachmann paper
Appendix 4.6 – Evolution and natural
Appendix 4.7 – Mizzi et al

5 Sub-project 5: Mptb shedding in vaccinates

Appendix Management factors with OJD