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Immune responses to *Campylobacter hepaticus* infection: impact on recovery from Spotty Liver Disease in chickens

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Project Summary

Project Title	Immune responses to *Campylobacter hepaticus* infection: impact on recovery from Spotty Liver Disease in chickens
Project No.	20-226
Date	Start: 15 April 2021 End: 1 December 2023
Project Leader(s)	A/ Prof. Thi Thu Hao Van
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Project Aim	The aims of this project are to investigate the ability of chickens to be reinfected following a first round of *C. hepaticus* exposure and recovery, to determine the role of immune responses (antibody, cytokines, and tight junction proteins) in resisting reinfection. A second aim is to study immunological responses of a large number of birds from commercial flocks to further our understanding of the epidemiology of the disease.
Background	Spotty Liver Disease (SLD) causes a serious risk to the egg production industry, reducing egg production 10–25% and increasing flock mortalities by up to 10% (Crawshaw & Young 2003). The cause of the disease had been a mystery for over 60 years, until a group in the UK reported the isolation of a new bacterium in chickens infected with SLD (Crawshaw et al. 2015) and our group characterised and formally named the bacterium that causes SLD, *Campylobacter hepaticus*, and then identified another new bacterium, *Campylobacter bilis* (Van et al. 2016; Van et al. 2023). Currently, antibiotic therapy is the most effective way to control SLD, but antibiotic resistant field isolates of *C. hepaticus* have already been identified. Understanding the epidemiology of SLD and how birds respond to *C. hepaticus* infection is important in designing and applying appropriate biosecurity standards and for developing ways to control the disease.
Research Outcome	This project demonstrates that birds infected with *C. hepaticus* two or three times provide a partial level of protection and do not develop any further liver lesions upon reinfection. Antibody levels increase six weeks after a single infection and significantly reduce nine weeks post infection. *C. hepaticus* was present in the gut of 50% of birds six weeks after a single infection. Changes in gene expression of tight junction proteins (ZO1 and ZO2) and pro-inflammatory cytokines were observed, particularly in birds challenged twice. A survey of the immune responses of *C. hepaticus* infected birds in commercial flocks revealed seroprevalence of between 2 and 64% in farms with a history of SLD. In non-SLD farms, one farm had a seroprevalence of 41%. No correlation was observed between the flock size or flock age and ELISA or PCR outcomes, and no significant difference between the seroprevalence of anti-*C. hepaticus* antibodies among flocks with or without a known history of SLD was established.
Impacts and Outcomes	The outcomes of this study provide a much better understanding of the immunological responses of birds infected with SLD. The assays developed in the project identified birds with current infections and also birds that had previously been infected with SLD. This informs future directions of research in relation to improvements to current biosecurity measures, vaccine and feed additive development, in order to control the spread of SLD in flocks. In particular, it

	indicates that it should be possible to develop a vaccine that could protect hens from the clinical outcomes of SLD.
Publications	Muralidharan C, Huang J, Anwar A, Scott PC, Moore RJ and Van TTH. (2022). Prevalence of *Campylobacter hepaticus* specific antibodies among commercial free range layers in Australia. *Veterinary Science,* 9:1058110. Eastwood S, Wilson TB, Scott PC, Moore RJ, Van TTH. Immune responses to *Campylobacter hepaticus* infections and the impact on recovery from Spotty Liver Disease in chickens (*in preparation*).

Executive Summary

The aim of this project was to investigate the immune responses produced by *C. hepaticus* infection and their effects on limiting disease development following subsequent infections. Our approach was to (i) assay a variety of genes to measure any differences in immune responses between birds via quantitative PCR (qPCR) and compare this to other methods such as commercialised ELISA kits; (ii) using our selected methodologies, investigate the ability of chickens to be reinfected, following a first round of *C. hepaticus* exposure and recovery; and (iii) to investigate the immune responses of *C. hepaticus* in large commercial flocks at varying times and locations.

For the first milestone, we have established two quantitative methods to measure aspects of the immune responses of birds. Assays for qPCR assessment of gene expression have been refined and validated for a collection of interleukins and housekeeping genes. The performance of commercial interleukin ELISA assays and the developed qPCR methods were compared by measuring differences in serum levels of IL-1β, IL-8. and IL-6. It was found that qPCR proved to be more sensitive. Given the results of the comparison of the two methods, and considering cost and throughput issues, it is proposed that qPCR should be the principal assay used in the next phase of the project.

For the second milestone, the animal trial demonstrated that birds exposed to *C. hepaticus* for the first time always developed SLD liver lesions, whereas after recovery and reinfection six weeks after the first infection, hens did not develop liver lesions. We observed a continued rise in anti-*C. hepaticus* antibody levels up to six weeks after a single infection and this acted as a marker indicating that birds would be resistant to liver lesion formation upon further reinfections. *C. hepaticus* was present in the gut of 50% of chickens six weeks post-infection, and decreased to 17% after nine weeks with reinfection not increasing persistence in the caeca. *C. hepaticus* could be recovered from the bile six weeks after a single infection and increased after a second infection. Cytokine gene expression analysis indicated that colonisation and translocation of *C. hepaticus* in the host gut induced significant inflammatory responses. Decreased gene expression of barrier forming CLDN5 suggests that paracellular migration across the intestinal epithelial barrier during early stages of infection may be possible whereas upregulation of tight junction proteins (ZO1 and ZO2) indicated an epithelial recovery phase that may inhibit the translocation of *C. hepaticus* across the gut barrier. Movement of *C. hepaticus* across the intestinal barrier may alternate between paracellular or transcellular modes depending on the stage of infection; similar to the seen with *C. jejuni.*

For the third milestone, *C. hepaticus* specific antibodies were detected from birds in four of the five farms that had no history of SLD, with seroprevalence as high as 41% in one of the farms. The findings of such high levels of both PCR positive and ELISA positive birds in the non-SLD flocks were unexpected. It indicates that there is a lot more *C. hepaticus* circulating across the egg industry than previously realised. Factors including the absence of predisposing factors or exposure to non-pathogenic *C. hepaticus* strains may be responsible for this finding. Among flocks that had active or previous SLD outbreaks, the presence of anti-*C. hepaticus* antibodies varied between 2 and 64%. Variability in immune responses could be attributed to different virulent strains of *C. hepaticus.* Lastly, no significant difference between the seroprevalence of anti-*C. hepaticus* antibodies among flocks with or without a known history of SLD was established. Distinguishing the cause of differences in immune response between flocks is important as it may influence how biosecurity measures and potential treatment options are applied.

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1. Introduction

Spotty Liver Disease (SLD) presents one of the most pressing challenges for the Australian poultry industry, especially among free range layer flocks but is also seen in other chickens. The disease is characterised by the formation of whitish-grey liver lesions, an increase in mortalities of up to 10% and a reduction in egg production between 10 and 25% (Crawshaw & Young 2003; Burch 2008). The cause of the disease had been a mystery for over 60 years, until several years ago when a group in the UK reported the isolation of a new bacterium in chickens infected with SLD (Crawshaw et al. 2021). Our group characterised and formally named the bacterium that causes SLD, *Campylobacter hepaticus* (Van et al. 2017). Several years later, we found that *C. bilis*, a second novel species of *Campylobacter* that we have identified, can also cause the disease (Phung et al. 2022).

Current strategies in place to reduce the incidence of SLD include maintaining high level biosecurity and animal husbandry (Groves 2010; Grimes & Reece 2011). The addition of probiotics and phytobiotics to feed have provided limited protection (Quinteros et al. 2021; Scott et al. 2020). Antibiotic treatment using chlortetracycline or Linco-Spectin® is the primary treatment of SLD, however, its efficacy has been compromised by the emergence of antibiotic resistance in some SLD outbreaks (Grimes & Reece 2011; Scott 2016). Conventional killed autogenous vaccines have shown limited efficacy (Scott et al. 2020). Thus, the control of the disease may be reliant on understanding the epidemiology of the disease and eliminating the vectors or predisposing factors, or the development of more effective vaccine strategies. To address this, the focus has shifted to understanding the pathogenesis of SLD and the immune responses of infected birds, with a particular emphasis on the role of cytokines during SLD reinfection and recovery.

Thanks to the support of Poultry Hub Australia (PHA) our SLD epidemiology project concluded that only 50% of birds in SLD infected flocks contained detectable levels of *C. hepaticus* DNA via PCR (Phung et al. 2020). Tools for monitoring SLD prevalence such as PCR can only detect active infections. We have further developed two enzyme-linked immunosorbent assays (ELISAs) to detect anti-*C. hepaticus* antibodies in chickens, therefore the results can inform past and current infections.

In this project we aimed to provide an extension to the developed monitoring tools (PCR and ELISA) and study the role of immune responses (antibodies, cytokines, and tight junction proteins) in resisting reinfections – aims (i) and (ii). The third aim was to use the developed monitoring tools to survey the immunological responses in large numbers of commercial flocks to investigate the incidence of SLD in symptomatic and asymptomatic birds. Understanding the immunological responses and epidemiology of the disease will influence how biosecurity measures and treatment options such as vaccination and/or colonisation with non-pathogenic strains of *C. hepaticus* could be implemented.

2. Objectives

The objectives of the project were:

1. Develop and optimise methods to study anti-*C. hepaticus* cytokine and tight junction protein responses.
2. Investigate the ability of chickens to be reinfected after *C. hepaticus* exposure and the role of immune responses to reinfection/fighting against future infections.
3. Large scale investigation of chicken antibody responses to *C. hepaticus* in commercial flocks.

3. Methodology

3.1. Development and optimisation of methods to study anti-*C. hepaticus* cytokine and tight junction protein responses

3.1.1. RNA extraction and cDNA synthesis

All of the samples used for the optimisation of cytokine/tight junction protein primers and antibody responses were collected from an unrelated probiotic trial, as the *C. hepaticus* challenge trial for this project had not started until the commencement of the second milestone.

Sections of chicken spleen, ileum, and bursa were stored in 5 mL of RNAlater and vials were stored at -20°C until processed. Approximately 10 mg of each tissue was thoroughly washed in 1 x PBS and was extracted using the RNA MiniPrep Kit (Monarch, New England Biolabs) as per the manufacturer's specifications. RNA quality was assessed using Qubit fluorescence (Thermo Fisher Scientific) and denaturing gel electrophoresis. Purified RNA was aliquoted and stored at -80°C until cDNA conversion. RNA (1 µg) was converted to cDNA using the SuperScript™ First-Stand Synthesis System (Invitrogen). cDNA was quantified using Qubit fluorescence, diluted 1:5 times in nuclease free water and stored at -80°C until qPCR was performed.

3.1.2. Optimisation of qPCR for the study of tight junction protein and cytokine expression in chicken tissues

End point PCR was first used to assess the primers used for amplification. End-point PCR was carried out in a final volume of 20 µL using Q5 High-Fidelity 2x Master Mix (New England Biolabs), with a primer concentration of 250 nM each and 50-100 ng of cDNA template. An Eppendorf Mastercycler Pro PCR instrument was used for amplification with cycling conditions of: 98°C for 1 min, 35 cycles of 98°C for 10 s; 57°C for 30 s and 72°C for 30 s, and final extension at 72°C for 10 min. PCR products were run on an agarose gel and visualised, and images captured using a Bio-Rad Gel Doc XR System.

After amplicon assessment via end-point PCR, genes were selected for further optimisation, i.e. efficacy and melting temperature analysis for selected genes. Targeted amplification efficiencies range from 90% to 110%. qPCR was carried out in a final volume of 10 µL using 2x KAPA SYBR® FAST mastermix (Roche) with a primer concentration of 200 nM each and 10 ng of cDNA isolated from spleen tissue. A CFX Connect Real-Time System (Bio-Rad) instrument was used with the following temperature cycling conditions: 95°C for 3 min, 39 cycles of 95°C for 10 s; 60°C for 30 s and 72°C for 30 s.

Table 1 Primers used for gene expression study

Gene	Primer sequence (5'>3')	Size of Amplicon	Accession Number	Reference
GAPDH	F:CCTAGGATACACAGAGGACCAGGTT R:GGTGGAGGAATGGCTGTCA	64	NM_204460.1	Calik et al. 2019
Actin-B	F:CCAGACATCAGGGTGTGATGG R:CTCCATATCATCCCAGTTGGTGA	137	AJ719605	Borowska et al. 2016
ZO1	F:GGAGTACGAGCAGTCAACATAC R:GAGGCGCACGATCTTCATAA	101	XM_413773	Emami et al. 2019
ZO2	F:GCGTCCCATCCTGAGAAATAC R:CTTGTTCACTCCCTTCCTCTTC	89	NM_204918	Emami et al. 2019
CLDN5	F:GCAGGTCGCCAGAGATACAG R:CCACGAAGCCTCTCATAGCC	162	NM_204201	Dao et al. 2022
IL-6	F:GCGAGAACAGCATGGAGATG R:GTAGGTCTGAAAGGCGAACAG	143	NM_204628	Jiang et al. 2011
IL-1B	F:GGTCAACATCGCCACCTACA R:CATACGAGATGCAAACCAGCAA	86	NM_204524.1	Guo et al. 2022

3.1.3. Interleukin enzyme-linked immunosorbent assay (ELISA) to compare with qPCR

Birds that showed differential interleukin expression compared to the control birds were selected for testing using commercial ELISA kits. Chicken Interleukin IL-6, IL-8 and IL-1β coated plates from Cusabio© were used. To optimise the dilution of serum required for the assays, serum samples were serially diluted and repeated over 2 experiments. The dilution that resulted in optical density (OD) values within the range of the kit's provided standards (0-1000 pg/mL) was selected as the optimal serum dilution. Serum and standard samples were incubated for 2 hours at room temperature in a total of 100 µL. Plates were incubated with 100 µL of biotin primary antibody for 1 hr at 37°C and incubated with 100 µL HRP-avidin for 1 hr at 37°C. 90 µL of TMB Substrate was added and incubated for 30 min. To stop colour development, 50 uL of stop solution (2 M Sulphuric acid) was added. Absorbances were read at 450 nm using an Omega Plate Reader (BMG Labtech).

3.2. Investigation of the ability of chickens to be reinfected after *C. hepaticus* exposure and the role of immune responses in limiting liver lesion development in reinfected hens

3.2.1. Animal trial and sample collection

The animal experimentation was approved by the Wildlife and Small Institutions Animal Ethics Committee of the Victorian Department of Economic Development, Jobs, Transport and Resources (approval no. 14.16). *C. hepaticus* HV10^T was grown in Brucella broth supplemented with L-cysteine (0.4 mM), and L-glutamine (4 mM) and sodium pyruvate (10 mM) in tissue culture T75 flasks at 37°C for 48 h in microaerophilic conditions (Phung et al. 2021) and used directly for the challenge. A total of 120 laying hens were used in this trial. Chickens were housed in groups of 3 birds per pen, with 4 pens per group; a total of 12 birds per group (n = 12) and 10 groups. Unchallenged control birds (Groups 2, 4 and 8) were orally inoculated with 1 mL of sterile Brucella broth, and challenged birds were orally dosed 1, 2 or 3 times (6 weeks apart each time) with 1 mL of Brucella broth containing

$1x10^9$ CFU/mL of *C. hepaticus.* The groups used in the trial are summarised in Table 2. Birds were sacrificed 6 days after inoculation and SLD lesions observed on the liver surface were enumerated. Blood was collected to monitor antibody levels and cloacal swabs were collected every 3 weeks to determine the presence of *C. hepaticus.* Bile samples were collected to determine recovery of *C. hepaticus* after each exposure timepoint. Spleen and jejunum samples were collected to measure cytokine and tight junction protein (TJP) responses in infected chickens, respectively.

Table 2 Design of infection and reinfection animal trial

Treatment	Group									
	1	2	3	4	5	6	7	8	9	10
1st challenge	+	-	+	-	+	-	+	-	+	-
1st necropsy	K	K								
2nd challenge			+	-	-	+	+	-	-	-
2nd necropsy			K	K	K	K				
3rd challenge							+	-	+	+
3rd necropsy							K	K	K	K

Positive control groups 1, 6 and 10 were challenged once.
Groups 2, 4 and 8 were negative control groups.
Test groups: + = infection with *C. hepaticus* HV10^T; - = inoculated with Brucella broth.
K = groups necropsied 6 days after inoculation with either sterile broth or *C. hepaticus*.
Each challenge was 6 weeks apart.
There were 12 hens per group.

3.2.2. DNA extractions

DNA from cloacal swabs, caecum contents, and bile samples were prepared using the DNeasy PowerSoil Kit (Qiagen) according to the manufacturer's instructions. Upon collection, cloacal swabs were resuspended in 150 µL of nuclease-free water and stored at -20°C until DNA was extracted. Total DNA concentrations were measured using NanoDrop and Qubit™ fluorometric quantitation (Thermo Fisher Scientific). Aliquots were stored at -20°C until further use.

3.2.3. Polymerase chain reaction (PCR)

Isolated DNA from cloacal swabs was subjected to PCR amplification to detect the presence of *C. hepaticus* DNA. PCR primers specific to *C. hepaticus* were used as previously described by Van et al. (2017). End point PCR was carried out in a final volume of 20 µL using MyTaq™ 2x Master Mix (Bioline), primers at a final concentration of 250 mM each and 1 µL of template DNA. An Eppendorf Mastercycler Pro PCR instrument was used for amplification with cycling conditions of: 95°C for 1 min, 35 cycles of 95°C for 30 s; 56°C for 30 s, 72°C for 10 s and final extension for 72°C for 5 min. PCR with universal primers targeting conserved bacterial 16S rRNA gene sequences was carried out as a positive control for PCR to demonstrate appropriate quality of all DNA templates.

3.2.4. Recovery, identification and quantitation of *C. hepaticus*

To isolate *C. hepaticus,* bile samples were directly streaked onto HBA plates. Remaining samples were stored at -20°C until DNA extraction. The identity of *C. hepaticus*-like colonies was confirmed by matrix assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS) using a Microflex LT mass spectrometer (Bruker MALDI Biotyper System, Bruker Daltonics) according to the manufacturer's instructions. Quantitation of *C. hepaticus* in bile was done using RT-PCR as described by Van et al. (2017) in a total of 10 µL in triplicates. DNA derived from a culture of known CFU of

C. hepaticus HV10^T was serially diluted 10-fold and used to generate a standard curve to determine the efficiency of the reaction.

3.2.5. Quantitative PCR (qPCR) to measure tight junction protein and cytokine gene expression in infected chickens

Spleen and jejunum samples were collected in 5 mL of RNAlater™ (Invitrogen) to measure tight junction protein and cytokine gene expression. Samples were stored at -20°C until ready for extraction. Total RNA was extracted using Maxwell® RSC simplyRNA Tissue Kit (Promega) as per the manufacturer's instructions and reverse transcribed to cDNA using SuperScript™ IV First-Strand Synthesis System (Thermo Fisher Scientific). Quantitative PCR was performed using 2x KAPA SYBR® FAST mastermix (Roche) on the CFX Connect Real-Time System (Bio-Rad) with the following temperature cycling conditions: 95°C for 3 min, 39 cycles of 95°C for 10 s; 60°C for 30 s and 72°C for 30 s. Gene expression was normalised against two reference genes using the formula:

$$Relative\ gene\ expression = \frac{(E_{\mathrm{GOI}})^{\Delta Ct\ GOI}}{GeoMean[(E_{REF})^{\Delta Ct\ REF}]}$$

The mRNA levels were analysed for the following genes: ZO1, ZO2, CLDN5, IL6, IL1B, GAPDH and actin-B (Table 1).

3.2.6. Preparation of total protein extracts (TPE) for ELISA

C. hepaticus HV10^T (NCBI Accession number CP031611) was grown on horse blood agar (HBA) plates (Brucella broth (Oxoid) supplemented with 1.5% agar (Oxoid) and 5% horse blood (Equicell)) under microaerophilic conditions using Campygen packs (Oxoid). The bacterial cells were harvested in 500 µL phosphate buffered saline (PBS) and stored at -20°C for 2 hours or overnight to enhance the cell disruption efficiency. The cell suspension was thawed on ice and sonicated to obtain the whole cell lysate (WCL). WCL was centrifuged at 8000 x g for 10 minutes to collect the supernatant, which was named the total protein extract (TPE). The standardised TPE solution (OD_{280}= 1) was used as the antigen to coat wells for the ELISA assay.

3.2.7. ELISA

Serum samples were collected in plain tubes every 3 weeks to monitor *C. hepaticus* antibodies over the course of the trial. The blood samples were centrifuged at 2000 x g for 30 min and the resulting serum layer was taken and stored at -20°C. The full outline of the assay is detailed in Muralidharan et al. (2020). Briefly, *C. hepaticus*-specific antibodies were measured by coating wells with *C. hepaticus* total protein extracts (TPE) diluted 1:100 to an OD_{280} of 0.01. Non-specific binding sites were blocked using 5% skim milk. Primary chicken sera were diluted 1:1000 and probed with goat anti-chicken secondary antibody diluted 1:2000. Cross reactivity antibodies to *C. jejuni* proteins were reduced by pre-absorbing sera with *C. jejuni* TPE diluted to OD=0.1. All wash steps were done using PBS with 0.05% Tween 20. Novex™ HRP Chromogenic Substrate was added and absorbances were measured at 652 nm. The assay was completed once, and all sera samples were tested in triplicate.

3.2.8. Statistical analysis

All statistical analyses were carried out using GraphPad Prism (9.3.1) software (San Diego, CA, USA). The p-values were taken to be significant at the 95% confidence interval. The significance of *C. hepaticus* numbers in bile was calculated using unpaired t-test with Welch's correction. Correlation of spleen and body weights was determined using Pearson correlation with a 95% confidence level. The variance in antibody levels between each infection group through the trial was determined using unpaired t-test

and one-way ANOVA with the Geisser-Greenhouse correction used when $p = 0.05$. Lastly, gene expression data were analysed using unpaired t-test with Welch's correction. For groups that were not normally distributed, a Mann Whitney's t-test was implemented.

3.3. Large scale investigation *C. hepaticus* detection and chicken antibody responses to *C. hepaticus* in commercial flocks

3.3.1. Experimental design

Blood and cloacal swab samples (1,419) from a total of 709 birds were collected from 11 commercial free range layer farms in Australia over a period of 9 months. Farms 1, 3, 4, 5, 7, 10 and 11 were in Victoria. Farm 2 was in New South Wales and Farms 6A, 6B, 8 and 9 were in Queensland. The age of the layer flocks at the time of sample collection ranged from 33 to 64 weeks in different farms, and the number of birds in each flock ranged between 4,000 and 22,500. Blood and cloacal swab sample pairs were collected from 50 to 52 birds in each farm except for Farm 6. One hundred blood and cloacal swab sample pairs each were collected from flocks housed in two well separated sheds/ranges in Farm 6, one with an active SLD outbreak at the time of sample collection (referred as Farm 6A), and the other with no known history of SLD (referred as Farm 6B).

3.3.2. Collection of blood, cloacal and bile samples

Blood samples were collected and centrifuged at 2,000 × g for 15 min at 4°C to separate blood clots from the sera. The sera were transferred to Eppendorf tubes and stored at -20°C until used. The faecal material on cloacal swabs (sterile dry cotton swabs) was resuspended in 200 µL sterile phosphate buffered saline (PBS) solution and stored at -20°C until used. Bile samples were collected aseptically using syringe and needles from 4 to 7 birds that showed clinical SLD manifestations in Farm 2 and Farm 6A. They were plated on Brucella agar (BD) supplemented with 5% horse blood (Equicell, Australia) and incubated at 37°C under microaerobic conditions provided by Campygen gas packs (Thermo Fisher Scientific) for 2–5 days. The egg production and mortality data were collected from the farms several weeks post sample collection.

3.3.3. PCR to detect *C. hepaticus* DNA from cloacal swabs

DNA was extracted using the method specified in Section 3.2.2. *C. hepaticus* specific end-point PCR was performed as described in Section 3.2.3. Reactions were carried out in a final volume of 20 µL at a final primer concentration of 250 nM each using 5 µL of extracted DNA preparation.

3.3.4. SLD-ELISA2 to detect *C. hepaticus* specific antibodies

Plate wells were coated with 50 µL per well of 0.5 µg/mL purified $FHA_{1,628–1,899}$ protein in PBS (Muralidharan et al. 2022). The plates were incubated for 2 h at room temperature (RT) and washed with 200 µL PBS containing 0.05% Tween 20 (PBST). Non-specific binding sites were blocked using 200 µL of blocking solution (5% skim milk powder in PBS) and incubated overnight at 4°C. The wells were washed twice with PBST and 100 µL of chicken sera diluted a thousand-fold in blocking solution was added. The plates were incubated for 2 h at RT followed by four PBST washes. Goat anti-chicken IgY-HRP (Thermo Fisher Scientific) antibody diluted two thousand-fold in blocking solution was added and incubated for an hour at RT. After four PBST washes, 50 µL of Novex 3,3' ,5,5' -tetramethylbenzidine chromogenic substrate (TMB, Invitrogen) was added and incubated for 15 min, followed by the addition of 50 µL of 2 M sulphuric acid to stop the reaction. The absorbance was measured at 450 nm using a POLARstar Omega Plate Reader (BMG LABTECH).

3.3.5. Statistical analysis

All statistical analysis was carried out using GraphPad Prism (9.3.1) software (San Diego, CA, USA). Correlation between the flock size, flock age, ELISA and PCR results was determined using multiple variable analysis correlation matrix by assuming Gaussian distribution of samples. The p-value was calculated at 95% confidence interval. Correlation between the sample collection time post first SLD outbreak and percentage of birds tested positive to SLD-ELISA2, and PCR was also calculated. The seroprevalence of anti-*C. hepaticus* antibodies among flocks with and without a known history of SLD was compared using the Mann-Whitney non-parametric t-test.

4. Results and discussions

4.1. Comparison of qPCR and commercial ELISA for the evaluation of interleukin expression

4.1.1. qPCR

To determine whether commercial interleukin assay ELISA kits were more efficient at differentiating immune responses on a large scale compared to qPCR assays, the IL-6, IL-8 and IL1-β genes were selected for further qPCR optimisation using RNA isolated from spleen tissue. Housekeeping or reference genes are a set of genes that are constantly expressed in cells/tissues and are essential for basic cellular function. β-actin and RPL30, as the best performing of the reference genes tested, were selected to be used as reference genes for the qPCR assays. These genes were used as internal controls to normalise the differential expression of cytokines and tight junction proteins. To ensure the reliability of the results the qPCR conditions were optimised to obtain amplification efficiencies of 90–110%, however, ideally the efficiency should be closer to 100%.

cDNA from spleen tissue was serially diluted 1:10 and assayed. The standard curve for β-actin generated an R^2 value of 0.9951% and a slope of -3.3977, translating to an efficiency of 97.03%. The standard curve for RPL30 generated an R^2 value of 0.9866% and a slope of -3.1677, translating to an efficiency of 106.9%. Based on both standard curves, a cDNA concentration of 10 ng was selected, as this concentration produced a Ct range of between 20-25 cycles (Figure 1).

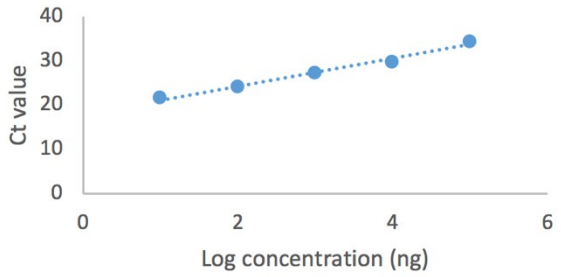

Figure 1 Standard curves for β-actin and RPL30, respectively

Similarly, the primers for cytokine genes IL-6, IL-8, and IL1-β were optimised. 10 ng of cDNA of each sample was used for the assays and expression was normalised to the reference genes RPL30 and β-actin. In summary, significant differences in gene expression levels between control and dosed birds could be observed. Dosed birds 1, 4, 5 and 6 showed upregulation of IL1-β gene expression compared to the control group ($p < 0.05$), whereas dosed bird 8 showed significantly higher IL-8 gene expression and dosed bird 6 showed significantly higher IL-6 gene expression compared to the control birds (Figure 2).

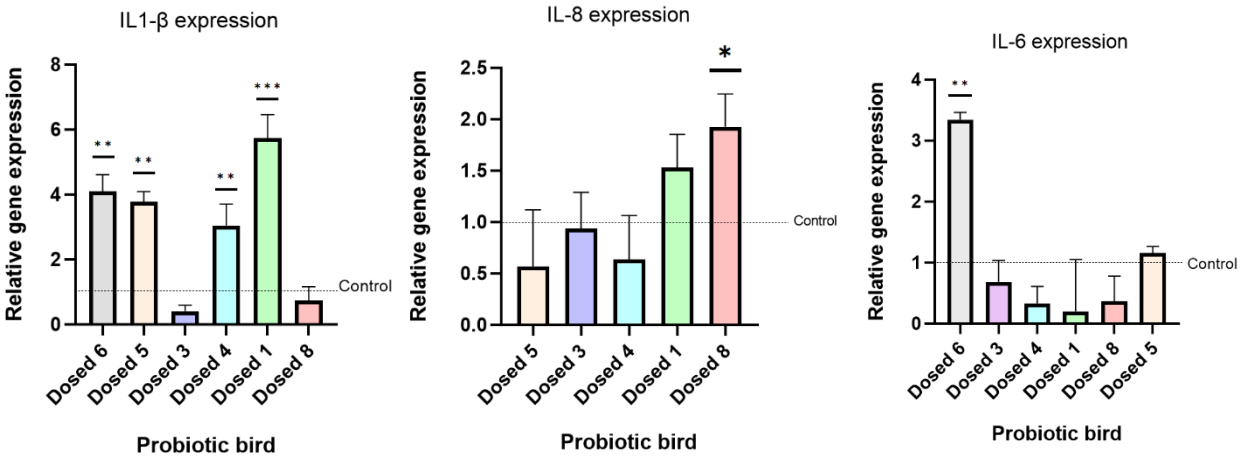

Figure 2 Relative gene expression of IL1-β, IL-8 and IL-6 in birds dosed with various probiotics

Expression was normalised to RPL30 and β-actin and data are represented as means ± SEM (n=3), relative to the control group (represented with a solid line, with a threshold of 1).

Significant difference between control and dosed birds (t-test) are represented as * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.005$).

4.1.2. Measurement of cytokine levels in sera using commercial ELISA kits

Dosed birds that showed differential cytokine expression compared to the control were selected for the ELISA assays. Optimisation of commercial ELISA assay plates was performed to provide the best resolution and comparison between control and dosed bird groups. For the IL-8 assay, a serum dilution of 1:125 was used across all samples; 1:100 serum dilution for IL1-β; and 1:2 for IL-6 (Figure 3). These dilutions were chosen to ensure that all the sample OD values were within the dynamic range of the assay based on the kits provided standards.

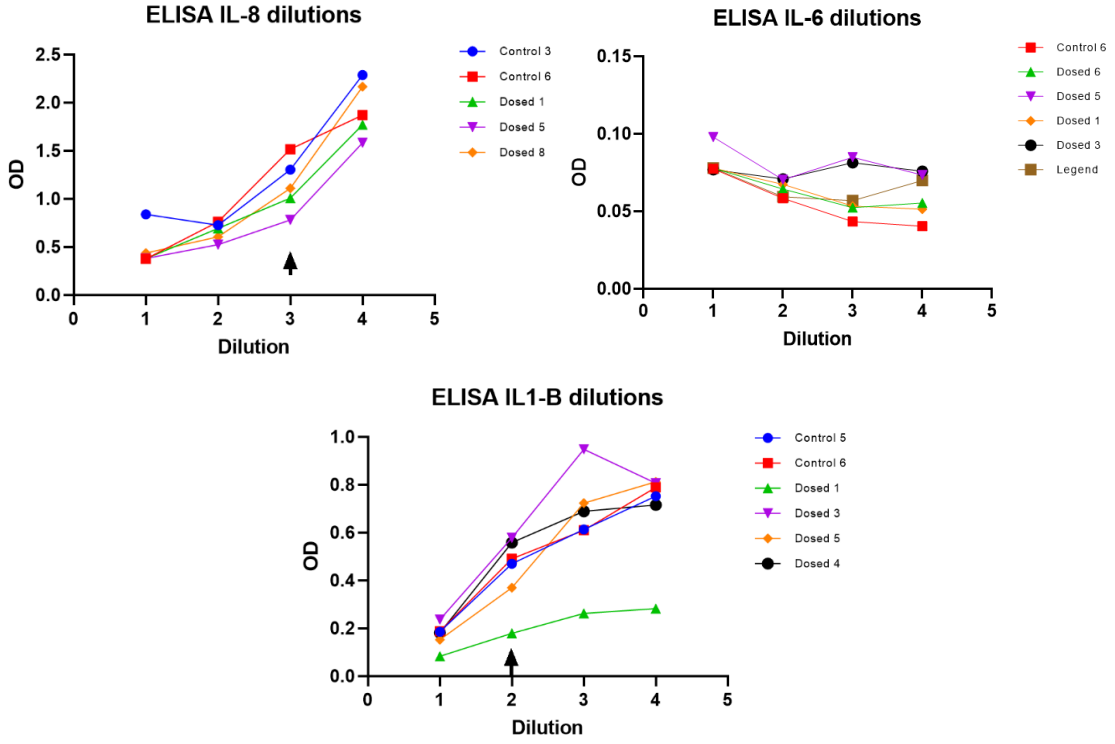

Figure 3 Optimisation of interleukin assays using ELISA kits

For IL-8, samples were serially diluted 1:5 from neat serum (axis mark 1) to 1:125 (axis mark 4).
For IL1-β, samples were serially diluted 1:10 (axis mark 1) to 1:10,000 (axis mark 4).
For IL-6, samples were serially diluted 1:2 (axis mark 1) to 1:16 (axis mark 4).
OD measurements are represented as means ± SEM (n=2), repeated in 2 independent experiments.

Based on the selected serial dilutions for each interleukin highlighted in Figure 3**,** sera were tested again, and values calculated based on the corresponding standard curve. Dosed bird 1 had a significant difference in IL1-β concentration (1873 pg/mL) compared to the controls (63.9 pg/mL) ($p = 0029$). Dosed bird 5 had the second highest concentration at 153 pg/mL, however, was borderline significant with a p-value of 0.0597 (Table 2). These results match the gene expression seen in the qPCR assays. However, qPCR showed that in addition to the dosed birds 1 and 5, dosed birds 4 also showed upregulation of IL1-β gene expression compared to the control group ($p < 0.05$).

Table 2 Averaged OD values and IL1-β concentrations calculated from standard curve above

Sample	OD	IL1-β concentration (pg/mL)
Control 5	0.4695	68.57
Control 6	0.49	59.20
Dosed 1	0.179	1872.8*
Dosed 3	0.578	32.93
Dosed 5	0.3695	153.04
Dosed 4	0.5575	37.55

For the IL-8 ELISA, dosed birds 1 and 8 had similar elevated concentrations, and dosed bird 5 had the highest IL-8 level of 31.05 pg/ml (p-value of 0.097, not statistically significant) (Table 3). The qPCR results also showed that dosed bird 8 had the highest IL-8 gene expression, statistically significantly higher than the controls.

Table 3 Averaged OD values and IL-8 concentrations interpolated from standard curve above

Sample	OD	IL-8 concentration (pg/mL)
Control 3	1.304	8.55
Control 6	1.515	5.025
Dosed 8	1.109	13.6
Dosed 1	1.007	17.365
Dosed 5	0.779	31.05

For the IL-6 assays, dosed bird 6 showed statistically significantly higher IL-6 protein levels compared to the control birds (Table 4). The result was in agreement with the qPCR result.

Table 4 Averaged OD values and IL-6 concentrations interpolated from standard curve above

Sample		OD	IL-6 concentration (pg/mL)
Control 5		0.0775	1.43
Control 6		0.078	1.47
Dosed 6		0.098	3.23*
Dosed 5		0.078	1.47
Dosed 1		0.077	1.39
Dosed 3		0.078	1.47

In summary, optimisation of commercial interleukin ELISA assays measured differences in serum levels of IL-1β, IL-8, and IL-6, however qPCR proved to be more sensitive. It is anticipated that using qPCR would be most beneficial and economical to measure immunological responses of *C. hepaticus* infected birds on a large population scale. Given the results of the comparison of the two methods and considering cost and throughput issues, it is proposed that qPCR should be the principal assay used in the next phase of the project. The advantage of using qPCR is the method can also be used to quantify the amount *C. hepaticus* in various tissues as well as measuring immune responses. The commercial ELISA kits may be used for 'spot' testing on samples of particular interest.

4.2. Investigation of the ability of chickens to be reinfected after *C. hepaticus* exposure and the role of immune responses in limiting liver lesion development in reinfected hens

4.2.1. *C. hepaticus* infection protects chickens from liver lesion development following subsequent *C. hepaticus* exposure

Six days after each challenge birds were necropsied, and their livers were scored for typical SLD lesions. The clinical presentation of SLD, prevalence of *C. hepaticus,* and spleen to body weight ratios are outlined in Table 5. All 12 birds in the positive control groups (Groups 1, 6 and 10) presented typical liver lesions, ranging in number from 10–1000+ lesions. As expected, no spots were observed in all negative control

groups (Groups 2, 4 and 8). After two reinfections, three birds from Group 3 presented with less than five spots. Birds challenged two and three times did not develop liver lesions.

Cloacal swabs were collected every three weeks to monitor the presence of *C. hepaticus* in infected birds. *C. hepaticus* was detected 7- and 9-weeks post challenge, as can be seen for Groups 5 and 9, respectively. Interestingly, the number of birds colonised with *C. hepaticus* reduced over time. In Group 9, at six weeks post-infection, 7 out of 12 birds had detectable *C. hepaticus* DNA in cloacal swabs whereas at nine weeks post-infection, only 2 out of 12 birds were positive. For birds reinfected three consecutive times (Group 7), only 5–6 birds had detectable *C. hepaticus* in their gut, however, *C. hepaticus* could be recovered from 11 birds in the bile collected 6 weeks after the first challenge. Similar results were observed for PCR conducted on caecum samples (data not shown).

Table 5 Prevalence of *C. hepaticus* from chickens during the reinfection trial spanning 12 weeks

For groups infected with *C. hepaticus,* I = Day of Infections (D) and K = days birds were killed and necropsied.

	Exposure combination	***Number of spots on liver/number of birds***	***Body weight to spleen ratio***	***Recovery from bile***	***PCR after 7 weeks***	***PCR after 9 weeks***
Group 1	I=D5 K=D11	10-500/12 birds	N/A	N/A	N/A	N/A
Group 2		0	N/A	N/A	N/A	N/A
Group 3	I=D5 & D47 K=D53	1-5/3 birds	0.12% ± 0.02780	10/12 (6 days)	N/A	N/A
Group 4		0	0.14% ± 0.02778	0/12	N/A	N/A
Group 5	I = D5 K=D53	0	0.10% ± 0.01567	11/12 (7 weeks)	7/12	N/A
Group 6	I = D47 K=D53	5-1000+/10 birds	0.15% ± 0.01476	8/12 (6 days)	N/A	N/A
Group 7	I=D5, D47 & D89 K=D53	0	0.11% ± 0.02314	11/12 (6 days)	6/12	5/12
Group 8		0	0.12% ± 0.02741	0/12	N/A	N/A
Group 9	I=D5 & D89 K=D95	0	0.10% ± 0.001567	12/12 (6 days)	7/12	2/12
Group 10	I=D89 K=D95	50-1000+/10 birds	0.18% ± 0.03562	11/12 (6 days)	N/A	N/A

12 birds/group.

N/A refers to appropriate methodology not being completed for that particular group.

For Groups 1 and 2, recovery of *C. hepaticus* was not performed via the plating method, however, was done by qPCR only.

Groups 1, 6 and 10: Positive control.

Groups 2, 4 and 8: Negative control.

Group 3: Challenge twice, 6 weeks apart between challenges and killed 6 days after the last challenge.

Group 5: Challenge once, killed 6 weeks and 6 days after the challenge.

Group 7: Challenged 3 times, killed 6 days after the last challenge.

Group 9: Challenged twice, 6 weeks apart between the challenges, and killed 6 days after the last challenge.

4.2.2. *C. hepaticus* reinfections reduces spleen body weight ratio

Spleen weights were calculated to evaluate whether there was an association between total body weight and organ weight during the reinfection stages. **T**he calculated ratio was represented as a percentage ± SD of the mean. Six days after the first infection, spleen weight ratio ($p = 0.0102$) significantly increased compared to the untreated groups. Two birds from Group 10, and 3 birds from Group 6 with the highest spleen weight also presented with the most liver lesions. Overall, there was no significant correlation between a single infection and spleen ratio (R^2 = 1.14% and 1.37% for Groups 6 and 10, respectively).

Secondary and third reinfections reduced spleen ratios. Twelve weeks between reinfection timepoints (Group 9) resulted in a mean average drop of 0.4% compared to untreated birds ($p = 0.0003$) (Figure 4) and a higher correlation (R^2 = 38%, $p = 0.032$). After 3 consecutive reinfections, the mean ratio for Group 7 decreased by 0.3% ($p = 0.0183$), however, the correlation for this group was not significant.

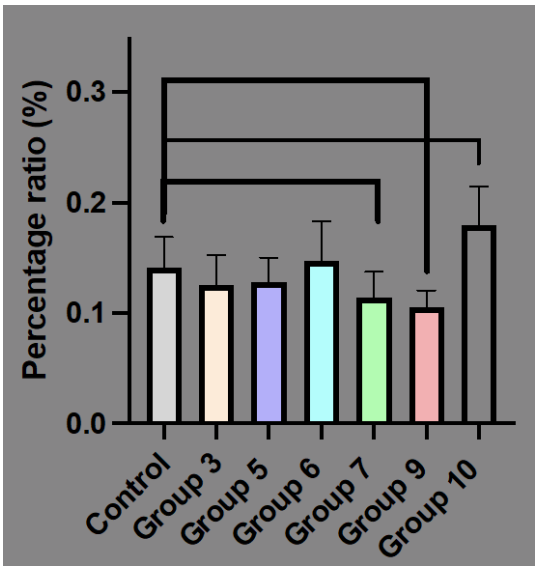

Figure 4 Spleen to body weight ratios presented as a percentage

Groups are as follows: untreated control group, secondary reinfections (Group 3), a 'long term' single infection (Group 5), two positive controls (Groups 6 & 10), third reinfections (Group 7) and staggered secondary reinfections (Group 9).

Percentages are represented as mean ± SD, n=12 birds per group.

Unpaired t-test using Welch's correction was used when $p<0.05$.

Correlation was determined using Pearson correlation with a 95% confidence level. * ($p<0.05$), *** ($p<0.005$).

4.2.3. *C. hepaticus* can be recovered from infected chickens 12 weeks after a challenge

To quantify the recovery of *C. hepaticus* from bile, quantitative RT-PCR was used. For Group 1, *C. hepaticus* was recovered from the bile of all birds 6 days after the initial infection. qPCR analysis showed counts ranging from 4.64×10^4 to 5.9×10^8 CFU/mL. The bird that indicated the lowest numbers of *C. hepaticus* (4.64×10^4 CFU/mL) also had the lowest growth on isolation plates, compared to other birds within the same group with higher numbers of *C. hepaticus.* Six weeks after the initial infection, a significant decrease in *C. hepaticus* counts was observed for all 12 birds in Group 5 (2.2×10^2 to 2.54×10^4 CFU/mL) ($p < 0.0001$). All birds had low growth of *C. hepaticus* on isolation plates compared to the recovery observed in all positive groups. In Group 9, a second infection occurred 12 weeks after the first exposure. Six days after the second exposure, *C. hepaticus* numbers

significantly increased for all birds in Group 9 (3.16 x 10^3 to 6.57 x 10^8 CFU/mL) ($p < 0.0001$) compared to six weeks after a single exposure for Group 5. One bird with the lowest *C. hepaticus* abundance (3.16 x 10^3 CFU/mL). Isolation on putative plates was difficult due to the overgrowth of other bacteria. Interestingly, for the plating method all birds had a higher number of *C. hepaticus* colonies formed after a second reinfection comparable to Group 5. After 3 consecutive infections (Group 7), the mean number of *C. hepaticus* was slightly higher than 2 infections with a range of 2.4 x1 0^4 to 6.8 x 10^8 CFU/ml. *C. hepaticus* numbers were significantly higher than Group 5 ($p < 0.0001$). Overall, *C. hepaticus* numbers in bile decreased over time but significantly increased again following reinfection (Figure 5).

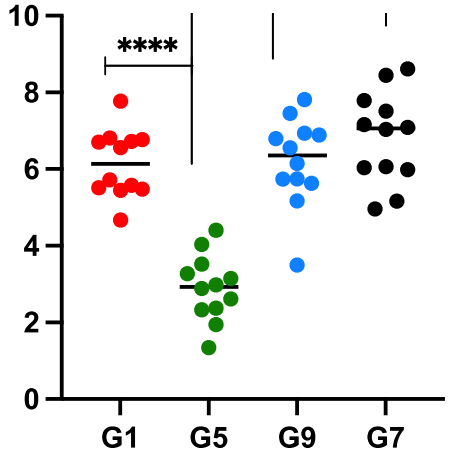

Figure 5 Quantification of *C. hepaticus* in bile using qPCR

CFU/mL are represented as a mean ± SD where n=3, over 2 independent experiments.
Unpaired t-test using Welch's correction was used when p=0.05 was used to measure statistical significance. ****: p<0.0001.

4.2.4. *C. hepaticus*-specific antibodies provide partial protection against reinfections

Sera samples from all birds were collected every 3 weeks to monitor *C. hepaticus*-specific antibody levels throughout reinfection periods using the SLD ELISA1 assay. Absorbance readings over 0.1 are considered positive. The assay was not conducted for Group 1 as levels may not be detected 6 days after a single infection. After 2 consecutive infections with *C. hepaticus,* antibody levels differed significantly between 3 and 6 weeks ($p = 0.0057$) for Group 3. The mean absorbance increased from 0.25 to 0.37. Generally, antibody levels increased for most birds after 6 weeks, however, the absorbance for one bird dropped below 0.1. One bird had similar absorbances 3 and 6 weeks after infection (0.180 and 0.154, respectively) (Figure 6a). A similar pattern was observed after 3 consecutive reinfections (Group 7). The mean absorbance raised from 0.36 to 0.52 ($p = 0.0001$) after the second reinfection. Six weeks after the reinfection, the mean plateaued to 0.57 (not statistically significant) (Figure 6b).

Overall, 12 weeks after a single infection, there was a significant reduction in antibody levels over time. At 3 weeks after infection, absorbances averaged to 0.39 and decreased to 0.24 ($p < 0.001$) and 0.19 ($p < 0.0001$) at 9 and 12 weeks, respectively (Figure 6c). However, levels were similar for one bird throughout the 12-week trial (absorbance of ~0.26). Three birds mounted and maintained the highest antibody levels 9 weeks after infection.

An overview of the differences in antibody levels between groups within the same time point is depicted in Figure 6d. ANOVA testing showed no significant difference in antibody levels at week 6 across all reinfection combinations. However, there was a significant reduction in antibody levels 3–6 weeks after two reinfections compared to a single infection ($p < 0.0001$).

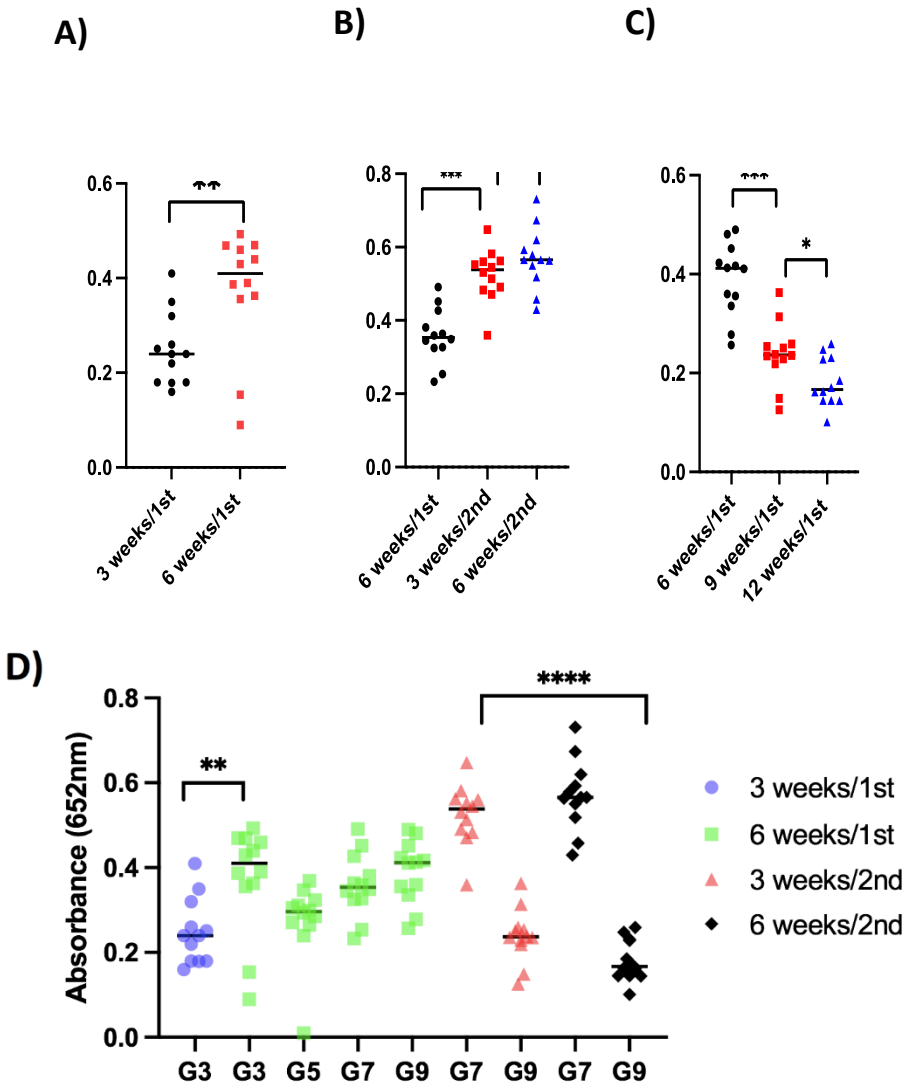

Figure 6 Measuring anti-*C. hepaticus* antibody levels using ELISA for (A) single exposure (Group 3), (B) three consecutive reinfections (Group 7), (C) two non-consecutive exposures (Group 9), and (D) the differences in antibody levels between groups within the same time point

Absorbances were measured in triplicates and mean calculated.

Absorbance values over 0.1 were considered positive.

For each group, unpaired t-test and One-way ANOVA with the Greenhouse-Geisser correction was used when $p<0.05$.

4.2.5. SLD reinfection leads to increased maintenance and integrity of the gut epithelium

The relative gene expression of three tight junction proteins was measured to elucidate the mechanism by which *C. hepaticus* may translocate across the intestinal lining to the liver during infection. The expression of the ZO1 gene significantly increased six days after a single infection ($p = 0.0005$) (Group 1). No significant difference in expression of ZO2 was observed for Group 1.

Interestingly, a secondary infection significantly increased both zonula occludens genes expression compared to a single infection. The mean fold change increases were 4.89 ($p < 0.0001$) and 4.94 ($p < 0.0001$) for ZO1 and ZO2, respectively. CLDN5 gene expression levels significantly decreased six days after a single infection, with the mean decreasing to 0.29 ($p < 0.0001$). CLDN5 levels increased to a mean of 1.61, seven weeks after this dose (Group 5) ($p < 0.0001$) (Figure 7). Some variation in CLDN5 expression in Group 5 was observed, whereby 5 birds had fold changes ranging between 1.49 and 3.25. The remaining 7 birds had similar gene levels to birds in Group 1. Secondary *C. hepaticus* infection (Group 9) resulted in a significantly higher expression of CLDN5 expression compared to a single dose ($p < 0.0001$) with a mean fold change of 1.23. One bird had a relative gene expression of two, the remaining 11 birds had fold changes between 0.73 and 1.39. No statistical significance of CLDN5 expression was observed between Group 9 and a long-term single infection of *C. hepaticus* (Group 5).

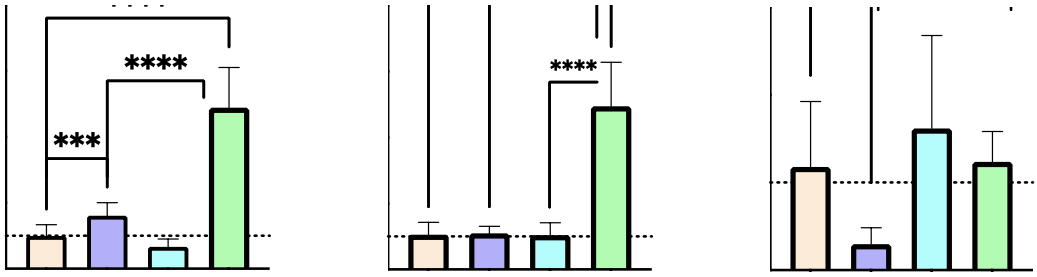

Figure 7 Changes in gene expression of various tight junction proteins from jejunum samples

Gene expression was normalised using ß-actin and GAPDH as the reference genes (represented as the dotted line for each graph).

The relative gene expression changes were calculated using the geometric mean for each reference gene.

Fold change is represented as mean ± SD where n=12 birds per group, assayed in triplicate.

Unpaired t-test using Welch's correction was used at $p<0.05$ was used to measure statistical significance. ***: $p \leq 0.001$; ****: $p<0.0001$.

For groups that were not normally distributed, a Mann Whitney's t-test was implemented.

4.2.6. SLD induces significant inflammatory responses in infected chickens

The expression of interleukins IL-6 and IL-1B from spleen samples was measured to understand the host's pro-inflammatory responses to SLD infection (Figure 8). The expression of IL-6 was significantly elevated six days after a single infection ($p = 0.0173$), with a mean fold change of 3.15. Two birds had relative expression values of 6.5 and 10, in which one presented with over 250 liver lesions and an enlarged spleen upon necropsy. Three birds had no increase in IL-6 expression. Further, there was no significant change in IL-1B expression after a single dose. Recovery of SLD led to a significant reduction in IL-6 levels for Group 5 with a relative mean of 0.88 ($p = 0.0068$).

Both pro-inflammatory cytokine gene expression levels increased after a secondary dose with mean fold changes of 9.32 ($p < 0.0005$) and 3.36 ($p < 0.0002$) for IL-1B and IL-6, respectively, compared to Group 5. Variability in IL-1B gene expression was observed amongst birds in Group 9. Specifically, two birds had fold changes of 24 and 29, whereas two birds had no significant change in IL-1B expression. One of these birds also had no response after a single infection.

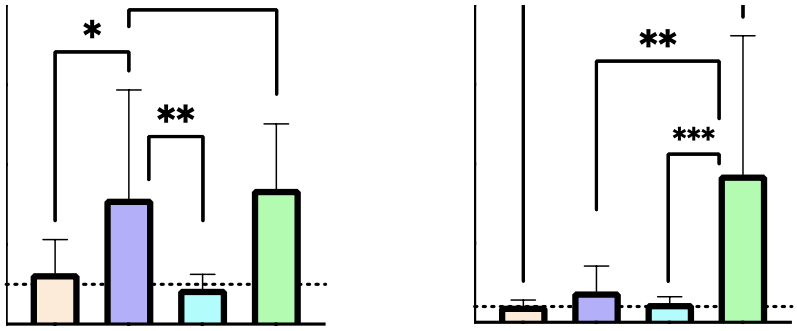

Figure 8 Changes in gene expression of pro-inflammatory cytokines IL-6 and IL-1ß in spleen samples

Gene expression was normalised using ß-actin and GAPDH as the reference genes (represented as the dotted line for each graph).

The relative gene expression changes were calculated using the geometric mean for each reference gene.

Fold change is represented as mean ± SD where n=12 birds per group, assayed in triplicate.

Unpaired t-test using Welch's correction at $p<0.05$ was used to measure statistical significance. *: $p\leq0.05$; **: $p\leq0.01$; ***: $p\leq0.001$.

For groups that were not normally distributed, a Mann Whitney's t-test was implemented.

In summary, the animal trial demonstrated that birds reinfected with *C. hepaticus* two or three times did not develop liver lesions following the reinfections. This finding contrasts with the on-farm experience of some egg producers, in so far as they do observe repeated outbreaks of SLD in some flocks. It is hypothesised that this may be because immune responses have waned in those birds that suffer a second (or third) episode of SLD on the farm, or that birds that were not infected in the first round of SLD are susceptible to a subsequent exposure event. Sera samples were collected to measure the abundance of anti-*C. hepaticus* antibodies in sera. We observed a continued rise in antibodies levels up to six weeks after a single infection. The antibodies, or other unassayed immune responses, induced by the first exposure were still present at the time of subsequent exposure and protected against the development of liver lesions. *C. hepaticus* persisted in the gut of 50% of chickens six weeks post-infection and decreased to 17% after nine weeks with reinfection not increasing persistence in the caeca. This is likely due to the elevated antibody levels clearing *C. hepaticus* from the gut; a similar pattern observed with *C. jejuni* strains (Hepworth et al. 2011; Jones et al. 2004). *C. hepaticus* could be recovered from the bile six weeks after a single infection of *C. hepaticus* suggesting the bacterium may be in a resting stage as indicated by the downregulation of genes involved in amino acid synthesis (Van et al. 2019). Downregulation of barrier forming CLDN5 suggests a paracellular migration across the intestinal epithelial barrier during early stages of infection comparative to an upregulation of scaffolding TJs; ZO1 and ZO2, indicative of a recovery phase to inhibit the translocation of *C. hepaticus* across the epithelial barrier. Based on our qPCR data, the *C. hepaticus* mode of translocation across the intestinal barrier may alternate between paracellular or transcellular modes depending on the stage of infection; similar to the pathogenicity of *C. jejuni* (Harvey et al. 1999; Kalischuk et al. 2009; Brás & Ketley 1999), but further work is required to confirm this.

4.3. Large scale investigation of chicken responses to *C. hepaticus* in commercial flocks

4.3.1. Prevalence of *C. hepaticus* DNA among Australian free range layers

C. hepaticus DNA was detected in three of the five farms (Farms 3, 5, 6B) with no known history of SLD. *C. hepaticus* DNA was detected in the cloacal swab samples in five of the seven farms (Farms 4, 6A, 7, 8, and 11) that had a current or previous SLD outbreak. Farms 4 and 7 had the highest proportion of birds that harboured *C. hepaticus* DNA. The results are presented in Table 6 along with the details of flock size, age, SLD status and ELISA results. Bile samples were collected from Farms 2 and 6A, which had active SLD outbreaks, at the same time as blood and swab sample collection. *C. hepaticus* was isolated from bile samples from Farm 6A but not from Farm 2.

Table 6 Prevalence of anti-*C. hepaticus* antibodies and *C. hepaticus* DNA among Australian free range layers

Farm ID (number of birds sampled)	Flock size (approx.)	Flock age at sampling (weeks)	Weeks before sample collection of the first (and recent) SLD bird death was reported	Percentage of birds tested positive in SLD-ELISA2	*C. hepaticus* DNA detected in pooled (n=10) cloacal swab samples by PCR
Farms with no known history of SLD					
Farm 1 (50)	9,500	65	N/A	2	No
Farm 3 (51)	8,500	34	N/A	41	Yes (4/5)
Farm 5 (50)	20,000	35	N/A	2	Yes (1/5)
Farm 6B (100)	4,300	51	N/A	9	Yes (2/10)
Farm 10 (52)	9,000	52	N/A	0	No
Farms with a known history of SLD					
Farm 2 (52)	13,000	58	21(15)	29	No
Farm 4 (50)	21,000	33	4	64	Yes (4/5)
Farm 6A (100)	4,000	64	41(13)	11	Yes (3/10)
Farm 7 (52)	22,500	60	7	62	Yes (5/5)
Farm 8 (50)	20,000	35	16	4	Yes (1/5)
Farm 9 (50)	19,000	38	6	2	No
Farm 11 (52)	17,700	46	11	35	Yes (1/5)

NA – not applicable.

4.3.2. Prevalence of anti-*C. hepaticus* antibodies among Australian free range layers

Anti-*C. hepaticus* antibodies were also detected in birds from four of five farms with no known history of SLD. Among them, Farm 3 had the highest number of birds (41%) that had seroconverted (Table 6). Anti-*C. hepaticus* antibodies were detected in birds from all seven farms with a known history of clinical SLD. Correlating with the PCR results, Farms 4 and 7 had the highest number of birds, 64% and 62% respectively, seropositive to *C. hepaticus*. SLD-ELISA2 also identified birds exposed to *C. hepaticus*

earlier in their life, that had cleared the bacteria in Farms 2, and 9. *C. hepaticus* DNA was not detected from birds in these farms.

4.3.3. Correlation between egg production, mortality, presence of *C. hepaticus* DNA and *C. hepaticus* immune status

Farms 1, 3, 5, 6B, and 10 had no known history of SLD outbreaks (Figure 9). In farm 3, a sudden rise in weekly mortalities of up to 2.4% was observed 7 weeks prior to sample collection, which was associated with a smothering event. The weekly egg production also dropped from 94% to 88%. Although the farm had no record of SLD, 41% of the birds had anti-*C. hepaticus* antibodies in their blood and four of the five pooled cloacal swabs had *C. hepaticus* DNA, suggesting an SLD outbreak that was not identified in the routine veterinary surveillance.

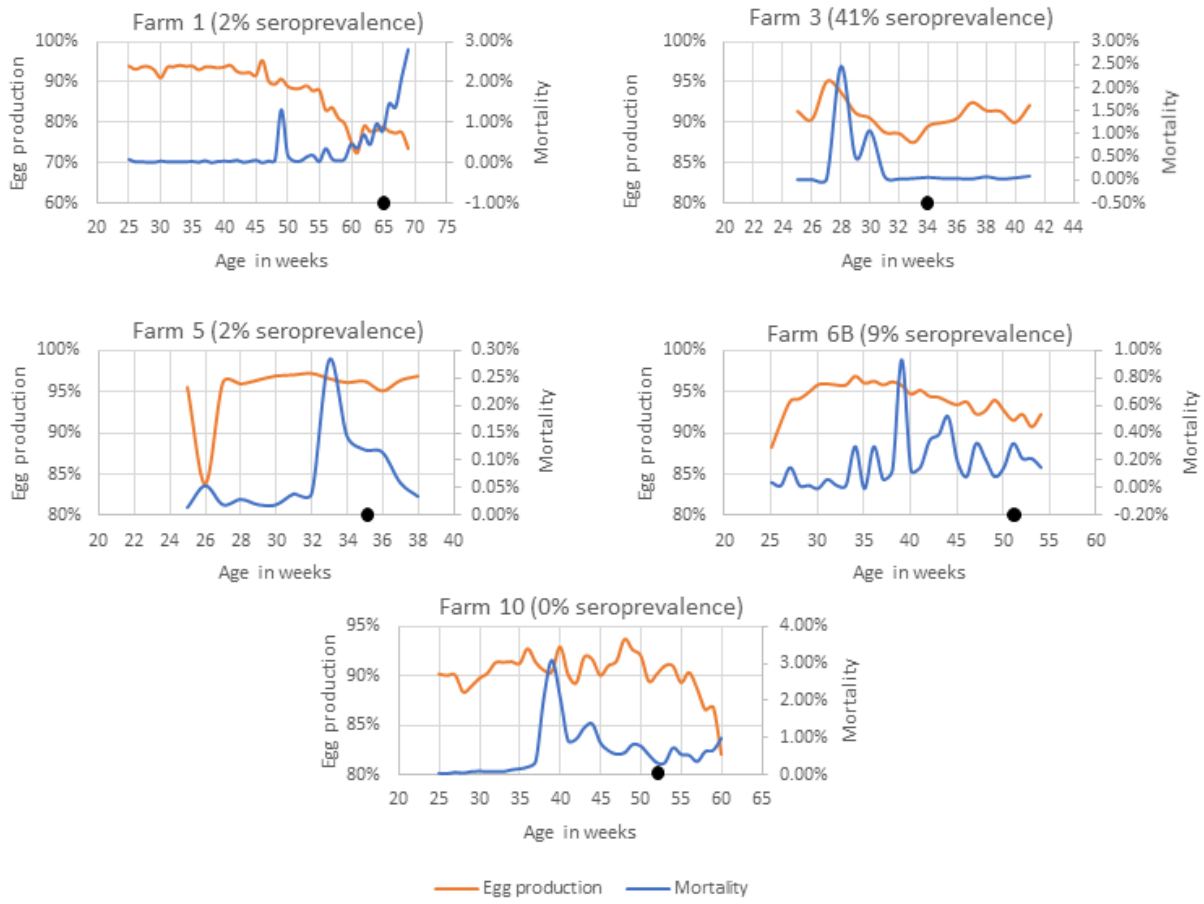

Figure 9 Mortality and egg production in farms with no known history of SLD

The black dot indicates the week of sample collection (Muralidharan et al. 2022b).

In Farms 1 and 5, 2% of birds (a single bird) were seropositive to *C. hepaticus*. *C. hepaticus* DNA was also detected in one of the five pooled cloacal swab samples from Farm 5. However, there was no notable production drop or mortalities in Farm 5.

In Farm 6B, 9% of the birds were seropositive to *C. hepaticus* and two of the 10 pooled cloacal swabs had *C. hepaticus* DNA. Mortalities peaked at 0.93% in week 39. However, no significant drop in egg production was noted. The birds in Farm 10 were *C. hepaticus* negative in SLD-ELISA2 and PCR. The

farm had high mortalities during weeks 38–44 and a drop in egg production from week 58 that was associated with injurious pecking and behavioural issues frequently reported within the flock.

Within Farm 6A, there were bird deaths associated with SLD at 22 weeks of age. The latest SLD death was documented 13 weeks prior to sample collection. Mortality rates were generally low and egg production was mostly above 85%. Only 11% of birds were positive in SLD-ELISA2. However, three of the five pooled cloacal swabs had detectable levels of C. hepaticus DNA. Furthermore, *C. hepaticus* was isolated from bile culture and confirmed active SLD infections in this flock.

Farms 2, 4, 6A, 7, 8, 9, and 11 had SLD outbreaks 4–41 weeks prior to sample collection (Figure 10). In Farm 4, an SLD outbreak occurred four weeks prior to sample collection and the birds were on water and in-feed antibiotic medication. An increase in mortality of up to 0.8% and an up to 18% drop in weekly egg production was observed, which correlated with the detection of anti-*C. hepaticus* antibodies in 64% of the birds and *C. hepaticus* DNA in four of the five pooled cloacal swab samples.

Farm 7 had high mortalities from week 15, as early as the birds were brought from rearing to the layer farm. A clinical SLD outbreak was reported when the flock was around 53 weeks of age. A steep rise in mortalities of up to 2% in week 54 coincided with a drop in egg production to 71%, which lasted for a period of six weeks that correlated well with the detection of *C. hepaticus* DNA in all five pooled cloacal swab samples and anti-*C. hepaticus* antibodies in 62% birds.

The egg production drop and mortalities in the QLD farms (Farms 6A, 6B, 8 and 9) were comparatively mild, concurrent with the low prevalence of *C. hepaticus* DNA and anti-*C. hepaticus* antibodies in flocks. SLD was confirmed in Farm 8 when the flock was 19 weeks of age. However, only 4% of the birds had anti-*C. hepaticus* antibodies and one of the five pooled cloacal swab samples had *C. hepaticus* DNA. Notably, the farm had very low mortalities. Only one bird out of 50 birds tested positive to SLD-ELISA2 in Farm 9 and *C. hepaticus* DNA was not detected in cloacal swabs. SLD outbreak was noticed 6 weeks prior to sample collection, marked by a sudden drop in egg production to 82%. The egg production recovered to a normal rate within 2 weeks.

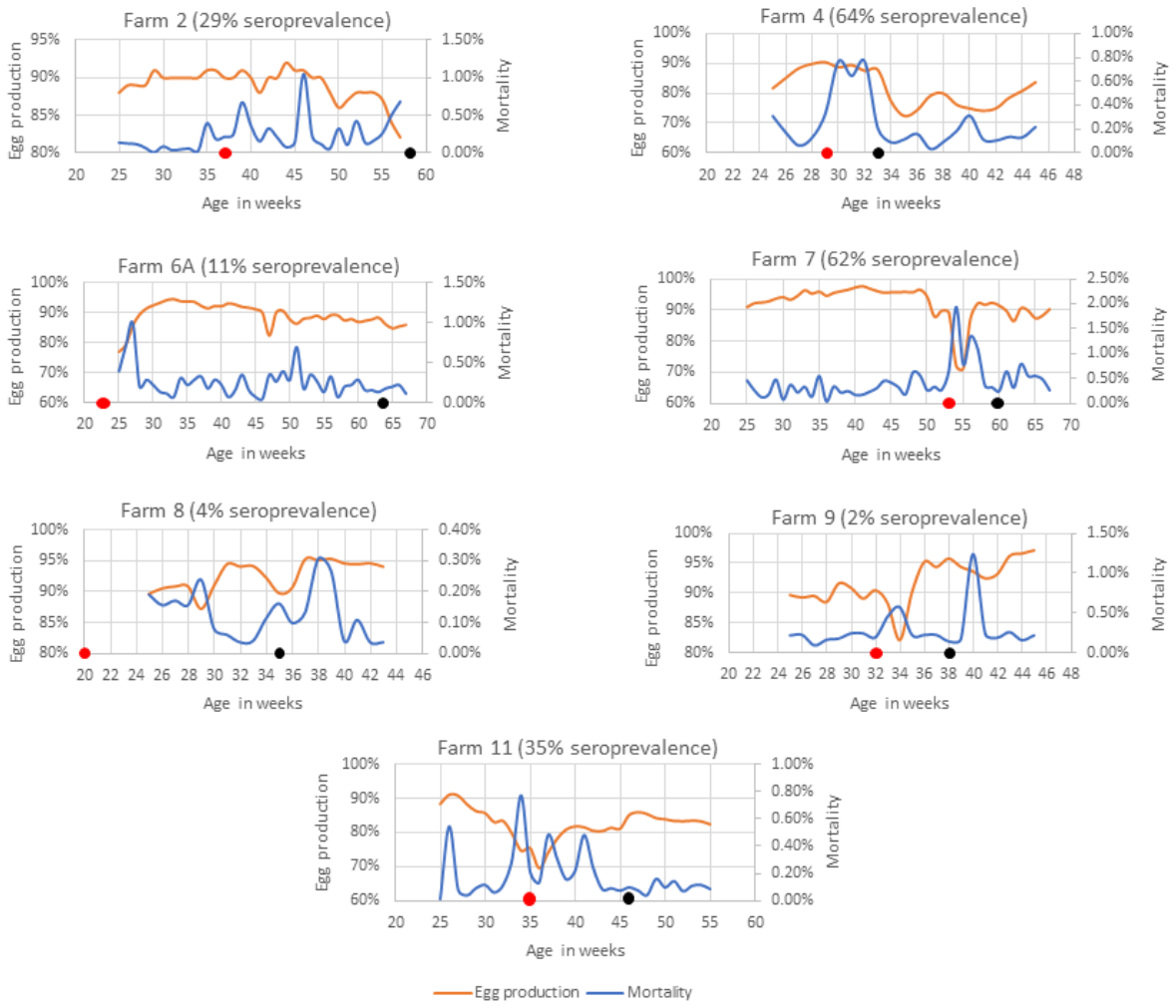

Figure 10 Mortality and egg production in farms with a known history of SLD outbreak

The black dot indicates the week of sample collection and the red dot indicates the week in which the first SLD associated death was recorded (Muralidharan et al. 2022b).

4.3.4. Statistical analysis

A good correlation was observed between SLD-ELISA2 and PCR results with a Pearson correlation coefficient, r value of 0.85 and a p-value of < 0.001. As expected, no correlation was observed between the flock size or flock age on ELISA and PCR outcomes (Figure 11). Although, the majority of seroconverted birds were in flocks with a known history of SLD, a statistically significant difference between the seroprevalence of anti-*C. hepaticus* antibodies among flocks with or without a known history of SLD could not be established (p-value = 0.143) (Table 7). Similarly, no significant difference was observed between the sample collection time post first SLD outbreak and the ELISA (p-value = 0.307) or PCR (p-value = 0.471) outcomes.

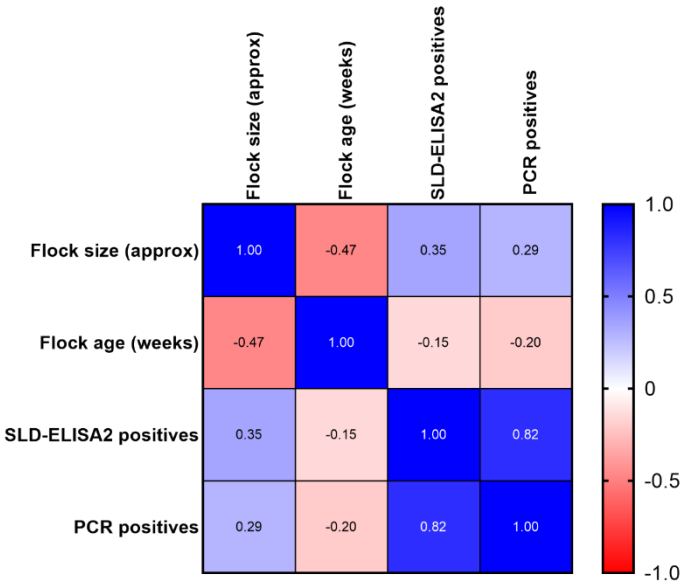

Figure 11 Pearson correlation, r between flock size, flock age, and percentage of birds positive to SLD-ELISA2 and PCR

Table 7 Seroprevalence of *Campylobacter hepaticus* in flocks with/without a known history of SLD

	Farm/shed (n)	Sample size (n)	ELISA positive (n)	Seroprevalence (mean)	Seroprevalence (median)	P-value
Flockes with a known history of SLD	7	406	111	27%	29%	0.1427
Flocks with no known history of SLD	5	303	32	11%	2%	
Total	12	709	143	20%		

In summary, this study highlights the usefulness of ELISA and PCR testing to determine the incidence of mild or sub-clinical SLD by identifying seroconverted birds in farms. The findings of such high levels of both PCR positive and ELISA positive birds in the non-SLD flocks was unexpected. It indicates that there is a lot more *C. hepaticus* circulating across the egg industry than previously realised. *C. hepaticus* has been shown to infect layers in rearing without presenting any clinical signs (Scott 2016; Phung et al. 2020), suggesting exposure during rearing may provide some level of protection from future infections. Farms with previous SLD history (6A, 8 and 9) presented with mild drops in egg production and mortalities, could be suggestive of the variability in virulence of *C. hepaticus* strains. Recently, strain QLD19L isolated from Queensland was found to be less pathogenic than the NSW44L strain isolated from New South Wales (Van et al. 2022). These farms may have been exposed to a less virulent *C. hepaticus* strain that is less immunologically recognised compared to more virulent strains. Finally, this study highlights that certain predisposing factors need to be present before clinical SLD manifests. Factors can include disruptions to bird husbandry such as availability of feed and water, birds accessing stagnant water in the range, hot-humid weather, wet litter, overcrowding in shelter houses on the range, inadequate feed space, cannibalism, round worm or coccidial damage to intestinal lining along with the hormonal changes, and stress in birds during lay (Grimes & Reece 2011; Scott et al. 2016). Furthermore, the occurrence of smothering events coinciding with reductions in egg

production may mask the onset of SLD in flocks, as observed for Farm 3. It is important to distinguish which of these aspects are contributing to differences in SLD occurrence as it will influence how biosecurity and treatments are applied, and what treatment option might be developed in the future, such as vaccination. The finding that not all birds seroconvert in a flock that has undergone an SLD outbreak indicates that there are birds in a flock that would likely remain susceptible to further infections and development of disease, potentially explaining why some flocks can experience more than one SLD outbreak despite the protection that was demonstrated in the experiment presented in Section 4.2.

Implications

Our study has shown that chickens are able to resist future infections and recover from the disease. This is based on our findings about liver lesions, which it is assumed to act as an indicator of clinical disease. Trials to determine the effects of infection and subsequent reinfection would need to be much larger if mortality and egg production effects were to be assessed. This knowledge of the response to reinfection and the antibody prevalence in the Australian flock provides an overview of factors affecting the epidemiology of the disease. In particular, by gaining an understanding of the incidence of the disease, in both symptomatic and asymptomatic birds, it is important to identify the factors that cause flocks to have different rates of recovery and then implement changes to immunologically control the disease. Changes include the development of appropriate biosecurity measures, potential vaccines, colonisation of non-pathogenic strains of *C. hepaticus* and feed additives.

Recommendations

It is recommended to perform an extensive immunological and molecular survey of birds in early lay to provide information on whether the birds are exposed to *C. hepaticus* in rearing or early lay and whether exposure in rearing provides any level of protection in the future. Research should also be directed to identifying whether non-pathogenic strains of *C. hepaticus* exist in flocks, and hence explain the seroconversion in flocks that have never shown signs of SLD, and whether these strains could be used to colonise chickens to reduce the severity of SLD.

Additionally, it is recommended to further investigate the mechanism by which *C. hepaticus* translocates from colonising the gut to the liver and bile. Also, the development of the qPCR assays could be implemented in a large-scale immunological survey to measure the efficacy of any future vaccine or treatment development.

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Media and Publications

Muralidharan C, Huang J, Anwar A, Scott PC, Moore RJ and Van TTH. (2022). Prevalence of *Campylobacter hepaticus* specific antibodies among commercial free-range layers in Australia. *Veterinary Science,* 9:1058110

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Intellectual Property Arising

No.

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