Assessment of the efficacy of autogenous vaccines in Spotty Liver Disease control

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Foreword

This project was undertaken to assess the efficacy of *Campylobacter hepaticus* autogenous vaccines as control options for Spotty Liver Disease in the field. The project included laboratory challenge studies and a commercial farm field component.

This project was funded from industry revenue, which is matched by funds provided by the Australian Government.

This report is an addition to Australian Eggs Limited's range of peer reviewed research publications and an output of our R&D program, which aims to support improved efficiency, sustainability, product quality, education and technology transfer in the Australian egg industry.

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Professor Robert Moore and Dr Thi Thu Hao Van are researchers at RMIT University with expertise in bacteriology, molecular microbiology, and advanced technologies such as next generation sequencing and bioinformatics. They work closely with industry partners. They have made significant contributions to poultry health research in areas such as Spotty Liver Disease in layers, necrotic enteritis in broilers, bacterial vaccines, and gut microbiota influences on health and productivity.

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Abbreviations

44L	*Campylobacter hepaticus* isolate (RMIT)
ANOVA	Analysis of variance
APVMA	Australian Pesticides and Veterinary Medicines Authority
CFU	Colony-forming unit
CH, *C. hepaticus*	*Campylobacter hepaticus*
CTC	Chlortetracycline
DAE	Days after exposure
DYF	Digital yolk fan
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
g	Gram
HV10	*Campylobacter hepaticus* isolate (RMIT)
LED	Light emitting diode
PCR	Polymerase chain reaction
R&D	Research and development
RMIT	Royal Melbourne Institute of Technology
SCARF	Scolexia Animal Research Facility
SLD	Spotty Liver Disease

Executive Summary

Spotty Liver Disease (SLD) is a serious condition affecting extensively housed laying hens, leading to losses through both bird mortality and reduced egg production. It is associated with peak-lay and management issues (such as interruptions to feed routine, range access, or fox attacks), but if introduced to previously negative farms can affect hens at any age during production. The causative agent has been described as a result of research performed by Crawshaw et al. (2015), and in Australia by Scolexia and RMIT University (Scott et al. 2016; Van et al. 2016). The causative agent of SLD was named *Campylobacter hepaticus.* It is hypothesised that changes in the intestinal health/microbiota balance allow a multiplication of *C. hepaticus* and the production of a toxin that causes the symptoms of the disease, including the liver lesions.

During the course of this project (September 2017 – April 2020), the exposure model previously developed by RMIT University and Scolexia Pty Ltd, which reproduces the liver lesions, was used to assess the efficacy of autogenous vaccines, using isolates HV10 and 44L, in amelioration of the impact of SLD.

Both field and laboratory studies were undertaken. Birds were vaccinated two or three times with killed vaccines prepared from *C. hepaticus* strains HV10 (GenBank accession number LUKK01000000) and 44L (WHMR00000000). A reduction in the extent of gross liver pathology was detected in the laboratory studies, with some of the studies showing a tendency towards reduction in the number of miliary lesions on the liver surface, and reduced lesion scores in vaccinated compared with unvaccinated hens after oral challenge with *C. hepaticus*.

A significant reduction of egg weights was detected one week after infection with *C. hepaticus* assessed in two independent studies. In both studies there was a significant reduction in the egg weight of the positive control groups at day 7 post-exposure. However, any drop in egg weights in the vaccinated groups at day 7 post-exposure was not significant. Other minor changes in production indicators were noted but were not consistent in all studies. A drop in egg production in exposed non-vaccinated hens was demonstrated in some studies but not others, and in one study vaccination significantly ameliorated that drop in production. However, that protection was not repeated with the vaccine manufactured using isolate 44L (noting that the challenge strain was HV10). A modest reduction in the weight of birds immediately post-challenge was seen in one study, and a reduction in the rate of weight gain in other studies. This difference in weight gain was overcome by vaccination.

Substantial progress has been made in overcoming major obstacles to providing an efficacious vaccine. This includes the ability to grow *C. hepaticus* in liquid cultures to enable simplified preparation of the culture volumes required for experimental challenge and vaccine manufacture. In independent work, outside of this vaccine project, RMIT University has developed an ELISA assay to measure serological responses to *C. hepaticus* infection and vaccination. Application of this assay has increased the ability to assess the likelihood of vaccination success or failure. The project has clearly demonstrated that there is a measurable humoral immune response to vaccination, and that response is related to some level of protection. This was demonstrated with low ELISA levels in the birds challenged in the field having no observable protection, compared to the two later laboratory studies where positive ELISA values were associated with some level of disease amelioration. Further investigation is urgently needed into the underlying mechanisms of disease pathogenesis, details of the immune response to vaccination, and methods to improve that response.

Overall Conclusions

Tools for the assessment of SLD control have been developed and refined during the course of this project, including the full description of the causal organism, the development of an effective challenge model, some understanding of the differences in virulence and the culture requirements of different strains, development of an ELISA assay to measure serological response, and increased understanding of the disease. In particular the experimental model can be used to induce disease, reproduce the disease impacts seen in the field such as reduction in egg weights and on occasions egg production, a hitherto un-noted impact on liveweight as well as inducing some clinical signs in a small portion of challenged birds (which mimics the field experience where many affected birds do not display clinical symptoms). A serological response to vaccination has been induced and where the response has been adequate, a degree of protection following the use of killed autogenous vaccines has been demonstrated. Further investigation is urgently needed into the underlying mechanisms of the disease pathogenesis, details of the immune response to vaccination and methods to improve that response.

1 Introduction

Spotty Liver Disease (SLD) is characterised by increased mortality, particularly around the time of peak egg production, the occurrence of multiple grey/white spots in the liver, and reduction in egg output. It is prevalent within the layer industry in Australia, especially within the free range sector of the industry (Grimes & Reece 2011). The clinical signs include a brief period of depression in laying birds (usually in good body condition and 'in-lay'). Often birds are found dead without any prior evidence of disease. The disease is less commonly found in barn and cage birds and parent stock (Scott et al. 2016). The recent identification of *Campylobacter hepaticus* (*C. hepaticus*), as the causative agent (Van et al. 2016), and the development of an experimental disease induction method (Van et al. 2017a), provide the tools to facilitate the study of disease pathogenesis and the evaluation of experimental vaccines.

The development of specific and sensitive PCR detection methods allows the detection of *C. hepaticus* in the gut of diseased birds. *C. hepaticus* occurs throughout the gut, increasing in abundance down the gut. To date *C. hepaticus* has only been detected in the gut of birds from sheds that have clinical signs of disease. *C. hepaticus* could not be detected in the gut of birds from other sheds, on the same farms, that have not had a history of clinical disease. Currently, a highly selective culture medium for *C. hepaticus* is not available, so the organism is most readily isolated from samples such as liver and bile, that usually do not contain other contaminating bacteria. In samples including other bacteria, the comparatively slow growing *C. hepaticus* tend to be rapidly overgrown. Recently, a filter penetration culture method has been shown to facilitate the recovery of *C. hepaticus* isolates from microbially complex faecal samples (Phung et al. 2020).

Disease cases with similar clinical presentations as modern day SLD were reported in the USA in the 1950s (Delaplane 1955; Tudor 1954). Bacteria described as 'vibrios' were cultured from diseased birds, initially by passage in chicken embryos and subsequently cultured on rich agar media of various compositions. In one case, cultured bacteria were fairly comprehensively characterised for fermentation and enzymatic activities, however, the bacterial genus was not identified and no subsequent study of the isolates has been reported (Peckham 1958). Other researchers have suggested the possible involvement of a number of bacterial species, including *Campylobacter jejuni* (*C. jejuni*, *Campylobacter coli* (*C. coli*), *Clostridium sordellii*, and *Helicobacter pullorum* (Burnens et al. 1996; Forsyth et al. 2005; Jennings et al. 2011) . The *Campylobacter* and *Helicobacter* species would be consistent with the previous findings of 'vibrio' like bacteria, but in no cases could the disease be experimentally reproduced with the candidate cultured bacteria.

Crawshaw et al. (2015) recovered a number of bacterial isolates from SLD affected hens from UK flocks and identified them as campylobacters. Van et al. (2016) isolated the bacterium from Australian cases of SLD, and went on to fully characterise the organism and identified it as a new species that they named *Campylobacter hepaticus*. The role of *C. hepaticus* as the etiological agent of SLD was confirmed by its ability to induce typical clinical signs of disease in experimentally infected birds (Van et al. 2017a).

C. hepaticus was first isolated from the livers of layer birds with typical indications of SLD. The groups who have reported successful isolation of the organism have used slightly different culturing methods (Crawshaw et al. 2015; Gregory et al. 2018; Van et al. 2016). In both cases aseptically collected internal fragments of liver were macerated in Preston broth and incubated under microaerophilic conditions at 37°C; the UK group cultured the bacteria for 7 days, while the Australian group for 2 days. Samples from the pre-enrichment step were plated onto 5% sheep blood agar (SBA) (UK group) or Brucella agar with 5% horse blood (BAB) (Australian group) and again incubated microaerophilically for several days.

The Australian isolates produced clearly visible colonies within 3–5 days, whereas the UK group reported that some isolates required up to 7 days before growth was obvious. An easier route to isolation of *C. hepaticus* from diseased birds, taken by both groups, is the direct plating of bile onto either SBA or BAB and incubation under microaerophilic conditions at 37°C for several days.

Following primary isolation, *C. hepaticus* can be reliably grown on BAB but grows poorly in liquid culture without blood supplementation. It grows at 37°C and 42°C but not at 25°C, and does not grow under aerobic conditions (Van et al. 2016). Electron microscopy (EM) showed that *C. hepaticus* has typical *Campylobacter* morphology. Cultures consist mainly of S-shaped cells and longer helical cells, but some coccoid forms are also present (Figure 1). Some cells have bipolar unsheathed flagella while many appear to have single polar flagella or no flagella; the variation observed under EM may be due to the sensitivity of the flagella to mechanical breakage as the scanning EM appears to show a lot of broken flagella fragments. Whole genome sequencing and comparison to the genomes of other *Campylobacter* species indicated that *C. hepaticus* is most closely related to *C. jejuni* and *C. coli* (Petrovska et al. 2017; Van et al. 2019).

Early attempts to induce pathology used some of the embryo passaged or cultured bacteria isolates from US cases in the 1950s. The 'vibrio' bacteria caused death in challenged chicken embryos and, in some cases, signs of clinical disease were reproduced in inoculated adult birds (Hofstad et al. 1958; Peckham 1958; Sevoian et al. 1958). More contemporary attempts to reproduce clinical disease, using the recent UK isolates in specific pathogen free chicks, resulted in microscopically visible lesions, but not typical miliary spots, in the liver of challenged birds (Crawshaw et al. 2015).

It is only with the use of the Australian *C. hepaticus* isolates in birds coming into lay that full-blown disease typical of field cases of SLD was successfully reproduced following experimental infections (Van et al. 2017a). Those studies fulfilled Koch's postulates (Grimes 2006) to unequivocally demonstrate that *C. hepaticus* causes SLD (Van et al. 2017a). Disease induction was achieved by inoculating birds from flocks with no history of SLD with 10^9 to 10^{10} CFU of *C. hepaticus* HV10^T via direct oral gavage. The severity of disease in the 24 challenged birds varied from no macroscopically obvious disease in one bird to severe disease covering the entire surface of all lobes of the liver in a few birds. Most of the birds had moderate numbers of macroscopically obvious lesions on the surface of the liver. No long-term trials to investigate the effect of experimental disease challenge on egg output have been previously reported. The success of the oral gavage in inducing the disease, and the finding that SLD in cage facilities usually affects birds on the lower cages, suggest that natural SLD infection probably occurs via the faecal-oral route.

Whole genome sequencing has shown that the genomes of 14 Australian isolates range in size from 1.48 to 1.57 Mb (Van et al. 2019). Sequencing of 10 British isolates showed a wider range of genome sizes from 1.50 to 1.80 Mb (Petrovska et al. 2017). The type strain, HV10 (=NCTC 13823; =CIP 111092), has a genome of 1,520,669 nucleotides and is predicted to contain 1494 protein coding sequences and 52 RNA coding genes (unpublished results). Overall whole genome comparison, on a single nucleotide polymorphism gene-by-gene basis of the core genome, showed that the Australian type-strain isolate differed from the three sub-clades of the British isolates. The Australian isolates had a lower GC content; 27.9% compared with an average of 28.4% for the British isolates (Petrovska et al. 2017). The *C. hepaticus* isolates have smaller genomes than typically found for the closely related species, *C. jejuni* and *C. coli*, with approximately 140 fewer genes encoded, including a notable reduction in the number of genes encoding products predicted to be involved in iron acquisition and general metabolism. There were also fewer putative virulence, disease, and defence subsystem genes predicted in the genomes of *C. hepaticus*. *C. hepaticus* genomes encoded more genes involved in carbohydrate, fatty acid, lipid, and isoprenoid metabolism than typically found in *C. jejuni* genomes (Petrovska et al. 2017). Petrovska et al. (2017) have suggested that the reduced genome of

C. hepaticus may result from the more specialised lifestyle that *C. hepaticus* has compared to *C. jejuni*, in particular, the reduction in iron acquisition may result from specialised niche adaptation to the iron rich environment within the liver.

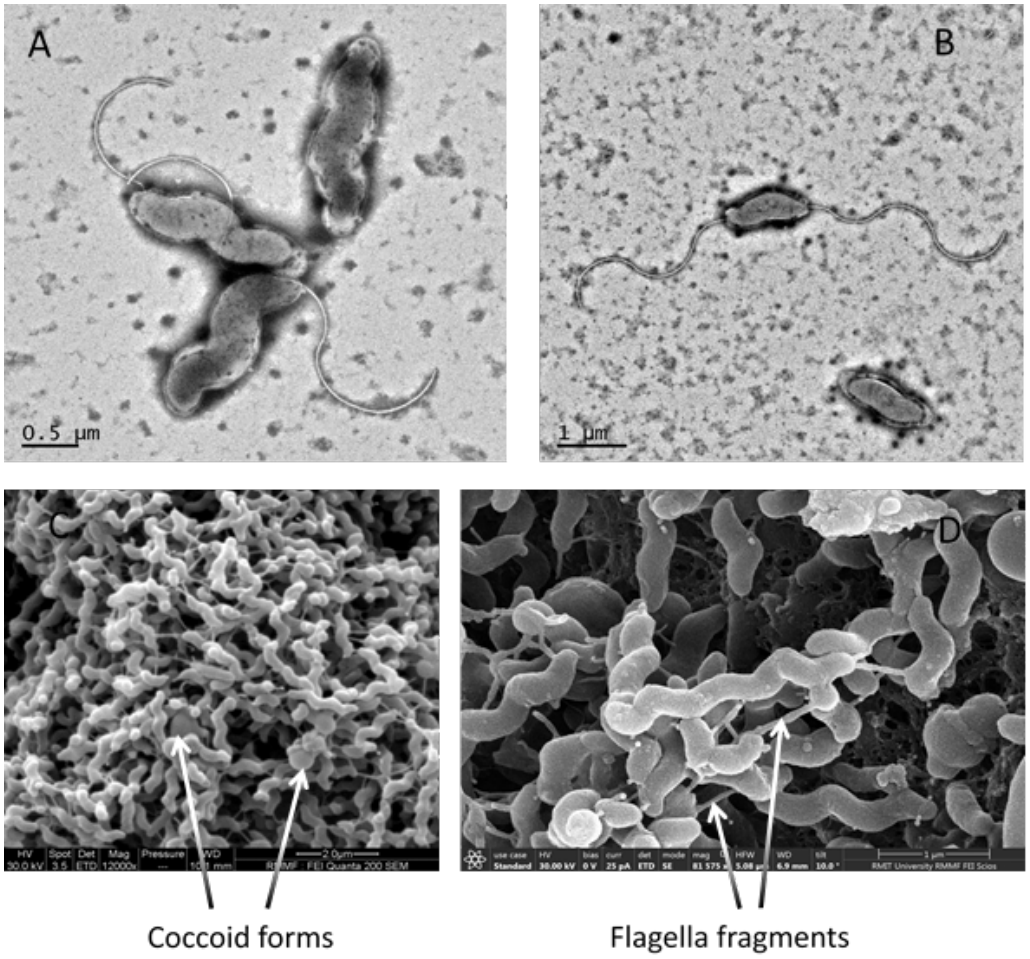

Figure 1 Electron micrographs of *C. hepaticus* cells

Panels A and B: Transmission electron micrographs of *C. hepaticus* cells.
Note the long bipolar flagella shown in Panel B.
Panels C and D: Scanning electron micrographs of the surface of a colony of C. hepaticus cells.
Note in panel D the variation in cell length, ranging from the S-shaped cell in the top centre of the panel to the long helical cell in the centre of the panel.

It was of particular interest to interrogate the genome of *C. hepaticus* for potential toxins that may be important in disease pathogenesis, in particular the pathology observed in the liver. To date no obvious toxin encoding genes have been identified although it should be noted that, like all genomes, the *C. hepaticus* genome contains many genes for which a function could not be predicted. There are only a few genes in other *Campylobacter* species that have been identified as encoding toxins that may play some role in disease pathogenesis. In *C. jejuni*, cytolethal distending toxin may play a role in disease pathogenesis but all the genes involved in its synthesis are absent from *C. hepaticus* (Petrovska et al. 2017).

Where not previously exposed to antibiotics, SLD responds to antibiotic therapy, with a decrease in the mortality within a few days after the commencement of treatment. In some flocks, antibiotic treatment fails; antibiotic resistance is the most likely cause of treatment failure, limiting the ability to effectively control the disease purely by antibiotic treatment. In any case rational antimicrobial

prescribing principles preclude the ongoing use of antibiotics for disease control. In previous research, a variety of isolates of *C. hepaticus* (HV10, DisRed, D4, 4L, 12L, 17L, 19L, 22L, 27L and 29L) exhibited resistance, or intermediate resistance, to nalidixic acid, and most of them showed resistance to cephalothin (Van et al. 2016). Research conducted in the UK (Petrovska et al. 2017) demonstrated the presence of a pTet tetracycline resistance plasmid in three *C. hepaticus* isolates from three separate farms. The plasmid was highly homologous to a previously characterised *C. coli* plasmid, pCC31. Some Australian isolates have also been shown to contain tetracycline resistance plasmids homologous to plasmids previously reported in *C. jejuni*, suggesting that other *Campylobacter* species may act as a genetic reservoir for *C. hepaticus* and vice versa (Phung et al. 2020; Van et al. 2019).

This project was commissioned by Australian Eggs Limited to test the efficacy of autogenous vaccines in the control of SLD. Concurrent studies have indicated that there are many different isolates of *C. hepaticus*, which appear to have varying virulence. Growing the organism is difficult, especially when growing the large volumes required for commercial vaccine production, and the growth characteristics vary with the particular isolate of the bacterium. These characteristics all added to the challenges of reliably producing a vaccine for the poultry industry; similar to those being experienced by other researchers who are working on poultry *Campylobacter* spp. associated with food poisoning.

2 Materials and methods

The challenge model had already been developed at the start of this project and is described below. Further progress with respect to the growth of the challenge organism was made during the course of the studies. Developments in the manufacture of autogenous vaccines allowed the conduct of two field studies and three laboratory studies to assess the efficacy of a vaccine made with the HV10 isolate, and one laboratory study with vaccine produced using the 44L isolate. During the course of this project several laboratory challenge studies were undertaken using different isolates and challenge doses of *C. hepaticus*, and some of the production aspects of those studies are reported here.

2.1 Autogenous vaccine production

The development and refinement of vaccine production methods was not a component of this project, but such work was undertaken in parallel with the assessment of vaccine efficacy. The first difficulty encountered in the manufacture of autogenous vaccines for SLD isolates was regulatory. Prior to production, a permit from the APVMA is required, and in this instance almost two years passed prior to the issue of the permit. *Campylobacter* organisms are microaerophilic (that is they have requirements for increased CO_2 and decreased O_2), are slow growing, and *C. hepaticus* growth requirements appear to be more exacting than for many other *Campylobacter* species. Other difficulties with the growth of strains direct from the field and in autogenous vaccine production, include the need to adjust the pH regularly, as well as the inherent limitations of *Campylobacter* growth in general. It is a regulatory requirement that autogenous vaccines be only grown in media that have *in vivo* permits issued by Biosecurity Australia. This limits what can be done to improve production conditions. We have also found that different isolates have different growth requirements and characteristics. Some grow better in one media compared to other isolates, which may grow better in another. Even light may inhibit growth in some isolates.

With respect to initiation of vaccine field and further laboratory challenge studies, the vaccine manufacturing facility had issues with the scale-up to commercial volumes and was unable to yield growth at target volumes between 250 mL and 10 L using the previously successful growth parameters (volumes of approximately 100 mL). A joint investigation with the manufacturer and RMIT was initiated to ensure the continued production of vaccine for further laboratory exposure studies and to move towards scale-up for the field studies.

Bioinformatics analysis of the *C. hepaticus* genome and a wide range of *C. hepaticus* growth conditions were examined to find the optimal *C. hepaticus* growing conditions in liquid culture, including:

- Growth of *C. hepaticus* in different media

  Brucella broth (BBL), Mueller Hinton (MH) broth (Oxoid), Brain Heart Infusion broth (BHI), Heart Infusion (HI) broth (Oxoid), Columbia media (Amyl Media), and *Campylobacter* Enrichment Hiveg™ Broth Base (HIMEDIA) were all tested.

- Investigation of the types of culture vessels on the growth of *C. hepaticus*

  Different size and shape of vessels including Costar® 24 Well Cell Culture Plates, Corning® 50 mL centrifuge tubes with a vented cap (0.2 µm pore size), Corning® cell culture flasks 75 cm^2 with a vented cap (0.2 µm pore size), and Erlenmeyer flasks (size: 250 mL) were used to determine the effects on the growth of *C. hepaticus*.

- Growth of *C. hepaticus* in static and shaking conditions

  C. hepaticus were grown in Costar® 24 Well Cell Culture Plates placed into the anaerobic jar to generate microaerobic conditions using CampyGen pack (Oxoid). The jars were incubated at 37°C, static and shaking conditions.

- Effect of pH on the growth of *C. hepaticus*

  A pH range of 6.0 to 10.0 in increments of 0.5 units was investigated.

- Effect of temperature on growth of *C. hepaticus*

  C. hepaticus was cultured in Brucella broth at 37°C and 42°C under microaerobic conditions to determine the effect of temperature on growth.

- Effects of carbohydrate, amino acids, and vitamins on the growth of *C. hepaticus.*

Genomic analysis of the metabolism of *C. hepaticus* showed that *C. hepaticus* was unable to biosynthesise L-cysteine and L-lysine and L-arginine. Therefore, a wide range of supplements including L-cysteine, L-lysine, L-arginine, L-glutamine, sodium pyruvate, L-methionine, L-histidine, L-valine, L-serine, L-leucine, L-threonine, choline chloride, niacinamide, myo-inositol, and Fe $(NO_3)_3$ were used to examine their effects on the growth of *C. hepaticus* in supplemented Brucella broth.

It was found that the maximum growth of *C. hepaticus* in modified Brucella broth was 10^9 CFU/mL after 48 hours of incubation, with the following conditions: pH adjusted to 7.5, 37°C, no shaking, supplemented with L-cysteine, L-glutamine, and sodium pyruvate in 75 cm^2 cell culture flasks. These optimal conditions allowed growth of *C. hepaticus* at a large scale suitable for vaccine production. Figure 2 illustrates the final packaging and labelling of the vaccine. The final vaccine formulation is a killed suspension of organisms together with a combination adjuvant comprising both aluminium hydroxide and oil-in-water.

Figure 2 Example of the finished product (*C. hepaticus* autogenous vaccine)

2.2 Growth of challenge culture

The optimal conditions to produce *C. hepaticus* challenge material growth in liquid culture for direct use in birds was achieved using the same methods described above for vaccine production. This replaced the previously used method of harvesting cells grown on HBA plates, which required more materials and much longer handling time.

2.3 Vaccine safety studies

Adverse reactions to autogenous bacterial vaccines occur rarely but frequently enough for the APVMA to require that safety testing of autogenous vaccines be undertaken prior to release. The basic protocol requires examination of 8 birds at 20, 40 and 60 minutes after vaccination, and then hourly observations for 4 hours. If no adverse effects are observed, up to a further 20 birds can be vaccinated if needed and all birds examined daily for 14 days. At the end of that period the birds are handled and the injection site palpated to check for any reaction at the injection site. Completed vaccination safety forms are required to be sent to the manufacturer prior to the release of the vaccine. Vaccines tested included those manufactured with HV10 (LUKK01000000), batch numbers KINCAM00317 and CAM01318, and a vaccine based on strain 44L (WHMR00000000), batch number RMTCAM03619.

2.4 General challenge model

The basic study design utilised a negative control group which was not vaccinated or exposed to *C. hepaticus*, a positive control group, also not vaccinated but exposed to *C. hepaticus*, and a vaccine group that was vaccinated generally at 8 and 12 weeks of age, and in some cases another vaccine group that received an additional vaccination at 19–23 weeks of age. Close to the peak of production (between 24 and 28 weeks), the birds were dosed orally with 1 mL of Brucella broth containing either 10^9 or 10^{10} *C. hepaticus* (HV10) as described by Van et al. (2017a). Negative control birds were dosed with 1 mL of sterile Brucella broth. The birds were assessed at least three times daily until they were autopsied between 5 and 7 days following the challenge. At autopsy, an assessment of the severity of liver lesions was undertaken, including a count of up to 50 miliary lesions (spots) and an estimation of the number for livers with more than 50 visible miliary lesions on the surface of the liver. An SLD liver lesion scoring system was developed based on the number of visible liver lesions using a logarithmic scale (Table 1). A similar scoring system has been previously applied to quantify liver damage caused by *C. hepaticus* (Van et al. 2017a). Assessment of the efficacy of vaccines was based on either the absence or presence of lesions, or a comparison of either lesion numbers or scores.

Table 1 Scoring system implemented to evaluate the liver damage induced by CH

Score	Number of spots on the liver
0	No visible spots
1	1–9 spots
2	10–99 spots
3	100–999 spots
4	More than 1000 spots

As the model rarely induces clinical symptoms, these were not used as a primary assessment of efficacy. During the project three separate strain, dose and virulence studies were undertaken, which confirmed that the challenge model challenge dose of 1×10^9 *C. hepaticus* organisms gave a reliable induction of disease.

Further details which were similar in all laboratory studies;

- Birds

  Healthy Hy-Line Brown pullets and hens were used in all trials.

- Inclusion/exclusion criteria

  Only healthy pullets or hens, from flocks that were PCR negative for *C. hepaticus* were included in the studies. Birds that were not fit, in the opinion of the attending veterinarian, were excluded from the study.

- Housing

  Pullets were housed in barns or cages on commercial rearing facilities in Victoria. The laboratory studies were carried out in group cages in the Scolexia Research Facility (SCARF). Field studies were carried out in commercial free range egg production sheds.

- Bird identification

  Vaccinated pullets were identified by the use of leg-tags and separate pens or cages. Positive and negative control birds were tagged at weighing on introduction to the research facility. Cages were clearly identified by both cage number and an added treatment code.

- Allocation

  Vaccinated birds were chosen based on location within the pullet rearing facility. All birds were weighed on arrival at the research facility. Allocation of birds to groups was undertaken after ranking by weight and use of previously generated random numbers generated using Microsoft® Excel® 2007 (© and trademarks Microsoft Corporation 2006). An analysis of variance was undertaken prior to finalisation of the groups to ensure no group had a significantly different mean weight to the others.

- Experimental unit

  For the purposes of disease negative or positive, liver lesion-scores, and in the field study, mortality, the individual bird was the experimental unit. For feed intake the cage or cage pair was the experimental unit. For egg production the cage was the experimental unit.

- Experimental groups (Table 2).

  Table 2 Basic experimental groups used in the studies

Group	Treatment	Exposure to *C. hepaticus*
A	Non-exposed – no treatment	No
B	Exposed – no treatment	Yes
C	Exposed – Autogenous vaccine 2 doses	Yes
[D	Exposed – Autogenous vaccine 3 doses	Yes]*

* Not included in all studies.

- Hypotheses

  $1H_0$: Group A [Spotty Liver] = Group (B or C) [Spotty Liver]
  $1H_1$: Group A [Spotty Liver] < Group (B or C) [Spotty Liver]
  and;
  $2H_0$: Group C, or D [Spotty Liver] = Group B [Spotty Liver]
  $2H_1$: Group C, or D [Spotty Liver] < Group B [Spotty Liver]
  Where [Spotty Liver] refers to birds being either positive or negative with respect to visible

SLD liver lesions. A further examination of SLD scores was also used to compare treatment groups.

Similar hypotheses were used for production parameters where these were assessed in 'Long' treatment groups (where production was measured for 4 weeks post-exposure), which were replicates of the groups listed in Table 2.

- Masking

  The treatments were not masked during the feeding and exposure periods.

- Criteria for a valid test

  At least 40% of challenged but untreated animals must contain SLD. A statistically significant difference in the proportion of SLD cases in the challenged control group compared to the unchallenged control group was required. The statistical assessment methods are listed below.

- Outcome criteria

  The outcome criteria involved comparison of the gross pathology of affected and unaffected birds in the treatment group with those in the challenged control group. Each bird with SLD was regarded as positive and those with none as negative. In addition, the severity of liver lesions (i.e., the number of lesions and/or the lesion scores) was examined, and the average or median results per group calculated and compared. Finally, in the later studies various production parameters were recorded and the average production parameter or change in production parameter per group was compared.

- Dose and route of administration

  Autogenous vaccine was administered intramuscularly (1 mL per bird per vaccination). A minimum of 0.3×10^9 *C. hepaticus* organisms per mL of vaccine was included in the autogenous vaccine.

  One mL of exposure or control broth was administered to the hens at between 24 and 28 weeks of age using a syringe inserted into the corner of the mouth and the birds allowed to naturally swallow the contents.

- Procedures
  - Daily husbandry

    Each day, birds were monitored for health as described below under monitoring and intervention. Eggs were collected and a record of eggs laid per cage was recorded. Birds were fed a commercial early lay ration *ad libitum*.

  - Vaccination

    The two autogenous vaccines were prepared from two Victorian strains of *C. hepaticus*, one strain per vaccine, at ACE Laboratory Services. The birds were sourced from an SLD negative farm in the same farming organisation that the isolate came from. The vaccine was administered to the pullets at approximately 9, 12, and, for the 3 times vaccinated birds, at 19–23 weeks of age.

  - Exposure material

    Exposure material (*C. hepaticus,* HV10 isolate) (Groups B, C and D) and control broth (Group A) produced by the RMIT University group was administered *per os* to the hens.

- Monitoring and intervention

  Birds were monitored for normal behavioural activity including drinking, feeding and egg laying prior to and after exposure. For specific clinical signs, birds were monitored for depression, inappetence, and any other abnormal signs. Birds were monitored a minimum of 3 times daily after the exposure.

- Euthanasia

  Intervention to remove affected birds was based on definitive signs of depression and recumbency being noted, as there was no requirement in this experiment to achieve mortality as an end-point. Intervention criteria as listed in the study protocols were used. Euthanasia was undertaken by cervical dislocation (as approved by the Model Code of Practice for the Welfare of Animals Domestic Poultry 4th Edition SCARM Report 83) at the defined examination points of the study.

- Autopsy examination

  The autopsy included visual examination of all the liver, spleen, gastrointestinal system, reproductive and renal systems. Samples of bile, liver, mid-intestinal and caecal content were taken for microbiology, and samples of liver, caeca and gut were taken for histopathology if required.

- Disposition of animals

  The birds were bagged, stored in a freezer and then disposed of by the appropriate method using a commercial contractor.

2.5 Modification of challenge model to assess production parameters.

As reduction in egg production (together with acute mortality) is the major impact of SLD, further treatment groups were added to the basic challenge model. The original groups (negative and positive control and vaccine groups) were retained and autopsied at 5–7 days post-challenge. These were referred to as 'Short' groups. These were replicated with groups that were kept for 4–5 weeks post-exposure to measure egg production and other parameters such as feed intake, egg weights, and in some cases yolk colour. These groups were also autopsied at the end of the period and livers examined for the presence of SLD.

2.6 Histology

Representative samples of livers were examined under light microscopy using routine processing with haematoxylin and eosin staining of the tissues.

2.7 Serology (ELISA)

The ELISA test used during this experiment was designed by RMIT to detect anti-*C. hepaticus* antibodies in chicken blood serum. These ELISA assays were carried out using *C. hepaticus* total protein extract (TPE) as the coating antigen, tested chicken sera as a primary antibody and goat anti-chicken Ig-Y-HRP as a secondary antibody. Novex 3,3',5,5'- tetramethylbenzidine (TMB) chromogenic substrate was used to develop the colour, and the intensity of the colour was measured at 652 nm in an ELISA plate reader. Samples with absorbance of more than the cut-off value (0.1) were considered as SLD positive. The cut-off value of 0.1 was established and validated testing the ELISA with positive and negative samples. The value of absorbance of 0.1 was able to clearly discriminate between positives and negatives.

2.8 Microbiology

During the project, the development of a selective culturing method for *C. hepaticus* was progressed as initially it was only possible to isolate the organism from bile and liver samples where there were no other organisms present. To that end the following activities were undertaken:

- *C. hepaticus* metabolic pathways, as predicted from the genome sequence, were bioinformatically studied to gain an understanding of the metabolic potential of the organism and determine if there were any metabolic processes that distinguished *C. hepaticus* from the other campylobacters commonly found in chickens; *C. jejuni* and *C. coli*. Such information could potentially be used to develop a specific selective media for *C. hepaticus*. A minimal media containing inorganic sources ($CaCl_2$, $Fe(NO_3)_3.9H_2O$, $MgSO_4$, KCl, $NaHCO_3$, NaCl, NaH_2PO_4), amino acid (L-cysteine) and αD-glucose as the carbon source was also used.
- Tested the optimisation of commercial selective media used for the isolation of other *Campylobacter* species to determine possible value in isolation of *C. hepaticus*. Media included: Brucella medium with horse blood (5%) and Skirrow supplement (Oxoid), Brucella medium with horse blood, Preston broth (Oxoid), Campy blood-free selective medium (CCDA) (Oxoid), blood free *Campylobacter* selective HivegTM agar base (Hiveg) plus Skirrow supplement, and tested the filter penetration method (using 0.65 µm membrane to filter mobile *C. hepaticus*). Colonies that were recovered were tested on a MALDI-Biotyper to determine if they were *C. hepaticus*. The optimised method was successfully used to isolate *C. hepaticus* from environmental samples (water and soil) spiked with *C. hepaticus.*

Due to the high similarity of the growth conditions and metabolisms of *C. hepaticus* and other *Campylobacter* (*C. jejuni* and *C. coli*), all the additives that were tested supported the growth of all three species. No clearly differentiating media could be identified. Therefore, the best method for primary isolation from microbiologically complex samples was to use a general *Campylobacter* selective medium and then differentiate the colonies that grew. The best method used Brucella medium with horse blood (5%) and Skirrow supplement (Oxoid), together with the use of the filter membrane technique in which the motile *Campylobacter* species can move through the membrane to the media beneath, whereas non-motile organisms are retained on the membrane surface and discarded when the membrane is removed. *C. hepaticus* could be differentiated from *C. jejuni* and *C. coli* based on a much slower growth rate and then subsequently by MALDI-Biotyper analysis. *C. hepaticus* was successfully reisolated from spiked soil and water samples; demonstrating the reliability of this method for the isolation of *C. hepaticus* from microbiologically complex samples. The method was subsequently used to isolate *C. hepaticus* from the faeces of experimentally challenged hens.

2.9 Yolk colour

In Study 2, the 15 eggs with weights closest to the mean weight of their group were opened and their contents placed on a white surface. The hue of the yolk was measured using a Digital Yolk Fan™ or DYF (DSM, powered by Nix Sensor Ltd.)[1], which is based on the modified Roche yolk colour fan, considering 16 grades of colour varying from pale yellow to deep orange. DYF is an automated system with a colour sensor based on a light emitting diode (LED) that objectively measures the hue of the yolk, reducing the subjectivity of the traditional yolk fan.

[1] For more information, visit: https://www.dsm.com/anh/en_US/solutions/dsm-color-fans/digital-yolkfan.html

2.10 Cloacal swabs and PCR

All pullets or hens enrolled in the studies were from flocks shown to be free from *C. hepaticus* infection both clinically and by use of either cloacal swabs or swabs of faeces taken prior to the study and examined for the presence of *C. hepaticus* by PCR.

DNA of the cloacal or faecal swab samples were prepared by either boiling of the sample resuspended in water and direct use of the supernatant or using DNeasy PowerSoil Kit (Qiagen) according to the manufacturer's instructions. For each batch of DNA extractions, cultured *C. hepaticus* cells were used as a positive control and water as a negative control. Isolated DNA was subjected to PCR amplification to detect the presence of C*. hepaticus* DNA. PCR primers specific to *C. hepaticus* were used as previously described (Van et al. 2017b). The PCR assay has been shown to be species-specific for *C. hepaticus*, with the limit of detection of the assay $1\times10^{0.9}$ (7.9) CFU/reaction.

2.11 Statistics

For non-parametric parameters and proportion of a treatment group with SLD and for comparison of the proportion of treatment groups below or above certain scores, a 2×2 contingency table was used as displayed in Table 3. Calculation of the χ^2 value was undertaken using either the Ausvet website tools (Sergeant, ESG, 2018. Epitools Epidemiological Calculators. Ausvet, available at: http://epitools.ausvet.com.au.), or by the use of the software package GraphPad Prism, version 8.4.2 (GraphPad Software, La Jolla California USA).

Table 3 Two by two contingency table

	Develop Spotty Liver	**No Disease**
Not exposed to the *C. hepaticus*	A	B
Exposed to the *C. hepaticus*	C	D

The odds ratio = (A x D) ÷ (B x C).
Probability was determined using the Chi-square ($\chi2$) distribution with a $P < 0.05$ being considered significant.

For data that were normally distributed, analysis of variance (ANOVA) was used. In pairwise analyses, a one-way ANOVA test was applied, while in multiple analyses, a two-way ANOVA test was applied and the multiple comparisons were made using the Tukey's multiple test. For data that were not normally distributed, a non-parametric ANOVA was used (one- or two-way, depending on the number of groups). In case of pairwise comparisons, a Mann-Whitney test was used. In the case of multiple comparisons, a Kruskal-Wallis test was used. Differences in proportions were compared using χ^2 and Fisher's exact tests. All these analyses were performed using the software package GraphPad Prism, version 8.4.2 (GraphPad Software, La Jolla California USA).

2.12 Animal ethics approvals

These studies were conducted using Scientific Procedures Fieldwork License SPFL20081, and under Animal Ethics approvals 14.16, 15.17, 16.17 and 19.17 issued by the Wildlife and Small Institutions Animal Ethics Committee.

2.13 Specific details of each study

2.13.1 Vaccine efficacy Study 1

In this study, *C. hepaticus* isolate HV10 was used for both the challenge and the vaccine manufacture. The vaccine batch was KINCAM00317 and it was stored at 3–8°C until use, as recommended by the manufacturer. Pullets were vaccinated on the rearing farm at 8 and 12 weeks of age. The birds in the three-times vaccine group were vaccinated at 23 weeks in the research facility. Eight birds were used in the negative control group, and 16 birds in each of the positive control and the two- and three-times vaccine groups. In this study all birds were euthanised five days after exposure. The timetable is displayed below.

Table 4 Timetable Study 1

Day (exact date)	Activity
-91 (11.12.17)	Administer first vaccination.
-66 (05.01.18)	Administer second vaccination.
-37 (13.03.18)	Pick up birds from a *C. hepaticus* free commercial layer farm (confirmed by negative PCR on cloacal swabs day -42) and transport to SCARF. Use Form 3 to record departure and arrival times, and examine and record bird health during transport every 2.5 hours. Only healthy birds (as determined by the veterinarian and considering the intervention criteria listed in Appendix A) are to be transported.
-36 (14.03.18)	Vaccinate birds in 3× vaccine group.
Daily	Monitor twice daily prior to exposure and 3 times daily after exposure, record egg production (Form 8) and observe general health of birds. Record monitoring results.
0 (19.04.18)	Administer sterile broth to Group A, and *C. hepaticus* broth to other groups. Record.
5 (24.04.18)	Autopsy examination of each group for evidence of SLD. Record autopsy results.

2.13.2 Vaccine efficacy Study 2

At the beginning of this study, a proportion of the pullet flock was vaccinated by injection using an autogenous vaccine of *C. hepaticus* isolate HV10 (ACE laboratories, Bendigo, Australia). The vaccine batch was CAM01318 and it was stored at 3–8°C until use, as recommended by the manufacturer. The pullets were vaccinated on two occasions, at 8 and 12 weeks of age in the rearing farm, using the intramuscular route of injection (pectoral muscle). The pullets to be used in the study were identified using leg-tags at the rearing farm and tested as negative to SLD. Vaccinated birds were separated from the rest of the flock by a fence during the complete rearing period.

At approximately 16 weeks of age, a sample group of vaccinated and unvaccinated birds (111 in total) was transported to the Scolexia Animal Research Facility (SCARF) for a controlled exposure of the birds to *C. hepaticus* under experimental conditions.

After their arrival at SCARF, pullets were weighed and initially allocated in individual cages equipped with feeders and nipple drinkers. Hens were distributed into three groups: negative control (30); positive control (39); and vaccinated (42). All groups were also divided into Short and Long groups, according of the time they were going to be euthanised. Short groups were euthanised 6 days after exposure, and Long groups at 41 days after exposure. The number of hens included in the negative control (NC), positive control (PC) and vaccinated Short groups were 6, 15 and 18, respectively, and 24 in all Long groups. Short groups were used to evaluate liver changes during the *post mortem* examination, while the Long groups were used to compare changes in production.

To assign pullets to each group, the weights initially recorded were considered, so the average weights of the hens per group were not statistically different, calculated using one-way analysis of variance (ANOVA) and Tukey's multiple comparison's test (Table 5).

Table 5 One-way ANOVA comparison of the weight of the pullets assigned per experimental group – Study 2

Source of Variation	SS	df	MS	F	P-value
Between Groups	0.016	6	0.003	0.334	0.914
Within Groups	0.276	35	0.008		
Total	0.292	41			

SS - sum of squares; df - degrees of freedom; MS - mean sum of squares; F - F-statistic.

After 48 hours in the individual cages, hens were transferred to their final cages according to their groups. All hens in the same cage shared the same egg collector. Every group of 3 or 4 hens were allocated to independent cages (having their own trough), or to two contiguous cages (sharing the same trough).

Hens were inspected two times a day during the complete duration of the experiment, except for the week after the exposure, when the hens were inspected three times a day to monitor any change in behaviour and health status.

Eggs were collected and the egg production recorded every day from every cage. The day before exposure to SLD and 7, 21 and 42 days after exposure (DAE), the eggs were individually weighed, and the weights recorded. The average weight of the eggs per group was calculated, and the 15 eggs with weights closest to their group average (below and above) were considered in the calculations. The objective of this selection was to eliminate the weight of double yolk eggs or extremely small eggs, which are of normal occurrence in egg production and their incidence is not related with SLD.

The amount of feed administered to each cage or pair of cages, depending of the distribution of the cages described above, was recorded. The residual feed in the troughs was collected with a vacuum machine at 9, 16, 23, 35, 45, 52, 66 and 87 days after the beginning of the experiment. The residual feed amounts were used to calculate the feed consumption during each period of time per cage-pair.

At 21 weeks of age, 62 blood samples were collected, 30 from vaccinated hens and 32 from unvaccinated hens. The blood sera were used to test seroconversion using ELISA. At the day of the challenge (11th of October) and 17 days after the challenge (28th of October), 10 hens per group were also bled and the blood serum used to test their anti-*C. hepaticus* antibody status using the ELISA test.

2.13.3 Vaccine efficacy Study 3 (field study)

The hens were sourced from the same flock of pullets in the rearing farm described above (Section 2.13.2 Vaccine efficacy Study 2). At approximately 16 weeks of age, vaccinated (1,910, HV10 vaccine) and unvaccinated (17,185) pullets were transferred to a commercial free range farm with a history of SLD. All the transferred pullets were housed in the same barn. At placement (8th of August, four weeks after the second vaccination), 45 and 46 blood samples were collected from the vaccinated and unvaccinated birds, respectively.

After transfer to the commercial farm, the flock was managed under normal husbandry conditions for a free range laying farm. All mortality in the shed was checked and recorded daily. Every dead hen was subjected to a *post mortem* examination by the shed manager, who was previously trained by Scolexia

personnel about *post mortem* techniques and pathological identification of SLD (basically, the presence of miliary spots on the liver surface). All mortalities were recorded on a Mortality Form, where birds were classified as with and without leg-tag (vaccinated and unvaccinated), and with and without SLD. The farm and the *post mortem* procedure were monitored by Scolexia at regular times.

2.13.4 Vaccine efficacy Study 4 (44L isolate used for vaccine production)

The study was conducted at the Scolexia Animal Research Facility (SCARF). The vaccine batch was RMTCAM03619 and it was prepared on 15th of May of 2019 and stored at 2–8°C until use, as recommended by the manufacturer. A total of 110 Hy-Line laying hens was distributed into 7 groups (Table 6). The weights of the hens were recorded on the day of arrival to SCARF. Both 'Short' (autopsied at 6 DAE) and 'Long' (29 DAE) treatment groups were used in the study. The Short groups were used to assess the *post mortem* changes of the hens (specifically, pathological changes of the liver), while the Long groups were used to compare production parameters between groups, such as egg production, egg weights, weight gain of the hens, feed consumption, and feed conversion efficiency (FCE). Also, there were two different treatment groups: the 2× and 3× vaccination groups (also described as vaccine × 2 and × 3). The 2× vaccine group received two vaccinations (at 8 and 12 weeks of age) using the *C. hepaticus* autogenous vaccine produced using the 44L isolate, while the 3× vaccine group was vaccinated using the same vaccine three times (at 8, 12 and 22 weeks of age). The negative control (NC) group comprised 28 hens (8 in the Short and 20 in the Long NC group), the positive control (PC) 36 (16 in the Short and 20 in the Long), the 2× vaccine 10 hens (Short group only) and the 3× vaccine group was comprised of 36 hens (16 in the Short and 20 in the Long group). Hens were allocated in groups of 4 hens per cage (with the exception of the 2× vaccine group, which had 2 cages with 3 hens each and one cage with 4 hens), each cage with an egg collector independent to that of other cages, but communal for the hens inside the cage. Contiguous cages shared a single feed trough, while cages separated from the other cages had an individual trough (important for feed consumption calculation). Hens were administered water using nipple lines of drinkers and town water, and were fed using commercial layer rations. Water and feed were administered *ad libitum*.

The weights of the hens were recorded on the day of arrival at SCARF and again at the end of the study (7 DAE for Short groups and 29 DAE for Long groups). During the experiment, the daily egg production per cage was collected and recorded. The amount of feed administered per cage/pair of cages was recorded. On the day of exposure of the hens to *C. hepaticus*, the residual amount of feed per trough was collected using a vacuum machine specially dedicated for this task, and the amount of residual feed per cage/pair of cages was recorded. The residual feed was collected and recorded at 6 DAE for the Short groups, and at 29 DAE for the Long groups. At 3, 6 and 14 DAE, all eggs were marked with the date and cage of origin, and were individually weighed. The average egg weight per group was calculated, and 30% of the egg weights that were most distant from the group average weight were not considered in the calculations.

In order to calculate the feed conversion efficiency (FCE), the data used were the egg weights collected on 3, 6, 14, 21 and 28 DAE and the average feed consumption per cage/pair of cages. The average feed consumption per cage calculated during the period was divided by the weight of the eggs collected from that cage. In order to exclude the outliers, those cages with an FCE more than mean ± 3 standard deviations (SD) were excluded from the calculations.

Table 6 Distribution by group of the hens included in Study 4 and the treatment received

Group	Treatment	Exposure to CH	Animals per group
A	Non-exposed – no treatment (Short)*- NC	No	8
B	Non-exposed – no treatment (Long)*- NC	No	20
C	Exposed – no treatment (Short) - PC	Yes	16
D	Exposed – no treatment (Long) - PC	Yes	20
E	Two-times vaccinated – 2× vaccine (Short)	Yes	10
G	Three-times vaccinated – 3× vaccine (Short)	Yes	16
H	Three-times vaccinated – 3× vaccine (Long)	Yes	20

* Short: autopsy conducted at 7 days after exposure; Long: autopsy conducted 29 days after exposure.

2.13.5 Vaccine efficacy Study 5 (field study)

A commercial quantity of *C. hepaticus* autogenous vaccine was prepared for use in the field. Pullets were placed in a divided aviary rearing shed with half (20,000) vaccinated at 12 and 16 weeks of age and the other half (20,000) left unvaccinated. Records of egg production and mortality due to SLD in each half of the shed were maintained throughout the life of the flock.

2.13.6 Strain and dose challenge model development studies

The general design of these studies was similar to the vaccine studies, with an unexposed negative control and the positive control group challenged with $1x10^9$ HV10 isolate *C. hepaticus* organisms. Other groups were challenged with either different isolates or different challenge doses, or with media grown in a different manner (plate or broth). As with the vaccine studies, the earliest of these studies were completed within a week of the challenge and the last study included Long groups, with autopsies conducted approximately one month after exposure.

3 Results

3.1 Safety studies

No reactions were observed except for slight depression in two birds, bird 1 at two- and 3-hour post-vaccination and bird 4 at two hours post-vaccination in the first safety study using vaccine batch KINCAM00317 (HV10 isolate) (ex 8 birds closely monitored). No other birds had visible signs of depression or endotoxic shock for the 7 or14 days following vaccination in any study during the three safety trials. No vaccine reactions were palpated when injection sites were examined at either 7- or 14-days post-vaccination in any of the studies. The lower density of birds in the cage to allow for careful observation is displayed in Figure 3 below.

Figure 3 Pullets being examined during the vaccine safety test

3.2 Autopsy (absence/presence of SLD)

In Table 7, the number of hens positive to SLD during the autopsy of Study 1 is presented. A hen was considered to be positive to SLD when presenting one or more liver lesions. All hens from the NC group were negative to SLD, as expected. From the challenged groups, the PC and 3× vaccine groups were 87.5% positive to SLD, while the 2× vaccine group had all the hens positive to SLD.

Table 7 Number of hens positive and negative to SLD and percentage of positives in hens unvaccinated or vaccinated with the HV10 *C. hepaticus* autogenous vaccine in Study 1

Treatment group	N° of hens without SLD	N° of hens with SLD	% positive
Negative control	8	0	0
Positive control	2	14	87.5
Vaccine × 2*	0	16	100
Vaccine × 3*	2	14	87.5

* Groups of birds vaccinated 2 or 3 times before the challenge with the HV10 C. hepaticus autogenous vaccine.

In Study 2, no negative control hens had SLD (Table 8). In contrast, all hens from both positive and vaccinated groups had characteristic SLD liver.

Of the birds that died during the field trial (Study 3), 13 vaccinated and 87 unvaccinated birds were recorded as SLD positive, while 17 non-SLD mortalities occurred in vaccinated and 136 non-SLD mortalities were recorded in unvaccinated birds (Table 9). Farm staff were trained and provided with photographic identification of SLD based on the presence of small discrete white/cream/grey/red lesions on the liver surface, and as distinct from larger amorphous lesions often associated with other bacterial infections. Due to the recurring nature of SLD on this farm, the farm staff were already familiar with our diagnostic recommendations but were formally briefed prior to the placement of the pullets. The diagnosis was based on the presence or absence of typical SLD liver lesions. The number of dead birds positive to SLD was compared with the total number of birds allocated to each group. The proportions were analysed using a contingency table, Chi-square analysis, and compared using Fisher's exact test. The results in Table 9 showed that there was no significant difference between the proportion of birds positive to SLD in the vaccinated and unvaccinated groups.

Table 8 Number of hens with SLD and percentage of positives in hens unvaccinated or vaccinated with the HV10 *C. hepaticus* autogenous vaccine in Study 2

Treatment group	N° of hens without SLD	N° of hens with SLD	% positive
Negative control	6	0	0
Positive control	0	14	100
Vaccine × 2*	0	16	100

* Number of times (2) these hens were vaccinated with the HV10 C. hepaticus autogenous vaccine in the rearing farm (at 8 and 12 weeks of age).

Table 9 Number of dead birds positive to SLD compared with the total of birds alive and those that died from a cause different to SLD, between vaccinated and unvaccinated birds in Study 3

Treatment group	N° of hens alive or dead without SLD	N° of dead hens with SLD	% positive
Unvaccinated group	1879	13	0.69%
Vaccine × 2*	17185	87	0.51%

* Number of times (2) these hens were vaccinated with the HV10 C. hepaticus autogenous vaccine in the rearing farm (at 8 and 12 weeks of age).
The Fisher's exact test was used comparing the proportions between vaccinated and unvaccinated birds, P = 0.55.

The farm was visited (4/10/2019) during an increase in the cases of SLD mortality. During the inspection, birds from the daily mortality were inspected, including those that died due to a smothering event. The vaccinated birds that were diagnosed as positive to SLD had visibly fewer lesion than the unvaccinated birds.

In Study 4, hens from the NC group had no SLD lesions (Table 10). A proportion of hens from the PC, 2× vaccine and 3× vaccine groups had visible SLD, ranging from 56.25% to 80.00%. The proportion of positives were significantly higher in the 2× and 3× vaccinated groups compared with the NC group, but not significantly different in both vaccinated groups compared with the PC group (Table 10 and Figure 4).

An outbreak of SLD occurred in the field (Study 5) when the hens were 27 weeks of age, and lasted for 6 weeks. A decrease in egg production of 11% was recorded in both vaccinated and unvaccinated groups. The mortality rate of hens showing *post mortem* findings attributable to SLD in the vaccinated group was 3.09%, while it was a 3.67% in the unvaccinated group.

Table 10 Number of hens with SLD in hens unvaccinated or vaccinated with the 44L *C. hepaticus* autogenous vaccine and challenged or not with the HV10 isolate in Study 4

Treatment groups	N° of hens without SLD	N° of hens with SLD	% positive
Negative control	8	0	0[a]
Positive control	5	11	68.75[b]
Vaccine × 2	2	8	80.00[b]
Vaccine × 3	7	9	56.25[b]

Different superscript in the same column represent significant differences, P < 0.05.

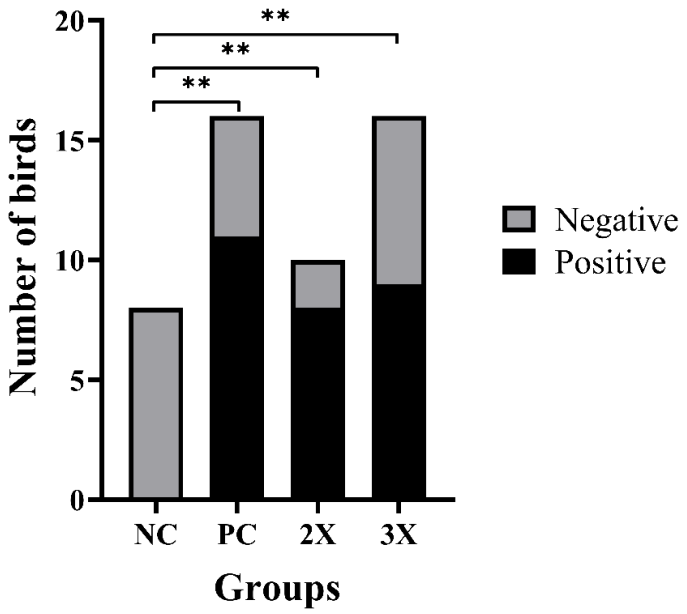

Figure 4 Proportions of hens positive and negative to SLD during the *post mortem* analysis of the Short groups in Study 4

NC - negative control; PC - positive control; 2X/3X - birds vaccinated 2 or 3 times.
** P < 0.01.

3.3 Autopsy (SLD lesion score and numbers)

The number of lesions and lesions scores are presented for those groups under laboratory conditions (Studies 1, 2 and 4). In the field studies (Studies 3 and 5), *post mortem* procedures were recorded by the personnel of the farm every day, determining those positives and negatives. However, liver lesions count and scoring were not performed.

Lesion scores of the hens in Study 1 are displayed in Table 11 and Figure 5 below.

Table 11 Average hepatic lesion scores in hens unvaccinated or vaccinated with a HV10 *C. hepaticus* autogenous vaccine – Study 1

Exposure	Number of birds	Median lesion score (range)
Negative control	8	0[a]
Positive control	16	2.5 (0-4)[b]
Vaccine × 2*	16	3 (1-4)[b]
Vaccine × 3*	16	3 (0-4)[b]

* Groups of birds vaccinated 2 or 3 times before the challenge with the HV10 C. hepaticus autogenous vaccine.

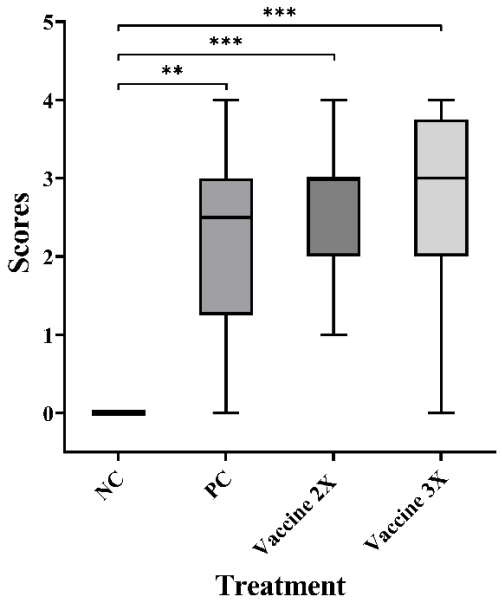

Figure 5 SLD lesion scores in birds either exposed or not to *C. hepaticus,* and vaccinated or not in Study 1

NC - negative control; PC - positive control; Vaccine 2X/Vaccine 3X - birds vaccinated 2 or 3 times.
** P < 0.01; *** P < 0.001.

In Study 2, the average number of lesions calculated from the positive control group was 638 ± 383 compared with the 331 ± 378 of the vaccinated group, and that difference was close to being statistically significant (Figure 6 and Table 12, P = 0.06). The mean number of lesions of the vaccinated group was not significantly higher than the NC group (P = 0.15). However, when the negative control is removed from the analysis and only the PC and the 2× vaccine groups are compared, the difference was significant, with a P = 0.04.

The median lesion score in the positive control was 3, with a range from 2 to 4. In the vaccinated group, the median score was 2.5, with a range from 1 to 4. The difference between the PC and the vaccinated group was significant (P = 0.045). The difference becomes even more significant when the NC control is not included in the analysis and only the PC and the vaccinated groups are compared (P = 0.02).

Table 12 Average numbers of lesions and lesion scores in hens unvaccinated or vaccinated with the HV10 *C. hepaticus* autogenous vaccine in Study 2, Short groups

Exposure	Number of birds	Average N° of lesions ± SD*	Median lesion score (range)
Negative control	6	0[a]	0[a]
Positive control	15	638 ± 383[b]	3 (2-4)[b]
Vaccine × 2*	18	331 ± 378[ab]	2.5 (1-4)[c]

* Number of times (2) these hens were vaccinated with the HV10 C. hepaticus autogenous vaccine in the brooding farm (at 8 and 12 weeks of age).
Different superscript letters in the same column represent significant differences, P < 0.05.

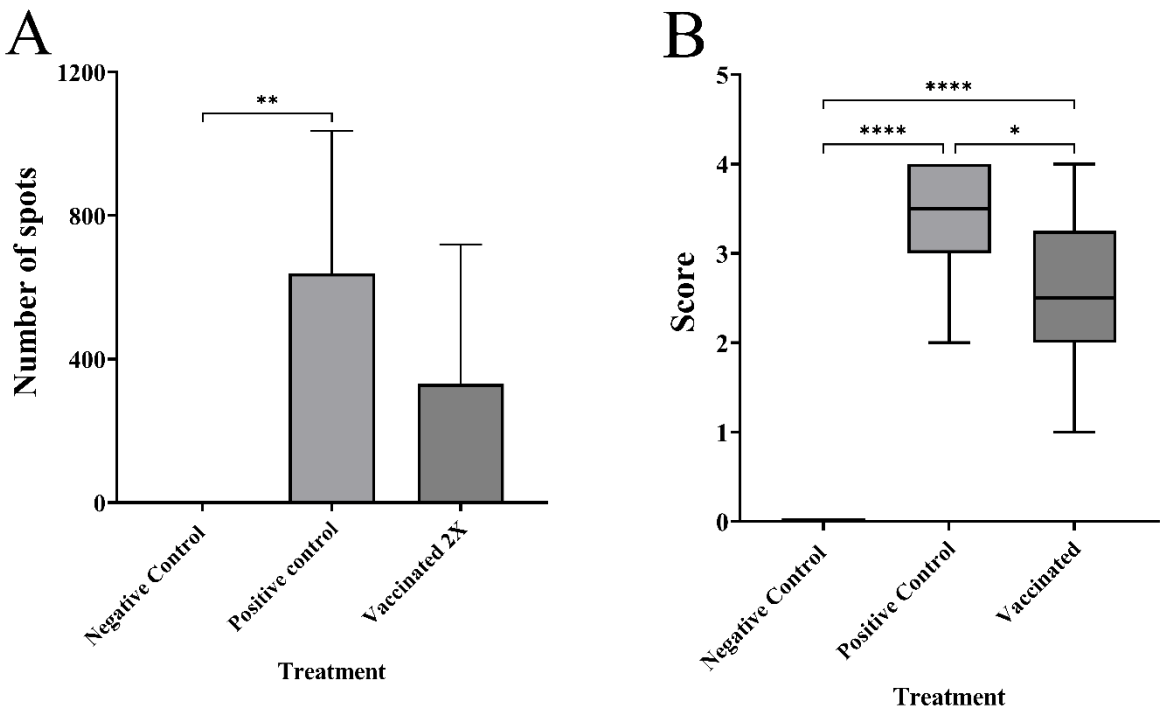

Figure 6 SLD lesion numbers (A) and lesion scores (B) in vaccinated and unvaccinated birds either exposed or not to *C. hepaticus*, and vaccinated or not in Study 2

The tops of the columns for the number of lesions represent the mean, and the error bars are the SD.
The middle line in the boxplots of the scores represent the median values, while the error bars the range (minimum and maximum values).
* P < 0.05; ** P < 0.01; **** P < 0.0001.

In Study 4, the average number of lesions compared with the negative control group, were significantly higher in both the PC and 2× vaccine groups (Table 13 and Figure 7, P = 0.02 and 0.003, respectively). However, the number of lesions remained low in the 3× vaccine group, which were not significantly higher compared with the negative control group either (P = 0.44), although the lesion was not significantly lower than those observed in the PC group (P > 0.999). The situation was similar with the lesion scores, where both PC and 2× vaccine groups had scores significantly higher than the NC group (Table 13 and Figure 7, P = 0.01 and 0.005, respectively). The scores in the 3× vaccine group were not different to the NC (P = 0.38) or the PC groups (P > 0.999).

Table 13 Average SLD lesion numbers and median lesion scores in hens vaccinated or not with a 44L *C. hepaticus* autogenous vaccine and challenged or not with *C. hepaticus* in Study 4

Treatment group	Number of birds	Average N° of lesions ± SD	Median lesion score (Min-Max)
Negative control	8	0[a]	0[a]
Positive control	16	201 ± 327[b]	2 (0-4)[b]
2× vaccine	10	271 ± 315[b]	3 (0-4)[b]
3× vaccine	16	37 ± 49[ab]	1 (0-3)[ab]

Different superscript letters in the same column represent significant differences, P < 0.05.

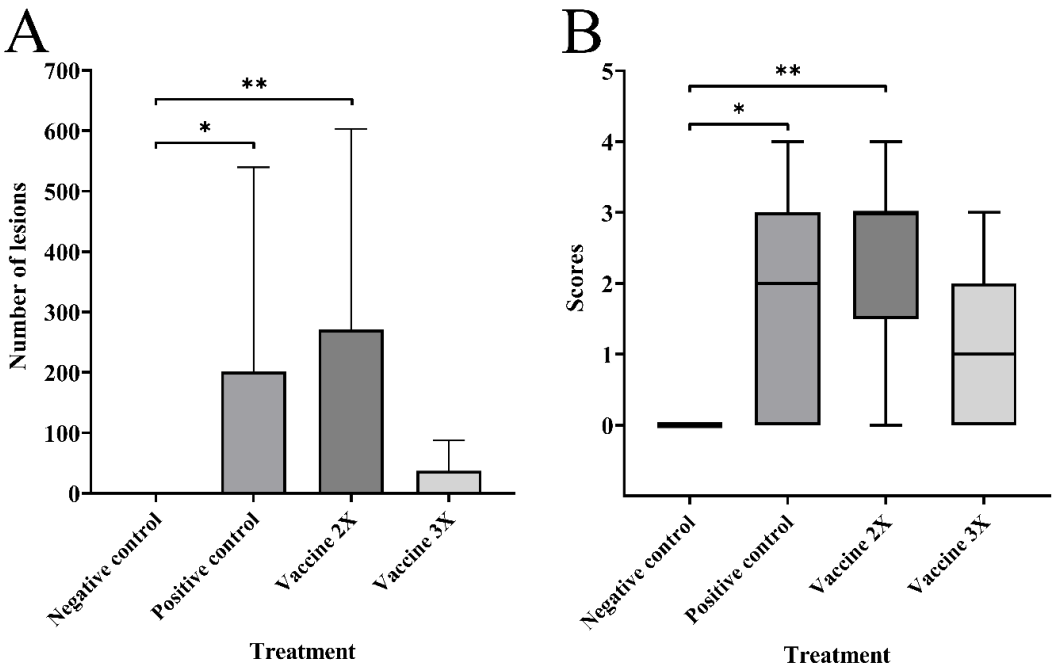

Figure 7 SLD lesion numbers (A) and lesion scores (B) in birds either exposed or not to *C. hepaticus*, and vaccinated or not in Study 4

The tops of the columns for the number of lesions represent the mean, and the error bars are the SD.
The middle line in the boxplots of the scores represent the median values, while the error bars the range (minimum and maximum values).
* P < 0.05; ** P < 0.01.

3.4 Histology

The primary lesion in SLD positive birds was well delineated multi-focal randomly dispersed areas of coagulative necrosis. Severe lesions (histological score of 3+) had a consistent finding of severe multi-focal subacute randomly distributed hepatic coagulative necrosis. This was characterised by degenerate, shrunken and necrotic hepatocytes, with lakes of fibrin with variable numbers of heterophils and macrophages. Mild to moderate lesions (histological score 1+ and 2+) were mainly aggregates of inflammatory cells, often macrophages and lymphocytes, and degenerate cells or disruption of hepatic cords.

3.5 Serology

Serology results in vaccine Study 2 showed that all the unvaccinated birds were ELISA negative and all the vaccinated birds were ELISA positive, showing a good increase in the antibody titres following vaccination. There was a slight decrease in the ELISA absorbance in the vaccinated group between the first and second sampling day, which was represented by a decrease in the percentage of positive samples (Table 14). However, the samples remained above the cut-off value of the test (Figure 8). Between the second and third sampling day, there was an increase in the mean absorbance of the vaccinated group, significantly higher than the mean absorbance from the previous sampling days.

The hens in Study 3 (field study) vaccinated with the HV10 SLD vaccine had an increase in antibody titres after vaccination, which decreased significantly approximately one month before the onset of clinical signs related with SLD recorded in the shed (Table 15 and Figure 9). The level of antibodies in the unvaccinated hens remained below the cut-off value of 0.1 (ELISA negative) in both ELISA tests and remained significantly lower than the vaccinated group.

Table 14 Proportion of sera above and below the positive ELISA threshold – Study 2

Group	ELISA	Weeks after second vaccination			
		5*	9	14**	16
Negative control	Positive	0	2	0	0
	Negative	46	30	30	30
	% ELISA positive	**0.0^{a,x}**	**6.3^{a,x}**	**0.0^{a,x}**	**0.0^{a,x}**
Positive Control	Positive	0	2	2	30
	Negative	46	30	28	0
	% ELISA positive	**0.0^{a,x}**	**6.3^{a,x}**	**6.7^{a,x}**	**100.0^{b,y}**
Vaccinated	Positive	39	16	28	30
	Negative	6	14	2	0
	% ELISA positive	**86.7^{a,y}**	**53.3^{b,y}**	**93.3^{a,y}**	**100.0^{a,y}**

Different superscript letters (a, b) represent significant differences within the same row, $P < 0.01$.
Different superscript letters (x, y) represent significant differences within the same column, $P < 0.0001$.
Note: The ELISA assays were conducted on different days and this can impact comparisons between time-points.
* Date of transfer to production; ** Date of exposure of hens to C. hepaticus.

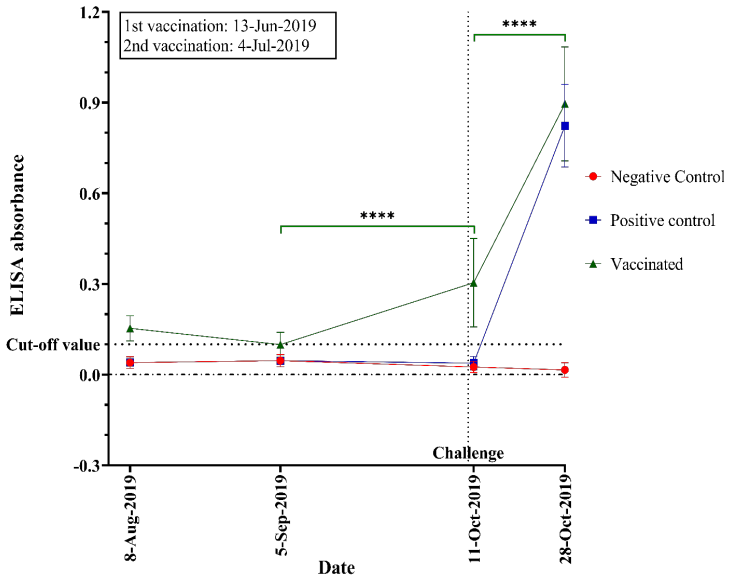

Figure 8 Serology (ELISA) from sera taken from vaccinated and unvaccinated hens at different time-points in Study 2

The horizontal dotted line represents the cut-off value of the test.
The vertical dotted line corresponds to the SLD challenge day.
Note: The ELISA assays were conducted on different days and this can impact comparisons between time-points.
**** $P < 0.0001$.

Table 15 Average ELISA absorbance of the hens in Study 3

Date	Negative control	Vaccinated	Event
08-Aug-19	0.041 ± 0.02^{a}	0.154 ± 0.04^{b}	Transfer to production
05-Sep-19	0.046 ± 0.02^{a}	0.100 ± 0.04^{a}	
03-Oct-19			Start of the outbreak*
15-Oct-19			End of the outbreak*

Different superscript letters in the same row represent significant differences ($P < 0.05$), calculated with a two-way ANOVA and Tukey's multiple comparisons test.
* These dates include 84.6% and 87.3% of the recorded mortalities with SLD liver lesions in the vaccinated and unvaccinated groups, respectively.

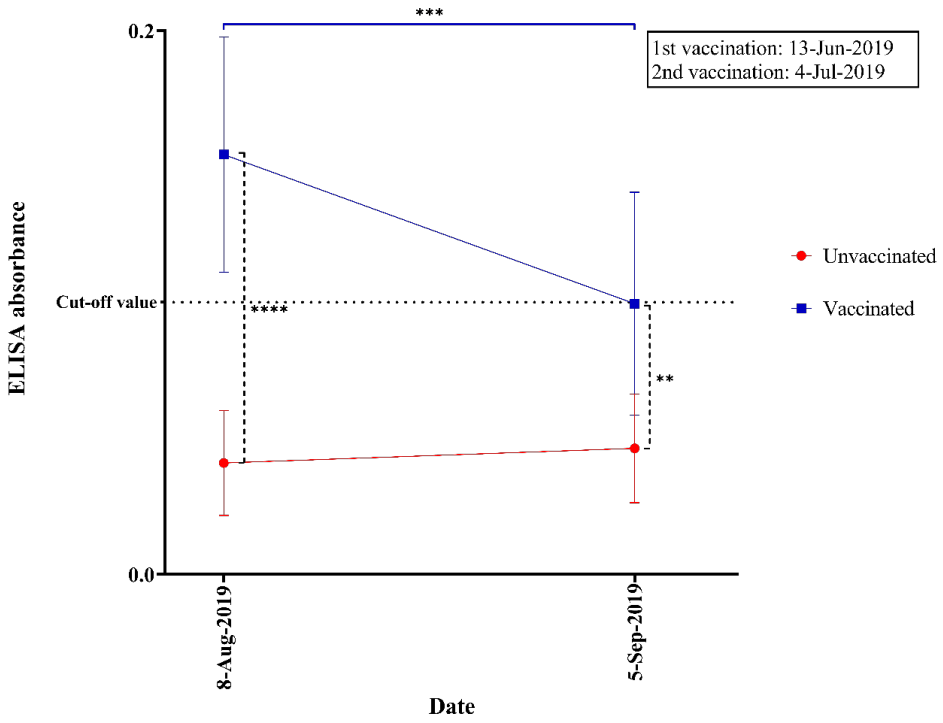

Figure 9 Serology (ELISA) from sera taken from vaccinated and unvaccinated hens at two different time-points in Study 3

The horizontal dotted line represents the cut-off value of the test.
Note: The ELISA assays were conducted on different days and this can impact comparisons between time-points.
** P < 0.01; *** P < 0.001; **** P < 0.0001.

Serology results in vaccine Study 4 showed that there was an increase in antibodies after the first vaccination, although there was no significant difference between vaccinated and unvaccinated hens (Figure 10). After the second vaccination there was a significant increase in the antibody level of the vaccinated hens (P < 0.0001) when compared with the unvaccinated hens. Before exposure to *C. hepaticus*, those hens that received a third vaccination (3× vaccine group) retained the level of serum antibodies, while those that were vaccinated 2 times (2× vaccine group) had a considerable decrease in serum antibodies, significantly lower than those of the hens in 3× vaccine group (P < 0.05). The antibody levels of the hens in the 2× vaccinated group were significantly higher than those in the unvaccinated hens (P < 0.05) and were above the cut-off level of the test. After challenge, all challenged groups had an increase in antibody levels, and there was not a significant different between those groups (P = 0.26), while the NC group hens remained negative, with an ELISA test reaction below the cut-off value, and significantly lower than that of the challenged hens (P < 0.0001).

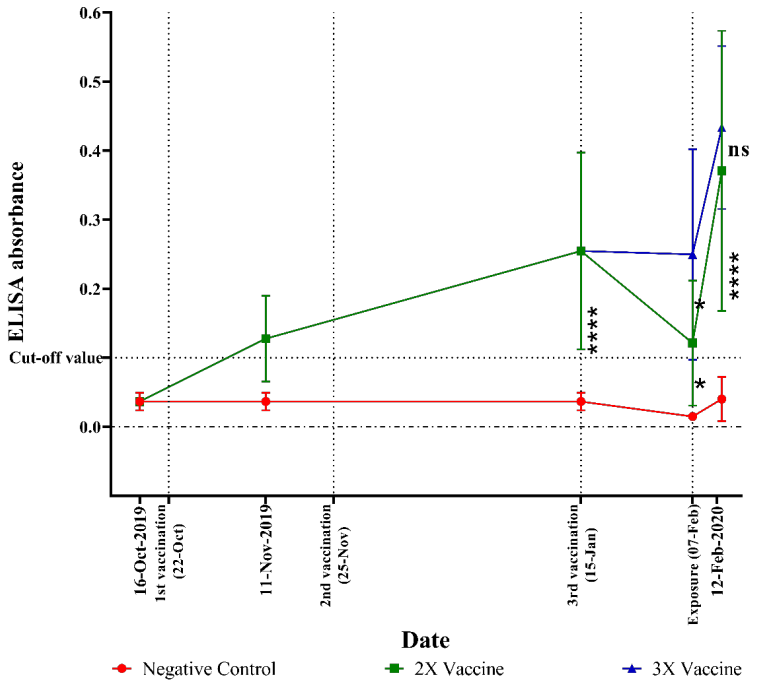

Figure 10 ELISA on serum samples collected before vaccination, before and after exposure of the hens to *C. hepaticus* during Study 4

ns - not significant.

Note: The ELISA assays were conducted on different days and this can impact comparisons between time-points.

* P < 0.05; **** P < 0.0001.

3.6 Production parameters (bird weight)

There were three independent dose and isolate studies undertaken prior to and during these vaccine studies. In the last of the dose and isolate studies where productivity was examined (Figure 11), the negative control birds gained an average of 68 g between exposure and the end of the study compared to the birds in the long-term exposed control group which lost on average 10 g of bodyweight (P < 0.02).

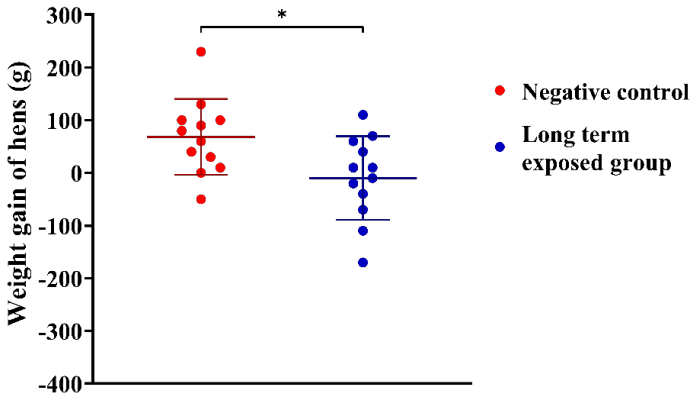

Figure 11 Weight gain of hens from the negative control group compared with the group of hens exposed to *C. hepaticus* (dose and isolate study)

* P = 0.02.

In vaccine Study 4, the hens in all groups had a significant increase of body weight during the study. However, that increase in weight appeared to be less significant in the PC group compared with the NC and 3× group (Figure 12-A). The weight gain was calculated per hen and group. As displayed in Figure 12-B, the mean weight gain in the hens from the 3× group was significantly higher than that of the PC group.

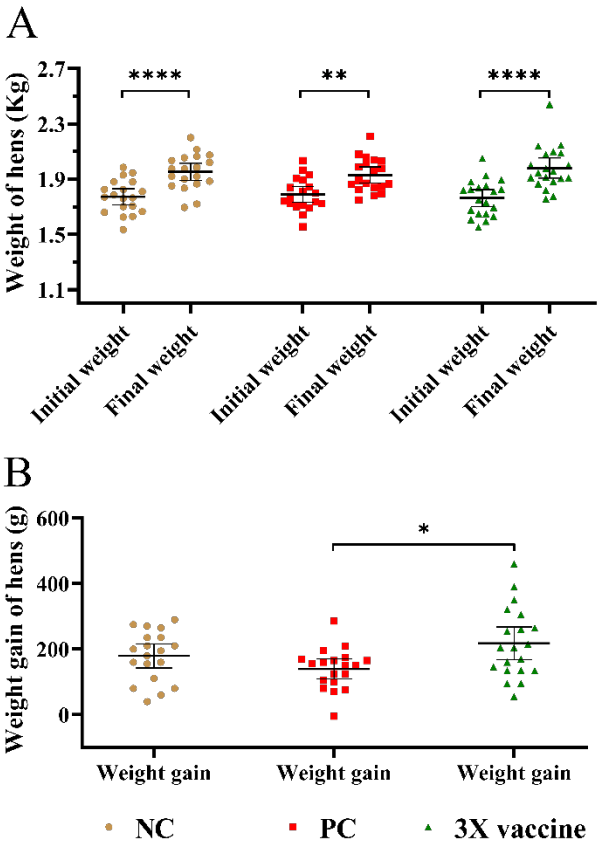

Figure 12 Weights of hens per group recorded in Study 4

Bars represent the mean and SD.
A - Initial and final weight of hens; B - weight gained per hen between the initial and final weights.
NC - negative control; PC - positive control.; 3X - hens vaccinated 3 times.
* P < 0.05; ** P < 0.01; **** P < 0.0001.

3.7 Production parameters (egg production)

In the final isolate and dose study (a preliminary study conducted before the vaccine studies referred to in this document), egg production was reduced compared to pre-exposure production in the exposed hens. After exposure, the hens exposed to *C. hepaticus* had a lower hen per-day egg production of 85.4% compared to the long-term negative control group (91.6%). Differences were calculated using contingency tables of the expected number of eggs based on pre-exposure production in each group and the difference in expected and actual production after exposure. In this study, the production was monitored for the first week post-exposure. This drop in production post-exposure was not replicated in vaccine Study 2, (Figure 13 and Table 16), where there was not a statistically significant difference in production pre-and post-exposure between the negative and positive control groups. However, there was a significant drop in production in the vaccinated group post-exposure.

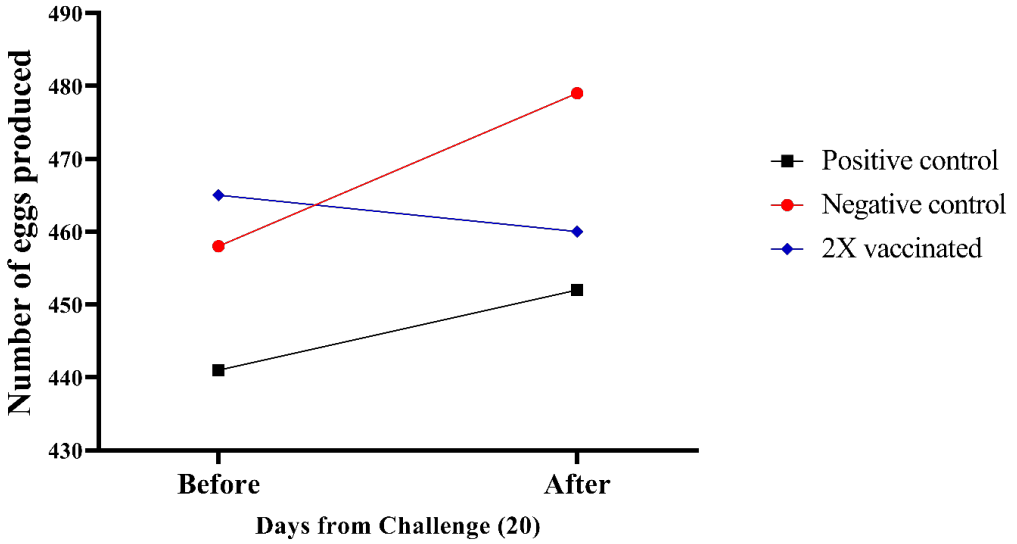

Figure 13 Egg production from hens in Study 2 which were either vaccinated or not (HV10 isolate) and exposed or not to *C. hepaticus* (HV10 isolate)

Table 16 Number of eggs expected for each group to be produced after exposure calculated using the egg production data before exposure in Study 2

	Negative control	Positive control	2× vaccine
Expected	458	441	465
Difference	21	11	-5
Significance*	A	A	B

* Different letters depict proportions that differ statistically, $P < 0.001$.

In Study 4, there was an increase in egg production in hens from PC, NC and 3× groups (Figure 14). However, the increase in production was less prominent in the hens from the PC group. Table 17 summarises the difference between the number of eggs produced during a period of 20 days after the exposure versus the expected egg production, which is based on the egg production during 20 days before the exposure. The increase in egg production obtained by the 3× vaccine group was significantly higher than that obtained by the PC control group.

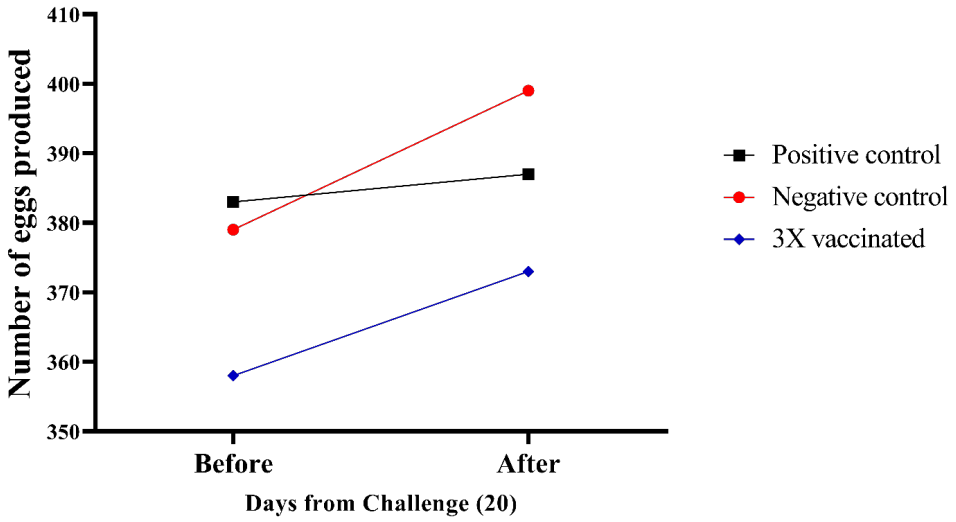

Figure 14 Egg production from hens in Study 4 which were either vaccinated or not (44L isolate) and exposed or not to *C. hepaticus* (HV10 isolate)

Total number of eggs produced from the same number of birds for 20 days prior to challenge and 20 days post-challenge.

Table 17 Number of eggs expected for each group to be produced after exposure calculated using the egg production data before exposure in Study 4

	Negative control	Positive control	3× vaccine
Expected	379	383	358
Difference produced cf. expected	20	4	15
Significance*	A	B	A

* Different letters between columns represent significant differences ($P < 0.01$), calculated by χ^2 and two-sided Fisher's exact test.

In Study 5, there was an equal decrease in egg production of 11% in both vaccinated and unvaccinated groups.

3.8 Production parameters (egg weight)

In Study 2, there was a constant increase in the weight of eggs collected from the negative control group from an average of 58.64 to 63.43 grams (Table 18 and Figure 15). In both groups exposed to *C. hepaticus*, there was a reduction in the average weight of the eggs one week after exposure. However, that decrease in the average egg weight was not significant in the vaccinated group. After that reduction, there was a significant increase in the average egg weight, and the average egg weights were also significantly higher when compared with the pre-exposure average egg weight.

Table 18 Average egg weights and their change between different sampling days in Study 2*

Days from challenge	Negative control (g)	Change (%)	Positive control (g)	Change (%)	Vaccinated (g)	Change (%)
-1	58.64[a]		59.86[a]		57.81[a]	
7	59.68[ab]	1.77%	56.45[b]	-5.70%	56.12[a]	-2.92%
21	61.30[b]	2.71%	60.07[a]	6.41%	59.88[b]	6.70%
42	63.43[c]	3.47%	61.36[a]	2.15%	60.82[b]	1.57%

* Only 70% of egg weights closest to their own group average weight were included.

Different superscript letters in the same column represent significant differences, $P < 0.05$.

The percentage change was calculated comparing the mean egg weight of that sample day with the mean egg weight from the previous sampling day.

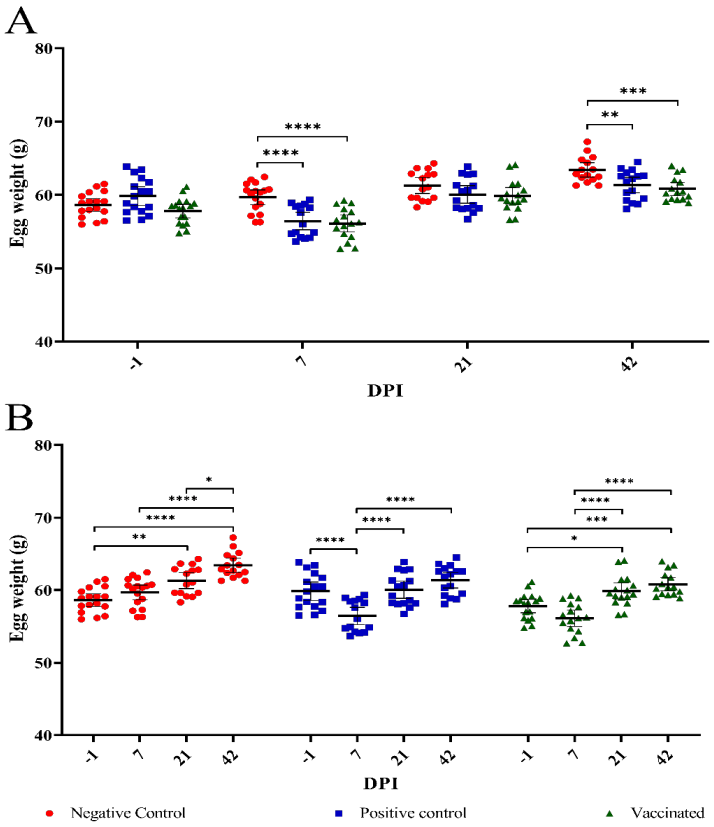

Figure 15 Plots depicting the egg weights from a sample taken from each treatment group the day before the challenge, and 7, 21 and 42 days from challenge in Study 2

Thirty percent of the egg weights that were more distant from the mean weight of their own groups were excluded from the analysis. DPI - Number of days from challenge (expressed as days post-infection).

A - Comparison of the egg weights per collection day between groups.

B - Comparison of the egg weights per collection day within each group.

Each dot represents an individual egg weight, the middle horizontal lines represent the mean values, and the top and bottom horizontal lines represent the 95% confidence interval.

The lines with asterisks show the significant difference: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

In Study 4, the weight of the eggs from the hens in the NC group increased significantly until 14 DAE, where it reached a plateau (Table 19 and Figure 16). On the other hand, there was a significant drop in the weight of the eggs in the PC group between 3 and 6 DAE. Even though there was a recovery in the weight of the eggs in the following sampling days, they were never statistically different to the egg weights 3 DAE. There was a slight decrease in the weight of the eggs in the vaccinated group between 3 and 6 DAE, but it was not significant. Unlike the PC group, egg weight in the vaccinated group at 28 DAE were significantly higher than at 3 DAE.

Table 19 Average egg weights and their change between different sampling days in Study 4*

Days from challenge	Negative control (g)	Change (%)	Positive control (g)	Change (%)	Vaccinated (g)	Change (%)
3	55.64^{a}		57.14^{ac}		57.16^{ab}	
6	57.97^{ab}	4.19%	54.38^{b}	-4.83%	55.38^{a}	-3.11%
14	59.70^{b}	2.98%	56.62^{abd}	4.12%	58.65^{b}	5.90%
21	60.19^{b}	0.82%	59.69^{c}	5.42%	59.67^{bc}	1.74%
28	59.96^{b}	-0.38%	58.73^{cd}	-1.61%	60.93^{c}	2.11%

* Only 70% of egg weights closest to their own group average weight were included.
Different superscript letters in the same column represent significant differences, P < 0.05.
The percentage change was calculated comparing the mean egg weight of that sample day with the mean egg weight from the previous sampling day.

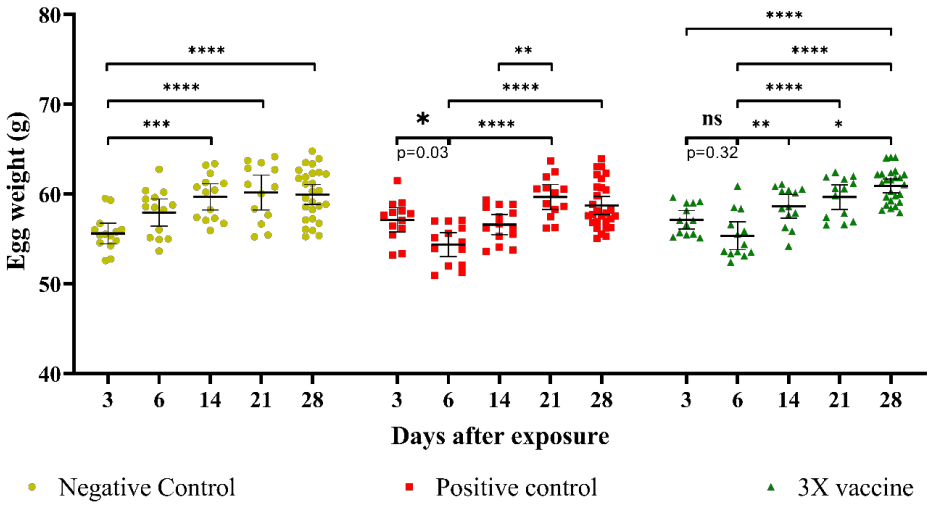

Figure 16 Egg weights in Study 4, hens vaccinated (isolate 44L) or not and challenged or not with *C. hepaticus* (VH10)

ns - not significant.
* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001.

3.9 Yolk colour

In Study 2, there were no significant differences in terms of yolk colour between the eggs laid by the unvaccinated and unchallenged hens, and those unvaccinated and vaccinated and challenged hens (Figure 17). The median score in the eggs from all the groups and at all the sampling times was 12, with a range that was in general between 11 and 13, with the exception of the negative control group at 7 DAE (11–12) and 21 DAE (12–13).

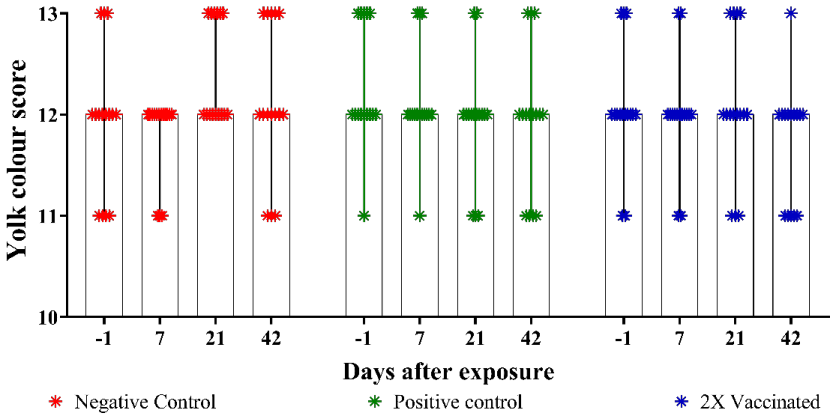

Figure 17 Median scores and quartile range of the egg yolk scores recorded per group and sampling day in Study 2

The scores from the 15 eggs with weights closer to the mean weight of their own group were included in the analysis. Each asterisk represents an individual score.
The tops of the columns represent the median value, and the black vertical lines represent the median range (minimum and maximum values).

3.10 Production parameters (feed intake)

In Study 2, feed consumption was, in general terms, increasing from the first sampling day until the date of challenge (Figure 18). There was an exception in the NC group between the 4th and 11th of September, where there was a drop in feed consumption, but this was not significant.

After the challenge with *C. hepaticus*, there was a drop in feed consumption in both the PC and vaccinated group, even though that drop was not statistically significant for either of them compared with their previous sampling day (P = 0.22 and 0.98 for both PC and vaccinated groups, respectively). However, in the vaccinated group the drop in feed consumption started from the sampling day before the challenge (30th of September), suggesting there could be other factors influencing the drop in feed consumption not related with SLD.

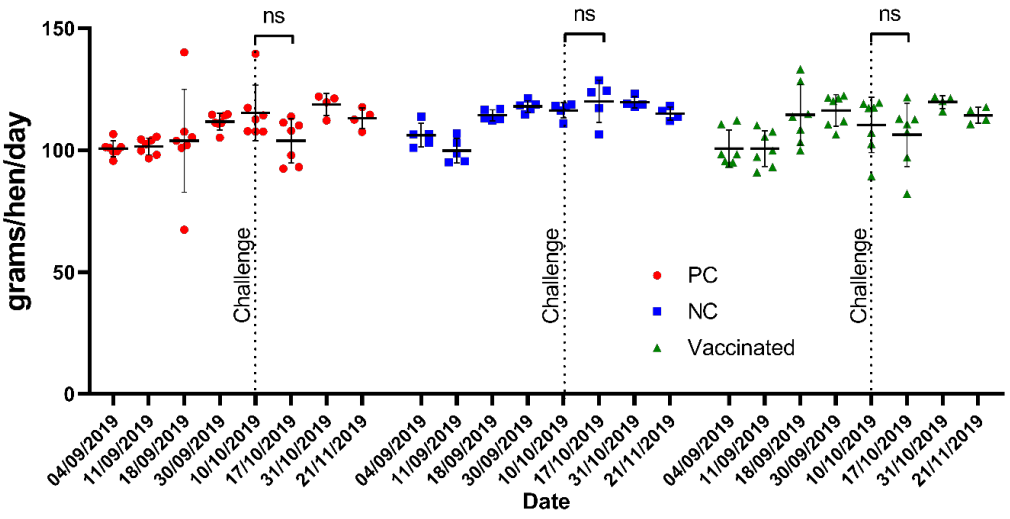

Figure 18 Average daily feed intake of hens of Study 2

Challenge: Day of the oral exposure of the PC and vaccinated groups with C. hepaticus.
ns - *not significant.*
PC - positive control; NC - negative control.

In Study 4, feed intake increased between pre-challenge and 29 DAE (Long groups), in the NC and 3× vaccine groups with a less pronounced increase in feed intake in the PC group (Figure 19). However, the differences were not statistically significant between groups nor between different times (before and after challenge) within groups.

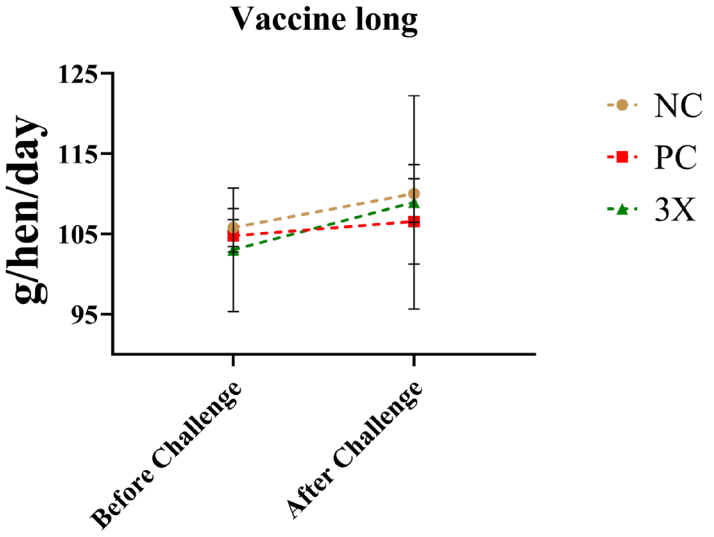

Figure 19 Average daily feed intake of hens belonging to the Long groups in Study 4

NC - negative control; PC - positive control.; 3X - hens vaccinated 3 times.
Differences were not statistically significant, P > 0.05.

3.11 Production parameters (feed efficiency)

The feed conversion efficiency of Study 2 is presented in Table 20 and Figure 20. Even though there was a decrease in the feed conversion efficiency in all the groups, this was not significant in the vaccinated group and slightly significant (P = 0.04) in the negative control group for reasons not related with SLD. However, the decrease in efficiency was highly significant in the positive control group (P = 0.0002). Also, the recovery from that decrease in efficiency was superior in those birds vaccinated compared with the positive control. While the difference in feed conversion between 7 and 21 and 42 DAE in the vaccinated group was highly significant (P < 0.0001), it was less significant in the positive control group (P < 0.001 and P < 0.01). Also, the feed conversion in the vaccinated group at day 21 was better than that observed before the challenge (P < 0.0001), and that was not the case with the PC group, where there was no statistically significant difference.

Table 20 Mean feed conversion efficiencies of hens in Study 2 (± SD)

DAE	NC	PC	2× vaccine
-1	1.96 ± 0.07^{a}	1.85 ± 0.08^{a}	2.00 ± 0.09^{ac}
7	2.04 ± 0.11^{c}	1.97 ± 0.08^{b}	2.05 ± 0.10^{c}
21	1.81 ± 0.05^{b}	1.85 ± 0.09^{a}	1.85 ± 0.05^{b}
42	1.82 ± 0.04^{b}	1.86 ± 0.07^{a}	1.87 ± 0.07^{b}

NC - negative control; PC - positive control.; 2x vaccine - hens vaccinated 2 times.
Different superscript letters in the same column represent significant differences, P < 0.05, calculated by two-way ANOVA and Tukey's multiple comparisons test.
DAE - days after exposure.

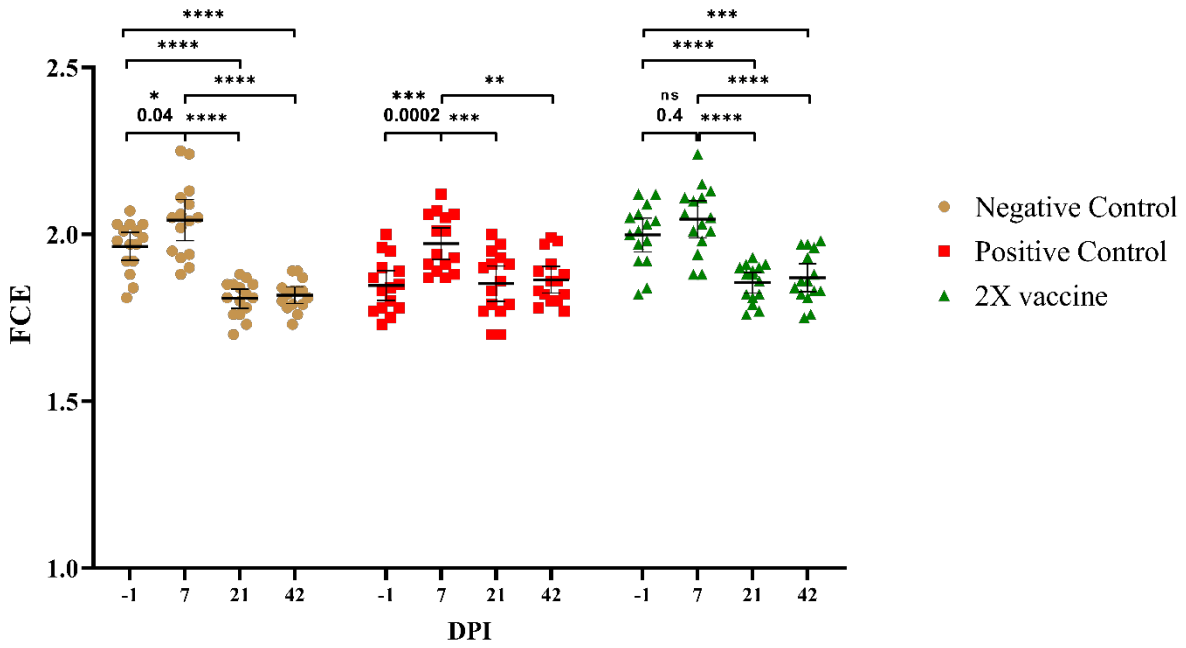

Figure 20 Feed conversion efficiency (FCE) calculated in the three Long groups in Study 2, using the egg weights and feed consumption data

DPI - Number of days from challenge (expressed as days post-infection
* P < 0.05; * P < 0.01; ** P < 0.001; **** P < 0.0001.

Data presented in Table 21 and Figure 21 show feed conversion efficiency (FCE), expressed as feed weight/egg weight ratio (grams of feed needed to produce one gram of egg) from hens in Study 4. The FCE improved over time in the hens from the NC group, with a decrease of the feed weight/egg weight ratio from 1.96 ± 0.13 to 1.86 ± 0.14. On the other hand, in the PC group there was a decrease in the FCE, with an increase in the feed weight/egg weight ratio between 3 and 6 DAE, but this difference was not statistically significant (P = 0.3). After day 6, the FCE improved gradually, and at 28 DAE it was significantly better compared with 6 DAE (1.96 ± 0.10 and 1.81 ± 0.10). Finally, in the 3× vaccine group, the FCE had a slight decrease in the FCE between 3 and 6 DAE, which was not statistically significant, and less prominent than that of the PC group. After that, the FCE improved gradually over time.

Table 21 Mean feed conversion efficiencies of the hens in Study 4 (± SD)

DAE	NC	PC	3× vaccine
3	1.96 ± 0.13	1.86 ± 0.14^{ab}	1.87 ± 0.22
6	1.91 ± 0.11	1.96 ± 0.10^{a}	1.90 ± 0.23
14	1.86 ± 0.11	1.86 ± 0.10^{ab}	1.82 ± 0.20
21	1.86 ± 0.12	1.80 ± 0.10^{ab}	1.81 ± 0.19
28	1.86 ± 0.14	1.81 ± 0.10^{b}	1.79 ± 0.22

NC - negative control; PC - positive control.; 3x vaccine - hens vaccinated 3 times.
Different superscript letters in the same column represent significant differences, P < 0.01, calculated by two-way ANOVA and Tukey's multiple comparisons test.
DAE - days after exposure.

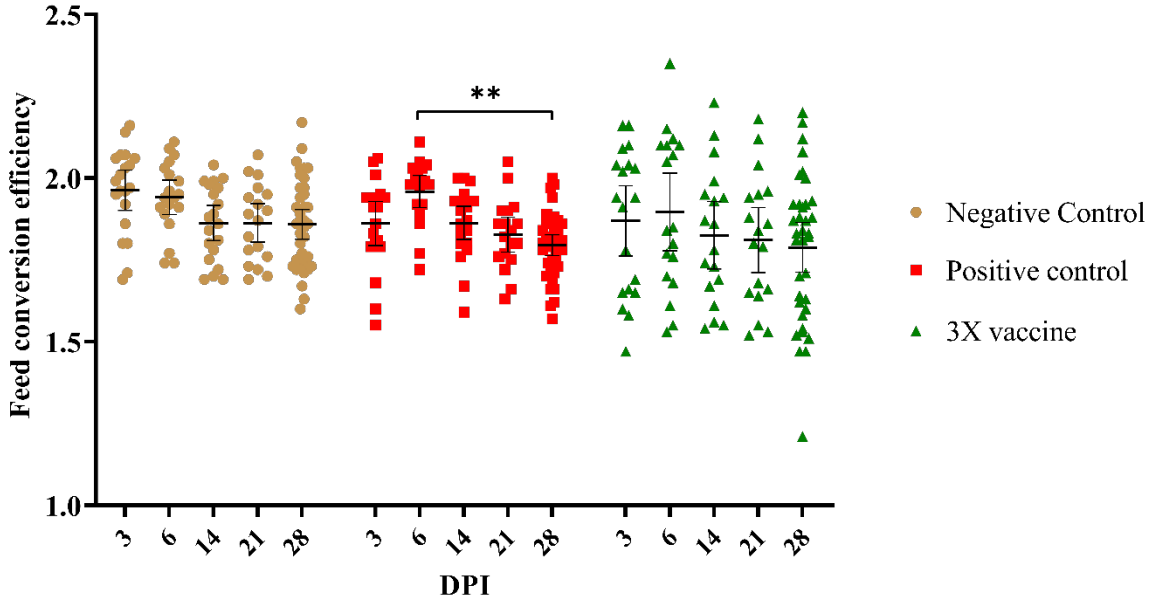

Figure 21 Feed conversion efficiency calculated in the three Long groups in Study 4, using the egg weights and feed consumption data

DPI - Number of days from challenge (expressed as days post-infection.
** P < 0.01.

4 Discussion

The difficulties of working with *Campylobacter* species are well known. *C. jejuni* is one of the most common causes of diarrhoea in humans world-wide and undercooked poultry meat is an important source of those infections (Kobierecka et al. 2016; Scott 1997). *Campylobacter* in poultry was ranked as the pathogen-food combination with the largest burden on public health in the USA in a study by Batz et al. (2012). Extensive attempts have been made to develop an effective vaccine for poultry to reduce the food safety risk of *Campylobacter* spp. in poultry meat. For example, in 1997 it was noted that an oral, killed whole cell vaccine was undergoing trials (Scott 1997) and yet 19 years later researchers were still investigating vaccination methods to reduce colonisation of chickens to an acceptable level (Kobierecka et al. 2016). There are currently no commercially available vaccines for the control of any *Campylobacter* spp. in poultry (Zhang & Sahin 2020). Some reduction of the levels of *Campylobacter* have been achieved but often with the use of multiple intramuscular vaccinations (Neal-McKinney et al. 2014). Difficulties with vaccine development have included growing the organism, the lack of knowledge of pathogenesis, the variety of strains, as well as the ability of the organisms to evade the host immune defences (Poly et al. 2019).

The difficulty of working with this species is emphasised by the fact that the only *Campylobacter* spp. vaccines registered in Australia are for reproductive disease in ruminants. *C. hepaticus* is quite closely related to *C. jejuni* and the same issues that make the development of other *Campylobacter* vaccines difficult, apply for SLD vaccines. In addition, *C. hepaticus* grows more slowly than other *Campylobacter* spp. and has added growth requirements (Crawshaw et al. 2015). During the project some of these difficulties were overcome, including the development of liquid culture for challenge media (thus enabling a larger number of birds to be challenged at one time), and in vaccine manufacture, which finally resulted in the production of commercial scale batches larger than that used in the field study reported in this project. However, issues with organism growth rate and therefore cost of production remain for further investigation and research to enable production of enough vaccine to meet industry requirements.

Autogenous vaccines are used where there are no registered vaccines that protect against the isolate(s) causing disease on the farm, and can only be manufactured by APVMA audited and approved facilities. A permit from the APVMA is required for each genus of organism, and there are numerous regulatory restrictions on the ingredients permitted for use in the manufacture of the vaccine, which can add to the difficulties of growing adequate numbers of organisms to produce commercial quantities of vaccine. The permit states the minimum titre of organism required per dose and that use must be authorised by a veterinarian and safety, sterility and potency tests must be undertaken by the manufacturer. Permits can take up to one year or longer to obtain. For an autogenous vaccine to be commercially sound it must be grown in large volumes consistently and reliably, at least at the minimum permit titre and at a sustainable cost per dose.

Autogenous vaccines manufactured from Gram-negative bacteria have been shown to be associated with endotoxins associated with the cell walls (particularly the lipopolysaccharide component) leading to endotoxic shock, tissue injury and lethality (Mergenhagen & Snyderman 1971; Stewart 1978; Walker 1992). In this project there was no indication of endotoxic shock or other adverse reaction to the vaccines following their administration. The autogenous *C. hepaticus* vaccine was shown to be safe for use in chickens.

C. hepaticus has been shown to have a far greater ability to invade liver cells than other *Campylobacter* species (Van et al. 2017a) and it is hypothesised that a toxin must be trafficked to the liver, and this may make it more amenable to protection by vaccines that produce a parenteral antibody response.

However, much is still to be investigated with respect to the pathogenesis of SLD. It has not been demonstrated that SLD is definitely caused by a toxin, or simply by the presence of the bacteria, or both. Due to the nature of the disease the involvement of a toxin is strongly suspected, however, the organism lacks the genes for the cytolethal distending toxins common in similar species. If the toxin could be isolated, the development of a toxoid vaccine may add to the efficacy of the killed-whole-bacteria approach reported in this project.

The challenge model has been shown to reliably reproduce many of the disease outcomes seen in the field, in that only a small proportion of birds die or show clinical signs, with infected birds that recover showing typical SLD liver lesions. As noted, the presence of a toxin in the pathogenesis of SLD is strongly suspected. It is probable that in the field birds are exposed to varying amounts of toxin depending on the extent of their exposure to the organism and the other factors involved in triggering the growth and production of toxin by the organism. The organism has been cultured from the liver, the bile, the intestinal contents and the caeca and it is not known in which organ toxin production occurs (if at all). It appears possible that variation in toxin production is responsible for the variation in the clinical outcome from liver lesions with no clinical signs and perhaps a temporary reduction in egg production (number of eggs and egg weight) through to extensive liver lesions, brief depression and death. A vaccine could impact *C. hepaticus* by enhancing immune response to either kill the bacteria, reduce the growth rate, reduce the production of a toxin or reduce some other aspect of the pathogenesis of the organism. A vaccine is unlikely to prevent the residence of the organism in the intestines or caeca but may enable the immune system to moderate the pathogenesis of the disease. In which case it may be that the absence or presence of some liver lesions is the last disease feature to be reduced by an effective vaccine. This may, in part, explain why there were no reductions in the number of birds with liver lesions in the laboratory studies.

Numerical reductions in lesion numbers were seen in some studies as well as reduction in lesion scores, which were not significantly different to the uninfected groups (even though they were not statistically lower compared to the positive control group), which suggests a level of protection conferred by the vaccine. This appeared to some extent to be associated with higher serological responses where they were measured. This suggestion was reinforced in Study 4 where the 2× vaccine group had a significantly lower ELISA value than the 3x vaccine group just prior to challenge, and actually had higher lesion numbers and lesion scores than the positive control group, whereas the 3× vaccine group had lower lesion scores and lesion numbers numerically than the positive control. There was statistical significance when the vaccine groups were compared to the positive control, including in Study 2, $P = 0.02$ for lesion score, $P = 0.04$ for lesion number, and a trend in Study 4, $P = 0.08$ and 0.09 (one-tailed t-test) for lesion number and scores respectively. These findings, combined with the finding of positive ELISA results around the time of challenge suggest that there is some relationship between immunity measured by the ELISA assay and protection with respect to the extent of pathology induced by *C. hepaticus* exposure.

This proposed relationship between ELISA values and partial protection is also emphasised by the lack of protection seen in the field study where the ELISA levels following vaccination dropped significantly prior to the outbreak of disease. Immunity to *Campylobacter* appears to be comprised of an innate component as well as adaptive, with both cellular and humoral components involved. With respect to innate components, *Campylobacter* evades TLR-5 recognition through glycosylation of its flagella (Howard et al. 2009), though is recognised through other receptors including TLR-21 (Meade et al. 2009). *Campylobacter* also induces an inflammatory response in the intestine, albeit of lesser magnitude than *Salmonella*, and is usually poorly invasive (Meade et al. 2009; Shaughnessy et al. 2009; Smith et al. 2008). Nevertheless, there is an inflammatory response to infection with *C. jejuni* in the gut and it is perhaps misleading to consider *Campylobacter* simply as a gut commensal (Hermans et al. 2012).

Secreted effector peptides such as β-defensins are important in the chicken. Initially such peptides were characterised as antimicrobial effectors of heterophils in the chicken and turkey (Evans et al. 1995). In recent years they have been shown to be key responders to infection at mucosal surfaces including *Salmonella* and *Campylobacter* infection of the intestinal tract (Akbari et al. 2008; Crhanova et al. 2011; Hasenstein & Lamont 2007; Meade et al. 2009; van Dijk et al. 2007), and *Salmonella* infection of the reproductive tract (Michailidis et al. 2012). They have also been shown to have considerable antimicrobial activity towards *Salmonella* both *in vivo* and *in vitro* (Cooper et al. 2019; Milona et al. 2007).

C. jejuni infection produces both systemic and secretory IgA antibody responses to a range of antigens including flagellin (Cawthraw et al. 1994; Widders et al. 1996), though as yet their role in clearance or protection is poorly defined. A feature of *C. jejuni* infection in chicken production is that it is usually detected only once birds reach around three weeks of age, though experimentally younger birds can be colonised. It is considered that the presence of maternally derived antibody prevents infection in young chicks and that colonisation correlates to the drop in these antibodies (Cawthraw & Newell 2010; Sahin et al. 2003). This was further demonstrated in a study by Ondrašovičová et al. (2012) who attempted to infect 3-day old broilers with *C. jejuni* with no infections resulting but were successful in using the same infectious dose to infect the broilers at 21 days. However, other factors such as changes in the microbiota and management practices may also have an impact. As yet the cellular responses to *Campylobacter* infection remain largely undefined. The ELISA used in this study only measures one aspect of humoral immunity and further understanding of immunity in *Campylobacter* spp. in general and *C. hepaticus* in particular are necessary to fully evaluate responses to vaccination.

The extension of the challenge model to include 'Long' groups up to around a month post-challenge has been successfully used to mimic the production effects noted in the field such as a reduction in egg production, and has also enabled the discovery of more modest changes that would generally be missed in a flock situation such as an initial reduction in egg weights following challenge. Whilst birds that die of SLD in the field are generally in good condition, a reduction in weight of challenged birds was noted in one of the studies, and this has not been reported in the field. This has also allowed the examination of the impact of vaccination in mitigating some of those effects.

With respect to egg production, the final isolate and dose study demonstrated a reduction in egg production in the immediate post-exposure period in exposed birds. Whilst this was not replicated in the two laboratory exposure studies, the increase in egg production in the positive control (PC) group was reduced compared to the negative control (NC), confirming the field findings of reduced egg production during SLD outbreaks. The impact of vaccination was not consistent. In study 4, the vaccinated group, which received the final vaccination within only a month prior to challenge, was able to ameliorate the negative impact of infection on egg production. This was not the case in Study 2, where the group vaccinated three months prior to challenge had a more significant decrease in expected egg production compared to both control groups. This is unlikely to be the result of the treatment, given the result in Study 4. In Study 5, the decrease in egg production of 11% in both vaccinated and unvaccinated groups shows that vaccination did not prevent the negative impact of SLD in this study either.

The project demonstrated for the first time that *C. hepaticus* does produce a reduction in the weight of eggs from infected hens. The negative effect on egg production appears to occur in the immediate days after exposure and for at least one week. However, it appears that this negative effect is transient, with a recovery of the egg weights in the following weeks. Autogenous vaccines conferred a partial protection against that decrease in egg weights. Even though there was a decrease in both the PC and vaccinated groups, the decrease was only significant in the PC group. It was also possible to see that, in the vaccinated group, there was a satisfactory recovery from the disease impact on the

weight of the eggs, which were significantly higher at 21 DAE than those before exposure. However, that was not the case with the eggs from the PC group, where the weight of the eggs at 21 and 42 DAE did not exceed the egg weight recorded before exposure. As expected, the weight of the NC group eggs increased throughout the study.

Yolk colour was unchanged when examined objectively in Study 2. The anecdotal reports of yolk colour changes during SLD outbreaks would in general have come from producers using subjective visual comparisons. This finding was a one-off and the colour monitoring equipment was not available during all the studies. Further studies are required to confirm this finding.

Where examined (in Study 4), the feed intake increased in all groups post-exposure (or sham exposure in the non-exposed groups) but to a lesser extent in the positive control group. Feed intake is expected to increase gradually during the laying period (Hy-Line Brown Commercial Layers Management Guide). Further investigation is required to examine whether the reduced increase in feed intake is a feature of SLD. In Study 2, there was a decrease in FCE consistent in all groups (including the NC), and the decrease was marked in the PC group and not significant in the vaccinated group. It is possible that a fraction of the decrease, the one that impacted the NC group, could be explained by changes in temperature in Melbourne before the sampling day. According to the registers of the Bureau of Meteorology, the temperature between the 10th and 16th of October varied from 14.6°C to 19.8°C, with a peak of 25.6°C on the 14th of October. Feed intake in hens can decrease from 111.9 g/day at 16.1°C to 99.0 g/day at 25.0°C, as shown under experimental conditions (Peguri & Coon 1991). Similarly, in Study 4, whilst there was a gradual improvement and then plateau in the FCE in the negative control group there was a decrease in efficiency 6 days post-exposure in the exposed groups. This increase was not pronounced in the vaccinated group in which, following the slight decrease 6 days post-exposure, the efficiency gradually increased over time. From this result, it is not expected that exposure to *C. hepaticus* will have a long-term impact on feed efficiency, however, further investigations are needed to confirm the transitory nature of the depression in FCE.

The moderate impacts of vaccination on lesion numbers and lesion scores and the impact of *C. hepaticus* exposure on some of the production parameters measured indicates that vaccination may provide a useful tool in the control of SLD. The lack of impact in the field study is likely due to low antibody levels, and this was confirmed in the fourth vaccine study in the laboratory where the vaccinated group with highest ELISA titres, vaccinated close to the time of exposure, had a positive impact on disease and production parameters compared to the 2x group with lower ELISA values. Further investigation into the pathogenesis of the disease and the protective antigens are urgently required to enable the production of more targeted vaccines. There is also a need to examine different adjuvants to ensure that antibody levels are high enough at the time of field exposure to enhance protection.

Whilst killed autogenous vaccines are able to be introduced with less regulatory restraints than other forms of vaccine, the use of other delivery systems such as oral or eyedrop vaccination need to be examined. The use of live oral or eyedrop vaccines would require much more research to ensure the organisms were sufficiently attenuated. However, studies examining the impact of combined killed parenteral and oral dosing systems should be considered. This project was able to examine the use of two different isolates used as autogenous killed vaccines and both strains helped ameliorate some of the negative effects of *C. hepaticus* infection. Further studies on different isolates may further elucidate particulars of isolates that are more likely to be protective, allowing examination of the differences in strains genetically to help identify protective genes.

5 Conclusion

Substantial progress has been made in the development of tools to investigate SLD, including the further description of the causal organism after its initial discovery by Crawshaw et al. (2015), the development of an effective challenge model, some understanding of the differences in virulence and the culture requirements of different strains, development of an ELISA assay to measure serological response, and increased understanding of the disease. In particular, the experimental model can be used to induce disease, reproduce the disease impacts seen in the field (such as reduction in egg weights and on occasions egg production), a hitherto un-noted impact on liveweight as well as inducing some clinical signs in a small portion of challenged birds (which mimics the field experience where many affected birds do not display clinical symptoms). A serological response to vaccination has been induced and where the response has been adequate, a degree of protection following the use of killed autogenous vaccines has been demonstrated. Further investigation is urgently needed into the underlying mechanisms of the disease pathogenesis, details of the immune response to vaccination and methods to improve that response.

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7 Plain English Summary

Project Title:	Assessment of the efficacy of autogenous vaccines in Spotty Liver Disease control
Australian Eggs Limited Project No	1BSO3SX
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Objectives	To assess the efficacy of autogenous vaccines (that is vaccines made from the organism causing disease on the farm) in the control of Spotty Liver Disease.
Background	Spotty Liver Disease (SLD) is a disease primarily affecting extensively housed chickens, especially free range. The disease is caused by the organism *Campylobacter hepaticus* and is clinically associated with egg production drops and mortality, with the prominent finding at autopsy being small white spots on the liver. Currently outbreaks of the disease are controlled by antibiotics. The organism is difficult to work with, being slow growing and with special growth requirements.
Research	Autogenous SLD vaccines, produced by ACE Laboratories, were tested in both laboratory and field studies. Four laboratory studies where birds were exposed to an oral dose of the organism were undertaken. A group of birds was left as unvaccinated and unexposed controls. Another group was exposed but not vaccinated, and finally vaccinated birds were exposed to the organism. Additional field studies examined the impact of autogenous vaccines in preventing deaths and production losses on commercial farms historically challenged with SLD. Additional work was also undertaken to develop methods for higher volume production of the organism. These were necessary for both experimental work and production of the vaccine. And also to look at the strain variation of the organisms recovered from field cases, ways of assessing the immune response of hens to the disease and vaccination, and to examine the differences in pathogenicity of different isolates of the organism.
Outcomes	The laboratory exposure protocol produced the typical spotty livers, but also for the first time demonstrated experimentally the reduction in egg production seen in the field. Other findings not previously reported included a slight weight loss or decrease in weight gain in exposed birds, and a slight reduction in egg weight in the week post-exposure. It was also demonstrated that no change in yolk colour occurred in response to SLD challenge. Vaccination of pullets and hens with autogenous vaccines resulted in a measurable immune response. This was associated with

	some improvements in the production impacts of SLD and a reduction in the severity of the liver lesions. In field studies the use of the autogenous vaccine did not reduce the rate of deaths due to SLD.
Implications	The use of autogenous vaccines may result in benefits in terms of reduction of some of the production impacts of SLD. Further research is needed to enable improvements in our understanding of the disease process and how immunity following vaccination can be improved, so that protection from mortality is enhanced. The production of commercial volumes of vaccine is proving challenging because of the fastidious conditions required to grow the organism and the growth characteristic variability with different strains.
Key Words	Spotty Liver Disease, *Campylobacter hepaticus*, autogenous vaccine, immunity
Publications	Moore, R.J., Scott, P.C., and Van, T.T.H. (2019) Spotlight on *Campylobacter hepaticus*, the cause of Spotty Liver Disease in layers. Avian Pathology 48:285-287. Van, T.T.H., Lacey, J.A, Vezina, B., Phung, C., Anwar, A., Scott, P.C., and Moore, R.J. (2019) Survival mechanisms of *Campylobacter hepaticus* identified by genomic analysis and comparative transcriptomic analysis of *in vivo* and *in vitro* derived bacteria. Frontiers in Microbiology 10:107. Van, T.T.H., Anwar, A., Scott, P.C., and Moore, R.J. (2018) Rapid and specific methods to detect foodborne pathogens *Campylobacter jejuni*, *C. coli* and the new species causing spotty liver disease in chickens, *C. hepaticus*. Foodborne Pathogens and Disease 15:526-530.

8 Appendix

Intervention criteria for Spotty Liver study

NB: The severity table includes responses not expected to be associated with study. During the first 4 hours of the study, experienced veterinarians will be conducting the monitoring. Farm staff will be involved in monitoring on subsequent days.

Intervention will occur when behaviour/symptoms are mild to moderate with the intervention of increasing observation times being the first response; if the observer is not a veterinarian a veterinarian will be contacted and will observe the affected animals. If hydration status is the issue involved, the animal will be weighed to check weight loss. If the condition observed is severe, the animal will be removed from the experimental group and treated according to veterinary direction.

Severity Table		
	Mild to Moderate	**Severe**
Alertness & responsiveness	Decreased alertness, decreased responsiveness	Obviously subdued, minimally or un-responsive to provocation
Posture	Slightly hunched, altered wing posture (poultry) or position	Significantly abnormal posture, hunched, recumbent
Movement/gait	Decreased use of a limb	Ataxic
Feathers	Slightly ruffled	Obviously ruffled
Respiratory signs	Slightly abnormal, mild nasal discharge	Respiratory distress, persistent nasal discharge
Appetite	Decreased appetite, without signs of weight or body condition loss	Decreased appetite, with signs of weight or body condition loss
Eyes	Mild ocular discharge or mild swelling of eyelids	Persistent and copious ocular discharge with swelling of eyelids
Diarrhoea	Slight, without obvious signs of dehydration (see below)	Marked diarrhoea, with obvious dehydration (see below)
Hydration status	Slightly shrunken comb (poultry with combs), weight loss under 8%, general loss of tissue turgidity	Severely shrunken comb, wrinkled skin on shanks, weight-loss over 8% Discolouration of beak (poultry), severe loss of tissue turgidity, sunken eyes (pigs)
Other signs	Seek advice from a staff veterinarian re: appropriate action, or euthanased if animal is in severe pain or distress	