Final report on the AE funded project to examine the effect of feed additives on Spotty Liver Disease

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Foreword

This project was conducted to evaluate the protective effect of various feed additives fed to commercial layers before exposure to the causative agent of Spotty Liver Disease (SLD), *Campylobacter hepaticus*.

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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1 Executive Summary

Spotty Liver Disease (SLD) is a serious condition extensively affecting laying hens (particularly in free range systems), leading to losses through both bird mortality and reduced egg production. It is hypothesised that changes in the intestinal health/microbiota balance allow a multiplication of the causative organism *Campylobacter hepaticus* and the production of a toxin that causes the symptoms of the disease, including the liver lesions.

We have recently developed an exposure model that reproduces the liver lesions, and have used this model to assess feed additives for their potential to reduce the impact of SLD. This report includes the results of both field and laboratory studies. No feed additive examined was able to cause a statistically significant improvement in the proportion of the treatment group with SLD liver lesions or in liver lesion scores, under the current laboratory exposure model. However, in the field there is some evidence that both the incidence and the severity of outbreaks can be reduced by the inclusion of feed additives, particularly a combination of oregano and sanguinarine feed additives.

The advantage of using feed additives include a reduction in the necessity to treat or to prevent SLD with antibiotics, a reduction in overall mortality during an outbreak and over the most common period of outbreaks (up to 35 weeks), and a trend towards reducing the negative production impact of SLD.

2 Introduction

Spotty Liver Disease (SLD) is a serious condition extensively affecting laying hens including free range laying hens, broiler breeders and less commonly caged birds, leading to losses through both bird mortality and reduced egg production. The disease responds to antibiotic treatment and we have recently shown that *Campylobacter hepaticus* can induce the disease and can be recovered from liver and bile samples from affected birds, although there are rarely organisms seen histologically in the liver lesions. The organism can be identified in the intestine and caeca using PCR. 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.

We have recently developed an exposure model using *C. hepaticus* to induce SLD in laying hens. Various nutraceutical products (feed additives) have shown promise in modifying the gut microbiota, which may provide protection against enteric avian pathogens. This report covers the initial trial of several classes of feed additives in this exposure model, and field studies to examine the following objectives as described in the extract from the full research proposal below. Note that treatment is defined as: "procedures concerned with remediation or prevention of disease" (https://www.online-medical-dictionary.org/definitions-t/therapeutics.html). So, whilst feed additives other than antibiotics have not been shown to be particularly useful in the face of an outbreak, this report examines their use in prevention and the term treatment will be used. In addition, it is normal to refer to different treatments in experimental studies.

Where statements about the nature of the disease, epidemiology or clinical manifestations related to SLD are not attributed in this report, the statements are based on the field observations of Scolexia veterinarians. The field studies reported were not undertaken based on the results of the laboratory studies. One field study reported involves an historical evaluation of data, and the other studies were undertaken using contemporary and previous field data.

2.1 Objectives

This project will evaluate the protective effect of various feed additives fed to commercial layers before exposure to the causative agent of Spotty Liver Disease (SLD), *Campylobacter hepaticus*. Feed additives include probiotics, prebiotics, organic acids and various nutraceuticals including essential oils. Various commercially available feed additive products will be evaluated to determine if any allow a sustainable approach to disease management and prevention. Currently antibiotics are used to treat and control SLD. Alternative control and treatment options are required and this study will examine some of the potential control compounds.

3 Materials and methods

3.1 General

Research licence and animal ethics approval

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

Test facility

The pen study was conducted at the Scolexia animal research facility and the field studies were conducted on a commercial poultry facility in Victoria.

Test system

The test system consisted of individual Hy-Line Brown layers between 20 and 25 weeks of age at the start of the studies.

Justification of the test system

Spotty Liver is a syndrome that has been noted for many decades, but has come to prominence in the Australian egg laying industry with the increase in the number of free range and barn flocks. Spotty Liver can cause significant mortalities and production losses in commercial layers held under alternative systems of housing compared to the caged system. Birds that are generally in good condition and die after a short illness of less than 24 hours, with the typical lesion of miliary hepatitis consisting of small 1 to 3 mm white to reddish foci in the hepatic parenchyma, which histologically are granulomatous foci of necrosis that have no relatedness to any of the liver architecture and no bacterial organisms can be seen in the lesions using light microscopy (with or without special stains). Grossly the liver may also have a capsular transudate and in severe cases there are ecchymotic haemorrhages on the abdominal serosal surfaces of the intestinal mesentery and organs. There is an obvious bacterial component as the flocks respond (at least initially) to treatment with antibiotics, although if repeated treatments are required the effectiveness of the antibiotic decreases, presumably due to the development of resistance in the causative bacteria.

Work by Cranshaw et al.[1] and our work with Professor Moore[2,3] demonstrated that Spotty Liver Disease (SLD) is caused by the bacterium *Campylobacter hepaticus*. The disease is causing significant productivity losses and welfare issues within the free range layer industry due to an inability to prevent, control or treat SLD flocks without antibiotic intervention. This has been exacerbated where the organism has become antibiotic resistant to the limited repertoire of antibiotics available to the Australian layer industry, there being only two antibiotics registered for use in layers with a nil withholding time for table eggs. The importance of SLD is increasing (in relation to both the incidence of outbreaks and the total number of laying hens involved) as the proportion of non-cage production sheds rises. Endemically affected sites have clinical disease in all new introductions of young layers and on some occasions ongoing reoccurrence of SLD. Epidemiological work using PCR technology indicates that on endemic sites the bacterium causing SLD can be identified in birds without clinical signs, with the clinical manifestation of the disease dependent on “stressors” such as approaching peak production, changes in rations, disruptive net nutritional intake, exposure to the range area and high ambient temperatures. It is a generally accepted hypothesis, based on the

epidemiology of the condition and diagnostics including microbiology and PCR, that the causal bacterium resides in the intestinal tract, and for reasons not yet understood, certain local enteric factors cause the organisms to replicate and directly and/or through the production of toxins, cause the clinical signs observed. Thus, control of SLD potentially can be achieved using additives that act within the intestinal tract to enhance the maintenance of microbiota unfavourable to the growth and multiplication of *C. hepaticus* in the layers.

Early field work with the application of medium chain organic acids and some phytogenic compounds well before the recognised induction stressors has demonstrated that the course of the condition can be subjectively delayed and/or modified, but overall the response in control and prevention is partial. None of the feed additives used within the field studies demonstrated any therapeutic effect and the classic growth-promotant antibiotics also have no impact on preventing or controlling the disease at normal low doses. More recently, Scolexia veterinarians have observed particular combinations of feed additives modifying the incidence and severity of SLD outbreaks.

To confirm the usefulness of botanical derivatives in preventing or ameliorating the disease it was necessary to use the affected species. The most common time of an outbreak is around the peak of lay (22 to 30 weeks of age) and so birds of that age were used. Some novel botanical products have been shown to positively impact gut health in the face of inflammatory, bacterial and protozoan challenge[4,5,6], and in mice have been shown to aid the post antibiotic treatment recovery of mice infected with *Clostridium difficile* infection[7]. We hypothesised that intestinal flora changes are a catalyst for the stimulation of *C. hepaticus* to induce SLD and therefore substances that influence gut microbiota population dynamics may be beneficial in reducing the incidence of SLD. Other factors to consider include the colonisation of the bacterium, the quantitative level of colonisation, the change in the dynamics of the *C. Hepaticus* population and finally the expression of any virulence factors.

Inclusion/exclusion criteria

Only healthy birds laying eggs regularly were included in the study. No birds were excluded from the study due to unsuitability or ill-health.

Test system ID

Each cage was identified with a unique number. For allocation purposes birds were identified with numbered leg bands attached at the initial weighing.

Experimental material

The exposure material consisted of 1 mL of a broth containing the organism (exposed groups) or 1 mL of the broth with no organism present (unexposed control group). The bacteria were grown on Brucella agar with 5% horse blood (HBA) and incubated at 37°C in microaerophilic conditions. The bacteria were then washed off the plates with Brucella Broth (BB). The resuspended cells were diluted to a concentration between $1x10^9$ and $1x10^{10}$ organisms per mL. BB contains water, yeast extract, sodium bisulfite, dextrose, sodium chloride, meat peptone and casein peptone.

The treatment materials in the laboratory studies consisted of a combination organic acid and medium chain fatty acid product, two novel botanical products, a yeast extract prebiotic, an oregano-based product, and a combination of the oregano and the prebiotic products.

Housing

The birds were housed in layer cages in the Scolexia Animal Research Facility (SCARF). The facility includes fogging-lines, mixing fans and an exhaust fan for cooling and ventilation.

Allocation

The birds were weighed and identified, ranked by weight and divided into groups using a random number generator. Allocation of treatments to groups was undertaken using the random numbers generated using Microsoft® Excel® 2007 (trademarks Microsoft Corporation 2006). Allocation of animals to groups was also undertaken by the use of previously generated random numbers. 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.

3.2 Experimental design

Experimental unit

The experimental unit was the individual bird.

Experimental groups

Each study involved a negative control group of 12 or 8 birds, which were not treated with feed additives and were not exposed to *C. hepaticus*, and a positive control group of 12 or 16 birds, which were also not treated with any feed additives but which were exposed to *C. hepaticus*. The feed additive groups consisted of 12 or 16 hens, which were treated with the feed additives for between 4 and 5 weeks prior to exposure to *C. hepaticus*. An extended period of pre-treatment when using feed additives is important as it allows the necessary time for the microflora population dynamics to change under the influence of the additive. The treatment groups included a combination of medium chain fatty acids and organic acids (one treatment, which included a mixture of propionic, formic and acetic acid and fatty acids), two different novel botanical products (extracts of *Phellodendron* spp., and *Eriobotrya* spp.), an oregano- based product (extract of the genus *Origanum*), a prebiotic yeast extract (derived from *Saccharomyces* spp.), and a combination of the latter two.

In the field we examined the use of two phytogenics, an oregano-based product combined with a sanguinarine product, and examined sheds against previous performance with respect to SLD outbreaks and severity. We have also examined the efficacy of the use of a feed additive containing medium chain fatty acids and monosaccharides in the field (two sheds) compared to the use of a Bacillus probiotic in cohort sheds on a farm where SLD is endemic.

Hypotheses for laboratory studies

Where [SLD] refers to the proportion of birds with SLD lesions and separately to the average SLD lesions scores for each treatment group:

$1H_0$: Negative control [SLD] = Positive control [SLD]
$1H_1$: Negative control [SLD] < Positive control [SLD]

and

$2H_0$: Feed additive group [SLD] = Positive control [SLD]
$2H_1$: Feed additive group [SLD] < Positive control [SLD]

Masking

Treatments were not masked during the feeding and exposure periods. It is not expected that this would influence the outcome of the laboratory study, with the absence or presence of visible lesions being the determining factor in classification of the outcome and in the field studies where all data is routinely collected and the comparison was with similar data from previous flocks.

Criteria for a valid test

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

Outcome criteria

The outcome criteria involved a comparison of the gross pathology of affected and unaffected birds in the treatment group with those in the challenged control group. Each bird with spotty liver lesions was regarded as positive and those with none as negative. Histology and microbiology were used as confirmatory tests.

Dose and route of administration

One mL of exposure or control broth was administered by mouth using a syringe inserted into the corner of the mouth and the birds allowed to naturally swallow the contents.

The treatments were administered in feed for four to five weeks prior to challenge and then post challenge for the duration of the study.

Labelling

Each treatment was mixed and homogenised with commercial layer feed at the prescribed rate, and stored in a labelled bin with the treatment group number and name affixed.

3.3 Procedures

Daily husbandry

Each day, birds were monitored for health as described below in monitoring and intervention. Eggs were collected and a record of eggs laid per pen pair was noted. Birds were fed *ad-lib* with the appropriate feed for their treatment group and the feed added recorded.

Treatment

The negative and positive control groups were fed untreated commercial bagged layer mash feed.

Treatment groups received various feed additives in the diet at a specified inclusion rate in feed. This was uniformly mixed at the study site.

Exposure material

The exposure material (*C. hepaticus* 1 x 10^9/mL) and the control broth (no *C. hepaticus*) (for the non-exposed controls) was administered *per os*, 1 mL per bird.

Monitoring and intervention

The hens 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.

Microbiology

Cloacal swabbing: dry cotton tip swabs were inserted into the cloaca and rotated over the mucosa, and placed into pre-numbered containers.

PCR examination: DNA from caecum of experimentally infected birds and control birds were prepared using the "Isolate Fecal DNA Kit" (Bioline) according to the manufacturer's instructions. For bile samples, boiled DNA was prepared by mixing 10 µL of bile with 90 µL of water and the mixture was boiled at 100°C for 5 minutes. End-point PCR was performed on these DNA samples using the method as described in Van et al. (2017)[8]. Controls, comprising a non-template negative control and a *C. hepaticus* DNA positive control, were also included in each run.

Euthanasia

Intervention to remove affected birds was to be based on definitive signs of depression and recumbency occurring. There was no requirement in these experiments to achieve mortality as an end point. The birds were monitored for any change in behaviour such as inappetence, reluctance to move, postural changes and general signs consistent with depression. 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 autopsies included visual examination of all the liver, spleen, gastrointestinal system, reproductive and renal systems with sampling for histopathology from the liver. A representative sample of liver approximately 10 mm^3 was placed in buffered 10% formalin solution in a labelled container. Fresh samples of caecal contents and bile were collected in sterile containers for microbiology.

Histology

From each treatment group, two liver sections which were grossly positive and two which were negative on gross appearance were prepared for histological examination. Only two negative livers were taken from the negative controls as there were no grossly positive livers in that group. The liver sections underwent routine processing and haematoxylin and eosin staining.

Disposal of animals

The birds were bagged and then disposed of by using a commercial medical waste contractor.

3.4 Field studies

Study 1 was undertaken utilising a shed with a prior history of regular SLD. The first year's flock included in this retrospective analysis had no prophylactic antibiotic but required treatment and further antibiotic prophylaxis during and after the SLD outbreaks. In the second year, a prophylactic antibiotic was included during the likely challenge period and in the test year no antibiotic was included or required but oregano and sanguinarine based products were combined in the feed from the time of transfer to the production shed.

In study 2 a further four sheds from two farms were also treated with the oregano and sanguinarine based products, and the performance and mortality data have been compared to that of the previous flock.

We have also compared two sheds treated with a feed additive containing medium chain fatty acids and monosaccharides with two sheds fed a Bacillus probiotic on the same farm during the same season with respect to the occurrence of SLD (reported as field study 3).

Hypothesis for shed comparisons

$S1H_0$: Hen day production% 2017 = Hen day production% 2018
$S1H_1$: Hen day production% 2017 < Hen day production% 2018

and

$S2H_0$: Weekly mortality% 2017 = Weekly mortality% 2018
$S2H_1$: Weekly mortality% 2017 > Weekly mortality% 2018

3.5 Statistics

The odds ratio for the occurrence of Spotty Liver Disease (as determined by obvious SLD lesions on autopsy) was calculated using a contingency table as follows:

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

The odds ratio = (A x D) ÷ (B x C)

Probability was determined using the χ^2 distribution with a $P < 0.05$ being considered significant. No measures of variance are reported for the primary laboratory studies because the comparisons are between ratios of positive and negative birds. Also for the field data we have not reported these measures in the main body of the report (they can be seen in the appendix). It should be noted that weekly hen day % is a repeat measure.

For the shed comparisons of mortality and hen day % production the previous year's results were compared using a Student's t-test assuming unequal variance to test the hypotheses listed below. This was undertaken for weeks 22-33 (the current age of two of the flocks) and for the period when SLD was observed in the 2017 flocks. A P value of <0.05 was considered significant for these one-tailed test procedures.

4 Results

4.1 Laboratory study

Valid test

The exposure studies met the requirements of a valid test as more than 40% of the positive control was SLD positive, and there was a statistically significant difference between the positive control group and the negative control group ($P<0.05$).

Gross SLD lesions

The outcomes of the challenge studies are listed below in Table 1. As well as describing the number of positive and negative birds per group, a "Disease Reduction Index" (DRI) has been listed, which compares the incidence of disease for the particular feed additive treatment group with the disease incidence in the positive control group used for that study. As this is a percentage of the difference between the treated and control group it can be a negative value. No statistically significant differences between the positive controls and feed additive groups were observed.

Table 1 Grossly visible SLD lesions in the study birds#

Treatment	% SLD lesions in treated group	% SLD lesions in control group	Disease Reduction Index*
Organic acid/MCFA	100.0	91.7	-9.1
Novel botanical 1**	50.0	75.0	33.3
Oregano product	93.8	87.5	-7.1
Novel botanical 2**	50.0	75.0	33.3
Yeast based prebiotic	93.8	87.5	-7.1
Oregano plus prebiotic##	81.25	87.5	7.1

No differences were significantly different ($P>0.05$).

* Disease Reduction Index represents the difference between the treated and the positive control groups.

** These ingredients are not available commercially in Australia but are based on traditional Chinese herbal extracts from *Phellodendron chinense* and *Eriobotrya japonica.*

A combination of an extract of the genus *Origanum*, and a *Saccharomyces* spp.

Histology of SLD lesions

All liver sections from lesion positive birds had histological evidence of SLD. The primary histological lesion was a well delineated multi-focal randomly disperse area of coagulative necrosis. Severe lesions 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 were mainly aggregates of inflammatory cells, often macrophages and lymphocytes and degenerate cells, or disruption of hepatic cords.

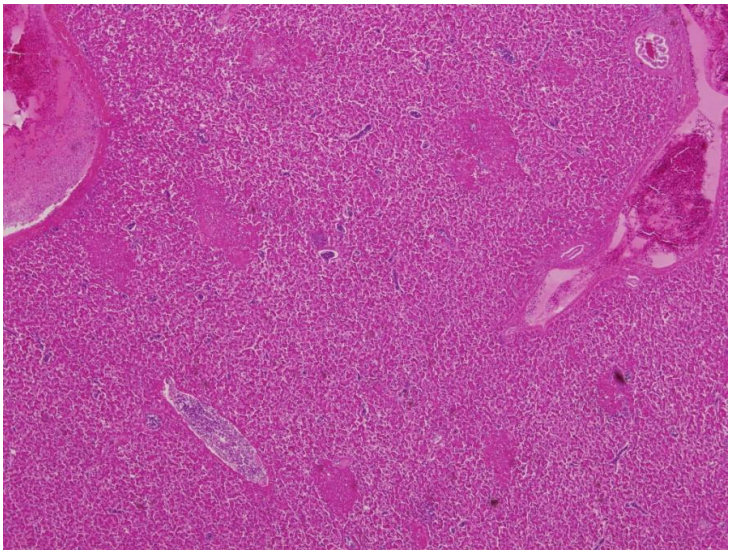

Figure 1 Multiple necrotic lesions in a bird affected by Spotty Liver
H & E stain, magnification 4x40

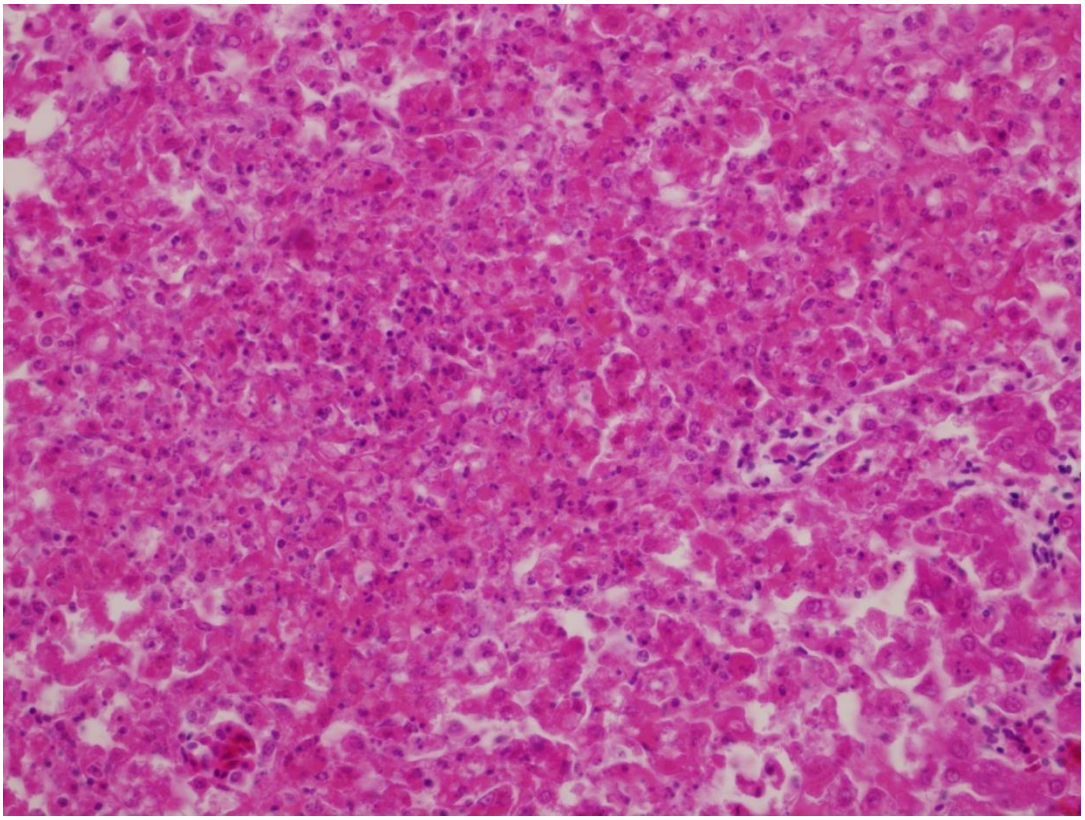

Figure 2 Hepatocellular dissolution necrosis
H & E stain, magnification 4x400

Production and health indicators of disease

There were few indicators of clinical disease (that is overt symptoms or egg production loss) in the exposure studies. In two studies, one bird would appear to be depressed on the fifth day post exposure, and those birds were lesion positive when examined. However, except for a separate exposure study examining the effects of different doses and isolates, we have not noted obvious differences in health or production indicators between the negative control and the other groups. The short time between exposure and autopsy does not allow for a rigorous examination of the effect of exposure on production. There were no significant differences between groups with respect to feed intake (between 105 and 111 g/hen/day on average) or in egg production (99.1-96.4%).

4.2 Field studies

Field study 1

A shed with a history of SLD was chosen to compare the impact of SLD on production and mortality between previous years, where antibiotic treatment and prevention were required, and with the use of an oregano and sanguinarine combination in-feed in the most recent flock. The results of the impact during the occurrence of SLD are listed below in Tables 2 and 3. During the most recent year Fowl Cholera was diagnosed in the flock so there would be some impact on the mortality and production indices.

Table 2 Weekly mortality % during Spotty Liver Disease outbreaks in flocks treated with or without in-feed oregano and sanguinarine and those requiring in-feed and in-water antibiotic medication

	Year 1 Required water medication and in-feed medication	**Year 2 Required in-feed medication**	**Year 3 No medication required. In-feed sanguinarine and oregano**
	Weekly mortality (%)	Weekly mortality (%)	Weekly mortality (%)*
	0.17	0.1	0.11
	0.49	0.08	0.1
	0.51	0.07	0.08
	0.31	0.11	0.09
Average#	0.37^{A}	0.09^{B}	0.095^{B}

* Note that some mortality in this group may have been due to concurrent Fowl Cholera.

Superscripts with different letters are statistically different ($P<0.05$).

Table 3 Weekly production during Spotty Liver Disease outbreaks in flocks treated with or without in-feed oregano and sanguinarine and those requiring in-feed and in-water antibiotic medication

	Year 1 Required water medication and in-feed medication	**Year 2 Required in-feed medication**	**Year 3 No medication required. In-feed sanguinarine and oregano**
	Weekly hen day %	Weekly hen day %	Weekly hen day %
	85	91	93
	89	87	91
	91	86	88
	92	88	89
Average	89.25	88	90.25#

Note there was a trend towards a difference between year 2 and year 3 (P<0.1, one tailed t-test). This group was also affected by Fowl Cholera during the SLD outbreak.

Overall weekly mortality % from weeks 22 to 35 averaged 0.148, 0.062 and 0.061% for years 1, 2 and 3 respectively. The difference between the first year and the subsequent years was statistically different (P<0.05) using a one-tailed t-test. The overall hen day production from weeks 22 to 35 was 90.22, 88.29 and 90.86% for years 1, 2 and 3 respectively. There was a trend to statistical significance between years 2 and 3 (P<0.099) using a one tailed t-test.

Field study 2

Two sheds from two farms were compared with respect to egg production (hen day %) over weeks 22-33 (the most common period for SLD outbreaks and the current age of two of the flocks) and over the period where SLD outbreaks occurred in the flocks in the same sheds in the previous year. The results are listed below in Tables 4 and 5.

Table 4 Average weekly hen-day % – flocks treated with oregano and sanguinarine in 2018

		Weeks 22-33			During outbreak period*		
Farm	Shed	2017	2018	P	2017	2018	P
1	3	90.39	91.47	0.333	90.97	94.58	0.0001
1	5	89.17	92.17	0.106	90.54	93.45	0.0006
2	5	91.53	91.95	0.378	91.89	93.68	0.0470
2	6	91.83	92.8	0.254	92.02	94.60	0.0074

* Where multiple outbreaks occurred the intervening 1 or 2 weeks data were included.

Table 5 Average weekly mortality % – flocks treated with oregano and sanguinarine in 2018

		Weeks 22-33			During outbreak period*		
Farm	Shed	2017	2018	P	2017	2018	P
1	3	0.168	0.057	0.016	0.207	0.075	0.034
1	5	0.110	0.173	0.232	0.180	0.081	0.129
2	5	0.078	0.128	0.089	0.101	0.159	0.174
2	6	0.150	0.098	0.150	0.207	0.097	0.047

* Where multiple outbreaks occurred the intervening 1 or 2 weeks data were included.

SLD occurred in the flocks in 2017 during the following weeks: farm 1, shed 3: 26-29 and 32 and 33; farm 1, shed 5: 29-33; farm2, shed 5: 27-28 and 30-33; and farm 2, shed 6: 25-32.

Smothers and fox attacks accounted for considerable mortalities overall and in particular in farm 1-shed 5 and farm 2-shed 5 during 2018. No occurrence of SLD was observed during the study period during 2018, however, some birds were affected in later weeks in both sheds on farm 1, and one of the sheds was treated with antibiotics. This compares to the necessity to treat more than once in the sheds during 2017.

Field study 3

In this retrospective study we observed the occurrence of SLD on a farm with four sheds, with flocks of similar ages treated with two different additives. The two sheds that were treated with a Bacillus based probiotic experienced outbreaks of SLD, whereas the sheds that had been treated with medium chain fatty acids and monosaccharides progressed throughout the batch with no SLD apparent.

5 Discussion

We have demonstrated that SLD is caused by the bacterium *Campylobacter hepaticus*[3], which resides in the intestine and caeca of laying hens. It is hypothesised that changes in the microbiota are a key component of the pathogenesis of SLD. Therefore, it is possible that some feed additives may have an impact on the incidence and severity of the disease through activity on the microbiota or in the case of plant extracts via direct antibacterial properties. It is currently thought that the disease involves production of a toxin due to the nature of the lesions and the absence of organisms associated with the lesions visible in histological sections. It is therefore possible that compounds that aid cellular recovery and responses to insults may further assist in ameliorating the effects of SLD.

Whilst no additive class was able to demonstrate a statistically significant reduction in SLD liver lesions in the exposure model, advantages, or at least equivalence with antibiotic prophylaxis, were demonstrated in the field using oregano and sanguinarine based products and to a lesser degree using medium chain fatty acids in conjunction with phosphorylated monosaccharides. In the laboratory study there were indications of some level of control by two novel botanically derived products, which are not yet commercially available.

The benefits of the use of feed additives that modify the extent of SLD mortality and production drop as demonstrated in this report include:

1. Reduction in the necessity to treat or to prevent SLD with antibiotics.
2. Reduction in overall mortality during an outbreak and over the most common period of outbreaks (up to 35 weeks).
3. A trend towards reducing the production impact of SLD even when ameliorated with prophylactic antibiotics up to 35 weeks of age.

The above benefits were seen particularly with the use of the combination of oregano and sanguinarine based additives. In field study 2, the observed benefit in preservation of egg production compared to the same period with SLD outbreaks in the prior year was noted in all four sheds examined, and was significantly higher in all four sheds. Mortality during the periods of SLD during 2017 was not always higher than 2018 due to other causes of mortality including smothers and fox attacks. This particular combination has not been assessed using our laboratory model, and given the apparent benefits in the field, this combination should be considered for inclusion in further studies. The relative cost of feed additive inclusion compared to treatment of an outbreak with antibiotics in water followed by in-feed antibiotics is not the major consideration in choosing feed additives to help ameliorate SLD.

Feed additives are likely to have additional benefits (for example, reduction in *Salmonella* shedding) and the antibiotics are likely to have additional negative issues (public perception of health risks associated with the use of antibiotics in agriculture, the actual need for prudent use, and especially the negative effect on performance of major alterations in gut microbiota caused by antibiotic use). It is also important to note that antibiotics are basically used to treat outbreaks when they occur and after the SLD associated losses have already been incurred, compared to the use of feed additives, which are designed to reduce the occurrence and impact of SLD outbreaks. However, Table 6 below gives an approximation of the relative cost of feed additives compared to antibiotic treatment and follow up prophylaxis, noting that it may be necessary to treat in-water more than once and then follow up with in-feed antimicrobial prophylaxis. There are also limitations to the length of preventative treatment with chlortetracycline due to residue issues.

An added difficulty in ascribing "cost-benefit analysis" to the treatment of SLD is that the severity and therefore impact on mortality and production of the disease appears to be moderated to some extent by the amount of "stress" to which the birds are exposed. Therefore, the benefit of treatment will be in part varied by the extent to which the birds are stressed. A possible biological explanation for this is the link between stress hormones and growth rate of some *Campylobacter* spp. as demonstrated) by Xu *et al* (2015)[9] who showed that the growth and invasiveness of *C. jejuni* were increased on exposure to the stress hormones epinephrine and norepinephrine. Thus, any cost benefit analysis of preventative treatments would need to consider the costs or reducing all possible stresses associated with the occurrence of SLD.

Table 6 Relative cost of feed of Spotty Liver Disease treatment and prophylaxis for 10,000 hens#

Additive or treatment	Length of treatment	Cost of treatment ($)*
CTC in water	5 days	600
Lincospectin in water	5 days	5,750
In feed CTC treatment	2 weeks	130
In feed CTC prophylaxis	4 weeks	130
Organic acid/MCFA	5 months	1,340
Oregano product	5 months	920
Yeast based prebiotic	5 months	590
Sanguinarine product	5 months	1,180

Note: Costs of combinations not listed as combinations are additive.
* GST exclusive.

The benefits of most feed additives are usually considered to be greatest when the challenge to the animals is greatest and that in low-challenge environments the benefit of additive inclusion is reduced. In the case of SLD, the inclusion of feed additives is undertaken in order to ameliorate the impact of SLD. Field experience, the field study presented here and the trend to reduction of SLD lesions associated with some feed additives in the exposure studies tend to suggest that the best combination of feed additives can ameliorate SLD.

It is also important to note that our understanding of the disease, how the organism causes the liver lesions, fever, depression and egg production loss is still limited. In the field it is possible to observe birds with SLD lesions from sheds without any prior obvious disease symptoms (deaths or egg production falls). It is therefore possible that birds can be infected, develop some liver lesions but not succumb to obvious disease. Therefore, it is probable that the presence of liver lesions, whilst being a definite indicator of the presence of *C. hepaticus* in the bird, are not necessarily an indicator of disease. The presence of a toxin or some other factor is probably critical for the organism to cause disease. Therefore, the current exposure model looking at the number of animals with visible SLD lesions in the liver after a one-off challenge may be too severe to detect a reduction in actual disease (that is the clinical signs of a production drop and increase in mortality). We have already undertaken one evaluation of the impact of different strains and doses in the exposure model and it may be possible to modify the model to better detect the benefits of feed additives in reducing the impact of SLD.

Further field and laboratory exposure studies should be undertaken in order to better define both the pathogenesis of the disease and the benefits of some feed additives in ameliorating SLD. It is imperative that further work be done to explain how the disease progresses. The way in which the

organism is involved in causing disease, and has changed from being a “normal” inhabitant of the gut microbiota to initiate toxin production (or whatever other mechanism it uses to cause disease), must be investigated to allow a better formulation of treatment and prevention feed additives, vaccines and management controls.

6 References

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7 Plain English Summary

Project Title:	Final report on the AE funded project to examine the effect of feed additives on Spotty Liver Disease
Australian Eggs Limited Project No	1BS804
Researchers Involved	Dr Peter C. Scott, Dr Timothy Wilson, Arif Anwar, Tyrone Scott, Nilhan Fernando (Scolexia Pty Ltd) Professor Robert Moore, Thi Thu Hao Van (RMIT University)
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Objectives	This project was conducted to evaluate the protective effect of various feed additives fed to commercial layers before exposure to the causative agent of Spotty Liver Disease (SLD), *Campylobacter hepaticus*.
Background	Spotty Liver Disease (SLD) is a serious condition extensively affecting laying hens (particularly in free range systems), leading to losses through both bird mortality and reduced egg production. It is hypothesised that changes in the intestinal health/microbiota balance allow a multiplication of the causative organism *Campylobacter hepaticus* and the production of a toxin that causes the symptoms of the disease, including the liver lesions. Therefore, it is possible that some feed additives may have an impact on the incidence and severity of the disease through activity on the microbiota or in the case of plant extracts via direct antibacterial properties.
Research	The researchers have recently developed an exposure model using *C. hepaticus* to induce SLD in laying hens. Various nutraceutical products (feed additives) have shown promise in modifying the gut microbiota, which may provide protection against enteric avian pathogens. Feed additives include probiotics, prebiotics, organic acids and various nutraceuticals including essential oils. Various commercially available feed additive products were evaluated to determine if any allow a sustainable approach to disease management and prevention. This investigation covered the initial trial of several classes of feed additives in the exposure model, as well as field studies. This report includes the results of both field and laboratory studies. One field study involved an historical evaluation of data, and the other studies were undertaken using contemporary and previous field data.

Outcomes	Whilst no additive class was able to demonstrate a statistically significant reduction in SLD liver lesions in the exposure model, advantages, or at least equivalence with antibiotic prophylaxis, were demonstrated in the field using oregano and sanguinarine based products and to a lesser degree using medium chain fatty acids in conjunction with phosphorylated monosaccharides. In the laboratory study there were indications of some level of control by two novel botanically derived products, which are not yet commercially available.
Implications	The advantage of using feed additives include a reduction in the necessity to treat or to prevent SLD with antibiotics, a reduction in overall mortality during an outbreak and over the most common period of outbreaks (up to 35 weeks), and a trend towards reducing the negative production impact of SLD.
Key Words	eggs; Spotty Liver Disease; *Campylobacter*; feed additives
Publications	Determining the cause and methods of control for 'Spotty Liver Disease' (Australian Eggs Publication No 1SX091)

8 Appendix – Statistics

Chi-squared testing of the most different additive results in the laboratory exposure model

Inputs	SLD	No lesn	Total
Controls	9	3	12
Treated (novel botanical)	6	6	12
Total	15	9	24
Results			
Expected values			
	Disease +	Disease -	Total
Exposure +	7.5	4.5	12
Exposure -	7.5	4.5	12
Total	15	9	24

	Chi-square	p-value
Uncorrected Chi-square	1.6	0.2059

Field study 1

Mort % Flock 1	Mort % Flock 2	Mort % Flock 3
0.08	0.05	0.03
0.03	0.02	0.04
0.17	0.01	0.03
0.49	0.06	0.03
0.51	0.05	0.07
0.31	0.1	0.11
0.19	0.08	0.1
0.06	0.07	0.08
0.01	0.11	0.09
0.03	0.05	0.03
0.01	0.03	0.03
0.09	0.09	0.05
0.02	0.07	0.09
0.07	0.08	0.08

Anova: Single Factor

SUMMARY mortality weeks 22-35 Farm 1 Shed 1

Groups	*Count*	*Sum*	*Average*	*Variance*
Mortality % Flock 1	14	2.07	0.147857	0.02927967
Mortality % Flock 2	14	0.87	0.062143	0.000864286
Mortality % Flock 3	14	0.86	0.061429	0.000905495

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.069148	2	0.034574	3.340523801	**0.045804**	3.238096
Within Groups	0.403643	39	0.01035			
Total	0.47279	41				

t-Test: Two-Sample Assuming Unequal Variances

	Mort % Flock 1	*Mort % Flock 2*
Mean	0.147857	0.062143
Variance	0.02928	0.000864
Observations	14	14
Hypothesised Mean Difference	0	
df	14	
t Stat	1.847214	
P(T<=t) one-tail	**0.042978**	
t Critical one-tail	1.76131	
P(T<=t) two-tail	0.085956	
t Critical two-tail	2.144787	

t-Test: Two-Sample Assuming Unequal Variances

	Mortality % Flock 1	*Mortality % Flock 3*
Mean	0.147857	0.061429
Variance	0.02928	0.000905
Observations	14	14
Hypothesised Mean Difference	0	
df	14	
t Stat	1.861335	
P(T<=t) one-tail	**0.041912**	
t Critical one-tail	1.76131	
P(T<=t) two-tail	0.083824	
t Critical two-tail	2.144787	

Anova: Single Factor

SUMMARY mortality during outbreak **Farm 1** **Shed 1**

Groups	*Count*	*Sum*	*Average*	*Variance*
SLD 1	5	1.67	0.334	0.02588
SLD 2	5	0.41	0.082	0.00057
SLD 3	4	0.38	0.095	0.000166667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.195243	2	0.097621	10.10193522	**0.003232**	3.982298
Within Groups	0.1063	11	0.009664			
Total	0.301543	13				

t-Test: Two-Sample Assuming Unequal Variances

	SLD 1	*SLD 2*
Mean	0.334	0.082
Variance	0.02588	0.00057
Observations	5	5
Hypothesised Mean Difference	0	
df	4	
t Stat	3.464756	
P(T<=t) one-tail	0.012853	
t Critical one-tail	2.131847	
P(T<=t) two-tail	**0.025706**	
t Critical two-tail	2.776445	

t-Test: Two-Sample Assuming Unequal Variances

	SLD 1	*SLD 3*
Mean	0.334	0.095
Variance	0.02588	0.000167
Observations	5	4
Hypothesised Mean Difference	0	
df	4	
t Stat	3.308718	
P(T<=t) one-tail	0.014845	
t Critical one-tail	2.131847	
P(T<=t) two-tail	**0.02969**	
t Critical two-tail	2.776445	

Age (wk)	Production % Shed 1	Production % Shed 2	Production % Shed 3
22	76	71	80
23	85	81	90
24	87	87	93
25	85	89	94
26	89	92	96
27	91	91	93
28	92	87	91
29	92	86	88
30	95	88	89
31	94	91	89
32	94	93	91
33	94	93	93
34	95	94	92
35	94	93	93

Production during outbreak

HD% Y1	HD% Y2	HD% Y3
85	91	93
89	87	91
91	86	88
92	88	89

Anova: Single Factor

SUMMARY Egg production to week 35 **Farm 1 Shed 1**

Groups	*Count*	*Sum*	*Average*	*Variance*
Production % Shed 1	14	1263	90.21	29.104
Production % Shed 2	14	1236	88.29	37.604
Production % Shed 3	14	1272	90.86	14.593

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	50.143	2	25.07	0.925	0.405	3.238
Within Groups	1056.929	39	27.10			
Total	1107.071	41				

Anova: Single Factor

SUMMARY Egg production during outbreak **Farm 1** **Shed 1**

Groups	*Count*	*Sum*	*Average*	*Variance*
HD% Y1	4	357	89.25	9.583
HD% Y2	4	352	88	4.667
HD% Y3	4	361	90.25	4.917

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	10.167	2	5.083333	0.796	0.481	4.256
Within Groups	57.500	9	6.388889			
Total	67.667	11				

Field study 2 (SLD occurred in the flocks in 2017 during the weeks highlighted in yellow)
Farm 1 Shed 3 Two sample t-tests assuming unequal variances

Week	Fm1 Sd3 18 Egg%	Fm1 Sd3 17 Egg%
33	92.22	90.52
32	94.3	91.65
31	94.07	93.89
30	94.35	91.55
29	95.47	89
28	94.75	89.11
27	95.39	90.36
26	96.06	91.71
25	94.85	91.53
24	92.65	91.86
23	86.61	90.24
22	66.95	83.21

	Fm1 Sd3 18 Egg%	*Fm1 Sd3 17 Egg%*
Mean	91.473	90.386
Variance	65.862	6.8681
Observations	12	12
Hypothesised Mean Difference	0	
df	13	
t Stat	0.4414	
P(T<=t) one-tail	0.3331	
t Critical one-tail	1.7709	
P(T<=t) two-tail	0.6662	
t Critical two-tail	2.1604	

Over period of outbreaks	*Fm1 Sd3 18 Egg%*	*Fm1 Sd3 17 Egg%*
Mean	94.576	90.974
Variance	1.3755	2.5411
Observations	8	8
Hypothesised Mean Difference	0	
df	13	
t Stat	5.1487	
P(T<=t) one-tail	**9E-05**	
t Critical one-tail	1.7709	
P(T<=t) two-tail	**0.0002**	
t Critical two-tail	2.1604	

week	Fm1 Sd3 17 Ttl Mort%	Fm1 Sd3 18 Ttl Mort%
33	0.44	0.08
32	0.46	0.03
31	0.07	0.05
30	0.09	0.01
29	0.22	0.04
28	0.09	0.04
27	0.20	0.29
26	0.08	0.05
25	0.02	0.03
24	0.08	0.01
23	0.09	0.02
22	0.18	0.03

	Fm1 Sd3 17 Ttl Mort%	*Fm1 Sd3 18 Ttl Mort%*
Mean	0.1681	0.0572
Variance	0.0208	0.0059
Observations	12	12
Hypothesised Mean Difference	0	
df	17	
t Stat	2.3486	
P(T<=t) one-tail	0.0156	
t Critical one-tail	1.7396	
P(T<=t) two-tail	**0.0312**	
t Critical two-tail	2.1098	

Over period of outbreaks	*Fm1 Sd3 17 Ttl Mort%*	*Fm1 Sd3 18 Ttl Mort%*
Mean	0.2066	0.0746
Variance	0.0257	0.0082
Observations	8	8
Hypothesised Mean Difference	0	
df	11	
t Stat	2.0282	
P(T<=t) one-tail	**0.0337**	
t Critical one-tail	1.7959	
P(T<=t) two-tail	0.0675	
t Critical two-tail	2.201	

Field study 2 (continued)

Farm 1 Shed 5 Two sample t-tests assuming unequal variances

Week	18 Egg%	17 Egg%
33	92.82	90.73
32	92.84	89.62
31	93.77	91.27
30	93.8	91.76
29	94.24	89.32
28	93.87	90.87
27	96.38	90.56
26	96.52	90.94
25	96.11	91.97
24	95.05	89.29
23	89.39	86.92
22	71.24	76.73

Hen day production %	***Fm1s5 18 Egg%***	***Fm1s5 17 Egg%***
Mean	92.16917	89.165
Variance	47.22919	17.21835
Observations	12	12
Hypothesised Mean Difference	0	
df	18	
t Stat	1.296318	
P(T<=t) one-tail	0.10562	
t Critical one-tail	1.734064	
P(T<=t) two-tail	0.211241	
t Critical two-tail	2.100922	

During outbreak	***Fm1s5 18 Egg%***	***Fm1s5 17 Egg%***
Mean	93.494	90.54
Variance	0.4021	1.0981
Observations	5	5
Hypothesised Mean Difference	0	
df	7	
t Stat	5.393	
P(T<=t) one-tail	**0.0005**	
t Critical one-tail	1.8946	
P(T<=t) two-tail	**0.001**	
t Critical two-tail	2.3646	

Week	Fm1s5 18 Ttl Mort%	Fm1s5 17 Ttl Mort%
33	0.09	0.14
32	0.10	0.48
31	0.14	0.14
30	0.04	0.07
29	0.04	0.07
28	0.12	0.03
27	0.99	0.06
26	0.05	0.05
25	0.17	0.06
24	0.23	0.05
23	0.08	0.10
22	0.03	0.08

	Fm1s5 18 Ttl Mort%	***Fm1s5 17 Ttl Mort%***
Mean	0.17313	0.11001
Variance	0.07039	0.01443
Observations	12	12
Hypothesised Mean Difference	0	
df	15	
t Stat	0.75071	
P(T<=t) one-tail	0.23222	
t Critical one-tail	1.75305	
P(T<=t) two-tail	0.46444	
t Critical two-tail	2.13145	

During outbreak	***Fm1s5 18 Ttl Mort%***	***Fm1s5 17 Ttl Mort%***
Mean	0.08086	0.18038
Variance	0.00198	0.02838
Observations	5	5
Hypothesised Mean Difference	0	
df	5	
t Stat	-1.2771	
P(T<=t) one-tail	0.12883	
t Critical one-tail	2.01505	
P(T<=t) two-tail	0.25766	
t Critical two-tail	2.57058	

Field study 2 (continued)

Farm 2 Shed 5 Two sample t-tests assuming unequal variances

2018 Week	Fm2 S5 18 Egg%	Fm2 S5 17 Egg%
33	93.76	87.4
32	93.66	91.91
31	93.95	90.21
30	93.1	92.83
29	93.58	94.4
28	93.88	93.13
27	93.81	93.32
26	93.47	93.5
25	92.94	93.56
24	91.71	92.35
23	88.72	90.22
22	80.86	85.5

	Fm2 S5 18 Egg%	*Fm2 S5 17 Egg%*
Mean	91.9533	91.528
Variance	14.3757	7.3987
Observations	12	12
Hypothesised Mean Difference	0	
df	20	
t Stat	0.31612	
P(T<=t) one-tail	0.37759	
t Critical one-tail	1.72472	
P(T<=t) two-tail	0.75518	
t Critical two-tail	2.08596	

During outbreak	*Fm2 S5 18 Egg%*	*Fm2 S5 17 Egg%*
Mean	93.6771	91.8857
Variance	0.08049	5.62483
Observations	7	7
Hypothesised Mean Difference	0	
df	6	
t Stat	1.98431	
P(T<=t) one-tail	**0.04723**	
t Critical one-tail	1.94318	
P(T<=t) two-tail	0.09446	
t Critical two-tail	2.44691	

2017 week	Fm2 S5 18 Ttl Mort%	Fm2 S5 17 Ttl Mort%
33	0.13	0.086
32	0.06	0.118
31	0.24	0.161
30	0.04	0.156
29	0.45	0.032
28	0.09	0.048
27	0.10	0.102
26	0.11	0.032
25	0.09	0.032
24	0.05	0.043
23	0.12	0.075
22	0.05	0.037

Ttl Mort%	*Fm2 S5 18*	*Fm2 S5 17*
Mean	0.12802	0.077
Variance	0.01335	0.0023
Observations	12	12
Hypothesised Mean Difference	0	
df	15	
t Stat	1.41402	
P(T<=t) one-tail	**0.08889**	
t Critical one-tail	1.75305	
P(T<=t) two-tail	0.17777	
t Critical two-tail	2.13145	

Ttl Mort% During outbreak	*Fm2 S5 18*	*Fm2 S5 17*
Mean	0.15894	0.10058
Variance	0.0211	0.00244
Observations	7	7
Hypothesised Mean Difference	0	
df	7	
t Stat	1.00646	
P(T<=t) one-tail	0.17385	
t Critical one-tail	1.89458	
P(T<=t) two-tail	0.34771	
t Critical two-tail	2.36462	

Field study 2 (continued)

Farm 2 Shed 6 Two sample t-tests assuming unequal variances

2018 Week	Fm2 S6 18 Egg%	Fm2 S6 17 Egg%
33	95.25	91.75
32	95.3	92.99
31	94.71	88.63
30	94.55	88.31
29	94.79	92.55
28	94.56	93.28
27	94.63	94.06
26	94.25	92.78
25	93.99	93.54
24	92.34	93.41
23	89.86	92
22	79.39	88.69

Egg%	***Fm2 S6 18***	***Fm2 S6 17***
Mean	92.8017	91.833
Variance	20.1429	4.3443
Observations	12	12
Hypothesised Mean Difference	0	
df	16	
t Stat	0.67845	
P(T<=t) one-tail	0.25359	
t Critical one-tail	1.74588	
P(T<=t) two-tail	0.50718	
t Critical two-tail	2.11991	

Egg% During outbreak	***Fm2 S6 18***	***Fm2 S6 17***
Mean	94.5975	92.018
Variance	0.14825	5.0165
Observations	8	8
Hypothesised Mean Difference	0	
df	7	
t Stat	3.21101	
P(T<=t) one-tail	0.00742	
t Critical one-tail	1.89458	
P(T<=t) two-tail	**0.01484**	
t Critical two-tail	2.36462	

2017 week	Fm2 S6 18 Mort%	Fm2 S6 17 Mort%
33	0.28	0.03
32	0.14	0.13
31	0.11	0.08
30	0.12	0.51
29	0.13	0.14
28	0.04	0.12
27	0.09	0.10
26	0.12	0.40
25	0.04	0.15
24	0.04	0.05
23	0.03	0.03
22	0.04	0.03

Mort%	***Fm2 S6 18***	***Fm2 S6 17***
Mean	0.09792	0.1498
Variance	0.00506	0.0231
Observations	12	12
Hypothesised Mean Difference	0	
df	16	
t Stat	-1.07073	
P(T<=t) one-tail	0.15009	
t Critical one-tail	1.74588	
P(T<=t) two-tail	0.30018	
t Critical two-tail	2.11991	

Mort% During outbreak	***Fm2 S6 18***	***Fm2 S6 17***
Mean	0.09687	0.2067
Variance	0.00147	0.0252
Observations	8	8
Hypothesised Mean Difference	0	
df	8	
t Stat	-1.90211	
P(T<=t) one-tail	**0.04683**	
t Critical one-tail	1.85955	
P(T<=t) two-tail	0.09366	
t Critical two-tail	2.306	