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Enhancing mucosal immunity in chickens

A report for the Rural Industries Research and Development Corporation

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Enhancing mucosal immunity in chickens by novel in-ovo and postnatal vaccination techniques.
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Foreword

In all animals the mucosal surfaces of, for example, the gastrointestinal tract and respiratory tracts, are the site of first contact between mucosal pathogens and the protective components of the immune system. Initially antigens and pathogens will encounter the non-specific defense mechanisms of the innate immune system. Activation of the acquired immune system will stimulate an antigen-specific immune response, which in the case of the mucosal immune system, results in the local production of IgA antibody. Following its translocation through the epithelial cells into the tract lumen, IgA antibody prevents pathogen contact with the host by interfering with microbial attachment, colonisation and virulence. This process is termed immune exclusion. Therefore, it is anticipated that the design of vaccination strategies that will increase the quantity of IgA antibody present at the mucosal surface will subsequently improve the efficiency of immune exclusion, reducing levels of pathogen infection, facilitating improved bird health and performance.

Studies undertaken in this project evaluated techniques designed to induce increased production of IgA antibody at the intestinal mucosa. The techniques investigated involved *in-ovo* and postnatal delivery of either:
1. Immunoregulators, in particular vitamin E and,
2. Exogenous cytokines, in particular interleukin-6.
This report focuses on the outcomes from this research work. The potential for these strategies to be applied to the Australian Poultry Industries are also discussed**.**

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

This report, a new addition to RIRDC's diverse range of over 700 research publications, forms part of our chicken meat and egg R&D programmes, which aims to develop strategies to more effectively control poultry diseases, such as vaccines, and to minimise the effects of disease in the layer flock, respectfully.

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Peter Core
Managing Director
Rural Industries Research and Development Corporation

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Contents

Abbreviations

CD	cluster determination
cfu	colony forming units
ChIFN-γ	chicken interferon-gamma
CTB	cholera toxin B subunit
D18E	day 18 embryonation
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
IgA	immunoglobulin A
IL	interleukin
IL-3	interleukin-3
IL-6	interleukin-6
ip	intraperitoneal
ISS	intestinal scrapings supernatant
LFU	limit flocculating units
PBS	phosphate buffered saline
pIL-3	porcine interleukin-3
pIL-6	porcine interleukin-6
T. toxoid	tetanus toxoid
TAGA	tryptose agar
TETR	tetrathionate broth
VE	vitamin E
VESD	vitamin E supplemented diet
XLD	xylose lysine desoxycholate medium

Executive Summary

Initial contact between many microbial pathogens and the chicken occurs at the intestinal surface. Locally produced IgA antibody present at the mucosal surface of the intestine interferes with microbial contact with the host, by binding to the pathogen. This excludes the pathogen from further interaction with the host, decreasing the incidence of infection. Stimulation and upregulation of local IgA antibody production strengthens the barrier between the intestinal surface and pathogens, further decreasing their opportunity to invade and cause disease. Improved resistance to disease will improve flock health and performance. Therefore, the focus of this research has been to assess the ability of novel vaccination strategies to enhance IgA antibody production at the intestinal surface. Strategies assessed have included the delivery of known mammalian immunostimulatory substances at 18 days of embryonation, that is *in-ovo,* or posthatch. In particular, the delivery of Vitamin E (VE) and the cytokine interleukin-6 (IL-6) have been assessed.

Studies assessing *in-ovo* (amniotic cavity), at day 18 embryonation, administration and oral administration at 7 days of age of 20 mg of VE together with *S. typhimurium* antigen increased (not statistically significant) the mean concentration of anti-*S. typhimurium* IgA antibody in the serum and intestinal scrapings at 14 days of age compared with chicks vaccinated with *S. typhimurium* in the absence of VE.

The impact of dietary supplementation with VE at levels above those required to meet nutritional requirements on IgA antibody production, both before and after immunisation, was evaluated. Supplementation of a maize-based diet (containing 50 mg/kg VE) with an additional 250 mg/kg VE, fed to broiler chickens for the duration of the study, significantly increased total IgA antibody titres in serum and at the intestinal surface. Following immunisation (intraperitoneal (ip) at 21 days and oral at 35 days) with either tetanus toxoid or whole killed *S. typhimurium*, there was a significant increase in the antigen-specific IgA antibody produced at the intestinal surface, when compared with chickens fed the basal diet.

Feeding a diet supplemented with 250 mg/kg VE (that is a VE supplemented diet; VESD) for shorter intervals was also investigated. Birds fed the VESD from day old until 21 days of age exhibited similar increases in IgA antibody titres in the intestinal scrapings, serum and bile, as birds fed the VESD from day 0-42. An important outcome from this study is the link between the timing of vaccination and the interval of feeding the VESD. Birds fed the VESD from day old until the first immunisation (day 21) had enhanced IgA antibody levels of similar amplitude to birds fed VESD from day 0 to 42. In contrast birds fed the VESD from day 21, which coincided with the start of the immunisation schedule and an ip immunisation of whole killed *S. typhimurium*, for the remainder of the study, had similar or lower (not statistically significant) IgA antibody titres compared to the birds fed the unsupplemented diet. Feeding VE from day old also appears to be important for upregulation of IgA antibody production.

The second series of studies assessed the ability of the recombinant protein of cytokines interferon-γ (IFN-γ) and interleukin-6 (IL-6) to enhance IgA antibody production. *In-ovo* delivery of IFN-γ without antigen failed to enhance IgA antibody production in young chicks. A number of experiments investigated the *in-ovo* delivery of IL-6 alone or in combination with antigen. It was evident that, in the absence of antigen, *in-ovo* delivery of IL-6 failed to stimulate IgA antibody production. However, when IL-6 was delivered in conjunction with killed *S. typhimurium* antigen *in-ovo*, followed with a second administration of antigen and IL-6 orally at seven days of age, there was an increase (not statistically significant) in the average amount of anti-*S. typhimurium* IgA antibody at the intestinal surface.

Studies in young chicks also demonstrated upregulation of IgA antibody titres at the intestinal mucosa when the primary ip immunisation at 8 days of age followed the oral delivery of IL-6 at 3 and 6 days of age. However, the most noteworthy increase in IgA antibody production at the intestinal site was observed when previously immunised birds received IL-6 for either two or four consecutive days after the delivery of a secondary oral booster immunisation. Following this protocol there was a trend for IL-

6 to enhance IgA antibody production to both T. toxoid and *S. typhimurium* in the serum, and, more notably, a statistically significant increase in anti-antigen IgA antibody titres at the intestinal surface.

The benefit of increased IgA antibody titres at the intestinal site as a result of oral IL-6 administration was assessed in vaccination/challenge studies with *S. typhimurium*. A *S. typhimurium* challenge model, which represented the trickle challenge normally experienced by chickens in the field, was replicated by housing the birds to be challenged with *S. typhimurium* infected seeder birds. That is, at 35 days of age birds to be challenged were placed in floorpens that had been housing *S. typhimurium* infected seeder birds for several days. Through co-habitation with the seeder birds the bacteria were gradually taken in, either orally or percloacally, by the challenged birds. Birds administered the *S. typhimurium* vaccine (ip at 14 days and orally at 28 days) and oral administration of IL-6 for the subsequent 4 days, had higher mean (not statistically significant) anti-*S. typhimurium* IgA antibody titres in serum, intestinal scrapings and bile prior to *S. typhimurium* challenge, compared with birds vaccinated with *S. typhimurium* alone. The higher antibody titres correlated with a reduced percentage of birds with *S. typhimurium* positive cloacal swabs at 14 days after challenge, and lower numbers of *S. typhimurium* isolated per gram of liver and spleen at 7 and 14 days after challenge. Within seven days of challenge these birds exhibited the highest mean anti-*S. typhimurium* IgA antibody titres in the bile and intestinal mucosa (not statistically significant) and had the lowest levels of *S. typhimurium* infection. Hence, IL-6 may benefit not only the immune response to vaccination, but also the immune response and resistance to a subsequent challenge.

The series of experiments undertaken during this project have identified a number of novel techniques for improving IgA antibody production at the intestinal site, which can reduce infection with bacteria such as *S. typhimurium*. The delivery of cytokine IL-6 either before or after immunisation or challenge will induce an increase in the anti-antigen IgA antibody titres at the intestinal surface. To date the most significant increase in antibody titre was observed when IL-6 was delivered to previously immunised chickens immediately following the secondary oral booster immunisation. VE upregulates the IgA antibody response to an antigen following the concurrent *in-ovo* delivery of VE and antigen with and without postnatal administration. Further, the supplementation of maintenance diets with approximately 250 mg/kg VE significantly increased total and antigen-specific IgA antibody titres. This can be achieved by feeding the VESD from day old for the 6 week duration of a broiler growout, or alternatively, from day old for the three week period prior to immunisation at 21 days of age

Further clarification and elucidation of the research outcomes is required before the identified immunoenhancing strategies can be successfully adopted by the poultry industry. These studies have identified the requirement to feed VESD from day old and prior to immunisation for immunoenhancement. However, the feasibility of reducing the pre-immunisation VESD feeding interval while maintaining an enhanced immune response to the antigen must be determined. These studies have also established the potential for IL-6 cytokine to activate the mucosal immune system in chickens, and in particular, IgA antibody production at the intestinal surface following immunisation, in 4-6 week old broiler chickens. However, the current delivery schedule of IL-6 is cumbersome and impractical. The potential for IL-6 to be delivered with antigen, either *in-ovo* or at hatch, to significantly enhance the antibody response, must be determined.

Introduction

The mucosal organs are the most common portal of entry of potential pathogens that cause diseases such as infectious bronchitis, campylobacteriosis, salmonellosis and coccidiosis. Under ideal conditions, contact with a potential pathogen at a mucosal surface will stimulate a local mucosal immune response. In all species the antigen-specific immune response at mucosal sites is characterised by a specialised set of effector cells and molecules, which induce the preferential production of IgA antibody (Husband, 1987; Muir *et al*., 2000). The presence of IgA antibody at the intestinal surface provides the first line of acquired immune defence against mucosal pathogens by interfering with their attachment to and colonisation of the intestinal mucosa. This barrier between potential pathogens and the host can be improved by stimulating increased levels of IgA antibody production. Further, the earlier that antigen-specific IgA antibody titres are generated at the mucosal surfaces in young chicks, the greater the opportunity to improve local mucosal immune defense, facilitating improved bird health and production.

To this end recent advances in antigen preparation and mechanisms for mucosal regulation, either via manipulation of biological response messengers (cytokines) and non-specific immunoregulators have opened the way for novel approaches in the design of vaccines that target immune responses at mucosal surfaces. Methods for the delivery of such substances are rapidly developing and readily lend themselves to *in-ovo* injection. Recently, work from this laboratory (Noor *et al*., 1995) has demonstrated the success of *in-ovo* delivery of heat-killed *Campylobacter jejuni,* which significantly increased IgA antibodies against *C. jejuni* in serum, bile and intestinal mucosa.

A number of studies in rodents have indicated the importance of cytokines in regulating mucosal immune responses (Husband *et al.,* 1994). Gene knockout mice, which lack important IgA regulatory cytokines such as interleukin (IL)-4, IL-5 and IL-6 exhibit a dramatic reduction in IgA plasma cells in the intestine and lungs, compared with normal mice. The exogenous supply of these cytokines can restore mucosal immune function. Similar techniques stimulate enhanced mucosal immunity in normal animals (Ramsay *et al*., 1994). In this way the co-delivery of cytokines, antigen and/or immunomodulators either *in-ovo* or postnatally may amplify the normal immune responses in chickens.

The immunoregulatory role of cytokine IL-6 on IgA antibody titres is one of the main focal points of studies undertaken in this project. In mammals IL-6 regulates local IgA production through its influence on late stage differentiation of B-cells into immunoglobulin-secreting plasma cells, and, its augmentation of the secondary immune responses (Lebman and Coffman, 1994). At the commencement of this project avian IL-6 had not been sequenced. However, porcine IL-6 (pIL-6) was a suitable candidate for use as an avian IL-6-cytokine equivalent, as it demonstrates cross-species reactivity and, can stimulate growth in a murine-IL-6-dependent cell-line. Therefore, in all experimental work presented in this report IL-6 refers to recombinant protein of porcine IL-6.

Vitamin E acts as a non-specific immunoregulator in a variety of animals. Studies in chickens have reported the modulatory influence of VE on cell-mediated immunity and IgG-based humoral immunity, macrophage function and phagocytosis and, improved resistance to disease (Finch and Turner, 1996). These effects have been observed following *in-ovo* delivery of VE (Gore and Qureshi, 1997), and after feeding VE supplemented diets (VESD) (Erf *et al*., 1998; Franchini *et al.,* 1986; Friedman *et al.,* 1998). However, the influence of VE on total or antigen-specific IgA antibody levels has not been evaluated in chickens. The potential for VE supplementation of the diet to increase IgA antibody production has been reported in rodents (Kaku *et al*., 1999; Gu *et al*., 1999). In view of this, several studies were undertaken to assess the immunoregulatory role of VE delivered *in-ovo* or as a supplement in the diet, to increase total and antigen-specific IgA antibody levels.

The research work presented in this report was designed to assess the ability of novel immunoregulatory agents to augment protective immunity at the intestinal surface in chickens. Vaccination procedures included *in-ovo* and postnatal immunisation, with an aim to induce

precocious stimulation of the mucosal immune system. In all cases the immune response was evaluated. The potential for increased levels of anti-*S. typhimurium* IgA antibody at the intestinal surface to protect the chicken from a challenge of *S. typhimurium* was also evaluated.

Objectives

The overall objective of this project was the induction of long-term immunoenhancement in chickens following early priming of the avian immune system via novel *in-ovo* and postnatal vaccination techniques.

All research was designed to assess the potential for immunomodulation of the avian intestinal immune system through the delivery of either immunoenhancing agents or cytokines. The research work is presented in the following two sections:

1. Assessment of the impact of mammalian immunomodulators on IgA antibody production in chickens, and
2. Assessment of the impact of cytokines on IgA antibody production in chickens.

Methodology

1. Birds.

Commercial outbred chickens (Cobb) obtained from Inghams hatchery, Casula, were used in all studies. Day 18 embryos (D18E) were obtained for *in-ovo* immunisations and day old chicks were used in studies involving only postnatal immunisation techniques. Typically, each treatment group consisted of 15-20 birds in the *in-ovo* studies and between 20-30 chickens in all other studies.

2. Ethical considerations.

All experimental procedures were approved by the University of Sydney Animal Ethics Committee, and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

3. Antigens and immunisation protocols.

Antigens: Two types of antigen were used throughout these studies. A protein antigen, tetanus toxoid, was used in studies designed for initial assessment of a novel vaccination technique. Where the technique was found to upregulate IgA antibody production, subsequent studies involved immunisation with a whole killed bacterial antigen, in this case, a field isolate of *S. typhimurium*.

Tetanus toxoid (T. toxoid), prepared by formalin treatment of *Clostridium tetani* culture supernatants, was obtained from Commonwealth Serum Laboratories, Melbourne, Australia. This was adjusted to 1500 limit flocculation units (LFU) by the addition of sterile phosphate buffered (pH 7.3) saline (PBS).

A recent field isolate of *Salmonella typhimurium* was obtained from Elizabeth Macarthur Agricultural Institute, Menangle. Cultures were grown overnight in Lemco broth at 37^0C in a shaking water bath. The concentration of *S. typhimurium* in the broth was evaluated by viable plate counts of serial dilutions onto xylose lysine desoxycholate (XLD) (Oxoid Australia Pty Ltd, West Heidelberg, Victoria) selective media and tryptose agar (TAGA) plates. For preparation of *S. typhimurium* immunogen, whole bacteria broth cultures were washed twice by centrifugation (11,000 x *g* for 20 minutes) and resuspended in PBS to the required final concentration. The bacteria were killed by the addition of 0.5% formalin.

Immunisation protocols: Fertile eggs at day 18 of embryonation were injected with either antigen, cytokine or immunoenhancer, placed directly into the amniotic cavity as described by Noor *et al.* (1995). Intraperitoneal immunisation (ip) was generally performed in 3 week old birds with antigen (T. toxoid or *S. typhimurium*) in Auspharm adjuvant (Husband, 1993), followed two weeks later with an oral booster of T. toxoid (Muir *et al*., 1995), or killed *Salmonella typhimurium* (Muir *et al.,* 1998b).

4. Challenge with live *S. typhimurium*.

Fresh floorpen litter was inoculated with *S. typhimurium* by placing 15, 30 day old infected seeder birds into the floorpens immediately after oral inoculation with live *S. typhimurium*. Five days later the challenged birds were placed into the same floorpens. The seeder and challenged birds were housed together in the floorpens for seven days, at which stage the seeder birds were removed. The challenged birds remained in the floorpen for a further seven days.

5. Isolation of viable *S. typhimurium* from challenged chickens.

Cloacal swabs: Cloacal swabs were collected from individual challenged birds seven and fourteen days after challenge. Swabs were selectively enriched in tetrathionate broth (TETR) (Oxoid Australia Pty Ltd.) for 48 hours at 37^0C followed by subculture onto XLD media which were then incubated at 37^0C for 48 hours.

Isolation and enumeration of S. typhimurium from spleen and liver: Isolation and enumeration of *S. typhimurium* in the spleen and liver was undertaken following homogenisation of 1 gram of tissue in a known volume of PBS. Serial dilutions were incubated for 48 hours at 37^0C in duplicate on XLD plates and the number of colony forming units (cfu) /gram were determined. To confirm the presence of *S. typhimurium* a 1 mL aliquot of the original homogenate was incubated in TETR and subcultures were incubated in duplicate on XLD plates.

The identity of *S. typhimurium* was confirmed biochemically by testing for the absence of lactose fermentation with o-Nitrophenyl-D-Galactopyranoside discs (Oxoid Australia Pty Ltd.), and serologically by slide agglutination using *Salmonella* O and H antisera (Oxoid Australia Pty Ltd.).

6. Sample collection.

Whole blood was collected from the jugular vein. At the end of each experiment all chickens were euthanased by intravenous administration of sodium pentobarbitone. Samples of intestinal scrapings were obtained from the length of jejunum after the serosal and mucosal surfaces were washed in ice cold PBS. Bile was retrieved from the gall bladder. Intestinal scrapings and bile were immediately frozen on dry ice and stored at -80^0C. The intestinal scrapings supernatant (ISS) was collected for antibody determination following ultracentrifugation at 24,000 x *g* for 60 minutes (Duncan *et al*., 1978).

7. Assay for detection of IgA-containing plasma cells.

Sections of intestine were fixed in cold ethanol, embedded in paraffin and then cut onto gelatin coated slides as described by Muir *et al*. (1995). To identify IgA-secreting plasma cells the sections were dewaxed in cold xylene and then rehydrated through graded dilutions of cold alcohol and finally PBS. Sections were stained with Lendrum's stain for 10 minutes at room temperature, to reduce non-specific fluorescein staining of eosinophils (Johnston and Bienenstock, 1974) and then washed in PBS until the colour ceased to elute from the tissues. IgA-secreting plasma cells were identified by fluorochrome immunofluorescence. The tissues were incubated with goat anti-chicken IgA conjugated with FITC for 20 minutes in a humid chamber, washed in PBS for one hour and then mounted in 90% glycerol in PBS. The number of IgA-containing cells in the intestine were counted using a X 40 objective and a X 10 eyepieces using a Ziess Axioskop 20 micropscope with incident light illumination. Each section of the intestine was scanned from the tips of the villi to the base of the mucosa and cells were counted in a total of 30 such scans. The average number of cells per scan was expressed as cells per linear centimetre of intestine by multiplying by 30.0, a correction factor determined from the field width of 330 μm.

8. Antibody detection by ELISA.

An indirect ELISA was used to determine either T. toxoid or *S. typhimurium*-specific IgA and total IgA in serum and ISS. To determine IgA antibodies to T. toxoid or *S. typhimurium* polysorb ELISA plates (Nunc Immuno, Medos Company, Sydney, Australia) were coated with 100 μl purified T. toxoid or 5 μg *Salmonella typhimurium* (diluted 1:100 in carbonate buffer), and incubated overnight at 4^0 C. All subsequent incubations were for 1 hour at 37^0C. Between each incubation, plates were

washed twice in washing buffer (0.05%Tween 20 and 0.5*M* sodium chloride in PBS) and PBS, then tapped dry. Plates were blocked with 0.25% gelatin in washing buffer. Optimal working dilutions for serum and ISS were determined for each experiment. The conjugate, horseradish peroxidase-conjugated goat anti-chicken IgA (Bethyl Laboratories, Montgomery, TX, USA) was diluted 1/200 in washing buffer containing 1% bovine serum albumin. Neat ABTS substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) was incubated for 30 minutes at room temperature and the reaction stopped with 1% sodium dodecyl sulphate (Kirkegaard and Perry Laboratories).

For total IgA analysis, maxisorb ELISA plates (Nunc Immuno, Medos Company, Sydney, Australia) were coated with mouse anti-chicken IgA (Southern Biotechnology Associates, Birmingham, Al, USA) diluted 1/1000 in carbonate buffer. Optimal working dilutions were determined for serum and ISS depending on the age of the birds and the immunisation schedule. All other steps were identical to the indirect ELISA for measuring anti-T. toxoid IgA.

Absorbance values were read at 405 nm in a Spectro Max 250 (Molecular Devices, Springvale, CA, USA) plate reader. A negative buffer blank and a hyperimmune positive reference standard were included in each plate, and all samples were analysed in triplicate. Sample dilutions were adjusted to provide optical density readings on the linear portion of the standard curve, approximately 20% below saturation point. Optical densities are expressed as a percentage of the hyperimmune positive control.

9. Hyperimmune antiserum.

Hyperimmune antisera were obtained from chickens immunised ip with 1500 LFU/ mL T. toxoid or 10^8 *S. typhimurium* in a vegetable oil-in-water adjuvant and Quil A (1mg/dose) at days 19 and 33 of age. A 0.5 mL oral booster of 1500 LFU/ mL T. toxoid or 10^8 whole killed *S. typhimurium* were delivered by gavage at 47 days of age. Blood was collected 7 days later, and serum prepared and stored at -20^0C.

10. Preparation of lymphocytes for flow cytometric analysis.

Blood taken from the jugular vein was collected into heparinised tubes. A total leucocyte count was determined manually by preparing a 1/200 dilution of whole blood in Natt and Herrick's solution (Campbell, 1995).

Heparinised blood was centrifuged at 1300 x *g* for 20 minutes. The buffy coat was diluted in an equal volume of PBS and then layered onto 5 mL of Ficoll Paque (Pharmacia Biotech, Sweden). Following centrifugation at 900 x *g* for 15 minutes the layer at the interface was removed and washed twice in PBS/5% foetal calf serum (FCS) by centrifugation at 300 x *g* for 5 minutes, prior to enumeration and viability assessment by trypan blue exclusion.

11. Flow cytometric analysis.

Approximately 10^7 lymphocytes in 100 µl PBS/FCS were incubated with 50 µl optimal dilution of either monoclonal mouse anti-chicken CD4, CD8, Ia or IgA (Southern Biotechnology Associates) at 4^0C for 20 minutes. Each sample was washed twice in 2 mL PBS, then incubated with 100 µl FITC conjugated affinity purified-goat anti-mouse immunoglobulin (Southern Biotechnology Associates) for 20 minutes at 4^0C in the dark, and washed as before. The cells were fixed in 0.1% paraformaldehyde in MilliQ water (Millipore, North Ryde, NSW, Australia), stored at 4^0C overnight and analysed within 24 hours of fixing. For each sample, data was collected from 10,000 events using an Argon ion laser providing light at 488 nm. Analysis was performed on the gated lymphoid population visually selected from the forward angle and 90^0 light scatter properties of the dot plot cloud. Both acquisition and analysis were undertaken on a FACScan flow cytometer (Becton Dickinson, Sydney, Australia) using Cell Quest software (Becton Dickinson). For each chicken a positive control tube was analysed to ensure minimal non-specific binding of the anti-mouse FITC secondary antibody.

12. Statistical analysis.

Statistical differences between treatment groups were determined using a one-way analysis of variance following $\log_e$ transformation of raw data. Significant differences between treatment group means were determined using Tukey's test at $p < 0.05$, $p < 0.01$ or $p < 0.001$.

Results

Section A: Assessment of the impact of mammalian immunomodulators on IgA antibody production in chickens.

All studies presented in section A assessed mammalian immunomodulatory agents in chickens. The aim of the work was to identify any immunomodulatory effect of these agents on IgA antibody production at the intestinal site in chickens.

Experiment 1: Assessment of the immunomodulatory effect of cholera toxin B-subunit (CTB), delivered *in-ovo,* on IgA antibody production.

Aim: To evaluate *in-ovo* delivery, that is at day 18 embryonation (D18E), of CTB subunit on the immune response to *in-ovo* delivered antigen (T. toxoid).

Experimental design: CTB was delivered at three rates; 1, 5, and 25 mg/embryo together with T. toxoid. At 14 days of age chicks received an oral delivery of CTB and T. toxoid at the same rates as they received them *in-ovo*. The impact of CTB on the anti-T. toxoid IgA antibody responses in serum and ISS was evaluated on day 21

Results: No significant upregulation of the immune response to T. toxoid was observed in birds administered CTB (data not shown).

Experiment 2: Evaluation of supplementary levels of dietary VE on the immune response

A number of publications have demonstrated the ability of VE (VE) to upregulate various components of cellular and humoral immunity (reviewed by Muir *et al*., 2000). However, despite facilitating improved resistance to disease, the impact of dietary VE supplementation on IgA antibody titres has not been determined. Experiments 2A, B and C investigate the impact of VE on the immune response in unimmunised and immunised chickens when it was included in the diet at levels above that required for nutritional maintenance. The impact of a number of supplementary rates of VE on the immune response to a protein antigen T. toxoid (experiment 2A) and a whole killed bacterial antigen, *S. typhimurium* (experiment 2B) were investigated. The influence of the interval of feeding vitamin E supplemented diets (VESD) in relation to the time of vaccination was also investigated (experiment 2C).

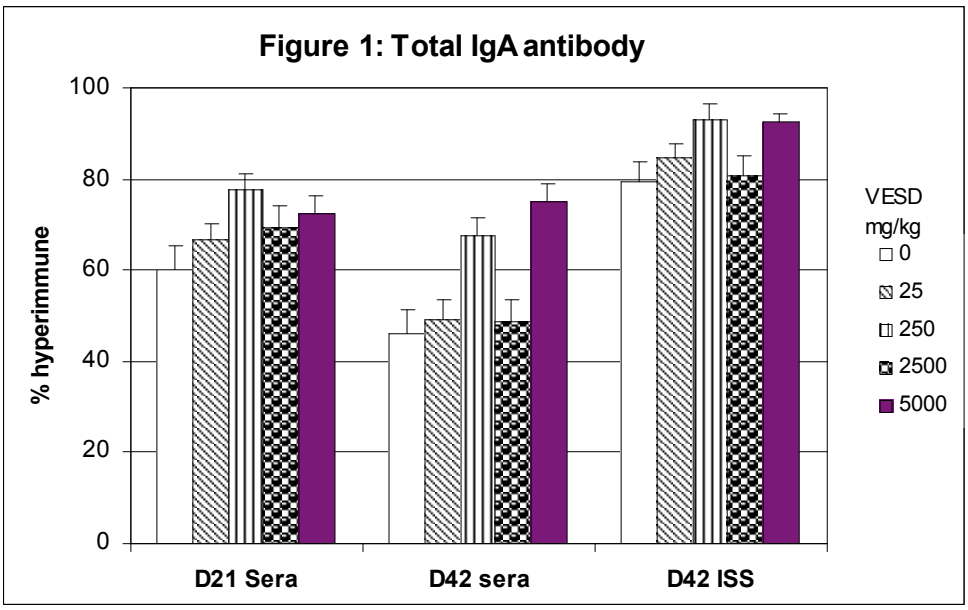
Experiment 2A: The effect of feeding VESD on the immune response in birds immunised with T. toxoid.

Aim: This study was designed to assess the influence of VE, included in graded doses in the diet, on IgA antibody titres in chickens with and without immunisation with T. toxoid.

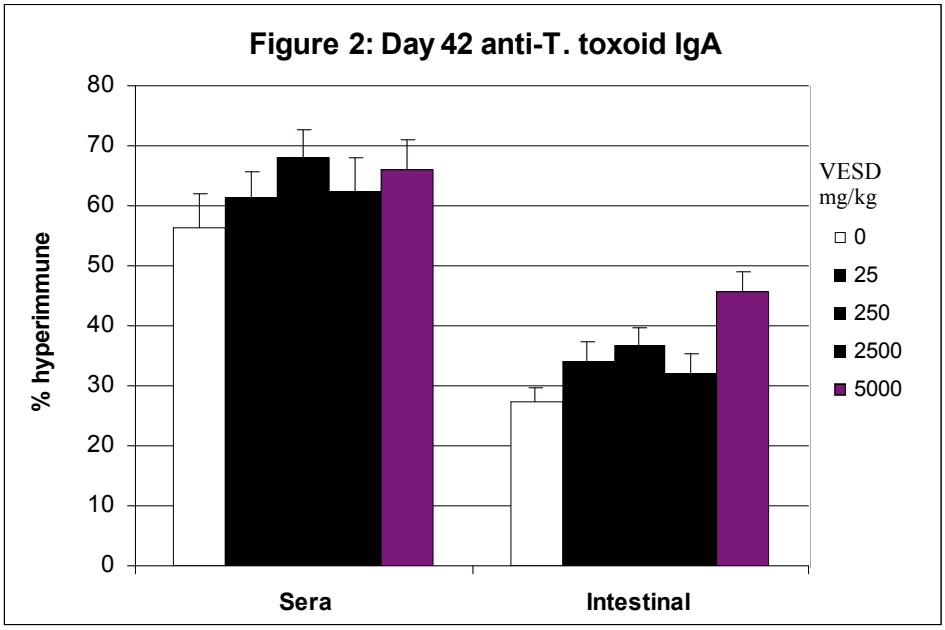
Experimental design: Chicks received a maize-based basal diet (containing 50 mg/kg VE) supplemented with either 0, 25, 250, 2500 or 5000 mg/kg VE (BASF Lutavit E 50 S) for the 42 days of the study. At 21 days of age birds received an ip immunisation of T. toxoid, and an oral booster of T. toxoid at 35 days of age. Average bird weight and feed conversion ratio was determined for the duration of the study. Serum samples were collected pre and post immunisation for assessment of

total and anti-T. toxoid IgA antibody levels respectively. Intestinal scrapings were collected on day 42 for antibody determination. Peripheral blood lymphocytes were analysed pre and post immunisation to determine the impact of VE on the percentage of T cell subsets (CD4+ and CD8+) and Ia+ cells by flow cytometry.

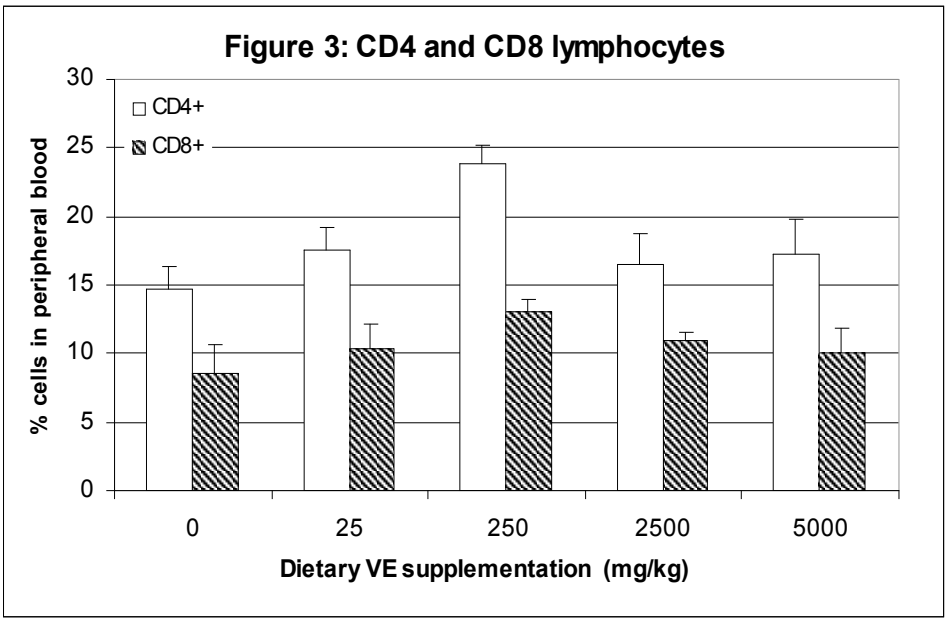
Results: Birds were clinically normal throughout the study and between days 21 to 42 there were no statistically significant differences between treatment groups in average body weight gain or feed conversion data. On day 21, prior to immunisation, birds receiving 250mg/kg VE supplementation had a statistically significant increase ($p < 0.03$) in serum total IgA compared with birds fed the basal diet (Figure 1). At day 42 birds fed 250 mg/kg ($p < 0.05$) and 5000 mg/kg ($p < 0.01$) VESD demonstrated significant increases in total serum IgA (Figure 1). Total IgA antibody in ISS at day 42 followed similar trends, with birds fed 250 and 5000 mg/kg supplementary VE demonstrating higher average titres, which was statistically significant ($p < 0.05$) in the latter group (Figure 1).

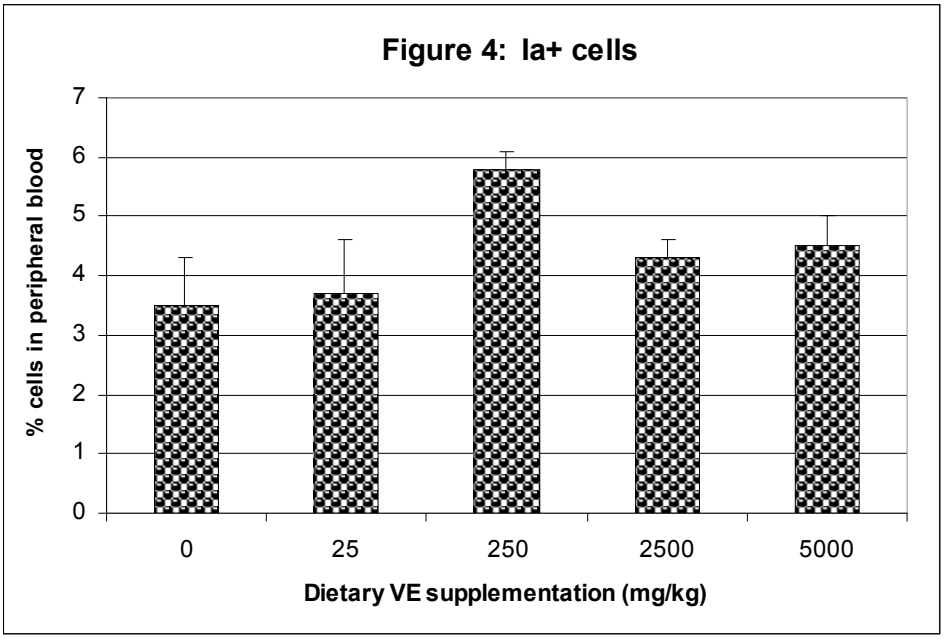


At day 42 average serum anti-T. toxoid IgA antibody was increased (not statistically significant) in birds fed 250 and 5000 mg/kg VESD compared with the unsupplemented group (Figure 2). In the ISS anti-T. toxoid IgA was increased in birds fed 250 mg/kg VESD and significantly increased ($p = 0.02$) in birds receiving 5000mg/kg VE supplementation (Figure 2).



On day 26, five days after the primary ip immunisation, the percentage of peripheral blood derived CD4+ helper T cells ($p<0.05$) (Figure 3) and Ia+ cells (that is, B cells and monocytes/macrophages) ($p< 0.06$) (Figure 4) were increased in birds receiving 250 mg/kg VE compared to the control birds. These results suggest increased activation of cell-mediated immunity in birds receiving 250 mg/kg VE supplementation, which may have influenced the IgA antibody response of these birds following immunisation. However, as birds fed 5000 mg/kg VESD experienced increased IgA antibody titres in the absence of any detectable alterations to T-cells and Ia+ cells, other mechanisms are likely to also be involved in the VE induced immune responses observed.





Outcome: Supplementation of the diet with 250 mg/kg VE induced an increase in serum total IgA antibody titres at day 21 and 42, compared with birds fed the basal diet. Supplementation of the diet with 5000 mg/kg VE significantly increased total IgA antibody in sera and ISS at day 42, and in anti-T. toxoid IgA at the intestinal site on day 42. However, no significant immunomodulatory effect was observed in birds fed 2500 mg/kg VE in either total or antigen-specific IgA antibody production. This observation is not entirely unexpected as a non-linear effect of VE supplementation on avian immunoglobulin production has been previously reported by Jackson *et al.* (1977) and Friedman *et al.* (1998). However, it is difficult to compare these studies as a number of variables, including the age and strain of the bird, the level of VE supplementation and the immunisation and/or challenge conditions, differ between experiments. It is interesting however, that in previously reported non-linear antibody responses, Friedman *et al.* (1998) observed a decline in VE induced upregulation of the immune system at the highest levels of VE supplementation (in that case at 30 and 150 mg/kg added VE), whereas Jackson *et al*. (1977) observed variation in the response across the range of levels of VE supplementation (in this case 150 and 450 ppm were immunostimulatory whereas 90, 300 and 900 ppm did not alter the antibody titres). The characteristics of the variation in immunostimulation observed by Jackson *et al.* (1977) is similar to the observations of experiment 2A.

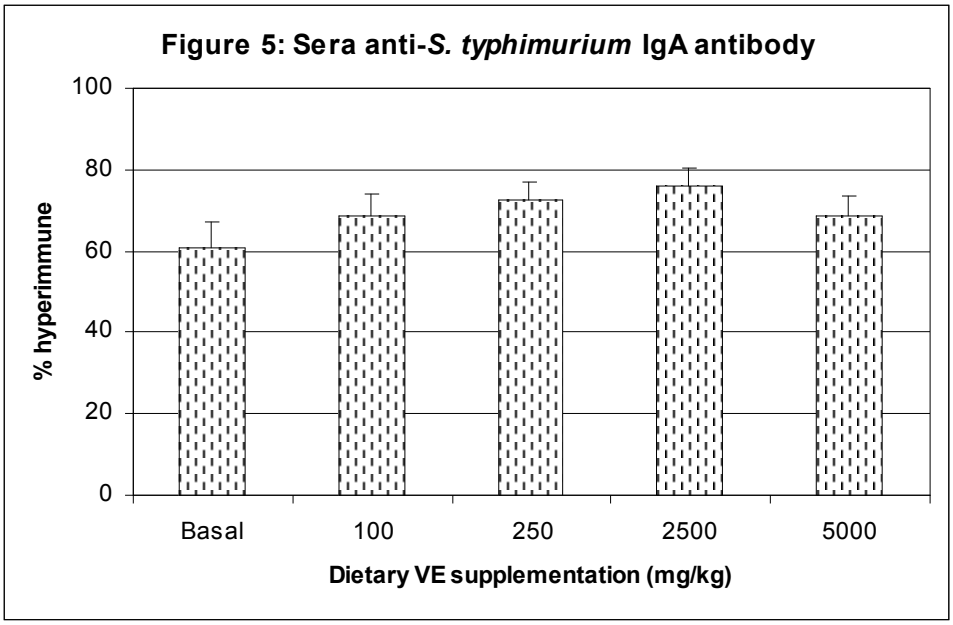
Experiment 2B: The effect of feeding VESD on the immune response in birds immunised with whole killed *S. typhimurium*.

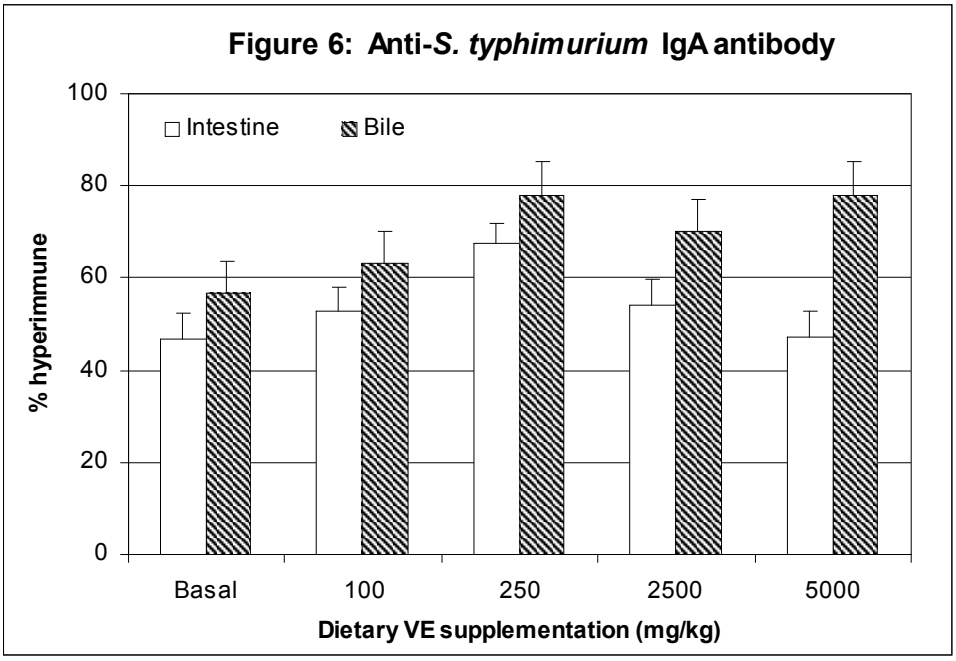
Aim: Experiment 2A illustrated that some VESD can significantly enhance total and anti-antigen IgA antibody titres. Experiment 2B was undertaken to determine whether VESD influence the immune response to *S. typhimurium* when fed to chickens immunised with whole killed *S. typhimurium*.

Experimental design: This experimental design was similar to experiment 2A. Briefly chickens were allocated to diets supplemented with either 0, 100, 250, 2500 and 5000 mg/kg VE (BASF, Lutavit E 50 S). These diets were fed from day old for the duration of the study. At 21 days of age each chicken received an ip immunisation of 10^9 whole killed *S. typhimurium* in a vegetable oil based adjuvant. At 35 days of age each chicken received an oral booster immunisation of 10^9 whole killed *S. typhimurium* in PBS. On day 42 samples of serum, intestinal scrapings and bile were collected and the anti-*S. typhimurium* IgA antibody titres were determined by ELISA. On days 20,

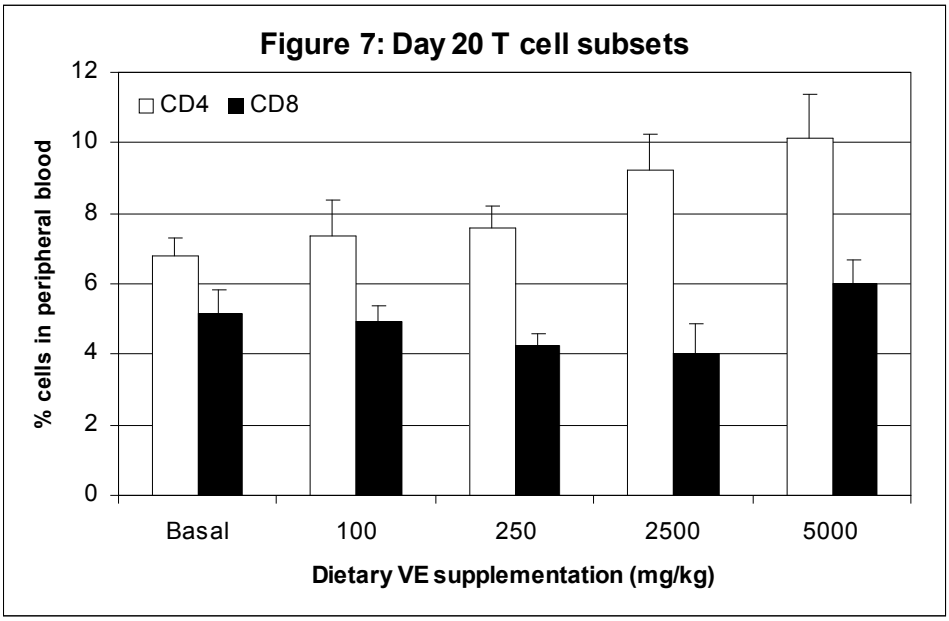
27 and 38 peripheral blood was collected and the percentage of T cells, Ia^+ and IgA^+ cells were determined by flow cytometry.

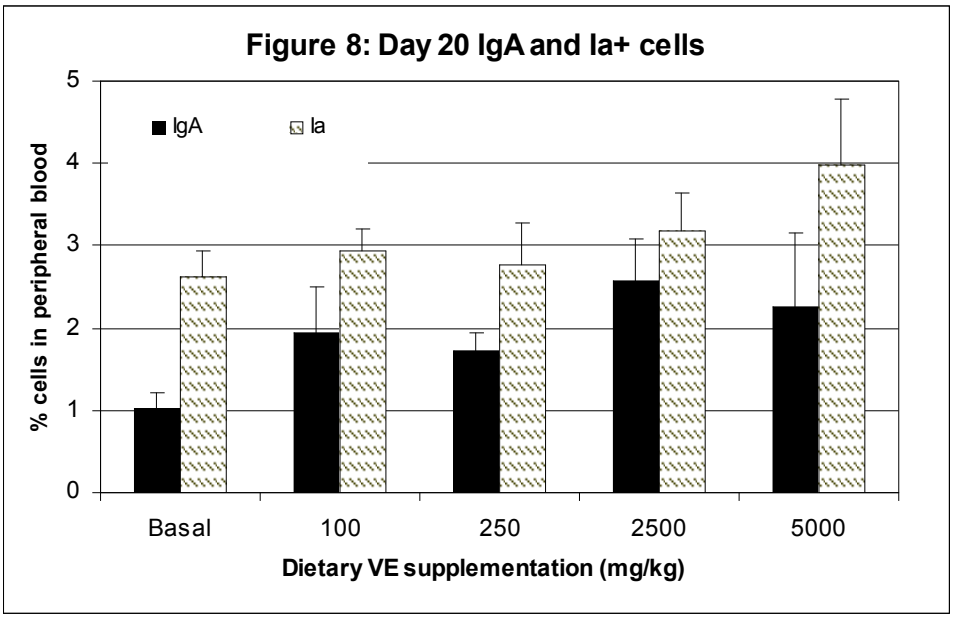
Results: Day 42 anti-*S. typhimurium* IgA antibody titre in serum (Figure 5) was significantly increased in birds fed the diets supplemented with either 250 mg/kg and 2500 mg/kg VE ($p < 0.05$), compared with birds receiving the basal diet. At the intestinal site (Figure 6), group average anti-*S. typhimurium* IgA antibody titres in bile were higher in all VE supplemented groups compared with the basal group, and in the ISS birds fed the 250 mg/kg VESD had a significant increase ($p<0.05$) in anti-*S. typhimurium* IgA antibody titres.





In contrast to experiment 2A, there were a number of significant alterations in peripheral blood T cell subsets, IgA and Ia^+ cells at day 20, that is prior to immunisation, in birds fed the VESD. Birds receiving 2500 and 5000 mg/kg supplementary VE demonstrated a significant increase in percentage of $CD4^+$ (helper) T cells (Figure 7). Further, as seen in Figure 8, birds fed all levels of supplementary VE had a higher (not statistically significant) average percentage of IgA and Ia^+ cells in peripheral blood compared with birds fed the basal diet.





On day 27, six days after the ip primary immunisation, no significant alterations in the percentage of $CD4^+$ or Ia^+ cells were observed (Figure 9 and 10), which is in contrast to experiment 2A. However, birds receiving 250 mg/kg VESD had a significant increase ($p < 0.05$) in the percentage of IgA cells in peripheral blood compared with birds fed the basal diet (Figure 10). No significant alterations were observed with any of the cellular subsets measured on day 38 (data not shown).

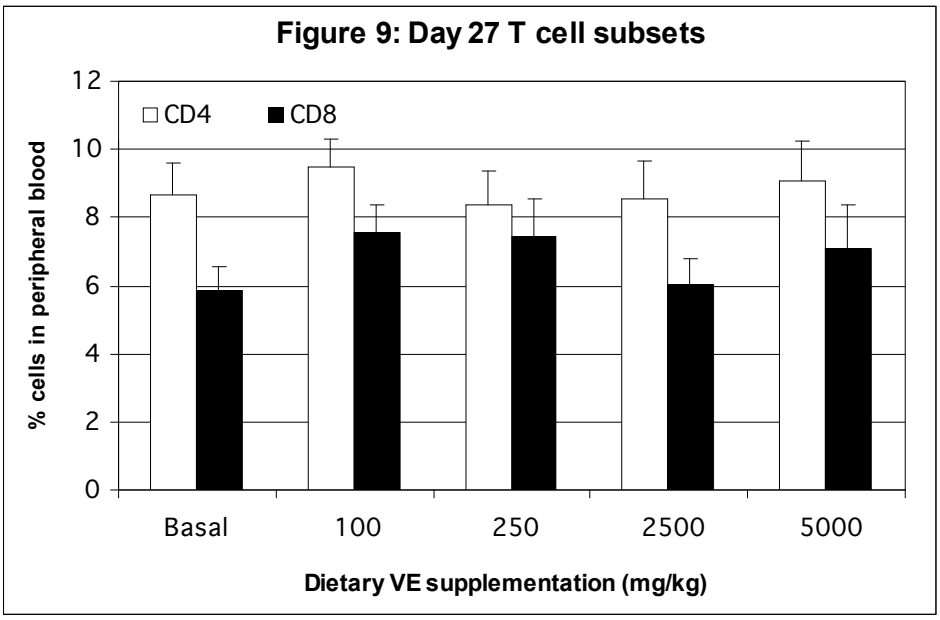


Figure 9: Day 27 T cell subsets
12
10
8
6
4
2
0
CD4
CD8
% cells in peripheral blood
Basal
100
250
2500
5000
Dietary VE supplementation (mg/kg)

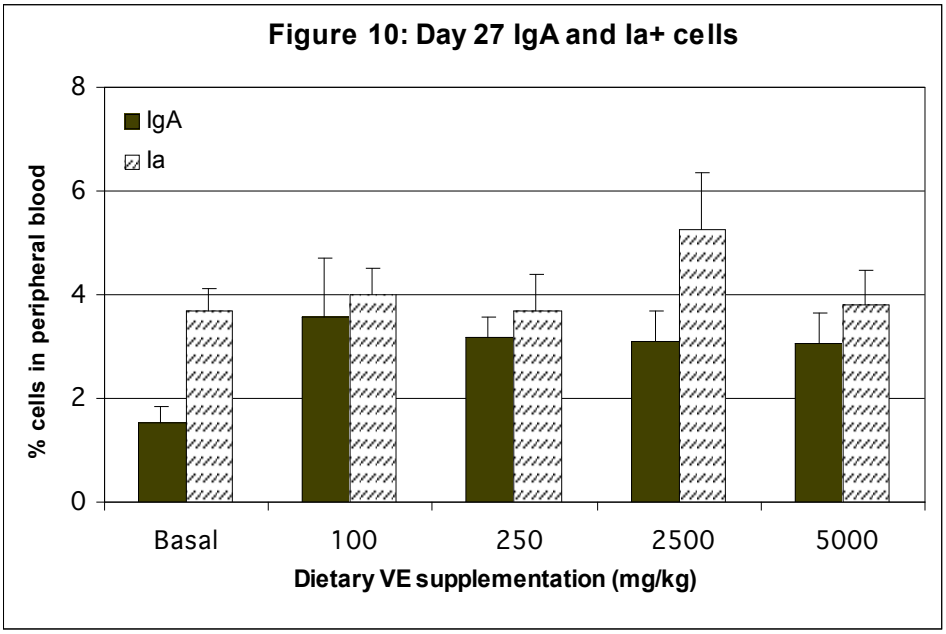


Figure 10: Day 27 IgA and Ia+ cells
8
6
4
2
0
IgA
Ia
% cells in peripheral blood
Basal
100
250
2500
5000
Dietary VE supplementation (mg/kg)

Outcome: Birds fed 250 mg/kg VESD and immunised with killed *S. typ*himurium had a significant increase in sera and intestinal anti-*S. typhimurium* IgA antibody titres at day 42. Further, birds fed 2500 mg/kg VESD also experienced a significant increase in serum derived anti-*S. typhimurium* IgA at day 42. Interestingly, birds fed 5000 mg/kg VESD did not exhibit any statistically significant increases in the immune response to *S. typhimurium*. This is in contrast to experiment 2A, but may be due to the different antigen.

The results of experiment 2A and 2B demonstrate the potential for some levels of dietary supplementation with VE to upregulate IgA antibody based immune responses following an immunisation protocol designed to stimulate a local immune response at the intestinal site. However this may vary depending on the antigen used and the levels of inclusion of VE in the diet. Further, the impact of the VESD on peripheral blood derived cellular subsets was variable between experiments, both pre and post immunisation.

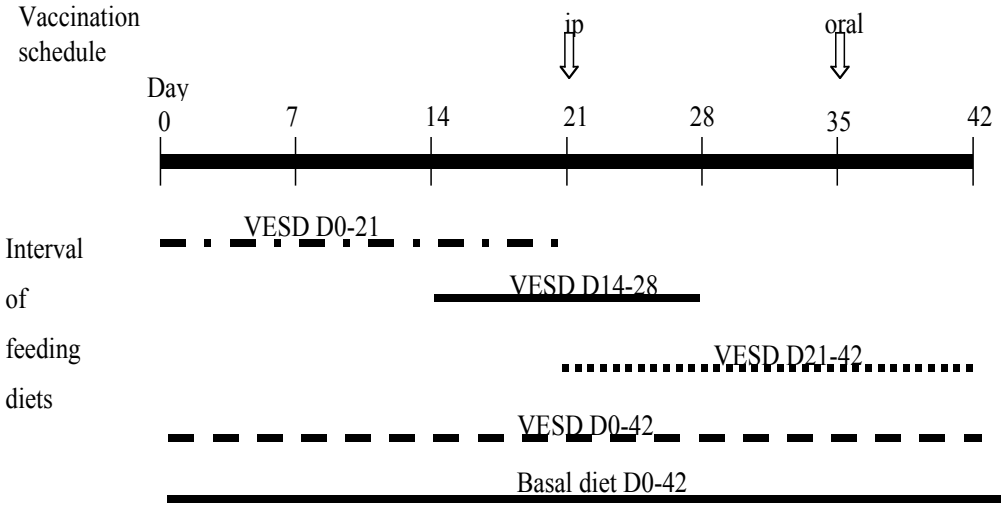
At this point no firm conclusions can be drawn regarding the mechanisms involved in VE induced increases in IgA antibody production observed in these studies, however, it is likely that a range of mechanisms are involved. The antioxidant action of VE is likely to play an important role in VE immunoregulation. In this capacity VE prevents peroxidation of unsaturated lipid materials by free radicals, avoiding damage to the cellular membrane (Moriguchi and Muraga, 2000). These reactions may be non-enzyme or enzyme catalysed, the latter resulting in the biosynthesis of prostaglandin E_2, which acts as an immunosuppressor (Meydani and Beharka, 1996). The high concentration of unsaturated lipid materials in immune cells makes them very susceptible to oxidation (Meydani, 1995) which indicates the potential for antioxidants as VE to maintain cellular homeostasis and function.

Experiment 2C: Investigation into the influence of the interval of feeding a VESD on the immune response.

Aim: Experiments 2A and 2B have illustrated the potential for a VESD to enhance the immune response, in terms of total and anti-antigen IgA antibody titres, when continually fed for the 42 day duration of a broiler growout. However, a few studies have shown VE induced immunoenhancement when VE is fed for shorter intervals (Chung and Boren, 1999), and, in the case of vaccination protocols, when VE supplementation occurs prior to immunisation (Franchini *et al*., 1986; Tengerdy, 1990). Experiment 2C investigated the influence of the interval of feeding a diet supplemented with 250 mg/kg VE on the induction of optimal anti-*S. typhimurium* IgA antibody titres. This rate of VE supplementation was chosen as it had demonstrated the most repeatable immunoregulation in experiments 2A and 2B.

Experimental design: Birds were allocated to treatments groups that received a diet supplemented with 250 mg/kg VE for the following intervals: days 0-21, days 14-28, days 21-42 and days 0-42. When not receiving the VESD the birds were fed the maize-based basal diet. A final group of birds were fed the maize-based basal diet for the duration of the study. The relationship between these intervals of VE supplementation and the immunisation schedule are outlined on the following timeline:

Timeline for bird age, immunisation and VE supplementation

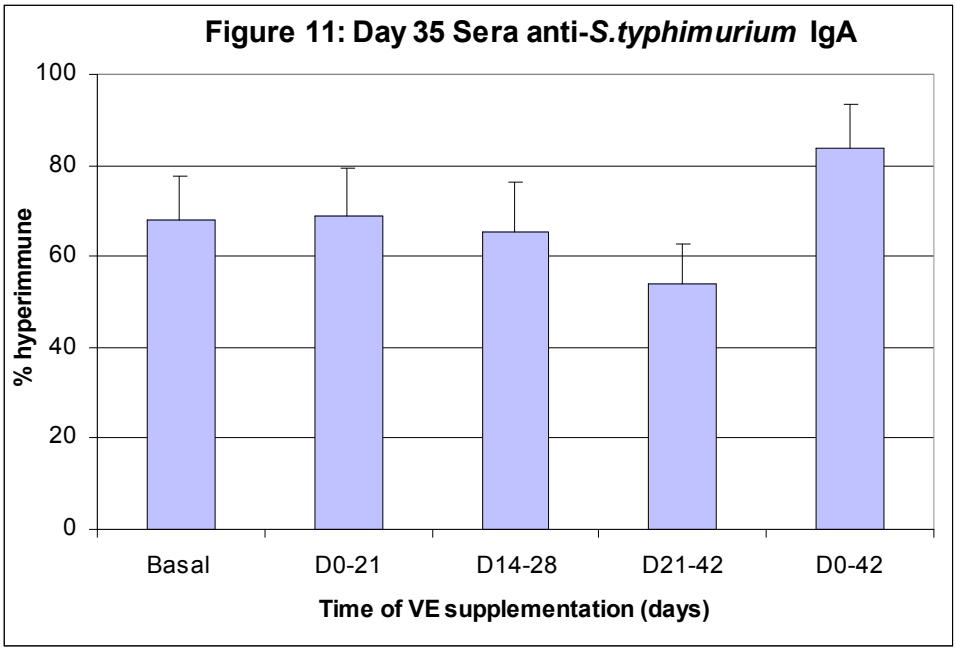


VESD: vitamin E supplemented diets.
D: days

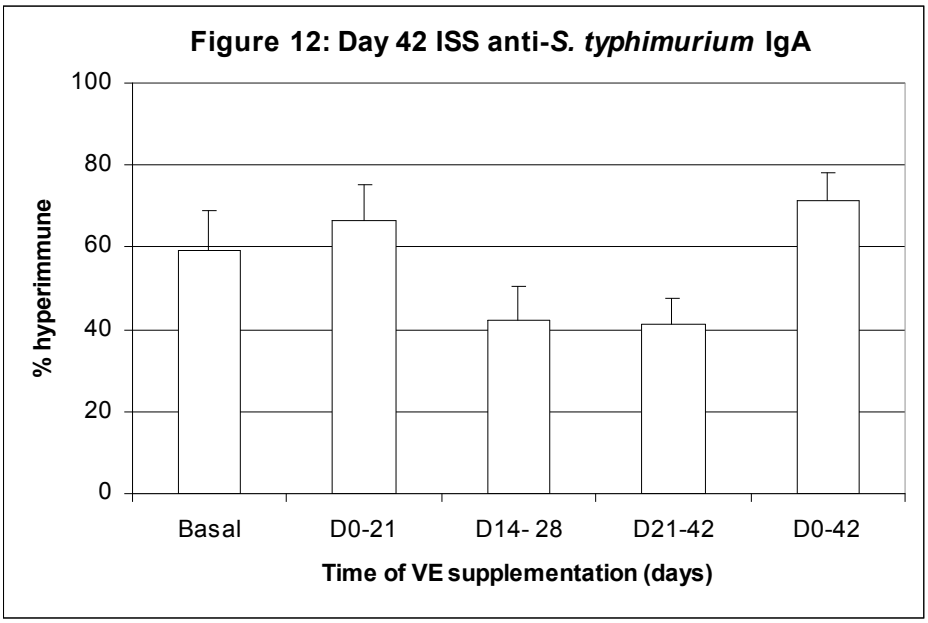
Immunisation protocol: At 21 days of age all birds received an ip immunisation of 10^9 whole killed *S. typhimurium* in a vegetable oil based adjuvant. An oral booster of the same dose of *S. typhimurium* was administered at 35 days of age.

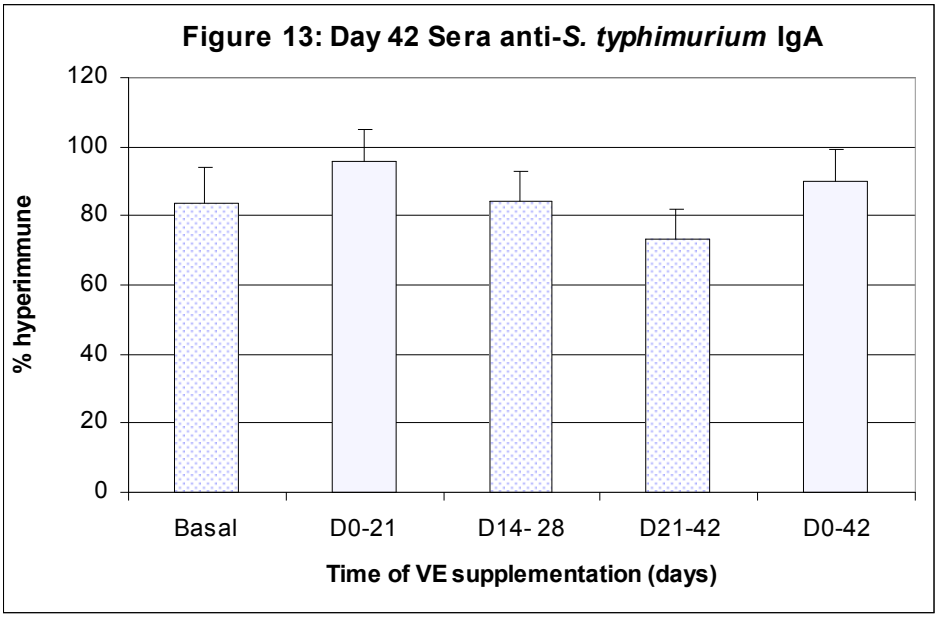
Observations: Average bird weight and feed conversion ratio was determined for the duration of the study. Blood samples were collected on days 35 and 42 pre and post oral booster immunisation, and the serum was retrieved to assess anti-*S. typhimurium* IgA antibody titres. Intestinal scrapings and bile were collected at day 42 for antibody assessment.

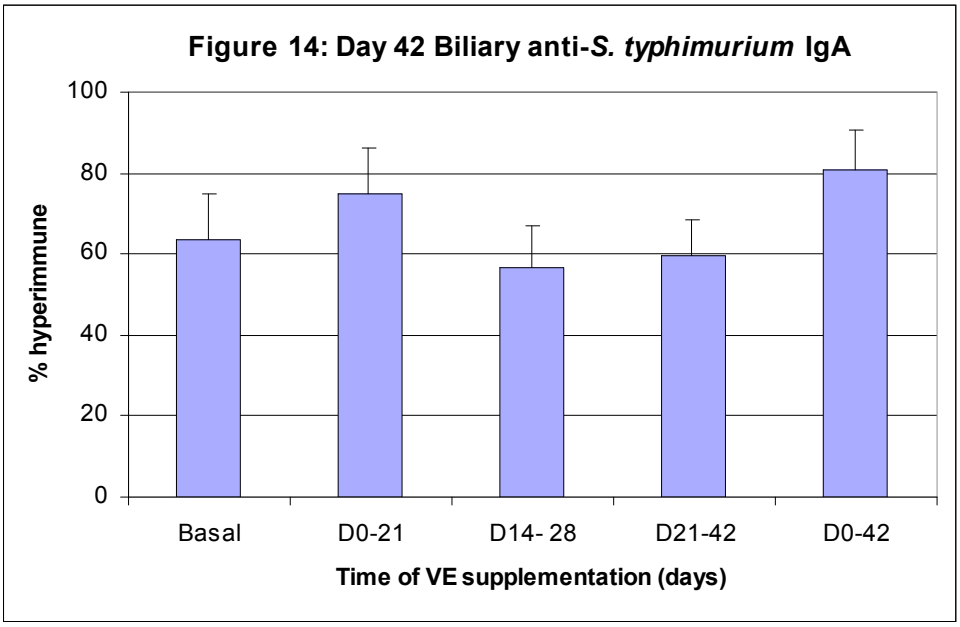
Results: Serum anti-*S. typhimurium* IgA antibody titre on day 35, that is 2 weeks after the primary ip immunisation, was increased (not statistically significant) in birds fed the VESD continuously from day 0 until day 35, that is birds in the treatment group receiving VESD D0-42, compared with birds receiving the basal diet continually from day 0 (Figure 11).

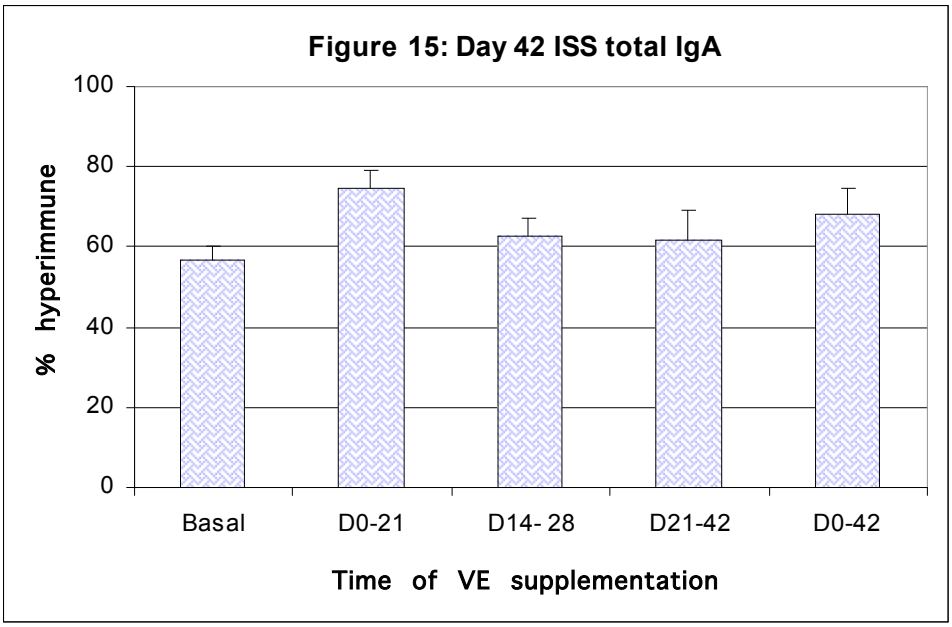


At day 42, seven days after the oral booster, birds receiving VESD from day 0-21 and days 0-42 demonstrated increased (not statistically significant) anti-*S. typhimurium* IgA antibody titres in ISS (Figure 12), serum (Figure 13) and bile (Figure 14). Notably, birds fed VESD from D14-28 and D21-42 had lower (not statistically significant) anti-*S. typhimurium* IgA antibody titres in ISS and bile on day 42. Total IgA antibody at the intestinal site on day 42 was highest (not statistically significant) in birds fed the VESD from days 0-21 and 0-42 (Figure 15).









Outcome: These results show some immunoenhancement of IgA antibody titres in broiler chickens fed a basal diet supplemented with 250 mg/kg VE, either from day old for the 42 days of life or from day old until 21 days of age. Birds fed 250mg/kg VESD for 42 days demonstrated an increase in total IgA antibody and, following vaccination, an increase in anti-*S. typhimurium* antigen IgA antibody titres. Further, comparable upregulation of anti-*S. typhimurium* IgA antibody titres was induced when the VESD was fed from day old for the first three weeks of life (that is the entire period prior to immunisation) or, continuously for six weeks of life. Interestingly, the benefit of feeding the VESD from day old and prior to immunisation, as opposed to only feeding the VESD after immunisation, concurs with the report of Franchini *et al*. (1986). However, when the VESD is fed for a short period before and after primary immunisation, that is D14-28, or for the entire period after but not before the primary immunisation, that is D21-42, the anti-antigen IgA antibody titre was compromised (this was not statistically significant). Franchini *et al.* (1986) also reported the

importance of feeding supplementary VE prior to immunisation to achieve increased antibody titres. Similarly, they observed no influence of VE on the immune response when it was fed from the time of vaccination. Franchini *et al*. (1986) related these observations to the key role of VE in assisting in the development of immune competence, and, in particular, in the development of the bursa of Fabricius (Marsh *et al*., 1986), the primary lymphoid organ involved in humoral immunity. However, a complete understanding of these observations is only likely to be gained when the mechanisms behind VE induced immunoregulation in chickens is itself better understood.

Experiment 3: An investigation into the effect of *in-ovo* delivered VE on IgA antibody production.

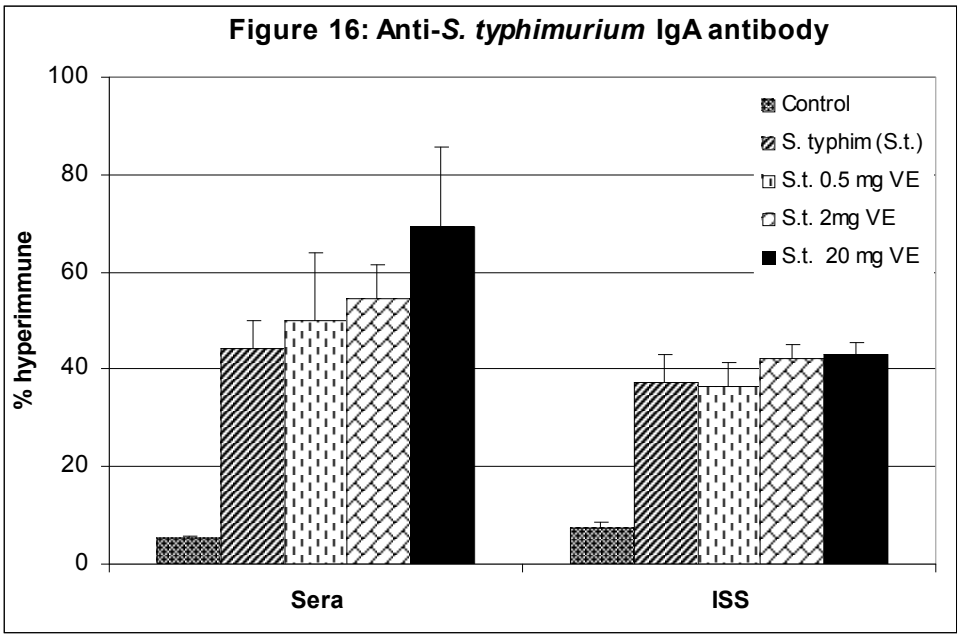
Experiment 3A: Assessment of the impact of *in-ovo* delivery of VE on IgA antibody production.

Aim: Throughout experiment 2 the immunomodulatory impact of some levels of dietary VE supplementation, was identified. As some poultry vaccines are delivered using *in-ovo* technology, an assessment of the potential for the *in-ovo* delivery of VE to alter IgA antibody production in the early posthatch period is required. Therefore, study 3A was designed to assess the impact of *in-ovo* delivered VE on IgA antibody titres in chicks immunised with killed *S. typhimurium*.

Experimental design: Whole killed *S. typhimurium* (10^5/embryo) was delivered with either 0.5, 2 or 20 mg VE into the amniotic cavity at day 18 of embryonation (D18E). Chicks received an oral booster of 10^5 whole killed *S. typhimurium* at 7 days of age. Total and antigen-specific IgA antibody titres in serum and ISS were determined at 14 days of age.

Results: There were no notable differences in total IgA antibody in the serum or ISS (data not shown). The anti-*S. typhimurium* IgA antibody titres in serum and ISS at 14 days of age are presented in Figure 16. Generally *in-ovo* delivery of 20 mg VE increased (not statistically significant) anti-*S. typhimurium* IgA antibody titres in the serum at 2 weeks of age, compared with embryos that received *S. typhimurium* in the absence of VE at D18E.

Figure 16: Anti-*S. typhimurium* IgA antibody

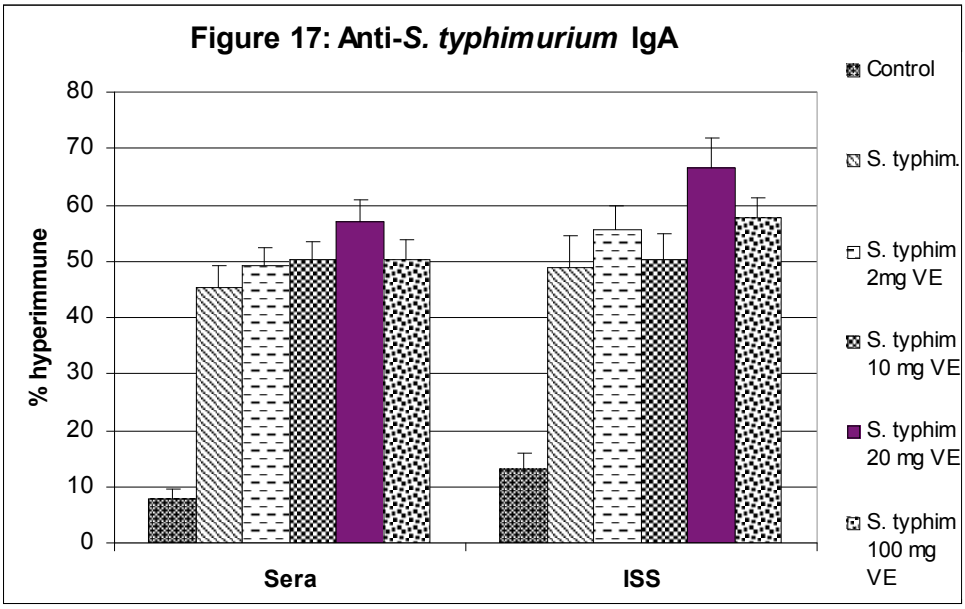


Experiment 3B: Evaluation of the influence of VE on IgA antibody production following its concurrent delivery with *S. typhimurium* both *in-ovo* and posthatch.

Aim: Experiment 3A indicated that *in-ovo* administration of VE may have some immunoregulatory effect on IgA antibody production during the early posthatch period. Experiment 3B was undertaken to evaluate the influence of co-delivery of VE with antigen, both *in-ovo* and at 7 days of age, on IgA antibody titres in young chicks.

Experimental design: Vitamin E (2, 10, 20 and 100 mg/embryo) and *S. typhimurium* (10^5/embryo) were delivered together at D18E and again at 7 days of age. Samples of serum and ISS were collected at 14 days of age and analysed for anti-*S. typhimurium* IgA antibody.

Results: Day 14 sera and ISS anti-*S. typhimurium* IgA antibody titres are presented in Figure 17. *In-ovo* and postnatal delivery of 20 mg VE in conjunction with the antigen, *S. typhimurium,* stimulated notable, but not statistically significant, increases in anti-*S. typhimurium* IgA antibody in serum and ISS compared to chicks immunised with *S. typhimurium* alone.



Outcome: The results of experiments 3A and 3B demonstrate a limited ability of VE (20 mg/administration) to increase (not statistically significant) anti-*S. typhimurium* IgA antibody production in the early posthatch period following the delivery of both VE and *S. typhimurium in-ovo* and at 7 days of age.

Section B: Assessment of the impact of cytokines on IgA antibody production in chickens.
All studies undertaken in section B evaluated the effect of *in-ovo* and postnatal cytokine delivery on IgA antibody production in chickens.

Cytokine delivery:
Collaboration with Dr J. Lowenthal and Dr D. Strom at CSIRO Livestock Industries, Geelong, facilitated the use of recombinant cytokine proteins of chicken interferon-gamma (ChIFN-γ), porcine interleukin-6 (pIL-6) and porcine interleukin-3 (pIL-3), in this project.

Experiment 4*: In-ovo* delivery of ChIFN-γ.

Aim: A number of studies have shown administration of ChIFN-γ to improve weight gain and resistance to disease challenge (Lowenthal *et al*., 1998). This study evaluated the effect of *in-ovo* delivery of ChIFN-γ on IgA antibody titres in chickens.

Experimental design: ChIFN-γ was delivered *in-ovo* at four rates; 1, 5, 25, and 125 μg/embryo. The influence of ChIFN-γ on the immune system was determined at day 7 and 21 posthatch by assessment of total IgA antibody titres in serum and enumeration of IgA secreting plasma cells in the intestinal duodenum.

Outcome: The *in-ovo* delivery of ChIFN-γ did not have any significant effect on total IgA antibody titre nor on the number of duodenal IgA secreting plasma cells during the first 3 weeks of life (data not shown).

Experiment 5: Delivery of pIL-6 posthatch

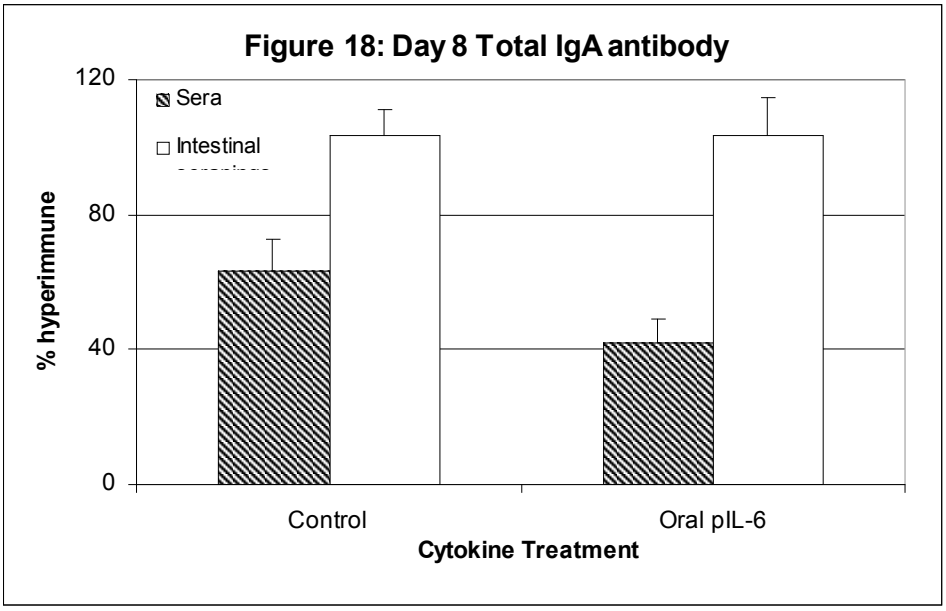
Aim: Experiment 5 was designed to establish the conditions required for IL-6 to augment IgA antibody production in chicks posthatch. The aims of this experiment were twofold and focus on determining whether:

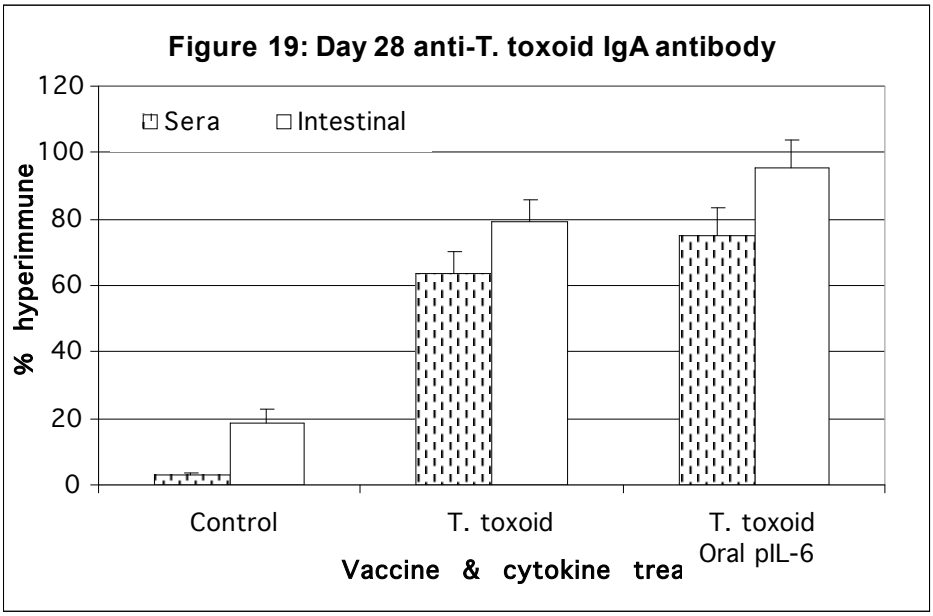
1) The oral delivery of pIL-6 alone will affect total IgA antibody titres in young chicks, and,
2) The delivery of pIL-6 prior to both the primary and secondary immunisation will alter the antigen-specific intestinal immune response.

Experimental design: Chickens received 15μg pIL-6 orally, via gavage, on days 3 and 6 of age. On day 8 half the chicks were euthanased from which samples of blood and ISS were collected for assessment of total IgA antibody titres. The number of IgA secreting plasma cells in the duodenum and spleen were also determined.

On day 8 all remaining birds (control and pIL-6 treated) received an ip immunisation of tetanus toxoid in a vegetable oil based adjuvant, with an oral booster at day 21. At day 21 and 28 anti-tetanus toxoid IgA antibody in serum and ISS was determined.

Results: Day 8 total IgA antibody titres of chicks administered pIL-6 on days 3 and 6 were no different from the control birds (Figure 18). However, the oral delivery of pIL-6 prior to ip immunisation with T. toxoid increased the anti-T. toxoid IgA antibody titre at the intestinal surface ($p = 0.07$), and in the serum (Figure 19) at 28 days of age, compared with birds immunised with T. toxoid in the absence of pIL-6.





Outcome: This experiment indicated an inability of pIL-6 to upregulate IgA antibody production in chicks, in the absence of an antigen challenge. Further, the increased titres of anti-T. toxoid IgA antibody production in ISS following immunisation suggest a link between the timing of pIL-6 delivery and the timing of the immunisation or antigen challenge to achieve IL-6 induced immune enhancement. Experiment 6 investigated this hypothesis by evaluating the influence of delivering pIL-6 on IgA antibody production to immunologically primed chickens.

Experiment 6: Oral delivery of pIL-6 to immunologically primed chickens.

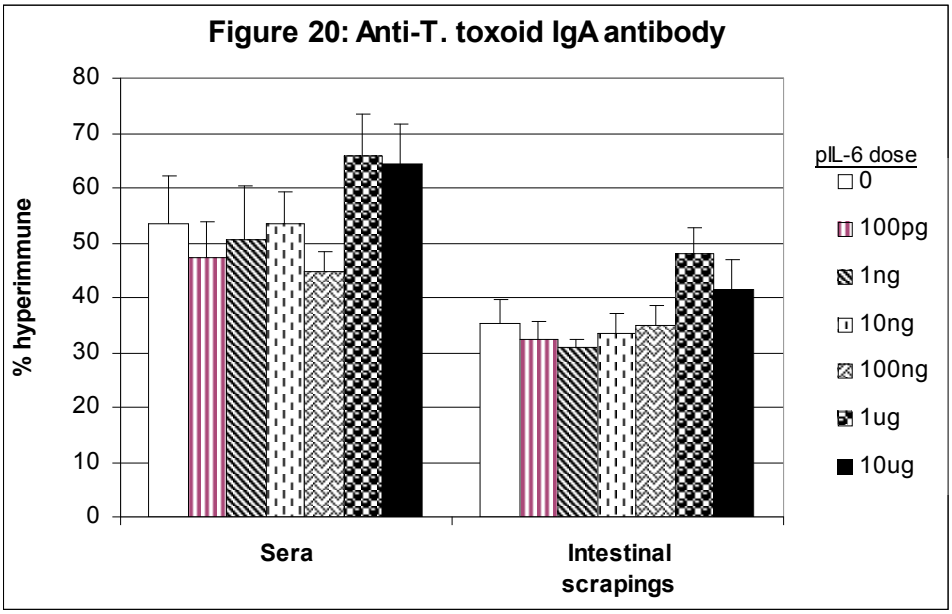
Experiment 6A: pIL-6 dose titration study in immunologically primed chickens.

Aim: The results of experiment 5 indicated that for effective upregulation of the intestinal immune response the timing of pIL-6 oral administration may need to closely coincide with antigen challenge.

This hypothesis is supported by reports from laboratory animals where IL-6 has been most effective in enhancing IgA antibody titres when delivered to immunologically primed animals after the oral booster immunisation (Husband *et al*., 1996). Therefore, in experiment 6A pIL-6 was delivered on a number of occasions immediately after the secondary booster vaccination. Titrated rates of IL-6 were delivered in an attempt to identify an optimal dose of pIL-6 for enhanced IgA antibody production.

Experimental design: At day 14 of age chickens were primed by ip immunisation with T. toxoid in a vegetable oil based adjuvant. Two weeks later the birds received an oral booster immunisation of T. toxoid in PBS. On each of the subsequent four days birds received pIL-6 orally via gavage, 20 minutes after a 0.5 ml oral dose of 0.1% sodium bicarbonate (for neutralisation of gut content). On the first day of pIL-6 delivery chicks were randomly assigned to one of seven groups which received pIL-6 at one of the following rates: 0, 100ρg, 1, 10 and 100ηg and, 1 and 10 μg. Seven days after the oral booster samples of serum and intestinal scrapings were collected for analysis of anti-T. toxoid IgA antibody titres.

Results: The repeated oral delivery of 1μg and 10μg pIL-6 following an oral booster immunisation of T. toxoid invoked an increase in the anti-T. toxoid IgA antibody titre in serum (not statistically significant) and in intestinal scrapings (Figure 20). This was particularly evident in the intestinal scrapings following the repeated delivery of 1μg pIL-6 ($p = 0.06$).



Outcomes: Taken together, experiments 5 and 6A highlight an important factor regarding the immunoregulatory role of pIL-6 in chickens. For immunoenhancement pIL-6 must be delivered in association with antigen, presumably enabling it to influence the processes involved in the generation of antigen-specific IgA antibody producing-cells and, their subsequent production of IgA antibody. Oral delivery of pIL-6 in association with an immunisation schedule designed to specifically induce IgA antibody production at the intestinal site demonstrated the most promise in increasing the local anti-antigen IgA antibody titre.

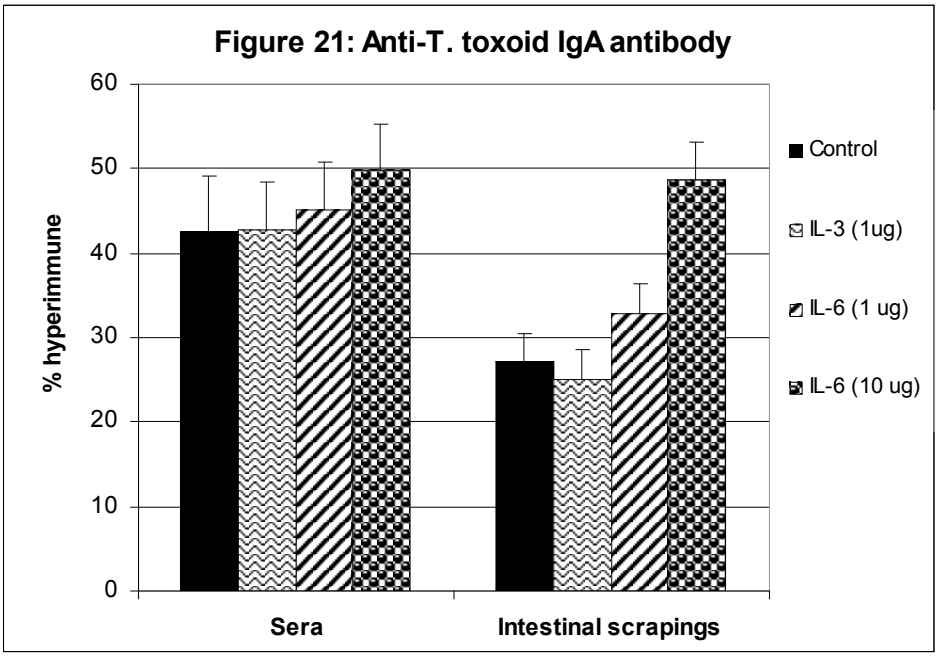
Experiment 6B: Comparison of the impact of pIL-6 and pIL-3 on IgA antibody.

Aim: In the previous experiment (6A) oral delivery of either 1 or 10 μg pIL-6 on each of the four days following an oral booster immunisation to chickens previously primed with T. toxoid, induced notable increases in anti-T. toxoid IgA antibody titres in ISS and serum. That experiment was repeated in a modified form which entailed the inclusion of cytokine control interleukin-3 (IL-3), to confirm that the antibody upregulation was induced by pIL-6. Unlike pIL-6, pIL-3 does not

demonstrate cross species reactivity and should not enhance local IgA antibody production. Further, pIL-3 and pIL-6 are produced using similar procedures and therefore delivery of pIL-3 is required to confirm that the upregulation seen in IgA antibody production observed in birds receiving pIL-6 is due to the activity of pIL-6 and not another substance inadvertently generated during cytokine production.

Experimental design: At 14 days of age chickens were primed by ip immunisation of T. toxoid. Two weeks later the birds received an oral booster immunisation of T. toxoid. On each of the subsequent four days birds received cytokine orally via gavage, 20 minutes after a 0.5 ml oral dose of 0.1% sodium bicarbonate. IL-6 was delivered at 1 and 10 μg and pIL-3 at 1μg per bird per day. Seven days after the oral booster samples of serum and ISS were collected for analysis of anti-T. toxoid IgA antibody titres.

Results: Birds receiving 10 μg pIL-6 demonstrate a statistically significant increase (p=0.002) in intestinal anti-T. toxoid IgA antibody titres (Figure 21). Anti-T. toxoid IgA antibody titres in birds receiving pIL-3 were not statistically different from the control birds. This suggests that IgA upregulation was due to the activity of pIL-6. However, as the more efficacious dose of pIL-6 in this case was 10 μg a group treated with 10μg pIL-3 was included as the cytokine control in experiment 6C.



Outcome: Four consecutive daily oral administrations of 10 μg pIL-6 in the four days immediately after the secondary oral booster significantly increased anti-antigen specific IgA antibody titres at the intestinal site. This is in contrast to the outcomes of experiment 6A, where the repeated oral delivery of 1μg IL-6 induced the highest anti-T. toxoid IgA antibody titres in both serum and intestinal scrapings. Therefore, a further study was undertaken to determine whether similar upregulation of IgA antibody to, in this case, a bacterial antigen, *S. typhimurium*, could be achieved following repeated oral delivery of either 1 or 10 μg pIL-6.

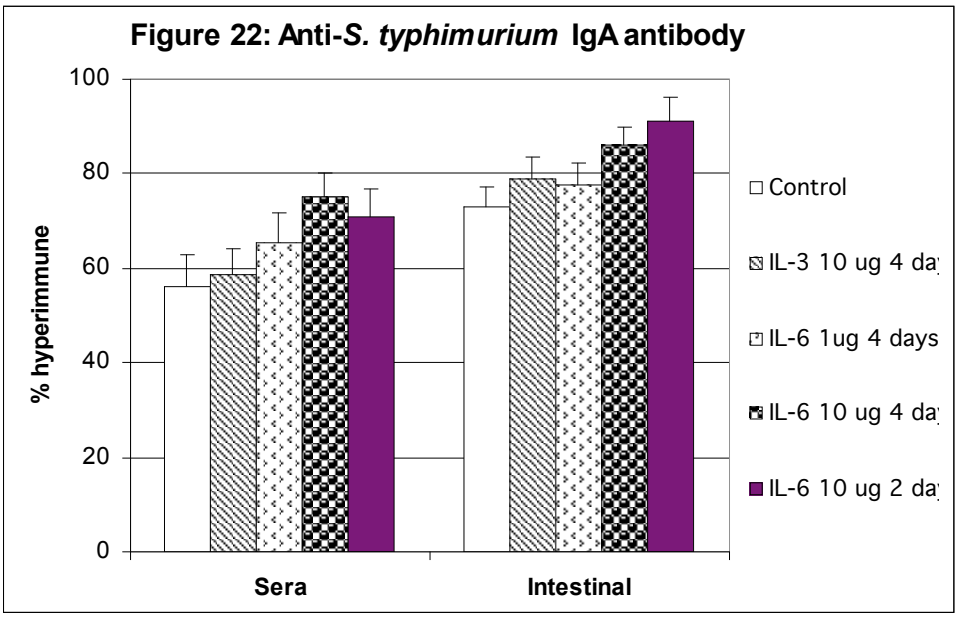
Experiment 6C: Oral delivery of pIL-6 to birds previously immunised with *S. typhimurium*.

Aim: The results from experiments 6A and 6B demonstrated the potential for repeated oral delivery of pIL-6 to enhance antigen-specific IgA antibody production in chickens immunised with T. toxoid.

However, the dose of IL-6 required to achieve this varied. Therefore in experiment 6C the potential for pIL-6 to enhance the immune response to another immunogen, in this case, whole killed *S. typhimurium,* was evaluated. The total dose and timing of pIL-6 delivery was also assessed by the inclusion of one treatment group which received 10 μg pIL-6 on each of 2 days only, for comparison with birds receiving 10 μg pIL-6 on each of 4 days. Further, IL-3, the cytokine negative control was administered at 10 μg on each of 4 days, the highest rate at which IL-6 enhanced anti-T. toxoid IgA antibody at the intestinal surface (experiment 6B).

Experimental design: At 14 days of age chickens were primed by ip immunisation of 10^9 whole killed *S. typhimurium* in a vegetable oil based adjuvant. Two weeks later the birds received an oral booster immunisation of 10^9 whole killed *S. typhimurium* in PBS. On each of the subsequent four days birds received cytokine orally via gavage, 20 minutes after a 0.5 ml oral dose of 0.1% sodium bicarbonate. PIL-6 was delivered at 1 and 10 μg and pIL-3 at 10 μg per bird per day. Another group of birds received 10 μg pIL-6 orally on two occasions only, the second and fourth days following the oral booster immunisation. Seven days after the oral booster samples of serum and ISS were collected for analysis of anti-*S. typhimurium* IgA antibody titres.

Results: As in experiment 6B repeated oral delivery of 1 μg IL-6 and, in this case 10 μg IL-3, did not induce any statistically significant changes to the IgA antibody titres compared with the control birds. Repeat oral delivery of 10 μg pIL-6 on each of the 4 days immediately following the oral booster immunisation increased anti-*S. typhimurium* IgA antibody titres in both serum and ISS ($p < 0.05$) (Figure 22). Interestingly birds receiving only two administrations of 10 μg pIL-6 had a significant increase in anti-*S. typhimurium* IgA antibody at the intestinal site ($p < 0.05$), compared with the control birds, which was higher than the average antibody titre observed in birds which received four consecutive doses of 10 μg pIL-6.



A total of 40 μg and 20 μg IL-6 both significantly increased anti-*S. typhimurium* IgA antibody at the intestinal surface. This indicates potential influence of and interaction between both the quantity and timing of IL-6 delivery on its immunoregulatory influence. Further research is required to fully understand these interactions.

Outcome: This study has shown the immunoregulatory effect of repeated oral delivery of 10 μg IL-6 on IgA-antibody production, particularly at the intestinal mucosa, following local immunisation with *S. typhimurium*. Subsequent studies were undertaken to evaluate the benefit of pIL-6-induced

IgA antibody production at the intestinal site utilising this vaccination schedule, in protecting birds from a live challenge of *S. typhimurium*.

Experiment 7: Vaccination/challenge studies with *S. typhimurium*.

Experiment 7A: Establishing a challenge model with live *S. typhimurium*.

Aim: To establish a *S. typhimurium* challenge model that represents the trickle challenge normally experienced by broiler birds housed on litter. A seeder bird based model, as described by Muir *et al.* (1998a) was utilised. An initial study was completed to determine the optimum number of *S. typhimurium* seeder birds required to infect co-habiting challenge birds.

Experimental design: Briefly, at 30 days of age seeder birds were infected with 10^9 live *S. typhimurium* and placed on fresh litter in floorpens in groups of either 5, 10 or 15. Five days later fifteen challenged birds (35 days old) were also placed in each floorpen. The seeder and challenged birds were held in the floorpen together for seven days, at which time all seeder birds were removed. The challenged birds remained on the *S. typhimurium* infected litter for a further seven days.

On days seven and fourteen postchallenge, half of the challenged birds were euthanased and a sample of spleen and liver was collected for enumeration of *S. typhimurium*. A cloacal swab was collected from each bird, selectively enriched in TETR broth before being plated onto XLD media for identification of *S. typhimurium*. A similar process of enrichment was undertaken for samples of spleen and liver, enabling isolation of *S. typhimurium*. At these times samples of blood, ISS and bile were collected from all euthanased birds for determination of anti-*S. typhimurium* IgA antibody titres by ELISA.

Results: Isolation of *S. typhimurium* from challenged birds co-habiting with either 15, 10 or 5 seeder birds days seven and fourteen after challenge are presented in Tables 1 and 2 respectively. By seven days postchallenge the majority of challenged birds cohabiting with 10 or 15 seeder birds were infected with *S. typhimurium*. The use of 5 seeder birds did not ensure infection of the majority of challenged birds (Table 1).

Table 1: Isolation of *Salmonella typhimurium* seven days postchallenge:

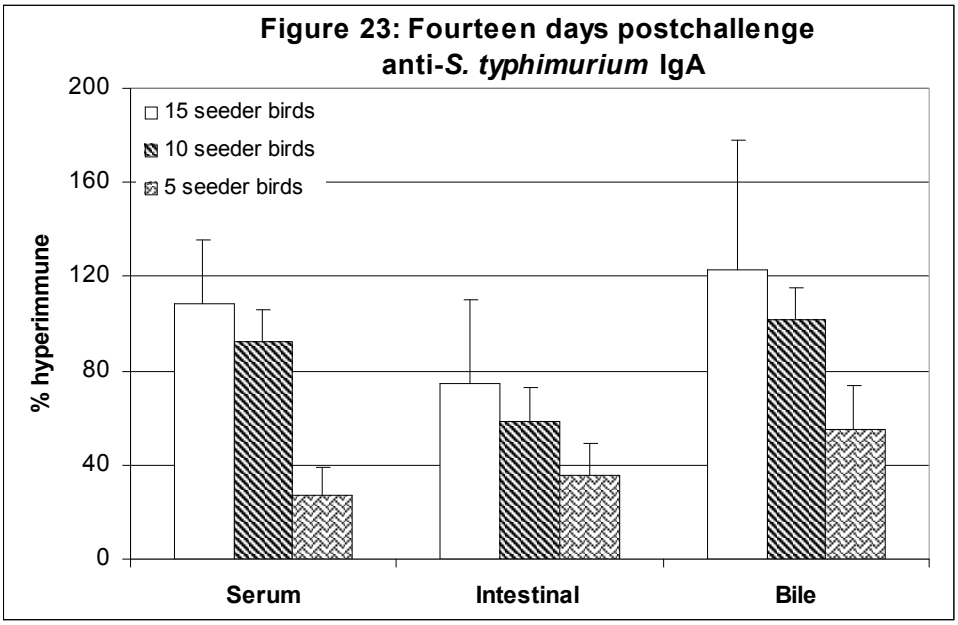
Challenge Regime	**Isolation of *Salmonella typhimurium* from**				
	Cloacal swabs	**Spleen**		**Liver**	
(n=7 in all groups).	% pos.	% pos.	cfu/g	% pos.	cfu/g
15 seeders	**86**	**86**	**489 +/- 220**	**86**	**137 +/- 99**
10 seeders	**71**	**86**	**1296 +/- 452**	**100**	**71 +/- 33**
5	**29**	**33**	**1311 +/- 1298**	**29**	**27 +/- 21**

At fourteen days postchallenge, the highest frequency and levels of *S. typhimurium* infection in challenged birds followed cohabitation with 15 seeder birds (Table 2).

Table 2: Isolation of *Salmonella typhimurium* fourteen days postchallenge:

Challenge Regime	**Isolation of *Salmonella typhimurium* from**				
	Cloacal swabs	**Spleen**		**Liver**	
(n=8 in all groups).	% pos.	% pos.	cfu/g	% pos.	cfu/g
15 seeders	**88**	**88**	**497 +/- 270**	**38**	**49 +/- 43**

The anti-*S. typhimurium* IgA antibody titres in serum, ISS and bile of the challenged birds at 14 days after challenge, are shown in Figure 23. Overall, cohabitation of challenged birds with 15 seeder birds stimulated higher average antibody titres, which correlates with the higher percentage of *S. typhimurium* infected birds in this group.



Outcome: The variation in the number of *S. typhimurium* isolated from the spleen and liver in the challenged birds is not unexpected as there is limited control over the dose of *S. typhimurium* received by each bird in this model. However, as compared with a challenge delivered by a single bulk, oral administration of live *S. typhimurium*, this model represents the natural process of infection, including the physiological processes involved in immunity and, the stimulation of protective mechanisms, at the intestinal surface.

In experiment 7B all groups of challenge birds were infected with *S. typhimurium* by cohabitation with 15 *S. typhimurium* infected seeder birds.

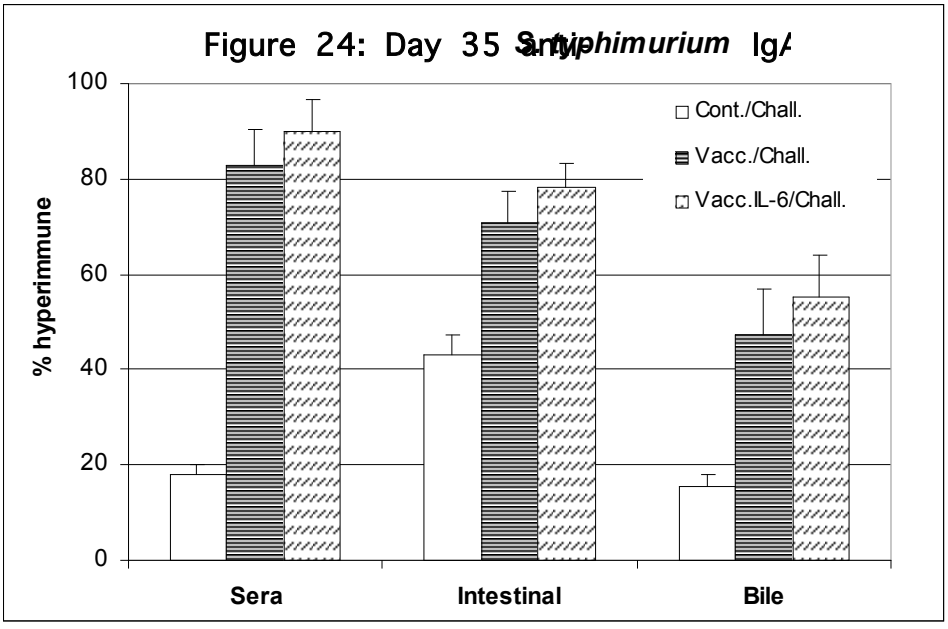
Experiment 7B: Assessment of the resistance of chickens immunised with *S. typhimurium* with a concurrent oral administration of pIL-6, to a live challenge of *S. typhimurium*

Aim: The results observed throughout experiments 6 illustrate the immunoregulatory role of pIL-6 in stimulating significant increases in post-vaccination IgA antibody titres at the intestinal site. Experiment 7B was designed to assess the protection from a challenge of live *S. typhimurium* in

chickens immunised with *S. typhimurium* along with the oral delivery of pIL-6 immediately after the secondary oral booster immunisation with *S. typhimurium*.

Experimental design: Chickens were randomly divided into three groups of 30 birds each. At 14 days of age two groups of chickens were primed by ip immunisation of 10^9 whole killed *S. typhimurium* in a vegetable oil based adjuvant. Two weeks later the birds received an oral booster immunisation of 10^9 whole killed *S. typhimurium* in PBS. On each of the subsequent four days one group of chickens received 10 μg pIL-6 orally via gavage, 20 minutes after a 0.5 ml oral dose of 0.1% sodium bicarbonate.
At 35 days of age half the birds in each treatment group were euthanased and samples of blood, intestinal scrapings and bile were collected for determination of anti-*S. typhimurium* IgA. The remaining birds, 15 from each treatment group, were placed in *S. typhimurium* infected floorpens, and cohabited with 15 *S. typhimurium* infected seeder birds for the following week. At 42 days of age all seeder birds were removed from the floorpens.
At seven and fourteen days postchallenge blood was collected from all challenged birds for antibody analysis. On each of these days, 7 and 8 birds respectively, were euthanased from each challenged group. Samples of ISS and bile were collected for antibody determination, and, the number of *S. typhimurium* in the spleen and liver were enumerated. Cloacal swabs were collected from all challenged birds to identify the presence of *S. typhimurium*.

Results: Serum, ISS and bile anti-*S. typhimurium* IgA antibody titres on day 35, seven days after the oral booster and just prior to being placed in the infected floorpens, were higher (not statistically significant) in Vaccinated.IL-6 treated birds as opposed to the birds which received the vaccination only (Figure 24). Unlike the response seen in experiments 6B and 6C, repeated oral delivery of 10 μg IL-6 did not induce a significant increase in anti-antigen IgA antibody titres in the intestinal scrapings. This is difficult to explain, but concurs with experiment 6A, where 10 μg IL-6 induced an increase in anti-T. toxoid IgA at the intestinal surface, which was not statistically significant, when compared with birds vaccinated in the absence of IL-6.



The percentage of birds with *S. typhimurium* positive cloacal swabs, spleen and liver samples seven days after being placed in the infected floorpens with seeder birds, are presented in Table 3. The number of *S. typhimurium* per gram of spleen and liver are also presented. The number of Vaccinated.IL-6/Challenged birds infected with *S. typhimurium* was similar to the Vaccinated/Challenged birds, however, there were fewer *S. typhimurium* per gram of spleen and liver in the former group. Birds immunised with *S. typhimurium* in conjunction with pIL-6 had

higher (not statistically significant) average anti-*S. typhimurium* IgA antibody titres at day 35 (Figure 24), which, by the process of immune exclusion may have reduced the incidence of *S. typhimurium* gaining access to internal organs such as the spleen and liver.

At seven days after challenge the Control/Challenged birds were more heavily infected with *S. typhimurium* in the spleen and liver, though the variation between birds was large, than the Vaccinated/Challenged and Vaccinated.IL-6/Challenged birds. The higher number of *S. typhimurium* in the control birds reflects their immunological naivity and therefore, their reduced resistance to *S. typhimurium.* This is not unexpected as they had not been previously exposed to or immunised with *S. typhimurium.* This is also evident in their low anti-*S. typhimurium* IgA antibody titres at day 35 (Figure 24). In contrast, the Vaccinated/Challenged and Vaccinated.IL-6/Challenged birds had notable anti-*S. typhimurium* IgA antibody titres at day 35 in ISS, bile and serum, which provided some defense against invading S. *typhimurium*.

Table 3: Isolation of *Salmonella typhimurium* seven days postchallenge:

Treatment Regime	**Isolation of *Salmonella typhimurium* from**				
	Cloacal swabs	**Spleen**		**Liver**	
(n=8 in all groups).	% pos.	% pos.	cfu/g	% pos.	cfu/g
Cont./Chall.	**100**	**88**	**3367+/- 2002**	**100**	**295 +/- 150**
Vacc./Chall.	**100**	**100**	**1091+/- 287**	**100**	**175 +/- 36**
Vacc.IL-6./Chall.	**100**	**100**	**833+/- 264**	**71**	**149 +/- 38**

Cont./Chall.: unimmunised control, *S. typhimurium* challenged treatment.
Vacc./Chall: *S. typhimurium* immunised, *S. typhimurium* challenged treatment.

The anti-*S. typhimurium* IgA antibody titres at seven days postchallenge are presented in Figure 25. Mean IgA antibody titres in the intestine and bile at this time were highest (not statistically significant) in the Vaccinated.IL-6/Challenged group, however the variation within the group was large. Further, the average sera anti-*S. typhimurium* IgA antibody titres in this group of birds was similar to the control birds and lower than the Vaccinated/Challenged birds. With no statistically significant differences in antibody titres between all three treatment groups at seven days after challenge it is interesting that the Vaccinated.IL-6/Challenged birds demonstrated the lowest numbers of *S. typhimurium* in the spleen and liver (Table 3).

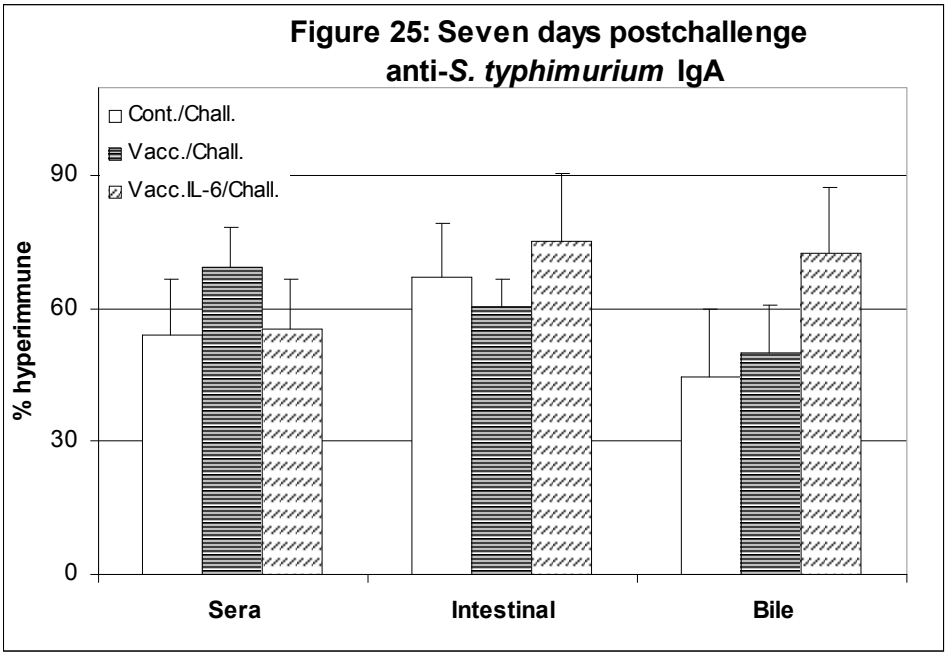


Figure 25: Seven days postchallenge
anti-S. typhimurium IgA
Cont./Chall.
Vacc./Chall.
Vacc.IL-6/Chall.
% hyperimmune
90
60
30
0
Sera
Intestinal
Bile

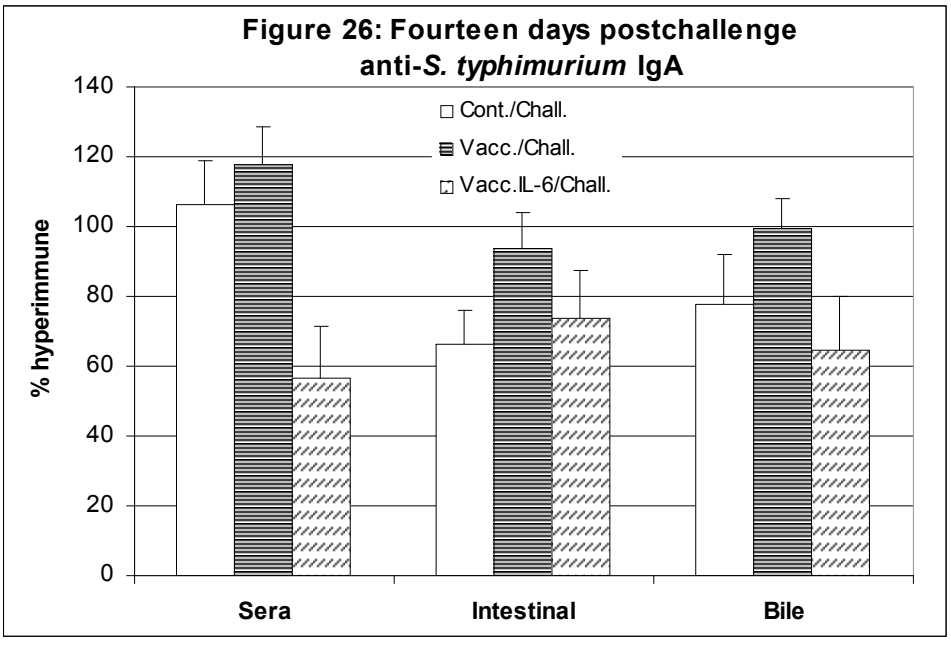
S. typhimurium infection levels fourteen days after challenge are shown in Table 4. At this time the improved protection against *S. typhimurium* provided to the chickens immunised in the presence of IL-6 is more apparent. These birds had a reduced percentage of *S. typhimurium* positive cloacal swabs, spleen and liver samples, in addition to reduced numbers of *S. typhimurium* per gram of liver and spleen, compared to the Control/Challenged and Vaccinated/Challenged birds.

Table 4: Isolation of *Salmonella typhimurium* fourteen days postchallenge:

Treatment Regime	**Isolation of *Salmonella typhimurium* from**				
	Cloacal swabs	**Spleen**		**Liver**	
(n=8 in all groups).	% pos.	% pos.	cfu/g	% pos.	cfu/g
Cont./Chall.	**72**	**72**	**166 +/- 116**	**57**	**17 +/- 9**
Vacc./Chall.	**100**	**86**	**801 +/- 444**	**43**	**13 +/- 9**
Vacc.IL-6./Chall.	**43**	**43**	**117 +/- 103**	**29**	**5 +/- 3**

Cont./Chall.: unimmunised control, *S. typhimurium* challenged treatment.
Vacc./Chall: *S. typhimurium* immunised, *S. typhimurium* challenged treatment.
Vacc.IL-6/Chall: *S. typhimurium* immunised with IL-6 administration, *S. typhimurium* challenged treatment

At fourteen days postchallenge, the Vaccinated/Challenged birds had the highest levels of *S. typhimurium* infection, which is likely to have stimulated the highest mean (not statistically significant) anti-*S. typhimurium* IgA antibody titres in the serum, intestine and bile (Figure 26).



As the Vaccinated/Challenged birds had been vaccinated with *S. typhimurium*, the high levels of *S. typhimurium* infection observed in these birds at fourteen days after challenge was unexpected. This is particularly so when compared to the Control/Challenged birds which had not been previously exposed to the *S. typhimurium* antigen. The Control/Challenged birds had the lowest levels of anti-*S. typhimurium* antibody titres at challenge (Figure 24) but had lower levels of infection than the

Vaccinated/Challenged birds fourteen days after challenge. Previous work has demonstrated that birds vaccinated and then challenged with *S. typhimurium* have reduced levels of *S. typhimurium* infection compared to unvaccinated/challenged birds at both seven and fourteen days after challenge (Muir *et al*., 1998b). The high level of *S. typhimurium* infection in the Vaccinated/Challenged birds in this study was also unexpected as their antibody titres were not statistically different from the Vaccinated.IL-6/Challenged birds at challenge, yet their levels of *S. typhimurium* infection at fourteen days after challenge were markedly different (though not statistically significant).

The Vaccinated.IL-6/Challenged birds demonstrated the highest anti-*S. typhimurium* IgA antibody titres in the sera, intestine and bile at challenge (Figure 24) and, in the intestine and bile at 7 days after challenge (Figure 25) with lower levels of *S. typhimurium* infection. However, their antibody titres were not statistically significant compared with the Vaccinated/Challenged or the Control/Challenged birds, making their lower levels of infection a little surprising. It may be that the IL-6 treatment continued to stimulate improved protection, possibly through improved antibody production during the challenge period, similar to the outcome of experiment 5, where increased anti-antigen titres were observed at the intestinal site when the antigen challenge occurred soon after IL-6 delivery. In this regard, the antibody data at day 7 postchallenge (Figure 25) illustrated higher average, though not statistically significant, anti-*S. typhimurium* IgA antibody titres in the bile and ISS of the birds immunised in association with IL-6. However, a more marked increase may be expected and, in the absence of more frequent measures of antibody titres, the detail of the changes in antibody production throughout the 14 day period following challenge is unclear. Further, IL-6 may have mediated improved protection through another mechanism. The inconsistencies observed between previous exposure to the antigen, antibody titres prior to challenge and the levels of infection postchallenge in this study raise questions about the mechanisms of improved protection in the Vaccinated.IL-6/Challenged birds.

Also requiring further clarification is the apparent reduction in the IgA antibody titres of the Vaccinated.IL-6/Challenged birds at day 14 postchallenge compared to the Control/Challenged and Vaccinated/Challenged birds. The decline in anti-*S. typhimurium* IgA antibody titres at 14 days postchallenge in the Vaccinated.IL-6/Challenged birds is apparent in the serum, ISS and bile. This observation may be testimony to the improved protection of these birds from *S. typhimurium.* As fewer *S. typhimurium* are gaining access to the host (Table 4), their immune system is less frequently stimulated and *anti-S. typhimurium* IgA antibody production is reduced. However, the timeframe for this observation is relatively small, given the *S. typhimurium* infection levels of these birds at seven days postchallenge, and further investigation into this observation may assist in understanding the processes involved.

Outcome: The reduced level of *S. typhimurium* infection observed in birds vaccinated *with S. typhimurium* in conjunction with IL-6 and then challenged with live *S. typhimurium* compared with either unvaccinated birds or birds vaccinated in the absence of IL-6, is evident throughout this experiment. Immunisation in conjunction with oral delivery of IL-6 induced an increase (though not statistically significant) in anti-*S. typhimurium* IgA antibody titres at challenge. Further, seven days after challenge these birds had higher average anti-*S. typhimurium* IgA antibody titres in the bile and intestine and reduced levels of *S. typhimurium* infection. *S. typhimurium* infection levels in the Vaccinated.IL-6/Challenged birds were further reduced by 14 days postchallenge, compared to the Control/Challenged and Vaccinated/Challenged birds, however, at this time their anti-*S. typhimurium* IgA antibody titres appeared to be declining. As the Vaccinated.IL-6/Challenged birds did not have statistically significant increases in anti-*S. typhimurium* IgA antibody titres at any of the observation points, their continued improved protection from *S. typhimurium* was a little surprising. It is possible that exposure to IL-6 facilitated continued benefits during challenge, which were not overtly apparent in terms of the anti-*S. typhimurium* IgA antibody titres at 7 and 14 days postchallenge.

In this study vaccination in conjunction with pIL-6 administration modulated the immune system, facilitating an increase in IgA antibody production and improved resistance to a *S. typhimurium* challenge.

Experiment 8*: In-ovo* delivery of pIL-6.

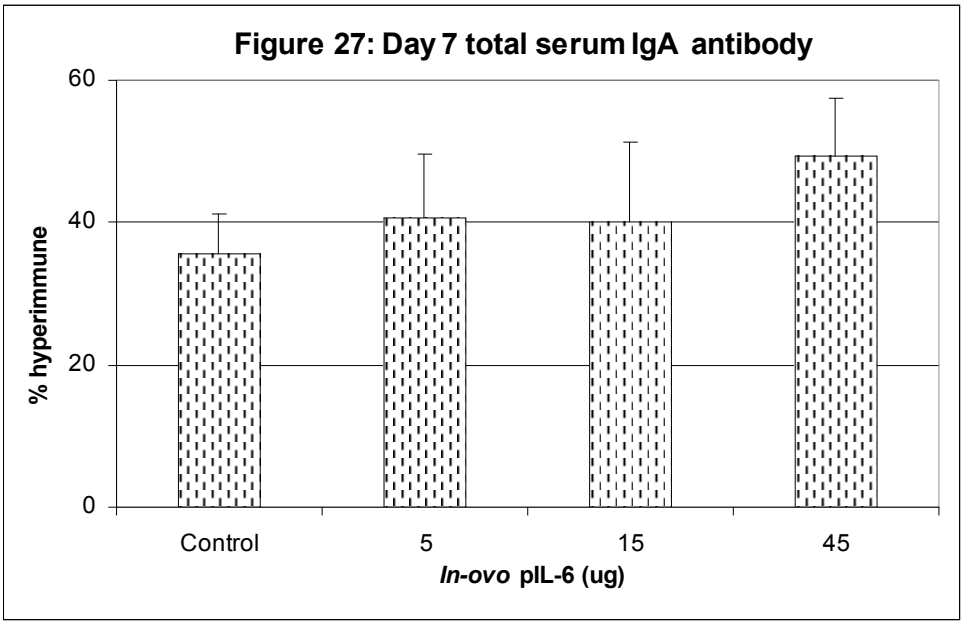
As experiments 5, 6 and 7 have identified the ability of pIL-6 to upregulate IgA antibody production in 4-6 week old broilers, the current study was designed to assess the impact of pIL-6 on IgA antibody production when administered *in-ovo* alone or in association with antigen. A number of dose rates of pIL-6 were assessed.

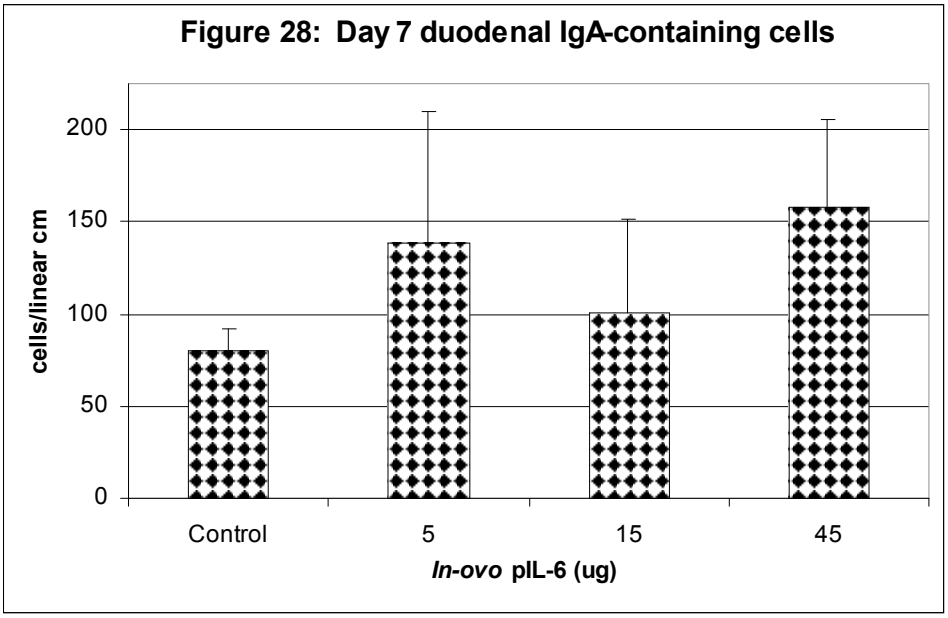
Experiment 8A: *In-ovo* delivery of pIL-6 alone.

Aim: To evaluate the effect of *in-ovo* (D18E) delivered pIL-6 protein on IgA antibody production in young chicks.

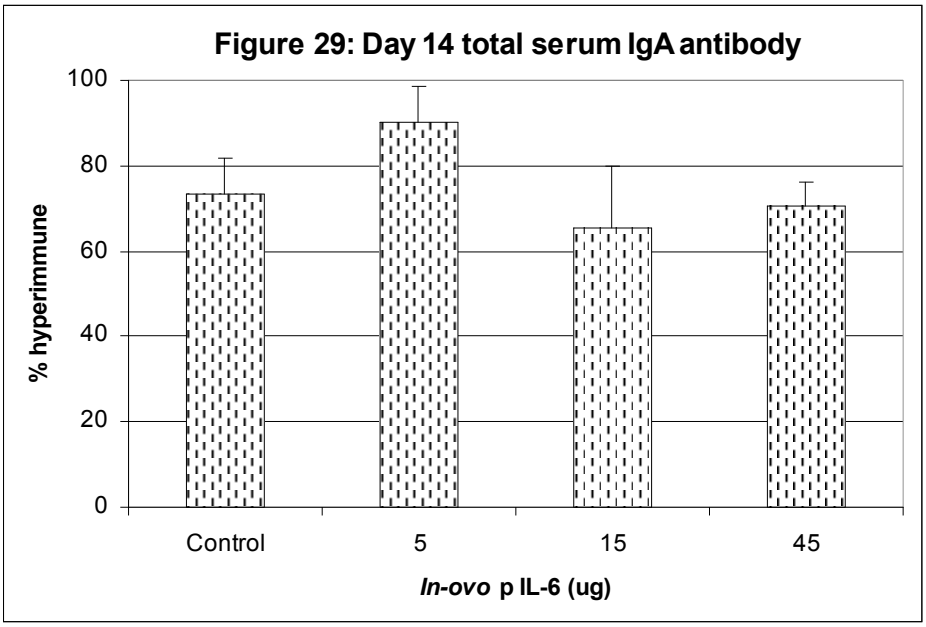
Experimental design: pIL-6 was delivered at three rates 5, 15, and 45 μg/embryo. The impact of pIL-6 on intestinal immunity was evaluated on day 7 and 14 posthatch by assessment of total IgA antibody titres in serum, ISS and bile and, enumeration of IgA secreting plasma cells in the duodenum and spleen.

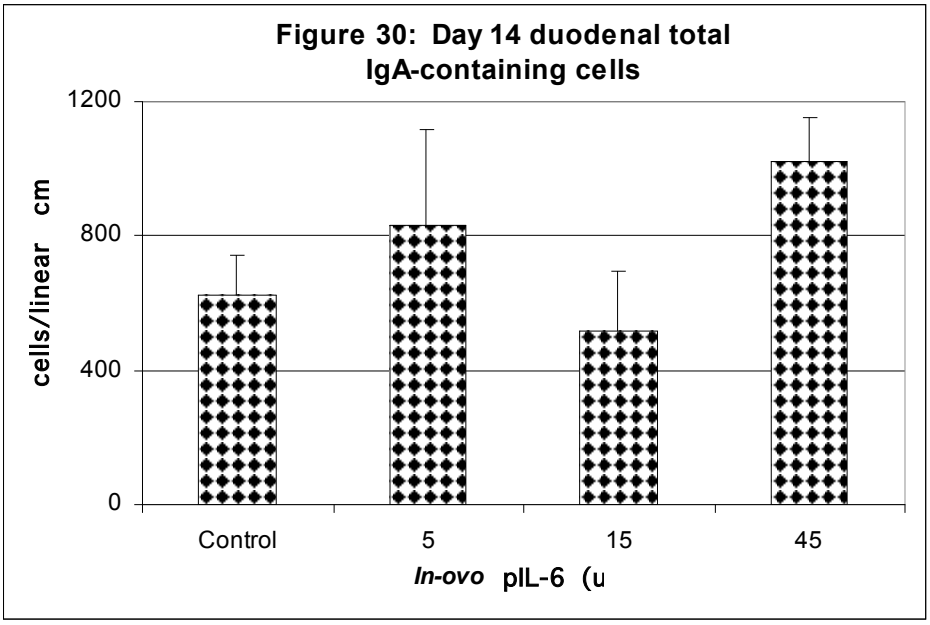
Results: As seen in Figure 27 *in-ovo* delivery of 45 μg pIL-6 invoked an increase (not statistically significant) in total IgA antibody in serum at 7 days of age. However, at this time the average number of IgA-containing plasma cells in the duodenum were variable (Figure 28), but 45 μg pIL-6 did invoke the greatest increase.





At day 14, birds administered 5 μg pIL-6 *in-ovo* demonstrated an increase in total serum IgA antibody (Figure 29), and, in the average number of IgA-containing plasma cells in the duodenum (Figure 30). However, none of these observations were statistically significant. Interestingly, on day 14 birds treated with 45 μg pIL-6 had an increase (not statistically significant) in the number of IgA-containing plasma cells in the duodenum but no increase in serum total IgA was detected.





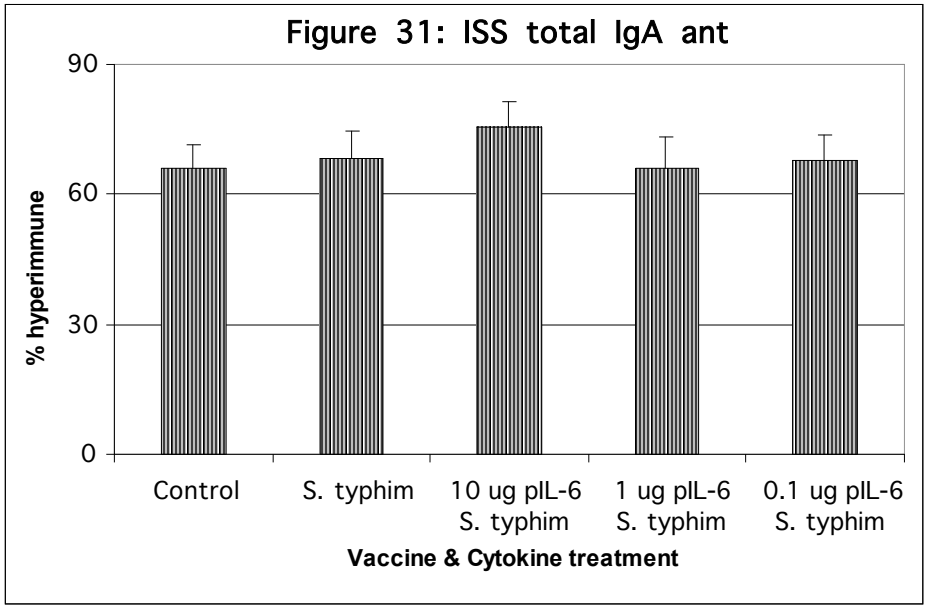
Outcome: The results of experiment 8A are inconclusive and do not provide a succinct indication of the influence of *in-ovo* delivered IL-6 on IgA antibody production in young chicks. On some occasions birds treated with pIL-6 had increased levels of total IgA antibody but the results were variable depending on the sample site (that is serum versus the intestine), pIL-6 dose and the age of the birds. During this experiment pIL-6 was delivered *in-ovo* in the absence of an antigen. These predisposing conditions may be contributing to the variation in results. At D18E, the time of *in-ovo* delivery, the immune system is relatively immature which may restrict the impact of IL-6. Further, and possibly more importantly, pIL-6 was delivered alone, in the absence of a specific antigen challenge. As IL-6 regulates post-switch B cells its administration in association with antigen may be necessary to optimise the potential for enhanced immunity. In view of this latter point, experiment 8B assessed the concurrent delivery of pIL-6 with antigen both *in-ovo* and posthatch.

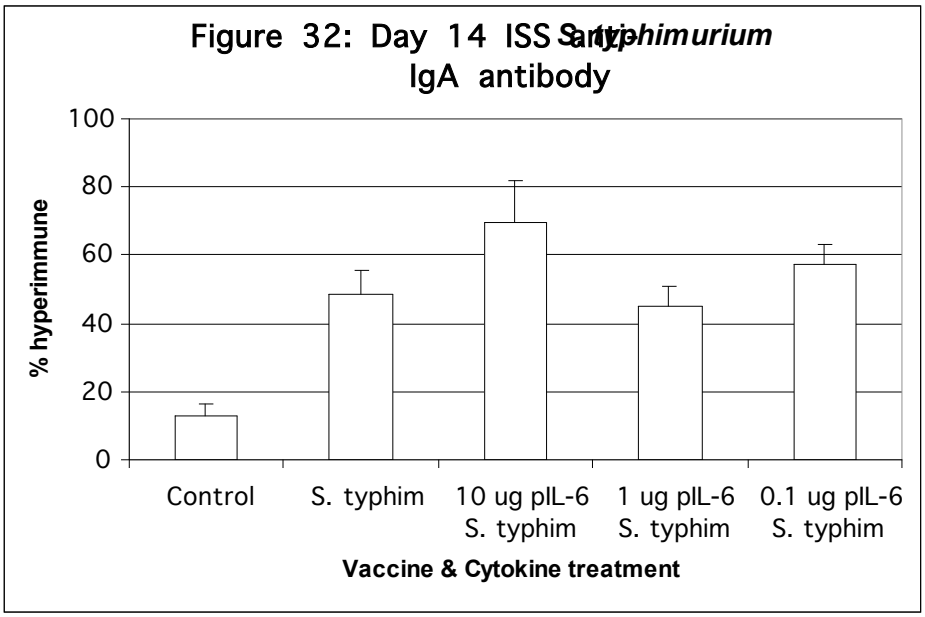
Experiment 8B: *In-ovo* and posthatch delivery of pIL-6 and antigen.

Aim: This study investigated the effect of pIL-6 on the IgA antibody response following its *in-ovo* delivery with antigen, and, a second administration of pIL-6 and antigen at 7 days of age.

Experimental design: At 18DE and 7 days of age chicks received pIL-6 at one of three dose rates, 0.1, 1 and 10 μg, and, whole killed *S. typhimurium* (10^5/dose). Total and anti-*S. typhimurium* IgA antibody levels were determined at fourteen days of age, seven days after the booster immunisation.

Results: The *in-ovo* and day 7 oral delivery of whole killed *S. typhimurium* immunogen with 10 μg pIL-6 induced a notable, but not statistically significant, increase in ISS total and anti-*S. typhimurium* IgA antibody titres at 14 days of age (Figures 31 and 32 respectively), compared with *in-ovo* delivery of the *S. typhimurium* antigen alone.





Outcomes: The concurrent delivery of 10μg pIL-6 and antigen *in-ovo* and at 7 days of age increased, though not significantly, IgA antibody titres. A number of factors including the relative immaturity of the immune system in chickens receiving cytokine *in-ovo,* and the antigen administered, may be limiting the effect of pIL-6 on IgA antibody production. Clearly more work is required to evaluate the potential for pIL-6 to upregulate IgA antibody levels during the early posthatch period.

Overall Discussion

Section A: Assessment of the impact of mammalian immunomodulators on IgA antibody production in chickens.

From the studies undertaken in this project the potential of VE to act as an immunoregulator is apparent. Delivered concurrently with antigen *in-ovo* and at 7 days of age, 20 mg VE augmented anti-antigen IgA immune responses in fourteen day old birds. (Experiment 3: Figures 16 and 17).

A number of the studies have also shown the immunoregulatory role of VE when included at supplementary levels in the diet. Initial studies identified supplementation of a maize-based diet, which contained 50 mg/kg VE, with an additional 250 or 5000 mg/kg VE, and fed for the duration of the study, as the most immunostimulatory (Experiment 2A: Figures 1 and 2). Birds fed 250 mg/kg VESD had statistically significant increases in total IgA in serum prior to immunisation at day 21, and on day 42 (Figure 1). Birds fed 5000 mg/kg VESD also had a significant increase in total IgA in the serum and intestine at day 42 and they also demonstrated a significant increase in anti-antigen IgA antibody titres at the intestinal surface at day 42 compared with the birds fed the basal diet. Significant increases in antigen-specific IgA antibody responses in the serum and intestinal scrapings were also observed in birds fed 250 mg/kg VESD and immunised with whole killed *S. typhimurium* (Experiment 2B: Figures 5 and 6 respectively).

Following proof of the concept that supplementation of the diet with 250 mg/kg VESD can modulate IgA immunity, a further study evaluated the importance of the time of the VESD feeding interval in relation to the immunisation schedule (Experiment 2C). Birds continuously fed 250 mg/kg VESD from day old and prior to immunisation only, exhibited similar anti-*S. typhimurium* IgA antibody titres at the intestinal surface and in serum as birds which received the VESD from day old for the 42 days of the study (Figures 12-14). However, in this case none of the observations were statistically significant when compared to the birds fed the basal diet. This outcome concurs with the work of Franchini *et al.* (1986) where increased antibody titres to Newcastle disease virus were observed when VESD were fed during the first weeks of life prior to vaccination. Further, in both studies there was no immunological benefit in feeding VESD beyond the point of initial vaccination. In fact, in this study birds which were fed the basal diet until immunisation, and were then transferred onto the VESD, had reduced (not statistically significant), anti-*S. typhimurium* IgA antibody titres, compared to birds fed the basal diet or the VESD for the duration of the study.

In terms of the objectives of this project these studies have identified that some levels of supplemental inclusion of VE in the diet can upregulate total and anti-antigen IgA antibody titres at the intestinal site. Diets supplemented with 250 mg/kg VE can improve the immune response when fed from day old for three weeks prior to immunisation, or, from day old for the duration of the study. Further studies are required to ascertain whether shorter feeding intervals of VESD prior to immunisation will invoke similar immunological benefits. VE (20 mg) also demonstrated some immunomodulatory potential when included in the *in-ovo* and early posthatch vaccine preparation.

Section B: Assessment of the impact of cytokines on IgA antibody production in chickens.

Studies involving *in-ovo* and postnatal delivery of pIL-6 described in this report have identified a number of important prerequisites for IL-6 induced upregulation of IgA antibody production in the chicken. From the studies undertaken in this project, pIL-6 must be delivered in close association with either immunisation or challenge. When administered alone, pIL-6 had no impact on total IgA antibody titres (Experiment 5: Figure 18 and Experiment 8A: Figure 27-30). However, when delivered *in-ovo* or orally to young chicks in close association with antigen some increase in IgA antibody titres was observed (Experiment 5: Figure 19 and Experiment 8B: Figures 31, 32).

In these studies pIL-6 was most effective when delivered orally to immunologically primed birds (Experiments 6: Figures 20-22). However, the dose of IL-6 required to increase IgA antibody titres to tetanus toxoid in the intestinal scrapings varied between experiments, either 1 or 10 μg. Both two and four consecutive doses of 10 μg IL-6 administered to birds immunised with whole killed *S.*

typhimurium induced significant increases in the anti-*S. typhimurium* IgA antibody titres in the intestinal scrapings (Experiment 6C: Figure 22).

The protection provided by IgA antibody production at the intestinal surface under the influence of an exogenous supply of pIL-6 was evaluated in *S. typhimurium* vaccination/challenge studies (Experiments 7). At challenge birds immunised with *S. typhimurium* and concurrent pIL-6 treatment demonstrated marginally higher *anti-S. typhimurium* IgA mean antibody titres at the intestinal surface and in bile (Figure 24). Consequently, at fourteen days after challenge, *S. typhimurium* were less frequently identified in their cloacal swabs and fewer bacteria were present in the spleen and liver than either the Control/Challenged or the Vaccinated/Challenged birds (Table 4). Interestingly, birds receiving the vaccination in combination with pIL-6 demonstrated a greater capacity to resist the *S. typhimurium* challenge in the absence of significantly higher anti-*S. typhimurium* IgA antibody titres at either day 7 (Figure 25) or 14 (Figure 26) postchallenge, compared with control birds or birds vaccinated in the absence of IL-6. It may be that the IgA antibody titres of the IL-6 treated birds were higher on days other than the two assessed in this study. The ability for pIL-6 to increase antibody production to an antigen challenge which occurred after the pIL-6 administration was observed in experiment 5 (Figure 19) indicating the possibility of a similar response in a vaccination/challenge study. Therefore, posthatch delivery of pIL-6 can modulate the immune response for a period of time, however, it is recommended that its initial delivery be closely linked with antigen delivery or challenge.

To provide practical benefits to the industry through the induction of improved immune responses in the early posthatch period, cytokines such as IL-6 need to be delivered at hatch in conjunction with current day old immunisation procedures. Further studies are required to investigate the application of this proposal. However, experiments completed during the current project have shown pIL-6 induced antigen-specific immunostimulation during the early posthatch period, the benefit of which may persist for a number of weeks. As chicken IL-6 is now sequenced and cloned, the delivery of species-specific chicken IL-6 to day old chicks is likely to have superior stimulatory impact, and may facilitate further upregulation of IgA antibody production and improved resistance to disease-causing pathogens.

Implications

This work has illustrated the immunological benefit to be gained from *in-ovo* delivery of VE, or, its inclusion in the diet at rates above those currently used for nutritional maintenance. Of these, the most promising technique involves the feeding of a basal diet (containing 50 mg/kg VE) supplemented with 250 mg/kg of VE from day old and prior to primary immunisation (in this case, a three week period). From this research work, feeding VESD for this interval facilitates similar immunological benefit as feeding it for the duration of a

broiler growout. However, further studies are required to ascertain whether the interval of feeding VESD prior to primary immunisation can be reduced below three weeks without compromising the immune response. At this stage the pivotal points are that the VESD must be fed from day old and prior to immunisation. Birds either fed the VESD from 3 weeks of age and after the primary immunisation, or, fed the VESD for several days both before and after the primary immunisation, but not for the duration of the growout, experienced notably reduced antibody titres.

The delivery of specific quantities of exogenous sources of the cytokine pIL-6 increased anti-antigen IgA antibody production. The successful immunoenhancement of IgA antibody production requires delivery of pIL-6 in close association with antigen. Delivery of pIL-6 alone does not enhance total IgA-antibody production. For practical application in an intensive industry as chicken meat or egg production, IL-6 must be delivered at hatch in conjunction with current immunisation protocols. The efficacy of this strategy requires investigation. However, pIL-6 did enhance antibody production in the early posthatch period following its delivery with antigen to young chickens, which augers well for day of hatch delivery. Further, as a biologically active form of recombinant chicken IL-6 is now available, the full potential of such a strategy can be ascertained. A research proposal involving this work has been submitted by this group to RIRDC.

Recommendations

A number of implications and outcomes from this research require further clarification before the proposed immunoenhancing strategies can be successfully adopted and exploited by the industry.

As mentioned under implications, the feasibility of reducing the interval of feeding VESD prior to immunisation, while maintaining enhanced immunity must be determined. The VESD must be fed from day old and prior to immunisation, but the length of time required for the VESD to prime the immune system needs further evaluation.

The establishment of a practical protocol involving the delivery of exogenous IL-6 to upregulate intestinal immune responses also requires further investigation. The concept that IL-6 can prime the avian immune system and, in particular, IgA antibody production at the intestinal surface, has been established. Further, birds receiving IL-6 in association with an immunisation programme for *S. typhimurium*, demonstrated improved protection from *S. typhimurium*. However, the delivery schedule for IL-6 must be manipulated to match the needs of the industry. The efficacy of IL-6 delivered either *in-ovo* or at hatch must be determined, ensuring that the antibody response to vaccines delivered at that time can be enhanced.

Bibliography

Campbell, T.W. (1995). *Avian hematology and cytology,* 2nd ed., Ames: Iowa State University Press.

Chung, T.K. and Boren, B. (1999). Vitamin E use in commercial flocks examined. *Feedstuffs.* **September 6 ,** 11-14.

Duncan, D.L., Smith, W.D. and Dargie, J.D. (1978) Possible relationship of levels of mucosal IgA and serum IgG to immune unresponsiveness of lambs to *Haemonchus contortus. Veterinary Parasitology.* **4**, 21-27.

Erf, G.F., Bottje, W.G., Bersi, T.K., Headrick, M.D. and Fritts, C.A. (1998). Effects of dietary vitamin E on the immune system in broilers: Altered proportions of CD4 T cells in the thymus and spleen. *Poultry Science*, **77**, 529-537.

Finch, J.M. and Turner, R.J. (1996). Effects of selenium and vitamin E on the immune responses of domestic animals. *Research in Veterinary Science.* **60**, 97-106.

Franchini, A., Bertuzzi, S. and Meluzzi, A. (1986). The influence of high doses of vitamin E on immune response of chicks to inactivated oil adjuvant vaccine. *The Clinical Veterinarian.* **109**, 117-127.

Friedman, A., Bartov, I., and Sklan, D. (1998). Humoral immune response impairment following excess vitamin E in the chick and turkey. *Poultry Science.* **77**, 956-962.

Gore, A.B. and Qureshi, M.A. (1997). Enhancement of humoral immunity and cellular immunity by vitamin E after embryonic exposure. *Poultry Science.* **76**, 984-991.

Gu, J-Y, Wakizono, Y., Sunada, Y., Hung, P., Nonaka, M., Sugano, M. and Yamada, K. (1999). Dietary effect of tocopherols and tocotrienols on the immune function of spleen and mesenteric lymph node lymphocytes in brown Norway rats. *Bioscience Biotechnology and Biochemistry.* **63**, 1697-1702.

Husband, A.J. (1987). Perspectives in mucosal immunity: A ruminant model. *Veterinary Immunology and Immunopathology.* **17**, 357 – 365.

Husband, A.J. (1993). Novel vaccination strategies for the control of mucosal infection. *Vaccine.* **11**, 107-112.

Husband, A.J., Bao, S., Muir, W.I., Ramsay, A.J. and Ramshaw, I.A. (1994). Cytokine regulation of mucosal responses: a rational basis for new vaccine delivery strategies. *Reproductive Fertility and Development.* **6**, 381-388.

Husband, A.J., Kramer, D.R., Bao, S., Sutherland, R.M. and Beagley, K.W. (1996). Regulation of mucosal IgA responses in vivo: cytokines and adjuvants. *Veterinary Immunology and Immunopathology.* **54**, 179-186.

Jackson, D.W., Law, G.R.J. and Nockels, C.F. (1977). Maternal vitamin E alters passively acquired immunity of chicks. *Poultry Science.* **57,** 70-73.

Johnston, N.W. and Bienenstock, J. (1974). Abolition of non-specific fluorescent staining of eosinophils. *Journal of Immunological Methods.* **4**, 189-194.

Kaku, S., Yunoki, S., Mori, M., Ohkura, K., Nonaka, M., Sugano, M. and Yamada, K. (1999). Effect of dietary antioxidants on serum lipid contents and immunoglobulin productivity of lymphocytes in Sprague-Dawley rats. *Bioscience Biotechnology and Biochemistry.* **63**, 575-576.

Lebman, D.A. and Coffman, R.L. (1994). Handbook of mucosal immunology. Eds: P.L. Ogra, M.E. Lamm, J.R. McGhee, J. Mestecky, W. Strober, J. Bienenstock. Academic Press Inc., San Diego.

Lowenthal, J.W., York, J.J., O'Neil, T.E., Steven, R.A., Strom, D.G. and Digby, M.R. (1998). Potential use of cytokine therapy in poultry. *Veterinary Immunology and Immunopathology.* **63**, 191-198.

Marsh, J.A., Combs, G.F., Whitacre. M.E. and Dietert, R.R. (1986). Effect of selenium and Vitamin E dietary deficiencies on chick lymphoid organ development. *Proceedings of the Society for Experimental Biology and Medicine.* **182**, 425-436.

Meydani, S.N. (1995). Vitamin E enhancement of T cell-mediated function in healthy elderly: mechanisms of action. *Nutrition Reviews.* **53**, S52-S58.

Meydani, S.N. and Beharka, A. (1996). Recent developments in vitamin E and immune response. *Nutrition Reviews.* **56**, S49-S58.

Moriguchi , S. and Muraga, M. (2000). Vitamin E and immunity. *Vitamins and Hormones***.** **59**, 305-336.

Muir, W.I., Bryden, W.L. and Husband, A.J. (1995). Intraperitoneal immunization promotes local intestinal immunity in chickens. *Avian Pathology.* **24**, 679-692.

Muir, W.I., Bryden, W.L. and Husband, A.J. (1998a). Comparison of *Salmonella typhimurium* challenge models in chickens. *Avian Diseases.* **42**, 257-264.

Muir, W.I., Bryden, W.L. and Husband, A.J. (1998b). Evaluation of the efficacy of intraperitoneal immunization in reducing *Salmonella typhimurium* infection in chickens. *Poultry Science.* **77**, 1874-1883.

Muir, W.I., Husband, A.J. and Bryden, W.L. (2000). *The impact of Vitamin E on immunity*, In Proceedings of the 6th Avian Immunology Research Group Meeting, Cornell University, Ithaca, October 8-10, 2000. pp45-49.

Muir, W.I., Bryden, W.L. and Husband, A.J. (2000). Immunity, vaccination and the avian intestinal tract. *Developmental and Comparative Immunology.* **24**, 325-342.

Noor, S.M., Husband, A.J. and Widders, P.R. (1995). *In ovo* vaccination with *Campylobacter jejuni* establishes early development of intestinal immunity in chickens. *British Poultry Science.* **36**, 563-573.

Tengerdy, P.R. (1990). Effect of vitamin E and A on humoral immunity and phagocytosis in *E. coli* infected chickens. *Poultry Science.* **56**, 957-963.

Ramsay, A.J., Husband, A.J., Ramshaw, I.A., Bao, S., Matthaei, K., Kohler, G., and Kopf, M. (1994). The role of IL-6 in mucosal IgA responses *in vivo*. *Science.* **264**, 561-563.