AUSTRALIAN POULTRY CRC

FINAL REPORT

Program 2

Project No: 04-15: US

PROJECT LEADER: Professor Tom A. Scott

Preparation of final report: Dr Wendy I Muir

Oral delivery system for poultry health products

DATE OF COMPLETION: January 2008.

ISBN 1 921010 15 0

Oral delivery system for poultry health products

Project No.04:15

Researcher Contact Details
Dr. Wendy I. Muir
Faculty of Veterinary Science
PMB #3
Camden, NSW 2570

Phone: 02 4655 0658
Fax: 02 4655 0693
Email: wmuir@camden.usyd.edu.au

In submitting this report, the researcher has agreed to the Australian Poultry CRC publishing this material in its edited form.

Australian Poultry CRC Contact Details
PO Box U242
University of New England
ARMIDALE NSW 2351

Phone: 02 6773 3767
Fax: 02 6773 3050
Email: info@poultrycrc.com.au
Website: http://www.poultrycrc.com.au

Published in August 2008

Executive Summary

Many pathogens make initial contact with the host across the mucosal surfaces of, for example, the gastrointestinal or respiratory tracts. Clusters of immune cells are located along the mucosal surface of the intestine, which are referred to as the gut associated lymphoid system or GALT, where they form the first line of immune defense against potential pathogens attempting to gain access to the host through the intestinal mucosa. It follows that stimulation of the GALT, through the oral delivery of antigen, would generate local immune responses that would protect the host from challenge. However, this is generally not the case, where the immune response following oral delivery of killed, native antigen induces immune responses of low amplitude and short duration. The limited response is due to several factors, including degradation of the antigen from the hydrolytic and proteolytic environment within the intestinal tract and difficulty with the antigen reaching and being sampled by the GALT. Alternative methods of vaccination, which typically involve injections, usually generate strong systemic immune responses, but generally poor mucosal immune responses. Immune responses at the mucosal surfaces typically require local immune activation and production. Therefore, the search for technology that will enable the efficacious delivery of antigen to the GALT, and the generation of significant local immune responses, is ongoing. In the poultry industry this is also driven by the need to deliver vaccines to large numbers of birds, in a way that does not require the handling of individual birds.

PerOs Systems Technologies Ltd (Canada) have recently developed an oral vaccine carrier diet (Oralject), which is designed to temporarily halt the hydrolytic and proteolytic activity of the gut, while also improving the permeability of the intestinal mucosa. This vaccine carrier has been used successfully for the oral delivery of vaccines in the aquaculture industry. Therefore, this research was a proof of concept project being designed to assess the ability of the Oralject technology to delivery orally administered antigens to the chicken GALT via an assessment of the local and systemic immune responses achieved following immunisation.

Oralject was tested as a vaccine carrier diet for the oral deliver of three different antigens, bovine serum albumen (BSA), heat inactivated epizootic haematopoietic necrosis virus (EHNV) and killed *Salmonella typhimurium* in four week old broiler chickens. Two different preparations of Oralject were also tested throughout this project. The first preparation used dry Oralject dietary ingredients which were delivered in either a wet form via gavage or a dry form directly in the feeder. Each of these was assessed with each of the three antigens. The second Oralject carrier diet was a liquid preparation, based on the extraction of chemicals from similar ingredients used in the dry diet preparation. It is identified as the Oralject extract solution, and was delivered by gavage. The Oralject extract solution was assessed using two antigens, BSA and killed *Salmonella typhimurium*. In some instances the Oralject carrier diet, both dry and extract solution, were delivered prior to the antigen, though most commonly the antigen and Oralject preparations were delivered together. The immune response in birds administered antigen/Oralject preparations were compared to those of control treatments which received neither antigen nor Oralject, or which received only antigen or Oralject alone. Positive control birds received antigen in adjuvant delivered via injection, and negative control groups received commercial broiler diet only. Birds were immunised with the Oralject/antigen preparation twice, two weeks apart. The subsequent immune response was assessed in terms of antigen-specific antibody production, both IgG and IgA isotypes, for five weeks following the primary immunisation. Samples of serum were collected throughout the study and at the end of the study birds were euthanased and samples of intestinal scrapings and bile were collected for determination of the local intestinal immune response.

Based on the antibody titres identified in serum samples, the delivery of antigen in association with the dry Oralject dietary ingredients did not present any benefits over and above the feeding of antigen alone. This was observed with all three antigens. However, the Oralject extract solution did generate significant immune responses, both of the IgG and IgA isotypes in serum, when assessed with BSA. This was seen when BSA and Oralject extract solution were administered together and also when the BSA was delivered 30 minutes after the oral administration of the Oralject extract solution. This was

most notable on day 22, one week after the booster immunisation, when both of the treatment groups that had received the Oralject extract solution had anti-BSA IgG and IgA titres that were not significantly different to those of the positive control treatment group, which had received BSA in adjuvant via injection. These responses were, however, relatively short lived and had declined by day 29. But, on days 29 and 36 the anti-BSA IgG titres of these groups remain significantly higher than all treatment groups except for the positive control group. Interestingly, this profile of antibody responses were not observed when the Oralject extract solution was used as the carrier for killed *S. typhimurium* antigen. In this instance the immune responses observed in birds administered *S. typhimurium* with the Oralject extract solution were generally no different to the low immune responses observed in the birds that received the antigen or Oralject alone. Unfortunately, unresolved problems with background colour in the ELISA assays for measuring anti-antigen IgA antibody titres in samples of intestinal scrapings and bile prevented their assessment, with the exception of the intestinal scrapings samples from birds immunised with *S. typhimurium.* However, again in the latter samples, the anti-*S. typhimurium* IgA titres were not different from those of the control groups.

In conclusion, this project has demonstrated the potential for Oralject, in the extracted solution form only, to generate a significant immune response to the antigen, BSA. However, it must be reiterated that birds receiving the Oralject extract solution with killed *S. typhimurium* failed to generate notable immune responses to *S. typhimurium*. Hence the features of an immune response achieved following the delivery of antigen to the GIT immune system with Oralject extract solution is likely to differ with each antigen. The dry Oralject carrier diet failed to generate any notable immune responses to any of the test antigens.

Acknowledgements

This project was made possible by funding from The Australian Poultry CRC, and in-kind support from PerOs Systems Technologies Ltd, Canada.

Significant contributions were also made by staff at the Poultry Research Unit, University of Sydney, Camden, NSW, Australia, for which we are very grateful. In particular Mrs Joy Gill, Mrs Melinda Hayter and Mrs Nishath Kowser Ganguli assisted with bird experimentation, Mrs Nishath Kowser Ganguli assisted with laboratory work and Mrs Jo-Ann Geist provided administrative assistance.

Contents

Introduction

"Oral vaccination of animals and man, to provide effective mucosal and/or systemic immunity, is largely ineffective. This is due mainly to the very small quantity of antigen that survives degradation in the intestine and that crosses the intestinal wall." (Russell-Jones, 2000).

Recently, PerOs Technologies (Canada) has developed, patented and commercially provided an oral vaccine carrier diet that has proven successful as a means of delivering vaccine to aquaculture species. The carrier formulation was developed from recognised food and feed ingredients, thereby reducing the difficulty in approval and implementation. This has spawned the present study to determine if a carrier diet can successfully suspend digestion of antigens, and potentially other bioactives, and thereby deliver them intact, in sufficient quantity and activity to improve the health and wellbeing of commercial poultry.

In all animals the mucosae of, for example the digestive and respiratory tracts, form part of the largest surface at the interface of an animal's internal and external environment. In this regard it is also the barrier across which many potential pathogens must cross to successfully infect the host. Despite the local protective mechanisms of the host, many pathogens do gain access to the host's internal environment by colonising these mucosal areas. Working to prevent breaches of the intestinal barrier by pathogens are concentrated areas of immune cells. These can be found along the length of the intestinal tract, being referred to as the gut-associated lymphoid tissues (GALT), and they form the first line of immune defence against potential pathogens. In this regard, the GALT are constantly distinguishing between non-harmful food-derived nutrients and potential pathogens. In the case of the latter the GALT and its associated cells mounts a defensive response to the antigen that ideally, precludes it from any further interaction with the host.

While the parenteral (administered in a manner other than through the digestive tract) vaccinations are effective in the development of systemic immunity, this protection may not necessarily be effective at the mucosal surface where the body is first likely to encounter "attack". Russell-Jones (2000) indicates that mucosal surface immunity is usually derived from local synthesis rather than from the systemic pool of antibodies generated by parenteral vaccination. Therefore, it follows that the induction of a local immune response at these sites as stimulated by oral delivery of antigen should be the most efficacious way to induce the required local immune protective mechanisms. However, following oral delivery of killed, non-replicating antigens, only small quantities of fully active antigen reach the GALT.

Explanations for lack of development of generic technology to administer oral vaccines were summarised by Russell-Jones (2000), and included:

- intestinal resistance to uptake of intact large molecules like vaccines;
- high hydrolytic and proteolytic environment (low pH and pepsin secretions of stomach/ proventriculus, and trypsin and chymotrypsin secretions of the pancreas);
- difficulty in material reaching and stimulating the 'M' cells/Peyer's patches of the intestinal epithelial and eliciting both mucosal and long-term serum antibody response (rather than tolerance); or alternatively
- binding to the intestinal epithelium and generating serum antibody responses directly.

A further complicating factor is the activation of cells that suppress the immune response when antigen is presented to GALT across the epithelial cells of the intestinal surface. This may be due in part to the prevention of a harmful immune response to food-derived antigens.

There are several reviews that address the challenges and opportunities for oral delivery of bioactive compounds in the literature (Andrianov and Payne 1998; Muir, 2000; Russell-Jones 2000; Senior 2001; Vila *et al.* 2002; Goldberg and Gomez-Orellana 2003; Hejazi and Amiji, 2003; Keegan *et al.* 2003; Johansson *et al.* 2004). Russell-Jones (2000) reviewed the four main methods currently used for oral vaccination of vertebrate hosts: 1) repeated large dose administration of vaccine antigens; 2)

vaccination using attenuated pathogenic bacteria-targeting to intestinal M cells; 3) detoxified toxins; and 4) mucosal binding proteins as carriers for oral vaccine delivery. Besides the obvious disadvantages of feeding high doses of a vaccine antigen, this costly treatment would be least likely to generate a high serum antibody response, a factor crucial for maternal antibody protection. Ongoing work to use bacterial vectors to target 'M' cells and stimulate antibody response have demonstrated potential, other work also suggests that indigestible chitin carriers of antigens can also target the Peyer's patches and allow sustained stimulation of antigenic response (Thanou *et al.* 2001; van der Lubben *et al.* 2001ab; Brayden and Baird 2004)).

The protective mechanisms, described above, of the digestive tract may still interfere with these delivery mechanisms. Enteric diseases such as necrotic enteritis are a result of toxins produced by bacteria (in this case *Clostridium perfringens*). These toxins can elicit antibody responses and may also be powerful mucosal adjuvants for the co-fed antigens. Work to detoxify and maintain adjuvant activity of these toxins is also underway. Direct antibody responses from proteins entering the "system" via the epithelial cells of the intestine, rather than via the Peyer's patches, include viral haemagglutinnins, bacterial toxins, lectins, plant toxins and bacterial invasins. Russell-Jones (2000) describes covalent linkage of potential vaccine antigens to the targeting molecules as a simple method of providing oral vaccination and antibody, rather than tolerance, stimulation. Lectin and some Vitamin B molecules, in particular Vitamin B12, may be useful for the delivery of "antigen-loaded" nanoparticles by facilitating attachment of these compounds to the epithelial cells of the gut and/or their transport across the gut wall (Russell-Jones *et al.*, 1999; Clark *et al.*, 2000).

The current project will initially investigate protection of bioactive compounds from hydrolytic, proteolytic, and "mechanical" destruction by the proventriculus and gizzard via temporary interruption of hydrolytic and proteolytic activity by simultaneous feeding of ingredients with antinutritional factors (ANFs) that limit these processes. PerOs Systems Technologies (personal communication with G. Vandenberg, 2004) use ANFs to temporarily suspend digestion for administration of vaccines in aquaculture, and have international patents pending on this technology. This expertise and experience will be applied in the present work.

Briefly, the vaccine carrier diet (Oralject) involves combinations of approved feed additives to raise gastric pH as well as buffer additional acid secretions thereby reducing acid hydrolysis of bioactive compounds. The elevated pH of the gut also reduces the activity of pepsin and prevents initial proteolysis. Subsequent proteolysis is countered by combining a number of potent plant and animal-derived inhibitors of protease activity. In this manner enterokinase and trypsin are inactivated. Lastly, a variety of naturally-occurring products can be employed that significantly increase the permeability of the intestinal mucosa. The strategy, besides increasing delivery of bioactive compounds to the intestinal mucosal and induction of local and systemic immunity, is to use naturally-derived plant and/or animal materials that currently have approval for use in animal feeds, thereby reducing the process associated with approval of the technology.

All bioactive compounds with gut health potential will likely benefit from protection of the harsh hydrolytic and proteolytic environment of the upper gut (proventriculus); however, in poultry we also have to be cognisant of the physical actions of the gizzard. It is apparent that antigens for Newcastle Disease Virus (NDV; Spradbrow and Samuel 1991), and certainly feed enzymes (Non Starch Polysaccharidases and Phytases) are capable of withstanding these forces and eliciting their response in the intestinal tract, although it may be interesting to determine if this could be enhanced by protection through the proventriculus and gizzard. It may also be that successful delivery of antigens, such as NDV via the feed may elicit their response via respiratory mucosae stimulation (occurring during feeding) and/or the upper digestive tract mucosae stimulation; likewise, enzymes such as phytase are principally only active while the digesta is retained in the crop and may benefit from protection through the upper digestive tract (personal communication with Dr Peter Selle, 2006).

One of the issues that will need to be addressed in delivery of intact bioactive compounds to the intestinal mucosae will be the dose rate and its ability to generate long-term antibody response rather

than systemic tolerance. Recent work specifically undertaken in chickens has highlighted some options for avoiding the induction of oral tolerance. Friedman *et al.* (2003) and Caldwell *et al.* (2004) identified time regimens for oral delivery of antigen that are likely to induce an immune response, as opposed to immune tolerance. The timing of both the first and second oral exposure to antigen, and the medium in which the antigen is delivered were all found to influence the generation of an immune response, as opposed to systemic tolerance, in chickens. Specifically, Friedman *et al.* (2003) recognised the induction of oral tolerance to orally administered antigens between hatch and 4 days of age. Interestingly, if exposure to this same antigen occurred 4-6 weeks later, the immune system subsequently responded to it, that is tolerance was "switched off". Additionally, Klipper *et al.* (2000 and 2001) found that orally delivered antigens suspended in an aqueous solution, as opposed to a "powder within the ration" (Klipper *et al*., 2000), will favour the generation of an immune response. Antigenic powder preparations are more likely to induce tolerance. Therefore, it is possible that the delivery of antigen in solution after chicks are 4 days of age may be a more efficient method of inducing an immune response in young broiler chickens. The success of these procedures is likely to vary with individual bioactive compounds and may require combinations of the alternative methods, as previously described, to ensure that long-term protection of the animal and that high levels of maternal antibodies are generated.

A unique feature of the avian species is the relatively short intestinal tract and high rate of passage that may limit the exposure time of bioactive compounds to elicit a desired response. A number of options may be applied to overcome this concern, including: targeting Peyer's patch uptake; co-factors that attach bioactive compound to intestinal epithelial and facilitate membrane penetration; and temporary reduction of passage rate through management practices (e.g. subsequent short-term feed restriction).

Stimulation of mucosal immunity and health directly by oral administration will enhance the health and well-being of poultry indirectly (by reducing handling of individual birds during vaccination and by reducing biosecurity concerns of vaccination crews moving from shed to shed) and directly (by stimulating mucosal immunity and health; and increasing the number of deliverable treatments to a larger community of birds). In particular, this will have advantages of ensuring optimum antibody titres to maintain health and insure maternal antibody transfer to chicks without necessitating individual bird treatments. Oral delivery will also facilitate administration of bioactive compounds to broilers and reduce the use of in-feed antimicrobials to prevent disease.

The consequence of maintenance of health of broilers without the use of in-feed antimicrobials would reduce concerns facing the industry with respect to development of pathogens with antimicrobial resistance and the direct possibility of the transfer of this resistance to human pathogens. Reductions of in-feed antimicrobials would also improve the sustainability of the industry by reducing contamination of meat or eggs, and excretion of potential toxins into the environment via the manure.

The animal friendly delivery of bioactive compounds without handling (injections, eye drop, aerosol spray) each chicken to generate health and promote antibody production would have direct benefits with regard to bird welfare. This would also be important in enabling "green" production of antibodies in eggs for human and other animal treatments.

Objectives

A significant proportion (~80%) of pathogenic challenge to poultry is via the mucosal surfaces of the digestive or respiratory tracts; however, much of our current health intervention requires alternative strategies to stimulate immune responses at these sites as the delivery of intact antigen to the mucosal surfaces, particularly the digestive mucosal surface, is currently neither practical nor possible. One of the main difficulties in achieving direct mucosal stimulation is providing delivery of intact bioactive compounds, in the required quantities, to the mucosal surface and specifically those mucosal cells capable of inducing an immune response. Our hypothesis is that direct stimulation of the intestinal mucosae with bioactive compounds would be possible if these compounds could be delivered without

digestive hydrolysis by by-passing and/or temporarily suspending this hydrolysis process. This will be tested by the simultaneous feeding of antigen with Oralject (PerOs Systems Technologies), a diet made up of ingredients with anti-nutritional factors that are designed to halt the hydrolytic processes. The success of this technology will be assessed in terms of the antibody titres to vaccine antigens, at designated times following both primary and secondary vaccinations.

Methodology

Bird care and management

The University of Sydney Animal Ethics Committee approved the project and all bird usage has been reported to that committee. At the beginning of the trial 380 broilers (Cobb mixed sex) at 4 weeks of age were randomly assigned to 95 broiler bioassay cages (2500 cm^2 floor space); there were four birds / cage in all cases except for one instance (EHNV as an antigen, EHNV adjuvant intramuscular injection) where only two birds were used to conserve antigen.

The birds were maintained at a constant room temperature of 22^oC and provided *ad libitum* access to feed (troughs) and water (2 nipple drinkers/cage), except for 8 hr prior to administration of antigen (feed was returned 2 hr post administration of antigen). All mortality was recorded; losses were typical of broilers of the age tested and has not been reported. The chicks were initially brooded in floor pens (shavings litter) and upon allocation to cages the birds were allowed one week to acclimatise to their new environment.

Antigens

Three antigens were used to assess the ability of the Oralject system to deliver antigen to the immune components of the intestinal mucosa of chickens, such that an antigen-specific immune response is generated. These antigens were bovine serum albumin (BSA, Tracebiosciences, New Zealand), heat inactivated epizootic haematopoietic necrosis virus (EHNV, Farm Animal Health Laboratory, Faculty of Veterinary Science, University of Sydney, Camden.) and killed *Salmonella typhimurium*, phage type 12, from the Baiada Salmonella vaccine (produced by Intervet Australia, Pty Ltd, Bendigo, Australia). This latter antigen was used as an example of a poultry vaccine antigen, and was kindly donated by Dr Peter Groves and Mr Tony Pavic, Birling Avian Laboratories, Bringelly, Australia. The dose of *S. typhimurium* delivered for the primary vaccination, 1 x 10^8, was selected as it equates to the dose of *S. typhimurium* included in the Salmonella vaccine (personal communication, Dr Mike McDermott, Intervet Australia Pty Ltd, 2006)

PerOs carrier diets

Two types of PerOs carrier diets (Oralject) were tested throughout these studies. One was based on dry dietary ingredients, which was delivered in either a wet form via gavage or a dry form directly in the feeder, and was assessed in all three studies. The second carrier diet was a liquid preparation, based on extraction of chemicals from similar ingredients used in the dry diet preparation. It is identified as the Oralject extract solution, which was delivered by gavage. The Oralject extract solution was assessed in Study 2 and 3, using two antigens, BSA and killed *Salmonella typhimurium* respectively. The formulation of all Oralject materials was maintained confidential by the PerOs Technologies technician who oversaw the administration of the treatments.

Experimental Design

Three studies were undertaken. The treatments assessed throughout the course of the three studies are outlined in Table 1. The specific treatments of each study are described separately. Briefly, Study 1 involved BSA as an antigen; Study 2 involved BSA and EHNV as antigens; and Study 3 involved killed *Salmonella typhimurium* as an antigen.

Table 1: Overview of treatments assessed throughout studies 1, 2 and 3.	
Treatment type	**Delivery method**
Commercial diet alone (fed)	In feeder
Oralject dry, alone (fed)	In feeder
Oralject dry + antigen (fed)	In feeder
Oralject in water alone	Gavage
Oralject in water + antigen	Gavage
Antigen dry alone	In feeder
Antigen in water , alone	Gavage
Oralject dry (fed) + 2 hours later antigen in water, gavage	In feeder (Oralject), Gavage (antigen)
Oralject in water + 30 minutes later antigen in water	Gavage
Oralject extract solution alone	Gavage
Oralject extract solution + antigen	Gavage
Oralject extract solution + 30 minutes later antigen in water	Gavage
Antigen in oil adjuvant	Injection

The treatments were administered in a set routine in all studies, as was the collection of blood, intestinal scrapings and bile for analysis of the antibody response. The routine is outlined in Table 2:

Table 2. Description of routine for administering antigens, collection of serum, intestinal scrapings and bile. -1 experimental day is one week after the birds were introduced to bioassay cages.	
Experimental Day	**Procedures**
-1	Collect blood from all birds.
0	Feed withheld 8 hr prior to start; water *ad libitum* throughout Administer antigen and Oralject treatments – primary immunisation Birds returned to *ad libitum* control diets 2 hrs following antigen administration
14	Collect blood from all birds.
15	Feed withheld 8 hr prior to start; water *ad libitum* throughout Administer antigen and Oralject treatments– secondary immunisation Birds returned to *ad libitum* control diets 2 hrs following antigen administration
22	Collect blood from all birds. Half of the birds from each pen euthanased; samples of bile and intestinal scrapings collected
29	Collect blood from all birds
36	Collect blood from all birds. Remaining birds euthanased; samples of bile and intestinal scrapings collected.

Study One: BSA as an antigen

In Study 1, BSA was the antigen which was evaluated with different types of the Oralject carrier diets (dry feed and wet feed) over different time and/or administration routes as described in Table 3. The consumption of diets during the administration of antigen was recorded, however, in almost all cases the total allotment was consumed since the amount was small, providing approximately 1% of diet relative to body weight of the birds.

Administration of BSA antigen

All of the following descriptions for antigen delivery are expressed on a per bird basis.

BSA delivered alone via gavage.

For the delivery of BSA alone via gavage, 75 mg BSA was dissolved in 12 ml water for the first challenge and 125mg BSA was dissolved in 15ml water for the second challenge. Delivery via gavage

to the crop was facilitated with a large syringe (60 ml) attached to a 10 cm length of tubing that was gently inserted via the oesophagus into the crop of each bird.

BSA delivered in dry diet (commercial or Oralject) carriers in feeder

In order to administer the BSA in the dry diets (commercial (Broiler finisher crumbles, Ridley AgriProducts) or Oralject carrier), the BSA crystals were first dissolved in water before mixing with the dry diets. In the first challenge 75 mg of BSA was dissolved in 6 ml of water and mixed with 15 g of dry diet yielding a damp mixture of feed, for each bird, volumes were adjusted for the number of birds / cage and feed provided in feed troughs. In the second challenge, due to increased body weight, the amount of BSA was increased to 125 mg and dissolved in 10 ml of water before adding to 25 g of dry diet, again amounts were adjusted to provide an equivalent of 25 g of dry feed per bird in each cage.

BSA delivered in wet feed carrier via gavage

The wet feed carrier was administered via gavage to the crop of each bird. For these treatments BSA crystals were dissolved in three times the amount of water used for the dry diet preparation, before being mixed with Oralject, giving a final dilution of Oralject 1:1.2 water g/bird. That is for the first challenge, 75mg of BSA was dissolved in 18ml water and mixed with 15g Oralject, and for the second challenge 125mg BSA was dissolved in 30ml water and mixed with 25 g Oralject. The slurry-like mixture was then administered with a large syringe (60 ml) attached to a 10 cm length of tubing that was gently inserted via the oesophagus into the crop of each bird.

BSA delivered via injection with adjuvant

The positive BSA treatment group received intramuscular injections of BSA in adjuvant. The vaccine consisted of 20mg BSA dissolved in 10 mL sterile phosphate buffered saline (PBS, pH 7.2), which was emulsified with an equivalent volume of Montanide ISA 50V oil adjuvant (Tall Bennett Group, Sydney) using an ultra tarrax (John Morris Scientific). The vaccine also contained thiomersal at 0.013%. 1 ml of vaccine /bird (1 mg BSA), was administered by intra-muscular injection, and delivered as 5, 0.2 mL volumes, injected into the breast muscle at several sites.

Oralject control administrations

For Oralject control treatments, Oralject carrier was delivered alone either as a dry or wet preparation. For dry feeding, 15g or 25g carrier / bird was mixed with 6 and 10ml water respectively, and then the resulting damp mixture was provided in the feeder for the first and second challenges respectively. For the wet feeding, Oralject was mixed with water (18 ml of water / 15 g of carrier or 30 ml of water / 25 g of carrier) for the first and second challenges respectively.

Commercial diet only

The birds receiving the commercial diet acted as the negative control treatment, receiving commercial diet (Broiler finisher crumbles, Ridley AgriProducts) only. No antigen or Oralject carrier was administered to these birds.

Timing of antigen and carrier delivery

In BSA administrations at 0 min after the carrier, the BSA was administered at the same time as the carrier. Alternatively if times were 30 or 120 min post administration of the Oralject carrier (delivered in feed as 6ml water mixed with 15g carrier or 10ml water mixed with 25 g carrier; or via gavage as 18 ml water / 15 g carrier or 30 ml of water / 25 g of carrier, for the first and second challenges respectively), the BSA was dissolved in water (75 or 125 mg/bird in 12 or 15 ml water respectively, for challenges 1 and 2) and administered by gavage at the designated time following delivery of the carrier.

Table 3. Description of BSA antigen treatments in Study 1, relative to carrier type, delivery route, administration time of antigen (gavage) following administration of carrier (if 0 min, the BSA was administered as part of the carrier), and amount of carrier/bird for the 1st and 2nd challenges, respectively, as well as the intended antigen delivery/bird for the 1st and 2nd challenges, respectively.

Antigen	Carrier Type (number of cages of 4 birds)	Delivery Route	Minutes after Carrier	g/bird carrier*		mg/bird antigen	
				1st	2nd	1st	2nd
No Antigen	Commercial Diet (n=2)	In feeder	0	15	25	0	0
No Antigen	Oralject dry (n=2)	In feeder	0	15	25	0	0
No Antigen	Oralject 1:1.2 water (n=2)	Gavage to crop	0	15	25	0	0
BSA	Adjuvant (n=2)	Intra muscular	0	1ml	1ml	1	1
BSA	Distilled water (n=3)	Gavage to crop	0	12	15	75	125
BSA	Commercial Diet (n=2)	In feeder	0	15	25	75	125
BSA	Oralject dry (n=2)	In feeder	0	15	25	75	125
BSA	Oralject 1:1.2 water (n=3)	Gavage to crop	0	15	25	75	125
BSA	Oralject 1:1.2 water (n=4)	Gavage to crop	30	15	25	75	125
BSA	Oralject dry (n=4)	Oralject in feeder, BSA gavage to crop	120	15	25	75	125

* Oralject 1:1.2 water g/bird carrier is expressed on a dry feed basis

Study two: EHNV and BSA as antigens

In the second study either BSA or EHNV were used as the vaccine antigen. As in study one, the antigens were administered via different carriers, administration routes or time after carriers were provided. Two antigen challenges were provided two weeks apart as previously discussed and outlined in Table 2.

Administration of BSA antigen

The first five BSA treatments in Study 2 (Table 4) were identical to those used in Study 1. Comparison of the results from these same treatment groups in Study 1 and 2 provided an assessment of the effect of day of delivery of the treatment on the immune response (see Results section).

BSA delivered with Oralject extract solution carrier

The last two BSA treatments were administered with the Oralject extract solution rather than the Oralject dry ingredient as the carrier. With the 0 min delivery, the Oralject extract solution and BSA were simultaneously delivered providing 75 or 125 mg of BSA in 15 or 25 ml Oralject extract solution, respectively for 1st and 2nd challenges, and delivered by gavage as described above.

Oralject control administrations

For Oralject control treatments, Oralject carrier was delivered alone either as a dry or wet preparation. For dry feeding, 15g or 25g carrier /bird was mixed with 6ml and 10ml water respectively and the resulting damp feed was provided in the feeder for the first and second challenges respectively. For the wet feeding, Oralject was mixed with water (18 ml of water / 15 g of carrier or 30 ml of water / 25 g of carrier) for the first and second challenges respectively. Similarly, for the Oralject extract solution control, 15ml and 25 ml for the first and second challenges, was delivered alone.

Commercial diet only

The birds receiving the commercial diet acted as the negative control treatment, receiving commercial diet (Broiler finisher crumbles, Ridley AgriProducts), only. No antigen or Oralject carrier was administered to these birds.

Timing of antigen and carrier delivery

In BSA administrations at 0 min after the carrier, the BSA was administered at the same time as the carrier. In the 30 min delivery Oralject extract solution treatment, 15 or 25 ml of Oralject extract solution with no BSA was initially administered. Thirty minutes later the BSA was delivered by gavage by dissolving 75 or 125mg of BSA/bird in 12 or 15 ml of water per bird, respectively for the 1st and 2nd challenge.

Table. 4. Description of EHNV and BSA antigen treatments, relative to carrier type, delivery route, administration time of antigen (gavage) following administration of carrier (if 0 min, the BSA was administered as part of the carrier), and amount of carrier/bird for the 1st and 2nd challenges, respectively, as well as the intended antigen delivery/bird for the 1st and 2nd challenges, respectively.

Antigen	Carrier Type (number of cages of 4 birds)	Delivery Route	Minutes after Carrier	g/bird carrier*		mg/bird antigen	
				1st	2nd	1st	2nd
No Antigen	Commercial Diet (n=2)	In feeder	0	15	25	0	0
No Antigen	Oralject dry (n=2)	In feeder	0	15	25	0	0
No Antigen	Oralject 1:1.2 water (n=2)	Gavage to crop	0	15	25	0	0
No Antigen	Oralject extract solution (n=2)	Gavage to crop	0	15	25	0	0
BSA	Distilled water (n=2)	Gavage to crop	0	12	15	75	125
BSA	Commercial Diet (n=3)	In feeder	0	15	25	75	125
BSA	Oralject dry (n=3)	In feeder	0	15	25	75	125
BSA	Oralject 1:1.2 water (n=2)	Gavage to crop	0	15	25	75	125
BSA	Oralject extract solution (n=3)	Gavage to crop	0	15	25	75	125
BSA	Oralject extract solution (n=3)	Gavage to crop	30	15	25	75	125
EHNV	Adjuvant (n=1 cage of 2 birds)	Intra muscular	0	0.5ml	0.5ml	0.0063	0.0063
EHNV	Distilled water (n=2)	Gavage to crop	0	15	15	0.01	0.01
EHNV	Commercial Diet (n=2)	In feeder	0	15	25	0.01	0.01
EHNV	Oralject dry (n=1)	In feeder	0	15	25	0.01	0.01
EHNV	Oralject 1:1.2 water (n=1)	Gavage to crop	0	15	25	0.01	0.01

* Oralject 1:1.2 water g/bird carrier is expressed on a dry feed basis

EHNV as an antigen

Preparation of inactivated EHNV antigen

The EHNV was produced by Professor R. Whittington, Farm Animal Health, Faculty of Veterinary Science, University of Sydney. EHNV was grown and partially purified using modifications of published methods (Steiner *et al.*, 1991; Whittington and Deece, 2004). Briefly, EHNV strain 86/8774 was inoculated onto BF-2 cell monolayers grown in a Nunc cell factory containing 1800 ml cell culture medium. Complete cytopathic effect was evident by day 5 and the viral preparation was decanted. The cell culture material was centrifuged at 12500 x g for 30 min at 4°C. The supernatant (SN1) was poured off and held. The pellet was resuspended in a small volume of supernatant, frozen on dry ice and thawed in a 37°C water bath. This was repeated twice. The pellet suspension was then

sonicated on wet ice using a probe sonicator for 1 minute then centrifuged at 6500 x g for 15 min at 4°C. The supernatant was pooled with SN1. A 5 ml aliquot was removed for viral enumeration and the remainder was stored overnight at 4°C. The supernatant was dispensed in 45 ml aliquots in 50 ml Falcon tubes and virus was inactivated by heating the tubes at 65°C in a water bath for 30 min. An aliquot of 1 ml was removed from three tubes and cultured to confirm inactivation. The Falcon tubes were then placed at -20°C for 1 month then -80°C for storage. The final volume of partially purified EHNV antigen was approximately 1.74 L.

Inactivation of EHNV was confirmed by inoculation of the antigen on BF-2 cell monolayers in 25 cm^2 flasks, with three passages at 1 week intervals. No cytopathic effect was observed. Cytopathic effect was observed in controls.

Batches of EHNV produced using Nunc cell factories described above contained a mean of 1.3 mg/1800ml viral protein, or 0.7 ug/ml, determined by Braford assay (Bradford, 1976). It can be assumed that the inactivated EHNV antigen preparation contains 0.7 ug/ml of EHNV protein.

Virus prior to inactivation was enumerated by end point titration in BF-2 cells and the count estimated using the method of Reed and Muench (Reed and Muench, 1938). The number of infectious viral particles was $10^{7.8}$ $TCID_{50}$/ml. The heat inactivated EHNV antigen can be assumed to contain about 100 million virions per ml.

Administration of EHNV antigen

Treatment groups using EHNV antigen are outlined in Table 4. On each occasion of oral administration of EHNV, each bird received 15mL of the viral preparation, which equated to approximately 10ug/ bird of EHNV protein. The dosage of EHNV was the same in both challenges 1 and 2.

EHNV delivered alone via gavage.

The oral EHNV challenge, with no carrier, was done by gavaging 15 ml of EHNV suspended solution per bird, again the same amount was used for the 1st and 2nd challenge.

EHNV delivered in dry diet (commercial or Oralject) carriers in feeder

EHNV delivery with feed (commercial diet (Broiler finisher crumbles, Ridley AgriProducts) or Oralject carrier) was conducted by mixing 15 ml of EHNV suspended solution in 15 and 25 g of feed, respectively for the 1st and 2nd challenges.

EHNV delivered in wet feed carrier via gavage

In the gavage treatments (wet feed carrier) the 15 ml/bird allotment of EHNV suspended solution was diluted with 3 and 15 ml of distilled water, respectively for the 1st and 2nd challenges and added to 15 or 25 g of feed (on a dry weight basis). The slurry like material was delivered via gavage as described in Study 1.

EHNV delivered via injection with adjuvant

The EHNV positive control treatment received a total of 6.3 ug EHNV/bird in oil adjuvant . To obtain this quantity of virus, one falcon tube containing 45 ml (31.5 ug EHNV protein) of heat inactivated EHNV antigen was thawed at room temperature and reduced to a volume of 2.5 ml by dialysis against carboxymethylcellulose. 1.25 ml of the concentrate was then made up to a final volume of 2.5 ml with sterile PBS. The antigen was mixed with an equal volume of Montainide ISA 50V oil adjuvant (Tall Bennett Group, Sydney), and an emulsion was prepared using repeated aspiration through a double syringe. The vaccine also contained thiomersal at 0.013%. Each bird received 1mL of vaccine which was delivered via intramuscular injection (approximately 0.2 ml at a time over five breast muscle points); as indicated previously to conserve EHNV antigen only two birds were intramuscularly injected.

Oralject control administration and commercial diet only

The negative control (commercial diet - Broiler finisher crumbles, Ridley AgriProducts)) and Oralject control (both dry and wet fed) treatment groups are the same as those described using BSA as an antigen in Study 2.

Timing of antigen and carrier delivery

All EHNV administrations were delivered at the same time as the carrier diet.

Study Three: *Salmonella typhimurium* as an antigen

Preparation of killed Salmonella typhimurium antigen

Killed *Salmonella typhimurium*, phage type 12, was the only antigen used in Study 3. A live broth culture of *S. typhimurium* (1 x 10^8/ml) was received from Birling Avian Laboratories, Bringelly. These samples were inactivated by the addition of formalin to a final concentration of 1:80 (1.2%). Inactivation was confirmed by plating onto tryptose agar plates and incubating for 48 hours at 37^0C. The samples were then washed twice, by centrifugation 11,000 g for 20 minutes. The supernatant was discarded and the bacteria were resuspended in sterile PBS to a final concentration of 1x 10^8 / 0.5 ml.

Administration of S. typhimurium antigen

Table 5 outlines the treatments utilised in Study 3. This includes details of the carrier used, delivery routes, administration time of antigen following administration of carrier, and the amount of carrier and antigen delivered to each bird in challenges 1 and 2. Treatment administrations for the Salmonella antigen materials were similar to those described previously for Study 1 and 2. Please note that for ease of expression, the preparation of *S. typhimurium* treatments described are for one bird only, and these numbers were adjusted depending on the number of birds in each cage.

S. typhimurium delivered alone via gavage.

For the delivery of *S. typhimurium* alone via gavage, 0.5 ml or 1 mL *S. typhimurium* preparation was mixed with in 11.5 mL water for the first challenge and 14 ml water for the second challenge. Delivery via gavage to the crop was as described in Study 1.

S. typhimurium delivered in dry diet (commercial or Oralject) carriers in feeder

To administer the dry diets (commercial diet (Broiler finisher crumbles, Ridley AgriProducts) or Oralject carrier) 0.5 ml (1 x 10^8) or 1 ml (2 x10^8) of *S. typhimurium* were first diluted with 5.5 or 9 ml of distilled water, before mixing with 15 or 25 g of dry ingredient, respectively for the 1st and 2nd challenges. This provided similar damp consistency as tested in Study 1 and 2.

S. typhimurium delivered in wet feed carrier via gavage

In the gavage delivered wet fed diets, the same volume of *S. typhimurium*, that is 0.5 ml and 1ml was mixed with 17.5 and 29 ml distilled water for each 15 or 25 g of dry feed, respectively for the 1st and 2nd challenges.

S. typhimurium delivered with Oralject extract solution carrier

For the Oralject extract solution, 0.5 or 1 ml of *S. typhimurium* was mixed with 14.5 or 24 ml of Oralject extract solution, respectively for the 1st and 2nd challenges, and delivered via gavage.

S. typhimurium delivered via injection with adjuvant

The positive *S. typhimurium* treatment group received an intramuscular injection of *S. typhimurium* in oil adjuvant. The vaccine consisted of 10 ml of *S. typhimurium* preparation at a concentration of 1 x 10^8 /0.5ml which was emulsified with an equivalent volume of Montanide ISA 50V oil adjuvant (Tall Bennett Group, Sydney), using an ultra tarrax (John Morris Scientific). The vaccine also contained thiomersal at 0.013%. 1 ml of vaccine (1x 10^8 *S. typhimurium*), was administered to each bird by intra-muscular injection, and delivered as 5, 0.2 mL volumes, injected into the breast muscle at different sites.

Oralject control administrations

For Oralject control treatments, Oralject carrier was delivered alone either as a dry or wet preparation. For dry feeding, 15g or 25g carrier / bird was mixed with 6 and 10ml water respectively, and then the resulting damp mixture was provided in the feeder for the first and second challenges respectively. For the wet feeding, Oralject was mixed with water (18 ml of water / 15 g of carrier or 30 ml of water / 25 g of carrier) for the first and second challenges respectively. Similarly, for the Oralject extract solution control, 15ml and 25 ml for the first and second challenges, was delivered alone.

Commercial diet only

The birds receiving the commercial diet acted as the negative control treatment, receiving commercial diet (Broiler finisher crumbles, Ridley AgriProducts) only. No antigen or Oralject carrier was administered to these birds.

Timing of antigen and carrier delivery

When *S. typhimurium* was administered at 0 min after the carrier, the bacterial preparation was administered at the same time as the carrier. Alternatively if times were 30 or 120 min post administration of the Oralject carrier (delivered in feed as 6ml water mixed with 15g carrier or 10ml water mixed with 25 g carrier; or via gavage as 18 ml water / 15 g carrier or 30 ml of water / 25 g of carrier, for the first and second challenges respectively), the *S. typhimurium* was mixed with water (0.5 ml or 1ml in 11.5 or 14 ml water respectively, for challenges 1 and 2) and administered by gavage at the appropriate time following carrier delivery.

In the 30 min delivery Oralject extract solution treatment, 15 or 25 ml of Oralject extract solution with no *S. typhimurium* was initially administered. Thirty minutes later the *S. typhimurium* was delivered by gavage by mixing 0.5ml or 1 ml *S. typhimurium* (10^8 /0.5ml) into 11.5 or 14 ml of water per bird, respectively for the 1^{st} and 2^{nd} challenge.

Table. 5. Description of killed *Salmonella* antigen treatments, relative to carrier type, delivery route, administration time of antigen (gavage) following administration of carrier (if 0 min, the BSA was administered as part of the carrier), and amount of carrier/bird for the 1st and 2nd challenges, respectively, as well as the intended antigen delivery/bird for the 1st and 2nd challenges, respectively.

Antigen	**Carrier Type (number of cages of 4 birds)**	**Delivery Route**	**Time after Carrier**	**g/bird carrier***		***Salmonella typhimurium*/bird**	
				1st	**2nd**	**1st**	**2nd**
No Antigen	Commercial Diet (n=2)	In feeder	0	15	25	0	0
No Antigen	Oralject dry (n=1)	In feeder	0	15	25	0	0
No Antigen	Oralject 1:1.2 water (n=1)	Gavage to crop	0	15	25	0	0
S. typhimurium	Adjuvant (n=2)	Intra muscular	0	0.5ml	0.5ml	$1x10^8$	$1x10^8$
S. typhimurium	Distilled water (n=5)	Gavage to crop	0	12	15	$1x10^8$	$2x10^8$
S. typhimurium	Commercial Diet (n=5)	In feeder	0	15	25	$1x10^8$	$2x10^8$
S. typhimurium	Oralject (n=5)	In feeder	0	15	25	$1x10^8$	$2x10^8$
S. typhimurium	Oralject 1:1.2 water (n=5)	Gavage to crop	0	15	25	$1x10^8$	$2x10^8$
S. typhimurium	Oralject 1:1.2 water (n=3)	Gavage to crop	30	15	25	$1x10^8$	$2x10^8$
S. typhimurium	Oralject dry (n=3)	Oralject in feeder, *S. typhimurium* via gavage	120	15	25	$1x10^8$	$2x10^8$
S. typhimurium	Oralject extract solution (n=3)	Gavage to crop	0	15	25	$1x10^8$	$2x10^8$
S. typhimurium	Oralject extract solution (n=3)	Gavage to crop	30	15	25	$1x10^8$	$2x10^8$

* Oralject 1:1.2 water g/bird carrier is expressed on a dry feed basis

Sample Collection

Blood samples (1.5ml) were collected from the jugular vein of each chicken on day -1, day 14 (pre-booster bleed), day 22, day 29 and day 36. Serum was prepared and stored at -20°C until assayed for antibody determination.

On day 22 half of the birds from each treatment group selected at random were euthanatized by intravenous administration of sodium pentobarbitone. Samples of intestinal scrapings were collected from the length of the jejunum after the serosal and mucosal surfaces had been washed in ice cold PBS. The samples were immediately frozen on dry ice and stored at -80°C. The intestinal scrapings supernatant (ISS) was collected after the samples were thawed and subjected to ultracentrifugation at 24,000 *g* for 90 min. The supernatant was stored at -80°C until assayed for antibody determination. Bile was aspirated from the gall bladder and was immediately frozen on dry ice and then stored at -80°C until assayed for antibody determination. These same procedures were used for the collection and storage of samples of serum, intestinal scrapings and bile from the remaining birds on day 36.

In all studies sera was analysed for anti-antigen IgA and IgG titres using an ELISA. Samples of bile and ISS from birds immunised with BSA were also assayed for anti-BSA IgA titres, however

problems with a non-specific binding and high background colour in the ELISA could not be resolved. Hence these results are not included in the overall review of project outcomes. Samples of ISS from birds immunised with *S. typhimurium* were analysed for anti-*S. typhimurium* IgA. Unfortunately assays for testing anti-*S. typhimurium* IgA in bile and anti-EHNV IgA in bile and ISS could not be run as significant non-specific background absorbance was experienced. Several procedures were tested to try to eliminate background colour but without success. Below the description of each ELISA assay methods used in an attempt to reduce the background absorbance and, the outcome of that procedure, are presented. Several different procedures were tested with varying levels of success. Please note that these techniques were initially tested with only a subset of randomly chosen samples, from which the technique which demonstrated the best reduction in background was selected for use with all test samples. However, in some instances, once assaying of all samples was completed and the results collated, it became apparent that background absorbance remained a problem.

Antibody detection by ELISA

Anti-BSA IgA

BSA specific antibody titres in serum, bile and intestinal scrapings supernatant (ISS) were determined by an ELISA. Nunc Immuno (Medos Company, Australia) plates were coated with 0.5 µg per well BSA in carbonate coating buffer (pH 9.6) and incubated overnight at 4^{o}C. All subsequent incubations were for 1hr at 37^{o}C unless otherwise stated. Between each incubation plates were washed twice in avian washing buffer (0.05% Tween 20 (Sigma-Aldrich, Inc., St Louis, MO, USA) and 0.5M sodium chloride in PBS) (AWB) and then double distilled water, before being tapped dry. Plates were blocked with 1% gelatine (Labtech, Ajax Chemicals, Australia) in carbonate coating buffer at 37 ^{o}C for 1.5 hours. To reduce non-specific binding due to cross-reactions between BSA and chicken serum albumin, all samples (serum, bile and ISS) were pre-incubated 1:1 overnight at 4^{0}C with 6% egg albumin (Sigma-Aldrich, Inc., St Louis, MO, USA) (Ameiss *et al*., 2005) in AWB. Serum was then diluted 1:10, bile 1:50 and ISS 1:10 in AWB for incubation on the plate. Horseradish peroxidise-conjugated goat anti-chicken IgA (Bethyl Laboratories, Montgomery, TX, USA) was diluted 1:250 in AWB. Neat 2.2'-azino-d:[3-ethyl-benzthiazoline sulfonate] (ABTS) substrate (Kirkegaard and Perry Laboratories (KPL), Gaithersburg, MD, USA) was incubated at room temperature for 30 min and the reaction stopped with 20% ABTS peroxidase stop solution (KPL, Gaithersburg, MD, USA) diluted in MilliQ water.

Absorbance values were read at 405nm using a Labsystems Multiscan ELISA Reader. A negative buffer blank, negative reference serum and a hyperimmune positive reference standard were included in each plate, and all samples were analysed in duplicate. The working sample dilution for each sample type and observation day was determined by the titrated assay of several samples from each treatment group, chosen at random, along with positive and negative control samples. The working dilution was selected due to its relative sensitivity between the optical density readings of the positive and negative samples. That is, there was low non-specific activity in the negative samples, but high specific activity in the positive samples, the latter corresponding with the linear portion of the hyperimmune positive control standard curve, from approximately 20% below saturation point. Optical densities of the test samples were expressed as a percentage of the anti-BSA IgA hyperimmune positive control. Typically absorbance values below 15% of the positive control are no longer on the linear portion of the curve due to the low concentration of antibody at these points. Samples with absorbance values below 15% hyperimmune are therefore, for the purposes of this assay, considered to be antibody negative. The absorbance of the negative reference serum was run within this range.

Investigation of techniques to reduce background in the anti-BSA IgA ELISA

In addition to titrating the dilution of the coating antigen, sample and conjugate, several techniques were assessed for their ability to reduce and eliminate the non-specific binding in the anti-BSA IgA ELISA for sera, bile and ISS samples. They are presented in the following tables which also outline the outcome of the technique. Please note that these techniques were tested with only a subset of randomly chosen samples.

Blocking buffers: The blocking buffers outlined in Table 6, were tested in place of 1% gelatine in coating buffer.

Table 6: Blocking buffers

Active	Concentration (%)	Outcome
PBS Tween 20 overnight at 4^{o}C and then PBS Tween 20 & Gelatine overnight at 4^{0}C.	Tween 20, 0.05% (Sigma-Aldrich Inc. St Louis, MO. USA. Gelatine 1% (Labtech, Ajax Chemicals, Sydney, Australia).	Increased absorbance in all samples.
PBST with rabbit serum (Donated by Dr Jeff Downing, Veterinary Science Faculty, University of Sydney) (Ogawa *et al.*, 2005).	10% rabbit serum	Reduced absorbance for positive controls, Increased absorbance in test samples and blanks.
Polyvinylpyrrolidone (PVP) (Sigma-Aldrich, Inc., St Louis, MO, USA), (Wang *et al.*, 2006)	1% PVP in PBST 2% PVP in PBST	Increased absorbance in all samples Increased absorbance in all samples.
AWB + skim milk powder solution (KPL, Gaithersburg, MD, USA) (Klipper *et al*., 2001)	1:20 incubated for 2 hrs	Increased absorbance in all samples

None of the tested blocking buffer combinations eliminated the non-specific binding.

Sample diluents: The samples diluents outlined in Table 7 were tested in place of AWB.

Table 7: Sample Diluents

1. AWB diluent containing		
Active	**Concentration**	**Outcome**
Gelatine (Labtech, Ajax Chemicals, Australia)	5%	No effect
Tween 20 (Sigma-Aldrich, Inc., St Louis, MO, USA)	5%	No effect
Coffee Mate (Nestle Australia Ltd, Sydney, Australia)	5%	All readings, including positive controls, very low
Egg Albumin (Sigma-Aldrich Inc., St Louis, MO, USA)	5%	All readings, including positive controls, very low
Skim milk powder (Woolworths Homebrand, Sydney, Australia) (Fukanoki *et al.*, 2000)	1, 2.5 and 5%	All readings, including positive controls, very low
Caesin (MPD Dairy Products, Sydney, Australia)	1, 2.5 and 5%	All readings, including positive controls, very low
2. PBS + 0.05% Tween 20 diluent containing:		
Active	**Concentration**	**Outcome**
Gelatine	10%	No effect

None of the tested diluents eliminated the non-specific binding and background.

Pre-incubation of conjugate:

Pre-incubation of the conjugate with solutions listed in Table 8 were also tested as a possible way of reducing non-specific binding in the anti-BSA IgA assay of serum, bile and ISS.

Table 8: Preincubation of conjugate with		
Active	**Concentration**	**Outcome**
BSA (Tracebiosciences, New Zealand)	0.1% in coating buffer for 1 hr @37^{o}C	No effect
Goat serum (donated by Dr V. Reeve, Faculty of Veterinary Science, University of Sydney)	Neat 1:1 for 1 hr @37^{o}C	Reduced absorbance in positive controls, increased absorbance in test samples.
Sheep serum (Farm Animal Health Laboratory, Faculty of Veterinary Science, University of Sydney).	Neat 1:1 for 1 hr @37^{o}C	Reduced absorbance in positive controls (but less than with goats serum), increased absorbance in test samples.

None of the tested pre-incubation protocols eliminated the non-specific binding.

Pre-incubation of samples overnight at 4^{0} C:

Pre-incubation of the sample overnight at 4^{0} C with egg albumin (Ameiss *et al*., 2005) (Table 9) were also tested as a possible way of reducing non-specific binding in the anti-BSA IgA assay of serum, bile and ISS.

Table 9: Pre-incubation of samples overnight at 4^{0}C		
Active	**Concentration**	**Outcome:**
Egg albumin (Sigma-Aldrich Inc., St Louis, MO, USA)	2%	Some reduced absorbance in test samples
Egg albumin	4%	Some reduced absorbance in test samples
Egg albumin	6%	Reduction in absorbance in test samples, and negative controls into negative range
Egg albumin	8%	Reduction in absorbance in test samples, and negative controls into negative range
Egg albumin	10%	Reduction in absorbance in test samples, and negative controls into negative range

As the pre-incubation of the samples with 6% egg albumin was the lowest concentration of egg albumin that reduced the non-specific binding in the test samples this was the selected concentration used with all serum, bile and ISS samples assayed for anti-BSA IgA titres. It is understood that, as there is some homology between BSA and egg albumin, pre-incubation of the samples with egg albumin will absorb the cross-reactive antibodies (Ameiss *et al*., 2005). However, as seen in Figures 11and 12this step failed to eliminate all non-specific binding in the assay for all ISS and bile samples tested respectively.

Anti-BSA IgG

Ant-BSA IgG was analysed only in the serum samples. The procedure was identical to that described for anti-BSA IgA, except that no pre-incubation of sera with egg albumin was required. Horseradish peroxidise-conjugated goat anti-chicken IgG (Bethyl Laboratories, Montgomery, TX, USA) was diluted 1:50000 with AWB. Test sample optical densities were expressed as a percentage of the hyperimmune positive control serum from birds specifically hyperimmunised for BSA IgG, following identical criteria to the anti-BSA IgA assay

Anti-EHNV IgA

EHNV specific antibody titre in serum was determined by a sandwich ELISA. Linbro 96 well microtitre plates (ICN Biomedicals Inc., Horsham, PA 19044) were coated with affinity purified rabbit

anti-EHNV antibody (freeze dried capture antibody was dissolved in 1ml sterile distilled water and 9ml TSGM (TS = TRIS Saline(50ml - pH 7.4), G = Glycerol (250ml)+ MQ water (500ml) , M = Merthiolate (1ml)) diluted 1:12800 in borate coating buffer and incubated overnight at 4^{o}C. All subsequent incubations unless otherwise mentioned were for 90min. at RT. Between each incubation, plates were washed five times with washing solution (RO water with 0.05% Tween 20) in a plate washer (Tecan 96 – PW), then tapped dry. After washing and drying the plates 100 µl neat EHNV control antigen (heat killed) / well was added to the plates in a Class II biological safety cabinet. After incubation the plates were hand washed twice (to avoid contamination of the plate washer) in the biological safety cabinet with wash solution and after flicking off the contents of the wells into a waste container having medol or 70% ethanol, plates were tapped dry and then washed in the normal way in the plate washer. Plates were then blocked with 1% gelatin in PBSTG (PBS + 0.05% Tween 20 + 0.1% gelatin), incubated for 30 min at RT. Serum was diluted 1:10 in PBSTG + 5%BSA. HRP conjugated goat anti-chicken IgA was diluted to 1:500 in PBSTG + 5%BSA. ABTS substrate {Citric acid 21g + disodium hydrogen orthophosphate 14g + 2 2'-azino–bis. (3-ethylbenzothiozolin–6–sulfonic acid) diammonium salt 0.55g and MQ water to 1000ml} with 0.045% H_2O_2 was added to the wells and the plates were incubated on a plate shaker (Platform shaker – Ratek Instruments Private Ltd, Bornia, Victoria, Australia) for 20 min at RT. ABTS stop solution (H_2O_2 - 30% extra pure – Riedel Haen) was added immediately and kept briefly on the plate shaker.

Absorbance values were read at 405nm using a Labsystems Multiscan ELISA Reader. A negative buffer blank and a hyperimmune positive reference standard were included in each plate, and all samples were analysed in duplicates. Sample dilutions were adjusted to provide optical density readings on the linear portion of the standard curve, approximately 20% below saturation point. Optical densities of the test samples were expressed as a percentage of the anti-EHNV IgA hyperimmune positive control. Typically absorbance values below 15% of the positive control are no longer on the linear portion of the curve due to the low concentration of antibody at these points. Samples with absorbance values below 15% hyperimmune are therefore, for the purposes of this assay, considered to be antibody negative. The absorbance of the negative reference serum was run within this range.

Investigation of techniques to reduce background in the anti-EHNV IgA ELISA.

In addition to titrating the dilution of the capture antibody, antigen, sample and conjugate, several other techniques were also tested for their ability to reduce background in the anti-EHNV IgA ELISA.

Sera Sample Diluents:

Several sample diluents were tested in place of PBSTG, for their ability to reduce non-specific binding in the anti-EHNV IgA ELISA for samples of sera. These are outlined in Table 10.

.

Table 10: Sample diluents

1. AWB as diluent with		
Active	**Concentration**	**Outcome**
BSA, (Tracebiosciences, New Zealand)	1%	No effect
BSA	5%	No effect
2. PBSTG as diluent with		
Active	**Concentration**	**Outcome**
BSA	1%	No effect
BSA	5%	Reduction in test samples and negative controls into negative range.

The inclusion of 5% BSA with the PBSTG as the diluent for serum samples was seen to reduce background colour in the test samples and therefore was the sera sample diluent used throughout the study as outlined in the above method. However, once all of the data on all sera samples was collated,

it was noted that the absorbance in what would be expected to be EHNV antibody negative samples, was higher than 15% hyperimmune.

For samples of bile and ISS another series of test samples were run to investigate possible methods of reducing background colour.

Samples of Bile and ISS

Sample diluents:

The samples diluents outlined in Table 11 were tested in place of PBSTG.

Table 11: Sample diluents

1. AWB as diluent with		
Active	**Concentration**	**Outcome**
BSA, (Tracebiosciences, New Zealand)	1% and 5%	No effect
2. PBSTG as diluent with		
Active	**Concentration**	**Outcome**
BSA	5%	No effect
3. PBSTG5% BSA as diluent with		
Active	**Concentration**	**Outcome**
Carnation skim milk powder (Nestle Australia Ltd. Sydney, Australia)	1% and 5%	No effect with bile samples All ISS samples negative Reduction in absorbance of positive controls.
AWB + skim milk powder solution (KPL, Gaithersburg, MD, USA)	1:10 and 1:20	No effect with bile samples All ISS samples very low readings Reduction in absorbance of positive controls.
Sheep serum (Farm Animal Health Laboratory, Faculty of Veterinary Science, University of Sydney).	1% and 5%	No effect with bile samples All ISS samples very low or negative readings, Reductions in absorbance of positive controls

None of the tested diluents eliminated the non-specific binding.

Pre-incubation of samples:

Due to the success of sample pre-incubation in the BSA assay in reducing non-specific binding, this was also tested for anti-EHNV IgA in bile and ISS. Test samples were pre-incubated with solutions listed in Table 12.

Table 12: Pre-incubation of samples

Active	**Concentration**	**Outcome**
Egg albumin ((Sigma-Aldrich Inc., St Louis, MO, USA)	6% , 1: 1 at room temp for 1 hr	No effect
Egg albumin ((Sigma-Aldrich Inc., St Louis, MO, USA)	6% , 1: 1, 4 C overnight	No effect
Rabbit serum (Donated Dr J. Downing, Veterinary Science Faculty, University of Sydney.)	neat, 1 : 0.5 at room temp for 1 hr	No effect
Rabbit serum	neat, 1 : 1 at room temp for 1 hr	No effect

None of the pre-incubation procedures showed any potential to reduce the background colour in the EHNV assay.

At this stage all of the serum samples had been tested for anti-EHNV IgG and IgA. As none of the Oralject treatments demonstrated any notable effects on the levels of circulating antibodies, and due to time limitations, it was decided that no further work would be undertaken to fine-tune the anti-EHNV IgA assay in bile and ISS.

Anti-EHNV IgG

Anti-EHNV IgG was analysed only in the serum samples. The procedure was identical to that described for anti-EHNV IgA in serum, except that serum was diluted in PBTSG only and the horseradish peroxidise-conjugated goat anti-chicken IgG was diluted 1:30000 with PBSTG . Optical densities were expressed as a percentage of the anti-EHNV IgG hyperimmune positive control, following identical criteria to the anti-EHNV IgA assay.

Anti-*S. typhimurium* IgA

The *Salmonella typhimurium* specific IgA antibody was assessed in samples of serum and intestinal scrapings supernatant (ISS) using an indirect ELISA. Nunc Immuno (Medos Company, Australia) plates were coated with 50µl per well with lipopolysaccharides from *S.enterica* serotype *typhimurium* as antigen (Sigma-Aldrich, Inc., St. Louis, MO, USA Sigma) (1:100 dilution of 2mg/ml of lipopolysaccharide stock) in carbonate coating buffer and incubated overnight at 4^{o}C. All subsequent incubations were for 1h at 37^{o}C unless otherwise mentioned. Between each incubation, plates were washed twice in AWB and double distilled water and then tapped dry. For serum samples, each plate was blocked with 1% gelatine in carbonate coating buffer and incubated at 37^{o}C for 1.5 hours. For intestinal scrapings samples each plate was blocked with 1% gelatine and 5% BSA in carbonate coating buffer and incubated at 37^{o}C for 1.5 hours Serum samples were diluted 1:15 and ISS were diluted 1:10 in AWB containing 1% BSA (AWB1%BSA). Horseradish peroxidise-conjugated goat anti-chicken IgA was diluted 1:200 with AWB1%BSA. Neat 2.2'-azino-d:[3-ethyl-benzthiazoline sulfonate] (ABTS) substrate was incubated at room temperature for 30 min and the reaction stopped with 20% ABTS peroxidase stop solution diluted in MilliQ water.

Absorbance values were read at 405nm using a Labsystems Multiscan ELISA Reader. A negative buffer blank and a hyperimmune positive reference standard were included in each plate, and all samples were analysed in duplicates. Sample dilutions were adjusted to provide optical density readings on the linear portion of the standard curve, approximately 20% below saturation point. Optical densities of the test samples were expressed as a percentage of the anti- *S. typhimurium* IgA hyperimmune positive control. Typically absorbance values below 15% of the positive control are no longer on the linear portion of the curve due to the low concentration of antibody at these points. Samples with absorbance values below 15% hyperimmune are therefore, for the purposes of this assay, considered to be antibody negative. The absorbance of the negative reference serum was run within this range.

Investigation of techniques to reduce background in the anti-*S. typhimurium* IgA ELISA for bile and ISS samples.

Problems with background colour in the *S. typhimurium* IgA ELISA assay were unexpected as identical assays have been run previously (Muir *et al.,* 1998). However, samples of bile and ISS demonstrated non-specific binding. Therefore, in addition to titrating the dilutions of the coating antigen, samples and conjugate, several techniques were investigated in an attempt to reduce background absorbance. Due to the relatively high levels of IgA in bile, the bile samples were tested at dilutions ranging from 1/50 to 1/500 with each of the following techniques.

Sample diluent:

Several sample diluents were tested in place of AWB1% BSA as a possible means of reducing non – specific binding in bile and ISS samples (Table 13).

Table 13: Sample dilutions		
AWB as a diluent with:		
Active	**Concentration**	**Outcome**
BSA, (Tracebiosciences, New Zealand	1, 2 and 5%	No effect
Carnation skim milk powder (Nestle Australia Ltd, Sydney, Australia)	0.5, 1, 2 and 5%	No effect with bile samples Some reduction in absorbance in ISS and reductions in control absorbance, little or no effect on negative controls.
Sheep serum, (Farm Animal Health Laboratory, Faculty of Veterinary Science, University of Sydney).	neat	Interference with absorbance in positive controls, low readings in all bile and ISS samples.

As seen in Table 13 none of the tested sample diluents successfully eliminated background.

Sample pre-incubation:

Samples were pre-incubated overnight at 4^0 C with egg albumin, as outlined in Table 14, without success.

Table 14: Pre-incubation of samples overnight at 4^0C with		
Active	**Concentration**	**Outcome:**
Egg albumin, (Sigma-Aldrich Inc., St Louis, MO, USA)	6%	No effect

Blocking buffers:

The blocking buffers outlined in Table 15 were tested in place of 1% gelatine in carbonate coating buffer.

Table 15 : Blocking buffers		
Coating buffer + 1% gelatine &		
Active	**Concentration (%)**	**Outcome:**
BSA	5%	No effect with bile samples Reduction in absorbance in test ISS samples, negative controls into negative range.
Egg albumin	5%	Increased absorbance in most bile samples Most ISS samples negative absorbance.

The addition of 5% BSA to the carbonate coating buffer with 1% gelatine successfully eliminated background colour in the anti-*S. typhimurium* IgA assay with samples of ISS.

Unfortunately neither of the above combinations of blocking buffers was successful with the bile samples. Again, due to time limitations and little indication of any notable effects of Oralject treatments on local antibody titres in ISS, no further analysis of bile samples was undertaken.

Anti-*S. typhimurium* IgG

Anti-*S. typhimurium* IgG was analysed only in the serum samples. Plates were blocked using 1% gelatine in carbonate coating buffer and incubated at 37^oC for 1.5 hours. Serum samples were diluted 1:10 with AWB1%BSA. Horseradish peroxidise-conjugated goat anti-chicken IgG was diluted 1:50000 with AWB1% BSA. Optical densities of the test samples were expressed as a percentage of the anti-*S. typhimurium* IgG hyperimmune positive control, following identical criteria to the anti-S. *typhimurium* IgA assay.

Generation of hyperimmune serum

Hyperimmunisation of chickens with BSA

Sixteen week old layer hens were immunised for the production of the serum and ISS containing high titres of anti-BSA IgG and IgA. BSA was dissolved in sterile phosphate buffer saline (1mg/ml of PBS) and emulsified using an Ultra Tarrx (John Morris Scientific) with an equal volume of Freund's incomplete adjuvant (Bacto Laboratories, Sydney, Australia). Prior to vaccination blood was collected from each bird and used as the negative reference serum in the ELISA assay. Birds received a 1mL intraperitoneal (IP) vaccination of the vaccine on two occasions, two weeks apart. One week later each bird received an oral booster (1mL in volume) of BSA dissolved in sterile PBS (1mg/ml). Prior to delivery of the oral booster blood was collected from each bird for retrieval of serum containing anti-BSA IgG antibody. One week after the delivery of the oral booster blood was collected for the retrieval of serum containing anti-BSA IgA antibody. Blood samples were collected from the jugular vein into an SST vacutainer, allowed to clot and then centrifuged at 3000rpm (at 4°C) for 10 minutes. The serum was separated and stored at -20°C until assayed by the ELISA.

One week after delivery of the oral booster all birds were euthanatized by intravenous administration of sodium pentobarbitone for the collection of ISS. Samples of intestinal scrapings were collected from the length of the jejunum after the serosal and mucosal surfaces had been washed in ice cold PBS. The samples were immediately frozen on dry ice and stored at -80^{o}C. The intestinal scrapings supernatant was collected after the samples were thawed and subjected to ultracentrifugation at 24,000 *g* for 90 min. The supernatant was stored at -80^{o}C until assayed for antibody determination

Hyperimmunisation of chickens with *S. typhimurium*

An identical protocol to that used for the hyperimmunisation of chickens with BSA antigen, was used for hyperimmunisation of chickens with *S. typhimurium*. With *S. typhimurium*, each dose of vaccine contained 1 x 10^{8} killed *S. typhimurium* suspended in PBS.

Hyperimmunisation of chickens with EHNV

Due to the limited amount of EHNV antigen, only six chickens were hyperimmunised, three for anti-EHNV IgG and three for anti-EHNV IgA titres. Prior to the primary immunisation blood was collected from each bird, and the serum from these samples was used as the negative reference serum.

For each group of three birds, one falcon tube containing 45 ml of heat inactivated EHNV antigen was thawed at room temperature and reduced to a volume of 2.5 ml by dialysis against carboxymethylcellulose then made up to a final volume of 5 ml with sterile PBS.

Vaccination for anti-EHNV IgG antibodies

The antigen was mixed with an equal volume of Montainide ISA 50V oil adjuvant (Tall Bennett Group, Sydney) then homogenised using repeated aspiration through a double syringe. Each hen was immunised at 4 sites in the breast muscle (0.5 ml/site) with adjuvanted EHNV (approximately 6.3 ug EHNV protein/bird) as the primary immunisation. Four weeks later two of the three birds were given a booster dose with a second batch of adjuvanted antigen which had been was prepared in a similar manner to the primary vaccine. The booster vaccine was also delivered via intramuscular injection.

Vaccination for anti-EHNV IgA antibodies

For the birds used to generate anti-EHNV IgA the two primary doses were prepared by emulsifying an equal volume of the antigen with Freund's incomplete adjuvant, and then 2 mL of that vaccine was delivered via intraperitoneal injection (approximately 6.3 ug EHNV protein/bird). For the booster immunisation 3 mL of the resuspended dialysed antigen was made up to a final volume of 6mL with sterile PBS, and each bird received 2 mL of the suspension, orally, delivered via gavage (approximately 10ug EHNV protein/bird).

For both anti-EHNV IgG and IgA birds, the third bird did not receive the booster vaccination, to provide a comparison of the antibody titres between birds receiving only the primary immunisations and those receiving the primary and booster immunisations. Blood samples were collected before vaccination and at weekly intervals after vaccination, for a total of six weeks after the booster immunisation. Serum was harvested, aliquots were diluted 1:10 in 50% glycerol (TSGM) and stored at -20°C pending analysis. On the final collection day all birds were euthanized and samples of intestinal scrapings collected and processed as described for hyperimmunisation with BSA.

Statistical analysis

Associate Professor Peter Thomson, Faculty of Veterinary Science, University of Sydney, Camden, generously donated his time to evaluate the statistical validity of the study prior to it starting (though several late changes to the experimental design were made at the request of PerOs Systems Technologies following the initial evaluation by Prof Thomson), and the actual analysis of data.

Due to the positive skewed distribution of each of the variables, the data were log-transformed prior to analysis. Each variable was analysed by fitting a mixed model, with random effects specified for Cage and Bird within Cage. A Restricted Maximum Likelihood (REML) procedure was used for fitting the mixed model. Comparison of treatments means on specific days was conducted by *t*-tests using the model-based predicted means and the outputted standard error of the difference between two means (SEDs). All analyses were undertaken using GenStat Release 10 (Lawes Agricultural Trust, 2007). Due to the large number of variables observed, the p-value was set at <0.01, to minimise the chance of identifying differences that are not biologically significant. Identification of treatments that induced a statistically significant difference for an observation on one day have been identified by the inclusion of superscripts above the columns on the relevant graph. Where no significant differences were identified no superscripts are included on the relevant graph.

Results

Immunisation with BSA (Studies 1 and 2)

Outline of treatment group abbreviations:

For ease of reference, Table 16 outlines the treatment groups included in BSA studies one and two of this project.

Table 16: Treatment group abbreviations

Abbreviation	**Treatment group**
BSA IM	BSA in adjuvant, intramuscular injection.
CBD	Commercial broiler diet alone
BSA F	BSA dry fed alone
BSA G	BSA wet, gavage alone
OJCF	Oralject control dry fed alone
OJ+BSA F	Oralject + BSA dry fed
OJF2hrsBSA G	Oralject dry fed, 2 hrs later BSA wet gavage
OJCG	Oralject control wet gavage alone
OJ+BSA G	Oralject + BSA wet gavage
OJG30minBSA G	Oralject wet gavage, 30 mins later BSA wet gavage
OJSnC	Oralject extract solution control gavage
OJSn+BSA G	Oralject extract solution + BSA wet gavage
OJSn30minBSA G	Oralject extract solution gavage, 30 mins later BSA wet gavage.

Serum anti-BSA antibody

Determining test sample working dilutions

As outlined in the Methodology section: Antibody detection by ELISA, anti-BSA IgG, the working sample dilution for each sample type and observation day were determined following the assaying of several samples from each treatment group, chosen at random, and positive and negative samples. The working dilution was selected because of its relative sensitivity between the optical density readings of the positive and negative samples. That is, there was low non-specific activity in the negative samples, but high specific activity in the positive samples, the latter falling within the linear portion of the standard curve, from approximately 20% below the upper saturation point. A typical hyperimmune positive control standard curve for anti-BSA IgG is presented in Figure 1. The linear portion of the curve (R^2 = 0.998), extends from absorbance reading of 1.418 OD (dilution 1:3200), to absorbance reading of 0.205 OD (dilution 1:51200), the lowest absorbance on the linear portion of the standard curve. The absorbance 1.121 OD (dilution 1:6400) was selected as the absorbance closest to 20% below saturation, and was therefore taken as 100% hyperimmune or the anti-BSA IgG hyperimmune positive control. The negative control sera generated absorbance values of 0.089 OD (dilution 1:3200) and 0.070 OD (dilution 1:51200).

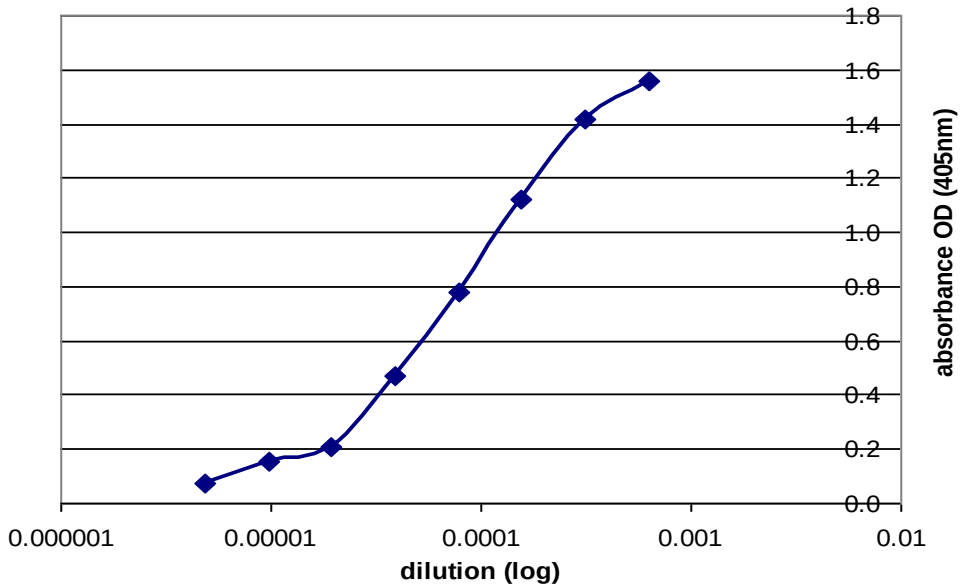

Figure 1: Titration curve for anti-BSA IgG hyperimmune positive control serum.

A series of titrated dilutions of test samples (typically including 1/5, 1/10, 1/25 and 1/50) were run for each treatment observation on each day to identify the ideal working dilution such that the absorbance value of positive samples corresponded to the linear portion of the standard curve, enabling direct comparison of antigen-specific antibody titres between treatment groups. The outcome of a subset of these from Day 22 sera tested for anti-BSA IgG are presented in Table 17.

Table 17 : Titrated sample dilutions of Day 22 sera for anti-BSA IgG				
Treatment group	**Absorbance (OD) at 405 nm for the following sample dilutions.**			
	1:5	**1:10**	**1:25**	**1:50**
BSA IM	1.342	1.003	0.578	0.299
CBD	0.095	0.089	0.078	0.081
OJ+BSA F	0.213	0.162	0.094	0.063
OJSn+BSA G	1.052	0.839	0.435	0.216
OJSn30minBSA G	0.628	0.456	0.243	0.177
Negative sera	0.070	0098	0.061	0.068

BSA IM: BSA in adjuvant, intramuscular injection; CBD: commercial broiler diet; OJ+BSA F: Oralject + BSA dry fed; OJSn+BSA G: Oralject extract solution + BSA wet gavage; OJSn30minBSA G: Oralject extract solution gavage, 30 mins later BSA wet gavage.

From Table 17, it can be seen that the 1:5 dilution generates OD readings for BSA IM birds (1.342) above 100% of the hyperimmune positive control reference standard (1.121 OD), and the 1:25 and 1:50 dilution reduces the absorbance of this and the other positive samples (OJSn+BSA G and OJSn30minBSA G), hampering the ability to make clear comparisons between relative levels of positivity. However, 1:10 allows distinction between the levels of absorbance in the positive birds, while the absorbance from negative samples essentially corresponds with absorbance from negative sera (and falls within the absorbance range considered to be negative i.e. 15% hyperimmune or less). Therefore, the day 22 sera samples were run at 1:10 for determination of anti-BSA IgG titres. Using this criteria 1:10 was suitable for measuring all sera samples for anti-BSA IgG from day -1 to day 36. This then allowed for comparison of treatment group titres across the entire study period, in addition to the between treatment group comparisons on any one day.

The procedure described above was utilised for establishing the working dilutions for all assays described in this report.

BSA studies one and two – comparison of day of treatment delivery.

Several treatment groups, some unimmunised and others immunised with BSA using a variety of carriers, were included in both studies one and two to directly compare any effect of day of treatment. The treatment groups and number of pens of each treatment group included in either study 1 or 2 are listed in Table 18.

Table 18: Treatment groups in study 1 and 2.		
Treatment	**Number of cages, of 4 birds each, in each study**	
	1	**2**
CBD	2	2
OJC F	2	2
OJC G	2	2
BSA F	2	3
BSA G	3	2
OJ+BSA F	2	3
OJ+BSA G	3	2

CBD: commercial broiler diet; OJC F: Oralject control dry fed; OJC G: Oralject control wet gavage; BSA F: BSA dry fed; BSA G: BSA wet gavage; OJ+BSA F: Oralject + BSA dry fed; OJ+BSA G: Oralject+BSA wet gavage.

The mean IgG and IgA antibody titres to BSA in serum were compared between study 1 and study 2 for each treatment group listed in Table 18 on observation days -1, 14, 22, 29 and 36. There was no significant ($p<0.01$) effect of day of treatment for all observations, with only one exception. This exception occurred with anti-BSA IgG in the BSA F treated groups on observation day 36 when the mean for birds in study 2 (4.6% hyperimmune) was significantly lower than that of the birds in study 1 (13.6% hyperimmune). However, both of these observations fall within the criteria for a negative antibody titre. Therefore, the results for each treatment group from studies 1 and 2 have been

combined for all observation days, and all of the data collected for birds immunised with BSA has been statistically analysed together, and the results are presented together in the following sections.

Serum anti-BSA IgG

As seen on Figure 2, at the start of the experiment, all birds had minimal (less than 10% hyperimmune) anti-BSA IgG antibody titres in serum. However, at this time BSA IM (9.6% hyperimmune) had a significantly higher anti-BSA IgG titre in serum than OJBSA F (2.2% hyperimmune) and OJSn+BSA G (1.8% hyperimmune). All of these measures are well below 15% which is the cut-off for a negative antibody titre.

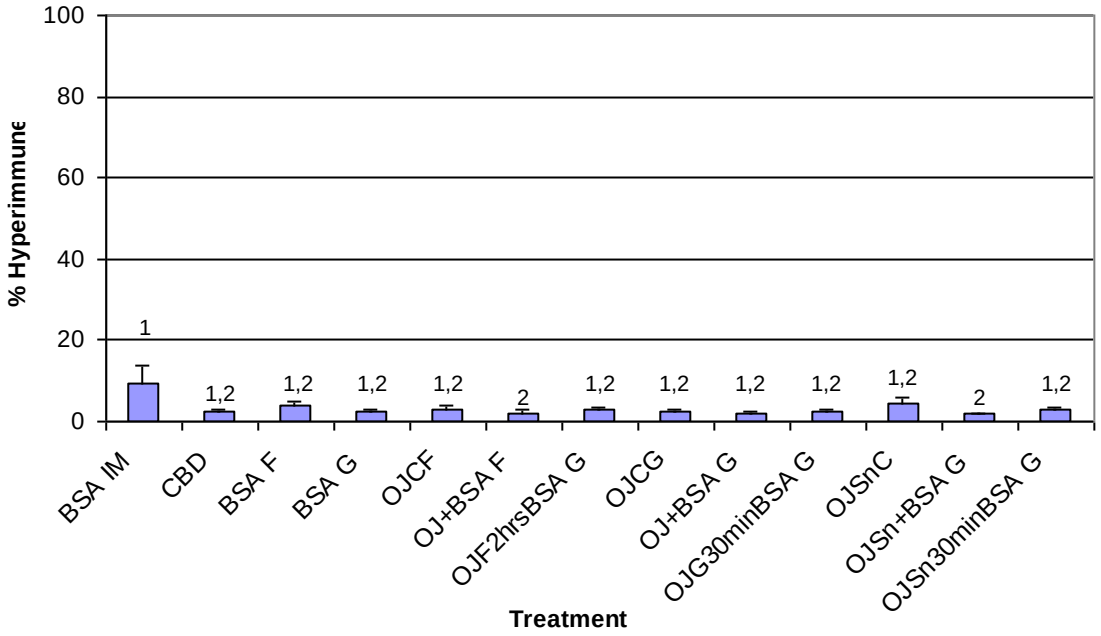

Figure 2: Day -1 anti-BSA IgG in serum

BSA IM: BSA in adjuvant, intramuscular injection; CBD: commercial broiler diet; BSA F: BSA dry fed; BSA G: BSA wet gavage; OJC F: Oralject control dry fed; OJ+BSA F: Oralject+BSA dry fed; OJF2hrsBSA G: Oralject dry fed 2 hrs later BSA wet gavage; OJC G: Oralject control wet gavage; OJ+BSA G: Oralject+BSA wet gavage; OJG30minBSAG: Oralject wet gavage 30 min later BSA wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+BSA G: Oralject extract solution + BSA wet gavage; OJSn30minBSA G: Oralject extract solution 30 min later BSA wet gavage.

Columns represent the treatment group mean and vertical bars are the standard error of the mean.

[1, 2] Columns with different superscripts differ significantly ($p<0.01$).

Figure 3 illustrates the day 14 anti-BSA IgG titres in serum 2 weeks after the primary immunisation. The positive control BSA IM treated chickens had a mean anti-BSA IgG titre in serum 85% of the hyperimmune birds which was statistically significant ($p<0.01$) when compared to all other treatment groups. Most notable of all the serum anti-BSA IgG titres from the other treatment groups at day 14 are from the birds which received BSA antigen in conjunction with the Oralject extract solution (OJSn + BSA G). The mean titre in this treatment group was almost 41 % of the hyperimmune serum, which is significantly higher than all other treatments with the exception of the positive control group (BSA IM) and OJSn30minBSA G (17.7% hyperimmune). Importantly, the mean titre for the OJSn+BSA G group is significantly higher than the very limited response of birds immunised with BSA alone either in the feed or via gavage (5 and 3% of the hyperimmune birds respectively). The extracted Oralject solution clearly had a greater impact on the immune response to BSA compared with feeding Oralject dry or wet fed in conjunction with or 2 hours prior to BSA administration. The anti-BSA IgG titres in serum on day 14 for both the BSA IM and OJSn + BSA G were significantly higher ($p<0.01$) than their day -1 titres, at which stage there was a significant difference between them. Therefore, both of there treatment groups have experienced a significant increase in BSA IgG antibody levels between day -1 and 14. In contrast, the OJBSA F group did not have a significant increase in its

mean anti-BSA IgG titre from day -1 to day 14, and the day 14 titre was significantly lower than the day 14 mean titre seen in both the BSA IM and OJSn+BSA G treatment groups.

On day 14, birds receiving OJSn and then 30 minutes later BSA wet gavage (OJSn30minBSA G), also had anti-BSA IgG titres in serum that were significantly higher ($p<0.01$) than BSA administered alone in feed (BSA F, 5.4% hyperimmune) or via gavage (BSA G, 3.1% hyperimmune) or together with Oralject in the feed (OJ+BSA F, 5.8% hyperimmune). The mean day 14 anti-BSA IgG titre in serum of the OJSn30minBSA G treated birds was also significantly higher than that of the birds receiving Oralject via gavage 30 minutes prior to gavage administration of the BSA (OJG30minBSA G, 7.79% hyperimmune) or when the Oralject was administered in the diet 2 hours prior to BSA being administered via gavage (OJF2hrsBSA G, 4.7% hyperimmune). Interestingly there was no significant difference between OJSn30minBSA G treatment generated anti-BSA IgG titres and the negative control group which received commercial broiler diet only (CBD), nor between OJSn30minBSA G and the three Oralject control groups, i.e. Oralject control gavage (OJC G) or in feed (OJC F) and Oralject Solution control (OJSn G). This places some questions over the repeatability of the significant differences observed between OJSn30minBSA G and other BSA treated groups outlined above on day 14.

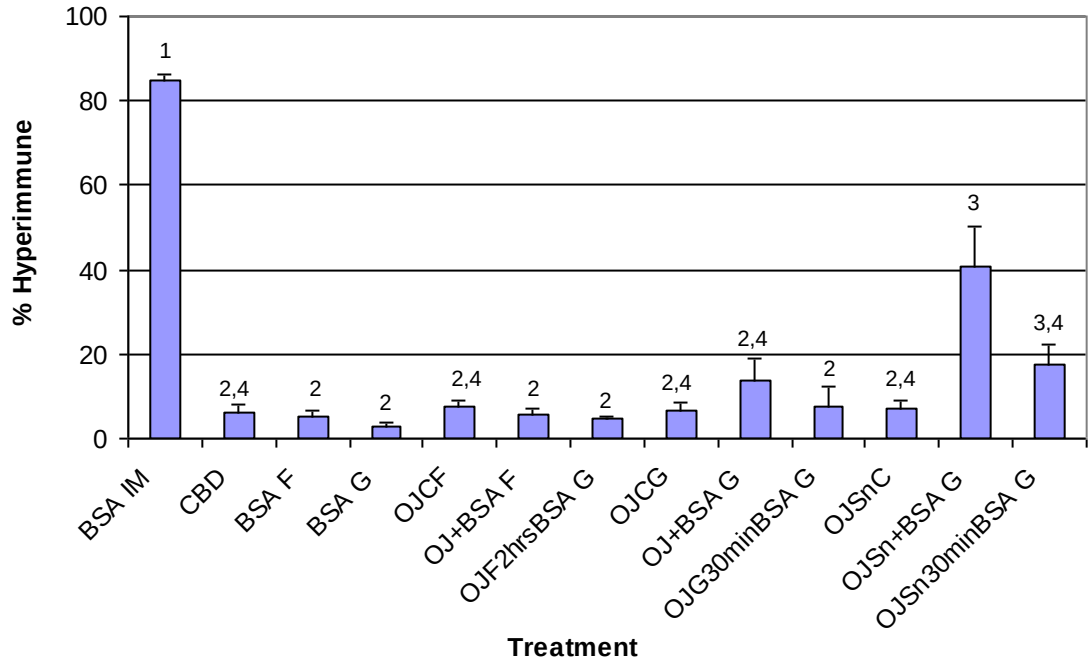

Figure 3: Day 14 anti-BSA IgG in serum
BSA IM: BSA in adjuvant, intramuscular injection; CBD: commercial broiler diet; BSA F: BSA dry fed; BSA G: BSA wet gavage; OJC F: Oralject control dry fed; OJ+BSA F: Oralject+BSA dry fed; OJF2hrsBSA G: Oralject dry fed 2 hrs later BSA wet gavage; OJC G: Oralject control wet gavage; OJ+BSA G: Oralject+BSA wet gavage; OJG30minBSAG: Oralject wet gavage 30 min later BSA wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+BSA G: Oralject extract solution + BSA wet gavage; OJSn30minBSA G: Oralject extract solution 30 min later BSA wet gavage.
Columns represent the treatment group mean and vertical bars are the standard error of the mean.
[1, 2,3,4] Columns with different superscripts differ significantly ($p<0.01$).

The mean anti-BSA IgG titre on Day 22 in the serum of the positive control birds (BSA IM), at 92% hyperimmune, is significantly higher ($p<0.01$) than all of the other treatment groups except for OJSn + BSA G (65% hyperimmune serum) and OJSn30minBSA G (49% hyperimmune serum) (Figure 4). Birds receiving OJSn + BSA G and OJSn30min BSA G had mean anti-BSA IgG titres that were significantly higher ($p<0.01$) than all other treatment groups except for BSA IM and each other. The day 22 mean BSA IgG titres in serum for the two treatment groups receiving OJSn in conjunction with BSA were the highest detected for these treatment groups throughout the experiment. It is also worth

noting that these birds had average anti-BSA IgG titres which did not differ statistically at $p<0.01$, from the BSA IM group and also they were significantly higher average titres than birds receiving BSA alone either via gavage or in the feed, or BSA administered with Oralject either dry in feed or wet via gavage.

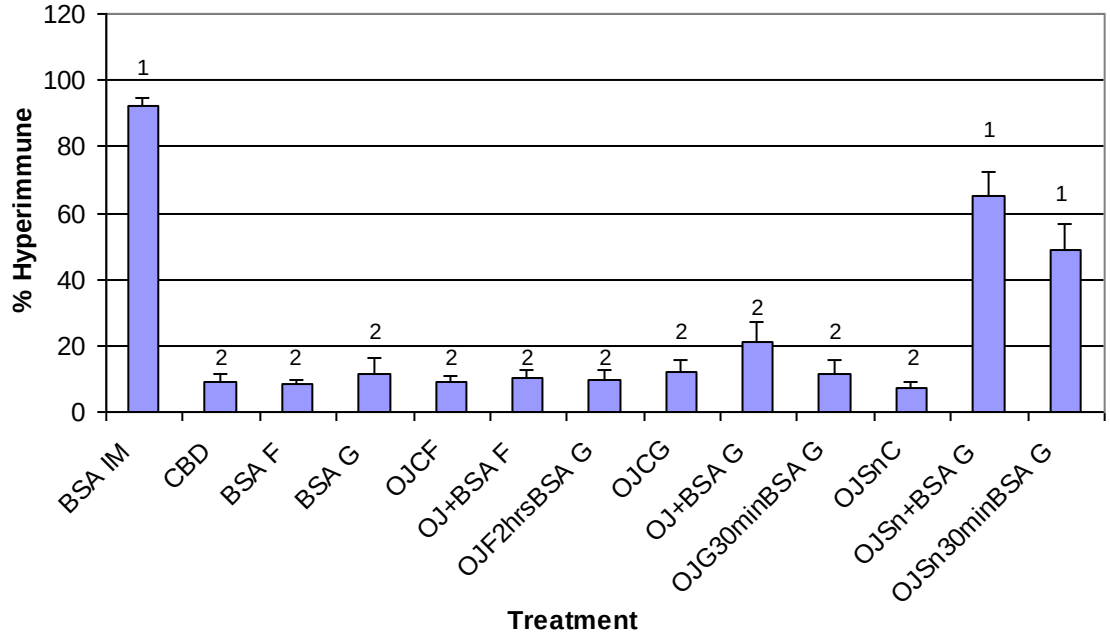

Figure 4: Day 22 anti-BSA IgG in serum

BSA IM: BSA in adjuvant, intramuscular injection; CBD: commercial broiler diet; BSA F: BSA dry fed; BSA G: BSA wet gavage; OJC F: Oralject control dry fed; OJ+BSA F: Oralject+BSA dry fed; OJF2hrsBSA G: Oralject dry fed 2 hrs later BSA wet gavage; OJC G: Oralject control wet gavage; OJ+BSA G: Oralject+BSA wet gavage; OJG30minBSAG: Oralject wet gavage 30 min later BSA wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+BSA G: Oralject extract solution + BSA wet gavage; OJSn30minBSA G: Oralject extract solution 30 min later BSA wet gavage.

Columns represent the treatment group mean and vertical bars are the standard error of the mean.

[1, 2,] Columns with different superscripts differ significantly ($p<0.01$).

On day 29, Figure 5, the two treatment groups which received the Oralject extract solution and BSA that is, OJSn + BSA G and OJSn30min BSA G had mean anti-BSA IgG titres in serum (40.5 % and 36.8% hyperimmune serum respectively) that were significantly higher ($p<0.01$) than all other treatment groups except BSA IM (93% hyperimmune serum). There were no significant differences in serum IgG for BSA between the two OJSn groups (OJSn + BSA G and OJSn30min BSA G). On day 29, BSA IM was significantly higher than all other treatment groups in the study and the day 29 serum BSA IgG titre was the highest detected for this treatment group throughout the study.

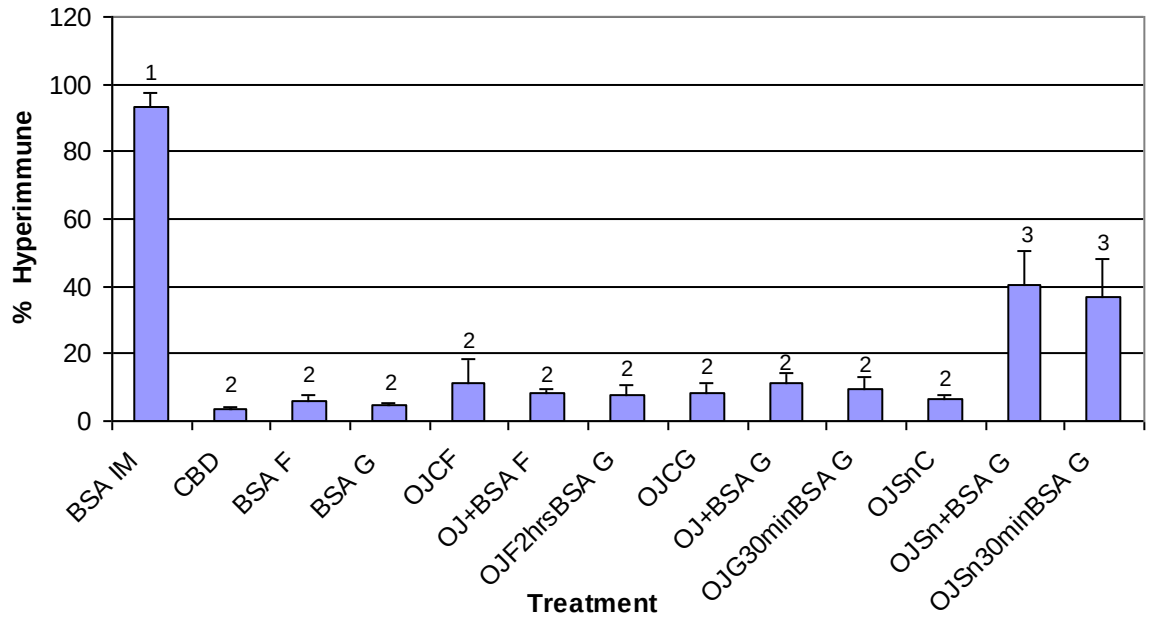

Figure 5: Day 29 anti-BSA IgG in serum

BSA IM: BSA in adjuvant, intramuscular injection; CBD: commercial broiler diet; BSA F: BSA dry fed; BSA G: BSA wet gavage; OJC F: Oralject control dry fed; OJ+BSA F: Oralject+BSA dry fed; OJF2hrsBSA G: Oralject dry fed 2 hrs later BSA wet gavage; OJC G: Oralject control wet gavage; OJ+BSA G: Oralject+BSA wet gavage; OJG30minBSAG: Oralject wet gavage 30 min later BSA wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+BSA G: Oralject extract solution + BSA wet gavage; OJSn30minBSA G: Oralject extract solution 30 min later BSA wet gavage.

Columns represent the treatment group mean and vertical bars are the standard error of the mean.

[1, 2, 3] Columns with different superscripts differ significantly ($p<0.01$).

As observed on day 29, on day 36, the OJSn + BSA G and OJSn30min BSA G had mean anti-BSA IgG titres in serum (38 % and 32% hyperimmune serum) which were significantly higher than all other treatment groups except BSA IM (90% hyperimmune) (Figure 6). Though, as can be seen when day 29 and 36 results are compared the mean anti-BSA IgG titres in these three treatment groups are now declining. There was no significant difference in the mean IgG titres observed between treatment groups OJSn + BSA G and OJSn30min BSA G. It is worth emphasising that both of the treatment groups which received Oralject extract solution together with BSA had anti-BSA IgG titres which are significantly higher than either BSA administered alone in feed or via gavage, or in conjunction with Oralject fed either wet via gavage or dry in feed. The birds treated with BSA IM had the highest mean anti-BSA IgG titre of all treatment groups on day 36, which was significantly different ($p<0.01$) from all other treatment group. A reminder that the BSA F group on day 36 demonstrated a significant difference between birds treated in study 1 and study 2. As can be seen from Figure 6, this observation would have had no impact on the statistically significant differences identified between treatment groups on day 36.

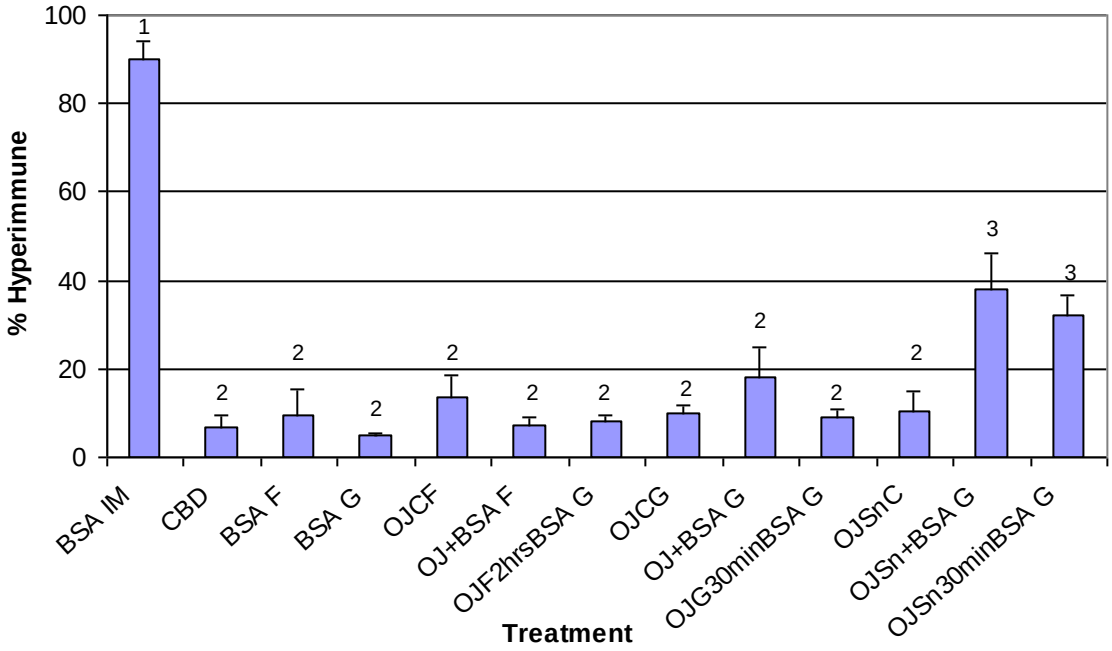

Figure 6: Day 36 anti-BSA IgG in serum

BSA IM: BSA in adjuvant, intramuscular injection; CBD: commercial broiler diet; BSA F: BSA dry fed; BSA G: BSA wet gavage; OJC F: Oralject control dry fed; OJ+BSA F: Oralject+BSA dry fed; OJF2hrsBSA G: Oralject dry fed 2 hrs later BSA wet gavage; OJC G: Oralject control wet gavage; OJ+BSA G: Oralject+BSA wet gavage; OJG30minBSAG: Oralject wet gavage 30 min later BSA wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+BSA G: Oralject extract solution + BSA wet gavage; OJSn30minBSA G: Oralject extract solution 30 min later BSA wet gavage.

Columns represent the treatment group mean and vertical bars are the standard error of the mean.

[1, 2, 3] Columns with different superscripts differ significantly ($p<0.01$).

Serum anti-BSA IgA

At the start of the experiment, day -1, all treatment groups had a mean IgA antibody titre to BSA less than 10% of the hyperimmune standard. There were no significant differences between any of the treatment groups at this time at $p<0.01$ (data not shown).

On day 14, 2 weeks after the delivery of the primary immunisation, the positive control group (BSA IM) had an anti-BSA IgA titre in serum that was 51% of the hyperimmune serum (Figure 7). This is significantly higher than all other treatment groups ($p<0.01$). The birds that had received BSA in conjunction with the Oralject solution (OJSn+BSA G) had an anti-BSA IgA mean titre (22.5% hyperimmune) which was significantly higher than birds in the negative control group (CBD; 10% hyperimmune), OJC F (10% hyperimmune), BSA G (9%) and OJF2hrsBSA G (14.5% hyperimmune).

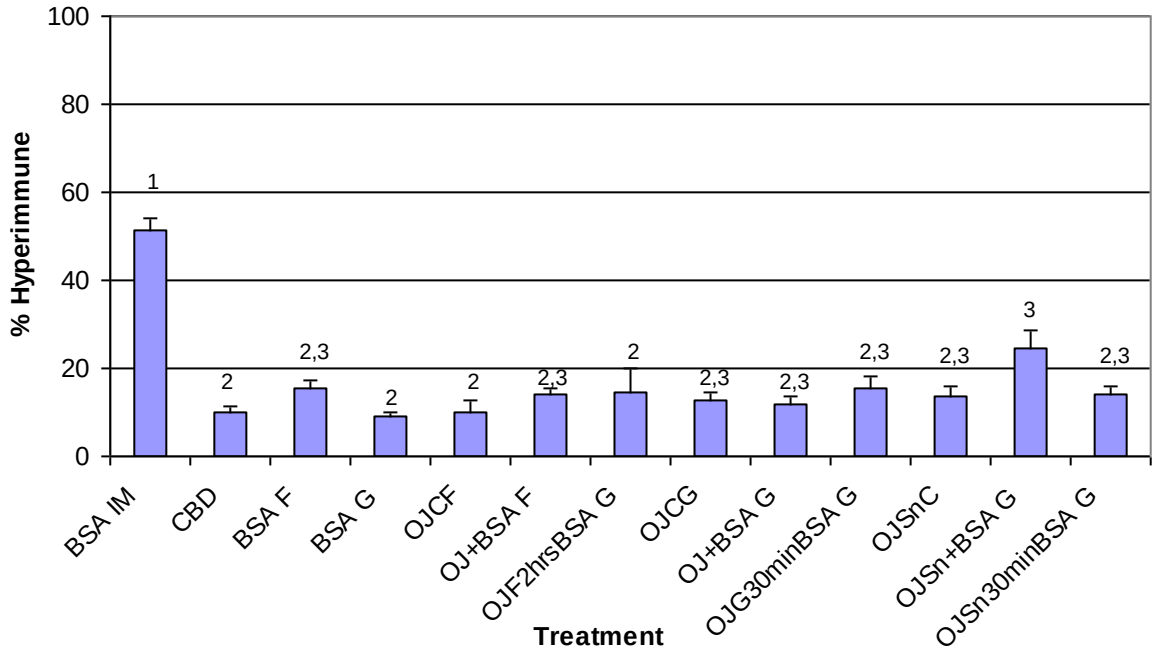

Figure 7 : Day 14 anti-BSA IgA in serum.
BSA IM: BSA in adjuvant, intramuscular injection; CBD: commercial broiler diet; BSA F: BSA dry fed; BSA G: BSA wet gavage; OJC F: Oralject control dry fed; OJ+BSA F: Oralject+BSA dry fed; OJF2hrsBSA G: Oralject dry fed 2 hrs later BSA wet gavage; OJC G: Oralject control wet gavage; OJ+BSA G: Oralject+BSA wet gavage; OJG30minBSAG: Oralject wet gavage 30 min later BSA wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+BSA G: Oralject extract solution + BSA wet gavage; OJSn30minBSA G: Oralject extract solution 30 min later BSA wet gavage.
Columns represent the treatment group mean and vertical bars are the standard error of the mean.
[1, 2, 3] Columns with different superscripts differ significantly ($p<0.01$).

One week later, on day 22, which was also one week after the booster immunisation delivered on day 14, the mean serum anti-BSA IgA of the birds receiving BSA in conjunction with the Oralject extract solution carrier, that is OJSn+BSA G and OJSn30minBSAG, were not significantly different from BSA IM, positive control birds, as seen on Figure 8. The titres as a percentage of the hyperimmune serum were OJSn+BSA G 47%, OJSn30minBSA G 41% and BSA IM 50%. Further, there was no significant difference between the titres of the two groups of birds treated with OJSn and BSA. The mean anti-BSA IgA titre of the OJSn+BSA G and OJSn30minBSA treated birds at day 22 were significantly higher than all other treatment groups except, as previously stated, the BSA IM treatment group. BSA IM had the highest mean anti-BSA IgA titre of all treatment groups, which was significantly higher than all treatment groups except for OJSn+BSA G and OJSn30minBSA.

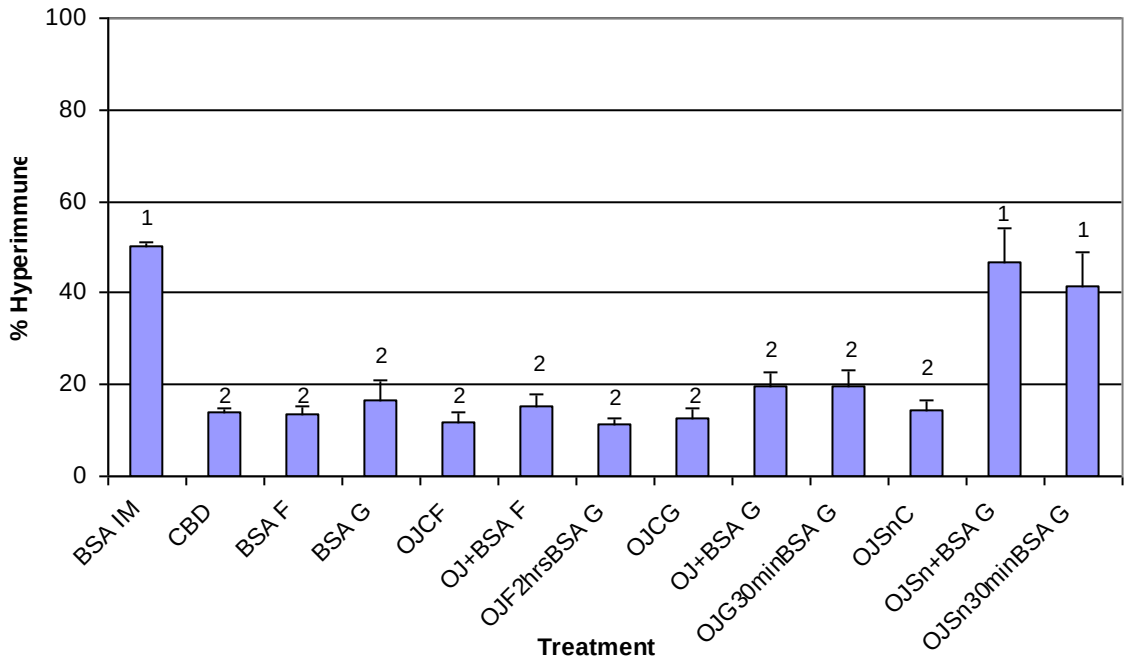

Figure 8: Day 22 anti-BSA IgA in serum

BSA IM: BSA in adjuvant, intramuscular injection; CBD: commercial broiler diet; BSA F: BSA dry fed; BSA G: BSA wet gavage; OJC F: Oralject control dry fed; OJ+BSA F: Oralject+BSA dry fed; OJF2hrsBSA G: Oralject dry fed 2 hrs later BSA wet gavage; OJC G: Oralject control wet gavage; OJ+BSA G: Oralject+BSA wet gavage; OJG30minBSAG: Oralject wet gavage 30 min later BSA wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+BSA G: Oralject extract solution + BSA wet gavage; OJSn30minBSA G: Oralject extract solution 30 min later BSA wet gavage.

Columns represent the treatment group mean and vertical bars are the standard error of the mean.

[1, 2,] Columns with different superscripts differ significantly ($p<0.01$).

On day 29, two weeks after the booster immunisations, the mean anti-BSA IgA in serum of the positive control group BSA IM (60% hyperimmune) was significantly higher ($p<0.01$) than all other treatment groups (Figure 9). This IgA titre of 60% hyperimmune in the BSA IM treated birds, is identical to that observed on day 36, and they are the highest anti-BSA IgA titres seen in the positive treatment group throughout the experiment. The birds that received Oralject extract solution in conjunction with BSA, (OJS30minBSA G, 26% hyperimmune; OJSn+BSA G, 25% hyperimmune) had an average IgA antibody titre in the serum to BSA that was significantly higher ($p<0.01$) than OJF2hrsBSA G (9% hyperimmune). As seen when comparing Figures 8 and 9, the mean anti-BSA IgA titre in both of the groups receiving the Oralject extract solution with BSA had declined between days 22 and 29.

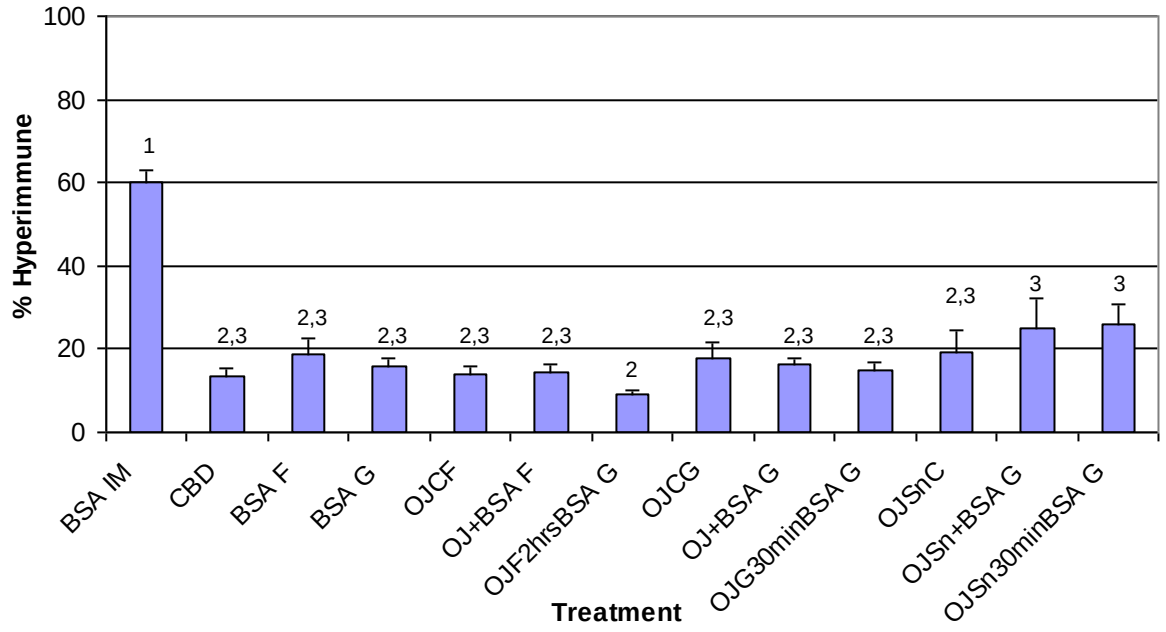

Figure 9: Day 29 anti-BSA IgA in serum

BSA IM: BSA in adjuvant, intramuscular injection; CBD: commercial broiler diet; BSA F: BSA dry fed; BSA G: BSA wet gavage; OJC F: Oralject control dry fed; OJ+BSA F: Oralject+BSA dry fed; OJF2hrsBSA G: Oralject dry fed 2 hrs later BSA wet gavage; OJC G: Oralject control wet gavage; OJ+BSA G: Oralject+BSA wet gavage; OJG30minBSAG: Oralject wet gavage 30 min later BSA wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+BSA G: Oralject extract solution + BSA wet gavage; OJSn30minBSA G: Oralject extract solution 30 min later BSA wet gavage.

Columns represent the treatment group mean and vertical bars are the standard error of the mean.

[1, 2, 3] Columns with different superscripts differ significantly ($p<0.01$).

On day 29 and 36, two of the negative treatment groups, that is OJC G and OJSn C, recorded mean anti-BSA IgA titres above 15% hyperimmune serum, that is, outside the negative range. At this time they were not significantly different from their BSA treatment groups, that is for OJC G, the treatments of OJ and BSA delivered via gavage, and for OJSn C the two groups treated with BSA and OJSnC. This increase in anti-BSA IgA titre is difficult to explain however, as the birds were not isolated from the antigen treated birds, it may be that they had been exposed to BSA in a manner that generated low, but positive responses. The other possibility is that the birds had been exposed to some other antigen that was causing cross-reactions in the anti-BSA IgA ELISA assay. In both of the above scenarios it is likely that all birds would have been affected, and when the mean titres of all the negative treatment groups are considered, they are higher on day 29 and 36 than they were at the start of the study. It may be that birds within the OJC G and OJSn C groups generated a higher response, explaining their higher mean BSA IgA titres at this stage.

Three weeks after the booster immunisation with BSA, that is on day 36, Figure 10, the positive control group maintained their significantly higher IgA titre to BSA in the serum (60% hyperimmune) ($p<0.01\%$) compared to all other treatment groups. Both of the groups of birds administered the OJSn carrier (OJSnBSA G and OJSn30minBSA G) also had anti-BSA IgA titres in serum (22% and 23.6 % hyperimmune respectively), that were significantly higher than OJ + BSA F (11% hyperimmune) and OJF2hrsBSA G (10% hyperimmune).

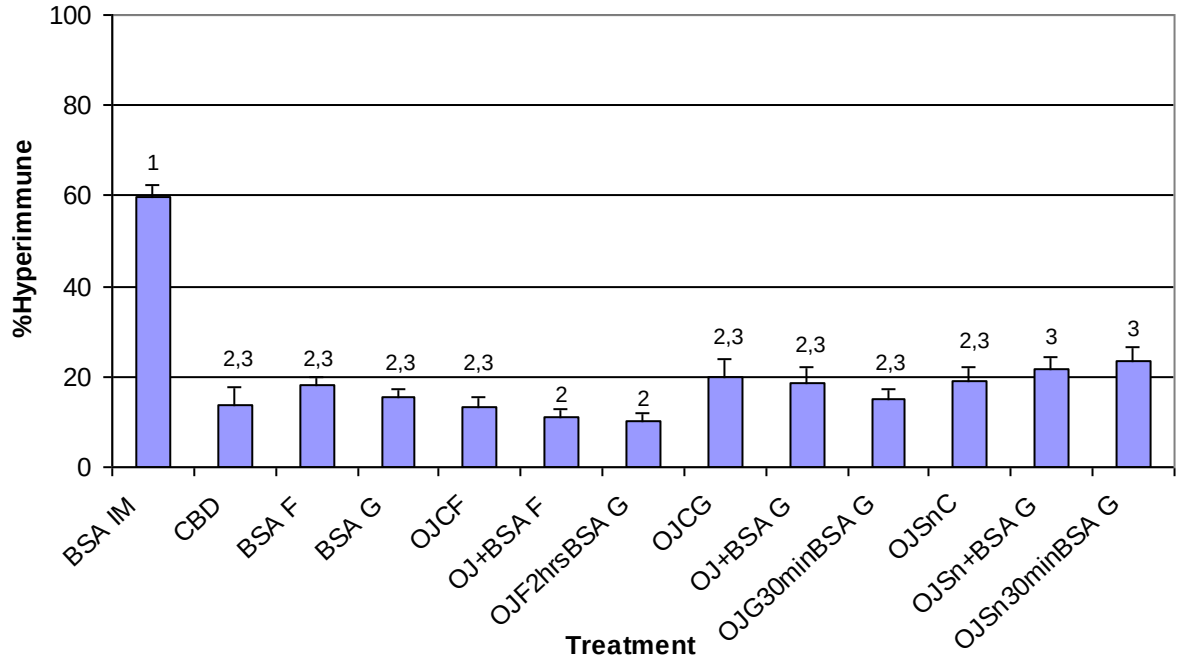

Figure 10: Day 36 anti-BSA IgA in serum

BSA IM: BSA in adjuvant, intramuscular injection; CBD: commercial broiler diet; BSA F: BSA dry fed; BSA G: BSA wet gavage; OJC F: Oralject control dry fed; OJ+BSA F: Oralject+BSA dry fed; OJF2hrsBSA G: Oralject dry fed 2 hrs later BSA wet gavage; OJC G: Oralject control wet gavage; OJ+BSA G: Oralject+BSA wet gavage; OJG30minBSAG: Oralject wet gavage 30 min later BSA wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+BSA G: Oralject extract solution + BSA wet gavage; OJSn30minBSA G: Oralject extract solution 30 min later BSA wet gavage.

Columns represent the treatment group mean and vertical bars are the standard error of the mean.

[1, 2, 3] Columns with different superscripts differ significantly ($p<0.01$).

Intestinal scrapings anti-BSA IgA.

As can be seen on Figure 11, the day 22 anti-BSA IgA titres in intestinal scrapings of all the control treatment groups, that is CBD, OJC F, OJC G, OJSnC indicate a considerable problem with non-specific binding in the assay. As described in the Methodology section under Investigation of techniques to reduce background in the anti-BSA IgA ELISA, several techniques were tested to try and reduce the background colour. Pre-incubation of samples with 6% egg albumin, which was used for all sera, bile and ISS samples, failed to eliminate background in the ISS and bile samples. Therefore, as the results are confounded by high levels of background colour, no statistical differences are outlined nor included on Figure 11. A similar scenario occurred with the day 36 samples. Again no statistical outcomes will be presented as they would most likely be inaccurate.

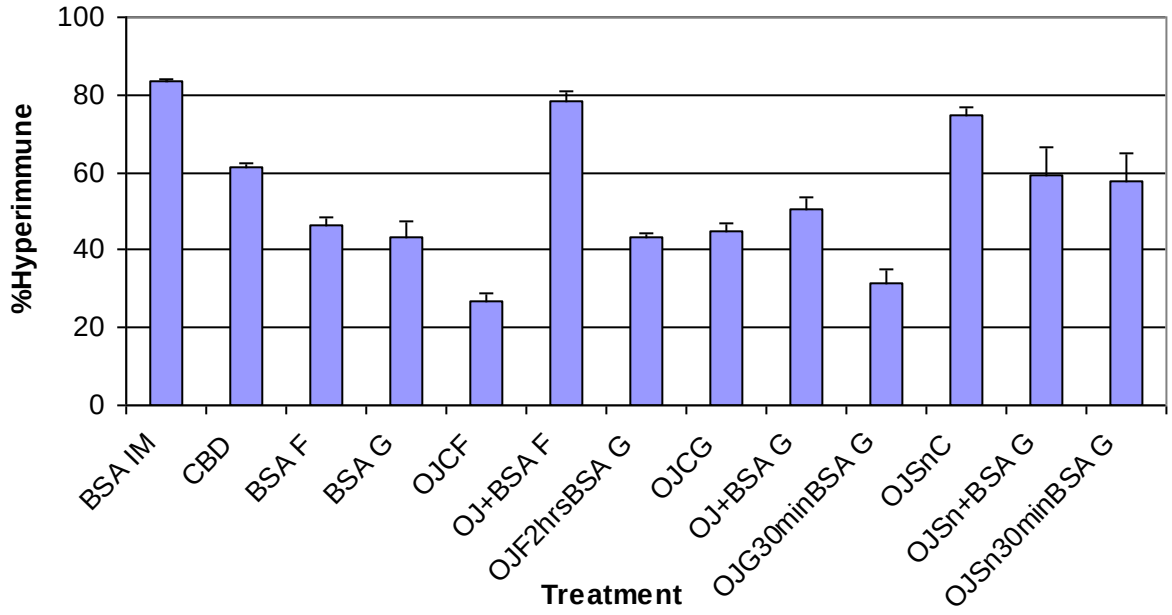

Figure 11: Day 22 anti-BSA IgA in intestinal scrapings supernatant
BSA IM: BSA in adjuvant, intramuscular injection; CBD: commercial broiler diet; BSA F: BSA dry fed; BSA G: BSA wet gavage; OJC F: Oralject control dry fed; OJ+BSA F: Oralject+BSA dry fed; OJF2hrsBSA G: Oralject dry fed 2 hrs later BSA wet gavage; OJC G: Oralject control wet gavage; OJ+BSA G: Oralject+BSA wet gavage; OJG30minBSAG: Oralject wet gavage 30 min later BSA wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+BSA G: Oralject extract solution + BSA wet gavage; OJSn30minBSA G: Oralject extract solution 30 min later BSA wet gavage.
Columns represent the treatment group mean and vertical bars are the standard error of the mean.

Bilary anti-BSA IgA.

As with the intestinal scrapings anti-BSA IgA ELISA, the bile samples also generated a high absorbance measure for control treatment groups, such as CBD, OJCG and OJSnC, indicating non-specific binding in the assay, which confounds the results. This occurred in samples from both days 22 (Figure 12) and 36, and therefore, no statistical outcomes are presented here or identified on Figure 12.

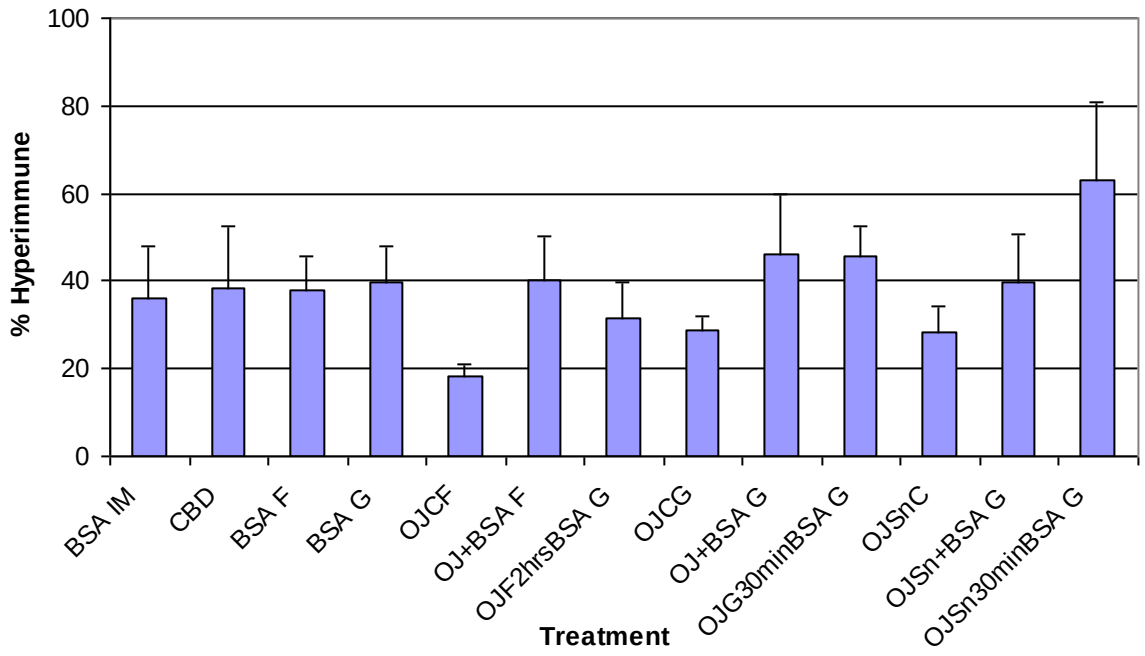

Figure 12: Day 22 anti-BSA IgA in bile
BSA IM: BSA in adjuvant, intramuscular injection; CBD: commercial broiler diet; BSA F: BSA dry fed; BSA G: BSA wet gavage; OJC F: Oralject control dry fed; OJ+BSA F: Oralject+BSA dry fed; OJF2hrsBSA G: Oralject dry fed 2 hrs later BSA wet gavage; OJC G: Oralject control wet gavage; OJ+BSA G: Oralject+BSA wet gavage; OJG30minBSAG: Oralject wet gavage 30 min later BSA wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+BSA G: Oralject extract solution + BSA wet gavage; OJSn30minBSA G: Oralject extract solution 30 min later BSA wet gavage.
Columns represent the treatment group mean and vertical bars are the standard error of the mean.

Immunisation with EHNV (Study 2)

Outline of treatment group abbreviations:

For ease of reference, Table 19 outlines the treatment groups included in EHNV study two of this project.

Table 19: Treatment group abbreviations

Abbreviation	Treatment
EHNV IM	EHNV in adjuvant, intramuscular injection
CBD	Commercial broiler diet alone
EHNV F	EHNV dry fed alone
EHNV G	EHNV, wet, gavage alone
OJCF	Oralject control dry fed
OJ+EHNV F	Oralject with EHNV dry, fed
OJCG	Oralject control, wet, gavage
OJ+EHNV G	Oralject with EHNV wet gavage

Serum anti-EHNV IgG

At the start of the experiment (day -1), prior to delivery of either EHNV antigen or any carrier substances, IgG antibody titres to EHNV in serum were low in all treatment groups (Figure 13) and there were no significant differences between them.

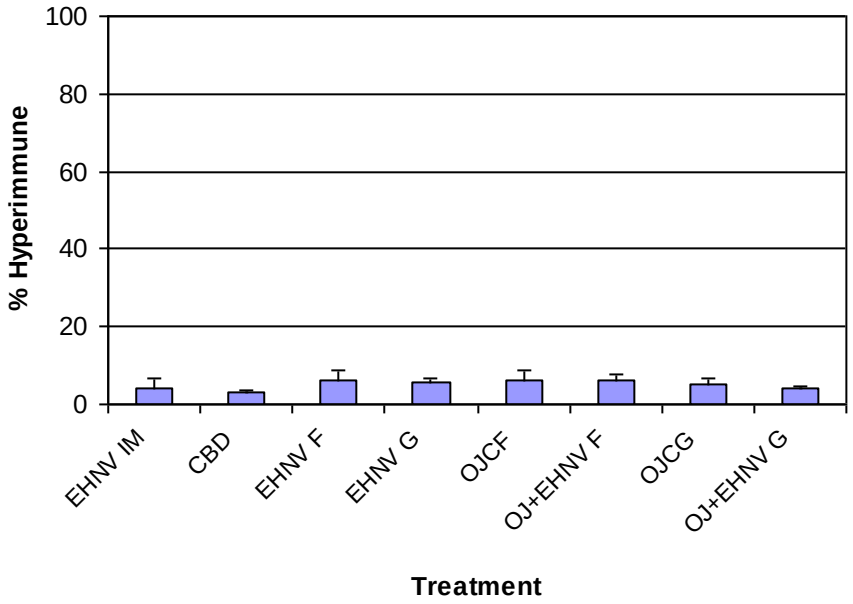

Figure 13 :Day -1 anti-EHNV IgG in serum
EHNV IM: EHNV in adjuvant, intramuscular injection; CBD: commercial broiler diet; EHNV F: EHNV dry fed; EHNV G: EHNV wet gavage; OJC F: Oralject control dry fed; OJ+EHNV F: Oralject+ EHNV dry fed; OJC G: Oralject control wet gavage; OJ+EHNV G: Oralject+EHNV wet gavage.
Columns represent the treatment group mean and vertical bars are the standard error of the mean.

However, by day 14, 14 days after the primary immunisation, birds immunised with EHNV in adjuvant, delivered via intramuscular injection (EHNV IM), had serum anti-EHNV IgG titres that were statistically higher ($p < 0.01$) than all other treatment groups (Figure 14). This titre remained statistically higher than that of all other treatment groups for the remainder of the experiment. At day 14 it was 79% of the hyperimmune serum (Figure 14), and, by day 22, 8 days after the secondary immunisation, it was 93% of the hyperimmune serum (Figure 15). It continued to increase to day 36, when it was 102% of the hyperimmune serum (Figure 17). Throughout the experiment there were no other statistically significant differences observed in serum anti-EHNV IgG titres.

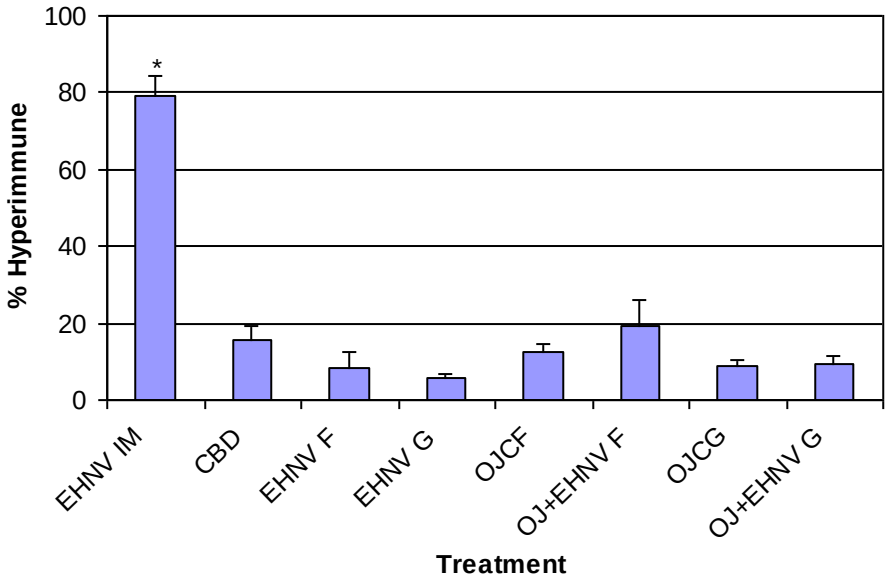

Figure 14: Day 14 anti-EHNV IgG in serum
EHNV IM: EHNV in adjuvant, intramuscular injection; CBD: commercial broiler diet; EHNV F: EHNV dry fed; EHNV G: EHNV wet gavage; OJC F: Oralject control dry fed; OJ+EHNV F: Oralject+ EHNV dry fed; OJC G: Oralject control wet gavage; OJ+EHNV G: Oralject+EHNV wet gavage.
Columns represent the treatment group mean and vertical bars are the standard error of the mean.
* significant difference at $p<0.01$.

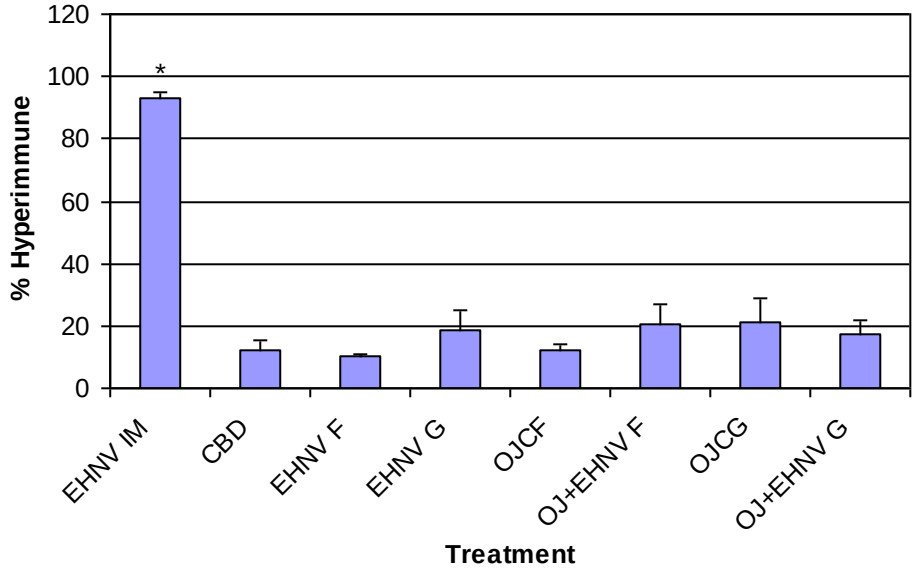

Figure 15: Day 22 anti-EHNV IgG in serum
EHNV IM: EHNV in adjuvant, intramuscular injection; CBD: commercial broiler diet; EHNV F: EHNV dry fed; EHNV G: EHNV wet gavage; OJC F: Oralject control dry fed; OJ+EHNV F: Oralject+ EHNV dry fed; OJC G: Oralject control wet gavage; OJ+EHNV G: Oralject+EHNV wet gavage.
Columns represent the treatment group mean and vertical bars are the standard error of the mean.
* significant difference at $p<0.01$.

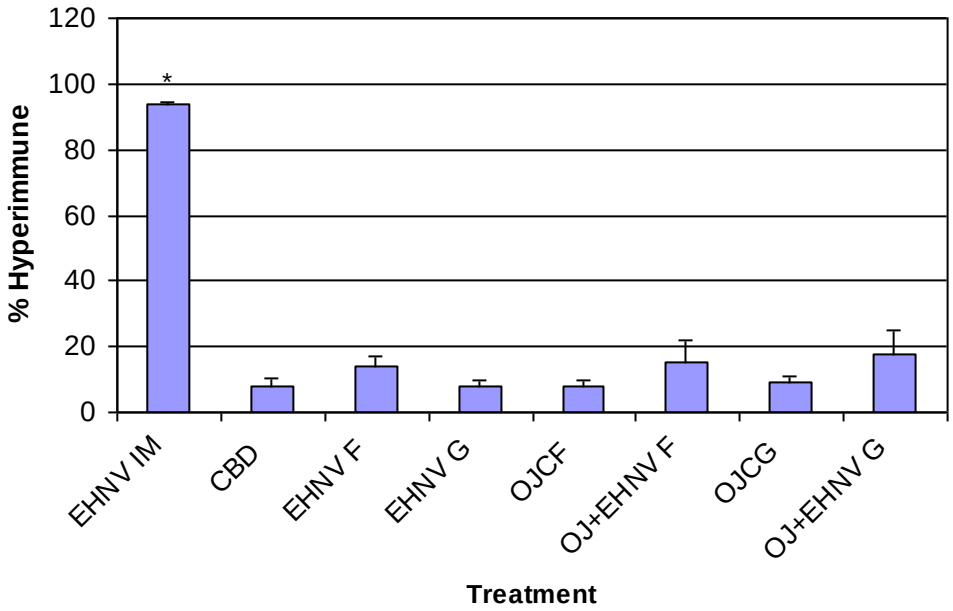

Figure 16 : Day 29 anti-EHNV IgG in serum
EHNV IM: EHNV in adjuvant, intramuscular injection; CBD: commercial broiler diet; EHNV F: EHNV dry fed; EHNV G: EHNV wet gavage; OJC F: Oralject control dry fed; OJ+EHNV F: Oralject+ EHNV dry fed; OJC G: Oralject control wet gavage; OJ+EHNV G: Oralject+EHNV wet gavage.
Columns represent the treatment group mean and vertical bars are the standard error of the mean.
* significant difference at $p<0.01$.

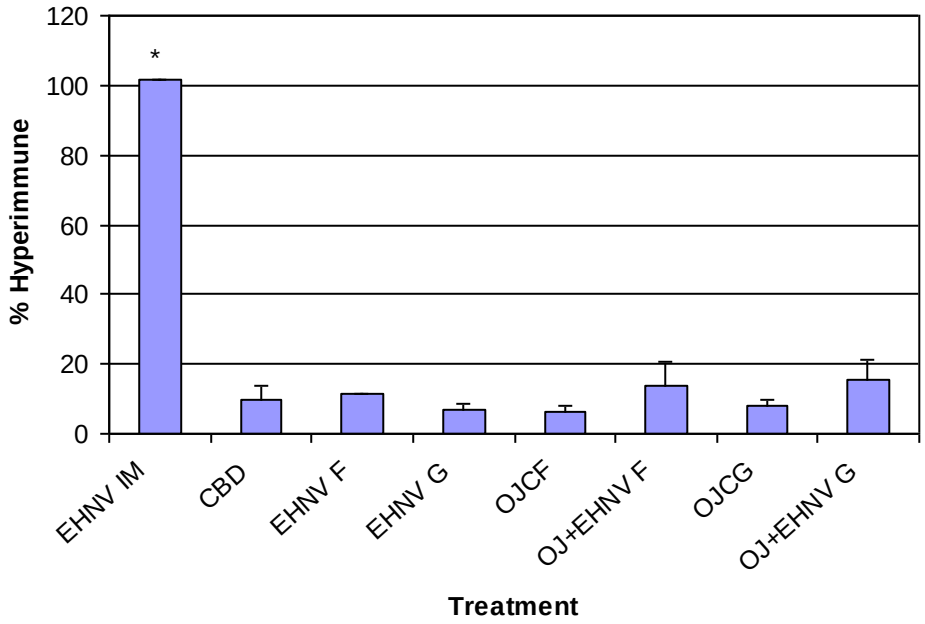

Figure 17: Day 36 anti-EHNV IgG in serum
EHNV IM: EHNV in adjuvant, intramuscular injection; CBD: commercial broiler diet; EHNV F: EHNV dry fed; EHNV G: EHNV wet gavage; OJC F: Oralject control dry fed; OJ+EHNV F: Oralject+ EHNV dry fed; OJC G: Oralject control wet gavage; OJ+EHNV G: Oralject+EHNV wet gavage.
Columns represent the treatment group mean and vertical bars are the standard error of the mean.
* significant difference at $p<0.01$.

As can also be seen on each of Figures 14, 15, 16 and 17, apart from birds treated IM with EHNV, there were no notable increases anti-EHNV IgG titres in any other treatment group, with the mean

titres remaining below 20% of the hyperimmune serum, for the duration of the study. Further, all EHNV negative treatments had mean anti-EHNV IgG titres of 15% or less of the hyperimmune serum throughout the study.

Serum anti-EHNV IgA

Day -1 serum anti-EHNV IgA titres were generally low in all treatment groups. However, as seen on Figure 18, some treatment groups demonstrated a titre greater than 15% of the hyperimmune standard serum at the start of the study, namely OJC F (17.8% hyperimmune) and OJ + EHNV G (16.8% hyperimmune), indicating that there may be some non-specific binding occurring in this assay, despite attempts to use a protocol which was designed to minimise background colour (see techniques tested to reduce non-specific binding under the EHNV IgA ELISA methodology).

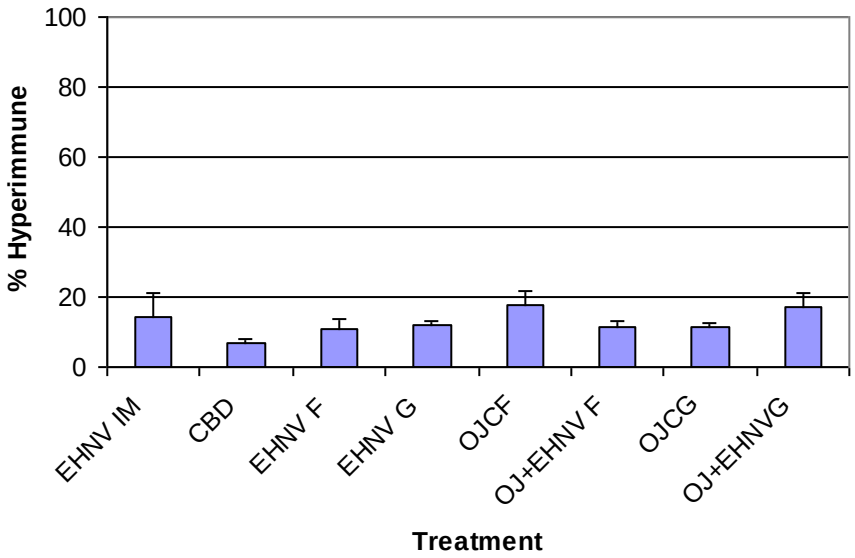

Figure 18: Day -1 anti-EHNV IgA in serum
EHNV IM: EHNV in adjuvant, intramuscular injection; CBD: commercial broiler diet; EHNV F: EHNV dry fed; EHNV G: EHNV wet gavage; OJC F: Oralject control dry fed; OJ+EHNV F: Oralject+ EHNV dry fed; OJC G: Oralject control wet gavage; OJ+EHNV G: Oralject+EHNV wet gavage.
Columns represent the treatment group mean and vertical bars are the standard error of the mean.

On day 14, 2 weeks after the primary immunisation, the interpretation of results is difficult, as birds in the negative control group, CBD, which received the commercial diet only, demonstrated an anti-EHNV IgA titre in the serum of 26%, which was just below that of the positive control group, EHNV IM (30% hyperimmune) (Figure 19). On each of the remaining observation days, that is day 22, 29 and 36, at least one of the control groups, for example Oralject dry fed alone (OJC F) or Oralject in water alone (OJC G), demonstrated an anti-EHNV IgA titre in serum that was greater than 15% hyperimmune serum (Figures 20 to Figure 22). In addition to these concerns, the anti-EHNV IgA response in the birds immunised IM, was lower than anticipated, reaching a maximum mean of only 48% hyperimmune on day 36. This may not be completely unexpected as the protocol used to immunise the birds in the EHNV IM treated group favours the generation of an IgG response over IgA. Clearly, the combination of these difficulties with the EHNV IgA titres, places some uncertainty over the accuracy of the results, and they should be interpreted with caution

The day 14 mean anti-EHNV IgA titre in the birds treated with EHNV IM was significantly higher ($p<0.01$) than EHNV F. Similarly, OJ+EHNV G treated birds had a mean anti-EHNV IgA titre which was significantly higher than EHNV F.

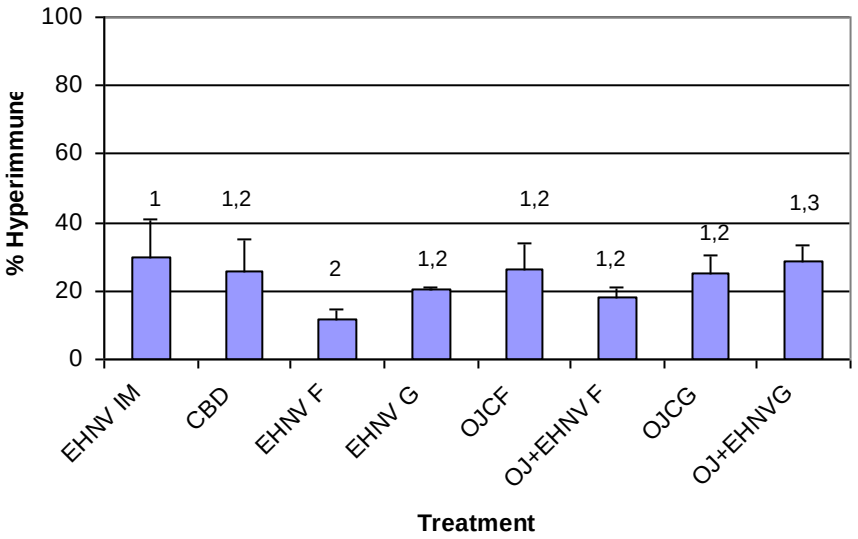

Figure 19: Day 14 anti-EHNV IgA in serum
EHNV IM: EHNV in adjuvant, intramuscular injection; CBD: commercial broiler diet; EHNV F: EHNV dry fed; EHNV G: EHNV wet gavage; OJC F: Oralject control dry fed; OJ+EHNV F: Oralject+ EHNV dry fed; OJC G: Oralject control wet gavage; OJ+EHNV G: Oralject+EHNV wet gavage.
Columns represent the treatment group mean and vertical bars are the standard error of the mean.
[1, 2] Columns with different superscripts differ significantly ($p<0.01$).

One week following the booster immunisation, that is on day 22, no significant differences ($p<0.01$) were observed between any of the treatment groups (Figure 20) for EHNV IgA titres in serum.

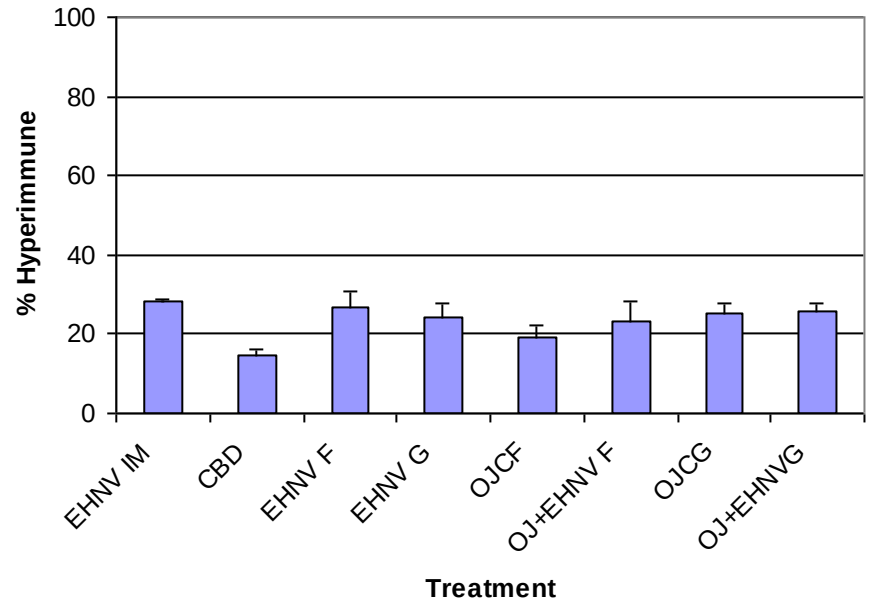

Figure 20: Day 22 serum anti-EHNV IgA
EHNV IM: EHNV in adjuvant, intramuscular injection; CBD: commercial broiler diet; EHNV F: EHNV dry fed; EHNV G: EHNV wet gavage; OJC F: Oralject control dry fed; OJ+EHNV F: Oralject+ EHNV dry fed; OJC G: Oralject control wet gavage; OJ+EHNV G: Oralject+EHNV wet gavage.
Columns represent the treatment group mean and vertical bars are the standard error of the mean.

On day 29, the only significant differences observed involved EHNV IM treated birds which had a mean anti-EHNV IgA titre that was higher than CBD and EHNV F ($p<0.01$) (Figure 21).

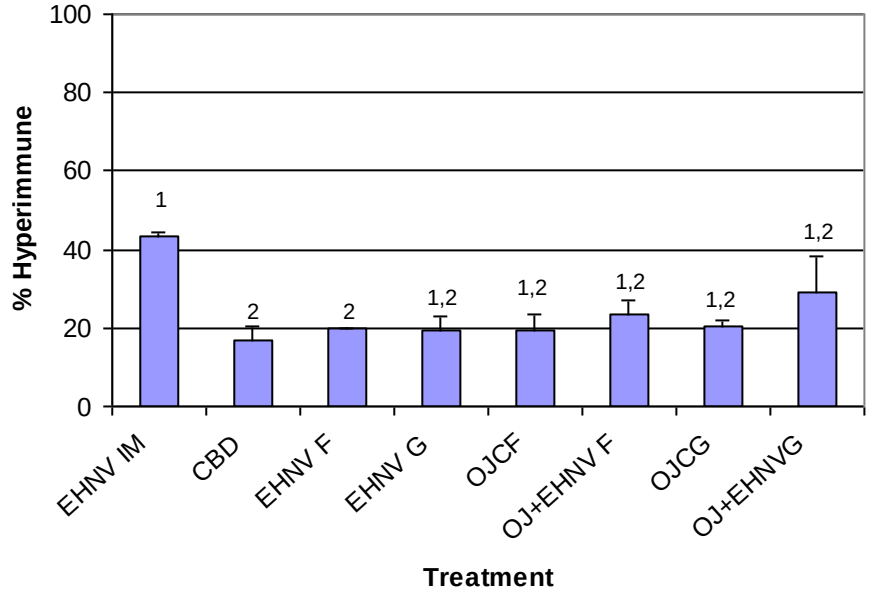

Figure 21: Day 29 serum anti-EHNV IgA.
EHNV IM: EHNV in adjuvant, intramuscular injection; CBD: commercial broiler diet; EHNV F: EHNV dry fed; EHNV G: EHNV wet gavage; OJC F: Oralject control dry fed; OJ+EHNV F: Oralject+ EHNV dry fed; OJC G: Oralject control wet gavage; OJ+EHNV G: Oralject+EHNV wet gavage.
Columns represent the treatment group mean and vertical bars are the standard error of the mean.
[1, 2] Columns with different superscripts differ significantly ($p<0.01$).

As illustrated in Figure 22, on day 36 a significant difference ($p<0.01$) in anti-EHNV IgA titre in serum was observed between EHNV IM and all other treatment groups except for OJC G and OJ+EHNV G.

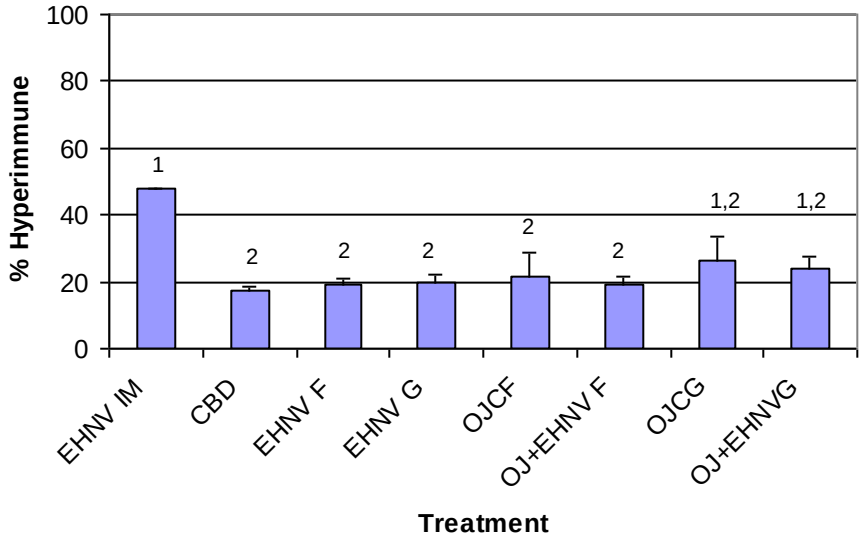

Figure 22: Day 36 anti-EHNV IgA in serum.
EHNV IM: EHNV in adjuvant, intramuscular injection; CBD: commercial broiler diet; EHNV F: EHNV dry fed; EHNV G: EHNV wet gavage; OJC F: Oralject control dry fed; OJ+EHNV F: Oralject+ EHNV dry fed; OJC G: Oralject control wet gavage; OJ+EHNV G: Oralject+EHNV wet gavage.
Columns represent the treatment group mean and vertical bars are the standard error of the mean.
[1, 2] Columns with different superscripts differ significantly ($p<0.01$).

Immunisation with *Salmonella typhimurium* (Study 3)

Outline of treatment group abbreviations

For ease of reference, Table 20 outlines the treatment groups included in *Salmonella typhimurium* study three of this project

Table 20: Treatment group abbreviations

Abbreviation	Treatment group
St IM	*S. typhimurium* in adjuvant, intramuscular injection.
CBD	Commercial broiler diet alone
St F	*S. typhimurium* dry fed alone
St G	*S. typhimurium* wet, gavage alone
OJCF	Oralject control dry fed alone
OJ+St F	Oralject + *S. typhimurium* dry fed
OJF2hrsSt G	Oralject dry fed, 2 hrs later *S. typhimurium* wet gavage
OJCG	Oralject control wet gavage alone
OJ+St G	Oralject + *S. typhimurium* wet gavage
OJG30minSt G	Oralject wet gavage, 30 mins later *S. typhimurium* wet gavage
OJSnC	Oralject extract solution control gavage
OJSn.+ St G	Oralject extract solution + *S. typhimurium* wet gavage
OJSn30minSt G	Oralject extract solution gavage, 30 mins later *S. typhimurium* wet gavage.

Serum anti-*Salmonella typhimurium* IgG

Day -1 serum anti-*S. typhimurium* IgG titres were low (less than 7% hyperimmune), and there were no significant differences ($p<0.01$) between any treatment groups, indicating no previous exposure of these birds to *S. typhimurium* (Figure 23).

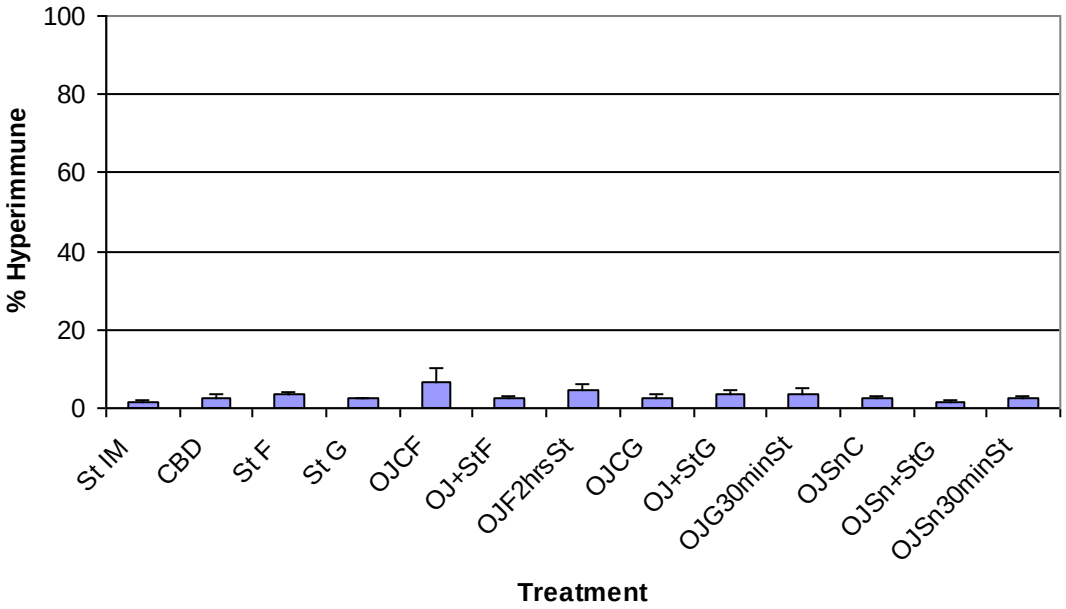

Figure 23: Day -1 anti-*S. typhimurium* IgG in serum.
St IM: *S. typhimurium* in adjuvant, intramuscular injection; CBD: commercial broiler diet; St F: *S. typhimurium* dry fed; St G: *S. typhimurium* wet gavage; OJC F: Oralject control dry fed; OJ+St F: Oralject+*S. typhimurium* dry fed; OJF2hrsSt G: Oralject dry fed 2 hrs later *S. typhimurium* wet gavage; OJC G: Oralject control wet gavage; OJ+St G: Oralject+*S. typhimurium* wet gavage; OJG30minStG: Oralject wet gavage 30 min later *S. typhimurium* wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+St G: Oralject extract solution + *S. typhimurium* wet gavage; OJSn30minSt G: Oralject extract solution 30 min later *S. typhimurium* wet gavage.
Columns represent the treatment group mean and vertical bars are the standard error of the mean.

However, on day 14, 2 weeks following the primary immunisation, the positive control group St IM, which received *S. typhimurium* in adjuvant, via intramuscular injection, demonstrated a mean titre which was 55% of the hyperimmune sera, which was significantly higher than all other treatment groups ($p<0.01$) (Figure 24). All other treatment groups had titres between 7-11% hyperimmune sera, which were not dissimilar to the titres observed on day -1

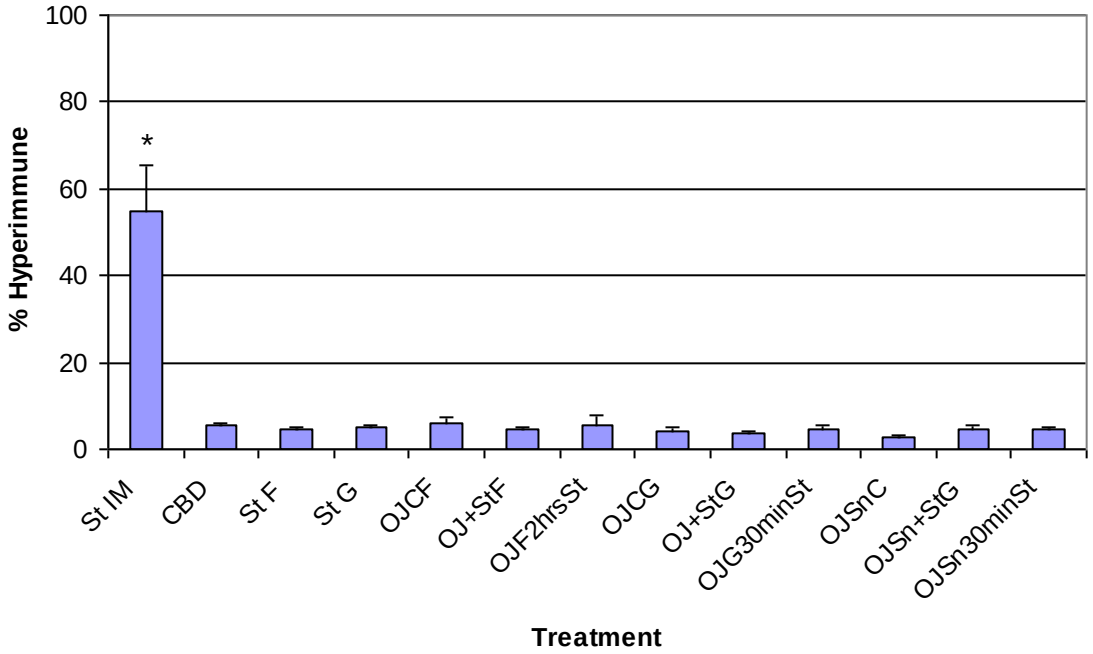

Figure 24: Day 14 anti-*S. typhimurium* IgG in serum

St IM: *S. typhimurium* in adjuvant, intramuscular injection; CBD: commercial broiler diet; St F: *S. typhimurium* dry fed; St G: *S. typhimurium* wet gavage; OJC F: Oralject control dry fed; OJ+St F: Oralject+*S. typhimurium* dry fed; OJF2hrsSt G: Oralject dry fed 2 hrs later *S. typhimurium* wet gavage; OJC G: Oralject control wet gavage; OJ+St G: Oralject+*S. typhimurium* wet gavage; OJG30minStG: Oralject wet gavage 30 min later *S. typhimurium* wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+St G: Oralject extract solution + *S. typhimurium* wet gavage; OJSn30minSt G: Oralject extract solution 30 min later *S. typhimurium* wet gavage.

Columns represent the treatment group mean and vertical bars are the standard error of the mean.

* significant difference at $p<0.01$.

As seen on Figure 25, on day 22, 8 days following the delivery of the booster immunisation, again it was only the positive control group (St IM) which exhibited notable anti-*S. typhimurium* IgG titres in serum, which were significantly ($p<0.01$) higher than all other treatment groups. All other treatment groups had negligible titres.

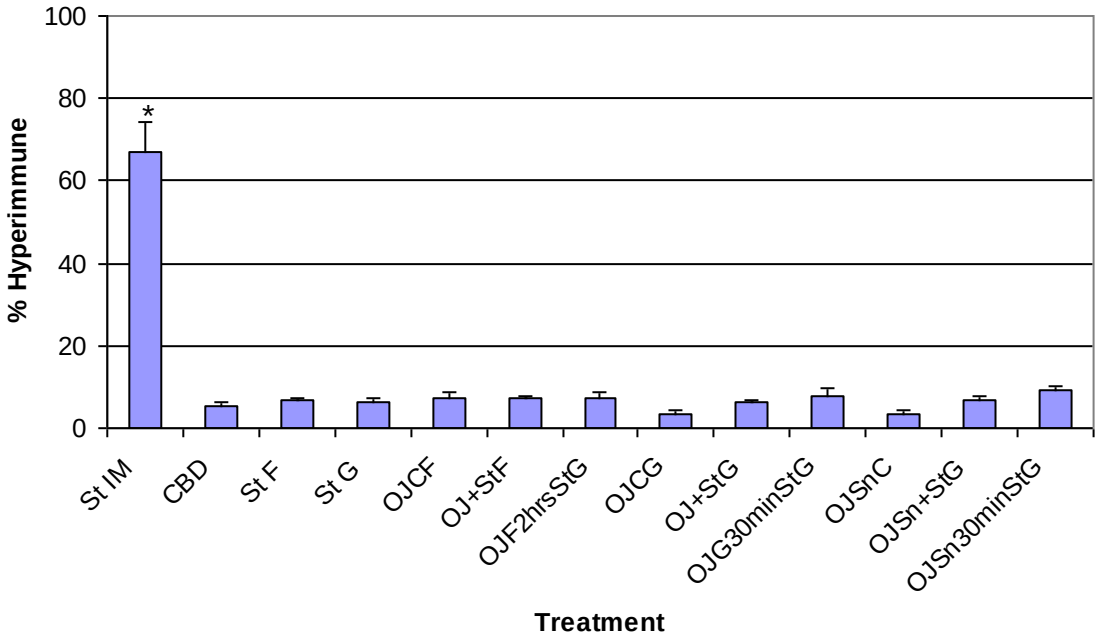

Figure 25: Day 22 anti-*S. typhimurium* IgG in serum

St IM: *S. typhimurium* in adjuvant, intramuscular injection; CBD: commercial broiler diet; St F: *S. typhimurium* dry fed; St G: *S. typhimurium* wet gavage; OJC F: Oralject control dry fed; OJ+St F: Oralject+*S. typhimurium* dry fed; OJF2hrsSt G: Oralject dry fed 2 hrs later *S. typhimurium* wet gavage; OJC G: Oralject control wet gavage; OJ+St G: Oralject+*S. typhimurium* wet gavage; OJG30minStG: Oralject wet gavage 30 min later *S. typhimurium* wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+St G: Oralject extract solution + *S. typhimurium* wet gavage; OJSn30minSt G: Oralject extract solution 30 min later *S. typhimurium* wet gavage.

Columns represent the treatment group mean and vertical bars are the standard error of the mean.

* significant difference at $p<0.01$.

Between days 22 and 29 (Figure 26) there was a notable increase in the anti-*S. typhimurium* IgG titre observed in the St IM positive control treatment group, increasing from 67% to 88%. The anti-*S. typhimurium* IgG titre of all other treatments groups remained below 11% on day 29 and were significantly lower ($p<0.01$) than St IM treated birds. However, of these groups, the negative control group (CBD) and the OJC F group mean anti-*S. typhimurium* IgG titres (11% hyperimmune serum in both cases) were significantly higher ($p<0.01$) than OJSnC (4% hyperimmune).

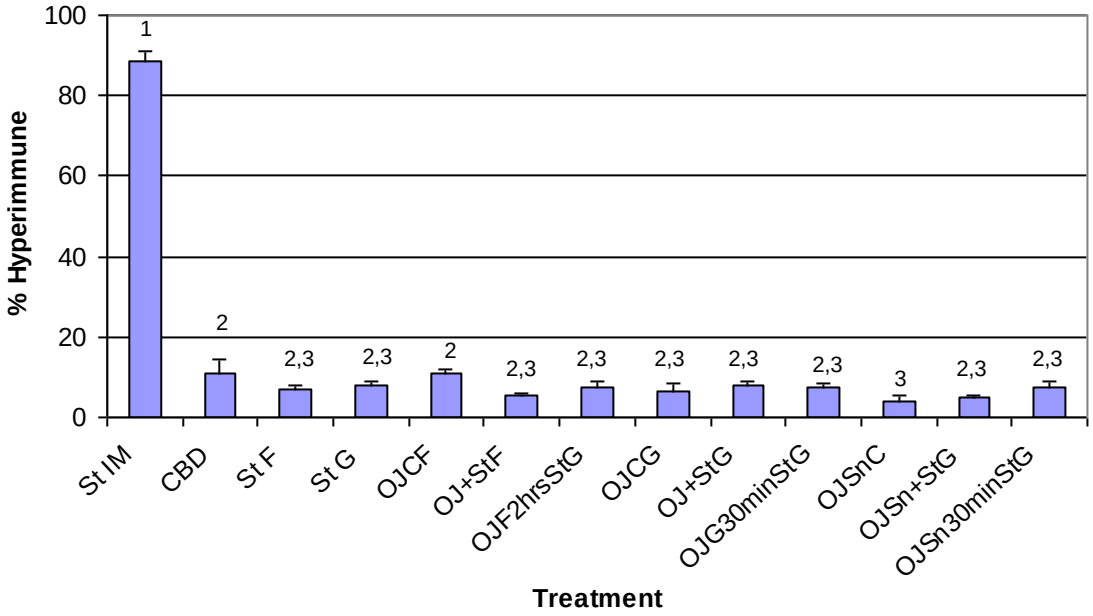

Figure 26: Day 29 anti-*S. typhimurium* IgG in serum

St IM: *S. typhimurium* in adjuvant, intramuscular injection; CBD: commercial broiler diet; St F: *S. typhimurium* dry fed; St G: *S. typhimurium* wet gavage; OJC F: Oralject control dry fed; OJ+St F: Oralject+*S. typhimurium* dry fed; OJF2hrsSt G: Oralject dry fed 2 hrs later *S. typhimurium* wet gavage; OJC G: Oralject control wet gavage; OJ+St G: Oralject+*S. typhimurium* wet gavage; OJG30minStG: Oralject wet gavage 30 min later *S. typhimurium* wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+St G: Oralject extract solution + *S. typhimurium* wet gavage; OJSn30minSt G: Oralject extract solution 30 min later *S. typhimurium* wet gavage.

Columns represent the treatment group mean and vertical bars are the standard error of the mean.

[1, 2, 3] Columns with different superscripts differ significantly ($p<0.01$).

On the final day of observation, day 36, the St IM treatment group had the highest anti-*S. typhimurium* IgG titre of 86% of the hyperimmune sera. This titre was significantly higher ($p<0.01$) than the average titre of all other treatment groups in study 3 (Figure 27).

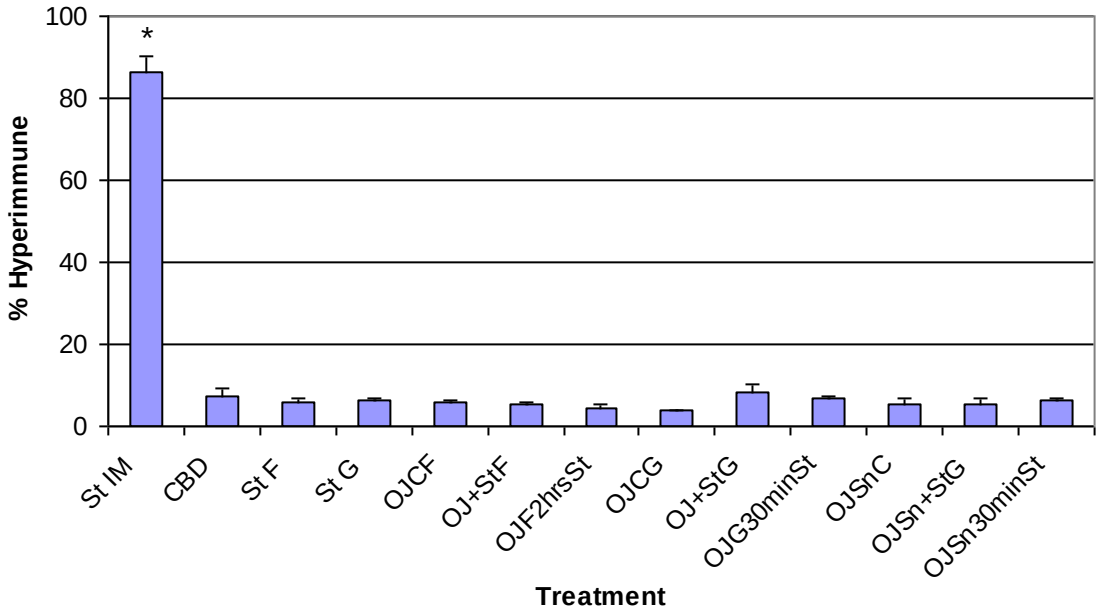

Figure 27: Day 36 anti-*S. typhimurium* IgG in serum
St IM: *S. typhimurium* in adjuvant, intramuscular injection; CBD: commercial broiler diet; St F: *S. typhimurium* dry fed; St G: *S. typhimurium* wet gavage; OJC F: Oralject control dry fed; OJ+St F: Oralject+*S. typhimurium* dry fed; OJF2hrsSt G: Oralject dry fed 2 hrs later *S. typhimurium* wet gavage; OJC G: Oralject control wet gavage; OJ+St G: Oralject+*S. typhimurium* wet gavage; OJG30minStG: Oralject wet gavage 30 min later *S. typhimurium* wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+St G: Oralject extract solution + *S. typhimurium* wet gavage; OJSn30minSt G: Oralject extract solution 30 min later *S. typhimurium* wet gavage.
Columns represent the treatment group mean and vertical bars are the standard error of the mean.
* significant difference at $p<0.01$.

Serum anti-*S. typhimurium* IgA

Day -1 sera anti-*S. typhimurium* IgA titres were below 10% hyperimmune sera in all treatment groups prior to the start of the study (Figure 28). It is worth noting here that on day -1, and despite these very low antibody titres for all treatment groups, the Oralject extract solution control group (OJSnC) had an average anti-*S. typhimurium* IgA antibody titre of 3.4% which was significantly lower ($p<0.01$) than all other treatment groups. This trend continued for this treatment group throughout the study, and is a reflection of the exceptionally low absorbance in the ELISA assay of the birds in this group throughout the study. On subsequent days significant differences between OJSnC and all other treatment groups except for St IM, reflect this very low mean titre of the OJSnC treatment group for *S. typhimurium* IgA, rather than any of the other treatment groups, again, apart from St IM, having notably high antibody titres.

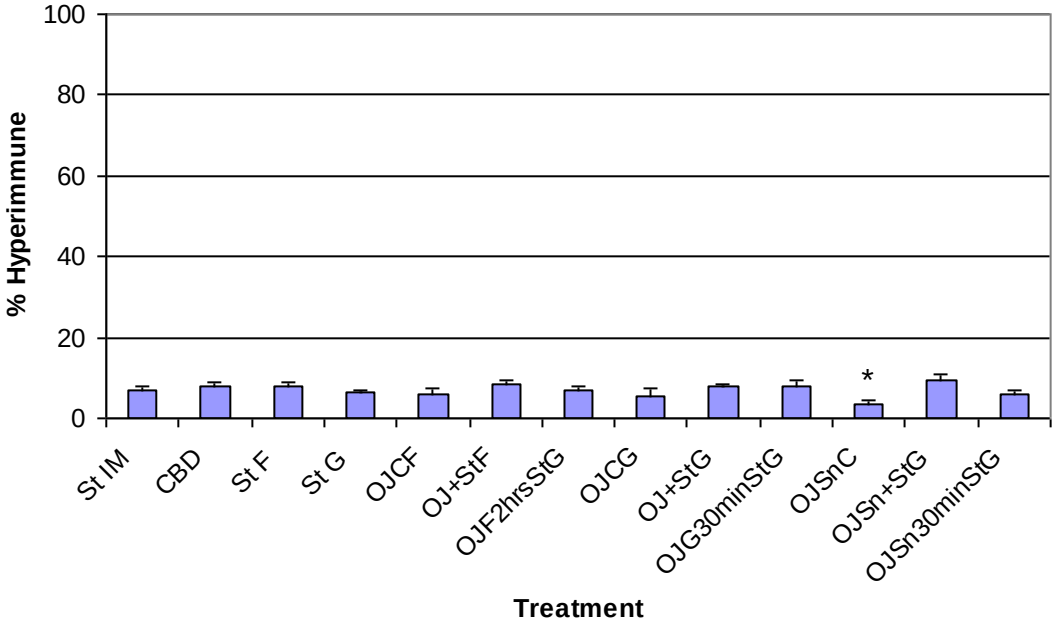

Figure 28: Day -1 anti-*S. typhimurium* IgA in serum.
St IM: *S. typhimurium* in adjuvant, intramuscular injection; CBD: commercial broiler diet; St F: *S. typhimurium* dry fed; St G: *S. typhimurium* wet gavage; OJC F: Oralject control dry fed; OJ+St F: Oralject+*S. typhimurium* dry fed; OJF2hrsSt G: Oralject dry fed 2 hrs later *S. typhimurium* wet gavage; OJC G: Oralject control wet gavage; OJ+St G: Oralject+*S. typhimurium* wet gavage; OJG30minStG: Oralject wet gavage 30 min later *S. typhimurium* wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+St G: Oralject extract solution + *S. typhimurium* wet gavage; OJSn30minSt G: Oralject extract solution 30 min later *S. typhimurium* wet gavage.
Columns represent the treatment group mean and vertical bars are the standard error of the mean.
* significant difference at $p<0.01$.

On day 14 (Figure 29) the St IM treatment group exhibited an anti-*S. typhimurium* IgA titre 95% of the hyperimmune sera, which was significantly higher ($p<0.01$) than all other treatment groups. The OJSnC group had a mean anti-BSA IgA titre that were significantly lower ($p <0.01$) than CBD, St F, OJC F, OJ+St F and OJF2hrsSt G. For birds treated with *S. typhimurium*, wet via gavage (St G) the mean anti- *S. typhimurium* IgA titre in serum on day 14 was significantly lower than CBD, OJC F and OJ+St F. While at this timepoint some of the *S. typhimurium* treated groups, apart from IM administration of *S. typhimurium* in adjuvant, had experienced small increases from day -1 to day 14, in the anti-*S. typhimurium* IgA titres, all remained below 20%. Further, two of the *S. typhimurium* untreated groups also had *S. typhimurium* specific IgA titres between 15 – 20%, (specifically CBD and OJC F, with OJC G and OJSnC recording titres below 15% hyperimmune), which, when compared with the day -1 measures, indicates that there may have been some low level exposure to *S. typhimurium* or some other antigen whose antibodies are cross-reacting with *S. typhimurium* in the IgA ELISA assay. However, as no notable increase was seen in the antibody titres of the control treatment groups for *S. typhimurium* IgG at day 14, it is most likely to be some non-specific binding that is occurring only in the *S. typhimurium* IgA ELISA. These higher background titres have been observed for anti-*S. typhimurium* IgA from day 14 onwards in this study.

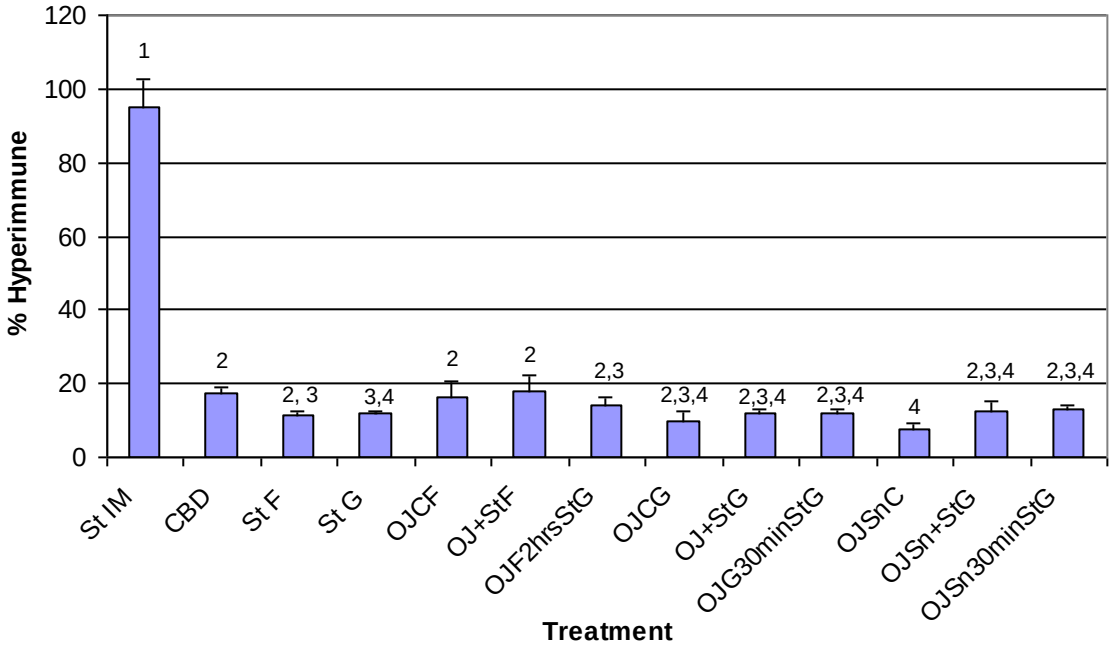

Figure 29: Day 14 anti-*S. typhimurium* IgA in serum

St IM: *S. typhimurium* in adjuvant, intramuscular injection; CBD: commercial broiler diet; St F: *S. typhimurium* dry fed; St G: *S. typhimurium* wet gavage; OJC F: Oralject control dry fed; OJ+St F: Oralject+*S. typhimurium* dry fed; OJF2hrsSt G: Oralject dry fed 2 hrs later *S. typhimurium* wet gavage; OJC G: Oralject control wet gavage; OJ+St G: Oralject+*S. typhimurium* wet gavage; OJG30minStG: Oralject wet gavage 30 min later *S. typhimurium* wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+St G: Oralject extract solution + *S. typhimurium* wet gavage; OJSn30minSt G: Oralject extract solution 30 min later *S. typhimurium* wet gavage.

Columns represent the treatment group mean and vertical bars are the standard error of the mean.

[1, 2, 3,4] Columns with different superscripts differ significantly ($p<0.01$).

There was a similar trend with the anti-*S. typhimurium* IgA titres on day 22 as seen on day 14, where the St IM treatment group has a high mean anti-*S. typhimurium* IgA titre, ($p<0.01$) while the mean titres of all other treatment groups remained below 20% hyperimmune (Figure 30). The anti-*S. typhimurium* IgA titres of all four control groups, that is CBD, OJC F, OJC G and OJSnC, were below 15% hyperimmune. However, the control group OJSnC was significantly lower ($p<0.01$) than OJ+St F and OJ+St G, OJF2hrsSt G, OJG30minSt G and OJSn+St G.

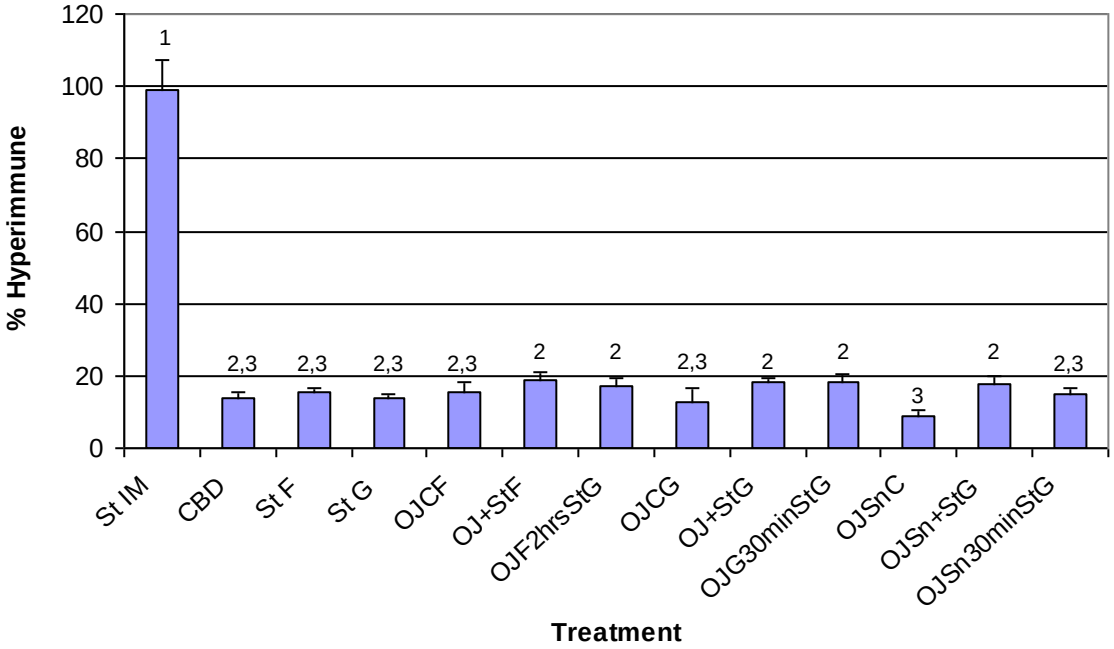

Figure 30: Day 22 anti-*S. typhimurium* IgA in serum

St IM: *S. typhimurium* in adjuvant, intramuscular injection; CBD: commercial broiler diet; St F: *S. typhimurium* dry fed; St G: *S. typhimurium* wet gavage; OJC F: Oralject control dry fed; OJ+St F: Oralject+*S. typhimurium* dry fed; OJF2hrsSt G: Oralject dry fed 2 hrs later *S. typhimurium* wet gavage; OJC G: Oralject control wet gavage; OJ+St G: Oralject+*S. typhimurium* wet gavage; OJG30minStG: Oralject wet gavage 30 min later *S. typhimurium* wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+St G: Oralject extract solution + *S. typhimurium* wet gavage; OJSn30minSt G: Oralject extract solution 30 min later *S. typhimurium* wet gavage.

Columns represent the treatment group mean and vertical bars are the standard error of the mean.

[1, 2, 3,] Columns with different superscripts differ significantly ($p<0.01$).

On day 29, the mean anti-*S. typhimurium* IgA titres of some treatment groups, apart from St IM, were above 20% hyperimmune. Some of these groups had been treated with *S. typhimurium*, for example OJ + St F (24% hyperimmune), but others had not been administered *S. typhimurium* as a part of their treatment, for example OJCF (21% hyperimmune) (Figure 31). In fact on day 29, the only negative control treatment group to have a *S. typhimurium* IgA titre below 15% was OJSn C. This raises the question again about the possibility of accidental exposure of all birds to *S. typhimurium*, or to an antigen that cross reacts with *S. typhimurium* IgA in the ELISA. However, as mentioned under day 14**,** as no notable increase was seen in the antibody titres of the control treatment groups for *S. typhimurium* IgG at day 29, (and they all remained below 15% at this time) it is most likely to be some non-specific binding that is occurring only in the *S. typhimurium* IgA ELISA. As on day 22, there were no statistically significant differences between the treatment groups except for St IM which had a significantly higher anti-*S. typhimurium* IgA titre than all other treatments ($p<0.01$) and OJSnC, which had a significantly ($p<0.01$) lower mean anti-*S. typhimurium* IgA titre than all other treatment groups.

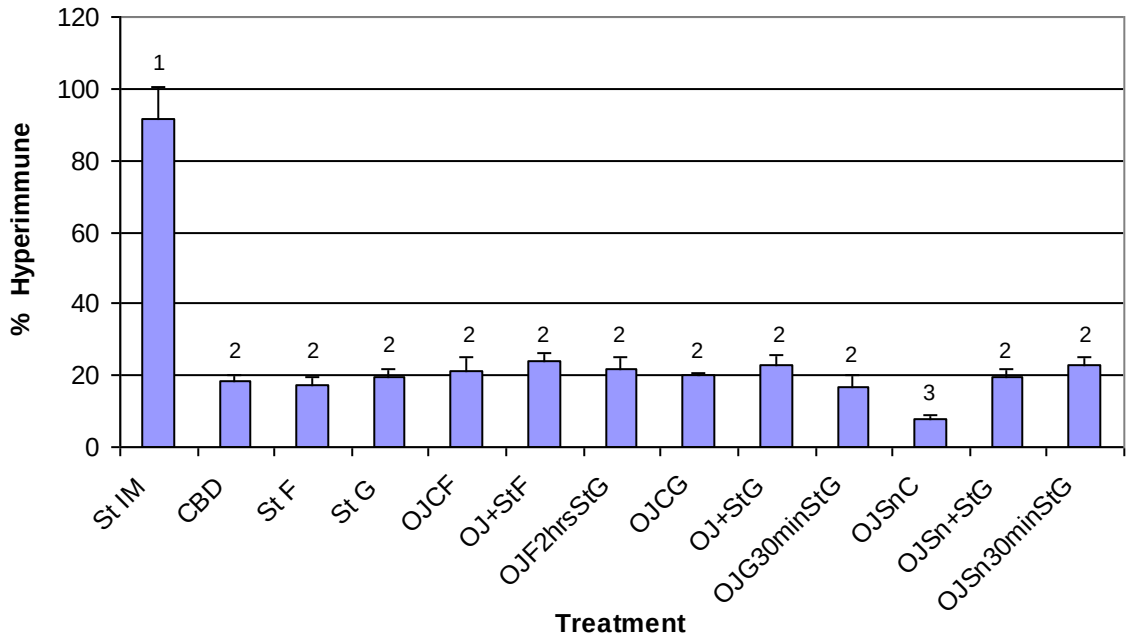

Figure 31: Day 29 anti-*S. typhimurium* IgA in serum

St IM: *S. typhimurium* in adjuvant, intramuscular injection; CBD: commercial broiler diet; St F: *S. typhimurium* dry fed; St G: *S. typhimurium* wet gavage; OJC F: Oralject control dry fed; OJ+St F: Oralject+*S. typhimurium* dry fed; OJF2hrsSt G: Oralject dry fed 2 hrs later *S. typhimurium* wet gavage; OJC G: Oralject control wet gavage; OJ+St G: Oralject+*S. typhimurium* wet gavage; OJG30minStG: Oralject wet gavage 30 min later *S. typhimurium* wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+St G: Oralject extract solution + *S. typhimurium* wet gavage; OJSn30minSt G: Oralject extract solution 30 min later *S. typhimurium* wet gavage.

Columns represent the treatment group mean and vertical bars are the standard error of the mean.

[1, 2, 3,] Columns with different superscripts differ significantly ($p<0.01$).

The observations made on day 36 are presented in Figure 32. St IM treated birds had a significantly higher mean anti-*S. typhimurium* IgA titre than all other treatment groups ($p<0.01$). All other groups had mean titres below 20% hyperimmune serum, and all negative control groups had titres below 15% hyperimmune serum.

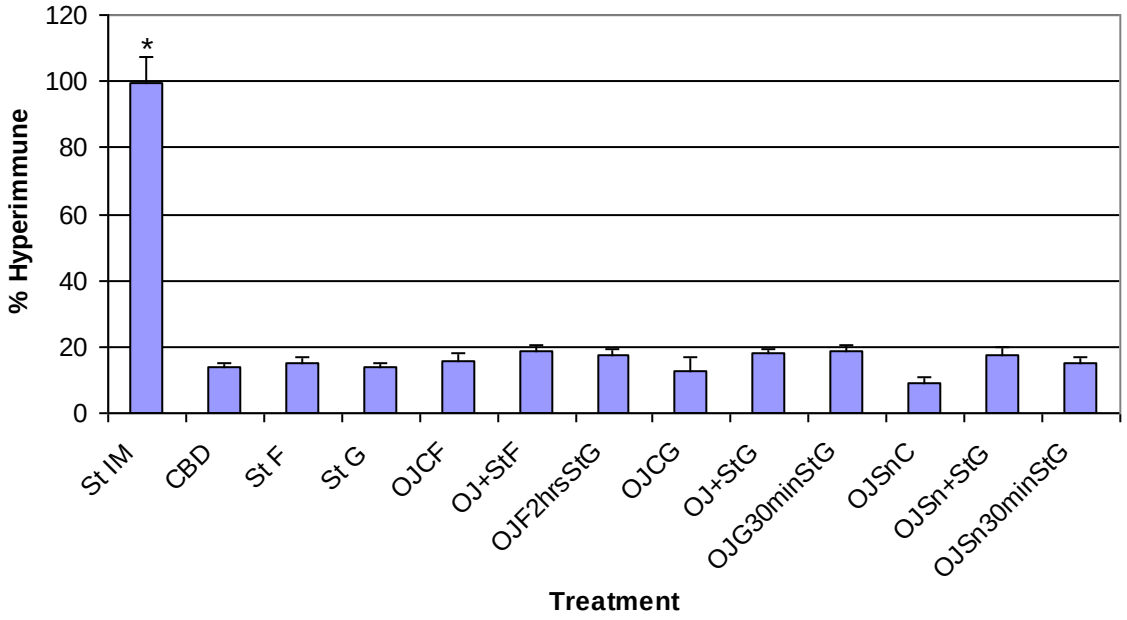

Figure 32: Day 36 anti-*S. typhimurium* IgA in serum

St IM: *S. typhimurium* in adjuvant, intramuscular injection; CBD: commercial broiler diet; St F: *S. typhimurium* dry fed; St G: *S. typhimurium* wet gavage; OJC F: Oralject control dry fed; OJ+St F: Oralject+*S. typhimurium* dry fed; OJF2hrsSt G: Oralject dry fed 2 hrs later *S. typhimurium* wet gavage; OJC G: Oralject control wet gavage; OJ+St G: Oralject+*S. typhimurium* wet gavage; OJG30minStG: Oralject wet gavage 30 min later *S. typhimurium* wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+St G: Oralject extract solution + *S. typhimurium* wet gavage; OJSn30minSt G: Oralject extract solution 30 min later *S. typhimurium* wet gavage.

Columns represent the treatment group mean and vertical bars are the standard error of the mean.

* significant difference at $p<0.01$.

Intestinal scrapings anti-*S. typhimurium* IgA

Day 22 *S. typhimurium* specific IgA antibody titres in ISS are depicted in Figure 33. Birds receiving the St IM treatment had a statistically significant titre (53% hyperimmune) ($p<0.01$) compared with all other treatment groups (highest titre was 11.5% for OJ+StG treatment). Unfortunately there was insufficient sample to measure anti-*S. typhimurium* IgA in the OJSnC group. At this time the mean anti-*S. typhimurium* IgA titre in intestinal scrapings of the OJC G treatment (4.3% hyperimmune) was significantly lower than the CBD, OJ+St G and OJSn+St G treatment groups.

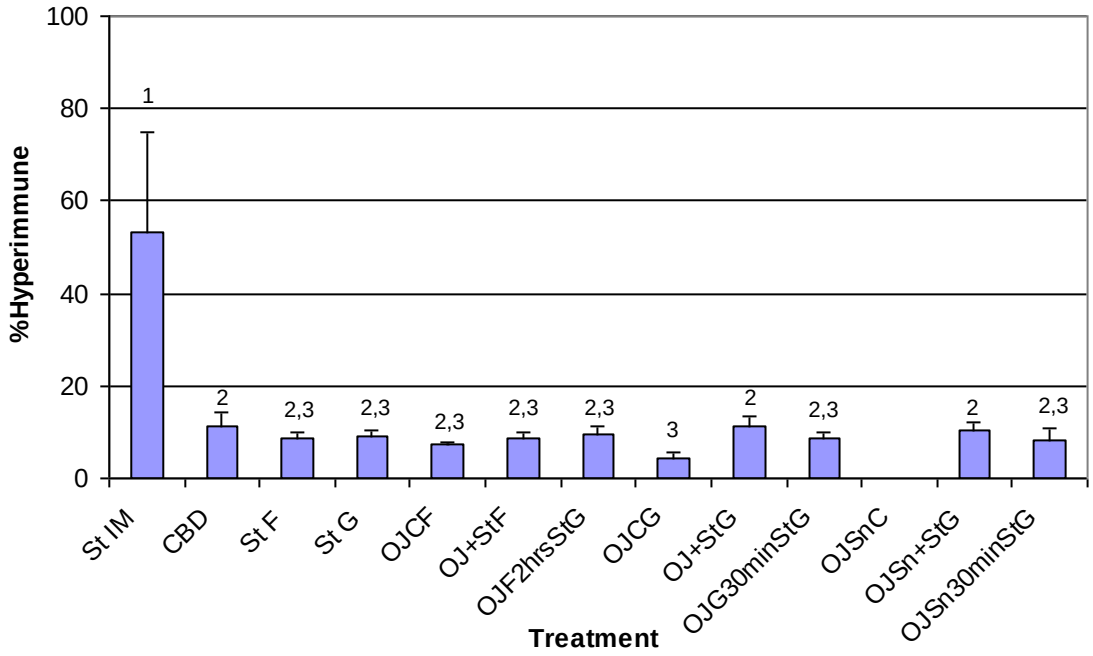

Figure 33: Day 22 anti-*S. typhimurium* IgA in intestinal scrapings supernatant

St IM: *S. typhimurium* in adjuvant, intramuscular injection; CBD: commercial broiler diet; St F: *S. typhimurium* dry fed; St G: *S. typhimurium* wet gavage; OJC F: Oralject control dry fed; OJ+St F: Oralject+*S. typhimurium* dry fed; OJF2hrsSt G: Oralject dry fed 2 hrs later *S. typhimurium* wet gavage; OJC G: Oralject control wet gavage; OJ+St G: Oralject+*S. typhimurium* wet gavage; OJG30minStG: Oralject wet gavage 30 min later *S. typhimurium* wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+St G: Oralject extract solution + *S. typhimurium* wet gavage; OJSn30minSt G: Oralject extract solution 30 min later *S. typhimurium* wet gavage.

Columns represent the treatment group mean and vertical bars are the standard error of the mean.

[1, 2, 3,] Columns with different superscripts differ significantly ($p<0.01$).

As seen on Figure 34, the only significant difference in day 36 anti-*S. typhimurium* IgA titres in intestinal scrapings was with the St IM treated birds where the mean titre was significantly higher than all other treatment groups.

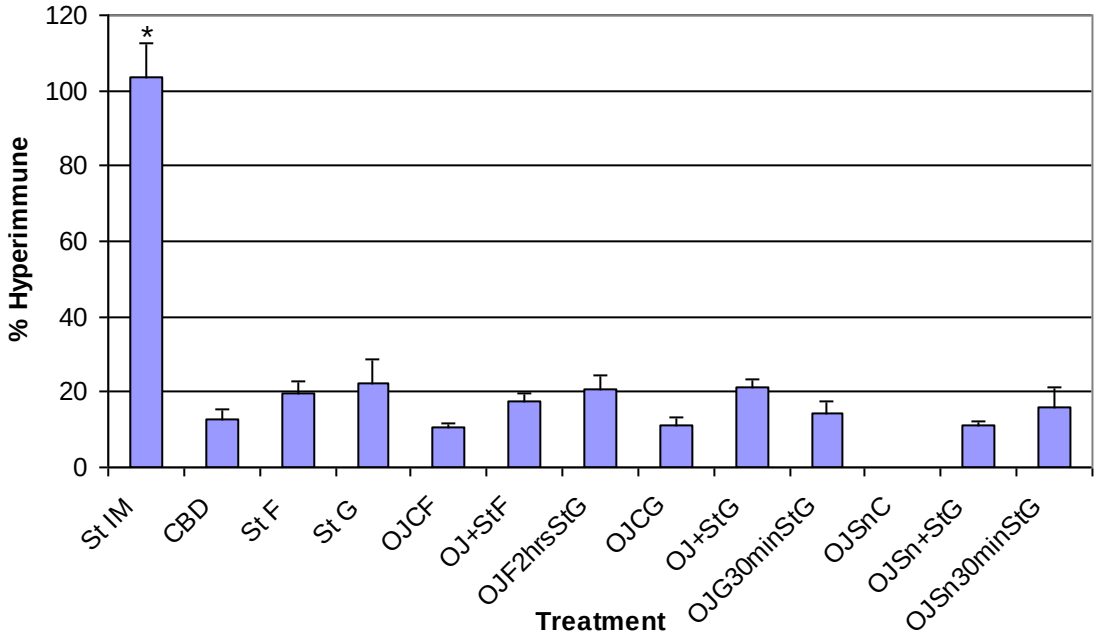

Figure 34: Day 36 anti-*S. typhimurium* IgA in intestinal scrapings supernatant
St IM: *S. typhimurium* in adjuvant, intramuscular injection; CBD: commercial broiler diet; St F: *S. typhimurium* dry fed; St G: *S. typhimurium* wet gavage; OJC F: Oralject control dry fed; OJ+St F: Oralject+*S. typhimurium* dry fed; OJF2hrsSt G: Oralject dry fed 2 hrs later *S. typhimurium* wet gavage; OJC G: Oralject control wet gavage; OJ+St G: Oralject+*S. typhimurium* wet gavage; OJG30minStG: Oralject wet gavage 30 min later *S. typhimurium* wet gavage; OJSnC: Oralject extract solution control gavage; OJSn+St G: Oralject extract solution + *S. typhimurium* wet gavage; OJSn30minSt G: Oralject extract solution 30 min later *S. typhimurium* wet gavage.
Columns represent the treatment group mean and vertical bars are the standard error of the mean.
* significant difference at $p<0.01$.

Discussion of Results

These studies were designed to assess the potential of Oralject, (PerOs Systems Technologies), a diet consisting of ingredients with anti-nutritional factors that will interrupt the hydrolytic processes, to deliver antigen orally, to the cells of the immune system along the intestinal mucosa of the chicken, thereby stimulating a local immune response. The most striking outcome of this series of studies is the ability of the Oralject extract solution to facilitate a significant immune response to BSA, following the concurrent delivery of BSA with the Oralject extract solution. When the Oralject extract solution and BSA were delivered together via gavage (OJSn+BSA G) they generated anti-BSA IgA titres, one week after the secondary immunisation (that is day 22 of the study), that were not significantly different from the injection of BSA in adjuvant (BSA IM). At this time OJSn+BSA G also had significantly higher BSA IgA titres than any of the BSA control groups i.e. the delivery of BSA alone in feed or via gavage, and also any of the BSA + Oralject dry or wet treatments delivered in feed or wet via gavage. Mean anti-BSA IgG titres which were not significantly different from the positive control group were also seen with OJSn+BSA G treated birds on day 22. However, it must be noted that the BSA IM treated birds had anti-BSA IgG titres which were significantly higher than the OJ+BSA F and OJSn+BSA G birds on day -1, though these were all within the range of a negative response. Further, the increase seen in the BSA IM group between day -1 and day 14 was statistically significant. The mean anti-BSA IgG titres of OJSn+BSA G treatment group were significantly higher than all other treatments, expect for the BSA IM treatment, on day 14, 29 and 36.

Interestingly, the OJSn30minBSA G treated birds, while also often generating anti-BSA titres of the IgG and IgA isotypes which were significantly higher than the control treatment groups, their titres were generally less than that of the OJSn+BSA G birds. This would tend to indicate that the Oralject

solution does have a very immediate effect on the local intestinal environment which favours antigen delivery, but that this effect may be relatively short-lived, so that within 30 minutes of the delivery of the Oralject extract solution the gut environment is returning towards its normal acidic state again. An assessment of the duration of the effects of the Oralject extract solution on the local gut environment, in particular gut pH, protease activity and gut permeability, the three pronged approach of Oralject, is required to better understand the physiological alterations within the intestinal lumen over time.

The success of the Oralject extract solutions compared with Oralject in either the dry or wet form, in association with the BSA antigen to generate BSA specific antibody titres may be related to the immediate availability and impact of the anti-nutritional components of the Oralject diet in the extracted solution. However, this requires investigation, as it may be expected that with time these nutritional factors would come into play in the Oralject wet or dry diets. But that appears not to be the case in these studies where treatments which involved the delivery of BSA either 2 hours after the delivery of Oralject in the dry form in the diet, or 30 minutes after the delivery of Oralject in the wet form administered via gavage, did not demonstrate any notable IgG or IgA antibody responses to BSA.

It was disappointing to see the absence of any effect of Oralject, delivered in the wet or dry form, on the humoral immune response of the chicken. This was the case with all antigens tested; BSA, EHNV and killed *S. typhimurium*, for the duration of the study. Clearly these three antigens are very different, which was a very deliberate decision by the investigators, in an attempt to assess a variety of antigen types with the Oralject technology. BSA is a small protein (molecular mass 69 kDa) antigen, EHNV a viral antigen, which was chosen as a model to reflect the effect of Oralject when used with avian viruses such as Marek's disease virus and *S. typhimurium*, a whole killed bacterium which is in the "Baiada" *Salmonella spp* vaccine, and was included as an example of the interaction of Oralject with a commercial vaccine antigen. It must be highlighted that the procedures used in these studies were specifically designed to meet the recommendations of Friedman *et al*. (2003) and Klipper *et al*., (2000) for the generation of a local immune response, while avoiding the generation of systemic tolerance.

Another variable to be considered with regard to the immune responses generated, is the antigen dose rate. In these studies there was limited opportunity to assess Oralject with several dose rates of one antigen. It may be that the concentration of antigen used may have been outside the range suitable for use with Oralject. Dose titration studies are an essential next step for each of these antigens with the dry and wet Oralject, and the Oralject extract solution preparations, to ensure that the Oralject technology has not been limited by the chosen antigen dose.

In a similar vein, it was curious to observe the difference in responses of the two antigens BSA and whole killed *S. typhimurium* when delivered with the Oralject extract solution. As discussed above, the gavage delivery of BSA together with Oralject extract solution generated antibody titres to BSA which were statistically higher than the BSA control treatments and, most notably, not dissimilar to the BSA IM treated birds at several observation times throughout the study. These are in outstanding contrast to the absence of any notable response of these same treatments when delivered with killed *S. typhimurium.* Again, these differences may be a consequence of the different antigens, the antigen dose rates used and possibly the timing of antigen and carrier delivery. Each of these factors requires further evaluation.

Of the antigens used, all of the positive control groups (where antigen in adjuvant was administered via injection) generated relatively high anti-antigen IgG titres in serum. Both the positive control groups for the BSA and *S. typhimurium* antigens also induced good anti-antigen IgA titres in serum. However, this was not the case with EHNV, where the anti-EHNV IgA titres in serum were very disappointing and indicated a potential limitation with the use of this antigen in chickens for the generation of IgA antibodies. However, it must also be remembered that the positive control groups were administered antigen in adjuvant via intramuscular injection, which typically generates a systemic, IgG dominant antibody response. This vaccination protocol is not ideal for the generation of IgA antibody titres. A more suitable protocol involves an intraperitoneal primary vaccination

followed with a local muscosal focussed booster immunisation, as was applied for hyperimmunisation of chickens (outlined under Methodology). This generated good anti-antigen IgA titres for all antigens, including EHNV, in the hyperimmunised birds.

Difficulties with the ELISA assays, and in particular, with the running of the ISS and bile samples, which were specifically collected to identify the local, intestinal immune response, has limited a thorough appreciation of the immune response at the intestinal surface in each of these studies. While previous work has shown that the ISS antibody titres typically follow the trends identified in serum IgA titres, (Muir *et al.*, 1998), and that was also the case here with the anti-*S. typhimurium* IgA titres in serum and ISS on day 22 (Figures 30 and 33 respectively) and 36 (Figures 32 and 34 respectively), this assumption should be treated with caution, and the samples need to be assayed to be able to accurately report on the intestinal response. High levels of background colour also presented challenges to the anti-antigen IgA titres in serum with all three antigens. Initial work to optimise the assays evaluated the use of a variety of agents and techniques to reduce background colour (outlined in the Methodology section for each ELISA). However, from the test results it would appear that the techniques identified and used to reduce this problem did not prove to be 100% effective for all samples, and may have compromised the statistical analysis of the results on some occasions.

In some instances statistically significant differences were observed between treatment groups at low percentages of the hyperimmune standard reference serum (15% hyperimmune or below) such that they are considered to be a negative measure. For example, this was seen in study 3, on day 29 anti-*S. typhimurium* IgG titres (Figure 26) and also with anti-*S. typhimurium* IgA titres – and was evident from day -1 (Figure 28), when the OJSnC group had a mean titre which was significantly lower than all other treatment groups, but the day -1 mean titres of all treatment groups were within the negative range. However, these differences have been reported to provide a complete review of the outcomes of the study.

Implications

These studies were designed as a proof-of-concept of the potential application of Oralject to the oral delivery of antigen to the chicken. They have demonstrated some potential for Oralject to deliver antigen to the intestinal immune system of the chicken, through evidence of an immune response to that antigen. But it must be reiterated that, of the three antigens used, this was seen with only one antigen, and further, it was achieved when the antigen was delivered with the extracted solution of Oralject only, not with Oralject in its original form. Therefore, further research and development is needed with the application of Oralject in poultry before it will be a viable option for the oral delivery of vaccines and bioactive compounds.

Technology such as Oralject, which would allow for the oral delivery of antigen to intensively housed poultry would have a significant impact on the industry. Stimulation of mucosal immunity with the oral administration of antigen will enhance the health and well-being of poultry both indirectly and directly. The indirect benefits include a reduction in the handling of individual birds during vaccination and a decline in biosecurity risks involved with the movement of vaccination crews between farms and sheds on farms. Direct benefits come from the direct stimulation of mucosal immunity and bird health; and the potential to increase the number of deliverable treatments administered to large populations of chickens. In particular, this will ensure optimum antibody titres to maintain health and the transfer of maternal antibody to chicks without necessitating individual bird treatments.

Effective methods of oral delivery will also facilitate the administration of bioactive compounds to broiler and layer hens and reduce the use of in-feed antimicrobials to prevent disease. A reduction in the use of in-feed antimicrobials would alleviate concerns about the potential to develop pathogens with antimicrobial resistance, and importantly, the transfer of this resistance onto human pathogens. Improvements in industry sustainability could also be realised through reductions in the contamination of meat or eggs and a decrease in the excretion of potential toxins in the manure.

Further, oral delivery of bioactive compounds without the need to handle individual animals for individual bird injections, will have benefits for bird welfare. Oral delivery avoids placing stress on the bird during handling and will also reduce the incidence of injury to birds while been handled for injections. This would also be important in enabling "green" production of antibodies in eggs for human and other animal treatments.

Recommendations

The Oralject carrier diet has been developed by PerOs Technology, and it is understood that they currently have filed for a provisional patent (personal communication G. Vandenberg, 2007). However, to further develop this technology for use in the poultry industry its application needs to be tested with a number antigen types and/or vaccines. The variation in immune responses when using Oralject extract solution with different antigens has been demonstrated in the studies presented here, and the reasons behind this need to be fully explored. Further, the feasibility of using Oralject with birds of different ages and in varying production systems, needs to be evaluated. The practical delivery of Oralject extract solution is also required as in this project it was delivered via gavage. Administration in the drinking water is the most desirable application, however, the suitability of the Oralject extract solution for this, and protocols that will ensure each bird consumes the required amounts of carrier and antigen, need to be established.

Plain English Compendium Summary

Project Title:	Oral delivery system for poultry health products
Poultry CRC Project No.:	04:14: US Scott
Researcher:	Professor Tom Scott and Dr Wendy Muir
Organisation:	University of Sydney
Phone:	(02) 46550658
Fax:	(02) 46 550693
Email:	wmuir@camden.usyd.edu.au
Project Overview	This project was designed to evaluate the ability of a vaccine carrier diet, Oralject (PerOs System Technologies Ltd, Canada), which has been used successfully for the oral delivery of vaccines in the aquaculture industry, to deliver orally administered antigen to chickens.
Background	Many pathogens come into contact with a host through the gastrointestinal tract (GIT). It is assumed that immunisation via the oral delivery of pathogen antigens would generate a protective immune response. However, generally the oral delivery of killed antigens generates poor immune responses. This is due to several factors including the damaging effect of the digestive processes of the GIT on the antigen, and poor antigen uptake by the immune cells along the GIT. Therefore, this study investigated the ability of the carrier diet, Oralject, which manipulates the environment within the GIT and also favours antigen delivery to immune cells along the GIT, to effectively deliver orally administered antigen to broiler chickens.
Research	Oralject was tested with the oral deliver of three different antigens, bovine serum albumen (BSA), heat inactivated epizootic haematopoietic necrosis virus (EHNV) and killed *Salmonella typhimurium*, in four week old broiler chickens. Two preparations of Oralject were tested, the first preparation used dry Oralject dietary ingredients which were delivered in either a wet form via gavage or a dry form directly in the feeder. The second preparation was a liquid retrieved following the extraction of chemicals from similar ingredients used in the dry diet preparation, known as Oralject extract solution, and was delivered by gavage. This was assessed with the BSA and *S. typhimurium* antigens only. The Oralject carrier diet was delivered prior to or together with the antigen. The antibody titres to the antigens were determined in serum, for 5 weeks following the initial immunisation.
Project Progress	In these studies the dry Oralject carrier diet did not demonstrate any benefits in terms of the resultant immune response, compared with the delivery of antigen alone. Birds receiving BSA with the Oralject extract solution had antibody titres that were significantly higher than the delivery of antigen alone, and they were, for a short period, comparable to the responses seen in the gold standard positive control group. However, this was only observed with the BSA antigen, it was not observed when killed *S. typhimurium* was delivered with the Oralject extract solution.
Implications	The outcomes of this study require further investigation – as both antigen and Oralject preparations influenced the characteristics of the immune response. The ability to deliver vaccines orally, to poultry would have significant benefits for the industry, including improved bird health, increased farm biosecurity and improved bird welfare. A reduction in the use of in-feed antimicrobials may also be realised, alleviating concerns about the potential to develop pathogens with antimicrobial resistance, and importantly, the transfer of this resistance onto human pathogens.
Publications	To date no part of this work has been published, at the request of PerOs Systems Technologies Ltd, Canada.

References

Ameiss, K.A., Attrache, J.El., Barri, E., McElroy, A.P. and Caldwell, D.J. 2005. Influence of orally administered CpG-ODN's on the humoral response to bovine serum albumin (BSA) in chickens. Veterinary Immunology and Immunopathology. 110:257-267.

Andrianov, A.K. and Payne, L.G. 1998. Polymeric carriers for oral uptake of microparticulates. Advanced Drug Delivery Reviews 34: 155-170.

Bradford, M.M. 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

Brayden, D.J. and Baird, A.W. 2004. Apical membrane receptors on intestinal M cells: potential targets for vaccine delivery. Advanced Drug Delivery Reviews 56(6) 721-726.

Caldwell, D.J., Danforth, H.D., Morris, B.C., Ameiss, K.A. and McElroy, A.P. 2004 Participation of the intestinal epithelium and mast cells in local mucosal immune responses in commercial poultry. Poultry Science 83: 591-599

Clark, M.A., Hirst, B.H. and Jepson, M.A. 2000. Lectin-mediated mucosal delivery of drugs and microparticles. Advanced Drug Delivery Reviews 43: 207-223.

Friedman, A., Bar-Shira, E. and Sklan, D. 2003. Ontogeny of gut associated immune competence in the chick. World's Poultry Science Journal 59; 209-219.

Fukanoki, S., Matsumoto, K., Mori, H. and Takeda, R. 2000. Relationship between antigen release and immune response of oil adjuvanted vaccines in chickens. Journal of Veterinary Medical Science 62: 571-574.

Goldberg, M. and Gomez-Orellana, I. 2003. Nature Reviews Drug Discovery 2: 289-295.

Hejazi, R. and Amiji, M. 2003. Chitosan-based gastrointestinal delivery systems – Review. Journal of Controlled Release 89: 151-165.

Johansson, E.-L. Bergquist, C., Edebo, A., Johansson, C. and Svennerholm, A.-M. 2004. Comparison of different routes of vaccination for eliciting antibody responses in the human stomach. Vaccine 22: 984-990.

Keegan, M.E., Whittum-Hudson, J.A. and Saltzman, W.M. 2003. Biomimetic design in microparticlate vaccines. Biomaterials 24: 4435-4443.

Klipper, E., Sklan, D. and Friedman, A. 2000. Immune responses of chickens to dietary protein antigens. I Induction of systemic and intestinal immune responses following oral administration of soluble proteins in the absence of adjuvant. Veterinary Immunology and Immunopathology 74: 209-223.

Klipper, E., Sklan, D. and Friedman, A. 2001. Response, tolerance and ignorance following oral exposure to a single dietary protein antigen in *Gallus domesticus.* Vaccine 19: 2890-2897.

Lawes Agricultural Trust, 2007. VSN International Ltd, Hemel Hempstead, UK.

Muir, W.I., Bryden, W.L. and Husband, A.J. 1998 Evaluation of intraperitoneal immunisation in reducing *Salmonella typhimurium* infection in chickens. Poultry Science 77: 1874-1883.

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.

Ogawa,Y., Asai, Y., Yasuda, K. and Sakamoto, H. (2004). Oral immunoadjuvant of a new symbiotic *Lactobacillus casei* subsp *casei* in conjunction with dextran in BALB/c mince. Nutrition Research 25: 295-304.

Reed, L.J. and Muench, H. 1938. A simple method of estimating fifty percent endpoints. American Journal of Hygiene 27: 493-497

Russell-Jones, G.J. 2000. Oral vaccine delivery. Journal of Controlled Release 65: 49-54

Russell-Jones, G.J., Veitch, H. and Arthur, L. 1999. Lectin-mediated transport of nanoparticles across caco-2 and OK cells. International Journal of Pharmacology 190(2): 165-174.

Senior, K. 2001. Bilosomes: the answer to oral vaccine delivery? Drug Discovery Today 6(20): 1031-1032.

Spradbrow, P.B. and Samuel, J.L. 1991. Oral Newcastle disease vaccination with V4 virus in chickens: comparison with other routes. Australian Veterinary Journal 68(3): 114-115.

Steiner, K.A., Whittington, R.J., Petersen, R.K., Hornitzky, C and Garnet, H. 1991 Purification of Epizootic Hematopoietic Necrosis Virus and its detection using ELISA. Journal of Virological Methods 33: 199-210

Thanou, M., Verhoef, J.C. and Junginger, H.E. 2001. Oral drug absorption enhancement by chitosan and its derivatives. Advanced drug delivery Reviews 52: 117-126.

Van der Lubben, I.M., Verhoef, J.C., Borchard, G. and Junginger, H.E. 2001a. Chitosan for mucosal vaccination. Advanced Drug Delivery Reviews 52: 139-144.

Van der Lubben, I.M., Verhoef, J.C., van Aelst, A.C., Borchard, G. and Junginger, H.E. 2001b. Chitosan microparticles for oral vaccination: preparation, characterization and preliminary in vivo uptake studies in murine Peyer's patches. Biomaterials 22: 687-694.

Vila, A., Sánchez, A., Tobío, M., Calvo, P. and Alonso, M.J. 2002. Design of biodegradable particles for protein delivery. Journal of Controlled Release 78: 15-24.

Wang, J., Meers, J., Spradbrow, B. and Robinson, W.F. 2006. Evaluation of immune effects of fowlpox vaccine strains and field isolates. Veterinary Microbiology 116: 106-119.

Whittington R.J. and Deece, K. 2004. Aquatic Animal Health Subprogram: Development of Diagnostic and Reference Reagents for Epizootic Haematopoietic Necrosis Virus of Finfish. The University of Sydney and Fisheries Research and Development Corporation, Sydney and Canberra.