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New Therapeutics for Poultry

Therapeutic applications of chicken interferon gamma (ChIFN-γ) in poultry

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by John W. Lowenthal

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Foreword

Over the past few decades control of infectious disease in humans and animals has largely relied on the use of vaccines, antibiotics and chemicals. However, livestock industries are now facing restricted usage of antibiotics and chemicals for food production animals. A predicted consequence of this withdrawal for the poultry industry is a reduction in productivity due to an increased incidence of infection by certain pathogens.

It is therefore crucial that safe, alternative vaccination strategies and therapeutics are developed in order to maintain productivity as well as maintaining the highest possible levels of animal health and welfare.

Cytokines are proteins that are naturally produced by the body's immune system immediately following an infection. Cytokines protect against disease by controlling the immune response to infection or vaccination and therefore represent excellent, naturally occurring therapeutics.

As part of a pro-active program involved in developing alternative therapeutics, the aim of this project was to assess the ability of one particular cytokine, chicken interferon-gamma, to enhance disease resistance and vaccine efficacy in commercial broilers.

This publication describes the development of reagents used for the identification and assessment of a new cytokine therapeutic. Results from poultry trials show that treatment with chicken interferon-gamma led to improvements in weight gain and also helped protect birds against coccidiosis infection. As a measure of the successful outcome of this project, it is anticipated that ChIFN-γ technology will be implemented in the Australian market in the near future. Evaluation of additional cytokines will provide an extended range of therapeutics.

This report, a new addition to RIRDC's diverse range of over 700 research publications, forms part of our Chicken Meat and Egg R&D programs. The Chicken Meat program aims to support increased sustainability and profitability in the chicken meat industry by focusing research and development on those areas that will enable the industry to become more efficient and globally competitive and that will assist in the development of good industry and product images. The Egg program aims to support improved efficiency, sustainability, product quality, education and technology transfer in the Australian egg industry.

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Peter Core
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Executive Summary

Newly hatched chicks are highly susceptible to infection by opportunistic pathogens during the first two weeks of life. Several factors contribute to this problem; broiler chickens are reared under crowded conditions, their immune system has not yet fully developed and the levels of maternal antibody are rapidly declining. A major problem faced by all livestock industries is reduced productivity due to disease and considerable resources must be applied in order to maintain the health status of animals.

The two main mechanisms by which disease is controlled involves the use of vaccines and antimicrobials. Vaccines aim to offer long term immunity and protection against a particular pathogen following a small number of immunisations. In contrast, broad spectrum protection provided by antimicrobials (as in-feed antibiotics and chemicals) requires their continual usage even in the absence of apparent disease. Antibiotics, in addition to being an antimicrobial, also have growth promoting activity which makes them even more attractive. Unfortunately, the extensive use of antibiotics and chemicals over a long period of time has resulted in the emergence of pathogens that have become resistant to such treatments. The World Health Organisation has now recommended restrictions in the type of antimicrobials used in food production animals and has recommended the development and use of alternative, environmentally-friendly methods to control disease.

Some countries have already implemented such restrictions and based on their experiences, it is anticipated that without appropriate substitutes for prophylactic antibiotics, particular micro-organisms may emerge as significant health problems. Faced with these restrictions and potential problems, the Australian poultry industry is supporting the development of alternative measures that will maintain productivity as well as ensuring the highest possible levels of animal health and welfare.

Another major problem faced by the poultry industry is related to vaccines which are designed to give high levels of protection to specific diseases. Vaccination strategies are the primary mechanism for the control of most parasitic, viral and bacterial pathogens. There are, however, concerns over the ability of current live vaccines to protect against emerging hyper-virulent strains of pathogens. For certain diseases, there is a need for alternative vaccines, however, killed and recombinant subunit vaccines do not usually offer an adequate level of long term protection and often require the use of adjuvants to enhance their activity. Oil-based adjuvants, however, induce adverse site reactions resulting in decreased meat quality and animal discomfort and are therefore not used. At this time there is a lack of suitable, cost effective adjuvants for use in both the broiler and egg industries.

Cytokines are proteins that control immune responses following infection or vaccination and represent excellent, naturally occurring therapeutics. The efficacy of cytokine therapy has been demonstrated in several human and animal studies. Cytokine therapy has been successfully used in humans for the treatment of immunodeficiencies, in particular, patients suffering from an impaired immune system as a result of cancer treatment have had their immune responses restored following administration of particular types of cytokines called colony stimulating factors. This enabled them to combat pathogenic organisms that would have otherwise overwhelmed them. This is a situation analogous to the immunodeficient nature of newly hatched chickens. The utilization of cytokines is becoming more feasible with the recent cloning of a number of cytokine genes and the establishment of commercially feasible methods of delivery. There are many different types of cytokines that perform different functions. Also, cytokines from one species cannot function in another species, therefore a number of chicken cytokines must be identified, characterized and assessed in order for the most beneficial ones to be identified.

In order to establish a proof-of-principal, we have assessed one of the most characterised chicken cytokines, interferon gamma (ChIFN-γ). ChIFN-γ is a member of a family of cytokines that share the capacity to modulate the immune response and inhibit viral replication. We have previously shown

that treatment with ChIFN-γ resulted in enhanced growth rates in healthy SPF chickens as well as in chickens infected with *Eimeria acervulina*. Furthermore, when co-administered with antigen, ChIFN-γ produced a prolonged secondary antibody response in SPF birds that persisted at higher levels and for longer periods compared to antigen injected alone.

The overall objective of this project was to assess the ability of ChIFN-γ to enhance disease resistance and increase vaccine efficacy in commercial broilers and layers. The first objective was to produce and optimize a potential commercial product (recombinant ChIFN-γ protein). The second objective was to perform pen trials under commercial conditions to assess the ability of ChIFN-γ to increase broiler growth performance, enhance vaccine efficacy and improve disease resistance.

In this project we have developed and compared several expression systems for the production of recombinant ChIFN-γ protein. Naturally-occurring ChIFN-γ can only be produced in very small amounts so more efficient methods of production are needed. ChIFN-γ was produced and purified from several established protein expression systems including plants, insect cells, yeast and bacteria. The *E coli* system was judged to be preferable as a potential commercial production system and was used in the animal trials.

We also developed several monoclonal antibodies and poly sera specific for ChIFN-γ that were used to develop a sensitive and specific ELISA for the detection and quantitation of ChIFN-γ. The ELISA was shown to detect only biologically active ChIFN-γ and it's sensitivity was greater than that of the standard bioassay. This ELISA proved to be an invaluable tool in the assessment of function and stability of ChIFN-γ *in vivo*. We showed that intravenous injection of ChIFN-γ protein into birds resulted in a rapid rise in circulating levels of protein followed by a fast decline. Intraperitoneal injection resulted in a slower rise in the level of serum ChIFN-γ followed by a slow decline. Both methods of delivery resulted in a biological response to ChIFN-γ as measured by increased expression of Class II antigen (a marker for enhanced immune activation by interferon) within 24-48 hr of treatment. For practical purposes, intraperitoneal injection was chosen as the preferred route of delivery for ChIFN-γ protein.

Several trials were performed in order to assess the ability of ChIFN-γ to enhance the growth performance of broilers reared under commercial conditions. Broilers were injected at day of hatch with ChIFN-γ protein and monitored for weight gain over a 8 week period. Treatment resulted in a 2.7% increase in mean body weight at day 56. Two similar trials also showed enhanced productivity following ChIFN-γ treatment.

We found that chemical modification of the ChIFN-γ protein by polyethylene glycol (PEG) enhanced its *in vivo* stability compared to unmodified ChIFN-γ. We then tested whether such modification was able to further enhance the growth promoting activity of ChIFN-γ. Broilers were injected with either PEG-modified or unmodified ChIFN-γ, and monitored for weight gain over a 6 week period. Both forms of ChIFN-γ were shown to be equally effective, producing a 1.3% increase in mean body weight.

CSIRO had previously developed and patented an effective way of delivering proteins to chickens via the use of fowl adenovirus (FAV) vectors. In a separate collaboration with Dr M Johnson, we have performed parallel experiments to assess the growth promoting activity of ChIFN-γ when delivered by FAV. The consistent finding was that broiler chickens treated with FAV::ChIFN-γ displayed enhanced weight gain (ranging from 1-7% relative to control birds) over periods of up to 8 weeks. Taken together, these results clearly indicate the potential use of ChIFN-γ as an effective, naturally occurring growth promoter. The underlying mechanisms are unknown, but may be due to the ChIFN-γ mediated enhancement of the immune system leading to decreased pathogen loads, resulting in healthier and more productive birds.

Several trials were performed in order to assess the effect of ChIFN-γ treatment to enhance the growth performance of broilers following a coccidiosis infection. Worldwide, coccidiosis represents the largest single threat to loss of productivity. Results from two representative trials are described in detail in this report. Infection of chickens with Eimeria, the causative agent for coccidiosis, results in a reduction in weight gain for several days. We assessed the ability of ChIFN-γ treatment to enhance protection against coccidial infection by measuring its ability to reduce the rate of weight loss associated with infection. Chickens were treated orally with FAV::ChIFN-γ at five days of age. A second group was treated with another cytokine, chicken myelomonocytic growth factor (cMGF) which was used as a negative control. All birds were then challenged with a dose of *E acervulina* that induces weight reduction. Birds treated with cMGF and non-treated birds had a significantly reduced rate of weight gain between days 7 and 11 post challenge, relative to ChIFN-γ treated birds. This is an indication that ChIFN-γ was able to reduce the severity of the infection. In a similar trial, treatment of infected birds with ChIFN-γ resulted in enhanced growth performance relative to non treated infected birds (7.1% and 2.9% increase in body weight at day 35 and 42, respectively).

Eimeriavax3 (Eimeria Pty. Ltd.) is a newly developed live vaccine that confers protection against challenge with *E. brunetti* in the absence of in-feed coccidial medication. The relatively high cost of production for this vaccine makes it not cost-effective for use in the broiler industry. If the effective dose could be significantly reduced by use of an adjuvant, then this vaccine could be used for broilers. Preliminary trials were conducted to assess the compatibility of ChIFN-γ co-treatment and vaccination. Birds were vaccinated with Eimeriavax3 either with or without ChIFN-γ co-treatment and then challenged 3 weeks later with *E. brunetti* at a dose that reduced growth rate in unprotected birds. Unvaccinated control birds displayed a decline in growth rate one week after Eimeria challenge. The growth rate of vaccinated birds on the other hand was not affected, as was the case for the vaccinated birds that were co-treated with ChIFN-γ, indicating that co-treatment did not reduce the protective effect of the vaccine. These results will allow future experiments to be performed to test whether treatment with ChIFN-γ will allow a lower dose of Eimeriavax3 to be used.

As expected with a live vaccination, there was a high output of oocysts beginning 5 days after vaccination and declining to very low levels within 12 days post-vaccination. ChIFN-γ did not alter oocyst output. Oocysts were not detected in the faeces of unvaccinated birds prior to challenge, but at 7 days post challenge there was a high output measured in this group. In contrast, the output in both of the vaccinated groups was approximately 1000-fold lower, indicating the effectiveness of the vaccination.

The results from this project have significant implications for the Australian poultry industry. Future restrictions in the use of in-feed antibiotics will have an economic impact on the industry unless alternative therapeutics are developed. Based on overseas experience, pathogens that are currently kept under control by antibiotics, such as Clostridium species, will become more prevalent, leading to severe cases of necrotic enteritis and other gastrointestinal problems. There is a risk that incidence of associated secondary infections will increase significantly, resulting in increase costs due to medication and vaccines and reduced productivity.

Alternative therapeutics such as cytokines and bacteriocins are currently being assessed as safe, naturally occurring alternatives to in-feed antibiotics. Cytokines offer a two pronged attack. They can strengthen and control immune responses resulting in the increase in the general health of animals. Secondly, they can improve the efficacy of vaccines, resulting in improved long term protection against specific pathogens. In this project we have made some very significant steps forward in the evaluation of chicken cytokines as alternative therapeutics. We have established proof-of-principal by showing that ChIFN-γ is a growth promoter and immunoenhancer under commercial conditions. The introduction of cytokines as commercial therapeutics should compensate for the reduction in the use of in-feed growth promotants. As a measure of the successful outcome of this project, it is anticipated that ChIFN-γ technology will be implemented in the Australian market in the near future. An

Australian poultry health company is currently negotiating with CSIRO for a licence to market ChIFN-γ technology to the Australian poultry industry.

To best make use of the outcomes of this project, the following recommendations are made for further work required in this area:

1. Evaluation of additional cytokines to provide an extended range of therapeutics.
2. Identification of the nature of protective immune responses to infection as a rational approach for identifying potential therapeutics.
3. Development of optimal methods for the safe and efficient delivery of therapeutics under commercial conditions.
4. Combined use of a variety of complimentary approaches (cytokines, bacteriocins and vaccines).

1. Introduction

1.1 Use of in-feed antibiotics

The sheer size of the global poultry broiler industry is staggering, almost 40 billion chickens are hatched worldwide every year. The Australian industry produces approximately 350 million broilers per annum. Chicken meat represents approximately 40% of all meat consumed and is a $150 billion per annum global retail market, as well as supporting a multi-billion dollar poultry health market. Chickens are reared under intensive conditions which are conducive to infection by opportunistic pathogens. This is especially critical during the first weeks of life, a time at which the immune system has not yet fully matured (Lowenthal et al., 1993) and when levels of maternal antibody are declining. A major problem faced by intensive livestock industries, such as the poultry industry, is loss of productivity due to disease, therefore considerable resources are required in order to maintain the health status of these animals.

The main mechanisms by which disease is controlled involves the use of vaccines and antimicrobials. Vaccines are used to provide long term specific protection against a particular pathogen following a small number of immunisations. In contrast, protection provided by antimicrobials (as in-feed antibiotics and chemicals) is broad spectrum and requires their continual usage even in the absence of apparent disease. Antibiotics have been used to treat and control diseases in livestock and poultry for more than 50 years. Low levels of antibiotics are used as feed supplements (in-feed) for their ability to enhance animal health and result in improved growth rates and feed-conversion efficiency (Williams, 2001). Because of this, in-feed antibiotics are often referred to as growth promoters. Unfortunately, the extensive use of antibiotics and chemicals over a long period of time has resulted in the emergence of pathogens that have become resistant to such treatments. The World Health Organisation has now recommended restrictions in the type of antimicrobials used in food production animals and has recommended the development and use of alternative, environmentally-friendly methods to control disease.

Some countries have already implemented such restrictions and based on their experiences, it is anticipated that without appropriate substitutes for prophylactic antibiotics, particular micro-organisms may emerge as significant health problems (Bedford, 2000). One example is the predicted increased prevalence of Clostridium species, leading to severe cases of necrotic enteritis and other gastrointestinal problems, resulting in decreased productivity and welfare in poultry. Replacements for current growth promoters in livestock are therefore a very high priority for the industry.
Faced with these restrictions and potential problems, the Australian poultry industry is supporting the development of alternative measures that will maintain productivity as well as ensuring the highest possible levels of animal health and welfare.

Another major problem faced by the poultry industry is related to vaccines which are designed to give high levels of protection to specific diseases. Vaccination strategies are the primary mechanism for the control of most parasitic, viral and bacterial pathogens. There are, however, mounting concerns over the effectiveness of current vaccines in the face of emerging of hyper-virulent strains. There is a need for alternative vaccines, however, killed and recombinant subunit vaccines do not usually offer an adequate level of long term protection and often require the use of adjuvants to enhance their activity. Oil-based adjuvants, however, induce adverse site reactions resulting in decreased meat quality and animal discomfort. At this time there is a lack of suitable, cost effective adjuvants for use in both the broiler and egg layers.

1.2 Alternatives to antibiotic growth promoters

A number of alternatives to in-feed antibiotics have been suggested, however, most of these will not by themselves fully compensate for the removal of antibiotics. One alternative is the use of in-feed enzymes. Enzymes such as Avizyme act by increasing the rate of diet digestibility and sugar provision, ultimately changing the substrate quality and quantity available to intestinal flora (Bedford 2000). Digested nutrients can then be absorbed by the chicken for energy and growth, instead of feeding the intestinal organisms. Another approach is to use bacteriocins which are small proteins produced by certain bacteria for the purpose of eliminating other competing bacteria (Jack et al., 1995). Particular types of bacteriocins have been identified that kill specific species of bacteria linked to necrotic enteritis in chickens. Treatment with recombinant bacteriocins may provide alternatives to in-feed antibiotics by limiting the growth of *Clostridium perfringens* and other disease-causing bacteria in chickens. Factors including increased nutrition, high quality feed, feed sterilisation and decreased nitrogen content in feed are all being considered as potential substitutes for antibiotics in chicken feed (Bedford 2000).

1.3 Cytokines

A novel approach in the search for alternative therapeutics has been to examine chicken cytokines as potential replacements for in-feed antibiotics in poultry. Cytokines are proteins that are naturally produced by the body's immune system immediately following infection or vaccination, resulting in protection from disease. Cytokines control and promote immune responses in all animal species and therefore represent excellent candidates as therapeutics as well as vaccine adjuvants. Cytokines from one species cannot function in another species, therefore a variety of chicken cytokines must be identified, characterized and assessed. In mammals, cytokines can be generally characterised according to the type of immune response they generate (Mosmann and Sad, 1996). Th1 type cytokines include interleukin-2 (IL-2) and interferon gamma (IFN-γ) and are mainly involved in the generation of cell mediated immunity. Th2 type cytokines are generally involved in the activation of B cells and therefore regulate antibody production and are represented by IL-4, IL-5, IL-6 and IL-10. Virtually all of the cytokines cloned in the chicken to date are Th1-like.

Table 1 lists 17 cytokine genes that have been cloned in the chicken to date. Recent advances have meant that some of these cytokines have also been cloned in other avian species including turkey, Japanese quail, duck, pheasant and guinea fowl. With the level of high amino acid identity between chicken and other avian species (70-98%) the cloning of these cytokines is a relatively easy process of using PCR with chicken primers. The procuring of these avian cytokines allows us to characterise and assess their ability to enhance immune responses to pathogens. Furthermore, studies in mammals have shown that cytokines have the potential to be developed as alternative forms of vaccine adjuvants (Heath and Playfair, 1992).

1.4 Chicken interferon gamma

One of the most characterised chicken cytokine genes is chicken (Ch) IFN-γ (Digby and Lowenthal, 1995). IFN-γ is a member of a family of cytokines that share the capacity to modulate the immune response and inhibit viral replication. Therefore, with these properties in mind, ChIFN-γ has been assessed as both an immunoenhancer and vaccine adjuvant. Treatment with ChIFN-γ resulted in enhanced growth rates in healthy SPF chickens as well as in chickens infected with *E. acervulina* (Lowenthal et al., 1997). Furthermore, when co-administered with antigen, recombinant ChIFN-γ produced a prolonged secondary antibody response in SPF birds that persisted at higher levels and for longer periods compared to antigen injected alone (Lowenthal et al., 1998 and 1999). These results may have important implications for the poultry industry if these effects can be repeated in broiler birds housed under commercial conditions.

Table 1. Cloned avian cytokine genes

Cytokine gene	Species cloned[a]	% identity[b]	References
IFN-α	C, T, D	18-22	Sekellick et al., 1994; Suresh et al., 1995; Schultz et al., 1995
IFN-β	C	18-20	Sick et al., 1996
IFN-γ	C, T, J, P, G, D	22-35	Digby and Lowenthal, 1995; Kaiser et al., 1998; Schultz and Chisari, 1999
Interleukin-1β	C	25-29	Weining et al., 1998
Interleukin-2	C, T	16-24	Sundick and Gill-Dixon, 1997; Lawson et al., 2000
Interleukin-6	C	32-39	Kaiser, P (unpublished results)
Interleukin-8	C	28-48	Bedard et al., 1987
Interleukin-15	C	34-36	Burnside & Sofer, GenBank AF152927
Interleukin-16	C	-	Kaiser, P (unpublished results)
Interleukin-18	C, D	30	Schneider et al., 2000
Stem cell factor	C, J	45-52	Zhou et al., 1993
MGF	C	20-30	Leutz et al., 1989
TGFβ	C	72-79	Jakowlew et al., 1988
Lymphotactin	C	25-28	Rossi et al., 1999
MIP-1β	C	75-80	Petrenko et al., 1995
CXC and CC chemokines	C	50	Sick et al., 2000

[a] Abbreviations: C, chicken; T, turkey; J, Japanese quail; P, pheasant; G, guinea fowl; D, duck;
[b] Percent amino acid identity to mammalian counterpart cytokine genes

2. Objectives

The overall objective of this project was to assess the ability of chicken interferon-gamma to enhance disease resistance and vaccine efficacy in commercial broilers and layers.

Objective 1: To produce and optimize a commercial product (recombinant ChIFN-γ protein).

Objective 2: To perform pen trials with ChIFN-γ to assess its ability to:

- increase broiler growth performance
- enhance vaccine efficacy
- improve disease resistance

3. Methodology

3.1 Animals

Specific Pathogen Free Hybrid White Leghorn (SPF) chickens were raised in flexible plastic isolators and fed fumigated feed and acidified water *ad libitum*. For commercial pen trials, sexed broiler chicks (Ross strain) were obtained at day of hatch from Steggles Pty Ltd and housed on deep litter in floor pens at the CSIRO Werribee animal facility. Broiler birds were maintained on a commercial feed (Ridleys Pty Ltd, Australia) regime consisting of 0.5 kg ration per bird of broiler starter between days 0-14, 1 kg of grower from days 15-30, 1 kg of finisher between days 31-40, and 2-3 kg of withdrawal between days 41-56. Drinking water was provided by commercial nipple drinkers. 6 week old Balb/C mice were used for the generation of monoclonal antibodies. Female New Zealand White rabbits were used for the generation of polyclonal antisera. All experimental procedures were approved by the CSIRO Animal Health Animal Experimental Ethics Committee.

3.2 Production of ChIFN-γ

Recombinant ChIFN-γ was produced in several expression systems as described elsewhere. COS-derived ChIFN-γ was produced by transfecting COS cells with pCDNA1-ChIFN-γ (Digby and Lowenthal, 1995). *E. coli*-derived, poly-HIS tagged ChIFN-γ was produced by cloning the mature coding region of ChIFN-γ into the pQE30 expression vector (Qiagen, CA), followed by Ni chromatography as described (Lowenthal et al., 1997). Native ChIFN-γ was obtained from supernatants from ConA-stimulated chicken splenic T cells (Lowenthal et al., 1995). Yeast expressed protein was produced using a commercial *P. pastoris* system and the *B. brevis* system was used as described previously (Yashiro et al., 2001). Expression in tobacco plants was done in collaboration with Biosource P/L (California, USA) – the details are commercial-in-confidence. PEG modification was performed according to manufacturers instructions (Sigma).

3.3 Assay for ChIFN-γ bio-activity

Production of nitric oxide by HD11 chicken macrophages was quantitated by accumulation of nitrite in the culture medium and was used as a measure of ChIFN-γ activity as described previously (Lowenthal et al., 1995). Briefly, two-fold serial dilutions of test supernatants were made in duplicate wells of 96 well plates in a volume of 100 ul of growth medium containing 10% FBS and 20 ng/ml LPS. HD11 cells were added to each well ($2x10^5$ in 100 ul) and the plates were incubated at 37°C. After 24 h, 50 ul of culture supernatant was added to 100 ul of Griess reagent and absorbance was read at 540 nm. The level of nitrite was determined using sodium nitrite as a standard. An International ChIFN-γ Standard of known activity was included in each assay. One unit/ml of IFN activity is defined as the reciprocal of the dilution giving half-maximum activity. Ability of antibodies to inhibit the biological activity of ChIFN-γ was determined by preincubating the antibody and ChIFN-γ for 1 h at room temperature prior to the addition of HD11 cells.

3.4 Production of monoclonal and polyclonal antibodies

Balb/C mice were immunized three times with *E. coli*-derived, poly-HIS tagged ChIFN-γ (10 ug in Freund's complete adjuvant sc; 10 ug in Freund's incomplete adjuvant ip; (10 ug in PBS ip). Hybridomas were generated by fusion of spleen cells and SP2/0 myeloma cells. Mabs were screened for their ability to bind to ChIFN-γ-coated ELISA plates. Antibody isotypes were determined using anti-Ig subclass-specific antibodies. Mab Ig was purified by protein G chromatography. Rabbit antisera was also raised against *E. coli* derived ChIFN-γ protein. Rabbits were immunized three times with 400 ug of protein. Sera were collected 10 days after the final injection. Specific reactivity of monoclonal and polyclonal antibodies

to ChIFN-γ was confirmed using Western blot analysis and ability to block ChIFN-γ induced nitrite secretion by HD11 macrophages.

3.5 ELISA for ChIFN-γ

Microtitre plates were coated with Mab 80 (1ug/ml in carbonate buffer, pH 8, 100 ul/well) overnight at 4^{o}C. All subsequent incubation steps were carried out at room temperature (RT) for 1 h with three washes (PBS with 0.5% tween) between each step. The plates were blocked (1% Powerblock , 100 ul/well) and serial dilutions of ChIFN-γ or control preparations (100 ul/well) were added. Biotinylated Mab 80 or polyclonal rabbit anti-ChIFN-γ sera (1 in 1000 dilution) was added to each well as the detection antibody, followed by the addition of HRP-labelled streptavidin (Amersham) or HRP-goat anti-rabbit Ig (Zymed). ChIFN-γ binding was revealed by the addition of tetra-methyl benzidine (TMB) peroxidase substrate followed by quenching with 0.5 M H_2SO_4. Plates were read on an ELISA reader at 450 nm. The ELISA titre is defined as the reciprocal of the dilution giving an OD reading above the cut off value which is calculated as [mean background ± 3SEM].

3.6 Weight gain trials

Several trials were performed in order to assess the ability of ChIFN-γ to enhance the growth performance of broilers reared under commercial conditions. Two typical trials are described.

3.6.1 Trial 1

One group of 80 one day-old broilers (40 males and 40 females per group) was injected ip with 100 ug of *E. coli* derived ChIFN-γ, a second group was injected with saline and used as untreated controls. All birds were fed as per normal commercial feed regime and monitored for weight gain over a 8 week period.

3.7.2 Trial 2

One group of 80 one day-old broilers (40 males and 40 females per group) was injected ip with *E. coli* derived ChIFN-γ, a second group was similarly treated with CC-PEG-modified ChIFN-γ. A third control group was injected with saline. All birds were fed as per normal commercial feed regime and monitored for weight gain over a 6 week period.

3.7 Coccidiosis challenge trials

Several trials were performed in order to assess the ability of ChIFN-γ to enhance the growth performance of broilers vaccinated against coccidiosis and reared under commercial conditions. Two typical trials are described.

3.7.1 Trial 3

One group of 80 five day-old chickens were treated orally with FAV::ChIFN-γ and a second group was treated with FAV::cMGF and a third group was left as untreated controls. All birds were then challenged orally with 5000 oocytes of *Eimeria acervulina* MCK strain (Medichick Pty Ltd, Australia), a dose that gives mild weight reduction. All birds were fed as per normal commercial feed regime and body weight was monitored until 12 days post challenge.

3.7.2 Trial 4

At 6 days post-hatch, two groups of 80 birds (40 males and 40 females per group) were vaccinated by eye drop with 100 oocysts of *E. brunetti* (Eimeriavax3, Eimeria Pty Ltd, Australia). A third group was left as unvaccinated controls. One of the vaccinated groups was also orally treated with FAV::ChIFN-γ at the same time while the second vaccinated group was not treated with ChIFN-γ. The unvaccinated control group was also not treated with ChIFN-γ. At 21 days post vaccination, all birds in each of the three groups were challenged with 6000 oocysts of *E. brunetti* (eye drop), a dose sufficient to induce a significant reduction in growth rate. Faecal samples were collected at various time points (10 samples per group). Weights of birds were monitored for 7 weeks. Birds were fed coccidiastat-free feed (Ridleys Pty Ltd, Australia) for the duration of the trial. A standard Students t-test was applied in analysis of the differences between mean weights of birds and mean oocyst output.

4. Results

4.1 Production of recombinant ChIFN-γ protein

We have developed several expression systems for the production of recombinant ChIFN-γ protein and have compared the relative amount of protein produced and the level of biological activity (Table 2). Production of natural ChIFN-γ from cultures of chicken spleen (ConA supernatant) yields low amounts of material. Production by transfected COS monkey cells is 100-fold more effective, however the yield is still too low to enable *in vivo* testing or for use in trials. Glycosylated ChIFN-γ was also produced in tobacco plants, insect cells (baculovirus) and yeast. For plant production the yield is too low and further improvements would be required, however, this may still be considered as a cost-effective alternative. The two bacterial systems (*E. coli* and *B. brevis*) produced comparable yields and activities. The *B. brevis* system requires more complex culture conditions and it's use is restricted due to commercial licensing conditions, therefore the *E. coli* system was judged to be preferable as a potential commercial production system.

Table 2. Characteristics of ChIFN-γ expression systems

System	Glycosylation	Yield (ug/ml)	Activity (Units/ml)
ConA Sn [a]	Yes	<0.1	$1x10^2$
COS	Yes	1.0	$1x10^4$
Tobacco plant	Yes	20	$5x10^4$
Insect cells [b]	Yes	50	$2x10^5$
Yeast	Yes	50	$3x10^5$
E. coli	No	100	$6x10^6$
B. brevis	No	200	$1x10^6$

a Supernatant from chicken spleen cells cultured 48h in the presence of ConA
b Baculovirus expression

4.2 Characterization of ChIFN-γ Mabs

We developed a panel of monoclonal antibodies (see Table 3) and polyclonal rabbit sera against ChIFN-γ. These Mabs differ in their ability to neutralize the biological activity of ChIFN-γ in the HD11 nitrite assay. Mabs 80.9 and 85.6 were able to neutralise the biological activity of recombinant ChIFN-γ derived from either *E. coli* or COS cells as well as native ChIFN-γ (derived from Con A activated chicken T cells). Some of the Mabs were able to detect ChIFN-γ in Western blots. Competition ELISAs were set up to determine whether these Mabs recognised different epitopes of the ChIFN-γ molecule. Biotinylated Mab 80.9 (bio80.9) was mixed with various concentrations of different anti-ChIFN-γ Mabs prior to their addition to plates coated with ChIFN-γ. Mabs 85.6 and 76.5 were able to block the binding of Mab 80.9 to ChIFN-γ indicating that they recognised the same or adjacent epitopes whereas others (9.1and 43.10) were not, indicating that they bind to different epitopes on the ChIFN-γ molecule.

Table 3. Characteristics of ChIFN-γ Mabs.

Clone	Isotype	Titre[a]	Bio-inhibition[b]		Western Blot[f]
			COS[c]	*E. coli*	
13.1	M	$2x10^5$	N[d]	N	++++
80.9	G1	$5x10^5$	200[e]	1200	-
149.1	G1	$1x10^5$	N	N	-
68.6	G1	$1x10^5$	N	N	-
85.6	G2a	$2x10^5$	50	160	-
31.9	G2a	$1x10^6$	N	N	+++
9.1	G2b	$1x10^5$	N	N	+++
19.10	G2b	$6x10^4$	N	N	++

a ELISA titre of purified Ig fraction
b Ability of Mab to neutralize ChIFN-γ activity in HD11 bioassay
c Inhibition of ChIFN-γ derived from transfected COS cells and *E. coli*
d Non inhibitory at 100 ug/ml
e Concentration of Ig (ng/ml) giving 50% inhibition of ChIFN-γ activity in the HD11 assay
f Relative ability to detect ChIFN-γ in Western blots

4.3 Development of an ELISA for ChIFN-γ

The Mabs were used to develop a sandwich ELISA to be used as an alternative to the bioassay. In order to validate the ELISA and test its ability to detect different forms of ChIFN-γ, a collection of different ChIFN-γ samples was produced by various expression systems and tested. ChIFN-γ was produced from Con A stimulated chicken T cells, fibroblasts infected with FAV-ChIFN-γ (provided by Dr M. Johnson; Johnson et al., 2000), COS cells transfected with pCNDA1-ChIFN-γ, Sf9 insect cells infected with baculovirus expressing ChIFN-γ and *E. coli*. In order to test whether the ELISA was able to detect denatured or non-functional molecules of ChIFN-γ, some samples were heated in order to reduce the biological activity of ChIFN-γ. All samples were quantitated in both the HD11 nitrite assay and the ELISA. A tight correlation between the level of biological activity (biological titre) and detectability by ELISA (ELISA titre) was observed (Figure 1). Furthermore, heating samples of ChIFN-γ or exposure to low pH conditions (not shown) resulted in a concomitant decrease in both biological and ELISA titres. Taken together, the data indicate that this ELISA detects only biologically active molecules (ie. homodimers) of ChIFN-γ and not inactive molecules. Therefore, it can be used to reliably quantitate the biological activity of ChIFN-γ with a sensitivity comparable to that of the conventional HD11 bioassay.

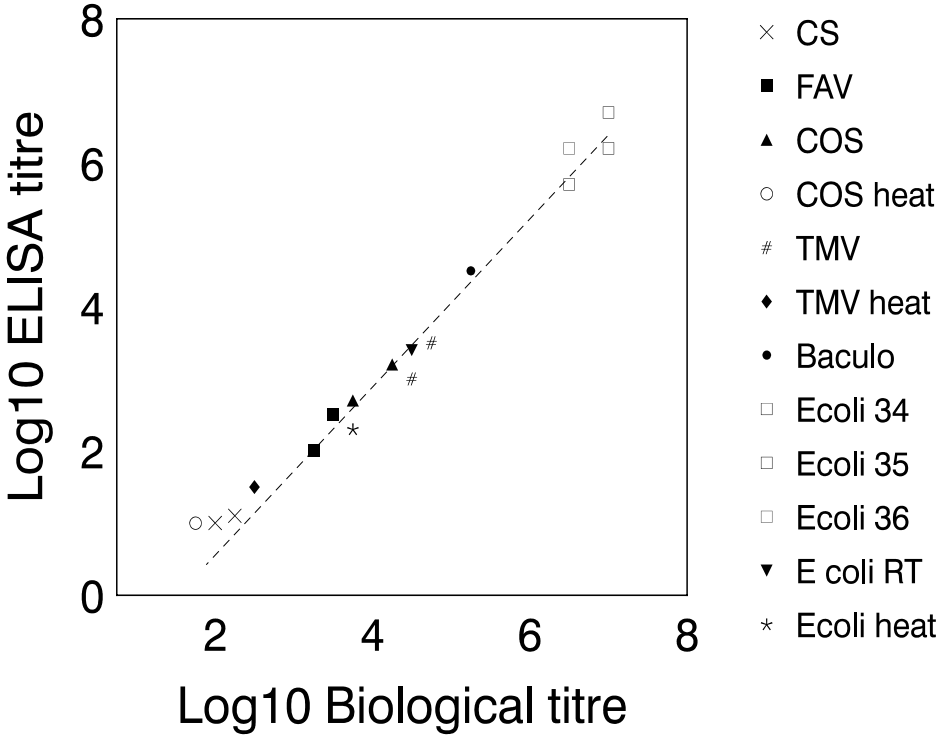

Figure 1. Correlation between biological function of ChIFN-γ and its ability to be detected in the ChIFN-γ ELISA. Samples of ChIFN-γ derived from ConA supernatant (CS), supernatant of chick kidney cells infected with FAV:: ChIFN-γ (FAV), COS cells, transformed tobacco plants (TMV), Baculovirus infected insect cells (Baculo), and different batches of *E coli*-derived protein. Some samples were heated to 65 degrees in order to reduce the level of biological activity of ChIFN-γ.

4.4 Pharmacokinetics of ChIFN-γ *in vivo*

The ELISA was used to measure the fate of recombinant ChIFN-γ following injection into chickens. ChIFN-γ derived from *E. coli* was injected either intra-venously (iv) or inta-peritoneally (ip) into birds. Blood samples were taken over a 24 hr period and serum ChIFN-γ levels were measured by ELISA. Following iv injection, the level of ChIFN-γ reached peak serum levels within 1-3 minutes and there after declined rapidly with about 90% clearance within 30 min (Figure 2). The initial $t_{½}$ of ChIFN-γ was approximately 5-10 min, a value similar to that reported for studies on mammalian IFN-γ in mice and humans. The remaining ChIFN-γ was cleared at a slower rate and persisted in the circulation over several hours. After 24 h about 0.3-1.0% of the initial level was still detectable. Following ip injection, ChIFN-γ appeared slowly in the serum, reaching peak levels between 1-7 h, however, the maximum serum levels were considerably lower than those observed following the same dose given iv (Figure 3).

Figure 4 compares the clearance rates of PEG-modified and unmodified ChIFN-γ following iv and ip injection. The PEG modification allowed ChIFN-γ to persist longer in the circulation, with approximately 100-fold higher levels detectable after 24 hrs compared to the unmodified form. For practical purposes, intraperitoneal injection was chosen as the preferred route of delivery for ChIFN-γ protein.

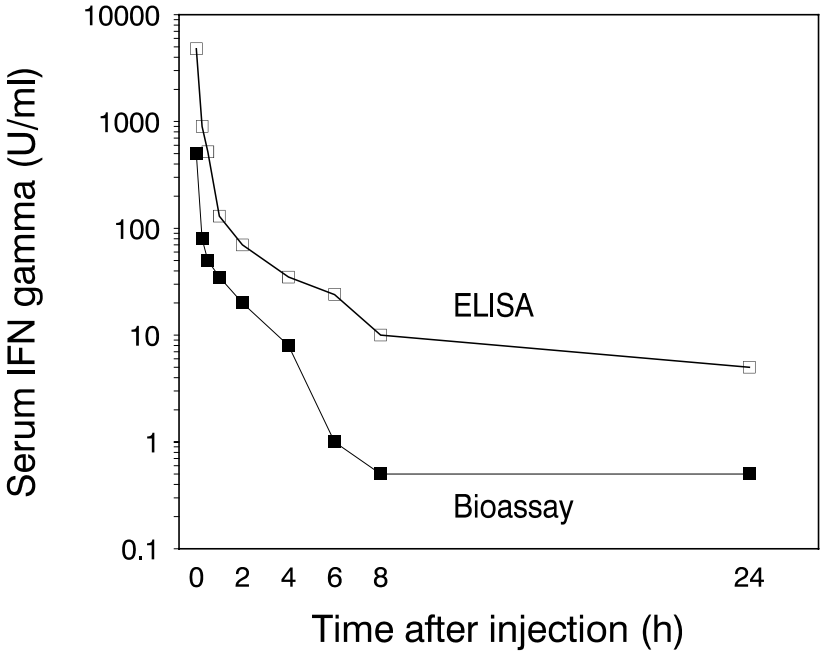

Figure 2. Detection of ChIFN-γ in the serum of chickens following iv injection of $1x10^5$ Units of *E coli*-derived ChIFN-γ by ELISA and by the HD11 bioassay.

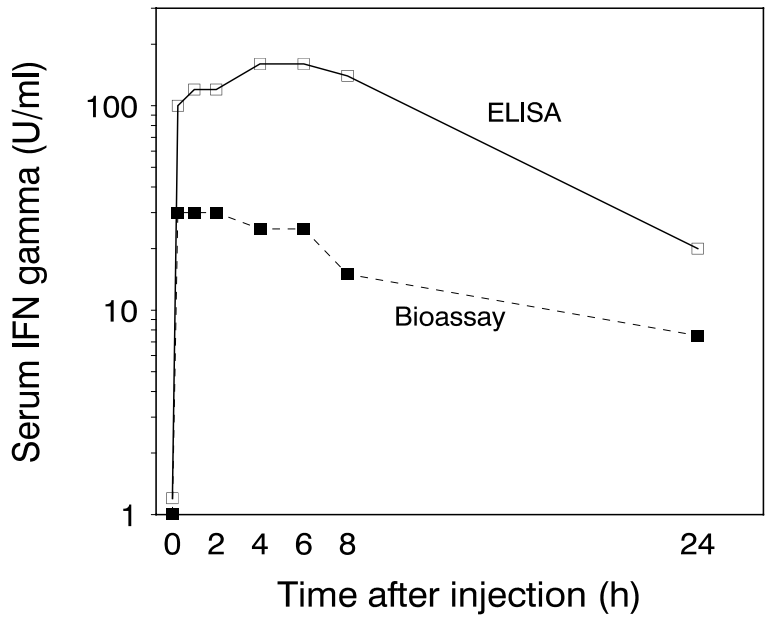

Figure 3. Detection of ChIFN-γ in the serum of chickens following ip injection of $1x10^5$ Units of *E coli*-derived ChIFN-γ by ELISA and by the HD11 bioassay.

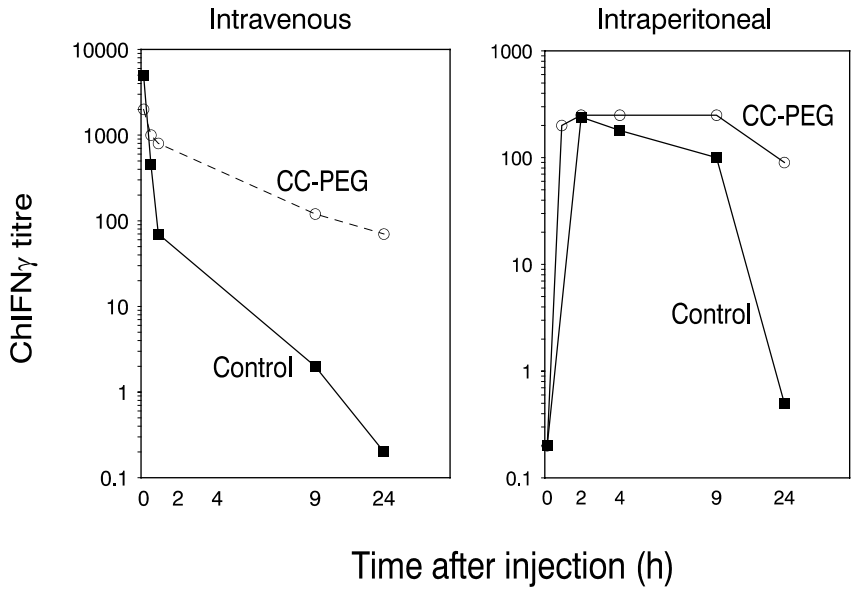

Figure 4. ELISA detection of ChIFN-γ in the serum of chickens following iv or ip injection of $1x10^5$ Units of unmodified or CC-PEG modified *E coli*-derived ChIFN-γ.

4.5 Induction of MHC class II antigen expression

HD11 cells were cultured for 24 h in the presence of various preparations of ChIFN-γ and then analyzed for the cell surface expression of MHC class II antigen (CIa). Approximately 15% of HD11 cells

constitutively express CIa, however, this proportion increased in a dose-dependent manner, following exposure to ChIFN-γ. ChIFN-γ expressed by COS cells, yeast and insect cells have similar dose-response curves to that produced by *E. coli* derived ChIFN-γ (Figure 5). The dose-response curves for class II expression and nitrite secretion are similar. Expression of class II by peripheral blood lymphocytes was also increased at 24 and 48 h after injection of ChIFN-γ (data not shown), indicating that ChIFN-γ mediated the same biological effects *in vivo* as it does *in vitro*.

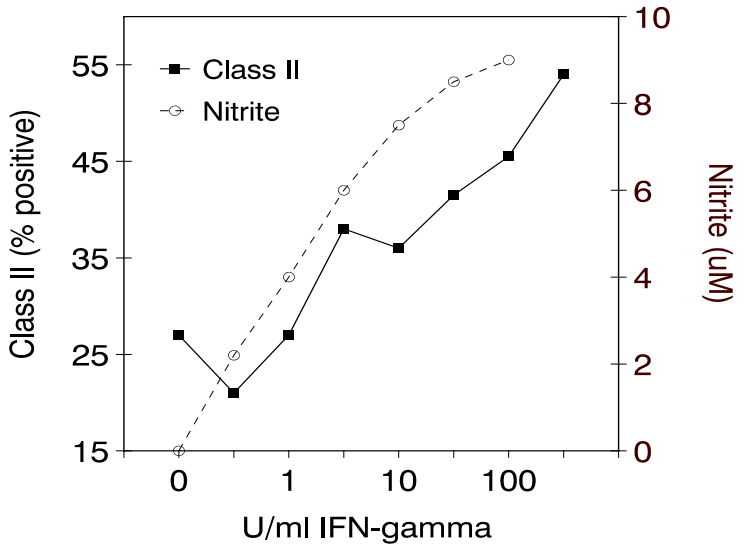

Figure 5. Induction of MHC class II expression on HD11 cells by ChIFN-γ. HD11 cells were cultured in the presence of various concentrations of E *coli* ChIFN-γ. After 24 h the level of MHC Class II expression (CIa) and nitrite secretion was measured.

4.6 Weight gain trials

Several trials were performed in order to assess the ability of ChIFN-γ to enhance the growth performance of broilers reared under commercial conditions. Results from two representative trials are shown.

4.6.1 Trial 1

In order to assess the effect of ChIFN-γ on growth performance, broilers were injected with ChIFN-γ, and monitored for weight gain over a 8 week period. Treatment resulted in a 2.7% increase in mean body weight at day 56 (Figure 6). Interestingly, in this particular trial, all of the weight gain was attributable to the males, with the females showing a marginal decrease in weight relative to controls. Two other trials showed similar trends in weight gain following ChIFN-γ treatment (data not shown).

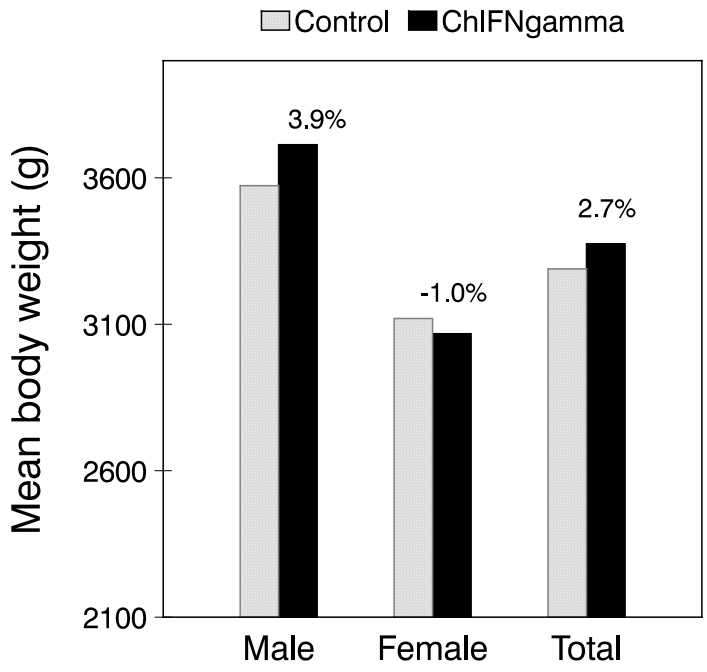

Figure 6. Trial 1. Enhancement of weight gain by broilers treated with ChIFN-γ. Groups of 80 chickens (40 of both sexes) were injected (ip) with 100 ug of *E coli*-derived ChIFN-γ or with saline (controls) and weighed over a 8 week period. Day 56 weights of males, females and total are shown. Numbers indicate the percent change relative to controls.

4.6.2 Trial 2

Since PEG modification enhanced the *in vivo* stability of ChIFN-γ, we tested whether such modification was able to enhance the growth promoting activity of ChIFN-γ. Broilers were injected with either modified or unmodified ChIFN-γ, and monitored for weight gain over a 6 week period. Figure 7 showed that both forms of ChIFN-γ were equally effective, producing a 1.3% increase in mean body weight.

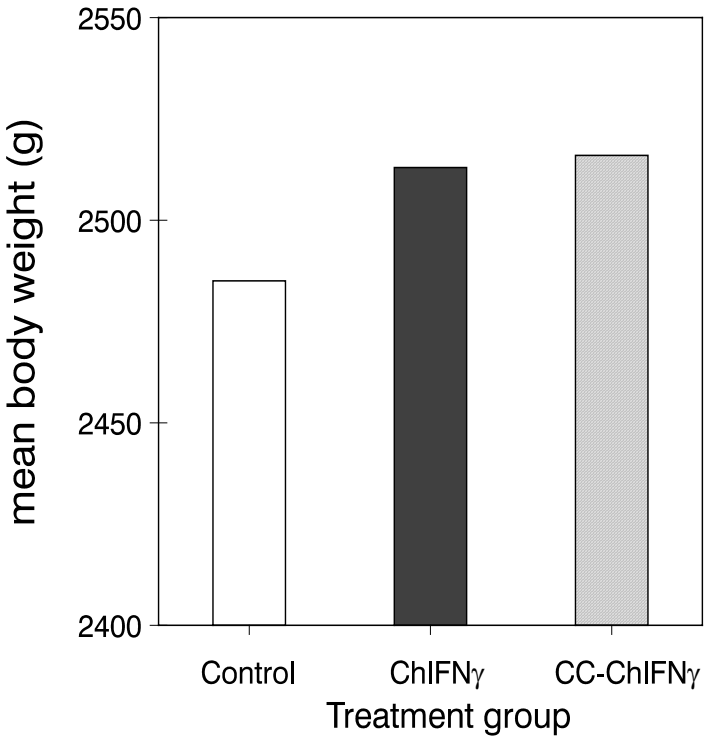

Figure 7. Trial 2. Enhancement of weight gain by broilers treated with ChIFN-γ. Groups of 80 chickens were injected (ip) with 100 ug of *E coli*-derived ChIFN-γ (unmodified or CC-PEG modified) and weighed 42 days later.

4.7 Coccidiosis challenge trials

Several trials were performed in order to assess the ability of ChIFN-γ to enhance the growth performance of vaccinated broilers reared under commercial conditions and challenged with *E. brunetti*. Results from two representative trials are shown.

4.7.1 Trial 3

We assessed the ability of ChIFN-γ to enhance protection against coccidial infection by measuring it's ability to reduce the rate of weight loss associated with infection. Chickens were treated orally with FAV::ChIFN-γ at five days of age. A second group was treated with FAV expressing a control cytokine, cMGF. All birds were then challenged with a dose of *E. acervulina* that gives mild weight reduction. Figure 8 shows that cMGF and non-treated birds had a significantly reduced rate of weight gain between days 7 and 11 post challenge, relative to ChIFN-γ treated birds. This is an indication that ChIFN-γ was able to reduce the severity of the infection. In a similar trial, treatment of infected birds with ChIFN-γ resulted in enhanced growth performance relative to non treated infected birds (7.1% and 2.9% increase in body weight at day 35 and 42, respectively, Table 4). Treated birds also had an improved feed conversion ratio, a further indication of protection from disease challenge.

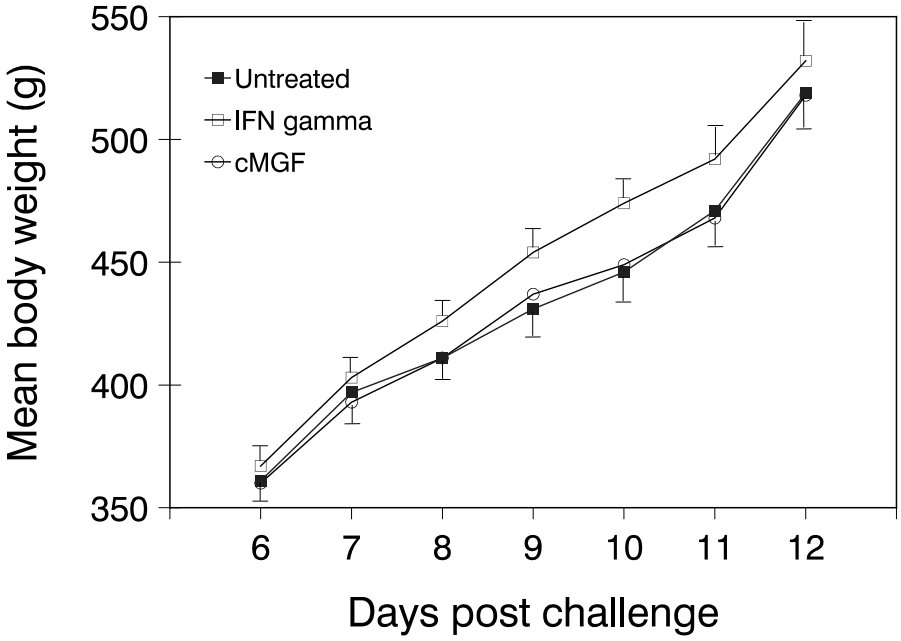

Figure 8. Trial 3. Enhancement of weight gain by broilers treated with ChIFN-γ prior to infection with *E acervulina*. Groups of 80 chickens were treated orally at day 5 of age with FAV::ChIFN-γ or FAV::cMGF or left as untreated controls. All bird were then challenged orally with 5000 oocytes of *E acervulina*. Body weights were measured until 12 days after challenge.

Table 4. Effect of ChIFN-γ on weight gain during coccidiosis infection.

	Body weight (g)				
Treatment	day21	day 28	day 35	day 42	FCR[c]
Control	800±9[a]	1302±18	1610±36	2270±40	1.95
ChIFN-γ	795±14 (-0.6%)[b]	1288±24 (-1.1%)	1725±43 (7.1%)	2335±58 (2.9%)	1.86

a Mean body weight ± SEM
b Percent difference in body weight relative to controls
c Feed conversion ratio

4.7.2 Trial 4

Eimeriavax3 is a newly developed live vaccine that confers protection against challenge with *E. brunetti* in the absence of in-feed coccidial medication. This trial was conducted to assess the compatibility of ChIFN-γ co-treatment and vaccination. Figure 9 shows that unvaccinated birds displayed a sharp decline (reduction by 50%) in growth rate one week after Eimeria challenge (week 4-5). The growth rate of vaccinated birds on the other hand was not affected, as was the case for the vaccinated birds that were co-treated with ChIFN-γ indicating that co-treatment did not reduce the protective effect of the vaccine. These results will allow future experiments to be performed to test whether treatment with ChIFN-γ will allow a lower dose of Eimeriavax3 to be used.

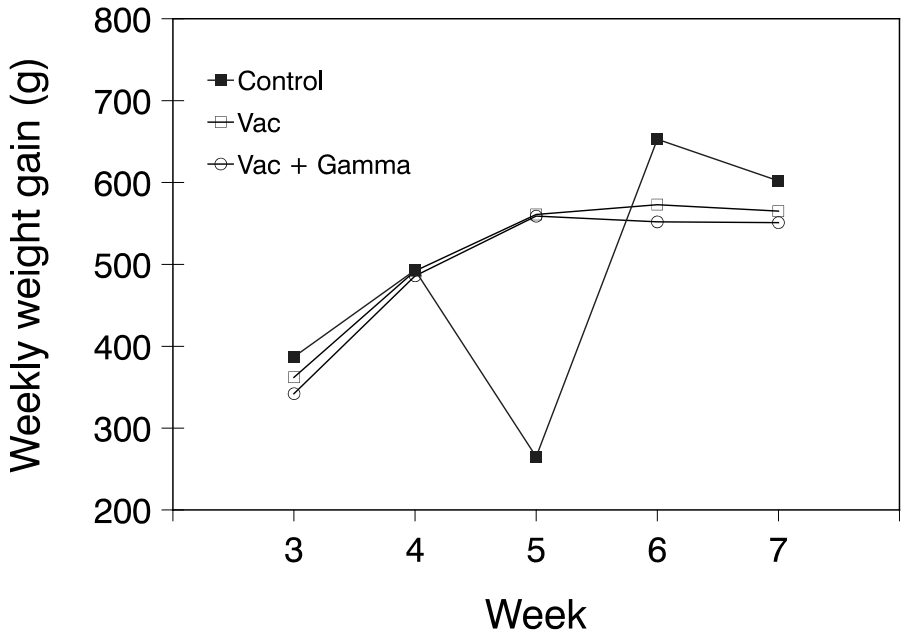

Figure 9. Effect of ChIFN-γ treatment of broilers in the face of coccidiosis vaccination. Two Groups of 80 chickens were vaccinated by eye drop with 100 oocysts of *E. brunetti* (Eimeriavax3) at day 6 of age. A third group served as unvaccinated controls. One of the vaccinated groups was at the same time treated with FAV::ChIFN-γ. All birds were challenged with *E. brunetti* 21 days later (Week 4). Mean weekly weight gains for each group is shown.

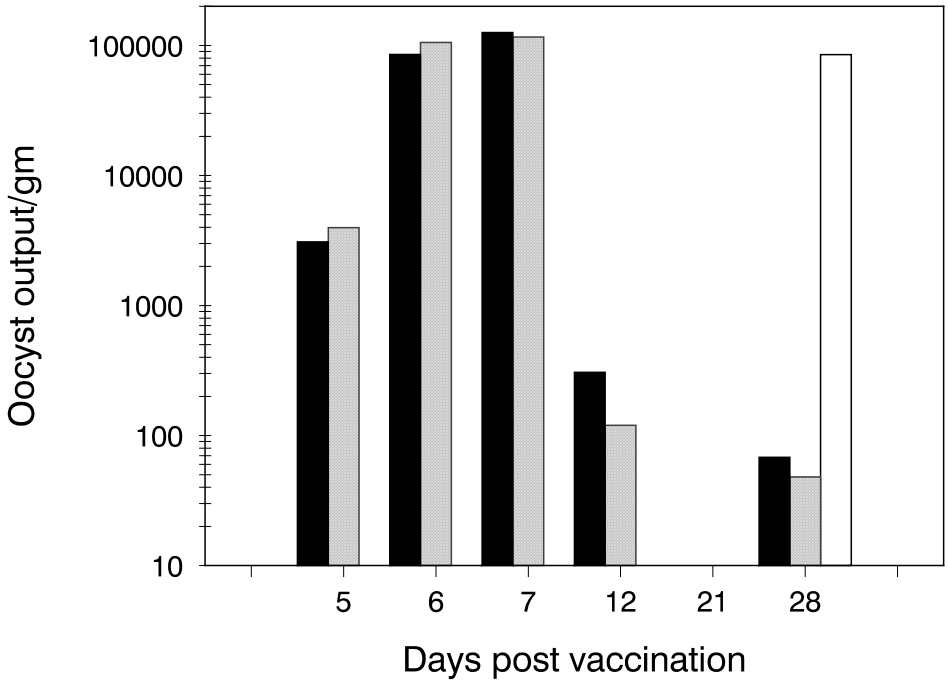

Figure 10. Effect of ChIFN-γ treatment on oocyst output in the face of coccidiosis vaccination and challenge. A group of 80 chickens was vaccinated with 100 oocysts of *E. brunetti* (Eimeriavax3) at day 6 of age (solid bars). A second group was similarly vaccinated but also treated with FAV::ChIFN-γ (hatched bars). A third group served as unvaccinated controls (open bar). Faecal samples were collected at various time points after challenge.

As expected with a live vaccination, there was a high output of oocysts beginning 5 days after vaccination and declining to very low levels within 12 days post-vaccination (Figure 10). ChIFN-γ did not alter oocyst output. Oocysts were not detected in the faeces of unvaccinated birds prior to challenge, but at 7 days post challenge there was a high output measured in this group. In contrast, the output in both of the vaccinated groups was approximately 1000-fold lower, indicating the effectiveness of the vaccination.

5. Discussion

5.1 ChIFN-γ as a growth promoter

Conditions of stress and illness have a negative impact on the ability of animals to grow at their optimal rate. It is also becoming evident that growth is influenced by interactions between the immune system and other systems within the body such as the neurological and endocrine systems and that cytokines may play a central role regulating this (Husband, 1995). It has been reasoned that reduction of stress and illness would allow a re-direction of the body's resources toward growth. There is now an emerging area of research examining the effects of utilising a combination of anti-inflammatory and immunostimulatory agents to achieve this, with cytokines being key players.

We have performed experiments to assess the growth promoting activity of ChIFN-γ in chickens (Lowenthal et al., 1999). The consistent finding is that broiler chickens treated with ChIFN-γ protein displayed enhanced weight gain over periods of up to 8 weeks. The increase in mean body weight ranged from 1-7% relative to control birds. Similar increases in growth rates were observed when ChIFN-γ was delivered via a FAV vector (Johnson et al., 2000). These results clearly indicate the potential use of ChIFN-γ as an effective, naturally occurring growth promoter. The underlying mechanisms are unknown, but may be due to the ChIFN-γ mediated enhancement of the immune system leading to decreased pathogen loads, resulting in healthier and more productive birds.

ChIFN-γ has been shown to prevent the development of Eimeria parasites *in vitro*, presumably through its ability to activate macrophages (Heriveau et al., 2000). The therapeutic potential of ChIFN-γ has also been demonstrated *in vivo* using a coccidial challenge model (Lowenthal et al., 1997; Lillehoj and Choi, 1998). Infection of young broilers with *Eimeria acervulina* normally results in weight loss beginning on day 4 after infection with weight gain resuming 2-3 days later. In these studies, broiler chickens were injected on two consecutive days with either *E. coli*-derived ChIFN-γ or diluent and then infected with *E. acervulina* oocytes one day later. Body weight was recorded daily for 12 days following challenge. ChIFN-γ treated birds lost less weight early in infection (day 4-5 post challenge) and recovered more quickly as indicated by a larger weight gain between day 6 and 8 post challenge when compared to controls. This result indicates that ChIFN-γ was effective in reducing the effect of coccidiosis on growth performance. In two separate trials, treatment of infected birds with ChIFN-γ resulted in a consistent increase in weight gain ranging from 2.7 to 12.5% relative to non-treated infected birds. In a later independent study, ChIFN-γ therapy was shown to also reduce oocyst production following Eimeria challenge (Lillehoj and Choi, 1998). These results have now been reproduced in larger trials performed in this current project under commercial farm conditions, where ChIFN-γ was also delivered by live FAV vectors.

5.2 ChIFN-γ as a vaccine adjuvant

There are concerns over the ability of certain live vaccines, currently used in the poultry industry, to adequately protect birds from infection by variant hyper-virulent strains of pathogens. There is a need for alternatives to such vaccines, however, killed and recombinant subunit vaccines usually do not offer an adequate level of protection and often require the use of adjuvants. Oil-based adjuvants, however, induce adverse site reactions resulting in decreased meat quality and animal discomfort. At this time there are no suitable, cost effective adjuvants for use in poultry, particularly broilers. ChIFN-γ is one cytokine that has been assessed for its ability to enhance antibody responses. When co-administered to birds with antigen, recombinant ChIFN-γ produced a prolonged secondary antibody response that persisted at higher levels and for longer periods compared to antigen injected alone (Lowenthal et al., 1998).

Infection with coccidiosis causes production losses through out the broiler industry. It is controlled by a combination of vaccination, in which low levels of the parasite in the litter are ingested by the birds

to which they develop a protective immune response, and chemicals via in-feed coccidiostats. Birds that are not exposed to the parasite at an early age remain susceptible and can be infected later, resulting in severe weight loss. In the coccidiosis challenge trials presented here in, we demonstrated that treatment of broilers with ChIFN-γ reduced weight loss following coccidial infection compared to control birds. This study also demonstrated the effectiveness of a low dose of Eimeriavax3 in affording protection of commercial broilers from challenge with *E. brunetti*. Vaccination of newly hatched chickens with as little as 100 oocysts prevented weight loss, improved feed conversion and reduced oocyst output by 99.9% following coccidial challenge at 4 weeks of age. Co-treatment with Eimeriavax3 and ChIFN-γ was equivalent to vaccination with Eimeriavax3 alone, indicating that this dose of vaccine by itself was sufficient to induced maximum levels of protection. Furthermore, these results show that ChIFN-γ treatment id not negatively impact on vaccine efficacy. In order for a poultry vaccine to be commercially viable it must be relatively inexpensive, particularly for the broiler industry. Even though Eimeriavax3 is highly effective, the high costs of production of live attenuated strains make it only suitable for the breeder/egg production market. If the effective dose could be decreased by ChIFN-γ co-administration, then this product would also be used in broilers. Further dose-response experiments need to be performed to test whether treatment with ChIFN-γ will allow a lower dose of Eimeriavax3 to be used.

5.3 Delivery methods for chicken cytokines

This project has focused on the application of recombinant ChIFN-γ protein. For cytokines to be used as commercial therapeutics we must consider certain aspects of the poultry, in particular the broiler, industry. The delivery methods for vaccines and other therapeutics are of prime importance and are required to be safe, easy to administer and cost-effective. Recombinant cytokine protein produced from baculovirus, yeast or *E. coli* expression systems have been successfully delivered via injection to larger high-value animals such as pigs and cattle (Heath and Playfair, 1992). For use in chickens, the recombinant protein must be manufactured cheaply on a large scale (tens of billions of doses annually) and ideally be given as a single dose to be cost-effective. The delivery of cytokines and vaccines via live viral vectors (York et al., 1996; Karaca et al., 1998) expressing these proteins has been successful and often eliminates the need for multiple boosts. Live viral vectors such a FAV expressing cytokine genes (Johnson et al., 2000) can overcome the short half-life of recombinant cytokines *in vivo* because the cytokines are expressed over a period of many days until the virus is cleared. FAV can be delivered via drinking water or aerosol sprays, making it very easy to administer. Further advantages of FAV vectors include the production of native proteins rather than prokaryotically-expressed proteins which may be less active. An additionally safety aspect is that the virus is chicken specific and will not replicate in other animal species.

5.4 Future directions and hurdles

There are several other cytokines that have a variety of functions that are equally important in terms of regulating immune responses. We now have a panel of cytokines, including interleukins-2, -15 and -18, that we can study using the same approach as used for the assessment of ChIFN-γ. These interleukins play a primary role in the generation of cell mediated immunity (Carson et al., 1994; Sugawara et al., 2000; Schneider et al., 2000), which is crucial for controlling diseases such as coccidiosis, Marek's disease, infectious bronchitis and Newcastle disease. Another example is a study by Marcus et al., 1999, which showed that administration of ChIFN-α protected birds against challenge with NDV. As we build on our understanding of how cytokines control the immune system, we will gain further insight on how to optimise immune responses to vaccination.

When considering therapeutic strategies, the concept of synergy is an extremely important one since it is fundamental to the way cytokines normally work in controlling the immune response. There have been some studies that showed the synergistic activity of cytokines in poultry (Sekellick et al., 1998; Schijns et al., 2000). The new generation delivery mechanisms such as FAV are compatible with this approach since

they permit the administration of multiple cytokines in combination with vaccine antigens. Furthermore, the choice of particular vectors will enable antigen and cytokine targeting to specific sites such as the gut or respiratory tract, thereby allowing the most appropriate type of immune responses to be generated at the correct site. The combined use of bacteriocins (the focus of a current RIRDC project) and cytokines offers an exciting opportunity to further improve the health status of Australian poultry.

One of the remaining challenges involves a closer understanding of the nature of protective immune responses. Acquired protection against pathogens in mammals generally falls into one of two types - cell mediated or antibody mediated. However, a combination of cell mediated, antibody and innate responses are often generated during phases of an infection, making it difficult to determine which of these responses is responsible for protection. In chickens it has not been established whether the same Th1/Th2 paradigm exists as it does in mammals. In order to rationally design therapeutics for a particular disease it is critical to first understand the nature of the protective immune response and then replicate that response during a vaccination strategy. This involves studying the cytokines produced during infection by the pathogen in question as well as the immune cell populations affected. With the growing accessibility to a number of avian cytokine genes, and the recent development of Real Time PCR and TaqMan® technology, cytokine profiles can now be accurately measured during the course of an infection.

Another remaining hurdle is the delivery of therapeutics and recombinant vaccines to poultry. Given that administration of recombinant cytokine proteins by injection is not feasible in commercial poultry, alternative methods are needed. FAV technology provides a simple, effective and inexpensive commercial delivery system. Therapeutics can also be directly administered to the embryo prior to hatching via *in ovo* injection of protein or targeted vectors. This allows cytokines and vaccines to be inexpensively delivered by an automated egg injector system and allows other potentially effective technologies such as *in ovo* DNA vaccination to be explored. These new generation delivery mechanisms permit the administration of single or multiple cytokines in combination with vaccine antigens. The choice of particular vectors will enable antigen and cytokine targeting to specific sites such as the gut or respiratory tract, thereby allowing the most appropriate type of immune responses to be generated at the correct site. Cytokines offer a natural approach to therapeutics particularly in relation to the enhancement of protective immune responses produced by vaccines. With the escalating number of chicken cytokines being cloned, only time will tell just how important these regulatory immune proteins will be for the poultry industry.

6. Implications

Restrictions in the use of in-feed antibiotics will have a very significant impact on the Australian poultry industry unless alternative therapeutics are developed. Base on overseas experiences, pathogens that are currently kept under control by antibiotics, such as Clostridium species, will become more prevalent, leading to severe cases of necrotic enteritis and other gastrointestinal problems. There is a risk that incidence of secondary infections will increase significantly, resulting in increased costs due to medication and vaccines and reduced productivity.

Alternative therapeutics such as cytokines and bacteriocins are currently being assessed as safe, naturally occurring alternatives to in-feed antibiotics. Cytokines offer a two pronged attack. They can strengthen and control immune responses resulting in the increase in the general health of animals. Secondly, they can improve the efficacy of vaccines, resulting in improved long term protection against specific pathogens. We have demonstrated that ChIFN-γ is capable of both of these types of activities.

The implications for the Australian poultry industry are very significant. The introduction of cytokines as therapeutics should compensate for the reduction in the use of in feed growth promotants. It is anticipated that ChIFN-γ technology will be implemented in the Australian market in the near future. An Australian poultry health company is currently negotiating with CSIRO for a licence to market ChIFN-γ technology to the Australian poultry industry.

There is now an opportunity to assess other types of cytokines that have different types of activities, for their therapeutic capability.

7. Recommendations

In this project we have made some very significant steps forward in the evaluation of chicken cytokines as alternative therapeutics. We have established proof-of-principal by showing that ChIFN-γ is a growth promoter and immunoenhancer under commercial conditions. The next step will be to make this technology available to Australian poultry producers as soon as possible. Infact, an Australian poultry health company is currently negotiating with CSIRO for a licence to develop ChIFN-γ technology to the Australian poultry market.

To best make use of the outcomes of this project, the following recommendations are made for further work required in this area:

1. Evaluation of additional cytokines.
Even though ChIFN-γ has shown great promise, there are other types of cytokines that need to be investigated. There are several other cytokines that have a variety of other functions that are equally important in terms of regulating immune responses. We now have a panel of cytokines (interleukins-2, -15 and -18) that play a primary role in the generation of cell mediated immunity, which is crucial for controlling diseases such as coccidiosis, Marek's disease, infectious bronchitis and Newcastle disease. We can apply the same approach as used for the assessment of ChIFN-γ.

2. Identification of the nature of protective immune responses.
One of the remaining challenges involves a closer understanding of the nature of protective immune responses. Acquired protection against pathogens in mammals generally falls into one of two types – cell mediated or antibody mediated. In order to rationally design therapeutics for a particular disease it is critical to first understand the nature of the protective immune response and then replicate that response during a vaccination strategy. This involves studying the cytokines produced during infection by the pathogen in question as well as the immune cell populations affected. With the growing accessibility to a number of avian cytokine genes, and the recent development of Real Time PCR and TaqMan® technology, cytokine profiles can now be accurately screened and measured during the course of a particular infection. This information can then be used to rationally determine which cytokine/s should be investigated for that particular disease.

3. Development of optimal delivery methods.
Another remaining hurdle involves the delivery of therapeutics and recombinant vaccines to poultry. Given that administration of recombinant cytokine proteins by injection is not feasible in commercial poultry, alternative methods are needed. In a separate project, CSIRO has developed FAV technology, which provides a simple, effective and inexpensive commercial delivery system. Therapeutics can also be directly administered to the embryo prior to hatching via *in ovo* injection of protein or targeted vectors. This allows cytokines and vaccines to be inexpensively delivered by an automated egg injector system.

4. Combined use of a variety of complimentary approaches.
When considering therapeutic strategies, the concept of synergy is an extremely important one since it is fundamental to the way cytokines normally work in controlling the immune response. The new generation delivery mechanisms such as FAV permit the administration of single or multiple cytokines in combination with vaccine antigens. The choice of particular vectors will enable antigen and cytokine targeting to specific sites such as the gut or respiratory tract, thereby allowing the most appropriate type of immune responses to be generated at the correct site. Furthermore, the combined use of bacteriocins (the focus of a current RIRDC project) and cytokines offers an exciting opportunity to further improve the health status of Australian poultry.

8. References

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