

ISSN 1682-8356  
ansinet.org/ijps



INTERNATIONAL JOURNAL OF  
**POULTRY SCIENCE**

**ANSI***net*

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## Effect of Dietary Fishmeal on Cell-Mediated Immune Response of Laying Hens

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**Abstract:** In this study, we examined the role of different amounts of dietary fishmeal, a component of chicken diet on cell-mediated immune response of chickens. Forty eight laying white Leghorn chickens were randomly divided into 4 groups and fed diets containing 0, 1.5, 3 or 6% fish-meal. Six chickens from each dietary group were euthanized 3 and 9 weeks later and the spleens were removed to isolate cells and assess immune response. Mitogenic response was determined by <sup>3</sup>H-thymidine incorporation and the cell subsets were established by flow cytometry. Three weeks of dietary treatment had no impact on Concanavalin A (Con A) or phytohemagglutinin (PHA) induced proliferation or cell subsets of splenic lymphocytes. However, 9 weeks post dietary treatment PHA-induced proliferation and CD4 cell population were elevated in 3% fishmeal fed hens compared to those fed the control (0% fishmeal) diet. Our results suggest that a moderate increase in dietary fish-meal may enhance the immune response in laying hens. This may prove useful in infections in which cell-mediated immunity plays a role.

**Key words:** Fishmeal, laying hens, cell-mediated immune response

### Introduction

Poultry is a common reservoir of *Salmonella* and a leading vehicle for outbreaks. Consumption of raw or partially cooked *Salmonella enteritidis* (SE)-contaminated eggs has been recognized as a significant source of human salmonellosis (Hope *et al.*, 2002). *Salmonella* is frequently found in the intestines of commercial broilers and laying hens. The organism colonizes primarily in the lower gastrointestinal tract of the chickens. Typically, these chickens appear healthy without any symptoms even though large numbers of *Salmonella* are excreted in their feces. One of the ways of eliminating or reducing the *Salmonella* contamination in the poultry products may be by improving the ability of the birds to avoid infection. Immunoenhancement could be one of the approaches since extensive mouse studies have shown the role for T cells, natural killer (NK) cells, macrophages, neutrophils and numerous cytokines in *Salmonella* resistance (Mastroeni *et al.*, 2001; Mittrucker and Kaufmann, 2000). Our recent findings in which a live-attenuated vaccine improved cell-mediated immunity (CMI) and also reduced SE shedding suggested that the immune system plays a role in *Salmonella* clearance (Babu *et al.*, 2004).

Diet has been shown to play an important role in modulating the resistance to infection and the immune system (Fraser *et al.*, 2000; Lesourd, 2004; Digby *et al.*, 2003; Klasing, 1998). With regard to *Salmonella* infections, increased protein, lactose, zinc-methionine, lactulose and calcium phosphate, capsaicin, mannose or palm kernel meal have been shown to improve

bacterial clearance (Otani *et al.*, 2003; Corrier *et al.*, 1990; Kidd *et al.*, 1994; Bovee-Oudenhoven and Van Der Meer, 1997; McElroy *et al.*, 1994; Allen *et al.*, 1997). Similarly, there have been reports of negative effects of forced molting and high doses of n-3 and n-6 fatty acids on *Salmonella* clearance (Holt, 2003; D'Ambola *et al.*, 1991). Dietary fish oil was shown to decrease kupffer cell phagocytosis and oxidative burst during the early part of *Salmonella typhimurium* infection as well as increase mortality rates of infected mice (Eicher and McVey, 1995; Anonymous, 1992; Chang *et al.*, 1992). Fish oil has also been shown to be immunosuppressive at high concentrations and immunostimulatory at lower concentration in chickens (Wang *et al.*, 2000; Korver and Klasing, 1997). Although fishmeal is a component of the commercial diet for laying hens (Gous and Swatson, 2000; Reddy *et al.*, 1997; Ponce and Gernat, 2002; Aletor, 1990), its role on immune response has received little attention. Therefore, we investigated the impact of different levels of fishmeal on the functions of splenic lymphocytes from laying hens. Results from this study could help the poultry industry in optimizing the levels of fishmeal in the feed for improving the immune response.

### Materials and Methods

**Animals and diets:** Forty eight specific pathogen-free White Leghorn laying hens were housed in individual laying wire cages and were provided feed and water *ad libitum*. The hens were randomly divided into 4 groups and were fed diets containing 0, 1.5%, 3% or 6% fishmeal. Composition of the diets is presented in

Table 1: Composition of Control and Test Diets

	Control	Test 1	Test 2	Test 3
Ground corn	61.30%	61.30%	61.30%	61.30%
Soy Meal	21.5%	20.0%	18.50%	15.50%
Soy Oil	2%	2%	2%	2%
Protein Blend	5%	5%	5%	5%
LimeStone (Ca)	7%	7%	7%	7%
Alfalfa Meal	2%	2%	2%	2%
Fish Meal	0.00%	1.50%	3%	6%
DL Methionine	0.50%	0.50%	0.50%	0.50%
Vitamin/Mineral Mix	0.50%	0.50%	0.50%	0.50%
NaCl	0.30%	0.30%	0.30%	0.30%

Table 1. The birds were euthanized by CO<sub>2</sub> asphyxiation after 3 and 9 weeks of dietary treatment. Spleens were removed immediately and used for isolating mononuclear cells (MNC).

**Isolation of mononuclear cells from spleen:** Spleens were washed and minced in HBSS and the residual tissue was removed using a cell strainer. Single cell suspension from the spleens was overlaid onto Histopaque (density 1.077 g/ml) and centrifuged at about 400xg for 30 min at room temperature. Mononuclear cells obtained from the interface were washed twice with HBSS and suspended in RPMI 1640 medium containing 5% heat inactivated fetal calf serum (Life Technologies, Rockville, MD). Cell concentrations were adjusted to 8x10<sup>6</sup> cells/ml.

**Mitogenic reactivity *in vitro*:** Aliquots (50 µl) of splenic MNC suspended in RPMI 1640 complete medium with 5% fetal calf serum were placed in 96 well plates. Another aliquot (50 µl) of either RPMI 1640 complete medium or medium with 3 different concentrations of Con A (0.4, 0.8 or 1.6 µg/well) or PHA (0.8, 1.6 or 3.2 µg/well). After 48 hours of incubation in air-5% CO<sub>2</sub> at 41°C, 1µCi <sup>3</sup>H-thymidine was added to each well. Cultures were incubated for an additional 24 hours. Cells were harvested using a 96 well automatic harvester and counted in a microbeta counter. Data are presented as cpm from stimulated cultures after subtracting the cpm from non-stimulated cultures.

**Antibodies and flow cytometric analysis:** Analysis of splenic lymphocyte populations was done by FACS analysis using an EPICS Elite flow cytometer (Beckman/Coulter, Miami FL). Splenic lymphocytes were suspended in phosphate buffered saline (PBS) containing 2% heat inactivated fetal bovine serum and 0.05% sodium azide (FACS diluent) at a concentration of 10<sup>7</sup> cells per ml. Monoclonal antibodies specific for chicken cell surface antigens were added to 50µl of FACS diluent in wells of a 96 well microtiter plate to achieve a predetermined optimal final concentration (0.1-1.0 µg/50µl). Ten microliters of spleen cell

suspension were added to each well. Antibodies (Southern Biotechnology Associates, Inc, Birmingham, AL) used for immunofluorescent staining were directed against chicken T lymphocytes (CD3, CD4, CD8). Direct FITC or R-phycoerythrin conjugated antibodies were used for staining. Matched isotype control antibody conjugates were also used to determine background staining. Immunofluorescent staining took place for 30 minutes at 4°C. Samples were then washed twice and re-suspended in 100ul FACS diluent. FACS analysis was conducted on the viable lymphocyte population as determined by forward light scatter versus 90 degree light scatter gating. Five thousand cells were analyzed for each antibody combination.

**Statistical analysis:** Data pertaining to proliferation and cell populations were compared between the control and the experimental groups by conducting one-way analysis of variance (ANOVA) followed by pair-wise multiple comparison procedures (Tukey test). These statistical analyses were done using Sigma Stat v2.03. Differences between the treatment groups were considered statistically significant at P < 0.05.

## Results and Discussion

In this study, we tested the impact of dietary fishmeal ranging from 0 to 6% (by weight) on cell-mediated immune parameters, such as splenic lymphocyte proliferation and cell subsets. This range of fishmeal included the amount present in commercial chicken feeds (1.5%) and at the highest level (6%), it did not cause any off flavor or taste as evidenced by no negative impact on food consumption or health of the hens (data not shown). Fishmeal has been used as a protein source for growing chicks for over three decades (Schumaier and McGinnis, 1969; Reddy *et al.*, 1997; Ponce and Gernat, 2002). Although dietary fishmeal has been shown to improve growth, performance and resistance to parasitic infections in chickens and young sheep, (Reddy *et al.*, 1997; Aletor, 1990; van Houtert *et al.*, 1995), its impact on the immune response has received little attention. Therefore, we assessed the role of moderate increase in dietary fishmeal on the splenic

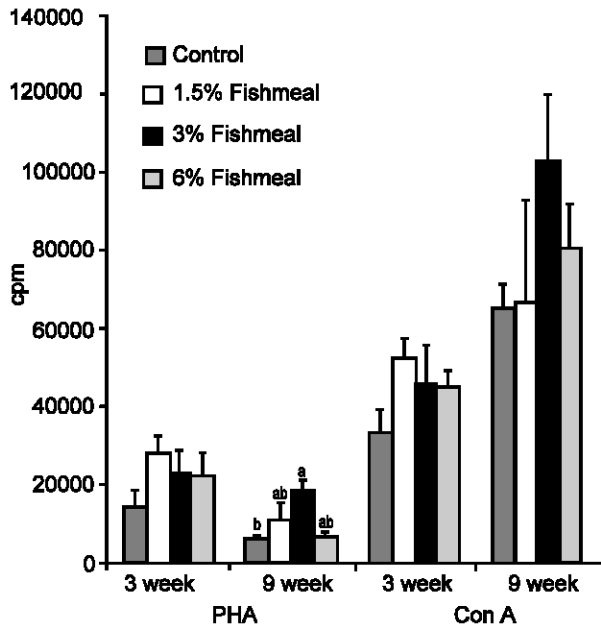


Fig. 1: Impact of dietary fishmeal (0-6% by weight) on Con A- and PHA- mediated spleen cell proliferation of laying hens. Results are presented as Total cpm – non-stimulated cpm. Mitogenic stimulation was carried out as described in Materials and Methods using different concentrations of Con A and PHA. Only optimal responses have been presented in this bar graph. Each bar represents mean±SEM (N=6). Bars with different superscripts differed at p<0.05.

lymphocyte function. Mitogen-induced proliferation and splenic lymphocyte cell subsets were not influenced by 3 weeks of dietary fishmeal. However, after 9 weeks of dietary regimen, splenic lymphocytes from 3% fishmeal fed hens responded significantly better to PHA than those from the control group (0% fishmeal group). Although response to Con A showed a similar trend, the difference was not statistically significant (Fig. 1). Fishmeal is known to contain several essential amino acids including lysine and methionine (van Niekerk and van Niekerk, 1997; Davenport *et al.*, 1990). Both quality and quantity of dietary protein have been shown to affect the immune responses (Nonnecke *et al.*, 2003; Yoshino *et al.*, 2003). Cell-mediated and humoral immunity of broiler chicks was shown to be enhanced by optimum levels of dietary methionine and cysteine, and at higher levels there was no immunoenhancement (Tsiagbe *et al.*, 1987). In our study, the immunoenhancement was not directly proportional to the amount of dietary fishmeal as seen from the lack of an impact at the 6% fishmeal level. It is possible that 3% fishmeal diet provided amino acids at more optimal levels for lymphocyte function than the 6% fishmeal diet.

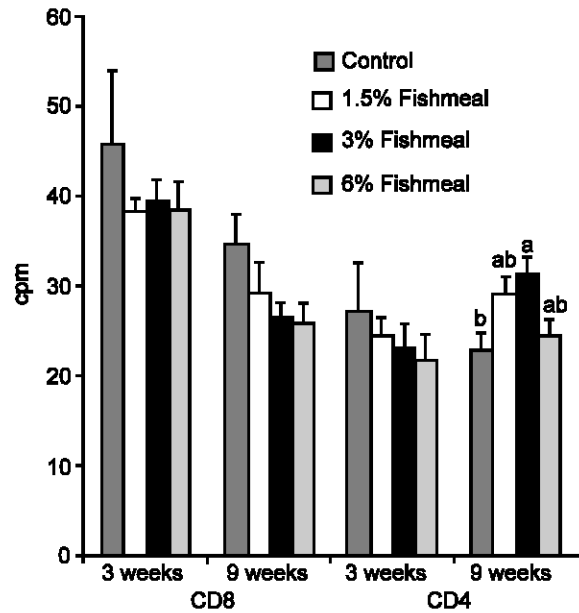


Fig. 2: Impact of dietary fishmeal (0-6% by weight) on splenic CD4 and CD8 cells from laying hens. Results are presented as percent total lymphocytes. Flow cytometry and the antibodies used for the cell subset analysis are described in Materials and Methods. Each bar represents mean±SEM (N=6). Bars with different superscripts differed at p<0.05.

In addition to increased proliferation of splenic lymphocytes, we observed a significant increase in the percentage of splenic CD4 cells from hens that were fed 3% fishmeal for 9 weeks (Fig. 2). Enhanced splenic lymphocyte proliferation to PHA in 3% fishmeal fed hens (and the lack there of in the 6% fishmeal fed hens) could be partially attributed to the increase in CD4 cells (Kasinrerk and Tokrasinwit, 1999), which are known to support lymphocyte activation, possibly by increased IL-2 production (Quade and Roth, 1999; Abdalla *et al.*, 2003). In addition to their role in lymphocyte activation, the CD4 cells are known to play a role in protection against intracellular pathogens by producing macrophage activating factors and by generating a response to *Salmonella* flagellar protein (Mastroeni *et al.*, 1992; Kaufmann 1993; McSorley *et al.*, 2000). Involvement of CD4 cells in protection against *Salmonella typhimurium* clearance from the spleens by *in vivo* treatment of mice with anti-CD4 monoclonal antibody (Nauciel, 1990). In general, role of cell-mediated immunity (CMI) in *Salmonella* elimination has been established in rodents (Ugrinovic *et al.*, 2003; Bumann, 2003), and one of our recent studies showed an association between improved CMI and reduced *Salmonella enteritidis* (SE) shedding in young vaccine

treated chickens (Babu *et al.*, 2004).

Overall, our results showed that moderate levels of dietary fishmeal had the potential to improve the immune response of laying hens. Upon further exploring, if there is a positive correlation between dietary fishmeal and SE clearance, it may be used in addition to other dietary regimens to reduce the incidence of *Salmonella* infection in chickens.

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