

**IMMUNOCOMPETENCE IN YOUNG AND OLD LAYING HENS IN RESPONSE
TO DIETARY FOLIC ACID AND *Escherichia coli* LIPOPOLYSACCHARIDE
CHALLENGE**

By

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A Thesis

Submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial Fulfilment of the Requirement

For the Degree of

MASTER OF SCIENCE

Department of Animal Science

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ABSTRACT

We investigated the effects of dietary folic acid and age, on immunological parameters in laying hens challenged with LPS. 48 Shaver White hens at different ages were fed 2 wheat-soybean based diets with 0 or 4 mg supplemental FA per kg of diet for 8 wk. 6 hens from each dietary treatment were injected with 8 mg/kg body weight of LPS or saline and observed for 4 h. A few diet X challenge interaction were observed in young hens, whereas FA and LPS independently influenced a number of parameters. In older hens, there were very few diet X challenge interactions as well as effects of FA, whereas LPS affected several parameters. In conclusion, dietary FA influenced some immune responses in younger hens, but no such effects were observed in older hens. FA may modulate immune responses in laying hens under acute LPS challenge which could be tissue and age dependent.

DEDICATION

This thesis is dedicated to my parents; Francis Munyaka and Beatrice Mweela for their warm love and moral support that has propelled me this far.

ACKNOWLEDGMENT

It is such a great pleasure to express my sincere gratitude to various people whose contribution in diverse ways made the completion of this thesis a success. First of all, my great appreciation and recognition goes to my advisor, Dr. J. C. Rodriguez-Lecompte for offering me the great opportunity and resources for my studies and also for his valuable advice, guidance, and encouragement throughout my entire Master's programme. His training on various techniques, patience and understanding, and desire to get this thesis done will forever be appreciated. I would like to extend my appreciation to Drs. Karmin, O, J. D. House, Rick Holley, and C. M. Nyachoti for accepting to be on my advisory committee and also for their suggestions and guidance throughout the programme. Statistical assistance and guidance received from Dr. G. Crow is highly appreciated.

I would like to thank members of our lab, Alex Yitbarek, for his help and training in RT-PCR, Angela Kroeker, for her assistance and training in Flow cytometry analysis, Harold Mauricio, for his help in the lab and assistance in all the injection and sampling processes. I would like to extend my heartfelt appreciation to all fellow graduate students, technical, and research staff at the Department of Animal Science for their friendship, company, great encouragements to me, and willingness to help me whenever I needed help. My thanks to Aurele, Jerry, and Harry for their special assistance with the animal trials. Administrative assistance received from Carol, Mei, Kathy, Margaret, and Cathy is highly appreciated. My special thanks to Dr. Augustine of Danisco Animal Nutrition, for his concern, friendship, encouragements, and valuable advice throughout my Masters programme.

This study was funded by the National Sciences and Engineering Research Council of Canada Collaborative Research and Development Grants Program, the Manitoba Egg Farmers and the Egg Farmers of Canada, and the Agri-Food Research and Development Initiative. Financial awards received from IGSES, UMGF, Manitoba government scholarship, Faculty of graduate studies conference travel award, and the Noval C. Young Graduate Fellowship in Animal Science, are highly appreciated. I am grateful for short-term jobs received from the Department of Animal Science.

I would like to express my special thanks and recognition to my family who have always been there for me. Special thanks to my parents to whom this thesis is dedicated for raising me in the best way they can and for their warm love and support; “Mum although you have just sadly left us, your inspiration will forever drive me”!. And to my sisters (Tryphena Monah, Bisciah Syombua, Faith Rose, and Alice Kavua) and brothers (Julius Mwendwa, Jeremiah Mwanza, Joseph Ndunda, and Caleb ndunga), who have been a great source of inspiration; their care, concern, support, and love will forever be appreciated. Of course to my nieces (Kasila, Mercy, and Beatrice) and nephews (Amos, Joshua, Peter, Baraka, and Ngala) for the great cheerful moments we share together.

Finally, to the only wise God our Saviour, be glory and majesty, dominion and power, both now and forever more. It is all about you!

FOREWORD

This thesis was written in a manuscript format and it is composed of two manuscripts. Manuscript I has been submitted to the Poultry Science Journal and manuscript II will be submitted to the same journal. Part of the work in manuscript I was presented at AVMA-PSA-AAAP meeting in July, 2011, manuscript II will be presented at PSA meeting in July, 2012 and parts of manuscript I and II will be presented at AAAP meeting in August, 2012. The authors in manuscript I are P. M. Munyaka, G. B. Tactacan, M. Jing, Karmin O, J. D. House, and J. C. Rodriguez Lecompte. The authors in Manuscript II are as indicated in manuscript I, including: Michael St. Paul, and Shayan Sharif. All manuscripts are formatted to meet the guidelines of Poultry Science.

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LIST OF ABBREVIATIONS

5, 10-MTHF	5,10 methylene tetra hydrofolate
ADCC	antibody-dependent cellular cytotoxicity
BMI	body mass index
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CON	concanavalin
CRP	C-reactive proteins
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DRI	daily recommended intake
dTMP	deoxythymidine monophosphate
dTTP	deoxythymidine triphosphate
dUMP	deoxyuridine monophosphate
dUTP	deoxyuridine triphosphate
ELISA	enzyme-linked immunosorbent assay
EU	European Union
FA	folic acid
FDA	food and drug administration
FITC	fluorescein isothiocyanate
H:L	heterophil to lymphocyte ratio

Hcy	homocysteine
HHcy	hyperhomocysteinemia
IFN- γ	interferon-gamma
IgG	immunoglobulin G
IL	interleukin
K ₂ EDTA	potassium ethylenediaminetetra-acetic acid
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
MAPKs	mitogen-activated protein kinases
MBL	Mannose-binding lectin
MCP-1	monocyte chemoattractant protein-1
mRNA	messenger ribonucleic acid
NAbs	natural antibodies
NADPH	nicotinamide adenine dinucleotide phosphate
NF-kB	nuclear factor kappa B
NK	natural killer
NO	nitric oxide
NRC	nutrient requirement council
PAMP	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
PHA	phytohaemagglutinin

PMA	phorbol myristate acetate
PRR	pattern recognition receptors
qRT-PCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
TGF	transforming growth factor
Th	T helper
TLR	toll-like receptor
TNF	tumor necrosis factor

CHAPTER ONE

1.0 GENERAL INTRODUCTION

Increasing emphasis has been placed on the need to optimize the efficiency of egg production in the recent years. This is due to various factors such as increasing the number of eggs produced per hen during production period, the need to lengthen the production period, and to meet the increasing demand for eggs and egg products, as well as reducing chances for diseases (infestations). As a result, several advancements over the last five decades have been made in various fields in the poultry industry including: genetic engineering, nutrition, and management (Havenstein et al., 2003). Regarding genetics, the selected, inbred, and congenic lines continue to be an essential component in industry breeding programs (Ellen et al., 2008). However, results of studies with selected lines of chicken strains indicate that genetic selection for group productivity and longevity alters the immunological and haematological profile (Cheng et al., 2001). Therefore, commercial chickens may diverge dramatically in immunocompetence and disease susceptibility attributable to genetic selection for production traits, environmental factors associated with commercial operation, and intense immunization (Cheng et al., 2001). In addition, genetic selection for immune traits can increase general and specific resistance to pathogens in chickens (Redmond et al., 2010). However, genetic resistance alone is not completely protective and the use of additional means to protect and enhance health such as dietary immunomodulation, are being pursued (Redmond et al., 2010).

A number of immune changes have been reported in animals with increasing age; indeed, the ageing of the immune system is associated with dramatic reductions in

immune responsiveness including impairment of physiological regulatory mechanisms (Burns, 2004). This in turn leads to a reduction in both innate and adaptive immune functions, increased incidences of diseases, and autoimmunity (Hale et al., 2002). One of the most remarkable changes with age is the decline of T cell function for both CD4+ and CD8+ T cells (Haynes et al., 2004; Effros, 2004), a condition that has been shown to be preceded by thymus gland involution and decreased levels of thymic hormone (Castle, 2000a, b). B cell function also suffers considerable changes with age, which may be in part related to changes in T cells and could also be independent of changes in T-cells (Burns, 2004; Linton and Dorshkind, 2004). Innate immunity precedes the antigen-specific T-cell and B-cell responses, and is also affected by advancing age (Hale et al., 2002). As a consequence, innate and adaptive immune responses become less effective with advanced age, and there exists an increased reactivity against self-antigens (autoimmunity) as well as increased incidences of infectious diseases (Zheng et al., 1998). In avian species, nutrient status is a central factor that contributes to the capacity of birds to respond to a pathogen challenge; indeed, nutritional status plays an important role in immune function and influences both the innate and adaptive components of the immune system (Kidd, 2004).

With respect to nutrition, folic acid (folate), a water soluble vitamin plays a central role in one carbon-transfer reactions and the synthesis of nucleic acid and proteins, hence poor folate status significantly alters the immune response (Troen et al., 2006; Wintergerst et al., 2007). Deficiency of folate has been shown to affect immune competence and resistance to infection, and have also been associated with a number of clinical abnormalities including reduced functioning of the immune system (Wintergerst

et al., 2007). In an in vitro culture system employing phytohaemagglutinin (PHA)-activated human T lymphocytes, folate deficiency was reported to reduce the proliferation of cytotoxic T lymphocytes and increased the CD4:CD8 ratio (Dhur et al., 1991a). Therefore, a reduction in circulating T lymphocytes and their proliferation can affect the ability to resist or respond to infections. In addition, folate supplementation in elderly individuals enhances the overall immune function by altering the age-associated decrease in NK cell activity supporting Th1 responses that are essential for the protection against viral infections (Troen et al., 2006). Furthermore, in a rodent model of aging, folate supplementation was found to enhance the ability of lymphocytes to proliferate in response to a mitogen challenge (Dhur et al., 1991b). These studies provide evidence that, despite the fact that animals were receiving diets containing “adequate” amounts of folate, as they age, additional folate may be required to optimize immune function.

To this end, information on the effects of dietary folic acid supplementation on immune competence in laying hens under acute *Escherichia coli* lipopolysaccharide (LPS) challenge is not available. Such information will help in assessing the potential for enhanced folic acid supply to reduce the adverse effects of LPS of gram negative bacteria in laying hens.

It was therefore hypothesized that: 1). Dietary folic acid supplementation in laying hens will exert a beneficial role by enhancing the immune competence. This will be achieved, to a considerable degree, by positively influencing a number of specific immune cells as well as enhancing the expression and regulation of immune-related genes in response to an immunological insult such as lipopolysaccharide (LPS) of gram negative bacteria. 2). Immune competence decreases with age and hence, the need for

dietary folic acid supplementation in laying hens increases with age. Additional inclusion of dietary folic acid in the diet of old laying hens will therefore counteract the age associated decline in immune competence.

The main objective of the research described in this thesis was to examine the relationship between dietary folic acid supplementation, LPS challenge, and age on the immune responses in laying hens. The specific objectives were:

- a). To examine immunomodulation in young laying hens by dietary folic acid and acute immune response after challenge with *Escherichia coli* lipopolysaccharide.
- b). To examine the response of old laying hens to an *Escherichia coli* lipopolysaccharide challenge when fed diets with or without supplemental folic acid.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 An overview of the avian immune system

In animal production, a functional and competent immune system is a requirement for a healthy life. The immune system is a multifaceted arrangement of membranes (skin, epithelial, mucus), cells, tissues, organs, and molecules whose function is to purge a host of invading pathogens or even more, to control cancer cells (Chadra, 1997). These components of the immune system work together ensuring a careful balance of being lethal enough to kill pathogens or cancer cells and yet exhibit a degree of specificity and tolerance so as not to cause extensive damage to healthy “self” tissues of the host (Kogut and Klasing, 2009). Once a host encounters any pathogenic insult, the first function of the immune response is to identify or recognize, and consequently eliminate the infection.

In all vertebrates, the immune system consists of two functional elements which interact in their mechanisms of pathogen recognition; the innate immune system and adaptive or specific immune system (Fearon and Locksley, 1996; Mak, 2003; Calder, 2007). The innate immune system is represented by cells and humoral factors that have a broad specificity with germ-line encoded receptors, referred to as pattern recognition receptors (PRR), that recognize the evolutionary conserved molecular components; pathogen-associated molecular patterns (PAMP) of infectious microbes (Fearon and Locksley, 1996; Medzhitov and Janeway, 1997a; Medzhitov and Janeway, 2000; Janeway and Medzhitov, 2002). The innate immune system is also characterized by usually a fast response with apparently no memory of a first exposure. On the other hand,

the specific immune system is represented by cells and humoral factors with very narrow specificity that uses highly specific antigen receptors on T and B lymphocytes that are generated by random processes by gene rearrangement, making it possible to respond to any kind of potential antigen (Fearon and Locksley, 1996; Carpenter and O'Neil, 2007). The rearrangement of the receptor genes results to a relatively slow response (days) with memory to the specific antigen.

The characteristics of the immune system may appear to be very simple in organization but rather complex in its operations and interactions (**Table 2. 1**). There are many cell types in the immune system that can be found in several different stages of maturation or differentiation, and several different sub-types of cells, molecules, and receptors are being found as more sophisticated techniques evolve with time (Noverr and Huffnagle, 2005). This has consequences on the understanding of the immune system.

2.1.1 Innate immune response

The innate immune system is a rapidly induced, phylogenetically conserved response of all multicellular organisms and it is dependent on PRR of PAMP on or in major groups of microbes including infectious bacteria, viruses, and parasites (Medzhitov and Janeway, 1997a; Medzhitov and Janeway, 2000; Janeway and Medzhitov, 2002; Mak, 2003). PRR are strategically expressed on cells which are first to encounter pathogens during infection: epithelial cells and effector cells of the innate immune system (dendritic cells and macrophages) (Berczi et al., 1998; Van Vliet et al., 2007).

The PAMPs are important for the replication and survival of the pathogens, and

Table 2.1 General characteristics of the immune system

	Innate/non-specific Immunity	Adaptive/specific/acquired immunity
Anatomical components	Skin, respiratory tract, gastrointestinal tract	Bone marrow, thymus, mucosal-associated lymphoid tissue, lymph nodes
Cells	Neutrophils/ Heterophils, monocytes, macrophages, dendritic cells, mast cells, eosinophils, basophils, natural killer cells	B lymphocytes, T lymphocytes
Protein	Interferons, complements, collectin and lysozymes	Immunoglobulins
Receptors	Pattern recognition receptors, encoded in germline	Antigen-specific receptors, rearranged during development (somatic recombination)
Distribution of receptors	Non-clonal	Clonal: clones of cells have distinct specificities and express different receptors
Specificity	Non-specific nature of activity	Discriminate and specific molecular entities
Rapidity	No induction time	Take days to activate
Immunological memory	Functioning is not increased as a result of previous exposure	Increased functioning as a result of previous exposure
Stage	Early stage of defence	Later stage of defence
Self-discrimination	Distinguish self from non-self components but not absolute	Distinguish self from non-self components but not absolute

(Partially adapted from Li et al., 2007)

are unique to large groups of microorganisms but not host cells, a property that enables the host to detect non-self invading pathogens (Akira, 2004; Kogut and Klasing, 2009; Kannaki et al., 2010). Examples of PAMPs includes lipopolysaccharides of gram-negative bacteria, lipoteichoic acids of gram-positive bacteria, flagellin, viral double stranded RNA, mannans of yeast cell walls, bacterial DNA, lipoproteins and peptidoglycans of all bacteria, and glycolipids of mycobacteria (Fearon and Locksley, 1996; Medzhitov and Janeway, 1997a,b; Kannaki et al., 2010).

Recognition of PAMP by PRR induces a number of extracellular activation cascades and intracellular signalling pathways, leading to either the inflammatory responses, recruitment of phagocytic cells for clearance of the pathogens, and or mobilization of professional antigen-presenting cells such as dendritic cells and macrophages (Medzhitov and Janeway, 1997a,b, 2000; Janeway and Medzhitov, 2002). Expression of several pro-inflammatory cytokines, including tumor necrosis factor (TNF)- α , IL-6, IL-12, IL-15, and type-1 interferons and subsequently inflammatory responses, are modulated by the release of IL-10 and transforming growth factor (TGF)- β (Medzhitov and Janeway, 1997a). The initiation of cytokine mRNA transcripts is regulated by molecular bridges known as transcription factors including nuclear factor kappa B (NF- κ B), activation protein-1, and interferon regulatory factors 3, 5, and 7 which mediates intracellular signalling that results in changes in the gene expression (Medzhitov and Janeway, 1997b, 2000). These endogenous signals orchestrate the recruitment of leukocytes to the site of infection and regulate the activation of the suitable effector mechanisms by controlling differentiation of T lymphocytes into effector cells of a particular type (Medzhitov and Janeway, 1997a, 2000; Janeway and Medzhitov, 2002),

and consequently leads to the development of highly specific memory cells (Romagnani, 1992; Song and Cerny, 2003). The innate immune system, therefore, acts as a link to the specific immune responses. In addition, pathogens are bound to specific receptors expressed on leukocytes, especially when these pathogens are opsonised by ligand molecules, like complement factors (C3), heat-shock proteins, and LPS-binding proteins (Berczi et al., 1998; Natea et al., 2005; Van Vliet et al., 2007; Barton, 2008). Such receptors include toll-like receptors (TLR) which are members of the large family of PRR systems, and chickens have been found to express a number of these TLR in various tissues and cells (Iqbal et al., 2005; Kogut et al., 2005a; Subedi et al., 2007; Kannaki et al., 2010). TLR are specific for structures present on bacteria, viruses and parasites, and enable cells of the innate immune system which express these TLR to react rapidly to these infections.

The recognition of conserved microbial or viral antigen via non-specific receptors such as TLR, heat-shock proteins or complement component C3 receptors present on the cells of the innate immune system (NK cells, macrophages, dendritic cells), or recognition of microbial antigens by natural antibodies (NAbs) from the first and probably most important defence mechanism against infection have been described previously (Berczi et al., 1998; Barton, 2008). Natural antibodies are antibodies present without intentional induction by means of either vaccination or immune challenge, and they are produced by B type cells which do not require T cell help and are considered to be part of the innate immune system rather than the adaptive immune system (Chou et al., 2008; Ochesnbein et al., 1999, 2000).

In chickens, heterophils, which are counterparts of mammalian neutrophils, and macrophages ingest and kill a variety of microbial pathogens. They display a number of activities including adhesion, chemotaxis, phagocytosis, and microbial activities of degranulation, and respiratory burst (Farnell et al., 2003; He et al., 2003a and b; Kogut et al., 2005b). Heterophils and macrophages are capable of inducing rapid changes in pro- and anti-inflammatory cytokines and chemokines gene expression following receptor mediated phagocytosis (He et al., 2003b; Kogut et al., 2005b, 2007).

2.1.2 Adaptive/ Acquired immune response (Specific Immunity)

Unlike the innate immune system, acquired immune response is limited to vertebrates and it is mediated by T (thymus-derived) and B (bursa-derived) lymphocytes (Fearon and Locksley, 1996; Carpenter and O'Neil, 2007). The ability of both T and B cells to respond to antigens is aided by the somatic rearrangement of immunoglobulin and T cell receptor genes to create clones of lymphocytes that express distinct antigen receptors (Yang et al., 1996; Wu et al., 2008). The receptors on lymphocytes are generated by somatic mechanisms during the embryonic development of each individual and thus generate a diverse repertoire of antigen receptors with random specificities on the lymphocytes (Yang et al., 1996; Wu et al., 2008). After clearing of an infection, the antigen-specific clones remain expanded as memory lymphocytes that provide a more rapid response to second exposure to the same antigen (Song and Cerny, 2003). Antigen recognition by a T or a B lymphocyte requires the antigen to be presented bound to cell surface proteins known as major histocompatibility complex (MHC) class I or II and co-stimulatory molecules (Avery et al., 2004; Korver, 2006).

Academically, two different types of responses mediate specific immune responses depending on the type of lymphocytes that primarily responds to the antigen: cell-mediated (T lymphocytes) or humoral (B lymphocyte) immunity (Avery et al., 2004). Cell-mediated immunity is characterized by recognition of infected host cells by T lymphocytes, whereas humoral immunity is characterized by the production of antibodies by the B lymphocytes in response to extracellular antigens recognized by T cells (Avery et al., 2004). In general, an adaptive immune response consists of various phases including antigen recognition, activation of lymphocytes, and elimination of antigen by effector cells (**Figure 2.1**). The response declines as antigen stimulated lymphocytes die by apoptosis, restoring homeostasis and the antigen-specific cells that survive are responsible for memory. With respect to T cells, two phenotypically different T lymphocytes exist that perform different effector functions that control immune responses: T cytotoxic (Tc; CD8+) and T helper (Th; CD4+) lymphocytes. Upon stimulation by relevant antigenic peptides presented by MHC class I on antigen presenting cells, activated CD8+ cells lyse virally infected host cells. On the other hand, naive CD4+ cells develop into functionally mature effector cells upon stimulation with relevant antigenic peptides presented by MHC class II molecules on antigen presenting cells (Natea et al., 2005). Depending on the kind of the cytokines that are produced upon stimulation of CD4+ cells, Th cells are generally segregated into two subsets: Th1 cells producing exclusively interleukin-2 (IL-2), interferon-gamma (IFN- γ), tumor necrosis factor-beta (TNF- β). On the other hand, Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13. The Th1 and Th2 subsets appear to cross-regulate each other's cytokine

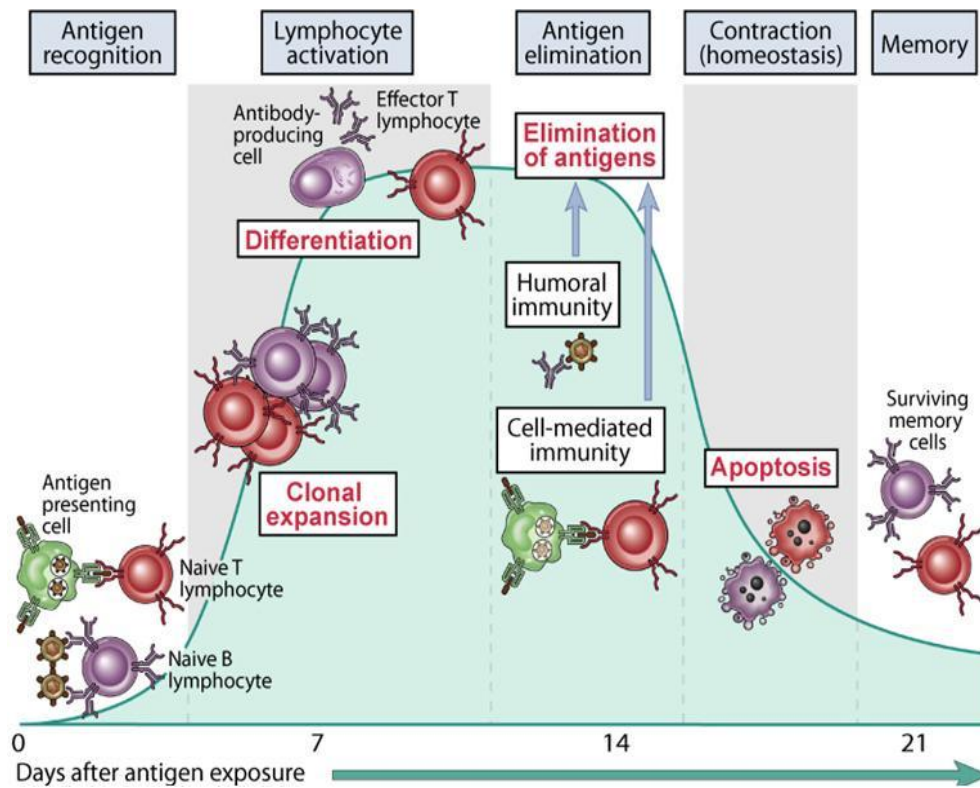


Figure 2.1 Phases of an adaptive immune response

<http://biology-forums.com/index.php?action=gallery;sa=view;id=1492>

production profiles, mainly through IFN- γ and IL-10 (Avery et al., 2004; Natea et al., 2005; Korver, 2006).

In addition to the Th1 and Th2 cells, other Th cell subsets have been described: regulatory T cells (Treg) which comprise of classical Th cells, capable of inhibiting Th1 responses and also other T cells, and mediate active suppression of various immune responses (Chen and O'Shea, 2008; Afzali et al, 2007). Recently, a novel Th cell subset has also been described; the Th17 which are induced by IL-23, and they produce IL-17 and IL-22 that both contribute to inflammatory responses (Bi et al., 2007; Stockinger et al., 2007; Jarnicki et al., 2008).

2.2 Nutrition- Immunity Interaction

A number of factors including genetics, the frequency of exposure to disease causing organisms, the virulence of the pathogens, management practises, and the efficacy of the vaccination programs are predominant determinants of the incidence of infectious diseases in birds and hence have a direct impact on the immune system (Klasing, 1998). Present strategies to control diseases in poultry include: vaccination, use of antibiotics, biosecurity, genetic selection for disease resistance, and husbandry programs (Kumar et al., 2011). However, alternative or additional strategies including dietary immunomodulation are being pursued (Chae et al., 2006). The interaction involving nutrition and immunity and how the host deals with infectious antigens is a strategic determinant in animal health (Dhur et al., 1991b; Scrimshaw and SanGiovanni, 1997). In addition, dietary characteristics such as the level of nutrients, or the types of ingredients used can modulate an animal's susceptibility to infectious challenges (Klasing, 1998). Furthermore, nutritional immunomodulation is thought to contribute to

survival of offsprings in ever-changing environmental conditions (Burdge et al., 2007); therefore, by understanding the mechanisms of nutritional effects on the immune system, specific interactions that occur between diet and infections can be well studied. For example, knowing how a diet or nutritional supplements induces temporal changes in host metabolism and the associated function of the immune cells, and expression of immune response genes during different stages of an immune response, could result in the formulation of affordable diets that optimize disease resistance to a variety of pathogenic organisms (Kogut and Klasing, 2009).

In summary, a number of mechanisms through which nutrition interacts with the immune system have been stipulated over the past: 1) engaging the immune system to induce differentiation of lymphocytes during development of an immune response, 2) acting as a substrate for cells of the immune system for example the amino acids as substrates for cytokine production by leukocytes, and production of acute phase proteins by the liver, 3) regulating the supply of critical nutrients to pathogens for example iron and biotin, 4) modulating signalling in leukocytes through up- or down-regulation of immune cell functions, 5) offering protection against immunopathology through management of inflammatory responses, 6) altering the hormonal milieu, and 7) influencing the microbial ecology of the gut for optimal growth and health, (Klasing, 1998, 2004, 2007; Kogut and Klasing, 2009).

As in other animals, immune responses are important in poultry for protection from various infections and maintaining health; indeed, any changes in the immune responses are likely to alter health status and influence poultry production (Wang et al., 2000). Folic acid (FA) a water soluble vitamin that belongs to the B-complex group

among other nutrients, has been shown to be a vital nutrient for optimal health, growth and development, however, exogenous intake is required since FA cannot be synthesised in the body (Lyer et al., 2009; Hoffbrand and Weir, 2001).

2.3 Stimulation of the immune system

Stimulation of the enteric immune system by plant lectins can result in most of the symptoms of an enteric infection, including diarrhoea, malabsorption, and poor growth and efficiency (Klasing, 2007). Plant lectins such as phytohaemagglutinins (PHA), concanavalins (CON), phorbol myristate acetate (PMA), and ionomycin are well-characterized immunostimulants (Dhur et al., 1991a,b; Gogal et al., 1997; Field et al., 2006; Stone et al., 2009). The activation of the immune system causes the release of pro-inflammatory cytokines and the accompanying acute phase responses that participate in clearance of infections and recruitment of other immunocompetent cells (Nii et al., 2011; Feng et al., 2011). On the other hand, stimulation of the immune system prior to exposure to a real pathogen improves disease resistance through development of immune memory to boost immune response upon exposure (Parmentier et al., 2010).

Lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria such as *Escherichia coli* and *Salmonella* is widely used as an immune stimulant (Chapman et al., 2005; Lorenzoni and Wideman, 2008). LPS is a potent inflammatory mediator and has been used to model bacterial infection experimentally in chickens and other species of farm animals (Yang et al., 2008). Many aspects of inflammation caused by infection with gram-negative bacteria can be mimicked by administration of LPS (Shini et al., 2008a; Gehad et al., 2002a, b). In chickens, LPS has been shown to influence natural and specific humoral immune competence (Star et al., 2007), as well as

inducing a variety of behavioural, hormonal, and physiological changes (Star et al., 2007; Shini et al., 2008b), such as an elevation in plasma corticosterone concentration with immune suppressing substances and consequently increase of the heterophil to lymphocyte ratio (H:L) (McFlane and Curtis, 1989; Altan et al., 2003; Shini et al., 2008b; Al-Ghamdi, 2008). In addition, administration of LPS has also been shown to influence the population and distribution of T cells in circulation, a condition that is associated with improved immunocompetence, and could also be detrimental to the health of the birds (Shini et al., 2008b; Gehad et al., 2002 a, b; Nii et al., 2011).

On the other hand, in some situations and with some pathogens, dietary stimulation of the immune system prior to exposure improves disease resistance due to an adjuvant-like effect caused by the diet (Lowry et al., 2005). However, increased protection in the absence of the pathogen comes at the cost of decreased productivity and general performance (Klasing, 2004; Huff et al., 2006). This therefore suggest that the inclusion of dietary components that stimulate immune system to respond should be kept at the very lower levels so as to ensure maximal growth, egg production, and general health, especially if the birds are not raised in a contaminated environment (Klasing, 2007). In light of this, diets or dietary components should only be used for engaging the immune system to induce differentiation of immune cells during development, nourish immune cells, modulate them, and facilitate establishment of commensal microflora rather than needlessly stimulating the immune system (Klasing, 2007). This may further suggest that immune stimulation should be limited to pathogens, plant lectins, and irritants that cause the immune system to respond.

2.4 Folic acid

2.4.1 An overview of the background of folic acid

Folic acid (FA) received its name in 1941 when it was isolated from spinach and was shown to be a growth factor for *Streptococcus lactis R (S. Faecalis)* (Michell et al., 1941). The compound was subsequently synthesized in pure crystalline form later in 1943 which provided a proof that FA is composed of a pteridine ring, paraminobenzoic acid and glutamic acid and was called 'pteroylglutamic acid' (**Fig 2.2**) (Angier et al., 1945). A review by Hoffbrand and Weir, (2001) indicates that, soon after its synthesis, it became apparent that natural folates usually differed from pteroylglutamic acid in three respects: a) presence of additional glutamate residues (polyglutamates), b) ability to be reduced into di- or tetra-hydroforms, and c) possession of additional single carbon units. However, FA (pteroylglutamic acid) is now used to denote the fully oxidized chemical compound not present in natural foods while the term "folate" is used to denote the large group of compounds possessing the same vitamin activity which includes natural folates and folic acid (Hoffbrand and Weir, 2001). Later on, it became apparent that FA was effective in the treatment of all types of megaloblastic anaemia as well. Vitamin B₁₂ was also found to have similar effects and it is therefore believed that, the availability of folic acid and vitamin B₁₂ in pure forms, both capable of producing a cure of megaloblastic anaemia, provided the basis for the present phase of studies in megaloblastic anaemia at the biochemical level (Hoffbrand and Weir, 2001). The 1950s and 1960s witnessed the elucidation of the biochemical reactions involving folates in single carbon unit transfer in amino acid conversions including homocysteine conversion to methionine, and purine

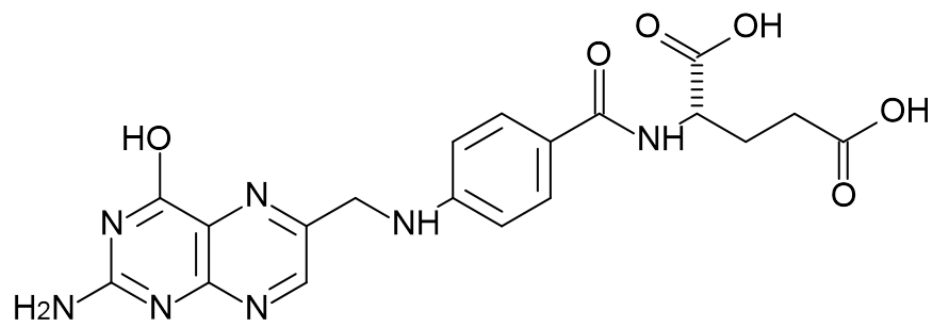


Figure 2.2 Structure of folic acid (Adapted from Angier et al., 1945)

and pyrimidine synthesis (Blakley, 1969). According to the authors, thymidylate synthesis in which deoxyuridine monophosphate (dUMP) is methylated by 5,10 methylene tetra hydrofolate (5, 10-MTHF) to thymidine monophosphate (dTMP), emerged important particularly in DNA synthesis.

2.4.2 Mandatory folic acid fortification

The daily recommended intake (DRI) as approved in the European Union (EU) is 400 µg/d for adults (FAO/WHO, 2002; IOM, 2004). Since folate deficiency has been associated with the incidence of neural tube defects during the embryonic development, a higher intake (600 µg/d) is recommended for women before and during pregnancy (Daly et al., 1995; Kim, 2004). In addition, low folate intake has also been associated with a number of health disorders including: Alzheimer's and coronary heart diseases, osteoporosis, increased risk of breast and colorectal cancer, increased level of homocysteine, a risk factor for development of cardiovascular diseases, (Morrisson et al., 1996; Boushey et al., 1996; Ames, 1999; Choi and Mason, 2002). This indicates that an exogenous supply of FA is required to prevent nutritional deficiency especially in view of the inability of mammalian cells to synthesize this vital biomolecule (Sarma and Duttagupta, 1995).

These health concerns including birth defects provided a basis for the mandatory fortification of cereal grains and grain products with FA in the United States, Canada, and other countries (Mills, 2000; Castilla et al., 2003; L'Abbé et al., 2008). As of January 1998, all cereal grain products in the United States labelled "enriched" (e.g., bread, pasta, flour, breakfast cereal, and rice) and mixed food items containing these grains were required by the Food and Drug Administration (FDA) to be fortified with FA (Mills,

2000). It is estimated that several thousand food items in the US food supply have been modified by fortification and now contain FA derived from enriched cereal grain ingredients (Lewis et al., 1999). Outside of the United States, mandatory fortification has also been implemented in Canada (L'Abbé et al., 2008), Chile, and some other Latin American countries (Castilla et al., 2003).

In addition to grain products' fortification with folic acid, other strategies for delivering dietary folic acid to the growing population including enrichment of eggs with folic acid through dietary supplementation in laying hens, have been explored and have proved to be effective as well (House et al., 2002; Hebert et al., 2005; Hoey et al., 2009; Dickson et al., 2010).

2.4.3 The use of folic acid in laying hens

To this end, studies have been conducted to investigate the effects of dietary folic acid on performance in laying hens. NRC (1994) recommends 0.25 mg FA per kg of diet in laying hens, however, studies have shown lack of dietary folic acid positivity and production performance in laying hens (House et al., 2002; Herbert et al., 2005; Roth-Maier and Bohmer, 2007; Bunchasak and Kachana, 2009; Dickson et al., 2010; Tactacan et al., 2012) and this has generally limited the inclusion of FA in laying hen diets. This is supported by surveys conducted on commercial feed mills with respect to FA supplementation in laying hen diet (BASF, 2000). However, most of the ingredients used for formulating laying hen diets have been found to contain higher amounts of natural folates that exceed the NRC (1994) recommendations (House et al., 2002; Herbert et al., 2005; Hoey et al., 2009; Dickson et al., 2010; Tactacan et al., 2010; 2012).

In addition to the mandatory fortification of cereal grains and grain products with FA in 1998 in the US and Canada (L'Abbé et al., 2008, Mills, 2008) (as discussed in section 2.2.1 of this chapter), other strategies such as the development of novel foods enriched with natural folates, and enhancement of folate content in fermented foods, have been pursued (Hjortmo et al., 2004; Buttriss, 2005). However, with respect to laying hens, the efficacy of enriching eggs with FA as a cheap and effective means for enhanced delivery of natural folates to the human population has been shown to be more successful (Sherwood et al., 1993; House et al., 2002; Herbert et al., 2005; Roth-Maier and Bohmer, 2007; Bunchasak and Kachana, 2009; Hoey et al., 2009; Dickson et al., 2010). Laying hens can convert high doses of FA added to the feed into natural folates in eggs thus providing a novel source of folate (House et al., 2002; Hebert et al., 2005; Hoey et al., 2009; Bunchasak and Kachana, 2009; Dickson et al., 2010). In support of this, a 2 to 2.5 fold increase in the level of folate concentration in the eggs has been reported in laying hens supplemented with 4 mg FA per kg of diet as compared to hens fed the basal diet (House et al., 2002; Hebert et al., 2005; Dickson et al., 2010). Furthermore, investigations on the effects of FA on the level of homocysteine in laying hens have reported a decreased level of plasma homocysteine concentration with increased dietary FA supplementation (Tactacan et al., 2012).

These reports have emphasized the vital role played by laying hens; acting as economical, safe, and reliable means of delivering natural folates to the human population, through supplementation with dietary FA. However, to this end, information on the effects of dietary FA in the immunocompetence of laying hens under challenged and unchallenged conditions remains to be known.

2.4.4 Folic acid and Immunity

2.4.4.1 Contributions of folic acid in innate immunity

Studies in humans and laboratory animals have shown the involvement of FA in innate immunity. For example, severe folate deficiency in rats was associated with a significantly impaired natural killer (NK)-mediated cytotoxicity, however, moderate folate deficiency had no significant influence (Kim et al., 2002). This study indicated that, although severe folate deficiency may have adverse effects on NK-mediated cytotoxicity, moderate folate deficiency appears not to affect NK-mediated cytotoxicity in rats. On the other hand, folate supplementation in elderly individuals (post-menopausal women) was also found to improve the overall immune function by altering the age-associated decrease in NK cell activity supporting a Th1 response through production of cytokines including IFN- γ , which in turn confers protection against infection (Troen et al., 2006); however, the presence of unmetabolized FA resulting from higher intakes of FA (folate-rich diet and supplements > 400 μg per day) was found to possibly impair NK cytotoxicity (Troen et al., 2006). This suggest that deficiency of FA as well as the presence of unmetabolized FA may have adverse effects on NK activities, and thus supplementation with FA should be offered at low levels where folate-rich diets are available. However, more studies are warranted to investigate whether excess consumption of FA could indeed interfere with normal functioning of the immune system with respect to NK cell activities.

2.4.4.2 Folic acid and cell-mediated immunity

Folic acid is necessary for normal functioning of the cell-mediated immune

system. Its deficiency has been associated with reduced ability of T cells to respond to mitogen stimulation or their inability to transform and take up ^3H -thymidine for DNA synthesis after exposure to PHA (a T cell mitogen), as well as decreased T cell cytotoxicity (Newberne and Gebhardt, 1973). The decreased response to PHA of lymphocytes from deficient animals can be explained by several mechanisms: a) inability of the T-lymphocytes to undergo blastic transformation, b) inability to synthesize DNA and to proliferate or divide after undergoing blast transformation, or c) overall reduction in the number of T-lymphocytes (Williams et al., 1975). Likewise, the reduced cytotoxicity could be due to inability of an animal's lymphocytes to recognize epitopes of virally infected cells presented by MHC class I on antigen presenting cells, or the inability to produce adequate numbers of cytotoxic cells to respond effectively upon antigenic attack (Williams et al., 1975).

In studies involving humans, rats and guinea pigs, folate deficiency have been shown to reduce the proportion of circulating T lymphocytes and their proliferation in response to mitogen activation, which in turn could decrease resistance to infections (Dhur et al., 1991b). However, the FA correction of these conditions was found to be more faster when done *in vitro* as opposed to *in vivo* conditions, suggesting that circulating unstimulated lymphocytes could be slow in incorporating FA *in vivo*, despite the vitamin deficiency (Dhur et al., 1991b). This may further indicate that a longer period of supplementation is paramount *in vivo* so as to provide adequate time for folate uptake by the cells. In an *in vitro* study employing human cells, folate deficiency reduced proliferation of T lymphocytes, induced cell cycle arrest in the S phase, induced apoptosis, and increased the CD4:CD8 ratio due to marked reduction of CD8⁺ cells

(Courtmanche et al., 2004). However, folate nucleoside repletion of folate-deficient cells rapidly restored these conditions. This data supported the hypothesis that dietary supplementation with FA may play a major role in enhancing the functionality of T cells by promoting their ability to proliferate.

2.4.4.3 Folic acid and humoral immunity

The reduced functionality of T cell mediated immunity due to FA deficiency can directly or indirectly lead to malfunctioning of the humoral arm of immunity, since Th2 cells are required for the activation of B cells. In addition, human studies have demonstrated a role of FA in cancer immunotherapy (Lu and Low, 2003). This is facilitated by the fact that, a variety of human and murine tumor cells over express cell surface receptors with high affinity for FA; haptens (small molecules that can elicit an immune response only when attached to a large carrier) have been specifically targeted to tumor cells by covalent ligation to FA (Zurawski and Kamen, 1992; Wang et al., 1997; Toffoli et al., 1997; Gates et al., 1996; Brigle et al., 1994). Lu and Low. (2002) demonstrated the efficacy of targeting cancer cells by linking FA to a model hapten hence decorating the folate receptor-expressing cancer cell surface with a higher amount of haptens per cell *in vivo* (**Figure 2. 3**). After marking the cancer cells with haptens, the cells are opsonised with autologous anti-hapten antibodies, leading to an antibody-mediated tumor cell regression mediated by antibody-dependent cellular cytotoxicity (ADCC) (Lu and Low, 2002, 2003). In this respect, FA acts as a carrier for delivering haptens to cancer targeted cells, this triggers humoral immune response via production

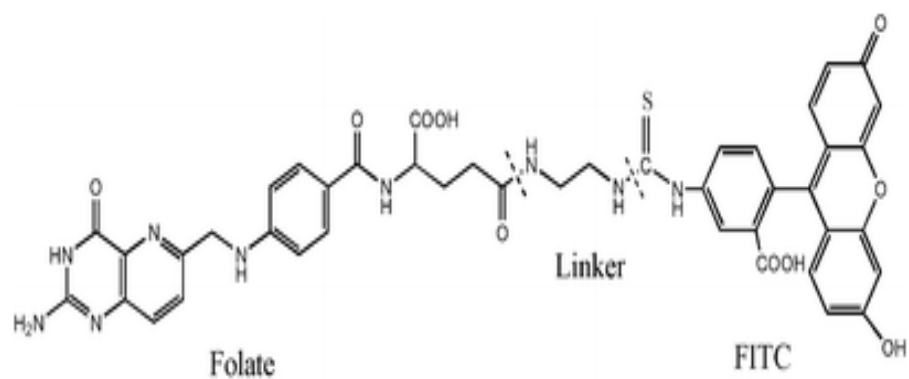


Figure 2. 3 Structure of the folate–hapten construct, (folate-FITC). Dashed lines indicate the boundaries between the three parts of the molecule; folate, linker, and hapten. (adapted from Lu and Low, 2002).

of antibodies against the haptens, which consequently clears the affected cells. Normal tissues are spared because they do not over express the folic acid cell surface receptors. However, high FA consumption is associated with possible enhancement of pre-existing cancers in humans (Cole et al., 2007), as discussed in section 2.4.5 in this chapter.

2.4.5 Clinical abnormalities associated with deficiency of folic acid

As a cofactor or co-enzyme, FA plays key biological roles in a variety of physiological processes, particularly in one carbon transfer reactions and the synthesis of nucleic acids and regeneration of methionine (Scott and Weir, 1998). Due to the crucial role of FA in nucleotide synthesis, it is clear that FA may affect immune cell proliferation and their responsiveness (Courtemanche et al., 2004). According to the existing literature, deficiency of FA can lead to many clinical abnormalities, including macrocytic anaemia, cardiovascular diseases, birth defects, carcinogenesis, poor cognitive disease, osteoporosis, increased level of homocysteine, (Morrisson et al., 1996; Boushey et al., 1996; Ames, 1999; Lucock, 2000; Mason and Choi, 2000; Choi and Mason, 2002).

Carcinogenesis emanating from FA deficiency is best studied in cases of colorectal cancer, in which several potential mechanisms through which folate may modulate carcinogenesis at the molecular and cellular levels including: DNA damage, impaired DNA repair, and aberrant DNA methylation have been proposed and studied (Kim et al., 2002). However, impaired NK cell activity has also been proposed to promote carcinogenesis (Kim et al., 2002).

On the other hand, contradictory findings in the use of FA on efforts to prevent or

reduce cancer recurrence have been reported (Kim, 2007; Cole et al., 2007; Figueiredo et al., 2009). Some studies on occurrence and incidences of cancer in humans suggest that supplementation with synthetic FA may promote growth of initiated cancer cells (Cole et al., 2007; Figueiredo et al., 2009). In addition, carcinogenesis appears to be accelerated if FA is supplemented after the emergence of lesions, probably through provision of DNA precursors for cancer cell growth (Song et al., 2000). However, reduction in early markers of colon cancer, such as aberrant crypt foci, has been reported in rodents when FA is supplemented before the onset of lesions (Song et al., 2000; Kim, 2007). Also, FA has been shown to be important in cancer immunotherapy in humans as discussed in section 2.4.4.3 in this chapter (Lu and Low, 2003). Overall, this may suggest a dual role of FA in development of cancer.

Hyperhomocysteinemia (HHcy) characterized by elevated levels of plasma homocysteine is a common leading risk factor for cardiovascular diseases, coronary artery disease and other arterial occlusive diseases, and has also been shown to increase under conditions of FA deficiency (Welch and Loscalzo, 1998; Refsum and Ueland, 1998; Wang et al., 2005). Supplementation with FA has therefore been shown to be a promising approach for prevention and treatment of cardiovascular diseases associated with HHcy and or with other risk factors (Woo et al., 1999; Doshi et al., 2002). In individuals with mild HHcy, oral FA supplementation was found to improve the arterial endothelium-dependent vascular function of the branchial artery (Woo et al., 1999). In addition, dietary FA supplementation was also found to prevent dietary-induced HHcy in rats as well as abolishing Hcy-stimulated chemokine expression in the aortic endothelium (Wang et al., 2002). This indicates that, FA has a significant contribution to general

health status and its deficiency can be detrimental. Furthermore, investigations on the effects of FA on the level of homocysteine in laying hens have also reported a decreased level of plasma homocysteine with increased dietary FA supplementation (Tactacan et al., 2012).

2.4.6 Folic acid and inflammatory responses

In addition to the well studied role of FA in lowering homocysteine and associated clinical disorders, current investigations have established an involvement of FA in inflammatory responses both in *in vivo* and *in vitro*. In an *in vitro* study, FA was reported to inhibit the LPS-induced production of NO, TNF- α and IL-1 β cytokines in the RAW264.7 macrophage cell line, through suppression of mitogen-activated protein kinases (MAPKs) and nuclear factor kappa B (NF- κ B) signal pathways (Feng et al., 2011). Similar findings were also reported where FA inhibited homocysteine-induced superoxide anion production and NF- κ B activation in human macrophages (Au-Yeung et al., 2006). These cytokines and superoxide anions are prominent pro-inflammatory mediators and if produced in high concentrations may be toxic to cells and tissues, leading to pathogenesis of various inflammatory associated diseases or autoimmunity (Beutler et, 1995; Detmers et al., 2000; Korhonen et al., 2005; Gabay et al., 2010). FA was also found to exert anti-inflammatory effects *in vivo*, through reduction of circulating levels of several inflammatory mediators in healthy overweight subjects and in patients with inflammatory bowel disease (Solini et al., 2006; Chiarello et al., 2009). In a study where a low-dose FA treatment (0.8 mg/kg) was used for 6 months, it was reported that plasma homocysteine (Hcy) levels were reduced and the hyper-responsiveness of

monocyte chemoattractant protein-1 (MCP-1) and IL-8 secreted by isolated monocytes was significantly reversed (Wang et al., 2005). In addition, the authors showed that FA supplementation at high concentrations (5 μ M) decreased the elevated levels of reactive oxygen species, NADPH oxidase activity and chemokines in response to Hcy in cultured human monocytes. FA may therefore be a promising candidate in attenuating the adverse effects of pro-inflammatory cytokines and oxygen reactive species; however, this remains to be explored in poultry and other farm animals.

2.4.7 Folic acid and Immunosenescence

Advanced age has been associated with overall decline in immunocompetence; however, nutritional intervention can attenuate age-related decline of immune competence. In a study involving young and older rats, folate supplementation was found to prevent the age-associated decline in the production of IFN- γ by unstimulated cells and the decrease in Th1/Th2- type response after stimulation with phorbol myristate acetate and ionomycin in the spleen (Field et al., 2006). However, the authors did not find any age-related changes in the mesenteric lymph nodes following dietary folate supplementation except an increased production of IL-4, a Th2 cytokine important for activation of B cells. This may provide information that different tissues may respond differently to nutrition and age-related decline in immune competence.

In elderly individuals, folate supplementation was found to improve the overall immune function by altering the age-associated decrease in NK cell activity supporting a Th1 response which in turn confers protection against infection (Troen et al., 2006). On

the other hand, other studies have reported contrasting findings. For example, Ravaglia et al. (2000) reported no correlation between total plasma folate concentration and NK cell cytotoxicity in elderly Italian subjects. However, these subjects were not under FA supplementation and their FA concentration was not followed up.

In addition, NK cytotoxicity was found to increase in healthy subjects aged over 70 years, who received over 4 months in addition to the regular diet a special nutritional formula providing, among other nutrients, 400 µg FA, 120 IU vitamin E and 3.8 µg vitamin B₁₂, but there was a decrease in NK cytotoxicity in the unsupplemented subjects (Bunout et al., 2004). Furthermore, supplemented subjects reported less infections indicating that these nutrients could increase innate immunity as well as conferring protection against infection in elderly individuals.

Further studies have also reported that dietary FA supplementation may also enhance immunocompetence in healthy elderly subjects. In apparently healthy elderly subjects carefully selected for the absence of protein-energy malnutrition (BMI > 23 kg/m², albumin > 38 g/l) (Lesourd, 2004), and who did not have a FA deficit, but only low erythrocyte FA contents that were in the lower part of normal ranges (200-780 µg/l), dietary FA supplemented at physiological doses (2 x Recommended dietary allowance), enhanced lymphocyte proliferation and IL-2 release which was related to increased erythrocyte FA (Lesourd, 2006). This may indicate that the immune system of the elderly is highly influenced by micronutrient (FA in this respect) deficiencies and could also be enhanced by supplementation even in older subjects without micronutrient deficiency. This therefore may raise the question whether dietary requirements for older individuals could be higher relative to that of young individuals.

2.5 Summary

In light of the existing information captured in this review, it is evident that FA is a vital nutrient with a significant impact on health status and functioning of the immune system, either alone and or in combination with other nutrients. Therefore, the question that arises is definitely not whether FA should be included in poultry diets, but instead, could dietary supplementation of poultry diets yield the same benefits in the immune system as observed in humans and laboratory animals particularly, the laying hens. This is due to the fact that, the laying hens have a long period of rearing and or production cycle, that provides an adequate time for exposure to varied management practises, and environmental conditions, that may consequently compromise their immunocompetence. It is therefore necessary to further extend research on the impacts of FA on the immune system of laying hens so as to further establish the relationship between FA supplementation and the immune response under both challenged and unchallenged conditions.

In addition, examining the effects of FA supplementation and its relationship with the age of laying hens may further provide information that could be useful in making decisions on the dietary composition of laying hens at late stages of the production cycle. Available literature has demonstrated the importance of laying hens as means of delivering FA to the human population through deposition in the egg; however, its relation to their immune system has not yet been established. The purpose of this study was therefore to determine the immunomodulation effects of dietary FA supplementation in young and old laying hens under challenge with *Escherichia coli* lipopolysaccharide.

CHAPTER THREE

3.0 MANUSCRIPT I

Immunomodulation in young laying hens by dietary folic acid and acute immune response after challenge with *Escherichia coli* lipopolysaccharide

3.1 ABSTRACT

We investigated the effects of dietary folic acid (FA) supplementation at 4 mg/kg of diet on immunological parameters in young laying hens under acute conditions of LPS challenge. In a completely randomized design, 48 Shaver White laying hens at 24 wk were fed 2 diets. The diets were wheat-soybean based, with either 0 or 4 mg supplemental FA per kg of diet. After 8 wk of feeding and at 32 wk of age, hens were injected intravenously with either 8 mg/kg body weight of LPS or saline. Four h after injection, blood was collected and the hens were euthanized to obtain spleen and cecal tonsils. Heterophil:lymphocyte (H:L) ratio, CD3+, CD4+, CD8+ T cells, and CD4+:CD8+ ratio in blood and spleen were not affected by dietary treatments. Relative to the saline-injected hens, LPS-injected hens had lower ($P < 0.05$) CD3+, CD4+, CD8+, and CD4+:CD8+ ratio, but no difference was found in the spleen. Serum total protein, albumin, and globulin were found to be higher ($P < 0.05$) in FA supplemented hens compared to the control. Total protein, albumin, and globulins decreased ($P < 0.05$) in LPS-injected hens compared to control. However, no significant effect was found on the level of fibrinogen among treatment groups. Expression of interleukin (IL)-1 β in cecal tonsils decreased ($P < 0.05$) in FA supplemented hens, however, no dietary influence was

found on the expression of toll-like receptor (TLR)-4, IL-8, IL-10, and interferon (IFN)- γ in cecal tonsils or TLR-4, IL-8, IL-10, and IFN- γ in the spleen. Expression of IL-10 and IFN- γ in the spleen and IL-1 β , IL-10, and IFN- γ in the cecal tonsils was higher ($P < 0.05$) in LPS-injected hens compared to the control, however, no effect was found on the expression of TLR-4 and IL-8 among the challenged groups in either the spleen or the cecal tonsils. Serum IgG, and cytokines IL-1 β and IL-18 in the spleen, and IL-18 in the cecal tonsils were influenced by a diet x LPS interaction. In conclusion, there were few interactions of dietary FA and LPS; however, FA increased biochemical constituents, enhanced generation of total IgG, as well as exhibiting pleiotropic effects in inflammatory responses.

3.2 INTRODUCTION

A functional immune system is a necessity for a healthy life in animal production; however, infectious diseases still represent a serious drain on the economics (reduced production, cost of therapeutics, and vaccines) and welfare of animal agriculture (Kogut and Klasing, 2009). The relationship between nutritional status and immunity has been well documented (Dhur et al., 1991a; Scrimshaw and SanGiovanni, 1997), and along with other measures, such as genetic selection for immune traits, improved management practices, and vaccinations, dietary immunomodulation is widely used to enhance health status (Klasing, 1998; Redmond et al., 2010). In poultry, nutritional status is a central factor that contributes to the capacity of birds to respond to pathogen challenge, by influencing both the innate and adaptive components of the immune system (Kidd, 2004).

Folic acid (FA), a water soluble vitamin has been demonstrated to be a nutrient

essential for immunity (Field et al., 2006; Troen et al., 2006). FA plays a key role in nucleic acid and protein synthesis; hence poor status significantly alters immune response. Deficiency of FA has been shown to affect immune competence and resistance to infections (Wintergerst et al., 2007). *In vitro* studies have associated FA deficiency with reduced ability of human T cells to divide following mitogen stimulation (Dhur et al., 1991b). This may suggest a decrease in the amount of circulating T lymphocytes due to a lack of cell proliferation. In addition, a number of clinical abnormalities including neonatal and cardiovascular disorders, elevated plasma homocysteine (hyperhomocysteinemia), macrocytic anaemia, osteoporosis, Alzheimer's disease, and carcinogenesis, have been linked to marginal levels of dietary FA (Dhur et al., 1991b; Morrisson et al., 1996; Boushey et al., 1996; Ames, 1999; Lucock, 2000; Mason and Choi, 2000; Choi and Mason, 2002;).

Lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria and a potent inflammatory mediator, has been used to model bacterial infection experimentally in chickens and in other species of farm animals (Yang et al., 2008). Immunological stress or challenge emanating from LPS or other stress factors affects the physiological and pathological processes of domestic animals and interferes with their normal functioning (Gehad et al., 2002a; Shini et al., 2008a; Huff et al., 2008). Administration of LPS has been shown to influence haematological, electrolyte, and serum biochemical values in turkeys and other animals (Simaraks et al., 2004; Huff et al., 2008). In addition, the level of natural antibodies thought to reflect the active status of innate humoral immunity, has been shown to be influenced by LPS in laying hens (Star et al., 2007). With respect to dietary supplementation, FA supplementation has been found

to influence the effects of LPS in laboratory animals as well as in humans. In mice, FA was found to inhibit the inflammatory response of the RAW 264.7 macrophage cell line to LPS, through inhibition of the mitogen-activated protein kinases (MAPK) and nuclear factor kappa B (NF- κ B) pathways (Feng et al., 2011). In patients with hyperhomocysteinemia, FA supplementation was found to reverse the hyper-responsiveness of LPS-induced chemokine; interleukin (IL)-8, and monocyte chemoattractant protein-1 (MCP-1) secretion from monocytes (Wang et al., 2005). This suggests that FA may exhibit anti-inflammatory properties important for the neutralization of the adverse effects of LPS.

Previous studies with laying hens have demonstrated that optimum deposition of folate in to the egg is attained when folic acid is supplemented at 4 mg/kg of diet (Herbert et al., 2005; Dickson et al., 2010). However, immunomodulatory effects of dietary FA supplementation at this level in young laying hens (24 to 32 wk of age) has not been established. One of the aims for dietary modulation of the immune system in domestic animals is to reduce disease susceptibility as well as to alleviate performance deficits following infection or immune stimulation (Klasing, 2007). The present study was designed to determine the potential for immunomodulation in young laying hens by dietary FA supplementation and systemic acute immune response after challenge with LPS.

3.3 MATERIALS AND METHODS

3.3.1 General

A total of 48 Shaver White laying hens (Manitoba Perfect Pullets, Winnipeg, Manitoba,

Canada) at 24 wk of age were used in this study. Hens were placed based on their performance (> 98 % egg laying) and housed in individual metabolic cage units (25.4 cm x 40.6 cm, providing 1,032 cm² space per bird) equipped with individual feeders, a nipple waterer, and a perch, and were exposed to 16 h of light. Water and feed were provided *ad libitum*. Animal usage and care approval was received from the University of Manitoba Animal Care Protocol Review Committee, and the hens were managed in accordance with the recommendations established by the Canadian Council on Animal Care (1984).

3.3.2 Experimental design

The hens were assigned to a completely randomized study design based on a 2 x 2 factorial arrangement of main factors. The main factors were: 1) diet; basal laying hen diet with no supplemental FA (n = 24) and basal laying hen diet with 4 mg/kg supplemental FA (n = 24), and 2) immunological challenge; injection with LPS or saline. The basal diet was a wheat-soybean based ration, formulated to meet the requirements of laying hens consuming 100 g of feed per day (NRC, 1994) (**Table 3. 1**). The basal diet contained 1.76 mg of total folate (from natural folate in feed ingredients) per kg of diet. The birds were maintained on the dietary treatments for 8 wk (the first 2 wk served as adaptation period), after which 6 hens were randomly selected from each dietary treatment and injected intravenously with either 8 mg/kg body weight of *Escherichia coli* LPS (Serotype 0111:B4, Sigma Aldrich Inc., St Louis MO, USA) or sterile saline. The LPS treatment was based on those of Gehad et al. (2002a) and Shini et al. (2008b). Feed was withdrawn after injection.

3.3.3 Blood and Tissue Sampling

Table 3.1. Composition of the basal wheat/soybean based laying hen diet.

Item	Composition
Ingredient %	
Wheat (13.5 % CP)	59.26
Soybean meal (45.8 % CP)	22.30
Vegetable oil (8,800 kcal/kg of ME)	5.22
Limestone (38% Ca)	9.90
Biophos (21/17)	1.61
Vitamin premix ¹	1.00
Mineral premix ²	0.50
DL- methionine	0.14
Lysine	0.06
Threonine	0.01
Total	100
Nutrient composition	
ME , kcal/kg	2850
CP, % (calculated)	18.5
CP, (analyzed)	18.77
Calcium, % (calculated)	4.2
Calcium, % (analyzed)	4.31
Available P, % (calculated)	0.45
Total P, % (analyzed)	0.65
Methionine, % (calculated)	0.43
Meth+cysteine, % (calculated)	0.80
Lysine, % (calculated)	0.95
Threonine, % (calculated)	0.70
Linoleic, % (calculated)	3.23
Folate, mg/ kg (analyzed)	1.76

¹Provided per kilogram of diet: 11,000 IU of vitamin A, 3,000 IU of vitamin D3, 20 IU of vitamin E, 3 mg of vitamin K3 (as menadione), 0.02 mg of vitamin B12, 6.5 mg of riboflavin, 10 mg of calcium pantothenate, 40.1 mg of niacin, 0.2 mg of biotin, 2.2 mg of thiamine, 4.5 mg of pyridoxine, 1,000 mg of choline, and 125 mg of ethoxyquin (antioxidant).

²Provided per kilogram of diet: 66 mg of Mn (as manganese oxide), 70 mg of Zn (as zinc oxide), 80 mg of Fe (as ferrous sulfate), 10 mg of Cu (as copper sulfate), 0.3 mg of Na (as sodium selenite), 0.4 mg of I (as calcium iodate), and 0.67 mg of iodized salt.

Four h after LPS or saline injection, blood samples were collected from the wing vein of each hen. Blood samples were divided into three aliquots (2-3 mL each); one 4 mL serum Vacutainer tube and two 4 mL Vacutainer tubes coated with K₂EDTA (BD Bio Sciences, Franklin Lakes, NJ, USA). The blood samples contained in K₂EDTA tubes were kept on ice during collection while the blood samples for serum were clotted at room temperature for approximately 2 h. Blood samples were then centrifuged at 12,000 x g for 5 min, and plasma and serum were obtained and stored at -80 °C until analysis. One K₂EDTA tube containing blood was retained for flow cytometry analysis. After the blood collection, birds were euthanized by cervical dislocation and the spleens were aseptically obtained and cut into 2 pieces. Cecal tonsils were also aseptically collected and together with one half of the spleen were immediately snap frozen in liquid nitrogen for 2 min and stored at -80 °C until analysis. One half of the spleen was kept on ice in 1 x PBS until extraction of splenocytes for flow cytometry analysis.

3.3.4 Determination of Heterophil:Lymphocyte ratio

The heterophil:lymphocyte (H:L) ratio was measured 4 h after LPS or saline injection using blood collected from the wing vein. Ratios were determined by preparing blood smears using glass slides stained according to Wright's method (Campbell, 1995). A total of 100 white blood cells were counted using oil immersion and light microscopy at 100 x magnification.

3.3.5 Flowcytometry Analysis

The percentages of CD3+, CD4+, and CD8+ cells in peripheral blood mononuclear cells (PBMC) and in the spleen were isolated using Ficoll-PaqueTM Plus (GE Health care, Quebec, Canada) following the manufacturer's procedure and prepared

as described previously (Gehad et al., 2002b; Shini and Kaiser, 2009) with minor modifications. Briefly, the blood from K₂EDTA vacutainer tubes was diluted 1:1 with 1 x PBS and held on ice. Each single suspension of splenocytes was prepared in 5 mL 1 x PBS by mashing the tissue using the end of a syringe plunger through a 100 µm nylon strainer (BD, Biosciences, USA). The blood and spleen suspensions were carefully layered into centrifuge tubes containing an equal volume of Ficoll to form a discrete layer above the Ficoll. The tubes were centrifuged at 220 x g for 30 min at room temperature, and the mononuclear layers (buffy coat) were removed, transferred to different centrifuge tubes, and washed twice in 1 x PBS. Immediately, the cells were counted on a haemocytometer using trypan blue exclusion assay (Sigma Chemical CO., St. Louis, MO) and the cell suspensions were adjusted to 1 x 10⁶ viable cells per mL of 1 x PBS.

Fluorescence monoclonal antibodies were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL), including mouse anti-chicken CD3-FITC, mouse anti-chicken CD4-PE, and mouse anti-chicken CD8-PE-CY5. Using 96-well round bottom plates, 100 µL of each cell suspension was plated in duplicate. Each of the fluorochrome labelled antibodies was added to their respective wells and the stained cells were incubated for 30 min at 4 °C in the dark. The cells were washed twice in 1 x PBS to remove any traces of unbound antibodies and transferred to 5 ml polystyrene round-bottom tubes for analysis. A total of 10,000 cells per sample were analysed using BD FACS Diva Software (Becton Dickinson Immunocytometry Systems, San Jose, CA) and the cells were evaluated using Flowjo Software (v.1.1.1, CyFlo Ltd., Turku, Finland).

3.3.6 Total IgG Determination

Serum samples obtained from the blood were used to measure the concentrations of total IgG via sandwich ELISA using chicken IgG ELISA quantitation and Starter Accessory Kits (Bethyl Laboratories, Montgomery, TX) following the manufacturer's procedure. A microtiter plate reader (Soft Max Pro 3.1.1) was used to measure the absorbance at 450 nm and a 4-parameter logistic curve fit was developed using the chicken reference serum absorbance.

3.3.7 Clinical biochemistry Analysis

Serum total protein, albumin, globulin, and plasma fibrinogen were determined using an automated analyzer (Cell-Dyn 3500 System, Abbot Laboratories, Abott Park, IL) at the Manitoba Veterinary Services Laboratory (Winnipeg, Manitoba, Canada).

3.3.8 Total RNA extraction and Reverse Transcription

Total RNA was extracted from spleen and cecal tonsil sections using Trizol Reagent (Invitrogen Canada Inc. Burlington, Ontario, Canada) following the manufacturer's protocol. Briefly, the ileal and cecal samples were removed from -80 °C storage and they were kept on ice until completely thawed. About 80 to 100 g of tissue was added to 1 mL of ice cold Trizol reagent (Invitrogen) and homogenized with a homogenizer at full speed for about 1 min. After extraction, the pellet was left to dry for 5 min and was dissolved in 300 µL of nuclease free water. Total RNA concentration was determined at an optical density at 260 nm and RNA purity was verified by evaluating the ratio of optical density of 260 nm to optical density at 280 nm. Total RNA was diluted to 2 µg/µL in nuclease-free water. Reverse transcription was done using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Mississauga, ON, Canada) following the manufacturers protocol, and the cDNA was stored at -20 °C.

3.3.9 *Quantitative Real-Time PCR (qRT-PCR)*

Quantitative Real-Time PCR was performed using the Step One Thermo Cycler (Applied Biosystem, Mississauga, ON, Canada). 2 μ L of cDNA was added to each well of a 48-well plate. Next, 23 μ L of real-time PCR master mix containing AmpliTag Gold DNA Polymerase LD SYBR Green I, dNTPs with dTTP/dUTP, (Applied Biosystems, Mississauga, ON, Canada) and nuclease free water were added to each well to a final volume of 25 μ L as outlined by Parvizi et al. (2009). Primer sequences for β -actin, toll-like receptor (TLR)-4, interleukin (IL)-8, IL-10, IL-1 β , IL-18, and interferon gamma (IFN- γ) were obtained from gene bank (**Table 3. 2**).

Primer concentrations were optimized depending on a standard curve using a gene target or endogenous gene previously cloned as a control for PCR efficiency, and different thermal cycling parameters were used for each target gene. Each reaction was run in duplicate using Step One Software (Applied Biosystems, Mississauga, ON, Canada).

3.3.10 *Quantitative Real-Time PCR Analysis*

Relative expression was calculated using Pfaffl's formula (Pfaffl, 2001) as described previously (Parvizi et al., 2009). Briefly, relative expression of all genes was calculated based on the expression of the housekeeping gene, β -actin. Absolute quantification of β -actin expression was estimated using the Step One Software (Applied Biosystems, Mississauga, ON, Canada). The absolute expression of all genes tested was then normalized to the express PCR efficiency: $E = 10^{-1/\text{slope of standard curve}}$ with the gene of

Table 3. 2. Toll-like receptors and cytokines primer sequences. The listed oligonucleotides were used to analyze gene expression via quantitative real-time polymerase chain reaction.

Gene	Primer sequence (5'-3')	Gene bank access	Annealing Temp, °C
chTLR-4	F: AGTCTGAAATTGCTGAGCTCAAAT R: GCGACGTTAAGCCATGGAAG	AY064697	60
chIL-1 β	F: GTGAGGCTAACATTGCGCTGTA R: TGTCCAGGCGGTAGAAAGATGAAG	Y15006.1	57
chIL-8	F: CCAAGCACACCTCTCTTCCA R: GCAAGGTAGGACGCTGGTAA	AJ009800	57
chIL-10	F: AGCAGATCAAGGAGACGTTC R: ATCAGCAGGTA C T C C T C G A T	AJ621614	55
chIL-18	F:GAAACGTCAATAGCCAGTTGC R:TCCCATGCTCTTTCTCACAACA	NM204608.1	53
chIFN- γ	F: CTGAAGAACTGGACAGAGAG R: CACCAGCTTCTGTAAGATGC	X99774	60
ch β -actin	F: CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	X00182	61

interest as the target and β -actin as the reference, the relative expression ratio (R) was determined as follows:

$$R = \frac{(E_{\text{target}})\Delta\text{CP}_{\text{target}}(\text{calibrator-sample})}{(E_{\text{ref}})\Delta\text{CP}_{\text{ref}}(\text{calibrator-sample})}$$

Where E_{target} and E_{ref} are the efficiencies of the target gene and β -actin, respectively, and the ΔCP is the difference of crossing points between calibrator and samples. The calculated R were used to determine differences in gene expression among different groups.

3.3.11 Statistical Analysis

A completely randomized design with 2 dietary treatments and 2 levels of immunological challenge in a 2 x 2 factorial arrangement was used. To test for the effects of each treatment combination, values were subjected to ANOVA using the PROC GLM procedure of SAS software (SAS institute, Cary, NC). Differences between means were determined using Tukey's procedure. Differences with an α level of $P < 0.05$ were considered to be statistically significant.

3.4 RESULTS

3.4.1 Heterophil:Lymphocyte Ratio

No significant difference was found in the H:L ratio in the FA supplemented hens compared to the unsupplemented hens. However, the LPS group showed a greater ($P < 0.05$) H:L ratio compared to the saline group (**Figure 3. 1**).

3.4.2 Flowcytometric Analysis

Percentages of CD3+, CD4+, and CD8+ cells in the blood and the spleen were not

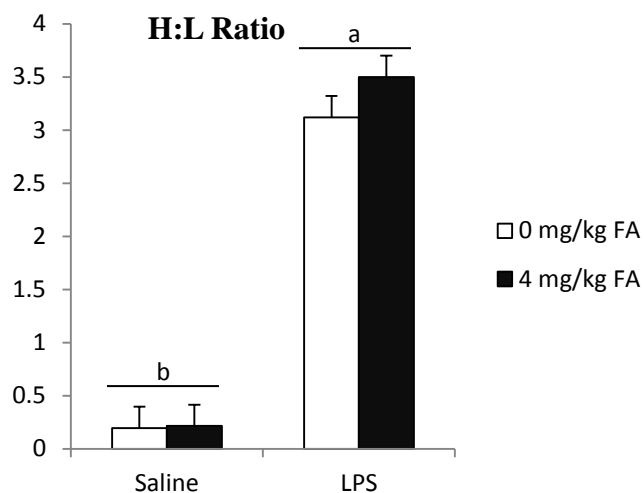


Figure 3.1. Heterophil:lymphocyte (H:L) ratio in young laying hens (24 to 32 wk of age) fed diets with or without supplemental dietary folic acid (FA) for 8 wk and at 4 h post-injection with 8 mg/ kg of body weight LPS or saline. Bar graphs represent LSmeans. Error bars represent standard error of the mean. Different superscripts (a, b) shows significant ($P < 0.05$) difference for the main effect of LPS. FA= folic acid. LPS= lipopolysaccharide.

influenced by dietary FA supplementation compared to the controls (**Table 3.3**). However, relative to saline injected hens, injection with LPS significantly reduced ($P < 0.05$) the level of CD3+, CD4+, and CD8+ T cells in the blood. No significant difference was found in the percentages of CD3+, CD4+, and CD8+ T cells in the spleen among the challenged groups (**Table 3.3**).

3.4.3 Total IgG

The level of serum IgG was influenced by a diet x challenge interaction. Serum IgG was found to be higher in FA supplemented hens that were injected with LPS compared to the control hens (**Figure 3.2**).

3.4.4. Clinical biochemistry

Total proteins, albumin, and globulin were found to be higher ($P < 0.05$) in FA supplemented hens compared to unsupplemented hens, however, no significant difference was found on the albumin:globulin ratio among the dietary treatment groups. On the other hand, injection with LPS reduced ($P < 0.05$) the levels of total proteins, albumin, globulin, and albumin:globulin ratio compared to the hens injected with saline solution. The amount of plasma fibrinogen was not influenced by either dietary supplementation or the immunological challenge (**Table 3. 4**).

3.4.5 Gene Expression in the spleen and the cecal tonsils

No effect of dietary FA was found on the expression of TLR-4 in the spleen and cecal tonsils among the treatment groups. Similarly, expression of cytokines IL-8, IL-10, and IFN- γ was not influenced by dietary FA supplementation in the spleen and cecal tonsils. However, the expression of cytokine IL-1 β in the cecal tonsils was found to be

Table 3. 3. T cell subsets in the peripheral blood mononuclear cells (PBMCs) and spleen (%) of young laying hens (24 to 32 wk) fed diets with or without supplemental dietary folic acid (FA) for 8 wk and at 4 h post- injection with 8 mg/ kg of body weight LPS or saline

Item	0 mg Folic Acid		4 mg Folic Acid		SEM	P Values		
	Saline	LPS	Saline	LPS		Diet	Challenge	Interaction
PBMCs								
CD3+	8.33	2.48	7.44	2.86	0.5	0.7369	<.0001	0.4137
CD4+	5.53	1.55	4.77	1.69	0.3	0.5759	<.0001	0.4094
CD8+	2.80	0.93	2.66	1.16	0.2	0.9056	<.001	0.6360
CD4+/CD8+	2.31	1.98	2.1	1.65	0.3	0.5202	0.3651	0.8855
Spleen								
CD3+	34.18	36.83	32.91	34.91	4.1	0.7846	0.6904	0.9559
CD4+	14.85	13.07	11.93	13.13	1.2	0.4190	0.8687	0.3975
CD8+	19.33	23.76	20.97	21.78	3.1	0.9699	0.5680	0.6914
CD4+/CD8+	0.77	0.67	0.59	0.65	0.04	0.1151	0.7340	0.2141

Data are presented as least squares means.

LPS = lipopolysaccharide

PBMC = peripheral blood mononuclear cells

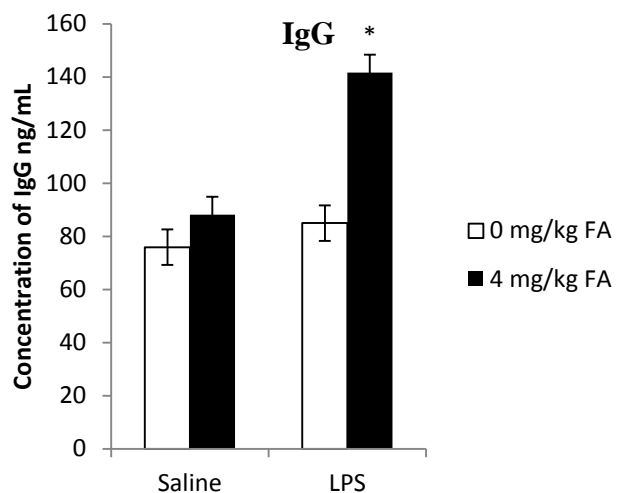


Figure 3.2. Serum IgG in young laying hens (24 to 32 wk of age) fed diets with or without dietary folic acid (FA) for 8 wk and at 4 h post- injection with 8 mg/kg of body weight LPS or saline. Bar graphs represent LSmeans. Error bars represent standard error of the mean. Bar with an asterisk show a significant ($P < 0.05$) FA x LPS interaction. LPS= lipopolysaccharide. FA= folic acid.

Table 3. 4. Clinical biochemistry constituents of young laying hens (24 to 32 wk) fed diets with or without supplemental dietary folic acid (FA) for 8 wk and at 4 h post- injection with 8 mg/ kg of body weight LPS or saline

Item	0 mg Folic Acid		4 mg Folic Acid		SEM	P Values		
	Saline	LPS	Saline	LPS		Diet	Challenge	Interaction
Total protein (g/L)	39	31	46	35	1.5	0.0276	0.0018	0.4520
Albumin (g/L)	14	9.6	18	11	0.7	0.0194	0.0003	0.2643
Globulin (g/L)	24	21	28	23	1.1	0.0505	0.0168	0.7273
Albumin:Globulin	0.58	0.46	0.65	0.48	0.02	0.1169	0.0003	0.3311
Fibrinogen (g/L)	4.8	3.7	4.4	3.7	0.6	0.8212	0.2986	0.8212

Data are presented as least squares means.

LPS = lipopolysaccharide

lower ($P < 0.05$) in the FA supplemented hens compared to the unsupplemented hens (**Figure 3.3**).

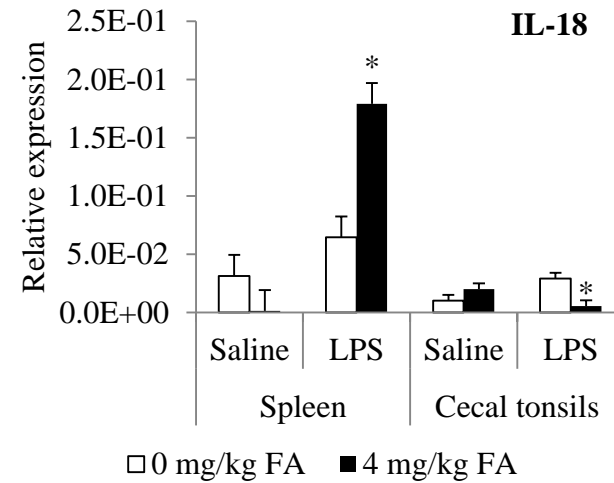
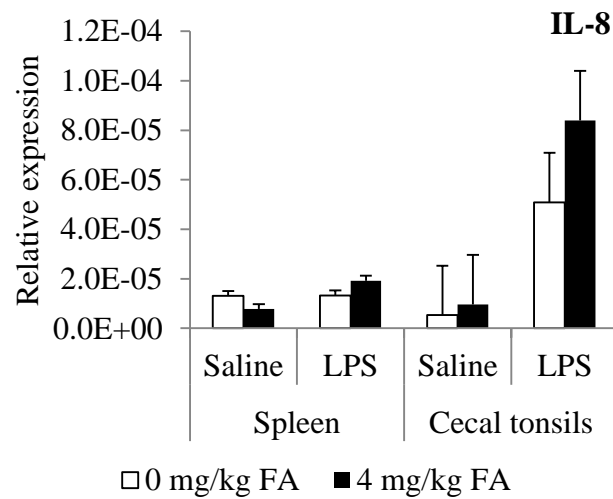
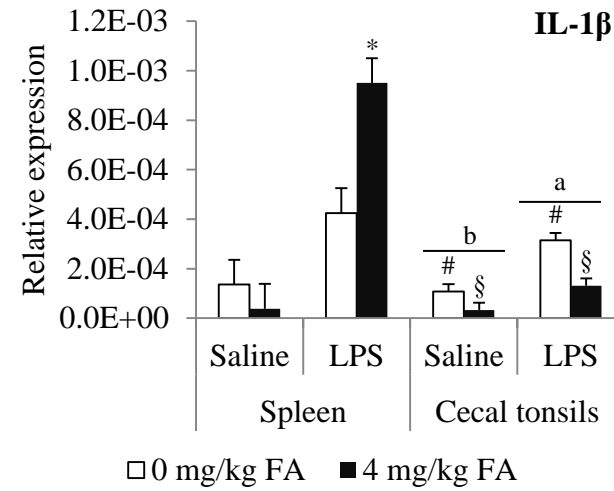
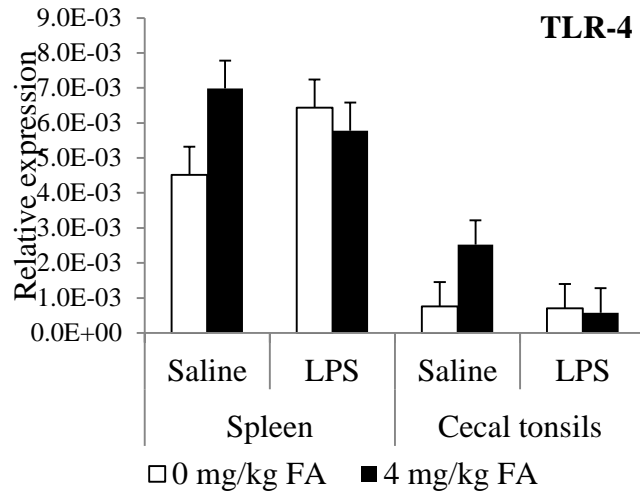
Infusion with LPS influenced the expression of cytokines in the spleen and cecal tonsils. Expression of IL-10 and IFN- γ in the spleen, and IL-1 β , IL-10, and IFN- γ in cecal tonsils was higher ($P < 0.05$) in LPS-injected hens compared to saline-injected hens. No significant difference was found in the expression of TLR-4 or IL-8 in the spleen and cecal tonsils among the challenged hens (**Figure 3.3**).

Diet x challenge interaction influenced the expression of cytokines IL-1 β and IL-18 in the spleen, and IL-18 in cecal tonsils. Expression of IL-1 β and IL-18 in the spleen was higher in FA supplemented hens that were injected with LPS, while expression of IL-18 in the cecal tonsils was lower in FA supplemented hens that were injected with LPS (**Figure 3.3**).

3.5 DISCUSSION

The present study was conducted to investigate the effects of dietary FA supplementation on the immune response in young laying hens, under systemic acute *Escherichia coli* (LPS) challenge. Previous studies have shown that optimum deposition of folate in eggs is achieved at 4 mg FA supplementation per kg of diet (House et al., 2002; Hebert et al., 2005; Dickson et al., 2010), and therefore in this study, we aimed to determine whether the same level could be optimal for immunocompetence and disease resistance in young laying hens.

Dietary supplementation with FA did not influence the H:L ratio. This indicates that FA did not alter the levels of heterophils and lymphocytes in circulation or their



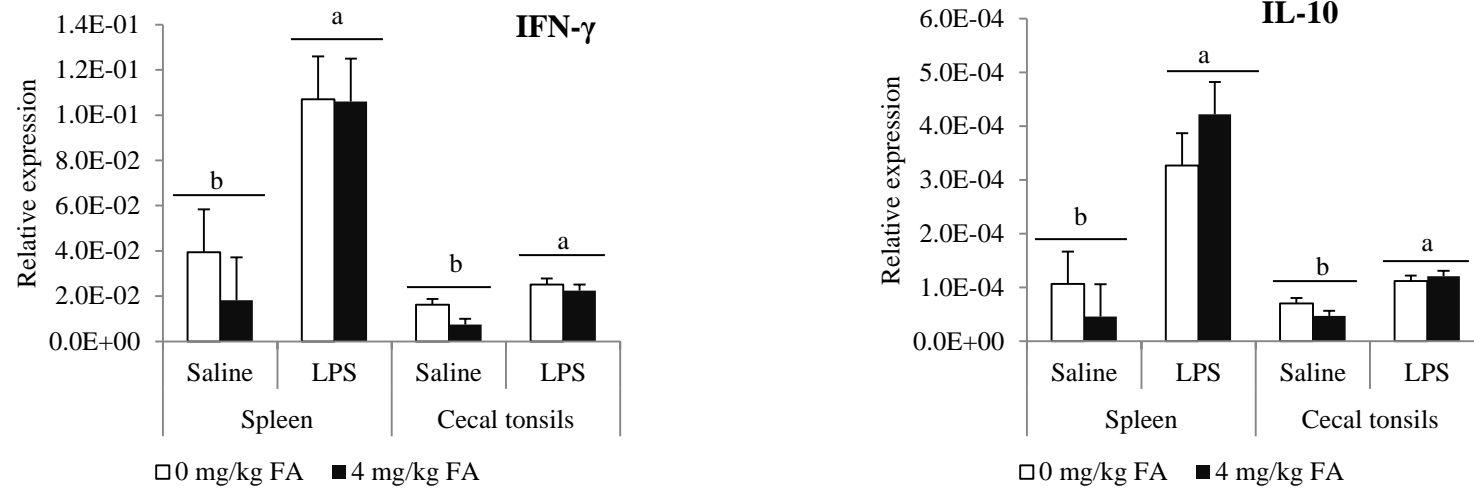


Figure 3. 3. Gene expression in the spleens and cecal tonsils of young laying hens (24 to 32 wk of age) fed diets with or without supplemental dietary folic acid (FA) for 8 wk and at 4 h post- injection with 8 mg/ kg of body weight LPS or saline. Gene expression was assessed using quantitative RT-PCR, and was calculated relative to the house keeping gene β -actin. Graphed values are expressed as relative expression ratios. Error bars represent standard error. Bars with asterisk show significant ($P < 0.05$) FA x LPS interactions, superscripts a,b, show significant main effects of LPS, and superscripts §,#, show significant main effect of FA. FA= folic acid. LPS = lipopolysaccharide.

distribution, and hence FA did not affect innate immune responses mediated by these cells. In addition, since H:L is used as an indicator of stress in birds, FA may not be a favourable way for helping laying hens to cope up with short-term stress. Exposure to *E. coli* and its lipopolysaccharide endotoxin with respect to elevated levels of H:L ratio and stress have been well documented (Gross and Siegel, 1983; Shini et al., 2008b). This was confirmed in our study where LPS injection caused a dramatic increase in the H:L ratio within 4 h. In chickens, heterophils are key components of the innate immune system and serve in the same role as mammalian neutrophils (Redmond et al., 2009). Increased number of heterophils could indicate stimulation of innate immune responses that are important for clearance of pathogens through phagocytosis and anti-microbial mechanisms, which involve the generation of reactive oxygen and nitrogen species, proteolytic enzymes and anti-microbial peptides as well as the recruitment of other cells of the immune system (Pae et al., 2011).

In this study, the percentages of CD3+, CD4+, and CD8+ T cells were not influenced by dietary FA supplementation in the blood. Previous studies in humans demonstrated the ability of FA to enhance T cell proliferation *in vitro* (Wintergerst et al., 2007). This was not observed in our study and the discrepancies could be due to the fact that, in the current study we examined the effects of FA supplementation *in vivo* while in the previous studies the experiments were conducted *in vitro*. In support of this, folate deficiency was also shown to reduce the proportion of T cells in circulation in rats and guinea pigs (Dhur et al., 1991b), and the authors indicated that folate correction of these conditions was faster *in vitro* as opposed to *in vivo* conditions. This suggests that

circulating T cells could be slow in incorporating FA *in vivo* and a longer period of supplementation may be more beneficial. Alternatively, the differences in species used could be a contributing factor. The lack of dietary FA effect on the T cell subsets in the spleen observed in this study is in agreement with the report of Field et al. (2006) who found no difference in young and old mice that were supplemented with folate compared to the unsupplemented mice.

The presence of LPS, a T-independent antigen reduced the percentages of the T-cell subsets (CD3+, CD4+, CD8+) in the blood. Similar results were reported by Gehad et al. (2002b) in young male chickens. LPS could have induced cell death in these cells leading to their massive reduction in circulation. Shini et al. (2008b) examined lymphocytes from LPS-treated chicken and reported destruction of ultra-morphological features 1 h and 3 h after treatment, and also stipulated that the cells experienced both induced apoptosis and necrosis. Alternatively, migration of T-lymphocytes to secondary lymphoid organs or other immune-related tissues could be a possible cause. Nii et al. (2011) reported increased frequency of CD4+ and CD8+ cells in to the lamina propria of both uterus and vagina in laying hens 3 h and 6 h after LPS injection.

No significant difference was observed in the percentages of CD3+, CD4+, and CD8+ cells in the spleen after LPS challenge. Similarly, Gehad et al. (2002b) found no differences in CD4+ and CD8+ T cells in the spleen in young male chickens. In our study, the 4 h period between LPS injection and sampling of splenic tissue may have caused a transient effect that was not detected.

Total IgG was found to be greater in hens that were supplemented with FA and injected with LPS suggesting that FA had beneficial effects on the level of IgG in hens

that were challenged with LPS, possibly through enhancement of the level of B type of lymphocytes that produce natural antibodies (Klasing, 2007; Ochsenbein et al., 1999, 2000). This also suggest the presence of natural (innate) antibodies specific to LPS which may be part of the active form of innate humoral immunity and may also act as a link to specific immune responses (Parmentier et al., 2004; Star et al., 2007). This is supported by the fact that, the hens used in this study were never vaccinated against LPS before, and the period of LPS exposure in our study was too short to initiate the development of specific immune responses. However, the hens might have come across environmental LPS at some point which again supports the presence of an active form of humoral innate immunity (Star et al., 2007). In addition, although not part of the scope of this study, deposition of maternal antibodies in the egg yolk could be enhanced. Furthermore, generation of specific antibodies against LPS of gram negative bacteria that can be administered to other farm animals (Sasse and Hlinak, 1998; Owusu-Asiedu et al., 2003; Dera-Tomaszewska et al., 2003), and humans (Amaral et al., 2002) may also be enhanced and help to boost immunity.

The biochemical role of folate in protein synthesis, repair, and DNA methylation is well documented (Selhub et al., 1996). In this study, an increased level of total protein, albumin, and globulins was found in FA supplemented hens. In accordance to our findings, greater levels of total proteins, albumin, and globulin were reported in rabbits and Japanese quails following dietary FA supplementation (Gursu et al., 2004; El-Demerdash et al., 2006). This may suggest a role of FA in supporting the levels of total proteins, globulins and albumin in young laying hens, important for good health and performance, since these constituents act as substrates for immune cells as well as

participating in the synthesis of acute phase proteins in the liver during inflammatory responses (Klasing, 1998, 2004, 2007; Kogut and Klasing, 2009).

Total proteins, albumin, globulin, and the albumin:globulin ratio were reduced following injection with LPS. In accordance to our findings, Hritcu et al. (2009) studied the effects of acute LPS administration in normal rats and reported a decrease in total protein. Nutritional costs of use particularly proteins during acute phase response has been reported to be higher in chicks and other species of animals, a condition that is coupled with an increased demand for these nutrients (Klasing, 2004). The decrease could therefore be due to loss of these proteins through synthesis of acute phase proteins in response to the acute effects of LPS. This could be correlated to a decrease in body weight that has been reported previously following LPS exposure, however, this was found to be strain and time-dependent (Cheng et al., 2004).

The level of plasma fibrinogen was not influenced by either dietary FA supplementation or LPS challenge. In addition to the role of fibrinogen in blood coagulation mechanism, fibrinogen is also classified as one of the acute phase proteins which are increased non-specifically in acute inflammatory disorders (Kaneko, 1980), and may be useful for haematological screening procedure for diagnosis and monitoring of bacterial infection and other inflammatory diseases (Schalm et al., 1975). A previous study reported a degree of hyperfibrinogenaemia in avian species with bacterial infections (Hawkey and Hart, 1988). However, the reasons why fibrinogen was not affected by LPS in the blood in our study, even though serum biochemical constituents were influenced, remains to be explained.

Expression of toll-like receptor (TLR)-4 mRNA in different tissues and immune cell subsets and cell lines has been reported in chickens (Iqbal et al., 2005). TLR-4 has also been shown to recognize lipopolysaccharides (LPS) of gram negative bacteria such as *E. coli* and *Salmonella* in laying hens (Subedi et al., 2007), however, the authors reported that the expression of TLRs in response to LPS in the ovarian follicles, liver, and kidney in laying hens was tissue- and time-dependent or their sensitivity to LPS may differ. Investigating the expression of TLR-4 in young laying hens under chronic conditions of LPS challenge, and at different end points could therefore enhance understanding in this topic.

Different mammalian species react more, or less, intensely to the effects of LPS (Tufveson and Alm, 1975). Chickens are relatively resistant to systemic administration of LPS and the nature of this resistance is unclear (Adler and DaMassa, 1979; Keestra and Van Putten, 2008). However, lipopolysaccharide binding protein (LBP), a soluble acute-phase protein that binds to bacterial LPS to elicit immune responses by presenting the LPS to important co-receptor CD14+ and cell surface pattern recognition receptor TLR-4, has been shown to be lacking in the chicken genome (Chiang et al., 2011). This may partly explain why chickens are said to be generally resistant to LPS exposure. However, LPS was found to bind to TLR-4 and induce the functional activation of chicken heterophils in the absence of LBP (Kogut et al., 2005a, b), and addition of chicken serum presumed to be the source of LBP increased heterophil degranulation response (Kogut et al., 2005a, b). More research is therefore warranted to further clarify the possible presence and functioning of LBP in chickens.

IL-18 and IL-1 β are related pro-inflammatory cytokines critical for the initiation of inflammatory responses (Kogut et al., 2005a, b). IL-18 is mainly produced by macrophages and stimulates the production of IFN- γ by NK cells and T- lymphocytes and thus induces cell mediated immunity while an increased level of IL-1 β stimulates infiltration of T cells and macrophages (Corwin, 2000). In this study, supplementation with FA down regulated the expression of IL-1 β in the cecal tonsils, and IL-18 was found to be lower in FA supplemented hens that were challenged with LPS compared to control hens. This may partly explain the beneficial anti-inflammatory effects of FA in young laying hens exposed to acute LPS challenge, a condition that has been previously reported in other species of animals under LPS challenge (Wang et al., 2005; Solini et al., 2006; Chiarello et al., 2009; Feng et al., 2011). However, the expression of IL-18 and IL-1 β in the spleen was found to be higher in hens that were supplemented with FA and injected with LPS compared to the other group of hens. This may suggest that dietary FA could exhibit both pro- and anti-inflammatory effects in young laying hens under acute conditions of LPS exposure which could be cytokine- and tissue- dependent.

IL-10 is a Th2 cytokine that serves to keep the immune responses in check by inhibiting cytokine production by Th1 cells, and down regulating the expression of the major histocompatibility antigens expressed by immune cells (Corwin, 2000; Min et al., 2001). This down regulation reduces the likelihood of an immune response to an antigen and serves to control inflammatory responses once the pathogen has been cleared. The upregulation of IL-10 in both the spleen and the cecal tonsils following LPS challenge suggest anti-inflammatory responses geared towards reduction of the effects of pro-inflammatory cytokines. On the other hand, the upregulation of cytokines IFN- γ , IL-1 β ,

and IL-18 in the spleen and cecal tonsils is an indicator of inflammatory responses. This therefore, shows that the balance between Th1 (IFN- γ) and Th2 (IL-10) inflammatory responses in response to LPS was maintained. However, since we only examined the effect of LPS at one end point, we might have missed an up regulation or down regulation of some cytokines earlier, before 4 h, or later, after the 4 h.

In summary, dietary FA supplementation may modulate immune responses in young laying hens; enhancing the level of proteins necessary for acute phase immune response in response to bacterial LPS infections, as well as the generation of natural antibodies such as IgG. Dietary FA may also convey pleiotropic effects, enhancing and repressing inflammatory cytokines, suggesting exhibition of both pro- and anti-inflammatory effects in young laying hens which could be tissue- and cytokine-dependent. However, dietary FA did not alter the level of T lymphocytes hence the effects of dietary FA on immune responses in young laying hens remains to be explored further in larger scales of production and under both acute and chronic conditions of LPS exposure.

CHAPTER FOUR

4.0 MANUSCRIPT II

Response of old laying hens to an *Escherichia coli* lipopolysaccharide challenge when fed diets with or without supplemental folic acid

4.1 ABSTRACT

Ageing is frequently claimed to result in an age-dependent deterioration in immune function, however, nutritional intervention may attenuate age-associated decline in immunocompetence. To this end, a study was conducted to investigate the effects of dietary folic acid (FA) supplementation in old (58 to 66 wk of age) laying hens challenged with *Escherichia coli* lipopolysaccharide (LPS). Forty eight Shaver White laying hens at 58 wk were fed 2 diets. The diets were wheat-soybean based, with either 0 or 4 mg supplemental FA per kg of diet. After 8 wk of feeding and at 66 wk, the hens were injected intravenously with either 8 mg/kg body weight of LPS or saline. Four h after injection, blood was collected and hens were euthanized to obtain spleen and cecal tonsils. T cell subsets in the blood and the spleen (CD4+, and CD8+), total IgG, and biochemical constituents (total protein, albumin, globulin, fibrinogen) were not influenced ($P > 0.05$) by dietary FA supplementation. However, LPS injection decreased ($P < 0.05$) biochemical constituents, CD4+, and CD8+ cells in the blood, whereas CD4+:CD8+ ratio and total IgG were increased ($P < 0.05$). Fibrinogen was not influenced among treatment groups ($P > 0.05$). Gene expression in the spleen and cecal tonsils was not influenced by dietary FA supplementation except the expression of IL-8. IL-8 was

affected by a diet x challenge interaction only in the spleen, whereas FA supplementation decreased the expression of IL-8 in cecal tonsils. Relative to saline-injected hens, expression of IL-1 β , IFN- γ , and IL-10, increased in the LPS-injected hens in the spleen and cecal tonsils, IL-8 increased in LPS-injected hens in the cecal tonsils only, while IL-4, IL-17, IL-18, and TLR-4 increased in the LPS-injected hens only in the spleen, however, LPS decreased expression of IL-13 in the cecal tonsils. In conclusion, FA did not influence immune responses in old laying hens but the hens were still able to mount immune responses following LPS challenge, showing no evidence of reduced immunocompetence.

4.2 INTRODUCTION

The ageing of the immune system is associated with dramatic reductions in immune responsiveness including impairment of physiological regulatory mechanisms (Burns, 2004). This in turn leads to reduction in both innate and adaptive immune functions, increased incidences of diseases, and autoimmunity (Hale et al., 2002). Remarkable changes with age have been reported in specific immune responses including the decline of T cell function for both CD4⁺ and CD8⁺ T cells, which has been shown to be preceded by thymus gland involution and decreased levels of thymic hormone (Castle, 2000a, b; Haynes et al 2004; Effros, 2004). B cell function also suffers considerable changes with age, which may be in part related to age-related changes in CD4⁺ cells, required for the activation of B cells, and could also be due to functional defects intrinsic to B cells (Burns, 2004; Linton and Dorshkind, 2004).

Similar to mammals, the immune system of birds is controlled by genes and the

interactions of genes with their environments (Cheng et al., 2001). With respect to immune changes, previous studies have demonstrated that in birds as reported in humans and other animals, cell-mediated immunity declines with age as indicated by a decrease in CD4⁺ cells (Fahey and Cheng, 2008). Moreover, age-associated decline in cell-mediated immunity has been associated with the great susceptibility of laying hens to *Salmonella* Enteritidis infection during induced molting (Holt, 2003).

In addition to the documented role of folate in biochemical reactions (Selhub and Rosenberg, 1996), dietary FA supplementation has been shown to be essential for immunity and is associated with improved immunocompetence in ageing subjects (Field et al., 2006; Troen et al., 2006). In rats, folate supplementation was found to prevent the age-associated decline in production of IFN- γ in the spleen by unstimulated cells, and the decrease in Th1/Th2- type responses after stimulation with phorbol myristate acetate and ionomycin (Field et Al., 2006). FA supplementation in elderly individuals has also been found to improve immune functions by altering the age-related decline in NK cell cytotoxicity supporting a Th1 response, and hence potentiating the ability of the immune system to respond (Bunout et al., 2004; Troen et al., 2006). However, differences in immunological responses in different tissues in response to nutritional supplementation at advanced age have also been reported (Field et al., 2006). Interestingly, in apparently healthy elderly subjects carefully selected for the absence of protein-energy malnutrition and who did not have a FA deficit, dietary FA supplementation enhanced lymphocyte proliferation and IL-2 release (Lesourd, 2004, 2006). This suggests that the immune system of the elderly is highly vulnerable to micronutrient deficiencies such as FA and may be enhanced by dietary supplementation even in absence of detectable deficiency.

Studies in laying hens on the influence of dietary FA supplementation in immunological responses at an advanced age and under challenged conditions have not been reported. This study was therefore designed to investigate the effects of dietary FA on the immune response parameters in old laying hens (58 to 66 wk of age) subjected to acute LPS challenge. T lymphocyte subsets in the blood and the spleen, biochemical constituents, total IgG, and immune-related genes were measured.

4.3 MATERIALS AND METHODS

4.3.1 General

48 Shaver White laying hens (Manitoba Perfect Pullets, Winnipeg, Manitoba, Canada) at 58 to 66 wk old were used in this study. Hens were placed based on their performance ($\geq 95\%$ egg laying) and were housed in a confined area under semi-controlled environmental conditions in individual cage units (25.4 cm x 40.6 cm to provide 1,032 cm² space per bird) equipped with individual feeders, a nipple waterer, and a perch and were exposed to 16 h of light. Water and feed were provided *ad libitum*. Animal usage and care approval was received from the University of Manitoba Animal Care Protocol Review Committee, and the hens were managed in accordance with the recommendations established by the Canadian Council on Animal Care (1984).

4.3.2. Experimental Design

The hens were assigned to a study design based on a 2 x 2 factorial arrangement of main factors. The main factors were: 1) diet; basal laying hen diet with no supplemental FA (n = 24) and basal laying hen diet + 4 mg supplemental FA/kg of diet (n = 24), and 2) immunological challenge; injection with LPS or saline. The basal diet was a

wheat-soybean based ration, formulated to meet the requirements of laying hens consuming 100 g of feed per day (NRC, 1994) (**Table 4. 1**). The basal diet contained 1.76 mg of total folate (from natural folate in feed ingredients) per kg of diet. The birds were maintained on the dietary treatments for 8 wk (the first 2 wk served as adaptation period) after which, 6 hens were randomly selected from each dietary treatment and injected intravenously with either 8 mg/kg body weight of *Escherichia coli* LPS (Serotype 0111:B4, Sigma Aldrich Inc., St Louis MO, USA) or sterile saline. Feed was withdrawn after injection.

4.3.3 Blood and Tissue Sampling

4 h after LPS or saline injection, blood samples were collected from the wing vein of each hen. Blood samples were divided into three aliquots (2-3 mL each); one 4 mL serum Vacutainer tube and two 4 mL Vacutainer tubes coated with K₂EDTA (BD Bio Sciences, Franklin Lakes, NJ, USA). The blood samples contained in K₂EDTA tubes were kept on ice during collection while the blood samples for serum were clotted at room temperature for approximately 2 h. Blood samples were then centrifuged at 12,000 x g for 5 min, and plasma and serum were obtained and stored at -80 °C until analysis. One K₂EDTA tube containing blood was retained for flow cytometry analysis. After the blood collection, birds were killed by cervical dislocation and spleens were aseptically obtained and cut into 2 pieces. Cecal tonsils were also aseptically collected and together with one half of the spleen were immediately snap frozen in liquid nitrogen for 2 min and stored at -80 °C until analysis. One half of the spleen was kept on ice in 1x PBS until extraction of splenocytes for flow cytometry analysis.

Table 4. 1. Composition of the basal wheat-soybean based laying hen diet.

Item	Composition
Ingredient %	
Wheat (13.5 % CP)	59.26
Soybean meal (45.8 % CP)	22.30
Vegetable oil (8,800 kcal/kg of ME)	5.22
Limestone (38% Ca)	9.90
Biophos (21/17)	1.61
Vitamin premix ¹	1.00
Mineral premix ²	0.50
DL- methionine	0.14
Lysine	0.06
Threonine	0.01
Total	100
Nutrient composition	
ME , kcal/kg	2850
CP, % (calculated)	18.5
CP, (analyzed)	18.77
Calcium, % (calculated)	4.2
Calcium, % (analyzed)	4.31
Available P, % (calculated)	0.45
Total P, % (analyzed)	0.65
Methionine, % (calculated)	0.43
Meth+cysteine, % (calculated)	0.80
Lysine, % (calculated)	0.95
Threonine, % (calculated)	0.70
Linoleic, % (calculated)	3.23
Folate, mg/ kg (analyzed)	1.76

¹Provided per kilogram of diet: 11,000 IU of vitamin A, 3,000 IU of vitamin D3, 20 IU of vitamin E, 3 mg of vitamin K3 (as menadione), 0.02 mg of vitamin B12, 6.5 mg of riboflavin, 10 mg of calcium pantothenate, 40.1 mg of niacin, 0.2 mg of biotin, 2.2 mg of thiamine, 4.5 mg of pyridoxine, 1,000 mg of choline, and 125 mg of ethoxyquin (antioxidant).

²Provided per kilogram of diet: 66 mg of Mn (as manganese oxide), 70 mg of Zn (as zinc oxide), 80 mg of Fe (as ferrous sulfate), 10 mg of Cu (as copper sulfate), 0.3 mg of Na (as sodium selenite), 0.4 mg of I (as calcium iodate), and 0.67 mg of iodized salt.

4.3.4 Flowcytometry Analysis

The percentages of CD4+, and CD8+ cells in peripheral blood mononuclear cells (PBMC) and in the spleen were isolated using Ficoll-PaqueTM Plus (GE Health care) following the manufacturer's instructions and were prepared as described previously (Gehad et al., 2002b; Shini and Kaiser, 2009), with minor modifications. Briefly, the blood from K₂EDTA vacutainer tubes was diluted 1:1 with 1x PBS and held on ice. Each single suspension of splenocytes was prepared in 5 mL 1 x PBS by mashing the tissue using the end of a syringe plunger through a 100 µm nylon strainer (BD, Biosciences, USA). The blood and spleen suspensions were carefully layered into centrifuge tubes containing an equal volume of Ficoll to form a discrete layer above the Ficoll. Tubes were centrifuged at 220 x g for 30 min at room temperature, and the mononuclear layers (buffy coat) were removed, transferred to different centrifuge tubes, and washed twice in 1 x PBS. Immediately, the cells were counted on a haemocytometer using trypan blue exclusion assay (Sigma Chemical CO., St. Louis, MO) and the cell suspensions were adjusted to 1 x 10⁶ viable cells per mL of 1 x PBS.

Fluorescence monoclonal antibodies were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL), including mouse anti-chicken CD4-PE, and mouse anti-chicken CD8-PE-CY5. Using 96-well round bottom plates, 100 µL of each cell suspension was plated in duplicate. Each of the fluorochrome labelled antibodies was added to their respective wells and the stained cells were incubated for 30 min at 4 °C in the dark. The cells were washed twice in 1 x PBS to remove any traces of unbound antibodies and transferred to 5 ml polystyrene round-bottom tubes for analysis. A total of 10,000 cells per sample were analysed using BD FACS Diva Software (Becton

Dickinson Immunocytometry Systems, San Jose, CA) and the cells were evaluated using Flowjo Software (v.1.1.1, CyFlo Ltd., Turku, Finland).

4.3.5 Total IgG Determination

Serum samples obtained from the blood were used to measure the concentration of IgG antibody via sandwich ELISA using chicken IgG ELISA quantitation and Starter Accessory Kits (Bethyl Laboratories, Montgomery, TX) following the manufacturer's procedure. A microtiter plate reader (Soft max pro 3.1.1) was used to measure the absorbance at 450 nm and a 4-parameter logistic curve fit was developed using the chicken reference serum absorbance.

4.3.6 Clinical biochemistry analysis

Serum total protein, albumin, and globulin, and plasma fibrinogen were determined using an automated analyzer (Cell-Dyn 3500 system; Abbot Laboratories, Abot Park, IL) at the Manitoba Veterinary Services Laboratory (Winnipeg, Manitoba, Canada).

4.3.7 Total RNA Extraction and Reverse Transcription

Total RNA was extracted from spleen and cecal tonsil sections using Trizol Reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada) following the manufacturer's protocol. Briefly the ileal and cecal samples were removed from -80 °C storage and they were kept on ice until completely thawed. About 80 to 100 g of tissue was added to 1 mL of ice cold Trizol reagent (Invitrogen) and homogenized with a homogenizer at full speed for about 1 min. After extraction, the pellet was left to dry for 5 min and was dissolved in 300 µL of nuclease free water. Total RNA concentration was determined at an optical density at 260 nm and RNA purity was verified by evaluating

the ratio of optical density of 260 nm to optical density at 280 nm. Total RNA was diluted to 2 µg/µL in nuclease-free water. Reverse transcription was done using the high capacity cDNA reverse Transcription kit (Applied Biosystems, Mississauga, Ontario, Canada) following the manufacturers protocol, and the cDNA was stored at – 20 °C.

4.3.8 Quantitative Real-Time PCR

Quantitative Real-Time PCR (qRT-PCR) was performed using the Step One Thermo Cycler (Applied Biosystem, Mississauga, Ontario, Canada). 1 µL of cDNA was added to each well of a 48-well plate. Next, 11.5 µL of real-time PCR master mix containing AmpliTag Gold DNA Polymerase LD SYBR Green I, dNTPs with dTTP/dUTP, (Applied Biosystems, Mississauga, Ontario, Canada) and nuclease free water were added to each well to a final volume of 12.5 µL as outlined by Parvizi et al. (2009). Primer sequences for β-actin, toll-like receptor (TLR)-4, interleukin (IL)-4, IL-8, IL-10, IL-1β, IL-13, IL-17, IL-18, and interferon gamma (IFN-γ) were obtained from gene bank (**Table 4. 2**)

Primer concentrations were optimized depending on standard curve using a gene target or endogenous gene previously cloned as a control for PCR efficiency, and different thermal cycling parameters were used for each target gene. Each reaction was run in duplicate using Step One Software (Applied Biosystems, Mississauga, ON, Canada).

4.3.9 Quantitative Real-Time PCR Analysis

Relative expression was calculated using Pfaffl's formula (Pfaffl, 2001) as described previously (Parvizi et al., 2009). Briefly, relative expression ratio of all genes

Table 4. 2. Toll-like receptors and cytokines primer sequences.

Gene	Primer sequence (5'-3')	Gene bank access	Annealing Temp, °C
chTLR-4	F: AGTCTGAAATTGCTGAGCTCAAAT R: GCGACGTTAAGCCATGGAAG	AY064697	60
chIL-1 β	F: GTGAGGCTAACATTGCGCTGTA R: TGTCCAGGCGGTAGAAGATGAAG	Y15006.1	57
chIL-4	F:TGTGCTTACAGCTCTCAGTG R:ACGCATGTTGAGGAAGAGAC	AJ621249.1	54
chIL-8	F: CCAAGCACACCTCTCTTCCA R: GCAAGGTAGGACGCTGGTAA	AJ009800	57
chIL-10	F: AGCAGATCAAGGAGACGTTT R: ATCAGCAGGTACTCCTCGAT	AJ621614	55
chIL-13	F:ACTTGTCCAAGCTGAAGCTGTC R:TCTTGCAGTCGGTCATGTTGTC	AJ621250.1	56
chIL-17	F: GGCTGCCAGCACTCGTACCG R: GGCCTGGCTCAGCTCTGTGG	AJ493595.1	60
chIL-18	F:GAAACGTCAATAGCCAGTTGC R:TCCCATGCTCTTTCTCACAACA	NM204608.1	53
chIFN- γ	F: CTGAAGAAGTGGACAGAGAG R: CACCAGCTTCTGTAAGATGC	X99774	60
ch β -actin	F: CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	X00182	61

was calculated based on the expression of the housekeeping gene, β -actin. Absolute quantification of β -actin expression was estimated using the Step One Software (Applied Biosystems, Mississauga, ON, Canada). The absolute expression of all genes tested was then normalized to the express PCR efficiency: $E = 10^{-1/\text{slope of standard curve}}$ with the gene of interest as the target and β -actin as the reference, the relative expression ratio (R) was determined as follows:

$$R = \frac{(E_{\text{target}})\Delta\text{CP}_{\text{target}}(\text{calibrator} - \text{sample})}{(E_{\text{ref}})\Delta\text{CP}_{\text{ref}}(\text{calibrator} - \text{sample})}$$

Where E_{target} and E_{ref} are the efficiencies of the target gene and β -actin, respectively, and the ΔCP is the difference of crossing points between calibrator and samples. The calculated R was used to determine differences in gene expression among different treatment groups.

4.3.10 Statistical Analysis

A completely randomized design with 2 dietary treatments and 2 levels of immunological challenge in a 2 x 2 factorial arrangement was used. To test for the effects of each treatment combination, values were subjected to ANOVA using the PROC GLM procedure of SAS software (SAS institute, Cary, NC). Differences between means were determined using Tukey's procedure. Significance statements were based on $P < 0.05$.

4. 4 RESULTS

4.4.1 T lymphocyte Subsets in the Blood and the Spleen

To establish the amount of T lymphocytes in circulation and in the spleen, CD4+ and CD8+ T cells were measured. Supplementation with dietary FA did not influence the level of T cell subsets in the blood as well as in the spleen ($P > 0.05$). However, injection

with LPS significantly influenced the amount of T cells in circulation but no significant effect was found on the level of T cells in the spleen. Compared to the saline-injected hens, LPS-injected hens had lower amount of CD4⁺ and CD8⁺ T cells (P=0.0004, and P=0.0003 respectively), however, the ratio of CD4⁺:CD8⁺ significantly increased (P=0.0376) in LPS-injected hens (**Table 4.3**).

4.4.2 Total IgG

The amount of serum IgG was not influenced by dietary FA; however, relative to the saline-injected hens, the LPS-injected hens had higher amount of IgG (P=0.0054) (**Figure 4.1**).

4.4.3 Clinical biochemistry

The level of total protein, albumin, globulin, as well as the albumin:globulin ratio was not influenced by dietary FA supplementation. However, there was a significant reduction in the levels of total protein (P=0.0006), albumin (P<.0001), globulin (P=0.0030), and albumin:globulin ratio (P=0.0049) in the LPS-injected hens compared to the saline-injected hens. No significant difference was found on the level of fibrinogen among the treatment groups. (**Table 4.4**).

4.4.4 Gene Expression in the Spleen and cecal tonsils

Dietary FA supplementation did not influence gene expression in the spleen and the cecal tonsils except the expression of IL-8. The IL-8 expression was influenced by a diet x challenge interaction only in the spleen. Expression of IL-8 in the spleen was found to be higher in the FA supplemented hens that were injected with LPS. Supplementation with FA also reduced the expression of IL-8 in the cecal tonsils compared to the

Table 4. 3. T cell subsets (%) in the Blood and the Spleen of old laying hens (56 to 66 wk) fed diets with or without supplemental dietary folic acid (FA) for 8 wk and at 4 h post- injection with 8 mg/ kg of body weight LPS or saline

Item	0 mg Folic Acid		4 mg Folic Acid		SEM	P Values		
	Saline	LPS	Saline	LPS		Diet	Challenge	Interaction
PBMCs								
CD4+	10	3.26	8.6	3.2	1.4	0.6391	0.0004	0.6448
CD8+	8.1	1.95	8.1	1.8	1.4	0.9509	0.0003	0.9490
CD4+/CD8+	1.4	2.2	1.1	2.3	0.4	0.9349	0.0376	0.6580
Spleen								
CD4+	11	10	11.5	10.5	0.6	0.6002	0.3071	0.9908
CD8+	35.5	37.1	32.9	38.3	3	0.8305	0.2700	0.5542
CD4+/CD8+	0.31	0.26	0.35	0.29	0.03	0.3041	0.1059	0.8060

Data presented as least squares means

LPS-lipopolysaccharide

PBMC = peripheral blood mononuclear cells

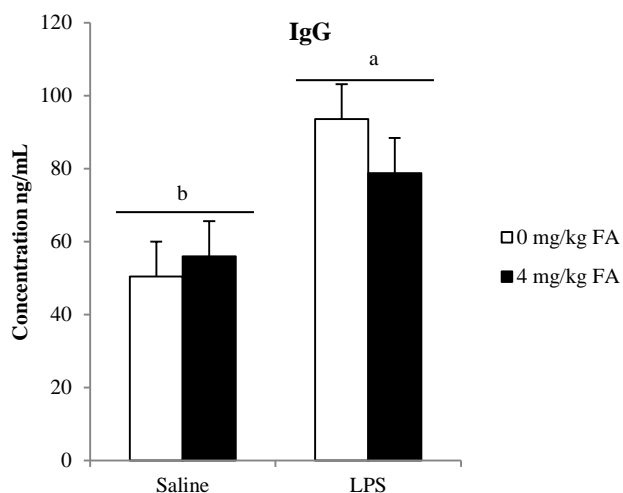


Figure 4. 1. Concentration (ng/mL) of serum IgG of old laying hens (56 to 66 wk of age) fed diets with or without supplemental dietary folic acid (FA) for 8 wk and at 4 h post-injection with 8 mg/ kg of body weight LPS or saline. Graphed values represent LSmeans. Error bars represent standard error of the mean. Different superscripts (a, b) show significant ($P < 0.05$) difference for the main effect of LPS. FA = folic acid. LPS= lipopolysaccharide.

Table 4. 4. Biochemical constituents of old laying hens (56 to 66 wk) fed diets with or without supplemental dietary folic acid (FA) for 8 wk and at 4 h post- injection with 8 mg/ kg of body weight LPS or saline

Item	0 mg Folic Acid		4 mg Folic Acid		SEM	P Values		
	Saline	LPS	Saline	LPS		Diet	Challenge	Interaction
Total protein (g/L)	46	37	46	35	2	0.4713	0.0006	0.6043
Albumin (g/L)	16	11.5	15.8	10.8	0.8	0.6379	< .0001	0.7717
Globulin (g/L)	30	26	30	24	1.5	0.5658	0.0030	0.5658
Albumin/Globulin	0.52	0.45	0.53	0.44	0.02	0.8959	0.0049	0.6021
Fibrinogen (g/L)	1.8	1.2	1.2	1.3	0.2	0.4469	0.3859	0.1988

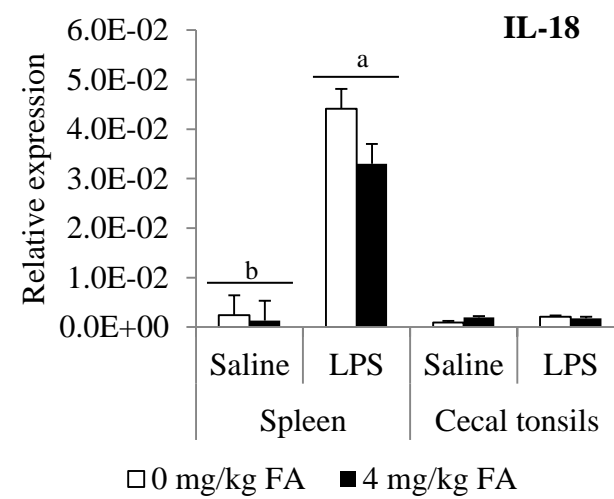
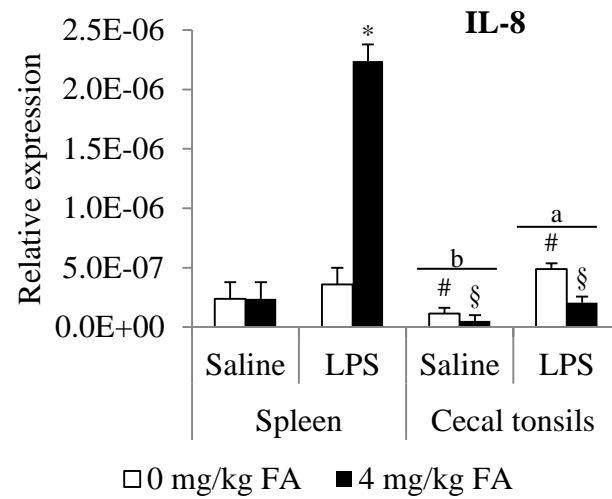
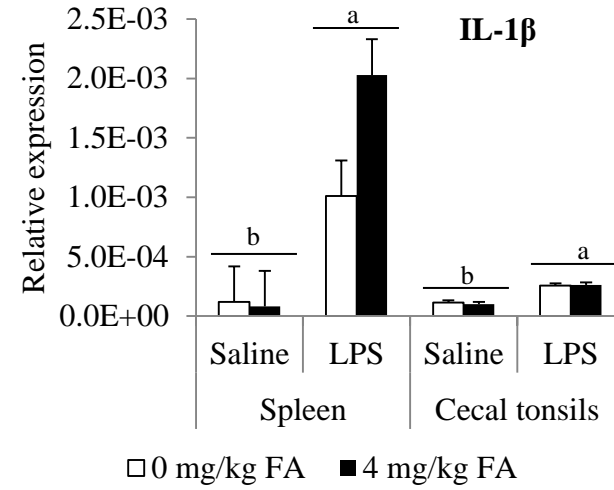
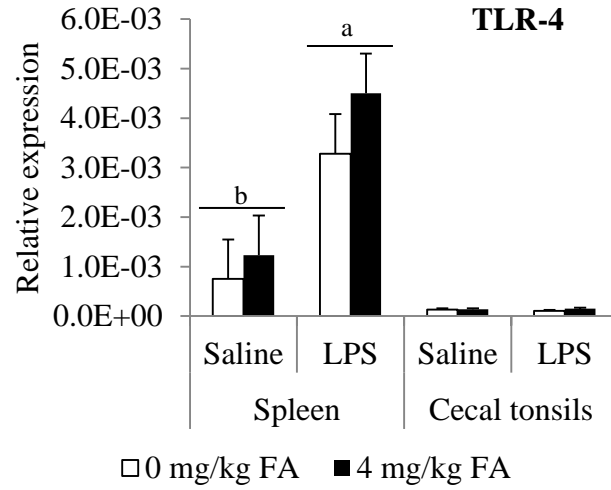
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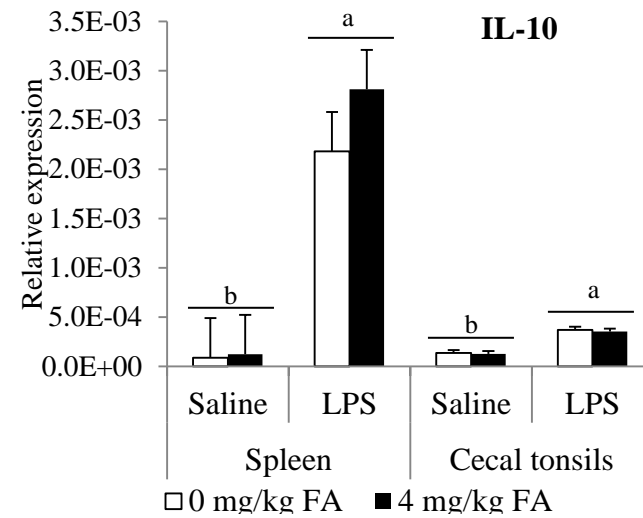
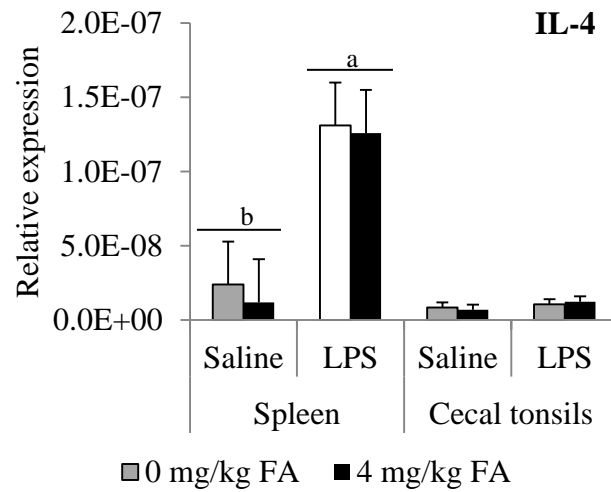
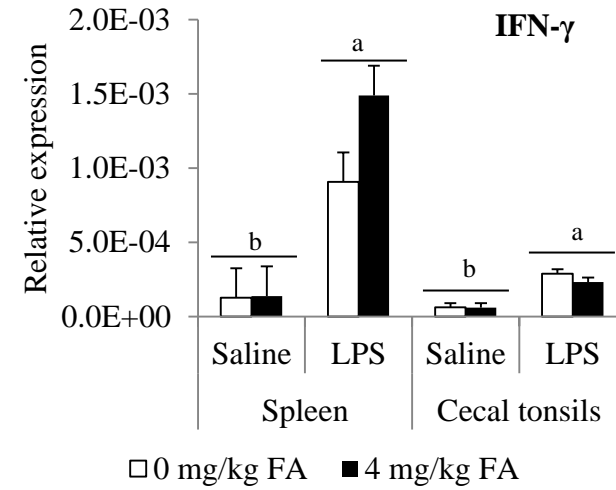
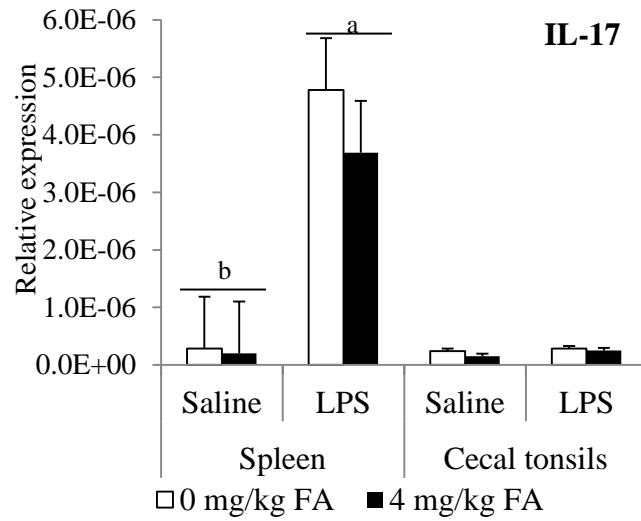
unsupplemented hens. On the other hand, expression of IL-8 in the cecal tonsils was significantly higher in the LPS-injected hens compared to the control (**Figure 4.2**).

Expression of IL-1 β , IL-10, and IFN- γ in the spleen and the cecal tonsils was higher in the LPS-injected hens compared to the control, whereas the expression of IL-4, IL-13, IL-17, IL-18, and TLR-4 was significantly higher in the LPS-injected hens only in the spleen, whilst the expression of IL-13 in the cecal tonsils decreased in LPS-injected hens ($P < 0.05$) (**Figure 4. 2**).

4. 5 DISCUSSION

Our aim in this study was to examine the effects of dietary folic acid (FA) supplementation at 4 mg/kg on the immune changes and responses in old laying hens challenged with *Escherichia coli* lipopolysaccharide (LPS). This is in connection with our previous work with young laying hens (chapter III) where we found that under acute conditions of LPS, dietary FA supplementation at 4 mg/kg of diet enhanced the level of total proteins, albumin, globulins, total IgG, as well as exhibiting pro- and anti-inflammatory effects which were cytokine- and tissue- dependent. However, FA did not alter the level of T cells in circulation and in the spleen. We therefore surmised that dietary FA supplementation at 4 mg/kg of diet in old laying hens under acute LPS challenge may elicit similar benefits as observed in young laying hens, or may be more beneficial with advancing age, thus counteracting the age-associated decline in immune competence. As shown in other species of animals, cell-mediated immunity declines with age in birds (Fahey and Cheng, 2008). Also, previous studies have hypothesised that, the age-associated decline in cell-mediated immunity may be involved in the great susceptibility of hens to *Salmonella* Enteritidis infection during





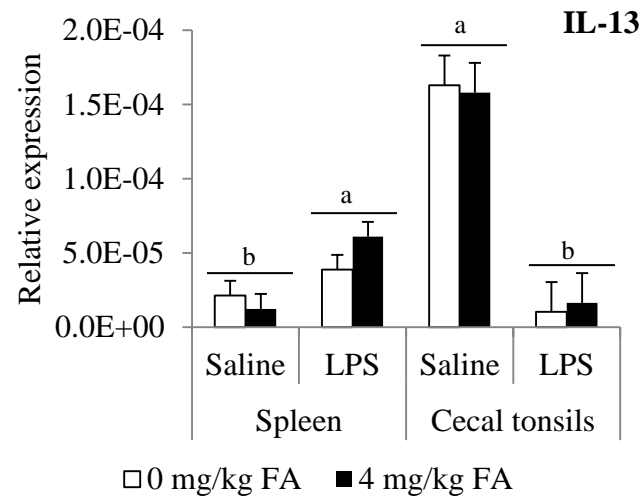


Figure 4. 2. Gene expression in the spleens and cecal tonsils of old laying hens (58 to 66 wk) fed diets with or without supplemental dietary folic acid (FA) for 8 wk and at 4 h post- injection with 8 mg/ kg of body weight LPS or saline. Gene expression was assessed using quantitative RT-PCR, and was calculated relative to the house keeping gene β -actin. Graphed values are expressed as relative expression. Error bars represent standard error. Bars with asterisk show significant ($P < 0.05$) FA x LPS interactions, superscripts a,b, show significant main effects of LPS, and superscripts \$,#, show significant main effect of FA. FA= folic acid. LPS = lipopolysaccharide.

molting (Holt, 2003). However, age-related differences in the response of chickens to *Salmonella enteric* serovar Enteritidis, vaccinations and other infections may also exist (Beal et al., 2005).

In this study, the percentages of T cell subsets (CD4+, and CD8+) in the blood and the spleen were not influenced by dietary FA supplementation. Similar findings were reported by Field et al. (2006) in young and old mice following dietary FA supplementation. This may be attributed to the duration of dietary FA supplementation that was employed in our study since previous studies have demonstrated a slow rate of FA uptake by cells in *in vivo* conditions (Dhur et al., 1991a; Kim et al., 2002). However, it is not known whether the amount of T cells found in this study were naive or memory cells since the functionality of T cells have been shown to decrease with advancing age (Song et al., 1993; Grubeck-loebenstein and Wick, 2002; Effros et al., 2003, 2004; Haynea et al 2004).

The lack of significant effect in the spleen following LPS challenge observed in this study has been reported previously in male leghorn birds (Gehad et al. 2002b), which could be partly explained by the general resistance of chickens to LPS (Adler and DaMassa, 1979). On the other hand, the reduction of T cell subsets in circulation following injection with LPS could be explained by the possible destruction of the cells by LPS toxicity leading to cell death (Gehad et al., 2002b; Shini et al., 2008b). In addition, induced massive apoptosis of T cell subsets and B cells following LPS challenge have also been reported in septic mice and in humans (Richardson et al., 1989; Hotchkiss et al., 1999). However, migration of the T cells from the blood to other

immune-related tissues and organs following LPS challenge to participate in immune responses could also reduce the amount of cells in circulation (Nii et al., 2011).

The ratio of CD4⁺ to CD8⁺ T cells may be used as an indicator of cell-mediated immune response. Previous studies in mammals have reported that the normal ratio of CD4⁺ to CD8⁺ T cells should be greater than 1.5; otherwise may suggest impaired cellular immune mechanisms (Levinson and Jawetz, 1996) and reduced survivability (Reid and Tervit, 1995). In addition, the CD4⁺ to CD8⁺ ratio reflects changes in T cell populations evaluated together at a particular time point, and can therefore serve as a good indicator of changes in the T cell subsets (Holt et al., 2010). Furthermore, a higher CD4⁺ to CD8⁺ ratio is also associated with increased immunocompetence in chickens (Bridle et al., 2006). This suggest that, the increased CD4⁺ to CD8⁺ ratio observed in this study following LPS injection in the blood could be an indicator of higher amount of CD4⁺ cells as well as improved immunocompetence.

Concentration of serum IgG was not influenced by dietary FA. This may suggest that dietary FA supplementation did not affect the level of, or the activities of B type lymphocytes that are involved in the secretion of natural antibodies (Ochsenbein et al., 1999, 2000; Klasing, 2007; Chou et al., 2008). However, the increased amount of IgG following LPS challenge may indicate the presence of an active form of innate (natural) humoral immunity that could aid in early recognition and clearance of invading pathogens (Ochsenbein et al., 1999, 2000; Klasing, 2007; Chou et al., 2008). These natural antibodies may in turn enhance specific immune responses (Parmentier et al., 2004; Star et al., 2007). In addition, it is also possible that the hens may have encountered

environmental LPS early in life which could have contributed to the spontaneous generation of memory antibodies that provided an immediate response to LPS challenge.

With respect to serum biochemical constituents, FA supplementation did not alter the levels of total protein, albumin, and globulin. Since FA has been shown to participate in protein and amino acid synthesis, it is not known why these constituents were not influenced by dietary FA supplementation; however, this could be due to tissue or muscle protein catabolism with advanced age for body maintenance and production purposes, that might have contributed to homeostatic levels of these constituents in the serum in both the FA supplemented and non-supplemented hens. On the other hand, LPS challenge significantly reduced the levels of total protein, albumin, globulin, and albumin:globulin ratio. The decrease may be due to loss of these proteins through synthesis of acute phase proteins by the liver following LPS challenge or utilization by the cells of the immune system, and production of cytokines (Klasing, 1998, 2004, 2007; Kogut and Klasing, 2009). It remains to be known why the level of fibrinogen was not influenced by LPS given the reduction of serum biochemical constituents. Apart from its function in blood coagulation mechanism, fibrinogen is classified as one of the acute phase proteins which are increased non-specifically in acute inflammatory disorders (Kaneko, 1980), and may be useful for haematological screening procedure for diagnosis and monitoring of bacterial infection and other inflammatory diseases (Schalm et al., 1975). However, other acute phase proteins including albumin could have been involved in a more active manner. A degree of hyperfibrinogenaemia in avian species with bacterial infections has been reported previously (Hawkey and Hart, 1988); however,

with the current developments and genetic manipulations, this might have been altered or it could be species- or strain-dependent.

TLR-4 recognizes LPS of gram negative bacteria such as *E. coli* and *Salmonella* and contributes to the initiation of inflammatory responses mediated by pro-inflammatory cytokines (Kogut et al., 2005a). In laying hens, a tissue and time-dependent expression of TLR-4 in the ovarian follicles, and other tissues has been reported following LPS challenge (Subedi et al., 2007). The upregulation of TLR-4 in the spleen observed in this study indicates a robust immune response following LPS challenge and may be associated with the upregulation of pro-inflammatory cytokines in the spleen such as IL-1 β and IL-8 that was observed in this study.

IL-1 β is a proinflammatory cytokine whose activities includes stimulation of T cells and macrophages, induction of fever, triggering of acute-phase response, and activation of the vascular endothelium (Lotz et al., 1988; Diehl et al., 2000; Corwin, 2000), however, most of these activities are also mediated by other proinflammatory cytokines and chemokines (IL-6, IL-8, IL-18 etc) (Staheli et al., 2001). The upregulation of IL-1 β in the spleen and cecal tonsils observed in this study therefore suggests induction of inflammatory responses in these tissues following LPS challenge. On the other hand, IL-17 is produced by activated and memory T cells and is involved in inflammatory responses as well as inducing the production of other pro-inflammatory cytokines (Hong et al., 2006). The current results demonstrate an upregulation of IL-17 in the spleen following LPS challenge; however, this was not observed in the cecal tonsils depicting tissue differences in immune responses.

IL-18 is a pro-inflammatory cytokine produced by macrophages and it operates in conjunction with IL-12 to induce a cell-mediated immune response following pathogenic insult (Corwin, 2000; Cox et al., 2010). It is mainly involved in the targeting of Th1-like cells which in turn secrete IFN- γ that plays an essential role in activating macrophages (Gobel et al., 2003). The upregulation of IL-18 in the spleen observed in this study could therefore be correlated with the increased level of IFN- γ , suggesting stimulation of both innate and cell-mediated immune responses following LPS challenge. In addition, IFN- γ plays a significant role in regulating the innate and adaptive immune responses (Cox et al., 2010) and it's generally involved in promoting Th1-like cell differentiation and enhancing the functions and activation of innate immune cells.

IL-8 also known as CXCLi2, is a chemokine produced mainly by macrophages and is an important mediator of innate immune responses (Cox et al., 2010). Its primary role involves acting as a chemoattractant that induces the migration of heterophils (Cox et al., 2010; Redmond et al., 2011), monocytes (Barker et al., 1993), and CD3+ T cells (Min et al., 2001) to inflammation or infection sites in birds. In this study, the upregulation of IL-8 in the spleen particularly in the FA supplemented group that was injected with LPS suggests that dietary FA enhanced immunocompetence in these hens, and hence influenced both innate and cell-mediated immune responses in old laying hens via enhancement of pro-inflammatory responses. On the other hand, dietary FA down regulated the expression of IL-8 in the cecal tonsils indicating a reduction in the rate of migration of heterophils, monocytes, and CD3+ cells to inflammation sites (Kogut, 2002). In this case, dietary FA act as an anti-inflammatory immunomodulator in the cecal tonsils, and consequently, suggesting pleiotropic form of effect in old laying hens under

acute LPS challenge; up regulating and down regulating expression of IL-8 in different tissues. Increased production of CXCLi2 along with other inflammatory signals by heterophils of some broiler genetic lines is associated with enhanced immune response to *Salmonella* Enteritidis (Ferro et al., 2004; Swaggerty et al., 2004; Chiang et al., 2008).

IL-4, a Th2- like cytokine plays a crucial role in the stimulation of B lymphocytes, proliferation of T lymphocytes, and the differentiation of CD4+ T cells into Th2 cells (Fietta and Delsante, 2009; Cox et al., 2010). However, the functions of IL-13 also characterized as a Th2 cytokine, overlap considerably with those of IL-4 (Cox et al., 2010). The increased levels of IL-4 and IL-13 in the spleen following LPS injection observed in this study suggests activation of T cell-mediated immune responses as well as enhanced humoral immune responses that are facilitated by the Th2 cells. However, expression of IL-4 was not affected by LPS challenge in the cecal tonsils while expression of IL-13 in the cecal tonsils was significantly reduced. This suggests a decrease or inhibition of Th2-like responses in the cecal tonsils.

IL-10 is also a Th2 cytokine that serves to keep the immune responses in check by inhibiting cytokine production by Th1 cells, and down regulating the expression of the major histocompatibility antigens expressed by immune cells (Corwin, 2000; Min et al., 2001). This down regulation reduces the likelihood of an immune response to an antigen. The up regulation of IL-10 in the spleen and cecal tonsils observed in this study therefore suggests a controlled or balanced immune response following LPS challenge in old laying hens. This is important for reducing chances of possible inflammatory damage, and reflects potential for enhanced homeostasis after an immune response.

In summary, dietary FA supplementation did not influence the level of T cell subsets, total IgG, and the serum biochemical constituents, as well as the expression of immune-related genes that were tested except the expression of IL-8. This indicates that dietary FA may not actively modulate immune responses in old laying hens under acute conditions of LPS. On the other hand, LPS challenge reduced the level of total proteins, albumin, and globulin in the serum, and the number of T cells in circulation; however, CD4+ to CD8+ ratio was increased indicating an enhanced immunocompetence. Serum IgG, as well as the expression of pro- and anti-inflammatory cytokines in the spleen and cecal tonsils was enhanced following LPS challenge; suggesting an improved innate and adaptive immunocompetence in old laying hens. This suggest that immunocompetence in old laying hens does not seem to be altered as previously reported; however, other factors including overall nutritional status may affect immunocompetence during ageing. Investigating the immunological role of dietary FA under chronic conditions of LPS, and at different end points will enhance understanding on the contributions of dietary FA on immune responses in old laying hens.

CHAPTER 5

5.0 GENERAL DISCUSSION

As highlighted in the literature review in chapter 2, the use of dietary folic acid (FA) in laying hens is not a new practice as evidenced by studies on folate deposition in eggs. However, in humans, dietary FA has also been employed as a tool for preventing many diseases and health disorders of economic importance for quite long. In addition, the role of FA in inflammatory responses, enhancement of both the innate and adaptive forms of immunity, as well as general improvement in immunocompetence with advancing age, has been demonstrated. The question that needs to be addressed therefore is whether dietary FA could enhance immunocompetence in laying hens under challenged and unchallenged conditions, and if this would be more beneficial at an advanced age. This is due to the fact that laying hens have a relatively longer rearing period, and or production cycle that provide adequate time for exposure to different management regimens and environmental conditions that may compromise their immune competence. The objective of this study was therefore to investigate the relationship between dietary FA supplementation, LPS challenge, and age, on the immune responses in laying hens. Previous studies have shown that folate deposition and saturation in the chicken egg is achieved when supplementation of FA in the laying hen diet is increased up to about 4 mg FA /kg of diet (House et al., 2002; Hebert et al., 2005; Dickson et al., 2010). In addition, because folate-enriched eggs continue to represent a cheap and viable method of providing natural folates to the human population, investigating whether the same amount of FA needed for optimum deposition in the egg is able to enhance

immunocompetence and disease resistance in laying hens deems necessary. Information generated from such a study could result in formulation of affordable diets that optimize disease resistance in laying hens while still serving as reliable sources of natural folates. In this thesis, two experiments were conducted to: 1) determine immunomodulation in young laying hens by dietary FA and acute immune responses after challenge with *Escherichia coli* lipopolysaccharide, and 2) to determine response of old laying hens to an *Escherichia coli* lipopolysaccharide challenge when fed diets with or without supplemental FA.

In manuscript I, young laying hens at 24 wk were fed a wheat soybean-based diet with 0 or 4 mg of supplemental FA per kg of diet. At 32 wk, 6 hens from each dietary treatment were either injected with 8 mg of *E. coli* lipopolysaccharide (LPS) per kg of body weight or saline solution to make 4 treatments; hens with no supplemental FA and injected with saline, hens supplemented with FA and injected with saline, hens with no supplemental FA and injected with LPS, and hens supplemented with FA and injected with LPS. Blood and tissue samples were collected 4 h after injection. In manuscript II, old laying hens at 58 to 66 wk were used and all the experimental procedures were carried out as indicated in manuscript I.

As reported in manuscript I, the percentages of CD3+, CD4+, and CD8+ cells both in circulation and in the spleen were not affected by dietary FA compared to the control and a similar observation was also reported in manuscript II. These observations contrast with some *in vitro* studies reported in literature review section of this thesis; however, a review by Dhur et al. (1991b) reported that the ability of T cells to take up folate could be faster *in vitro* as compared to *in vivo* conditions despite the vitamin

deficiency, and this might have been the case in our study where we investigated the effect of FA under *in vivo* conditions. Alternatively, since we only used one level of dietary FA supplementation based on results from studies measuring different parameters, it is not known whether this amount was sufficient or insufficient for improved immunocompetence, or there could be some other endogenous sources of folates (Rong et al., 1991; Kim et al., 2004; Asar and O'Connor, 2005), that might have contributed to enhanced availability of folates to the unsupplemented hens; which might be responsible for the lack of significant differences among dietary treatment groups. LPS challenge as reported in manuscript I and II reduced the amount of T cell subsets in circulation but had no influence in the spleen. The loss of T cells following LPS challenge might indicate a massive destruction of these cells or their migration to other tissues to participate in immune responses; however, since the LPS challenge used in our study was so acute, investigating the effects of LPS under chronic conditions may be better to see how the cells will behave in both FA supplemented and unsupplemented hens. It remains to be known why the T cell subsets were not influenced by LPS challenge in the spleen; however, tissue and genetic factors including species and strain effects may contribute to this observation.

Statistical analysis of the level of CD4⁺ and CD8⁺ cells in the blood in young (manuscript I) and old (manuscript II) hens revealed that the percentage of CD4⁺ cells in circulation was not affected by dietary FA, however, LPS significantly reduced the level of CD4⁺ cells, whereas CD8⁺ cells and the CD4:CD8 ratio were influenced by a age x challenge interaction with higher amount of CD8⁺ cells observed in old hens that were not challenged with LPS, and a lower CD4:CD8 ratio in the same group (**Table 5. 1**). On

the other hand, T cell subsets in the spleen were not influenced by diet and immunological challenge, however, CD4⁺ cells were higher in young hens compared to old hens whereas the percentage of CD8⁺ cells and the CD4:CD8 ratio were higher and lower respectively in old hens compared to young hens (**Table 5.1**). The fewer CD4⁺ cells reported in our study in old hens is in agreement with previous studies which have reported that the amount of T cells decrease with age (Fahey and Cheng, 2008); however, it is not known whether the higher amount of CD8⁺ cells found in the old hens were naive or memory cells since the functionality of T cells have been shown to decrease with advancing age despite the increase or homeostatic maintenance of these cells (Song et al., 1993; Grubeck-loebenstein and Wick, 2002; Effros et al., 2003). In addition a lower CD4:CD8 ratio is an indicator of reduced immunocompetence (Bridle et al., 2006), hence the immune competence in old hens might appear to be compromised.

In manuscript I, the concentration of serum IgG was enhanced in FA supplemented hens that were challenged with LPS, implying that FA had extra beneficial effects in the hens that were exposed to LPS challenge. This may indicate an ability of FA to enhance the amount of B type cells or their ability to produce natural antibodies as indicated in the literature review. Surprisingly, this was not observed in old laying hens (manuscript II) where the level of IgG was only increased following injection with LPS but no effect of dietary FA was found. FA supplementation could therefore be more useful in young laying hens as opposed to old laying hens with respect to the active form of humoral innate immunity that is mediated by B cells. The enhanced level of IgG following injection with LPS observed in manuscript I and II is in agreement with previous studies (Parmentier et al., 2004; star et al., 2007) and explain the ability of the

Table 5. 1 T cell subsets in the blood (PBMC) and the spleen of young and old laying hens fed diets with or without supplemental dietary folic acid (FA) for 8 wk and at 4 h post- injection with 8 mg/ kg of body weight LPS or saline (3-factorial analysis)¹

Diet	Challenge	Age	PBMC			Spleen		
			CD4	CD8	CD4:CD8	CD4	CD8	CD4:CD8
0 FA	Saline	Y	8.57	3.71	2.31	14.8	19.3	0.77
		O	10	8.09	1.36	11	35.5	0.31
	LPS	Y	1.55	0.93	1.98	13.1	23.8	0.67
		O	3.26	1.95	2.18	10	37.1	0.27
4 FA	Saline	Y	4.77	2.66	2.1	11.9	20.9	0.59
		O	8.67	8.1	1.1	11.5	32.9	0.35
	LPS	Y	1.69	1.16	1.65	13.1	21.8	0.65
		O	3.25	1.77	2.35	10.5	38.3	0.29
SEM ²			1.52	1.09	0.44	1.35	3.77	0.05
P values								
Diet			0.2498	0.7476	0.6186	0.6326	0.8778	0.303
Challenge			<.0001	<.0001	0.312	0.5179	0.2663	0.2943
Age			0.0527	0.0006	0.4105	0.0141	<.0001	<.0001
Diet x Challenge			0.2257	0.725	0.816	0.4411	0.9963	0.2933
Diet x Age			0.5944	0.836	0.7064	0.3269	0.928	0.0553
Challenge x Age			0.6331	0.0114	0.0286	0.7257	0.8753	0.6679
Diet x Challenge x Age			0.545	0.638	0.6691	0.4476	0.5022	0.2063

¹Values were expressed as LSMeans

²SEM= standard error of the mean. Y = young hens. O = old hens. FA= folic acid. LPS = lipopolysaccharide

hens to respond to T-independent antigens via the production of natural antibodies.

Statistical analysis of the data from manuscript I and II indicated that, the level of serum IgG was influenced by a diet x age x challenge interaction. Serum IgG was higher in young hens that were supplemented with FA and challenged with LPS (**Table 5. 2**). The young hens (manuscript I) therefore seems to have more natural antibodies binding to LPS as compared to the old hens (manuscript II), an observation that is in contrast with the findings of Parmentier et al. (2004), who reported that the amount of natural antibodies binding to LPS increase with increasing age. However, this was associated with reduced survivability due to increased chances of autoimmunity. The less amount of IgG binding to LPS in old hens reported in this study, could therefore be an important mechanism of maintaining low amount of natural antibodies for protection while reducing chances of autoimmunity and thus enhancing survivability.

The hens fed the FA supplemented diet as reported in manuscript I had higher levels of total protein, albumin and globulin. This is in agreement with previous studies that have elaborated a role of FA in protein and nucleotide synthesis (Selhub et al., 1996). During the peak period of egg production, laying hens have a generally high requirement for amino acids and nucleotide biosynthesis and FA supplementation could therefore be crucial; otherwise the hens might end up utilizing their body reserves so as to meet production requirements and consequently compromising health status. In manuscript II, FA supplementation did not affect the level of total protein, albumin, and globulin compared to the control. This may indicate that active synthesis of nucleotides and

Table 5. 2 Clinical biochemistry and natural antibody (IgG) in young and old laying hens fed diets with or without supplemental dietary folic acid (FA) for 8 wk and at 4 h post- injection with 8 mg/ kg of body weight LPS or saline (3-factorial analysis)¹

Diet	Challenge	Age	Total			Albumin:globulin		IgG
			protein	Albumin	Globulin	ratio	Fibrinogen	
0 FA	Saline	Y	39	14.3	24.7	0.56	4.8	76.8
		O	46.5	16	30.3	0.52	1.8	50.4
	LPS	Y	31.6	10.2	21.2	0.48	3.7	82.9
		O	37.8	11.5	26	0.45	1.2	93.6
4 FA	Saline	Y	43.5	16.5	27.2	0.62	4.4	91.9
		O	46	15.8	30.3	0.53	1.2	56
	LPS	Y	32.6	10.4	22	0.5	3.7	141
		O	34.8	10.8	24.2	0.44	1.3	72.8
SEM ²			2.32	0.95	1.46	0.02	0.67	10.3
P values								
Diet			0.7662	0.5826	0.7192	0.2977	0.6347	0.0505
Challenge			<.0001	<.0001	<.0001	<.0001	0.1927	0.0003
Age			0.0082	0.325	0.0005	0.0041	<.0001	0.0002
Diet x Challenge			0.3643	0.3616	0.4035	0.4398	0.5112	0.5574
Diet x Age			0.1776	0.24	0.2262	0.3884	0.9708	0.004
Challenge x Age			0.8127	0.7972	0.6605	0.5539	0.4657	0.8894
Diet x Challenge x Age			0.8838	0.5994	0.9809	0.9636	0.8262	0.0211

¹Values were expressed as LSMeans

²SEM= standard error of the mean. Y = young hens. O = old hens. FA= folic acid. LPS = lipopolysaccharide

proteins might have slowed down at this time. Alternatively, this could have been due to tissue, muscle, and protein catabolism by the hens to meet production requirements and for body maintenance that contributed to homeostatic levels of these constituents in the serum in both the FA supplemented and unsupplemented hens. It is therefore tempting to surmise that FA may promote the levels of total protein, albumin, and globulin in young laying hens but not in old laying hens.

In manuscript I and II, LPS challenge reduced the levels of total protein, albumin and globulin. This could be explained by the fact that the LPS exposure was so acute and lead to the loss of these constituents for the synthesis of inflammatory cytokines and acute phase proteins by the liver (Klasing, 2004, 2007). Most of the immune cells are made up of proteins or various kinds of amino acids, hence depletion of these components might have direct adverse effects on the integrity of the immune system. However, dietary FA may be useful in attenuating the effects of acute LPS in young laying hens. Investigating the effects of dietary FA under chronic conditions of LPS challenge, and at different end points might therefore shed more light on whether the supplemented hens will have an added advantage during the recovery period than the unsupplemented hens.

Statistical analysis of the data from manuscript I and II, indicated that dietary FA had no influence on the levels of total protein, albumin, and globulin in both young and old laying hens; however, LPS challenge was found to reduce the level of these constituents. On the other hand, total protein and globulin was found to be higher in old hens compared to the young hens but the amount of albumin was not influenced by age (**Table 5. 2**). The lack of dietary influence on the levels of these constituents could also

be attributed to the possible sources of endogenous folate; however, the possible catabolism effects with age as discussed in manuscript II could also be responsible, and could also explain why old hens had higher levels of total protein and globulin compared to the young hens; it is also possible that the level of these constituents may differ with age.

Although the level of fibrinogen was neither influenced by dietary FA nor the LPS challenge in both manuscript I and II, it is worth noting that the levels reported in manuscript I (young hens) were higher as compared to the levels reported in manuscript II (old hens) (**Table 5. 2**). This might raise the question whether young laying hens have got higher levels of fibrinogen compared to the old hens, or other factors not considered in our study might have been responsible for this. However, since fibrinogen is classified as one of the acute phase proteins which are increased non-specifically in acute inflammatory disorders (Kaneko, 1980), it is even more surprising that LPS challenge did not alter the levels of fibrinogen at any moment as reported in manuscript I and II. This could be attributed to the general finding that for unknown reasons chickens are more resistant to systemic administration of LPS (Adler and DaMassa, 1979; Keestra and Van Putten, 2008). Alternatively, genetic selection and improvement might have a role to play in this observation.

As reported in manuscript I, dietary FA modulated the inflammatory responses following LPS challenge by exhibiting pleiotropic effects on the expression of IL-1 β , and IL-18 in the spleen as well as IL-18 in the cecal tonsils. However, similar immunomodulation effects were exhibited via the expression of IL-8 in the spleen and the cecal tonsils as reported in manuscript II. Anti-inflammatory effects of FA have been

reported in human and mice studies under infection or challenged conditions (as discussed in chapter 2), but there are no reports on inflammatory effects of FA in the literature hence, this study revealed the pleiotropic effect of FA under acute conditions of LPS in laying hens. This observation is in agreement with some of the mechanisms for dietary immunomodulation highlighted in the literature review (Klasing, 1998, 2004, 2007; Kogut and Klasing, 2009) whereby diets or dietary components should modulate leukocyte signalling through up- or down-regulation of immune cell functions as well as protecting against immunopathology through management of inflammatory responses.

The expression of TLR-4 as reported in manuscript I was not influenced by the LPS challenge in both the spleen and the cecal tonsils 4 h post-injection. This was not expected given the fact that IL-1 β a cytokine that is associated with TLR-4 was up regulated in the spleen following LPS challenge; however, IL-8 a cytokine that is also associated with TLR-4 was also not influenced. This might suggest a possibility of a different pathway through which IL-1 β was up regulated. However, other factors including the duration of exposure to the LPS challenge and the tissue under investigation could also be responsible (Subedi et al., 2007). In manuscript II, TLR-4 was significantly expressed only in the spleen following LPS challenge. In addition to the time and tissue factors behind the expression of TLRs after LPS challenge (as discussed in manuscript I), the expression of TLR-4 in laying hens could also be age-dependent.

As shown in the literature review, the Th1-like and Th2-like cell subsets cross-regulate each other's cytokine production profiles, mainly through IFN- γ and IL-10 respectively. Therefore, with the up regulation of both IFN- γ and IL-10, it appears that the immune responses that are mediated by the Th1 and Th2 cell subsets are balanced and

hence the immune system is behaving normally. In addition, an up regulation of cytokines implies the ability of the immune system to respond after a pathogenic insult in efforts to clear the infection.

Statistical analysis of the expression of cytokines in manuscript I and II, showed that the expression of TLR-4 in the spleen was higher in old hens compared to the young hens whereas IL-18 was influenced by a diet x challenge x age interaction in both the spleen and the cecal tonsils. The expression of IL-10 and IFN- γ in the spleen and cecal tonsils were influenced by age x challenge interaction. IL-10 was higher in old hens that were injected with LPS compared to young hens, while IFN- γ was higher in young hens that were injected with LPS compared to old hens. This indicates that young hens may mount strong inflammatory responses compared to the old hens while on the other hand; old hens could have strong anti-inflammatory responses. However, the up-regulation of other Th2 cytokines; IL-4 and IL-13 may serve to strengthen the Th2-like responses in the spleen in old hens thus enhancing the activities of B cells. On the other hand, the expression of IL-1 β in both the young and old hens in the spleen and cecal tonsils was higher in LPS-injected hens, and was also influenced by the diet x age interaction in the cecal tonsils being lower in young hens that were supplemented with FA compared to the supplemented old hens (**Table 5. 3 and 5. 4**). Therefore, the redundancy and pleiotropic nature of various cytokines could serve to strengthen pro- and anti-inflammatory responses in old and young laying hens respectively thereby balancing the inflammatory responses. In addition, IL-1 β seems to be an important pro-inflammatory cytokine in laying hens during acute LPS challenge.

Table 5. 3 Gene expression in the spleen of young and old laying hens fed diets with or without supplemental dietary folic acid (FA) for 8 wk and at 4 h post- injection with 8 mg/ kg of body weight LPS or saline (3-factorial analysis)¹.

Diet	Challenge	Age	Spleen					
			IL-1 β	IL-8	IL-10	IL-18	IFN- γ	TLR-4
0 FA	Saline	Y	0.136	0.013	0.107	31.25	39.4	0.14
		O	0.19	0.0002	0.088	298.6	0.13	19
	LPS	Y	0.425	0.013	0.326	1.17	106.9	0.19
		O	0.936	0.0004	2.18	104.3	0.81	4.75
4 FA	Saline	Y	0.039	0.008	0.046	64.39	18.15	0.2
		O	0.081	0.0002	0.122	1.34	0.14	10.16
	LPS	Y	0.95	0.019	0.422	178.8	106.7	0.014
		O	4.158	0.0022	2.81	33	1.49	4.5
SEM ²			0.7	0.002	0.4	20	10	4
P values								
Diet			0.1119	0.7415	0.586	0.092	0.6916	0.4873
Challenge			0.009	0.0638	0.0001	0.0051	0.0053	0.1319
Age			0.0996	<.0001	0.0017	0.1034	<.0001	0.0059
Diet x Challenge			0.0882	0.0766	0.5577	0.1213	0.6944	0.5162
Diet x Age			0.2221	0.8513	0.6233	<.0001	0.6727	0.4847
Challenge x Age			0.1042	0.1948	0.0022	0.7901	0.0065	0.1327
Diet x Challenge x Age			0.2419	0.2022	0.7298	0.0058	0.7128	0.5039

¹Values were expressed as LSMeans of relative expression ratios and were all divided by 10⁻³

²SEM= standard error of the mean. Y = young hens. O = old hens. FA= folic acid. LPS = lipopolysaccharide

Table 5. 4 Gene expression in the cecal tonsils of young and old laying hens fed diets with or without supplemental dietary folic acid (FA) for 8 wk and at 4 h post- injection with 8 mg/ kg of body weight LPS or saline (3-factorial analysis)¹

Diet	Challenge	Age	IL-1 β	IL-8	IL-10	IL-18	IFN- γ	TLR-4
0 FA	Saline	Y	0.108	0.005	0.07	9.97	16.1	0.025
		O	0.114	0.0001	0.14	22.8	0.06	1.89
	LPS	Y	0.315	0.051	0.11	20	25.3	0.032
		O	0.256	0.0005	0.37	2.1	0.29	0.107
4 FA	Saline	Y	0.033	0.0096	0.05	29	7.4	0.033
		O	0.1	0.00005	0.125	6.9	0.061	0.276
	LPS	Y	0.13	0.25	0.12	5.3	22.5	0.012
		O	0.26	0.002	0.35	11.7	0.23	12.2
SEM ²			0.04	0.006	0.03	7	2	2
P values								
Diet			0.0299	0.2543	0.6751	0.93	0.1361	0.1893
Challenge			<.0001	0.1121	<.0001	0.1855	0.0023	0.2039
Age			0.2192	0.082	<.0001	0.3485	<.0001	0.0735
Diet x Challenge			0.4509	0.2739	0.8027	0.7077	0.4291	0.0879
Diet x Age			0.0376	0.2527	0.8882	0.6329	0.1401	0.1884
Challenge x Age			0.9869	0.1134	0.0017	0.9156	0.0031	0.2026
Diet x Challenge x Age			0.2798	0.2729	0.7076	0.0103	0.4207	0.0867

¹Values were expressed as LSMeans of relative expression ratios and were all divided by 10⁻³

²SEM= standard error of the mean. Y = young hens. O = old hens. FA= folic acid. LPS = lipopolysaccharide

In conclusion, although the expression of IL-10 and IFN- γ showed a shift towards Th1 responses in young hens and Th2 responses in old hens, this may not be the case given the support from other cytokines as well as the pleiotropic and redundancy nature of cytokines. Dietary FA modulated immune responses in young laying hens, but had little effect in old hens, hence dietary FA cannot be said to have extra beneficial effects in older hens as compared to the young hens. In addition, given the fact that the old hens were able to mount an immune response following LPS challenge, same way as the young hens; the results reported in our study do not support the general decline in immunocompetence with age that has been previously reported in the literature. This may indicate genetic or strain differences in immune responses in response to acute LPS challenge (Cheng et al., 2001); however other factors not analyzed in our study including nutritional status may also influence immunocompetence with age (Lesourd et al., 2006).

6. 0 SUMMARY AND CONCLUSIONS

1. Dietary supplementation at 4 mg of FA per kg of laying hen diet did not cause any adverse effect on the immune system in young and old laying hens.
2. T cell subsets in the blood and the spleen, for both young and old laying hens were not affected by dietary FA supplementation, as well as the H:L ratio in young laying hens.
3. Dietary supplementation of FA increased the level of clinical biochemical constituents (total proteins, albumin, and globulin) in young laying hens; however, dietary FA did not influence the level of these constituents in old laying hens.
4. Synergistic effects of dietary FA supplementation enhanced the level of serum IgG in young laying hens; however, total IgG in old laying hens was only influenced by LPS.
5. Supplementation of FA in laying hen diet affected the expression of IL-1 β , and IL-18 in young laying hens, and IL-8 in old laying hens, in the spleen and cecal tonsils by exhibiting pleiotropic effects through expression of both pro- and anti-inflammatory effects. However, dietary FA supplementation did not influence the expression of other genes that were tested.
6. LPS challenge at 8 mg per kg of body weight influenced expression of a number of genes, as well as the H:L ratio in young laying hens.

7. LPS challenge reduced the levels of T cell subsets in circulation but did not influence these cells in the spleen in both young and old laying hens.
8. LPS challenge reduced the levels of clinical biochemical constituents (total protein, albumin, and globulin) in both young and old laying hens; however, the levels of fibrinogen were not influenced.
9. Tissue differences in immune responses were observed with respect to gene expression.
10. Dietary FA influenced some immune responses in young hens under LPS challenge; however, no such effects were observed in old hens.
11. The old hens were still able to mount immune responses following LPS challenge indicating no evidence of reduced immunocompetence with age. However, other factors including overall nutritional status may influence immunocompetence with age.

7.0 FUTURE STUDIES

In the present study, dietary FA was used to determine the potential for FA supplementation in laying hen diet to enhance immune competence under challenged conditions. Our results demonstrate a significant role of FA in enhancing total IgG and biochemical constituents in young laying hens, as well as participating in both pro- and anti-inflammatory responses under acute conditions of LPS challenge in laying hens. However, further investigations on this topic may enhance understanding on the immunomodulation role of FA in laying hens.

1. *Dietary FA supplementation under chronic conditions of LPS, and multilevel supplementation with dietary FA.*

Some of the beneficial effects of dietary FA in human and mice studies reported in chapter 2 employed a long period of supplementation (Troen et al., 2006; Field et al., 2006). In support of this, a slow rate of FA uptake by immune cells particularly T cells under *in vivo* conditions have been reported compared to *in vitro* conditions (Dhur et al., 1991a). In the present study, the immunomodulation properties of dietary FA were investigated under acute exposure to LPS challenge; dietary FA did not attenuate the effects of LPS on the levels of T cells as well as the biochemical constituents. However, if chronic conditions of LPS exposure could be employed, investigating the immune responses at both cellular and molecular levels and at different end points could provide more detailed results. *In vitro* studies have supported the role of FA in enhancing T cell proliferation (Wintergerst et al., 2007), however, since *in vitro*

conditions may differ from *in vivo*, consecutive administration of a secondary immunological challenge could aid in determining the potential for FA to boost memory in immune cells particularly through cell proliferation upon secondary infection *in vivo*. This will consequently help in determining the functionality differences between cells from the young and those from old hens.

On the other hand, since in the present study only one level of dietary FA supplementation was investigated, supplementing FA at several, and different levels might give a clear indication on the impacts of this novel nutrient on immune competence. This may therefore help in answering the hypothetical question on whether the dietary requirement values, that maximize productivity and or other parameters of economic importance in healthy unchallenged birds, are optimal for immunocompetence and disease resistance (Klasing, 1998).

2. *Severe or moderate FA deficiency?*

Although feed industries do not include FA in laying hen diet during feed formulations due to the general lack of impact in performance (House et al, 2002; Herbert et al., 2005), feed ingredients used for formulating laying hen diets have been found to contain natural folates that even exceeds the level that is recommended by NRC (1994) (House et al., 2002; Herbert et al., 2005; Hoey et al., 2009; Dickson et al., 2010; Tactacan et al., 2010; 2012). This is in line with the feed analysis results done in our study where the basal diet used was found to contain 1.76 mg FA /kg of diet. In addition, bacterially synthesised folate in the large intestines have been reported in pigs, rats, and humans, and this folate can be absorbed and incorporated into the hosts' tissues (Rong et al., 1991; Kim et al.,

2004; Asar and O'Connor. 2005). Based on these results, it is possible that the level of natural folates derived from the basal diet and the microbial-derived folate (if any) is still sufficient to confer positive effects on the immune status of the unsupplemented hens; a condition that might have contributed to the lack of dietary difference on the levels of most of the measured parameters among the supplemented and the unsupplemented hens. Investigating the presence of microbial-derived folate and its possible absorption in laying hens could enhance understanding on the impacts of FA supplementation on the immune responses in challenged and unchallenged conditions. This may help in deciding on the best levels for FA supplementation if need be, with respect to immune competence.

3. *Determination of enhanced deposition of IgG in the egg*

In addition to the well studied deposition of natural folates in the egg, laying hens have been used for the generation of antibodies in the egg that are used in other species of farm animals as well as in humans for enhancing protection against specific pathogens. With respect to the fact that the serum IgG increased in FA supplemented hens that were injected with LPS (manuscript I), a further investigation on the possible beneficial effects of FA in LPS challenged hens through enhanced protection, via generation of antibodies, and their subsequent deposition in the egg, could yield results that may be a novel finding.

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