

**IMMUNE RESPONSE AND *Salmonella* CLEARANCE IN BROILER CHICKENS
AFTER FED ARGININE, VITAMIN E AND PREBIOTICS**

A Thesis

by

XIAO LIU

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2011

Major Subject: Poultry Science

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Vitamin E and Prebiotics

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Approved by:

Chair of Advisory Committee:	John B. Carey
Committee Members:	Morgan Farnell
	James A. Byrd
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ABSTRACT

Immune Response and *Salmonella* Clearance in Broiler Chickens after Fed Arginine, Vitamin E and Prebiotics. (December 2011)

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Chair of Advisory Committee: Dr. John B Carey

Four experiments were conducted to evaluate effects of arginine (**ARG**), vitamin E (**VE**) and mannanoligosaccharide (**MOS**) on immune response and clearance of *Salmonella* in broiler chickens. There were 4 groups in Exp.1 (**E.1**) and **E.2**: antibiotic-free diet (**CTL-**); diet with antibiotic (**CTL+**); ARG and VE (**AVE**); ARG, VE and MOS (**AVM**). Birds were infected with 10^6 CFU/ml of a novobiocin and nalidixic acid resistant *Salmonella* enteric Serovar Typhimurium (**ST**) at d 7 (E.1) or at d 3 (E.2). In E.3 and E.4, there were three groups: CTL, AVE and AVM; at d 3, birds in E.3 were infected with 10^2 CFU/ml ST and birds in E.4 were infected with 10^6 CFU/ml ST.

The following were analyzed for four experiments: Oxidative burst heterophils response (**OBHR**) and monocytes response (**OBMR**); Lymphocyte proliferative response (**LP**); antibodies concentrations in serum; chicken body weight (BW), cecal *Salmonella* concentration; gut parameters. In E.1, both AVM and AVE diet decreased OBHR; birds fed AVM showed the highest IgA level, birds fed AVM had higher IgM than CTL+ and had the lowest *Salmonella* population. Birds fed AVE showed higher LP than CTL- in E.1 and E.2. From E.1 and E.2, AVM decreased chicken innate immune

response at a younger age and improved adaptive immune response when birds were older than 16 d. AVM reduced ST when administrated later (d 7) versus early (d 3).

In E.3, no BW and *Salmonella* counts differences were found; birds fed AVM had a higher IgA than AVE but similar to CTL at d 17. Chickens fed CTL had the highest OBHR at d 17, chickens fed AVM had the highest LPR at d 17.

In E.4, chickens fed AVM showed higher BW than CTL at d 17 and d 24. Also no *Salmonella* counts differences were found. Chickens fed AVE had higher LPR than chickens fed AVM at d 17. These results are consistent with results from E.1 and E.2.

Overall, diet AVM has different effects on chicken immune response when chicks were infected at different age; higher level of ARG and VE improved immune response and improve gut health.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Carey, and my committee members, Dr. Farnell, Dr. Byrd for their support and patience throughout this research. I also would like to thank my colleagues and friends, Stringfellow, Kendres and Bautista-Ortega, Jaime who were always there to provide help unconditionally. I feel grateful for the faculty and staff in the department of Poultry Science in Texas A&M University for providing the lab facilities and technical support for this research, also feel grateful for the instructors in Microscopy and Imaging center in Texas A&M University and millions of help from Mrs. Denise Caldwell in USDA-Southern Plains Agriculture Research Center in College Station.

Finally, thanks to my parents for their endless love.

NOMENCLATURE

ARG	L-Arginine
VE	Vitamin E
MOS	Mannan oligosaccharide
CTL-	Control negative, antibiotic diet
CTL+	Control positive, with antibiotic
AVE	Diet with arginine and vitamin
AVM	Diet with arginine, vitamin E and MOS
ST	<i>Salmonella</i> enteric Serovar Typhimurium
OBHR	Oxidative burst heterophils response
OBMR	Oxidative burst monocyte response
LP	Lymphocyte proliferation
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
VH	Villi height
VB	Villi base
LPR	Lamina propria
CD	Crypt depth
PI	Post infection
IBDV	Infectious bursal disease virus

NO	Nitric oxide
NOS	Nitric oxide synthase
CP	Crude protein
RPMI	Roswell Park Memorial Institute
DCF-DA	2,7-dichlorofluorescein
PMA	Phorbol-12-myristate-13 acetate
Con-A	Concanavalin A
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium abromide

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INTRODUCTION

***Salmonella* and Immune System**

Salmonellosis is a worldwide health problem. About 95% of cases of human salmonellosis are associated with the consumption of contaminated products such as meat, poultry, eggs, milk and seafood, and fresh produce (Foley and Lynne, 2008). According to the Center for Disease Control and Prevention, more than 2,300 types of *Salmonella* have been identified, but the two most common are *Salmonella* Enteritidis and *Salmonella* Typhimurium, which are to blame for more than half of the infections in humans. *Salmonella* Enteritidis infections are mainly from the consumption of contaminated eggs, and *Salmonella* Typhimurium infections are mainly from the consumption of contaminated meat.

Typhoid fever is a unique disease associated with *Salmonella enterica* Serovar Typhimurium and typically involves human-to-human transmission. Typical symptoms include gastroenteritis, diarrhea, abdominal cramps, vomiting and fever which develop within 6 to 72 h after infection and are self-limiting within 2 to 7 days (CDC, 2001; Pegues and Miller, 2005). More severe cases may lead to septicemia, osteomyelitis, pneumonia and meningitis which need antimicrobial therapy (Benenson et al., 1995).

In chickens, *Salmonella* Typhimurium (ST) usually causes no disease. Modern strains of chickens are intensively raised to maximize productivity at high stocking densities

This thesis follows the style of *Poultry Science*.

under environmental conditions that are often less than optimal, increasing the risk of disease (Klasing et al., 1998). In order to improve production, antibiotics are commonly used in commercial poultry production. However, the use of prophylactic levels of antibiotics has been linked to the development of antibiotic-resistant bacteria which have become a public concern. Therefore, it is necessary to find alternative ways to replace antibiotics in the poultry industry. Nutritional supplements in poultry feed have been shown to play an important role in improving chicken immune response (Klasing, 1998; Kidd, 2004).

Immune System

In mammals and birds, the defense against microbial infection is mediated by the early reactions of innate immunity and the later responses of adaptive immunity. Innate immunity (also called natural or native immunity) provides the early line of defense against microbes. It consists of cellular and biochemical defense mechanisms that are in place even before infection and are poised to respond rapidly to infections (Abul et al. 2006). These mechanisms react only to microbes (and to the products of injured cells), and they respond in essentially the same way to repeated infections. The principal components of innate immunity are 1) physical and chemical barriers, such as epithelial surfaces; 2) phagocytic cells (neutrophils, macrophages) and natural killer (NK) cells; 3) blood proteins, including members of the complement system and other mediators of inflammation; and 4) proteins called cytokines that regulate and coordinate many of the activities of the cells of innate immunity.

Polymorphonuclear leukocytes (PMNs) are vital cellular components of innate immunity and function by killing pathogenic microbes following phagocytosis. The primary PMN in poultry is the heterophil, the avian equivalent to the mammalian neutrophil. Like the neutrophil, avian heterophils are involved in the phagocytosis of invading microbes and foreign particles (Kogut et al., 2003). Heterophils are essential cellular components of the avian innate immune system. Other white blood cells such as monocytes, basophils also have the ability to kill pathogens. These granulocytic phagocytes kill pathogens by the release of toxic oxygen metabolites (oxidative burst) and the release of lytic enzyme and antimicrobial peptides (degranulation) (Farnell et al., 2003).

Monocytes-macrophages are important regulators with decisive functions in both innate and acquired immunity. They are the sensors and scavengers of microbial invasion and the amplifiers of signals by cytokine secretion and T-cell activation (Yamate et al., 2000; O' Mahony et al., 2008). Production of NO by activated monocytes and macrophages is an important innate immune response, critical for bactericidal activity (Bogdan et al., 2001). Induction of iNOS in chicken macrophages has been shown to associate with different TLR4 expression in chicken macrophages.

Recognition of potential pathogenic microbes by the innate immune system is the function of a class of cellular receptors known as the pattern recognition receptors (PRRs), which include the toll-like receptors (TLRs) (Kogut et al., 2003). Toll like receptors have been shown to be functionally and structurally conserved among species. Eleven TLRs have been identified in humans and mice, with each member recognizing

and responding to different microbial components. Chicken TLR 4 has been cloned and its allelic variation has been linked to resistance to infection with *Salmonella enterica* Serovar Typhimurium in chickens (Leveque et al., 2003).

Intestinal Mucosa

Reducing *Salmonella* levels in the intestine of broiler chickens requires understanding of the interactions between *Salmonella* and the intestinal barriers that represent the first line of defense (Fasina et al., 2010). Such barriers include the mucus layer (composed of mucins secreted by goblet cells) and the underlying epithelium. Both the mucus layer and the underlying epithelium are components of the intestinal mucosal innate immune system and are the first line of defense against invading microorganisms (Bland et al., 2006; Delbridge and O' Riordan, 2007; Werling and Coffey, 2007; Xiao et al., 2004). The intestinal epithelium serves as a barrier that prevents uncontrolled passage of partially digested food, bacteria, and bacterial products into the host and also regulates fluid and electrolyte absorption and secretion.

The mucosa of small intestinal mucosa is arranged into two fundamental structures: villus and crypt. Villi are projections into the lumen covered predominantly with mature, absorptive enterocytes, along with occasional mucus-secreting goblet cells (Bowen, 2006). Crypts are tubular invaginations of the epithelium around the villi, lined largely with younger epithelial cells, which are involved primarily in secretion. The lamina propria contains capillaries and a central lacteal (lymph vessel) in the small intestine, as well as lymphoid tissue. Lamina propria also contains glands with the ducts

opening on to the mucosal epithelium that secrete mucus and serous secretion (Bowen, 2006). According to Uni et al. (1998) and Applegate et al. (1999) mucosa development consists in the increase in the height and density of the villi, which corresponds to an increase in the number of their epithelium cell. Longer villi represent bigger absorption area.

Fasina et al. (2010) showed that *Salmonella* Typhimurium infection increased goblet cell density, reduced villi surface area, increase the incidence of epithelial exfoliation and increased the incidence of heterophil influx into the lamina propria.

Adaptive Immunity

In contrast to the innate immunity, other immune responses are stimulated by exposure to infectious agents and increase in magnitude and defensive capabilities with each successive exposure to a particular microbe (Sinha, 2006). Because this form of immunity develops as a response to infection and adapts to the infection, it is called adaptive immunity. The defining characteristics of adaptive immunity are exquisite specificity for distinct molecules and an ability to remember and respond more vigorously to repeated exposure to the same microbe (Abul, 2006). The adaptive immune system is able to recognize and react to a large number of microbial and non-microbial substances. The main components of adaptive immunity are cells called lymphocytes and their secreted products, such as antibodies (Abul, 2004). Foreign substances that induce specific immune responses or are the targets of such responses are called antigens.

There are two types of adaptive immune responses, called humoral immunity and cell-mediated immunity. Humoral immunity is mediated by B lymphocytes or B cells. Antibodies recognize microbial agents, neutralize the infectivity of the microbes, and target microbes for elimination by various mechanisms (Zoher, 2006). Humoral immunity is the principal defense mechanism against extracellular microbes and their toxins because secreted antibodies can bind to these microbes and toxins and assist in their elimination. Different from innate immunity, these antibodies can remember and produce memory cells to this particular microbe. Antibodies themselves are specialized, and different types of antibodies may activate different effector mechanisms (Zoher, 2006). Different from mammals, birds produce B-lymphocytes in the bursa of Fabricius (Michael, 2008). Cell mediated immunity, also called cellular immunity, and is mediated by T lymphocytes (also called T cells). Intra cellular microbes, such as virus and some bacteria survive and proliferate inside phagocytes and other host cells, where there are inaccessible to circulating antibodies. Defense against such infections is a function of cell-mediated immunity, which promotes the destruction of microbes residing in phagocytes of the killing of infected cells to eliminate reservoirs of infection (Abul, 2004).

Antibodies

An antibody, also known as an immunoglobulin, is a large Y-shaped protein used by the immune system to identify and neutralize foreign objects like bacteria and virus (Janeway, 2001). Evidence from laboratory models and from epidemiological

observations in humans and other animals indicates a crucial role for antibody in protection against lethal septicemic infections (Goh et al., 2011). Immunoglobulin A (IgA), immunoglobulin M (IgM) and immunoglobulin G (IgG) have already been detected in serum, intestinal tract, oviduct or bile of birds challenged with different *Salmonella* strains and are usually considered as a measure of active immunity (Berndt et al., 2007). In mucosal secretory immune system, IgA play an important role in defense against disease. Studies of humans with mucosal humoral immunodeficiency suggest that the absence of secretory IgA leads to an increase in mucosal infection. In chicken, immunoglobulin A inhibits the attachment and penetration of bacteria in the lumen, increases the production of mucus (McKay and Perdue, 1993) and prevents inflammation that could cause epithelial tissue damage (Russell et al., 1989). Berndt and Methner. (2004) found out that IgM and IgA secretory cells are of importance in the cecal immune response of chickens against *Salmonella* strains.

IgG molecules are key players in the anti-*Salmonella* antibody response. In enteric infections, IgG bind to bacteria in their transient extracellular phase and enhance the antibacterial functions of phagocytes when the microorganisms are recaptured by these cells. IgG is the most abundant antibody class in human serum, and is also the dominant antibody class in human immune serum from patients in areas of endemic typhoid fever (Goh et al., 2011). In chicken, IgY is equivalent to human IgG, and is also an important antibody against disease, although IgY differs from mammalian IgG in terms of structural and immunological properties (Higgins, 1975).

Nutritional Supplementation

Since *Salmonella* causes serious health problem and economic losses and antibiotic treatment has caused public concerns, nutritional supplementation could play an important role in the control of bacteria. The main problem from antibiotic treatment is antibiotic-resistant bacteria, which is a huge threat to human beings. Many studies about functional food such as probiotics and prebiotics against *Salmonella* are well documented (Zubillaga et al., 2001). Also, there are many studies about the effect of amino acid and antioxidant against *Salmonella*.

L-Arginine on Chicken Immune Response and Chicken Performance

Arginine (ARG) is an essential amino acid in chickens and has been shown to boost humoral and cellular immune response to experimental infection challenges. Tayade et al. (2006) found that ARG supplementation increased the antibody response to infectious bursal disease virus (IBDV), reduced the immunosuppressive effects of IBDV vaccines, and significantly enhanced the proliferative response to IBDV stimulation. Kwak et al. (1999) found that ARG influenced lymphoid organ development with a more pronounced effect to the thymus and spleen than on the bursa of Fabricius. Qiao et al. (2005) found that ARG can improve the intestinal immunity and reduce bacterial and endotoxin translocation in rats. The percentage and absolute number of heterophils and the heterophil to lymphocyte ratio in the peripheral blood of birds infected with infectious bronchitis virus significantly increased as dietary ARG increased (Lee et al., 2002). Also, in the same study, the results showed that ARG supplementation altered the

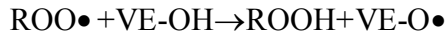
percentage of CD8⁺ cells under infectious bronchitis challenge. All these different animal and human experiments convincingly suggest that ARG is an important modulator of immunity.

The importance of ARG supplementation is mainly because of nitric oxide. Nitric oxide (NO) has also been described as a potent agent capable of limiting the growth of not only *Salmonella* Typhimurium but also that of other intracellular parasites (Eriksson et al., 2003). Previous works suggest that NO contributes in a critical way to host defense during *Salmonella* infection. In the last two decades, studies already showed that growth of *Salmonella* can be inhibited in vitro by compounds that generate NO or its metabolites (Saito et al., 1991). Also, in vitro experiments reported by Vazquez-Torres et al. (2000) demonstrated that macrophage killing of *Salmonella* involves coordinated actions of reactive oxygen intermediates and NO. Higher ARG level produces more NO, which increase the ability of killing *Salmonella*. Therefore, supplemented ARG in poultry feed at higher levels than those recommended by the NRC (1994) may be necessary.

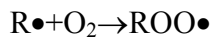
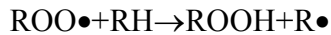
Vitamin E on Chicken Immune Response and Chicken Performance

Vitamin E (**VE**) is essential for the integrity and optimal function of the reproductive, muscular, circulatory, nervous, and immune system. As a primary antioxidant of cell membranes, VE is particularly important for the prevention of fatty acid peroxidation (Benedich, 1990; Young and Woodside, 2001). When lipid hydroperoxides (ROOH) are oxidized to peroxy radicals (ROO•), as could occur in the

presence of free metals such as iron or copper, the ROO• react faster with α -tocopherol (VE-OH) than with polyunsaturated fatty acids as shown in the following equation:



In the absence of vitamin E the reaction will be as follows:



In this way, α -tocopherol acts as a chain breaking antioxidant, preventing the further auto-oxidation of PUFAs in membranes or lipoproteins.

It is most likely that VE, like other nutritional factors, affects the development and maintenance of immunocompetence through multiple functions, by acting directly on the immune cell or by indirectly altering metabolic and endocrine parameters which in turn influence immune function (Gershwin et al., 1985). In poultry there are evidences that VE improve immune response. Erf et al. (1998) found that 7 wk old chickens fed with VE showed a higher percentage of CD4+CD8- thymocytes and a higher CD4+CD8- to CD4-CD8+ thymocyte ratio. Niu et al. (2009) fed broiler chickens with VE at 0, 100 or 200 mg / kg of feed, and found that the numbers of abdominal exudate cells, percentage of macrophages, phagocytic macrophages and internalized opsonized and unopsonized sheep red blood cells (SRBC) were increased with higher levels of VE. Singh et al. (2006) reported that chickens receiving 200 mg VE / kg of feed and 0.2 mg of selenium produced significantly higher hemagglutination inhibition antibody titers. Boa-Amponsem et al. (2000) showed that after feeding high levels of VE, 6 and 20 days after injection with SRBC, the ratio of heterophil to lymphocytes

increased, suggesting an improved phagocytic ability of the immune system.

However, VE supplementation does not always improve immune response. The effects of VE appear to be influenced by several factors including age and dietary levels. For instance, Leshchinsky and Klasing. (2001) fed chickens with VE at 0, 10, 17.5, 25, 37.5, 50, 100 and 200 IU/kg of feed, and immunity was assessed as antibody production to infectious bronchitis virus (IBV), SRBC, and *Brucella abortus* (BA) antigens; they found that moderate (25 to 50 IU/kg) levels of VE supplementation were most effective than higher levels. Friedman et al. (1998) reported that high levels of VE worsened the antibody production to Newcastle disease virus and *E. coli* in chickens and turkeys. Qureshi et al. (1993) and Marsh et al. (1981) reported that VE supplementation (100 or 250 IU/kg of diet) did not affect antibody production of chicks, although it increased the number of macrophages (Qureshi et al., 1993).

Prebiotics on Chicken Immune Response and Chicken Performance

Prebiotics are non-digestible feed ingredients which have the capacity to stimulate the growth or metabolic activity of some beneficial intestinal microorganisms, improve the integrity of intestines, and also improve the live performance of chickens (Ferket et al., 2002; Baurhoo et al., 2009).

There is currently a great deal of interest in the use of prebiotic as functional feed ingredients to manipulate the composition of colonic microflora in order to improve health (Aryana and McGrew, 2007; Coppa et al., 2006). Prebiotics are considered a favorable alternative over antibiotics for poultry, because they promote competitive

exclusion of pathogenic microbes and selective colonization by beneficial microbes (Janardhana et al., 2009). Of the known prebiotics, mannanoligosaccharides (MOS) have been tested extensively in poultry (Janardhana et al., 2009).

Mannanoligosaccharides are present in the cell wall of yeast, and contains high affinity ligands for bacteria (Ofek et al., 1977). Mannose is the main component of MOS and is unique because it is bound by the type 1 fimbriae used by many enteric bacteria to attach to host cells, therefore, mannose can result in the movement of undesirable bacteria through the intestine without colonization (Newman, 1994). Janardhana et al. (2009) showed that MOS-fed broilers exhibit significantly greater weight gain, improved feed conversion and lower mortality. Pelicano et al. (2004) reported an improvement in weight gain and feed efficiency when MOS (1.1g/kg) was supplemented to broiler chicken from 1 to 21d. Prebiotics also have important role in modulating chicken immune response. Verduzco et al. (2009) found that dietary supplementation of 0.05% of yeast cell walls increased local mucosal IgA secretions, humoral and cell-mediated immune responses and reduced parasite excretion in feces. Cetin et al. (2005) reported that IgG level were increased by MOS supplementation in turkeys and Woo et al. (2007) reported that IgG was increased by MOS supplementation in layers. However, Janardhana et al. (2009) found that the addition of MOS to the diet resulted in a significant reduction in the proportion of B cells and in mitogen responsiveness of lymphocytes in cecal tonsil; in addition, he also found that MOS supplementation reduced the level of IgA, IgM and IgG. Recent studies on broilers (Kim et al., 2011) showed that 0.05% MOS supplementation reduced the heterophil to lymphocyte ratio

compared to a control diet, whereas the concentration of plasma IgA and IgG was not affected by the diet.

Spring et al. (2000) utilized bacterial isolation chambers to detect the effects of MOS on *Salmonella* inoculated birds, the results showed that upon challenge with *Salmonella* Typhimurium 29E the number of bacteria in the MOS treatment group were lower than those in the control group. In a similar series of trials performed with *S. dublin*, which typically colonizes the intestine at low concentrations, MOS decreased the number of *Salmonella*-positive birds from 89.8% to 55.7%. *Salmonella* counts in the cecum, liver and spleen were all lower with the use of MOS though no differences between 0.1 and 0.2% inclusion rates were observed. Overall, from previous studies, the effects of MOS on immune response are not consistent. However, those studies refer to different types of birds at different age, and there are other factors such as stress, that may also play an important role in the effect of MOS on broiler chicken performance, immune response and intestinal morphology (Burkholder et al., 2008).

Abdukalykova and Ruiz-Feria (2006) and Abdukalykova et al. (2008) showed that ARG and VE have complementary or synergistic effects on the immune response against several antigens, suggesting that the combination of ARG and VE could be used to boost the immune response and improve the health of broilers and potentially reduce the prophylactic use of antibiotics. We hypothesize that ARG, VE and prebiotics (MOS) will improve the immune response and will accelerate *Salmonella* clearance after an experimental challenge. The objectives of the present experiments were to evaluate the effects of ARG, VE and prebiotics on *Salmonella* levels in the ceca, monocyte and

heterophil oxidative burst, lymphocyte proliferation in vitro, improve intestinal health and antibody titers after an experimental challenge in broiler chickens.

MATERIALS AND METHODS OF EXP.1 AND EXP.2

Animals and Treatments

Two experiments were conducted. One-day-old commercial broiler chicks (Cobb 500) were obtained from a local hatchery and housed in a bio safety facility at the USDA-Southern Plains Agriculture Research Center in College Station. Chicks were grown on fresh pine litter. The birds were brooded following standard temperature regimes, which gradually decreased from 32°C to 24°C, and under a 16:8 light:dark cycle throughout the studies. All the birds were fed a corn-soybean meal-based diet formulated to meet or exceed all of the NRC (1994) requirements (Table 1); the basal diet contained 23% of CP, 3,200 kcal ME / kg, 1.54% ARG and 40.3 IU VE / kg. A completely randomized design with four dietary treatments (800 birds in Exp.1, 200 birds in each treatment; 160 birds in Exp.2, 40 birds in each treatment) was used, as follows: the basal diet free of antibiotics (negative control diet, CTL-), the basal diet plus 40 mg bacitracin / kg feed (positive control, CTL+), the basal diet free of antibiotics supplemented with ARG (0.4% in Exp.1 and 0.8% in Exp.2) and 40 IU / kg of VE (for a total content of 1.94 [Exp.1] and 2.34% [Exp.2] ARG and 80 IU of VE / kg of feed, AVE), or the basal diet free of antibiotics supplemented with ARG and VE at the same levels plus 0.2 % MOS (BioMos, Alltech Co., Lexington KY; AVM). The experimental procedures were approved by the institutional Animal Care Committee.

Table 1. Composition of basal diet and calculated analysis (Exp.1, Exp.2, Exp.3 and Exp.4)

Ingredients	Percentage
Composition	
Corn	50.523
Soybean	37.892
FAT	7.218
Limestone	1.624
Biofos	1.624
Salt	0.451
Vitamins ²	0.253
Lysine HCl	0.090
Trace Minerals ³	0.054
Calculated analysis	
Energy (kcal/kg)	3,265
CP (%)	24.4%
Arg (%)	1.53%
Met(%)	0.53
Lys (%)	1.36
Cal (%)	0.95
Available P (%)	0.45

¹ Basal diets were completed to 1,000g by adding the feed additives, and inert filler, or both, for a total of 15g/kg.

² Provided the following per kilogram: Vitamin D₃: 3856.12IU, Vitamin K: 1.56mg; Vitamin A: 10.35 KIU; Vitamin E: 39.02 IU.

³ Provided the following per kilogram of feed: Mg, 299.8g; Zn: 251.3g; Fe: 33.07g; Cu: 3.3g; I: 2101g; Se: 500.45g; Ca: 12.57g.

Bacteria

A primary poultry isolate of ST was obtained from the National Veterinary Services Laboratory (Ames, IA) and selected for resistance to novobiocin and nalidixic acid, and maintained in media containing 25 µg of novobiocin and 20 µg of nalidixic acid / ml. Portions (1 to 2 ml) of cultures grown overnight in tryptic soy broth (Difco Laboratories, Detroit, MI) were used as inocula for challenging broilers.

Cecal Colony Counts

At d 7 (Exp. 1) or d 3 (Exp. 2), all chicks were orally challenged with 10^6 CFU/ml of *Salmonella* Typhimurium. Ten birds per treatment were euthanized at 3, 10 and 14 days after challenge (Exp. 1) or at 7, 14 and 21 days after challenge (Exp. 2). All broilers were killed by cervical dislocation, Cecal contents (0.25 g) were aseptically collected and deposited in 2.25 ml of phosphate buffer (pH 6.5). Samples were thoroughly mixed, serially diluted in phosphate buffer, and then spread plated onto XLT4 Agar (Becton, Dickinson and Company sparks, USA) containing 20 µg of nalixidic acid / ml and 25 µg novobiocin / ml. Plates were incubated for at least 24h at 37 °C, and the number of *Salmonella* CFU per gram was determined. *Salmonella* colony number was log-transformed prior to statistical analysis. For qualitative enrichment, cecal contents were enriched in Rappaport-Vassiliadis Broth (Difco) for 24 h at 37 °C, streaked on XLT4 agar plates, and after 24 h of incubation at 42° C, plates were read as positive or negative.

Blood Collection

Blood samples were taken from the jugular vein at 5 and 9 d (Exp.1) after challenge or at 7 and 14 d after challenge (Exp.2). In each experiment, 180 ml of blood from each experimental group were collected using EDTA (EMD Chemicals Inc., Gibbstown, N.J) as anticoagulant, the blood samples were separate evenly into four 50 ml tubes as four gradients, and the gradients were used for oxidative burst and lymphocytes proliferation assays. All blood samples were kept on ice until analysis.

Leukocyte Isolation

In this study, we use the same leukocyte isolation and oxidative burst assay as Stringfellow et al. (2011) did in their research. Briefly, the blood was mixed with a solution of 1% methylcellulose (Sigma-Aldrich), dissolved in Roswell Park Memorial Institute (**RPMI**) 1640 media (Mediatech Inc., Herndon, VA) at a ratio of 1:1.5 and then centrifuged at 250 x g for 15 min. The supernatant was removed and resuspended in Hank's balanced salt solution (HBSS) without Ca and Mg (Mediatech Inc.). The resulting suspension was layered over a 1.077/1.119 histopaque (Sigma-Aldrich) discontinuous gradient and centrifuged at 500 x g for 60 minutes. The top layer was collected for the mononuclear cell fraction and the interface of the 2 gradients was collected for heterophils. Cells types were then separated into different conical tubes and washed with 45 ml of RPMI-1640 by centrifugation at 500 x g for 30 min. Following the RPMI-1640 wash, a "Complete" solution of RPMI-1640 was then added to reconstitute the cell. The solution consisted of 5% fetal bovine serum (Atlanta

Biologicals, Lawrenceville, GA); 4 mM L-glutamine, 10,000 U penicillin/ml, 10 mg streptomycin / ml (JR Scientific Inc., Woodland, CA); 1 mM sodium pyruvate (Mediatech Inc.) and 0.1 mM non essential amino acids (MP Biomedicals, LLC, Solon, OH). All agonists and substrates were suspended in this complete RPMI-1640. Working concentration of 4×10^6 heterophils / ml and 1×10^7 mononuclear cells / ml and kept in ice until use. Cell viability was determined by a commercially prepared trypan blue solution (Sigma-Aldrich).

Oxidative Burst Assay

Oxidative burst activity of heterophils and monocytes was measured using a Wallac fluorescent plate reader (Perkin Elmer, Boston, MA) and an indicator of reactive oxygen species, 2,7-dichlorofluorescein (**DCF-DA**; Sigma-Aldrich) as previously described (Xie et al., 2002). Cells (1 ml) were preincubated for 30 minutes with 125 μ L of phorbol-12-myristate-13-acetate (**PMA**; 20 μ g / ml of cells, Calbiochem, La Jolla, CA) at 42°C in a water-jacketed 5% CO₂ incubator. An equivalent volume of complete RPMI-1640 was added for the negative control treatments. Immediately after the preincubation period 125 μ l of DCF-DA (0.2 mg / ml) was added and samples were mixed and aliquoted into a clear 96-well flat-bottomed plate (24 wells per treatment). Oxidative burst was then measured at an excitation/emission wavelength at 485/ 530 nm.

Lymphocyte Proliferation

After isolation, heterophil and monocyte cells were placed into plates with equal volumes of the lymphocyte suspension and Concanavalin A (Con-A) (Sigma-Aldrich) which was used as a mitogen. An equivalent volume of complete RPMI-1640 was added in replacement of the Con-A solution for the negative control treatment. Plates were then incubated in 5% CO₂ at 42 °C for 24 hours. Fifteen µL of stock 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5mg/ml) was added to each well and plates were incubated for 4 h. Immediately after incubation a MTT substrate releasing compound was added to the cells, resulting in a color change which allows to assess the viability and the proliferation of cells. The color change was analyzed by a colorimetric plate reader (Wallac Victor-2 1420 Multilabel Counter) at 550nm.

ELISA

Eight blood serum samples per treatment, from 19 d-old-chickens (Exp.2), were used to measure the concentrations of IgA, IgG and IgM isotypes. Serum samples were allowed to clot at room temperature for 4 h, samples were then centrifuged at 400x g for 8 min and the serum was collected and stored at -80°C until assayed. The serum samples were used to measure the concentrations of IgA, IgG, and IgM isotypes, using chicken IgG, IgA, or IgM ELISA quantitation kits (Bethyl Laboratories, Montgomery, TX). Briefly, flat-bottomed microtiter plates were coated for 60 min with capture antibody (Goat anti-chicken IgG-Fc or IgM or IgA affinity purified) and coating buffer (0.05 M

carbonate-bicarbonate, pH 9.6). Plates were washed three times with wash solution (50 mM Tris buffered saline, 0.14 M NaCl, 0.05% Tween 20, pH 8.0), and wells were incubated with blocking (postcoat) solution (50 mM Tris buffered saline, 0.14 M NaCl, 1% BSA, pH 8.0) for 30 min, then rinsed three times with wash solution. The calibrator (Chicken reference serum) and sample/conjugate diluent (50 mM Tris buffered saline, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0) were used to do standards, whereas serum samples, thawed at 4°C overnight, were diluted at 1:1000 in the sample/conjugate diluent. Then, they were incubated in wells for 60 min, and washed five times with wash solution. Detection antibody HRP (Goat anti-chicken IgG-Fc or IgA or IgM) diluted in sample/conjugate diluent was added to wells, incubated for 60 min, and rinsed five times with wash solution. Enzyme substrate (3,3',5,5'-Tetramethyl benzidine (TMB) peroxidase substrate & peroxidase solution B) was added and incubated for 15 min (IgA or IgM) or 30 min (IgG). Finally, 2 M H₂SO₄ was used to stop the TMB reaction. A microtiter plate reader (Wallac Victor-2 1420 Multilabel Counter) was used to measure the absorbance at 450 nm. To calculate the immunoglobulin (IgG, IgA or IgM) concentration a four parameter logistic curve-fit was develop using the chicken reference serum absorbance.

Morphology of Jejunum

At d 10 and 14 (Exp.1), 10 birds from each treatment were randomly selected, and were killed by cervical dislocation. Tissue samples for histology were taken from the jejunum at the junction of the Meckel's diverticulum and fixed in 4% buffered

formalin, processed and embedded in paraffin. Tissue sections (5- μm thick, 6 cross-sections per sample) were cut, fixed and stained with eosin and hematoxylin on slides for further measurements using a light microscope (Zeiss Axiophot, USA). The microscope was equipped with color and monochrome CCD cameras. Image acquisition was controlled via the MetaView software and also by the free Micro-Manager software. Ten readings per sample for each parameter were recorded including villi height (μm , VH), villi base (μm , VB), lamina propria (μm , LPr), and crypt depth (μm , CD). VH was measured from the villi tip to the villi-crypt junction, and the VB was measured from the middle space between two villi to the next space. LPr was measured from the villi tip to the muscle layer, and CD was defined as the depth of the invagination between 2 villi. The mean VH, VB, LPr and CD from 10 birds were expressed as a mean VH, VB, LPr and CD for 1 treatment. To record measurements the samples were light-micro photographed at three different fields, and the Image J software was used to measure the photographs. The unit of the measurement from the software is pixel, and according to the microscopes manufacturer's instruction, measures were calculated as follows: 2.5X: 1.4731 μm per pixel; 5X: 0.7746 μm per pixel; 10X: 0.3865 μm per pixel.

Statistic Analysis

All data were analyzed using a one-way ANOVA (Sigma Stat software) and the means were separated using the Tukey's multiple comparison method. Significance was declared at* $P < 0.05$.

RESULTS AND DISCUSSION OF EXP.1 AND EXP.2

The objectives of the experiments were to evaluate the effects of ARG, VE and MOS on 1) Chicken performance by measuring chicken body weight and feed conversion; 2) heterophil and monocyte oxidative burst; 3) lymphocyte proliferation; 4) immunoglobulin A (IgA), immunoglobulin G (IgG) and immunoglobulin M (IgM) concentration in serum; 5) morphology of intestine in birds after an experimental challenge of *Salmonella*; and 6) *Salmonella* colony counts in the cecal content of infected birds.

Chicken Body Weight and Feed Conversion

Based on previous studies, there are some studies of the effect of ARG and VE on chicken performance and immune system, but there are no studies of the combination of arginine (ARG), vitamin E (VE) and MOS on chicken body weight, cecal *Salmonella* population, intestinal morphology, and immune response compare with the effect of antibiotics in the diet. We hypothesized that diets with supplemental ARG, VE, and MOS will improve chicken immune response and will reduce *Salmonella* colonization in the ceca of broiler chickens. Two experiments were conducted to investigate the effects of ARG, VE, and MOS, alone or in combination, on chicken performance, intestinal morphology and immune response, using antibiotic-free diets. Baurhoo et al. (2007) reported that neither MOS (0.2% or 0.5%) nor antibiotics improved live performance characters (BW, FI, and FCR) of broilers compared with a non-supplemented die

Table 2. Effect of arginine, vitamin E, and mannanoligosaccharides on broiler chicken body weight (BW, gram) and feed conversion (FC) after challenged with 10^6 CFU/ml *Salmonella* Typhimurium (Exp.1)

Treatments		CTL-	CTL+	AVE	AVM
Day 1	BW	40.9±0.2	40.2±0.5	40.0±0.3	40.8±0.4
Day 7	BW	152.7±7.9	168.4±8.4	162.0±4.0	163.8±1.7
Day 14	BW	337.1±9.9	373.0±11.9	357.3±14.1	366.5±6.5
	FC	2.24±0.1	2.12±0.08	2.03±0.08	2.04±0.03
Day 21	BW	681.5±20.9	724.4±13.0	705.6±26.3	699.5±10.1
	FC	2.01±0.02	2.06±0.04	2.04±0.05	2.1±0.04
Day 28	BW	1163.7±27.0	1148.8±37.2	1260.0±21.1	1157.1±31.6
	FC	2.5±0.02	2.75±0.22	2.5±0.07	2.6±0.16
Mortality		4%	1%	6%	1.5%

N=4

Mean±SEM of 200 chickens per treatment.

CTL-=antibiotic-free diet; CTL+=commercial-type diet with 40 mg bacitracin/kg

AVE= antibiotic-free diet plus 0.8% L-Arginine and 80 IU/kg Vitamin E

AVM=AVE diet supplemented with 0.2% manna oligosaccharides (Alltech Inc., Nicholasville, KY)

The use of antibiotics to enhance growth and feed efficiency and reduce mortality in broiler production was introduced without rigorous testing as to efficacy some 50 years ago (Libby and Schaible, 1955; Stokstad and Jukes, 1958; Waibel et al., 1954). However in order to make antibiotic work effectively, there are many factors to consider; for instance, Roura et al. (1992) reported that the lack of growth improvement due to MOS or antibiotic supplementation was due to a clean environment. In this study, the nutritional supplementation of ARG, VE, and MOS (AVM) or the use of antibiotics in the feed (CTL+) reduced the mortality compared with birds fed the CTL- or the AVE diet (Table 2).

Oxidative Burst Heterophil

Heterophils play critical roles in the innate immunity as the first line of cellular defense against microbial infection. Activation of heterophils has been shown to protect against *Salmonella* organ invasion and *Salmonella*-induced mortality in neonatal poultry (Haiqi et al., 2003). Basically, like their mammalian counterpart neutrophils, heterophils destroy invading microorganisms by producing toxic Table1 Composition of basal diet and calculated analysis (Exp.1, Exp.2, Exp.3 and Exp.4).

In Exp.1, birds fed the AVE diet had a decreased heterophil oxidative burst (HOB) both 5 and 9 d after challenge compared to birds fed the CTL- diet; also, birds fed the AVM diet also had a lower HOB both 5 and 9 d after challenge, compared to birds fed the CTL- diet (Figure 1). ARG is the precursor of nitric oxide (NO) through the NO synthesis pathway improving macrophage function (Eriksson et al., 2003). Lee et al.

(2002) reported that the percentage and absolute number of heterophils and the heterophil to lymphocyte ratio in the peripheral blood 1 d post infection during infectious bronchitis challenge significantly increased as dietary ARG increased. However, the effects of ARG supplementation on immune function are not consistent; the inconsistent effects of ARG supplementation on immune function are due to numerous factors, such as the amount and timing of ARG supplementation, the animal species or strain of species, and the experimental model (Carmelo and Henken, 2002). Some researchers have shown that IL-2 can directly activate chicken heterophils to exert effector functions, but rChIL-2 is also an age-dependent event. IL-2 significantly increased the phagocytosis and bactericidal activity of heterophils from 7- and 14 d-old chickens (Kogut et al., 2002). Other researchers found that dietary ARG supplementation does not enhance lymphocyte proliferation or interleukin-2 production in young or aged rats (Ronnenberg et al., 1991). This might be another explanation why birds fed the AVE or the AVM diet had reduced heterophil function 5 and 9 d after challenge in Exp.1.

In Exp.2, birds were challenged at d 3. Seven d after challenge birds fed the AVE diet had a higher HOB than birds fed the CTL+ or the AVM diet, whereas birds fed the CTL- diet had an intermediate HOB (Figure 1).

Fourteen d after challenge, birds fed AVE had the highest HOB response compared with the other treatments. These results are in agreement with previous reports (Abdukalykova and Ruiz-Feria, 2006) indicating that the combination of ARG and VE improve cellular and humoral immune response. In these two experiments, MOS

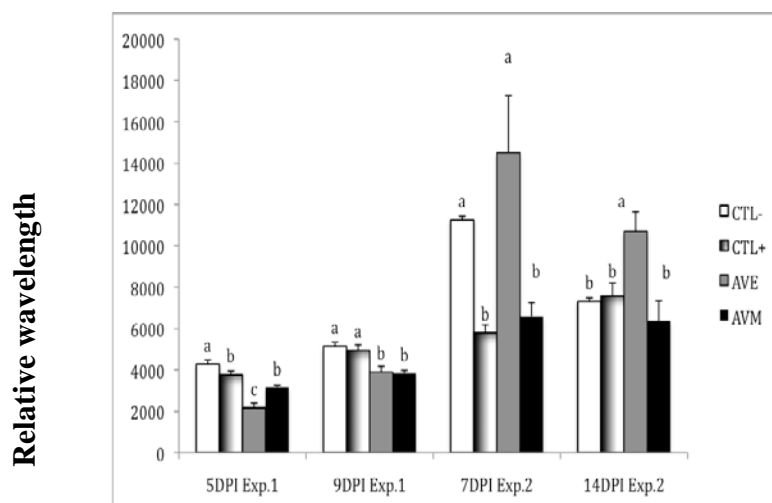


Figure 1. Effects of feeding an antibiotic-free diet (CTL-), an antibiotic-free diet plus 40 mg bacitracin / kg feed (CTL+), a CTL- plus L-Arginine (0.4% in Exp.1, 0.8% in Exp.2) and 80 IU of vitamin E /kg (AVE) feed, or AVE plus 0.2% mannanoligosaccharides (AVM) on chicken oxidative burst of heterophiles of Exp.1 and Exp.2. Heterophils were isolated from pooled peripheral blood of 10 broiler chickens per treatment at 5 and 9 days (Exp. 1, challenged at day 7 , or at 7 and 14 days (Exp. 2, challenged at day 3) after a challenge with 10^6 CFU of *Salmonella* Typhimurium. PMA was used as a positive control treatment, whereas an equal volume of RPMI 1640 media was used as a negative control treatment. Eight observations were read per gradient and each treatment had four gradients. Data is presented as the mean relative fluorescence difference from the positive control to the negative control with the SEM. ^{a-c} Values with different letters within a sampling period are different (Tukey test, $P < 0.05$).

somehow negated the effect of ARG and VE, suggesting that MOS may decrease innate immunity. Further studies need to be done to further clarify the effect of MOS on the innate immune system.

Oxidative Burst Monocyte

Monocytes typically respond to microbes nearly as rapidly as heterophils do. Macrophages (monocytes in tissue) are involved in phagocytosis through oxygen independent mechanisms, in which NO formation is effective in helping macrophages to get rid of bacteria (Abul, 2006).

In Exp.1, 5 d after challenge, birds fed the CTL- diet had a similar monocyte oxidative burst response (MOB) compared with birds fed AVE, but these birds had a higher MOB than birds fed the CTL+ or the AVM diets (Figure 2). Birds fed CTL+, AVE and AVM had a similar MOB. It is well known that antibiotics are used for controlling bacteria, also the idea to use MOS in poultry feeds evolved from the concept that certain sugars, particularly mannose, could be used to largely block the colonization of intestinal pathogens such as *Salmonella* species and *E. coli* (Ferket et al., 2002). Byrd et al., (1998) stated that normal gut flora of the chick is established within the first week of age; this flora is important for the control of ingested pathogens, especially food-borne pathogens like *Salmonella* (Guarner and Malagelada, 2003). It is possible that normal flora in chicken intestine had not established very well 5 d after challenge, and antibiotic reduced the amount of bacteria, therefore reducing activation of the immune

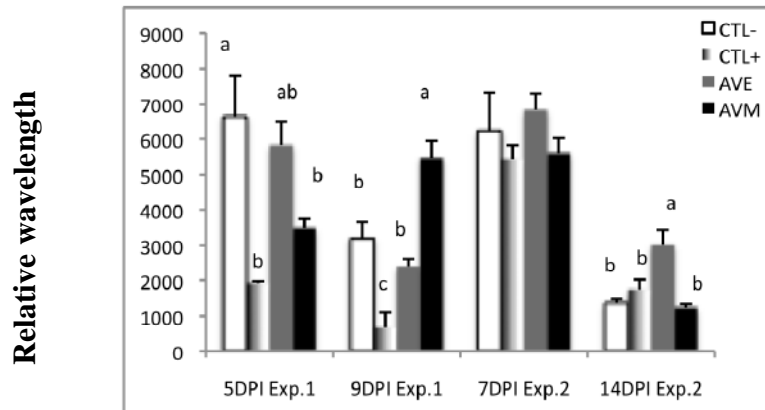


Figure 2. Effects of feeding an antibiotic-free diet (CTL-), an antibiotic-free diet plus 40 mg bacitracin / kg feed (CTL+), a CTL- plus L-Arginine (0.4% in Exp.1, 0.8% in Exp.2) and 80 IU of vitamin E /kg (AVE) feed, or AVE plus 0.2% mannanoligosaccharides (AVM) on chicken oxidative burst monocytes of Exp.1 and Exp.2. Monocytes isolated from pooled peripheral blood of 10 broiler chickens per treatment on 5 and 9 days (Exp. 1, challenged at day 7) or 7 and 14 days (Exp. 2, challenged at day 3) after a challenge with 10^6 CFU of *Salmonella* Typhimurium. An equal volume of RPMI 1640 media was used as a negative control treatment. Eight observations were read per gradient and each treatment has four gradients. PMA was used as a positive control treatment *in vitro*. Data is presented as the mean relative fluorescence difference from the positive control to the negative control with the SEM.

^{a-c} Values with different letters within a sample period are different (Tukey test, $P < 0.05$).

system compared with birds fed the CTL- diet, explaining why fed CTL+ and AVM had lower MOB than CTL-. In addition, the MOB was not different between birds fed CTL- and AVE diets (Figure 2). Abdukalykova and Ruiz-Feria (2006) showed that ARG and VE improve the cellular and humoral immune response of broiler chickens, but they did not show that ARG and VE improved humoral and cellular immune response when birds were challenged at an early age (d 7). Adding MOS to the AVE diet (AVM) may cause *Salmonella* to bind to the MOS, reducing activation of the immune system, explaining why birds fed CTL+ or AVM diets had lower MOB than birds fed the CTL+ diets.

However, 9 d after challenge, birds fed AVM diet had the highest MOB whereas birds fed CTL+ had the lowest MOB (Exp.1). Birds fed the CTL- diet had a similar MOB compared with birds fed the AVE diet. At this age normal flora established compared to 5 d after challenge, and MOS could bind *Salmonella* while other bacterial populations kept growing. Mannan oligosaccharides have also been shown to enhance macrophage response in different animal species (Spring and Privulesu, 1998). At the same time, antibiotic still controlled all the bacteria in the intestine which created a lower activation of the immune system compared to birds fed CTL-.

In Exp.2, no difference was found 7 d after challenge in which birds were challenged at d 3. With the same concept, flora was not established at an early age, and it is possible that early *Salmonella* challenge destroyed the immune system and the challenge caused the immune system to develop slowly. With numerous pathogens to be bound and with small numbers of beneficial flora, it is reasonable that no difference was detected 3 d after challenge.

Fourteen d after challenge, birds fed AVE had the best MOB compared to the other treatments; no difference was detected among birds fed CTL-, CTL+ and AVM treatments. As birds were challenged at d 3, no MOB difference could be found. From the two experiments and the experiment from Abdukalykova and Ruiz-Feria in 2006, ARG and VE may not improve MOB at an early age, but they may improve MOB when birds are older than 17 d. Also, AVM may improve MOB at a relatively older age but not an early age of broiler chickens.

Lymphocyte Proliferation

Adaptive immune responses consist of distinct phases; the first three are recognition of antigen, activation (proliferation), and elimination (Abul, 2006). Lymphocyte proliferation stimulated by foreign antigens is the central event in adaptive immune response. An increased lymphocyte proliferation therefore refers to the ability of clones of antigen-specific lymphocytes to rapidly proliferate and differentiate into effector cells (Quan et al., 2007).

In Exp.1, birds were challenged at d 7. Five d after challenge birds fed AVE had a lower LP than those from other treatments, while birds fed with CTL-, CTL+ and AVM showed a similar LP (Figure 3). Nine d after challenge, birds fed AVE and AVM showed a significantly higher LP than birds fed CTL- and CTL+. No studies have reported that the combination of ARG and VE reduce LP. The inconsistent effect of ARG and VE on LP observed in Exp.1 may be due to the inconsistent effects of VE on immune function reported by others. The effect of VE was reported to be highly

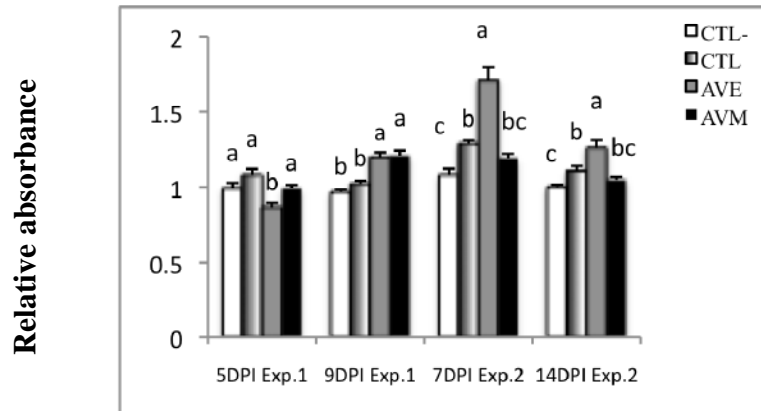


Figure 3. Effects of an antibiotic-free diet (CTL-), an antibiotic-free diet plus 40 mg bacitracin /kg feed (CTL+), a CTL- plus L-Arginine (0.4% in Exp.1, 0.8% in Exp.2) and 80 IU of vitamin E /kg (AVE) feed, or AVE diet plus 0.2% mannanoligosaccharides (AVM) on chicken lymphocyte proliferation response of Exp.1 and Exp.2. Lymphocytes were isolated from pooled peripheral blood of 10 broiler chickens per treatment on 5 and 9 days (Exp. 1, challenged at day 7) or 7 and 14 days (Exp. 2, challenged at day 3) after a challenge with 10^6 CFU of *Salmonella* Typhimurium. An equal volume of RPMI 1640 media was used as a negative control treatment. 8 observations were read per gradient and each treatment has four gradients. Concovalin A was used as a positive control treatment *in vitro*. Data is presented as the mean relative fluorescence ratio from the positive control to the negative control with the SEM.

^{a-c} Values with different letters within a sample period are different (Tukey test, $P < 0.05$).

variable, depending on the dose, the strain and age of the bird (Friedman et al., 1998; Leshchinsky and Klasing, 2001).

In Exp.2, in which birds were challenged at d 3, birds fed AVE had the highest LP activity both 7 and 14 d after challenge. In addition, there were no differences in LP among CTL-, CTL+ and AVM. This result suggested that diet AVE improved LP compared with diet CTL-, however, compared with the effect of AVE, additional MOS (AVM) reduced LP. This finding agreed with Finucane et al. (1999) and Spring et al. (2000) who reported that MOS reduces the pathogenic bacteria numbers in the intestine and prevents the acute immune response against such bacteria. Some research suggests that MOS supplementation results in significant improvement in antibody responses in broiler and layers (Cotter et al., 2000; Raju and Devegowda, 2002). However, other found that MOS enhances the protective antibody response to improve disease resistance while at the same time suppressing the acute phase response (Ferket et al., 2002). Studies of the immunosuppressive effects of mannan on human lymphocytes are well documented. These studies have shown that mannan can directly or indirectly block T or B cell receptor or depress proliferative response of T cells and inhibit the generation of B cell colonies in spleen or lymph nodes (Muchmore et al., 1990; Nelson et al., 1991; Jelinek et al., 1985).

In general, in birds fed AVE, LP was improved 9 d after challenge in Exp.1, and was improved at both 7 and 14 d after challenge in Exp. 2. At the two sampling points in Exp.2, and 5 d after challenge in Exp.1, diet AVM had no effect on LP compared with diet CTL-. However in Exp.2 birds fed AVM had a lower LP than birds fed AVE. This

is the first time that the combined effects of ARG, VE and MOS on LP are reported in the young chicken. The diet AVM had no effect on improving LP, which was against our hypothesis; however, further studies are needed in order to draw any conclusions.

ELISA of IgA, IgG and IgM

Innate immune mechanisms are not sufficient to prevent bacterial multiplication, thus adaptive and specific immunity is initiated to completely eradicate bacterial infections. Adaptive immune mechanisms are divided into cell mediated immunity, which acts predominantly through effector T cells, and humoral immunity, which operates by means of specific antibodies (Abul, 2006). Infected chickens are known to produce a humoral immune response of mainly immunoglobulin (Ig) A, IgG, and IgM. IgA, IgG and IgM were measured using ELISA 16 d after challenge in Exp.2.

Table 3 shows that birds fed AVM had the highest IgA concentration, whereas the rest of the treatments did not have significant differences. These results suggest that additional MOS increased IgA antibody titer which agrees with the findings of Savage et al. (1996) and Savage et al. (1997) who showed that phosphorylated mannan oligosaccharide (MOS) elevated plasma IgA in turkeys. MOS has been shown to increase the production of IgA in rats (Kudoh et al., 1999).

Table 3. Effects of arginine, vitamin E, and mannanoligosaccharides on serum antibody concentrations (ng / ml) 16days after an experimental challenge with 10^6 CFU/ml *Salmonella* Typhimurium of broiler chickens¹ determined by ELISA in Exp.2

Treatments ²				
Isotypes	CTL-	CTL+	AVE	AVM
IgA	127.1±38.1 ^b	97.3±10.9 ^b	138.3±37.3 ^b	310.3±68.7 ^a
IgG	1249.3±229.5	936.04±143.3	943.6±81.3	674.3±65.0
IgM	121.3±17.1 ^{ab}	97.6 ±9.2 ^b	116.2±15.6 ^{ab}	152.3±11.0 ^a

^{a,b} Values with different superscripts within the same row are different ($P<0.05$)

¹Absorbance were read using a micro titer plate reader (Wallac Victor-2 1420 Multiabel Counter). Mean ± SEM of 8 chickens per treatment.

²CTL-=antibiotic-free diet; CTL+=commercial-type diet with 40 mg bacitracin/kg
 AVE= antibiotic-free diet plus 0.8% L-Arginine and 80 IU/kg Vitamin E
 AVM=AVE diet supplemented with 0.2% manna oligosaccharides (Alltech Inc., Nicholasville, KY)

Field et al. (1999) found that MOS may enhance the secretion of plasma serum intestinal mucosa IgA, increasing the number of lymphocytes in the gut associated lymphoid tissue and in peripheral blood. The production of IgA is important to immunity because it inhibits the attachment and the penetration of bacteria in the lumen, increases the production of mucus (Mckay and perdue, 1993), and prevents inflammation that would cause epithelial tissue damage. Results from Exp.2 also suggest that diet AVM may have increased the population of beneficial bacteria in the ceca such as lactobacillis and bifidobacteria; these bacteria may improve IgA production. Intestinal microflora is one of the fundamental boosters for both local and systemic immune responses (Isolauri, 2001; Cross and Gill, 2001). Baurhoo et al. (2007) reported that birds fed MOS had a comparative advantage over birds fed antibiotics by increasing populations of lactobacilli and bifidobacteria and lowering *E. coli* loads after challenge. Lactobacillis and bifidobacteria were reported to induce the secretion of cytokine production by human enterocyte like cells (He et al., 2006). This may also happen in the chicken. IgA plays a critical role in mucosal immunity. Diet AVM may modulate chicken intestinal flora thus increasing IgA production and enhance mucosal immunity.

IgG is the most abundant immunoglobulin (Junqueira, 2003). Higher IgG concentrations help bind virus, bacteria, and fungi, and protects the body against them by agglutination and immobilization, complement activation, opsonization for phagocytosis and neutralization of their toxins (Junqueira et al., 2003). In the present experiment, no differences were detected among treatments but the concentrations of IgG were relatively higher than those of IgA and IgM. Hassan et al. (1990) reported that

after infection with *Salmonella* Typhimurim, chickens had a high concentration of IgG whereas the specific IgM response was transitory. Abdukalykova and Ruiz-Feria (2006) found that ARG and VE may have a complementary effect and improve humoral immune responses; however in this experiment antibodies were measured at 19 d, whereas Abdukalykova and Ruiz-Feria. (2006) measured antibody titers when the chickens were more than 29 d old. Bailey et al. (2007) reported that IgG titers decreased at 13 d and were no different from controls by 34 d after *Salmonella* challenge; thus the production of IgG may be related to chicken age. Further studies are needed to measure chicken IgG at different ages.

IgM is primarily found in serum, it possesses high avidity, and is particularly effective at complement activation. Because IgM is a large molecule, it is found in serum only in very low quantities. It is the primary antibody against antigens on red blood cells (Wiersma et al., 1998). Table 3 shows that IgM concentrations were found higher in birds fed AVM than those fed CTL+ but were similar with birds fed CTL- and AVE. Bacitracin was found to decrease *E.coli* in pigs (Walton and Wheeler, 1987) but had no effect on *Salmonella* (Lin et al., 2009) when fed at 50g / ton. It is possible that diet CTL+ which contained 40g / ton bacitracin controlled bacteria other than *Salmonella*; it is also possible that less antibodies were produced to fight the pathogens. Also, diet AVM may have increased IgM antibody. Baurhoo et al. (2007) reported that feeding MOS resulted in higher lactobacilli and bifidobacteria numbers in ceca of 42 d old chickens. It is possible that birds fed AVM improved the population of lactobacilli

and bifidobacteria in ceca thus inducing cytokine production resulting in increased IgM production.

Although the combination of ARG, VE and MOS had no effect on innate immune response compared with CTL+, birds fed AVM showed a higher IgA and IgM antibody concentration than birds fed CTL+, and so diet AVM may have a promising future in replacing antibiotic. AVE had been reported to improve humoral immune response but had no effect in these experiments. However, diet AVM improved adaptive immune response, ARG, VE and MOS may have a complementary effect on adaptive immune response or the additional MOS may have an indirect effect on immune response by increasing the beneficial bacteria, such as lactobacilli and bifidobacteria therefore inducing higher immune response.

Gut Parameters

Research on intestinal structure is very important as the intestine is the digestive and absorptive organ (Yamauchi, 2002). According to Cera et al. (1988), maximal absorption and digestion capacity, provided by a large luminal area, with high villi and mature enterocytes, are essential for the animal development. Table 4 shows the villi height (VH), villi base (VB), lamina propria (LPr) and crypt depth (CD) in chicken jejunum 3 and 7 d after challenge respectively of Exp.1. An increased villus height is paralleled by an increased digestive and absorptive function of the intestine due to increased absorptive surface area, expression of brush border enzymes and nutrient

Table 4. Effects of arginine, vitamin E, and mannanoligosaccharides on the length (μm) of villi height (VH), villi base (VB), lamina propria (LPr) and crypt depth (CD) under light-microscope in chicken jejunum 3 days and 7 days after an experimental challenge with 10^6 CFU/ml *Salmonella* Typhimurium of broiler chickens¹ in Exp 1 (challenged at day 7)

Treatment ²					
PI		CTL-	CTL+	AVE	AVM
VH	3d	576.7 \pm 70.6 ^a	386.9 \pm 26.4 ^b	508.1 \pm 19 ^{ab}	405.0 \pm 20.4 ^b
	7d	542.0 \pm 28.7 ^a	448.0 \pm 40.7 ^{ab}	403.0 \pm 33.6 ^b	550.2 \pm 16.1 ^a
VB	3d	188.8 \pm 30.3	163.0 \pm 14.2	224.3 \pm 14.2	194.3 \pm 10.6
	7d	205.9 \pm 9.8	176.3 \pm 21.1	209.2 \pm 23.8	228.2 \pm 18.4
LPr	3d	186.9 \pm 19.9	151.7 \pm 10.3	158.0 \pm 6.0	137.8 \pm 12.4
	7d	157.0 \pm 9.2	141.8 \pm 13.2	148.1 \pm 10.1	176.9 \pm 7.2
CD	3d	157.2 \pm 23.7	104.0 \pm 10.1	153.7 \pm 14.5	134.7 \pm 11.8
	7d	160.5 \pm 18.3 ^a	106.0 \pm 13.3 ^b	162.8 \pm 12.7 ^a	167.4 \pm 11.6 ^a

^{a,b} Values with different superscripts within the same row are different ($P<0.05$).

¹ Mean \pm SEM of 6 birds per treatment.

² CTL-=antibiotic-free diet; CTL+=commercial-type diet with 40 mg bacitracin/ kg; AVE=antibiotic-free diet plus 0.8% L-Arginine and 80IU/kg Vitamin; AVM=AVE diet supplemented with 0.2% Bio-Mos (Alltech Inc., Nicholasville, KY). PI=post infection.

transport systems (Amat et al., 1996). Three d after challenge, the VH of birds fed the CTL- was higher than that of birds fed the CTL+ or AVM, but they were not different from the VH of birds fed the AVE diet. Birds fed the CTL+, AVE or AVM diet had similar VH values. At 7 d after challenge, birds fed the CTL- or AVM had higher VH than birds fed the AVE diet, whereas birds fed CTL+ had intermediate VH values. Thus, birds fed diets with antibiotics or ARG and VE had smaller VH value than birds fed the CTL- diet or the diet supplemented with the prebiotic. Baurhoo et al., (2007) found that MOS did not have any effect on the VH of 14 d old birds, but it had positive effects on 28 and 42 d old birds. Our results show that the combination of ARG and VE reduced VH in 14 d old chickens compared with birds fed the CTL- and the AVM diet. In mammals it has been documented that ARG plays an important role in the maintenance of intestinal integrity under viral infections (Rhoads and Guoyao, 2009) and that VE maintains intestinal integrity through antioxidant mechanisms (Drew et al., 2004). Further research is necessary to elucidate the effects of ARG and VE on intestinal integrity. The CD was not affected by the dietary treatments 3 d after challenge. Seven ds after challenge birds fed AVM had a higher CD value than birds fed the CTL+ diet, but were not different from birds fed the CTL- or AVE diet.

Table 5. Effects of arginine, vitamin E, and mannanoligosaccharides on the length (μm) of villi height (VH), villi base (VB), lamina propria (LPr) and crypt depth (CD) under light-microscope in chicken jejunum 7 days and 17 days after an experimental challenge with 10^6 CFU/ml *Salmonella* Typhimurium of broiler chickens¹ in Exp 2 (challenged at day 3)

Treatment ²					
PI		CTL-	CTL+	AVE	AVM
VH	7d	1042.6±39.4	975.7±41.6	956.9±31.7	1075.1±44.0
	17d	717.0±35.4 ^c	1258.6±35.8 ^a	1035.6±42.6 ^b	1163.3±37.0 ^a
VB	7d	247.1±11.1 ^b	282.9±8.7 ^a	237.6±10.4 ^b	263.4±9.8 ^{ab}
	17d	271.1±9.0	248.2±9.6	257.5±8.9	247.8±7.5
LPr	7d	234.0±6.8	228.6±10.5	217.0±7.9	235.1±9.9
	17d	288.4±8.9 ^a	274.8±10.0 ^a	228.5±6.8 ^b	212.8±6.7 ^b
CD	7d	241.3±8.9 ^a	218.4±9.3 ^{ab}	186.3±7.2 ^b	222.9±7.3 ^a
	17d	257.3±10.7 ^a	246.9±10.9 ^a	213.3±8.3 ^b	214.6±6.5 ^b

^{a,b} Values with different superscripts within the same row are different ($P<0.05$).

¹ Mean±SEM of 6 birds per treatment.

² CTL-=antibiotic-free diet; CTL+=commercial-type diet with 40 mg bacitracin/ kg;

AVE=antibiotic-free diet plus 0.8% L-Arginine and 80IU/kg Vitamin;

AVM=AVE diet supplemented with 0.2% Bio-Mos (Alltech Inc., Nicholasville, KY).

PI=post infection.

Deeper crypts indicate fast cellular turnover to permit renewal of the villus as needed in response to normal sloughing or inflammation from pathogens or their toxins and high demands for tissue (Rehman et al., 2007). The effects of prebiotics on CD are highly variable; Savage et al. (1996) observed a reduction in CD when MOS was included in a turkey diet, and Loddi et al. (2002) measured the highest CD values in the ileum of turkeys fed a normal diet compared with those of turkeys fed diet supplemented with MOS, whereas Spring (1996) reported higher CD values in broilers fed MOS.

In Table 5, birds fed CTL+ diet tend to improve chicken intestinal health by improving VH (17d PI), VB (7d PI), Lpr (17d PI) and CD (7d PI and 17d PI) in Exp.2. Antibiotic is well known as growth promoter that improves animal performance and health status (Coates et al., 1955), while this seemed not true based on Exp.1 in which antibiotic diet lower intestinal health. The purpose of putting supplementation in poultry feed is to improve chicken health and immune system instead of antibiotic, apparently, although AVE diet consistently improve chicken immune parameters in Exp.2, the diet has no effect on or has a negative effect on the intestine health. Interestingly, in Exp.1, the result was opposite. A wide range of factors associated with diet, infectious disease agents, environment, and management practices can negatively affect gut health (Hughes, 2005). In Exp.2, diet AVE and AVM have negative effect on chicken gut health.

***Salmonella* Colony Counts**

Since the ceca are the primary site of *Salmonella* colonization in poultry (Fanelli et al., 1971), it has been considered to be the primary source of *Salmonella* contamination of broilers.

In Exp.1 (Table.6), the *Salmonella* CFU per gram of cecal content 3 d after challenge were lower in chickens fed the AVM diet compared with chickens fed the CTL- diet, but were not different from chickens fed the CTL+ or AVE diet. Nevertheless 10 and 17 d after challenge there were no differences in *Salmonella* levels among treatments. In Exp.2 the *Salmonella* levels were not different among treatments at any sampling period. Thus, when birds were challenged at an early age (3d) there was no effect of diet, but when birds were challenged at d 7, the *Salmonella* colonization was reduced in birds fed the AVM compare to CTL-. According to Oyofe et al. (1989), MOS might affect colonization of such strains through blocking bacterial attachment to the gut mucosa. MOS affects bacterial concentrations in the gastrointestinal tract by adsorbing bacteria and keeping them from adhering to the gut wall (Spring et al., 2000). Further studies are needed to investigate if MOS can reduce *Salmonella* colonization at an older age. The levels of dietary MOS varied by trial and by feed phase in different studies, but in most research with poultry, dietary MOS was used in small doses (0.05% to 0.2%), probably for economical reasons (Hooge et al., 2003).

Table 6. Effects of arginine, vitamin E and mannanoligosaccharides on cecal *Salmonella* Typhimurium concentrations (Colony Forming Units, log₁₀) and on number of *Salmonella* positive samples after an experimental challenge with 10⁶ CFU/ml *Salmonella* Typhimurium of broiler chickens¹ in Exp.1 and Exp.2

Treatments ²				
Days PI ³	CTL-	CTL+	AVE	AVM
	CFU /+	CFU /+	CFU/+	CFU /+
Experiment 1				
3	3.0±0.5 ^a /8	2.1±0.5 ^{ab} /5	1.6±0.5 ^{ab} /3	0.8±0.4 ^b /1
10	0.8±0.4/1	1.5±0.4/1	0.2±0.2 /0	0.7±0.4/0
17	0.5±0.4/1	0.7±0.3/0	1.0±0.6/2	0.6±0.4/1
Experiment 2				
7	4.4±0.6/10	4.0±1.1/10	4.4±1.1/10	4.4±1.14/9
14	1.5±0.5/7	1.9±0.6/2	1.9±0.6/7	0.9±0.6/7
21	0.3±0.3/1	0.0 ±0/1	0.8±0.4/0	0.4±0.4/0

^{a,b} Values with different superscripts within the same row are different ($P<0.05$).RV= Number of positive out of 10 birds

¹ Mean of 10 observation ± SEM / number of birds tested positive, out of 10, after RV enrichment for 24 h.

²CTL-=antibiotic-free diet; CTL+=commercial-type diet with 40 mg bacitracin / kg
AVE=antibiotic-free diet plus 0.8% L-Arginine and 80IU/kg Vitamin E
AVM=AVE diet supplemented with 0.2% mannanoligosaccharides (Alltech Inc., Nicholasville, KY)

³ PI: post infection

In summary, we found that the combination of ARG, VE and MOS have different effects on the chicken immune response when challenged at different ages. When birds were challenged at d 7, the combination of ARG, VE and MOS decreased innate immune response by decreasing oxidative burst of heterophils and of monocytes while improving adaptive immune response as showed from lymphocyte proliferation. When birds were challenged at d 3, the AVM diet improved broiler chickens immune response by increasing IgA and IgM antibody concentration compared with antibiotic diet. Also, we found that the combination of ARG, VE and MOS decreased *Salmonella* colony number at a young age, when challenged at d 7, but had no effect on broiler chickens when challenged at d 3. All of the above suggest that the combination of ARG, VE and MOS may have complementary effect in promoting birds' immunity and health when birds were infected at an early age.

INTRODUCTION OF EXP.3 AND EXP.4

In Exp.1 (challenged *Salmonella* at day 7) the results indicated that the combination of ARG, VE and MOS decreased innate immune response, and may improve adaptive immune response. Also, including VE in the diet tended to decrease chicken immune response in Exp.1. In Exp.2 (challenged *Salmonella* at day 3), contrary to the result from Exp.1, diet AVE had positive effect both on innate and adaptive immune response. The combination of ARG, VE and MOS has no immune effect on chickens compared to CTL- and CTL+. We can conclude that additional MOS negated the effect of AVE on chickens. From these two experiments, chicken immune response were improved by a higher level of ARG supplementation and benefited from an earlier *Salmonella* infection. This may result from the un-established cecal microflora at that young age, which is a major factor in the susceptibility of chicks to bacterial infection (Barrow, 1992), or, because of the synergic effect between AVE and MOS, or some other unknown reasons. In Exp.1 and Exp.2, it is likely that a challenge dose of 10^6 CFU/ml *Salmonella* was too high for young chicks' immune system. Thus, in Exp.3, 10^2 CFU/ml was used and 10^6 CFU/ml was used in Exp.4. We hypothesis that when infected at two different levels, the impact of diet AVE and AVM on immune function can be further evaluated.

MATERIALS AND METHODS OF EXP.3 AND EXP.4

Animals and Treatments

Three hundred day-old broiler chicks (Cobb 500) were obtained from a local hatchery and were housed in a bio safety facility at the USDA-Southern Plains Agriculture Research Center in College Station. The chicks were placed on fresh pine litter and were brooded following standard temperature regimes, which gradually decreased from 32°C to 24°C, and under a 16:8 light: dark cycle throughout the study. All the birds were fed a corn-soybean meal-based diet formulated to meet or exceed all of the NRC (1994) requirements (Table 1). The basal diet contained 23% of CP, 3,200 kcal ME/kg, 1.54 % ARG and 40.3 IU VE/kg. Two simultaneous experiments were conducted using these diets. Each experiment consisted of completely randomized design with three treatments (50 birds in each treatment) was used, as follows: the basal diet (CTL) constituted treatment 1; the basal diet supplemented with ARG (0.8%) and 80 IU/kg of VE constituted treatment 2, the basal diet supplemented with ARG and VE at the same level plus 0.2% MOS constituted treatment 3 (BioMos, Alltech Co., Lexington KY; AVM). At day 3, chickens from experiment 3, were orally challenged with 10^2 CFU of *Salmonella* Typhimurium. Chickens from experiment 4 were orally challenged at day 3 with 10^6 CFU of *Salmonella* Typhimurium. The Texas A&M University Institutional Animal Care Committee approved the experimental procedures.

Bacteria

A primary poultry isolate of ST was obtained from the national Veterinary Services Laboratory (Ames, IA) and selected for resistance to novobiocin and nalidixic acid, and maintained in media containing 25 µg of novobiocin and 20 µg of nalidixic acid/ml. Portions (1 to 2 ml) of cultures grown overnight in tryptic soy broth (Difco Laboratories, Detroit, MI) were used as inocula for challenging the broiler chickens.

Colony Counts

Ten birds per treatment from each experiment were euthanized at day 10, 17 and 24. All broilers were killed by cervical dislocation and the cecal contents (0.25g) were aseptically collected and deposited in 2.25ml of phosphate buffer (pH 6.5). Samples were thoroughly mixed, serially diluted in phosphate buffer, and then streaked on XLT 4 agar plates (Becton, Dickinson and Company sparks, USA) containing 20 µg of nalidixic acid/ml and 25 µg novobiocin/ml. Plates were incubated for at least 24 h at 37 °C, and the number of *Salmonella* CFU per gram was determined. *Salmonella* colony numbers was log-transformed, the number of four plates was averaged and then analyzed. For qualitative enrichment, cecal contents were enriched in Rappaport-Vassiliadis Broth (Difco) for 24 h at 37 °C and were then streaked on XLT 4 agar plates, after 24 h, 42°C incubation, plates were read as positive or negative.

Blood Collection

Blood samples were taken from the jugular vein of birds from each treatment in both experiments at day 10, 17 and 24. Ninety ml of blood from each treatment group were collected at day 10 using EDTA (EMD Chemicals Inc., Gibbstown, N.J) as anticoagulant, whereas 180ml blood samples were collected at day 17 and day 24. Blood samples were then separated evenly into three 50 ml tubes as three gradients, each gradient were used for oxidative burst and lymphocytes proliferation. All blood samples were kept in ice until used.

Immune Response and Intestinal Architecture

Peripheral blood was collected by decapitation and EDTA was used as an anticoagulant. Blood was pooled from 10 birds per treatment in each experiment. Heterophils and mononuclear cell fractions were isolated as previously described (Kogut et al., 1995). Oxidative activity of heterophils and monocytes, lymphocyte proliferation, determination of serum levels of IgG, IgM, and IgA (10 d-old, 17 d-old and 24 d-old chickens), and intestinal integrity of the jejunum (day 10, 17 and 24) were conducted following the methods previously described for Experiments 1 and 2.

RESULTS OF EXP.3 AND EXP.4

Body weight data for chicks in all treatments for Exp.3 and Exp.4 are presented in Table 7. Throughout Exp.3, there were no differences in the BW of chicks. However, in Exp.4, birds fed AVM showed higher body weight than birds fed CTL both at d 14 and d 21. Body weight was not different at d 1 and d 7 in Exp.4. Total mortality was 6% in Exp.3 and 2% in Exp.4 (Table 7).

Levels of *Salmonella* Typhimurium from the ceca of challenged chicks were not affected by dietary treatment or by challenge dose at any of the sampling times (Table 8). Antibody concentrations were measured at day 10, 17 and 24 using ELISA. At day 10 and d 24, no differences in the levels of immunoglobulin A (IgA), immunoglobulin G (IgG) and immunoglobulin M (IgM) were found among treatments in either Experiment 3 or 4. At d 17, in Exp.3, birds fed AVM showed a higher IgA concentration than birds fed AVE, but have a similar IgA concentration compare with birds fed CTL. No IgG difference was found at day 17. Regarding IgM concentration, in Exp.3, birds fed AVE has a higher IgM concentration than birds fed CTL and AVM, however, in Exp.4, birds fed AVM has a higher IgM concentration than birds fed AVE and AVM (Table 9 and Table 10). Immune response was further measured by evaluating oxidative burst heterophil response (OBHR), oxidative monocyte response (OBMR) and lymphocyte proliferation response (LPR). Figure 4 shows at day 17, chicks in Exp.3 has a higher OBHR when fed CTL than when fed the AVE or AVM diet; however, at d 24 there were no difference among treatments. Figure 6 shows that the OBMR was not affected by

Table 7. Effects of arginine, vitamin E, and mannanoligosaccharides on broiler chicken body weight (BW, gram) of Exp.3 and Exp.4

Treatments		CTL	AVE	AVM
<u>Experiment 3</u>				
Day 1	BW	58.70±1.69	58.85±1.09	58.67±0.54
Day 7	BW	141.56±5.87	133.05±5.18	141.41±4.68
Day 14	BW	375.37±13.52	343.29±14.59	355.87±10.65
Day 21	BW	714.15±25.88	623.76±31.88	674.17±24.74
Mortality		0%	2%	4%
<u>Experiment 4</u>				
Day 1	BW	59.37±1.19	59.41±0.48	60.68±0.88
Day 7	BW	138.72±5.35	144.34±1.58	142.34±1.29
Day 14	BW	307.85±31.02 ^b	357.54±10.94 ^{ab}	378.22±5.95 ^a
Day 21	BW	655.32±22.34 ^b	693.26±13.32 ^{ab}	731.80±18.41 ^a
Mortality		0%	0%	2%

^{a,b} Data are presented as means ± SEM. Values within Experiment bearing different superscript letters within a row are significantly different ($P < 0.05$).

Chickens in Exp.3 were challenged with 10^2 CFU/ml *Salmonella* Typhimurium at day 3.

Chickens in Exp.4 were challenged with 10^6 CFU/ml *Salmonella* Typhimurium at day 3.

¹CTL= control chicks fed corn-soybean meal basal diet with no supplementation

AVE=chicks fed corn-soybean meal basal diet supplemented with 0.8% of L-Arginine and 80IU/kg of Vitamin E;

AVM=chicks fed corn-soybean meal basal diet ² Values are based only on weight of live birds.

Table 8. Effects of dietary L-arginine, vitamin E and mannanoligosaccharides on the concentration of *Salmonella* Typhimurium in the ceca of chicks (Exp.3 and Exp.4)

<u>Treatments²</u>			
Days PI ³	CTL	AVE	AVM
<u>Experiment 3</u>			
10	0.66±0.36	0.0±0	0.0±0
17	0.64±0.37	0.0±0	0.35±0.35
24	0.68±0.46	0.11±0.11	0.4±0.4
<u>Experiment 4</u>			
10	1.85±0.59	2.29±0.57	1.79±0.64
17	0.69±0.38	0.0±0	0.49±0.33
24	1.08±0.08	1.01±0.52	1.75±0.64

Chickens in Exp.3 were challenged with 10^2 CFU/ml *Salmonella* Typhimurium;
 Chickens in Exp.4 were challenged with 10^6 CFU/ml *Salmonella* Typhimurium;
¹CTL= control chicks fed corn-soybean meal basal diet with no supplementation
 AVE=chicks fed corn-soybean meal basal diet supplemented with 0.8% of L-Arginine
 and 80IU/kg of Vitamin E; AVM=chicks fed corn-soybean meal basal diet ² Values are
 based only on weight of live birds.

Table 9. Effects of arginine, vitamin E, and mannanoligosaccharides on serum antibody concentrations (mg/ml) at day 10, day 17 and day 24 after an experimental challenge (Exp.3)

Treatments ²		CTL	AVE	AVM
Day				
IgA	Day 10	0.21±0.03	0.24±0.03	0.23±0.03
	Day 17	0.34±0.04 ^{ab}	0.23±0.023 ^b	0.4±0.06 ^a
	Day 24	1.03±0.03	0.98±0.02	1.01±0.03
IgG	Day 10	5.84±0.06	5.9±0.03	5.76±0.03
	Day 17	5.7±0.46	5.4±0.37	5.3±0.48
	Day 24	4.3±0.15	4.0±0.08	4.14±0.18
IgM	Day 10	1.94±0.22	1.64±0.33	1.79±0.35
	Day 17	1.9±0.6 ^b	3.9±0.3 ^a	1.0±0.3 ^b
	Day 24	0.56±0.08	0.56±0.08	0.39±0.04

^{a,b} Values with different superscripts within the same row are different ($P<0.05$)

Chickens were challenged with 10^2 CFU/ml *Salmonella* Typhimurium;

¹Absorbance were read using a micro titer plate reader (Wallac Victor-2 1420 Multiabel Counter), Mean±SEM of 8 chickens per treatment. ²CTL =Corn soybean diet; AVE= antibiotic-free diet plus 0.8% L-Arginine and 80 IU/kg Vitamin E
AVM=AVE diet supplemented with 0.2% manna oligosaccharides (Alltech Inc., Nicholasville, KY)

Table 10. Effects of arginine, vitamin E, and mannanoligosaccharides on serum antibody concentrations (mg/ml) at day 10, day 17 and day 24 after an experimental challenge (Exp.4)

Treatments ²		CTL	AVE	AVM
Day				
IgA	Day 10	0.3±0.04	0.2±0.03	0.34±0.05
	Day 17	0.37±0.05	0.24±0.03	0.33±0.04
	Day 24	1.03±0.02	1.05±0.02	1.03±0.02
IgG	Day 10	5.87±0.05	5.89 ±0.03	5.93±0.04
	Day 17	4.5±0.38	5.4±0.35	4.6±0.41
	Day 24	4.4±0.07	4.4±0.08	4.3±0.1
IgM	Day 10	2.9±0.6	2.09±0.3	2.2±0.2
	Day 17	1.1±0.2 ^b	0.32±0.11 ^c	4.01±0.23 ^a
	Day 24	0.42±0.04	0.42±0.03	0.37±0.03

^{a,b} Values with different superscripts within the same row are different ($P<0.05$)

Chickens were challenged with 10^6 CFU/ml *Salmonella* Typhimurium;

¹Absorbance were read using a micro titer plate reader (Wallac Victor-2 1420 Multiabel Counter), Mean±SEM of 8 chickens per treatment. ²CTL =Corn soybean diet; AVE= antibiotic-free diet plus 0.8% L-Arginine and 80 IU/kg Vitamin E
AVM=AVE diet supplemented with 0.2% manna oligosaccharides (Alltech Inc., Nicholasville, KY)

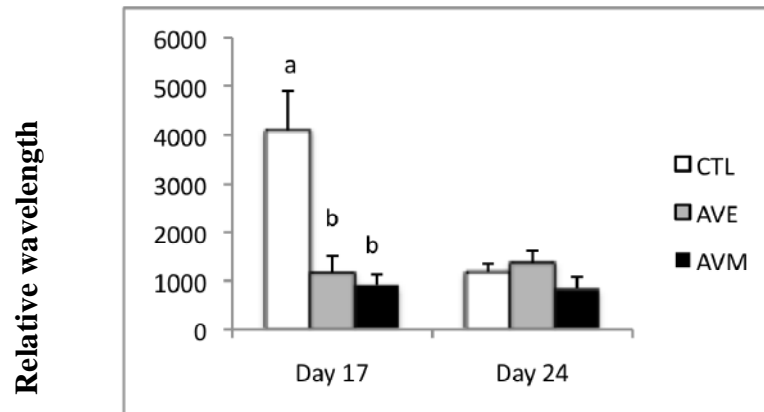


Figure 4. Effects of a corn soy-bean basal diet (CTL), a CTL plus 0.8% L-Arginine and 80 IU of vitamin E /kg (AVE) feed, an AVE plus 0.2% mannanoligosaccharides on chicken oxidative burst of heterophiles of Exp.3. Heterophils were isolated from pooled peripheral blood of 10 broiler chickens per treatment on day 17 and 24 days of experiment 3 (challenged at day 3) when chicks were challenged at $10^2 \log_{10}$ CFU/ml of *Salmonella* Typhimurium. An equal volume of RPMI 1640 media was used as a negative control treatment. 8 observations were read per gradient and each treatment has three gradients. PMA was used as a positive control treatment. Data is presented as the mean relative fluorescence difference from the positive control to the negative control with the SEM. ^{a-c} Values with different letters within a sample period are different (Tukey test, $P < 0.05$).

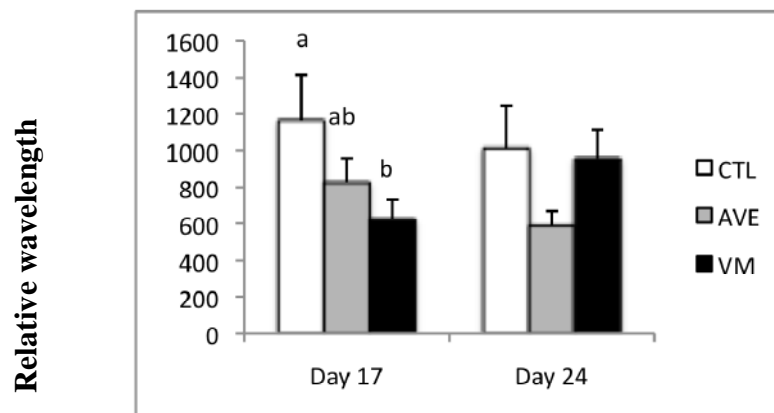


Figure 5. Effects of a corn soy-bean basal diet (CTL), a CTL plus 0.8% L-Arginine and 80 IU of vitamin E /kg (AVE) feed, an AVE plus 0.2% mannanoligosaccharides on chicken oxidative burst of heterophils of Exp.4. Heterophils were isolated from pooled peripheral blood of 10 broiler chickens per treatment on day 17 and 24 days (Exp. 4, challenged at day 3) when chicks were challenged at $10^6 \log_{10}$ CFU/ml of *Salmonella* Typhimurium. An equal volume of RPMI 1640 media was used as a negative control treatment. 8 observations were read per gradient and each treatment has three gradients. PMA was used as a positive control treatment. Data is presented as the mean relative fluorescence difference from the positive control to the negative control with the SEM. ^{a-c} Values with different letters within a sample period is different (Tukey test, $P < 0.05$).

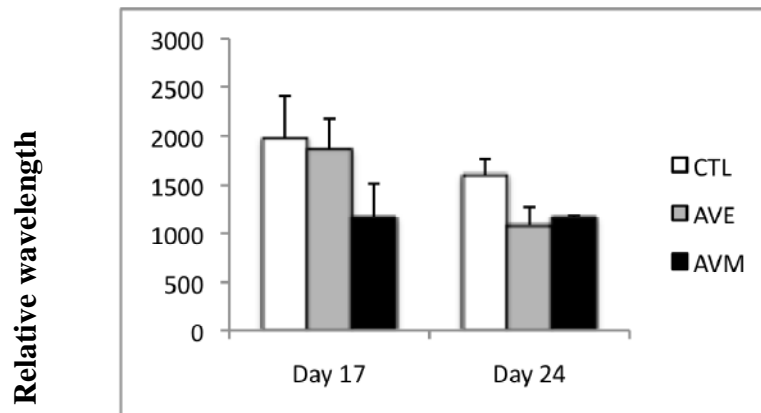


Figure 6. Effects of a corn soy-bean diet (CTL), a CTL plus 0.8% L-Arginine and 80 IU of vitamin E/kg (AVE) feed, an AVE plus 0.2% mannanoligosaccharides (AVM) on chicken monocytes oxidative burst of Exp.3. Monocytes were isolated from pooled peripheral blood of 10 broiler chickens per treatment on day 17 and 24 days (Experiment 3, challenged at day 3) after a challenge with 10^2 CFU/ml of *Salmonella* Typhimurium. An equal volume of RPMI 1640 media was used as a negative control treatment. 8 observations were read per gradient and each treatment has three gradients. PMA was used as a positive control treatment *in vitro*. Data is presented as the mean relative fluorescence difference from the positive control to the negative control with the SEM. ^{a-c} Values with different letters within a sample period is different (Tukey test, $P < 0.05$).

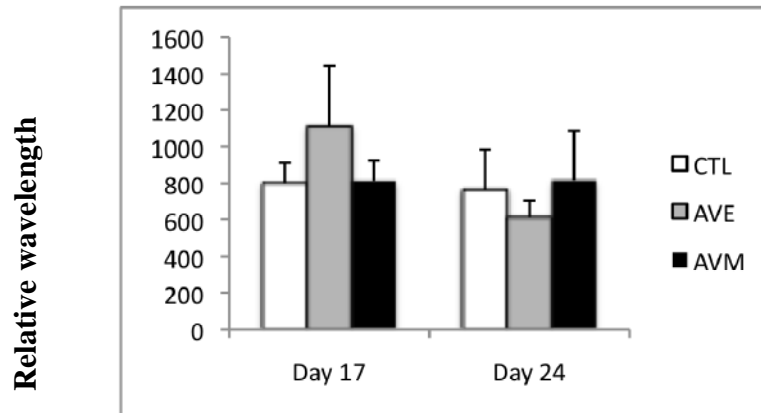


Figure 7. Effects of a corn soy-bean diet (CTL), a CTL plus 0.8% L-Arginine and 80 IU of vitamin E/kg (AVE) feed, an AVE plus 0.2% mannanoligosaccharides (AVM) on chicken oxidative burst monocytes of Exp.4. Monocytes were isolated from pooled peripheral blood of 10 broiler chickens per treatment on day 17 and 24 days (Experiment 4, challenged at day 3) after a challenge with 10^6 CFU/ml of *Salmonella* Typhimurium. An equal volume of RPMI 1640 media was used as a negative control treatment. 8 observations were read per gradient and each treatment has three gradients. PMA was used as a positive control treatment *in vitro*. Data is presented as the mean relative fluorescence difference from the positive control to the negative control with the SEM. ^{a-c} Values with different letters within a sample period is different (Tukey test, $P < 0.05$)

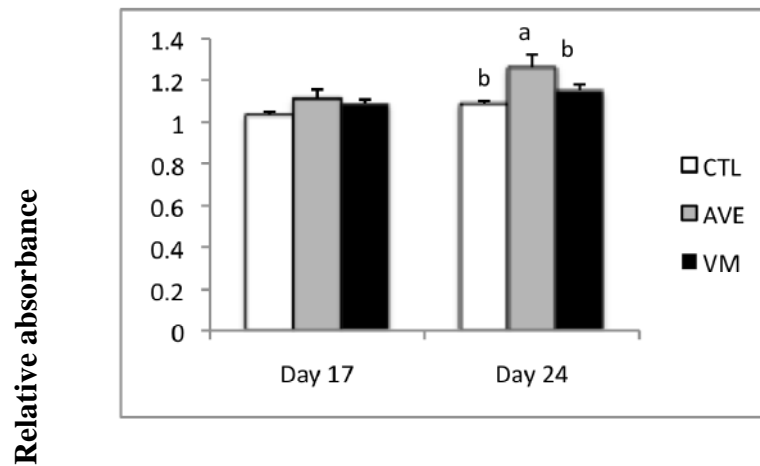


Figure 8. Effects of a corn soy-bean diet (CTL), a CTL plus 0.8% L-Arginine and 80 IU of vitamin E /kg (AVE) feed, or an AVE diet plus 0.2% mannanoligosaccharides (AVM) on chicken lymphocyte proliferation response of Exp.3. Lymphocytes were isolated from pooled peripheral blood of 10 broiler chickens per treatment at day 10, 17 and 24 after a challenge with 10^2 CFU/ml of *Salmonella* Typhimurium in Experiment 3. An equal volume of RPMI 1640 media was used as a negative control treatment. 8 observations were read per gradient and each treatment has three gradients. Concovalin A was used as a positive control treatment *in vitro*. Data is resented as the mean relative fluorescence ratio from the positive control to the negative control with the SEM.

^{a-c} Values with different letters within a sample period are different (Tukey test, $P < 0.05$).

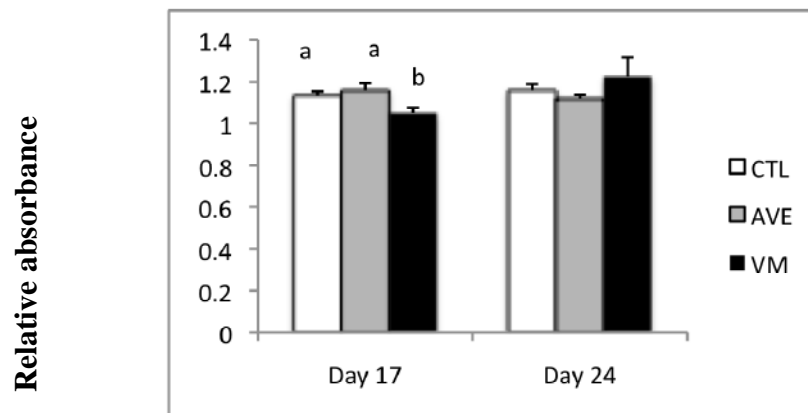


Figure 9. Effects of a corn soy-bean diet (CTL), a CTL plus 0.8% L-Arginine and 80 IU of vitamin E /kg (AVE) feed, or an AVE diet plus 0.2% mannanoligosaccharides (AVM) on chicken lymphocyte proliferation response of Exp.4. Lymphocytes were isolated from pooled peripheral blood of 10 broiler chickens per treatment at day 10, 17 and 24 after a challenge with 10^6 CFU/ml of *Salmonella* Typhimurium in experiment 4. An equal volume of RPMI 1640 media was used as a negative control treatment. 8 observations were read per gradient and each treatment has three gradients. Concovalin A was used as a positive control treatment *in vitro*. Data is resented as the mean relative fluorecence ratio from the positive control to the negative control with the SEM.

^{a-c} Values with different letters within a sample period are different (Tukey test, $P < 0.05$).

treatment at d 17 or d 24 in Exp.3. On the other hand Figure 8 shows that chicks fed AVM had a higher LPR than the other treatments at d 24, but not at d 17 in Exp.3. In Exp.4, dietary treatments did not affect the OBHR or the OBMR regardless of age (Figure 5 and Figure 7). However the LPR was higher in chicks fed the AVE diet compared with birds fed the AVM diet, although at d 24 there was not difference due to treatment (Figure 9).

For intestine parameters including villi height (VH), villi base (VB), lamina propria (LP) and crypt depth (CD) which are shown in Table 11, in Exp.3, we can see that at day 10 chickens in AVE and AVM had similar VH, VB, LPr value between each other but had higher VH, VB and LPr value than the values from chickens in CTL. Chickens in AVM had a deeper CD than chicken in CTL or AVE at d 10. At d 17, chickens in AVE had a similar VH with CTL, but higher VH than birds in AVM. Chickens in AVM had a wider VB than chickens in CTL, whereas chickens from AVE had a similar LPr than chickens in AVM, but higher LPr than birds in CTL, and chickens from AVE had a deeper CD than chickens from CTL. At day 24, chickens from AVE had higher VH than birds in CTL and AVM; birds in the AVM had the widest VB. Chickens from CTL had a similar LPr compared with chickens from AVM but higher value than those of chickens from AVE. No CD differences were found in Exp.3. In Exp.4, no significant VH differences were found among treatments in all sampling days (Table 12). The VB was not different among treatments at d 10 or 17, but at d 24 birds in the AVM had higher VB than birds in the CTL treatment. The LPr was not affected by treatment at d 10, but at d 17 birds in the AVE had a wider LPr than birds

Table 11. Effects of arginine, vitamin E, and mannanoligosaccharides on villi height (VH) (μm), villi base (VB), lamina propria (LPr) and crypt depth (CD) under light-microscope in chicken jejunum at day 10, day 17 and day 24 after an experimental challenge (Exp.3)

		CTL	AVE	AVM
Day				
VH	Day 10	430.9 \pm 12.7 ^b	484.6 \pm 11.9 ^a	473.0 \pm 11.8 ^a
	Day 17	672.3 \pm 29.1 ^{ab}	760.1 \pm 27.7 ^a	658.3 \pm 34.1 ^b
	Day 24	702.6 \pm 26.8 ^b	801.4 \pm 27.9 ^a	724.5 \pm 22.4 ^b
VB	Day 10	149.0 \pm 9.5 ^b	194.4 \pm 10.4 ^a	193.8 \pm 12.4 ^a
	Day 17	131.3 \pm 5.4 ^b	139.7 \pm 6.5 ^{ab}	160.4 \pm 9.7 ^a
	Day 24	133.9 \pm 12.5 ^b	120.7 \pm 8.1 ^b	184.1 \pm 11.5 ^a
LPr	Day10	120.3 \pm 8.7 ^b	147.5 \pm 7.14 ^a	163.8 \pm 6.1 ^a
	Day17	242.2 \pm 9.5 ^b	282.6 \pm 11.1 ^a	248.6 \pm 15.4 ^{ab}
	Day 24	229.5 \pm 10.2 ^a	191.2 \pm 9.6 ^b	218.2 \pm 9.2 ^{ab}
CD	Day10	115.7 \pm 4.9 ^b	121.0 \pm 6.7 ^b	142.9 \pm 8.3 ^a
	Day 17	248.9 \pm 7.3 ^b	284.5 \pm 19.0 ^a	255.1 \pm 14.9 ^b
	Day 24	176.6 \pm 18.9	185.8 \pm 16.3	216.9 \pm 11.2

^{a,b} Data are presented as means \pm SEM. Values bearing different superscript letters within a row are significant different ($P < 0.05$).

Chickens were challenged with 10^2 CFU/ml *Salmonella* Typhimurium at day 3.

¹CTL= control chicks fed corn-soybean meal basal diet with no supplementation
²AVE=chicks fed corn-soybean meal basal diet supplemented with 0.8% of L-Arginine and 80IU/kg of Vitamin E; avm=chicks fed corn-soybean meal basal diet
² Values are based only on weight of live birds.

Table 12. Effects of arginine, vitamin E, and mannanoligosaccharides on villi height (VH) (μm), villi base (VB), lamina propria (LPr) and crypt depth (CD) under light-microscope in chicken jejunum at day 10, day 17 and day 24 after an experimental challenge (Exp.4)

		CTL	AVE	AVM
Days				
VH	Day 10	487.7 \pm 13.4	496.7 \pm 13.7	480.9 \pm 13.7
	Day 17	603.6 \pm 15.1	640.2 \pm 19.1	640.4 \pm 20.1
	Day 24	800.1 \pm 25.4	865.1 \pm 44.2	816.9 \pm 41.5
VB	Day 10	116.7 \pm 6.7	126.6 \pm 8.0	130.2 \pm 5.2
	Day 17	141.3 \pm 6.4	135.3 \pm 8.8	143.7 \pm 8.6
	Day 24	121.7 \pm 5.7 ^b	155.6 \pm 18.1 ^{ab}	170.3 \pm 7.9 ^a
LPr	Day10	151.1 \pm 6.3	156.9 \pm 5.4	163.7 \pm 5.6
	Day17	241.5 \pm 6.4 ^b	270.7 \pm 10.3 ^a	233.3 \pm 7.2 ^b
	Day 24	261.3 \pm 12.1 ^a	242.7 \pm 10.2 ^a	179.3 \pm 15.6 ^b
CD	Day10	138.9 \pm 10.8 ^b	161.7 \pm 7.7 ^{ab}	167.8 \pm 5.6 ^a
	Day 17	237.5 \pm 9.0 ^{ab}	266.1 \pm 14.1 ^a	217.6 \pm 11.3 ^b
	Day 24	265.9 \pm 15.2 ^a	241.9 \pm 12.7 ^a	185.2 \pm 10.8 ^b

^{a,b} Data are presented as means \pm SEM. Values bearing different superscript letters within a row are significant different ($P < 0.05$).

Chickens were challenged with 10^6 CFU/ml *Salmonella* Typhimurium at day 3.

¹ CTL= control chicks fed corn-soybean meal basal diet with no supplementation
 AVE=chicks fed corn-soybean meal basal diet supplemented with 0.8% of L-Arginine and 80IU/kg of Vitamin E; avm=chicks fed corn-soybean meal basal diet ² Values are based only on weight of live birds.

in the other treatments, and at d 24 birds in the AVM treatment had a thinner LPr than birds in the other two treatments. At day 10, CD was deeper in chickens from AVM than those chickens from CTL. No CD difference was found at day17, but CD value of AVM at day 24 was lower than both CTL and AVE.

DISCUSSION OF EXP.3 AND EXP.4

This study was conducted to investigate the effects of ARG, VE and MOS on broiler chicken's body weight, serum antibodies, ceca *Salmonella* concentrations, jejunum villi height, width, lamina propria thickness, crypt depth and immune responses after challenged with 2 different levels of *Salmonella* Typhimurium. Our results showed that body weight gain were unaffected by any of the treatment in Exp.3, but in Exp.4, at day 14 and day 21, birds in AVM treatment showed a higher BW gain compared with birds in the control group (Table 7). This may indicate that supplementation with AVM improves chicken body weight when chickens have a higher bacteria level. Studies showed that higher doses of *Salmonella* are more toxic than lower doses of *Salmonella* (Asheg et al., 2001). However, Wijburg et al. (2006) reported that at an oral dose of 10^6 or 10^5 CFU/ml *Salmonella* Typhimurium mice were equally susceptible to infection, but infected mice with 10^4 CFU/ml of *Salmonella* Typhimurium resulted in a 100% mortality which means 10^4 CFU/ml tend to be more toxic than 10^6 or 10^5 CFU/ml. Our results agreed with this finding that chickens infected with 10^2 CFU/ml *Salmonella* had a higher overall mortality than those chickens challenged with 10^6 CFU/ml *Salmonella*. The effect of MOS on chicken body weight is inconsistent. Benites et al. (2008) reported that birds fed MOS at 1.0/0.5/0.5 (starter/grower/finisher) kg / ton diets had significantly greater BW at 42 d than birds fed control diet; Ozpinar et al. (2010) fed chicken in 4 groups: 1) control group; 2) 1.5g/kg MOS (0.5%); 3) 500 μ g/kg VE and 500 μ g/kg and no significant body weight difference were found among treatments. Baurhoo et al. (2009)

also reported that the body weight in MOS (0.2% and 0.5%) groups did not show significant difference compared with control group. Studies like Kubena et al. (2001) reported that it is possible that supplementation with higher levels of MOS (1% in starter diet) at an early age could increase chicken body weight, because younger chicks are more susceptible to *Salmonella*. Sims et al. (2004) fed turkeys 1 g/kg of MOS from 1 to 6 wk and 0.5 g/kg of MOS from 7 to 18 wk and reported that live weight was improved at 18 wk of age. These results are contradictory. This may indicate that although many studies reported that MOS may improve chicken body weight, the effectiveness of MOS on chicken body weight depends on many factors, including birds age, the bacteria levels, stress etc.

Also, the combination of ARG, VE and MOS has a different effect compared to MOS alone. Further work is necessary to establish the effect of the combination of ARG, VE and MOS supplementation and the doses of *Salmonella* Typhimurium on chicken growth performance.

In Exp.3, the increase in VH in birds fed the AVM diet was not correlated with chicken body weight (Table 8). Similar findings have been reported by van Leeuwen et al. (2004) that *Salmonella* Typhimurium infection reduced villous surface area, but did not affect chicken growth.

In this study, the AVM supplemented diet only had a positive effect on VH at day 10 in Exp.3, but in Exp.4 (Table 9), AVM and AVE diets did not have any positive effect on jejunum VH compared to chickens fed the control diet. Probably, the high level of bacteria damaged the intestinal mucosa; however when birds were challenged at

lower level of *Salmonella*, diet of AVE had a positive effect on jejunum VH. Previous studies have reported that birds fed AVE can improve chicken body weight and chicken performance and have a higher immune response than control group (Abdukalykove et al., 2008). No studies have reported the effect of ARG and VE on chicken intestinal morphology. In this study, we found that AVE diet can increase chicken villi height at day 7, day 14 and day 21 in Exp.3; additional MOS may negate the effect of AVE on chicken villi height.

Salmonella numbers in ceca didn't show any differences among treatments both in Exp.3 and Exp.4 (Table 10). Some *in vitro* studies have suggested that ARG and VE have effect on chicken immune response when infected with *Eimeria* (Carbajal et al., 2010), but no studies have reported that the effect of ARG, VE and MOS on the clearance of ceca *Salmonella* concentration in chickens when challenged with *Salmonella*. On previous studies we found that *Salmonella* concentration in ceca was lower in AVM supplemented chickens compared to chickens fed control diet only at day 10, followed by AVE supplemented chickens. It is possible that the level of 10^6 CFU/ml was too high for broiler chickens when challenged at an early age, in which the immune organs may be damaged which made chickens unable to clear *Salmonella*, so in the present study, chickens were orally challenged birds with two different levels of *Salmonella*. One level remained the same as in previous studies (10^6) (Exp.4), and the second was lower (10^2) (Exp.3). We find that chickens responded the same among treatments in chickens both in Exp.3 and Exp.4. Some *in vitro* studies suggested that ARG can help to clear *Salmonella*, and VE can help to maintain the enterocyte in the

villi, therefore help to maintain chicken gut health. However, no differences were found in both experiments.

The large intestine and ceca develop a dense, predominantly anaerobic, microbial community, while the small intestine supports a less dense community that is dominated by lactic acid bacteria. In a modern hatchery, eggshells and the hatching rooms are kept as bacteria-free as possible. This is likely to affect the development of the micro flora, immune system and the intestinal physiology of the broiler chickens. Overall, in this study, the AVE and AVM diet did not help chicken to clear *Salmonella* concentration in ceca.

Chickens fed the AVM diet had higher IgM concentration than the rest treatments in Exp.4 (Table 12). Tohid et al. (2010) reported that MOS increased antibody titers against Avian influenza disease vaccination in the fourth, fifth and sixth weeks of age. Also Vesna et al. (2007) indicated that broilers fed MOS based prebiotics had significantly higher titers of antibodies than observed in the other two groups. Cetin et al. (2005) found that the addition of MOS significantly increased serum IgG levels at the end of a 15 weeks trial period in turkeys. However, Swanson et al. (2002) reported that the addition of MOS did not significantly influence IgG concentration after 2 weeks in dogs. Midilli et al. (2008) also found that dietary probiotic or prebiotic (both 0.1% and 0.2%) did not have significant effect on IgG concentration in chickens. Kim et al. (2011) reported that the 0.05% MOS has no effect on plasma IgA and IgG level among treatments. IgM and IgA are important in the ceca immune response of chickens against *Salmonella* strains (Berndt et al., 2004).

In this study, AVM diet increased chicken serum antibody concentrations of IgA at day 17 in Exp.3 (Table 11), and IgM at day 17 in Exp.4 (Table 12). The AVE diet also have some positive effect on serum antibody concentration based on the data that chickens fed AVE showed an increase IgM at day 17 in Exp.3

Heterophils are decisive component of chicken innate immunity (Kogut et al., 1994). Chicken TLR 1, TLR2, TLR3, TLR 4, TLR5 and TLR 7 have previously been described and been shown to be expressed in chicken heterophils (Higgs et al., 2006), and are important in pathogen recognition which initiate the pathways controlling expression of cytokines and chemokines, representing a link between innate and acquired immunity (Higgs et al., 2006). Our results suggest that ARG, VE or MOS down-regulated the expression and function of toll like receptors in this study. Kim et al. (2011) reported that birds fed 0.05% MOS had a significantly lower heterophil: lymphocyte ratio compare to birds fed a control diet. On the other hand, Huff et al. (2010) reported that the percentage of heterophils in peripheral blood was increased and their oxidative burst activity was stimulated by yeast cell walls. Other studies have also reported that supplementation with MOS can improve neutrophil function (Gómez-Verduzco et al., 2009; Palic et al., 2006; Sauerwein et al., 2007). However, in our study, heterophil activity was reduced by AVE and AVM diets at day 17. Maybe at day 17, when chickens are fed diets with AVE and AVM, nitric oxide might help to kill *Salmonella* while heterophil was less. Similar results were found in our previous study. Foley and Farrell (2003) reported that NO is an inducer of the innate immune response and concluded that NO can up-regulate the innate immune response. However, Wu et al.

(2005) did a study about nitric oxide on TLR 2 and TLR 4 gene expression in rats with acute lung injury complicated by acute hemorrhage necrotizing pancreatitis and found that NO markedly inhibited TLR 2 and TLR 4. To our knowledge, this is the first study about the combination of ARG, VE and MOS on chicken heterophil function and we found that the diets AVE and AVM down-regulated the heterophil function in chicken.

Macrophages are originated from bone marrow, entering the blood circulation as monocytes and subsequently differentiating into macrophages upon migrating to various tissues (Qureshi et al., 2000; Taylor et al., 2006). Monocyte oxidative burst response is another indicator of innate immune response and also indicator of adaptive immune response of chickens in this study. As shown in Figure 6 and Figure 7, no significant differences among treatments were found both in Exp.3 and Exp.4. These results suggest that supplementation with AVE and AVM have no effect on chicken monocytes activity compare to control group. No studies have ever reported about the effects of AVE and AVM on monocytes activity after challenged with *Salmonella*. Monocyte/macrophage were reported to induce nitric oxide synthase and produce increased amounts of NO during bacterial infection (Rydstrom and Wick, 2007). As shown in Table.1, basal diet already contain 1.53% L-Arginine, maybe this level is enough for monocyte's activity, and the extra ARG in AVE and AVM may not be necessary to improve monocyte activity. Also, Woollard et al. (2006) reported that alpha-tocopherol supplementation does not affect human monocytes. According to Rydstrom and Wick (2007), monocyte activity may be suppressed by *Salmonella* infection and has a poor capacity to process and present an antigen from *Salmonella*. Therefore in this study, it is possible that no

matter what is the supplementation in the diet, the signal of monocyte expression is blocked by *Salmonella* infection. This result is supported by Mastroeni (2002) who reported that monocytes can be further activated after arrival in the tissue; the reason why they delay arriving in tissue after *Salmonella* infection is because monocytes requires additional signals that are either not present or at too low concentrations in the blood.

Furthermore, chickens fed AVE showed a higher lymphocyte proliferative response in Exp.3 and had a higher LPA response at day 17 in Exp.4. These results are consistent with our previous studies, in which chicken fed AVE also showed a higher LPA response compare to control group. This suggests that AVE have a positive effect on chicken adaptive immune response. Many studies have showed that AVE has positive effect on immune response. Carbajal et al. (2010) reported that the combination of ARG and VE have an important role in improving immune response in chickens vaccinated and later challenged with *Eimeria*. Abdukaylkova et al. (1998) reported that ARG and VE improved T lymphocytes.

CONCLUSION

In conclusion, based on this study and our previous study, supplementation with AVE have positive effect on chicken immune response supported by the increase body weight, increased villi height and increased lymphocyte proliferate response, however, diet AVM may have a positive effect on adaptive immune response supported by increased IgA and IgM, but has a negative effect on innate immune response. The results from AVM in this study is inconsistent, maybe it is due to the birds age, the addition effect from MOS and *Salmonella* level, the different effect between MOS and ARG and vitamin E, or the concentration of MOS supplementation.

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