EFFECTS OF YEAST PRODUCT ON MODULATING THE ADAPTIVE IMMUNE FUNCTION IN BROILERS

A Thesis

by

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ABSTRACT

In this study, *Saccharomyces cerevisiae* fermentation yeast product was fed to broilers to investigate the effect of yeast products on the broiler immune function. The broilers were vaccinated for Newcastle Disease Virus on day 1 (B1 strain) and day 21 (LaSota strain) via eye drop. Body weights, feed consumption, feed conversion ratio, and mortality were recorded to monitor growth performance. There was no significant difference in Body weights (Probability < 0.05) between the control and treated groups. Since there was only one pen per group, no statistical analysis was conducted for feed consumption or feed conversion. At day 14, 21, 28, 35(blood only), and 42 blood and immune organ samples (thymus, bursa of Fabricius, and spleen) were collected to evaluate immune system development. All data were considered significantly different at Probability < 0.05. The control group had greater thymus indices at day 28, and 42. The control group had greater bursa of Fabricius indices on day 14 and 42. The control group had greater spleen index on day 21. The result of Newcastle Disease Virus antibody titer measurement showed that the population of treated group Immunoglobulin G was higher than control group after 1st vaccination at day 14 and increased faster than the control group after boost at day 21. Cell proliferation results from blood showed similar trend with Newcastle Disease Virus antibody titer results. The control group of simulation index of thymus was higher than the treated group at day 14. Through histology observation, there was no significant difference (Probability > 0.05) in the thymus medulla and cortex ratio between the control and treated groups. In the spleen, the

control group contained more nodules at day 14. All these results demonstrated that feeding the yeast product does influence broiler live immune system development.

DEDICATION

This thesis is dedicated to God, my family and friends who helped me to stand during this course.

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NOMENCLATURE

BW	Body weight
BF	Bursa of Fabricius
ConA	Concanavalin A
D	Day(s)
ELISA	Enzyme Linked Immuno-Sorbent Assay
FC	Feed consumption
FCR	Feed conversion Ratio
H	Hour(s)
IgG	Immunoglobulin G
µl	Microliter
ml	Milliliter
N	Normal
NDV	Newcastle Disease Virus Vaccination
WK	Week(s)

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CHAPTER I

INTRODUCTION

There are many organic types of animal feed additives being investigated to reduce the utilization of antibiotics in poultry production. Yeast is a unicellular fungi (Schneiter, 2004) that has historically been utilized as an animal feed supplement. There are more than 600 different species of yeast in the world (Baking industry research trust). Yeast products were used in human society from ancient times. However, the scientific study of yeast and the establishment of yeast fermentation were first accomplished by Louis Pasteur in late 1860's; resulting in processes to produce and isolate pure strains of yeast (Baking industry research trust). Currently most yeast products are utilized for production of bread, beer, and wine. However, yeast is also used as an animal feed additive to improve live production and host body immune system.

Saccharomyces cerevisiae is not only the most well-known yeast product, but it is also the most common yeast species (Schneiter, 2004); *'Saccharo'* means sugar and *'myces'* means fungus (Baking industry research trust). *Saccharomyces cerevisiae* accounts for quite a large proportion in the dried yeast product for animal feed additives (Chaucheyras-Durand et al., 2008). The *Saccharomyces* strains use a budding proliferation system to make multiple cells from single cell (Schneiter, 2004). Currently dried *Saccharomyces cerevisiae* is registered as an animal feed additive in Europe (EU Regulation 1831/2003) and Generally Recognized As Safe (GRAS) in US (Chaucheyras-Durand et al., 2008). The yeast-derived animal feed additives are used in a

variety of different animal species. Hippen et al. (2010) observed that yeast product had beneficial effects on milk production and milk fat percentage of dairy cows, and Abdelhamid et al. (2014) observed yeast product positive effects on energy utilization and live production of Nile tilapia. The effect of yeast product on poultry live production has been also reported many different ways. For many years yeast products have been used as a feed additive to increase efficiencies of meat and egg production in the poultry industry (Grau and Kamei, 1949). In the poultry industry, the yeast products are a dried product which ensures yeast cell viability and metabolic activity (Chaucheyras-Durand et al., 2008). The dried yeast product is mainly composed of extracted yeast cell wall; the yeast cell wall is well known as a non-specific stimulator of the immune system for both human and animals (Huang et al., 2009).

Yeast's mode of action in the poultry gut has not been fully revealed but yeast cell wall components are known to stimulate gut microflora in a manner similar to probiotic products. According to Barrow (1992), some of swallowed microorganisms can survive the low pH of gizzard and surviving microorganisms can multiply in the small intestine of normal (*N)* chicken. The crop, which is usually mentioned as the first alimentary organ, can be colonized by swallowed microorganisms in the chicken. Therefore, enteropathogens have more of a chance to colonize the crop. Smith (1965) reported that the yeast could also be viable Log_{10} 2.7 in the crop and Log_{10} 1.7 in the feces. That report showed that yeast may inhibit enteropathogens colonization on the host gut epithelium environment. *S. cerevisiae* is well-known as a species of yeast that does not colonize the host (Schneiter, 2004). There are two main purposes of using yeast

products: 1) the yeast may act as beneficial organisms in the alimentary tract and 2) the yeast may work with beneficial bacteria located in the alimentary tract (Barrow, 1992). Using yeast culture products has been shown to improve health status and longevity (Barrow, 1992). Yeast has been shown to positively impact growth performance in a variety of poultry species. Fathi et al. (2012) demonstrated BW improvements and decreasing mortality rate using yeast in broilers, Lensing et al. (2012) reported feed intake, laying rate, and egg weight improvement using yeast in laying hens, Firman et al. (2013) reported BW and feed efficiency improvement, and better gut parameters using yeast in turkeys. Gao et al. (2008) showed that yeast improved diet digestibility, and villus height in duodenum, jejunum, and ileum in broilers. Secondly, yeast also improves the poultry immune system. Osweiler et al. (2010) showed that yeast improved immune function against aflatoxin in broilers, Gao et al. (2008) showed that yeast improved antibody titer against Newcastle Disease Virus (NDV), and the population of immunoglobulin G, A, and M, and secretory IgA in duodenum in broiler. Lensing et al. (2012) reported that yeast improved immune function against coccidiosis in laying hens.

The purpose of the present study was to measure the effects of yeast in broilers. BW, feed intake, feed conversion ratio, and mortality were monitored. Spleen, BF, and thymus (primary and secondary immune organs) indices were measured at specific time points. ELISA was performed to measure anti-NDV antibody titer (IgG). Cell proliferation was performed to check activation, proliferation and differentiation of both CD4 and CD8 T cells from peripheral blood, and the primary and secondary immune organs. Hematoxylin and eosin staining was performed to count follicle numbers in the

primary and secondary immune organs. These experiments were conducted to evaluate immune system improvement in broilers.

CHAPTER II

REVIEW OF LITERATURE

Introduction

Over the past several decades, knowledge of the avian immune system has been greatly augmented. To begin with, a chicken has two different immune systems that are similar to that of other vertebrates. The innate immune system is usually known as the first barrier of the immune system. The innate immune system does not have memory, but it does act fast and short. However, the innate immune system does not work alone, as the adaptive immune system works to protect it from exposures to diseases. The adaptive immune system is known as long-term immunity and consists of humoral and cell-mediated immunity. The immunity is accomplished by many different cells; T and B cells (lymphocytes) take up the majority of host body immune system, and are either produced by or matured at primary and secondary immune organs. In order to understand a chicken's immune system, functions of primary and secondary immune organs of chicken need to be well understood.

Bursa of Fabricius (BF)

B lymphocytes are produced by bone marrow in most mammalian species (Sharma, 1997). In avian species, the BF is one of the primary lymphoid organs, and is a very important place for differentiation and maturation of B lymphocytes (Tirziu and Seres, 2010); there are some T lymphocytes located on follicular cortex area of the BF

but not many in the follicular medulla area of BF. Even if the BF is experimentally removed, some of the B lymphocytes can be made by alternative sites. However, B lymphocytes produced by alternative sites will not be fully developed to the same level as the B lymphocytes produced by the BF (Tirziu and Seres, 2010). The BF is located near the cloaca, and has a muscle layer that wraps around the folded BF epithelium surface; the appearance of BF is a folded wrinkle that contains many follicles. BF follicles consist of follicular medulla and cortex areas; those two areas are divided by the cortico-medullary junction. The B lymphocytes in BF proliferate at both the cortex and medulla area.

There are two developmental phases of BF in a chicken: 1) In the embryonic phase, each of follicles in BF starts to connect with bursal epithelium, and bursal lumen attaches to gut lumen, and 2) after the hatching phase, the growth rate of BF is slow, but the cortex and medulla divide rapidly (Tirziu and Seres, 2010). After common lymphoid progenitors get into the BF mesenchyme of base-membrane, immunoglobulin gene (heavy and light chain) rearrangement starts before progenitor cells colonize the BF during the early and middle WK of embryonic development (Ratcliffe and Hartle, 2013); when the progenitor cells in the BF begin rearrangement into heavy and light chains, only about 3% of 30,000 precursors of B lymphocytes will have undergone rearrangement that contains both heavy and light chains (Tirziu and Seres, 2010). Next, progenitor B cells migrate from the bursal mesenchyme, and colonize BF lymphoid follicles where they express immunoglobulin on their surfaces (Ratcliffe and Hartle, 2013). The number of immunoglobulin expressing B cells increases during the final WK

of embryonic development (Ratcliffe and Hartle, 2013). Prior to hatching, the BF starts to become well-structured. Gut-derived antigens enter the medulla area through the follicle associated epithelium and these antigens are presented by dendritic cells to the B lymphocytes (Ratcliffe and Hartle, 2013). The BF grows in 8-10 WK until it starts to involute (Olah et al., 2013). The antigen presented naïve B lymphocytes migrate from medulla to cortex. The follicular cortex is the most cell-proliferating area in juvenile BF (Ratcliffe and Hartle, 2013). Finally, the naïve B lymphocytes in the follicular cortex immigrate to the secondary lymphoid immune organ through peripheral blood (Ratcliffe and Hartle, 2013).

Thymus

Thymus lobes (4-7) are located on each side of the neck and covered by connective tissue. The thymus lobes have a bean shape. Similar to BF, the thymus grows in 3-4 months, and its growth rate is especially increased around hatching time (Olah et al., 2013). The thymus is known to produce T lymphocytes, and it also is the place where activation, proliferation, and differentiation of T lymphocytes occur. The cortex and medulla area are the main structures of the thymus and cortico-medullary junction that divides them. Densely packed T lymphocytes are located in the cortex area and smaller numbers of T lymphocytes are located in the medulla area (Olah et al., 2013). Some heterophils can be found in the medulla. Macrophage and dendritic cells stay in the cortico-medullary junction to help differentiation of T lymphocytes (Olah et al., 2013).

When hematopoietic cells enter the thymus through post capillary venules, they stay in the cortico-medullary junction (Meis et al., 2012). When hematopoietic cells interact with the thymus, TCR arrangement and cell proliferation begins (Meis et al., 2012). Progenitor T cells generate as double negative ($CD4^-CD8^-$) cells, and then migrate to the cortex area (Meis et al., 2012). In the cortex area, the double negative T cells start the TCR beta chain rearrangement and proliferation (Meis et al., 2012). After finishing the TCR arrangement, double negative T cells change to double positive T cells. Positive selection occurs to determine which double positive T cells have high affinity with antigenic peptides (Li et al., 2007). Positive selected T lymphocytes migrate to medulla area, and then negative selection occurs to sort out self-reacting T lymphocytes (Olah et al., 2013; Meis et al., 2012). After negative selection, $CD4^+CD8^-$ and $CD4^-CD8^+$ lymphocytes are produced as a result. Finally, antigen non-experienced $CD4^+CD8^-$ and $CD4^-CD8^+$ lymphocytes are sent out to the secondary immune organs through blood vessels that are located near the cortico-medullary junction to finish the maturation of T lymphocytes.

Spleen

The spleen is not only an important secondary immune organ, but also is the site for all immature lymphocytes to mature in a chicken. The bean shaped spleen is covered by collagen and reticular fibers (Olah et al., 2013). A chicken does not have a well-developed immune system (such as lymphatic vessels and nodes), so the spleen can be a critically vital immune organ (Olah et al., 2013). There are two primary differences

between the avian and mammalian spleen. First, the chicken spleen uses a 'closed circulation system', which means capillaries continuously enter the red pulps through the venous sinusoid of spleen (Olah et al., 2013). Second, the mammalian spleen has a marginal zone around white pulps not present in the avian spleen (Hajime, 2004; Nagy et al., 2005). The avian spleen starts to form at 48 H of embryo development and can be fully functioning as a secondary immune organ near the time of hatch (Olah et al., 2013).

The spleen consists of central arterioles, red pulps, and white pulps. However, clear division of these structural elements is difficult. The main splenic artery connects with the pulps, and divides into central arterioles, then further divide as penicillar capillaries (Olah et al., 2013). Most of T lymphocytes (T helper cells), the area called periarteriolar lymphoid sheaths (PALS), surround the arterioles for maturation of B lymphocytes; PALS contains mainly $CD3^{+}CD4^{+}$ expressed T lymphocytes with some other types of T lymphocytes (Nagy et al., 2005; Olah et al., 2013). The penicillar capillaries are covered by ellipsoids. The ellipsoids are also covered by white pulps. The penicillar capillary connects with sinuses of the red pulp (Nagy et al., 2005). The red pulps contain primarily $CD8^{+}$ T lymphocytes, but some of $CD4^{+}$ and plasma cells also reside at red pulps (Olah et al., 2013). Antigens can reach the spleen white pulps of ellipsoids within 25-30 H. After an antigen enters the spleen, a germinal center is formed within 72 H that contains follicular dendritic cells from the ellipsoids which capture antigens and introduce them to naïve B lymphocytes in the germinal center; the germinal center keeps their form until 6 D (White et al., 1975).

When the naïve B lymphocytes from the BF and the immature T lymphocytes from the thymus enter the spleen through the splenic artery, they start to inhabit each different place in the spleen to mature. At first, antigens moving around in the host enter the spleen through the peripheral blood flow, and are captured by macrophage or dendritic cells (Hajime, 2004). The captured antigens are then introduced to naïve T and B lymphocytes by macrophage or dendritic cells. Lastly, T and B lymphocytes mature after they have experienced an antigen once. Then those matured T and B lymphocytes leave the spleen through the splenic vein (Hajime, 2004).

CHAPTER III

DIETARY EFFECTS OF THE YEAST PRODUCT ON GROWTH PERFORMANCE OF BROILERS

Introduction

Since 2006, when the Europeans Union has banned the use of antibiotics as an animal growth promoter, the animal industry sought alternative feed additives to improve and protect farm animals. Antimicrobial agents (Costa et al., 2011; Torok et al., 2011), organic acids (Menconi et al., 2013; Liem et al., 2008), minerals (Manangi et al., 2012; Nollet et al., 2007), and herbs (Yasar et al., 2011; Al-Kassie et al., 2008) have been used and reported worldwide in the poultry industry recently. Yeast, one of the alternative feed additives, has been also widely used to avoid or minimize use of antibiotics in animal industry. For example, the yeast products were shown to have a variety of positive effects on different animals, such as dairy cow (Hippen et al., 2010), swine (Price et al., 2010), and dairy ewe (Firmin et al., 2011). The effect of yeast products on poultry live production had been also reported many different effects of ways. For example, a positive effect of yeast products was reported on broiler and turkey weight (Fathi et al., 2012; Firman et al., 2013), feed efficiency (Adebiyi, et al., 2012; Firman et al., 2013), and egg production (Hashim et al., 2013). Therefore, yeast products bring many physical positive effects on growth performance to help the poultry industry.

According to Gourbeyre et al. (2011), the definition of probiotic is ‘live microorganisms which, when consumed in adequate amounts, confer a health benefit on

the host'. Therefore probiotics are usually used as feed additives to improve live production and immune function in animal industries. Probiotics improve intestinal microbial balance to secrete bacteriocin or defensin, inhibit bacterial adhesion, reduce pH of lumen, and increase mucus production (Otutumi et al., 2012).

Similar to most probiotic feed additive products, yeast culture products (mainly *Saccharomyces cerevisiae*) were used as growth promoters. Yeast products have long been used as a feed additive to increase efficiencies of meat and egg production in the poultry industry (Grau and Kamei, 1949). Many reports have discussed the immune system enhancing effect of yeast product on various poultry species, turkey (Cetin et al., 2005), broiler (Gao et al., 2009), and laying hen (Lensing et al., 2012). Most of the yeast product used in the poultry industry is dried product. The dried yeast product mainly consists of extracted yeast cell wall, such as β-1,3 (30-45 % of wall mass)/1,6-glucans (5-10 % of wall mass), mannan-oligosaccharides (MOS, 30-50 % of wall mass), or nucleotides (Klis et al., 2006). At first, β-1,3/1,6-glucans can bind to specific receptor CR3 on surface of macrophages that activates phagocytosis and production of cytokines (Swiatkiewicz et al., 2014). Following that, innate and acquired immune responses are increased by T lymphocyte proliferation (Swiatkiewicz et al., 2014). Second, gram-negative pathogen bacteria bind the MOS instead of the binding on host intestinal epithelial cell and move through host intestine without colonization (Ferket et al., 2002). Third, the immune function of nucleotide is not clear, but there is some evidence that nucleotide supplementation may enhance concanavalin A (ConA) and antigen-specific induced spleen cell proliferation (IL-2 and IFN-γ) in mice (Adjei et al., 1999).

The mechanism of probiotics in the broiler gut immune flora has not yet been established completely. However, the probiotic action in mucosa can be explained briefly. At first, the probiotics interact with M cell. In this case, the M cell stimulates an immune response 2 different ways using transcytosis processes; 1) presentation to macrophage or dendritic cell, 2) directly activating B lymphocytes. Both ways stimulate T lymphocyte activation in lamina propria. The second probiotic action in the mucosa is to interact with intestinal intra-epithelial lymphocytes directly to stimulate T lymphocytes in the lamina propria. Third, the probiotics also interact with intra-epithelial cells to stimulate T lymphocytes in the lamina propria using endocytosis (Otutumi et al. 2012). Therefore, the interaction of probiotics with the host mucosa derives differentiation and results in the migration of T lymphocytes and proliferation of IgA, IgM and IgG.

Therefore we hypothesize that feeding a yeast product to broilers will improve productive performance and enhance immune function.

Materials and Methods

Birds, Housing, and Diets

This study was performed at Texas A&M University Poultry Research, Teaching and Extension Center (TAMUPRC). A total 180 of broiler (Ross 708, Aviagen, Huntsville, AL) 18 d of incubation embryos were obtained from a local commercial broiler hatchery and were incubated to hatch at the TAMUPRC. Only healthy broiler

chicks (no leg, naval, abdominal, or deformities issues) were selected. Excess birds were euthanized at the beginning of the study. A total of 150 randomly selected birds were separated to control and treated groups. Each group of birds was allocated to one of two 3.05m X 3.66m of floor pens and space per bird 0.185m^2/bird at initial placement. The dietary treatments consisted of 0 (Control), and 1.25 kg/metric ton (Treated) of *Saccharomyces cerevisiae* fermentation product (Original XPC™, Diamond V, Cedar Rapids, IA). Starter, grower, and finisher complete diets were manufactured at the TAMUPRC feed mill (Table 3.1). All diets were fed in pelleted form. Each floor pen was provided *ad libitum* supply of feed and water. There were 2 feeders and total 2 lines of 6 nipple drinkers in each pen. Each pen had a 150 watt heat lamp to provide supplemental heat for the first 7 d. Room temperature was controlled at 32.2°C by central heating furnace (23939, L.B. White, Onalaska, WI) and pen temperature was 35°C for first 3 d. Pen temperature was controlled 48 H before placing hatched birds in the pen. Pen temperature was decreased 5 degrees each WK until 25°C was reached. Light was provided 24 H throughout the study. During the animal trial, there were no replaced birds. During the trial, broilers did not receive any vaccination other than the NDV.

Table 3.1. Experimental diets and nutrient composition

	Starter 0-14 d	Grower 15-28 d	Finisher 29-42 d
Ingredients, %			
Corn, yellow grain	58.46	64.73	68.53
Soybean meal, dehulled solvent	34.48	29.33	24.64
Bio Phos 16/21 P	1.56	1.59	1.29
Limestone	1.49	1.46	1.60
Salt, plain	0.51	0.49	0.46
Mineral mix[1]	0.05	0.05	0.05
DL Methionine	0.28	0.08	0.16
Lysine	0.17	0.02	0.12
Vitamin premix[2]	0.25	0.25	0.25
Fat, A/V blend	2.75	2.00	2.90
Original XPC[3]	0.125	0.125	0.125
Nutrient Composition			
CP, %	22.02	19.80	17.99
ME, kcal/lb	3048	3055	3151
Crude Fat, %	5.30	4.74	5.73
Lysine, %	1.30	1.05	1.00
Methionine (M), %	0.61	0.39	0.44
M+Cysteine, %	0.97	0.72	0.75
Tryptophan, %	0.26	0.23	0.20
Threonine, %	0.82	0.74	0.67
Arginine, %	1.45	1.29	1.14
Valine, %	1.00	0.91	0.82
Calcium, %	0.92	0.90	0.89
Available Phosphorus, %	0.45	0.45	0.38
Sodium, %	0.22	0.21	0.20
Chloride, %	0.39	0.34	0.35

[1] Trace mineral premix added at this rate yields 149.6 mg manganese, 55.0 mg zinc, 26.4 mg iron, 4.4 mg copper, 1.05 mg iodine, 0.25 mg selenium, a minimum of 6.27 mg calcium, and a maximum of 8.69 mg calcium per kg of diet. The carrier is calcium carbonate and the premix contains less than 1% mineral oil.

[2] Vitamin premix added at this rate yields 11,023 IU vitamin A, 3,858 IU vitamin D_3, 46 IU vitamin E, 0.0165 mg B_{12}, 5.845 mg riboflavin, 45.93 mg niacin, 20.21 mg d-pantothenic acid, 477.67 mg choline, 1.47 mg menadione, 1.75 mg folic acid, 7.17 mg pyroxidine, 2.94 mg thiamine, 0.55 mg biotin per kg diet. The carrier is ground rice hulls.

[3] Diamond V Original XPC, Cedar Rapids, Iowa

Growth Performance

Group BW, feed weights, and feed consumption of each pen were recorded on d 1, 14, 21, 28, and 42. Individual BW of 15 birds per pen was recorded on d 14, 21, 28, 35, and 42. Mortality was recorded daily.

Animal Immunization

Birds were vaccinated with Newcastle Disease Vaccine (NDV ND-0620, Merial, Duluth, GA) on d 1, followed by (NDV ND-1820, Merial, Duluth, GA) on d 21 via eye drop.

Collecting Samples

At d 14, 21, 28, 35, and 42, peripheral blood samples were collected from 15 randomly selected birds from each pen. Blood samples were collected from vein located on wing and were mixed with one volume sodium citrate to prevent blood coagulation. The blood plasma serum samples from 5 out of 15 randomly selected broilers were chosen for antigen-specific titer assay. All plasma serum samples were stored at -20 °C until analysis. Birds were euthanized via CO2 chamber, and BW data were recorded. Immune organs, BF, and spleen were harvested and weighed immediately from the euthanized birds. Immune organs from 5 out of 15 randomly selected broilers were also chosen for lymphocyte proliferation assay. Other remaining harvested organs were fixed in buffered 10% neutral formalin (16004-112, VWR international, Radnor, PA) and

were transferred into 70% alcohol (61509-5000, Acros, Geel, Belgium) for cell immune-histology analysis.

Humoral Immunity Measurement of NDV Antibody Titer

For measuring the effect of dietary XPC supplementation on NDV vaccination, Newcastle Disease Virus Antibody Test Kit (96-6547, Synbiotics Corp., San Diego, CA) was used. Serum dilution preparation plates were prepared by adding 300 µl of dilution buffer to each well of an uncoated 96 well micro-titer plate, then 6 µl of blood sample serum was added per well. Negative and positive controls were made on specific wells of the plate. Before being transferred to NDV antigen coated ELISA plates, the preparation plate was incubated 5 minutes at room temperature to allow equilibration of the dilution buffer. Then 50 µl of dilution buffer was added to all wells on NDV antigen test plate. Fifty µl of positive control and all other diluted serum samples were transferred from the dilution plate to the corresponding wells of the NDV antigen test plate, the plate was incubated 30 minutes at room temperature. Liquid from each well was discarded then 300 µl of wash solution (20 ml concentrated wash solution was applied in 380 ml laboratory grade (distilled or R.O.) water (1:20), then mixed well) was applied to each well. After 3 minutes, all liquids in the plate were discarded. This washing procedure was repeated 2 more times. 100 µl stock conjugate solution (horseradish peroxidase (HRP) conjugated anti-chicken IgG(H+L) supplied in HRP stabilizer.) was diluted in 10ml dilution buffer (1:100 dilution), then were mixed well. This 100 µl of diluted anti-chicken IgG peroxidase conjugate solution was applied to

NDV antigen test plate. The test plate was incubated for 30 minutes at room temperature. Washing procedure was repeated the same as above 3 times. Then 100 µl of room temperature stayed substrate solution (provided from test kit) was applied to each assay well. The test plate was incubated for 15 minutes at room temperature followed by 100 µl stop solution applied to each assay well. The test plate was read using a microplate reader set 405-410 nm for antibody titer measurement. After reading, the average of positive and *N* control serum absorbance (Optical Density as O.D.) was calculated. Each samples was analyzed and antibody titers will calculate using the formula: SP (Sample to positive ratio) = (Absorbance of sample – Average of *N* control absorbance)/(Average of positive control absorbance – Average of *N* control absorbance), Final Titer = ANTILOG(1.464 X (Log_{10}SP) + 3.740). Gradient value data were analyzed using trend-line by Microsoft Excel 2010 for Windows (Microsoft, Redmond, WA).

Lymphocyte and Blood Leukocyte by Cell Proliferation

In order to harvest single lymphocytes containing mononuclear cells suspension, immune organs (spleen, BF, and thymus) were passed through 70 µm cell strainer (REF 352350, BD Falcon, Franklin Lakers, NJ) into a 50 ml tube and were re-suspended in RPMI-1640 (12-115F, Lonza, Walkersvill, MD). Lymphocytes containing mononuclear cells were harvested using Histopaque 1077 (10771, Sigma-Aldrich, St. Louis, MO) gradient separation method. Briefly, one volume of cell suspension was overlaid onto Histopaque 1077 and was centrifuged at 400 x g for 20 min at room temperature. Lymphocytes at the interface was collected and washed twice with PBS by centrifuged

at 400 x g for 10 min at 4 °C. Lymphocytes were re-suspended in RPMI 1640 medium supplemented with 1% of Glutamax (35050-061, Life Technologies, Grand Islaand, NY), 1% Sodium Pyruvate (S8636, Sigma-Aldrich, St. Louis, MO), 8% of fetal bovine serum (S11150, Atlanta Biological, Lawrenceville, GA), 5% of chicken serum (C5405, Sigma-Aldrich, St. Louis, MO), 1% of penicillin with streptomycin (P4333, Sigma-Aldrich, St. Louis, MO). 95% of the viability and number of cells were determined by trypan blue (RNBC7180, Sigma-Aldrich, St. Louis, MO) exclusion method. For peripheral blood mononuclear cells separation, one volume of whole blood was first diluted with sterile PBS and separated by Histopaque 1077 gradient method as previous mentioned. Cell Counting Kit-8 (CCK8) (96992, Sigma-Aldrich, St. Louis, MO) was used to assess lymphocyte proliferation rate. Briefly, lymphocytes ($2X10^6$) were seeded onto the 96-well tissue culture plates then were stimulated with 10 µg/ml ConA (72H7195, Sigma-Aldrich, St. Louis, MO) for 48 H at 37 °C in a 5% CO2, 95% humidity incubator. Ten microliters of CCK-8 solution was added and incubated for 4 h at 37 °C. The optical density (OD) was measured at 450 nm with a microplate reader (1420-832, Perkin-Elmer, Waltham, MA). Each samples were analyzed in triplicate and the stimulation index (SI) was calculated using the formula: SI = (mean OD of mitogen-, OD antigen-stimulated proliferation)/(mean OD of non- stimulated proliferation).

Histology

BF, thymus and spleen were freshly isolated from 14, 21, 28, and 42 d-old broilers. The harvested samples stored in 70% alcohol were transferred into mega-

cassettes (25608-844, VWR, Randor, PA). All samples were stained with Hematoxylin and eosin stain at the Texas A&M Veterinary Medical Histopathology Laboratory (TVMHL). Stained sections were analyzed under a microscope (SZH10, Olympus, Center Valley, PA) with a imaging camera (RT2500, Sterling Heights, MI) and program (SPOT^{TM} 5.0, Sterling Heights, MI). The variation in area shape of BF, the cortex and medulla area size of ratio (Ratio= Medulla/Cortex) of thymus, and the splenic nodules of spleen were measured as an indicator (ImageJ, National Institutes of Health, Bethesda, MD) of the effect of yeast as feed additive on BF, thymus, and spleen maturation or immunological system development.

Statistical Analysis

All statistical analysis data were analyzed using one-way analysis of variance by JMP® 11 for Windows (SAS INC., Cary, NC). All data (BW, organ indices, ELISA titer, and lymphocyte population results) were presented as mean and the pooled standard error of the mean. All data were considered significantly different at $P < 0.05$.

Results and Discussion

Live Production

In this study, BW, feed consumption, and cumulative feed conversion ratio were observed. Significant differences were not observed ($P > 0.05$) between the control and the treated group in BW (Table 3.2). This trend was also observed by others (Al-

Mansour et al., 2011; Adebiyi et al., 2012). Statistical analysis was not conducted for FC and FCR between the control and treated groups because only one pen per treatment was utilized (Table 3.3 and 3.4). Mortality was 2 birds in the control group and 3 birds in the treated group. There was no significant difference ($P > 0.05$) in mortality between the control and the treated group. In conclusion, yeast dietary supplementation is expected to bring many positive effects on live production but there were no differences noted in this experiment as it was not designed to examine these factors closely but focused on the adaptive immune system.

Table 3.2. Effect of supplementation of yeast product on Broiler Body Weights (g) from Day 1-42

Days	1	14	21	28	35	42
Control	42.3	309.3±11.0	659.6±15.6	1203.3±30.0	1989.3±41.8	2527.3±51.2
Treated	43.0	298.6±8.4	699.1±13.4	1165.7±21.2	1956.7±43.3	2511.1±56.0

Table 3.3. Effect of supplementation of yeast product on Broiler Feed Consumption (kg/bird) from Day 1-42

Days	1 to 14	14 to 21	21 to 28	28 to 42	1 to 42
Control	0.433	0.593	0.957	2.545	4.528
Treated	0.413	0.557	0.882	2.375	4.227

Table 3.4. Effect of supplementation of yeast product on Broiler Cumulative Feed Conversion Ratio from Day 1-42

Days	1 to 14	1 to 21	1 to 28	1 to 42
Control	1.533	1.552	1.602	1.705
Treated	1.523	1.517	1.570	1.683

Primary and Secondary Immune Organ Index

In the primary immune organs, BF and thymus have similar trends. The decreasing trend of the thymus index (Figure 3.1) as the bird ages has been also observed through another experiment (Zhang et al. 2008) that used a variety concentrations of yeast product (0, 25, 50, 75, 100, 125 mg/kg) in Arbor Acres broiler. However, Li et al. (2012) used 2 g/kg of the yeast product in Arbor Acres broiler and reported that the thymus index value increased as the birds aged. Index value of decreasing the BF (Figure 3.2) and increasing spleen (Figure 3.3) trends with age has been observed through other experiments (Zhang et al., 2008; Li et al., 2012).

Significant differences ($P > 0.05$) were not observed on D 14 and 21 thymus, and D 14 and 42 BF (Figure 3.1 and 3.2). Significant differences were observed on D 28 ($P < 0.05$) and 42 ($P < 0.05$) of the thymus, and D 21 ($P < 0.05$) and 28 ($P < 0.05$) of the BF. The control group had greater thymus index on D 28, and 42, and also the control group had greater BF index on D 14 and 42. The control group of the thymus reached a peak at D 28 (0.3793) then the index value decreased. However, the treated group of thymus reached the peak at D 14 (0.3375). Similar to the thymus index trend, the control group reached the peak of the BF index value at D 21 (0.2443) then decreased. However, the treated group highest BF index value point was D 14 (0.1882). These results gave a hypothesis that the peak of the treated group thymus index value might be reached even before D 14. Throughout this experiment, the possibility that the yeast product stimulated immune function at a very early age was deduced.

In the results from the spleen, there were no significant differences (P > 0.05) at D 14, 28, and 42 but D 21(P < 0.05) (Figure 3.3); the control group had greater spleen index at D 21. The spleen had a different trend from the primary immune organs. The treated group spleen index value peaked once at D 14 (0.1047), and then index value decreased slightly (0.1004). However, after boost, the spleen index value of the treated group was increased again until D 42. This result gave a hypothesis that the yeast product could stimulate the secondary immune organ of broiler much earlier after first vaccination and faster after boost. Therefore, further experiments also need to check even before D 14 for secondary immune organ.

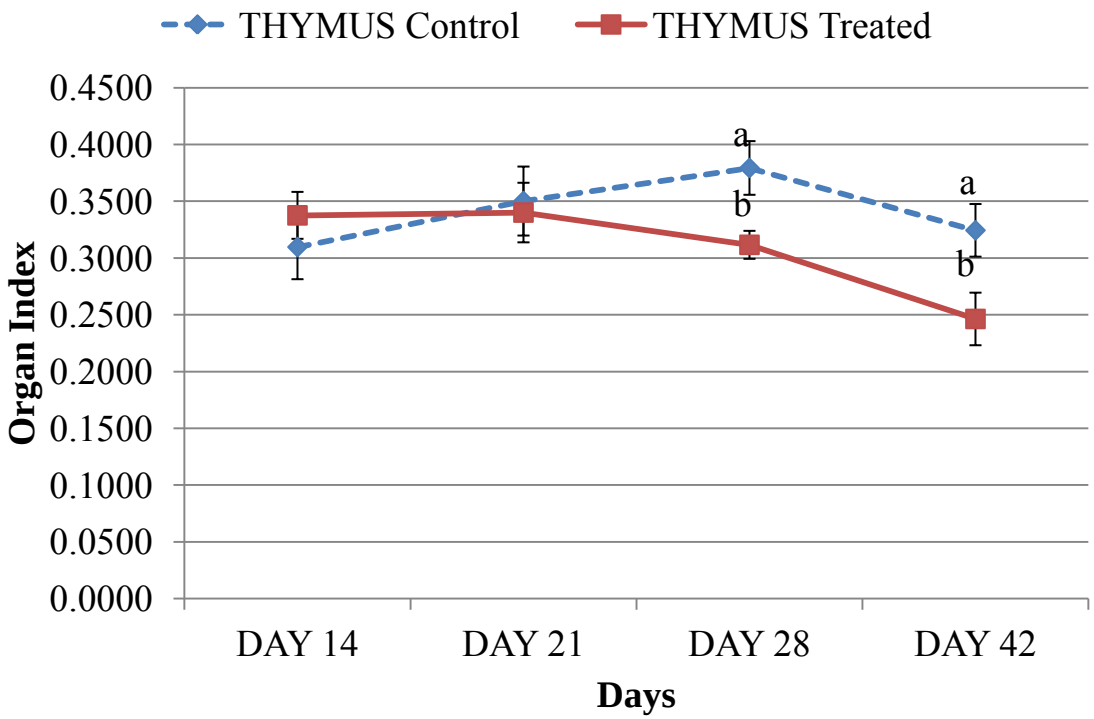

Figure 3.1. Effect of supplementation of yeast product on thymus indices

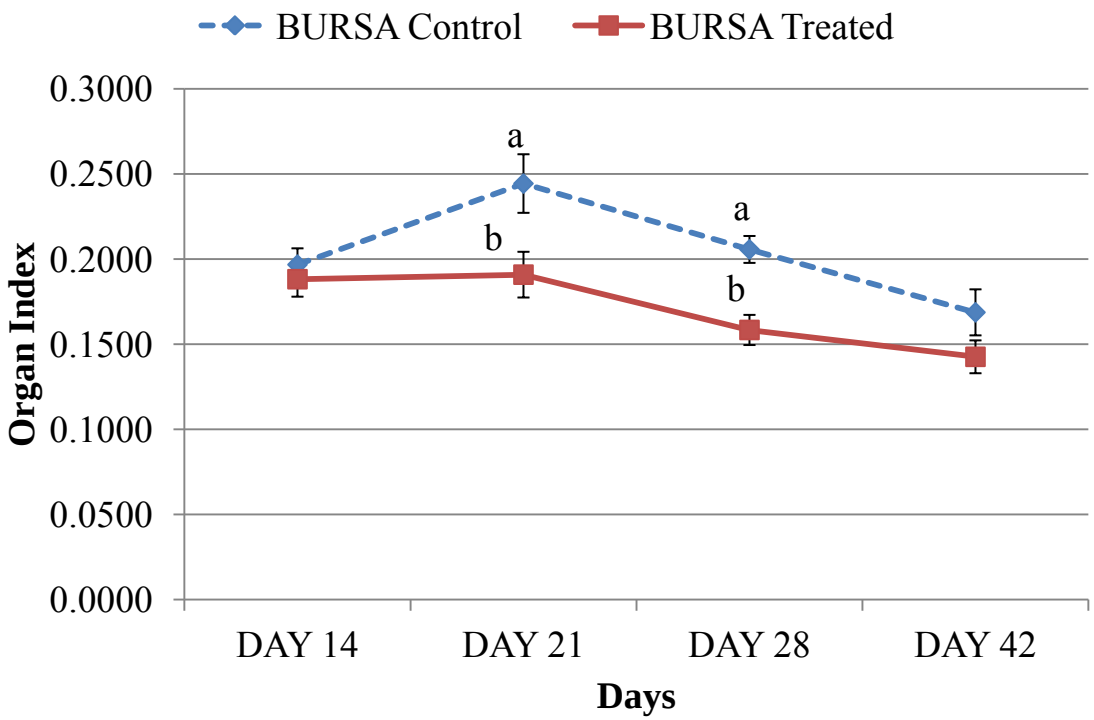

Figure 3.2. Effect of supplementation of yeast product on bursa of Fabricius indices

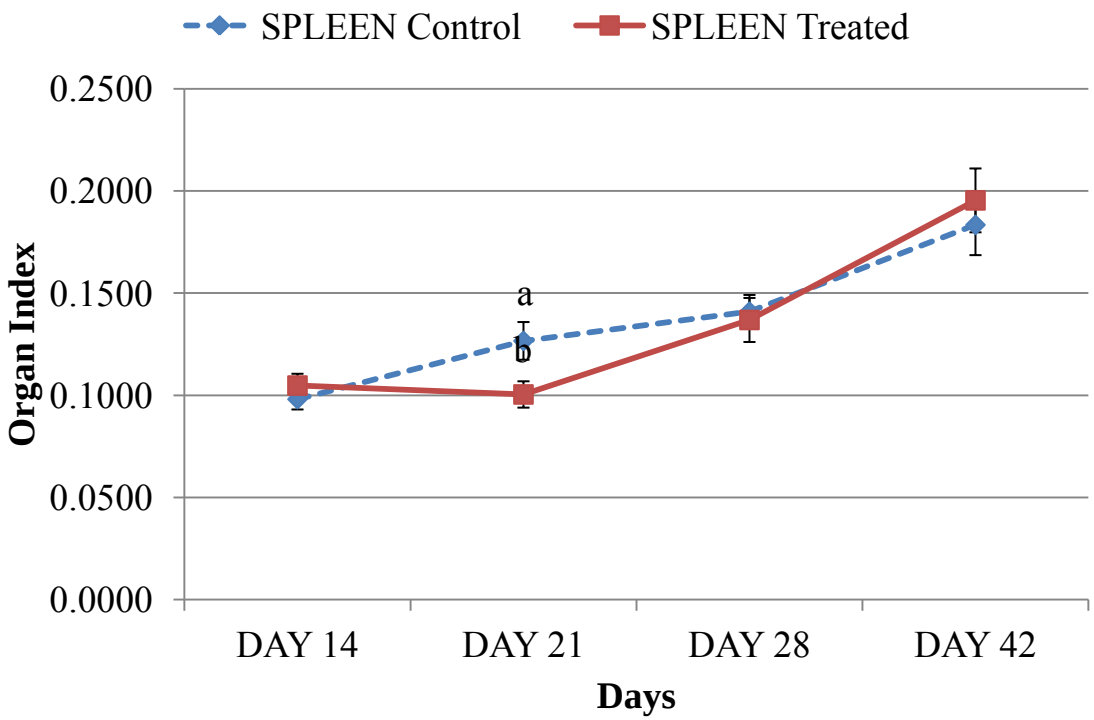

Figure 3.3. Effect of supplementation of yeast product on spleen indices

Antibody Titer against to NDV

Both the treated and control group antibody titer showed a decreasing trend through D 14 to 42 in this study (Figure 3.4). This trend was observed by Li et al. (2012). However, other studies (Fathi et al., 2012; Gao et al., 2008) observed that antibody titer increased as the bird aged. These and Li studies were vaccinated on D 1 and 7, and boosted on D 21 and showed high antibody titer in the early stages. Fathi et al. (2012) and Gao et al. (2008) studies vaccinated the birds on D 10 and 7, respectively, and boosted vaccine on D 28. These studies showed a high antibody titer in later stages.

In this study, the treated group IgG antibody titers were significantly different from the control on D 21 ($P < 0.05$) and 28 ($P < 0.05$). Titers on other days were not significantly different (Figure 3.4). After the first vaccination, the antibody titer in both groups was increased but the antibody titer decreased soon. After boost, antibody titer increased again but the treated group increased faster (The gradient value of the treated and control group were 1610.7 and 747.92 respectively) and stronger (The antibody titer of the treated and control group were 2506.289 and 1840.428) than the control group. Therefore, the yeast products stimulated the broiler immune system at an early age and provided for a response of the immune system after the boost to be faster and greater.

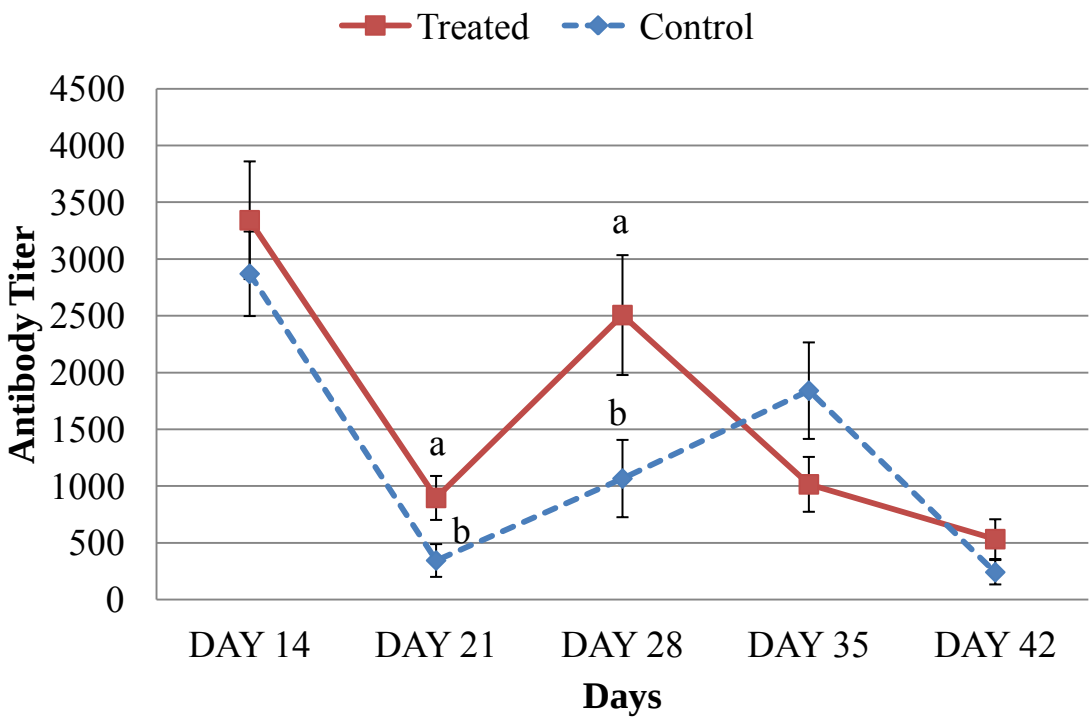

Figure 3.4. Effect of supplementation of yeast product on IgG antibody titer against NDV in broiler

Lymphocyte and Blood Leukocyte by Cell Proliferation

The cell proliferation assay was executed to measure abilities of T lymphocytes' proliferation, differentiation, and activation. Significant differences were observed at D 28 ($P < 0.05$) and 42 ($P < 0.05$) of blood. In the thymus and spleen, significant differences ($P > 0.05$) were not observed (Figure 3.6 and 3.7). There were no statistical data on both the treated and control groups of BF because ConA is a T lymphocyte mitogen. Therefore, both groups of BF did not contain enough T lymphocyte population (at least 1000 cells are necessary per well, according to sigma CCK-8 manual) to measure T lymphocyte abilities.

The blood results (Figure 3.5) were very similar to the results of IgG antibody titer against NDV. For example, the treated group simulation index was significantly ($P < 0.05$) higher than the control group at D 28. However, simulation index of the treated group was significantly ($P < 0.05$) lower than the control group at D 42. Throughout this experiment, there was no significantly difference at D 14 (after 1st vaccination). However, the treated group T lymphocytes' proliferation, differentiation, and activation abilities were increased (The simulation index value of the treated and control group were 1.0903 and 0.9513) than the control group after boost. As a result, the yeast products stimulated the broiler immune system when the host body was stimulated by boost.

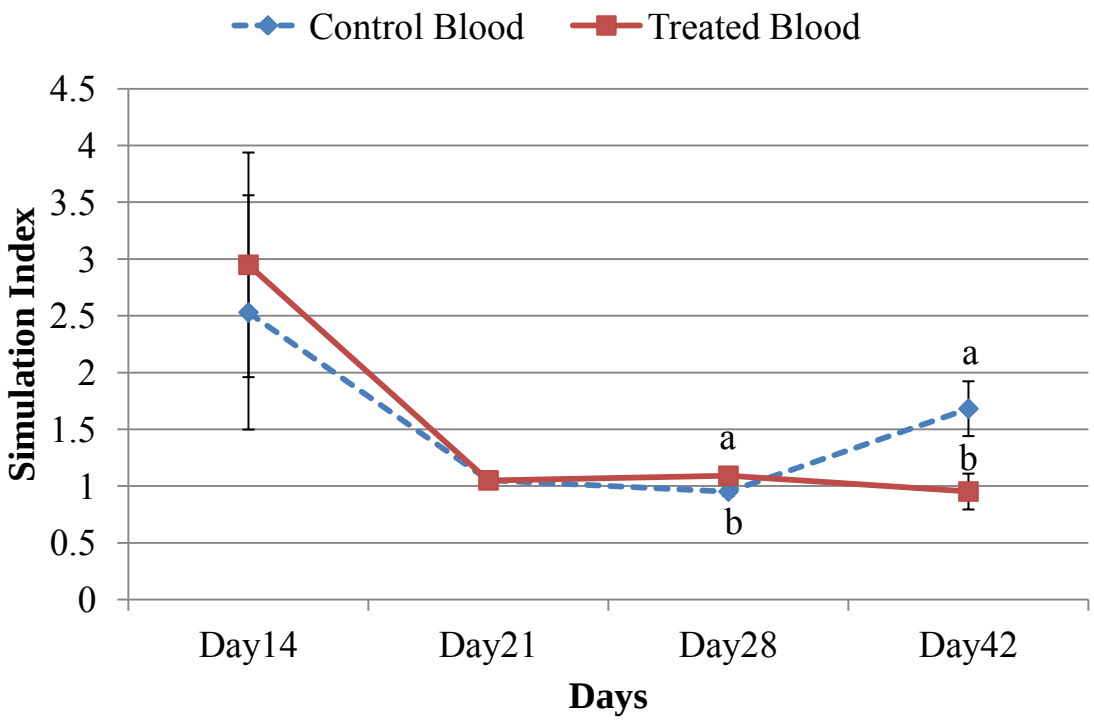

Figure 3.5. Effect of supplementation of yeast product on T cell abilities in blood

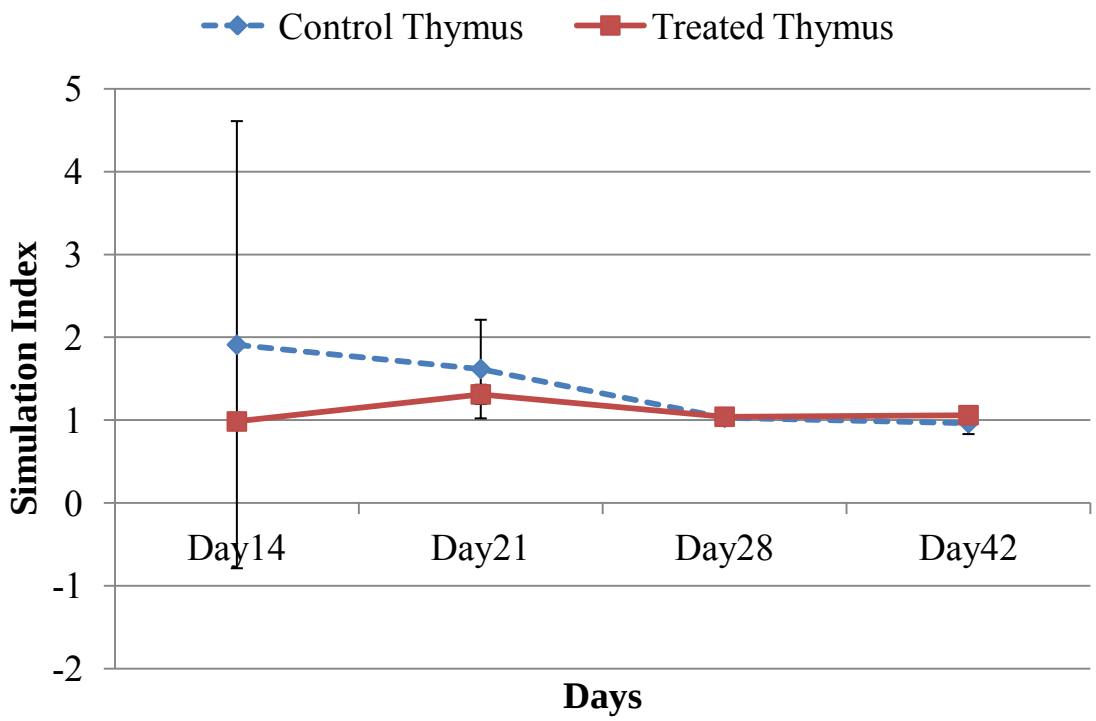

Figure 3.6. Effect of supplementation of yeast product on T cell abilities in thymus

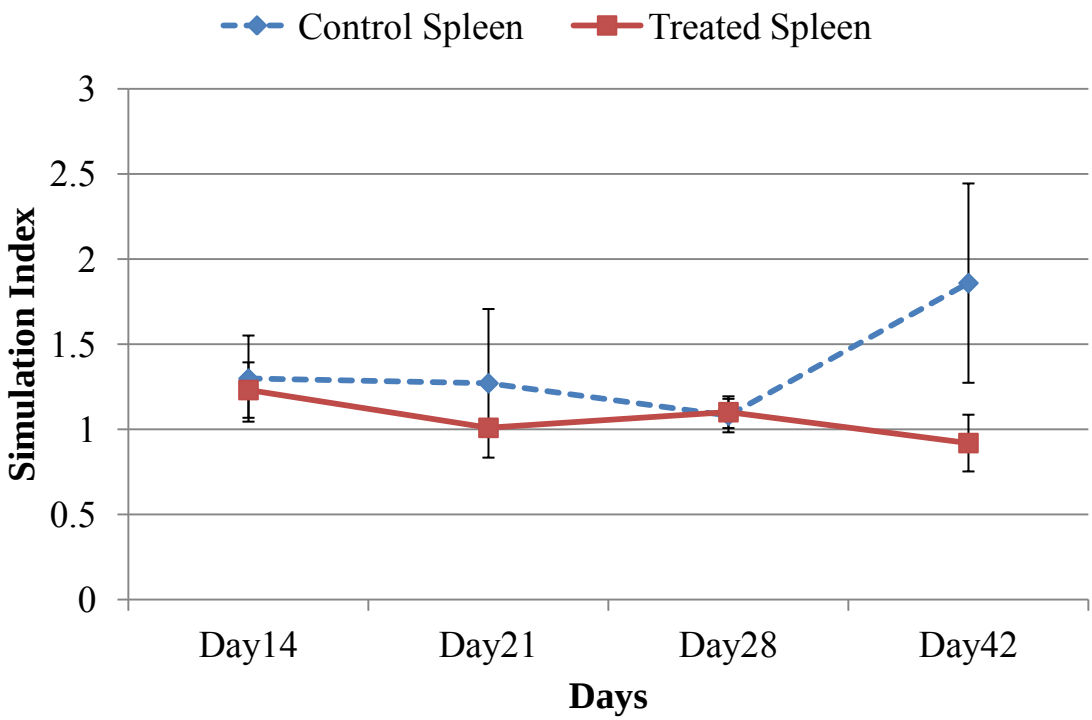

Figure 3.7. Effect of supplementation of yeast product on T cell abilities in spleen

Histology

In the BF, there were no differences between the control and treated group from typical view observation (Figure 3.8.1 and 2).

The thymus, medulla and cortex area ratio was measured. The medulla and cortex are very important structures in the thymus because T lymphocyte maturation occurs through those two areas. However, significant differences were not observed ($P > 0.05$) in thymus medulla and cortex ratio between the control and treated group (Figure 3.9).

For the spleen, the number of white pulp in the spleen was counted. The splenic nodule, which is locates inside of white pulp, is very important site for B lymphocyte maturation. The splenic nodules only appear when antigens enter into the spleen. Significant differences in the number of nodules between the control and treated group were observed at D 14 only ($P < 0.05$) (Figure 3.10); the treated group contained more nodules in the spleen. Through this result, the yeast product had an effect on the early stages of the spleen development.

The yeast product affected spleen immunological maturation but not the BF and thymus. This was a somewhat predictable result because the mechanism of nucleotides on immune function is not fully understood. Adjei et al. (1999) discussed the possibility that nucleotide supplementation might be able to induce cell proliferation in spleen of mice. In a similar fashion, yeast product affected the spleen in this experiment.

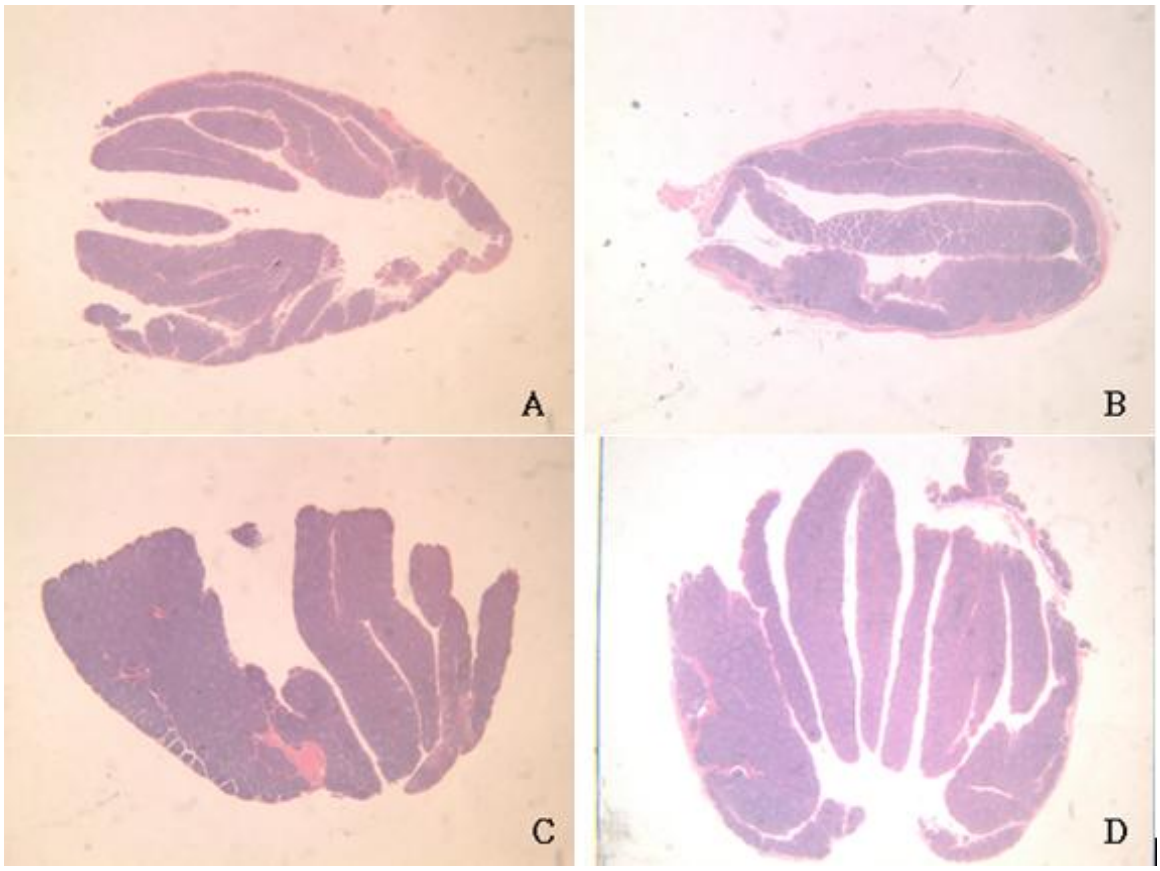

Figure 3.8. A) H&A stained bursa of Fabricius of day 14 treated group (4X), B) H&A stained bursa of Fabricius of day 14 control group (4X), C) H&A stained bursa of Fabricius of day 21 treated group (4X), D) H&A stained bursa of Fabricius of day 21 control group (4X).

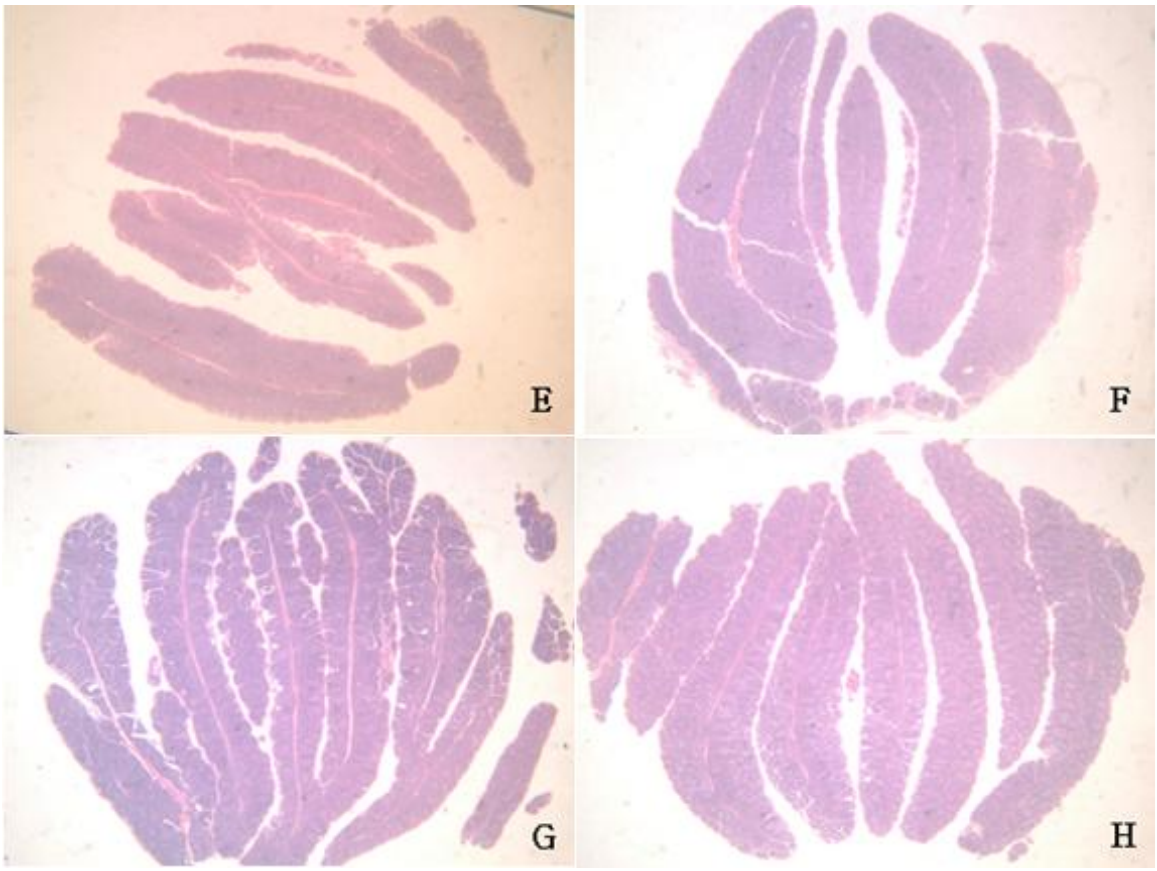

Figure 3.9. A) H&A stained bursa of Fabricius of day 28 treated group (4X), B) H&A stained bursa of Fabricius of day 28 control group (4X), C) H&A stained bursa of Fabricius of day 42 treated group (4X), D) H&A stained bursa of Fabricius of day 42 control group (4X).

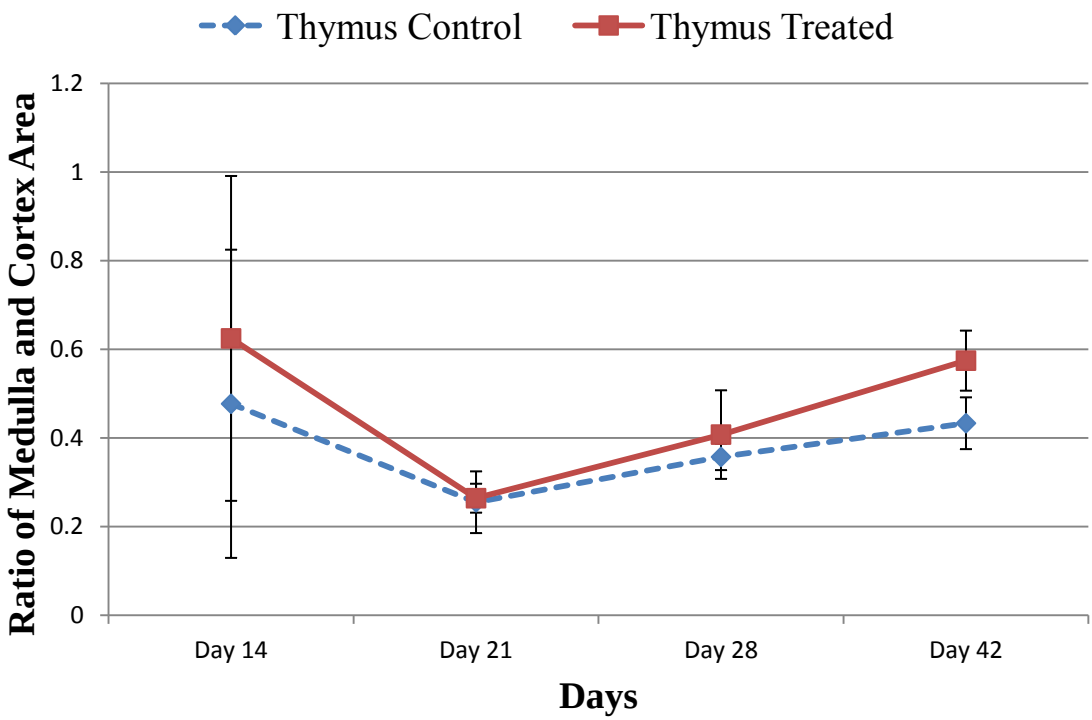

Figure 3.10. Effect of supplementation of yeast product on ratio of medulla and cortex area of thymus

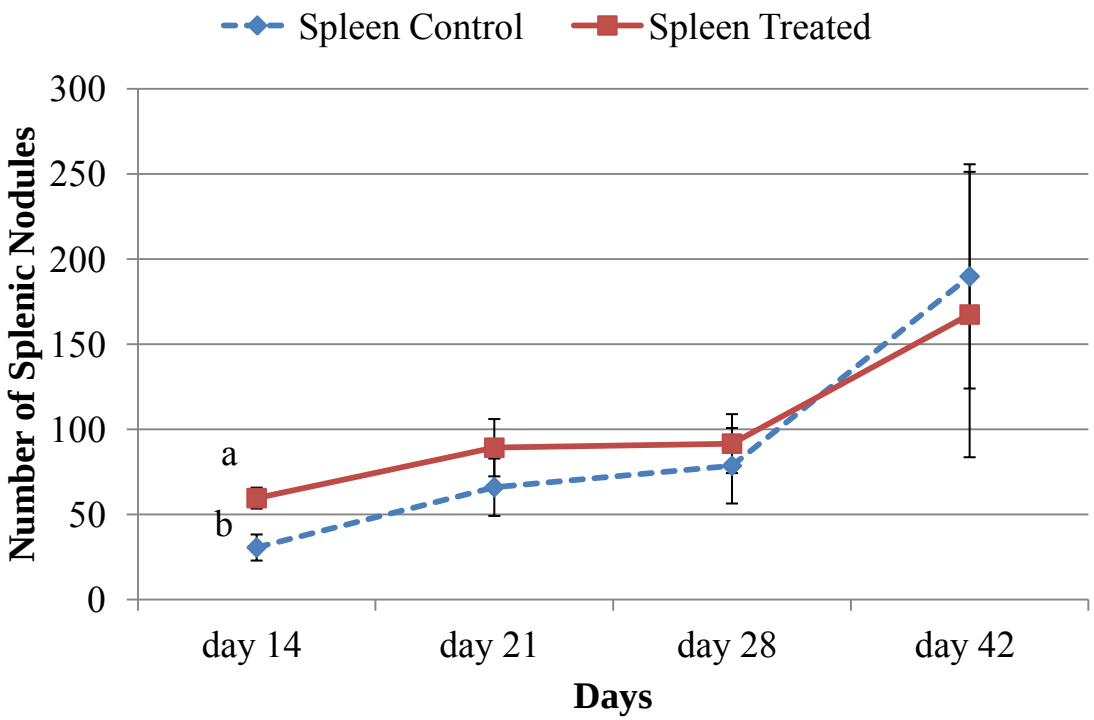

Figure 3.11. Effect of supplementation of yeast product on number of splenic nodules in spleen

CHAPTER IV

SUMMARY

According to Barrow (1992), new born chicks are normally more susceptible to get intestinal infection through the oral route. That statement makes sense through this experiment because most of this experiment result showed that improvement of immune system in early stage of chicken (around D 14). However, the pathogen colonization inhibition mechanism by yeast products is still not fully understood. When chicks intake the yeast-derived products, the yeast may encourage or stimulate host intestinal flora. For example, chicken alimentary tract can resist Salmonella infection through young to adult phase by probiotics (Barrow, 1992). The probiotics inhibit or interrupt pathogens directly or indirectly; in this case, the yeast culture product, *S. cerevisiae* encourages host beneficial microorganisms to kill or prevent pathogens (Otutumi et al., 2012). However, using the probiotic as feed additive may reduce host growth rate. According to Erf (1997), BW and immune function are negatively correlated. Therefore, the yeast-derived products may reduce on host growth performances. However, there was no difference on the growth performance between the control and treated group through this experiment. This experiment's data showed that three important immune organs indices of the treated group were smaller (D 28, 42 of thymus, D 21, 28 of BF, and D 21 of spleen) than the control group, there were many results (IgG population, T cell abilities in blood and thymus, and B cell maturation rate in spleen) that improved immune system in early age of chicken.

The probiotic also reduces pH of intestinal mucosa. The probiotics also stimulate a release of cytokines. Those cytokines induce the secretion of Immunoglobulin A on the intestinal mucosa, then IgA release mucins (Otutumi et al., 2012). The mucins form a physical barrier against pathogens (Otutumi et al., 2012). Therefore, further experiments need to investigate the IgA population in the esophagus or trachea. According to Barrow (1992), appropriate diet formulation can help colonization of the probiotic in the intestine. Therefore, further experiments need to investigate that feed additive is able to make ultimate immunological effect with the yeast product on chicken.

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