EVALUATION OF THE IMMUNOMODULATORY CAPACITY OF THE FUNCTIONAL METABOLITES OF A *SACCHAROMYCES CEREVISIAE* FERMENTATION PRODUCT IN BROILERS

A Thesis

by

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ABSTRACT

Our lab has identified the functional metabolites of Diamond V Original XPC™ (XPC) as a potential strategy to be used by the poultry industry to enhance immune function and poultry health. Three experiments were conducted using Ross 308 birds to determine the immunomodulatory capacity of XPC in the adaptive immune system of broilers. In the first experiment (n=240), birds were randomly assigned to either a control or XPC diet. Newcastle Disease Virus (NDV)-specific humoral immune response to a live attenuated vaccine was significantly ($P<0.05$) higher in XPC at d42. Flow cytometry showed no significant differences in cell populations ($P>0.05$) between treatments on the same sampling days. In Experiment Two (n=640), birds were randomly assigned to an unvaccinated or vaccinated group and further assigned to XPC or control diets. At d1, live NDV LaSota strain vaccine was used as a primary immunization to evaluate its impact on adaptive immunity. Remarkably, no substantial NDV-specific humoral immune response was established. Body weights were significantly higher ($P<0.05$) in the vaccinated birds on days 4 and 7. Spleen index of the vaccinated birds was significantly ($P<0.05$) lower at day 28 and 35. In Experiment Three (n=180), the role of maternal antibodies on XPC's immunomodulatory capacity was evaluated. A factorial arrangement of three vaccination protocols and two diets (XPC or Control) was used. Protocol 1 used live B1 strain as primary immunization whereas Protocol 2 used live LaSota strain. Protocol 3 used live LaSota strain after maternal antibodies had decayed. In Protocol 1, XPC birds' NDV titers were significantly higher

($P<0.001$) at day 28. Protocol 3 resulted in the highest NDV-titer level during the trial; XPC birds reached a significantly ($P<0.001$) higher level than control birds on d35. Protocol 2 had the lowest NDV-titer. Feed conversion was significantly higher ($P<0.05$) in Protocol 3 compared to 1 and 2. Overall, the results indicate that XPC supplementation positively modulates the broiler immune system, allowing it to have a robust and significantly higher NDV-specific humoral immune response compared to a control diet; its immunomodulatory capacity appears to be potentiated by the level of maternal antibodies and the vaccination schedule.

DEDICATION

I dedicate this thesis work to God, and my family.

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Contributors

This work was supervised by a thesis committee consisting of Dr. John Carey serving as a chair of committee and Dr. Christine Alvarado, and Dr. Luc Berghman as members of the committee. Also, Dr. David Caldwell head of the Department of Poultry Science at Texas A&M University.

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1. INTRODUCTION

The poultry industry has evolved to become an effective, high yield, and economically feasible industry through the development of better ventilation, housing, animal care, and intense research. However, due to high levels of production and use of antibiotics in combination with consumer demand and media pressure the poultry industry has been forced to develop new strategies for pathogen control, animal health, and food safety without the use of antibiotics. Dietary supplementation with the functional metabolites from a *Saccharomyces cerevisiae* fermentation product, Diamond V (Cedar Rapids, Iowa) Original XPC^{TM} (XPC), have been identified by our lab as a potential strategy to be used by the poultry industry, particularly in antibiotic free programs (ABF). The need to find effective, economically feasible and practical strategies in order to maintain high levels of production in the poultry industry while continuing appropriate animal care has become a top priority in the industry around the world and therefore it is imperative to continue thorough research in that field.

Yeast products have been studied for a long time for their properties as natural growth promoters and potential immune modulators. Initially, the use of *Saccharomyces cerevisiae* as a growth promoter in ruminants was reported by Eckles and Williams, 1925. Gao et al., 2008 described a linear increase in antibody titers to Newcastle Disease Virus (NDV) when the level of yeast extract fed to birds increased and suggested its potential modulation of systemic immunity. Efficient poultry production cannot be accomplished without birds achieving their full genetic potential and one crucial piece in

order to accomplish a high level of success is good animal health. The immune system in broilers plays a major role in every aspect of a broiler's life. However, much more research is necessary in order to achieve a better understanding of the processes and effector mechanisms of the avian immune system. This important system allows a modern broiler to successfully defend itself from pathogens and maintain a high level of growth and excellent feed conversion which allows for economic gains and sustainable practices. The strategies that aim to achieve a strong immune system and as a result provide strong animal health allow for the preservation of high production levels without the use of antibiotics. These strategies rely on their ability to modulate the immune system or enhance immune response to a particular antigen.

The objectives of this study are: 1) to further study the immunomodulatory capacity of the functional metabolites of *Saccharomyces cerevisiae* fermentation product XPC on the adaptive immune system of broilers. 2) Analyze the overall Newcastle Disease Virus-specific antibody serum IgY titers of broilers in response to vaccination throughout the duration of a typical production cycle. 3) Measure immune-associated parameters in early stages of immune system development (day 4 and 7 post-hatch) as well as early post-boost (day 24 instead of day 28). 4) Evaluate the impact of a 1x dose of live LaSota strain NDV vaccine administered oculo-nasally on days 0 and 21 on early development of humoral and cell-mediated immune response in broilers. 5) Assess the influence of maternal derived antibodies on the efficacy of XPC in birds vaccinated with live NDV vaccine.

The information obtained at the end of this study will help provide a deeper understanding of the immunomodulatory capacity of XPC in broilers. The results will help determine the extent of the immunomodulatory capacity of XPC. This study will help improve the understanding of the multiple factors that could influence its function and interaction with the broiler's adaptive immune system. In addition, the results will aid the research and agricultural community to evaluate the potential use of XPC as an effective strategy to help maintain animal health and enhance immune function.

2. LITERATURE REVIEW

2.1 Newcastle Disease

The development of the different effector mechanisms of the chicken adaptive immune system have been further studied in recent decades. In order to maintain a high level of production while upholding animal health at all levels of the production cycle one has to understand the way the early development of the chicken adaptive immune system will be able to effectively combat pathogens in a commercial production setting. Modern poultry production results in birds being exposed to multiple immunological challenges starting at the hatchery.

A disease of poultry that was first described in 1926 is Newcastle Disease (ND) and ever since it has been a major problem in the poultry industry primarily because of its worldwide distribution and economic impact. As a result, multiple strategies exist to combat this disease which can be effectively managed with good biosecurity programs and effective vaccination programs. The causative agent of ND is Newcastle Disease Virus (NDV) and all strains of NDV belong to the family Paramyxoviridae, genus Avulavirus, and they are part of one serotype avian paramyxovirus serotype-1 (APMV-1); this virus is an enveloped negative single-stranded RNA virus (Alexander and Senne, 2008).

NDV was isolated for the very first time in 1926 from chickens in Newcastle, England and it was through the use of serological and structural tests, and genetic properties that nine distinct groups of avian paramyxoviruses were identified (Poultry Diseases, 2008). In modern poultry, producers develop vaccination programs that aim,

among many other objectives, to protect against NDV and reduce potential economic losses as result of loss of live birds, loss of performance or loss due to condemnations at the processing plant. Antibodies will prevent NDV growth in the visceral organs but the virus will still be able to replicate at the mucosal surfaces and thus resulting in the virus still being excreted by infected birds (McFerran and McCracken, 1988). Currently, three main types of commercially available vaccines for Newcastle disease: live lentogenic, live mesogenic and inactivated are available to the poultry industry.

The lentogenic strains are of low pathogenicity but are capable of inducing a successful immune response. In contrast, mesogenic strains are derived from fully virulent strains and inactivated vaccines are developed by the use of killed virus. It is important to mention that different strains of NDV can cause different signs depending on the age of the host, susceptibility, and environmental stress, which can result in different rates of mortality and different levels of economic impacts. Mortality can be as high as 100% in velogenic NDV infections. The clinical signs of ND are not specific and can be associated with other diseases; clinical signs can range from depression and diarrhea, to a deterioration or cessation in egg production (Alexander and Senne, 2008).

The purpose of vaccination is not only to generate a humoral immune response against a specific pathogen but also to establish cell-mediated immunity. In commercial poultry production, vaccination programs of breeder hens are intended to hyper immunize hens to allow for the passive transfer of immunity to the progeny which then is able to establish passive immunity from day of hatch (Qureshi et al., 1998b). It is important to mention that such vaccination regimens may vary from region to region of

the world as they are dependent upon the type of breeder hen and, very importantly, on the endemic diseases and current immunological challenges present in that particular region.

Additionally, it is important to mention that in order to maximize immunological benefit by assuring proper function of the vaccines they have to be delivered properly and effectively. It is also imperative to understand that the immunological response may vary depending on the nature of the challenge or infection. In modern poultry, the efficacy of vaccines and the health of any flock at any given time are directly related to management practices.

2.2 Avian Adaptive Immune System

The avian immune system has been characterized for being functionally and structurally complex. It is very clear that in commercial poultry production its performance, establishment and development is undoubtedly influenced by genetic, nutritional, and environmental factors that allows it to be a sensitive and very important gauge of management practices on avian health and as a result on production performance (Qureshi et al., 1998b).

The importance of understanding the mechanisms in which the adaptive immune system works in order to combat pathogens commonly found in a commercial poultry setting forms the basis of any plan that aims to maintain animal health and economic feasibility in a commercial setting. The immune system of birds is divided into innate and adaptive immunity where the innate immune system has low specificity with no memory and in contrast the adaptive immune system has high specificity, develops

immunological memory and it often times provides life-long protection against a previously encountered pathogen (Davidson et al., 1996). Even though the adaptive immune system is quite different from the innate immune system, both components are intrinsically connected and are fundamentally important for the generation of effective immune responses. Innate immune responses are primarily mediated by cells such as macrophages and natural killer cells that can prevent the growth and replication of an antigen source or, if required, initiate a cascade of events that will trigger an adaptive immune response.

The correct processing and presentation of an antigen is essential for the optimal development and establishment of an adaptive immune response and it constitutes the beginning of the latter response. It is important to mention that the cellular components of blood including red blood cells, platelets, and white blood cells have the same precursor cells; it is because hematopoietic stem cells located in the bone marrow have the ability to generate any cellular component of blood they are often referred to as pluripotent hematopoietic stem cells (Janeway et al., 1999).

An important distinction is made when the pluripotent hematopoietic stem cells generate stem cells with a more limited potential and those stem cells become the immediate creators of red blood cells and the two types of white blood cells (Janeway et al., 1999). The stem cells which give origin to the two types of white blood cells are myeloid and lymphoid stem cells. The myeloid stem cells give origin to multiple important cells like macrophages, dendritic cells, and granulocytes. All these cells play essential roles in the immune system.

Macrophages, which are part of the mononuclear phagocytic system play an essential role in both adaptive and innate immune responses and are part of the first line of defense against microbial infections (Qureshi and Miller, 1991). Macrophages are the mature form of monocytes which are constantly circulating in the blood. When they migrate into the tissue they become macrophages capable of killing bacteria and tumor cells; they perform phagocytosis like dendritic cells which are essential cells that can phagocytose, be macropynocytic, and present antigens to lymphocytes (Janeway et al., 1999). In addition to macrophages being phagocytic as well as bacteriostatic, chicken macrophages are highly bactericidal as they are capable of killing more than 80% of internalized *Salmonella* in the first 15 min of engulfment (Qureshi et al., 1998b). Macrophages are also capable of producing numerous types of cytokines and chemokines.

Cytokines produced by cells that belong to the immune system receive the name of leukocytic cytokines and they serve as regulators of the immune responses and can have several effector functions on many cells. For instance, macrophages following activation secrete Interleukin-1, which is a proinflammatory cytokine. Its effector mechanism may result in activation, deactivation, cell death, anabolism, catabolism, chemotaxis, migration inhibition, or differentiation (Qureshi, 1998a).

Interferon gamma is a cytokine produced predominantly by natural killer cells upon activation of an innate immune response and by CD4 and CD8 T-cells as part of the activation of the adaptive immune system. Interferon gamma is an extremely important cytokine with immunostimulatory and immunomodulatory effects on the

entire immune system that among many different effector mechanisms, it can inhibit viral and intracellular bacterial infections, control tumor development by enhancing their immunogenicity and very importantly, it can activate macrophages, an important cell in both innate and adaptive immunity (Schoenberg and Wilson, 2007).

The adaptive immune system relies on many different cells and effector mechanisms that provide the host organism with a complex defense system against innumerable pathogens. It can do all this by utilizing many effector mechanisms and cells like the B or T lymphocytes. B cells when stimulated differentiate into plasma cells that secrete antibodies that are highly specific towards an antigen and are able to combat pathogens (Davidson et al., 1996). T-cells are able to react and destroy host cells that have been invaded by pathogens (Davidson et al., 1996).

The lymphoid stem cell originated from pluripotent hematopoietic stem cells gives rise to both B-lymphocytes and T lymphocytes (Janeway et al., 1999). These two major types of lymphocytes mature in the primary lymphoid organs thymus and bursa of Fabricius. The T-lymphocytes mature in the thymus and B lymphocytes in the bursa of Fabricius. The adaptive immune system can be divided into humoral immune responses, which relies on antibodies to combat extracellular pathogens, or cell-mediated immune responses, which rely on T-cells and cytokines in order to respond to intracellular pathogens (Kapczynski, 2013).

The humoral immune arm of the adaptive immune system relies on the ability of antibodies to interact with extracellular antigens which results in the activation of

effector mechanisms, for example phagocytosis or activation of the classical pathway of the complement system which results in the elimination of antigen (Erf, 2004).

Moreover, the ability of antibodies to activate natural killer cells (NK) by forming a bridge between infected cells expressing antigens and killer cells through the bonding of the Fc region on the antibodies with Fc receptors on killer cells allows the organism to ensure the elimination of infected cells in a cellular mechanism known as antibody-dependent cellular-cytotoxicity (Abbas et al., 2000). Most immune responses that fall into the adaptive immune system are triggered when T cells in circulation are able to identify its specific antigen on the surface of a dendritic cell found on peripheral lymphoid tissues which include spleen, lymph nodes, and mucosal associated lymphoid tissues which are constantly collecting antigens from the epithelial surfaces of the tissues. The constant collection of antigen and its further presentation to specialized cells that many organisms exposed to a direct stimulus in any lymphoid organ experience can result in alterations in immune system functions. This direct tropism often times results in organ necrosis or loss of function; it can result in specific lymphocyte subpopulations to be affected (Qureshi et al., 1998b).

2.3 Immunoassays

A substantial development on the amount of immunoassays that one can utilize in order to study the responses generated by the immune system of any organism has been advanced throughout the years. Among the many different types of immunoassays that are used to measure cell-mediated immune responses, the most valuable is possibly

flow cytometry because of its ability to identify and quantify in a rapid manner functional subpopulations of cells in a heterogeneous cell culture.

The use of flow cytometry provides valuable information like quantification of cytokines from a specific cell type in a heterogeneous cell population (Jung, 1993). Flow cytometry is a valuable technique that allows for rapid measurements of cells as they pass in a fluid stream one by one through a sensing laser. One could argue that the most notable advantage about flow cytometry is that it provides measurements from each cell within a heterogeneous suspension and not just an average value of the whole population. Because of its ability to measure multiple cell parameters using fluorescence and light scatter, flow cytometry's applications in research and medicine are innumerable. The ability of flow cytometry to study individual types of cells via cell sorting allows quantifying, studying, and identification of specific functional subpopulations of cells quickly and reliably; making it ideal for the study of cell-mediated immune responses for example.

Other common tools used to measure immune responses include enzyme-linked immunoassay (ELISA), detection of cytokines like interferon gamma, which plays an important role in the activation of cell-mediated immunity, and use of sandwich ELISA (Bercovici, 2000).

2.4 *Saccharomyces cerevisiae*

The benefits of non-pathogenic yeast *Saccharomyces cerevisiae* as a growth promoter in the animal industry are very well documented. Yeast products are regarded as a viable alternative for the use of antibiotics in the poultry industry due to their

modulating ability in the immune system as they can activate the immune system and provide competitive binding sites for pathogenic bacteria (Gao et al., 2008).

In the poultry industry, *Saccharomyces cerevisiae* has received a lot of interest throughout the years and it has been the subject of many research programs and has been used among many other areas as a way to improve immune response, protect against aflatoxicosis, enhance intestinal health, and as a replacement strategy for the use of antibiotics in the poultry industry.

Stanley et al., (1993) reported the benefits of *Saccharomyces cerevisiae*, which included restoring to control levels the activities of the enzymes alamine transaminase and creatine phosphokinase, as well as having a positive effect on mean body weights of chicks fed aflatoxin-contaminated feed. It has been reported that yeast could be a potential strategy in the replacement of antibiotic-based drugs in feed of broiler chicks in chicken houses with new litter (Hooge et al., 2003) or in houses with recycled litter (Stanley et al., 2004).

The inclusion of yeast culture residue derived from *Saccharomyces cerevisiae* has been found to be useful in controlling intestinal coliforms and, to some extent, coccidial oocysts as well as in effecting growth rate in broiler chickens on recycled litter using a rapid turnover schedule (Stanley et al., 2004).

The effector mechanisms and effects of *Saccharomyces cerevisiae* on other animals have been studied and are widely reported in the literature. Van der Peet-Schwering et al., (2007) reported that weanling piglets fed Diamond V XPCLS (Diamond V Mills, Cedar Rapids, IA) improved their feed efficiency and growth rate in

comparison to control groups and these effects were comparable to those from Avilamycin-fed piglets. The supplementation of *Saccharomyces cerevisiae* in laying hens has also been reported in the literature, and it has been shown to improve phosphorus utilization, feed efficiency, and egg quality (Tangendjaja and Yoon, 2002).

Multiple mechanisms in which yeast can have an effect on the immune system have been intensely studied and it has recently been suggested by Alizadeh, (2016) that yeast cell wall products can stimulate the expression of pattern recognition receptors and are able to maintain an immune homeostasis through the regulation of inflammatory activities.

Morales-Lopez and Brufau, (2013) reported a positive effect of yeast cell wall on feed efficiency in chickens predominantly under conditions of an immunological challenge. The report also suggested that inclusion of yeast cell wall in the diets of broilers may avoid the reduction of the bursa of Fabricius caused by a challenge with *Escherichia coli* lipopolysaccharide by helping them increase tolerance to microbial challenge.

The number of *Saccharomyces cerevisiae* derived products is considerable and the development of more products as well as more research has evolved recently. However, the need to keep studying yeast products is still highly relevant as its mode of action has not been completely identified, nor the extent of its immunomodulatory capacities.

It is important to mention that not all *Saccharomyces cerevisiae* derived products are the same. Thus, their positive effects can vary significantly. Some products are

primarily cell walls and others like XPC, are a mixture of a fermentation product and yeast components. More information about XPC can be found in the Appendix section.

3. THE EFFECT OF XPC™ SUPPLEMENT IN DIET ON EARLY DEVELOPMENT OF ADAPTIVE IMMUNE RESPONSE IN BROILERS

3.1 Introduction

The poultry industry is currently searching for new strategies to maintain production levels while eliminating the use of antibiotics and maintaining bird health. Our lab as a potential strategy to enhance immune function and poultry health has identified the functional metabolites of a Saccharomyces cerevisiae fermentation product, Diamond V Original XPC™ (XPC). The overall goal of this study was to evaluate the effect of dietary XPC supplementation on the early development of the adaptive immune response in broilers in response to a live attenuated NDV vaccine.

A total of 240 one-day old Ross 308 broilers were randomly assigned to one of two diets: 1) XPC, 2) control group. Both treatments consisted of 4 pens per diet and 30 birds per pen. Antigen-specific humoral immune response was assessed by NDV-specific IgY using ELISA on days 4, 7, 14, 21, 24, 28, 35, and 42. Feed conversion ratio, feed consumption, and body weights were also assessed. Cumulative feed conversation ratio, body weights, and feed consumption were calculated.

In a previous experiment conducted in our lab using B1 strain NDV vaccine as primary immunization, Park et al., (2014), we observed a strong antigen-specific humoral immune response following secondary immunization with live LaSota strain NDV vaccine in both control and XPC birds. However, XPC birds reached a peak NDV

specific humoral immune response by day 28, 7 days earlier than control birds. All antibody titers were in response to a live attenuated NDV vaccine and not infection.

In the current experiment, we observed an establishment of NDV specific humoral immune response by day 28 on both dietary treatments in contrast to a previous experiment, Park et al., (2014). However, we observed a humoral immune response with slower decaying of NDV-specific antibodies in XPC supplemented birds. This leads us to believe that the difference in humoral response between this experiment and our previous data could be a result of interference by maternal antibodies.

3.2 Materials and Methods

With the intention to have a better understanding of previous results obtained in our lab involving the immunomodulatory capacity on the adaptive immune system of broilers fed XPC, several immune parameters were measured. The present experiment studied the overall NDV-specific antibody serum IgY titers of broilers throughout the duration of the trial and measured immune-associated parameters in early stages of immune system development (day 4 and 7 post-hatch) as well as earlier post-boost (day 24 instead of day 28). Differences in the development of cell-mediated immunity were also measured. Birds were housed at the Texas A&M University Poultry Research, Teaching and Extension Center (TAMUPRC) located in College Station, TX.

A total of 240 one-day old broiler chicks Ross 308 type were randomly assigned to either a control diet (120 broiler chicks) or XPC supplemented diet (120 broiler chicks). XPC was included into treatment diet at 1.25 kg/metric ton for starter, grower

and finisher diets. Feed was stored in plastic lined 30 gallon barrels labeled with date, diet, treatment code and experiment identification number.

Starter (0-14d), grower (15-28d), and finisher (29-42d) diets were manufactured at TAMUPRC feed mill. Feed and water were provided ad libitum during the entire trial. A 2x batch of the control diet was mixed and split into two equal parts prior to pelleting. The control feed was pelleted and placed in barrels. The treated feed was returned to the mixer and an appropriate amount of XPC was added and allowed to mix for 10 minutes prior to pelleting and storage in barrels. Composite feed samples from each feed mixing were collected and stored at -20^{o}C until submitted for proximate analysis. The experimental design consisted of 8 pens, 4 pens per diet. All diets had a corn/soybean meal base (Table 1).

On day one, chicks in the vaccinated group received oculo-nasally live B1 strain NDV vaccine (USDA Product code: 1711.10; Merial Select Laboratories, Gainesville, Georgia) followed by live LaSota strain NDV vaccine (USDA Product code: 1721.11; Merial Select Laboratories, Gainesville, Georgia) at day 21 administered via the ocular-nasal route. The level of NDV-specific IgY was measured by ELISA on days 1, 4, 7, 14, 21, 24, 28, 35, and 42 using a Newcastle Disease Antibody Test Kit (Synbiotics Corp., San Diego, CA) to monitor the humoral immune response. The average of positive and normal control serum absorbance was calculated. Each blood sample collected was examined; the antibody titers were calculated using the formula: S/P (Sample to positive ratio) = (Absorbance of sample – Average of *N* control absorbance)/ (Average of positive control absorbance – Average of *N* control absorbance). The final titer was calculated based

on the following formula provided in manufacturer's instructions: Final Titer = ANTILOG (1.464 x (Log_{10}S/P) + 3.740).

Blood samples (0.5ml) were obtained by venipuncture. Cumulative feed conversion ratio, feed consumption as well as body weights were recorded from all treatments.

Because a strong adaptive immune system involves both effective humoral and cell-mediated immune responses, the effect of XPC on vaccine-induced cell-mediated immunity was evaluated by obtaining peripheral blood leukocytes. 0.5ml of blood samples were collected from the wing by venipuncture from each group and transferred to heparinized tubes on day 7 (n=5 birds from each dietary treatment) and 24 (n=5 birds from each dietary treatment) post-immunization.

The subpopulation of CD4+ (T-helper cell) and CD8+ (T-cytotoxic cells) T lymphocytes in peripheral blood of control and XPC groups served as the immune-competence indicator of the adaptive immune response. The non-coagulated blood samples were diluted with PBS in a 1:1 ratio and 6 ml of diluted blood was carefully layered onto 6 ml of Histopaque 1077 medium (Sigma, St. Louis, MO.) per the manufacturer's instructions.

Lymphocyte subpopulations were measured using flow cytometry on day 7 and 24. Peripheral blood leukocytes were used for peripheral lymphocyte subset analysis. Cells were blocked with mouse whole IgG (Jackson ImmunoResearch, West Grove, PA) to block Fc receptors. Four-color immunostaining was performed. Blocked cell suspensions were incubated with FITC-conjugated-anti-CD3, R-PE-conjugated anti-

CD4, Cy5-conjugated anti-CD8, and biotinylated anti-Bu-1. The detection of biotinylated-anti-Bu-1 was performed by incubation with quantum-red labeled streptavidin. After washing and fixation, the cell suspensions were analyzed at the Texas A&M College of Medicine-Cell Analysis Facility and data were analyzed using Flowjo software (Flowjo LLC, Ashland, OR.).

An important interaction that occurs as part of the adaptive immune response is the interaction between follicular T-helper cells and B cells in the spleen. This interaction is of importance as it has a major effect on the development of antigen-specific B-cell responses during vaccination. From each group, 5 birds were euthanized by CO_2 asphyxiation for the collection of splenocytes. Single-cell splenocyte suspensions were obtained by pushing the spleen through a 70-μm cell strainer (BD Falcon, Franklin Lakes, NJ). Splenocytes were used for splenic lymphocyte subset analysis. Cells were blocked with mouse whole IgG (Jackson ImmunoResearch, West Grove, PA) to block Fc receptors. Four-color immunostaining was performed. Blocked cell suspensions were incubated with FITC-conjugated-anti-CD3, R-PE-conjugated anti-CD4, and Cy5-conjugated anti-CD8, and biotinylated anti-Bu-1. The detection of biotinylated-anti-Bu-1 was performed by incubation with quantum-red labeled streptavidin. After washing and fixation, the cell suspensions were analyzed at Texas A&M College of Medicine-Cell Analysis Facility and data was analyzed using Flowjo software.

No drug therapy was used during the study. When any study personnel entered a pen (i.e., to collect birds for study procedures), plastic boots and gloves were utilized.

The plastic boots and gloves were removed by the person who was inside the pen as they stepped out to avoid cross-contamination throughout the facility. Plastic boots and plastic gloves were discarded in an appropriate container after use.

All pens were monitored three times daily for general flock condition, temperature (recorded on log sheets), lighting, water, feed, litter condition, and unanticipated house conditions/events. Findings were documented on log sheets. Mortality was monitored in each pen daily.

Statistical analysis was completed with the SAS (SAS Institute, Cary, NC) statistical software package. All data were analyzed via one-way ANOVA using the GLM procedure. Differences were deemed significant at $P<0.05$ and means were separated by the PDIFF procedure. All animal handling and care practices were in accordance with an approved animal use protocol, IACUC 2014-0017 reference number 047580.

Table 1 Experiment 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	34.48	29.33	26.64
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
Calculated 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	0.74	1.14
Valine, %	1.00	0.91	0.82
Calcium, %	0.92	0.90	0.89
Available Phosphorous,	0.45	0.45	0.38
Sodium	0.22	0.21	0.20
Chloride, %	0.39	0.34	0.35

[1]Vitamin premix added at this rate yields 11,023 IU vitamin A, 3,858 IU vitamin D3, 46 IU vitamin E, 0.0165 mg B12, 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. [2]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.
[3]XPC is Diamond V Original XPC (Cedar Rapids, Iowa); corn will replace XPC in control diet.

3.3 Results

Assessment of live production performance was measured using mean body weights and feed conversion ratio at day 42 as comparison parameters. Results of the effects of a diet containing XPC on body weights are summarized in Table 2. There were no significant differences in body weights or feed conversion (P>0.05) between the dietary treatments during the experimental period (1 to 42 d). Feed consumption and feed conversion results are summarized on table 3. No significant differences (P>0.05) in feed consumption were observed.

Table 2 Experiment 1 - Effects of diet containing XPC on body weight (BW)

Day of age*	Diets[1]		SEM
	Control BW (g)	XPC BW (g)	
4	104.30	106.15	2.11
7	179.65	178.65	4.15
14	491.50	422.65	32.48
21	944.00	907.15	25.59
24	1132.10	1137.75	33.17
28	1414.95	1393.60	11.84
35	1963.85	1942.45	63.2
42	2724.60	2621.00	36.35

No significant differences were observed (P>0.05)
* n= 15 birds per day, Ross 308.
[1] Diets with no XPC served as Control and XPC diets had inclusion of 1.25 kg of Original XPC /metric ton

Table 3 Experiment 1 - Effects of a diet containing XPC on cumulative feed consumption and feed conversion ratio (FC) at day 42

Day of age*	Diets[1]		SEM
	Control Feed Consumption (g)	XPC Feed Consumption (g)	
14[2]	652.81	631.17	14.20
28[3]	2488.18	2421.08	27.87
42[4]	4938.83	4689.30	36.35
FC at day 42	1.64	1.58	0.06

No significant differences were observed (P>0.05)
* n= 15 birds per day, Ross 308.
[1]Diets with no XPC served as Control and XPC diets had inclusion of 1.25 kg of Original XPC /metric ton
[2]End of starter diet; [3]End of grower diet; [4]End of finisher diet

The results of the effect of XPC on serum levels of NDV-specific IgY are presented in table 4. There was no indication of a faster establishment of NDV-specific humoral immune response on XPC supplemented birds compared to the control group. The dietary control treatment achieved its peak NDV-specific humoral immune response on day 28, 7 days after the secondary immunization, as well as XPC supplemented birds which was only seen on treated birds in previous experiments by our lab. The differences observed on days 28 and 35 were not statistically significant (P>0.05) and a rapid decline of NDV titers was observed in the control group. However, on day 42 XPC supplemented birds had a significantly (P<0.05) higher NDV titer and a much slower decline of NDV-specific antibodies was observed following secondary immunization.

Table 4 Experiment 1 – Effect of XPC on mean anti-NDV titers (IgY)

	Diets[1]		
Day of age*	Control	XPC	SEM
4	4405.68	3424.53	581.65
7	2798.41	3609.55	603.22
14	857.37	865.01	254.97
21	1251.64	682.99	261.74
24	1100.88	1051.36	351.26
28	2524.50	2198.61	406.29
35	1596.19	2197.35	397.92
42	**1032.40[b]**	**2026.09[a]**	381.55

Shown in table are the calculated antibody titers (IgY) from the optical density measured by ELISA reader for the samples that were run through ELISA test specific for anti-Newcastle disease (ND) virus IgY production.
On day of hatch the level of NDV-specific maternal antibodies was calculated and determined to be 6886.58
[a, b] Means within a row lacking a common superscript differ significantly ($P<0.05$). Each value represents the mean value for each group.
* n= 15 birds per day, Ross 308.
[1]Diets with no XPC served as Control and XPC diets had inclusion of 1.25 kg of Original XPC /metric ton

Cell-mediated immunity was also measured during this trial. Results of peripheral lymphocyte and splenic lymphocyte subset analysis are summarized in table 5. On days 7 and 24, cell-mediated immunity was measured via flow cytometry. There were no significant differences ($P>0.05$) observed between the dietary treatments on the same sampling day however, the percentage of CD3+, CD4+, and B-cells in the spleen were significantly ($P<0.05$) higher for both treatments on day 7 compared to day 24. Flow cytometry was conducted on peripheral blood leukocytes and it showed no significant differences ($P>0.05$) between the dietary treatments on the same sampling day. The number of CD3+ cells was significantly higher in both dietary treatments at day 7

compared to day 24. B-cell population in circulation at day 24 was significantly (P<0.05) higher in the control group compared to both treatments at day 7.

Table 5 Experiment 1 – Flow cytometry: Peripheral lymphocyte and splenic lymphocyte subset frequencies

	Day of age				
	7		24		
	Diets[1]				
	Control *	XPC *	Control *	XPC *	SEM
Spleen					
(%) CD3+	**26.24** [a]	**35.52**[a]	**9.69**[b]	**10.92**[b]	3.15
(%) CD4+	18.64	22.38	25.24	26.04	3.07
(%) CD8+	**23.62**[a]	**23.46**[a]	**13.96**[b]	**10.53**[b]	1.90
(%) Bu-1+	**29.86**[ab]	**30.30**[a]	**19.18**[ab]	**17.29**[b]	3.12
Circulation					
(%) CD3+	**37.63**[a]	**34.31**[a]	**2.08**[b]	**2.71**[b]	4.29
(%) CD4+	7.60	6.52	8.60	5.63	1.98
(%) CD8+	4.29	2.58	11.11	4.54	3.24
(%) Bu-1+	**1.54**[a]	**1.06**[a]	**14.81**[b]	**8.85**[ab]	4.39

[a, b] Means within a row lacking a common superscript differ significantly (P<0.05). Each value represents the mean value for each group.
* n= 5 birds per day, Ross 308.
[1]Diets with no XPC served as Control and XPC diets had inclusion of 1.25 kg of Original XPC /metric ton

3.4 Discussion

The overall goal of this study was to evaluate the effect of dietary XPC supplementation on the early development of the adaptive immune response in broilers. Live production performance results showed no significant differences (P>0.05) between the dietary treatments in body weights, feed consumption or feed conversion ratio during the experimental period (1 to 42d). The feed conversion ratio showed a tendency to improve in the XPC group and on day 42 a lower feed conversion ratio was observed.

These results are in agreement with Alizadeh et al., (2016) where yeast-derived products were utilized as a dietary treatment, and it was reported that diets containing yeast-derived product tended to improve feed conversion ratio compared to the control group.

Morales-Lopes and Brufau (2013) showed that supplementation with *Saccharomyces cerevisiae* cell walls did not result in any significant differences in feed intake, body weight gain but feed conversion ratio on days 21 and 28 were better compared to the control group. Gao et al., (2008) reported that dietary supplementation of yeast culture, Diamond V XP (Yeast Culture), at appropriate levels resulted in improved average daily gain and feed conversion in broilers. Some studies have had similar results to the present experiment.

It has been reported in the literature that birds receiving yeast-derived products tend to have an improvement in feed conversion ratio; Ghosh et al., (2012) suggested as well as Jung and Batal (2012) that the effector mechanism of these products is the result of an establishment of gut development and exclusion of pathogenic bacteria. This experiment did not monitor gut development or presence of pathogenic bacteria in the broilers utilized.

In this experiment, the establishment of cell-mediated immunity was measure on days 7 and 24. Cell-mediated immune response following NDV infection can be detected as early as two or three days after infection (Ghumman et al., 1976). There were no significant differences ($P>0.05$) observed between the dietary treatments on the same sampling day however, CD3+, CD4+ and B-cell populations on the spleen were significantly ($P<0.05$) larger in both treatments on day 7 compared to day 24. Flow

cytometry on peripheral blood leukocytes did not show significant differences ($P>0.05$) between the dietary treatments in the same sampling day. CD3+ was significantly higher on both dietary treatments at day 7 compared to day 24. B-cell population in circulation at day 24 was significantly ($P<0.05$) higher in the control group compared to both treatments at day 7. Overall, a higher percent of lymphocytes in circulation and spleen was observed on day 7 compared to day 24, three days after the secondary immunization. Lambrecht et al., (2004) compared NDV obtained from chicken splenocytes that received live or inactivated NDV vaccines and reported an increased cell-mediated immune response with the live NDV vaccination. However, no stimulation of cell-mediated immunity was observed in this experiment following the secondary immunization with live LaSota strain NDV vaccine.

Protection by NDV-specific antibodies has been reported throughout the literature to be fundamental against a live NDV challenge. In birds, measurements of antibody responses have been used to analyze their humoral immune status (Sklan et al., 1994). Gao et al., (2008) reported a linear increase in NDV antibody titers when the level of yeast extract fed to birds increased and suggested its potential modulation of humoral immunity. During the entirety of this experiment, a thorough monitoring of NDV-specific humoral immune response was conducted. In immature chickens, immunoglobulin Y is the functional homolog to immunoglobulin G found in humans and is considered the major antibody in blood circulation as well as an important activator of the classical complement system, which is involved in pathogen elimination (Larsson et al., 1993). *Saccharomyces cerevisiae* has been identified in multiple

literature reviews as an effective modulator of the immune system capable of reducing susceptibility of animals to infections. Ghosh et al., (2012) reported that *Saccharomyces cerevisiae* had a beneficial impact on the humoral immune response against Newcastle disease in broilers.

The ELISA results obtained from this experiment did not show any significant difference between the treatments until day 42. It was hypothesized based on a previous experiment by our lab, Park et al., (2014), that XPC supplemented birds are able to establish a faster antigen-specific humoral immune response, 7 days after receiving a secondary-immunization, compared to control birds. However, both dietary treatments achieved a fast-acquired humoral immune response. A significant difference in NDV titers between the dietary treatments was not observed until day 42 when XPC supplemented birds reached a significantly ($P<0.05$) higher NDV titer level.

The acquired humoral immune response observed in XPC supplemented birds not only resulted in a significantly ($P<0.05$) higher NDV titer level at day 42. It also remained at a high titer level from day 28 until day 42, which could indicate that the rate of decay of the acquired protective antibodies against NDV was slower than the one observed in the control treatment. These results could be the indication that the immune function of broilers was modified by XPC during this trial. It has been reported by Gao et al., (2008) that immune function can be modified with dietary yeast culture supplementation.

The ELISA results observed in this experiment suggest that the level of maternal antibodies could have influenced the extent or origin of the potential immunomodulatory

capacity of XPC. There are no current reports in the literature with similar results on acquired humoral NDV response or the influence of maternal antibodies on the effect of XPC in broilers. Cell-mediated immunity development in both dietary treatments was minimal following secondary immunization at day 21. None of the two dietary groups established a high cell-mediated immune response on day 24 compared to day 7. Despite the lack of cell-mediated immune response in both dietary treatments a robust humoral immune response was observe in both dietary treatments following the secondary immunization.

3.5 Conclusion

The results observed in this trial are different from those observed in a previous experiment conducted in our lab by Park et al., (2014). The results show XPC supplemented birds did not establish a faster NDV-specific humoral immune response than control birds like it was observed in our previous experiment; however, both groups achieved their highest NDV-titer at day 28, 7 days after the secondary immunization. XPC supplemented birds reached a significantly ($P<0.05$) higher NDV-specific humoral immune response on day 42.

The observed NDV-specific humoral immune response on birds receiving XPC as a dietary treatment indicates that XPC could have modulated the adaptive immune system and as a result it established a different response that allowed them to have NDV-specific antibodies for a more extended time. The achievement of a robust antigen-specific humoral immune response in combination with a prolonged presence of

antibodies in circulation represent an excellent defense mechanism and a desired modulation of the development of the adaptive immune system by the poultry industry.

No significant differences ($P>0.05$) in the development of cell-mediated immunity were observed between the treatments on the same sampling day. The absence of cell-mediated immune response following a secondary immunization at day 21 in addition to the development of a strong humoral immune response in both dietary treatments could indicate that the mechanism of replication and infection of live NDV LaSota strain virus may be moving faster than expected; its presence in the spleen and circulation could be developing prior to 3 days post-secondary immunization.

Despite the absence of a faster establishment of antigen-specific humoral immune response in treated birds there was a noticeable effect that resulted in a slower decay of NDV-specific antibodies which represents a more robust and protective humoral immune response compared to control birds.

In this experiment, XPC supplemented birds did obtain a significant difference in live performance compared to birds fed a control diet. However, feed conversion ratio at day 42 was better in XPC treatment compared to the control treatment. Multiple reports in the literature like Gao et al., (2008); Alizadeh et al., (2016); Morales-Lopes and Brufau, (2013) have shown similar results with the use of comparable products.

A considerable difference existed in the level of maternal antibodies on birds from experiment one compared to the level observed in our previous experiment where a faster establishment of NDV-specific humoral immune response was observed in XPC supplemented birds. The difference in maternal antibodies leads us to believe that

maternal antibodies could be influencing the extent of XPC's modulation on the adaptive immune system and combined with NDV LaSota strain could also influence its development post-secondary immunization.

4. EVALUATION OF THE IMPACT OF LIVE LASOTA NEWCASTLE DISEASE VIRUS VACCINE ON EARLY DEVELOPMENT OF HUMORAL AND CELL-MEDIATED IMMUNE RESPONSE IN BROILERS

4.1 Introduction

Newcastle Disease (ND) has been a major problem in the poultry industry primarily because of its worldwide distribution and economic impact. Thus, multiple strategies exist to combat this disease, which can be effectively managed with good biosecurity programs and effective vaccination programs. The purpose of vaccination is not only to generate humoral immune response against a specific pathogen but also to establish cell-mediated immunity.

The main objective of this experiment was to evaluate the impact of a 1x dose of live LaSota strain Newcastle Disease Virus vaccine administered ocular-nasal on days 0 and 21 on early development of humoral and cell-mediated immune response in broilers and analyze the impact of this immunization schedule on the immunomodulatory capacity of Diamond V Original XPC^{TM} (XPC). We hypothesized that the use of a more virulent lentogenic strain of NDV vaccine at day 1 could aid in a faster establishment of antigen specific humoral response without compromising the development of NDV specific adaptive immunity and possibly potentiate the immunomodulatory effect of XPC on broilers.

A total of 640 Ross 308 broilers were randomly assigned to one of two treatments: 1) vaccinated, 2) unvaccinated, control group. Both groups will have 320

birds each. Each group will be further assigned to one of two treatments either supplemented with XPC or control diet. Spleen and bursa samples were excised for organ index analysis.

Cumulative feed conversation ratio and body weights were measured. The level of NDV specific IgY was measured by ELISA which was evaluated on days 4, 7, 14, 21, 24, 28, 35, and 42. Remarkably, no humoral response was established after receiving the booster (day21-42). Spleen index of the vaccinated group was significantly lower than control which is consistent with the ELISA data. The levels of interferon gamma in the circulation and in the spleen were measured on days 7 and 24. The results show considerably low amounts of detectable interferon gamma present in the circulation and secreted by splenocytes.

Altogether, the results indicate that the use of a more virulent strain of NDV vaccine before the immune system develops could compromise the establishment of NDV specific adaptive immunity and it seems to have a detrimental effect on the modulation and establishment of adaptive immunity in XPC birds.

4.2 Materials and Methods

The main objective of this trial was to evaluate the impact of a 1x dose of live LaSota strain NDV vaccine administered oculo-nasally on days 1 and 21 on early development of humoral and cell-mediated immune responses in broilers. In addition to the main objective, a secondary purpose was to analyze the impact of this immunization schedule on the immunomodulatory capacity of XPC. Based on previous data obtained in our lab, Park et al., (2014), we identified that B1 strain NDV vaccine provides a

strong antigen-specific humoral immune response, and when used as primary immunization on birds fed a control diet it will result in birds reaching peak NDV-specific humoral immune response by day 35.

In a previous experiment using B1 strain NDV vaccine as primary immunization, Park et al., (2014), we observed a strong antigen-specific humoral immune response following secondary immunization with live LaSota strain NDV vaccine in both control and XPC birds but XPC birds reaching a peak NDV specific humoral immune response by day 28, 7 days prior to control birds.

A total of 640 one-day old Ross 308 broilers were randomly assigned to one of two groups of 320 birds each: 1) vaccinated, 2) unvaccinated, control group. Each group was further assigned to one of two treatments either supplemented with XPC or control diet. The vaccinated birds received a live LaSota strain NDV vaccine (USDA Product code: 1721.11; Merial Select Laboratories, Gainesville, Georgia) at day 1 and 21. XPC was included into treatment diet at 1.25 kg/metric ton for starter, grower and finisher diets. Feed was stored in plastic lined 30 gallon barrels labeled with date, diet, treatment code and experiment identification number.

All diets had a corn/soybean meal base (Table 6). Starter (0-14d), grower (15-28d), and finisher (29-42d) diets were manufactured at TAMUPRC feed mill. Feed and water was provided ad libitum during the entire trial. A 2x batch of the control diet was mixed and split into two equal parts prior to pelleting. The control feed was pelleted and placed in barrels. The treated feed was returned to the mixer and an appropriate amount of XPC was added and allowed to mix for 10 minutes prior to pelleting and storage in

barrels. Composite feed samples from each feed mixing were collected and stored at -20^{o}C until submitted for proximate analysis. Each group with its treatment had a replicate pen for a total of 16 pens.

Spleen and bursa samples were excised for organ index analysis on days 4, 7, 14, 21, 24, 28, 35. Chickens were weighed prior to euthanasia by CO_2 asphyxiation. Spleen and bursa of Fabricius from each group were harvested and weighed to determine organ indexes, defined as organ weight/body weight ratio.

Cumulative feed conversion and feed consumption of all treatments were calculated during each dietary phase (1-14d, 15-28d, 29-42d) and body weights were obtained from all treatments as well.

As previously described, a strong adaptive immune system involves both an effective humoral and cell-mediated immune responses. The effect of XPC on vaccine-induced cell-mediated immunity was evaluated by obtaining peripheral blood leukocytes. 0.5ml of blood samples were collected from the wing by venipuncture from vaccinated groups only and transferred to heparinized tubes on day 7 (n=5 birds from each dietary treatment) and 24 (n=5 birds from each dietary treatment) post-immunization.

The subpopulation of CD4+ (T-helper cell) and CD8+ (T-cytotoxic cells) T lymphocytes in peripheral blood of control and XPC groups served as the immune-competence indicator of the cell-mediated immune response. The non-coagulated blood samples were diluted with PBS in a 1:1 ratio and 6 ml of diluted blood was carefully

layered onto 6 ml of Histopaque 1077 medium (Sigma, St. Louis) per the manufacturer's instructions.

Lymphocyte subpopulations were measured using flow cytometry on day 7 and 24. Peripheral blood leukocytes were used for peripheral lymphocyte subset analysis. Cells were treated with mouse whole IgG (Jackson ImmunoResearch, West Grove, PA) to block Fc receptors. Four-color immunostaining was performed. Blocked cell suspensions were incubated with FITC-conjugated-anti-CD3, R-PE-conjugated anti-CD4, and Cy5-conjugated anti-CD8, and biotinylated anti-Bu-1. The detection of biotinylated-anti-Bu-1 was performed by incubation with quantum-red labeled streptavidin. After washing and fixation, the cell suspensions were analyzed at the Texas A&M College of Medicine-Cell Analysis Facility and data was analyzed using Flowjo software (Flowjo LLC, Ashland, OR.).

Interferon Gamma release by T-cells after *in vitro* stimulation has been used as an efficient way to evaluate cell-mediated immunity in the chicken after vaccination. Splenocytes and peripheral blood leukocytes harvested at 7 and 24 days of age were used in this assay. Combined with flow cytometry data, this provided us with valuable information about the activation status of the T-lymphocytes. Interferon Gamma secreted by splenocytes was measured by sandwich ELISA on day 7 and 24.

The level of NDV-specific IgY was measured by ELISA on days 4, 7, 14, 21, 24, 28, 35, and 42 using a Newcastle Disease Antibody Test Kit (Synbiotics Corp., San Diego, CA). The average of positive and normal control serum absorbance was calculated. The antibody titers were calculated using the formula: S/P (Sample to

positive ratio) = (Absorbance of sample – Average of N control absorbance)/ (Average of positive control absorbance – Average of N control absorbance). The final titer was calculated based on the following formula provided in manufacturer's instructions: Final Titer = ANTILOG (1.464 x (Log10S/P) + 3.740).

An important interaction that occurs as part of the adaptive immune response is the interaction between follicular T-helper cells and B cells in the spleen. This interaction is of importance as it has a major effect on the development of antigen-specific B-cell responses during vaccination. From each group, 5 birds were euthanized by CO_2 asphyxiation for the collection of splenocytes. Single-cell splenocyte suspension was obtained by pushing the spleen through a 70-μm cell strainer (BD Falcon, Franklin Lakes, NJ). Splenocytes were used for splenic lymphocyte subset analysis. Cells were treated with mouse whole IgG (Jackson ImmunoResearch, West Grove, PA) to block Fc receptors. Four-color immunostaining was performed. Blocked cell suspensions were incubated with FITC-conjugated-anti-CD3, R-PE-conjugated anti-CD4, and Cy5-conjugated anti-CD8, and biotinylated anti-Bu-1. The detection of biotinylated-anti-Bu-1 was performed by incubation with quantum-red labeled streptavidin. After washing and fixation, the cell suspensions were analyzed at Texas A&M College of Medicine-Cell Analysis Facility and data was analyzed using Flowjo software.

Blood samples were obtained on days 4, 7, 14, 21, 24, 28, 35, and 42 by venipuncture; 0.5ml blood samples were collected. NDV-titers were obtained and calculated as described by the manufacturer. The measurement of all these parameters aimed to evaluate the impact of a more virulent strain of NDV vaccine on the

establishment of NDV specific adaptive immunity and to analyze the immune response generated by broilers supplemented with XPC.

No drug therapy was used during the study. When any study personnel entered a pen (i.e., to collect birds for study procedures), plastic boots and gloves were utilized. The person who was inside the pen as they stepped out to avoid carrying over any fecal material throughout the facility removed the plastic boots and gloves. Plastic boots and plastic gloves were discarded in an appropriate container after use.

All pens were monitored three times daily for general flock condition, temperature (recorded on log sheets), lighting, water, feed, litter condition, and unanticipated house conditions/events. Findings were documented on log sheets. Mortality was monitored in each pen daily.

The statistical analysis was completed with the SAS (SAS Institute, Cary, NC) statistical software package. All data were analyzed via one-way ANOVA using the GLM procedure. Differences were deemed significant at $P<0.05$ and means were separated by the PDIFF procedure. All animal handling and care practices were in accordance with an approved animal use protocol, IACUC 2017-0003.

Table 6 Experiment 2 - 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	34.48	29.33	26.64
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
Calculated 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	0.74	1.14
Valine, %	1.00	0.91	0.82
Calcium, %	0.92	0.90	0.89
Available Phosphorous,	0.45	0.45	0.38
Sodium	0.22	0.21	0.20
Chloride, %	0.39	0.34	0.35

[1]Vitamin premix added at this rate yields 11,023 IU vitamin A, 3,858 IU vitamin D3, 46 IU vitamin E, 0.0165 mg B12, 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. [2]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.
[3]XPC is Diamond V Original XPC (Cedar Rapids, Iowa); corn will replace XPC in control diet.

4.3 Results

Results of mean body weights are summarized in Table 7. There were no significant differences (P>0.05) among the dietary treatments during the experimental period (1 to 42 d) but feed conversion ratio was numerically better at day 42 in vaccinated birds from the control diet when compared to XPC supplemented birds. Results of feed consumption and feed conversion ratio at day 42 are summarized on table 8.

Table 7 Experiment 2 - Effects of diet containing XPC on body weight (BW)

	Treatments[1]				
	Vaccinated		Not vaccinated		
	Diets[2]		Diets[2]		
Day of age	Control * BW (g)	XPC * BW (g)	Control * BW (g)	XPC * BW (g)	SEM
1	48.06	47.50	46.13	47.75	0.44
7	143.12	151.44	119.92	126.09	7.78
14	331.01	368.23	327.61	313.63	11.66
21	636.68	749.03	690.13	641.11	25.87
28	1124.51	1269.68	1169.39	1130.72	50.34
35	1742.91	1892.19	1777.90	1699.47	82.80
42	2437.73	2600.13	2403.86	2332.70	110.13

No significant differences were observed (P>0.05)
* n= 15 birds per day, Ross 308.
[1]Vaccinated birds received live NDV LaSota strain vaccine at day 1 and 21; the not vaccinated birds receive no vaccination during the entire experimental period (1 to 42d)
[2]Diets with no XPC served as Control and XPC diets had inclusion of 1.25 kg of Original XPC /metric ton

Table 8 Experiment 2 - Effects of a diet containing XPC on cumulative feed consumption and feed conversion ratio (FC) at day 42

	Treatments[1]				
	Vaccinated		Not vaccinated		
	Diets[2]		Diets[2]		
Day of age	Control* Feed consumption (g)	XPC* Feed consumption (g)	Control* Feed consumption (g)	XPC* Feed consumption (g)	SEM
14[3]	428.29	456.58	423.97	435.97	0.16
28[4]	1690.93[c]	1863.60[a]	1749.96[bc]	1730.28[ab]	0.02
42[5]	4115.60[b]	4299.2[a]	3983.34[b]	4059.3[ab]	0.03
FC at d42	1.72	1.70	1.75	1.73	0.02

[ab] Means within a row lacking a common superscript differ significantly (P<0.05).
* n= 15 birds per day, Ross 308.
[1]Vaccinated birds received live NDV LaSota strain vaccine at day 1 and 21; the not vaccinated birds receive no vaccination during the entire experimental period (1 to 42d)
[2]Diets with no XPC served as Control and XPC diets had inclusion of 1.25 kg of Original XPC /metric ton.
[3]End of starter diet; [4]End of grower diet; [5]End of finisher diet.

Vaccinated XPC supplemented birds had a significantly (P<0.05) higher cumulative feed consumption compared to the vaccinated control birds at days 28 and 42. There were no significant differences (P>0.05) between vaccinated birds and unvaccinated birds of the same dietary treatment at days 14, 28, and 42.

The results of NDV-specific IgY levels are summarized in table 9. There was no evidence of a robust NDV-specific humoral immune response in either control or XPC supplemented birds. The highest NDV titer level observed during the experimental period was reached by birds in the control dietary treatment from the vaccinated group at

day 28, 7 days following secondary immunization, and it was significantly (P<0.05) higher than XPC supplemented birds.

Table 9 Experiment 2 – Effect of XPC on mean anti-NDV Titers (IgY)

	Treatments[1]				
	Vaccinated		Not vaccinated		
	Diets[2]		Diets[2]		
Day of age	Control * (g)	XPC * (g)	Control * (g)	XPC * (g)	SEM
4	8267.94	9140.09	10451.64	10175.64	814.67
7	6524.16	5697.84	6948.80	6210.25	665.61
14	2480.81	2077.51	2136.25	1971.13	363.72
21	434.20	442.73	311.11	313.87	122.82
24	625.12	364.90	262.40	57.43	190.18
28	**1048.25[a]**	**543.94[b]**	**2.04[c]**	**0[c]**	150.57
35	**663.90[a]**	**454.78[a]**	**0[b]**	**0[b]**	198.68
42	**439.60[a]**	**691.10[a]**	**0[b]**	**0[b]**	108.64

Calculated antibody titers (IgY) from the optical density measured by ELISA reader for the samples that were run through ELISA test specific for anti-Newcastle disease (ND) virus IgY production.
On day of hatch the level of NDV-specific maternal antibodies was calculated and determined to be 11744.02
[abc] Means within a row lacking a common superscript differ significantly (P<0.05).
Each value represents the mean value for each group.
* n= 15 birds per day, Ross 308.
[1]Vaccinated birds received live NDV LaSota strain vaccine at day 1 and 21; the not vaccinated birds receive no vaccination during the entire experimental period (1 to 42d)
[2]Diets with no XPC served as Control and XPC diets had inclusion of 1.25 kg of Original XPC /metric ton

Cell-mediated immunity was also measured during this trial. Results of peripheral lymphocyte and splenic lymphocyte subset analysis are summarized on table 10. On days, 7 and 24 cell-mediated immunity was measured via flow cytometry. There were

no significant differences (P>0.05) observed between the dietary treatments on the same sampling day however, CD3+, CD4+ and B-cell populations on the spleen were significantly (P<0.05) higher in both treatments on day 7 compared to day 24.

Flow cytometry conducted on peripheral blood leukocytes showed no significant (P>0.05) differences between the dietary treatments on the same sampling day. The number of CD3+ cells was significantly higher on both treatments at day 7 compared to day 24. B-cell population in circulation at day 24 was significantly (P<0.05) higher in the control group compared to day 7.

Table 10 Experiment 2 – Flow cytometry: Peripheral lymphocyte and splenic lymphocyte subset frequencies of vaccinated birds

	Day of age *				
	7		24		
	Diets[1]				
	Control	XPC	Control	XPC	SEM
Spleen					
(%) CD3+	5.99[a]	4.17[a]	**43.76[b]**	**39.64[b]**	3.89
(%) CD4+	1.65[a]	0.82[a]	5.55[b]	5.63[b]	0.58
(%) CD8+	2.90[a]	2.38[a]	**23.73[b]**	**18.38[b]**	2.15
(%) Bu-1+	**46.44[a]**	**43.36[a]**	15.42[b]	14.88[b]	2.08
Circulation					
(%) CD3+	3.24[a]	3.35[a]	**9.97[b]**	6.20[ab]	1.63
(%) CD4+	2.53[a]	1.37[a]	**5.48[b]**	2.50[a]	0.98
(%) CD8+	1.51	0.81	2.41	1.41	0.49
(%) Bu-1+	4.65	6.43	1.67	3.56	1.69

* n= 5 birds per day/treatment, Ross 308. Vaccinated birds only.
[ab] Means within a row lacking a common superscript differ significantly (P<0.05).
Each value represents the mean value for each group.
Vaccinated birds received live NDV LaSota strain vaccine at day 1 and 21
[1]Diets with no XPC served as Control and XPC diets had inclusion of 1.25 kg of Original XPC /metric ton

Interferon-gamma release by T-cells after *in vitro* stimulation has been shown to be a good index of cell-mediated immunity in the chicken after vaccination. In this experiment, splenocytes and peripheral blood leukocytes harvested at 7 and 24 days of age were used to conduct this assay using sandwich ELISA and the mitogen concanavalin A. The optical densities obtained following the completion of this assay are summarized on table 11.

Optical densities of interferon gamma in circulation both in XPC supplemented birds and control birds were considerably low at 7 and 24 days of age. On day 24, the level of interferon gamma of control birds was significantly ($P>0.05$) higher than both dietary treatments at day 7. Interferon gamma in the spleen of birds from the control dietary treatment was significantly ($P<0.05$) higher at day 7 compared to both dietary treatments at day 24 and XPC supplemented birds at day 7. A higher level of interferon gamma in the spleen was detected at day 7 and 24 of the experimental period in both dietary treatments compared to the levels of this cytokine detected in circulation.

Table 11 Experiment 2 –Interferon Gamma levels of vaccinated birds – ELISA optical densities

	Day of age *				
	7		24		
	Diets[1]				
	Control	XPC	Control	XPC	SEM
Circulation	0.10[a]	0.099[a]	0.138[b]	0.109[ab]	0.01
Spleen	0.876[b]	0.44[a]	0.179[a]	0.640[ab]	0.22

[ab] Means within a row lacking a common superscript differ significantly (P<0.05).
Each value represents the mean value for each group.
Interferon gamma secretion was measured using sandwich ELISA and concanavalin A as a mitogen. * n= 5 birds per day/treatment, Ross 308. Vaccinated birds only.
Vaccinated birds received live NDV LaSota strain vaccine at day 1 and 21
[1]Diets with no XPC served as Control and XPC diets had inclusion of 1.25 kg of Original XPC /metric ton

Our lab has observed in a previous experiment, Park et al., (2014), that central and peripheral immune organs produce different relative growth curves in XPC groups compared to a control dietary treatment. In this project, immune organ indexes defined as organ weight/body weight ratio were measured at 4, 7, 14, 21, 24, 28 and 35 days of age. In table 12, the effect of XPC on spleen index is summarized. On days 28 and 35 of the experimental period, unvaccinated birds had significantly (P<0.05) higher spleen index than vaccinated birds.

Table 12 Experiment 2 – Effect of XPC on spleen index

	Treatments[1]				
	Vaccinated		Not vaccinated		
	Diets[2]		Diets[2]		
Day *	Control[2]	XPC[2]	Control[2]	XPC[2]	SEM
4	0.09	0.09	0.09	0.09	0.006
7	0.11	0.11	0.12	0.12	0.007
14	0.12	0.11	0.14	0.12	0.013
21	0.15	0.16	0.16	0.14	0.01
24	0.16	0.13	0.14	0.16	0.009
28	**0.14[a]**	**0.13[a]**	**0.18[b]**	**0.18[b]**	0.014
35	**0.15[a]**	**0.13[a]**	**0.18[b]**	**0.19[b]**	0.011

Spleen index (defined as organ weight/body weight ratio) was determined.
[a, b] Means within a row lacking a common superscript differ significantly (P<0.05).
Each value represents the mean value for each group.
* n= 15 birds per day, Ross 308.
[1]Vaccinated birds received live NDV LaSota strain vaccine at day 1 and 21; not vaccinated birds receive no vaccination during the entire experimental period (1 to 42d)
[2]Diets with no XPC served as Control and XPC diets had inclusion of 1.25 kg of Original XPC /metric ton

In table 13, the effect of XPC on the bursa of Fabricius index is summarized. Significant differences (P<0.05) were observed on days 4 and 21. XPC supplemented birds had a significantly higher bursa of Fabricius index on day 4 compared to the control dietary treatment of the vaccinated group. On day 21, XPC supplemented birds of the unvaccinated treatment were significantly lower compared to both treatments of the vaccinated group.

Table 13 Experiment 2 – Effect of XPC on bursa of Fabricius index

	Treatments[1]				
	Vaccinated		Not vaccinated		
	Diets[2]		Diets[2]		
Day *	Control	XPC	Control	XPC	SEM
4	**0.14[b]**	**0.19[a]**	**0.16[ab]**	**0.18[ab]**	0.009
7	0.18	0.17	0.19	0.17	0.009
14	0.20	0.18	0.19	0.17	0.013
21	**0.23[a]**	**0.21[a]**	**0.20 [ab]**	**0.16[b]**	0.018
24	0.21	0.19	0.20	0.20	0.021
28	0.20	0.17	0.19	0.17	0.011
35	0.18	0.16	0.18	0.17	0.015

Bursa of Fabricius index (defined as organ weight/body weight ratio) was determined.
[a, b,] Means within a row lacking a common superscript differ significantly ($P<0.05$).
Each value represents the mean value for each group.
* n= 15 birds per day, Ross 308.
[1]Vaccinated birds received live NDV LaSota strain vaccine at day 1 and 21; not vaccinated birds receive no vaccination during the entire experimental period (1 to 42d)
[2]Diets with no XPC served as Control and XPC diets had inclusion of 1.25 kg of Original XPC /metric ton

4.4 Discussion

In order to expand the evaluation and further understanding of the immunomodulatory capacity of XPC on the adaptive immune system of broilers, a different NDV primary immunization schedule was utilized in this trial. Thus, the main objective of this trail was to evaluate the impact of a 1x dose of live LaSota strain NDV vaccine administered oculo-nasally on days 0 and 21 on early development of humoral and cell-mediated immune response in broilers and analyze the impact of this immunization schedule on the immunomodulatory capacity of XPC.

The results obtained in a previous experiment conducted by our lab, Park et al., (2014), allowed us to observe differences in activation of cell-mediated immune response between the period following primary immunization and secondary immunization. In this experiment, an incomplete activation of cell-mediated immune response in birds from both dietary treatments was observed. There are no reports in the literature describing a similar development of CD4+ and CD8+ cells like the one observed in the current experiment.

In the literature, there are reports of the influence of the virulence of a vaccine on the establishment and development of cell-mediated immunity. Rauw et al., 2009 reported that an earlier and shorter cell-mediated immunity was generated from an NDV vaccine containing a less virulent strain, in comparison to vaccines containing more virulent strains, which resulted in a stronger and longer cell-mediated immunity. However, the results obtained in this experiment which used live LaSota strain NDV vaccine, a more virulent lentogenic strain compared to B1 strain, as a primary immunization indicate that a negative effect on the development of the adaptive immune system of broilers is generated.

In the poultry industry, live vaccines used in the field are designed to induce a strong humoral immune response in order to adequately establish adaptive immunity and establish immunological memory in the flock. A significantly different immunological response can be observed depending on the type of vaccine used, live or inactivated NDV vaccines.

In this experiment, cell-mediated immune responses were measured on days 7 and 24. Recent studies have established that it is possible to detect cell-mediated immune responses to NDV soon after vaccination when a live NDV vaccine is used (Reynolds and Maraqa, 2000). The results obtained in this experiment show an absence of a strong development of cell-mediated immunity in both dietary treatments. Overall, a significantly ($P<0.05$) higher percent of lymphocytes in both the spleen and in circulation was seen at day 24 compared to day 7; CD3+ was significantly ($P<0.05$) higher at day 24 in both dietary treatments compared to day 7. However, B cells present in the spleen were significantly ($P<0.05$) higher in both treatments at day 7 compared to day 24.

These results may indicate that live LaSota strain NDV vaccine may be causing an ineffective and incomplete activation of cell-mediated immune responses when used as primary immunization on the first day of age of a broiler as it was observed in the current experiment. Another potential explanation for the incomplete activation observed in the flow cytometry assay could be that the migration of T-cells and B cells occurs faster than we hypothesize and the cells might be undergoing proliferation somewhere in in the organism's lymphoid aggregate by the time the assay is conducted.

It is important to notice that within the complexity of the adaptive immune system it is often times difficult to draw clear direct cause and effect conclusions because many factors are involved in the immune response. The vaccine used in this experiment, live NDV LaSota strain, is more virulent compared to the B1 strain but still lentogenic and is widely used in the poultry industry typically only as a secondary

immunization. In the immune system, many different types of cytokines constitute the major instrument for immune cell communication and they act as immune modulating agents regulating both innate and adaptive immunity response to antigens and infectious agents (Stenger and Rollinghoff, 2001).

More research is needed to better understand the specific cell-mediated immunity mechanisms that aid in the immunological response against NDV. In prior studies, birds that were determined to have cell-mediated immunity specific for NDV via blastogenesis microassay with inactivated NDV, were not protected from lethal challenge in the absence of HI antibodies but birds with NDV-specific antibodies were shown to be protected (Alizadeh, 2012). Russell et al., (1997) indicated that antibodies are the key modulators of protection, but that cell-mediated immunity likely contributes to decrease viral shedding through target killing of NDV infected cells.

A good measurement of activation of cell-mediated immunity includes analyzing the expression of interferon gamma. Interferon gamma plays an important role in the activation of macrophages and has effector functions in the entire immune system. In this experiment, interferon gamma secreted by splenocytes and interferon gamma expression detected in circulation were calculated by using a common mitogen, concanavalin A, a classical T-cell mitogen (Ginsburg et al., 1971). The results are summarized in table 11. A higher level of interferon gamma in the spleen was detected at day 7 and 24 of the experimental period on both dietary treatments compared to the levels of this cytokine detected in circulation.

Overall, the expression of interferon gamma was low in both dietary treatments. These results further strengthen the observations made in this experiment and the hypothesis that the use of live LaSota strain NDV vaccine resulted in an incomplete activation and development of a successful humoral and cell mediated immune response. However, there is a possible explanation for the low levels of interferon gamma and the incomplete activation of adaptive immunity, which caused an incomplete development of humoral and cell mediated immunity in all birds from both dietary treatments. The primary vaccination could have triggered the activation of high levels of interlueukin-10, which has been described by Zhiguang et al., (2016) as being immunosuppressive in chickens as it is in mammals.

Interleukin-10, a multifunctional cytokine, is able to inhibit activation and effector function of T cells, monocytes, and macrophages. The principal routine function of IL-10 appears to be to limit and ultimately terminate inflammatory responses as well as to regulate B cells and helper T cells, which makes it play a key role in differentiation and function of T regulatory cells (Moore et al., 2001). Further study of the specific mechanisms that will trigger the inhibition seen in this trail is needed; this will also aid in the understanding of interleukin-10 role in broilers and the potential to use this understanding to enhance the immunological benefit of vaccines.

Protection by NDV-specific antibodies has been reported to be fundamental against a live NDV challenge. The results of NDV-specific humoral immune response observed in this trial are summarized in table 7. An absence of a robust humoral immune response was observed in vaccinated birds from both dietary treatments. At day 28, 7

days post-secondary immunization, only the control birds generated a clear NDV-specific humoral immune response. This weak antigen-specific humoral immune response was significantly ($P<0.05$) higher than the one observed on XPC supplemented birds. Unvaccinated birds did not show any NDV-specific humoral immune response, as expected, and its maternal antibodies followed a natural decline. This group showed an absence of a humoral response to any potential natural immunological challenge present at the farm that could have interfered with the establishment of adaptive immunity in the vaccinated birds. There are no literature reports under a similar experimental design showing comparable results of the antigen-specific humoral immune response observed in this trial.

The results of XPC supplementation in the diet of Ross 308 broilers on body weight and feed conversion ratio at day 42 are summarized in table 6. No significant differences ($P>0.05$) in body weights were observed throughout the experimental period (1-42d) between the dietary treatments. There were no significant differences ($P>0.05$) between vaccinated birds and unvaccinated birds of the same dietary treatment at days 14, 28, and 42 which indicates that the vaccine did not have an effect on cumulative feed consumption. Feed conversion ratio at day 42 was numerically improved in XPC supplemented birds but no significant differences ($P>0.05$) were observed.

Baurhoo et al., (2009) reported that inclusion of yeast-derived carbohydrates in broiler diets did not result in a significant difference on growth performance in a non-infectious setting. Our lab has consistently found no significant differences ($P>0.05$) in body weights of Ross 308 birds from XPC-containing diet compared to birds

supplemented a control diet but feed conversion ratio has tended to improve by day 42 in XPC supplemented birds.

4.5 Conclusion

In a previous experiment by this lab, Park et al., (2014), the immunomodulatory capacity of XPC was measured by immunizing birds on day 1 using live B1 strain NDV vaccine followed by a secondary immunization at day 21 with live LaSota strain NDV vaccine. The purpose of vaccination is not only to generate humoral immune response against a specific pathogen but also to establish cell-mediated immunity.

The main objective of this study was to evaluate the impact of a 1x dose of live LaSota strain NDV vaccine administered oculo-nasally on days 0 and 21 on early development of humoral and cell-mediated immune response in broilers and analyze the impact of this immunization schedule on the immunomodulatory capacity of XPC.

The results indicate that there was a detrimental effect on the development of the adaptive immune system of broilers vaccinated with live LaSota strain NDV vaccine. No robust NDV-specific humoral immune response was observed in birds from any of the dietary treatments. Control vaccinated birds reached a non-robust but significantly ($P<0.05$) higher antigen-specific humoral immune response compared to XPC supplemented birds on day 28, 7 days after secondary immunization.

Cell mediated immunity was also measured and the results indicated that there was an incomplete activation of T-cell lymphocytes on both the spleen and in circulation. B-cell population in the spleen and in circulation had a similar trend.

Overall, a higher percent of T-cell subpopulations in the spleen and in circulation were observed on day 7 compared to day 24.

The results of the organ index analysis showed an overall higher organ development in the unvaccinated birds compared to the vaccinated treatment except on day 21 where bursa of Fabricius was significantly ($P<0.05$) higher in the vaccinated birds compared to the unvaccinated treatment. The level of interferon gamma detected in circulation and in spleen was low and consistent with ELISA and flow cytometry data.

The results of this experiment strongly suggest that the use of live LaSota strain NDV vaccine at day 1 of age in broilers results in a severe detrimental effect on the development and establishment of adaptive immunity. The effects of the immunization schedule used in this experiment resulted in an absence of a robust NDV-specific humoral immune response in vaccinated birds; XPC supplemented birds suffered a more severe effect on the development of their adaptive immunity.

The unvaccinated birds showed a normal decaying of maternal antibodies. No significant differences were observed in live production parameters between the dietary treatments. The absence of an antigen-specific humoral immune response in unvaccinated birds shows the absence of a natural immunological challenge in the farm during the experimental period and allows for the further reliability of the results observed in vaccinated birds.

The results of this experiment indicate that the use of a more virulent strain of NDV vaccine compared to B1 strain can have a severe impact on the establishment of a

broiler's adaptive immunity. This is practical information for the poultry industry as it relates to immunization schedules.

5. EVALUATING THE INFLUENCE OF MATERNAL ANTIBODIES ON THE EFFICACY OF LIVE NEWCASTLE DISEASE VIRUS VACCINE ON XPC™ SUPPLEMENTED BROILERS

5.1 Introduction

The poultry industry needs new strategies to maintain production levels while eliminating the use of antibiotics and maintaining bird health. Our lab as a potential strategy to combat the problems faced in antibiotic free production has identified the functional metabolites of Diamond V Original XPC™ (XPC). This potential strategy can result in enhanced immune function and poultry health.

This study's objective was to evaluate the role of maternal antibodies on XPC modulation of antigen-specific humoral immune response in broilers. One-day old Ross 308 broilers (180) were randomly assigned to a factorial arrangement of three vaccination protocols and two diets (XPC or control (CON)). In the first protocol broiler chicks were immunized with live Newcastle Disease Virus (NDV) B1 strain vaccine at day 1. In the second protocol broiler chicks were vaccinated with live LaSota strain NDV vaccine at day 1.

Both protocols received a secondary immunization with live LaSota strain NDV vaccine at day 21. Protocol three consisted of delaying immunizing the chicks until the level of maternal antibodies decayed below 2000 NDV titer level. Once the level of maternal antibodies was verified, broilers were immunized with live LaSota strain NDV vaccine. NDV-specific IgY was measured using ELISA on days 1, 7, 14, 21, 28, and 35.

Feed consumption, cumulative feed conversion ratio and body weights were also measured. XPC birds reached a higher and faster titer level one-week following secondary immunization in protocol 1. Protocol 3 resulted in birds reaching the highest NDV-titer level during the trial with XPC birds reaching the highest NDV-titer level. Protocol 2 had the lowest NDV-titer level observed in the trial. Feed conversion was higher in both dietary treatments for protocol 3 compared to protocols 1 and 2.

The results indicate that XPC favorably modulated the broiler immune system against NDV specific adaptive immunity. The level of maternal antibodies appears to have a role on the impact of XPC on the adaptive immune system which is consistent with previous data from our lab.

5.2 Materials and Methods

In order to evaluate the influence of maternal derived antibodies on the efficacy of live NDV vaccine on XPC supplemented birds a 35-day trial was conducted. The main objective of this study was to evaluate the role of maternal antibodies on the immunomodulatory capacity of XPC on antigen-specific humoral immune response in broilers.

A total of 180-one-day old Ross 308 broilers were randomly assigned to a factorial arrangement of three vaccination protocols and two diets (XPC or control). XPC was included into treatment diet at 1.25 kg/metric ton for starter, and grower diets. Feed was stored in plastic lined 30 gallon barrels labeled with date, diet, treatment code and experiment identification number. A 2x batch of the control diet was mixed and split into two equal parts prior to pelleting. The control feed was pelleted and placed in

barrels. The treated feed was returned to the mixer and an appropriate amount of XPC was added and allowed to mix for 10 minutes prior to pelleting and storage in barrels. Composite feed samples from each feed mixing were collected and stored at -20^{o}C until submitted for proximate analysis.

In the first protocol, broiler chicks were immunized with live NDV B1 strain vaccine on day 1 (USDA Product code: 1711.10; Merial Select Laboratories, Gainesville, Georgia). In the second protocol broiler chicks were vaccinated with live LaSota strain NDV vaccine on day 1 (USDA Product code: 1721.11); Merial Select Laboratories, Gainesville, Georgia). In both protocols, chicks received a secondary immunization with live LaSota strain NDV vaccine at day 21. Protocol three consisted of delaying immunization of the chicks until the level of maternal antibodies decayed below 2000 NDV titer level. Once the level of maternal antibodies was verified, broilers were immunized with live LaSota strain NDV vaccine. Each treatment group of 15 birds had a replicate pen for a total of 12 pens.

Antigen-specific humoral immune response was assessed by NDV-specific IgY using ELISA on days 1, 7, 14, 21, 28, and 35. Blood samples were obtained by venipuncture; 0.5ml blood samples were collected. A Newcastle Disease Antibody Test Kit (Synbiotics Corp., San Diego, CA) was used to assess the establishment of humoral immune response. The average of positive and normal control serum absorbance (Optical Density as O.D.) was calculated. Each sample was analyzed, and antibody titers were calculated using the formula: S/P (Sample to positive ratio) = (Absorbance of sample – Average of *N* control absorbance)/ (Average of positive control absorbance – Average of *N*

control absorbance). The final titer was calculated based on the following formula provided in manufacturer's instructions: Final Titer = ANTILOG (1.464 x (Log_{10}S/P) + 3.740).

Cumulative feed conversion ratio, feed consumption, and body weights were obtained. All birds were housed at TAMUPRC. Birds were fed a corn/soybean meal base diet (table 14). Starter (0-21d), grower (22-35d) diets were manufactured at TAMUPRC feed mill. Feed and water was provided *ad libitum* during the entire trial.

No drug therapy was used during the study. When any study personnel entered a pen (i.e., to collect birds for study procedures), plastic boots and gloves were utilized. The person who was inside the pen as it stepped out to avoid carrying any fecal material throughout the facility removed the plastic boots and gloves. Plastic boots and plastic gloves were discarded in an appropriate container after use.

All pens were monitored three times daily for general flock condition, temperature (recorded on log sheets), lighting, water, feed, litter condition, and unanticipated house conditions/events. Findings were documented on log sheets. Mortality was monitored in each pen daily.

All data were analyzed with the SAS (SAS Institute, Cary, NC) statistical software package and analyzed via one-way ANOVA using the GLM procedure. Differences were deemed significant at $P<0.05$ and means were separated by the PDIFF procedure. All animal handling and care practices were accordance with an approved animal use protocol, IACUC 2016-0103 reference number 047515.

Table 14 Experiment 3 – Experimental diets and nutrient composition

	Starter 0-21 d	Grower 22-35 d
Ingredients, %		
Corn, yellow grain	58.46	64.73
Soybean meal, dehulled solvent	34.48	29.33
Bio/Phos 16/21 P	1.56	1.59
Limestone	1.49	1.46
Salt, plain	0.51	0.49
Mineral mix[1]	0.05	0.05
DL Methionine	0.28	0.08
Lysine	0.17	0.02
Vitamin Premix[2]	0.25	0.25
Fat A/V blend	2.75	2.00
Original XPC[3]	0.125	0.125
Calculated Nutrient Composition		
CP, %	22.02	19.80
ME, kcal/lb.	3048	3055
Crude Fat, %	5.30	4.74
Lysine, %	1.30	1.05
Methionine (M), %	0.61	0.39
M+Cysteine, %	0.97	0.72
Tryptophan	0.26	0.23
Threonine	0.82	0.74
Arginine	1.45	0.74
Valine, %	1.00	0.91
Calcium, %	0.92	0.90
Available Phosphorous, %	0.45	0.45
Sodium	0.22	0.21
Chloride, %	0.39	0.34

[1]Vitamin premix added at this rate yields 11,023 IU vitamin A, 3,858 IU vitamin D3, 46 IU vitamin E, 0.0165 mg B12, 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. [2]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.
[3]XPC is Diamond V Original XPC (Cedar Rapids, Iowa); corn will replace XPC in control diet.

5.3 Results

The results for serum levels of NDV-specific IgY are presented in table 15. There was a robust and faster establishment of NDV-specific humoral immune response of XPC supplemented birds in protocol 1 compared to control birds on day 28, ($P<0.05$). The lowest level of NDV titers were observed in protocol 2, LaSota strain as primary immunization. This protocol also resulted in an absence of a robust NDV-specific humoral immune response. The highest level of NDV titers was seen in birds from protocol 3; XPC supplemented birds reached a significantly ($P<0.001$) higher level of NDV titers at day 35 compared to control birds and were able to mount the highest NDV specific humoral immune response compared to all other treatments in the trial.

The results obtained in this experiment concerning the effect of XPC on mean body weights are summarized in Table 16. There was a significant ($P<0.05$) difference observed in feed conversion ratio between dietary treatments of protocol 3 compared to 1 and 2 on day 35. This however, was determined to be a vaccination protocol effect and not a dietary effect. Feed consumption and feed conversion ratio at day 35 are summarized in table 17.

Table 15 Experiment 3 – Effect of XPC on mean NDV Titers (IgY)

Vaccination Protocols[1]							
	Protocol 1 Diets[2]		Protocol 2 Diets[2]		Protocol 3 Diets[2]		
Day *	Control	XPC	Control	XPC	Control	XPC	SEM
7	6991.03	7700.39	6856.71	8289.03	7297.21	7260.82	489.29
14	1620.01	2174.68	1923.39	2255.66	1873.11	1996.01	287.95
21	901.09	821.43	836.25	1106.71	557.12	451.93	145.51
28	**1041.34^{bA}**	**1805.01^{aA}**	740.67^{B}	966.51^{B}	60.20^{B}	250.21^{B}	187.06
35	**1662.45^{B}**	**1436.20^{B}**	1135.21^{B}	785.11^{B}	**2020.76^{Ab}**	**2829.34^{aA}**	439.83

Calculated antibody titers (IgY from the optical density measured by ELISA reader for the samples that were run through ELISA test specific for anti-Newcastle disease (ND) virus IgY production.

On day of hatch the level of NDV-specific maternal antibodies was calculated and determined to be 11363.83

$^{A, B}$ Means within a row lacking a common superscript differ significantly ($P<0.001$).

a, b Means within a row lacking a common superscript differ significantly ($P<0.05$).

Each value represents the mean value for each group.

* n= 15 birds per day, Ross 308

[1]Vaccination Protocol 1: B1 at day 1, LaSota at day 21; Protocol 2: LaSota at day 1 and day 21; Protocol 3: B1 at day 21, once the level of maternal antibodies decayed below 2000 NDV titer.

[2]Diets with no XPC served as Control and XPC diets had inclusion of 1.25 kg of Original XPC /metric ton

Table 16 Experiment 3 - Effects of diet containing XPC on body weight (BW)

Vaccination Protocols[1]							
	Protocol 1 Diets[2]		Protocol 2 Diets[2]		Protocol 3 Diets[2]		
Day *	Control	XPC	Control	XPC	Control	XPC	SEM
	BW (g)	BW (g)	BW (g)	BW (g)	BW (g)	BW (g)	
1	38.13	39	39.53	38.50	39.03	38.33	0.47
35	2281.90	2140.63	2224.40	2206.88	2168.80	2175.01	127.69

* n= 15 birds per day, Ross 308.
[a, b] Means within a row lacking a common superscript differ significantly (P<0.05).
[1]Vaccination Protocol 1: B1 at day 1, LaSota at day 21; Protocol 2: LaSota at day 1 and day 21; Protocol 3: B1 at day 21, once the level of maternal antibodies decayed below 2000 NDV titer.
[2]Diets with no XPC served as Control and XPC diets had inclusion of 1.25 kg of Original XPC /metric ton

Table 17 Experiment 3 - Effects of diet containing XPC on cumulative feed consumption (CFC) and feed conversion ratio (FC) at day 35

Vaccination Protocols[1]							
	Protocol 1 Diets[2]		Protocol 2 Diets[2]		Protocol 3 Diets[2]		
Day *	Control	XPC	Control	XPC	Control	XPC	SEM
	CFC (g)	CFC (g)	CFC (g)	CFC (g)	CFC (g)	CFC (g)	
35[3]	3604.19	3437.92	3520.38	3599.13	4074.75	3818.63	100.80
FC	**1.61[B]**	**1.64[B]**	**1.61[B]**	**1.66[B]**	**1.82[A]**	**1.80[A]**	0.07

* n= 15 birds per day, Ross 308.
[A, B] Means within a row lacking a common superscript differ significantly (P<0.001).
[1] Vaccination Protocol 1: B1 at day 1, LaSota at day 21; Protocol 2: LaSota at day 1 and day 21; Protocol 3: B1 at day 21, once the level of maternal antibodies decayed below 2000 NDV titer.[2] Diets with no XPC served as Control and XPC diets had inclusion of 1.25 kg of Original XPC /metric ton. [3]End of grower diet.

5.4 Discussion

The main objective of this experiment was to evaluate the role of maternal antibodies on XPC modulation of antigen-specific humoral immune response in broilers. The factorial arrangement of three vaccination protocols and two diets (XPC or control (CON)) described in detail in the materials and methods section allowed for the identification of a vaccination protocol that allows for a faster and robust establishment of NDV-specific humoral immune response in broilers.

Feed conversion ratio was measured during the experimental period. A significantly ($P<0.05$) higher feed conversion ratio at day 35 for protocol 3 was observed compared to protocols 1 and 2. This was determined to be a vaccination protocol effect. There are no reports in the literature utilizing the same vaccination protocol used in this experiment that studied the effect on feed conversion ratio. We hypothesize that the differences in feed conversion at day 35 are the result of birds from vaccination protocol 3 losing gain to feed consumption ratio as a result of the vaccination they received on day 21, which was the first time these birds encountered NDV in addition to having lost their protective level of maternal antibodies.

Body weight results showed no significant differences ($P>0.05$) during the experimental period (1 to 35d) as a result of a dietary treatment effect or vaccination protocol effect. Morales-Lopes and Brufau, (2013) showed that supplementation of *Saccharomyces cerevisiae* cell walls did not result in any significant differences in feed intake and body weight gain, but feed conversion ratio on days 21 and 28 was better compared to the control group.

The experimental design allowed for the study of the effect of maternal antibodies on XPC and control birds as well as the interaction between vaccination protocols and maternal antibodies on NDV-specific humoral immune response. On day 28 a significantly ($P<0.001$) higher anti-NDV titer level between XPC birds from protocol 1 and the birds from both dietary treatments from protocols 2 and 3 was observed. On day 28 for protocol 1 a significantly ($P<0.05$) higher titer was observed for XPC supplemented birds compared to controls.

There are reports in the literature (Haghighi et al., 2005; Ghosh et al., 2012) that have shown that supplementation of diet with yeast-derived carbohydrates can enhance humoral immune responses by increasing the antibody production. The ELISA results in this experiment indicate that the level of maternal antibodies and vaccination schedule do have an effect on the development of antigen-specific humoral immune response in broilers fed a control diet.

Additionally, they suggest that the level of maternal antibodies on day 1 appears to modulate the effect of XPC on the adaptive immune system of broilers. The results of this experiment also strongly indicate that vaccination schedule also has a significant impact on the ability of XPC to modulate the adaptive immune system of broilers.

A more robust NDV-specific humoral immune response was observed on birds from vaccination protocol three. Protocol three consisted of delaying immunization of the chicks until the level of maternal antibodies decayed below 2000 NDV titer level. Once the level of maternal antibodies was verified, broilers were immunized with live LaSota strain NDV vaccine, day 21. XPC supplemented birds from this group reached

the highest NDV titer level observed in the trial and their titer level was significantly ($P<0.05$) higher from control birds. This vaccination protocol only included one immunization, after the level of maternal antibodies had dropped to baseline levels. Maternal antibodies are antibodies that allow for the establishment of passive immunity in chicks that receive these antibodies from the hen through the egg yolk. The interference in vaccine-induced humoral immune response by maternal antibodies is well studied. It can result in a significant difference in the development of antigen-specific humoral immune response in birds as was seen in this experiment.

An immunosuppressive effect of maternal antibodies on humoral immune response following vaccination has been attributed to neutralization of vaccine virus by these antibodies (Siegrist et al., 1998). Naqi et al. (1983) showed that birds with high levels of IBDV maternal antibodies had no immune response activation to IBDV vaccines, whereas birds with low maternal antibodies showed delayed response to vaccines. It has been reported also that vaccination of birds against Infectious Bronchitis Virus in the presence of maternal antibodies at d1 of age quickens the clearance rate of maternal antibodies (Mondal and Naqi, 2001).

5.5 Conclusion

The results observed in this experiment concur with the results obtained in a previous experiment conducted in our lab by Park et al., (2014). In addition, they corroborate the hypothesis that the level of maternal antibodies influences the immunomodulatory effect of XPC on the adaptive immune system of broilers. On day 28, a significantly ($p>0.05$) higher humoral response was observed in XPC birds in

comparison to the control group, in vaccination protocol 1. The results seen in vaccination protocol 2 are comparable to the ones obtained in experiment 2. The results restate the conclusion from experiment 2 about the negative impact that NDV live LaSota strain vaccine has on the development of adaptive immune response.

Vaccination protocol 3 resulted in the highest level of NDV titers following immunization at day 21. Vaccination protocol 3 allowed for the measurement of the immunomodulatory effect of XPC to be seen without the interference by maternal antibodies. It is important to mention that maternal antibodies are pathogen specific and are able to act similarly to naturally induced antibodies.

No significant differences ($P>0.05$) in body weights were observed but feed conversion ratio at day 35 was significantly ($P<0.05$) higher in birds from protocol 3 compared to protocols 1 and 2. This was concluded to be a vaccination protocol effect and not a dietary effect.

6. SUMMARY

The poultry industry has evolved to become an efficient, high yield, and economically lucrative industry through the development of better ventilation, housing, animal care, use of antibiotics and intense research. However, due to high levels of production and the use of antibiotics in combination with consumer demand, media pressure and growing concern over antibiotic-resistant bacteria, and the potential for their residual presence in poultry products; the poultry industry has been forced to seek and develop new strategies for pathogen control, animal health, and food safety without the use of antibiotics.

The functional metabolites of a *Saccharomyces cerevisiae* fermentation product from Diamond V (Cedar Rapids, Iowa) Original XPC^{TM} (XPC) have been identified by our lab as a potential strategy for use in the poultry industry, particularly in antibiotic free production. The need to find effective, economically sound and practical strategies in order to maintain high levels of production in the poultry industry while continuing appropriate animal care has become a top priority in the industry around the world. Therefore, it is imperative to continue thorough research in that field.

This study involving three different experiments had the objective to 1) evaluate the effect of dietary XPC supplementation on the early development of the adaptive immune response in broilers, 2) assess the impact of a 1x dose of live LaSota strain NDV vaccine administered oculo-nasally on days 0 and 21 on early development of humoral and cell-mediated immune response in broilers and analyze the impact of this

immunization schedule on the immunomodulatory capacity of XPC, 3) to evaluate the role of maternal antibodies on XPC modulation of antigen-specific humoral immune response in broilers.

The most robust antigen specific humoral immune response observed at the completion of this series of three experiments was observed in XPC supplemented birds vaccinated only at day 21; once maternal antibodies had been cleared. The results also indicate that if the level of maternal antibodies is relatively high at day of vaccination XPC supplemented broilers can reach a faster and more robust NDV-specific humoral immune response when the primary immunization is live B1 NDV vaccine as opposed to LaSota.

The results obtained from this series of three experiments that overall aimed to assess the impact of the immunomodulatory capacity of XPC on the adaptive immune system of broilers indicate that the functional metabolites of XPC have a positive effect on the establishment of antigen-specific humoral immune response in broilers. The results show a more robust response, and depending on the primary immunization and level of maternal antibodies, a faster establishment of antigen-specific humoral response by XPC supplemented birds.

However, the results from the flow cytometry conducted to measure the development of cell-mediated immunity did not show any significant differences between the control and XPC supplemented diets nor clear trends amongst the dietary treatments. It is unclear what specific mechanisms or genes in the adaptive immune system are being modulated by XPC. Further research is needed in the area of gene

expression in order to identify the genes that are being upregulated and downregulated and are being responsible for the development of a more robust adaptive immune response.

Supplementation of XPC did not result in a significant improvement of growth performance. Throughout the literature there are inconsistent reports concerning the effects of yeast-derived products on broiler growth. One potential explanation for the difference in results could be associated with experimental and environmental conditions, as well as the type and concentrations of yeast products used in different studies and the genetic background of the birds utilized.

It is clear that after the completion of this series of experiments, more research is needed to better understand the mechanisms in the establishment of adaptive immunity that XPC modulates in order to fully assess its potential value for the industry.

One of the objectives of this study was to evaluate the role of maternal antibodies on XPC modulation of antigen-specific humoral immune response in broilers. It is clear that the efficacy of NDV vaccine can be enhanced if the level of maternal antibodies is low prior to the immunization day (day 21 of age in this study). One cost associated to this immunization schedule however, can be the dysregulation of feed consumption and as a result a negative effect in feed conversion ratio; this as a result of a natural reaction from the birds to the vaccine as they have never been exposed to the antigen previously.

The inconsistent results regarding the effects of probiotics and prebiotics on adaptive humoral immune response suggest that the immunomodulatory effects of these products cannot be generalized due to a number of factors such as genetic background of

the host, the type of the probiotic and prebiotic used, immunization regimen as well as experimental conditions.

The results of this study allow for the identification of live LaSota strain NDV vaccine as potentially detrimental to the development of adaptive immunity in broilers when used at one-day of age.

This study reinforces the widely accepted theory that maternal antibodies can interfere with vaccine efficacy and as a result fail to induce a full immunological benefit. In addition, this study provides practical information to the poultry industry regarding vaccination protocols and use of XPC in broilers, and it supplies valuable information about XPC that can result in a better understanding of its immunomodulatory capacity in broilers. It assess its potential value as a strategy to be used by the poultry industry to enhance immune function and poultry health.

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APPENDIX

XPC™ by Diamond V

Diamond V's Original XPC™ is an all-natural fermentation product that contains bioactive components produced using proprietary anaerobic fermentation technology of *Saccharomyces cerevisiae*; it is composed of metabolites, beta-glucans, and mannans that promote animal health and performance (Diamond V Original XPC™, n.d.).

The metabolites in Original XPC™ help to balance the gut microbiota in order to support optimum digestive health, and the antioxidant activity of XPC™ ads immune function (Diamond V Original XPC™, n.d.).

Research has shown that XPC supports production performance for all poultry, specifying in egg production, quality and efficiency for layers. (Diamond V Original XPC™, n.d.) It is used in all classes of livestock, poultry, equine and pet diets.