YEAST PRODUCTS AS POTENTIAL SOURCES OF IMMUNOMODULATORY AND GROWTH PROMOTING ACTIVITY FOR BROILER CHICKENS

By

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ABSTRACT

The use of antibiotic growth promoters has been limited all around the world because of the concerns about antibiotic resistant bacteria and the presence of antibiotic residues in poultry products. Yeast-derived products are rich sources of ß1,3-1,6-glucan, mannan polysaccharides, and nucleotides and are considered as possible antibiotic alternatives due to their potential intestinal health benefits, growth promotion, and immune system stimulation. The objectives of the current research were: (1) to the evaluate effect of yeast products derived from yeast *Saccharomyces cerevisiae* on growth performance, gut histomorphology, and innate immune response of broiler chickens; (2) to investigate the effect of yeast products, including distillers dried grains with solubles (DDGS), on innate and antibody-mediated immune response following immunization with different antigens; and (3) to examine the effect of yeast-derived products and DDGS on growth performance, incidence of necrotic enteritis (NE), and local innate immunity in broiler chickens challenged with *Clostridium perfringens*. Overall, supplementation of diets with yeast products did not affect growth performance of broilers. However, the diets containing yeast cell walls (YCW) and nucleotides increased the villus height in the jejunum and enhanced the number of goblet cells in the ileum. Inclusion of diets with yeast products did not activate the innate immune response of birds under non-pathogen challenge conditions. However, the diet containing YCW activated Th2 cell-mediated immune response in birds immunized with sheep red blood cells and bovine serum albumin. Furthermore, supplementation of diets with YCW and DDGS in birds challenged with *Escherichia coli* lipopolysaccharide, activated the systemic innate immune response. Regarding antibody-mediated immune response, when compared to the control, serum antibody titer and specific antibody response against

different antigens were not affected by dietary treatments. In the *C. perfringens* challenge study, growth performance, NE lesions and *C. perfringens* counts in the intestine were not affected by yeast-derived products. However, diets containing YCW and nucleotides stimulated the local innate immune response of birds by upregulation of cytokines and receptors involved in innate immunity. Such findings suggest that the immune-adjuvant like properties of YCW and nucleotides activate the innate immunity of broiler chickens following immunization or challenge with different antigens.

DEDICATION

This thesis is dedicated to my lovely parents for

their sacrifice and support throughout my life

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FOREWORD

This thesis was prepared in a manuscript format with four manuscripts to be submitted. All manuscripts have been formatted to meet the guidelines of Poultry Science.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AGPs	Antibiotic growth-promoters
ANOVA	Analysis of variance
AP-1	Activator protein 1
BCR	B cell receptor
BSA	Bovine serum albumin
BWG	Body weight gain
C. perfringens	*Clostridium perfringens*
CD 14	Cluster of differentiation 14
cDNA	Complementary deoxyribonucleic acid
CFU	Colony-forming unit
Ch TLR	Chicken TLR
CpG	Cytosine-phosphate-guanine
CWP	Cell wall polysaccharide
DCs	Dendritic cells
DDGS	Distillers dried grains with solubles
DNA	Deoxyribonucleic acid
DSS	Dextran Sulfate Sodium
E.coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
EU	European union
FCR	Feed conversion ratio

FI	Feed intake
GlcNAc	N-acetylglucosamine
GLM	Generalized linear mode
HI	Hemagglutination-inhibition
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IRFs	Interferon regulatory factors
ITAM	immunoreceptor tyrosine-based activation like motif
LPS	lipopolysaccharide
LRR	Leucine-rich repeats
MAMP	Microbe-associated molecular patterns
MBL	Mannose-binding lectin
MBP	Mannose binding protein
MD-2	Myeloid differentiation protein 2
MHC	Major Histocompatibility Complex
MMR	Monocyte mannose receptor
MR	Mannose receptor
Myd88	Myeloid differentiation factor 88
MΦ	Macrophages
NCBI	National Centre for Biotechnology

NE	Necrotic Enteritis
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cells
NOD	Nucleotide-binding oligomerization domain
NSP	Non-starch polysaccharides
ODN	Oligodeoxynucleotides
PAMP	Pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
PRRs	Pattern recognition receptors
QRT-PCR	Quantitative real-time pcr
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S. cerevisiae	*Saccharomyces cerevisiae*
SRBC	Sheep red blood cells
ß-glucan	Beta-glucan
TCR	T cell receptor
Th	T helper cells
Th1	T helper type 1
Th2	T helper type 2
TIR	Toll/IL-1 receptor

TIRAP	Toll-interleukin 1 receptor domain containing adaptor protein
TLR2a	Toll-like receptor 2 type a
TLR2b	Toll-like receptor 2 type b
TLRs	Toll-like receptors
TNFα	Tumor necrosis factor alpha
TRAM	TRIF-related adaptor molecule
Tregs	Regulatory T cells
TRIF	TIR-domain-containing adapter-inducing interferon-β
YCW	Yeast cell wall

CHAPTER 1: GENERAL INTRODUCTION

Over the past 50 years, antibiotic growth-promoters (AGPs) have been used in poultry feed to prevent bacterial infections, reduce mortality rate, and improve growth performance and production (Casewell et al., 2003; Castanon, 2007). However, inappropriate and excessive use of AGPs in animal feeds has led to antibiotic resistance development which is one of the major public health concerns (Cogliani et al., 2010). Many countries especially in Europe, have banned the use of AGPs in poultry production (Castanon, 2007). However, in many cases AGP removal has been associated with impaired growth performance and increased mortality rate in broiler chickens (Dibner and Richards, 2005). Different strategies including alternative dietary supplements have therefore been introduced in the broiler industry to reduce the consequences of AGP removal. Yeast-derived products including ß1,3-1,6-glucan, mannan polysaccharides and nucleotides are considered as potential alternative supplements because of their growth promoting effects and immunomodulatory properties (Gao et al., 2008; Brummer et al., 2010). The results of some studies demonstrated that cell wall polysaccharides derived from the yeast *Saccharomyces cerevisiae* can improve growth performance by facilitating gut development and providing competitive binding sites for pathogenic bacteria (Spring et al., 2000; Muthusamy et al., 2011). Yeast cell wall polysaccharides have been shown to improve digestion and absorption of nutrients by increasing the intestinal villus height in some of the studies (Sims et al., 2004; Refstie et al., 2010). It has also been reported that yeast cell wall polysaccharides can improve gut health by increasing the number of goblet cells, the special epithelial cells involved in mucus production (Solis de los Santos et al., 2007; Reisinger et al., 2012). Mucus released by goblet cells is a glycoproteins-rich product that can trap bacteria and prevent bacterial attachment to the

epithelial layer (Nochi and Kiyono, 2006; Sheehan et al., 2007). Furthermore, mannose-based oligosaccharides derived from the cell wall of *Saccharomyces cerevisiae* have the ability to control enterobacteria colonization and prevent bacterial attachment to the epithelium through binding to the type 1 fimbriae, which serve as an adhesion organelle expressed by many gram-negative bacteria such as *Salmonella enterica* and *Escherichia coli* (Ferket et al., 2002; Spring et al., 2000).

In addition to intestinal health benefits, yeast cell wall polysaccharides are known for their ability to modulate the immune system (Staykov et al., 2007; Novak and Vetvicka, 2008). It has been hypothesized that yeast cell wall polysaccharides including ß1,3-1,6-glucan, manno oligo- or polysaccharides have the ability to act as microbe-associated molecular patterns (MAMP) and stimulate the innate immunity by activation of pattern recognition receptors (PRRs) expressed by cells of innate immunity and epithelial cells (Shashidhara and Devegowda, 2003; Mogensen, 2009). Recognition of yeast cell wall polysaccharides as MAMP by PRRs may result in activation of the cells of innate immunity and lead to the production of cytokines that develop further immune response (Akira et al., 2006). Other than cell wall polysaccharides, nucleotide components of yeast products are involved in intestinal gut development and immune system activation. Nucleotides can be synthesized endogenously and are not considered as essential nutrients (Carver and Walker, 1995; Cosgrove, 1998). However, the intestinal epithelium and lymphocytes of the gastrointestinal tract have rapid cell turnover and are unable to produce all the necessary nucleotides *de novo* to satisfy their own requirements (Carver, 1999; Li et al., 2005; Low et al., 2003). Therefore, intestinal development is highly dependent on the presence of dietary nucleotides. The results of some human and animal studies have shown that nucleotides can increase the development of the villi, intestinal wall thickness and protein

content in the gastrointestinal tract (Uauy, 1994; Bueno et al., 1994; Mateo and Stein, 2004). Considering the role of nucleotides in the development of cells with rapid turnover (i.e., epithelial cells and lymphocytes) dietary nucleotides can probably modulate the immune response of broiler chickens (Gil, 2002; Hess and Greenberg, 2012). There is also a possibility that yeast nucleotides can activate innate immunity by binding to TLR21, the receptor that is expressed by epithelial cells and cells of innate immunity, and is involved in recognition of CpG oligodeoxynucleotides (Brownlie et al., 2009). Distillers dried grains with solubles (DDGS) is a by-product of grain-based ethanol production that has been used as a protein source in swine and poultry diets (Loar et al., 2010). Over the last few years, extensive research has been conducted on the chemical and nutritive evaluation of corn and wheat DDGS and development of nutrient availability data for poultry and swine (Slominski et al., 2008). To our knowledge, what has not yet been considered in the DDGS research is the fact that as co-products of brewer's yeast (*Saccharomyces cerevisiae*) fermentation, they contain a significant quantity (6%) of yeast biomass that could be beneficial for gut development and effective in immune system stimulation (Slominski, 2012). As a result, we hypothesized that yeast products derived from *Saccharomyces cerevisiae* and DDGS could become a significant source of yeast components that can probably stimulate the immune response of broiler chickens and improve growth performance by facilitating gut development and providing competitive binding sites for pathogenic bacteria.

Therefore, the objectives of this research were:

1. To investigate the effect of corn and wheat DDGS and yeast-derived products of different nucleotides, ß1,3-1,6-glucan, and mannan oligo- or polysaccharides on growth performance, gastrointestinal tract development and innate immune response of broiler

chickens.

2. To evaluate the effect of different yeast-derived products and DDGS on innate and antibody-mediated immune response in broiler chickens immunized with non-pathogenic antigens such as sheep red blood cells and bovine serum albumin.
3. To examine the effects of different yeast-derived products and DDGS on innate and antibody-mediated immune response in broiler chickens challenged with *Escherichia coli* lipopolysaccharide.
4. To use a *Clostridium perfringen*s challenge model to induce a mild form of NE in broiler chickens in a commercial operation, to evaluate the effects of different yeast-derived products and DDGS on growth performance and immune response.

CHAPTER 2: LITERATURE REVIEW

2.1 Antibiotic Growth Promoters

Antibiotic growth promoters (AGPs) are defined as a group of antimicrobial agents that are administered at subtherapeutic levels to inhibit bacterial growth at intestinal levels and improve animal growth (Dibner and Richards, 2005). The exact mechanism underlying the growth promoting effects of antibiotics is still unknown. However, it has been hypothesized that AGPs can show their beneficial effects through different mechanisms such as the reduction of opportunistic pathogens and their metabolites and toxins and increased nutrient absorption via thinning of the small intestine (Anderson et al., 1999; Gaskins et al., 2002; Martel et al., 2004). Despite all beneficial effects of AGPs, there are some concerns over APGs used in farm animals. In the long term, the excessive use of antibiotics in animal feeds can contribute to the development of antibiotic-resistant bacteria (Turnidge, 2004; Wegener, 2003). There is some evidence that antibiotic use in food animal production is associated with antibiotic-resistant bacterial infections in humans. Human exposure to antibiotic residues in animal products makes their microbiota more resistant to antibiotics and would make it more expensive and difficult to treat human bacterial infections (Mathew et al., 2007; Engberg et al., 2001). Because of these health concerns regarding the use of AGPs, many European countries have restricted or banned the use of AGPs in farm animals (Castanon, 2007). However, the ban on AGPs has resulted in impaired growth performance and enhanced mortality on poultry farms (Diarra and Malouin, 2014). Therefore, the feed industry and nutritionists have been looking for non-antibiotic or natural growth promoters to maintain the health and efficient poultry production under commercial conditions (Huyghebaert et al., 2011).

2.2 Yeast-derived Products

Yeast cell walls derived from yeast *Saccharomyces cerevisiae* are mainly composed of mannoproteins and β-Glucans (Klis et al., 2002; Lipik and Ovalle, 1998). The outer layer of the yeast cell wall is composed of mannoproteins that contains mannan polysaccharides as the major constituent (Zlotnik et al., 1984; Brul et al., 1997; Levin, 2005). Mannan polysaccharides present in yeast cell walls are composed of the sugar mannose and are linked to proteins to form a mannoprotein layer that is located at the external surface and acts as a filter for large molecular weight materials (De Nobel et al., 1990). Mannan poly/oligosaccharides have the ability to reduce the colonization of pathogenic bacteria in the gastrointestinal tract (Sims et al., 2004). Many enteropathogenic bacteria including *Escherichia coli* (66% of strains), *Salmonella Typhimurium* (80% of strains) and *Salmonella* Enteritidis (67% of strains) use the adhesion organelles called "type 1 fimbriae" to attach to the intestinal lining (Spring et al., 2000). Type 1 fimbriae specifically recognizes mannan-based sugar residues and facilitates the colonization and adhesion of bacteria to mucosal surfaces (Firon et al., 1987). Mannan poly/oligosaccharides of yeast cell walls have the ability to block bacterial attachments and reduce pathogen colonization by inhibiting the adhesion of mannose-specific fimbriae to mucosal surfaces (Baurhoo et al., 2009; Yang et al., 2008). Furthermore, it has been reported that mannan components of yeast cell walls are involved in modulation of the immune system by activation of pattern recognition receptors such as mannose-binding lectin (MBL) and monocyte mannose receptor (MMR) present on the surface of cells of innate immunity (Takahashi and Ezekowitz, 2005; Eddie LP et al., 2009; Martinez-Pomares, 2012). Yeast cell wall ß1,3- and ß1,6-glucans are polysaccharides consisting of a linear backbone of β-1,3 linked D-glucose with β-1,6 linked side chains of varying distribution and length (Kollar et al., 1997; Klis et al., 2002). Beta 1,3- and 1,6-glucans

derived from *Saccharomyces cerevisiae* are known as natural biological modifiers because of their ability to activate the immune system through binding to the PRRs such as TLR2 and dectin-1 that are expressed by epithelial cells and leukocytes (Brown et al., 2007; Yadav et al., 2006; Lee et al., 2001).

Nucleotides derived from yeast *Saccharomyces cerevisiae*, are low-molecular-weight intracellular compounds which make up the structural units of DNA and RNA (Cosgrove, 1998). Nucleotides synthesized endogenously are considered as non-essential nutrients; however, under certain conditions such as food restriction, rapid growth, and certain diseases, their availability could limit the maturation of fast dividing tissues such as brain, bone marrow, and intestine, therefore, making nucleotides semi-essential nutrients (Vanburen et al., 1994; Sánchez-Pozo and Gil, 2002). It has been reported that nucleotides have beneficial effects on the health and development of intestine (as a fast dividing tissue), intestinal microflora, and mucosal immunity in humans and animals (Carver, 1994; Grimble, 1994; Maldonado et al., 2001; Gil, 2002).

2.3 Distillers Dried Grains with Solubles (DDGS)

Considering the concerns over environmental pollution, the federal and provincial governments in Canada mandated that gasoline in Canada must contain a minimum of 5% ethanol on average. Therefore, the governments allocated millions of dollars for the development of the ethanol industry and expansion of ethanol plants across Canada (Olar et al., 2004). Distillers dried grains with solubles (DDGS) is a by-product of grain-based ethanol production that can be used as an ingredient (protein source) in animal and poultry diets (Behnke, 2007). During the process of ethanol production, grains go through yeast fermentation and distillation that lead to the production of ethanol and residual grain co-products called wet spent-mash. Following centrifugation and evaporation, the wet spent-mash is converted to Solubles (liquid)

and Distillers Grains (semi-dry). Then, the Solubles and Distillers Grains get blended and dried to produce DDGS (Bothast and Schlicher, 2005; Liu, 2011). In order to derive the maximum value from DDGS as co-products, they should be used as feed ingredients by the livestock industry in appreciative amounts. Over the last few years, extensive research has been conducted on the chemical and nutritive evaluation of corn and wheat DDGS and the development of nutrient availability data on DDGS for poultry and swine (Parsons et al., 1983; Slominski et al., 2008; Wang et al., 2008; Min et al., 2009). To our knowledge, what has not yet been considered in DDGS research is the fact that as co-products of brewer's yeast (*Saccharomyces cerevisiae*) fermentation, they would contain a significant quantity of yeast components that might be beneficial for gut development and health, and effective in immune system stimulation (Slominski, 2012). Based on the analysis and taking into account the mannose content (which is a unique sugar component of yeast cell walls and as such is present only in small quantities in both wheat and corn grains) the residual yeast biomass content has been determined to average 6.2 and 5.6% for wheat and corn DDGS, respectively (Slominski, 2012). This could be of importance in light of a variety of *Saccharomyces cerevisiae* yeast-based products currently being offered to the poultry industry as growth promoters and natural alternatives to antibiotics (Hooge, et al, 2003; Stanley et al., 2004; Solis de los Santos *et al.*, 2007).

2.4 Innate Immunity

Innate immunity is the non-specific part of the immune system and is the first line of defense that protects a host against invading pathogens (Janeway, 2001). Innate immunity produces a fast and strong response against infection, however, unlike adaptive immunity, it does not form an immunological memory. Therefore it does not have the ability to elicit an improved response following the second exposure to an antigen (Akira et al., 2006). Activation of innate

immunity happens through recognition of small molecules motifs called "microbe-associated molecular patterns (MAMP)" by the pattern recognition receptors (PRRs) expressed by the cells of innate immunity (Basset et al., 2003). Recognition of MAMP by PRRs triggers the activation and maturation of cells of innate immunity such as dendritic cells, and macrophages production of cytokines by these cells that links innate immunity to the adaptive immune response (Medzhitov, 2000; Janeway, 2001).

2.4. 1 Toll-like Receptors

Toll-like receptors (TLRs) are a type of PRRs which play a critical role in the innate immune response through recognition of pathogen associated molecular patterns (PAMPs) (Akira, 2001). Toll-like receptors are a type 1 transmembrane proteins consist of an extracellular domain containing leucine-rich repeats (LRRs) and the intracellular domain containing the Toll/IL-1 receptor (TIR) domain. The LRRs domains are involved in protein-protein interactions required for TLR pathogen recognition, whereas TIR domains are involved in signal transduction in signalling pathways (Takeda and Akira, 2005; Albiger et al., 2007). In broiler chickens, TLRs are mainly expressed by epithelial cells and cells of the innate immune system in tissues involved in immune function such as the spleen, bursa of Fabricius, and cecal tonsils (Kogut et al., 2005; Yilmaz et al., 2005).

2.4.2 TLRs Signaling Pathways

Upon recognition of PAMPs by LRRs in the extracellular domain of the TLRs, the activation of the TLR signaling pathways begins in the cytoplasmic TIR domains of the TLRs (Mogensen, 2009; Lim et al., 2013). Depending on the TLRs, one or more adaptor molecules, including myeloid differentiation factor 88 (MyD88), Toll/IL-1 receptor domain containing adaptor protein (TIRAP), Toll/IL-1 receptor domain containing adaptor inducing interferon-beta

(TRIF) and TRIF-related adaptor molecule (TRAM) get activated and lead to the activation of transcription factors in the downstream signal transduction pathway (Takeda and Akira, 2004; O'Neill and Bowie, 2007). Activation of transcription factors such as nuclear factor (NF)-kappaB, interferon regulatory factors (IRFs) and activator protein 1 (AP-1), would lead to the production of pro-inflammatory cytokines in response to the specific pathogens and adaptor molecules (Akira and Takeda, 2004; Takeda and Akira, 2004). Activation of TLRs signaling pathways occurs through two different pathways, the MyD88-dependent pathway (for all TLRs) and the MyD88-independent pathway (particular for TLR3 and TLR4) (Akira and Takeda, 2004).

2.4.3 TLRs and Yeast-derived products

Ten TLRs have been identified in broilers; each of them has specific ligands which are absent in the host but are present within microbial community (Fukui et al., 2001). Chicken TLR2 (Ch TLR2) appears to be more diverse yet less specific than mammalian TLR2 (Boyd et al., 2001). Chicken TLR2 can recognize peptidoglycans from gram-positive and gram-negative bacteria, and zymosan (β-1,3-glycosidic linkage) from *Saccharomyces cerevisiae* (Fukui et al., 2001; Sato et al., 2003). Recognition of ligands by TLR2 would result in the activation of the MyD88 signalling pathway and transcription factor NF-κB, and production of inflammatory cytokines (Barton and Medzhitove, 2003). Two different types of TLR2 have been identified in broiler chickens, TLR 2 type 1 (TLR2a) and TLR2 type 2 (TLR2b) which both appear to be highly homologous to human TLR2. It has been reported that TLR2b appears to be expressed mainly in the spleen, bursa, tonsils, and intestine (Iqbal et al., 2005).

Chicken TLR4 is the principal receptor for lipopolysaccaride, a major component of the outer membrane of gram-negative bacteria (Keestra and Putten, 2008). In the mammalian immune

system, TLR4 becomes functional after getting complexed with MD-2 co-receptor (Fitzgerald et al., 2004). MD-2 in the TLR4/MD-2 complex is the protein that enables TLR4 to respond to LPS (Keestra and Putten, 2008). It has been reported that human CD14 enhances the chTLR4/chMD-2-mediated response to LPS (Kim et al., 2007). However, there is no evidence regarding the existence of a functional CD14 in chickens (Keestra and Putten, 2008). In mammals, activation of TLR4/MD-2 complex occurs through two different intracellular signalling pathways, the MyD88/TIRAP pathway and the TRAM-TRIF-mediated pathway. Activation of the MyD88/TIRAP pathway results in early NF-κB activation and production of inflammatory cytokines, whereas activation of the TRAM-TRIF-mediated pathway leads to the late NF-κB activation and activation of IRF3. Stimulation of IRF3 (as a transcription factor) enhances the production of type l interferons that are involved in endotoxic shock (Keestra et al., 2008). However, broiler chickens do not develop endotoxic shock in respond to LPS and they are generally resistant to the toxic effects of LPS (Adler and DaMassa, 1979). This might be associated with the absence of TRAM in broiler chickens as an adaptor molecule that links TLR4 and TRIF and contributes to late NF-κB activation and production of type l interferons (Keestra et al., 2013).

Chicken TLR21 is expressed by various cells of innate immunity such as monocytes, dendritic cells and natural killer (NK) cells and is located within the endosomal compartments of cells. TLR21 is considered as the functional homologue to mammalian TLR9 and is involved in the recognition of synthetic and microbial unmethylated CpG sequences (Kesstra et al., 2010). Unmethylated CpG motifs are regions of DNA mainly found in bacterial DNA, and are relatively rare in vertebrate genomes (Bauer and Wagner, 2002). It has been reported that recognition of

CpG DNA by TLR21 as an innate DNA sensing receptor, results in NF-κB activation and production of inflammatory cytokines (Kesstra et al., 2010). Therefore, CpG DNA might be used in broiler diets as an immune adjuvant. In broiler chickens, the immune stimulatory effects of synthetic CpG- oligodeoxynucleotides have been proven in different studies (Brownlie et al., 2009; He et al., 2011). However, further research is required to prove the immune stimulating effects of yeast nucleotides in broiler chickens.

2.4.5 C-type Lectin Receptors and Yeast-derived Products

C-type lectin receptors are a large family of receptors that bind to carbohydrates through carbohydrate-recognition domains in a calcium dependent manner (Cambi et al., 2005; Zelensky and Gready, 2005). Monocyte mannose receptor (MMR), mannose binding lectin (MBL) and dectin-1 are examples of C-type lectins that play a critical role in antimicrobial and antifungal innate immunity (Fraser et al., 1998; Zelensky and Gready, 2005).

Monocyte mannose receptor is a type l transmembrane protein mostly expressed by dendritic cells, macrophages and epithelial cells; and it is involved in activation of innate immunity through recognition of carbohydrates on the surface of infectious agents (Linehan et al., 2002). It has been reported that MMR can selectively bind to carbohydrates such as terminal mannose, fucose and N-Acetyl-D-Galactosamine (GalNAc) as well as the carbohydrates on the outer walls of microorganisms (Zamze et al., 2002). Upon recognition of a mannose structure, MMR mediates the process of phagocytosis, endocytosis and pinocytosis of microbial pathogens through dendritic/macrophage cells, followed by presentation of the antigen complex to the lysosomal compartment where they get destroyed by lysosomal enzymes (Gazi and Martinez-Pomares, 2009). After internalization, MMR transports antigens to MHC class II-containing compartments in immature antigen presenting cells (APC) for antigen processing and

presentation to T helper cells. This would lead to the activation of T helper cells and production of cytokines (Zamze et al., 2002; Gazi and Martinez-Pomares, 2009).

Mannose-binding lectin (MBL) is a serum soluble PRR protein synthesized by hepatocytes and mostly expressed on the surface of monocytes/macrophages (Worthley et al., 2005). Mannose-binding lectin belongs to the C-type lectin super family which has a critical role in the first line of defence against invading pathogens by the host immune system and its function appears to be similar to the mannose receptor on macrophages (Seyfarth et al., 2005). It is believed that the main function of MBP is to bind to carbohydrate components of microorganisms (such as mannan, GlcNAc or glucose molecules), which results in activation of the lectin pathway of the complement system (Seyfarth et al., 2005). The mannose-binding lectin pathway is a non antibody-dependent pathway, which along with the classic complement pathway and alternative complement pathway has a pivotal role in innate immunity, and is the link between the innate immunity and acquired immunity (Takahashi and Ezekowitz, 2005).

Dectin-1 is a type ll transmembrane protein and the major β-glucans receptor in mammals that is mainly expressed on the surface of monocyte/macrophage, neutrophil and dendritic cells (Brown and Gordon, 2001; Taylor et al., 2002). Upon recognition of β-glucans, dectin-1 triggers internalization and phagocytosis of β-glucans by cells of innate immunity and activates the downstream signalling pathway through the immunoreceptor tyrosine-based activation like motif (ITAM). This would lead to the production of reactive oxygen species (ROS) and activation of the NF-κB pathway leading to production of inflammatory cytokines (Taylor et al., 2007). It has been reported that there is collaboration between dectin-1 signaling and TLR2 signaling that increases the responses triggered by each receptor against infectious agents (Gantner et al., 2003; Yadav and Schorey, 2006). In broiler chickens, there is some evidence regarding the presence of

dectin-1 or dectin-1 receptor on avian heterophils, however, the presence of functional dectin-1 in broiler chickens awaits future investigation (Nerren and Kogut, 2009).

2.5 Cytokines

Cytokines are defined as soluble proteins that are produced by a variety of cells and play a key role in cell signaling and communication (Dinarello, 2007). Binding of a cytokine to its specific receptor on the surface of the target cell, triggers intercellular signalling cascades (such as the protein kinase transduction cascade) resulting in changes in gene expression of the target cells that leads to proliferation, differentiation, and activation of the target cell (Stenger and Röllinghoff, 2001). In the immune system, cytokines are considered as the major factor for immune cell communication and they act as immunomodulating agents regulating both innate and adaptive immunity response to antigens and infectious agents (Stenger and Röllinghoff, 2001). Some cytokines such as interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), and interleukin IL-1 induce inflammation and are called proinflammatory cytokines; whereas, others such as IL-10, IL-4, and IL-13 suppress inflammatory response and are called anti-inflammatory cytokines (Opal and DePalo, 2000; Dinarello, 2000). Cytokines produced by T helper cells or $CD4^+$ T cells play a critical role in the immune system. Cytokines produced by T helper type 1 (Th1) cells such as IFNγ, and IL-12 promotes cell-mediated immunity; whereas, cytokines released by T helper type 2 (Th2) cells such as IL-4, IL-10, and IL-13 induce antibody-mediated or humoral immunity (Dong and Flavell 2000; Romagnani, 2000).

Interferon gamma is produced mainly by Th1 cells and the natural killer cells (NK) and enhances the expression of MCH II molecules on the surface of T cells and is involved in activation of macrophages (MΦ) as antigen presenting cells. Furthermore, IFN-γ induces the

differentiation of naive T cells to Th1 cells and inhibits the production of IL-4 by Th2 cells (Young and Hardy, 1995; Dinarello, 2000).

Interleukin 12 is mostly produced by phagocytic cells such as MΦ and DCs and to some degree by B cells. Interleukin 12 stimulates further production of IFN-γ by T cells and NK cells and induces differentiation of naive T cells to Th1 cells. Besides, IL-12 promotes differentiation and development of CD8+ T cells (cytolytic T cells) and thus forms a link between innate and acquired immune responses. There is a cross-regulatory effect between IL-12 and IL-4 through which IL-12 reduces the suppressing effect of IL-4 on Th1 cytokines (Trinchieri and Scott, 1994; Wolf et al., 1994).

Interleukin-6 is regarded as a pro-inflammatory cytokine that is primarily produced by monocytes and macrophages during acute inflammatory response and by T cells in chronic inflammation (Xing et al., 1998). Interleukin 6 along with transforming growth factor (TGF-β1) is involved in differentiation of naive cells to either regulatory T cells (Tregs) cells or Th17 cells. Under normal conditions and without any infection, when the level of IL-6 is low, TGF-β induces the proliferation and differentiation of naive T cells to Tregs. However, following infection and inflammation, increased IL-6 would be produced and would shift the differentiation of naive T cells to Th17. The regulatory T cells have anti-inflammatory activities and suppress activation of the immune system, while Th17 induces further inflammatory response. Therefore, IL-6 plays a key role in differentiation of naive T cells to become activators (Th17) or suppressors (Treg) of the adaptive immunity. In addition to inflammatory activities, IL-6 is involved in maturation and differentiation of B cells to antibody-producing B cells, and can works as an anti-inflammatory cytokines through inhibition of TNFα and activation of IL-10 (Dinarello, 2000; Naugler et al., 2008).

Interleukin 4 is an anti-inflammatory cytokine that induces differentiation of naive T cells to Th2 cells. The initial cellular source of IL-4 has not been convincingly identified yet, however, it has been reported that mast cell/basophils, eosinophils, NK cells, and naive CD^+ T cells have been identified as the initial sources of IL-4. Considering the cross-regulating effect between Th1 and Th2 cytokines, IL-4 suppresses the production of IFN-γ by Th1 cells and thus directs the differentiation of naive T cells to Th2 cells. Furthermore, IL-4 is involved in differentiation and proliferation of native B cells to antibody-producing plasma cells and promotes immunoglobulin class switching (Urban et al., 1991; Opal and DePalo, 2000; Noben-Trauth et al., 2000).

Interleukin 10 is an important immunoregulatory cytokine that is initially produced by monocytes, however, it is released by Tregs cells, Th2 cells, and activated cytotoxic T cells as well. As an anti-inflammatory cytokine, IL-10 can block NF-κB activation and inhibits the synthesis of pro-inflammatory cytokines such as IFN-γ, IL-2, and TNFα, and downregulates the expression of co-stimulatory molecules on macrophages. Interleukin 10 also down-regulates cell-mediated immune response by downregulation of the expression of Th1 cytokines and shifting the differentiation of the naive T cells towards Th2 cells. In spite of anti-inflammatory activities, IL-10 is considered a potent activator of B lymphocytes that promotes B cell proliferation and antibody production and induces antibody-mediated immune response (Akdis and Blaser, 2001; Opal and DePalo, 2000).

2.6 Humoral Immunity

Humoral immunity or antibody-mediated immunity is the part of the immune system which is mostly mediated by antibodies released by B cells or B lymphocytes (Shishido et al.,

2012). In the immune system, B cells are considered important antigen presenting cells (APC) because of their ability to recognize pathogens via B cell receptors (BCR) (Do et al., 2000). Furthermore, B lymphocytes express TLRs on their surface which can recognise PAMP. Recognition of antigens by the receptors would be followed by activation of B cells and production of antibody (Hua and Hou, 2013).

2.6.1 B cell Activation

Activation of B cells occurs through a T cell-dependent and T cell-independent manners (Parker, 1993). Some antigens such as soluble proteins (bovine serum albumin) require T helper cells to activate B cells; however, there are some antigens such as lipopolysaccharides, peptidoglycan, and lipoprotein that can activate B cells without T cells involvement (Endres et al., 1983; Monroe and Cambier, 1983; Snow, 1990). During T cell-dependent B cell activation, B cells as antigen presenting cells bind to the antigens through B-cell receptors (BCR), membrane immunoglobulins (Ig) with different antigen binding sites that are present on the outer surface of B cells. Following the internalization and degradation of the antigen, B cells present peptides from the antigen to the molecules on their surface called "major histocompatibility complexes (MHCs)". Finally, recognition of processed peptides on MHCs molecules by the T cell receptor (TCR) on naive T helper cells would lead to the mutual activation of B and T helper cells. Once B cells present the same antigens to the T helper cells, they release cytokines that induce B cell activation, proliferation and differentiation to antibody-plasma cells (Parker, 1993; Obukhanych and Nussenzweig, 2006; Janeway et al., 2001). Depending on antigen, the T cell-independent B cell activation happens through binding of antigens to either toll-like receptors or cross-linking a number of B-cell receptors expressed by B cells; this induces signaling

transduction cascades and leads to transcription of cytokine genes and antibodies (Dullforce et al., 1998; Vos et al., 2000; Hess et al., 2013).

2.6.2 Antibodies

Antibodies or immunoglobulins are glycoproteins produced by B lymphocytes which play a critical role in innate and adaptive immune response through neutralization, immobilization, and opsonisation of pathogenic bacteria and viruses (Janeway et al., 2001; Scott et al., 2012). The Y shaped structure of antibodies consists of four polypeptides, two heavy chains and two light chains. Each antibody chain has a constant and variable region (Morea et al., 1997; Janeway et al., 2001; Schroeder, 2010). The ends of the light and heavy chains form the variable region of antibody (or Fab portion) which serves as antigen binding site, while the constant region of the antibody (Fc portion) determines the antibody isotype. During the opsonisation process, the Fab portion of the antibody binds to the antigen and neutralizes the biological effects of the antigen while the Fc portion of the antibody binds to the Fc receptor on the surface of phagocyte cells (such as macrophages and dendritic cells), facilitating ingestion and destruction by phagocytes (Schroeder, 2010; Janeway et al., 2001; Nimmerjahn and Ravetch, 2010). Based on the constant region antibodies are divided into five major classes including IgG, IgM, IgA, IgE and IgD. These antibody isotypes perform different roles in the immune system (Morea et al., 1997; Nimmerjahn and Ravetch, 2010).

In broiler chickens, immunoglobulin Y is the functional equivalent to human IgG and is considered as the major antibody in blood circulation (Larsson et al., 1993; Sugita-Konishi et al., 1996). Immunoglobulin G has very high affinity compared to other antibody isotypes and can stay in blood circulation for a long time. In addition, IgG is an important activator of the classical complement system that consists of a cascade of immune proteins involved in pathogen

elimination (Larsson et al., 1993 Janeway et al., 2001). Furthermore, IgG plays a key role in antibody-dependent cell-mediated cytotoxicity through which IgG binds to the pathogen-infected target cells and facilitates the process of destruction and lysis by natural killer cells (Mallery et al., 2010). Immunoglobulin M is the first antibody isotype produced during B cell development (mainly by CD+B-cells or B1 cells) and plays an essential role in the innate immune response against early infection (Vollmers and Brändlein, 2002). In the immune system, IgM antibodies are the significant source of "natural antibodies" because of their high avidity which allows them to bind to a specific antigen without previous exposure (Vollmers and Brändlein, 2005). Immunoglobulin M antibodies have low affinity because they are produced before somatic hypermutation of B cells; however, because of their pentameric structure (they have 10 week binding sites for antigens), they have high avidity for antigens and are considered strong activators of the complement system (Bose, 2000; Vollmers and Brändlein, 2005). Immunoglobulin A is considered as the major antibody isotype in the mucosal immune system (Goldblum, 1990; Lamm et al., 1995). Immunoglobulin A has two isotypic forms, IgA1 and IgA2. In blood circulation IgA is mostly found as a monomer and the ratio of IgA1 to IgA2 is about 4:1, while in mucosal secretion, IgA is present as a dimer and the ratio of IgA1 to IgA2 is about 3:2 (Janeway et al., 2008; Macpherson et al., 2008). The dimeric form of IgA is called secretory IgA (sIgA) and is mostly found in mucus epithelium of the interstitial and respiratory tracts such as saliva, mucus, and tears (Goldblum, 1990). Although the monomeric form of IgA is susceptible to proteolytic enzymes, the sIgA is highly resistant to digestion and can survive the harsh gastrointestinal environment (Woof and Kerr, 2006). Unlike IgG, IgA is a week activator of complement systems and is considered as a poor opsonin; certainly because of the fact that

IgA mostly works on the epithelial surface where phagocytes and complement proteins are not generally present (Goldblum, 1990; Woof and Kerr, 2006).

2.7 Necrotic Enteritis and Yeast-derived Products

Necrotic enteritis (NE), the major economic disease of poultry industry, is an enteric disease caused by proliferation of *Clostridium perfringenes* in the small intestine (Craven et al., 2001; Immersee et al., 2004). *Clostridium perfringenes* is a gram-positive, ubiquitous anaerobic bacterium that is part of the normal intestinal microbiota in broilers (10^4 cfu/g). Nevertheless, under certain conditions such as changes in diet, or exposure to additional pathogens, it may proliferate rapidly (to 10^7-10^9 cfu/g) and cause NE (Gholamiandekhordi et al., 2006; Van Immersee et al., 2009). There is some controversy regarding the pathogenesis of NE. Although initially it was reported that α-toxin produced by *Clostridium perfringens* is the major virulence factor involved in intestinal damage, further investigations revealed that other factors such as *NetB* t toxin and proteolytic enzymes released by the bacteria are the major contributors to the pathogenesis of NE (Keyburn et al., 2008; Olkowski et al., 2008). The disease can be effectively controlled by antibacterial drug administration at prophylactic levels (Martel et al., 2001). However, the concerns over antibiotic resistant bacteria and antibiotic residues in animal products have led to the reduced use of antibacterial products in broiler diets (Bedford, 2002). It has been shown that diet composition has a great effect on the incidence of NE. Diets rich in water-soluble non-starch polysaccharides (NSPs) have been found to be associated with NE outbreaks in broiler flocks (Langhout et al., 1999; Annet et al., 2002). As a result, feeding strategies have been introduced to the broiler industry to control the occurrence of NE in the absence of antibiotics (Timbermont et al., 2010). Yeast-derived products as food additives have been known for their potential immune stimulating properties and intestinal health benefits (Gao

et al., 2008; Muthusamy et al., 2011). Therefore, application of yeast-derived products in broiler diets might be effective to control the incidence of NE associated with *Clostridium perfringens*.

CHAPTER 3: MANUSCRIPT I

Effect of yeast-derived products and distillers dried grains with solubles (DDGS) on growth performance, gut morphology, and gene expression of pattern recognition receptors and cytokines in broiler chickens

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3.1 Abstract

An experiment was carried out to investigate the effect of yeast-derived products and distillers dried grains with solubles (DDGS) on growth performance, small intestinal morphology, and innate immune response in broiler chickens from 1 to 21 d of age. A total of 315 one-day-old male broiler chickens (Ross 308) were randomly assigned to seven dietary treatments with nine replicates (cage) of 5 birds each. Dietary treatments consisted of a Control diet without antibiotic (C), and diets containing 11 mg/kg of antibiotic "virginiamycin", 0.25% of yeast cell wall (YCW), 0.2% of a commercial product "Maxi-Gen Plus" containing processed yeast and nucleotides, 0.025% of nucleotides, 0.05% of nucleotides, or a diet containing 10% of DDGS. Body weight gain, feed intake, feed conversion ratio, and mortality were recorded. On d 21, 5 birds per treatment were euthanized and approximately 5 cm long duodenum, jejunum, and ileum segments were collected for intestinal morphology measurements. For gene expression analysis, cecal tonsils and spleen were removed and samples were collected to measure the gene expression of toll-like receptors TLR2b, TLR4, and TLR21, macrophage mannose receptor (MMR), and cytokines IFN-γ, IL-12, IL-10, and IL-4. No significant difference was observed for growth performance parameters. However, diets containing 0.05% of nucleotides and YCW significantly increased (P<0.05) villus height in the jejunum of broilers. Furthermore, the number of the goblet cells per unit area in the ileum was increased (P<0.05) in diets supplemented with yeast-derived products compared to the Control. With regards to gene expression analysis in the spleen, the expression of TLR2b was downregulated in the diets supplemented with nucleotides and antibiotic compared with the control. In addition, lower expression of TLR21 and MMR was observed in the spleen of birds receiving yeast-derived products and antibiotic. However, expression of TLR4 in the spleen was upregulated in diets

supplemented with YCW and nucleotides compared to the control diet. With regards to the gene expression of cytokines, the expression of IFNγ and IL-12 was down-regulated in the spleen of birds fed diet supplemented with yeast-derived products. In addition, inclusion of diets with either YCW, Maxi-Gen Plus, or 0.05% of nucleotides down-regulated the expression of IL-10 and IL-4 in the cecal tonsils. In conclusion, downregulation of receptors and cytokines in the spleen and cecal tonsils of birds fed diets supplemented with yeast-derived products suggests that yeast products do not exert immune stimulating effects in the absence of pathogen challenge.

Key Words: Yeast-derived products, gut histomorphology, pattern recognition receptors, cytokines, broilers

3.2 Introduction

In the poultry industry, antibiotics have been widely used as growth promoters to protect the birds from the adverse effects of pathogenic and non-pathogenic enteric microorganisms and to improve growth performance and production (Dibner and Richards, 2005; Engberg et al, 2000). However, repeated use of antibiotics in poultry diets can result in problems such as pathogens resistant to antibiotics, as well as increased concerns in consumers for antibiotic residues in poultry products (Capita and Alonso-Calleja, 2011; McEwen et al., 2002; Bedford, 2000). In order to avoid such problems and maintain the health and the efficient production of broilers under commercial conditions, several alternatives have been proposed to the broiler industry by feed manufacturers and research communities. Among those, yeast-derived products have received considerable attention because of their growth promoting and immunomodulatory effects (Huyghebaert et al., 2011; Yang et al., 2009). Yeast-derived products have been used in the broiler industry for years in the form of yeast by-products from breweries and distilleries, or

live yeast commercially produced for animal feed (Gao et al., 2009). Although the composition of the yeast-derived components are variable, they are essentially rich sources of ß1,3-1,6-glucans, mannan polysaccharides and nucleotides (Lipke and Ovalle, 1998). It has been suggested that the beneficial effects of yeast-derived products are due to development of the intestinal mucosa, improvement of gut health, and their immunomodulatory properties (Savage et al., 1996; Reisinger et al., 2012).

It has also been demonstrated that distillers dried grains with solubles (DDGS) as co-products of brewer's yeast (*Saccharomyces cerevisiae)* fermentation contains a significant quantity of yeast biomass (approximately 6%) which can be potentially beneficial for growth performance and gut development of broilers (Slominski, 2012). It has been reported that supplementation of broiler diets with yeast-derived products improved body weight gain (BWG) and feed conversion ratio (FCR) by establishing beneficial microflora in the gastrointestinal tract (GI), and facilitating gut development (Yang et al., 2007; Santin et al., 2001). Baurhoo et al. (2007) showed that inclusion of yeast-derived products in broiler diets increased villus height and enhanced goblet cell numbers. Furthermore, it has been found that yeast-derived products are able to modulate the innate immune response of broiler chickens. The innate immune system, known as the non-specific immune system, depends mainly on the recognition of conserved microbial structures called pathogen associated molecular patterns (PAMPs), by the pattern recognition receptors (PRRs) expressed by a variety of cells of the innate immune system (Kawai and Akira, 2010; Abbas et al., 2012) . Modulation of PRRs (including toll-like receptors, and C-type lectins) by yeast-derived products as PAMP would be followed by presentation of antigens to T- and B-lymphocytes, and production of cytokines which induce further immune responses (Stenger and Rollinghoff, 2001). Some studies have confirmed that yeast products are able to

stimulate the innate immune response of broilers through modulation of the gene expression of PRRs and cytokines (Yitbarek et al., 2013). However, others reports indicated that yeast products failed to elicit innate immunity associated gene expression of broilers under unchallenged conditions (Cox et al., 2010; Munyaka et al., 2012).

In broiler studies, inconsistent results have been reported about the effect of yeast products on performance, gut morphology and immune response of broiler chickens. A number of studies have confirmed the effects of yeast products in development of gut cells, suppression of pathogenic bacteria, and modulation of the immune system (Ghosh et al., 2012; Thanissery et al., 2010; Gao et al., 2009; Zhang et al., 2008). However these effects were not reported by other researchers (Deng et al., 2005; Cox et al., 2010; Jung and Batal, 2012). Therefore, the objective of the present study was to evaluate the effects of different yeast-derived products on growth performance, histo-morphology of the small intestine, and local and systemic innate immunity associated gene expression of broiler chickens under an unchallenged condition.

3.3 Materials and Methods

Yeast-derived Products Used in the Study

Yeast-derived products used in the present study contained different levels of potentially active components including mannan polysaccharides, β1,3 and β1,6-glucans and nucleotides. As illustrated in Tables 3.1, 3.2, and 3.3, distinct differences in cell wall polysaccharides, nucleotides, and carbohydrate contents exist among the products, which should assist in identification of the active yeast components involved in innate immunity and gut health modulation and data interpretation when these products are used along with wheat/corn (1:1 wt/wt) DDGS in broiler studies. The analysis of the Brewer's yeast showed that it contained

0.32% of nucleotides and 18.9% of cell wall polysaccharides (CWP) with component sugars mannose and glucose accounting for 99.0%. Such amounts would reflect the composition of yeast biomass present in DDGS. Other products investigated included a yeast cell wall product containing 43.3% of CWP with mannose and glucose accounting for 99% and a commercial product "Maxi-Gen Plus" which contained 21.6% of CWP and 1.13% of nucleotides. The nucleotide product contained 9.3% of nucleotides and very little CWP. It contained, however, 6.8% of mannose derived from yeast cell walls with very little glucose, indicating the presence of free mannans but not β 1,3 and 1,6-glucans.

Experimental Design and Diets

This experiment was conducted in electrically heated batteries under a protocol approved by the University of Manitoba Animal Care Protocol Management and Review Committee. All animal procedures were conducted according to the guidelines of the Canadian Council of Animal Care (1993). A total of 315 one-day-old male broiler chickens (Ross-308) were weighed and randomly assigned to 7 dietary treatments with 9 replicate cages of 5 birds each. Birds were fed ad libitum, and feed intake and body weight were measured on d 21. Mortality was recorded on a daily basis; and BWG, feed consumption, and FCR were calculated. Dietary treatments consisted of a Control diet without antibiotic (C), and diets containing 11 mg/kg of antibiotic "virginiamycin", 0.25% of yeast cell wall (YCW), 0.2% of a commercial product "Maxi-Gen Plus" containing processed yeast and nucleotides, 0.025% of nucleotides, 0.05% of nucleotides, or a diet containing 10% of DDGS. Basal diets were formulated to meet the requirements of broiler chickens as recommended by NRC (1994). The experimental diets were formulated to contain 3,050 kcal of ME/kg and 23% of CP. In the diet supplemented with DDGS, 10% of

wheat-corn DDGS replaced wheat and barley in the basal diet and was formulated with the same energy and CP content (Table 3.4).

Relative Weight of Immune Organs and Tissue Sampling for Gene Expression Analysis

On d 21, 5 birds per treatments were euthanized by cervical dislocation and the bursa of Fabricius and spleen were removed and weighed and their relative weights were expressed as a percentage of live body weight. Samples from spleen and cecal tonsils were rinsed in saline, snap frozen, and stored at -80 °C for gene expression analysis.

Gut Histomorphology Analysis

Gut histomorphology was performed as previously described (Baurhoo et al., 2011). Briefly, five-centimeter sections of duodenum (from the pylorus to the distal point of entry of the bile ducts), jejunum (from entry of the bile ducts to Meckel's diverticulum) and ileum (from Meckel's diverticulum to the ileocecal junction) were collected and gently rinsed with saline solution to remove the intestinal content. The tissue samples were fixed in 10 % formalin for 18h. The samples were then rinsed 3 times with deionized water and placed in 70 % ethanol. Each sample then was cut into five 5-mm sections and embedded in paraffin. Embedded samples were cut at 5 μm, mounted onto slides, and stained with Alcian blue (pH 2.5). The stained samples were used to measure the villus height and crypt depth. The goblet cells density as the number of the cells per mm^2 was measured in the ileum. Villus height was measured from the tip of the villus to the top of the crypt, and crypt depth was measured from the villus-crypt axis to the base of the specific crypt. Stained slides were examined with an Axiplan-2 optical microscope (Carl Zeiss Jena GmbH, Oberkochen, Germany) coupled with an QImaging Retiga 4000R digital camera (QImaging, Surrey, BC, Canada) with a charge-coupled device detector.

The images were analyzed using image software of MetaMorp Imaging System (Molecular Devices Ltd., Sunnyvale, CA).

Total RNA Extraction and Reverse Transcription

Total RNA was extracted from individual spleen and cecal tonsil samples using the Trizol extraction method as described by the Trizol manufacturer (Invitrogen Canada Inc., Burlington, ON, Canada). The quantity and purity of the RNA samples was measured by using NanoDrop spectroscopy (Thermo Scientific) with the ratio of absorbance at 260 nm and 280 nm. In this procedure, a ratio of ~2.0 is generally accepted as "pure' for RNA, and the ratio lower than 2.0 (i.e., ~1.59 and under) may indicate the presence of phenol, protein and other contaminants. Reverse-Transcription was performed by using a High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Mississauga, ON, Canada) according to the manufacturer's protocol.

Quantitative Real-time PCR

Primer sequences for β-Actin, TLR2 type 2 (henceforth known as TLR2b), TLR4, TLR21, macrophage mannose receptor (MMR), interleukin (IL)-12p35, IL10, IL-4, and interferon gamma (IFNγ), were designed using Genebank database sequences from the National Center for Biotechnology Information (Bethesda, MD) corresponding to each quantified gene (Table 3.5). Quantitative real-time (qRT) PCR was performed using the Step One Thermo Cycler (Applied Biosystems) on a 48-well plate with 25 µL of total reaction volume as described by Pfaffl and Hageleit (2001). The iTaq Universal SYBR Green Supermix was used as the QRT-PCR master mix and each reaction was run in duplicate. The PCR cycling protocol included an initial denaturation step at 95°C, followed by amplification for 40 cycles at 95°C for 10 s, an

annealing step at a temperature described in Table 3.5 for each of the primer pairs, and extension at 72°C for 10 s.

Statistical Analysis

Growth performance and gut histomorphology parameters were analyzed according to a completely randomized design by ANOVA using the GLM procedure of SAS (SAS Institute, 2003). The significance of differences between means was determined using Scheffe's procedure. The effect of diets on mortality was compared using Chi-square test. For the gene expression analysis, the cycle threshold values were recorded and uploaded to the REST-2009 Software (Qiagen, Valencia, CA). Relative expression levels of all genes were calculated relative to a housekeeping gene (β-actin in the present study), and gene expression was presented as fold changes relative to the control diet. Gene expression fold changes, standard error, and statistical significance were calculated by the software based on the formula developed by Pfaffl et al. (2002). A *P*-value of less than 0.05 was considered as significant.

3.4 Results

Growth Performance and Gut Morphology

Results for feed intake (FI), body weight gain (BWG), feed conversion ratio (FCR), mortality rate, and relative weight of immune organs (i.e., bursa and spleen) are summarized in Table 3.6. There were no significant differences in FI and BWG gain among the dietary treatments. However, FCR was lower for birds receiving diets supplemented with antibiotic or Maxi-Gen Plus compared with birds fed the diet containing DDGS ($P < 0.01$). Mortality rate and relative weight of bursa and spleen (Table 3.7) were not affected by dietary treatments ($P > 0.05$). Histomorphology measurements, including villus height, crypt depth, and villus height to

crypt ratio in the all three section of intestine, and the number of the goblet cells per mm^2 in the ileum are presented in Table 3.7. The diets supplemented with YCW and 0.05% of nucleotides both significantly increased villus height in the jejunum of broilers compared to the Control diet; and inclusion of 0.05% of nucleotides increased crypt depth in the jejunum ($P < 0.05$) relative to the Control diet. Furthermore, supplementation of diets with yeast-derived products increased the number of goblet cells in the ileum relative to the Control ($P < 0.05$).

Cecal Tonsils and Spleen Gene Expression

The fold change gene expression of pattern recognition receptors relative to the expression in the Control diet, acquired from QRT-PCR are presented in Fig. 3.1. Expression of TLR2 in cecal tonsils was not affected by dietary treatments; however, the diet supplemented with antibiotic and nucleotides at two levels (i.e., 0.025 and 0.05%) down-regulated the expression of TLR2 in spleen ($P < 0.05$). Inclusion of 0.05% of nucleotides into the diet, upregulated the expression of TLR4 in cecal tonsils and the spleen. Birds fed diets containing YCW and 0.025% of nucleotides showed upregulation of TLR4 in the spleen. Expression of TLR21 and MMR in cecal tonsils was not affected by dietary treatments. However, diets containing YCW, Maxi-Gen Plus and nucleotides (i.e., 0.025 and 0.05%) down-regulated the expression of both TLR21 and MMR in spleen. In addition, the expression of MMR in spleen was downregulated in the YCW group.

The results of cytokine gene expression by QRT-PCR analysis are presented in Fig. 3.2. In cecal tonsils, no significant difference was observed in IFNγ expression; however, all dietary treatments except DDGS diet downregulated the expression of IFNγ in the spleen. Expression of IL-12 was downregulated by diet supplemented with YCW, Maxi-Gen Plus and nucleotides (i.e.,

0.025 and 0.05%) in both spleen and cecal tonsils, and broiler chicken fed antibiotic-containing diet showed downregulation of IL-12 expression in cecal tonsils. Diets supplemented with Maxi-Gen Plus and nucleotides (i.e., 0.025 and 0.05%) down-regulated the expression of IL-10 in both spleen and cecal tonsils. Birds that consumed diets containing YCW and Maxi-Gen Plus showed downregulation of the IL-10 in spleen. Supplementation of diets with 0.05% of nucleotides, YCW, and Maxi-Gen Plus downregulated the expression of IL-4 in cecal tonsils; and diets containing nucleotides (i.e., 0.025 and 0.05%), Maxi-Gen Plus, and antibiotic downregulated the expression of IL-4 in spleen.

3.5 Discussion

In the current study, overall performance and mortality rate of broilers chickens were not affected by dietary treatments. Various results have been reported regarding the effects of yeast or yeast-derived products on the performance and mortality in broiler chickens. Some researchers have found that inclusion of yeast-derived products in the diet improved growth performance of broiler chickens (Ghosh et al., 2012; Muthusamy et al., 2011). On the other hand, other studies demonstrated no significant difference in growth performance parameters (Munyaka et al., 2012; Cox et al., 2010a). The inconsistent results found in different studies could be due to differences in the source and concentration of the yeast products, duration of the trial, or presence and type of challenge used. In the current study, the lack of response of broilers to the antibiotic growth promoter and yeast-derived products might be related to the highly sanitized research facility environment. Experimental conditions employed in this study may not be comparable with rearing conditions in the poultry industry, where chickens are raised on the floor pens, have access to their excreta and may be under stress by different factors such as

overcrowding, chilling and overheating. It has been reported that under commercial farm conditions, supplementation of diets with antibiotic and yeast-derived products can be more effective (Miles and Bootwalla, 1991). Under challenge conditions, yeast-derived products might be able to stabilize and protect the intestinal tract from colonization by harmful organisms, and improve the bird's defence mechanism (Cruickshank, 2002).

With regards to morphology analysis, diets containing 0.05% of nucleotides increased villus height and crypt depth in the jejunum of broiler chickens. It is assumed that an increased villus height is associated with greater digestion and absorption of nutrients due to increased surface area of the villus (Gao et al., 2008). The villus crypt is considered as the villus factory and deeper crypts indicate fast tissue turnover that permit renewal of the villus in response to normal sloughing or inflammations resulting from pathogens or their toxins (Yason et al., 1987). Higher villus height and deeper crypt depth observed in the jejunum of birds receiving diets containing nucleotides (i.e., 0.025 and 0.05%) could be because of the role the nucleotides play in the development and proliferation of tissues with a rapid cell turnover such as that in the intestine and bone marrow (Grimble, 1994; Maldonado, et al., 2001). Under conditions of rapid growth, the *de novo* purine nucleotide synthesis may not be sufficient to support an increased need for nucleic acids. Therefore, under these conditions, dietary nucleotides may serve as precursors of DNA and RNA in rapidly growing tissues via the salvage pathway thus sparing the organism from the cost of their de novo synthesis (Carver, 1999). The diet supplemented with YCW increased villus height in the jejunum of broiler chickens where maximum absorption of nutrient takes place. These results are in agreement with Bradley et al. (1994), Savage et al. (1997), and Shane (2001) who demonstrated that YCW can increase the villus height in the intestine of broiler chicken. The increase in villus height observed in the jejunum of birds

receiving YCW could be related to the effects of mannans and glucans in the digestive tract of these animals. It has been reported that mannans and β-glucans present in the yeast cell wall are capable of binding and blocking enteropathogens and thus improving nutrient utilization by stimulation of specific microbial communities in the gastrointestinal tract (Guo et al., 2003; Yang et al., 2008).

In the ileum, diets supplemented with yeast-derived products increased the number of goblet cells compared to the control. Goblet cells are known as special epithelial cells which are involved in intestinal mucus production (Specian and Oliver, 1991; Uni et al., 2003). Mucus, a viscous secretion produced by goblet cells, can protect underlying epithelial cells by trapping and neutralizing bacteria by providing several potential attachment sites and colonization niches (Singh et al., 2002; Sheehan et al., 2007). Considering the role of mucin as a first line of defence against invading pathogens in the intestine, higher numbers of goblet cells observed in diets supplemented with yeast-derived products can be an advantage for the birds as it will lead to a greater removal of intestinal pathogens and improvement of gut health. In agreement with our results, Baurhoo et al. (2007, 2009) also showed that supplementation of a diet with "mannanoligosaccharide" increased the goblet cell numbers in broilers. The mechanism by which yeast product increases the number of goblet cells is still unknown. However, it has been reported that mucin genes can be regulated at the transcriptional level by growth factors, bacterial products and cytokines (Temann et al., 1997; Dohrman et al., 1998; Takeyama et al., 1999).

The innate immune system is known as the first line of defence that protects the host against invading pathogens in a non-specific manner (Turvey and Broide, 2010). Toll-like receptors (TLRs), a group of membrane receptors expressed by innate immunity cells, play a key role in

activation of the innate immune system through recognition of conserved microbial-associated molecular patterns (MAMP) (Ramasamy et al., 2010). In addition to TLRs, macrophage mannose receptor (MMR), a PRRs belonging to the C-type lectin family, plays a critical role in modulation of the innate immune system through recognition of mannose structures on the surface of potentially pathogenic bacteria and fungi (Stahl and Ezekowitz, 1998). In the current study, the mRNA expression of TLR2, TLR21 and MMR in the cecal tonsils of broiler chickens was not influenced by dietary treatments. However, diets containing antibiotic and nucleotides (i.e., 0.025 and 0.05%) down-regulated the expression of TLR2, TLR21 and MMR in the spleen of broiler chickens relative to the Control. Furthermore, the expression of TLR21and MMR was downregulated in the spleen of birds receiving antibiotic and all yeast-derived products. In agreement with our results Munyaka et al. (2012) reported that supplementation of diets with yeast products down-regulated the expression of PRRs in the Ileum of broiler chickens. In contrast, Yitbarek et al. (2013) showed that inclusion of yeast products in broiler diets upregulated the expression of TLR2 and TLR21 in cecal tonsils. In addition, Cheled-Shoval et al., (2011) demonstrated that administration of "mannan oligosaccharide" to chicken embryos 3 days before hatch increased the intestinal TLR2 and TLR4 gene expression. Considering the strong antibacterial effects of virginiamycin on gram-positive bacteria, downregulation of PRRs in the spleen of birds receiving antibiotic might be related to the decreased amount of gram-positive bacteria such as *Clostridium, Streptococcus, and Enterococcus* in the intestine of these birds. The downregulation of TLR2, TLR21, and MMR in spleens of birds fed diets containing yeast-derived product might be related to the higher number of goblet cells in the intestine of these birds. It has been reported that commensal bacteria play an essential role in activation and development of the immune system (Jiang et al., 2004; Rakoff-Nahoum et al., 2004; Kelly et al.,

2005). Regarding the role of mucus in trapping and neutralizing bacteria (by providing several potential attachment sites) and given the fact that the number of goblet cells is an indicator of mucus production, downregulation of PRRs (TLR2, TLR21, and MMR) in birds fed yeast-derived products might be associated with the high number of the goblet cells in the intestine of these birds (Uni et al., 2003). On the other hand, the expression of TLR4 as a receptor which detects lipopolysaccharide from Gram-negative bacteria was upregulated in the spleen of birds receiving diets supplemented with YCW and nucleotides. This might be explained by the fact that lysozyme as an antibacterial enzyme that is abundant in mucus, has been found to be more effective against Gram-positive bacteria because of their greater accessibility for breaking specific chemical bonds present in the peptidoglycan (Janeway et al., 2001). However, further research may be required to clarify the possible effect of goblet cells and mucus production on the intestinal gene expression of PRRs in broiler chickens.

In addition to PRRs, cytokines play a critical role in regulation of innate immunity. Cytokines are known as small cell-signalling protein molecules that are secreted by numerous cells of the immune system and used for inter-cell communication (Belardelli et al., 1995; Van der Meide and Schellekens, 1996). In the current study we measured the expression of T helper type 1 and T helper type 2 cytokines in spleen and cecal tonsils. Interferon gamma (IFNγ) and IL-12 are produced by T helper type 1 cells and are considered as inflammatory cytokines; conversely IL-4 and IL-10 are produced mostly by T helper type 2 cells and are considered as anti-inflammatory cytokines (Rosenzweig and Holland, 2005; Fietta and Delsante, 2008). Diets supplemented with antibiotic or yeast products downregulated the expression of IFNγ and IL-12 in spleen. Interferon gamma plays a critical role in activation of macrophages and is generally involved in differentiation of naive T cells to Th1 cell (Schoenborn and Wilson, 2007); and IL-12 stimulates

the production of IFN-γ and reduces IL-4 mediated suppression of IFN-γ (Hsieh et al., 1993). Downregulation of inflammatory cytokines in the spleen of birds fed antibiotic and yeast-derived products might be explained by the reduced expression of PRRs (TLR2, TLR21, and MMR) in the spleen of these birds, and suggests that in the absence of an immune challenge yeast products have anti-inflammatory effects. The lack of immune activation in birds fed yeast products might be due to the stage of the development of the immune system. It has been reported that 2 to 3 week old broiler chickens that have already lost the protection from maternal antibodies and are not fully immunocompetent (cannot develop strong immune response) are more vulnerable towards infections (Mc Corkle and Glick, 1980; Pitcovski et al., 1987). Therefore, during 2-3 weeks post-hatching when the immune system is still under development, yeast products might be more effective in immune system activation. However, after microflora establishment and maturation of the immune system these products may not be as effective as they are at the early stages of growth (Cox, et al. 2010a). In addition to the suppression of inflammatory cytokines, diets supplemented with antibiotic and yeast-derived products down-regulated the expression of IL-4 and IL-10 in the spleen. Interleukin-4 is an anti-inflammatory mediator which induces the differentiation of naive $CD4^+$ T cells to effector Th2 cells and inhibits the production of pro-inflammatory cytokines such as IFNγ and IL-12 (Martinez et al., 2009; Luzina et al., 2012); and IL-10 is considered as the immunoregulatory and anti-inflammatory cytokine mostly produced by monocytes, Th2 and Treg cells that regulate the differentiation and proliferation of immune cells. IL-10 limits the inflammatory response through a different process such as down-regulation of the expression of Th1 cytokines (INF-γ) and co-stimulatory molecules on macrophages (Mocellin et al., 2005; O'Garra and Vieira, 2007; Ng et al., 2013). Downregulation of both inflammatory and anti-inflammatory cytokines in the spleen of birds

receiving yeast products may be explained by the balance and cross-regulatory effect between Th1 and Th2 cytokines (Kidd, 2003). Following immune system activation, IL-12 stimulates the production of IFN-γ and reduces IL-4 mediated suppression on IFN-γ (Trinchieri and Scott, 1994; Wolf et al., 1994). On the contrary, IL-4 as an anti-inflammatory mediator, downregulates the expression of Th1 cytokines such as IFN-γ and IL-12 (King et al., 1993). These results are in agreement with Cox et al. (2010a) and Munyaka et al. (2012) who demonstrated that supplementation of diets with yeast products downregulated the expression of Th1 and Th2 cytokines in the absence of immune challenge. However, Yitbarek et al. (2013) demonstrated that inclusion of yeast-derived macromolecules in broiler diets upregulated the expression inflammatory and anti-inflammatory cytokines. The inconsistent and conflicting results reported in different studies suggests that yeast products may exert different immunomodulatory effects under different experimental conditions.

In conclusion, diets with various yeast-derived products did not affect growth performance of broilers. Furthermore, diets supplemented with nucleotides improved gut development by increasing villus height and the density of the goblet cells in the ileum. Regarding the local innate immunity, gene expression of the PRRs and cytokines was downregulated in the spleen and cecal tonsils of birds receiving yeast-derived products. This may indicate the lack of immune stimulating effects of these products in the absence of an immune challenge. However, further research is needed to determine whether the lack of immune activation observed in this study is because of the yeast products or the absence of immune system challenge.

Table 3.1. Chemical composition of yeast-based products (%, as-is basis)

Sample	Sample description	Cell wall polysaccharides (CWP)	Protein (N x 6.25)	Nucleotides[1]	Carbohydrates[2]
Brewer's Yeast	*Saccharomyces cerevisiae*	18.9	35.3	0.32	19.8
Wheat/corn DDGS	Contain 6% of yeast biomass	21.8-24.2	32.0-37.4	0.13[3]	4.1
Nucleotide	Nucleotide-rich product	7.2	52.0	9.32	5.4
Yeast Cell Wall	CWP-rich product	43.3	17.2	0.33	9.4
Maxi-Gen Plus	CWP/nucleotide-rich product	21.6	32.7	1.13	14.3

[1]Includes CMP, AMP, UMP, GMP, and IMP; [2] Other than the call wall polysaccharides; [3] Corn DDGS: 0.12%, wheat/corn DDGS: 0.13%.

Table 3.2. Cell wall polysaccharide component sugar content (mg/g, as is basis)

Sample	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acids	Total
Brewer's Yeast	1.5	0.0	102.1	0.0	84.7	0.4	188.6
Wheat/corn DDGS	52.6	84.9	16.1	11.4	65.6	11.9	242.4
Nucleotide[1]	0.5	0.0	67.7	0.0	3.1	0.5	71.8
Yeast Cell Wall	0.9	0.0	229.9	0.0	200.4	1.9	433.2
Maxi-Gen Plus	21.8	33.2	65.4	7.3	81.7	6.5	215.8

[1]Note high mannose and low glucose content of nucleotide-rich product.

Table 3.3. Oligosaccharide component sugar and simple sugar content (mg/g, as is basis)

	Oligosaccharides (80% EtOH solubles)				Simple (free) sugars			
Sample	Arabinose	Mannose	Glucose	Total	Arabinose	Mannose	Glucose	Total
Brewer's Yeast	3.7	nd	161.7	165.4	nd	nd	33.0	33.0
Wheat/corn DDGS	2.5	nd	10.4	18.2[2]	2.2	nd	20.5	22.7
Nucleotide	7.9	2.9	36.0	46.8	nd	nd	7.6	7.6
Yeast Cell Wall	2.2	nd	25.2	27.4	nd	nd	66.7	66.7
Maxi-Gen Plus	4.2	2.0	51.6	57.8	nd	nd	85.1	85.1

[nd] Not detected; [2] Includes 5.3 mg/g of xylose

Table 3.4. Composition and calculated analysis of the basal diet and the diet containing 10% of distillers dried grains soluble (DDGS) (%)

Item/Ingredient	Basal diet	DDGS diet
Wheat	43.0	40.3
Soybean meal	26.0	20.0
DDGS	0.0	10.0
Barley	13.1	13.8
Fish meal	5.0	5.0
Canola oil	6.0	5.5
Limestone[1]	0.81	1.0
Dicalcium phosphate[2]	1.10	0.9
DL-Methionine	0.1	0.1
L-Lysine-HCL	0.0	0.2
Mineral premix[3]	0.5	0.5
Vitamin premix[4]	1.0	1.0
Chromic oxide	3	3
Total	100	100
Calculated analysis		
ME, kcal/kg	3,055	3,055
CP, %	22.0	22.0
Calcium, %	1.02	1.00
Available phosphorous, %	0.45	0.45
Lysine, %	1.11	1.12
Methionine, %	0.54	0.54
Methionine + cystine, %	0.90	0.90
Threonine, %	0.80	0.80

[1]Contained 38% calcium.
[2]Contained 21% calcium and 18% phosphorous.
[3]Mineral premix provided per kilogram of diet: Mn, 70 mg; Cu 10 mg; Fe, 80 mg, Zn, 80 mg; Se, 0.3 mg; I, 0,5 mg; and Na, 1.7 g.
[4]Vitamin premix provided per kilogram of diet: A, 8,255 IU; vitamin D_3, 3000 IU; vitamin E, 30.0 IU; vitamin B_{12}, 0.013 mg; vitamin K, 2.0 mg; niacin, 24.5; choline, 1081 mg; folic acid 4.0 mg; biotin, 0.25 mg; riboflavin, 6.0 mg

Table 3.5. Primer sequences used for real-time quantitative PCR[1]

Gene[2]	Primer sequence (5'-3')[3]	Fragment size, (bp)	Annealing temperature, (°C)	GenBank accession number
TLR2b	F: CGCTTAGGAGAGACAATCTGTGAA R:GCCTGTTTTAGGGATTTCAGAGAATTT	90	59	NM204278
TLR4	F: AGTCTGAAATTGCTGAGCTCAAAT R: GCGACGTTAAGCCATGGAAG	190	55	AY064697
TLR21	F: TGGCGGCGGGAGGAAAAGTG R: CACCGTGCTCCAGCTCAGGC	106	59	NM_001030558
MMR	F: GCAGGGCACGTTCAGGTGGG R: GCCACACAGCCTGGCTCCCT	90	60	XM001235105
IFN-γ	F: CTGAAGAACTGGACAGAGAG R: CACCAGCTTCTGTAAGATGC	264	60	X99774
IL-12p35	F: CTGAAGGTGCAGAAGCAGAG R: CCAGCTCTGCCTTGTAGGTT	217	64	NM213588
IL-10	F: AGCAGATCAAGGAGACGTTC R: ATCAGCAGGTACTCCTCGAT	103	55	AJ621614
IL-4	F: TGTGCCCACGCTGTGCTTACA R: CTTGTGGCAGTGCTGGCTCTCC	193	57	GU119892
β-Actin	F: CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	205	61	X00182

[1]The listed oligonucleotides were used to analyze gene expression via real-time quantitative PCR.

[2]TLR = Toll-like receptor; MMR = monocyte/macrophage mannose receptor; IFN = interferon; IL=interleukin.

[3]F = forward; R = reverse.

Table 3.6. Effects of diets containing yeast-derived products and distillers dried grains with solubles (DDGS) on growth performance of broiler chickens from 1 to 21 d of age

	Treatment[1]								
Item	Control	Antibiotic	Yeast Cell Wall	Maxi-Gen Plus	Nucleotides (0.025%)	Nucleotides (0.05%)	DDGS	SEM	*P*-value
Feed intake (g/bird)	1180	1139	1175	1155	1200	1184	1197	6.67	0.15
Body weight gain (g/bird)	942.4	921.6	945.9	937.5	953.9	943.9	928.1	4.92	0.65
Feed conversion ratio	1.25[ab]	1.23[b]	1.24[ab]	1.23 [b]	1.25[ab]	1.25[ab]	1.29[a]	0.004	0.01
Mortality (%)	2.2	4.4	4.4	4.4	4.3	4.4	4.4	.	0.99

[a,b] Means within a column with no common superscripts differ significantly (P< 0.05).

[1]Experimental diets included: Control (C) = with no antibiotic; Antibiotic = C + 11mg/kg of virginiamycin; Yeast Cell Wall = C + 0.25% of yeast cell wall polysaccharides; Maxi-Gen Plus = C + 0.2 % of a commercial product containing processed yeast and nucleotides; Nucleotides (0.025%) = C + 0.025% of nucleotide-rich products; Nucleotides (0.05%) = C + 0.05% of a nucleotide-rich products; DDGS = 10% of wheat/corn distillers dried grains with solubles.

[2]Chi-squared analysis was used for significant differences in mortality

Table 3.7. Effects of dietary treatments on gut histomorphology and relative weigh of immune organs

Item	Treatment[1]								
	Control	Antibiotic	Yeast Cell Wall	Maxi-Gen Plus	Nucleotides (0.025%)	Nucleotides (0.05%)	DDGS	SEM	*P*-value
Duodenum									
Villus height, mm	2.53	2.47	2.66	2.5	2.68	2.65	2.51	0.037	0.67
Crypt dept, mm	0.16	0.17	0.19	0.18	0.18	0.17	0.20	0.005	0.55
Ratio, mm	15.68	14.41	13.85	13.68	15.18	15.07	12.58	0.36	0.38
Jejunum									
Villus height, mm	1.16^{b}	1.25^{ab}	1.51^{a}	1.44^{ab}	1.35^{ab}	1.67^{a}	1.32^{ab}	0.038	0.0001
Crypt dept, mm	0.12^{a}	0.18^{ab}	0.16^{ab}	0.17^{ab}	0.16^{ab}	0.19^{b}	0.15^{ab}	0.005	0.009
Ratio, mm	9.3	7.02	9.66	8.25	8.3	8.74	8.5	0.25	0.18
Ileum									
Villus height, mm	1.12	1.08	0.99	1.05	1	1.07	0.96	0.027	0.76
Crypt dept, mm	0.21	0.2	0.2	0.24	0.22	0.25	0.18	0.008	0.2
Ratio, mm	5.3	5.59	4.83	4.45	4.55	4.42	5.23	0.162	0.33
Goblet cells/mm^2	1110^{b}	1314^{ab}	1507^{a}	1527^{a}	1409^{a}	1521^{a}	1215^{ab}	60.25	0.03
Immune organ weight									
Spleen*	0.27	0.27	0.3	0.21	0.24	0.21	0.24	0.009	0.12
Bursa of Fabricius*	0.09	0.08	0.12	0.09	0.1	0.09	0.1	0.003	0.27

[a,b] Means within a column with no common superscripts differ significantly (P< 0.05).

[1]Experimental diets included: Control (C) = with no antibiotic; Antibiotic = C + 11mg/kg of virginiamycin; Yeast Cell Wall = C + 0.25% of yeast cell wall polysaccharides; Maxi-Gen Plus = C + 0.2 % of a commercial product containing processed yeast and nucleotides; Nucleotides (0.025%) = C + 0.025% of nucleotide-rich products; Nucleotides (0.05%) = C + 0.05% of a nucleotide-rich products; DDGS = 10% of wheat/corn distillers dried grains with solubles.

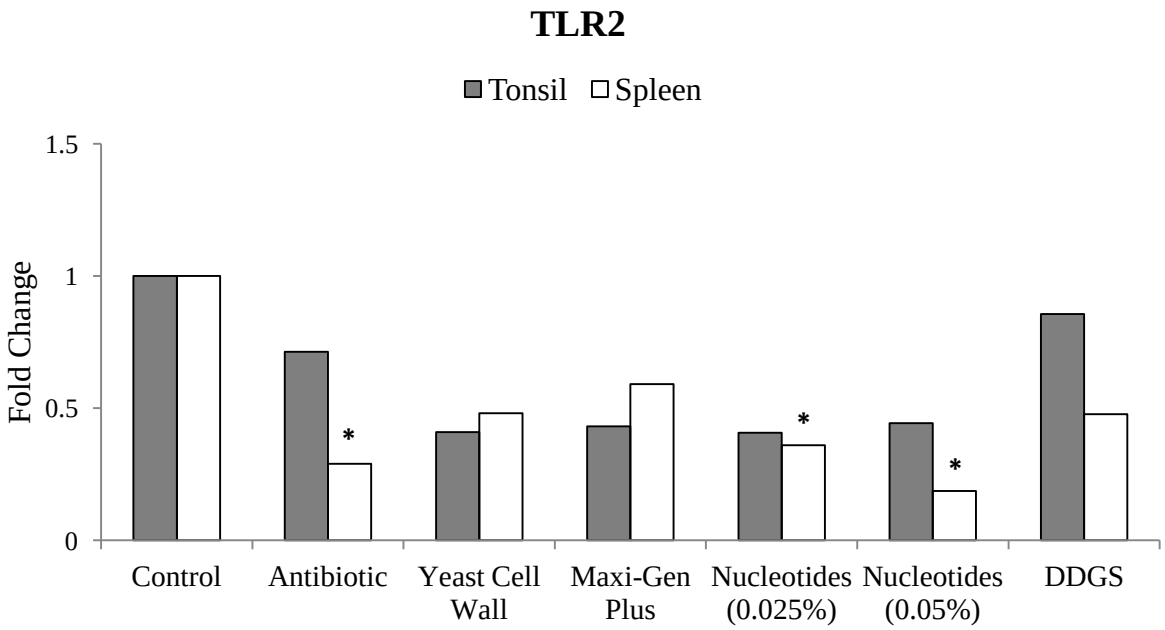
* Represents percentage organ weight relative to body weight.

3.6 Figure Legend

Figure 3.1 Fold change expression of Toll-like receptors TLR2b, TLR4, TLR21, and macrophage mannose receptor (MMR) in the cecal tonsil and spleen of broiler chickens fed the following diets: Control (C) = control diet with no antibiotic; Antibiotic = C + 11mg/kg of virginiamycin; Yeast Cell Wall = C + 0.25% of yeast cell wall polysaccharides; Maxi-Gen Plus = C + 0.2% of a commercial product containing processed yeast and nucleotides; Nucleotides (0.025%) = C + 0.025% of nucleotides; Nucleotides (0.05%) = C + 0.05% of nucleotides; DDGS = 10% of DDGS. *Bars with an asterisk differ significantly from the control group. Results were considered statistically significant from the control group if $P < 0.05$.

Figure 3.2 Fold change expression of IFNγ, IL-12, IL-4, and IL-10, in spleen and cecal tonsil of broiler chickens fed the following diets: Control (C) = control diet with no antibiotic; Antibiotic = C + 11mg/kg of virginiamycin; Yeast Cell Wall = C + 0.25% of yeast cell wall polysaccharides; Maxi-Gen Plus = C + 0.2% of a commercial product containing processed yeast and nucleotides; Nucleotides (0.025%) = C + 0.025% of nucleotides; Nucleotides (0.05%) = C + 0.05% of nucleotides; DDGS = 10% of DDGS. *Bars with an asterisk differ significantly from the control group. Results were considered statistically significant from the control group if $P < 0.05$.

Figure 3.1. Fold change gene expression of pattern recognition receptors in spleen and cecal tonsils



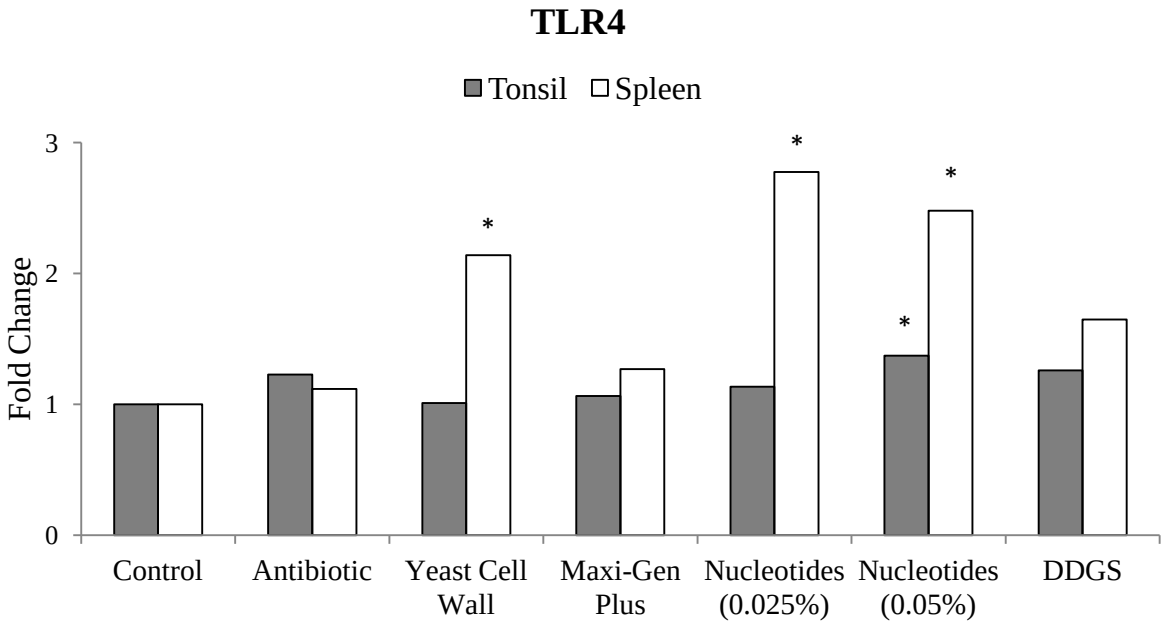
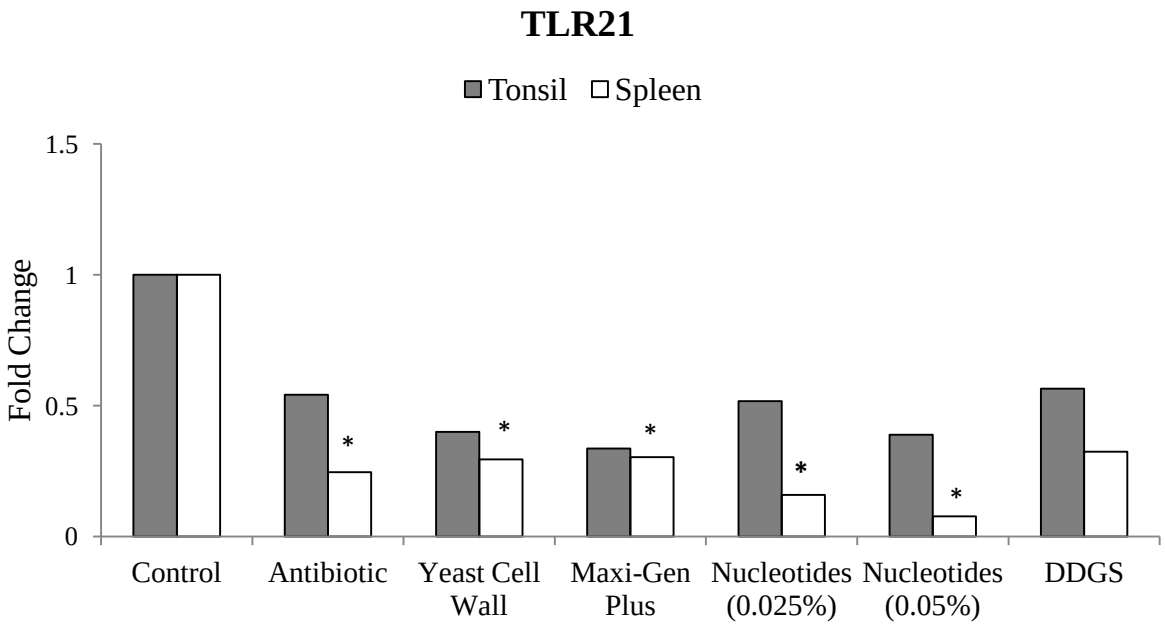


Figure 3.1 Continued. Fold change gene expression of pattern recognition receptors in spleen and cecal tonsils



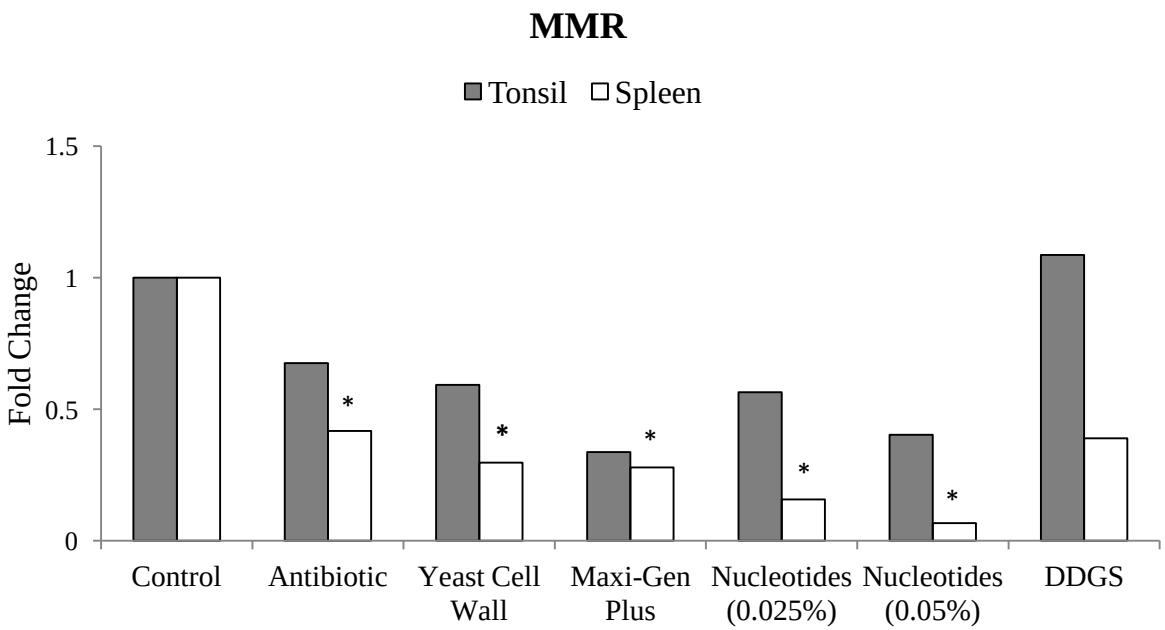


Figure 3.2. Fold change gene expression of cytokines in spleen and cecal tonsils

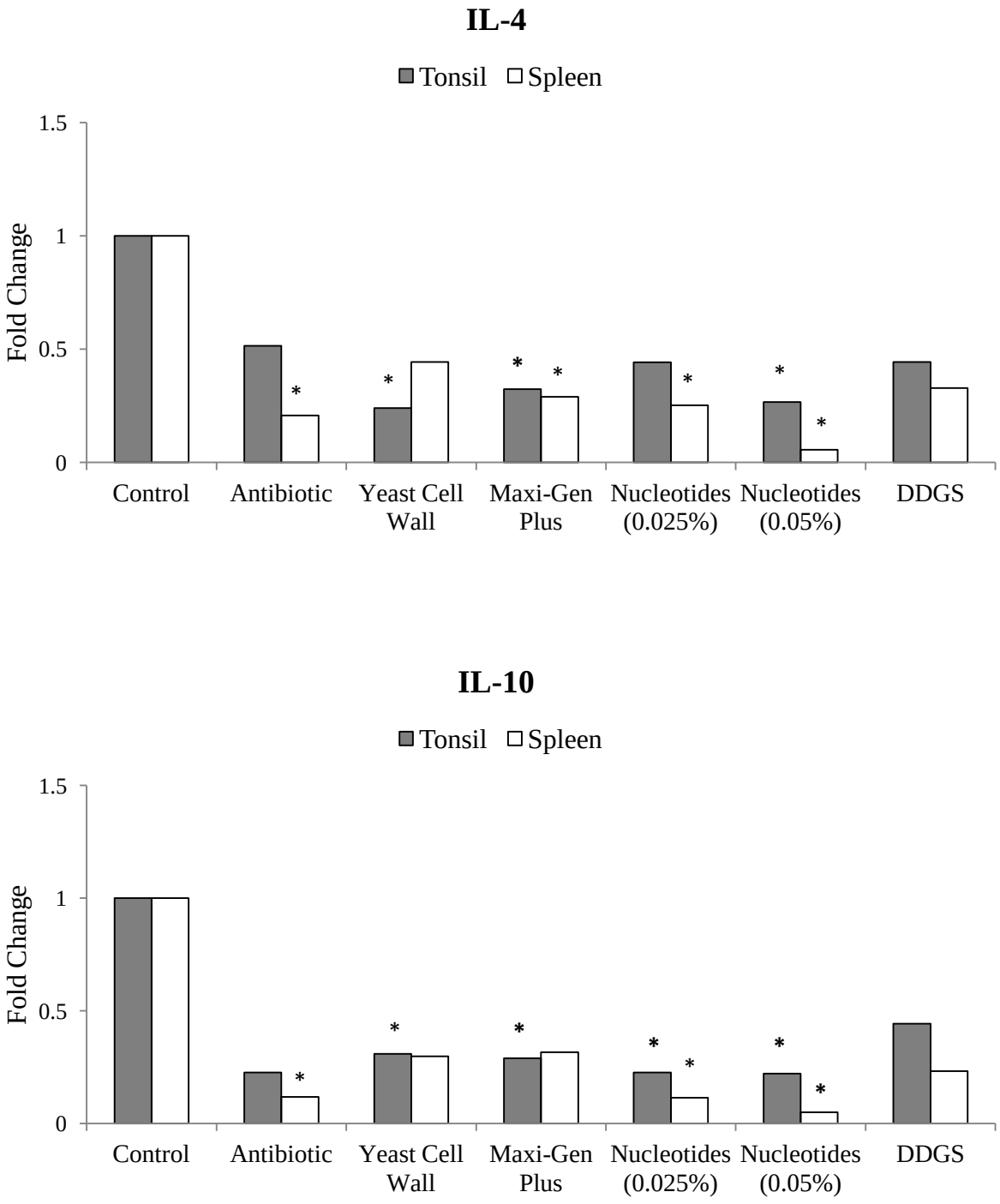
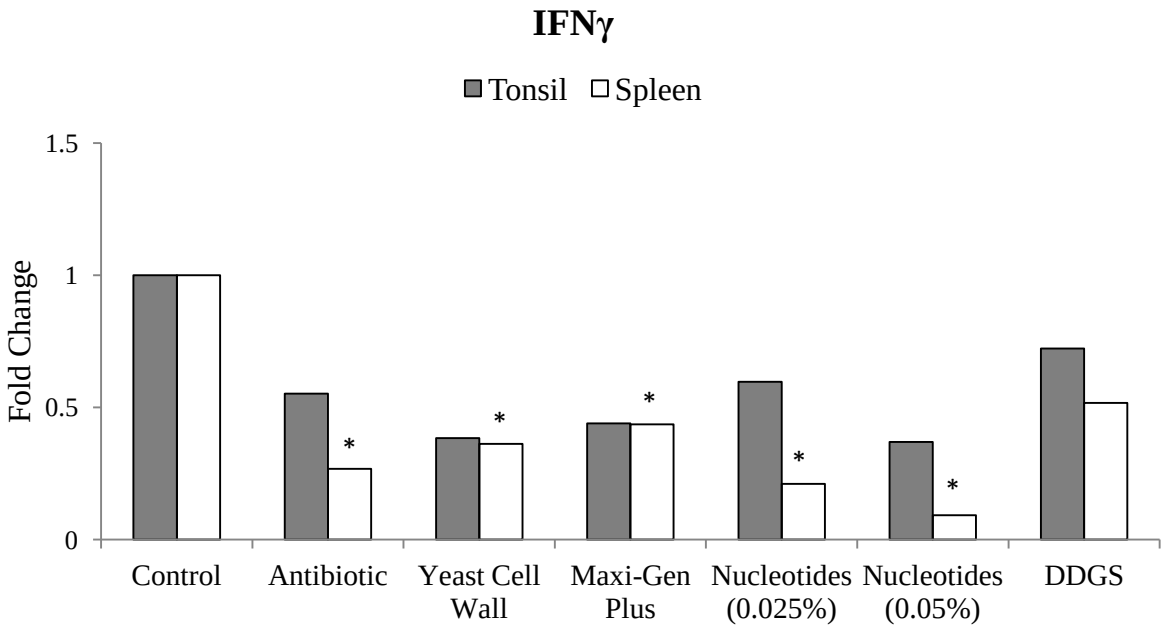
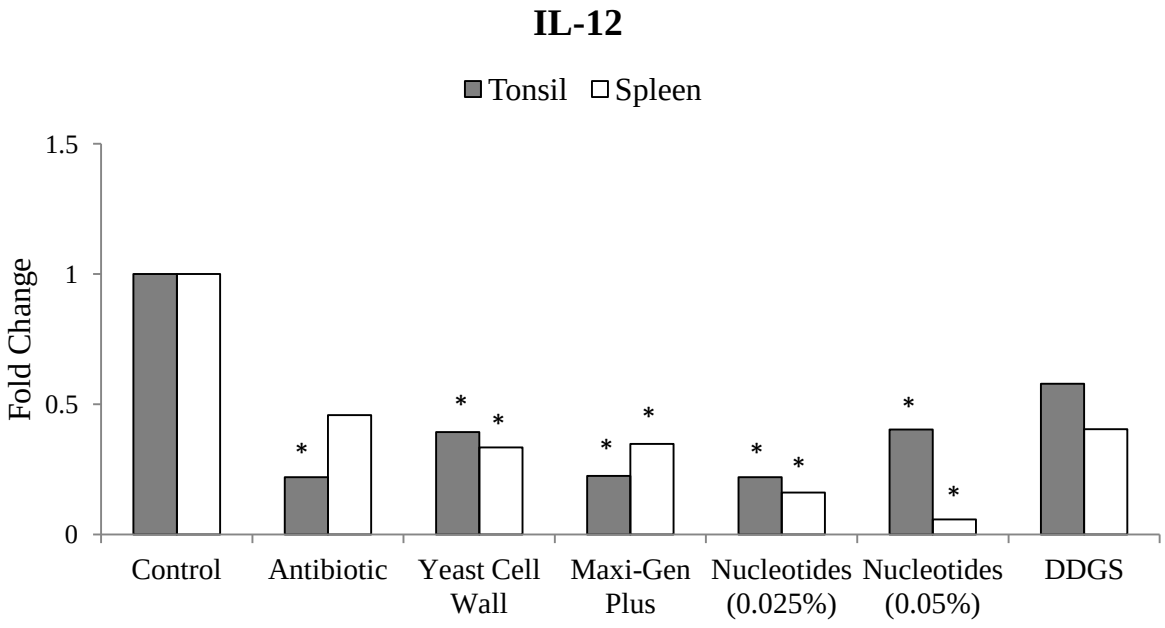


Figure 3.2 Continued. Fold change gene expression of cytokines in spleen and cecal tonsils





CHAPTER 4: MANUSCRIPT II

Effect of yeast-derived products and distillers dried grains with solubles (DDGS) on antibody-mediated immune response and gene expression of pattern recognition receptors and cytokines in broiler chickens immunized with T-cell dependent antigens

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4.1 Abstract

This study was conducted to investigate the effect of yeast-derived products on innate and antibody mediated immune response in broiler chickens following immunization with the sheep red blood cells (SRBC) and bovine serum albumin (BSA). Sixty one-day old male broiler chickens (Ross-308) were randomly assigned to six dietary treatments consisting of a Control diet without antibiotic (C), and diets containing 11 mg/kg of antibiotic "virginiamycin", 0.25% of yeast cell wall (YCW), 0.2% of a commercial product "Maxi-Gen Plus" containing processed yeast and nucleotides, 0.05% of nucleotides, or a diet containing 10% of DDGS. On days 21 and 28 post-hatching, birds were immunized intramuscularly with SRBC and BSA. The unimmunized group was injected with saline solution. One week after each immunization, blood samples were collected. Serum samples were analyzed by hemagglutination (HA) test for antibody response to SRBC, and by ELISA for serum IgM and IgG response to BSA. On d 35, 5 birds per treatment were euthanized and the tissue samples from the cecal tonsils were collected to assess the gene expression of toll-like receptors TLR2b, TLR4, and TLR21, monocyte mannose receptor (MMR), and cytokines IL-10, IL-13, IL-4, IL-12p35, and IFN-γ. With regards to gene expression analysis in cecal tonsils, the expression of TLR21 and MMR was not affected by dietary treatments; however, the diet with YCW increased the expression of TLR2b relative to the Control; while the diet containing antibiotic showed a significant downregulation of TLR2b in cecal tonsils. Furthermore, in birds receiving the nucleotide-containing diet the expression of TLR4 was significantly upregulated compared to the control treatment. Regarding the cytokine analysis, the diet supplemented with YCW upregulated the expression of T helper (Th) 2 cytokines IL-10, IL-4, and IL-13; and birds fed the diet supplemented with nucleotides showed upregulation of IL-13 in cecal tonsils. The diets with antibiotics or Maxi-Gen Plus

downregulated the expression of IFN-γ; however, no significant difference was observed for gene expression of IL-12. The results of the HA test showed that although the primary antibody response to SRBC was not affected by dietary treatments, the diet containing YCW increased ($P<0.05$) secondary antibody response to SRBC (from $\log_2$ 4.6 to 8.2 geometric mean titer) compared to the antibiotic treatment. The results of the ELISA test demonstrated that neither primary nor secondary IgG and IgM response against BSA were affected by dietary treatments. Additionally, the relative weight of immune organs (bursa and spleen) was not influenced by the diets. In conclusion, supplementation of the diet with YCW stimulated Th2 cell-mediated immune response by increasing the expression of anti-inflammatory cytokines and improving antibody production. This may indicate immunomodulatory effects of YCW products occours following immunization with non-inflammatory antigens.

Key Words: Yeast-derived products, innate immunity, humoral immunity, broiler chicken

4.2 Introduction

Newly hatched chickens are vulnerable to invading pathogens because of their immature and underdeveloped immune system (Dibner et al., 1998; Cox et al., 2010a). Antibiotic growth promoters (AGP) have been used in the broiler industry in order to suppress the populations of bacteria in intestine and protect birds from the harmful effects of pathogenic enteric microorganisms (Dibner and Richards, 2005; Ganan, et al., 2012). However, the use of AGP in the diet has been limited in many countries because of bacterial resistance to antibiotics as well as the potential for antibiotic residues in broiler meat (McEwen and Fedorka-Cray, 2002; Capita and Alonso-Calleja 2013). Dietary immunomodulation has been introduced to the broiler industry as a strategy to control the pathogens and maintain the health of broilers in the absence

of AGP (Klasing, 2007; Huyghebaert et al., 2011). The results of some studies demonstrated that supplementation of a diet with yeast products derived from *Saccharomyces cerevisiae* can modulate both the innate and humoral immune system in broilers (Gao et al., 2008; Muthusamy et al., 2011; Yitbarek *et a*l., 2013). Although the composition of the yeast-derived components are variable, they essentially are rich sources of ß1,3-1,6-glucan, mannan polysaccharides and nucleotides (Lipke and Ovalle, 1998). It has been reported that yeast cell wall polysaccharides including β-glucans and mannan can act as microbe associated molecular patterns (MAMPs) and modulate the immune system through pattern recognition receptors (PRRs) (Ferket et al., 2002; Shashidhara and Devegowda, 2003; Jawhara et al., 2012). Modulation of PRRs expressed by cells of the innate immune system including macrophages and dendritic cells would be followed by production of cytokines, some of which are involved in B cell development and antibody production (Reis e Sousa, 2004).

The immunomodulatory effects of yeast cell wall components have been confirmed in several human and animal studies. Gomez-Verduzco et al. (2009) demonstrated that supplementation of 0.05% of YCW in the diet increased humoral and cell-mediated immune response in broiler chickens following natural exposure to *Eimeira* spp. Lowery et al. (2005) showed that a diet containing purified β-glucans induced an innate immune response against *Salmonella enterica* serovar *Enteritidis* in immature chickens. In addition to YCW, nucleotides of yeast *Saccharomyces cerevisiae* have been demonstrated to have immunomodulatory properties (Maldonado et al., 2001; Frankic et al., 2006; Hess et al., 2012). Dietary nucleotides are not considered essential nutrients, however, under conditions of rapidly growing tissues and cells such as intestinal epithelium and lymphocytes), the de novo purine nucleotide synthesis may not be sufficient to support the increased need for nucleic acids (Adjel

et al., 1995; Sauer et al., 2011). Therefore, dietary nucleotides may serve as precursors of DNA and RNA in such tissues via the salvage pathway, sparing the organism from the cost of their synthesis (Carver, 1999; Grimble, 1994). Some in vitro as well as in vivo studies in humans and animals demonstrated that dietary nucleotides have the ability to increase humoral immunity and cell-mediated immunity, and improve the host resistance to bacterial infections (Jyonouchi, 1994, Maldonado et al., 2001; Frankic et al., 2006; Hess et al., 2012). However, further research is needed to explain the mechanism by which nucleotides can contribute to the modulation of the immune system.

Biochemical analysis in our laboratory demonstrated that distillers dried grains with solubles (DDGS) as co-products of brewer's yeast (*Saccharomyces cerevisiae)* fermentation contain a significant quantity of yeast biomass (6%) which can be potentially beneficial for immune system modulation (Slominski, 2012). Therefore, the objective of the present study was to evaluate the effects of different yeast-derived products on local innate immunity gene expression and antibody-mediated immune response in broilers chickens following immunization with sheep red blood cells (SRBC) and bovine serum albumin (BSA).

4.3 Materials and Methods

Experimental Design and Diets

This experiment was conducted in electrically heated batteries under the University of Manitoba Animal Care Protocol Management and Review Committee. All animal procedures were handled according to the guidelines of the Canadian Council of Animal Care (1993). A total of sixty one-day-old male broiler chickens (Ross-308) were weighed and randomly assigned to six dietary treatments including a control diet without antibiotic (C), and diets containing 11

mg/kg of antibiotic “virginiamycin”, 0.25% of yeast cell wall (YCW), 0.2% of a commercial product “Maxi-Gen Plus” containing processed yeast and nucleotides, 0.05% of nucleotides, or a diet containing 10% of DDGS. Basal diets were formulated to meet the requirements of broiler chickens as recommended by NRC (1994). The experimental diets were formulated to contain 3,050 kcal of ME per kg and 23% of CP. In diet supplemented with DDGS, 10% of wheat-corn DDGS replaced wheat and barley in the basal diet and was formulated with the same energy and CP content (Table 4.1).

Immunization, Blood Collection, and Tissue Sampling

To evaluate the antibody mediated immune response, on days 21 and 28 post-hatching 5 birds per treatment were immunized with 0.25 ml of 2% sheep red blood cells (SRBC) (Bethyl Laboratories Inc, Montgomery, TX, U.S.A) in phosphate buffered saline (PBS) and 0.25 ml of PBS containing 100 µg of bovine serum albumin (BSA) (Hy Clone Laboratories, Logan, UT, U.S.A). The unimmunized group was fed the control diet and injected with the saline solution. One week after each immunization, on d 28 and 35 post-hatching blood samples were collected from the wing vein of 5 birds per treatment and transferred into serum tubes. Blood samples were kept at room temperature for 2 hours and centrifuged at 12000 × g for 5 min to isolate serum. Serum samples were stored at -80ºC for antibody analysis. On day 35, after blood collection, 5 birds per treatment were euthanized by cervical dislocation and weighed. Spleen and bursa of Fabricius were removed and weighed, and cecal tonsils were collected, rinsed in saline, snap frozen, and stored at -80°C for gene expression analysis.

Serological Analysis

Detection of the total antibody response to SRBC in sera was performed by a direct hemagglutination-inhibition (HI) assay as previously described (Haghighi et al., 2005). Serum samples were heat treated at 56°C for 30 min. Then, 50 µL of phosphate buffered saline (PBS) containing 0.05% of BSA was added into each well of a round-bottomed 96-well microplate. Serum samples were added in an equal amount of PBS in the first column of the plate and serially double diluted in the wells. Subsequently, 50 µL of 2% SRBC in PBS was added to each well and the plates were shaken for 1 min followed by incubation for 24 hours at 37°C. A positive result for agglutination titer was recorded when at least 50% of SRBC agglutination was observed.

Detection of specific systemic antibody response (IgG and IgM,) against BSA in sera was performed by indirect ELISA technique. Briefly, each well of a flat-bottomed 96-well microplate was coated overnight with 100 µL of coating buffer (0.1 M $NaHCO_3$, pH 9.6) containing BSA (30 µg/mL) at 4ºC. Wells were then washed five times with 200 µL of PBS with 0.05% of Tween 20 (PBST) as washing solution and were completely decanted between each washing step. Subsequently, the wells were covered by 100 µL of blocking buffer (PBST containing 0.25% of gelatine) and the plate was incubated for 2 hours at room temperature. Washing was repeated, by addition of 100 µL of chicken serum (diluted 1:500 v/v in blocking buffer) to each well. Plates were incubated 2 hours at room temperature and then were washed five times with the washing solution. One hundred µL of detection antibody (goat anti-chicken IgG-Fc and IgM-Fc) conjugated with horseradish peroxidase (diluted in 50 mL of blocking buffer) was added to each well and incubated for 1 h at room temperature. Washing was repeated, followed by addition of 100 µL of tetramethyl benzidine solution as substrate to each

well and incubation for 15 min in the dark. Finally, stop solution was added to prevent further color development, and absorbance was measured at 450 nm using the micro plate reader.

Total RNA Extraction and Reverse Transcription

Total RNA was extracted from individual cecal tonsil samples using the Trizol extraction method as described by Trizol manufacturer (Invitrogen Canada Inc., Burlington, ON, Canada). The quantity and purity of the RNA samples was measured by using NanoDrop spectroscopy (Thermo Scientific, Boston, MA, USA) with the ratio of absorbance at 260 nm and 280 nm. In this procedure, a ratio of ~2.0 is generally accepted as "pure' for RNA, and the ratio lower than 2.0 (i.e., ~1.59 and under) may indicate the presence of phenol, protein and other contaminants. Reverse-transcription was performed by using High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol.

Quantitative Real-Time PCR

Primer sequences for β-Actin, TLR2 type 2 (henceforth known as TLR2b), TLR4, TLR21, macrophage mannose receptor (MMR), interleukins IL-12p35, IL10, IL-13, IL-4, and interferon gamma (IFNγ), were designed using GeneBank database sequences from the National Center for Biotechnology Information (Bethesda, MD) (Table 4.2). Quantitative real-time (qRT) PCR was performed using the Step One thermo cycler (Applied Biosystem, Mississauga, ON, Canada) on 48-well plate with 25 µL of total reaction volume as described by Pfaffl and Hageleit (2001). The iTaq Universal SYBR Green Supermix was used as QRT-PCR master mix and each reaction was run in duplicate. The PCR cycling protocol included an initial denaturation step at

95°C, followed by amplification for 40 cycles at 95°C for 10 s, an annealing step at a temperature described in Table 4.2 for each of the primer pairs, and extension at 72°C for 10 s.

Statistical Analysis

The relative weight of immune organs, were analyzed according to a completely randomized design by ANOVA using GLM procedures of SAS (SAS Institute, 2003). The significance of differences between means was determined using Scheffe's procedure. For the blood parameters, *P-value* are presented after log transformation when error deviations did not have homogenous variance across the treatments, and the differences among the treatment means were determined using Duncan's multiple comparison procedure. For the gene expression analysis, the cycle threshold values were recorded and uploaded to the REST-2009 Software (Qiagen, Valencia, CA). Relative expression levels of all genes were calculated relative to a housekeeping gene (β-actin in the present study), and gene expression was presented as fold changes relative to the control diet. Gene expression fold changes, standard error, and statistical significance were calculated by the software based on the formula developed by Pfaffl et al. (2002). A *P*-value of less than 0.05 was considered as significant.

4.4 Results

Gene Expression Analysis

The effect of dietary treatments on fold change gene expression of PRRs in cecal tonsils is presented in Fig.4.1. No significant difference ($P > 0.05$) was observed for gene expression of TLR21, and MMR in cecal tonsils. However, the diet supplemented with YCW upregulated the expression of TLR2b compared to the control ($P < 0.05$); and the expression of TLR2b was downregulated in the antibiotic treatment compared to the control diet ($P < 0.05$). Furthermore,

birds fed the diet containing nucleotides showed a significant upregulation of TLR4 compared to the control diet. The results for the gene expression of cytokines in cecal tonsils are presented in Fig.4.2. The diet supplemented with YCW increased gene expression of IL-4, IL-10, and IL-13 compared to the control diet. Expression of IL-13 was upregulated in cecal tonsils of birds receiving nucleotides. As compared to the control diet, the expression of IFNγ was downregulated in birds receiving diets supplemented with antibiotic, DDGS, and Maxi-Gen Plus. However, no significant difference between treatments was observed for gene expression of IL-12.

Antibody-mediated Immune Response and Relative Weight of Immune Organs

The results for specific antibody-mediated immune response against SRBC are presented in Fig.4.3a. Immunization of birds with SRBC increased the primary total antibody response in the diet supplemented with YCW compared to the unimmunized group ($P < 0.05$). In addition, all immunized groups demonstrated higher total secondary antibody titres against SRBC compared to the unimmunized birds. However, regardless of immunization, no significant difference was observed for primary and secondary anti-SRBC among treatments. The results for the specific antibody-mediated immune response against BSA are presented in Fig. 4.3b. Birds receiving the Control diet and diets containing antibiotic, YCW, and Maxi-Gene Plus showed significantly higher primary anti-BSA IgM compared to the unimmunized group. However, no significant difference was observed for secondary anti-BSA IgM. No significant difference was also observed for primary anti-BSA IgG, while immunization with BSA significantly increased secondary anti-BSA IgG compared to the unimmunized group. Regardless of immunization, anti-BSA IgG and IgM were not affected by dietary treatments. The effect of dietary treatments

on relative weight of immune organs is presented in Table 4.3. No significant difference was observed in relative weight of spleen and bursa of Fabricius among treatments.

4.5 Discussion

Pattern recognition receptors (PRRs) expressed by most immune cells play a key role in host defense through recognition of MAMPs present on the surface and cell walls of microorganisms (Gordon, 2002). Toll-like receptors, the most widely studied type of PRRs, are transmembrane proteins expressed by cells of innate immunity as well as epithelial cells (Takeda et al., 2003; Brownlie and Allan, 2011). In addition to TLRs, the macrophage mannose receptor (MMR) as a transmembrane carbohydrate binding protein belonging to the C-type lectin family, is involved in recognition of carbohydrates such as mannose, fucose, glucose, GlcNAc, as well as carbohydrates on the outer membrane of microorganisms (Apostolopoulos and McKenzie, 2001; Gazi and Martinez-Pomares, 2009). In the present study, gene expression of TLR21 as a functional homologue to mammalian TLR9 which is involved in recognition of unmethylated CpG oligonucleotides (Brownlie et al., 2009) was not affected by dietary treatments. Furthermore, no significant difference was observed in gene expression of MMR among treatments. However, diet supplemented with YCW products upregulated the expression of TLR2b compared to the control. These findings are in agreement with those of Yitbarek et al. (2013) who demonstrated that the diets containing yeast-derived products increased TLR2b expression in cecal tonsils of birds. In broiler chickens, TLR2 can recognize a variety of substances, including peptidoglycans and lipoproteins from gram-positive and gram-negative bacteria, and zymosan found on the surface of fungi (Higuchi et al., 2008; Schenk et al., 2009). Upregulation of TLR2 in birds receiving YCW product might be associated with β- ß1,3-1,6-

glucan and mannan components of this product. It has been reported that the recognition of zymosan as the yeast cell wall component containing significant quantities of β-glucans and mannan polysaccharides, is mainly mediated by TLR2 (Sato et al., 2003). In addition, downregulation of TLR2b in birds fed the antibiotic could be related to antibacterial activity of this product which prevents bacterial over-growthing and reduces bacterial immune stimulation at the intestinal level (Collier et al., 2003; Rhee et al., 2004). Expression of TLR4 was upregulated in birds fed the diet containing nucleotides. In broilers, TLR4 detects lipopolysacharide of gram-negative bacteria and plays a key role in intestinal mucosal innate immunity (Warren et al., 2005). It has been reported that upregulation of TLR4 at the intestinal level is associated with enhanced gastroepithelial barrier function against pathogens. In the current study, increased TLR4 expression in the cecal tonsil of birds receiving nucleotides might be explained by the role of this product in the development and proliferation of tissues and cells with the high rate of turnover such as intestinal epithelium and lymphocytes (Bueno et al., 1994; Maldonado et al., 2001).

Cytokines as small cell-signalling molecules modulate the balance between humoral and cell-mediated immune response (Shrum, 1996). In broiler chickens, effector CD4+ T-helper (Th) cells play an important regulatory role in immune response. Upon activation, CD4+ Th cells differentiate into Th1 and/or Th2 effector cells. These two main subsets of T cells are characterized based on their cytokine production (Paul and Seder, 1994; Van de Veerdonk and Netea, 2010). Effector Th1 cells release proinflammatory cytokines such as IL-1β and IFNγ which are involved in cell-mediated immunity; while effector Th2 cells produce anti-inflammatory cytokines such as IL-10 and IL-4 that stimulate B cells proliferation and differentiation into antibody-secreting plasma cells (Dong and Flavell, 2000; Haghighi et al,

2005). In the present study, birds fed the diet containing YCW showed upregulation of IL-10, IL-4, and IL-13 in cecal tonsils; and supplementation of the diet with nucleotides upregulated the expression of IL-13.

Interleukin-10 is an anti-inflammatory and immunoregulatory cytokine that suppresses the production of Th1 cytokines (INF-γ), and is involved in B cells activation and antibody production (Saraiva and O'Garra, 2010; Fietta and Delsante, 2009). Upregulation of IL-10 in birds receiving YCW might be associated with anti-inflammatory properties of the β-glucan component of YCW. In an animal study, Jawhara et al. (2012) evaluated the immunomodulatory effects of yeast cell wall β-glucans on intestinal inflammation in a dextran sulfate sodium (DSS)-induced mice model. The results demonstrated that admistration of yeast β-glucans displayed a strong anti-inflammatory effect by reduction in TNF-α expression (inflammatory cytokine) and increased IL-10 production. Interleukin-4 is an anti-inflammatory mediator which induces the differentiation of naive T cells to Th2 lymphocytes and stimulates B cell proliferation and differentiation to antibody producing plasma cells (Swain et al., 1990; Lee et al., 2002). The function of IL-13 as a cytokine mostly produced by Th2 cells is closely related to IL-4 (Wynn, 2003). Upregulation of IL-4 and IL-13 in the cecal tonsils of birds receiving YCW suggests that supplementation of YCW supports Th2 cell-mediated immune response following immunization by T-dependent antigens. Furthermore, the high level of IL-13 expression in cecal tonsils of birds receiving nucleotides is probably associated with higher expression of TLR4 in this group and may indicate anti-inflammatory properties of these products (Cox et al., 2010a).

Regarding inflammatory cytokines, diets supplemented with either antibiotic, Maxi-Gen Plus, or DDGS downregulated the expression of IFNγ in cecal tonsils. However, no significant

difference was observed for gene expression of IL-12 among treatments. Interferon gamma is produced by Th1 cells and is defined as a major macrophages activator which is involved in differentiation of naive Th cells to Th1 cells; and IL-12 is secreted predominantly by activated macrophages and induces further production of IFNγ by Th1 cells (Trinchieri, et al., 2003; Wolf et al, 1994). Downregulation of IFNγ in cecal tonsils of birds receiving either Maxi-Gen Plus or DDGS may indicate anti-inflammatory effects of these products. The anti-inflammatory effects of yeast products have already been proven in an in vitro study, where a diet with cell wall-free soluble extract of yeast culture downregulated the expression of IFNγ (Jensen et al., 2008). Furthermore, Munyaka et al. (2012) demonstrated that adding yeast-derived carbohydrates to the diet downregulated the expression of IFNγ and IL-12 in the cecal tonsils of birds. In the present study, diet supplemented with YCW increased gene expression of anti-inflammatory cytokines IL-4, IL-10, and IL-13; however the expression of inflammatory cytokines IFNγ and IL-12 was not affected by YCW. The findings of the current study are contrary to the results described by Yitbarek et al. (2012) where a diet containing yeast-derived carbohydrates increased gene expression of IFNγ and IL-12 in the ileum of birds challenged with *Clostridium perfringens*. This might be explained by the difference in immunogens applied in these studies. Immunogens used in the current study are not considered pathogenic Immunogens and would not cause inflammation in host animals.

Humoral immunity is a component of the immune system that is mostly mediated by antibodies (Scott, 2004). Antibodies as an important facet of B cells contribute to host defence through neutralization, immobilization and opsonisation of foreign subjects such as pathogenic bacteria and viruses (Janeway, 2001). Activation of B cells as the precursor of antibody secreting cells may occur through T cell-dependent or T cell-independent pathways (Parker, 1993).

Antigens such as SRBC and BSA are considered T-dependent antigens and require T helper cells cooperation to stimulate B cells into becoming antibody-producing B cells and memory cells (Gehad et al., 2002). During T cell-dependent B cell activation, presentation of antigens by B cells (as antigen presenting cells) to T helper cells would be followed by the production of cytokines (by T helper cells) which are involved in B cell activation and antibody production.

In the current study, immunization of birds with SRBC did not change the primary total antibody response. However, the total secondary antibody titres against SRBC were significantly increased compared to the unimmunized group. This is probably because of immunological memory which allows the birds to mount stronger and faster immune response following a second exposure to the antigen (Ahmed and Gary, 1996). Furthermore, birds receiving diet containing YCW mounted a significantly greater secondary antibody repose to SRBC compared to the birds fed the diet containing antibiotic. Improvement in the antibody-mediated immune response observed in the YCW treatment might be associated with the polysaccharide components of the yeast cell wall that can activate B cells in a T cell-dependent manner. Yeast cell wall polysaccharides, including mannan and β-glucans, can exert immune adjuvant activities through binding to the carbohydrate receptors such as C-type lectins, dectin-1, TLR2, and MMR, and enhance cellular and humoral immunity following immunization with antigens (Tsoni and Brown, 2008; Petrovsk and Cooper, 2011; Martinez-Pomares, 2012). In a broiler study, Guo et al. (2003) demonstrated that dietary supplementation of *β*-1,3/1,6 glucan increased the percentages of $CD4^+$ and $CD8^+$ lymphocytes and improved antibody titers against SRBC. Moreover, in a study by Ghosh et al. (2012) a diet containing YCW components increased antibody response against Newcastle disease. However, Cheng et al. (2004) found that dietary β-glucans (0.2 or 0.5 g/kg) did not affect antibody titers against Newcastle disease vaccine. In the

current study, beneficial effects of YCW on humoral immunity were confirmed by upregulation of TLR2 and cytokines including IL-4, IL-10 and IL-13 which can enhance B cell survival and proliferation, and induce B-cell differentiation and antibody production. Furthermore, the diet supplemented with nucleotides did not affect primary and secondary antibody response against SRBC. Similarly to our results, Deng et al., (2005), demonstrated that yeast nucleotide supplementation had no effect on humoral immune response to SRBC in broiler chickens. In contrast, Jyonouchi et al. (1994) showed that inclusion of dietary nucleotides to mice previously fed a nucleotide-free diet restored the humoral immune response to T-dependent antigens. Nucleotides are considered as semi-essential nutritional components (Cosgrove, 1998). Under normal conditions where birds are not under metabolic stress or challenge, supplementation of yeast nucleotides may not affect humoral immune response. However, these products might be able to bring more beneficial effects under pathogen challenged conditions.

In the current study, we also measured specific IgM and IgG response in broilers following immunization with BSA as a T cell-dependent soluble peptide antigen as previously used by Gehad et al. (2002). Immunoglobulin M is the major component of natural antibodies and is considered as the first class of antibodies found to bind to specific antigens in the absence of apparent antigenic stimulation (Vollmers and Brandlein, 2005). Immunoglobulin G is the main antibody isotope in blood circulation, and plays a critical role in humoral immunity by immobilization and opsonisation of pathogens (Kapur et al., 2014). In birds receiving the Control diet and the diets containing antibiotic, YCW, and Maxi-Gen Plus, immunization with BSA significantly increased the primary anti-BSA IgM compared to the unimmunized group. However, immunization with BSA significantly increased secondary anti-BSA IgG compared to the unimmunized group. This might be explained by the fact that unlike IgG antibody which is

detected later in infections, IgM is considered the first antibody to appear in response to the initial exposure to an antigen (Gronwall and Silverman, 2012). Regardless of immunization, primary and secondary anti-BSA IgG and IgM responses were not affected by dietary treatments. These findings are in agreement with those of Silva et al. (2009) who demonstrated that supplementation of the broiler diet with yeast extract did not result in a significant difference in IgG titers against Newcastle disease virus and infectious bursal diseases in broiler chickens. Conversely, Muthusamy et al. (2011) showed that a diet containing with yeast cell wall increased antibody titres against Newcastle disease after vaccination of broiler chickens. Inconsistent and conflicting results have been reported regarding the effects of yeast-derived products on humoral immune response in broiler chickens. This might be related to the type and concentration of the yeast products, immunization regimen, and experimental and environmental conditions applied in the different studies.

Immune organs, including the spleen and bursa of Fabricius are considered as important sites for antigen recognition and activation of immune cells (Akter et al., 2006); and relative weight of the spleen and bursa reflect the host's ability to produce T and B lymphocytes during immune response. In the present study, the relative immune organ weights were not significantly affected by dietary treatments. These findings are contrary to what Zhang et al. (2012) showed, where supplementation of the broiler diet with YCW (3 g/kg) significantly increased the relative weights of bursa.

In conclusion, our results demonstrated that although serum IgG and IgM levels and relative weights of immune organs, were not affected by dietary treatments, supplementation of diets with an antibiotic, Maxi-Gen Plus or DDGS demonstrated anti-inflammatory effects by reducing the expression of IFNγ as a Th1 cytokine. Furthermore, dietary supplementation with

YCW increased the gene expression of Th2 cytokines IL-10, IL-4, and IL-13 compared to the control diet and improved total antibody production over the antibiotic group. However, no significant difference was observed in Th1 cytokines IFNγ and IL-12. This suggests that YCW has immune adjuvant-like properties and a combination of YCW with non-pathogenic immunogens may enhance Th2 cell-mediated and humoral immune response in broilers.

Table 4.1. Composition and calculated analysis of the basal diet and the diet containing 10% of distillers dried grains soluble (DDGS) (%)

Item	Basal diet	DDGS diet
Ingredient		
Wheat	43.0	40.3
Soybean meal	26.0	20.0
DDGS	0.0	10.0
Barley	13.1	13.8
Fish meal	5.0	5.0
Canola oil	6.0	5.5
Limestone[1]	0.81	1.0
Dicalcium phosphate[2]	1.10	0.9
DL-Methionine	0.1	0.1
L-Lysine-HCL	0.0	0.2
Mineral premix[3]	0.5	0.5
Vitamin premix[4]	1.0	1.0
Chromic oxide	3	3
Total	100	100
Calculated analysis		
ME, kcal/kg	3,055	3,055
CP, %	22.0	22.0
Calcium, %	1.02	1.00
Available phosphorous, %	0.45	0.45
Lysine, %	1.11	1.12
Methionine, %	0.54	0.54
Methionine + cystine, %	0.90	0.90
Threonine, %	0.80	0.80

[1]Contained 38% calcium.
[2]Contained 21% calcium and 18% phosphorous.
[3]Mineral premix provided per kilogram of diet: Mn, 70 mg; Cu 10 mg; Fe, 80 mg, Zn, 80 mg; Se, 0.3 mg; I, 0,5 mg; and Na, 1.7 g.
[4]Vitamin premix provided per kilogram of diet: A, 8,255 IU; vitamin D_3, 3000 IU; vitamin E, 30.0 IU; vitamin B_{12}, 0.013 mg; vitamin K, 2.0 mg; niacin, 24.5; choline, 1081 mg; folic acid 4.0 mg; biotin, 0.25 mg; riboflavin, 6.0 mg

Table 4.2. Primer sequences used for real-time quantitative PCR[1]

Gene[2]	Primer sequence (5'-3')[3]	Fragment size, (bp)	Annealing temperature, (°C)	GenBank accession number
TLR2b	F: CGCTTAGGAGAGACAATCTGTGAA R:GCCTGTTTTAGGGATTTCAGAGAATTT	90	59	NM204278
TLR4	F: AGTCTGAAATTGCTGAGCTCAAAT R: GCGACGTTAAGCCATGGAAG	190	55	AY064697
TLR21	F: TGGCGGCGGGAGGAAAAGTG R: CACCGTGCTCCAGCTCAGGC	106	59	NM_001030558
MMR	F: GCAGGGCACGTTCAGGTGGG R: GCCACACAGCCTGGCTCCCT	90	60	XM001235105
IFN-γ	F: CTGAAGAACTGGACAGAGAG R: CACCAGCTTCTGTAAGATGC	264	60	X99774
IL-12p35	F: CTGAAGGTGCAGAAGCAGAG R: CCAGCTCTGCCTTGTAGGTT	217	64	NM213588
IL-10	F: AGCAGATCAAGGAGACGTTC R: ATCAGCAGGTACTCCTCGAT	103	55	AJ621614
IL-4	F: TGTGCCCACGCTGTGCTTACA R: CTTGTGGCAGTGCTGGCTCTCC	193	57	GU119892
IL-13	F: ACTTGTCCAAGCTGAAGCTGTC R: TCTTGCAGTCGGTCATGTTGTC	129	55	Gu119894
β-Actin	F: CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	205	61	X00182

[1]The listed oligonucleotides were used to analyze gene expression via real-time quantitative PCR.

[2]TLR = Toll-like receptor; MMR = monocyte/macrophage mannose receptor; IFN = interferon; IL=interleukin.

[3]F = forward; R = reverse.

Table 4.3. Effect of dietary treatments on relative weight of immune organs

Item	Treatment[1]						SEM	*P*-value
	Control	Antibiotic	Yeast Cell Wall	Maxi-Gen Plus	Nucleotides	DDGS		
Spleen (g/kg body weight)	0.85	1.10	0.99	0.84	1.12	1.02	0.03	0.34
Bursa of Fabricius (g/kg body weight)	1.99	2.18	2.04	1.95	2.3	1.86	0.07	0.94

[ab]Means within a column with no common superscripts differ significantly (P< 0.05).

[1]Experimental diets inculuded: Control (C) = with no antibiotic; Antibiotic = C + 11mg/kg of virginiamycin; Yeast Cell Wall= C + 0.25% of yeast cell wall polysaccharides; Maxi-Gen Plus = C + 0.2 % of a commercial product containing processed yeast and nucleotides; Nucleotides = C + 0.05% of a nucleotide-rich products; DDGS = 10% of wheat/corn distillers dried grains with solubles.

4.6 Figure Legend

Figure 4.1 Fold change expression of Toll-like receptors TLR2b, TLR4, TLR-21, and macrophage mannose receptor (MMR) in cecal tonsils of broiler chickens immunized with sheep red blood cells and bovine serum albumin and fed the following diets: Control (C) = with no antibiotic; Antibiotic = C + 11mg/kg of virginiamycin; Yeast Cell Wall = C + 0.25% of yeast cell wall polysaccharides; Maxi-Gen Plus = C + 0.2% of a commercial product containing processed yeast and nucleotides; Nucleotides = C + 0.05% of nucleotides; and DDGS = 10% of wheat/corn distillers dried grains with solubles. *Bars with asterisks differ significantly from the control group. Results were considered statistically significant from the control group if $P < 0.05$.

Figure 4.2 Fold change expression of cytokines including IL-10, IL-4, IL-13, IFNγ, and IL-12 in cecal tonsils of broiler chickens immunized with sheep red blood cells and bovine serum albumin and fed the following diets: Control (C) = with no antibiotic; Antibiotic = C + 11mg/kg of virginiamycin; Yeast Cell Wall = C + 0.25% of yeast cell wall polysaccharides; Maxi-Gen Plus = C + 0.2% of a commercial product containing processed yeast and nucleotides; Nucleotides = C + 0.05% of nucleotides; and DDGS = 10% of wheat/corn distillers dried grains with solubles. *Bars with asterisks differ significantly from the control group. Results were considered statistically significant from the control group if $P < 0.05$.

Figure 4.3 Total antibody titer against sheep red blood cells as determined by hemagglutination assay, and serum anti-BSA titer (IgG and IgM) as determined by indirect ELISA at d 28 and 35 in birds fed following diets: Control (C) = with no antibiotic; Antibiotic = C + 11mg/kg of virginiamycin; Yeast Cell Wall = C + 0.25% of yeast cell wall polysaccharides; Maxi-Gen Plus = C + 0.2% of a commercial product containing processed yeast and nucleotides; Nucleotides = C + 0.05% of nucleotides; and DDGS = 10% of wheat/corn distillers dried grains with solubles. Birds in the unimmunized group were fed the control diet and injected with saline solution. *Bars with asterisks show significant downregulations compared to the immunized treatments. Error bars represent standard errors. ^{ab}Means on the bars with no common superscripts are significantly different ($P < 0.05$).

Figure 4.1. Fold change gene expression of pattern recognition receptors in cecal tonsils of birds immunized with sheep red blood cells (SRBC) and bovine serum albumin (BSA)

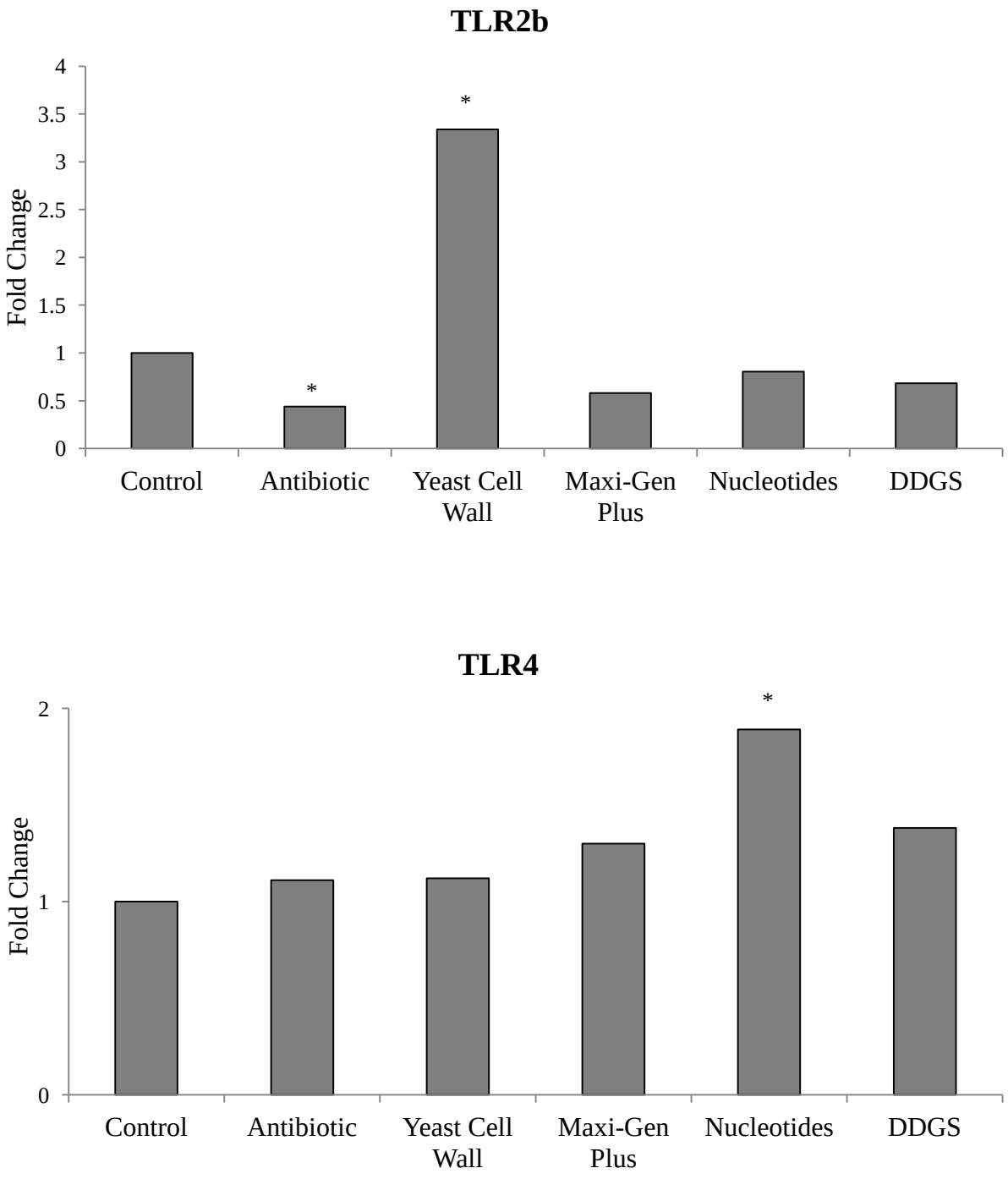
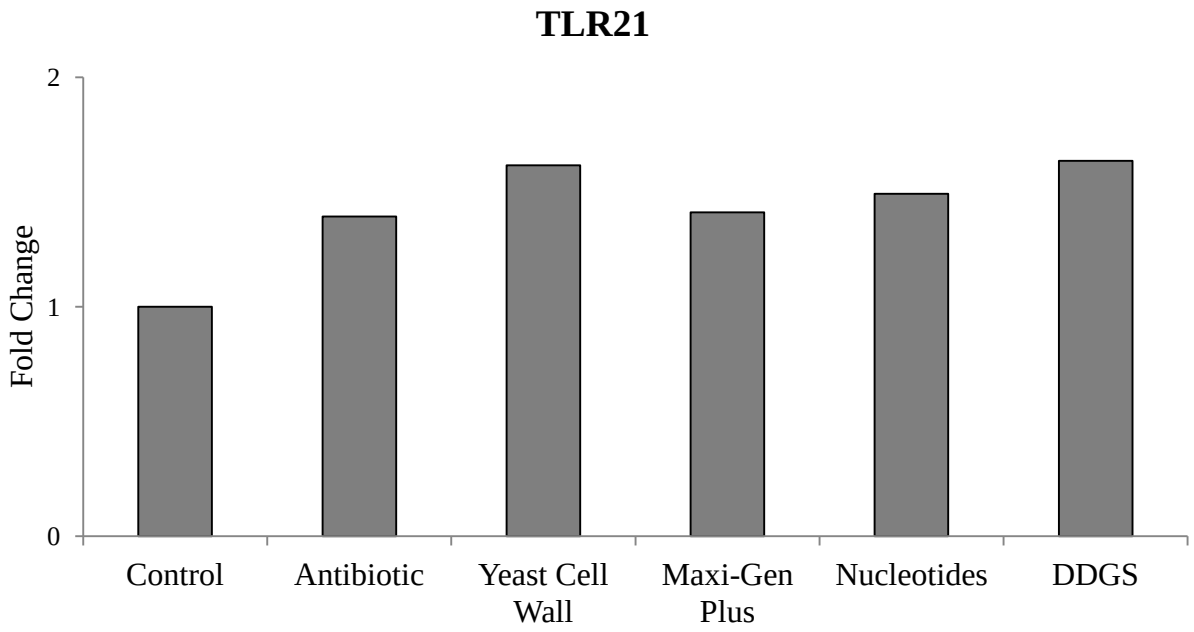


Figure 4.1 Continued. Fold change gene expression of pattern recognition receptors in cecal tonsils of birds immunized with sheep red blood cells (SRBC) and bovine serum albumin (BSA)



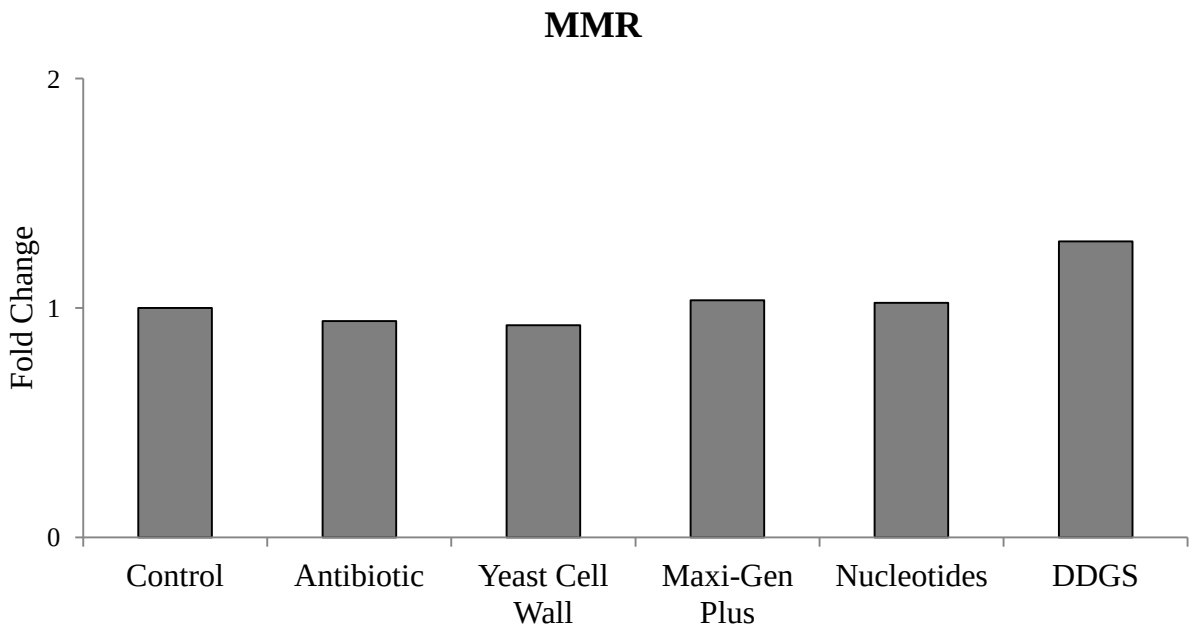
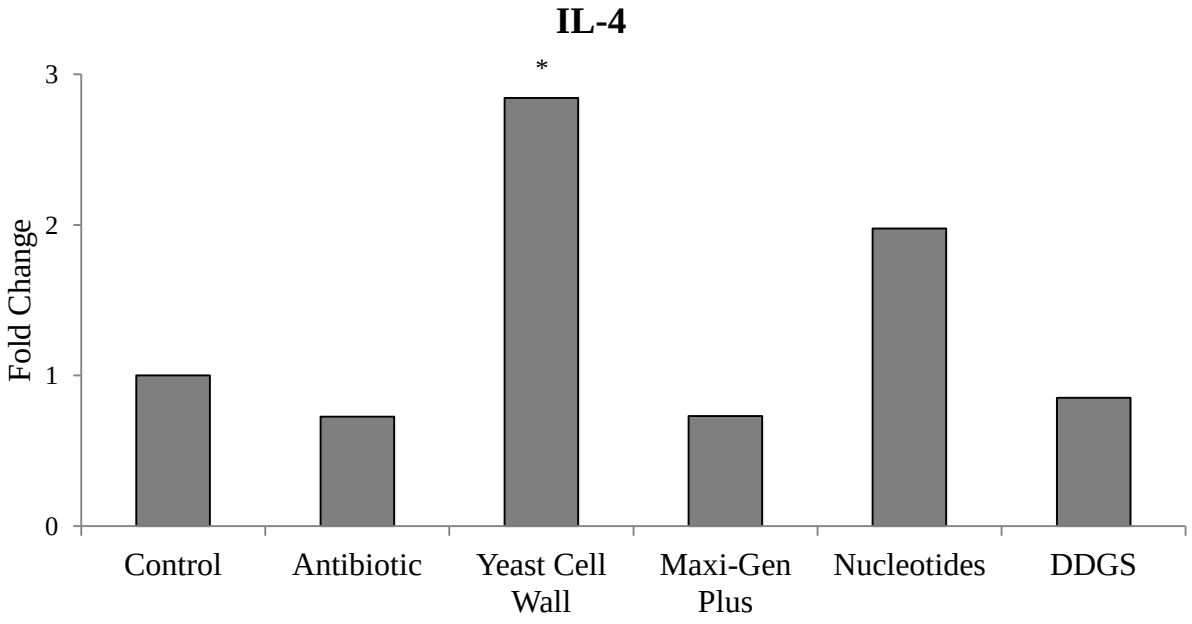


Figure 4.2. Fold change gene expression of cytokines in cecal tonsils of birds immunized with sheep red blood cells (SRBC) and bovine serum albumin (BSA)



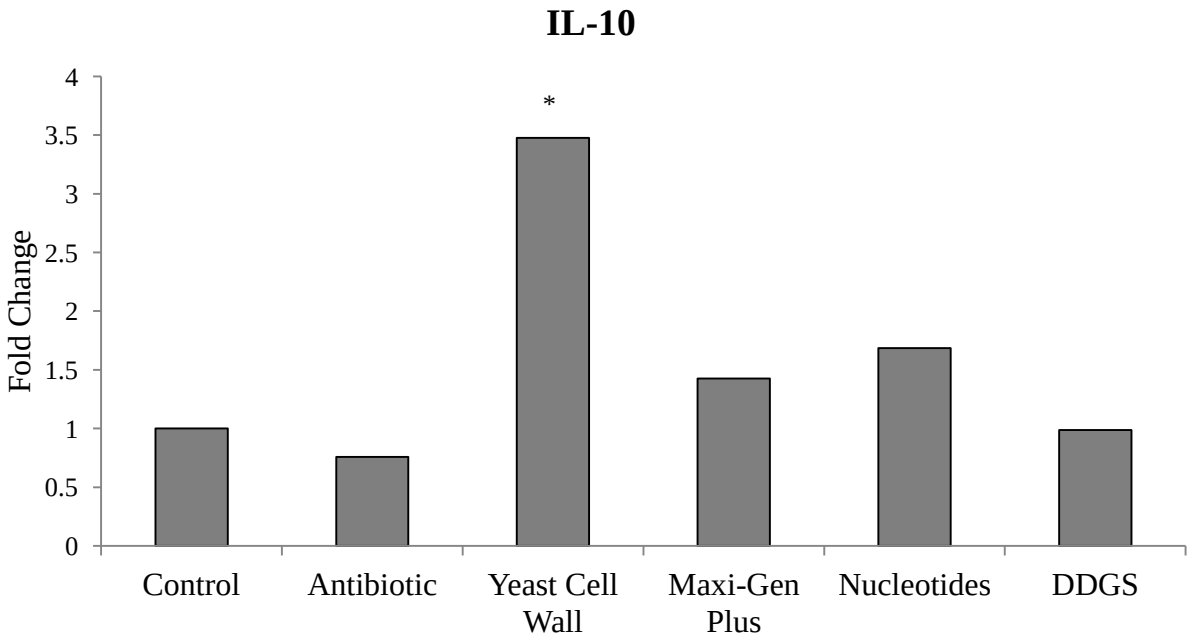
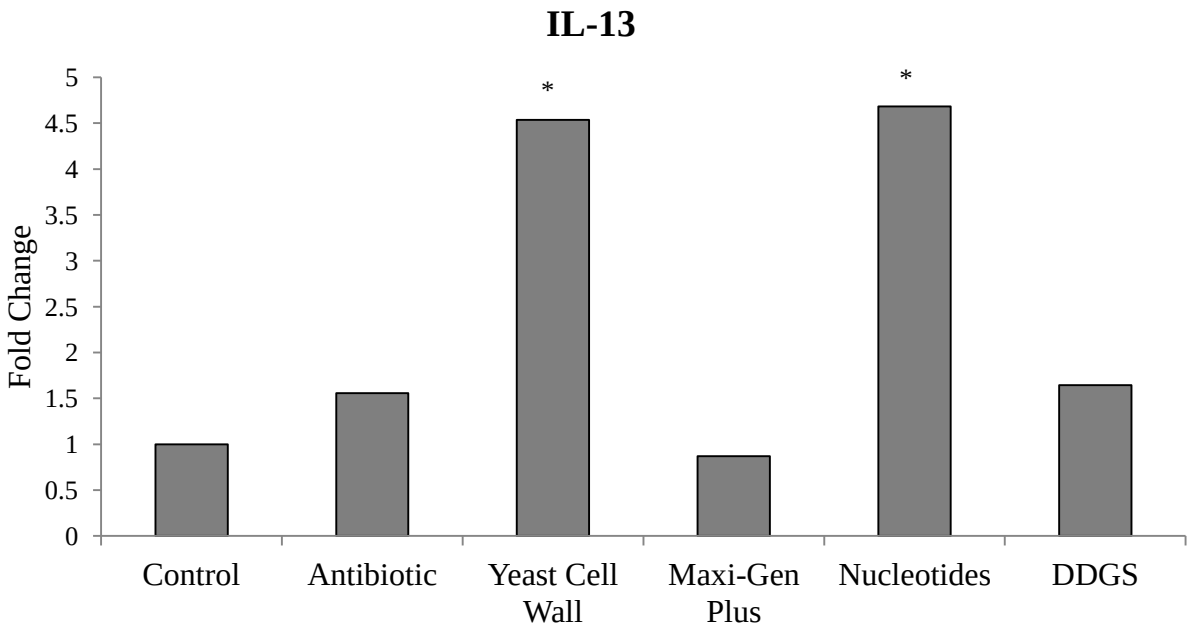


Figure 4.2 Continued. Fold change gene expression of cytokines in cecal tonsils of birds immunized with sheep red blood cells (SRBC) and bovine serum albumin (BSA)



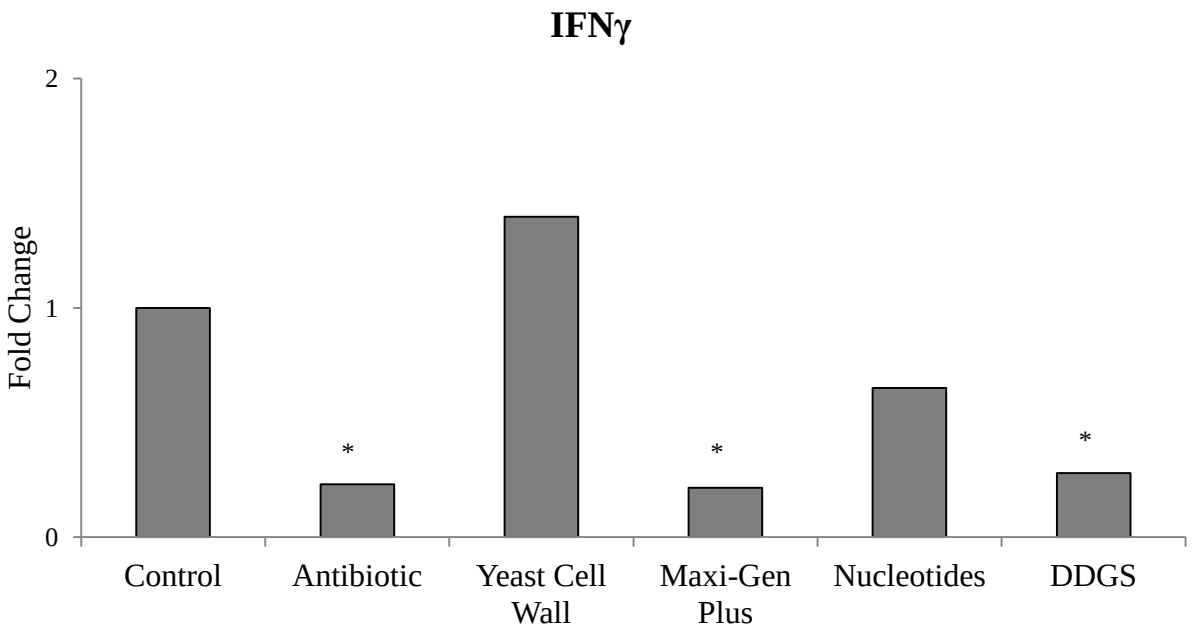


Figure 4.2 Continued. Fold change gene expression of cytokines in cecal tonsils of birds immunized with sheep red blood cells (SRBC) and bovine serum albumin (BSA)

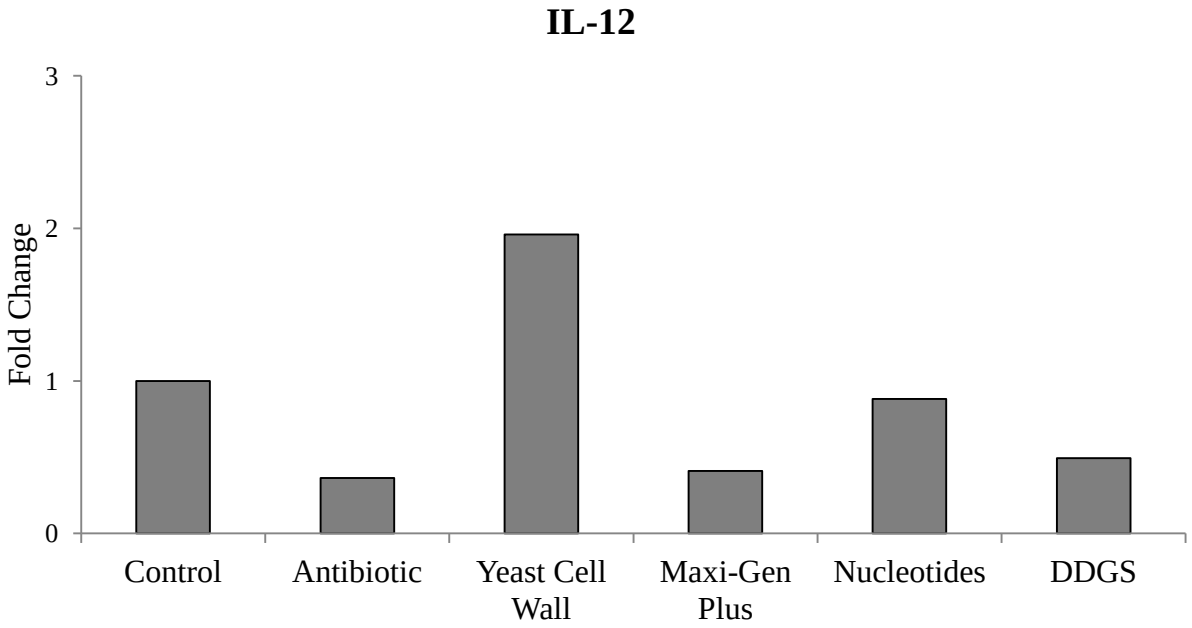


Figure 4.3a. Specific antibody-mediated immune response against sheep red blood cells (SRBC)

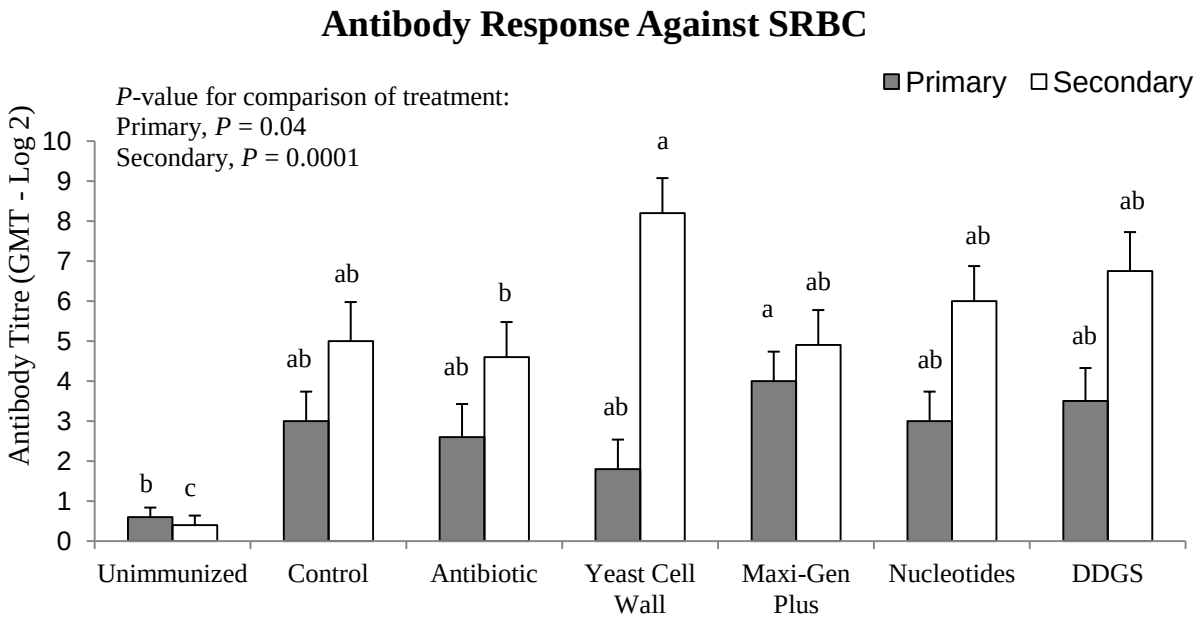
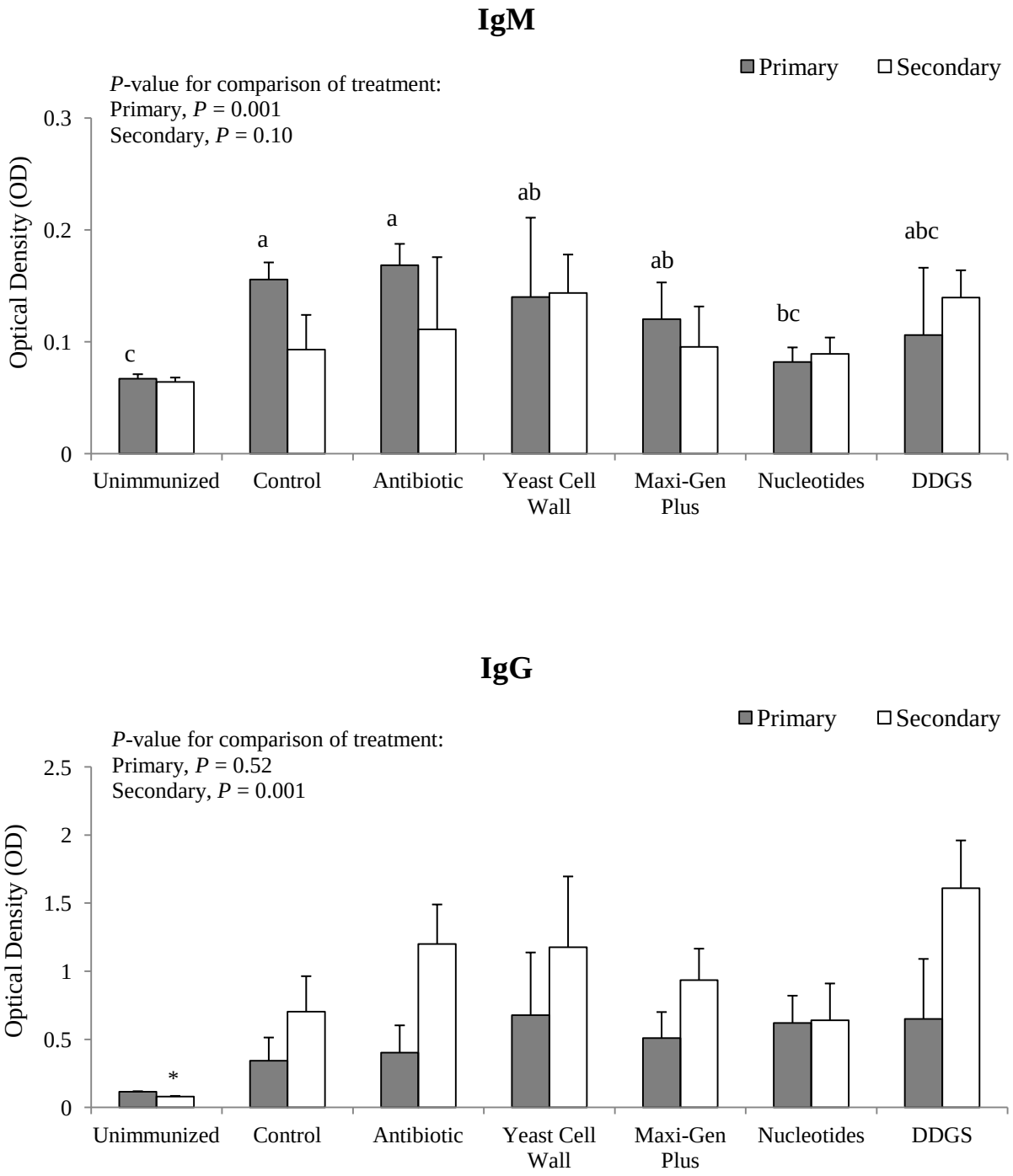


Figure 4.3b. Specific antibody-mediated immune response against bovine serum albumin (BSA)



CHAPTER 5: MANUSCRIPT III

Effect of yeast-derived products on innate and antibody mediated immune response of broiler chickens challenged with *Escherichia coli* lipopolysaccharide

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5.1 Abstract

This study was conducted to assess the effect of yeast-derived products on growth performance, antibody-mediated immune response and mRNA gene expression of pattern-recognition receptors, and cytokines in broiler chickens. Two hundred sixteen one-day old male broiler chickens (Ross-308) were randomly assigned to six dietary treatments with six replicates (cage) of 6 birds per cage. Dietary treatments consisted of a Control diet without antibiotic (C), and diets containing 11 mg/kg of antibiotic "virginiamycin", 0.25% of yeast cell wall (YCW), 0.2% of a commercial product "Maxi-Gen Plus" containing processed yeast and nucleotides, 0.05% of nucleotides, or a diet containing 8% of DDGS. On d 21 posthatch, blood samples were collected from 6 birds per treatment to evaluate humoral immune response. To assess innate immune response and specific systemic antibody response against *Escherichia coli* lipopolysaccharide (LPS), 6 birds per treatment were injected intraperitoneally with 3 mg/kg of BW of LPS on d 21. The unchallenged group was injected with saline solution. Blood samples were collected at 12 h post-injection, and spleen samples were collected to measure the gene expression of toll-like receptors TLR2b, TLR4, and TLR21, macrophage mannose receptor (MMR), and cytokines including interleukin IL-12, IL-10, IL-4, IL-6, IL-18 and interferon IFN-γ. No significant difference in body weight gain, feed intake and FCR were observed among treatments. With regards to systemic innate immune response, no significant difference was observed in mRNA gene expression of TLR2 and TLR4, whereas the expression of TLR21 and MRR was upregulated in diets containing YCW and DDGS. No significant difference was observed for cytokine gene expression in the antibiotic and nucleotides groups. However, the diet supplemented with YCW increased the expression of all cytokines, and expression of IFN-γ

was upregulated in the DDGS group. Regarding humoral immunity, the diet supplemented with YCW increased serum IgA level compared with the antibiotic group; however, serum concentrations of IgG and IgM were not affected by dietary treatments. Furthermore, the specific IgG, IgM, and IgA against LPS were not influenced by dietary treatments. In conclusion, although the growth performance was not affected by yeast-derived products, supplementation of diet with YCW stimulated the humoral and cell-mediated innate immune responses of broiler chickens following challenge with LPS.

Key Words: Yeast-derived products, lipopolysaccharides, innate immunity, humoral immunity, broilers

5.2 Introduction

In the broiler industry, antibiotics have been widely used as growth promoters to protect birds from pathogenic microorganisms and to improve growth performance (Gaskins et al., 2002; Ganan et al., 2012). However, the use of antibiotics as feed additives has been gradually restricted all over the world because of increasing prevalence of antibiotic-resistant bacteria, and their possible residual presence in poultry products (McEwen et al., 2002; Huyghebaert et al., 2011). Considering the vulnerability of the immune system of broiler chickens to invading pathogens during the first 2 weeks post-hatching, banning antibiotic growth promoters in broiler diets could result in increased incidence of diseases such as necrotic enteritis (Dibner et al., 1998; Cox et al., 2010a). Therefore, over the past decade, substantial efforts have been made to find alternatives to antimicrobial feed additives to maintain health and efficiency of broiler performance under commercial conditions (Dahiya et al., 2006; Emborg et al., 2001).

Yeast products are considered as potential "antibiotic alternatives" due to their ability to activate the immune system and provide competitive binding sites for pathogenic bacteria (Ferket et al., 2002; Gao et al., 2008). The exact mechanisms that mediate immunomodulatory activities of yeast-derived products are still unclear. However, it has been suggested that yeast cell wall polysaccharides (YCW) including mannan and β-Glucans can act as pathogen associated molecular patterns (PAMP) and modulate the expression of pattern recognition receptors (PRRs) expressed by cells of innate immunity (Ferket et al., 2002; Mogensen et al., 2009; Shashidhara and Devegowda, 2003). Considering the interaction between innate and humoral immune response, it has been suggested that the recognition of YCW by PRRs could lead to activation of cells of innate immunity such as macrophages and dendritic cells followed by production of cytokines, some of which such as IL-4 and IL-10 are involved in antibody production (Haghighi et al., 2006; Reis e Sousa, 2004). With regards to yeast nucleotides, several studies in humans, and animals demonstrated that dietary nucleotides are capable of increasing humoral immunity, cell-mediated immunity, and improving host resistance to bacterial infections (Frankic et al 2006; Hess et al., 2012; Maldonado et al., 2012). Dietary nucleotides have an essential role in the development and proliferation of tissues and cells with a rapid cell turnover such as the intestine and lymphocytes where de novo synthesis of nucleotides cannot meet their demand in such rapidly proliferating tissues. Therefore, adding nucleotides to diets may spare the energetic cost of de novo synthesis (Bueno et al., 1994; Uauy et al., 1994). Domeneghin et al. (2004) showed that adding 0.05 % of nucleotides to the weanling piglet diet, improved growth and maturation of the intestinal mucosa which was indicated by higher percentages of macrophages and intra-epithelial lymphocytes in the intestine. It has been reported that supplementation of piglets diets with nucleotides resulted in changes in gene expression of

inflammatory and anti-inflammatory cytokines, and increased plasma IgA concentration (Sauer et al., 2011; Superchi et al., 2012). Inconsistent results have been reported regarding the effects of yeast-derived products on growth performance and immune response of broiler chickens (Cox et al., 2010a; Muthusamy et al., 2011; Munyaka et al., 2012); and there is limited information about the effect of yeast nucleotides on innate and humoral immune response of broiler chickens following a systemic immune challenge condition. Therefore, the present study was designed to investigate the effects of yeast nucleotides and YCW on performance, gene expression of PRRs and cytokines, and antibody-mediated immune responses in broiler chickens challenged with *E. coli* lipopolysaccharide (LPS).

5.3 Materials and Methods

Experimental Design and Diets

This experiment was conducted in electrically heated batteries under the University of Manitoba Animal Care Protocol Management and Review Committee. All animal procedures were handled according to the guidelines of the Canadian Council of Animal Care (1993). A total of 216 one-day-old male broiler chickens (Ross-308) were weighed and randomly assigned to six dietary treatments with 6 replicate cages of 6 birds each. Birds were fed ad libitum, and feed intake and body weight were measured on d 21. Mortality was recorded on a daily basis; and body weight gain, feed consumption, and FCR were calculated. Dietary treatments consisted of a Control diet without antibiotic (C), and diets containing 11 mg/kg of antibiotic "virginiamycin", 0.25% of yeast cell wall (YCW), 0.2% of a commercial product "Maxi-Gen Plus" containing processed yeast and nucleotides, 0.025% of nucleotides, 0.05% of nucleotides, or a diet containing 8% of DDGS. Basal diets were formulated to meet the requirements of

broiler chickens as recommended by NRC (1994). The experimental diets were formulated to contain 3,030 kcal of ME/kg and 22% of CP. In the diet supplemented with DDGS, 8% of corn-wheat DDGS replaced wheat and canola meal and was formulated with the same energy and CP content as the other diets (Table 5.1).

Blood Collection, lipopolysaccharide Administration, and Tissue Sampling for Gene Expression Analysis

On d 21 post-hatching blood samples were collected from the wing vein of 6 birds per treatment and transferred into serum tubes to evaluate systemic antibody mediated immune response. Blood samples were kept at room temperature for 2 h and centrifuged at 12000 × *g* for 5 min to isolate serum. Serum samples were stored at -80ºC for antibody analysis. To assess specific systemic antibody response against LPS, on d 21, 6 birds per treatment were injected intraperitoneally with 3 mg/kg of BW of *Escherichia coli* lipopolysaccharide (LPS) (serotype o111:B4, Sigma Aldrich Inc., St. Louis, MO). The unchallenged group was injected with saline solution. Twelve hours after LPS/saline injection, blood samples were collected and serum was isolated and stored as described above. To measure systemic innate immune response of broilers challenged with LPS, 6 birds per treatment were euthanized by cervical dislocation, and the spleen was rinsed in saline, snap frozen, and stored at -80°C for gene expression analysis.

Serological Analysis

Serum levels of immunoglobulin were analyzed by the Sandwich ELISA (enzyme-linked immunosorbent assay) technique using the starter accessory kit and chicken IgG, IgM, and IgA ELISA Quantitation Set (Bethyl Laboratories, Montgomery, TX) following the manufacturer's instructions. The plates were read using an ELISA micro plate reader (Bio-Tek Instruments Inc., Winooski, VT) at 450 nm, and serum antibody concentration was calculated using Gen 5

software. Detection of specific systemic antibody response (IgG, IgM, and IgA) against LPS in sera was performed by indirect ELISA. Briefly, each well of a flat-bottomed 96-well microplate was coated overnight with 100 µl coating buffer (0.1 M $NaHCO_3$, pH 9.6) containing LPS (30 µg/ml) at 4ºC. Wells were then washed five times with 200 µl of washing solution (50 mM Tris, 0.14 M NaCl, pH 8.0, 0.05% Tween 20) and were completely decanted between each washing step. Afterwards the wells were covered by blocking buffer (50 mM Tris, 0.14 M NaCl, pH 8.0, 1% BSA) and the plate was incubated for 2 h at room temperatures. Washing was repeated, by addition of 100 µl of chicken serum (diluted 1:10 in blocking buffer) to each well. Plates were incubated 2 hours at room temperature and then were washed five times with washing solution. One hundred µl of detection antibody (goat anti-chicken IgG-Fc, IgM-Fc, and IgA-Fc) conjugated with horseradish peroxidase diluted in 30 ml of blocking buffer and was added to each well and incubated for 1 h at room temperature. Washing was repeated, and was followed by addition of 100 µl of tetramethyl benzidine solution as substrate to each well and incubatted for 15 min in the darkness. Finally, the stop solution was added to prevent further color development, and absorbance was measured at 450 nm using the micro plate reader.

Total RNA Extraction and Reverse Transcription

Total RNA was extracted from individual spleen and cecal tonsil samples using the Trizol extraction method as described by the manufacturer (Invitrogen Canada Inc., Burlington, ON, Canada). The quantity and purity of the RNA samples was measured by using NanoDrop spectroscopy (Thermo Scientific) and the ratio of absorbance at 260 nm and 280 nm. In this procedure, a ratio of ~2.0 is generally accepted as "pure' for RNA, and a ratio lower than 2.0 (i.e., ~1.59 and under) may indicate the presence of phenol, protein and other contaminants.

Reverse-Transcription was performed by using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol.

Quantitative Real-Time PCR

Primer sequences for β-Actin, TLR2 type 2 (henceforth known as TLR2b), TLR4, TLR21, macrophage mannose receptor (MMR), interleukins IL-12p35, IL10, IL-18, IL-6 IL-4, and interferon gamma (IFNγ), were designed using GeneBank database sequences from the National Center for Biotechnology Information (Bethesda, MD) (Table 5.2). Quantitative real-time (qRT) PCR was performed using the Step One Thermo Cycler (Applied Biosystem, Mississauga, ON, Canada) on 48-well plates with 25 µL of total reaction volume as described by Pfaffl and Hageleit (2001). The iTaq Universal SYBR Green Supermix was used as the QRT-PCR master mix and each reaction was run in duplicate. The PCR cycling protocol included an initial denaturation step at 95°C, followed by amplification for 40 cycles at 95°C for 10 s, an annealing step at a temperature described in Table 5.2 for each of the primer pairs, and extension at 72°C for 10 s.

Statistical Analysis

Growth performance and gut histomorphology parameters were analyzed according to a completely randomized design by ANOVA using the GLM procedures of SAS (SAS Institute, 2003). The significance of differences between means was determined using Scheffe's procedure. For the blood parameters, *P-value* are presented after log transformation when errors deviation did not have homogenous variance across the treatments, and the differences among the treatment means were determined using Duncan's multiple comparison procedures. For the gene expression analysis, the cycle threshold values were recorded and uploaded to the REST-

2009 Software (Qiagen, Valencia, CA). Relative expression levels of all genes were calculated relative to housekeeping gene (β-actin in the present study), and gene expression was presented as fold changes relative to the control diet. Gene expression fold changes, standard error, and statistical significance were calculated by the software based on the formula developed by Pfaffl et al. (2002). A *P*-value of less than 0.05 was considered significant.

5.4 Results

Growth Performance

Results for feed intake (FI), body weight gain (BWG), FCR and relative weight of immune organs bursa and spleen are summarized in Table 5.3. There were no significant differences in FI, BWG, and FCR among the dietary treatments during the experimental period (1 to 21 d). However, diets containing YCW, Maxi-Gen Plus and nucleotides tended (P = 0.06) to improve FCR compared with the Control diet. Furthermore the relative weight of bursa and spleen were not affected by diets ($P > 0.05$).

Gene Expression Analysis

The fold change gene expression of pattern recognition receptors acquired from QRT-PCR, are presented in Fig. 5.1. Immunization with LPS did not change the expression of PRRs in the spleen ($P > 0.05$). The expression of TLR2b and TLR4 was not affected by dietary treatments. In addition, diets supplemented with antibiotic or Maxi-Gen Plus did not influence the expression of PRRs (TLRs and MMR) in the spleen of broiler chickens ($P > 0.05$). However, expression of TLR21 and MMR were significantly upregulated in the DDGS and YCW groups compared to the Control ($P < 0.05$). The results for cytokine gene expression analysis obtained from QRT-PCR are shown in Fig. 5.2. The expression of IL-6 and IL-18 was significantly upregulated in spleen samples of the control group versus the unchallenged group. The

expression of IFNγ, IL-6 and IL-18 was increased in birds fed the diet containing DDGS. Furthermore, birds receiving the diet containing Maxi-Gen Plus showed upregulation of IL-6 and IL-18. The diet containing YCW significantly increased the expression of all the cytokines in the spleen of the birds. No significant difference was observed in gene expression of cytokines in birds fed diets supplemented with antibiotic or nucleotides compared with control.

Serum Antibody and Specific Antibody Response

The results for serum IgG, IgM, and IgA levels are presented in Fig. 5.3. No significant difference was observed for serum IgG and IgM levels among the diets ($P > 0.05$). However, the diet supplemented with YCW significantly increased serum concentration of IgA compared with the antibiotic group ($P < 0.05$). The results for serum specific IgG, IgM, and IgA against LPS are shown in Fig. 5.4. The birds receiving the control diet and challenged with LPS showed higher serum IgG and IgA levels compared to the unchallenged group. However, no significant difference was observed for serum IgM level for the Control challenged group vs. the unchallenged group. Regardless of immunization, serum levels of specific IgG, IgM, and IgA against LPS were not affected by dietary treatments.

5.5 Discussion

In the present study, inclusion of diets with yeast-derived products did not significantly influence the FI and BWG of broiler chickens. However, diets containing YCW, Maxi-Gen Plus and nucleotides tended to improve FCR over the Control group. This finding is in agreement with Morales-Lopes and Brufau (2013) who showed that supplementation of *Saccharomyces cerevisiae* cell walls did not affect the FI and BWG, however FCR at both 21 and 28 d of age was improved compared to the Control group. In contrast, Baurhoo et al. (2009) demonstrated

that dietary inclusion of yeast-derived carbohydrates did not significantly influence growth performance parameters of broiler chickens in a non-infectious setting. The inconsistent results regarding the effects of yeast-derived products on growth performance of broiler chickens could be associated with experimental and environmental conditions as well as the type and concentrations of yeast products used in different studies. In the current study, the improvement in FCR of birds receiving yeast-derived products might be explained by the effects of these products on gut development and in removal of pathogenic bacteria such as *E. coli* and *Salmonella* (Ghosh et al., 2012; Jung and Batal, 2012). The lack of a significant effect of yeast-derived products on FI and BWG observed in this study suggests that these products do not exert any strong growth-promoting effect under non-challenged conditions. However, yeast-derived products might be able to yield more beneficial effects under pathogen challenge conditions.

Considering the fact that the spleen, as an immune organ, can act both as reservoir and activation site for leukocytes, gene expression profiling of the spleen can monitor a systemic immune response (Redmond et al., 2010). In the current study, we measured the expression of toll-like receptors TLR2, TLR4, and TLR21, and MMR in the spleen of birds following immunization with LPS. Toll-like receptors are PRRs expressed by cells of the innate immune system such as macrophages and dendritic cells and play an essential role in innate immunity by recognition of conserved molecular patterns known as microbe-associated molecular patterns (MAMP) (Cormican et al., 2009; Blasius and Beutler, 2010; Brownlie and Allan, 2011). In addition to TLRs, MMR is a transmembrane protein belonging to the C-type lectins which is involved in recognition of high mannose structures on the surface of potentially pathogenic bacteria and fungi (Stahl and Ezekowitz, 1998; Apostolopoulos and McKenzie, 2001; Gazi and Martinez-Pomares, 2009). Recognition of MAMP by TLRs and MMR leads to the activation of cells of

innate immunity and production of cytokines that develop further activation of immune response. (Trinchieri and Sher, 2007; Gaiz and Martinez-Pomares, 2009). In the present study, higher expression of TLR21 and MR was observed in birds receiving YCW or DDGS diets compared to the Control. Upregulation of TLR21 and MR in birds receiving DDGS or YCW might be associated with a significant quantity of yeast cell wall components (i.e., mannan, β-glucans) in these products (Pelizon, et al., 2005; Morales-Lopez et al., 2009; Shashidhara and Devegowda, 2003). The results of previous studies demonstrated that cell wall components of yeast including mannan and ß1,3-1,6-glucan are able to stimulate the immune response of broiler chickens (Morales-Lopez et al., 2009; Gomez-Verduzco et al., 2009; Guo et al., 2003). Yeast cell wall polysaccharides can possibly act as immune-adjuvants and stimulate innate immune response following microbial challenge. In agreement with our results, Yitbarek et al. (2012) showed that dietary inclusion of yeast-derived carbohydrates in diets of broilers challenged with *Clostridium perfringens,* upregulated the expression of TLR2 and TLR4 in the ileum compared to the Control. In addition to PRRs, cytokines play a critical role in regulation of innate immunity. Cytokines are small cell-signalling protein molecules which are secreted by numerous cells of the immune system and used for inter-cell communication (Dinarello, 2007; Plat and Mensink, 2005). The results of cytokine gene expression analysis demonstrated that regardless of diet, challenging with LPS upregulated the expression of inflammatory cytokines IL-6 and IL-18 compared to the unchallenged group. Interleukin-6, an important mediator of fever, is a pro-inflammatory cytokine mainly produced by macrophages and involved in innate immune response by stimulation of acute phase protein synthesis; and IL-18 is a potent pro-inflammatory cytokine mostly produced by macrophages that together with IL-12 have a profound effect on T-cell activation following exposure to pathogens (Akira, 2000). Upregulation of IL-6 and IL-18 in

the spleen of birds challenged with LPS suggests that injection of birds with LPS (3 mg/kg of BW) successfully challenged the birds and caused an inflammatory response. In agreement with our results, Shen et al. (2010) showed that the plasma interleukin-6 level of the LPS-treated group (3 mg/kg of BW) was significantly increased compared to that of the unchallenged group. In addition to IL-6 and IL-18, we measured the expression of Th1 cytokines including IFNγ, IL-12 as inflammatory cytokines and Th2 cytokines including IL-4, and IL-10 as anti-inflammatory cytokines in the spleen of birds. Interferon-gamma and IL-12 represent T helper1-like response and support the differentiation of naive Th0 to Th1 cells (Hsieh et al., 1993; Young et al., 1995; Schoenborn and Wilson, 2007). On the other hand, IL-10 and IL-4 represent Th2- like response and induce differentiation of naive $CD4^+$ T helper cells to effector Th2 (Luzina et al., 2012; Saraiva and O'Garra, 2010). In this context the diet containing YCW upregulated the expression of inflammatory cytokines IFN-γ, IL-12, IL-6, and IL-18, and anti-inflammatory cytokines IL-10 and IL-4 compared with Control group. Furthermore, birds receiving the DDGS diet showed upregulation of IFNγ, IL-6 and IL-18 compared to the Control group. Upregulation of cytokines in the spleen of birds fed the diets supplemented with the YCW and DDGS might be associated with higher expression of MMR and TLR21 in these birds. It has been demonstrated that activation of PRRs by MAMP would result in the production of cytokines that can link innate immunity to acquired immunity (Akira et al., 2006). Upregulation of both inflammatory and anti-inflammatory cytokines in the YCW group might be explained by the immunological balance between Th1 and Th2 cytokines. The balance between IL-4 and IL-12 determines Th1 or Th2 responses. Interleukin-12 stimulates the production of IFN-γ and reduces IL-4 mediated suppression of IFN-γ (Trinchieri and Scott, 1994; Wolf et al., 1994). On the other hand, IL-4 is an anti-inflammatory mediator that downregulates the expression of Th1 cytokines such as IFN-γ

and IL-12 (King et al., 1993). As with our results, Yitbarek, et al. (2012) showed that supplementation of diets with yeast-derived products in broiler chickens under a *Clostridium perfringens* challenge increased the expression IFN-γ and IL-12. In contrast, Munyaka et al. (2012) reported that supplementation of diets with yeast-derived products reduced the expression of both Th1 and Th2 cytokines in broiler chickens. The variations in immunomodulatory properties of yeast-derived products might be related to the different experimental models (challenged vs. non-challenged) applied in these studies.

Humoral immunity is defined as a component of the immune system which is mainly mediated by antibodies. Antibodies are involved in host defence immune response through different strategies including immobilization and opsonisation of pathogenic bacteria (Janeway, 2001). In the immune system, IgG is considered as the most abundant antibody isotype in blood circulation and has a very high affinity for antigens compared to the other antibody isotypes (Schwab and Nimmerjahn, 2013); whereas IgM is the first immunoglobulin released by B cells in response to initial exposure to antigens and plays a key role in innate immune response against early infection (Boes, 2000). In the present study, serum levels of IgG and IgM were not affected by dietary treatments. However, the serum level of IgA, an antibody that plays a critical role in mucosal immunity (Lamm et al, 1995), was increased in the diet supplemented with YCW compared to antibiotic. This suggests that supplementation with yeast cell wall polysaccharides (mannan and β 1,3-1,6-glucan) can improve mucosal immune response in broiler chickens. Yeast cell wall polysaccharides can bind directly to the receptors on the surface of B cells and activate these cells in a T cell-independent manner (Parker, 1993; Mond et al., 1995). Furthermore yeast cell walls can induce antibody-mediated immune response through binding to C-type lectin receptors such as the mannose receptor and dectin-1 on the surface of macrophages/dendritic

cells (Porcaro et al., 2003; Dillon et al., 2006). Recognition of yeast cell wall by C-type lectin receptors would activate the macrophages/dendritic cells and facilitate the process of phagocytosis, and presentation of these antigens to naive B cells (Gazi and Martinez-Pomares 2009). In agreement with these findings, the gene expression of IL-10 and IL-4 as cytokines involved in activation of B cells and antibody production was also upregulated in the spleen of birds fed the diet containing YCW. Considering the fact that yeast cell wall polysaccharides are not considered as invasive antigens, presentation of these components as antigens by cells of innate immunity generates IgA-switched B cells. This might be the reason why serum IgA, a noninflammatory and poor opsonin antibody, was present at a higher level in birds receiving YCW compared to the antibiotic group (Cerutti and Rescigno, 2008). Regarding the role of commensal bacteria in shaping and development of the immune system, and the fact that germ-free animals showed impaired development of the immune system and weaker immune responses compared to conventional animals (Macpherson et al., 2000; Rhee et al., 2004), lower IgA concentration in the serum of birds fed the antibiotic-containing diet may be explained by the antibacterial activity of such products that limit bacterial over-growth and reduce bacterial immune stimulation at the intestinal level (Collier et al., 2003; Rhee et al., 2004).

In the current study, specific antibody response in broilers following challenge with LPS, as a major component of the outer membrane of Gram-negative bacteria, was also measured (Xie et al., 2000). Naive B cells can be activated in a T-cell dependent or independent manner (Parker et al., 1993; Vos et al., 2000). Polysaccharides such as LPS are considered as T-independent antigens because they can directly stimulate the B cells to release antibody without T cell involvement (Gehad et al, 2002). Following specific antibody-mediated immune response, antibodies identify and neutralize specific antigens (LPS in this study) and cause agglutination

and precipitation of an antibody-antigen complex that facilitates the process of phagocytosis by antigen presenting cells (Vos et al., 2000; Xie et al., 2000). It has been reported that macrophages and monocytes play an essential role in the inflammatory response induced by LPS and activation of B cells in a T-independent manner (Xie et al., 2000; Gehad et al., 2002). Interaction of LPS with the cluster of differentiation 14 (CD14) receptors expressed by macrophages would result in the production of pro-inflammatory cytokines such as IL-6 and TNFα that mediate the host immune response to LPS (Shen et al., 2010; Lu et al., 2008). In the current study, stimulation of the immune system by LPS increased the fold change gene expression of IL-6 and IL-18 in the spleen of broiler chickens. Regarding the specific antibody response, although immunization increased specific IgG and IgA response to LPS, the specific IgM response was not different between the Control, challenged vs. unchallenged groups. This is may be explained by the high avidity nature of IgM that allows it to bind to specific antigens, even without prior immunization (Vollmers and Brandlein, 2005). Regardless of immunization, specific antibody-mediated immune responses, including IgG, IgM, and IgA, were not affected by dietary treatments. This may indicate that yeast-derived products may not be involved in B cell activation following immunization with LPS. In the other words, a combination of yeast-derived products and LPS as a T cell-independent antigen did not lead to further activation of the immune system. It is also possible that with high variability and the dynamic nature of antibodies, and given the fact that antibody production was only measured at one end point (d 21) in this study, we may have missed any possible initial effects of yeast products on specific antibody production. In agreement with our results, Silva et al., (2009) did not observe any difference in antibody response against Newcastle disease virus (NDV) or infectious bursal disease (IBD) in broiler chickens fed a diet supplemented with YCW. In contrast, Gao et al.

(2008) reported that diets supplemented with the yeast extract influenced humoral immune responses of birds and increased antibody titre against Newcastle disease.

In conclusion, our results showed that supplementation of diets with yeast-derived products did not influence feed intake and body weight gain. However, diets containing yeast-derived products tended to improve FCR over the Control diet suggesting the potential growth promoting effects of these products. In addition, inclusion of the diet containing YCW stimulated cell-mediated innate immune response by increasing the expression of PRRs TLR21 and MMR and inflammatory cytokines IFN-γ, IL-6 and IL-18. Furthermore, the DDGS diet that contained a significant quantity of YCW components increased the expression of PPRs MMR, TLR21 and cytokines IL-6, IL-18, and IFN-γ. Regarding humoral immunity, although the specific antibody response was not affected by dietary treatments, supplementation of the diet with YCW increased the serum IgA level and the gene expression of the cytokines IL-10 and IL-4 involved in B cell activation and antibody production. This suggests that YCW products may have immune adjuvant-like properties and are able to stimulate immune response in broilers under pathogen challenge conditions.

Table 5.1. Composition and calculated analysis of the basal diet and the diet containing 8% of distillers dried grains with solubles (DDGS) (%)

Item	Basal diet	DDGS diet
Ingredient		
Wheat	19.5	15.3
Corn	35.0	35.2
Canola meal	5.0	3.0
Soybean meal	31.2	29.5
DDGS	-	8.0
Canola oil	4.4	4.1
Calcium carbonate[1]	1.41	1.53
Dicalcium phosphate[2]	1.6	1.40
DL-Methionine	0.10	0.10
L-Lysine-HCL	-	0.07
Mineral premix[3]	0.5	0.5
Vitamin premix[4]	1.0	1.0
Titanium oxide	0.3	0.3
Total	100.0	100.0
Calculated analysis		
ME, kcal/kg	3034	3030
CP, %	21.9	22.0
Calcium, %	1.0	1.0
Available phosphorous, %	0.45	0.45
Lysine, %	1.19	1.18
Methionine, %	0.49	0.49
Methionine + cystine, %	0.84	0.84
Threonine, %	0.84	0.83

[1]Contained 38% calcium.
[2]Contained 21% calcium and 18% phosphorous.
[3]Mineral premix provided per kilogram of diet: Mn, 70 mg; Cu 10 mg; Fe, 80 mg, Zn, 80 mg; Se, 0.3 mg; I, 0,5 mg; and Na, 1.7 g.
[4]Vitamin premix provided per kilogram of diet: A, 8,255 IU; vitamin D_3, 3000 IU; vitamin E, 30.0 IU; vitamin B_{12}, 0.013 mg; vitamin K, 2.0 mg; niacin, 24.5; choline, 1081 mg; folic acid 4.0 mg; biotin, 0.25 mg; riboflavin, 6.0 mg

Table 5.2. Primer sequences used for the real-time quantitative PCR[1]

Gene[2]	Primer sequence (5'-3')[3]	Fragment size (bp)	Annealing temperature (°C)	GenBank accession number
TLR2b	F: CGCTTAGGAGAGACAATCTGTGAA R:GCCTGTTTTAGGGATTTCAGAGAATTT	90	59	NM204278
TLR4	F: AGTCTGAAATTGCTGAGCTCAAAT R: GCGACGTTAAGCCATGGAAG	190	55	AY064697
TLR21	F: TGGCGGCGGGAGGAAAAGTG R: CACCGTGCTCCAGCTCAGGC	106	59	NM_001030558
MMR	F: GCAGGGCACGTTCAGGTGGG R: GCCACACAGCCTGGCTCCCT	90	60	XM001235105
IFN-γ	F: CTGAAGAACTGGACAGAGAG R: CACCAGCTTCTGTAAGATGC	264	60	X99774
IL-12p35	F: CTGAAGGTGCAGAAGCAGAG R: CCAGCTCTGCCTTGTAGGTT	217	64	NM213588
IL-10	F: AGCAGATCAAGGAGACGTTC R: ATCAGCAGGTACTCCTCGAT	103	55	AJ621614
IL-4	F: TGTGCCCACGCTGTGCTTACA R: CTTGTGGCAGTGCTGGCTCTCC	193	57	GU119892
IL-18	F:GAAACGTCAATAGCCAGTTGC R:TCCCATGCTCTTTCTCACAACA	213	53	AY628648.2
IL-6	F: CAGGACGAGATGTGCAAGAA R: TAGCACAGAGACTCGACGTT	233	59	AJ309540
β-Actin	F: CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	205	61	X00182

[1]The listed oligonucleotides were used to analyze gene expression via real-time quantitative PCR.

[2]TLR = Toll-like receptor; MMR = monocyte/macrophage mannose receptor; IFN = interferon; IL= interleukin.

[3]F = forward; R = reverse.

Table 5.3. Effects of diets containing yeast-derived products and distillers dried grains with solubles (DDGS) on growth performance and relative weight of immune organs of broiler chickens from 1 to 21 d of age

Item	Treatment[1]						SEM	*P*-value
	Control	Antibiotic	Yeast Cell Wall	Maxi-Gen Plus	Nucleotides	DDGS		
Feed intake (g/bird)	1042.2	1039.2	1030.0	996.5	991.4	1045	5.21	0.95
Body weight gain (g/bird)	750.7	764.6	783.4	762.4	751.1	762.1	9.02	0.23
Feed conversion ratio	1.39	1.36	1.32	1.31	1.32	1.37	0.009	0.06
Spleen*	0.08	0.09	0.08	0.08	0.09	0.07	0.003	0.74
Bursa of Fabricius*	0.25	0.25	0.28	0.22	0.23	0.27	0.008	0.25

[a,b] Means within a column with no common superscripts differ significantly (P< 0.05).

[1]Experimental diets included: Control (C) = with no antibiotic; Antibiotic = C + 11mg/kg of virginiamycin; Yeast Cell Wall = C + 0.25% of yeast cell wall polysaccharides; Maxi-Gen Plus = C + 0.2 % of a commercial product containing processed yeast and nucleotides; Nucleotides = C + 0.05% of a nucleotide-rich products; DDGS = 8% of wheat/corn distillers dried grains with solubles.

* Represents percentage organ weight relative to body weight.

5.6 Figure and Legend

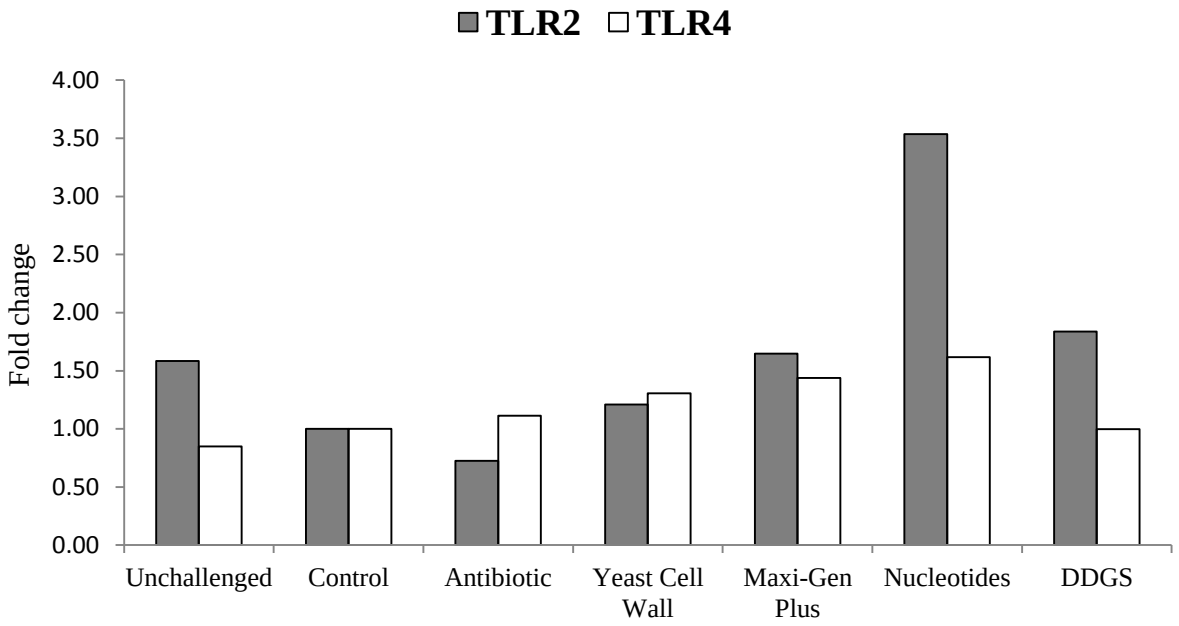
Figure 5.1 Fold change expression of Toll-like receptors TLR2b, TLR4, TLR-21, and macrophage mannose receptor (MMR) in the spleen of broiler chickens immunized intraperitoneally with 3 mg/kg of BW of *Escherichia coli* LPS when fed the following diets: Control (C) = no antibiotic; Antibiotic = C + 11 mg/kg of virginiamycin; Yeast Cell Wall = C + 0.25% of yeast cell wall polysaccharides; Maxi-Gen Plus = C + 0.2% of a commercial product containing processed yeast and nucleotides; Nucleotides = C + 0.05% of nucleotides; and DDGS = 8% of wheat/corn distillers dried grains with solubles. *Bars with asterisks differ significantly from the control group. Results were considered statistically significant from the control group if $P < 0.05$.

Figure 5.2 Fold change expression of cytokines IL-10, IL-4, IL-18, IL—6, IFNγ, and IL-12 in the spleen of broiler chickens immunized intraperitoneally with 3 mg/kg of BW of *Escherichia coli* LPS when fed the following diets: Control (C) = no antibiotic; Antibiotic = C + 11 mg/kg of virginiamycin; Yeast Cell Wall = C + 0.25% of yeast cell wall polysaccharides; Maxi-Gen Plus = C + 0.2% of a commercial product containing processed yeast and nucleotides; Nucleotides = C + 0.05% of nucleotides; and DDGS = 8% of wheat/corn distillers dried grains with solubles. *Bars with asterisks differ significantly from the control group. Results were considered statistically significant from the control group if $P < 0.05$.

Figure 5.3 Serum IgG, IgM, and IgA antibody titers as determined by ELISA on d 21 in birds fed the following diets: Control (C) = no antibiotic; Antibiotic = C + 11 mg/kg of virginiamycin; Yeast Cell Wall = C + 0.25% of yeast cell wall polysaccharides; Maxi-Gen Plus = C + 0.2% of a commercial product containing processed yeast and nucleotides; Nucleotides = C + 0.05% of nucleotides; and DDGS = 8% of wheat/corn distillers dried grains with solubles. Error bars represent standard errors. ^{ab}Means with no common superscripts differ significantly ($P < 0.05$).

Figure 5.4 Serum level of IgG, IgM, and IgA antibodies against *Escherichia coli* LPS as determined by indirect ELISA on d 21 in birds fed the following diets: Control (C) = no antibiotic; Antibiotic = C + 11 mg/kg of virginiamycin; Yeast Cell Wall = C + 0.25% of yeast cell wall polysaccharides; Maxi-Gen Plus = C + 0.2% of a commercial product containing processed yeast and nucleotides; Nucleotides = C + 0.05% of nucleotides; and DDGS = 8% of wheat/corn distillers dried grains with solubles. Error bars represent standard errors. ^{ab}Means with no common superscripts differ significantly ($P < 0.05$).

Figure 5.1. Fold change gene expression of pattern recognition receptors in spleen of birds challenged with lipopolysaccharide (LPS)



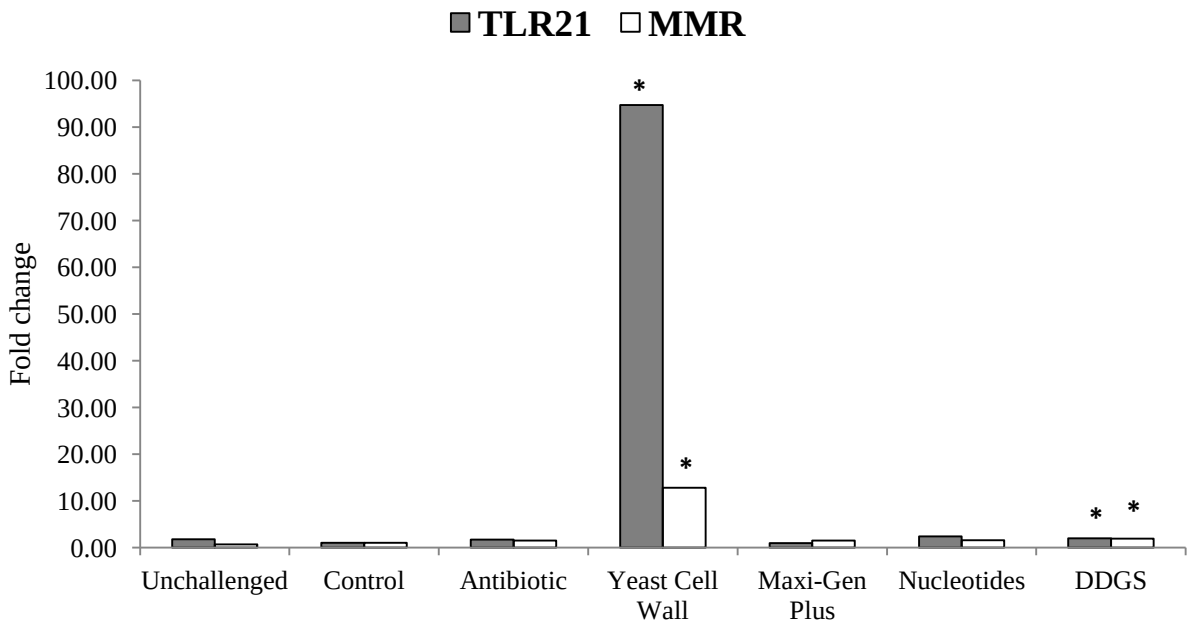
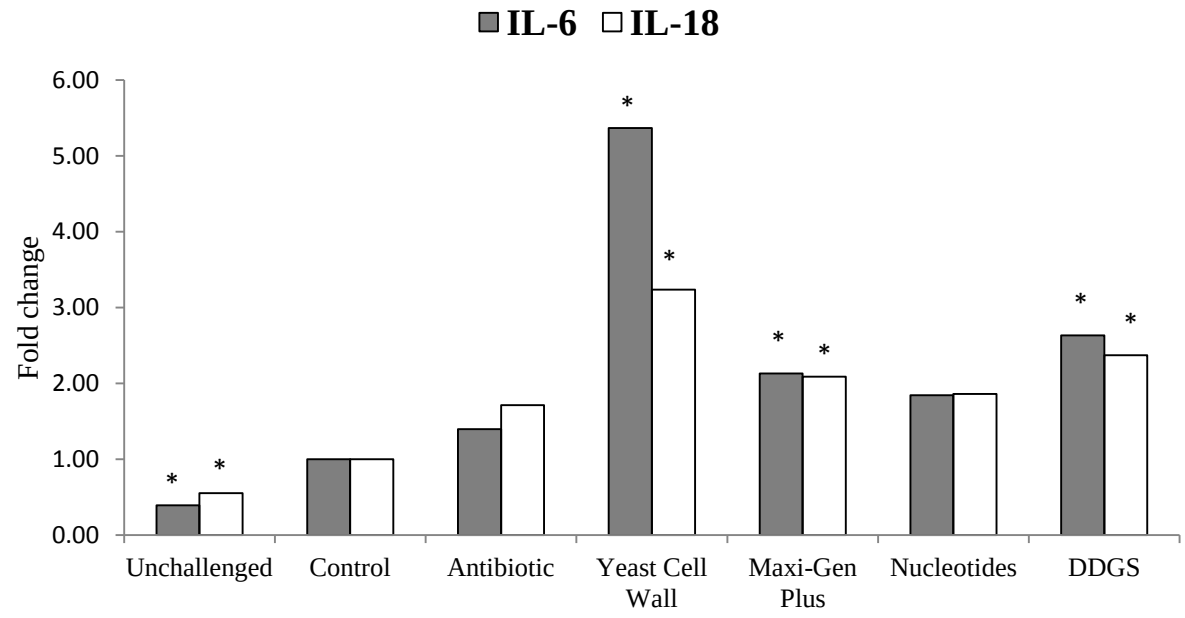


Figure 5.2. Fold change gene expression of cytokines in spleen of birds challenged with lipopolysaccharide (LPS)



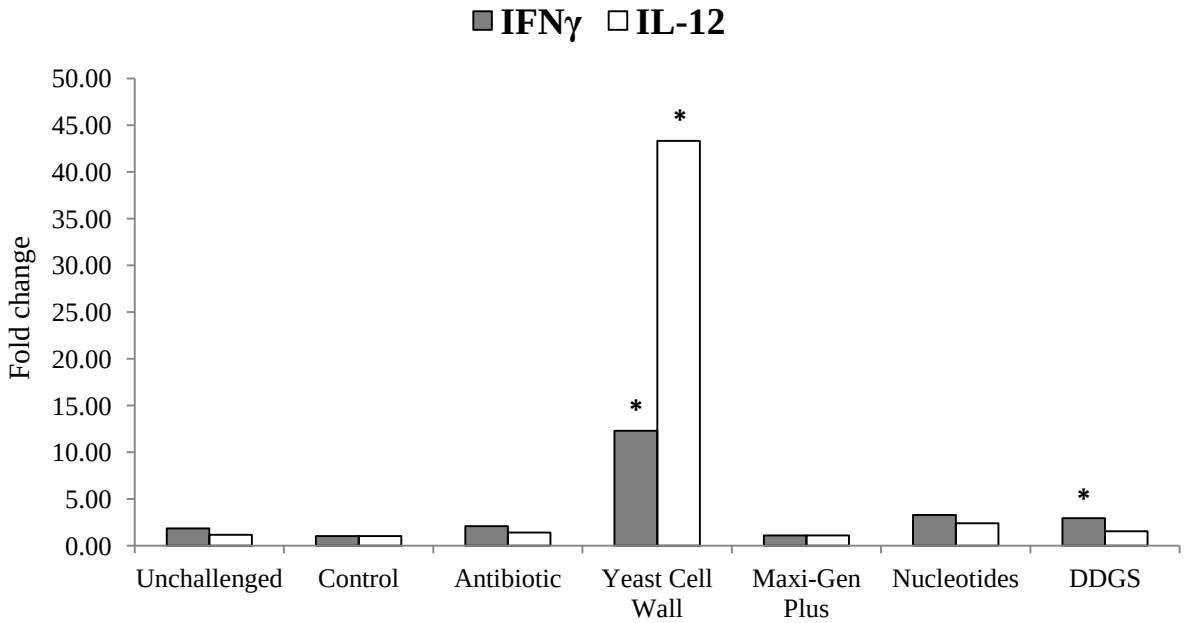


Figure 5.2 Continued. Fold change gene expression of cytokines in spleen of birds challenged with lipopolysaccharide (LPS)

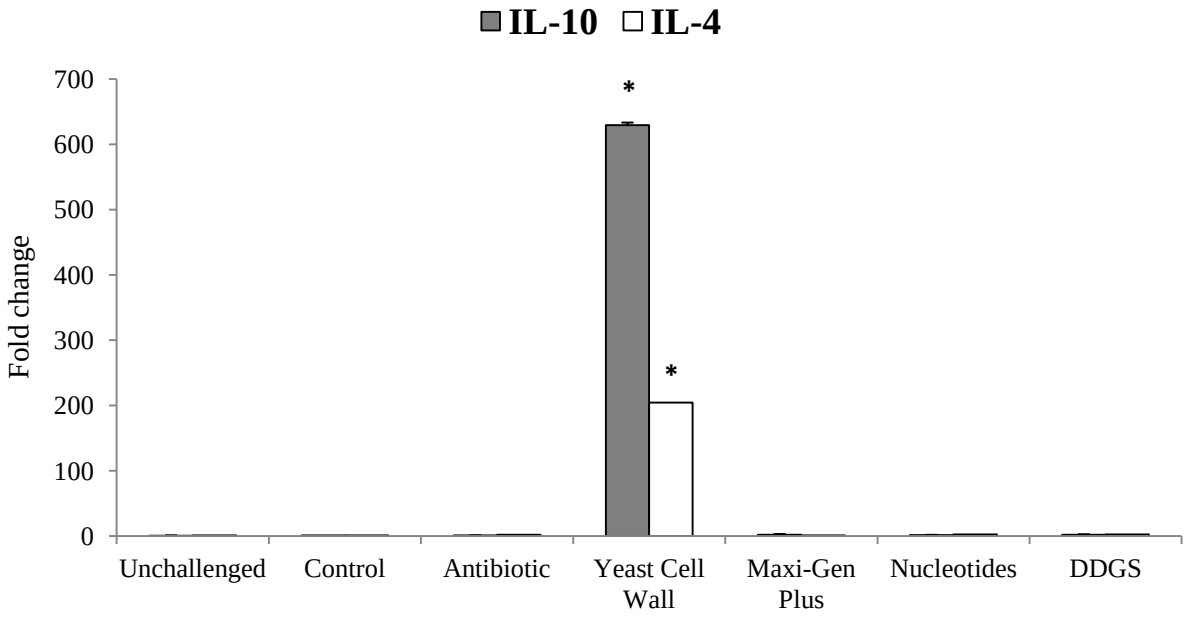


Figure 5.3. Serum IgG, IgM, and IgA levels of birds fed the diets containing yeast-derived products and DDGS (d 21)

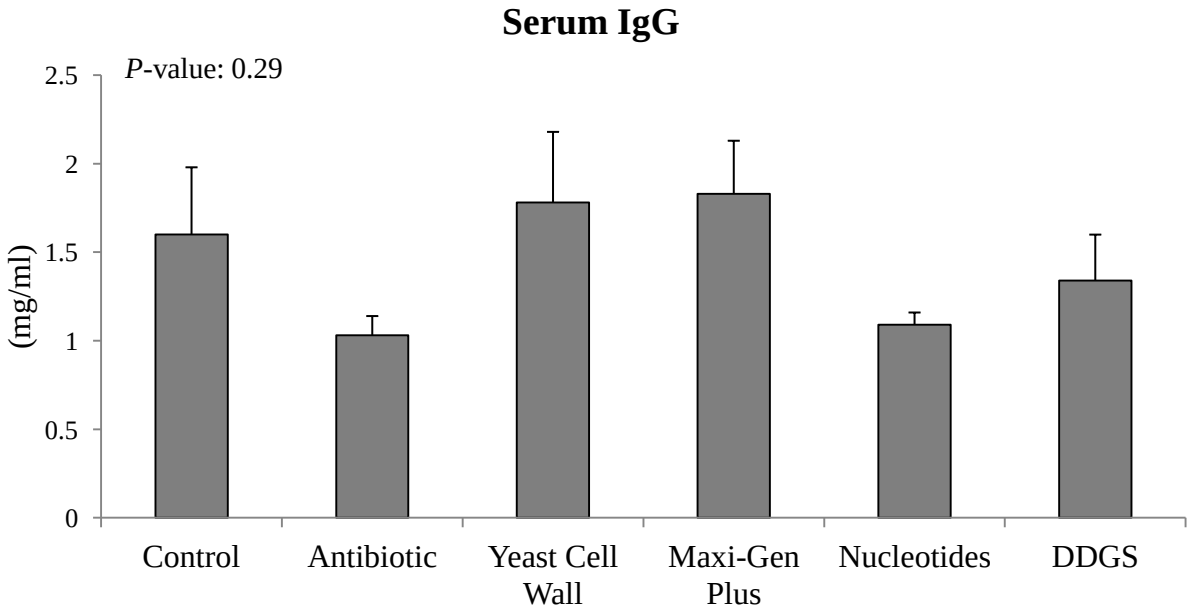
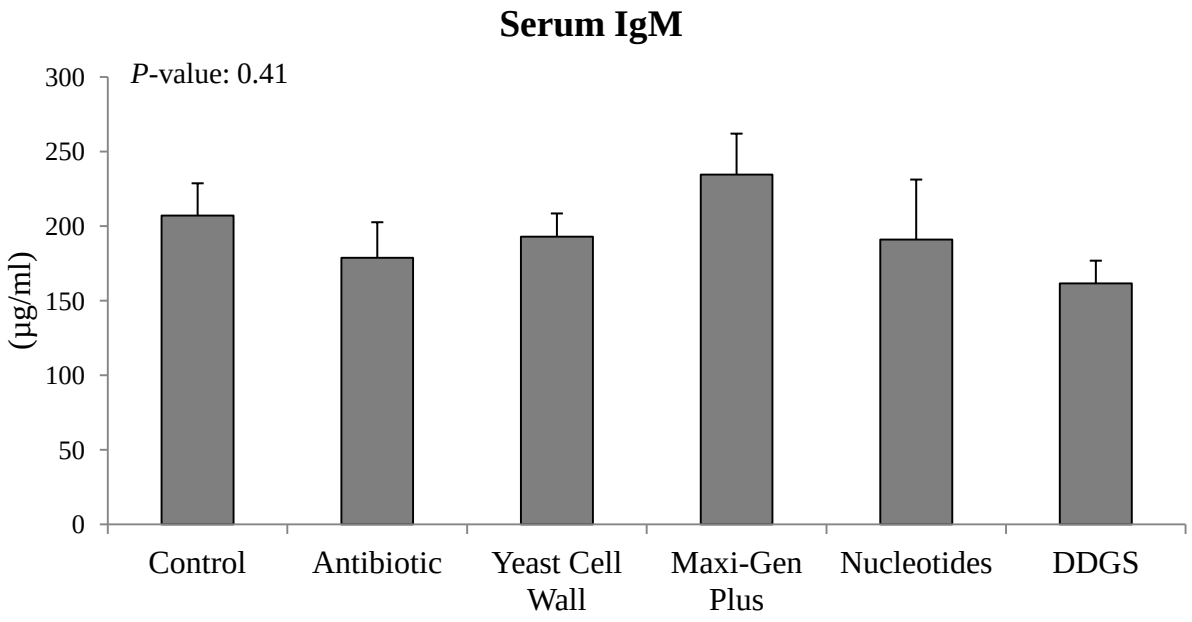


Figure 5.3 Continued. Serum IgG, IgM, and IgA levels of birds fed the diets containing yeast-derived products and DDGS (d 21)



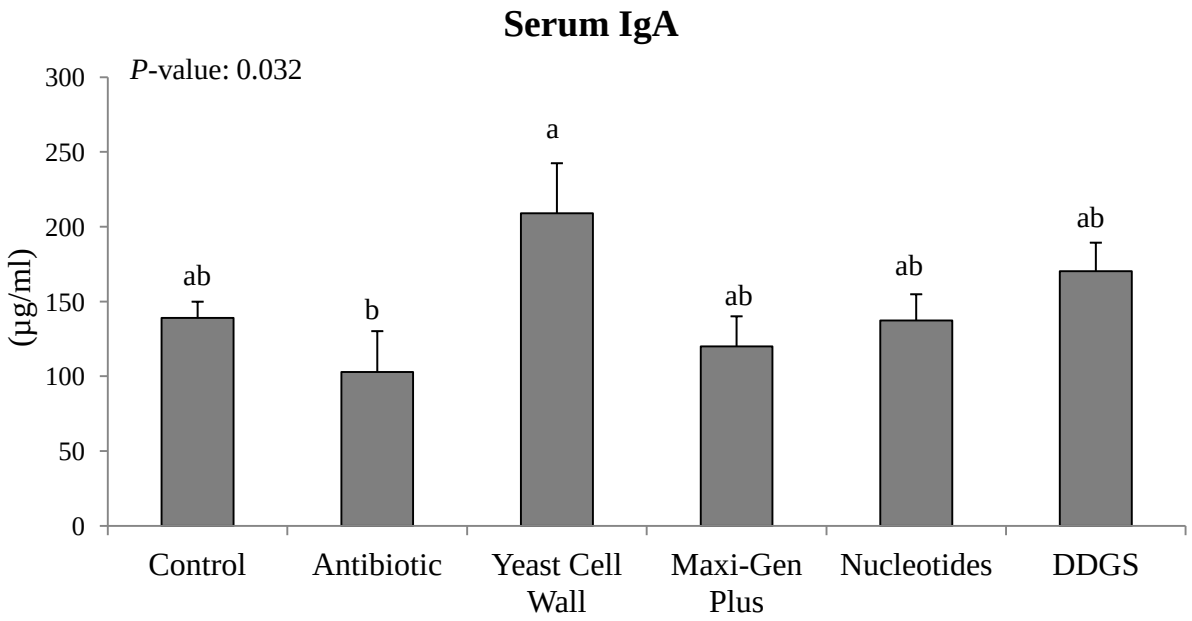
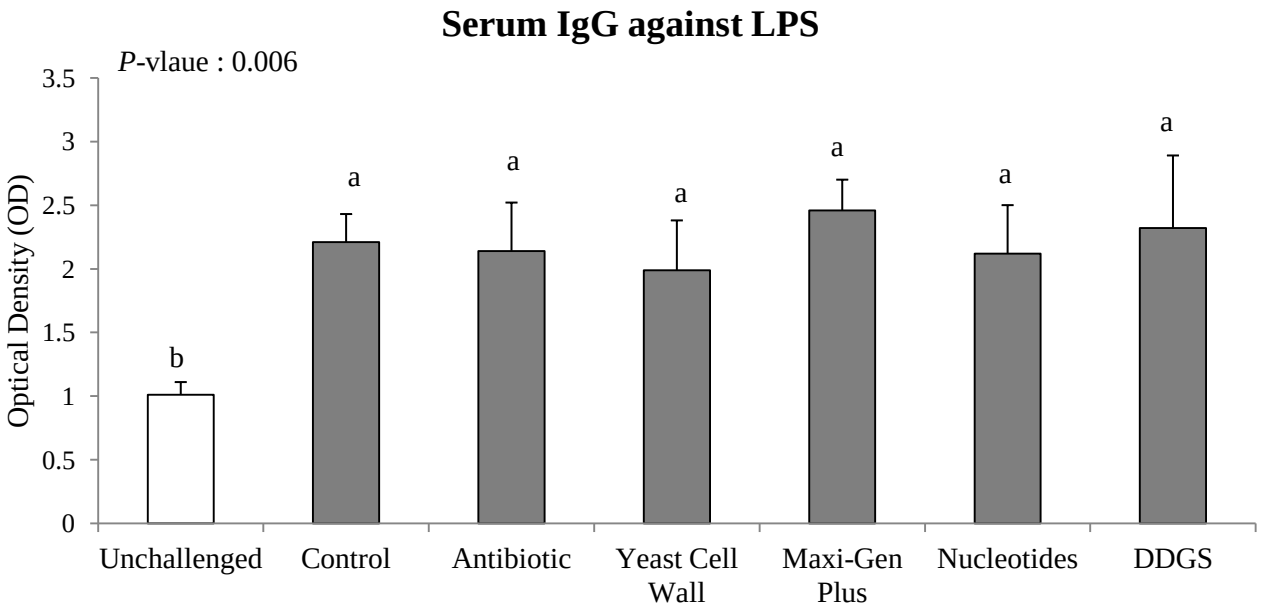


Figure 5.4. Serum specific IgG, IgM, and IgA responses against lipopolysaccharide (LPS) in birds fed diets containing yeast-derived products and DDGS (d 21)



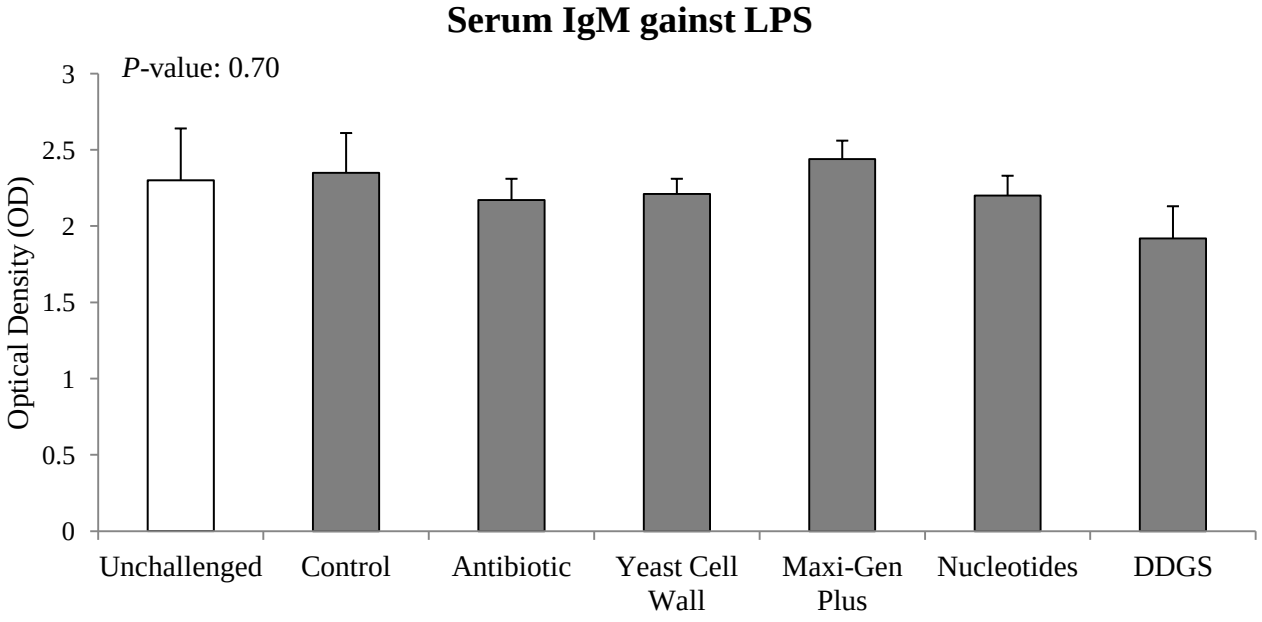
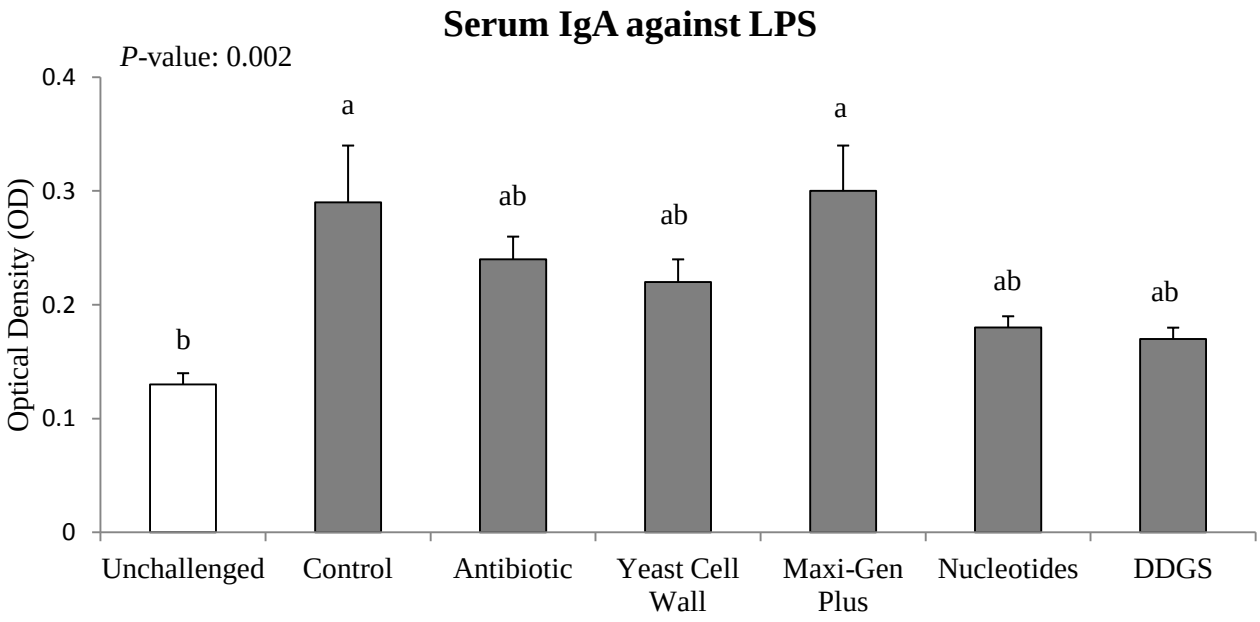


Figure 5.4 Continued. Serum specific IgG, IgM, and IgA responses against lipopolysaccharide (LPS) in birds fed diets containing yeast-derived products and DDGS (d 21)



CHAPTER 6: MANUSCRIPT IV

Effect of yeast-derived products and distillers dried grains with solubles (DDGS) on growth performance and local innate immune response of broiler chickens challenged with *Clostridium perfringens*

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6.1. Abstract

An experiment was conducted to assess the effect of yeast-derived products and DDGS on growth performance, gut lesion score, intestinal population of *Clostridium perfringens*, and local innate immunity of broiler chickens challenged with *C. perfringens*. Male Ross-308 chickens were assigned to 8 dietary treatments in a randomized complete block design providing 6 replicate pens of 55 birds each per treatment. The antibiotic and coccidiostat -free corn/SBM/canola meal diets were formulated to meet the NRC requirements of broiler chickens. Dietary treatments consisted of two Control corn/SBM diets without and with *C. perfringens* challenge, and diets containing antibiotic (bacitracin methylene disalicylate (BMD), 55 g/tonne), nucleotides (150 g/tonne), yeast cell wall (300 g/tonne), and a commercial product Maxi-Gen Plus (1 kg/tonne) fed to chickens challenged with *C. perfringens*. Corn/SBM diets containing 10% of DDGS without and with *C. perfringens* challenge were also used. The experiment lasted for 35 d and consisted of 3 phases (0-14 d, starter; 14 to 28 d, grower; 28-35 d, finisher). Birds were orally challenged with *C. perfringens* (none or 10^8 CFU/bird) on d 14. On d 21, 6 birds per treatment were euthanized by CO_2 asphyxiation and tissues from the ileum and cecal tonsils were collected to determine the mRNA expression of toll-like receptors TLR2, TLR4, TLR21, macrophage mannose receptor (MMR), and cytokines IL-4, IL-10, IL-13, IL-12p35, and IFN-γ, IL-6, and IL-18. The pathogen challenge significantly ($P<0.05$) impaired feed intake, body weight gain, and feed conversion ratio shortly after the challenge (14-21 d). Increased *C. perfringens* counts and intestinal lesion score were observed for challenged birds except the BMD-containing diet. Over the entire trial, no difference in growth performance was found among the treatments except for the BMD diet which somewhat improved feed conversion ratio over the Control, challenged diet. Regarding gene expression analysis, the diet supplemented

with nucleotides upregulated the expression of TLRs; and expression of MMR was upregulated in the ileum of the birds receiving YCW. Expression of IL-18 in the ileum was upregulated in diets supplemented with YCW and nucleotides; and the diet containing nucleotides upregulated the expression of IL-4 compared to the Control, challenge group. No significant difference was observed for expression of receptors and cytokines in the cecal tonsils, except the BMD containing diet which downregulated the expression of MMR compared to the Control, challenge group. In conclusion, although inclusion of yeast-derived products in the diet was not as an effective as antibiotic with regards to *C. perfringens* counts and lesion scores, diets supplemented with nucleotides and YCW were able to modulate the expression of intestinal cytokines and receptors in the ileum. This suggests that nucleotides and YCW components may be involved in activation of the local innate immune response in broilers under *C. perfringens* challenge condition.

Key Words: Yeast-derived products, *Clostridium perfringens*, lesion score, innate immunity, broilers

6.2 Introduction

Necrotic enteritis (NE) associated with *Clostridum perfringens* infection in the small intestine is considered the major economic disease in broiler production (Van der Sluis 2000; Van Immerseel et al., 2004). It has been reported that *C. perfringens* is part of the normal intestinal microbiota and is normally found in small numbers (i.e., 10^4 cfu/g of digesta) in the intestine of most avian species. However, overgrowth of *C. perfringens* often leads to NE which is characterized by accumulation of multiple extracellular toxins that damage the intestinal wall (Stevens and Bryant, 1997; Timbermont et al., 2011). The clinical form of the disease leads to

high mortality in broiler flocks, whereas the damaged intestinal mucosa observed in the subclinical form of *C. perfringens* infection can result in decreased production output due to decreased nutrient digestion and absorption and thus reduced weight gain and impaired feed conversion ratio (Kaldhusal and Hofshagen, 1992; Kaldhusal et al., 1999; Loveland and Kaldushal, 2001). Because the sub-clinical form of NE is usually undetected and the birds remain untreated, it can negatively affect productivity and cause economic losses for the broiler industry. Necrotic enteritis can be controlled effectively with the use of in-feed antibiotic growth promoters; however, it would be considered a risk in broiler operation where antibiotic use is not practiced (Bedford, 2000). In light of concerns about bacterial resistance and antibiotic residues in poultry products, several strategies have been introduced in the broiler industry to control the disease in the absence of antibiotic growth promoters (Bedford, 2000; Dahiya et al., 2006). Feeding strategies including dietary manipulation and admistration of probiotics and prebiotics have been used as tools to control the incidence and severity of NE in broiler chickens (Branton et al., 1997; Engberg et al., 2004; Thanissery et al., 2010).

Yeast cell wall components, including mannan and beta 1,3-1,6 glucans (β-glucans), have been known for their purported, immunomodulatory properties, intestinal health benefits, and anti-pathogenic effects (Zdunczyk et al., 2005; Solis de los Santos et al., 2007; Zhang et al., 2008; Morales-Lopez and Brufau, 2013). It has been reported that yeast cell walls (YCW) are involved in innate immune system modulation by acting as "pathogen-associated molecular patterns" (PAMPs) (Shashidhara, and Devegowda, 2003). In addition, YCW can improve gut health (i.e., increase mucus production) and development by providing favorable conditions for beneficial intestinal bacteria such as *Bifidobacterium* and *Lactobacillus* spp.; and competitive binding sites for pathogenic bacteria (Spring, et al., 2000; Huff et al., 2006; Halder et al., 2011).

In addition to YCW, nucleotides of *S. cerevisiae* might be useful in reducing clinical signs associated with NE in broilers. Dietary nucleotides play an essential role in development and proliferation of tissues with rapid cell turnover such as enterocytes, and lymphocytes (Grimble, 1994; Sauer et al., 2011). Therefore, yeast nucleotides might be helpful in reducing the intestinal damage caused by *C. perfringens* in broiler chickens.

Distillers dried grains with soluble (DDGS) as co-products of brewer's yeast (*Saccharomyces cerevisiae*) fermentation contain a significant quantity of yeast biomass components (6%) potentially beneficial for gut health and development, and immune system modulation (Slominski, 2012). Considering the potential growth-promoting and immunomodulatory effects of yeast-derived products, these products might be useful as feed additives to control *C. perfringens*-associated necrotic enteritis in broiler chickens. Therefore, the present study evaluated the effects of DDGS and different yeast products derived from *S. cerevisiae* on growth performance, gut lesions, and innate immunity associated gene expressions in broiler chickens following challenge with *C. perfringens*.

6.3 Materials and Methods

The research facility at Nutreco Canada Agresearch in Burford, ON, Canada was used to conduct the study. All animal procedures used in this study were conducted according to the guidelines of the Canadian Council on Animal Care (1993).

Experimental Design and Diets

This experiment was conducted as a randomized complete block design, with 8 dietary treatments assigned randomly within 6 replicate blocks of 8 pens (48 pens total). Location within the facility was considered as blocking factor and each pen of 55 male broiler chickens (Ross-

308) was considered an experimental unit. The antibiotic-and coccidiostat-free corn/SBM/canola meal diets were formulated to meet the NRC requirements for broiler chickens (Table 6.1). Diets supplemented with 10% DDGS were formulated to a similar specification as other diets. Dietary treatments consisted of two Control corn/SBM diets without and with *C. perfringens* challenge, and diets containing antibiotic (bacitracin methylene disalicylate, 55 g/tonne), nucleotides (150 g/tonne), yeast cell wall (300 g/tonne), and a commercial product Maxi-Gen Plus containing processed yeast plus nucleotides (1 kg/tonne) fed to chickens challenged with *C. perfringens*. Corn/SBM diets containing 10% DDGS without and with *C. perfringens* challenge were also used.

Experimental Procedures, Challenge Model and Sample Collections

The experiment lasted for 35 d and consisted of 3 phases (0-14 d, starter; 14 to 28 d, grower; 28-35 d, finisher). The *C. perfringens* challenge model used in this study was as described by Prescott el al. (1978), and Brennan et al. (2001). The *C. perfringens* strain used in this study was isolated from an outbreak of NE in commercial flocks in Ontario and was known to produce lesions typical of NE, with mild suppression of growth rate and minimal mortality. On d 14, feed was withdrawn from all birds approximately 7 h prior to challenge. Feed was mixed with inoculum (bacterial broth mixed) and offered on the afternoon of d 14. Inoculation lasted for 16 h, the remaining inoculum-containing feed was weighted and discarded. The concentration of *C. perfringens* in the inoculum ranged from 6.7×10^8 to 8.9×10^8 cfu/bird. During inoculation, the birds in control treatments were fed their regular feed.

Feed intake and body weight were measured on a pen basis on d 0, 14, 21, 28, and 35, while mortality was recorded daily. Average daily feed intake, average daily gain, and feed

conversion ratio (FCR) were calculated for each period (d 0 to 14, d 14 to 21, d 21 to 28, d 28 to 35, and d 0 to 35). On d 17, and 21, 2 birds per pen (12 birds per treatment) were randomly selected and euthanized by CO_2 asphyxiation. The small intestine from each bird was removed, opened and subjected to scoring for NE lesions by the same poultry pathologist. Lesions were scored using the following scale: 0, no gross lesions; 1, thin friable small intestine; 2, focal necrosis; 3, patchy necrosis; and 4, severe extensive mucosal necrosis (Johnson and Reid, 1970; Prescott et al., 1978). On d 35, 6 birds per treatment were euthanized by CO_2 asphyxiation and ileum and cecal tonsils were removed, snap frozen, and stored at -80 °C for gene expression analysis.

***C. perfringens* Enumeration**

Clostridium perfringens enumeration was performed as described by Jia et al. (2009). Briefly, on d 17 and 21, ileal contents from 2 birds per pen (12 birds per treatment) selected for NE lesion scoring were used for *C. perfringens* enumeration. Pooled digesta (10 g) were transferred into 90 ml of sterile peptone and serially diluted. The dilutions were then plated on Perfringens agar base (OPSP, Oxoid Inc., Nepean, ON, Canada) containing supplements SR 76 and SR 7 (Oxoid Inc.) and were incubated anaerobically at 38°C for 48 h in jars containing gas generation kits (BBL GasPak Plus, Becton Dickinson, Sparks, MD). Each sample was plated in duplicate. The concentration of *C. perfringens* was expressed as $\log_{10}$ colony forming units per gram of intestinal content.

Total RNA Extraction and Reverse Transcription

Total RNA was extracted from individual spleen and cecal tonsil samples using the Trizol extraction method as described by the manufacturer (Invitrogen Canada Inc., Burlington, ON,

Canada). The quantity and purity of the RNA samples was measured by using NanoDrop spectroscopy (Thermo Scientific) with the ratio of absorbance at 260 nm and 280 nm. In this procedure, a ratio of ~2.0 is generally accepted as "pure' for RNA, and a ratio lower than 2.0 (i.e., ~1.59 and under) may indicate the presence of phenol, protein and other contaminants. Reverse-transcription was performed by using a High-capacity cDNA Reverse Transcription Kit (Applied Bisystems, Foster City, CA) according to the manufacturer's protocol.

Quantitative Real-time PCR

Primer sequences for β-Actin, TLR2 type 2 (henceforth known as TLR2b), TLR4, TLR21, macrophage mannose receptor (MMR), interleukin IL-12p35, IL10, IL-4, , IL-18, IL-6 and interferon gamma (IFNγ), were designed using GeneBank database sequences from the National Center for Biotechnology Information (Bethesda, MD) (Table 6.2). Quantitative real-time (qRT) PCR was performed using the Step One Thermo Cycler (Applied Biosystems, Mississauga, ON, Canada) on 48-well plate with 25 µL of total reaction volume as described by Pfaffl and Hageleit (2001). The iTaq Universal SYBR Green Supermix was used as the QRT-PCR master mix and each reaction was run in duplicate. The PCR cycling protocol included an initial denaturation step at 95°C, followed by amplification for 40 cycles at 95°C for 10 s, an annealing temperature described in Table 6.2 for each of the primer pairs, and extension at 72°C for 10 s.

Statistical Analysis

All statistical analysis (except gene expression analysis) was performed using SAS software (SAS Institute, 2003). Growth performance parameters and lesion scores were analyzed according to a Randomized Complete Block (RCB) design using the MIXED procedures, with

fixed effect of treatments and random effect of block, while analysis based on the pooled samples (bacteria enumeration) was conducted using the GLM procedure. The significance of differences between means was determined using Scheffe's procedure. Analysis of mortality was done by GLIMMIX procedures and differences between treatment means were adjusted by Tukey multiple comparison test. For the gene expression analysis, the cycle threshold values were recorded and uploaded to the REST-2009 Software (Qiagen, Valencia, CA). Relative expression levels of all genes were calculated relative to a housekeeping gene (β-actin in the present study), and gene expression was presented as fold changes relative to the control diet. Gene expression fold changes, standard error, and statistical significance were calculated by the software based on the formula developed by Pfaffl et al. (2002). A *P*-value of less than 0.05 was considered significant.

6.4 Results

Growth Performance

The results for the growth performance parameters are presented in Table 6.3. Before *C. perfringens* challenge (0-14 d) feed intake, body weight gain, and FCR were not affected by dietary treatments ($P > 0.05$). After *C. perfringens* challenge (14-21 d), impaired feed intake, body weight gain, and FCR were observed in challenged groups, except the BMD group in which performance parameters were not affected by pathogen challenge. In the grower phase (21-28 d), the pathogen challenge no longer affected the feed intake, and increased body weight gain was observed in birds receiving BMD, nucleotides, and YCW compared to the control, unchallenged group; and FCR was significantly decreased for challenged groups versus the Control, unchallenged group. In the finisher phase (28-35 d), pathogen challenge no longer

affected the growth performance parameters. Over the entire trial (0-35 d), feed consumption was not affected by dietary treatments, however, the BMD group had significantly increased body weight gain compared to the YCW group, and improved FCR was observed in the BMD group compared to the Control, challenged group.

Lesion Score, Mortality, and Intestinal C. perfringens Numbers

The results for lesion score, mortality and bacterial enumeration are listed in Table 6.4. No intestinal lesions were observed in birds from the unchallenged groups. The *Clostridium perfringens* challenge significantly increased average lesion score on d 17, except for the BMD group in which no intestinal lesions were observed. Additionally, the average lesion score on d 21 was significantly reduced in the BMD group compared with nucleotide group. The mortality rate caused by NE and the total mortality rate were not affected by dietary treatments. Pathogen challenge significantly increased intestinal *C. perfringens* numbers, except in the BMD group where *C. perfringens* counts were not affected.

Gene Expression of Pattern Recognition Receptors (PRRs) and Cytokines

Quantitative RT-PCR analysis was performed on tissue collected from the ileum and cecal tonsils to evaluate the effects of yeast-derived products on gene expression of PRRs and cytokines in 21-d-old birds after challenge with *C. perfringens*. The fold change gene expression of pattern recognition receptors, acquired from QRT-PCR are presented in Fig. 6.1. In the ileum, diets supplemented with nucleotides significantly upregulated the expression of TLR2, TLR4 and TLR21 compared to the Control, challenged group. The diet supplemented with DDGS upregulated the expression of TLR21 in the ileum and expression of MMR was upregulated in birds receiving YCW. However, no significant difference in TLR expression was observed in

cecal tonsils. The expression of MMR was downregulated in cecal tonsils of birds receiving the BMD and DDGS, unchallenged groups compared with the Control, challenged group. The fold change gene expression of cytokines, acquired from QRT-PCR is presented in Fig. 6.2. No significant differences were observed for cytokine expression in cecal tonsil of birds. However, diet containing nucleotides upregulated the expression of IL-4 and IL-18 in the ileum. In addition, the expression of IL-18 was upregulated in the ileum of birds receiving the diet supplemented with YCW.

6.5 Discussion

Yeast-derived products including mannan, ß1,3-1,6-glucan, and nucleotides are well known for modulation of the immune system and improvement of gut health (Huff et al., 2006; Haldar et al., 2011). In the current study, the effects of yeast-derived products supplementation on growth performance and local innate immunity-associated gene expression of broiler chickens challenged with *C. perfringens* was investigated. Following the *C. perfringens* challenge (14-21 d) impaired body weight gain and FCR were observed in all challenged groups except the BMD. Impaired growth performance observed in challenged groups might have been associated with overgrowth of *C. perfringens* which could lead to the accumulation of extracellular toxins and damage to the intestinal mucosa (Kulkarni et al., 2007). In agreement with our results Liu et al. (2010) and Mikkelsen et al. (2009) reported that *C. perfringens* challenge decreased body weight gain in broiler chickens. In contrast, Pedersen et al. (2008) did not observe any significant difference in body weight gain of broilers following *C. perfringens* challenge. In the current study, growth performance parameters were not influenced by *C. perfringens* challenge in birds receiving BMD. It is likely that antibacterial properties of this antibiotic minimized bacterial overgrowth in the intestinal contents (Dumonceaux et al., 2006; Lin et al., 2013). Over the entire

trial, growth performance was not affected by diets supplemented with yeast-derived products. These results are in agreement with the observation of Hofacre et al. (2003) who showed that supplementation of diets with mannan oligosaccharides had no effect on growth performance of chickens challenged with *C. perfringens*. Furthermore, Thanissery et al. (2010) demonstrated that inclusion of yeast extract in the diet did not affect growth performance of *C. perfringens*-challenged birds. However, Shawkat et al. (2015) reported that a diet containing yeast cell walls was effective in curbing performance decline in birds challenged with *C. perfringens* and *Eimeria*.

In the current study, increased *C. perfringens* counts and higher intestinal lesion scores were observed for all challenged groups except the BMD-containing diet. Similarly, Ao et al. (2012) showed that mannan oligosaccharide administration to broiler chickens was not effective in reducing NE lesion score development in the small intestine. Owing to the fact that C. *perfringens* is a gram-positive bacteria, lower bacteria counts and lesion scores observed in the BMD-containing diet in the current study is probably associated with the antibacterial activity of BMD against these bacteria (Engberg et al., 2000; Sims et al., 2004). However, the lack of a positive effect of the yeast-derived products in minimizing *C. perfringens* counts and NE lesion score development in the current was perhaps due to the distinctly better antibacterial action of yeast cell wall polysaccharides against bacteria with type-1 fimbriae such as *Salmonella* and *E. coli* (Spring et al., 2000; Ferket et al., 2002). In the present study, the diet supplemented with nucleotides showed the highest *C. perfringens* counts and lesion score among dietary treatments. In contrast, Thanissery et al. (2010) demonstrated that the diet containing nucleotides reduced *C. perfringens* level compared with the Control diet on d 1 and 7 post-challenge. The high *C. perfringens* counts and lesion score observed in the current study for the diet supplemented with

nucleotides might be because of the ability of nucleotides to increase the growth of both commensal and pathogenic bacteria which has already been proven in an *in vitro* study by Sauer et al. (2010). There is a possibility that dietary nucleotides can be used as nutrient source for pathogenic bacteria. In some human and animal studies it has been shown that dietary nucleotides can modify the gastrointestinal microflora (Brunser et al., 1994; Mateo et al., 2004). Balmer et al. (1994) demonstrated that in infants fed nucleotide supplemented milk, increased colonisation of *Escherichia coli* was observed compared to infants receiving the standard milk-based formula.

The mucosal immune system plays a key role in host defence against pathogenic bacteria (MacDonald, 2003). The ileum and cecal tonsils are considered important components in the avian mucosal immune system that are involved in immune protection of the intestine (Davison, 2008). At the intestinal level, pattern recognition receptors (PRRs), which are expressed by epithelial cells and cells of the innate immune system, can recognize microbe-associated molecular patterns (MAMP) as small molecular motifs found on the most classes of microbes. The recognition of MAMP by PRRs would lead to the stimulation of immune systems (Trinchieri and Sher, 2007; Kawai et al., 2010). Toll-like receptors are considered the most important PRRs which can recognize MAMP and facilitate the initiation of immune response against pathogen invasion (Takeda and Akira, 2005; Akira et al, 2006). In broiler chickens, TLR2b is the principal receptor for peptidoglycan from gram negative and gram positive bacterial, while TLR4 recognizes lipopolysacharide as the major component of outer membrane of gram-negative bacteria (Warren et al., 2005; Higuchi et al., 2008). Chicken toll-like receptor TLR21 was shown to act as a functional homologue to mammalian TLR9 and is involved in recognition of unmethylated CpG oligonucleotides (Keestra et al., 2010). In addition to TLRs,

macrophage mannose receptor (MMR) is a transmembrane protein that belongs to C-type lectins which are involved in modulation of the innate immune system through recognition of high mannose structures on the surface of potentially pathogenic bacteria and fungi (Gazi and Martinez-Pomares, 2009). In the current study, the expression of PRRs was not affected by *C. perfringens* challenge. Similar results were reported in the study of Lu et al. (2009), where no significant difference was observed in expression of TLR2 and TLR4 following *C. perfringens* challenge. We found that the diet supplemented with nucleotides upregulated the expression of TLRs in the ileum of broilers. It has been reported that upregulation of TLRs in the ileum is associated with an enhanced gastroepithelial barrier against pathogenic bacteria (Chen et al., 2007). Although the exact molecular mechanisms by which nucleotides modulate the immune system are not completely understood, upregulation of toll-like receptors by the a nucleotide-containing diet might be related to the role of these products in the development and proliferation of tissues with rapid cell turnover such as enterocytes, and lymphocytes (Carver, 1999). Dietary nucleotides might influence protein synthesis by modulation of the intercellular nucleotide pool. (Bueno et al., 1994; Uauy et al., 1994; Holen and Jonsson, 2004). It has been reported that dietary nucleotides are capable of increasing cell-mediated immunity, and improving host resistance to bacterial infections (Maldonado et al., 2001; Frankic et al., 2006; Hess et al., 2012). Therefore, inclusion of nucleotides in the diet of broiler chickens might be beneficial for activation of the local innate immunity of broilers under microbial challenge. In the current study, the diet supplemented with YCW upregulated the expression of MMR in the ileum. Mannans which are considered the major constituent of yeast cell wall derived from *Saccharomyces cerevisiae* can bind to MMR on the cells of the innate immune system and initiate the activation of innate immunity (Paulovicová, et al., 2005). In agreement with these

results, Yitbarek et al., (2012) found that supplementation of the diet with mannan-oligosacharide increased the expression of PRRs in the ileum of broilers. In the current study, no significant changes were observed in the expression of PRRs in cecal tonsils of birds fed yeast-derived products. However, the diet supplemented with BMD downregulated the expression of MMR in the cecal tonsils. This might be related to the antibacterial activity of BMD which decreased the bacterial population and thus MAMPs associated with bacteria and in turn the MMR response (Rakoff-Nahoum et al., 2004).

In addition to PRRs, cytokines as small cell-signalling molecules play an essential role in innate immunity regulation (Swaggerty et al., 2008). In the current study, we measured the expression of inflammatory cytokines including IFN-γ, IL-12, IL-6, and IL-18 and anti-inflammatory cytokines including IL-4 and IL-10 in the ileum and cecal tonsils. No significant difference was observed in gene expression of cytokines as a result of *C. perfringens* challenge. In contrast, Collier et al. (2008), and Park et al. (2008) found that upregulation of IFNγ was associated with NE in chickens. The conflicting results regarding the expression of cytokines following *C. perfringens* infection might be related to the differences in the challenge models used in the different studies. Regarding the inflammatory cytokines, the expression of IFN-γ as a macrophage activator and the cytokine involved in differentiation of naive Th cells to Th1 cells, and IL-12 as a pro-inflammatory cytokine which promotes the secretion of IFN-γ by Th1 cells (Trinchieri et al., 2003) were not influenced by dietary treatments in both the ileum and cecal tonsils of birds. In contrast, Yitbarek et al. (2012) showed that following *C. perfringens* challenge the expression of IFN-γ and IL-12 were upregulated in the ileum of birds receiving yeast-derived products. They concluded that inclusion of yeast-derived products in the diet of birds challenged with *C. perfringens* might support a pro-inflammatory response via T-helper

type-1 associated pathways to control early stages of infection. The diet supplemented with YCW and nucleotides upregulated the expression of IL-18 in the ileum. Interleukin-18 is considered an important mediator of Th1 response and together with IL-12 induces cell-mediated immunity following microbial infections (Biet et al., 2002). It has been reported that activation of PRRs by PAMPs mediates the production of cytokines necessary for innate immune system activation (Trinchieri and Sher, 2007). Therefore, upregulation of IL-18 in the ileum of birds receiving nucleotides and YCW might be associated with increased expression of TLRs and MMR observed in the ileum of these birds. In the current study the expression of IL-10 and IL-6 were not affected by dietary treatments. Interleukin-10 is a T helper 2 type cytokine which inhibits secretion of IFN-γ by Th1 cells (Liu et al., 2010); and IL-6 is a cytokine mainly produced by macrophages and involved in stimulation of innate immune response via activation of acute phase proteins following tissue damage (Diehl et al., 2002). Similar results were found in the study of Yitbarek et al. (2012), where no significant difference was observed for gene expression of IL-10 and IL-6 in *C. perfringens*-challenged broilers fed yeast-derived products. This suggests that yeast-derived products may not be involved in IL-6 and IL-10 immunoregulation following *C. perfringens* challenge. However, more research may be needed to characterize and clarify immunoregulatory effects of IL-10 and IL-6 under microbial challenge conditions. The expression of IL-4 was upregulated in the ileum of birds receiving nucleotides. It has been suggested that dietary nucleotides may favour the balance of T cell differentiation to Th-2 cells, which are mainly involved in production of anti-inflammatory cytokines and suppression of pro-inflammatory cytokines (Jyonouchi et al., 1994). Interleukin-4 is an anti-inflammatory mediator which inhibits the production of pro-inflammatory cytokines such as IFNγ and IL-12 (King et al., 1993). Therefore, the upregulation of IL-4 in the ileum of

birds receiving nucleotides may indicate that yeast nucleotides could have anti-inflammatory properties and perhaps inhibit any further inflammation caused by *C. perfringens.*

In conclusion, supplementation of diets with yeast-derived products was not as effective as the antibiotic with regard to *C. perfringens* counts and lesion scores in the gut. This is probably due to the fact that antibiotic growth promoters have been found to be more effective against gram-positive bacteria (i.e., *Clostridium*) (Engberg et al., 2000; Sims et al., 2004); while yeast-derived components, especially mannans, are more effective against bacteria with type-1 fimbriae such as *Salmonella* (Spring et al., 2000; Ferket et al., 2002). Regarding the innate immune response, challenging the birds with *C. perfringens* did not activate the receptors and cytokines involved in innate immune responses. However, supplementation of diets with nucleotides and YCW resulted in stimulation of the local innate immune system in broiler chickens. This suggests that YCW and nucleotides are able to stimulate the innate immune response of broilers and help birds to mount faster and stronger immune responses under pathogen challenge conditions.

Table 6.1. Composition and calculated analysis of experimental diets

Item/ Ingredient (% of diet)	Starter (1-14)		Grower (14-28)		Finisher (28-35)	
	Basal Diet	DDGS Diet	Basal Diet	DDGS Diet	Basal Diet	DDGS Diet
Corn	58.9	53.7	59.4	54.3	60.6	55.2
Soybean meal	31.3	25.9	27.2	21.8	25.6	20.4
Canola meal	5.0	5.0	7.5	7.5	7.5	7.5
Calcium carbonate	1.56	1.61	1.38	1.43	1.22	1.27
Dicalcium phosphate	1.13	1.08	0.89	0.84	0.66	0.61
Fat	0.97	1.50	2.60	3.87	3.60	4.81
Salt	0.42	0.37	0.42	0.37	0.42	0.37
DL-Methionine	0.25	0.25	0.20	0.20	0.12	0.12
Mineral and Vitamin premix [1]	0.22	0.23	0.17	0.17	0.14	0.14
L-Lysine	0.12	0.23	0.10	0.22	0.10	0.22
L-Treonine	0.04	0.07	0.01	0.04	0.00	0.03
Ronozyme	0.03	0.03	0.03	0.03	0.03	0.03
Choline	0.02	0.02	0.02	0.02	0.02	0.02
Corn distillers dried grain	-	10.0	-	10.0	-	10.0
Total	100.0	100.0	100.0	100.0	100.0	100.0
Calculated analysis						
CP (%)	21.2	21.2	20.2	20.2	19.5	19.5
ME (kcal / kg)	2919	2930	3024	3036	3105	3116
Calcium (%)	0.9	0.9	0.8	0.8	0.7	0.7
Available phosphorus (%)	0.45	0.45	0.40	0.40	0.34	0.35
Methionine (%)	0.56	0.57	0.50	0.50	0.46	0.47
Methionine + cystine (%)	0.93	0.94	0.85	0.86	0.81	0.82
Lysine (%)	1.1	1.1	1.04	1.04	1.0	1.0
Sodium (%)	0.16	0.16	0.16	0.16	0.16	0.16

[1]Mineral and vitamin premix provided: Mn, 89 mg; Zn, 88 mg; Fe, 34 mg; Cu, 63 mg; Se, 0.3 mg; I, 1.8 mg; vitamin A, 6238 IU; vitamin D_3, 2275 IU; vitamin E, 20 IU; vitamin B_{12}, 0.013 mg; vitamin K, 2.9 mg; niacin, 75 mg; folic acid, 0.86 mg; biotin, 0.1 mg; riboflavin, 5.5 mg kg^{-1} of the starter diet; and Mn, 71 mg; Zn, 71 mg; Fe, 27 mg; Cu, 50 mg; Se, 0.24 mg; I, 1.4 mg; vitamin A, 4990 IU; vitamin D_3, 1820 IU; vitamin E, 16 IU; vitamin B_{12}, 0.011 mg; vitamin K, 2.3 mg; niacin, 60 mg; folic acid, 0.69 mg; biotin, 0.08mg; riboflavin, 4.4 mg kg^{-1} of the grower diet; and Mn, 44.5 mg; Zn, 44 mg; Fe, 17 mg; Cu, 31.5 mg; Se, 0.15 mg; I, 0.9 mg; vitamin A, 1138 IU; vitamin D_3, 2275 IU; vitamin E, 10 IU; vitamin B_{12}, 0.006 mg; vitamin K, 1.45 mg; niacin, 37.5 mg; folic acid, 0.43 mg; biotin, 0.05 mg; riboflavin, 2.75 mg kg^{-1} of the finisher diet

Table 6.2. Primer sequences used for the real-time quantitative PCR[1]

Gene[2]	Primer sequence (5'-3')[3]	Fragment size (bp)	Annealing temperature (°C)	GenBank accession number
TLR2b	F: CGCTTAGGAGAGACAATCTGTGAA R:GCCTGTTTTAGGGATTTCAGAGAATTT	90	59	NM204278
TLR4	F: AGTCTGAAATTGCTGAGCTCAAAT R: GCGACGTTAAGCCATGGAAG	190	55	AY064697
TLR21	F: TGGCGGCGGGAGGAAAAGTG R: CACCGTGCTCCAGCTCAGGC	106	59	NM_001030558
MMR	F: GCAGGGCACGTTCAGGTGGG R: GCCACACAGCCTGGCTCCCT	90	60	XM001235105
IFN-γ	F: CTGAAGAACTGGACAGAGAG R: CACCAGCTTCTGTAAGATGC	264	60	X99774
IL-12p35	F: CTGAAGGTGCAGAAGCAGAG R: CCAGCTCTGCCTTGTAGGTT	217	64	NM213588
IL-10	F: AGCAGATCAAGGAGACGTTC R: ATCAGCAGGTACTCCTCGAT	103	55	AJ621614
IL-4	F: TGTGCCCACGCTGTGCTTACA R: CTTGTGGCAGTGCTGGCTCTCC	193	57	GU119892
IL-18	F:GAAACGTCAATAGCCAGTTGC R:TCCCATGCTCTTTCTCACAACA	213	53	AY628648.2
IL-6	F: CAGGACGAGATGTGCAAGAA R: TAGCACAGAGACTCGACGTT	233	59	AJ309540
β-Actin	F: CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	205	61	X00182

[1]The listed oligonucleotides were used to analyze gene expression via real-time quantitative PCR.

[2]TLR = Toll-like receptor; MMR = monocyte/macrophage mannose receptor; IFN = interferon; IL= interleukin.

[3]F = forward; R = reverse.

Table 6.3. Effects of dietary treatments and *C. perfringens* challenge on growth performance of broiler chickens

	Average daily feed intake (g/bird/d feed)					Average daily gain (g/bird/d)					Feed conversion ratio (g feed / g gain)				
Item	0-14 d	14-21 d	21-28 d	28-35 d	0-35 d	0-14 d	14-21 d	21-28 d	28-35 d	0-35 d	0-14 d	14-21 d	21-28 d	28-35 d	0-35 d
Treatment[1,2]															
Control, unchallenged	37	93^{a}	125	181	92	27.2	61.3^{a}	69.0^{b}	91.7	53.8^{ab}	1.36	1.51^{a}	1.83^{a}	1.99	1.70^{ab}
Control, challenged	37	83^{b}	128	187	91	27.7	50.8^{b}	75.2^{ab}	92.5	53.2^{ab}	1.35	1.63^{ab}	1.71^{b}	2.02	1.71^{a}
Antibiotic, challenged	37	95^{a}	130	182	93	27.7	62.3^{a}	78.0^{a}	91.4	55.7^{a}	1.35	1.52^{a}	1.67^{b}	2.00	1.67^{b}
Nucleotides, challenged	37	82^{b}	131	180	90	26.8	49.2^{b}	78.5^{a}	92.3	53.0^{ab}	1.38	1.67^{b}	1.67^{b}	1.95	1.69^{ab}
Yeast Cell Wall, challenged	38	81^{b}	129	180	90	28.0	47.8^{b}	77.3^{a}	91.0	52.2^{b}	1.34	1.69^{b}	1.68^{b}	1.98	1.70^{ab}
Maxi-Gen Plus, challenged	37	83^{b}	128	181	90	27.5	51.5^{b}	76.8^{a}	91.0	53.0^{ab}	1.34	1.62^{ab}	1.69^{b}	1.99	1.69^{ab}
DDGS, unchallenged	36	92^{a}	126	176	90	26.2	60.5^{a}	72.8^{ab}	90.0	53.4^{ab}	1.39	1.51^{a}	1.75^{ab}	1.98	1.69^{ab}
DDGS, challenged	37	82^{b}	129	177	89	27.0	48.8^{b}	76.5^{ab}	92.3	52.5^{b}	1.36	1.68^{b}	1.70^{b}	1.92	1.69^{ab}
SEM	0.16	0.86	0.63	1.06	0.41	0.18	0.91	0.73	0.69	0.27	0.006	0.014	0.012	0.011	0.003
P-value	0.58	0.0001	0.29	0.37	0.071	0.28	.0001	0.003	0.97	0.02	0.52	0.0001	0.001	0.29	0.006

[1]Dietary treatments consisted of two Control corn/SBM diets without and with *C. perfringens* challenge, and diets containing antibiotic (bacitracin methylene disalicylate, 55 g/tonne), nucleotides (150 g/tonne), yeast cell wall (300 g/tonne), and a commercial product Maxi-Gen Plus (1 kg/tonne) fed to chickens challenged with *C. perfringens*. Corn/SBM diets containing 10% of DDGS without and with *C. perfringens* challenge were also used.

[2]Birds in the challenged groups received an in-feed *C. perfringens* inoculation on d 14 that lasted for 16 h, and the calculated dose ranged from 9.26×10^{8} to 9.31×10^{8} cfu/bird.

Table 6.4. Effects of dietary treatments and *C. perfringens* challenge on average necrotic enteritis (NE) lesion score, NE and total mortality and *C. perfringens* numbers in broiler chickens

Item	Average intestinal lesion score[3]		Mortality (%)[4]		*C. perfringens* (log_{10} cfu/g)
	17 d	21 d	NE	Total	
Treatment[1,2]					
Control, unchallenged	0.17^{cd}	0.22^{ab}	1.52	2.12	1.95^{a}
Control, challenged	1.44^{ab}	0.89^{ab}	0.91	3.33	7.99^{b}
Antibiotic, challenged	0.00^{d}	0.00^{b}	0.30	2.73	0.00^{a}
Nucleotides, challenged	1.78^{a}	1.11^{a}	3.05	4.24	7.44^{b}
Yeast Cell Wall, challenged	1.39^{ab}	0.56^{ab}	4.05	6.36	7.66^{b}
Maxi-Gen Plus, challenged	1.33^{ab}	1.00^{ab}	1.86	4.24	7.09^{b}
DDGS, unchallenged	0.00^{d}	0.33^{ab}	0.62	3.03	0.77^{a}
DDGS, challenged	0.95^{bc}	0.67^{ab}	1.83	3.03	7.28^{b}
SEM	0.11	0.08	0.18	0.25	0.43
P-value	0.0001	0.002	0.043	0.18	0.0001

[1]Dietary treatments consisted of two Control corn/SBM diets without and with *C. perfringens* challenge, and diets containing antibiotic (bacitracin methylene disalicylate, 55 g/tonne), nucleotides (150 g/tonne), yeast cell wall (300 g/tonne), and a commercial product Maxi-Gen Plus (1 kg/tonne) fed to chickens challenged with *C. perfringens*. Corn/SBM diets containing 10% of DDGS without and with *C. perfringens* challenge were also used.

[2]Birds in the challenged groups received an in-feed *C. perfringens* inoculation on d 14 that lasted for 16 h, and the calculated dose ranged from 9.26×10^8 to 9.31×10^8 cfu/bird.

[3]The following scale was used: 0 = no gross lesions; 1 = thin, friable small intestine; 2 = focal necrosis, ulceration or both; 3 = patchy necrosis; 4 = severe, extensive mucosal necrosis.

[4]Mortality was recorded from start to end of the experiment, and Glimmix procedure was used for significant differences.

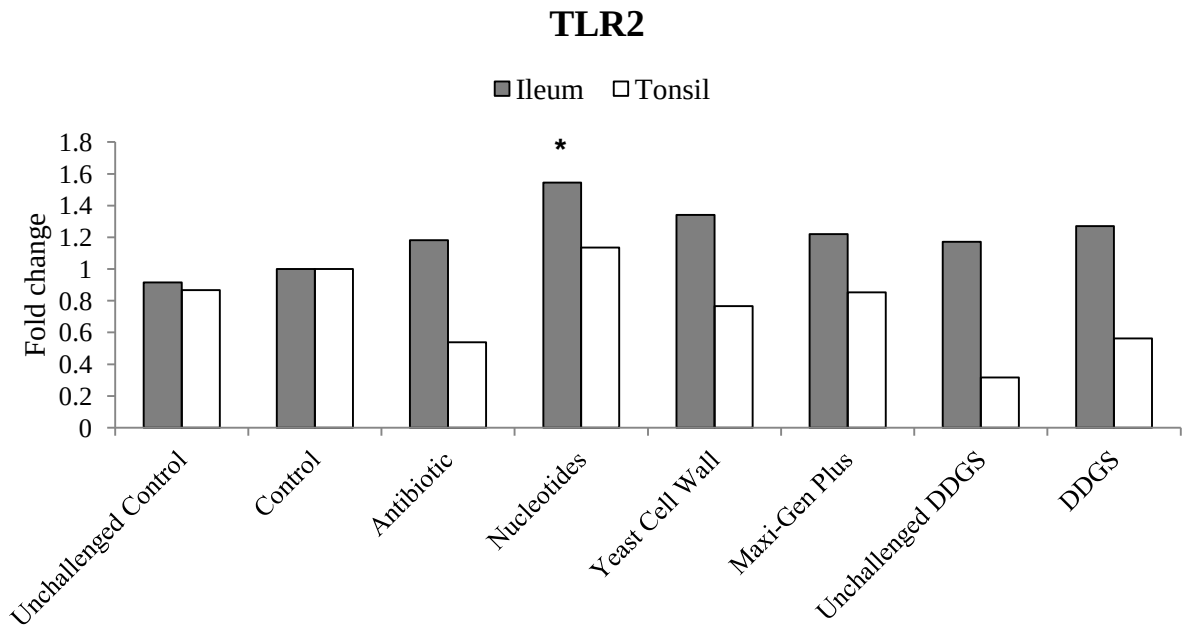
[a,-c] Means within a column with no common superscripts differ significantly ($P < 0.05$).

6.6 Figure and Legend

Figure 6.1 Fold change expression of Toll-like receptors TLR2b, TLR4, TLR21, and macrophage mannose receptor (MMR) in the ileum and cecal tonsils of broiler chickens fed the two Control corn/SBM diets without and with *C. perfringens* challenge, and diets containing antibiotic (bacitracin methylene disalicylate, 55 g/tonne), nucleotides (150 g/tonne), yeast cell wall (300 g/tonne), and a commercial product Maxi-Gen Plus (1 kg/tonne) fed to chickens challenged with *C. perfringens*. Corn/SBM diets containing 10% of DDGS without and with *C. perfringens* challenge were also used.
*Bars with asterisks differ significantly from the Control, challenged group. Results were considered statistically significant from the control group if $P < 0.05$.

Figure 6.2 Fold change expression of cytokines IL-10, IL-4, IL-12, IL-6 IFNγ, and IL-18 in the ileum and cecal tonsils of broiler chickens fed the two Control corn/SBM diets without and with *C. perfringens* challenge, and diets containing antibiotic (bacitracin methylene disalicylate, 55 g/tonne), nucleotides (150 g/tonne), yeast cell wall (300 g/tonne), and a commercial product Maxi-Gen Plus (1 kg/tonne) fed to chickens challenged with *C. perfringens*. Corn/SBM diets containing 10% of DDGS without and with *C. perfringens* challenge were also used.
*Bars with asterisks differ significantly from the Control, challenged group. Results were considered statistically significant from the control group if $P < 0.05$.

Figure 6.1. Fold change gene expression of pattern recognition receptors in the ileum and cecal tonsils of birds challenged with *C. perfringens*



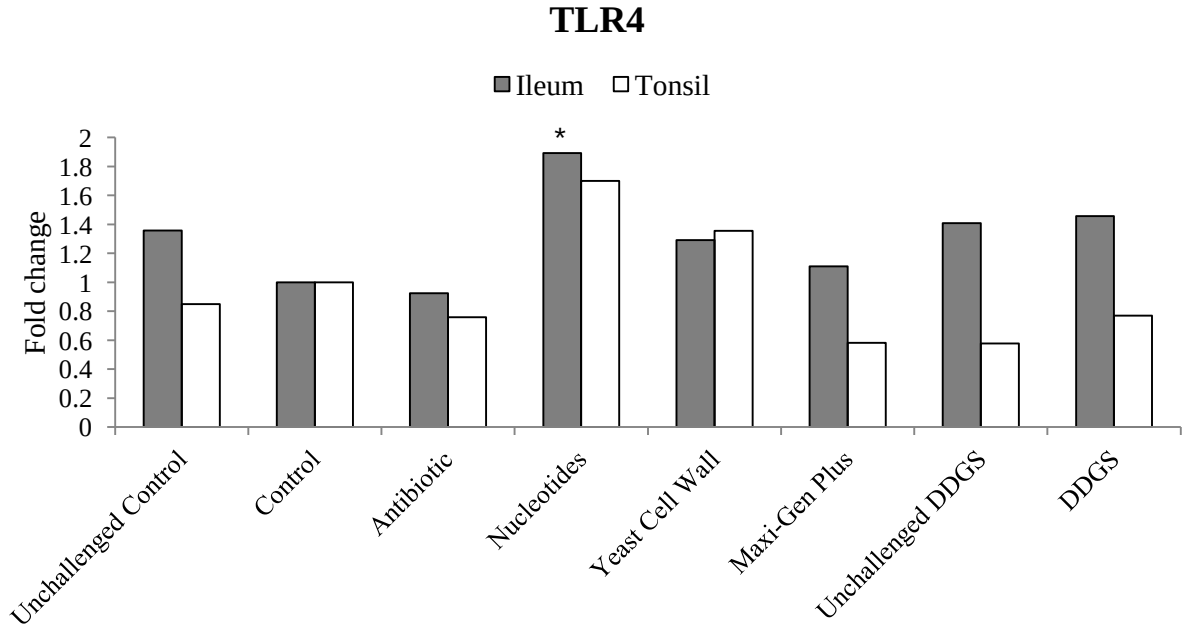
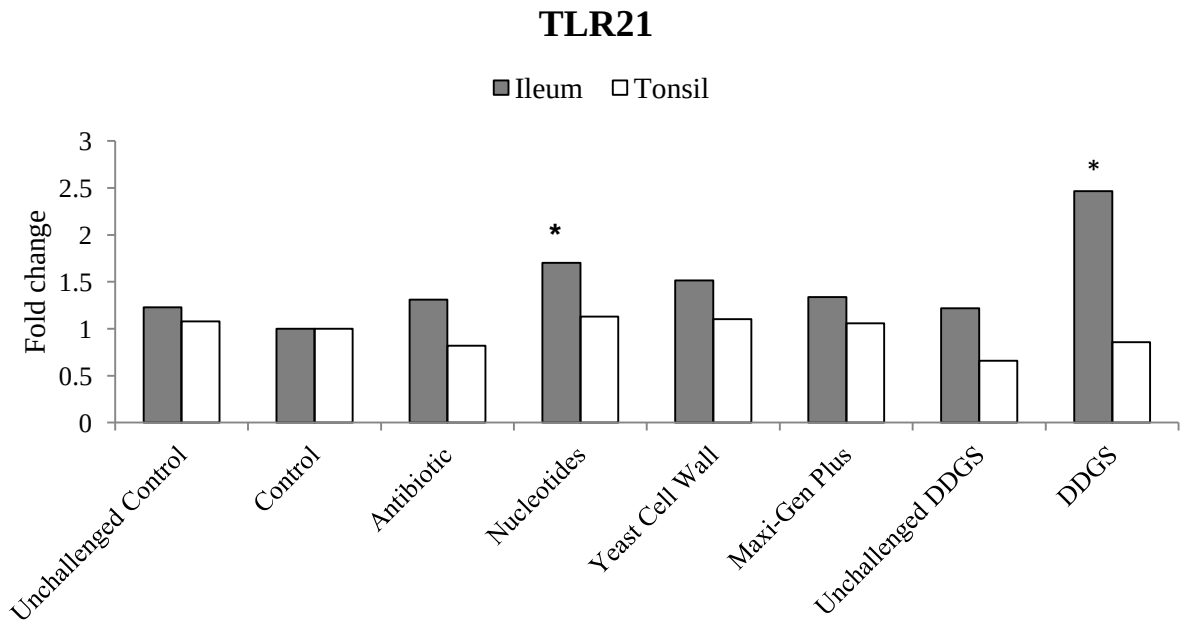


Figure 6.1 Continued. Fold change gene expression of pattern recognition receptors in the ileum and cecal tonsils of birds challenged with *C. perfringens*



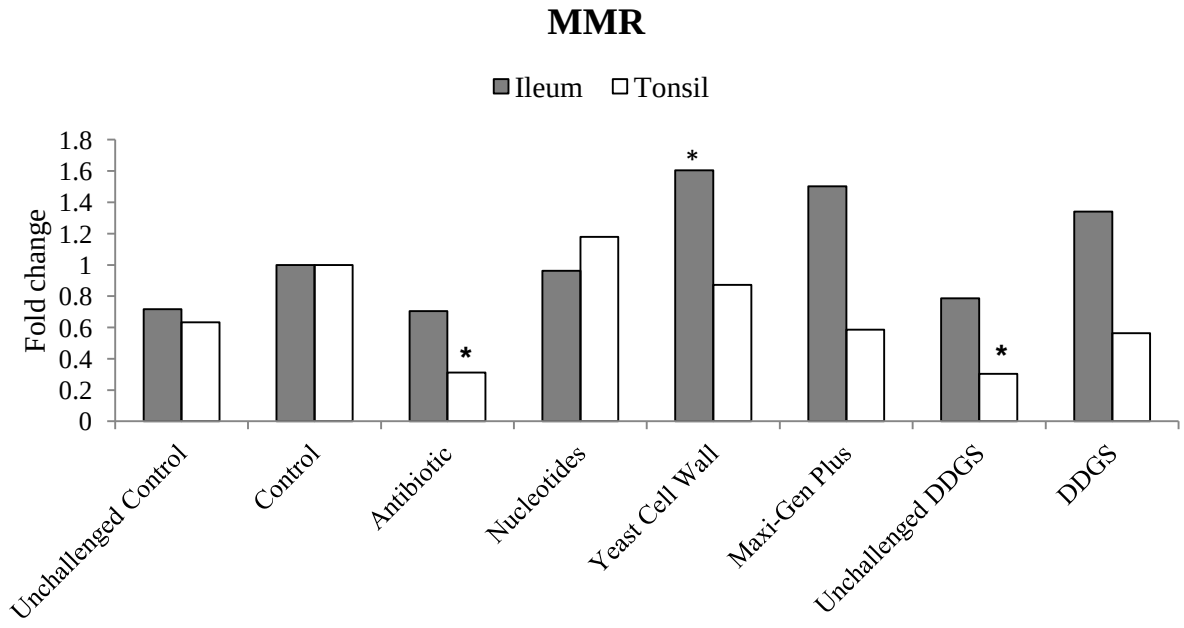
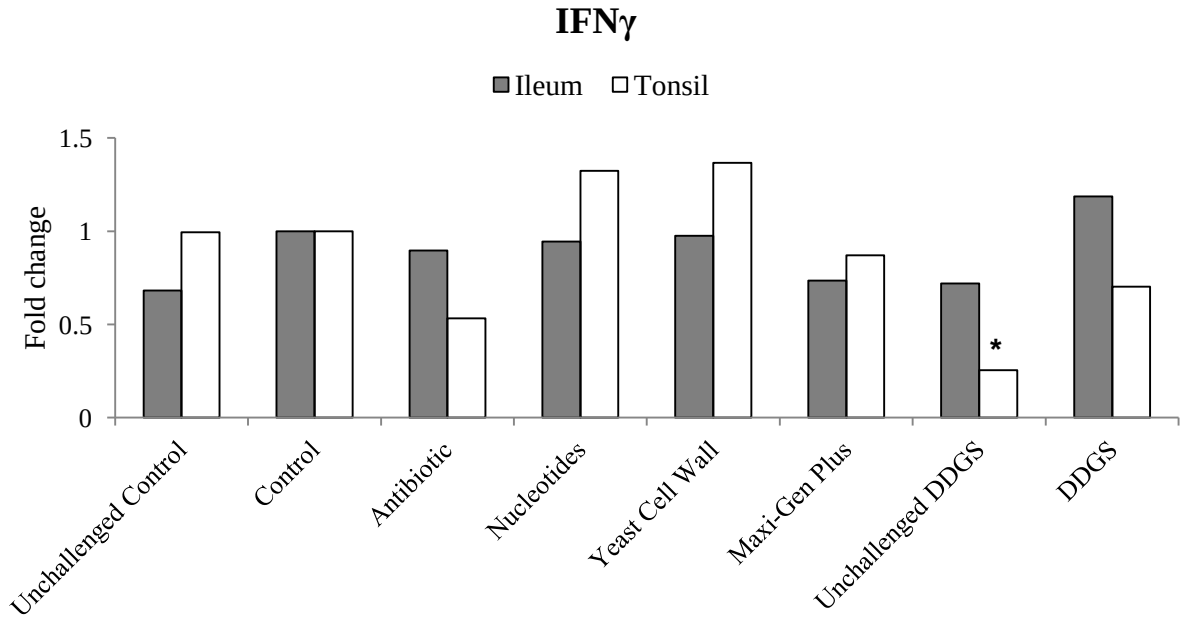


Figure 6.2. Fold change gene expression of cytokines in the ileum and cecal tonsils of birds challenged with *C. perfringens*



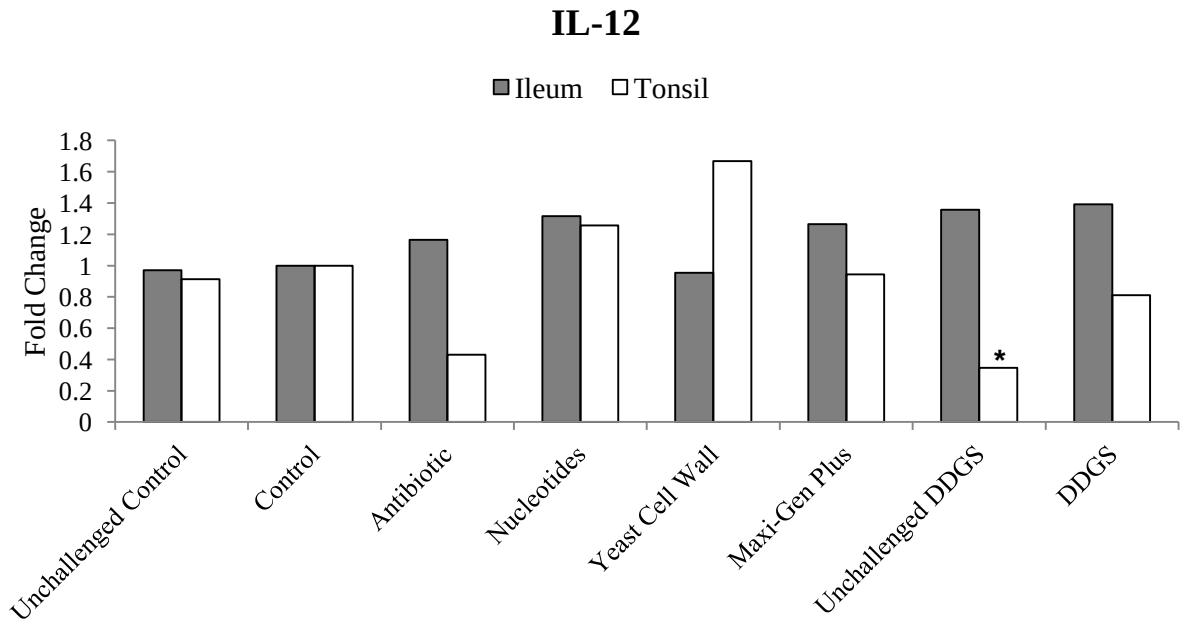
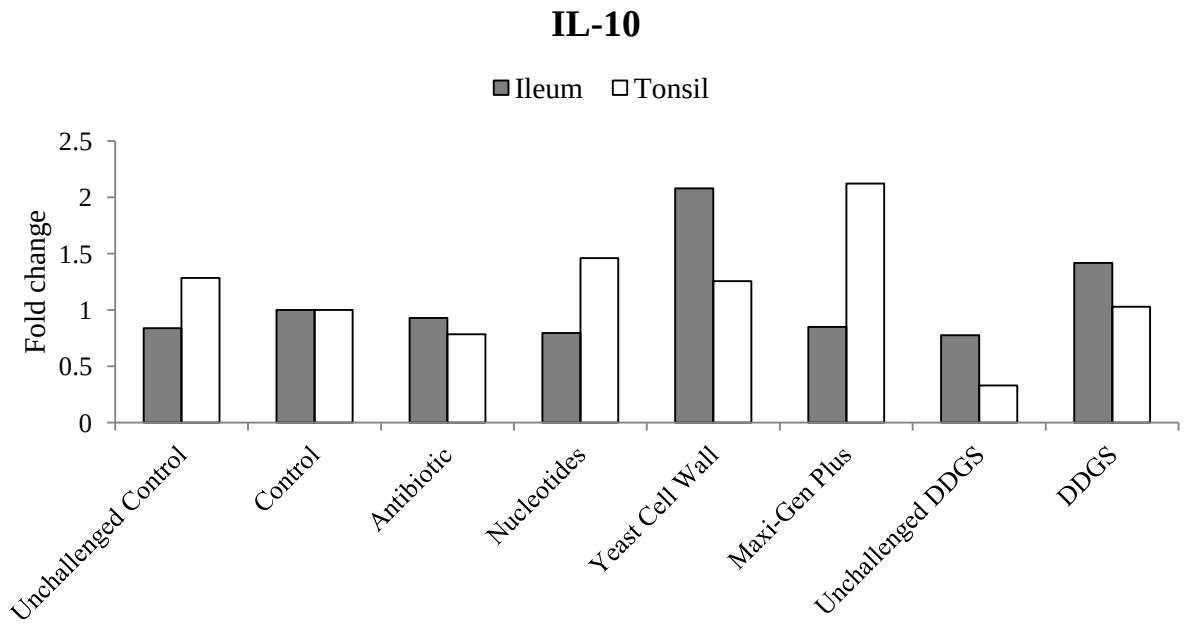


Figure 6.2 Continued. Fold change gene expression of cytokines in the ileum and cecal tonsils of birds challenged with *C. perfringens*



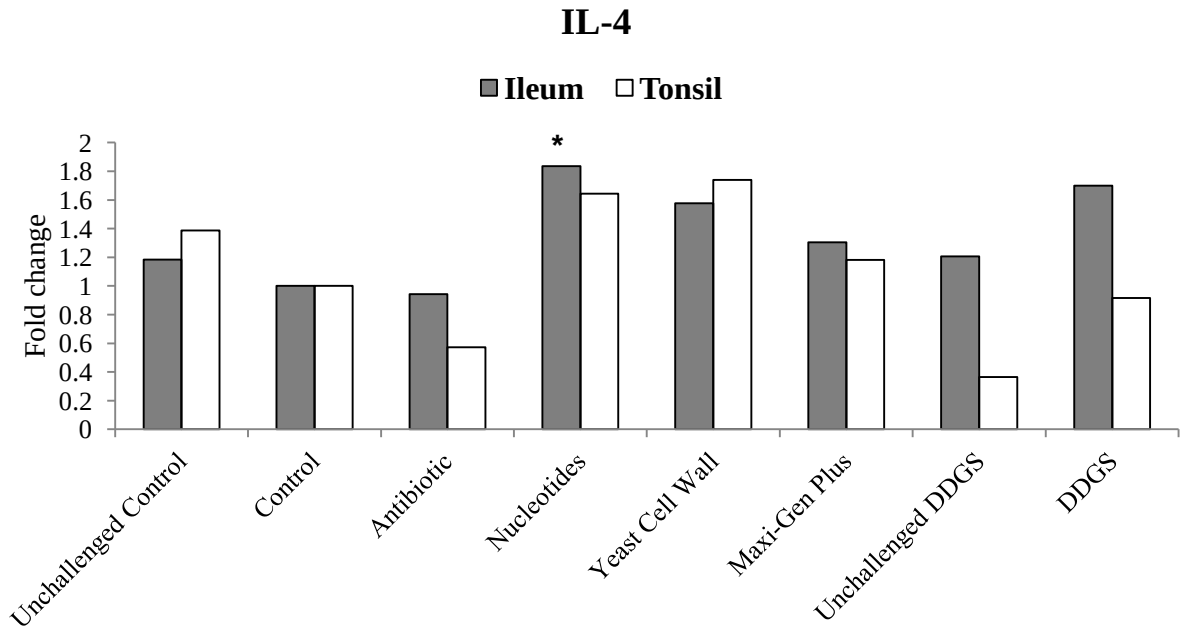
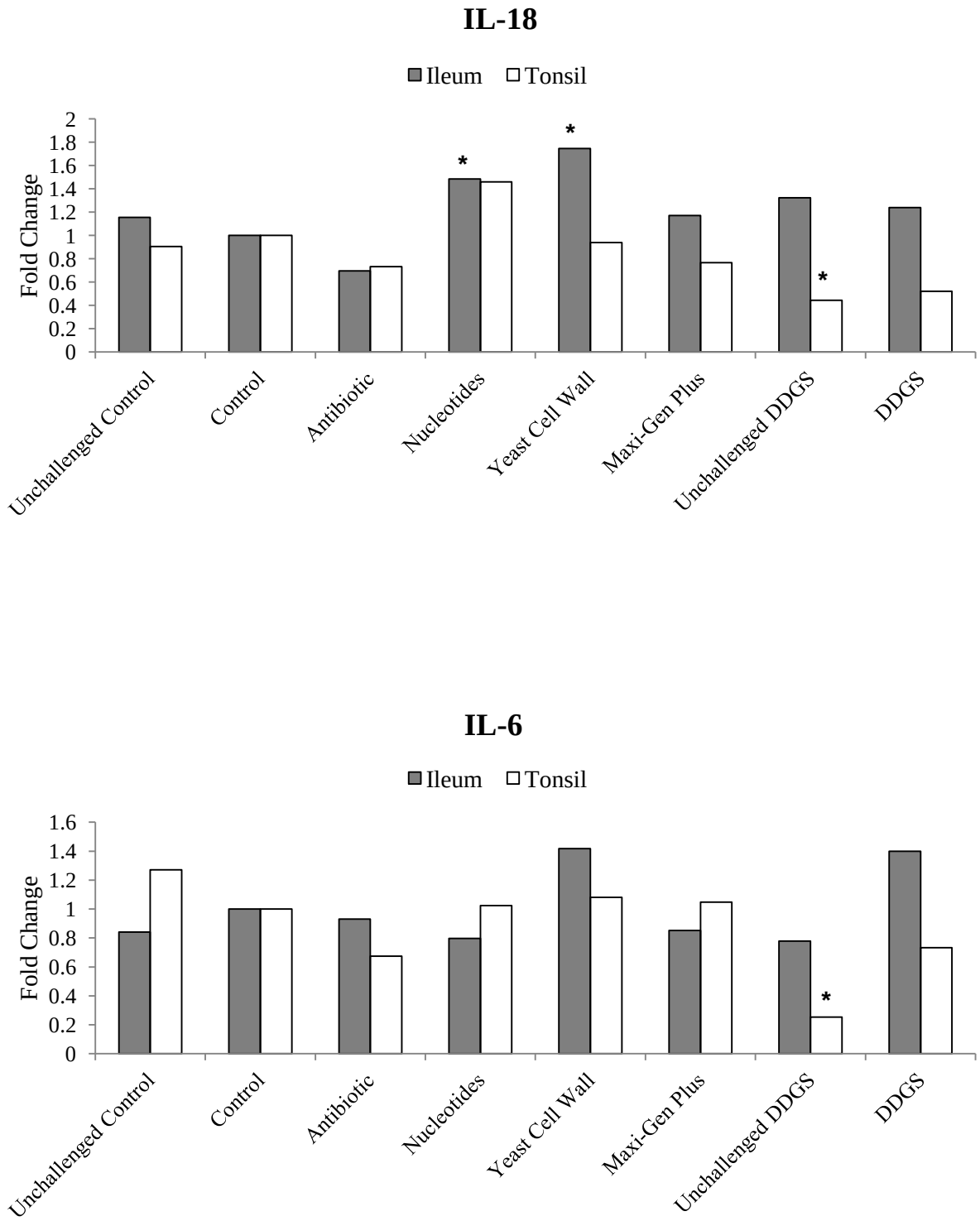


Figure 6.2 Continued. Fold change gene expression of cytokines in the ileum and cecal tonsils of birds challenged with *C. perfringens*



CHAPTER 7: GENERAL DISCUSSION

Growth Performance and Gut Morphology

Yeast cell wall polysaccharides including ß1,3-1,6-glucan and mannan polysaccharides have been shown to have growth promoting properties through improving the integrity of the intestinal epithelium and providing competitive binding sites for pathogenic bacteria such as *Salmonella* and *E. coli* (Spring et al., 2000; Huff et al., 2006). In addition, yeast nucleotides have the potential to increase digestion and absorption of nutrients and improve growth performance due to their critical role in the development and proliferation of tissues with rapid cell turnover such as intestinal epithelium (Grimble, 1994; Maldonado, et al., 2001). The results of the first and third study of this thesis (i.e., Chapters 3 and 5) demonstrated that supplementation of the diet with yeast-derived products did not affect growth performance parameters under non-pathogen challenge conditions. Furthermore, in the last study (Chapter 6), the effect of yeast-derived products on growth performance of birds under *Clostridium perfringens* challenge was investigated. The results demonstrated that over the entire trial (0-35) supplementation of the diet with yeast products did not significantly influence the growth performance of birds compared to the control group. These findings are in agreement with some previous studies showing no significant changes in feed intake, body weight gain, and FCR of broilers fed diets containing yeast products (Cox et al., 2010a; Munyak et al., 2012; Baurhoo et al., 2009; Yitbarek et al., 2013).

In contrast, other studies demonstrated that yeast-derived products can improve growth performance of broiler chickens (Baurhoo et al., 2009; Ghosh et al., 2012; Muthusamy et al., 2011). The inconsistent results regarding the effect of yeast products on growth performance parameters might be ascribed to the source, concentration, and the type of yeast products,

experimental conditions, duration of the trial and the presence and type of challenge conditions used in the different studies.

In the first study of this thesis (i.e., Chapter 3) involving gut histomorphology, supplementation of diets with YCW and nucleotides (i.e., 0.025 and 0.05%) increased villus height in the jejunum of broiler chickens. Similarly to our results, Shane et al. (2001) and Savage et al. (1997) showed that supplementation of YCW increased villus height in broiler chickens. It has been reported that the increase in villus height is associated with increased surface area of villus and improved digestion and absorption of nutrients (Izat et al., 1989; Gao et al., 2008). However, in the current study increased villus height did not translate into an improvement in growth performance. This could be associated with the duration of our first trial which lasted for 21 d. There is a possibility that improvement in gut morphology could lead to the enhanced growth performance of birds in grower phase (21-42 d). Furthermore, birds receiving the yeast-derived products (i.e., YCW, Maxi-Gen Plus, and 0.025 and 0.05% of nucleotides) had an increased number of goblet cells in the ileum compared to the control group. Goblet cells are special epithelial cells that are involved in mucus production. Considering the role of mucus in trapping and removal of pathogenic bacteria, an enhanced number of goblet cells in birds receiving yeast-derived products could lead to an improvement of gut health (Specian and Oliver, 1991; Uni et al., 2003).

Innate Immunity

In addition to their potential growth promoting properties, it has been reported that yeast cell wall polysaccharides can modulate the innate immune response. Innate immunity is considered the first line of defense against invading pathogens (Turvey and Broide, 2010). Pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and c-type lectin

receptors play a critical role in innate immunity by recognition of conserved microbial-associated molecular patterns (MAMP) present in bacteria and fungi. Recognition of MAMP by PRRs expressed by epithelial cells and cells of innate immunity (i.e., macrophages dendritic cells and natural killer cells) leads to the activation of such cells and the production of cytokines that regulate the immune response and link innate immunity to acquired immunity (Kawai and Akeira, 2010; Kannaki et al., 2010). It has been reported that mannan polysaccharides and ß1,3-1,6-glucan present in yeast cell walls can act as MAMP and modulate innate immunity by activation of the PRRs (Shahinian and Bussey, 2000; Shashidhara and Devegowda, 2003; Cox et al., 2010a; Yitbarek et al., 2012). In the first study (Chapter 3), the effects of yeast-derived products on gene expression of PPRs and cytokines in cecal tonsils and the spleen of broiler chickens was evaluated. The results demonstrated that supplementation of diets with yeast products downregulated the expression of PRRs and cytokines in broiler chickens. Considering the role of bacteria in activation of immune system, downregulation of PRRs and cytokines in birds receiving yeast-derived products might be explained by the higher number of goblet cells observed in the ileum of these birds. Mucus produced by goblet cells is a glycoprotein-rich secretion that is able to trap and neutralize bacteria and reduce bacterial immune stimulation at the intestinal level (Singh et al., 2002; Sheehan et al., 2007). The results of the first study showed that yeast-derived products did not exert growth promoting and immunostimulating activity under non-pathogen challenge conditions. Furthermore, the decreased expression of inflammatory cytokines such as IFNγ and IL-12 in birds receiving yeast-derived products suggests anti-inflammatory properties of these products under non-pathogen challenge conditions. Based on the results of the first study, we designed three more studies to evaluate the

effects of yeast-derived products on the immune response of birds following immunization and challenge with different antigens.

In the second study (Chapter 4), the effect of yeast-derived products on the innate immune response of broilers immunized with sheep red blood cells (SRBC) and bovine serum albumin (BSA) was investigated. The results of this study demonstrated that YCW supplementation of the diet in birds immunized with SRBC and BSA upregulated the expression of TLR2 and Th2 cytokines in cecal tonsils. However, expression of Th1 cytokines was not affected by dietary treatments. Toll-like receptor 2 is involved in recognition of "zymosan" as a yeast cell wall component that contains β-1,3-glycosidic linkages. Therefore, upregulation of TLR2 in birds receiving the diet containing YCW might have been associated with significant quantities of β-glucans component in this product (Sato et al., 2003). Furthermore, birds receiving the diet containing YCW showed an increased expression of Th2 cytokines. T helper 2 cytokines, including IL-10, IL-4, and IL-13, are considered anti-inflammatory cytokines that are involved in activation, maturation and proliferation of B cells into antibody-producing plasma cells and that enhance production of antibodies (Romagnani, 2000; Opal and DePalo, 2000). However, in the second study, the expression of proinflammatory cytokines was not affected by yeast products. This was probably due to the fact that SRBC and BSA were not identifiable as pathogenic immunogens (Gehad et al., 2002), and immunization of birds with these antigens did not lead to an inflammatory response. The results of the second study suggested that a combination of YCW and non-pathogenic immunogens can increase Th2 cell-mediated immune response in broiler chickens.

In the third study (Chapter 5), the effect of yeast products on the innate immune response of birds challenged with *Escherichia coli* lipopolysaccharide was evaluated. The results

demonstrated that supplementation of the diet with YCW and DDGS upregulated the expression of PRRs (i.e., TLR21 and MMR) and both inflammatory and anti-inflammatory cytokines in the spleen of birds. Increased expression of both Th1 and Th2 cytokines in this study might be explained by the cross-regulatory effects between these two types of cytokines. Interleukin-12 is a Th1 cytokine that is involved in the inflammatory response and induces the production of IFN-γ while it decreases IL-4 mediated suppression of IFN-γ (Trinchieri and Scott, 1994; Wolf et al., 1994). On the other hand, IL-4 as a Th2 cytokine reduces the differentiation of naive T cells to Th1 cells and downregulates the expression of Th1 cytokines (King et al., 1993). The increased expression of PRRs and cytokines observed in birds receiving diets supplemented with YCW and DDGS suggested that yeast cell wall polysaccharides including ß1,3-1,6-glucan and mannan can work as immune-adjuvants and help birds to mount faster immune responses following immunization with a pathogenic antigen.

Humoral Immunity

In addition to cell-mediated innate immunity, humoral immunity plays an essential role in innate and acquired immune response. Antibodies produced by B cells are the most important components of humoral immunity that are involved in host immune response through immobilization, neutralization, and opsonisation of antigens and pathogenic bacteria (Scott, 2004; Janeway, 2001). Following the humoral immune response, activation of B cells as the precursor of antibody producing cells happens through T cell-dependent or T cell-independent pathways (Parker, 1993). Antigens such as SRBC and BSA are considered T-dependent antigens that require T helper cells cooperation for activation of B cells and the production of antibodies. On the other hand, antigens such as LPS are considered as T-independent antigens because they can activate B cells without T helper cells involvement (Gehad et al, 2002).

The results of the second study (Chapter 4) demonstrated that supplementation of the diets with yeast-derived products in birds immunized with SRBC and BSA did not change the antibody-mediated immune response (i.e., IgG and IgM) compared to the control. However, the diet containing YCW increased the total antibody response against SRBC compared to the antibiotic group. This might be explained by the action of polysaccharide components including ß1,3-1,6-glucan and mannan polysaccharide in this product that have immune adjuvant properties and can stimulate the humoral immune response following immunization with a T-dependent antigen (Martinez-Pomares, 2001; Petrovsk and Cooper, 2011; Tsoni and Brown, 2008).

In the third study (Chapter 5) the effects of yeast product on antibody-mediated immune response in broiler chickens before and after immunization with LPS was also investigated. Prior to immunization, supplementation of diets with yeast products did not affect serum IgG and IgM level compared to the control. However, the diet containing YCW increased serum IgA compared to the antibiotic group. Considering the role of commensal bacteria in shaping and development of the immune system, a lower level of IgA in the serum of birds fed the antibiotic-containing diet might be explained by the antibacterial activities of this product that limit bacterial over-growth and decrease bacterial immune stimulation at the intestinal level (Collier et al., 2003; Rhee et al., 2004). Nevertheless, supplementation of the diets with yeast-derived products did not significantly affect the antibody-mediated immune response against LPS. This suggests that supplementation of yeast-derived product may not be involved in further activation of B cells in birds under challenge of a T-independent antigen (LPS). In agreement with our results, Silva et al. (2009) did not report any significant difference in antibody response against Newcastle disease virus or infectious bursal disease in birds fed diets containing yeast products.

Clostridium perfringens Counts*, *Lesion Score and Local Innate Immunity

In the last study, the effects of yeast products on gut lesion score, *C. perfringens* counts in the gut and local innate immune response of birds infected with mild necrotic enteritis caused by *C. perfringens* was investigated. The results demonstrated that supplementation of the diet with yeast-derived products was not as effective as antibiotic with regards to gut lesion score and *C. perfringens* counts. This is probably is because of the fact that *C. perfringens* is a gram positive bacteria and antibiotics such as BMD have been found to be most effective against gram positive bacteria (Engberg et al., 2000; Sims et al., 2004). However, yeast-derived products and particularly mannan polysaccharides of YCW have been found to be more effective against bacteria with type-1 fimbriae such as *Salmonella and E.coli* (Spring et al., 2000; Ferket et al., 2002). Regarding the innate immune response, the diet containing YCW increased the expression of MMR and IL-18 in the ileum suggesting some immune stimulatory activity of the YCW. In addition, birds receiving the diet containing nucleotides showed upregulation of TLRs and IL-4 in the ileum. It has been demonstrated that enhanced expression of TLRs in the ileum is associated with an improved mucosal epithelial barrier against pathogenic bacteria (Chen et al., 2007). Upregulation of TLRs and IL-4 in birds receiving nucleotides might be related to the role of these products in development of epithelial cells and lymphocytes (Carver, 1999), suggesting that dietary nucleotides are capable of activation of the local innate immune response under microbial challenge.

CHAPTER 8: CONCLUSIONS

1. Supplementation of diets with YCW and nucleotides increased villus height in the jejunum and enhanced the number of goblet cells in the ileum. However, this did not translate into improvement in growth performance.
2. Yeast products did not exert immune stimulating activity under non-pathogen challenge conditions, however, they may exhibit some anti-inflammatory properties by down-regulating the inflammatory cytokines IFNγ and IL-12
3. Diets containing YCW and fed to birds immunized with T-dependent antigens (sheep red blood cells and bovine serum albumin) activated the Th2-cell mediated immune response compared to the control and increased the antibody-mediated immune response compared to the antibiotic group. This suggested that yeast cell wall polysaccharides have immune-adjuvant activities and are able to stimulate the innate and humoral immune responses following immunization with T-dependent antigens.
4. Birds receiving YCW and challenged with LPS, a T-independent antigen, increased the expression of PRRs and cytokines involved in innate immune responses. However, humoral immunity was not affected by any of the yeast products. This may indicate that YCW containing ß1,3-1,6-glucan, mannan polysaccharides can help birds to mount faster and stronger innate immune response under pathogen challenge conditions.
5. Yeast-derived products were not as effective as BMD in reducing the lesion scores and *C. perfringens* counts in the gut. However, diets containing YCW and nucleotides increased the expression of PRRs and cytokines in the ileum suggesting the beneficial effect of these products in further activation of local innate immunity during pathogen challenge.

CHAPTER 9: FUTURE DIRECTIONS

The present studies demonstrated that the diet supplemented with yeast-cell wall polysaccharides, including ß1,3-1,6-glucan and mannan, was able to stimulate the innate immune response by increasing the expression of pattern recognition receptors and cytokines involved in innate immunity, and improved gut health and development by enhancing the number of goblet cells in ileum and increasing villus height in the jejunum.

In addition to the beneficial effects of yeast cell wall polysaccharides on gut development and immune system activation, it has been reported that mannans in the yeast cell wall have the ability to bind to the receptor sites (type 1 fimbriae) of some pathogenic bacteria such as *Salmonella* and *E.coli* to prevent bacterial adhesion to the epithelial cells. Therefore, it would be of interest to conduct additional animal experiments to evaluate the effects of the yeast cell wall polysaccharides on gut health, immune response, and gut microbiota of broiler chickens under *Salmonella* and *E. coli* challenge conditions.

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