EFFECT OF DIFFERENT CARBOHYDRATE AND NUCLEOTIDE DERIVED FROM YEAST PRODUCTS, INCLUDING DISTILLERS DRIED GRAINS WITH SOLUBLE (DDGS), ON INNATE IMMUNITY USING A CHICKEN B CELLS MODEL: *in vitro studies*

by

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ABSTRACT

The poultry industry has been using antibiotics to enhance bird performance through the control of microbial agents in the gastrointestinal tract; however antibiotic alternatives must be investigated. The effect of yeast-derived products such as brewer's yeast (Y), yeast cell wall polysaccharide-rich product (YCW), wheat/corn distiller dried grains with solubles (DDGS), nucleotide-rich product (N), processed yeast/nucleotide-rich product (PY+N) and D+mannose (M) was assessed using chicken B cells (DT40 cell line) and a LPS challenge model. Relative gene expression of toll-like receptors (TLR) and cytokines was then characterized.. These results show that yeast and yeast-derived products stimulate LPS-challenged B cells to produce Th2-associated cytokines which may lead to in vivo antibody production and gram-negative bacteria clearance. Furthermore, the up-regulation of IL-10 caused by the presence of Y, DDGS, N and PY+N is an important finding as it illustrates the regulatory activity stimulated by these treatments in the presence of Escherichia coli-derived LPS.

FOREWARD

This thesis was written in a manuscript format and it is composed of one manuscript. The manuscript will be submitted to the Journal of Animal Science and for that reason it is formatted according to journal guidelines. The results from this manuscript will be presented at the American Association of Avian Pathologists meeting in August 2012 in San Diego, CA. The authors of the manuscript are Harold Echeverry, Alexander Yitbarek, Mohammadali Alizadeh, Peris Munyaka, Bogdan Slominski and Juan C. Rodriguez-Lecompte.

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LIST OF ABREVIATIONS

APC	Antigen presenting cell
ATCC	American Type Culture Collection
BALT	Bronchial-associated lymphoid tissue
BCR	B cell receptor
CALT	Conjuntival-associated lymphoid tissue
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
COX	Cyclooxygenase
CpG	Cytosine-phosphate-guanine
CRD	Carbohydrate recognition domain
CTLD	C-type lectin-like domain
CTLR	C-type lectin receptor
CWP	Cell wall polysaccharide
DAMP	Damaged-associated molecular patterns
DC	Dendritic cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DDGS	Distillers dried grains with solubles
DNA	Deoxyribonucleic acid

dsRNA	double-stranded Ribonucleic Acid
E. coli	*Escherichia coli*
FN II	Fibronectin type II
GalNAc	N-Acetyl-D-Galactosamine
GALT	Gut-associated lymphoid
GC	Germinal centers
iDCs	immature DCs
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IKK	IkB Kinase
IL- R	Interleukin Receptor
IL	Interleukin
IRAK	IL-1R-associated Kinase
JAKs	Janus tyrosine kinases
JNK	c-Jun N-terminal Kinase
LPS	Lipopolysaccharide
LRR	Leucine-reach repeats
MAL	Toll-interleukin 1 receptor domain containing adaptor protein
MASP	Mannose-binding lectin -associated protease
MBL	Mannose-binding lectin
MHC	Major Histocompatibility Complex
MKK3	MAPK Kinase 3

MR	Mannose receptor
MYD88	Myeloid differentiation primary response gene (88)
NFkB	Nuclear Factor-κβ
NK	Natural killer cell
NOD	Nucleotide-binding oligomerization domain
NOS	Nitric oxide synthase
ODN	Oligodeoxynucleotides
PAMP	Pathogen-associated molecular patterns
pDCs	plasmocytoid-Dendritic Cells
PRR	Pattern recognition receptor
qRT-PCR	quantitative real time-polymerase chain reaction
RIP1	Receptor-interacting protein 1
RNA	Ribonucleic acid
ssRNA	single-stranded Ribonucleic Acid
STATs	Signal transducers and activators of transmition
TAK1	Activating transforming growing factor-activated protein kinase 1
TCR	T cell receptor
TICAM-1	TIR-domain-containing adapter-inducing interferon-β
TIR	Toll/IL-1 receptor
TIRAP	Toll-interleukin 1 receptor domain containing adaptor protein
TLR	Toll-like receptors
TNF	Tumor Necrosis Factor

TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
TSH	Thyroid-stimulating hormone
YDC	Yeast-derived carbohydrates

1. GENERAL INTRODUCTION

Yeast and yeast-derived products are rich sources of mannan oligo- or polysaccharides, ß1,3- ß1,6-glucans and nucleotides that can act as prebiotics (Lipke and Ovalle, 1998). They have been shown to stimulate the immune system as well as gastrointestinal tract development (de los Santos et al., 2007; Gao et al., 2008; Zdunczyk et al., 2005) and provide favourable conditions for growth of *Lactobacillus spp*., a genera of beneficial intestinal microbiota (An et al., 2008). Moreover, they compete for binding sites with pathogens containing mannose-specific type I fimbriae receptors, thus decreasing attachment and colonization (Krogfelt et al., 1990; Nurin et al., 1982). Based on preliminary results, distillers dried grains with solubles (DDGS) from corn and wheat contain 5.6 and 6.2% residual yeast biomass, respectively (Slominski et al., 2011). In addition, yeast cell wall ß1,3- and ß1,6-glucans and manannoligosaccharydes have been reported to be effective, at least to some extent, in binding mycotoxins and to providing protection from the effects of *Escherichia coli* challenge in broiler chickens (Biagi, 2009; Huff et al., 2010). Mannose is a unique sugar component of yeast cell wall and is only present in small quantities in wheat and corn grains. Therefore, given the variability of mannose content in DDGS products 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 antimicrobial alternatives to replace conventional antibiotics (de los Santos et al., 2007; Gao et al., 2008; Stanley et al., 2004).

Little is known about yeast-derived nucleotides because as they are not considered to be essential nutrients. However, research in the last several years has indicated that this consideration may not be completely clear because under rapid growth conditions and metabolic stress, demands for nucleotides may exceed the capacity of *de novo* synthesis (Adjei et al., 1996; Carver, 1994; Grimble, 1994; Sauer et al., 2011). Under such conditions, dietary nucleotides may spare the energetic cost of *de novo* synthesis (Grimble, 1994). In young chickens, the gastrointestinal tract exhibits rapid cell turnover and is unable to produce *de novo* all the necessary nucleotides needed to satisfy its own requirements (Uauy et al., 1994). Therefore, intestinal development is highly dependent on the presence of dietary nucleotides. Dietary nucleotides have shown to increase villi development, intestinal wall thickness, protein content, and DNA and RNA contents (Nunez et al., 1990).

The innate immune system is activated by pathogens or environmental antigens once they are recognized by receptor molecules such as mannan-binding lectin, DC-SIGN and the mannose receptor (East and Isacke, 2002; Geijtenbeek et al., 2009; Kilpatrick, 2003). Antigen-presenting cells (APC) such as macrophages, dendritic cells and B cells represent a central and important part of the immune defence against invading microorganisms. APCs participate in the initial capture and processing of microbial antigens (innate immunity) and present the antigen to specific T and B cell for activation of effector mechanisms (acquired immunity) (Rodríguez-Pinto, 2005; Siamon, 2002). Recognition of microbial molecules by antigen-presenting cells occurs through pattern recognition receptors (PRRs), which recognize conserved structures that are present in

microorganisms called pathogen-associated molecular patterns (PAMPs) (Kedziora and Słotwiński, 2009). Toll-like receptors are the most extensively studied PRRs, but accumulating evidence shows that other PRRs, such as scavenger receptors, C-type lectin receptors and NOD-like receptors (nucleotide-binding oligomerization domain), also play important roles in innate immune defence (Fabriek et al., 2005; Mathews et al., 2008; Willment and Brown, 2008).

In addition to their role in humoral immunity, B lymphocytes are important antigen presenting cells; in the same way as other APCs, B cells, have TLRs and produce cytokines upon activation with the potential to modulate T cell responses (Berglová et al., 2011; Mosman, 2001). This data highlights the potentially unique nature of immune modulation when B cells act as APC. Furthermore, B cell TLRs have shown to be of key importance in cooperation with BCR for reaching a rapid and lasting humoral activity (Cerutti et al., 2011).

The objective of the proposed research was to investigate the effect of corn and wheat DDGS, yeast and yeast-derived products with different concentration of mannan, ß-glucan and nucleotides on innate immune response of chicken B cells using a LPS challenge model .

2. LITERATURE REVIEW

2.1. INTRODUCTION

Under natural conditions animals are being exposed to multiple environmental microorganisms. Initially, the microorganisms must deal with the first line of defence of the organism involving physical, mechanical and chemical barriers belonging to the innate immune system. Immediately after pathogens trespass these barriers, the cellular parts of the innate immune system are activated (Turvey and Broide, 2010). Heterophils and antigen presenting cells (APCs) such as B cells, macrophages and dendritic cells (DC) are able to capture and phagocytise invader pathogens, leading to their destruction. APCs stimulate T and B cells via antigen presentation and cytokines release, therefore activating a second line of defence known as acquired immunity (Banchereau et al., 2003; Blander and Medzhitov, 2006). In addition, other cells such as natural killer cells (NK) participate in the initial elimination of pathogens (Turvey and Broide, 2010).

APCs have the capability to recognize several conserved structures common to pathogens through pathogen-associated molecular patterns (PAMPs), such as proteins, saccharides, lipids and nucleic acids, which are essential parts of the pathogen for their survival and infectivity (Uematsu and Akira, 2006). One of the most characterized PAMP is lipopolysaccharide (LPS), an important component of the external membrane of gram-negative bacteria responsible for endotoxin activity and immune system activation

(Seydel et al., 2000). This recognition process of PAMPs is carried out by the presence of a large group of germline-encoded surface molecules on cells known as pattern recognition receptors (PRR) (Siamon, 2002). Some of these PRRs, following contact with the corresponding PAMP, assist in the activation of APCs phagocytosis process of the pathogen. First, pathogens are internalized into a phagosome, which fuses with lysosomes to form phago-lysosomes, in which pathogens are digested and processed for further presentation and activation of the acquired immune system (Stuart and Ezekowitz, 2005). Once PRRs are activated by the recognition of a specific PAMP, they initiate a downstream signalling pathway which will lead to the production of different cytokines and chemokines, directing the immune response to a pro-inflammatory process (Aderem, 2003). LPS, a PAMP of gram-negative bacteria, is recognized by TLR4 on APCs, activating a signalling pathway leading to the production of pro-inflammatory cytokines (Lu et al., 2008). TLRs can also recognize mannan structures from cell walls of fungi species (Levitz, 2004; Tada et al., 2002; Van der Graaf et al., 2006).

It is estimated that ethanol plants in Manitoba will generate 227,000 tonnes of distilled grains to be consumed by the swine, livestock and poultry industry every year (Mannes, 2002). In order to derive maximum value from these co-products, their natural advantage as feed ingredients must be presented to the poultry industry. Over the last few years extensive research on the chemical and nutritional content of corn and wheat DDGS for poultry and swine has been completed (Lan et al., 2008; Lumpkins et al., 2004; Rogiewicz et al., 2010). To our knowledge, what has not been considered yet in DDGS research is the fact that as co-products of brewer's yeast (*Saccharomyces*

cerevisiae) fermentation, DDGS will contain a significant quantity of components which are beneficial to gut development, health and effective modulation of the immune system. Because of the antimicrobial and immuno-modulatory activity of DDGS, it is thought that they may serve as alternatives to antibiotics for growth promotion and disease resistance in poultry production.

2.2. YEAST (*Saccharomyces cerevisiae*)-DERIVED PRODUCTS

Yeast cells are surrounded by an outer hard cell wall and inner membrane together commonly known as the yeast cell wall, which protect the cell from environmental factors, selectively allows or limits molecule entrance into the cell and gives consistency to the cell (Kang et al., 2008). Yeast cell wall is mainly composed of ß1.3-glucan (50-55%), mannoproteins (35-40%), ß1,6-glucan (5-10%) and N-Acetylglucosamine (1-3%) in chitin (Blazejak et al., 2007; Cabib, 2001). On the other hand, nucleotides are part of internal yeast cell components that could be of importance for animal production systems (Li, 2004). In general terms, the previously mentioned components of yeast and/or yeast-derived products have been found to function as prebiotics (Lipke and Ovalle, 1998). The term prebiotic makes reference to non-digestible dietary elements which stimulate the development of beneficial bacteria (microbiota) in the digestive tract, which in turn aid in pathogens control and modulate the immune system (Manning and Gibson, 2004). Prebiotics have been shown to provide favourable conditions for beneficial intestinal *Lactobacillus* spp. and competitive binding sites for pathogens with mannose-specific fimbriae receptor Type I (such as *Salmonella*),

thus decreasing attachment and colonization (de los Santos et al., 2007; White et al., 2002). In addition, they have been shown to stimulate both the immune system and gastrointestinal tract development (Gao et al., 2008; Solis de los Santos et al., 2005; Zdunczyk et al., 2005). Yeast cell wall ß1,3- and ß1,6-glucans have been reported to be at least somewhat effective in binding mycotoxins and in providing protection from the effects of *E. coli* challenge in broiler chickens (Biagi, 2009; Huff et al., 2010). Little is known about yeast nucleotides since they are not considered essential nutrients. Recent research, however, indicates that this may not be the case because under conditions of rapid growth and metabolic stress, demands may exceed the capacity of *de novo* synthesis (Adjei et al., 1996; Carver, 1994; Grimble, 1994; Sauer et al., 2011). Under such conditions, dietary nucleotides may spare the energetic cost of *de novo* synthesis. Cells and tissues with a high rate of proliferation such as immune cells, bone marrow cells and gastrointestinal tract cells may not be able to produce enough endogenous nucleotides through the *de novo* synthesis pathway, so they may use external nucleotides to satisfy their own requirements (Maldonado et al., 2001). Nucleotides have proven to improve gut morphology in mice by increasing villi development, intestinal wall thickness, protein content and DNA and RNA contents (Carver, 1994).

Yeast and yeast-derived products have a large variation in terms of their chemical composition (Table. 2) and sugar content (Table. 3) (Slomisnki, et al. 2011, unpublished data) and it is thought that they are going to benefit the immune system in different fashions.

Table. 1 Chemical composition of yeast-based products (%, as is basis)

Sample ID	Sample Description	Cell Wall Polysaccharides (CWP)	Protein (N x 6.25)	Nucleotides[1]	Carbohydrates[2]
Brewer's Yeast	Active yeast S. 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	0.133	4.1
Hi-Yeast 751	Nucleotide-rich product	7.2	52	9.32	5.4
Yeast Cell Wall	CWP-rich product	43.3	17.2	0.33	9.4
Maxi-Gen Plus	Process yeast/nucleotide-rich product	21.6	32.7	0.13	14.3

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

(Slomisnki, et al. 2011, unpublished data)

Table. 2 Cell wall polysaccharide component sugar content (mg/g, as is basis)

Sample ID	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acids	Total
Brewer's Yeast	1.5	0.0	102.0	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
Wheat DDGS	51.0	82.0	12.5	9.6	53.6	8.4	218.0
Hi-Yeast 751[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 thus mannan content of Hi-yeast 751 and very little glucose content

(Slomisnki, et al. 2011, unpublished data)

2.3. DISTILLERS DRIED GRAINS WITH SOLUBLES (DDGS)

An increased interest in environmental sustainability has created an opportunity for expanding ethanol production as a motor fuel additive, which has a successive production history in the United States (Patzek et al., 2005). In Canada, federal and provincial governments have mandated that all fuels sold must contain a certain percentage of alcohol, that being 10% in the province of Manitoba. As a result of this, the Government of Canada has allocated millions of dollars towards the construction and expansion of ethanol plants across Canada, examples being Commercial Alcohols Inc., Husky Energy, Integrated Grain Processors, Iogen Corporation and Commercial Alcohols Inc. Existing ethanol plants and plants under construction bring total Canadian ethanol production to approximately 1,200 million litres per year, almost six times the amount produced prior to the launch of the Ethanol Expansion Program (Olar et al., 2004). The ethanol industry is using two different approaches to obtain ethanol from the fermentation of cereal grains: wet milling and dry grind processes. In the wet milling process, cereal grains are divided into components including starch, fibre, gluten and germ. Only the starch solution separated from the solids is fermented with yeast to produce ethanol, which is later distilled from the total fermented mixture. In the dry grind process, grains are grounded, mixed with water, enzymes and later cooked. After cooking they are fermented with yeast to produce the ethanol which is then separated by distillation. The final residue is dried and later used as feedstuff in the animal feed industry – it is this final dried product known as distilled dried grains with solubles DDGS (Bothast and

Schlicher, 2005; Liu, 2011). It has been estimated that ethanol plants in Manitoba will generate 227,000 tonnes per year of distilled grains to be used by the livestock industry (Mannes, 2002). Aside from the nutritional value that DDGS offer as a feedstuff, it is important to consider that they consist of around 6% of yeast biomass (Slominski et al, 2010. Unpublished data), which could have implications on the health and immune system of animals in production.

2.4. AVIAN IMMUNE SYSTEM

In general, the avian immune system works in a similar fashion to that of their mammalian counterparts; however, due to evolution, birds developed anatomical and physiological differences in order to accomplish immunological tasks (Schat, 2008).

The avian immune system is formed by primary lymphoid organs such as the Bursa of Fabricius and the Thymus; as well as secondary lymphoid organs including the spleen, bone marrow, conjuntival-associated lymphoid tissue (CALT), bronchial-associated lymphoid tissue (BALT) and gut-associated lymphoid tissue (GALT) (Bar-Shira et al., 2003; Fagerland and Arp, 1993; van Ginkel et al., 2012). Chickens do not have lymph nodes; however, they possess lymphoid tissue distributed throughout the parenchyma of several organs including the liver, pancreas, spleen and kidney. These consist of one or more germinal centers (GCs) which are separated from adjacent tissue by a fine capsule of connective tissue (Pastoret Paul-Pierre, 1998). GCs are mainly formed by B cells after antigen stimulation in the lymphoid tissue (Yasuda et al., 2003).

The Bursa of Fabricius is found only in birds; it is a small, rounded organ located in the hind gut, between the sacrum and the cloaca and is the site of B cell differentiation (Glick, 1991, 1985). The Bursa is connected to the cloaca by a small duct which allows the passage of products and antigens to the bursal lumen and puts them in contact with B cells. Precursor of B cells from the yolk sac, spleen and bone marrow begin colonizing the Bursa around day 10-12 of embryonic life, where the cells begin to proliferate (Reynaud et al., 1992). Later, these B cell precursors begin to rearrange immunoglobulin heavy and light chain gene segments to create a functional cell surface IgM receptor. However, due to out-of-frame rearrangements in both heavy and light chains as well as self-reactivity, approximately 75% of these B cells undergo apoptosis (Aliahmad et al., 2005; Ollila and Vihinen, 2005). Surviving cells express surface B cell receptor (BCR) (Ratcliffe, 2006). Around day 18 of embryonic life, B cells are mature enough to migrate to peripheral lymphoid locations such as the spleen, cecal tonsils and Peyers' patches (Kajiwara et al., 2003; Linna et al., 1969). Thus, once stimulation with antigens occurs, cells begin differentiation and class switching (Narabara et al., 2009). With multiple antigens, B cells have shown to be able to stimulate and prime T CD4+ cells independently of DCs presence, stating their antigen presentation capabilities (Rodríguez-Pinto, 2005). B cells are generally known to be able to recognize pathogens via the BCR, nevertheless it has been shown that Toll-like receptors also play an important role in the recognition of pathogens, activation of B cells and final production of cytokines, chemokines and co-stimulatory molecules (Booth et al., 2010; Fillatreau and Manz, 2006; Peng, 2005).

The first line of defence the body has against pathogens consists of physical (epithelial surfaces), physiological (mucociliary movements, peristaltic movements) and chemical barriers (low stomach pH, lysozymes in saliva and tears) (Turvey and Broide, 2010). Antigen-presenting cells, such as B cells, macrophages and dendritic cells, represent a central and important part of the immune defence against invading microorganisms, as they participate in the initial capturing and processing of microbial antigens (innate immunity) that were able to trespass the first line of defence of the body (Janeway et al., 2008; Rodríguez-Pinto, 2005). Recognition of microbial molecules by antigen presenting cells occurs through pattern recognition receptors (PRRs), which recognize conserved structures, or pathogen-associated molecular patterns (PAMPs), in both non-pathogenic and pathogenic microbes (Kumar et al., 2011). Toll-like receptors are the most extensively studied type of receptors (Siamon, 2002), though research now shows that other PRRs, such as scavenger receptors, C-type lectin receptors and NOD-like receptors, also play important roles in the innate immune defence (Fabriek et al., 2005; Mathews et al., 2008; Stuart and Ezekowitz, 2005). After pathogen recognition and processing by APCs, presentation occurs to prime and stimulate clonal expansion of antigen specific T and B cells, as well as to activate T and B cell effector mechanisms (acquired immunity) (Janeway et al., 2008). However, it is important to highlight the fact that B lymphocytes are not only important in humoral immunity, but also important antigen presenting cells. Similar to other APCs, B lymphocytes contain TLRs, produce cytokines upon activation and also have the potential to modulate T cell responses (Berglová et al., 2011; Mosman, 2001).

2.5. CARBOHYDRATES AND IMMUNE SYSTEM

The cell wall of microbial organisms is composed of polysaccharides and glycol-conjugates which are essential for microbial survival and virulence (Knirel, 2011). These carbohydrates have been shown to interact with receptors of the immune system, participating in processes such as opsonisation, phagocytosis, cell activation, cell differentiation and apoptosis (Ni and Tizard, 1996).

Mannose is a sugar component of yeast cell wall, that is also present in both wheat and corn grains but just in small quantities. The residual yeast biomass content has been determined to average 6.2 and 5.6% for wheat and corn DDGS, respectively (Slominski et al., 2011). These facts could be of importance due to the variety of *Saccharomyces cerevisiae* yeast-based products currently being offered to the poultry industry as growth promoters and natural alternatives to antibiotics (de los Santos et al., 2007; Stanley et al., 2004).

2.5.1. Recognition of Carbohydrates by the Immune System

Considering the fact that microbial organisms are composed of different glycol-conjugates and polysaccharides on their external surfaces, and that they are common to groups of pathogens, it was thought that the immune system would develop different receptors which would be able to recognize these foreign carbohydrate molecules (Buzás et al., 2006). In addition, due to evolution, these receptors should be germ-line encoded

so future generations will have the mechanisms necessary to detect and fight these pathogens.

Pathogen carbohydrate molecule recognition is essential for understanding the way the immune system interacts with different yeast and yeast-derived products, including those used in the present study. Therefore, the main receptors involved in this process are going to be discussed.

2.5.2. C-Type Lectin Receptors

The term “C-type lectin” was first used to determine a group of $Ca2^{+}$-dependent (C-type) carbohydrate-binding (lectin) proteins. Their carbohydrate-binding activity is directed by a carbohydrate recognition domain (CRD) which was shown to be Ca dependent (Zelensky and Gready, 2005).

The C-type lectin receptors (CTLRs) are a diverse family of proteins that contain at least one structurally related C-type lectin-like domain (CTLD) with a broad range of activities for carbohydrate recognition; it is now known that it is not always $Ca2^{+}$ dependent (Willment and Brown, 2008).

Members of the CTLR family are PRRs that participate in pathogen recognition by the immune system, with some even mediating the phagocytosis of pathogens. Specifically, dendritic cell-specific intercellular adhesion molecule-grabbing non-integrin (DC-SIGN), mannose receptor, Dectin-1 and mannose binding lectin (MBL) will be discussed (Kerrigan and Brown, 2009).

2.5.2.1. DC-SIGN

DC-SIGN is a type II trans-membrane C-type lectin that is Ca^{+} dependent for carbohydrate binding. It is composed of an intracellular domain with the responsibility of signal transduction and, due to the presence of internalization motifs, it is thought to be an important endocytic receptor. DC-SIGN also contains extracellular domains called the neck region which are characterized by the presence of a C-type carbohydrate recognition domain (CRD) (Khoo et al., 2008). CRD can recognize and bind high-mannose-type glycans (Fidgor et al., 2009). In addition, DC-SIGN can act as PRR detecting viruses, bacteria and parasites. Viruses include human immunodeficiency virus 1, Ebolavirus, Hepatitis C virus, Denguevirus, Cytomegalovirus, and SARS coronavirus. Bacteria include *Mycobacterium tuberculosis, Helicobacter pylori*, while parasites include *Leishmaniapifanoi, Schistosomamansoni*, and yeast include *Candida albicans* (Geijtenbeek et al., 2009; van Kooyk and Geijtenbeek, 2003). Furthermore, DC-SIGN can bind to the intracellular adhesion molecules-3 (ICAM3), allowing the TCR to detect small amounts of peptides from pathogens presented in the context of MHC II, finally allowing T cells to start the appropriate immune response (Ralph M, 2000).

2.5.2.2. Mannose Receptor

The mannose receptor (MR, CD206), previously known as the macrophage mannose receptor due to its initial discovery in only macrophages, is now known to be present in immature DCs (iDCs) and several epithelial cells (Linehan, 2005). The MR belongs to the family of CTLs; it is a trans-membrane receptor that differs from other

CTL family members by the presence of multiple CTLDs tandemly arranged. The MR is made up of an N-terminal cysteine-rich domain, a Fibronectin type II (FN II) domain, a trans-membrane domain and a cytoplasmic domain (Sturge et al., 2007). MR has the capability to bind either a carbohydrate with terminal mannose or fucose residues with its CTLDs. Furthermore, MR mediates the binding of N-Acetyl-D-Galactosamine (GalNAc) residues sulphated at position 4 or 3 with its cysteine-rich domain, and fibronectin type II (FNII) domain can bind collagens (Martinez-pomares, 2001). MR also mediates previous steps than antigen presentation delivering lipoarabinomannan through CD1b vesicles that are MCH and MHC-like molecules that participate in antigen presentation. In addition, MR can bind 4'-sulfated GalNAc ligands such as thyroid-stimulating hormone (TSH thyrotropin), removing it from circulation due to its cysteine-rich domain (East and Isacke, 2002).

2.5.2.3. Dectin-1

Dectin-1 is a Type II trans-membrane protein of the CTLRs family. It consists of an extracellular CTLD, a trans-membrane region and an intracellular tail containing signalling motifs. This receptor is present in macrophages, monocytes, dendritic cells, neutrophils, microglia and in some T cells. It also has been found in human B cell lines (Harnack et al., 2011; Meyer-Wentrup et al., 2007). Dectin-1 is able to recognize β-Glucans from fungi, plants and bacteria (Reid et al., 2009). Even though Dectin-1 has only been associated with the recognition of β-Glucans, it can also recognize mycobacteria, whose lack of β-Glucans allows us to infer that there are other ligands for

this receptor (Kimberg and Brown, 2008). B-Glucan recognition by Dectin-1 activates a downstream signalling pathway through the immunoreceptor tyrosine-based activation like motif (ITAM-like). This pathway has phosphorylation stages and a final oxidative burst response, COX activation and pro-inflammatory cytokine production. In most cases, Dectin-1 activity is mediated in collaboration with TLRs (Tsoni and Brown, 2008). Dectin-1 activation by β-Glucans has been shown to induce acquired immunity by driving differentiation of Th17 (*in vitro*) and Th1 (*in vivo*) CD4+ T cells in a TLR-independent manner (Vautier et al., 2010). Furthermore, Dectin-1 induces T cytotoxic activity and antibody responses in vivo (Reid et al., 2009).

2.5.2.4. Mannose-Binding Lectin (MBL)

MBL is a serum soluble PRR protein produced in the liver by IL-1β, IL-6 and TNF-β stimulus (Janeway et al., 2005). This binds specific rich-mannose, GlcNAc or glucose molecules present on the surfaces of a variety of microorganisms, leading to the activation of the lectin pathway of the complement system (Arlaud and Thielens, 2001; Sato et al., 1994). MBL consists of a collagenous tail that keeps the integrity of the protein, and a lectin domain that is responsible for carbohydrate recognition. MBL forms a part of the group of acute phase proteins that are increased after an infection, trauma or surgery (Babovic-Vuksanovic et al., 1999). MBL can activate the classical complement pathway through C3 convertase, binding to carbohydrate molecules from pathogens (Gunesacar et al., 2011). MBL can also activate C2 and C4 by their cleavage with MBL-

associated proteases 1 and 2 (MASP1, MASP2) and following activation of C3. In this way, MBL works like an opsonin covering carbohydrate PAMPs facilitating pathogen elimination (Jack and Turner, 2003). MBL recognizes not only PAMPs, but can also bind damaged-associated molecular patterns (DAMPs) from self-altered cells. For instance, MBL can bind self-altered sugars from apoptotic or damaged cells facilitating their elimination by phagocytosis (Eddie Ip et al., 2009).

2.5.3. Toll-like Receptors (TLRs)

Initial studies in Drosophila species showed that these flies displayed a lack of adaptive immune responses, relying only on the innate immune system (Blasius and Beutler, 2010; H. Bilak, 2003). This fact makes Drosophila a viable research alternative in studying the functions of innate immunity. Drosophila Toll receptor was found to protect these flies against fungal and gram-positive bacterial infections through up-regulation and antimicrobial peptides production (Valanne et al., 2011). Further studies in mammals showed the presence of homologous receptors to Toll receptor found in Drosophila, described by their capability of recognizing different PAMP. Thus, these receptors were named Toll-like receptors (TLR) (Medzhitov et al., 1997; Takeda et al., 2003). Initial studies eventually led to the first TLR being described in mammals: TLR4, which is involved in recognition of gram-negative bacteria LPS (Poltorak et al., 1998; Qureshi et al., 1999; Warren, 2005).

The structure of TLRs consists of a leucin-rich repeat domain (LRR) in the extracellular domain and a Toll/IL-1 receptor domain (TIR domain) in the intracellular domain (Akira and Takeda, 2004). LRRs are known to be important in protein-protein

interactions and are essentials for TLR pathogen recognition (Kobe and Deisenhofer, 1994). TIR domains are responsible for signal transduction in downstream signalling pathways (Yamamoto, 2004).

According to their localization on the cells, TLRs are divided into different cell surface TLRs: TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10; and endosomal TLRs: TLR3, TLR7, TLR8 and TLR9 (Blasius and Beutler, 2010).

In spite of the fact that mainly TLR2 and TLR4 are involved in pathogen recognition via carbohydrates, in the present document a general review of TLRs will be performed due to their importance for the immune response.

2.5.3.1. Cell surface TLRs:

TLR2, 1 and 6: TLR2 is able to recognise different particles present in bacteria such as lipoproteins from gram-negative bacteria, peptidoglycans and lipoteichoic acid from gram-positive bacteria, Zymosan from fungi and also some atypical types of LPS that are not recognized by TLR4 (Schenk et al., 2009).

TLR1 and TLR6 activation depends on their coupling with TLR2 as they work as together as co-receptors, forming heterodimers with different functions. For example, TLR2/TLR1 heterodimer recognizes triacylated bacterial lipoproteins. On the other hand TLR2/TLR6 heterodimer can recognize diacylated bacterial lipoproteins (Reynaud et al., 1992; Schumann and Tapping, 2007).

TLR4 recognizes LPS present in the external membrane of gram-negative bacteria, and the main PAMP found in LPS is Lipid A (Miller et al., 2005). For an adequate TLR4 recognition of LPS, previous recognition of LPS by LPS-binding protein

(LBP) and the formation of a complex with CD14 and MD2 are required. However, in chickens there is no evidence of LBP. After this complex is formed, TLR4 is able to begin signal transduction (Lu et al., 2008). TLR4 is the only TLR able to activate two different signalling pathways and produce pro-inflammatory cytokines and Type I IFN (later discussed) (Lu et al., 2008). Besides TLR4 function in the recognition of LPS, it has also been known to participate in recognition of fungi, specifically mannan from cell wall of different fungi species (Levitz, 2004; Tada et al., 2002; Van der Graaf et al., 2006).

TLR5 recognizes Flagellin from bacteria such as Salmonella species, *E. coli* and *Campylobacter jejuni*; however, some of these pathogens have found ways to escape recognition from this TLR by producing altered Flagellin molecules (Andersen-Nissen et al., 2005; de Zoete et al., 2010; Hayashi et al., 2001). Flagellin is the main structural protein of flagellar filaments found in motile species (Joys, 1988). After motile bacteria recognition by TLR5, the signalling pathway leads production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 (McDermott et al., 2000; Ramos et al., 2004).

Phylogenetic analysis of the TLR10 receptor revealed its similarity with TLR1 and TLR6. It recognizes triacylated lipopeptides and other bacterial and fungal particles also recognized by TLR1, TLR2 and TLR6. However, the function of TLR10 is dependent upon TLR2 activation. (Guan et al., 2010).

TLR11 is mainly present in urinary tract epithelial cells, though it is also expressed in DCs and macrophages (Cai et al., 2009). Initially TLR11 activity was just attributed to the recognition of uro-pathogenic *E. coli*. However, further studies have

shown that it is also able to react against *Toxoplasma gondii* Profilin molecules, triggering the production of pro-inflammatory cytokines like IL12 (Bird, 2005).

2.5.3.2. Endosomal TLRs

TLR3 recognizes viral double stranded (ds) RNA that is normally produced during the replicative cycle of the virus. Once TLR3 is activated by its ligand, it promotes an antiviral state through the TRIF signalling pathway leading to Type I IFN production. (Matsumoto et al., 2011).

TLR7-TLR8 are able to recognize ssRNA from viruses; once activated, stimulate the production of pro-inflammatory cytokines. Moreover, it has been shown that activation of these TLRs is mainly associated with ssRNA location within the cell rather than in its structure (Crozat, 2004).

TLR9 recognizes cytosine-phosphate-guanine (CpG) di-nucleotides from microbial DNA sequences, differing from eukaryotic DNA by being mainly un-methylated. Recognition of CpG DNA by TLR9 leads the immune response to the production of pro-inflammatory Th1 cytokines such as IL-12 and IFN-γ (Gupta and Agrawal, 2010; Krieg, 2007).

2.5.4. Chicken TLRs

Chicken TLRs are similar in structure to mammals TLRs, consisting of several Leucine-rich repeats (LLRs) in the extracellular domain, a trans-membrane domain and a TIR-1 receptor domain. (Yilmaz et al., 2005). Chickens have a set of 10 TLRs

(Temperley et al., 2008) from which TLR3, 4, 5 and 7 are orthologous to other vertebrate TLRs (Kannaki et al., 2010). The chicken TLR2 was found to be duplicated in TLR2a (also called type1) and TLR2b (also called type2), both showing high homology with human TLR2; the pattern of chicken TLRs gene duplication is also observed in TLR1 (Cormican et al., 2009; Fukui et al., 2001). Chickens possess TLR15, which appears exclusive to avian species (Higgs et al., 2006). Chickens also posses TLR21, which is not present in mammals, however it is orthologous to the TLR21 found in fish and amphibians (Kannaki et al., 2010). The lack of TLR8 and TLR9 in chickens was determined by phylogenetic analysis studies (Temperley et al., 2008). In addition, chickens do not express TLR6 or TLR10. This deficiency appears to be compensated by gene duplication of TLR2 and TLR1 (Brownlie and Allan, 2010).

According to the literature, chicken TLR2.1, 2 or TLR1.1, 2 expressed on HEK293 cells do not activate NFkB when individually stimulated with *Mycobacterium avium* cell wall or its cell lysate (Higuchi et al., 2008; Keestra et al., 2007). Conversely, all combinations of chicken TLR1 and TLR2 were able to activate NFkB. Furthermore, TLR2.1 together with TLR1.2 activated NFkB expression when they are stimulated with peptidoglycan (Higuchi et al., 2008; Keestra et al., 2007). Additionally, a high activation of NFkB was observed in HELA cells transfected with chicken TLR2.2 and TLR1.1 and stimulated with FSL-1 and PAM-3. This suggests that chicken TLR1, which has homology with human TLR1, TLR6 and TLR10, replaces their function in chickens, and therefore assists in TLR2 pathogen recognition (Keestra et al., 2007).

As mentioned before, chickens do not have an orthologous gene of the mammalian TLR9, which is involved in recognition of un-methylated CpG DNA (CpG-Oligodeoxynucleotides) (ODNs). However, chickens do possess TLR21, which is a functional homologous receptor to mammals TLR9 responsible for ODNs recognition (Brownlie et al., 2009; Keestra et al., 2010). Furthermore, TLR15 in chickens has been shown to be up-regulated in macrophages when stimulated with ODNs, and in this test up-regulation of TLR21 was also observed. When an inhibitor of CpG-ODNs was used, TLR15 was down-regulated; however, in this study TLR21 was not affected (Ciraci and Lamont, 2011).

2.5.5. TLR Signalling

Once TLRs have recognized their specific PAMP, one or more adaptor molecules such as Myeloid differentiation primary response gene (88) (MyD88), TIR-domain-containing adapter-inducing interferon-β (TRIF or TICAM-1), toll-interleukin 1 receptor domain containing adaptor protein (TIRAP or MAL), TRIF-related adaptor molecule (TRAM) are activated by the specific TIR domain of the TLR (Kumar et al., 2011). After recruitment of these adaptor molecules, a downstream signal transduction pathway is activated. This leads to the activation of transcription factors for a specific immune response to the pathogen, according to the adaptor molecule or molecules recruited. These responses include induction and production of pro-inflammatory cytokines, stimulation of co-stimulatory molecules on DCs or inducing Type I IFNs (Kawai and Akira, 2007).

Two main down-stream signalling pathways are recognized to be stimulated by TLRs, leading to cytokine production: MyD88 dependent signalling pathway is activated by all TLRs excuding TLR3. MyD88 independent signalling pathway or TRIF dependent that is activated by TLR3 and TLR4 (Mogensen, 2009).

MyD88 dependent signalling pathway is activated with all TLRs except TLR3. Downstream signalling through the MyD88 adaptor molecule takes two possible routes; if signal stimulation is coming from cell surface TLRs such as TLR2, TLR4, TLR5, the final outcome is going to be production of pro-inflammatory cytokines. On the other hand if stimulation is coming from endosomal TLRs such as TLR7 or TLR9, the final outcome will be the production of Type I IFN (Mogensen, 2009).

In the case of surface TLR stimulation by the presence of a specific PAMP, MyD88 is recruited to the TIR domain of the TLR, followed by the recruitment of IL-1R-associated Kinase (IRAK) family proteins IRAK4 and IRAK1/2, which are sequentially phosphorylated. Later, on IRAK1 or IRAK2 associates with TRAF6 and E2 ubiquitin-conjugating enzymes (Ubc13 and Uev1A), ubiquitinating IRAKs and activating transforming growing factor-activated protein kinase 1 (TAK1) (Kumar et al., 2011). Activated TAK1 participates in the activation of IkB Kinase (IKK) with further phosphorylation of IkB protein, which will undergo proteosomal degradation facilitating activation of NF-kB for transcription of pro-inflammatory cytokines. Activated TAK1 can phosphorylate MAPK Kinase 3 (MKK3) and MKK6, triggering activation of P38 or phosphorylate MKK4 and MKK7, further triggering activation of c-Jun N-terminal

Kinase (JNK); with a final outcome in both cases of pro-inflammatory cytokines production (Mogensen, 2009).

Endosomal TLRs (such as TLR7 and TLR9) in plasmocytoid-dendritic cells (pDCs) lead the immune system to the production of Type I IFN in response to viral infections, via the recruitment of adaptor molecule MyD88. This differs from TLR3, which instigates a similar final outcome; however, via the recruitment of TRIF. Recognition of specific PAMP by TLR7 or TLR9 is followed by recruitment of MyD88 and activation of adaptor molecules such as IRAK1, IRAK4, TRAF6, TRAF3 and IKKa which mediate phosphorylation of IRF7 and final Type I IFN production. Moreover, TLR7 and TLR9 can activate NFkB via MyD88 pathway by MAPKS, similar in fashion to the TLR3 cascade ending in the production of pro-inflammatory cytokines (Gough, 2008; Mogensen, 2009).

MyD88 independent (TRIF dependent signalling pathway): TRIF dependent signalling pathway is activated by TRL3 and TLR4-TRAM association. They activate TRIF with a subsequent recruitment of TRAF6 and receptor-interacting protein 1 (RIP1). This last molecule is poly-ubiquitinated to form a complex with TRAF6, allowing activation of TAK1 with further NFkB activation via IKK or activation of the MAPK pathway (Ermolaeva et al., 2008). Activated TRIF recruits TRAF3 and TANK which mediate signal transduction to TBK1 and IKKe. These two IKK-related Kinases facilitate phosphorylation of IRF3 and IRF7. Finally, IRF3 and IRF7 translocate to the nucleus for Type I IFN production (Sotolongo et al., 2011).

2.5.6. Chicken TLR Signalling

In general terms, the chicken TLR signalling pathway is similar to what is found in mammals with presence of MyD88 and TRIF signalling adaptor molecules (Wheaton et al., 2007). However, in chickens MyD88 is present in three isoforms, MyD88-1, MyD88-2 and MyD88-3; with only MyD88-2 able to activate NFkβ (Qiu et al., 2008). Chickens have similar adaptor molecules to mammals, and since these molecules are involved in signalling pathways, this indicates that signalling pathways are conserved (Lynn et al., 2003). Conversely, TRAM which in mammals is involved in TRIF-dependent signalling pathway of TLR4, is not present in the birds genome(Brownlie and Allan, 2010).

2.5.7. Cytokines

The term “cytokine” makes reference to small protein molecules secreted by numerous immune and epithelial cells, which have regulatory functions of the immune system via intercellular signalling communication (Shrum, 1996). Cytokines involve immuno-modulating molecules such us interleukins, interferons and colony-stimulating factors (CSF) (Glick, 1985). Interleukins are cytokines produced by leukocytes that participate in their intercommunication process; being interferons, cytokines that participate in viral clearance (Glick, 1991). Binding of cytokines with their specific receptors on the target cell triggers a signal transduction pathway through activation of Janus tyrosine kinases (JAKs), which bridge the cytosolic domain of the receptor (Ihle, 1995). Later, JAKs sequentially phosphorylate signal transducers and activators of

transmition (STATs) with a final translocation to the nucleus to turn on the production of products needed for an appropriate response (Levy and Darnell, 2002).

IL-4 is produced by Th2 cells, mast cells and activated basophils (Seder and Paul, 1994). IL-4 stimulates differentiation of naïve T cells into TH2 cells (Ihle, 1997); it also stimulates growth and differentiation of B cells, promoting immunoglobulin class switching to IgE, which may have implications in allergies (Gascan et al., 1991). On B cells, IL-4 causes up-regulation of the expression of MHC II (Hudak et al., 1987; Noelle et al., 1984). The function of this cytokine is blocked by the presence of high levels of IFN-γ, moreover IL-4 can interfere with the activity of IFN-γ (King et al., 1993).

IL-6 is produced by macrophages, T cells, B cells, mast cells, vascular endothelial cells, fibroblast, keratinocytes, mesangial cells and muscle cells (Stevan R, 2011). This interleukin promotes IL-2 and IL-2R. IL-6 in association with IL-4 promotes Th2 cells differentiation (Diehl and Rincón, 2002). Additionally, IL-6 participates in the final process of maturation of B cells into plasma cells and is an important stimulator of acute phase responses (Gunesacar et al., 2011).

IL-10 is mainly produced by memory Th1, Th2 and Tho; however, Treg cells, B lymphocytes, activated macrophages, cytotoxic T cells, mast cells, keratinocytes and eosinophils can produce it as well (O'Garra et al., 2004; Sauder and Dytoc, 1997). This regulatory IL-10 has been referred to as an anti-inflammatory cytokine, which has a large effect on monocytic cells and other APCs, inhibiting expression of MHC II, co-stimulatory molecules and production of IL-1β and TNF-α (Fridman and Tartour, 1997; Shrum, 1996). Additionally, IL-10 has been reported to affect Th1 cell function by

inhibiting IL-12 and IFN-γ production (Seder and Paul, 1994). However, Th1 cells have been reported to produce IL-10 (King et al., 1993), and in some cases IL-10 can inhibit the production of IL-6 and TNF-α (Jack and Turner, 2003). In addition to the anti-inflammatory effect above mentioned for IL-10, it has also been reported to stimulate the cytotoxic effect of NK cells (Masteller and Thompson, 1994).

IL-18 is produced in response to LPS by APCs and activated T cells (Tripodi et al., 2011). IL-18 enhances production of Th1 cytokines with subsequent stimulation of cytotoxic cell-mediated activity; which can be explained by the induction of IFN-γ that this cytokine stimulates (Biet et al., 2002; Torigoe et al., 1997).

IFN-γ is produced primarily by T cells and NK cells. Macrophages that recognize viral, intra-cellular bacteria or protozoa produce IL-12, which in turn stimulates T cells and NK cells to produce IFN-γ (Sen, 2001). IFN-γ stimulates production of NOS_2 in macrophages enhancing their microbial degradation process (Chung et al., 2011). This cytokine has also been shown to promote production of MHC I molecules in T cells and differentiation of Th1 cells in different experimental models (Baldeon et al., 1997; Geldhof et al., 1996; Martini et al., 2010). Furthermore, it inhibits Th2 cells production of IL-4 and expression of IL-4R (Byron et al., 1991).

2.5.8. Summary

Yeast cell wall components have been shown to assist in the development and pathogen clearance from the gastrointestinal tract. DDGS are rich sources of yeast biomass; and due to expansion of ethanol production as a fuel additive, a large quantity of

these DDGS is offered in the market for use as poultry feed ingredient. As described previously, the immune system has a large repertoire of carbohydrate recognition molecules which mediate recognition and clearance of pathogens by stimulating phagocytosis, promoting cytokines and chemokines production which in turn will drive the immune response towards pathogen clearance. Priming the immune system with the presence of yeast cell wall compounds such as mannose and ß–Glucans or yeast macromolecules (nucleotides) may potentiate the immune response against invader pathogens, allowing for their easier control.

This research will contribute to our better understanding of the immunological value of yeast and yeast-derived products and will promote the use of wheat/corn DDGS, an important Canadian feed resource, especially in poultry rations. The proposed research will contribute to increased productivity through improved bird health and nutrition by developing tools to prevent, manage and eradicate disease, and recommending strategies that will aid in the prudent use of antibiotics in poultry production.

3. MANUSCRIPT

Toll-like receptors and cytokines profile of chicken B cells in the presence of yeast-derived carbohydrates

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3.1. Abstract:

It has been documented that yeast or yeast-derived products enhance bird immune systems; however it has not been very well characterized. The effect of different yeast and yeast-derived carbohydrates such as brewer's yeast (Y), yeast cell wall polysaccharide (CWP) - rich product (YCW), distillers dried grains with solubles (DDGS), nucleotide-rich product (N), processed yeast + nucleotide-rich product (PY+N) and D-mannose (M) was assessed on chicken B cells response using an LPS challenge model. Relative gene expression of toll-like receptors (TLRs) and cytokines was then investigated, and statistical analysis was performed using REST-2009 software. TLR2 gene expression was not up-regulated ($p>0.05$) in any treatment with either challenged or unchallenged conditions; however, TLR2 with DDGS and M in unchallenged cells or N and M in challenged cells down-regulated this gene ($p<0.05$). The addition of DDGS up-regulated TLR4 gene expression with or without LPS; furthermore, Y, N and PY+N also up-regulated TLR4, but only when LPS was present. TLR21 was not modified in the presence of N ($p>0.05$); however, DDGS was able to up-regulate it ($p<0.05$). Even though PY+N showed the highest increase in fold change under unchallenged conditions, it was found to not be statistically different ($p>0.05$). After LPS challenge all treatments except M were able to up-regulate TLR21 ($p>0.05$). In general, the signalling adaptor molecules MyD88 and TRIF were not affected by treatments in unchallenged conditions with the exception of DDGS and N that caused up-regulation of MyD88 ($p<0.05$). However, in LPS challenged cells, a dramatic up-regulation ($p<0.05$) of TRIF was caused

by most treatments with the exception of N and M. Cytokine profile in unchallenged conditions showed a pattern of up-regulation of Th1-associated cytokines (IFN-γ and IL-18) caused mainly by YCW, DDGS and PY+N ($p<0.05$). There was also up-regulation of IL-4 by these same treatments ($p<0.05$), and when cells were challenged with LPS, most of the products (except M) caused different degrees of up-regulation of Th2-associated cytokines (IL-4, IL-6, IL-10) ($p>0.05$). Our results let us infer that yeast and yeast-derived products regulate the immune response of chicken B cells towards the production of Th2-associated cytokines which could lead *in vivo* to induce pathogen clearance via antibody production.

Key Words: B cells, cytokines, lipopolysaccharide, toll-like receptors, yeast.

3.2. Introduction

The commercial poultry industry has been using antibiotics to promote bird performance through the control of microbial agents in the gastrointestinal tract (Dibner and Richards, 2005). However, the growing concern about antibiotic resistance by pathogenic microorganisms is urging customers to demand antibiotic-free animal products (Smith, 2005). Yeast and yeast-derived products are rich sources of ß1,3- and ß1,6-glucans, nucleotides, and mannan oligo- or polysaccharides which can function as prebiotics (Lipke and Ovalle, 1998). They have been shown to stimulate the immune system and gastrointestinal tract development (de los Santos et al., 2007; Gao et al., 2008;

Zdunczyk et al., 2005), as well as aid in control of fimbria-containing gram-negative bacteria (Krogfelt et al., 1990; Nurin et al., 1982). Antigen-presenting cells (APC) such as macrophages, dendritic cells and B cells represent an important part of the immune defence, participating in the initial capture and processing microbial antigens, and presenting them to specific T and B cells (Rodríguez-Pinto, 2005; Siamon, 2002). Recognition of microbial molecules by antigen-presenting cells occurs through the pattern recognition receptors (PRRs), which recognize conserved structures in groups of microorganisms called pathogen-associated molecular patterns (PAMPs) (Kedziora and Słotwiński, 2009). In addition to their role in humoral immunity, B lymphocytes are important antigen presenting cells; in the same way as other APCs, B cells contain TLRs, make cytokines upon activation and have the potential to modulate T cell responses (Berglová et al., 2011; Mosman, 2001). This data highlights the potentially unique nature of immune modulation when B cells act as APC. Furthermore, B cell TLRs have been shown to be of key importance in cooperation with BCR for reaching rapid and lasting humoral activity (Cerutti et al., 2011). The objective of the proposed research was to investigate the effect of wheat/corn DDGS, yeast and yeast-derived products rich in ß-glucans, mannan-oligosaccharides and nucleotides on the innate immune response of chicken B cells using an LPS challenge model through relative gene expression.

3.3. Materials and Methods:

3.3.1. Cell culture:

The chicken B cell line (DT40 cell line ATCC CRL-2111) (American Type Culture Collection, Manassas, VA, USA) was grown in Dulbecco's Modified Eagle's Medium (ATCC 30-2002) supplemented with 10% fetal bovine serum heat inactivated, 5% chicken serum heat inactivated, 10% tryptose phosphate broth and 0.05mM 2-mercaptoethanol in a 5% CO_2 incubator at 37^{o}C as described by Rodriguez-Lecompte (Rodríguez-Lecompte et al., 2005). Cells were centrifuged at 1400rpm for 5min, media was discarded and the cell pellet was reconstituted to obtain a final concentration of $2x10^{6}$cells/ml.

3.3.2. Treatments:

CONTROL **(C)**: Cells without any treatment

Treatment 1 **(Y)**: Brewer's yeast (Husky Energy. Minnedosa, MB, R0J 1E0 Canada)

Treatment 2 **(YCW)**: Yeast cell wall rich product (Cell wall polysaccharide-rich product) (Hi-Yeast 751. Canadian Bio-Systems Inc. Calgary, AB, T2C 0J7 Canada)

Treatment 3 **(DDGS)**: Wheat/corn DDGS (Husky Energy. Minnedosa, MB, R0J 1E0 Canada)

Treatment 4 **(N)**: Nucleotide-rich product (Hi-Yeast 751. Canadian Bio-Systems Inc. Calgary, AB, T2C 0J7 Canada)

Treatment 5 **(PY+N)**: Processed Yeast + nucleotide-rich product (Maxi-Gen Plus. Canadian Bio-Systems Inc. Calgary, AB, T2C 0J7 Canada)

Treatment 6 **(M)**: D+ mannose (Sigma-Aldrich, St. Louis, MO 63178 US. Cat # 63580-25)

A total of 7 test tubes were labelled with treatment and control names. 120mg of each treatment product was added to the corresponding tube, and later 6ml of fresh media was added and mixed in each test tube. An extra test tube containing just fresh media was used as a control.

3.3.3. Treatment application:

A total of two 24-well plates were used in the experiment, one for product treatments test only (Unchallenged) and the other for treatments plus the addition of *Escherichia coli* O111:B4-derived lipopolysaccharide (LPS) challenge (Challenged) (Sigma-Aldrich Oakville, ON, Canada. Cat. L2630). Each plate contained a control group consisting of cells without treatment. Challenged cells also contained LPS. One millilitre of cells was added to 21 of the 24 wells of each plate. Immediately, 1ml of Y was added to 3 wells of each plate (3 technical replicates); followed by YCW, the other treatments and the control. The final concentration of cells per well was $1x10^6$cells/ml and the final concentration of each product per well was 10mg/ml. Both plates were placed in an incubator at 37^oC and 5% CO_2. Unchallenged cells were incubated undisturbed for 8 hours. Challenged cells were initially incubated for 4 hours and then 200µl of LPS with a concentration of 0.1µg/µl were added to each well to achieve a final concentration of

10µg/ml of LPS. Following the challenge, cells were placed in incubation for additional 4 hours.

At the end of the incubation process, one ml of supernatant from each well was carefully removed and discarded, and the remaining supernatant and cells were transferred to micro-centrifuge tubes for centrifugation at 13,000rpm for 20sec. Later, supernatant was discarded and cell pellets were disrupted by flicking tubes and before being placed in -80ºC freezer for further RNA extraction.

3.3.4. Gene expression:

RNA was isolated using Invitrogen Trizol® Reagent (Life Technologies Inc. Burlington, ON, Canada) according to the manufacturer's protocol. From the RNA, cDNA was produced using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Mississauga, ON, Canada) according to the manufacturer's protocol. Gene expression for β-actin, TLR2, TLR4, TLR21, IL-4, IL-6, IL-10, IL-18 and IFN-γ was measured using qRT-PCR Thermal Cycler Step One Applied Biosystems (Applied Biosystems, Mississauga, ON, Canada) in a 48-well plate using a 12.5µl total reaction volume. Primer concentrations for the target and housekeeping genes were adjusted according to the results obtained from a standard curve for each specific case. Power SYBR Green PCR Master Mix was used according to the Applied Biosystems instruction manual (Mississauga, ON, Canada). The thermal cycling protocol consisted of an initial denaturation at 95^{o}C for 10 min, followed by amplification for 40 cycles at 95^{o}C for 10s, annealing as described in Table 1 for each of the primer pairs, and elongation at 72^{o}C for

15s. The specificity of amplification for each product was determined by melting curve analysis at 95^{o}C for 15s and 65^{o}C for 1min; this was followed by progressive increase of the temperature to 95^{o}C. The plates were then cooled at 40^{o}C for 30s. Along with each qRT-PCR assay, a 10^{-2} dilution of DNA plasmid encoding related genes and a water control were run to serve as a calibrator and a negative control, respectively. After qRT-PCR runs, data was exported to Excel and the average cycle threshold value (CT value) of the three technical replications completed per sample was used for gene expression analysis.

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

Gene	Primer sequence (5'-3')	Annealing Temp, ^{o}C	Accession no.
chβ-actin	F: CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	61	X00182
chTLR-2b	F: CGCTTAGGAGAGACAATCTGTGAA R: CCTGTTTTAGGGATTTCAGAGAATTT	59	NM204278
chTLR-4	F: AGTCTGAAATTGCTGAGCTCAAAT R: GCGACGTTAAGCCATGGAAG	60	AY064697
chTLR-21	F: TGGCGGCGGGAGGAAAAGTG R: CACCGTGCTCCAGCTCAGGC	59	NM_001030558
chIL-4	F: CCTGCGTCAAGATGAACGTG R: GCAGGTTCTTGTGGCAGTGCT	55	GU119892.1
chIL-6	F: CAGGACGAGATGTGCAAGAA R: TAGCACAGAGACTCGACGTT	59	AJ309540
chIL-10	F: AGCAGATCAAGGAGACGTTC R: ATCAGCAGGTACTCCTCGAT	55	AJ621614
chIL-18	F: GAAACGTCAATAGCCAGTTGC R: TCCCATGCTCTTTCTCACAACA	55	AY628648.2
chIFN-γ	F: CTGAAGAACTGGACAGAGAG R: CACCAGCTTCTGTAAGATGC	60	X99774
chMyD88	F: AGAAGGTGTCGGAGGATGGT R: TGTGCAGTGAGATGCTGTAGGA	55	EF011109.1
chTRIF	F: GCTGACCAAGAACTTCCTGTGC R: AGAGTTCTCATCCAAGGCCACC	57	EF025853

3.3.5. Statistical analysis

Statistical analysis of the relative gene expression in the present study was performed using REST-2009 Software (Technical University Munich. Qiagen, Germantown, MD, USA) which compares each treatment individually with the control group and expresses the relative gene expression as a fold change of the target gene, taking into consideration a reference gene (the housekeeping gene, β-actin in the present work) and the efficiency of reference and target genes as previously described by Pfaffl et all and Steiger et al (Pfaffl et al., 2002; Steiger et al., 2010) . Results from this analysis are given as fold change with a respective p-value. In the present work, significant difference is considered when the p-value is less than 0.05. Values of fold change higher or lower than 1 with $p<0.05$ are considered statistically significant in both up-regulation or down-regulation, respectively.

3.4. Results

Gene expression analysis for TLR2 of unchallenged cells cultured with yeast or yeast-derived products showed that this gene is not significantly up-regulated by any of the treatments; however, down-regulation with respect to the control group (cells + media) ($p<0.05$) was observed when DDGS and M were added to the cells (Figure 1A). Although N treatment was not significantly down-regulated, a trend was observed ($p=0.051$). Furthermore, when cells were cultured with the same treatments and later

challenged with LPS (Challenged) a similar pattern was observed where down-regulation ($p<0.05$) was found when N and M were used and compared to the control group (cells + media + LPS) (Figure 1B).

Most of the treatments in unchallenged conditions did not influence TLR4 gene expression when compared to the control group (cells + media), and only the Y group appeared to down-regulate this gene ($p<0.05$) (Figure 2A). Furthermore, when cells were challenged with LPS treatments, DDGS, N and PY+N were able to cause an up-regulation ($p<0.05$) of TLR4 (Figure 2B).

TLR21 gene expression was up-regulated ($p<0.05$) by the addition of DDGS to cells in unchallenged conditions. Even though PY+N displayed a 9.2 fold change, this being the highest of all treatments when compared to control group, it was still not significantly different compared to the control ($p>0.05$) (Figure 3A). On the other hand, challenged cells in all treatments, with the exception of M, caused a up-regulation of TLR21 ($p<0.05$) compared to the control group (cells + media + LPS) (Figure 3B).

Myeloid differentiation primary response gene (MyD) 88 expression with the addition of products in unchallenged cells was up-regulated ($p<0.05$) under the presence of DDGS and N. In addition, YCW showed a tendency to cause up-regulation for MyD88 in unchallenged cells, however it was not significantly different from the control group ($p=0.054$) (Figure 4A). Consequently, the profile of gene expression of MyD88 in LPS challenged cells and in those treated with DDGS and N was also up-regulated ($p<0.05$) (Figure 4B).

Gene expression analysis for TIR-domain-containing adapter-inducing interferon-β (TRIF) was not affected by treatments in unchallenged cells ($p>0.05$) (Figure 5A). However, Y, YCW, DDGS and PY+N caused up-regulation ($p<0.05$) of TRIF in challenged cells (Figure 5B).

In unchallenged cells, gene expression of IL-4 was found to be up-regulated by the use of treatments YCW, DDGS and PY+N ($p<0.05$), moreover from fold change calculations, PY+N had the highest value (Figure 6A). Even more, in LPS challenged cells treated with YCW, DDGS and PY+N, this pattern of up-regulation ($p<0.05$) of IL-4 was also observed. In this case, however, Y also caused up-regulation of this gene ($p<0.05$) (Figure 6B).

IL-6 gene expression analysis showed that Y was able to cause statistical down-regulation when compared to the control group (Unchallenged). Despite the fact that this cytokine showed an 8.6 fold change under the addition of PY+N to cells, this increase in gene expression was not enough to make it significantly different from the control group ($p=0.07$) (Figure 7A). Cells treated with different products and challenged with LPS presented up-regulation ($p<0.05$) when Y, YCW, DDGS and N were used compared to the control group. Results from analysis of PY+N and LPS challenge of cells was similar to those found in unchallenged conditions because even though it had the highest fold change, this was not significantly different from the control group ($p=0.07$) (Figure 7B)

IL-10 gene expression was not affected by the presence of any treatments when compare to the control group in unchallenged conditions (Figure 8A); nevertheless when

cells were cultured with treatments Y, DDGS, N, PY+N and challenged with LPS, the gene expression of IL-10 was up-regulated ($p<0.05$) (Figure 8B).

Most treatments used on cells in unchallenged conditions caused up-regulation of IL-18 ($p<0.05$) when compared to the control group, with the exception of Y and M (Figure 9A). This up-regulation pattern in gene expression ($p<0.05$) was also observed with most treatments in LPS challenged cells, except with Y (Figure 9B).

IFN-γ gene expression was found to be significantly up-regulated in the presence of YCW, DDGS and PY+N ($p<0.05$) when compared to the control group in unchallenged cells (Figure 10A). Furthermore, when cells were challenged with LPS and treated with these same products, statistical up-regulation ($p<0.05$) was also found for this gene. In the case of brewer's yeast in unchallenged conditions, IFN-γ gene expression was not affected, although after challenged with LPS, significantly up-regulation ($p<0.05$) was observed (Figure 10B).

3.5. Discussion

Carbohydrate structures present on the cell wall of different microbial agents have been shown to interact as ligands agonistic to the host immune system, allowing for their recognition and initiation of an adequate response. Pattern recognition receptors such as mannose-binding lectins, mannose receptor, DC-SIGN and TLRs such as TLR2 and TLR4 also participate in this recognition process of carbohydrates (Buzás et al., 2006).

B lymphocytes are mostly known for their important role in humoral immune responses; however, in the same way as other APCs, B cells have TLRs which upon activation, produce cytokines and have the potential to modulate T cell responses (Berglová et al., 2011; Mosman, 2001)

In the present study, the effect of yeast and yeast-derived products on TLR2, TLR4 and TLR21 gene expression was assessed using a chicken B cell and LPS challenge model. Cytokine gene expression was also determined in order to evaluate the effect of these products as possible immuno-modulators of chicken B cell activity and general downstream immune responses.

It has been reported that TLR2 is able to recognize different components of the yeast cell wall such as β-Glucans and mannan (Brownlie and Allan, 2010; Dillon et al., 2006; Gantner et al., 2003). B cells have shown to express TLRs such as TLR2 and TLR4 (Lanzavecchia and Sallusto, 2007; Pasare and Medzhitov, 2005). In addition, chicken B cells and Bursa of Fabricius gene expression of TLR2 and TLR4 has been reported (Iqbal et al., 2005). However, even though we found the presence of TLR2 in chicken B cells (DT40 cell line); it seems that chicken B cell TLR2 gene expression against these yeast cell wall components is not completely affected. In this research, none of the yeast and/or yeast-derived treatments used induced up-regulation of this gene, both in unchallenged or LPS challenged cells. Furthermore, the addition of wheat/corn DDGS, D+mannose (M) in unchallenged conditions and nucleotide-rich product (N) and D+mannose (M) in LPS challenged chicken B cells caused down-regulation of this gene. Our findings allow us to infer that these products can control activation of TLR2 with a subsequent decrease in

local inflammation, and decrease the spend energy that could be caused by a possible immune response. It is important to stress that the immune system has more PRRs with the capability to recognize these yeast cell wall molecules, such as DC-SIGN, mannose receptor, Dectin-1 (Fidgor et al., 2009; Martinez-pomares, 2001; Tsoni and Brown, 2008). It has been stated that B cell TLR activation is necessary for adequate acquired immune responses (Pasare and Medzhitov, 2005).

Toll-like receptor 4 is well known for its ability to recognize lipopolysaccharides (LPS) from gram-negative bacteria (Warren, 2005) and also to recognize mannan in the cell wall of different fungi species (Levitz, 2004; Tada et al., 2002; Van der Graaf et al., 2006). According to this, it would be expected to find TLR4 gene expression up-regulation when the cells were treated with different yeast or yeast-derived products; however, gene expression was not up-regulated. Furthermore, cells treated with brewer's yeast showed down-regulation for TLR4, suggesting that this gene in chicken B cells is not completely active in recognizing yeast structures. The results obtained from LPS challenged cells allow us to infer that wheat/corn DDGs, nucleotide-rich product and processed yeast + nucleotide-rich product activate TLR4 in the presence of LPS as per the up-regulation that was observed.

In humans, the recognition of microbial un-methylated cytosine-phosphate-guanine (CpG) di-nucleotides is performed by TLR9 (Gupta and Agrawal, 2010; Latz et al., 2004). It has been determined that chickens do not have a gene orthologous to TLR9; however, the function of recognition of these compounds in chickens is carried out by a functional homologous gene referred to as TLR21 (Keestra et al., 2010). Unlike

mammals, in which a high percentage of CpG dinucleotides are methylated, fungi species have a low level of CpG residues methylated, ranging from 0.1% to 0.5% cytosine methylation (Antequera et al., 1984; Ehrlich et al., 1982) which may make them easy to recognize by PRRs such as mammalian TLR9 or chicken TLR21. Our results are in agreement with previously reported results showing that in particular, DT40 cell line gene expression of TLR21 is not affected by the presence of nucleotides (Han et al., 2010), as we did not find up-regulation of this gene when cells were treated with nucleotide-rich product. Cells treated with wheat/corn DDGS in unchallenged or LPS challenged conditions showed up-regulation for this gene. This may have been due to the presence of contaminant bacteria debris present in this treatment, as it has been reported that contaminant bacteria are commonly found in alcohol fermentation processes (Meneghin et al., 2008; Skinner and Leathers, 2004). Furthermore, this fact may explain the up-regulation caused by this same treatment in TLR4 gene expression. Further studies must be conducted in the field of TLR21 agonist molecules as is not clear how LPS challenge of cells treated with brewer's yeast, yeast cell wall, nucleotide-rich product and processed yeast + nucleotide-rich product was able to cause up-regulation of TLR21.

The patterns of MyD88 gene expression in both, unchallenged and LPS challenged cells were similar and only affected by the presence of wheat/corn DDGS and nucleotides-rich product causing up-regulation for this gene. This may be related to the recognition of these products by the immune system since all TLRs except TLR3 use the MyD88 dependent signalling pathway to activate NFkβ for cytokines production (Mogensen, 2009). However, TLR2, TLR4 and TLR21 were not up-regulated with these

products in unchallenged conditions, and only TLR4 and TLR21 were up-regulated in LPS challenged cells. It is possible that other TLRs such as TLR15 may be involved in the recognition of the products, as TLR15 has been shown to be able to recognize microbial nucleotides as well (Ciraci and Lamont, 2011). Under general conditions, it would be expected to find high fluctuations in MyD88 gene expression since large changes in the gene expression of cytokines analysed were found in the present work, all of which were induced in a MyD88 dependent signalling pathway (Mogensen, 2009). This indicates PRRs other than TLRs may be involved in the recognition of yeast and yeast-derived products in chicken B cells and could be responsible for the activation of gene expression of these cytokines.

As expected, TRIF gene expression was not affected by yeast or yeast-derived products under unchallenged conditions, as this signalling adaptor molecule is just activated by the engagement of viral double stranded RNA to TLR3 (Matsumoto et al., 2011). In addition, this gene may also be activated by TLR4 (Weighardt et al., 2004), which would explain the up-regulation seen in TLR4 and TRIF gene expression of challenged cells treated with DDGS and processed yeast + nucleotide-rich product. However, cytokines related to the TRIF signalling pathway such as type I IFNs were not analysed in the present work to corroborate this idea.

IL-4 gene expression was up-regulated by the presence of yeast cell wall, wheat/corn DDGS, processed yeast + nucleotide rich product in both unchallenged and challenged conditions. However, in challenged conditions the fold changed was much more dramatic, ranging from 80 to 136 compared to 3.7 to 11.6 in unchallenged cells.

These patterns of up-regulation of IL-4 may lead to the *in vivo* stimulation of naïve Th cells to differentiate into Th2 cells (Ihle, 1997). In addition, IL-4 may stimulate B cell development and differentiation, promoting immunoglobulin class switching to IgE (Gascan et al., 1991) and stimulating expression of MHC II molecules on B cells (Hudak et al., 1987; Noelle et al., 1984).

When chicken B cells were treated with yeast and yeast-derived carbohydrates, IL-6 gene expression was not up-regulated, and only yeast was able to cause its down-regulation. However, LPS challenged cells treated with yeast, yeast cell wall, wheat/corn DDGS and nucleotides-rich product were able to induce IL-6 gene up-regulation. Even though processed yeast + nucleotide-rich product caused the highest fold change with respect to the control group, it was not statistically significant. Greater production of IL-6 by LPS challenged B cells treated with yeast and yeast-derived products could be seen as a positive feedback for B cell proliferation, stimulating B cell activation (Gunesacar et al., 2011). Furthermore, IL-6 has two different mechanisms in order to induce the differentiation of naïve $CD4^+$ T cells to Th2: the first by stimulating expression of IL-4 during activation of $CD4^+$ T cells, and the second by blocking production of IFN-γ and Th1 cell differentiation (Diehl and Rincón, 2002).

The up-regulation of IL-10 gene expression caused in LPS challenged cells by yeast, wheat/corn DDGS, nucleotides and processed yeast + nucleotide treatments is an important finding of the immuno-modulatory effect of these compounds related with the activity of IL-10 as an anti-inflammatory cytokine, regulating T cell responses and its participating in mucosal homeostasis (Bouaziz et al., 2010; Liu et al., 2010; Pillai et al.,

2011). The regulatory function of IL-10 on the gastrointestinal tract has been determined in gene knock-out mice. Mice developed chronic gastrointestinal inflammation in the presence of normal intestinal flora compared to similar mice kept in pathogen-free conditions (Kuhn et al., 1993). In homeostatic conditions, IL-10 regulates APCs by affecting their maturation, decreasing their production of co-stimulatory molecules and the release of pro-inflammatory cytokines (Mantovani et al., 2002; Moore et al., 2001). IL-10 can also act as pro-inflammatory cytokine due to its stimulatory activity on NK cells with further pathogen clearance and even tumor control (Parato et al., 2002; Zheng et al., 1996).

The cytokine gene expression profile of chicken B cells treated with yeast or yeast-derived products was shown in most cases to be a Th1 response. This Th1 response is due to the fact that yeast cell wall, wheat/corn DDGS and processed yeast + nucleotide-rich product caused an up-regulation of IFN-γ. It has been reported that IFN-γ as a pro-inflammatory cytokine stimulates Th1 cells differentiation (Martini et al., 2010). Furthermore, IL-18 which is also involved in stimulation of Th1 cytokines with subsequent stimulation of cytotoxic cell-mediated activity (Biet et al., 2002) was found to be up-regulated by the presence of these products. Even though IL-4 which stimulates Th2 differentiation (Ihle, 1997), was found to be up-regulated by these products in unchallenged conditions, other cytokines involved in Th2 responses such as IL-6 and IL-10 (Adachi et al., 1999; Diehl and Rincón, 2002) were not affected. This research showed that under LPS challenged conditions, wheat/corn DDGS and brewer's yeast were able to cause up-regulation in the gene expression of Th2 cytokines such as IL-4, IL-6 and IL-10.

Moreover, the other treatments with exception of D+mannose, also increased gene expression of these cytokines in a moderate way. These patterns of cytokine gene expression allow us to infer that yeast and yeast-derived products are able to stimulate the immune system to respond to gram-negative bacteria via the stimulation of Th2 responses.

Overall, our results show that chicken B cell gene expression of TLR2, TLR4 and TLR21 was not increased by the presence of yeast or yeast-derived products. Only wheat/corn DDGS were able to cause up-regulation ($p<0.05$), which is correlated with a similar pattern of up-regulation of MyD88. However, changes seen in cytokines gene expression allow us to infer that additional receptors might be participating in recognition of these products by chicken B cells.

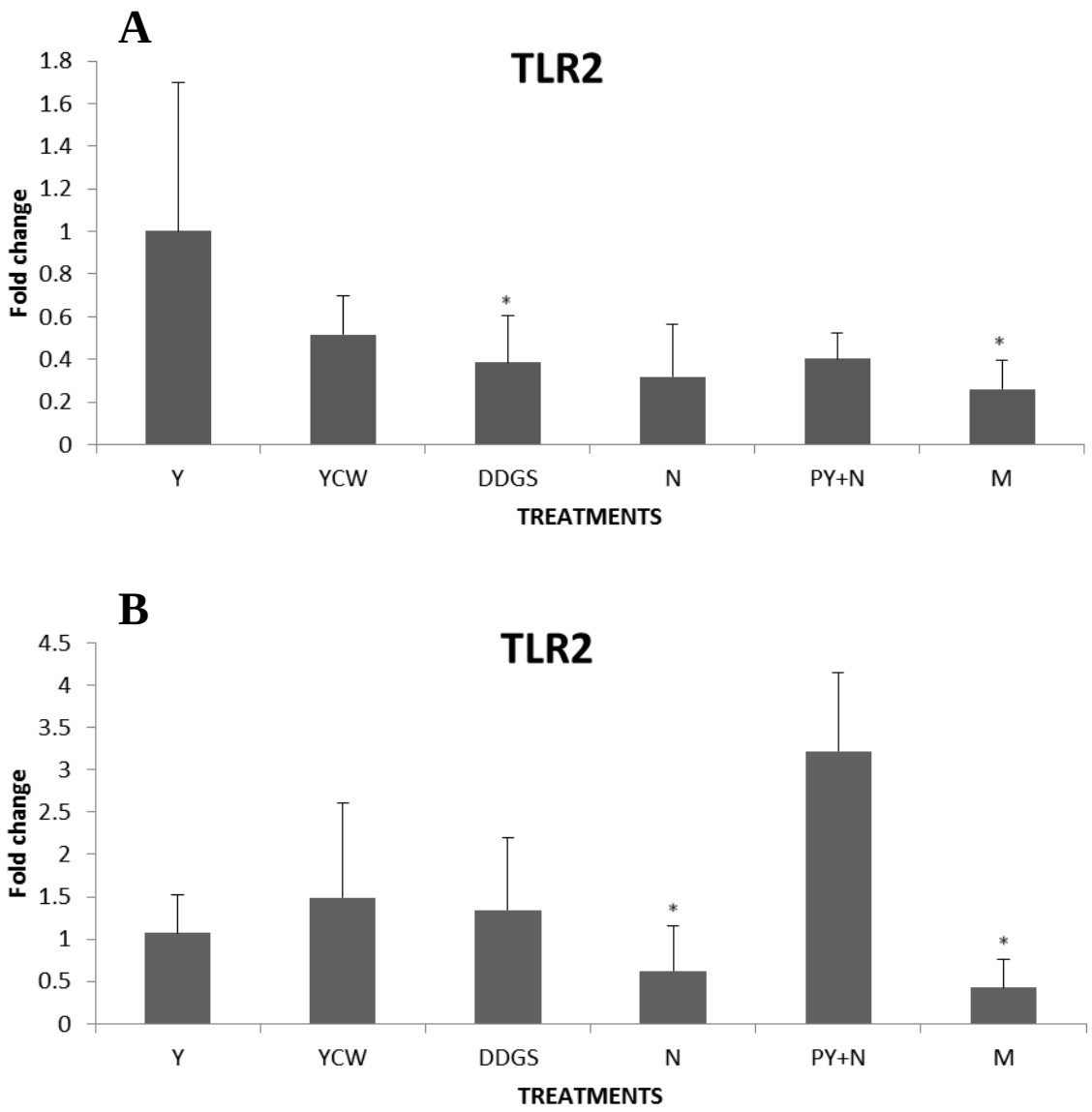

Figure 1. Relative TLR2 gene expression of DT40 cell line

(A) In presence of yeast-derived products (B) In presence of yeast-derived products and LPS challenge. Gene expression was assessed using quantitative RT-PCR and was calculated relative to the house keeping gene β-actin. Bar graph values are expressed as fold change relative to control group receiving only basal diet, as calculated by REST 2009. Error bars represent standard error. Results were considered statistically significant from the control group if $p \leq 0.05$ (*).

Y= Brewer's yeast; **YCW**= Yeast cell wall rich product; **DDGS**= Wheat/corn DDGS; **N**= Nucleotide-rich product; **PY+N**= Processed yeast + nucleotide-rich product; **M**= D+ mannose.

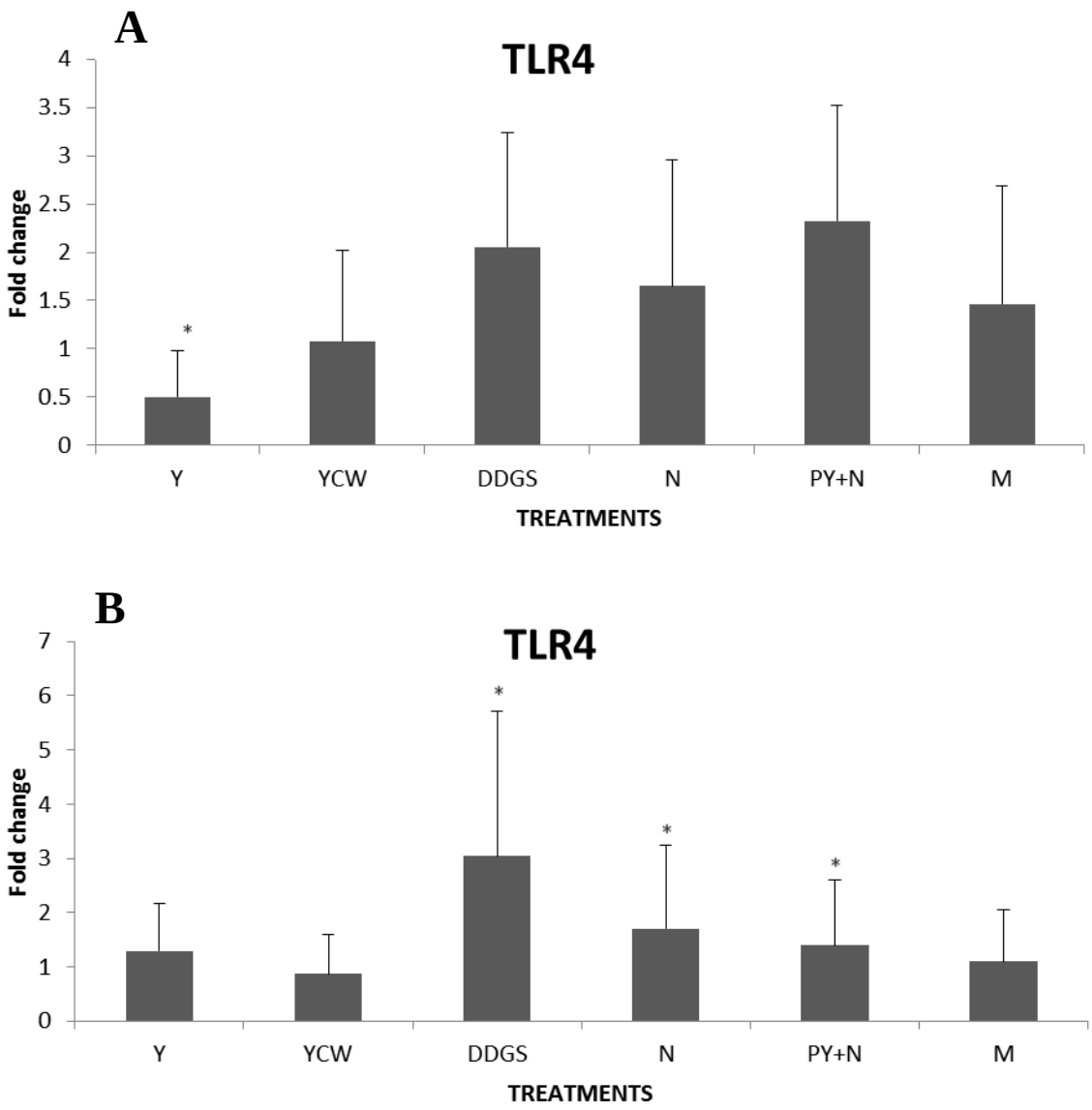

Figure 2. Relative TLR4 gene expression of DT40 cell line

(A) In presence of yeast-derived products (B) In presence of yeast-derived products and LPS challenge. Gene expression was assessed using quantitative RT-PCR and was calculated relative to the house keeping gene β-actin. Bar graph values are expressed as fold change relative to control group receiving only basal diet, as calculated by REST 2009. Error bars represent standard error. Results were considered statistically significant from the control group if $p \leq 0.05$ (*).

Y= Brewer's yeast; **YCW**= Yeast cell wall rich product; **DDGS**= Wheat/corn DDGS; **N**= Nucleotide-rich product; **PY+N**= Processed yeast + nucleotide-rich product; **M**= D+ mannose.

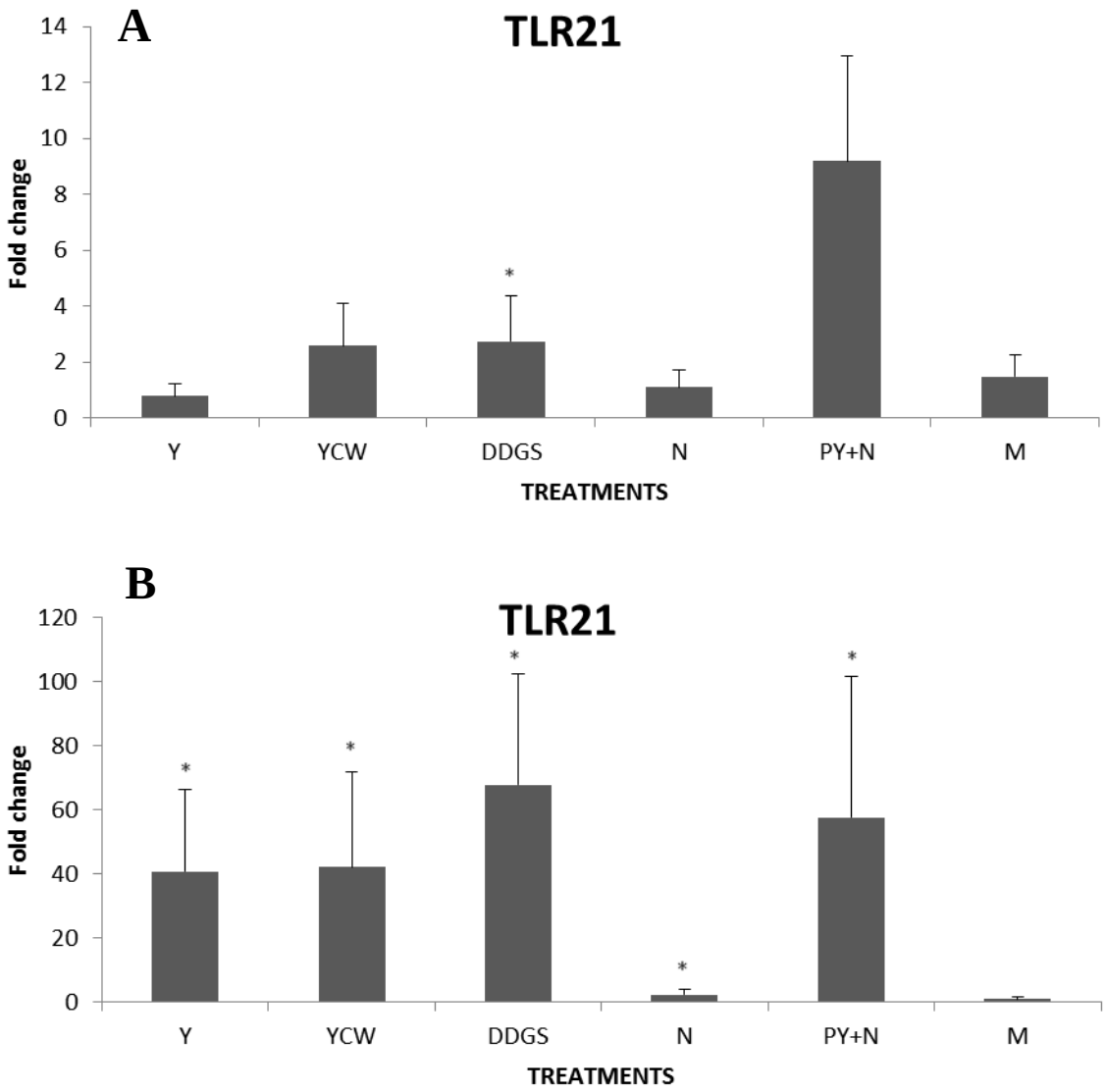

Figure 3. Relative IL-21 gene expression of DT40 cell line

(A) In presence of yeast-derived products (B) In presence of yeast-derived products and LPS challenge. Gene expression was assessed using quantitative RT-PCR and was calculated relative to the house keeping gene β-actin. Bar graph values are expressed as fold change relative to control group receiving only basal diet, as calculated by REST 2009. Error bars represent standard error. Results were considered statistically significant from the control group if $p \leq 0.05$ (*).

Y= Brewer's yeast; **YCW**= Yeast cell wall rich product; **DDGS**= Wheat/corn DDGS; **N**= Nucleotide-rich product; **PY+N**= Processed yeast + nucleotide-rich product; **M**= D+ mannose.

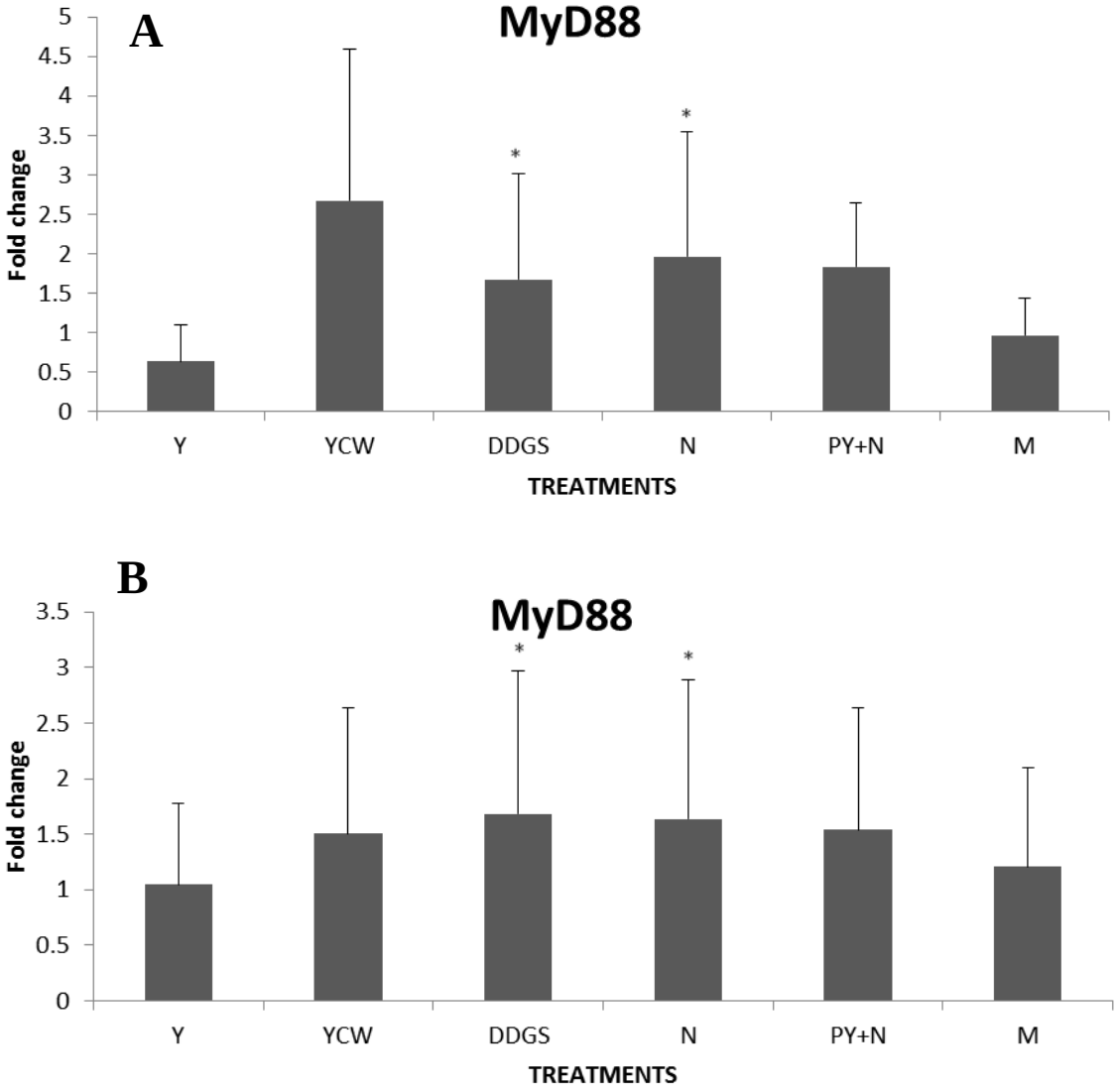

Figure 4. Relative MyD88 gene expression of DT40 cell line

(A) In presence of yeast-derived products (B) In presence of yeast-derived products and LPS challenge. Gene expression was assessed using quantitative RT-PCR and was calculated relative to the house keeping gene β-actin. Bar graph values are expressed as fold change relative to control group receiving only basal diet, as calculated by REST 2009. Error bars represent standard error. Results were considered statistically significant from the control group if $p \leq 0.05$ (*).

Y= Brewer's yeast; **YCW**= Yeast cell wall rich product; **DDGS**= Wheat/corn DDGS; **N**= Nucleotide-rich product; **PY+N**= Processed yeast + nucleotide-rich product; **M**= D+ mannose.

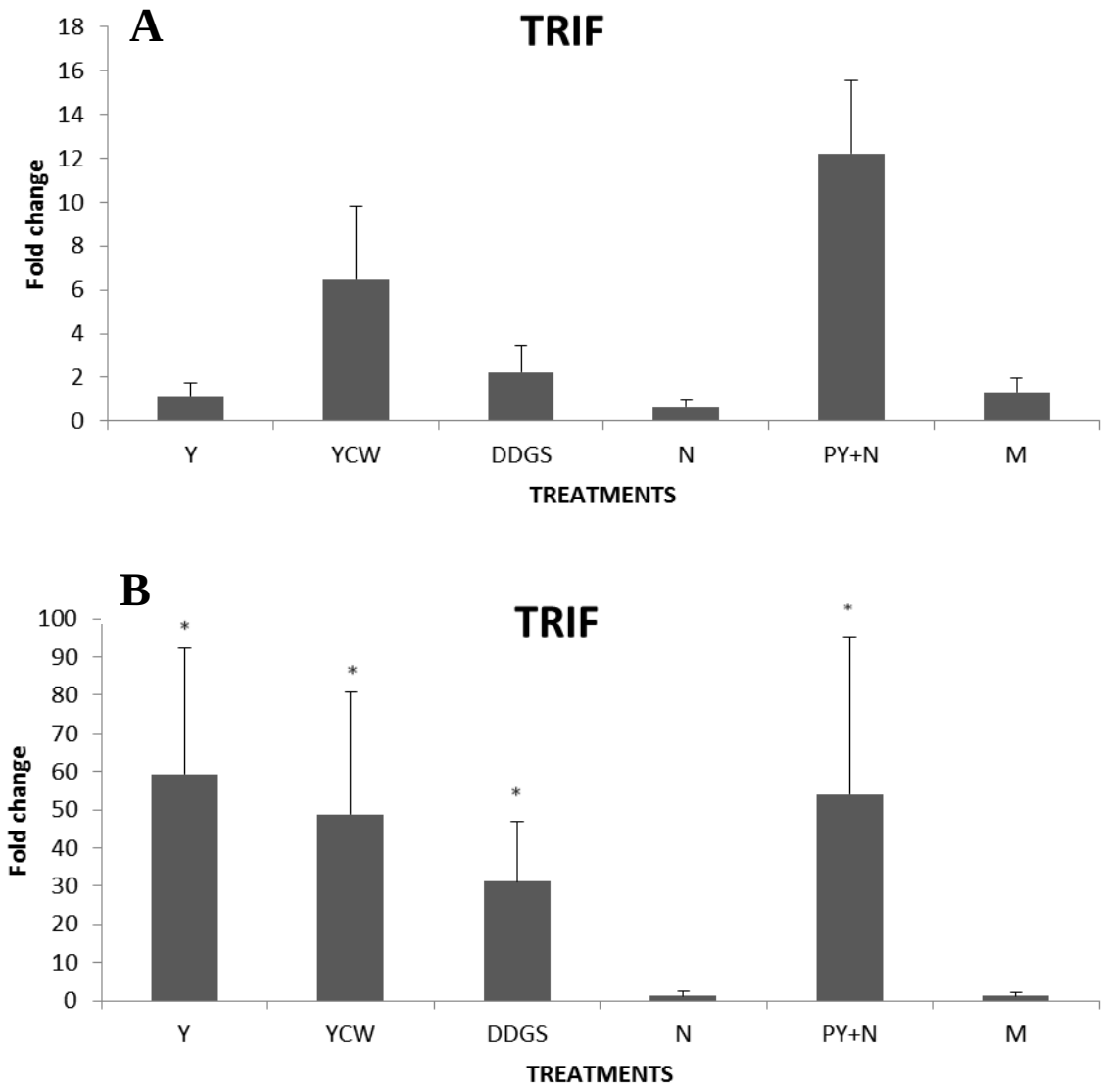

Figure 5. Relative TRIF gene expression of DT40 cell line

(A) In presence of yeast-derived products (B) In presence of yeast-derived products and LPS challenge. Gene expression was assessed using quantitative RT-PCR and was calculated relative to the house keeping gene β-actin. Bar graph values are expressed as fold change relative to control group receiving only basal diet, as calculated by REST 2009. Error bars represent standard error. Results were considered statistically significant from the control group if $p \leq 0.05$ (*).

Y= Brewer's yeast; **YCW**= Yeast cell wall rich product; **DDGS**= Wheat/corn DDGS; **N**= Nucleotide-rich product; **PY+N**= Processed yeast + nucleotide-rich product; **M**= D+ mannose.

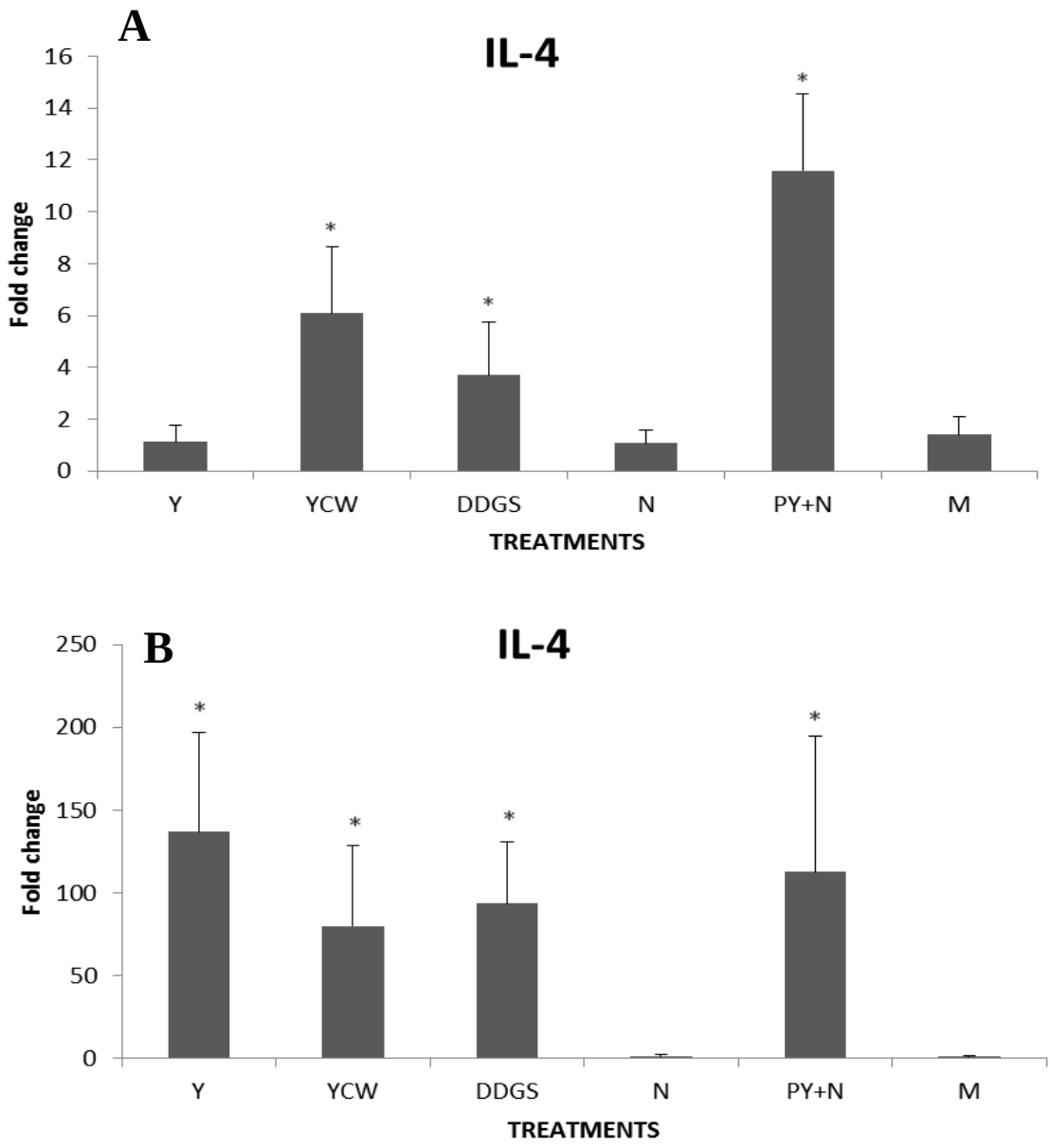

Figure 6. Relative IL-4 gene expression of DT40 cell line

(A) In presence of yeast-derived products (B) In presence of yeast-derived products and LPS challenge. Gene expression was assessed using quantitative RT-PCR and was calculated relative to the house keeping gene β-actin. Bar graph values are expressed as fold change relative to control group receiving only basal diet, as calculated by REST 2009. Error bars represent standard error. Results were considered statistically significant from the control group if $p \leq 0.05$ (*).

Y= Brewer's yeast; **YCW**= Yeast cell wall rich product; **DDGS**= Wheat/corn DDGS; **N**= Nucleotide-rich product; **PY+N**= Processed yeast + nucleotide-rich product; **M**= D+ mannose.

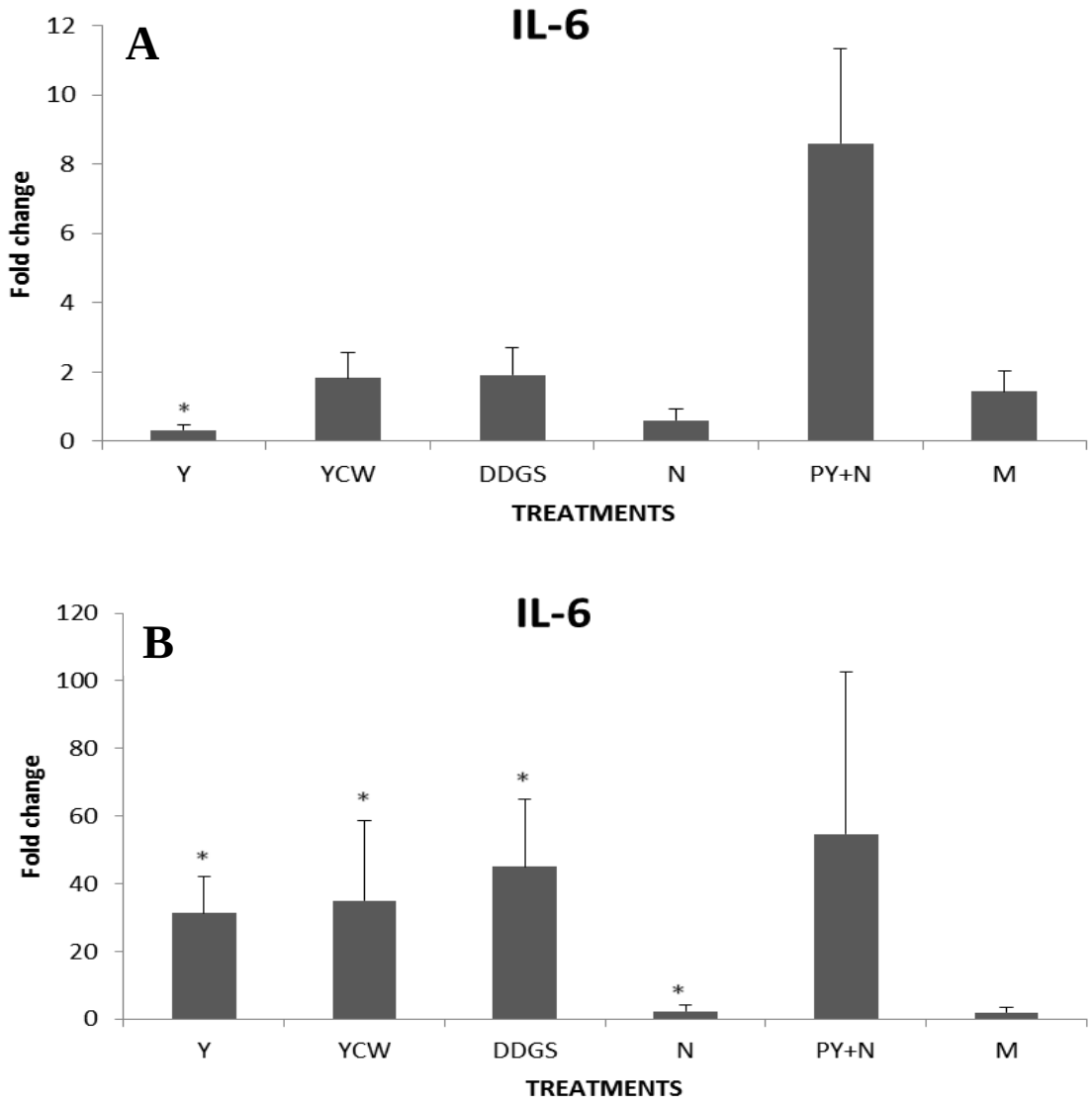

Figure 7. Relative IL-6 gene expression of DT40 cell line

(A) In presence of yeast-derived products (B) In presence of yeast-derived products and LPS challenge. Gene expression was assessed using quantitative RT-PCR and was calculated relative to the house keeping gene β-actin. Bar graph values are expressed as fold change relative to control group receiving only basal diet, as calculated by REST 2009. Error bars represent standard error. Results were considered statistically significant from the control group if $p \leq 0.05$ (*).

Y= Brewer's yeast; **YCW**= Yeast cell wall rich product; **DDGS**= Wheat/corn DDGS; **N**= Nucleotide-rich product; **PY+N**= Processed yeast + nucleotide-rich product; **M**= D+ mannose.

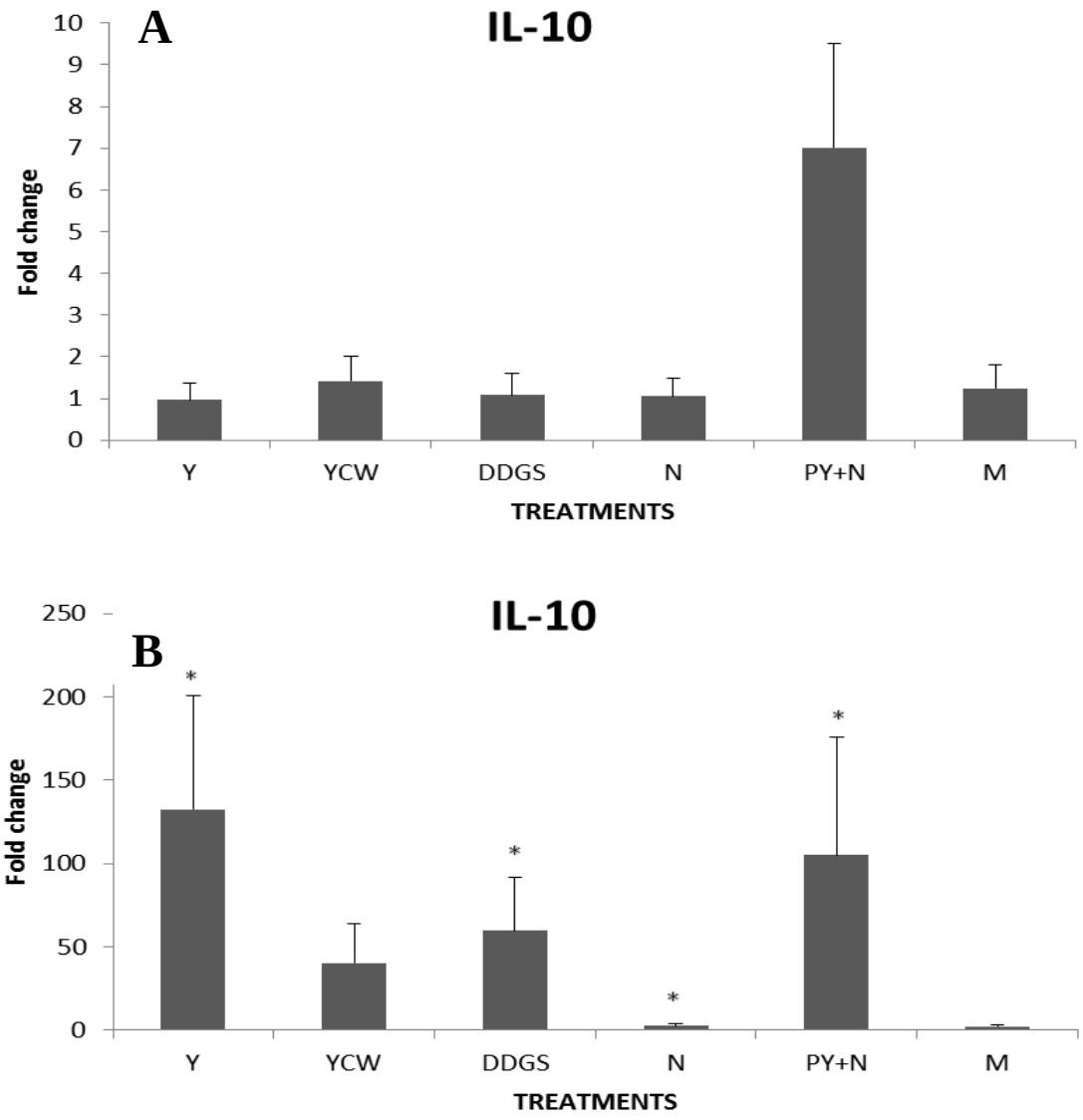

Figure 8. Relative IL-10 gene expression of DT40 cell line

(A) In presence of yeast-derived products (B) In presence of yeast-derived products and LPS challenge. Gene expression was assessed using quantitative RT-PCR and was calculated relative to the house keeping gene β-actin. Bar graph values are expressed as fold change relative to control group receiving only basal diet, as calculated by REST 2009. Error bars represent standard error. Results were considered statistically significant from the control group if $p \leq 0.05$ (*).

Y= Brewer's yeast; **YCW**= Yeast cell wall rich product; **DDGS**= Wheat/corn DDGS; **N**= Nucleotide-rich product; **PY+N**= Processed yeast + nucleotide-rich product; **M**= D+ mannose.

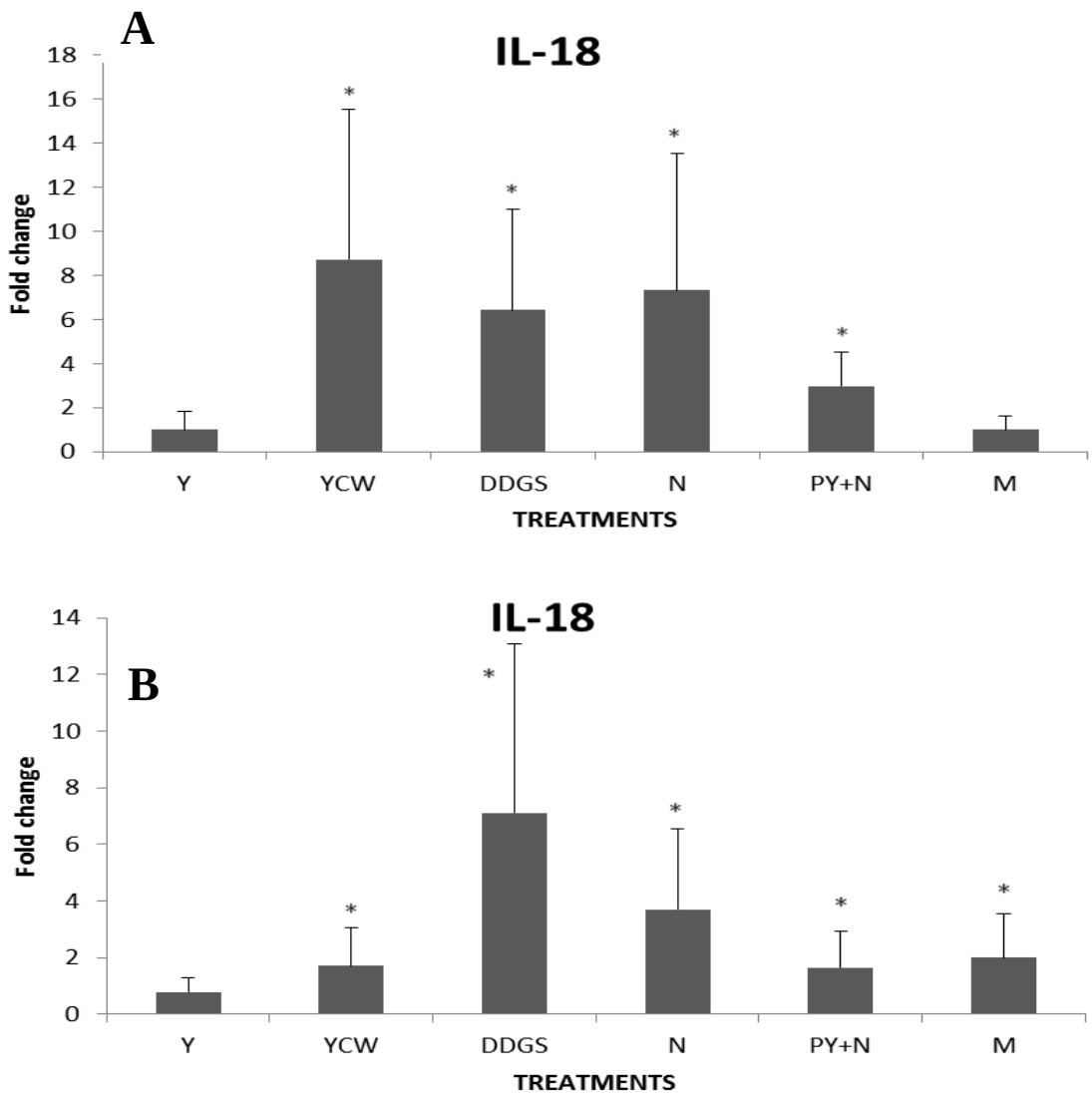

Figure 9. Relative IL-18 gene expression of DT40 cell line

(A) In presence of yeast-derived products (B) In presence of yeast-derived products and LPS challenge. Gene expression was assessed using quantitative RT-PCR and was calculated relative to the house keeping gene β-actin. Bar graph values are expressed as fold change relative to control group receiving only basal diet, as calculated by REST 2009. Error bars represent standard error. Results were considered statistically significant from the control group if $p \leq 0.05$ (*).

Y= Brewer's yeast; **YCW**= Yeast cell wall rich product; **DDGS**= Wheat/corn DDGS; **N**= Nucleotide-rich product; **PY+N**= Processed yeast + nucleotide-rich product; **M**= D+ mannose.

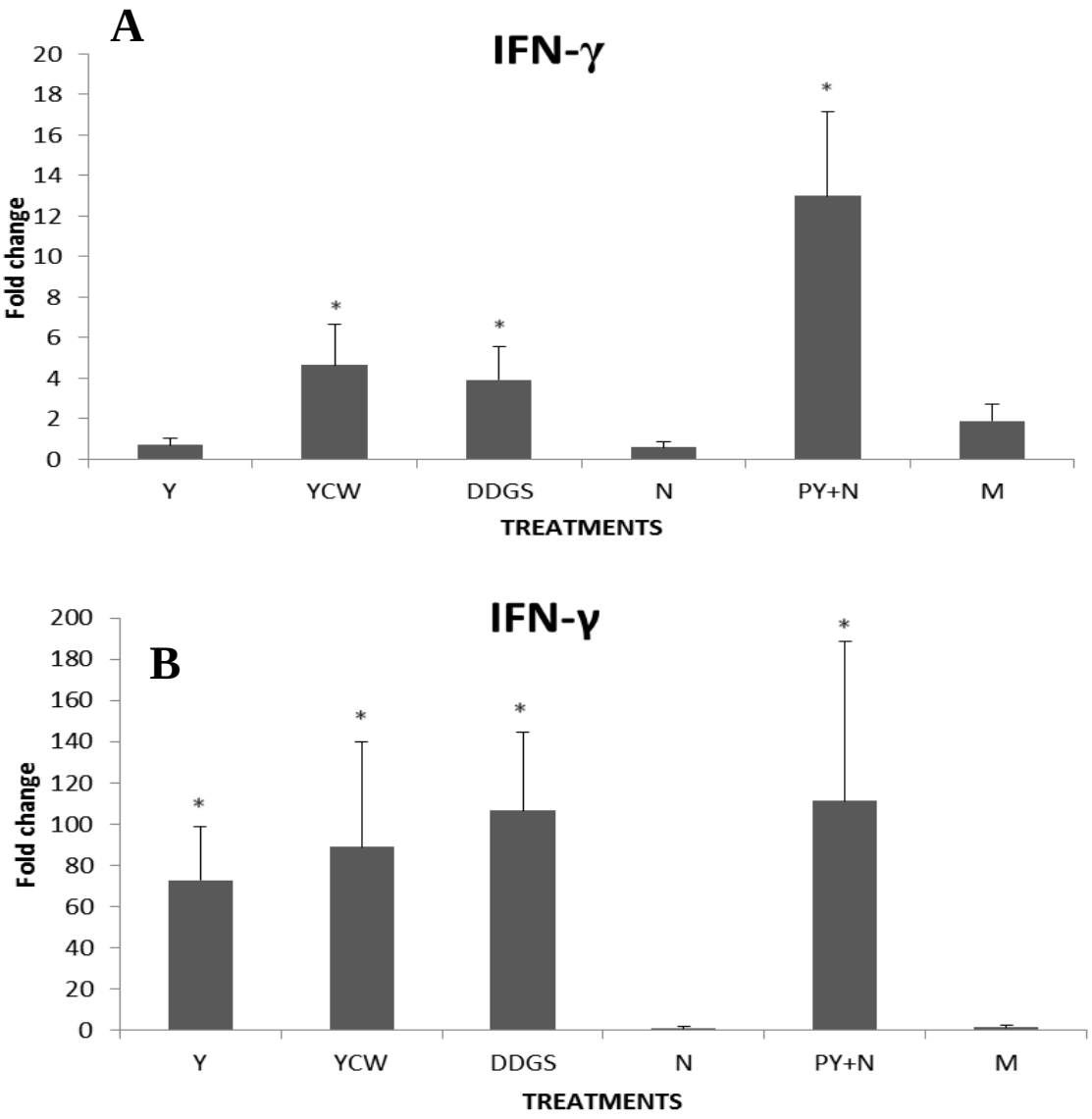

Figure 10. Relative IFN- γ gene expression of DT40 cell line

(A) In presence of yeast-derived products and (B) In presence of yeast-derived products and LPS challenge. Gene expression was assessed using quantitative RT-PCR and was calculated relative to the house keeping gene β-actin. Bar graph values are expressed as fold change relative to control group receiving only basal diet, as calculated by REST 2009. Error bars represent standard error. Results were considered statistically significant from the control group if $p \leq 0.05$ (*).

Y= Brewer's yeast; **YCW**= Yeast cell wall rich product; **DDGS**= Wheat/corn DDGS; **N**= Nucleotide-rich product; **PY+N**= Processed yeast + nucleotide-rich product; **M**= D+ mannose.

4. GENERAL DISCUSSION

It has been reported that APCs can recognize conserved structures common to pathogens through pathogen-associated molecular patterns (PAMPs), which generally are essential parts of the pathogen, required for survival or infectivity (Uematsu and Akira, 2006). Additionally, they can activate acquired immunity through stimulation of specific T and B cells via antigen presentation and for APC cytokines production and release (Banchereau et al., 2003; Blander and Medzhitov, 2006). To some extent, our results show the activity of B cells as APCs, as B cells were able to recognize in different levels the presence of the yeast or yeast products and LPS from gram-negative bacteria. This was shown through up-regulation or down-regulation of TLR2, TLR4 and TLR21. In response to those stimuli, they responded with increased gene expression of cytokines such as IL-4, IL-6, IL-10, IL-18 and IFN-γ that could stimulate specific B and T cell subsets. However, further studies should be performed in order to completely support this idea.

Yeast cell wall is mainly composed of ß1,3-glucan (50-55%), mannoproteins (35-40%), ß1,6-glucan (5-10%), and N-Acetylglucosamine (1-3%) in chitin (Blazejak et al., 2007; Cabib, 2001). It has been found to function as prebiotics (Lipke and Ovalle, 1998) and is able to modulate the immune system (Gao et al., 2008; Solis de los Santos et al.,

2005; Zdunczyk et al., 2005). Our results are in agreement with this immune-modulatory function of these compounds as different yeast and yeast-derived products were able to polarize DT40 chicken B cell line response towards the production of Th2 associated cytokines when challenged by a gram-negative bacteria derived LPS.

The present research provides important data supporting the consumption of DDGS as they have proven to modulate the immune system, and may be of importance in light of a variety of *Saccharomyces cerevisiae* yeast-based products currently available to the poultry industry as growth promoters and natural alternatives to antibiotics (de los Santos et al., 2007; Stanley et al., 2004).

TLR2 has been reported to recognize peptidoglycans and lipoteichoic acid from gram-positive bacteria, Zymosan derived from fungi and also some atypical types of LPS that are not recognized by TLR4 (Schenk et al., 2009). However, it seems that this does not always happen, as DT40 chicken B cell line gene expression for this gene was not up-regulated by the presence of yeast or yeast-derived products.

Unlike higher organisms in which DNA has a high degree of methylation in CpG dinucleotides as a way of protection to confer stability of gene expression patterns of cells, micro-organisms such as bacteria, viruses and yeast have been shown to have a low level of methylation of their CpG dinucleotides (Antequera et al., 1984; Ehrlich et al., 1982; Hoelzer et al., 2008; Krieg, 2002; Mogensen, 2009). It has been reported that bacterial and viral DNA can be recognized by TLR9 in mammals and TLR21 in chickens, which is a functional homologue to the mammalian gene (Brownlie et al., 2009; Keestra et al., 2010). Even though DNA from fungal species has been said to be

recognized by mammalian TRL9, to our knowledge the recognition of these molecules by chicken TLR21 has not been assessed (Nakamura et al., 2008). In this research the up-regulation of TLR21 in DT40 chicken B cell line after treatment with nucleotide-rich product was not found, as it has been reported that IL-15 in chickens can also detect these molecules (Ciraci and Lamont, 2011), and it is possible that in chicken B cells IL-15 is taking the responsibility of recognizing these molecules.

This research is shows that Brewer's yeast, wheat/corn DDGS, nucleotide-rich product and YCW + nucleotide-rich product are able to stimulate DT40 chicken B cell line gene expression of IL-10. This is important due to the anti-inflammatory activity of IL-10 which has been reported to affect Th1 cell function by inhibiting IL-12 and IFN-γ production (Seder and Paul, 1994). Moreover, in some cases IL-10 can inhibit the production of IL-6 and TNF-α (Jack and Turner, 2003). In addition to the anti-inflammatory effects of IL-10 in Th1 and Th2 cells, it has also been reported that it can stimulate the cytotoxic effect of NK cells (Masteller and Thompson, 1994).

5. CONCLUSIONS

In general terms, yeast and yeast-derived products such as brewer's yeast, yeast cell wall rich product (YCW), wheat/corn DDGS, nucleotide-rich product, YCW + nucleotide rich product and D+ mannose are not able to stimulate DT40 chicken B cell line TLR2 and TLR4 gene expression. However, when cells were challenged with gram-negative bacteria derived LPS, increases in TLR4 gene expression was observed with the addition of wheat/corn DDGS, nucleotide rich product, as well as YCW + nucleotide rich product.

Gram-negative bacteria derived LPS was able to boost TLR21 recognition in DT40 chicken B cell line of brewer's yeast, yeast cell wall rich product (YCW), wheat/corn DDGS, nucleotide-rich product, YCW + nucleotide-rich product.

Yeast and yeast-derived product recognition by DT40 chicken B cell line stimulates gene expression of mainly Th1-related cytokines. However, when faced with gram-negative bacteria derived LPS challenge, yeast and yeast-derived products trigger a switch in gene expression towards mainly Th2-related cytokines and thus, regulating the immune response.

Brewer's yeast, wheat/corn DDGS, nucleotide-rich product and YCW + nucleotide-rich product are able to stimulate DT40 chicken B cell line gene expression of anti-inflammatory IL-10.

6. FUTURE DIRECTIONS

Further studies should include the effect of yeast and yeast-derived products in other APCs, such as macrophages, as they may also be present in the gastrointestinal tract and be in contact with these products. Furthermore, future studies should include assays of interaction with T and antibody-producing B cells in order to fully characterize these immune responses and measuring cell-to-cell interaction.

It is important to perform an *in vivo* validation of the immuno modulatory effects of yeast and yeast-derived products, as the present study displayed a tendency to stimulate Th2-related cytokine production in which appears the regulatory IL-10. Furthermore, the inclusion of a bacterial challenge, rather than just toll-like receptor agonists as a method of characterizing the immune modulatory effects of yeast and yeast-derived products should be examined.

Additional work in the recognition of yeast and yeast-derived products should include the use of other PRRs such as c-type lectins which may be present in B cells.

In order to maximize the value of different signalling pathways in future research, it is important to include also type I IFNs.

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