Effects of Whole Yeast Cell Product Supplementation in Chickens Post-coccidial and Post-*Salmonella* Challenge

Thesis

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By

Ashley D. Markazi, B.S.

Graduate Program in Animal Sciences

The Ohio State University

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Master’s Examination Committee:

Ramesh K. Selvaraj, Advisor

Michael S. Lilburn

Renukaradhya Gourapura
ABSTRACT

This project studied the effects of whole yeast cell product supplementation in (1) layer pullets and hens post-coccidial challenge and (2) in broiler chickens post-
*Salmonella enterica* serotype Enteritidis challenge. The whole yeast product is a
commercial-killed whole yeast consisting of all its inner components and the outer yeast
cell wall.

The first study consisted of two experiments. In the first experiment, day-old
layer chicks were supplemented with 0, 0.1 or 0.2% whole yeast cell product. After
21 days of whole yeast cell product feeding, the birds were challenged with live
coccidial oocysts. Whole yeast cell product supplementation improved body weight
gain and had no effect on feed consumption at 9 days post-coccidial challenge.
Whole yeast cell product supplementation decreased fecal oocyst count at 7, 8 and 9
days post-coccidial challenge. Whole yeast cell product supplementation did not
significantly alter CD4⁺ or CD8⁺ cell populations in the cecal tonsils or spleen.
In the second experiment, 40-week-old layer hens were supplemented with 0, 0.05 or 0.1% whole yeast cell product and challenged with live coccidial oocysts after 21 days of whole yeast feeding. Whole yeast cell product supplementation did not significantly alter body weight or feed consumption post-challenge. Supplementation with whole yeast cell product decreased intestinal content ooocyst count at 5, 12 and 28 days post-coccidial challenge. Whole yeast cell product supplementation increased CD8+ cell populations in the cecal tonsils post-coccidial challenge.

The second study evaluated the effects of whole yeast cell product supplementation on broiler production parameters, S. Enteritidis colony forming units, relative proportions of Salmonella, Bifidobacteria and Lactobacillus within the cecal contents and immune parameters at 9 days post-Salmonella challenge. Day-old broiler chicks were supplemented with 0, 0.1 or 0.2% whole yeast cell product. At 21 d of feeding, birds were challenged with S. Enteritidis. Whole yeast cell product did not significantly alter body weight of feed consumption post-challenge. Supplementation with whole yeast cell product numerically increased (P = 0.11) relative proportion of Salmonella at 7 and 14 days post-Salmonella challenge and significantly decreased relative proportion of Lactobacillus 7 days post-Salmonella challenge in the cecal contents. Whole yeast cell product supplementation did not significantly alter relative proportion of Bifidobacteria in the cecal contents at 7 or 14 days post-Salmonella challenge. Whole yeast cell product supplementation downregulated IL-10 and TNF-α and upregulated IL-1β mRNA amounts in the cecal tonsils post-Salmonella challenge. There were no significant differences in IL-6 mRNA amounts in challenged birds.
supplemented with whole yeast cell product. Whole yeast cell product supplementation decreased CD8⁺ cell populations in the cecal tonsils 14 days post-challenge. Whole yeast cell product supplementation decreased jejunum villi lengths and crypt depths 7 days post-Salmonella challenge.

This study demonstrated that whole yeast cell product supplementation may lower infection in layer pullets and hens post-coccidial challenge. Whole yeast cell product may lower infection in broiler chickens post-Salmonella challenge.
Dedication

I dedicate my thesis to my parents and my sister. Thank you for your endless love, support and belief in me.
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I would like to give a most sincere thank you to my advisor, Dr. Ramesh Selvaraj, for giving me this opportunity to further my education and strengthen my knowledge in science. I thank Dr. Michael Lilburn for his continual support, and for the invaluable advice and knowledge he has given me in many subjects including experimental layouts, classes, the poultry industry and nutrition. I also would like to thank Dr. Renukaradhya Gourapura for the very appreciated support and advice he has given me about graduate school, my experiments and explaining to me the importance of balancing both research and lab work.

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Vita

2008.................................................................Barrington High School
Barrington, Illinois

2012.................................................................B.S. Natural Resources and
Environmental Sciences,
University of Illinois at Urbana-
Champaign

2013-2015............................................................Graduate Research Associate,
Graduate program in Animal
Sciences, The Ohio State University

Fields of Study

Major Field: Animal Sciences
Specialization: Poultry Nutrition Immunology
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Chapter 1: Introduction

Whole yeast cell products are derived from yeast species including “Saccharomyces cerevisiae”, “Torula utilis” and “Pichia guilliermondii” (Shanmugasundaram and Selvaraj, 2012; Sohail et al., 2012). The whole yeast product is a commercial-killed whole yeast consisting of all its inner components and the outer yeast cell wall. The yeast cell wall contains the polysaccharides mannan oligosaccharides and β-glucans (Lipke and Ovalle, 1998). These polysaccharides can have immunomodulatory effects in several species including poultry (Shanmugasundaram et al, 2013). Whole yeast cell products may become important in the poultry industry for their potential, especially as anticoccidials and antibiotics decrease in use due to the public’s concerns of antibiotic and drug resistance (Gustafson and Bowen, 1997).

Mannan oligosaccharides and β-glucans facilitate conditioning for gut immunity through several different mechanisms. Mannan oligosaccharides can prevent bacterial adhesion and colonization (Becker and Galletti, 2008). Pathogens such as Salmonella and Escherichia coli have type-1 fimbriae, which are appendages used to attach to other cells. Type-1 fimbriae adhere to MOS due to its high affinity for mannose residues of glycoproteins present on the host’s cell surfaces (Badia et al., 2013). Mannose-rich products such as whole yeast cell products have shown to inhibit pathogenic colonization by adhering to type-1 fimbriae as opposed to the host’s enterocytes (Badia et al., 2013).
The specific structure of β-glucan monomers, which are linked at the 1,3 position with branches at the 1,6 position, influences the activity of leukocytes by resembling pathogen-associated molecular patterns (PAMPs) that stimulates innate immunity (Brown and Gordon, 2003). The β-glucan PAMPs are recognized by the receptor dectin-1 on macrophages (Brown and Gordon, 2003). This recognition signals dectin-1 receptor, which then triggers the innate immune system to recruit leukocytes such as macrophages, neutrophils and natural killer (NK) cells to the site of infection (Brown and Gordon, 2003).

Both mannann oligosaccharides and β–glucans are considered to be prebiotics (Janardhana et al., 2009). Prebiotics are indigestible foods that stimulate the selective growth and activity of beneficial microorganisms such as Bifidobacteria and Lactobacillus in the gut, thus improving overall tissue health (Cummings and Macfarlane, 2002).

Coccidiosis is a major disease in poultry caused by protozoan parasites of the genus Eimeria (Dalloul and Lillehoj, 2006). Eimeria parasites produce lesions that destroy the intestinal epithelia, thereby reducing feed efficiency and body weight gain (Paris and Wong, 2013). Coccidiosis is spread through the chicken’s consumption of coccidial oocysts within the feces (Allen & Fetterer, 2002). Each year coccidiosis causes a loss of about $800 million in the United States alone (Sharman et al., 2010). Current treatment protocols, such as the use of anticoccidial medications, are susceptible to resistance and vaccination by itself is not always enough to control the problem (Chapman, 1998).
Salmonella enterica serotype Enteritidis, one of the most commonly isolated nontyphoidal serotypes of Salmonella, can lead to salmonellosis infection in humans, with symptoms including diarrhea, fever and vomiting (Fabrega & Vila, 2013). Approximately 40,000 cases of salmonellosis are reported each year in the United States, although the real number may be 30-fold greater (Fabrega & Vila, 2013). Acute salmonellosis in humans causes 400 deaths a year in the United States (Fabrega & Vila, 2013). Salmonella can be spread by feces or infected intestinal contents spilling and contaminating the meat (Humphrey, 1988). Because vaccines are not effective in clearing the persistence of S. enterica infections of chickens, the commercial poultry production industry attempts to control S. enterica infections by supplementing vaccination programs with several other procedures such as prebiotics (EFSA Scientific Panel, 2004).

The objectives of the present study are as follows:

Specific Aim 1
To study the effects of whole yeast cell product supplementation in layer chickens post-coccidial challenge

Specific Aim 2
To study the effects of whole yeast cell product supplementation in broiler chickens post-Salmonella challenge
Chapter 2: Review of Literature

Introduction
Yeast cell wall products are derived from yeast species including “Saccharomyces cerevisiae”, “Torula utilis” and “Pichia guillermondii” (Shanmugasundaram and Selvaraj, 2012; Sohail et al., 2012). The yeast cell wall contains the polysaccharides mannan oligosaccharides and β-glucans (Lipke and Ovalle, 1998).

These polysaccharides can have immunomodulatory effects in several species including poultry (Shanmugasundaram et al., 2013). Yeast cell wall products may become important in the poultry industry for their potential, especially as anticoccidials and antibiotics are decreasing in use due to the public’s concerns with fear of antibiotic and drug resistance (Gustafson and Bowen, 1997).

Composition of yeast cell wall products
Polysaccharides make up the majority of the yeast cell wall (Kogan et al., 2008). Mannan oligosaccharides (MOS) represent about 40% of the cell wall’s dry matter, β-glucans represent about 60% and chitin represents about 2% (Kogan et al., 2008). The yeast cell wall’s composition and structure have immunomodulatory implications (Lesage and Bussey, 2006).
**Immunomodulatory Effects of MOS and β-glucans**

MOS and β-glucans facilitate conditioning for gut immunity through several different mechanisms. MOS can prevent bacterial adhesion and colonization (Becker and Galletti, 2008). Pathogens such as *Salmonella* and *E. coli* have type-1 fimbriae, which are appendages used to attach to other cells. Type-1 fimbriae adhere to the due to its high affinity for mannose residues of glycoproteins present on the host’s cell surfaces (Badia et al., 2013). Products derived from mannan have shown to inhibit pathogenic colonization by adhering to type-1 fimbriae as opposed to the host’s enterocytes (Badia et al., 2013).

β-glucans derived from the yeast cell wall have been shown to increase immune signaling for macrophages to migrate to the site of the infection (Brown and Gordon, 2003). The specific structure of these glucose monomers, linked at the 1,3 position with branches at the 1,6 position, influences the activity of immune cells by resembling pathogen-associated molecular patterns (PAMPs) that stimulates innate immunity (Brown and Gordon, 2003). The β-glucan PAMPs are recognized by the receptor dectin-1 on macrophages (Brown and Gordon, 2003). This recognition signals dectin-1 receptor and toll-like receptor 2, which then triggers the innate immune system to recruit leukocytes such as macrophages, neutrophils and natural killer (NK) cells to the site of infection (Brown and Gordon, 2003).

Both β-glucans and MOS are considered to be prebiotics (Janardhana et al., 2009). Prebiotics are indigestible foods that stimulate the selective growth and activity of
beneficial microorganisms such as *Bifidobacteria* and *Lactobacillus* in the gut, thus improving overall tissue health (Cummings and Macfarlane, 2002).

**Gut microflora**

The gastrointestinal (GI) tract is populated with bacteria, fungi, and protozoa, with bacteria as the highest populated microorganism within the intestine (Gabriel et al, 2006). The intestinal tract of newly hatched chicks contain few if any microorganisms. Over time, microbes colonize the gut through feeding, forming stable microflora populations in two to four weeks (Mead and Adams, 1975). These microbes can be classified as generally pathogenic or beneficial (Yegani and Korver, 2008). Pathogenic microbes cause localized or systemic infections and toxin production, which ultimately lead to disruption of the integrity of the gut and therefore less efficient production of the animal (Jeurissen et al., 2002). Beneficial microbes can have effects such as vitamin production, stimulation of the immune system, and inhibition of pathogenic microbe colonization (Jeurissen et al, 2002).

**Villus and crypts**

Intestinal villi are finger-like projections that form the lining of the lumen of the GI tract (McLin et al., 2009). Intestinal villi increase surface area for nutrient absorption and longer villi are often associated with improved gut health and nutrient absorption (Sims et al, 2004). Intestinal crypts surround the base of villi and contain stem cells that continually divide and provide the sources of the epithelial cells on both the crypts and on the villi (Barker and Clevers, 2012).
Both coccidial infection and *S. Enteritidis* can result in lower villus height and greater crypt depths (Eustis and Nelson, 1981; Andrade et al., 2013). Greater crypt depth can be related to the turnover of epithelial cells during an infection, suggesting a compensatory response to the effects of *S. Enteritidis* (Morris et al., 2004). Higher cell proliferation rate may cause the intestine to respond by reducing villus height (Andrade et al., 2013).

Multiple studies have observed that MOS and β-glucan supplementation can increase villus height and decrease crypt depth, suggesting that MOS and β-glucan supplementation improves the intestinal gut health (Baurhoo et al., 2007; Shao et al., 2013).

**Innate Immune System**

The innate immune system is the first line of defense against pathogens (Akira et al., 2006). The innate immune system uses a limited number of germ-line encoded pattern recognition receptors (PPRs) that recognize pathogen-associated molecular patterns (PAMPs) (Akira et al., 2006). PRRs such as toll-like receptors recognize these patterns on antigens such as foreign microbes, leading to direct activation of immune cells (Akira et al., 2006). Toll-like receptors (TLRs) are expressed on macrophages, dendritic cells, B cells, and some T cells. When these immune cells are exposed to the receptors’ ligands, adaptor proteins containing a TIR domain, such as myeloid differentiation factor 88 (MyD88), are recruited to the cytoplasmic region portion of the TLR through interaction of the TIR domains. The resulting complex activates downstream signaling
cascades, resulting in the production of proinflammatory cytokines and chemokines (Akira et al., 2006).

Macrophage and dendritic cells, known as antigen presenting cells (APCs), activate the adaptive immune response by presenting antigens to naïve CD4+ T cells. Additionally, dendritic cells can activate T cells, resulting in the transformation of naïve CD4+ T cells to into T helper (Th1) cells. Th1 cells are essential in eliminating bacterial and viral infection, as they produce interferon-γ (IFN-γ) (Akira et al., 2006). Th2 cells produce IL-4 and IL-13, which are cytokines important in eliminating helminth infection. TLR stimulation more often leads to Th1 differentiation rather than Th2 differentiation. As a result, innate immunity is an important activator of inflammatory response and important for the immune response against pathogens (Akira et al., 2006).

The innate immune response is activated by foreign bacteria through molecules collectively termed as modulins (Henderson et al., 1996). These modulins include lipopolysaccharides (LPS), peptidoglycans, lipoteichoic acid, lipoarabinomannan, lipoproteins, and lipopeptides. Monocytes and macrophages respond to modulins by producing proinflammatory cytokines such as IL-1β, IL-6, and TNF-α (Henderson et al., 1996). These cytokines play a critical role in both the innate immune response and the initiation of the adaptive immune response (Moors et al., 2001.)

**Adaptive Immune System**

The adaptive immune system has a more specific mechanism than the innate immune system for recognizing self and non-self-antigens (Bonilla and Oettgen, 2010).
The adaptive immune system uses antigen-presenting cells and T and B lymphocytes to signal pathogen-specific immunologic pathways.

Lymphocytes are white blood cells such as T cells and B cells. During lymphocyte development, genes encode a diverse array of specific antigen receptors. The interaction also generates immunologic memory (Bonilla and Oettgen, 2010). Long-lived memory T and B cells are established during the first encounter with an antigen. When these memory cells encounter the same antigen again, the memory cells respond more rapidly (Bonilla and Oettgen, 2010).

T lymphocytes are comprised of two sub-populations: cytotoxic T lymphocytes and helper T lymphocytes. These sub-populations are defined by their surface phenotypes (Yun et al., 2000). Cytotoxic T lymphocytes recognize foreign antigens in the context of MHC class I molecules, whereas T helper cells recognize antigens in the context of MHC II molecules (Yun et al., 2000).

Intestinal lymphocytes within the intestinal mucosa are located in either the epithelium (intestinal intraepithelial lymphocytes) or the lamina propria (lamina propria lymphocytes). Intestinal intraepithelial lymphocytes (IELs) are present in the basal part of the epithelium near the basement membrane (Yun, 2000). The number of these cells increase in the presence of antigens in the intestine (Yun, 2000).

Although cytotoxic T cells are evident in the intestine of mammals, MHC-restricted intestinal epithelial lymphocytes (IELs) demonstrating cytotoxic activity have not been seen in chickens (Yun, 2000). IELs are capable of processing antigens, although the process is slower than in APCs (Brandeis et al., 1994). Additionally, IELs are
categorized as non-professional APCs since they do not express class II MHC (Guehler et al., 1999). IELs secrete cytokines including IL-1, IL-2, IL-6, TGF-β, TNF-α, and IFN-γ. IELs can also induce other intestinal cells to express class II molecules (Yun, 2000).

Cytokines

Cytokines are peptides involved in cell signaling and can affect the activity behavior of other cells through autocrine, paracrine, and endocrine pathways (Cannon, 2000). Cytokines can be produced by almost all nucleated cells. Endothelial cells, epithelial cells, and macrophages are especially strong producers of IL-1β and TNF-α (Cannon, 2000). Cytokines can be produced in response to a wide a variety of stresses including pathogenic microbes, endocrine stimuli, mechanical factors and environmental factors (Cannon, 2000). T lymphocytes and macrophages are most likely the major sources of cytokine production in the intestine (Lillehoj, 1994). Intestinal lymphocytes have shown to be in direct contact with parasitized epithelial cells (Choi et al., 1999).

Chicken homologues to mammalian cytokines include IFN-γ, TGF-β, TNF-α, and IL-2 (Lillehoj, 1998). IFN-γ stimulates proliferation and differentiation of hematopoietic cells, and enhances non-specific immunity to tumors, bacteria, viruses and parasites (Yun et al., 2000). Chicken IFN-γ (ChIFN-γ) regulates adaptive immunity by activating lymphocytes and enhancing expression of MHC class II antigens (Kaspers et al., 1994). Lymphocytes from Eimeria-infected chickens produce higher levels of IFN-γ compared to uninfected controls (Martin et al., 1994). TGF-β is also produced by lymphocytes in response to Eimeria (Jakowlew et al., 1997).
**IL-1**

Interleukin-1 cytokines induce inflammatory responses to infection (Dinarello, 2011). Both interleukin 1-α and interleukin 1-β bind to the IL-1 receptor (IL-1R) resulting in the accessory protein 1RAcP to form a heterodimic complex with IL-1R1 and IL-1 (Dinarello, 2011). The complex signals the adaptor protein MyD88 to join the IL-1 receptor domain, resulting in phosphorylated kinases and NF-kB translocation into the nucleus (Dinarello, 2011). Translocation of NF-kB to the nucleus leads to transcription of inflammatory genes (Dinarello, 2011).

**IL-10**

Interleukin-10 is an anti-inflammatory cytokine (Sabat et al., 2010). In mammals, IL-10 cytokines are synthesized mainly by monocytes, macrophages and T helper cells (Murai et al., 2009: Roers et al., 2004). IL-10 can additionally be produced from almost all leukocytes, including dendritic cells, B cells, NK cells and mast cells (Wolk et al., 2002). IL-10 targets monocytes and macrophages by suppressing antigen presentation, phagocytosis, and cytokine production. IL-10 regulates these immune cells via the transmembrane receptor complex (Sabat et al., 2010). The transmembrane receptor complex is composed of IL-10R1 and IL-10R2 (Sabat et al., 2010). When IL-10 binds to its receptors, it activates members of the Janus kinase family (Jak1 and Tyk2). These kinases phosphorylate tyrosines of IL-10R1, which cause the transcription factor STAT3 to bind and become phosphorylated. STAT molecules are involved with inhibiting the production of inflammatory molecules (Sabat et al., 2010).
Chicken IL-10 (chIL-10) has been cloned and isolated from the cecal tonsils of chickens infected with *Eimeria Tenella* (Rothwell et al., 2004). ChIL-10 mRNA expression is prominent within the cecal tonsils and bursa of fabricus, but is also expressed in the thymus liver and lung (Rothwell et al., 2004). ChIL-10 downregulates inflammatory responses by inhibiting the synthesis of proinflammatory cytokines (including IL-1β, TNF-α, and IL-12), nitric oxide production, and MHC class II expression (Rothwell et al., 2004).

**IL-6**

IL-6 has a wide variety of functions in regulating the immune system (Schneider et al., 2001). IL-6 is produced by many different cell types and acts on B cells and T cells (Hirano et al., 1986). IL-6 can modulate the Th1/Th2 response by inhibiting Th1 differentiation through upregulating the suppression of cytokine signaling (Diehl et al., 2000). The IL-6 receptor alpha chain is responsible for ligand binding (Schneider et al., 2001). Binding of IL-6 results in the activation of JAK/STAT and Ras/Raf signaling pathway (Hibi et al., 1996).

**TNF-α**

TNF-α acts as the major inflammatory response to parasites, bacteria and viruses (Hong et al., 2006). Hong et al. (2006) isolated a full-length cDNA encoding the chicken homologue of LPS-induced TNA- α factor (LITAF) and concluded the chicken LITAF (chLITAF) can serve as a homologue to the mammalian TNF-α. Upregulation of chLITAF mRNA levels were observed after macrophages were stimulated with
Escherichia coli, Salmonella enterica serotype Typhimurium endotoxin, and coccidial parasites Eimeria acervulina, Eimeria maxima, and Eimeria tenella (Hong et al., 2006).

**Coccidiosis**

There are seven species of Eimeria in chickens: Eimeria acervulina, Eimeria maxima, Eimeria tenella, Eimeria necatrix, Eimeria mitis, Eimeria brunette, and Eimeria praecox (McDougald, 1998). Each Eimeria species infects a specific part of the intestines with a different lesion appearance (Paris and Wong, 2013). Five of the species, E. acervulina, E. brunette, E. maxima, E. necatrix, and E. tenella are well known and easily identified, as they produce characteristic gross lesions which range from moderate to severe (Allen and Fetterer, 2002). Species such as E. praecox and E. miti are considered to be benign as they do not kill chickens or produce lesions. However, even with these two species, symptoms can include diarrhea and reduced feed efficiency, therefore leading to potential commercial losses as well. Prophylactic use of anticoccidial feed additives were the main way to control coccidiosis for many years however; anticoccidial resistance has become a concern within the industry (Allen and Fetterer, 2002).

Avian Eimeria spp have homogenous life cycles (Allen and Fetterer, 2002). Oocysts shed in feces undergo sporogony in the external environment and this process takes about 23 hours. Sporogony is a meiotic process. The sporulated oocysts contain four sporocysts, each of which contains two sporozoites. After the chicken ingests the oocysts, the oocysts excyst within the intestinal lumen, while being aided by trypsin, bile and CO₂. The oocysts release sporozoites which can then penetrate the villous epithelial cells. Some species such as E. brunette and E. praecox have sporozoites that
develop within the villous epithelial cells that were penetrated. Other species (*E. acervulina, E. maxima, E. necatrix, and E. tenella*) have sporozoites that are transported to other sites, such as the crypt epithelium, to undergo development (Allen and Fetterer, 2002).

Sporozoites undergo asexual reproduction which results in merozoites breaking free and penetrating other host cells. The merozoites may carry out more merogenic generations. Eventually, merozoites enter host cells and develop into either male (microgamonts) or female (macrogamonts) forms. The microgametes fertilize macrogamonts, which then results in oocysts. The oocysts are shed with the feces. Prepatent periods generally range from 4 to 5 days after the infection, with the maximum oocyst output ranges being from 6 to 9 days post infection (Allen and Fetterer, 2002).

**Anticoccidial drugs**

The poultry industry has been largely dependent on anticoccidial drugs (Allen et al., 1997). Despite various anticoccidial drugs used in rotation, resistance has developed to all of the anticoccidial drugs that have been introduced (Peek and Landman, 2011). There are fewer new drugs being developed due to high cost, strict testing and regulatory requirement (Peek and Landman, 2011).

Live anticoccidial vaccines are increasingly being incorporated to anticoccidial drug rotation programs, resulting in an increase of drug-sensitive *Eimeria* spp (Peek and Landman, 2011). This may result in ameliorated efficacy of anticoccidial drugs (Peek and Landman, 2011). Despite this, anticoccidials are continuing to be banned due to public fear of drug residues in the food supply and resistance to antibiotics (Chapman, 1994).
Management and biosecurity play a major role in lowering *Eimeria* spp. introduction to the farm; however, they alone do not prevent coccidiosis outbreaks (Peek and Landman, 2011).

Anticoccidial drugs can be classified in three categories: synthetic compounds, polyether antibiotics or ionophores, and mixed products (Allen and Fetterer, 2002). Synthetic compounds are produced by chemical synthesis and have a specific mode of action against the parasite metabolism. An example of this is amprolium, which competes for the absorption of thiamine by the parasite (Allen and Fetterer, 2002). Polyether antibiotics or ionophores are produced by the fermentation of *Streptomyces* spp. or *Actinomadura* spp. and destroy coccidia by interfering with the balance of ions such as sodium and potassium (Peek and Landman, 2011). Mixed products are drug mixtures consisting of either a synthetic compound and ionophore or two synthetic compounds can also be used again coccidiosis (Peek and Landman, 2011). Resistance to all three types of anticoccidial drug products has been seen in *Eimeria* spp. (Peek and Landman, 2011).

**Coccidial Vaccines**

Due to anticoccidial drug resistance developed in *Eimeria* spp, vaccines are becoming a popular alternative in large-scale production (Chapman, 2002). Types of vaccines include Coccivac, Immucox, and Advent. These live vaccines are not attenuated, and therefore can cause some lesions and occurrence of cocidiosis in birds (Peek and Landman, 2011). Coccidiosis vaccines used in Europe and attenuated—not disease-causing, and are more expensive than nonattenuated vaccines. These vaccines include Paracox, Livacox, and Viracox (Peek and Landman, 2011). Anticoccidial
vaccines include mixtures of species of *Eimeria* that affect chickens, especially *Eimeria acervulina, Eimeria maxima,* and *Eimeria tenella,* as these types cause the most damage to chickens (Peek and Landman, 2011). Vaccines should be given at the hatchery or by the first week of age, and can be applied through spray cabinets, edible gel, feed spray, or drinking water (Chapman, 2002).

**Immune Responses to Coccidiosis**

Due to *Eimeria* parasites complex life cycle with both intracellular and extracellular stages, the host immune response is complex, involving both innate and adaptive immune responses, and both cellular and humoral immune responses within the adaptive response (Dalloul and Lillehoj, 2005). Both innate and adaptive immune responses react to *Eimeria* invasion of the host intestine (Dalloul and Lillehoj, 2005). Physical barriers, phagocytes, leukocytes, chemokines and complement components make up the innate immune response to *Eimeria* (Dalloul and Lillehoj, 2005). Antibodies, lymphocytes and cytokines mediate the adaptive immune response to *Eimeria* (Dalloul and Lillehoj, 2005). In the naïve host, coccidia activates the antigen-presenting cells (APCs) dendritic cells and macrophages (Lillehoj, 1998). The APCs then produce chemokines and cytokines (Lillehoj, 1998). In hosts that have previously been infected with coccidia, after *Eimeria* enters the gut, the adaptive immunity inhibits further development (Trout and Lillehoj, 1994).

A number of studies have shown that T cells are the primary effectors of immunity to *Eimeria* (Lillehoj, 1987). T cells in adaptive immune response to coccidia have been shown to be very important (Lillehoj, 1998). Cd8⁺ IELs were detected in
chickens given both a primary and secondary infection (Lillehoj and Bacon, 1991). The increase in CD8$^+$ cells correlated with reduced oocyst excretion (Lillehoj and Bacon, 1991). Additionally, studies have shown that CD4$^+$ IELs increased 7 days after a primary infection, and a higher number of CD8$^+$ IELs were observed after a secondary infection, suggesting that CD8$^+$ IELs may contribute to adaptive immunity to *Eimeria* (Lillehoj, 1994).

**Salmonella**

The genus *Salmonella* is a gram-negative, non-spore-forming, rod-shaped bacteria from the *Enterobacteriaceae* family (Fabrega & Vila, 2013). These bacteria have diameters of .7 to 1.5 μm, lengths from 2 to 5 μm and peritrichous flagella that are all around the cell body (Fabrega & Vila, 2013). *Salmonella* are chemoorganotrophs, meaning that they get energy from oxidation and reduction reactions using organic sources (Fabrega & Vila, 2013).

There are two species of *Salmonella*: *Salmonella bongori* and *Salmonella enterica*. *S. enterica* can be divided into six subspecies: 


Almost all *Salmonella* organisms that cause disease in humans and domestic animals belong to *S. enterica* subspecies *enterica* (I). *S. enterica* serotypes can be divided into two groups: typhoidal and nontyphoidal. The typhoidal serotype can cause enteric
fever in humans. The more common nontyphoidal serotype causes diarrheal disease and infect a wide range of animal hosts (Ohl and Miller, 2001).

*S. Enteritidis* and *S. Typhimurium* are the most commonly isolated nontyphoidal serotypes in clinical practice (Fabrega and Vila, 2013). *S. Enteritidis* and *S. Typhimurium* rarely cause clinical disease in chickens, but can colonize the gut of poultry (Barrow et al., 1987).

Approximately 40,000 cases of the *Salmonella*-caused disease salmonellosis are reported each year in the United States, although the real number may be 30-fold greater (Fabrega & Vila, 2013). The illness occurs most commonly in young children, the elderly, and those with compromised immune system. Acute salmonellosis causes 400 deaths in humans a year in the United States (Fabrega and Vila, 2013). Infection with salmonellosis is caused by the interactions of the pathogen with multiple environments, beginning from the hen house, to the poultry plant facility, and finally to the poultry product in the customer’s home (Guard-Petter, 2001). *Salmonella* is shed in the feces and can be spread by horizontal transmission to other birds by fecal-oral routes (Humphrey, 1988). *Salmonella* can also be spread by feces or infected intestinal contents spilling and contaminating the meat. Additionally, *Salmonella* can colonize the reproductive tract, leading to the contamination of eggs (Humphrey et al., 1988).

Infection from *S. Enteritidis* and *S. Typhimurium*, which begins from the ingestion of the organism, have similar virulence mechanisms in regards to cellular invasion, survival and growth within the host (Guard-Petter, 2001). To protect itself from acidity of the stomach, *S. Enteritidis* activates an acid tolerance response which results in
a pH-homeostatic function that maintains the intracellular pH at values higher than the extracellular environment (Foster & Hall, 1991).

The *salmonellae* travels through the intestinal mucus layer before adhering to intestinal epithelial cells (Fabrega and Vila, 2013). After the adhesion, the engaged host cell signals pathways that lead to cytoskeletal rearrangement (Francis et al., 1992). These rearrangements disrupt the epithelial brush border, inducing the formation of membrane ruffles that engulf adherent bacteria vesicles called *Salmonella*-containing vacuoles (SCVs) where the *Salmonella* cells can survive and replicate (Garcia-del Portillo and Finlay, 1995). The SCVs transports to the basolateral membrane and releases salmonella cells into the submucosa. Phagocytes internalize the bacteria and are moved within an SCV again. These phagocytes can move through the lymph and bloodstream, facilitating a systemic infection (Fabrega and Vila, 2013).

In mammalian hosts, type III secretion systems play an important role in host cell invasiveness and the production of proinflammatory cytokines (Darwin and Miller, 1999). The type III secretion results in an influx polymorphonuclear cell to the infection. In chicken cells infected with *S. Typhimurium*, *S. Enteritidis* or *S. Gallinarum*, IFN-γ production differed little compared to uninfected cells. IL-1β production had little effect on *S. Typhimurium* and had a slight reduction with *S. Gallinarum* and *S. Enteritidis*. *S. Typhimurium* and *S. Enteritidis* caused an eight to tenfold increased in IL-6 expression, suggesting that that both *S. Typhimurium* and *S. Enteritidis* produced a strong inflammatory effect. *S. Gallinarum*, in contrast, did not cause an increase in IL-6 (Kaiser et al., 2000).
**S. Enteritidis flagellin (FliC)** activates monocytes to produce proinflammatory cytokines (Moors et al., 2001). Lipopolysaccharides (LPS), a well-studied immune stimulatory component of gram-negative bacteria, signals Toll-like receptor 4 (TLR4). HeNC2 cells stimulated with LPS in the presence of a neutralizing anti-TLR4 monoclonal antibody reduced inflammatory TNF-α and nitric oxide production (Moors et al., 2001). In contrast, FliC in the presence of anti-TLR4 has minimal effect on TNF-α and nitric oxide production, suggesting that Flic may signal a toll-like receptor distinct from TLR4 (Moors et al., 2001).

**Immune Responses to *Salmonella***

Leghorn chicks inoculated on day of hatch with *S. Enteritidis* had an increase in *S. Enteritidis*-specific mucosal immunoglobulin A (IgA) 14 and 28 days post-challenge and an increase in number of gut-associated T cells 14, 28 and 56 days post-challenge (Sheela et al., 2003). These results suggest that *S. Enteritidis* in chickens induces both mucosal and systemic responses (Sheela et al., 2003).

*S. Enteritidis* infects chickens most commonly through contaminated feed and feces. The infection is initiated by extensive colonization of *S. Enteritidis* in the intestine (Carter and Collins, 1974). *S. Enteritidis* colonization can persist for as long as 18 weeks in the intestine postinoculation in hens (Gast and Benson, 1995). After colonization within the intestine, *S. Enteritidis* can spread to the liver, spleen, ovary and oviduct postinoculation (Barrow, 1991; Gast and Beard, 1990). The persistence of *S. Enteritidis* in infected laying hens contributes to the contamination of shell eggs (Gast and Beard, 1990).
The development of effective vaccines in chickens has been hindered due to limited knowledge of the immune responses against *Salmonella* in chickens (Sheela et al., 2003). The mucosal immune system of the intestine, which includes mucosal immunoglobulin A (IgA) and mucosa-associated lymphocytes and leukocytes, form the first line of defense against *S. Enteritidis* infection. Humoral and cell-mediated responses are critical for the resistance and clearance of *S. Enteritidis* infection (Barrow, 1992). Immune suppression of B cell precursors and B cells showed an increase of *S. Enteritidis* intestinal shedding rate (Arnold and Holt, 1995). Secretory IgA limits mucosal colonization of *S. Enteritidis* by preventing adherence of the bacteria (Shroff et al., 1995). However, several studies have shown the number of IgA cells and secretory IgA in the intestine increased slowly over time, suggesting the mucosal humoral immunity may not be fully developed in newly hatched chicks and take time to mature (Sheela et al., 2003). Furthermore, IgA response returned to baseline 56 days postinoculation, despite *S. Enteritidis* infection still persisting (Sheela et al., 2003). Lymphocytes in the peripheral blood and spleen were not altered by *S. Enteritidis* infection, suggesting that the mucosal IgA response by *S. Enteritidis* may be a primary humoral response (Sheela et al., 2003).

*S. Enteritidis* is a facultative intracellular bacterium, suggesting that cell-mediated immunity additionally plays a major role during *Salmonella* infection (George et al., 1987). CD3+, CD4+ and CD8+ T cells have shown to proliferate in the reproductive tract of hens infected with *S. Enteritidis* (Withanage et al., 1998). The increase in CD3+ T cells may suggest that *S. Enteritidis* infection either stimulates gut-associated T cells to expand or recruits more T cells to the infection (Sheela et al., 2003). However, T-cell
immunosuppression showed no significant effect of *S. Enteritidis* infection in chickens, therefore the role of T cells in response to *S. Enteritidis* infection is still unclear (Arnold and Holt, 1995). Sheela et al. (2003) suggests that the ineffectiveness of T cells in *S. Enteritidis* infection may be due to *S. Enteritidis* avoiding or suppressing the activation of T cells.

Although *S. Enteritidis* increases titers in the serum, the infection does not increase mononuclear cells in the blood and spleen, suggesting that *S. Enteritidis* does not activate peripheral or circulating lymphocytes specific for *S. Enteritidis* (Sheela et al., 2003). These results may explain why *Salmonella* can persist in the chickens for their lifetime without elimination from the immune system (Dunlap et al., 1991).

**Salmonella Vaccines**

*S. Enteritidis*, which does not normally cause a systemic disease, stimulates high production of IL-1 and IL-6 from epithelial cells within the intestine (EFSA Scientific Panel, 2004). *S. Gallinarum* and *S. Pullorum*, in contrast, suppresses production by these cells (EFSA Scientific Panel, 2004). A Th1-type response against *S. Enteritidis* may result in immune clearance, whereas a predominantly Th2-response may lead to prolonged infection in *S. Pullorum*. Vaccines are required to induce a Th-1 type response that leads to immunity and clearance after a challenge (EFSA Scientific Panel, 2004).

*Salmonella* vaccines are rarely used for broiler flocks due the cost of treatment and short lifespan of the birds (EFSA Scientific Panel, 2004). In Germany, it has been obligatory to vaccinate all layer chickens during rearing period using live
S. Typhimurium or S. Enteritidis vaccines. The use of inactivated *Salmonella* vaccines in flocks for poultry meat is more preferable, since it eliminates possibility of live *Salmonella* vaccine organisms in the end product. Some live vaccines can persist in birds for several weeks (EFSA Scientific Panel, 2004).

Oral administration of vaccines in drinking water is more preferable than parenteral administration by injection, as handling poultry can be time consuming and the injection itself can be painful (EFSA Scientific Panel, 2004). Previous or simultaneous live vaccine treatment with antimicrobials given to the chickens may affect the efficacy of the vaccination (EFSA Scientific Panel, 2004). For reducing shedding and egg contamination in layers, only inactivated vaccines can be used due to possible egg contamination by the vaccine strain (EFSA Scientific Panel, 2004). Additionally, another possible disadvantage of live vaccines is the spread of the strain to the environment or to humans (EFSA Scientific Panel, 2004).
Chapter 3: Effects of Whole Yeast Product Post-Coccidial Challenge

Abstract

Two experiments were conducted to study the effects of whole yeast cell product supplementation in layer chicken production parameters, fecal and intestinal coccidial oocyst counts and immune parameters following an experimental coccidial infection. In experiment I, one-day-old layer chicks were fed three experimental diets with 0, 0.1 or 0.2% whole yeast cell product. At 21 days of whole yeast cell product feeding, birds were challenged with live coccidial oocysts. In experiment II, 40-week-old layer hens were fed three experimental diets with 0, 0.05 or 0.1% whole yeast cell product and challenged with live coccidial oocysts at 21 d of whole yeast cell product feeding.

In experiment I, supplementation with whole yeast product numerically increased (P = 0.08) body weight gain post-coccidial challenge and had no significant effect on feed consumption. Supplementation with whole yeast cell product decreased (P < 0.05) the fecal coccidial oocyst count at 7 and 8 days post-coccidial challenge. Supplementation with whole yeast cell product did not alter CD4+ and CD8+ cell populations in the spleen or cecal tonsils post-coccidial challenge. In experiment II, supplementation with whole yeast cell product did not significantly alter body weight
gain or feed consumption post-coccidial challenge. Supplementation with whole yeast cell product decreased intestinal coccidial oocyst count (P < 0.05) at 5, 12 and 28 days post-coccidial challenge. Supplementation with whole yeast cell product significantly increased (P < 0.05) CD8+ cell populations and numerically (P = 0.08) increased CD4+ cell populations in the cecal tonsils at 5 days post-coccidial challenge. It could be concluded that supplementing whole yeast cell product in layer diets can improve body weight gain, decrease fecal and intestinal oocyst count and increase CD8+ cell populations in cecal tonsils post-coccidial challenge.
Introduction

Whole yeast cell products are derived from yeast species including “Saccharomyces cerevisiae”, “Torula utilis” and “Pichia guillermondii” (Shanmugasundaram & Selvaraj, 2012; Sohail et al., 2012). The yeast cell wall contains the polysaccharides mannan oligosaccharides and $\beta$-glucans (Lipke and Ovalle, 1998). These polysaccharides can have immunomodulatory effects in several species including poultry (Shanmugasundaram et al., 2013). Yeast products may become important in the poultry industry for their potential, especially as anticoccidials and antibiotics are decreasing in use due to the public’s concerns with fear of antibiotic resistance (Gustafson and Bowen, 1997).

Coccidiosis is a major disease in the poultry industry caused by protozoan parasites of the genus *Eimeria* (Dalloul and Lillehoj, 2006). Coccidiosis impairs growth and feed utilization in poultry, resulting in major losses in productivity (Dalloul and Lillehoj, 2006). *Eimeria* parasites causes lesions that impair the intestinal epithelia, thereby reducing feed efficiency and body weight gain. This leads to severe economic losses in the poultry industry (Dalloul and Lillehoj, 2006).

$\beta$-glucans are glucose polymers that form the structural components in the cell wall of yeast, fungi, algae and cereal grains including oat and barley (Cox et al., 2010). The structure of $\beta$-glucans from different sources results in differing functions (Volman et al., 2008). The highly branched structure of 1,3/1,6-$\beta$-D-glucans are considered to be the most effective of the $\beta$-glucans in modulating
the immune system (Vetvicka and Vetvickova, 2007). β-glucans influence the activity of immune cells by resembling pathogen-associated molecular patterns (PAMPs) that stimulate innate immunity (Brown and Gordon, 2003). The β-glucan PAMPs are recognized by the receptor dectin-1 on macrophages (Brown and Gordon, 2003). This recognition activates a cascade system leading to the production of proinflammatory cytokines and chemokines (Brown and Gordon, 2003).

β-glucan supplementation in broilers has resulted in enhanced cell-mediated and humoral immune responses (Chae et al., 2006; Cox et al., 2010). β-glucan supplemented birds had lower intestinal coccidial lesion severity and upregulated nitric oxide synthase (iNOS) expression 14 days post-coccidial challenge (Cox et al., 2010). Upregulated iNOS mRNA implies increased nitric oxide production, which is an important product of macrophages for defense against a wide scope of microbes (Ljungman et al., 1998).

Mannan oligosaccharide supplementation has additionally been shown to lower coccidial infection in broilers (Elmusharaf et al., 2007). The anticoccidial properties of mannan oligosaccharides may result from its ability to improve gut integrity and uniformity due to lower colonization of harmful pathogens (Hooge, 2004, Huyghebaert et al., 2011).

Shanmugasundaram et al. (2013) previously studied the effect of whole yeast cell product supplementation post-coccidial challenge in broiler chickens and identified that whole yeast cell product supplementation can improve production parameters, lower fecal oocyst count and increase inflammatory cytokines production post-coccidial infection. Layer chickens have shown to differ from broilers in their immunological
responses to antigens (Koenen et al., 2001). The present experiment was conducted to study the effects of whole yeast cell wall product supplementation on layer production performance, fecal and intestinal oocyst count and CD4⁺ and CD8⁺ cell populations in the spleen and cecal tonsils following an experimental coccidial infection in unvaccinated layer chickens.
Materials and Methods

Coccidiosis Experiment

Two experiments were conducted to study the effects of whole yeast cell product supplementation on production parameters and intestinal immune parameters during an experimental coccidial infection model. All animal protocols were approved by the Institutional Animal Care and Use Committee at The Ohio State University.

Coccidiosis Experiment I

Birds

One day-old specific pathogen-free White Leghorn birds were randomly distributed to one of three dietary treatments with 0, 0.1 or 0.2 % whole yeast cell product (CitriStim, ADM, Quincy, IL). At 21 days of age, the birds were weighed individually to identify birds of similar sizes to be used in the coccidial challenge studies. Six birds per treatment (n = 6) were challenged with coccidiosis after 21 days of whole yeast cell product feeding. Immediately after coccidial challenge, birds were placed in individual battery cages (total of 36 battery cages).

Coccidial infection and production parameters post-coccidial infection

At 21 days of age, six birds from each treatment group were orally gavaged with 1 X 10⁵ live coccidial oocysts (Inovocox, Pfizer Animal Health, NY) in 200 µl PBS (Annamalai and Selvaraj, 2012). The birds were weighed individually at 0 and 9 d post-
coccidial challenge to analyze body weight gain of different treatment groups. Feed consumption was measured at 9 d post-coccidial challenge.

*Effect of whole yeast cell product supplementation on fecal coccidial oocyst count post-coccidial challenge*

Feces were collected daily from six battery cages per treatment group (n = 6) at six to nine days post-coccidial challenge. The fecal coccidial oocysts were counted using a salt flotation technique and analyzed for the total number of coccidial oocysts using a McMaster chamber as described previously (Annamalai and Selvaraj, 2012).

*Effect of whole yeast cell product supplementation on CD4+ and CD8+ cell populations in the cecal tonsils post-coccidial challenge*

CD4+ and CD8+ cell populations in cecal tonsils were analyzed. At nine days post-coccidial challenge, cecal tonsils were collected from six birds per treatment group (n = 6). Cells were collected from cecal tonsils using a 0.45 μm cell strainer (Fisher Scientific). Cells from each sample were placed into 5 ml of RPMI, centrifuged at 3000 RPM for 5 minutes, resuspended and centrifuged again at 3000 RPM for 5 minutes. Cells were then resuspended in wash buffer (1xPBS, 2mM EDTA, 1.5% FBS) at a concentration of 10⁶ cells per well. For the CD4+/CD8+ plate, 10⁶ cells were added to each well containing CD4⁺ (1:200 dilution) and CD8⁺ (1:450 dilution) and then incubated for 20 minutes at 4 °C. Plates were then centrifuged at 2000 RPM for 3 minutes. Cells were washed 3 times and
then analyzed by flow cytometry using cytosoft software (Guava Easycyte, Millipore, Billerica, MA) as described previously (Shanmugasundaram and Selvaraj, 2012).

_Coccidiosis Experiment II_

_Birds_

Specific pathogen-free 40-week-old layer hens were fed experimental diets with 0, 0.05 or 0.1% whole yeast cell product (Citristim, ADM, Quincy, IL). At 28 days of whole yeast cell product feeding, 15 birds per treatment were challenged with coccidiosis. The experimental design, animal husbandry, and coccidial challenge protocols were similar to Coccidiosis Experiment I.

_Effect of whole yeast cell product supplementation on intestinal content coccidial oocyst count post-coccidial challenge_

At 5, 12 and 28 days post-coccidial challenge, intestinal content were collected from five birds per treatment group (n = 5), enriched for coccidial oocysts using a salt flotation technique and analyzed for total number of coccidial oocysts using a McMaster chamber as described previously (Annamalai and Selvaraj, 2012).
Effect of whole yeast cell product supplementation on CD4+ and CD8+ cell populations in the spleen and cecal tonsils post-coccidial challenge

At 5, 12 and 28 d post-coccidial challenge, cecal tonsils were collected from five birds per treatment group (n = 5). The protocol for analysis of CD4+ and CD8+ cell populations were as described in Coccidial Experiment I.

Statistical analysis

A one-way ANOVA (JMP, SAS, Cary, NC) was used to examine the effect of whole yeast cell product supplementation on dependent variables. When main effects were significant (P <0.05), differences between means were analyzed by Tukey’s least square means comparison. Data was trimmed for outliers.
**Results**

*Coccidiosis Experiment I.*

*Effect of whole yeast product supplementation on production parameters pre- and post-coccidial challenge*

Supplementation with whole yeast cell product did not significantly increase the body weight gain between 0-21 d of age. Supplementation with whole yeast cell product numerically (P = .08) increased body weight gain by 32% between 0 and 9 days post-coccidial challenge (Fig. 1). Feed consumption was not significantly different between the experimental groups at 9 days post-coccidial challenge.

*Effect of whole yeast cell product supplementation on fecal oocyst count post-coccidial challenge*

Supplementation with whole yeast cell product decreased (P = 0.05) the fecal coccidial oocyst count at seven through eight days post-coccidial challenge (Fig. 2). Supplementation with whole yeast cell product numerically (P = 0.18) lowered fecal oocyst count by 75% at nine days post-coccidial challenge.

*Effect of whole yeast cell product supplementation on CD4+ and CD8+ cell populations the cecal tonsils*

Supplementation with whole yeast cell product did not alter (P > 0.05) CD4+ and CD8+ populations in the cecal tonsils at nine days post-coccidial infection.
Coccidiosis Experiment II.

Effect of whole yeast product supplementation on production parameters pre- and post-coccidial challenge

Supplementation with whole yeast cell product did not significantly increase body weight gain at 21 days of feeding. Body weight gain and feed consumption were not significantly different between the experimental groups at 5, 12 and 28 d post-coccidial challenge.

Effect of whole yeast cell product supplementation on intestinal content oocyst count post-coccidial challenge

Supplementation with whole yeast cell product decreased (P < 0.01) the intestinal content coccidial oocyst count at 5, 12 and 28 days post-coccidial challenge compared to the challenged birds supplemented with 0% whole yeast cell product supplementation (Fig. 3).

Effect of whole yeast cell product supplementation on CD4+ and CD8+ cell populations in the cecal tonsils

Supplementation with whole yeast cell product increased CD8+ cell populations (P < 0.05) at 5 days post-coccidial challenge (Fig. 4). Supplementation with whole yeast cell product increased CD4+ cell populations numerically (P = 0.13) at 5 days post-coccidial challenge (Fig. 4). Supplementation with whole yeast cell product did not alter (P > 0.05) CD4+ and CD8+ cell populations at 12 and 28 days post-coccidial challenge.
Discussion and Conclusion

This experiment studied the effects of whole yeast cell product supplementation on layer chicken production parameters, fecal and intestinal content coccidial oocyst count and immune parameters following an experimental coccidial infection in unvaccinated layer chickens. Whole yeast cell product supplementation improved the production performance, decreased the fecal and intestinal content oocyst count and increased CD8\(^+\) cell populations in the cecal tonsils.

The results of this experiment are similar to a study by Elmusharaf et al. (2007), in which day-old broilers were supplemented with a yeast cell wall product and challenged at eight days of age with *Eimeria tenella*. The study found that birds supplemented with yeast cell wall product had reduced oocyst excretion, indicating that the yeast cell wall may have anticoccidial characteristics (Elmusharaf et al., 2007). Anticoccidial effects due to mannan oligosaccharides within the yeast cell wall may be caused by increased villi length and improved integrity and uniformity of the gut, as seen in previous studies with chickens fed mannan oligosaccharide-supplemented diets (Hooge, 2004). Mannan oligosaccharide supplementation may counteract the symptoms of coccidial infection such as reduced weight gain and compromised intestinal wall (Mcdougald, 2003; Chapman et al., 2004).

The results of our experiment observed a significant increase in CD8\(^+\) cell populations and a numerical increase in CD4\(^+\) cell populations 5 days post-coccidial challenge. Studies have shown that T cells in the adaptive immune response are the
primary response to *Eimeria* (Lillehoj et al., 1998). CD8+ T intraepithelial lymphocytes (IELs) were detected in chickens after both a primary and secondary infection (Lillehoj and Bacon, 1991). The increase in CD8+ cells correlated with reduced oocyst excretion (Lillehoj and Bacon, 1991). Additionally, studies have shown that CD4+ IELs increased seven days after a primary infection and a higher number of CD8+ IELs were observed after a secondary infection, further suggesting that CD8+ IELs may contribute to adaptive immunity to *Eimeria* (Lillehoj and Trout, 1994).

The increase in CD4+ and CD8+ T cells in the cecal tonsils of the whole yeast cell product supplemented chickens may be due to both the ability of β-glucans and mannan oligosaccharides to enhance immunity (Brown and Gordon 2003; Ferket, 2002). β-glucans derived from yeast resembles pathogen-associated molecular patterns (PAMPs) that stimulate innate immunity (Brown & Gordon, 2003). Additionally some studies have shown that mannan oligosaccharides stimulate gut associated and systemic immunity by acting as a non-pathogenic microbial antigen, giving an adjuvant-like effect (Ferket et al., 2002).

It could be concluded that supplementing whole yeast cell products to layer diets can improve production parameters, decrease fecal oocyst count and increase CD8+ cell populations in the cecal tonsils post-coccidial infection.

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Chapter 4: Effects of Whole Yeast Cell Product Supplementation post-Salmonella Challenge

Abstract

This experiment studied the effects of whole yeast cell product supplementation on broiler production parameters, S. Enteritidis colony forming units from cecal content, relative proportions of Salmonella, Bifidobacteria and Lactobacillus within the cecal contents and immune parameters following an experimental S. Enteritidis infection. Day-old birds were fed experimental diets with 0, 0.1 or 0.2% whole yeast cell product. At 21 days of age, birds were challenged with 1x10⁹ CFU/ml S. Enteritidis. Supplementation with whole yeast cell product had no significant effect on body weight gain or feed consumption post-Salmonella challenge. Supplementation with whole yeast cell product increased (P < 0.05) relative proportion of Lactobacillus in the cecal content 7 days post-Salmonella challenge and did not significantly alter relative proportion of Bifidobacteria post-Salmonella challenge. Whole yeast cell product supplementation decreased (P < 0.05) CD4⁺ and CD8⁺ cell populations in the cecal tonsils 14 days post-Salmonella infection. Whole yeast cell product supplementation decreased (P < 0.05) villi lengths and crypt depths in the jejunum 7 days post-Salmonella challenge. It could be concluded that supplementing whole yeast cell product to broiler diets can significantly alter gut
microflora in the cecal contents, cytokine mRNA amounts and CD4⁺ and CD8⁺ cell populations in the cecal tonsils post-\textit{Salmonella} challenge.
Introduction

Whole yeast cell products are derived from several species of yeast such as Saccharomyces cerevisiae or Pichia guilliermondii. Whole yeast cell products are rich in mannan oligosaccharides, β-glucan, D-mannose, alpha methyl-D-mannoside and several other compounds (Shanmugasundaram and Selvaraj, 2012). The polysaccharides mannan oligosaccharide and β-glucan have immunomodulatory effects when supplemented either individually or as a mixture with other compounds as immune stimulators in several species including poultry (Shanmugasundaram et al., 2013).

Mannan oligosaccharides have the ability to agglutinate gram-negative pathogenic bacteria containing type-2 fimbriae (including Salmonella and E. Coli), resulting in the pathogenic bacteria attaching to the mannan oligosaccharide product and moving out of the intestine as opposed to adhering to the host’s intestinal epithelial cells (Badia et al., 2013). β-glucans derived from yeast resemble pathogen-associated molecular patterns (PAMPs) that when recognized by the innate immune system can trigger a cascade reaction that results in an increase in inflammatory cytokines such as IL-1β and TNF-α (Brown and Gordon, 2003).

Salmonella enterica serotype Enteritidis is one of the most commonly isolated nontyphoidal serotypes of Salmonella (Fabrega and Vila, 2013). S. Enteritidis rarely causes clinical disease in chickens, but can colonize the gut of poultry, eventually affecting the poultry plant facility and the final poultry product in the customer’s home (Barrow et al., 1987; Humphrey, 1988). Approximately 40,000 cases of Salmonella-caused disease salmonellosis are reported in the United States (Fabrega and Vila, 2013).
Acute salmonellosis in humans causes 400 deaths a year in the United States (Fabrega and Vila 2013). *Salmonella* can be spread by feces or infected intestinal contents spilling and contaminating the meat (Humphrey, 1988). Because most *Salmonella* vaccines do not completely clear *S. Enteritidis* colonization in chickens, the commercial poultry production industry attempts to control *S. Enteritidis* infections by supplementing vaccination programs with probiotics or prebiotics (Davies and Breslin, 2003; Patterson and Burkholder, 2003). Additionally, prebiotics may serve as a substitute for antibiotics, as antibiotic use is decreasing due to concern of antibiotic resistance (Gustafason and Bowen, 1997).

Fernandez et al. (2002) observed that day-old broiler chicks supplemented with 2.5% mannose-oligosaccharide and challenged at one week of age with *S. Enteritidis* had lower *S. Enteritidis* caecal colonization compared to birds fed the control diet at two weeks post-challenge. In broilers supplemented with whole yeast cell product and challenged with coccidiosis, increased anti-inflammatory cytokines and decreased inflammatory cytokines in the cecal tonsils post-coccidial infection were observed (Shanmugasundarm and Selvaraj, 2012a). The present experiment was conducted to study the effects of whole yeast cell product supplementation on broiler production performance, jejunum villi length and crypt depth, *S. Enteritidis* colony forming units, relative proportions of gut microflora in the cecal content, inflammatory and anti-inflammatory cytokine expression in the cecal tonsils, and CD4+ and CD8+ cell populations in the cecal tonsils following an experimental *S. Enteritidis* infection in unvaccinated broiler chickens.
Materials and Methods

Salmonella Experiment

An experiment was conducted to study the effects of whole yeast cell product supplementation on production parameters and intestinal immune parameters during an experimental Salmonella infection model. All animal protocols were approved by the Institutional Animal Care and Use Committee at The Ohio State University.

Birds

Day-old specific pathogen-free broiler chickens were provided (Gerber Farms, Kidron, OH). All birds were given ad libitum water and feed, housed in battery cages and raised using standard animal husbandry practices. All animal protocols were approved by the Institutional Animal Care and Use Committee at The Ohio State University. 72 birds were individually weighed, wing banded, and distributed evenly into 18 battery cages. Birds were assigned one of three diets: 0, 0.1 or 0.2% whole yeast cell product (n = 6 battery cages). At 21 days of age, 24 birds per diet group were challenged with S. Enteritidis. Birds that received the challenge were placed in a separate room to avoid contamination of control group. For both rooms, birds were placed in pairs into a total of 18 separate cages and continued their previous assigned diets of 0, 0.1 or 0.2% whole yeast cell product supplementation (n = 6).
Salmonella infection

At 21 days of age, 24 birds from each treatment group were orally challenged with 150 ul of 1x10⁹ CFU/ml *S*. Enteriditis to induce a *Salmonella* infection. Birds were previously tested negative for *Salmonella* colonization by cloacal swab sampling. The birds were weighed 0, 7 and 14 days post-*Salmonella* challenge to analyze body weight gain of different treatment groups. Feed consumption was measured at 7 and 14 days post-*Salmonella* challenge.

Effect of whole yeast cell product on *Salmonella* colonization formation units in cecal contents post-*Salmonella* challenge

Culture plates with XLD agar were prepared on the day of collection. During sample collection, cecal contents were placed in a separate plastic bag containing two ml of peptone water. Samples were brought to the lab directly after the collection. Each cecal content sample was massaged in the plastic bag to spread bacteria around as much as possible. Serial dilutions were done for each sample. The dilutions 10⁰, 10¹, 10², and 10⁴ were plated for each sample (100 ul of each solution were put onto each plate). Plates were then incubated at 37 °C for 24 hours. At 24 hours, colonies were counted (Desmidt et al., 1998).

Effect of whole yeast cell product supplementation gut microflora post-*Salmonella* challenge

At 7 and 14 days post-*Salmonella* challenge, cecal content was collected from six birds per treatment (n = 6). Cecal contents (0.2 g) were diluted in 10 mL of sterile PBS
and centrifuged at 14000 RPM for two minutes to remove debris. The pellet was then resuspended in EDTA and treated with lysozyme (20 mg/ml) for 45 minutes at 37°C. After incubation, samples were centrifuged at 14000 RPM for two minutes and supernatant discarded. Samples were then treated with lysis buffer and Proteinase K (20mg/ml) for five minutes at 80°C. 6M NaCl and isopropanol were added to the cell lysate and centrifuged at 14000 RPM for two minutes. DNA pellets were resuspended and washed in 70% ethanol and then resuspended in TE buffer. Extracted DNA samples were stored at -20°C (Amit-Romach et al., 2004). Cecal microflora was analyzed by real-time PCR as described earlier (Selvaraj et al., 2010). Bacterial primers are described in Table 1.

*Effect of whole yeast cell product supplementation on CD4*, *CD8* and *CD4*CD25* cell populations in the cecal tonsils post-Salmonella challenge*

Regulatory T cells, CD4* and CD8* populations of cecal tonsils were analyzed. Cecal tonsils were stored in RPMI + Penicillin-Streptomycin at 4°C overnight. CD4* and CD8* cells were prepared as described in the Coccidiosis Experiment. To analyze regulatory T cells, 10⁶ cells were added to each well containing CD25* antibodies (1 μg per well) and then incubated for 40 minutes at 4 °C. Plates were then centrifuged at 2000 RPM for three minutes. Cells were washed three times, then analyzed by flow cytometry. Protocol for analysis of CD4*, CD8* and CD25* cells using flow cytometry were as described in the Coccidiosis Experiment.
Effect of whole yeast cell product supplementation on IL-1β, IL-10, TNF-α and IL-6 mRNA expression in the cecal tonsils post-Salmonella challenge

At 7 and 14 days post-Salmonella challenge, total RNA was collected from cecal tonsils (n = 6). RNA was then reverse transcribed into cDNA (Selvaraj and Klasing, 2006). The mRNA was analyzed for IL-1β, IL-10, IL-6 and TNF-α by real-time PCR (iCycler, BioRad, Hercules, CA) using SyBr green after normalizing for β-actin mRNA. Primers are described in Table 2. The annealing temperature for IL-10 was 57.5°C. The annealing temperature for IL-1β, TNF-α and β-actin was 57°C. Fold change from the reference was calculated using the comparative Ct method so that \(2^{(Ct\ Sample - Housekeeping)}\)/\(2^{(Ct\ Reference - Housekeeping)}\), where Ct is the threshold cycle (Schmittgen and Livak, 2008). The Ct was determined by iQ5 software (Biorad) when the fluorescence rises exponentially 2-fold above the background.

Effect of whole yeast cell product supplementation on jejunum villi length and crypt depth post-Salmonella challenge

Six jejunum samples per treatment group were collected (cut from the end of the duodenal loop and before the Meckel’s diverticulum) and stored in 10% formalin at 7 and 14 d post-Salmonella challenge. Samples were dehydrated at room temperature in a graded series of alcohols (15 min in 50% ethanol, 15 min in 70% ethanol, 15 min in 96% ethanol, 30 min in 100% ethanol with one change at 15 min), cleared in Pro-par (Anatech, Battle Creek, MI) for 45 min with two changes at 15 and 30 min and infiltrated with paraffin at 60 °C overnight with one change at 15 min using a Leica TP 1020 tissue
processor (GMI Inc., Ramsey, MN). Paraffin blocks were cut into 5 μm cross-sections and mounted on frosted slides. Slides were then stained with hematoxylin and eosin (Velleman et al., 1998). Cross sections were viewed using CellSense Imaging software (Olympus America, Central Valley, PA) to measure villi length and crypt depth. Five villi and crypts per section and five sections per sample were analyzed (total of 25 villi and crypts per bird).

Statistical analysis

A one-way ANOVA (JMP, SAS, Cary, NC) was used to examine the effect of CitriStim supplementation on dependent variables. When main effects were significant ($P < 0.05$), differences between means were analyzed by Tukey’s least square means comparison. Data was trimmed for outliers.
Results

Effect of whole yeast cell product supplementation on production parameters pre- and post-Salmonella challenge

Supplementation of whole yeast cell product did not significantly increase the body weight gain between 0-21 days of age. Supplementation of whole yeast cell product did not significantly increase body weight gain or feed consumption 7 or 14 days post-Salmonella challenge.

Effect of whole yeast cell product on Salmonella colonization formation units in cecal content post-Salmonella challenge

Supplementation of whole yeast cell product did not significantly alter Salmonella colonization formation units in the cecal contents post-Salmonella challenge.

Effect of whole yeast cell product supplementation on gut microflora in the cecal content post-Salmonella challenge

In the absence of a Salmonella infection, supplementation with whole yeast cell product decreased relative proportion of Lactobacillus (P < 0.05) compared to the control (Fig. 5). Supplementation with 0.2% whole yeast cell product increased relative proportion of Lactobacillus (P < 0.05) compared to the control group supplemented with 0% whole yeast cell product at 7 days post-Salmonella challenge (Fig. 5). Supplementation with whole yeast cell product did not alter relative proportion of Lactobacillus (P > 0.5) compared to the control at 14 days post-Salmonella challenge. Supplementation with whole yeast cell product did not alter relative proportion of
**Bifidobacteria** (P > 0.5) compared to the control at 7 and 14 days post-**Salmonella** challenge. Supplementation with whole yeast cell product numerically increased (P = 0.11) relative proportion of *S*. Enteritidis compared to the control at 7 days post-**Salmonella** challenge (Fig. 6).

*Effect of whole yeast cell product supplementation on CD4+, CD8+, and CD4+CD25+ cell populations in the cecal tonsils*

In the absence of a *Salmonella* infection, supplementation with whole yeast cell product decreased (P < 0.5) CD4+ and CD8+ populations compared to the control at 7 days post-**Salmonella** challenge (Fig. 7). In the absence of a *Salmonella* infection, supplementation with 0.1% whole yeast cell product increased CD4+ populations compared to the control at 14 days post-**Salmonella** challenge (Fig. 8). Supplementation with whole yeast cell product did not alter (P > 0.05) CD8+ cell populations at 14 days post-**Salmonella** challenge. Supplementation with whole yeast cell product numerically (P = 0.08) increased CD4+CD25+ cell populations at 14 days post-**Salmonella** challenge compared to both the control and the challenged group supplemented with 0% whole yeast cell product (Fig. 9).
Effect of whole yeast cell product supplementation on IL-1β, IL-10, TNF-α and IL-6 mRNA amounts in the cecal tonsils post-Salmonella challenge

Supplementation with whole yeast cell product did not alter (P > 0.05) IL-1β mRNA amounts 7 days post-Salmonella challenge. In the absence of a Salmonella infection, 0.2% whole yeast cell product supplementation decreased (P < 0.05) IL-1β mRNA amounts in the cecal tonsils at 14 days post-Salmonella challenge (Fig. 10). Supplementation with 0.1% whole yeast cell product increased (P < 0.05) IL-1β mRNA amounts in the cecal tonsils compared to both the control and the challenged group supplemented with 0% whole yeast cell product at 14 days post-Salmonella challenge (Fig. 10) In the absence of a Salmonella infection, whole yeast cell product supplementation increased (P < 0.05) IL-10 mRNA amounts in the cecal tonsils compared to the control at 7 days post-Salmonella infection (Fig. 11). Whole yeast cell product supplementation increased (P < 0.05) IL-10 mRNA amounts in the cecal tonsils compared to the control at 7 days post-Salmonella infection (Fig. 11). In the absence of a Salmonella infection, whole yeast cell product supplementation increased (P < 0.05) IL-10 mRNA amounts in the cecal tonsils compared to the control at 14 days post-Salmonella infection (Fig. 11). Whole yeast cell product supplementation decreased (P < 0.05) IL-10 mRNA amounts in the cecal tonsils compared to the challenged group supplemented with 0% whole yeast cell product at 14 days post-Salmonella infection (Fig. 11). In the absence of a Salmonella infection, 0.2% whole yeast cell product supplementation increased (P < 0.05) TNF-α mRNA amounts in the cecal tonsils compared to the control at 7 days post-Salmonella challenge (Fig. 12). Supplementation
with 0.2% whole yeast cell product decreased (P < 0.05) TNF-α mRNA amounts in the cecal tonsils compared to both the control and the challenged group supplemented with 0% whole yeast cell product at 7 days post-*Salmonella* challenge (Fig. 12). In the absence of a *Salmonella* infection, whole yeast cell product supplementation increased (P < 0.05) IL-6 mRNA amounts in the cecal tonsils compared to the control at 7 and 14 days post-*Salmonella* challenge (Fig. 13).

*Effect of whole yeast cell product supplementation on jejunum villi length and crypt depth post-Salmonella challenge*

In the absence of a *Salmonella* infection, supplementation with 0.2% whole yeast cell product increased (P < 0.05) jejunum villi length at 7 days post-*Salmonella* challenge compared to the control (Fig. 14). Supplementation with whole yeast cell product decreased (P < 0.05) jejunum villi length compared to both the control and the control group supplemented with 0% whole yeast cell product at 7 days post-*Salmonella* (Fig. 14). In the absence of a *Salmonella* infection, 0.1% whole yeast cell product supplementation increased (P < 0.05) jejunum crypt depth compared to the control (Fig. 15). Supplementation with whole yeast cell product decreased (P < 0.05) jejunum crypt depth compared to both the control and the control group supplemented with 0% whole yeast product at 7 days post-*Salmonella* challenge (Fig. 15). Supplementation with whole yeast cell product did not alter jejunum villi length or crypt depth at 14 days post-*Salmonella* challenge (P > 0.05).
Discussion and Conclusion

This experiment studied the effects of whole yeast cell product supplementation on production parameters, gut microflora and immune parameters following an experimental *Salmonella* infection in unvaccinated broiler chickens. In the absence of infection, whole yeast cell product supplementation increased villi lengths and crypt depths in the jejunum, increased IL-10, TNF-α and IL-6 and lowered IL-1β mRNA amounts in the cecal tonsils compared to the control. Whole yeast cell product supplementation decreased villi lengths and crypt depths in the jejunum, increased IL-1β compared to the challenged group supplemented with 0% whole yeast cell product, and decreased TNF-α, IL-6, and IL-10 mRNA amounts compared to the challenged group supplemented with 0% whole yeast cell product. Whole yeast cell product supplementation increased *Lactobacillus post-Salmonella* infection. In the absence of infection, whole yeast cell product supplementation decreased CD4+ and CD8+ cell populations in the cecal tonsils compared to the control. Supplementation with whole yeast cell product did not significantly alter CD4+ or CD8+ cell percentage in the cecal tonsils compared to the challenged group supplemented with 0% whole yeast cell product 7 days post-*Salmonella* challenge.

Broilers challenged with *S. Enteritidis* can result in lower weight gain due to a compromised intestinal wall (Gorham et al., 1994; Dhillon et al., 1999). Prebiotics have had varied results on body weight gain in broilers. Markovic et al. (2009) observed that day-old broilers supplemented with yeast cell wall product for 42 days had higher body weight gain than unsupplemented broilers. Bozkurt et al. (2012) observed similar results
with laying hens supplemented with mannan oligosaccharides. Smith et al. (2010) supplemented mice with mannan oligosaccharides for 12 weeks and did not see significant differences in body weight gain. In a study by Midilli et al. (2008), day-old broilers supplemented for 42 days with 0.1% yeast cell wall product did not significantly increase in feed intake or body weight gain.

Commensal bacteria within the gut, such as *Lactobacillus* and *Bifidobacteria*, protect the host from colonization of pathogenic bacteria such as *Salmonella* through several mechanisms. These include competing for epithelial binding sites and nutrients, strengthening the intestinal immune response and producing antimicrobial bacteriocins (Guarner and Malagelada, 2003). Prebiotics act as a source of nutrients for commensal bacteria, therefore reducing pathogen colonization within the gut (Patterson and Burkholder, 2003). Additionally, the fermentation of prebiotics by intestinal microbes produces volatile fatty acids, which promote epithelial cell proliferation, enhancing the integrity of the intestinal wall (Macfarlane et al., 2008). Mannan oligosaccharides have the ability to agglutinate gram-negative bacteria containing type-1 fimbriae (such as *Salmonella*), resulting in the pathogens adsorbing to the mannan oligosaccharide product and moving out of the intestine as opposed to adhering to the host’s intestinal wall (Badia et al., 2013). However in the current study, whole yeast cell product supplementation numerically increased relative proportions of *S. Enteritidis* in the cecal contents at 7 days post-challenge. Whole yeast cell products increases IL-10 and decreases IL-1β mRNA amounts in the cecal tonsils in the absence of an infection (Shanmugasundaram and Selvaraj, 2012). Increased levels of IL-10 and decreased levels of IL-1β present in the
cel tonsils during the 21 days of whole yeast cell product feeding prior to the 
Salmonella infection may have contributed to the increase of S. Enteritidis in the cecal 
contents at 7 days post-challenge.

Chicken cell cultures infected with S. Enteritidis showed an eight to tenfold 
increased in IL-6 mRNA expression, concluding that S. Enteritidis results in a strong 
inflammatory response (Kaiser et al., 2000). The study also showed a slight down-
regulation of IL-1β during the Salmonella infection. This is interesting, since IL-1β is a 
pro-inflammatory cytokine that may act to inhibit Salmonella crossing the intestinal 
epithelium (Kaiser et al., 2000). In study by Setta et al. (2012), chickens infected with S. 
Enteritidis were associated with an increase in macrophage percentage and an 
upregulation of TNF-α and IL-10 in the cecal tonsils. S. Enteritidis results in an increase 
in CD4⁺ cells in the cecal tonsils 4-6 days post-infection in chickens (Setta et al., 2012). 
In addition, CD4⁺ and CD8⁺ cells have been observed to proliferate in the reproductive 
tract of S. Enteritidis infected layer hens (Withanage et al., 1998). The current experiment 
observed an increased inflammation in challenged birds supplemented with whole yeast 
cell product at 14 days post-challenge. β-glucans and mannoligosaccharides can 
enhance immunity and increase proinflammatory cytokines to the site of infection 
(Brown and Gordon 2003; Ferket, 2002).

The current experiment observed that whole yeast cell product supplementation 
increased villi lengths in the absence of an infection. In the challenged birds, whole yeast 
cell product supplementation decreased villi lengths and crypt depths in infected birds at 
7 days post-Salmonella challenge. The current study additionally showed as increase in
relative proportions of *S*. Enteritidis in the cecal tonsils of the challenged birds supplemented with whole yeast cell products, which may correlate to the shorter villi lengths and crypt depths that were observed. Broiler chicks hatched from eggs inoculated with *S*. Enteritidis had a decrease in villi length and an increase in crypt depth in the duodenum and ileum during early infection with *S*. Enteritidis (Andrade et al., 2013).

High crypt depth is related to the turnover of epithelial cells, which may be due to compensation for cell destruction during an infection (Rose et al., 1992). However, lower crypt depths in birds infected with *Salmonella* has also been observed (Rieger et al., 2014). This may be due to the stress of *Salmonella* decreasing the absorptive epithelium of the intestine, resulting in both the reduction of villus height and crypt depth (Yamauchi et al., 1996). Whole yeast cell product supplementation in unchallenged birds has lead to in an increase in villi length and decrease in crypt depth in several studies (Morales-Lopez et al., 2009; Shanmugasundaram et al., 2012; M’Sadeq et al., 2015).

It could be concluded that supplementing whole yeast cell products to broiler diets can improve immune parameters and alter gut microflora post-coccidial infection.

**Acknowledgements**

Animal Husbandry help from K. Patterson and J. Sidle and technical help from R. Shanmugasundaram are acknowledged.
Chapter 5: Conclusions

Whole yeast cell products are derived from yeast species such as “Saccharomyces cerevisiae” and “Pichia guillermondii” (Shanmugasundaram and Selvaraj, 2012; Sohail et al., 2012). Whole yeast cell products contains mannan oligosaccharides, β-glucan, D-mannose, alpha methyl-D-mannoside and several other compounds (Shanmugasundaram and Selvaraj, 2012). Mannan oligosaccharides and β-glucans have immunomodulatory effects in several species including poultry (Shanmugasundaram et al., 2013). Mannan oligosaccharides can agglutinate gram-negative pathogenic bacteria containing type-1 fimbriae (including Salmonella and E. Coli), resulting in the pathogenic bacteria attaching to mannan oligosaccharides and moving out of the intestine as opposed to adhering to the host’s intestinal epithelial cells (Badia et al., 2013). β-glucans derived from yeast resembles pathogen-associated molecular patterns (PAMPs) that when recognized by the innate immune system triggers a cascade reaction that results in an increase in proinflammatory cytokines (Brown and Gordon, 2003). The immunomodulatory effects of whole yeast cell products have important implications in the poultry industry, especially as anticoccidial and antibiotic use decreases due to the public’s concern of antibiotic and drug resistance (Gustafson and Bowen, 1997).
Coccidiosis is a major disease in poultry caused by protozoan parasites of the genus *Eimeria* (Dalloul and Lillehoj, 2006). *Eimeria* parasites produce lesions that destroy the intestinal epithelia, thereby reducing feed efficiency and body weight gain. This leads to severe economic losses in the poultry industry (Paris and Wong, 2013). Coccidiosis causes the poultry industry a loss of about $800 million a year in the United States (Sharmen et al., 2010). Anticoccidial drugs are commonly used in the poultry industry; however *Eimeria* resistance has developed to all of the anticoccidial drugs that have been introduced (Allen et al., 1997; Peek and Landman, 2011).

Salmonellosis is a disease caused by the gram-negative bacteria, *Salmonella*, with symptoms ranging from diarrhea and fever to life threatening systemic illness (Giannella, 1979). *Salmonella enterica* serotype Enteritidis is one of the most commonly isolated nontyphoidal serotypes of *Salmonella* (Fabrega and Vila, 2013). Although *S. Enteritidis* does not commonly cause clinical disease in chickens, the bacteria can colonize the gut of poultry, which can then affect the poultry plant facility and the final poultry product in the customer’s home (Barrow et al., 1987; Humphrey, 1988). Approximately 40,000 cases of salmonellosis are reported in the United States annually (Fabrega & Vila, 2013). Acute salmonellosis in humans causes 400 deaths a year in the United States (Fabrega & Vila 2013).

The objectives of this thesis were to

1. Study the effects of whole yeast cell product supplementation in layer chickens post-coccidial challenge
(2) Study the effects of whole yeast cell product supplementation in broiler chickens post-\textit{Salmonella} challenge

The study presented in chapter 3 identified production parameters, relative T cell populations, and coccidial oocyst count in layer pullets and hens supplemented with whole yeast cell product and challenged with coccidiosis. The objectives of the study were to evaluate the effects of whole yeast cell product supplementation post-coccidial challenge on, (1) body weight gain and feed consumption, (2) CD4$^+$ and CD8$^+$ cell populations in the cecal tonsils and spleen and (3) coccidial oocyst count within the fecal and intestinal content.

Whole yeast cell product supplementation numerically increased body weight gain in layer pullets 9 days post-coccidial challenge. Whole yeast did not alter feed consumption. Whole yeast product supplementation did not have an effect on body weight gain or feed consumption post-coccidial challenge in layer hens.

In layer pullets, whole yeast cell product supplementation lowered the fecal coccidial oocyst count at 7, 8 and 9 days post-coccidial challenge. In layer hens, whole yeast cell product supplementation lowered intestinal content coccidial oocyst count at 5, 12 and 28 days post-coccidial challenge.

In layer pullets, whole yeast cell product supplementation did not alter CD4$^+$ and CD8$^+$ populations in the cecal tonsils at 9 days post-coccidial infection. In layer hens, whole yeast product supplementation increased CD4$^+$ and CD8$^+$ populations in the cecal tonsils 5 days post-coccidial challenge.
The study presented in chapter 4 identified production parameters, gut microflora, relative T cell populations, and cytokine levels in broiler chickens supplemented with whole yeast cell product and challenged with *Salmonella*. The objectives of the study were to evaluate the effects of whole yeast cell product supplementation post-*Salmonella* challenge on, (1) body weight gain and feed consumption, (2) *Salmonella* colony formation units in the cecal contents, (3) relative proportions of *Lactobacillus*, *Bifidobacteria* and *Salmonella* in the cecal contents, (4) regulatory T cells, CD4+ and CD8+ cell populations in the cecal tonsils, (5) inflammatory and anti-inflammatory cytokine expression in the cecal tonsils and (6) jejunum villi length and crypt depth.

Supplementation of whole yeast cell product did not significantly increase body weight gain or feed consumption post-*Salmonella* challenge. In the absence of infection, whole yeast cell product supplementation increased villi lengths and crypt depths in the jejunum. Whole yeast cell product supplementation decreased villi lengths and crypt depths in the jejunum 7 days post-*Salmonella* challenge. Whole yeast cell product supplementation did not significantly alter villi lengths or crypt depths at 14 days post-*Salmonella* challenge.

Supplementation of whole yeast cell product increased IL-10, TNF-α and IL-6 and lowered IL-1β mRNA amounts in the cecal tonsils in the absence of a *Salmonella* infection. Whole yeast product supplementation decreased IL-10 and TNF-α in birds challenged with *Salmonella*. Whole yeast product supplementation increased IL-1β mRNA amounts in the cecal tonsils post-*Salmonella* challenge. Whole yeast cell product supplementation decreased CD4+ and CD8+ cell populations in the cecal tonsils 14 days
post-Salmonella infection. Supplementation of whole yeast cell product increased relative proportion of S. Enteritidis 7 and 14 days post-Salmonella challenge. Supplementation of whole yeast cell product increased Lactobacillus in the cecal contents 7 days post-Salmonella challenge.

In conclusion whole yeast cell product supplementation,

1. Improved body weight gain in layer pullets post-coccidial challenge
2. Lowered fecal and intestinal content oocyst count in layer chickens post-coccidial challenge
3. Lowered CD8+ and CD4+ cell populations in the cecal tonsils post-coccidial challenge
4. Increased Lactobacillus post-Salmonella infection
5. Decreased CD4+ and CD8+ cell populations in the cecal tonsils post-Salmonella infection
6. Increased IL-10, TNF-α and IL-6 and lowered IL-1β mRNA amounts in the cecal tonsils in the absence of a Salmonella infection, while decreasing IL-10, TNF-α and increasing IL-1β expression in the cecal tonsils post-Salmonella challenge
7. Increased villi lengths and crypt depths in the jejunum in the absence of a Salmonella infection and decreased villi lengths and crypt depths post-Salmonella challenge
References


Appendix A: Tables

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<tr>
<th>Bacterial Group</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
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<tbody>
<tr>
<td><em>Universal</em></td>
<td>Uni-f</td>
<td>CGTGCAGCCGCTGCTTAATACG</td>
</tr>
<tr>
<td></td>
<td>Uni-R</td>
<td>GGGTTGCGGCTGCTGCGGGACTTAACCAACAT</td>
</tr>
<tr>
<td><em>Lactobacillus</em></td>
<td>LAA-F</td>
<td>CATCCAGTGCAACCTAAGAG</td>
</tr>
<tr>
<td></td>
<td>LAA-R</td>
<td>GATCCGCTTGCCTCGCA</td>
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<tr>
<td><em>Bifidobacterium</em></td>
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<td>GGGTGGTAATGCGGATG</td>
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<tr>
<td></td>
<td>Bif662-r</td>
<td>CCACCGTTACCCGGAAG</td>
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<tr>
<td><em>Salmonella</em></td>
<td>Sal201-f</td>
<td>CGGGCCTGTGGCACCATCAGGTG</td>
</tr>
<tr>
<td></td>
<td>Sal597-r</td>
<td>CACATCCGACTTGACAGACCG</td>
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Table 1. Bacterial primers
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<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
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<tr>
<td>IL-1β-F</td>
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<td>IL-10-R</td>
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<td>IL-6-F</td>
<td>CGCGGATCCCCGCTGCCCGCCGCGCCGCG</td>
</tr>
<tr>
<td>IL-6-R</td>
<td>CCCAAGCTCTTCAGGGGACTGAACTCCT</td>
</tr>
<tr>
<td>TNF-α-F</td>
<td>TGTGTATGTGCAGCAACCGGTAGT                                                       (Hong et al., 2006)</td>
</tr>
<tr>
<td>TNF-α-R</td>
<td>GGCATTGCAATTTTGGACAGAAGT</td>
</tr>
<tr>
<td>β-actin-F</td>
<td>ACCGGACTGTACACCCACC                                                          (Shanmugasundaram and Selvaraj, 2010)</td>
</tr>
<tr>
<td>β-actin-R</td>
<td>GACTGCTGCTGACACCTTCA</td>
</tr>
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**Table 2.** Cytokine primers
Figure 1. Effect of whole yeast cell product supplementation on body weight gain at 9 d post-coccidial challenge. Day-old layer birds were fed 0, 0.1 or 0.2 % whole yeast cell product. At 21 d of age, birds were challenged with 0 (control) or $1 \times 10^5$ live coccial oocysts (Cocci). n = 6. P values Yeast cell $P < 0.05$, Cocci $P = 0.09$, Yeast cell X Cocci $P = 0.09$. 
Figure 2. Effect of whole yeast cell product supplementation on fecal oocyst count at 7, 8 and 9 d post-coccidial challenge. Day-old layer chicks were fed 0, 0.1 or 0.2% whole yeast cell product. At 21 d of age, birds were challenged with 0 (control) or $1 \times 10^5$ live coccidial oocysts (Cocci). Feces were collected daily from six battery cages per treatment group ($n = 6$) at 7 through 9 d post-coccidial challenge. Bars (± SEM) without a common superscript differ significantly within a day ($P < 0.05$). $n = 6$. $P$ values, 7 d: Yeast cell $P = 0.05$, Cocci $P < 0.01$, Yeast cell X Cocci $P = 0.05$, 8 d: Yeast cell $P < 0.01$, Cocci $P < 0.01$, Yeast cell X Cocci $P < 0.01$, 9 d: Yeast cell $P < 0.18$, Cocci $P < 0.01$, Yeast cell X Cocci $P = 0.18$. 
Figure 3. Effect of whole yeast cell product supplementation on intestinal content oocyst count at 5, 12 and 28 d post-coccidial challenge. 40-week-old layer hens were fed 0, 0.05 or 0.1% whole yeast cell product. At 21 d of whole yeast cell product feeding, birds were challenged with 0 (control) or $1 \times 10^5$ live coccidial oocysts (Cocci). Intestinal content were collected at 5, 12 and 28 d post-coccidial challenge from five battery cages per treatment group ($n = 5$). Bars ($\pm$ SEM) without a common superscript differ significantly within a day ($P < 0.05$). $n = 5$. $P$ values, 5 d: Yeast cell $P < 0.01$, Cocci $P < 0.01$, Yeast cell X Cocci $P < 0.01$, 12 d: Yeast cell $P < 0.01$, Cocci $P < 0.01$, Yeast cell X Cocci $P < 0.01$, 28 d: Yeast cell $P < 0.01$, Cocci $P < 0.01$, Yeast cell X Cocci $P < 0.01$. 
Figure 4. Effect of whole yeast cell product supplementation on CD4+ and CD8+ cell populations in the cecal tonsils at 5 d post-coccidial challenge. 40-week-old layer hens were fed 0, 0.05 or 0.1% whole yeast cell product. At 21 d of whole yeast cell product feeding, birds were challenged with 0 (control) or $1 \times 10^5$ live coccial oocysts (Cocci). Cecal tonsils were processed for isolating lymphocytes by density centrifugation and incubated with a fluorescent-conjugated anti-chicken CD4 and CD8 mAb and analyzed using flow cytometry. Bars (± SEM) without a common superscript differ significantly within a day ($P < 0.05$). $n = 5$. $P$ values, CD4+: Yeast cell $P = 0.30$, Cocci $P < 0.01$, Yeast cell X Cocci $P = 0.13$; CD8+: Yeast cell $P = 0.02$, Cocci $P < 0.01$, Yeast cell X Cocci $P < 0.01$. 

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Figure 5. Effect of whole yeast cell product supplementation on relative proportion of *Lactobacillus* in the cecal content at 7 d post-*Salmonella* challenge. Day-old broilers were fed 0, 0.1 or 0.2% whole yeast cell product. At 21 d of age, birds were challenged with 0 (control) or $1 \times 10^9$ CFU/ml *S. Enteritidis* (*Salmonella*). The relative proportion of *Lactobacillus* in the cecal content was measured by real-time PCR after normalizing to the total DNA content of the cecal content. The proportion of *Lactobacillus* is presented where the total of the examined bacteria was set at 100%. Bars (± SEM) without a common superscript differ significantly within a day ($P < 0.05$). n = 6. $P$ values Yeast cell $P = 0.48$, *Salmonella* $P = 0.01$, Yeast cell X *Salmonella* $P < 0.05$. 

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Figure 6. Effect of whole yeast cell product supplementation on relative proportion of S. Enteritidis in the cecal contents at 7 and 14 d post-Salmonella challenge. Day-old broilers were fed 0, 0.1 or 0.2% whole yeast cell product. At 21 d of age, birds were challenged with 0 (control) or of 1x10⁹ CFU/ml S. Enteritidis (Salmonella). The relative proportion of S. Enteritidis in the cecal content was measured by real-time PCR after normalizing to the total DNA content of the cecal content. The proportion of S. Enteritidis is presented where the total of the examined bacteria was set at 100%. n = 6. P values, 7 d: Yeast cell \( P = 0.11 \), Salmonella \( P = 0.04 \), Yeast cell X Salmonella \( P = 0.11 \), 14 d: Yeast cell \( P = 0.28 \), Salmonella \( P = 0.05 \), Yeast cell X Salmonella \( P = 0.28 \).
Figure 7. Effect of whole yeast cell product supplementation on CD4+ and CD8+ cell populations in the cecal tonsils at 7 d post-<em>Salmonella</em> challenge. Day-old broilers were fed 0, 0.1 or 0.2% whole yeast cell product. At 21 d of age, birds were challenged with 0 (control) or 1x10⁹ CFU/ml <em>S. Enteritidis</em> (<em>Salmonella</em>). Cecal tonsils were processed for isolating lymphocytes by density centrifugation and incubated with a fluorescent-conjugated anti-chicken CD4 and CD8 mAb and analyzed using flow cytometry. Bars (± SEM) without a common superscript differ significantly within a day (P < 0.05). n = 6. P values, CD4+: Yeast cell P < 0.01, <em>Salmonella</em> P < 0.01, Yeast cell X <em>Salmonella</em> P = 0.01, CD8+: Yeast cell P < 0.01, <em>Salmonella</em> P < 0.01, Yeast cell X <em>Salmonella</em> P = 0.01.
Figure 8. Effect of whole yeast cell product supplementation on CD4⁺ and CD8⁺ cell populations in the cecal tonsils at 14 d post-*Salmonella* challenge. Day-old broilers were fed 0, 0.1 or 0.2% whole yeast cell product. At 21 d of age, birds were challenged with 0 (control) or of 1x10⁹ CFU/ml *S. Enteritidis* (*Salmonella*). Cecal tonsils were processed for isolating lymphocytes by density centrifugation and incubated with a fluorescent-conjugated anti-chicken CD4 and CD8 mAb and analyzed using flow cytometry. Bars (± SEM) without a common superscript differ significantly within a day (*P* < 0.05). *n* = 6. *P*
values, CD4+ : Yeast cell $P = 9.48$, Salmonella $P = 0.04$, Yeast cell X Salmonella $P < 0.01$, CD8+ : Yeast cell $P < 0.01$, Salmonella $P = 0.05$, Yeast cell X Salmonella $P = 0.09$. 
Figure 9. Effect of whole yeast cell product supplementation on CD4⁺CD25⁺ cell populations in the cecal tonsils at 7 and 14 d post-Salmonella challenge. Day-old broilers were fed 0, 0.1 or 0.2% whole yeast cell product. At 21 d of age, birds were challenged with 0 (control) or of 1x10⁹ CFU/ml S. Enteritidis (Salmonella). Cecal tonsils were processed for isolating lymphocytes by density centrifugation and incubated with a fluorescent-conjugated anti-chicken CD4 and CD25 mAb and analyzed using flow cytometry. n = 6. P values, 7 d: Yeast cell P = 0.74, Salmonella P = 0.01, Yeast cell X Salmonella P = 0.16; 14 d: Yeast cell P < 0.01, Salmonella P = 0.74, Yeast cell X Salmonella P = 0.08.
Figure 10. Effect of whole yeast cell product on IL-1β mRNA amounts in the cecal tonsils at 14 d post-Salmonella challenge. Day-old broilers were fed 0, 0.1 or 0.2% whole yeast cell product. At 21 d of age, birds were challenged with 0 (control) or 1x10⁹ CFU/ml S. Enteritidis (Salmonella). At 14 d post-Salmonella challenge, mRNA amount was analyzed by real time PCR, correct for β-Actin and normalized to the mRNA content for the uninfected birds so all bars represent fold change compared to the control group. Bars (± SEM) without a common superscript differ significantly within a day (P < 0.05). n = 6. P values, Yeast cell P = 0.09, Salmonella P = 0.07, Yeast cell X Salmonella P = 0.03.
Figure 11. Effect of whole yeast cell product on IL-10 mRNA amounts in the cecal tonsils at 7 and 14 d post-Salmonella challenge. Day-old broilers were fed 0, 0.1 or 0.2% whole yeast cell product. At 21 d of age, birds were challenged with 0 (control) or 1x10⁹ CFU/ml S. Enteritidis (Salmonella). At 7 and 14 d post-Salmonella challenge, mRNA amount was analyzed by real time PCR, corrected for β-Actin and normalized to the mRNA content for the uninfected birds so all bars represent fold change compared to the control group. Bars (± SEM) without a common superscript differ significantly within a day (P < 0.05). n = 6. P values, 7 d: Yeast cell P = 0.10, Salmonella P = 0.15, Yeast cell X Salmonella P = 0.02, 14 d: Yeast cell P = 0.18, Salmonella P = 0.20, Yeast cell X Salmonella P = 0.02.
Figure 12. Effect of whole yeast cell product on TNF-α mRNA amounts in the cecal tonsils at 7 d post-Salmonella challenge. Day-old broilers were fed 0, 0.1 or 0.2% whole yeast cell product. At 21 d of age, birds were challenged with 0 (control) or 1x10⁹ CFU/ml S. Enteritidis (Salmonella). At 7 d post-Salmonella challenge, mRNA amount was analyzed by real time PCR, corrected for β-Actin and normalized to the mRNA content for the uninfected birds so all bars represent fold change compared to the control group. Bars (± SEM) without a common superscript differ significantly within a day (P < 0.05). n = 6. P values Yeast cell P = 0.15, Salmonella P = 0.12, Yeast cell X Salmonella P = 0.03.
Figure 13. Effect of whole yeast cell product on IL-6 mRNA amounts in the cecal tonsils at 7 and 14 d post-Salmonella challenge. Day-old broilers were fed 0, 0.1 or 0.2% whole yeast cell product. At 21 d of age, birds were challenged with 0 (control) or 1x10⁹ CFU/ml S. Enteritis (Salmonella). At 7 and 14 d post-Salmonella challenge, mRNA amount was analyzed by real time PCR, corrected for β-Actin and normalized to the mRNA content for the uninfected birds so all bars represent fold change compared to the control group. Bars (± SEM) without a common superscript differ significantly within a day (P < 0.05). n = 6. P values, 7 d: Yeast cell P = 0.05, Salmonella P = 0.13, Yeast cell X Salmonella P = 0.01; 14 d: Yeast cell P = 0.07, Salmonella P = 0.18, Yeast cell X Salmonella P < 0.01.
Figure 14. Effect of whole yeast cell product supplementation on jejunum villi length 7 d post-*Salmonella* challenge. Day-old broiler chickens were randomly distributed to one of three dietary treatments with 0, 0.1 or 0.2% whole yeast cell product. At 21 d of age, birds were challenged with 0 (control) or $1 \times 10^9$ CFU/ml *S. Enteritidis* (*Salmonella*). Jejunum villi lengths were measured. Bars (± SEM) without a common superscript differ significantly within a day ($P < 0.05$). $n = 6$. $P$ values Yeast cell $P = 0.47$, *Salmonella* $P < 0.01$, Yeast cell X *Salmonella* $P = 0.01$. 
**Figure 15.** Effect of whole yeast cell product supplementation on jejunum crypt depth 7 d post-*Salmonella* challenge. Day-old broiler chickens were fed 0, 0.1 or 0.2% whole yeast cell product. At 21 d of age, birds were challenged with 0 (control) or $1 \times 10^9$ CFU/ml *S. Enteritidis* (*Salmonella*). Jejunum crypt lengths were measured. Bars (± SEM) without a common superscript differ significantly within a day ($P < 0.05$). $n = 6$. $P$ values Yeast cell $P = 0.01$, *Salmonella* $P < 0.01$, Yeast cell X *Salmonella* $P = 0.01$. 

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The bar graph shows the effect of yeast cell product supplementation on jejunum crypt depth 7 days post-*Salmonella* challenge. Different superscripts indicate significant differences within a day ($P < 0.05$).