

IMMUNOMODULATORY EFFECTS OF WHOLE YEAST CELLS AND CAPSICUM IN
WEANLING PIGS CHALLENGED WITH PATHOGENIC *ESCHERICHIA COLI*

BY

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THESIS

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ABSTRACT

Post-weaning diarrhea, caused by enterotoxigenic *Escherichia coli*, is one of the most common causes of mortality and morbidity in commercial pig production. With recent global concern over antibiotic resistance, the development of nutritional technologies to support animal health during times of infection is more important than before. An experiment was conducted to evaluate the combined dietary effects of a whole yeast cell product, CitriStim, and an essential oil, capsicum, on performance and immune indices in weanling pigs inoculated with an F18, enterotoxigenic *E. coli* strain. Growth performance, fecal bacteria counts, frequency of diarrhea, clinical blood outcomes, intestinal cytokine gene expression, T cell profiles, and recall nitric oxide (NO) production from isolated peripheral blood mononuclear cells (PBMC) stimulated with *E. coli* lipopolysaccharide (LPS) were evaluated throughout the experiment. The pigs challenged with ETEC showed clear signs of infection, such as diarrhea and F18 *E. coli* at 5 days post-inoculation (DPI), while the control animals showed no signs of infection and had no detectable levels of F18 *E. coli* throughout the study. Both capsicum and whole yeast cells had minimal impact on body weight gain, however pigs fed the capsicum only diet were more efficient than those fed the whole yeast cell only diet. Total fecal bacterial counts were higher during the acute phase of infection (0 – 5 DPI) in pigs fed the combination of additives ($P = 0.05$) compared with all other experimental groups. Pigs fed the capsicum-only diet also had higher total fecal bacterial counts ($P = 0.05$) compared with pigs fed the control or whole yeast cell-only diet. The combination of additives elicited lower total bacteria counts than either additive alone ($P = 0.03$) at 10 DPI, suggesting a possible return to gut homeostasis. Blood leukocyte counts at 7 DPI were increased in pigs fed the combination of additives ($P = 0.04$), while cytotoxic and memory T cell proportions were lower in these pigs at the same post-inoculation time-point ($P = 0.02$). To better

quantify extent of the inflammatory response mounted against *E. coli* infection, PBMC were isolated from each pig, exposed to purified *E. coli* LPS, and measured for NO production, an indicator of macrophages reactivity. Nitric oxide production was higher ($P = 0.02$) in macrophages from pigs fed the whole yeast cell-only diet, while capsicum was effective at reducing the amount of NO produced by macrophages challenged with LPS after 24 h compared to cells isolated from pigs fed the whole yeast cell-only diet. Cytokine mRNA expression for 4 different cytokines did not differ among all ETEC-challenged groups. This indicates that cytokine levels were down regulated to low levels by the recovery phase of infection. In conclusion, these findings indicate that while feeding whole yeast cells and capsicum alone is not effective at improving growth performance in weanling pigs, these additives have potential to more efficiently reduce the untoward effects of enterotoxigenic *E. coli* infection, as evidenced by the lower bacterial counts and lower T cell proportions. The ability of these additives to reduce mortality and loss of growth makes them a viable option as alternatives to antibiotics for producers, and can be marketed as natural feed ingredients.

For my grandma

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CHAPTER 1: INTRODUCTION

Post-weaning diarrhea is a major disease concern in large swine production systems. It can cause decreased weight gain, feed intake, and feed efficiency as well as mortality in severe cases, leading to major economic losses by the producer (Fairbrother et al., 2005). The cause of post-weaning diarrhea is most often enterotoxigenic *Escherichia coli* (ETEC) and, more specifically, the F18-fimbriated strain of ETEC is a leading cause of outbreaks in post-weaning age pigs (Gonzalez-Ortiz et al., 2013). Traditionally, a wide variety of antibiotics, both injectable and in water/feed, have been effectively used to manage, treat, and eradicate outbreaks of ETEC infection in post-weaning pigs (Fairbrother et al., 2005). The use of in-feed antibiotic growth promoters has also become common practice as it has been shown to reduce mortality by approximately 50% (Stein and Kil, 2006). However, since the 2006 ban of antibiotics in Europe, and the upcoming Veterinary Feed Directive (VFD) that will begin in January 2017 requiring veterinarian oversight for the use of therapeutic drugs that can impact human medicine, there has been a move away from antibiotic use (Le Bon et al., 2010; FDA, 2016). In 2015, U.S. consumption per capita for pork was 49.7 pounds, making it the second most consumed meat in the U.S. (USDA, 2016) and a target for consumer concern over antibiotics in their pork products. With this much pork being consumed, it is important for producers to cultivate a healthy herd, while also pleasing consumers. A consumer demand for never-fed antibiotic pork, as well as concern over antibiotic resistant bacteria transfer has driven pork producers to find alternatives to antibiotics to treat infections like post-weaning diarrhea. One of the alternatives with the greatest potential are prebiotics, which have been shown stimulate the growth of microbes already present in a healthy gut, that can then outcompete invading microbes for attachment on the intestinal epithelium (Gibson et al., 2004, McNeil et al., 1978). With invading microbes

unable to attach to the epithelium, they are unable to colonize and subsequently unable to cause infection (McNeil et al., 1978). Prebiotics have also been shown to influence the immune system of pigs, helping to more quickly alleviate infection in order to avoid large losses in growth, leading to increased profitability for the producer. Due to prebiotics being a relatively new alternative to antibiotics for swine, results of research has been varied. To determine if a combination of prebiotic and bioactive substances may serve to support growth performance and animal health during an enteric challenge, the current research was carried out with the objective to quantify combined effects of feeding a yeast cell wall product and an essential oil on the immune and growth response of pigs. These products may serve as natural feed ingredients to replace antibiotics and prevent enteric infection in swine production, effectively putting consumer minds at ease while also following the new VFD restrictions, and the move away from in-feed antibiotics.

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CHAPTER 2: LITERATURE REVIEW

Escherichia Coli Infection

Introduction

Escherichia coli is one of the most studied bacteria today. Many of the differing strains of *E. coli* reside in the gut at all times as non-harmful bacteria, while others are the cause of intestinal infection and diarrhea in both animals and humans. *Escherichia coli* strains responsible for diarrhea can be categorized into six different groups; enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli*, enterhemorrhagic *E. coli*, enteroinvasive *E. coli*, diffusely adhering *E. coli*, and enteroaggregative *E. coli* (Torres et al., 2005). The primary cause of post-weaning diarrhea in pigs is ETEC and will be the *E. coli* focused on here.

The most problematic bacteria during the weaning phase in a piglet's life is ETEC, and can lead to large economic losses due to morbidity, mortality, and decreased growth performance (Fairbrother et al., 2005; Gonzalez-Ortiz et al., 2013). With weaning already being one of the most stressful times during a pig's life, the infection by ETEC only exacerbates decreased growth and feed intake (Sugiharto et al., 2014). The ability of ETEC to express fimbriae, either F4, associated with nursing pig and pre-weaning diarrhea, or F18, associated with post-weaning diarrhea provides ETEC with a highly specific means of attachment in the gut (Gonzalez-Ortiz et al., 2013). Antibiotic resistance and the possible impact on human health is a major cause for concern in today's agricultural world. This review will focus on the pathogenesis of ETEC, the following immune response, and possible alternative feed ingredients that could prevent post-weaning diarrhea through the blockage of ETEC adhesion.

Pathogenesis

Enterotoxigenic Escherichia coli causing post-weaning diarrhea enter the gut through ingestion and are able to effectively colonize in the small intestine when present in sufficient numbers (Fairbrother et al., 2005). During an ETEC infection, the bacteria are able to proliferate rapidly mainly in the mid-jejunum and ileum. The degree at which the bacteria are able to colonize then determines if diarrhea will result from infection. Colonization is dependent not only on number of bacteria, but also on the major virulence factors of the ETEC strain; the expression of fimbriae and the production of enterotoxins. The expression of specific fimbriae allows bacteria to adhere to receptors along the epithelial cells of the small intestine, while the enterotoxins produce increased water and electrolyte secretion in the small intestine and decreased fluid absorption, resulting in hyper-secretory diarrhea (Holland, 1990; Nataro and Kaper, 1998; Fairbrother et al., 2005). The pathogenicity and virulence of ETEC are influenced by the antigens and fimbriae on the bacteria surface recognizing highly specific receptors along the mucosal wall and closely adhering to the intestinal wall (Francis et al., 1999).

Fimbriae

Fimbriae are proteinaceous appendages found on the outer membrane of bacterial cells and help to adhere and facilitate the colonization of ETEC in the small intestine (Nagy et al., 1976; Morris et al., 1982; Sarrazin and Bertschinger, 1997). Fimbriae can be classified as either mannose-sensitive or mannose-resistant. Mannose-sensitive strains have mannose-rich glycoproteins as receptors and are also called type-1 fimbriae (Ofek and Beachey, 1980). Approximately 70% of 77 different *E. coli* strains were discovered to possess type 1 fimbriae that were mannose-sensitive (Finuance et al., 1999). F18 fimbriae are associated with post-

weaning diarrhea, while the F4 fimbriae are more common in nursing pigs (Fairbrother et al., 2005; Frydendahl, 2002). This review will be focusing on the F18 fimbriae, as it is associated with post-weaning diarrhea.

The F18 fimbriae are long and flexible and have a defining zig-zag pattern (Nagy et al., 1997). There are two antigenic variants of the F18 fimbriae, F18ab and F18ac. Prior to 1995 these fimbriae were designated F107 (now F18ab), 2134P (now F18ac), and 8813 (now F18ac) (Imberechts et al., 1992, 1994; Rippinger et al., 1995). F18ab fimbriae are poorly expressed *in vitro*, while the F18ac fimbriae are more readily expressed *in vitro* and are more often associated with ETEC strains expressing enterotoxins (Nagy et al., 1997). F18 fimbriae are typically found on ETEC strains that produce heat-stable enterotoxins STa and STb, and infrequently on those strains producing heat-labile enterotoxin (Rippinger et al., 1995; Francis 2002). The F18 fimbriae are expressed at 37°C but not at 18°C, and only mediate adherence to the intestinal wall of pigs older than three weeks of age and not younger pigs (Fairbrother et al., 2005). Receptors for the F18 fimbriae are found to increase with age, and are not found in newborn pigs, in part possibly explaining when ETEC strains with F18 fimbriae are more prevalent in post-weaned pigs (Nagy et al., 1992). Adhesion of the bacteria to the epithelium of the small intestine is in part mediated by the fimbriae. A close adhesion is necessary for the bacteria to have access to nutrition, as well as easier delivery of toxins into the tissue and better migration of the bacteria into the tissues. A close adhesion also prevents bacteria from being carried to other parts of the intestine and eventually being expelled by any cleaning and protection mechanisms of the host animal. The detailed mechanism of this binding is still not well known.

Enterotoxins

Diarrhea caused by ETEC infection is induced through the release of enterotoxins. These toxins stimulate a significant increase of fluid excretion in the small intestine and cause a failure of the bowel to reabsorb the excess fluid. Uptake of these toxins along with antigens through transcellular and paracellular pathways then triggers inflammation and contributes to diarrhea (Kim et al., 2012). *Enterotoxigenic Escherichia coli* produces a two different types of enterotoxins, heat-stable toxin (ST) and heat-labile toxin (LT). Different strains of ETEC can produce ST alone or both ST and LT. ST is characterized as non-antigenic, while LT is antigenic and is thought to be a potent modulator of the immune response (Salmond et al., 2002; Moeser et al., 2007). In addition to ST and LT, ETEC, a Gram negative bacteria possesses lipopolysaccharide (LPS) on the surface which is responsible for activation of the innate immune system. Some ETEC also have genes which allow it to express Shiga toxin, which is responsible for edema disease in pigs. While ST and LT produce functional changes in the small intestine through the secretion of H₂O, Na⁺ and Cl⁻, and cause decreased fluid absorption and increased fluid secretion, LPS and Shiga toxin induce diarrhea and disease through the release of cytokines and the direct killing of cells and other bacteria (Fairbrother et al., 2005; Kim et al., 2012).

Heat stable toxins are further classified as STa, which is found in porcine and bovine isolates, and STb, found in porcine isolates only (Gyles, 1994; Nataro and Kaper, 1998). STa only induces fluid secretion in newborn pigs, while STb can induce fluid secretion in both newborn and weaned pigs (Nagy and Fekete, 1999).

Heat stable toxin a is a small, non-immunogenic protein that is resistant to proteolytic enzymes and exposure to acids (Lazure et al., 1983; Fairbrother et al., 2005). Produced within the intestine, STa binds to guanylyl cyclase C a protein spanning the membrane with an

intracellular protein kinase and catalytic domain and an extracellular binding domain. Binding activates guanylate cyclase which leads to an increase of cyclic guanosine monophosphate (cGMP) inside enterocytes (Fairbrother et al., 2005; Moeser et al., 2007). The increased cGMP accumulation then leads to activation of CFTR and elevated secretion of Cl^- and H_2O in the crypt cells and inhibition of Na^+ and Cl^- absorption from the villi (Forte et al., 1992).

Heat stable toxin b is almost exclusively associated with porcine ETEC and is poorly immunogenic, meaning it is unlikely to provoke an immune response (Dubreuil 1997). The mechanism by which STb stimulates accumulation of fluid in the small intestine is not well known. It does not increase intracellular levels of cyclic adenosine monophosphate (cAMP) or cGMP (Weikel et al., 1986), but does stimulate intestinal epithelial cells to secrete HCO_3^- (Weikel and Guerrant, 1985) and may involve elevation of prostaglandin E2 (PGE2) in the intestine (Menard and Dubreuil, 2002). STb causes some histological damage in the epithelium of the intestine, such as villous atrophy. Damage such as the loss of villous epithelial cells by STb may be responsible for lower absorption of fluids (Whipp et al., 1987). It has also been found that STb may open a G-protein linked calcium channel in the plasma membrane, which would elevate levels of Ca^{2+} , activating the prostaglandin endoperoxidase synthetase system, leading to the formation of prostaglandins (Dreyfus et al., 1993).

Heat-labile toxin is highly related to cholera toxin, with 77% similar identity at the nucleotide level (Fairbrother et al., 2005). It is composed of a single enzymatic A subunit which is non-covalently associated with five B subunits that bind the toxin to its receptor (de Haan and Hirst, 2004). After the B subunits bind the toxin to the cell surface, a fragment of the A subunit will translocate into the cell and activate the cAMP system which leads to increased fluid and electrolyte secretion and decreased absorption. The cAMP-stimulated protein kinase A

phosphorylates the cystic fibrosis transmembrane conductance regulator and causes chloride secretion (O'Brien and Holmes, 1996). Other cAMP mediated changes include activation of an apical chloride channel and a basolateral Na/K/2Cl transporter, the loosening of tight junctions, and release of PGE2 (de Haan and Hirst, 2004). All of the above contributes to the irreversible increased chloride secretion, reduced sodium absorption, and a massive loss of water in the small intestinal lumen.

Lipopolysaccharide (LPS) is a surface component found on the outer membrane of many gram negative bacteria, including ETEC. Binding of LPS require both CD14 (a pattern recognition receptor) and LPS binding protein (LBP). The receptors that respond to LPS are mainly located within cells of the innate immune system which causes the release of cytokines during infection (Raetz and Whitfield, 2002). CD14 are found mainly on monocytes or macrophages. After binding to CD14, LPS can be transported to other LPS receptors such as toll-like receptors (TLR), mainly TLR4 (Wiese et al., 1999). The target cells then activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) which stimulates the release of tumor necrosis factor alpha (TNF- α), and the interleukins IL-1 and IL-6 (Bannerman and Goldblum, 2003).

Shiga toxin is similar to LT, as it has a single A subunit and five B subunits, however the mechanism of infection is very different from that of LT. Shiga toxin is able to kill cells after binding to the cell surface of the host. Shiga toxin binds only to cells that possess Gb3 and Gb4, the glycolipid receptors for Shiga toxin. After binding the Shiga toxin will enter the epithelial cells and release an enzymatically active A subunit fragment into the cytosol. This fragment will then cause apoptosis through inhibition of protein synthesis. Shiga toxin also have the ability to induce cytokine synthesis, such as IL-8, IL-6, IL-1 and TNF- α (Paton and Paton, 1998).

With many different toxins being produced by a variety of ETEC strains, the immune system must respond accordingly to rid the host of infection. Understanding how the immune system responds to an ETEC infection can lead to novel approaches of disease prevention.

The Immune System

Innate vs. Adaptive Immunity

The immune system is in place to protect the host from infection agents and pathogens that exist in the environment. There are two divisions of the immune system; innate immunity and adaptive immunity. The innate immune system is the “natural” immune system and is universal among organisms. It is the first line of defense against invading pathogens and responds non-specifically to antigens. The innate immune response is composed of physical barriers, soluble factors such as complement factors, and phagocytic cells including granulocytes, monocytes, and macrophages (Janeway and Medzhitov, 2002; Yaqoob and Calder, 2011). The phagocytic cells are the main effectors of the innate immune system. These cells possess surface receptors that recognize specific structures present on bacteria, and eliminate invading infectious agents by phagocytosis followed by degradation by toxic compounds such as superoxide radicals and hydrogen peroxide (Yaqoob and Calder, 2011). The innate immune response also contains natural killer cells that destroy infectious agents through the release of cytotoxic proteins and interferon gamma (IFN- γ). The innate immune response is characterized by quick activation and response of effectors, recognition of conserved molecular patterns of microbes, and highly-effective self/non self-recognition. Though the phagocytic cells have the main job of destroying invading pathogens, they are also able to present antigens to highly specific lymphocytes (Jenkins et al., 2001; Trombetta and Mellman, 2005). This provides

coordinated action between the two divisions of the immune response to more effectively rid the host of infection.

The adaptive, or acquired, immune response involves the specific recognition of antigens, and is more complex than the innate immune response. It is the second line of defense and is able to create immunological memory of infection, leading to a faster response if the host is ever infected with the same pathogen again. The adaptive immune response is made up of T lymphocytes and B lymphocytes. The adaptive immune response is highly specific, but the high specificity leads to low lymphocyte numbers, meaning it takes a few days for the adaptive response to clonally expand enough to fight the pathogen after initial infection (Yaqoob and Calder, 2011). Small lymphocyte numbers are dealt with through clonal expansion, giving rise to an “army” of lymphocytes all able to recognize the same antigen with high specificity.

B lymphocytes are characterized by their ability to produce antibodies specific to individual antigens. They are an antigen presenting cell containing immunoglobulins that bind antigens to their cell surface. The other antigen presenting cells are dendritic cells and macrophages. All three of these cells will bind antigens in the peripheral tissues, process the antigens to proteolytic peptides, and then present these peptides to major histocompatibility complex (MHC) class I and II molecules (McHeyzer-Williams and McHeyzer-Williams 2005; Parham, 2015). Macrophages and dendritic cells present antigens to T helper 1 (Th) cells via MHCI, and present antigens to Th2 cells. This occurs in the secondary lymphoid tissues and initiates antigen-specific immune response or tolerance (Trombetta and Mellman, 2005; Parham 2015). Mature T lymphocytes will recognize specific antigen-MHC complexes and will then give rise to clonal expansion.

T lymphocytes, unlike B lymphocytes are only able to recognize antigens that are presented to them by antigen presenting cells. T lymphocytes express specific T cell receptors (TCR) on their surface which have a large antigen catalogue. Activation of the TCR will activate the T lymphocyte to start proliferation of clone T cells (Yaqoob and Calder, 2011; Parham 2015). There are three types of T lymphocytes; cytotoxic T cells, T helper cells, and regulatory T cells. T lymphocytes expressing CD8 surface molecules are cytotoxic T lymphocytes. These T cells secrete cytotoxic enzymes to kill infected cells (Yaqoob and Calder, 2011). Regulatory T cells express CD4 and CD25, and are activated to suppress rogue activity of B and T cells. T lymphocytes that express CD4 are helper T cells (Th cells). These cells secrete cytokines that recruit neutrophils and monocytes to the site of infection and inflammation. Cytokines are small proteins that bind to receptors on the surface of cells and induce changes in development or activity of the target cell. They also act as chemical messengers and can act to modulate the activity of the cell producing the cytokine (Yaqoob and Calder, 2011). This is the primary defense against intracellular bacteria (Jenkins et al., 2001). T helper cells can be further divided into Th1 and Th2 cells, each with specific cytokines they produce. Th1 cytokines are mainly pro-inflammatory and are responsible for killing intracellular pathogens. Interferon gamma (IFN- γ) is the main Th1 cytokine which activate macrophage, natural killer cells, and cytotoxic T lymphocytes. Th1 cells are mainly activated during infection by bacteria and fungi. Too many pro-inflammatory cytokines can lead to unnecessary tissue damage (Oswald, 2005; Lippolis, 2008). Th2 cytokines have more of an anti-inflammatory effect, unless an allergic reaction is occurring. These cytokines include IL-4, IL-5, and IL-10 and IL-13. IL-10 and IL-4 together can suppress cell-mediated immunity (Yaqoob and Calder, 2015). In order to maintain an effective immune response, the host must produce a good balance of both Th1 and Th2 cells.

Immune Response to ETEC Infection in Weanling Pigs

During weaning pigs are exposed to various new pathogens in both their changing feed and environment. This comes at a time when the passive immunity given by the sow's milk antibodies has diminished almost completely. The microbial community in the gut of the pig is also changing during weaning due to stress and the switch from milk to solid feed. This leaves newly weaning pigs with an increased susceptibility to infection (Pié et al., 2004; Sekirov and Finlay, 2009; Campbell et al., 2013). The immediate immune response to ETEC is excretion of cell fluid and electrolytes into the lumen through activation of the cAMP system and a reduced ability to absorb fluid due to ST toxin effects on the cGMP system (Hopwood et al., 2006). Proliferation also stimulates nitric oxide synthase expression, producing nitric oxide (NO) and further stimulating chloride secretion (Kim et al., 2012). Infection by ETEC also loosens tight junctions within the intestinal epithelium allowing bacterial antigens to enter tissues and the circulatory system. This triggers the production of cytokines and recruitment of inflammatory cells to the site of infection (Berkers et al., 2003).

Innate Immune Response

In response to ETEC infection the first goal of the innate immune response is to rapidly clear or prevent attachment of bacteria to alleviate any prolonged inflammation and sepsis. The first line of defense is the physical barrier of the epithelial cells in the small intestine, as well as the mucosal layer. The intestinal epithelial cells act to separate the contents of the luminal environment from the inner tissue layers (Medzhitov and Janeway, 1997; Gerwitz et al., 2002). Tight junctions between the epithelial cells play an important role in maintain the integrity of the epithelial cell barrier, allowing only specific molecules to pass through the barrier, and keeping

infectious bacteria out. The mucus layer is also an important part of the physical barrier within the gut. The mucins associate with other proteins and lipids to form a continuous gel that is in a state of constant turnover. The mucins can trap ETEC, prevent its adhesion, and with turnover of the mucin molecules, the pathogenic ETEC can be carried out of the small intestine and eventually expelled from the host. The mucin layer also tightly adheres to the epithelium and is important for lubrication of the gut as well as allowing commensal bacteria to adhere to the epithelium, thereby outcompeting pathogenic bacteria and preventing infection (Desseyn et al., 2000; Montagne et al., 2004; Oswald, 2005). Commensal bacteria in the gut are essential to prevent the attachment, growth, and migration into tissues of pathogenic microorganisms in the intestine. These commensal bacteria help with pathogen resistance through direct interaction with invading microorganisms and by influencing the immune system (Tlaskalova-Hogenova et al., 2004; Kamada et al., 2013). Commensal bacteria can interact with intestinal epithelial cells to help induce the secretion of antimicrobial peptides, as well as allow cross-talk between the immune system and commensal microbiota, thereby assisting the immune system to more effectively identify pathogenic bacteria (Louis and O’Byrne, 2010; Kamada et al., 2013). Some commensal bacteria can drive T lymphocyte development, both helper T cells and regulatory T cells (Kamada and Nunez, 2013).

The intestinal epithelial cells can also secrete antimicrobial peptides that act to disrupt the integrity of the bacterial membrane of ETEC, destroying it before it can attach to the epithelium (Hooper et al., 2003). If *Enterotoxigenic Escherichia coli* is allowed to attach to the epithelium, then the innate immune response is activated through the recognition of bacteria fimbriae and antigens, distinguishing them from host “self” cells (Janeway and Medzhitov, 2002). The binding of ETEC to the intestinal epithelium also begins the release of the toxins, promoting

fluid accumulation and hyper-secretory diarrhea. These receptors are deemed pattern recognition receptors and are very diverse. One such receptor, TLR-4, recognizes LPS on the bacterial surface, while mannose receptors identify a variety of bacteria, yeasts, and mycobacteria (Stahl and Ezekowitz, 1998; Parham, 2015). This binding of ETEC to specific receptors along the intestinal wall then activates epithelial and sub-epithelial immune cells such as macrophages, mast cells, and dendritic cells (Malaviya et al., 1994; van der Windt et al., 2010; Qian and Cao, 2012). This triggers intracellular signaling cascades that lead to the synthesis and release of cytokines such as TNF- α , IL-10, IL-1 β , IL-6, and IL-8 (Jung et al., 1995; Stadnyk, 2002; Pié et al., 2004). Binding of ETEC to TLR-4 and TLR-5 leads to the secretion of IL-1 β , IL-8, and TNF- α by the intestinal epithelial cells (Alexander and Rietschel, 2001; Moue et al., 2008; Devriendt et al., 2010). These cytokines signal inflammation and communicate to the adaptive immune system to create a more robust immune response to infection.

Lipopolysaccharide on the bacterial outer membrane, as well as cytokine exposure, stimulates NO production from macrophages, which contributes to the inflammatory response (Xie and Nathan, 1992). NO is an inorganic gas that function in a number of mammalian systems, one of the major functions being immune regulation (Schneemann et al., 1993). When NO is regulated and controlled it serves as modulator for signaling pathways and participates as an immunotoxin, but prolonged exposure, or uncontrolled rises in NO levels, can lead to cellular and tissue damage (Babior and Peters, 1981; Moncada et al., 1991; Colton and Gilbert, 1999). Nitric oxide synthase has three isoforms that produce NO, two constitutive forms and one inducible form, each differing in function (Forstermann et al., 1991). The constitutive forms are expressed mainly in neurons and endothelial tissues and function to regulate vascular tone and release short bursts of NO in a calcium dependent manner (Griffith and Stuehr, 1995; Thomas et

al., 2008). The inducible form of nitric oxide synthase produces large amounts of NO and can be upregulated in immune cells. It is considered the pathophysiological form of the enzyme because it is associated with the inflammatory response and formation of carcinogenic *N*-nitrosamines (Moncada et al., 1991; Marletta, 1993; Schmidt et al., 1993). The inducible form is also not spatially restricted, so it can participate in widespread killing of invading pathogens (Wink et al., 2011).

Adaptive Immune Response

Neutrophils and natural killer cells are typically the first to arrive at the sight of infection during the early stages of inflammation with marked increase in both neutrophils and lymphocytes 5 days after initial infection (Kohlmeier and Woodland, 2009; Song et al., 2012). These cells are recruited to the intestinal tissue by cytokines or cytokine mediated chemokine production, primarily IL-6 and IL-8, which increase dramatically in elevation during ETEC infection (Mclamb et al., 2013). IL-6 has been shown to increase neutrophil killing, while IL-8 is critical for neutrophil recruitment. TNF- α has a remarkable increase during ETEC infection and is released by a variety of cells including macrophages and monocytes in response to bacterial cell wall components such as LPS and toxins released by ETEC (Jesmok et al., 1992; Frank et al., 2003). TNF- α has been shown to help recruit more neutrophils to the site of infection (Bischoff, 2009; Abraham and St John, 2010; Mclamb et al., 2013). However, TNF- α has also been shown to increase Cl⁻ secretion in the ileum, along with IL-1 β , which may exacerbate post-weaning diarrhea (Kandil et al., 1994; McKay and Baird, 1999). Chronic inflammation can then follow the acute inflammatory response if the initial response is not sufficient to clear the ETEC infection from the host. Inflammation will promote further protection and tissue repair, as the

pro-inflammatory cytokines, and neutrophils can cause changes in the morphology and enzymatic activity of the small intestine (Pluske et al., 1997; Thacker, 2006). These pro-inflammatory cytokines can cause gaps in the tight junctions of the intestinal epithelial cells, allowing pathogens to penetrate tissues, compromising tissue integrity (Williams, 2001). IL-1 β , however has been shown to increase epithelial cell turnover, allowing the small intestine to expel more pathogenic bacteria in the feces. This rapid cell turnover can also lead to more commensal bacteria being shed, allowing pathogenic bacteria to proliferate during time of infection (Panja et al., 1998; Wolf et al., 2000).

Lymphocytes are present mainly in the gut associated lymphoid tissues (GALT), which includes Peyer's patches and the lamina propria and are capable of responding to antigens in the lumen of the small intestine (Craig and Cebra, 1971). During an ETEC infection B cells in GALT produce antibodies such as immunoglobulins (Ig), like IgA, an antibody with antimicrobial properties that also helps to combat toxins produced by ETEC (Fubera and Freter, 1973). A majority of B cells are located within the Peyer's patches, which are known as the predominant structures to take up antigens (Binns and Licence, 1985; Rothkotter and Pabst, 1989). GALT also contains T lymphocytes, and both cytotoxic and helper T cells are released to help combat infection.

Overall ETEC activates both the innate and adaptive immune response. Each system has components to recognize and eliminate the invading bacteria. The inflammatory response is both beneficial as it is effective in ridding the host of infection, but can be damaging if not controlled, or if is allowed to become severe for prolonged periods of time. Because these immune responses can be damaging, and ETEC infection can cause death and severe growth performance reductions, actions must be taken to prevent post-weaning diarrhea from happening. In the past

antibiotics have been used as a prevention method to ETEC, however in recent years there has been a shift away from using antibiotics and instead moving toward alternative feed ingredients that provide some of the same benefits as antibiotics. Some of the feed ingredients gaining the most interest are pro-biotics, prebiotics, and plant extracts.

Antibiotic Alternatives

Overview

The use of antibiotics in food animals and the possible impact of human health have been a major concern in the past decade. Since the 2006 European ban on the use of in-feed antibiotic growth promoters, there has been increased interest, especially in the United States, in finding alternatives to antibiotics to both prevent and treat against infectious challenges, especially in food-producing animals (Le Bon et al., 2010). The concern is that antibiotic resistant bacteria or genes will be transferred to humans through the ingestion of antibiotic fed animal products (Gold and Moellering, 1996; Collignon, 1997). Some of the alternatives to antibiotics that have been showing the most promise are prebiotics and plant extracts.

Prebiotics

Prebiotics are defined as a food ingredient that is “composed of oligosaccharides that are not digestible by the host and that has a beneficial effect on host health through selective stimulation of the growth and/or activity of specific members of the gut microbiota” (Gibson et al., 2004). Studies of prebiotic substrates usually focus on non-digestible oligosaccharides and dietary fibers, as these feed ingredients are thought to be selectively fermented by the gut microbiota into short-chain fatty acids (SCFA) such as butyrate and propionate. An increase in

SCFA can lead to increased numbers of beneficial bacteria in the gut, which will help to competitively drive out pathogenic bacteria (McNeil et al., 1978; Yaqoob and Calder, 2011). Prebiotics can also directly affect the mucosal immune system to improve and alleviate inflammatory disease and the adaptive immune response (Vieira et al., 2013). These anti-inflammatory effects are most likely from the production of SCFA. Butyrate has been associated with increased production of T regulatory cells, as well as reduced production of pro-inflammatory cytokines and reduction of the NF- κ B pathway, suggesting it may be advantageous to controlling inflammation (Su et al., 1999; Luhrs et al., 2002; Klampfer et al., 2003). Receptors on eosinophils and neutrophils have also been identified as recognizing SCFA, suggesting that SCFA may help bolster the immune response during infection (Maslowski et al., 2009; Kim et al., 2013; Smith et al., 2013). Prebiotics may also provide protection from infection through the inhibition of the adherence of pathogens to the epithelium in the intestine. Prebiotics, especially those containing mannans, can bind to the epithelial surface of the intestine and provide a competitive binding site for some bacteria, like ETEC, as described above. Once bound to the mannan receptor of the prebiotic, pathogenic bacteria are prevented from colonizing the hold and instead are expelled in the feces. Two prebiotics that will be better focused on here are mannan oligosaccharides and β -glucans, both derived from the cell walls of yeast.

Mannan Oligosaccharides

Mannan oligosaccharide (MOS) derived from the yeast cell wall of *Saccharomyces cerevisiae* has shown the potential to be a viable alternative to antibiotics. *Saccharomyces cerevisiae* is a non-commensal and non-pathogenic yeast commonly used in the food industry as brewer and baker's yeast (Zanello et al., 2011). MOS has been shown to improve animal health

and growth performance through the modulation of the immune system, the prevention of pathogenic bacteria from adhering the epithelium, and the alteration of microbial populations (Newman 1994; Newman and Newman, 2001; O'Quinn et al., 2001; Davis et al., 2004). In swine the addition of MOS to a diet has resulted in variation in the growth performance of nursery pigs. Some studies have shown MOS to be as effective as antimicrobial growth promoters when fed to pigs (Davis et al., 1999; Pettigrew, 2000), while others have reported little or no response on growth performance when MOS was included in the diet (Cromwell et al., 1991; Davis et al., 2004). The growth performance response to MOS is highly variable, and seems to be dependent on growth rate, environment, and stressors (Miguel et al., 2004). When pigs already have a high growth rate, MOS seems to be ineffective, while pigs with a low growth rate benefit from the addition of MOS to the diet, especially in the first weeks after weaning (Miguel et al., 2004).

MOS is also believed to help promote gut health by preventing the attachment of pathogenic bacteria to the epithelial wall (Spring et al., 2000). The mannan component of MOS provides a competitive binding site for pathogenic bacteria containing Type 1 (i.e., mannan-sensitive) fimbriae (Baumler et al., 1997). This prevents colonization of pathogenic bacteria and subsequent inflammation and infection (Firon et al., 1983). Spring et al. (2000) showed 5 of 7 strains of *E. coli* were agglutinated by MOS and yeast cells, while Kogan and Kocher (2007) showed that 56% of 258 pathogenic bacterial strains were agglutinated by MOS, with the highest activity against the widest spread pathogens such as *E. coli*. These studies confirm that MOS has potential for inhibiting pathogen colonization in the host. The ability of MOS to reduce pathogenic bacteria numbers may result in a "healthier" gut microbiota, adding to claims that MOS changes microbial populations in the intestinal tract of young pigs. Reduction in

pathogenic bacteria in the intestine and higher counts of “beneficial” bacteria such as lactobacilli may help to prevent diarrhea associated with ETEC infection (White et al., 2002).

Several studies have shown that MOS influences both the innate and adaptive immune response. Recent studies have shown that MOS has the ability to alter the cytokine response of macrophages (Che et al., 2008), as well as stimulate the phagocytic capability of macrophages in a dose-dependent manner (Newman, 1995). Results from Davis et al. (2004) suggested that MOS-fed pigs tended to have lower neutrophil numbers and a higher proportion of lymphocytes, suggesting that MOS may contribute to a reduced inflammatory response. The exact mechanism of how MOS reduces inflammation is not yet known, but may be involved with increasing the number of pattern recognition receptors. White et al. (2002) showed the addition of MOS led to increased levels of both IgG and IgA. An immune response to the yeast products is something that cannot be ruled out, however the authors did not observe a direct activation of a T cell mediated mechanism or an increase in neutrophils, which is common during an immune response to fungi specifically. Many benefits of the addition of MOS to feed have been shown and discussed, but because of the large variability between studies, more research needs to be done before MOS can be declared a true alternative to antibiotics.

β-glucans

A group of carbohydrates that has also shown potential to replace in-feed antibiotics are β-glucans. β-glucans constitute 50-60% of the total yeast cell wall polysaccharides (Bohn and BeMiller, 1995). β-glucan has been shown to possess antimicrobial activities by enhancing the immune response (Hetland et al., 2000). Specifically in weaned pigs, it has been shown to

increase the innate immune response and tolerance to new oral antigens presented from a changing environment and diet during weaning (Mowat, 1987; Stokes et al., 1987).

β -glucans may improve growth performance by improving intestinal health. Adding β -glucan to the diet of pigs has been shown to increase mucosal barrier function, preventing pathogenic bacteria from entering tissues, while also increasing the nutrients absorbed and digested, therefore improving growth performance (Vetvicka et al., 2014). Dritz et al. (1995) showed inclusion of β -glucan at 0.025% of the diet improved ADG after weaning. However, experiments conducted by Schoenherr et al. (1994) and Hahn et al. (2014) showed that β -glucan added to the diet had no effect on growth parameters of weanling pigs. Few studies have focused on how β -glucans influence the gut microbiota, though β -glucans may help to proliferate commensal bacteria, therefore alleviating disease symptoms such as diarrhea (Vetvicka et al., 2014).

β -glucans may enhance the function of macrophages and neutrophils, modify immunosuppression, and increase resistance to gram-negative bacteria (Browder et al., 1990; Pretus et al., 1991; Williams et al., 1996). β -glucans may bind to specific receptors on immune cells such as macrophages and monocytes to elicit increased production of anti-inflammatory cytokines and chemokines to enhance the immune response (Vetvicka et al., 2014). Li et al. (2006) showed that pig diets including β -glucan partially down-regulated the synthesis of pro-inflammatory cytokines. These pigs also had increasing levels of the anti-inflammatory cytokine IL-10 which inhibits T cell proliferation, and macrophage activation, leading to increased growth performance. On the other hand Eicher et al. (2006) concluded that pigs fed β -glucan derived from *S. cerevisiae* exhibited increased levels of pro-inflammatory cytokines in the small intestine. Several other studies have also shown that β -glucans that bind to macrophages lead to

increased antibody production and cytokine release, as well as increased prostaglandin E2 production (Ganter et al., 2003; Herre et al., 2004; Willment et al., 2005; Brown 2006). Supplementation of β -glucans given to pigs during *E. coli* infection showed increased concentrations of both cytotoxic and helper T cells (Vetvicka et al., 2014). β -glucans have been shown to have a multitude of biological and immunological effects, however mixed results have been documented. Effects of β -glucans may be highly dependent on growth performance, infection status, and structure of the β -glucan used within the diet. Overall, β -glucan seem to have beneficial effects on pigs and may be a good alternative to antibiotics.

Plant Extracts

Plant extracts are defined as “secondary plant metabolites that can be naturally obtained from plant materials or chemically synthesized” (Liu et al., 2013). Certain plant extracts such as capsicum (an extracted oleoresin) are believed to have antimicrobial, anti-inflammatory, antioxidant, and antiviral effects (Baydar et al., 2004; Lang et al., 2004; Sökmen et al., 2004; Dundar et al., 2008). Many plant extracts have also been shown to have beneficial effects on growth performance and diarrhea reduction in pigs, possibly due to their immunomodulatory effects (Sads and Bilkei, 2003; Janz et al., 2007; Michiels et al., 2010). Shoba and Thomas (2001) and Liu et al. (2013) showed supplementation of plant extracts lead to a reduction in diarrhea in post-weaning pigs. This may be due to plant extracts increasing the water absorption in the intestine. The reduction in diarrhea could have also been caused by plant extracts directly killing ETEC or inhibiting toxin secretion from ETEC (Hammer et al., 1999).

Plant extracts have been shown to affect the immune system through inhibition of pro-inflammatory cytokines from LPS stimulated macrophages (Liu et al., 2012). While the mode of

anti-inflammatory activity of plant extracts has yet to be defined, it may involve the blockage of the NF- κ B pathway, which is a prominent signaling cascade associated with immune system activation, and which leads to activated B cells (Jobin et al., 1999; Lee et al., 2005; Choi et al., 2007). Liu et al. (2013) showed that during an ETEC infection, pigs provided dietary plant extracts exhibited a decrease in total white blood cells, as well as individual fractions of neutrophils and lymphocytes, indicating that the inflammatory response was less severe in supplemented pigs. Plant extracts may be able to prevent overstimulation of the inflammatory response and early adaptive immune response in pigs infected with ETEC. In many of these studies different plant extracts produced different results, especially in the reduction of pro-inflammatory cytokines and the increased stimulation of anti-inflammatory cytokines. Due to these differing results, more studies are needed to be able to identify the best plant extract, or combination of extracts to elicit the same responses as antibiotics.

In summation, enterotoxigenic *E. coli* is a large problem in the swine industry. It causes reduced growth performance and mortality which can lead to large losses in profit for the producer. Due to recent movement away from using in-feed antibiotics, alternative feed ingredients have generated a lot of interest for treating infections such as ETEC. Some of these alternatives showing the most promise for treating ETEC infection are mannan oligosaccharides from yeast cells, plant extracts, and β -glucans.

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CHAPTER 3: EFFECTS OF WHOLE YEAST CELLS AND CAPSICUM ON GROWTH PERFORMANCE, IMMUNE RESPONSE, AND CLINICAL OUTCOMES IN WEANLING PIGS INFECTED WITH ENTEROTOXIGENIC ESCHERICHIA COLI

Abstract

An experiment was conducted to evaluate growth performance, fecal bacteria counts, frequency of diarrhea, complete blood cell counts, and serum cytokine concentrations in weanling pigs inoculated with enterotoxigenic *Escherichia coli* (ETEC), who were fed Citristim, a whole yeast cell product, and capsicum, an plant essential oil. Weanling pigs (34 barrows and 30 gilts, 21 d of age, 5.90 ± 1.03 kg BW) were allotted to experimental treatments in a randomized complete block design based on litter, sex, and initial BW. Four pigs were individually housed within each containment chamber and assigned to 1 of 4 dietary treatments ($n = 13$), which included a control diet without or with 0.2% whole yeast cell product, CitriStim (ADM, Decatur, IL) or 10 ppm of capsicum, provided either alone or in combination. After receiving diets for 13 d, pigs were orally inoculated with F18⁺ ETEC and maintained on the same diets for an additional 10 d; a separate cohort of pigs ($n = 12$) receiving the control diet was sham-inoculated using PBS. Body and feeder weights were recorded, and fecal swabs collected, on 0, 5, and 10 d post-inoculation (DPI), with blood sampled at 0, 2, 7, and 10 DPI for quantification of cytokine concentrations and clinical outcomes. While no interactive effects were observed for growth performance, main effects revealed that whole yeast cells increased ($P < 0.05$), and capsicum decreased ($P < 0.05$), ADFI 0-10 DPI; reciprocal effects were observed for gain:feed due to a lack of differences in average daily gain. The challenge ETEC strain was undetectable in fecal swabs on 0 and 10 DPI, but differed between unchallenged and challenged control-fed pigs at 5 DPI. Challenged pigs fed either whole yeast cells (main effect, $P = 0.01$) or capsicum (main effect, $P = 0.02$) alone had lower frequency of diarrhea when compared to those

fed the combination diet. Total bacterial counts in pigs fed the combination of additives were highest when compared with either additive alone (interaction, $P = 0.03$) at 10 DPI. Blood leukocyte counts were increased in infected pigs receiving the combination of additives compared with all other infected treatment groups (interaction, $P = 0.04$). The addition of whole yeast cells increased (main effect, $P = 0.01$) lymphocyte counts at 7 DPI. In conclusion, these data indicate that whole yeast cells and capsicum have different effects on ADFI in pigs infected with ETEC. The combination of the two additives, however, elicited higher bacterial and circulating leukocyte counts than either additive alone. Therefore, these data indicate that while whole yeast cells and capsicum have small effects on growth performance, they may have an impact on the immune system of weanling pigs infected with ETEC.

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) infection in weanling pigs is a major problem for producers due to economic losses caused by morbidity, mortality, and reduced growth performance (Fairbrother et al., 2005). Due to weaning already being one of the most stressful times during the pork production cycle, an ETEC infection causes decreased feed intake, and sometimes growth, that a pig may be experiencing due to changes in diet and environment. Typically, antibiotics are used to minimize incidence and severity ETEC infection, but due to recent moves away from the use of antibiotics in food producing animals, alternatives must be found to treat and prevent bacterial infection (Le Bon et al., 2010). Two potential alternatives include prebiotics and plant essential oils, but relatively little is known about how these compounds may interact to influence the immune system and growth response of weanling pigs when challenged with ETEC.

Infection caused by ETEC causes inflammation and changes in gut microbiota populations that lead to reductions in nutrient absorption and growth performance. Prebiotics and plant essential oils both have immunomodulatory effects that may combat the deleterious effects of ETEC (Baydar et al., 2004; Lang et al., 2004; Vieira et al., 2013). Prebiotics have been shown to improve and alleviate effects from the inflammatory immune response through the production of short-chain fatty acids (SCFA) (Vieira et al., 2013). These SCFA help promote the growth of beneficial bacteria in the gut, which effectively outcompetes infectious *E. coli* and other harmful bacteria for colonization in the host (McNeil et al., 1978; Yaqoob and Calder, 2011). Receptors for SCFA on immune cells, such as eosinophils and neutrophils, may also help to strengthen the immune response, contributing to the immunomodulatory effects of prebiotics (Maslowski et al., 2009; Kim et al., 2013). Prebiotics also contain mannan fractions that can bind to invading microbes, preventing infectious *E. coli* from attaching to the intestinal epithelium and colonizing, and instead being expelled in the feces (Newman 1994; Newman and Newman, 2001).

Essential oils have previously been shown to increase growth performance and reduce incidence of diarrhea in pigs (Shoba and Thomas, 2001; Liu et al., 2013). Essential oils may increase water absorption in the gut, which during *E. coli* infection may help reduce the untoward effects of diarrhea. Plant essential oils have also been shown to decrease white blood cell counts through the inhibition of pro-inflammatory cytokine productions (Liu et al., 2012). This inhibition causes less energy diverted away from growth and minimizes damage resulting from an inflammatory response. Both prebiotics and essential oils have the potential to replace in-feed antibiotics used to alleviate ETEC infection, though their effects on the immune system have been varied, and the combination of these two additives has yet to be fully researched.

Due to differing results among these studies, more research needs to be done to determine if a combination of additives is the best alternative to antibiotics to combat ETEC infection. Thus, we conducted an experiment to evaluate the combined effects of a whole yeast cell prebiotic, CitriStim, and a plant essential oil, capsicum, on growth performance, frequency of diarrhea, fecal bacterial counts, and blood clinical outcomes in weanling pigs experimentally challenged with ETEC.

Materials and Methods

All animal care and experimental procedures were approved by the University of Illinois Institutional Animal Care and Use Committee before initiation of the experiment.

Animal Husbandry

A total of 64 weaned pigs (21 days of age), were used in in a single experiment with 2 cohorts of pigs (32 pigs per experiment) and analyzed together. A total of 34 barrows and 30 gilts (initial BW 5.91 ± 1.03 kg) were selected and allotted to experimental treatments in a randomized complete block design by evenly distributing initial BW, litter, and sex. Pigs were housed in the Edward R. Madigan Laboratory (ERML) large animal biocontainment facility at the University of Illinois. A suite of 8 chambers was used to house both the challenged and unchallenged (sham-inoculated) pigs, and strict biosecurity measures were followed to prevent cross-contamination. Each chamber consisted of 4 individual pens (0.84m^2) each equipped with a nipple drinker and a feeder, allowing pigs to be individually housed.

Four diets were prepared from a common basal in a 2×2 factorial arrangement. Diet 1 (negative control) was a nursery diet formulated to meet or exceed NRC (2012) requirements for weanling pigs; this diet did not contain antibiotics, spray-dried plasma, or zinc oxide as growth promoting or prophylactic agents (Table 3.2). Diet 2 was formulated as diet 1, but was

supplemented with whole yeast cells added directly on top of the control diet at 0.2%. Diet 3 was formulated as diet 1, but was supplemented with an essential oil (capsicum, 10 ppm) added directly on top of the control diet. Diet 4 was formulated as diet 1, but was supplemented with whole yeast cells (0.2%) and capsicum (10 ppm) added directly on top of the control diet. Pigs remained on the same diet throughout the entire study.

Upon arrival, pigs were allotted to one of 5 different experimental groups. Treatment 1 was the control group, fed diet 1 and without *E. coli* challenge. The rest of the treatments were challenged with enterotoxigenic *E. coli* and were fed one of the four diets listed above.

Enterotoxigenic Escherichia coli Challenge

The pathogenic *Escherichia coli* (isolate number UI-VDL 05-27242) was an F-18 fimbriated *E. coli* strain that produces heat-labile toxin, heat stable toxin b, and Shiga-like toxin 2 (Almeida et al., 2014). Pigs were orally inoculated with *E. coli* (10^{10} CFU per 3 mL dose) in PBS daily for 3 consecutive days, and the sham group was orally inoculated with a 3 mL dose of PBS as described for the challenged pigs. Actual *E. coli* concentrations used in this study were 1.23×10^{10} , 1.03×10^{10} , and 1.07×10^{10} CFU for cohort 1, and 9.75×10^{11} , 2.13×10^{11} , and 3.42×10^{11} CFU for cohort 2 on consecutive inoculation days [0, 1, and 2 d post-inoculation (DPI), respectively]. There were never detectable levels of ETEC quantified in the control (uninfected) group throughout the study, proving that biosecurity measures were effective in preventing contamination, yet all challenged pigs were confirmed positive for the inoculation strain of ETEC administered.

Growth Performance and Frequency of Diarrhea Analyses

Pigs and feeders were weighed at weaning (-13 DPI), the day of the first inoculation (0 DPI), and every 5 d thereafter (i.e., 5 and 10 DPI). Growth performance was calculated during

the pre-inoculation period (-13 to 0 DPI), and in two post-inoculation periods [0 to 5 DPI (acute phase), and 5 to 10 DPI (recovery phase)]. Beginning at study initiation, pigs were observed twice daily and subjectively scored for the incidence of diarrhea as previously outlined by Ivers and Veum (2012). Daily observations were conducted by two individuals independently. Fecal consistency was recorded as normal (no diarrhea) or diarrhea (i.e., watery feces compared with the normal feces). The incidence of diarrhea was reported as the number of experimental days of diarrhea per pig as a proportion of total study days (i.e., frequency of diarrhea).

Blood Cytokines and Clinical Outcomes

Blood samples were collected from the jugular vein of each pig on 0, 2, 7, and 10 DPI. Serum was collected from each of these days to permit later analysis of both pro-inflammatory cytokines (TNF- α and IL-1 β) and anti-inflammatory cytokines (IL-10 and TGF- β) by ELISA (Liu et al., 2013). For serum analysis, blood was collected into 3-mL evacuated tubes containing no anti-coagulant with a 21 gauge, 31.75 millimeter needle. Blood was then allowed to clot at room temperature for 30 minutes before blood samples were centrifuged at $1160 \times g$ at 4°C for 15 minutes. The serum portion was removed using a pasture pipette and stored in sterile microcentrifuge tubes at -80°C pending analysis. A second blood sample was collected from each pig on 7 DPI, for submission to the Veterinary Clinical Pathology Laboratory at the University of Illinois at Urbana-Champaign for measuring total and differential blood cell counts.

Fecal Bacterial Counts

Fecal samples were collected from the rectum of each pig on 0, 5, and 10 DPI using a 22.86 cm, plastic fecal loop with a slotted end (Lambert Vet Supply, NE). The samples were placed into a sterile 1.5-mL microcentrifuge tube and snap frozen in liquid nitrogen before being

analyzed for total bacteria, total *E. coli*, and F18-fimbriated *E. coli* using validated qPCR procedures as follows. Total nucleic acid was extracted from 100-120 mg of fecal material using a Biosprint 96 Work Station (Qiagen, Hayward, CA). Fecal samples were re-suspended in 500 μ l of RNEasy lysis buffer and vortexed for 15 seconds to produce homogeneous mixtures. Then, 100 μ l of this mixture was processed according to manufacturer's recommendations using the One-For-All-Vet Kit (Qiagen, Hayward, CA), and nucleic acids were eluted in 75 μ l of elution buffer. Real time PCR was performed on an Eppendorf Mastercycler[®] ep Realplex System (Eppendorf North America, Westbury Road, NY). The reaction mixture contained 1X Quanta PerfeCTa SYBR Green Supermix (Quanta Biosciences, Gaithersburg, MD 2087); 0.3 μ M each of the forward and reverse primers (*gad AB* and *rrs*) or 0.625 μ M of primers for F18 and 1 μ l of DNA in a total volume of 20 μ l. The challenge *E. coli* strain used for inoculating the animals served as a positive control.

The following thermal cycling profile was used for all amplifications: an initial denaturation at 95°C for 180 seconds, plus 45 repeating cycles with samples at 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Finally, a post-amplification thermal dissociation curve analysis of the amplification products was conducted using a thermal gradient of 60°C to 90°C in increments of 0.2°C/sec to further facilitate data collection. All primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA) as described in Table 3.1.

Statistical Analyses

All data involving infected groups were analyzed as a 2-way ANOVA using SAS (SAS Institute Inc., Cary, NC), with a separate comparison (independent t-test) between unchallenged and challenged control-fed pigs. Because frequency of diarrhea was recorded as a binomial

variable, these data were analyzed using a Chi-square test. Individual pig served as the experimental unit for all outcomes, and significance among means was assessed using an alpha level of 0.05, with trends being recognized when the alpha level was between 0.05 and 0.10.

Results

Growth Performance and Diarrhea Frequency

All ETEC-challenged pigs showed signs of infection, but were able to recover from loss of growth in the last 5 days of the experiment so that all treatments had similar final body weight to the control animals. All of the control animals remained healthy and free of ETEC infection and met expectations for growth performance in the biocontainment facility (Table 3.3). There were a total of 8 pig deaths throughout the study. While there were no interactive effects observed for growth performance outcomes, main effects revealed that whole yeast cells increased ($P < 0.05$), and capsicum decreased ($P < 0.05$), ADFI from 0 to 10 PDI. Reciprocal effects were observed for feed efficiencies (i.e., gain:feed) due to a lack of difference in ADG between dietary treatments within the infected group of pigs.

None of the sham-inoculated (i.e., uninfected) pigs exhibited diarrhea post-infection, so a comparison between the negative and positive control pigs was not made for this outcome (Table 3.7). Pigs challenged with ETEC and fed diets containing whole yeast cells or capsicum alone had a lower frequency of diarrhea when compared with pigs fed the combination diet, yet ETEC-challenged pigs fed the control diet had the lowest diarrhea frequency overall (main effect of whole yeast cells, $P = 0.01$; main effect of essential oil, $P = 0.02$).

Fecal Bacterial Counts

The inoculation *E. coli* strain was non-detectable in challenged pigs on either 0 or 10 DPI (Table 3.4). However, on 5 DPI there were detectable levels of the F18 *E. coli* strain in

challenged pigs only, confirming there was no cross-contamination of the chosen *E. coli* strain with the un-challenged pigs. Total fecal bacterial counts were highest ($P = 0.05$) in pigs fed the combination diet at 5 DPI when compared with all other infected groups. Total fecal bacteria were highest ($P = 0.02$) in pigs fed the capsicum diet at 5 DPI when compared to those fed whole yeast cells alone. The whole yeast cell product, either alone or in combination with capsicum, had no significant effect on total bacteria counts at 0, 5, or 10 DPI. A trend was observed for pigs fed the capsicum-only diet to have the highest ($P = 0.09$) fecal counts of total *E. coli* at 5 DPI when compared with all other infected groups. At 10 DPI, pigs fed the combination diet had the lowest ($P = 0.03$) total bacteria counts when compared with either additive alone, but trended toward having the highest ($P = 0.07$) total fecal *E. coli* counts at 10 DPI.

Clinical Blood Outcomes

No significant dietary effects were observed for hematology measures from blood collected on 7 DPI (Table 3.5). However, dietary treatment effects were observed for differential leukocyte counts in infected pigs on 7 DPI (Table 3.6). Blood leukocyte counts were increased in infected pigs receiving the combination of whole yeast cells and capsicum compared with all other infected groups (interaction, $P = 0.04$). Pigs fed whole yeast cells exhibited increased lymphocyte counts (main effect, $P = 0.02$), however, pigs fed the combination of additives also tended to exhibit increased lymphocyte counts (interaction, $P = 0.09$). Similar neutrophil levels were observed across all the infected groups.

Discussion

Based on previous research indicating that prebiotics and plant essential oils had beneficial effects on pigs challenged with *E. coli*, we hypothesized that whole yeast cells and capsicum, alone or in combination, would influence detriments in growth performance and

support a more effective immune response. Studies by Davis et al. (1999) and van der Peet-Schwering et al. (2007) both observed that yeast products served as effective antimicrobial growth promoters in weanling pigs, while Sads and Bilkei (2003) reported plant extracts to improve growth performance of immune challenged pigs. However, growth performance responses to yeast products and plant extracts appear to be highly variable. In our study, both the whole yeast cell product and capsicum had little effect on growth performance of ETEC-challenged pigs, opposite to what we hypothesized, but similar to results reported by Kornegay et al. (1995), LeMieux et al. (2003), and Liu et al. (2013). However, whole yeast cells did increase feed intake post-inoculation while capsicum elicited an opposite effect. Considering ending pig body weights were similar between treatments, these data indicate that ETEC-challenged pigs fed capsicum were more efficient and needed less feed to achieve growth comparable to challenged pigs fed the control diet. Lack of increases in growth performance may be due to the levels of additives being too low to elicit large enhancements in growth, as White et al. (2002) included dried yeast at 3% and reported no increases in growth performance, while the levels used in our study were much lower at 0.2%.

While previous studies have shown that capsicum may reduce the incidence of diarrhea in *E. coli*-challenged pigs (Liu et al., 2013), data from our study suggested that neither capsicum nor the whole yeast cell product reduced the frequency of diarrhea when compared with challenged pigs fed the control diet. Instead, there was a higher frequency of diarrhea in challenged pigs fed either whole yeast cells or capsicum alone. Surprisingly, a combination of the two additives yielded the highest incidence of diarrhea overall, which is certainly counter to our hypothesis. While the frequency of diarrhea in these challenged pigs was higher, it did not appear to adversely affect growth performance of the ETEC-challenged pigs. The lack of effect

on growth performance may indicate the ETEC infection was not severe enough to impact growth while still causing diarrhea. However, these results may also indicate that while the pigs had high frequency of diarrhea, the additives were able to combat any negative growth performance effects by more quickly alleviating ETEC infection and allowing a rebound of growth in the last days of the study.

Because ingredients with potential to serve as prebiotic substrates have been shown to compete for ETEC binding sites, thereby allowing more ETEC to be shed in the feces instead of binding to the intestinal epithelium (Baumler et al., 1997; Kogan and Kocher, 2007), we quantified fecal bacterial counts. No significant differences were observed between treatments for excretion of F18 *E. coli*, however challenged pigs fed the combination of additives tended to have higher fecal shedding of total bacteria than pigs fed either additive alone at 5 DPI. While no significant effect was observed in pigs fed whole yeast cells alone, the effectiveness of the feed additive combination to increase total fecal bacteria may have implication for either gut health or food safety. The increased shedding of total bacteria on 5 DPI from pigs fed the combination diet may be due to a combined effects of the mannan fraction of the whole yeast cell product to competitively bind pathogenic ETEC and prevent colonization (Firon et al., 1983; Baumler et al., 1997; Kogan and Kocher, 2007) and capsicum's potential to directly kill pathogenic bacteria (Hammer et al., 1999). At 10 DPI, pigs fed the combination of additives had lower total fecal bacterial shedding, but trended to exhibit higher *E. coli* shedding compared with all other infected groups. This rapid, 5-day reduction in fecal ETEC shedding may indicate a return to intestinal homeostasis. With the return to homeostasis, shedding of bacteria would decrease as the gut recovered from ETEC infection, and only small amounts of *E. coli* still lingering in the gut would need to be shed in the feces.

We also hypothesized that whole yeast cells and capsicum would enhance the immune response of pigs challenged with ETEC to lessen the severity of infection and allow pigs to restore homeostasis in a shorter period of time. Challenged pigs fed whole yeast cells, either alone or in combination with capsicum, had higher leukocyte counts at 7 DPI, when compared to other ETEC-challenged groups. Those pigs fed the combination diet had higher lymphocyte counts, while neutrophil counts remained similar overall. This increase in the lymphocyte counts, paired with the similar neutrophil counts, may indicate that the combination of feed additives elicited a reduced inflammatory response in those pigs. This corroborates with research reporting that both yeast products like mannan oligosaccharides and beta-glucan, as well as plant essential oils, may down-regulate the production of pro-inflammatory cytokines (Li et al., 2006; Che et al., 2008; Liu et al., 2012). Davis et al. (2004) also reported that pigs fed mannan oligosaccharides had lower neutrophil counts and higher lymphocytes counts, corroborating observations of our own study. The pigs that were fed capsicum alone had a numerically decreased number of leukocytes, though the numbers were not significant. A reduction in the inflammatory response, and the immune response in general may have been beneficial as pigs may have been able to divert less energy toward immune processes, and instead use resources for supporting growth.

In conclusion, we observed minimal effects of feeding whole yeast cells and capsicum on growth performance. However, since all ETEC challenged pigs had similar body weights at 10 DPI, these feed additives may have prevented loss of growth due to an inflammatory response. The feed additives may have influenced the inflammatory response, allowing less resources to be diverted to immune processes and instead be used for growth. A possible reduction in the inflammatory response, combined with increased bacterial shedding earlier post-inoculation in

pigs fed the combination of additives indicates that there may have been a quicker alleviation of ETEC infection. Further research is needed to investigate the mechanism by which the combination of these additives modulates the immune response to determine if they make a good alternative to antibiotics when ETEC is present.

Tables

Table 3.1. Analytical details regarding quantification of bacteria by RT-PCR

Outcome	Gene	Primer Sequences¹	Amplicon	Reference
F18 ⁺ <i>E. coli</i>	F18	5'-CTC AAA AGA CTA GTG TTT ATT TC-3' 5'-CTT GTA AGT AAC CGC GTA AGC-3'	510 bp	DebRoy and Maddox, 2001
Total <i>E. coli</i>	gadAB	5'-GGA TTA GAT ACC CTG GTA GTC-3' 5'-CGT CAC AGG CTT CAA TCA TGC GTT-3'	305 bp	Chen et al., 2006
Total bacteria	rrs	5'-GGA TTA GAT ACC CTG GTA GTC-3' 5'-TCG TTG CGG GAC TTA ACC CAA C-3'	300 bp	Kariyama et al., 2000

¹Forward and reverse sequences are shown on the top and bottom line, respectively, for each gene.

Table 3.2. Ingredient composition of diets fed to weanling pigs¹

Item	Value²
Ingredient, % of diet	
Corn	39.12
Dried whey permeate ³	23.99
Soybean meal	15.05
Fish meal ⁴	10.00
Processed soy product ⁵	6.00
Dried whey	3.50
Limestone	0.70
Monocalcium phosphate	0.60
L-Lys HCl	0.32
DL-Methionine	0.06
L-Thr	0.21
Vitamin premix ⁶	0.30
Mineral premix ⁷	0.10
Calculated energy and nutrient composition	
Dry matter, %	92.86
ME, kcal/kg	3,561
Crude fiber, %	1.13
Fat, EE, %	3.60
Lactose, %	23.41
Calcium, %	1.08
Phosphorus, %	0.83
SID Lys, %	1.31
SID Ile, %	0.71
SID Leu, %	1.40
SID Met, %	0.40
SID Met + Cys, %	0.63
SID Thr, %	0.85
SID Trp, %	0.19
SID Val, %	0.81

¹Diets were fed 13 d prior to inoculation and for 10 days post inoculation. Abbreviations: CP = crude protein, ME = metabolizable energy, SID = standardized ileal digestibility.

²All values were obtained using swine NRC 2012, excluding the processed soy product where values were obtained from supplier.

³Dairylac 80; International Ingredients Corp., St. Louis MO.

⁴Select menhaden; Omega Protein, Houston, TX.

⁵HP 300; Hamlet Protein, Findlay, OH.

⁶Provided per kilogram of complete diet: vitamin A, 6600 IU; vitamin D3, 700 IU; vitamin E, 70 IU; vitamin K, 4 mg; vitamin B2, 4 mg; vitamin B3, 50 mg; vitamin B5, 24 mg; vitamin B6, 10 mg; vitamin B7, 0.2 mg; vitamin B9, 5 mg; and vitamin B12, 0.04 mg.

⁷Provided per kilogram of complete diet: 100 mg Zn from ZnSO₄; 100 mg Fe from FeSO₄; 10 mg Mn from MnSO₄; 6 mg Cu from CuSO₄; 0.3 mg I from Ca(IO₃)₂; and 0.3 mg Se from (Na₂SeO₃).

Table 3.3. Effects of whole yeast cells and capsicum on growth performance of weanling pigs challenged with a pathogenic *E. coli*¹

Treatment	Un-challenged		Challenged			Pooled SEM	Control, Challenged vs. Unchallenged	P -values		
	1	2	3	4	5			Whole yeast cells	Capsicum	Interaction
Whole yeast cells	-	-	+	-	+					
Capsicum	-	-	-	+	+					
<i>Pre-challenge</i>										
Initial BW, kg	6.02	6.15	6.16	6.13	6.13	0.31	0.35	0.97	0.84	0.97
ADG, g	243	240	214	231	248	30.45	0.91	0.81	0.50	0.24
ADFI, g	787	808	1040	646	819	138.6	0.91	0.13	0.15	0.82
G:F, g/kg	328	364	298	449	412	56.91	0.65	0.33	0.07	0.79
<i>Post-challenge</i>										
Initial BW, kg	9.24	9.27	8.94	9.14	9.31	0.65	0.92	0.75	0.65	0.31
ADG, g										
0-5 DPI	485	435	371	408	248	63.80	0.56	0.05	0.19	0.40
5-10 DPI	675	602	622	626	539	53.36	0.20	0.39	0.45	0.17
0-10 DPI	583	535	503	516	389	45.91	0.44	0.07	0.13	0.27
ADFI, g										
0-5 DPI	1258	1289	1629	893	1506	296.2	0.91	0.01	0.17	0.47
5-10 DPI	1374	1488	1645	982	1170	216.9	0.65	0.32	0.01	0.93
0-10 DPI	1308	1282	1637	896	1288	226.6	0.91	0.02	0.03	0.91
G:F, g/kg										
0-5 DPI	445	378	290	475	262	96.37	0.48	0.02	0.59	0.34
5-10 DPI	560	457	423	718	490	64.85	0.25	0.04	0.01	0.12
0-10 DPI	500	415	322	582	345	68.07	0.27	0.003	0.08	0.18
Final BW, kg	15.04	14.58	14.04	14.18	13.21	0.82	0.57	0.19	0.30	0.70

¹Values represent least squares means of 12 replicates for the unchallenged control animals and 13 replicates per challenged treatment. Pigs were inoculated on 0, 1, and 2 DPI with either a 3 ml dose of PBS (Unchallenged) or F-18+ *E. coli* (Challenged). Treatment annotations: ‘-’ indicates the treatment does not contain the ingredient while ‘+’ indicates the ingredient is present in the treatment. Double ‘-’ indicates the control treatment (i.e., treatment 1 and 2). Abbreviations: DPI = days post inoculation.

Table 3.4. Effects of whole yeast cells and capsicum on fecal bacterial profiles [cycle threshold (Ct) values] and in weanling pigs challenged with a pathogenic *E. coli*¹

Treatment	Un-challenged	Challenged				Pooled SEM	P -values			
	1	2	3	4	5		Control, Challenged vs. Unchallenged	Whole yeast cells	Capsicum	Interaction
Whole yeast cells	-	-	+	-	+					
Capsicum	-	-	-	+	+					
0 DPI										
n	11	13	13	13	11					
F18 <i>E. coli</i>	ND	ND	ND	ND	ND	-	-	-	-	-
Total <i>E. coli</i>	26.80	26.96	25.73	26.58	27.06	0.992	0.91	0.69	0.61	0.36
Total Bacteria	15.05	12.87	13.47	13.97	13.27	0.843	0.06	0.96	0.57	0.42
5 DPI										
n	11	11	12	11	10					
F18 <i>E. coli</i>	ND	22.71	24.82	29.49	27.40	5.65	-	0.99	0.19	0.55
Total <i>E. coli</i>	27.19	22.99	22.30	21.47	18.94	1.78	0.04	0.26	0.09	0.51
Total Bacteria	14.54	14.29	14.44	14.11	12.90	0.655	0.60	0.13	0.02	0.05
10 DPI										
n	11	9	12	12	9					
F18 <i>E. coli</i>	ND	ND	ND	ND	ND	-	-	-	-	-
Total <i>E. coli</i>	28.25	26.78	29.99	29.61	28.78	1.16	0.35	0.28	0.46	0.07
Total Bacteria	13.95	14.31	13.78	13.55	14.09	0.460	0.29	0.99	0.36	0.03

¹Values represent least squares means with the number of pigs per treatment denoted. Pigs were inoculated on 0, 2, and 2 DPI with either a 3 ml dose of PBS (Unchallenged) or F-18+ *E. coli* (Challenged). Treatment annotations: A ‘-’ indicates the treatment does not contain the ingredient while a ‘+’ indicates the ingredient is present in the treatment. Double ‘-’ indicates the control treatment. Abbreviations: DPI = days post inoculation, ND = non-detectable levels.

Table 3.5. Effects of whole yeast cells and capsicum on red blood cell measures on 7 DPI in weanling pigs challenged with a pathogenic *E. coli*¹

Treatment	Un-challenged	Challenged				Pooled SEM	P-values			
	1	2	3	4	5		Control, Challenged vs. Unchallenged	Whole yeast cells	Capsicum	Interaction
Whole yeast cells	-	-	+	-	+					
Capsicum	-	-	-	+	+					
RBC, × 10 ⁶ /μL	6.89	6.98	6.88	6.67	7.01	0.230	0.76	0.57	0.67	0.30
HB, g/dL	12.36	12.45	12.54	12.03	13.17	0.421	0.87	0.10	0.78	0.16
HT, %	39.20	39.02	39.52	37.76	40.72	1.34	0.90	0.12	0.98	0.25
MCV, fl	56.94	55.85	57.13	56.83	58.61	2.29	0.52	0.22	0.32	0.84
MHC,	17.95	17.80	18.13	18.10	18.90	0.670	0.77	0.14	0.16	0.53
MCHC, pg	31.52	31.88	31.76	31.87	32.32	0.229	0.20	0.43	0.19	0.16

¹Values represent least squares means of 12 replicates for the unchallenged control animals and 13 replicates per challenged treatment. Pigs were inoculated on 0, 1, and 2 DPI with either a 3 ml dose of PBS (Unchallenged) or F-18+ *E. coli* (Challenged). Treatment annotations: A ‘-’ indicates the treatment does not contain the ingredient while a ‘+’ indicates the ingredient is present in the treatment. Double ‘-’ indicates the control treatment. Abbreviations: DPI = days post inoculation, RBC = red blood cells; HB = hemoglobin; HT = packed cell volume; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration.

Table 3.6. Effects of whole yeast cells and capsicum on differential leukocyte counts on 7 DPI in weanling pigs challenged with a pathogenic *E. coli*¹

Treatment	Un-challenged		Challenged			Pooled SEM	P -values			
	1	2	3	4	5		Control, Challenged vs. Unchallenged	Whole yeast cells	Capsicum	Interaction
Whole yeast cells	-	-	+	-	+					
Capsicum	-	-	-	+	+					
Count, 10 ³ /μL										
Platelets	622.7	664.0	582.6	553.2	584.1	89.3	0.56	0.62	0.28	0.27
WBC	19.80	23.24	24.01	18.92	27.63	3.14	0.21	0.01	0.86	0.04
NEU	8.26	9.05	8.81	6.78	8.36	1.18	0.53	0.45	0.14	0.32
LYMPH	10.07	12.21	13.36	10.80	17.44	2.52	0.34	0.02	0.40	0.09
NEU/LYMPH	0.89	0.95	0.78	0.69	0.58	0.16	0.75	0.34	0.11	0.82
MONO	1.10	1.51	1.35	0.92	1.28	0.31	0.23	0.68	0.18	0.28
EOS	0.26	0.31	0.22	0.28	0.10	0.08	0.60	0.07	0.32	0.58
BASO	0.11	0.12	0.17	0.10	0.10	0.07	0.88	0.73	0.43	0.65
% WBC										
NEU	41.8	40.9	37.2	35.8	32.3	4.04	0.85	0.32	0.17	0.99
LYMPH	50.6	50.4	55.0	56.8	61.3	3.79	0.97	0.20	0.08	0.99
MONO	5.6	6.3	5.8	5.0	4.8	1.58	0.55	0.70	0.18	0.85
EOS	1.2	1.4	1.0	1.6	0.4	0.35	0.74	0.01	0.63	0.21
BASO	0.5	0.6	0.6	0.6	0.4	0.27	0.74	0.66	0.57	0.79

¹Values represent least squares means of 12 replicates for the unchallenged control animals and 13 replicates per challenged treatment. Pigs were inoculated on 0, 1, and 2 DPI with either a 3 ml dose of PBS (Unchallenged) or F-18+ *E. coli* (Challenged). Treatment annotations: A ‘-’ indicates the treatment does not contain the ingredient while a ‘+’ indicates the ingredient is present in the treatment. Double ‘-’ indicates the control treatment. Abbreviations: DPI = days post inoculation, WBC = white blood cells; NEU = neutrophils; LYMPH = lymphocytes; MONO = monocytes; EOS = eosinophils; BASO = basophils.

Table 3.7. Effects of whole yeast cells and capsicum on frequency of diarrhea in weanling pigs challenged with a pathogenic *E. coli*¹

Treatment	Un-challenged	Challenged				P -values			
	1	2	3	4	5	Control, Challenged vs. Unchallenged	Whole yeast cells	Capsicum	Interaction
Whole yeast cells	-	-	+	-	+				
Capsicum	-	-	-	+	+				
<i>Pre-challenge</i>									
Pig days ²	152	169	169	169	167				
Diarrhea days ³	3.5	3	4	2.5	1				
Frequency ⁴ , %	2.30	1.78	2.37	1.48	0.60	0.74	0.70	0.23	0.18
<i>Post-challenge</i>									
Pig days	110	117	128	121	111				
Diarrhea days	0	20	23.50	21.50	23.50				
Frequency, %	0	17.09	18.36	17.77	21.17	<0.0001	0.01	0.02	0.59

¹Values represent least squares means of 12 replicates for the unchallenged control animals and 13 replicates per challenged treatment. Pigs were inoculated on 0, 1, and 12 DPI with either a 3 ml dose of PBS (Unchallenged) or F-18+ *E. coli* (Challenged). Treatment annotations: A ‘-’ indicates the treatment does not contain the ingredient while a ‘+’ indicates the ingredient is present in the treatment. Double ‘-’ indicates the control treatment. Abbreviations: DPI = days post inoculation.

²Pig days = number of pigs × the number of days of diarrhea scoring.

³Diarrhea days = number of pig days with diarrhea.

⁴Frequency = (diarrhea days × 100)/pig days.

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CHAPTER 4: EFFECTS OF WHOLE YEAST CELLS AND CAPSICUM ON CYTOKINE EXPRESSION IN TISSUES, IMMUNOPHENOTYPING AND NITRIC OXIDE CONCENTRATION IN CELLS ISOLATED FROM PIGS INFECTED WITH ENTEROTOXIGENIC *ESCHERICHIA COLI*

Abstract

An experiment was conducted to evaluate cytokine mRNA expression in tissues, identify different phenotypes of T cells from peripheral blood mononuclear cells (PBMC), and quantify nitric oxide content of PBMC challenged with *E. coli* lipopolysaccharide (LPS). PBMC were isolated from weanling pigs inoculated with enterotoxigenic *Escherichia coli* (ETEC), who were fed CitriStim, a whole yeast cell product, and capsicum, an essential oil. Weanling pigs (34 barrows and 30 gilts, 21 d of age, 5.90 ± 1.03 kg BW) were allotted to experimental treatments in a randomized complete block design based on litter, sex, and initial BW. Four pigs were individually housed within a containment chamber and assigned to 1 of 4 dietary treatments ($n = 13$), which included a control diet without or with 0.2% whole yeast cells product, CitriStim (ADM, Decatur, IL) or 10 ppm of capsicum, provided either alone or in combination. After receiving diets for 13 d, pigs were orally inoculated daily for 3 consecutive days with F18+ ETEC and maintained on the same diets for an additional 10 d; a separate cohort of pigs ($n = 12$) receiving the control diet was sham-inoculated using PBS. At 10 DPI pigs were euthanized and intestinal tissues were collected and snap frozen at -80°C . Tissues collected from the ileum and colon were analyzed by real-time PCR for cytokine mRNA expression. Blood samples were collected at 7 DPI for immunophenotyping by multi-color flow cytometry. Peripheral blood mononuclear cells were isolated from blood collected on 10 DPI and used for nitric oxide (NO) quantification. While no interactive effects were observed for NO production, main effects revealed that the cells isolated from challenged pigs fed the capsicum diet, expressed lower NO production when compared to cells isolated from pigs fed whole yeast cells alone. Proportions of

CD8⁺ and CD4⁺CD8⁺ cells were lower in pigs fed the combination of additives compared with pigs fed either additive alone at 0 and 7 DPI. Gene expression of cytokine mRNA at 10 DPI was similar across all ETEC-challenged groups. These data indicate that capsicum alone may be effective at reducing NO expression, and therefore beneficially influence the inflammatory response. The combination of additives reduced the proportion of cytotoxic (CD8⁺) and memory (CD4⁺CD8⁺) T cells, suggesting a lessened adaptive immune response to ETEC infection. Similar cytokine mRNA expression in the intestinal tissues of challenged pigs suggests that by 10 DPI, pigs across all ETEC-challenged groups may have already recovered from infection, or that 10 DPI is too late to observe significant differences in intestinal cytokine mRNA expression.

Introduction

Weaning piglets are abruptly exposed to a variety of new stressors including a new environment, new conspecifics and the establishment of a new social hierarchy, and changes in diet that can lead to the immune system being less effective, as well as reduced growth performance (Fairbrother et al., 2005; Moeser et al., 2007). If the immune system becomes compromised, or is less effective, it allows for pigs to be infected by a variety of viral and bacterial diseases, one of the most common being enterotoxigenic *Escherichia coli* (ETEC). Infection by ETEC may cause reductions in growth performance due partly to energy being diverted away from growth and towards immune related processes (Spurlock, 1997). Most commercial farms use antibiotics to treat ETEC infection, however there have been recent concerns about antibiotic levels in food producing animals, so alternatives must be found to treat these bacterial infections (Barton, 2000). Two alternatives that have been showing recent promise are plant essential oils and prebiotics. Further research is needed to determine how these additives influence the immune response of weanling pigs both before and after bacterial infection.

During an ETEC infection, the energy that a pig may use for growth gets diverted towards combating an infective agent. The immune system responds to infection by first sending phagocytic cells to recognize specific structures present on bacteria (i.e. using host-derived pattern recognition receptors), and eliminate invading infectious agents by phagocytosis followed by degradation by toxic compounds such as superoxide radicals and hydrogen peroxide (Yaqoob and Calder, 2011). The phagocytic cells also release nitric oxide, which helps to kill the infectious agents, but if left unchecked or produced in large quantities in a severe infection, can start to damage tissue and slow down repair (Wink et al., 2011). If the first line of defense in the immune system is not enough to quell the infection, an adaptive immune response will be initiated and B lymphocytes will produce antigen-specific antibodies, while T lymphocytes will secrete cytokines and cytotoxic enzymes to kill invading pathogens (Trombetta and Mellman, 2005; Yaqoob and Calder, 2011). Pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interferon- γ (IFN- γ), and interleukin 1 β (IL-1 β), and anti-inflammatory cytokines like interleukin 10 (IL-10) and transforming growth factor β (TGF- β) play important parts in regulating the immune response to ETEC infection, as well as regulation of tissue damage caused by the immune response (Johnson, 1997; Al-Sadi et al., 2009). This response acts as the main defense against intracellular bacterial infection. (Jenkins et al., 2001).

Both prebiotics and essential oils have been shown to reduce the inflammatory response and alleviate detrimental effects either through the production of short chain fatty acids (prebiotics) or the inhibition of pro-inflammatory cytokines and decreased white blood cell levels (essential oils) (Yaqoob and Calder, 2011; Liu et al., 2012; Vieira et al., 2013). Certain immune cells may have receptors for SCFA, so when they are produced they recruit immune cells such as neutrophils to their production site, which can help to strengthen the immune response

(Maslowski et al., 2009; Kim et al., 2013). The inhibition of cytokines causes a smaller immune response, which can help to divert energy back toward growth and minimize tissue damage caused by an abundance pro-inflammatory cytokines (Liu et al., 2012).

Few studies have investigated tissue gene expression of inflammatory cytokines in newly-weaned pigs, and few have looked at immunomodulatory effects of antibiotic alternatives on ETEC-infected weanling pigs. Thus, we conducted an experiment to evaluate the combined effects of a whole yeast cell prebiotic, CitriStim, and a plant essential oil, capsicum, on tissue gene expression of cytokines, and NO production and immunophenotyping of blood-derived T cells in weanling pigs experimentally challenged with ETEC.

Materials and Methods

All animal care and experimental procedures were approved by the University of Illinois Institutional Animal Care and Use committee before initiation of the experiment.

Animal Husbandry

A total of 64 weaned pigs (21 days of age), were used in in a single experiment with 2 cohorts of pigs (32 pigs per experiment) and analyzed together. A total of 34 barrows and 30 gilts (initial BW 5.91 ± 1.03 kg) were selected and allotted to experimental treatments in a randomized complete block design by evenly distributing initial BW, litter, and sex. Pigs were housed in the Edward R. Madigan Laboratory (ERML) large animal biocontainment facility at the University of Illinois. A suite of 8 chambers was used to house both the challenged and unchallenged (sham-inoculated) pigs, and strict biosecurity measures were followed to prevent cross-contamination. Each chamber consisted of 4 individual pens (0.84m^2) each equipped with a nipple drinker and a feeder, allowing pigs to be individually housed.

Four diets were prepared from a common basal in a 2×2 factorial arrangement. Diet 1 (negative control) was a nursery diet formulated to meet or exceed NRC (2012) requirements for weanling pigs; this diet did not contain antibiotics, spray-dried plasma, or zinc oxide a growth promoting or prophylactic agents. Diet 2 was formulated as diet 1, but was supplemented with whole yeast cells added directly on top of the control diet at 0.2%. Diet 3 was formulated as diet 1, but was supplemented with an essential oil (capsicum, 10 ppm) added directly on top of the control diet. Diet 4 was formulated as diet 1, but was supplemented with whole yeast cells (0.2%) and capsicum (10 ppm) added directly on top of the control diet. Pigs remained on the same diet throughout the entire study.

The pigs were allotted to one of 5 different experimental treatments. Treatment 1 was the control group, fed diet 1 and without *E. coli* challenge. The rest of the treatments were challenged with *E. coli* and were fed one of the four diets listed above.

Enterotoxigenic Escherichia coli Challenge

The pathogenic *Escherichia coli* (isolate number UI-VDL 05-27242) was an F-18 fimbriated *E. coli* strain that produces heat-labile toxin, heat stable toxin b, and Shiga-like toxin 2 (Almeida et al., 2014). Pigs were orally inoculated with *E. coli* (10^{10} CFU per 3 mL dose) in PBS daily for 3 consecutive days, and the sham group was orally inoculated with a 3 mL dose of PBS as described for the challenged pigs. Actual *E. coli* concentrations used in this study were 1.23×10^{10} , 1.03×10^{10} , and 1.07×10^{10} CFU for cohort 1, and 9.75×10^{11} , 2.13×10^{11} , and 3.42×10^{11} CFU for cohort 2 on consecutive inoculation days [0, 1, and 2 d post-inoculation (DPI), respectively]. There were never detectable levels of ETEC quantified in the control (uninfected) group throughout the study, proving that biosecurity measures were effective in preventing

contamination, yet all challenged pigs were confirmed positive for the inoculation strain of ETEC administered.

Immunophenotyping

Blood samples were collected from the jugular vein of each pig on 0 and 7 DPI. Blood was collected into a 4mL evacuated tube containing 5.4mg of EDTA and placed on ice for 45 minutes until cell isolation was conducted using the Sepmate procedure (Stemcell Technologies, Vancouver, Canada). Briefly, blood was diluted with an equal volume of PBS containing 2% fetal bovine serum (FBS) and pipetted into 15mL Sepmate tubes containing 4.5mL of density gradient medium. The Sepmate tubes were then centrifuged at $1160 \times g$ for 10 minutes. The top layer, containing PBMC was then poured into a 15mL conical tub, washed, and then cells were counted using the Moxi 2 mini automated cell counter (ORFLO, Ketchum, ID) and the concentration was adjusted to 1×10^6 cells/mL using high glucose Dulbecco's modified eagle media (DMEM) supplemented with HEPES, sodium pyruvate, non-essential amino acids, sodium bicarbonate and penicillin-streptomycin (ThermoFisher Scientific, Waltham, MA).

Cells were then stained for surface markers CD3, a T cell marker, CD4, or CD8 using porcine validated monoclonal antibodies conjugated to a distinct fluorochrome (BD, San Jose, CA). Once stained with these surface markers, the cells were washed and permanently fixed following manufacturer's instructions using the Cytofix/Cytoperm with Golgi stop kit (BD, San Jose, CA). Finally the fixed cells were stained for the intracellular cytokine IFN- γ using a porcine validated monoclonal antibody conjugated to a distinct fluorochrome (BD, San Jose, CA). The relative percentage of different phenotypes of T cells (i.e. CD4, CD8 single or double positive T-cells secreting IFN- γ) were determined using multi-color flow cytometry (BD LSR II Flow cytometry analyzer, UIUC W.M. Keck Center).

Tissue Collection

At 10DPI pigs were euthanized for tissue and blood collection. Pigs were anesthetized using an intramuscular injection of telazol:ketamine:xylazine administered at 0.03mL/kg BW. Pain reflex and eye blink response were tested to ensure anesthesia before euthanizing animals with an intracardiac injection of Euthasol (Virbac, Fort Worth, TX) at 1 mL/4.5kg BW. Blood was collected immediately after euthanasia into 50 mL conical tubes containing 5 mL EDTA and left on ice pending analyses. Tissue samples were excised from the ileum, 30 cm anterior to the ileocecal junction, and from the colon, at the junction between ascending and descending colon (i.e. the mid-colon). All tissue was collected in 2 cm pieces, rinsed free of contents and blood in Millipore water, and snap frozen at -80°C.

Recall Nitric Oxide Analysis

For recall NO analysis, blood was collected on 10 DPI immediately after euthanasia via the carotid artery, placed into 50mL conical tubes containing 5% EDTA, and set on ice for 1 hour pending isolated procedures. Subsequently, PMNC were isolated using the Sepmate procedure described above. At the final step, isolated cells were counted using the Moxi 2 mini automated cell counter (ORFLO, Ketchum, ID) and the concentration was adjusted to 2×10^6 cells/mL using the same DMEM described above (ThermoFisher Scientific, Waltham, MA). Cells were plated in triplicate in 24-well cell culture plates in DMEM (ThermoFisher Scientific, Waltham, MA) and allowed to grow and adhere for 24-h. Due to complications with adherence in the first cohort, NO data were only collected from pigs in the second cohort. After the 24-h growth period, adherent cells were considered to be of monocytic lineage, and the non-adherent cells were discarded along with the original medium. The adhered monocytes were then stimulated with 0 or 50µg/mL of *Escherichia coli* 0111:B4 lipopolysaccharide (Sigma-Aldrich,

St. Louis, MO) in monocyte medium for 24- or 48-h. At the appropriate time, supernatant from each well was pooled into two 1.5mL microcentrifuge tubes and stored at 4°C pending NO analysis.

Samples were analyzed using a nitrate/nitrite colorimetric assay kit (Cayman Chemical, Ann Arbor, MI). Briefly, the kit follows a two-step process, with the first step being the conversion of nitrate to nitrite utilizing nitrate reductase. The second step uses Griess reagent which converts nitrite into a purple azo compound. The photometric measurement of absorbance of the azo compound is what accurately determines nitrite concentration. Samples were diluted 2:1 with an assay buffer, plated in a 96-well plate in triplicate in a total volume of 80µL. The nitrate reductase enzyme cofactor and enzyme mixture were added to each well. The plate was allowed to incubate at room temperature for 2h and then Griess reagents R1 and R2 were added to each well per manufacturer's instructions. The color was allowed to develop for 10 minutes at room temperature, and then the plate was read using a Synergy HT micro-plate reader at 540nm (BioTek, Winooski, VT).

Inflammatory Cytokine mRNA Expression

Frozen colonic and ileal tissue samples, (50 to 100 mg), were placed into a 2mL microcentrifuge tube along with 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA) and a 5mm steel bead. Samples were homogenized through tissue disruption for 5 minutes at 30 Hz using a TissueLyser II (Qiagen, Valencia, CA) and RNA was extracted following manufacturer instructions. Extracted RNA was quantified using a spectrophotometer, with all samples having a 260:280 absorbance ratio of 1.8 or higher (NanoDrop ND-1000, Nano-Drop Technologies, Wilmington, DE), with all samples having a 260/280 ratio of 1.8 or higher.

The extracted RNA was transcribed to complementary DNA (cDNA) using a high capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). The samples were placed in a thermocycler (Bio-Rad, Hercules, CA) set to 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 4 minutes, and then cooled at 4°C and held there until the cDNA samples were removed and stored at -20°C. The TaqMan Gene Expression Assay (Applied Biosystems, Foster, CA) was used to perform quantitative real-time PCR to quantify relative gene expression of pro-inflammatory cytokines IL-1 β and TNF- α and anti-inflammatory cytokines IL-10 and TGF- β . Amplifications was achieved by PCR for both target (IL-1 β , TNF- α , IL-10, TGF- β) and reference (PPIA; Jacobson et al., 2011; Daudelin et al., 2011) porcine genes. Sample cDNA was amplified using TaqMan (Invitrogen, Carlsbad, CA) oligonucleotide probes containing 5' fluorescent reporter dye (6-FAM) and 3' non-fluorescent quencher dye, and fluorescence was determined using an ABI PRISM 7900HT-sequence detection system (Applied Biosystems, Foster City, CA). Gene expression was normalized through parallel amplification of endogenous PPIA for each sample. Relative gene expression was calculated with the comparative threshold cycle method, with results expressed as fold-change relative to the infected, control fed pigs (Livak and Schmittgen, 2001).

Statistical Analyses

All data involving infected groups were analyzed as a 2-way ANOVA using SAS (SAS Institute Inc., Cary, NC), with a separate comparison (independent T-test) between unchallenged and challenged control-fed pigs. Gene expression data for cytokines were log transformed to achieve normal distribution, and experimental treatment 2 served as the calibrator group, with all values regulated to 1. Individual pig served as the experimental unit for all outcomes, and

significance among means was assessed using an alpha level of 0.05, with trends being recognized when the alpha level was between 0.05 and 0.10.

Results

T Cell Immunophenotyping

Pigs fed the combination of additives had significantly lower percentages of CD8⁺ ($P = 0.05$) and CD4⁺CD8⁺ ($P = 0.02$) T cells when compared to pigs fed either additive alone on 0 DPI (Table 4.2). Pigs fed the combination of additives also had lower percentages of CD8⁺ ($P = 0.02$) and CD4⁺CD8⁺ ($P = 0.02$) cells on 7 DPI, showing that there were less cytotoxic T cells (CD8⁺) and less effector memory T cells (CD4⁺CD8⁺) present in the blood after inoculation when compared with either additive alone. The whole yeast cell product alone tended to increase the proportion of CD4⁺ cells on 7DPI ($P = 0.09$). No differences were observed for the proportion of effector memory T cells that secrete IFN- γ between infected groups.

Nitric Oxide Production

Cells isolated from pigs fed capsicum alone exhibited lower NO production when compared to PBMC from pigs fed whole yeast cells alone at 24 h, without or with LPS challenge (Table 4.1). Pigs fed the capsicum only diet had less NO production at 24 hr in both the LPS challenged ($P = 0.02$) and non-LPS cells ($P = 0.04$) than those fed whole yeast cells alone. There were no differences in NO production from PBMC isolated from infected groups after 48 h of culture.

Gene Expression

Cytokine mRNA expression was not measured in the un-challenged, control fed pigs. The level of expression of mRNA encoding the cytokines TNF- α , IL-1 β , TGF- β , and IL-10, in both the ileal and colonic tissues, did not vary between the ETEC-challenged groups.

Discussion

Few studies have quantified at the intestinal immune response to pigs challenged with ETEC infection, so our goal was to determine if whole yeast cells and capsicum, either alone or in combination, would allow ETEC-inoculated pigs to mount a more effective immune response. Vetvicka et al. 2014 previously reported that supplementation of β -glucan, which can be found in yeast products like CitriStim, increased concentrations of both cytotoxic and helper T cells in pigs infected with *E. coli*, while Liu et al. (2013) reported that plant extracts could reduce pro-inflammatory cytokine production, which may indicate a decrease in helper T cell populations. Data from our study indicated that neither additive alone affected T cell subsets, and only when fed in combination did we observe a decrease in both cytotoxic and memory T cells. Passive immunity that pigs acquire from the sow's milk are depleted by the time the pigs are weaned (King and Pluske, 2003; Gallois et al., 2009). This leaves the immune system of the weanling pig under developed at the time of weaning. When combined with weaning stressors, not limited to, a new environment and a new diet, the immune system can be overwhelmed with new antigens and can cause intestinal inflammation which can cause an increase in both CD4⁺ and CD8⁺ T cells directly after weaning (McCracken et al., 1999; Moeser et al., 2007). The combination of capsicum and whole yeast cells decreased CD8⁺ cells before inoculation making this combination a viable nutritional intervention to post-weaning inflammation.

The continued reduction in cytotoxic and helper T cell sub-populations at 7 DPI in pigs fed the combination diet suggests that a lesser adaptive immune response may have been mounted against ETEC infection. This could be due to the innate response being more effective in these animals, which would mean less of an adaptive immune response was needed. The observed response may indicate that the combination of feed additives allowed pigs to be more effective at clearing ETEC infection (i.e. more efficient immune response) than when either additive was fed alone.

Research indicates that both weaning, and ETEC infection leads to an upregulation of inflammatory cytokines in both the small intestine and colon (Pié, et al., 2004; Hu, et al., 2014). Both IL-1 β and TNF- α are major inflammatory cytokines that mediate acute inflammation, while IL-10 and TGF- β are anti-inflammatory cytokines acting to keep inflammation controlled and prevent tissue damage (Zhao et al., 2008). Importantly, IL-1 β and TNF- α may increase permeability of the intestinal epithelium, thereby allowing microorganisms and cytokines to infiltrate tissues and cause untoward effects, including diarrhea (Piè et al., 2004; Al-Sadi and Thomas, 2007). Anti-inflammatory cytokines help to protect the intestine from inflammation by preserving barrier function and repairing epithelial injuries (Madsen et al., 1997; Blikslager et al., 2007). We hypothesized that whole yeast cells, capsicum, or the combination of both would reduce inflammatory cytokines, while possibly up-regulating anti-inflammatory cytokines. Data from our study indicated that neither the whole yeast cell product, or capsicum, alone or in combination had an effect on intestinal cytokine gene expression at 10 DPI. Both the inflammatory and anti-inflammatory cytokines were expressed at similar levels between the ETEC-challenged groups, contrary to what we hypothesized. The lack of fold change between the ETEC-challenged groups may indicate that though an adaptive immune response may have

been mounted against ETEC infection, cytokine levels were downregulated quickly and 10 DPI is too late to observe large differences among the ETEC-challenged groups. This is contrary to what Li et al. (2006) observed, as they presented that β -glucans increased IL-10 to inhibit T cell proliferation. While we did observe a reduction in T cell proliferation, we may have collected tissue too late to observe if tissue cytokine mRNA expression contributed to T cell reduction.

Nitric oxide production is necessary for a robust immune response, as it has cytotoxic properties capable of damaging pathogenic microbes through oxidation of DNA, proteins, and lipids (Korhonen et al., 2005; Li et al., 2006). An overabundance or overproduction of NO can have potent cytotoxic effects that cause tissue damage to the host, so the immune system has to tightly regulate NO production (Li et al., 2006; Wink et al., 2011). Li et al., 2006 and Lee et al., 2002 provided evidence that plant extracts can inhibit NO production due to plant extracts being a source of bioactive chemicals and chemotherapeutic reagents (Lee et al., 2000). We observed that PBMC isolated from pigs fed the capsicum diet had less NO production when challenged with LPS, compared to cells isolated from pigs fed the whole yeast cell only diet. The reduction in NO in these PBMC may indicate that capsicum is effective at controlling NO production to prevent oxidative damage, but such effects need to be confirmed *in vivo*. The lack of differences between infected groups after 48 h of culture could be due to 48 h being too long after initial LPS challenged to observe changes in NO production. Because we only quantified the total amount of NO produced, we cannot definitively claim that capsicum inhibited the iNOS protein directly, or if capsicum is inhibiting NO production through another route.

In conclusion, our results demonstrate that whole yeast cells and capsicum may have downregulating effects on the immune system, especially the T cell immune response. While neither feed additive alone was able to alter T cell sub-populations, the combination elicited

decreased cytotoxic and memory T cells proportions. At the same time, pigs challenged with *E. coli* exhibited similar expression of cytokine mRNA in both the ileum and colon, possibly indicating that the additives had little effect on tissue cytokine expression, or that 10 DPI is too late to observe large differences in tissue cytokine expression. Reduced NO production from PBMC isolated from pigs fed the capsicum only diet suggest this feed additive may be a used as part of a strategy to reduce oxidative damage caused by excess NO during inflammation responses. Further research is needed to determine why cytokine mRNA levels were lower in infected pigs, as well as possibly mechanisms for the combination of additives reducing T cell sub-populations.

Tables

Table 4.1. Effects of LPS on NO production in porcine macrophage cells isolated from weanling pigs challenged with a pathogenic *E. coli*¹

Treatment	Un-challenged	Challenged				Pooled SEM	P-values			
	1	2	3	4	5		Control, Challenged vs. Unchallenged	Whole yeast cells	Capsicum	Interaction
Whole yeast cells	-	-	+	-	+					
Capsicum	-	-	-	+	+					
<i>No LPS Challenge</i> ²										
NO production, μ M										
24 h	419	483	617	330	255	123.3	0.68	0.81	0.04	0.40
48 h	227	233	229	210	193	38.5	0.91	0.79	0.45	0.87
<i>LPS Challenge</i> ²										
NO production, μ M										
24 h	341	399	581	303	238	93.1	0.62	0.53	0.02	0.19
48 h	217	215	225	199	200	39.7	0.97	0.88	0.61	0.90

¹Values represent least squares means of 12 replicates for the unchallenged control animals and 13 replicates per challenged treatment. Pigs were inoculated on 0, 1, and 2 DPI with either a 3 ml dose of PBS (Unchallenged) or F-18+ *E. coli* (Challenged). Treatment annotations: A ‘-’ indicates the treatment does not contain the ingredient while a ‘+’ indicates the ingredient is present in the treatment. Double ‘-’ indicates the control treatment. Abbreviations: DPI = days post inoculation, LPS = lipopolysaccharides, NO = nitric oxide.

²Cells were treated with either 1mL of complete media (no LPS Challenged) or with 1mL of completed media containing LPS at a dose of 0.5 μ g/mL (LPS Challenge). Media was then collected for NO analysis at either 24 h or 48 h after the time of challenge with LPS.

Table 4.2. Effects of whole yeast cells and capsicum on T cell proportions (% of total isolated CD3⁺ T cells) in weanling pigs challenged with a pathogenic *E. coli*¹

Treatment	Un-challenged	Challenged				Pooled SEM	P -values			
	1	2	3	4	5		Control, Challenged vs. Unchallenged	Whole yeast cells	Capsicum	Interaction
Whole yeast cells	-	-	+	-	+					
Capsicum	-	-	-	+	+					
<i>0 DPI</i>										
n	11	13	13	12	12					
%CD4CD8 ⁻ cells	54.5	54.0	54.9	48.6	56.0	5.05	0.90	0.16	0.47	0.28
% CD4 ⁺ cells	24.7	23.0	20.1	22.4	23.9	1.98	0.52	0.70	0.38	0.24
% CD8 ⁺ cells	17.8	19.9	21.2	23.6	16.9	4.99	0.49	0.19	0.89	0.05
%CD4 ⁺ CD8 ⁺ cells	3.1	3.1	3.7	5.3	3.1	0.83	0.99	0.18	0.20	0.02
%IFN- γ ⁺ cells	63.0	61.0	64.2	60.3	67.2	10.6	0.77	0.27	0.80	0.69
<i>7 DPI</i>										
n	10	11	13	12	10					
%CD4CD8 ⁻ cells	57.8	59.3	57.5	56.6	61.8	3.97	0.63	0.42	0.71	0.10
% CD4 ⁺ cells	23.0	23.1	20.4	21.8	19.4	3.81	0.95	0.09	0.46	0.92
% CD8 ⁺ cells	15.9	14.4	17.8	17.0	15.0	1.30	0.37	0.53	0.96	0.02
%CD4 ⁺ CD8 ⁺ cells	3.2	3.2	4.4	4.7	3.8	0.47	0.93	0.66	0.32	0.02
%IFN- γ ⁺ cells	46.8	55.3	49.8	48.0	50.4	7.79	0.17	0.70	0.42	0.35

¹Values represent least squares means of 12 replicates for the unchallenged control animals and 13 replicates per challenged treatment. Pigs were inoculated on 0, 1, and 2 DPI with either a 3 ml dose of PBS (Unchallenged) or F-18+ *E. coli* (Challenged). Treatment annotations: A ‘-’ indicates the treatment does not contain the ingredient while a ‘+’ indicates the ingredient is present in the treatment. Double ‘-’ indicates the control treatment. Abbreviations: DPI = days post inoculation.

Table 4.3. Effects of whole yeast cells and capsicum on intestinal gene expression of cytokines in weanling pigs challenged with a pathogenic *E. coli*¹

Treatment	Challenged				Pooled SEM	P-values ² Challenged		
	2	3	4	5		Whole yeast cells	Capsicum	Interaction
Whole yeast cells	-	+	-	+				
Capsicum	-	-	+	+				
<i>10 DPI</i>								
Ileum								
n	9	11	12	10				
TNF- α	1.00	2.17	3.61	5.42	3.51	0.98	0.17	0.37
IL-1 β	1.00	3.42	4.70	4.95	3.56	0.44	0.16	0.24
TGF- β	1.00	1.50	1.11	1.05	1.03	0.88	0.33	0.17
IL-10	1.00	0.93	0.80	0.51	0.25	0.42	0.20	0.25
Colon								
n	10	11	12	9				
TNF- α	1.00	0.88	0.58	2.26	1.08	0.76	0.85	0.45
IL-1 β	1.00	0.60	0.37	0.98	0.46	0.73	0.85	0.10
TGF- β	1.00	0.95	0.63	2.89	1.44	0.70	0.90	0.48
IL-10	1.00	0.74	0.48	2.87	1.49	0.75	0.81	0.42

¹Values represent least squares means with the number of pigs per treatment denoted. Values are based on $2^{-\Delta\Delta C_T}$ method, with treatment 2 serving as the calibrator. Cytokine gene expression was not measured for the un-challenged, control pigs (treatment 1). Pigs were inoculated on 0, 1, and 2 DPI with either a 3 ml dose of PBS (Unchallenged) or F-18+ *E. coli* (Challenged). Treatment annotations: A ‘-’ indicates the treatment does not contain the ingredient while a ‘+’ indicates the ingredient is present in the treatment. Double ‘-’ indicates the control treatment. Abbreviations: DPI = days post inoculation.

²P – values represent log-transformed data to achieve a normal distribution.

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